Extraction and utilization of olive waste biophenols; antioxidant and anti-pancreatic cancer activity

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BFSHN(Hons)

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Originality

I hereby certify that to the best of my knowledge and belief this thesis is my own work and contains no material previously published or written by another person except where due references and acknowledgements are made. It contains no material which has been previously submitted by me for the award of any other degree or diploma in any university or other tertiary institution.

Chloe Desiree Goldsmith

30/11/2017

Thesis by Publication

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 statement from each co-author, endorsed in writing by the Faculty Assistant Dean (Research Training), attesting to my contribution to any jointly authored papers.
*Refer to clause 39.2 of the Rules Governing Research Higher Degrees for acceptable papers.

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List of publications included as a part of this thesis

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Major research papers in peer reviewed journals

1. **Chloe D. Goldsmith**, Quan V. Vuong, Costas E. Stathopoulos, Paul D. Roach, Christopher J. Scarlett: *Optimization of the Aqueous Extraction of Phenolic Compounds from Olive Leaves*. **Antioxidants**. 12/**2014**; 3(4-4):700-712. DOI:10.3390/antiox3040700
2. **Chloe D. Goldsmith**, Quan V. Vuong, Costas E. Stathopoulos, Paul D. Roach, Christopher J. Scarlett: *Ultrasound Increases the Aqueous Extraction of Phenolic Compounds with High Antioxidant Activity from Olive Pomace*. **LWT-Food Science and Technology**. 3/**2018**; 89: 284-290). DOI: 10.1016/j.lwt.2017.10.065
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3. **Chloe D. Goldsmith**, Quan V. Vuong, Elham Sadeqzadeh, Costas E. Stathopoulos, Paul D. Roach, Christopher J. Scarlett: *Phytochemical Properties and Anti-Proliferative Activity of Olea europaea L. Leaf Extracts against Pancreatic Cancer Cells*. **Molecules.** 07/**2015**; 20:12992-13004. DOI:10.3390/molecules200712992
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Statement of contribution of others

30/11/2017

To whom it may concern,

This is to confirm that Chloe Desiree Goldsmith has contributed to the series of major research papers, which are submitted as a part of her PhD thesis as shown in each paper below.

Sincerely,

Date: 29/11/17
A/Prof. Christopher J. Scarlett

Date: 29/11/17
Dr Paul D. Roach

Date: 29/11/17
Prof. Costas E. Stathopoulos

Date: 29/11/2017
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Date: 30/11/2017
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Other publications related to this thesis

1. Benjamin Munro, Quan V Vuong, Anita C Chalmers, **Chloe D Goldsmith**, Michael C Bowyer, Christopher J Scarlett: *Phytochemical, Antioxidant and Anti-Cancer Properties of Euphorbia tirucalli Methanolic and Aqueous Extracts*. 10/**2015**; 4(4):647-662. DOI:10.3390/antiox4040647
2. Tiffany L.K. Chuen, Quan V. Vuong, Sathira Hirun, Michael C. Bowyer, **Chloe D. Goldsmith**, Christopher J. Scarlett: *Optimum aqueous extraction conditions for preparation of a phenolic-enriched Davidson's plum (Davidsonia pruriens F. Muell) extract*. International Journal of Food Science & Technology 08/**2015**; 50(11). DOI:10.1111/ijfs.12915
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1. Chloe D Goldsmith, Quan V Vuong, Costas E Stathopoulos, Paul D Roach, Christopher J Scarlett. Utilization of industrial food waste: Olive pomace. Australian Institute of Food Science and Technology (2017).
2. Chloe D Goldsmith[1], Helen Jankowski, Danielle Bond[1], Costas E. Stathopoulos[2], Paul D. Roach[1] and Christopher J Scarlett. The olive biophenol oleuropein selectively induces apoptosis in pancreatic cancer cells *in vitro.* American Association of Cancer Research. Washington DC, The United States of America (April, 2017).
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Abstract

Olive oil processing leads to thousands of tonnes of waste being produced each year. These waste products (olive pomace and leaf) are usually dumped in landfill; this is causing environmental concerns due to their high concentration of biophenols including oleuropein, hydroxytyrosol, luteolin and apigenin. However, these compounds have known health benefits such as anti-atherogenic, anti-inflammatory as well as anti-cancer properties. Therefore, the extraction and utilization of these biophenols constitutes a viable use for olive waste products while simultaneously reducing the environmental impact of olive processing. Additionally, the anti-cancer potential of olive biophenols has been previously described *in vitro* for cancers of the breast, colon and prostate. However, there has not yet been any investigation into the activity of olive biophenols in pancreatic cancer. Therefore, the objectives of the present study were: to optimize the aqueous extraction of biophenols from olive waste products, to determine if the aqueous olive waste extracts were a source of major olive biophenols and to assess the anti-pancreatic cancer potential of olive waste extracts and major olive waste biophenols. Water extracts and methanol extracts were prepared from olive leaf and olive pomace waste. All extracts contained a high concentration of phenolic compounds with high antioxidant capacity. The olive leaf extracts (water extract and methanol extract) both contained high concentrations of the major biophenol oleuropein, however, only the methanol extract contained luteolin or apigenin. Additionally, oleuropein was identified in the methanol pomace extract but not in the water extract. Crude olive leaf and pomace extracts displayed anti-cancer activity. Treatment with all extracts (0-200 μg/mL) dose-dependently reduced the proliferation of pancreatic cancer cells (MIA PaCa-2, BxPC-3, CFPAC-1), while a protective effect was observed in non-tumorigenic (HPDE) cells at low doses (50 μg/mL). Furthermore, pure olive waste biophenols displayed selective toxicity. Oleuropein and hydroxytyrosol reduced proliferation, induced cell cycle arrest and induced apoptosis in MIA PaCa-2 cells by increasing the Bax/Bcl-2 ratio and subsequent activation of caspase 3/7. Gene expression analysis suggested that *JUN* and *FOS* are involved in oleuropein and hydroxytyrosol induced apoptosis. Additionally, oleuropein exhibited an apparent protective effect on HPDE cells by a decrease in the Bax/Bcl-2 ratio and decrease in expression of *ADAMTS-1*. Luteolin and apigenin caused cell cycle arrest at G2 and increased expression of caspase 3/7 inducing apoptosis in a number of different pancreatic cancer cell lines (BxPC-3, CFPAC-1, MIA PaCa-2 and ASPC-1); however, toxicity to HPDE cells was higher than cancer cells. In conclusion, water is an effective extraction solvent for the recovery of certain biophenols from olive leaves; however, organic solvents are more suitable for the extraction of major biophenols from pomace waste. Crude olive waste extracts as well as major biophenols displayed selective toxicity towards pancreatic cancer cells while displaying limited toxicity/ a protective effect towards non-tumorigenic pancreas cells. These data highlight the potential of olive biophenols as anti-pancreatic cancer agents, which, warrant further investigation in other models of pancreatic cancer.

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Abbreviations

5-FU, 5-fluorouracil

AMPK, Adenosine monophosphate-activated protein kinase

AP-1, Activator Protein 1

Bax, Bcl-2 like protein 4

Bcl-2, B-cell lymphoma protein 2

COX-2, Cyclooxygenase 2

EGRF, Epidermal Growth Factor Receptor

EMT, epithelial to mesenchymal transition

ER, Endoplasmic Reticulum

FASN, Fatty Acid Synthase

GBM, Glioblastoma Multiform

HGF, Hepatocyte Growth Factor

HPLC, High Pressure Liquid Chromatography

iNOS, Nitrous Oxide Synthanse

LLP, Liquid Liquid Partitioning

MAPK, mitogen activated protein kinase)

MGMT, Methyltransferase

MMP Matrix

NF-kB. nuclear factor kappa-light-chain-enhancer of activated B cells

OMWW, olive mill wastewater

PLE, Pressurised Liquid Extraction

PP2A, protein phosphatase 2 activity

ROS, Reactive Oxygen Species

RSM, Response Surface Methodology

SPE, Solid Phase Extraction

TNF-α, Tumour Necrosis Factor Alpha

CHAPTER 1

1. INTRODUCTION

1.1 Research background

Pancreatic cancer is a devastating disease with a 5-year survival rate of less than 8% (Wolfgang, Herman et al. 2013). Resistance to conventional treatment and toxicity of currently available chemotherapy drugs highlight the need for the development of new therapies for pancreatic cancer.

Epidemiological data suggests that adherence to a Mediterranean style diet is linked with a reduced risk for pancreatic cancer (Bosetti, Turati et al. 2013). One characteristic difference between this diet and other healthy diets is the high consumption of olives and olive oil (Trichopoulou, Lagiou et al. 2000). Oleuropein and hydroxytyrosol, the main biophenols in olive products, and luteolin and apigenin, the main flavonoids in olive products, have been investigated for their health applications including anti-atherogenic (Covas 2007), anti-inflammatory (Fito, Cladellas et al. 2008, Lin, Shi et al. 2008) and anti-cancer (Hassan, Elamin et al. 2012, Li, Han et al. 2014); hence, these phenolic compounds constitute a viable starting place for compounds with anti-pancreatic cancer activity.

Olive oil production has grown rapidly in Australia in recent decades. However, alongside this thriving industry is a growing waste problem. Currently, olive wastes (pomace, wastewater and leaves) remain unutilized and are often dumped in landfill or remain in large dams/piles on production plants. This causes significant environmental concerns due, in part, to the high concentration of biophenols in olive wastes (Goldsmith, Stathopoulos et al. 2014). Therefore, the extraction of olive biophenols constitutes a potential use for olive waste products. While there are a number of published extraction protocols for phenolic compounds from olive leaves and olive pomace waste, the majority use organic solvents (Şahin and Şamlı 2013) or elaborate extraction systems (Rubio-Senent, Martos et al. 2015) which can be difficult or expensive to scale-up to an industrial scale. Moreover, solvents can often be more difficult to dispose of than olive waste itself. This highlights the need for the development of simple, “green” extraction protocols. Furthermore, the anti-cancer properties of oleuropein and hydroxytyrosol have been investigated *in vitro* and *in vivo* for cancers of the breast (Elamin, Elmahi et al. 2017), prostate (Acquaviva, Di Giacomo et al. 2012, Samara,

Christoforidou et al. 2017) and colon (Giner, Recio et al. 2016). However, there has not yet been any investigation into the anti-pancreatic cancer potential of these olive biophenols.

1.2. Research content

This research has been divided into 3 major parts: (1) optimization of the extraction of biophenols with antioxidant activity from *Olea europaea* leaf and pomace waste using green extraction methods; (2) biophenol content and cytotoxicity of crude *Olea europaea* leaf and pomace extracts; (3) anti-pancreatic cancer potential of major biophenols from *Olea europaea* leaf and pomace.

1.3.1 Research hypothesis

Olive biophenols display antioxidant and cytotoxic activity *in vitro.*

1.3.2 Research aims

The specific aims and outcomes of the project are as follows:

AIM 1: To investigate the effect of different extraction parameters on the aqueous extraction of biophenols with high antioxidant capacity from olive waste products (pomace and leaves)

EXPECTED OUTCOMES: Determination of the optimal combination of parameters for the highest retention of phenolic compounds with high antioxidant capacity from olive pomace and olive leaves using water as an extraction solvent.

AIM 2: To determine if crude aqueous olive leaf and pomace extracts optimized in Aim 1 have potential as a source of major biophenols (oleuropein, hydroxytyrosol, tyrosol, luteolin or apigenin).

EXPECTED OUTCOMES: The presence of major biophenols in aqueous and methanol olive leaf and olive pomace extracts will be identified, and the efficacy of water as an extraction solvent for the extraction of major biophenols from olive pomace and olive leaf will be determined.

AIM 3: To determine the cytotoxicity of biophenol containing crude olive leaf and pomace extracts identified in Aim 2.

EXPECTED OUTCOMES: The growth inhibition of pancreatic cancer cells when treated with biophenol containing crude olive leaf and pomace extracts will be determined.

AIM 4: To determine the *in vitro* anti-pancreatic cancer potential of the major olive waste biophenols.

EXPECTED OUTCOMES: Determination of the effect of the major olive waste biophenols on pancreatic cancer cells *in vitro.*

2. LITERATURE REVIEW

2.1. Introduction

2.1.1. The Mediterranean Diet

The "Mediterranean diet" is a well-known example of a diet where consumption is associated with a reduced risk for chronic disease. Studies have shown that adherence to a Mediterranean diet is associated with a reduced risk of morbidity and mortality from cardiovascular disease (de Lorgeril, Salen et al. 1999, Cicerale, Conlan et al. 2009), atherosclerosis (Visioli, Borsani et al. 2000) and certain types of cancer (Fortes, Forastiere et al. 2003, Kapiszewska, Soltys et al. 2005).

Epidemiological studies have consistently shown that a diet high in fruits and vegetables is strongly associated with a reduced risk for chronic diseases such as cancer. A large randomised controlled trial found that a Mediterranean diet was more effective at reducing complications after myocardial infarction than a traditionally administered low fat diet (de Lorgeril, Salen et al. 1999). Another study estimated that up to 25% of the incidence of colorectal cancer, 15% of the incidence of breast cancer and approximately 10% of the incidence of prostate, pancreas, and endometrial cancer could potentially be prevented if developed Western countries were to consume a traditional Mediterranean diet (Trichopoulou, Lagiou et al. 2000). Additionally, adherence to a Mediterranean diet has been positively associated with reduced risk for pancreatic cancer (Bosetti, Turati et al. 2013).

Similar to most "healthy" diets, the Mediterranean diet is characterized by a high consumption of fruits, vegetables, fish, legumes and whole grains. However, there is one main difference; fat consumption is high. It accounts for approximately 40% of caloric intake with the main source of which being olive oil (Stark and Madar 2002). Originally it was thought that the health benefits associated with the consumption of olive oil were attributed to oleic acid (the main fatty acid found in olive oil). However, it is now believed to be, in part, due to the presence of a unique combination of biophenols found in olive oil (Serra-Majem, Ngo de la Cruz et al. 2003).

2.2. Pancreatic cancer

Presently many cancers are still incurable, especially at advanced stages when cancer cells metastasise throughout the body. Pancreatic cancer is considered as one of the most devastating cancers as it presents late and is rapidly progressive (Scarlett, Smith et al. 2006). Having a dismal 5-year survival rate of less than 8%, it remains as the third most common gastrointestinal cancer and the fourth most common cause of cancer death (Chang, Merrett et al. 2008, Siegel, Naishadham et al. 2012, Vuong, Scarlett et al. 2012). Molecular analysis of pancreatic cancer has shown that a compilation of genetic lesions was found to be associated with known cancer-related genes and well-known cancer signalling pathways (Figure 1). Changes at genomic, transcriptomic, epigenetic and proteomic levels are responsible for molecular abnormalities that occur in the different stages of pancreatic cancer. Alterations of cancer-related genes may drive carcinogenesis by providing cells with specific advantages that allow them to survive and proliferate in their microenvironment (Scarlett, Salisbury et al. 2011). Pancreatic cancer is largely resistant to conventional therapeutic strategies; thus, it is essential to control and manage its development (Biankin, Waddell et al. 2012). The use of natural bioactive components may additionally serve as a useful strategy to prolong or block the process of carcinogenesis (Vuong, Scarlett et al. 2012).

2.2.1. Resistance to Conventional Treatment

Pancreatic cancer is one of the most molecularly diverse malignancies which results in its clinical heterogeneity (Biankin, Waddell et al. 2012, Smith 2012). Its rapid progression and metastatic nature along with specific genetic characteristics cause a high degree of resistance to conventional treatments. This makes it difficult to identify specific factors that may contribute to the disease itself and subsequently the selection of an effective treatment, as each treatment may react differently in different individuals (Cowley, Chang et al. 2013).

The effect and severity of metastasis is thought to be a result of multiple genetic, epigenetic and post-translational events in the lifetime of a cancer (Yachida and Iacobuzio-Donahue 2013). While surgery aims to remove the bulk of ductal adenocarcinoma tumours, it may not entirely remove all of the affected cells. Distinct stem cells have demonstrated high resistance to chemotherapy (Hermann, Huber et al. 2007). The over-expression of tissue transglutaminase genes were also shown to be a contributing factor in the metastatic nature of pancreatic cancer (Verma, Guha et al. 2008). Therefore, remaining cancer cells may continue

to proliferate and rapidly metastasise, even with further chemotherapeutic treatment. Although radiotherapy and chemotherapy may control the spread of metastases from a localised tumour, these cells may still continue to divide and spread, thus mortality rates remain high (Haeno, Gonen et al. 2012). Despite significant advances in conventional therapies, there has been little improvement in patient survival within the last few decades (Sheahan, Phillips et al. 2013). Therefore, there is a critical need for the development of novel and effective therapeutic strategies for pancreatic cancer.

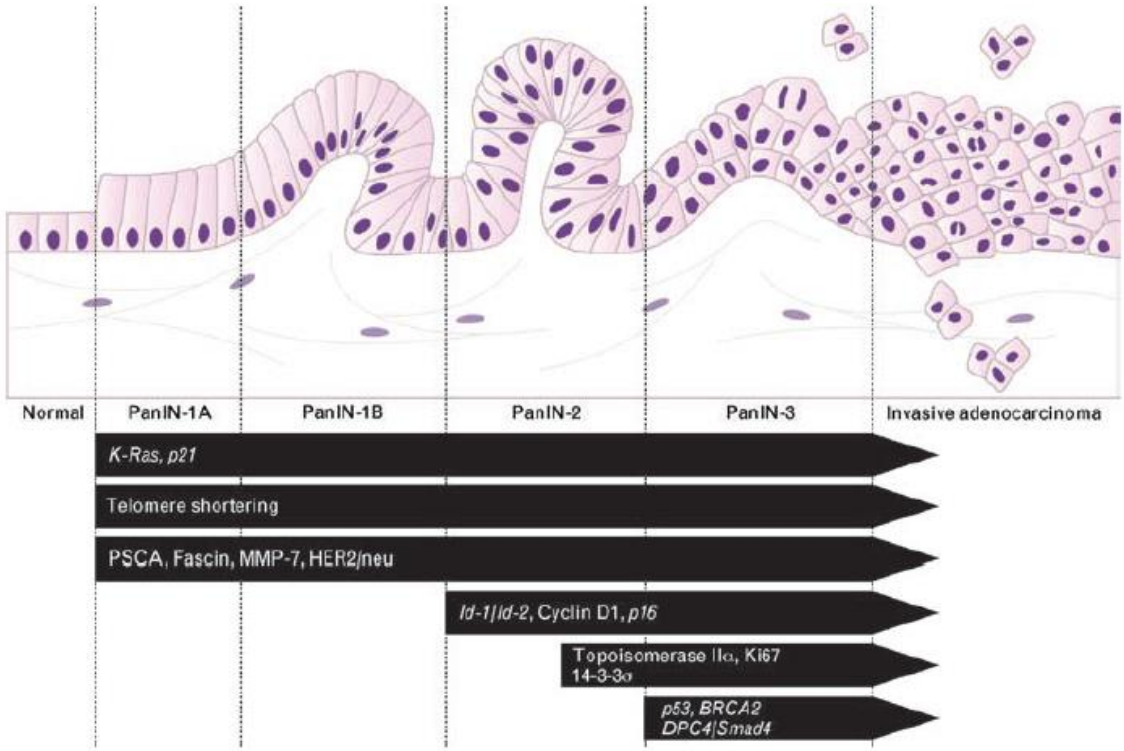


Figure 1. Schematic representation of the molecular aberrations in pancreatic ductal adenocarcinoma (Scarlett, Salisbury et al. 2011).

2.3. Phytochemicals

Fruit and vegetables are constantly exposed to environmental stressors including UV radiation and high temperatures, therefore, needing a variety of compounds, such as antioxidants to preserve their physicochemical integrity. This set of chemical reactions makes up the plants secondary metabolism and results in a large reservoir of natural chemical diversity that encompasses an enormous range of mechanisms driving gene regulation and transport of metabolites and enzymes. Levels of secondary metabolites are controlled by both genetic factors and external environmental triggers. Secondary metabolism is considered an

integral part of the plant biology with the accumulation on certain compounds often defining the onset of certain developmental stages (Surh 2003).

The term phytochemicals is often used interchangeably with biophenols despite having a slightly different definition. Phytochemicals refer to bioactive non-nutrient plant compounds found in fruits, vegetables, grains and other plant foods (Liu 2004), while, biophenols are phenolic compounds, isolated from plant tissues or products, that are derived from shikimate-phenylpropanoid and / or polyketide pathway(s) including their derivatives, conjugates, degradation products and metabolites. Hence, "biophenols" is an umbrella term that refers to all phenolic compound molecules derived from botanical origin (Obied 2013).

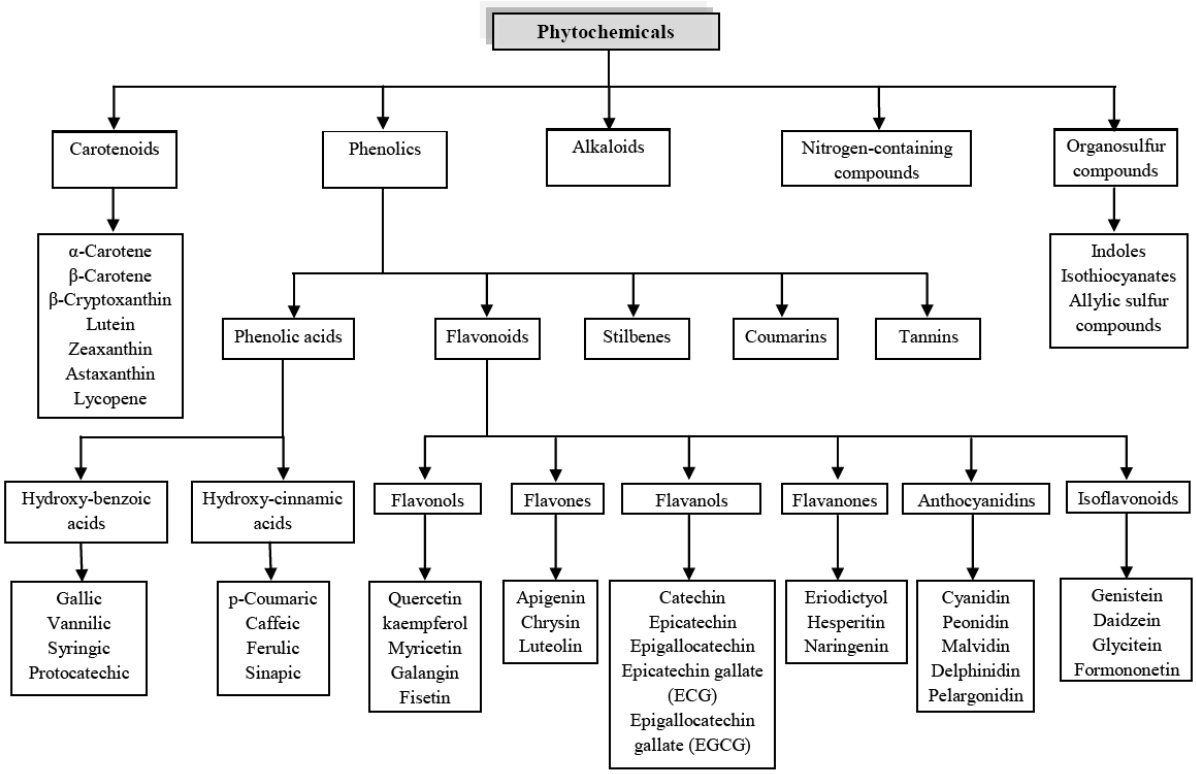


Figure 1.2. Classification of dietary phytochemicals (Liu 2004)

2.3.1. Phenolic compounds as chemotherapeutic agents

Plants have formed the basis for many traditional medicines throughout the world for centuries and still remain the source of structurally important chemicals from which novel therapeutic drugs are developed. Originally the use of medicinal plants was based on folk medicine from different parts of the world. Today, the search for new anti-inflammatory and anti-cancer therapies derived from plant sources is intensifying.

Natural products have long contributed to the development of modern therapeutic drugs (Table 1). Drugs derived from natural products have been shown to modulate various inflammatory mediators (e.g. peptides, cytokines, excitatory amino acids), the production or action of secondary messengers (cAMP, cGMP, calcium and protein kinases), the expression of transcription factors such as AP-1, NFkB and proto-oncogenes as well as the expression of key pro-inflammatory molecules such as inducible NO synthase (iNOS), COX-2, cytokines, neuropeptides and proteases (Gautam and Jachak 2009, Kim, Young et al. 2009).

Over the last 30 years, approximately 45% of all anti-cancer drugs have been derived from plant phenolic compounds, of which 12% come from natural products and 32% are semisynthetic modifications of naturally derived compounds (Newman and Cragg 2012). A significant percentage of anti-cancer drugs are entirely dependent on natural products, their structures and bioactivity (Newman and Cragg 2007); however, only 10% of the estimated 250,000 species worldwide have been screened for evidence of bioactivity and elucidation of their bioactive components (Mohanty and Cock 2012). As such, there is great potential for using bioactives unique to fruits as anti-cancer therapies, and continued research into this field is warranted.

Table 1.1. Examples of current clinical anti-cancer agents derived from natural products.

Derived Agent	Cancer Treatment	Natural Source	References
Vinorelbine	Breast Lung	Madagascar rosy periwinkle, *(Catharanthus roseus)*	(Cragg and Newman 2005, Barni, Visini et al. 2007)
Etoposide	Lymphomas Bronchial Testicular	Mayapple *(Podophyllum peltatum),* Himalayan mayapple *(Podophyllum emodii*)	(Kong, Goh et al. 2003)
Paclitaxel	Ovarian Lymphomas Testicular Breast Lung	Pacific yew *(Taxus brevifolia)*	(Kong, Goh et al. 2003, Cragg and Newman 2005)
Vindesine	Leukemias Lymphomas Testicular Breast Lung	Madagascar rosy periwinkle *(Catharanthus roseus)*	(Kong, Goh et al. 2003, Cragg and Newman 2005)
Teniposide	Lymphomas Bronchial Testicular	Mayapple *(Podophyllum peltatum),* Himalayan mayapple *(Podophyllum emodii*)	(Kong, Goh et al. 2003, Cragg and Newman 2005)
Topotecan	Ovarian Lung	Happy tree *(Camptotheca acuminata*)	(Kong, Goh et al. 2003, Guarneri, Piacentini et al. 2010, Spigel 2012)
Irinotecan	Colorectal	Happy tree *(Camptotheca acuminata*)	(Kong, Goh et al. 2003)
Elliptinium	Breast	*Bleekeria vitensis* (No common name)	(Kong, Goh et al. 2003)

2.4. Influences on phenolic compound profile of olive products

From Table 2 it can be seen that there is much variation in the phenolic concentration and composition of olive products. This is due to a number of factors, including: the maturity of the fruit at harvest, the cultivar (the type of olive), the region in which the olives were grown, the cultivation methods as well as the olive oil extraction and processing methods (Cicerale, Conlan et al. 2009). Phenolic compounds accumulate in the fruit and leaf of olives predominately as complex molecules (glycosides and esters). Among these, oleuropein is by far the most abundant compound, reaching up to 14% of the dry weight in unripe olives (Ranalli, Lucera et al. 2003). Other major phenolic structures present in olives include oleuropein aglycone, hydroxytyrosol, tyrosol, luteolin-7-glucodise, apigenin-7-glucoside as well as luteolin and apigenin (Obied, Allen et al. 2005)

2.4.1. Maturation

The phenolic composition and concentration of olives has been shown to change as the fruit matures (Malik and Bradford 2006). Different phenolic compounds are present at different stages of olive maturity. As olives mature the breakdown of phenolic compounds begins to outweigh their synthesis (Dag, Kerem et al. 2011). One study found the mean total phenolic concentration in virgin olive oil obtained from green olives (immature) to be much higher than that obtained from mature (ripe) olives (98.38 mg/kg compared to 61.48 mg/kg, respectively) (Lopez-Sabater, Gimeno et al. 2002). However, the concentrations of certain phenolic compounds will increase as the fruit ripens, due to the breakdown of higher molecular weight compounds. For example, the concentrations of tyrosol and hydroxytyrosol have been shown to increase as the olive fruit ripens (Brenes, Garcia et al. 1999), while oleuropein has been shown to decrease as the fruit ripens (Malik and Bradford 2006). This is because tyrosol and hydroxytyrosol, derivatives of oleuropein, are formed following its degradation. Additionally, tyrosol can be hydroxylated into hydroxytyrosol (Boss, Bishop et al. 2016).

2.4.2. Cultivar and growing conditions

The cultivar and the region in which the olives are grown have been demonstrated to greatly influence the concentration and composition of phenolic compounds in olives. Obeid et al., (2008) assessed the impact of cultivar and seasonal variation on the phenolic content of

Australian olive pomace waste. They found that the phenolic content and antioxidant activity at different harvesting times were mainly a function of the cultivar. This Australian olive pomace was also found to have a unique phenolic profile compared to Mediterranean olive pomace waste.

Seabra et al., (2005) also demonstrated the impact of the cultivar on the phenolic compound profile. They found that olives of different cultivars possessed unique phenolic profiles. However, geographic region was found to be an even more influential factor. Olives from the same geographic region, at similar maturities but from different cultivars were found to have very similar phenolic profiles.

2.4.3. Olive processing methods

The processing method employed also has a significant effect on the phenolic concentration of olive oil and olive waste products. Olive oil extraction begins with clean olives that are first crushed in order to break the vegetable structure and liberate the oil from the cells forming a paste that contains liquid and solid phases (Vekiari, Papadopoulou et al. 2002). There are two main methods of separating these phases that are currently employed in commercial olive oil mills: continuous two-phase centrifugation, three phase centrifugation and the less common discontinuous traditional press.

Centrifugation is a process that involves the use of centrifugal force to separate mixtures by sedimentation. This separation method relies on the principle that any combination of immiscible solvents of differing densities will separate into layers of the constituent solvents (Digiovacchino, Solinas et al. 1994). Three phase centrifugation requires the paste to be quite fluid to facilitate separation of the oil. In order to achieve this, lukewarm water equating to approximately 40-60% (v/w) of the weight of the olives is added. This process produces three phases: oil, vegetable water mixed with olive mill wastewater (OMWW) and an olive pomace (Digiovacchino, Solinas et al. 1994). Two-phase centrifugation requires no additional water and produces two phases, the oil and a pomace. The pomace obtained via this method has a higher moisture content (60-70%) than the three-phase pomace (Digiovacchino, Solinas et al. 1994). In the traditional press method, the olive paste is spread onto mats that are then subjected to hydraulic pressure of up to 400 bar which forces out the liquid phase. This phase

is then left to settle in settling tanks before the oil is siphoned off the top. This process produces three phases: oil, solid pomace and wastewater.

2.4.4. Phenolic content of olives compared to olive oil

The phenolic compound content is highly dependent on the extraction process as this determines the partitioning behaviour of the phenolic compounds and hence their distribution between the oil and waste fractions. Most of the phenolic compounds present in olives have low-partitioning coefficients ranging from 6×10^{-4} to 1.5 and hence have a high-water solubility and favour partitioning into the aqueous waste products. In fact, only 1-2% of the phenolic compounds originally in the olive usually ends up in the olive oil (Rodis, Karathanos et al. 2002). The amount of water added will therefore greatly affect the destination of the phenolic compounds. When more water is added, as for the three-phase process, more of the phenolic compounds are partitioned into the waste fractions (Obied, Allen et al. 2005).

2.5. Olive waste products as a source of phenolic compounds

Olives are approximately 10-15% oil. Hence, in processing olives into olive oil a large amount of waste is produced. One of the biggest issues for the olive oil industry is the disposal and non-utilization of olive waste. The rapid expansion of this industry has increased the production of noxious waste, which needs to be disposed of, and this has become a serious environmental concern (Obied, Allen et al. 2005).

Olive waste is claimed to be one of the most polluting effluents among those produced by the agriculture/food industries. This is due to its high content of organic substances (14-15%) including sugars, nitrogenous compounds, volatile fatty acids, polyalcohols, pectins and fats (Lafka, Lazou et al. 2011) and its high concentration of phenolic compounds (up to 10 g/L) (Ranalli, Lucera et al. 2003). Therefore, olive pomace waste is characterized by having a high specific chemical oxygen demand (COD) reaching values as high as 220 g/L (Agalias, Magiatis et al. 2007).

Disposal of olive waste is already a major environmental issue in a number of olive growing countries. The practice of spreading the solid waste on farmland has caused enormous pollution to the land and air. Unsuitable disposal of olive waste has been shown to leach

nitrates and other pollutants into the ground water (Sierra, Marti et al. 2001). The phenolic compounds present in olive waste have also been found to be phytotoxic, cause salinity and have a low pH (Capasso, Cristinzio et al. 1992). With this in mind, the extraction of the phenolic compound fraction from olive waste products not only has potential in the realm of cancer research but it also has the potential to utilise an environmentally damaging effluent.

2.5.1. Extraction of phenolic compounds

Extraction aims to maximise the recovery of phenolic compounds from the sample while minimising the co-extraction of matrix components. Some of the phenolic compounds are in the cytoplasmic vacuoles in the olives, while others are attached to the cell walls (Obied, Allen et al. 2005). Therefore, the choice of extraction method, solvent, time and temperature is critical. Furthermore, it is imperative that the extraction method is compatible with subsequent analysis procedures or uses for the phenolic compounds (Obied, Allen et al. 2005, Fernandez, Espino et al. 2018).

2.5.2. Olive pomace and waste water fractions

The recovery and analysis of the phenolic compounds is difficult for a number of reasons. The phenolic compounds are reactive chemical species and are vulnerable to oxidation, conjugation, hydrolysis, polymerisation and complexation. This is further complicated by the likely contact with degradation enzymes which are released when cells are no longer intact due to the mechanical pressing of the olives (Obied, Allen et al. 2005).

Olive pomace waste is a complex medium that offers substrates, catalysts and a reaction medium, water. Substrates include proteins, polysaccharides, metals, small molecular weight reactive compounds and phenolic compounds. The catalysts consist of enzymes and organic acids. There is a vast array of phenolic compounds in olives, with different structures and physiochemical properties (solubility and partitioning) which makes optimizing the extraction process a difficult task (Obied, Allen et al. 2005).

Some studies adapted methods previously used to analyse the phenolic fraction of virgin olive oil to analyse olive waste (De Tommasi, Cioffi et al. 2010, Cicerale, Conlan et al. 2011). These often involve a combination of solvents to extract compounds with different polarities. To date, over 50 phenolic compounds have been identified in olive waste products

(Obied, Allen et al. 2005), some of which are now commercially available such as oleuropein, tyrosol, hydroxytyrosol, luteolin, apigenin and myricetin.

Liquid-liquid partitioning (LLP) is a method used in a number of studies to extract phenolic compounds to be analysed by High Pressure Liquid Chromatography (HPLC). This method separates compounds based on their relative solubilities in two different immiscible liquids. Hexane is commonly used initially to remove any oil (Impellizzeri and Lin 2006, Robards, Obied et al. 2007, Obied, Prenzler et al. 2008, Cicerale, Conlan et al. 2011, Lafka, Lazou et al. 2011); however, petroleum ether has also been used (De Tommasi, Cioffi et al. 2010). The commonly used extraction solvents include acetonitrile, methanol or a mixture of either with water (Servili, Baldioli et al. 1999, Impellizzeri and Lin 2006, Cicerale, Conlan et al. 2011, Lafka, Lazou et al. 2011). However, other solvents include chloroform (De Tommasi, Cioffi et al. 2010), ethyl ether, ethanol, ethyl acetate or a combination (Servili, Baldioli et al. 1999, Obied, Allen et al. 2005, Angelino, Gennari et al. 2011, Kalogerakis, Politi et al. 2013, Rubio-Senent, Martos et al. 2015, Rubio-Senent, Martos et al. 2015, Aggoun, Arhab et al. 2016).

Polar extraction solvents have been found to yield the highest concentration of phenolic compounds. Lafka et al., (2011) selected ethanol as the most appropriate solvent for the extraction of phenolic compounds from olive pomace waste and they were able to produce extracts with a high content of phenolic compounds and a high antioxidant activity. However, others found methanol to be a more effective extraction solvent (Obied, Allen et al. 2005, De Tommasi, Cioffi et al. 2010), most likely due to its ability to disrupt cell walls and inhibit enzyme activity (Obied, Allen et al. 2005). Another advantage of methanol over ethanol is its lower boiling point, 65°C *vs.* 95°C, respectively. This enables it to be evaporated easier at a more moderate temperature, especially under vacuum, thus facilitating the concentration and drying of the extracted phenolic compounds (Lopez-Sabater, Gimeno et al. 2002).

Ethyl acetate has not been found to have a high recovery of phenolic compounds, with extracts containing only 43 - 45% of total phenolic compounds (Obied, Allen et al. 2005), even as low as 33.3% (Lafka, Lazou et al. 2011) . However, ethyl acetate has demonstrated a high selectivity for polyphenolic compounds, since at least 50% of the extractable matter is

comprised of polyphenolic compounds (Obied, Allen et al. 2005, Kalogerakis, Politi et al. 2013, Rubio-Senent, Martos et al. 2015).

Solid Phase Extraction (SPE) has also been used to separate the phenolic fraction from water extracts obtained from pomace samples (Servili, Baldioli et al. 1999, Delisi, Saiano et al. 2016). The solvents used to elute the phenolic compounds off the SPE columns include ethyl ether, ethyl acetate and methanol. After evaporation of the organic solvents, the sample can then be injected onto the HPLC. Servili et al., (1999) found that SPE was significantly ($p<0.05$) more effective for recovering the phenolic compounds than LLP (94.4% vs. 60.2% recovery, respectively). The yield of oleuropein was particularly increased (SPE = 2805.1 mg/L vs. LLP = 1732.8 mg/L), while oleocanthal was not (SPE = 29.7 mg/L and LLP=31.2mg/L).

Suarez et al., (2009) first separated the water and solid residues of the pomace by centrifugation in order to apply a different method for each phase and maximise the extraction of the different phenolic compounds. They found that ethyl acetate was a more appropriate solvent for the extraction of the phenolic compounds from the vegetable waters. However, the solid residue was best separated using methanol, indicating the more hydrophilic nature of the phenolic compounds remaining in the solid residue.

The use of advanced technologies for the extraction of phenolic compounds has grown in recent years. Additionally, since the focus has shifted towards more green extraction techniques, novel methods have been developed. Technology including ultrasound and microwave have become useful for improving the extraction efficiency of solvents (Vuong, Goldsmith et al. 2014, Jha, Das et al. 2017). However, there has been limited research into their use in olive waste materials.

Fernandez et al. (2018) combined an ultrasound method with a novel solvent they developed. They tested its ability to extract phenolic compounds in a number of different plants, including olive pomace. The solvent was a combination of lactic acid, glucose and 15% water and it was able to efficiently extract 14 phenolic compounds from olive pomace, including hydroxytyrosol and tyrosol. Unfortunately, oleuropein was not detected; however, this was likely due to a limited presence of oleuropein in the pomace, not the solvent properties. This novel method was innovative, effective and environmentally friendly and boasted increased

stability of phenolic compounds when stored in this solvent. However, this method was still complex in nature, requiring expensive reagents and multiple centrifugation steps.

The authors (Fernandez, Espino et al. 2018) also conducted a stability assay, whereby individual phenolic compounds were dissolved in their novel solvent and stored at -18°C, 4°C and 25°C; with water was used as the control solvent. Certain compounds were much more stable in the novel solvent including apigenin and quercetin; however, oleuropein and hydroxytyrosol behaved similarly in both the novel solvent and water. In fact, oleuropein and hydroxytyrosol were both stable in water for up to 60 days at 4°C and hydroxytyrosol was relatively stable for 60 days at 25°C.

These data highlight the potential of water as an extraction solvent for olive products. However, there has yet to be any investigation into the aqueous extraction of phenolic compounds form olive pomace. Considering that the phenolic compounds in olive products are mostly hydrophilic, the stability of the major phenolic compounds (oleuropein and hydroxytyrosol) in water, and that water is a cheap and safe solvent, the aqueous extraction of phenolic compounds from pomace warrants investigation.

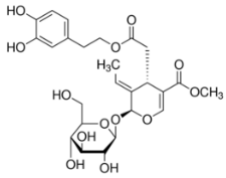
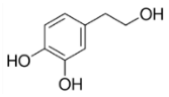
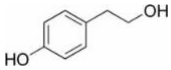
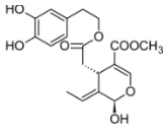
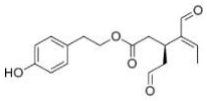
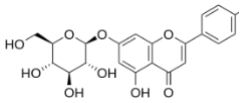
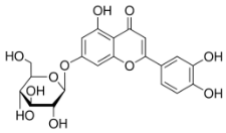
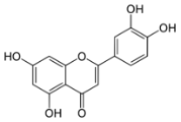
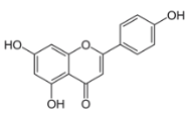
2.5.3. Olive leaves

Olive leaves are seen in the olive oil industry as a waste product, obtained as biomass after the pruning of olive trees (Schieber, Stintzing et al. 2001). This waste is an abundant vegetative material and a potential source of phenolic compounds. As with olives there are many environmental parameters that can affect the phenolic compound profile of olive leaves. Factors such as the position of the leaf on the tree, the tree's geographic location, time of year, the soil content and how much water the olive tree received can all have a dramatic effect on the levels of phenolic compounds generated/degraded in the leaf and hence the phenolic compound profile of the olive leaf extract (Brahmi, Mechri et al. 2013). In addition to these environmental considerations, the extraction methods may also influence this phenolic compound profile. Experimental parameters including; sample-to-solvent ratios, solvent type/concentration, temperature and time can all influence the extractability of the reactive, labile phenolic compounds in olive leaves.

Since there is no standard method for the extraction of phenolic compounds from plants, the literature can yield conflicting results. A number of studies have optimised the extraction of bioactives using specific technologies such as ultrasound (Japón-Luján, Luque-Rodríguez et al. 2006, Sahin and Samli 2013), pre-treatment blanching (Stamatopoulos, Katsoyannos et al. 2012), pressurised liquid extraction (PLE) (Xynos, Papaefstathiou et al. 2014) and microwave assisted technologies (Taamalli, Arraez-Roman et al. 2012). However, it is difficult to compare the results from these separate studies since they do not control for the same parameters. For example, Sahin & Samli (Şahin and Şamlı 2013) optimised the extraction of olive leaves using ultrasound with Response Surface Methodology (RSM). The extraction parameters they used were: sample-to-solvent ratio, solvent concentration and extraction time; while another study optimizing the extraction of olive leaves via PLE, also using RSM, used the solvent concentration, temperature and the number of extraction cycles as their parameters (Xynos, Papaefstathiou et al. 2014). This outlines the need for a comprehensive comparison of extraction conditions and methods that control and account for all biological parameters.

Despite this lack of consistency amongst studies there have still been a number of important phenolic compounds identified in olive leaves including: oleuropein, hydroxytyrosol, tyrosol, luteolin, apigenin and rutin (Stamatopoulos, Katsoyannos et al. 2012, Ahmad-Qasem, Barrajón-Catalán et al. 2013, Dermeche, Nadour et al. 2013, Rahmanian, Jafari et al. 2015). Additionally, there has yet to be comprehensive investigation into aqueous extraction protocols. For the reasons discussed previously, future research into green extraction technologies, like water extraction, is warranted.

Table 1.2. Major olive biophenols and quantity range in different olive products.

Compound name	Structure and class	olive leaf (mg/kg)	olive (mg/kg)	olive oil (mg/kg)	olive waste (mg/kg)	Reference
Oleuropein	 Secoiridoids	1.02-22610	1.87-159	0.09 - 2	0.39 - 489	(Obied, Allen et al. 2005, Seabra, Vinha et al. 2005, Japón-Luján, Luque-Rodríguez et al. 2006, Lee, Lee et al. 2009, Goldsmith, Stathopoulos et al. 2014)
Hydroxytyrosol	 Phenolic alcohol		302	37 - 41	8.4 - 1258	(Obied, Allen et al. 2005, Seabra, Vinha et al. 2005, De Tommasi, Cioffi et al. 2010, Bouallagui, Han et al. 2011, Taamalli, Arraez-Roman et al. 2012)
Tyrosol	 Phenolic alcohol			23.8 - 34.6	20.7-1373	(Obied, Allen et al. 2005, De Tommasi, Cioffi et al. 2010)
Oleuropein-aglycone	 Secoiridoids			19.9 - 24.9	23.3-24	(De Tommasi, Cioffi et al. 2010, Taamalli, Arraez-Roman et al. 2012)
Oleocanthal	 Secoiridoids			0.02 – 152.22	62.35-128.25	(Impellizzeri and Lin 2006, Cicerale, Conlan et al. 2011)
Apigenin-7-glucoside	 Flavanoids	1072				(Japón-Luján, Luque-Rodríguez et al. 2006)
Luteolin-7-glucoside	 Flavanoids	970				(Japón-Luján, Luque-Rodríguez et al. 2006)
Luteolin	 Flavonoids	0.0046				(Fu, Arráez-Roman et al. 2010)
Apigenin	 Flavonoids	199.3				(Fu, Arráez-Roman et al. 2010, Nashwa and Abdel-Aziz 2014)

2.6. Anti-cancer activity of crude extracts from olive products

The crude extracts are a mixture of phenolic compounds that is largely dependent on the extraction protocol as well as the environmental conditions the plant was subjected to. The exact mechanism by which these extracts exhibit any anti-cancer activity is difficult to determine. Despite this, there has been some investigation into the *in vitro* and *in vivo* effects of crude extracts from olive products on cancers of the colon, breast and prostate as well as glioblastoma.

2.6.1. Colon cancer

Although the exact mechanism by which olive crude extracts exert their anti-cancer actions is unknown, the down-regulation of COX-2 (Cyclooxygenase 2) and Bcl-2 (B-cell lymphoma protein 2) expression, which play a critical role in colorectal carcinogenesis, has been suggested (Corona, Deiana et al. 2007). COX-2 is overexpressed in colorectal cancer cells and this overexpression has a strong association with colorectal neoplasia by promoting cell survival, cell growth, migration, invasion and angiogenesis (Chu, Lloyd et al. 2003). In addition, the MAPK (mitogen activated protein kinase) signalling pathway, which is known to regulate COX-2 activity (Tsatsanis, Androulidaki et al. 2006), has long been viewed as an attractive pathway for anti-cancer therapies due to its central role in regulating the growth and survival of cells from a broad spectrum of human cancers (Sebolt-Leopold and Herrera 2004).

Previously, crude olive extracts, containing high concentrations of hydroxytyrosol, tyrosol and oleuropein, exerted an inhibitory effect on colon cancer cell proliferation. This was linked to the cell cycle arrest at the G2/M phase. This G2/M block was mediated by the ability of olive oil phenolic compounds (50 μg/ml) to exert rapid inhibition of p38 MAPK/cAMP response element-binding signalling and CREB phosphorylation which led to a downstream reduction in COX-2 expression (Corona, Deiana et al. 2007).

