Phytochemicals derived from Australian eucalypts as anticancer agents for pancreatic malignancies

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

January 2018

School of Environmental and Life Sciences

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Statement of Originality

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Acknowledgements

This project could not have been completed without the help and support of a number of people. First of all, I would like to acknowledge my supervisor **Associate Professor Christopher Scarlett** for giving me the opportunity to undertake this exciting and challenging project, for his invaluable guidance, insightful feedback, motivation, continual support and endless patience throughout the course of this work. I would like to express my sincerest gratitude to my co-supervisors **Associate Professor Michael Bowyer** and **Dr Ian A. van Altena** and my advisory team- **Dr Quan Vuong, Dr Anita Chalmers, Dr Danielle Bond** and **Dr Jennette Sakoff** for sharing their knowledge, insights and technical expertise during my research. I would also like to thank Quan for his constant guidance, support and encouragement especially in the initial stages of my PhD when I was still new to natural product chemistry. Also, special thanks to Chris and Mike for putting up with my naive questions every now and then.

I wish to express my sincerest gratitude to Professor Brad Potts and Dr Paul Tilyard from the School of Biological Science, University of Tasmania, Australia for providing the *Eucalyptus globulus* sample and their valuable comments. I would also like to acknowledge all the members of the Pancreatic Cancer Research Group for their friendship, advice and help in so many ways throughout my PhD; Tang and Thanh for their technical assistance in phytochemical analysis of my samples during my initial days, Dani, Mel, Ben, Josh, Helen and Chloe for their help in cell culture lab. A big thank you goes to Mrs Katy Marrotte, Mrs Merrilee Pettit, Mrs Leonie Holmesby and Mrs Kiran Sharma for their technical support in the labs. I would like to thank all my colleagues and friends from the School of Environmental and Life Sciences for making it an enjoyable and productive workplace to go to every day. Thank you, Bahar, Hang, Hebah, Rahul, Dharmesh, Vahid, Thida, Dipankar, Adelle, Thuy, Tang, Thanh, Kostas, Stella and Quynh for all those parties, BBQs, dinners, lunches, day-outs and night-outs and making my PhD a truly enjoyable international experience as well as helping me maintain a perfect work-life balance. I would also like to thank my friends Rose, Dani, Meenal, Ivan, Adam (and his family), Lauren, Chris and Dean for introducing me to the true-blue Australian culture of friendship. Special thanks

to Rose for listening to me patiently every day about how my day went as well as my problems, successes and failures. I feel very honoured to have made so many lifelong friends during my time in Australia. I will forever be grateful to Cherie, my “Aussie Grand Mother” for her love, care and support throughout my days in Australia and introducing me to the nitty-gritty of Australian culture and lifestyle. Special thanks to Jo for being a caring landlady and making my stay at Ourimbah comfortable in every possible way. I am thankful to Mrs Nicole Day, Mrs Pam Steenkamp, and Mrs Fiona Adams for their administrative support in organising my conference travels, chemical/labware orders and HDR funds. A big thank you to Karen O'Neill for teaching Microbiology, Food safety and Immunology lab classes with me. I will always treasure these moments. I am indebted to my supervisor Chris for giving me the opportunity to deliver lectures and conduct laboratory classes in food microbiology for BSc FSHN students and helping me discover my passion for teaching. I am also extremely thankful to Dr Emma Beckett for spreading the word about my research on the ABC radio, Australia.

I would also like to acknowledge The University of Newcastle for providing the International Postgraduate Research Scholarship and Central Postgraduate Research Scholarship to accomplish my dream of pursuing PhD research in Australia as well as for supporting my attendance at numerous national and international conferences through conference grants and HDR funds. My sincerest gratitude to the Hunter Medical Research Institute, the Hunter Cancer Research Alliance and the Ramaciotti Foundation for their financial assistance in making my PhD research possible.

Last but not the least, I would like to thank my parents, brother, friends including Amrita, Manoj, Disha, Ghanashyam, Sumith, Sunti da and his family, Rinku, Sunil, Reema, Rineesha and Neha as well as my previous supervisors- Professor Mridula Saikia Barooah and Dr Jayshree Das for believing in me. Special thanks to Professor Barooah and Dr Das for their numerous recommendation letters to support my previous PhD applications. Maa and Papa, thank you so much for being there for me always, for your love, sacrifice and support in whatever path in life I

choose to follow whether personally and professionally. Thank you, Maa for taking care of Papa's health and taking all the responsibilities single-handedly when I am away.

This thesis is dedicated to my parents, brother, friends and the most supportive team of people who believed in me and my dreams. Maa and Papa, without you I could not have accomplished this.

Thank you.

List of publications included as part of the thesis

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Major research and review papers either published or under review

1. **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Michael C. Bowyer, Christopher J. Scarlett. An array of bioactive compounds from Australian eucalypts and their relevance in pancreatic cancer therapeutics (Review article). In Press **Pancreas (2018).**
2. **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. Microwave-assisted extraction of *Eucalyptus robusta* leaf for the optimal yield of total phenolic compounds. **Industrial Crops and Products (2015),** 69: 1–10. DOI: 10.1016/j.indcrop.2015.02.044
3. **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. Development of the ultrasonic conditions as an advanced technique for extraction of phenolic compounds from *Eucalyptus robusta*. **Separation Science and Technology (2016),** 52(1): 100-112.
   DOI: 10.1080/01496395.2016.1250777
4. **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. Investigation of phytochemicals and antioxidant capacity of selected *Eucalyptus* species using conventional extraction. **Chemical Papers (2015)**, 70(5) 567–575. DOI: 10.1515/chempap-2015-0237
5. **Deep Jyoti Bhuyan**, Quan V. Vuong, Danielle Bond, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. Exploring the least studied Australian Eucalypt genera: Corymbia and Angophora for phytochemicals with anticancer activity against pancreatic malignancies. **Chemistry & Biodiversity (2017).** DOI: 10.1002/ cbdv.201600291

6. **Deep Jyoti Bhuyan**, Jennette Sakoff, Danielle Bond, Melanie Predebon, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. *In vitro* anticancer properties of selected *Eucalyptus* species. ***In vitro* Cellular & Developmental Biology – Animal (2017).** DOI: 10.1007/s11626-017-0149-y
7. **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, and Christopher J. Scarlett. Phytochemical, antibacterial and antifungal properties of an aqueous extract of *Eucalyptus microcorys* leaves. **South African Journal of Botany (2017),** 112: 180-185. DOI: 10.1016/j.sajb.2017.05.030
8. **Deep Jyoti Bhuyan**, Quan V. Vuong, Danielle R. Bond, Anita C. Chalmers, Michael C. Bowyer, Christopher J. Scarlett. *Eucalyptus microcorys* leaf extract derived HPLC-fraction reduces viability of MIA PaCa-2 cells by inducing apoptosis and arresting cell cycle. Review completed **Biomedicine and Pharmacotherapy (2018).**

Statement of contribution of others

18th January 2018

To Whom It May Concern

This is to confirm that Deep Jyoti Bhuyan has contributed to the series of major research papers, which are submitted as a part of his PhD thesis as shown in each paper below.

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Publications included in the thesis

1. **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Michael C. Bowyer, Christopher J. Scarlett. An array of bioactive compounds from Australian eucalypts and their relevance in pancreatic cancer therapeutics (Review article). In Press **Pancreas (2018).**

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2. **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. Microwave-assisted extraction of *Eucalyptus robusta* leaf for the optimal yield of total phenolic compounds. **Industrial Crops and Products (2015),** 69: 1–10. DOI: 10.1016/j.indcrop.2015.02.044

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Christopher J. Scarlett	Wrote the manuscript (5%) Critically reviewed the manuscript (20%)

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Contributor	**Statement of Contribution**
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8. **Deep Jyoti Bhuyan**, Quan V. Vuong, Danielle R. Bond, Anita C. Chalmers, Michael C. Bowyer, Christopher J. Scarlett. *Eucalyptus microcorys* leaf extract derived HPLC-fraction reduces viability of MIA PaCa-2 cells by inducing apoptosis and arresting cell cycle. Review completed **Biomedicine and Pharmacotherapy (2018).**

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Other publications related to the thesis

Book Chapter

1. **Deep Jyoti Bhuyan** and Amrita Basu. Phenolic compounds: Potential health benefits and toxicity. In: Utilization of bioactive compounds from agricultural and food waste. Edited by Quan V. Vuong. ISBN: 9781498741316. **CRC Press, Taylor & Francis Group (2017).**

Additional research papers in peer-reviewed journals

1. Quan V. Vuong, Chloe D. Goldsmith, Trung Thanh Dang, Van Tang Nguyen, **Deep Jyoti Bhuyan**, Elham Sadeqzadeh, Christopher J Scarlett, Michael C Bowyer: Optimisation of Ultrasound-Assisted Extraction Conditions for Phenolic Content and Antioxidant Capacity from *Euphorbia tirucalli* Using Response Surface Methodology. **Antioxidants (2014),** 3(3): 604-617. DOI: 10.3390/antiox3030604
2. Quan V. Vuong, Van Tang Nguyen, Thanh Trung Dang, **Deep Jyoti Bhuyan**, Chloe D. Goldsmith, Elham Sadeqzadeh, Christopher J. Scarlett, Michael C. Bowyer. Optimization of ultrasound-assisted extraction conditions for euphol from the medicinal plant, *Euphorbia tirucalli*, using response surface methodology. **Industrial Crops and Products (2015),** 63: 197–202. DOI: 10.1016/j.indcrop.2014.09.057
3. Quan V. Vuong, Anita C. Chalmers, **Deep Jyoti Bhuyan**, Michael C. Bowyer, Christopher J. Scarlett. Botanical, Phytochemical, and Anticancer Properties of the *Eucalyptus* Species. **Chemistry & Biodiversity (2015),** 12(6): 907–924. DOI: 10.1002/cbdv.201400327
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5. Thanh Trung Dang, **Deep Jyoti Bhuyan**, Danielle R. Bond, Michael C. Bowyer, Ian A. Van Altena & Christopher J. Scarlett. Fucoxanthin content, isolation and cytotoxic

activity against pancreatic cancer from brown alga *Hormosira banksii* (Turner) Decaisne. Under review **Algal Research (2017).**

Conference oral presentations / posters / proceedings

1. Elham Sadeqzadeh, Quan V. Vuong, Chloe D. Goldsmith, Van Tang Nguyen, **Deep J. Bhuyan**, Trung Thanh Dang, Anita C. Chalmers, Ian A. van Altena, Troy F. Gaston, Natalie Moltschaniwskyj, Michael C. Bowyer, Rick F. Thorne, Judith Weidenhofer, Phuong Thien Thuong, Nguyen Minh Khoi, Christopher J Scarlett. A natural product drug discovery pipeline for novel pancreatic cancer therapies: A new cancer research hub for the hunter region of NSW. Hunter Cancer Research Alliance Annual Symposium, NSW, Australia 12/2014. **Asia-Pacific Journal of Clinical Oncology (2014),** 10: 18-18. DOI: 10.1111/ajco.12335/full
2. **Deep Jyoti Bhuyan,** Quan Van Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. Optimisation of microwave-assisted extraction parameters for total phenolic content from *Eucalyptus robusta* using response surface methodology. International Journal of Food Science and Technology Conference, Lincoln, New Zealand 02/2015. Oral presentation.
3. Danielle Bond, Alexandra Turner, Rebecca Richmond, Elham Sadeqzadeh, Quan Vuong, **Deep Bhuyan**, Yusnita Rifai, Anita Chalmers, Ian van Altena, Troy Gaston, Michael Bowyer, Joshua Brzozowski, Helen Jankowski, Judith Weidenhofer, Jennette Sakoff, Phuong Thien Thuong, Do Thi Ha, Nguyen Minh Khoi, Christopher Scarlett. The search for novel treatment agents for pancreatic cancer: Tales from the land and sea. Hunter Cancer Research Alliance Annual Symposium, NSW, Australia 12/2015. **Asia-Pacific Journal of Clinical Oncology (2015),** 11 (S5), 6-19. DOI: 10.1111/ajco.12444
4. **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, and Christopher J. Scarlett. Phytochemical, antibacterial and antifungal properties of an aqueous extract of *Eucalyptus microcorys* leaves*.* IFT16 (Institute of Food Technologists), Chicago, Illinois, USA 07/2016. ePoster presentation.

5. **Deep Jyoti Bhuyan**, Quan V. Vuong, Danielle R. Bond, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, and Christopher J. Scarlett. Aqueous *Eucalyptus microcorys* extract derived HPLC fractions with antioxidant and anti-pancreatic cancer activity. Australian Society of Medical Research Newcastle Satellite Scientific Meeting, Newcastle, NSW, Australia 06/2017.
6. **Deep Jyoti Bhuyan**, Quan V. Vuong, Danielle R. Bond, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, and Christopher J. Scarlett. Aqueous *Angophora floribunda* extract as a source of phenolics and antioxidants with anti-pancreatic cancer and antimicrobial activity. International Conference on Scientific Frontiers in Natural Product Based Drugs 2017, Pharmacological Society of Singapore, NUS, Singapore 07/2017. Oral presentation.

Table of contents

Abstract

The poorest prognostic outcome for pancreatic cancer (PC) patients, among all gastrointestinal malignancies, can be attributed to the molecular heterogeneity and lack of specific therapeutic strategies. The emergence of resistance against the common chemotherapeutic drug gemcitabine has also been widely reported. Several studies have demonstrated improved efficacy using gemcitabine in conjunction with plant polyphenolics and antioxidants for PC treatment. This suggests that plant secondary metabolites should be investigated further in a search for adjuncts to current PC treatments. Moreover, plant-derived bioactive compounds have played a key role in the development of anticancer drugs over many decades.

Eucalypts dominate the Australian landscape with over 800 distinct species. Eucalypt-derived phytochemicals have been associated with a wide range of bioactivity, both in traditional indigenous Australian bush medicine and in the scientific literature. However, a few eucalypt species and their essential oils have to date been exploited for their anticancer properties. An extensive review (Chapter 1) confirmed that more research was required to gain an improved understanding of the anticancer potential of Australian eucalypt phytochemicals with activity specific to PC. Therefore, the research reported herein was designed to address two main aspects, namely; (1) determining the optimal extraction conditions for phenolics and antioxidants from eucalypts, and (2) assessing their antiproliferative activity against PC cells including the delineation of potential molecular mechanisms of action responsible for this activity. Conventional extraction with water was employed to prepare crude extracts from eight different eucalypt species and was shown to be the most efficient method for extracting phenolics and antioxidants when compared to microwave-assisted (MAE) and ultrasound-assisted extractions (UAE) (Chapter 2). Crude extracts derived from *Angophora floribunda, Angophora hispida* and *Eucalyptus microcorys* were demonstrated to possess the most potent phytochemical profile, exhibiting statistically similar cytotoxicities against MIA PaCa-2 cells as

discussed in Chapter 3. In addition, *E. microcorys* crude extracts exerted significantly greater cytotoxicity against glioblastoma, neuroblastoma and lung cancer cells than the other extracts. In MIA PaCa-2 cells, *E. microcorys* crude extracts induced caspase 3/7-mediated apoptosis. Therefore, the aqueous *E. microcorys* extract was subjected to further investigation to obtain a greater depth of understanding of their bioactivity. Chapter 4 focuses on the significant antioxidant, antifungal and antibacterial properties of aqueous *E. microcorys* extract. Subsequent bioassay-guided fractionation of *E. microcorys* aqueous crude extract using semi-preparative Reversed-Phase (RP) HPLC revealed that fraction-1 was significantly more efficacious in terms of its antioxidant and antiproliferative activity against MIA PaCa-2 cells in comparison to other four fractions, as stated in Chapter 5. Flow cytometry analyses validated that the cytotoxicity was mediated by induction of apoptosis and abrogation of the cell cycle in the G2/M phase. Western blot analysis showed that the active fraction significantly downregulated the antiapoptotic protein B-cell lymphoma 2 (Bcl-2) and upregulated the proapoptotic proteins Bcl-2 homologous antagonist killer (Bak) and Bcl-2-associated protein (Bax) and cleaved Poly (ADP-ribose) polymerase (PARP) in MIA PaCa-2 cells. Combination treatment of the active fraction with gemcitabine increased apoptosis and cell cycle abrogation of MIA PaCa-2 greater than either mono treatment, indicating a potential additive/synergistic effect against the PC cells. Untargeted metabolomics using High performance/pressure liquid chromatography/electrospray ionisation/mass spectroscopy/mass spectroscopy (HPLC-ESI/MS/MS) revealed the tentative identities of the phytochemicals in the active fraction to be mostly phenolic compounds, of which several have previously been described to possess antipancreatic cancer activity.

The findings presented in this thesis provide further scientific evidence of the antipancreatic cancer activity of extracts from Australian eucalypts. This is the first report to optimise the MAE and UAE techniques and parameters for extracting phenolic compounds and antioxidants from *Eucalyptus robusta* and establish the antiproliferative activity of species belonging to all three main genera of Australian eucalypts against the PC cells. Bioassay-guided fractionation of *E.*

microcorys aqueous crude extract, investigation of bioactive compounds in the most potent fraction by liquid chromatography-mass spectroscopy (LC-MS) based-metabolomics and studies to obtain a mechanistic explanation of antiproliferative activity against PC cells are other key contributions of this project.

List of Figures*

*Represents only the figures used in the overview sections of the chapters and does not include figures from the published or submitted manuscripts.

List of Abbreviations

5-FU	5-Fluorouracil
7-AAD	7-Aminoactinomycin D
ABTS	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
AE	Aescin equivalents
ALDH1	Aldehyde dehydrogenase isoform 1
ANOVA	Analysis of variance
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated protein
BBD	Box–Behnken design
Bcl-2	B-cell lymphoma 2
BiA	Betulinic acid
BoA	Betulonic acid
BPE	Bovine pituitary extract
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BrdU	Bromodeoxyuridine
CAE	Catechin equivalents
CAPE	Caffeic acid phenethyl ester
CCK-8	Cell counting kit-8
cdc2	Cell division cycle 2
cdc25	Cell division cycle 25
cdc25c	Cell division cycle 25 homolog c
CDKN2A	Cyclin-dependent kinase Inhibitor 2A
CFU	Colony forming units
CG	Catechin gallate
Chk-1	Checkpoint kinase-1

Chk-2	Checkpoint kinase-2
CUPRAC	Cupric reducing antioxidant capacity
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EAC	Ehrlich ascites carcinoma
EBV-EA	Epstein-Barr virus early antigen
ECG	Epicatechin gallate
EGCG	Epigallocatechin-3-gallate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FPG	Formylated phloroglucinol
FX	FOLFIRINOX
GAE	Gallic acid equivalents
GC	Gas chromatography
GSK-3β	Glycogen synthase kinase-3β
HPDE	Human pancreatic ductal epithelial cell
HPLC	High performance/pressure liquid chromatography
HPLC-ESI/MS/MS	High performance/pressure liquid chromatography, electrospray ionisation, mass spectroscopy, mass spectroscopy
IC_{50}	The half-maximal inhibitory concentration
IL-8	Interleukin 8
IMDM	Iscove's modified Dulbecco's media
IPMN	Intraductal papillary mucinous neoplasm

JNK	c-Jun N-terminal kinase
KRAS	Kirsten rat sarcoma
KSFM	Keratinocyte serum-free media
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography mass spectroscopy, mass spectroscopy
LPS	Lipopolysaccharides
MAE	Microwave assisted extraction
MCNs	Mucinous cystic neoplasms
MHA	Mueller Hinton agar
MIC	Minimum inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Nutrient agar
NB	Nutrient broth
NF-*κ*B	Nuclear factor κB
p16	Protein 16
p17	Protein 17
p21	Protein 21
p27	Protein 27
p50	Protein 50
p53	Protein 53
p65	Protein 65
PanIN	Pancreatic intraepithelial neoplasia
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PC	Pancreatic cancer
PCA	Protocatechuic acid

PDAC	Pancreatic ductal adenocarcinoma
PG	Phloroglucinol
PI	Propidium iodide
PI3 Kinase	Phosphatidylinositol 3-kinase
PRESS	Predicted residual sum of squares
PS	Phosphatidylserine
RE	Rutin equivalents
rEGF	Recombinant epidermal growth factor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase high performance/pressure liquid chromatography
RSM	Response surface methodology
RT	Room temperature
SD	Standard deviation
SDA	Sabouraud dextrose agar
SDL	Sabouraud dextrose liquid
SFE	Supercritical fluid extraction
SRE	Sideroxylonal-rich extract
TAC	Total antioxidant capacity
TE	Trolox equivalent
TFC	Total flavonoid content
TNF-α	Tumour Necrosis Factor α
TPA	12-O-tetradecanoylphorbol-13-acetate
TPC	Total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UAE	Ultrasound assisted extraction

uPA	urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
WST-8	Water Soluble tetrazolium salt-8

CHAPTER 1

Introduction and literature review

1.1 Foreword

Pancreatic cancer (PC) has a five-year survival rate of 7% even after treatment and it is the twelfth most common type of cancer worldwide (American Cancer Society, 2017). However, the treatment of PC has not improved much over the last five decades. Eucalypts play an important role in both indigenous Australian bush medicine and Chinese folk medicine. Several (*in vitro* and *in vivo*) published over the last few years have shown strong evidence regarding the anticancer acivity of eucalypt phytochemicals (Bhagat et al., 2012; Tian et al. 2012, Topçu et al., 2011; Ashour, 2008; Benyahia et al., 2004; Doll-Boscardin et al., 2012; Hrubik et al., 2012; Wang et al., 2012; Islam et al. 2012). However, very few studies have attempted to demonstrate a relationship between the biological activity of eucalypt extracts and PC thus far (Vuong et al., 2015b). Hence, the current literature was comprehensively assessed and all relevant studies reported in this area were collected to present a review on the potential antipancreatic cancer activity of eucalypts. In this review, we have not only analysed the recent studies performed in the area of PC therapeutics but also discussed how phytochemicals present in plants particularly in relation to Australian eucalypts can be useful as mono or combination therapies for PC. In 2014, we published a review article (Vuong et al., 2015a, included in the appendix) about the general bioactivity and anticancer properties of *Eucalyptus* species. Therefore, the current review significantly adds to the body of existing knowledge in establishing a beneficial link specifically between PC and the three main genera of eucalypts: *Angophora, Corymbia* and *Eucalyptus*. This review article has recently been accepted for publication in the journal ***Pancreas*** (Wolters Kluwer).

Review Paper: **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Michael C. Bowyer, Christopher J. Scarlett. An array of bioactive compounds from Australian eucalypts and their relevance in pancreatic cancer therapeutics. In Press **Pancreas (2018).**

An Array of Bioactive Compounds From Australian Eucalypts and Their Relevance in Pancreatic Cancer Therapeutics

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Abstract: Pancreatic cancer (PC) is one of the most devastating human cancers, and despite the significant advances in the current therapeutic options, the overall survival rate for PC has remained static for the past 50 years. Plant-derived bioactive compounds play a vital role in cancer therapeutics by providing new lead compounds for future drug development. Therefore, the isolation, characterization, and identification of new bioactive compounds for the prevention and treatment of cancer continue to be an important aspect of natural product research. Many in vitro and in vivo studies published in the last few decades have established strong links between the phytochemical profile of eucalypts and anticancer activity. However, only a small number of these reports have attempted to demonstrate a relationship between the biological activity of eucalypt extracts and PC. This review focuses on potential anti-PC effects of an array of bioactive compounds present in various species of eucalypts. It also highlights the necessity for further in vitro and in vivo studies to develop a complete understanding of the potential this group of plants has for the development of potent and specific chemotherapeutic drugs for PC.

Key Words: pancreatic adenocarcinoma, Australian eucalypt, anticancer drugs, phenolic compounds, natural products, apoptosis

(*Pancreas* 2018;00: 00–00)

Pancreatic adenocarcinoma, also known as pancreatic cancer (PC), is a type of gastrointestinal cancer and one of the most lethal forms of human cancers.[1,2] Based on the GLOBOCAN 2012 estimates, it is the twelfth most common type of cancer worldwide, with 338,000 new cases diagnosed in 2012.[3,4] The vigorous attempts and efforts that have been made to improve the survival rate of PC for the past 15 years have been unsuccessful; with at best, only very minor advances in treatment in comparison with that of other cancers.[5,6] Bioactive compounds derived from natural sources comprise more than 60% of the anticancer drugs commercially available today.[7] Furthermore, various natural and semisynthetic compounds have been evaluated specifically for their activity against PC. A number of published articles were comprehensively reviewed to obtain an acute understanding of the anticancer activity of bioactive compounds present in eucalypts and their further applications in developing chemotherapeutic drugs against PC.

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Received for publication November 7, 2017; accepted April 16, 2018.
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This study was supported by University of Newcastle.
The authors declare no conflict of interest.

DOI: 10.1097/MPA.0000000000001074

Pancreatic Cancer

The pancreas is a compound gland composed of exocrine and endocrine epithelia with the following three areas: head, body, and tail.[8] According to the American Cancer Society,[9] PC has an average 5-year survival rate of approximately 7% after treatment for stage IIA, whereas, the stage IV PC has a 5-year survival rate of approximately 1%. It has a median survival of less than 5 months after diagnosis.[1] Pancreatic cancer is the seventh leading cause of cancer-related death worldwide and fifth in Australia because of poor prognosis and few effective therapies.[4,10] According to the Australian Cancer Research Foundation,[11] PC has the highest mortality rate of any cancer and 3364 new diagnoses are estimated in 2018 in Australia. The incidence and mortality statistics of PC are similar throughout the world.[12] Approximately 60% of PCs occur in the head of the pancreas with 15% and 5% occur in the body and tail, respectively, and approximately 20% tumors are diffused throughout the pancreas.[13] Pancreatic cancer can be divided into serous cystadenomas, mucinous cystic neoplasms, endocrine neoplasms, and exocrine tumors.[14] Serous cystadenoma is the most common benign pancreatic neoplasm, with resection carried out as the usual treatment.[15] Mucinous cystic neoplasms (MCNs) of the pancreas are uncommon tumors that occur almost exclusively in the pancreas body and tail of middle-aged women and are benign.[16,17] Endocrine neoplasms are also uncommon tumors that comprise less than 3% of all pancreatic neoplasms and are more indolent with a better prognosis.[18] Pancreatic ductal adenocarcinoma (PDAC) composed of up to 90% of all exocrine tumors develops in the cells that produce digestive enzymes in the pancreas.[19] Various confirmed risk factors such as age, heredity, cigarette smoking, obesity, and type II diabetes (diabetes mellitus) have been found to be associated with PC, whereas alcohol, chronic pancreatitis, a high intake of carbohydrate, meat and other animal products, and *Helicobacter pylori* or hepatitis B infections are suspected risk factors.[12]

Pancreatic cancer has a complex mutational landscape with four most common oncogenic events occurring in well-known cancer genes such as *KRAS*, *TP53*, *SMAD4*, and *CDKN2A* along with a number of other genes mutated at low incidence.[20] Pancreatic cancer is by nature highly heterogeneous in its profile, which makes it extremely difficult to identify a single tumor marker that can serve as a sensitive and definitive marker for all other tumor types. Moreover, the heterogeneity occurs at the pathological, molecular, and clinical level.[21] It is estimated that PC will become the second cause of cancer death in Western societies within a decade.[20,22]

Genetic Basis of PC

Inherited and acquired mutations in specific cancer-associated genes are the main causes of PC.[23] In addition, it has been proven that PDAC originates through a multistep progression rather than de novo.[24] Patients with a strong familial history of PC inherit

genetic alterations that predispose them to develop PC.[23] Pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm, and MCN are the three major subtypes of precursor lesions of PC that have been identified to date.[23,24] Pancreatic intraepithelial neoplasias are small, not readily detectable, and the most common precursor lesions. It is extremely important to identify and characterize these lesions to be able to treat pancreatic neoplasia before it becomes invasive.[23] Significant progress has been made in characterizing the genetic alterations found in these lesions. For instance, PanIN lesions have been found to harbor many similar genetic alterations present in invasive PC.[23] A number of mutated genes have been associated with PC. For example, PanIN lesions have been associated with the mutations of *KRAS*, *p16/CDKN2A*, or *BRAF*.[23] Of these, an activating point mutation in *KRAS* is seen in 80% to 95% of PCs as well as PanINs.[24–26] The *KRAS2* oncogene is located on chromosome 12p, and these mutations are among the earliest genetic mutations observed during pancreatic carcinogenesis in intraductal papillary mucinous neoplasm and MCN.[24] Alterations of *P53* and *SMAD4* (*DPC4*, *MADH4*) are also found to be highly associated with PC, whereas mutation in the mismatch repair genes, *BRCA2*, *LKB1*, *STK11*, *AKT2*, and telomerase are considered as low-frequency genetic alterations that also can give rise to PC.[26] A study by Waddell et al[27] analyzed 100 PDACs using whole-genome sequencing and copy number variation and established that PDACs can be divided into the following 4 subtypes: stable, locally rearranged, scattered, and unstable based on the patterns of structural variations in the chromosome. The stable subtype had 50 or less structural variation events, whereas the locally rearranged subtype displayed a significant focal event on one or two chromosomes. By contrast, the scattered subtype exhibited less than 200 and the unstable subtype exhibited greater than 200 structural variation events. These subtypes are crucial for treatment directing against specific molecular targets. A recent breakthrough study by Bailey et al[20] analyzed 456 PDACs and identified 32 recurrently mutated genes linked to the following 10 pathways: KRAS, TGF-β, WNT, Notch, ROBO/SLIT signaling, G1/S transition, SWI-SNF chromatin modification, DNA repair, and RNA processing. They were able to divide PDACs into the following 4 subtypes: squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine correlating with histopathological characteristics based on key transcriptional networks and histopathology. This study has further delineated the molecular evolution of PC subtypes and has thereby opened up numerous opportunities for targeted therapy for PC.

