Extraction of Bioactives and Oil from Gac Seeds

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M. Sc. In Food Science and Technology

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Food Science

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STATEMENT OF ORIGINALITY

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

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I hereby certify that this thesis is in the form of a series of papers. I have included as part of the thesis a written declaration from each co-author, endorsed in writing by the Faculty Assistant Dean (Research Training), attesting to my contribution to any jointly authored papers.

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Physicochemical properties of Gac (*Momordica cochinchinensis* Spreng) seeds and their oil extracted by supercritical carbon dioxide and Soxhlet methods.

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Paper III. Anh V. Le, Sophie E. Parks, Minh H. Nguyen and Paul D. Roach (2018).

Effect of solvents and extraction methods on recovery of bioactive compounds from defatted Gac (*Momordica cochinchinensis* Spreng) seeds.

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Paper IV. Anh V. Le , Sophie E. Parks, Minh H. Nguyen and Paul D. Roach (2018).

Optimised extraction of trypsin inhibitors from defatted Gac (*Momordica cochinchinensis* Spreng) seeds for production of a trypsin inhibitor-enriched freeze-dried powder.

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Paper VII. Anh V. Le , Tien T. Huynh, Sophie E. Parks, Minh H. Nguyen and Paul D. Roach (2018).

Bioactive composition, antioxidant activity and anticancer potential of freeze-dried extracts from defatted Gac (*Momordica cochinchinensis* Spreng) seeds.

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STATEMENT OF CONTRIBUTION BY OTHERS

To whom it may concern,

This statement outlines Anh Van Le's contribution to the series of papers that are submitted as a part of her PhD. All papers that are contributing to her thesis are listed below, with a statement of her contribution for each.

Regards,

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LIST OF ABBREVIATIONS AND UNITS OF MEASUREMENT

Abbreviations	**Full forms**
AC	Antioxidant capacity
ABTS	2,2'-azino-bis(3-ethylbenzothiozoline-6-sulfonic acid)
AE	Aecsin equivalents
ANOVA	Analysis of variance
AV	Acid value
BAPNA	N-α-benzoyl-D,L-arginine-4-nitroanilide
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCD	Central composite design
DI	Deionised
DW	Dry weight
DPPH	2,2'-diphenyl-1-picrylhydrazyl
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
MAE	Microwave-assisted extraction
MCoTI	*Momordica Cochinchinensis* trypsin inhibitor
PNA	Para-nitroaniline
PV	Peroxide value
RSM	Response surface methodology
RT	Room temperature
SC-CO_2	Supercritical carbon dioxide
SD	Standard deviation
SFE	Supercritical fluid extraction
SPSS	Statistical package for social science
TE	Trolox equivalents
TIA	Trypsin inhibitor activity
TPC	Total phenolic content
TSC	Total saponin content
UAE	Ultrasound-assisted extraction
v/v	Volume per volume

w/v	Weight per volume
w/w	Weight per weight

Units	**Full forms**
%	Percent
^{o}C	Degree Celsius
× *g*	g force
g	Gram
g^{-1}	Per gram
g/g	Gram per gram
g/L	Gram per litre
g/mL	Gram per millilitre
h	Hour
kPa	Kilopascal
mbar	Millibar
meq/kg	Milliequivalents of active oxygen per kg
mg	Milligram
mg/g	Milligram per gram
mL	Millilitre
mL/min	Milliliter per minute
mM	Millimolar
min	Minute
rpm	Revolutions per minute
W	Watt
μmol/g	Micromole per gram
μL	Microliter

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ABSTRACT

Gac (*Momordica cochinchinensis* Spreng) seeds are a by-product of Gac fruit processing. The increase in growing Gac and the processing of the Gac fruit for its aril, the red-orange covering around the seeds, has lead to thousands of tonnes of Gac seeds being produced or under-utilised in Gac factories each year. They are usually dumped in landfill whole or after being pressed for their oil; this is causing environmental concerns and it is a waste of their other valuable components such as trypsin inhibitors, saponins and phenolic compounds. However, in traditional medicine, Gac seeds are alleged to have a wide array of therapeutic effects for a wide variety of conditions, including fluxes, liver and spleen disorders, hemorrhoids, wounds, bruises, inflammation, swelling and infections. Recently, the bioactive compounds in Gac seeds have been reported to have health benefits such as anti-inflammatory, anti-cancer and antioxidant properties, to name a few. Therefore, the extraction and utilisation of these bioactive compounds may constitute a viable use for Gac seeds while simultaneously reducing the environmental impact of the Gac aril processing. Although several constituents have been identified, which could be involved in the medicinal effects of Gac seeds, studies on how to efficiently extract these various components from Gac seeds are scarce and they are vital for facilitating future applications for these bioactives. The effective extraction of these bioactives is not only important in order to add value to an underestimated resource but also meaningful in the context of the growing interest for natural-based medicines and the development of new markets in the field of nutraceuticals.

The working hypotheses for this thesis were that 1) Gac seeds contain high levels of extractable oil, trypsin inhibitors and saponins and their yield can be optimised using different extraction methods and solvents and 2) the Gac seed extracts are of high quality

and possess biological activity, including antioxidant and anticancer properties. Therefore, in order to test the hypotheses, the main aim was to extract oil, trypsin inhibitors and saponins from Gac seeds with high yields. For the extraction of oil, the supercritical carbon dioxide (SC-CO_2) method was optimised in relation to the extraction time, pressure and flowrate of CO_2 and the oil produced was compared to oil produced using the Soxhlet hexane extraction method. For the extraction of trypsin inhibitors, the conventional solvent extraction method was optimised in relation to the type and concentration of solvent, extraction time and the ratio of powder to solvent and a freeze dried trypsin inhibitor-enriched powder was produced using the optimal extraction conditions. For the extraction of saponins, the microwave assisted extraction method was optimised in relation to the seed material used (defatted powder or full-fat powder), the ethanol concentration, irradiation time, irradiation power and ratio of powder to solvent. The antioxidant activity of extracts were determined using three assays: 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay; 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay; and ferric reducing antioxidant power (FRAP) assay and their anticancer potential was assessed using two melanoma cell lines (MM418C1 and D24).

The results showed that, in comparison to other oilseeds, Gac seed kernels have a very high oil content (53%). However, when extracted with the optimised SC-CO_2 method, the oil could not be considered an edible virgin oil due to its high percentage of unsaponifiable matter (33.2 g/kg). The oil yield was higher with the Soxhlet hexane extraction than with the SC-CO_2 apparatus, but the SC-CO_2 oil had better qualities than the Soxhlet oil, including unsaponifiable matter, peroxide value, free fatty acid value, antioxidant capacity and colour. Therefore, although further refining of both extracted Gac seed oils would be needed to achieve the safety criteria prescribed for edible oils, the SC-CO_2 would require less effort. However, some of the unsaponifiable matter in both

oils may make them useful for medicinal purposes. In this context, the Soxhlet oil may have more potential than the SC-CO_2 oil due to its higher unsaponifiable matter content. However, further composition analysis of the unsaponifiable matter and studies on the biological activities of the oils are needed to confirm the feasibility of their use as a medicinal ingredient.

For the extraction of trypsin inhibitors, the conventional solvent extraction method was more efficient than the microwave-assisted and the ultrasound-assisted methods; and saline was a suitable extraction media. Therefore, optimisation of the trypsin inhibitor extraction was conducted by varying the NaCl concentration, extraction time and ratio of Gac seed kernel powder to solvent. The results revealed that Gac seed trypsin inhibitors were best extracted for 1 h at the ratio of 2 g of Gac seed kernel powder in 30 mL of 0.05M NaCl (1:15 g/mL).

For the extraction of saponins, microwave-assisted extraction proved to be more efficient than ultrasound-assisted and conventional methods and ethanol was the superior solvent. Therefore, the microwave-assisted saponin extraction was optimised for microwave power and irradiation time, concentration of ethanol and ratio of powder to solvent. The optimal parameters for the extraction of saponins were a ratio of 1 g of full-fat Gac seed kernel powder in 30 mL of 100% absolute ethanol with the microwave set at 360 W for three irradiation cycles of 10 s power ON and 15 s power OFF per cycle. The results also showed that a four-fold higher total saponin content (TSC) was obtained in extracts from full-fat Gac seed kernel powder than from defatted powder (100 vs. 26 mg aescin equivalents (AE) per gram of Gac seed kernel powder).

The antioxidant activity analysis of freeze dried powders prepared from defatted Gac seed powder extracted using several solvents indicated that the DI water extract had a high

antioxidant activity (213 µmol TE/g crude extract powder) and that the ABTS antioxidant activity was correlated with the phenolic compound content of the extracts ($r = 0.97$, $p < 0.001$).

The anticancer potential analysis of the freeze dried powders prepared from defatted Gac seed powder extracted using the various solvents indicated that the DI water extract was the most effective; it reduced the viability of the MM418C1 and D24 melanoma cells by 75.5±1.3 and 66.9±2.2%, respectively. Additionally, the anticancer potential against the MM418C1 cells was highly correlated with the trypsin inhibitor activity ($r = 0.92$, $p < 0.05$). However, there was no direct correlation between the antioxidant activity and anticancer potential of the different extracts.

In summary, the aim of the thesis was achieved and the hypotheses were supported. High yields of oil, trypsin inhibitors and saponins were obtained from Gac seed kernel powder using different optimised extraction methods for each of the components. These results confirmed the potential of Gac seeds as an effective source for the recovery of oil and valuable bioactive compounds. Further investigations in terms of the potential applications of the Gac seed extracts in the food, nutraceutical and pharmaceutical industries are warranted.

SYNOPSIS

Gac is a tropical fruit claimed to have many medicinal properties. The aril (the coating on the seeds) is the most used part of the fruit. The seeds are mainly discarded after the aril is separated from the seeds. However, the seeds are relatively rich in oil, which has potential to be used as an edible oil. The seeds have also long been used in traditional remedies and have been reported lately to contain a wide range of bioactive compounds with potential medicinal properties, such as trypsin inhibitors and saponins. Although these components have been identified in the seeds, to date, effective approaches for their recovery from the Gac seeds have not been determined. Therefore, to address this issue, a series of comparative studies of the methods, which could be used for the effective extraction of oil, trypsin inhibitors and saponins from Gac seeds, were conducted in this thesis.

Due to the high oil content of Gac seeds, it is practical to separate the oil before other water-soluble bioactives are extracted. Therefore, in the initial studies (**Papers I and II**), the extraction of the oil and its quality characteristics were investigated in order to determine the conditions for effectively recovering the oil from Gac seed kernels ground into a powder.

The defatted Gac seed kernel powder was then used as the starting material to screen for suitable solvents and methods for the extraction of trypsin inhibitors, saponins and phenolic compounds (**Paper III**). The results from this study indicated that the conventional extraction with DI water was suitable for the extraction of the Gac seed trypsin inhibitors and therefore, in the next study, the aqueous extraction of the trypsin inhibitors was optimised and a trypsin inhibitor-enriched freeze dried powder was produced (**Paper IV**).

In **Paper III**, it was also found that water-saturated butanol and methanol were the best solvents for saponin recovery followed by 70% ethanol in water. Despite the lower effectiveness of the 70% ethanol compared to butanol and methanol, ethanol costs less and is easier to evaporate than butanol and is safer than methanol; therefore, ethanol with MAE was chosen for optimisation of the Gac seed saponin extraction in **Paper VI**. Of note, it was found that the extraction of saponins was better from full-fat (not defatted) Gac seed kernel powder.

However, before doing **Paper VI** on the extraction of saponins, the original assay for measuring saponins was investigated and modified (**Paper V**) in order to eliminate the observed interference of solvents in the assay and therefore, improve the reliability of the TSC determination.

Finally, an examination of the antioxidant activity and anticancer potential of freeze dried powders of extracts prepared with different solvents was performed (**Paper VII**) in order to examine the biological capacities of these Gac seed extracts and to try to match these properties with bioactive compounds contained in the extracts.

Chapter 1: LITERATURE REVIEW

This review covers the literature available on Gac fruits and Gac seeds, their bioactive compounds, biological properties and extraction methods. Note that the referencing system follows the American Psychological Association referencing style (APA) 6th.

1.1. Gac (*Momordica cochichinensis* Spreng)

Gac (*Momordica cochinchinensis* Spreng) is botanically classified as: Family *Cucurbitaceae*, Genus *Momordica*, Species *Cochichinensis*. Gac has an Asian origin (Schaefer et al., 2009) and occurs wild in India, the Philippines, Vietnam, Thailand and is cultivated in Vietnam, Japan and other Asian countries for its fruit, which is thought to be endowed with many medicinal properties (Behera et al., 2011). Besides the name 'Gac' used in Vietnam, the fruit is also known by other common names in English such as Chinese Cucumber, Cochinchin Gourd, Giant Spine Gourd, Spiny Bitter Cucumber, Spiny Bitter Gourd and Sweet Gourd (Lim, 2012).

The plant is a vine, which can be cultivated either from seeds or root tubers (Parks et al., 2013). The plant starts flowering about 2 months after root tubers have been planted. In Vietnam, flowering usually occurs in April and continues to July or August and sometimes until September. On average, it takes about 18-20 days for a fruit to mature from emergence of the bud of the female flower.

As it matures, the green fruit first increases in size (Figure 1A) and then becomes a dark red/orange colour upon ripening (Figure 1B). It is typically round or oblong, maturing to a size of about 13 cm in length and 10 cm in diameter. In some countries, Gac fruit can be used as a vegetable at the immature green stage (Figure 1A) for cooking. However, in Vietnam, fruits weighing 350-600 g or more are usually harvested when fully ripe, when

the fruit is bright red and seeds are hardened, and used for food or medicine (Behera et al., 2011; Lim, 2012). A plant produces 30 to 60 fruits on average in one season (Wimalasiri et al., 2016).

The exterior skin of the fruit is covered with small spines while its interior consists of fleshy yellow pulp and clusters of seeds surrounded by a dark red aril (Figures 1C and D). The biggest anatomical component of a Gac fruit (Figure 2) is yellow pulp (49% by weight), whereas the aril, which contains the highest level of carotenoids, accounts for 18% of the fruit's weight on average (Kha et al., 2010) but it can vary from 10% to 24.6% (Ishida et al., 2004; Nhung et al., 2010). The skin and seeds account for 17% and 16% of the fruit's total weight, respectively (Kha et al., 2013a). These values may vary according to the variety, growth stage and length of storage (Nhung et al., 2010; Tran et al., 2015).

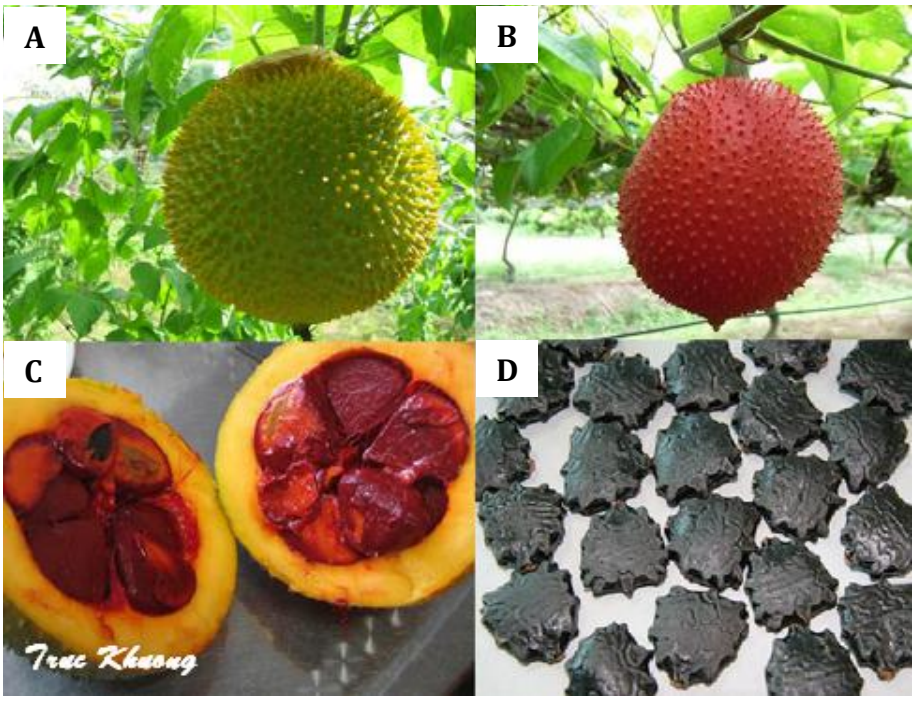


Figure 1: Components of Gac fruit.
(A) Green Gac fruit; (B) Ripe Gac fruit; (C) Yellow pulp and red aril; (D) Gac seeds

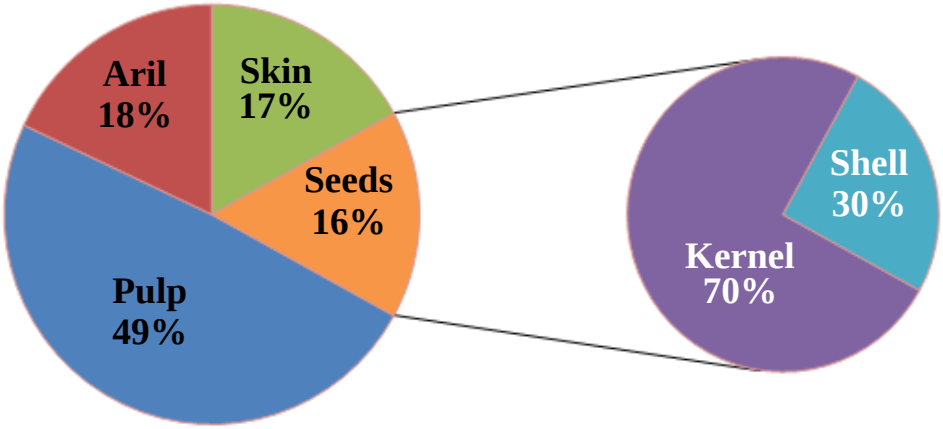


Figure 2: Components of Gac fruit.
(Data from Kha et al. (2013a))

Among the components of the ripe Gac fruit, Gac aril is, up until now, the only component, which has been extensively used commercially and domestically. The aril has been considered as the most precious component of the fruit due to its high content of carotenoids, especially β-carotene and lycopene, and its high content of polyunsaturated fatty acids (Aoki et al., 2002; Ishida et al., 2004). Traditionally, the red aril of the fully ripe Gac fruit is used as a natural colourant for food. In Vietnam, it is most commonly used to prepare a dish called 'xôi gấc' in which the aril of the fruit, with or without its seeds, is cooked in glutinous rice - imparting both its colour and flavour. In Thailand, Gac fruit is used immature as vegetable. The fruit meat is cooked or boiled to consume with chili paste, or used to cook a curry (Kubola & Siriamornpun, 2011). Commercially, Gac aril has recently been marketed in the form of juice, dietary supplements, frozen Gac aril and dried Gac powder (Chuyen et al., 2014).

During the last few years, many studies have been carried out in order to enhance the usage of Gac aril in the food industry. In this attempt, Gac aril has been processed into forms that are easier to disperse in various food systems (Kha et al., 2010; Kha et al., 2014b). Emphasis has also been put on pre-treatment and extraction techniques that help

increase the yield of the oil and bioactives extracted from the Gac aril and on encapsulation techniques that help prolong the storage time of the extract (Kha et al., 2013b, 2014a; Kha et al., 2014c).

As for the pulp and skin of the Gac fruit, some studies have reported that they contain a significant content of carotenoids, especially lutein (Ishida et al., 2004; Kubola & Siriamornpun, 2011) and therefore, they are promising sources for the extraction of this carotenoid (Chuyen et al., 2017).

1.2. Gac seeds

Gac seeds are the dried ripe seed of the Gac fruit. In Gac fruit, the seeds are surrounded by the aril. In traditional medicinal usage, the seeds are used fully ripened (Figure 1D); they are large, compressed and ovoid with a wavy outline, are blackish-brown and have an irregularly sculptured seed coat (Lim, 2012).

Each fruit has on average between 15 to 20 round, compressed and sculptured seeds. The seeds, which represent about 16% of the total weight (Figure 2) of the fruit (Ishida et al., 2004), are one of the by-products when the red pulp (aril) around them is recovered. According to preliminary analyses, the kernel of the Gac seeds accounts for about 80% of the weight in comparison to the whole seed.

As for the seeds of the Gac fruit, it is estimated that the amount of Gac seeds produced in Vietnam is around 760 tons per year; this is based on 16% of the estimated annual yield of Gac fruit of around 4,750 tons being seeds (Figure 2). These seeds are often discarded in Vietnam (Chuyen et al., 2014), although there are some factories that extract oil by pressing. This oil is used for massaging and for treatment of some skin diseases according

to traditional uses. However, the resulting residue, including the hydrophilic components of the seeds, is discarded.

Gac seeds contain a significant amount of oil, which is comparable to oil-rich sources such as sesame seeds and peanuts (Wang et al., 2010; Yermanos et al., 1972), with one report showing that fresh Gac seeds contain up to 60% oil (Ishida et al., 2004) with the long chain saturated fatty acid, stearic acid (60%) and the essential ω-6 polyunsaturated fatty acid, linoleic acid (20%) being the major components (Ishida et al., 2004).

This fatty acid composition provides Gac seed oil with the potential to be utilised in the confectionary industry and also as a frying oil, where the oil needs to have a high melting point in order to minimise the melting of confectionary products and to increase the stability of oil at high frying temperatures. Hydrogenated oils are often used for confectionaries and frying. However, hydrogenated oils are a primary source of trans fatty acids, which increase blood cholesterol levels and therefore, the risk of coronary heart disease (Ascherio et al., 1999). In contrast, the major Gac oil fatty acids, stearic acid (Grundy, 1994; Hunter et al., 2010) and linoleic acid (Farvid et al., 2014; Rassias et al., 1991) have no effect or lower blood cholesterol, respectively. Therefore, Gac seed oil has the potential of being a good replacement for hydrogenated oils in these areas of the food industry.

The hydrophilic components of the Gac seeds include a group of trypsin inhibitors, many of which are cyclotides. Unlike those from other plants, the trypsin inhibitors in Gac seeds are low molecular weight compounds with compact conformations. These features make it easier for them to penetrate into tumour cells. Many studies have confirmed this anticancer activity of trypsin inhibitors from Gac seeds (Zhao et al., 2010a; Zheng et al., 2013; Zhizhong, 2007).

Cyclotides are bioactive mini-proteins from plants that have the unique topological feature of a head-to-tail cyclic backbone combined with a cystine knot. Because of this structure they are ultra-stable and have attracted interest as peptide-based templates for drug design applications. They have a range of pharmaceutically relevant activities, including anti-HIV, antimicrobial and uterotonic activity. Their exceptional stability lends them to uses as pharmaceutical templates into which bioactive peptide sequences could be grafted (Smith et al., 2011).

In addition to trypsin inhibitors and cyclotides, Gac seeds are also a source of saponins, phenolics, flavonoids and tocopherols at significant levels (Lin et al., 2012; Matthaus et al., 2003). There have been many studies confirming biological activities of Gac seeds relating to these components (Jung et al., 2013a; Jung et al., 2013b). Due to containing a range of these bioactives, Gac seeds have long been used in traditional medicine in many Asian countries where the Gac vine is grown, particularly in China.

According to the usual method of exploitation, the hydrophilic components such as trypsin inhibitors, phenolics, flavonoids and some types of saponins are still present in the discarded Gac seed residue. This leads to the wastage of potentially valuable bioactive compounds. The high content of these bioactives in the residue also decreases the possibility of utilising the Gac waste as animal feed because these bioactives can be toxic to animals in high amounts (Tsoi et al., 2005). Some of these bioactives can also cause environmental damage to land and waterways if waste containing them is dumped without prior treatment and for the same reason the material cannot be used as fertiliser (Capasso et al., 1992).

If the Gac seeds can be used as a resource for the extraction of bioactive compounds and oil, it will definitely add value to the Gac fruit industry via the production of new saleable

bioactive and oil products whilst at the same time lessening the environmental problems caused by the industry's waste. Furthermore, once the bioactive compounds are removed, the 'cleaner' waste material may even become a resource if it can be used by the fertiliser and animal feed industries. Therefore, the results from this project may benefit not only Vietnam but also other countries who have or are developing a Gac fruit industry, including Australia.

1.2.1. Oil and fatty acids in Gac seeds

Several studies have demonstrated that the kernel of Gac seeds contains a high proportion of oil that is composed of several types of fatty acids. The reported oil concentration in Gac seeds varies according to the different studies due to the differences in seed varieties, growing conditions, sample preparations, extraction techniques and measurement methods. However, in general, the oil content of Gac seeds is comparable with other oil-rich seeds like peanuts, sunflower, sesame. Table 1 shows the oil content and fatty acid composition of Gac seeds reported by the various studies.

Stearic acid was identified as principle among the fatty acids represented in Gac seeds. Unlike the seeds of other tropical plants, which predominantly have palmitic acid, Gac seeds are somewhat unusual in having a higher level of stearic acid. Increasing the stearic acid content of an oil relative to palmitic acid generally raises its melting point (62.9 °C for palmitic acid to 69.3 °C for stearic acid). This characteristic can contribute to producing a solid and oxidatively stable fat for shortenings and margarines used for frying. Such a fat could reduce the need for the partial hydrogenation of liquid oils, a process which is commonly used to obtain solid fats but which has the effect of producing trans fatty acids that have unfavourable health effects (Ishida et al., 2004). The oil of Gac

seeds has also been reported to have a reasonable amount of Vitamin E (274 mg/kg oil) and the omega-3 linolenic acid (Matthaus et al., 2003).

Some unsaponifiable compounds have also been reported to present in Gac seed oil, including karounidiol, β-sitosterol, pentacyclic triterpene and their derivatives (Kan et al., 2006). Another study by Akihisa et al. (1986) showed that there was 218 mg of sterol in 100 g of dried Gac seeds, and most of these were Δ7-sterols.

Table 1. Oil content and composition of main fatty acids (as % of total fatty acid) in Gac seeds.

% Oil (w/w)	% Palmitic (16:0)	% Stearic (18:0)	% Oleic (18:1Δ^{9})	% Linoleic (18:2$\Delta^{9,12}$)	References
35	4.8	51.7	17.4	12.6	(Huijuan et al., 2000)
53	2.1	18.0	7.9	10.5	(Matthaus et al., 2003)
15.7-36.6	5.0-6.0	60.5	9.0	20.0	(Ishida et al., 2004)
-	8.4	33.5	21.6	19.9	(Ding et al., 2005)

Gac seeds are not only rich in oil but are also a rich source of phytochemicals, with high levels of trypsin inhibitors, saponins, phenolics and flavonoids. Although these compounds were traditionally considered to be antinutritional and/or toxic factors (Anderson & Wolf, 1995; George, 1965), later research has indicated that these compounds may significantly contribute to medicinal and antioxidant activities and therefore, may be related to the health benefits attributed to these seeds. However, research has yet to focus on the effect of growing, storage and processing conditions on the quantity and quality of these phytochemical compounds in Gac seeds and techniques and varieties are yet to be developed to protect and/or enhance their content and quality of desired bioactives.

1.2.2. Bioactive compounds in Gac Seeds

1.2.2.1. Trypsin inhibitors

Trypsin inhibitors (TIs) are low molecular weight peptides, which can inhibit the hydrolase activity of many kinds of serine proteases. They are commonly found in the storage organs of plants, such as seeds, roots and tubers. Three major sub-types of TIs have been reported and identified in plants: the Bowman-Birk-type inhibitors, the Kunitz-type inhibitors and the squash family inhibitors. Their molecular weights are about 7500, 20000 and 3500 kDa, respectively. The first two types were isolated from leguminous plants while the third one was obtained from Cucurbitaceous species (Wong et al., 2004). Table 2 shows the different types of protease inhibitors that have been identified in plants (De Leo et al., 2002) including the squash inhibitor family, which is the latest established family.

Table 2. Families of protease inhibitors in plant tissues.
(Source: *De Leo et al. (2002)*)

1	Bowman-Birk serine proteinase inhibitors
2	Cereal trypsin/α-amylase inhibitors
3	Cystein proteinase inhibitors
4	Metallocarboxypeptidase inhibitors
5	Mustard trypsin inhibitors
6	Potato type I inhibitors
7	Potato type II proteinase inhibitors
8	Serpin
9	Soybean trypsin inhibitors (Kurnitz)
10	Squash inhibitors

The anticancer activity of protease inhibitors was first reported by Troll and Kennedy (1993) in relation to trypsin inhibitors suppressing two-stage carcinogenesis and breast cancer. Since then, extensive investigations on trypsin inhibitors as cancer chemo-

preventive agents have been conducted. Several TIs from *Momordica cochinchinensis* (MCoTIs) have been characterised (Chan et al., 2009; Felizmenio-Quimio et al., 2001; Hernandez et al., 2000; Wong et al., 2004) and proposed to be among the most important bioactives in Gac seeds. They are classified as protease inhibitors and belong to the squash trypsin inhibitor family (Laskowski Jr & Kato, 1980). They serve as storage proteins and may also be involved in the regulation of endogenous proteases during seed dormancy (Birk, 1996). Nine MCoTIs have been isolated and sequenced from the seeds of the Gac fruit (Hernandez et al., 2000; Huang et al., 1999; Wong et al., 2004) (Table 3). Structurally, MCoTIs consist of 28 – 34 amino acid residues, 6 of which are cysteine residues that form 3 disulfide bonds. The Gac seed TIs have a very small molecular weight of 3-5 kDa and in comparison to other TI families, they are more compact in structure and exceptionally stable (Cascales et al., 2011; Contreras et al., 2011; Greenwood et al., 2007). Among these, MCoTI-I and MCoTI-II are cyclic peptides (Figure 3) and as such, they have a very compact and stable structure (Hernandez et al., 2000; Mahatmanto et al., 2014). This enables them to penetrate into cells and therefore, they are attractive candidates for use as scaffolds for the development of novel intracellularly-targeted drugs (Craik et al., 2012; Wang et al., 2013). Moreover, the activity of Gac seed TIs is very high, at least 50-fold more potent than those from different *Cucurbitaceous* seeds (Huang et al., 1999).

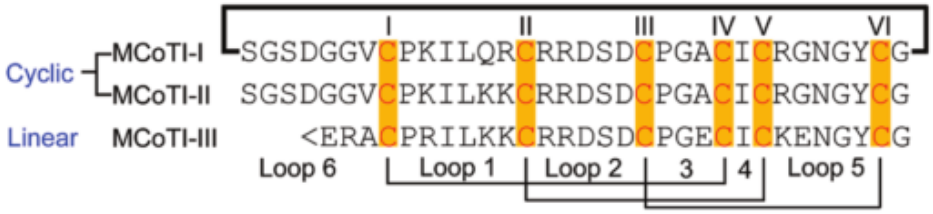


Figure 3: Amino acid sequences of cyclic peptides MCoTI-I and MCoTI-II, and acyclic peptide MCoTI-III.

Phytochemical activities of protease inhibitors have been investigated by several studies. They have been shown to prevent carcinogenesis in a wide variety of *in vitro* and *in vivo* model systems (Birk, 2003; Hocman, 1992; Kennedy, 1998a; Troll & Kennedy, 1993) due to their ability to irreversibly suppress the carcinogenic process (Kennedy, 1998b). Due to their clinical potential, Gac seed TIs could be used in a variety of applications in medicine and agriculture. However, studies on the efficient extraction of MCoTIs are very scare.

The structure and phytochemical activity of MCoTIs have been investigated by several studies. They have been shown to prevent carcinogenesis in a wide variety of *in vitro* and *in vivo* model systems (Birk, 2003; Kennedy, 1998b), and thus may have clinical potential.

Table 3. Mini peptides in Gac seeds

No	Peptide	Backbone	Enzyme inhibitor	Number of amino acid residues	Molecular weight (Da)	Medicinal activity	Reference
1	MCCTI-1	Open chain	Trypsin inhibitor	NA	3479	NA	(Huang et al., 1999)
2	MCoTI-I	Cyclotide	Trypsin inhibitor	34	3453.0	NA	(Hernandez et al., 2000)
3	MCoTI-II	Cyclotide	Trypsin inhibitor	34	3480.7	NA	(Hernandez et al., 2000)
4	MCoTI-III	Open chain	Trypsin inhibitor	30	3379.6	NA	(Hernandez et al., 2000)
5	MCoTI-IV	NA	Trypsin inhibitor	NA	5100	NA	(Wong et al., 2004)
6	MCoTI-V	NA	Trypsin inhibitor	NA	4800	NA	(Wong et al., 2004)
7	MCoTI-VI	NA	Trypsin inhibitor	NA	4400	NA	(Wong et al., 2004)
8	MCoTI-VII	NA	Trypsin inhibitor	NA	4100	NA	(Wong et al., 2004)
9	MCoTI-VIII	NA	Trypsin inhibitor	NA	3900	NA	(Wong et al., 2004)
10	MCoCI	NA	Chymotrypsin inhibitor	23	7514	Immuno-enhancing and anti-inflammatory	(Tsoi et al., 2004)
11	MCoCC-1	Cyclotide	Not trypsin inhibitor	33	3286.5	toxic against three cancer cell lines	(Chan et al., 2009)
12	MCoCC-2	NA	NA	32	3168.9	Toxic against three cancer cell lines	(Chan et al., 2009)
13	MCo-3	Cyclotide	Trypsin inhibitor, not potent	N/A	N/A	Not to have medicinal activities	(Chan et al., 2013)
14	MCo-4	Cyclotide	Trypsin inhibitor, not potent	N/A	N/A	Not to have medicinal activities	(Chan et al., 2013)
15	MCo-5	Cyclotide	Trypsin inhibitor, not potent	N/A	N/A	Not to have medicinal activities	(Chan et al., 2013)
16	MCo-6	Cyclotide	Trypsin inhibitor, not potent	N/A	N/A	Not to have medicinal activities	(Chan et al., 2013)

1.2.2.2. Saponins

Saponins are complex compounds consisting of nonpolar triterpenoid aglycones linked to one or more polar oligosaccharides (Wei, 2011), which results in molecules with

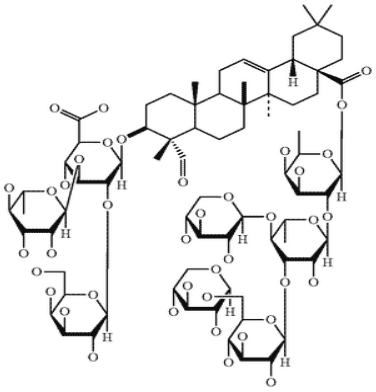
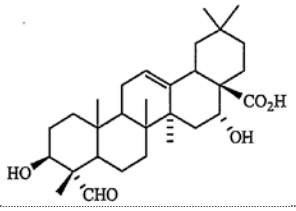
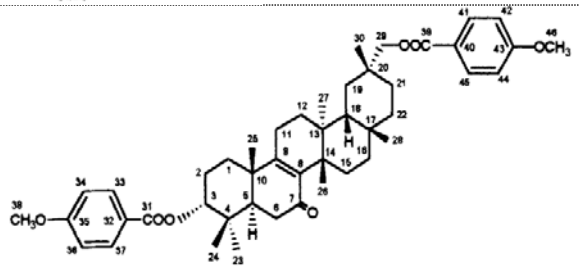
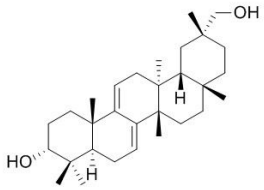
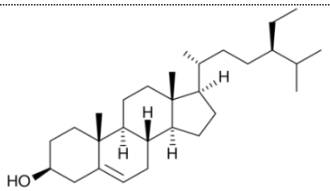
amphiphilic properties similar to detergents. Although saponins have antibiotic activity and are toxic to fish and insects, they appear to be practically non-toxic to man, remaining within the gastrointestinal tract. Dietary saponins, either isolated or as saponin-containing food plants, lower plasma cholesterol levels in several mammalian species. They are therefore potentially important in human diets to reduce the risk of coronary heart disease (Oakenfull, 1981). Recently, they have also been recognised to be outstanding candidates in the development of new adjuvants for new generation vaccines (Song & Hu, 2009). Many saponins are biologically active compounds of medicinal plants and play a variety of physiological functions. In a review by Rao and Gurfinkel (2000), the biological activities of saponins included anti-tumour, immunomodulatory, anti-inflammatory, cholesterol-lowering, anti-hepatotoxic, hypoglycemic, antimicrobial and anti-cardiovascular disease activity.

Many studies have reported the presence of several saponins in Gac seeds (Gao, 2005; Lin et al., 2012), of which *momordica* saponin I and II were found to be the major active components (Jung et al., 2013a; Jung et al., 2013b; Kubota et al., 1971). Recently, *M. cochinchinensis* was used in a wound-healing agent (Kim et al., 2010) and in an anti-gastritis and anti-ulcer agent (Kim et al., 2012) and as the critical ingredient in the recipe, *momordica* saponin I was the major bioactive component in it. A list of saponins that have been found in Gac seed is shown in Table 4.

Other researchers have revealed that *M. cochinchinensis* saponins have antitumour activity (Tien et al., 2005). The *M. cochinchinensis* saponins are oleanane type triterpene saponins, which have two saccharic chains. Masayo et al. (1985) was the first to isolate three saponins from the seeds and roots of *M. cochinchinensis,* which were named as momordicasaimin I, momordicasaimin II and momordicasaimin III. However, although saponins preparations can be readily isolated from plant materials by solvent extraction,

it has proven very difficult to isolate individual saponins from the crude extracted mixtures and this has rarely been achieved. Therefore, there is still limited information on the structure of the individual saponins in Gac seeds.

Table 4. Saponins in Gac seeds.

Name	Chemical Structure and Formula		Reference
Momordica Saponin I (Gypsoside)		3-O-β-D-galactopyranosyl(1->2)-[α-L-rhamnopyranosyl(1->3)]-β-D-glucuronopyranosido-28-O-β-D-xylopyranosyl(1->3)-β-D-glucopyranosyl(1->3)-[β-D-xylopyranosyl(1->4)]-α-L-rhamnopyranosyl(1->2)-β-D-fucopyranosylgypsogenin	(Kim et al., 2012)
Momordica Saponin II (Quillaic acid)		$C_{30}H_{46}O_5$	(Lin et al., 2012)
Multiflorane Triterpenoid Ester	 3,29-di-*O*-(*p*-methoxy)benzoylmultiflora-8-ene-3*α*,29-diol-7-one		(Shan et al., 2001)
Stigmast-7-en-3β-ol Multiflorane Triterpenoid Ester	NA		(Jung et al., 2013b)
Karounidiol		$C_{30}H_{48}O_2$	(Jung et al., 2013b)
β-sitosterol		$C_{29}H_{50}O$	(Jung et al., 2013b)
Gypsogenin 3-O-β-D-galactopyranosyl(1→2)-[αL-rhamnopyranosyl(1→3)]-β-D-glucuronopyranoside			(Jung et al., 2013b)

1.2.2.3. Phenolic compounds

Phenolic compounds are common dietary phytochemicals found in fruits, vegetables and grains. Epidemiological evidence has suggested that food phenolics may have protective effects against degenerative diseases (Fukumoto & Mazza, 2000). Several beneficial effects derived from phenolic compounds are mainly due to their antioxidant activity (Tagliazucchi et al., 2010; Viswanath et al., 2009). Health benefits linked to polyphenols and their application have been already well discussed in several earlier reviews (Del Rio et al., 2010; Terao, 2008). According to Chung and Champagne (2008); Singh et al. (2010) and Viswanath et al. (2009), phenolic compounds exhibit a wide range of beneficial properties for health, such as: anti-allergenic, anti-inflammatory, anti-microbial, anti-oxidant, antithrombotic, cardioprotective, and vasodilatory effects.

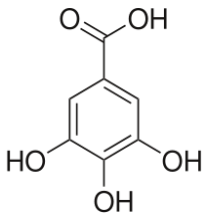
The antioxidant properties of polyphenolics are mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Rice-Evans et al., 1996). Some also act as chelators of metal divalent cations, preventing metal ion-catalysed formation of free radical species (Salah et al., 1995). Essentially, phenolic antioxidants interfere with the oxidation of lipid and other molecules by the rapid donation of a hydrogen atom to free radicals, which stabilises them.

Structurally, phenolic compounds are comprised of an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds (Bravo, 1998). Most naturally occurring polyphenolic compounds are present as conjugates with mono or polysaccharides linked to one or more of the phenolic groups but they also may occur as functional derivatives, such as esters and methyl esters (Harborne & Dictionary, 1999; Shahidi & Naczk, 1995). Phenolic compounds can be classified into several classes as shown in Table 5.

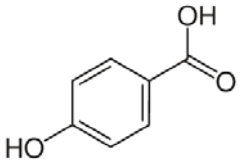
Table 5. Classes of phenolic compounds from natural sources.
(Source: Balasundram et al. (2006)).

Class	Structure
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	$C_6 – C_1$
Hydroxycinnamic acids, phenylpropanoids	$C_6 – C_3$
Naphthoquinones	$C_6 – C_4$
Xanthones	$C_6 – C_1 – C_6$
Flavonoids, isoflavanoid	$C_6 – C_3 – C_6$
Lignans, neolignans	$(C_6 – C_3)_2$
Bioflavonoid	$(C_6 – C_3 – C_6)_2$
Lignin	$(C_6 – C_3)_n$
Condensed tannins	$(C_6 – C_3 – C_6)_n$

Gallic acid and p-hydroxybenzoic acid are two phenolics (Figure 4) that have been found in Gac seeds (Kubola & Siriamornpun, 2011). Gallic acid is a trihydroxybenzoic acid, a type of organic acid, also known as 3,4,5-trihydroxybenzoic acid (Chevallier, 1996). Gallic acid has been reported to possess anti-inflammatory activity (Kroes et al., 1992) and both antioxidant and pro-oxidant actions (Aruoma et al., 1993). Therefore, this compound can be considered for extraction if it is present in Gac seeds at a reasonable level. As for p-hydroxybenzoic acid, there is little literature on its bioactive activities.



Gallic acid



p-hydroxybenzoic acid

Figure 4: Chemical structure of phenolic compounds in Gac seeds.

1.2.2.4. Flavonoids

Flavonoids are polyphenolic compounds that are also widely distributed in fruits, vegetables, teas and medicinal plants and most commonly known for their antioxidant

activity. The basic structures of flavonoid molecules are composed of three rings with various substitutions, including glycosylation, hydrogenation, hydroxylation, malonylation, methylation and sulfation (Beecher, 2003; Onyilagha et al., 2004). Flavonoids are divided into classes according to their substitution and oxidation level on the middle ring. The main subclasses and their respective food sources are anthocyanidins (red, purple and blue berries), flavanols (teas, red grapes and red wines), flavones (green leafy species), flavonols (ubiquitous in foods), flavanones (citrus), and isoflavones (soybeans). In nature, they are present principally as glycosylated, esterified and polymerized derivatives. Sugar moieties attached to flavonoids increase the polarity of the molecules for their storage in plant cell vacuoles (Beecher, 2003; Onyilagha et al., 2004).

For humans, several beneficial health properties of dietary flavonoids have been recognised and linked to their antioxidant (Rice-Evans et al., 1996) and anti-proliferative effects; they may protect the body from various diseases, such as cancers, cardiovascular disease and inflammatory diseases (Middleton et al., 2000; Nijveldt et al., 2001).

Myricetin is a member of the flavonoid class of polyphenolic compounds, which has been found in Gac seeds (Kubola & Siriamornpun, 2011). Its chemical structure is shown in Figure 5.

The antioxidant and pro-oxidant actions of myricetin have been highlighted (Laughton et al., 1989; Ong & Khoo, 1997). However, the concentration of this component in Gac seeds has not been reported. Therefore, more research needs to be carried out in order to evaluate the potential of extracting this bioactive compound from the seeds.

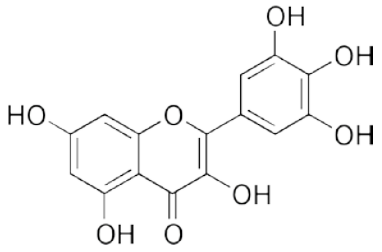


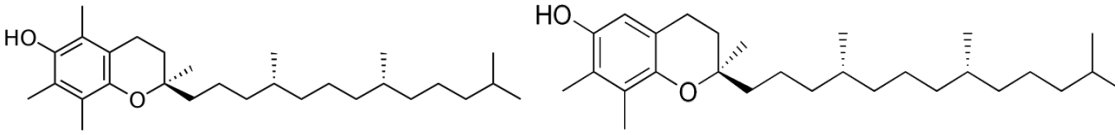
Figure 5: Chemical structure of myricetin.

The characterisation of the phenolic and flavonoid content and the antioxidant activity of the Gac seeds may give more insight into their functionality. Whereas, bitter gourd (*Momordica charantia*), a close relative of Gac fruit, has been assayed for antioxidant activity by many researchers (Benitez & De Guzman, 2012; Kubola & Siriamornpun, 2008; Semiz & Sen, 2007; Tan et al., 2014a; Wu & Ng, 2008), this has not been documented for Gac seeds. Therefore, the quantification of phenolic and flavonoid compounds in Gac seeds and their antioxidant capacity needs to be done.

1.2.2.5. Tocopherols

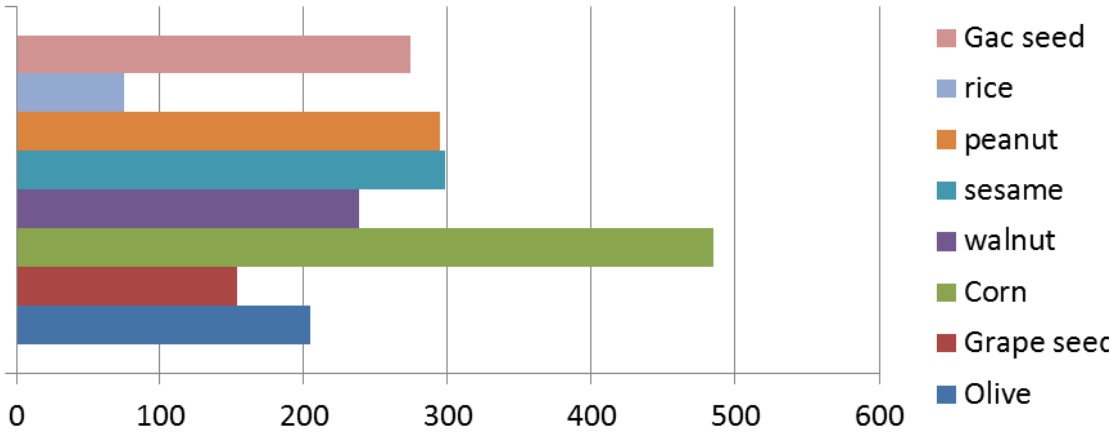
Extraction of tocopherols from natural sources has received increasing interest due to the high antioxidant activity associated with this family of compounds. Besides its well-known antioxidant activity, studies have demonstrated that synthetic vitamin E is less effective than natural vitamin E (Hadolin et al., 2001). Vitamin E has been extracted from several natural sources using supercritical fluid extraction with carbon dioxide (SC-CO_2) (Ge et al., 2002; Gelmez et al., 2009; Nyam et al., 2010).

Matthaus et al. (2003) found that the total tocopherol content in Gac seeds is 274 mg/kg oil with α-tocopherol and γ-tocopherol (Figure 6) accounting for 176 mg/kg oil (64%) and 93 mg/kg oil (34%), respectively; this is comparable to tocopherol-rich oils such as soybean, sunflower, corn, and olive oils. A comparison of the tocopherol content among different tocopherol-rich oils is shown in Figure 7.



α-tocopherol ***γ-tocopherol***

Figure 6: Chemical structures of the tocopherols in Gac seeds.



mg tocopherols/kg oil

Figure 7: Total tocopherol content in different oils.
(Data from Matthaus et al. (2003)).

In conclusion, a wide range of phytochemical components have been found in Gac seeds. Of these, trypsin inhibitors and saponins are the most distinguishing components and their phytochemical activities have been reported in detail. However, optimising the extraction of these components is still necessary in order to take full advantage of an underestimated resource.

To facilitate the extraction of these bioactive compounds, knowledge about the macro components (proximate) of Gac seeds is also necessary. However, the proximate composition of Gac seeds has not been reported in the available references. Therefore, it is necessary to carry out a comprehensive analysis of the macro components in the seeds: total carbohydrate, protein, fat and moisture content. Knowledge about these components

in the seeds will play an important role in the selection of the extraction and purification methods to be used for the analysis of the bioactive phytochemicals.

1.2.3. Biological properties of bioactive compounds in Gac seeds

Many bioactives have been reported to be present in Gac seeds at significant levels, including trypsin inhibitors (Chan et al., 2013), saponins (Lin et al., 2012), phenolics, flavonoids (Bharathi et al., 2014) and tocopherols (Matthaus et al., 2003). These bioactives are considered to contribute to the medicinal properties of Gac seeds (Lim, 2012). In addition, the seeds also contain high levels of fatty acids (Huijuan et al., 2000; Ishida et al., 2004; Matthaus et al., 2003) and therefore, they might potentially be a new source of edible oil.

As presented in Section 1.2.2, Gac seeds contain a variety of bioactive components. It is important, therefore, to review the biological activities of these constituents.

1.2.3.1. Anticancer and antiviral activities

Cancer is one of the most severe health problems worldwide, in both developing and developed countries. An estimated 12.7 million people were diagnosed with cancer across the world in 2008 and 7.6 million people died from cancer during the same year (WHO, 2011). Conventional treatment of cancer includes interventions such as surgery, radiotherapy, chemotherapy and psychosocial support (WHO, 2010). However, due to the increasing rate of mortality associated with cancer and adverse or toxic side effects of cancer chemotherapy and radiation therapy, discovery of new anticancer agents derived from nature, especially plants, is currently under intensive investigation. Cultured cancer-derived cell lines with comparison to normal healthy cell lines are commonly used to

assess the anticancer properties of isolated phytochemicals and extracts of medicinal plants.

In recent years, there have been many studies demonstrating that Gac seeds contain many anticancer phytochemicals. These constituents have inhibitory effects against breast cancer, lung cancer and renal cancer (Choi et al., 2012; Kang et al., 2010; Liu et al., 2012; Meng et al., 2012; NewsBank, 2012; Rajput et al., 2010; Sakwiwatkul et al., 2010; Zhao et al., 2012; Zheng et al., 2013).

Chuethong et al. (2007) found that Cochinin B, a novel ribosome-inactivating protein (RIP) purified from the seeds of *Momordica cochinchinensis*, displayed a strong inhibitory activity on protein synthesis in the cell-free rabbit reticulocyte lysate system with an IC50 of 0.36nM. Furthermore, it exhibited N-glycosidase activity and cytotoxicity against the Vero cell line with an IC50 of 1.54μM. Also, Cochinin B manifested strong anti-cancer activities against human cervical epithelial carcinoma (HeLa), human embryonic kidney cancer (HEK293) and human small cell lung cancer (NCI-H187) cell lines with IC50s of 16.9, 114 and 574nM, respectively.

Zhao et al. (2010a) showed that an ethanol extract of *M. cochinchinensis* seeds significantly inhibited the proliferation of A549, MDA-MB-231, TE-13 and B16 tumour cell lines in a dose-dependent manner by arresting the cell cycle and inducing apoptosis of the cancer cells. The ethanol extract of the *M. cochinchinensis* seeds (10–100 mg/l) also strongly and dose-dependently inhibited the proliferation of the melanoma B16 cells through the induction of differentiation and the promotion of apoptosis of the B16 cells (Zhao et al., 2010b).

Another Gac seed compound, karounidiol (2mg/mouse), markedly suppressed the promoting effect of TPA (12-O-tetradecanoylphorbol-13-acetate) (1mg/mouse) on skin

tumour formation in mice following initiation with 7,12-dimethylbenz[α]anthracene (50mg/mouse) (Yasukawa et al., 1994). Karounidiol and other Gac seed compounds, isokarounidiol, 5-dehydrokarounidiol and 7-oxodihydrokarounidiol, also showed an inhibitory effect against EBV-EA activation by TPA (Akihisa et al., 2001). In this study, karounidiol exhibited inhibitory activity against several human cancer cell lines and it demonstrated cytotoxicity especially against a human renal cancer cell line.

Lai et al. (2009) isolated and characterised, from *M. cochinchinensis* seeds, two novel peptides, MCoCC-1 and MCoCC-2 containing 33 and 32 amino acids, respectively. These two compounds were toxic against three cancer cell lines; of the cell lines tested, MCoCC-1 was the most toxic against a human melanoma cell line and was non-hemolytic to human erythrocytes.

1.2.3.2. Immuno-enhancing anti-inflamatory activity

A report by Jung et al. (2013b) showed that a triterpenoidal glycoside in Gac seeds had anti-inflammatory properties in the LPS-stimulated macrophage assay. A chymotrypsin-specific potato type I inhibitor from *Momordica cochinchinensis* (MCoCI) was also shown to possess immuno-enhancing and anti-inflammatory effects (Tsoi et al., 2006). The MCoCI stimulated the proliferation of different cells of the immune system, including splenocytes, splenic lymphocytes and bone marrow cells, in a manner comparable to that of Concanavalin A. Furthermore, MCoCI suppressed the formation of hydrogen peroxide in neutrophils and macrophages. Karounidiol and 7-oxodihydrokarounidiol from *M. cochinchinensis* seed oil inhibited the inflammatory activity induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) and the 50% inhibitory doses for TPA-induced inflammation were 0.3 and 0.4 mg/ear, respectively (Yasukawa et al., 1994).

Other studies have shown that ovalbumin (OVA, 10µg) co-administered in Balb/c mice with an extract from *Momordica cochinchinensis* seeds (ECMS), induced a significantly higher specific antibody production than OVA used alone (Xiao et al., 2007a; Xiao et al., 2007b). Analysis of antibody isotypes indicated that the ECMS could promote the production of both IgG1 and IgG2a, but favoured the IgG2a isotype. Splenocyte proliferative responses to concanavalin A, lipopolysaccharides or OVA were also significantly higher in mice immunised with OVA mixed with ECMS than with OVA alone or mixed with aluminum hydroxide. No local reactions and negative effects such as body weight gain occurred after the injection of OVA mixed with various amounts of ECMS in mice. The ECMS was found to be safe for injection and could be used as a potential vaccine adjuvant biasing the production of IgG2a in mice.

1.2.3.3. Antioxidant activity

Antioxidants present in fruits and vegetables prevent or inhibit the damage and tissue deterioration caused by oxidation. Dietary antioxidant capacity is related mainly to the total polyphenol content of fruits and vegetables. Consistent with this, antioxidant activity in extracts from Gac seeds has been shown to increase from the mature stage to the ripe stage as the content of the total phenolic and total flavonoid compounds in the seeds increased during the fruit development stages (Kubola & Siriamornpun, 2011).

Some studies have demonstrated that the phytochemicals in Gac seeds could also stimulate the enzymatic antioxidant system in cells. In a study conducted by Tsoi et al. (2005), pre-treatment of rat hepatocytes with chymotrypsin-specific potato type I inhibitor from *Momordica cochichinensis* (MCoCI) for 24 h significantly reversed tert-butyl hydroperoxide-induced cell damage, and the associated glutathione depletion and

lipid peroxidation. The activities of glutathione-S-transferase and superoxide dismutase were also increased.

1.2.3.4. Gastroprotective and antiulcerogenic activities

Kang et al. (2009) have reported that SK-MS10, an extract from Gac seeds, exhibited gastroprotective effects against acute gastric mucosal damage by suppressing pro-inflammatory cytokines, down-regulating cytosolic phospholipase A2 (cPLA2) and 5-lipoxygenase (5-LOX) and enhancing the synthesis of mucus in an acute gastric mucosal damage model in rats. It was also demonstrated that the calcitonin gene-related peptide (CGRP)-nitricoxide (NO) pathway played an important role in the gastroprotective effects of SK-MS10.

In a further study, Kang et al. (2010) reported that treatment with SK-MS10 for 7 and 14 days significantly accelerated ulcer healing and increased the expression of mRNA (at day 7) as well as vascular endothelial growth factor (VEGF) protein (at day 14) compared to the vehicle-treated rats. The microvasculature density for factor VIII was also higher in the SK-MS10 treatment group compared to the vehicle-treated rats. The results suggested that SK-MS10 treatment accelerated the healing of gastric ulcers via upregulation of VEGF and angiogenesis in the acetic acid rat model that was used.

Another study by Jung et al. (2013a) confirmed that a Gac seed extract had anti-gastritis effects in various rodent models. The study found that the extract from Gac seeds provided remarkable protective effects, comparable to those of rebamipide, a gastroprotective drug, in ethanol- and diclofenac-induced acute gastritis. In addition, it demonstrated protective effects in a *Helicobacter pylori*-insulted chronic gastritis model. The extract also showed a wound healing effect on cutaneous injuries in mice: it stimulated the calcitonin gene-related peptide and somatostatin receptors, which may be

related to its anti-gastritis effects. Moreover, momordica saponin I, a major ingredient of the Gac seed extract, decreased gastric mucosal damage indices in the ethanol- and diclofenac-induced acute gastritis models. The results suggested that Gac seed extract could be a promising gastroprotective herbal medicine and momordica saponin I might be the active marker compound in the extract.

1.2.3.5. Ribosome inactivating protein activity

A ribosome-inactivating protein, momorcochin-S, has also been purified from the seeds of *Momordica cochinchinensis* (Bolognesi et al., 1989). This protein could be considered as an isoform of the previously purified momorcochin from the roots of *M. cochinchinensis*. Momorcochin-S inhibited protein synthesis by a rabbit-reticulocyte lysate, phenylalanine polymerisation by isolated ribosomes and altered rRNA in a similar manner to the A-chain of ricin and related toxins (Endo et al., 1987). Momorcochin-S was linked to a monoclonal antibody (8A) against human plasma cells and the resulting immunotoxin was selectively toxic to target cells.

1.2.3.6. Trypsin inhibitory activity

A trypsin inhibitor, MCCTI-1, with a molecular weight of 3,479 Da, was isolated from *Momordica cochinchinensis* seeds (Huang et al., 1999). Three other trypsin inhibitors, MCoTI-I, MCoTI-II and MCoTI-III were isolated, purified and characterised from the seeds of *Momordica cochinchinensis* (Hernandez et al., 2000). In the case of MCoTI-I and -II, it was shown that their polypeptide backbones were cyclic. They were found to contain 34 amino acid residues with 3 disulfide bridges and measured molecular masses of 3,453.0 and 3,480.7 Da, respectively. They represented the largest known macrocyclic peptides containing disulfide bridges and their sequences showed strong homology to other squash trypsin inhibitors.

The MCoTI-III was found as a minor species containing 30 amino acid residues with a molecular mass of 3,379.6 Da. This component was found to possess a linear backbone with a blocked N-terminus. MCoTI-II, a macrocyclic squash trypsin inhibitor from *Momordica cochinchinensis* seeds was found to have a "knottin" fold in its structure (Heitz et al., 2001). The "knottin" fold represented a stable cysteine-rich scaffold, in which one disulfide bond crosses the macrocycle made by two other disulfides and the connecting backbone segments. However, MCoTI-II displayed no significant antibacterial activity, unlike other cyclic knottins, i.e., kalata B1 or circulin A.

Felizmenio-Quimio et al. (2001) found MCoTI-II, a novel trypsin inhibitor from *Momordica cochinchinensis*, to have a different sequence and a different biological activity from known cyclotides. Based on its structural similarity, cyclic backbone and plant origin, the authors proposed that MCoTI-II could be classified as a new member of the cyclotide class of proteins. While the cyclic inhibitors MCoTI-I and MCoTI-II were found to be very potent trypsin inhibitors, the open-chain variants displayed an approximately 10-fold lower affinity (Avrutina et al., 2005). The data suggested that the formation of a circular backbone in the MCoTI squash inhibitors resulted in an enhanced affinity for trypsin and therefore was a determinant of anti-trypsin biological activity.

Recently, Sommerhoff et al. (2010) reported on the design, chemical and recombinant synthesis and functional properties of a series of novel inhibitors of human mast cell tryptase beta, a protease of considerable interest as a therapeutic target for the treatment of allergic asthma and inflammatory disorders. These inhibitors were derived from a linear variant of the cyclic cysteine knot miniprotein MCoTI-II, originally isolated from the seeds of *Momordica cochinchinensis*. These cystine knot miniproteins may therefore become valuable scaffolds for the design of a new generation of tryptase inhibitors.

Five trypsin inhibitors, with N-terminal sequences demonstrating homology to each other and exhibiting a molecular weight of 5,100, 4,800, 4,400, 4,100, and 3,900, respectively, have also been isolated from *Momordica cochinchinensis* seeds (Wong et al., 2004). Tsoi et al. (2004) also isolated an inhibitor, named MCoCI, that belonged to the potato I inhibitor family from *M. cochinchinensis* seeds. The MCoCI possessed remarkable thermostability and was stable from pH 2 to 12. The MCoCI also inhibited subtilisin, but had at least a 50-fold lower inhibitory activity towards trypsin and elastase. Amino acid sequencing of a peptide fragment of MCoCI revealed a sequence of 23 amino acids.

1.2.3.7. Traditional medicinal uses

In traditional Chinese medicine, the dried and ripe seeds, which are also named *Mubiezhi* (Tang & Eisenbrand, 1992) or *Semen Momordicae* (Huijuan et al., 2000), are considered to have cooling and resolvent properties, and are used as a remedy for fluxes, liver and spleen disorders, haemorrhoids, wounds, bruises, swelling and pus (Masayo et al., 1985).

The seeds of the *Momordica cochinchinensis* fruit, known in traditional Chinese medicine as "*Mubiezi*", has been utilised in China for more than 1,200 years. It is traditionally used for a variety of internal and external purposes that include the treatment of inflammatory swelling, scrofula, tinea and diarrhoea as well as suppurative skin infections such as sores, carbuncles, furuncles and boils in both humans and animals (Rajput et al., 2007; Xiao et al., 2007a). In Chinese medicine, the seeds are referred to as having 'resolvent and cooling' properties, and are used for liver and spleen disorders, wounds, hemorrhoids, bruises, swelling, and pus (Shan et al., 2001).

In Vietnam, Gac is prized by natives for promoting longevity and vitality (Burke et al., 2005). The National Institute of Materia Medica in Hanoi (1999) has listed a comprehensive list of medicinal uses of *M. cochinchinensis*. The oil is believed to

invigorate the spleen and stomach and improve eyesight. The oil is recommended for the treatment of children with rickets, xerophthalmia, nyctalopia, poor appetite and general weakness. It is believed to be beneficial for pregnant and breast feeding women. It is also a laxative and used for constipation but is also used for diarrhoea.

The oil is applied externally for wounds, burns and sores. The oil is also used in combination with antibiotics for acne. The oil is also considered as a good source of the vitamin A precursors, the carotenoids. The seeds are principally prescribed for external application and are recommended for furunculosis, scrofula, mastitis, galactophoritis and haemorrhoids. However, the powdered seeds are also administered internally with warm rice wine for malaria with splenomegaly. The ground seed extract is also applied externally for boils, impetigo and scabies as a liniment.

In the Philippines, the pulverised seeds are used as a natural expectorant for coughs but also for haemorrhoids (Stuart, 2010). The Gac seeds and leaves are also considered to be aperient (purgative) and abstergent (cleansing) and in peninsular Malaysia, the ethnic Chinese have been reported to use the seeds as an aperient for treating tumours, malignant ulcers and for obstructions of the liver and spleen (Burkill, 1966).

Although potential medicinal compounds have been found in the seeds, the true benefits of the various traditional preparations and what they have been used for remains to be scientifically tested and clarified.

1.2.3.8. Other uses

In Indo-China, Gac seed oil is burnt as an illuminant. The Gac roots are rich in saponins and are used as soap in laundering. Studies have reported that an extract of *M. cochinchinensis* seeds (ECMS) has also been used as a supplement for enhancing the

serological immune response to a foot-and-mouth disease (FMD) vaccine (Xiao et al., 2007a; Xiao et al., 2007b). The FMD is a highly contagious disease affecting cloven-hoofed animals. Vaccination against FMD is a routine practice in many countries where the disease is endemic. The results indicated that the ECMS and an oil emulsion acted synergistically as adjuvants to promote the production of FMDV- and VP1-specific immunoglobulin G (IgG) and subclasses in guinea pigs. Supplementing a commercial FMD vaccine with ECMS also significantly enhanced FMDV-specific indirect hemagglutination assay titers as well as VP1-specific IgG and subclasses in pigs. Therefore, ECMS could be an alternative approach to improving swine FMD vaccination to induce an effective immune response when the vaccine is poor. In further studies, supplementing a Newcastle disease vaccine with an extract of *Momordica cochinchinensis* seeds (ECMS) was found to enhance immune responses in chickens (Xiao et al., 2009). Results indicated that the humoral immune response was enhanced by ECMS 14 days post-immunisation and 80 mg of ECMS was the best dose with the Newcastle disease vaccine.

1.2.4. Processing of Gac seeds

In order to take advantage of the medicinal benefits of Gac seeds, it is necessary that appropriate methods of processing be applied. The aim is to get the highest yields and activities of the bioactive compounds in forms that are stable and easy to use. Figure 8 illustrates the ways in which Gac seeds can be processed for their bioactive constituents to produce extracts for potential medicinal supplements.

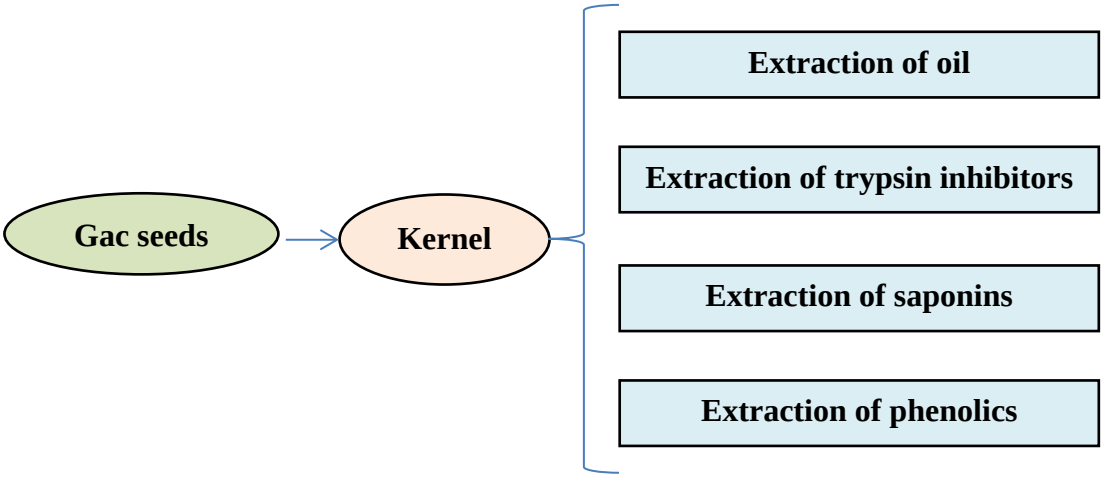


Figure 8: A potential processing scheme for Gac seeds.

1.3. Extraction of oil and bioactives from Gac seeds

1.3.1. Extraction of oil

A range of methods has been used for the extraction of oil from various plant seeds. Traditional extraction using potentially harmful organic solvents has largely been abandoned due to health concerns, environmental problems and quality degradation and it is important to find an alternative extraction method using non-organic food-grade solvents.

Generally, the conventional industrial methods of producing seed oil include 1) expeller pressing and 2) conventional solvent extraction mainly using hexane. The pressing process yields extracts of high-quality but, in most cases, the yield is low and the active components can be subject to thermal degradation. The latter achieves almost complete recovery of the oil; however, it can cause unacceptable solvent contamination quite harmful to human health and the environment, which may restrict its use in the food, cosmetic and pharmaceutical industries.

Many reports have shown that plant oils can be extracted using other methods such as SC-CO_2, aqueous enzymatic extraction, microwave-assisted extraction and ultrasound-assisted extraction. These methods are environmentally-friendly and organic solvent-free. The advantages and drawbacks of ultrasound-assisted pressing extraction (Chemat & Khan, 2011; Soria & Villamiel, 2010; Vilkhu et al., 2008) and microwave-assisted pressing extraction (Desai et al., 2010; Kaufmann & Christen, 2002; Tatke & Jaiswal, 2011) in food extraction have been reviewed.

1.3.1.1. Soxhlet extraction

Soxhlet has been the leaching technique mostly used for a long time. This assertion is supported by the fact that Soxhlet has been a standard technique during more than one century and, at present, it is the main reference to which the performance of other leaching methods is compared.

Extraction mechanisms

In conventional Soxhlet, the sample is placed in a thimble-holder, and during operation gradually filled with condensated fresh solvent from a distillation flask. When the liquid reaches the overfow level, a siphon aspirates the solute of the thimble-holder and unloads it back into the distillation flask, carrying the extracted analytes into the bulk liquid. This operation is repeated until complete extraction is achieved (De Castro & Garcıa-Ayuso, 1998).

Applications of Soxhlet extraction for vegetable oil

For decades, this traditional method of Soxhlet extraction have been used everywhere for many different purposes. In terms of efficiency, the Soxhlet extraction is described as an

universal chemical extraction process. However, this method requires large extraction time and quantity of solvent.

1.3.1.2. Supercritical carbon dioxide (SC-CO_2) extraction

Extraction mechanisms

In the supercritical fluid extraction (SFE), the fluid is in its supercritical state, having its pressure and temperature above their critical values. In this state, unique properties of the supercritical fluid such as density, viscosity and diffusivity are intermediate between those of a gas and a liquid. In particular, lower viscosity and higher diffusion coefficient are evident, as compared to that of a liquid. The density of the fluid is similar to that of a liquid. Those properties depend on the pressure, temperature and composition of the fluid (Camel, 2001). Importantly, the density, the dissolving power of the fluid, can be adjusted by changing both temperature and pressure of the fluid. As a process, SFE offers numerous potential advantages over conventional extraction processes, including: reduced extraction time, reduced solvent used and more selective extractions (Taylor, 1996). In addition, it is also considered to offer benefits such as non-solvent residues, higher extraction yields and better retention of nutritional and valuable bioactive compounds (Herrero et al., 2006).

It is important to select the most suitable supercritical fluid in this extraction technique. In general, many solvents can be used as supercritical fluids such as ethylene, methane, nitrogen, xenon and fluorocarbons. Among those, carbon dioxide (CO_2) has been a preferred solvent for SFE, known as supercritical carbon dioxide (SC-CO_2) extraction. The main reasons are the low critical temperature of CO_2 (31^oC) and the low critical pressure (74 bar), which enables the extraction process at low temperature and moderate pressures. Due to its low polarity, CO_2 is good for extraction of low or non-polar

compounds, but not suitable for polar compounds. To overcome this drawback, the use of a small amount of chemical modifier or co-solvent can significantly enhance the solubility of the polar compounds in SC-CO_2. A diagram of phase transitions of carbon dioxide is shown in Figure 9.

In SC-CO_2, the CO_2 is vented after the extraction process and it is not present in the extract. Therefore, CO_2 extracts are generally recognized as safe (GRAS) to be used in food production (Gerard & May, 2002). Furthermore, the extracted products are generally of good quality and may not need particular refining operations.

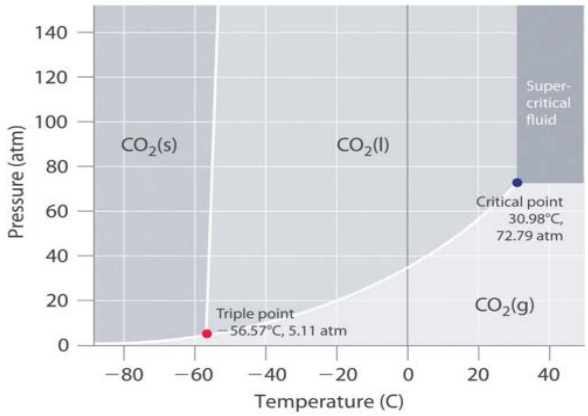


Figure 9: Phase diagram of carbon dioxide.
(Adapted from Silberberg (2007)).

Applications of SC-CO_2 extraction for vegetable oil

The SC-CO_2 technology has been applied for the extraction of oil from a large number of materials in food and pharmaceutical processing with a high potential for future applications (King, 2000; Reverchon & Marrone, 2001). In recent years, the SC-CO_2 technique has been employed to extract essential oils (Caredda et al., 2002; Guan et al., 2007; Mitra et al., 2009), fatty acids (Garlapati & Madras, 2010; Toribio et al., 2011), carotenoids (Mattea et al., 2009; Nobre et al., 2009) and vitamin E (Nyam et al., 2010; Pereira & Meireles, 2010) from fruits and vegetables. Some comparisons have been conducted between SC-CO_2 and solvent extraction in terms of characteristics of the oils

extracted. Generally, oils extracted by SC-CO_2 is reported to have a lighter colour, a milder odour, more tocopherols and less impurities such as unsaponifiables, gossypol and phosphorous (Friedrich & Pryde, 1984).

Although for vegetable oils, hydraulic pressing and/or traditional solvent extraction have been commonly used. High extraction efficiency is usually achieved using the hazardous solvent extraction, however, solvent elimination after the extraction is an inconvenient step. Furthermore, the main drawbacks of the traditional solvent extraction are thermal degradation of the bioactive compounds and the incomplete solvent elimination. Since CO_2 is the main solvent used in the SC-CO_2 extraction, it is most effective when the desirable compounds are nonpolar. Importantly, separation of the solute from the CO_2 solvent can be easily obtained by depressurising the SC-CO_2 (Martínez & Carolina de Aguiar, 2014). Therefore, the SC-CO_2 extraction can be employed as an alternative to traditional extraction methods with hazardous solvent. In recent years, vegetable oils from plant materials have been extracted using SC-CO_2 extraction technique (Santos et al., 2013; Tomita et al., 2013). According to those studies, material characteristics (particle size and moisture content) and SC-CO_2 extraction conditions (pressure, temperature, time and flow rate) significantly influenced the extraction yield of the vegetable oils. In fact, a faster rate of the CO_2 diffusion is achieved when using a smaller particle size because of increasing surface area to volume ratio of material and rupturing cell membranes (del Valle & Uquiche, 2002). As a result, grinding the sample to an appropriate particle size is recommended. This is because there can be a problem with channelling inside the extraction bed if very fine particles are used.

Furthermore, it should be noted that when filling the vessel, it is necessary to ensure a homogeneous bed of material to avoid channelling. Moisture content of the material is also an important parameter in the SC-CO_2 extraction. High moisture content can cause

mechanical problems such as restrictor clogging due to ice formation. To overcome this, addition of anhydrous Na_2SO_4 and silica gel to the wet plant sample to capture the moisture can be used (Lang & Wai, 2001). However, the preferred pre-treatment is to dry the plant materials to appropriate moisture content before the extraction. It has been reported that the moisture content of the plant materials should not be higher than 12% because water can cause unwanted difficulties such as ice formation in pipelines (Fornari et al., 2012).

There are numerous extraction conditions such as pressure, temperature, time and flow rate influencing the extraction efficiencies. It is generally agreed that applying higher pressure and temperature increases mass transfer and release of bioactive compounds from the plant matrix. However, high pressure and temperature also produces more undesirable compounds in the extract. Generally pressure and temperature can be controlled to optimise the oil yield. Many studies reported that the extraction pressures between 30 and 40 MPa resulted in the maximal oil yield from different plant matrices. For extraction temperature, the low temperature of 60 ^{o}C gave the highest extraction efficiencies of saponins, which were reported in many studies (Cheok et al., 2014). Furthermore, the high extraction temperature of 80 ^{o}C also favours saponin extraction from plant materials (Cheok et al., 2014). However, the degradation at temperature higher than 80 ^{o}C is promoted, the lower temperature is recommended. Therefore, it is necessary to investigate the most important factors affecting the efficiencies and then optimise the conditions.

1.3.2. Extraction of bioactive compounds

Bioactive compounds phytochemicals found in plant materials that are capable of modulating metabolic processes and resulting in the promotion of better health

(Galanakis, 2016). Bioactive compounds can be classified by their functional groups such as hydroxyl (-OH), carbonyl (-CO), carboxyl (-COOH), amine ($-NH_2$), or by their functional properties, for example, antioxidant, anticancer, anti-diabetic, anti-cardiovascular, anti-bacterial, and anti-inflammatory activities. The major categories of natural bioactive compounds include polyphenols, phenolic acids, flavonoids, flavanols, flavanones, flavones, flavonols, isoflavones, chalcones, anthocyanins, stilbenes, resveratrol, carotenoids and phytosterols (Naumovski, 2014), which link directly to their potential biological activities (Cragg & Newman, 2005; Newman et al., 2003; Rao, 2012).

Over the last few decades, many bioactive compounds have been studied, isolated, identified, purified and applied in the fields of nutraceuticals and pharmaceuticals for the treatment and prevention of numerous diseases. However, the development of novel techniques for extraction, isolation, identification and purification is also essential in retaining bioactive levels and reducing production costs.

In recent years, a range of conventional extraction methods have been used to extract bioactives from plant materials such as Soxhlet extraction, maceration, serial exhaustive extraction, decoction, infusion, digestion, percolation and hydrodistillation. However, these methods have major disadvantages such as long extraction times, requirement of high purity solvent, evaporation of a huge solvent amount, low extraction selectivity, low efficiency, potential environmental pollution and thermal decomposition of thermolabile compounds (Cheok et al., 2014). Therefore, many advanced or non-conventional extraction techniques have been successfully developed and applied to optimize the extraction of bioactive compounds from plant materials. For example, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pulsed-electric field extraction (PEF), enzyme-assisted extraction (EAE), counter-current extraction (CCE), subcritical water extraction (SWE), supercritical fluid extraction (SFE), pressurized fluid extraction

(PFE) or accelerated solvent extraction (ASE), coupled supercritical fluid extraction - supercritical fluid chromatography/gas or liquid chromatography (SFE-SFC/GC/LC), and phytonic extraction (PE), with some techniques have been considered as “green techniques” (Cheok et al., 2014). These techniques have proven to reduce significantly extraction time as well as achieve improved extraction efficiency.

1.3.2.1. Extraction solvents

When other factors are kept constant, the extraction solvent plays a key role in obtaining the target constituents in a desired quality and quantity (Samuelsson et al., 1985; Sasidharan et al., 2011). The choice of the solvent is mainly done based on the chemical properties, i.e. polarity or hydrophobicity of the target compounds. For samples selected on the basis of traditional use, the choice is often done to mimic the traditional extraction method as much as possible (Sasidharan et al., 2011). Decoction with water is the method of extraction for most of the traditionally prepared remedies (Samuelsson et al., 1985). For example, the traditional healers of the Lulua tribe use a decoction with water to extract the cyclotide containing plant Kalata-Kalata (Gran et al., 2000).

Organic solvents with medium or high polarity have been most commonly used in laboratories dealing with natural products; and there is evidence that some extracts from these solvents have better activity compared with the aqueous extracts (Samuelsson et al., 1985). The characterisation of complex structures such as peptides and proteins from aqueous or organic solvent extracts has been overlooked in the past. However, during the last decades, interest in this class of natural products has increased with the development of automated and affordable techniques for their isolation (Franco, 2011; O'Keefe, 2001).

1.3.2.2. Ultrasound assisted extraction (UAE)

Extraction mechanisms

Ultrasound technology is mechanical waves at frequencies above human hearing (16 Hz to 16-20 kHz). The applications of ultrasound in the food industry can be divided basically into two different categories: low-intensity and high-intensity ultrasound. The power levels used in low-intensity ultrasound are very small, typically less than 1 W cm^{-2}. This ultrasonic wave is most commonly applied in the food analysis due to no physical and chemical modifications in the physicochemical properties of the materials, such as firmness, sugar content and acidity (McClements, 1995). In contrast, the high-intensity ultrasound uses the large power levels, typically from 10 to 1000 W cm^{-2}. The power ultrasound can alter the properties of food physically and chemically. During the past years the high-intensity ultrasound, which is applied at higher frequencies up to 2.5 MHz, has attracted attention due to strong physical disruption of tissues for extraction purposes.

The main principle of the ultrasound power used in the extraction is the propagation of ultrasound pressure waves, resulting in cavitation. In liquid systems, longitudinal waves are formed via a series of compression and rarefaction waves of elastic materials (Soria & Villamiel, 2010). A sufficiently high intensity results in the local pressure waves below the vapour pressure of the liquid, generating a constant growth of gas bubbles being distributed throughout the liquid. These bubbles will reach a critical size, then become unstable and violently collapse (cavitation). High temperature of 5,000 ^{o}K and high pressure up to 100 atm are momentarily generated when the bubbles collapse, thus producing very high shear energy waves and turbulence in the cavitation zone (Pingret et al., 2013). Therefore, it can be seen that the combination of heat, pressure and turbulence are responsible for a variety of effects of high-intensity ultrasound in the extraction. For

instance, the release of extractable compounds is facilitated due to the fast changes in pressure and temperature (cavitation phenomena), which cause shear disruption of cell membranes of the plant matrix. They also enhance the mass transfer by disrupting the plant cell walls (Azmir et al., 2013) and hence extraction rate.

Applications of UAE for bioactive compound extraction

UAE is also known as a novel technique for extraction of bioactive compounds from plants. Reducing extraction time, increasing yield and producing high quality of the plant extracts are the main benefits of this method. In order to achieve an efficient and effective ultrasound-assisted extraction, it is of interest to understand influence of the extraction conditions and the plant characteristics.

For the conditions of ultrasound-assisted extraction, proper choice of the solvent is the key to successful extraction. The important criterion is the extent of ultrasound cavitation in the solvent. For instance, the physical properties such as surface tension, viscosity and vapour pressure of the solvent affect the intensity of cavitation in a liquid medium. In addition, the solubility of the analytes of interest and the interactions between the solvent and plant matrix should also be considered. Other important factors including ultrasound power, temperature and extraction time also need to be taken into account. It is necessary to control the extraction temperature during UAE because ultrasound generates heat. As a result, it is also important to minimise the sonication time to avoid degradation of bioactive compounds.