Despite these positive *in vitro* findings, one study investigated the influence of a diet containing olive oil which was high in phenolic compounds compared to olive oil without the phenolics and corn oil in rats *in vivo*. They found that there was no difference in the development of either pre-cancerous lesions or tumours over the experimental period and concluded that in their experimental model, olive oil, irrespective of phenolic content, had no effect on colon carcinogenesis in F344 rats compared to corn oil (Femia, Dolara et al. 2008).

This highlights the need for future studies to elucidate the molecular mechanisms behind the effectiveness of olive phenolic compounds in *in vitro* cancer models. Moreover, additional research is required to investigate the mechanisms behind the role of olive oil and colon cancer prevention.

2.6.2. Breast cancer

The first study to investigate the anti-proliferative effects of olive phenolic compounds against breast cancer was by Goulas et al., (2009). These authors demonstrated that extracts containing high levels of hydroxytyrosol and oleuropein were able to reduce the proliferation of breast cancer cells *in vitro*. This was soon confirmed by Han et al., (2009) who demonstrated that after treatment with olive phenolic compound extracts, the percentage of MCF-7 cells that had entered the G0/G1 phase of the cell cycle had increased. Following phenolic compound treatment, the sub G1 DNA content and caspase activity of the MCF-7 cells was investigated and revealed that apoptotic pathway activation was the reason for the observed cell death.

Later research into MCF-7 breast cancer cells and olive leaf phenolic compounds by Bouallagui et al, (2011) showed that after treatment with olive leaf extract, Pin1 was down-regulated, which in turn decreased the level of Cyclin D1 and arresting the cell cycle at the G1 phase. However, the authors also found that olive leaf extract treatment up-regulated the AP1 transcription factor member, c-jun. C-jun regulates the transcriptional level of cyclin D1 and hence allowes cell cycle progression. That is, c-jun is required for progression through the G1 phase of the cell cycle by up regulation of cyclin D1. This conflicting data highlights the need for further investigation into these mechanisms of action to establish a clearer understanding of what specifically olive phenolic compounds are acting upon. Alternatively, this confusion could be attributed to the phenolic compound mixture, underlining the need for the purification of extracts before testing.

More recently, a new molecular mechanism activated by crude olive phenolic extracts in JIMT-1 cells was discovered. Treatment of JIMT-1 breast cancer cells with complex polyphenols induced a strong up-regulatory effect in G2/M checkpoint sensor growth arrest and DNA-damage inducible nuclear protein (GADD45) genes leading to the selective transcription of potent inhibitors of cyclin-dependant kinases (including; $p21^{WAaf1/Cip1}$ and

p57^{Kip2}) (Oliveras-Ferraros, Fernandez-Arroyo et al. 2011). This result, in combination with the finding that olive phenolic extracts functionally restore the p53 pathway in p53 deficient cells (Oliveras-Ferraros, Fernandez-Arroyo et al. 2011), led authors to investigate a possible role of olive phenolic compounds as modulators of chromatin topology through the regulation of histone deacetylase activity (Besson, Dowdy et al. 2008). They revealed that olive phenolic compounds induced the expression of acetylated H3 histone proteins, revealing molecular aspects related to epigenetic events that are regulated by these bioactive compounds (Oliveras-Ferraros, Fernandez-Arroyo et al. 2011). These results warrant further exploration to open up new therapeutic approaches based on naturally occurring compounds, such as olive phenolic compounds.

The survival of breast cancer cells is also highly dependent on lipid metabolism; HER2 overexpressing breast cancer cells increase translation of fatty acid synthase (FASN). FASN overexpression increases EGRF (Epidermal Growth Factor Receptor) and HER2 signalling resulting in enhanced growth. Menendez, Vazquez-Martin et al. (2008) were able to show that olive oil phenolic compound extracts, that were high in lignans, flavonoids and secoiridoids, may have induced anti-cancer effects by suppressing the expression of the lipogenic enzyme FASN in HER2-overexpressing breast carcinoma cells. This discovery offers a previously unrecognized mechanism for the preventative effects of olive phenolic compound extracts on breast cancer cells.

More recently, a metabolite profiling approach was used to assess the uptake and metabolism of phenolic compounds from an olive leaf extract in the breast cancer cell line SKBR3 to evaluate the compound or compounds responsible for the observed cytotoxicity. HPLC was used to evaluate the intracellular phenolic compounds with 16 original compounds and 4 metabolites being identified. The major compounds found within the cells were oleuropein, luteolin-7-*O*-glucoside and its metabolites luteolin aglycone and methyl-luteolin glucoside, as well as apigenin, and verbascoside. Neither hydroxytyrosol nor any of its metabolites were found within the cells at any incubation time (Quirantes-Piné, Zurek et al. 2013). It is proposed that the major compounds responsible for the cytotoxic activity of the olive-leaf extract in SKBR3 cells are oleuropein and the flavones luteolin and apigenin, since these compounds showed high uptake and their antitumour activity has previously been reported and is discussed further in sections **2.7.1.** and **2.8.2**.

2.6.3. Prostate cancer

Wild olive leaf extracts harvested from Portugal have displayed selective toxicity towards prostate cancer cells. The crude extracts contained high concentrations of oleuropein as well as flavonoids luteolin and apigenin; these extracts were able to selectively reduced the proliferation of prostate cancer cells DU-145 and PC-3, while not displaying toxicity towards the normal skin fibroblasts (BJ) and prostate epithelial cells (PNT2) (Makowska-Was, Galanty et al. 2017). This study supports the chemopreventative and chemotherapeutic potential of olive leaf biophenols.

2.6.4. Glioblastoma

Glioblastoma multiform (GBM) is the most aggressive cancer that originates in the brain. Olive leaf extracts have recently been investigated as a combination treatment for glioblastoma; treatment of olive leaf extracts combined with chemotherapy drug bevacizumab reduced tumour volume, angiogenesis and migration by reducing the expression of VEGFA (Tezcan, Taskapilioglu et al. 2017). Moreover, the combination of olive leaf extract with chemotherapy drug temozolomide induced MGMT methylation which reduced p53 expression in glioblastoma tumours (Tezcan, Tunca et al. 2017). These data highlight the chemotherapeutic potential of olive leaf extracts.

2.7. Anti-cancer activity of olive phenolic compounds

As discussed earlier, consumption of the unique combination of phenolic compounds in olive oil has been linked to the health benefits associated with adherence to a Mediterranean diet. A small number of compounds unique to olive products have also been investigated for their anti-cancer potential. Namely, oleuropein and its degradation products hydroxytyrosol and tyrosol; however, other compounds including oleocanthal and the flavonoids, luteolin and apigenin, have also shown promise in the search for novel chemotherapeutic agents from natural products.

2.7.1. Oleuropein

Oleuropein (Table 2) is the most abundant phenolic compound in olives, reaching up to 14% of the dry matter in immature olives. It is responsible for the characteristic bitterness of the olive fruit (Servili, Baldioli et al. 1999). A number of the beneficial effects of virgin olive oil

have been attributed to oleuropein. It has been found to have anti-atherogenic (Covas 2007), anti-inflammatory (de la Puerta, Ruiz Gutierrez et al. 1999), anti-cancer (Menendez, Vazquez-Martin et al. 2007) and antimicrobial (Bisignano, Tomaino et al. 1999) properties. Oleuropein levels have been found to change during fruit development and maturation (Malik and Bradford 2006); hence, time of harvest will greatly influence the levels of oleuropein detected.

The majority of the research into the anti-cancer effects of olive products conducted so far has been on oleuropein or its degradation product, hydroxytyrosol. Hamdi and Castellon (2005) extensively investigated the anti-cancer effects of oleuropein. It was found to inhibit proliferation and migration of a number of advanced grade human tumour cell lines in a dose dependant manner (LN-18, glioblastoma; TF-1a, erythroleukaemia; 786-o, renal cell adenocarcinoma; T-47D, infiltrating ductal adenocarcinoma of the breast-pleural effusion; RPMI-795, malignant melanoma of the skin- lymph node metastasis; LoVo, colorectal adenocarcinoma-supraclavicular region metastasis). Oleuropein was also found to irreversibly round cancer cells in a novel tube disruption assay and preventing replication, invasiveness and motility of LoVo cells, effects that were reversible in normal cells.

Additionally, the authors (Hamdi and Castellon 2005) studied the anti-cancer effects of oleuropein *in vivo*. Oleuropein was administered orally to mice that developed spontaneous soft tissue sarcomas (H_2RC). Their drinking water was spiked with oleuropein to a final concentration of 1%, which they consumed *ad libitum* until their tumours regressed. In treated mice, complete tumour regression was observed in 9-12 days while the untreated mice exhibited 100% mortality by the 10^{th} day (Hamdi and Castellon 2005).

There has also been some analysis of the effect of oleuropein on breast cancer cells. It has been shown to induce apoptosis in MCF-7 breast cancer cells via a p53 dependant pathway that is mediated by Bax and Bcl-2 genes (Hassan, Elamin et al. 2013). Oleuropein has also shown anti-cancer activity against prostate cancer cells. By exposing cell cultures to oleuropein, a pro-oxidant effect was seen in cancer cells (LNCap and DU145) while an anti-oxidant effect was seen in non-malignant cells (BPH-1). Oleuropein reduced cell viability, induced thiol group modification, γ –glutamylcysteine synthetase, ROS, heme oxygenase and pAkt (Acquaviva, Di Giacomo et al. 2012). These results potentiate the use of oleuropein in

the treatment of prostatitis in order to prevent the transformation of hypertrophic cells to cancerous cells.

2.7.2. Hydroxytyrosol

Hydroxytyrosol (Table 2) comes from the hydrolysis of oleuropein. This process commences during maturation of the olives, processing and storage of olive oil as well as processing of table olives. These processes give rise the compounds; oleuropein aglycone, hydroxytyrosol and elanolic acid (Figure 6). Due to its polar nature, hydroxytyrosol is usually found in large quantities in olive waste (Fernandez-Bolanos, Rodriguez et al. 2002). However, hydroxytyrosol exhibits amphipathic behaviour and this molecule is therefore found in the oil as well ranging from 113.7-381.2 mg/kg (Brenes, Garcia et al. 2000) (Table 2). In addition, hydroxytyrosol is located in the olive leaf accompanied by other compounds including oleuropein (Briante, Patumi et al. 2002). This makes hydroxytyrosol an interesting candidate for study since it is present in the diet as well as easily available for extraction in waste products.

Hydroxytyrosol has been shown to elicit antitumour effects in a number of different cell lines via diverse mechanisms. In HL60 leukaemia cells, hydroxytyrosol induced apoptosis by releasing cytochrome-c at concentrations of 50-100 μM, which activated the effector caspase-3. These authors further concluded that the results excluded the death receptor pathway FAS, since the release of cytochrome-c precedes caspase-8 activation without affecting death receptor activation, including the FAS receptor. It was also suggested that hydroxytyrosol also induced apoptosis in HL60 cells through oxidative stress by primarily generating extracellular H_20_2 (Della Ragione, Cucciolla et al. 2002).

Moreover, hydroxytyrosol induced growth arrest and apoptosis in colon adenocarcinoma HT29 cells by modulating the ER stress dependant signalling involving the Ire1/JNK/c-jun/AP-1/Nox4 pathway (Guichard, Pedruzzi et al. 2006). These authors also showed that hydroxytyrosol altered the phosphorylation status of the extracellular kinase-1/2 (ERK1/2) and phosphosphinositol 3 kinase/AKT pathways by enhancing protein phosphatase 2 activity (PP2A) as well as preventing TNF-α-dependant NKkB activation. Another apoptotic pathway hydroxytyrosol has been shown to trigger, is the activation of c-jun by c-jun NH2-terminal

kinase (Della Ragione, Cucciolla et al. 2002), which under certain circumstances causes cell death and inactivates the anti-apoptotic protein Bcl 2.

Fabiani et al, (Fabiani, De Bartolomeo et al. 2002) studied HL60 cells, and demonstrated that hydroxytyrosol inhibited cell proliferation by blocking the G1 phase of the cell cycle with a proportional increase of cells in the G0/G1 phase and a concomitant decline in the S and G2/M phases. The mechanisms they proposed for this inhibition were: an induction of the cyclin-dependant kinase inhibitors; a direct blockage of hydroxytyrosol on cyclin dependant kinases; blockage of messengers involved in cell proliferation, such as ROS, which caused apoptosis and favoured differentiation of these HL60 cells (Fabiani, De Bartolomeo et al. 2002).

The effect of hydroxytyrosol on breast cancer cells has also been studied. Han, Talorete et al. (2009) showed that 25 μg/ml of hydroxytyrosol and 100 μg/ml of oleuropein exhibited a G1 to S phase transition blockade which was manifested by an increase in the number of MCF-7 breast cancer cells in the G0/G1 phase. These studies show the *in vitro* activity of hydroxytyrosol against leukaemia, breast and colon cancers. Although this data cannot be extrapolated to form conclusions about the efficacy of hydroxytyrosol in a more complex biological system, it does provide a platform for future work. In addition, the activity of hydroxytyrosol against pancreatic cancer cells has yet to be investigated. This is warranted, considering the efficacy of this compound against the other types of cancers discussed.

2.7.3. Oleocanthal

Oleocanthal (Table 2) is a naturally occurring secoiridoid found in olive products including olive oil and olive pomace waste. It first became known for its potent anti-inflammatory activity (Beauchamp, Keast et al. 2005, Lucas, Russell et al. 2011). In more recent years, oleocanthal has been investigated for its biological effects in cancer and Alzheimer's disease (Elnagar, Sylvester et al. 2011, Busnena, Foudah et al. 2013, Scotece, Gomez et al. 2013).

Oleocanthal has also been shown to inhibit the growth of breast cancer cells (MDA-MB-231, MCF-7 and BT-474) without affecting normal cell (MCF10A) growth (Akl, Ayoub et al. 2014). In addition, oleocanthal treatment caused a dose-dependent inhibition of HGF-induced cell migration, invasion and G1/S cell cycle progression in the breast cancer cell lines.

Moreover, the oleocanthal treatment effects were shown to be mediated via inhibition of HGF-induced c-Met activation and its downstream mitogenic signalling pathways. This growth inhibitory effect is associated with blockade of epithelial to mesenchymal transition (EMT) and reduction in cellular motility.

EMT is a process by which epithelial cells lose their polarity and cell-cell adhesion while gaining migratory and invasive properties to become mesenchymal stem cells. EMT is essential for a number of different developmental processes but in this context it is involved in the initiation of metastasis for cancer progression. Further results from *in vivo* studies showed that oleocanthal treatment suppressed tumour cell growth in an orthotopic model of breast cancer in athymic nude mice (Akl, Ayoub et al. 2014). Collectively, these findings suggest that oleocanthal is a promising compound for therapeutic use to control malignancies with aberrant c-Met activity.

Adenosine monophosphate-activated protein kinase (AMPK) is a sensor of cellular energy status and is involved in cancer cell apoptosis. Since tumour suppressor genes, including TSC2 and LKB1, function as upstream kinases of AMPK, AMPK is known as an anti-proliferative molecule. AMPK signalling may facilitate growth inhibition and cell death and therefore, may serve as a new therapeutic target in cancer research. A recent study showed oleocanthal inhibited cell viability and induce apoptosis in HT-29 colon cancer cells through the activation of AMPK and inhibition of COX-2 expression. Importantly, oleuropein suppressed the colony formation of HT-29 cells in soft agar. In the same study, oleocanthal also inhibited tumour promoter-induced cell transformation in mouse skin cells (JB6 Cl41 cells) (Khanal, Oh et al. 2011). These findings revealed that the targeted activation of AMPK and inhibition of COX-2 expression could contribute to the chemopreventative and chemotherapeutic potential of oleocanthal, thus suggesting that AMPK activation by this naturally occurring compound has potential for cancer therapy.

2.8. Anti-pancreatic cancer activity of olive phenolic compounds

While there has been some research into olive phenolic compounds and their chemopreventative or therapeutic effects on a number of different cancers, there has yet to be any investigation into the major olive phenolic compounds (oleuropein, hydroxytyrosol and tyrosol) and pancreatic cancer. However, there has been some investigation into the minor

constituents oleanolic acid and the flavonoids luteolin and apigenin in pancreatic cancer cells. Additionally, these compounds are not found exclusively in olive products.

2.8.1. Oleanolic acid

Oleanolic acid belongs to the group of pentacyclic triterpenes, which are widely distributed in fruits and some medicinal herbs. Oleanolic acid has attracted attention due to its antioxidant, anti-inflammatory, anti-mutagenic and anti-diabetic properties (Aparecida Resende, de Andrade Barcala et al. 2006, Teodoro, Zhang et al. 2008, Martin, Carvalho-Tavares et al. 2010, Wang, Ye et al. 2010). More recently, the anti-tumour activity of oleanolic acid has attracted more attention due to its marked effects as well as its pharmacological safety.

One study revealed that oleanolic acid inhibited proliferation of pancreatic cancer cells (Panc-1) in a concentration dependant manner (IC_{50} value 46.35 μg/ml); the cell cycle was arrested in the G2/M phase. Furthermore, this study revealed that oleanolic acid also induced ROS generation, mitochondrial depolarization and lysosomal membrane permeabilization (Wei, Liu et al. 2012). Another study investigated the effects of oleanolic acid combined with 5-fluorouracil (5-FU), a chemotherapy agent widely used for the treatment of solid tumours such as pancreatic cancer. The results showed that the combined use of oleanolic acid and 5-FU synergistically potentiated cell death effects as well as increasing pro-apoptotic signalling of Panc-28 cells (Wei, Liu et al. 2012). Similar to their previous study on oleanolic acid alone, the combined treatment resulted in mitochondrial depolarization and lysosomal membrane permeabilization in Panc-1 cells. The expression of apoptotic proteins was also increased, including the activation of caspase-3 and the expression of Bax/Bcl-2, survivin and NF-kB.

2.8.2. Luteolin and apigenin

Luteolin and apigenin (Table 2) are flavonoids that exist in many different fruits, vegetables and medicinal herbs. While luteolin and apigenin are found in small quantities in olive pomace or oil, larger amounts have been identified in olive leaves (Herrero, Temirzoda et al. 2011, Moudache, Colon et al. 2016). Plants rich in luteolin and apigenin have been used in Chinese medicine for the treatment of a number of ailments, including inflammatory disorders and hypertension.

The anti-cancer potential of luteolin and apigenin has been associated with the inhibition of cell proliferation, metastasis and angiogenesis as well as the induction of apoptosis, (Lin, Shi et al. 2008). In pancreatic cancer specifically, a small number of studies have been conducted *in vitro* to determine their effects. Luteolin has induced apoptosis in Panc-1, CoLo-357 and BxPC-3 cells by increasing the expression of the Bax/Bcl-2 ratio and caspase 3 by inhibiting NFkB (Cai, Lu et al. 2012). Luteolin has also been found in inhibit the invasiveness of pancreatic cancer cell lines PANC-1 and SW1990 by inhibiting EMT and MMP secretion (Huang, Dai et al. 2015). Additionally, luteolin and apigenin have increased the growth inhibition and apoptotic potential of gemcitabine in BxPC-3 cells.

These data highlight the potential of flavonoids in pancreatic cancer prevention and treatment. However, it is important to investigate how normal cells would respond to treatment with luteolin and apigenin, in order to better understand the potential toxicity of these compounds for *in vivo* applications.

2.9. Potential of olive phenolic compounds in pancreatic cancer

Over the last decade interest has grown considerably in the compounds discussed, with investigation into their anti-cancer activity at a number of different tissue sites being studied. Although this research has mainly been conducted *in vitro*, it has provided a platform for future investigation into the activity of these compounds in multicellular systems. Moreover, certain mechanisms which olive phenolic compounds have influenced in other cancers, are also important for pancreatic cancer. This highlights the potential for investigation into the chemopreventative and anti-cancer activity of olive biophenols against pancreatic cancer.

As discussed, pancreatic cancer is a devastating malignancy. Resistance to conventional treatments and toxicity of current chemotherapy drugs to normal cells requires a search for novel treatments. Considering the epidemiological link between adherence to the Mediterranean diet and reduced risk for pancreatic cancer, the high consumption of olives and olive oil in the Mediterranean and the anti-cancer potential of olive phenolic compounds, it is justified to investigate olive phenolic compounds. More specifically, with oleuropein, hydroxytyrosol and tyrosol; these compounds have been demonstrated to induce apoptosis in cells from cancers of different origins. Additionally, in certain situations, they have not

displayed toxicity towards normal cells. The *in vivo* evidence for the anti-cancer and chemo-preventative potential of these compounds is also mounting. For these reasons, investigation into the anti-pancreatic cancer potential of oleuropein and hydroxytyrosol is warranted.

p53 is an important molecular aberration underlying pancreatic cancer progression (Figure 1) (Scarlett, Salisbury et al. 2011). The p53 gene encodes for a tumour suppressor protein that functions as a short-lived transcription factor, crucial in cell cycle regulation and apoptosis, and is activated and stabilised in response to a wide variety of genotoxic cellular stresses. Since Hassan, Elamin et al. (2013), have shown that oleuropein induces apoptosis by altering the Bax/Bcl-2 ratio in MCF-7 breast cancer cells via a p53 dependant pathway this pathway could be a potential target for oleuropein in pancreatic cancer.

Moreover, hydroxytyrosol induced apoptosis in colon cancer cells by modulating ER stress dependant signalling involving the Ire1/JNK/c-jun/AP-1/Nox4 pathway (Guichard, Pedruzzi et al. 2006) and again by activation of c-jun by c-jun NH2-terminal kinase (Della Ragione, Cucciolla et al. 2002). The activation of the proto-oncogene c-jun and the JNK/ c-jun dependant apoptosis pathway have also been linked to the induction of apoptosis in pancreatic cancer cells (Shankar, Suthakar et al. 2007, Marchand, Arsenault et al. 2015). Therefore, these pathways are also potential targets for hydroxytyrosol in pancreatic cancer. Hydroxytyrosol also increased the Bax/Bcl-2 ratio and subsequently induced apoptosis in prostate cancer cells by induction of the inhibitory p21/p27. Loss of p21 is believed to be an early mutation leading to the development of pancreatic cancer. Therefore, p21 is a potential target for hydroxytyrosol in pancreatic cancer.

2.10. Conclusion

This review has highlighted that the majority of the research conducted on the extraction of biophenols from olive waste products has been on a small/laboratory sized scale and the processes often lack the capacity for upscaling due to technical constraints or hazardous waste production. This shows a need for the development of more environmentally friendly extraction methods that can be easily applied on an industrial scale.

From this review, it is clear that olive waste products are a potential source of biophenols with potent anti-cancer activity. In utilising this effluent by extracting the phenolic compounds, some damage to the environment caused by the olive oil production industry

could be reduced. However, the methodology behind the extraction protocol is important. Some methods require the use of organic solvents or corrosive acids, the disposal of which can be more difficult than the disposal of the olive waste material itself. Additionally, in order to make an impression on industry, the extraction methods need to be able to be applied to an industrial scale. Therefore, the optimisation of simple and "green" extraction protocols is paramount.

In addition, while the anti-cancer activity of some olive biophenols has been investigated, there has not yet been any investigation into olive biophenols and pancreatic cancer. Considering the link between adherence to a Mediterranean diet and a reduced risk for pancreatic cancer, this warrants further exploration.

CHAPTER 3

OPTIMISATION OF THE AQUEOUS EXTRACTION OF BIOPHENOLS FROM OLIVE WASTE PRODUCTS

3.1. Introduction

Olive pomace and olive leaves are waste products of the olive oil extraction process. A number of health benefiting biophenols such as oleuropein, hydroxytyrosol, tyrosol, luteolin and apigenin have been identified in olive waste products (Rubio-Senent, Martos et al. 2015, Aggoun, Arhab et al. 2016). Extraction of these biophenols constitutes a viable use for olive pomace and leaf waste.

There are a number of extraction methods that have been published optimising extraction conditions using organic solvents (Şahin and Şamlı 2013) or advanced technologies such as microwave (Taamalli, Arraez-Roman et al. 2012) ultrasound (Şahin and Şamlı 2013) and pressurised liquid extraction (Xynos, Papaefstathiou et al. 2014).

Water is classified as a safe and "green" solvent, which is inexpensive, accessible and considered an environmentally friendly alternative to harmful organic solvents (Hartonen and Riekkola 2017). Moreover, the majority of olive biophenols are highly water soluble (Dermeche, Nadour et al. 2013); however, there has been little investigation into the use of water as an extraction solvent.

Variation in extraction parameters including: solvent, sample-to-solvent ratio, extraction duration, temperature and the use of advanced technologies can dramatically affect the type

and yield of biophenols. Therefore, it is important to optimise extraction conditions to maximise the retention of target biophenols. Additionally, it is important to determine the interaction of extraction parameters.

From our thorough review of the literature, we have identified a number of extraction methods for extracting biophenols from olive leaves and pomace waste. While water extraction is sometimes used as a control to compare to solvent extraction systems, the optimisation of aqueous parameters for the extraction of biophenols from olive wastes has not yet been investigated. Considering that the purchasing of organic solvents, the significant costs associated with the disposal and removal of this industrial waste as well as the implementation of advanced technologies to an industrial scale, the use of water as an extraction solvent for the recovery of biophenols from waste materials can be viable if properly optimised. In this study, the use of water as an extraction solvent for the recovery of biophenols from olive leaves and pomace waste was investigated.

3.2. Experimental design

The design of experiments for the optimisation of the aqueous extraction conditions from olive waste products are shown in Figure 3.1.

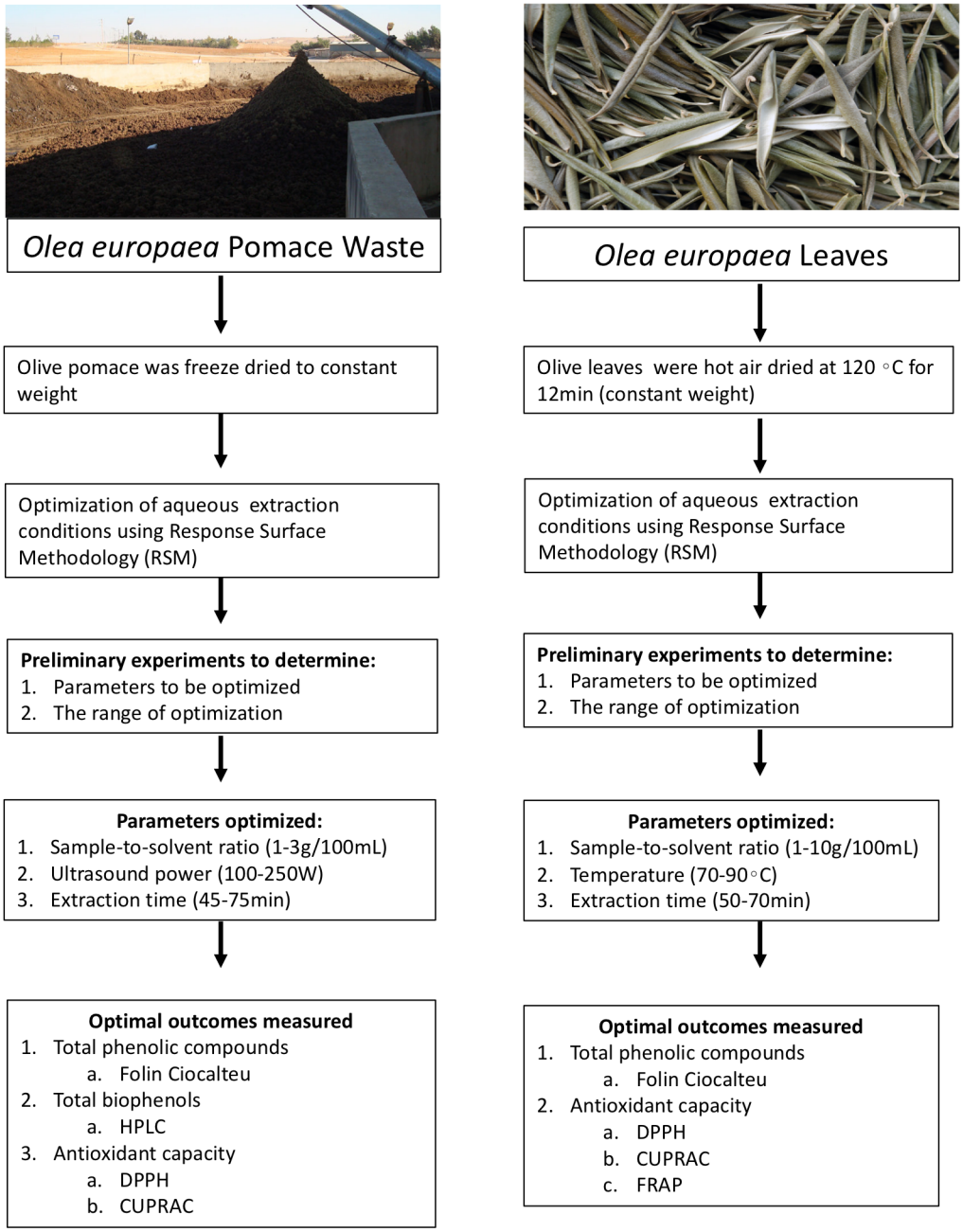


Figure 3.1. Experimental design for the optimization of the extraction of phenolic compounds from olive pomace and olive leaves.

3.3 Results and Discussion

The results and detailed discussion of the optimisation of the aqueous extraction of phenolic compounds from olive leaves, is presented in research paper 1 entitled "**Optimization of the aqueous extraction of phenolic compounds from olive leaves"** which was published by **Antioxidants** in 2014 (DOI:10.3390/antiox30407000). Additionally, the discussion of the

optimisation of the aqueous extraction of phenolic compounds from olive pomace waste, is presented in a research paper 2 entitled "**Ultrasound increases the aqueous extraction of phenolic compounds with high antioxidant activity from olive pomace"** which was published by **LWT-Food Science** in 2017 (DOI:10.1016/j.lwt.2017.10.065).

3.3.1. Research Paper 1

Research paper 1 entitled "**Optimization of the aqueous extraction of phenolic compounds from olive leaves"** which was published by **Antioxidants** in 2014 (DOI:10.3390/antiox30407000)

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Article

Optimization of the Aqueous Extraction of Phenolic Compounds from Olive Leaves

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Abstract: Olive leaves are an agricultural waste of the olive-oil industry representing up to 10% of the dry weight arriving at olive mills. Disposal of this waste adds additional expense to farmers. Olive leaves have been shown to have a high concentration of phenolic compounds. In an attempt to utilize this waste product for phenolic compounds, we optimized their extraction using water—a "green" extraction solvent that has not yet been investigated for this purpose. Experiments were carried out according to a Box Behnken design, and the best possible combination of temperature, extraction time and sample-to-solvent ratio for the extraction of phenolic compounds with a high antioxidant activity was obtained using RSM; the optimal conditions for the highest yield of phenolic compounds was 90 °C for 70 min at a sample-to-solvent ratio of 1:100 g/mL; however, at 1:60 g/mL, we retained 80% of the total phenolic compounds and maximized antioxidant capacity. Therefore the sample-to-solvent ratio of 1:60 was chosen as optimal and used for further validation. The validation test fell inside the confidence range indicated by the RSM output; hence, the statistical model was trusted. The proposed method is inexpensive, easily up-scaled to industry and shows potential as an additional source of income for olive growers.

Keywords: olive leaves; phenolic compounds; green extraction solvents; waste valorisation; *Olea europaea*; response surface methodology (RSM)

1. Introduction

Adherence to a Mediterranean-style diet has been associated with a reduced risk for cardiovascular disease and certain types of cancers [1]. These associations have been linked, in part, to the high consumption of olive oil, more specifically, the consumption of the unique phenolic compounds found in olive oil [2–4]. The same compounds believed to be responsible for the health-promoting properties attributed to olive oil consumption have also been identified in olive leaves [5]. Hence, the potential applications for the health promoting compounds extracted from olive leaves are extensive. These include their use as food additives or health supplements, as well as their continued use in future research into potential anti-cancer [6], anti-inflammatory [7] or anti-fungal [8] agents. It is therefore important to optimize the extraction of these compounds. An understanding of the parameters affecting the extraction of phenolic compounds is paramount to establishing the foundations for this future work.

Mediterranean countries account for around 98% of the world's olive cultivation (approximately ten million hectares); they produce about 1.9 million metric tonnes per annum of olive oil and 1.1 million tonnes of table olives [9]. Olive leaves are an agricultural waste of the olive oil and table olive production industry. This waste is produced as a result of pruning olive trees during the growing season, as well as accounting for approximately 10% of the weight of materials received by olive mills. Currently, this by-product is not profitable, given that in many countries, olive leaves are used as animal feed or simply burned with excess branches gathered from pruning [10,11]. Many olive oil producers even charge a fee to the olive farmer for the disposal of olive leaves.

The market for natural additives and ingredients is rapidly growing, with some natural products obtaining high prices. Moreover, the possible toxicity of certain synthetic compounds [5,12] has led to an increased interest in natural product research from the cosmetic, pharmaceutical and food additive industries. This has led to improved extraction, fractionation and purification technologies being developed in the last few years. However, these modern purification and separation technologies can be expensive and sometimes hazardous, rendering it near impossible for farmers to profit directly.

A number of methods have been proposed for the extraction of phenolic compounds from olive leaves, including the use of advanced technologies, such as microwave, pressurized liquid extraction and ultra-sonic extraction methods [13–15]. However, these practices can often have high energy costs and lead to the production of excessive solvent waste, which can be more hazardous to dispose of than the actual agricultural waste itself. Therefore, there is a need for the development of "green" extraction procedures. Water is a cheap, non-hazardous polar extraction solvent. It has been shown to efficiently extract a vast array of phenolic compounds with high antioxidant activities from a number of plant materials [16–18].

Therefore, in the present study, we aimed to optimize the extraction of phenolic compounds from olive leaves using the inexpensive, non-hazardous and easily obtainable solvent, water. The parameters of time, temperature and sample-to-solvent ratio were chosen for optimization, as they are easy for farmers or processors to control. The influence of these extraction parameters on antioxidant activity was also investigated.

2. Experimental Section

2.1. Materials and Reagents

Folin–Ciocalteu's reagent, sodium carbonate, gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), ferric chloride, sodium acetate, acetic acid, copper (II) chloride, ammonium acetate (NH_4Ac), neocuproine methanol and ethanol were purchased from Sigma Aldrich (Castle Hill, Australia). Ultra-pure (type 1) de-ionized (DI) water was prepared by reverse osmosis and filtration using a Mili-Q direct 16 system (Milipore Australia Pty Ltd., North Ryde, Australia).

2.2. Sample Preparation

Corregiola olive leaves were obtained from Houndsfield Estate in the Hunter Valley of NSW Australia. The leaves were dried at 120 °C for 90 min according to [19], ground to a size of 0.1 mm and stored at −20 °C until further analysis.

2.3. Response Surface Methodology (RSM)

The RSM with the Box–Behnken design was then employed to design the experiment to investigate the influence of three independent parameters, temperature, time and sample-to-solvent ratio, on the extraction of total phenolic compounds (TPC) and on the antioxidant activity of the resultant extracts. The optimal ranges of temperature (70–90 °C), time (50–70 min) and sample-to-solvent ratio (1:10–1:100 g/mL) were determined based on preliminary experiments. The independent variables and their code variable levels are shown in Table 1. To express the TPC or antioxidant capacity as a function of the independent variables, a second-order polynomial equation was used as follows and as previously described by Vuong *et al.* [20]: $Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k=1} \sum_{j}^{k} \beta_{ij} X_i X_j + \sum_{i=1}^{k} \beta_{ii} X_i^2$, where various X_i values are independent variables affecting the response Y; β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for the intercept and the linear, quadratic and interaction terms, respectively, and k is the number of variables.

2.4. Total Phenolic Compounds

The TPC was determined according to Thaipong *et al.* [21]. Briefly, the appropriately diluted samples (300 μL) were added to Folin–Ciocalteu's reagent (300 μL) and left to equilibrate for 2 min before adding 2.4 mL of 5% sodium carbonate solution and incubating in the dark for 1 h. Absorbance was then read at 760 nm using a UV spectrophotometer (Varian, Melbourne, Australia). Gallic acid was used as the standard, and results were expressed as mg of gallic acid equivalents per g of sample (mg GAE/g).

Table 1. Values of the independent parameters and their coded forms with their symbols employed in RSM for optimization of olive leaf extraction using water.

Independent Parameters	Symbols of the Parameters	Original Values of the Parameters	Parameter Coded Forms *
Temperature (°C)	X_1	70	−
		80	0
		90	+
Time (min)	X_2	50	−
		60	0
		70	+
Ratio (mg/mL)	X_3	10	−
		55	0
		100	+

* Parameter coded forms −, 0 and + are the minimum point, centre point and maximum point (respectively) for the independent parameters temperature, time and ratio.

2.5. Antioxidant Activity Assays

Three assays were employed in order to assess the antioxidant activity of the olive leaf extracts:

For the ferric reducing antioxidant power (FRAP) assay, the extract was diluted within the appropriate range, and then, their ferric ion reducing capacity was determined according to Thaipong *et al.* [21].

Stock solutions were: (1) 300 mM acetate buffer; (2) 10 mM TPTZ solution in 40 mM HCL; (3) 20 mM $FeCl_3$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3$ and then warming to 37 °C. Olive leaf extracts, standards and blanks (150 μL) were then added to 2.85 mL of the working FRAP solution and left to incubate in the dark at 37 °C for 30 min. Absorbance was read at 593 nm. Results were expressed as mg trolox equivalents per gram of sample dry weight (mg Trolox Equivalents (TE)/g).

For the cupric reducing antioxidant capacity (CUPRAC) assay, the extracts were diluted within the appropriate range, and their cupric ion reducing capacity was determined as described by Apak *et al.* [22].

The stock solutions were: (1) 10 mM $CuCl_2$ solution; (2) ammonium acetate buffer at pH 7.0; (3) 7.5 mM neocuproine (Nc) solution in 95% ethanol. A working solution of the three reagents (1:1:1 v/v) was prepared, 3 mL of which was added to 1.1 mL of the diluted extracts, standards and blanks and left to react in the dark for 1 hour. Absorbance was read at 450 nm. Results were expressed as mg of trolox equivalents per gram of sample dry weight (mg TE/g).

The DPPH free radical scavenging activity of the extracts was analysed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, as described by Vuong *et al.* [23]. Briefly, the appropriately diluted samples, standards and blank (150 μL) were added to 2.85 mL of DPPH working solution (made to an absorbance of 1.1 ± 0.01 at 760 nm) and left to react in a dark at room temperature for 3 h. Trolox was used as a standard. The results were expressed as mg of trolox equivalents per g of sample dry weight (mg TE/g).

2.6. Statistical Analysis

The RSM experimental design and analysis was conducted using JMP software (Version 11, SAS, Cary, NC, USA). The software was also used to establish the model equation, graph the 3D plot with 2D contour of the responses and to predict the optimum values for the three response variables in order to obtain the maximum TPC level. All experiments were carried out in triplicate.

3. Results and Discussion

3.1. Fitting the Models for the Prediction of Total Phenolic Compounds and Antioxidant Capacity

The experimental design is presented in Table 1, while Table 2 indicates the effects of temperature, time and the ratio of sample-to-solvent on the extraction of TPC from olive leaves using water. The predicted yield of TPC ranged from 22.36 to 38.25 mg GAE/g depending on the combination of extraction parameters.

Table 2. Analysis of variance for the determination of the fit of the model. TPC, total phenolic compounds; FRAP, ferric reducing antioxidant power; CUPRAC, cupric reducing antioxidant capacity; PRESS, predicted residual sum of squares.

Sources of Variation	TPC	Antioxidant Capacity		
		FRAP	CUPRAC	DPPH
Lack of fit (*p*-value)	0.1991	0.0168 *	0.1369	0.1377
R^2	0.8	0.95	0.97	0.92
Adjusted R^2	0.44	0.87	0.92	0.78
PRESS	1149.1	1500.72	1097.5	1988.1
F-ratio of model	2.2025	11.54	19.6	6.639
p of model > F	0.1991	0.0075 *	0.0022 *	0.0258 *

* Significant difference with $p < 0.05$.

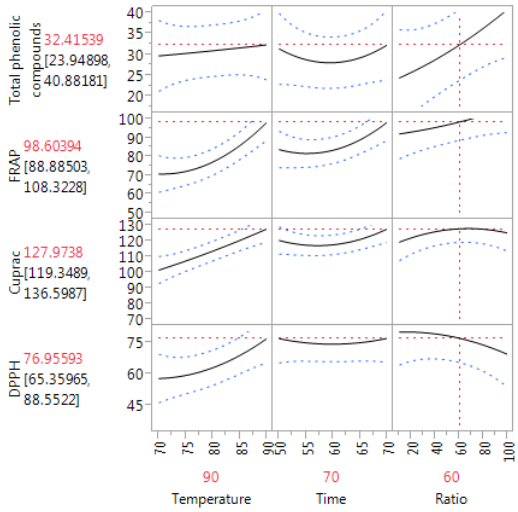
Table 2 shows the reliability of the RSM mathematical model in predicting optimal variances and accurately representing the real interrelationships between the selected parameters. The results for the analysis of variances of the Box–Behnken design are shown in Table 2. Figure 1 indicates the correlation between the predicted and experimental values.

Figure 1 and Table 2 indicate that there was no significant difference between the actual and predicted values for TPC ($p > 0.05$). Furthermore, the coefficient of determination (R^2) value for the correlation between the predicted and actual values was 0.8, indicating that the model can predict 80% of the actual data for TPC. Table 2 also showed that the "lack of fit" for the model was also not significant ($p = 0.1991$). In addition, the PRESS (predicted residual sum of squares) was 1149.1 and the F-ratio was 2.2025. PRESS is a measure of how well each point fits into the experimental design, further identifying the appropriateness of the model's fit.

It was therefore concluded that the second-order polynomial equation for the following three independent variables could be used: temperature (X_1), time (X_2) and sample-to-solvent ratio (X_3). The predictive equation for the response of total phenolic compounds (Y) was as follows:

$$Y = 26.02 + 1.31\,X_1 + 0.42\,X_2 + 4.88\,X_3 - 0.14\,X_1\,X_2 + 1.42\,X_1\,X_3 + 1.91\,X_2\,X_3 + (0.09\,X_1)^2 + (3.79\,X_2)^2 + (1.23\,X_3)^2 \quad (1)$$

Figure 1. Prediction profiler plots for the effects of the test parameters on the extraction of phenolic compounds from olive leaves.



The model fit for the antioxidant activity of the olive leaf extract was also investigated. Figures 2–4 show the relationship between the actual and predicted values, while Table 2 represents the analysis of variance results for the determination of the fit of the model. The *p*-values for the model fit were 0.0168, 0.1369 and 0.1377 for FRAP, CUPRAC and DPPH, respectively. This shows that there was no difference between actual and predicted values for CUPRAC and DPPH. However, there was a significant difference between the actual and predicted values for FRAP.

The coefficients of determination were 0.95, 0.97 and 0.92 for FRAP, CUPRAC and DPPH, respectively. This highlighted the close correlation between the actual and predicted values. This relationship is further supported with the values for PRESS and the F-ratios of the model: 1500.72 and 11.54 for FRAP, 1097.5 and 19.6 for CUPRAC and 1988.1 and 6.639 for DPPH, respectively. This indicated that the mathematical models were reliable predictors of the antioxidant activity of the olive leaf water extracts. Therefore, the following second order polynomials could be used:

FRAP:

$$Y = 64.66 + 10.51\,X_1 + 4.58\,X_2 + 7.45\,X_3 + 3.05\,X_1X_2 + 2.16\,X_1X_3 - 2.66\,X_2X_3 + (7.39\,X_1)^2 + (7.64\,X_2)^2 + (1.4\,X_3)^2 \quad (2)$$

CUPRAC:

$$Y = 104.53 + 11.76\,X_1 + 1.91\,X_2 + 11.31\,X_3 + 2.06\,X_1\,X_2 - 6.14\,X_1\,X_3 - 2.27\,X_2\,X_3 + (1.01\,X_1)^2 + (6.45\,X_2)^2 - (5.33\,X_3)^2 \quad (3)$$

DPPH:

$$Y = 60.08 + 9.29\,X_1 + 0.39\,X_2 + 7.02\,X_3 + 0.68\,X_1\,X_2 - 3.4\,X_1\,X_3 - 8.98\,X_2\,X_3 + (4.43\,X_1)^2 + (2.71\,X_2)^2 - (3.03\,X_3)^2 \quad (4)$$

Figure 2. Correlation between the actual and the predicted values for the total phenolic compounds (TPC) and antioxidant capacity of olive leaf water extract (FRAP, DPPH and CUPRAC).

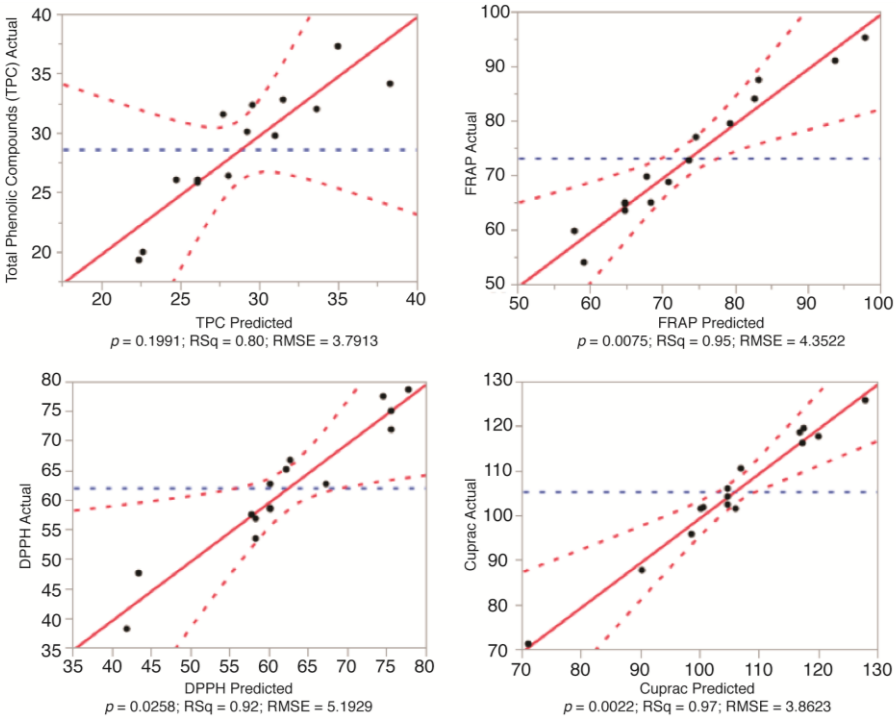


Figure 3. 3D response surface and 2D contour plots for the effects of the test parameters on total phenolic compounds.