TREATMENT OF PC

Pancreatic cancer presents a daunting challenge for oncologists. It has a fatal prognosis due to its late diagnosis and high resistance to both chemotherapy regimens and radiotherapy.[26,28] The combination of having a 7% survival rate even after treatment, and it being the fifth most common cause of cancer death in Australia clearly suggests that the treatment of PC remains in a state of infancy. Despite the advances in oncology, such as better understanding of the biology of PC, surgical skills, improved imaging systems, radiation, and molecular targeted therapies, the overall survival rate has not improved for the last 5 decades.[28,29]

Resection

The single curative treatment for PDAC is surgical resection, also known as the Kausch-Whipple pancreaticoduodenectomy. However, only 10% to 15% of patients are potential candidates, and there is a 5% mortality rate accompanying the procedure.[28,30,31] Moreover, most patients are diagnosed in their advanced stages of the disease and are not suitable for resection.[30]

Chemotherapeutic Agents

The chemotherapeutic agent gemcitabine has been the drug of choice more than others such as 5-fluorouracil for the management of advanced PC in terms of overall clinical benefit and survival.[5,28] Intracellularly, gemcitabine hydrochloride is metabolized to a nucleoside analogue that inhibits DNA replication and induces apoptosis.[5] It can provide an advantage of disease-free survival to patients who have undergone resection when given as adjuvant therapy.[28] Adjuvant therapy is a treatment that is administered along with the primary therapy to increase effectiveness, hence maximizing the chances of long-term patient survival. When agents that target the epidermal growth factor receptor (EGFR), matrix metallo proteases, farnesyl transferase, or vascular endothelial growth factor (VEGF) were used in combination with gemcitabine, no significant improvements were observed.[6] Even molecularly targeted agents, which can act on pathways that play major roles in PC development and progression, have been found to be ineffective with the exception of a tyrosine kinase inhibitor viz erlotinib, which inhibits the EGFR.[5,6,32] A randomized phase III trial conducted by Moore et al[32] found that a statistically significant improvement in survival rate for advanced PC was obtained when erlotinib was given in combination with gemcitabine compared with gemcitabine monotherapy. However, the high cost of erlotinib and an increased risk of toxicity are some of its shortcomings.[33] Recent studies have also identified that the oral fluoropyrimidine, capecitabine either alone or in combination with gemcitabine, provided similar results to that of a gemcitabine + erlotinib therapy in patients with PDAC.[34,35] Adjuvant gemcitabine with capecitabine had a statistically significant improvement in survival compared with gemcitabine monotherapy in patients with PDAC within 12 weeks of surgery.[36] Moreover, because capecitabine is more cost-effective than erlotinib, it is considered to be a good alternative therapy for patients with lower economic background, especially in developing countries.[5] Platinum agents in conjunction with gemcitabine have also shown promising results in recent studies.[28] For instance, cisplatin, an inorganic and water-soluble platinum complex, has been suggested to improve the median overall survival of patients with *BRCA*-mutated or familial PC.[37] FOLFIRINOX (FX), a combination therapy for advanced stages of PC using chemotherapy agents the following: oxaliplatin, irinotecan, fluorouracil, and leucovorin, has been shown to be associated with survival advantage with significant increase in the overall survival, progression-free survival, and higher response rate compared with gemcitabine alone in randomized phase II and III trials.[38,39] However, FX exhibited more toxicity in patients than gemcitabine.[38] Another population-based cohort study by Papneja et al[40] involving 119 patients with advanced PC found that FX and nab-paclitaxel had comparable survival rates with different safety profiles.

Other Approaches

Various other approaches such as gene therapy, vaccine therapy, and immunotherapy have generated considerable recent interest, but are still in their early stages of development, and face many challenges and shortcomings.[29,41] In gene therapy, exogenous nucleic acids are introduced into cancer cells of the patient so that they can either express proteins, restore genetic mutations, or inhibit the expression of specific oncoproteins to kill or reverse the cancer cells to normal.[29] Wong and Lemoine[29] have commented that even though promising results have been demonstrated in animal models by many studies, clinical trial results remain

unsatisfactory. Furthermore, the anatomical location of the pancreas makes it difficult to deliver the current gene therapeutic protocols intratumorally. Immunotherapy is a promising approach that uses genetically modified cells or viral particles to stimulate the immune system to destroy cancer cells.[42] However, identification of new PC-associated antigens and immune checkpoints that inhibit immune cell activation is required for effective immunotherapy against PC.[41] Zhu et al[43] demonstrated that functional macrophage responses that enhance antigen presentation and productive antitumor T-cell responses can be reprogrammed by inhibiting signaling by the myeloid growth factor receptor CSF1R in a mouse model of PDAC. Recently, a phase Ib trial of a CC chemokine receptor type 2 antagonist (CCX872) in patients with nonresectable PC has been initiated by Chemocentryx.[44]

ROLE OF NATURAL BIOACTIVE COMPOUNDS IN CANCER THERAPEUTICS

Natural sources of bioactive compounds such as plants, marine sponges, and microorganisms have been extensively studied and used for their anticancer properties in modern and traditional medicine.

Compounds From Marine Sources

Gemcitabine, a fluorinated nucleoside derived from the marine sponge *Tectitethya crypta* (formerly known as *Cryptotethya crypta* and *Tethya crypta*), is currently administered against a number of cancers including pancreatic, bladder, breast, and non-small cell lung cancers.[45,46] Cytarabine (ara C, cytosine arabinose), another nucleoside analogue derived from *T. crypta*, is used for treatment of leukemias and non-Hodgkin lymphomas.[46,47] As per Food and Drug Administration guidelines, conventional cytarabine can be used for the treatment of acute lymphocytic leukemia, acute myelocytic leukemia, and the blast crisis phase of chronic myelogenous leukemia and meningeal leukemia 2 and 3.[48] In 2007, the European Union approved trabectedin (yondelis/ecteinascidin-743/ET-743) isolated from *Ecteinascidia turbinata*, as an antitumor agent against soft-tissue sarcoma.[47,48] Similarly, halichondrin B, also known as eribulin mesylate, isolated from the marine sponge *Halichondria okadai*, has been approved for the treatment of refractory metastatic breast cancer by the Food and Drug Administration in 2010.[48,49] Soblidotin (auristatin PE, TZT-1027), an antimicrotubule agent isolated from a marine bacterium, is in phase III clinical trial currently for evaluation of its antitumor properties.[48,50] Other marine-derived experimental anticancer drugs include didemnin B, bryostatin 1, ecteinascidin-743, dolastatin 10, halomon, aplidine, aplyronine A, kahalalide F, mycaperoxide B, thiocoraline, and granulatimide.[46] Phase II clinical anticancer trials are currently in progress for a range of other marine natural products including plitidepsin, elisidepsin, PM00104, synthadotin, and pseudopterosins.[48]

Phytochemicals

Although more than 5000 phytochemicals have been identified to date, there remains immense scope for identification and characterization of new compounds, with less than 10% of the estimated 250,000 species of plants having been formally profiled.[51,52] Ethnopharmacology remains an integral part of indigenous medicine worldwide, being employed by approximately 75% to 90% of rural-based populations worldwide.[51] Many bioactive compounds derived from plants have been demonstrated to exert anticancer properties both in vitro and in vivo.[46,53] Approximately, 50% to 60% of patients with cancer in the United States use products isolated from different plant components, either alone or in conjunction with commercial chemotherapy and/or radiation therapy.[53]

Approximately 45% of all anticancer drugs currently in commercial use are derived from plants (12% are natural products and 32% are semi-synthetic derivatives).[51,54] Vinca alkaloids, vinblastine, vinorelbine, and vincristine isolated from the Madagascan periwinkle *Catharanthus roseus* exhibit potent cytotoxic activity against Hodgkin lymphoma, non-Hodgkin lymphoma, germ-cell tumors, Kaposi sarcoma, breast cancer, acute leukemia, rhabdomyosarcoma, Wilm tumor, and nonsmall-cell lung cancer.[46] The alkaloid elliptinium, a semisynthetic compound derived from *Bleekeria vitiensis*, a Fijian medicinal plant, belonging to the Apocynaceae family, is used to treat breast cancer.[55,56] Two *Taxus* species, *T. brevifolia* and *T. baccata*, contain the cytotoxic compounds paclitaxel and docetaxel, respectively, which have proven efficacy against ovarian, breast, lung, and other cancers.[46] In addition, plants such as *Podophyllum peltatum* and *Podophyllum emodi* are the sources of etoposide, a topoisomerase II inhibitor, which is used for the treatment of testicular tumors, small-cell lung cancer, and brain cancer.[46,57,58] A second compound-teniposide derived from these two plant species is known for its activity against lymphomas, bronchial, and testicular cancers.[51] Both etoposide and teniposide are phase-specific cytotoxic drugs that act in the late S and early G2 phases of the cell cycle, with the latter exhibiting more effective cell uptake and binding ability due to its higher protein binding affinity.[59]

Topotecan and irinotecan isolated from the Chinese ornamental plant *Camptotheca acuminata* have been used for the management of ovarian, colorectal, and small-cell lung cancers.[51] Moreover, phytochemicals from common food-based plant materials such as tea polyphenols (green tea), diallyl sulfide (garlic), gingerol (gingers), resveratrol (grapes), curcumin (turmeric), isothiocyanates (cruciferous vegetables), genistein (soybean), lycopene (tomato), sulforaphane (broccoli), rosmarinic acid (rosemary), apigenin (parsley), and silymarin (milk thistle) are used in addition to chemotherapy and radiation therapy.[53] Other naturally derived antiproliferative drugs such as doxorubicin, daunomicin, bleomycin, mytomicin C, taxanes, and campthothecins also play a significant role in cancer chemotherapy.[7]

ROLE OF NATURAL AND SEMISYNTHETIC COMPOUNDS IN THE TREATMENT OF PC

With regard to the management of PC, various natural and semi-synthetic compounds, including gemcitabine, have been identified and characterized in several studies.[51,60] Vuong et al[51] reviewed the anti-PC activity of different groups of fruit-derived phenolic compounds both in vitro and in vivo and highlighted the potential of bioactive compounds particularly from Australian native fruits in the treatment of PC.

Flavonoids such as quercetin have been shown to possess strong antioxidant activity with potent anti-inflammatory and antiproliferative properties.[51] Zhou et al[61] demonstrated the anticancer activity of quercetin using in vitro and in vivo models of PC stem cells. They showed that the compound successfully diminished aldehyde dehydrogenase isoform 1 activity, proliferation, and angiogenesis, induced apoptosis, and prevented the expression of proteins involved in the epithelial-mesenchymal transition (EMT) and binding of nuclear factor κB (NF-κB) when used alone or in combination with sulforaphane (an isothiocyanate found in broccoli). Similar observations were made by Fan et al[62] when applied to BxPC-3 cells and a xenograft mouse model. They reported that continuous exposure of BxPC-3 PC cells to sulforaphane or quercetin did not induce resistance in surviving cells but reduced tumorigenicity by inhibition of tumor progression

markers. However, tumor progression markers were enriched in gemcitabine-exposed cells.

Phillips et al[63] demonstrated that the flavonoid myricetin inhibited PC both in vitro and in vivo. They showed that myricetin was nontoxic to both the normal pancreatic ductal cells and the orthotopic mouse model but it induced apoptosis and reduced the phosphatidylinositol 3-kinase activity in PC cells, which led to tumor regression and a decrease in the metastatic spread of orthotopic pancreatic tumors in vivo. Kaempferol, a flavonoid found in *Ginkgo biloba*, has been shown to inhibit cell proliferation and induce apoptosis in the PC cell lines MIA PaCa-2 and PANC-1 in vitro.[64] Kaempferol significantly inhibited MIA PaCa-2 cell proliferation by 79% and 45.7% compared with control as assessed by direct cell counting and the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, respectively, with similar observations made for PANC-1.

The antiproliferative and antiangiogenic effects of the flavonoid apigenin were illustrated by Melstrom et al.[65] They established that apigenin inhibits HIF-1α, GLUT-1, and VEGF mRNA and protein expression in PC cells in both normoxic and hypoxic conditions, proving the potential of this compound as a therapeutic agent for PC. Johnson et al[66] suggested that citrus flavonoids such as luteolin, apigenin, and quercetin can inhibit glycogen synthase kinase 3β, leading to decreased cancer cell proliferation and survival by reducing NF-κB activity. Likewise, apigenin has been linked with inhibition of PC cell proliferation by G2/M cell cycle arrest, downregulation of the overexpressed protein geminin, an increase in growth inhibitory effects of gemcitabine, and abrogation of gemcitabine resistance.[67–69] A study by Lee et al[70] proposed that gemcitabine in combination with apigenin resulted in enhanced apoptosis and growth inhibition by downregulation of NF-κB activity with suppression of Akt activation in PC cell lines in vitro, making similar observations in vivo. Apigenin and luteolin were also shown to improve the efficacy of gemcitabine, cisplatin, 5-fluorouracil, and oxaliplatin in terms of their antiproliferative activity against BxPC-3 human PC cells.[71]

Lou et al[72] demonstrated that naringenin, a type of flavanone, downregulated markers of EMT by inhibiting TGF-β1/Smad3 signaling in PC cells. A moderate activity against multidrug-resistant PC cell line (EPP85-181) was also observed by Duarte et al.[73] The TGF-β1/Smad3 signaling pathway (an important pathway also driving pancreatic carcinogenesis) was inhibited in HeLa cells by the flavanone hesperetin.[74] Alpinetin, a novel plant flavonoid found in *Alpinia katsumadai* Hayata, also exhibited an antiproliferative effect in BxPC-3 cells.[75] Other plant flavonoids, such as daidzein and genistein, have also been documented to possess activity against PC cell lines.[51,76–81] Guo et al[78] suggested that daidzein has antiproliferative effects on human estrogen-receptor–positive and negative PC cells, whereas Han et al[79] showed that genistein can effectively inhibit transforming growth factor β1–induced invasion and metastasis in the PANC-1 human PC cell line. Another study also concluded that genistein inhibited cell growth and induced apoptotic processes in PC cells by inhibition of Notch-1 pathway through upregulation of miR-34a.[81] Another study by Buchler et al[82] reported that genistein-induced caspase-3 mediated apoptosis in all PC cell lines tested in vitro and improved survival, inhibited metastasis, and increased apoptosis in an orthotopic model of PC in vivo. Moreover, genistein derived from soy has been proposed to inactivate or downregulate NF-κB and Akt and increase growth inhibition and apoptosis induced by cisplatin, docetaxel, doxorubicin, gemcitabine, and the combination of gemcitabine + erlotinib in PC cells.[76,80,83]

Baicalein, a component of the traditional Chinese and Japanese herbal remedy, derived from *Scutellaria baicalensis* was found to inhibit PC cell growth by reducing the expression of antiapoptotic proteins, inducing mitochondrial-based apoptosis, and inhibiting the lipoxygenase pathway that stimulates cell proliferation.[84] Epigallocatechin-3-gallate (EGCG), a polyphenolic compound found in green tea, was shown to inhibit growth and induce apoptosis in human PC cells by Shankar et al.[85] It was illustrated that, in a xenograft model system, when AsPC-1 xenografted tumors were treated with EGCG, a significant reduction in volume, proliferation, angiogenesis, metastasis, and induction in apoptosis, caspase-3 activity, and growth arrest were observed. Epigallocatechin-3-gallate bound to the C-terminal domain of the Hsp90, which impairs the association of Hsp90 with its co-chaperones, thus induced degradation of Hsp90 client proteins resulting in antiproliferative effects in MIA PaCa-2 cells.[86] Epigallocatechin-3-gallate was also shown to decrease the expression of *KRAS* in the human pancreatic adenocarcinoma cell line.[87] Qanungo et al[88] also suggested that EGCG can induce mitochondrial membrane depolarization and caspase-dependent apoptosis in MIA PaCa-2 cells in vitro. Similarly, EGCG exhibited anticancer effects in human pancreatic carcinoma cells via the inhibition of both focal adhesion kinase and the insulin-like growth factor-I receptor.[89] In addition, this study showed that EGCG induces apoptosis without the activation of caspase-3 in PC cells, unobserved in other studies. Another study by Takada et al[90] also validated the anticancer properties of EGCG against the PANC-1, MIA PaCa-2, and BxPC-3 cells. A significant suppression of pancreatic carcinoma cells by EGCG in a dose-dependent manner was observed in this study with the destruction of the invasive ability of the cell lines without affecting the cell cycle protein cyclin D1. Epigallocatechin-3-gallate, together with catechin gallate (CG) and epicatechin gallate (ECG), two minor green tea catechins, were evaluated for their anticancer properties on the human PC cells PANC-TU-I, PANC-1, PANC-89, and BxPC-3.[91] All 3 catechins inhibited proliferation of the PDAC cells in a dose- and time-dependent manner. However, this study also stated that the antiproliferative effects exhibited by CG and ECG were more significant than EGCG. Epicatechin gallate was also shown to inhibit tumor necrosis factor α (TNFα)-induced activation of NF-κB and consequently secretion of proinflammatory and invasion promoting proteins such as IL-8 and uPA. Härdtner et al[92] demonstrated that EGCG and celecoxib synergistically diminished the metabolic activity of Colo357 cells via apoptosis induction and downregulated release of proangiogenic VEGF and invasiveness-promoting matrix metalloproteinase (MMP)-2. Epigallocatechin-3-gallate reduced the levels of celecoxib required to stimulate beneficial effects on tumorigenic mediators by a factor of ten.[92]

A study by Tang et al[93] demonstrated that EGCG inhibited the expression of pluripotency maintaining transcription factors such as Nanog, c-Myc, and Oct-4 and self-renewal capacity of PC stem cells. In addition, EGCG inhibited cell proliferation and induced apoptosis by preventing the expression of Bcl-2 and XIAP and activating caspase-3. It also inhibited the EMT (by inhibiting the expression of Snail, Slug and ZEB1, and TCF/LEF transcriptional activity), which correlated with significantly reduced migration and invasion of PC stem cells, suggesting the blockade of signaling involved in early metastasis.

Phenolic acids such as gallic acid, protocatechuic acid, and caffeic acid phenethyl ester (CAPE) have been shown to possess anticancer properties against PC in various studies.[94–97] Liu et al[96] suggested that gallic acid can induce apoptosis in MIA PaCa-2 cells via mitochondria-mediated pathways and thus can function as a cancer-selective agent. According to Chen et al,[95] CAPE can induce apoptosis of BxPC-3 and PANC-1 human PC cell lines by activation of caspase and mitochondrial dysfunction. They further suggested that CAPE can inhibit the orthotopic growth and EMT of PANC-1 cells with downregulation of vimentin and Twist 2 expression.[94] In terms of in vivo studies, Nakamura et al[97]

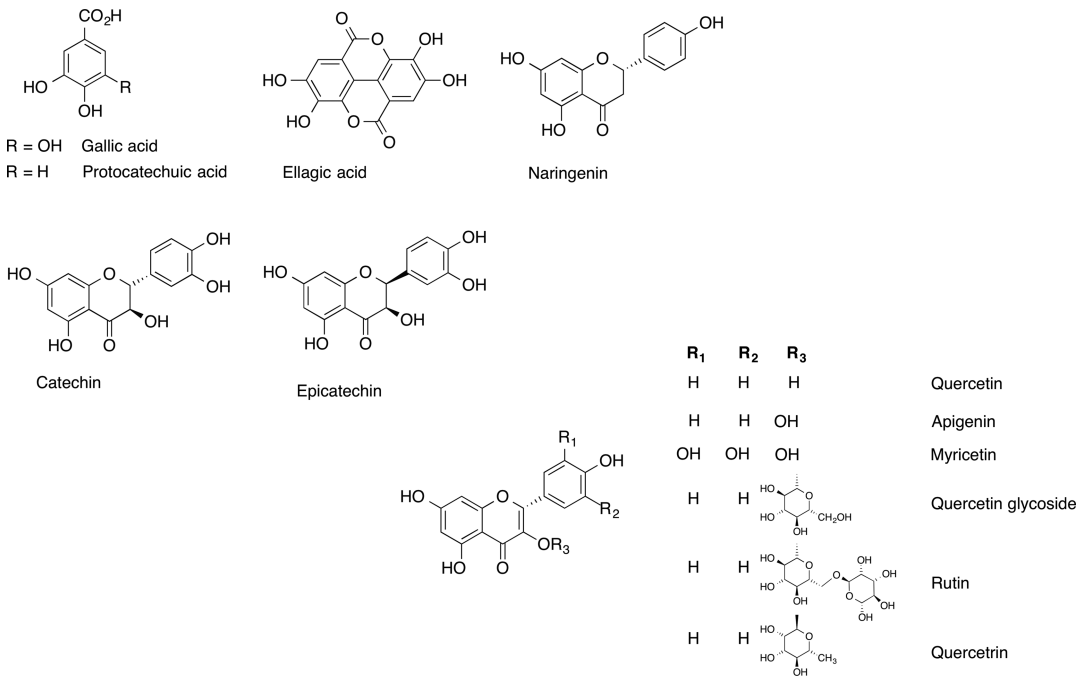

FIGURE 1. Phenolic compounds and acids isolated from eucalypts.

suggested that protocatechuic acid can inhibit the late postinitiation or progression phase of *N*-nitrosobis(2-oxopropyl) amine-induced pancreatic carcinogenesis in hamsters. Ellagic acid, a polyphenol found in several plants and fruits, has also been demonstrated to have anti-PC activity both in vitro and in vivo.[98–100] Zhao et al[98] showed that when PANC-1 xenografted mice were treated with ellagic acid, it significantly suppressed tumor growth through reduced cell proliferation and caspase-3 activation, as well as induction of poly (ADP-ribose) polymerase cleavage. Ellagic acid also inhibited the expression of Bcl-2, cyclin D1, CDK2, and CDK6 and induced the expression of Bax. Various markers of angiogenesis (COX-2, HIF1α, VEGF, VEGFR, IL-6, and IL-8) and metastasis (MMP-2 and MMP-9) in tumor tissues were also inhibited by ellagic acid. Moreover, a significant inhibition in phospho-Akt, Gli1, Gli2, Notch1, Notch3, and Hey1 was also observed in the treated mice. Ellagic acid also reversed EMT by upregulating E-cadherin and inhibiting the expression of Snail, MMP-2, and MMP-9.[98]

Furthermore, a study by Zhang et al[101] stated that anthocyanins found in Chinese bayberry extract can protect pancreatic β cells (INS-1) against H_2O_2-induced necrosis and apoptosis. Other phenolic compounds such as stilbenes and lignans have similarly been linked with anti-PC activity.[51] The trans-stilbene resveratrol (trans-3,4',5-trihydroxystilbene) has been shown to exhibit both antiproliferative and proapoptotic effects in vitro and in vivo.[102–107] In addition, Harikumar et al[108] emphasized the role of resveratrol

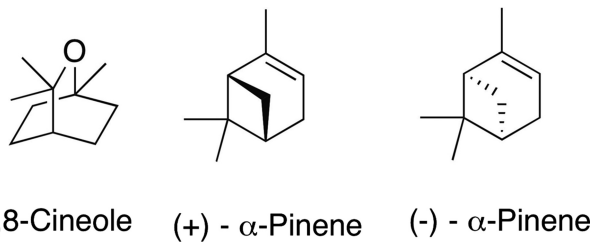

FIGURE 2. Compounds found in eucalypt essential oils.

in enhancing the antitumor activity of gemcitabine in vitro and in an orthotopic mouse model of human PC. Inhibition of glycogen synthesis and turnover by resveratrol in a recent study by Harris et al[109] explained the underlying mechanism of controlling tumor cell proliferation. Pterostilbene, a naturally occurring analogue of resveratrol found in blueberries, has likewise been associated with antiproliferative and proapoptotic effects against PC both in vitro and in vivo.[110,111] Lignans such as honokiol and arctigenin isolated from the *Magnolia officinalis* var. *officinalis* and *Arctium lappa*, respectively, have also been shown to possess preventive potential against pancreatic carcinogenesis in various studies.[112,113]

Triptolide, a diterpenoid triepoxide, was demonstrated to induce apoptosis mediated via the reduction of the Hsp70 level in PANC-1 and MIA PaCa-2 cell lines.[114] In vivo results also showed a decrease in PC growth and local-regional tumor spread. Similarly, minnelide, a synthetic water-soluble analogue of triptolide, was shown to be effective in decreasing tumor burden, tumor-associated morbidity, and local-regional spread in multiple animal models of PC.[115]

Ursolic acid, a pentacyclic triterpenoid compound extracted and purified from various herbs, edible vegetables, and medicinal plants including eucalypts,[116–118] has been found to inhibit growth and induce apoptosis in MIA PaCa-2, PANC-1, and Capan-1 PC cell lines in a dose-dependent manner via activation of c-Jun N-terminal kinase (JNK) and inactivation of phosphatidylino-sitol 3-kinase (PI3K)/Akt/NF-κB pathways.[119] Similar observations were also made by Prasad et al[120] where ursolic acid inhibited proliferation, induced apoptosis, and suppressed NF-κB activation and its regulated proliferative, metastatic, and angiogenic proteins in vitro. The authors also confirmed that ursolic acid can inhibit the growth of human pancreatic tumors and sensitize them to gemcitabine by suppressing inflammatory biomarkers linked to proliferation, invasion, angiogenesis, and metastasis.