Saponins and bioactive peptides from plant materials have been successfully extracted using UAE technique (Hua, 2007; Kadam et al., 2015; Wu et al., 2001; Zhao et al., 2007). Results indicated that UAE conditions such as ultrasound power, temperature and extraction time, type of solvent, ratio of solvent to solid positively affected the extraction

yield of the bioactive compounds. Reduction in extraction time and improvement in quality of bioactive components are also the two main advantages of this technique. Thus, it is interesting to note that implementation of UAE may improve throughput in commercial bioactive compound production process.

1.3.2.3. Microwave assisted extraction (MAE)

Extraction mechanisms

Microwaves are non-ionising electromagnetic waves within the frequency band of 300 MHz to 300 GHz in the electromagnetic spectrum, corresponding to wavelengths between 1 mm to 1 m. Generally, microwave applications are performed at 915 MHz (United States), 896 MHz (United Kingdom) and 2450 MHz (worldwide) for all practical purposes. According to Singh and Heldman (2001), as the velocity of light is 3 x 108 m/s, the microwave wavelengths at 915 MHz and 2450 MHz are calculated as 0.328 and 0.122 m, respectively. Therefore, the frequency of 915 MHz is considered as most useful for industrial applications with its greater penetration depth, whereas domestic microwave ovens use the frequency of 2450 MHz.

When microwaves penetrate deeply into a material, the absorption of microwaves by the dielectric component of this material results in release of their thermal energy to it. The two main mechanisms of heating using microwaves are ionic polarisation and dipole rotation (Kaufmann & Christen, 2002). Plant materials contain water molecules, which are the most common, and generally have a random orientation. When an electric field is applied, the molecules orient themselves according to the polarity of this field. As the electric field is alternated rapidly, the polar molecules rotate to follow the applied field. This leads to friction with the surrounding medium, resulting in heat being generated. For ionic polarisation, this happens when ions in food solutions move due to their inherent

charges as the electric field is applied. Kinetic energy of the moving ions is converted into heat by the resulting collisions between ions; there is a rapid increase in temperature as a consequence (Kadam et al., 2013; Zhang et al., 2011).

Recently, the development of microwave-assisted extraction has been applied to heating the moisture inside a plant cell using microwave radiation, thus evaporation and high pressure on the cell wall are generated. The physical properties of the biological tissues (cell membrane and organelles) are modified by the generated pressure inside the material. The cell membrane is ruptured; the bioactive compounds from the ruptured cells are released into the medium. The penetration of extraction solvent through the porous biological matrix is facilitated. Therefore, the extraction yield of desired compounds could be increased. In addition, the use of this extraction also offers several benefits such as less chemical solvent use, reduced processing time and uniform heating (Azmir et al., 2013). These advantages have recently led to microwave-assisted extraction to be considered as one of the most novel techniques for the extraction of bioactive substances from different plant materials.

Applications of MAE for bioactive compounds

Recent interest in microwave-assisted extraction for plant bioactives has been highlighted owing to their benefits. Different microwave treatment conditions (intensities and times) were studied, and an increase in bioactive yield was found in comparison to untreated samples.

According to Desai et al. (2010), optimisation of microwave assisted extraction conditions has been reported in the literature. Several parameters and their levels including solvent type or composition, solvent volume or solid loading, power applied or extraction temperature and extraction time need to be studied.

1.3.2.4. Comparison of different novel extraction methods

The different extraction techniques including the conventional, Soxhlet, SC-CO_2, UAE and MAE have been presented in the previous sections. Depending on the availability of equipment, the target bioactive compounds and the processing cost, a proper choice of the extraction technique or a combination of different extraction methods can be made.

It is well known that the conventional or Soxhlet extraction methods take a very long time to complete the extraction process of desired bioactive compounds. Another drawback of the conventional methods are the likely degradation of thermolabile compounds due to the high temperature applied. In addition, the large amounts of chemical solvent used and wasted in the solid-liquid extraction incur costs for solvent disposal and environmental control measures, and contribute to the criticism of the conventional extraction methods (De Castro & Garcıa-Ayuso, 1998).

To overcome these limitations of the conventional methods, the development of innovative technologies for extracting bioactive compounds from plant materials addresses specific human requirements for health and safety. The main advantages of the novel extraction methods including SC-CO_2, UAE and MAE are shorter extraction time, higher extraction yield and better retention of valuable bioactive compounds. The use of SC-CO_2 extraction in the extraction of the bioactive compounds, which can be used as nutraceuticals and pharmaceuticals, has been reported. The extracts containing high bioactive compounds can be used to treat or prevent disease (Henry & Yonker, 2006). SC-CO_2 extraction method uses nil or a small amount of chemical solvent (as a co-solvent) in the extraction, so is considered as more environmentally friendly than the conventional extraction method. However, the main drawbacks of SC-CO_2 extraction

technique are economics and onerous operating conditions, thus its use so far is limited to areas such as essential oil extraction and coffee decaffeination (Wang & Weller, 2006).

There have been many studies reporting the benefits of MAE and UAE methods for extracting bioactive compounds. Similar to SC-CO_2, the MAE and UAE methods can operate at low temperature, allowing the extraction of thermolabile compounds from various plants. Between them, UAE device is cheaper and its operating process is easier compared with MAE. Like Soxhlet extraction, UAE can be used with any solvent, in contrast, the extraction solvent used in the MAE must absorb microwave energy. Overall, MAE and UAE techniques are comparable to other innovative extraction techniques such as SC-CO_2 and are considered as strong novel extraction methods in terms of process simplicity, low investment cost and practicality (Wang & Weller, 2006).

Although these methods are very promising as alternatives to the conventional extraction method, they have been used only at laboratory or bench scale, except for several industrial applications of SC-CO_2 extraction (Wang & Weller, 2006). It is important to conduct more research to up-scale these novel extraction methods.

Potentially, from understanding the advantages and drawbacks of different extraction methods as presented above, there are opportunities to combine different extraction methods to overcome the limitations and retain the advantages.

1.3.3. Extraction of trypsin inhibitors

Trypsin inhibitors (TIs) are low molecular weight peptides, which are known highly hydrophilic. As for all plant-derived natural products, extraction is the first critical step in the isolation of TIs from their sources. However, despite many achievements on the

structure determination of Gac seed trypsin inhibitors, there is still very limited information on the techniques, which can be used to extract them.

For extraction of trypsin inhibitors from various plant tissues, most studies used solid-liquid conventional extraction, with or without the assistance of shaking/stirring, using different extraction solvents. The extraction solvents were chosen based on the solubilities of the trypsin inhibitors to be extracted and the types of plant tissues from which the trypsin inhibitors were derived. As a result, the extraction methods differ in the type of solvents used and the procedures performed. In one study (Mahatmanto, 2014), a mixture of acetonitrile, water and formic acid (ACN/Water/FA) was found to be optimal for extracting cysteine knot peptides from Gac seeds, some of which are trypsin inhibitors. For other plant sources of TIs, water was also the optimal solvent for their extraction from Thai mung beans (Klomklao et al., 2011) but 0.1 M NaCl was the best for their extraction from *Chenopodium quinoa* seeds (Pesoti et al., 2015) and 0.02 M NaOH was the best for their extraction from grass pea (Deshpande & Campbell, 1992).

Therefore, a comparative study assessing the extraction solvents mentioned above for trypsin inhibitors from *Gac* seeds is necessary.

1.3.4. Extraction of saponins

Saponins are amphiphilic compounds with either a steroid or triterpenoid component (hydrophobic part) and one or more glycosides (hydrophilic) (Oakenfull, 1981). Due to their amphiphilic properties, strongly polar aqueous solvents and strongly non-polar organic solvents are both not ideal for extracting saponins. Therefore, the different polarities of the solvents used in extractions will result in differences in the composition of the saponins extracted (Du et al., 2004).

Most studies use methanol or ethanol as general solvents for the extraction of saponins from various plant materials (Güçlü Üstündağ & Mazza, 2007). However, to better characterise the types of saponins present in a sample, both a polar (e.g. water) and a non-polar (e.g. n-butanol) solvent are commonly used to separately extract the more hydrophilic and the more hydrophobic saponin fractions, respectively.

Various techniques have been implemented in saponin extraction such as microwaved-assisted extraction (Chen et al., 2007; Kerem et al., 2005; Li et al., 2010), pressurised low polarity water extraction (Güçlü Üstündağ et al., 2007), ultrasound-assisted extraction (Wu et al., 2001) and aqueous extraction (Tan et al., 2014b).

Gac seed saponins have been reported to be critical constituents in Gac seed extracts, which were responsible for their medicinal properties (Yu et al., 2017a; Yu et al., 2017b). These constituents of Gac seeds have been investigated by several investigators: two saponins, referred to as momordica saponin I and II, have been isolated and characterised (Masayo et al., 1985), in which momordica saponin I is a major gastroprotective ingredient (Jung et al., 2013a) and momordica saponin II shows an anti-inflammatory activities in RAW 264.7 cells (Jung et al., 2013b). Another saponin, karounidiol, a compound possessing cytotoxic activity against human cancer cell lines (Akihisa et al., 2001), has been reported to be present in Gac seeds (Kan et al., 2006). The potential valuable pharmaceutical properties of the Gac seed saponins warrants investigating how they are best extracted from the seeds i.e., which extraction technique(s) will maximise the yield of saponins.

When it comes to extraction of saponins from seeds, defatting is often carried out before the saponins are extracted (Cheok et al., 2014). Although the defatting might make it simpler for the saponin extraction in terms of technique, and does not greatly affect the

saponin yield for some type of seeds, it can cause a great loss of saponin for others. Therefore, studies on the suitable material Gac seeds and how to efficiently extract their saponins are important.

1.3.5. Response surface methodology for optimising extraction conditions

Response surface methodology (RSM) is the most popular optimisation technique used in recent years. It is used to determine the relationship between the response variable(s) and more than one independent variable when optimising processes or products (Myers et al., 2014). The statistical technique allows the simultaneous evaluation of the effect of multiple parameters and their interactions on the output variable(s) with a reduced number of trials (Lee et al., 2000); essentially, the statistical package eliminates the need to do all the possible combinations and permutations. Therefore, it is faster and more economical than other approaches used to optimise a process or product.

In general, the quality of a product is affected by numerous parameters. It is impossible to control the effects of all variables; hence the major effects of parameters on the process must be identified. It is useful to use RSM to develop, improve and optimise the effects on processes such as the extraction and the spray drying of the extract.

According to Baş and Boyaci (2007) and Bezerra et al. (2008), optimisation studies using RSM include three main stages, (1) screening of the independent variables and their levels; (2) selection of the experimental design and the prediction and verification of the model equation; and (3) graphical representation of the model equation and determination of the optimal operating conditions.

According to Baş and Boyaci (2007), it is important to screen all variables, which may affect the process. Factorial designs may be used for determining which of the

independent parameters have the major effects. After this, the levels of the key parameters need to be identified. This is because the success of the optimisation process is directly related to these levels. To make sure the response is affected more evenly, each variable is coded to range from −1 (for x_{min}) to +1 (for x_{max}), and expressed as follows:

$$X = \frac{x - (x_{max} + x_{min})/2}{(x_{max} - x_{min})/2}$$

where x is the natural variable, X is the coded variable and x_{max} and x_{min} are the maximum and minimum values of the natural variable, respectively.

The second stage of the RSM is to select the experimental design and then predict and verify the model equation. The experimental designs are usually generated from RSM computer packages based on the criteria input. The experimental points, number of runs and block types are identified by the package. However, it is very important to choose the appropriate type of design. For example, if the experimental data shows curvature, the experimental designs for first-order models cannot be used and the experimental designs for quadratic response surfaces, which include the three-level factorial, Box-Behnken, central composite or Doehlert designs (Bezerra et al., 2008) should be used. The details for these individual designs can be found elsewhere in the literature.

After selection of the appropriate design, the model equation is defined and coefficients of the model equation are predicted by the package. The estimated response is easily calculated from the model equation after obtaining the regression coefficients. To verify whether the model fits well to the determined experimental data, prediction error sum of squares residuals are normally used. In addition, other techniques such as residual analysis, scaling residuals or testing of the lack of fits can be used (Baş & Boyacı, 2007).

The optimal operating conditions can then be determined from the response surface plot and contour plot generated by the computer package. The response surface plot is a three-dimensional plot, which shows the relationship between the response and the independent variables, whilst the contour plot is a two-dimensional plot, which helps to visualise the shape of the response surface. Therefore, it is useful to use the plots to evaluate the best operating conditions that fit of model (Baş & Boyacı, 2007).

As reviewed above, the RSM has been demonstrated to be a powerful tool for determining the simultaneous effects of more than one factor and their interactions, which allows for process optimisation to be conducted effectively. This method is the preferred experimental design for fitting polynomial models to analyse the response surface of multi-factor combinations. For the extraction of seed oils, many studies have been carried out to investigate the effect of supercritical carbon dioxide extraction operating conditions and to find optimal process conditions by RSM (Bhattacharjee et al., 2007; Oliveira et al., 2002; Özkal et al., 2005b), (Özkal et al., 2005a). However, this remains to be done for the extraction and encapsulation of Gac seed oil and bioactives.

1.4. Spectrophotometry method for quantification of bioactive compounds

Spectrophotometry is a method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution. The basic principle is that each compound absorbs or transmits light over a certain range of wavelength. This measurement can also be used to measure the amount of a known chemical substance. Spectrophotometry is one of the most useful methods of quantitative analysis in various fields such as chemistry, physics, biochemistry, material and chemical engineering and clinical applications. Colorimetric reactions are widely used in the

UV/VIS spectrophotometric method, which is easy to perform, rapid and applicable in routine laboratory use, and low-cost (Pelozo et al., 2008).

1.4.1. Principle of spectrophotometry measurement

Every chemical compound absorbs, transmits, or reflects light (electromagnetic radiation) over a certain range of wavelength. Spectrophotometry is a measurement of how much a chemical substance absorbs or transmits. Therefore, spectrophotometry is used to estimate the level of an analyte in solution.

Beer's Law states that the amount of light of a particular wavelength absorbed by a substance across a constant distance (light path) is proportional to the concentration of that substance: $A_n = L \times C$, where A_n is the absorbance at *n* nm; L is the light path (cm) and C is the concentration of the analyte in solution.

If the absorbance of a solution containing a known concentration of an analyte is measured, this value can be used to estimate the concentration of the analyte in an unknown solution by comparing the two absorbance values. The range over which absorbance is proportional to concentration varies according to the analyte and the wavelength of light used. To ensure that there is a direct relationship between absorbance and concentration, a standard curve must be prepared using a reference substance. Despite its name, the part of the standard curve that gives a proportional relationship is a straight line.

To build a standard curve, a series of dilutions are prepared of a standard solution of the reference substance (standard) of known concentration. The first tube always contains none of the standard (eg. a concentration of 0 g/L) and this is called a blank. This blank is used to calibrate the spectrophotometer to take into account the natural absorbance of

the diluents. Each of the following tubes contain increasing concentrations of the analyte. The absorbance of each of the standards are read using the spectrophotometer.

To create the standard curve, a line graph of Absorbance (Y axis) vs Concentration (X Axis) is plotted for each of the standards. A line of best fit is then drawn through the points.

To estimate the concentration of an unknown solution of the analyte, the absorbance of the unknown solution is read, and then the standard curve linear equation is used to estimate the concentration of the analyte in the sample extract.

1.4.2. Devices and mechanism for spectrophotometry measurement

A spectrophotometer is a device which measures the absorbance of a solution as light of a specified wavelength is passed through it (Figure 10).

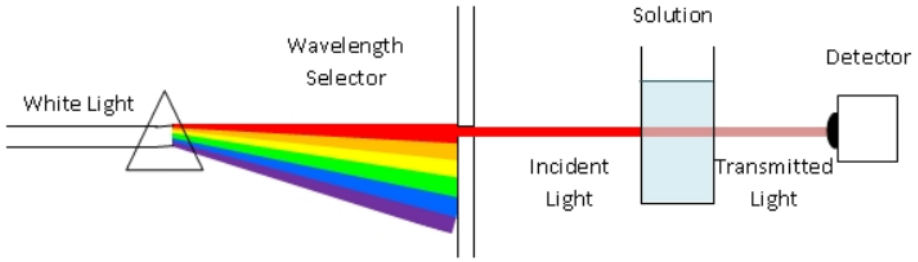


Figure 10: The mechanism for spectrophotometry measurement.
The difference between the incident and transmitted light indicates the absorbance.
(Source: https://di.uq.edu.au/community-and-alumni/sparq-ed/sparq-ed-services/spectrophotometry)

A spectrophotometer, in general, consists of two devices; a spectrometer and a photometer. A spectrometer is a device that produces, typically disperses and measures light. A photometer indicates the photoelectric detector that measures the intensity of light (Figure 10).

The spectrometer produces a desired range of wavelength of light. First a collimator (lens) transmits a straight beam of light (photons) that passes through a monochromator (prism)

to split it into several component wavelengths (spectrum). Then a wavelength selector (slit) transmits only the desired wavelengths, as shown in Figure 10.

After the desired range of wavelength of light passes through the solution of a sample in cuvette, the photometer detects the amount of photons that is absorbed and then sends a signal to a galvanometer or a digital display, as illustrated in Figure 10.

1.5. Oxidation and antioxidant activity of medicinal plants

The oxidation process in the biological systems depends greatly on oxygen, which is the most important acceptor of electrons, leading to the formation of active oxygen and free radical species. A free radical can be defined as any molecular species with one or more unpaired electrons, which can exist in an independent state (Naumovski, 2014).

Oxygen is generally sourced from two active oxygen species, namely Reactive Oxygen Species (ROS) include superoxide anion ($\bullet O_2^-$), free radicals: hydroxyl (•OH), peroxyl (ROO•) and alkoxyl ($RO^\bullet$), hydrogen peroxide (H_2O_2), organic hydroperoxide (ROOH) and singlet oxygen (O_2^*), and Reactive Nitrogen Species (RNS) such as nitric oxide (NO•), peroxynitrite (ONOO-), peroxynitrous acid (ONOOH) and nitrogen dioxide (NO2). These species may attack biological macromolecules in cells, giving rise to protein, lipid, nucleic acid and DNA damage, resulting in cellular aging and oxidative stress-originated diseases such as cardiovascular, diabetic and neurodegenerative diseases, and cancer (Apak et al., 2013).

Therefore, it is necessary to prevent the radical chain reactions of oxidation or to inhibit the initiation and propagation step leading to the termination of the reaction and delay the oxidation process (Doughari, 2012). One of the most popular ways is to use natural antioxidant compounds or bioactive compounds, which are taken into the body via daily

consumption of vegetables, fruits, drinks, or may be extracted and purified from various plant materials in either an individual form or in a combined form.

A large number of bioactive compounds and their antioxidant activity have been reported in recent years. For example, antioxidant activity of Radix Trichosanthis saponins was determined by Chen et al. (2014), of plant phenolics was determined by Dai and Mumper (2010), or a wide variety of free radical scavenging molecules such as phenols, flavonoids, flavanols, proanthocyanidins, vitamins, terpenoids, and carotenoids with potential antioxidant activity have been identified in the plants (Doughari, 2012).

Evaluation of total antioxidant capacity of bioactive compounds is of great interest to researchers. A number of chemical assay techniques have been recently developed to assess antioxidant capacity. However, each antioxidant assay only displays a limited level of total antioxidant capacity, it is therefore necessary to use several different antioxidant assays.

The most common methods that have been used to evaluate the total antioxidant capacity belong to two major reaction groups, namely single electron transfer (SET) and hydrogen atom transfer (HAT) reactions. The SET reaction is observed through the change in colour of the reagent solution when the oxidant is reduced. The assay of ABTS radical scavenging capacity or DPPH radical scavenging capacity is based on the single electron transfer capacity from the antioxidants for ABTS or DPPH radical to form an electron pair, which leads to the colour of the reagent solution being decreased (lighter colour, level depends on concentration of antioxidants). The assay of cupric ion reducing antioxidant capacity (CUPRAC) or ferric reducing antioxidant power (FRAP) is based on the copper ion or ferric reduction ability of antioxidants, which leads to the colour of the reagent solution being increased (darker colour, level depends on concentration of

antioxidants) (Apak et al., 2013). The HAT reaction measures the capacity of an antioxidant when it quenches free radicals (generally considering to peroxyl radicals) by hydrogen atom donation, such as crocin bleaching, β-carotene bleaching, oxygen radical absorbance capacity (ORAC) and total peroxyl radical-trapping antioxidant parameter (TRAP) assays. In addition, some other methods not included in these two groups have been developed, such as total oxidant scavenging capacity (TOSC), chemiluminescence and electrochemiluminescence assays (Apak et al., 2013; Apak et al., 2007).

1.6. Cancer and anticancer activity of medicinal plants

Cancer is a disease characterised by uncontrolled cell growth and proliferation initiated by inappropriate cell division. It is categorised as the second leading cause of death, with 18 million estimated new cases in 2018 and causing a major health problem in both developed and developing countries in the world (Bray et al., 2018). Melanoma is the fourth most common cancer diagnosed in Australia, which along with New Zealand has the world's highest incidence rate for melanoma, mainly due to the exposure of skin to high UV radiation (Cancer Councel Australia, 2017). A rise in the incidence and mortality rates of cancer and skin melanoma has led to an increased emphasis on drug development, public health policies and awareness programs for reducing cancer (Stracci et al., 2005).

More than 80% of the world’s population consider traditional plant derived medicine as their source of primary health care (Bhanot et al., 2011). Thus, there is a continuing need for the development of new anticancer drugs and/or drug combinations, through methodical and scientific exploration of the enormous pool of plant based products. Currently, the most common approaches for treating cancer include chemotherapy, surgery and radiotherapy (National Cancer Institute, 2017). However, non-surgical therapies are associated with toxicity due to non-selective targets (Mukherjee et al., 2001).

Plant-derived medicines have a long history of use in the treatment of cancer and over 60% of currently used anti-cancer agents are from natural sources (Bhanot et al., 2011).

Medicinal plants are rich sources of phytochemicals with antioxidant, immune-modulatory and anti-cancerous properties (Greenwell & Rahman, 2015).

Gac seeds are rich in phytochemicals such as saponins, trypsin inhibitors and tocopherols, with potential vitamin E, antimicrobial and anticancer activities. These bioactive compounds possess antioxidant, anti-inflammatory and anticancer effects (Lim, 2012). An ester extract of Gac seeds are known to have anticancer activity against melanoma cells (Zhao et al., 2012) and an ethanolic extract inhibits the proliferation of lung carcinoma cells, breast carcinoma cells, esophageal carcinoma cells and melanoma cells (Zhao et al., 2010a; Zhao et al., 2010b). However, so far, it is unknown whether the water extract of defatted Gac seeds has the same activity on other cancer cells including melanoma. Understanding the responsible compounds in anticancer activity of Gac seeds, will be important for optimising the extraction, purification and isolation for the production medicinal products with high anti-cancer activity.

1.7. Experimental rationale

Plants and plant extracts have been used as traditional cures and herbal remedies for centuries throughout the world. Recently, there has been a renewed interest in secondary plant metabolites because of their potential preventative and therapeutic effects on chronic diseases such as cardiovascular disease and cancer (Rowland, 1999). Hence, the isolation, identification and quantification of phytochemicals in plant resources and the evaluation of their potential health benefits have been in focus. However, *in vitro* and animal studies have shown that the action of some isolated phytochemicals are likely to be achieved only at doses much higher than those that may be obtained from eating plants

(Rowland, 1999). Therefore, the extraction of active ingredients is essential if these kinds of preparations are to be of prophylactic or therapeutic value in human subjects (Rowland, 1999).

Gac is an Asian tropical fruit, which is being planted in an increasing number of countries around the world. The aril, the flesh surrounding the seeds, of Gac fruits is the main part utilised for food; it contains high levels of nutrients, such as carotenoids, α-tocopherol (vitamin E) and polyunsaturated fatty acids (Kha et al., 2013a). The seeds, which account for 16-18% of the weight of the fruit, are normally discarded and can be an environmental issue. For example, in Vietnam alone, the amount of discarded Gac seeds is estimated to be around 750 tons per year. If this amount of seeds can be used for the extraction of oil and bioactives, it may add value to the Gac fruit industry whilst reducing an environmental problem.

Gac seeds contain a significant amount of oil (Section 1.2.1), which needs to be further investigated for use as a source of edible oil or medicinal oil. The total fatty acid content in Gac seeds is between 15.7% and 36.6% of the total weight of the seed (Ishida et al., 2004). Its fatty acid composition includes stearic acid (54.5–71.7% by weight), linoleic acid (11.2–25.0%), α-linolenic acid (0.5–0.6%) and several other types of fatty acids in smaller amounts (Ishida et al., 2004). This oil can be extracted from Gac seeds but its extraction needs to be optimised.

Gac seed preparations have also long been used in traditional Chinese medicine for the treatment of many diseases, including some skin disorders. Modern studies have also shown that Gac seeds have antitumour and anticancer properties. Therefore, they are purported to contain medicinal compounds and some of the potential bioactive compounds that have been identified among others in Gac seeds are trypsin inhibitors and

saponins as described in detail in Section 1.2.2. These bioactive compounds are enriched in Gac seeds and therefore, it is worth optimising their extraction.

Many factors, such as the method of extraction, solvent composition, extraction time, extraction temperature (Wettasinghe & Shahidi, 1999a), solid to solvent ratio (Cacace & Mazza, 2003b) and extraction pressure (Cacace & Mazza, 2002), are known to significantly influence the efficacy of extracting oil and bioactives from plant materials, including seeds.

In recent years, supercritical carbon dioxide (SC-CO_2) extraction of vegetable oil has attracted considerable attention as a promising alternative to the conventional solvent extraction and mechanical pressing processes (Gomes et al., 2007; Lu et al., 2007). The main reasons are that SC-CO_2 has a higher extraction rate than the mechanical pressing process and that the solvent, CO_2, is non-flammable, nonexplosive, cost-efficient, readily available and, because it is a gas, it is easy to remove from the extracted oil.

For bioactive compounds from plant materials, the conventional solvent extraction technique, in which the solid material is suspended in a solvent like water with no assistance for breaking the cell structure of the solid material, is often associated with a long heating time, which risks the degradation of bioactive compounds. This has led to the proposed use of advanced techniques to assist extractions by breaking the cell structure of the solid material. Microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE) are two such methods, which can increase the efficiency of extractions especially in terms of decreasing the extraction time and the consumption of solvent. In MAE, microwave heating is able to disrupt the plant cell structure via an increase in the internal pressure of the cell, thereby releasing the bioactive compounds (Tatke & Jaiswal, 2011). Similarly, ultrasonic cavitation during UAE produces

shockwaves that are also capable of disrupting the plant cell structure and releasing the plant bioactives (Pingret et al., 2013). These two advanced extraction methods have been widely used for the recovery of bioactive compounds from plant materials and are considered as dominant trends in “green chemistry” extractions (Wang & Weller, 2006). For example, they have been reported to be more efficient than the conventional extraction method for recovering carotenoids from Gac peel (Chuyen et al., 2018).

To maximise the yield of Gac seed oil and bioactive compounds, optimisation of the extraction processes is needed. This optimisation could be achieved by either empirical or statistical methods. However, the former method has limitations for achieving complete optimisation; the traditional one-factor-at-a-time approach to process optimisation is time consuming and the interactions among various factors are ignored and hence, the chance of approaching a true optimum is unlikely. The one-factor-at-a-time procedure assumes that various parameters do not interact and thus, the experimental results are only valid if a process response is a direct function of the single varied parameter. However, the actual response of a process is more likely to result from the interactive influence of various variables and therefore, for optimising a process, a statistical optimisation procedure like the RSM, which allows the interaction of variables to be taken into consideration, is preferable (Haaland, 1989).

The RSM, originally described by Box and Wilson (1951), enables evaluation of the effects of several process variables and their interactions on response variables. Thus, the RSM is a collection of statistical and mathematical techniques that has been successfully used for developing, improving and optimising processes (Myers & Montgomery, 2002). The RSM has been successfully used to model and optimise biochemical and biotechnological processes related to food systems (Cacace & Mazza, 2003a, 2003b; Parajó et al., 1995), including the extraction of phenolic compounds from berries (Cacace

& Mazza, 2003a) and evening primrose meal (Wettasinghe & Shahidi, 1999b), anthocyanins from black currants (Cacace & Mazza, 2003b) and sunflower hull (Gao & Mazza, 1996) and vitamin E from wheat germ (Ge et al., 2002), among others.

In this thesis, the effect of SC-CO_2 extraction conditions on the yield and the quality of Gac seed oil was first investigated and optimised using RSM. The effectiveness of the SC-CO_2 extraction method was also compared with the conventional Soxhlet extraction technique in terms of oil yield and oil characteristics. Secondly, the effectiveness of various solvents and of the assisted-extraction methods, MAE and UAE, on the yield of trypsin inhibitors, saponins and phenolic compounds was investigated. This was followed by the optimisation of the extraction of trypsin inhibitors and saponins using one-factor-at-a-time and RSM methodologies. The antioxidant activity and anticancer potential were also evaluated for extracts produced using different solvents. Finally, a freeze dried trypsin inhibitor-enriched powder was prepared using the optimal conditions for extracting the Gac seed trypsin inhibitors.

1.8. Hypotheses, Aims and Objectives

The working hypotheses for this study were that:

1) Gac seeds contain high levels of extractable oil, trypsin inhibitors and saponins and their yield can be optimised using different extraction methods and solvents.

2) The Gac seed extracts are of high quality and possess biological activity, including antioxidant and anticancer properties.

The aim of the thesis was to extract oil, trypsin inhibitors and saponins from Gac seeds with high yields.

In order to test the hypotheses and achieve the aim, the objectives of this thesis were as follows:

1. To optimise the extraction of oil from the dried Gac seeds using SC-CO_2. The conditions evaluated were the critical temperature, pressure and flow rate of the fluid CO_2.

2. To characterise the Gac seed oil extracted by SC-CO_2 and compare it with the oil extracted by the conventional Soxhlet method.

3. To determine suitable extraction methods for Gac seed trypsin inhibitors and saponins.

4. To optimise the extraction of Gac seed trypsin inhibitors and produce a freeze dried trypsin inhibitor-enriched powder. The conditions evaluated were 1) type of solvent, 2) time of extraction and 3) the ratio of Gac seed material to solvent.

5. To determine the optimal method and conditions for the ethanol extraction of Gac seed saponins. The conditions evaluated were 1) microwave conditions (power, irradiation time) and 2) the ratio of ethanol to Gac seed material.

6. To evaluate the antioxidant activity of the Gac seed oils and bioactive extracts from different extraction methods and solvents.

7. To evaluate the anticancer potential of Gac seed bioactives extracted using various solvents.

A summary of the research design used to achieve the aims and objectives of this thesis is shown in Figure 11.

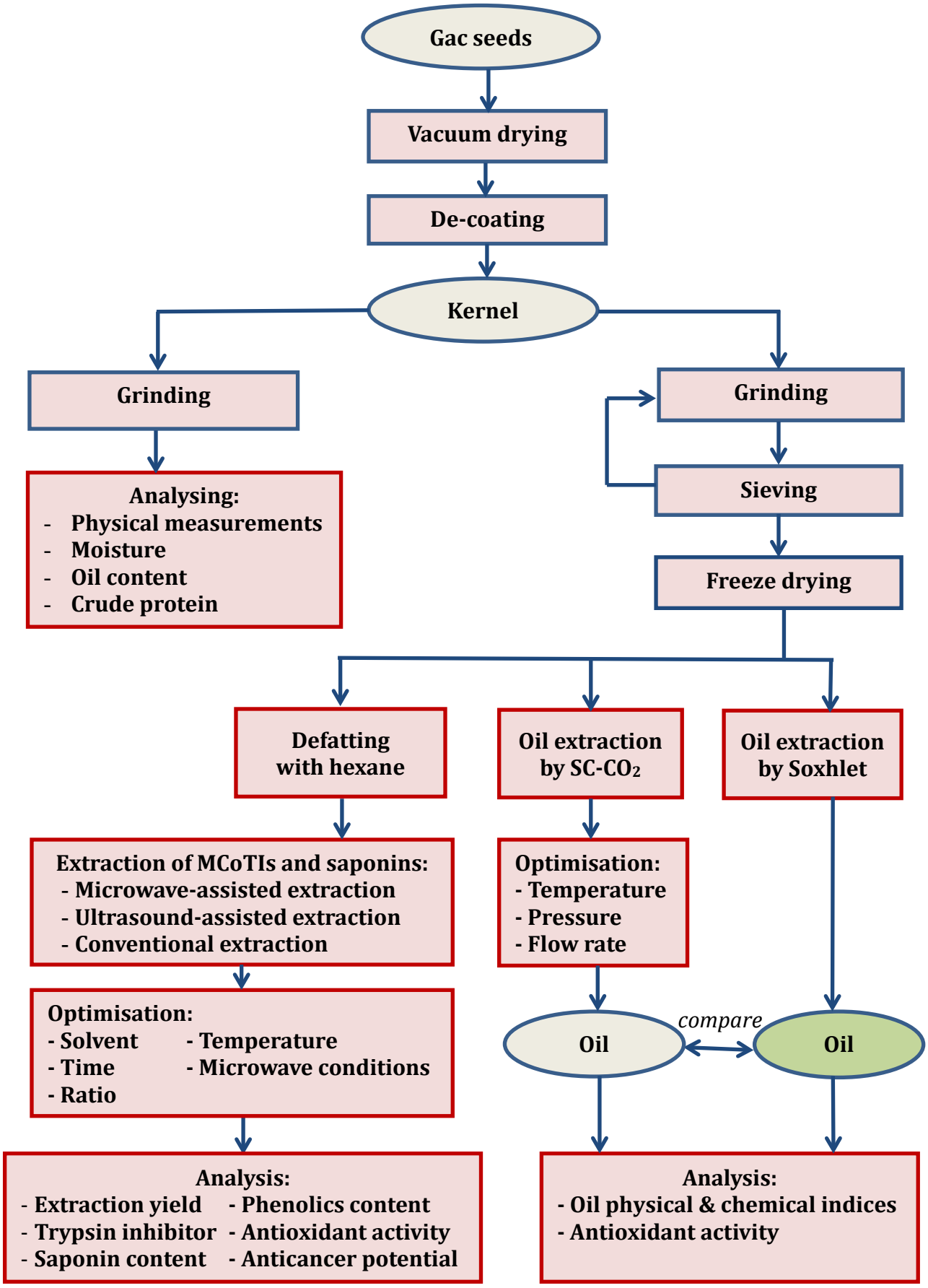


Figure 11: Diagram summarizing the research design.

Chapter 2: MATERIALS AND METHODS

2.1. Gac seeds

Fully ripe Gac fruits (450 kg) were purchased at farms in Ho Chi Minh (HCM) city – Vietnam (Latitude: 10.757410; Longitude: 106.673439) (Figure 12). Traditionally classified, Gac fruit has three varieties, namely ordinary Gac, sticky Gac and hybrid Gac. Gac fruits used in this project belong to the sticky variety, which has a bigger size of fruit and more seeds. According to the classification by Wimalasiri et al. (2015), the fruits belong to accession VS7 with a global-oval shape, dense spikes (Figure 13A) and blackish-brown seeds (Figure 13D).

Figure 12: Geographical locations of *Momordica cochinchinensis* in Vietnam. *(Adapted from Wimalasiri et al. (2015)).*

The fruits were harvested in May 2015 at the fully ripened stage, 20 days after emergence, when the whole skin had turned orange. Just after purchasing, the fruits were washed, cut into halves (Figure 13B) and the seeds were removed from the aril (Figure 13C). The resulting seeds were washed with water and left to air-dry before vacuum drying.

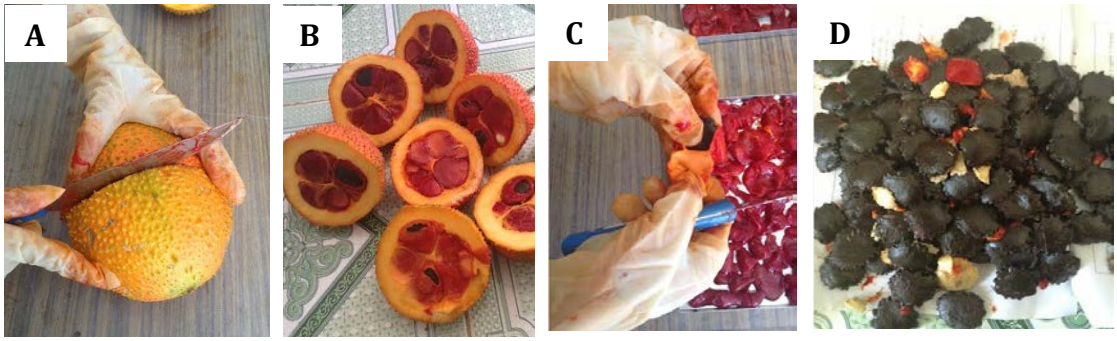


Figure 13: Collecting of Gac seeds.
(A)Gac fruit in global-oval shape; (B)Half-cut; (C)Removing aril; (D)Blackish brown seeds.

2.1.1. Drying of the Gac seeds

To facilitate temporary storage and de-coating, the fresh Gac seeds were vacuum dried at 40 ^{o}C until a moisture content below 7% (w/w) was obtained. The dried seeds were then de-coated to remove the seed shells (Figure 14).

2.1.2. De-coating of the Gac seeds

The shells of the dried seeds were removed manually using the back of a heavy knife (Figure 14A). The kernels (24 kg), along with their thin silver green skins (Figure 14B) were collected and packaged in aluminium bags of 1 kg, vacuum sealed (Figure 14C), air shipped to Australia and stored in a fridge (4 °C) until required.

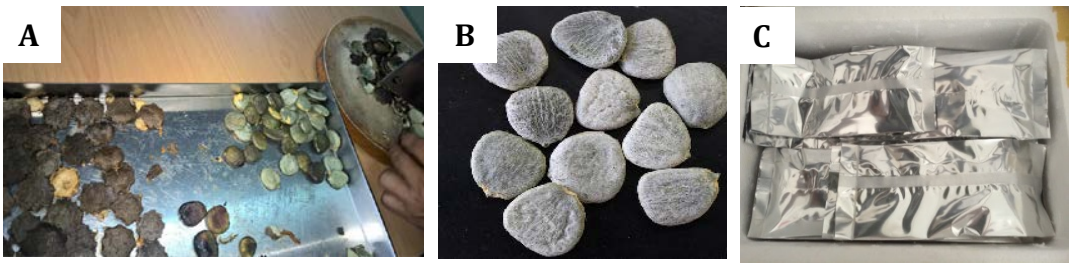


Figure 14: De-coating of Gac seeds.
(A) Decoating; (B) Whole Gac kernel; (C) Bags of Gac kernel.

2.1.3. Grinding of the Gac seeds

Prior to extraction, the seeds will be ground into powder of particle sizes less than 1.4mm or 0.5mm using the 100g Mulry disintergrater ST-02A (China) (Figure 15A). The size of the ground seeds will be measured using the Endecotts Test Sieve (London, England) (Figure 15B). Theoretically, the smaller the particle size, the higher the yield of extraction is. However, preliminary trials showed that having particle sizes under 0.5 mm was suitable for SC-CO_2 extraction and under 1.4 mm was suitable for other liquid-solid extractions. This may be because, at too small particle sizes, powders will coagulate and give a mud-like consistency, which will lead to a decrease in extraction yields (Snyder et al., 1984). Therefore, the powder that went through the 500 µm sieve was used for oil extraction with SC-CO_2 and powder that went through the 1.4 mm sieve was used for all other experiments in this study. The grinder and sieves are shown in Figure 15A and Figure 15B, respectively.

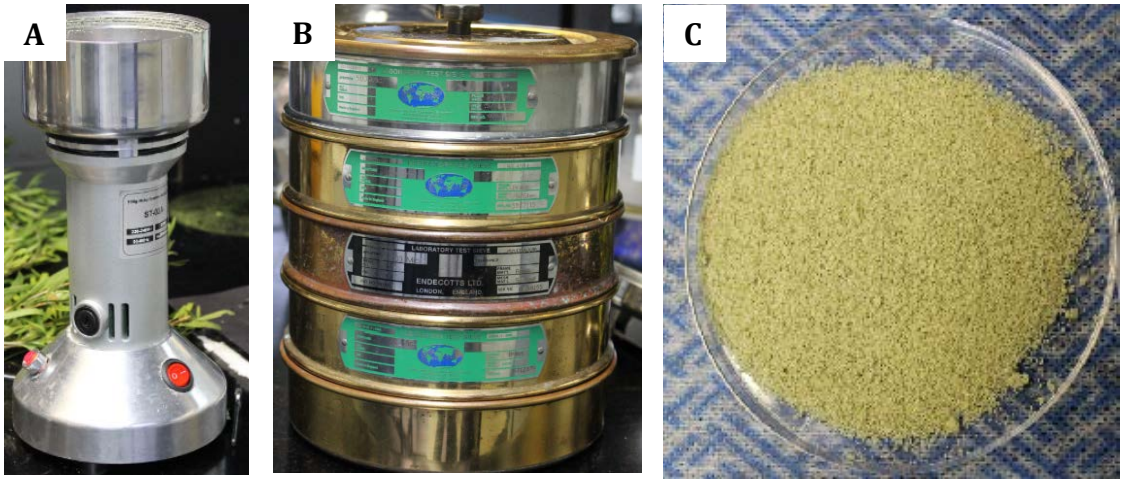


Figure 15: Grinding of Gac seeds.
(A) Grinder; (B) Sieve set; (C) Ground Gac seeds

2.1.4. Freeze-drying of the Gac seed powder

After grinding, the Gac seed powder will need to be dried. The aim of this process is to reduce the moisture content in the material in order to facilitate oil extraction. The elimination of moisture from oil-containing materials is known to increase the quality and yield of the oil extracted (Kha et al., 2013b). Due to the heat sensitive properties of bioactives in general and antioxidants in particular, the freezed-drying method was chosen for this treatment. Moreover, in relation to the Soxhlet extraction of oils, preliminary trials revealed that oil yields were significantly higher from freeze-dried samples than from air-dried ones.

Gac seed powder will be put in aluminium foil pans, placed on drying trays and dried in a freeze dryer (FD3, Dynavac, Sydney, NSW, Australia) (Figure 16) for 24 h. The moisture content of the dried powder will then be measured using a moisture determination balance (AD-4712, A&D Company Limited, Japan). The dried seed powder will then be stored in plastic boxes and kept frozen (-20 °C) until needed for extraction.

Figure 16: Freeze dryer (FD3 Dynavac).

2.2. Optimisation the extraction of oil

Gac seed oil extraction was optimised with SC-CO_2 method, using RSM. There were three parameters investigated: 1) Extraction temperature, 2) CO_2 pressure and 3) CO_2

flow rate. The optimised oil extracted by SC-CO_2 was compared with the oil extracted by the standard method – Soxhlet, in terms of oil yield, physical and chemical properties and antioxidant capacity.

2.2.1. Supercritical carbon dioxide extraction of Gac seed oil

2.2.1.1. Extraction procedure

A laboratory-scale SFE system (SFX 2-10, Teledyne Isco, USA; Syringe Pump 260D Teledyne Isco, Lincoln, Nebraska, USA) (Figure 17 and Figure 18) was used in this study. Dried Gac seed powder was accurately weighed around 4 g, packed in a sample cartridge with glass sand (1:10 w:w). The filled cartridge (Figure 19A) was inserted into the thermal-controlled extraction cell. Liquefied CO_2 was introduced into the sample cartridge through a piston pump with a cooling jacket. Both the pressure and temperature of the cartridge were automatically reached and maintained by a control unit according to the settings. The system was held for 30 min under the desired conditions when the oil had stopped coming out. The flow rate of CO_2 was regulated by both the pressure-releasing valve and a thermal-controlled restrictor and monitored by a flow metre. Oil was finally separated from the CO_2 phase and collected in the collector vial, which was at ambient temperature and atmospheric pressure (Figure 19B). Oils obtained by SC-CO_2 at the different conditions was then weighed to obtain the yield. The Gac seed oil yield was expressed as g of oil per 100 g of Gac seeds.

Figure 17: Supercritical fluid extraction system.

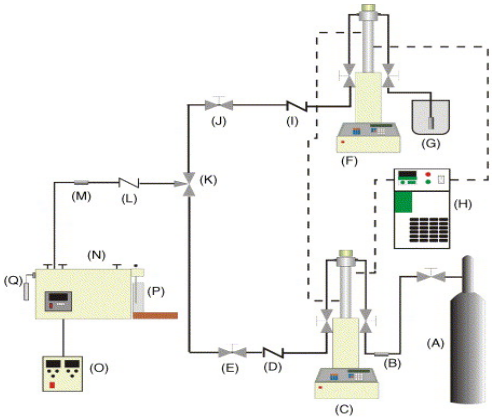


Figure 18: Schematic of the supercritical extraction (SC-CO_2) apparatus

(A) CO_2 cylinder; (B & M) filters; (C) Isco 260D syringe pump; (D, L & I) check valves; (E & J) gate valves; (F) Isco 260D syringe pump; (G) modifier vessel with in-line filter; (H) chiller/circulator; (K) valco mixing tee; (N) Isco SFX 2-10 unit; (O) restrictor temperature controller; (P) trapping vessel; (Q) vent valve. *(Wood et al., 2006).*

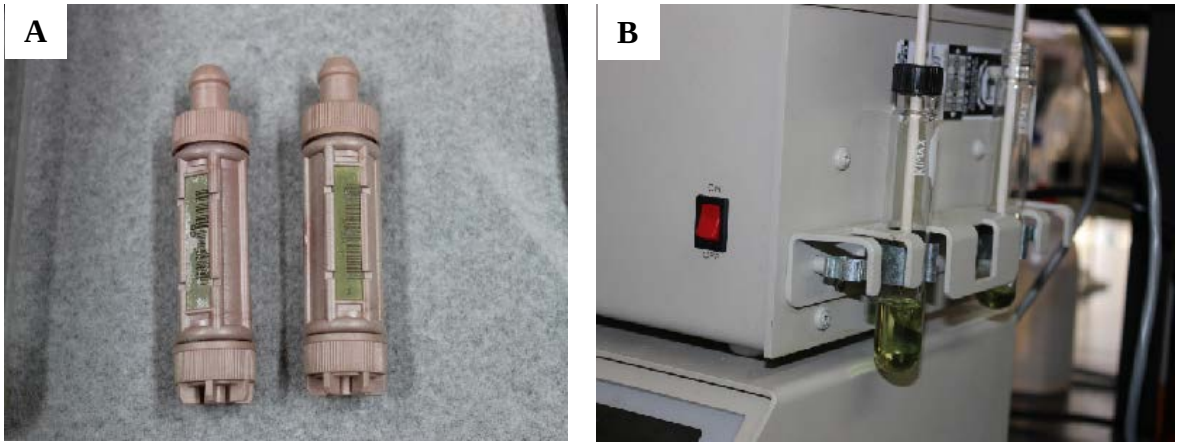


Figure 19: Cartridges of seed powder (A) and extracted oils (B).

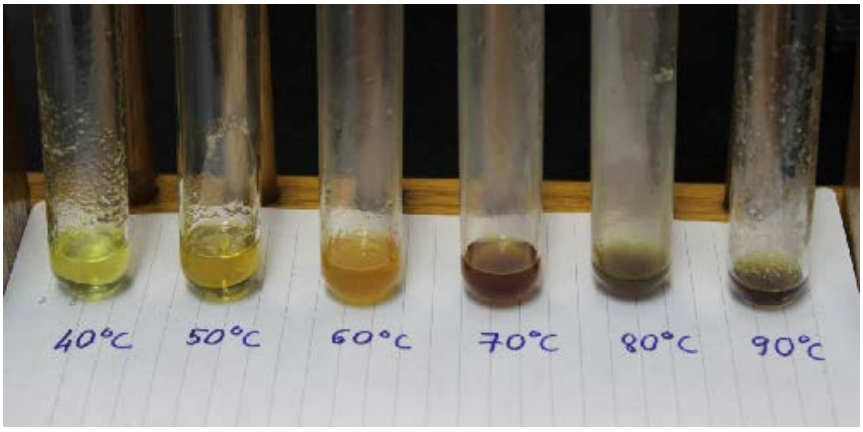


Figure 20: Changes in colour of extracted oil samples at various extraction temperatures.

2.2.1.2. Optimisation process

The efficiency of SC-CO_2 extraction can be affected by many factors including pressure, temperature and flow rate. In most of the previous studies, the process conditions have been merely optimised by conducting one factor-at-a-time experiments. The results of one-factor-at-a-time experiments do not reflect actual changes in the environment as they ignore interactions between factors that are present simultaneously. Therefore, these factors may be collectively studied to validate the optimal extraction conditions using the RSM.

The RSM with central composite design (CCD) was employed to investigate the effect of SC-CO_2 extraction on the yield of oil from the Gac seed powder. Three independent parameters namely, extracting pressure, extracting temperature and supercritical fluid flowrate at three different levels each, were tested. The parameters chosen and their levels were based on preliminary experiments. For designing and analysing the RSM experiment, including generating the three-dimensional (3D) surface and two-dimensional (2D) contour plots, the JMP software version 11.0 (SAS, Cary, NC, USA) was used. The adequacy of the RSM second-order polynomial model was determined based on the lack of fit and the coefficient of determination (R^2).

2.2.2. Soxhlet extraction of Gac seed oil

A conventional method, the Soxhlet extraction, was performed to compare the extraction performance of the SC-CO_2. The oil content of ground Gac seeds was determined according to the standard method AOCS: Ab 3-49 (AOCS, 1998). Gac seed kernel powder (7 g) was added to a cellulose thimble, plugged with glass wool and put into a Soxhlet extractor. Approximately 250 mL n-hexane were added into a pre-weighed boiling flask, which was fitted to the extractor and condenser (Figure 21). The solvent flow rate was recorded as 14 min/cycle, and the extraction process was terminated after 8 h. After extraction, the n-hexane was removed under reduced pressure at 50 ^{o}C using a Buchi Rotavapor B480 evaporator (Buchi Australia, Noble Park, VIC, Australia). The remaining traces of hexane were then removed using a flow of compressed nitrogen blowing on the surface of the oil until constant weight achieved (≈3 min).

Figure 21: Soxhlet system for the extraction of Gac seed oil.

The oils extracted by the Soxhlet method was compared with that extracted by SC-CO_2 at optimal conditions, in terms of: 1) oil yield, 2) antioxidant activity, 3) colour and 4) physicochemical characteristics.

2.3. Optimisation the extraction of trypsin inhibitors

For the extraction of Gac seed trypsin inhibitors, firstly we compared three extraction methods (conventional, MAE and UAE) using water and organic solvents (butanol, methanol and ethanol) to screen the suitable extraction method and solvent for Gac seed trypsin inhibitors (Paper III). Then, the convention extraction method with various aqueous solvents were optimised for the extraction of trypsin inhibitors (Paper IV).

2.3.1. MAE procedure

MAE was tested for the extraction of trypsin inhibitors from defatted Gac seeds with DI water as the extraction solvent. MAE was performed using a R395YS Sharp Carousel microwave oven (1200 W, Sharp Corporation, Bangkok, Thailand) at the set radiation power of 600 W. This level of power was chosen based on preliminary trials to make sure the mixture did not blow up during irradiating. An amount of 1.5 g of defatted Gac seed powder was mixed with 30 mL DI water in a 100 mL conical flask. The flask mouth was tightly wrapped with plastic film and the suspension was left soaking for 45 min at ambient temperature of 22 ± 1 °C before the microwave treatment was applied (four cycles of 10 s power ON and 15 s power OFF per cycle). The temperature of the suspension was recorded as 66 ± 1 °C at the end of the extraction process.

2.3.2. UAE procedure

UAE was tested for the extraction of trypsin inhibitors from defatted Gac seeds with DI water as the extraction solvent. Defatted Gac seed powder (1.5 g) was suspended with 30 mL of DI water. UAE was carried out in an ultrasonic bath (Soniclean, 220 V, 50 Hz and 250 W, Soniclean Pty Ltd., Thebarton, SA, Australia) with the working power and

temperature were set at 250 W and 40 °C, respectively. The maximum power of 250 W was chosen based on previous studies on ultrasound-assisted extraction of phenolic compounds (Bhuyan et al., 2017) and saponins (Wu et al., 2001). The suspensions were sonicated for 30 min in covered vessels to avoid evaporation. To measure the temperature of the ultrasonic bath, an external digital thermometer was also used. In the case that the ultrasonic bath exceeded the designated temperature, tap water was used to maintain the required temperature.

2.3.3. Optimised conventional extraction

2.3.2.1. Extraction procedure

There were four aqueous solvents being used that vary in the properties (i.e. based on polarity, pH, and salinity). These aqueous solvents were the best for extraction of trypsin inhibitors from various plant materials. The four extraction solvents were as follows:

1. The acetonitrile (ACN)/water/formic acid (FA) (25:24:1), which was found to be optimal for extracting cysteine knot peptides from Gac seeds, some of which are trypsin inhibitors (Mahatmanto, 2014).

2. The 0.1M NaCl solution, which was the optimal for extraction of trypsin inhibitors from *Chenopodium quinoa* seeds (Pesoti et al., 2015).

3. The 0.02M NaOH solution, which was the optimal for extraction of trypsin inhibitors from grass pea (Deshpande & Campbell, 1992).

4. Water, which was the optimal solvent for the extraction of trypsin inhibitors from Thai mung bean (Klomklao et al., 2011) and Gac seeds (Le et al., 2018).

Finally, the optimisation for the conventional extraction was performed to obtain an extract rich in trypsin inhibitors, which was finally used to produce a freeze-dried powder.

2.3.2.2. Optimisation process

The efficiency of the conventional extraction can be affected by many factors including the solvent used, extraction time, extraction temperature and the ratio of sample to solvent. In previous studies for the extraction of trypsin inhibitors, the process conditions have been merely optimised by conducting one factor-at-a-time experiments. The results of one-factor-at-a-time experiments do not reflect actual changes in the environment as they ignore interactions between factors that are present simultaneously. Therefore, these factors may be collectively studied to validate the optimal extraction conditions using the RSM.

The RSM with Box Behnken design was employed to investigate the effect of extraction conditions on the yield of trypsin inhibitors from the defatted Gac seed kernel. Three independent parameters namely, NaCl concentration of the extraction solvent, extraction time and the ratio of Gac seed material to solvent, at three different levels each, were tested. The parameters chosen and their levels were based on preliminary experiments. For designing and analysing the RSM experiment, including generating the three-dimensional (3D) surface and two-dimensional (2D) contour plots, the JMP software version 13.0 (SAS, Cary, NC, USA) was used. The adequacy of the RSM second-order polynomial model was determined based on the lack of fit and the coefficient of determination (R^2).

2.4. Optimised MAE for extraction of Gac seed saponins

A range of methods has been used for the extraction of saponins from various plant tissues. These methods were developed based on the solubilities of the saponins to be extracted and the types of plant tissues from which they were derived (Cheok et al., 2014). For the extraction of Gac seed saponins, firstly we tested with the defatted material and compared three extraction methods (conventional, MAE and UAE) using water and organic solvents (butanol, methanol and ethanol) (Paper III). Then, MAE method with ethanol was used to optimised the extraction of Gac seed saponins (Paper V) as described in Section 2.4.1 below.

2.4.1. Extraction procedure

Firstly, the effectiveness of MAE for saponins was tested with the material was defatted Gac seeds. The extraction was performed using a R395YS Sharp Carousel microwave oven (1200 W, Sharp Corporation, Bangkok, Thailand) at the set radiation power of 600 W. An amount of 1.5 g of defatted Gac seed powder was mixed with 30 mL DI water in a 100 mL conical flask. The flask mouth was tightly wrapped with plastic film and the suspension was left soaking for 45 min at ambient temperature of 22 ± 1 °C before the microwave treatment was applied (four cycles of 10 s power on and 15 s power off per cycle). The temperature of the suspension was recorded as 66 ± 1 °C at the end of the extraction process.

Consequently, optimisation for saponin extraction was carried out as figured in Section 2.4.2.

2.4.2. Optimisation process

The optimisation process for the extraction of Gac seed saponins is shown in Figure 22 and described in more detail in Paper VI.

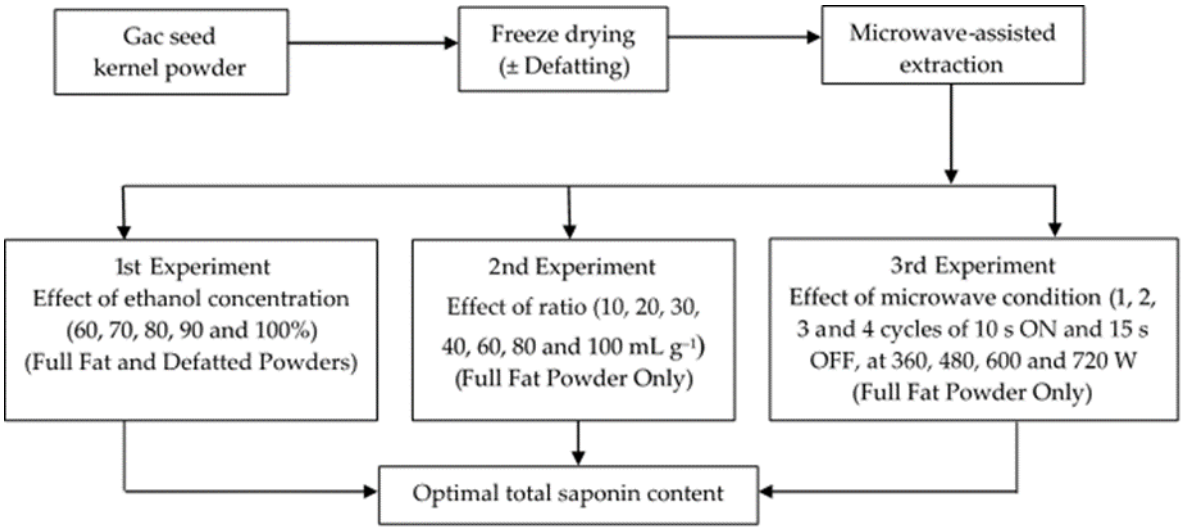


Figure 22: Experimental design for optimisation of saponin yield from Gac seeds.

2.5. Evaporation of solvents from extracts

The extracts of trypsin inhibitors and saponins, were concentrated at 40 ^{o}C while the oil hexane extract was concentrated at 50 ^{o}C, using a rotary vacuum evaporator (Buchi Rotavapor B-480, Buchi Australia, Noble Park, Victoria, Australia) to remove the solvent(s). The concentrated extracts containing the bioactives were then be freeze-dried. The content of saponins and phenolic compounds were analysed according to the methods presented in sections 2.6.6 and 2.6.7. The trypsin inhibitor activity and protein content were analysed according to the methods presented in sections 2.6.4 and 2.6.5. The total antioxidant capacity and anticancer potential of the crude extracts or powders were analysed according to the methods presented in sections 2.6.8 and 2.6.9.

2.6. Analysing methods

This section covers all of the methods which were used for analysing Gac seed oil and bioactive compounds.

2.6.1. Oil characterising

Characteristics of an oil greatly define its applications. Therefore, it is necessary to analyse the characteristics of Gac seed oil extracted in this thesis, in order to better see its potential uses. The physical and chemical indices of Gac seed oils extracted using SC-CO_2 and Soxhlet methods were determined according to AOCS methods (AOCS, 1998) as described in following subsections 2.6.1.1 to 2.6.1.10.

2.6.1.1. Specific gravity

This index determines the ratio of the mass of a unit volume of the oil sample at 25 ^oC to the mass of a unit volume of water at 20 ^oC (AOCS, 1998). The specific gravity of Gac seed oil samples was measured according to AOCS Cc 10a-25. Ten millilitres of the oil sample was put in a pre-weighed test tube and warmed up 25 ^oC in a water bath. The tube was then weighed using an analytical balance. The weighing procedure was carried out similarly for DI water except being warmed up to 20 ^oC. The specific gravity of Gac seed oil samples was calculated according to the Equation 1.

$$Specific\ gravity\ = \frac{mass\ of\ oil\ sample}{mass\ of\ equal\ volume\ of\ water}$$

Equation 1: The calculation for the specific gravity of Gac seed oil.

2.6.1.2. Refractive index

The refractive index of an oil is defined as the ratio of the speed of light in a vacuum to the speed of light in the oil. However, for practical measurements, the scales of standard instruments indicate refractive index with respect to air, rather than vacuum (AOCS, 1998). The refractive index is a parameter that relates to molecular weight, fatty acid chain length, degree of unsaturation, and degree of conjugation (Gunstone, 2011).

The refractive index of Gac seed oil samples was measured according to AOCS Cc 7-25. Oil sample (5 g) was weighed into a test tube and heated to 40 ^{o}C in a water bath. Two drops of the heated oil sample was placed on the lower prism of a refractometer. The prisms were closed tightly and left stand for 2 min before reading.

2.6.1.3. Slip melting point

This index indicates the temperature at which fat softens and becomes sufficiently fluid to slip in an open capillary tube (AOCS, 1998).

The slip melting point of Gac seed oil samples was measured according to AOCS Cc 3-25. Oil sample (10 g) was weighed into a test tube and completely melted in a water bath set at 40 ^{o}C. Three clean capillary tubes were dip in to the oil so that the oil rose about 10 mm high in the capillary tubes. The ends of the tubes were chilled by holding against a piece of ice until the oil solidified then the tubes were kept in a refrigerator at 4 ^{o}C overnight. The tubes were removed from the refrigerator and attached to a thermometer whose mercury bulb was then suspended in a beaker containing DI water. The beaker was heated slowly in a water bath initially set at 15 ^{o}C and its temperature was increased 1^{o}C per minute until the fat column rose in each tube. The average temperature at which three columns rose was reported as slip point.

2.6.1.4. Iodine value

The iodine value (IV) is a chemical constant and a measure of the unsaturation of fats and oils (Codex, 2001). This value reflects the ability of an unsaturated carbon-to-carbon bond to absorb halogen atoms (Uquiche et al., 2008).

The IV of Gac seed oil samples was measured according to AOCS Cd 1-25. Oil sample, which had been completely melt at 35 ^{o}C in a water bath and filtered through a piece of Whatman No.1 filter paper, was accurately weighed (600.0 mg) into a flask to which had been added 20 mL of CCl_4. 25 mL of the Wijs solution of 13.0 g of iodine in 1 L of glacial acetic acid was pipetted into the flask containing the sample and swirled. The flask was stoppered and left in the dark for 30 min at ambient temperature. The flask was removed from storage and 20 mL of KI solution was added, followed by 100 mL of DI water. The solution was titrated with 0.1N $Na_2S_2O_3$ solution until the yellow colour had almost disappeared. Two millilitre of starch indicator solution was added the titration was continued until the blue colour had just disappeared. A blank was carried out simultaneously and similar in all respects, using DI water instead of oil sample. The results were calculated according the Equation 2, where *B* is the quantity of $Na_2S_2O_3$ used for blank (mL), *S* is the quantity of $Na_2S_2O_3$ for oil sample (mL) and N is the normality of $Na_2S_2O_3$ solution.

$$IV = \frac{(B - S) \times N \times 12.69}{mass\ of\ oil\ sample\ (g)}$$

Equation 2: The calculation for the iodine value (IV) of Gac seed oil.

2.6.1.5. Saponification value

The saponification value (SV) is a measure of the alkali-reactive groups in oils and can usually be used for predicting the type of glycerides in a sample. The higher the SV the lower is the molecular weight of the oil (Kasote et al., 2013).

The SV of Gac seed oil samples was measured according to AOCS Cd 3-35. Oil sample (1.0 g) was weighed into a conical flask to which was then added with 15 mL 1N KOH and 10 mL of distilled water and heated under a reserved condenser for 30–40 min to ensure that the sample was fully dissolved. After this sample was cooled to ambient temperature, phenolphthalein was added and titrated with 0.5 M of HCl until a pink endpoint was reached. A blank was determined with the same conditions. The results were calculated as Equation 3, where *B* is the quantity of sodium hydroxide used for blank, *S* is the quantity of sodium hydroxide used for sample and *W* is the weight (g) of the oil sample.

$$SV = \frac{(B - S) \times 28.2}{W}$$

Equation 3: The calculation of saponification value of Gac seed oil.

2.6.1.6. Free fatty acid

The free fatty acid (%FFA) is defined as the mass percentage of free oleic acid (mg) in oil (g). The %FFA value of Gac seed oil samples was measured according to AOCS Ca 5a-40. Ethanol (25 mL) was added to 1.5 g of each oil sample contained in the different conical flasks. The mixture was brought to boil in a water bath then cooled. Two drops of phenolphthalein indicator were added to the solution. 0.1 M NaOH was used to titrate the mixture with constant shaking for proper mixing. %FFA was calculated as the Equation 4, where V is titre value (mL).

$$\%FFA = \frac{V \times 0.0282}{Weigh\ of\ the\ sample} \times 100$$

Equation 4: The calculation for the free fatty acid value of Gac seed oil.

2.6.1.7. Peroxide value

The peroxide value (PV) is an important parameter used to evaluate the quality of oil, measures the quantity of hydroperoxides in the oil, which are formed by the reaction between oxygen and unsaturated fatty acids. This value is used to evaluate the initial stages of the oxidation process (Van Hoed et al., 2010).

The PV of Gac seed oil samples was measured according to AOCS Cd 8-53. Extracted oils (5 g) from the Gac seeds were placed in a 250 mL erlenmeyer flask and dissolved in 30 mL of a mixture containing acetic acid and chloroform (3:2, v/v), and then the mixture was stirred for a few seconds to ensure thorough mixing. Thereafter, 0.5 mL of saturated potassium iodide solution was added. After exactly one minute, deionized water (30 mL) was added and it was titrated with 0.01N sodium thiosulfate using starch solution as an indicator until the solution became colorless. The mixture was magnetically stirred during the titration procedure. The results were calculated as milliequivalents (meq) of active oxygen per kg oil as Equation 5, where *S* and *B* are the titration amount of 0.01 N sodium thiosulfate for sample and blank (in mL), respectively. *N* is the normality of the sodium thiosulfate solution.

$$PV\left(\frac{meq}{kg}\right) = (S - B) \times N \times \frac{1000}{mass\ of\ oil\ sample(g)}$$

Equation 5: The calculation for the peroxide value (PV) of Gac seed oil.

2.6.1.8. Unsaponifiable matter

The unsaponifiable matter measures those substances frequently found dissolved in fats and oils, which cannot be saponified by the usual caustic treatment, but are soluble in ordinary fat and oil solvents. Included in this group of compounds are higher aliphatic alcohols, sterols, pigments, and hydrocarbons (AOCS, 1998).

The unsaponifiable matter of Gac seed oil samples was measured according to AOCS Ca 6a-40. Gac seed oil sample (5.0 g) was added with 30 mL of 95% ethanol and 5 mL of 50% KOH solution. The mixture was mixed and boiled gently under reflux for 1 h then completely transferred to an extraction cylinder to the 40 mL mark with 95% ethanol. The transfer was completed with warm and then cold water until the total volume was 80 mL. The flash was washed out with 5 mL of petroleum ether and added to the cylinder. The contents of the cylinder was cooled down to ambient temperature and then 50 mL of petroleum ether was added. The cylinder was stoppered and shaken vigorously for 1 min and allowed to settle until both layers were clear. The upper layer was removed completely using a glass siphon. The extraction was repeated six times and the petroleum ether fractions were combined in a 500 mL separatory funnel. The combined extracts in the separatory funnel was washed three times, using 25 mL portions of 10% ethanol in DI water (v/v). After each washing, the aqueous ethanolic layer was drawn off. The petroleum ether extract was then transferred to a tared beaker and evaporated to dryness in a fume hood, using a stream of nitrogen. The beaker was placed in a vacuum oven set at 70 ^{o}C and 60 KPa for 2 h for completely drying. The beaker was cooled down to ambient temperature in a desiccator and weighed. The value became “A” in the calculation (Equation 6).

After weighing, the residue was taken up in 50 mL of warm (50 ^{o}C) 95% alcohol, containing phenolphthalein indicator previously neutralised to the phenolphthalein end point. The solution was titrated with 0.02M NaOH to the same final colour. The weight of the residue was corrected for free fatty acid content using the following relationship: 1 mL of 0.02 NaOH was equivalent to 0.0056 g of oleic acid. The grams of fatty acid determined by this titration became "B" in the calculation (Equation 6).

A blank was carried out simultaneously and similar in all respects, using DI water instead of oil sample. The blank determined by this procedure became "C" in the calculation Equation 6.

The unsaponifiable matter of the oil sample was calculated according to the Equation 6.

$$Unsaponifiable\ matter, \% = \frac{A - (B + C)}{mass\ of\ oil\ sample\ (g)} \times 100$$

Equation 6: The calculation of the unsaponifiable matter of Gac seed oil.

2.6.1.9. Moisture and volatile matter

This index is defined as the percentage of the moisture and any other material volatile under the conditions of the test (AOCS, 1998). The moisture and volatile matter of Gac seed oil samples was measured according to AOCS Ca 2c-25. Five grams of each oil sample were weighed into a tared moisture dish that had been dried and cooled previously in a desiccator. The dish was placed in air oven and dried for 30 min at 130 ± 1^{o}C and then cooled to ambient temperature in a desiccator and weighed. The drying procedure was repeated three times until the loss in weight did not exceed 0.05% per 30 min of drying. The moisture and volatile matter was calculated according to the Equation 7.

$$Moisture\ and\ volatile\ matter, \% = \frac{loss\ in\ mass\ (g)}{mass\ of\ test\ portion\ (g)} \times 100$$

Equation 7: The calculations for the percentage of moisture and volatile matter of Gac seed oil.

2.6.1.10. Insoluble impurities

This test determines dirt, meal, and other foreign substances insoluble in kerosene and petroleum ether. The insoluble impurities of Gac seed oil samples was measured according to AOCS Ca 3a-46. The residue from the moisture and volatile matter determination was used (Section 2.6.1.9). 50 mL of petroleum ether was added to the residue and heated in a water bath set at 50 ^{o}C. The mixture was filtered through a pre-dried and weighed Gooch crucible with the aid of vacuum. The crucible was washed five times with 10 mL hot petroleum ether portions. The crucible and contents were dried at 101±1^{o}C for 2 h, cooled to ambient temperature and weighed.

The insoluble impurities of Gac seed oil sample was calculated according to Equation 8.

$$Insoluble\ impurities, \% = \frac{gain\ in\ mass\ of\ crucible}{mass\ of\ test\ portion\ for\ moisture} \times 100$$

Equation 8: The calculation for the insoluble impurities of Gac seed oil.

2.6.2. Oil antioxidant activity

The Gac seed oils were analysed for their antioxidant activity using the DPPH and the ABTS radical-scavenging assays.

- ***DPPH radical-scavenging assay***

The scavenging activity of Gac seed oils towards DPPH radicals was determined by the method of Tan et al. (2014a) with some modifications; the main modification was that

ethyl acetate was used as the solvent instead of methanol to ensure the oil samples were well dissolved. Each of the oil samples (0.20 g) was diluted in ethyl acetate (10 mL). A stock solution of 0.6 mM DPPH in ethyl acetate was prepared and kept at 4 ^{o}C to be used within a week. Fresh working solution was prepared for each assay by mixing 8 mL of stock solution with 42 mL of ethyl acetate to obtain an absorbance of 1.1 ± 0.02 units at 515 nm. The reaction was initiated by mixing the diluted oil sample (0.15 mL) with the DPPH working solution (3.85 mL). Absorbance was measured at λ_{max} of 515 nm in a Cary 60 UV–vis spectrophotometer (Agilent Technologies, Mulgrave, VIC, Australia) after 6 h storage in the dark (experimentally determined as the steady state of reaction). Trolox was used as a standard and the anti-oxidant activity was expressed as µmol trolox equivalents (TE) per g of oil (µmol TE/g).

A standard curve of trolox was prepared for the determination of DPPH antioxidant activity of Gac seed oil samples. A stock solution of 1,000 µmole/L trolox was prepared triplicate in ethyl acetate. A serial dilution of the stock solution in ethyl acetate is displayed in Table 6.

Table 6. Preparation of the trolox standard curve for the determination of DPPH antioxidant activity of Gac seed oils.

Tube No.	Dilution	Concentration (µmole/mL)	Absorbance (515 nm)
(1)	4 mL stock solution + 1 mL EA	800	0.3093 ± 0.0146
(2)	2 mL stock solution + 2 mL EA	500	0.5885 ± 0.0113
(3)	2 mL (1) + 2 mL EA	400	0.6955 ± 0.0026
(4)	2 mL (3) + 2 mL EA	200	0.8749 ± 0.0055
(5)	2 mL (4) + 2 mL EA	100	0.9606 ± 0.0062
(6)	2 mL (5) + 2 mL EA	50	1.0021 ± 0.0048
(7)	2 mL (6) + 2 mL EA	25	1.0301 ± 0.0129
(8)	2 mL (7) + 2 mL EA	12.5	1.0317 ± 0.0061
Control	Only EA	0	1.0332 ± 0.0043

EA: Ethyl acetate.

The difference between the absorbance of trolox solutions with the absorbance of the control was plotted to build the standard curve to determine the DPPH antioxidant activity of Gac seed oil samples (Figure 23).

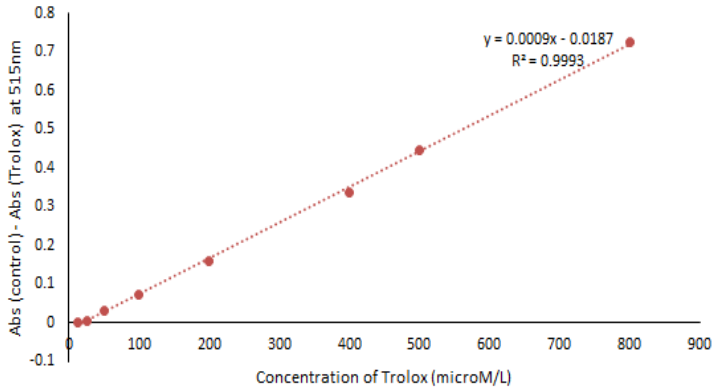


Figure 23: The Trolox standard curve for DPPH assay for Gac seed oils.

- ***ABTS assay***

The ABTS assay was conducted according to Christodouleas et al. (2015) with several modifications to ensure it worked with Gac seed oil samples. The ABTS was dissolved in pure methanol and potassium persulfate ($K_2S_2O_8$) was dissolved in 50% methanol to a final concentration of 5.92mM and 2.08mM, respectively. These two solutions were mixed at a 1 : 1 (v/v) ratio and the mixture was allowed to stand in the dark at ambient temperature for 24 h in order to produce the ABTS free radical ($ABTS^{\bullet+}$). Then, the solution was diluted with ethanol at a ratio of 1:15 (v/v) solution: ethanol and left in the dark for another 3 h to obtain an absorbance of 1.00 ± 0.02 units at 734 nm. Each oil sample (0.2 g) was dissolved in 10 mL of dichloromethane. The reaction was initiated by mixing the dissolved oil sample (0.15 mL) with the diluted $ABTS^{\bullet+}$ solution (2.85 mL) left to stand in the dark for 2 h. The absorbance was then measured at the λ_{max} of 734 nm in a Cary 60 UV–vis spectrophotometer (Agilent Technologies, Mulgrave, VIC, Australia). Trolox was used as a standard and the antioxidant activity was expressed as μmol trolox equivalents (TE) per g of oil (μmol TE/g).

A standard curve of trolox was prepared for the determination of ABTS antioxidant activity of Gac seed oil samples. A stock solution of 1,000 µmole/L trolox was prepared triplicate in dichloromethane. A serial dilution of the stock solution was prepared using dichloromethane as displayed in Table 7.

Table 7. Preparation of the trolox standard curve for the determination of ABTS antioxidant activity of Gac seed oils.

Tube No.	Dilution	Concentration (µmole/mL)	Absorbance (734 nm)
(1)	Stock solution	1,000	0.0476 ± 0.0429
(2)	4 mL (1) + 1 mL DCM	800	0.2296 ± 0.0789
(3)	2 mL (1) + 2 mL DCM	500	0.4939 ± 0.0649
(4)	2 mL (2) + 2 mL DCM	400	0.5621 ± 0.0447
(5)	2 mL (4) + 2 mL DCM	200	0.7253 ± 0.0236
(6)	2 mL (5) + 2 mL DCM	100	0.8052 ± 0.0148
(7)	2 mL (6) + 2 mL DCM	50	0.8532 ± 0.0227
(8)	2 mL (7) + 2 mL DCM	25	0.8705 ± 0.0080
Control	Only DCM	0	0.8860 ± 0.0248

DCM: Dichloromethane.

The difference between the absorbance of trolox solutions with the absorbance of the control was plotted to build the standard curve to determine the ABTS antioxidant activity of Gac seed oil samples (Figure 24).

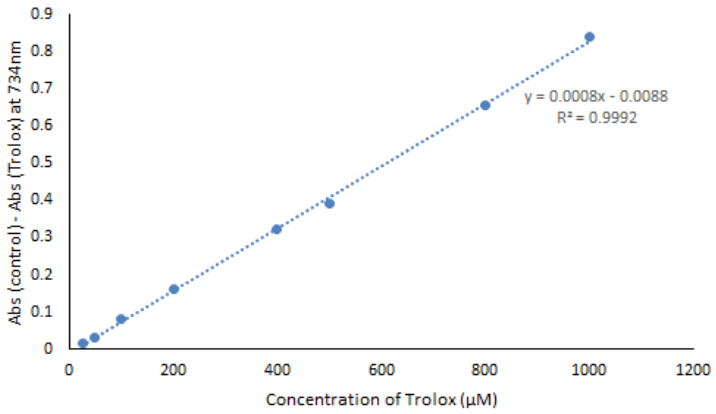


Figure 24: The Trolox standard curve for ABTS assay for Gac seed oils.

2.6.3. Oil colour measurement

The colour of Gac seed oils was conducted according to (Xu, 2003) with several modifications, using a chroma meter (Minolta CR-400, Minolta Camera Co., Ltd, Osaka, Japan). Oil samples were placed in standard disposable cuvettes (1 cm optical path), and a white calibration plate was used as the background (for illuminants D65, Y = 93.5, x = 0.3140, y = 0.3318). Before the measurements, the instrument was calibrated with deionized water. Measurements were performed on liquid oil samples at ambient temperature (27 ^{o}C). For each sample, three points along the height of the cuvette were measured, and the colour result of each sample was the average of the three measurements. The absolute measurements were displayed in L*, a* and b* co-ordinates as defined by CIE (Commission Internationale de l'Eclairage). The L* value represents the lightness-darkness dimension; the a* value represents the red-green dimension; and the b* value represents the yellow-blue dimension.

2.6.4. Determination of trypsin inhibiting activity using spectrometric method

2.6.4.1. Principle of the assay

Spectrometric methods has been established and adopted by AACC 71-10 (AACC International, 2000) and ISO 14902 (2001) for determining the TI activity. There are two different spectrometric methods known as Smith (Smith et al., 1980) and Kuntiz (Kunitz, 1946) methods, which used different substrates to assay the TI activity. The method of Smith used N-α-benzoyl-D,L-arginine-4-nitroanilide (BAPNA) as the substrate, while Kunitz used casein. Most spectrophotometric methods (Kowalska et al., 2007; Kreutzmann et al., 2004; Kubiak et al., 2009) used BAPNA as the substrate, which produced *p*-nitroanilide (PNA) that has the maximum UV adsorption at 410. The reaction

mechanism is shown in Figure 25. The presence of TIs can suppress the activity of trypsin and reduce the amount of reaction product PNA.

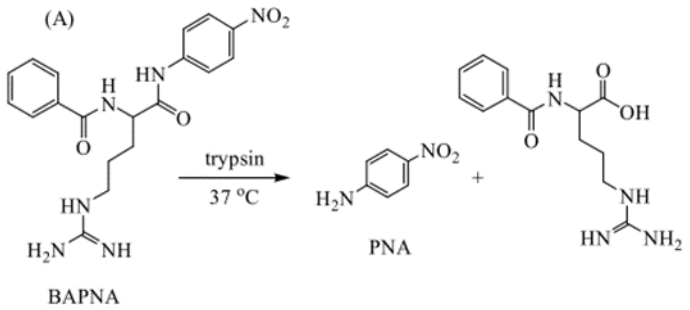


Figure 25: Enzymatic reaction mechanism of BAPNA.

Although the measurement at 410 nm is used widely, the wavelength of 385 nm was found to improve the measurement of TI activity (Stauffer, 1990).

2.6.4.2. Determination of trypsin inhibiting activity of Gac seed extracts

In the course of this study, trypsin-inhibitory activity (TIA) of the extracts was determined according to the method of Makkar et al. (2007) and Stauffer (1990). The inhibitor activity due to trypsin inhibitors of Gac seed extracts was measured indirectly by inhibiting the activity of pure trypsin. A synthetic substrate, benzyl-DL-arginine-para-nitroanilide (BAPNA) was subjected to hydrolysis by trypsin to produce the yellow-coloured *p*-nitroanilide. The degree of inhibition by the extracts of the yellow-color production, a measure of trypsin inhibitor activity, was measured at 385 nm.

Reagent preparation:

Tris-buffer (0.05M, pH 8.2) containing 0.02M $CaCl_2$: 6.05 g Tris and 2.94 g $CaCl_2.2H_2O$ dissolved in 900 mL of water. The pH was adjusted to 8.20 using 25 mL of HCl 1N, and the volume was brought to 1 L with water.

Substrate solution: A substrate solution of 92mM BAPNA was 40.0 mg BAPNA dissolved in 1.00 mL DMSO and diluted to 100 mL with the buffer solution pre-warmed to 37 ^{o}C. This solution was prepared daily and kept at 37 ^{o}C while in use.

Trypsin solution: 20 mg of trypsin (type I) from bovine pancreas was dissolved in 0.001M HCl to make 1 L, stored at 4 ^{o}C for use within 2-3 weeks. When subjected to the analytical procedure for the standard, 2 mL of this solution gave an absorbance value in the range of 0.576 ± 0.026 after subtracting the reagent blank at 385 nm.