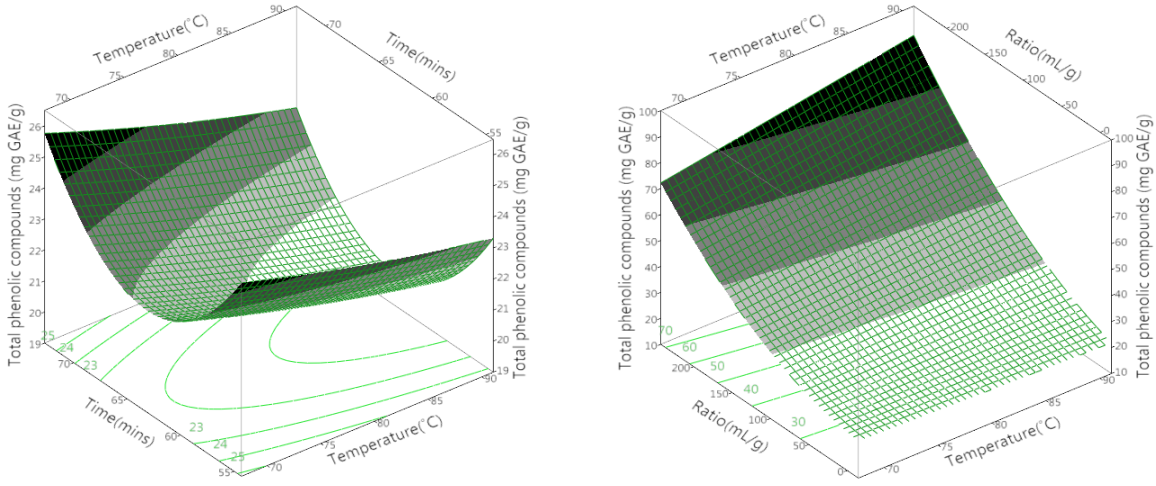


Figure 3. *Cont.*

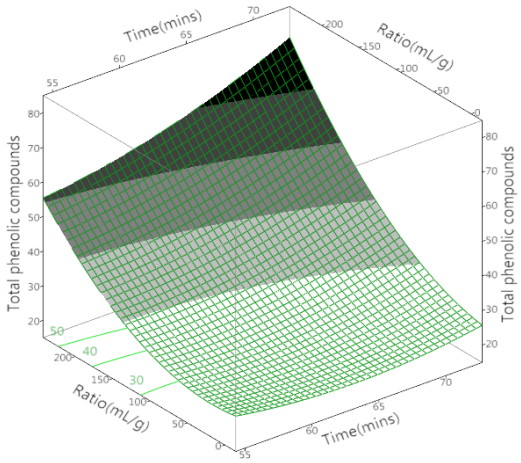


Figure 4. 3D response surface and 2D contour plots for the effects of the test parameters on antioxidant activity.

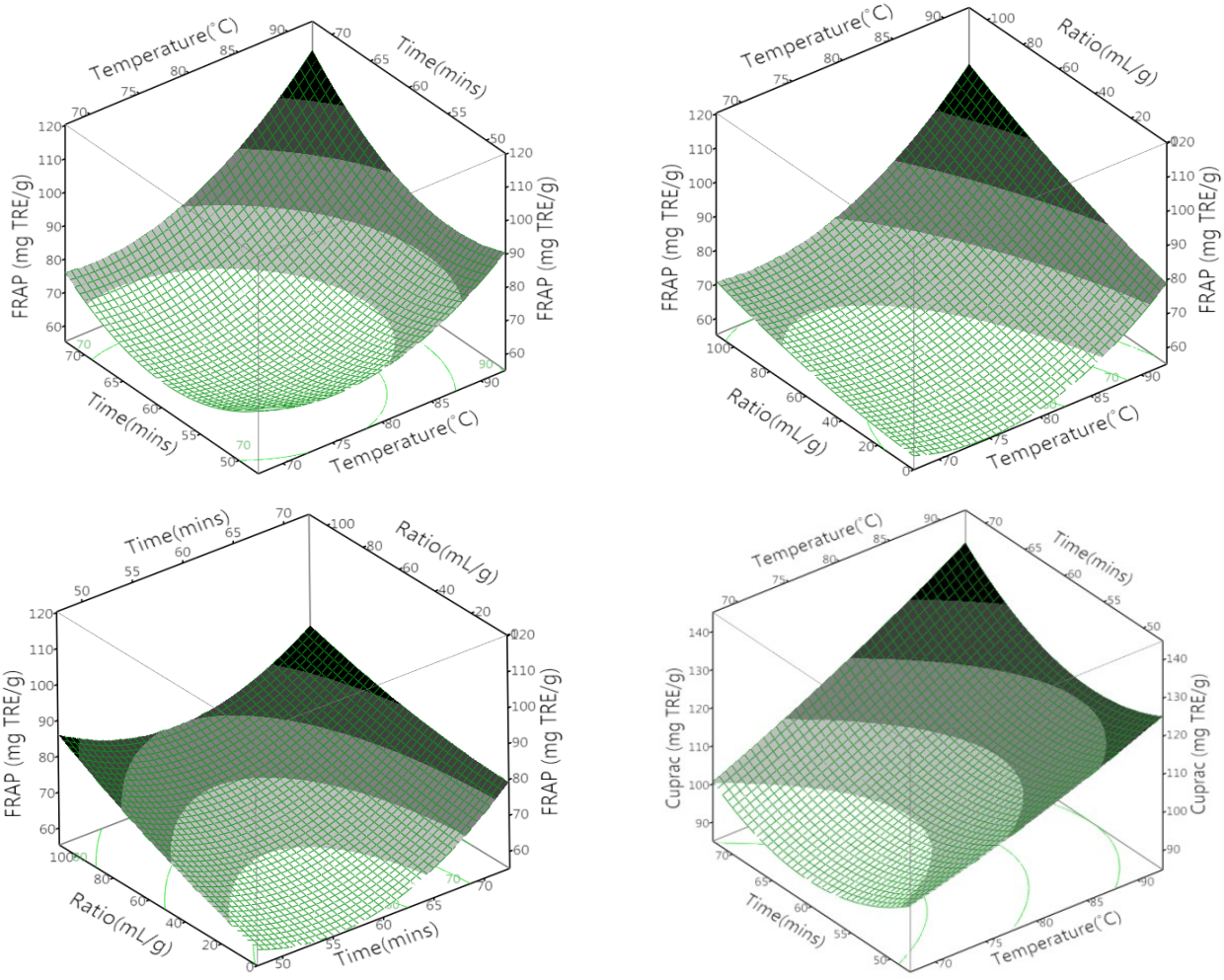
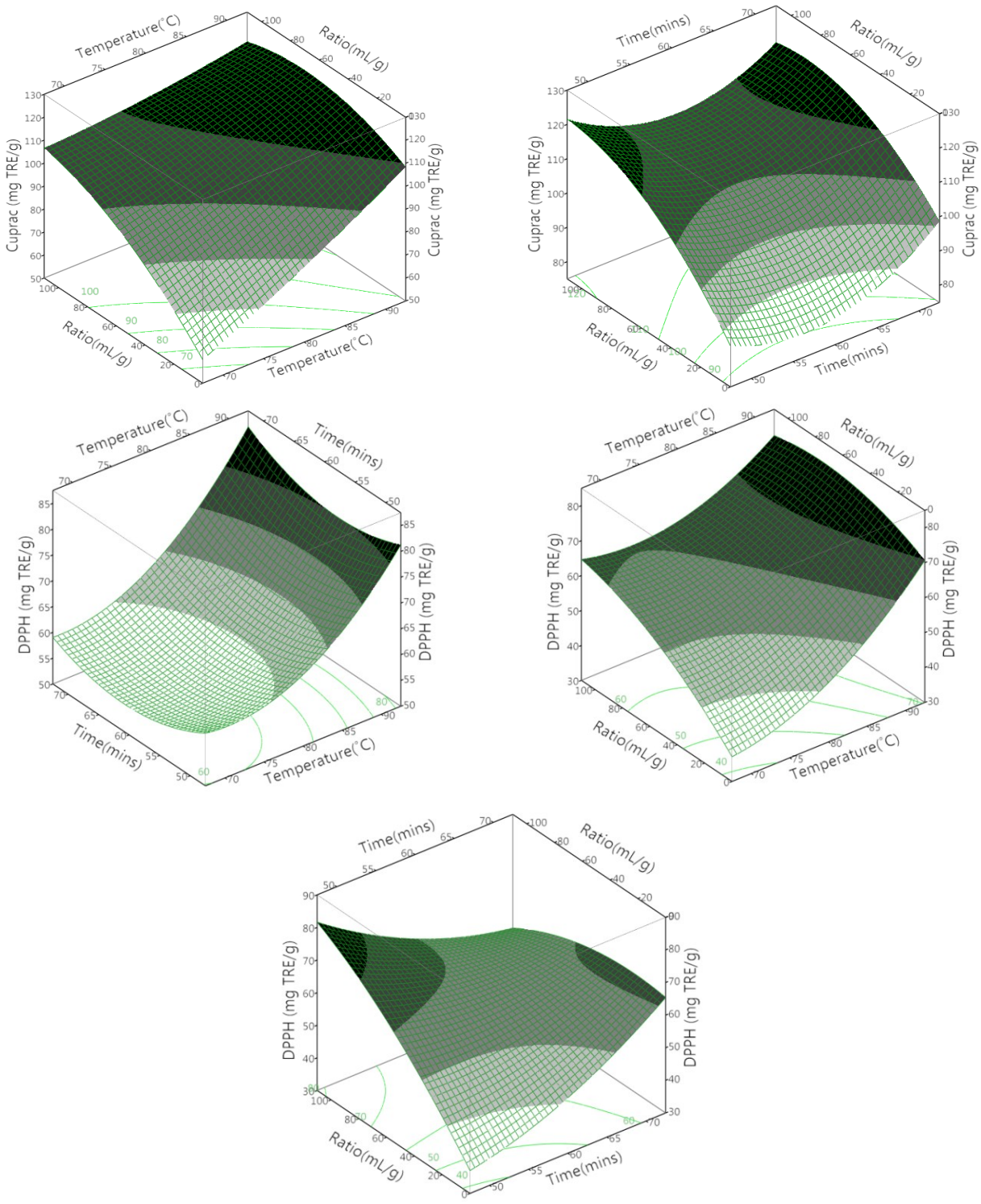


Figure 4. *Cont.*



3.2. The Effect of the Different Variables on the Total Phenolic Compounds

Table 3 presents the linear regression coefficients and indicates their statistical significance. Temperature, time and ratio were all shown to have a positive influence on the extraction of TPC. However, the only parameter to significantly affect the extraction efficiency was the sample-to-solvent ratio ($p = 0.01$). Temperature and time had no significant effect on TPC ($p > 0.05$), nor did any of the various

combinations of factors (temperature × time, temperature × ratio or time × ratio) (Table 3). This was unexpected, since time has previously been shown to have a significant effect on the extraction of TPC from olive leaves when using ultrasonic assistance [15]. Extraction time has also been identified as a significant extraction parameter for the extraction of natural polyphenols from wheat bran [24]. However, in both of these studies, the use of advanced technologies could account for the observed differences.

Table 3. The analysis of variance for the experimental results.

Parameter	DF	TPC		Antioxidant Capacity					
				Frap		DPPH		CUPRAC	
		F	Prob > F	F	Prob > F	F	Prob > F	F	Prob > F
β_0	1	26.02	<0.0001	64.66	<0.0001	60.08	<0.0001	104.53	<0.0001
β_1	1	1.31	0.37	10.51	0.001 *	9.29	0.004 *	11.76	0.0003 *
β_2	1	0.42	0.77	4.58	0.031 *	0.39	0.84	1.91	0.22
β_3	1	4.88	0.01 *	7.45	0.005 *	7.02	0.01 *	11.31	0.0004 *
β_{12}	1	−0.14	0.94	3.05	0.22	0.68	0.8	2.06	0.34
β_{13}	1	1.42	0.49	2.16	0.37	−3.4	0.25	−6.14	0.02 *
β_{23}	1	1.91	0.36	−2.66	0.28	−8.98	0.02 *	−2.27	0.29
β_{11}	1	0.09	0.96	7.39	0.02 *	4.43	0.16	1.01	0.63
β_{22}	1	3.79	0.11	7.64	0.02 *	2.71	0.36	6.45	0.02 *
β_{33}	1	1.23	0.56	1.4	0.56	−3.03	0.31	−5.33	<0.05 *

* Significantly difference with $p < 0.05$; β_0: intercept; β_1, β_2 and β_3: linear regression coefficients for temperature, time and ratio; β_{12}, β_{13} and β_{23}: regression coefficients for interaction between temperature × time, temperature × ratio and time × ratio; β_{11}, β_{22} and β_{33}: quadratic regression coefficients for temperature × temperature, time × time and ratio × ratio; Prob = probability.

The sample-to-solvent ratio was shown to have a significant effect on the extraction of TPC. This is consistent with mass transfer principles, which outline that the concentration gradient (the driving force) is higher when there is more solvent present, leading to higher diffusion rates.

3.3. The Effect of the Different Variables on Antioxidant Activity

The temperature and ratio were both found to significantly impact the antioxidant activity of the olive leaf extract measured via FRAP, CUPRAC and DPPH (p = 0.001, 0.004, 0.0003, respectively). However, time was only shown to significantly affect the antioxidant capacity measured via FRAP. The temperature × ratio had a negative influence on the DPPH measurements ($p < 0.05$).

3.4. Optimization of Aqueous Extraction Conditions for Maximizing the Total Phenolic Content and Antioxidant Capacity of Olive Leaf Extract

Based on the predictive models shown in Figures 3 and 4 the optimal conditions for the aqueous extraction of phenolic compounds were a temperature of 90 °C for 70 min at a sample-to-solvent ratio of 1:100 g/mL. These conditions were the same for the optimization of antioxidant capacity via FRAP. However, the optimal conditions for CUPRAC and DPPH varied slightly (CUPRAC: temperature 90 °C, time 70 min, sample-to-solvent ratio of 1:60 g/mL, DPPH: temperature 90 °C, time 70 min, sample-to-solvent ratio of 1:20 g/mL). Therefore, the extraction conditions of a temperature at 90 °C for

70 min and at a sample-to-solvent ratio of 1:60 g/mL were chosen for the extraction of phenolic compounds, as the extracts also displayed a high level of antioxidant activity. Furthermore, consuming less extraction solvent is practical from an economic point of view. For this reason, the sample-to-solvent ratio of 1:60 g/mL was used for validation. Increases in antioxidant activity with increasing temperature have previously been linked to the thermal degradation of higher molecular weight compounds into lower molecular weight ones [25,26]. This is one example of the non-specificity of the Folin–Ciocalteu method.

In order to validate the conditions predicted by the models, these extraction conditions (temperature 90 °C, time 70 min, sample-to-solvent ratio of 1:60 g/mL) were tested. The resulting values fell inside of the predicted ranges for TPC and all three antioxidant capacity assays (Table 4). These conditions are therefore proposed as optimal for the aqueous extraction of phenolic compounds with a high antioxidant capacity from olive leaves.

Table 4. Validation of the experimental model. GAE, gallic acid equivalents.

Assay	Values of TPC and Antioxidant Capacity	
	Predicted	**Experimental (*n* = 3)**
TPC (mg GAE/g)	32.42 ± 8.66	32.4 ± 2.06
FRAP (mg TE/g)	98.6 ± 9.71	91.03 ± 6.13
DPPH (mg TE/g)	76.96 ± 11.56	85.26 ± 3.54
CUPRAC (mg TE/g)	127.97 ± 8.62	121.97 ± 5.45

4. Conclusions

The optimal conditions for the aqueous extraction of phenolic compounds from olive leaves were proposed to be at 90 °C for 70 min at a sample-to-solvent ratio of 1:60 g/mL. Using olive leaves as a starting material for the extraction of phenolic compounds via this simple and inexpensive method constitutes a viable use for this agricultural waste product and may potentially serve as an additional source of income for olive growers/olive oil producers.

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Author Contributions

Chloe Goldsmith participated in the experimental design and completion, as well as interpretation, manuscript design and preparation. Quan Vuong and Costas Stathopoulos participated in the experimental design and data interpretation. Chris Scarlett and Paul Roach participated in manuscript design and preparation. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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3.3.2. Research Paper 2

Research paper 2 entitled "Ultrasound increases the aqueous extraction of phenolic compounds with high antioxidant activity from olive pomace**" published by LWT-Food Science in 2017** (DOI:10.1016/j.lwt.2017.10.065).

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Ultrasound increases the aqueous extraction of phenolic compounds with high antioxidant activity from olive pomace

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ABSTRACT

Olive pomace is a waste produced by the olive oil industry in massive quantities each year. Disposal of olive pomace is difficult due to high concentrations of phenolic compounds, which is an environmental concern. However, phenolic compounds have applications in the health industry. Therefore, extraction of phenolic compounds from olive pomace has the potential to remove an environmentally hazardous portion of pomace while creating an additional source of income for farmers and producers. Using advanced technologies including Ultrasound Assisted Extraction (UAE), combined with water as an extraction solvent, has recently gained popularity. The present study outlines the optimal UAE conditions for the extraction of phenolic compounds with high antioxidant activity from olive pomace. Optimal conditions were developed using RSM for parameters power, time and sample-to-solvent ratio. Total phenolic compounds determined by Folin Ciocalteu method and total major bioactive compounds determined by HPLC as well as antioxidant capacity (DPPH and CUPRAC) were investigated. The optimal conditions for the extraction of phenolic compounds with high antioxidant activity were 2 g of dried pomace/100 mL of water at 250 W power for 75 min. UAE improved the extraction efficiency of water and yielded extracts with high levels of phenolic compounds and strong antioxidant activity.

1. Introduction

Olive pomace is the solid waste product of the olive oil extraction process, which retains high amounts of organic substances (14–15%), including sugars, nitrogenous compounds, volatile fatty acids, polyalcohols, pectins and fats (Lafka, Lazou, Sinanoglou, & Lazos, 2011) as well as a high concentration of phenolic compounds (Goldsmith, Vuong, Stathopoulos, Roach, & Scarlett, 2014a; Ranalli, Lucera, & Contento, 2003). Thousands of tonnes of olive waste are produced each year; these waste products are often dumped in landfill, which is causing a number of environmental concerns due to the presence of phenolic compounds. Therefore, the disposal of olive waste products has been a major environmental issue in a number of olive growing countries (Capasso, Cristinzio, Evidente, & Scognamiglio, 1992).

Extraction of the phenolic compounds from olive pomace has the potential to somewhat limit the environmental damage that can be caused by this waste fraction and may even provide an additional source of income for olive oil producers (Obied, Allen, Bedgood, Prenzler, & Robards, 2005). For example, the extraction of oleuropein, the most abundant phenolic compound in olive products, would add value to the olive oil production process. This is because a number of the beneficial health effects of virgin olive oil have been attributed to consumption of oleuropein, including anti-atherogenic (Covas, 2007), anti-inflammatory (de la Puerta, Ruiz Gutierrez, & Hoult, 1999), anti-cancer (Ahmad Farooqi et al., 2017; Fayyaz et al., 2016; Hadrich et al., 2016; Liu, Wang, Huang, Chen, & Li, 2016; Maalej, Bouallagui, Hadrich, Isoda, & Sayadi, 2017; Morana et al., 2016; Secme, Eroglu, Dodurga, & Bagci, 2016; Sepporta et al., 2016; Xu & Xiao, 2017) and anti-microbial (Bisignano et al., 1999) properties and therefore oleuropein is a valuable product in itself. A number of advanced techniques to extract phenolic compounds have gained popularity in recent years including Microwave Assisted Extraction (MAE), Pressurised Liquid Extraction (PLE) and Solid Phase Extraction (SPE). However, Ultrasound Assisted Extraction (UAE) is considered one of the simplest and most cost-effective techniques to scale up for industrial production.

The UAE method has been used to improve the extraction efficiency of phenolic compounds from a variety of plant matrices (Vuong et al., 2015). The method has a number of benefits, including as an add on step to existing processes with minimum alteration, as an application in

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Table 1
Values of the independent parameters and their coded forms with their symbols employed in RSM for optimisation of UAE conditions for phenolic compounds from olive pomace.

Independent Parameters	Symbols of the Parameters	Original Values of the Parameters	Parameter Coded Forms[a]
Power (W)	X_1	100	–
		150	0
		250	+
Time (min)	X_2	45	–
		60	0
		75	+
Ratio (g/100 mL)	X_3	1	–
		2	0
		3	+

[a] Parameter coded forms -, 0 and + are the minimum point, centre point and maximum point (respectively) for the independent parameters temperature, time and ratio.

Table 2
Analysis of variance for determination of the model fit. Total Phenolic Compounds (TPC) and antioxidant capacity (CUPRAC and DPPH).

Sources of Variation	TPC	Antioxidant Capacity	
		CUPRAC	DPPH
Lack of fit (*p*-value)	> 0.0001*	> 0.0001*	0.0076*
R^2	0.8	0.81	0.69
PRESS	3128	3001	1566
F-ratio of model	15.1	6.56	6.15
p of model > F	> 0.0001*	> 0.0001*	> 0.0001*

*Denotes significant result ($p < 0.05$).

the aqueous extraction of phenolic compounds therefore reducing the need for harmful organic solvents, which can be difficult and expensive to dispose of. The UAE method often results in shorter extraction times and high yields; importantly, UAE has been shown to improve extraction yield up to 35% (Vilkhu, Mawson, Simons, & Bates, 2008).

Despite the clear benefits of UAE, the use of high power levels with the method can lead to the degradation of phenolic compounds. For example, in one of our previous studies we observed a 25% decrease in the extraction of Euphol from Euphorbia Tirucalli when the power was increased from 150 to 250 W (2015). Therefore, it is important to optimise the UAE extraction parameters to ensure the maximum retention of valuable compounds.

Water is classified as a safe and "green" solvent, which is inexpensive, accessible and considered an environmentally friendly alternative to harmful organic solvents (Hartonen & Riekkola, 2017). Therefore, water was the solvent of choice for the recovery of bioactive compounds from olive pomace in the present study. This study, for the first time, optimised the Ultrasound Assisted Extraction (UAE) conditions for maximum recovery of phenolic compounds with high antioxidant activity from olive pomace using water. Our study is the first to investigate water as an extraction solvent and determine the optimal conditions for the extraction of bioactive compounds from olive pomace.

2. Materials and methods

2.1. Materials and reagents

Folin–Ciocalteu's reagent, sodium carbonate, gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,4,6-Tris (2-pyridyl)-*s*-triazine (TPTZ), ferric chloride, sodium acetate, acetic acid, copper (II) chloride, ammonium acetate (NH_4Ac), neocuproine methanol and ethanol were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Ultra-pure (type 1) de-ionized (DI) water was prepared by reverse osmosis and filtration using a Milli-Q direct 16 system (Millipore Australia Pty Ltd., North Ryde, NSW, Australia).

2.2. Sample collection and preparation

Green olives of the Manzanilla cultivar were harvested at Houndsfield Estate (Hunter Valley, NSW, Australia) in July 2015 and processed on-site the next day using a semi-continuous Enorossi 150 traditional olive oil pressing system (Enoagricola Rossi, Calzolaro di Umbertide, Perugia, Italy) standardised to press a maximum of 150 kg of olives at a time. Olive pomace was collected and stored at −20 °C until further analysis. Olive pomace was freeze dried until constant weight was achieved before blending in a blender and being passed through a 0.1 mm sieve and stored at −20 °C until further analysis. Dried pomace was then de-fatted 3 times by adding 100 mL of hexane to 10 g of pomace and filtering with a Buchner funnel apparatus. For extraction yields, the water was removed from a certain quantity of extract in a vacuum drier (Memmert, Schwabach, Germany) at 50 °C and vacuum pressure of 65 mb until constant weight was achieved (total aqueous extract yield = 208.35 ± 35 mg/g dried sample).

2.3. Response surface methodology (RSM)

The RSM with the Box–Behnken design was used to investigate the influence of three independent parameters; power, time and sample to

Table 3
The analysis of variance for the experimental results.

Parameter	DF	TPC		Antioxidant Capacity			
				DPPH		CUPRAC	
		Estimate	Prob > |F|	Estimate	Prob > |F|	Estimate	Prob > |F|
β_0	1	8.26	< 0.0001*	22.4	< 0.0001*	37.14	< 0.0001*
$\beta_{1\ power}$	1	2.4	< 0.0001*	2.53	0.0288*	6.79	< 0.0001*
β_{2time}	1	0.068	0.89	0.29	0.7921	1.61	0.2950
β_{3ratio}	1	−0.70	0.11	5.68	< 0.09	−0.91	0.4832
$\beta_{12power.time}$	1	1.59	0.025*	3.64	0.0209*	4.81	0.0250*
$\beta_{13power.ratio}$	1	−0.97	0.10	−0.92	0.4758	−1.60	0.3650
$\beta_{23time.ratio}$	1	−1.12	0.065	−2.96	0.0272*	−6.33	0.0009*
$\beta_{11power2}$	1	6.24	< 0.0001*	1.26	0.4260	19.93	< 0.0001*
$\beta_{22time2}$	1	3.53	< 0.0001*	−3.79	0.0210*	10.37	< 0.0001*
$\beta_{33ratio2}$	1	−2.62	0.0026*	−0.24	0.8914	−5.06	0.0445*

*Significantly different at $p < 0.05$; β_0: intercept; β_1, β_2 and β_3: linear regression coefficients for power, time and ratio; β_{12}, β_{13} and β_{23}: regression coefficients for interaction between power × time, power × ratio and time × ratio; β_{11}, β_{22} and β_{33}: quadratic regression coefficients for power × power, time × time and ratio × ratio.

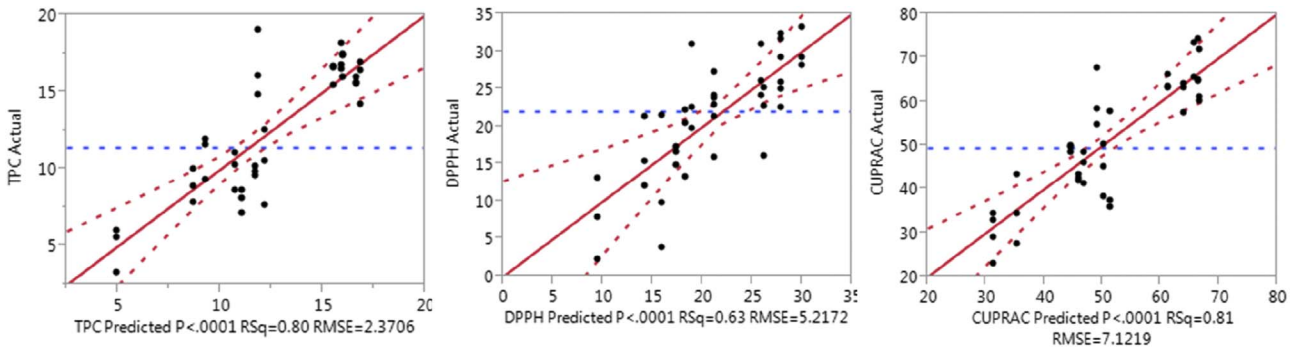

Fig. 1. Correlation between the actual and predicted values for TPC, DPPH and CUPRAC of the aqueous olive pomace extract.

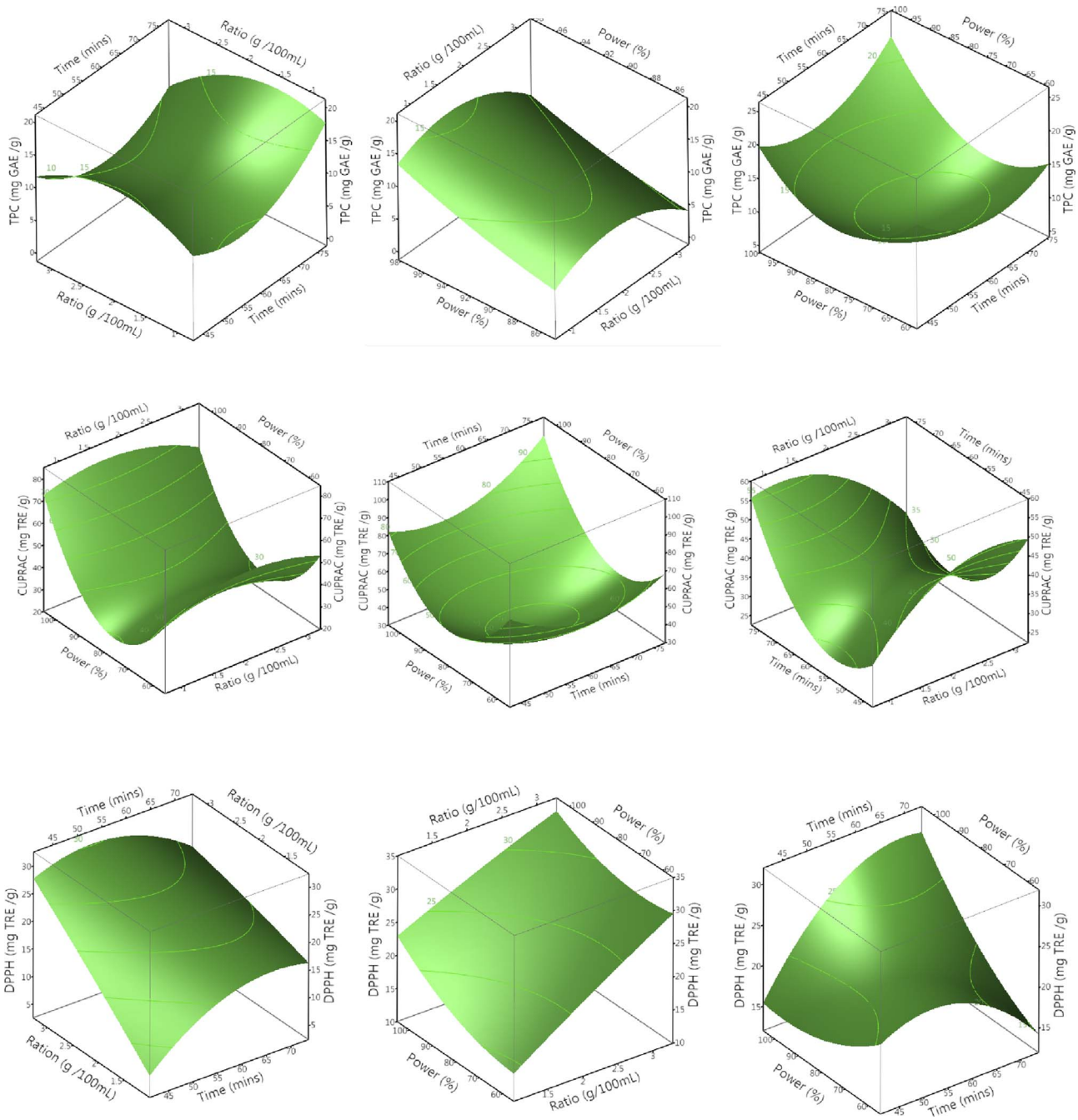

Fig. 2. 3D response surface and 2D contour plots for the effect of the test parameters on the total phenolic compounds (TPC) and antioxidant activity (DPPH and CUPRAC) of the aqueous olive pomace extracts.

solvent ratio, on the extraction of total phenolic compounds (TPC) and the antioxidant activity of the extracts. An ultrasonic bath was used (Soniclean, 220 V, 50 Hz and 250 W model 250HD, Soniclean, Pty Ltd, Thebarton, SA, Australia). The optimal ranges of power (150–250 W), time (45–75 min) and sample-to-solvent ratio (1–3 g/100 mL) were determined based on preliminary experiments (data not shown). A control extraction was conducted at the same optimal time and sample to solvent ratio without ultrasound. Temperature was maintained at 40 °C by the ultrasound baths temperature regulator. The independent variables and their code variable levels are shown in Table 1.

To express the TPC or antioxidant capacity as a function of the independent variables, a second-order polynomial equation was used as follows and as previously described by Vuong, Stathopoulos, Golding, Nguyen, and Roach (2011):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k=1} \sum_{j}^{k} \beta_{ij} X_i X_j + \sum_{i=1}^{k} \beta_{ii} X_i^2 ,$$

Where various X_i values are independent variables affecting the response Y; β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for the intercept and the linear, quadratic and interaction terms, respectively, and k is the number of variables.

2.4. Total phenolic compounds

The TPC were determined according to Thaipong et al. (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006). Briefly, samples were added to Folin–Ciocalteu's reagent before adding 5% sodium carbonate solution and incubating in the dark for 1 h. Absorbance was then read at 760 nm using a UV spectrophotometer (Varian, Melbourne, VIC, Australia). Results were expressed as mg of gallic acid equivalents per gram of dried olive pomace (mg GAE/g).

2.5. Total major bioactive compounds

For determination of total major bioactive compounds, HPLC was performed according to Goldsmith et al. (2014a) with minor modifications. The extracts were analysed using a Shimadzu HPLC system (Shimadzu Australia, Rydalmere, NSW Australia) and a 250 ± 4.6 mm Synergi 4 μm Fusion-RP 80A reversed-phase column (Phenomenex Australia Pty. Ltd., Lane Cove, NSW Australia) with detection at 254 nm. The column was maintained at 30 °C, the flow rate was 1 ml/min and three solvents were used for the mobile phase Solvent **A**: 0.1% orthophosphoric acid, Solvent **B**: 100% Methanol, Solvent **C**; 100% Ethanol. A gradient elution schedule was used according to the following: 0–40 min A 96%, B 2%, C 2%; 40–60 min A 40%, B 30%, C 30%; 60–62 min A 96%, B 2%, C 2%. Syringic acid was used as internal standard. Values for total major bioactive compounds were determined using a tyrosol standard curve; they were expressed as μg Tyrosol equivalents (TYE) per gram of dried olive pomace.

2.6. Antioxidant activity assays

Two assays were employed to assess the antioxidant activity of the pomace extracts:

The cupric reducing antioxidant capacity (CUPRAC) assay was conducted as previously described by Apak, Guclu, Ozyurek, and Karademir (2004). Results were expressed as mg of trolox equivalents per gram of dried olive pomace (mg TRE/g).

The DPPH free radical scavenging capacity of the extracts were analysed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, as described by Goldsmith, Vuong, Stathopoulos, Roach, and Scarlett (2014b). The results were expressed as mg of trolox equivalents per gram of dried olive pomace (mg TRE/g).

2.7. Statistical analysis

The RSM was designed and analysed using JMP Version 11 (SAS Cary, NC, USA). JMP was also used to develop the model equation, graph the 2D and 3D prediction profiler plots to predict the optimum values of the response variables in order to maximise the TPC and antioxidant capacity of the extracts. The original values and ranges of the parameters under investigation as well as their parameter symbols and codes are presented in Table 1.

3. Results and discussion

3.1. Fitting the models for the prediction of TPC and antioxidant capacity

Based on preliminary experiments (not shown), time, power and sample-to-solvent ratio were identified as important parameters which could impact upon the extraction of phenolic compounds from olive pomace, the ranges for each variable were determined and are listed in Table 1.

Table 2 shows the reliability of the mathematical model in predicting variances between actual and predicted values. The analysis of variance for the experimental results for the Box Behenkin design showed the coefficient of determination (R^2) for the fit of the model of TPC was 0.8, CUPRAC was 0.81 and DPPH was 0.69; suggesting that 80%, 81% and 69% of the actual TPC, CUPRAC and DPPH values could be predicted by the model, respectively. This relationship is further supported by the values for Predicted Residual Sum of Squares (PRESS is a measure of how well each point fits the experimental design) and the F-ratio of the model: 3128 and 15.1 for TPC, 3001 and 6.56 for CUPRAC and 1566 and 6.15 for DPPH (respectively). In summary, analysis of variance showed that the models are reliable for prediction of TPC and antioxidant capacity.

3.2. The effect of the test parameters on the extraction of TPC

The effect of the test parameters (coded variables in Table 1) on the response variable (Y) TPC is shown in the following equation:

$$Y = 8.3 + 2.4\ X_1 + 0.1\ X_2 - 0.7\ X_3 + 1.6\ X_1 X_2 - 1.0\ X_1 X_3 - 1.1\ X_2 X_3 + (6.2\ X_1)^2 + (3.5\ X_2)^2 - (2.6\ X_3)^2$$

Table 3 presents the linear regression coefficients for each variable and indicates their statistical significance. Power and time both had positive relationships with the extraction of TPC, while the sample-to-solvent ratio had a negative effect; that is, as we increased the amount of sample while keeping the amount of solvent that same, we saw a decrease in TPC. Therefore, as power and time were increased and as the amount of solvent/g of sample were increased, the extraction of TPC also increased. However, the only individual variable that had a significant influence on the extraction of TPC within the ranges tested

Table 4
Validation of the RSM models; the predicted values and the actual values obtained at the maximum desirability for the UAE conditions of 2 g of dried pomace/100 mL of water at 100% power for 75 min maintained at 30 °C.

	Phenolic compounds	Antioxidant activity	
	TPC	DPPH	CUPRAC
	(mg GAE g^{-1})	(mg TRE g^{-1})	(mg TRE g^{-1})
Predicted	22.02 ± 2.66[a]	26.37 ± 5.85[a]	80.57 ± 7.99a
Actual (UAE)	19.71 ± 1.41[a]	31.23 ± 1.42[a]	73.54 ± 2.54a
Control (no UAE)	13.76 ± 0.91[b]	28.07 ± 3.24[a]	65.36 ± 1.77b

[a, b]Values in the same column with a different superscript are significantly different from one another ($p < 0.05$)
Total yield of extracts (UAE = 222.2 ± 48.1, Control = 194 ± 39.6).

Table 5
Quantification of selected HPLC peaks expressed as μM Tyrosol Equivalents (TYE)/g of dried pomace. Peak numbers correspond to the peaks in Fig. 3.

Peak number	Retention time (mins)	UAE (μM TYE/g)	Control (μM TYE/g)
1	7.20	0.95 ± 0.1[a]	0.46 ± 0.07[b]
2	8.46	13.65 ± 0.84[a]	10.01 ± 0.12[b]
3	10.14	1.38 ± 0.02[a]	0.64 ± 0.06[b]
4	12.27	0.08 ± 0.03[a]	0.00[b]
5	16.26	6.24 ± 1.01[a]	4.99 ± 0.03[b]
6	16.89	1.29 ± 0.01[a]	0.61 ± 0.04[b]
7	17.41	0.00[a]	0.69 ± 0.07[b]
8	19.47	20.01 ± 0.04[a]	15.87 ± 0.09[b]
9	19.98	5.68 ± 0.07[a]	4.22 ± 0.03[b]
10	22.74	2.24 ± 0.12[a]	1.58 ± 0.15[b]
11	23.86	3.95 ± 0.01[a]	2.86 ± 0.49[b]
12	24.69	0.80 ± 0.01[a]	0.76 ± 0.31[a]
IS	26.11	na	Na
13	31.46	3.76 ± 0.25[a]	0.72 ± 0.24[b]
14	42.91	5.78 ± 0.05[a]	5.21 ± 0.73[a]
Total		**62.05 ± 1.87[a]**	**49.98 ± 2.27[b]**

[a, b] Values are means ± SD in the same row with a different superscript are significantly different from one another ($p < 0.05$).

was power ($p = 0.0001$). Power has previously been shown to increase the extraction of phenolic compounds from a variety of sources (Altemimi, Watson, Choudhary, Dasari, & Lightfoot, 2016). Moreover, the combination of power and time also had a significant influence on the extraction of TPC ($p = 0.03$); this is also in accordance with the literature (Falleh, Ksouri, Lucchessi, Abdelly, & Magné, 2012). In addition, the interaction between power and time within the ranges tested had a significant impact on extraction of TPC whereas, there was no interactive relationship between power and ratio or time and ratio (Table 3); indicating that increasing both power and time can result in a higher TPC being extracted from the olive pomace.

3.3. The effect of the test parameters on antioxidant activity

The effect of the test parameters (coded variables in Table 1) on the response variable DPPH scavenging capacity (Y) is shown in the following equation:

$$Y = 22.4 + 2.5\,X_1 + 0.3\,X_2 + 5.7\,X_3 + 3.6\,X_1X_2 - 0.9\,X_1X_3 - 3.0\,X_2X_3 + (1.3\,X_1)^2 - (3.8\,X_2)^2 - (0.2\,X_3)^2$$

Similarly, the effect of the test parameters (coded variables in Table 1) on the response variable cupric reducing antioxidant capacity (Y), is shown in the following equation:

$$Y = 37 + 6.8\,X_1 + 1.6\,X_2 - 0.9\,X_3 + 4.8\,X_1X_2 - 1.6\,X_1X_3 - 6.3\,X_2X_3 + (19.9\,X_1)^2 + (10.4\,X_2)^2 - (5.1\,X_3)^2$$

The results showed that the individual variables of power and time had a positive influence on both the DPPH scavenging capacity and the cupric reducing antioxidant capacity of the extracts. Sample-to solvent ratio on the other hand, had a positive influence on the DPPH scavenging capacity but had a negative influence on the cupric reducing antioxidant capacity. In addition, power and time as well as time and sample to solvent ratio, in the tested ranges, had a significant interactive effect on DPPH scavenging capacity and the cupric reducing

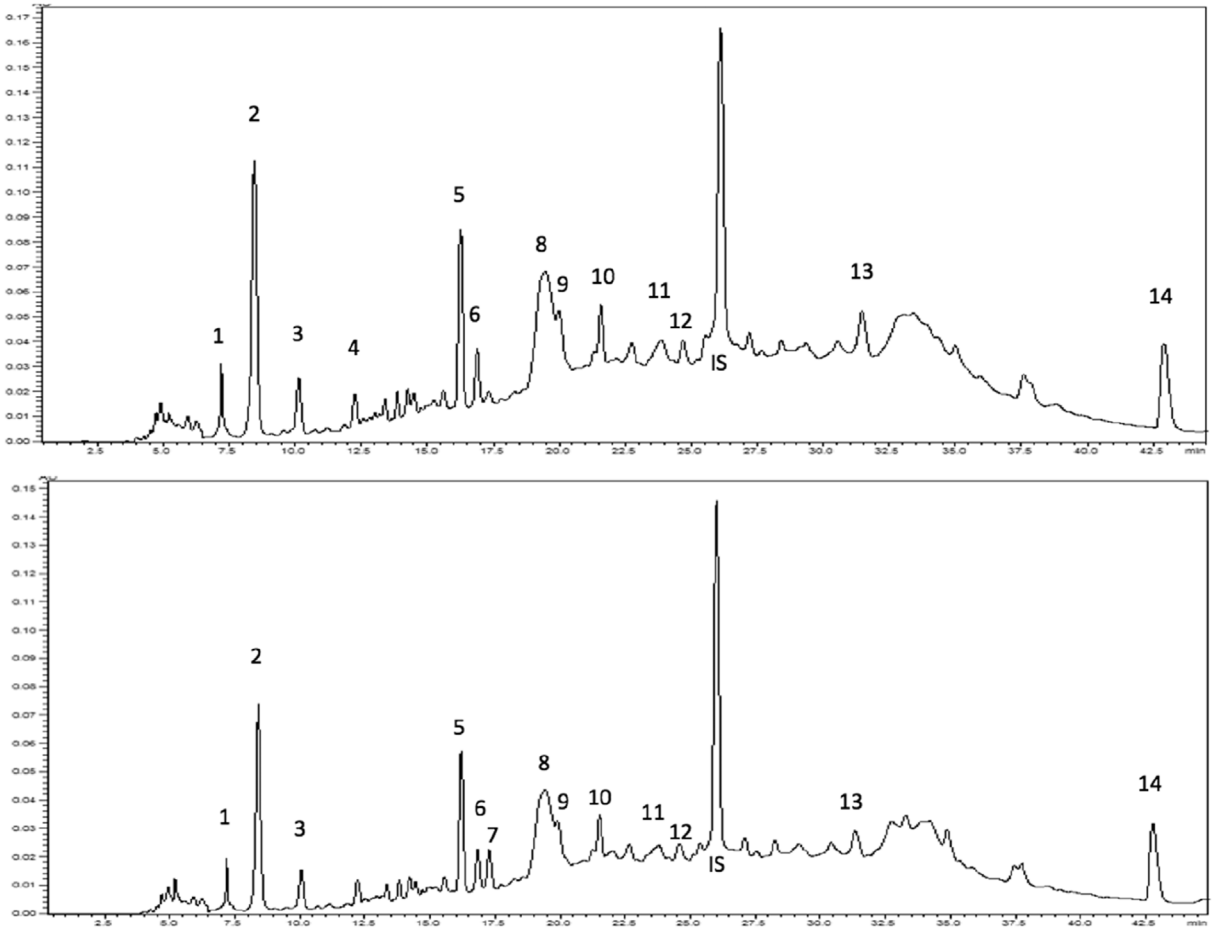

Fig. 3. Typical HPLC chromatogram at 254 nm of; (Top) optimal UAE extract (Bottom) control extract. The internal standard (IS) was syringic acid.
*Axes on chromatograms are not the same.

antioxidant capacity of the extracts. Of interest, power and ratio in the tested ranges did not show a significant interactive effect on DPPH scavenging capacity and cupric reducing antioxidant capacity of the extracts.

3.4. Optimisation of the extraction conditions for maximum extraction of TPC with high antioxidant activity from olive pomace

Based on the predictive models (Figs. 1 and 2), the optimal conditions for the extraction of phenolic compounds from olive pomace were 2 g of dried pomace/100 mL of water at 250 W power for 75 min. These conditions were the same for the optimisation of antioxidant activity via DPPH and CUPRAC; therefore, these conditions were used for further validation (Table 4). The resulting values fell inside the proposed ranges for TPC and antioxidant activity. As such, these conditions were proposed as optimal for the extraction of phenolic compounds with high antioxidant activity from olive pomace waste.

3.5. Optimal UAE conditions compared to control conditions

The principle of UAE extraction is to disrupt plant cell walls and increase mass transfer of intracellular components into the extraction solvent (Yingngam, Monschein, & Brantner, 2014). To assess the efficacy of ultrasound in extracting phenolic compounds with high antioxidant activity from olive pomace, validation was also conducted comparing the optimal conditions with and without ultrasound. The optimised UAE conditions increased the extraction of TPC by 24% (Table 4). This was also reflected in the HPLC results where by the UAE improved total peak area by 20.4% (Table 5). Typical chromatograms produced from optimised UAE extracts as well as control extracts are pictured in Fig. 3. The UAE conditions yielded a higher level of TPC as well as antioxidant activity compared to the control. Fig. 3 shows that the optimised UAE extracts had a higher area for most of the peaks compared to the control extracts; however, the UAE extracts did not have any additional peaks. This suggests that UAE enhanced the ability of water to extract compounds from the pomace without extracting any additional compounds. This increase can be attributed to the ability of Ultrasound to impact the microstructure of plant materials; since ultrasonic cavitation creates shear forces that disrupt cell walls, which enabled the extraction solvent to penetrate the pomace tissue and extract the phenolic compounds. Similar results have been reported previously (Chen et al., 2018; Feng, Luo, Tao, & Chen, 2015; Tian, Xu, Zheng, & Martin Lo, 2013).