Curcumin, phytopolylphenol pigment isolated from turmeric, has been widely studied for its activity against PC. Curcumin was demonstrated to suppresses NF-κB binding and IkappaB kinase activity, downregulate COX-2, PGE2, and IL-8,

TABLE 1. Anticancer Activity of Extracts and Compounds Directly Isolated From Eucalypt Species

Type of Eucalypt Extracts/Compounds	In Vitro/In Vivo Studies and Observed Effect/Mechanism	References
E. robusta leaf - 70% ethanol *E. microcorys* leaf and fruit - water and 70% ethanol *E. saligna* leaf - 70% ethanol	HT29 (colon); U87, SJ-G2, SMA (glioblastoma); MCF-7 (breast); A2780 (ovarian); H460 (lung); A431 (skin); Du145 (prostate); BE2-C (neuroblastoma). Growth inhibition was observed (GI_{50} values in 33 to >200 μg/mL range). Ethanolic extract of *E. saligna* leaf exhibited the greatest growth inhibition against ovarian (GI_{50} = 33 μg/mL) and skin (GI_{50} = 47 μg/mL) cancer cells.	Bhuyan et al[158]
E. camaldulensis bark *p*-Menth-1-ene-4,7-diol	EAC cells and EAC-bearing mice. Cell growth inhibition was observed using the MTT assay (IC_{50} = 32 μg/mL). The mRNA expressions of p53 and Bax genes were increased whereas and negative expressions of Bcl-2 and Bcl-X were observed which confirmed induction of apoptosis. Inhibited the cellular proliferation of EAC cells by suppressing the synthesis of DNA in S phase.	Islam et al[159]
*E. citriodora** root-citriodora A(3β,25-dihydroxy-5α, 6α-epoxy-7-oxocucurbita-(23E)-en-19-al)	Osseous tumor cell line. Significant cytotoxic activity with IC_{50} value of approximately 2 μM/L.	He et al[160]
E. woodwardi, *E. stricklandii*, *E. salubris*, *E. sargentii*, *E. torquata*, *and E. wandoo* leaf - essential oil	MCF7, T47D (breast); Caki, A498 (kidney); PC3 (prostate); Raji, BJAB (lymphoma); Caco-2 (colon); HeLa (cervix). Potent cytotoxicity was evident against BJAB and Raji lymphoma tumor cell lines. Weak to moderate cytotoxicity was reported against nonlymphoma tumor cell lines. A decrease in cellular DNA content suggested that the cytotoxicity was most likely mediated by apoptotic pathways.	Bardaweel et al[161]
E. camaldulensis bark-methanol	EAC cells and EAC-bearing mice. Significant cell growth inhibition (96%), reduced tumor burden (81.4%), increased lifespan (71.36%) of EAC-bearing mice were observed. Altered haematological and biochemical parameters were restored to towards normal level. Treated EAC cells showed membrane blebbing, chromatin condensation, nuclear fragmentation (apoptotic features) in Hoechst 33342 staining under the fluorescence microscope. Cell death by apoptosis was demonstrated by DNA fragmentation of treated EAC cells by agarose gel electrophoresis.	Islam et al[162]
E. nitens bark-dichloromethane, betulonic acid, betulinic acid	HCT116 (colon). Inhibition of cell proliferation and cell cycle arrest. Cell death by apoptosis through intrinsic mitochondrial pathway was observed which is probably via JNK activation but not via p53 as the extracts/compounds significantly decreased the levels of p53 in cells. The potency of the triterpenoids enriched fraction (F2) was two times to that of the lipophilic crude extract (CE) (IC_{50} values 1.3 *vs.* 2.2 μg/mL), whereas isolated betulonic acid (BoA) was around four times more potent than betulinic acid (BiA) (IC_{50} values 0.8 *vs.* 3.9 μM). In spite of the lower IC_{50} value, BiA was found to be a more potent inducer of apoptosis than BoA.	Pereira[163]
E. camaldulensis leaf - ethyl acetate, *n*-butanol, methanol, and water	MCF7, MDAMB-231 (Breast). Cytotoxic effect was observed with IC_{50} in the range of 3.2–250.70 μg/mL in the MTT and SRB tests. Water extract showed the lowest IC_{50} value of 3.2 μg/mL against MDAMB-231 cells in SRB test, whereas, ethyl acetate extract displayed the lowest IC_{50} value of 26.7 μg/mL against MCF7 cells in the MTT test.	Hrubik et al[156]
E. camaldulensis leaf - petroleum ether	EAC cells and EAC-bearing mice. Tumor growth rate was reduced and life span of EAC-bearing mice was enhanced significantly. Reversed the haematological parameters back to normal and reduced the transplantability as treatment of the EAC cells with the extract decreased the cell viability. In addition, showed immunomodulatory effects by increasing the number of macrophages significantly.	Islam et al[164]

(Continued on next page)

TABLE 1. *(Continued)*

Type of Eucalypt Extracts/Compounds	In Vitro/In Vivo Studies and Observed Effect/Mechanism	References
E. globulus bark - 80% ethanol	MDAMB-231 (Breast). Antiproliferative effects were observed with an IC_{50} value of 85.83 (SD = 19.05) μg/mL after 24 h using the MTT assay.	Mota et al[145]
E. citriodora leaf - various organic solvent and water	SW-620 (colon); HEp-2 (liver); OVCAR-5 (ovary); PC-3 (prostate); HeLa (cervix); IMR-32 (neuroblastoma); HOP-62(lung) and In vivo EAC model. Antiproliferative effects were displayed by the ethyl acetate extract against all tested human cancer cell lines with significant growth inhibition (71-92%) at 100 μg/mL. Aqueous extract inhibited the growth (72-85%) of three human cancer cell lines (OVCAR-5, PC-3, HOP-62) at the concentration of 100 μg/mL. Ethyl acetate and aqueous extracts also suppressed the growth of EAC in vivo by 29.79% and 18.48%, respectively.	Bhagat et al[150]
E. globulus fruit - eucalyptal D, eucalyptal E, euglobal-In-3	BGC-823, KE-97 (gastric); Huh-7 (liver); Jurkat (T lymphoblast). Cytotoxic effects were observed with IC_{50} values in 4.63 (SD = 0.86) - 24.57 (SD = 4.45) μM/mL range as measured by the CellTiter-GloTM luminescent cell viability assay. Euglobal-In-3 was most potent (IC_{50} = 4.63 [SD = 0.86] μM/mL) followed by eucalyptal D (IC_{50} = 5.20 [SD = 1.16] μM/mL) and E (IC_{50} = 7.22 [SD = 2.06] μM/mL) against KE-97 cells.	Wang et al[157]
E. maidenii† branches - resveratrol, piceatannol, gallic acid, and macrocapal G	MCH-7 (Breast); SMMC-7721 (liver); HL-60 (leukemia); SW480 (colon); A-549 (lung). Exhibited moderate inhibitory effects with IC_{50} values of 22.05, 22.05, 7.75, and 31.93 μM/mL, respectively, against HL-60 cells in the MTT assay. Only macrocapal G inhibited the growth of SMMC-7721 cells with an IC_{50} value of 26.75 μM. Tested compounds displayed no activity against breast, colon and lung cancer cell lines at 40 μM/mL.	Tian et al[151]
E. camaldulensis leaf - aqueous acetone	MCF-7 (breast); HEp-2 (larynx); HepG-2 (liver); HeLa (cervix); HCT-116, Caco-2 (colon). Reduction of cell viability in a dose-dependent manner was observed after a continuous exposure during a 48 h period. The extract was more potent against MCF-7 and HCT-116 cell lines with IC_{50} values of 36.5, and 33.3 μg/mL, respectively.	Singab et al[165]
E. camaldulensis fruit extracts - methanol, hexane, and dichloromethane Compounds - 3b-acetoxy-urs-11, 13(18)-dien-28-oic acid, 3b-hydroxy-urs-11-en-28,13b-olide, 3b-acetoxy-urs11-en-28,13b-olide, 3-acetylbetulinic acid, oleanolic acid, ursolic acid, b-amyrin acetate	A2780 (ovary). The fruit extracts showed cytotoxic effects with IC_{50} values from 17.2 - 17.5 μg/mL. All the tested triterpenoids exhibited moderate activity with ursolic and oleanolic acids showed most potency with IC_{50} values of 9.5 and 11.9 μg/mL, respectively.	Topçu et al[152]
E. torquata and *E. sideroxylon* stem, leaf and flower - essential oils	MCF7 (breast). The essential oils from *E. torquata* stem had a significant cytotoxic effect (IC_{50} = 1.34 mg/mL) followed by *E. torquata* leaf (IC_{50} = 5.22 mg/mL) and *E. sideroxylon* leaf (IC_{50} = 6.76 mg/mL). However, essential oils from leaf, stem, and flower of *E. sideroxylon* and *E. torquata* had no notable effect on HepG-2 cell line.	Ashour[153]
E. camaldulensis resin - methanolic solution	ECV-304 (bladder). Exerted cytotoxic effects with an IC_{50} value of 20.7 μg/mL in the MTT assay.	Al-Fatimi et al[142]

(Continued on next page)

TABLE 1. *(Continued)*

Type of Eucalypt Extracts/Compounds	In Vitro/In Vivo Studies and Observed Effect/Mechanism	References
E. cladocalyx leaf - Cladocalol (1) and its derivatives (2 and 3)	HL-60 (leukaemia). Compounds 1 and 2 showed similar cytotoxic effects with IC_{50} values of 42 ± 4 and 51 ± 1 μM/mL. These results indicated that the b-hydroxyl group might play an important role in cytotoxic activity of compound 1 and 2 as acetylation of cladocalol to give the b-monoacetate derivative (compound 3) led to a twofold increase in its IC_{50} value. It also hypothesised that the formyloxy group is not important for the cytotoxicity of cladocalol. The lower cytotoxicity of compound 3 could also be explained by the possible decrease in the binding affinity of the compound to the target molecule/s due to steric hindrance as a result of the addition of a more bulky group (i.e., an acetyl group).	Benyahia et al[143]
E. globulus leaf - essential oil	THP-1 (leukaemia). Inhibition of nuclear translocation of nuclear factor κB (NF-κB) induced by lipopolysaccharides (LPS) in THP-1 cells in a concentration-dependent manner. FITC-label NF-κB/p65 was only detected in the nuclei of LPS induced untreated THP-1 cells by indirect immunofluorescence and laser scanning confocal microscope.	Zhou et al[166]
E. benthamii leaf - essential oils, α-pinene, terpinen-4-ol, and γ-terpinene	Jurkat (T leukemia); J774A.1 (murine macrophage tumor); HeLa (cervix). Essential oils exerted more cytotoxic effect than α-pinene and γ-terpinene particularly against Jurkat and HeLa cells in the MTT assay. Cytotoxicity against Jurkat cells was probably mediated by apoptosis as demonstrated by lactate dehydrogenase activity assay. A decrease in cellular DNA content was observed in treated Jurkat cells.	Döll-Boscardin et al[155]
E. cypellocarpa leaf - Cypellocarpins A, B, and C, and chromene glucoside	Raji (lymphoma) and In vivo mouse skin tumor. Inhibited 12-*O*-tetradecanoyl-13-*O*-acetylphorbol (TPA) induced Epstein-Barr virus Early Antigen activation in Raji cells at 100 mol ratio/TPA. Cypellocarpins B and C and chromene glucoside also suppressed the in vivo 2-stage carcinogenesis induced with nitric oxide and TPA on mouse skin by reducing the percentage of tumor-carrying mice to 30% after 10 wk at 50 mol ratio of TPA relative to control.	Ito et al[144]
E. grandis leaf - Euglobal-G1 (EG-1)	In vivo mouse skin and mouse pulmonary tumor. Euglobal-G1, a PG derivative with a monoterpene in its skeleton, inhibited the 2-stage carcinogenesis induced by both TPA-type and non-TPA–type promoter (fumonisin B1) and inhibited the pulmonary tumorigenesis induced by 4-nitroquinoline-*N*-oxide and glycerol in vivo.	Takasaki et al[148]

[*] *Eucalyptus citriodora* is currently accepted as *C. citriodora.*

[†] *Eucalyptus maidenii* is currently accepted as *Eucalyptus globulus* subspecies *maidenii.*

C. citriodora indicates Corymbia citriodora ; E. cladocalyx, Eucalyptus cladocalyx; E. globulus, Eucalyptus globulus; E. robusta, Eucalyptus robusta.

and inhibit STAT3 signaling in PC cells.[121–124] It also caused G2/M cell cycle arrest and induced significant apoptosis (cleavage of caspase-3 and poly [ADP-ribose] polymerase) due to activation of ATM/Chk1 in BxPC-3 cells at 2.5 μM after 24 h of treatment.[125] Jutooru et al[126] reported that curcumin inhibited the growth of Panc-28 and L3.6pl cells in both in vitro and in xenograft models by downregulating the expression of p50 and p65 along with the downregulation of Sp1, Sp3, and Sp4 transcription factors, which are overexpressed in PC cells. The decrease in mitochondrial membrane potential and induction of reactive oxygen species in PC cells due to curcumin treatment were also observed.[126] Curcumin was also reported to inhibit PC tumor growth and aggressiveness by targeting a histone methyltransferase EZH2-miRNA regulatory circuit for epigenetically controlled gene expression and alter miRNA expression in BxPC-3 cells by upregulating miRNA-22 and downregulating miRNA-199a*.[127,128] In phase II clinical trial, 2 of 25 patients showed clinical biological activity after treatment with curcumin.[129] One had the ongoing stable disease for more than 18 months and the other had a brief yet marked tumor regression by 73% accompanied by 4- to 35-fold increases in serum cytokine levels (IL-6, IL-8, IL-10, and IL-1 receptor antagonists). In addition, peripheral blood mononuclear cells from patients exhibited downregulation of NF-κB, cyclooxygenase-2, and phosphorylated signal transducer and activator of transcription 3 expressions by curcumin.[129] In another phase II clinical trial, median survival time after initiation of curcumin (8 g/d) was 161 days and the 1-year survival rate was 19% in 21 patients.[130]

Capsaicin, an alkylamide found in chili peppers, has been reported to inhibit cell viability in a dose-dependent manner and induces apoptosis mediated through reactive oxygen species generation and mitochondrial death pathway in AsPC-1 and BxPC-3 cells in vitro.[131] It also markedly suppressed the growth of AsPC-1 pancreatic tumor xenografts in athymic nude mice by activation of JNK and increased cytosolic protein expression of

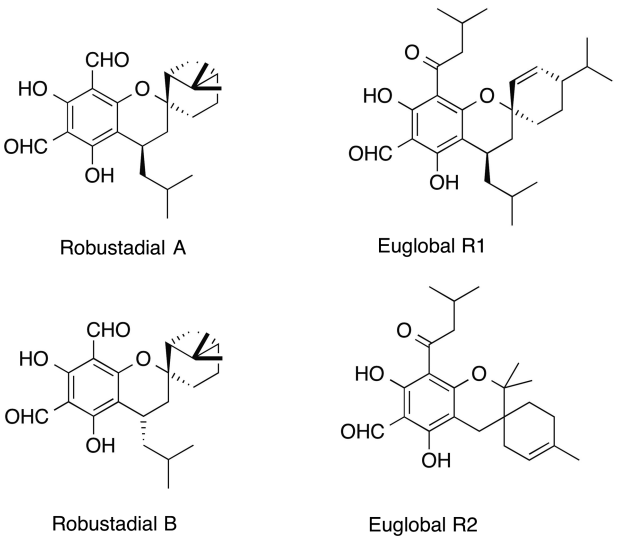

FIGURE 3. Structures of euglobals isolated from eucalypts.

Bax, cytochrome c, apoptosis-inducing factor, and cleaved caspase-3.[131] Bai et al[132] demonstrated that capsaicin can inhibit the progression of PanIN-1 to high-grade PanIN-2 and PanIN-3 by significantly reducing the phosphorylation of ERK, c-JUN, and Hedgehog/GLI1 activation in *LSL-KRAS(G12D)/Pdx1-Cre* mice. Similarly, it reduced Trx expression and dissociated Trx-ASK1 complex, which led to the activation of ASK1 and downstream effectors resulting in apoptosis in pancreatic tumor cells both in vitro and in vivo.[133]

Benzyl isothiocyanate, an agent found in cruciferous vegetables such as watercress, cabbage, cauliflower, mustard, and horseradish, has been reported to inhibit initiation, growth, and metastasis of PC by targeting key signaling pathways including Akt, Stat3, HDAC, and NF-κB both in vitro and in vivo.[60,134–141] These findings clearly underline the importance of plants as a source of bioactive compounds in developing novel therapeutic strategies against pancreatic and other forms of cancers.

RELEVANCE OF EUCALYPTS IN PC RESEARCH

Anticancer Properties of Eucalypts

Many volatile and nonvolatile constituents found in the eucalypt family of plants have been reported to possess a wide range of biological activities (Figs. 1, 2). In addition to their anti-inflammatory, antibacterial, antifungal, antiviral, larvicidal, and adulticidal properties, eucalypt extracts and their isolated compounds have displayed potent antitumor and cytotoxic properties in various studies.[142–148] Vuong et al[149] recently reviewed the anticancer activities of different eucalypt oils and extracts against a range of cancer cell lines in vitro including the following: human gastric (BGC-823, KE-97), colon (SW-620), liver (HEp-2, SMMC-7721, Huh-7), ovary (OVCAR-5, A2780), prostate (PC-3), cervix (HeLa), neuroblastoma (IMR-32), lung (HOP-62), breast (MDAMB-231, MCF7), human bladder carcinoma (ECV-304), HeLa, HL-60, murine macrophage (J774A.1), and Jurkat T lymphoma.[142,143,145,150–157] An updated list of key eucalypt species with anticancer activity is shown in Table 1. Mota et al[145] illustrated the potential of *Eucalyptus globulus* bark as a source of polyphenolic compounds with antiproliferative activity. Crude extracts using 80% ethanol showed significant antiproliferative activity against a breast cancer cell line (MDAMB-231). In vivo studies showed that *Corymbia citriodora* (synonym: *Eucalyptus citriodora*) and *E. camaldulensis* leaf extracts possess antitumor properties against Ehrlich ascites carcinoma (EAC).[150,164] Topçu et al[167] have demonstrated the DNA damaging and cytotoxic activities of the *E. camaldulensis* extract against the A2780 human ovarian cell line. The cytotoxic activity of a methanolic solution of *E. camaldulensis* was also illustrated against human ECV-304 cells (concentration that inhibits cell growth by 50% [IC50] = 20.7 μg/mL).[142]

Phloroglucinols (PGs) are an important class of polyphenolic natural products containing the 1,3,5-trihydroxy benzene moiety.[168] They are extensively reported in eucalypts. Grandinol, isolated from mature leaves of *Eucalyptus grandis (E. grandis)*, is the most studied acyl PG contains isovaleryl, formyl, and methyl substituents in its ring structure.[169] Grandinol has been shown to exert strong inhibitory effects on Epstein-Barr virus Early Antigen, a feature suggestive of antimelanoma activity.[170] In addition, naturally occurring PGs have shown a diverse range of biological activities including cancer chemopreventive, antitumor, antimalarial, and antifouling activities.[146] A newly identified PG group called the formylated PG compounds (FPGs) have gained increasing interest among the phenolic compounds found in eucalypt species.[146] Soliman et al[146] also stated that FPGs include the subtypes known as euglobals, macrocarpals, and sideroxylonals (Figs. 3, 4). The FPGs are almost exclusive to eucalypt species. Loxophlebal A, a type of FPG, was isolated after separation of sideroxylonals from the chloroform-methanol extract from leaves of *E. loxophleba* subspecies lissophloia by Sidana et al[171] (Fig. 4). The authors spectroscopically (1D- and 2D-NMR) determined the structure of loxophlebal A to be 3-desformyl sideroxylonal A. Loxophlebal A also exhibited greater antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermis*, and *Staphylococcus aureus* in comparison with sideroxylonals A, B, and C. They postulated that the removal of the formyl group from position 3 of sideroxylonal nucleus leads to an increase in antibacterial activity against both gram- and gram-negative strains (Fig. 4). The cytotoxic activity of 2 acyl PGs-sideroxylonal B and macrocarpal A isolated from the sideroxylonal-rich extract of the juvenile leaves of *Eucalyptus cinerea* was evaluated against 3 human cancer cell lines: MCF7 (breast carcinoma), HEp-2 (laryngeal carcinoma), and CaCo (colonic adenocarcinoma), and one normal human cell line: 10 FS (fibroblast).[146] Results indicated that sideroxylonal-rich extract, sideroxylonal B, and macrocarpal A possessed antiproliferative properties against the 3 tested human cancer cell lines and exhibited low cytotoxicity against the normal cell line indicating their selectivity. An in vivo study by Takasaki et al[148] reported that euglobal-G1 isolated from *E. grandis* leaf inhibited the 2-stage carcinogenesis in mouse skin and mouse pulmonary tumors. Different compounds identified from eucalypt such as euglobals (I, III, and G1), cypellocarpins A, B, and C have been demonstrated to exhibit strong antitumor promoting activity on 12-*O*-tetradecanoylphorbol-13-acetate–induced Epstein-Barr virus Early Antigen in vitro and on the 2-stage carcinogenesis in mouse skin tumors in vivo.[144,147,148,172] Cytotoxic effects of cladocalol and its

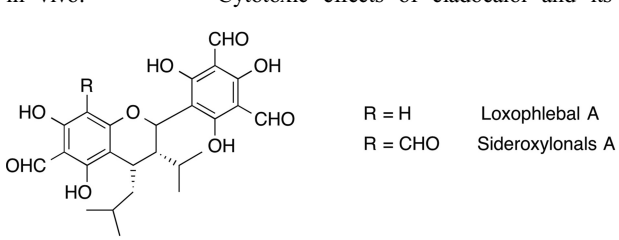

FIGURE 4. Structures of FPGs isolated from *Eucalyptus loxophleba*.

TABLE 2. Eucalypt Extracts, Phenolic Compounds, and Acids Reported in the Literature With Anti-PC Activity

	Anti-PC Activity	References	Also Reported in Pure or Derivative Form in Eucalypts
Type of eucalypt extracts			
Eucalyptus microcorys leaf and fruit - water and 70% ethanol	The leaf (aqueous) and fruit (ethanolic) extracts inhibited the growth of MIA PaCa-2 PC cells by at least 80% at 100 μg/mL in the MTT assay. Statistically similar IC_{50} values were observed in aqueous leaf (86.05 [SD = 4.75] μg/mL), aqueous fruit (64.66 [SD = 15.97] μg/mL) and ethanolic leaf (79.30 [SD = 29.45] μg/mL) extracts in MIA PaCa-2 cells in the CCK-8 assay. After 24 h of treatment, the MIA PaCa-2 cells displayed caspase 3/7-mediated apoptosis and morphological changes.	Bhuyan et al[158]	—
Angophora floribunda, *A. hispida*, *C. citriodora, and C. maculata* leaf - water	*A. floribunda* extract exerted significantly greater cell growth inhibition of 77.9 % followed by *A. hispida* with 62% at 100 μg/mL in MIA PaCa-2 cells with IC_{50} values of 75.58 and 87.28 μg/mL respectively. *C. citriodora* and *C. maculata* showed low cytotoxicity with cell growth inhibition values of 8.21 and 24.50%, respectively, at 100 μg/mL.	Bhuyan et al[178]	—
Phenolic compounds/acids			
Ellagic acid	Decreased NF-κB activity, thereby activated the mitochondrial death pathway, which is associated with loss of mitochondrial membrane potential (Δψm), cytochrome C release, and caspase-3 activation in MIA PaCa-2 and PANC-1 cells.	Edderkaoui et al[100]	Conde et al,[180] Osawa et al,[181] Kim et al,[182] Guo and Yang,[183] Santos et al[184]
	In vivo, dietary ellagic acid alone or in combination with embelin decreased tumor size and tumor cellularity in a subcutaneous xenograft mouse model of PC.	Edderkaoui et al[99]	
	Inhibited pancreatic tumor growth, angiogenesis and metastasis by suppressing Akt, SHh and Notch pathways in Balb C nude mice.	Zhao et al[98]	
Quercetin	Inhibited pancreatic tumor growth in vitro and in vivo via an increase in apoptosis.	Mouria et al,[185] Aghdassi et al,[186] Borska et al,[187] Angst et al[188]	Conde et al,[189] Okamura et al,[190] Conde et al,[191] Okamura et al[192] Abd-Alla et al[193] Ali et al[194] Proestos et al[195]
	Alone or in combination with sulforaphane, affected self-renewal potential, ALDH1 activity, induction of apoptosis, and inhibition of angiogenesis, NF-κB and EMT processes in in vitro and in vivo models of PC.	Zhou et al[61]	
	Reduced tumorigenicity by inhibition of tumor progression markers when PC cells were continuously exposed to quercetin.	Fan et al[62]	
Myricetin	Induced apoptosis through inhibition of the PI3K signaling pathway in PC cell both in vitro and in vivo.	Phillips et al[63]	Okamura et al[190] Abd-Alla et al[193] Hillis and Hingston,[196] Hillis[197]
Kaempferol	Inhibited cell proliferation and induced apoptosis in MIA PaCa-2 and PANC-1 cell lines in vitro.	Zhang et al[64]	Benyahia et al,[154] Okamura et al,[190] Conde et al,[191] Okamura et al,[192] Abd-Alla et al,[193] Hillis and Carle,[198] Venkateswara et al,[199] Hillis and Isoi[200]

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TABLE 2. *(Continued)*

	Anti-PC Activity	References	Also Reported in Pure or Derivative Form in Eucalypts
Apigenin	G2/M cell cycle arrest, downregulation of the overexpressed protein geminin, an increase in growth inhibitory effects of gemcitabine and abrogation of gemcitabine resistance, enhanced apoptosis and growth inhibition by downregulation of NF-κB activity with suppression of Akt activation.	Salabat et al,[67] Strouch et al,[68] Ujiki et al,[69] Lee et al[70]	Conde et al,[189] Conde et al,[191] Zhukovich et al,[201] Hillis and Carle,[202] Al-Sayed et al[203]
	Inhibited HIF-1α, GLUT-1, and VEGF mRNA and protein expression in PC cells in both normoxic and hypoxic conditions.	Melstrom et al[65]	
	Improved the efficacy of the chemotherapeutic drugs gemcitabine, cisplatin, 5-fluorouracil, and oxaliplatin	Johnson and Gonzalez de Mejia[71]	
Naringenin	Downregulated markers of EMT by inhibiting TGF-β1/Smad3 signaling pathway in the PC cells and thus decreased the invasiveness and metastasis	Lou et al[72]	Conde et al,[180] Conde et al,[189] Conde et al,[191] Ali et al,[194] Hillis and Carle,[202] Santos et al[204]
Genistein	Inhibited TGF-β1–induced invasion and metastasis in PANC-1 cells.	Han et al[79]	Mohamed and Ibrahim,[205] Ibrahim et al,[206] González et al[207]
	Inhibited Notch-1, NF-κB activity and Akt, increased growth inhibition and apoptosis induced by cisplatin, docetaxel, doxorubicin, gemcitabine and the gemcitabine + erlotinib combination in PC cells.	Banerjee et al,[76] Li et al,[80] Xia et al,[81] El-Rayes et al[83]	
Resveratrol	Damaged mitochondrial function leading to increased ROS, causes cell G0/G1 cycle arrest and apoptosis in PC cell lines.	Mo et al,[104] Sun et al,[208] Cui et al[209] Ding and Adrian,[210] Bortolotti et al[211]	Dhandayuthapani et al,[212] Saraf and Kaur[213]
	Targeted different signaling pathways in PC such as Hedgehog, FOXO, leukotriene A4 hydrolase, macrophage inhibitory cytokine-1, Src, and STAT3.	Boreddy and Srivastava,[60] Golkar et al,[102] Mo et al,[104] Roy et al,[105] Kotha et al,[214] Oi et al,[215] Chen et al[216]	
Epicatechin	Decreased in the proliferation, guanosine triphosphate-bound RAS protein, Akt phosphorylation and NF-κB transcriptional activity of premalignant and malignant *KRAS*-activated pancreatic ductal epithelial cells but induced no effect on normal cells.	Siddique et al[217]	Santos et al,[184] Kahla et al,[218] Vázquez et al[219]
	4-*O*-methyl-epicatechin and 3-*O*-methyl-epicatechin exhibit antiproliferative activity in BxPC-3 PC cells.	Delgado et al[220]	
	Stimulated mitochondrial respiration and oxygen consumption in PANC-1 cells but not in human normal fibroblasts. Sensitized PANC-1, U87, and MIA PaCa-2 cells but not the normal fibroblasts suggesting cancer cell selectivity. Also, enhanced Chk2 phosphorylation and p21 induction when combined with radiation in cancer cells but not in normal cells.	Elbaz et al[221]	

ALDH1 indicates aldehyde dehydrogenase isoform 1; C. citriodora indicates Corymbia citriodora; ROS, reactive oxygen species.

derivatives (from *Eucalyptus cladocalyx*) against human tumor cell line (HL-60) were also observed.[143,155] Hasegawa et al[173] also illustrated the inhibitory effect of globulusin A and eucaglobulin on melanogenesis in cultured murine melanoma B16F1 cells without any significant toxicity. In addition, they demonstrated the concentration-dependent suppression of inflammatory cytokine production, TNF-α, and IL- 1β in cultured human myeloma THP-1 cells co-stimulated with phorbol myristate acetate by globulusin A and eucaglobulin.