Determination of TIA

From each filtrate 4 test tubes were prepared according to the compositions shown in Table 8. All the prepared test tubes were kept in a water bath at 37 ^{o}C for 10 min to promote the formation of an enzyme-inhibitor complex; and 5.0 mL of BAPNA solution pre-warmed to 37 ^{o}C was added into each tube at 30 s intervals. The contents of the tubes were well mixed after each addition. The tubes were then left in the water bath at 37 ^{o}C for incubation for another 10 min before 1 mL of 30% acetic acid solution was added to each tube to stop the reaction at 30 s intervals. Then 2.0 mL of trypsin solution was added into each blank tube. After thorough mixing, the absorbance of the reaction mixture due to the release of *p*–nitroanilide was measured at 385 nm.

Table 8. Reagent composition in extracted filtrate for the determination of trypsin inhibitory activity.

Component (mL)	Reagent blank (a)	Standard (b)	Sample blank (c)	Sample (d)
DI water	2	2	1	1
Trypsin solution	-	2	-	2
Diluted extract	-	-	1	1
Trypsin solution after reaction inactivation	2	-	2	-

Calculation:

The change in absorbance (A_I) due to trypsin inhibitor per mL diluted extract is ($A_b - A_a$) – ($A_d - A_c$), where the subscripts refer to tubes (a) to (d) above. Since 1µg pure trypsin would give an absorbance of 0.0190, the weight of pure trypsin inhibited per mL diluted extract is A_I/0.019µg. From this value, the trypsin inhibitor activity (TIA) is calculated in terms of miligrams of pure trypsin per gram of dried defatted Gac seed powder (mg/g) as per Equation 9.

$$TIA = \frac{A_I \times V \times D}{19 \times S \times (1 - m\%/100)}$$

Equation 9: The calculation for the trypsin inhibitor activity of Gac seed extracts.

where, A_I : Change in absorbance due to inhibition per 1 mL of diluted extract ($A_I = (A_b - A_a) - (A_d - A_c)$)

V: Volume of original extract (mL)

D : Dilution factor of the original extract

S : Sample weight (g)

19: Constant figure based on the absorbance given by 1mg of pure trypsin

m%: moisture content of defatted Gac seed powder

2.6.5. Determination of protein content

In this thesis, estimation of protein concentration is necessary for the determination of the specific activity of Gac seed trypsin inhibitor extracts. There are a wide variety of protein assays available including Bradford, Lowry, bicinchoninic acid (BCA) and Biuret. Each assay has its own advantages and limitations. However, the Lowry assay was chosen in this thesis for quantifying the protein concentration in the Gac seed trypsin inhibitor extract due to the availability of the chemicals and also the method is sensitive and simple.

2.6.5.1. Principle of the Lowry assay

In the Lowry assay, the protein solution is mixed with an alkaline solution of copper salt. Under alkaline conditions, cupric ions (Cu^{2+}) chelate with the peptide bonds resulting in the reduction of cupric (Cu^{2+}) to cuprous ions (Cu^{+}) (Figure 26). The cuprous ions are detected either with BCA or Folin Reagent (phosphomolybdic/ phosphotungstic acid). Cuprous ions (Cu^{+}) reduction of Folin Reagent produces a blue color that can be read at 650-750nm. The amount of colour produced is proportional to the amount of peptide bonds, i.e. size as well as the amount of protein/peptide.

The Lowry assay are reliable and has little variation among different protein. However, it has some limitations including being interfered by many substances (detergents, carbohydrate, glycerol,...) and requiring a longer reaction time.

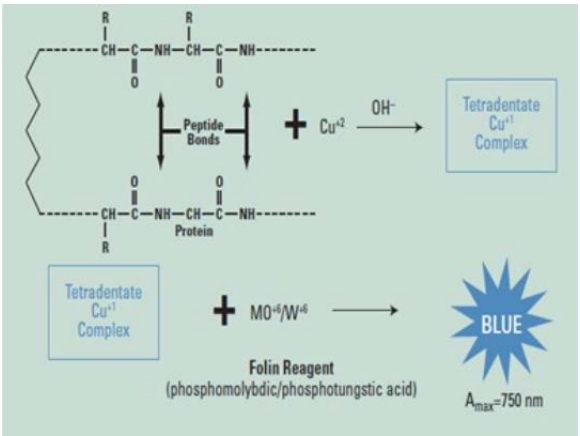


Figure 26: The interaction of proteins with copper ions and Folin reagent in the Lowry assay.

2.6.5.2. Building the standard curve

Standard curves of BSA were prepared in four media, which were used to extract trypsin inhibitors from defatted Gac seeds. A stock solution of 1 mg/mL was prepared triplicate in each media and then diluted in water as displayed in Table 9.

Table 9. Preparation of bovine serum albumin (BSA) standard.

Tube No.	µmol BSA stock (1 mg/mL)	DI water added (µL)	Lowry solution (mL)	Folin 0.5N (mL)	Final concentration (µg/mL)
1	0	500	2.5	0.25	0
2	10	490	2.5	0.25	20
3	20	480	2.5	0.25	40
4	30	470	2.5	0.25	60
5	40	460	2.5	0.25	80
6	50	450	2.5	0.25	100
7	100	400	2.5	0.25	200
8	150	350	2.5	0.25	300

The absorbance of the BSA after reaction was measured at 750 nm to build up the standard curves (Figure 27).

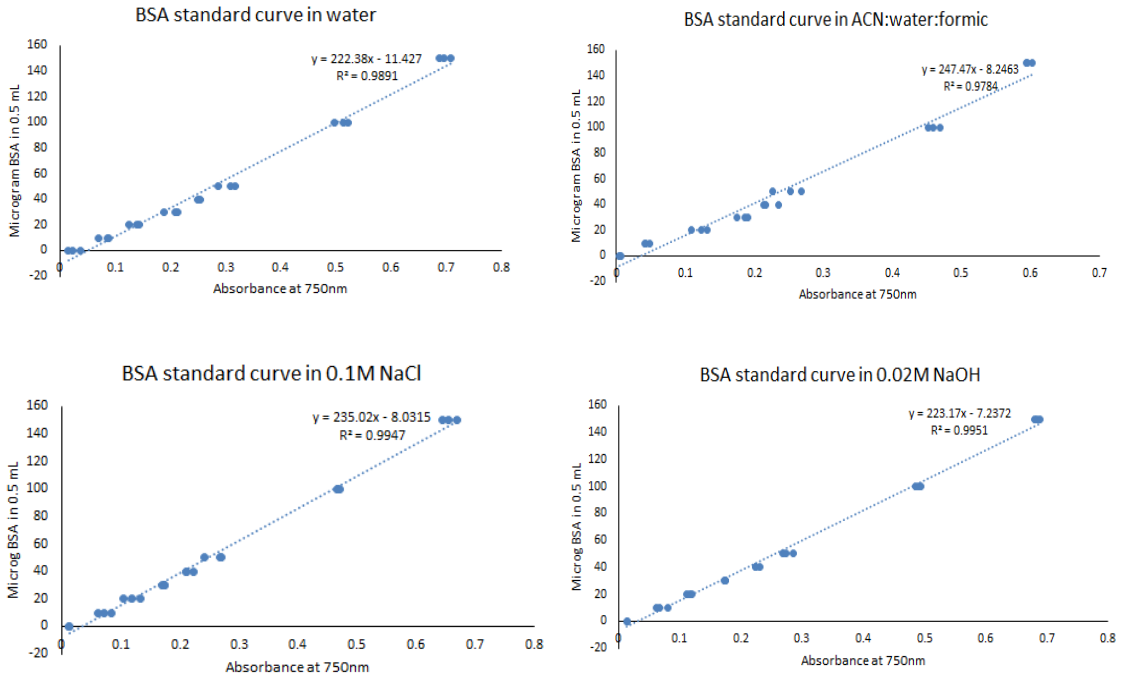


Figure 27: Standard curves of bovine serum albumin (BSA) prepared in various media.

2.6.5.3. Determination of soluble protein content

The soluble protein content was measured by the method of Lowry et al. (1951) as described by Klomklao et al. (2011), with some modifications. Briefly, 0.5 mL of diluted

sample or standard was mixed with 2.5 mL of reagent A containing 2 mL of 0.5% $CuSO_4$ in 1% sodium citrate and 100 mL of 2% sodium carbonate in 0.1 N NaOH for 10 min at ambient temperature. Then, 0.25 mL of 0.5 N Folin-Ciocalteu phenol reagent was added while vortexing. After incubating for 30 min, the protein concentration was measured by the absorbance at 750 nm using a Cary 50 Bio UV–VIS spectrophotometer (Agilent Technologies, Mulgrave, VIC, Australia). Bovine serum albumin (BSA) was used as a standard and the result was expressed as mg BSA per gram of the protein material (mg BSA/g DW).

2.6.6. Determination of total saponin content

The total saponin content (TSC) was determined for liquid extracts or reconstituted crude powder extracts from Gac seed.

2.6.6.1. Principle of the vanillin-sulfuric acid assay for TSC analysis

For TSC analysis, a spectrophotometric method proposed by Hiai et al. (1975a) has become popular for the detection and quantification of saponins in plant materials (Cheok et al., 2014), largely because it is simple, fast and inexpensive. The method is known as the vanillin-sulfuric acid assay because the basic principle of the method is the reaction of sulfuric acid-oxidised triterpene saponins with vanillin, which gives a distinctive red-purple colour measured at wavelengths ranging from 473 to 560 nm (Cheok et al., 2014) using a spectrophotometer (Hiai et al., 1975b). The TSC of a plant sample is determined from a calibration curve with a standard saponin (e.g. aescin, oleanolic acid, diosgenin, quillaja saponin) (Cheok et al., 2014; Tan et al., 2014b) and it is expressed in terms of the standard's equivalence (e.g. mg standard equivalents per g sample; or g standard equivalents per 100 g sample).

2.6.6.2. Building the standard curve of aescin

The standard curve of aescin was prepared with ethanol and DI water. A stock solution of 15 mg/mL aescin was prepared triplicate in ethanol. A serial dilution of the stock solution was prepared using water as displayed in Table 10.

Table 10. Preparation of the aescin standard curve for determination of total saponin content.

Tube No.	Dilution	Concentration (mg/mL)	Absorbance (560 nm)
(1)	4 mL stock + 2 mL DI water	1.00	1.5551 ± 0.0096
(2)	4 mL (1) + 1 mL DI water	0.80	0.2278 ± 0.0131
(3)	2 mL (1) + 2 mL DI water	0.50	0.7700 ± 0.0133
(4)	2 mL (3) + 2 mL DI water	0.25	0.3778 ± 0.0101
(5)	2 mL (4) + 3 mL DI water	0.10	0.1542 ± 0.0149
Blank	DI water only	0.00	Used for zeroing

The absorbance of the aescin standard solutions after reaction was measured at 560 nm to build up the standard curve (Figure 28).

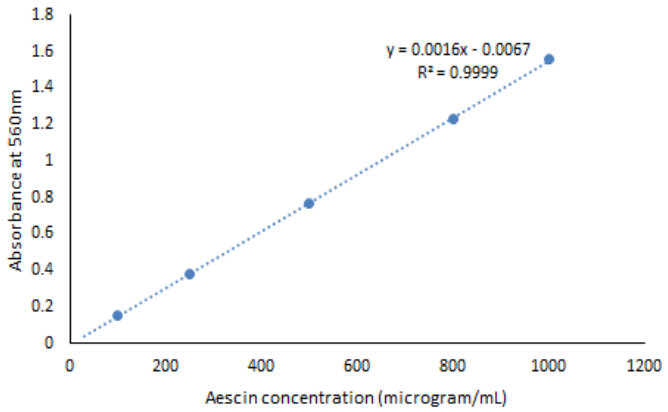


Figure 28: Standard curve of aescin for the determination of total saponin content of Gac seed extracts.

2.6.6.3. Determination of total saponin content

The TSC assay was performed according to Tan et al. (2015) with some modifications. The sample solution (0.25 mL) was mixed with 0.25 mL of 8% (w/v) vanillin in ethanol and 2.5 mL of 72% (v/v) sulfuric acid. The mixture was vortexed and incubated at 60 °C for 15 min and then cooled on ice for 10 min. The absorption of the mixture was measured

at 560 nm using a spectrophotometer (Carry 50 Bio, Varian Pty. Ltd., Mulgrave, VIC, Australia). Aecsin was used as a standard and the result was expressed as aecsin equivalents (AE) per g dry weight (DW) of the material containing saponins (mg AE/g DW).

2.6.7. Determination of total phenolic content (TPC)

The Folin-Ciocalteau assay is the simplest method available for the measurement of phenolic content in products. It is a development of Folin Denis reagent used in the early 19th century for the determination of tyrosine in proteins (Folin O, 1927).

2.6.7.1. Principle of the Folin-Ciocalteu method for determination of TPC

The Folin-Ciocalteu method is described in several pharmacopoeias (Council of Europe, 2007). Folin-Ciocalteu′s phenol reagent reacts with phenolic (-OH containing amino acids, such as L-tyrosine) compounds and non-phenolic reducing substances (including ascorbic acid) to form a blue complex that can be quantified spectrophotometrically. The reaction forms a blue chromophore constituted by a phosphotungsticphosphomolybdenum complex, where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds (Schofield et al., 2001).

Many phenolic compounds can be used as standards, such as gallic acid, catechin, chlorogenic acid and ferulic acid. The criteria to consider in the choice of the standard to be used are the stability, the price, the solubility in the solvent used, however, the most important criteria is the abundance in the concerned sample (Singleton et al., 1999).

2.6.7.2. Building the standard curve for TPC assay

Because gallic acid is one of the prominent phenolic compounds in Gac seeds (Kubola & Siriamornpun, 2011), therefore it was chosen as the standard to estimate the TPC of Gac seed extracts. The standard curve of gallic acid was prepared with DI water. A stock solution of 0.5 mg/mL gallic acid was prepared triplicate in water. A serial dilution of the stock solution was carried out using water as displayed in Table 11.

Table 11. Preparation of gallic acid standard curve for determination of total phenolic content.

Tube No.	Dilution	Concentration (µg/mL)	Absorbance (765 nm)
(1)	1 mL Stock + 4 mL DI water	100	1.1173 ± 0.0110
(2)	2 mL (1) + 2 mL DI water	50	0.5729 ± 0.0121
(3)	2 mL (1) + 3 mL DI water	40	0.4713 ± 0.0132
(4)	3 mL (1) + 7 mL DI water	30	0.3553 ± 0.0101
(5)	2 mL (1) + 8 mL DI water	20	0.2374 ± 0.0149
(6)	2 mL (5) + 2 mL DI water	10	0.1140 ± 0.0141

The standard curve of gallic acid for TPC determination is shown in Figure 29.

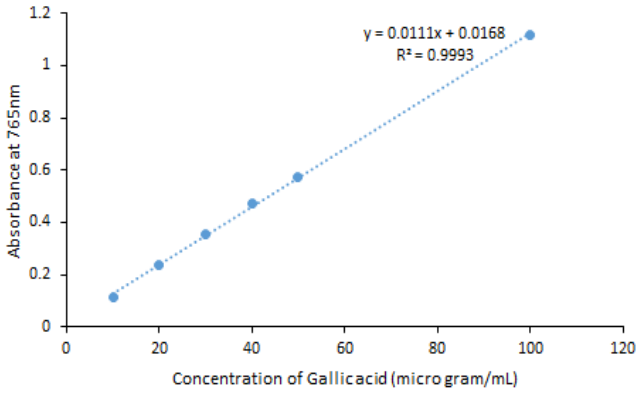


Figure 29: Standard curve of gallic acid for the determination of total phenolic content (TPC) Gac seed extracts.

2.6.7.3. Determination of TPC

The total phenolic content (TPC) of Gac seed extracts and crude extract powders was determined according to the method of Tan et al. (2014b) with some modifications.

Briefly, 0.5 mL of sample was mixed with 2.5 mL of 10% (v/v) Folin-Ciocalteu reagent in water and incubated at room temperature for 2 min to equilibrate. Then, 2 mL of 7.5% (w/v) sodium carbonate solution in water was added and the mixture was incubated at ambient temperature for 1 h. The absorption of the reaction mixture was measured at 765 nm against a reagent blank using a Cary 50 UV–Vis spectrophotometer (Carry 50 Bio, Varian Pty. Ltd., Mulgrave, VIC, Australia). Gallic acid was used as a standard and result was expressed as gallic acid equivalents (GAE) per g dry weight (DW) of the material containing phenolics (mg GAE/g).

2.6.8. Determination of total antioxidant capacity of Gac seed extracts

There are several *in vitro* methods for evaluating the antioxidant activity. This study used three methods: 1) The 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic (ABTS) acid assay; 2) The 1,1-diphenyl-2-pycryl-hydrazyl (DPPH) assay and 3) The Ferric reducing antioxidant power (FRAP) assay.

2.6.8.1. The 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic (ABTS) acid assay

In the ABTS assay, the green–blue stable radical cationic chromophore, 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) is produced by oxidation (Figure 30), and has absorption maxima at 414, 645, 734, and 815 nm (Prior et al., 2005).

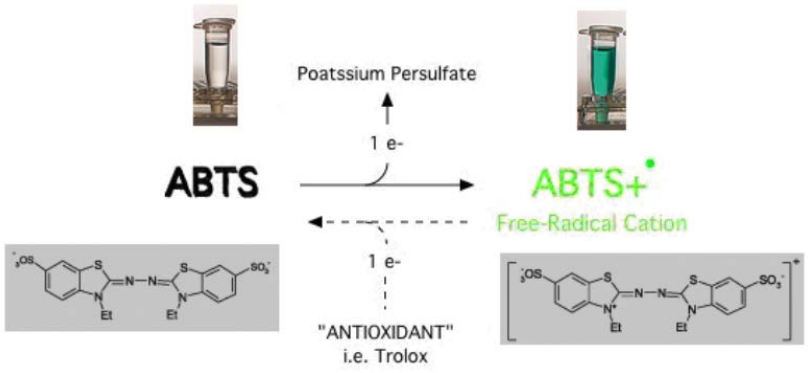


Figure 30: ABTS chemical reaction.
(Source: Boligon et al. (2014)).

Over the course of this study, the ABTS assay was performed as described by Tan et al. (2014b). Stock solutions of 7.4 mM ABTS and 2.6 mM potassium persulfate was prepared and kept at 4 °C until use. The working solution was prepared by mixing the 2 stock solutions in equal quantities and incubating them for 15 to 16 h in the dark at RT. Then, 1 mL of the working solution was mixed with 60 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a spectrophotometer. Fresh working solution was prepared for each assay. For the samples, different concentrations was prepared in deionized water and the sample solutions was mixed with 2.85 mL of the working solution and incubated for 2 h in the dark at RT. The absorption of the solution at 734 nm was measured using a spectrophotometer. Trolox was used as a standard and results will be expressed as mili mole trolox equivalents (TE) per gram of original sample (mM TE/g).

A standard curve of trolox was prepared for the determination of ABTS antioxidant activity of Gac seed extracts. A stock solution of 1,000 µmole/L trolox was prepared triplicate in methanol. A serial dilution of the stock solution in methanol is displayed in Table 12.

Table 12. Preparation of the trolox standard curve for the determination of DPPH antioxidant activity of Gac seed extracts.

Tube No.	Dilution	Concentration (µmole/mL)	Absorbance (734 nm)
(1)	2 mL stock solution + 2 mL Me	500	0.6594 ± 0.0617
(2)	2 mL (1) + 2 mL Me	400	0.8164 ± 0.0392
(3)	2 mL (3) + 2 mL Me	200	0.9399 ± 0.0069
(4)	2 mL (4) + 2 mL Me	100	0.9639 ± 0.0386
(5)	2 mL (5) + 2 mL Me	50	0.9853 ± 0.0180
(6)	2 mL (6) + 2 mL Me	25	0.9830 ± 0.0160
Control	Only Methanol	0	0.9956 ± 0.0045

Me: Methanol.

The difference between the absorbance of trolox solutions with the absorbance of the control was plotted to build the standard curve to determine the ABTS antioxidant activity of Gac seed extracts (Figure 31).

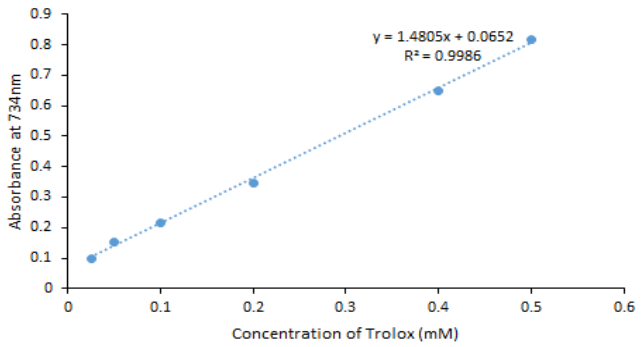


Figure 31: The Trolox standard curve for the ABTS assay for Gas seed extracts.

2.6.8.2. The 1,1-diphenyl-2-pycryl-hydrazyl (DPPH) assay

The DPPH assay is popular in natural product antioxidant studies. One of the reasons is that this method is simple and sensitive. This assay is based on the theory that a hydrogen donor is an antioxidant. It measures compounds that are radical scavengers. Figure 32 below, shows the mechanism by which DPPH• accepts hydrogen from an antioxidant. DPPH• is one of the few stable and commercially available organic nitrogen radicals (1). The antioxidant effect is proportional to the disappearance of DPPH• in test samples. Monitoring DPPH• with a UV spectrometer has become the most commonly used method because of its simplicity and accuracy. DPPH• shows a strong absorption maximum at 517 nm (purple). The colour turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm.

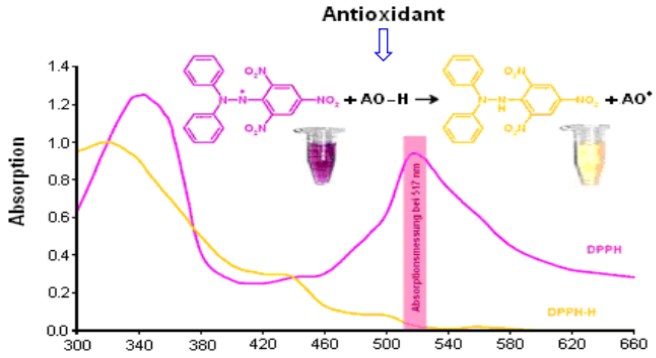


Figure 32: DPPH• free radical conversion to DPPH by anti-oxidant compound. (Source: Boligon et al. (2014))

In the course of this thesis, the DPPH assay was conducted as described by Tan et al. (2014b). A stock solution of 0.6 M DPPH in methanol was prepared and kept at –20 °C until use. The working solution was prepared by mixing 10 mL of stock solution with 45 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm using a spectrophotometer. For the Gac seed extract samples, appropriately dilution was carried out in DI water and was then be mixed with the working solution. The sample was be allowed to stand for 30 min and the absorption at 515 nm was then be measured using a spectrophotometer. Trolox was used as a standard and results was expressed as trolox equivalents (TE) per gram of original sample (µmole TE/g).

A standard curve of trolox was prepared for the determination of DPPH antioxidant activity of Gac seed extracts. A stock solution of 1,000 µmole/L trolox was prepared triplicate in methanol. A serial dilution of the stock solution in methanol is displayed in Table 13.

Table 13. Preparation of the trolox standard curve for the determination of DPPH antioxidant activity of Gac seed extracts.

Tube No.	Dilution	Concentration (µmole/mL)	Absorbance (515 nm)
(1)	4 mL stock solution + 1 mL Me	800	0.2334 ± 0.0043
(2)	2 mL stock solution + 2 mL Me	500	0.5095 ± 0.0070
(3)	2 mL (1) + 2 mL Me	400	0.6422 ± 0.0032
(4)	2 mL (3) + 2 mL Me	200	0.8110 ± 0.0171
(5)	2 mL (4) + 2 mL Me	100	0.9121 ± 0.0113
(6)	2 mL (5) + 2 mL Me	50	0.9604 ± 0.0118
(7)	2 mL (6) + 2 mL Me	25	0.9703 ± 0.0103
(8)	2 mL (7) + 2 mL Me	12.5	0.9778 ± 0.0046
Control	Only Methanol	0	0.9831 ± 0.0011

Me: Methanol.

The difference between the absorbance of trolox solutions with the absorbance of the control was plotted to build the standard curve to determine the DPPH antioxidant activity of Gac seed extracts (Figure 23).

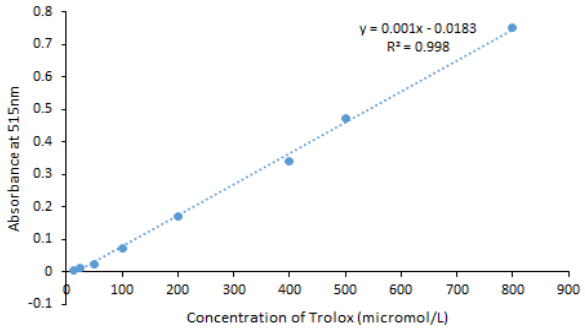


Figure 33: Standard curve of Trolox for the determination of DPPH antioxidant activity of Gac seed extracts.

2.6.10.3. The Ferric reducing antioxidant power (FRAP) assay

The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method employing an easily reduced oxidant, Fe(III). Reduction of a ferric tripyridyltriazine complex to ferrous-(2,4,6-tripyridyl-s-triazine)$_2$ ie. Ferric (III) (colourless) to Ferrous (II)

(blue) (Figure 34) can be monitored by measuring absorbance at 593 nm. The absorbance readings are related to the reducing power of the electron-donating antioxidants present in the test sample. Therefore, the FRAP assay can rank the reducing power and the antioxidant potential of a wide range of test compounds.

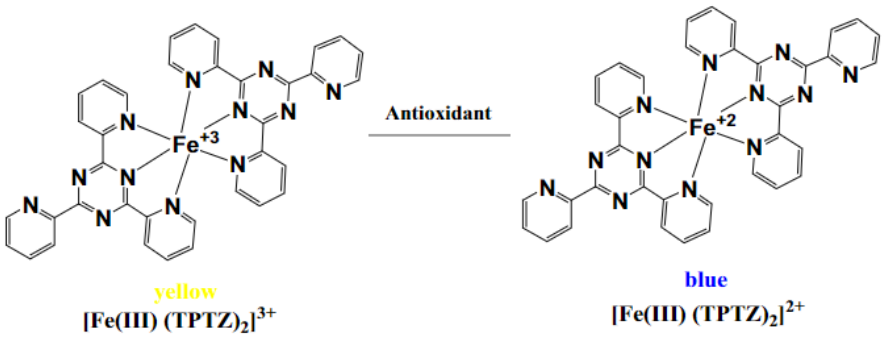


Figure 34: Redox reaction for ferric complex in the FRAP assay.
(Source: Pérez-Cruz et al. (2018)).

In the course of this thesis, FRAP assay was estimated following the method of Benzie and Strain (1996). The FRAP reagent was initially prepared consisting of 300 mM acetate buffer, pH 3.6, 10 mM iron reagent (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was warmed at 37 °C before using. After incubation for 4 min, the absorbance was read at 593 nm using a spectrophotometer. Trolox was used as a standard and results was expressed as milimole Trolox equivalents (TE) per gram of original sample (mM TE/g).

A standard curve of trolox was prepared for the determination of FRAP antioxidant activity of Gac seed extracts. A stock solution of 1,000 µmole/L trolox was prepared triplicate in methanol. A serial dilution of the stock solution in methanol is displayed in Table 14.

Table 14. Preparation of the trolox standard curve for the determination of FRAP antioxidant activity of Gac seed extracts.

Tube No.	Dilution	Concentration (µmole/mL)	Absorbance (593 nm)
(1)	4 mL stock solution + 1 mL Me	800	1.6663 ± 0.0172
(2)	2 mL stock solution + 2 mL Me	500	1.0506 ± 0.0112
(3)	2 mL (1) + 2 mL Me	400	0.8214 ± 0.0086
(4)	2 mL (3) + 2 mL Me	200	0.4031 ± 0.0110
(5)	2 mL (4) + 2 mL Me	100	0.1976 ± 0.0046
(6)	2 mL (5) + 2 mL Me	50	0.0903 ± 0.0076
(7)	2 mL (6) + 2 mL Me	25	0.0496 ± 0.0044
(8)	2 mL (7) + 2 mL Me	12.5	0.0248 ± 0.0022

Me: Methanol.

The absorbance of trolox solutions (Table 14) was plotted to build the standard curve to determine the FRAP antioxidant activity of Gac seed extracts (Figure 35).

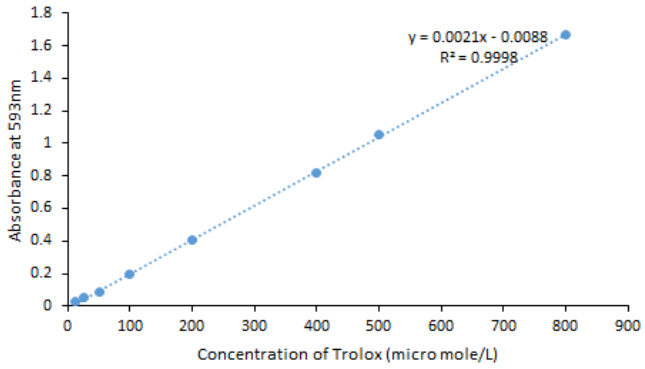


Figure 35: Standard curve of Trolox for the determination of FRAP antioxidant activity of Gac seed extracts.

2.6.9. Determination of anticancer activity

Cell Counting Kit-8 (CCK-8) is a very convenient assay for evaluating preliminary anticancer activity of both synthetic derivatives and natural products and natural product extracts by utilizing Dojindo's highly water-soluble tetrazolium salt. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,

monosodium salt] produces a water-soluble formazan dye upon reduction in the presence of an electron mediator, as shown in Figure 36.

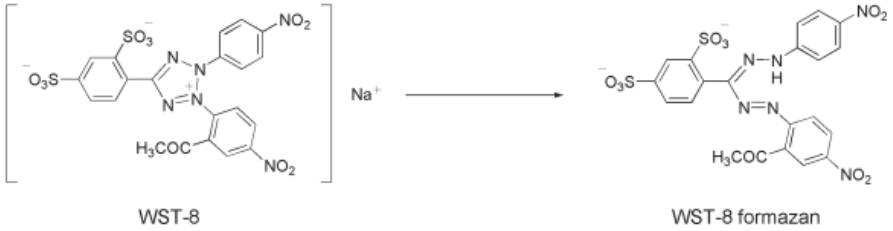


Figure 36: Structures of WST-8 and WST-8 formazan.
(Source: https://www.dojindo.com/TechnicalManual/Manual_CK04.pdf).

Being nonradioactive, CCK-8, allows sensitive colorimetric assays for the determination of the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 is reduced by dehydrogenases in cells to give an orange coloured product (formazan), which is soluble in the tissue culture medium (Figure 37). The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells (Ishiyama et al., 1997; Tominaga et al., 1999).

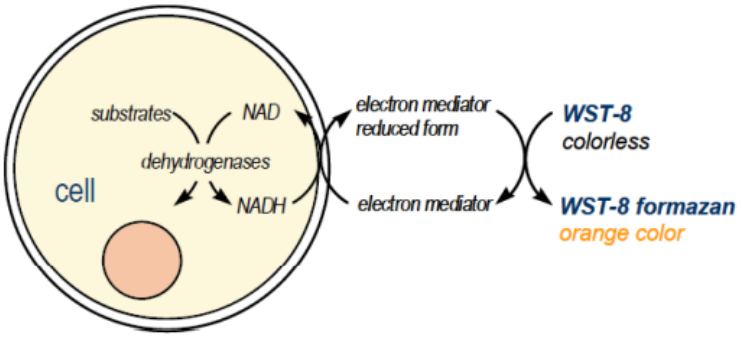


Figure 37: Principle of the cell viability detection with Cell Counting Kit-8.
(Source: https://www.dojindo.com/TechnicalManual/Manual_CK04.pdf)

The anticancer potential against melanoma cells of the freeze-dried powdered extracts from defatted Gac seed kernel was carried out as follows:

Cell lines and culture

The human melanoma MM418C1 (C1, wild type BRAF oncogene) and D24 (mutated BRAF oncogene) cell lines were maintained in RPMI-1640 media supplemented with 10% (*v*/*v*) FBS, 1% (*v*/*v*) streptomycin and penicillin at 37 °C in 5% CO_2. HaCat keratinocytes were used as normal untransformed cells and grown in the same media.

In vitro cytotoxicity assay

All cells were seeded in 96 well plates (Greiner Bio-One, Labfriend, Sydney, NSW, Australia), 5000 cells/well along with 100 μL of fresh media. FD powdered extracts were dissolved in RPMI-1640 cell culture media at a concentration of 2 mg/mL and UV-sterilised for 10 min in a laminar flow hood before use on cells. The cells were allowed to attach for 4 h before being treated with 10 μL of the extract and incubated for 48 h.

The effect of the extracts on cell growth was determined using the CCK-8 (Cell Counting Kit-8) assay (Sigma-Aldrich, St Louis, MO, USA). The assay measured cytotoxicity based on the conversion of a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-8), to a water-soluble formazan dye upon reduction by dehydrogenases in the presence of an electron carrier (Ishiyama et al., 1996).

To determine cell viability, 10 μL of CCK-8 solution was added to each well of the 96-well plate containing treated and control samples. The plates were incubated at 37 °C for 2 h and the absorbance was measured spectrophotometrically at 450 nm using a CLARIOstar® High Performance Monochromator Multimode Microplate Reader (BMG LABTECH, Mornington, VIC, Australia) and the results were analysed using the MARS data analysis software (version 3.00R2, BMG LABTECH, Mornington, NSW, Australia). The data were presented as a proportional viability (%) by comparing the treated cells with the untreated cells (control) using Equation 10.

$$\text{Cell viability} = \frac{At - Ab}{Ac - Ab} \times 100$$

Equation 10: The calculation for cell viability.

where *At* was the absorbance value of the treated cells, *Ab* was the absorbance of CCK-8 only, and *Ac* was the negative control which included cells and CCK-8 only. Two types of controls were used: the media control consisted of cultured cells in 10% (*v*/*v*) FBS containing medium alone and the vehicle control consisted of cells in 10% (*v*/*v*) FBS containing medium, to which 10 μL of RPMI-1640 media without FBS was added. However, as both controls did not cause cytotoxicity, the media control was used to calculate cell viability.

Cell morphology was analysed at 48 h using a Nikon Eclipse TS100 (Nikon, Tokyo, Japan) phase contrast inverted microscope and the images were captured using a Nikon DS-Fi1digital camera.

2.7. Statistical analysis

All experiments were carried out in triplicate and results were presented as mean values ± standard deviations (SD). Differences in mean values were analysed using the analysis of variance (ANOVA) post-hoc test using SPSS software version 24.0 (IBM Corp., Armonk, NY, USA). Differences in means were considered to be significant at $p < 0.05$.

Correlations and graphs of mean values and error bars were created using Excel version 2016 (Microsoft Corp., Seattle, WA, USA) or and Principle Component Analysis (Minitab 17.1.0, Sydney, NSW, Australia).

For designing and analysing the RSM experiments, including generating the three-dimensional (3D) surface and two-dimensional (2D) contour plots, the JMP software version 11.0 or 13.0 (SAS, Cary, NC, USA) was used. The adequacy of the RSM second-

order polynomial model was determined based on the lack of fit and the coefficient of determination (R^2). A $p < 0.05$ value was used for determining the statistical significance for model fitting and for the effects of the independent variables.

Chapter 3: RESULTS

3.1. Synopsis of research result papers

In this thesis, the results are presented in a series of seven research papers, including five which are already published and two which are currently under review.

The first research paper (**Paper I**) entitled "*Optimisation of process parameters for supercritical carbon dioxide extraction of oil from Gac seed kernel powder*" investigated the influence of three different extraction factors (temperature, pressure and flow rate) on the oil yield of Gac seeds. The physicochemical characteristics of the optimised oil was also investigated. The results indicated that there were two extraction parameters significantly affected the oil yield. The highest oil yield was obtained at the temperature of 73°C, pressure of 5,900psi and CO_2 flow rate of 1.5 mL/min. Using these conditions, the optimal Gac seed oil yield (34%) was achieved. The oil was likely high in saturated fat, being solid at room temperature and having a low iodine value, with 33 % being unsaponifiable matter.

The second research paper (**Paper II**) entitled "*Physicochemical properties of Gac (Momordica cochinchinensis Spreng.) seeds and their oil extracted by supercritical carbon dioxide and Soxhlet methods*" elaborated the physical characteristics of Gac seeds and investigated the physicochemical properties of Gac seed oils extracted with two methods: supercritical carbon dioxide extraction and Soxhlet extraction with hexane. The results indicated that Gac seed kernel accounted for 60 % of Gac seed weight, had 53 % of total lipid and 17 % of crude protein. Oil analysing revealed that the SCE oil had higher quality than its SOX counterpart. The SCE oil also had a higher antioxidant capacity. However, the Soxhlet method obtained a higher yield in comparison to the supercritical extraction method (53 % vs. 34 %).

The third research paper (**Paper III**) entitled “Effect of solvents and extraction methods on recovery of bioactive compounds from defatted Gac (*Momordica cochinchinensis* Spreng.) seeds” determined a suitable solvent and extraction technique for recovery of trypsin inhibitors, saponins, and phenolics from Gac seeds. The antioxidant capacity and total solids of derived extracts were also measured. The results indicated that water with conventional extraction method was the best for recovery trypsin inhibitors (118 mg trypsin /g) while water-saturated n-butanol and methanol extracts were the best for saponin extraction (40 and 39 mg AE /g, respectively). Water with microwave assistance achieved the highest phenolics (3.18 mg GAE /g). This extract also had the highest ABTS antioxidant activity while the water-saturated n-butanol and 70% ethanol extracts had highest FRAP antioxidant activity. The ultrasound treatment did not improve any extractions.

The fourth research paper (**Paper IV**) entitled “Optimized extraction of trypsin inhibitors from Gac (*Momordica cochinchinensis* Spreng) seeds for production of a trypsin inhibitor-enriched powder” investigated the extraction conditions for the optimal yield of trypsin inhibitors from defatted Gac seeds; and then produced a freeze-dried powder from the optimal extract. The results indicated that that extraction with 0.05 M NaCl for 1 h at a solvent to seed ratio of 1:15 (g/mL) represents an optimum extraction for Gac seed trypsin inhibitors. The optimal conditions were applied to produce a trypsin inhibitor-enriched powder with the dry mass yield of 16.2±0.1 g powder /100 g defatted seeds. The resulting powder had a high trypsin inhibitor activity (695.6±77.2 mg trypsin/g) with a recovery yield of trypsin inhibitors of 66.7% and a specific trypsin inhibitor activity of 1.57±0.17 mg trypsin inhibited/mg protein. The powder had a total saponin content of 43.6±2.3 mg aescin equivalents/g, total phenolic content of 10.5±0.3 mg gallic acid equivalents/g, and displayed a good physical characteristics.

The fifth research paper (**Paper V**) entitled “Improving the vanillin-sulphuric acid method for quantifying total saponins” investigated the interference of solvent to the measurement of total saponin content in the vanillin-sulphuric acid assay. The results showed that that acetone, methanol and n-butanol caused a great deal of interference for the assay. To improve the method, a solvent evaporation step was added prior to the colorisation reaction to prevent undesired solvent interference during the reaction step. This modified protocol eliminated any solvent interference.

The sixth research paper (**Paper VI**) entitled “Optimisation of the microwave-assisted ethanol extraction of saponins from Gac (*Momordica cochinchinensis* Spreng.) seeds” investigated the MAE conditions for the optimal yield of saponins from Gac seeds. The results indicated that the optimal parameters for the extraction of saponins were a ratio of 30 mL of 100% absolute ethanol per g of full-fat Gac seed kernel powder with the microwave set at 360 W for three irradiation cycles of 10 s power ON and 15 s power OFF per cycle. Therefore, Gac seed saponins could be efficiently extracted using MAE. Full-fat powder of the seed kernels is recommended to be used for a better yield of saponins.

The seventh research paper (**Paper VII**) entitled “Bioactive composition, antioxidant activity and anticancer potential of freeze-dried extracts from defatted Gac (*Momordica cochinchinensis* Spreng.) seeds” investigated the solvent extraction of compounds that could be responsible for antioxidant activity and anticancer potential. The results indicated that water yielded a Gac seed extract, rich in trypsin inhibitors (581.4±18.5 mg trypsin/mg), which had high anticancer potential against two melanoma cell lines, reducing the viability of MM418C1 and D24 melanoma cells (75.5±1.3 and 66.9±2.2%, respectively); the anticancer potential against the MM418C1 cells was highly correlated with trypsin inhibitors ($r=0.92$, $p<0.05$). The results also showed that the water saturated

butanol had the highest saponins 30 (71.8±4.31 mg AE/g), phenolic compounds (20.4±0.86 mg GAE/g) and antioxidant activity but these measures were not related to anticancer potential.

3.2. Extraction of oil – the research papers

3.2.1. Optimisation of process parameters for supercritical carbon dioxide extraction of oil from Gac seed kernel powder

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Research Article

Optimisation of Process Parameters for Supercritical Carbon Dioxide Extraction of Oil from Gac Seed Kernel Powder

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Abstract: This study aimed to maximize the oil yield from Gac seed kernels using supercritical carbon dioxide (SC-CO_2) extraction. Gac seed kernel powder (4 g) with particle diameters <500 μm was extracted for 32 min. Response surface methodology with central composite design was used to optimize the SC-CO_2 extraction parameters: temperature (60-80°C), pressure (5,000-7,000 psi (34,474-48,263 kPa)) and SC-CO_2 flow rate (1-2.5 mL/min). The oil yield, accurately represented by a second order equation (R^2 = 0.99, p<0.0001), was predicted to be most substantially and significantly influenced by temperature (p<0.0001), followed by pressure (p<0.02) but not by the CO_2 flow rate (p = 0.20). The optimum conditions were predicted to be: temperature of 73°C, pressure of 5,900psi (40,679kPa) and CO_2 flow rate of 1.5 mL/min. The optimum oil yield was predicted to be 34.1±0.8% (g oil/100 g Gac seed kernel powder) and experimentally validated at 33.9±0.5%. The oil was likely high in saturated fat, being solid at room temperature and having a low iodine value, with 33.2±1.1% being unsaponifiable matter.

Keywords: Extraction, *Momordica cochinchinensis*, oil, response surface methodology, supercritical carbon dioxide

INTRODUCTION

Momordica cochinchinensis Spreng. (Gac), a plant of the Cucurbitaceae family, is widely distributed in Asian countries. The seeds of Gac are used as a Chinese traditional medicine called Mubezhi. These seeds are believed to have anticancer, antiviral, immuno-enhancing, anti-inflammatory, antioxidant, gastroprotective, antiulcerogenic, ribosome inactivating and trypsin inhibiting activities (Lim, 2012).

Vietnam is one of the world's largest producers of Gac fruit. A small percentage of the Vietnamese produce is sold in markets/supermarkets but most of it is processed by industry into commercial products such as Gac aril oil and Gac aril powder. The Gac seeds are usually discarded as a waste product after being separated from the aril of the fruit.

Gac seeds contain 35-53% oil (Ishida *et al*., 2004; Matthaus *et al*., 2003), with the long chain saturated fatty acid, stearic acid (60%) and the omega-6 polyunsaturated fatty acid, linoleic acid (20%), being the major components (Ishida *et al*., 2004). Unlike other long chain saturated fatty acids, which increase blood cholesterol levels, stearic acid has been shown to have a neutral effect on blood total and Low-Density Lipoprotein (LDL) cholesterol levels (Grundy, 1994; Hunter *et al*., 2010). In addition, the high percentage of stearic acid in the oil raises its melting point, which is desirable if it is to be used as frying oil or in the confectionary industry. Stearic acid, as a saturated fatty acid, is also relatively stable to oxidative processes (Leyton *et al*., 1987) linoleic acid is also an important component of the oil; It is an essential dietary fatty acid for humans, which is vital to human metabolic processes and it also lowers blood cholesterol levels (Rassias *et al*., 1991).

Vegetable oil is conventionally extracted physically using a mechanical pressing process or chemically with solvents (Norris, 1982). Although the properties of oil extracted by the mechanical pressing process are better than when solvents are used because the oil is less likely to be contaminated with chemical solvents, the extraction rate is low. The extraction rate is high using solvent extraction but the solvent is mixed

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with the oil, which results in a need for further purification.

In recent years, Supercritical Carbon dioxide (SC-CO_2) extraction of vegetable oil has attracted considerable attention as a promising alternative to the conventional solvent extraction and mechanical pressing processes (Gomes *et al*., 2007; Lu *et al*., 2007). The main reasons are that SC-CO_2 has a higher extraction rate than the mechanical pressing process and that the solvent, CO_2, is non-flammable, non-explosive, cost-efficient, readily available and, because it is a gas, it is easy to remove from the extracted oil.

To maximize the yield of oil by SC-CO_2 extraction, the pressure, temperature and flow rate of the SC-CO_2 need to be optimized. The classical method of optimizing such processes is the single dimensional search, which involves changing one variable while fixing the others at a certain level (one-factor-at-a-time experiment). This is laborious and time consuming especially when the number of variables is large. More importantly, the results of one-factor-at-a-time experiments do not reflect actual changes in the environment as they ignore interactions between factors presented simultaneously. An alternative and more effective approach, which is increasingly being used, is based on statistical methods. One such method is the Response Surface Methodology (RSM), which has been well described (Baş and Boyacı, 2007; Bezerra *et al*., 2008). The RSM has been demonstrated to be a fast, economical and powerful tool for determining the effects of several factors and their interactions, which allows process optimization to be conducted effectively (Baş and Boyacı, 2007).

For the extraction of seed oils, many studies have used RSM to investigate the effect of the SC-CO_2 operating conditions on the yield of oil in order to determine the optimal process conditions for the extraction of oil from a number of seeds, including hemp seed (Da Porto *et al*., 2012), flaxseed (Jiao *et al*., 2008; Özkal, 2009), pomegranate seed (Liu *et al*., 2009a), *Passiflora* seed (Liu *et al*., 2009b; Zahedi and Azarpour, 2011), apricot kernel (Özkal *et al*., 2005a), hazelnut (Özkal *et al*., 2005b) and *Nigellaglandulifera* freyn seed (Zhang *et al*., 2012).

However, to the best of our knowledge, there is no data in the literature on the optimization of the yield of oil from Gac seeds using SC-CO_2 extraction. Thus, the present study aims to determine the optimum SC-CO_2 process parameters, in terms of pressure, temperature and flow rate of SC-CO_2, for the yield of oil from Gac seeds using RSM. Knowledge of the total oil yield with SC-CO_2 extraction will be useful for the design and development of a process for the extraction of oil from Gac seeds in a commercial setting.

MATERIALS AND METHODS

Materials and chemicals: Gac seeds, accession VS7 according to the classification by Wimalasiri *et al*. (2016), were collected from fresh Gac fruits grown in Ho Chi Minh (HCM) city, Vietnam (Latitude: 10.757410; Longitude: 106.673439). After their separation from the fresh fruit, the seeds were vacuum dried at 40°C, de-coated and the kernels were packaged in vacuum-sealed bags and stored at 4°C. Before conducting experiments, the Gac seed kernels were ground into powder of particle sizes less than 500 μm using the 100 g ST-02A Mulry Disintegrator. The powdered Gac kernel particle sizes were measured using the Endecotts Test Sieve (Endecotts, London, England). The powder was then dried in a Dynavac FD3 Freeze Dryer (Sydney, NSW, Australia) for 24 h at -45°C under vacuum at a pressure loading of 10^{-2} m bar (1Pa). There was no detectable moisture in the freeze-dried product as measured according to the standard AOCS Ab 2-49 method (AOCS, 1998). Carbon dioxide (99.9%) was purchased from Coregas Pty. Ltd. (Mayfield, NSW, Australia).

Supercritical carbon dioxide extraction of Gac seed oil: The extraction of Gac seed oil with SC-CO_2 was performed using a laboratory-scale Supercritical Fluid Extraction System (Teledyne Isco, Lincoln, NE, USA), which consisted of an SFX 2-10 extractor and two 260D syringe pumps. The extractor was a 10 mL cartridge in which SC-CO_2 flowed downwards. The operation parameters of the system range between 10 and 7,500psi (68.9 and 51,711kPa) for pressure, between ambient and 150°C for temperature and between 0.001 and 107 mL/min for CO_2 flow rate.

Four grams of Gac seed kernel powder, with particle diameters less than 500 μm was added to a 10 mL extraction vial, which was then placed in the extraction vessel. Based on the literature (Zhang *et al*., 2012), the mass ratio of powder to CO_2, ranges from 1:8 to 1:10 and therefore, it was fixed at a ratio of 1:10 for these experiments. The extraction time for each run was determined from the known mass of CO_2 which needed to be passed through the system (~40 g) and this was based on the CO_2 flow rate of the run and the density of the CO_2 at given temperatures and pressures according to the Benedict-Webb-Rubbin (BWR) equation of state proposed by Span and Wagner (1996).

The oil-CO_2 extract was passed through the coaxially heated adjustable restrictor set at 70°C to evaporate the CO_2 and the oil was collected in a test tube. After the desired extraction time (~32 min), the extraction was manually stopped - at which time the oil had stopped flowing. The oil extracts obtained from the SC-CO_2 extractions, done under the different conditions, were then weighed to obtain the Gac seed oil yield for each extraction and expressed as g of oil per 100 g of Gac seed kernel powder.

EXPERIMENTAL DESIGN

The RSM with Central Composite Design (CCD) was employed to investigate the effect of the SC-CO_2

extraction parameters on the yield of oil from the Gac seed kernel powder (Myers *et al*., 2014). Based on preliminary experiments, the three independent parameters namely, extraction temperature (X_1), extraction pressure (X_2) and supercritical CO_2 flow rate (X_3), were tested at three different levels each, as shown in Table 1.

A total of 16 experiments were carried out. The experimental design consisted of eight (2^3) factorial points, six axial points (star points) to form a central composite design and two replicates for the center point. Optimization was performed using an on-face central composite design with an alpha value of ±1.00 for the three factors (Table 1). The experiments were run in random order to minimize the effects of unexpected variability in the observed responses due to extraneous factors. When the optimum extraction conditions were predicted by RSM from the experimental data, three Gac seed kernel powder samples (4 g) were extracted by SC-CO_2 using the predicted optimum conditions for the temperature, pressure and CO_2 flow rate and the oil yield was compared to the predicted oil yield.

The experimental plan was designed and the results were analysed using JMP software version 11 (SAS, Cary, NC, USA). The software was also used to establish the model equation for graphing the three dimensional and two dimensional contour plots of the variable responses and to predict the optimum values for the three response variables. The Student's *T*-test, conducted using the SPSS statistical software version 20 (IBM, Armonk, NY, USA), was used to compare the observed oil yields to the predicted oil yields, after optimization. Values were taken to be statistically significant at $p<0.05$.

Physical and chemical properties of Gac seed oil: Oil extracted from the Gac seed kernel powder at the optimum conditions was subjected to physical and chemical characterization. The state (solid or liquid) of the oil at room temperature and the color when liquid were noted by visual inspection. The procedures for determination of the other physical and chemical indices were carried out following the official AOCS methods (AOCS, 1998) as follows: Specific gravity: AOCS Cc 10a-25, Refractive index: AOCS Cc 7-25, Slip melting point: AOCS Cc 3-25, Free fatty acids: AOCS Ca 5a-40, Peroxide value: AOCS Cd 8-53, Saponification value: AOCS Cd 3-35, Unsaponifiable matter: AOCS Ca 6a-40, Iodine value: AOCS Cd 1-25, Insoluble impurities: AOCS Ca 3-46, Moisture and volatile matter: AOCS Ca 2c-25.

RESULTS AND DISCUSSION

Fitting of the model for prediction of oil yield: The RSM model generated the following second-order polynomial formula:

$$Y_{(\%)} = 33.726 + 2.184X_1 - 0.722X_2 - 0.317X_3 + 0.129X_1X_2 - 0.561X_1X_3 - 0.961X_2X_3 - 3.938X_1^2 - 2.778X_2^2 - 0.733X_3^2 \quad (1)$$

where,
$Y_{(\%)}$ = Oil yield (g oil/100 g Gac seed)
X_1 = Temperature (°C)
X_2 = Pressure (psi)
X_3 = CO_2 flow rate (mL/min)

It is necessary to test the reliability of the RSM mathematical model in predicting variances and

Table 1: Uncoded and coded levels of independent variables used in RSM design

Coded levels (*X*)	Temperature (X_1, °C)	Pressure (X_2, psi)	Flow rate (X_3, mL/min)
-1	60	5,000	1.00
0	70	6,000	1.75
+1	80	7,000	2.50

Table 2: Experimental and predicted data for the yield of Gac seed oil obtained from the central composite experiment design

	Factors			Oil yield (g oil/100 g powder)	
Trial no.	X_1	X_2*	X_3	Experimental	Predicted
1	60	7,000	1.00	24.51	23.96
2	60	7,000	2.50	21.96	22.52
3	60	5,000	1.00	23.43	23.74
4	60	5,000	2.50	26.28	26.15
5	60	6,000	1.75	27.79	27.60
6	70	7,000	1.75	29.95	30.26
7	70	6,000	1.00	32.52	33.31
8	70	6,000	1.75	33.80	33.73
9	70	6,000	1.75	34.10	33.73
10	70	6,000	2.50	33.24	32.68
11	70	5,000	1.75	31.72	31.67
12	80	7,000	1.00	29.63	29.71
13	80	7,000	2.50	26.39	26.03
14	80	6,000	1.75	31.56	31.97
15	80	5,000	2.50	28.64	29.14
16	80	5,000	1.00	29.59	28.97

X_1: temperature (°C); X_2: pressure (psi); X_3: CO_2 flow rate (mL/min); * 5,000, 6,000 and 7,000 psi are 34,474, 41,369 and 48,263 kPa, respectively

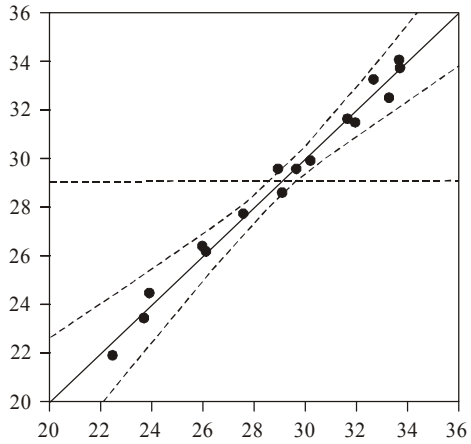

Fig. 1: Correlation (p<0.0001; R^2 = 0.99) between the predicted and actual total oil yields from Gac seed kernel powder extracted under the conditions listed in Table 2

Table 3: Analysis of variance values for model fitting

Sources	Values
Lack of fit	0.56
R^2	0.99
Adjusted R^2	0.97
PRESS	41.80
F Ratio of model	12.57
p of Model > F	0.21
X_1	p<0.0001
X_2	p<0.02
X_3	p = 0.20
X_1X_2	p = 0.62
X_1X_3	p = 0.06
X_2X_3	p<0.01
X_1^2	p<0.0001
X_2^2	p<0.001
X_3^2	p = 0.14

accurately representing the real interrelationships between the selected parameters. Therefore, a goodness of fit analysis was undertaken using the oil yields obtained from all the experiments listed in Table 2 in relation to the RSM design.

The results from the analysis of variance of the central composite design are shown in Fig. 1 and Table 3. Figure 1 shows the correlation between the predicted and the experimental values listed in Table 2 while Table 3 presents the values for the analysis of variance of the model.

Figure 1 shows that the fit between the experimental values and the model oil yield outputs was highly significant (p<0.0001). Furthermore, the coefficient of determination (R^2) for the model was 0.99, which indicated that 99% of the experimental data for the oil yield predictively matched against the model data.

Table 3 shows that the lack-of-fit value (0.56) was also not significant (p>0.05), which indicated that the generated model adequately explained the variation in the experimental data and that the model was an accurate representation of the actual relationship between the extraction parameters and the oil yield.

In addition, the Predicted Residual Sum of the Squares (PRESS) for the model, which is a measure of how well the predictive model fits each point in the design, was 41.80, the F value of the model was 12.57 and the experimental and predicted values did not differ significantly (p = 0.21) from each other (Table 3). This further showed that the mathematical model accurately predicted the amount of oil that could be extracted from the dried Gac seed kernel powder using the SC-CO_2 system when the values for the temperature in ^oC (X_1), the pressure in psi (X_2) and the CO_2 flow rate in mL/min (X_3) were varied as shown in Table 2.

Response surface analysis: From Eq. (1), it can be seen that the oil yield from the Gac seed kernel powder had a complex relationship with the three independent variables. This can also be seen in the three-dimensional response surface curves and their corresponding contour plots as shown in Fig. 2A to 2C, with each illustrating the relationship between two of the independent variables and the oil yield.

Figure 2A shows the response surface curve and its contour plot for the combined effects of temperature and pressure on the oil yield and their interaction at a fixed CO_2 flow rate of 1.75 mL/min. The effect showed a response that could typically be modelled using a quadratic Eq. (1) with temperature having a more marked effect on the oil yield than pressure with both having significant effects (Table 3). However, the interaction between the temperature and the pressure was not significant (p = 0.62) (Table 3).

At the low end of the temperature levels, the model predicted that the oil yield would substantially increase as the temperature increased but that the effect would plateau at approximately 73°C and that the oil yield would then decrease with further increases in temperature (Fig. 2A). At the low end of temperatures, the positive effect is most likely due to an increased mass transfer speed of the solutes into the liquid CO_2 as the temperature is increased. However, at high temperatures, the density of the CO_2 is likely to be reduced, with a consequent reduction in solute solubility (Clifford and Clifford, 1999).

At the low end of the pressure values, the model predicted that the oil yield would increase as the pressure was increased but that the effect would plateau at approximately 5,900psi (40,679 kPa) and that the oil yield would then decrease with further increases in pressure (Fig. 2A). At the low end of pressures, the positive effect is most likely due to an improvement in solute solubility in the liquid CO_2, which results from an increase in the CO_2 density as the pressure is increased in the SC-CO_2 system (Zhang *et al*., 2012). However, when the pressure is increased to high levels, a reduction in the diffusivity and mass transfer

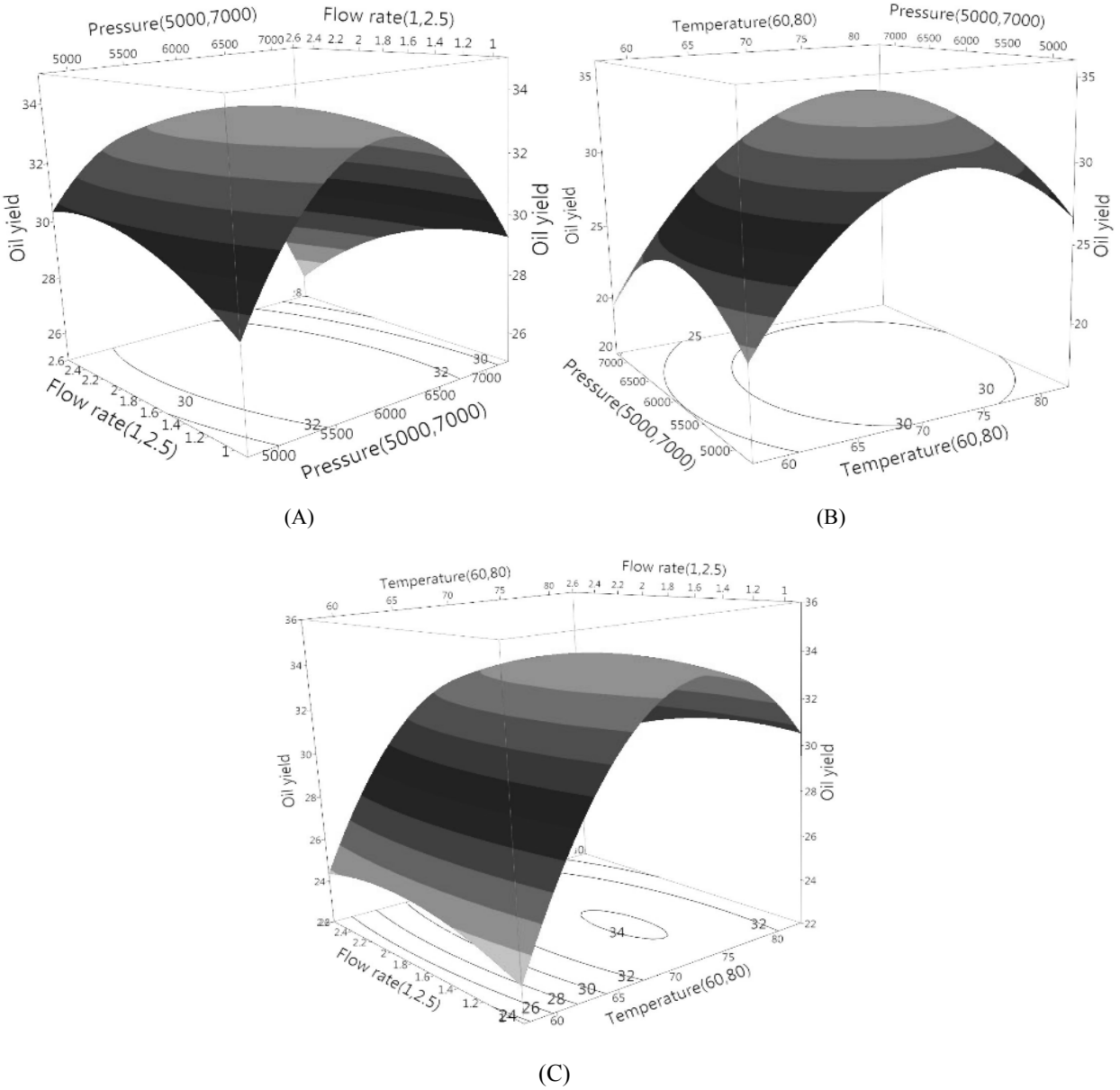

Fig. 2: Response surface curve and its contour plot for; (A): the effects of temperature and pressure at a constant CO_2 flow rate of 1.75 mL/min; (B): the effects of pressure and CO_2 flow rate at a constant temperature of 70°C; (C): the effects of temperature and CO_2 flow rate at a constant pressure of 6,000psi (41,369 kPa) on the oil yield

coefficient of the liquid CO_2 will occur, which can offset the increase in the extraction rate caused by higher CO_2 densities (Clifford and Clifford, 1999).

Figure 2B shows the response surface curve and its contour plot for the combined effects of pressure and CO_2 flow rate on the oil yield and their interaction at a fixed temperature of 70°C. The effect of the extraction pressure and flow rate showed a response that could typically be modelled using a quadratic Eq. (1) with pressure having a significant effect (p<0.02) on the oil yield while the CO_2 flow rate did not (p = 0.20) (Table 3). The interaction between the pressure and the CO_2 flow rate was also not significant (p = 0.62) (Table 3).

The model predicted that the oil yield would increase as the pressure was increased at the low end of values but that the effect would plateau at approximately 5,900psi (40,679 kPa) and that the oil yield would then decrease with further increases in pressure (Fig. 2B). At the low end of the pressure values, the model predicted that the oil yield would increase as the CO_2 flow rate was increased but that the effect would plateau at approximately 1.5 mL/min (Fig. 2B). However, at the median values for the pressure around 5,900psi (40,679kPa), the model predicted that the CO_2 flow rate would have little effect on the oil yield and that at the high end of the pressure values, the

oil yield would decrease as the CO_2 flow rate was increased (Fig. 2B).

Figure 2C shows the response surface curve and its contour plot for the combined effects of temperature and CO_2 flow rate on the oil yield and their interaction at a fixed pressure of 6,000psi (41,369kPa). The effect showed a response that could typically be modelled using a quadratic Eq. (1) with temperature having a significant effect on the oil yield while the CO_2 flow rate did not (Table 3). In addition, the interaction between the temperature and the CO_2 flow rate was not significant ($p = 0.062$) (Table 3).

The model predicted that the oil yield would substantially increase as the temperature increased but that the effect would plateau at approximately 73°C and that the oil yield would then decrease with further increases in temperature (Fig. 2C). However, at the low and optimal values (~73°C) for the temperature, the model predicted that the CO_2 flow rate would have little effect on the oil yield and that at the high end of the temperature values, the oil yield would decrease as the CO_2 flow rate was increased (Fig. 2C).

From these three-dimensional response surface curves (Fig. 2A to 2C) and the analysis of variance values for the model (Table 3), it is evident that the extraction temperature was predicted to have the most substantial and significant effect on the oil yield during the SC-CO_2 extraction of the Gac seed kernel powder, followed by the extraction pressure. However, the predicted effect of the CO_2 flow rate was not significant (Table 3). Furthermore, there were no significant interactions between the extraction temperature with the pressure or the CO_2 flow rate but the interaction between the pressure and the CO_2 flow rate was significant (Table 3).

Optimization of supercritical carbon dioxide extraction of Gac seed oil: Based on the predictive model in Eq. (1), the response surface curves (Fig. 2) and the predictive plots shown in Fig. 3, the optimum conditions for the extraction of the oil from the Gac seed kernel powder were determined to be: temperature = 73°C, pressure = 5,900psi (40,679kPa) and CO_2 flow rate = 1.5mL/min. Under these conditions, the maximum predicted oil yield was 34.1±0.8% (g oil/100 g Gac seed kernel powder).

To validate the optimum conditions predicted by the model, three Gac seed kernel powder samples (4 g) were extracted by SC-CO_2 for 32 min at 73°C, 5,900psi (40,679kPa) and 1.5 mL/min. The result showed that the experimental value for the oil yield was 33.9±0.5%, which was not significantly different ($p = 0.72$) from the predicted value of 34.1±0.8%.

Physical and chemical properties of the Gac seed oil: The Gac seed oil, extracted using the optimum SC-CO_2 extraction conditions, was light green yellow in colour

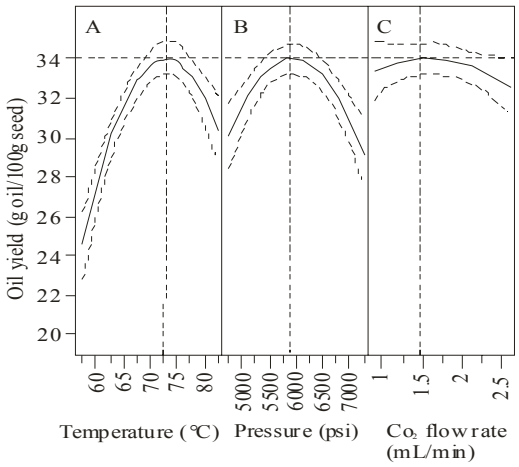

Fig. 3: Determination of the optimum values for temperature (A, 73°C), pressure (B, 5,900psi) and CO_2 flow rate (C, 1.5 mL/min).

as observed visually when it eluted in the liquid state from the extraction system. However, the oil was solid at room temperature and had a slip melting point of 24.8±0.2°C. The specific gravity of the oil was 0.90±0.01 and its refractive index was 1.45±0.01. Its free fatty acid content, expressed in terms of acid value, was 1.47±0.02 mgKOH/g oil, a value that is within the allowable limits for edible oils (CODEX, 1999).

The peroxide value of the oil was very low at 0.12±0.01meqO_2/kg oil, which showed that oxidization was largely avoided during the course of the SC-CO_2 extraction. The moisture and volatile matter was also very low at 0.08±0.01% and insoluble impurities were not detectable in the oil. These results also demonstrated the advantage of the SC-CO_2 extraction over conventional extraction methods. The low peroxide and moisture values also suggest that the SC-CO_2 extracted Gac seed oil may be able to be stored for long periods without deterioration.

The saponification value was 189.4±3.1 mgKOH/g oil and its content of unsaponifiable matter was relatively high at 33.2±1.1%. Therefore, relatively high amounts of non-glyceride fat-soluble matter was extracted from the Gac seed komel powder under the conditions used for the SC-CO_2 extraction. This is in agreement with the findings of Akihisa *et al*. (1986, 1988), which revealed that there are high percentages of sterols and triterpene alcohols in the Gac seed; these compounds may well be extracted by the SC-CO_2 process. Another study by Kan *et al*. (2006) also reported the presence of potentially important bioactive compounds, such as karounidiol, β-sitosterol, pentacyclic triterpene and their derivatives, as constituents of the unsaponifiable matter of Gac seed oil.

The iodine value of the oil was 55.2±1.7 g I_2/100 g oil, which indicated that the oil was likely to be high in

saturated fatty acids as it had an iodine value similar to that of palm oil (Haryati *et al*., 1997), which is high in the saturated fatty acid, palmitic acid. However, unlike palm oil, the Gac seed oil is more likely to primarily contain stearic acid as shown previously (Ishida *et al*., 2004). Therefore, in comparison to palm oil, Gac seed oil, if eaten, is less likely to contribute to the buildup of LDL cholesterol in humans (Grundy, 1994). However, like palm oil, its low iodine value further adds to its likely stability by making it a non-drying oil, an oil which is not likely to harden on exposure to air. Also, the high content of unsaponifiable matter is likely to have contributed to its low iodine value.

CONCLUSION

The RSM was successfully applied to the SC-CO_2 parameters to optimize the extraction of oil from Gac seed kernel powder. A statistically significant multiple regression relationship between the independent variables of the SC-CO_2 extraction (temperature, pressure and CO_2 flow rate) and the response variable (oil yield) was established using a second order polynomial model to represent the relationship among the selected parameters.

The model, the response surface plots and the analysis of variance indicated that two of the three SC-CO_2 parameters, the temperature and pressure but not the CO_2 flow rate, significantly and mainly independently influenced the oil yield. The optimum process parameters were predicted to be: a temperature of 73°C, a pressure of 5,900psi (40,679 kPa) and a CO_2 flow rate of 1.5 mL/min. Under these conditions, the maximum predicted oil yield was 34.1±0.8% (g oil/100 g Gac seed kernel powder). The adequacy of the predictive model was verified by validation experiments, which showed that the experimental values agreed with the predicted values for the oil yield under these optimum SC-CO_2 extraction conditions.

The extracted oil was likely to be high in saturated fatty acids because it was solid at room temperature and had a low iodine value. It also had a high percentage of unsaponifiable matter. Therefore, it would most likely need to be further refined to remove this matter before it can be used as an edible oil. Alternatively, the oil can be further analyzed to confirm the presence of bioactive compounds, which may make it useful as a medicinal oil.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this study.

REFERENCES

Akihisa, T., P. Ghosh, S. Thakur, F.U. Rosentein and T. Matsumoto, 1986. Sterol compositions of seeds and mature plants of family *Cucurbitaceae*. J. Am. Oil Chem. Soc., 63(5): 653-658.

Akihisa, T., Y. Inada, P. Ghosh, S. Thakur, F.U. Rosenstein, T. Tamura and T. Matsumoto, 1988. Compositions of triterpene alcohols of seeds and mature plants of family *Cucurbitaceae*. J. Am. Oil Chem. Soc., 65(4): 607-610.

AOCS, 1998. Official Methods and Recommended Practices of the AOCS. 5th Edn., AOCS Press, Champaign, IL.

Baş, D. and İ.H. Boyacı, 2007. Modeling and optimization I: Usability of response surface methodology. J. Food Eng., 78(3): 836-845.

Bezerra, M.A., R.E. Santelli, E.P. Oliveira, L.S. Villar and L.A. Escaleira, 2008. Response Surface Methodology (RSM) as a tool for optimization in analytical chemistry. Talanta, 76(5): 965-977.

Clifford, A. and T. Clifford, 1999. Fundamentals of Supercritical Fluids. Oxford University Press, New York.

CODEX, S., 1999. Codex Standard for Named Vegetable Oils. 1999. 210. Food and Agriculture Organization of the United Nations, Roma.

Da Porto, C., D. Voinovich, D. Decorti and A. Natolino, 2012. Response surface optimization of hemp seed (*Cannabis sativa* L.) oil yield and oxidation stability by supercritical carbon dioxide extraction. J. Supercrit. Fluid., 68: 45-51.

Gomes, P.B., V.G. Mata and A.E. Rodrigues, 2007. Production of rose geranium oil using supercritical fluid extraction. J. Supercrit. Fluid., 41(1): 50-60.

Grundy, S.M., 1994. Influence of stearic acid on cholesterol metabolism relative to other long-chain fatty acids. Am. J. Clin. Nutr., 60(6): 986S-990S.

Haryati, T., Y.B.C. Man, A. Asbi, H.M. Ghazali and L. Buana, 1997. Determination of iodine value of palm oil by differential scanning calorimetry. J. Am. Oil Chem. Soc., 74(8): 939-942.

Hunter, J.E., J. Zhang and P.M. Kris-Etherton, 2010. Cardiovascular disease risk of dietary stearic acid compared with trans, other saturated, and unsaturated fatty acids: A systematic review. Am. J. Clin. Nutr., 91(1): 46-63.

Ishida, B.K., C. Turner, M.H. Chapman and T.A. McKeon, 2004. Fatty acid and carotenoid composition of Gac (*Momordica cochinchinensis* Spreng) fruit. J. Agr. Food Chem., 52(2): 274-279.

Jiao, S.S., D. Li, Z.G. Huang, Z.S. Zhang, B. Bhandari, X.D. Chen and Z.H. Mao, 2008. Optimization of supercritical carbon dioxide extraction of flaxseed oil using response surface methodology. Int. J. Food Eng., 4(4): 1-17.

Kan, L.D., Q. Hu, Z.M. Chao, X. Song and X.L. Cao, 2006. Chemical constituents of unsaponifiable matter from seed oil of *Momordica cochinchinensis*. J. Chin. Mater. Med., 31(17): 1441-1444.

Leyton, J., P.J. Drury and M.A. Crawford, 1987. Differential oxidation of saturated and unsaturated fatty acids *in vivo* in the rat. Brit. J. Nutr., 57(3): 383-393.

Lim, T.K., 2012. *Momordica cochinchinensis*. In: Lim, T.K. (Ed.), Edible Medicinal and Non-Medicinal Plants. Springer, Dordrecht, The Netherlands, pp: 369-380.

Liu, G., X. Xu, Q. Hao and Y. Gao, 2009a. Supercritical CO_2 extraction optimization of pomegranate (*Punica granatum* L.) seed oil using response surface methodology. LWT-Food Sci. Technol., 42(9): 1491-1495.

Liu, S., F. Yang, C. Zhang, H. Ji, P. Hong and C. Deng, 2009b. Optimization of process parameters for supercritical carbon dioxide extraction of *Passiflora* seed oil by response surface methodology. J. Supercrit. Fluid., 48(1): 9-14.

Lu, T., F. Gaspar, R. Marriott, S. Mellor, C. Watkinson, B. Al-Duri, J. Seville and R. Santos, 2007. Extraction of borage seed oil by compressed CO_2: effect of extraction parameters and modelling. J. Supercrit. Fluid., 41(1): 68-73.

Matthaus, B., K. Vosmann, L.Q. Pham and K. Aitzetmüller, 2003. FA and tocopherol composition of Vietnamese oilseeds. J. Am. Oil Chem. Soc., 80(10): 1013-1020.

Myers, C.M. Anderson-Cook and D.C. Montgomery, 2014. Response Surface Methodology: Process and Product Optimization Using Designed Experiments. 3rd Edn., Wiley, Somerset, NJ, USA.

Norris, F., 1982. Extraction of fats and oils. Bailey's Ind. Oil Fat Prod., 2(4): 179-188.

Özkal, S.G., 2009. Response surface analysis and modeling of flaxseed oil yield in supercritical carbon dioxide. J. Am. Oil Chem. Soc., 86(11): 1129-1135.

Özkal, S.G., M.E. Yener and L. Bayındırlı, 2005a. Response surfaces of apricot kernel oil yield in supercritical carbon dioxide. LWT-Food Sci. Technol., 38(6): 611-616.

Özkal, S.G., M.E. Yener, U. Salgın and Ü. Mehmetoğlu, 2005b. Response surfaces of hazelnut oil yield in supercritical carbon dioxide. Eur. Food Res. Technol., 220(1): 74-78.

Rassias, G., M. Kestin and P.J. Nestel, 1991. Linoleic acid lowers LDL cholesterol without a proportionate displacement of saturated fatty acid. Eur. J. Clin. Nutr., 45(6): 315-320.

Span, R. and W. Wagner, 1996. A new equation of state for carbon dioxide covering the fluid region from the triple-point temperature to 1100 K at pressures up to 800 MPa. J. Phys. Chem. Ref. Data, 25(6): 1509-1596.

Wimalasiri, D., T. Piva, S. Urban and T. Huynh, 2016. Morphological and genetic diversity of *Momordica cochinchinenesis* (*Cucurbitaceae*) in Vietnam and Thailand. Genet. Resour. Crop Ev., 63(1): 19-33.

Zahedi, G. and A. Azarpour, 2011. Optimization of supercritical carbon dioxide extraction of *Passiflora* seed oil. J. Supercrit. Fluid., 58(1): 40-48.

Zhang, J.P., X.L. Hou, T. Yu, Y. Li and H.Y. Dong, 2012. Response surface optimization of *Nigella glandulifera freyn* seed oil yield by supercritical carbon dioxide extraction. J. Integr. Agr., 11(1): 151-158.

3.2.2. Physicochemical properties of Gac (*Momordica cochinchinensis* (Lour.) Spreng) seeds and their oil extracted by supercritical carbon dioxide and Soxhlet methods

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Physicochemical Properties of Gac (*Momordica cochinchinensis* (Lour.) Spreng) Seeds and Their Oil Extracted by Supercritical Carbon Dioxide and Soxhlet Methods

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Abstract: Gac seeds are high in oil, but there are few studies on its extraction and characteristics. This study aimed to characterise Gac seeds and investigate the physicochemical properties of Gac seed oil extracted with supercritical carbon dioxide (SC-CO_2) and hexane (Soxhlet). The Gac seed kernel accounted for 66.4 ± 2.7% of the seed weight, and 53.02 ± 1.27% of it was oil. The SC-CO_2 oil had a higher quality than the Soxhlet oil for important criteria, such as peroxide (0.12 ± 0.02 vs. 1.80 ± 0.01 meq O_2/kg oil), free fatty acid (1.74 ± 0.12 vs. 2.47 ± 0.09 mg KOH/g oil) and unsaponifiable matter (33.2 ± 1.5 vs. 52.6 ± 2.4 g/kg) values, respectively. It also had a better colour (light yellow vs. dark greenish brown) and a higher antioxidant capacity measured with the DPPH (52.69 ± 0.06 vs. 42.98 ± 0.02 µmol Trolox equivalent/g oil) and ABTS (2.10 ± 0.12 vs. 1.52 ± 0.06 µmol Trolox equivalent/g oil) assays. However, a higher yield (53.02 ± 1.27 vs. 34.1 ± 0.8%) was obtained for the Soxhlet oil. Unless refined, the oils would not be edible due to their high unsaponifiable matter, but the SC-CO_2 oil would need less refining. Alternatively, the high unsaponifiable matter in the oil, especially in the Soxhlet oil, may make it useful for medicinal purposes.

Keywords: *Momordica cochinchinensis*; Gac seeds; oil; characteristics; supercritical carbon dioxide; Soxhlet

1. Introduction

Gac (*Momordica cochinchinensis* (Lour.) Spreng), also known as red melon, baby jackfruit, spiny bitter gourd, sweet gourd or cochinchin gourd, is found throughout the Southeast Asian region from South China to Northeastern Australia, including Thailand, Laos, Myanmar, Cambodia and Vietnam [1]. The most important part of the fruit is the red flesh surrounding the seeds, the aril, which is used as a colorant in rice or as a material for further processing into functional food ingredients and supplements since the Gac aril is well known by its high content of lycopene and β-carotene [2]. In factories, after the aril is recovered, the remaining seeds, pulp and peel are mostly considered waste and have very little use [3]. However, in the traditional medicine of countries like China and Vietnam, Gac seeds have been used as a treatment for a range of diseases such as fluxes, liver and spleen disorders, haemorrhoids, wounds, bruises, swelling and pus [4,5].

Several studies have demonstrated that the kernel of Gac seeds contains a high proportion of oil, which ranges from 35% [6] to 53% w/w [7]. Two common methods used for recovering oil from seeds are expeller pressing and conventional solvent extraction, mainly using hexane. The pressing process yields extracts of high quality; however, in most cases, the yield is low and the oil can be subject to thermal degradation. The latter achieves almost complete recovery of the oil as determined using the closed circuit continuous reflux system of the Soxhlet apparatus. However, the solvent extraction method can cause unacceptable solvent contamination, which can be harmful to human health and/or the environment, and thus, the use of oil produced in this way may be restricted in the food, cosmetic and pharmaceutical industries for safety reasons [8].

Many reports have shown that seed oils can be extracted using supercritical carbon dioxide (SC-CO_2), which is environmentally friendly and organic solvent-free. This technique has been employed to extract oil from many kinds of seeds with oil yields that are comparable to the conventional solvent extraction methods [9,10]. Although the optimal conditions for extracting Gac seed oil using SC-CO_2 have been reported recently [11], to our knowledge, there are no reports comparing the physicochemical characteristics of Gac seed oil extracted with SC-CO_2 with Gac seed oil extracted using the conventional hexane extraction method with the Soxhlet apparatus.

Therefore, the aim of this study was to characterise the oil extracted from Gac seed kernels using a SC-CO_2 system and compare it to oil extracted using the conventional solvent extraction method with hexane and the Soxhlet apparatus (Soxhlet). The extracted oils were compared in terms of extraction yield and physiochemical indices, including their antioxidant capacity.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Organic solvents including hexane, methanol and ethanol were obtained from Merck Pty Ltd. (Kilsyth, VIC, Australia). 2,2′-azinobis-(3-ethylbenzothiozoline-6-sulfonic) acid (ABTS), 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and Trolox were purchased from Sigma-Aldrich Pty Ltd. (Castle Hill, NSW, Australia). Carbon dioxide (99.9%) was purchased from Coregas Pty. Ltd. (Mayfield, NSW, Australia). All chemicals were of analytical grade.