The antioxidant activity of the UAE extracts (Table 4) was also higher than the controls (an increase of 11% and 12% for the DPPH and CUPRAC assays respectively). The application of UAE has been shown to increase the antioxidant activity of extracts from a variety of plant materials, including olive leaves (Sahin & Samli, 2013), peach, pumpkin (Altemimi et al., 2016) and green tea (Nkhili et al., 2009). This is likely due to the improvement in the extraction of total phenolic compounds. In the present study, no new peaks were identified in the chromatograms from the UAE extracts (Fig. 3) when compared to the controls; therefore, the increase in antioxidant activity is likely due a larger quantity of each compound being extracted. However, since the peak area (mg TYE equivalents) increased by 26% with the application of UAE (Table 4) the peaks that were significantly increased must correspond to compounds with high antioxidant activity. Therefore, UAE can be considered as an effective technique to increase the levels of the extracted compounds with high antioxidant activity in olive pomace extracts.

4. Conclusions

UAE increased the quantity of phenolic compounds extracted from olive pomace. The proposed optimal conditions for the extraction of phenolic compounds with high antioxidant activity from olive pomace were 2 g of dried pomace/100 mL of water at 100% power (250 W) for 75 min maintained at 30 °C. This simple and inexpensive method could be readily up-scaled to add a source of income to olive farmers and olive oil processors, a viable use for this agricultural waste product.

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3.4 Conclusions

Outcomes of this study have identified the best possible combination of parameters for using water as an extraction solvent for the extraction of phenolic compounds with antioxidant activity from olive leaves and pomace. For olive leaves, the optimal conditions were sample-to-solvent ratio 1:100 (g/ mL), heated to 90°C for 70min. However, at a sample-to-solvent ratio of 1:60 (g/ mL), we retained 80% of the total phenolic compounds and maximized the antioxidant activity of the extracts. For olive pomace, optimal conditions were sample-to-solvent ratio 1:50 (g/ mL), at 250W of ultrasound power for 75min. These aqueous extraction methods are simple and potentially easily scaled up to an industrial setting. Moreover, these findings set a baseline for researchers working on the development of novel green extraction technologies for the recovery of phenolic compounds from olive waste.

CHAPTER 4

MAJOR BIOPHENOL CONTENT AND CYTOTOXICITY OF CRUDE OLIVE WASTE EXTRACTS

4.1. Introduction

Crude olive waste materials are a complex system of compounds with varying polarities. In the previous chapter, we optimised the aqueous extraction conditions for the recovery of biophenols from olive waste materials. However, there are some important compounds found in olive materials which have been shown to favour partitioning into organic solvents. In fact, the major biophenols from olive products, which have displayed anti-cancer activity previously (namely; oleuropein, hydroxytyrosol, tyrosol, luteolin and apigenin) are often extracted with organic solvents. Therefore, in order to test the efficacy of water as an extraction solvent it is important to compare the major biophenols obtained to that of solvent extracts. Therefore, this chapter investigates the water extracts optimised in CHAPTER 3, as a source of the major olive biophenols oleuropein, hydroxytyrosol, tyrosol, luteolin and apigenin, comparing to solvent extracts in order to establish the efficacy of water as an extraction solvent for biophenols from olive waste material.

The extraction of biophenols is an important area of research. However, extracting compounds which have no application is pointless. Therefore, the determination of applications for biophenols is important. The major olive biophenols (oleuropein, hydroxytyrosol, tyrosol, luteolin and apigenin) were selected because they have been shown to exhibit anti-cancer effects in different tissues. Furthermore, luteolin and apigenin have previously exhibited activity in a pancreatic cancer cell line (BxPC-3). Therefore, the cytotoxicity of crude olive waste extracts containing major biophenols was investigated.

4.2. Experimental design

The design of experiments for the determination of the phytochemical properties and anti-cancer potential of olive pomace and olive leaf extracts are shown in Figure 4.1. and Figure 4.2.

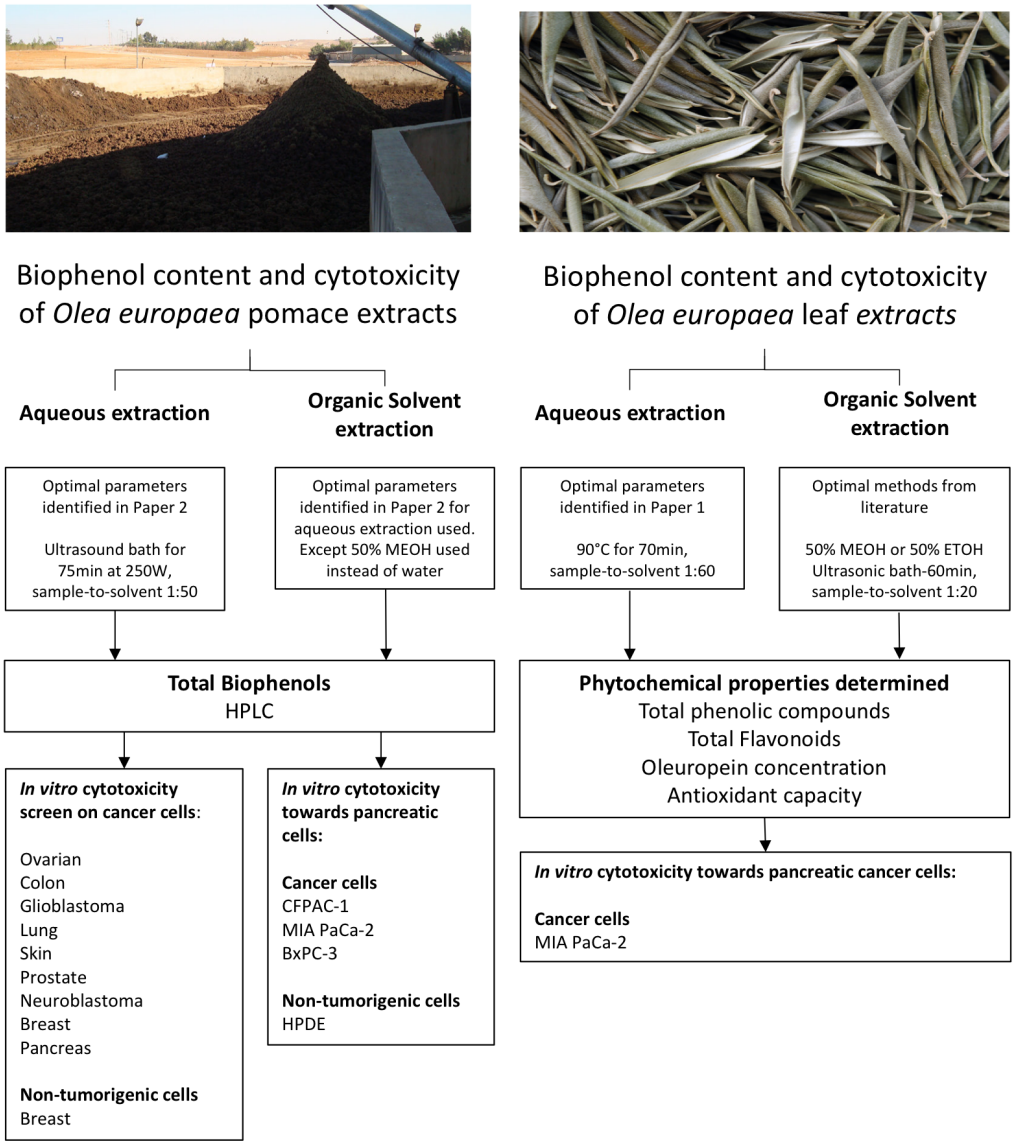

Figure 4.1. Experimental design for the investigation of the biophenol content and cytotoxicity of olive pomace and olive leaf extracts.

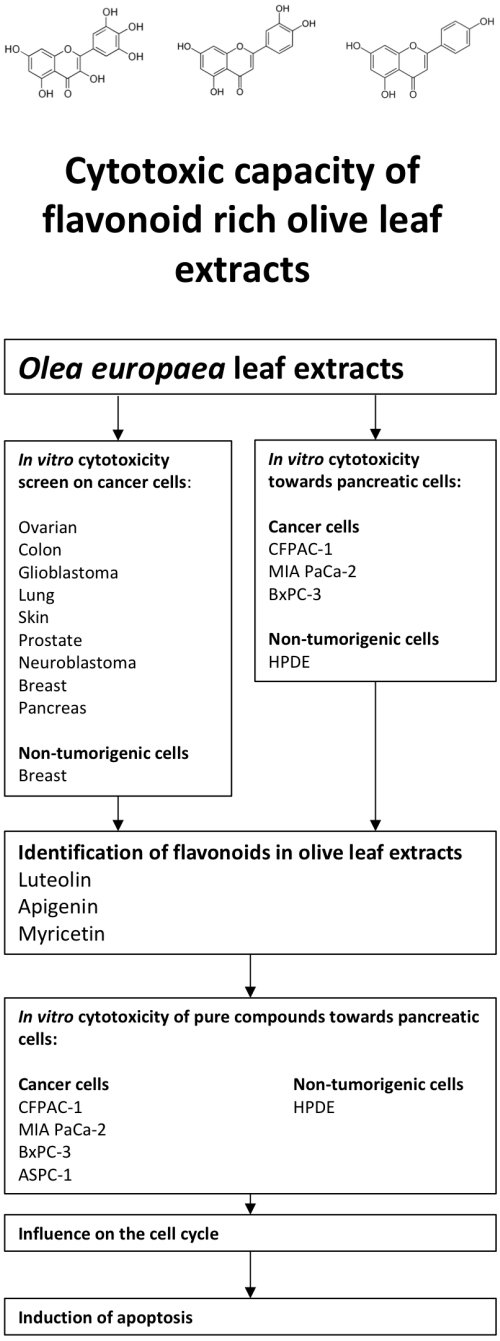

Figure 4.2. Schematic detailing the experimental design for the investigation of the cytotoxicity and identification of major biophenols in olive leaf extracts.

4.3. Results and discussion

The results and detailed discussion of the investigation of the phytochemical and anti-cancer properties of crude olive leaf extracts is presented in research paper 3 entitled "**Phytochemical properties and anti-proliferative activity of *Olea europaea* L. leaf extracts against pancreatic cancer cells"** published in **Molecules** in 2015 (DOI:10.3390/molecules200712992). Additionally, the biophenol content and anti-cancer activity of olive pomace extract is presented in research paper 4 entitled "**Cytotoxicity of olive pomace extract towards pancreatic cancer cells" submitted to Herbal Medicine in 2017.**

Furthermore, the results and detailed discussion of the major biophenols and cytotoxicity of olive leaf extracts is presented in research paper 5 entitled "**Flavonoids in *Olea europaea* leaf extracts and their cytotoxicity towards pancreatic cancer cells" submitted to Food & Function in 2017.**

4.3.1. Research paper 3

Research paper 3 entitled "**Phytochemical properties and anti-proliferative activity of *Olea europaea* L. leaf extracts against pancreatic cancer cells"** published in **Molecules** in 2015 (DOI:10.3390/molecules200712992).

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Article

Phytochemical Properties and Anti-Proliferative Activity of *Olea europaea* L. Leaf Extracts against Pancreatic Cancer Cells

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Abstract: *Olea europaea* L. leaves are an agricultural waste product with a high concentration of phenolic compounds; especially oleuropein. Oleuropein has been shown to exhibit anti-proliferative activity against a number of cancer types. However, they have not been tested against pancreatic cancer, the fifth leading cause of cancer related death in Western countries. Therefore, water, 50% ethanol and 50% methanol extracts of *Corregiola* and *Frantoio* variety *Olea europaea* L. leaves were investigated for their total phenolic compounds, total flavonoids and oleuropein content, antioxidant capacity and anti-proliferative activity against MiaPaCa-2 pancreatic cancer cells. The extracts only had slight differences in their phytochemical properties, and at 100 and 200 μg/mL, all decreased the viability of the pancreatic cancer cells relative to controls. At 50 μg/mL, the water extract from the *Corregiola* leaves exhibited the highest anti-proliferative activity with the effect possibly due to early eluting HPLC peaks. For this reason, olive leaf extracts warrant further investigation into their potential anti-pancreatic cancer benefits.

Keywords: olive leaf; oleuropein; pancreatic cancer; phenolic compounds; *Olea europaea* L.; antioxidant activity; phytochemicals; biophenols

1. Introduction

Olea europaea L. leaf (olive leaf) is a waste product of the olive oil extraction process, weighing up to 10% of the material arriving at the mill. Currently, this by-product is not profitable; olive leaves are often used as animal feed or simply burned with excess branches gathered from pruning [1,2]. Many olive oil producers even charge a fee to the olive farmer for the disposal of olive leaves. The interest in olive leaf has grown in recent years due to the high concentration of phenolic compounds, of which oleuropein is the most abundant. A number of the health benefits of virgin olive oil consumption have been attributed to oleuropein. It has been found to have anti-atherogenic [3], anti-inflammatory [4] and antimicrobial [5] properties. More recently, oleuropein has been investigated for its potent anti-cancer activity. It has been shown to inhibit proliferation and migration of a number of advanced grade human tumour cell lines in a dose dependent manner [6–11]. However, the effect of olive phenolic compounds has yet to be investigated for pancreatic cancer.

Pancreatic cancer is a devastating heterogeneous disease with significant resistance to the limited conventional treatment options and the current chemotherapy agents are highly toxic [12,13–15]. Thus, it is essential to control and manage the development of pancreatic cancer [16] as well as to develop novel therapeutic strategies against it. The use of olive phenolic compounds may serve as a useful strategy to inhibit carcinogenesis [14]. To our knowledge there has not been any investigation into the effect of olive leaf phenolic compounds on pancreatic cancer cells.

It is important to understand the effect different extraction conditions have on phenolic compound yield. A number of methods have been proposed for the extraction of phenolic compounds from olive leaves [17–19]. However, it is difficult to compare these studies since they use very different methods including advanced technologies and an array of different solvents. Furthermore, the use of advanced technologies, including microwave and ultrasound-assisted extraction methods, are difficult to scale up to an industrial setting and organic solvents can be expensive and difficult to dispose of. This has led to a push from industry and researchers for the development of more environmentally friendly, or "green" extraction techniques, for example, using water as an extraction solvent. However, it is important to understand the efficacy of these "green" extraction protocols compared to organic solvent extraction methods and advanced technologies.

We hypothesised that water is an effective extraction solvent for preparing oleuropein rich olive leaf extracts with anti-pancreatic cancer activity. Therefore, this study aimed to characterise the phytochemical properties of olive leaf extracts obtained from two different cultivars of olive leaves via different previously optimised extraction methods. A water extraction method was compared to two ultrasound-assisted extraction methods with 50% ethanol or 50% methanol as the solvent. The anti-pancreatic cancer effect of these extracts was also assessed.

2. Results and Discussion

There were no differences between the Corriola and Frantoio varieties in their TPC, total flavonoids and oleuropein content (Table 1) and in their antioxidant capacity (Table 2). Many variables can affect the phenolic compound content of olive products including the position on the tree, cultivar, soil mineral content as well as sun exposure. However, the geographic location of the tree has been shown to have the largest effect on the phenolic compound profile of olive products [20]. Therefore, it is likely that the reason that no difference was seen between the two different varieties of olive leaves was that they were from the same location.

Table 1. Phytochemical properties of olive leaf extracts. Total phenolic compounds (TPC) are expressed as gallic acid equivalents (GAE)/g of extract, total flavonoids are expressed as rutin equivalents (RE)/g of extract and oleuropein is expressed as mmol/g of dried extract.

Solvent	Cultivar	TPC (mg GAE/g)	Total Flavonoids (mg RE/g)	Oleuropein (μmol/g)
Water	*Corregiola*	230.15 ± 6.85 [a]	345.45 ± 85.71 [a]	86.33 ± 1.41 [a]
Ethanol (50%)	*Corregiola*	238.70 ± 11.85 [a]	828.13 ± 47.82 [b]	114.54 ± 1.14 [b]
Methanol (50%)	*Corregiola*	231.05 ± 11.15 [a]	539.53 ± 18.16 [a]	109.54 ± 3.92 [b]
Water	*Frantoio*	233.45 ± 0.20 [a]	442.95 ± 16.52 [a]	85.11 ± 1.65 [a]
Ethanol (50%)	*Frantoio*	241.60 ± 23.5 [a]	1035.79 ± 121.25 [b]	111.93 ± 5.80 [b]
Methanol (50%)	*Frantoio*	236.20 ± 11.02 [a]	528.51 ± 43.87 [a]	105.01 ± 1.13 [b]

[a,b] Values in the same column not having the same superscript letter are significantly different from each other ($p < 0.05$).

Table 2. Antioxidant capacity of olive leaf extracts measured using three different antioxidant activity assays. DPPH is expressed as % inhibition and FRAP and CUPRAC are expressed as mg trolox equivalents (TRE)/g of dried extract.

Solvent	Cultivar	DPPH (% Inhibition)	FRAP (mg TRE/g)	CUPRAC (mg TRE/g)
Water	*Corregiola*	74.75 ± 5.85 [a]	22.85 ± 19.17 [a]	308.65 ± 36.83 [a]
Ethanol (50%)	*Corregiola*	70.97 ± 12.9 [a]	218.51 ± 49.34 [a]	322.32 ± 32.99 [a]
Methanol (50%)	*Corregiola*	84.25 ± 4.31 [a]	237.81 ± 35.49 [a]	302.54 ± 6.75 [a]
Water	*Frantoio*	75.61 ± 2.73 [a]	232.12 ± 4.89 [a]	318.07 ± 59.76 [a]
Ethanol (50%)	*Frantoio*	86.34 ± 4.27 [a]	303.44 ± 19.81 [a]	326.62 ± 21.71 [a]
Methanol (50%)	*Frantoio*	86.63 ± 8.19 [a]	216.15 ± 55.66 [a]	303.92 ± 22.17 [a]

[a] All values in the same column were not significantly different from each other ($p > 0.05$).

2.1. The Influence of Extraction Methods on Phytochemical Properties

The different solvents and extraction conditions had no effect on the TPC but did influence the total flavonoids and the oleuropein content in the extracts (Table 1). The water extract had a lower level of total flavonoids and oleuropein while the methanol extract had a lower level of total flavonoids, when compared to the ethanol extract. Flavonoids are the largest group of phenolic compounds and include both polar and non-polar moieties. The 50% ethanol extract contained more than double the total

flavonoids compared to the water extract (Table 1). This suggests that the majority of the compounds present in the olive leaves were either less polar flavonoids or were potentially heat sensitive compounds which were degraded during the water extraction process conducted at 90 °C for 70 min [21]. Consistent with this, acetone is well known to be the best solvent for the extraction of flavonoids [21] and acetone is less polar than 50% ethanol.

The typical HPLC chromatograms for the different extraction protocols (Figure 1) showed similar peak profiles. However, the earlier-eluting less polar peaks (1–7) were slightly larger in the water extract while the later-eluting more polar peaks (9–17) were larger in the ethanol and methanol extracts. The greatest effect was seen in peak 12, for which the area was more than tripled in the ethanol and methanol extracts compared to the water extract.

Since oleuropein (peak 13) is one of the less polar compounds in the HPLC chromatograms (Figure 1), it was not surprising to see that the organic solvents extracted more of it from the olive leaves. However, the water method still extracted approximately 80% of the oleuropein compared to the ethanol extraction method. Moreover, there was no difference in the level of TPC between the different extracts. Therefore, when considering the current push towards "green" extraction protocols, the water extraction method [21] is an excellent candidate for industrialisation.

2.2. The Influence of Extraction Methods on Antioxidant Capacity

Despite the differences in the phytochemical properties of the extracts obtained with the different solvents, there was no significant difference in their antioxidant capacity as measured via the DPPH, FRAP or CUPRAC assays (Table 2). This was not surprising since although individual compounds did vary depending on the extraction conditions, there was no difference in the TPC of the different extracts (Table 1). This further highlights the effectiveness of this "green" water extraction protocol. Additionally, previous reports have shown that antioxidant activity can increase in extracts whose high molecular weight compounds have degraded into more active lower molecular weight compounds. One example of this is the degradation of oleuropein into hydroxytyrosol and tyrosol. However, this seems unlikely since tyrosol (peak 5) was not detected in significant amounts and hydroxytyrosol was not detected at all in the HPLC chromatograms (Figure 1).

2.3. The Influence of Extraction Methods on Growth Inhibition of Pancreatic Cancer Cells in Vitro

At a concentration of 200 μg/mL, all of the crude olive leaf extracts for both the *Corregiola* and *Frantoio* cultivars were able to reduce the viability of the MiaPaCa-2 cells to less than 1% relative to controls, and they were significantly more toxic (47.8%) than the chemotherapy drug gemcitabine at its IC_{50} of 50 nm (Table 3). At 100 μg/mL, the water extract of the *Frantoio* variety (0.47%), the 50% ethanol extract of the *Corregiola* variety (4.26%) and the water extract of the *Corregiola* variety (14.59%) demonstrated a significantly greater effect on the cell viability of the MiaPaCa-2 cells compared to the 50% ethanol (30.37%) and the 50% methanol (41.13%) extracts of the *Frantoio* variety and the 50% methanol extract of the *Corregiola* variety (32.83%). However, all the extracts at 100 μg/mL were still more toxic than gemcitabine at its IC_{50} (Table 3).

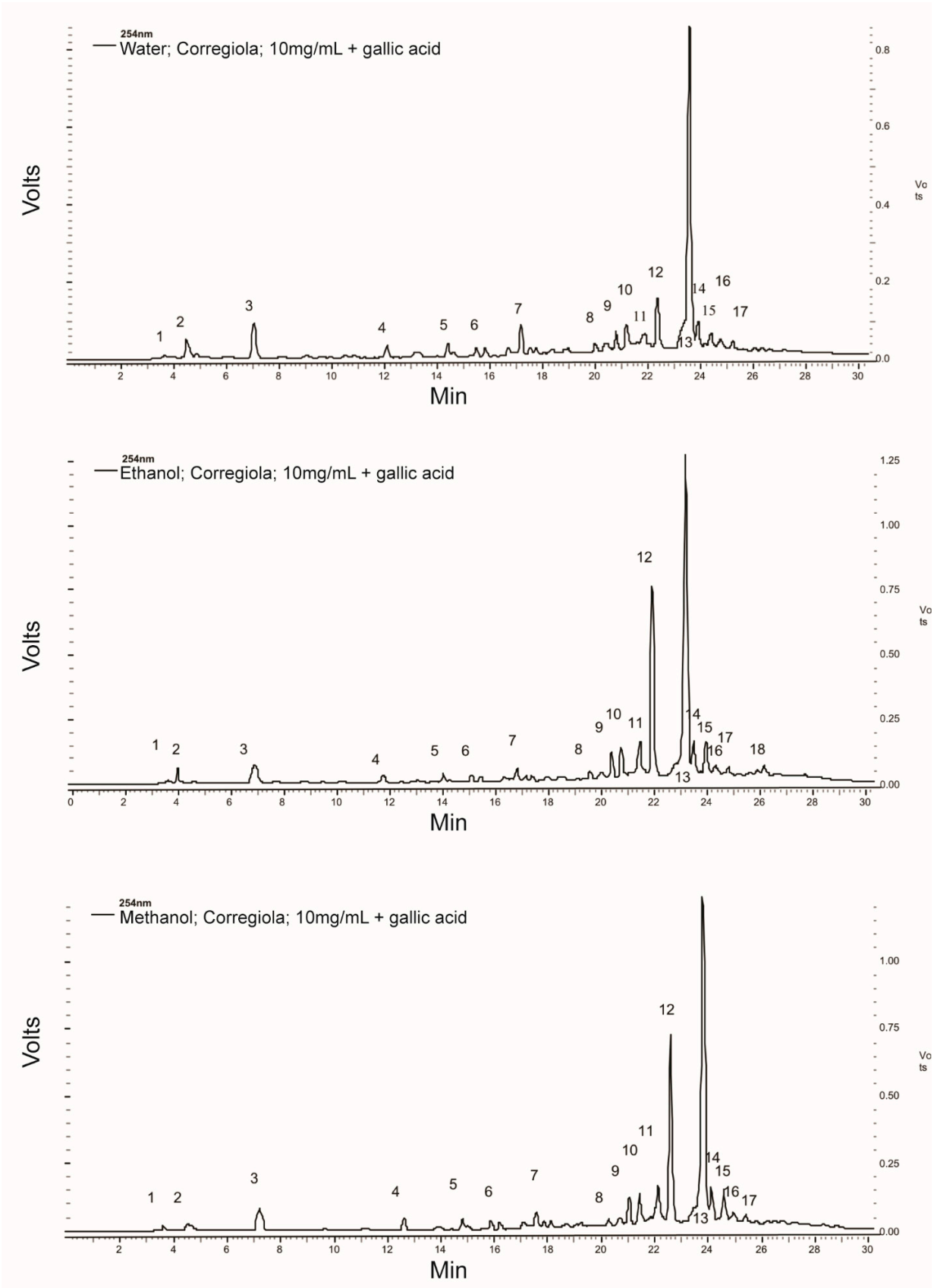

Figure 1. Typical HPLC chromatograms for the water, 50% ethanol and 50% methanol extracts from olive leaves. Peaks identified were: (3) gallic acid (internal standard), (5) tyrosol and (13) oleuropein.

Table 3. Anti-proliferative activity of olive leaf extracts (0–200 μg/mL) on MiaPaCa-2 pancreatic cancer cells. Results are expressed as % viability compared to controls ± standard deviation.

Solvent	Cultivar	Gemcitabine (50 nM)	Concentration of Olive Leaf Extract (μg/mL)			
			0 (Controls)	50	100	200
water	*Corregiola*		100 ^{a,i}	55.89 ± 3.53 ^{b,i}	14.59 ± 0.5 ^{c,i}	0.63 ± 0.29 ^{c,i}
ethanol	*Corregiola*		100 ^{a,i}	121.59 ± 13.7 ^{a,ii}	4.26 ± 2.6 ^{b,ii}	0.44 ± 2.08 ^{b,i}
methanol	*Corregiola*		100 ^{a,i}	73.57 ± 9.33 ^{b,ii}	32.83 ± 10.41 ^{c,iii}	0.87 ± 0.17 ^{d,i}
water	*Frantoio*		100 ^{a,i}	103.19 ± 27.9 ^{a,ii}	0.47 ± 0.13 ^{b,ii}	0.61 ± 0.17 ^{b,i}
ethanol	*Frantoio*		100 ^{a,i}	122.78 ± 21.1 ^{a,ii}	30.37 ± 4.48 ^{b,iii}	0.87 ± 0.22 ^{c,i}
methanol	*Frantoio*		100 ^{a,i}	120.26 ± 9.22 ^{b,ii}	41.13 ± 16.02 ^{c,iii}	0.98 ± 0.56 ^{c,i}
	control		100 ^{a,i}			
		47.8 ± 0.1				

^{a,b,c,d} Values in the same row not having the same superscript letter are significantly different from each other. ^{i,ii,iii} Values in the same column not having the same superscript roman numeral are significantly different from each other. Values are expressed as percentage growth compared to controls with no extracts or gemcitabine. Therefore, the lower the value in response to olive leaf extract (50–200 μg/mL), the greater the anti-proliferative effect. Values greater than 100% represent cell growth greater than controls. Time = 96 h.

Interestingly, at 50 μg/mL, the water extract of the *Corregiola* variety (55.89%) had a significantly greater negative impact on the MiaPaCa-2 cells' viability than all the other extracts (Table 3). The compounds eluting as peaks 2 and 3 in the HPLC chromatogram (Figure 1) for the *Corregiola* variety are of interest because they appear more prominent in the water extract than in the other two extracts. However, the water extract from the *Corregiola* olive tree leaves may also have other compounds which are not detected at 254 nm.

Figure 1 shows the phenolic compound profile of the olive leaf samples and it appears that oleuropein (peak 13) is by far the most abundant compound present. However, at a concentration of 200μg/mL, the crude olive leaf extracts only contained approximately 20 nM of oleuropein. Despite this very low dose, the crude leaf extracts were still able to significantly reduce the viability of the pancreatic cancer cells compared to gemcitabine at its IC_{50} ($p < 0.05$). The anti-proliferative capacity of the olive leaf extracts in the present study against pancreatic cancer cells is better than what has been observed in previous studies on cancers of different origins. Han *et al*. [22] showed that 200 μg/mL of pure oleuropein was able to dramatically reduce the cell viability of MCF-7 human breast cancer cells. Further investigation discovered that oleuropein decreased the number of MCF-7 cells by inhibiting the rate of proliferation and inducing cell apoptosis. However, the results suggest that the water extract from the *Corregiola* leaves may have other active compounds, which are more potent against this pancreatic cell line than oleuropein, since the water extracts from both olive varieties were significantly lower, not higher, in oleuropein than the 50% ethanol and 50% methanol extracts (Table 1). Therefore, the present study provides a platform for further research into olive leaf phenolic compounds and their efficacy.

3. Experimental Section

3.1. Materials

Folin Ciocalteu's reagent, sodium carbonate, gallic acid, Sodium Nitrite, aluminium chloride, sodium hydroxide, rutin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), ferric chloride, sodium acetate, acetic acid, copper(II) chloride, ammonium acetate (NH_4Ac) , neocuproine methanol and ethanol were purchased from Sigma Aldrich (Castle Hill, NSW, Australia).

Human pancreatic cancer (Mia-PaCa2) cells, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), horse serum and L-glutamine.

3.2. Sample Preparation and Extraction of Phenolic Compounds

Corregiola and *Frantoio* olive leaves were obtained from Houndsfield Estate in the Hunter Valley of NSW Australia. Leaves were dried at 120 °C for 90 min according to Ahmad-Qasem *et al*. [23], ground to a size of 0.1 mm and stored at −20 °C until further analysis. Water extracts were prepared according to Goldsmith *et al*. [24] while 50% methanol and 50% ethanol extracts were prepared according to Sahin *et al*. [19] (Figure 2). Extracts were concentrated (their volume reduced) using a rotary evaporator, freeze dried and then stored at −20 °C until further analysis.

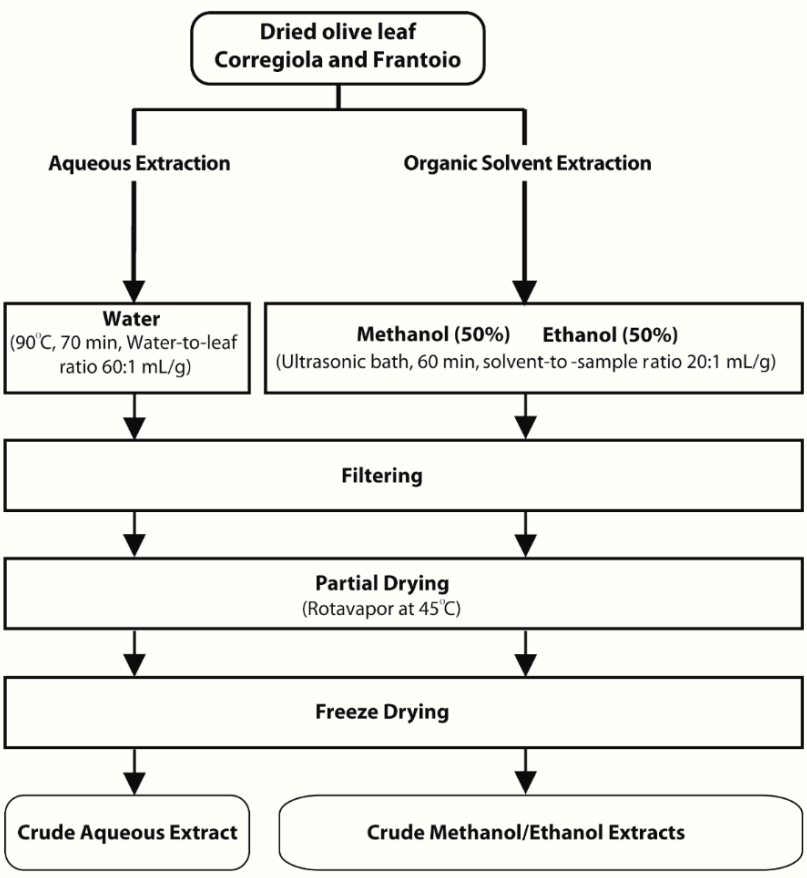

Figure 2. Methods for the preparation of olive leaf extracts.

3.3. Total Phenolic Compounds

The total phenolic compounds (TPC) were determined according to Thaipong *et al*. [25]. Briefly, diluted samples (300 μL) were added to Folin Ciocalteu's reagent (300 μL) and left to equilibrate for 2 min before adding 2.4 mL of 5% sodium carbonate solution and incubated in the dark for 1 h. Absorbance was then read at 760 nm using a UV spectrophotometer (Varian, Melbourne, VIC, Australia). Gallic acid was used as the standard and results were expressed as mg of gallic acid equivalents per g of sample dry weight (mg GAE/g).

3.4. HPLC

The olive leaf extracts were re-dissolved at 10 mg/mL and analysed using high performance liquid chromatography (HPLC) according to Goldsmith *et al*. [26] with some minor modifications. A Shimadzu HPLC system was used (Shimadzu Australia, Rydalmere, NSW Australia) with a 250 × 4.6 mm Synergi 4 μm Fusion-RP 80A reversed-phase column (Phenomenex Australia Pty. Ltd., Lane Cove, NSW Australia) with UV detection at 254 nm. The column was maintained at 30 °C, flow rate 1 mL/min and the three solvents used for the mobile phase were: solvent A—1% acetonitrile in 0.2% H_3PO_4 (*v*/*v*); solvent B—100% methanol; and solvent C—100% acetonitrile. A gradient elution schedule was used. The initial solvent system at the time of injection was 96% A, 2% B and 2% C. The eluting solvent was then changed, in a linear gradient manner, to 40% A, 30% B and 30% C by 20 min and held there for 20 min. From 40–42 min, the solvent was then returned to 96% A, 2% B and 2% C and maintained there for 10 min to re-equilibrate the column with the initial solvent system before the next injection. Gallic acid was used as an internal standard.

3.5. Determination and Quantification of Oleuropein

The HPLC peak corresponding to oleuropein was identified using an internal standard. The quantity of oleuropein in the extracts was determined using a standard curve of oleuropein prepared in methanol, which was linear between 0.05 and 0.925 mM, with the results expressed as mmol oleuropein per g dry weight (mmol/g).

3.6. Flavonoids

Total flavonoids were determined according to Vuong *et al*. [27]. Briefly, powdered extracts were re-dissolved at a concentration of 1 mg/mL in their respective solvents and 0.5 mL was added to 0.15 mL of 5% sodium nitrite, incubated for 6 min before adding 0.15 mL of 10% aluminium chloride and incubating for an additional 6 min. Finally, 2 mL of sodium hydroxide was then added before incubating for a further 15 min. Absorbance was then read at 510 nm using a UV spectrophotometer (Varian, Melbourne, VIC, Australia). Rutin was used as a standard and results were expressed as mg of rutin equivalents per g of sample dry weight (mg RE/g).

3.7. Assessment of Antioxidant Capacity

Three assays were employed to assess the antioxidant activity of the olive leaf extracts:

3.7.1. FRAP

For the ferric reducing antioxidant power (FRAP) assay, the extract was diluted and then the ferric ion reducing capacity was determined according to Thaipong *et al*. [25]. Stock solutions were: (1) 300 mM acetate buffer pH, (2) 10 mM TPTZ solution in 40 mM HCl, (3) 20 mM $FeCl_3$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3$ and then warming to 37 °C. Olive leaf extracts, trolox standards and blank (150 µL) were then added to 2.85 mL of the working FRAP solution and left to incubate in the dark at 37 °C for 30 min. Absorbance was read at 593 nm. Results were expressed as mg trolox equivalents per g of sample dry weight (mg TE/g).

3.7.2. CUPRAC

For the cupric reducing antioxidant capacity (CUPRAC) assay, the extracts were diluted and their cupric ion reducing capacity was determined as described by Apak *et al*. [28]. The stock solutions were: (1) 10 mM $CuCl_2$ solution, (2) ammonium acetate buffer at pH 7.0, (3) 7.5 mM neocuproine (Nc) solution in 95% ethanol. A working solution of the three reagents (1:1:1 *v*/*v*) was prepared, 3 mL of which was added to 1.1 mL of the diluted extracts, trolox standards and blanks and left to react in the dark for 1 h. Absorbance was read at 450 nm. Results were expressed as mg of trolox equivalents per g of sample dry weight (mg TE/g).

3.7.3. DPPH

The free radical scavenging activity of the extracts was analyzed using the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay as described by Vuong *et al*. [29]. Briefly, the appropriately diluted samples, trolox standards and blank (150 µL) were added to 2.85 mL of DPPH working solution (made to an absorbance of 1.1 ± 0.01 at 520 nm) and left to react in the dark at room temperature for 3 h. The results were expressed as % inhibition.

3.8. Effect of Olive Leaf Extracts on Pancreas Cells

3.8.1. Pancreas Cell Culture

Human pancreatic cancer (Mia-PaCa2) cells were cultured at 37 °C under 5% CO_2. Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2.5% horse serum and L-glutamine (100 µg/mL), was used.

3.8.2. Assessment of Cell Growth Inhibition of Olive Leaf Extracts

Cell growth inhibition was determined using the Dojindo Cell Counting Kit-8 (CCK-8: Dojindo Molecular Technologies Inc., Rockville, MD, USA). Cells were seeded into a 96 well plate at 5×10^3 cells per well and allowed to adhere for 24 h. The cells were then treated with 50–200 µg/mL of crude olive leaf extracts, positive control gemcitabine (IC_{50} = 50 nM) or vehicle control. The concentration 50–200 µg/mL was chosen in order to show the range in which the extracts had activity on the cells. This was based on previously published data on the anti-proliferative activity of olive leaf extracts for breast cancer [30]. After 96 h, 10 µL of CCK-8 solution was added before incubating at 37 °C for 120 min.

The absorbance was measured at 450 nm and cell growth inhibition was determined as a percentage of control. All experiments were performed in triplicate.

3.9. Statistical Analysis

The one-way ANOVA and the LSD post-hoc test were used to assess mean differences in TPC levels, antioxidant capacity and cell viability between extracts using the JMP statistical software (Version 11). Data are represented as means ± standard deviations for triplicate experiments. Differences between the means were taken to be statistically significant at $p < 0.05$.

4. Conclusions

The development of novel extraction techniques to obtain bioactive compounds from biomass is gaining the interest of researchers as well as industry. The current study compared the phytochemical properties of six olive leaf extracts obtained from three different optimised extraction protocols: a "green" extraction method using water as a solvent, a 50% methanol extraction method and a 50% ethanol extraction protocol with the latter two also being ultrasound-assisted extraction techniques. While the TPC and antioxidant capacity of the extracts did not change depending on the extraction conditions, it is important to note that the levels of specific compounds did slightly vary and that there was a suggestion that the water extract of the *Corregiola* variety had the highest cytotoxicity of the leaf extracts against the MiaPaCa-2 pancreatic cancer cells. Although, the specific compounds causing cytotoxicity were not identified, it can be concluded that olive leaf extracts are a good source of phenolic compounds, including oleuropein. Furthermore, the olive leaf extracts at 100 and 200 μg/mL were found to significantly decrease the growth of the pancreatic cancer cells compared to the standard chemotherapeutic agent gemcitabine at its IC_{50}.

This study is the first to show the anti-pancreatic cancer activity of olive leaf extracts and provides a foundation for further study of the activity of olive leaf compounds in pancreatic cancer. Moreover, this study shows the effectiveness and justifies the use of an environmentally friendly or "green" extraction method, which uses water, to extract bioactive compounds from olive leaves, including oleuropein and other phenolic compounds. This method could be easily scaled up and therefore shows great potential to benefit the olive oil production industry.

This is a preliminary study which aimed to assess the effectiveness of water as an extraction solvent for phenolic compounds from olive leaves and investigate olive leaf extracts as anti-pancreatic cancer agents. Limitations of this study include it being limited to one pancreatic cancer cell line and that the molecular mechanisms underlying activity were not investigated. Moreover, the individual compounds responsible for the anti-pancreatic cancer activity have not yet been identified. Nevertheless, this study provides a platform for further work to delineate the phenolic compound profile of olive leaf extracts as well as assess the molecular mechanisms involved in the anti-cancer activity of olive leaf extracts in pancreatic cancer cells.

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Author Contributions

Chloe D. Goldsmith participated in the experimental design and completion, as well as interpretation, manuscript design and preparation. Quan V. Vuong, Elham Sadeqzadeh, Costas E. Stathopoulos and Christopher J. Scarlett participated in the experimental design and data interpretation. Christopher J. Scarlett and Paul D. Roach participated in manuscript design and preparation. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the olive leaf extracts are available from the authors.

4.3.2. Research Paper 4

Research paper 4 entitled "**Cytotoxicity of olive pomace extract towards pancreatic cancer cells" submitted to Herbal Medicine in 2017.**

*Title page with Author details

Cytotoxicity of olive pomace extract towards pancreatic cancer cells *in vitro*.

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Keywords: Olea europaea; olive oil; biophenols; pancreatic cancer; phenolic compounds; waste utilisation

ABSTRACT

Adherence to a Mediterranean diet is associated with a reduced risk for most cancers, including those of pancreatic origin. Pancreatic cancer is a devastating disease with a 5-year survival rate of less than 8%, and as such the search for novel treatments for pancreatic cancer is vital. Olive leaf extracts have been shown to induce apoptosis in certain cancer cells while displaying a protective effect towards non-tumorigenic cells. Olive pomace contains some similar biophenols to olive leaves, therefore the aim of this work was to determine the anti-cancer potential of olive pomace extract. Moreover, the efficacy of water as an extraction solvent for the recovery of biophenols from olive pomace was determined. Methanol pomace extract (100 µg/mL) caused growth inhibition in all cells tested; HT29 (colon) 35%, A2780 (ovarian) 53%, H460 (lung) 22%, A431 (skin) 38%, Du145 (prostate) 32%, BE2-C (neuroblastoma) 31%, MCF-7 (breast) 57%, and U87, SJ-G2, SMA (glioblastoma) 49%, 43% and 46% respectively, and one non-cancer derived cell line MCF-10A (normal breast) 36% growth inhibition and reduced the viability of pancreatic cancer cells in a dose-dependent manner. Low concentrations (50 µg/mL) of methanol pomace extract increased the viability of non-tumorigenic cells (HPDE) compared to vehicle controls (20% increase). Additionally, the methanol extract contained a higher concentration of total biophenols compared to the water extract (63.34 ± 4.82 and 51.55 ± 4.23 µg tyrosol equivalents/mL, respectively). Further work on the cytotoxicity of olive pomace extracts towards pancreatic cancer cells, and the protective effect on non-tumorigenic cells is warranted.

INTRODUCTION

Epidemiological data has shown that consumption of a Mediterranean-style diet is associated with a reduced risk for most cancers (Bosetti et al., 2013; de Lorgeril et al., 1998; Fortes et al., 2003; Trichopoulou et al., 2000). One characteristic difference between Mediterranean diets and other healthy diets is the high consumption of olive oil. Accumulating evidence suggests that micro-constituents found in olive oil, including oleic acid and phenolic compounds, provide health benefits (Akl et al., 2014; Liu et al., 2011; Omar, 2010). Interestingly, only approximately 2% of phenolic compounds originally present in the pre-processed olive actually end up in the olive oil (Goldsmith et al., 2014; Jerman Klen and Mozetič Vodopivec, 2012). As such, olive waste products constitute a wealth of available phenolic compounds with known health benefits.

The concentration and type of phenolic compounds in olive waste extracts will vary widely due to a number of factors, including growing conditions, location of the tree, time of harvest as well as extraction conditions (Lopez-Sabater et al., 2002; Obied et al., 2008). Compounds that have been identified in olive waste extracts include oleuropein, hydroxytyrosol, oleocanthal, tyrosol and the flavonoids luteolin and apigenin (Cardinali et al., 2010; Cicerale et al., 2011; Suarez et al., 2009). Olive waste extracts have displayed numerous health benefiting properties including reducing hyperlipidemia (Liu et al., 2011), antioxidant activity and reduction of oxidative stress and hypoglycemia (Hamden et al., 2009),

The anti-cancer potential of olive waste biophenols has been previously described. Olive waste phenolic extracts have displayed protective effects against cancers of the brain (Schaffer et al., 2007), colon (Gill et al., 2005) and breast (Ramos et al., 2013). However, there has yet to be investigations into the activity of olive pomace extracts on pancreatic cancer. Pancreatic cancer has a 5-year survival rate of less than 8%. Resistance to conventional treatment and its complex molecular heterogeneity contributes significantly to these dismal statistics. Current treatment options only increase survival by approximately 6 months. Moreover, chemotherapeutic drugs (such as gemcitabine) are highly toxic to the patient's healthy tissue. Therefore, the search for novel drugs which selectively induce apoptosis in cancer cells without being toxic to healthy tissue is a key strategy. Considering the link between the consumption of a Mediterranean diet and reduced cancer incidence and mortality, as well as the protective effects of olive phenolic compounds observed previously, olive pomace extracts warrant detailed investigation.

We previously identified an optimal method for the extraction of phenolic compounds from olive pomace using water as the extraction solvent. While water is a cheaper and more green alternative to organic solvents, the selectivity of water for phenolic compounds is low in comparison. Methanol (50-80%) has been identified as the most selective solvent for the preparation of olive extracts high in desirable compounds including oleocanthal, oleuropein as well as the flavonoids luteolin and apigenin. Therefore, the aims of this study were to determine the anti-cancer activity of methanol pomace extracts and compare the biophenol content of aqueous pomace extracts to methanol pomace extracts.

METHODS

Materials

HPLC Methanol, HPLC ethanol, orthophosphoric acid, syringic acid and tyrosol were purchase from Sigma-Aldrich (Temecula, MS, USA). CCK-8 reagent was purchased from Dojindo Molecular Technologies Inc., Rockville, MD, USA. Dulbecco's Modified Eagle Medium (DMEM), Keratinocyte Serum-Free Media (K-SFM), Roswell Park Memorial Institute medium (RPMI), Iscove's Modified Dulbecco's Medium (IMDM), trypsin-EDTA, L-glutamine and Phosphate Buffered Saline (PBS) were purchased from Invitrogen (Carlsbad, CA, USA). Foetal Bovine Serum (FBS) and horse serum (HS) was purchased from Interpath (Heildberg West VIC, Australia). Luminata Classico western Horse Radish Peroxidase substrate was purchased from MERCK-Millipore (Temecula, MA, USA). Pancreatic cancer cells (MIA PaCa-2, BxPC-3 and CFPAC-1) were purchased from ATCC (Manassas, VA, USA) and immortalised normal pancreatic ductal epithelial cells (HPDE) were a gift from the lab of Dr. M. Tsao (MD, FRCPC, University of Health Network, Tronto, ON, Canada). All cells have been authenticated by CellBank Australia (Westmead, NSW, AUS).