The oils and extracts of leaves, stems, and flowers of *Eucalyptus sideroxylon (E. sideroxylon)* and *Eucalyptus torquata (E. torquata)* were found to have antimicrobial and cytotoxic activities.[153] A sulphorhodamine B assay was used to evaluate the in vitro cytotoxic activities of oils and extracts against the human hepatocellular carcinoma cell line (HepG-2) and the human breast adenocarcinoma cell line (MCF7). Oils of *E. torquata* stems were the most cytotoxic against MCF7 cells followed by oils of *E. torquata* and *E. sideroxylon* leaves. Although oils from the species

did not exert cytotoxic effects on the HepG-2 cells, the report clearly demonstrated the potential that *E. sideroxylon* and *E. torquata* possess in terms of their antimicrobial and antitumor properties. Moteki et al[174] reported the ability of 1,8-cineole, a major constituent of *Eucalyptus* essential oils (Fig. 2), to suppress the growth of human leukemia cell lines by induction of apoptosis. Specific induction of apoptosis was also observed in Molt 4B, HL-60 human leukemia cell lines by fragmenting the DNA into oligonucleosomal-sized fragments in a concentration- and time-dependent manner.[174] Similarly, Murata et al[175] evaluated the antiproliferative effect of 1,8-cineole in human colon cancer cell lines HCT116 and RKO by the WST-8 and BrdU assays showing that it successfully suppressed the proliferation of human colorectal cancer cells by inducing apoptosis. In addition, they established that in xenotransplanted SCID mice, the 1,8-cineole group had significantly inhibited tumor progression compared with the control group. This compound has also been linked to inhibition of production of TNF-α, IL-1β, leukotriene B4, and thromboxane B2 in vitro.[176] Cytotoxic activity of another volatile compound terpinen-4-ol from *E. benthamii* against Jurkat J774A.1 has also been reported.[155]

Eucalypts as a Resource for Anti-PC Drug Development

Australia is home to more than 800 different species of eucalypts, which includes 9 *Angophora* species, 113 *Corymbia* species, and more than 750 *Eucalyptus* species. To date, however, biologically active constituents in only a few eucalypt species have been exploited for product development by the pharmaceutical industry.[177] Eucalypt research has largely focused on the exploitation of volatile compounds present in the essential oils. As a consequence, there is an immense opportunity of finding both volatile and nonvolatile novel anticancer compounds specific to PC, from Australian eucalypts.

As described previously, a number of in vitro and in vivo studies have reported the antitumor and cytotoxic properties of extracts and isolated individual phytochemicals from eucalypts. Although most of these studies explored the anticancer properties of eucalypt extracts and essential oils, there are only a few reports currently in the literature that underline the cytotoxicity of eucalypt extracts against PC cells. For instance, we have recently investigated the cytotoxic activity of 4 less explored eucalypt species (*Angophora floribunda [A. floribunda]*, *Angophora hispida [A. hispida]*, *Corymbia citriodora*, and *Corymbia maculata [C. maculata]*) against both primary and secondary pancreatic adenocarcinoma cells.[178] We found that aqueous extracts of *A. floribunda* and *A. hispida* inhibited the growth of MIA PaCa-2 cells by 77.91% (IC_{50} = 75.58 μg/mL) and 62.04% (IC_{50} = 87.28 μg/mL), respectively, at 100 μg/mL. In another study, we showed that aqueous extract of *Eucalyptus microcorys (E. microcorys)* leaf and ethanolic extracts of *E. microcorys* fruit inhibited the cell growth of glioblastoma, neuroblastoma, lung, and PC cells by more than 80% at 100 μg/mL.[158] It was also observed that aqueous *E. microcorys* leaf and fruit extracts at 100 μg/mL exerted cell growth inhibition of 89.96% (IC_{50} = 86.05 μg/mL) and 93.75 % (IC_{50} = 64.66 μg/mL), respectively, in MIA PaCa-2 cells. Caspase-3/7 mediated apoptosis and morphological changes of cells were also witnessed in MIA PaCa-2 cells after 24 h of treatment with aqueous *E. microcorys* leaf and fruit extracts. In addition, Vuong et al[179] previously showed that *Eucalyptus robusta* aqueous extract decreased cell viability of MIA PaCa-2, ASPC-1, and HPAF-II PC cells by 86%, 62%, and 47%, respectively, at 100 μg/mL.

As discussed earlier, an array of phenolic compounds such as ellagic acid, gallic acid, quercetin, naringenin, daidzein, myricetin, epicatechin, and apigenin, isolated from natural sources have been shown to exhibit anti-PC activity both in vitro and in vivo. It should be noted that these particular compounds are also reported extensively in eucalypts, which emphasize its potential as a source of novel therapeutic agents for the treatment of PC. Table 2 represents the phenolic compounds that have been investigated thus far both in vitro and in vivo for the development of possible chemotherapeutic drugs against PC. Studies showing the prevalence of some of these bioactive compounds in eucalypts are also listed in Table 2.

CONCLUSION AND FUTURE DIRECTIONS

Despite the fact that phenolic and volatile compounds that are also commonly found in eucalypts have been shown to exhibit anticancer activity against PC both in vitro and in vivo, only a limited number of studies in the current literature have directly demonstrated the cytotoxic properties of eucalypt extracts against PC cells and are most often limited to an in vitro experimental context. Moreover, the mechanisms of action of eucalypt extracts and their isolated compounds against PC are not entirely understood yet. Therefore, more in vitro and in vivo studies exploring the numerous anti-PC compounds present in Australian eucalypts are crucial in order to precisely understand their potential in PC therapy. Important aspects such as bioavailability, side effects, and toxicity of the eucalypt extracts and the isolated compounds should also be considered for further research. In addition, further investigations should be carried out to define the optimal conditions for the extraction and purification of bioactive compounds from eucalypts. Chemical modification of potent compounds to achieve better efficacy and specificity against PC should also be taken into account.

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1.2 Research hypothesis and aims

The literature review summarised various studies that have demonstrated the potential of phenolic and volatile compounds, which are also commonly found in Australian eucalypts, in the development of chemotherapeutic agents against PC. However, only a limited number of studies in the current literature have directly emphasised the cytotoxic properties of eucalypt extracts against PC cells. While a number of investigations have been directed towards the genus *Eucalyptus,* very little importance has been given to the other two eucalypt genera: *Corymbia* and *Angophora*. Therefore, the following research hypothesis was proposed:

Phytochemicals derived from Australian eucalypts can be used for the development of anticancer agents for pancreatic malignancies

This PhD project was conducted with the following approaches:

Aim 1

To optimize the extraction conditions for total phenolic compounds and antioxidants from the genus *Eucalyptus* using different solvents and extraction techniques.

A comparison between various extraction conditions and solvents for highest yields of TPC and antioxidants from *Eucalyptus robusta* was drawn and further implementation of the optimal extraction conditions on other selected *Angophora, Corymbia* and *Eucalyptus* species was carried out. This aim was further divided into the following studies:

i. Optimisation of microwave assisted extraction (MAE) conditions for total phenolic compounds (TPC) from *E. robusta* leaves using water as the solvent.
ii. Optimisation of ultrasound assisted extraction (UAE) conditions for TPC from *E. robusta* using water and different organic solvents.

iii. Conventional extraction of TPC, TFC, antioxidants, proanthocyanidins and saponins from *E. robusta* using water and different organic solvents and further implementation of the optimal solvent on other species of eucalypts.

Aim 2

To evaluate and screen the crude extracts for antiproliferative properties.

This aim was further divided in to the following studies:

i. Evaluation of crude extracts from selected species of *Eucalyptus* for cell growth inhibition against a panel of cancer cell lines using the MTT assay and against pancreatic cancer cell lines using the CCK-8 assay.
ii. Evaluation of crude extracts from selected species from the genera *Angophora* and *Corymbia* for cell growth inhibition against pancreatic cancer cell lines using the CCK-8 assay.

Aim 3

To evaluate the most promising crude extract (from Aim 2) for additional antimicrobial properties against different bacterial and fungal pathogens.

Aim 4

To study the phytochemical profile and molecular mechanisms of action of the most promising species of eucalypt.

This aim was further divided in to the following studies:

i. Bioassay guided fractionation of crude extracts using RP-HPLC.
ii. Antioxidant activity of the fractions using the ABTS, DPPH and CUPRAC assays.
iii. Antiproliferative activity of the fractions against pancreatic cancer cell lines.
iv. Mechanisms of action against the pancreatic cancer cells by using the cell cycle, Annexin V & Dead Cell, and Western blot assays.
v. Tentative identification of compounds in the active fraction by HPLC-ESI/MS/MS.

CHAPTER 2

Optimisation of extraction conditions for total phenolic compounds and antioxidants from the genus *Eucalyptus* using different solvents and extraction techniques

2.1 Introduction

For comprehensive characterisation of the phytochemical profile and associated bioactivity of eucalypts, it is crucial to optimise the extraction conditions and techniques. The literature provides very limited information about the optimisation of extraction conditions of bioactive compounds from eucalypts.

MAE is a novel extraction method that has gained significance recently, as it is reported rapid, simple, eco-friendly and economical due to the minimal amount of solvents, energy and extraction time required (Gharekhani et al., 2012; Ince et al., 2013; Verma et al., 2011). It is one of the most dominant trends of the 'green chemistry' movement (Saifuddin et al., 2014). Gharekhani et al. (2012) reported the efficiency of MAE over UAE and traditional extraction methods for extracting phenolic and flavonoid compounds from *E. camaldulensis* Dehn leaves. The authors reported that the extraction of phenolic and flavonoid compounds with MAE for 5 min was equivalent with 60 min of UAE and 24 h of traditional extraction. In contrast, ultrasound is a non-thermal process and hence, the thermal decomposition of heat-sensitive compounds during the extraction process can be minimised. Due to the two main principles - cavitation phenomena and the mechanical mixing effect, the extraction efficiency in UAE increases whereas the extraction time decreases (Ince et al., 2013). Compared to conventional extraction, UAE is more economical, rapid, simple and efficient. Therefore, it has been applied to extract bioactive compounds from various materials in the food, chemical and material industries (Liu et al., 2013). Ultrasonic parameters such as temperature, time and power highly impact the extraction yields of phytochemicals and antioxidants (Vuong et al., 2014). Conventional techniques involve different solid-liquid extraction procedures such as Soxhlet or reflux using water and other organic solvents (Ollanketo et al., 2002). In many cases conventional extraction can be quite effective in obtaining greater yields of phytochemicals than other modern techniques like MAE and UAE. Vuong et al. (2015b) optimised the extraction conditions for total phenolic content from *E. robusta* using water with the aid of response surface methodology (RSM). Similarly, the reliability of RSM in optimizing the

extraction of phenolic compounds from *E. globulus* bark has also been emphasized by Mota et al. (2012). Therefore, this aim was proposed to conduct three different studies to optimise various extraction parameters of MAE, UAE and conventional extraction of phenolic compounds from *E. robusta* using water and other organic solvents, as well as to determine the most efficient extraction technique. As *E. robusta* was readily available in the region, it was used as the representative of all three genera of eucalypts for the optimisation processes. Moreover, this approach was more economical and efficient as it minimised the time and resources required to optimise extraction conditions for every species individually. Easy, low-cost and widely accepted electron transfer based methods such as the ABTS, DPPH and CUPRAC assays were used to evaluate the antioxidant content of extracts.

2.2 Results and Discussion

The overall experimental design of the three studies are shown in Figure 1. The results obtained from the studies were published in the following three Research Papers:

Research Paper 1: **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. Microwave-assisted extraction of *Eucalyptus robusta* leaf for the optimal yield of total phenolic compounds. **Industrial Crops and Products (2015),** 69: 1–10. DOI: 10.1016/j.indcrop.2015.02.044

Research Paper 2: **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. Development of the ultrasonic conditions as an advanced technique for extraction of phenolic compounds from *Eucalyptus robusta*. **Separation Science and Technology (2016),** 52(1): 100-112. DOI: 10.1080/01496395.2016.1250777

Research Paper 3: **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. Investigation of phytochemicals and antioxidant capacity of selected *Eucalyptus* species using conventional extraction. **Chemical Papers (2015)**, 70(5) 567–575. DOI: 10.1515/chempap-2015-0237

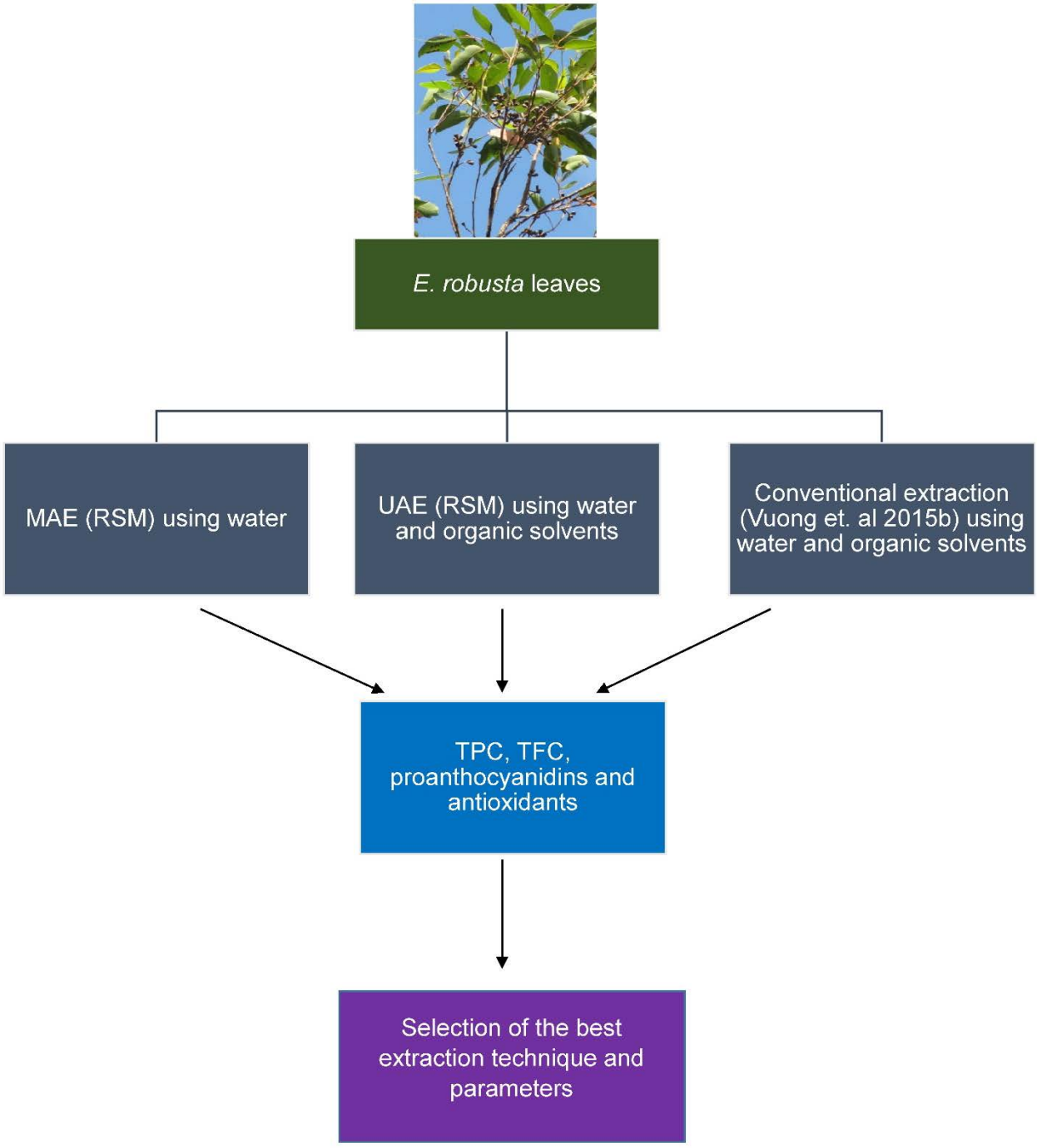

Figure 1: The overall experimental design of the three studies to optimise the extraction techniques and parameters for total phenolic content and antioxidants from *E. robusta.*

2.3 Conclusions

The effects of various MAE parameters such as power, extraction time, solvent, sample to solvent ratio and temperature; and UAE parameters such as ultrasonic temperature, time and power on the liberation of TPC, TFC, antioxidants, proanthocyanidins and saponins from *E. robusta* were evaluated. Water was found to be the best extraction solvent compared to other organic solvents such as ethanol, acetone, acetonitrile and ethyl acetate for extracting TPC, TFC, antioxidants and proanthocyanidins from *E. robusta* leaves both in UAE and conventional extraction. Furthermore, efficiency of conventional extraction was significantly greater than MAE and UAE in terms of TPC, TFC and antioxidants yields from *E. robusta* using water as the solvent. It was also established that *E. robusta* leaf had the highest TPC, TFC and antioxidant contents compared to the leaves of *E. saligna, E. microcorys, E. globulus* and *E. microcorys* fruit. Therefore, conventional extraction with its optimal parameters was selected as the best technique for the preparation of polyphenolic-rich crude extracts from all three genera of eucalypts: *Eucalyptus*, *Corymbia* and *Angophora* for further investigations into their potential bioactivity.

Industrial Crops and Products 69 (2015) 1–10

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Microwave-assisted extraction of *Eucalyptus robusta* leaf for the optimal yield of total phenolic compounds

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ARTICLE INFO

Article history:
Received 27 November 2014
Received in revised form 2 February 2015
Accepted 20 February 2015

Keywords:
Eucalyptus robusta
Microwave-assisted extraction
Optimisation
RSM
BBD

ABSTRACT

Eucalyptus robusta (*E. robusta*) has a significant value in traditional medicine and recently has been shown to possess many pharmacological properties *in vitro*. This study was designed to utilise microwave-assisted extraction (MAE) to yield optimal total phenolic content (TPC), total flavonoid content (TFC), proanthocyanidin levels and antioxidant capacity from *E. robusta* using water as the solvent, facilitated by the use of response surface methodology (RSM). A three-level-three-factor Box–Behnken design was implemented to elucidate the effect of irradiation time, power and sample-to-solvent ratio on the yields of these phytochemicals. The results highlighted the accuracy and reliability of RSM as a tool for predicting the yields of TPC, TFC, proanthocyanidins and total antioxidants using MAE. Sample-to-solvent ratio had the greatest impact on the TPC yield followed by power and irradiation time. The optimal MAE conditions for TPC and TFC were 3 min, 600 W power and 2 g/100 mL sample-to-solvent ratio. The experimental yield of TPC was 58.40 ± 1.03 mg GAE/g, and 19.15 ± 1.06 mg RE/g of TFC was obtained under these optimal conditions. These conditions, optimised for maximum TPC yield also liberated 62%, 64.6%, 66.3% and 67% of the maximum proanthocyanidins, ABTS, DPPH and CUPRAC values, respectively. This study revealed that MAE is a reliable and efficient method for extracting high yields of phytochemicals from *E. robusta*, with significant potential to be up-scaled for industrial, nutraceutical or pharmaceutical applications.

1. Introduction

Eucalyptus robusta is native to a narrow coastal area in south-eastern Australia (King and Skolmen, 1990). Due to the widely adaptable nature of the species, it has been introduced into many climatic regions around the world including tropical, subtropical and warm-temperate (King and Skolmen, 1990). Although, *E. robusta* is utilised in a range of ways including timber production, fuel, watershed protection, and windbreaks (King and Skolmen, 1990). *E. robusta* has been reported to possess many pharmacological properties that has seen it employed in traditional medicinal formulations. In Chinese traditional medicine, only the leaves are used to treat malaria (Konoshima and Takasaki, 2002), while in other parts of the world, both the leaves and bark have been used to treat an array of ailments including fever, skin diseases, dysentery, malaria and bacterial diseases (Nagpal et al., 2010).

The major non-volatile compounds found abundantly in *Eucalyptus* are phenolic compounds which contribute significantly to the antioxidant activities of extracts (Al-Sayed et al., 2012; Almeida et al., 2009). In general, several phenolic compounds such as gallic acid, protocatechuic acid, ellagic acid, quercetin, quercetin glycoside, naringenin, catechin, epicatechin, rutin, quercitrin, apigenin, and myricetin have been isolated from *Eucalyptus* extracts (Al-Sayed et al., 2012; Vázquez et al., 2012). Santos et al. (2012) identified epicatechin, catechin, quercetin-glucuronide, ellagic acid-rhamnoside, ellagic acid, galloyl-bis-hexahydroxydiphenoyl (HHDP)-glucose, gallic acid, chlorogenic acid and methyl-ellagic acid-pentose in *E. grandis, E. urograndis* and *E. maidenii* bark extracts. These results emphasized the high potential of *Eucalyptus* species as source of biologically active phenolic compounds (Santos et al., 2012). The anti-proliferative activity of the phenolic compounds present in *E. globulus* bark was illustrated by Mota et al. (2012). In addition, Santos et al. (2012) demonstrated that

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http://dx.doi.org/10.1016/j.indcrop.2015.02.044
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Table 1
Box–Behnken design and observed responses.

Run	Microwave conditions			Experimental values ($n = 3$)					
	Irradiation Time	Power	Ratio	TPC	TFC	Proanthocyanidins	Antioxidant capacity (mg TE/g)		
	(min)[a]	(%)[b]	(g:100 mL)	(mg GAE/g)	(mg RE/g)	(mg CAE/g)	ABTS	DPPH	CUPRAC
1	1	40	5	37.97	13.16	8.42	104.85	99.76	96.52
2	1	50	8	42.05	17.32	12.68	97.81	49.94	92.04
3	1	50	2	43.76	15.05	7.96	65.45	66.06	169.28
4	1	60	5	45.42	14.05	15.07	104.72	85.45	106.17
5	2	50	5	30.05	11.11	10.03	91.15	53.51	100.26
6	2	60	8	38.32	9.47	16.67	98.01	77.23	164.57
7	2	50	5	29.08	12.52	13.75	104.53	57.87	132.48
8	2	40	2	39.45	15.64	8.57	64.19	68.11	148.85
9	2	50	5	30.21	11.86	10.53	104.79	56.36	100.33
10	2	40	8	33.18	15.83	12.72	97.82	55.32	116.35
11	2	60	2	48.59	16.38	11.76	65.49	67.56	153.35
12	3	60	5	45.91	18.11	9.52	104.75	100.96	224.56
13	3	40	5	40.53	18	15.78	104.64	108.66	225.93
14	3	50	2	55.26	22.22	8.5	65.56	69.52	148.66
15	3	50	8	45.33	21.09	15.77	70.88	65.33	236.92

[a] Extraction time is 2X of irradiation time as 10 s ON and 10 s OFF strategy was implemented.
[b] 40, 50 and 60% power were equivalent to 480 W, 600 W and 720 W, respectively.

there is a positive correlation between phenolic contents and the antioxidant activities of *E. grandis, E. urograndis* and *E. maidenii* bark extracts. Antioxidants such as flavonoids and other phenolics have gained more attention in recent years as potential agents for their therapeutic values and cardioprotective, anticarcinogenic and antimutagenic properties (Fu et al., 2010; Gharekhani et al., 2012; Luis et al., 2014).

A recent study by Fu et al. (2010) illustrated the significant antioxidant capacity and total phenolic contents of the fruits of the *E. robusta,* indicating a strong correlation between the two parameters. In addition, essential oil from *E. robusta* has been shown to possess anti-microbial properties against various bacterial and fungal pathogens *in vitro* (Cimanga et al., 2002; Sartorelli et al., 2007) as well as larvicidal and adulticidal activity (Lucia et al., 2012). These findings clearly suggest strong bioactivity in the phytochemical profile of *E. robusta* and as such, prospective application in other fields of medicine. Comprehensive characterization of the phytochemical profile and associated bioactivity of *E. robusta* is, therefore, crucial. Optimal extraction conditions for polyphenolics have not yet been established to allow for further study of *E. robusta*. This manuscript presents the first comprehensive study assessing parameters associated with optimal extraction conditions.

Although, a number of organic solvents are used for extraction of phenolic compounds from *Eucalyptus*, water is inarguably the safest, cheapest and most environmentally friendly and accessible polar extraction solvent. Moreover, it is traditionally employed for plant bioactive extractions in the form of decoction and infusions (Goldsmith et al., 2014; Vuong et al., 2011a). Water has also been employed in the extraction of phenolic compounds from *E. globulus* and *Eucalyptus* hybrids (Almeida et al., 2009; Hasegawa et al., 2008; Santos et al., 2011; Chapuis-Lardy et al., 2002).

Microwave assisted extraction (MAE) is a novel extraction method that has gained significance recently due to its shortened extraction time and reduced solvent consumption (Gharekhani et al., 2012; Ince et al., 2013). It is one of the dominant trends of the 'green chemistry' movement (Saifuddin et al., 2014). Conventional Soxhlet extraction usually requires long extraction times leading to thermal degradation of phyto-constituents (Gharekhani et al., 2012). In MAE, the internal pressure of solid media is increased by microwaves resulting in enhanced extraction efficiency (Bayramoglu et al., 2008). This also reduces the deterioration of phenolic compounds (Ince et al., 2013). The current literature does not provide any information regarding the optimal utilization of water and MAE for extraction of phenolic compounds from *E. robusta*. Therefore, the present study was undertaken to optimize the three MAE parameters of irradiation time, power and sample-to-solvent ratio for extracting maximal levels of phenolic compounds from *E. robusta* using response surface methodology (RSM) and water as the solvent system (Yemis and Mazza, 2012).