2.1.2. Gac Seed Kernels

Twenty kilograms of seeds were separated from fresh Gac fruits collected in Ho Chi Minh (HCM) city, Vietnam (latitude: 10.757410; longitude: 106.673439). The average weight of the fresh Gac seeds was determined immediately after they were separated from the fruit arils. Three samples of 200 fresh seeds were randomly taken from the well-mixed 20-kg lot of seeds and weighed using an analytical balance (ε = 0.0001 g).

For prolonged preservation and to facilitate the removal of their shells, the seeds were vacuum dried at 40 °C for 24 h to reduce their moisture and to increase the breakability of their shells. The dried seeds were then de-shelled manually using a knife. The average weight of the dried whole seeds and the dried de-shelled kernels was determined before and after de-shelling. Three samples of 300 dried seeds were taken randomly from the well-mixed dried seeds and weighed using an analytical balance (ε = 0.0001 g). The seeds in the three samples were then de-shelled, and the resulting three samples of kernels were weighed. Equation (1) was used to calculate what percentage the weight of the kernels was in terms of the weight of the dried seeds before they were deshelled. The results were expressed as the mean value ± the standard deviation.

$$Kernel\ (\%) = \frac{mass\ of\ kernels}{mass\ of\ whole\ seeds} \times 100 \quad (1)$$

The dried kernels were packaged in vacuum-sealed aluminium bags and stored at 4 °C before further investigation.

2.2. Proximate Analysis of the Gac Seed Kernels

The moisture, protein and fat contents of the Gac seed kernels were determined in triplicate samples using the official methods and recommended practices of the American Oil Chemists' Society (AOCS) [12]. Moisture was measured using the AOCS Ab 2-49 method and using a MOC63u moisture analyser (Shimadzu Corp., Kyoto, Japan) at 160 °C; crude protein was measured using the AOCS Ba 4d-90 method and a Kjeldahl apparatus; and oil content was measured using the AOCS Am 2-93 method using a Soxhlet apparatus.

2.3. Preparation of Gac Seed Kernel Powder for Oil Extraction

Before conducting extractions, the Gac seed kernels were ground into a powder of less than a 500-µm particle size, using the 100 g ST-02A Mulry Disintegrator (Taiwan Machinary, Sydney, NSW, Australia). The powder was then dried in a Dynavac FD3 freeze dryer (Dynapumps, Seven Hills, NSW, Australia) for 48 h at −45 °C under vacuum at a pressure loading of 10^{-2} mbar (1 Pa). The remaining moisture of the freeze-dried powder was 1.21 ± 0.02%, as measured at 160 °C using a Shimadzu MOC63u moisture analyser (Shimadzu Corp., Kyoto, Japan).

2.4. Methods for the Extraction of Gac Seed Oil

2.4.1. SC-CO_2 Extraction

The extraction of Gac seed oil with SC-CO_2 was performed using a laboratory-scale supercritical fluid extraction system (Teledyne Isco, Lincoln, NE, USA), which consisted of an SFX 2-10 extractor and two 260D syringe pumps. The extractor was a 10-mL cartridge through which SC-CO_2 flowed downwards. The operation parameters of the system ranged between 10 and 7500 psi for pressure, ambient, 150 °C for temperature and 0.001 and 107 mL/min for CO_2 flow rate. Based on the results from optimisation investigations on the Gac seed oil yield [11], the extraction conditions were set at a temperature of 73 °C, a CO_2 pressure of 5900 psi and a CO_2 flow rate of 1.5 mL/min.

For each extraction (n = 3), 4 g of Gac seed kernel powder were added to the 10-mL extraction cartridge, which was then placed in the extraction vessel. The oil-CO_2 mixture was passed through the coaxially-heated adjustable restrictor set at 70 °C to evaporate the CO_2, and the oil was collected in a pre-weighed glass tube (≈10 min). The tube was then weighted to calculate the oil yield, and the oil was transferred into dark sealed vials and stored at −20 °C before further analysis.

2.4.2. Soxhlet Extraction

For quantitative and qualitative comparisons with the SC-CO_2 extracted oil, oil was extracted with the conventional Soxhlet extraction method as described by Da Porto et al. [13] with some modifications. Briefly, for each extraction (n = 3), 7 g of Gac seed kernel powder were added to a cellulose thimble, plugged with glass wool and put into a Soxhlet extractor. Approximately 250 mL n-hexane were added into a pre-weighed boiling flask, which was fitted to the extractor and condenser. The solvent flow rate was recorded as 14 min/cycle, and the extraction process was terminated after 8 h. After extraction, the n-hexane was removed under reduced pressure at 50 °C using a Buchi Rotavapor B480 evaporator (Buchi Australia, Noble Park, VIC, Australia). The remaining traces of hexane were then removed using a flow of compressed nitrogen blowing on the surface of the oil until the weight of the flask was constant (≈3 min). The weight of the flask containing the oil was then recorded to calculate the oil yield, and the oil was transferred to dark sealed vials and stored at −20 °C before analysis.

2.5. Oil Yield Calculation

The 3 oil samples extracted by each method were precisely weighed using a 2-digit digital balance, which had also been used for weighing the initial Gac seed kernel powder samples. The oil yield was expressed in terms of mass percentage as formulated in Equation (2).

$$Oil\ yield\ (\%) = \frac{mass\ of\ oil\ extracted\ (g)}{mass\ of\ sample\ (g)} \times 100\% \quad (2)$$

2.6. Methods for Characterisation of Oils

2.6.1. Determination of Physicochemical Indices

Oils extracted from the Gac seed kernel powder using SC-CO_2 or and the Soxhlet method with hexane were subjected to physical and chemical analyses following the official AOCS methods [12] as follows: specific gravity: AOCS Cc 10a-25; refractive index: AOCS Cc 7-25; slip melting point: AOCS Cc 3-25; free fatty acids: AOCS Ca 5a-40; peroxide value: AOCS Cd 8-53; saponification value: AOCS Cd 3-35; unsaponifiable matter: AOCS Ca 6a-40; iodine value: AOCS Cd 1-25; insoluble impurities: AOCS Ca 3-46; moisture and volatile matter: AOCS Ca 2c-25.

2.6.2. Colour Measurement

The colour of Gac seed oils was conducted according to Xu [14] with several modifications, using a chroma meter (Minolta CR-400, Minolta Camera Co., Ltd, Osaka, Japan). Oil samples were placed in standard disposable cuvettes (1 cm optical path), and a white calibration plate was used as the background (for illuminants $D65$, Y = 93.5, x = 0.3140, y = 0.3318). Before the measurements, the instrument was calibrated with deionized water. Measurements were performed on liquid oil samples at ambient temperature (27 °C). For each sample, three points along the height of the cuvette were measured, and the colour result of each sample was the average of the three measurements. The absolute measurements were displayed in L*, a* and b* co-ordinates as defined by CIE (Commission Internationale de l'Eclairage). The L* value represents the lightness-darkness dimension; the a* value represents the red-green dimension; and the b* value represents the yellow-blue dimension.

2.6.3. Determination of Antioxidant Activity

The seed oils obtained by the two techniques were subjected to analysis of their antioxidant activity using the DPPH and the ABTS radical-scavenging assays. All data were averages (± standard deviations) of triplicate determinations of three independent oil samples for each extraction method.

- DPPH radical-scavenging assay

The scavenging activity of Gac seed oils towards DPPH radicals was determined by the method of Tan et al. [15] with some modifications; the main modification was that ethyl acetate was used as the solvent instead of methanol to ensure the oil samples were well dissolved. Each of the oil samples (0.20 g) was diluted in ethyl acetate (10 mL). A stock solution of 0.6 mmol/L DPPH in ethyl acetate was prepared and kept at 4 °C to be used within a week. Fresh working solution was prepared for each assay by mixing 8 mL of stock solution with 42 mL of ethyl acetate to obtain an absorbance of 1.1 ± 0.02 units at 515 nm. The reaction was initiated by mixing the diluted oil sample (0.15 mL) with the DPPH working solution (3.85 mL). Absorbance was measured at λ_{max} of 515 nm in a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Mulgrave, VIC, Australia) after 6 h storage in the dark (experimentally determined as the steady state of reaction). Trolox was used as a standard, and the anti-oxidant activity was expressed as μmol trolox equivalents (TE) per g of oil (μmol TE/g).

- ABTS assay

The ABTS assay was conducted according to Christodouleas et al. [16] with several modifications to ensure it worked with the oil samples. The ABTS was dissolved in pure methanol, and potassium persulfate ($K_2S_2O_8$) was dissolved in 50% methanol to a final concentration of 5.92 mM and 2.08 mM, respectively. These two solutions were mixed at a 1:1 (v/v) ratio, and the mixture was allowed to stand in the dark at ambient temperature for 24 h in order to produce the ABTS free radical ($ABTS^{\bullet+}$). Then, the solution was diluted with ethanol at a ratio of 1:15 (v/v) solution: ethanol and left in the dark for another 3 h to obtain an absorbance of 1.00 ± 0.02 units at 734 nm. Each oil sample (0.2 g) was dissolved in 10 mL of dichloromethane. The reaction was initiated by mixing the dissolved oil sample (0.15 mL) with the diluted $ABTS^{\bullet+}$ solution (2.85 mL) left to stand in the dark for 2 h. The absorbance was then measured at the λ_{max} of 734 nm in a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Mulgrave, VIC, Australia). Trolox was used as a standard, and the antioxidant activity was expressed as µmol trolox equivalents (TE) per g of oil (µmol TE/g).

2.7. Statistical Analyses

Oil extractions and other measurements were performed in triplicate, and the results were expressed as the means ± SD. Student's *t*-test, conducted using Microsoft Excel 2010, was used to compare the difference between the mean values for the SC-CO_2 and Soxhlet oils and statistical significance was taken to be at $p < 0.05$.

3. Results and Discussion

3.1. Characteristics of Gac Seeds

The characteristics of the Gac seeds and their macro constituents are shown in Table 1. The results of the weight analysis of the Gac seeds revealed that the weight of the fresh and dried Gac seeds was quite consistent, with the variation being within 4% of the mean seed weight. The average weight of these fresh seeds was similar to the weight reported by Ishida et al. [17]. This is likely because these seeds were sourced from the same country (Vietnam) and were possibly from the same variety. During drying, the seeds lost 38% of their weight (Table 1). After de-shelling, the dried kernels were 34% lower in weight than the whole dried seeds, meaning that 66% of the dried seed weight was in the kernel and 34% in the shell. This is the first report of the Gac kernel to shell ratio, and the shell weight was found to be much higher than for other dried oil seeds. For example, the hull ranged from 9–15% for *Mucuna* seeds [18] and from 20–30% for sunflower seeds [19].

Table 1. Proximate analysis of dried Gac seeds.

Characteristics	Component	Mean ± SD
Average seed weight (g) †	Fresh seeds	5.07 ± 0.19^{a}
Average seed weight (g) †	Dried seeds	3.15 ± 0.12^{b}
Average kernel weight (g) †	Dried kernels	2.09 ± 0.33^{c}
Moisture (%) Moisture Analyser ‡	Dried kernels	3.34 ± 0.10^{z}
Moisture (%) AOCS: Ab 2-49 ‡	Dried kernels	3.47 ± 0.11^{z}
Crude protein (%) ‡	Dried kernels	17.33 ± 0.47^{y}
Oil content (%) ‡	Dried kernels	53.02 ± 1.27^{x}

† The mean ± SD (standard deviation) weights for 3 random samples of 200 fresh seeds or 300 dried seeds or kernels, and values not sharing the same superscript letter (^{a}, ^{b}, ^{c}) are significantly different ($p < 0.05$). ‡ The mean ± SD percentage for 3 independent sample determinations, and values not sharing the same superscript letter (^{x}, ^{y}, ^{z}) are significantly different ($p < 0.05$).

Due to the high relative weight of the Gac seed shell, the shell should be removed before oil extraction. Besides reducing the volume of material to be extracted, removing the seed shell is likely to improve the quality of the oil in terms of colour and purity [20]. However, the shell represents

a substantial component of the Gac seeds, and therefore, studies on the constituents in the shell should be done to determine whether this part of the seeds can be utilised, because otherwise, it still represents a considerable waste burden.

The seed weight may be important for the industrial processing of Gac seeds as observed by Saiedirad et al. [21] for seeds in general in that not all seeds required the same grinding conditions and that variability in weight can affect the grinding time and the uniformity of the resulting powder.

Drying at 40 °C for 24 h resulted in the moisture content of the Gac seed kernels, measured using two methods (Table 1), being much lower than the safe moisture upper limit of 6% suggested by Brooker et al. [22] for oilseed storage. Therefore, the vacuum drying conditions of 40 °C for 24 h used in this study can be applied to sufficiently dry Gac seeds so that they can be safely stored for a longer time without spoilage than the fresh seeds. However, from the energy-saving perspective, studies on the optimisation of the drying methods and the drying conditions used to dry Gac seeds are needed.

The moisture content of the dried kernels was not significantly different (p = 0.2) between the two methods used (Table 1), and therefore, the infrared moisture determination balance can be used instead of the more time-consuming AOCS method for analysing the moisture content of Gac seeds.

The protein content of the Gac seed kernels (Table 1) was lower than for other common oilseeds (23.1–33.0%) such as soybeans, cowpeas, pigeon peas, melon, pumpkin and gourd seeds [23]. The crude protein constituent of an oilseed can also affect the oil extraction process. In oilseeds, proteins form a membrane around the lipid bodies, which needs to be broken down before the oil is able to be extracted [24,25]. Therefore, the low content of protein in Gac seed kernels can be an advantage for the extraction of their oil. However, the known presence of trypsin inhibitor proteins in Gac seeds [26], which can have anti-nutritive effects [27], might hinder the possibility of using Gac seed protein in human food or animal feed.

The oil content of the Gac seed kernels (Table 1) was similar to that reported previously (52.7%) for Gac seed kernels [7] and comparable to the values for other oil-rich seeds like peanuts (45–52%) [28], sunflower kernels (45–55%) [29] and sesame seeds (30-50%) [30] and higher than for linseeds (33.3%), soybeans (18.4%) and palm kernels (44.6%) [31]. Therefore, Gac seed kernels are one of the richest sources of oil, which should make it easy to recover for analysis and potential use, such as edible oil, medicinal or cosmetic ointment.

3.2. Oil Yield

Table 2 shows the oil yield obtained from the two studied extraction methods. On a mass per mass basis (%, m/m), the Soxhlet method with hexane was more effective at extracting oil from the Gac seed kernels; a 36% higher oil yield was obtained for the Soxhlet method than for the SC-CO_2 method. This is likely because hexane is more lipophilic than CO_2, and based on the chemical analysis (Table 2), it was able to extract more triglycerides and other non-polar compounds from the kernels. The oil extracted with hexane had a higher unsaponifiable matter component and a lower saponifiable matter component than its SC-CO_2 counterpart (Table 2), but this did not explain much of the difference in the amount of oil extracted. Thus, the extraction method can affect the yield of oil from Gac seeds.

For other types of seeds, the oil yield also tends to be higher when the oil is extracted using hexane than when the SC-CO_2 method is used. However, the differences are not usually as great as was observed for the Gac seeds in the present study; Zhao et al. [32] reported 24.1% and 27.2% oil yields for radish seeds when extracted by SC-CO_2 and hexane, respectively, and Molero et al. [33] reported close values of 36.0% and 38.4% for oil yields from sunflower seeds and 39.3% and 40.1% from grape seeds.

3.3. Characterisation of the Extracted Oils

Several characteristics are usually measured to give information about the structure, stability and quality of seed oils. The physicochemical and quality characteristics of the two oils extracted by the SC-CO_2 and the Soxhlet methods are shown in Table 2. This is the first time that these characteristics

have been reported for Gac seed oil. The results revealed that all the measured physical and chemical characteristics were significantly different ($p < 0.05$) between the oils extracted by the two methods.

The specific gravity (or relative density) of the SC-CO_2 oil was higher than for the Soxhlet oil, suggesting that the SC-CO_2 oil contained less long chain saturated fatty acids and/or more unsaturated fatty acids than the Soxhlet oil [31]. This may be due to hexane having a higher lipophilicity than SC-CO_2, and thus, it was more efficient at extracting long chain saturated fatty acids. At the same temperature, the higher the density of an oil is, the shorter the average fatty acid chains it contains [34]. Generally, the density of an oil decreases with the molecular weight of its fatty acids, but its density increases with the degree of unsaturation of the fatty acids [35].

One of the factors that affects an oil's specific gravity, the degree of unsaturation, is measured by the refractive index and the iodine value, which decrease and increase, respectively, with the degree of unsaturation of the fatty acids in the oil [36,37]. Consistent with its higher specific gravity, the refractive index value of the SC-CO_2 oil was lower than that of the Soxhlet oil (Table 2), which suggests that there were less unsaturated fatty acids in the former than in the latter. The iodine value of the SC-CO_2 oil was also consistent with its higher specific gravity; it was higher than that of the Soxhlet oil (Table 2), indicating that the SC-CO_2 oil contained more unsaturated fatty acids.

Table 2. Yield, physicochemical characteristics and antioxidant activity of Gac seed oil obtained by supercritical (SC)-CO_2 and Soxhlet.

Indices	Method Standard [9]	Unit	Mean ± SD		Standards for Vegetable Oil [38]
			SC-CO_2	Soxhlet *	
Yield		% (m/m)	34.1 ± 0.8 ^{b}	53.0 ± 2.3 ^{a}	NA
Specific gravity	Cc 10a-25	25 °C/water at 20 °C	0.895 ± 0.001 ^{a}	0.885 ± 0.001 ^{b}	0.881–0.927
Refractive index at 40 °C	Cc 7-25		1.455 ± 0.002 ^{b}	1.462 ± 0.002 ^{a}	1.448–1.477
Iodine value (Wijs)	Cd 1-25	g I_2/100 g oil	54.15 ± 0.62 ^{a}	51.66 ± 0.55 ^{b}	6–135, specific to oil
Saponification value	Cd 3-35	mg KOH/g oil	189.4 ± 2.1 ^{a}	167.2 ± 4.6 ^{b}	168–265, specific to oil
Unsaponifiable matter	Ca 6a-40	g/kg	33.2 ± 1.5 ^{b}	52.6 ± 2.4 ^{a}	≤28
Slip melting point	Cc 3-25	°C	24.8 ± 0.30 ^{b}	26.8 ± 0.65 ^{a}	NA
Free fatty acids	Ca 5-40	mg KOH/g oil	1.74 ± 0.12 ^{b}	2.47 ± 0.09 ^{a}	≤10
Peroxide value	Cd 8-53	meq O_2/kg oil	0.12 ± 0.02 ^{b}	1.80 ± 0.01 ^{a}	≤15
Moist and volatile matter	Ca 2c-25	% (m/m)	0.08 ± 0.01 ^{b}	0.12 ± 0.01 ^{a}	≤0.2
Insoluble impurities	Ca 3-46	% (m/m)	Not detected	0.04 ± 0.01	≤0.05
Antioxidant activity					
DPPH		µmol TE/g oil	52.69 ± 0.06 ^{a}	42.98 ± 0.02 ^{b}	NA
ABTS		µmol TE/g oil	2.10 ± 0.12 ^{a}	1.52 ± 0.06 ^{b}	NA

The mean ± SD values for each measurement in the SC-CO_2 and Soxhlet columns not sharing the same superscript letter are significantly different ($p < 0.05$) according to Student's *t*-test, and the letter '^{a}' denotes a higher value than '^{b}'. NA: not available; TE: Trolox equivalents.

The range of iodine values measured for the Gac seed oil samples, whether they were extracted with the SC-CO_2 or the Soxhlet method, was 51.11–54.77 g I_2/100 g oil. This range was similar to the range of 50.0–55.0 I_2/100 g oil reported for palm oil [38], which suggests that Gac seed oil is very similar to this vegetable oil and, therefore, may have similar applications in the food, cosmetic and biodiesel industries [39–41]. However, the iodine values for the Gac seed oil were low in comparison to other oils, such as canola (188–193), corn (103–128), cottonseed (99–199), linseed (>177), peanut (80–106), rapeseed (94–120), soybean (120–143) and sunflower seed (110–143) oils [36]. The low iodine values for the Gac seed oil were likely due to a high content (60.5%) of the saturated fatty acid, stearic acid, as measured previously by Ishida et al. [17]. The low iodine values of Gac seed oil place it in the non-drying oil group [32], and being a saturated oil, it is fairly stable to auto-oxidation.

The saponification values obtained for the Gac seed oil samples (Table 2) showed that the value for the SC-CO_2 oil was higher than for the Soxhlet oil. This suggests that the fatty acids in the SC-CO_2 oil were likely to be shorter than those in the Soxhlet oil because the saponification value is an index of the average molecular mass of the fatty acids in an oil sample [42]. This was consistent with the specific gravity of the SC-CO_2 oil being higher than that of the Soxhlet oil, which also suggested that the SC-CO_2 oil contained less long chain fatty acids [31].

The range of saponification values for the Gac seed oil samples, whether they were extracted with the SC-CO_2 or the Soxhlet method, was 167–189 mg KOH/g oil. The range of values was similar to those for mustard seed oil (168–184) [38] and rapeseed oil (168–187) [36]. However, they were lower than for most of the other common oils, such as coconut (248–265), palm (190–209), corn (187–195), cottonseed (189–198), linseed (189–195), peanut (187–196), soybean (189–195) and sunflower seed (188–194) [36]. The low saponification values of the Gac seed oil were likely caused by the high proportion of the C18 long chain fatty acids, stearic (60.5%), linoleic (20.3%) and oleic (9.0%), as measured previously by Ishida et al. [17]. However, its low saponification value is an indication that the oil may not be suitable for soap making, for which shorter fatty acids like lauric (C12) and myristic (C14) are preferable [43].

The unsaponifiable matter values obtained for the Gac seed oil samples (Table 2) showed that the value for the SC-CO_2 oil was much lower than for the Soxhlet oil, which is an indication of a higher percentage of oil soluble substances other than triglycerides in the Soxhlet oil. This suggests that the SC-CO_2 was not lipophilic enough to extract all the non-polar substances from the Gac seeds that hexane was able to extract. This is consistent with the findings of Friedrich and Pryde [44], who observed that oils extracted from soybeans, cottonseed, corn and wheat germ or bran by SC-CO_2 had less impurities, such as unsaponifiables, gossypol and phosphorous, than oils extracted with hexane.

The unsaponifiable matter values for the Gac seed oil (3.3–5.3%) were very much higher than those for many edible oils, such as soybean oil (0.015%), rapeseed oil (0.02%), sesame seed oil (0.02%) and palm oil (0.012%) [45]. More importantly, the unsaponifiable matter values for the Gac seed oil extracts, especially the values for the Soxhlet extracted oil, were higher than the upper limit accepted for edible vegetable oils, which is 28 g/kg (2.8%) [38]. Therefore, the oils as extracted in the current study could not be considered to be edible virgin oils and would need to be refined as for most crude seed oils [46].

Gac seed oil is known to contain triterpenoids, steroids and tocopherols, which can be part of the unsaponifiable matter of oils [47]. These compounds do not contain glyceride linkages, and thus, they are unable to be hydrolysed during saponification [48]. Kan et al. [49] have reported the presence of seven triterpenoids in the unsaponifiable matter of Gac seed oil: karounidiol, isokarounidiol, 5-dehydrokarounidiol, 7-oxodihydrokarounidiol, beta-sitosterol, stigmast-7-en-3beta-ol and stigmast-7, 22-dien-3beta-ol. Therefore, it is likely that the unsaponifiable substances in the Gac oil extracted in the present study were due to these triterpenoid saponins [50–52]. For example, Le et al. [52] found that a considerable proportion (≈75%) of the Gac seed saponins was deposited in its oil body. Tocopherols, which have been found in Gac seed oil at a concentration of 274 mg/100 g oil [7], may also have contributed to the unsaponifiable matter.

Although the present Gac seed oil samples would not be acceptable for food applications in terms of their high unsaponifiable matter, they may still be useful in terms of medicinal applications. Their high unsaponifiable matter content may be advantageous as there have been reports on potential beneficial medicinal properties of Gac seed triterpenoids due to their anti-inflammatory [53], anticancer [54] and tumour-inhibitory [55] activities. In this context, the Gac seed oil extracted using the Soxhlet method may be more advantageous than the SC-CO_2 oil because the unsaponifiable matter for the Soxhlet oil was 58% higher than for the SC-CO_2 oil.

The slip melting point of the SC-CO_2 oil was significantly lower than that of the Soxhlet oil (Table 2), again confirming that the SC-CO_2 was less able to extract saturated or long chain lipids [31]. This was consistent with the iodine values, a measure of unsaturation, which were higher for the SC-CO_2 oil than for the Soxhlet oil (Table 2). Compared to other oils, the slip point values for the Gac seed oils (24.5–27.5 °C) were similar to those of coconut (23–26 °C), babassu (24–26 °C) and palm kernel (23–30 °C) oils [56] but lower than for palm oil (31.1–37.6 °C). The melting point of an oil defines its hardness and thermal behaviour and is directly affected by the structure of its fatty acids in that

unsaturated fatty acids give an oil a lower slip melting point than saturated fatty acids and short chain fatty acids give lower values than long chain fatty acids [31].

The free fatty acid values for the SC-CO_2 oil were lower than for the Soxhlet oil (Table 2), and this may simply be due to the difference in the length of the two extractions. Although the temperatures used for the two methods were similar, 73 °C for the SC-CO_2 extraction and 68 °C for the Soxhlet extraction, the extraction time for the Soxhlet extraction (8 h) was much longer than for the SC-CO_2 extraction (≈10 min). Therefore, the possibility that the fatty acids were hydrolysed from triglycerides during the extraction process was higher for the Soxhlet oil than for the SC-CO_2 oil.

The free fatty acid values for both Gac seed oils varied from 1.47–2.47, indicating that the range for the free fatty acid content was higher than for edible oils like soybean oil (0.38–0.54) and palm oil (0.17–1.06), but lower than for mustard oil (3.65–4.5) [57]. The presence of free fatty acids in an oil can promote auto-oxidation reactions, and they should be as low as possible. However, the acceptable limit for free fatty acids is 10 mg KOH/g oil [38], and therefore, the values for the Gac seed oils were far below this limit.

The primary oxidation products of fatty acids are usually peroxides, which are measured by the peroxide value [35]. The peroxide value for the SC-CO_2 oil was 93% lower than for the Soxhlet oil (Table 2). As for the free fatty acid values, the lower peroxide value for the SC-CO_2 oil than for the Soxhlet oil may simply be due to the difference in the length of the two extractions; the extraction time for the Soxhlet extraction (8 h) was much longer than for the SC-CO_2 extraction (≈10 min), and therefore, there was more time for the fatty acids to be oxidised during the Soxhlet extraction than during the SC-CO_2 extraction. Furthermore, because the SC-CO_2 apparatus is sealed from the atmosphere, exposure to oxygen is avoided during the short SC-CO_2 extraction.

Compared to the acceptable limit of 15 meq O_2/ kg oil [38], the peroxide values for the Gac seed oils were both well below this threshold, and therefore, the oils were very stable and not susceptible to oxidation. This is consistent with the low iodine values observed for the Gac seed oils (Table 2) and their high content of saturated fatty acids (60.5% stearic), as measured previously [17]. The presence of tocopherols in Gac seed oil [7] may also contribute to the oil's resistance to the lipid oxidation processes [58].

The moist and volatile matter value for the SC-CO_2 oil was lower than for the Soxhlet oil (Table 2), but the values for both Gac seed oils were lower than the acceptable standard level (0.2%) for vegetable oils [38]. The higher value of the Soxhlet oil, compared to the SC-CO_2 oil, was consistent with this oil also having the higher free fatty acids value (Table 2); the observed higher free fatty acids value was likely the consequence of an increased hydrolysis of fatty acids from the triglycerides in the presence of a higher moisture content in the Soxhlet oil.

In terms of insoluble impurities, the Gac seed oil extracted by SC-CO_2 had no detectable impurities, while the values for the Soxhlet oil were measurable (Table 2). This insoluble impurities value is a measure of unwanted materials suspended in the oil, such as oxidized fatty acids and alkaline soaps of palmitic and stearic acids, among other materials [38]. Therefore, the observed higher insoluble impurities were consistent with both the higher peroxide value (e.g., oxidised fatty acids) and the higher free fatty acid values (e.g., stearic acid) for the Soxhlet oil compared to the SC-CO_2 oil (Table 2). Nonetheless, the level of insoluble impurities in the Soxhlet oil was still below the acceptable threshold of 0.05% for edible oils [38].

The oil extracted by SC-CO_2 had a higher antioxidant capacity than the Soxhlet oil for both of the antioxidant assays performed (Table 2). This may be because of the shorter extraction time needed for the SC-CO_2 extraction (≈10 min) than for the Soxhlet extraction (8 h), and therefore, the former may have more heat-sensitive antioxidant compounds, such as tocopherols, saponins and phenolics, remaining than the latter.

The values for the DPPH assay were much higher (>25 times) than for the ABTS assays for both of the extracted Gac seed oils (Table 2). The antioxidant activity values for the Gac seed oils (DPPH: 1076–1319 and ABTS: 38–53 mg trolox/100 g oil) were also much higher for the DPPH assay,

but similar for the ABTS assay when compared to the DPPH and ABTS antioxidant activity values reported for some seed oils, such as hemp (62 and 40 mg trolox/100 g oil, respectively), terebinth (52 and 47 mg trolox/100 g oil, respectively), radish (53 and 36 mg trolox/100 g oil, respectively), stinging nettle (46 and 33 mg trolox/100 g oil, respectively) and laurel (86 and 85 mg trolox/100 g oil, respectively) [59].

The very high DPPH antioxidant activity measured for the Gac seed oils in this study are likely due to the modifications used for this assay in the present study and, therefore, may not be able to be compared with other studies. The main modification was that ethyl acetate was used as the solvent for the DPPH and the oil samples instead of methanol [15], and therefore, it suggests that ethyl acetate may be better than methanol at solubilising the antioxidant compounds in the Gac seed oils. For the ABTS assay, the oil samples were dissolved in dichloromethane, and the ABTS was dissolved in methanol instead of 1-butanol and water, respectively [16], but the values were similar to those obtained for other oils [59].

In summary, the SC-CO_2 oil appeared to have a higher quality than the Soxhlet oil in terms of free fatty acids, peroxide value, insoluble impurities, moist and volatile matter and antioxidant activity (Table 2). However, both oils, as extracted in this study, could not be used as edible virgin oil because of their high unsaponifiable matter [38], but for the same reason, they may have more potential in medicinal applications. In this context, the Soxhlet oil may have more potential because it had a higher unsaponifiable matter value and a much higher yield than the SC-CO_2 oil.

3.4. Colour Characterisation

As seen in Table 3, the SC-CO_2 oil had a light yellow colour, and therefore, it had a much better colour than the Soxhlet oil, which had a much darker colour and would not require bleaching during the refining process. The CIELab colour measurements reflected this colour difference as significant differences ($p < 0.05$) were found for the L*, a* and b* values between the two oils (Table 3); a higher value for L*, reflecting more brightness, a lower and negative value for a*, reflecting more green than red, and a higher value for b*, reflecting more yellow than blue, were found for the SC-CO_2 oil than for the Soxhlet oil (Table 3). According to Kraujalyte et al. [60], the colour of a vegetable oil depends mainly on the presence of carotenoids and chlorophylls in the oil. Therefore, the darker colour of the Gac seed oil extracted with hexane is probably due to this solvent's capacity to extract carotenoids and chlorophylls [61–63] compared to the more polar solvent carbon dioxide. This is also consistent with the Soxhlet oil having a higher unsaponifiable matter than the SC-CO_2 oil (Table 2), part of which could be carotenoids and chlorophyll. However, based on the bright green colour of the Soxhlet extract observed before the hexane was evaporated, chlorophyll is likely to be a major pigment in this oil.

Table 3. Colour (CIELab) of Gac seed oils extracted by SC-CO_2 and Soxhlet.

Extraction Method	Colour		Colour Measurements		
			L*	a*	b*
SC-CO_2	Light yellow		62.65 ± 7.14^{a}	-1.73 ± 0.23^{a}	47.32 ± 2.38^{a}
Soxhlet	Dark greenish brown		27.12 ± 5.59^{b}	11.46 ± 0.29^{b}	5.88 ± 0.86^{b}

The mean ± SD values for each measurement not sharing a superscript letter indicate significant ($p < 0.002$) differences according to Student's *t*-test.

There are currently no colour standards for Gac seed oil, and the L* a* b* measurements could be useful for developing a colour classification system.

4. Conclusions

Compared to other oilseeds, Gac seed kernel was found to possess a very high oil content. However, when extracted, the oil could not be considered an edible virgin oil due to its high percentage of unsaponifiable matter. The oil yield was higher with the Soxhlet extraction using hexane than with the SC-CO_2 apparatus, but the Soxhlet oil had a considerably higher unsaponifiable matter content than the SC-CO_2 oil. The Gac seed oil extracted by SC-CO_2 had better oil qualities, including antioxidant capacity, colour, peroxide value, free fatty acid value and unsaponifiable matter than the Soxhlet oil. Therefore, although further refining of both extracted Gac seed oils would be needed to achieve the safety criteria prescribed for edible oils, the SC-CO_2 would require less effort to bring it within the criteria.

Some of the unsaponifiable matter in the oil may make it useful for medicinal purposes. In this context, the Soxhlet oil may have more potential than the SC-CO_2 oil due to its higher unsaponifiable matter content. However, further composition analysis of the unsaponifiable matter and studies on the biological activities of the hexane-extracted Gac seed oil are needed to confirm the feasibility of using this oil as a medicinal ingredient.

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References

1. Tran, X.T.; Parks, S.E.; Roach, P.D.; Golding, J.B.; Nguyen, M.H. Effects of maturity on physicochemical properties of Gac fruit (*Momordica cochinchinensis* Spreng.). *Food Sci. Nutr.* **2016**, *4*, 305–314. [CrossRef] [PubMed]
2. Kha, T.C.; Nguyen, M.H.; Roach, P.D.; Parks, S.E.; Stathopoulos, C. Gac fruit: Nutrient and phytochemical composition, and options for processing. *Food Rev. Int.* **2013**, *29*, 92–106. [CrossRef]
3. Chuyen, H.V.; Nguyen, M.H.; Roach, P.D.; Golding, J.B.; Parks, S.E. Gac fruit (*Momordica cochinchinensis* Spreng): A rich source of bioactive compounds and its potential health benefits. *Int. J. Food Sci. Technol.* **2015**, *50*, 567–577. [CrossRef]
4. Masayo, I.; Hikaru, O.; Tatsuo, Y.; Masako, T.; Yoshie, R.; Shuji, H.; Mihashi, K.; Higuchi, R. Studies on the constituents of *Momordica cochinchinensis* Spreng. I. Isolation and characterization of the seed saponins, Momordica saponins I and II. *Chem. Pharm. Bull.* **1985**, *33*, 464–478. [CrossRef]
5. Behera, T.; John, K.J.; Bharathi, L.; Karuppaiyan, R. Wild Crop Relatives: Genomic and Breeding Resources: Vegetables. In *Wild Crop Relatives: Genomic and Breeding Resources*; Kole, C., Ed.; Springer: New York, NY, USA, 2011; pp. 217–246.
6. Shang, H. Studies on Fatty Acid Composition in the Oil of *Momordica cochinchinensis*. *Chin. Tradit. Herbal Drugs* **2000**, *31*, 727–728.
7. Matthaus, B.; Vosmann, K.; Pham, L.Q.; Aitzetmüller, K. FA and tocopherol composition of Vietnamese oilseeds. *J. Am. Oil Chem. Soc.* **2003**, *80*, 1013–1020. [CrossRef]
8. Q3C—Tables and List Guidance for Industry. Available online: https://www.fda.gov/downloads/drugs/guidances/ucm073395.pdf (accessed on 10 October 2012).
9. Stahl, E.; Schuetz, E.; Mangold, H.K. Extraction of seed oils with liquid and supercritical carbon dioxide. *J. Agric. Food Chem.* **1980**, *28*, 1153–1157. [CrossRef]

10. Venturi, F.; Sanmartin, C.; Taglieri, I.; Andrich, G.; Zinnai, A. A simplified method to estimate Sc-CO_2 extraction of bioactive compounds from different matrices: Chili pepper vs. tomato by-products. *Appl. Sci.* **2017**, *7*, 361. [CrossRef]
11. Anh, V.L.; Paul, D.R.; Minh, H.N.; Sophie, E.P. Optimisation of process parameters for supercritical carbon dioxide extraction of oil from Gac seed kernel powder. *Adv. J. Food Sci. Technol.* **2017**, *13*, 170–177. [CrossRef]
12. Firestone, D. *Official Methods and Recommended Practices of the American Oil Chemists Society*, 5th ed.; AOCS Press: Champaign, IL, USA, 1998; ISBN 0935315977.
13. Da Porto, C.; Porretto, E.; Decorti, D. Comparison of ultrasound-assisted extraction with conventional extraction methods of oil and polyphenols from grape (*Vitis vinifera* L.) seeds. *Ultrason. Sonochem.* **2013**, *20*, 1076–1080. [CrossRef] [PubMed]
14. Xu, X.Q. A chromametric method for the rapid assessment of deep frying oil quality. *J. Sci. Food Agric.* **2003**, *83*, 1293–1296. [CrossRef]
15. Tan, S.P.; Stathopoulos, C.; Parks, S.; Roach, P. An optimised aqueous extract of phenolic compounds from bitter melon with high antioxidant capacity. *Antioxidants* **2014**, *3*, 814–829. [CrossRef] [PubMed]
16. Christodouleas, D.C.; Fotakis, C.; Nikokavoura, A.; Papadopoulos, K.; Calokerinos, A.C. Modified DPPH and ABTS assays to assess the antioxidant profile of untreated oils. *Food Anal. Methods* **2015**, *8*, 1294–1302. [CrossRef]
17. Ishida, B.K.; Turner, C.; Chapman, M.H.; McKeon, T.A. Fatty acid and carotenoid composition of Gac (*Momordica cochinchinensis* Spreng) fruit. *J. Agric. Food Chem.* **2004**, *52*, 274–279. [CrossRef] [PubMed]
18. Ezeagu, I.; Maziya-Dixon, B.; Tarawali, G. Seed characteristics and nutrient and antinutrient composition of 12 Mucuna accessions from Nigeria. *Trop. Subtrop. Agroecosyst.* **2003**, *1*, 129–139.
19. Gupta, R.; Das, S. Fracture resistance of sunflower seed and kernel to compressive loading. *J. Food Eng.* **2000**, *46*, 1–8. [CrossRef]
20. Applewhite, T.H. *Proceedings of the World Conference on Oilseed Technology And Utilization*; The American Oil Chemists Society: Urbana, IL, USA, 1993; ISBN 0935315454.
21. Saiedirad, M.; Tabatabaeefar, A.; Borghei, A.; Mirsalehi, M.; Badii, F.; Varnamkhasti, M.G. Effects of moisture content, seed size, loading rate and seed orientation on force and energy required for fracturing cumin seed (*Cuminum cyminum* Linn.) under quasi-static loading. *J. Food Eng.* **2008**, *86*, 565–572. [CrossRef]
22. Brooker, D.B.; Bakker-Arkema, F.W.; Hall, C.W. *Drying and Storage of Grains and Oilseeds*; Springer Science and Business Media: Berlin/Heidelberg, Germany, 1992; ISBN 0442205155.
23. Olaofe, O.; Adeyemi, F.; Adediran, G.O. Amino acid and mineral compositions and functional properties of some oilseeds. *J. Agric. Food Chem.* **1994**, *42*, 878–881. [CrossRef]
24. Frandsen, G.I.; Mundy, J.; Tzen, J.T. Oil bodies and their associated proteins, oleosin and caleosin. *Physiol. Plant* **2001**, *112*, 301–307. [CrossRef] [PubMed]
25. Stahl, E.; Quirin, K.W.; Blagrove, R.J. Extraction of seed oils with supercritical carbon dioxide: Effect on residual proteins. *J. Agric. Food Chem.* **1984**, *32*, 938–940. [CrossRef]
26. Wong, R.C.; Fong, W.; Ng, T. Multiple trypsin inhibitors from *Momordica cochinchinensis* seeds, the Chinese drug mubiezhi. *Peptides* **2004**, *25*, 163–169. [CrossRef] [PubMed]
27. Makkar, H.; Becker, K.; Schmook, B. Edible provenances of Jatropha curcas from Quintana Roo state of Mexico and effect of roasting on antinutrient and toxic factors in seeds. *Plant Foods Hum. Nutr.* **1998**, *52*, 31–36. [CrossRef] [PubMed]
28. Raheja, R.; Batta, S.; Ahuja, K.; Labana, K.; Singh, M. Comparison of oil content and fatty acid composition of peanut genotypes differing in growth habit. *Plant Foods Hum. Nutr.* **1987**, *37*, 103–108. [CrossRef]
29. Zheljazkov, V.D.; Vick, B.A.; Ebelhar, M.W.; Buehring, N.; Baldwin, B.S.; Astatkie, T.; Miller, J.F. Yield, oil content, and composition of sunflower grown at multiple locations in Mississippi. *Agron. J.* **2008**, *100*, 635–642. [CrossRef]
30. Were, B.A.; Onkware, A.O.; Gudu, S.; Welander, M.; Carlsson, A.S. Seed oil content and fatty acid composition in East African sesame (*Sesamum indicum* L.) accessions evaluated over 3 years. *Field Crops. Res.* **2006**, *97*, 254–260. [CrossRef]
31. Gunstone, F. *The Chemistry of Oils and Fats: Sources, Composition, Properties and Uses*; John Wiley & Sons: Hoboken, NJ, USA, 2009; ISBN 1405150025.
32. Zhao, G.; Ren, Y.; Ma, H. Extraction and characterization of radish seed oils using different methods. *Trop. J. Pharm. Res.* **2017**, *16*, 165–169. [CrossRef]

33. Gómez, A.M.; López, C.P.; de la Ossa, E.M. Recovery of grape seed oil by liquid and supercritical carbon dioxide extraction: A comparison with conventional solvent extraction. *Chem. Eng. J. Biochem. Eng. J.* **1996**, *61*, 227–231. [CrossRef]
34. Noureddini, H.; Teoh, B.; Clements, L.D. Densities of vegetable oils and fatty acids. *J. Am. Oil Chem. Soc.* **1992**, *69*, 1184–1188. [CrossRef]
35. Gunstone, F.D. *Rapeseed and Canola Oil: Production, Processing, Properties and Uses*; CRC Press: Boca Raton, FL, USA, 2004; ISBN 0849323649.
36. Knothe, G. Structure indices in FA chemistry. How relevant is the iodine value? *J. Am. Oil Chem. Soc.* **2002**, *79*, 847–854. [CrossRef]
37. Kyriakidis, N.B.; Katsiloulis, T. Calculation of iodine value from measurements of fatty acid methyl esters of some oils: Comparison with the relevant American oil chemists society method. *J. Am. Oil Chem. Soc.* **2000**, *77*, 1235–1238. [CrossRef]
38. Codex, A.C. *Codex Alimentarius: Fats, Oils and Related Products*; Food and Agriculture Organisation: Quebec, QC, Canada, 2001; ISBN 9251046824.
39. Koh, L.P.; Wilcove, D.S. Cashing in palm oil for conservation. *Nature* **2007**, *448*, 993. [CrossRef] [PubMed]
40. Mekhilef, S.; Siga, S.; Saidur, R. A review on palm oil biodiesel as a source of renewable fuel. *Renew. Sust. Energy Rev.* **2011**, *15*, 1937–1949. [CrossRef]
41. Mba, O.I.; Dumont, M.-J.; Ngadi, M. Palm oil: Processing, characterization and utilization in the food industry—A review. *Food Biosci.* **2015**, *10*, 26–41. [CrossRef]
42. Patterson, H.B.W. Quality and Control. In *Hydrogenation of Fats and Oils: Theory and Practice*, 2nd ed.; List, G.R., King, J.W., Eds.; Elsevier Inc.: Urbana, IL, USA, 2011; pp. 329–350.
43. Kumar, A.; Sharma, S. An evaluation of multipurpose oil seed crop for industrial uses (*Jatropha curcas* L.): A review. *Ind. Crops. Prod.* **2008**, *28*, 1–10. [CrossRef]
44. Friedrich, J.; Pryde, E. Supercritical CO_2 extraction of lipid-bearing materials and characterization of the products. *J. Am. Oil Chem. Soc.* **1984**, *61*, 223–228. [CrossRef]
45. Paul, B.; Munshi, M.; Ahmed, M.; Saha, G.; Roy, S. The fatty acid composition and properties of oil extracted from fresh rhizomes of turmeric (*Curcuma longa* Linn.) cultivars of Bangladesh. *Bangladesh J. Sci. Ind. Res.* **2011**, *46*, 127–132. [CrossRef]
46. Hamm, W.; Hamilton, R.J.; Calliauw, G. *Edible Oil Processing*; John Wiley & Sons: Hoboken, NJ, USA, 2013; ISBN 1118541782.
47. Fontanel, D. *Unsaponifiable Matter in Plant Seed Oils*; Springer: New York, NY, USA, 2013; ISBN 3642357091.
48. Bailey, A.E. *Industrial Oil and Fat Products*; Interscience Publishers, Inc.: New York, NY, USA, 1945; ISBN 9780471678496.
49. Kan, L.; Hu, Q.; Chao, Z.; Song, X.; Cao, X. Chemical constituents of unsaponifiable matter from seed oil of *Momordica cochinchinensis*. *China J. Chin. Mater. Med.* **2006**, *31*, 1441–1444.
50. Shan, M.D.; Hu, L.H.; Chen, Z.L. A new multiflorane triterpenoid ester from *Momordica cochinchinensis* Spreng. *Nat. Prod. Lett.* **2001**, *15*, 139–145. [CrossRef] [PubMed]
51. Lin, Z.Y.; Liu, X.; Yang, F.; Yu, Y.Q. Structural characterization and identification of five triterpenoid saponins isolated from *Momordica cochinchinensis* extracts by liquid chromatography/tandem mass spectrometry. *Int. J. Mass Spectrom.* **2012**, *328*, 43–66. [CrossRef]
52. Le, A.V.; Parks, S.E.; Nguyen, M.H.; Roach, P.D. Optimisation of the microwave-assisted ethanol extraction of saponins from Gac (*Momordica cochinchinensis* Spreng) seeds. *Medicines* **2018**, *5*, 70. [CrossRef] [PubMed]
53. Jung, K.; Chin, Y.-W.; Yoon, K.D.; Chae, H.-S.; Kim, C.Y.; Yoo, H.; Kim, J. Anti-inflammatory properties of a triterpenoidal glycoside from *Momordica cochinchinensis* in LPS-stimulated macrophages. *Immunopharmacol. Immunotoxicol.* **2013**, *35*, 8–14. [CrossRef] [PubMed]
54. Akihisa, T.; Tokuda, H.; Ichiishi, E.; Mukainaka, T.; Toriumi, M.; Ukiya, M.; Yasukawa, K.; Nishino, H. Anti-tumor promoting effects of multiflorane-type triterpenoids and cytotoxic activity of karounidiol against human cancer cell lines. *Cancer Lett.* **2001**, *173*, 9–14. [CrossRef]
55. Yasukawa, K.; Akihisa, T.; Tamura, T.; Takido, M. Inhibitory effect of karounidiol on 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion. *Biol. Pharm. Bull.* **1994**, *17*, 460–462. [CrossRef] [PubMed]
56. Firestone, D. *Physical and Chemical Characteristics of Oils, Fats, and Waxes*; AOCS press: Urbana, IL, USA, 2013; ISBN 0983079196.

57. Mowlah, G.; Sheikh, N.M.; Kamal, A.S.M. *A Hand Book on Edible Oils and Fats, with Special Reference to Bangladesh*, 1st ed.; University of Dhaka: Dhaka, Bangladesh, 1990; pp. 9–172.
58. Kamal-Eldin, A.; Appelqvist, L.Å. The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* **1996**, *31*, 671–701. [CrossRef] [PubMed]
59. Uluata, S.; Özdemir, N. Antioxidant activities and oxidative stabilities of some unconventional oilseeds. *J. Am. Oil Chem. Soc.* **2012**, *89*, 551–559. [CrossRef] [PubMed]
60. Kraujalyte, V.; Andjelkovic, M.; Verhé, R.; Venskutonis, P.R. Determination of volatiles, tocopherols and colour changes in aromatised oils with marjoram. *FOODBALT-2011* **2011**, *2011*, 84. [CrossRef]
61. Vernon, L.P.; Seely, G.R. *The Chlorophylls*; Academic Press: Cambridge, MA, USA, 2014; ISBN 1483267725.
62. Boutaoui, N.; Zaiter, L.; Benayache, F.; Benayache, S.; Carradori, S.; Cesa, S.; Giusti, A.M.; Campestre, C.; Menghini, L.; Innosa, D.; et al. Qualitative and quantitative phytochemical analysis of different extracts from *Thymus algeriensis* aerial parts. *Molecules* **2018**, *23*, 463. [CrossRef] [PubMed]
63. Boutaoui, N.; Zaiter, L.; Benayache, F.; Benayache, S.; Cacciagrano, F.; Cesa, S.; Secci, D.; Carradori, S.; Giusti, A.M.; Campestre, C.; et al. *Atriplex mollis* Desf. aerial parts: Extraction procedures, secondary metabolites and color analysis. *Molecules* **2018**, *23*, 1962. [CrossRef] [PubMed]

3.3. Extraction of trypsin inhibitors – the research papers

3.3.1. Effect of solvents and extraction methods on recovery of bioactive compounds from defatted Gac (*Momordica cochinchinensis* Spreng) seeds

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Effect of Solvents and Extraction Methods on Recovery of Bioactive Compounds from Defatted Gac (*Momordica cochinchinensis* Spreng.) Seeds

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Abstract: Gac (*Momordica cochinchinensis* Spreng.) seeds contain bioactive compounds with medicinal properties. This study aimed to determine a suitable solvent and extraction technique for recovery of important compounds, namely, trypsin inhibitors, saponins, and phenolics. The antioxidant capacity and total solids of derived extracts were also measured. Water with conventional extraction method gave the highest value of trypsin inhibitor activity (118.45 ± 4.90 mg trypsin g^{-1}) while water-saturated *n*-butanol and methanol extracts were characterized by their highest content of saponins (40.75 ± 0.31 and 38.80 ± 2.82 mg AE g^{-1}, respectively). Aqueous extract with microwave assistance achieved the highest phenolics (3.18 ± 0.04 mg GAE g^{-1}). As a measure of antioxidant capacity, the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assay gave highest value to the aqueous microwave extract (23.56 ± 0.82 µmol TE g^{-1}) while the ferric reducing antioxidant power (FRAP) assay gave highest values to water-saturated *n*-butanol and 70% ethanol extracts (5.25 ± 0.04 and 4.71 ± 0.39 µmol TE g^{-1}, respectively). The total solids value was highest using water with microwave assistance (141.5 g kg^{-1}) while ultrasound treatment did not improve any extractions. Therefore, trypsin inhibitors are suitably recovered using water while water-saturated *n*-butanol or methanol is for saponins, both using a conventional method. Microwave extraction is suitable for phenolics recovery. These conditions are recommended for an efficient recovery of bioactive compounds from defatted Gac seeds.

Keywords: Gac seeds; MAE; UAE; *Momordica cochinchinensis*; trypsin inhibitors; saponins; phenolics

1. Introduction

Gac (*Momordica cochinchinensis* Spreng.) is a plant species of the family *Cucurbitaceae*, whose fruit is also known as red melon, baby jackfruit, spiny bitter gourd, sweet gourd and cochinchin gourd. It is native to the Southeast Asian region and is commonly grown as a crop in countries like Vietnam, Thailand, Laos, Myanmar, and Cambodia [1,2]. The most important part of the mature fruit is the red flesh surrounding the seeds, called the aril, which is used as a colourant in rice or as a material for further processing into functional food ingredients [3].

The seeds are not eaten; they are removed from the aril and are mostly considered waste [3]. However, in traditional medicine, Gac seeds are purported to have a wide array of therapeutic effects

on a wide variety of conditions, such as fluxes, liver and spleen disorders, haemorrhoids, wounds, bruises, swelling, and pus [2,4]. Several constituents have been identified, which could be involved in the medicinal effects of Gac seeds. These include trypsin inhibitors, such as MCoTI-I, MCoTI-II, and MCoTI-III [5–7]; saponins, such as Momordica Saponin I (Gypsoside), Momordica Saponin II (Quillaic acid) [8,9]; and phenolic compounds such as gallic acid and *p*-hydroxybenzoic acids [10]. However, studies on how to efficiently extract these various components from Gac seeds are scarce and they are vital for facilitating future applications for these bioactives.

For any given plant bioactive, extractive yield depends on the extraction solvent, the chemical nature of the targeted component, and the characteristics of the extraction procedure. When other factors are kept constant, the extraction solvent plays a key role in obtaining the target constituents in terms of desired quality and quantity [11,12]. The choice of the solvent is mainly done based on the chemical properties, that is, polarity or hydrophobicity, of the target compounds.

Organic solvents with medium or high polarity have been most commonly used in laboratories dealing with natural products and there is evidence that some extracts from these solvents have better activity compared with aqueous extracts [11]. However, organic solvents present safety and environmental issues. Due to the hydrophylic nature of the trypsin inhibitors and phenolics, and the amphiphilic properties of saponins in Gac seeds, aqueous solvents and the alcohols are the safest and the most environmentally-friendly solvents for the extraction of these compounds [13,14].

The conventional aqueous solvent extraction technique, in which the solid material is suspended in water with no assistance for breaking the cell structure of the solid material, is often associated with a long heating time, which risks the degradation of bioactive compounds. This has led to the proposed use of advanced techniques such as microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE) that are efficient in terms of extraction time and water consumption. In MAE, microwave heating is able to disrupt the plant cell structure via an increase in the internal pressure of the cell, thereby releasing the bioactive compounds [15]. Similarly, ultrasonic cavitation during UAE produces shockwaves that are also capable of disrupting the plant cell structure and releasing the plant bioactives [16]. These two advanced extraction methods were reported to be more efficient than the conventional method for recovering carotenoids in Gac peel [17]. They have been widely used for the recovery of bioactive compounds from plant materials and are considered the dominant trends in "green chemistry" extractions [18].

In the present study, it was hypothesised that MAE and UAE would be better than the conventional aqueous extraction technique for the recovery of the important bioactive compounds from Gac seeds. Therefore, the extraction of trypsin inhibitors, saponins, and phenolic compounds, as well as the antioxidant activity of extracts from Gac seeds, using MAE and UAE was compared to conventional extraction with water. Furthermore, the efficiency of extraction for the MAE and UAE techniques was also compared to other alcohol solvents, namely methanol, 50% methanol, ethanol, 70% ethanol, and water-saturated *n*-butanol.

To date, no study has focused on assisted systems of aqueous extraction for the recovery of bioactive compounds from Gac seeds. The findings will be useful in the selection of best extraction methods specifically for the recovery of trypsin inhibitors, saponins, and phenolic compounds.

2. Materials and Methods

2.1. Materials

2.1.1. Solvents, Reagents, and Chemicals

Solvents, including ethanol, methanol, and *n*-butanol, and chemicals, including vanillin, sulphuric acid, and potassium persulfate were purchased from Merck (Darmstadt, Germany). Folin-ciocalteu's phenol reagent, anhydrous sodium carbonate, sodium nitrite, ferric chloride, gallic acid, 2,4,6-Tris (2-pyridyl)-s-triazine; (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2carboxylic acid (Trolox), aecsin, 2,2′-azino-bis(3ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), trypsin (type I) from

bovine pancreas, benzyl-DL-arginine-para-nitroanilide (BAPNA), tris, and dimethylsulfoxide (DMSO) were from Sigma-Aldrich Co. (Castle Hill, NSW, Australia). Sodium acetate trihydrate was purchased from Government Stores Department (Kingswood, NSW, Australia). Acetic acid was obtained from BDH Laboratory Supplies (Poole, UK). Sodium hydroxide was from Ajax FineChem (Taren Point, NSW, Australia) and hydrochloride acid was obtained from Lab-scan Ltd. (Bangkok, Thailand).

2.1.2. Gac Seeds

Gac seeds were collected from 450 kg of fresh Gac fruit from accession VS7 as classified by Wimalasiri et al. [1]. These fruits were bought at Gac fruit fields in Dong Nai province, Ho Chi Minh city, Vietnam (Latitude: 10.757410; Longitude: 106.673439). After their separation from the fresh fruit, the seeds were vacuum dried at 40 °C for 24 h to reduce moisture and increase the crispness of the shell, which would facilitate shell removal. The dried seeds were de-coated to get the kernels, which were then packaged in vacuum-sealed aluminium bags and stored at −18 °C until used.

Preparation of Defatted Gac Seed Powder

Gac seed kernels were ground in an electric grinder (100 g ST-02A Mulry Disintegrator), to pass through a sieve of 1.4 mm. The powder was then freeze-dried (Dynavac FD3 Freeze Dryer (Sydney, NSW, Australia)) for 48 h at −45 °C under vacuum at a pressure loading of 10^{-2} mbar (1 Pa), to reduce the moisture content to 12.1 ± 0.2 g kg^{-1}. The powder was then defatted using hexane (1:5 w/v, 30 min, ×3) on a magnetic stirrer at room temperature. The resulting slurry was suction filtered and the residue (defatted powder) was air-dried for 12 h and stored in a desiccator at room temperature until used. The moisture content of the meal was recorded prior to weighing for extraction, using a MOC63u moisture analyzer (Shimazdu, Kyoto, Japan).

2.2. Methods

2.2.1. Extraction Methods

Initially, for the conventional extraction (CE) method, there were six extraction solvents: water (DIW), pure methanol (Methanol), 50% methanol (Methanol50%), pure ethanol (Ethanol), 70% ethanol (Ethanol70%), and water-saturated *n*-butanol (Butanol), examined for the extraction of bioactive compounds from Gac seeds. For the two assisting methods of UAE and MAE, only water was chosen to investigate because in the CE method it was much better than the other solvents in terms of the extraction yield of trypsin inhibitors and phenolic compounds. As such, the aqueous extract obtained using the CE method was used as a control for comparing among the extracts obtained from the different solvents using the same CE method, and also for comparing the extracts obtained with the UAE- and MAE-assisted aqueous extractions.

Based on preliminary experiments on the suitable ratio of solvent to Gac seed powder (data not shown), the ratio of 20 mL solvent per g of defatted Gac seed powder was chosen for the following extraction procedures. Preliminary experiments were also done for extraction conditions (time, temperature, microwave, and ultrasonic power) to assure these conditions were around the optimal area.

- *Conventional extraction (CE)*

1.5 g of defatted Gac seed powder was suspended with 30 mL of each solvent in a covered tube. The mixture was kept under constant shaking in a shaking water bath at 40 ± 1 °C for 30 min.

- *Ultrasonic-assisted extraction (UAE)*

UAE was investigated with only water because it was the best solvent for both trypsin inhibitors and phenolics in the conventional extraction.

1.5 g of defatted Gac seed powder was suspended with 30 mL of deionized water (DIW). UAE was carried out in an ultrasonic bath (Soniclean, 220 V, 50 Hz and 250 W, Soniclean Pty Ltd., Thebarton, SA, Australia) with the working power and temperature were set at 250 W and 40 °C, respectively. The maximum power of 250 W was chosen based on previous studies on ultrasound-assisted extraction of phenolic compounds [19] and saponins [20]. The suspensions were sonicated for 30 min in covered vessels to avoid evaporation. To measure the temperature of the ultrasonic bath, an external digital thermometer was also used. In the case that the ultrasonic bath exceeded the designated temperature, tap water was used to maintain the required temperature.

- *Microwave-assisted extraction (MAE)*

MAE was investigated with only water because it was the best solvent for both trypsin inhibitors and phenolics in the conventional extraction.

MAE was performed using a R395YS Sharp Carousel microwave oven (1200 W, Sharp Corporation, Bangkok, Thailand) at the set radiation power of 600 W. This level of power was chosen based on previous studies on microwave-assisted extraction of phenolic compounds [21] and saponins [22]. An amount of 1.5 g of defatted Gac seed powder was mixed with 30 mL DIW in a 100 mL conical flask. The flask mouth was tightly wrapped with plastic film and the suspension was left soaking for 45 min at ambient temperature of 22 ± 1 °C before the microwave treatment was applied (four cycles of 10 s power on and 15 s power off per cycle). The temperature of the suspension was recorded as 66 ± 1 °C at the end of the extraction process.

- *Filtration*

After all extractions, the suspensions were rapidly cooled to ambient temperature in an ice water bath, and filtered through a Whatman No.1 filter paper. The clear extracts were collected and kept at −20 °C for less than a week before analysis. Before analysis, the different filtrates needed to be diluted appropriately with DIW.

2.2.2. Determination of Trypsin Inhibitor Activity (TIA)

The TIA assay was performed as described by Makkar et al. [23] and Stauffer [24]. A synthetic substrate, benzyl-DL-arginine-para-nitroanilide (BAPNA), was subjected to hydrolysis by trypsin to produce the yellow-coloured *p*-nitroanilide. The degree of inhibition of the development of the yellow colouring by the extracts, a measure of trypsin inhibitor activity, was measured at 385 nm.

Reagent preparation:

- Substrate solution: A substrate solution of 92 mmol L^{-1} BAPNA was made in 0.05 mol L^{-1} Tris-buffer (pH 8.2) containing 0.02 mol L^{-1} $CaCl_2$. The BAPNA was first dissolved in DMSO and then diluted with the buffer solution pre-warmed to 37 °C. This solution was prepared daily and kept at 37 °C while in use.
- Trypsin solution: 20 mg of trypsin (type I) from bovine pancreas was dissolved in 0.001 mol L^{-1} HCl to make 1 L, stored at 4 °C for use within 2–3 weeks. When subjected to the analytical procedure for the standard, 2 mL of this solution gave an absorbance value in the range of 0.576 ± 0.026 after subtracting the reagent blank at 385 nm.

Determination of TIA

From each appropriately diluted filtrate, 4 test tubes were prepared according to Table 1. All the prepared test tubes were kept in a water bath at 37 °C for 10 min to promote the formation of an enzyme-inhibitor complex; 5.0 mL of BAPNA solution pre-warmed to 37 °C was then added into each tube. The contents of the tubes were well mixed after each addition. The tubes were then incubated in the water bath at 37 °C for another 10 min before 1 mL of 30% acetic acid solution was added to each tube to stop the reaction. Then 2.0 mL of trypsin solution was added into each blank tube (Table 1).

After thorough mixing, the absorbance of the reaction mixture (due to the release of *p*-nitroanilide) was measured at 385 nm using a Cary 50 UV–vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

Table 1. Reagent composition in extracted filtrate.

Component (mL)	Reagent Blank (a)	Standard (b)	Sample Blank (c)	Sample (d)
Deionised water	2	2	1	1
Trypsin solution	-	2	-	2
Diluted extract	-	-	1	1
Trypsin solution after reaction inactivation	2	-	2	-

Calculation

The change in absorbance (A_I) due to the trypsin inhibitor per mL of diluted extract is ($A_b - A_a$) − ($A_d - A_c$), where the subscripts refer to tubes (*a*) to (*d*) above. Since 1 µg pure trypsin would give an absorbance of 0.0190, the weight of pure trypsin inhibited per mL of diluted extract is $A_I/0.019$ µg. From this value, the trypsin inhibitor activity (TIA) is calculated in terms of milligrams of pure trypsin per gram of defatted Gac seed powder on a dry-weight basis (mg g^{-1}) (Equation (1)).

TIA = mg of pure trypsin inhibited per gram of dried defatted Gac seed powder

$$TIA = \frac{A_I \times V \times D}{19 \times S \times (1 - m\%/100)} \quad (1)$$

where,

A_I: Change in absorbance due to inhibition per 1 mL of diluted extract $(A_I = (A_b - A_a) - (A_d - A_c))$
V: Original volume of solvent (mL)
D: Dilution factor for the filtered extract
S: Weight of defatted Gac seed powder sample extracted (g)
19: Constant figure based on the absorbance given by 1 mg of pure trypsin
m%: moisture content of defatted Gac seed powder

2.2.3. Determination of Total Saponin Content (TSC)

The TSC was determined according to Tan et al. [25] with some modifications. Briefly, 0.25 mL of diluted extract was mixed with 0.25 mL of 8% vanillin solution in ethanol (w/v) and 2.5 mL 72% sulfuric acid (v/v). The mixture was vortexed and incubated in a water bath at 60 °C for 15 min and then cooled on ice for 10 min. The absorption of the mixture was measured at 560 nm using a Cary 50 UV–vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) against a reagent blank. Aecsin was used as a standard and the results were expressed as Aecsin equivalents per gram dry weight of defatted Gac seed (mg AE g^{-1}).

2.2.4. Determination of Total Phenolic Content (TPC)

The total phenolic content of Gac seed extracts was determined according to the method of Tan et al. [26] with some modifications. Briefly, 0.5 mL of diluted extract was mixed with 2.5 mL of 10% (v/v) Folin-Ciocalteu reagent in water and incubated at room temperature for 2 min to equilibrate. Then, 2 mL of 7.5% (w/v) sodium carbonate solution in water was added and the mixture was incubated at room temperature for 1 h. The absorption of the reaction mixture was recorded at 765 nm using a Cary 50 UV–vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) against a reagent blank. Gallic acid was used as a standard and results were expressed as Gallic acid equivalents per gram dry weight of defatted Gac seed (mg GAE g^{-1}).

2.2.5. Determination of Antioxidant Capacity

Two methods, namely the 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) assays, were performed to assess the antioxidant capacity of the Gac seed extracts.

- *ABTS*

The ABTS assay was as described by Tan et al. [26] with slight modifications. A stock solution of 7.4 mmol L^{-1} ABTS was freshly prepared and 2.6 mmol L^{-1} potassium persulfate solution was prepared and kept at 4 °C in dark bottle for use within a month. A fresh working solution was prepared for each assay by mixing the previous stock solutions in equal quantities and incubating them for 15 to 16 h in the dark at room temperature. Then, 1 mL of the working solution was diluted with approximately 30 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a UV spectrophotometer (Varian Australia Pty. Ltd., Mulgrave, VIC, Australia). An amount of 0.15 mL of each sample was mixed with 2.85 mL of the working solution and incubated for 2 h in the dark at room temperature. The absorption of the reaction mixture at 734 nm was measured using the spectrophotometer. Trolox was used as a standard and results were expressed as mg Trolox equivalents per gram of dry defatted Gac seed sample (mg TE g^{-1}).

- *FRAP*

FRAP was estimated following the method of Thaipong et al. [27], based on the increase in absorbance at 593 nm. The fresh FRAP working solution was initially prepared by mixing 300 mmol L^{-1} acetate buffer (pH 3.6), 10 mmol L^{-1} iron reagent (TPTZ) in 40 mmol L^{-1} HCl, and 20 mmol L^{-1} $FeCl_3.6H_2O$ in the ratio of 10:1:1. The fresh working solution was warmed at 37 °C before using. An amount of 2.85 mL of the working FRAP solution was added to 0.15 mL of diluted sample and incubated at room temperature in the dark for 30 min before its absorbance was read at 593 nm using a Cary 50 UV–vis spectrophotometer (Varian Australia Pty. Ltd., Mulgrave, VIC, Australia). Trolox was used as a standard and the antioxidant capacity of each sample, based on its ability to reduce ferric ions, was expressed as mg Trolox equivalents per gram of dry defatted Gac seed sample (mg TE g^{-1}).

2.2.6. Determination of Total Solids

To determine the total solids, 10 mL of each filtered extract was transferred to a tared flat-bottomed glass vial and then dried at 95 °C and vacuum pressure of 60 KPa for 24 h in a vacuum oven (Thermoline, Wetherill Park, NSW, Australia) until constant weight was achieved. These vials were cooled in a desiccator for 30 min and weighed. The total solids were calculated in g dried extract per kg of dried defatted Gac seed powder, using Equation (2), where *TS* is the total solids, *DE* (g) is the mass of dried extract after the drying, and *DW* (g) is the mass of dried defatted powder which had been used for the extraction.

$$TS\ (\text{g kg}^{-1}) = \frac{DE}{DW} \times 1000 \quad (2)$$

2.2.7. Statistical Analyses

Experiments were performed in triplicate, and means ± SD were assessed using one-way ANOVA and Tukey's *Post Hoc* Multiple Comparisons test with the IBM SPSS Statistics 24 program (IBM Corp., Armonk, NY, USA). Differences in means were considered statistically significant at $p < 0.05$. The correlations and significance of correlations were tested using the same software at the level of 0.05.

3. Results and Discussion

3.1. Effect of Extraction Methods on the Trypsin Inhibitor Yield

The extraction yield of trypsin inhibitors was measured by the trypsin inhibitor activity (TIA) of the extracts. The highest TIA (118.45 ± 4.90 mg g^{-1}) was found in the extract produced using the conventional aqueous extraction method (Figure 1). This value is higher compared with the ones that have been reported for other seeds, such as 0.34–12.50 mg g^{-1} for peas [28], 3 mg g^{-1} for soybean [29], and 18.9 mg g^{-1} for fresh soya meal [30], revealing that Gac seeds can be an important source for recovery trypsin inhibitors.

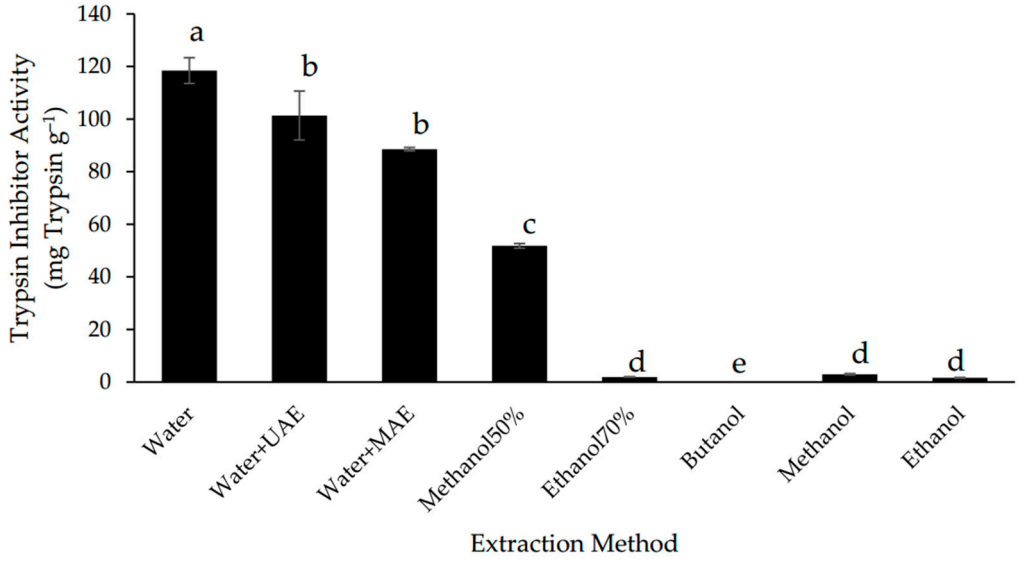

Figure 1. Trypsin inhibitor activity of defatted Gac seed extracts affected by extraction method. The values are the means of three replicates for each extraction. Columns not sharing the same superscript letter are significantly different at $p < 0.05$.

Of the aqueous extracts, the extract with the conventional method gave the highest TIA value, followed by UAE and MAE. Although these two assisted extraction techniques have been reported to improve the extraction of bioactives from plant materials [18], it was not the case for the extraction of trypsin inhibitors from the Gac seeds (Figure 1). In fact, it appears that the ultrasound and microwave treatments had a negative impact on the Gac seed trypsin inhibitors as has previously been observed for trypsin inhibitors in soybeans [31,32] and in bean seeds [33].

The temperature achieved during MAE (66 °C) may have caused some denaturation of the trypsin inhibitors and therefore may explain the loss of TIA when using this method; however, temperature was unlikely to be an issue with UAE as the same temperature (40 °C) was used as for the conventional aqueous extraction. Additionally, two of the known trypsin inhibitors in Gac seeds, MCoTI-I and MCoTI-II, are known to be cysteine knot peptides, which can withstand being extracted with boiling water for 1 h [14], which means that the temperature is not a crucial factor.

As shown in Figure 1, the TIA values of all the aqueous extracts were significantly higher than for the organic solvent extracts. This is due to trypsin inhibitors being polypeptides, which are likely to be more soluble in aqueous media than in organic solvents [5–7].

Of the alcohol solvents, only 50% methanol was able to extract appreciable TIA but this was less than 50% of the activity observed with the conventional water extraction (Figure 1). The TIA for the extract with saturated *n*-butanol was undetectable while the values for the extracts of methanol, ethanol, and 70% ethanol were low and insignificantly different from each other (Figure 1).

It is well known that alcohols can denature and precipitate proteins and the Gac seed trypsin inhibitors may be similarly affected. This is consistent with the observations by Nicholls, Sharp, and Honig [34], who found that the conformation of trypsin inhibitors from soybean was changed due to the effect of n-propanol. Others have also reported on the use of organic solvents to deactivate trypsin inhibitors in soybeans [35–37]. Furthermore, a more hydrophobic solvent, dichloromethane/methanol (1:1), was not suitable for the extraction of the two known trypsin inhibitors in Gac seeds, MCoTI-I, and MCoTI-II [14].

3.2. *Effect of Extraction Methods on the Total Saponin Content (TSC)*

In this study, MAE increased the extraction of saponins from Gac seeds by 40% in comparison to the conventional aqueous extraction method but UAE did not have a significant effect (Figure 2). Despite of MAE improved the recovery of saponins, the extraction was less efficient than for most of the non-water solvents; the highest TSC was found in the butanol and methanol extracts (Figure 2), which were 2-fold higher than the TSC of the MAE extract and not significantly different from each other.

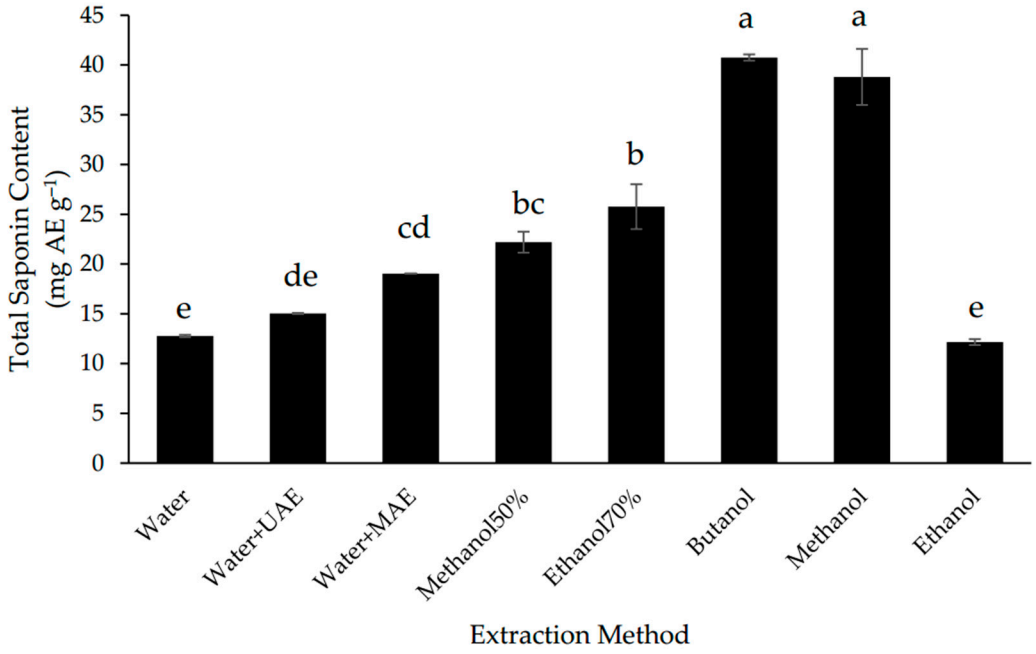

Figure 2. Total saponin content of defatted Gac seed extracts affected by extraction method. The values are the means of three replicates for each extraction. Columns not sharing the same superscript letter are significantly different at $p < 0.05$. AE: aescin equivalents.

Due to their amphiphylic nature, saponins are usually extracted with water or alcohols using many different techniques [13,38]. The present results indicate that the Gac seed saponins are more soluble in alcohols than in water (Figure 2), which is consistent with these saponins being triterpenoids [8,9]. The finding that butanol and methanol were the best solvents for the extraction of the Gac seed saponins (Figure 2) is also consistent with previous studies. For example, butanol has been reported to be the solvent of choice for the extraction of saponins from the hull of Chenopodium quinoa seeds [39] while methanol has been used widely to extract saponins from a wide range of plant matrices [13,38]. Nonetheless, if the safety and economical characteristics of the solvent extraction system are a high priority, then water with MAE could be a reasonable choice for the extraction of Gac seed saponins.

3.3. Effect of Extraction Methods on Total Phenolic Content (TPC)

The MAE system was the best way to extract the phenolics from the defatted Gac seed powder. MAE increased the TPC of the Gac seed extract by 25% compared to the conventional water extraction but UAE had no effect (Figure 3). However, the three water-based extractions were better than all the organic solvent extractions tested and the TPC for the MAE was 2.2 times higher than for the best of the alcoholic extractions tested, 70% methanol (Figure 3). These findings suggest that the extractable phenolic compounds from Gac seeds are very hydrophilic, which is consistent with gallic acid and *p*-hydroxybenzoic acids being previously identified in Gac seeds [10].

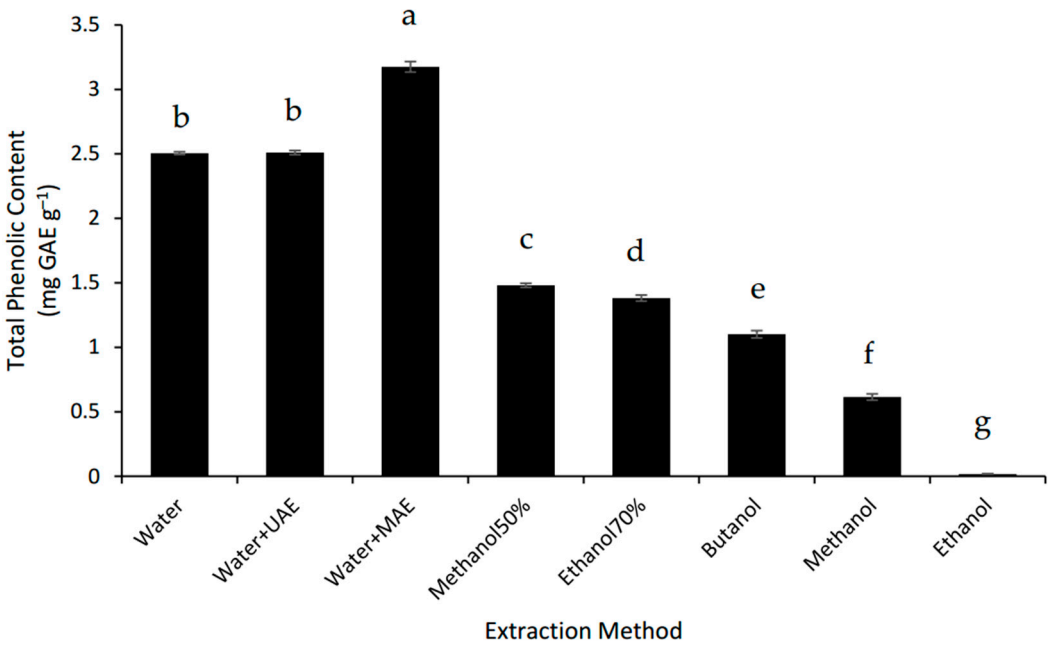

Figure 3. Total phenolic content of defatted Gac seed extracts affected by extraction method. The values are the mean of three replicates for each extraction. Columns not sharing the same superscript letter are significantly different at $p < 0.05$. GAE: gallic acid equivalents.

3.4. Effect of Extraction Methods on Total Solids and Antioxidant Capacity

Among the three extraction techniques using water as extracting solvent (CE, MAE, and UAE), MAE gave the highest TS value, followed by the conventional method, and then UAE (Table 2). The three aqueous extraction methods also gave higher TS values than all the alcohol extraction methods. These results suggest that the extractable material in the defatted Gac seed powder is highly polar, which is consistent with the finding that the aqueous extractions are enriched in trypsin inhibitors [6,40] (Figure 1) and phenolics [10] (Figure 3).

Table 2. Effect of extraction method on total solids and antioxidant capacity of defatted Gac seed powder.

Extraction Method	Total Solids (g kg^{-1})	ABTS (μmol TE g^{-1})	FRAP (μmol TE g^{-1})
Water	120.50 ± 2.16 ^{b}	21.74 ± 0.84 ^{a,b}	3.73 ± 0.40 ^{b}
Water + UAE	116.31 ± 0.40 ^{c}	19.86 ± 0.91 ^{b,c}	3.13 ± 0.10 ^{b}
Water + MAE	141.46 ± 1.08 ^{a}	23.56 ± 0.82 ^{a}	3.76 ± 0.12 ^{b}
50% methanol	83.13 ± 1.00 ^{d}	18.55 ± 1.09 ^{c}	3.76 ± 0.26 ^{b}
70% ethanol	79.37 ± 0.68 ^{e}	18.61 ± 0.96 ^{c}	4.71 ± 0.39 ^{a}
Butanol	43.79 ± 0.58 ^{g}	17.36 ± 1.49 ^{c}	5.25 ± 0.04 ^{a}
Methanol	48.76 ± 0.55 ^{f}	10.87 ± 0.57 ^{d}	3.36 ± 0.21 ^{b}
Ethanol	28.90 ± 0.19 ^{h}	6.05 ± 0.35 ^{e}	1.97 ± 0.08 ^{c}

The results display mean values ± standard deviation (n = 3). In a same column, values not sharing the same superscript letter are significantly different at $p < 0.05$. ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay; FRAP: ferric reducing antioxidant power assay; MAE: Microwaved-assisted extraction; UAE: Ultrasound-assisted extraction; TE: Trolox equivalents.