Sample preparation and extraction

Olive pomace was collected in April 2015 from Houndsfield Estate, Lochinvar, the Hunter Valley, NSW. The olive paste came from fruit of the Frantoio cultivar which was picked on Friday 17th April, stored overnight at 14°C before milling on Saturday 18th April. The paste temperature during the malaxing phase was 22-23°C. Olive pomace was stored for 12h at -20°C before freeze drying. Phenolic compound extracts were prepared according to Goldsmith et al. (2018). Briefly, 5 g of dried pomace was added to 250 mL of water or 50% methanol and placed in ultrasound bath for 75 min at 250 W power. Extracts were concentrated (their volume reduced) using a rotary evaporator, freeze dried and then stored at -20 °C until further analysis

Determination of total biophenols

The extracts were analysed according to (Goldsmith et al., 2015) with minor modifications. Briefly, a Shimadzu HPLC system (Shimadzu Australia, Rydalmere, NSW Australia) and a 250 ± 4.6mm Synergi 4 µm Fusion-RP 80A reversed-phase column (Phenomenex Australia Pty. Ltd., Lane Cove, NSW Australia) with detection at 320nm (Tasioula-Margari and Tsabolatidou, 2015) was used. The column was maintained at 30°C, the flow rate was 1 mL/min and three solvents were used for the mobile phase Solvent **A**: 0.1% orthophosphoric acid, Solvent **B**: 100% Methanol, Solvent **C**; 100% Ethanol. A gradient elution schedule was

used according to the following: 0min A96%, B 2%, C 2%; 0-40 min A 40%, B 30%, C 30%; 40-60 min A 40%, B 30%, C 30%; 60-62 min A 96%, B 2%, C 2%. Peaks were identified at 320 nm. Syringic acid was used as an internal standard. A tyrosol standard curve was developed which was linear between 0-500 µg/mL and total peak areas of chromatograms expressed as µg tyrosol equivalents/ mL.

Growth inhibition screening of cancer cell lines

The cytotoxic potential of methanol olive pomace extract was assessed. Briefly, the growth inhibition of eleven cancer cell lines including MIA PaCa-2 (pancreas), HT29 (colon), A2780 (ovarian), H460 (lung), A431 (skin), Du145 (prostate), BE2-C (neuroblastoma), MCF-7 (breast), and U87, SJ-G2, SMA (glioblastoma) and one non-cancer derived cell line MCF-10A (normal breast) was determined. Briefly, all cancer cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 50 IU/mL penicillin, 50 µg/mL streptomycin, and 2 mM L -glutamine. The MCF-10A cells were cultured in DMEM: F12 (1:1) cell culture media, 5% heat inactivated horse serum, supplemented with 50 IU/mL penicillin, 50µg /mL streptomycin, 20 mM Hepes, 2 mM L glutamine, 20 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 µg/mL insulin. Cells were plated in triplicate in 100 µL DMEM on a 96 well plate, at a density of 2500–4000 cells per well. When cells were at logarithmic growth after 24 h, medium without (control), and medium with methanol olive pomace extract (100 µg/mL) was added to each well to give a final concentration of 100 µg/mL (day 0). The MTT assay was employed whereby absorbance was read at 540 nm to determine growth inhibition after 72 h of incubation based on the difference between the optical density values on day 0 and those at the end of drug exposure. Cell growth inhibition as a percentage was determined where a value of 100% is indicative of total growth inhibition.

Viability of pancreatic cell lines treated with methanol olive pomace extract

Human pancreatic cancer (MIA PaCa2) cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), 2.5% horse serum and L-glutamine (100 µg/mL), BxPC-3 cells were cultured in RPMI supplemented with 10% FBS and L-glutamine (100 µg/mL) and CFPAC-1 cells were cultured in IMDM supplemented with 10% FBS and L-glutamine (100 µg/mL). Human pancreatic ductal epithelial cells (HPDE) were cultured in KSFM supplement free media. All cells were maintained at 37 °C under 5% CO_2.

Pancreas cell viability was determined using the Dojindo Cell Counting Kit-8. Human pancreatic cancer (MIA PaCa2, BxPC-3 and CFPAC-1) cells and non-tumorigenic pancreas (HPDE) cells were seeded into a 96 well plate at 3000-10000 cells per well and allowed to adhere for 24 h. Cells were then treated within the range of 0-200 µg/mL of crude extract, gemcitabine (0-50 nM), a combination of gemcitabine and methanol pomace extract or vehicle control. After 72 h, a 10% CCK-8 solution in media, was added before incubating at 37°C for 180 min. Absorbance was measured at 450 nm and cell growth inhibition. All experiments were performed in triplicate.

Statistical Analysis

The student's t-test was conducted on HPLC total biophenols comparing Methanol and Water total biophenols. Repeated Measures two-way ANOVA and the Dunnett's multiple comparisons test were used to compare combination treatment cells to gemcitabine only controls. Significance value was set at $p < 0.05$.

RESULTS AND DISCUSSION

Total biophenol content of olive pomace extracts.

There are numerous methods that can be used for the determination of biophenols in crude extracts; the gold standard of which is HPLC. In this study, total olive biophenols were evaluated using RP-HPLC by identifying the main peaks at the wavelength of 320nm (Figure 1). Peaks were quantified using a tyrosol standard curve. The methanol extract had a higher content of biophenols (63.34 ± 4.82 µg tyrosol equivalents/mL; Table 1) compared to the water extract (51.55 ± 4.23 µg tyrosol equivalents/mL; Table 1). Methanol has been confirmed as an ideal solvent for the extraction of biophenols from olive materials (Cicerale et al., 2011; Lafka et al., 2011; Suarez et al., 2009; Tasioula-Margari and Tsabolatidou, 2015), with 80% methanol the official method for the identification of biophenols in olive oil (Burns, 2010). However, due to the different chemical composition and the low oil content of olive pomace compared to olive oil, authors have previously argued that a lower percentage of methanol or ethanol (50%) is better able to effectively extract the biophenols present (Lozano-Sánchez et al., 2014).

Cytotoxicity of olive pomace extracts

Considering that the methanol pomace extract contained a higher quantity of total biophenols, the cytotoxicity of the methanol extract was determined. The growth inhibition of 100 µg/mL of methanol pomace extract was determined in a panel of different cancer cells. The strongest cytotoxicity was observed in breast cancer (MCF-7) and ovarian cancer cells (A2780) followed closely by glioblastoma cells (U87 and SJ-G2) and pancreatic cancer cells (MIA PaCa-2) (57%, 53%, 49%, 43% and 42% growth inhibition respectively; Table 2). Crude olive waste extracts have been shown to confer toxicity towards colon cancer and gastric cancer cells *in vitro* (Obied et al., 2009), with olive leaf extracts have been shown to reduce proliferation in glioblastoma (Tezcan et al., 2017a; Tezcan et al., 2017b), colon cancer cells (Zeriouh et al., 2017), mesothelioma (Marchetti et al., 2015), breast cancer (Barrajón-Catalán et al., 2015) and pancreatic cancer cells (Goldsmith et al., 2015). However, olive leaf and olive pomace extracts have very different biophenol contents (Obied et al., 2005; Rahmanian et al., 2015; Sahin and Samli, 2013). In fact, a number of compounds are formed during olive oil processing. Therefore, it is important to also determine the toxicity of olive pomace extracts. Moreover, the cytotoxicity of olive pomace extracts has not been investigated in pancreatic cancer previously.

Dose response of pancreatic cells treated with olive pomace extract

To further explore the activity of olive pomace extracts against pancreatic cancer cells, we tested the response of different pancreatic cancer cells (MIA PaCa-2, BxPC-3 and CFPAC-1) as well as non-tumorigenic cells (HPDE) treated with different concentrations of methanol pomace extract (0-200 µg/mL). Methanol pomace extract reduced the viability of all pancreatic cancer cells at each dose within the treatment range (Figure 2). Interestingly, the viability of HPDE cells when treated with 50 µg/mL of olive pomace extract increased compared to the vehicle control (20% increase). While the protective activity of olive pomace extracts has not been observed previously, crude olive leaf extracts have displayed protective action against the DNA damage of leukocytes (Cabarkapa et al., 2014), UV induced skin cancer in mice (Kimura and Sumiyoshi, 2009) as well as renal toxicity in diabetic rats (Hamden et al., 2009). These data highlight the potential of olive pomace extracts as protective agents for pancreatic cells.

Combination treatment of olive pomace extracts and gemcitabine

Gemcitabine is a highly toxic chemotherapy drug used to treat pancreatic cancer. We analyzed the effects of the methanol pomace extract combined with gemcitabine to evaluate the utility of olive pomace extracts as a supplement to chemotherapy (Figure 3); the rationale for this is to explore whether the concentration of gemcitabine required to inhibit the proliferation of pancreatic cancer cells could be reduced, thereby decreasing toxicity and providing a protective effect from gemcitabine to non-tumorigenic cells.

Pancreatic cancer cells (MIA PaCa-2, BxPC-3 and CFPAC-1), as well as non-tumorigenic pancreas cells (HPDE), were treated with 50 μg/mL of crude extract combined with 0-50 nM of gemcitabine. Interestingly, the methanol pomace extract displayed a protective effect from gemcitabine in the pancreatic cancer cells and not the non-tumorigenic pancreas cells (Figure 3). The combination of olive pomace extract and 10 nM gemcitabine caused an increase in the viability of MIA PaCa-2 (20% increase, $p<0.0001$, Figure 3A) and CFPAC-1 (16% increase, $p<0.0001$, Figure 3C) cells compared to 10 nM of gemcitabine alone, the combination of extract and 25 nM gemcitabine increased the viability of BxPC-3 (7% increase, $p = 0.0194$, Figure 3B) and MIA-PaCa-2 (14% increase, $p<0.0001$, Figure 3A) compared to the 25mN of gemcitabine alone and the combination of extract and 50 nM of gemcitabine increased the proliferation of CFPAC-1 cells (11% increase, $p = 0.0066$, Figure 3C) compared to the 50 nM of gemcitabine alone. Additionally, there was no difference between the combination-treatment HPDE cells and the gemcitabine only cells (Figure 3D).

This has not been observed previously. In fact, olive leaf extracts have previously displayed a synergistic effect with the breast cancer chemotherapy agent doxorubicin (Elamin et al., 2017) as well as increasing the efficacy of temozolomide (Tezcan et al., 2017b) and bevacizuman (Tezcan et al., 2017a) therapy in glioblastoma cells. These differences are likely due to the unique profile of biophenols in olive pomace extracts compared to olive leaves. The process of olive oil extraction causes the formation of various compounds (Jerman Klen and Mozetič Vodopivec, 2012), with their activity in cancer yet to be investigated. Moreover, the activity of olive pomace extracts combined with lower doses of gemcitabine warrant investigation.

CONCLUSION

Olive pomace extracts are a rich source of biophenols. Methanol extraction increased the yield of biophenols from olive pomace compared to extraction with water. Olive pomace methanol extracts displayed cytotoxicity towards glioblastoma, breast, ovarian and pancreatic cancer cells. While olive leaf extracts have previously displayed toxicity towards breast cancer and glioblastoma cells, this study is the first to determine the cytotoxicity of olive pomace extracts in lung, ovarian, neuroblastoma and pancreatic cancer cells. Moreover, the protective effect of low doses of olive pomace extracts on non-tumorigenic pancreas cells warrants further investigation.

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Table 1

Table 1. Total biophenols of the methanol and water pomace extracts determined by HPLC. Peak numbers correspond to chromatogram peaks identified in Figure 1.

[a, b] different letter denotes significant difference ($p<0.05$).

Peak number	***Retention time***	***Methanol extract*** *(µg tyrosol equivalents/mL)*	***Water extract*** *(µg tyrosol equivalents/mL)*
1	17.615	3.09 ± 0.1	4.86 ± 0.1
2	18.185	1.54 ± 0.67	2.29 ± 0.32
3	19.233	2.07 ± 0.23	3.37 ± 0.06
4	20.281	4.58 ± 0.06	6.11 ± 0.65
5	20.982	2.40 ± 0.36	3.39 ± 0.4
6	22.924	-	-
7	23.372	2.99 ± 0.34	
8	23.781	3.44 ± 0.72	4.24 ± 0.87
9	24.559	6.42 ± 0.03	7.36 ± 0.01
10	25.017	6.68 ± 0.5	-
11	27.881	1.48 ± 0.66	-
12	28.125	5.46 ± 0.38	5.68 ± 0.4
13	29.219	9.21 ± 0.01	6.49 ± 0.97
14	29.607	4.84 ± 0.54	
15	31.415	9.14 ± 0.22	7.77 ± 0.51
Total		**63.34 ± 4.82[a]**	**51.55 ± 4.23[b]**

Table 2. Dose screen: Cells from cancer tissues of different organs of origin as well as non-cancer derived breast cells (MCF10A). Percentage (%) cell growth in response to 100 µg/mL of methanol pomace extract (the higher the value the greater the growth inhibition).

Tissue	*Cell line*	*Growth Inhibition (%)*
Colon	HT29	35 ± 7
Glioblastoma	U87	49 ± 5
Breast	MCF-7	57 ± 3
Ovarian	A2780	53 ± 3
Lung	H460	22 ± 3
Skin	A431	38 ± 4
Prostate	Du145	32 ± 8
Neuroblastoma	BE2-C	31 ± 9
Glioblastoma	SJ-G2	43 ± 1
Pancreas	MIA	42 ± 1
Glioblastoma	SMA	46 ± 16
Breast	MCF10A	36 ± 2

Figure 1

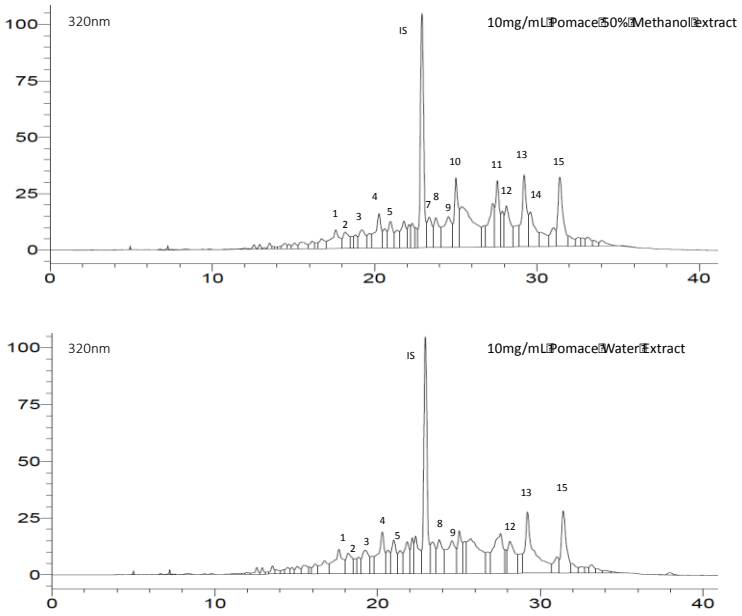

Figure 1. Typical HPLC chromatograms, the methanol (top) and water (bottom) extracts from olive pomace with detection at 320nm. Peaks identified include oleuropein (Peak 10) and Internal Standard (IS) syringic acid.

Figure 2

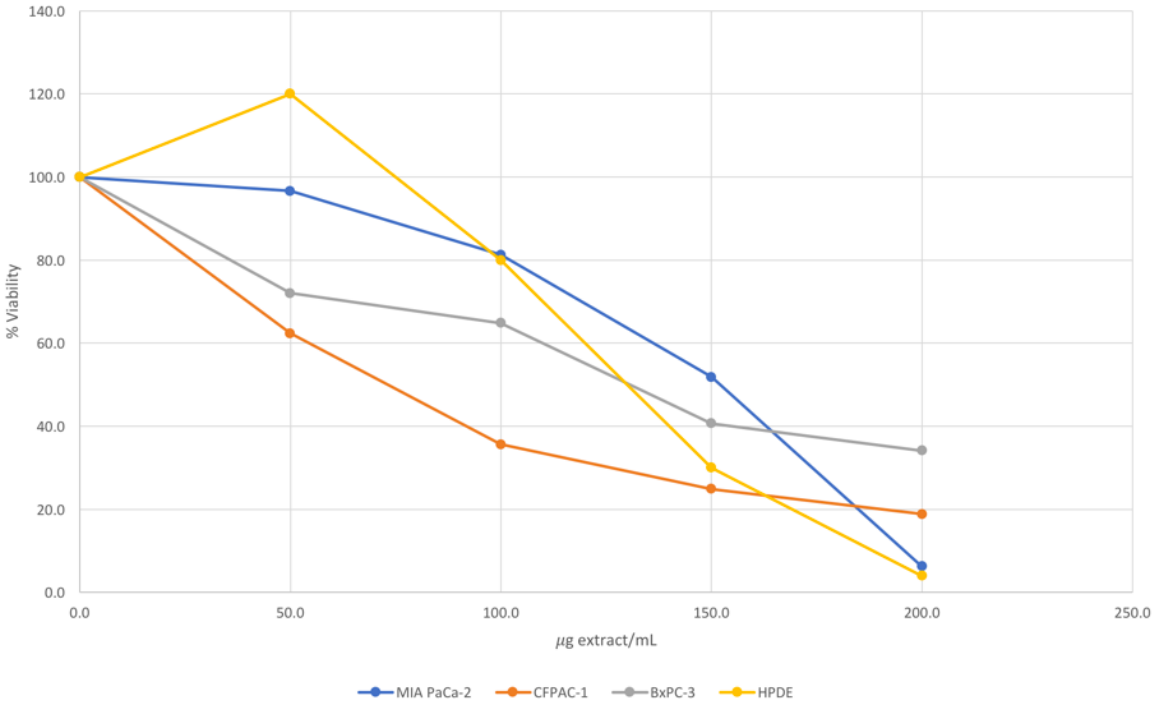

Figure 2. Dose response of pancreatic cancer cells (CFPAC-1, MIA PaCa-2 and BxPC-3) and non-tumorigenic cells (HPDE) when treated with 0-200 µg/mL of crude methanol pomace extract.

Figure 3

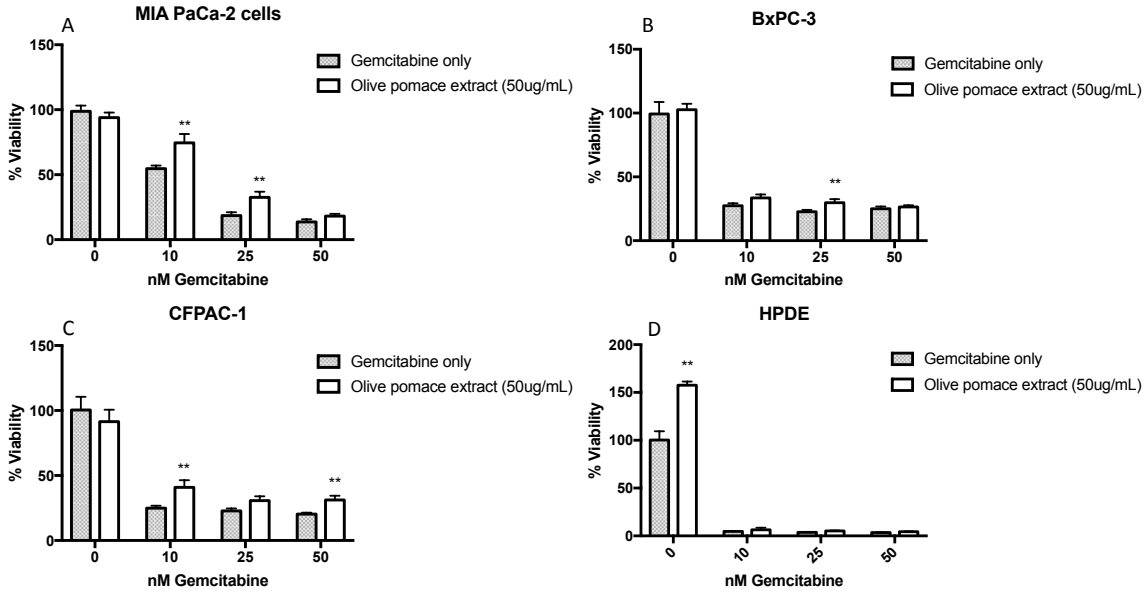

Figure 3. Combination treatment of cancer cells (MIA PaCa-2, CFPAC-1 and BxPC-3) and non-tumorigenic cells (HPDE) with 50 µg/mL of methanol pomace extract and a range of gemcitabine concentrations (0-50 nM).
** denote significant difference from gemcitabine only controls ($p<0.05$).

***Conflict of Interest Statement**

AUTHOR DECLARATION

On behalf of my co-authors, I wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

I confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

I further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Signed

Chloe Goldsmith
29/11/2017

4.3.3. Research Paper 5

Research paper 5 entitled "**Flavonoids in *Olea europaea* leaf extracts and their cytotoxicity towards pancreatic cancer cells" submitted to Food & Function in 2017.**

Flavonoids in *Olea europaea* leaf extracts and their cytotoxicity towards pancreatic cancer cells.

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Keywords: olive leaf; pancreatic cancer; apoptosis; myricetin; biophenols; cytotoxicity.

ABSTRACT

The chemopreventative and therapeutic potential of olive leaf extracts has been previously described in cancers of the breast, prostate and colon. However, there has been little evaluation of the cytotoxic activity of olive leaf extracts in pancreatic cancer; with the exception of luteolin, apigenin and myricetin. These structurally similar flavonoids have previously been investigated for their anti-pancreatic cancer potential in BxPC-3 cells. Luteolin and apigenin have been identified in olive leaf extracts. Therefore, the aims of this study were to investigate the anti-cancer potential of olive leaf extracts and to determine if olive leaf extracts were a source of luteolin, apigenin or myricetin, as well as to further investigate the anti-pancreatic cancer activity of these flavonoids. Olive leaf extract reduced the proliferation of pancreatic cancer cells (MIA PaCa-2, BxPC-3 and CFPAC-1) in a dose dependent manner and displayed a protective effect towards non-tumorigenic cells (HPDE). Combination of olive leaf extract with gemcitabine exerted a protective effect on the HPDE cells; however, they also exerted a protective effect on the cancer cells. Luteolin, apigenin and myricetin augmented the cell cycle and induced apoptosis in pancreatic cancer cells, while displaying less toxicity towards non-tumorigenic cells when compared to gemcitabine. The flavonoids, luteolin, apigenin and myricetin displayed potential as anti-pancreatic cancer agents and continued investigations on their mechanisms of action in pancreatic cancer cells is warranted.

INTRODUCTION

Cancer is a major health and economic burden and a leading cause of death worldwide, accounting for millions of deaths each year. The World Health Organization reported that the most common cancers include: lung, liver, stomach, colorectal, breast and oesophageal cancers (WHO, 2015). However, pancreatic cancer is one of the deadliest cancers with a 5-year survival rate of less that 8%. In fact, pancreatic cancer is predicted to be the second leading cause of cancer related death by 2030 [1]. Moreover, resistance to conventional treatment and toxicity of current chemotherapy agents (such as gemcitabine) makes pancreatic cancer an important target for the development of novel therapeutic agents [2, 3].

The chemopreventative and therapeutic potential of olive leaf extracts has been previously described in cancers of different origins [4-7]. However, there has been little evaluation of the cytotoxic activity of olive leaf extracts in pancreatic cancer. Moreover, crude extracts are a

complex mixture of compounds and while the activity of crude extracts is an important first step in the development of new cancer therapeutics, it is difficult to determine the compound/s responsible for the observed activity. In order to establish biological relevance, it is important to determine the individual compounds responsible for biological effects observed with crude extracts.

Olive leaves have proven to be a robust source of flavonoids (~2% of olive leaf polyphenols) including luteolin, apigenin, rutin and catechin [8, 9]. Flavonoids display a number of activities *in vitro* and *in vivo,* such as anti-inflammatory, anti-cancer and antioxidant properties [8, 10]. Additionally, the flavonoid compounds myricetin, luteolin and apigenin have demonstrated ability to induce apoptosis in some pancreatic cancer cells [11-13]. Moreover, we have previously shown that water and methanol olive leaf extracts were a source of flavonoids and that these extracts were able to reduce the viability of pancreatic cancer cells *in vitro* [6].

The structures of luteolin, apigenin and myricetin differ only by the number of hydroxyl groups on their B-ring (Figure 1). Despite the similarity between these flavonoid structures, their biological properties vary considerably. The number and specific positions of hydroxyl groups and the nature of the substitutions determine whether flavonoids function as strong antioxidants, anti-inflammatory or anti-proliferative agents [10]. While there has been some investigation into the anti-pancreatic cancer activity of the individual flavonoids myricetin, luteolin and apigenin, there has not been a direct comparison of the activity of these structurally similar compounds on different pancreatic cancer cells. Additionally, their effect on non-tumorigenic pancreas cells has yet to be determined. Therefore, the aims of the present study were to determine the ability of luteolin, apigenin and myricetin to influence the cell cycle and induce apoptosis in pancreatic cancer cells as well as determine their effects on non-tumorigenic pancreas cells. Additionally, we aimed to determine the cytotoxicity of our methanol and water olive leaf extracts and investigate if they were a source of the flavonoids, myricetin, luteolin and apigenin.

METHODS

Materials

Myricetin, luteolin and apigenin were purchased from Sigma-Aldrich (Temecula, MS, USA). CCK-8 reagent was purchased from Dojindo Molecular Technologies Inc., Rockville, MD,

USA. Dulbecco's Modified Eagle Medium (DMEM), Keratinocyte Serum-Free Media (K-SFM), Roswell Park Memorial Institute medium (RPMI), Iscove's Modified Dulbecco's Medium (IMDM), trypsin-EDTA, L-glutamine and Phosphate Buffered Saline (PBS) were purchased from Invitrogen (Carlsbad, CA, USA). Foetal Bovine Serum (FBS) and horse serum (HS) was purchased from Interpath (Heildberg West VIC, Australia). Luminata Classico western Horse Radish Peroxidase substrate and MUSE™ cell cycle and caspase 3/7 reagent kits were purchased from MERCK-Millipore (Temecula, MA, USA). Pancreatic cancer cells (MIA PaCa-2, BxPC-3, CFPAC-1 and ASPC-1) were purchased from ATCC (Manassas, VA, USA) and immortalised normal pancreatic ductal epithelial cells (HPDE) were a gift from the lab of Dr. M. Tsao (MD, FRCPC, University of Health Network, Toronto, ON, Canada). All cells were authenticated.

Sample collection

Olive leaves of the Frantoio cultivar were collected from olives harvested on the 17th April 2015 from Houndsfield Estate, Lochinvar, NSW, Australia. All samples were stored at -20°C for 48h before hot air drying at 120°C according to the methods of Ahmad-Qasem et al. [14]. Dried samples were then stored in airtight containers in the dark at -20°C until further analysis.

Extraction conditions

Water extracts were generated according to Goldsmith et al. [15] and methanol extracts according to Goldsmith et al. [6].

HPLC

The extracts were analysed according to Goldsmith et al. [6] with minor modifications. Briefly, a Shimadzu HPLC system (Shimadzu Australia, Rydalmere, NSW Australia) and a 250 ± 4.6mm Synergi 4 µm Fusion-RP 80A reversed-phase column (Phenomenex Australia Pty. Ltd., Lane Cove, NSW Australia) with detection at 320nm [16] was used. The column was maintained at 30°C, the flow rate was 1 mL/min and three solvents were used for the mobile phase Solvent **A**: 0.1% orthophosphoric acid, Solvent **B**: 100% Methanol, Solvent **C**; 100% Ethanol. A gradient elution schedule was used according to the following: 0min A96%, B 2%, C 2%; 0-20 min A 40%, B 30%, C 30%; 20-40 min A 40%, B 30%, C 30%; 40-42 min

A 96%, B 2%, C 2%. Myricetin, luteolin and apigenin were identified using commercial standards (Sigma-Aldrich, Castle Hill, NSW, Australia).

Pancreas Cell Culture

Human pancreatic cancer (MIA PaCa2) cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), 2.5% horse serum and L-glutamine (100 µg/mL), BxPC-3 and ASPC-1 cells were cultured in RPMI supplemented with 10% FBS and L-glutamine (100 µg/mL) and CFPAC-1 cells were cultured in IMDM supplemented with 10% FBS and L-glutamine (100 µg/mL). Human Pancreas cells (HPDE) were cultured in KSFM supplement free media. All cells were maintained at 37°C under 5% CO_2. All cell lines were validated.

Growth Inhibition of Cancer Cell Lines

The cytotoxic potential of olive leaf extracts was assessed. Briefly, the growth inhibition of olive leaf extracts on eleven cancer cell lines including MIA PaCa-2 (pancreas), HT29 (colon), A2780 (ovarian), H460 (lung), A431 (skin), Du145 (prostate), BE2-C (neuroblastoma), MCF-7 (breast), and U87, SJ-G2, SMA (glioblastoma) and one non-cancer derived cell line MCF-10A (normal breast). Briefly, all cancer cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 50 IU/mL penicillin, 50 µg/mL streptomycin, and 2 mM L -glutamine. The MCF-10A cells were cultured in DMEM:F12 (1:1) cell culture media, 5% heat inactivated horse serum, supplemented with 50 IU/mL penicillin, 50µg /mL streptomycin, 20 mM Hepes, 2 mM L glutamine, 20ng/mL epidermal growth factor, 500 ng/mL hydrocortisone, 100 ng/mL cholera toxin, and 10µg/mL insulin. Cells were plated in triplicate in 100µL DMEM on a 96 well plate, at a density of 2500–4000 cells per well. When cells were at logarithmic growth after 24 h, medium without (control), and medium with the olive leaf extracts (100 µL) was added to each well to give a final concentration of 100µg/mL (day 0). The MTT assay was employed where absorbance was read at 540 nm to determine growth inhibition after 72 h of incubation based on the difference between the optical density values on day 0 and those at the end of drug exposure. Cell growth inhibition as a percentage was determined where a value of 100% is indicative of total growth inhibition.

Growth Inhibition of Pancreatic Cell Lines

Pancreas cell growth inhibition was determined using the Dojindo Cell Counting Kit-8 (CCK-8: Dojindo Molecular Technologies Inc., Rockville, MD, USA). Human pancreatic cancer (MIA PaCa2, BxPC-3 and ASPC-1) cells and non-tumorigenic pancreas (HPDE) cells were seeded into a 96 well plate at 3000-10000 cells per well and allowed to adhere for 24 h. Cells were then treated within the range of 0-200 μM of each compound, 0-200 μg/mL of crude extract, 0- 1 μM gemcitabine or vehicle control. After 72 h, a 10% CCK-8 solution in media, was added before incubating at 37 °C for 180 min. Absorbance was measured at 450 nm and cell growth inhibition was determined and IC_{50} values calculated. All experiments were performed in triplicate.

Cell cycle analysis

Cell cycle analysis was determined by DNA staining with propidium iodide. Cells were seeded into 12 well plates at 30,000 – 100,000 cells/well. After 24 h cells were treated with the IC_{50} of either luteolin, myricetin or apigenin for 24h before washing with PBS and dislodging with 0.25% trypsin EDTA. Cells were washed twice with 1 x PBS before fixing in ice cold 70% ethanol and storing at -20°C for a minimum of 3 h prior to staining. For staining, cells were centrifuged at 300 g for 5 min; the supernatant was removed and discarded. Cells were washed in 1 x PBS before re-suspending in 200 μL of cell cycle reagent and incubation for 30 min at room temperature in the dark. All assays were performed in triplicate. Fluorescence was read on a MUSE flow cell analyzer and DNA content profile histograms were produced. Results are expressed as percent of cells in the G0/1, S and G2/M phases of the cell cycle.

Expression of caspase 3/7

The induction of apoptosis was evaluated by assessing caspase 3/7 activation. Cells were seeded into 12 well plates at 30,000 – 100,000 cells/well. After 24 h cells were treated with the IC_{50} of either luteolin, myricetin or apigenin for 48 h before washing with PBS and dislodging with 0.25% trypsin EDTA. Cells were diluted to 300 cells/μL prior to staining. MUSE caspase 3/7 reagent working solution was prepared by diluting the stock solution with 1x PBS at a ratio of 1:8. MUSE Caspase 7-AAD reagent was diluted with 1x assay Buffer at a ratio of 1:74. Cells were stained by adding 5 μL of caspase 3/7 working solution to 50 μL of diluted cells and incubating for 30 min at 37°C and 5% CO_2 before adding 150 μL of Caspase 7-AAD working solution and incubating for 5 min at room temperature. All assays were

performed in triplicate. Fluorescence was read on a MUSE cell analyzer. Results are expressed as percent of live cells, early and late apoptotic cells as well as percent of dead cells.

Statistical analysis

Values are expressed as means ± standard deviation from 3 independent experiments. Statistical analysis was performed by ordinary two-way ANOVA followed by Dunnett's multiple comparisons test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Cytotoxicity of crude olive leaf extracts

To determine the cytotoxicity of crude olive leaf extracts, different cancer cell lines and one non-cancer derived cell line were treated (Table 1). At a dose of 100 µg/mL, the cell growth inhibition of the water and methanol extracts was similar in all cells. The highest inhibition for both extract was observed in the ovarian cells (A2780), followed closely by the pancreatic cancer cells (MIA PaCa-2). Weak activity in glioblastoma (SMA, U87 and SJ-G2) and breast (MCF-7) cancer cells was observed and very weak activity in neuroblastoma (BE2-C) lung (H460) and prostate (Du145) cancer cells was demonstrated. Olive leaf extracts also displayed weak activity towards the non-tumorigenic breast cells (MCF10A).

The cytotoxicity of olive leaf extracts against pancreatic cancer cells was explored further. The viability of pancreatic cancer cells BxPC-3, CFPAC-1, MIA PaCa-2 and non-tumorigenic pancreas cells HPDE was determined following treatment with 0-200 µg/mL of olive leaf extracts. All extracts reduced the viability of the pancreas cells in a dose dependent manner (Figure 2). Methanol extracts had the strongest effect on pancreatic cancer cells, with 100 µg/mL reducing the viability of BxPC-3 cells to 8.97%. The aqueous extract had a toxicity similar to the methanol extract towards CFPAC-1 cells (Figure 2C). However, the aqueous extract was more toxic towards the MIA PaCa-2 cells (Figure 2B) and less towards the BxPC-3 cells (Figure 2A) than the methanol extract. Interestingly, both water and methanol extracts increased the proliferation of the non-tumorigenic cells (HPDE) at a dose of 50-100 µg (water extract = 119.2%, methanol extract = 138.7% viability) (Figure 2D).

Combination treatment of olive leaf extracts with gemcitabine

Considering the increase in the viability of the HPDE cells when they were treated with olive leaf extracts, we hypothesized that olive leaf extracts could have a protective effect on HPDE cells when treated with gemcitabine. Hence, pancreatic cancer cells (MIA PaCA-2, BxPC-3 and CFPAC-1) and non-tumorigenic cells (HPDE) were treated with a combination of 100 μg/mL of either methanol or water extract and 0-10 nM of gemcitabine (Figure 3D).

Overall, the olive leaf extracts did not have a strong protective effect on HPDE cells when combined with the pancreatic cancer therapeutic agent gemcitabine within the treatment ranges tested. However, treatment of HPDE cells with the methanol extract did slightly increase the viability at each dose of Gemcitabine, with the highest increase observed when extracts were combined with 2.5nM gemcitabine (21% increase). However, the protective effect decreased with increasing gemcitabine concentrations (5nM = 17%, 7.5nM = 14% and 10nM = 8% increase compared to gemcitabine only controls).

Although the water extract increased the viability of HPDE cells 26% more than methanol extract in the absence of gemcitabine, its effect when combined with gemcitabine was weaker than the methanol extract. In fact, the combination with 2.5nM of gemcitabine was the only significant difference, compared to the gemcitabine only viability values. While the olive leaf extract exerted a weak protective effect on HPDE cells, they displayed a stronger protective effect on the cancer cells when combined with gemcitabine. The largest effect observed was in BxPC-3 cells treated with 10 nM of Gemcitabine (Figure 3A); when combined with the methanol extract, viability increased 51% compared to the gemcitabine only controls.

Identification of luteolin and apigenin in olive leaf extracts

Biophenols were detected in olive leaf extracts by HPLC. The major biophenol in the extracts, oleuropein, had been quantitated previously [6]. Hence the focus of this work was to identify other compounds that could be responsible for the anti-proliferative and the protective effects observed. The methanol extracts had a stronger protective effect on HPDE cells. Additionally, luteolin and apigenin were only detectable in the methanol extracts.

Viability of pancreatic cells treated with flavonoids

Considering luteolin has been previously shown to induce apoptosis in BxPC-3 cells, the viability, influence on cell cycle and induction of apoptosis of pancreatic cells when treated with luteolin and apigenin was determined. Moreover, the activity of myricetin, a structurally similar compound to luteolin and apigenin, was also investigated (Figure 1). To determine the cytotoxicity of the flavonoids, myricetin, luteolin and apigenin, a CCK-8 viability assay was undertaken. The dose required to achieve an IC_{50} for each compound in pancreatic cancer cells (MIA PaCA-2, BxPC-3, CFPAC-1 and ASPC-1) was compared to non-tumorigenic cells (HPDE) (Table 2), with gemcitabine used as the positive control. The lowest IC_{50} observed for pancreatic cancer cells was with luteolin and apigenin-treated BXPC-3 and CFPAC-1 cells (BxPC-3, IC_{50} = 24.6 and 22.2 μM respectively; CFPAC-1, IC_{50} = 22.5 and 27 μM respectively). However, luteolin and apigenin were also highly toxic to the non-tumorigenic cells (HPDE) (IC_{50} = 6.3 and 5.7μM respectively). Additionally, myricetin achieved the lowest IC_{50} in MIA PaCa-2 cells (IC_{50} = 36.7 μM) and it was also the least toxic to the HPDE cells (IC_{50} = 55.7 μM).

Influence of flavonoid treatment on the cell cycle of pancreatic cells.

One of the first cell regulatory mechanisms that can be affected prior to apoptosis, is the cell cycle. Therefore, the effect of myricetin, luteolin or apigenin treatment of pancreatic cancer cells (MIA PaCA-2, BxPC-3, CFPAC-1 and ASPC-1) and non-tumorigenic cells (HPDE) was determined by propidium iodide staining and subsequent flow cytometry (Figure 5). Luteolin and apigenin treatment of BxPC-3, MIA PaCa-2, ASPC-1 and CFPAC-1 cells caused G2 cell cycle arrest with a significant increase in the percentage of cells in the G2 phase (luteolin: 15.3%, 28.3%, 20.1% and 8% increase respectively, $p<0.0001$; apigenin: 22.1%, 26.3%, 7% and 27% increase, respectively, $p <0.0001$) and a decrease in the percentage of cells in G0/1 (luteolin: 7.7%, 21.4%, 32.7% and 8.7% decrease, respectively, $p <0.0001$; apigenin: 7.4%, 18.8%, 6% and 18.1% decrease, respectively, $p <0.0001$). However, the opposite affect was observed in HPDE cells treated with luteolin and apigenin (Figure 5D); that is, no significant effect on the percentage of cells in G0/1 (3.1% and 2.8% increase, $p = 0.052$ and 0.11, respectively), and a decrease in G2 (8.1% and 4.2% decrease, $p<0.0001$ and 0.004, respectively).

Interestingly, myricetin treatment did not have a significant effect on the percentage of HPDE cells in any stage of the cell cycle (p values: G0/1 = 0.14; S = 0.47; G2 = 0.75) (Figure 5D). However, myricetin treatment did cause cell cycle arrest at G2 phase in MIA PaCa-2 (Figure

5B) and ASPC-1 cells (Figure 5E), with an increase in the percentage of cells in G2 (13% and 21% increase respectively, $p<0.0001$) and a decrease in G0/1 (18.8% and 36% decrease, respectively, $p<0.0001$).

Influence of flavonoid treatment on pancreatic cells expressing caspase 3/7

Caspase 3 and 7 are activated downstream in the apoptosis cascade and result in the cleavage of protein substrates and the disassembly of the cell [17]. Hence, the activation of caspase 3/7 measured by fluorescent tagging and subsequent flow cytometry was used to determine the induction of apoptosis in the present study (Figure 6). Pancreatic cancer cells (MIA PaCa-2, CFPAC-1, BxPC-3 and ASPC-1) and non-tumorigenic cells (HPDE) were treated with myricetin, luteolin or apigenin. In cells expressing caspase 3/7, the fluorescent dye (MUSE caspase 3/7 reagent) was able to bind to the DNA, while the dead cell marker (7-AAD) entered the compromised membrane of later stage apoptotic and dead cells. The number of fluorescently labelled cells expressing caspase 3/7 was counted by MUSE flow cytometry.

Luteolin and apigenin treatment of MIA PaCa-2 cells (Figure 6B) resulted in the largest increase in the percentage of cells expressing caspase 3/7 (66.6% and 68.6% increase, $p<0.0001$ respectively); while myricetin did not affect MIA PaCa-2 cells at all ($p = 0.99$). However, myricetin, luteolin and apigenin treatment of CFPAC-1 cells (Figure 6C) all caused an increase in the expression of caspase 3/7 (10.8%, 41.2% and 23.2% increase, $p = 0.03$, 0.0001 and 0.0001, respectively). Similar effects were also observed in BxPC-3 cells (Figure 6A) treated with myricetin, luteolin and apigenin (13.3%, 7.2% and 11% increase, $p = 0.0001$, 0.002 and 0.0001, respectively). Interestingly, only myricetin treatment influenced the percentage of ASPC-1 (Figure 6E) cells expressing caspase 3/7 (12.3%, $p = 0.0001$). Additionally, myricetin, luteolin and apigenin all displayed a small yet significant effect on the percentage of the non-tumorigenic cells (HPDE) (Figure 6D) expressing caspase 3/7 (7.5%, 5.9% and 2.6% increase, $p = 0.0001$, 0.0001 and 0.001 respectively).

DISCUSSION

Pancreatic cancer is one of the most aggressive malignancies due to its late diagnosis, low resection rate and resistance to therapy. Available chemotherapeutic agents only result in an average survival advantage of 3-6 months [3]; hence, new treatments are highly sought after. Olive leaves are a rich source of polyphenols and flavonoid compounds which have been

shown to elicit different activities *in vitro* and *in vivo*. Olive leaves can be a source of the flavonoids luteolin and apigenin containing up to 0.33mg/g of apigenin and up to 0.58mg/g of luteolin [18-20]; however, the flavonoid content of olive leaves can vary widely due to different growing and extraction conditions [21].

We have previously shown that olive leaves high in flavonoids were able to reduce the proliferation of pancreatic cancer cells (MIA PaCa-2) *in vitro* [6]. Considering the anti-pancreatic cancer activity previously reported by others for myricetin, luteolin and apigenin, the aims of this study were to determine the anti-cancer potential of water and methanol olive leaf extracts and to determine if they were a source of myricetin, luteolin or apigenin as well as investigate the activity of these flavonoids in pancreatic cancer cells and non-tumorigenic cells.

Crude olive leaf extracts displayed weak toxicity towards a panel of cancer cells in this study. The highest toxicity observed was on ovarian cancer cells (A2780) (64% growth inhibition) followed closely by pancreatic cancer cells (MIA PaCa-2) (47% growth inhibition). Overall, the methanol leaf extract had slightly higher inhibition values in each cell line. However, this was only significant in glioblastoma (U87), ovarian (A2780) and pancreas (MIA PaCa-2) cancer cells. Olive leaf extracts have been shown to induce apoptosis in colon [22], liver [23] and breast cancer cells [24]. However, the present study is the first to test olive leaf extracts on glioblastoma or ovarian cancer cells.

We have previously shown that olive leaf extracts high in flavonoids were able to reduce the viability of pancreatic cancer cells (MIA PaCa-2) *in vitro* [6]. To further explore the potential of olive leaf biophenols in pancreatic cancer, we tested these extracts on a panel of pancreatic cancer cells (BxPC-3, MIA PaCa-2 and CFPAC-1) as well as non-tumorigenic pancreas cells (HPDE). Both methanol and water olive leaf extracts were able to dose-dependently reduce the proliferation of pancreatic cancer cells (BxPC-3, MIA PaCa-2 and CFPAC-1) within the treatment range (0-200 μg/mL).

Interestingly, at a low dose (50 μg/mL), the methanol extract exhibited a proliferative effect on MIA PaCa-2 cells, increasing cancer cell proliferation to 109.5%; this effect was also observed in the non-tumorigenic cells (HPDE) with the methanol and the water extract causing an increase in proliferation at 50 μg/mL (165.5% and 120.1% respectively) and 100 μg/mL (138.7 and 119.2%, respectively). Although polyphenols are well-known as potent

antioxidants, low doses of antioxidants have previously been shown to exhibit pro-oxidant effects which have been associated with increased proliferation in leukocytes [5] and gastric mucosal cells [7]. While these reports demonstrate the protective effect of olive leaf extracts on normal tissues/cells, increased proliferation after treatment with olive leaf biophenols has not been previously reported in cancer cells.

The pro-oxidant activity of certain polyphenols outlines their potential to sensitize cancer cells, which may be prone to developing resistance [25]. To explore the potential of olive leaf extracts as chemo-sensitizing agents for cancer cells and as a chemo-protective agent for non-tumorigenic cells, pancreatic cells were treated with olive leaf extracts and a range of gemcitabine concentrations. Olive leaf extracts displayed a protective effect towards all cells in this study. That is, olive leaf extracts appeared to protect the non-tumorigenic as well as the cancer cells from gemcitabine treatment. While this result is not ideal, it is important to understand the effects of cyto-protective compounds on both cancer cells and non-tumorigenic cells.

Myricetin, luteolin and apigenin are naturally occurring structurally similar flavonoid compounds, with their structures differing only by the number of hydroxyl groups on their B ring. Despite this small difference in structure, these compounds have markedly different biological properties. In this study, myricetin reduced the proliferation and caused G2 cell cycle arrest in MIA PaCa-2 and ASPC-1 cells, however, it only increased the percentage of ASPC-1 cells expressing caspase 3/7 within the treatment range. This is in contrast to a previous report which found that myricetin induced apoptosis by increasing the expression of caspase 3 and 9 in MIA PaCa-2 cells [11]. Interestingly, the IC_{50} of myricetin on MIA PaCa-2 cells (36.7 μM) fits within the previous dose response observed by Phillips et al., (2011); additionally, the dose used for our apoptosis assay was lower than in the previous study and the time-points we used were longer. Therefore, considering this previous report, as well as the effect of myricetin in the present study on the cell cycle of MIA PaCa-2 cells, it is likely that myricetin could have exhibited an apoptotic effect in the present study if administered in larger doses for shorter time points. Additionally, Phillips et al., (2011) used a spectrophotometric assay to determine the expression of caspase 3 and 9. However, in the present study flow cytometry was used; this method requires washing steps before dislodging the cells with trypsin. Combined with the 48h time point, it is possible that cells undergoing apoptosis were weak and were lost during washing. This study is the first to show that

myricetin induces apoptosis in ASPC-1, BxPC-3 and CFPAC-1 cells and had a limited effect on HPDE cells within the treatment ranges tested.