2. Materials and methods

2.1. Plant materials

Fresh leaves of *E. robusta* were collected on 2nd April, 2014 from cultivated plants located at Ourimbah, Central Coast, NSW, Australia (latitude of 33.4°S, longitude of 151.4°E). The plants were authenticated by one of the authors (A.C.C.) and a voucher specimen deposited at the Don McNair Herbarium (Accession number 10492), at the University of Newcastle, NSW, Australia. The leaves were immediately transferred to the laboratory and stored at −20 °C to limit the degradation of phenolic compounds. Using a dry air oven, the leaves were dried at 70°C for 5 h before commencing the experiments. The leaves then were ground to a fine powder using a commercial grade blender (Rio™ Commercial Bar Blender, Hamilton Beach) and stored at −20 °C until required.

2.2. Microwave-assisted extraction (MAE)

A household microwave equipped with inverter technology (1200 W, Frequency 2450 MHz, Sharp Carousel, Japan) was used for optimizing the MAE conditions. Water was used as the solvent system, and parameter permutations as designed by the response surface methodology software were implemented. The extraction was carried out in sealed vessels and no evaporation was observed.

2.3. Response surface methodology (RSM)

JMP software (version 11) was utilized for RSM experimental design and analysis. A three-level-three-factor, Box–Behnken design was applied with three central point replicates for designing experimental conditions based on the results of preliminary single-factor-test (Liu et al., 2013) to elucidate the influence of the three primary independent microwave parameters: irradiation time (1–3 min), power (40–60% or 480–720 W) and sample-to-solvent ratio 2–8 (g/100 mL). Prior to irradiation, pre-leaching was performed for each suspension for 5 min. The leaf samples were irradiated with different power settings (40–60% or 480–720 W)

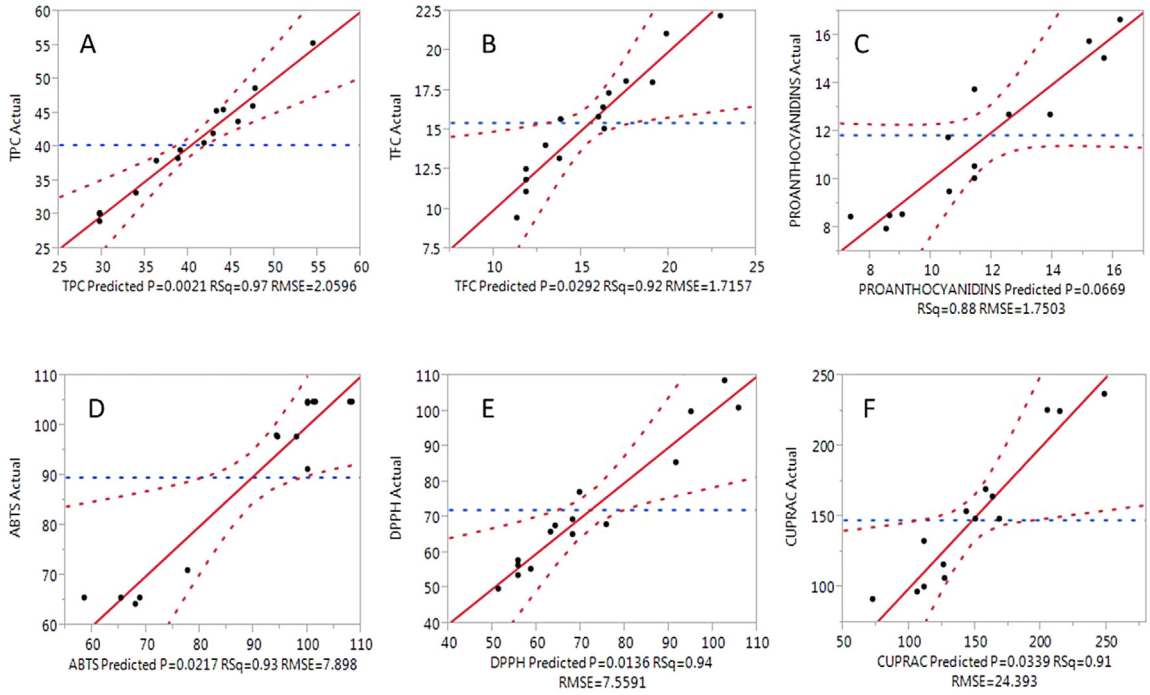

Fig. 1. Correlations between predicted and experimental total phenolic content (A), total flavonoids content (B), proanthocyanidins (C), ABTS total antioxidant capacity (D), DPPH free radical scavenging capacity (E) and Cupric reducing antioxidant power (F).

with a 10 s ON and 10 s OFF strategy. Thus, the total extraction time was in the range of 2–6 min. The extracts were immediately cooled to room temperature (ice bath), whereupon the solution volume was made up to 250 mL using distilled water. All extracts were filtered using 10 mL syringe fitted with a 0.45 μm cellulose syringe filter (Phenomenex Australia Pty. Ltd., NSW, Australia) prior to further investigation.

The independent variables and their code variable levels are shown in Table 1. Six experimental responses are presented in this work:

Y_{TPC} = total phenolic content (mg GAE/g), Y_{TFC} = total flavonoid content (mg RE/g), $Y_{PROANTHOCYANIDINS}$ = proanthocyanidins (mg CAE/g), Y_{ABTS} = ABTS (mg TE/g), Y_{DPPH} = DPPH (mg TE/g), Y_{CUPRAC} = CUPRAC (mg TE/g). A second order polynomial equation was postulated for each experimental response Y, as follows (Vuong et al., 2011b):

$$Y = \beta_0 + \sum_{i=1}^{k}\beta_i X_i + \sum_{\substack{i=1 \\ i<j}}^{k-1}\sum_{j=2}^{k}\beta_{ij}X_iX_j + \sum_{i=1}^{k}\beta_{ii}X_i^2$$

where various X_i values are independent variables affecting the responses Y; β_0, β_1, β_2, and β_3 are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively; and k is the number of variables. The three independent microwave parameters (Table 1) were assigned as: X_1 (irradiation time, min), X_2 (power, %) and X_3 (sample-to-solvent ratio, g/100 mL). Thus, the function containing these three independent variables can be expressed as follows:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}{X_1}^2 + \beta_{22}{X_2}^2 + \beta_{33}{X_3}^2$$

2.4. Determination of total phenolic content (TPC)

Colorimetric assessment of TPC was determined according to the method of Vuong et al. (2013). The extracts were diluted up to tenfold to fit within the optimal absorbance range (OD value 0.1–0.9). A calibration curve was constructed using Gallic acid as standard, with the results expressed as mg of gallic acid equivalents (GAE) per g of dry weight (mg GAE/g).

2.5. Determination of total flavonoid content (TFC)

Total flavonoid content (TFC) was determined as per the method described by Tan et al. (2014). Rutin was used as a standard and TFC expressed as mg of rutin equivalents (RE) per g of dry weight (mg RE/g).

Table 2
Analysis of variance for determination of model fit.

	TPC	TFC	Proanthocyanidins	Antioxidant capacity		
				ABTS	DPPH	CUPRAC
Lack of fit	0.052	0.10	0.67	0.52	0.0512	0.32
R^2	0.97	0.92	0.88	0.93	0.94	0.91
Adjusted R^2	0.92	0.76	0.66	0.79	0.83	0.75
PRESS	329.01	221.67	133.35	3317.61	4435.98	38109.59
F ratio of Model	19.98	6.20	4.11	7.13	8.84	5.77
P of model > F	0.0021	0.0292	0.0669	0.0217	0.0136	0.0339

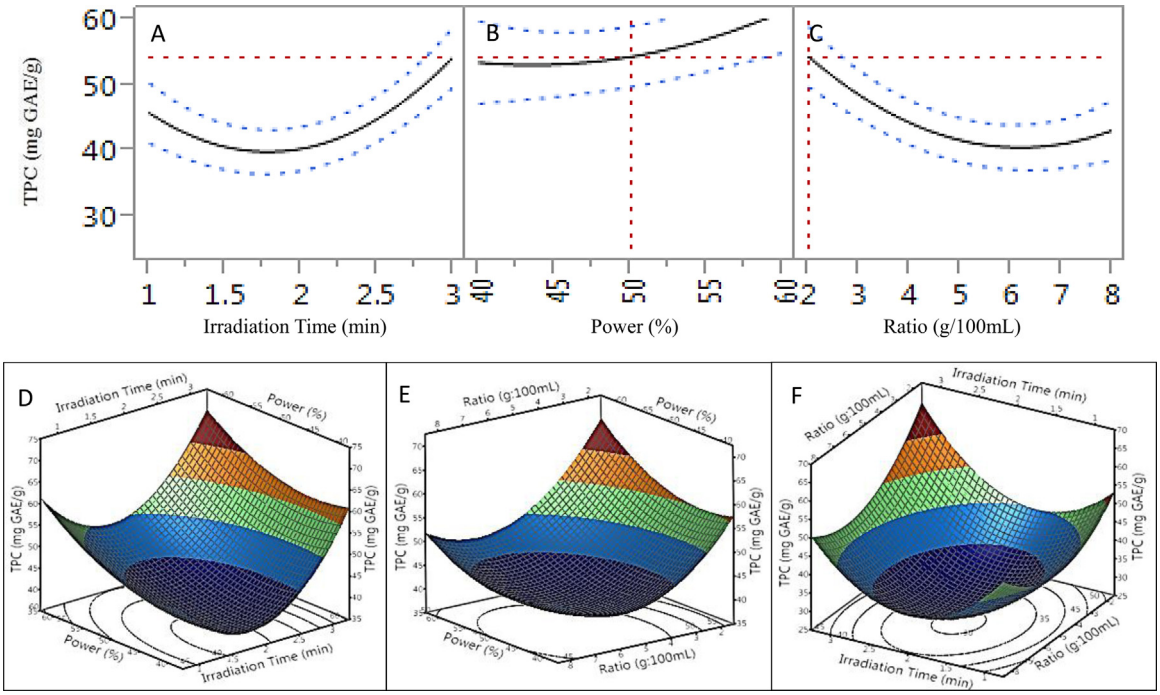

Fig. 2. Impact of irradiation time (1–3 min), microwave power (40–60%) and sample-to-solvent ratio (2–5 g/100 mL) on total phenolic content. The 2D impact of irradiation time, power and sample-to-solvent ratio were expressed in A–C; while their 3D effects were shown in D–F.

2.6. Determination of proanthocyanidins

The vanillin-HCl method was employed to estimate the proanthocyanidin content of the extract, as described by Broadhurst and Jones (1978). Catechin was used as the standard with proanthocyanidins concentration expressed as catechin equivalents (CAE) per g of dry weight (mg CAE/g).

2.7. Determination of antioxidant capacity

2.7.1. ABTS total antioxidant capacity (TAC)

TAC was measured using ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assay as described by Thaipong et al. (2006). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard

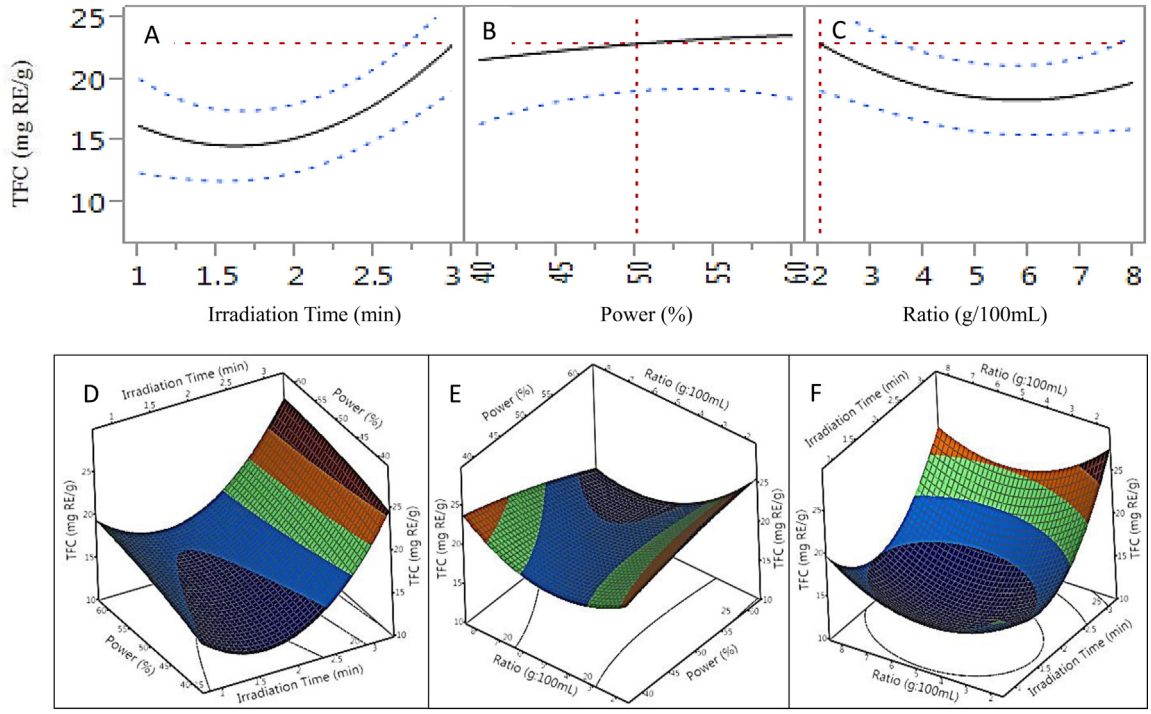

Fig. 3. Impact of irradiation time (1–3 min), microwave power (40–60%) and sample-to-solvent ratio (2–5 g/100 mL) on total flavonoid content. The 2D impact of irradiation time, power and sample-to-solvent ratio were expressed in A–C; while their 3D effects were shown in D–F.

for constructing the calibration curve. Results were expressed in mg trolox equivalents (TE) per g of dry weight (mg TE/g).

2.7.2. Free radical scavenging capacity

The free radical scavenging activity of the extract was analysed using the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay as described by Vuong et al. (2013). Results were expressed in mg trolox equivalents (TE) per g of dry weight (mg TE/g).

2.7.3. Cupric reducing antioxidant capacity (CUPRAC)

The iron chelating capacity of the extract was analysed using the cupric ion reducing antioxidant capacity (CUPRAC) assay as described by Apak et al. (2004). Trolox was used as the calibration standard, with results expressed in mg trolox equivalents (TE) per g of dry weight (mg TE/g).

2.8. Statistical analysis

For establishing the model equation to graph the 3D- and 2D-contour plots of variable response, and to predict optimal values for the three response variables, JMP software was used. All experiments were performed in triplicate and the results averaged. The significance of the interactions between the variables can be visualized by the circular or elliptical shapes of the contour plot (Bai et al., 2010). SPSS statistical software (version 16.0) was utilised to compare the means analysis by independent samples *t*-test. At $p < 0.05$, the differences between the mean values in the performed experiments were taken to be statistically significant.

3. Results and discussion

3.1. Fitting the models

Fitting the models for TPC, TFC, proanthocyanidins and antioxidant capacity of the *E. robusta* extracts is crucial to elucidate how precisely the RSM mathematical model can predict ideal variances and represent the correlations between the selected parameters of microwave extraction. The analysis of variance of the Box–Behnken design for determining the model fit is illustrated in Table 1 and Fig. 1.

In the case of TPC, the coefficient of determination (R^2) of the model was 0.97, signifying a 97% match between the predicted and experimental data (Fig. 1A). The *p*-value for lack of fit was estimated to be 0.052 further specifying that the lack of fit of the model was not significant ($p > 0.05$). Moreover, the predicted residual sum of squares (PRESS) value (which is useful when comparing the predictive power of multiple models) was found to be 329.01. The *F* value of the model was estimated to be 19.98 which in turn indicated the competency of the model in predicting the extraction yields of TPC. *p*-Value inferior to 0.05 or 0.01 gives the significance of the model considering a confidence interval of 95% or 99%, respectively (Mota et al., 2012). The *p*-value of the model was found to be 0.0014.

Fitting the model for TFC was also established. The results indicated that R^2 value of the model was 0.92 (Fig. 1B) suggesting a strong correlation between predicted and experimental data (92% data match). Likewise, the *p*-value for lack of fit, PRESS, *F* value and *p*-value of the model were 0.10, 221.67, 6.20 and 0.0292, respectively, further verifying the reliability of the model in predicting the yields of TFC in MAE.

The RSM mathematical model was also successful in predicting the amount of proanthocyanidins liberated during predefined MAE conditions with $R^2 = 0.88$ (Fig. 1C) and an insignificant lack of fit value of 0.67 (Table 2). Additionally, the PRESS and *F* values of the model were estimated as 133.35 and 4.11, respectively, which provided concrete evidence in favor of reliability of the model.

In a similar manner, the results for three antioxidant assays: ABTS, DPPH and CUPRAC also supported the efficiency of the model. The R^2 values of ABTS, DPPH and CUPRAC were 0.93, 0.94 and 0.91, respectively (Fig. 1D–F). The *p*-values for lack of fit for all the assays were found insignificant ($p > 0.05$, Table 2). The *p*-values (0.0217, 0.0136 and 0.0339 respectively), *F* values (7.13, 8.84 and 5.77 respectively) and PRESS values (3317.61, 4435.98 and 38,109.59

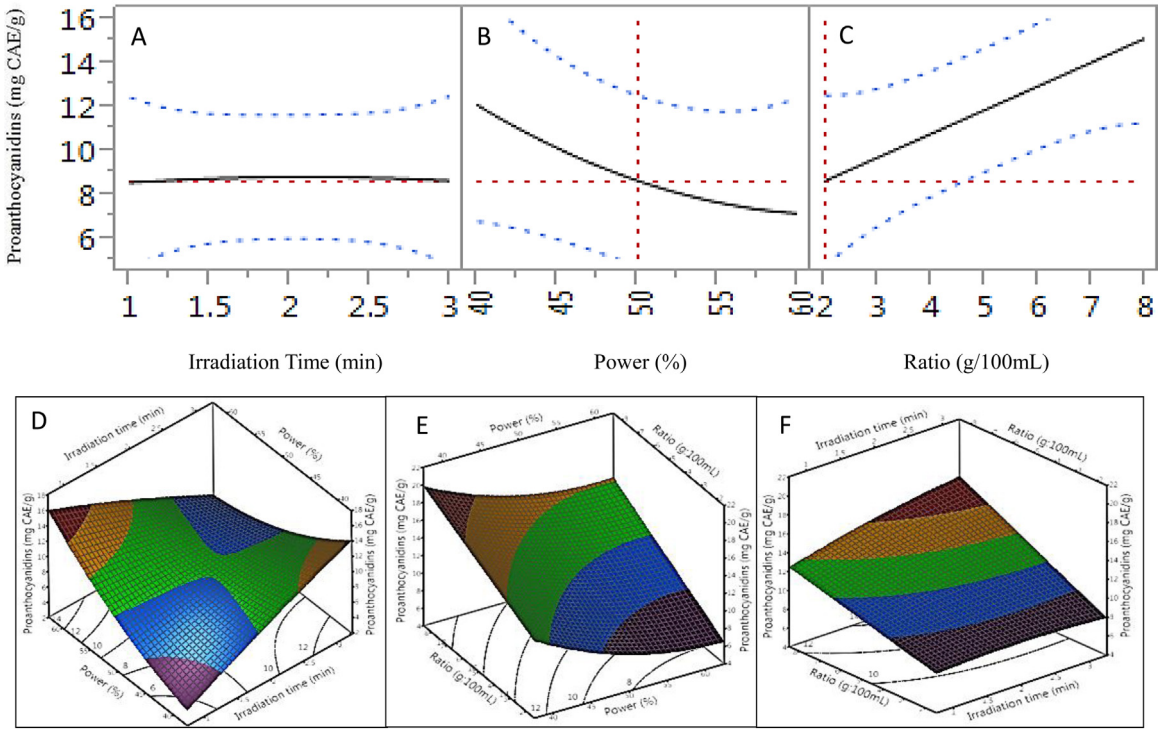

Fig. 4. Impact of irradiation time (1–3 min), microwave power (40–60%) and sample-to-solvent ratio (2–5 g/100 mL) on proanthocyanidins. The 2D impact of irradiation time, power and sample-to-solvent ratio were expressed in A–C; while their 3D effects were shown in D–F.

respectively) of the model also established the competency of these models in accurately predicting the antioxidant capacity of *E. robusta* extracts.

By applying multiple regression analysis on the experimental data, a predicted response, *Y* for the TPC, TFC, proanthocyanidins and antioxidant capacity of the *E. robusta* extracts can be expressed by the following second-order polynomial equations:

$$Y_{TPC} = 29.78 + 2.22X_1 + 3.38X_2 - 3.52X_3 - 0.51X_1X_2 - 2.05X_1X_3 - 1.00X_2X_3 + 9.69{X_1}^2 + 2.98{X_2}^2 + 7.12{X_3}^2$$

$$Y_{TFC} = 11.83 + 2.47X_1 - 0.57X_2 - 0.69X_3 - 0.19X_1X_2 - 0.84X_1X_3 - 1.177X_2X_3 + 4.29{X_1}^2 - 0.29{X_2}^2 + 2.79{X_3}^2$$

$$Y_{PROANTHOCYANIDINS} = 11.44 + 0.67X_1 + 0.94X_2 + 2.63X_3 - 3.22X_1X_2 + 0.63X_1X_3 + 0.18X_2X_3 - 0.21{X_1}^2 + 0.98{X_2}^2 + 0.01{X_3}^2$$

$$Y_{ABTS} = 100.16 - 3.37X_1 + 0.18X_2 + 12.97X_3 + 0.05X_1X_2 - 6.76X_1X_3 - 0.27X_2X_3 - 0.93{X_1}^2 + 5.51{X_2}^2 - 24.29{X_3}^2$$

$$Y_{DPPH} = 55.91 + 5.40X_1 - 0.08X_2 - 2.93X_3 + 1.65X_1X_2 + 2.98X_1X_3 + 5.61X_2X_3 + 19.22{X_1}^2 + 23.56{X_2}^2 - 12.42{X_3}^2$$

$$Y_{CUPRAC} = 111.02 + 46.50X_1 + 7.62X_2 - 1.28X_3 - 2.75X_1X_2 + 41.37X_1X_3 + 10.93X_2X_3 + 34.10{X_1}^2 + 18.16{X_2}^2 + 16.59{X_3}^2$$

3.2. Effect of microwave independent variables on TPC of E. robusta extracts

The *p*-values are used to check the significance of each coefficient, which consecutively may indicate the pattern of the interaction between the variables (Bai et al., 2010). All the three independent microwave variables: irradiation time (1–3 min), power (40–60% or 480–720 W) and sample-to-solvent ratio (2–8 g/100 mL) had significant impact on the extraction yield of TPC within *E. robusta* extracts ($p < 0.05$, Table 3). These observations are indicative of findings illustrating the correlations between irradiation/extraction time, microwave power and solvent to raw material ratio in previous studies relating to the extraction of TPC from prune (*Prunus domestica*) (Haddadi-Guemghar et al., 2014) and apple pomace (Bai et al., 2010) using MAE. In our study, however, we observed that the sample-to-solvent ratio had the greatest impact on the TPC yields, followed by power and time (based on the *p*-value). An increase in sample-to-solvent ratio resulted in a decrease in TPC yield (Fig. 2). Parallel observations were made by Ince et al. (2013) who indicated that a decrease in solid-to-solvent ratio increased total phenolic content significantly in the microwave-based extraction of *Melissa officinalis*. In the case of irradiation time (*t*), the TPC yield initially dropped from $t = 1$ to 1.75 min and then increased again reaching a maximum of $t = 3$ min (Fig. 2). The level of TPC also increased with an increase in microwave power from 40 to 50% (Fig. 2). Interaction between independent variables irradiation time × power, irradiation time × ratio and power × ratio had no significant impact on the extraction yield of TPC ($p > 0.05$, Table 3). The negative impact of the sample-to-solvent ratio on the extraction yields can be explained by the inhibitory effect that a highly dense suspension possesses on the liberation of contents within a cell. As the sample-to-solvent ratio increases, suspension density increases, resulting in less effective solvation of the liberated cellular contents.

3.3. Effect of microwave independent variables on TFC of E. robusta extracts

In the case of TFC, irradiation time was the only single factor found to have a significant impact ($p < 0.05$, Table 3). These findings are in agreement with previous studies on wheat bran (*Triticum* spp.) (Singh et al., 2012) and *Inula helenium* (Guo et al., 2013) which concluded that increased microwave irradiation time increases flavonoid yield. Wang et al. (2012) also suggested that repeated extraction times can increase the yields of TFC in *Toona sinensis* leaves. Similar to TPC, the interaction between independent variables – irradiation time × power, irradiation time × ratio and power × ratio had no significant influence on the yields of TFC ($p > 0.05$, Table 3).

3.4. Effect of microwave independent variables on proanthocyanidis of E. robusta extracts

Sample-to-solvent ratio is the only independent variable found to exert significant influence on the extraction yield of proanthocyanidins ($p < 0.05$, Table 3), increasing as the sample-to-solvent ratio increased, reaching a maximum at a concentration of 8 g/100 mL (Fig. 4). Interaction between factors irradiation

Table 3
Analysis of variance for the experimental results.