The antioxidant capacity of the extracts was measured using two assays—ABTS and FRAP. The extracts from the MAE and conventional aqueous method gave the highest value for ABTS (Table 2). For the FRAP assay, MAE and UAE did not improve the activity compared to the conventional extraction. Furthermore, the water-saturated butanol and the 70% ethanol extracts gave the highest FRAP values. Therefore, it is likely that the ABTS assay measured the antioxidant activity due to hydrophilic compounds, such as the Gac seed TPC (Figure 3), while the FRAP assay measured antioxidant activity due to more hydrophobic compounds, such as the Gac seed saponins (Figure 2). However, it may also be due to the FRAP assay measuring ferric reducing power of the extract rather than the free radical scavenging activity measured by the ABTS assay [27].

3.5. Correlations between Bioactive Compounds and Total Solids and Antioxidant Activity in the Extracts

As seen in Table 3, TIA was highly correlated with the amount of total solids across all the extracts. This could suggest that the trypsin inhibitors contributed to the total solids in the aqueous extracts (Figure 1). However, it is more likely that different proteins, not just the trypsin inhibitors, contributed to the total solids in the aqueous extracts [41]. In contrast, there were no correlations between TIA and the ABTS or FRAP values across the extracts (Table 3), which suggest that Gac seed trypsin inhibitors are unlikely to have antioxidant activity. However, in another study [42], trypsin inhibitors from *Cajanus cajan* and *Phaseolus limensis* were observed to possess FRAP antioxidant activities comparable to that of ascorbic acid. The difference in molecule structure of these trypsin inhibitors and the purity of the extracts possibly explained their different contributions to antioxidant activity [43].

Table 3. Correlations between bioactive compounds with total solids and antioxidant activity of defatted Gac seed extracts.

Bioactive Compounds	*R*-Squared Value		
	TS	ABTS	FRAP
TIA	0.80 †	0.50	0.02
TSC	0.24	0.02	0.38
TPC	0.96 †	0.85 †	0.05

TIA: Trypsin inhibitor activity; TSC: Total saponin content; TPC: Total phenolic content; TS: Total solids; ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay; FRAP: ferric reducing antioxidant power assay. † p value < 0.01.

There was also a very strong correlation between TPC and TS (Table 3), confirming the highly polar nature of the phenolic compounds in Gac seeds [10]. Phenolic compounds in plant materials are found to be well correlated with antioxidant potential [44,45]. The results in Table 3 also show that the TPC values correlated strongly with the ABTS antioxidant activity but not with the FRAP values. This suggests that the phenolic compounds present in Gac seed extracts acted as antioxidants through the mechanism of free radical scavenging (of ABTS) rather than the mechanism of reducing of

the oxidised intermediate in the FRAP chain reaction or through chelation [27]. Polyphenols that can neutralise free radicals by donating an electron or hydrogen atom [46] result in their strong antioxidant capacity possibly explained the correlation between TPC and ABTS in Gac seed extracts.

There were no significant correlations between the TSC and the TS, ABTS, and FRAP values across the extracts (Table 3), revealing that the saponins did not relate to the amount of material extracted from the defatted Gac seed powder and that they were not the main drivers of the ABTS or the FRAP antioxidant activity. However, a report of Tan et al. [26] showed that saponins from bitter melon correlated as strongly as phenolics with antioxidant capacity (R^2 ranged from 0.87 to 0.93). The structural diversity of different saponins from different materials possibly explains their variations in antioxidant activity.

4. Conclusions

MAE proved to be the best method for extracting phenolic compounds and the best aqueous extraction method for recovering saponins but it did not improve the extraction of trypsin inhibitors, for which the conventional water extraction was the best method. For saponins, water-saturated *n*-butanol and methanol were more efficient than MAE. On the other hand, UAE did not show any improvement in comparison to the tested conventional extraction methods.

Therefore, this study demonstrated that the choice of solvent and extraction method plays an important role in the recovery of bioactive compounds from defatted Gac seeds. The MAE method is recommended for the extraction of phenolic compounds and saponins, if an aqueous extraction is preferred due to the safety and environmental concerns when organic solvents are used. However, the conventional water extraction method is the best option for the extraction of trypsin inhibitors.

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References

1. Wimalasiri, D.; Piva, T.; Urban, S.; Huynh, T. Morphological and genetic diversity of *Momordica cochinchinenesis* (Cucurbitaceae) in Vietnam and Thailand. *Genet. Resour. Crop Evol.* **2016**, *63*, 19–33. [CrossRef]
2. Behera, T.; John, K.J.; Bharathi, L.; Karuppaiyan, R.; Momordica. *Wild Crop Relatives: Genomic and Breeding Resources*; Kole, C., Ed.; Springer: Heidelberg, NY, USA, 2011; pp. 217–246. ISBN 978-3-642-20449-4.
3. Chuyen, H.V.; Nguyen, M.H.; Roach, P.D.; Golding, J.B.; Parks, S.E. Gac fruit (*Momordica cochinchinensis* Spreng.): A rich source of bioactive compounds and its potential health benefits. *Int. J. Food Sci. Technol.* **2015**, *50*, 567–577. [CrossRef]
4. Masayo, I.; Hikaru, O.; Tatsuo, Y.; Masako, T.; Yoshie, R.; Shuji, H.; Kunihide, M.; Ryuichi, H. Studies on the constituents of *Momordica cochinchinensis* Spreng. I. Isolation and characterization of the seed saponins, Momordica saponins I and II. *Chem. Pharm. Bull.* **1985**, *33*, 464–478.
5. Chan, L.Y.; Wang, C.K.L.; Major, J.M.; Greenwood, K.P.; Lewis, R.J.; Craik, D.J.; Daly, N.L. Isolation and characterization of peptides from *Momordica cochinchinensis* seeds. *J. Nat. Prod.* **2009**, *72*, 1453–1458. [CrossRef] [PubMed]
6. Wong, R.C.; Fong, W.; Ng, T. Multiple trypsin inhibitors from *Momordica cochinchinensis* seeds, the Chinese drug mubiezhi. *Peptides* **2004**, *25*, 163–169. [CrossRef] [PubMed]
7. Chan, L.Y.; He, W.; Tan, N.; Zeng, G.; Craik, D.J.; Daly, N.L. A new family of cystine knot peptides from the seeds of *Momordica cochinchinensis*. *Peptides* **2013**, *39*, 29–35. [CrossRef] [PubMed]
8. Gao, X.M.; Mu, B.Z. (Eds.) Semen momordicae. In *Chinese Materia Medicia*; Traditional Chinese Materia Medica Press: Beijing, China, 2005; pp. 601–602.

9. Lin, Z.Y.; Liu, X.; Yang, F.; Yu, Y.Q. Structural characterization and identification of five triterpenoid saponins isolated from *Momordica cochinchinensis* extracts by liquid chromatography/tandem mass spectrometry. *Int. J. Mass Spectrom.* **2012**, *328*, 43–66. [CrossRef]
10. Kubola, J.; Siriamornpun, S. Phytochemicals and antioxidant activity of different fruit fractions (peel, pulp, aril and seed) of Thai gac (*Momordica cochinchinensis* Spreng). *Food Chem.* **2011**, *127*, 1138–1145. [CrossRef] [PubMed]
11. Samuelsson, G.; Kyerematen, G.; Farah, M.H. Preliminary chemical characterization of pharmacologically active compounds in aqueous plant extracts. *J. Ethnopharmacol.* **1985**, *14*, 193–201. [CrossRef] [PubMed]
12. Sasidharan, S.; Chen, Y.; Saravanan, D.; Sundram, K.; Latha, L.Y. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *Afr. J. Tradit. Complement. Altern. Med.* **2011**, *8*, 1–10. [CrossRef] [PubMed]
13. Cheok, C.Y.; Salman, H.A.K.; Sulaiman, R. Extraction and quantification of saponins: A review. *Food Res. Int.* **2014**, *59*, 16–40. [CrossRef]
14. Mahatmanto, T.; Poth, A.G.; Mylne, J.S.; Craik, D.J. A comparative study of extraction methods reveals preferred solvents for cystine knot peptide isolation from *Momordica cochinchinensis* seeds. *Fitoterapia* **2014**, *95*, 22–33. [CrossRef] [PubMed]
15. Tatke, P.; Jaiswal, Y. An overview of microwave assisted extraction and its applications in herbal drug research. *Res. J. Med. Plant* **2011**, *5*, 21–31. [CrossRef]
16. Pingret, D.; Fabiano-Tixier, A.S.; Chemat, F. Ultrasound-assisted extraction. In *Natural Product Extraction: Principles and Applications*; Mauricio, R., Juliana, P., Eds.; RSC Pub: Cambridge, UK, 2013; pp. 89–111, ISBN 1849736065, 9781849736060.
17. Chuyen, H.V.; Nguyen, M.H.; Roach, P.D.; Golding, J.B.; Parks, S.E. Microwave-assisted extraction and ultrasound-assisted extraction for recovering carotenoids from Gac peel and their effects on antioxidant capacity of the extracts. *Food Sci. Nutr.* **2018**, *6*, 189–196. [CrossRef] [PubMed]
18. Wang, L.; Weller, C.L. Recent advances in extraction of nutraceuticals from plants. *Trends Food Sci. Technol.* **2006**, *17*, 300–312. [CrossRef]
19. Bhuyan, D.J.; Vuong, Q.V.; Chalmers, A.C.; van Altena, I.A.; Bowyer, M.C.; Scarlett, C.J. Development of the ultrasonic conditions as an advanced technique for extraction of phenolic compounds from *Eucalyptus robusta*. *Sep. Sci. Technol.* **2017**, *52*, 100–112. [CrossRef]
20. Wu, J.; Lin, L.; Chau, F.T. Ultrasound-assisted extraction of ginseng saponins from ginseng roots and cultured ginseng cells. *Ultrason. Sonochem.* **2001**, *8*, 347–352. [CrossRef]
21. Bhuyan, D.J.; Vuong, Q.V.; Chalmers, A.C.; van Altena, I.A.; Bowyer, M.C.; Scarlett, C.J. Microwave-assisted extraction of *Eucalyptus robusta* leaf for the optimal yield of total phenolic compounds. *Ind. Crops Prod.* **2015**, *69*, 290–299. [CrossRef]
22. Anh, V.L.; Sophie, E.P.; Minh, H.N.; Paul, D.R. Optimisation of the microwave-assisted ethanol extraction of saponins from Gac (*Momordica cochinchinensis* Spreng.) seeds. *Medicines* **2018**, *5*, 70. [CrossRef]
23. Makkar, H.P.; Siddhuraju, P.; Becker, K. Trypsin Inhibitor. In *Plant Secondary Metabolites*; Humana Press: New York, NY, USA, 2007; pp. 1–6. ISBN 1588299937.
24. Stauffer, C.E. Measuring trypsin inhibitor in soy meal: Suggested improvements in the standard method. *Cereal Chem.* **1990**, *67*, 296–302.
25. Tan, S.P.; Kha, T.C.; Parks, S.E.; Stathopoulos, C.E.; Roach, P.D. Effects of the spray-drying temperatures on the physiochemical properties of an encapsulated bitter melon aqueous extract powder. *Powder Technol.* **2015**, *281*, 65–75. [CrossRef]
26. Tan, S.P.; Vuong, Q.V.; Stathopoulos, C.E.; Parks, S.E.; Roach, P.D. Optimized aqueous extraction of saponins from bitter melon for production of a saponin-enriched bitter melon powder. *J. Food Sci.* **2014**, *79*, E1372–E1381. [CrossRef] [PubMed]
27. Thaipong, K.; Boonprakob, U.; Crosby, K.; Cisneros-Zevallos, L.; Byrne, D.H. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J. Food Compost. Anal.* **2006**, *19*, 669–675. [CrossRef]
28. Boye, J.I.; Ma, Z. Impact of Processing on Bioactive Compounds of Field Peas. In *Processing and Impact on Active Components in Food*; Preedy, V., Ed.; Elsevier: Boston, MA, USA, 2015; pp. 63–70. ISBN 978-0-12-404699-3.
29. Serrano, M.; Rebollar, P.; Sueiro, S.; Hermida, M.; Mateos, G. Influence of duration of storage on protein quality traits of soybean meals. *J. Appl. Poult. Res.* **2013**, *22*, 423–429. [CrossRef]

30. Smith, C.; van Megen, W.; Twaalfhoven, L.; Hitchcock, C. The determination of trypsin inhibitor levels in foodstuffs. *J. Sci. Food Agric.* **1980**, *31*, 341–350. [CrossRef] [PubMed]
31. Huang, H.; Kwok, K.C.; Liang, H.H. Inhibitory activity and conformation changes of soybean trypsin inhibitors induced by ultrasound. *Ultrason. Sonochem.* **2008**, *15*, 724–730. [CrossRef] [PubMed]
32. Vagadia, B.H.; Vanga, S.K.; Raghavan, V. Inactivation methods of soybean trypsin inhibitor—A review. *Trends Food Sci. Technol.* **2017**, *64*, 115–125. [CrossRef]
33. Pysz, M.; Polaszczyk, S.; Leszczyńska, T.; Piątkowska, E. Effect of microwave field on trypsin inhibitors activity and protein quality of broad bean seeds (*Vicia faba* var. major). *Acta. Sci. Pol. Technol. Aliment.* **2012**, *11*, 193–198. [PubMed]
34. Nicholls, A.; Sharp, K.A.; Honig, B. Protein folding and association: Insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins Struct. Funct. Bioinform.* **1991**, *11*, 281–296. [CrossRef] [PubMed]
35. Pacheco, W.; Stark, C.; Ferket, P.; Brake, J. Effects of trypsin inhibitor and particle size of expeller-extracted soybean meal on broiler live performance and weight of gizzard and pancreas. *Poult. Sci.* **2014**, *93*, 2245–2252. [CrossRef] [PubMed]
36. Jirgensons, B. Effects of *n*-propyl alcohol and detergents on the optical rotatory dispersion of α-chymotrypsinogen, β-casein, histone fraction F1, and soybean trypsin inhibitor. *J. Biol. Chem.* **1967**, *242*, 912–918. [PubMed]
37. Pesoti, A.R.; Oliveira, B.M.; Oliveira, A.C.; Pompeu, D.G.; Gonçalves, D.B.; Marangoni, S.; Silva, J.A.; Granjeiro, P.A. Extraction, purification and characterization of inhibitor of trypsin from Chenopodium quinoa seeds. *Food Sci. Technol.* **2015**, *35*, 588–597. [CrossRef]
38. Güçlü-Üstündağ, Ö.; Mazza, G. Saponins: Properties, applications and processing. *Crit. Rev. Food Sci. Nutr.* **2007**, *47*, 231–258. [CrossRef] [PubMed]
39. Madl, T.; Sterk, H.; Mittelbach, M.; Rechberger, G.N. Tandem mass spectrometric analysis of a complex triterpene saponin mixture of Chenopodium quinoa. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 795–806. [CrossRef] [PubMed]
40. Hernandez, J.F.; Gagnon, J.; Chiche, L.; Nguyen, T.M.; Andrieu, J.P.; Heitz, A.; Trinh, H.T.; Pham, T.T.; Le, N.D. Squash trypsin inhibitors from *Momordica cochinchinensis* exhibit an atypical macrocyclic structure. *Biochemistry* **2000**, *39*, 5722–5730. [CrossRef] [PubMed]
41. Sastry, M.; Murray, D.R. The contribution of trypsin inhibitors to the nutritional value of chick pea seed protein. *J. Sci. Food Agric.* **1987**, *40*, 253–261. [CrossRef]
42. Shamsi, T.N.; Parveen, R.; Afreen, S.; Azam, M.; Sen, P.; Sharma, Y.; Fatima, S. Trypsin inhibitors from *Cajanus cajan* and *Phaseolus limensis* possess antioxidant, anti-inflammatory, and antibacterial activity. *J. Diet. Suppl.* **2018**, *15*, 939–950. [CrossRef] [PubMed]
43. Zou, T.B.; He, T.P.; Li, H.B.; Tang, H.W.; Xia, E.Q. The structure-activity relationship of the antioxidant peptides from natural proteins. *Molecules* **2016**, *21*, 72. [CrossRef] [PubMed]
44. Kähkönen, M.P.; Hopia, A.I.; Vuorela, H.J.; Rauha, J.P.; Pihlaja, K.; Kujala, T.S.; Heinonen, M. Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* **1999**, *47*, 3954–3962. [CrossRef] [PubMed]
45. Pham, H.N.T.; Nguyen, V.T.; Vuong, V.Q.; Bowyer, M.C.; Scarlett, C.J. Bioactive compound yield and antioxidant capacity of *Helicteres hirsuta* Lour. stem as affected by various solvents and drying methods. *J. Food Process. Preserv.* **2017**, *41*, e12879. [CrossRef]
46. Tsao, R. Chemistry and biochemistry of dietary polyphenols. *Nutrients* **2010**, *2*, 1231–1246. [CrossRef] [PubMed]

3.3.2. Optimised extraction of trypsin inhibitors from defatted Gac (*Momordica cochinchinensis* Spreng) seeds for production of a trypsin inhibitor-enriched freeze-dried powder.

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Optimised Extraction of Trypsin Inhibitors from Defatted Gac (*Momordica cochinchinensis* Spreng) Seeds for Production of a Trypsin Inhibitor-Enriched Freeze Dried Powder

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Abstract: The seeds of the Gac fruit, *Momordica cochinchinensis* Spreng, are rich in trypsin inhibitors (TIs) but their optimal extraction and the effects of freeze drying are not established. This study aims to (1) compare aqueous solvents (DI water, 0.1 M NaCl, 0.02 M NaOH and ACN)/water/FA, 25:24:1) for extracting TIs from defatted Gac seed kernel powder, (2) to optimise the extraction in terms of solvent, time and material to solvent ratio and (3) to produce a TI-enriched freeze-dried powder (FD-TIP) with good characteristics. Based on the specific TI activity (TIA), the optimal extraction was 1 h using a ratio of 2.0 g of defatted powder in 30 mL of 0.05 M NaCl. The optimisation improved the TIA and specific TIA by 8% and 13%, respectively. The FD-TIP had a high specific TIA (1.57 ± 0.17 mg trypsin/mg protein), although it also contained saponins (43.6 ± 2.3 mg AE/g) and phenolics (10.5 ± 0.3 mg GAE/g). The FD-TIP was likely stable during storage due to its very low moisture content (0.43 ± 0.08%) and water activity (0.18 ± 0.07) and its ability to be easily reconstituted in water due to its high solubility index (92.4 ± 1.5%). Therefore, the optimal conditions for the extraction of TIs from defatted Gac seed kernel powder followed by freeze drying gave a high quality powder in terms of its highly specific TIA and physical properties.

Keywords: Gac; seeds; *Momordica cochinchinensis*; extraction; trypsin inhibitors; optimisation; freeze drying; response surface methodology

1. Introduction

Trypsin inhibitors (TIs) are low molecular weight peptides which can inhibit the hydrolase activity of many kinds of serine proteases. They are commonly found in the storage organs of plants, such as seeds, roots and tubers. Three major sub-types of TIs have been reported and identified in plants [1]: the Bowman–Birk-type inhibitors, Kunitz-type inhibitors and squash family inhibitors. Their molecular weights are about 7500, 20000 and 3500 kDa, respectively. The first two types were isolated from leguminous plants while the third was obtained from Cucurbitaceous species [1].

This study focuses on the seeds from Gac (*Momordica cochinchinensis* Spreng), a plant that belongs to the Cucurbitaceae family. The aril around the seeds of the Gac fruit is widely used as a food

ingredient but the seeds are mainly discarded. However, the seeds have long been used as a traditional Chinese medicine (Mu bie zi) to treat many common diseases such as boils, pyodermatitis, mastitis, tuberculous cervical lymphadenitis, ringworm infections, freckles, sebaceous hemorrhoids and hemangiomas [2,3].

Several TIs from *Momordica cochinchinensis* (MCoTIs) have been characterised [1,3–5] and proposed to be among the most important bioactives in Gac seeds. They serve as storage proteins and may also be involved in the regulation of endogenous proteases during seed dormancy [6]. Nine MCoTIs have been isolated and sequenced from the seeds of Gac fruit [1,2,4]. Structurally, MCoTIs consist of 28–34 amino acid residues, six of which are cysteine residues that form three disulfide bonds. The Gac seed TIs have a very small molecular weight of 3–5 kDa [1,2,4] and in comparison to other TI families, they are more compact in structure and exceptionally stable [7–9]. Among these, MCoTI-I and MCoTI-II are cyclic peptides and as such, they have a very compact and stable structure [4,10]. This enables them to penetrate into cells and, therefore, they are attractive candidates for use as scaffolds for the development of novel intracellularly-targeted drugs [11,12]. Moreover, the activity of Gac seed TIs is very high, at least 50-fold more potent than those from different *Cucurbitaceous* seeds [2]. Due to their clinical potential, Gac seed TIs could be used in a variety of applications in medicine, agriculture and food technology.

As for all plant-derived natural products, extraction is the first critical step in the isolation of TIs from their sources. However, up to date, there are only a few papers dealing with the suitability of different solvents and the extraction conditions for the extraction of these valuable compounds from Gac seeds. In one study [13], a mixture of acetonitrile, water and formic acid (ACN/Water/FA) was found to be optimal for extracting cysteine knot peptides from Gac seeds, some of which are trypsin inhibitors. In our previous study [14], we showed that trypsin inhibitors were able to be effectively extracted from defatted Gac seed kernel powder using conventional solvent extraction with deionised (DI) water only. For other plant sources of TIs, water was also the optimal solvent for their extraction from Thai mung beans [15] but 0.1M NaCl was the best for their extraction from *Chenopodium quinoa* seeds [16] and 0.02 M NaOH was the best for their extraction from grass peas [17]. Clearly, aqueous media are the best solvents for the extraction of TIs.

Apart from the type of solvent used, the efficiency of extractions can be affected by other factors, such as extraction time and the ratio of sample to solvent. For the extraction of trypsin inhibitors, the extraction conditions have so far been studied by conducting one-factor-at-a-time experiments [15–17]. However, one-factor-at-a-time experiments cannot fully determine the interactions between different factors [18] and to overcome this deficiency, the response surface methodology (RSM) is used to determine the simultaneous effects of several factors on extractions.

Therefore, the present study aimed to determine the suitability of different aqueous media (DI water, 0.1 M NaCl, 0.02 M NaOH and ACN)/water/FA) as extraction solvents and to further determine the optimal conditions for the extraction of TIs from defatted Gac seed kernel powder by using RSM. The optimised extraction conditions were then used to produce a TI-enriched freeze-dried powder and the physicochemical properties of the freeze-dried powder were determined.

2. Materials and Methods

2.1. Materials

2.1.1. Reagents and Chemicals

Trypsin (type I) from bovine pancreas, benzyl-DL-arginine-para-nitroanilide (BAPNA), dimethyl sulfoxide (DMSO), tris(hydroxymethyl)aminomethane, bovine serum albumin, Folin-Ciocalteu's phenol reagent, cupric sulphate, sodium carbonate, sodium tartrate and formic acid were products of Sigma-Aldrich (Castle Hills, NSW, Australia). Sodium hydroxide and calcium chloride were from Merck (Bayswater, VIC, Australia) and sodium chloride and acetic acid were from Chem-Supply (Port Adelaide, SA, Australia).

2.1.2. Gac Seeds

Gac seeds were collected from 450 kg of fresh Gac fruit from *Momordica cochinchinensis* accession VS7 as classified by Wimalasiri et al. [19]. These fruits were obtained at Gac fruit fields in Dong Nai province, Ho Chi Minh City, Vietnam (Latitude: 10.757410; Longitude: 106.673439). The seeds were separated from the seed pulp and then vacuum dried at 40 °C for 24 h to reduce their moisture and to increase the crispness of the shells to facilitate their removal. The dried seeds were de-shelled to get the kernels, which were then packaged in vacuum-sealed aluminium bags and stored at −18 °C for use within 4 years.

2.1.3. Preparation of Defatted Gac Seed Kernel Powder

The Gac seed kernels were ground using an electric grinder (100 g ST-02A Mulry Disintegrator) to a powder that could pass through a 1.4-mm sieve. The powder was then frozen with liquid nitrogen and freeze-dried using a Dynavac FD3 Freeze Dryer (Sydney, NSW, Australia) for 48 h at −45 °C under vacuum at a pressure loading of 10^{-2} mbar (1 Pa), to reduce the moisture content to 1.21 ± 0.02%. The powder was then defatted using hexane (1:5 w/v, 30 min, ×3) on a magnetic stirrer at ambient temperature. The resulting slurry was suction filtered using a Buchner funnel and Whatman No. 1 filter paper (Sigma-Aldrich, Castle Hills, NSW, Australia). The residue was placed in a fume hood at ambient temperature until dry and free of hexane odour (~12 h) and stored in an air-tight jar at ambient temperature for use within a year. This defatted Gac seed kernel powder was referred to as "defatted powder" and its moisture content was measured, using a Shimazdu MOC63u moisture analyser (Gallay Medical & Scientific, Mulgrave, VIC, Australia), prior to weighing for extractions so that the results could be expressed in terms of the defatted powder's dry weight (DW).

2.2. Methods

2.2.1. Experiment Design

A summary of the experimental design for the study is shown in Scheme 1.

2.2.2. Extraction with Four Aqueous Media

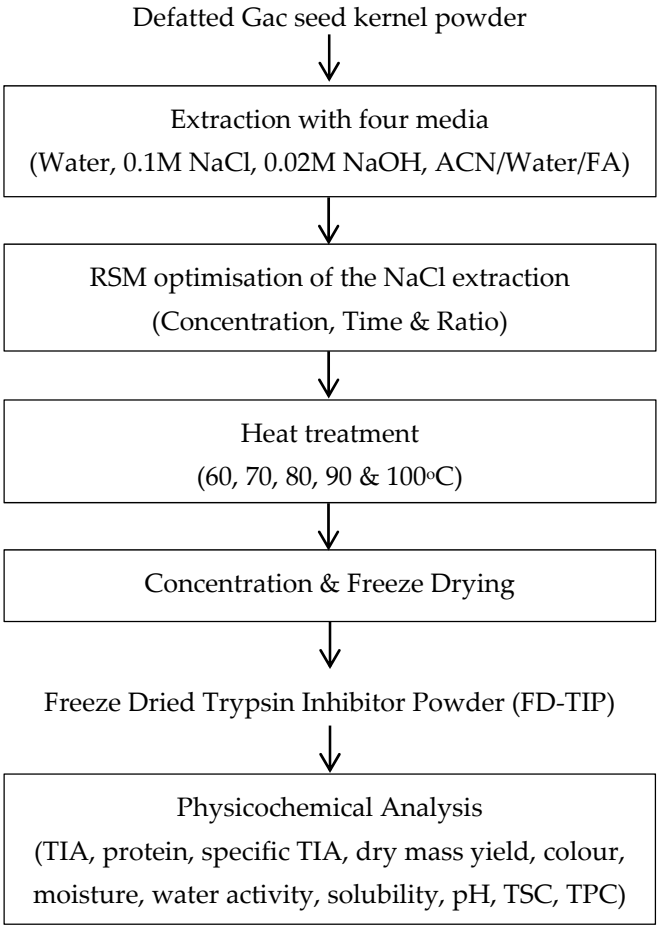
Due to the known hydrophilic nature of the Gac seed TIs, aqueous solutions were investigated as solvents for their extraction. Four different aqueous media were compared for their efficiency to extract TIA from the defatted powder: (1) DI water, (2) 0.1 M NaCl, (3) 0.02 M NaOH and (4) ACN)/Water/FA, (25:24:1, v/v/v).

Defatted powder (1.5 g) was suspended in 30 mL of each medium in a 100-mL conical flask and shaken for 1 h at 110 rpm at ambient temperature (17 °C) using a Citenco KQ-606 shaking water bath (Citenco Ltd., London, UK). The suspensions were filtered through a 0.45 µm syringe filter (Phenomenex, Lane Cove, NSW, Australia). The clear filtrates were collected and their TIA (mg trypsin/g defatted powder DW), protein content (mg/g defatted powder DW) as well as their specific TIA (TIA/protein content) were analysed. Each extraction was done in triplicate.

2.2.3. Optimisation of the Extraction Conditions Using RSM

The results from the extractions in Section 2.2.2 showed that the 0.1 M NaCl solution was the best media for extracting TIA from the defatted powder. Therefore, the NaCl solution was selected for further optimisation of the extraction using the response surface methodology (RSM) with three independent factors: the NaCl concentration of the extraction media, the extraction time and the ratio of defatted powder to the volume of NaCl solution. Based on the results from preliminary experiments (data not shown), three levels (Table 1) were selected for a Box–Behnken RSM design [20] to test the NaCl concentration (X_1, mol/L), the extraction time (X_2, hour) and the amount of defatted powder (X_3, g) extracted with 30 mL of the extraction NaCl solution. Therefore, 15 experimental combinations representing 12 factorial points and three central points were performed randomly in duplicate and the experimental values for TIA (mg trypsin/g powder DW), protein

content (mg/g defatted powder DW) and specific TIA (mg trypsin/mg protein) were determined for each of the 15 combinations.



Scheme 1. Experimental design for the study. ACN: acetonitrile; FA: formic acid; TIA: trypsin inhibitor activity, TSC: total saponin content, TPC: total phenolic content.

Table 1. Independent factors and their levels for the RSM Box–Behnken design.

Levels	**Independent factors**		
	X_1	X_2	X_3
	NaCl concentration (mol/L)	**Extraction time (h)**	**Ratio (g/ 30 mL)**
−1	0.050	1.0	1.0
0	0.175	2.5	3.0
+1	0.300	4.0	5.0

The data obtained for the selected combinations was used to generate the second-order polynomial equation/quadratic model shown in Equation 1, which was then used to predict the optimal parameters for the extraction process [21]. To test the predicted optimal parameters, these parameters were used for a TI extraction from defatted powder done in triplicate.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y is the response variable; X_1, X_2, X_3 are the coded independent variables for the NaCl concentration, the extraction time and the ratio of sample to solvent (Table 1), respectively. β_0, β_1, β_2, β_3 and β_{11}, β_{22}, β_{33}, β_{12}, β_{13}, β_{23} are the regression coefficients for the constant (β_0), the linear effects (β_1, β_2, β_3), the quadratic effects (β_{11}, β_{22}, β_{33}) and the interactions (β_{12}, β_{13}, β_{23}), respectively.

2.2.4. Heat Treatment of the Optimal TI Extract

The extract obtained with the optimal NaCl extraction conditions determined in Section 2.2.3. (2.0 g of defatted powder in 30 mL of 0.05 M NaCl for 1 h) was then heated at different temperatures to determine whether any unstable proteins could be removed as described by Klomklao et al. [15]. Triplicate samples (5 mL) of the extract were heated at different temperatures (60, 70, 80, 90 and 100 °C) for 10 min and then cooled with ice water. As controls, triplicate samples were kept at ambient temperature. To remove any heat coagulated debris, the extracts were filtered through a 0.45 μm syringe filter (Phenomenex, Lane Cove, NSW, Australia). The TIA (mg trypsin/ g powder DW), protein content (mg/g defatted powder DW) and specific TIA (mg trypsin/mg protein) were then determined for the clear filtrates.

2.2.5. Concentrating and Freeze Drying the Extract Prepared with the Optimal Parameters

Based on the results in Section 2.2.4, which showed that heat treatment between 60 and 100 °C had no effect on the specific TIA of the extract, heat treatment was not included as a step in the production of a freeze-dried powder with high specific TIA. The optimal extraction parameters determined in Section 2.2.3. were applied to extract the defatted powder but the relative amounts were increased by 60 in order to obtain an amount of freeze-dried material sufficient for the many physicochemical analyses to be undertaken.

The defatted powder (120 g) was stirred with 1,800 mL of 0.05 M NaCl for 1 h at ambient temperature and then the suspension was filtered through 3 layers of cheese cloth and finally through a No. 1 Whatman filter paper. The collected filtrate was equally transferred into three evaporating flasks (1 L) and concentrated using a rotary evaporator (Buchi Rotavapor B480, Buchi Australia, Noble Park, VIC, Australia) at 45 °C under vacuum, until around 70 mL of the concentrated extract was left in each flask (~4 h). The three concentrates were transferred into three 250 mL beakers, frozen using liquid nitrogen and freeze-dried using a Scitek BenchTop Pro freeze dryer (Lane Cove, NSW, Australia) at −60 °C and 30 mbar for 48 h.

The three beakers with the freeze dried trypsin inhibitor powder (FD-TIP) were placed in a desiccator, quickly weighed and kept at ambient temperature until analysed. The difference in weight between each beaker with powder and the empty beakers was taken to be the mass recovered in each beaker for the extract.

2.2.6. Determination of Trypsin-Inhibiting Activity (TIA)

The TIA assay was performed as described by Makkar et al. [22] except that the absorbance was measured at 385 nm, as suggested by Stauffer [23], instead of at 410 nm.

- *Reagent preparation:*

Substrate solution: A substrate solution of 92 mM BAPNA was made in 0.05 M Tris-buffer (pH 8.2) containing 0.02 M $CaCl_2$. The BAPNA was first dissolved in DMSO (40 mg/mL) and then diluted with the buffer solution pre-warmed to 37 °C (1:100 v/v). This BAPNA solution was prepared daily and kept at 37 °C while in use.

Trypsin solution: 20 mg of trypsin (type I) from bovine pancreas was dissolved in 1 mM HCl to make 1 L, stored at 4 °C for use within 2 weeks.

- *Determination of TIA*

The liquid extracts from Section 2.2.2, 2.2.3 and 2.2.4 or the freeze-dried trypsin-inhibitor powder (FD-TIP) from Section 2.2.5 was dissolved and diluted in water at a concentration to give an inhibition of Trypsin between 40 and 60%. The assay was setup as shown in Table 2 with four test tubes (a, b, c and d) prepared for each sample. All the prepared test tubes were kept in a water bath at 37 °C for 10 min to promote the formation of an enzyme-inhibitor complex; 2.5 mL of BAPNA solution pre-warmed to 37 °C was then added into each tube. The contents of the tubes were well mixed after each addition. The tubes were then incubated in the water bath at 37 °C for another 10 min before 0.5 mL of 30% acetic acid solution was added to each tube to stop the reaction. Then, 1.0 mL of trypsin

solution was added into each blank tube (Table 2). After thorough mixing, the absorbance of the reaction mixture (due to the release of *p*–nitroanilide) was measured at 385 nm.

- *Calculations*

The TIA was calculated in terms of milligrams of pure trypsin inhibited per gram on a dry-weight basis (mg/g DW) of the defatted Gac seed kernel powder or the FD-TIP (Equation 2) [14].

$$TIA = \frac{A_I \times V \times D}{19 \times S \times (1 - m\%/100)} \quad (2)$$

where, A_I: Change in absorbance due to inhibition per 1 mL of diluted extract

$A_I = (A_b - A_a) - (A_d - A_c)$, subscripts as per Table 2.

V: Volume of undiluted extract (mL)

D: Dilution factor of the extract

S: Weight of defatted Gac seed kernel powder or FD-TIP in V mL (g)

19: Constant figure based on the absorbance given by 1 mg of pure trypsin

m%: moisture content of defatted Gac seed kernel powder or freeze-dried extract powder

Table 2. Reagent composition in extracted filtrate.

Component (mL)	Reagent Blank (a)	Standard (b)	Sample Blank (c)	Sample (d)
DI water	1	1	0.5	0.5
Trypsin solution	-	1	-	1
Trypsin-inhibitor sample	-	-	0.5	0.5
Trypsin solution after reaction inactivation	1	-	1	-

2.2.7. Determination of Total Protein Yield (TPY)

Total protein yield (TPY) was defined as the amount (mg) of water-soluble protein obtained per 100 g of ground defatted seed kernel powder on dry weight (DW) basis. TPY was measured by the method of Lowry et al. [24] as described by Klomklao et al. [15], with some modifications. Briefly, 0.5 mL of diluted sample or standard was mixed with 2.5 mL of reagent A containing 2 mL of 0.5% $CuSO_4$ in 1% sodium citrate and 100 mL of 2% sodium carbonate in 0.1 N NaOH for 10 min at ambient temperature. Then, 0.25 mL of 0.5 N Folin-Ciocalteu phenol reagent was added while vortexing. After incubating for 30 min, the absorbance of the reaction mixture was measured at 750 nm using a Cary 50 Bio UV–VIS spectrophotometer (Agilent Technologies, Mulgrave, VIC, Australia). Bovine serum albumin (BSA) was used as a standard and the result was expressed as mg BSA per gram of the defatted Gac seed kernel powder or the FD-TIP (mg BSA/g DW).

2.2.8. Physicochemial Analyses on the FD-TIP

Along with the determination of TIA (Section 2.2.6) and protein content (Section 2.2.7), the following physicochemical analyses were done on each of the FD-TIP in the three beakers described in Section 2.2.5.

- *Dry Mass Yield, Moisture Content, Water Activity, Water Solubility Index, pH and Colour*

The dry mass yield was defined as the amount (g) of FD-TIP produced per 100 g of dried defatted Gac seed kernel powder. Equation 3 was used to calculate the dry mass yield (DM), in which FD (g) is the weight of the FD-TIP obtained after the extraction and DS (g) is the mass of the dried defatted Gac seed kernel powder used for the extraction.

$$DM\ (g/100g) = \frac{FD}{DS} \times 100 \quad (3)$$

The moisture content of the FD-TIP was determined by weight difference after drying at 80 °C for 24 h in a vacuum oven drier (Thermoline Scientific, Wetherill Park, NSW, Australia). The water activity of the freeze dried powder was measured using a Pawkit water activity meter (Graintec, Toowoomba, QLD, Australia).

The water solubility index of the FD-TIP was determined according to Anderson [25] with some modifications. The FD powder (2.5 g) was dispersed in 25 mL of DI water and stirred constantly for 10 min at ambient temperature. The solution was centrifuged at 3,000 rpm for 10 min in a Clements 2000 centrifuge (Clements Medical Equipment Pty Ltd., Somersby, NSW, Australia). The supernatant was vacuum oven dried at 80 °C and −70 kPas for 44 h. The water solubility index is expressed as a percentage of the dry solids obtained after drying of the extracted material compared to the original 2.5 g FD powder sample.

To determine the pH of the powders, 2.5 g of powder was dissolved in 25 mL of deionized water and the pH was measured using a labCHEM pH meter (TPS Pty Ltd., Brendale, QLD, Australia) calibrated with standard pH 4 and 7 buffers.

The colour of the powder was measured using a CR-400 chroma meter (Thermo Fisher Scientific, North Ryde, NSW, Australia) calibrated with a white standard tile. Each of the three FD-TIPs were packed into a polyethylene pouch for colour measurements, and the results for each sample were the average of five measurements expressed as the Hunter colour values for the L*, a* and b* co-ordinates as defined by the CIE (Commission Internationale de l'Eclairage). The L* value represents the lightness–darkness dimension, the a* value represents the red–green dimension and the b* value represents the yellow–blue dimension.

- *Total Saponin Content (TSC)*

The FD-TIP crude extract was dissolved in water at a concentration of 2 mg/mL and vortexed before the TSC was determined according to Le et al. [26]. Briefly, 0.25 mL of each extract was mixed with 0.25 mL of 8% (w/v) vanillin solution and 2.5 mL of 72% (v/v) sulphuric acid. The mixture was vortexed and incubated in a water bath at 60 °C for 15 min and then cooled on ice for 10 min. The absorption of the mixture was measured at 560 nm using a Cary 50 Bio UV–VIS spectrophotometer (Agilent Technologies, Mulgrave, VIC, Australia). Aecsin was used as a standard and the results are expressed as milligram aecsin equivalents (AE) per gram of the FD-TIP (mg AE/g DW).

- *Total Phenolic Content (TPC)*

The FD-TIP was dissolved in water at a concentration of 2 mg/mL and vortexed before the TPC was determined according to the method of Le et al. [26]. Briefly, 0.5 mL of each extract was mixed with 2.5 mL of 10% (v/v) Folin-Ciocalteu's phenol reagent in water and incubated at ambient temperature for 2 min to equilibrate. Then, 2 mL of 7.5% (w/v) sodium carbonate solution in water was added and the mixture was incubated at ambient temperature for 1 h. The absorption of the reaction mixture was measured at 765 nm using a Cary 50 UV–Vis spectrophotometer. Gallic acid was used as a standard and the results are expressed as milligram gallic acid equivalents (GAE) per gram of the FD-TIP (mg GAE/g DW).

2.2.9. Statistical Analyses

For designing and analysing the RSM experiment, including generating the three-dimensional (3D) surface and two-dimensional (2D) contour plots, the JMP software version 13.0 (SAS, Cary, NC, USA) was used. The adequacy of the RSM second-order polynomial model was determined based on the lack of fit and the coefficient of determination (R^2).

For extractions performed in triplicate, the means ± SD were assessed with the IBM SPSS Statistics 24 program (IBM Corp., Armonk, NY, USA) using the Student's *t*-test, when only two means were compared, or the one-way analysis of variance (ANOVA) and Tukey's Post Hoc multiple comparisons test, when more than two means were compared. Differences in means were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Extraction with four Aqueous Media

The highest TIA was obtained with the ACN/water/FA extraction media (Table 3). This result was in accordance with the findings of Mahatmanto [13], who reported that the extraction mixture of ACN/water/FA yielded the highest concentration in cyclotides from Gac seeds, some of which are trypsin inhibitors. However, in the present study, a high TPY was also found for this solvent mixture, which reduced the specific TIA to the second lowest value (Table 3).

The solution of 0.1 M NaCl achieved the second highest TIA. However, this solvent extracted a low protein content, which therefore resulted in the highest specific TIA value. The effectiveness of 0.1 M NaCl is consistent with the extraction of TIs from *Chenopodium quinoa* seeds [16]. DI water also achieved the highest specific TIA; however, it yielded a 35% lower TIA in comparison to 0.1 M NaCl (Table 3) and, therefore, water was less effective than 0.1 M NaCl in the recovery of Gac seed trypsin inhibitors.

Table 3. Effect of extraction media on the extraction of Gac seed trypsin inhibitors [†].

Extraction Media	Mean ± SD		
	TIA (mg trypsin/g defatted powder)	TPY (mg BSA/g defatted powder)	Specific TIA (mg trypsin/ mg protein)
DI Water	85.22 ± 0.47 [c]	42.55 ± 3.15 [d]	2.01 ± 0.14 [a]
0.1 M NaCl	140.39 ± 8.33 [b]	80.58 ± 0.65 [c]	1.74 ± 0.11 [a]
ACN/Water/FA	168.11 ± 0.50 [a]	152.44 ± 2.08 [b]	1.10 ± 0.01 [b]
0.02 M NaOH	81.43 ± 2.02 [c]	245.19 ± 1.06 [a]	0.33 ± 0.01 [c]

[†] The defatted powder was extracted in different media at ambient temperature (17 ± 1 °C) for 1 h. Trypsin-inhibitor activity (TIA) was analysed using BAPNA as a substrate [23,24] and total protein yield (TPY) was determined using the Lowry assay [25]. Different letter superscripts for the values in the same column denote statistically significant differences ($p < 0.05$).

The 0.02 M NaOH showed the lowest capacity for extracting trypsin inhibitors from Gac seeds, as evidenced by the lowest specific activity, particularly with the highest protein concentration (Table 3). This is consistent with a study on the extractability of protein from flamboyant seeds [27], which found that their protein was most soluble in NaOH followed by NaCl and then water. The result was also in agreement with Benjakul et al. [28], who reported that the extraction of proteins from cowpea and pigeon pea was markedly increased when alkaline solution was used, compared to NaCl. The increase of protein content was observed when grass pea was solubilised with NaOH solution [17]. There are many factors involved in protein solubility and recovery including protein meal and solvent ratio, particle size of flour, temperature, length of extraction time, pH, ionic strength, type and concentration of extraction as well as the hydration properties of proteins [29]. From the results, 0.1 M NaCl was selected as the extractant for Gac seeds.

3.2. Optimisation of the Extraction Conditions Using RSM

3.2.1. Fitting the Response Surface Model

Table 4 shows the experimental values (Exp.) measured for TIA and TPY and the Exp. values calculated for specific TIA in the extracts prepared as described by the RSM Box–Behnken design in Table 2. From the Exp. TIA values, the response surface model described in Equation 4 was generated and the values predicted (Pred.) for the specific TIA values by this model are also shown in Table 4.

$$Y = 1.557 - 0.154X_1 + 0.041X_2 - 0.043X_3 + 0.014X_1^2 + 0.234X_2^2 - 0.153X_3^2 + 0.020X_1X_2 + 0.063X_1X_3 + 0.038X_2X_3; \quad (R^2 = 0.98) \tag{4}$$

Table 4. The experimental values for trypsin-inhibiting activity (TIA), total protein yield (TPY) and specific TIA and the predicted values for specific TIA for the extracts produced using a 15 run RSM Box–Behnken design.

Run	Pattern	X_1 NaCl (mol/L)	X_2 Time (h)	X_3 Ratio (g/30 mL)	TIA	TPY	Specific TIA	
					Exp.	Exp.	Exp.	Pred.
1	− − 0		1	3	155.04	79.85	1.94	1.94
2	− + 0	0.05	4	3	156.19	79.18	1.97	1.98
3	+ − 0	0.3	1	3	164.01	102.43	1.60	1.59
4	+ + 0	0.3	4	3	189.26	110.69	1.71	1.71
5	0 − −	0.175	1	1	152.39	93.85	1.62	1.68
6	0 − +	0.175	1	5	143.55	91.79	1.56	1.52
7	0 + −	0.175	4	1	178.60	108.97	1.64	1.68
8	0 + +	0.175	4	5	177.72	102.65	1.73	1.67
9	− 0 −	0.05	2.5	1	142.63	82.31	1.73	1.68
10	+ 0 −	0.3	2.5	1	168.29	130.12	1.29	1.24
11	− 0 +	0.05	2.5	5	115.65	81.72	1.42	1.47
12	+ 0 +	0.3	2.5	5	145.57	118.48	1.23	1.28
13	000	0.175	2.5	3	167.90	105.47	1.59	1.56
14	000	0.175	2.5	3	147.02	99.21	1.48	1.56
15	000	0.175	2.5	3	163.60	102.10	1.60	1.56

The determination coefficient (R^2 = 0.98) and the p value for the lack of fit (0.42), shown in Table 5, indicated that the model for the specific TIA of the 15 extracts (Equation 4) had a very high and statistically significant fit; there was a 98% fit of the predicted values with the experimental values (Table 4). Therefore, the response surface model for the specific TIA (Equation 4) was adequate and suitable for describing the effects of and the interactions between the three independent factors, the NaCl concentration of the extraction media, the extraction time and the ratio of defatted powder to extraction media and could be used to determine the optimum values for these extraction parameters.

Table 5. The regression coefficients and their significance values generated from the fitted 2nd order equation for the Box–Behnken design.

Regression coefficient	Specific Trypsin-Inhibitor Activity (mg trypsin/mg protein)		
	Regression coefficient values	*t* ratio	*p* value
β_0	1.56	35.23	<0.0001
Linear			
β_1	−0.15	−5.68	0.002
β_2	0.04	1.52	0.188
β_3	−0.04	−1.57	0.177
Quadratic			
β_{11}	0.01	0.36	0.74
β_{22}	0.23	5.88	0.002
β_{33}	−0.15	−3.85	0.012
Interaction			
β_{12}	0.02	0.52	0.623
β_{13}	0.06	1.63	0.163
β_{23}	0.04	0.98	0.372
R^2	0.98		
p value of lack of fit	0.42		

3.2.2. Effects of the Extraction Parameters on the Specific TIA

It can be seen in Table 5 that all three of the extraction parameters had a significant effect on the specific TIA. The NaCl concentration of the extraction media (X_1) had a significant inverse linear effect, the extraction time (X_2) had a significant positive quadratic effect and the ratio of defatted powder to extraction media (X_3) had a significant inverse quandratic effect on the specific TIA of the

extracts. However, there were no significant interactive effects between the three parameters. The predicted effects of the three independent extraction parameters are presented visually in the 3D surface and 2D contour plots of Figure 1A–C.

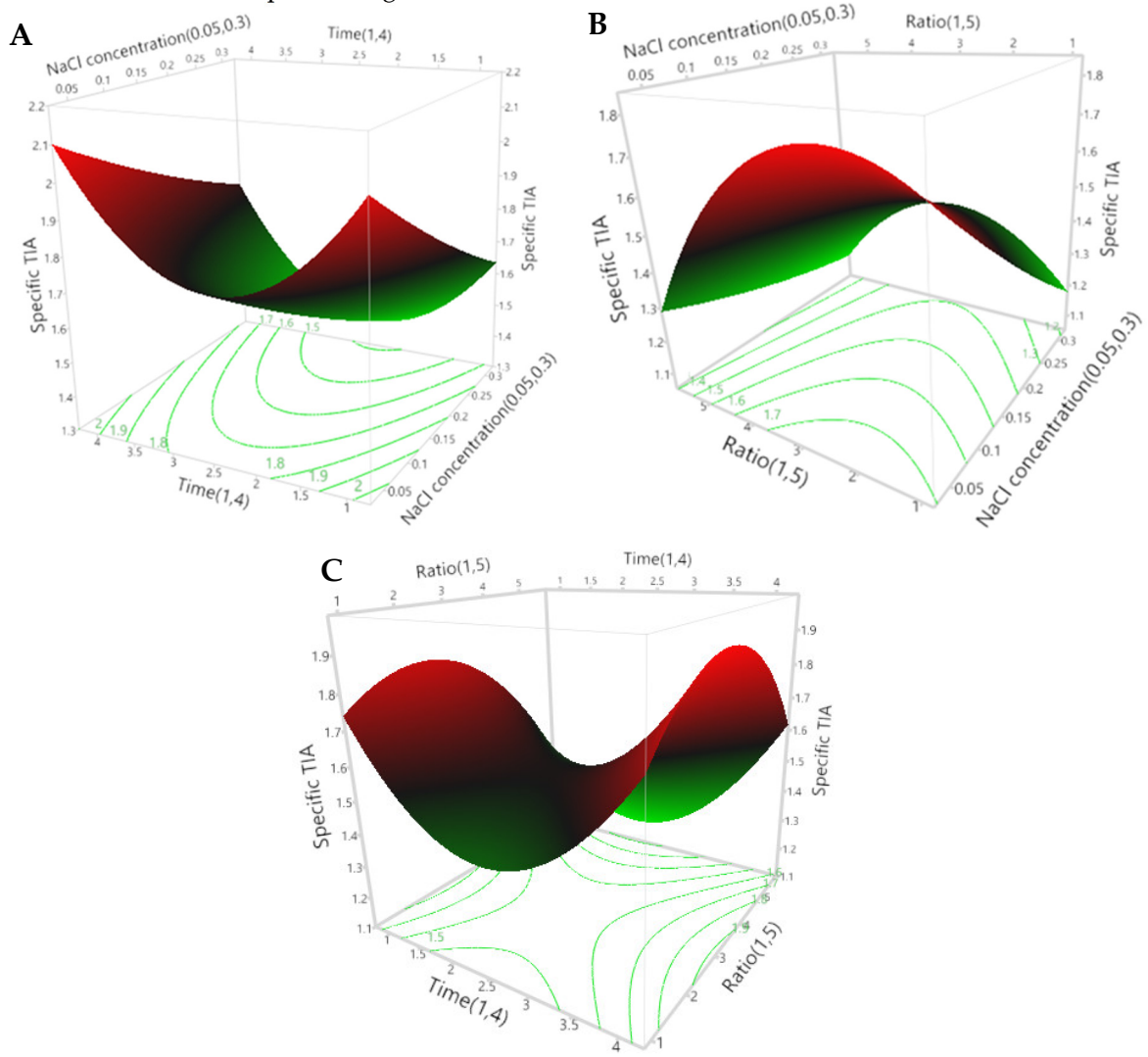


Figure 1. The 3D response surface and 2D contour plots for the specific trypsin-inhibitor activity (TIA) of the extracts. The effects of the NaCl concentration and the extraction time (**A**), the NaCl concentration and the ratio of defatted powder to solvent (**B**) and the extraction time and ratio of defatted powder to solvent (**C**) on the predicted specific TIA of the extracts are plotted according to the 2nd-order polynomial equation generated by the RSM (Equation 4).

3.2.3. Optimal Extraction Parameters and Validation of the Model

The extraction conditions for obtaining the highest TIA, protein and specific TIA from the defatted Gac kernel powder were generated using the prediction profiler as seen in the plots presented in Figure 2. There were two sets of theoretical maximum values predicted for the extraction parameters to achieve the highest specific TIA. The first optimum set of extraction conditions was 0.05 M NaCl, an extraction time of 1 h and ratio of 2.0 g of defatted powder in 30 mL of the extraction media (Figure 2, left panels). The second optimum set of conditions was 0.05 M NaCl, an extraction time of 4 h and a ratio of 2.5 g/30 mL (Figure 2, right panels). However, for the first set, a much shorter extraction time (1 h) was predicted in comparison to the second set (4 h). Therefore, the first set was chosen as the optimal conditions for the extraction of trypsin inhibitors from the defatted Gac kernel powder (0.05 M NaCl, 1 h extraction with a ratio of 2.0 g/ 30 mL). Under these optimal conditions, the TIA and specific TIA improved by 8% and 13%, respectively, in comparison to the un-optimal conditions.

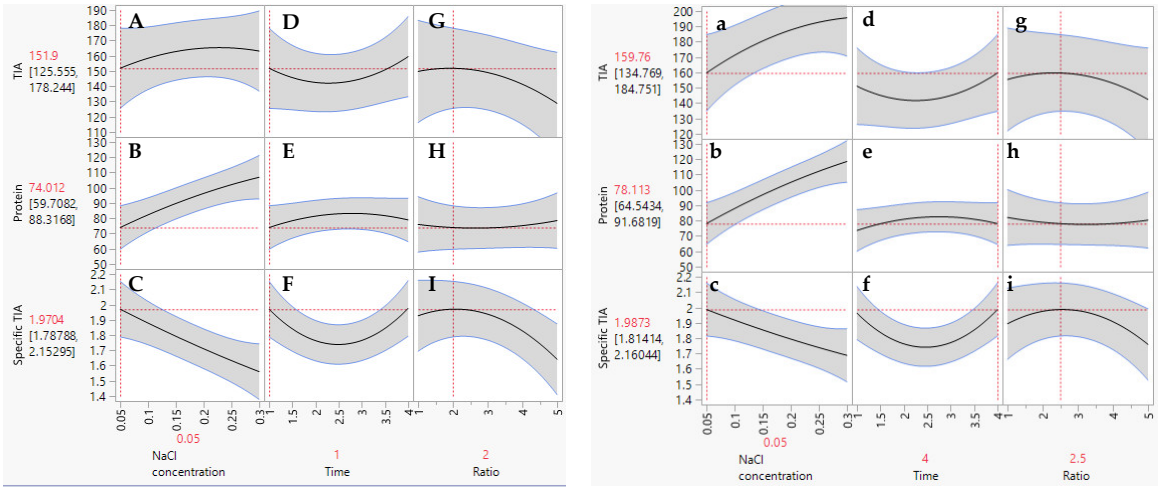


Figure 2. Prediction profiler plots for TIA, protein and specific TIA relative to NaCl concentration, time, and ratio of defatted powder to solvent. Two sets of optimal conditions were predicted as per the panels on the left (capital letters) and the panels on the right (small letters).

To validate the response model (Equation 3), three independent extractions were conducted for 1 h using a ratio of 2.0 g of defatted powder in 30 mL of 0.05 M NaCl. There was no difference (p = 0.45) between the measured (1.86 ± 0.12 mg trypsin/mg protein) and predicted (1.97 ± 0.19 mg trypsin/mg protein) values for the specific TIA of the extracts. This indicated that the response model (Equation 3) was valid and reliable, and thus, the conditions (1 h at a ratio of 2.0 g/30 mL 0.05 M NaCl) were used in subsequent studies for the extraction of trypsin inhibitors from the defatted Gac seed kernel powder.

3.3. Heat Treatment of the Optimal TI Extract

The optimal TI extract was subjected to heat treatment (Table 6) to determine whether this preparation was resistant to heat as previously observed for Gac seed TIs [13] and to determine whether its specific TIA could be increased because heat treatment has been used previously to remove protein without loss of TIA [30]. Table 6 shows that the TI extract was mostly resistant to heat treatment with only 13% of the TIA lost. However, there was also only an 11% loss of protein and, therefore, the heat treatment between 60 and 100 °C did not improve the specific TIA of the optimal TI extract (Table 6). This meant that the trypsin inhibitors as well as the other proteins in the extract were stable at these elevated temperatures and suggests that the trypsin inhibitors may be the dominant proteins in the preparation.

The heat stability of the TIs in the present study is consistent with the findings of Mahatmanto [13], who showed that boiling water could be used to successfully extract trypsin inhibitors from Gac seeds. This attribute of the Gac seed trypsin inhibitors is most likely due to their small size and their compact cyclical conformation [4]. Other TIs are also heat stable; for example, the trypsin inhibitor from barley has also been found to be heat stable when exposed to 100 °C for 15 min [31].

Table 6. Trypsin-inhibitory activity (TIA), total protein yield (TPY) and specific TIA of Gac seed extracts after heating for 10 min at diferent temperatures.

Temperature (°C)	Mean ± SD		
	TIA (mg trypsin/g DW defatted powder)	**TPY (mg BSA/g DW defatted powder)**	**Specific TIA (mg trypsin/ mg protein)**
Control	169.46 ± 4.73 ^{a}	91.17 ± 2.05 ^{a}	1.86 ± 0.09 ^{a}
60	155.28 ± 2.29 ^{a}	84.96 ± 2.64 ^{ab}	1.83 ± 0.07 ^{a}
70	149.24 ± 2.58 ^{b}	83.00 ± 3.09 ^{b}	1.80 ± 0.06 ^{a}
80	157.34 ± 6.84 ^{ab}	84.29 ± 2.15 ^{ab}	1.87 ± 0.13 ^{a}
90	147.76 ± 7.98 ^{b}	81.19 ± 3.34 ^{b}	1.82 ± 0.05 ^{a}
100	149.13 ± 7.31 ^{b}	83.25 ± 2.76 ^{b}	1.79 ± 0.08 ^{a}

The values are means ± SD of triplicate determinations for each temperature. Values in a column not sharing the same superscript letter are significantly different at $p < 0.05$.

3.4. The Physicochemical Properties of the Freeze Dried Trypsin-Inhibitor Powder (FD-TIP)

The physicochemical properties of the powder obtained by freeze-drying the optimal TI extract are shown in Table 7. The dry mass yield for the FD-TIP (16.3 ± 0.1) was higher than that obtained for extracts in previous studies using water (13.1 ± 0.1 g/100 g) [26] and ACN/water/FA (14.3 g/100 g) [13] as the extraction solvents. However, the high value in the present study is likely due to the retention, through the freeze-drying process, of the NaCl in the 0.05 M NaCl extraction media used, which could have added approximately 4.6 g/100 g to the dry mass yield.

As is often seen with freeze-drying [21,32], the moisture content of the FD-TIP was very low (Table 7), which ensure the stability of the powder. Nonetheless, moisture content alone is insufficient to predict the stability and quality of dried products. For example, according to Mai et al. [33], dried Gac fruit powder with a moisture content between 15% and 18% had better physicochemical properties than powder with a moisture content of 6%. Therefore, it is important to understand whether the water in a dried product is available for microbial growth or for enzyme or chemical activity, which can all lead to degradation of the product. Water activity (a_w) of a product reflects the free water available for the growing of microbials and chemical reactions. The a_w level of 0.18 for the powder in this study can be considered as a low-moisture product with the a_w level lower than 0.70, hence having a long shelf life [34]. It is well known that reduced a_w protects against microbiological growth and degradation-causing chemical reactions [35]. Generally, the rate of deterioration can be reduced if the water activity is below 0.6 because the growth of moulds and bacteria is inhibited at those levels [36]. The powder prepared in the present study had water activity less than 0.18 (Table 7), and thus, was likely to be microbiologically stable during storage.

The FD-TIP had a high water solubility index (Table 7), which means the powder could be used as a water-based preparation or in water-based products. Its high water solubility is likely due to the aqueous nature of the 0.05M NaCl extraction media; the ionic strength of the proteins thus extracted and the presence of NaCl in the powder most likely both contributed to the powder's high water solubility [37]. It also suggests that the FD-TIP may have had a small particle size, which also helps solubility [32]. The water solubility index of the FD-TIP was higher than for bitter melon freeze-dried powder (69–79%) [21] and Gac aril spray-dried powder (37%) [38], which suggests it contained more hydrophilic components than these other powders. However, the powder was white in colour and had an acidic pH of 5.3 (Table 7), which is similar to those of the freeze-dried powders from bitter melon [21].

The results on the TIA of the FD-TIP (Table 7) showed that the recovery of trypsin inhibitors and protein were ~67% and 79% compared to the values shown in Tables 6 for the control samples, respectively. The 33% and 21% losses may have been due to degradation of the trypsin inhibitors and other proteins occurring during the lengthy concentration (~4 h) and freeze-drying processes or to incomplete resolubilisation of the trypsin inhibitors and other proteins in the FD-TIP powder in water prior to the TIA and protein assays. Compared to the starting material, the defatted powder, the FD-

TIP was 4.1-fold and 4.9-fold more concentrated in trypsin inhibitors and protein, respectively (Table 7 vs. Table 6). This means that the specific TIA of the FD-TIP was 16% lower (1.57 ± 0.17 vs. 1.86 ± 0.09*)* than for the starting material (Table 7 vs. Table 6). This specific TIA was very similar to the activity of the trypsin inhibitor from bovine pancreas (1.5 mg trypsin/mg protein), which is commercially available from Sigma-Aldrich (CAS number 9035-81-8).

The total saponin content of the FD-TIP (Table 7) was comparable to a comercial bitter melon powder (43.6 ± 2.3 vs. 40.2 ± 1.6) [21] but it was higher than for a Gac seed powder previously extracted using deionised water (43.6 ± 2.3 vs. 34.0 ± 1.4) [26]. In contrast, the total phenolic content of the FD-TIP (Table 7) was lower than for the Gac seed powder extracted with the deionised water (10.5 ± 0.3 vs. 17.8 ± 0.5) [26]. This suggests that the saline water used in the present study promoted the extraction of saponins but hindered the extraction of phenolics from the defatted Gac seed kernel powder.

Table 7. Physicochemical properties the trypsin-inhibitor freeze-dried powder.

Properties	**Mean ± SD**
Dry mass yield (g FD-TIP/100g DW defatted powder)	16.3 ± 0.1
Moisture content (%, w/w)	0.43 ± 0.08
Water activity	0.18 ± 0.07
Water solubility (%)	92.4 ± 1.5
pH	5.33 ± 0.01
Colour	
L*	96.73 ± 0.18
a*	−0.50 ± 0.03
b*	8.22 ± 0.15
Trypsin-ininbitor activity (mg trypsin/g DW FD-TIP)	695.6 ± 77.2
Protein content (mg protein/g DW FD-TIP)	444.0 ± 21.5
Specific trypsin-inhibitor activity (mg trypsin/mg protein)	1.57 ± 0.17
Total saponin content (mg AE/g DW FD-TIP)	43.6 ± 2.3
Total phenolic content (mg GAE/g DW FD-TIP)	10.5 ± 0.3

FD-TIP: freeze dried trypsin-inhibitor powder; AE: Aescin equivalents; GAE: gallic acid equivalents.

4. Conclusion

Using the RSM and the Box–Behnken system, the optimal conditions for the extraction of trypsin inhibitors from defatted Gac seed kernel powder were determined to be 1 h using a ratio of 2.0 g of defatted powder in 30 mL of 0.05 M NaCl. The powder obtained by freeze-drying the extract prepared using these optimal conditions had a highly specific TIA, although it also contained some saponins and phenolics. The powder was likely to be stable during storage due to its very low moisture content and water activity and to be easily reconstituted in water due to its high water solubility. Therefore, the optimal conditions for the extraction of trypsin inhibitors from defatted Gac seed kernel powder followed by freeze drying gave a high quality trypsin inhibitor-enriched powder.

Abbreviations

ACN	Acetonitrile	MCoTI inhibitor	*Momordica cochinchinensis* trypsin
a_w	water activity	RSM	Response surface methodology
BAPNA	Benzyl-DL-arginine-para-nitroanilide	TI	Trypsin inhibitor
DI	Deionised	TIA	Trypsin-inhibitor activity
DMSO	Dimethyl sulfoxide	TPC	Total phenolic content
DW	Dry weight	TPY	Total protein yield
FA	Formic acid	TSC	Total saponin content
FD-TIP	Freeze dried trypsin-inhibitor powder		

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wong, R.C.; Fong, W.; Ng, T. Multiple trypsin inhibitors from *Momordica cochinchinensis* seeds, the Chinese drug mubiezhi. *Peptides* **2004**, *25*, 163–169, DOI: 10.1016/j.peptides.2004.01.002.
2. Huang, B.; Ng, T.; Fong, W.; Wan, C.; Yeung, H. Isolation of a trypsin inhibitor with deletion of N-terminal pentapeptide from the seeds of Momordica cochinchinensis, the Chinese drug mubiezhi. *Int. J. Biochem. Cell Biol.* **1999**, *31*, 707–715, DOI: 10.1016/S1357-2725(99)00012-6.
3. Chan, L.Y.; Wang, C.K.L.; Major, J.M.; Greenwood, K.P.; Lewis, R.J.; Craik, D.J.; Daly, N.L. Isolation and characterization of peptides from *Momordica cochinchinensis* seeds. *J. Nat. Prod.* **2009**, *72*, 1453–1458, DOI: 10.1021/np900174n.
4. Hernandez, J.F.; Gagnon, J.; Chiche, L.; Nguyen, T.M.; Andrieu, J.P.; Heitz, A.; Hong, T.T.; Pham, T.T.C.; Nguyen, D.L. Squash trypsin inhibitors from *Momordica cochinchinensis* exhibit an atypical macrocyclic structure. *Biochemistry* **2000**, *39*, 5722–5730, DOI: 10.1021/bi9929756.
5. Felizmenio-Quimio, M.E.; Daly, N.L.; Craik, D.J. Circular Proteins in Plants: Solution structure of a novel macrocyclic trypsin inhibitor from *Momordica Cochinchinensis. J. Biol. Chem.* **2001**, *276*, 22875–22882, DOI: 10.1074/jbc.M101666200.
6. Birk, Y. Protein proteinase inhibitors in legume seeds-overview. *Arch. Latinoam. Nutr.* **1996**, *44*, 26S–30S.
7. Greenwood, K.P.; Daly, N.L.; Brown, D.L.; Stow, J.L.; Craik, D.J. The cyclic cystine knot miniprotein MCoTI-II is internalized into cells by macropinocytosis. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 2252–2264, DOI: 10.1016/j.biocel.2007.06.016.
8. Contreras, J.; Elnagar, A.Y.; Hamm-Alvarez, S.F.; Camarero, J.A. Cellular uptake of cyclotide MCoTI-I follows multiple endocytic pathways. *J. Control. Release* **2011**, *155*, 134–143, DOI: 10.1016/j.jconrel.2011.08.030.
9. Cascales, L.; Henriques, S.T.; Kerr, M.C.; Huang, Y.-H.; Sweet, M.J.; Daly, N.L.; Craik, D.J. Identification and characterization of a new family of cell-penetrating peptides cyclic cell-penetrating peptides. *J. Biol. Chem.* **2011**, *286*, 36932–36943, DOI: 10.1074/jbc.M111.264424.
10. Mahatmanto, T.; Mylne, J.S.; Poth, A.G.; Swedberg, J.E.; Kaas, Q.; Schaefer, H.; Craik, D.J. The evolution of Momordica cyclic peptides. *Mol. Biol. Evol.* **2014**, *32*, 392–405, DOI: 10.1093/molbev/msu307.
11. Craik, D.J.; Swedberg, J.E.; Mylne, J.S.; Cemazar, M. Cyclotides as a basis for drug design. *Exp. Opin. Drug Discov.* **2012**, *7*, 179–194, DOI: 10.1517/17460441.2012.661554.
12. Wang, C.K.; Gruber, C.W.; Cemazar, M.; Siatskas, C.; Tagore, P.; Payne, N.; Sun, G.; Wang, S.; Bernard, C.C.; Craik, D.J. Molecular grafting onto a stable framework yields novel cyclic peptides for the treatment of multiple sclerosis. *ACS Chem. Biol.* **2013**, *9*, 156–163, DOI: 10.1021/cb400548s.
13. Mahatmanto, T.; Poth, A.G.; Mylne, J.S.; Craik, D.J. A comparative study of extraction methods reveals preferred solvents for cystine knot peptide isolation from *Momordica cochinchinensis* seeds. *Fitoterapia* **2014**, *95*, 22–33, DOI: 10.1016/j.fitote.2014.02.016.
14. Le, A.V.; Parks, S.E.; Nguyen, M.H.; Roach, P.D. Effect of solvents and extraction methods on recovery of bioactive compounds from defatted Gac (*Momordica cochinchinensis* Spreng.) seeds. *Separations* **2018**, *5*, 39, DOI: 10.3390/separations5030039.
15. Klomklao, S.; Benjakul, S.; Kishimura, H.; Chaijan, M. Extraction, purification and properties of trypsin inhibitor from Thai mung bean (*Vigna radiata* (L.) R. Wilczek). *Food Chem.* **2011**, *129*, 1348–1354, DOI: 10.1016/j.foodchem.2011.05.029.
16. Pesoti, A.R.; de Oliveira, B.M.; de Oliveira, A.C.; Pompeu, D.G.; Gonçalves, D.B.; Marangoni, S.; da Silva, D.A.; Granjeiro, P.A. Extraction, purification and characterization of inhibitor of trypsin from Chenopodium quinoa seeds. *Food Sci. Technol.* **2015**, *35*, 588–597, DOI: 10.1590/1678-457X.6655.

17. Deshpande, S.S.; Campbell, C.G. Effect of different solvents on protein recovery and neurotoxin and trypsin inhibitor contents of grass pea (*Lathyrus sativus*). *J. Sci. Food Agric.* **1992**, *60*, 245–249, DOI: 10.1002/jsfa.2740600213.
18. Baş, D.; Boyacı, İ.H. Modeling and optimization I: Usability of response surface methodology. *J. Food Eng.* **2007**, *78*, 836–845, DOI: 10.1016/j.jfoodeng.2005.11.024.
19. Wimalasiri, D.; Piva, T.; Urban, S.; Huynh, T. Morphological and genetic diversity of *Momordica cochinchinenesis* (Cucurbitaceae) in Vietnam and Thailand. *Genet. Resour. Crop Evol.* **2016**, *63*, 19–33, DOI: 10.1007/s10722-015-0232-8.
20. Box, G.E.P.; Behnken, D.W. Some New Three Level Designs for the Study of Quantitative Variables. *Technometrics* **1960**, *2*, 455–475.
21. Tan, S.P.; Vuong, Q.V.; Stathopoulos, C.E.; Parks, S.E.; Roach, P.D. Optimized aqueous extraction of saponins from bitter melon for production of a saponin-enriched bitter melon powder. *J. Food Sci.* **2014**, *79*, E1372–E1381, DOI: 10.1111/1750-3841.12514.
22. Makkar, H.P.; Siddhuraju, P.; Becker, K. Trypsin Inhibitor. *Plant Secondary Metabolites*; Humana Press: New York, NY, USA, 2007; pp. 1–6.
23. Stauffer, C.E. Measuring trypsin inhibitor in soy meal: Suggested improvements in the standard method. *Cereal Chem* **1990**, *67*, 296-302.
24. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
25. Anderson, R. Water absorption and solubility and amylograph characteristics of roll-cooked small grain products. *Cereal Chem.* **1982**, *59*, 265–269.
26. Le, A.; Huynh, T.; Parks, S.; Nguyen, M.; Roach, P. Bioactive Composition, Antioxidant Activity, and Anticancer Potential of Freeze-Dried Extracts from Defatted Gac (*Momordica cochinchinensis* Spreng) Seeds. *Medicines* **2018**, *5*, 104, DOI: 10.3390/medicines5030104.
27. Marfo, E.; Oke, O. Effect of sodium chloride, calcium chloride and sodium hydroxide on Denolix regia protein solubility. *Food Chem.* **1989**, *31*, 117–127, DOI: 10.1016/0308-8146(89)90022-8.
28. Benjakul, S.; Visessanguan, W.; Thummaratwasik, P. Isolation and characterization of trypsin inhibitors from some Thai legume seeds. *J. Food Biochem.* **2000**, *24*, 107–127, DOI: 10.1111/j.1745-4514.2000.tb00689.x.
29. Sathe, S.; Salunkhe, D. Solubilization and electrophoretic characterization of the Great Northern bean (*Phaseolus vulgaris* L.) proteins. *J. Food Sci.* **1981**, *46*, 82–87, DOI: 10.1111/j.1365-2621.1981.tb14536.x.
30. Hamato, N.; Koshiba, T.; Pham, T.-N.; Tatsumi, Y.; Nakamura, D.; Takano, R.; Hayashi, K.; Hong, Y.-M.; Hara, S. Trypsin and elastase inhibitors from bitter gourd (*Momordica charantia* LINN.) seeds: purification, amino acid sequences, and inhibitory activities of four new inhibitors. *J. Biochem.* **1995**, *117*, 432–437, DOI: 10.1093/jb/117.2.432.
31. Mikola, J.; Suolinna, E.M. Purification and properties of a trypsin inhibitor from barley. *Eur. J. Biochem.* **1969**, *9*, 555–560.
32. Lee, C.-W.; Oh, H.-J.; Han, S.-H.; Lim, S.-B. Effects of hot air and freeze drying methods on physicochemical properties of citrus 'hallabong'powders. *Food Sci. Biotechnol.* **2012**, *21*, 1633–1639, DOI: 10.1007/s10068-012-0217-8.
33. Mai, H.C.; Truong, V.; Haut, B.; Debaste, F. Impact of limited drying on Momordica cochinchinensis Spreng. aril carotenoids content and antioxidant activity. *J. Food Eng.* **2013**, *118*, 358–364, DOI: 10.1016/j.jfoodeng.2013.04.004.
34. Blessington, T.; Theofel, C.G.; Harris, L.J. A dry-inoculation method for nut kernels. *Food Microbiol.* **2013**, *33*, 292–297, DOI: 10.1016/j.fm.2012.09.009.
35. Tuyen, C.K.; Nguyen, M.H.; Roach, P.D. Effects of pre-treatments and air drying temperatures on colour and antioxidant properties of Gac fruit powder. *Int. J. Food Eng.* **2011**, *7*, DOI: 10.2202/1556-3758.1926.
36. Fellows, P.J. *Food Processing Technology: Principles and Practice*, 4th ed.; Woodhead Publishing: Cambridge, UK, 2009; ISBN: 9780081019078.
37. Vojdani, F. Solubility. In *Methods of Testing Protein Functionality*, Hall, G.M., Eds.; Blackie Academic & Professional: London, UK, 1996; pp. 11–60.

38. Tan, S.P.; Tuyen, K.C.; Parks, S.E.; Stathopoulos, C.E.; Roach, P.D. Effects of the spray-drying temperatures on the physiochemical properties of an encapsulated bitter melon aqueous extract powder. *Powder Technol.* **2015**, *281*, 65–75, DOI: 10.1016/j.powtec.2015.04.074.

3.4. Extraction of saponins – the research papers

3.4.1. Improving the vanillin-sulphuric acid method for quantifying total saponins

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Improving the Vanillin-Sulphuric Acid Method for Quantifying Total Saponins

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Abstract: The colorimetric assay used for saponin quantification in plant extracts is subject to interference by common solvents used to extract the saponins from plant materials. Therefore, the degree of interference of ten common solvents was investigated. It was found that the presence of acetone, methanol and n-butanol in the reaction solution caused an intense darkening of the reaction solution in the absence of saponins, which likely could lead to erroneous saponin content values. Using aescin to construct standard curves with different solvents—such as water, ethanol, and methanol— also showed significant differences in the standard curves obtained, which led to different values when they were applied to quantify the saponin content of an ethanol extract from dried and powdered Gac (*Momordica cochinchinensis* Spreng) seed kernels. To improve the method, a solvent evaporation step was added prior to the colorisation reaction to prevent undesired solvent interference during the reaction step. Using this modified protocol for the aescin standard curve and the Gac seed kernel extract eliminated any solvent interference. Thus, this improved protocol is recommended for the quantification of the saponin content of plant extracts irrespective of which extraction solvent is used.

Keywords: saponin; aescin; vanillin-sulphuric acid assay; spectrophotometry; colorimetric assay; solvents; interference

1. Introduction

Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are characterised by their structure containing a triterpenoid (C30) or steroid (C27) aglycone, and one or more carbohydrate chains [1]. Their physicochemical (e.g., surfactant) properties [1], coupled with mounting evidence on their biological activities (e.g., anticancer and anti-cholesterol activity) [2], have led to the emergence of saponins as significant compounds with expanding applications in the food, cosmetics and pharmaceutical sectors [3].

As for other plant bioactives, the realisation of the potential of saponins requires robust methods for their effective extraction from plant materials, for their easy quantification in extracts and for the ultimate identification of individual saponins. Many methods have been developed for the detection and measurement of saponins in plant products; they can be classified into two main categories [4], (i) spectrophotometric methods [5–7], which give a total saponin content (TSC) value, and (ii) chromatographic methods, which quantify specific saponin compounds [8,9].

For TSC analysis, a spectrophotometric method proposed by Hiai et al. [7] has become popular for the detection and quantification of saponins in plant materials [4], largely because it is simple, fast and inexpensive. The method is known as the vanillin-sulfuric acid assay because the basic principle of the method is the reaction of sulphuric acid-oxidised triterpene saponins with vanillin, which gives a distinctive red-purple colour measured at wavelengths ranging from 473 to 560 nm [4] using a spectrophotometer [10]. The TSC of a plant sample is determined from a calibration curve with a standard saponin (e.g., aescin, oleanolic acid, diosgenin, quillaja saponin) [4,11] and it is expressed in terms of the standard's equivalence (e.g., mg standard equivalents per g sample; or g standard equivalents per 100 g sample).

The original method was used to measure saponins in aqueous and ethanol extracts of ginseng [10,12]. However, since then, a number of other solvents have been used to extract saponins from a wide range of plant materials [3], including butanol [13], methanol [14], ethyl acetate [15], dichloromethane [16], acetone [17], and hexane [18], among others. In order to appropriately eliminate any colour reactions that are not due to the saponins, each solvent needs to be included in the reagent blank and the standard solutions at the same concentration as in the sample extracts. However, in initial experiments, it was noticed that solvents such as methanol and acetone gave high reagent blank absorbance values, which may not be able to be effectively used to zero a spectrophotometer or subtract from readings obtained for the samples and standards. Therefore, in this study, the effect of ten different solvents, used for the extraction of saponins from plant materials, on the vanillin-sulphuric acid assay [7] was investigated. The possible interference of the solvents was addressed by introducing some modifications to the assay. The modified assay was verified by quantifying the TSC of a sample extract—the ethanolic extract of Gac (*Momordica cochinchinensis* Spreng) seeds.

2. Materials and Methods

2.1. Materials

The solvents acetonitrile, acetone, methanol, ethanol, ethyl acetate, n-butanol, dichloromethane, diethyl ether, chloroform and hexane and chemicals including vanillin and sulphuric acid were products of Merck (Bayswater, VIC, Australia). Aescin was purchased from Sigma-Aldrich Co. (Castle Hill, NSW, Australia).

Dried seed kernel powder from the Gac fruit (*Momordica cochinchinensis* Spreng), a material rich in triterpenoid saponins [19], was used for the extraction and analysis of TSC using the vanillin-sulphuric acid assay. The Gac seeds were collected from fresh Gac fruit bought at Gac fruit fields in Dong Nai province, Ho Chi Minh city, Vietnam (latitude: 10.757410; longitude: 106.673439). After separation from the fresh fruit, the seeds were vacuum dried at 40 °C for 24 h and the dried seeds were de-coated to get the kernels. These kernels were ground to pass through a 1.4 mm sieve. The powder was then freeze-dried using a Dynavac FD3 freeze dryer (Sydney, NSW, Australia) for 48 h at −45 °C, under vacuum at a pressure loading of 10^{-2} mbar (1 Pa), to reduce the moisture content to 1.21 ± 0.02%.

2.2. Methods

2.2.1. The Usual Vanillin-Sulphuric Acid TSC Assay

The vanillin-sulphuric acid assay for determining the TSC of plant materials [12] is usually done [11,20] by incubating 0.25 mL of plant sample extracts, standards or reagent blank (Table 1) with 0.25 mL of 8% (w/v) vanillin in ethanol and 2.50 mL of 72% (v/v) sulphuric acid in water for 15 min at 60 °C in a shaking water bath, with the standards and the reagent blank made up with the solvent used for extracting the plant samples (extraction solvent) as shown in Table 1. After cooling in water at the ambient temperature for 5 min, the absorbance of the standards and extracts are measured at 560 nm using a Cary 50 UV–VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA)

after zeroing it with the reagent blank (Table 1). The TSC of the samples is then expressed in mg of standard equivalents per gram of plant sample (mg SE g^{-1}).

Table 1. The vanillin-sulphuric acid assay setup procedure.

Component	Reagent Blank (mL)	Standards (mL)	Samples (mL)
Extraction Solvent	0.25	-	-
Standards in Extraction Solvent	-	0.25	-
Sample in Extraction Solvent	-	-	0.25
Vanillin 8% (w/v) in Ethanol	0.25	0.25	0.25
Sulphuric acid 72% (v/v) in Water	2.50	2.50	2.50

2.2.2. Solvents and the Reagent Blank

To investigate whether any colour development occurred, due to the extraction solvent in the vanillin—sulphuric acid assay, ten common extraction solvents were tested: Acetonitrile, acetone, methanol, ethanol, ethyl acetate, n-butanol, dichloromethane, diethyl ether, chloroform, and hexane. These solvents were used as absolute. Accordingly, ten reagent blanks and a zeroing blank with water were prepared as described in Table 2. The reagent blanks and the zeroing blank were vortexed, and incubated in a water bath at 60 °C for 15 min, and then cooled down in a water bucket for 5 min. The absorbance of each solution was then measured at 560 nm, using a Cary 50 UV–VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) after the spectrophotometer was zeroed with the water blank (Table 2).