Luteolin and apigenin were identified in our methanol olive leaf extracts but not the water extracts. Since the use of water as an extraction solvent is uncommon we could not find reports of a water extract that contained luteolin or apigenin; however, based on their structures, it is highly unlikely that they would have been soluble in water. Previous studies have identified methanol as the optimal solvent for extraction of luteolin from *Vitex nigungo* linn. when compared to ethanol, chloroform and dichloromethane [26]. This supports the use of methanol as an extraction solvent for luteolin.

This study is the first to show that luteolin and apigenin display anti-cancer activity towards CFPAC-1, MIA PaCa-2 and ASPC-1 cells as well as the non-tumorigenic HPDE cells. Luteolin and apigenin decreased the viability of BxPC-3, CFPAC-1, MIA PaCa-2 and ASPC-1 cells, and exhibited low IC_{50} values; at these concentrations luteolin and apigenin were both able to augment the cell cycle and increase the percentage of cells expressing caspase 3/7 in BxPC-3, CFPAC-1 and MIA PaCa-2 cells. Similar activity has been observed previously in BxPC-3 cells [27-29]. Moreover, the authors highlight that the inhibition of NF-kB is a potential mechanism of action of luteolin [30] and apigenin [31] on BxPC-3 cells.

Additionally, while luteolin and apigenin were more toxic to the non-tumorigenic cells, it was less toxic than gemcitabine. In fact, based on the IC_{50} values, luteolin was 4 and 3.5 times more toxic and apigenin was 3.8 and 4.7 times more toxic towards HPDE cells than BxPC-3 and CFPAC-1 cells, respectively. However, gemcitabine was 900 and 650 times more toxic towards BxPC-3 and CFPAC-1 cells, respectively than the HPDE cells. Since the induction of apoptosis in cancer cells while limiting impact on surrounding cells is a chemotherapeutic goal [32], these data highlight the chemotherapeutic potential of luteolin and apigenin in pancreatic cancer.

The number of hydroxyl groups on the B ring of a flavonoid has been correlated with antioxidant activity. As such, myricetin is a stronger antioxidant followed by luteolin then apigenin [10]. In this study, myricetin had the weakest activity against pancreatic cancer cells. However, although polyphenols are well known for their antioxidant effects, a pro-oxidant effect is more often associated with anti-proliferative and pro-apoptotic activity [25]. This could

explain why the strongest antioxidant tested, myricetin, had the weakest toxicity. However, myricetin also had the lowest cytotoxic activity on the non-tumorigenic cells. Increased levels of ROS are associated with key aspects of carcinogenesis including the induction of genetic alterations and resistance to apoptosis. Moreover, it has been shown than decreasing the levels of ROS by using antioxidants, malignant phenotypes can be reversed [33]. This highlights the potential of myricetin as a chemo-preventative agent.

Therefore, further studies investigating the combination treatment of myricetin and toxic chemotherapy drugs, as well as pre-treatment of cancer cells with myricetin warrants attention.

CONCLUSION

Olive leaf extracts displayed limited toxicity towards cancer cells and a protective effect on non-tumorigenic cells. However, the doses required are high for a biological context. Additionally, crude extracts are a complex system and it is difficult to determine the compound/s responsible for observed activities. However, in this study, the anti-pancreatic cancer activity and combination treatment efficacy (with gemcitabine) of water and methanol olive leaf extracts on cancer cells BxPC-3, CFPAC-1 and MIA PaCa-2 and non-tumorigenic cells HPDE was investigated for the first time. The methanol olive leaf extract did protect non-tumorigenic cells from the toxicity of gemcitabine; however, they also exhibited a protective effect on cancer cells. Therefore, more studies are needed to potentiate olive leaves as a combination treatment with gemcitabine for pancreatic cancer. Moreover, the anti-cancer activity of methanol and water extracts was similar; however, luteolin and apigenin were only identified in the methanol extracts. Therefore, the activity of olive leaf extracts was most likely not due to the presence of luteolin or apigenin, and more likely due to a combinational effect of the mixture of compounds not able to be identified in this study. Additionally, the anti-pancreatic potential of luteolin, apigenin and the chemo-preventative potential of myricetin as pure compounds warrant further investigation.

Conflicts of Interest

The Authors declare no conflict of interest.

Acknowledgements

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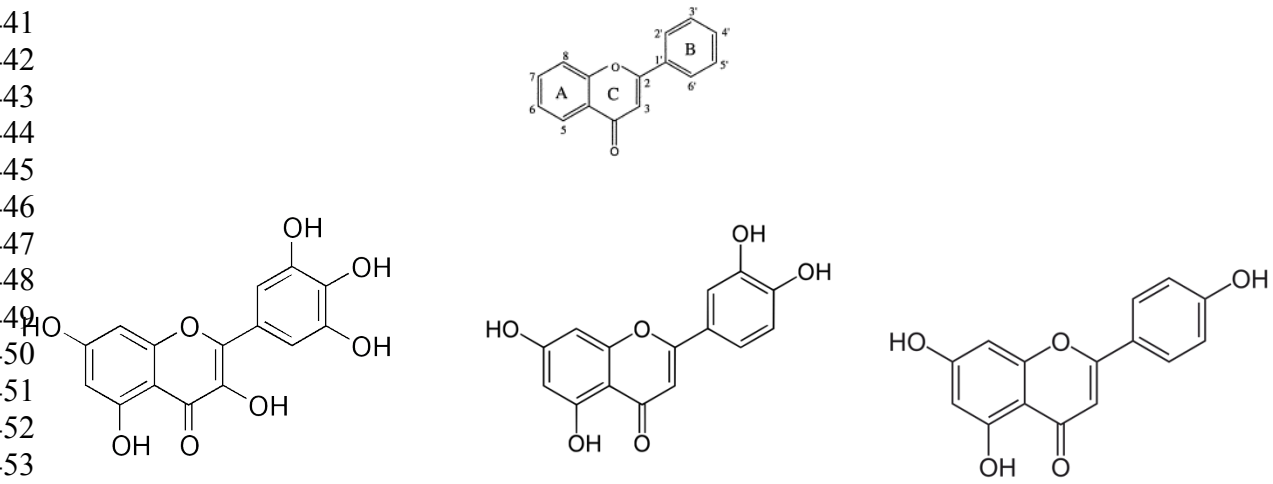

Figure 1. Basic structure of flavonoids (Top); (Bottom: left to right) myricetin, luteolin and apigenin.

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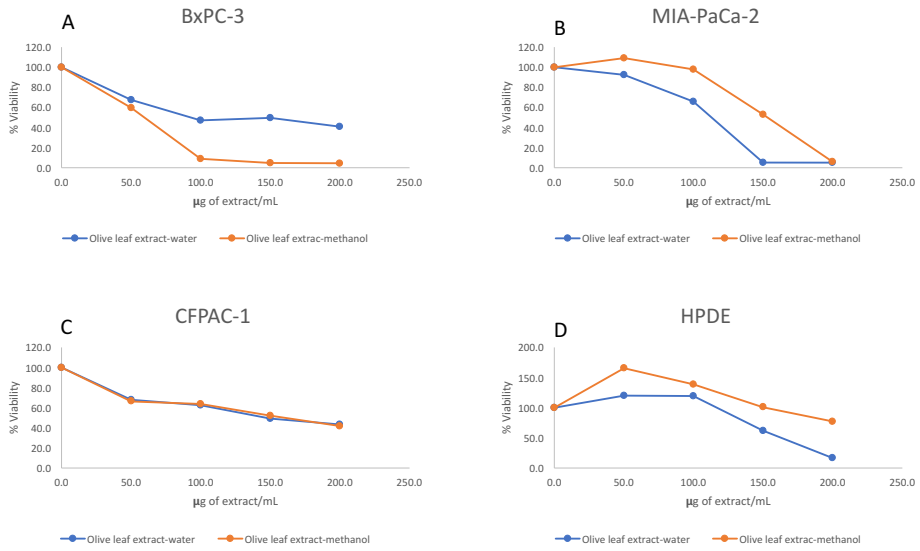

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462 **Figure 2**. Dose response of pancreatic cancer cells (A: BxPC-3, B: MIA PaCa-2 and C:
463 CFPAC-1) and non-tumorigenic cells (D: HPDE) when treated with 0-200 µg/mL of water or
464 methanol crude olive leaf extracts.
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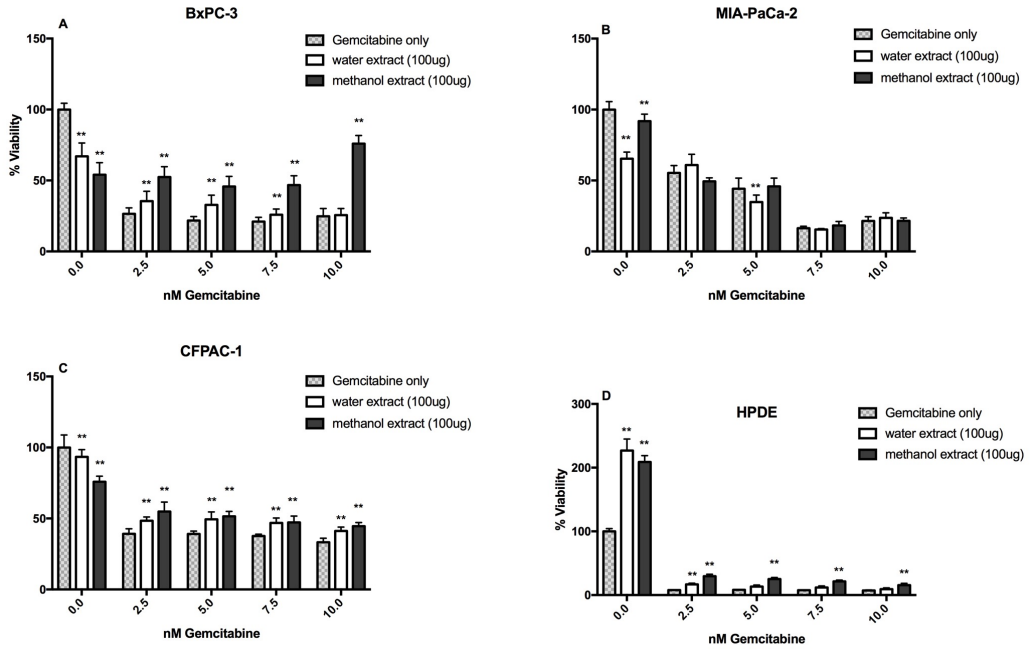

Figure 3. Effect of the combination of olive leaf extracts (100 µg/mL) with varying doses of gemcitabine (0-10nM) on pancreatic cancer cells (A: BxPC-3, B: MIA PaCa-2 and C: CFPAC-1) and non-tumorigenic cells (D: HPDE).
** denotes significantly different from gemcitabine only control at each dose.

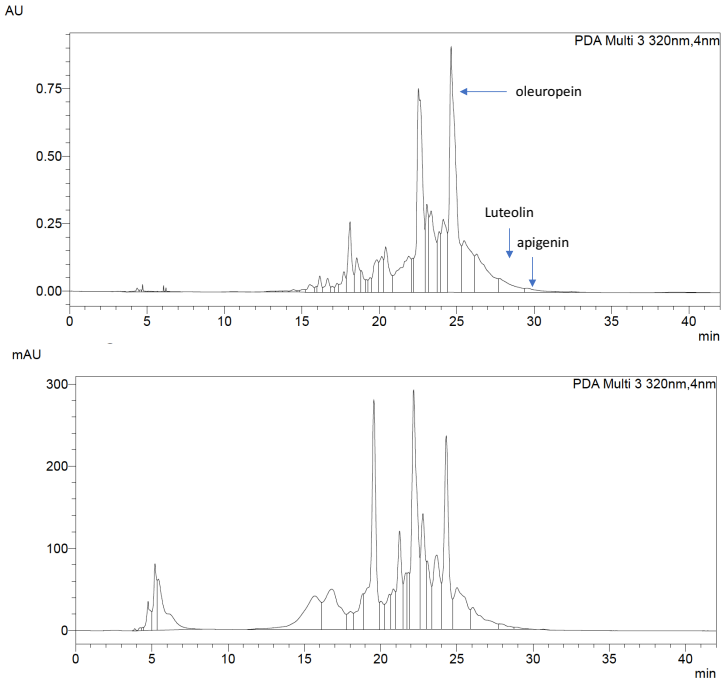

Figure 4. Representative HPLC chromatogram for olive leaf extracts. Compounds identified include oleuropein, luteolin and apigenin.

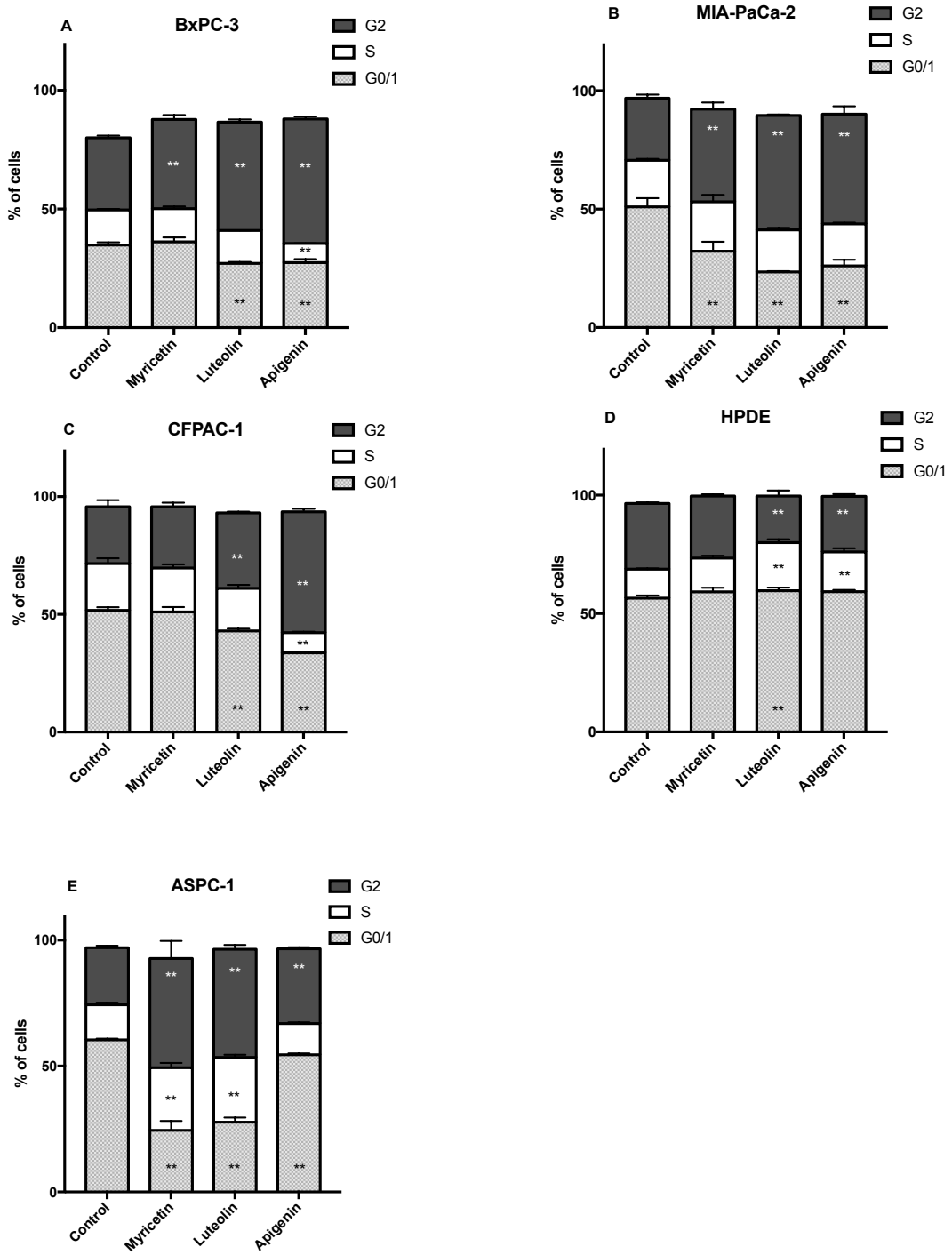

Figure 5. Cell cycle analysis of pancreatic cancer cells (A: BxPC-3, B: MIA PaCa-2 C: CFPAC-1 and E: ASPC-1) and non-tumorigenic cells (D: HPDE) when treated with flavonoids myricetin, luteolin and apigenin. Results are expressed as percentage of cells in each stage of the cell cycle ± SD.
** Denotes significantly different from control cells ($p<0.05$).

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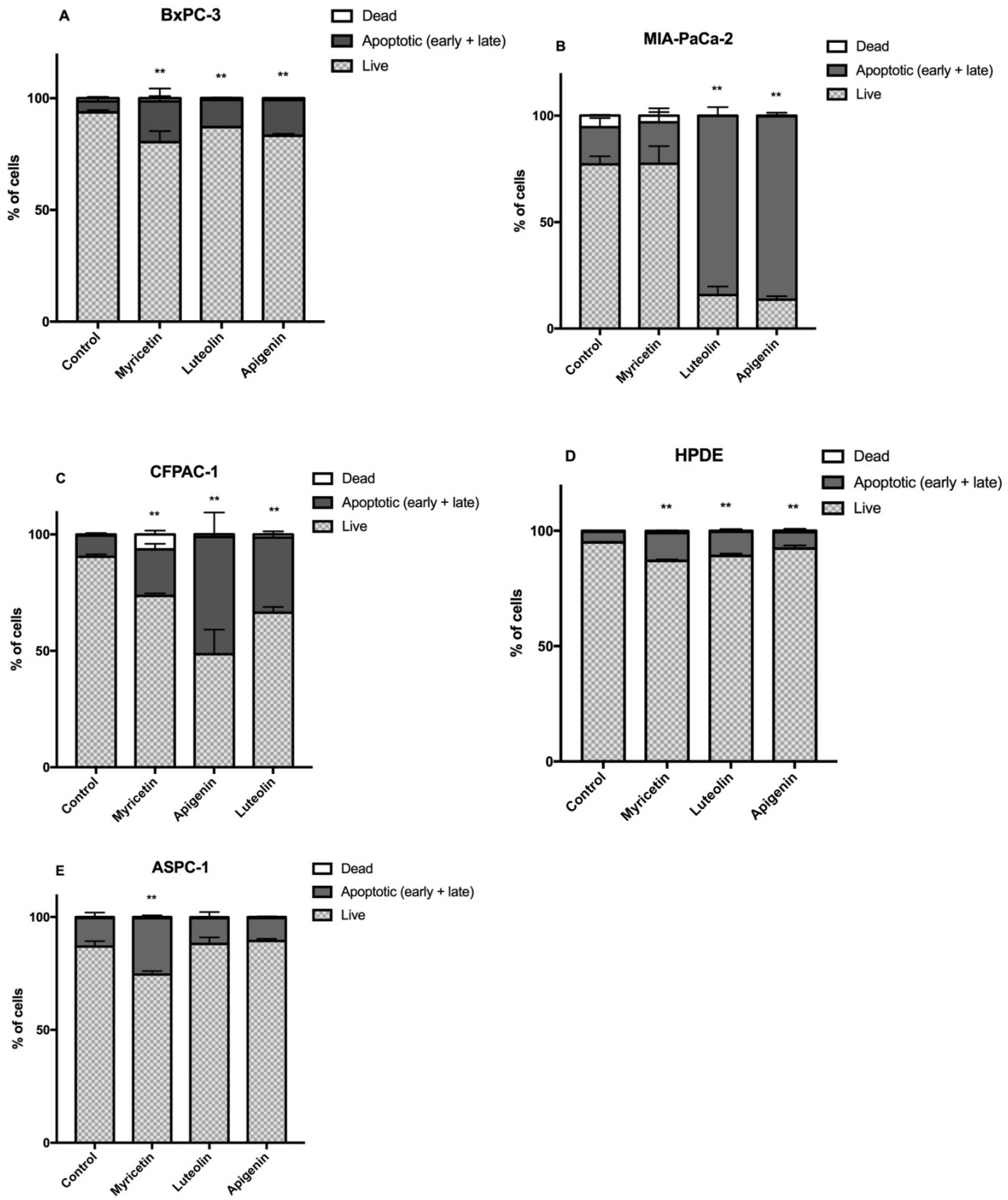

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507 **Figure 6**. Expression of caspase 3/7 by pancreatic cancer cells (A: BxPC-3, B: MIA PaCa-2
508 C: CFPAC-1 and E: ASPC-1) and non-tumorigenic cells (D: HPDE) when treated with
509 flavonoids myricetin, luteolin and apigenin. Results are expressed as percentage of: live cells,
510 cells expressing caspase 3/7 (early and late apoptosis) and dead cells ± SD.
511 ** Denotes cells expressing caspase 3/7 is significantly different from control cells ($p<0.05$).

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516 **Table 1**. Dose screen: Cells from cancer tissues of different organs of origin. Percentage (%)
517 cell growth in response to 100µg/mL of crude olive leaf and pomace extracts (the **higher** the
518 value the greater the growth inhibition).
519

Extract	*HT29 Colon*	*U87 Glioblastoma*	*MCF7 Breast*	*A2780 Ovarian*	*H460 Lung*	*A431 Skin*	*Du145 Prostate*	*BE2-C Neuroblastoma*	*SJ-G2 Glioblastoma*	*MIA Pancreas*	*SMA Glioblastoma*	*MCF10A Breast*
Aqueous leaf extract	17 ± 2	28 ± 5	26 ± 6	43 ± 4	2 ± 7	18 ± 5	6 ± 6	-1 ± 4	23 ± 6	25 ± 2	27 ± 11	17 ± 1
Methanol leaf extract	23 ± 4	10 ± 5	30 ± 7	64 ± 1	2 ± 5	23 ± 8	< 0	12 ± 5	27 ± 6	47 ± 3	37 ± 10	20 ± 4

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Table 2. IC_{50} for flavonoids myricetin, luteolin and apigenin, as well as gemcitabine on pancreatic cancer cells (MIA PaCa-2, BxPC-3, CFPAC-1 and ASPC-1) and non-tumorigenic cells (HPDE).

Compound	*MIA PaCa-2*	*CFPAC-1*	*BxPC-3*	*ASPC-1*	*HPDE*
Myricetin (μM)	36.71	120	66.73	108.14	55.7
Luteolin (μM)	50.75	22.46	24.58	66.2	6.3
Apigenin (μM)	75.63	26.96	22.18	62.54	5.7
Gemcitabine (nM)	31.02	2.6	3.6	12.0	0.004

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4.3. Conclusions

The water and the methanol olive leaf extracts both contained high concentrations of oleuropein. However, luteolin and apigenin were only identified in the methanol olive leaf extracts. Olive pomace was a poor source of major olive biophenols compared to olive leaves; in fact, the only compound identified in olive pomace extracts was oleuropein in the methanol extract. The cytotoxicity of the olive leaf methanol and water extracts as well as the pomace methanol extract was determined. All extracts dose dependently reduced the proliferation of pancreatic cancer cells (MIA PaCa-2, CFPAC-1 an BxPC-3); interestingly, at a low dose of extract (50 μg/mL), a significant increase in the viability of non-tumorigenic cells (HPDE) was observed. While the cytotoxicity of these extracts was low, subtle cellular toxicity is not unexpected. The slight reduction in viability observed in cancer cells compared to the increase in viability of non-tumorigenic cells would be beneficial with prolonged dietary exposure. Therefore, these data highlight the chemo-preventative potential of crude olive waste extracts.

CHAPTER 5

ANTI-CANCER ACTIVITY OF MAJOR OLIVE BIOPHENOLS

5.1. Introduction

Crude extracts are a complex mixture of compounds. It is difficult to determine the compound/compounds responsible for the effects observed when treating cancer cells with crude extracts. Therefore, the individual compounds identified in the olive waste extracts were investigated for their anti-pancreatic cancer potential.

The major olive biophenols that were identified in olive waste in chapter 4 were oleuropein, hydroxytyrosol, luteolin and apigenin. Oleuropein and hydroxytyrosol are the major biophenols found exclusively in olive products. While luteolin and apigenin are often identified in olive products, they are also found in the fruit of other plants.

As discussed previously, oleuropein and hydroxytyrosol have displayed anti-cancer activity in cancers from a number of different origins (**Section 2.7.**). However, their activity in pancreatic cancer has yet to be determined. Therefore, the anti-pancreatic cancer potential of biophenols identified in our olive waste, oleuropein and hydroxytyrosol, was investigated.

Luteolin and apigenin have displayed activity towards cancers of different origins. Moreover, these compounds have also previously induced apoptosis in a pancreatic cancer cell line (BxPC-3) by inhibiting the transcription factor NF-kB (Cai, Lu et al. 2012, Johnson and Gonzalez de Mejia 2013). However, more work on a broader panel of pancreatic cancer cells as well as an investigation into the effect of these compounds on non-tumorigenic pancreas cells is necessary in order to establish the anti-pancreatic cancer potential of luteolin and apigenin.

5.2. Experimental design

The design of experiments for the investigation of the anti-pancreatic cancer potential of the biophenols luteolin and apigenin is outlined in Figure 4.2., with the biophenols oleuropein, hydroxytyrosol and tyrosol pictured in Figure 5.1.

5.3. Results and discussion

The results and detailed discussion of the anti-cancer activity of flavonoids luteolin and apigenin is presented in research paper 5 entitled "**Flavonoids in *Olea europaea* leaf extracts and their cytotoxicity towards pancreatic cancer cells" submitted to Food & Function in 2017.**

The results and detailed discussion of the anti-pancreatic cancer activity of olive biophenols oleuropein, hydroxytyrosol and tyrosol is presented in research paper 6 entitled "**Oleuropein and hydroxytyrosol selectively augment the cell cycle and induce apoptosis in pancreatic cancer cells *in vitro*" submitted to the Journal of Nutritional Biochemistry in 2017.**

5.3.1. Research Paper 6

Research paper **6 entitled "Oleuropein and hydroxytyrosol selectively augment the cell cycle and induce apoptosis in pancreatic cancer cells *in vitro*"** submitted to the **Journal of Nutritional Biochemistry** in 2017**.**

Oleuropein and hydroxytyrosol selectively reduce proliferation, augment the cell cycle and induce apoptosis in pancreatic cancer cells *in vitro*.

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Keywords

Olive; phenolic compound; biophenols; chemoprevention; anti-cancer.

ABSTRACT

Current chemotherapy drugs for pancreatic cancer only offer an increase in survival of up to 6 months. Additionally, they are highly toxic to normal tissues, drastically affecting the quality of life of patients. Therefore, the search for novel agents, which induce apoptosis in cancer cells while displaying limited toxicity towards normal cells, is paramount. The olive biophenols, oleuropein, hydroxytyrosol and tyrosol, have displayed cytotoxicity towards cancer cells without effecting non-tumorigenic cells in cancers of the breast and prostate. However, their activity in pancreatic cancer has not been investigated. Therefore, the aim of this study was to determine the anti-pancreatic cancer potential of oleuropein, hydroxytyrosol and tyrosol. Pancreatic cancer cells (MIA PaCa-2, BxPC-3 and CFPAC-1) and non-tumorigenic pancreas cells (HPDE) were treated with oleuropein, hydroxytyrosol and tyrosol to determine their effect on cell viability. Oleuropein displayed selective toxicity towards MIA PaCa-2 cells and hydroxytyrosol towards MIA PaCa-2 and HPDE cells. Subsequent analysis of Bcl-2 family proteins and caspase 3/7 activation determined that oleuropein and hydroxytyrosol induced apoptosis in MIA PaCa-2 cells, while oleuropein displayed an apparent protective effect on HPDE cells. Gene expression analysis revealed putative mechanisms of action, which suggested that c-Jun and c-Fos are involved in oleuropein and hydroxytyrosol induced apoptosis of MIA PaCa-2 cells.

1. INTRODUCTION

Adherence to a Mediterranean diet is associated with a reduced risk for heart disease and most cancers, including pancreatic cancer (Trichopoulou, Lagiou et al. 2000, Fortes, Forastiere et al. 2003, Kapiszewska, Soltys et al. 2005, Bosetti, Turati et al. 2013). One of the major differences between Mediterranean diets and other healthy diets is the high intake of olives and olive oil; the annual intake of olive oil in Mediterranean countries can range between 15.3 - 23kg per capita (Serra-Majem, Ngo de la Cruz et al. 2003, Burns 2010). Many of the health benefits associated with consuming olive oil have been attributed to its high concentration of biophenols (Serra-Majem, Ngo de la Cruz et al. 2003).

Oleuropein is the main biophenol in olive products (olives, olive oil and olive leaves). While values vary in the literaure, oleuropein can reach up to 14% of the dry weight of olives; olive

55 oil can contain up to 2.8 mg/kg and olive leaf extracts can contain up to 61.56 g/ kg (Obied,
56 Allen et al. 2005, Seabra, Vinha et al. 2005, Goulas, Exarchou et al. 2009, Lee, Lee et al.
57 2009, Goldsmith, Stathopoulos et al. 2014, Goldsmith, Vuong et al. 2015).
58
59 Hydroxytyrosol is one of the most potent antioxidants in olive oil. Hydroxytyrosol and
60 tyrosol are degradation products of oleuropein both in the olive fruit and in the body (Figure
61 1). Hydrolysis of oleuropein occurs in the fruit during maturation and processing (Gomez
62 Caravaca, Carrasco Pancorbo et al. 2005) and after ingestion of oleuropein, it is broken down
63 by lipase activity and converted to hydroxytyrosol (Carrera-González, Ramírez-Expósito et
64 al. 2013). The hydroxytyrosol and tyrosol content of olive products can also vary.
65 Interestingly, the hydrolysis of oleuropein during olive oil processing often results in higher
66 concentrations of hydroxytyrosol present in olive oil compared to olives (Jerman Klen and
67 Mozetič Vodopivec 2012, Goldsmith, Stathopoulos et al. 2014).
68
69 The activity of oleuropein and hydroxytyrosol *in vitro* and *in vivo* has been well characterised
70 for certain cancers. For example, oleuropein has been shown to induce apoptosis in colon
71 cancer and breast cancer cells via activation of the p53 pathway (Cardeno, Sanchez-Hidalgo
72 et al. 2013, Hassan, Elamin et al. 2013). Additionally, oleuropein inhibited the proliferation
73 and induced thiol modifications, γ-glutamylcysteine synthetase and reactive oxygen species
74 in prostate cancer cells (Acquaviva, Di Giacomo et al. 2012). Oleuropein also activated
75 apoptosis in hepatocellular carcinoma cells by suppression of the phosphatidylinositol 3-
76 kinase/protein kinase B pathway (Yan, Chai et al. 2015). Hydroxytyrosol has also
77 demonstrated a range of activities *in vitro*. Hydroxytyrosol treatment of leukaemia cells
78 resulted in the induction of apoptosis while not affecting primary human cells, including
79 lymphocytes and polymorphonuclear cells (Fabiani, De Bartolomeo et al. 2002), highlighting
80 the chemo-preventative potential of hydroxytyrosol. Hydroxytyrosol also exhibited cardio-
81 protective effects; treatment of vascular endothelial cells resulted in an upregulation of
82 PI3K/Akt and Erk 1/ 2 and the subsequent activation of the Nrf-2 pathway - inducing
83 proliferation and protection against H_20_2 (Zrelli, Matsuoka et al. 2011).
84
85 Oleuropein and hydroxytyrosol have also been shown to have anti-cancer activity *in vivo*.
86 Oleuropein treatment in combination with doxorubicin significantly reduced tumour volume
87 of breast cancer xenografts (Elamin, Elmahi et al. 2017) and melanoma tumour volumes
88 (Samara, Christoforidou et al. 2017) in mice and prevented ultraviolet B radiation-induced

carcinogenesis in nude mice at a dose of 25mg/kg/day (Kimura and Sumiyoshi 2009). Moreover, hydroxytyrosol exhibited anti-inflammatory properties in mice at a dose of 5mg/kg (Silva, Sepodes et al. 2015), inhibited the growth of cholangiocarcinoma xenografts in mice after a dose of 250mg/kg/day (Li, Han et al. 2014) and a dose of 20mg/kg of hydroxytyrosol significantly inhibited tumour growth, angiogenesis and the activation of the AKT and NF-κB pathways in an orthotopic model of human hepatocellular carcinoma *in vivo* in nude mice (Zhao, Ma et al. 2014).

While oleuropein and hydroxytyrosol have both been investigated for their anti-cancer potential in a variety of different tissues, including breast, colon, prostate and melanoma as discussed above, their activity has not been determined against pancreatic cancer cells. Considering the link between adherence to a Mediterranean diet and reduced pancreatic cancer risk (Bosetti, Turati et al. 2013), the anti-pancreatic cancer potential of oleuropein and hydroxytyrosol clearly warranted investigation. Moreover, pancreatic cancer is a devastating disease with a 5-year survival rate of less that 8%. Resistance to conventional treatment options and the toxicity of current chemotherapy agents, such as gemcitabine, makes pancreatic cancer a vital target for the development of novel therapeutic agents (Vincent, Herman et al. 2011).

We have previously shown that olive leaf extracts, containing high concentrations of oleuropein, reduced the viability of pancreatic cancer cells (MIA PaCa2) in a dose dependant manner (Goldsmith, Vuong et al. 2015). This was the first-time olive biophenols had been investigated in relation to pancreatic cancer. However, crude extracts are a complex mixture of compounds and it was not possible to determine the compound/s responsible for the observed effects. Considering the desperate need for new treatment options for pancreatic cancer, the potential exhibited by the major olive biophenols against cancers from different origins and our previous observations of oleuropein-rich extracts reducing the viability of pancreatic cancer cells, the aim of this study was to investigate the effects of the major olive biophenols, oleuropein and hydroxytyrosol, on pancreatic cancer cells *in vitro*.

2. METHODS

2.1. Materials

Oleuropein, hydroxytyrosol, tyrosol, isopropranol, glycogen, ethanol, 2-mercaptoethanol, Roche cOmplete protease inhibitor cocktail as well as the reagents for the RIPA lysis buffer (made from 150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 50 mM Tris-HCl, pH 8.0) were purchased from Sigma Aldrich (Temecula, MS, USA). CCK-8 reagent was purchased from Dojindo Molecular Technologies Inc., (Rockville, MD, USA). Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12), Keratinocyte Serum-Free Media (K-SFM), Roswell Park Memorial Institute medium (RPMI), Iscove's Modified Dulbecco's Medium (IMDM), trypsin-EDTA, L-glutamine, phosphate buffered saline (PBS) and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA). Foetal Bovine Serum (FBS) was purchased from Interpath (Heildberg West VIC, Australia). Luminata Classico western Horse Raddish Peroxidase substrate and MUSE™ cell cycle and caspase 3/7 reagent kits were purchased from MERK-Millipore (Temecula, MA, USA). Gene Chip WT PLUS reagent kits were purchased from Affymetrix (Carlsbad, CA, USA). RIPA lysis buffer and bicinchoninic acid (BCA) protein assay kit was purchased from Thermofisher (Mulgrave VIC, Australia). Pancreatic cancer cells, MIA PaCa-2, CFPAC-1, BxPC-3 and ASPC-1, were purchased from ATCC (Manassas, VA, USA) and immortilised normal pancreatic ductal epithelial cells (HPDE) were a gift from the lab of Dr. M. Tsao (MD, FRCPC, University of Health Network, Toronto, ON, Canada). All cell lines were authenticated by CellBank Australia (Westmead, NSW, AUS).

2.2. Pancreas Cell Culture

Human pancreatic cancer cells, MIA PaCa2, were cultured in DMEM-F12, supplemented with 10% FBS, 2.5% horse serum and L-glutamine (100 µg/mL). BxPC-3 and ASPC-1 were cultured in RPMI supplemented with 10% FBS and L-glutamine (100 µg/mL). CFPAC-1 cells were cultured in IMDM supplemented with 10% FBS and L-glutamine (100 µg/mL). Human Pancreas (HPDE) cells were cultured in K-SFM supplemented with BPE and EGF. All cells were grown and maintained at 37 °C under 5% CO_2.

2.3. Assessment of Cell Growth Inhibition

Cell growth inhibition was determined using the Dojindo Cell Counting Kit-8. Cells were seeded into a 96 well plate at 3000-10000 cells per well and allowed to adhere for 24 h. Cells were then treated within the range of 0-300 μM of each compound, 0-50 nM gemcitabine (positive control) or 0.5% DMSO (vehicle control). After 72 h, 10% CCK-8 solution in media was added before incubating at 37 °C for 180 min. The absorbance was measured at 450 nm and cell growth inhibition was determined as the IC_{50}. All experiments were performed in triplicate.

2.4. Apoptosis Assay

The induction of apoptosis was evaluated by assessing caspase 3/7 activation. MIA PaCa-2, and HPDE cells were seeded into 12 well plates at 30000 and 100000 cells/well, respectively. After 24 h, cells were treated with oleuropein (200 μM) or hydroxytyrosol (100 μM) for 48 h before washing with PBS and dislodging with 0.25% trypsin EDTA. Cells were diluted to 300 cells/μL prior to staining. MUSE caspase 3/7 reagent working solution was prepared by diluting the stock solution with 1x PBS. The MUSE Caspase 7-AAD reagent was diluted with 1x assay Buffer at a ratio of 1:74. Cells were stained by adding 5 μL of the caspase 3/7 working solution to 50 μL of diluted cells and incubating for 30min at 37 °C and 5% CO_2 before adding 150 μL of Caspase 7-AAD working solution and incubating for 5mins at room temperature. All assays were performed in triplicate. Fluorescence was read on a MUSE cell analyser (MERCK Millipore, Sydney, NSW Australia). Results were expressed as percentage of live cells, early and late apoptotic cells as well as percentage of dead cells.

2.5. Cell cycle analysis

The effect of oleuropein on the cell cycle was analysed by DNA staining with propidium iodide (MERCK Millipore cell cycle kit). MIA PaCa-2 and HPDE cells were seeded into 12 well plates at 30000 and 100000 cells/well, respectively. After 24 h, cells were treated with oleuropein (200 μM) or hydroxytyrosol (100 μM) for 24 h before washing with PBS and dislodging with 0.25% trypsin EDTA. Cells were washed with PBS before fixing in ice cold 70% ethanol and storing at -20 °C for a minimum of 3 h prior to staining. For staining, cells were centrifuged at 300 g for 5 min, washed in PBS before resuspending in 200 μL of cell cycle reagent and incubated for 30 min at ambient temperature in the dark. Fluorescence was read on a MUSE flow cell analyser and DNA content profile histograms were produced.

Results were expressed as percentage of cells in G0/1, S and G2/M phases of the cell cycle. All assays were performed in triplicate.

2.6. Assessment of the effect of oleuropein and hydroxytyrosol on gene expression

HPDE and MIA PaCa-2 cells were seeded into 12 well plates at a concentration of 100000 or 30000 cells/well, respectively, and allowed to adhere for 24 h before treatment with either oleuropein (200 μM), hydroxytyrosol (100 μM) or vehicle control for 24 h. Total RNA was extracted after 18 h using TRIZOL reagent (Invitrogen) as per the manufacture's guidelines, with an overnight -30 °C isopropanol and glycogen precipitation. The quantity and integrity of the RNA preparation was analysed using a 6000 Nano Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA integrity number (RIN) integrity scores were all over 7. 100 ng of total RNA was used as starting material. The WT PLUS amplification and labelling process was prepared according to Affymetrix protocols. Fragmented and biotinylated ss-cDNA preparations (5.5 μg) were hybridized to HTA 2.0 Arrays, which were subsequently washed, stained, and scanned using a GeneChip Fluidics station and GeneChip Scanner 3000 (Affymetrix) according to the Affymetrix protocol. Array images were processed using the Affymetrix GeneChip command console (AGCC) to produce probe intensity data (*cel* files). After using the Expression console to normalise and examine data quality, only the microarrays meeting acceptable Affymetrix quality control criteria were considered for further analysis. The Expression console was then used to create probe level summarisation files (CHP) from the *cel* files. The Transcriptome analysis console (TAC) was used to convert the CHP files into a visual representation of the differentially expressed genes. Statistical analysis was preformed using One-Way ANOVA (unpaired) on genes displaying a significant fold change [(linear) < -2 or > 2, and p-value < 0.05)].

2.7. Protein expression

HPDE and MIA PaCa-2 cells were seeded into 12 well plates at a concentration of 100000 or 30000 cells/well, respectively, and allowed to adhere for 24 h before treatment with either oleuropein (200 μM), hydroxytyrosol (100 μM) or vehicle control (0.5% DMSO) for 24 h. Whole cell lysates were collected by lysis with RIPA lysis buffer containing protease inhibitor for 30 min and subsequent centrifugation at 10000 g at 4 °C for 30 min. Protein concentration was determined by a micro BCA protein assay kit according to the manufacturer's instructions (Thermofisher, Mulgrave VIC, Australia. Lysates were dissolved

in sample buffer (0.35 M Tris-HCl pH 6.8, 30% glycerol, 10% SDS and bromophenol blue) and reducing agent (9% 2-mercaptoethanol) before heating at 75° C for 10 min. Samples were separated on NuPAGE® Novex® Midi Bis-Tris 4-12% gels followed by transfer onto nitrocellulose membrane (GE Healthcare, Sydney, NSW, Australia). The membranes were blocked using 10% skim milk in TBST, washed with TBST and probed with primary antibodies for 2 h and secondary antibodies for 1 h before addition of a chemiluminescent substrate (Luminata Classico western Horse Radish Peroxidase substrate, MERCK-Millipore, Temecula, MA, USA). Bands were revealed using an Amersham Imager 600 (GE Healthcare, Sydney, NSW, Australia). The following primary antibodies were used: rabbit polyclonal anti-Bax (dilution 0.125 µg/mL; abc11; MERK Millipore), rabbit monoclonal anti-Bak (dilution 1:1000; 06-536 ; MERK Millipore), mouse monoclonal anti-Bcl-2 (dilution 2 µg/mL; 05-729: MERK Millipore), rabbit polyclonal anti-ADAMTS1 antibody (dilution 1µg/mL; ab113847; abcam), rabbit polyclonal anti-c-Fos antibody (dilution 1:500; ab209795; abcam), rabbit monoclonal anti-c-Jun antibody (dilution 1:2000; [E254] ab32137; abcam), rabbit polyclonal anti-EGR-1 antibody (dilution 1:500; ab208780; abcam), rabbit polyclonal anti-GAPDH (dilution 1:1000; 00-18231; Biovision). The secondary antibodies were rabbit anti-goat (dilution 1:5000; 172-1034; BioRad), mouse anti-goat (dilution 1:5000; 170-6516; BioRad).

2.8. Statistics

GraphPad Prism Version 7.0 was used to determine the IC_{50} of normalised and transformed viability data. Ordinary two-way ANOVA followed by Tukey's multiple comparisons test was conducted on cell cycle, caspase 3/7 expression and expression of apoptosis proteins data to compare treated verses vehicle control cells; ordinary one-way ANOVA followed by Tukey's multiple comparisons test was conducted on Bax/Bcl-2 ratio and gene expression data to compare treated verses vehicle control cells. Significance was set at $p<0.05$.

3. RESULTS

3.1. Treatment with olive biophenols reduces pancreatic cancer cell viability

The viability of pancreas cancer (MIA PaCa-2, BxPC-3 and CFPAC-1) and non-tumorigenic pancreas (HPDE) cells treated with oleuropein, hydroxytyrosol or tyrosol was assessed using a CCK8 viability assay to determine effective doses for each drug. Neither oleuropein or hydroxytyrosol had any effect on the viability of BxPC-3 or CFPAC-1 cells (Table 1) in the treatment range tested (0-300 μM). However, both compounds inhibited the proliferation of MIA PaCa-2 cells in a dose dependant manner; the IC_{50} for oleuropein and hydroxytyrosol were 150.1 μM and 75.1 μM, respectively (Table 1).

In contrast to its effects on MIA PaCa-2 cells, oleuropein did not reduce the viability of HPDE cells, even at the concentration of 300 μM (Table 1). Considering the usual sensitivity of HPDE cells to cytotoxic drugs (Gemcitabine, IC_{50} = 0.04 nM), the activity of oleuropein and hydroxytyrosol was investigated further. However, tyrosol did not have any influence on the viability of any of the pancreatic cells within the treatment range tested (Table 1) and hence, the activity of tyrosol was not explored further.

3.2. Oleuropein and hydroxytyrosol induce morphological changes in pancreatic cells

Significant morphological changes were observed following treatment with oleuropein and hydroxytyrosol (Figure 2). Cell shrinkage and the formation of apoptotic bodies were identified in MIA PaCa-2 cells treated with oleuropein or hydroxytyrosol; also, hydroxytyrosol treatment of HPDE cells caused similar effects. Importantly, oleuropein did not induce any morphological changes in HPDE cells when compared to vehicle control (Figure 2).

3.3. Olive biophenols cause G2/M cell cycle arrest in pancreatic cells

The cell cycle is one of the first cell regulatory mechanisms that can be affected prior to apoptosis. Therefore, the effect of oleuropein and hydroxytyrosol on the cell cycle was determined by propidium iodide staining and subsequent MUSE flow cytometry analysis. Treatment of MIA PaCa-2 cells with oleuropein or hydroxytyrosol caused cell cycle arrest at the G2 phase (Figure 3A); there was a significant increase in the percentage of cells in G2 (10.1% and 23.1% increase, $p < 0.0001$ and < 0.0001, respectively), coupled with a decrease in

the percentage of cells in G0/1 (11.9% and 22.3% decrease, $p < 0.0001$ and < 0.0001, respectively) when compared to cells treated with the vehicle control (Figure 3A).

In HPDE cells, oleuropein did not have a significant effect (Figure 3B) on the number of cells in G0/1 or G2 phase ($p = 0.058$ and 0.3088, respectively). However, hydroxytyrosol treatment of HPDE cells did cause a significant increase in in the percentage of cells in G2 (7.3% increase, $p < 0.0001$) and a decrease in the percentage of cells in G0/1 (11.8% decrease, $p < 0.0001$) when compared to the vehicle control (Figure 3B). Importantly, this effect was much smaller than that observed for MIA PaCa-2 cells.