Parameter	DF	TPC		TFC		Proanthocyanidins		Antioxidant capacity					
					ABTS		DPPH		CUPRAC				
		Estimate	Prob > |t|	Estimate	Prob > |t|	Estimate	Prob > |t|	Estimate	Prob > |t|	Estimate	Prob > |t|	Estimate	Prob > |t|
β_0	1	29.78	<.0001[a]	11.83	<.0001[a]	11.44	<.0001[a]	100.16	<.0001[a]	55.91	<.0001[a]	111.02	0.0005[a]
β_1	1	2.22	0.0281[a]	2.47	0.0095[a]	0.67	0.32	−3.37	0.28	5.4	0.09	46.5	0.0030[a]
β_2	1	3.38	0.0056[a]	−0.57	0.38	0.94	0.18	0.18	0.94	−0.082	0.97	7.62	0.41
β_3	1	−3.52	0.0047[a]	−0.69	0.3	2.63	0.0081[a]	12.34	0.0056[a]	−2.93	0.32	−1.28	0.88
β_{12}	1	−0.51	0.6364	−0.19	0.82	−3.22	0.0142[a]	0.05	0.98	1.65	0.68	−2.75	0.83
β_{13}	1	−2.054	0.1026	−0.84	0.36	0.63	0.49	−6.76	0.14	2.98	0.46	41.37	0.0194[a]
β_{23}	1	−1	0.3756	−1.77	0.09	0.18	0.83	−0.27	0.94	5.61	0.19	10.93	0.42
β_{11}	1	9.69	0.0003[a]	4.29	0.0048[a]	−0.21	0.81	−0.93	0.82	19.22	0.0045[a]	34.1	0.0435[a]
β_{22}	1	2.98	0.0388[a]	−0.29	0.75	0.98	0.33	5.51	0.23	23.56	0.0019[a]	18.16	0.21
β_{33}	1	7.12	0.0012[a]	2.79	0.0259[a]	0.011	0.99	−24.29	0.0020[a]	−12.42	0.0251[a]	16.59	0.24

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

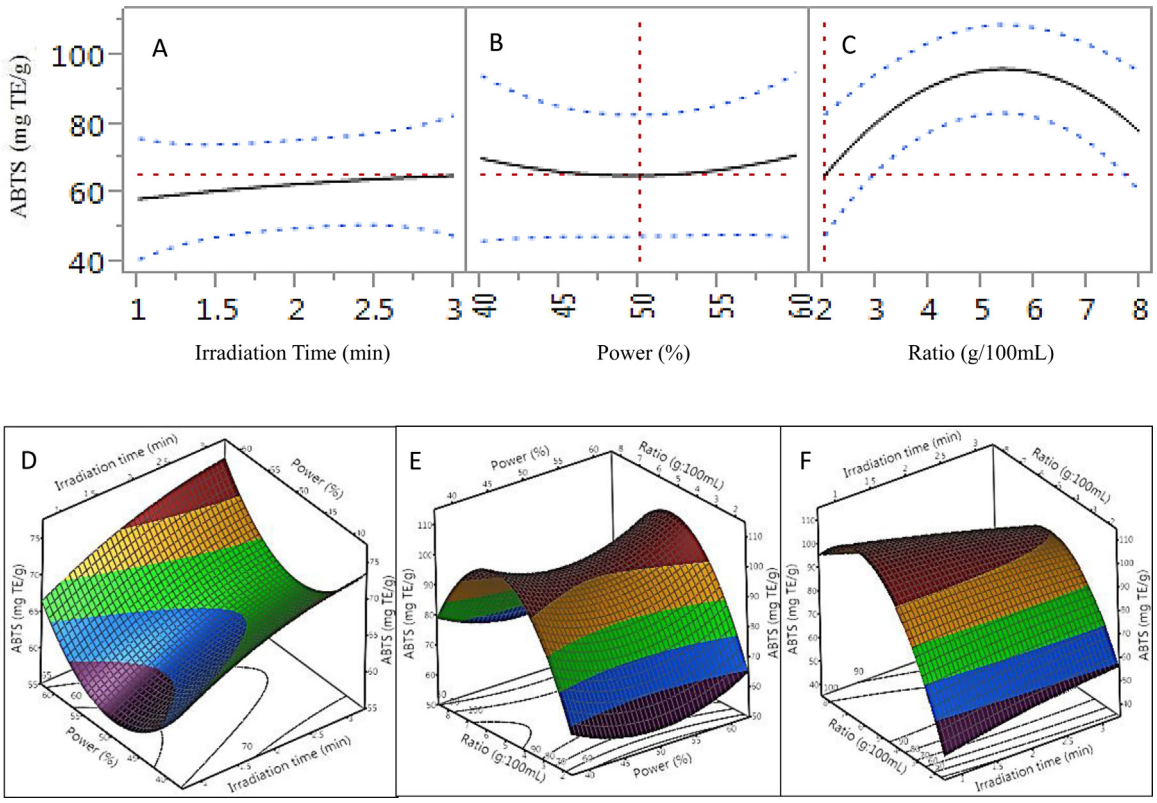

Fig. 5. Impact of irradiation time (1–3 min), microwave power (40–60%) and sample-to-solvent ratio (2–5 g/100 mL) on ABTS total antioxidant capacity. The 2D impact of irradiation time, power and sample-to-solvent ratio were expressed in A–C; while their 3D effects were shown in D–F.

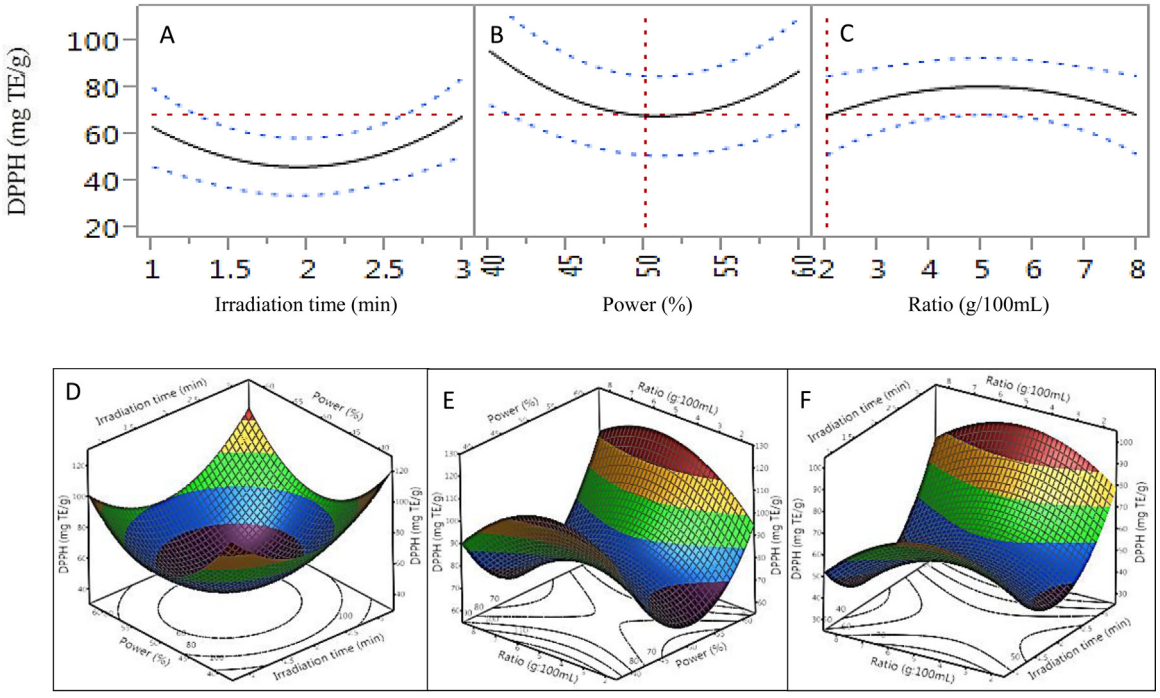

Fig. 6. Impact of irradiation time (1–3 min), microwave power (40–60%) and sample-to-solvent ratio (2–5 g/100 mL) on DPPH free radical scavenging capacity. The 2D impact of irradiation time, power and sample-to-solvent ratio were expressed in A–C; while their 3D effects were shown in D–F.

time × power was also shown to impact the yield of proanthocyanidins ($p < 0.05$, Table 3). Previous studies on the *Larix gmelini* bark, indicated that very high microwave power can cause the plant samples to be thermally compromised, leading to decreased extraction yield (Yang et al., 2012). This supported our observations of a negative correlation between microwave power and proanthocyanidin extraction yield. The proanthocyanidin content plateaued between 1 and 3 min of irradiation time (Fig. 4).

3.5. Effect of microwave independent variables on antioxidant capacity of E. robusta extracts

The impact of microwave conditions on the antioxidant capacity of *E. robusta* was estimated using three different antioxidant assays: ABTS, DPPH and CUPRAC, as the antioxidant capacity of a sample can fluctuate depending on the assays used (Thaipong et al., 2006). Sample-to-solvent ratio was the only factor that influenced the antioxidant capacity significantly, as revealed by the ABTS assay ($p < 0.05$, Table 3). Based on the *p*-values, the degree of effect of the microwave independent variables can be represented as following order: ratio > time > power. The interaction between variables time × power, irradiation time × ratio, and power × ratio was shown to have no noteworthy consequences on total antioxidant capacity *via* the ABTS assay. An increase in total antioxidant capacity was observed with an increase in sample-to-solvent ratio that reached a maximum in between the ratio of 5–6 (g/100 mL) and then decreased, reaching the minimum value at a ratio of 8 g/100 mL (Fig. 5). Previous studies on star anise oil from *Illicium verum* Hook.f by Cai et al. (2013) suggested that the ABTS scavenging activity of microwave extracted oil initially improved with increasing concentration of the oil, then plateaued, reaching equilibrium, resulting in no further change.

Based on the observations made in the DPPH assay, it was concluded that none of the independent variables had a significant influence on the free radical scavenging capacity of *E. robusta* extracts ($p > 0.05$, Table 3). On the basis of *p*-values, the magnitude of influence of the independent variables was determined to be: time > ratio > power. The antioxidant value initially decreased, reaching a minimum at $t = 2$ min before improving between $t = 2$–3 min (Fig. 6). A similar pattern was observed for power, whereas an increase in sample-to-solvent ratio (2–5 g/100 mL) led to increase in free radical scavenging capacity (Fig. 6).

Haddadi-Guemghar et al. (2014) also reported that DPPH scavenging capacity increases with an increase in irradiation time in prune extracts. Only the influence of solvent concentration on the free radical scavenging capacity of extracts from potato peel, as assessed by DPPH, was illustrated earlier but not the other microwave parameters (Singh et al., 2011). Interestingly, the CUPRAC assay confirmed that the cupric reducing antioxidant capacity (CUPRAC) was influenced by time ($p < 0.05$, Table 3) but not the other microwave independent parameters. Based on the *p*-values, the order of influence on CUPRAC was determined as: time > power > ratio. The interaction between independent variables time × ratio was also shown to be significantly influencing the CUPRAC of *E. robusta* ($p < 0.05$, Table 3). CUPRAC was observed to increase with time (2–3 min) with an initial decline (1–2 min) and ratio 2–8 g/100 mL, (Fig. 7). An increase in power from 50 to 60% seemed to slightly improve the CUPRAC (Fig. 7).

3.6. Optimization and validation of the models

On the basis of the RSM predictive models and values shown in Figs. 2 and 3, the ideal MAE conditions for the extraction of TPC and TFC from *E. robusta* were estimated to be: irradiation time = 3 min, power 50% (600 W) and sample-to-solvent ratio of 2 g/100 mL; optimal conditions for proanthocyanidins (Fig. 4) extraction were: 2 min – 40% power – 8 (g/100 mL) sample-to-solvent ratio; and 3 min – 40% – 5 (g/100 mL) sample-to-solvent ratio for the ABTS and DPPH antioxidant capacity (Figs. 5 and 6). Estimation of optimal conditions for CUPRAC were: 3 min – 40% – 8 sample-to-solvent ratio (Fig. 7). Under the optimal extraction conditions for TPC and TFC, 62%, 64.6%, 66.3% and 67% of the maximum proanthocyanidins, ABTS, DPPH and CUPRAC values could be obtained, respectively, under their respective ideal conditions as described above. Hence, it was concluded that the optimal MAE conditions for extraction of TPC, TFC, proanthocyanidins and antioxidant activity of *E. robusta* were as follows: irradiation time 3 min, microwave power 50% (600 W) and sample-to-solvent ratio of 2 (g/100 mL).

Validation experiments were performed under the above-mentioned optimal conditions to validate the adequacy of the models. Predicted and experimental ($n = 3$) results are depicted

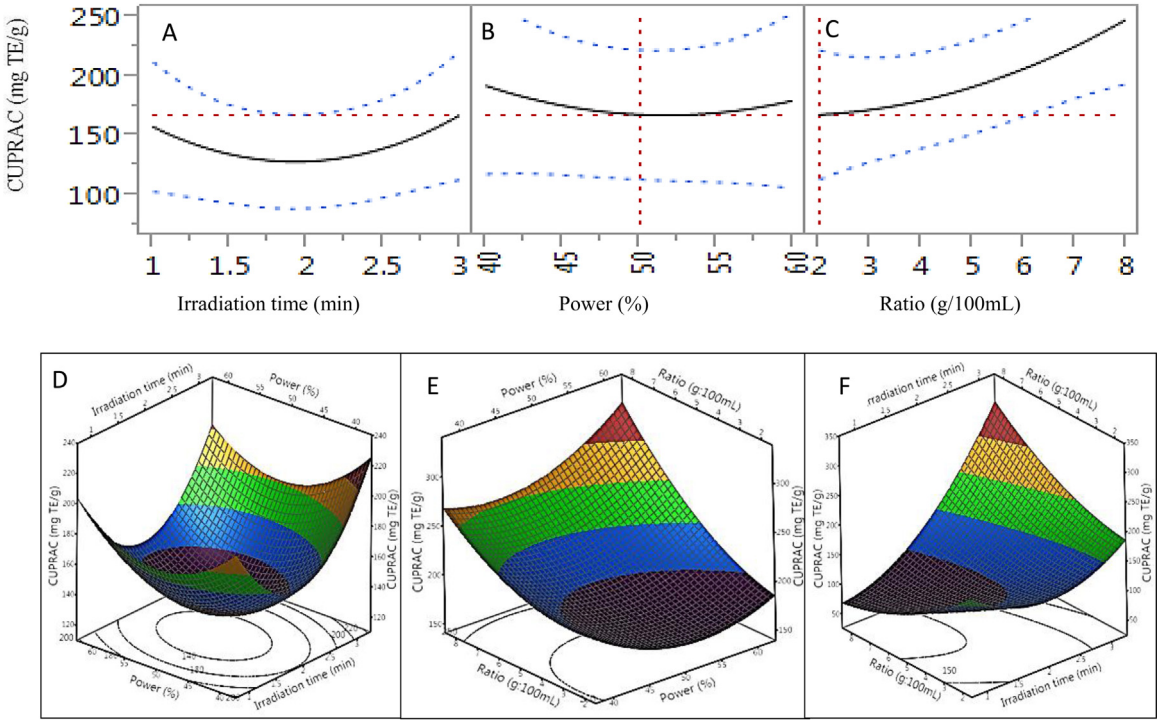

Fig. 7. Impact of irradiation time (1–3 min), microwave power (40–60%) and sample-to-solvent ratio (2–5 g/100 mL) on cupric reducing antioxidant capacity. The 2D impact of irradiation time, power and sample-to-solvent ratio were expressed in A–C; while their 3D effects were shown in D–F.

Table 4
Validation of the predicted value for TPC, TFC, Proanthocyanidins and antioxidant capacity.

	Values	
	Predicted	Experimental ($n = 3$)
TPC (mg GAE/g)	54.40 ± 6.48^a	58.40 ± 1.03^a
TFC (mg RE/g)	22.94 ± 5.40^a	19.15 ± 1.06^a
Proanthocyanidins (mg CAE/g)	8.64 ± 5.51^a	6.23 ± 0.47^a
ABTS (mg TE/g)	65.33 ± 24.86^a	74.95 ± 0.33^a
DPPH (mg TE/g)	68.07 ± 23.79^a	67.95 ± 0.038^a
CUPRAC (mg TE/g)	168.14 ± 76.79^a	143.70 ± 5.42^a

All the values are means ± standard deviations and those in the same row not sharing the same superscript letter are significantly different from each other ($p < 0.05$).

in Table 4. These results showed close correlations between the predicted and experimental values indicating the adequacy of the models to predict the optimal MAE conditions. Therefore, these conditions were recommended for future extractions of TPC, TFC, proanthocyanidins and antioxidants of *E. robusta* leaves. These findings also justified that RSM is a reliable and effective tool for modelling and optimizing extraction conditions. In addition, the findings validated that water is an efficient solvent system for extracting phenolic compounds from *E. robusta* in terms of its non-hazardous, economical, environment friendly and accessible nature in comparison to organic solvents.

4. Conclusion

Box–Behnken Design was successfully implemented for optimizing the MAE parameters. RSM was established to be an appropriate and reliable tool in evaluating the influence of three independent parameters (irradiation time, power and sample-to-solvent ratio) for extractions of TPC from *E. robusta*. Sample-to-solvent ratio had the greatest impact on the TPC yield of *E. robusta* followed by power and irradiation time. Sample-to-solvent ratio was also found to have the major influence on the yield of proanthocyanidins and ABTS antioxidant capacity. From the results obtained, it can be concluded that the optimal extraction conditions for TPC, TFC, proanthocyanidins and antioxidant activity of *E. robusta* were: irradiation time 3 min, microwave power 50% (600 W) and sample-to-solvent ratio of 2 g/100 mL. This study has the potential to be particularly valuable for industrial scale MAE of phytochemicals from *E. robusta* using water. The findings highlight the efficiency of MAE and its importance as an alternative method of extraction to the conventional procedures. This work also emphasises the need for further study to isolate and characterize the phytochemical profile of *E. robusta* leaves.

5. Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We acknowledge the following funding support: Ramaciotti Foundation (ES2012/0104); Cancer Australia and Cure Cancer Australia Foundation (1033781); the Hunter Cancer Research Alliance (HCRA); the Hunter Medical Research Institute (HMRI) and the University of Newcastle.

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SEPARATION SCIENCE AND TECHNOLOGY
2017, VOL. 52, NO. 1, 100–112
http://dx.doi.org/10.1080/01496395.2016.1250777

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Development of the ultrasonic conditions as an advanced technique for extraction of phenolic compounds from *Eucalyptus robusta*

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ABSTRACT

This study is designed to develop ultrasonic conditions as an advanced technique for optimal recovery of phenolics and antioxidants from *Eucalyptus robusta* leaf and to evaluate the impact of solvents, temperature, sonication time and power on ultrasound-assisted extraction of these compounds. Temperature has the greatest impact on the total phenolic content (TPC) yield followed by time and power. A yield of 163.68 ± 2.13 mg GAE/g of TPC is observed using 250 W ultrasonic power for 90 min at 60°C with water. This study validates UAE as an efficient, green, and sustainable technique for extracting phenolics from *E. robusta*.

ARTICLE HISTORY
Received 18 March 2016
Accepted 17 October 2016

KEYWORDS
BBD; *Eucalyptus robusta*; RSM; TPC; ultrasound-assisted extraction

Introduction

Eucalyptus robusta, commonly known as swamp mahogany, is a native Australian tree known for its widely adaptable nature.[1] Previous studies have suggested strong biological activity in the phytochemical profile of *E. robusta* and, as such, prospective application in various fields of medicine.[2–6] Traditional Chinese medicine employs the leaves of *E. robusta* for the treatment of dysentery, malaria, and other bacterial diseases.[7] Various studies have also emphasized the high potential of other eucalyptus species including *E. robusta* as sources of biologically active phenolic compounds.[8–13] Phenolic compounds are major bioactive compounds present in many medicinal plants with powerful antioxidant and health-promoting properties.[14] Antioxidants such as flavonoids and other phenolics have garnered increased interest in recent years for the prevention and treatment of cancer and other oxidative stress-related diseases.[15, 16]

We previously established the optimal extraction conditions for total phenolic compounds from *E. robusta* using microwave-assisted extraction (MAE) with the aid of response surface methodology (RSM).[13] Optimal extraction conditions for polyphenolics from *E. robusta* using other modern and green extraction techniques, such as ultrasound-assisted extraction (UAE), have yet to be characterized. Although various extraction techniques are used in the isolation and purification of phytochemicals from different eucalyptus species, it is crucial to determine the optimal technique in terms of its efficiency, affordability, and ease of use with the least impact on the environment. Moreover, these optimal techniques will eventually aid in the characterization of the phytochemical profile and the bioactivity of *E. robusta*.

UAE is a nonthermal process, and therefore, the thermal decomposition of heat-sensitive compounds is avoided in the extraction process.[17] The two main principles—cavitation phenomena and the mechanical mixing effect—increase the extraction efficiency and reduce the extraction time.[18] UAE is economical, rapid, simple, and efficient, and it has been applied to extract bioactive compounds from various materials due to its high reproducibility at shorter time, simplified manipulation, significant reduction in solvent consumption, lower extraction temperatures, and lower energy input.[17, 19, 20] Hence, UAE has been used in many industries such as the food, chemical, and material industries.[19–21] Extraction yields of plant phytochemicals are highly influenced by ultrasonic parameters such as temperature, time, and power.[14] There is little information available in regard to the optimal conditions for UAE of phenolic compounds from *E. robusta*.

RSM is a collection of mathematical and statistical techniques that overcomes the disadvantages of single-factor optimization in analytical chemistry.[22] It is an efficient and economical means of evaluating the functional relationship

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Table 1. Yields of TPC, TFC, and proanthocyanidins from *E. robusta* using UAE and different solvent systems (n = 3).

	TPC (mg GAE/g)	TFC (mg RE/g)	Proanthocyanidins (mg CAE/g)
Water	93.21 ± 0.66	5.29 ± 1.11	1.69 ± 0.42
Ethanol 70%	57.31 ± 2.31	3.57 ± 0.10	1.44 ± 0.16
Ethanol 100%	37.55 ± 0.89	4.88 ± 0.22	1.47 ± 0.19
Acetone	33.32 ± 3.06	4.98 ± 1.76	0.60 ± 0.08
Acetonitrile	10.51 ± 0.36	3.37 ± 0.83	1.28 ± 0.11
Ethyl acetate	4.70 ± 0.62	1.02 ± 0.10	0.08 ± 0.01

between a response of interest or a set of responses of interest and a number of input variables.[22, 23] It has been extensively employed in the optimization of the extraction conditions, including concentration of solvent, extraction time, and solvent-to-mass ratio in extraction chemistry.[24] Although RSM has been utilized to optimize UAE of phenolic compounds in a number of studies,[14, 24–27] this study is the first to optimize the UAE parameters: temperature, sonication time, and power in order to extract total phenolic compounds from *E. robusta* using the most efficient solvent system. A preliminary single-factor test was performed to determine the most efficient solvent system prior to the RSM analysis. The aim of this study was to develop UAE conditions for optimal extraction of total phenolic compounds from *E. robusta*.

Materials and methods

Plant materials

E. robusta fresh leaves were collected on April 2, 2014, from cultivated plants located at Ourimbah, Central Coast, NSW, Australia (latitude of 33.4°S, longitude of 151.4° E). The plants were authenticated by one of the authors (A.C.C.) and a voucher specimen deposited at the Don McNair Herbarium (Accession number 10492), at the University of Newcastle, NSW, Australia. The leaves were then immediately transferred to the laboratory and stored at −20°C to limit the degradation of phenolic compounds. Using a dry air oven, the leaves were dried at 70°C, ground to a fine powder with the help of a commercial grade blender (Rio™ Commercial Bar Blender, Hamilton Beach) and stored at −20°C until required.

Ultrasound-assisted extraction (UAE)

To evaluate the effect of solvents on the extraction of total phenolic content, ground and dried leaves of *E. robusta* were extracted with four different solvents with varying polarity: water, ethanol (70% and 100%), acetone, acetonitrile, and ethyl acetate at a sample-to-solvent ratio of 2 g/100 mL. Extraction was carried out in a tunable ultrasonic bath (Soniclean, 220 V, 50 Hz and 250 W, Soniclean Pty Ltd, Thebarton, SA, Australia) at room temperature for 60 min at a power of 150 W.

For optimizing the UAE conditions, the most effective solvent system (water, Table 1) was used at a sample-to-solvent ratio of 2 g/100 mL, and parameter permutations as designed by the RSM software were implemented. Extraction was carried out in sealed vessels to eliminate evaporation. To measure the temperature of the ultrasonic bath, an external digital thermometer was also used. In the instance that the ultrasonic bath exceeded the designated temperature, tap water was used to maintain the required temperature.

Response surface methodology (RSM)

JMP software (version 11) was utilized for RSM experimental design and analysis. Based on the results of preliminary single-factor test using different solvent systems (Table 1), a three-level-three-factor, Box–Behnken design was applied with three central point replicates for designing experimental conditions to elucidate the influence of the three primary independent ultrasonic parameters: temperature (30, 45, 60°C), time (30, 60, 90 min), and power (60%, 80%, 100% or 150, 200, 250 W). The extracts were then immediately cooled to room temperature (ice bath) and filtered using 10-mL syringe fitted with a 0.45-µm cellulose syringe filter (Phenomenex Australia Pty. Ltd, Lane Cove, NSW, Australia) prior to further investigation.

Table 2 represents the independent variables and their code variable levels. The following six experimental responses are presented in this work:

Y_{TPC} = Total phenolic content (mg GAE/g), Y_{TFC} = Total flavonoid content (mg RE/g), Y_{PRO} = Proanthocyanidins (mg CAE/g), Y_{ABTS} = ABTS (mg TE/g), Y_{DPPH} = DPPH (mg TE/g), Y_{CUPRAC} = CUPRAC (mg TE/g). A second-order polynomial equation was postulated for each experimental response Y, as follows:[13]

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1_{i<j}}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \sum_{i=1}^{k} \beta_{ij} X_1^2$$

where the various X_i values are independent variables affecting the response Y; β_0, β_i, β_{ij}, and β_{ii} are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively; and k is the number of variables. The three independent ultrasonic parameters (Table 2) were assigned as: X_1 (temperature),

Table 2. Box–Behnken design for independent variables and their response.

	Microwave conditions			Experimental values (n=3)					
	Temperature	Time	Power	TPC	TFC	Proanthocyanidins	Antioxidant capacity (mg TE/g)		
Run	(°C)	(min)	(%)*	(mg GAE/g)	(mg RE/g)	(mg CAE/g)	ABTS	DPPH	CUPRAC
1	30	30	80	59.18	4.61	0.53	151.05	189.12	492.86
2	30	60	60	72.8	6.95	0.49	148.65	187.72	553.39
3	30	60	100	49.21	10.05	1.73	162.30	168.3	511.3
4	30	90	80	48.97	1.52	0.6	138.32	94.1	412.72
5	45	30	60	46.75	4.4	2.4	167.58	174.41	544.12
6	45	30	100	52.53	8.14	0.75	165.68	228.84	595.72
7	45	60	80	63.15	2.86	0.68	178.25	141.18	531.04
8	45	60	80	65.8	3.08	0.65	185.27	129.57	520.11
9	45	60	80	63.35	3.48	0.63	169.42	124.32	539.82
10	45	90	60	56.83	2.76	0.79	162.16	158.15	605.46
11	45	90	100	84.26	2.84	0.91	221.45	113.54	509.48
12	60	30	80	76.48	5.27	1.59	232.26	187.76	523.58
13	60	60	60	98.24	9.09	10.98	249.28	185.19	622.31
14	60	60	100	139.37	8.04	5.81	250.99	286.47	629.23
15	60	90	80	152.69	5.75	6.12	252.96	284.35	710.67

* 60%, 80%, and 100% power were equivalent to 150, 200, and 250 W, respectively.

X_2 (time), and X_3 (power, %). Therefore, the function containing these three independent variables can be expressed as following polynomial equation:

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

where β_1, β_2, and β_3 represent β_i, β_{ij}, and β_{ii}, respectively.

Determination of total phenolic content (TPC)

Total phenolic content (TPC) was determined according to the method of Škerget et al.[28] Extracts were diluted up to 40 times to fit within the optimal absorbance range. A calibration curve was constructed using gallic acid as the standard, with the results expressed as mg of gallic acid equivalents (GAE) per g of dry weight (mg GAE/g).

Determination of total flavonoid content (TFC)

Colorimetric assessment of TFC was determined as per the method described by Tan et al.[29] TFC was expressed as mg of rutin equivalents (RE) per g of dry weight (mg RE/g) using rutin as the standard.

Determination of proanthocyanidin content

For determining the proanthocyanidin content of the extract, the vanillin–HCl method as described by Broadhurst and Jones[30] was used. Catechin was used as the standard with the concentration of proanthocyanidins expressed as mg of catechin equivalents (CAE) per g of dry weight (mg CAE/g).

Determination of antioxidant capacity

ABTS total antioxidant capacity (TAC)

The extracts were diluted up to 40×, and TAC was measured using ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assay as described by Arnao et al.[31] with some modification. In 10 mL of distilled water, 7.4 mM of ABTS was dissolved to prepare the ABTS stock solution. Potassium persulfate stock solution was prepared by dissolving 2.6 mM of potassium persulfate in 100 mL of distilled water. Both the stock solutions were then mixed in equal quantities and allowed to react for 15 h in the dark at room temperature to obtain the mixture solution. The working solution was then prepared by mixing 1 mL of mixture solution with 60 mL of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using the UV spectrophotometer. For each assay, fresh working solution was prepared. To 0.150 mL of extracts, 0.285 mL of the working ABTS solution was added and then allowed to react in the dark for 2 h at room temperature before measuring the absorbance at 734 nm. Results were expressed in mg of trolox equivalents (TE) per g of dry weight (mg TE/g) using trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as the calibration standard.