Table 2. The setup procedure for testing different extraction solvents in the reagent blank.

Component	Zeroing Blank (mL)	Reagent Blank (mL)
Deionised Water	0.25	-
Extraction Solvent (absolute)	-	0.25
Vanillin 8% (w/v) in Ethanol	0.25	0.25
Sulphuric acid 72% (v/v) in Water	2.50	2.50

2.2.3. Solvents Used to Prepare Saponin (Aescin) Solutions

To investigate whether solvents had an effect on the colour development with increasing concentrations of saponins in the vanillin (sulphuric acid assay, aescin—a natural mixture of triterpenoid saponins) was used. However, of the ten solvents investigated with the reagent blank, aescin could only be dissolved in methanol and ethanol, as well as in water, to produce a clear solution. Therefore, aescin was initially dissolved in three solvents (methanol, ethanol and water) and four sets of serially diluted aescin solutions were prepared as follows:

- Set 1: A stock solution of aescin in ethanol (1500 mg L^{-1}) was prepared. The ethanol stock solution was diluted with ethanol to 1000, 800, 500, 250 and 100 mg L^{-1}.
- Set 2: A stock solution of aescin in water (1500 mg L^{-1}) was prepared. The water stock solution was diluted with water to 1000, 800, 500, 250 and 100 mg L^{-1}.
- Set 3: The ethanol stock solution (1500 mg L^{-1}) was diluted with water to 1000, 800, 500, 250 and 100 mg L^{-1}.
- Set 4: A stock solution of aescin in methanol (1500 mg L^{-1}) was prepared. The methanol stock solution was diluted with water to 500, 250, 125, 62.5 and 31.25 mg L^{-1}. The range of concentrations for this set was adjusted downwards because the initial concentrations (as in Sets 1 to 3) gave absorbance values that were too high for the Cary 50 UV-VIS spectrophotometer.

The four sets of aescin solutions were set up with two reagent blanks, one with water and the other with ethanol, as described in Table 3. The solutions were mixed and incubated at 60 °C in a water bath for 15 min and then cooled in water at the ambient temperature for 5 min. The absorbance was measured at 560 nm using the UV–VIS spectrophotometer after the spectrophotometer was zeroed with the ethanol reagent blank (Table 3) for Set 1 of aescin solutions and with the water reagent blank (Table 3) for Sets 2, 3, and 4 of aescin solutions. The resulting absorbance values were plotted against the aescin concentrations to generate four regression lines.

Table 3. The setup procedure for testing different solvents used to prepare saponin (aescin) solutions.

Component	Reagent Blank		Aescin Solutions (mL)
	Water Blank (mL)	Ethanol Blank (mL)	
DI water	0.25	-	-
Ethanol	-	0.25	-
Aescin solution	-	-	0.25
Vanillin 8% (w/v) in Ethanol	0.25	0.25	0.25
Sulphuric acid 72% (v/v) in Water	2.50	2.50	2.50

2.2.4. The Modified Vanillin-Sulphuric Acid TSC Assay

The setup procedure for the TSC assay was modified as shown in Table 4. Based on the experiments with the different solvents in the reagent blank, and the preparation of the saponin (aescin) solutions, it was determined that the extraction solvent should be removed from the extracts before the vanillin-sulphuric acid reaction was performed. In the usual assay (Table 1), the volume of extract used is 0.25 mL. However, the extracts typically need to be diluted to fit within the linear range of the standard curve. Therefore, to obviate the need to dilute the extracts, and especially to make it easier to remove the extraction solvent, only 25 µL of the extract was used in the modified assay (Table 4).

To also make the removal of the standards' solvent easier, the standards were also made at 10 times higher concentrations than for the usual method (Table 1), and only 25 µL of the standard solutions were used in the modified assay (Table 4). A corresponding reagent blank was also prepared with 25 µL of the extraction solvent (Table 4), which was also removed before the vanillin, and the sulphuric acid solutions were added. Furthermore, to keep the total volume in the assay at 3 mL (Table 1) with the concentration of vanillin. and sulphuric acid unchanged, the concentration of the vanillin solution was decreased by half to 4% (w/v), and the volume was doubled to 0.50 mL (Table 4).

Table 4. The modified vanillin-sulphuric acid assay setup procedure.

Component	Reagent Blank (mL)	Standards (mL)	Samples (mL)
Extraction Solvent	0.025 (removed)	-	-
Standards in Methanol	-	0.025 (removed)	-
Sample in Extraction Solvent	-	-	0.025 (removed)
Vanillin 4% (w/v) in Ethanol	0.500	0.500	0.500
Sulphuric acid 72% (v/v) in Water	2.500	2.500	2.500

2.2.5. Determining the TSC of a Gac Seed Extract Using the Modified Method

- Preparation of the Standard Curve

Aescin, a natural mixture of triterpenoid saponins, was used to produce a standard curve. A 15,000 mg L^{-1} aescin stock solution was prepared by dissolving 150.0 mg of aescin in 10 mL of methanol. A serial dilution was then done in triplicate using methanol, and methanol was used as the solvent blank for the standards, as presented in Table 5.

As described in Table 4, 25 µL of each aescin standard solution (Table 5) was used in the modified assay. A reagent blank for the standards was also prepared using 25 µL of methanol (Table 4). All tubes

were placed in a water bath set at 65 °C until the methanol was evaporated to dryness (~5 min). Then, 0.5 mL of 4% vanillin in ethanol (w/v) was added to each tube followed by 2.5 mL of 72% H_2SO_4 (v/v) (Table 4). The tubes were covered, vortexed, incubated in a water bath at 60 °C for 15 min and then cooled for 5 min in water at ambient temperature. The absorbance of the solutions was measured at 560 nm using the Cary 50 UV-VIS spectrophotometer after zeroing with the blank. The absorbance values obtained (Table 5) were plotted against the concentrations to construct a standard curve.

Table 5. Preparation of the aescin standard curve for the modified method.

Standard	Dilution	Concentration (mg L^{-1})	Absorbance (560 nm)
(1)	Stock solution (no dilution)	15,000	1.7283 ± 0.0321
(2)	2 mL (1) + 2 mL methanol	7500	0.8674 ± 0.0062
(3)	2 mL (2) + 2 mL methanol	3750	0.4352 ± 0.0124
(4)	2 mL (3) + 2 mL methanol	1875	0.2069 ± 0.0265
(5)	2 mL (4) + 2 mL methanol	937.5	0.1151 ± 0.0075
Blank	Methanol only	0	Used for zeroing

- Preparation of a Gac Seed Kernel Powder Extract

One gram of freeze-dried Gac seed kernel powder was mixed with 30 mL of ethanol in a 100 mL conical flask. The suspension was left standing for 30 min pre-leaching at ambient temperature before extraction was done for three cycles of 10 s power ON and 15 s power OFF (per cycle), with the power set at 360 W, using a R395YS Sharp Carousel microwave oven (Sharp Corporation, Bangkok, Thailand). The suspension was then rapidly cooled to ambient temperature in an iced water bath, and filtered through a 0.45 µm membrane filter. The clear extract was collected and its TSC was determined using the usual (Table 1) and the modified (Table 4) vanillin-sulphuric acid methods.

- Determination of the Total Saponin Content of the Gac Seed Kernel Powder Extract

The modified method was applied to measure the TSC for the ethanol Gac seed kernel powder extract following the composition as described in Table 4, where the extraction solvent was ethanol and the standard was aescin. The saponin content of the sample extract was calculated from the aescin standard curve equations (Figures 2 and 3). The TSC was calculated relative the mass of the sample used as per Equation (1):

$$Total\ saponin\ content = \frac{weight\ of\ saponins\ in\ the\ extract\ (mg)}{weight\ of\ Gac\ seed\ powder\ (g) \times \left(1 - \frac{moisture\ content\ (\%)}{100}\right)}. \quad (1)$$

The TSC was expressed as mg aescin equivalents per gram dry weight of Gac seed kernel powder (mg AE g^{-1}).

2.2.6. Statistical Analyses

Experiments were performed in triplicate and means ± SD were assessed using the Student *t*-test when comparing two means only, and the one-way ANOVA and Tukey's Post Hoc Multiple Comparisons for more than two means, using IBM SPSS Statistics 24 (IBM Corp., Armonk, NY, USA). Differences in means were considered statistically significant at $p < 0.05$. The aescin standard curves and regression equations were generated using the Microsoft Excel software.

3. Results

3.1. The Effect of Different Solvents in the Reagent Blank

It can be seen in Figure 1 that the presence of acetone in the reagent blank for the vanillin-sulphuric acid method resulted in the darkest solution, which obstructed the transmission of most of the

spectrophotometer's light in the absence of any saponins. Besides acetone, n-butanol, and methanol also led to substantially high absorbance values (Figure 1), followed by ethyl acetate and hexane, and then by dichloromethane, diethyl ether and chloroform to a lesser extent. Only ethanol did not have a significant effect on the absorbance of the reaction mixture compared to water. It was also observed that chloroform and hexane also caused some cloudiness in the reagent blank solution.

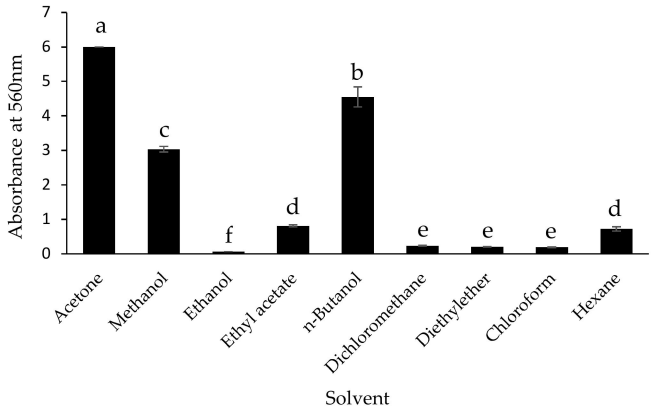

Figure 1. The effect of the indicated solvents on the absorbance of the reagent blank at 560 nm for the vanillin-sulphuric acid method. The values are the means of three replicates for each reaction. Columns not sharing the same superscript letter are significantly different at $p < 0.05$.

3.2. Effect of Solvents on the Aescin Standard Curve

Following the procedure described in Section 2.2.3, four aescin standard curves and their regression equations were obtained as shown in Figure 2. Except for curves 2 and 3, it was observed that the aescin standard curves varied between the solvents used to prepare the aescin solutions.

Notably, when the aescin was dissolved in methanol and diluted in water, the slope of the resulting standard curve was substantially higher (Line 4) than for to the other 3 standard curves. This effect was likely, due to the increasing concentrations of methanol in the aescin solutions that were less diluted with water to give the increasing aescin concentrations, and was consistent with the increased absorbance methanol was observed to have in the reagent blank (Figure 1).

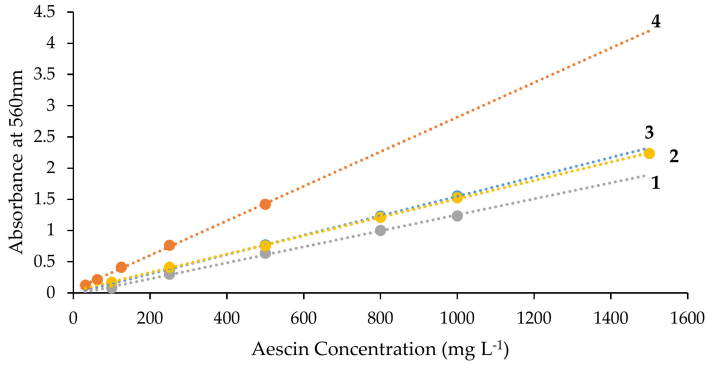

Figure 2. Standard curves for aescin as prepared in four ways as described in Section 2.2.3, and assayed using the unmodified vanillin-sulphuric acid method described in Section 2.2.1. Line 1: Aescin dissolved and diluted in ethanol; Y = 0.0013X – 0.0302 (R^2 = 0.9984); Line 2: Aescin dissolved and diluted in water; Y = 0.0015X + 0.0309 (R^2 = 0.9997); Line 3: Aescin dissolved in ethanol and diluted in water; Y = 0.0016X – 0.0067 (R^2 = 0.9999); Line 4: Aescin dissolved in methanol and diluted in water; Y = 0.0028X + 0.0491 (R^2 = 0.9991).

3.3. Aescin Standard Curve for the Modified Method

The aescin standard curve and its regression equation, obtained using the modified method described in Sections 2.2.4 and 2.2.5, is shown in Figure 3. It can be seen that over the concentration range of the aescin standard (937.5 to 15,000 mg L^{-1}), a very highly linear response in absorbance was achieved as the aescin concentration was increased.

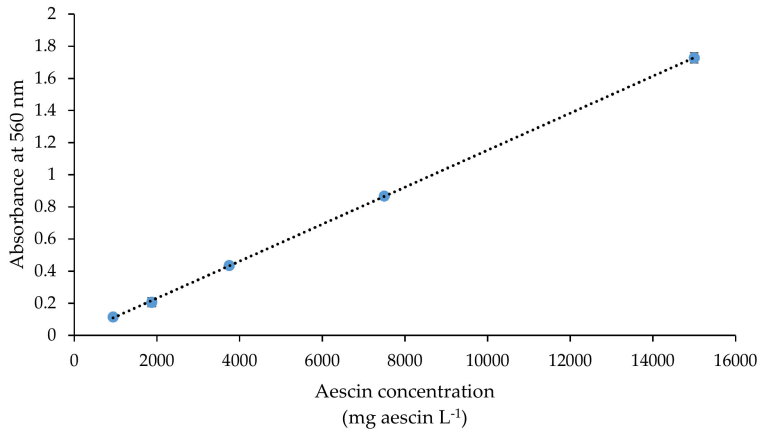

Figure 3. Standard curve for aescin using the modified vanillin-sulphuric acid method (Y = 0.00012X + 0.0008 (R^2 = 0.9999)).

3.4. Saponin Content of the Gac Seed Kernel Powder

The TSC of the Gac seed kernel powder was determined using an ethanol extract and the modified vanillin-sulfuric acid method using the aescin standard curve in Figure 3. The results were compared with the unmodified method using the four aescin standard curves in Figure 2.

As seen in Figure 4, the values for the TSC of the Gac seed kernel powder were significantly lower (25%, 23% and 62% lower, respectively) when aescin standard curves 2, 3 and 4 in Figure 2 were used to measure the saponins in the ethanol extract compared to when the value was derived from aescin standard curve 1.

Using the modified vanillin-sulphuric acid method and the aescin standard curve in Figure 3, the value for the TSC of the Gac seed kernel powder was the same for the ethanol extract as when the value was derived using the unmodified method and aescin standard curve 1 (Figure 4).

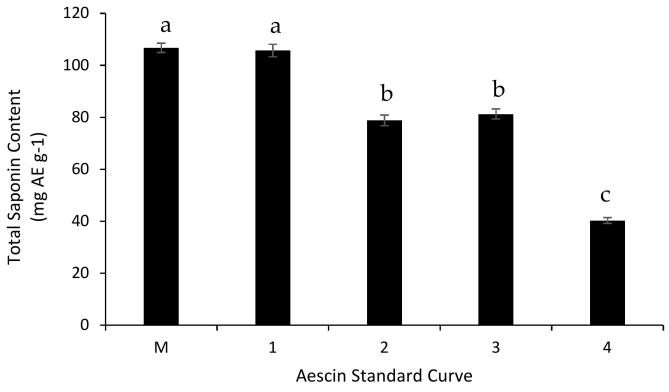

Figure 4. The total saponin content (TSC) of the Gac (*Momordica cochinchinensis* Spreng) seed kernel powder determined using ethanol as the extraction solvent and the modified method with the aescin standard curve in Figure 3 (M) and the unmodified method with the four aescin standard curves in Figure 2 (1–4). The values are the means of three replicates for each extraction. Columns not sharing the same superscript letter are significantly different at $p < 0.05$.

Although the regression equations for the modified method and the unmodified method with aescin standard curve 1 were different, the absorbance and the saponin concentration obtained for the ethanol extract of the Gac seen kernel powder were not significantly different, which led to the TSC for the Gac seed kernel powder also not being significantly different between the two methods (Table 6). In other words, the modified method gave results that were entirely consistent with the unmodified vanillin-sulphuric acid method when ethanol was used as the extracting solvent and included in the standard curve, a feature of the original methodology [7,10,12].

Table 6. Saponin values for the Gac seed kernel powder using ethanol as the extraction solvent and the unmodified and modified vanillin-sulphuric acid methods.

Method	Absorbance ‡	Standard Curve	Saponin ‡ (mg AE L^{-1})	TSC § (mg AE g^{-1})
Unmodified †	0.409 ± 0.010 [a]	Y = 0.0013X − 0.0302	3377 ± 77 [a]	105.69 ± 2.40 [a]
Modified	0.410 ± 0.008 [a]	Y = 0.00012X + 0.0008	3411 ± 57 [a]	106.77 ± 1.79 [a]

† The Unmodified method and aescin standard curve 1 in Figure 2. ‡ Absorbance and Saponin concentration for the ethanol extract. § The TSC for the Gac seed kernel powder. In each column, values not sharing the same superscript letter are significantly different at $p < 0.05$.

4. Discussion

4.1. Effect of Common Extraction Solvents on the Vanillin-Sulphuric Acid Method

Of the ten solvents investigated, acetone caused the most intense darkening of the reaction solution in the absence of saponins, followed by butanol and methanol, which also caused very substantial colour development in the reagent blank (Figure 1). Therefore, these three solvents were found to be incompatible with the vanillin-sulphuric acid method. Ethyl acetate and hexane also caused moderate absorbance in the reagent blank, and could also significantly interference with the accurate measurement of saponins using this assay. Of note, ethanol, which was used in the original assay [7,10,12], was the only solvent not to cause a significant colour reaction in the absence of saponins.

For acetone, it is known that its reaction with concentrated sulphuric acid at an elevated temperature creates the carbonaceous resin, mesitylene, which has a dark colour [21,22]. Development of this coloured product, during the vanillin-sulphuric acid assay, could obviously result in an artificially high saponin result, and may explain why acetone could give the highest TSC values when compared to several other extraction solvents [23].

While the mechanism for the colour development with n-butanol remains unknown, the mechanism for the chroma effect of methanol is most likely, due to the esterification reaction between methanol and concentrated sulphuric acid to create methyl hydrogen sulphate [24,25]. Methanol is often used to dissolve aescin because, unlike for other solvents, the saponin mixture used as an external saponin standard [11,20,26], dissolves very well in the alcohol. For example, aescin cannot be efficiently dissolved in acetone and n-butanol and therefore these solvents could not be tested for their effects on the aescin standard curve in the present study.

However, the interfering effects of methanol were also seen (Figure 2) when it was used to dissolve aescin to construct the standard curve. Therefore, the effects of methanol on the reagent blank, and the standard curve may explain why methanol extracts have often been observed to give higher TSC values than extracts derived from other solvents [26–33], and why the TSC values increase as the methanol concentration is increased [20,33].

The presence of ethanol in the reaction solution is acceptable because it does not cause a significant difference in the absorbance readings in comparison to its aqueous counterpart. This means that the unmodified vanillin-sulphuric acid method is still acceptable for ethanol extracts providing that the standard curve is prepared with ethanol only.

However, it can be concluded that, except for ethanol, the extraction solvents evaluated in this study can interfere with the TSC measurements and thus, they should be eliminated from the extracts before the vanillin-sulphuric acid reaction step as proposed in this study. Furthermore, even for

ethanol extracts, the modified method does not require the extract to be diluted, and therefore, it is an improvement because it saves on both labour and dilution solvent.

The construction of the standard curves also revealed that the linear range of the absorbance value measured by a spectrophotometer can be as high as 2.2, or more (Figure 2), instead of 1.0, as usually recommended. This means a higher range of saponin concentration can be measured by this method.

4.2. Modifications of the Vanillin-Sulphuric Acid Method

In the modified assay, the major change was the elimination of the extraction solvent by evaporation from the sample extract before the vanillin-sulphuric acid reaction step. To facilitate this change, other minor modifications were also made:

- To make it easier to remove the solvent from the extract by evaporation, the volume of the sample extract used in the assay was decreased ten times (from 0.25 mL to 25 μL);
- To keep the total volume of the solution, and the concentration of the reagents during the reaction step the same as for the unmodified assay, the concentration of the vanillin solution in ethanol was decreased by half from 8% to 4% (w/v), and the volume of the 4% (w/v) vanillin solution added was doubled (from 0.25 mL to 0.5 mL).

4.3. Advantages of the Modified Vanillin-Sulphuric Acid Method

The modified method for the quantification of the TSC of plant materials has the following advantages over the unmodified protocol:

- Because the chemical reaction in the modified protocol is not interfered with by the presence of extraction solvent, it does not require the standard curve to be constructed with a new solvent every time the extraction solvent is changed. Moreover, once the standard curve has been proven to be reproducible over time, then it will not need to be done for every assay; the same standard curve can be used for subsequent assays, and for extracts derived from all types of solvents, which means that labour and reagents can be is saved.
- In the unmodified protocol, the standards and the reagent blank needs to be prepared (dissolved and diluted) in the same solvent that has been used to extract the material. However, this is difficult in practice because every saponin standard dissolves only in a limited number of solvents. This often leads to the use of a solvent other than that used for extraction of the material to dissolve the saponin standard, which can result in some errors and inconsistencies in the results obtained. In contrast, the results obtained with the modified protocol are not affected by the nature of the solvent used to dissolve the standard because whatever the solvent is, it is eliminated from the reaction system in the same way the extraction solvent is removed. Therefore, the modified protocol can be applied not only for aescin, used in the present study as the external saponin standard, but also for any other selected saponin standard.
- In the unmodified protocol, the sample extract needs to be diluted so that its saponin content does not cause an absorbance reading that is too high, and therefore, out of the assay's linear range. In contrast, in the modified protocol, the appropriate concentration of saponin in the reaction mixture can simply be achieved by changing the volume of the sample aliquot added to the test tube prior to evaporating the extraction solvent. Therefore, the modified protocol does not require the sample dilution step, which means that labour and solvents can be saved.
- For the unmodified protocol, when the sample extract contains a highly non-polar solvent, such as hexane or chloroform, it can form a cloudy emulsion with the highly polar vanillin-sulphuric acid solution, and therefore, the saponin content cannot be accurately measured spectrophotometrically. The modified method avoids that problem because the solvent contained in the extract is evaporated away and not present when the polar vanillin-sulphuric acid solution is added and therefore possible emulsification is prevented.

4.4. Implementation of the Modified Vanillin-Sulphuric Acid Method

For the modified protocol to best work and be consistently performed across laboratories, the following should be considered:

- To facilitate the removal of the solvent from the sample extract, a small volume of undiluted sample (high concentration) is recommended.
- If the saponins in the sample extract are heat- and/or oxidation-sensitive, a stream of an inert gas (e.g., nitrogen or helium) can be used to evaporate the extraction solvent instead of using a hot water bath.
- When building up the standard curve for saponin standards other than aescin, the concentration of the standard stock solution and the volume of each diluted standard solution can be adjusted so that the absorbance readings fall within the absorbance linear range of the spectrophotometer used.
- When the volume of the sample used (V μL) is not 25 μL, the concentration value calculated from the calibration curve needs to be multiplied by a normalisation factor of 25/V to obtain the final saponin concentration in the extract, which can then be used to determine the TSC of the extracted plant material.

5. Conclusions

This study demonstrated that the current method for the vanillin-acid sulphuric assay was not appropriate to use for extracts in solvents such as methanol, acetone, butanol, acetonitrile, ethyl acetate, dichloromethane, diethyl ether, chloroform and hexane. Therefore, to improve the existing method, a solvent evaporation step was added prior to the reaction step to eliminate any undesired effects of the extraction solvent. The improved protocol is simpler, requires less labour and solvent for dilutions, and most importantly, eliminates any possible interference by the extraction solvent. Therefore, this improved method is recommended for quantifying the TSC of extracted plant materials.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hostettmann, K.; Marston, A. *Chemistry and Pharmacology of Natural Products, Saponin*; Cambridge University Press: Cambridge, UK, 1995; ISBN 0-521-32970-1.
2. Sparg, S.; Light, M.; Van Staden, J. Biological activities and distribution of plant saponins. *J. Ethnopharmacol.* **2004**, *94*, 219–243. [CrossRef] [PubMed]
3. Güçlü-Üstündağ, Ö.; Mazza, G. Saponins: Properties, applications and processing. *Crit. Rev. Food Sci. Nutr.* **2007**, *47*, 231–258. [CrossRef] [PubMed]
4. Cheok, C.Y.; Salman, H.A.K.; Sulaiman, R. Extraction and quantification of saponins: A review. *Food Res. Int.* **2014**, *59*, 16–40. [CrossRef]
5. Gestetner, B.; Birk, Y.; Bondi, A.; Tencer, Y. Soya bean saponins—VII: A method for the determination of sapogenin and saponin contents in soya beans. *Phytochemistry* **1966**, *5*, 803–806. [CrossRef]
6. Baccou, J.; Lambert, F.; Sauvaire, Y. Spectrophotometric method for the determination of total steroidal sapogenin. *Analyst* **1977**, *102*, 458–465. [CrossRef] [PubMed]
7. Hiai, S.; Oura, H.; Hamanaka, H.; Odaka, Y. A color reaction of panaxadiol with vanillin and sulfuric acid. *Planta Med.* **1975**, *28*, 131–138. [CrossRef] [PubMed]

8. Oleszek, W.; Bialy, Z. Chromatographic determination of plant saponins—an update (2002–2005). *J Chromatogr. A* **2006**, *1112*, 78–91. [CrossRef] [PubMed]
9. Oleszek, W. Chromatographic determination of plant saponins. *J. Chromatogr. A* **2002**, *967*, 147–162. [CrossRef]
10. Hiai, S.; Oura, H.; Odaka, Y.; Nakajima, T. A colorimetric estimation of Ginseng saponins. *Planta Med.* **1975**, *28*, 363–369. [CrossRef] [PubMed]
11. Tan, S.P.; Vuong, Q.V.; Stathopoulos, C.E.; Parks, S.E.; Roach, P.D. Optimized aqueous extraction of saponins from bitter melon for production of a saponin-enriched bitter melon powder. *J. Food Sci.* **2014**, *79*, E1372–E1381. [CrossRef] [PubMed]
12. Hiai, S.; Oura, H.; Nakajima, T. Color reaction of some sapogenins and saponins with vanillin and sulfuric acid. *Planta Med.* **1976**, *29*, 116–122. [CrossRef] [PubMed]
13. Wu, J.; Lin, L.; Chau, F.T. Ultrasound-assisted extraction of ginseng saponins from ginseng roots and cultured ginseng cells. *Ultrason. Sonochem.* **2001**, *8*, 347–352. [CrossRef]
14. Oleszek, W.A. Composition and quantitation of saponins in alfalfa (*Medicago sativa* L.) seedlings. *J. Agric. Food Chem.* **1998**, *46*, 960–962. [CrossRef]
15. Habicht, S.D.; Kind, V.; Rudloff, S.; Borsch, C.; Mueller, A.S.; Pallauf, J.; Yang, R.; Krawinkel, B.M. Quantification of antidiabetic extracts and compounds in bitter gourd varieties. *Food Chem.* **2011**, *126*, 172–176. [CrossRef]
16. Masullo, M.; Calabria, L.; Gallotta, D.; Pizza, C.; Piacente, S. Saponins with highly hydroxylated oleanane-type aglycones from *Silphium asteriscus* L. *Phytochemistry* **2014**, *97*, 70–80. [CrossRef] [PubMed]
17. Zhao, Y.; Wang, X.; Wang, H.; Liu, T.; Xin, Z. Two new noroleanane-type triterpene saponins from the methanol extract of *Salicornia herbacea*. *Food Chem.* **2014**, *151*, 101–109. [CrossRef] [PubMed]
18. Abubakar, E.-M.M. Antibacterial activity of crude extracts of Euphorbia hirta against some bacteria associated with enteric infections. *J. Med. Plants Res.* **2009**, *3*, 498–505.
19. Masayo, I.; Hikaru, O.; Tatsuo, Y.; Masako, T.; Yoshie, R.; Shuji, H.; Kunihide, M.; Ryuichi, H. Studies on the constituents of *Momordica cochinchinensis* Spreng. I. Isolation and characterization of the seed saponins, Momordica saponins I and II. *Chem. Pharm. Bull.* **1985**, *33*, 464–478. [CrossRef]
20. Nguyen, V.T.; Vuong, Q.V.; Bowyer, M.C.; Van Altena, I.A.; Scarlett, C.J. Microwave-assisted extraction for saponins and antioxidant capacity from Xao Tam Phan (*Paramignya trimera*) Root. *J. Food Process. Preserv.* **2017**, 41. [CrossRef]
21. Kane, S.M.; Timonen, R.S.; Leu, M.-T. Heterogeneous chemistry of acetone in sulfuric acid solutions: implications for the upper troposphere. *J. Phys. Chem. A* **1999**, *103*, 9259–9265. [CrossRef]
22. Reichardt, C.; Welton, T. *Solvents and Solvent Effects in Organic Chemistry*; John Wiley & Sons: Weinheim, Germany, 2011; ISBN 9783527324736.
23. Bhuyan, D.J.; Vuong, Q.V.; Chalmers, A.C.; van Altena, I.A.; Bowyer, M.C.; Scarlett, C.J. Investigation of phytochemicals and antioxidant capacity of selected Eucalyptus species using conventional extraction. *Chem. Pap.* **2016**, *70*, 567–575. [CrossRef]
24. Van Loon, L.L.; Allen, H.C. Methanol reaction with sulfuric acid: A vibrational spectroscopic study. *J. Phys. Chem. B* **2004**, *108*, 17666–17674. [CrossRef]
25. Ota, K.I.; Nakagawa, Y.; Takahashi, M. Reaction products of anodic oxidation of methanol in sulfuric acid solution. *J. Electroanal. Chem. Interface Electrochem.* **1984**, *179*, 179–186. [CrossRef]
26. Pham, H.N.T.; Bowyer, M.C.; van Altena, I.A.; Scarlett, C.J. Influence of solvents and novel extraction methods on bioactive compounds and antioxidant capacity of Phyllanthus amarus. *Chem. Pap.* **2016**, *70*, 556–566. [CrossRef]
27. Van Tang, N.; Bowyer, M.C.; Van Vuong, Q.; Van Altena, I.A.; Scarlett, C.J. Phytochemicals and antioxidant capacity of Xao tam phan (*Paramignya trimera*) root as affected by various solvents and extraction methods. *Ind. Crops Prod.* **2015**, *67*, 192–200. [CrossRef]
28. Pham, H.N.T.; Nguyen, V.; Vuong, Q.; Bowyer, M.C.; Scarlett, C.J. Bioactive compound yield and antioxidant capacity of *Helicteres hirsuta* Lour. stem as affected by various solvents and drying methods. *J. Food. Process Preserv.* **2017**, *41*, e12879. [CrossRef]
29. Pham, H.N.T.; Nguyen, V.T.; Vuong, Q.V.; Bowyer, M.C.; Scarlett, C.J. Effect of extraction solvents and drying methods on the physicochemical and antioxidant properties of *Helicteres hirsuta* Lour. leaves. *Technologies* **2015**, *3*, 285–301. [CrossRef]
30. Pham, H.N.T.; Vuong, Q.V.; Bowyer, M.C.; Scarlett, C.J. Effect of extraction solvents and thermal drying methods on bioactive compounds and antioxidant properties of *Catharanthus roseus* (L.) G. Don (Patricia White cultivar). *J. Food Process. Preserv.* **2015**, *3*, 285–301. [CrossRef]

31. Nguyen, V.T.; Sakoff, J.A.; Scarlett, C.J. Physicochemical properties, antioxidant and anti-proliferative capacities of dried leaf and its extract from Xao tam phan (*Paramignya trimera*). *Chem. Biodivers.* **2017**, 14. [CrossRef] [PubMed]
32. Barve, K.; Laddha, K.; Jayakumar, B. Extraction of saponins from Safed Musli. *Pharmacogn. J.* **2010**, *2*, 561–564. [CrossRef]
33. Ngo, T.V.; Scarlett, C.J.; Bowyer, M.C.; Ngo, P.D.; Vuong, Q.V. Impact of different extraction solvents on bioactive compounds and antioxidant capacity from the root of *Salacia chinensis* L. *J. Food Qual.* **2017**, 2017. [CrossRef]

3.4.2. Optimisation of the microwave-assisted ethanol extraction of saponins from Gac (*Momordica cochinchinensis* Spreng) seeds

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Optimisation of the Microwave-Assisted Ethanol Extraction of Saponins from Gac (*Momordica cochinchinensis* Spreng.) Seeds

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Abstract: BACKGROUND: Gac (*Momordica cochinchinensis* Spreng.) seeds contain saponins that are reportedly medicinal. It was hypothesised that the extraction of saponins from powdered Gac seed kernels could be optimised using microwave-assisted extraction (MAE) with ethanol as the extraction solvent. The aim was to determine an appropriate ethanol concentration, ratio of solvent to seed powder and microwave power and time for extraction. Whether or not defatting the Gac seed powder had an impact on the extraction of saponins, was also determined. RESULTS: A four-fold higher total saponin content (TSC) was obtained in extracts from full-fat Gac seed kernel powder than from defatted powder (100 vs. 26 mg aescin equivalents (AE) per gram of Gac seeds). The optimal parameters for the extraction of saponins were a ratio of 30 mL of 100% absolute ethanol per g of full-fat Gac seed kernel powder with the microwave set at 360 W for three irradiation cycles of 10 s power ON and 15 s power OFF per cycle. CONCLUSIONS: Gac seed saponins could be efficiently extracted using MAE. Full-fat powder of the seed kernels is recommended to be used for a better yield of saponins. The optimised MAE conditions are recommended for the extraction of enriched saponins from Gac seeds for potential application in the nutraceutical and pharmaceutical industries.

Keywords: Gac seeds; *Momordica cochinchinensis*; saponins; microwave-assisted extraction; optimization

1. Introduction

Momordica cochinchinensis Spreng. is a perennial climber, which belongs to the Cucurbitaceae family. It ranges from China to the Moluccas and has been used in food and traditional medicine in East and Southeast Asia [1]. The most important part of the mature fruit is the red flesh surrounding the seeds, called the aril, which is used as a colorant in rice or as a material for further processing into functional food ingredients. The seeds are not eaten and they are removed from the aril and are mostly considered waste [2]. However, in traditional medicine, Gac seeds are alleged to have a wide array of therapeutic effects for a wide variety of conditions, including fluxes, liver and spleen disorders, hemorrhoids, wounds, bruises, inflammation, swellling and infections [1,3]. Modern science has reported biological activities for Gac seed extracts, including being a gastroprotective agent [4,5] and accelerating the healing of gastric ulcers in rats [6], and possessing antitumour [7], anticancer [8] and anti-inflammatory [9,10] activities.

Gac seed saponins have been reported to be critical constituents in Gac seed extracts, which were responsible for their medicinal properties [9,11]. These constituents of Gac seeds have been investigated by several investigators: two saponins, referred to as momordica saponin I and II, have been isolated and characterised [12], in which momordica saponin I is a major gastroprotective ingredient [5]. Another saponin, karounidiol, a compound possessing cytotoxic activity against human cancer cell lines [13], has been reported to be present in Gac seeds [14]. The potential valuable pharmaceutical properties of the Gac seed saponins warrants investigating how they are best extracted from the seeds i.e., which extraction technique(s) will maximise the yield of saponins.

The conventional extraction technique, in which the solid material is suspended in extraction solvent with no assistance for breaking the cell structure of the solid material, is often associated with a long heating time, which risks the degradation of bioactive compounds. This has led to the proposed use of advanced techniques such as microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE) that are efficient in terms of extraction time and solvent consumption. Microwave heating or ultrasonic cavitation is able to disrupt the plant cell structure via an increase in the internal pressure of the cell and thereby, release the bioactive compounds [15,16]. However, in a comparative study being carried out by the same authors [17], it was found that while the MAE significantly improved Gac seed saponin extraction in comparison to the conventional method, UAE did not. MAE, therefore, is the technique which needs to be further optimised. The MAE method is likely to be effective for the extraction of saponins from the Gac seeds, as it has been reported that microwave assistance significantly improved the recovery of saponins from a wide range of plant sources such as *Phyllanthus amarus* [18], yellow horn [19], *Ganoderma atrum* [20], chick pea [21] and ginseng [22], among others.

The choice of the extraction solvent is also important. Low alcohols such as methanol and ethanol have usually been used as effective solvents for the extraction of saponins from plant materials. However, according to the US Food and Drug Administration [23], methanol belongs to the Class 2 solvents, which should be limited in pharmaceutical products because of their inherent toxicity. Ethanol, on the other hand, belongs to the Class 3 solvents [23], which are less toxic and of lower risk to human health and therefore, should be used instead of methanol for the extraction of plant bioactive compounds. Moreover, ethanol in form of wines has been traditionally used for maceration of Gac seeds, therefore, it is reasonable to investigate the efficiency of this solvent for modern extraction methods. In addition, ethanol is also an excellent microwave absorbing solvent and has been used to advantage in MAE [16].

When it comes to extraction of saponins from seeds, defatting is often carried out before the saponins are extracted [24]. Although the defatting might make it simpler for the saponin extraction in terms of technique, and does not greatly affect the saponin yield for some type of seeds, it can cause a great loss of saponin for others.

Therefore, in this study, the extraction of saponins from powdered Gac seed kernels was optimised using MAE with ethanol as the extraction solvent. The aim was to determine an appropriate ethanol concentration, ratio of solvent to seed powder and microwave power and time for saponin extraction. Whether or not defatting the Gac seed powder had an impact on the extraction of saponins, was also determined.

2. Materials and Methods

2.1. Materials

2.1.1. Solvents, Reagents and Chemicals

Absolute ethanol (≥99.8%), methanol and chemicals including vanillin, sulphuric acid, and potassium persulfate were products of Merck (Bayswater, VIC, Australia) and 2,4,6-tris(2-pyridyl)-*s*-triazine; (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2carboxylic acid (trolox),

aescin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were products of Sigma-Aldrich Co. (Castle Hill, NSW, Australia).

2.1.2. Gac Seed Kernel Powder

Gac seeds, were collected from 450 kg of fresh Gac fruit, from accession VS7 as classified by Wimalasiri, Piva, Urban and Huynh [25]. These fruits were bought at Gac fruit farms in Dong Nai province, Ho Chi Minh city, Vietnam (Latitude: 10.757410; Longitude: 106.673439). After their separation from the fresh fruit, the seeds were vacuum dried at 40 °C for 24 h to reduce moisture and increase the crispness of the shell to facilitate shell removal. The dried seeds were de-coated to obtain the kernels, which were then packaged in vacuum-sealed aluminum bags and stored at −18 °C prior to use.

2.1.3. Preparation of Gac Seed Kernel Powder

The Gac seed kernels were ground in an electric grinder (100 g ST-02A Mulry Disintegrator), to produce powder, which could pass through a sieve of 1.4 mm. The powder was then freeze-dried using a Dynavac FD3 freeze dryer (Sydney, NSW, Australia) for 48 h at −45 °C under vacuum at a pressure loading of 10^{-2} mbar (1 Pa) to reduce the moisture content to 1.21 ± 0.02%, as determined using a MOC63u moisture analyser (Shimazdu, Kyoto, Japan). This Gac seed kernel powder was referred to as 'full-fat powder' and was stored in vacuum-sealed polyethylene bags under vacuum at −20 °C until used.

2.1.4. Preparation of Defatted Gac Seed Kernel Powder

To prepare defatted Gac seed kernel powder, the freeze-dried kernel powder was extracted three times for thirty minutes with hexane (1:5 w/v) on a magnetic stirrer at room temperature. Each time, the resulting slurry was suction-filtered and the final residue was air-dried for 12 h and stored in a desiccator at ambient temperature until used. This Gac seed kernel powder was referred to as 'defatted powder'.

2.2. Methods

The experimental design for the study is shown in Figure 1.

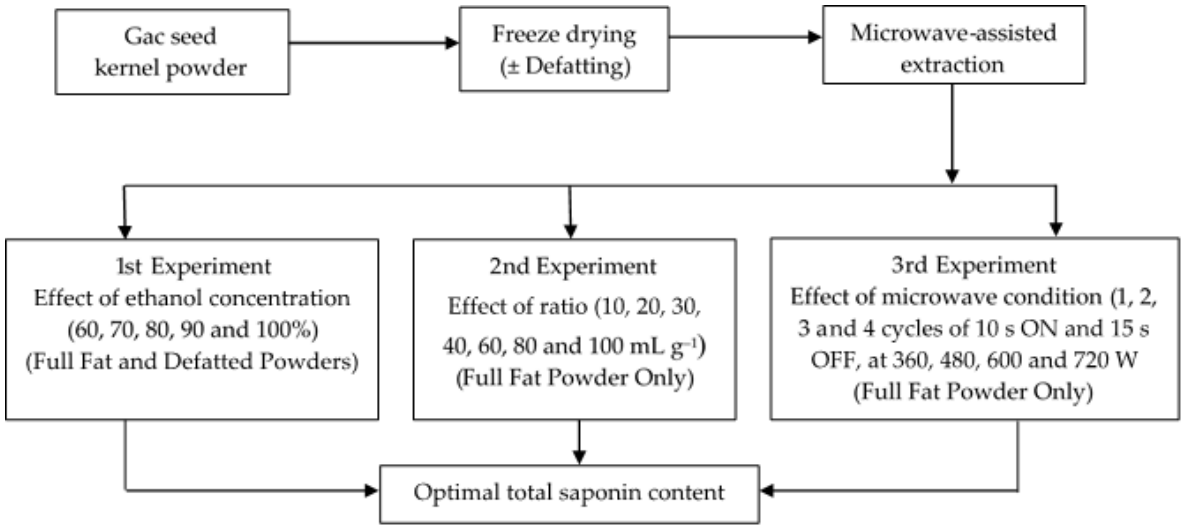

Figure 1. Experimental design for optimisation of saponin yield from Gac seeds.

2.2.1. Microwave Assisted Extraction (MAE)

The MAE was performed using a R395YS Sharp Carousel microwave oven (Sharp Corporation, Bangkok, Thailand) bought from a local Target store (Tuggerah, NSW, Australia). Gac seed kernel powder was mixed with ethanol of various concentrations with water in a 100 mL conical flask. The suspension was left pre-leaching for 30 min at ambient temperature before microwave treatment was applied for varying number of cycles, which consisted of 10 s power ON and 15 s power OFF per cycle. The temperature of the suspension was recorded at the end of the MAE process.

2.2.2. Extraction of Saponins from Full-Fat and Defatted Gac Seed Kernel Powders

Prior to weighing for extraction, the moisture content of the powder samples was measured using a MOC63u moisture analyser (Shimazdu, Kyoto, Japan), which was used in the determination of saponin yield.

The effect of the ethanol concentration was investigated for the MAE of saponins from both the full-fat and the defatted powders. The concentration of ethanol was varied (60%, 70%, 80%, 90% and 100%) but the solvent to powder ratio and the microwaving conditions were kept constant at 30 mL g^{-1} and 600 W for four cycles, respectively (1st experiment in Figure 1). After finishing the extractions, the suspensions were rapidly cooled to $\leq$20 °C in an ice water bath and filtered through a 0.45 µm membrane filter. The clear extracts were collected and kept at −20 °C for analysis within a week.

2.2.3. Extraction of Saponins from the Full-Fat Seed Kernel Powder

The full-fat powder was selected for the following two experiments since it resulted in a higher extraction of saponins for all the concentrations of ethanol; and 100% absolute ethanol was chosen because it resulted in the highest extraction of saponins from the full-fat powder.

Two experiments (Figure 1) were done using the full-fat powder and 100% ethanol as the extraction solvent to determine the effect of three individual parameters, (i) the ratio of solvent to powder (10, 20, 30, 40, 60, 80 and 100 mL g^{-1}) (Figure 1, 2nd Experiment), (ii) microwave radiation power (360, 480, 600, 720 and 840 W) and (iii) microwave irradiation time (1, 2, 3 and four cycles) (Figure 1, 3rd Experiment), on the recovery of saponins from the Gac seed kernel powder was investigated. When one parameter was examined, the other was maintained constant; for the 2nd experiment (Figure 1), the microwave conditions were 600 W with four cycles and for the 3rd experiment, the ratio of ethanol to powder was 30 mL g^{-1}. After finishing the extractions, the suspensions were rapidly cooled to $\leq$20 °C in an ice water bath and filtered through a 0.45 µm membrane filter. The clear extracts were collected and kept at −20 °C for less than a week before analysis.

2.2.4. Verifying Optimal Conditions for Gac Seed Saponin Extraction

From the findings in the 3rd experiment, two possible optimal sets of microwave parameters were chosen for the extraction of saponins from the full-fat powder. Therefore, these two sets of microwave parameters were repeated to validate the findings. A control (no microwave) extract was also run with 100% ethanol and the optimal solvent to powder ratio but where the heat was provided using a water bath instead of the microwave oven. The water bath temperature was chosen to be 76 °C and the incubation was done for 100 s because it was the maximum temperature and incubation time achieved during the MAE using the two sets of microwave parameters. These three extracts were analysed for TSC and antioxidant capacity–measured with two assays, ABTS and DPPH. The energy consumption for these extracts was also estimated according to the Equation (1) as follows:

$$W_i = P_i \times t_i \quad (1)$$

where W_i is the consumed electrical energy for the extraction method (kWh), P_i is the electrical power supplied for the extraction method (kW) and t_i is the electricity consumption time for the extraction method (h).

2.3. Analytical Methods

2.3.1. Determination of Total Saponin Content (TSC)

Determination of the total saponin content was conducted using the colorimetric method of Hiai, Oura and Nakajima [26] with slight modifications. The principle of this method is the reaction of sulphuric acid-oxidised saponins with vanillin to produce a distinctive red-purple colour, which is measured at 560 nm using a spectrophotometer.

To 0.25 mL of the appropriately diluted Gac seed ethanol extract samples, 0.25 mL 8% vanillin in ethanol (w/v) was added followed by 2.5 mL of 72% H_2SO_4 (v/v). The test tube was vortexed, covered, incubated at 60 °C for 15 min and cooled to ambient temperature in an iced-water bucket for 2 min. With a reagent blank as reference, the absorbance was measured at 560 nm using a Carry 50 Bio spectrophotometer (Varian Pty. Ltd., Mulgrave, VIC, Australia).

A standard curve of aescin (100–1000 μg/mL) was constructed to determine the saponin concentrations. The results were expressed as mg aescin equivalents (AE) per gram dry weight of Gac seed kernel powder (mg AE g^{-1}).

2.3.2. Determination of Antioxidant Capacity

The antioxidant capacity was tested for the optimal and control extracts using two assays: ABTS and DPPH.

ABTS Assay

The ABTS assay [27] was used as described by Tan et al. [28] with slight modifications. Stock solutions of 7.4 mM ABTS and 2.6 mM potassium persulfate were prepared and kept at 4 °C until use. Fresh working solution was prepared for each assay by mixing the 2 stock solutions in equal quantities and incubating them for 15 h in the dark at ambient temperature. Then, 1 mL of the working solution was diluted with ~30 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm. To 0.15 mL of each standard, blank and appropriately diluted extract sample, 2.85 mL of the working solution was added. The tubes were incubated for 2 h in the dark at ambient temperature and the absorption was measured at 734 nm using a Carry 50 Bio spectrophotometer (Varian Pty. Ltd., Mulgrave, VIC, Australia). Trolox was used as the standard and the results were expressed as mg Trolox equivalents per gram dry weight of Gac seed kernel powder (mg TE g^{-1}).

DPPH Assay

The DPPH assay [29] was used as described by Tan et al. [28]. A stock solution of 0.6 M DPPH in methanol was prepared and kept at −20 °C until use. The working solution was prepared by mixing 10 mL of stock solution with ~45 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm. To 0.15 mL of each standard, blank and appropriately diluted extract sample, 2.85 mL of the working solution was added. The tubes were allowed to stand for 3 h in the dark at ambient temperature and the absorption was measured at 515 nm using a Carry 50 Bio spectrophotometer (Varian Pty. Ltd., Mulgrave, VIC, Australia). Trolox was used as the standard and results were expressed as mg Trolox equivalents per gram of dry weight Gac seed kernel powder (mg TE g^{-1}).

2.4. Statistical Analyses

Experiments were performed in triplicate and values were expressed as means ± SD and were assessed for statistical significance using the one-way ANOVA and Tukey's *Post Hoc* Multiple Comparison test using the IBM SPSS Statistics 24 program (IBM Corp., Armonk, NY, USA). Correlation and regression analyses were done using Microsoft Excel 2016. Differences between means, correlations and regressions were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of the Ethanol Concentration on the MAE of Saponins from Full-Fat and Defatted Gac Seed Kernel Powders

The full-fat and defatted Gac seed kernel powders were extracted using MAE with the ethanol concentration ranging from 60% to 100% (in water) in the extraction solvent. Figure 2 shows that at the lower ethanol concentrations, from 60% to 80%, there was no significant difference in the measured TSC for the full-fat powder. The measured TSC was higher with 90% ethanol and the highest (100.3 mg AE g^{-1}) with 100% ethanol as the extraction solvent. In contrast, changing the ethanol concentration from 60% to 100% did not increase the measured TSC of the defatted Gac seed kernel powder, which was lower than for the full-fat powder for all the ethanol concentrations. Therefore, the full-fat Gac seed kernel powder and 100% absolute ethanol, as the extraction solvent, gave the best MAE extraction of saponins and they were used in the subsequent experiments.

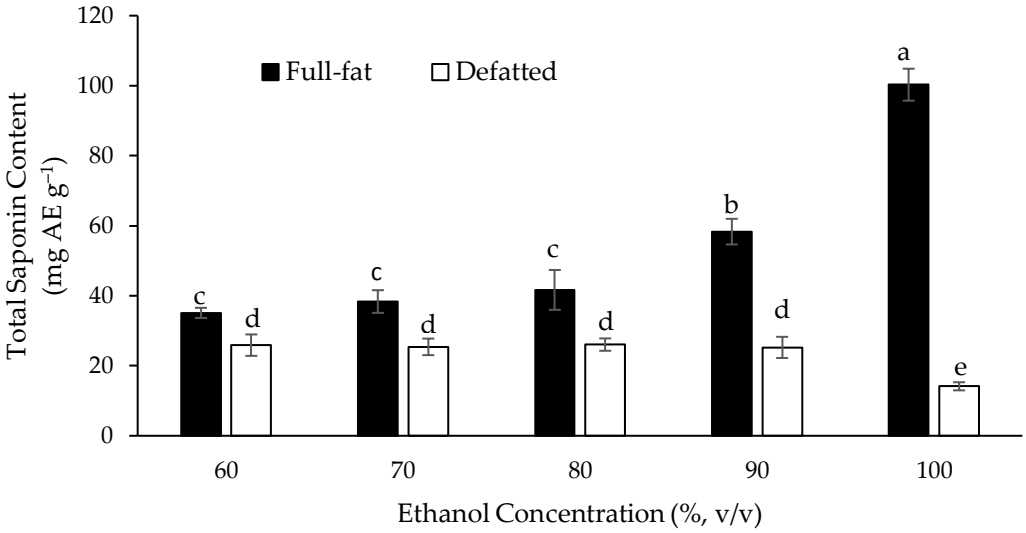

Figure 2. Effect of the ethanol concentration, in the extraction solvent used for microwave-assisted extraction (MAE), on the measured total saponin content (TSC) of the full-fat and defatted Gac seed kernel powders. The values are the means of three replicates for each extraction and columns not sharing the same superscript letter are significantly different at $p < 0.05$.

3.2. Effect of the Ethanol to Sample Ratio on the MAE of Saponins from the Full-Fat Gac Seed Kernel Powder

Seven ratios of 100% absolute ethanol to full-fat powder, from 10 to 100 mL g^{-1}, were investigated. Figure 3 shows that increasing the ratio from 10 to 30 mL g^{-1} had a significant effect on the measure TSC value after MAE, which increased by 30% from 70.4 to 100.8 mg AE g^{-1}. However, increasing the ratio from 30 to 100 mL g^{-1} resulted in less pronounced increases in the measured TSC. Therefore, although the measured TSC was slightly and significantly higher with the ratio of 100 mL g^{-1} compared to 30 mL g^{-1} (Figure 3), the ratio of ethanol to powder of 30 mL g^{-1} was deemed to be the better ratio, from the conservation of solvent perspective, and it was chosen for investigating the microwave parameters.

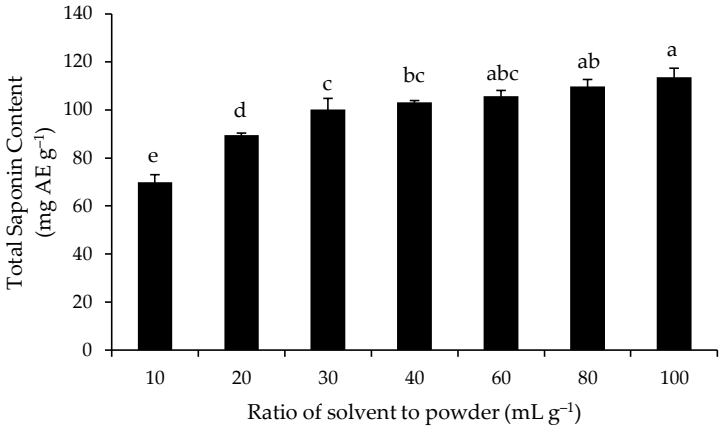

Figure 3. Effect of the ethanol to powder ratio on the TSC of the full-fat Gac seed kernel powder measured using MAE. The values are the means of three replicates for each extraction and columns not sharing the same superscript letter are significantly different at $p < 0.05$.

3.3. Effect of the Microwave Parameters on the MAE of Saponins from the Full-Fat Gac Seed Kernel Powder

Four levels of microwave power (360, 480, 600 and 720 W) were investigated and at every power level, the number of irradiation cycles was also varied (1, 2, 3 and four cycles). Each cycle consisted of 10 s power ON (irradiation) followed by 15 s power OFF (no irradiation). The full-fat powder was used and the ratio of 100% ethanol to powder was 30 mL g^{-1}. In general, Figure 4 shows that the measured TSC gradually increased as the power and irradiation time were increased for the MAE but that many of the values were not significantly different from each other. Notably, from 600 W to two cycles upwards (to the right in Figure 4), there was no significant increase in the measured TSC values. However, the two sets of parameters, which only shared the a superscript in Figure 4, 360 W and three cycles and 480 W and four cycles, were selected as possibly optimal for the MAE extraction of saponins from the full-fat Gac seed kernel powder.

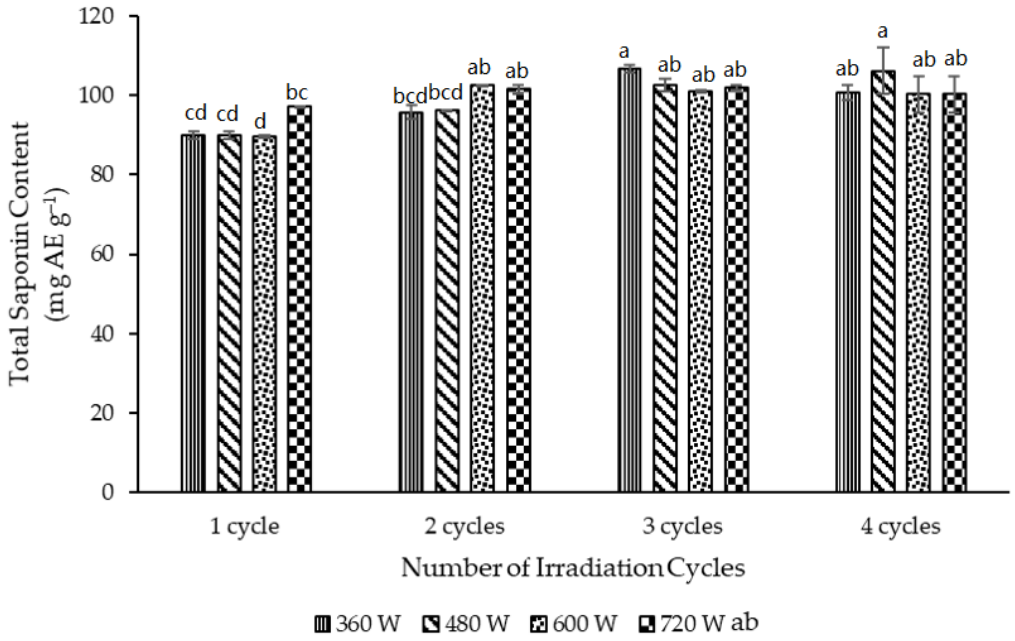

Figure 4. Effect of microwave power and irradiation time (cycles) on the TSC of the full-fat Gac seed kernel powder measured using MAE. The values are the means of three replicates for each extraction and columns not sharing the same superscript letter are significantly different at $p < 0.05$.

3.4. Correlations between the TSC and the MAE Temperature

The temperature of the extracts at the end of each MAE in Figure 4 was recorded using a digital thermometer. Their temperature ranged from 43.4 to 75.6 °C. Correlation analysis revealed that the measured TSC of the extracts was positively correlated with the temperature of the extraction mixture at the end of the MAE (Figure 5).

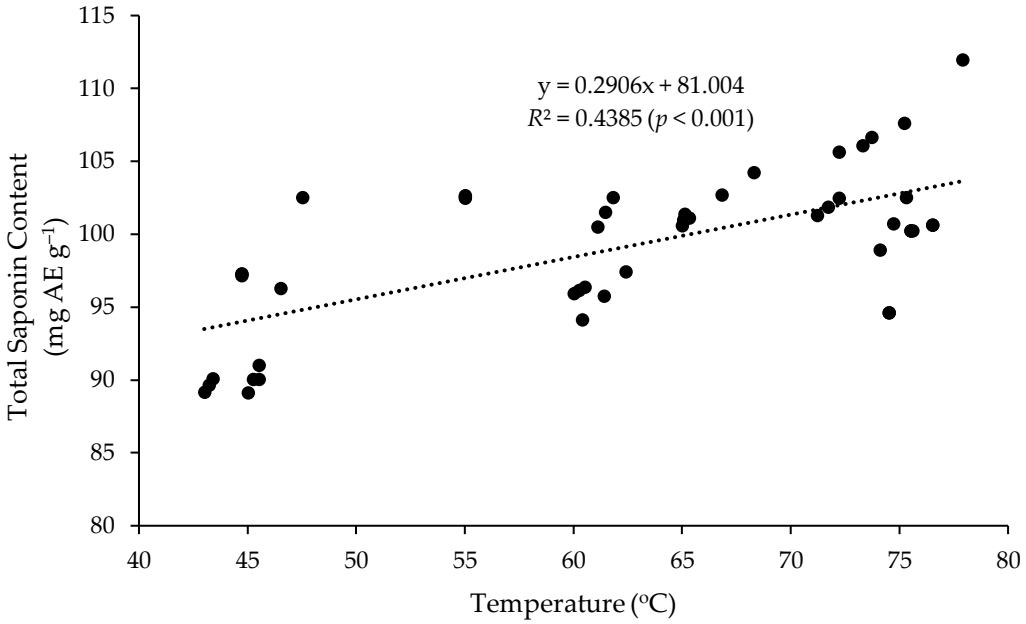

Figure 5. Correlation between the TSC and the temperature of the extract at the end of various MAE treatments. The black dots: TSC at different temperature of the extracts.

Table 1 shows that the temperature of the extracts at the end of the MAE was almost all due (92.5%) to the number of irradiation cycles (length of the microwave irradiation time) during the MAE; in contrast, there was no correlation between the temperature and the microwave power. Consisted with this, the measured TSC of the extracts was positively correlated with the number of microwave irradiation cycles but wasn't correlated with the power used during the MAE. Moreover, there was no interaction between the microwave power and the number of irradiation cycles.

Table 1. Correlations between the TSC and the MAE parameters.

	R^2 (p Value)			
	TSC	**Number of Cycle**	**Power**	**Cycle and Power**
Temperature	0.439 ($p < 0.001$)	0.925 ($p < 0.001$)	0.002 ($p > 0.5$)	0.926 ($p < 0.001$)
Number of cycle	0.362 ($p < 0.001$)	-	-	-
Power	0.000 ($p > 0.5$)	-	-	-
Cycle and Power	0.188 ($p > 0.1$)	-	-	-

3.5. Verification of the Optimal MAE Conditions for the Extraction of Saponins from Full-Fat Gac Seed Kernel Powder

Two possible optimal sets of microwave parameters (360 W and three cycles, 480 W and four cycles) were chosen for the extraction of saponins from the full-fat powder (Figure 4). Notably, the temperature measured at the end of the MAE using the two sets of microwave parameters (360 W and three cycles, 480 W and four cycles) was 72.2 ± 1.2 and 75.6 ± 1.9 °C, respectively, and they were not significantly different from each other.

These two sets of microwave parameters were repeated to validate the findings. A control (no microwave) set of extracts was also run with 100% ethanol and the optimal solvent to powder ratio where the temperature measured at the 480 W and four cycles MAE (76 °C) was provided using a water bath instead of the microwave oven. Also, the time used for the control extraction was chosen to be 100 s in order to match the length of time used for the 480 W and four cycles MAE.

These three sets of extracts were analysed for their saponin content and their ABTS and DPPH antioxidant activities. The results revealed that there was no difference among the three extracts in saponin content and antioxidant capacity (Table 2). However, the ABTS values were low and the DPPH assay did not detect any antioxidant activity for any of these extracts (Table 2).

Table 2. Saponin content, antioxidant activities and energy consumption of the optimal MAE and control extracts.

Extract	TSC (mg AE g^{-1})	ABTS (μmol TE g^{-1})	DPPH (μmol TE g^{-1})	Energy Consumption (kWh)
Optimal treatment 1 †	105.69 ± 2.40 [a]	1.47 ± 0.12 [a]	Undetected	0.003
Optimal treatment 2 ‡	109.23 ± 2.69 [a]	1.80 ± 0.31 [a]	Undetected	0.005
Control (no microwave) §	109.64 ± 4.79 [a]	1.63 ± 0.10 [a]	Undetected	0.325

The results are mean values ± standard deviations (n = 3) and the values not sharing the same superscript letter in the same column, are significantly different at $p < 0.05$. † Ethanol + Full-fat powder (30 mL g^{-1}); MAE at 360 W, three cycles for 75 s. ‡ Ethanol + Full-fat powder (30 mL g^{-1}); MAE at 480 W, four cycles for 100 s. § Ethanol + Full-fat powder (30 mL g^{-1}); Shaking water bath at 76 °C for 100 s.

From the point of view of saving energy, the optimal treatment 1 MAE parameters (Table 2) of 360 W with three irradiation cycles of 10 s power ON and 15 s power OFF per cycle (total of 75 s), were the best microwave conditions for the extraction of saponins from full-fat Gac seed kernel powder (0.003 kWh). The conventional extraction for 100 s in a shaking water bath at the same temperature (76 °C) as at the end of the optimal treatment 2 MAE settings also gave the same results but this also required more energy than the optimal treatment 1 MAE parameters because of the energy needed (0.325 kWh) to bring the temperature of the 5 L water bath from 20 °C up to 76 °C.

4. Discussion and Conclusions

The full-fat Gac seed kernel powder was the more suitable material to use as defatting caused a considerable loss of saponins (~75%). The highest TSC of extracts were obtained with 100% absolute ethanol, a 30 mL g^{-1} ratio of ethanol to full-fat Gac seed kernel powder and several sets of MAE conditions. Furthermore, when two sets of the MAE parameters, which gave the highest measured TSC in the extracts, were re-tested, the two extracts had the same TSC. However, from the point of view of saving energy, the optimal 1 MAE parameters of 360 W with three irradiation cycles of 10 s power ON and 15 s power OFF per cycle (total of 75 s), were the best conditions for the extraction of saponins from the full-fat powder.

It was concluded that, to extract Gac seed saponins, it was better to use the full-fat seed kernel powder rather than kernel powder from which the fat had been extracted. The Gac seed saponins appeared to be mainly associated with the fat component of the seeds because they were largely lost during the defatting process with hexane. Undoubtedly, most of the Gac seed saponins (75%) were highly non-polar, which is consistent with a previous finding that saponins were found in the unsaponifiable matter from Gac seed oil [14]. The saponin content of the full-fat Gac seed kernels was also similar to that reported for other oily plant extracts, such as eucalyptus [30] and *Phyllanthus amarus* [31], and significantly higher than the non-oily extract from the flesh of bitter melon [28].

Absolute ethanol was found to be the best concentration of ethanol for extracting the saponins from the full-fat Gac seed kernel powder. This is also consistent with the Gac seed saponins being hydrophobic in nature and consistent with Gac seeds having a high fat content [32]. This result

is also consistent with the earlier findings that Gac seed oil has a high content of unsaponifiable matter [33] and that this unsaponifiable material contains triterpenoid saponins [14]. It may be that more non-polar class 3 solvents, such as 1-propanol, isobutyl alcohol and n-butanol, could further improve the extraction of saponins from full-fat Gac seed kernels. However, because of the higher costs of these solvents, ethanol would be the solvent of choice for recovery of the Gac seed saponins on economic grounds.

The ratio of 30 mL ethanol per 1 g of Gac seed powder was the ratio of choice because, at this ratio, the saponin yield was improved significantly compared to the two lower ratios and there was not much improvement at the higher ratios. Although it varies for different plant materials, in the conventional extraction method, the higher the ratio of solvent volume to solid sample the better the extraction of compounds is. However, in the case of MAE, a higher solvent: sample ratio may not necessarily give a better yield due to non-uniform distribution and exposure to microwaves [16].

Varying the MAE parameters did not greatly affect the saponin extraction, mostly likely due to ethanol being a very good solvent [16] for the extraction of the Gac seed saponins and for increasing in temperature even under mild microwave conditions. When the optimal MAE conditions were compared with a control extraction (no microwave), it was found that the same level of saponin extraction was achieved irrespective of the heating source. However, from the point of view of saving energy, the optimal 1 MAE parameters were the best conditions for the extraction of saponins from full-fat Gac seed kernel powder. The energy saving characteristic of MAE has been confirmed in numerous reports [15]. This is due to the heat is generated inside the materials and then comes outwards, whereas in conventional heating the surface is heated first. Thus, the microwave heating is rapid and effective as heat is transferred directly to the material.

The antioxidant capacity of the Gac seed saponin extracts was low. This is possibly due to the more lipophilic nature of the Gac seed saponins, which is consistent with the findings that lipophilic compounds such as carotenoids, which do not show DPPH-radical scavenging activity [34], and tocopherols, which do not show much activity in the ABTS assay [35].

In conclusion, this study demonstrated that the extraction parameters play an important role in the extraction of saponins from Gac seeds. Accordingly, the MAE optimal parameters for the extraction of saponins were a ratio of 30 mL of 100% absolute ethanol per g of full-fat Gac seed kernel powder with the microwave set at 360 W for three irradiation cycles of 10 s power ON and 15 s power OFF per cycle. These parameters are recommended for the extraction of enriched saponins from Gac seeds for potential application in the nutraceutical and pharmaceutical industries.

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References

1. Perry, L.M.M. *Medicinal Plants of East Southeast Asia: Attributed Properties and Uses*; MIT Press: Cambridge, MA, USA, 1980.
2. Chuyen, H.V.; Nguyen, M.H.; Roach, P.D.; Golding, J.B.; Parks, S.E. Gac fruit (*Momordica cochinchinensis* Spreng.): A rich source of bioactive compounds and its potential health benefits. *Int. J. Food Sci. Technol.* **2015**, *50*, 567–577. [CrossRef]
3. Yin, M.H.; Kang, D.G.; Choi, D.H.; Kwon, T.O.; Lee, H.S. Screening of vasorelaxant activity of some medicinal plants used in Oriental medicines. *J. Ethnopharmacol.* **2005**, *99*, 113–117. [CrossRef] [PubMed]
4. Kang, J.M.; Kim, N.; Kim, B.; Kim, J.-H.; Lee, B.-Y.; Park, J.H.; Lee, M.K.; Lee, H.S.; Jang, I.-J.; Kim, J.S. Gastroprotective action of *Cochinchina momordica* seed extract is mediated by activation of CGRP and inhibition of cPLA2/5-LOX pathway. *Dig. Dis. Sci.* **2009**, *54*, 2549–2560. [CrossRef] [PubMed]

5. Jung, K.; Chin, Y.-W.; Chung, Y.H.; Park, Y.H.; Yoo, H.; Min, D.S.; Lee, B.; Kim, J. Anti-gastritis and wound healing effects of *Momordicae* semen extract and its active component. *Immunopharmacol. Immunotoxicol.* **2013**, *35*, 126–132. [CrossRef] [PubMed]
6. Kang, J.M.; Kim, N.; Kim, B.; Kim, J.-H.; Lee, B.-Y.; Park, J.H.; Lee, M.K.; Lee, H.S.; Kim, J.S.; Jung, H.C. Enhancement of gastric ulcer healing and angiogenesis by *Cochinchina momordica* seed extract in rats. *J. Korean Med. Sci.* **2010**, *25*, 875–881. [CrossRef] [PubMed]
7. Tien, P.G.; Kayama, F.; Konishi, F.; Tamemoto, H.; Kasono, K.; Hung, N.T.K.; Kuroki, M.; Ishikawa, S.-E.; Van, C.N.; Kawakami, M. Inhibition of tumor growth and angiogenesis by water extract of Gac fruit (*Momordica cochinchinensis* Spreng.). *Int. J. Oncol.* **2005**, *26*, 881–889. [CrossRef] [PubMed]
8. Zheng, L.; Zhang, Y.-M.; Zhan, Y.-Z.; Liu, C.-X. *Momordica cochinchinensis* seed extracts suppress migration and invasion of human breast cancer ZR-75-30 cells via down-regulating MMP-2 and MMP-9. *Asian Pac. J. Cancer Prev.* **2014**, *15*, 1105–1110. [CrossRef] [PubMed]
9. Yu, J.S.; Kim, J.H.; Lee, S.; Jung, K.; Kim, K.H.; Cho, J.Y. Src/Syk-Targeted Anti-inflammatory actions of *Triterpenoidal saponins* from Gac (*Momordica cochinchinensis*) seeds. *Am. J. Chin. Med.* **2017**, *45*, 1–15. [CrossRef] [PubMed]
10. Jung, K.; Chin, Y.-W.; Yoon, K.D.; Chae, H.-S.; Kim, C.Y.; Yoo, H.; Kim, J. Anti-inflammatory properties of a triterpenoidal glycoside from *Momordica cochinchinensis* in LPS-stimulated macrophages. *Immunopharmacol. Immunotoxicol.* **2013**, *35*, 8–14. [CrossRef] [PubMed]
11. Yu, J.S.; Roh, H.-S.; Lee, S.; Jung, K.; Baek, K.-H.; Kim, K.H. Antiproliferative effect of *Momordica cochinchinensis* seeds on human lung cancer cells and isolation of the major constituents. *Rev. Bras. Farmacogn.* **2017**, *27*, 329–333. [CrossRef]
12. Masayo, I.; Hikaru, O.; Tatsuo, Y.; Masako, T.; Yoshie, R.; Shuji, H.; Kunihide, M.; Ryuichi, H. Studies on the constituents of *Momordica cochinchinensis* Spreng. I. Isolation and characterization of the seed saponins, Momordica saponins I and II. *Chem. Pharm. Bull.* **1985**, *33*, 464–478.
13. Akihisa, T.; Tokuda, H.; Ichiishi, E.; Mukainaka, T.; Toriumi, M.; Ukiya, M.; Yasukawa, K.; Nishino, H. Anti-tumor promoting effects of multiflorane-type triterpenoids and cytotoxic activity of karounidiol against human cancer cell lines. *Cancer Lett.* **2001**, *173*, 9–14. [CrossRef]
14. Kan, L.; Hu, Q.; Chao, Z.; Song, X.; Cao, X. Chemical constituents of unsaponifiable matter from seed oil of *Momordica cochinchinensis*. *China J. Chin. Mater. Med.* **2006**, *31*, 1441–1444.
15. Desai, P.J.; Parikh, P. Extraction of natural products using microwaves as a heat source. *Sep. Purif. Rev.* **2010**, *39*, 1–32. [CrossRef]
16. Tatke, P.; Jaiswal, Y. An overview of microwave assisted extraction and its applications in herbal drug research. *Res. J. Med. Plant* **2011**, *5*, 21–31. [CrossRef]
17. Le, A.V.; Roach, P.D.; Nguyen, M.H.; Parks, S.E. The microwave- and ultrasonic-assisted aqueous extraction of bioactive compounds from Gac (*Momordica cochinchinensis* S.) seeds. In Proceedings of the 4th World Congress on Medicinal Plants and Natural Products Research, Osaka, Japan, 8–9 August 2018.
18. Nguyen, V.T.; Bowyer, M.C.; Van Altena, I.A.; Scarlett, C.J. Microwave-assisted extraction as an advanced technique for optimization of saponin yield and antioxidant potential from *Phyllanthus amarus*. *Sep. Sci. Technol.* **2017**, *52*, 2721–2731. [CrossRef]
19. Li, J.; Zu, Y.-G.; Fu, Y.-J.; Yang, Y.-C.; Li, S.-M.; Li, Z.-N.; Wink, M. Optimization of microwave-assisted extraction of *Triterpene saponins* from defatted residue of yellow horn (*Xanthoceras sorbifolia* Bunge.) kernel and evaluation of its antioxidant activity. *Innov. Food Sci. Emerg. Technol.* **2010**, *11*, 637–643. [CrossRef]
20. Chen, Y.; Xie, M.-Y.; Gong, X.-F. Microwave-assisted extraction used for the isolation of total *Triterpenoid saponins* from *Ganoderma atrum*. *J. Food Eng.* **2007**, *81*, 162–170. [CrossRef]
21. Kerem, Z.; German-Shashoua, H.; Yarden, O. Microwave-assisted extraction of bioactive *saponins* from chickpea (*Cicer arietinum* L.). *J. Sci. Food Agric.* **2005**, *85*, 406–412. [CrossRef]
22. Kwon, J.-H.; Belanger, J.M.; Pare, J.J.; Yaylayan, V.A. Application of the microwave-assisted process (MAP™☆) to the fast extraction of ginseng *saponins*. *Food Res. Int.* **2003**, *36*, 491–498. [CrossRef]
23. Food and Drug Administration. Guidance for Industry: Q3C—Tables and List. In *USHaH Services*; Food and Drug Administration: Silver Spring, MD, USA, 2012.
24. Cheok, C.Y.; Salman, H.A.K.; Sulaiman, R. Extraction and quantification of *saponins*: A review. *Food Res. Int.* **2014**, *59*, 16–40. [CrossRef]

25. Wimalasiri, D.; Piva, T.; Urban, S.; Huynh, T. Morphological and genetic diversity of *Momordica cochinchinenesis* (Cucurbitaceae) in Vietnam and Thailand. *Genet. Resour. Crop Evol.* **2016**, *63*, 19–33. [CrossRef]
26. Hiai, S.; Oura, H.; Nakajima, T. Color reaction of some *sapogenins* and *saponins* with vanillin and sulfuric acid. *Planta Med.* **1976**, *29*, 116–122. [CrossRef] [PubMed]
27. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical *Cation decolorization* assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]
28. Tan Vuong, Q.V.; Stathopoulos, C.E.; Parks, S.E.; Roach, P.D. Optimized aqueous extraction of *saponins* from bitter melon for production of a saponin-enriched bitter melon powder. *J. Food Sci.* **2014**, *79*, E1372–E1381.
29. Blois, M.S. Antioxidant determinations by the use of a stable free radical. *Nature* **1958**, *181*, 1199. [CrossRef]
30. Bhuyan, D.J.; Vuong, Q.V.; Chalmers, A.C.; van Altena, I.A.; Bowyer, M.C.; Scarlett, C.J. Investigation of phytochemicals and antioxidant capacity of selected Eucalyptus species using conventional extraction. *Chem. Pap.* **2016**, *70*, 567–575. [CrossRef]
31. Pham, H.N.T.; Bowyer, M.C.; van Altena, I.A.; Scarlett, C.J. Influence of solvents and novel extraction methods on bioactive compounds and antioxidant capacity of *Phyllanthus amarus*. *Chem. Pap.* **2016**, *70*, 556–566.
32. Ishida, B.K.; Turner, C.; Chapman, M.H.; McKeon, T.A. Fatty acid and carotenoid composition of Gac (*Momordica cochinchinensis* Spreng.) fruit. *J. Agric. Food Chem.* **2004**, *52*, 274–279. [CrossRef] [PubMed]
33. Le, A.V.; Roach, P.D.; Nguyen, M.H.; Parks, S.E. Optimisation of process parameters for supercritical carbon dioxide extraction of oil from Gac seed kernel powder. *Adv. J. Food Sci. Technol.* **2017**, *13*, 170–177. [CrossRef]
34. Müller, L.; Fröhlich, K.; Böhm, V. Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical scavenging assay. *Food Chem.* **2011**, *129*, 139–148. [CrossRef]
35. Aguilar-Garcia, C.; Gavino, G.; Baragano-Mosqueda, M.; Hevia, P.; Gavino, V.C. Correlation of tocopherol, tocotrienol, γ-oryzanol and total polyphenol content in rice bran with different antioxidant capacity assays. *Food Chem.* **2007**, *102*, 1228–1232. [CrossRef]

3.5. Anticancer and antioxidant activities of Gac seed extracts – the research paper

3.5.1. Bioactive composition, antioxidant activity, and anticancer potential of freeze-dried extracts from defatted Gac (*Momordica cochinchinensis* Spreng) seeds

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Bioactive Composition, Antioxidant Activity, and Anticancer Potential of Freeze-Dried Extracts from Defatted Gac (*Momordica cochinchinensis* Spreng) Seeds

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Abstract: Background: Gac (*Momordica cochinchinensis* Spreng) seeds have long been used in traditional medicine as a remedy for numerous conditions due to a range of bioactive compounds. This study investigated the solvent extraction of compounds that could be responsible for antioxidant activity and anticancer potential. **Methods:** Defatted Gac seed kernel powder was extracted with different solvents: 100% water, 50% methanol:water, 70% ethanol:water, water saturated butanol, 100% methanol, and 100% ethanol. Trypsin inhibitors, saponins, phenolics, and antioxidant activity using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the ferric reducing antioxidant power (FRAP) assays; and anticancer potential against two melanoma cancer cell lines (MM418C1 and D24) were analysed to determine the best extraction solvents. **Results:** Water was best for extracting trypsin inhibitors (581.4 ± 18.5 mg trypsin/mg) and reducing the viability of MM418C1 and D24 melanoma cells (75.5 ± 1.3 and 66.9 ± 2.2%, respectively); the anticancer potential against the MM418C1 cells was highly correlated with trypsin inhibitors ($r = 0.92$, $p < 0.05$), but there was no correlation between anticancer potential and antioxidant activity. The water saturated butanol had the highest saponins (71.8 ± 4.31 mg aescin equivalents/g), phenolic compounds (20.4 ± 0.86 mg gallic acid equivalents/g), and antioxidant activity, but these measures were not related to anticancer potential. **Conclusions:** Water yielded a Gac seed extract, rich in trypsin inhibitors, which had high anticancer potential against two melanoma cell lines.

Keywords: *Momordica cochinchinensis*; Gac; seeds; saponins; trypsin inhibitors; phenolics; anticancer; antioxidant; extraction; freeze dried extract

1. Introduction

Momordica cochinchinensis Spreng, commonly called Gac, is a plant species of the family *Cucurbitaceae*, which is also known as red melon, baby jackfruit, spiny bitter gourd, sweet gourd, and cochinchin gourd. It is native to Southeast Asia and is commonly grown as a food crop in Vietnam,

Thailand, Laos, Myanmar, and Cambodia [1,2]. From the food and commercial perspectives, the most commonly used part of the mature fruit is the red flesh surrounding the seeds, called the aril, which is traditionally used as a colourant in rice or, more recently, as a material that is processed into functional food ingredients or supplements [3].

The seeds are not eaten; they are removed from the aril and are mostly considered as waste [3,4]. However, in traditional medicine, Gac seeds are purported to have an array of therapeutic effects on a variety of conditions, such as fluxes, liver and spleen disorders, haemorrhoids, wounds, bruises, swelling, and pus [2,5]. In modern research, several constituents have been identified, which could be involved in the putative medicinal properties of Gac seeds, including trypsin inhibitors (e.g., MCoTI-I, MCoTI-II and MCoTI-III) [6–8], saponins (e.g., Momordica Saponin I and Momordica Saponin II) [9,10], and phenolic compounds (e.g., gallic acid and *p*-hydroxybenzoic acid) [11]. Gac seed extracts have been linked with many medicinal properties, such as gastroprotective [12–14], anti-inflammatory [15,16], anticancer [17], and antitumor [18] activities. Most of these properties are linked to the seed's saponins [12,13,15,16]. Furthermore, karounidiol, a triterpenoid compound, likely a saponin, present in Gac seeds [19], possess cytotoxic activity against human cancer cell lines [20]. Protease inhibitors, like the trypsin inhibitors in Gac seeds [7,8,21], have diverse biochemical functions [4,22], including acting as anticancer agents by inhibiting the growth of transformed cells [23–26]. Some trypsin inhibitors, such as those from *Cajanus cajan* and *Phaseolus limensis*, possess antioxidant, anti-inflammatory, and anti-bacterial activity [27].