3.4. Treatment with Oleuropein and Hydroxytyrosol promotes caspase 3/7 dependent apoptosis

Caspase 3 and 7 are activated downstream in the apoptosis cascade and result in the cleavage of protein substrates and the disassembly of the cell (Mandavilli, Yan et al. 2018). Therefore, the activation of caspase 3/7 measured by fluorescent tagging and subsequent flow cytometry was used to determine the induction of apoptosis. In cells expressing caspase 3/7, the fluorescent dye (MUSE caspase 3/7 reagent) was able to bind to the DNA, while the dead cell marker (7-AAD) entered membrane-compromised, later-stage apoptotic and dead cells. The number of fluorescently labelled cells expressing caspase 3/7 was counted by MUSE flow cytometry. Treatment of MIA PaCa-2 cells with either oleuropein or hydroxytyrosol caused a significant increase in the percentage of cells expressing activated caspase 3/7 (Figure 4A) with the total percentage of cells (early + late apoptosis) increasing from 7.93% (vehicle control) to 40.63% after oleuropein treatment ($p < 0.0001$) and 47.17% after hydroxytyrosol treatment ($p < 0.0001$). The effect on HPDE cells was much smaller, with the total percentage of HPDE cells with caspase 3/7 activation only increasing from 4.6% (vehicle control) to 10% after oleuropein ($p = 0.613$) and 22.01% after hydroxytyrosol ($p < 0.0001$) treatment (Figure 4B).

3.5. Differential expression of Bcl2 family proteins following treatment with oleuropein and hydroxytyrosol

The Bcl-2 family of proteins are involved in the regulation of apoptosis (Thandapani and Aifantis 2017). In order to determine if Bcl-2 family members were involved in oleuropein-

induced apoptosis, the expression of Bax, Bak and Bcl-2 were determined by Western blot. Results were normalised to GAPDH expression and expressed as fold change compared to vehicle control cells. Interestingly, expression of the pro-apoptotic protein Bax, decreased in MIA PaCa-2 cells (Figure 5A) after oleuropein and hydroxytyrosol treatment (23.4% and 26.6% decrease, $p = 0.035$ and 0.017, respectively). Expression of the anti-apoptotic protein Bcl-2 also decreased (51.4% and 33.7% decrease, $p < 0.0001$ and 0.0027, respectively). However, there was no significant change (Figure 5A) in the expression of Bak (oleuropein $p = 0.302$ and hydroxytyrosol $p = 0.105$). Additionally, oleuropein or hydroxytyrosol treatment of HPDE cells significantly decreased the expression of Bax (31.5% and 20.3% decrease, $p = 0.016$ and 0.013, respectively) and Bak (25.6% and 29.5% decrease, $p = 0.052$ and 0.024, respectively) while increasing the expression of Bcl-2 (28.9% and 69.2% increase, $p = 0.027$ and <0.0001, respectively) (Figure 5B).

A more important indicator of survivability, than the individual expression levels of each apoptosis protein, is the ratio of Bax to Bcl-2 expression. The Bax/Bcl-2 ratio in MIA PaCa-2 cells treated with oleuropein was almost double that of vehicle control cells (control = 2.5, oleuropein = 4.3, $p = 0.007$) (Figure 5C). For hydroxytyrosol-treated cells the Bax/Bcl-2 ratio was not significant different compared to controls (control = 2.5, hydroxytyrosol = 2.7, $p = 0.72$) (Figure 5C). Interestingly, in HPDE cells treated with either oleuropein or hydroxytyrosol, the Bax/Bcl-2 ratio was more than halved (control = 1.02, oleuropein = 0.55, $p = 0.012$ and hydroxytyrosol = 0.47, $p = 0.0063$) (Figure 5D). These data suggest that oleuropein may have induced apoptosis in MIA PaCa-2 cells via the regulation of the expression of mitochondrial proteins while oleuropein and hydroxytyrosol may have had a protective effect on HPDE cells.

3.6. Gene and protein expression changes in MIA PaCa-2 cells following treatment with oleuropein and hydroxytyrosol

To gain a better understanding of the mechanism by which oleuropein and hydroxytyrosol induce apoptosis in MIA PaCa-2 cells, and the protective role of oleuropein in HPDE cells, the changes in gene expression were determined. An mRNA microarray was utilised to determine the changes in gene expression after treatment with oleuropein or hydroxytyrosol (Table 2). Genes with significant, high fold changes that have a known role in pancreatic cancer were selected for further validation at the protein level by Western blot.

Studies suggest that Early Growth Response-1 (EGR-1) is a cancer suppressor gene and accordingly, it was found that EGR-1 was significantly upregulated in oleuropein (8-fold, $p = 0.018$) and hydroxytyrosol (20-fold, $p = 0.018$) treated MIA PaCa-2 cells in the microarray assay (Table 2). Considering EGR-1 has previously been shown to induce apoptosis in pancreatic cancer (Wang, Husain et al. 2015), EGR-1 was chosen for further validation at the protein level to determine if this change in gene expression resulted in a functional effect. However, despite the increased expression observed in the microarray (Table 2), this was not reflected at the protein level (Figure 6A). In fact, the protein expression of EGR-1 was significantly reduced after treatment with oleuropein (55% decrease $p = 0.006$) and hydroxytyrosol (50% decrease, $p = 0.008$).

JUN and FOS are known proto-oncogenes which dimerize to form the transcription factor AP-1. More specifically, forced expression of c-Jun and c-Fos has previously been linked to the induction of apoptosis in pancreatic cancer cells *in vitro* (Ren, Zhao et al. 2016). In the present study (Table 2), the expression of JUN significantly increased 4.6-fold after oleuropein treatment ($p = 0.000126$) and 4.7-fold after hydroxytyrosol treatment ($p = 0.000041$). The expression of FOS also increased 2.4-fold after oleuropein treatment ($p = 0.007736$) and 5-fold after hydroxytyrosol treatment ($p = 0.000103$) (Table 2). Due to the relationship between c-Jun, c-Fos and apoptosis in pancreatic cancer cells, the effects on c-Jun and c-Fos were further investigated at the protein level. The protein expression of c-Jun increased in both oleuropein (291% increase, $p = 0.008$) and hydroxytyrosol-treated cells (242% increase, $p = 0.029$) (Figure 6C). This trend was also observed with c-Fos; c-Fos protein expression increased in MIA PaCa-2 cells treated with oleuropein (289% increase, $p = 0.0002$) and hydroxytyrosol (182% increase, $p = 0.015$) (Figure 6B).

3.7. Reduced expression of ADAMTS1 in HPDE cells following treatment with oleuropein

Expression of ADAMTS1 has been positively correlated with disease progression in cancers of different origins (de Arao Tan, Ricciardelli et al. 2013). The gene expression of ADAMTS1 in HPDE cells after treatment with oleuropein was reduced 2.2-fold ($p = 0.00003$) (Figure 6D). Therefore, in order to gain an insight into the potential protective role of oleuropein in HPDE cells, ADAMTS1 was validated at the protein level by western blot. The protein expression of ADAMTS1 was also significantly reduced in cells treated with

368 oleuropein (50% decrease, $p = 0.003$) (Figure 6D). However, the effect of hydroxytyrosol on
369 the expression of ADAMTS1 by HPDE cells was not statistically significant (26% decrease,
370 $p = 0.055$) (Figure 6).

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4. DISCUSSION

Pancreatic cancer is one of the most aggressive malignancies due to its late diagnosis, low resection rate and resistance to therapy. Available chemotherapy agents only result in an average survival advantage of 3-6 months; hence, new treatments are highly sought after. The epidemiological link between the Mediterranean diet and pancreatic cancer (Bosetti, Turati et al. 2013) offers a potential avenue for the exploration of biophenols which may possess anti-pancreatic cancer potential. Oleuropein and hydroxytyrosol are the most abundant biophenols in olives and olive oil and their consumption has been linked to health benefits (Granados-Principal, Quiles et al. 2010, Omar 2010). Additionally, the anti-cancer potential of oleuropein and hydroxytyrosol has previously been described in cancers of different tissues (Cardeno, Sanchez-Hidalgo et al. 2013, Chimento, Casaburi et al. 2013, Hassan, Elamin et al. 2013); however, the present study is the first to investigate their anti-pancreatic cancer activity.

The toxicity of oleuropein towards cancer cells (MIA PaCa-2) and non-toxicity towards non-tumorigenic cells (HPDE) observed in the current study augments oleuropein's potential as a chemotherapeutic agent for pancreatic cancer; however, elucidation of the underlying mechanisms is important. Similarly, oleuropein has been previously observed to selectively reduce the proliferation of prostate cancer cells while displaying an antioxidant effect on non-malignant prostate cells (Acquaviva, Di Giacomo et al. 2012). The authors highlighted that cancer cells were more sensitive to ROS generation within the cells. Many human cancer cell types exist in a highly oxidative state compared to their normal tissues and therefore, the selective activity of oleuropein on MIA PaCa-2 cells could be due to their increased sensitivity towards ROS. Additionally, hydroxytyrosol is a more potent antioxidant than oleuropein (Angelino, Gennari et al. 2011), and could explain the lower IC_{50} of hydroxytyrosol in MIA PaCa-2 cells compared to oleuropein.

Changes in the cell cycle are often an early indicator of cellular stress leading to apoptosis. In the present study, treatment of MIA PaCa-2 cells with oleuropein or hydroxytyrosol caused cell cycle arrest at G2 phase of the cell cycle. Oleuropein has been shown to cause cell cycle arrest in neuroblastoma cells by down-regulation of Cylin-D1, 2 and 3, and CDK4 and 6 while up-regulating p53 (Secme, Eroglu et al. 2016). Hydroxytyrosol has also previously caused cell cycle arrest in prostate cancer cells by inhibiting cyclins D1/E and CDK 2/ 4 and inducing inhibitory p21/ p27 (Zubair, Bhardwaj et al. 2017). Therefore, oleuropein and

hydroxytyrosol may be exerting cell cycle arrest via changing the expression of specific cyclins and CDKs involved in G2/M phase. However, this was not investigated in the present study.

Resistance to chemotherapy occurs primarily due to cellular evasion of apoptosis. This evasion can lead to deregulated cell proliferation and subsequent tumour formation. Therefore, the induction of tumour cell apoptosis without displaying toxicity towards surrounding normal cells is an effective chemotherapy strategy (Baudino 2015). In the present study, oleuropein reduced the viability of pancreatic cancer cells (MIA PaCa-2) without displaying toxicity towards the non-tumorigenic cells (HPDE). This has not been reported previously. In fact, a direct comparison of the effect of any drug on MIA PaCa-2 and HPDE cells could not be found in the literature. This may be due to the high concentrations that were required to achieve a reduction in viability (150μM). While this concentration is quite high in a biological context, these results are useful to determine the appropriate dose of oleuropein for *in vivo* models of pancreatic cancer. Additionally, it is important to understand how drugs behave *in vitro*. Cellular toxicity can arise via a number of mechanisms. For this reason, the ability of oleuropein and hydroxytyrosol to induce apoptosis was determined.

The Bcl-2 family of proteins regulate apoptotic mitochondrial events such as the ability of ceramide to form channels in the mitochondrial outer membrane. Anti-apoptotic proteins (Bcl-2) inhibit ceramide channels while the pro-apoptotic proteins (Bax, Bak) enhance these channels and lead to the release of pro-apoptotic proteins into the cytosol, which initiates the execution phase of apoptosis (Ganesan and Colombini 2010). For this reason, the Bax/Bcl-2 ratio is an important indicator of cell survivability. In this study, the treatment of pancreatic cancer cells with oleuropein or hydroxytyrosol increased the Bax/Bcl-2 ratio and led to caspase 3/7-dependent apoptosis. In contrast, this ratio was decreased by these olive biophenols in the non-tumorigenic HPDE cells. These results demonstrate the selective activity of oleuropein and hydroxytyrosol in pancreatic cells.

Olive biophenols have previously been shown to induce apoptosis in cancer cells. Oleuropein caused an increase in the Bax/Bcl-2 ratio in lung cancer cells (A549) (Cao, Zhu et al. 2017), breast cancer cells (Hassan, Elamin et al. 2014) and neuroblastoma cells (SH-SY5Y) (Secme, Eroglu et al. 2016), in each case activating the caspase cascade causing cells to undergo apoptosis. Hydroxytyrosol has also increased the Bax/Bcl-2 ratio in prostate cancer cells

(Zubair, Bhardwaj et al. 2017). The previous studies credit varying upstream mechanisms for the change in expression of Bcl-2 family proteins, which appear to be cell type specific.

In order to elucidate the mechanisms underlying the selective activity of oleuropein and hydroxytyrosol, changes in gene expression after treatment were assessed. Major genes that were upregulated in MIA PaCa-2 cells after treatment included EGR-1, JUN and FOS. EGR-1 has been linked with apoptosis in MIA PaCa-2 cells treated with vitamin E δ-tocotrienol by causing an increase in the Bax/Bcl-2 ratio and subsequent caspase cascade inducing apoptosis (Wang, Husain et al. 2015). In the present study, oleuropein and hydroxytyrosol treatment also increased the gene expression of EGR-1 and the Bax/Bcl-2 ratio as well as caspase 3/7 activation in MIA PaCa-2 cells. However, EGR-1 protein expression decreased as a result of treatment with oleuropein and hydroxytyrosol and therefore, the role of EGR-1 in oleuropein and hydroxytyrosol-induced apoptosis of MIA PaCa-2 cells is still to be determined. This would suggest that transcription of the mRNA may be inhibited or that degradation of the protein is increased, which in turn causes the cells to put out more mRNA.

The proteins c-Jun and c-Fos dimerize to form Activator Protein-1 (AP-1). The AP-1 site is a ubiquitous regulatory element that is found in a wide range of promoter and enhancer regions. They bind to AP-1 DNA recognition elements and control cell proliferation, transformation, survival and death. The functions of Fos-Jun family proteins depend on the specific cell type in which they are expressed (Chinenov and Kerppola 2001, Shaulian 2010). We found that oleuropein and hydroxytyrosol treatment of MIA PaCa-2 cells caused an increase in the gene expression of JUN and FOS and an increase in the protein expression of c-Jun and c-Fos. Previously, the forced expression of c-Jun and c-Fos has been demonstrated to cause the induction of apoptosis in neuronal cells (Estus, Zaks et al. 1994, Ham, Babij et al. 1995). Additionally, treatment of myelodysplastic cells with the plant-derived compound, withaferin A, caused an increase in the mRNA expression of JUN and FOS and their subsequent protein expression and resulted in downstream activation of apoptosis (Oben, Alhakeem et al. 2017). More specifically, Ren et al., (2016) were able to show that increasing the expression of c-Jun and c-Fos in MIA PaCa-2 cells caused the downstream induction of apoptosis via activation of Bim and the subsequent effect on Bcl-2 family proteins. These previous studies support the theory that the induction of apoptosis in this study could be due to an increased expression of c-Jun and c-Fos, which dimerize into AP-1 and result in

activation of the AP-1/JNK pathway. However, more work is needed to substantiate this hypothesis.

The effects observed in MIA PaCa-2 cells in the present study and the previous studies showcase the ability of oleuropein and hydroxytyrosol to induce apoptosis via a number of different pathways. However, in this study, oleuropein and hydroxytyrosol decreased the Bax/Bcl-2 ratio in HPDE cells, suggesting a protective effect on these non-tumorigenic cells. In addition to their potent cytotoxic activities previously described, these olive biophenols have also displayed cyto-protective effects. Kalaiselvan et al.,(2016) found that oleuropein, hydroxytyrosol and even tyrosol exhibited a protective effect on rat liver cells exposed to the harsh environmental toxin, 2,3,7,8-tetrachlorodibenzo-P-dioxin (TCDD); all the olive compounds increased the expression of Bcl-2 and decreased the expression of Bax and hence, decreased the Bax/Bcl-2 ratio. Additionally, olive oil treatment of hippocampus CA1 neurons following ischemia in mice also reduced apoptosis by decreasing the expression of Bax and increasing the expression of Bcl-2 (Zamani, Hassanshahi et al. 2013). The cyto-protective activity of oleuropein was also observed by Geyikoglu et al., (2017), who found that the administration of 200mg/kg/day of oleuropein for 3 days modulated oxidative stress and completely reversed 8-OHdg production in mouse kidneys after treatment with the chemotherapy drug, cisplatin. These studies support the observed protective effects of oleuropein and hydroxytyrosol in HPDE cells.

ADAMTS1 is a metalloprotease which remodels the ECM (extracellular matrix) through the proteolytic degradation of key substrates such as collagen (Kuno, Okada et al. 2000). Low ADAMTS1 expression has been linked to tumorigenesis in some cancers. However, the specific role of ADAMTS1 in pancreatic cancer is yet to be determined. In the present study, lower levels of ADAMTS1 were expressed in oleuropein and hydroxytyrosol-treated HPDE cells than in control cells. Low expression of ADAMTS1 in pancreatic cancer tissue compared to normal pancreas tissue has previously been studied (de Arao Tan, Ricciardelli et al. 2013); however, large variability in the data resulted in the relationship being non-significant ($p = 0.206$). Despite this, low ADAMTS1 expression in primary tumours compared to normal tissue is a common trend that can be correlated with disease progression. Reports on prostate (Gustavsson, Wang et al. 2009), colon (Lind, Kleivi et al. 2006) and lung cancer (Choi, Kim et al. 2008) have shown lower expression of ADAMTS1 in primary cancers compared to non-tumorigenic tissue. Further investigations have revealed that

epigenetic silencing through promoter hyper-methylation to be the key mechanism underlying ADAMTS1 suppression during tumour development. Therefore, the methylation state of ADAMTS1 has been suggested as a potential early biomarker for colon, prostate and non-small cell lung cancer (Choi, Kim et al. 2008). As mentioned, oleuropein treatment of the non-tumorigenic pancreas cells (HPDE) decreased the expression of ADAMTS1 in the present study; this result conflicts the protective activity exhibited by oleuropein. Moreover, considering the previous reports of ADAMTS1 and disease progression, it is possible that the observed protective effects of oleuropein on the HPDE cells could instead be the first stages of cellular transformation leading to tumorigenesis. However, more research is needed to determine the role of oleuropein and hydroxytyrosol on ADAMTS1 functions as well as the role of ADAMTS1 on pancreatic cancer disease progression. Due to the complex nature of tumorigenesis, these data highlight the importance of determining chemo-preventative mechanisms.

5. CONCLUSION

For the first time, the anti-pancreatic cancer properties of oleuropein and hydroxytyrosol have been determined *in vitro*. Oleuropein and hydroxytyrosol arrested the cell cycle, increased the Bax/Bcl-2 ratio, increased activation of caspase 3/7 and induced apoptosis in pancreatic cancer cells (MIA PaCa-2). Increased expression of c-Jun and c-Fos was also observed in oleuropein and hydroxytyrosol-treated cells and therefore, dimerization of c-Jun and c-Fos into AP1 is a potential underlying mechanism for oleuropein and hydroxytyrosol-induced apoptosis in MIA PaCa-2 cells. However, more work is needed to validate these findings. Additionally, oleuropein did not display toxicity towards non-tumorigenic cells (HPDE); in fact, a putative protective effect was observed. However, the downregulation of ADAMTS1 in oleuropein treated cells conflicts its protective label. Therefore, more work is also needed to determine if the observed protective effects of oleuropein on non-tumorigenic pancreas cells could lead to cancer prevention.

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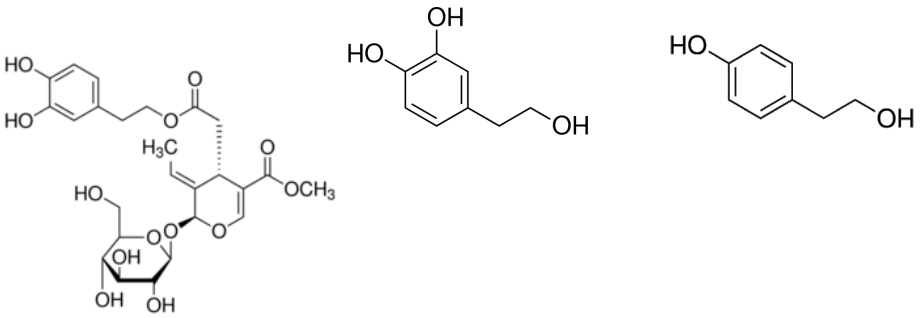


Figure 1. Structure of oleuropein (left) and hydroxytyrosol (middle) and tyrosol (right).

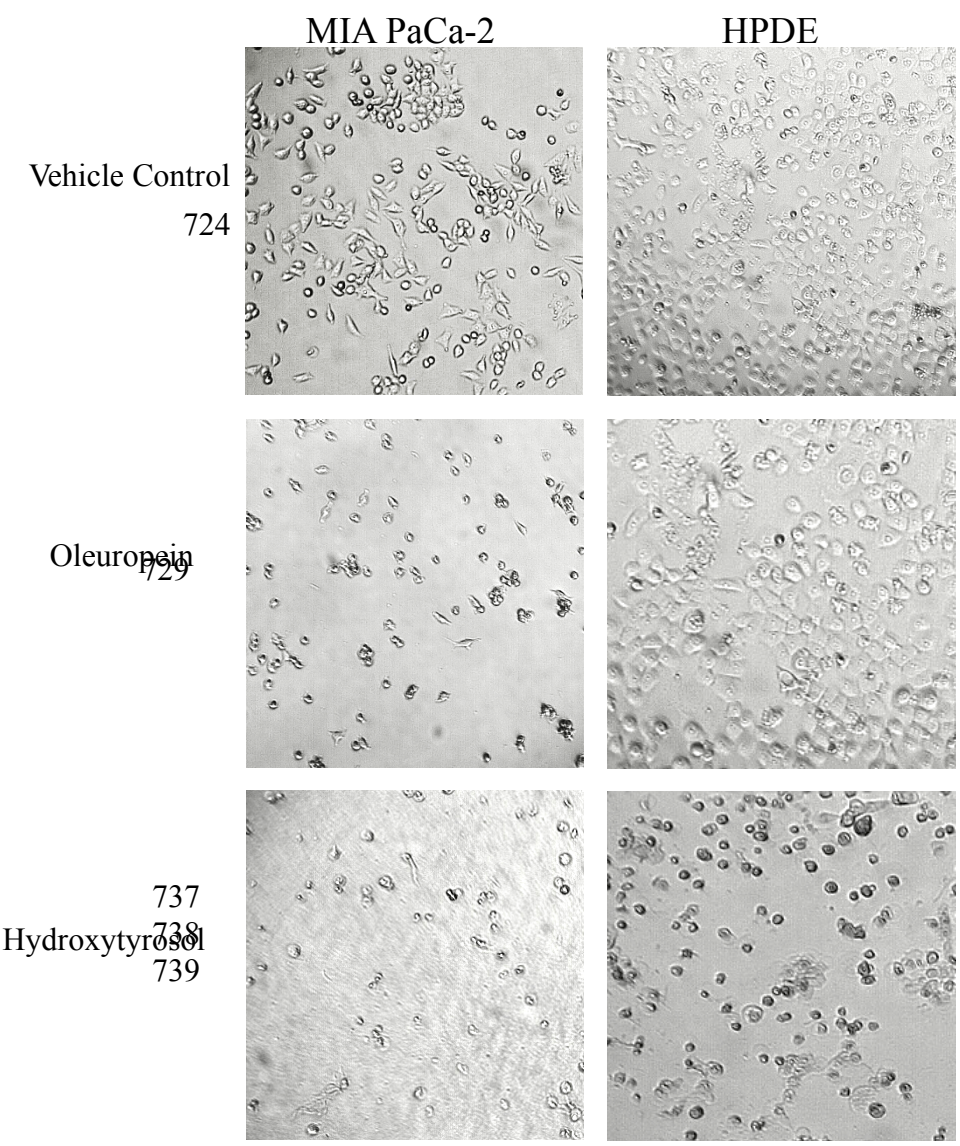


Figure 2. Morphological changes of MIA PaCa-2 and HPDE cells when treated with oleuropein (200µM) and hydroxytyrosol (100µM) for 24h at x100 magnification.

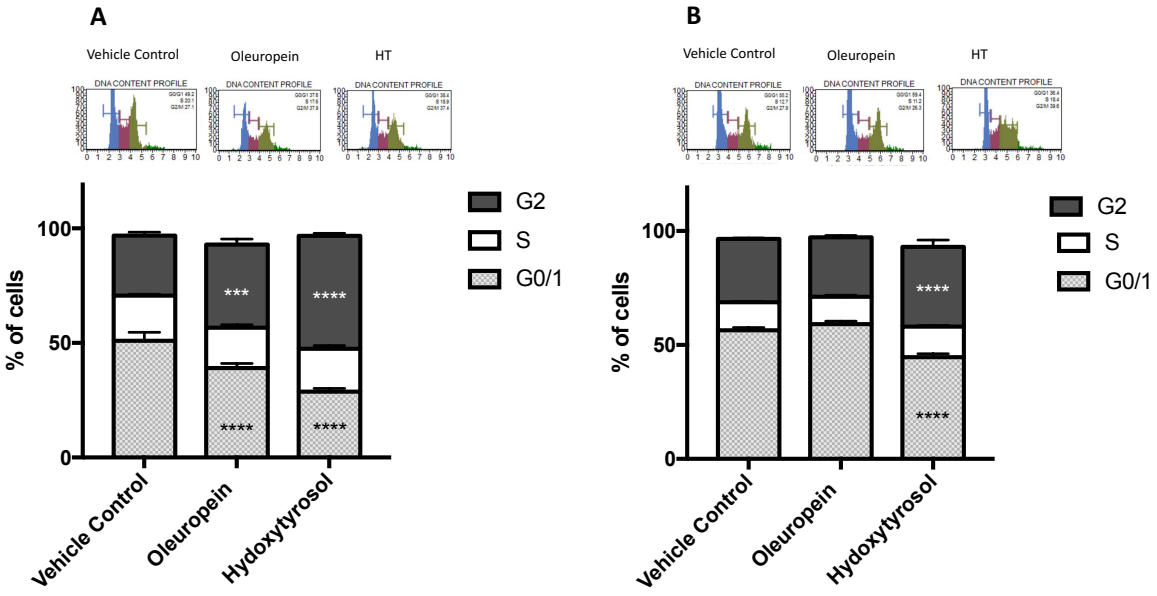


Figure 3. Cell cycle analysis of MIA PaCa-2 (A) and HPDE (B) cells treated with oleuropein (200µM) and hydroxytyrosol (100µM) for 24h. Bar graphs show the percentage of cells in G0/1, S and G2 phase of the cell cycle measured by MUSE cell cycle analysis kit. A representative DNA content profile for vehicle control, oleuropein and hydroxytyrosol (HT) treatment is pictured for MIA PaCa-2 (A) and HPDE (B) cells. Ordinary two-way ANOVA and tukey's multiple comparisons test compare the percentage of treated cells (oleuropein or hydroxytyrosol) in each stage of the cell cycle to vehicle control. **** $p<0.0001$.

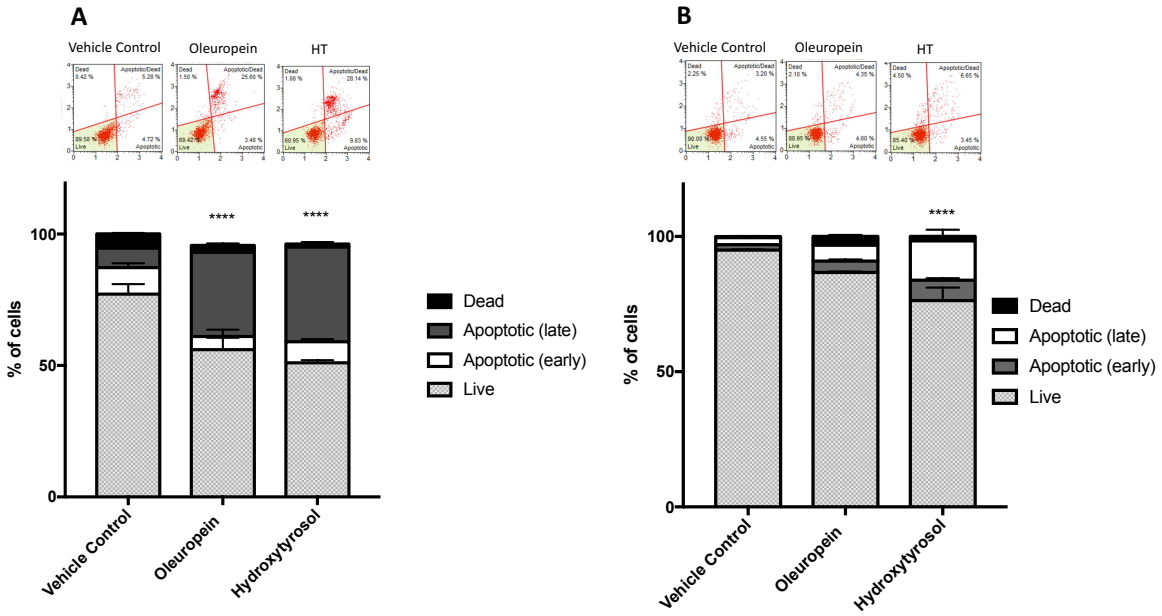


Figure 4. Induction of caspase 3/7-dependent apoptosis of MIA PaCa-2 (A) and HPDE (B) cells treated with oleuropein (200µM) and hydroxytyrosol (100µM) for 48h. Bar graphs show the percentage of live, early apoptotic, late apoptotic and dead cells determined by analysis of the activation of caspase 3/7. Ordinary two-way ANOVA and tukey's multiple comparisons test compare total apoptotic cells in treated cells (oleuropein or hydroxytyrosol) to vehicle control.
**** $p<0.0001$.

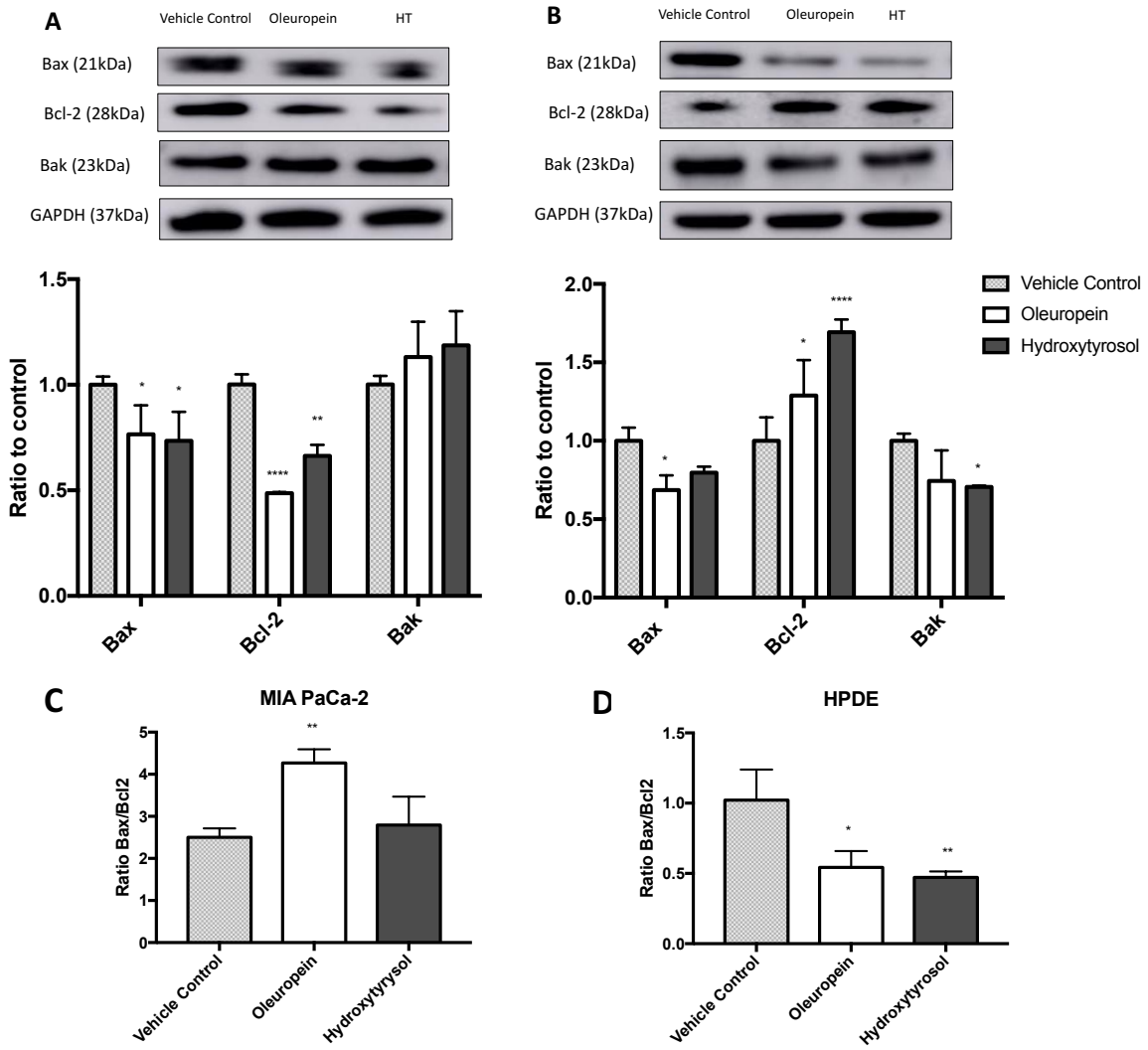
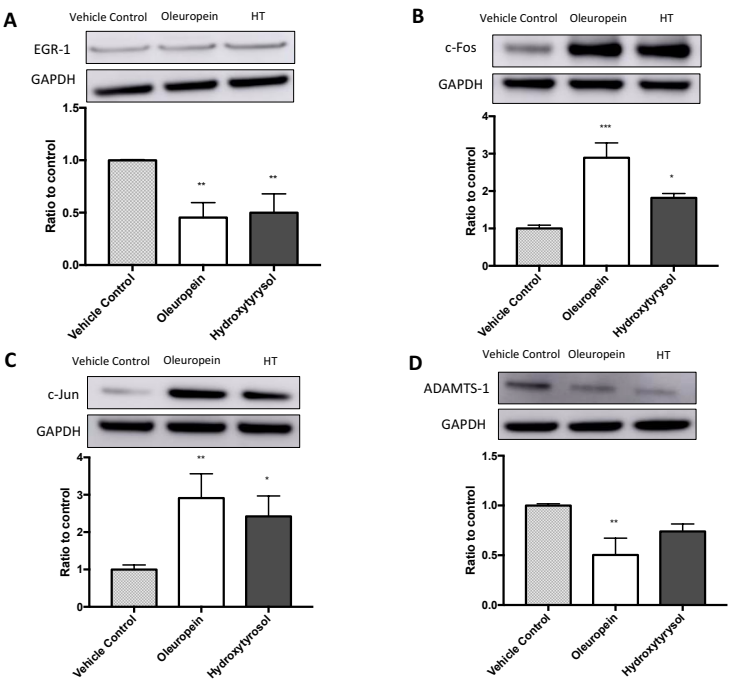


Figure 5. Expression of Bax, Bak and Bcl-2 in MIA PaCa-2 (**A**) and HPDE (**B**) cells treated with oleuropein (200µM) and hydroxytyrosol (HT) (100µM) or vehicle control as assessed using gel electrophoresis and western blotting. GAPDH was used as a loading control. Results displayed as optical density measurements of target antibody/GAPDH/control average; hence, results are represented as fold change. Ratio of the expression of Bax to Bcl-2 in MIA PaCa-2 (**C**) and HPDE (**D**) cells. For **A** and **B**, ordinary two-way ANOVA with tukey's multiple comparisons test compares protein expression of treated cells (oleuropein or hydroxytyrosol) to vehicle control; for **C** and **D**, ordinary one-way ANOVA with tukey's multiple comparisons test compares Bax/Bcl-2 ratio of treated cells to vehicle control. * p 0.05, ** p 0.01, *** p 0.001, **** $p < 0.0001$.



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793 **Figure 6**. Protein expression of EGR-1 (**A**), c-Fos (**B**) and c-Jun (**C**) in MIA PaCa-2 cells and
794 expression of ADAMTS-1 (**D**) in HPDE cells. GAPDH was used as a loading control.
795 Results displayed as optical density measurements of target antibody/GAPDH/control
796 average; hence, results are represented as fold change. Ordinary one-way ANOVA with
797 tukey's multiple comparisons test compares the expression of protein from treated cells
798 (oleuropein or hydroxytyrosol) to vehicle control.
799 * p 0.05, ** p 0.01, *** p 0.001, **** $p < 0.0001$.
800
801

802 **Table 1**. Viability of pancreatic cancer cells (MIA PaCa-2, BxPC-3, CFPAC-1 and ASPC-1)
803 and non-tumourigenic pancreas cells (HPDE) when treated with 0-300μM of oleuropein,
804 hydroxytyrosol or tyrosol. Values represent concentration required to achieve a 50%
805 reduction in viability (IC_{50}).
806

Cell line	*Oleuropein (μM)*	*Hydroxytyrosol (μM)*	*Tyrosol (μM)*	*Gemcitabine (nM)*
MIA PaCa-2	150.1	75.1	>300	31.02
BxPC-3	>300	>300	>300	3.6
CFPAC-1	>300	>300	>300	2.6
ASPC-1	>300	>300	>300	12
HPDE	>300	65.5	>300	0.04

807

808 **Table 2**. Fold change in the gene expression of JUN, FOS and EGR-1 in pancreatic cancer
809 cells (MIA PaCa-2) and ADAMTS-1 in non-tumorigenic cells (HPDE).

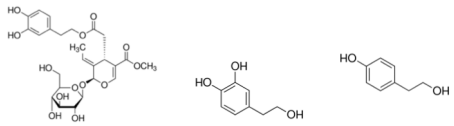
810

811

CELL LINE	*GENE SYMBOL*	*TREATMENT*	*FOLD CHANGE (LINEAR) (VS. CONTROL)*	*ANOVA P-VALUE (VS. CONTROL)*
MIA PaCa-2	**JUN**	Oleuropein	4.64	**0.000126**
		Hydroxytyrosol	4.68	**0.000041**
	FOS	Oleuropein	2.41	**0.007736**
		Hydroxytyrosol	4.98	**0.000103**
	EGR-1	Oleuropein	8.01	**0.00083**
		Hydroxytyrosol	20.75	**0.000019**
HPDE	**ADAMTS1**	Oleuropein	-2.19	**0.00003**
		Hydroxytyrosol	-	-

5.4. Conclusions

Luteolin and apigenin induced apoptosis in BxPC-3, CFPAC-1 and MIA PaCa-2 cells. However, luteolin and apigenin were more toxic to non-tumorigenic cells (HPDE). Oleuropein and hydroxytyrosol increased the Bax/Bcl-2 ratio and subsequent caspase cascade inducing apoptosis in pancreatic cancer cells (MIA PaCa-2). Increased expression of c-Jun and c-Fos was observed in oleuropein and hydroxytyrosol treated cells. c-Jun and c-Fos dimerization into AP1 is proposed as a potential underlying mechanism for oleuropein and hydroxytyrosol induced apoptosis in MIA PaCa-2 cells. Importantly oleuropein did not display toxicity towards the non-tumorigenic cells (HPDE); in fact, a decrease in the Bax/Bcl-2 ratio was observed after treatment with oleuropein and hydroxytyrosol. However, gene expression analysis revealed that ADAMTS1 was also decreased in HPDE treated cells and the loss of ADAMTS1 has previously been linked to tumorigenesis in cancers of different origins. Therefore, the observed protective effects on HPDE cells could instead be the initiation of unchecked replication; however, more work is needed to determine if these effects will lead to cancer prevention or development. These findings support the potential of olive biophenols as chemotherapeutic agents for pancreatic cancer.



Anti-pancreatic cancer potential of olive biophenols:

Oleuropein, hydroxytyrosol and tyrosol

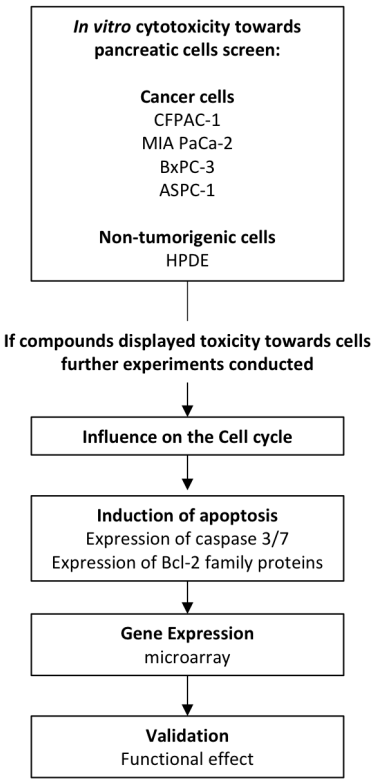


Figure 5.1. Schematic detailing the experimental design for the investigation of the anti-pancreatic cancer potential of olive biophenols oleuropein, hydroxytyrosol and tyrosol.

6. Discussion

This project aimed to investigate the anti-pancreatic cancer potential of the major olive biophenols and determine the efficacy of water as an extraction solvent for their recovery from leaves and pomace. The hypothesis and aims of the project were addressed through three major studies. We revealed the following significant findings, each one addressing the hypothesis and aims postulated in **Section 1.2**.

To address Aim 1, the optimal parameters for aqueous extraction of biophenols with antioxidant capacity from olive waste products (leaves and pomace) were investigated.

To optimize the extraction of phenolic compounds with antioxidant activity from olive leaves, we used a Box Behnken central composite design to determine the best possible combination of the parameters temperature, extraction time and sample-to-solvent ratio; the optimal conditions were sample-to-solvent ratio 1:100 (g/ mL), heated to 90°C for 70min. However, at a sample-to-solvent ratio of 1:60 (g/ mL) we retained 80% of the total phenolic compounds and maximized the antioxidant activity of the extracts. This work is presented in **Paper 1 (Section 3.3.1.)**. For olive pomace, we optimised the parameters of ultrasound power, time and sample-to-solvent ratio. The optimal conditions for the extraction of phenolic compounds with high antioxidant activity were sample-to-solvent ratio 1:50 (g/ mL) at 250W of ultrasound power for 75min. This work is presented in **Paper 2 (Section 3.3.2.)**.

The preparation of plant extracts for food industry applications, such as natural antioxidants and preservatives as well as health supplements is increasing in popularity and out of necessity. As previously discussed, olive waste is a polluting effluent due in part to the high concentration of phenolic compounds (Lafka, Lazou et al. 2011). However, olive waste has been identified as a source of health benefiting compounds such as oleuropein, hydroxytyrosol, luteolin and apigenin. As such, the extraction of phenolic compounds from olive waste constitutes a viable use for these compounds for further investigations and utilization of their reported health benefiting properties (Dermeche, Nadour et al. 2013). Moreover, water is a cheap and non-hazardous substitute to organic solvents; therefore, extracts prepared with water have wide industrial applications. In addition to industry uses, our optimized water extraction methods also have research applications. By optimizing the parameters for using water as an extraction solvent we were able to establish a baseline for the development of more elaborate extraction technologies.

While water as an extraction solvent is useful for many purposes, certain compounds with anti-cancer activity, identified in olive waste products, are not highly soluble in water, including flavonoids luteolin and apigenin. Methanol has been identified as an ideal solvent for the maximum extraction of luteolin and apigenin from different plant matrices (Abidin, Mujeeb et al. 2014, Al-Quraishy, Othman et al. 2017). **To determine the efficacy of water as an extraction solvent for the recovery of major biophenols (AIM 2), we investigated the presence of the major olive biophenols (oleuropein, hydroxytyrosol, tyrosol, luteolin and apigenin) in our water extracts and compared these to solvent extracts.**

Our water and methanol olive leaf extracts contained high concentrations of oleuropein (**Paper 3, Section 4.3.1.**). Oleuropein has been identified as the major biophenol in methanol olive leaf extracts (Goulas, Exarchou et al. 2009, Taamalli, Arraez-Roman et al. 2012, Dermeche, Nadour et al. 2013, Al-Quraishy, Othman et al. 2017). While our methanol olive leaf extracts contained higher amounts, our study revealed that water was able to extract ~80% of the oleuropein from olive leaves when compared to our methanol extracts (50% methanol). Therefore, we can conclude that water is an effective extraction solvent for the recovery of oleuropein from olive leaves.

Moreover, we have shown that aqueous olive leaf extracts contain approximately 65% of the total flavonoid content of methanol olive leaf extracts (**Paper 3, Section 4.3.1.**). However, the individual flavonoids of interest, luteolin and apigenin, were only identified in the methanol olive leaf extract (**Paper 5, Section 4.3.3.**). Based on the structures of luteolin and apigenin it is unlikely that they would have been extracted using water alone. Additionally, methanol has been identified as the most efficient solvent for extracting luteolin from plant materials (Abidin, Mujeeb et al. 2014). Hence, we determined that water is not an appropriate solvent for the recovery of the flavonoids luteolin or apigenin from olive leaves and methanol is confirmed as a more appropriate solvent.

Oleuropein was identified in the olive pomace methanol extracts, while the pomace water extracts did not contain any major biophenols (**Paper 4, Section 4.3.2.**). The phenolic compound fraction of olives and olive oil has been shown to vary widely due to many factors including seasonal variation, time of harvest, maturation index and geographic location (Obied, Bedgood et al. 2008). Previously, olive pomace has been identified as an excellent source of biophenols including oleuropein, hydroxytyrosol, tyrosol (Leouifoudi, Harnafi et al. 2015). Interestingly, we have also shown olive pomace to be an excellent source of

oleuropein. In fact, in olive pomace prepared from olives of the same cultivar, on the same processing equipment and harvested from the same farm 2 years earlier, oleuropein represented ~10% of the total biophenols extracted (Goldsmith, Stathopoulos et al. 2014). However, the extraction solvent used in our previous study was 80% methanol while in the present study 50% methanol was used; hence, we are unable to discern if the differences between these observations are due to seasonal variation or the different methanol concentrations. Although compounds extracted with water from olive pomace displayed antioxidant activity (**Paper 2, Section 3.3.2.**), the compounds of interest were not detected (**Paper 4, Section 4.3.2.)**. This was unexpected, particularly considering the efficacy of water as an extraction solvent for oleuropein from olive leaves (**Paper 3, Section 4.3.1.).** During the maturation of olives, the oleuropein content of olives decreases while in the olive leaves, it increase (Ortega-Garcia, Blanco et al. 2008). Our olive leaves were collected as a waste product during the olive harvest; a time when olives are at their most mature. Therefore, the limited content in our olive pomace extracts could be due to a limited starting concentration of oleuropein in the original olives. However, the oleuropein content of the original olives would need to be determined in order to corroborate our findings. Nevertheless, we have shown that the extraction of oleuropein from olive leaves using water, constitutes a valuable use for this agricultural waste product because of the high concentrations of the valuable compound oleuropein.

Olive leaf water and methanol extracts as well as olive pomace methanol extracts were found to be sources of the major biophenols of interest. Therefore, the cytotoxicity of the extracts was determined. Our pomace water extracts did not contain any major biophenols hence, it was not investigated further.