Free radical scavenging capacity

For measuring the free radical scavenging activity of the extracts, DPPH (1,1-diphenyl-2-picrylhydrazyl-hydrate) assay as described by Brand-Williams et al.[32] with some modifications was employed. DPPH (24 mg) was dissolved in 100 mL of methanol to prepare the DPPH stock solution. This solution was then stored at −20°C until required. The working solution was prepared fresh by mixing the 10 mL of stock solution with

45 mL of methanol to obtain an absorbance of 1.1 ± 0.02 at 515 nm using the UV spectrophotometer. Extracts (0.150 mL) were allowed to react with 0.285 mL of working solution in darkness at room temperature for 3 h. Then, the absorbance was measured at 515 nm. Results were expressed in mg of trolox equivalents (TE) per g of dry weight (mg TE/g).

Cupric reducing antioxidant capacity (CUPRAC)

CUPRAC assay was used to analyze the cupric ion chelating capacity of the extracts as described by Apak et al.[33] Trolox was used as the calibration standard, with results expressed in mg of trolox equivalents (TE) per g of dry weight (mg TE/g).

Statistical analysis

To predict optimal values for the three response variables and for establishing the model equation to graph the variable response, JMP software (version 11, SAS Institute Inc., Cary, USA) was used. Experiments were performed in triplicate and the results averaged. The circular or elliptical shapes of the contour plot illustrate the significance of the interactions between the variables.[34] The responses in function of two factors are presented by these 3D contour plots, and they keep the other variable constant at its middle level.[35] To compare the means analysis by independent samples t-test, SPSS statistical software (version 16.0, SPSS Inc., Chicago, IL, USA) was used. At $p < 0.05$, the differences between the mean values in the performed experiments were taken to be statistically significant.

Results and discussion

Impact of solvents on extraction efficiency of TPC, TFC and proanthocyanidins

This study evaluated the impact of six different solvent systems with varied polarities on the extraction yields of TPC, TFC, and proanthocyanidins from *E. robusta*. The aim was to determine the most optimal solvent for further optimizing the UAE methodology using RSM. Table 1 indicated that the yields of TPC, TFC, and proanthocyanidins were greatest in the case of the aqueous extracts and lowest in the case of ethyl acetate. These differences are explained by the variations in polarities of the solvent systems utilized.[23] Based on these findings, water was selected as the extraction solvent for optimizing the three UAE parameters of temperature, time, and power for extracting TPC, TFC, proanthocyanidins, and antioxidants from *E. robusta* leaf. Previously, Pinto et al.[36] also illustrated the efficiency of water in the extraction of TPC from the bark of *E. globulus*.

Fitting the models for TPC, TFC, proanthocyanidins, and antioxidant capacity

To evaluate the reliability of RSM mathematical model in accurately predicting the optimal variances and representing the correlations between the selected parameters, fitting the models for TPC, TFC, proanthocyanidins, and antioxidant capacity of the *E. robusta* leaf extracts was carried out. The Box–Behnken design representing the analysis of variances for determination of the model fit is shown in Figure 1 and Table 2.

The coefficient of determination (R^2) of the model for TPC was 0.99, indicating that 99% of similarity can be obtained between the experimental and predicted data (Figure 1a). The p-value for lack of fit was estimated to be 0.057 (Table 3), a statistically insignificant value ($p > 0.05$). The F-value and the p-value of the model were estimated to be 70.94 and < 0.0001, respectively, further demonstrating the reliability of the mathematical model in precise prediction of TPC yields. In addition, the predicted residual sum of squares (PRESS) value was 1729.96. The PRESS value is particularly useful to draw a comparison between the predictive powers of multiple mathematical models.[13]

In the case of TFC, the p-value for lack of fit was found to be 0.051, indicating the statistical relevance of the model ($p > 0.05$). Furthermore, the R^2 value of the model was 0.94, illustrating a 94% match between the experimental and predicted values (Figure. 1b). The F ratio, p-value, and PRESS of the model were 8.85, 0.0136, and 89.52, respectively. These observations validated the competence of the RSM model in predicting TFC yields from *E. robusta* using UAE.

Fitting the model for proanthocyanidins was also conducted. The results indicated the adequacy of the RSM model in predicting the amount of proanthocyanidin liberated during predefined UAE conditions with a R^2 value of 0.90 (Figure 1c). Although the lack-of-fit value of the model was statistically significant ($p < 0.05$), the F ratio, p-value, and PRESS were 4.76, 0.05, and 212.40, respectively, providing evidence favoring the model's reliability.

The results obtained from three antioxidant assays —ABTS, DPPH, and CUPRAC—also strongly supported the reliability of the model in predicting the antioxidant yields from *E. robusta* leaf using UAE with R^2 values of 0.98, 0.92, and 0.93, respectively (Figure 1d–f). The p-values for lack of fit for all the assays were statistically insignificant ($p > 0.05$,

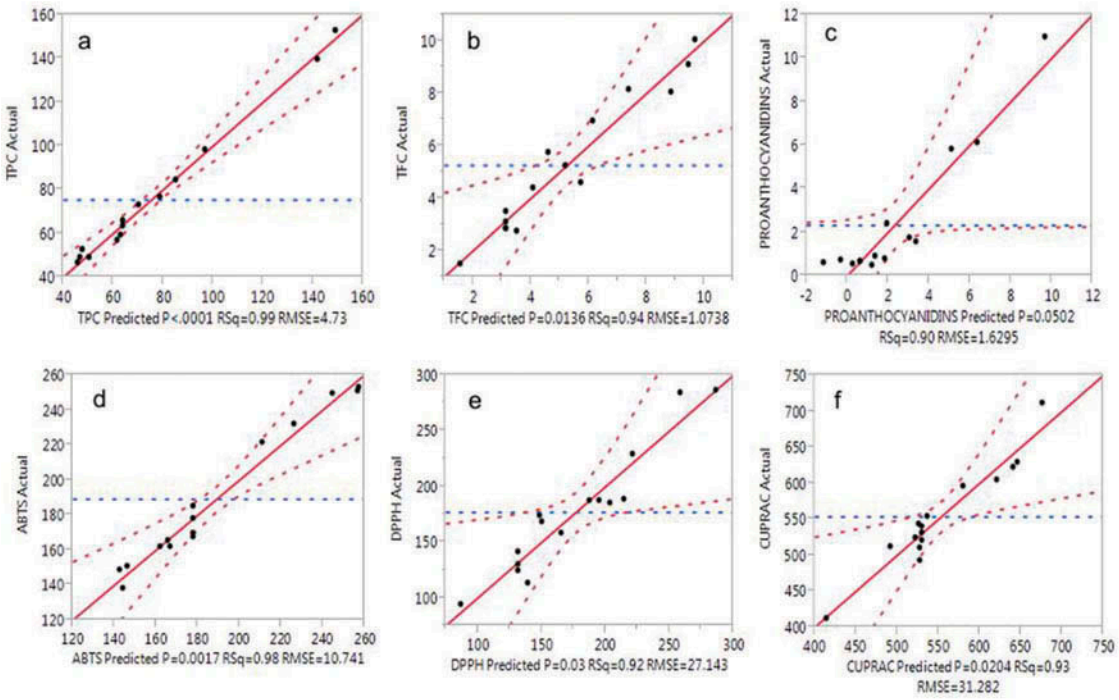

Figure 1. Correlations between predicted and experimental total phenolic content (a), total flavonoid content (b), proanthocyanidins (c), ABTS antioxidant capacity (d), DPPH free radical scavenging capacity (e) and cupric reducing antioxidant power (f).

Table 3. Analysis of variance for determination of model fit.

				Antioxidant capacity		
	TPC	TFC	Proanthocyanidins	ABTS	DPPH	CUPRAC
Lack of fit	0.057	0.051	0.0001*	0.30	0.06	0.059
R^2	0.99	0.94	0.90	0.98	0.92	0.93
Adjusted R^2	0.97	0.83	0.70	0.93	0.76	0.80
PRESS	1729.96	89.52	212.40	7494.30	56892.80	75603.09
F ratio of model	70.94	8.85	4.76	21.67	6.12	7.35
P of model > F	<0.0001	0.0136	0.05	0.0017	0.03	0.0204

* Significant difference with $p < 0.05$.

Table 3). Moreover, the F ratio (21.67, 6.12, and 7.35, respectively), p-values (0.0017, 0.03, and 0.0204, respectively), and PRESS values of the model (7494.30, 56892.80, and 75603.09, respectively) demonstrate the consistency of the model in prediction of antioxidant capacity of *E. robusta* aqueous extracts.

The models for TPC, TFC, proanthocyanidins, and antioxidant capacity of the *E. robusta* extracts can be represented by the following second-order polynomial equations:

$$Y_{TPC} = 64.10 + 29.57X_1 + 13.47X_2 + 6.34X_3 + 21.60X_1X_2 + 16.18X_1X_3 + 5.41X_2X_3 + 25.02{X_1}^2 - 4.79{X_2}^2 + 0.78{X_3}^2$$

$$Y_{TFC} = 3.14 + 0.62X_1 - 1.19X_2 + 0.73X_3 + 0.89X_1X_2 - 1.03X_1X_3 - 0.91X_2X_3 + 2.57{X_1}^2 - 1.42{X_2}^2 + 2.82{X_3}^2$$

$$Y_{PRO} = 0.65 + 2.64X_1 + 0.39X_2 - 0.68X_3 + 1.11X_1X_2 - 1.60X_1X_3 + 0.44X_2X_3 - 2.54{X_1}^2 - 0.99{X_2}^2 + 1.55{X_3}^2$$

$$Y_{ABTS} = 177.64 + 48.14X_1 + 7.29X_2 + 9.09X_3 + 8.35X_1X_2 - 2.98X_1X_3 + 15.29X_2X_3 + 19.79{X_1}^2 - 3.79{X_2}^2 + 5.36{X_3}^2$$

$$Y_{DPPH} = 131.69 + 38.06X_1 - 16.24X_2 + 11.46X_3 + 47.90X_1X_2 + 30.17X_1X_3 - 24.76X_2X_3 + 47.66{X_1}^2 + 9.47{X_2}^2 + 27.566{X_3}^2$$

$$Y_{CUPRAC} = 530.32 + 64.44X_1 + 10.25X_2 - 9.94X_3 + 66.80X_1X_2 + 12.25X_1X_3 - 36.89X_2X_3 + 9.99{X_1}^2 - 5.36{X_2}^2 + 38.73{X_3}^2$$

Effect of ultrasonic independent variables on TPC of aqueous E. robusta *extracts*

The extraction yield of TPC from aqueous *E. robusta* extracts was significantly influenced by all three ultrasonic independent variables: temperature, time, and power ($p < 0.05$, Table 4). The p-value is particularly important in verifying the significance of each independent variable, which may also indicate the pattern of interaction between multiple variables.[34] The results indicated that temperature had the greatest positive impact on the TPC yield followed by time and power ($p < 0.05$, Table 4). An increase in the level of TPC was observed with individual increases in temperature (from 30 to 60°C), time (from 30 to 90 min), and power (from 60 to 100%) (Figure 2). Ince and colleagues[17] also reported similar findings on the significant positive impact of time and power on the yield of TPC from nettle using UAE. Likewise, Bashi and colleagues[25] demonstrated that increases in sonication time and temperature led to increased TPC extraction from yarrow (*Achillea beibrestinii*). The positive effect of longer extraction time on the TPC yield was also

Table 4. Analysis of variance for the experimental results.

								Antioxidant capacity					
		TPC		TFC		Proanthocyanidins		ABTS		DPPH		CUPRAC	
Parameter	DF	Estimate	Prob>|t|	Estimate	Prob>|t|	Estimate	Prob>|t|	Estimate	Prob>|t|	Estimate	Prob>|t|	Estimate	Prob>|t|
Model													
β_0	1	64.1	<.0001*	3.14	0.0039*	0.6533333	0.5183	177.64667	<.0001*	131.69	0.0004*	530.32333	<.0001*
Linear term													
β_1	1	29.5775	<.0001*	0.6275	0.1593	2.64375	0.0059*	48.14625	<.0001*	38.06625	0.0107*	64.44	0.0021*
β_2	1	13.47625	0.0005*	−1.19375	0.0255*	0.39375	0.5247	7.29	0.1130	−16.24875	0.1512	10.25625	0.3963
β_3	1	6.34375	0.0127*	0.73375	0.1111	−0.6825	0.2894	9.09375	0.0620	11.46	0.2860	−9.94375	0.4098
Interactions													
β_{12}	1	21.605	0.0003*	0.8925	0.1573	1.115	0.2294	8.3575	0.1804	47.9025	0.0167*	66.8075	0.0079*
β_{13}	1	16.18	0.0010*	−1.0375	0.1111	−1.6025	0.1064	−2.985	0.6023	30.175	0.0768	12.2525	0.4689
β_{23}	1	5.4125	0.0708	−0.915	0.1491	0.4425	0.6104	15.2975	0.0359*	−24.76	0.1277	−36.895	0.0648
Quadratic													
β_{11}	1	25.02125	0.0002*	2.5725	0.0058*	2.5483333	0.0299*	19.794167	0.0165*	47.66375	0.0198*	9.9983333	0.5660
β_{22}	1	−4.79125	0.1092	−1.425	0.0513	−0.991667	0.2950	−3.793333	0.5275	9.47875	0.5319	−5.364167	0.7551
β_{33}	1	0.78375	0.7631	2.82	0.0039*	1.5508333	0.1270	5.3641667	0.3813	27.56625	0.1085	38.735833	0.0632

* Significantly different at $p < 0.05$; $\beta 0$: intercept; $\beta 1$, $\beta 2$, and $\beta 3$: linear regression coefficients for temperature, time, and power; $\beta 12$, $\beta 13$, and $\beta 23$: regression coefficients for interaction between temperature × time, temperature × power, and time × power; $\beta 11$, $\beta 22$, and $\beta 33$: quadratic regression coefficients for temperature × temperature, time × time, and power × power.

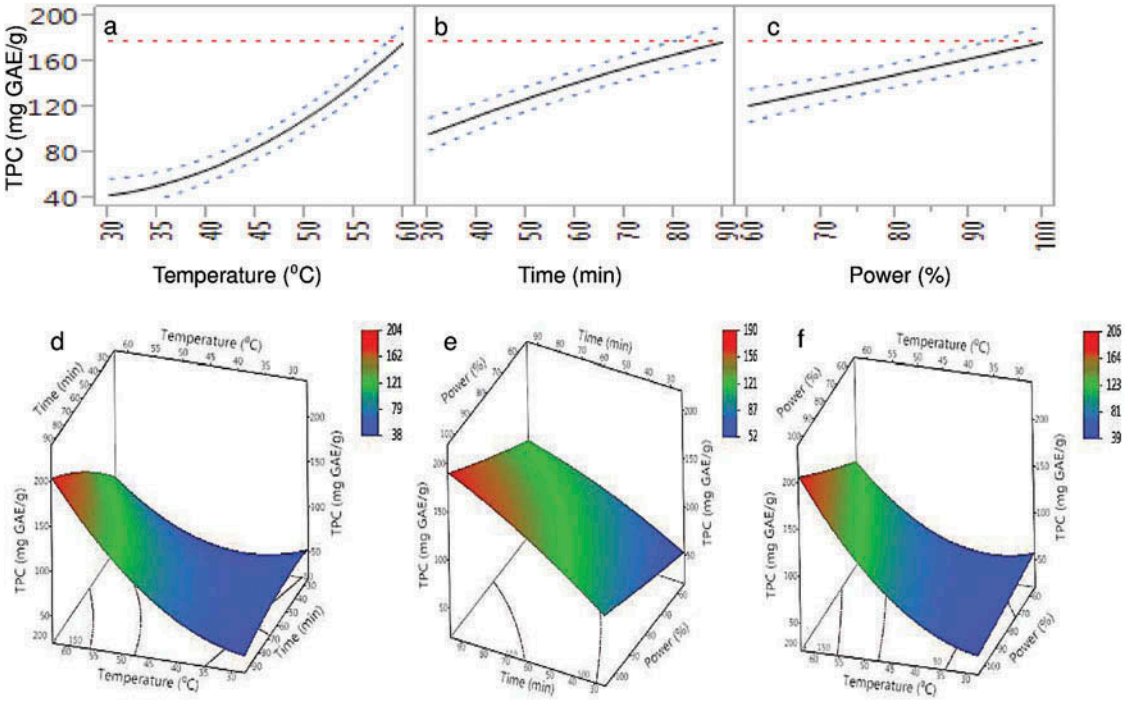

Figure 2. Impact of temperature (30–60°C), time (60–90 min) and power (60–100%) on total phenolic content. The 2D impact of temperature, time and power is expressed in Fig. 2a–c, while their 3D effects are shown in Fig. 2d–f.

illustrated by Tabaraki and Nateghi[35] in rice bran. Moreover, we previously established the significant impact of irradiation time and power on the extraction of TPC from *E. robusta* using MAE and RSM.[13] This positive impact can be explained by the accelerated liberation and solubility of cell contents with increasing temperature, time, and power of UAE due to greater solvent penetration into cellular materials, microstreaming-assisted improvement in mass transfer, and disruption of biological cell walls.[19] Pinto and colleagues[36] coined the term H-factor as a kinetic parameter to combine the two ultrasonic factors: time and temperature into a single factor for elucidating the impact of extraction media on the extraction yield of TPC from *E. globulus* bark using water. However, they concluded that the H-factor had no significant influence on TPC yield. In the case of interaction between independent variables, temperature × time had the most statistically significant impact on the TPC yield followed by temperature × power ($p < 0.05$, Table 4). However, TPC yield was not significantly impacted by the interaction between time and power ($p > 0.05$, Table 4).

Effect of ultrasonic independent variables on TFC of aqueous *E. robusta* extracts

Time was the only independent variable found to significantly impact the extraction yield of TFC from *E. robusta* using UAE ($p < 0.05$, Table 4). Extraction time is one of the major factors that can influence the flavonoid yield from plant materials.[37, 38] Similarly, Lu et al.[39] showed that sonication time had a linear relationship with extraction yield of flavonoids from *Cryptotaenia japonica* using UAE. On the other hand, Ghasemzadeh et al.[40] reported that only temperature significantly influenced the extraction yields of three main flavonoids: catechin, myricetin, and quercetin from curry leaf (*Murraya koenigii*) when UAE was coupled with RSM. In the case of MAE, we observed comparable results indicating that the microwave irradiation time significantly influences the liberation of TFC from *E. robusta*.[13] Interactions between independent variables—temperature × time, temperature × power, and time × power—were found to possess a statistically insignificant effect on the TFC yield ($p > 0.05$, Table 4). Yang and colleagues [38] also determined that interactions between temperature and time had no significant impact on TFC extraction ($p > 0.05$) from *Citrus aurantium*, further validating our finding. As shown in Figure 3b, the TFC yield steadily increased from $t = 30$ to 40 min, then reached a plateau at $t = 50$ min and sharply dropped from $t = 50$ to 90 min. An almost identical effect of extraction time on TFC was also observed by Yang and colleagues.[38] They postulated that within a certain period of time, equilibrium is achieved between solutions within and external to cells. As the extraction time is extended, negative reactions could lead to decline in the yield.

Effect of ultrasonic independent variables on proanthocyanidins of aqueous E. robusta extracts

Both linear and quadratic regression coefficients for temperature had a significant impact on the extraction yield of proanthocyanidins ($p < 0.05$, Table 4). The proanthocyanidin content initially dropped between 30 and 40°C and then increased significantly between 40 and 60°C (Figure 4a). Interactions between the independent variables were not found to possess any significant impact on proanthocyanidin content of the extracts ($p > 0.05$, Table 4). Previously, the significant impact of temperature on the yield of proanthocyanidins from the seeds of grape pomace using UAE was reported by Andjelkovic et al.[41] They also observed that the total proanthocyanidin content improved between 40 and 50°C, which somewhat supported our observation.

Effect of ultrasonic independent variables on antioxidant capacity of aqueous E. robusta extracts

Significant linear and quadratic effects of temperature were observed on the TAC of aqueous *E. robusta* extracts, as revealed by the ABTS assay ($p < 0.05$, Table 4). An increase in temperature can lead to an increase in acoustic cavitation, surface contact area, and reduction in viscosity and density of the media, which in turn induces rupture of the plant cells releasing phenolics and antioxidants.[42] Individual impacts of time and power on the TAC of extracts were found to be statistically insignificant in our study. However, the interaction between time and power influenced the TAC significantly ($p < 0.05$, Table 4). The degree of effect of the ultrasonic independent variables on TAC can be represented as follows: temperature > power > time on the basis of the *p*-values. The statistical insignificance of extraction time on the TAC and DPPH scavenging capacity of rapeseed cultivars and *Clerodendrum cyrtophyllum* leaves extracts has been highlighted in earlier studies.[42, 43] However, Şahin et al.[44] demonstrated that higher antioxidant capacity can be obtained from *Artemisia absinthium* extract using higher temperatures and longer extraction times. An increase in TAC was observed with an increase in temperature (Figure 5a). Similar trends were also recorded in the case of time and power (Figure 5b and c). These observations were comparable with the findings reported by Aybastıer et al.[45] in blackberry leaf extract.

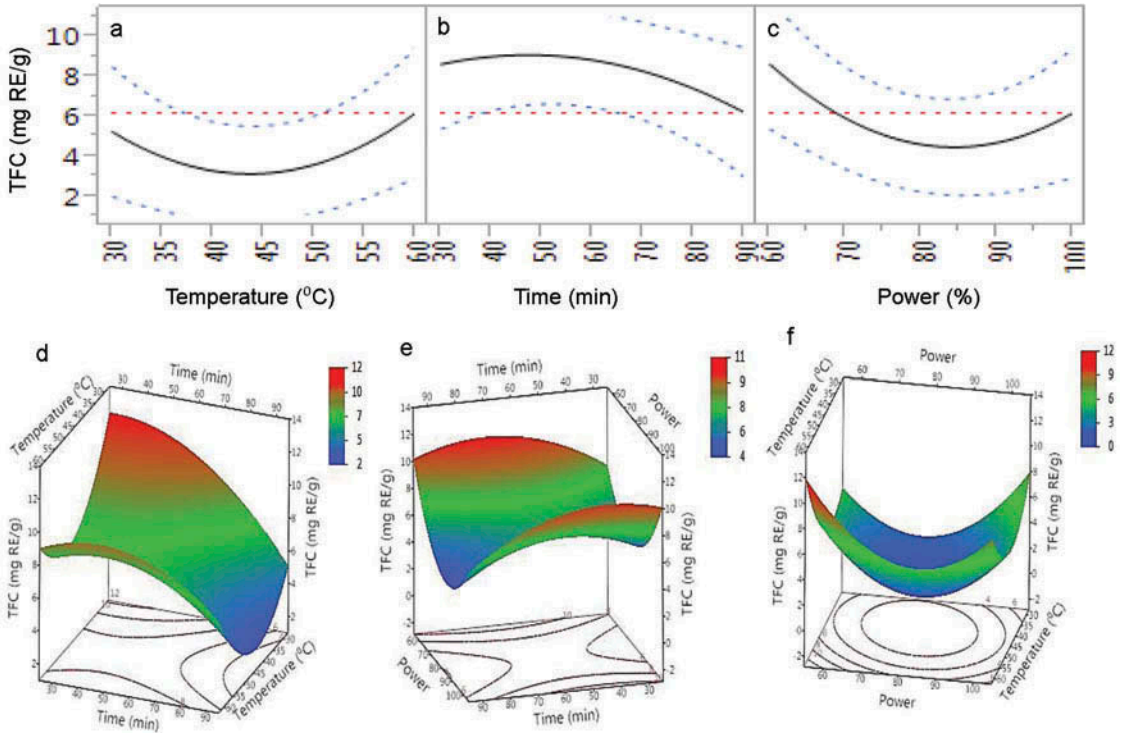

Figure 3. Impact of temperature (30–60°C), time (60–90 min) and power (60–100%) on total flavonoid content. The 2D impact of temperature, time and power is expressed in Fig. 3a–c, while their 3D effects are shown in Fig. 3d–f.

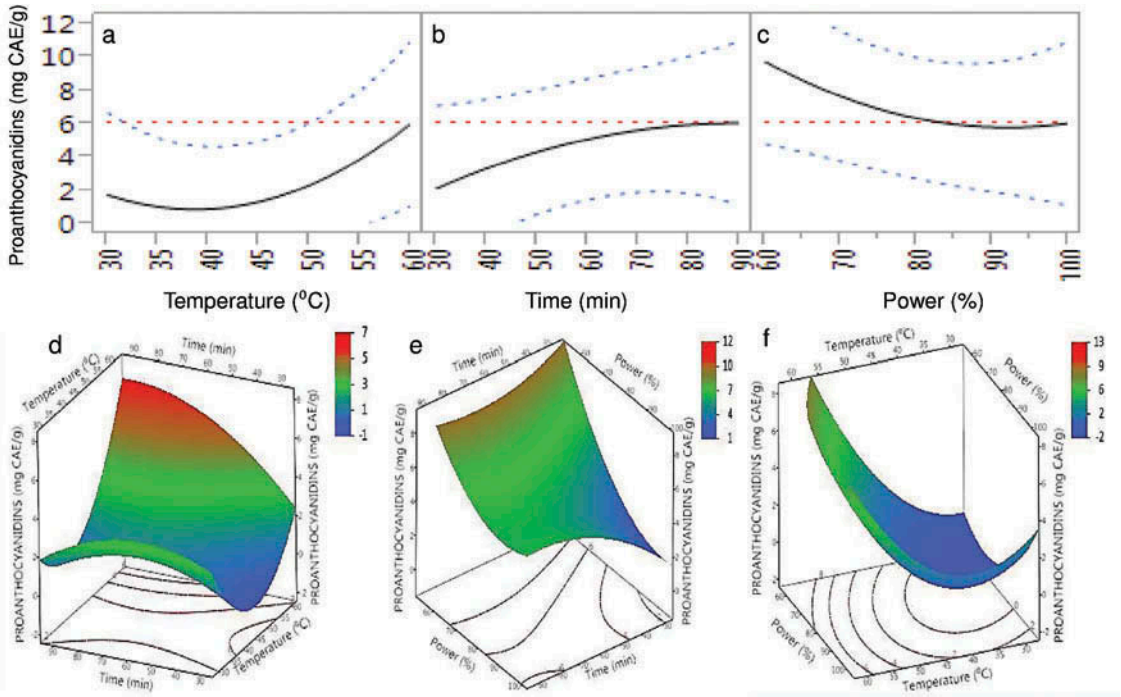

Figure 4. Impact of temperature (30–60°C), time (60–90 min) and power (60–100%) on proanthocyanidin content. The 2D impact of temperature, time and power is expressed in Fig. 4a–c, while their 3D effects are shown in Fig. 4d–f.