There is limited information on how to best recover the bioactive compounds from Gac seeds, particularly to optimise anticancer potential. Efficient extraction and preservation methods are important for their immediate and long-term use. For any given plant bioactive, extractable yield depends on the extraction solvent, the chemical nature of the targeted component, and the characteristics of the extraction procedure. When other factors are kept constant, the extraction solvent plays a key role in obtaining the desired quality and quantity of the target constituents [28,29]. The choice of solvent is mainly based on the chemical properties of the targeted compounds, such as their polarity or hydrophobicity.

Due to the hydrophilic or amphiphilic nature of the trypsin inhibitors, phenolics, and saponins in Gac seeds, aqueous solvents and the low alcohols are likely to be the best extraction solvents for these bioactive compounds. They are also the safest and the most environmentally-friendly solvents for the extraction of bioactive compounds from plant materials [30,31]. For drying after solvent extractions, numerous methods have been developed; however, freeze drying is considered to be superior for preserving the medicinal qualities of botanical extracts and it is therefore widely used [32]. Additionally, to make it easier for the extraction of trypsin inhibitors [33] and to avoid interference of sticky oil during the freeze-drying procedure, the Gac seeds need to be defatted before they are extracted with solvents.

Thus, this study aimed to investigate the effectiveness of different solvents (water, alcohols, and mixtures) to extract the bioactive compounds of interest (trypsin inhibitors, saponins, and phenolics) from defatted Gac seed kernel powder. The relationships between the extracted compounds and the antioxidant activity and the anticancer potential of the extracts were also investigated.

2. Materials and Methods

2.1. Materials

2.1.1. Solvents, Reagents, and Chemicals

Solvents (ethanol, methanol and butanol) and chemicals (vanillin, sulphuric acid and potassium persulfate) were purchased from Merck (Bayswater, VIC, Australia). Folin-ciocalteu's phenol reagent, anhydrous sodium carbonate, sodium nitrile, ferric chloride, gallic acid, catechin, 2,4,6-tris(2-pyridyl)-s-triazine, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), aescin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

diammonium salt (ABTS), trypsin (type I) from bovine pancreas, benzyl-DL-arginine-para-nitroanilide (BAPNA), Tris, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (Castle Hill, NSW, Australia). Sodium acetate trihydrate was purchased from Government Stores Department (Sydney, NSW, Australia). Aluminium chloride was a product of J. T. Baker Chem. Co. (Thermo Fisher Scientific, North Ryde, NSW, Australia). Acetic acid was a product of BDH Laboratory Supplies (Bio-Strategy, Tingalpa, QLD, Australia). Sodium hydroxide was a product of Ajax FineChem (Thermo Fisher Scientific, North Ryde, NSW, Australia) and hydrochloride acid a product of Lab-Scan Ltd. (Bacto, Mt Pritchard, NSW, Australia).

Human melanoma MM418C1 (mutated BRAF oncogene), referred to as C1 melanoma cells, and D24 (wild type BRAF oncogene) and human keratinocyte (HaCat) cell lines were provided by the School of Health and Biomedical Sciences, RMIT University (Bundoora, VIC, Australia). RPMI-1640 media, streptomycin, and penicillin were Gibco products (Thermo Fisher Scientific, North Ryde, NSW, Australia). Fetal bovine serum (FBS) was from Serana (Melbourne, VIC, Australia).

2.1.2. Gac Seeds

Gac seeds, from accession VS7 as classified by Wimalasiri et al. [1], were collected from 450 kg of fresh Gac fruit. These fruits were bought at Gac fruit fields in Dong Nai province, Ho Chi Minh City, Vietnam (Latitude: 10.757410; Longitude: 106.673439). After their separation from the fresh fruit, the seeds were vacuum dried at 40 °C for 24 h to reduce moisture and increase the crispness of the shell, which facilitated shell removal. The dried seeds were de-coated to get the kernels, which were then packaged in vacuum-sealed aluminium bags and stored at −18 °C until used.

Preparation of Defatted Gac Seed Kernel Powder

Defatted Gac seed kernel powder was prepared as described by Le et al. [34]. Briefly, the Gac seed kernels were ground in an electric grinder (100 g ST-02A Mulry Disintegrator, Taiwan Machinary, Sydney, NSW, Australia) to a powder that could pass through a sieve of 1.4 mm. The powder was then freeze-dried using a Dynavac FD3 Freeze Dryer (Dynapumps, Seven Hills, NSW, Australia) for 48 h, at −45 °C under vacuum at a pressure loading of 10^{-2} mbar (1 Pa), to reduce the moisture content to 1.21 ± 0.02%. The powder was then defatted using three 30-min extractions with hexane at a powder to hexane ratio of 1:5 w/v. The resulting slurry was suction filtered and the residue (defatted meal) was air-dried for 12 h and stored in a desiccator at ambient room temperature until used. The moisture content of the defatted powder, measured using a Shimadzu MOC63u moisture analyser (Rydalmere, NSW, Australia), was 8.61 ± 0.15%.

2.2. Methods

2.2.1. Extraction

Based on a previous study [34], six solvents (deionised water, 100% methanol, 50% methanol in water, 100% ethanol, 70% ethanol in water, and 90% *n*-butanol in water), were used for the extraction of bioactive compounds from the defatted and dried Gac seed kernel powder. Twenty grams of the powder were added to 400 mL of each solvent and the suspension was kept under constant magnetic stirring for 30 min at 40 ± 1 °C. Following extraction, the mixtures were filtered through two layers of cheese cloth and then through a Whatman No. 1 filter paper (Thermo Fisher Scientific, North Ryde, NSW, Australia), and the clear filtrates were collected in 500 mL evaporating flasks. Triplicate extractions were done for each solvent.

2.2.2. Freeze Drying Extracts

The filtrates collected from the extractions were freeze-dried into powders as summarized in Figure 1. First, the liquid extracts were concentrated using a rotary evaporator (Buchi Rotavapor B480, Buchi Australia, Noble Park, VIC, Australia) at 40 °C under vacuum until thick but not completely

dried in the 500 mL evaporating flasks. Then, to transfer the extracts into pre-weighed 100 mL evaporating flasks, three different solvents were used. For each of the butanol, methanol, and 50% methanol extracts, 50 mL of 50% methanol in water was used, for each of the ethanol and 70% ethanol extracts, 50 mL of 50% ethanol in water was used, and for the water extract, 30 mL of water was used. The suspensions were mixed well, and the evaporation resumed until around 20 mL of the concentrated extracts were left. The concentrates were then frozen using liquid nitrogen before freeze drying with a BenchTop Pro freeze dryer (Scitek, Lane Cove, NSW, Australia) at −60 °C and 30 mbar for 48 h. The flasks with residue were placed in a desiccator and quickly weighed. The difference in weight between the flasks with residue and the empty flasks was taken to be the mass recovered for each extract. These freeze dried (FD) crude extracts were then stored in air-tight containers at −20 °C for use within 3 months.

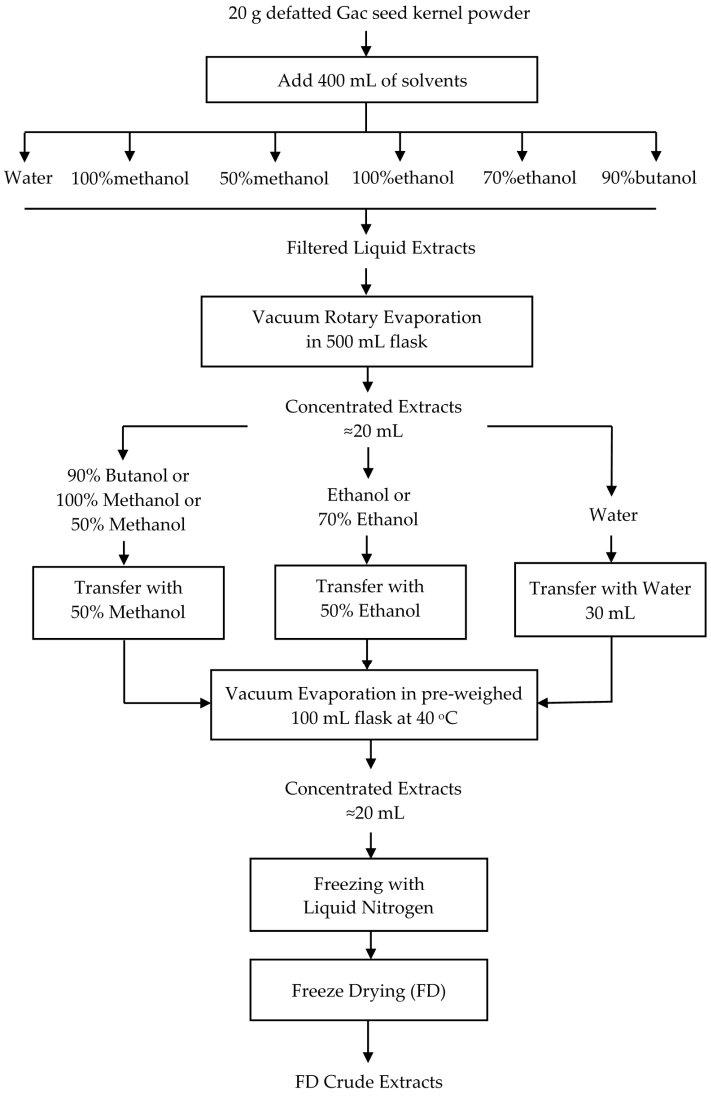

Figure 1. Procedure for producing freeze dried (FD) crude extracts from defatted Gac seed kernel powder.

2.2.3. Determination of Extractable Yield

To determine the extractable yield, 10 mL of each filtered liquid extract, resulting from Section 2.2.1, was transferred into a tared flat-bottomed glass vial and then dried at 105 °C with a vacuum pressure of 60 kPa for 24 h in a vacuum oven (Thermoline, Wetherill Park, NSW, Australia) until a constant weight was achieved. These vials were cooled in a desiccator for 30 min and weighed. The extractable yield was calculated, in g dried extract per 100 g of dried defatted Gac seed kernel powder, using Equation (1),

where EY was the extractable yield, DE (g) was the mass of dried extract after the vacuum oven drying, 40 was the ratio of the 10 mL used for the vacuum oven drying to the 400 mL originally used for the extract, and DS (g) was the mass of dried defatted Gac seed kernel powder used for the extraction.

$$\text{EY (g/100 g)} = \frac{\text{DE} \times 40}{\text{DS}} \times 100 \quad (1)$$

2.2.4. Determination of Dry Mass Yield

Dry mass yield was defined as the amount (g) of FD crude extract, produced as described in Section 2.2.2, per 100 g of dried defatted Gac seed kernel powder. Equation (2) was used to calculate the dry mass yield (DM), in which FD (g) was the weight of the FD crude extract, DS (g) was the mass of dried defatted Gac seed kernel powder used for the extraction, and V was the volume of the filtrate collected after extraction.

$$\text{DM (g/100 g)} = \frac{\text{FD} \times \text{V}/(\text{V} - 10)}{\text{DS}} \times 100 \quad (2)$$

2.2.5. Determination of Trypsin Inhibitor Activity (TIA)

The TIA assay was performed as described by Makkar et al. [35] except that the absorbance was measured at 385 nm, as suggested by Stauffer [36], instead of at 410 nm.

Reagent Preparation

Substrate solution: A substrate solution of 92 mM BAPNA was prepared as follows. First, 40.0 mg BAPNA was dissolved in 1.00 mL DMSO and diluted to 100 mL with 0.05 M Tris-buffer (pH 8.2) containing 0.02 M $CaCl_2$ pre-warmed to 37 °C. This solution was prepared daily and kept at 37 °C while in use.

Trypsin solution: 20.0 mg of trypsin (type I) from bovine pancreas was dissolved in 1 mM HCl to make 1 L and stored at 4 °C for use within a week. In the analytical procedure with 92 mM BAPNA, this solution gave an absorbance value in the range of 0.900 ± 0.010 after subtracting the reagent blank at 385 nm.

Determination of TIA

Each FD crude extract from Section 2.2.2 (Figure 1) was dissolved in water at a concentration to give an inhibition of Trypsin between 40% and 60% and the assay was setup as shown in Table 1 with four test tubes prepared for each FD crude extract. All the prepared test tubes were kept in a water bath at 37 °C for 10 min to promote the formation of an enzyme–inhibitor complex and then 5.0 mL of BAPNA solution, pre-warmed to 37 °C, was added into each tube and the tubes were incubated in a water bath at 37 °C for 10 min. One mL of 30% acetic acid solution was added to each tube to stop the reaction. Then, 2.0 mL of trypsin solution was added into each reagent and sample blank (Table 1). After thorough mixing, the absorbance of the reaction mixture due to the release of *p*–nitroaniline was measured at 385 nm.

Table 1. The trypsin inhibitor activity assay setup.

Component	Reagent Blank (a)	Standard (b)	Sample Blank (c)	Sample (d)
Deionised water (mL)	2	2	1	1
Trypsin solution (mL)	-	2	-	2
Diluted extract (mL)	-	-	1	1
BAPNA (mL)	5	5	5	5
Acetic acid (mL)	1	1	1	1
Trypsin solution after reaction inactivation (mL)	2	-	2	-

BAPNA (benzyl-DL-arginine-para-nitroanilide) was added to start the reaction and acetic acid for inactivation.

Calculation

The change in absorbance (A_I) due to the trypsin inhibitor per mL of diluted extract was $(A_b - A_a) - (A_d - A_c)$, where the subscripts referred to tubes (a) to (d) in Table 1. Since 1 µg of trypsin gave an absorbance of 0.0190, the weight of trypsin inhibited per mL of extract was $A_I/0.019$ µg. From this value, The TIA of the FD crude extracts was calculated using Equation (3) and expressed as mg of pure trypsin inhibited per mg of FD crude extract.

$$\text{TIA} = \frac{A_I}{0.019 \times S \times \left(1 - \frac{m\%}{100}\right)} \quad (3)$$

where,

A_I: Change in absorbance due to inhibition per 1 mL of extract;
$A_I = (A_b - A_a) - (A_d - A_c)$, subscripts as per Table 1;
S: Weight (mg) of the FD crude extract dissolved in 1 mL;
m%: Moisture content of the FD crude extract powder.

2.2.6. Determination of Total Saponin Content (TSC)

The FD crude extracts from Section 2.2.2 were dissolved in water at a concentration of 2 mg/mL and vortexed before the TSC was determined according to Tan et al. [37] with some modifications. Briefly, 0.25 mL of each extract was mixed with 0.25 mL of 8% (w/v) vanillin solution and 2.5 mL of 72% (v/v) sulphuric acid. The mixture was vortexed and incubated in a water bath at 60 °C for 15 min and then cooled on ice for 10 min. The absorption of the mixture was measured at 560 nm using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Mulgrave, VIC, Australia). Aecsin was used as a standard and the results were expressed as milligram aecsin equivalents (AE) per gram of the FD crude extract powder (mg AE/g).

2.2.7. Determination of Total Phenolic Content (TPC)

The FD crude extracts from Section 2.2.2 were dissolved in water at a concentration of 2 mg/mL and vortexed before the TPC was determined according to the method of Tan et al. [38] with some modifications. Briefly, 0.5 mL of each extract was mixed with 2.5 mL of 10% (v/v) Folin–Ciocalteu reagent in water and incubated at room temperature for 2 min to equilibrate. Then, 2 mL of 7.5% (w/v) sodium carbonate solution in water was added and the mixture was incubated at ambient temperature for 1 h. The absorption of the reaction mixture was measured at 765 nm using a Cary 60 UV-Vis spectrophotometer. Gallic acid was used as a standard and the results were expressed as milligram gallic acid equivalents (GAE) per gram dry weight of the FD crude extract powder (mg GAE/g).

2.2.8. Determination of Antioxidant Capacity

The FD crude extracts from Section 2.2.2 were dissolved in water at the concentration of 2 mg/mL and mixed before analysing antioxidant capacity using three assays: the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the ferric reducing antioxidant power (FRAP).

DPPH

The DPPH assay measures the total free radical scavenging capacity of the extracts. The assay was performed as described by Tan et al. [38]. A stock solution of 0.6 M DPPH in methanol was prepared and kept at −20 °C until use. The working solution was prepared by mixing 10 mL of stock solution with 45 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm using a spectrophotometer. Each extract (0.15 mL) was mixed with 2.85 mL of the working solution and the mixture was allowed to stand for 3 h, after which the absorption was measured at 515 nm using a

Cary 60 UV-Vis spectrophotometer. Trolox was used as a standard and the results were expressed as milligram Trolox equivalents (TE) per gram of the FD crude extract powder (mg TE/g).

ABTS

The ABTS assay measures the total free radical scavenging capacity of the extracts. The assay was performed as described by Tan et al. [38] with slight modifications. Stock solutions of 7.4 mM ABTS and 2.6 mM potassium persulfate were freshly prepared or kept at 4 °C in a dark bottle for use within a month, respectively. A fresh working solution was prepared for each assay by mixing equal quantities of the two stock solutions and incubated for 15 h to 16 h in the dark at ambient temperature. Then, 1 mL of the working solution was diluted with approximately 30 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a Cary 60 UV-Vis spectrophotometer. Each extract (0.15 mL) was mixed with 2.85 mL of the working solution and the mixture was incubated for 2 h in the dark at ambient temperature. The absorption of the reaction mixture was measured at 734 nm using a Cary 60 UV-Vis spectrophotometer. Trolox was used as a standard and the results were expressed as milligram Trolox equivalents (TE) per gram of the FD crude extract powder (mg TE/g).

FRAP

The FRAP assay measures the ferric reducing power. The assay was performed following the method of Thaipong et al. [39], based on the increase in absorbance at 593 nm. A fresh FRAP working solution was initially prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM iron reagent (TPTZ) in 40 mM HCl, and 20 mM $FeCl_3 \bullet 6H_2O$ in the ratio of 10:1:1 ($v/v/v$). The fresh working solution was warmed to 37 °C before using. Each extract (0.15 mL) was mixed with 2.85 mL of the working FRAP solution and the mixture was incubated at ambient temperature in the dark for 30 min before its absorbance was measured at 593 nm using a Cary 60 UV-Vis spectrophotometer. Trolox was used as a standard and the antioxidant capacity of each sample, based on its ability to reduce ferric ions, was expressed as milligram Trolox equivalents (TE) per gram of the FD crude extract powder (mg TE/g).

2.2.9. Determination of Cytotoxicity

Cell Lines and Culture

The human melanoma MM418C1 (C1, wild type BRAF oncogene) and D24 (mutated BRAF oncogene) cell lines were maintained in RPMI-1640 media supplemented with 10% (v/v) FBS, 1% (v/v) streptomycin and penicillin at 37 °C in 5% CO_2. HaCat keratinocytes were used as normal untransformed cells and grown in the same media.

In Vitro Cytotoxicity Assay

All cells were seeded in 96 well plates (Greiner Bio-One, Labfriend, Sydney, NSW, Australia), 5000 cells/well along with 100 µL of fresh media. The FD crude extracts from Section 2.2.2 were dissolved in RPMI-1640 cell culture media at a concentration of 2 mg/mL and UV-sterilised for 10 min in a laminar flow hood before use on cells. The cells were allowed to attach for 4 h before being treated with 10 µL of the extract and incubated for 48 h.

The effect of the extracts on cell growth was determined using the CCK-8 (Cell Counting Kit-8) assay (Sigma-Aldrich, St Louis, MO, USA). The assay measured cytotoxicity based on the conversion of a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-8), to a water-soluble formazan dye upon reduction by dehydrogenases in the presence of an electron carrier [40].

To determine cell viability, 10 µL of CCK-8 solution was added to each well of the 96-well plate containing treated and control samples. The plates were incubated at 37 °C for 2 h and the absorbance was measured spectrophotometrically at 450 nm using a CLARIOstar® High Performance Monochromator Multimode Microplate Reader (BMG LABTECH, Mornington, NSW, Australia) and

the results were analysed using the MARS data analysis software (version 3.00R2, BMG LABTECH, Mornington, NSW, Australia). The data were presented as a proportional viability (%) by comparing the treated cells with the untreated cells (control) using Equation (4):

$$\text{Cell viability} = \frac{At - Ab}{Ac - Ab} \times 100 \quad (4)$$

where *At* was the absorbance value of the treated cells, *Ab* was the absorbance of CCK-8 only, and *Ac* was the negative control which included cells and CCK-8 only. Two types of controls were used: the media control consisted of cultured cells in 10% (*v*/*v*) FBS containing medium alone and the vehicle control consisted of cells in 10% (*v*/*v*) FBS containing medium, to which 10 µL of RPMI-1640 media without FBS was added. However, as both controls did not cause cytotoxicity, the media control was used to calculate cell viability.

Cell morphology was analysed at 48 h using a Nikon Eclipse TS100 (Nikon, Tokyo, Japan) phase contrast inverted microscope and the images were captured using a Nikon DS-Fi1digital camera.

2.2.10. Statistical Analyses

Extractions were performed in triplicate and means ± standard deviation (SD) were assessed with the one-way Analysis of Variance (ANOVA) and Tukey's *Post Hoc* Multiple Comparisons test using the IBM SPSS Statistics 24 program (IBM Corp., Armonk, NY, USA). Differences in means were considered statistically significant at $p < 0.05$. Correlations and their significance were determined using the Microsoft Excel 2016 (Microsoft Corp., Seattle, WA, USA) and Principle Component Analysis (Minitab 17.1.0, Sydney, NSW, Australia).

3. Results

3.1. Effect of Solvent on the Extractable Yield and the Dry Mass Yield

Extractable yield and dry mass yield were calculated for each extract. The extractable yields ranged from 4.4 g/100 g for the ethanol extract to 15.5 g/100 g for the aqueous extract while the dry mass yield ranged from 3.7 g/100 g for the ethanol extract to 13.1 g/100 g for the aqueous extract (Table 2). For both the extractable yield and the dry mass yield, the values were substantially higher for the aqueous extracts than for the organic solvents, whether they were mixed with water or not. Furthermore, the extractable yield (before drying) was higher than the dry mass yield (after freeze drying) for all extracts. The observed loss during the drying process was highest for the 70% ethanol extract (−31%) followed by the methanol extract (−28%) and the butanol extract (−20%). The aqueous and ethanol extracts had the same loss (−15%), while the lowest lost was for the 50% methanol extract (−11%).

Table 2. Effect of solvent on the extractable yield (EY) and the dry mass yield (DM).

Solvent	EY (g/100 g)	DM (g/100 g)	Original Volume (mL)	Collected Volume (mL)	Yield Loss (%)
Water	15.5 ± 0.1 ^{a}	13.1 ± 0.1 ^{b}	400 ± 3	355 ± 0	15.3 ± 0.1 ^{d}
50% Methanol	10.2 ± 0.0 ^{c}	9.1 ± 0.3 ^{d}	400 ± 3	348 ± 3	10.9 ± 0.2 ^{f}
70% Ethanol	10.2 ± 0.0 ^{c}	7.1 ± 0.4 ^{e}	400 ± 3	340 ± 5	30.6 ± 0.3 ^{a}
90% Butanol	6.9 ± 0.1 ^{e}	5.5 ± 0.1 ^{f}	400 ± 3	350 ± 0	19.9 ± 0.1 ^{c}
Methanol	6.7 ± 0.2 ^{e}	4.8 ± 0.2 ^{g}	400 ± 3	330 ± 0	27.8 ± 0.2 ^{b}
Ethanol	4.4 ± 0.0 ^{g}	3.7 ± 0.0 ^{h}	400 ± 3	345 ± 0	14.9 ± 0.0 ^{e}

The values were the means ± SD of three replicate extractions for each solvent. All the values for EY and DM were compared to each other, the values for yield loss were compared separately and the values not sharing the same superscript letter for EY and DM, and separately for yield loss, were significantly different at $p < 0.05$.

3.2. Effect of Solvents on the Content of Bioactive Compounds

3.2.1. Trypsin Inhibitors

The yield of trypsin inhibitors was measured as the trypsin inhibitor activity (TIA) of the FD crude extracts (Figure 2). The water extract had the highest TIA (581.4 mg/mg) and the yield decreased relative to the concentration of water in the water and low alcohol mixtures: −40% for the 50% methanol (≈50% water) extract, −54% for the 70% ethanol (≈30% water) extract and −97% for the water-saturated butanol (≈10% water) extract. The 100% methanol and 100% ethanol extracts had much lower TIA values than the water extract, −96% and −95%, respectively (Figure 2). Therefore, water was the best solvent for extracting trypsin inhibitors from the defatted Gac seed kernel powder.

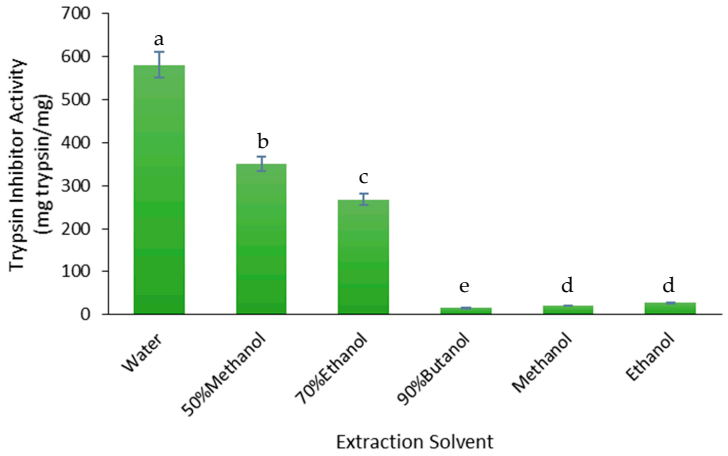

Figure 2. Effect of solvent on the trypsin inhibitor activity (TIA) of the FD crude extracts. The values are the means ± SD of three replicate extractions for each solvent. Columns not sharing the same letter are significantly different at $p < 0.05$.

3.2.2. Saponins

The yield of saponins was measured as the total saponin content (TSC) of the FD crude extracts (Figure 3). The highest TSC was found in the butanol and methanol extracts, which were 24% higher than the TSC in the 50% methanol and 70% ethanol extracts, 48% higher than the ethanol extract, and 53% higher than the water extract. Therefore, butanol and methanol were the best solvents for extracting the saponins from the defatted Gac seed kernel powder.

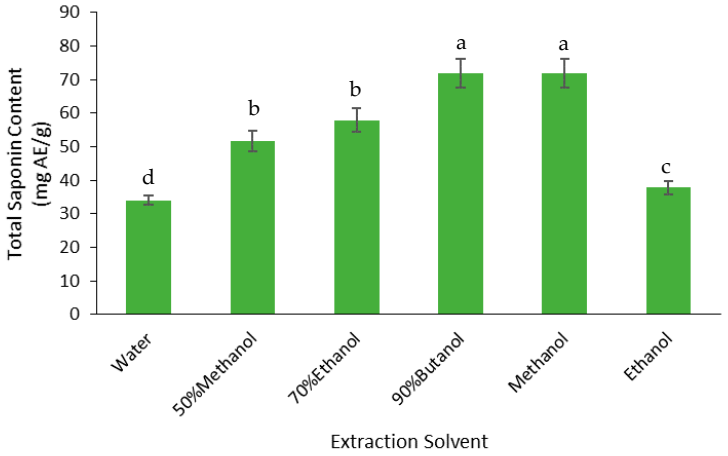

Figure 3. Effect of solvent on the total saponin content (TSC) extraction from Gac seed. The values were the means ± SD of three replicate extractions for each solvent. Columns not sharing the same letter were significantly different at $p < 0.05$.

3.2.3. Phenolics

The yield of phenolics was measured as the total phenolic content (TPC) of the FD crude extracts (Figure 4). The highest TPC was found in the butanol extract; the TPC in this extract was 13% higher than the water extract, 29% higher than the 50% methanol and 70% ethanol extracts, 35% higher than the methanol extract, and 63% higher than the ethanol extract. Therefore, butanol extracted the highest phenolics from the defatted Gac seed kernel powder.

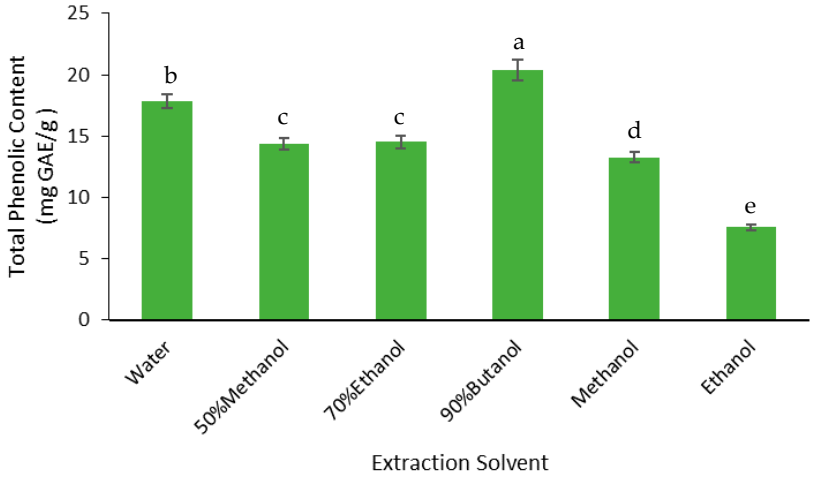

Figure 4. Effect of solvent on the total phenolic content (TPC) of the FD Gac seed crude extracts. The values were the means ± SD of three replicate extractions for each solvent. Columns not sharing the same letter were significantly different at $p < 0.05$. GAE, Gallic acid equivalents.

3.3. Effect of Solvents on Antioxidant Activity

The antioxidant activity of the extracts was measured using three assays: DPPH, ABTS, and FRAP. The butanol extract gave the highest values for ABTS and DPPH assays and the ethanol extract gave the highest value for FRAP assay (Figure 5). These two extracts produced significantly higher antioxidant activity based on the ABTS, DPPH, and FRAP assays (Figure 5).

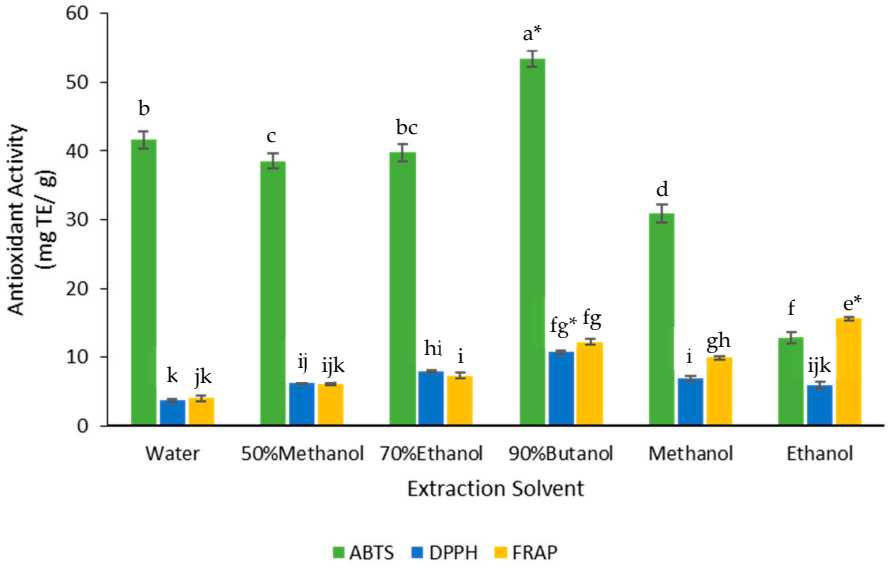

Figure 5. Effect of solvent on the antioxidant capacity of the FD crude extracts. The values were the means ± SD of three replicate extractions for each solvent. Columns not sharing the same superscript letter were significantly different at $p < 0.05$. * indicated the highest antioxidant activity. TE, Trolox equivalents.

3.4. Effect of Extraction Solvent on Cancer Cell Viability

The FD crude extracts were tested for cell toxicity using two melanoma cell lines (D24 and C1) and a normal keratinocyte line (HaCat). The water extract was the most cytotoxic towards both cancer cell lines compared to the other extracts (Figure 6); the extract decreased the cell viability by 67% for D24 and 75% for C1 melanoma cells. The 70% ethanol and 50% methanol extracts also showed cytotoxic activity towards the C1 melanoma cells, decreasing their viability by 69% and 46%, respectively, while the butanol, methanol, and ethanol extracts had no effect. Besides the water extract, no other FD crude extract had any effect on the viability of the D24 melanoma cells.

In contrast, all the FD crude extracts decreased the cell viability for the normal keratinocytes (HaCat). Except for the ethanol extract, which only decreased the HaCat cell viability by 24%, all the extracts reduced the viability by between 64% (water) and 75% (methanol).

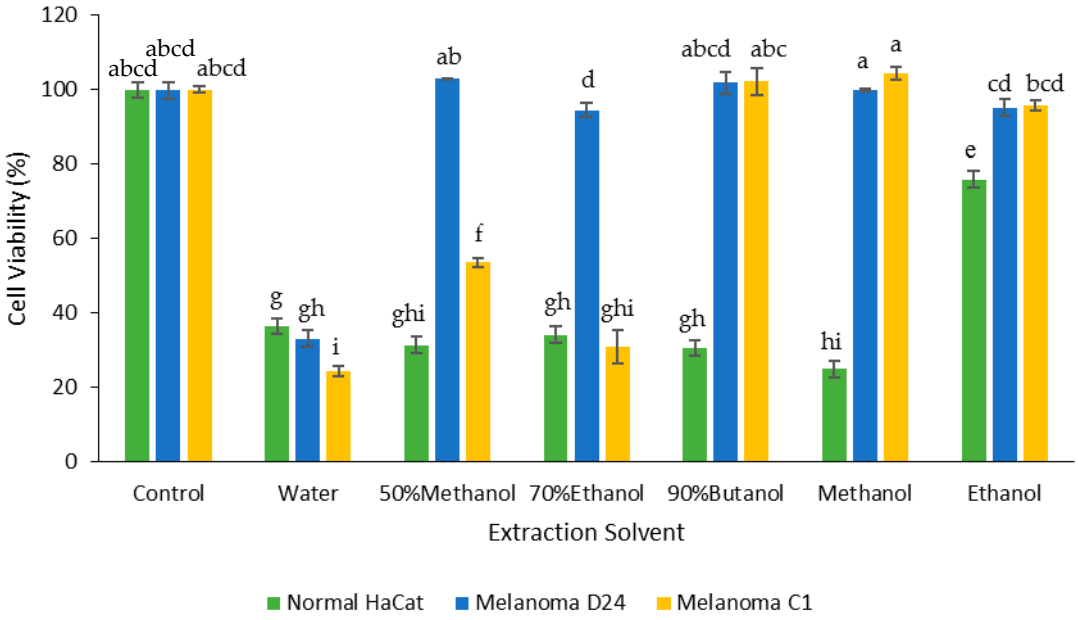

Figure 6. Effect of the FD crude extracts prepared with the different solvents on the cell viability of normal (HaCat) and melanoma (D24 and C1) cell lines after 48 h treatment. The values were the means ± SD of three replicate extractions for each solvent. Columns not sharing the same letter were significantly different at $p < 0.05$.

These results were consistent with the cellular changes observed using a phase contrast microscope. As seen in Figure 7, the control untreated HaCat keratinocytes were firmly attached with flattened oblong shapes (Figure 7A), the D24 melanoma cells were lightly attached with advanced elongations (Figure 7B), and the C1 melanoma cells were firmly attached and tightly packed with elongated processes (Figure 7C). In comparison to the untreated cells (Figure 7A–C), exposure of the cells to the various Gac seed extracts induced typical changes of cell death, such as cytoplasmic condensation, detached cells (red arrows in Figure 7), and cell disruption, to form apoptotic bodies (blue arrows in Figure 7).

The cellular changes observed with the water extract (Figure 7D–F) were consistent with the viability results (Figure 6) in that signs of morphological changes were seen for all three cell types. The 50% methanol and 70% ethanol extracts also caused substantial morphological changes to the C1 melanoma cells (Figure 7I,L) and HaCat cells (Figure 7G,J). The butanol and methanol extracts also caused substantial morphological changes to the HaCat cells (Figure 7M,P). In contrast, the ethanol extract had the least effect on the morphology of all three cells (Figure 7S–U) akin to controls.

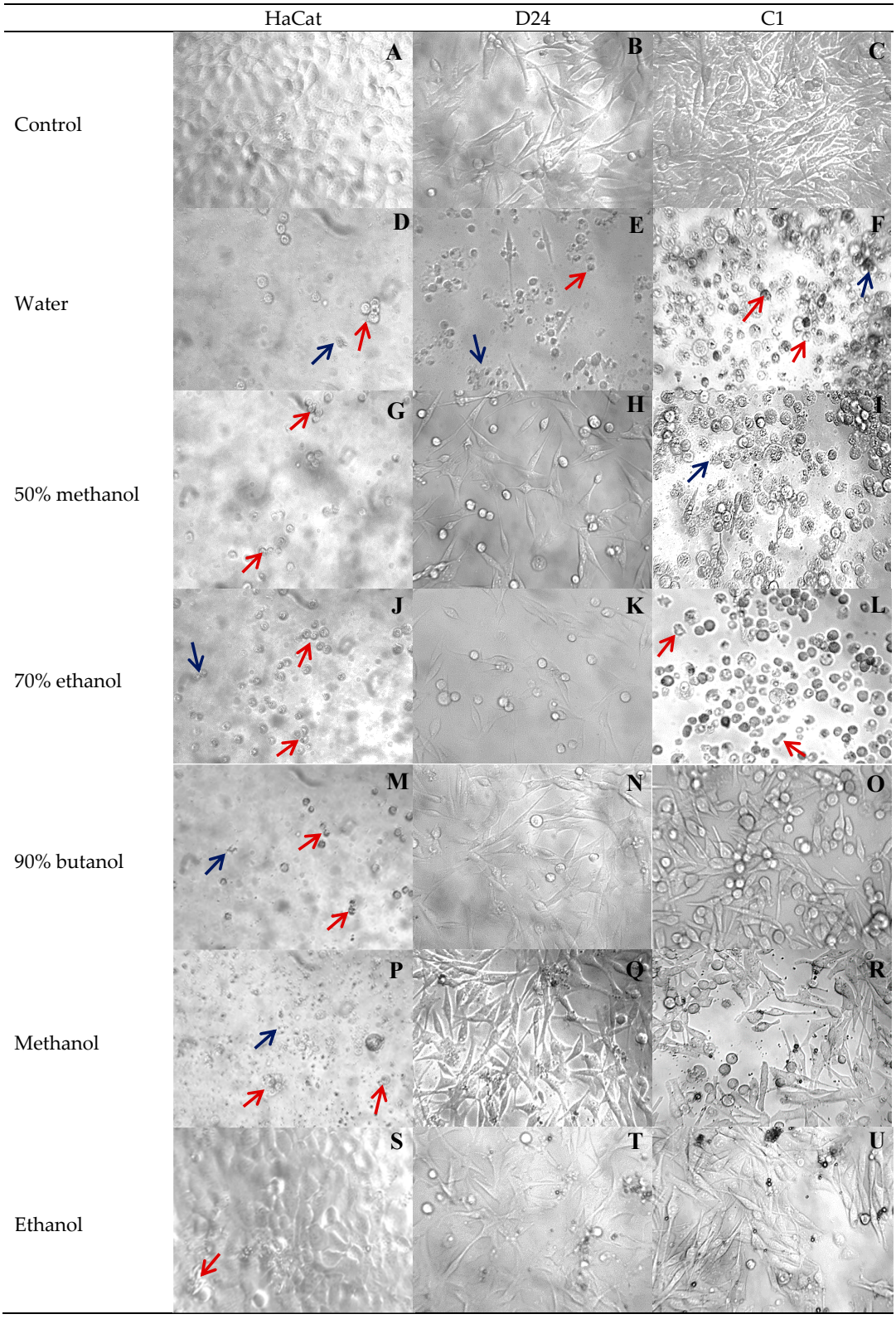

Figure 7. Morphological effects of different extraction solvents of Gac seed on HaCat (control) and melanoma D24 and C1 cell lines observed under a phase contrast microscope after 48 h treatment. Cytotoxicity is indicated by red arrows pointed to condensation and detached cells; blue arrows pointed to apoptotic bodies. Magnification: 100×.

The most sensitive cells were HaCat keratinocytes, while the least sensitive were the D24 melanoma cells, which had the least morphological changes when treated with all the solvent extracts, except for the water extract (Figure 7).

3.5. Correlations between Extract Yields, Bioactive Compounds, Antioxidant Activity, and Cancer Cell Viability across the FD Crude Extracts

TIA of the extracts was strongly and positively correlated with the extractable yield and the dry mass yield (Table 3, Figure 8). Essentially, the more material extracted, the higher the TIA, with the water extract having the highest values for both. TIA and extracted yields were also negatively correlated with the FRAP antioxidant activity and the viability of the C1 melanoma cells (Table 3, Figure 8). For example, almost 85% (r^2) of the variability in the C1 cell viability could be explained by variability in the TIA. In contrast, the C1 cell viability was positively correlated with the FRAP antioxidant activity. Therefore, the negative effect of the water extract on the C1 cell viability may be related to its high TIA but not FRAP activity.

Table 3. Coefficient of correlations (r) between yields, bioactive compounds, antioxidant activity, and cell viability across the Gac seed crude extracts.

	Yield		**Bioactive Compound**			**Antioxidant Activity**			**Cell Viability**		
	EY	**DMY**	**TIA**	**TSC**	**TPC**	**ABTS**	**DPPH**	**FRAP**	**HaCat**	**D24**	**C1**
EY	1.00										
DMY	0.98 †	1.00									
TIA	0.96 ‡	0.97 ‡	1.00								
TSC	−0.42	−0.50	−0.61	1.00							
TPC	0.51	0.49	0.31	0.36	1.00						
ABTS	0.48	0.43	0.29	0.44	0.97 †	1.00					
DPPH	−0.52	−0.58	−0.66	0.81 §	0.37	0.46	1.00				
FRAP	−0.93 ‡	−0.89 §	−0.88 §	0.19	−0.48	−0.50	0.44	1.00			
HaCat	−0.42	−0.35	−0.22	−0.63	−0.73	−0.78	−0.28	0.63	1.00		
D24	−0.79	−0.80	−0.77	0.65	−0.28	−0.13	0.69	0.56	−0.01	1.00	
C1	−0.88 §	−0.83 §	−0.92 §	0.53	−0.23	−0.26	0.50	0.83 §	0.18	0.61	1.00

† $p < 0.001$, ‡ $p < 0.01$, § $p < 0.05$, EY: Extraction yield; DMY: Dry mass yield; TIA: Trypsin inhibitor activity; TSC: Total saponin content; TPC: Total phenolic content; ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay; DPPH: 2,2-diphenyl-1-picrylhydrazyl assay; FRAP: ferric reducing antioxidant power assay.

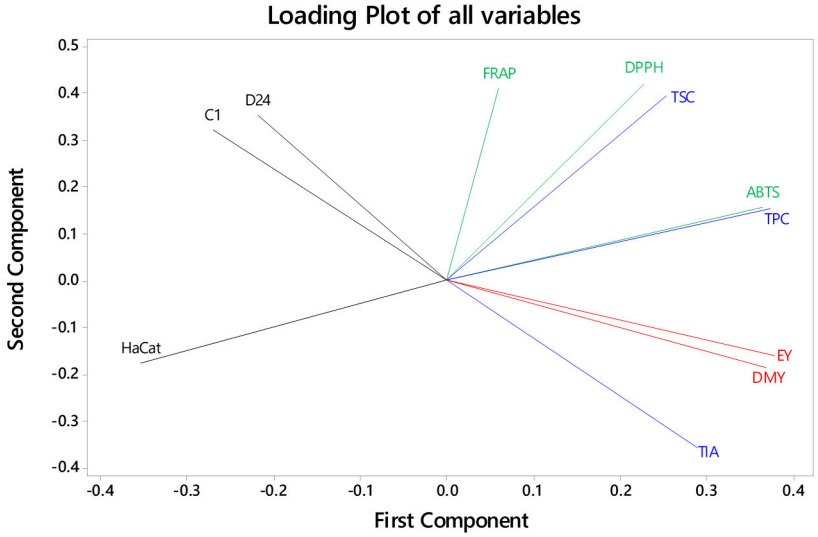

Figure 8. Correlations between extraction yields (red lines), bioactive compounds (blue lines), antioxidant activity (green lines), and cell viability (black lines) from Gac seed crude extracts.

There were positive correlations between the TSC with DPPH antioxidant activity, and TPC with ABTS antioxidant activity (Table 3, Figure 8). However, there were no significant correlations between these bioactive compounds and any other variables, including the effects of the extracts on cytotoxicity for both C1 and D24 cancer cells (Table 3, Figure 8). Furthermore, there were no other significant correlations between any of the other measured parameters.

4. Discussion

Gac seeds have long been used in traditional medicine as a remedy for numerous conditions. Several bioactive constituents have been identified in Gac seeds, such as trypsin inhibitors, saponins, and phenolics. In this study, the extraction of these bioactive constituents with different solvents was investigated and their relationships with antioxidant activity and anticancer potential were explored. The results revealed that the water extract had the highest anticancer potential, which may be related to its high content of trypsin inhibitors but not to its antioxidant activity.

The water extract from defatted Gac seeds had the highest anticancer potential, reducing the growth of the melanoma cells compared to the control. This is consistent with the finding that a water extract from Gac seeds was the best extract for supressing the migration and invasion of a breast cancer cell line [18]. Water extracts from Gac aril have also been shown to suppress the viability of colon, liver [17], and melanoma [41] cancer cell lines. Compared to the previous study [17], where the Gac aril water extract at 1.24 mg/mL inhibited colon and liver cancer cells by 38 and 45%, respectively, in the present study, the water extract at the much lower concentration of 0.2 mg/mL had a significantly higher anticancer activity of 67% and 75% for the D24 and C1 melanoma cells, respectively. This suggests that water is not only safe and inexpensive, but it is also a highly efficient solvent for extracting compounds with anticancer potential from Gac, especially Gac seeds. The use of water as the extraction solvent also means that the methodology is widely accessible to more people, particularly in rural and underdeveloped countries that do not have access to organic solvents and processing facilities.

The water extract also had the highest TIA value, which was likely due to the presence of trypsin inhibitor proteins because Gac seeds are known to contain trypsin inhibitor peptides that are soluble in buffered aqueous solvents [31,33,42]. The strong inverse correlation between TIA and the viability of the C1 melanoma cells also suggests that trypsin inhibitors were involved in the water extract's anticancer potential against these cells. The known Gac seed trypsin inhibitors have a low molecular weight of 3–4 kDa with a compact cyclic conformation [6,21], which can make it easy for them to penetrate into cancer cells and illicit cytotoxicity [43]. However, this is also consistent with the known anticancer potential of bigger proteins from Gac aril (35 kDa) [17] and other seeds, such as soybeans [44], which has been studied extensively [26,45–47].

However, although the water extract was the only extract to decrease the viability of the D24 melanoma cells, an inverse correlation between TIA and the viability of the D24 cells was not seen ($p = 0.07$) mainly because the 50% methanol and the 70% ethanol extracts had no activity against this cell line while they had activity against the C1 melanoma cell line. This could be due to the D24 cells, which have a mutated BRAF oncogene, being more resistant [48] to the presence of trypsin inhibitors, and therefore, they could only be killed when the trypsin inhibitors reach a high enough concentration, for example, in the water extract compared to the lower concentrations in the other extracts. However, it could also be that the D24 cells, and maybe also the C1 cells, were affected by other water soluble constituents of the Gac seeds, for example cyclotides which do not have trypsin inhibition activity [6]. For example, although many Gac seed trypsin inhibitors are cyclotides, MCoCC-1, a 3.3 kDa cyclotide unique to Gac seeds, which does not have trypsin inhibitor activity, has been shown to exhibit high cytotoxicity against the human melanoma MM96L cell line [6]; cell survival was decreased 43% in the presence of 2 μM MCoCC-1. The TIA is a measure of trypsin inhibitors, which may be cyclotides because they are found in Gac seeds [21], but other cyclotides are also present in Gac seeds that are

purported to have anticancer potential [6,22]; however, these may not have antitrypsin activity and therefore would not be measured by the TIA.

The higher antioxidant activity of the butanol extract was likely due to its high content of saponin and phenolic compounds; this solvent had the highest TSC and TPC values among the extracts. There were strong positive correlations between saponins with DPPH antioxidant activity and phenolics with ABTS antioxidant activity, but neither saponins nor phenolic compounds were related to the FRAP antioxidant activity. Therefore, the extracted Gac seed saponins and phenolic compounds acted as antioxidants through the mechanism of scavenging the free radicals produced by DPPH and ABTS [39] rather than the reduction of oxidised intermediates in the FRAP chain reaction or through chelation [39]. A similar correlation was also reported by Chan et al. [49] for saponins and phenolic compounds extracted from defatted kenaf seeds. In that study, butanol was the most effective solvent for the extraction of saponins and phenolics. This solvent was non-polar enough to dissolve the saponin aglycones [50] and the phenolic rings [11], yet polar enough to also interact with the carbohydrate end of the saponin molecules and the carboxyl groups of gallic acid and p-hydroxybenzoic acid, which are reported to be present in Gac seeds [11]. Because butanol is classified by the FDA as a Class 3 solvent [51] with no known human hazards and approved for pharmaceutical applications, the use of it as an extraction solvent is regarded as less toxic and of low risk to human health.

Despite the high saponins, phenolics, and antioxidant activity of the butanol extract, it did not exhibit any anticancer potential against the two melanoma cell lines. This observation was not consistent with previous findings, which showed that one of the triterpenoid compounds found in Gac seed oil, karounidiol [20], had anticancer potential [19] and tumor inhibition effects [52]. This compound, likely a saponin, may not have been at a high enough concentration in the present extracts because the Gac seed kernels were defatted before they were extracted with the various solvents, which was consistent with a study by Le et al. [53], who found that Gac seed saponins were mainly associated with the fat component of the seeds.

5. Conclusions

Gac seeds, which are mostly considered as a waste product, could be a potentially useful source of anticancer candidates due to their cytotoxic activity against specific melanoma cells. The utilisation of Gac seeds to produce an anticancer product could reduce the waste burden on the environment and add value to the Gac fruit, which is cultivated in an increasing number of countries. This study found that water is suitable for the recovery of trypsin inhibitors and the preparation of an extract with significant anticancer potential. Butanol was found to be suitable to produce an extract enriched in saponins and phenolic compounds and with a high antioxidant activity. However, further studies are needed to fully understand what specific compounds are involved in the bioactivity of Gac seeds and their mechanism of action in order to better define their potential applications in the nutraceutical and pharmaceutical industries.

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References

1. Wimalasiri, D.; Piva, T.; Urban, S.; Huynh, T. Morphological and genetic diversity of *Momordica cochinchinenesis* (Cucurbitaceae) in Vietnam and Thailand. *Genet. Resour. Crop Evol.* **2016**, *63*, 19–33. [CrossRef]
2. Behera, T.; John, K.J.; Bharathi, L.; Karuppaiyan, R. Momordica. In *Wild Crop Relatives: Genomic and Breeding Resources*; Kole, C., Ed.; Springer: Heidelberg, Germany; New York, NY, USA, 2011; pp. 217–246.
3. Chuyen, H.V.; Nguyen, M.H.; Roach, P.D.; Golding, J.B.; Parks, S.E. Gac fruit (*Momordica cochinchinensis* Spreng): A rich source of bioactive compounds and its potential health benefits. *Int. J. Food Sci. Technol.* **2015**, *50*, 567–577. [CrossRef]
4. Huynh, T.; Nguyen, M.H.; Dao, N. Biomedical importance of *Momordica cochinchinensis* (GAC) fruit and future applications. *BJSTR* **2018**, *8*. in press.
5. Masayo, I.; Hikaru, O.; Tatsuo, Y.; Masako, T.; Yoshie, R.; Shuji, H.; Kunihide, M.; Ryuichi, H. Studies on the constituents of *Momordica cochinchinensis* Spreng. I. Isolation and characterization of the seed saponins, Momordica saponins I and II. *Chem. Pharm. Bull.* **1985**, *33*, 464–478. [CrossRef]
6. Chan, L.Y.; Wang, C.K.L.; Major, J.M.; Greenwood, K.P.; Lewis, R.J.; Craik, D.J.; Daly, N.L. Isolation and characterization of peptides from *Momordica cochinchinensis* seeds. *J. Nat. Prod.* **2009**, *72*, 1453–1458. [CrossRef] [PubMed]
7. Wong, R.C.; Fong, W.; Ng, T. Multiple trypsin inhibitors from *Momordica cochinchinensis* seeds, the Chinese drug mubiezhi. *Peptides* **2004**, *25*, 163–169. [CrossRef] [PubMed]
8. Chan, L.Y.; He, W.; Tan, N.; Zeng, G.; Craik, D.J.; Daly, N.L. A new family of cystine knot peptides from the seeds of *Momordica cochinchinensis*. *Peptides* **2013**, *39*, 29–35. [CrossRef] [PubMed]
9. Gao, X.M. (Ed.) Mu Bie Zi (*Semen momordicae*). In *Chinese Materia Medicia Beijing*; Traditional Chinese Materia Medica Press: Beijing, China, 2005; Volume 2, pp. 601–602.
10. Lin, Z.Y.; Liu, X.; Yang, F.; Yu, Y.Q. Structural characterization and identification of five triterpenoid saponins isolated from *Momordica cochinchinensis* extracts by liquid chromatography/tandem mass spectrometry. *Int. J. Mass Spectrom.* **2012**, *328*, 43–66. [CrossRef]
11. Kubola, J.; Siriamornpun, S. Phytochemicals and antioxidant activity of different fruit fractions (peel, pulp, aril and seed) of Thai gac (*Momordica cochinchinensis* Spreng). *Food Chem.* **2011**, *127*, 1138–1145. [CrossRef] [PubMed]
12. Kang, J.M.; Kim, N.; Kim, B.; Kim, J.-H.; Lee, B.-Y.; Park, J.H.; Lee, M.K.; Lee, H.S.; Jang, I.-J.; Kim, J.S.; et al. Gastroprotective action of cochinchina Momordica seed extract is mediated by activation of CGRP and inhibition of cPLA2/5-LOX pathway. *Dig. Dis. Sci.* **2009**, *54*, 2549–2560. [CrossRef] [PubMed]
13. Jung, K.; Chin, Y.-W.; Chung, Y.H.; Park, Y.H.; Yoo, H.; Min, D.S.; Lee, B.; Kim, J. Anti-gastritis and wound healing effects of Momordicae Semen extract and its active component. *Immunopharmacol. Immunotoxicol.* **2013**, *35*, 126–132. [CrossRef] [PubMed]
14. Kang, J.M.; Kim, N.; Kim, B.; Kim, J.-H.; Lee, B.-Y.; Park, J.H.; Lee, M.K.; Lee, H.S.; Kim, J.S.; Jung, H.C.; et al. Enhancement of gastric ulcer healing and angiogenesis by cochinchina Momordica seed extract in rats. *J. Korean Med. Sci.* **2010**, *25*, 875–881. [CrossRef] [PubMed]
15. Yu, J.S.; Kim, J.H.; Lee, S.; Jung, K.; Kim, K.H.; Cho, J.Y. Src/Syk-targeted anti-inflammatory actions of triterpenoidal saponins from Gac (*Momordica cochinchinensis*) Seeds. *Am. J. Chin. Med.* **2017**, 1–15. [CrossRef] [PubMed]
16. Jung, K.; Chin, Y.-W.; Yoon, K.D.; Chae, H.-S.; Kim, C.Y.; Yoo, H.; Kim, J. Anti-inflammatory properties of a triterpenoidal glycoside from Momordica cochinchinensis in LPS-stimulated macrophages. *Immunopharmacol. Immunotoxicol.* **2013**, *35*, 8–14. [CrossRef] [PubMed]
17. Tien, P.G.; Kayama, F.; Konishi, F.; Tamemoto, H.; Kasono, K.; Hung, N.T.; Kuroki, M.; Ishikawa, S.E.; Van Nguyen, C.; Kawakami, M. Inhibition of tumor growth and angiogenesis by water extract of Gac fruit (*Momordica cochinchinensis* Spreng). *Int. J. Oncol.* **2005**, *26*, 881–889. [CrossRef] [PubMed]
18. Zheng, L.; Zhang, Y.-M.; Zhan, Y.-Z.; Liu, C.-X. *Momordica cochinchinensis* seed extracts suppress migration and invasion of human breast cancer ZR-75-30 cells via down-regulating MMP-2 and MMP-9. *Asian Pac. J. Cancer Prev.* **2014**, *15*, 1105–1110. [CrossRef] [PubMed]
19. Akihisa, T.; Tokuda, H.; Ichiishi, E.; Mukainaka, T.; Toriumi, M.; Ukiya, M.; Yasukawa, K.; Nishino, H. Anti-tumor promoting effects of multiflorane-type triterpenoids and cytotoxic activity of karounidiol against human cancer cell lines. *Cancer Lett.* **2001**, *173*, 9–14. [CrossRef]

20. Kan, L.; Hu, Q.; Chao, Z.; Song, X.; Cao, X. Chemical constituents of unsaponifiable matter from seed oil of *Momordica cochinchinensis*. *China J. Chin. Mater. Med.* **2006**, *31*, 1441–1444.
21. Hernandez, J.F.; Gagnon, J.; Chiche, L.; Nguyen, T.M.; Andrieu, J.P.; Heitz, A.; Trinh Hong, T.; Pham, T.T.; Le Nguyen, D. Squash trypsin inhibitors from *Momordica cochinchinensis* exhibit an atypical macrocyclic structure. *Biochemistry* **2000**, *39*, 5722–5730. [CrossRef] [PubMed]
22. Weidmann, J.; Craik, D.J. Discovery, structure, function, and applications of cyclotides: Circular proteins from plants. *J. Exp. Bot.* **2016**, *67*, 4801–4812. [CrossRef] [PubMed]
23. Sever, N.; Filipic, M.; Brzin, J.; Lah, T.T. Effect of cysteine proteinase inhibitors on murine B16 melanoma cell invasion in vitro. *Biol. Chem.* **2002**, *383*, 839–842. [CrossRef] [PubMed]
24. Heidtmann, H.-H.; Salge, U.; Abrahamson, M.; Bencina, M.; Kastelic, L.; Kopitar-Jerala, N.; Turk, V.; Lah, T.T. Cathepsin B and cysteine proteinase inhibitors in human lung cancer cell lines. *Clin. Exp. Metastasis* **1997**, *15*, 368–381. [CrossRef] [PubMed]
25. Clawson, G.A. Protease inhibitors and carcinogenesis: A review. *Cancer Investig.* **1996**, *14*, 597–608. [CrossRef]
26. Hocman, G. Chemoprevention of cancer: Protease inhibitors. *Int. J. Biochem.* **1992**, *24*, 1365–1375. [CrossRef]
27. Shamsi, T.N.; Parveen, R.; Afreen, S.; Azam, M.; Sen, P.; Sharma, Y.; Haque, Q.M.R.; Fatma, T.; Manzoor, N.; Fatima, S. Trypsin inhibitors from *Cajanus cajan* and *Phaseolus limensis* possess antioxidant, anti-Inflammatory, and antibacterial activity. *J. Diet. Suppl.* **2018**, 1–12. [CrossRef] [PubMed]
28. Samuelsson, G.; Kyerematen, G.; Farah, M.H. Preliminary chemical characterization of pharmacologically active compounds in aqueous plant extracts. *J. Ethnopharmacol.* **1985**, *14*, 193–201. [CrossRef]
29. Sasidharan, S.; Chen, Y.; Saravanan, D.; Sundram, K.; Latha, L.Y. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *Afr. J. Tradit. Complement. Altern. Med.* **2011**, *8*, 1–10. [CrossRef] [PubMed]
30. Cheok, C.Y.; Salman, H.A.K.; Sulaiman, R. Extraction and quantification of saponins: A review. *Food Res. Int.* **2014**, *59*, 16–40. [CrossRef]
31. Mahatmanto, T.; Poth, A.G.; Mylne, J.S.; Craik, D.J. A comparative study of extraction methods reveals preferred solvents for cystine knot peptide isolation from *Momordica cochinchinensis* seeds. *Fitoterapia* **2014**, *95*, 22–33. [CrossRef] [PubMed]
32. Abascal, K.; Ganora, L.; Yarnell, E. The effect of freeze-drying and its implications for botanical medicine: A review. *Phytother. Res.* **2005**, *19*, 655–660. [CrossRef] [PubMed]
33. Klomklao, S.; Benjakul, S.; Kishimura, H.; Chaijan, M. Extraction, purification and properties of trypsin inhibitor from Thai mung bean (*Vigna radiata* (L.) R. Wilczek). *Food Chem.* **2011**, *129*, 1348–1354. [CrossRef]
34. Le, A.V.; Parks, S.E.; Nguyen, M.H.; Roach, P.D. Effect of solvents and extraction methods on recovery of bioactive compounds from defatted Gac (*Momordica cochinchinensis* Spreng.) seeds. *Separations* **2018**, *5*, 39. [CrossRef]
35. Makkar, H.P.; Siddhuraju, P.; Becker, K. Trypsin Inhibitor. In *Plant Secondary Metabolites*; Humana Press: New York, NY, USA, 2007; pp. 1–6.
36. Stauffer, C.E. Measuring trypsin inhibitor in soy meal: Suggested improvements in the standard method. *Cereal Chem.* **1990**, *67*, 296–302.
37. Tan, S.P.; Tuyen, K.C.; Parks, S.E.; Stathopoulos, C.E.; Roach, P.D. Effects of the spray-drying temperatures on the physiochemical properties of an encapsulated bitter melon aqueous extract powder. *Powder Technol.* **2015**, *281*, 65–75. [CrossRef]
38. Tan, S.P.; Vuong, Q.V.; Stathopoulos, C.E.; Parks, S.E.; Roach, P.D. Optimized aqueous extraction of saponins from bitter melon for production of a saponin-enriched bitter melon powder. *J. Food Sci.* **2014**, *79*, E1372–E1381. [CrossRef] [PubMed]
39. Thaipong, K.; Boonprakob, U.; Crosby, K.; Cisneros-Zevallos, L.; Byrne, D.H. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J. Food Compost. Anal.* **2006**, *19*, 669–675. [CrossRef]
40. Ishiyama, M.; Tominaga, H.; Shiga, M.; Sasamoto, K.; Ohkura, Y.; Ueno, K. A combined assay of cell vability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biol. Pharm Bull.* **1996**, *19*, 1518–1520. [CrossRef] [PubMed]
41. Wimalasiri, D. Genetic Diversity, Nutritional and Biological Activity of *Momordica cochinchinensis* (Cucurbitaceae). Ph.D. Thesis, RMIT University, Melbourne, Australia, 2015.

42. Arimatsu, P.; Sawangsook, O. Purification and characterization of Trypsin inhibitor from *Momordica cochinchinensis* Spreng. seed and its effects to Spodoptera litura. *Asian J. Plant Sci.* **2012**, *11*, 195–199. [CrossRef]
43. Cascales, L.; Henriques, S.T.; Kerr, M.C.; Huang, Y.-H.; Sweet, M.J.; Daly, N.L.; Craik, D.J. Identification and characterization of a new family of cell-penetrating peptides cyclic cell-penetrating peptides. *J. Biol. Chem.* **2011**, *286*, 36932–36943. [CrossRef] [PubMed]
44. Anderson, R.L.; Wolf, W.J. Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *J. Nutr.* **1995**, *125*, 581S. [CrossRef] [PubMed]
45. Birk, Y. *Plant Protease Inhibitors as Cancer Chemopreventive Agents*; Springer: Berlin, Germany, 2003; ISBN 978-3-642-05514-0.
46. Troll, W.; Kennedy, A.R. *Protease Inhibitors as Cancer Chemopreventive Agents*; Plenum Pub Corp: New York, NY, USA, 1993; ISBN 0306443902.
47. Kennedy, A.R. Chemopreventive agents: Protease inhibitors. *Pharmacol. Ther.* **1998**, *78*, 167–209. [CrossRef]
48. Housman, G.; Byler, S.; Heerboth, S.; Lapinska, K.; Longacre, M.; Snyder, N.; Sarkar, S. Drug resistance in cancer: An overview. *Cancers* **2014**, *6*, 1769–1792. [CrossRef] [PubMed]
49. Chan, K.W.; Iqbal, S.; Khong, N.M.; Ooi, D.-J.; Ismail, M. Antioxidant activity of phenolics–saponins rich fraction prepared from defatted kenaf seed meal. *LWT-Food Sci. Technol.* **2014**, *56*, 181–186. [CrossRef]
50. Hostettmann, K.; Marston, A. *Chemistry and Pharmacology of Natural Products, Saponin*; Cambridge University Press: Cambridge, NY, USA, 1995; ISBN 0-521-32970-1.
51. US Food and Drug Administration (FDA). *Guidance for Industry*; USHaH Services, Ed.; US Food and Drug Administration (FDA): Silver Spring, MD, USA, 2012.
52. Yasukawa, K.; Akihisa, T.; Tamura, T.; Takido, M. Inhibitory effect of karounidiol on 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion. *Biol. Pharm Bull.* **1994**, *17*, 460–462. [CrossRef] [PubMed]
53. Le, A.V.; Parks, S.E.; Nguyen, M.H.; Roach, P.D. Optimisation of the microwave-assisted ethanol extraction of saponins from Gac (*Momordica cochinchinensis* Spreng) seeds. *Medicines* **2018**, *5*, 70. [CrossRef] [PubMed]

Chapter 4: GENERAL DISCUSSION AND CONCLUSIONS

4.1. General discussion

As indicated in Section 1.8., the working hypotheses for this study were that 1) Gac seeds contain high levels of extractable oil, trypsin inhibitors and saponins and their yield can be optimised using different extraction methods and solvents and 2) the Gac seed extracts possess biological activity, including antioxidant and anticancer properties. Therefore, the aim of the thesis was to extract oil, trypsin inhibitors and saponins from Gac seeds with high yields (Section 1.8.).

The aim of the thesis was achieved and the main findings supported the hypotheses in that the seed kernel powder prepared from Gac fruit collected in Vietnam was found to have a high content of oil, a high trypsin inhibitor activity (TIA) and a high total saponin content (TSC), when it was extracted using different methodologies. The yield of oil was optimised for the SC-CO_2 extraction method and the extraction of the bioactives, trypsin inhibitors and saponins, was optimised using different extraction solvents and conditions. The Gac seed bioactive extracts were also shown to possess antioxidant activity and anticancer potential. The implications of these findings for the preparation of Gac seed oil and bioactives, including trypsin inhibitors and saponins, are discussed in the following sections.

4.1.1. Gac seed oil

The extraction of Gac seed oil using the unconventional method of SC-CO_2 was optimised in Paper I (Objective 1, Section 1.8.) and the general characteristics of this oil was compared to the oil generated from the standard method of Soxhlet in Paper II (Objective

2, Section 1.8.). In Paper II, the proximate components of dried Gac seed kernels were also determined, including oil, protein and moisture.

The findings in Papers I and II supported the hypothesis that Gac seeds contain high levels of extractable oil. Using the RSM in Paper I, the optimum oil yield for the SC- CO_2 extraction was predicted and validated to be 33.90 ± 0.47g oil/100g Gac seeds when the extraction was done at 73 ^oC, 5,900 psi and 1.5 mL/min CO_2 flow rate for 10 min using 4 g of Gac seed kernel powder with particle diameters less than 500 μm (Paper I). Using the conditions determined in Paper I, the oil yield was virtually identical in Paper II (34.1 ± 0.8%), an indication that the SC- CO_2 extraction was reproducible. In comparison to other seeds extracted with the SC-CO_2 method, the Gac seeds used in this thesis gave a higher oil yield (33.9%) than soybeans (16.4-16.6%) and a yield similar to sunflower seeds (35.0-36.0%) and rapeseeds (38.2 39.3%) (Stahl et al., 1980).

In Paper II, the SC-CO_2 method gave a lower yield of oil than the conventional Soxhlet method (34.1 vs 53.0%) but the SC-CO_2 oil had a higher quality than the Soxhlet oil for important criteria, such as peroxides (0.12±0.02 vs 1.80±0.01 meq O_2/kg oil), free fatty acids (1.74±0.12 vs 2.47±0.09 mg KOH/g oil), unsaponifiable matter (33.2±1.5 vs 52.6±2.4 g/kg), colour (light yellow vs dark greenish brown) and antioxidant capacity measured with two methods, the DPPH (52.69±0.06 vs 42.98±0.02 μmol Trolox equivalents/g oil) and ABTS (2.10±0.12 vs 1.52±0.06 μmol Trolox equivalents/g oil) assays.

Furthermore, although both oils would need to be further refined because they would not be considered edible due to their high content of unsaponifiable matter (>28 g/kg), the SC-CO_2 oil would need less refining. However, if either or both of the Gac seed oils were to be used as an edible oils, their oxidative stability and hydrolysis reactions under food

processing conditions would also need to be determined as these characteristics are critical for maintaining the quality of an edible oil (Tan et al., 2002).

Alternatively, the high unsaponifiable matter in the oil, especially in the Soxhlet oil, may make it useful for medicinal purposes. Bioactive compounds, such as karounidiol, isokarounidiol, 5-dehydrokarounidiol, 7-oxodihydrokarounidiol, beta-sitosterol, stigmast-7-en-3beta-ol and stigmast-7,22-dien-3beta-ol have been identified in Gac seeds (Kan et al., 2006), which may have anticancer (Zhao et al., 2010a; Zhao et al., 2010b) and anti-inflammatory (Jung et al., 2013b; Yu et al., 2017a) properties. However, whether the unsaponifiable matter in the Gac seed oil extracted in this thesis contains any of these bioactive compounds and/or has any medicinal properties remains to be determined. Clearly, this is an interesting area for further research.

Overall, the successful recovery of oil from Gac seeds shown in this thesis supports the utilisation of this by-product of the Gac fruit processing industry to produce potentially viable products whilst also reducing the waste burden to the environment. However, other oil extraction methods such as pressing and maceration also need to be investigated. Because different extraction methods also vary in their costs, their ability to be scaled up and the effects they have on the physicochemical properties of the produced oil (Swern, 1982), further investigations of extraction methods need to include economical evaluations, suitability for large scale production and physicochemical analyses in order to determine the most effective and feasible method for the recovery of Gac seed oil. As a result of such investigations, the feasibility of producing Gac seed oil and its possible applications should be better known.

4.1.2. Gac seed bioactives

Trypsin inhibitors and saponins are two important types of bioactives in Gac seeds. The initial experiments to determine suitable extraction methods for these two types of bioactives were done in Paper III (Objective 3, Section 1.8), using six extraction solvents (DI water, methanol, 50% methanol, ethanol, 70% ethanol and water-saturated butanol) and three extraction methods with DI water (conventional, MAE and UAE). These experiments revealed that the conventional extraction method with DI water gave the highest TIA values (118.45 ± 4.90 mg trypsin/g) while the conventional extraction method with water-saturated *n*-butanol and methanol extracted the highest amounts of saponins (40.75 ± 0.31 and 38.80 ± 2.82 mg AE/g, respectively). Water being the best solvent for the Gac seed trypsin inhibitors was consistent with these bioactives having been shown to be peptides (Hernandez et al., 2000; Wong et al., 2004). Water-saturated *n*-butanol and methanol being the best solvents for the Gac seed saponins was consistent with previous studies showing that the low alcohols are good extraction solvents for these compounds (Cheok et al., 2014) and suggested that the Gac seed saponins were triterpenoid in nature (Lin et al., 2012).

The assisted extraction methods (MAE and UAE) using DI water did not improve the extraction of trypsin inhibitors (Paper III). Given the protein nature of the trypsin inhibitors, this may have been due to the extraction conditions; the microwave and ultrasound power may have been too high or the extraction time too long and may have destroyed or inactivated some of the trypsin inhibitors (Huang et al., 2008; Pysz et al., 2012). These methods were not pursued further for the extraction of the trypsin inhibitors in this thesis but future studies could be done to determine whether lower UAE and MAE power levels and/or shorter extraction times would improve the extraction.

The assisted extraction methods (MAE and UAE) using DI water also did not improve the extraction of the Gac seed saponins (Paper III) and water-saturated *n*-butanol and methanol remained the best solvents for this extraction followed by 70% ethanol in water. This indicated that the Gac seed saponins were more lipophilic than the usual saponins from other sources (Nguyen et al., 2017; Tan et al., 2014b). However, methanol belongs to the Class 2 solvents (Food and Drug Administration, 2012) and therefore, it should be limited in food and pharmaceutical products because of its inherent toxicity. Butanol and ethanol both belong to the Class 3 solvents (Food and Drug Administration, 2012), which are less toxic and of lower risk to human health. However, ethanol cost less than butanol and it is easier to evaporate than butanol because of its lower boiling point (78.5 °C vs 117.7 °C). Therefore, on safety and economic grounds, ethanol would be the solvent of choice if the recovery of the Gac seed saponins could be improved. Ethanol is also an excellent microwave absorbing solvent and has been used to advantage in MAE (Tatke & Jaiswal, 2011). For these reasons, the subsequent experiments on the Gac seed saponins focused on optimising their extraction using ethanol at different concentrations in water with MAE.

Therefore, the findings in Papers III supported the hypothesis that Gac seeds contain high levels of extractable trypsin inhibitors and saponins and for subsequent optimisation studies, DI water and ethanol (with MAE) were used as the base solvents for the extraction of the trypsin inhibitors (Objective 4, Section 1.8) and saponins (Objective 5, Section 1.8), respectively. The MAE DI water method gave the highest extraction for phenolics (3.18 ± 0.04 mg GAE/g) but the extraction of these bioactives was not pursued further in this thesis.

4.1.2.1. Gac seed trypsin inhibitors

Subsequent to the findings in Paper III, the extraction of the Gac seed trypsin inhibitors was optimised in Paper IV (Objective 4, Section 1.8). Using the conventional solvent extraction method with the assistance of stirring or shaking, the most suitable of four water-based solvents (DI water, 0.1 M NaCl, 0.02 M NaOH and ACN)/water/FA, 25:24:1) was first determined to be 0.1 M NaCl. The RSM was then used to determine the best combination of NaCl concentration, extraction time and ratio of Gac seed material to solvent. It was thus determined that the optimal conditions for extracting the Gac seed trypsin inhibitors were 1 h at the ratio of defatted (not full-fat) Gac seed kernel powder to solvent of 2g/30mL of 0.05 M NaCl. Therefore, the findings in Paper IV supported the hypothesis that the yield of Gac seed trypsin inhibitors could be optimised.

In addition to optimising the extraction efficiency for the Gac seed trypsin inhibitors, the production of a trypsin inhibitor-enriched powder was also explored in Paper IV. Due to its high protein content, the trypsin inhibitor water extract is likely to have been susceptible to degradation during storage. Therefore, it was deemed desirable to use an effective method to preserve the bioactive compounds in the extract. Freeze drying was chosen because it has often been shown to be superior to other preservation methods in that it is able to properly preserve the medicinal qualities of plant extracts while also enabling their solubility in an aqueous medium (Abascal et al., 2005).

Therefore, in paper IV, freeze drying was applied to produce a powder from the optimised Gac seed trypsin inhibitor extract (Objective 4, Section 1.8). The freeze-dried trypsin inhibitor-enriched powder displayed good physical characteristics right after its production. However, further studies on its long term stability during storage under different environment conditions are needed in order to determine its shelf-life. Although

freeze drying is often the preferred choice for preserving a wide range of pharmaceutical formulations, other less expensive methods for powder production, such as spray drying (Gharsallaoui et al., 2007) and spray freeze drying (Ishwarya et al., 2015) also need to be investigated in future research to determine whether these methods are able to further improve the shelf-life without compromising the quality of the Gac seed trypsin inhibitor-enriched powder they produce.

Further purification of the trypsin inhibitors in the optimised Gac seed trypsin inhibitor extract should also be attempted in future studies; techniques such as ammonium sulphate precipitation and Sephadex fractionation could be tried in order to purify the Gac seed trypsin inhibitors (Scopes, 2013). In addition, the stability of the extracted and purified tripsin inhibitors under different conditions, such as at different pHs and at different salt concentrations (Becktel & Schellman, 1987), should also be studied in order to determine the conditions under which the Gac seed trypsin inhibitors are the most stable.

4.1.2.2. Gac seed saponins

Subsequent to the findings in Paper III, the extraction of the Gac seed saponins was optimised using ethanol at different concentrations in water and the MAE method (Paper VI) (Objective 5, Section 1.8). Various ethanol concentrations, microwave conditions and ratios of Gac seed material to solvent were tested to achieve the optimal yield of saponins. Overall, the saponins were best extracted from full-fat Gac seed powdered kernels (not defatted Gac seed kernel powder) with absolute ethanol at the ratio of Gac seed material to solvent of 1:30 (g/mL) with the microwave set at 360 W for three irradiation cycles of 10 s power ON and 15 s power OFF per cycle (Paper VI). The TSC of the optimised extraction was 75% higher than prior to optimisation (105.7±2.4 vs 25.8±2.3 mg AE/g), which demonstrated the efficiency of the optimisation process. This TSC value for the

optimised extract was higher than for other plant materials also extracted with ethanol, such as 6.73±1.55 mg AE/g from *H. Hirsuta* L. stem (Pham et al., 2017a) and 42.43±4.10 mg AE/g from *C. Roseus* leaf (Pham et al., 2017b). Therefore, the findings in Paper VI supported the hypothesis that the yield of Gac seed saponins could be optimised.

In addition, an improved assay for the quantification of the total saponin content of extracts was proposed in Paper V. In the studies with the different extraction solvents, it became apparent that some solvents substantially interfered with the original saponin assay (Hiai et al., 1975b). To prevent this interference by extraction solvents from occurring, a step was included in which the extraction solvent was evaporated before the ethanol-vanillin reagent of the saponin assay was added (Paper V).

Studies on scaling up the MAE process for the extraction of the Gac seed saponins are still needed to determine whether the utilisation of Gac seeds for this purpose is feasible on an industrial scale. Furthermore, other innovative extraction methods such as SC-CO_2 extraction or liquid pressurised extraction, which have been used for the extraction of saponins from various plant sources (Güçlü Üstündağ et al., 2007; Zhen, 2006), could also be tested for their efficacy and feasibility in recovering the Gac seed saponins.

Unlike the extraction of trypsin inhibitors, which was done from defatted Gac seed kernel powder (Paper IV), the saponins were best extracted from full-fat Gac seed kernel powder (Paper VI). Therefore, if the same Gac seed material is to be used for the extraction of both saponins and trypsin inhibitors, the saponin extraction would need to be done before the trypsin inhibitor extraction. However, whether the residue from the saponin extraction could be used directly for the trypsin inhibitor extraction or whether the residue would need to be defatted first remains to be determined; enough oil could remain in the residue to interfere with the aqueous extraction of the trypsin inhibitors.

Alternatively, it may be possible to extract the saponins from the oil after the Gac seed kernel powder has been defatted and the trypsin inhibitors could then be extracted from the defatted kernel powder. In this sequence, the Gac seed oil would be extracted first and somewhat refined by the saponin extraction, which could make it more likely to be edible, and the residue from the oil extraction could be used to extract the trypsin inhibitors. However, this sequence of extractions would need to be thoroughly investigated for the yield and quality of the oil, saponins and trypsin inhibitors extracted.

Like saponins from other sources (Tava et al., 2003), the Gac seed saponins are likely to be stable in the 100% ethanol they were extracted in using the MAE method (Paper VI) but this was not studied. Therefore, the shelf-life of the Gac seed saponin ethanol preparation remains to be determined. Alternatively, as for the Gac seed trypsin inhibitors, a powder could be produced by evaporating the ethanol, for example by vacuum evaporation. However, simply evaporating the ethanol could leave the saponins in the powder vulnerable to oxidation and therefore encapsulation, including by spray drying, may be needed to protect the saponins from degradation (Tan et al., 2014b; Tuyen et al., 2014). As a third alternative, the saponin extract could be used to produce an ointment by suspending it in a suitable oil (Saeki & Matsukawa, 1976).

Finally, due to the reported toxicity of the Gac seed saponins (Kubota et al., 1971), the biological potential of the saponin extract produced in this thesis could be determined for uses such as antimicrobial (Merve & Oguz, 2016) and antibacterial (Parekh et al., 2006) agents. Liquid fractionation of the crude saponin extract could also be done in order to purify individual saponins and determine which have the highest activity in these biological tests.

4.1.2.3. Gac seed phenolic compounds

Although the extraction of the Gac seed phenolic compounds was not optimised in this thesis, they were quantified in various extracts and their relation to the antioxidant activity of the extracts was determined (Papers III, IV and VII). For example, the findings in Paper VII showed that the water-saturated butanol extract from the defatted Gac seed kernel powder had a TPC similar to the highest TPC achieved for extracts from bitter melon (20.35±0.8 vs 22.4±0.4 mg GAE/g), which is known to be a rich source of phenolic compounds (Tan et al., 2014b). Moreover, a strong direct correlation was found between the TPC and the ABTS antioxidant activity of the various Gac seed extracts in Papers III and VII, thereby providing evidence for the antioxidant capacity of the Gac seed phenolic compounds. Therefore, because the phenolic compounds are at a high level in Gac seed extracts and contribute to their antioxidant capacity, it may also be worth optimising their extraction in future studies.

4.1.3. Antioxidant activity and anticancer potential of Gac seed extracts

In Paper VII, the antioxidant activity (Objective 6, Section 1.8.) and anticancer potential (Objective 7, Section 1.8.) were evaluated for Gac seed extract powders prepared by freeze drying after extraction of a defatted Gac seed kernel powder with six solvents (DI water, methanol, 50% methanol, ethanol, 70% ethanol and water-saturated butanol). The findings supported the hypothesis that Gac seed extracts possess biological activities, including antioxidant and anticancer properties (Section 1.8.).

For the antioxidant activity, when measured with the ABTS assay, the freeze dried powder from the water-saturated butanol extract of the defatted Gac seed kernel powder generally displayed the highest activity (213 µmole TE/g) (Paper VII), which was similar to the activity reported for a freeze dried powder from an ethanol extract of bitter melon

(190 µmole TE/g) (Tan et al., 2014b). This suggests that Gac seeds possess a high antioxidant capacity and therefore, Gac seed extracts could be investigated further for applications in therapeutics and functional foods for which antioxidant capacity is important.