The cytotoxicity of crude olive waste extracts towards cancer cells was investigated in Aim 3. Briefly, the growth inhibition of water and methanol olive leaf extracts (**Paper 5, Section 4.3.3.)** as well as methanol olive pomace extracts (**Paper 4, Section 4.3.2.)** on eleven cancer cell lines [MIA PaCa-2 (pancreas), HT29 (colon), A2780 (ovarian), H460 (lung), A431 (skin), Du145 (prostate), BE2-C (neuroblastoma), MCF-7 (breast), and U87, SJ-G2, SMA (glioblastoma)] and one non-cancer derived cell line [MCF-10A (normal breast)] was determined. Cells were treated with 100 µg/mL of each crude extract. The highest growth inhibition was observed in the ovarian cancer cells treated with methanol leaf extracts causing 64% growth inhibition (GI), methanol pomace extracts (57% GI) and water leaf extracts (43% GI). This was closely followed by breast cancer growth inhibition (methanol

pomace 57% GI, methanol leaf 30% GI and water leaf 26% GI). Cytotoxicity towards pancreatic cancer cells was also high with crude methanol leaf extracts causing 47% GI, followed by methanol pomace extract (42% GI) and water leaf extract (25% GI). Olive leaf extracts have been reported to display cytotoxicity towards breast cancer cells (Goulas, Exarchou et al. 2009), however we are the first to report the cytotoxicity of crude olive leaf or pomace extracts on the remaining 10 cell lines. By identifying the cytotoxicity of crude olive leaf and pomace extracts we provide a platform for the investigation of the anti-cancer activity of the major biophenols identified in our extracts.

Considering the growth inhibition of pancreatic cancer cells observed in our cytotoxicity screen and the link between consumption of olive oil and reduced pancreatic cancer risk (Soler, Chatenoud et al. 1998), the anti-pancreatic cancer potential of crude olive waste extracts was investigated further. Pancreatic cancer cells (MIA PaCa-2, CFPAC-1 and BxPC-3) and non-tumorigenic pancreas cells (HPDE) were treated with varying concentrations of olive waste extracts. All olive waste extracts reduced the viability of pancreatic cells in a dose dependent manner. However, at a low dose (50 μg/mL) the proliferation of non-tumorigenic cells increased; this trend was consistent between both the methanol and water leaf extracts (**Paper 5, Section 4.3.3.),** as well as the methanol pomace extract (**Paper 4, Section 4.3.2.**). Interestingly, despite the very different biophenol profiles of leaf and pomace extracts, the cytotoxicity was similar. The activity of the olive leaf extracts is likely attributed to the presence of oleuropein and luteolin and apigenin. However, more work needs to be performed to identify the compound/s responsible for the activity of the methanol pomace extracts. This can be a difficult task due to the complex system of different compounds existing within crude extracts. This work provides a platform for future studies on the synergistic activity of olive biophenols as well as the potential protective effect of crude olive leaf and pomace extracts on normal cells.

The combination of olive biophenols and chemotherapy agents has been investigated in different cancers with positive results. The biophenols have been shown to work synergistically to either reduce the proliferation of cancer cells, thus requiring a lower dose of toxic chemotherapy drugs (Elamin, Elmahi et al. 2017, Tezcan, Taskapilioglu et al. 2017, Tezcan, Tunca et al. 2017), or by protecting normal cells from harmful chemotherapy agents (Geyikoglu, Emir et al. 2017). Therefore, the effect of the combination of olive biophenols with gemcitabine (chemotherapy drug for pancreatic cancer) on pancreas cells was

determined. Cells were treated with varying doses of gemcitabine combined with either 100 µg/mL of olive leaf extract or 50 µg/mL of olive pomace extract. Despite the promising activity of the crude extracts on the non-tumorigenic (HPDE) cells alone, the crude extracts displayed a protective effect towards the pancreatic cancer cells when administered with gemcitabine; hence protecting the cancer cells from the gemcitabine and not displaying any effect on HPDE cells compared to the gemcitabine only controls. However, HPDE cells are highly sensitive to gemcitabine; therefore, more work is needed to investigate the ability of crude olive waste extracts to protect HPDE cells from lower doses of gemcitabine (<1nM). These results also highlight the difficulty of working with crude extracts in *in vitro* models, due to the complex nature of the crude extract systems. The combination of different compounds with varying antioxidant and pro-oxidant capabilities can result in unpredictability and makes discerning the compounds responsible for the observed activity an arduous task. For these reasons, mechanistic investigations *in vitro* are better focused on individual compounds.

The anti-pancreatic cancer activity of major olive biophenols *in vitro* was investigated in Aim 4.

The flavonoids luteolin and apigenin caused cell cycle arrest at G2 and induced apoptosis in pancreatic cancer (BxPC-3, CFPAC-1 and ASPC-1) cells. However, cytotoxicity towards non-tumorigenic (HPDE) cells was also observed at low IC_{50} values (**Paper 5, section 4.3.3**). Luteolin and apigenin have been shown to induce apoptosis in BxPC-3 cells by inhibiting the transcription activity of nuclear transcription factor NF-kB (Cai, Lu et al. 2012, Johnson and de Mejia 2013). Our investigation of the anti-pancreatic cancer activity of luteolin and apigenin supports these findings while also adding to this work by establishing the cytotoxicity of these compounds in additional pancreatic cancer cells as well as the non-tumorigenic cells. Therefore, this work contributes to the larger body that has investigated the anti-cancer potential of luteolin and apigenin. While the activity of luteolin and apigenin has been investigated in pancreatic cancer cells previously, the activity of biophenols found exclusively in olive products (oleuropein and hydroxytyrosol) has not.

The activity of oleuropein, hydroxytyrosol and tyrosol on pancreatic cancer cells (MIA PaCa-2, BxPC-3 and CFPAC-1) and non-tumorigenic cells (HPDE) is presented in **Paper 6 (section 5.3.1)**. Oleuropein exhibited selective toxicity towards MIA PaCa-2 cells without displaying any toxicity towards CFPAC-1, BxPC-3 and importantly, HPDE cells.

Hydroxytyrosol displayed selective toxicity towards MIA PaCa-2 and HPDE cells, however, toxicity towards HPDE cells was minimal. Subsequent analysis of Bcl-2 family proteins and caspase 3/7 activation determined that oleuropein and hydroxytyrosol induced apoptosis in MIA PaCa-2 cells while exhibiting a protective effect on HPDE cells. Increased expression of *JUN* and *FOS* and subsequent protein expression of c-JUN and c-FOS were identified as potential mechanisms of action; these two proteins dimerise to form the transcription factor AP-1 which controls proliferation and apoptosis (Ameyar, Wisniewska et al. 2003, Ren, Zhao et al. 2016). However, more work is needed to potentiate these findings as mechanisms of oleuropein and hydroxytyrosol induced apoptosis in pancreatic cancer cells.

As discussed thoroughly in our literature review (**sections 2.7.1. and 2.7.2.**), oleuropein and hydroxytyrosol have displayed anti-cancer activity in a range of different cancer types. By investigating the activity of olive biophenols in pancreatic cancer we have established the potential of olive biophenols as anti-pancreatic cancer agents. Moreover, we have added to the body of work which has established olive biophenols as anti-cancer agents.

7. Conclusions

In conclusion, we have shown that olive waste products have the potential to be important sources of major olive biophenols. Water has been established as an effective solvent for the extraction of oleuropein from olive leaves, however more work is required to determine if water is appropriate for the recovery of biophenols from olive pomace. Crude extracts from olive leaves and pomace display potential as cytotoxic agents towards pancreatic cancer cells and protective agents towards non-tumorigenic cells. Major olive biophenols oleuropein, hydroxytyrosol, luteolin and apigenin selectively induced apoptosis in different pancreatic cancer cells, hence establishing their anti-pancreatic cancer potential. Moreover, oleuropein displayed a potential protective effect on non-tumorigenic cells however more work is necessary to determine the specific role of oleuropein in this process. This work provides a platform for the continued investigation of olive biophenols as chemo-preventative or therapeutic agents for pancreatic cancer.

8. Future Directions

Based on the findings obtained from this study, recommendations for further study include the following areas.

Investigation into the stability of aqueous olive waste crude extracts warrants attention. While studies have previously revealed the stability of certain purified compounds in water, investigations into the stability of olive biophenols in aqueous crude extract systems has not been determined. Previous studies have revealed that compounds in crude extracts do not always simply degrade. In fact, as oleuropein and tyrosol decrease, hydroxytyrosol content often increases. By understanding the stability of biophenols in aqueous crude extract systems, we would be able to understand how other compounds might degrade or change during storage and handling. This information would allow for very specific expiry dates to be determined for each compound in crude olive waste extracts. This is valuable information for scientists working with crude extracts as well as for commercial producers of crude olive waste extracts. Moreover, this information is necessary in order to establish the efficacy of water as an extraction solvent.

The mechanisms underlying oleuropein and hydroxytyrosol induced apoptosis also require further investigation. While we have shown that *JUN* and *FOS* were upregulated as a result of treatment, as well as the protein expression of c-JUN and c-FOS, and hence displaying a functional effect, more work is needed to potentiate that *JUN* and *FOS* are involved in the mechanism underlying oleuropein and hydroxytyrosol induced apoptosis in MIA PaCa-2 cells. This is important to determine the pathway/s involved in the activation of apoptosis. Understanding the mechanism of action of oleuropein and hydroxytyrosol in cancer cells to determine their appropriateness as a cancer treatment, is a rational subsequent experimental step. This information will aid researchers working on *in vivo* models of pancreatic cancer by providing information on how these compounds behave in single cell culture systems and the mechanisms underlying their activity.

The role of *ADAMTS-1* in non-tumorigenic pancreas cells requires clarification. As discussed in section 6, it is unclear if *ADAMTS-1* is a tumour suppressor or promoter gene for pancreatic cancer. It is important to determine the role of this gene in pancreatic cancer to develop more specific treatments. In our study, oleuropein treatment of HPDE cells caused a decrease in the expression of *ADAMTS-1*. If ADAMTS-1 is involved in tumour suppression,

oleuropein shows potential as a preventative treatment for pancreatic cancer. This has implications for patient survival or the development of preventative treatments for high risk patients.

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Appendices

Other journal articles related to this thesis

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Physicochemical, antioxidant and anti-cancer activity of a *Eucalyptus robusta* (Sm.) leaf aqueous extract

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ABSTRACT

Eucalyptus robusta (Sm.) (ER) is a widely distributed tree native to the east coast of Australia, which has also been established in numerous other countries. ER leaves contain high levels of essential oils and are rich in total phenolic compounds (TPC), which have been linked with health benefits; however, there is limited information on the bioactivity of ER leaf extracts. This study aimed to optimise water extraction conditions for TPC, prepare a spray-dried powdered extract and test its physicochemical, antioxidant and anti-proliferative properties. The results showed that optimal water extraction conditions for TPC were 85 °C, 15 min and a water-to-leaf ratio of 20:1 mL/g. Under these conditions, spray-dried powdered extract was prepared with a recovery yield of 85%. The extract was water-soluble and had a TPC level of 407 mg GAE/g. It also possessed potent antioxidant capacity, comparable to pure ascorbic acid, but higher than pure α-tocopherol. In addition, the powdered extract demonstrated significant activity against a panel of cancer cell lines, which included cancers of the pancreas, breast, lung, brain, skin, colon and ovary. Of note, the ER extract exerted a more significant toxic effect on pancreatic cancer (PC) cells compared to gemcitabine, the first line chemotherapeutic agent for PC. We suggest that future studies should purify individual bioactive compounds from ER for further investigation of its potential health promoting and anti-cancer activity.

1. Introduction

The natural distribution of *Eucalyptus robusta* (Sm.) (ER), also known as swamp mahogany, is in a narrow belt along the east coast of Australia (Boland et al., 2006). However, it has been well established in other countries and reportedly occupies a total area of 2.3 million hectares worldwide (Rejmanek and Richardson, 2011). The eucalyptus leaf contains high levels of essential oils and is also rich in total phenolic compounds (TPC), with several studies extracting phenolics from the eucalyptus leaf using either organic solvents or a mixture of organic solvents and water (Amakura et al. 2002; Bachir and Benali, 2012; Bhagat et al., 2012; Rejmanek and Richardson, 2011; Takasaki et al., 2000). Numerous methods have been employed for the extraction of phenolic compounds, with many procedures often associated with high-energy costs and the production of excessive solvent waste, increasing hazard potential as well as increased expenses associated with its disposal. As such, there is a need for the development of "green" extraction procedures with water being the ideal solvent of choice. Importantly water is a safe, inexpensive, and environmentally friendly solvent and several studies have extracted polyphenols from eucalyptus leaf under aqueous conditions (Chapuis-Lardy et al., 2002; Hasegawa et al., 2008); however, no previous studies have optimised conditions for water extraction to yield maximal quantities of phenolic compounds from eucalyptus leaf, nor assessed the activity of *aqueous* extracts as potential anti-cancer agents.

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Optimization of ultrasound-assisted extraction conditions for euphol from the medicinal plant, *Euphorbia tirucalli*, using response surface methodology

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ABSTRACT

Euphol identified in *Euphorbia tirucalli* (*E. tirucalli*) has been linked with various health benefits. This study aimed to optimize ultrasonic extraction conditions for euphol from *E. tirucalli* leaf. Different solvents were tested to determine the most effective solvent for extraction of euphol. Then, response surface methodology (RSM) was employed to optimize ultrasound-assisted extraction conditions including temperature time and power for maximal extraction of euphol. Our results showed that ethyl acetate:ethanol (4:1 v/v) was the most effective solvent for the extraction of euphol. Ultrasonic temperature and time had a positive impact, whereas, ultrasonic power had a negative effect on the extraction efficiency of euphol. The optimum ultrasonic extraction conditions for euphol were identified as: solvent-to-fresh sample ratio of 100:32 mL/g; ultrasonic temperature of 60 °C; ultrasonic time of 75 min and ultrasonic power of 60% (150 W). Under these optimum conditions, approximately 4.06 mg of euphol could be obtained from one gram of fresh *E. tirucalli* leaf. This extract also contained phenolic compounds (2.5 mg GAE/g FW) and possessed potent antioxidant capacity. These optimal conditions are applicable for a larger scale to extract and isolate euphol for potential utilization in the pharmaceutical industry.

1. Introduction

Euphorbia tirucalli (*E. tirucalli*), also known as the pencil tree, sticks-on-fire or milk bush, is a small tree native to Madagascar and Africa; however, it has been widely distributed across the globe because of its tolerance to a wide range of climatic conditions (Mwine and Damme, 2011). *E. tirucalli* has been used as a traditional medicine in the Middle East, India, Africa and South America for the treatment of a range of ailments including syphilis, asthma, cancer, colic, intestinal parasites and leprosy (Cataluna and Rates 1997; Gupta et al., 2013).

The traditional use of *E. tirucalli* for purported health benefits has prompted scientific interest in the exploration of its bioactive constituents for pharmacological utilization. A range of di- and triterpene compounds has been identified in *E. tirucalli*. Of those identified, euphol (Fig. 1) is the most prominent and has been found to exhibit anti-cancer activity against human gastric cancer and breast cancer *in vitro* (Sadeghi-Aliabadi et al., 2009; Zhang et al., 2012). Consequently, optimization of conditions for maximal extraction of euphol and other bioactives from *E. tirucalli* is of interest and worthy of further investigation.

To date however, no formal studies in this area have been undertaken. Response Surface Methodology (RSM) is a statistical technique that aims to develop a functional relationship between a response of interest and a number of input variables (Khuri and Mukhopadhyay, 2010). In comparison with single variable optimization methods, RSM is a time and cost effective means of simultaneously evaluating the key experimental parameters

Abbreviations: RSM, response surface methodology; UAE, ultrasound-assisted extraction.

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Optimum aqueous extraction for preparation of phenolic-enrich extract and determination of its proliferation against selected cancer cell lines from Davidson' plum (*Davidsonia pruriens* F. Muell)

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Article

Phytochemical, Antioxidant and Anti-Cancer Properties of *Euphorbia tirucalli* Methanolic and Aqueous Extracts

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Abstract: *Euphorbia tirucalli* is a succulent shrub or small tree that is native to the African continent, however, it is widely cultivated across the globe due to its use in traditional medicines to treat ailments, ranging from scorpion stings to HIV. Recent studies have identified compounds present in the latex of the plant, including a range of bi- and triterpenoids that exhibit bioactivity, including anticancer activity. This study aimed to optimize water extraction conditions for high-yield total phenolic content recovery, to prepare methanol and aqueous extracts from the aerial sections of the plant, and to test the phytochemical, antioxidant, and anti-cancer properties of these extracts. Water extraction of total phenolic compounds (TPC) was optimized across a range of parameters including temperature, extraction time, and plant mass-to-solvent ratio. The water extract of the *E. tirucalli* powder was found to contain TPC of 34.01 mg GAE (gallic acid equivalents)/g, which was approximately half that of the methanol extract (77.33 mg GAE/g). The results of antioxidant assays showed a uniform trend, with the methanol extract's antioxidant reducing activity exceeding that of water extracts, typically by a factor of 2:1. Regression analysis of the antioxidant assays showed the strongest correlation between extract TPC and antioxidant

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Article

Optimisation of Ultrasound-Assisted Extraction Conditions for Phenolic Content and Antioxidant Capacity from *Euphorbia tirucalli* Using Response Surface Methodology

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Abstract: *Euphorbia tirucalli* (*E. tirucalli*) is now widely distributed around the world and is well known as a source of traditional medicine in many countries. This study aimed to utilise response surface methodology (RSM) to optimise ultrasonic-assisted extraction (UAE) conditions for total phenolic compounds (TPC) and antioxidant capacity from *E. tirucalli* leaf. The results showed that ultrasonic temperature, time and power effected TPC and antioxidant capacity; however, the effects varied. Ultrasonic power had the strongest influence on TPC; whereas ultrasonic temperature had the greatest impact on antioxidant capacity. Ultrasonic time had the least impact on both TPC and antioxidant capacity. The optimum UAE conditions were determined to be 50 °C, 90 min. and 200 W. Under these conditions, the *E. tirucalli* leaf extract yielded 2.93 mg GAE/g FW of TPC and exhibited potent antioxidant capacity. These conditions can be utilised for further isolation and purification of phenolic compounds from *E. tirucalli* leaf.

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Research Article Open Access

Phenolic Compounds, Antioxidant and Anti-Cancer Properties of the Australian Maroon Bush *Scaevola spinescens* (Goodeniaceae)

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Abstract

Scaevola spinescens (Goodeniaceae) has been traditionally used by indigenous Australians to treat various ailments including cancer, thus it is necessary to identify optimum extraction conditions for bioactive components from this plant. This study investigated the effects of different extraction conditions on Total Phenolic Content (TPC), antioxidant capacity (ABTS, DPPH, CUPRAC, FRAP assays) and anti-cancer activity (MTT assay) of S. spinescens. The results showed that optimal extraction conditions for TPC using water were 80°C, 15 min and ratio of 20:1 mL/g. However, the aqueous extract prepared under optimal conditions had lower TPC and less antioxidant capacity than those of the organic solvent extracts. The acetone extract displayed the greatest TPC as well as the highest antioxidant capacity and anti-cancer activity against a panel of cancer cell lines, including cancers of the pancreas, breast, lung, brain, skin, colon and ovary. Therefore, further investigations should be conducted to identify key bioactive compounds as potential anti-cancer agents.

Keywords: *Scaevola spinescens;* Maroon bush tea; Phenolic content; Antioxidant; Anti-cancer

Introduction

Scaevola spinescens (maroon bush, currant bush, or fanflower) belongs to Goodeniaceae family and is native to Australia. It has been traditionally used by the Aboriginal community for the treatment of various ailments such as colds, stomach-ache, urinary problems, boils, sores and rashes [1]. The first claim for the cancer curing capacity of this plant was reported in 1946 in Western Australia, where a patient claimed that he was cured following continued ingestion of an aqueous extract of the *S. spinescens* root bark combined with ashes of the desert poplar *Codonocarpus cotinifolius* [1]. Currently there has been limited research undertaken to elucidate the phytochemical profile and anti-cancer properties of *S. spinescens* [2].

The majority of bioactive compounds in plant materials are phenolic compounds [3]. The structural hydroxyl groups within the phenolic compounds results in the capability of these compounds in scavenging reactive oxygen species, such as the superoxide radical, singlet oxygen, hydroxyl radical, nitric oxide, nitrogen dioxide, and peroxynitrite, pertaining to their antioxidant and potential anti-cancer capacities [4,5]. Therefore, optimised extraction conditions for the maximum yield of phenolic compounds from *S. spinescens* is essential for ongoing assessment of potential biological and anti-cancer activity.

This study aimed to investigate the impact of extraction conditions on total phenolic content and antioxidant capacity from *S. spinescens*, and is the first to identify optimal conditions for the increased extraction yield of phenolic compounds from *S. spinescens*. In addition, the anti-cancer effects of *S. spinescens* extracts were tested against numerous cancer cell lines *in vitro*, providing strong preliminary evidence for further assessment of the putative anti-cancer activity of *S. spinescens*.

Materials and Methods

Plant material

Scaevola spinescens (Goodeniaceae) (SC) was donated by Jeanie Crago (Outback Books Australia; and commercial supplier of Maroon bush tea sold in ground-dried form). Dried and harvested SC was packed in PE bags and stored in freezer at -18°C until required.

Experimental design

SC has been traditionally prepared by brewing in water, with the decoction consumed like tea. The current study determined the impact of aqueous extraction conditions (including temperature, time and water-to-SC ratio) on extraction efficiency of Total Phenolic Compounds (TPC), and optimized these extraction conditions to maximize the extraction yield of TPC. In addition, three organic solvents of high to modest polarity were used to extract TPC. SC was extracted in acetone, methanol or ethanol at the ratio of 20:1 mL/g and agitated at room temperature (RT) for 72 h. The solutions were then filtered twice using 11 µm medium filter paper (Lomb Scientific, Taren Point, NSW, Australia), and concentrated under reduced pressure at 45°C (Buchi Rotavapor B-480, Buchi Australia, Noble Park, Vic., Australia). The concentrated extract was then dried using a FD3 freeze drier (Thomas Australia Pty. Ltd., Seven Hills, NSW, Australia) at -40°C for 48 h and the resulting crude powdered extracts then stored at -18°C until required. The extraction scheme is illustrated in Figure 1.

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Original Research Article

Physicochemical composition, antioxidant and anti-proliferative capacity of a lilly pilly (*Syzygium paniculatum*) extract

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ABSTRACT

Lilly pilly (LP) fruit (*Syzygium paniculatum* Gaertn.) is widely grown in eastern Australia and has been used as food by indigenous Australians. However, there is limited information on its bioactivity. This study investigated the physicochemical and antioxidant properties of the crude fruit extract, identified its bioactive compounds and also assessed its potential anti-proliferative effect on pancreatic cancer cells. Our data showed that the LP extract was water-soluble and possessed a total phenolic content of 96 mg of gallic acid equivalents (GAE)/g, flavonoid levels of 52 mg catechin equivalents (CAE)/g, proanthocyanidin levels of 29 mg CAE/g. Several phenolic compounds such as gallic acid, chlorogenic acid, catechin and epicatechin were identified in the LP extract with levels of 0.39, 2.35, 0.47 and 2.9 mg/g, respectively. Results from six different antioxidant assays revealed that the LP extract pocessed potent antioxidant and free radical scavenging capacity. Although antioxidant capacity of the extract was lower than that of vitamin E, vitamin C and BHT, it could be significantly improved if the extract was to be further purified. We also showed that the LP extract (200 μg/mL) significantly reduced the viability of MiaPaCa-2 and ASPC-1 pancreatic cancer cells to levels comparable to that of the chemotherapeutic agent gemcitabine. For this reason lilly pilly should be further investigated for its health promoting and potential anti-cancer benefits, particularly for pancreatic cancer.

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Original article

Antioxidant and anticancer capacity of saponin-enriched *Carica papaya* leaf extracts

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Summary The papaya (*Carica papaya*) leaf (PL) contains high levels of saponins and polyphenolic compounds, and historically, it has been used as a folk medicine for numerous ailments, including cancer. PL is traditionally prepared by hot water extraction; however, optimised extraction conditions have not been assessed. This study optimised conditions for the extraction of saponins from PL and assessed their antioxidant capacity and antipancreatic cancer activity. Optimisation was achieved using response surface methodology. Saponins and total phenolic compounds were assessed for their antioxidant, free radical scavenging, ion-reducing capacity, and antipancreatic cancer activity. Optimal aqueous extraction conditions were 85 °C, 25 min. and a water-to-leaf ratio of 20:1 mL g^{-1}. Ethanol extracts demonstrated higher antioxidant, free radical scavenging and ion-reducing capacity, as well as antipancreatic cancer activity. This study revealed that the PL contains numerous bioactive compounds, with significant anticancer activity warranting further studies on the isolation and characterisation of individual bioactive compounds from the PL.

Keywords Antioxidant, *Carica papaya* leaf, pancreatic cancer, saponins.

Introduction

In many parts of the world, especially in remote areas of Asian countries, *Carica papaya* L. (papaya or paw paw) leaf has been used as a folk medicine for a variety of ailments such as healing of burns, relief of asthma symptoms, treatment of intestinal worms, treatment of digestion problems, fever control and treatment of amoebic dysentery (Starley *et al.*, 1999; Canini *et al.*, 2007; Zunjar *et al.*, 2011). Papaya leaf has also been used to increase appetite, ease menstrual pain and relieve nausea (Aravind *et al.*, 2013). Furthermore, papaya leaf juice has been consumed by people living on the Gold Coast of Australia, with some anecdotes of successful cases being reported for its purported anticancer activity (Otsuki *et al.*, 2010). Additionally, the tender leaf has been consumed as an alternative to traditional leafy vegetables and as an additive to tenderise meat (Aravind *et al.*, 2013).

Recent scientific reports suggest that papaya leaf extract and its latex can be utilised to treat skin lesions (Mahmood *et al.*, 2005; Gurung & Škalko-Basnet, 2009), lower the risk of cardiovascular disease (Runnie *et al.*, 2004), act as an anti-inflammatory (Owoyele *et al.*, 2008) and an anthelmintic against intestinal nematode (Satrija *et al.*, 1995). A recent study (Otsuki *et al.*, 2010) found that papaya leaf extract could prevent growth of cancer cells, including pancreatic cancer – one of the most devastating forms of cancer (Scarlett *et al.*, 2011). This result suggests that papaya leaf may contain compounds that limit the proliferation of pancreatic cancer cells. However, because the study investigated only one pancreatic epithelioid carcinoma cell line (Panc-1), further study on other types of pancreatic cancer cells is required to substantiate this claim.

We recently revealed that the papaya leaf not only contained phenolic compounds but it also had a

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Review

Fruit-derived phenolic compounds and pancreatic cancer: Perspectives from Australian native fruits

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ABSTRACT

Ethnopharmacological relevance: Pancreatic cancer is a devastating cancer that presents late, is rapidly progressive and has current therapeutics with only limited efficacy. Bioactive compounds are ubiquitously present in fruits and numerous studies *in vitro* are addressing the activity of these compounds against pancreatic cancer, thus studies of specific bioactive compounds could lead to new anti-pancreatic cancer strategies. Australian native fruits have been used as foods and medicines by Australian Aboriginals for thousands of years, and preliminary studies have found these fruits to contain rich and diversified bioactive components with high antioxidant activity. Thus, Australian native fruits may possess key components for preventing or delaying the onset of tumorigenesis, or for the treatment of existing cancers, including pancreatic cancer.
Materials and methods: Numerous databases including PubMed, SciFinder, Web of Knowledge, Scopus, and Sciencedirect were analysed for correlations between bioactive components from fruits and pancreatic cancer, as well as studies concerning Australian native fruits.
Results: In this review, we comprehensively highlight the proposed mechanisms of action of fruit bioactives as anti-cancer agents, update the potential anti-pancreatic cancer activity of various major classes of bioactive compounds derived from fruits, and discuss the existence of bioactive compounds identified from a selection Australian native fruits for future studies.
Conclusion: Bioactive compounds derived from fruits possess the potential for the discovery of new anti-pancreatic cancer strategies. Further, Australian native fruits are rich in polyphenols including some flora that contain unique phenolic compounds, thereby warranting further investigations into their anti-cancer properties.

Contents

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Fate of the phenolic compounds during olive oil production with the traditional press method

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Abstract

In the traditional press method for olive oil production, olives are crushed and malaxed into a paste, which is spread on mats. Pressure is applied to squeeze out the oil and wastewater, leaving a material on the mats called pomace. The oil and wastewater are then separated by gravity. The fate of the olive phenolic compounds, including oleuropein, and antioxidant activity was investigated at each stage of the process and the waste products (pomace and wastewater) were evaluated as potential sources of valuable phenolic compounds and antioxidant activity. The largest loss of phenolic compounds was seen at the crushing stage (60% of phenolic compounds, 70% of oleuropein) but only 21% of antioxidant activity was lost. Malaxation did not cause significant losses of phenolic compounds but the antioxidant activity was affected (43% loss). Pomace retained 26% of the phenolic compounds, 21% of the oleuropein and 33% of the antioxidant activity. When dried, the phenolic compounds and oleuropein were 3.5-fold concentrated in the wastewater and it exhibited a 2.7-fold increase in antioxidant activity compared to whole olives. The olive waste products from the traditional press method, pomace and wastewater, are good sources of valuable phenolic compounds and antioxidant activity.

Introduction

Adherence to a Mediterranean diet and consumption of olive oil has been associated with a number of health benefits including a reduced risk of morbidity and mortality (Cicerale *et al.*, 2009), particularly by reducing the risk of cardiovascular disease (de Lorgeril *et al*., 1999), atherosclerosis (Visioli *et al*., 2000) and certain types of cancer (Kapiszewska *et al*., 2005). The Mediterranean diet is characterized by a high consumption of fruits, vegetables, fish, legumes and whole grains. However, fat consumption is also high; it accounts for approximately 40% of caloric intake, the main source of which is olive oil (Stark and Madar, 2002).

Historically, the healthful properties of olive oil have been attributed to its high proportion of monounsaturated fatty acids (MUFAs), in particular oleic acid which represents 70-80% of the total fatty acids present in virgin olive oil (Cicerale *et al*., 2009). In addition to MUFAs, virgin olive oil contains a minor yet significant phenolic compound fraction, which has garnered much interest in relation to the health promoting properties of olive oil (Cicerale *et al*., 2009).

Olive oil extraction aims to separate the liquid oil phase from the other constituents of the fruit. Currently, commercial olive oil production is carried out using both continuous (centrifugation) and batch (traditional press) approaches. However, centrifugal systems face larger waste disposal issues and produce oils, which can be of lower quality especially in terms of phenolic compound content (Di Giovacchino *et al*., 2002; Issaoui *et al*., 2009; Torres and Maestri, 2005).

The traditional press method works by first grinding the olives in a hammer mill followed by malaxing the pulp into a paste, which is then spread on spherical mats before pressure is applied using a hydraulic piston press to squeeze the oil and the water from the paste and leaving a solid material on the mats referred to as pomace. The oil and water phases are then separated by gravity and collected by decantation. Therefore, the traditional press method produces three fractions, olive oil plus large amounts of two waste products – a relatively dry and solid pomace and wastewater (Jerman Klen and Mozetic Vodopivec, 2012).

The wastes, especially the pomace, possess high amounts of organic substances (14-15%) including sugars, nitrogenous compounds, volatile fatty acids, polyalcohols, pectins and fats (Lafka *et al*., 2011) and high concentrations of phenolic compounds (up to 10 g L^{-1}) (Ranalli *et al*., 2003). Therefore, disposal of these waste products has been a major environmental issue in a number of olive growing countries (Capasso

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Conference Abstracts

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Utilization of industrial food waste: Olive pomace

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Olive oil production has grown rapidly in Australia in recent years. However, alongside this growing industry, is a rising waste problem. Olive pomace is the waste product of the olive oil extraction process; this waste is currently dumped in landfill which is causing a number of environmental concerns due to the high concentration of volatile fatty acids and phenolic compounds. Most of the phenolic compounds present in olives are highly water solubility and favour partitioning into the aqueous waste products. In fact, only 1-2% of the phenolic compounds originally in olives, end up in the olive oil. A number of phenolic compounds found in olives, including oleueopein and hydroxytyrosol, have applications in the food and health industries. Moreover, these compounds are very expensive. Therefore, by extracting the phenolic fraction of this waste it could limit its environmental impact while creating an additional source of income for farmers and producers. The aim of the present study was to develop a method for the aqueous extraction of phenolic compounds from olive pomace. Response Surface Methodology (RSM) was used to determine the optimal combination for parameters time, ultrasound power and sample to solvent ratio. Total phenolic compounds (Folin Ciocalteu and HPLC) were investigated. The optimal conditions for the extraction of phenolic compounds were 2 g of dried pomace/ 100mL of water at 250W power for 75mins. The proposed method yielded extracts with high levels of phenolic compounds.

American Association of Cancer Research. Washington DC, The United States of America (April, 2017).

The olive biophenol Oleuropein and its degradation product Hydroxytyrosol induce apoptosis in pancreatic cancer cells *in vitro.*

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Pancreatic cancer is a devastating disease with a 5-year survival rate of less than 5%. Resistance to conventional treatment options and toxicity of current chemotherapy agents (gemcitabine) makes pancreatic cancer a target for the development of novel therapeutic agents. Oleuropein and Hydroxytyrosol are the most abundant biophenols found in olive products; both compounds have anti-atherogenic and anti-inflammatory properties as well as anti-cancer activity in cancers of the breast, colon and prostate. However, there has yet to be any investigation into the effects of Oleuropein or Hydroxytyrosol on pancreatic cancer. Consequently, this study aimed to assess the anti-pancreatic cancer activity of Oleuropein and Hydroxytyrosol *in vitro.* Two cell lines were investigated; a pancreatic cancer cell line (MiaPaCa-2) and a normal pancreas cell line (HPDE). The viability of cells after treatment with 0-200μM of Oleuropein or Hydoxytyrosol was assessed using the Dojindo CCK-8 viability assay and compared to gemcitabine. The induction of apoptosis was measured by way of caspase 3/7 activation using a MUSE flow cell analyser. Cell cycle analysis was conducted using the MUSE flow cell analyser. RNA expression was assessed using Affymetrix GeneChip® Whole Transcript (WT) expression arrays. The IC_{50} values of Oleuropein and Hydroxytyrosol against MiaPaCa-2 cells were 148μM and 75μM respectively. However, importantly, Oleuropein did not decrease the viability of HPDE cells within the treatment range. Moreover, 20nM of gemcitabine reduced the viability of MiaPaCa-2 cells to 21% and HPDE cells to 2%. An increase in the expression of caspase 3/7 was seen in MiaPaCa-2 cells when treated with Oleuropein and Hydroxytyrosol, but no effect on the HPDE cells was observed when treated with Oleuropein. When treated with Oleuropein, an increase in the expression of genes involved in the NRF-2 (oxidative stress) pathway was observed in MiaPaCa-2 cells, an effect not observed in HPDE cells.

Conclusion

Oleuropein and Hydroxytyrosol induced apoptosis in pancreatic cancer cells (MiaPaCa-2), with Oleuropein appearing non-toxic to normal pancreas cells (HPDE) within the treatment ranges; this is significant, since gemcitabine was significantly more toxic to HPDE cells. Furthermore, the link between Oleuropein and the NRF-2 pathway in MiaPaCa-2 cells justifies further study into the mechanisms of action of Oleuropein and its potential as a novel therapeutic approach to pancreatic cancer.

Hunter Cancer Research Association, Newcastle, Australia (November, 2016).

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The olive phenolic compounds apigenin, luteolin and oleuropein induce cell cycle arrest and apoptosis in pancreatic cancer cells *in vitro*

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Background

Pancreatic cancer is a devastating disease with a 5-year survival rate of less than 5%. The heterogeneity of the disease, resistance to conventional treatment options and toxicity of current chemotherapy agents (FOLFIRINOX, gemcitabine) makes pancreatic cancer an important target for the development of novel therapeutic agents. Individual compounds isolated from olive products have been investigated extensively for their anti-cancer activity in cancers of the breast, colon, prostate and leukaemia however there is limited research into their effects against pancreatic cancer.

Aims

This study aimed to assess the anti-pancreatic cancer potential of individual olive phenolic compounds.

Methods

Pancreatic cancer (BxPC-3, CFPAC-1, MiaPaCa-2), and normal human pancreatic ductal epithelial (HPDE) cells were treated with oleuropein, hydroxytyrosol, myrictin, luteolin and apigenin. Cell viability was assessed using the CCK-8 viability assay. The induction of apoptosis was assessed by way of caspase 3/7 activation and cell cycle analysis was performed using a MUSE flow cell analyser.

Results

Most notable results include the IC_{50} values for luteolin and apigenin on BxPC-3 cells (10 and 12μM respectively) and CFPAC-1 cells (22 and 25 μM respectively). Apigenin also induced G2-phase arrest in both CFPAC-1 and BxPC-3 cells. Interestingly, MiaPaCa-2

Australian Pancreatic Club Annual Scientific meeting, Sydney (November, 2016).

The olive biophenol Oleuropein and its degradation product Hydroxytyrosol induce apoptosis in pancreatic cancer cells *in vitro.*

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Pancreatic cancer is a devastating disease with a 5-year survival rate of less than 5%. Resistance to conventional treatment options and toxicity of current chemotherapy agents (gemcitabine) makes pancreatic cancer a target for the development of novel therapeutic agents. Oleuropein and Hydroxytyrosol are the most abundant biophenols found in olive products; both compounds have anti-atherogenic and anti-inflammatory properties as well as anti-cancer activity in cancers of the breast, colon and prostate. However, there has yet to be any investigation into the effects of Oleuropein or Hydroxytyrosol on pancreatic cancer. Consequently, this study aimed to assess the anti-pancreatic cancer activity of Oleuropein and Hydroxytyrosol *in vitro.* Two cell lines were investigated; a pancreatic cancer cell line (MiaPaCa-2) and a normal pancreas cell line (HPDE). The viability of cells after treatment with 0-200μM of Oleuropein or Hydoxytyrosol was assessed using the Dojindo CCK-8 viability assay and compared to gemcitabine. The induction of apoptosis was measured by way of caspase 3/7 activation using a MUSE flow cell analyser. Cell cycle analysis was conducted using the MUSE flow cell analyser. RNA expression was assessed using Affymetrix GeneChip® Whole Transcript (WT) expression arrays. The IC_{50} values of Oleuropein and Hydroxytyrosol against MiaPaCa-2 cells were 148μM and 75μM respectively. However, importantly, Oleuropein did not decrease the viability of HPDE cells within the treatment range. Moreover, 20nM of gemcitabine reduced the viability of MiaPaCa-2 cells to 21% and HPDE cells to 2%. An increase in the expression of caspase 3/7 was seen in MiaPaCa-2 cells when treated with Oleuropein and Hydroxytyrosol, but no effect on the HPDE cells was observed when treated with Oleuropein. When treated with Oleuropein, an increase in the expression of genes involved in the NRF-2 (oxidative stress) pathway was observed in MiaPaCa-2 cells, an effect not observed in HPDE cells.

Conclusion

Oleuropein and Hydroxytyrosol induced apoptosis in pancreatic cancer cells (MiaPaCa-2), with Oleuropein appearing non-toxic to normal pancreas cells (HPDE) within the treatment ranges; this is significant, since gemcitabine was significantly more toxic to HPDE cells. Furthermore, the link between Oleuropein and the NRF-2 pathway in MiaPaCa-2 cells justifies further study into the mechanisms of action of Oleuropein and its potential as a novel therapeutic approach to pancreatic cancer.

10th World Congress on Polyphenols Applications. Porto, Portugal (June, 2016).

THE OLIVE BIOPHENOL 'OLEUROPEIN' DISPLAYS SELECTIVE TOXICITY TOWARD PANCREATIC CANCER CELLS

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Introduction: Pancreatic cancer is a devastating disease with a 5-year survival rate of less than 5%. Resistance to conventional treatment options and toxicity of current chemotherapy agents (gemcitabine) makes pancreatic cancer an important target for the development of novel therapeutic agents. Oleuropein is the most abundant biophenol found in olive products which has anti-cancer activity in cancers of the breast, colon and prostate. However, there has yet to be any investigation into the effects of oleuropein against pancreatic cancer.

Aim: This study aimed to assess the anti-pancreatic cancer activity of oleuropein against pancreatic cells *in vitro.*

Methods: The viability of cells was assessed using CCK-8 viability assay, with results compared to the standard chemotherapeutic agent gemcitabine (20nM). The induction of apoptosis was measured by way of Caspase 3/7 activation using a MUSE flow cell analyser.

Results: Treatment of MiaPaCa-2 cells with 200 μM of oleuropein resulted in viability of 4%, while no effect was observed for HPDE cells at this concentration. At 20nM gemcitabine, the viability of MiaPaCa-2 cells was 21% with HPDE cell viability reduced to 2%. Moreover, after treatment of MiaPaCa-2 cells with 150μM of oleuropein, a significant induction of apoptosis, as measured by increased activation of caspase 3/7, was observed (17% to 79%).

Conclusions: Oleuropein induced apoptosis in pancreatic cancer cells (MiaPaCa-2), however, was non-toxic to normal pancreas cells (HPDE) at the same concentrations. As the chemotherapeutic agent gemcitabine is extremely toxic to HPDE cells, further investigation into oleuropein is warranted.

Hunter Cancer Research Association, Newcastle, Australia (November, 2015).

Anti-proliferative capacity of oleuropein rich olive leaf extracts against pancreatic cancer cells.

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Background: Olive leaves are an agricultural waste product with a high concentration of phenolic compounds namely oleuropein. Oleuropein has been shown to exhibit anti-proliferative activity against a number of different cancer types. Pancreatic cancer is the 5th leading cause of cancer related death in western countries. Resistance to conventional treatment options and toxicity of current chemotherapy agents (gemcitabine) makes pancreatic cancer a target for the development of novel therapeutic agents.

Aims: Therefore, in the present study, olive leaf phenolic compounds were investigated for their anti-proliferative activity against pancreatic cancer cells.

Methods: The oleuropein content of two varieties of olive leaf extracts prepared from 3 different extraction protocols have been elucidated and their anti-proliferative capacity against primary pancreatic cancer cells (MiaPaCa-2) and normal pancreas cells (HPDE).

Results: Despite differences in the oleuropein content of the different olive leaf extracts, there was no difference between their anti-proliferative capacity ($p>0.05$). Furthermore, all extracts (200µg/mL) significantly decreased the viability of pancreatic cancer cells (MiaPaCa-2) compared to gemcitabine ($p<0.05$). However, there was no difference in the toxicity of the extracts towards the normal pancreas cells (HPDE) compared to gemcitabine ($p>0.05$).

Conclusions: Olive leaf phenolic compounds warrant further investigation into their potential anti-pancreatic cancer activity.

Translational research aspect: This research fits into T1 of the translational pipeline.

International Symposium on Phytochemicals in Medicine and Food. Shanghai, China (June, 2015).

Phytochemical compounds from *Olea Europaea.* leaf extracts have anti-pancreatic cancer activity

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ABSTRACT

Pancreatic cancer is the 5th leading cause of cancer related death in western countries. Resistance to conventional treatment options and toxicity of current chemotherapy agents (e.g. gemcitabine) makes pancreatic cancer a target for the development of novel therapeutic agents. Oleuropein is the most abundant phytochemical compound found in olive leaf products, which has purported anti-cancer, anti-atherogenic and anti-inflammatory properties. Most of the literature regarding olive products has been focused on this compound; however, there are as yet no investigations reporting on olive leaf phytochemicals and pancreatic cancer.

Aims: To analyse olive leaf extracts for their phytochemical properties (including oleuropein content) and assess their anti-pancreatic cancer activity.

Methods: Extracts were prepared with previously optimised methods using water, ethanol or methanol. Phenolic compounds in the extracts were measured with the Folin Ciocalteu's total phenolic compound (TPC) assay and HPLC while the antioxidant capacity was assessed using the FRAP, DPPH and CUPRAC assays. The CCK-8 viability assay was used to assess the cell viability of pancreas cells after treatment with olive leaf extracts compared to controls.

Results: The methanol and ethanol extracts contained higher levels of oleuropein than the water extracts ($p<0.05$). However, there was no difference in the levels of TPC or antioxidant activity in the organic solvent extracts compared to the water extracts ($p>0.05$). At 100 and 200 µg/mL, all leaf extracts significantly decreased ($p<0.05$) cell viability of the pancreatic cancer cells compared to control. However, at 50 µg/mL, the water extract exhibited the highest anti-proliferative activity ($p>0.05$) but the effect could not be explained by the phytochemical properties.

Conclusion: Water represents a potentially viable alternative to organic solvents for the extraction of biologically active phenolic compounds from olive leaves. Furthermore, olive leaf compounds possess anti-pancreatic cancer activity and additional investigations are needed to identify these biologically active compounds.

8th World Congress on Polyphenol Applications. Lisbon, Portugal (June, 2014).

OPTIMISATION OF THE EXTRACTION OF PHENOLIC COMPOUNDS FROM OLIVE LEAVES USING RESPONSE SURFACE METHODOLOGY

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Introduction: Olive leaves are agricultural residue resulting from the pruning of olive trees. They also account for approximately 10% of the total weight of olives arriving at the olive mill. Olive leaves have been shown to have a high concentration of phenolic compounds. In the present study, we optimised the extraction of phenolic compounds from this agricultural and industrial waste; olive tree leaves (*Olea europaea*).

Objectives: To investigate various extraction parameters, including: solvent to sample ratio, time and temperature, to obtain the highest total phenolic yield through response surface methodology (RSM).

Methodology: Preliminary experiments were conducted to obtain optimal rages for the parameters to be investigated. These were temperature 70-90°C, time 50-70mins and sample to solvent ratio 1:10-1:100. These parameters were assessed using a Box Behnken RSM analysis. A total of 15 runs with different combinations of optimisation parameters were performed and then the final optimised conditions were validated. Total phenolic compounds were measured using Folin Ciocalteu's total phenolic compound assay. Results were expressed as mg Gallic acid equivalents/g of dried sample (mg GAE/g).

Results: The optimal conditions for the highest yield of phenolic compounds was obtained on a shaking water bath at 90°C, for 70mins at sample to solvent ratio 1:100 (40.92 ± 9.50mg GAE/g). However, at a sample-to-solvent ratio of 1:60 we retained 80% of the total phenolic compounds (32.41 ± 8.47mg GAE/g). This retention was considered satisfactory and was used for further validation. The validation run fell inside the confidence range indicated by the RSM output hence the statistical model was trusted (32.43 ± 2.06mg GAE/g).

Conclusion: The optimal conditions for the aqueous extraction of phenolic compounds from olive leaves were 90°C, for 70mins at sample to solvent ratio 1:60. Using olive leaves as a starting material for the extraction of phenolic compounds via this simple, inexpensive method constitutes a viable use for this agricultural waste product, potentiating an additional source of income for olive growers.