For the anticancer potential, the freeze dried powder of the DI water extract from the defatted Gac seed kernel powder showed the highest potential against melanoma cells, reducing the viability of MM418C1 and D24 cells by 75.5±1.3 and 66.9±2.2%, respectively (Paper VII). This activity was likely related to the DI water extract's high content of trypsin inhibitors, which is consistent with the reported anticancer potential of other trypsin inhibitors (Bai et al., 2015; Birk, 2003). However, although promising, more work clearly needs to be done to reproduce this finding and to determine the mechanism of action by which the Gac seed trypsin inhibitors could exhibit cytotoxicity against these cancer cells. Understanding the mechanisms by which cell death is induced by phytochemicals, including trypsin inhibitors, is a key step in aiding the development of chemo-preventive or chemo-therapeutic drugs (Wimalasiri, 2015).

Besides the antioxidant activity and anticancer potential of the Gac seed extracts presented in this thesis (Paper VII), other biological properties could be evaluated in future studies. For example, trypsin inhibitors from other plant materials have been reported to have antiproliferative (Ho & Ng, 2008), anti-inflamatory and antibacterial (Shamsi et al., 2018) activities.

4.1.4. Scheme for the research presented and recommendations for the future

Although the outcomes of the current thesis are substantial, they can only be used as a reference point for future work in terms of the usefulness of the end products. A summary of the research reported in this thesis and the recommendations for future studies, some

of which have been proposed and discussed in the above discussion sections, is shown in Figure 38.

For example, in this thesis, the ground Gac seed kernels were freeze-dried before they were extracted for oil or bioactive compounds, the extracts from different solvents were freeze dried before they were tested for antioxidant activity and anticancer potential in Paper VII and the optimised trypsin inhibitor-enriched extract was freeze dried in Paper IV. Although freeze-drying is known to be a good method for preserving the biological activities of bioactive compounds and therefore often used in research, it is a costly option, which may not be affordable in the industrial context (Müller & Heindl, 2006). Therefore, studies on different drying methods that are more economical, including spray drying, are also warranted.

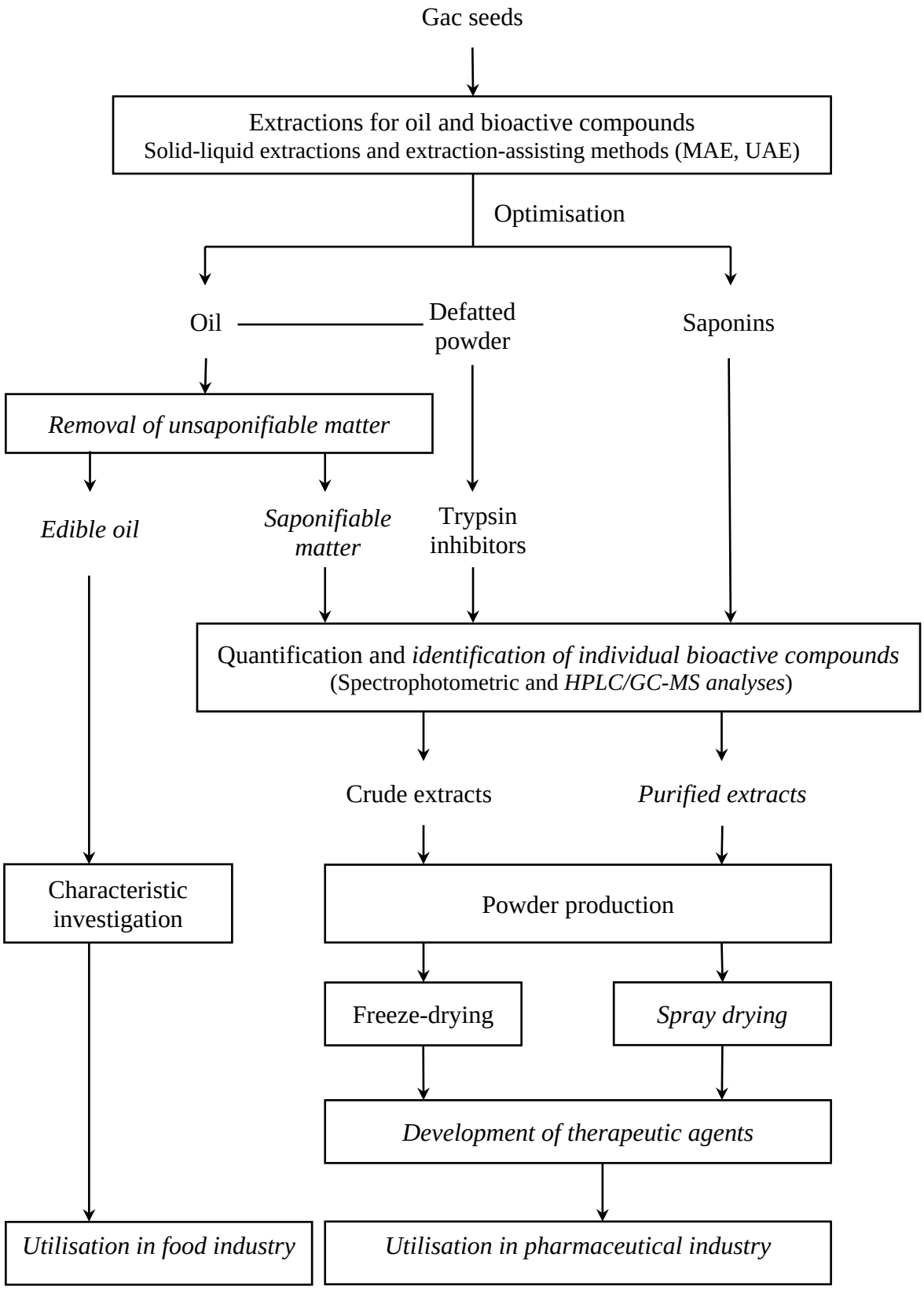

Figure 38: A diagram of the research presented in this thesis and recommendations for future studies (in *italics*).

4.2. Conclusions

In this thesis, the hypotheses were supported and the aim and objectives were achieved. Gac seeds were found to be a rich source of oil and bioactive compounds, including trypsin inhibitors and saponins. The extraction of oil, trypsin inhibitors and saponins from the Gac seeds was improved by investigating the extraction methods and optimising the extraction conditions. The Gac seed oil yield was optimised for the SC-CO_2 extraction method and the oil produced was of higher quality that the oil produced using the Soxhlet method, although both oils contained high levels of unsaponifiable matter and would need further refining to be considered edible. The extraction of Gac seed trypsin inhibitors using aqueous solvents was optimised and the optimum conditions were used to successfully produce a trypsin inhibitor-enriched freeze dried powder with good physicochemical properties. The extraction of Gac seed saponins was also optimised using ethanol and the MAE. Gac seed extracts prepared using various solvents displayed varying antioxidant activity and anticancer potential against melanoma cells. The antioxidant activity of the extracts was correlated with their content of phenolic compounds and the anticancer potential was related to their content of trypsin inhibitors. In summary, oil, trypsin inhibitors and saponins were successfully extracted from Gac seeds. The SC-CO_2 extraction of oil, the conventional extraction of trypsin inhibitors and the ethanol MAE of saponins were improved after optimisation.

BIBLIOGRAPHY

AACC International. (2000). *American Association of Cereal Chemists. Approved methods of the AACC* (10th ed.). St. Paul, MN, U.S.A.: AACC International.

Abascal, K., Ganora, L., & Yarnell, E. (2005). The effect of freeze-drying and its implications for botanical medicine: a review. *Phytotherapy Research, 19*(8), 655-660.

Akihisa, T., Ghosh, P., Thakur, S., Rosentein, F. U., & Matsumoto, T. (1986). Sterol compositions of seeds and mature plants of family Cucurbitaceae. *Journal of the American Oil Chemists Society, 63*(5), 653-658.

Akihisa, T., Tokuda, H., Ichiishi, E., Mukainaka, T., Toriumi, M., Ukiya, M., . . . Nishino, H. (2001). Anti-tumor promoting effects of multiflorane-type triterpenoids and cytotoxic activity of karounidiol against human cancer cell lines. *Cancer Letters, 173*(1), 9-14.

Anderson, R. L., & Wolf, W. J. (1995). Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *Journal of Nutrition, 125*(3), 581S.

AOCS. (1998). *Official Methods and Recommended Practices of the AOCS* (5th ed.). Champaign, IL: AOCS Press.

Aoki, H., Kieu, N. T. M., Kuze, N., Tomisaka, K., & Chuyen, N. V. (2002). Carotenoid pigments in GAC fruit (Momordica cochinchinensis SPRENG). *Bioscience, Biotechnology, and Biochemistry, 66*(11), 2479-2482.

Apak, R., Gorinstein, S., Böhm, V., Schaich, K. M., Özyürek, M., & Güçlü, K. (2013). Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). *Pure and Applied Chemistry, 85*(5), 957-998.

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S. E., Bektaşoğlu, B., . . . Özyurt, D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules, 12*(7), 1496-1547.

Aruoma, O. I., Murcia, A., Butler, J., & Halliwell, B. (1993). Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives. *Journal of Agricultural and Food Chemistry, 41*(11), 1880-1885.

Ascherio, A., Katan, M. B., Zock, P. L., Stampfer, M. J., & Willett, W. C. (1999). Trans fatty acids and coronary heart disease. *New England Journal of Medicine, 340*, 1994-1998.

Avrutina, O., Schmoldt, H.-U., Gabrijelcic-Geiger, D., Le Nguyen, D., Sommerhoff, C. P., Diederichsen, U., & Kolmar, H. (2005). Trypsin inhibition by macrocyclic and open-chain variants of the squash inhibitor MCoTI-II. *Biological Chemistry, 386*(12), 1301-1306.

Azmir, J., Zaidul, I., Rahman, M., Sharif, K., Mohamed, A., Sahena, F., . . . Omar, A. (2013). Techniques for extraction of bioactive compounds from plant materials: a review. *Journal of Food Engineering, 117*(4), 426-436.

Bai, C. Z., Feng, M. L., Hao, X. L., Zhao, Z. J., Li, Y. Y., & Wang, Z. H. (2015). Anti-tumoral effects of a trypsin inhibitor derived from buckwheat in vitro and in vivo. *Molecular Medicine Reports, 12*(2), 1777-1782.

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry, 99*(1), 191-203.

Baş, D., & Boyacı, İ. H. (2007). Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering, 78*(3), 836-845.

Becktel, W. J., & Schellman, J. A. (1987). Protein stability curves. *Biopolymers: Original Research on Biomolecules, 26*(11), 1859-1877.

Beecher, G. R. (2003). Overview of dietary flavonoids: nomenclature, occurrence and intake. *The Journal of nutrition, 133*(10), 3248S-3254S.

Behera, T., John, K. J., Bharathi, L., & Karuppaiyan, R. (2011). Momordica. In C. Kole (Ed.), *Wild Crop Relatives: Genomic and Breeding Resources* (pp. 217-246). Heidelberg, New York: Springer.

Benitez, M. M., & De Guzman, C. C. (2012). *Antioxidant content of bitter gourd (Momordica charantia L.) grown in different nutrient amendments.* Paper presented at the UMT 11th international annual symposium on sustainability science and management.

Benzie, I. F., & Strain, J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry, 239*(1), 70-76.

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta, 76*(5), 965-977.

Bhanot, A., Sharma, R., & Noolvi, M. N. (2011). Natural sources as potential anti-cancer agents: A review. *International journal of phytomedicine, 3*(1), 09-26.

Bharathi, L. K., Singh, H. S., Shivashankar, S., Ganeshamurthy, A. N., & Sureshkumar, P. (2014). Assay of Nutritional Composition and Antioxidant Activity of Three Dioecious Momordica Species of South East Asia. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 84*(1), 31-36.

Bhattacharjee, P., Singhal, R. S., & Tiwari, S. R. (2007). Supercritical carbon dioxide extraction of cottonseed oil. *Journal of Food Engineering, 79*(3), 892-898.

Bhuyan, D. J., Vuong, Q. V., Chalmers, A. C., van Altena, I. A., Bowyer, M. C., & Scarlett, C. J. (2017). Development of the ultrasonic conditions as an advanced

technique for extraction of phenolic compounds from Eucalyptus robusta. *Separation Science and Technology, 52*(1), 100-112.

Birk, Y. (1996). Protein proteinase inhibitors in legume seeds-overview. *Archivos Latinoamericanos de Nutrición, 44*(4 Suppl 1), 26S-30S.

Birk, Y. (2003). *Plant protease inhibitors as cancer chemopreventive agents*. Berlin, Germany: Springer.

Boligon, A. A., Machado, M. M., & Athayde, M. L. (2014). Technical evaluation of antioxidant activity. *Medicinal Chemistry (Shariqah, United Arab Emirates), 4*(7), 517-522.

Bolognesi, A., Barbieri, L., Carnicelli, D., Abbondanza, A., Cenini, P., Falasca, A. I., . . . Stirpe, F. (1989). Purification and properties of a new ribosome-inactivation protein with RNA N-glycosidase activity suitable for immunotoxin preparation from the seeds of Momordica cochinchinensis. *Biochimica et Biophysica Acta (BBA)-General Subjects, 993*(2), 287-292.

Box, G. E., & Wilson, K. (1951). On the experimental attainment of optimum conditions. *Journal of the Royal Statistical Society. Series B (Methodological), 13*(1), 1-45.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*.

Burke, D., Smidt, C., & Vuong, L. (2005). Momordica cochinchinensis, Rosa roxburghii, wolfberry, and sea buckthorn-highly nutritional fruits supported by tradition and science. *Current Topics in Nutraceutical Research, 3*(4), 259.

Burkill, I. H. (1966). A dictionary of the economic products of the Malay Peninsula. *A Dictionary of the Economic Products of the Malay Peninsula., 2*(2nd edition).

Cacace, J., & Mazza, G. (2002). Extraction of anthocyanins and other phenolics from black currants with sulfured water. *Journal of Agricultural and Food Chemistry, 50*(21), 5939-5946.

Cacace, J., & Mazza, G. (2003a). Mass transfer process during extraction of phenolic compounds from milled berries. *Journal of Food Engineering, 59*(4), 379-389.

Cacace, J., & Mazza, G. (2003b). Optimization of extraction of anthocyanins from black currants with aqueous ethanol. *Journal of Food Science, 68*(1), 240-248.

Camel, V. (2001). Recent extraction techniques for solid matrices—supercritical fluid extraction, pressurized fluid extraction and microwave-assisted extraction: their potential and pitfalls. *Analyst, 126*(7), 1182-1193.

Cancer Councel Australia. (2017). Understanding Melanoma: A guide for people with cancer, their families and friends [Press release]. Retrieved from

https://www.cancer.org.au/about-cancer/types-of-cancer/skin-cancer/melanoma.html#jump_1

Capasso, R., Cristinzio, G., Evidente, A., & Scognamiglio, F. (1992). Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. *Phytochemistry, 31*(12), 4125-4128.

Caredda, A., Marongiu, B., Porcedda, S., & Soro, C. (2002). Supercritical carbon dioxide extraction and characterization of Laurus nobilis essential oil. *Journal of Agricultural and Food Chemistry, 50*(6), 1492-1496.

Cascales, L., Henriques, S. T., Kerr, M. C., Huang, Y.-H., Sweet, M. J., Daly, N. L., & Craik, D. J. (2011). Identification and characterization of a new family of cell-penetrating peptides cyclic cell-penetrating peptides. *Journal of Biological Chemistry, 286*(42), 36932-36943.

Chan, He, W., Tan, N., Zeng, G., Craik, D. J., & Daly, N. L. (2013). A new family of cystine knot peptides from the seeds of *Momordica cochinchinensis*. *Peptides, 39*, 29-35.

Chan, Wang, C. K., Major, J. M., Greenwood, K. P., Lewis, R. J., Craik, D. J., & Daly, N. L. (2009). Isolation and characterization of peptides from *Momordica cochinchinensis* seeds. *Journal of Natural Products, 72*(8), 1453-1458.

Chemat, F., & Khan, M. K. (2011). Applications of ultrasound in food technology: processing, preservation and extraction. *Ultrasonics Sonochemistry, 18*(4), 813-835.

Chen, Y., Miao, Y., Huang, L., Li, J., Sun, H., Zhao, Y., . . . Zhou, W. (2014). Antioxidant activities of saponins extracted from Radix Trichosanthis: an in vivo and in vitro evaluation. *BMC Complementary and Alternative Medicine, 14*(1), 86.

Chen, Y., Xie, M.-Y., & Gong, X.-F. (2007). Microwave-assisted extraction used for the isolation of total triterpenoid saponins from Ganoderma atrum. *Journal of Food Engineering, 81*(1), 162-170.

Cheok, C. Y., Salman, H. A. K., & Sulaiman, R. (2014). Extraction and quantification of saponins: A review. *Food Research International, 59*, 16-40.

Chevallier, A. (1996). The encyclopedia of medicinal plants. *London: Dorling Kindersley 336p. ISBN, 751303143*.

Choi, K. S., Kim, E. H., Hong, H., Ock, C. Y., Lee, J. S., Kim, J. H., & Hahm, K. B. (2012). Attenuation of cysteamine-induced duodenal ulcer with Cochinchina momordica seed extract through inhibiting cytoplasmic phospholipase A2/5-lipoxygenase and activating γ-glutamylcysteine synthetase. *Journal of Gastroenterology and Hepatology, 27*(s3), 13-22.

Christodouleas, D. C., Fotakis, C., Nikokavoura, A., Papadopoulos, K., & Calokerinos, A. C. (2015). Modified DPPH and ABTS assays to assess the antioxidant profile of untreated oils. *Food Analytical Methods, 8*(5), 1294-1302.

Chuethong, J., Oda, K., Sakurai, H., Saiki, I., & Leelamanit, W. (2007). Cochinin B, a novel ribosome-inactivating protein from the seeds of Momordica cochinchinensis. *Biological and Pharmaceutical Bulletin, 30*(3), 428-432.

Chung, S., & Champagne, E. (2008). Using phenolic compounds to reduce the allergenic properties of peanut extracts and peanut butter slurries. *Journal of Allergy and Clinical Immunology, 121*(2), S249.

Chuyen, H. V., Nguyen, M. H., Roach, P. D., Golding, J. B., & Parks, S. E. (2014). Gac fruit (*Momordica cochinchinensis* Spreng.): a rich source of bioactive compounds and its potential health benefits. *International Journal of Food Science and Technology*.

Chuyen, H. V., Nguyen, M. H., Roach, P. D., Golding, J. B., & Parks, S. E. (2018). Microwave-assisted extraction and ultrasound-assisted extraction for recovering carotenoids from Gac peel and their effects on antioxidant capacity of the extracts. *Food science & nutrition, 6*(1), 189-196.

Chuyen, H. V., Roach, P. D., Golding, J. B., Parks, S. E., & Nguyen, M. H. (2017). Optimisation of extraction conditions for recovering carotenoids and antioxidant capacity from Gac peel using response surface methodology. *International Journal of Food Science & Technology, 52*(4), 972-980.

Codex, A. C. (2001). *Codex Alimentarius: fats, oils and related products* (Vol. 8): Food and Agriculture Organisation.

Contreras, J., Elnagar, A. Y., Hamm-Alvarez, S. F., & Camarero, J. A. (2011). Cellular uptake of cyclotide MCoTI-I follows multiple endocytic pathways. *Journal of Controlled Release, 155*(2), 134-143.

Council of Europe. (2007). Determination of tannins in herbal drugs. In *European pharmacopoeia* (6th ed., pp. A 286). Strasbourg, France: European Directorate for the Quality of Medicines ….

Cragg, G. M., & Newman, D. J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology, 100*(1-2), 72-79.

Craik, D. J., Swedberg, J. E., Mylne, J. S., & Cemazar, M. (2012). Cyclotides as a basis for drug design. *Expert opinion on drug discovery, 7*(3), 179-194.

Dai, J., & Mumper, R. J. (2010). Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules, 15*(10), 7313-7352.

De Castro, M. L., & Garcıa-Ayuso, L. (1998). Soxhlet extraction of solid materials: an outdated technique with a promising innovative future. *Analytica Chimica Acta, 369*(1-2), 1-10.

De Leo, F., Volpicella, M., Licciulli, F., Liuni, S., Gallerani, R., & Ceci, L. R. (2002). PLANT-PIs: a database for plant protease inhibitors and their genes. *Nucleic Acids Research, 30*(1), 347-348.

Del Rio, D., Costa, L., Lean, M., & Crozier, A. (2010). Polyphenols and health: what compounds are involved? *Nutrition, Metabolism and Cardiovascular Diseases, 20*(1), 1-6.

del Valle, J. M., & Uquiche, E. L. (2002). Particle size effects on supercritical CO 2 extraction of oil-containing seeds. *Journal of the American Oil Chemists' Society, 79*(12), 1261-1266.

Desai, Parikh, J., & Parikh, P. (2010). Extraction of natural products using microwaves as a heat source. *Separation & Purification Reviews, 39*(1-2), 1-32.

Deshpande, S. S., & Campbell, C. G. (1992). Effect of different solvents on protein recovery and neurotoxin and trypsin inhibitor contents of grass pea (*Lathyrus sativus*). *Journal of the Science of Food and Agriculture, 60*(2), 245-249.

Ding, X.-g., Zhang, J.-l., Zheng, J., Zhu, Y.-q., & Hou, D.-y. (2005). Analysis of Fatty Acid in Seed of Momordica Cochinchinensis by GC/MS. *LiShiZhen Medicine and Materia Medica Research, 3*, 018.

Doughari, J. H. (2012). Phytochemicals: extraction methods, basic structures and mode of action as potential chemotherapeutic agents. In V. Rao (Ed.), *Phytochemicals-A global perspective of their role in nutrition and health* (pp. 1-32). Rijeka, Croatia: InTech.

Du, X., Wills, R., & Stuart, D. (2004). Changes in neutral and malonyl ginsenosides in American ginseng (Panax quinquefolium) during drying, storage and ethanolic extraction. *Food Chemistry, 86*(2), 155-159.

Endo, Y., Mitsui, K., Motizuki, M., & Tsurugi, K. (1987). The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in 28 S ribosomal RNA caused by the toxins. *Journal of Biological Chemistry, 262*(12), 5908-5912.

Farvid, M. S., Ding, M., Pan, A., Sun, Q., Chiuve, S. E., Steffen, L. M., . . . Hu, F. B. (2014). Dietary linoleic acid and risk of coronary heart disease: a systematic review and meta-analysis of prospective cohort studies. *Circulation*, CIRCULATIONAHA. 114.010236.

Felizmenio-Quimio, M. E., Daly, N. L., & Craik, D. J. (2001). Circular Proteins in Plants: Solution structure of a novel macrocyclic trypsin inhibitor from Momordica Cochinchinensis. *Journal of Biological Chemistry, 276*(25), 22875-22882.

Folin O, C. V. (1927). On tyrosine and tryptophan determinations in proteins. *J. biol. Chem, 73*, 627-650.

Food and Drug Administration. (2012). Guidance for Industry. In U. Services (Ed.), *Q3C–Tables and List*. USA: Silver Spring, MD.

Fornari, T., Vicente, G., Vázquez, E., García-Risco, M. R., & Reglero, G. (2012). Isolation of essential oil from different plants and herbs by supercritical fluid extraction. *Journal of Chromatography A, 1250*, 34-48.

Franco, O. L. (2011). Peptide promiscuity: an evolutionary concept for plant defense. *FEBS Letters, 585*(7), 995-1000.

Friedrich, J., & Pryde, E. (1984). Supercritical CO_2 extraction of lipid-bearing materials and characterization of the products. *Journal of the American Oil Chemists' Society, 61*(2), 223-228.

Fukumoto, L., & Mazza, G. (2000). Assessing antioxidant and prooxidant activities of phenolic compounds. *Journal of Agricultural and Food Chemistry, 48*(8), 3597-3604.

Galanakis, C. M. (2016). *Nutraceutical and Functional Food Components: Effects of Innovative Processing Techniques*: Academic Press.

Gao. (2005). Mu Bie Zi (Semen momordicae). *Gao XM, ed. Chinese Materia Medicia. Beijing: Traditional Chinese Materia Medica Press, 2*, 601-602.

Gao, L., & Mazza, G. (1996). Extraction of anthocyanin pigments from purple sunflower hulls. *Journal of Food Science, 61*(3), 600-603.

Garlapati, C., & Madras, G. (2010). Solubilities of palmitic and stearic fatty acids in supercritical carbon dioxide. *The Journal of Chemical Thermodynamics, 42*(2), 193-197.

Ge, Y., Ni, Y., Yan, H., Chen, Y., & Cai, T. (2002). Optimization of the supercritical fluid extraction of natural vitamin E from wheat germ using response surface methodology. *Journal of Food Science, 67*(1), 239-243.

Gelmez, N., Kıncal, N. S., & Yener, M. E. (2009). Optimization of supercritical carbon dioxide extraction of antioxidants from roasted wheat germ based on yield, total phenolic and tocopherol contents, and antioxidant activities of the extracts. *The Journal of Supercritical Fluids, 48*(3), 217-224.

George, A. (1965). Legal status and toxicity of saponins. *Food and Cosmetics Toxicology, 3*, 85-91.

Gerard, D., & May, P. (2002). Herb and spice carbon dioxide extracts-versatile, safe ingredients for premium food and health food. *Food Tech*, 1-5.

Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A., & Saurel, R. (2007). Applications of spray-drying in microencapsulation of food ingredients: An overview. *Food Research International, 40*(9), 1107-1121.

Gomes, P. B., Mata, V. G., & Rodrigues, A. E. (2007). Production of rose geranium oil using supercritical fluid extraction. *The Journal of Supercritical Fluids, 41*(1), 50-60.

Gran, Sandberg, F., & Sletten, K. (2000). Oldenlandia affinis (R&S) DC: a plant containing uteroactive peptides used in African traditional medicine. *Journal of Ethnopharmacology, 70*(3), 197-203.

Greenwell, M., & Rahman, P. (2015). Medicinal plants: their use in anticancer treatment. *International journal of pharmaceutical sciences and research, 6*(10), 4103.

Greenwood, K. P., Daly, N. L., Brown, D. L., Stow, J. L., & Craik, D. J. (2007). The cyclic cystine knot miniprotein MCoTI-II is internalized into cells by macropinocytosis. *The international journal of biochemistry & cell biology, 39*(12), 2252-2264.

Grundy, S. M. (1994). Influence of stearic acid on cholesterol metabolism relative to other long-chain fatty acids. *The American journal of clinical nutrition, 60*(6), 986S-990S.

Guan, W., Li, S., Yan, R., Tang, S., & Quan, C. (2007). Comparison of essential oils of clove buds extracted with supercritical carbon dioxide and other three traditional extraction methods. *Food Chemistry, 101*(4), 1558-1564.

Güçlü Üstündağ, Ö., Balsevich, J., & Mazza, G. (2007). Pressurized low polarity water extraction of saponins from cow cockle seed. *Journal of Food Engineering, 80*(2), 619-630.

Güçlü Üstündağ, Ö., & Mazza, G. (2007). Saponins: properties, applications and processing. *Critical Reviews in Food Science and Nutrition, 47*(3), 231-258.

Gunstone, F. (2011). *Vegetable oils in food technology: composition, properties and uses*: John Wiley & Sons.

Haaland, P. D. (1989). *Experimental design in biotechnology* (Vol. 105): CRC press.

Hadolin, M., Škerget, M., Knez, Z. e., & Bauman, D. (2001). High pressure extraction of vitamin E-rich oil from Silybum marianum. *Food Chemistry, 74*(3), 355-364.

Harborne, J., & Dictionary, B. H. M. G. P. (1999). A Handbook of Bioactive Compounds from Plants, 1999. In: Taylor & Francis, London.

Heitz, A., Hernandez, J.-F., Gagnon, J., Hong, T. T., Pham, C. T., Nguyen, T. M., . . . Chiche, L. (2001). Solution Structure of the Macrocyclic Squash Trypsin Inhibitor MCoTI-II, the first member of a new family of cyclic Knottins. In *Peptides: The Wave of the Future* (pp. 387-388): Springer.

Henry, M. C., & Yonker, C. R. (2006). Supercritical fluid chromatography, pressurized liquid extraction, and supercritical fluid extraction. *Analytical Chemistry, 78*(12), 3909-3916.

Hernandez, J. F., Gagnon, J., Chiche, L., Nguyen, T. M., Andrieu, J. P., Heitz, A., . . . Le Nguyen, D. (2000). Squash trypsin inhibitors from *Momordica cochinchinensis* exhibit an atypical macrocyclic structure. *Biochemistry, 39*(19), 5722-5730.

Herrero, M., Cifuentes, A., & Ibanez, E. (2006). Sub-and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review. *Food Chemistry, 98*(1), 136-148.

Hiai, S., Oura, H., Hamanaka, H., & Odaka, Y. (1975a). A color reaction of panaxadiol with vanillin and sulfuric acid. *Planta Medica, 28*(06), 131-138.

Hiai, S., Oura, H., Odaka, Y., & Nakajima, T. (1975b). A colorimetric estimation of Ginseng saponins. *Planta Medica, 28*(4), 363-369.

Ho, V. S., & Ng, T. (2008). A Bowman-Birk trypsin inhibitor with antiproliferative activity from Hokkaido large black soybeans. *Journal of peptide science: an official publication of the European Peptide Society, 14*(3), 278-282.

Hocman, G. (1992). Chemoprevention of cancer: protease inhibitors. *International Journal of Biochemistry, 24*(9), 1365-1375.

Hua, L. (2007). Ultrasound-assisted extraction of soybean saponins from waste residue of the soybeans [J]. *Science and Technology of Food Industry, 5*, 049.

Huang, B., Ng, T., Fong, W., Wan, C., & Yeung, H. (1999). Isolation of a trypsin inhibitor with deletion of N-terminal pentapeptide from the seeds of Momordica cochinchinensis, the Chinese drug mubiezhi. *The international journal of biochemistry & cell biology, 31*(6), 707-715.

Huang, H., Kwok, K.-C., & Liang, H.-H. (2008). Inhibitory activity and conformation changes of soybean trypsin inhibitors induced by ultrasound. *Ultrasonics Sonochemistry, 15*(5), 724-730.

Huijuan, S., Wei, W., & Chunfang, Y. (2000). Studies on Fatty Acid Composition in the Oil of Momordica cochinchinensis. *Chinese Traditional and Herbal Drugs, 31*(10), 727-728.

Hunter, J. E., Zhang, J., & Kris-Etherton, P. M. (2010). Cardiovascular disease risk of dietary stearic acid compared with trans, other saturated, and unsaturated fatty acids: a systematic review. *The American journal of clinical nutrition, 91*(1), 46-63.

Ishida, B. K., Turner, C., Chapman, M. H., & McKeon, T. A. (2004). Fatty acid and carotenoid composition of Gac (*Momordica cochinchinensis* Spreng) fruit. *Journal of Agricultural and Food Chemistry, 52*, 274-279.

Ishiyama, M., Miyazono, Y., Sasamoto, K., Ohkura, Y., & Ueno, K. (1997). A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. *Talanta, 44*(7), 1299-1305.

Ishiyama, M., Tominaga, H., Shiga, M., Sasamoto, K., Ohkura, Y., & Ueno, K. (1996). A combined assay of cell vability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biological and Pharmaceutical Bulletin, 19*(11), 1518-1520.

Ishwarya, S. P., Anandharamakrishnan, C., & Stapley, A. G. (2015). Spray-freeze-drying: a novel process for the drying of foods and bioproducts. *Trends in Food Science & Technology, 41*(2), 161-181.

ISO 14902. (2001). Animal feeding stuffs -- Determination of trypsin inhibitor activity of soya products.

Jung, K., Chin, Y.-W., Chung, Y. H., Park, Y. H., Yoo, H., Min, D. S., . . . Kim, J. (2013a). Anti-gastritis and wound healing effects of Momordicae Semen extract and its active component. *Immunopharmacology and Immunotoxicology, 35*(1), 126-132.

Jung, K., Chin, Y.-W., Yoon, K. d., Chae, H.-S., Kim, C. Y., Yoo, H., & Kim, J. (2013b). Anti-inflammatory properties of a triterpenoidal glycoside from Momordica cochinchinensis in LPS-stimulated macrophages. *Immunopharmacology and Immunotoxicology, 35*(1), 8-14.

Kadam, S. U., Tiwari, B. K., Álvarez, C., & O'Donnell, C. P. (2015). Ultrasound applications for the extraction, identification and delivery of food proteins and bioactive peptides. *Trends in Food Science & Technology, 46*(1), 60-67.

Kadam, S. U., Tiwari, B. K., & O'Donnell, C. P. (2013). Application of novel extraction technologies for bioactives from marine algae. *Journal of Agricultural and Food Chemistry, 61*(20), 4667-4675.

Kan, L., Hu, Q., Chao, Z., Song, X., & Cao, X. (2006). Chemical constituents of unsaponifiable matter from seed oil of *Momordica cochinchinensis*. *China journal of Chinese materia medica, 31*(17), 1441-1444.

Kang, J. M., Kim, N., Kim, B., Kim, J.-H., Lee, B.-Y., Park, J. H., . . . Kim, J. S. (2009). Gastroprotective action of cochinchina momordica seed extract is mediated by activation of CGRP and inhibition of cPLA2/5-LOX pathway. *Digestive Diseases and Sciences, 54*(12), 2549-2560.

Kang, J. M., Kim, N., Kim, B., Kim, J.-H., Lee, B.-Y., Park, J. H., . . . Jung, H. C. (2010). Enhancement of gastric ulcer healing and angiogenesis by cochinchina Momordica seed extract in rats. *Journal of Korean Medical Science, 25*(6), 875-881.

Kasote, D. M., Badhe, Y. S., & Hegde, M. V. (2013). Effect of mechanical press oil extraction processing on quality of linseed oil. *Industrial Crops and Products, 42*, 10-13.

Kaufmann, B., & Christen, P. (2002). Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction. *Phytochemical Analysis, 13*(2), 105-113.

Kennedy, A. R. (1998a). The Bowman-Birk inhibitor from soybeans as an anticarcinogenic agent. *The American journal of clinical nutrition, 68*(6), 1406S-1412S.

Kennedy, A. R. (1998b). Chemopreventive agents: protease inhibitors. *Pharmacology & Therapeutics, 78*(3), 167-209.

Kerem, Z., German-Shashoua, H., & Yarden, O. (2005). Microwave-assisted extraction of bioactive saponins from chickpea (Cicer arietinum L). *Journal of the Science of Food and Agriculture, 85*(3), 406-412.

Kha, T. C., Nguyen, M. H., & Roach, P. D. (2010). Effects of spray drying conditions on the physicochemical and antioxidant properties of the Gac (Momordica cochinchinensis) fruit aril powder. *Journal of Food Engineering, 98*(3), 385-392.

Kha, T. C., Nguyen, M. H., Roach, P. D., Parks, S. E., Murray, C. T., Gale, D. L., . . . Stathopoulos, C. E. (2013a). Gac fruit: nutrient and phytochemical composition, and options for processing. *Food Reviews International, 29*(1), 92-106.

Kha, T. C., Nguyen, M. H., Roach, P. D., & Stathopoulos, C. E. (2013b). Effects of Gac aril microwave processing conditions on oil extraction efficiency, and β-carotene and lycopene contents. *Journal of Food Engineering, 117*(4), 486-491.

Kha, T. C., Nguyen, M. H., Roach, P. D., & Stathopoulos, C. E. (2014a). Effect of drying pre-treatments on the yield and bioactive content of oil extracted from Gac aril. *International Journal of Food Engineering, 10*(1), 103-112.

Kha, T. C., Nguyen, M. H., Roach, P. D., & Stathopoulos, C. E. (2014b). Microencapsulation of gac oil by spray drying: Optimization of wall material concentration and oil load using response surface methodology. *Drying Technology, 32*(4), 385-397.

Kha, T. C., Phan, H. T., & Nguyen, M. H. (2014c). Effects of pre-treatments on the yield and carotenoid content of Gac oil using supercritical carbon dioxide extraction. *Journal of Food Engineering, 120*, 44-49.

Kim, B. C., Kim, J.-h., Yun, S.-J., Jang, G.-U., & Pyo, S. (2010). Wound-healing agent containing momordicae semen extract. In: Google Patents.

Kim, B. C., Kim, J. H., Yun, S. J., Noh, E. J., Jang, G. U., Han, C.-k., . . . Kwak, W.-j. (2012). Anti-gastritis and anti-ulcer agent containing Momordicae semen extract and momordica saponin I isolated from the same. In: Google Patents.

King, J. (2000). Advances in critical fluid technology for food processing. *Food Science and Technology Today, 14*(4), 186-191.

Klomklao, S., Benjakul, S., Kishimura, H., & Chaijan, M. (2011). Extraction, purification and properties of trypsin inhibitor from Thai mung bean (*Vigna radiata* (L.) R. Wilczek). *Food Chemistry, 129*(4), 1348-1354.

Kowalska, J., Pszczoła, K., Wilimowska-Pelc, A., Lorenc-Kubis, I., Zuziak, E., Ługowski, M., . . . Lesner, A. (2007). Trypsin inhibitors from the garden four o'clock (Mirabilis jalapa) and spinach (Spinacia oleracea) seeds: isolation, characterization and chemical synthesis. *Phytochemistry, 68*(11), 1487-1496.

Kreutzmann, P., Schulz, A., Ständker, L., Forssmann, W.-G., & Mägert, H.-J. (2004). Recombinant production, purification and biochemical characterization of domain 6 of LEKTI: a temporary Kazal-type-related serine proteinase inhibitor. *Journal of Chromatography B, 803*(1), 75-81.

Kroes, B., Van den Berg, A., Quarles, v. U. H., Van Dijk, H., & Labadie, R. (1992). Anti-inflammatory activity of gallic acid. *Planta Medica, 58*(6), 499-504.

Kubiak, A., Jakimowicz, P., & Polanowski, A. (2009). A Kazal-type serine proteinase inhibitor from chicken liver (clTI-1): purification, primary structure, and inhibitory properties. *International Journal of Biological Macromolecules, 45*(2), 194-199.

Kubola, & Siriamornpun, S. (2008). Phenolic contents and antioxidant activities of bitter gourd (Momordica charantia L.) leaf, stem and fruit fraction extracts in vitro. *Food Chemistry, 110*(4), 881-890.

Kubola, & Siriamornpun, S. (2011). Phytochemicals and antioxidant activity of different fruit fractions (peel, pulp, aril and seed) of Thai gac (*Momordica cochinchinensis* Spreng). *Food Chemistry, 127*(3), 1138-1145.

Kubota, K., Sato, M., Murakami, T., & Yamagishi, T. (1971). Pharmacological studies on the saponin isolated from the seed of Momordica cochinchinensis Sprenger. *Pharm Soc Jap J*.

Kunitz, M. (1946). A spectrophotometric method for the measurement of ribonuclease activity. *J. biol. Chem, 164*, 563-568.

Lang, Q., & Wai, C. M. (2001). Supercritical fluid extraction in herbal and natural product studies—a practical review. *Talanta, 53*(4), 771-782.

Laskowski Jr, M., & Kato, I. (1980). Protein inhibitors of proteinases. *Annual Review of Biochemistry, 49*(1), 593-626.

Laughton, M. J., Halliwell, B., Evans, P. J., Robin, J., & Hoult, S. (1989). Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin: effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. *Biochemical Pharmacology, 38*(17), 2859-2865.

Le, A. V., Parks, S. E., Nguyen, M. H., & Roach, P. D. (2018). Effect of solvents and extraction methods on recovery of bioactive compounds from defatted Gac (*Momordica cochinchinensis* Spreng.) seeds. *Separations, 5*(3), 39.

Le, V., & Nguyen, G. (1999). Selected medicinal plants in Vietnam. *National Institute of Materia Medica Science and Technology Publishing House, Hanoi*.

Lee, J., Ye, L., Landen, W. O., & Eitenmiller, R. R. (2000). Optimization of an extraction procedure for the quantification of vitamin E in tomato and broccoli using response surface methodology. *Journal of Food Composition and Analysis, 13*(1), 45-57.

Li, J., Zu, Y.-G., Fu, Y.-J., Yang, Y.-C., Li, S.-M., Li, Z.-N., & Wink, M. (2010). Optimization of microwave-assisted extraction of triterpene saponins from defatted residue of yellow horn (Xanthoceras sorbifolia Bunge.) kernel and evaluation of its antioxidant activity. *Innovative Food Science & Emerging Technologies, 11*(4), 637-643.

Lim, T. K. (2012). Momordica cochinchinensis. In T. K. Lim (Ed.), *Edible Medicinal And Non-Medicinal Plants* (Vol. 2, pp. 369-380). Dordrecht, the Netherlands: Springer.

Lin, Z. Y., Liu, X., Yang, F., & Yu, Y. Q. (2012). Structural characterization and identification of five triterpenoid saponins isolated from *Momordica cochinchinensis* extracts by liquid chromatography/tandem mass spectrometry. *International Journal of Mass Spectrometry, 328*, 43-66.

Liu, H.-R., Meng, L.-Y., Lin, Z.-Y., Shen, Y., Yu, Y.-Q., & Zhu, Y.-Z. (2012). Cochinchina momordica seed extract induces apoptosis and cell cycle arrest in human gastric cancer cells via PARP and p53 signal pathways. *Nutrition and Cancer, 64*(7), 1070-1077.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265-275.

Lu, T., Gaspar, F., Marriott, R., Mellor, S., Watkinson, C., Al-Duri, B., . . . Santos, R. (2007). Extraction of borage seed oil by compressed CO2: effect of extraction parameters and modelling. *The Journal of Supercritical Fluids, 41*(1), 68-73.

Mahatmanto, T., Mylne, J. S., Poth, A. G., Swedberg, J. E., Kaas, Q., Schaefer, H., & Craik, D. J. (2014). The evolution of Momordica cyclic peptides. *Molecular Biology and Evolution, 32*(2), 392-405.

Mahatmanto, T., Poth, A. G., Mylne, J. S., Craik, D. J. . (2014). A comparative study of extraction methods reveals preferred solvents for cystine knot peptide isolation from *Momordica cochinchinensis* seeds. *Fitoterapia, 95*, 22-33.

Makkar, H. P., Siddhuraju, P., & Becker, K. (2007). Trypsin Inhibitor. In *Plant secondary metabolites* (pp. 1-6). New York: Humana Press.

Martínez, J., & Carolina de Aguiar, A. (2014). Extraction of triacylglycerols and fatty acids using supercritical fluids-review. *Current Analytical Chemistry, 10*(1), 67-77.

Masayo, I., Hikaru, O., Tatsuo, Y., Masako, T., Yoshie, R., Shuji, H., . . . Ryuichi, H. (1985). Studies on the constituents of *Momordica cochinchinensis* Spreng. I. Isolation and characterization of the seed saponins, Momordica saponins I and II. *Chem. Pharm. Bull, 33*(2), 464-478.

Mattea, F., Martín, Á., & Cocero, M. J. (2009). Carotenoid processing with supercritical fluids. *Journal of Food Engineering, 93*(3), 255-265.

Matthaus, B., Vosmann, K., Pham, L. Q., & Aitzetmüller, K. (2003). FA and tocopherol composition of Vietnamese oilseeds. *Journal of the American Oil Chemists' Society, 80*(10), 1013-1020.

McClements, D. J. (1995). Advances in the application of ultrasound in food analysis and processing. *Trends in Food Science & Technology, 6*(9), 293-299.

Meng, L.-Y., Liu, H.-R., Shen, Y., Yu, Y.-Q., & Tao, X. (2012). Cochinchina momordica seed extract induces G2/M arrest and apoptosis in human breast cancer MDA-MB-231 cells by modulating the PI3K/Akt pathway. *Asian Pacific Journal of Cancer Prevention, 13*, 3483-3488.

Merve, D. K., & Oguz, B. (2016). Extraction of Saponins from Soapnut (Sapindus Mukorossi) and Their Antimicrobial Properties. *World Journal of Research and Review, 2*(5), 89-93.

Middleton, E., Kandaswami, C., & Theoharides, T. C. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews, 52*(4), 673-751.

Mitra, P., Ramaswamy, H. S., & Chang, K. S. (2009). Pumpkin (Cucurbita maxima) seed oil extraction using supercritical carbon dioxide and physicochemical properties of the oil. *Journal of Food Engineering, 95*(1), 208-213.

Mukherjee, A. K., Basu, S., Sarkar, N., & Ghosh, A. C. (2001). Advances in cancer therapy with plant based natural products. *Current Medicinal Chemistry, 8*(12), 1467-1486.

Müller, J., & Heindl, A. (2006). Drying of medicinal plants. *Frontis*, 237-252.

Myers, Anderson-Cook, C. M., & Montgomery, D. C. (2014). *Response Surface Methodology : Process and Product Optimization Using Designed Experiments* (3rd ed.). Somerset, NJ, USA: Wiley.

Myers, & Montgomery, D. (2002). Response Surface Methodology, John Wiley&Sons. *Inc., USA*.

National Cancer Institute. (2017). Types of Cancer Treatment. Retrieved from https://www.cancer.gov/about-cancer/treatment/types

Naumovski, N. (2014). Bioactive composition of plants and plant foods. *Plant Bioactive Compounds for Pancreatic Cancer Prevention and Treatment; Scarlett, CJ, Vuong, QV, Eds*, 81-115.

Newman, D. J., Cragg, G. M., & Snader, K. M. (2003). Natural products as sources of new drugs over the period 1981− 2002. *Journal of Natural Products, 66*(7), 1022-1037.

NewsBank. (2012, 2012 Nov 19). Australia: SK Chemicals Receives Patent for 'Anti-gastritis and anti-ulcer agent containing Momordicae semen extract and momordica saponin I isolated from the same'. *Gov Australia Live (Australia)*.

Nguyen, V. T., Vuong, Q. V., Bowyer, M. C., Van Altena, I. A., & Scarlett, C. J. (2017). Microwave-Assisted Extraction for Saponins and Antioxidant Capacity from Xao Tam Phan (Paramignya trimera) Root. *Journal of Food Processing and Preservation, 41*(2).

Nhung, D. T. T., Bung, P. N., Ha, N. T., & Phong, T. K. (2010). Changes in lycopene and beta carotene contents in aril and oil of gac fruit during storage. *Food Chemistry, 121*(2), 326-331.

Nijveldt, R. J., Van Nood, E., Van Hoorn, D. E., Boelens, P. G., Van Norren, K., & Van Leeuwen, P. A. (2001). Flavonoids: a review of probable mechanisms of action and potential applications. *The American journal of clinical nutrition, 74*(4), 418-425.

Nobre, B. P., Palavra, A. F., Pessoa, F. L., & Mendes, R. L. (2009). Supercritical CO2 extraction of trans-lycopene from Portuguese tomato industrial waste. *Food Chemistry, 116*(3), 680-685.

Nyam, K. L., Tan, C. P., Karim, R., Lai, O. M., Long, K., & Man, Y. B. C. (2010). Extraction of tocopherol-enriched oils from Kalahari melon and roselle seeds by supercritical fluid extraction (SFE-CO 2). *Food Chemistry, 119*(3), 1278-1283.

O'Keefe, B. R. (2001). Biologically Active Proteins from Natural Product Extracts 1. *Journal of Natural Products, 64*(10), 1373-1381.

Oakenfull, D. (1981). Saponins in food—a review. *Food Chemistry, 7*(1), 19-40.

Oliveira, R., Rodrigues, M. F., & Bernardo-Gil, M. G. (2002). Characterization and supercritical carbon dioxide extraction of walnut oil. *Journal of the American Oil Chemists' Society, 79*(3), 225-230.

Ong, K. C., & Khoo, H.-E. (1997). Biological effects of myricetin. *General Pharmacology: The Vascular System, 29*(2), 121-126.

Onyilagha, J. C., Grotewold, E., & Pandalai, S. (2004). The biology and structural distribution of surface flavonoids. *Recent research developments in plant science. Vol. 2*, 53-71.

Özkal, S., Yener, M., & Bayındırlı, L. (2005a). Response surfaces of apricot kernel oil yield in supercritical carbon dioxide. *LWT-Food Science and Technology, 38*(6), 611-616.

Özkal, S., Yener, M., Salgın, U., & Mehmetoğlu, Ü. (2005b). Response surfaces of hazelnut oil yield in supercritical carbon dioxide. *European Food Research and Technology, 220*(1), 74-78.

Parajó, J., Santos, V., Dominguez, H., Vázquez, M., & Alvarez, C. (1995). Protein concentrates from yeast cultured in wood hydrolysates. *Food Chemistry, 53*(2), 157-163.

Parekh, J., Karathia, N., & Chanda, S. (2006). Evaluation of antibacterial activity and phytochemical analysis of Bauhinia variegata L. bark. *African Journal of Biomedical Research, 9*(1).

Parks, S. E., Murray, C. T., Gale, D. L., Al-Khawaldeh, B., & Spohr, L. J. (2013). Propagation and production of Gac (Momordica cochinchinensis Spreng.), a greenhouse case study. *Experimental Agriculture, 49*(02), 234-243.

Pelozo, M. I. d. G., Cardoso, M. L. C., & Mello, J. C. P. d. (2008). Spectrophotometric determination of tannins and caffeine in preparations from Paullinia cupana var. sorbilis. *Brazilian Archives of Biology and Technology, 51*(3), 447-451.

Pereira, C. G., & Meireles, M. A. A. (2010). Supercritical fluid extraction of bioactive compounds: fundamentals, applications and economic perspectives. *Food and Bioprocess Technology, 3*(3), 340-372.

Pérez-Cruz, K., Moncada-Basualto, M., Morales-Valenzuela, J., Barriga-González, G., Navarrete-Encina, P., Núñez-Vergara, L., . . . Olea-Azar, C. (2018). Synthesis and antioxidant study of new polyphenolic hybrid-coumarins. *Arabian Journal of Chemistry, 11*(4), 525-537.

Pesoti, A. R., Oliveira, B. M. d., Oliveira, A. C. d., Pompeu, D. G., Gonçalves, D. B., Marangoni, S., . . . Granjeiro, P. A. (2015). Extraction, purification and characterization of inhibitor of trypsin from Chenopodium quinoa seeds. *Food Sci Technol, 35*(4), 588-597.

Pham, H. N. T., Nguyen, V., Vuong, Q., Bowyer, M. C., & Scarlett, C. J. (2017a). Bioactive compound yield and antioxidant capacity of Helicteres hirsuta Lour. stem as affected by various solvents and drying methods. *Journal of Food Processing and Preservation, 41*(1).

Pham, H. N. T., Vuong, Q. V., Bowyer, M. C., & Scarlett, C. J. (2017b). Effect of extraction solvents and thermal drying methods on bioactive compounds and antioxidant properties of Catharanthus roseus (L.) G. Don (Patricia White cultivar). *Journal of Food Processing and Preservation, 41*(5).

Pingret, D., Fabiano-Tixier, A. S., & Chemat, F. (2013). Ultrasound-assisted extraction. In R. Mauricio & P. Juliana (Eds.), *Natural product extraction: principles and applications* (Vol. 21, pp. 89-111). Cambridge, UK: RSC Pub.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry, 53*(10), 4290-4302.

Pysz, M., Polaszczyk, S., Leszczyńska, T., & Piątkowska, E. (2012). Effect of microwave field on trypsin inhibitors activity and protein quality of broad bean seeds (*Vicia faba* var. major). *Acta Scientiarum Polonorum Technologia Alimentaria, 11*(2), 193-198.

Rajput, Xiao, C.-w., Hu, S.-h., Arijo, A. G., & Soomro, N. M. (2007). Improvement of the efficacy of influenza vaccination (H5N1) in chicken by using extract of Cochinchina momordica seed (ECMS). *Journal of Zhejiang University Science B, 8*(5), 331-337.

Rajput, Xiao, C., Hu, S., Habib, M., & Soomro, N. (2010). Enhancement of immune responses to infectious bursal disease vaccine by supplement of an extract made from Momordica cochinchinensis (Lour.) Spreng. seeds. *Poultry Science, 89*(6), 1129-1135.

Rao, V. (2012). *Phytochemicals: a global perspective of their role in nutrition and health*: InTech Rijeka.

Rao, A., & Gurfinkel, D. (2000). The bioactivity of saponins: triterpenoid and steroidal glycosides. *Drug Metabolism and Drug Interactions, 17*(1-4), 211-236.

Rassias, G., Kestin, M., & Nestel, P. (1991). Linoleic acid lowers LDL cholesterol without a proportionate displacement of saturated fatty acid. *European Journal of Clinical Nutrition, 45*(6), 315-320.

Reverchon, E., & Marrone, C. (2001). Modeling and simulation of the supercritical CO 2 extraction of vegetable oils. *The Journal of Supercritical Fluids, 19*(2), 161-175.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*(7), 933-956.

Rowland, I. (1999). Optimal nutrition: fibre and phytochemicals. *Proceedings of the Nutrition Society, 58*(02), 415-419.

Saeki, K., & Matsukawa, H. (1976). Process for hardening microcapsules containing hydrophobic oil droplets. In: Google Patents.

Sakwiwatkul, K., Li, R. l., Song, X. m., & Hu, S. h. (2010). Effect of the extract made from Cochinchina momordica seeds on the humoral immune responses of mice to a commercial foot-and-mouth disease vaccine (serotypes O and Asia 1). *Microbiology and Immunology, 54*(1), 31-37.

Salah, N., Miller, N. J., Paganga, G., Tijburg, L., Bolwell, G. P., & Riceevans, C. (1995). Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Archives of Biochemistry and Biophysics, 322*(2), 339-346.

Samuelsson, G., Kyerematen, G., & Farah, M. H. (1985). Preliminary chemical characterization of pharmacologically active compounds in aqueous plant extracts. *Journal of Ethnopharmacology, 14*(2), 193-201.

Santos, O., Corrêa, N., Carvalho Jr, R., Costa, C., & Lannes, S. (2013). Yield, nutritional quality, and thermal-oxidative stability of Brazil nut oil (Bertolletia excelsa HBK) obtained by supercritical extraction. *Journal of Food Engineering, 117*(4), 499-504.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K., & Latha, L. Y. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary, and Alternative Medicines, 8*(1), 1-10.

Schaefer, H., Heibl, C., & Renner, S. S. (2009). Gourds afloat: a dated phylogeny reveals an Asian origin of the gourd family (Cucurbitaceae) and numerous oversea dispersal events. *Proceedings of the Royal Society of London B: Biological Sciences, 276*(1658), 843-851.

Schofield, P., Mbugua, D., & Pell, A. (2001). Analysis of condensed tannins: a review. *Animal Feed Science and Technology, 91*(1-2), 21-40.

Scopes, R. K. (2013). *Protein purification: principles and practice*: Springer Science & Business Media.

Semiz, A., & Sen, A. (2007). Antioxidant and chemoprotective properties of Momordica charantia L.(bitter melon) fruit extract. *African journal of biotechnology, 6*(3).

Shahidi, & Naczk, M. (1995). Antioxidant properties of food phenolics. *Food phenolics: Sources, chemistry, effects and applications*, 235-277.

Shamsi, T. N., Parveen, R., Afreen, S., Azam, M., Sen, P., Sharma, Y., . . . Fatima, S. (2018). Trypsin inhibitors from *Cajanus cajan* and *Phaseolus limensis* possess antioxidant, anti-Inflammatory, and antibacterial activity. *Journal of Dietary Supplements*, 1-12.

Shan, M. D., Hu, L. H., & Chen, Z. L. (2001). A new multiflorane triterpenoid ester from *Momordica cochinchinensis* Spreng. *Natural Product Letters, 15*(2), 139-145.

Silberberg, M. S. (2007). *Principles of general chemistry*: McGraw-Hill Higher Education New York.

Singh, R., Akhtar, N., & Haqqi, T. M. (2010). Green tea polyphenol epigallocatechi3-gallate: Inflammation and arthritis. *Life Sciences, 86*(25), 907-918.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In *Methods in Enzymology* (Vol. 299, pp. 152-178): Elsevier.

Smith, A. B., Daly, N. L., & Craik, D. J. (2011). Cyclotides: a patent review. *Expert Opinion on Therapeutic Patents, 21*(11), 1657-1672.

Smith, C., Van Megen, W., Twaalfhoven, L., & Hitchcock, C. (1980). The determination of trypsin inhibitor levels in foodstuffs. *Journal of the Science of Food and Agriculture, 31*(4), 341-350.

Snyder, J., Friedrich, J., & Christianson, D. (1984). Effect of moisture and particle size on the extractability of oils from seeds with supercritical CO2. *Journal of the American Oil Chemists' Society, 61*(12), 1851-1856.

Sommerhoff, C. P., Avrutina, O., Schmoldt, H.-U., Gabrijelcic-Geiger, D., Diederichsen, U., & Kolmar, H. (2010). Engineered cystine knot miniproteins as potent inhibitors of human mast cell tryptase β. *Journal of Molecular Biology, 395*(1), 167-175.

Song, X., & Hu, S. (2009). Adjuvant activities of saponins from traditional Chinese medicinal herbs. *Vaccine, 27*(36), 4883-4890.

Soria, A. C., & Villamiel, M. (2010). Effect of ultrasound on the technological properties and bioactivity of food: a review. *Trends in Food Science & Technology, 21*(7), 323-331.

Stahl, E., Schuetz, E., & Mangold, H. K. (1980). Extraction of seed oils with liquid and supercritical carbon dioxide. *Journal of Agricultural and Food Chemistry, 28*(6), 1153-1157.

Stauffer, C. E. (1990). Measuring trypsin inhibitor in soy meal: Suggested improvements in the standard method. *Cereal Chemistry, 67*(3), 296-302.

Stracci, F., Minelli, L., D'Alo, D., Fusco-Moffa, I., Falsettini, E., Cassetti, T., . . . La Rosa, F. (2005). Incidence, mortality and survival trends of cutaneous melanoma in Umbria, Italy. 1978-82 and 1994-98. *Tumori, 91*(1), 6-8.

Stuart, G. (2010). Philippine alternative medicine. Manual of some Philippine medicinal plants. In.

Swern, D. (1982). Bailey's industrial oil and fat products.

Tagliazucchi, D., Verzelloni, E., Bertolini, D., & Conte, A. (2010). In vitro bio-accessibility and antioxidant activity of grape polyphenols. *Food Chemistry, 120*(2), 599-606.

Tan, Kha, T. C., Parks, S. E., Stathopoulos, C. E., & Roach, P. D. (2015). Effects of the spray-drying temperatures on the physiochemical properties of an encapsulated bitter melon aqueous extract powder. *Powder Technology, 281*, 65-75.

Tan, Stathopoulos, C. E., Parks, S. E., & Roach, P. D. (2014a). An optimised aqueous extract of phenolic compounds from bitter melon with high antioxidant capacity. *Antioxidants, 3*(4), 814-829.

Tan, C., Man, Y. C., Selamat, J., & Yusoff, M. (2002). Comparative studies of oxidative stability of edible oils by differential scanning calorimetry and oxidative stability index methods. *Food Chemistry, 76*(3), 385-389.

Tan, S. P., Vuong, Q. V., Stathopoulos, C. E., Parks, S. E., & Roach, P. D. (2014b). Optimized aqueous extraction of saponins from bitter melon for production of a saponin-enriched bitter melon powder. *Journal of Food Science, 79*(7), E1372-E1381.

Tang, W., & Eisenbrand, G. (1992). Momordica cochinchinensis (Lour.) Spreng. and M. grosvenori Swingle. In *Chinese Drugs of Plant Origin* (pp. 665-668): Springer.

Tatke, P., & Jaiswal, Y. (2011). An overview of microwave assisted extraction and its applications in herbal drug research. *Res. J. Med. Plant, 5*(1), 21-31.

Tava, A., Mella, M., Bialy, Z., & Jurzysta, M. (2003). Stability of saponins in alcoholic solutions: ester formation as artifacts. *Journal of Agricultural and Food Chemistry, 51*(7), 1797-1800.

Taylor, L. T. (1996). *Supercritical fluid extraction* (Vol. 4): Wiley-Interscience.

Terao, J. (2008). Highlight on polyphenols and health. *Archives of Biochemistry and Biophysics, 476*(2), 101.

Tien, P. G., Kayama, F., Konishi, F., Tamemoto, H., Kasono, K., Hung, N. T. K., . . . Kawakami, M. (2005). Inhibition of tumor growth and angiogenesis by water extract of Gac fruit (*Momordica cochinchinensis* Spreng). *International Journal of Oncology, 26*(4), 881-889.

Tominaga, H., Ishiyama, M., Ohseto, F., Sasamoto, K., Hamamoto, T., Suzuki, K., & Watanabe, M. (1999). A water-soluble tetrazolium salt useful for colorimetric cell viability assay. *Analytical Communications, 36*(2), 47-50.

Tomita, K., Machmudah, S., Quitain, A. T., Sasaki, M., Fukuzato, R., & Goto, M. (2013). Extraction and solubility evaluation of functional seed oil in supercritical carbon dioxide. *The Journal of Supercritical Fluids, 79*, 109-113.

Toribio, L., Bernal, J. L., Nozal, M. J., Arnaiz, E., & Bernal, J. (2011). Sequential supercritical fluid extraction of lipids. Application to the obtention of the fatty acid profile of some genetically modified varieties of corn. *Food Analytical Methods, 4*(2), 196-202.

Tran, X. T., Parks, S. E., Roach, P. D., Golding, J. B., & Nguyen, M. H. (2015). Effects of maturity on physicochemical properties of Gac fruit (Momordica cochinchinensis Spreng.). *Food Science & Nutrition*.

Troll, W., & Kennedy, A. R. (1993). *Protease inhibitors as cancer chemopreventive agents*: Plenum Pub Corp.

Tsoi, A. Y., Ng, T. B., & Fong, W. P. (2005). Antioxidative effect of a chymotrypsin inhibitor from Momordica cochinchinensis (Cucurbitaceae) seeds in a primary rat hepatocyte culture. *Journal of Peptide Science, 11*(10), 665-668.

Tsoi, A. Y., Ng, T. B., & Fong, W. P. (2006). Immunomodulatory activity of a chymotrypsin inhibitor from Momordica cochinchinensis seeds. *Journal of Peptide Science, 12*(9), 605-611.

Tsoi, A. Y., Wong, R. C., Ng, T.-B., & Fong, W.-P. (2004). First report on a potato I family chymotrypsin inhibitor from the seeds of a Cucurbitaceous plant, Momordica cochinchinensis. *Biological Chemistry, 385*(2), 185-189.

Tuyen, C. K., Nguyen, M. H., Roach, P. D., & Stathopoulos, C. E. (2014). Microencapsulation of gac oil: optimisation of spray drying conditions using response surface methodology. *Powder Technology, 264*, 298-309.

Uquiche, E., Jeréz, M., & Ortíz, J. (2008). Effect of pretreatment with microwaves on mechanical extraction yield and quality of vegetable oil from Chilean hazelnuts (Gevuina avellana Mol). *Innovative Food Science & Emerging Technologies, 9*(4), 495-500.

Van Hoed, V., Ali, C. B., Slah, M., & Verhé, R. (2010). Quality differences between pre-pressed and solvent extracted rapeseed oil. *European Journal of Lipid Science and Technology, 112*(11), 1241-1247.

Vilkhu, K., Mawson, R., Simons, L., & Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry—A review. *Innovative Food Science & Emerging Technologies, 9*(2), 161-169.

Viswanath, V., Urooj, A., & Malleshi, N. (2009). Evaluation of antioxidant and antimicrobial properties of finger millet polyphenols (Eleusine coracana). *Food Chemistry, 114*(1), 340-346.

Wang, C. K., Gruber, C. W., Cemazar, M. a., Siatskas, C., Tagore, P., Payne, N., . . . Craik, D. J. (2013). Molecular grafting onto a stable framework yields novel cyclic peptides for the treatment of multiple sclerosis. *ACS Chemical Biology, 9*(1), 156-163.

Wang, L., & Weller, C. L. (2006). Recent advances in extraction of nutraceuticals from plants. *Trends in Food Science & Technology, 17*(6), 300-312.

Wang, M., Chen, C., Davis, J., Guo, B., Stalker, H., & Pittman, R. (2010). Assessment of oil content and fatty acid composition variability in different peanut subspecies and botanical varieties. *Plant Genetic Resources, 8*(01), 71-73.

Wei, M. Z. (2011). Saponins. In *Introduction to Natural Products Chemistry* (pp. 125-145): CRC Press.

Wettasinghe, M., & Shahidi, F. (1999a). Antioxidant and free radical-scavenging properties of ethanolic extracts of defatted borage (Borago officinalis L.) seeds. *Food Chemistry, 67*(4), 399-414.

Wettasinghe, M., & Shahidi, F. (1999b). Evening primrose meal: a source of natural antioxidants and scavenger of hydrogen peroxide and oxygen-derived free radicals. *Journal of Agricultural and Food Chemistry, 47*(5), 1801-1812.

WHO. (2010). Cancers, NMH Facts Sheet.

WHO. (2011). CancerStats cancer worldwide. *England, Wales and Scotland: Cancer Research UK*.

Wimalasiri, D. (2015). *Genetic diversity, nutritional and biological activity of Momordica cochinchinensis (Cucurbitaceae).*

Wimalasiri, D., Piva, T., Urban, S., & Huynh, T. (2015). Morphological and genetic diversity of Momordica cochinchinenesis (Cucurbitaceae) in Vietnam and Thailand. *Genetic resources and crop evolution*, 1-15.

Wimalasiri, D., Piva, T., Urban, S., & Huynh, T. (2016). Morphological and genetic diversity of *Momordica cochinchinenesis* (Cucurbitaceae) in Vietnam and Thailand. *Genetic Resources and Crop Evolution, 63*(1), 19-33.

Wong, R. C., Fong, W., & Ng, T. (2004). Multiple trypsin inhibitors from *Momordica cochinchinensis* seeds, the Chinese drug mubiezhi. *Peptides, 25*(2), 163-169.

Wood, J. A., Bernards, M. A., Wan, W.-k., & Charpentier, P. A. (2006). Extraction of ginsenosides from North American ginseng using modified supercritical carbon dioxide. *The Journal of Supercritical Fluids, 39*(1), 40-47.

Wu, & Ng, L.-T. (2008). Antioxidant and free radical scavenging activities of wild bitter melon (Momordica charantia Linn. var. abbreviata Ser.) in Taiwan. *LWT-Food Science and Technology, 41*(2), 323-330.

Wu, J., Lin, L., & Chau, F.-t. (2001). Ultrasound-assisted extraction of ginseng saponins from ginseng roots and cultured ginseng cells. *Ultrasonics Sonochemistry, 8*(4), 347-352.

Xiao, Bao, G., & Hu, S. (2009). Enhancement of immune responses to Newcastle disease vaccine by a supplement of extract of Momordica cochinchinensis (Lour.) Spreng. seeds. *Poultry Science, 88*(11), 2293-2297.

Xiao, C., Hu, S., & Rajput, Z. I. (2007a). Adjuvant effect of an extract from Cochinchina momordica seeds on the immune responses to ovalbumin in mice. *Frontiers of Agriculture in China, 1*(1), 90-95.

Xiao, C., Rajput, Z. I., Liu, D., & Hu, S. (2007b). Enhancement of serological immune responses to foot-and-mouth disease vaccine by a supplement made of extract of Cochinchina momordica seeds. *Clinical and Vaccine Immunology, 14*(12), 1634-1639.

Xu, X. Q. (2003). A chromametric method for the rapid assessment of deep frying oil quality. *Journal of the Science of Food and Agriculture, 83*(13), 1293-1296.

Yasukawa, K., Akihisa, T., Tamura, T., & Takido, M. (1994). Inhibitory effect of karounidiol on 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion. *Biological and Pharmaceutical Bulletin, 17*(3), 460-462.

Yermanos, D., Hemstreet, S., Saleeb, W., & Huszar, C. (1972). Oil content and composition of the seed in the world collection of sesame introductions. *Journal of the American Oil Chemists' Society, 49*(1), 20-23.

Yu, J. S., Kim, J. H., Lee, S., Jung, K., Kim, K. H., & Cho, J. Y. (2017a). Src/Syk-targeted anti-inflammatory actions of triterpenoidal saponins from Gac (*Momordica cochinchinensis*) Seeds. *The American journal of Chinese medicine*, 1-15.

Yu, J. S., Roh, H.-S., Lee, S., Jung, K., Baek, K.-H., & Kim, K. H. (2017b). Antiproliferative effect of Momordica cochinchinensis seeds on human lung cancer cells and isolation of the major constituents. *Revista Brasileira de Farmacognosia, 27*(3), 329-333.

Zhang, H.-F., Yang, X.-H., & Wang, Y. (2011). Microwave assisted extraction of secondary metabolites from plants: current status and future directions. *Trends in Food Science & Technology, 22*(12), 672-688.

Zhao, Han, L.-n., Shan, B.-e., Pan, X.-m., Liu, L.-h., & Li, X.-g. (2010a). Anti-tumor activity of cochinchina momordica seed extract. *Carcinogenesis, Teratogenesis & Mutagenesis, 1*, 005.

Zhao, Han, L.-n., Shang, X.-h., & Shan, B.-e. (2010b). Inhibitory effect of cochinchina momordica seed ethanol extract on proliferation of melanoma B16 cells and its possible mechanism. *Chinese Journal of Cancer Biotherapy, 1*, 003.

Zhao, Han, L. N., Ren, F. Z., Chen, S. H., Liu, L. H., Wang, M. X., . . . Shan, B. E. (2012). An Ester Extract of Cochinchina Momordica Seeds Induces Differentiation of Melanoma B16 F1 Cells via MAPKs Signaling. *Asian Pacific Journal of Cancer Prevention, 13*(8), 3795-3802.

Zhao, S., Kwok, K.-C., & Liang, H. (2007). Investigation on ultrasound assisted extraction of saikosaponins from Radix Bupleuri. *Separation and Purification Technology, 55*(3), 307-312.

Zhen, W. (2006). Extraction of Saponins From Asterias amurensis with Supercritical Carbon Dioxide. *Fine Chemicals, 7*.

Zheng, L., Zhang, Y.-M., Zhan, Y.-Z., & Liu, C.-X. (2013). Momordica cochinchinensis seed extracts suppress migration and invasion of human breast cancer ZR-75-30 cells via down-regulating MMP-2 and MMP-9. *Asian Pacific journal of cancer prevention: APJCP, 15*(3), 1105-1110.

Zhizhong, C. (2007). Progress on Studies and Application of Anticancer Activity of Active Components of Cochinchina Momordicae Seed, Rhubarb, Liquorice Root and Their Preparation. *Chinese Journal of Ethnomedicine and Ethnopharmacy, 2*, 000.

APPENDICES

CONFERENCE ABSTRACT

Anh V. Le*, Paul D. Roach, Minh H. Nguyen and Sophie E. Parks (2015).

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Seeds from the Gac fruit (*Momordica cochinchinensis* L. Spreng.), a rich source of oil and bioactive compounds

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***Abstract*— Seeds from the Gac fruit (*Momordica cochinchinensis* L. Spreng.) have long been used in traditional Chinese medicine and some other countries in Asia for treatment of some skin diseases. Modern studies also show that Gac seeds have some antitumor and anticancer capacity. The important bioactive compounds in Gac seeds that have been identified are phenolics, flavonoids, saponins and trypsin inhibitors. In addition, Gac seeds also contain a significant amount of oil, which needs to be further investigated for use as a source of edible oil. Until now, only the aril surrounding the seeds of the Gac fruit has been utilised for food. The seeds, the fruit flesh (pulp) and the skin, which account for the bulk of the weight of the fruit, are normally discarded and can be an environmental issue. In Vietnam, the amount of discarded Gac seeds is estimated to be around 950 tons per year. If this amount of seeds can be used for the extraction of oil and bioactives, it may add value to the Gac fruit whilst reducing an environmental problem. This paper reviews the components of Gac seeds, its oil, its bioactive compounds and their bioactive activities and the possible application of various extraction techniques for the extraction, purification and production of valuable food and medicinal commercial products.**

***Keywords*— Gac seed; *Momordica cochinchinensis* Spreng; bioactives; oil.**

CONFERENCE POSTER AND ABSTRACT

Anh V. Le*, Paul D. Roach, Minh H. Nguyen and Sophie E. Parks (2016).

Optimisation of Process Parameters for Supercritical Carbon Dioxide Extraction of Oil from Gac Seeds. The 49th Annual AIFST convention, 27 & 28th June, 2016, Brisbane, Australia. Poster Presentation. (E3).

australian institute of food science & technology

CERTIFICATE OF ATTENDANCE

49TH ANNUAL AIFST CONVENTION 2016

PRESENTED TO

Ahn Le

Georgie Aley, Chief Executive Officer
6 July 2016

Optimisation of process parameters for supercritical carbon dioxide extraction of oil from Gac seeds

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Introduction

Seeds from the Gac fruit (*Momordica cochinchinensis* Spreng.) are a rich source of oil and bioactive compounds, such as trypsin inhibitors and saponins. The pharmaceutical properties of Gac seed oil extracts and their use in traditional treatments have long been reported[1]. However, the seeds are currently underutilised as a byproduct from the Gac aril recovery. Supercritical carbon dioxide extraction (SC-CO_2) is considered a promising alternative to conventional solvent extraction and mechanical pressing processes. This extraction technique provides high selectivity, short extraction times and does not use toxic organic solvents[2]. This study aims to optimise the process of oil extraction from Gac seeds, using SC-CO_2. Three parameters, namely temperature, pressure and the flow rate of carbon dioxide, have been optimised using response surface methodology (RSM).

Materials and Methods

Materials

Gac seeds were separated from fresh Gac fruits, which were grown in Ho Chi Minh City, Vietnam. The seeds were vacuum dried at 40°C, de-coated, ground into powder of particle sizes less than 500µm using the 100g Multi-function Disintegrater ST-02A (China). Gac seed particles were measured using the Endecotts Test Sieve (London, England). The powder was then dried in a freeze dryer (FD3, Dynavac, Sydney, NSW, Australia) for 24 hours at -18°C and pressure loading of 10^{-2}Pa. The freeze dried powder contained 53.02±0.10% oil and 0.00±0.01% moisture. Carbon dioxide (99.9%) was supplied by Coregas Pty. Ltd, NSW, Australia.

Methods

Extraction method: A Supercritical Fluid Extraction System (SFX 2-10, Teledyne Isco, USA) was used as shown in Figure 1. Four grams of Gac seed powder were extracted for 10 minutes. Extracts obtained at different conditions were weighed to obtain the yield.

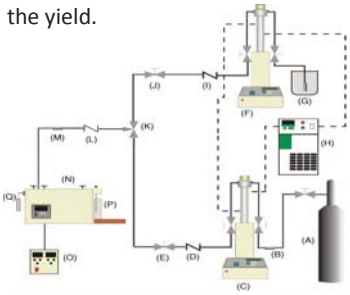


Figure 1: Schematic of the Supercritical Extraction (SFE) Apparatus[3]

(A) CO_2 cylinder; (B & M) filters; (C) Isco 260D syringe pump; (D, L & I) check valves; (E & J) gate valves; (F) Isco 260D syringe pump; (G) modifier vessel with in-line filter; (H) chiller/circulator; (K) valco mixing tee; (N) Isco SFX 2-10 unit; (O) restrictor temperature controller; (P) trapping vessel; (Q) vent valve.

Experiment design: RSM with central composite design was employed to investigate the effect of SFE-CO_2 extraction parameters on the yield of oil. Extracting temperature (60, 70 and 80°C), extracting pressure (5,000, 6,000 and 7,000psi) and supercritical CO_2 flow rate (1.00, 1.75 and 2.50mL/min), were tested. The experimental plan was designed and the results obtained were analyzed using JMP software (Version 11, SAS, Cary, NC, USA). The Student's *T*-test (conducted using the SPSS statistical software version 20 (IBM, Armonk, NY, USA) was used for comparison of the mean analysis. Optimisation was performed using a rotatable central composite design. Sixteen experiments were run in random order. Each experiment was performed in triplicate.

Results and Discussion

Different extraction conditions resulted in significant difference in oil yield. The response variable and the test variables were related by the second-order polynomial equation:

$$Y_{(\%)} = 33.726 + 2.184X_1 - 0.722X_2 - 0.317X_3 + 0.129X_1X_2 - 0.561X_1X_3 - 0.961X_2X_3 - 3.938X_1^2 - 2.778X_2^2 - 0.733X_3^2$$

Equation 1: Mathematical model for prediction of oil yield extracted from Gac seeds, where Y = oil yield (g oil/ 100g Gac seed); X_1 = temperature (°C); X_2 = pressure (psi); X_3 = CO_2 flow rate (mL/min).

Fitting of the model for prediction of oil yield

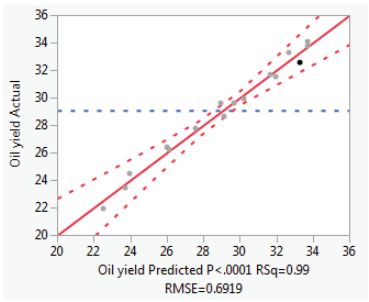
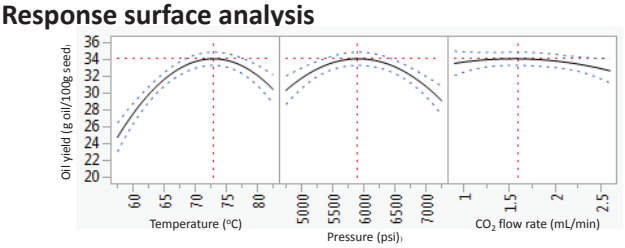


Figure 2: Correlation between predicted and experimental total oil yield of Gac seeds.

As shown in Figure 2, the model oil yield output did not differ significantly from the experimental values (p > 0.05). The coefficient of determination (R^2) of the model was 0.99, indicating that 99% of the experimental data can be predictively matched against the model data for the oil yield. Therefore, the mathematical model was adequate for prediction of the oil yield.

Response surface analysis



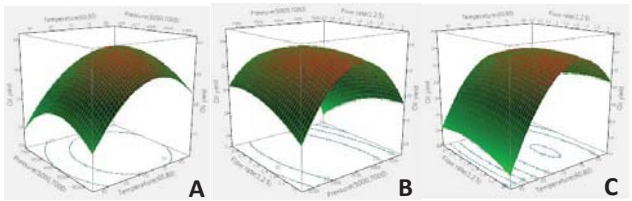


Figure 3: Response surface curve and its contour plot for **(A)** the effects of pressure and temperature at a constant CO_2 flow rate of 1.75mL/min, **(B)** the effects of CO_2 flow rate and pressure at a constant temperature of 70°C, **(C)** the effects of CO_2 flow rate and temperature at a constant pressure of 6,000 psi on the oil yield.

As shown in Figure 3, the temperature is the most significant factor affecting the oil yield in SC-CO_2 extraction, followed by the extraction pressure and the CO_2 flow rate. The interaction between extraction pressure and temperature was significant, for the oil extraction from Gac seeds, but the interactions between temperature and the CO_2 flow rate and between the extraction pressure and the CO_2 flow rate were not.

Conclusions

- RSM was successfully applied to optimise the SC-CO_2 extraction parameters for Gac seed oil yield.
- Pressure, temperature and CO_2 flow rate significantly influenced the oil yield, independently and interactively.
- The optimum process parameters were: 73°C, 5,900 psi, and 1.5mL CO_2/min. Under these conditions, the maximum predicted oil yield was between 33.3 – 34.9%

References

1. Lim, T. K. (2012). Momordica cochinchinensis. In T. K. Lim (Ed.), *Edible Medicinal And Non-Medicinal Plants* (pp. 369-380). Dordrecht, the Netherlands: Springer.

2. Herrero, M., Cifuentes, A., & Ibanez, E. (2006). Sub-and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review. *Food Chemistry, 98*(1), 136-148

3. Wood, J. A., Bernards, M. A., Wan, W.-k., & Charpentier, P. A. (2006). Extraction of ginsenosides from North American ginseng using modified supercritical carbon dioxide. *The Journal of supercritical fluids, 39*(1), 40-47

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Optimisation of process parameters for supercritical carbon dioxide extraction of oil from Gac seeds

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Abstract

Seeds from the Gac fruit (*Momordica cochinchinensis* L. Spreng.) have long been used in traditional Chinese medicine and some other countries in Asia for treatment of many diseases. Modern studies also show that Gac seeds contain a significant amount of oil and possess a wide range of biological activities such as anticancer and antiviral, immuno-enhancing and anti-inflammatory, antioxidant, gastroprotective and antiulcerogenic, ribosome inactivating protein, and trypsin inhibition. Up until now, only the aril surrounding the seeds of the Gac fruit has been utilised for food. The seeds are normally discarded and can be an environmental issue. In Vietnam, the amount of discarded Gac seeds is estimated to be around 750 tons per year. If this amount of seeds can be used for the extraction of oil and bioactives, it may add value to the Gac fruit whilst reducing an environmental problem. In this paper, the optimized oil extraction and characterization of the extracted oil were performed. Supercritical fluid extraction with carbon dioxide technique was implemented. Effects of pressure, temperature and the flow rate of supercritical carbon dioxide (SC-CO_2) were investigated. Response surface methodology was used to determine the effects of pressure, temperature and CO_2 flow rate on Gac seed oil yield. The oil yield was represented by a second order response surface equation (R^2 = 0.986) using the central composite design of experiments. The oil yield increased significantly with increasing pressure ($p<0.05$), temperature ($p<0.01$) and SC-CO_2 flow rate ($p<0.01$). The maximum oil yield from the response surface equation was predicted as 42.85g oil/100g Gac seeds for 10min extraction of 4g Gac seed particles (particle diameter<500µm) at 6,000 psi pressure and 63°C temperature, with 2mL/min CO_2 flow rate. Total extraction time at these conditions was predicted to be 10 minutes. The physical and chemical characterization of the oil suggested that it may contain a significant percentage of bioactives.

Key words: *Momordica cochinchinensis,* oil, supercritical carbon dioxide, extraction, response surface methodology