Similarly, in the case of the DPPH assay, temperature was found to exert significant linear and quadratic effects on the free radical scavenging capacity of *E. robusta* extracts ($p < 0.05$, Table 4). The steep slope indicated that an increase in temperature from 30 to 60°C led to an increase in DPPH scavenging capacity (Figure 6a). However, time and power had no noteworthy consequences on the DPPH scavenging

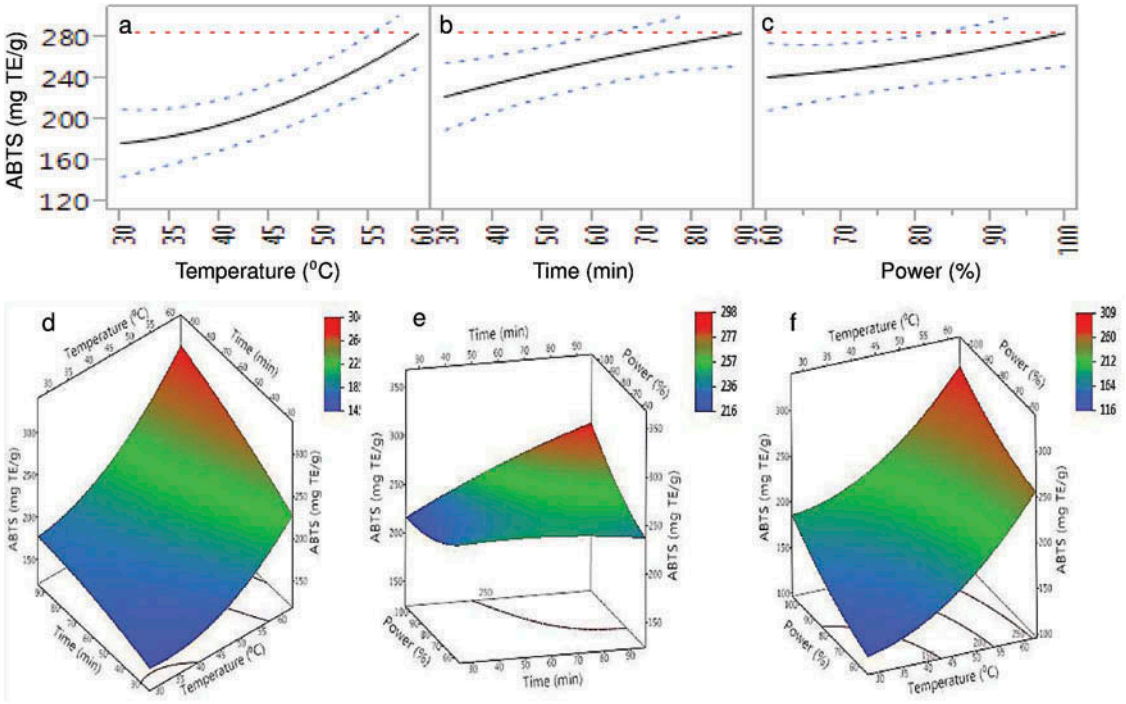

Figure 5. Impact of temperature (30–60°C), time (60–90 min) and power (60–100%) on ABTS antioxidant capacity. The 2D impact of temperature, time and power is expressed in Fig. 5a–c, while their 3D effects are shown in Fig 5d–f.

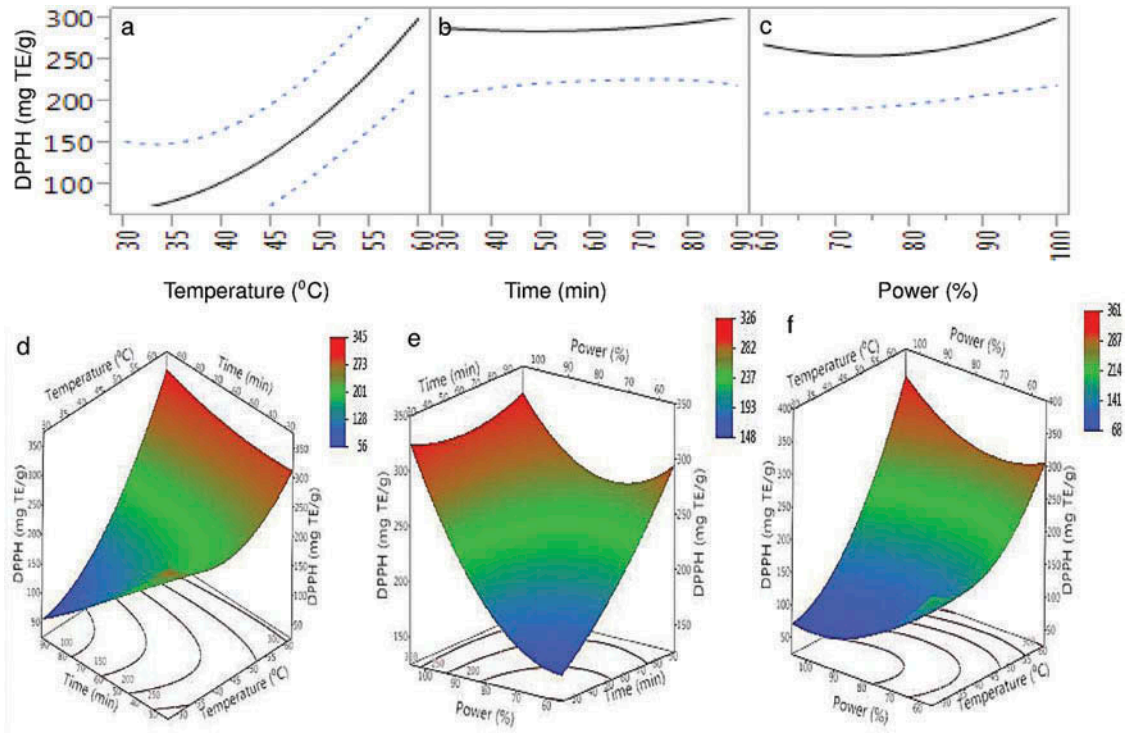

Figure 6. Impact of temperature (30–60°C), time (60–90 min) and power (60–100%) on DPPH free radical scavenging capacity. The 2D impact of temperature, time and power is expressed in Fig. 6a–c, while their 3D effects are shown in Fig. 6d–f.

capacity. Similar to our study, Ghasemzadeh and colleagues[40] reported that temperature exerted both significant linear and quadratic effects on the DPPH scavenging capacity of curry leaf (*Murraya koenigii*) extracts when UAE was utilized. They also demonstrated that ultrasonic power had no significant impact

on the free radical scavenging capacity, further validating our results. However, they did not consider extraction time as one of the independent variables of the study. Likewise, another study by Szydłowska-Czerniak and Tułodziecka[27] established that higher DPPH values can be obtained from rapeseed when extracted for a longer time with 50% methanol, which has lower dielectric constant than water. On the contrary, effects of temperature and time were found to be insignificant in rice bran extracts when UAE was utilized.[35] Based on the *p*-values obtained from our study, the magnitude of influence of the ultrasonic independent variables was determined to be: temperature > time > power. Additionally, temperature × time interaction had a statistically significant effect on the DPPH scavenging capacity of the extracts ($p < 0.05$, Table 4).

Based on the observations made in the CUPRAC assay, it was concluded that temperature influenced the cupric reducing antioxidant capacity (CUPRAC) of the extracts in a statistically significant manner ($p < 0.05$, Table 4). The CUPRAC was found to be directly proportional to the extraction temperature, that is, an increase in temperature led to an increase in CUPRAC of the extracts (Figure 7a). Identical observations were made by Şahin and colleagues[44] in *A. absinthium* extract. Therefore, temperature was identified to be the single most important factor that significantly affected all three antioxidant assays performed in our study. Similar to the DPPH assay, the CUPRAC assay also revealed that the interaction between temperature and time influenced the CUPRAC of the extracts significantly ($p < 0.05$, Table 4). Aybastıer and colleagues[45] also reported that higher extraction time and temperature improved the CUPRAC values in blackberry leaf extracts. Other ultrasonic independent variables—time and power—did not exhibit any statistically significant influence on CUPRAC ($p > 0.05$, Table 4).

Correlation analyses

The positive correlation between the TPC and antioxidant activity of the *E. robusta* fruit, *E. grandis, E. urograndis*, and *E. maidenii* bark extracts has been reported previously.[11, 15] Regression analysis was performed to evaluate the correlation between TPC and ABTS, DPPH, and CUPRAC values obtained. Our data also revealed the statistically significant correlation between TPC and antioxidant capacity of *E. robusta* supporting the previous findings. Pearson's correlation coefficients (r) of 0.846 ($p = 0.01$), 0.709 ($p = 0.003$), and 0.728 ($p = 0.002$) were observed between TPC and ABTS, TPC and DPPH, and TPC and CUPRAC, respectively. The differences in the correlation values between TPC and all three antioxidant assays can be attributed to the fact that these assays are quite different from each other in regard to the ways they function

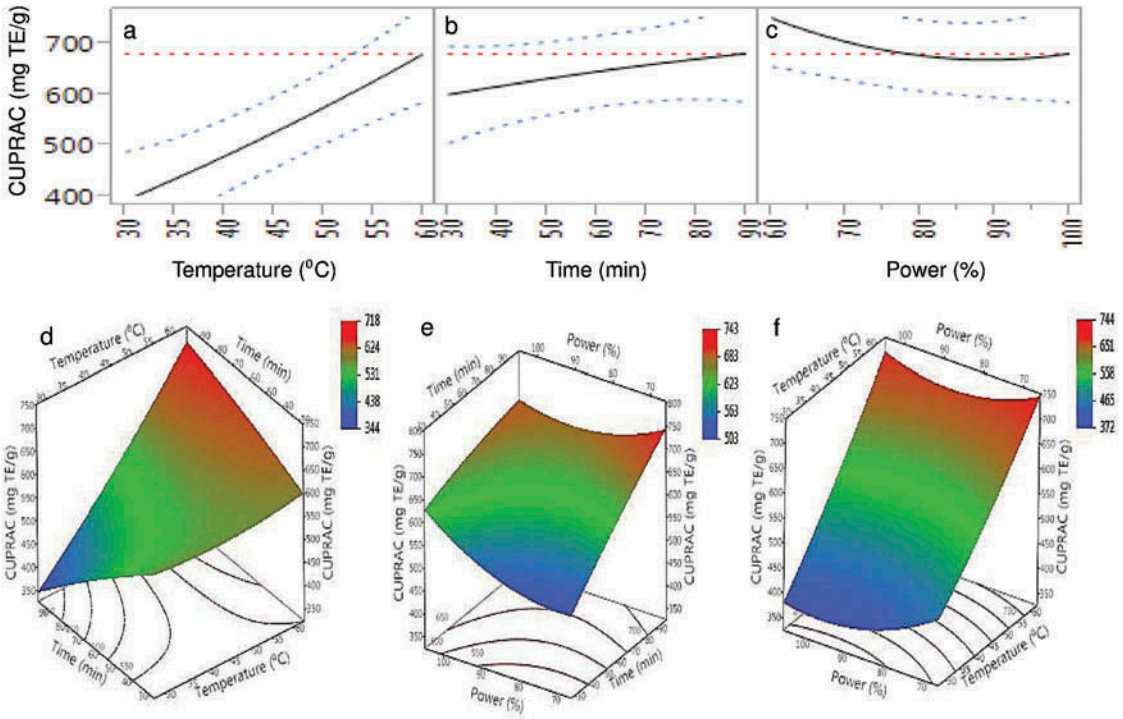

Figure 7. Impact of temperature (30–60°C), time (60–90 min) and power (60–100%) on cupric reducing antioxidant power. The 2D impact of temperature, time and power is expressed in Fig. 7a–c, while their 3D effects are shown in Fig. 7d–f.

and their measurement techniques. Nevertheless, significant positive correlations were observed between ABTS and DPPH (r = 0.533, p = 0.041), ABTS and CUPRAC (r = 0.628, p = 0.012), and DPPH and CUPRAC (r = 0.789, p = 0.01).

Optimization and validation of the models

The RSM mathematical model predicted the optimal UAE conditions for liberation of TPC from *E. robusta* was as follows: 60°C, t = 90 min and 100% power (250 W) as shown in Figure 2. These conditions were also found to be optimum for yielding maximum ABTS and DPPH values (Figure 5 and 6). Optimal conditions for the extraction of TFC were as follows: 60°C–50 min–60% power (Figure 3); and 60°C–90 min–60% power for proanthocyanidins (Figure 4) and CUPRAC (Figure 7). Under the optimal conditions for TPC, ABTS, and DPPH, 64.72%, 68.30%, and 79.21% of the maximum TFC, proanthocyanidins, and CUPRAC values could be obtained, respectively, under their respective ideal conditions as described above. Therefore, the optimal UAE conditions for extracting TPC, TFC, proanthocyanidins, and antioxidants from *E. robusta* using water as the solvent system were selected to be the temperature of 60°C, sonication time of 90 min, and power of 100% (250 W).

To validate the optimal conditions predicted by the models, *E. robusta* leaf was extracted using water under the UAE conditions of 60°C–90 min–100% power. The results indicated that was no statistically significant difference between the predicted and experimental (n = 3) values of TPC, TFC, proanthocyanidins, ABTS, DPPH, and CUPRAC from aqueous *E. robusta* leaf extract (p > 0.05; Table 5). Therefore, these UAE conditions are suggested for further characterization and isolation of TPC and antioxidants from *E. robusta* leaf. Moreover, these findings validated the accuracy and reliability of the RSM mathematical model as an appropriate tool for optimizing extraction conditions for recovery of phenolics from plant material. Additionally, the efficiency of water as the solvent system in extracting phenolic compounds and antioxidants from *E. robusta* has also been highlighted by these findings.

Conclusions

The impact of solvents on extraction efficiency of TPC, TFC, and proanthocyanidins was elucidated, and water was found to be the most efficient solvent system for their extraction from *E. robusta*. Furthermore, RSM coupled with Box–Behnken design was effectively utilized for optimizing the UAE parameters for TPC from *E. robusta* using water as the solvent system. It was validated that RSM is a very reliable and accurate tool for elucidating the impact of three independent variables: temperature, sonication time, and power on the extraction yield TPC from *E. robusta*. Temperature had the greatest impact on the TPC yield followed by time and power. The extraction yields of proanthocyanidins and antioxidants were also significantly influenced by temperature. In the case of TFC, sonication time was the only independent variable that affected the yield. Based on the results, it can be concluded that the optimal UAE conditions for extracting TPC, TFC, proanthocyanidins, and antioxidants were as follows: 60°C, 90 min sonication time, and 100% power (250 W). These conditions can be applied for further isolation and purification of phenolic compounds from *E. robusta* for their potential applications in food, pharmaceutical, and nutraceutical industry. Additionally, the UAE was validated as an efficient, green, and sustainable technique for extracting phenolics and antioxidants from *E. robusta* leaf.

Table 5. Validation of the predicted value for TPC, TFC, proanthocyanidins, and antioxidant capacity.

	Values	
	Predicted	Experimental (n = 3)
TPC (mg GAE/g)	170.70 ± 20.32[a]	163.68 ± 2.13[a]
TFC (mg RE/g)	6.21 ± 4.61[a]	5.89 ± 0.065[a]
Proanthocyanidins (mg CAE/g)	6.06 ± 6.99[a]	6.66 ± 0.89[a]
ABTS (mg TE/g)	284.21 ± 46.13[a]	253.95 ± 3.14[a]
DPPH (mg TE/g)	302.98 ± 116.58[a]	285.50 ± 4.32[a]
CUPRAC (mg TE/g)	680.60 ± 134.35[a]	593.95 ± 3.88[a]

All the values are means ± standard deviations and those in the same row not sharing the same superscript letter are significantly different from each other (p < 0.05).

Conflict of interest

The authors declare no conflict of interest.

Funding

We acknowledge the following funding support: Ramaciotti Foundation (ES2012/0104); the Hunter Cancer Research Alliance (HCRA); the Hunter Medical Research Institute (HMRI) and the University of Newcastle.

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DE GRUYTER

Chemical Papers
DOI: 10.1515/chempap-2015-0237

ORIGINAL PAPER

Investigation of phytochemicals and antioxidant capacity of selected *Eucalyptus* species using conventional extraction

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Received 29 June 2015; Revised 21 September 2015; Accepted 10 October 2015

Eucalyptus species have found their place in traditional medicine and pharmacological research and they have also been shown to possess a large number of phenolic compounds and antioxidants. The present study sought to implement conventional extraction to yield maximal total phenolic content (TPC), total flavonoid content (TFC), proanthocyanidins, antioxidants, and saponins from *E. robusta* using different solvents. The most suitable extraction solvent was further employed for extracting phytochemicals from *E. saligna*, *E. microcorys*, and *E. globulus* to select the *Eucalyptus* species with the greatest bioactive compound content. The results emphasised the efficiency of water in extracting TPC ((150.60 ± 2.47) mg of gallic acid equivalents per g), TFC ((38.83 ± 0.23) mg of rutin equivalents per g), proanthocyanidins ((5.14 ± 0.77) mg of catechin equivalents per g), and antioxidants ABTS ((525.67 ± 1.99) mg of trolox equivalents (TE) per g), DPPH ((378.61 ± 4.72) mg of TE per g); CUPRAC ((607.43 ± 6.69) mg of TE per g) from *E. robusta*. Moreover, the aqueous extract of *E. robusta* had the highest TPC, TFC and antioxidant values among the other *Eucalyptus* species tested. These findings highlighted the efficiency of conventional extraction in extracting natural bioactive compounds from *Eucalyptus* species for pharmaceutical and nutraceutical applications.

Keywords: *Eucalyptus*, conventional extraction, TPC, antioxidants, saponins

Introduction

Eucalyptus is a diverse genus belonging to the family Myrtaceae (Rozefelds, 1996). The term *Eucalyptus* was coined by the French botanist, Charles Louis L'Héritier de Brutelle, in 1788, meaning 'well covered' with reference to the operculate nature of the flower which lacks conspicuous petals and sepals (Rozefelds, 1996; Vuong et al., 2015a). *Eucalyptus* is mostly indigenous to Australia, with a small number found in the adjacent areas of New Guinea and Indonesia (Gupta et al., 2013; Konoshima & Takasaki, 2002). A wide variety of secondary chemicals occurring in large concentrations are found in *Eucalyptus* and its relatives (Sidana et al., 2011). The major non-volatile compounds found abundantly in *Eucalyptus* are phenolic compounds, which contribute significantly to the antioxidant activities of extracts (Al-Sayed et al., 2012; Almeida et al., 2009; Bhuyan et al., 2015). Previous reports have stated that phenolic compounds such as gallic acid, protocatechuic acid, ellagic acid, quercetin, quercetin glycoside, naringenin, catechin, epicatechin, rutin, quercitrin, apigenin, and myricetin have all been isolated from *Eucalyptus* extracts (Al-Sayed et al., 2012; Bhuyan et al., 2015; Vázquez et al., 2012). Santos et al. (2012) identified a number of phenolic compounds in *E. grandis*, *E. urograndis*, and *E. maidenii* bark extracts. *E. grandis* and *E. urograndis* bark were found to possess epicatechin and quercetin-glucuronide, followed by ellagic acid-rhamnoside. Ellagic acid was the major phenolic compound in *E. grandis,* whereas *E. urograndis* mainly

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contained galloyl-bis-hexahydroxydiphenoyl (HHDP)-glucose and gallic acid. In *E. maidenii* bark, the major compounds were catechin, chlorogenic acid, and methyl-ellagic acid-pentose. These reports demonstrated the high potential of *Eucalyptus* as a source of bioactive phenolic compounds.

Traditionally, the indigenous people of Australia have used many *Eucalyptus* species leaves to heal wounds and cure fungal infections (Gilles et al., 2010). In Anatolia, *Eucalyptus* plants have also been used in folk medicine for antiseptic, antimicrobial, astringent, and deodorant purposes, as well as anti-fever, stimulant, antispasmodic, and haemostatic agents (Ashour, 2008; Mulyaningsih et al., 2010; Topçu et al., 2011). *Eucalyptus* also contains a wide variety of phenolic compounds with antioxidant, antiproliferative, anti-inflammatory, anti-hyperglycemic, and anti-thrombotic properties (Gilles et al., 2010; Mota et al., 2012; Santos et al., 2012). In recent years, the area of natural antioxidants has developed significantly due to increasing limitations on the use of synthetic antioxidants and enhanced public awareness of health issues (Vázquez et al., 2012). Because of their potential health benefits, natural antioxidants are regarded as a better alternative to the synthetic products (Fu et al., 2010). Hence, the identification of antioxidants from natural sources has become a main research focus in natural product development.

Conventional techniques such as maceration, percolation, infusion, decoction, and hot continuous extraction have been used in the extraction of phytochemicals for many decades (Dhanani et al., 2013). In addition, the conventional extraction of plant material often entails different solid–liquid extraction procedures such as Soxhlet or reflux using water and other organic solvents (Ollanketo et al., 2002). Sultana et al. (2009) suggested that the manner of extraction of the solvent influences the extract yields and the resultant antioxidant activities of the plant materials. This is because different antioxidant compounds possess varied polarities and chemical properties that may or may not be soluble in a particular solvent. Different polar and non-polar solvents such as water, ethanol, methanol, acetone, and ethyl acetate are often used for the recovery of polyphenols, carotenoids, and saponins from a plant matrix (Arvayo-Enríquez et al., 2013; Cheok et al., 2014; Sultana et al., 2009). Even though advanced procedures such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), and enzyme-assisted extraction have recently attracted interest worldwide for the extraction of phytochemicals (Dahmoune et al., 2014; Domingues et al., 2012; Ma et al., 2014; Zuorro & Lavecchia, 2013), in many cases conventional extraction may be quite efficient in achieving enhanced yields of phytochemicals. Accordingly, the present study was designed to employ solid–liquid conventional extraction to extract TPC, TFC, antioxidants, proanthocyanidins, and saponins from *E. robusta* leaves using water and different organic solvents. Furthermore, the optimal extraction solvent was then implemented to yield TPC, TFC, proanthocyanidins, antioxidants, and saponins from other *Eucalyptus* species such as *E. saligna*, *E. microcorys*, and *E. globulus*. *E. microcorys* fruit was also included. The study sought to determine the *Eucalyptus* species that possess the highest amount of TPC, TFC, proanthocyanidins, antioxidants, and saponins for their potential industrial use.

Experimental

Fresh leaves of *E. robusta, E. saligna*, and *E. microcorys* were collected on 2nd April, 2014 from cultivated plants located at Ourimbah, Central Coast, NSW, Australia (latitude 33.4° S, longitude 151.4° E). E. globulus fresh leaves were collected from Tinderbox, Tasmania, Australia (latitude 43° S, longitude 147.2° E) on 6th May, 2014. The plants were authenticated by the author (A.C.C.). The leaves were then immediately transferred to the laboratory and stored at –20 ℃ to avoid potential degradation of the phenolic compounds. Using a dry air oven, the leaves were dried at 70 ℃ for 5 h prior to commencing the experiments. The leaves were ground to a fine powder using a commercial grade blender (Rio^{TM}Commercial Bar Blender, Hamilton Beach, Australia) and stored at –20 ℃ until required.

The powdered leaf samples were extracted using water, 70 vol. % water–ethanol mixture, ethanol, acetone, acetonitrile, and ethyl acetate. These solvents represented a polarity ranging from high to low. The conventional extraction parameters, such as time and temperature for *E. robusta* using water, were previously optimised by Vuong et al. (2015b). The sample-to-solvent ratio was modified by mixing 2 g of powdered leaf sample with 100 mL of distilled water and refluxing at 85 ℃ for 15 min. For the organic solvents, 2 g of the powdered sample was mixed with 100 mL of the solvent and macerated at ambient temperature for 72 h. The extractions were carried out in 250 mL conical flasks sealed with a stopper and covered with aluminium foil in a shaking water bath (Ratek instruments Pty, Victoria, Australia). The aqueous extracts were immediately cooled on ice to ambient temperature (AT). All the extracts were filtered using a 0.45 μm cellulose syringe filter (Phenomenex Australia Pty, NSW, Australia).

Determination of extracted components content and antioxidant, radical scavenging and iron chelating capacities

The colorimetric assessment of TPC was determined following the method proposed by Škerget et al. (2005). The extracts were diluted up to 40× to

fit within the optimal absorbance range. A calibration curve was constructed using gallic acid as standard, with the results expressed as mg of gallic acid equivalents (GAE) per g of dry mass (mg of GAE per g).

Total flavonoid content (TFC) was determined as per the method described by Tan et al. (2014). Rutin was used as a standard and TFC expressed as mg of rutin equivalents (RE) per g of dry mass (mg of RE per g).

The vanillin–HCl method was employed to estimate the proanthocyanidins content of the extract, as described by Broadhurst and Jones (1978). 3 mL of vanillin solution (40 g per L in methanol) was added to 0.5 mL of the diluted sample. 1.5 mL of concentrated HCl was then added to the mixture and left at AT for 15 min prior to measurement of the absorbance at 500 nm. Catechin was used as a standard and the amount of proanthocyanidins was expressed as mg of catechin equivalents (CAE) per g of dry mass (mg of CAE per g).

TAC was measured using ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assay as described by Arnao et al. (2001) with some modifications. The ABTS stock solution was prepared by dissolving 7.4 mM of ABTS in 10 mL of distilled water. The potassium persulphate stock solution was prepared by dissolving 2.6 mM of potassium persulphate in 100 mL of distilled water. Both the stock solutions were then mixed together in equal quantities and allowed to react for 15 h at ambient temperature in the dark to obtain the mixture solution. The working solution was then prepared by mixing 1 mL of the mixture solution with 60 mL of methanol to obtain an absorbance of (1.1 ± 0.02) units at 734 nm using the UV spectrophotometer (Cary® 50 Bio, Varian, Victoria, Australia). A fresh working solution was prepared for each assay. To 150 μL of extracts, 2850 μL of the working ABTS solution was added and then allowed to react in the dark at ambient temperature for 2 h before measuring the absorbance at 734 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard for constructing the calibration curve. The results were expressed in mg of Trolox equivalents (TE) per g of dry mass (mg of TE per g).

The free-radical scavenging activity of the extracts was analysed using the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay as described by Brand-Williams et al. (1995) with some modifications. The DPPH stock solution was prepared by dissolving 24 mg of DPPH in 100 mL of methanol. It was then stored at –20 ℃ until required. The working solution was prepared afresh by mixing 10 mL of the stock solution with 45 mL of methanol to obtain an absorbance of (1.1 ± 0.02) at 515 nm using the UV spectrophotometer. The extracts (150 μL) were allowed to react with 2850 μL of working solution in darkness at ambient temperature for 3 h. The absorbance was measured at 515 nm and the results were expressed in mg of Trolox equivalents (TE) per g of dry mass (mg of TE per g).

The iron-chelating capacity of the extract was analysed using the cupric ion-reducing antioxidant capacity (CUPRAC) assay as described by Apak et al. (2004). Trolox was used as the calibration standard, with the results expressed in mg of Trolox equivalents (TE) per g of dry mass (mg of TE per g).

The saponin content of the extracts was determined by the method described by Hiai et al. (1976). Aescin was used as the standard for the calibration curve, with the results expressed as mg of aescin equivalents (ASE) per g of dry mass (mg of ASE per g).

All of the analyses were carried out in triplicate. The SPSS statistical software (version 16.0) was used to compare the means by the one-way ANOVA, independent samples *t*-test and the LSD post-hoc test. Values were expressed as means ± standard deviations. At $p < 0.05$, the differences between the mean values in the experiments were taken to be statistically significant.

Results and discussion

Selection of optimal solvent

Water, 70 vol. % water–ethanol mixture, ethanol, and acetone extracts of *E. robusta* were found to be more efficient than acetonitrile and ethyl acetate extracts for TPC yield (Fig. 1A). Moreover, the one-way ANOVA test revealed that the TPC yield of the aqueous extract was not statistically different ($p > 0.05$) from that of the 70 vol. % water–ethanol mixture, ethanol, and acetone extracts. Vuong et al. (2015b) recently reported that 125.8 mg of GAE per g of TPC could be obtained from *E. robusta* using a sample-to-solvent ratio of 1 g per 20 mL, which is similar to the present finding. A TPC yield of 150.60 mg of GAE per g was obtained using a sample-to-solvent ratio of 2 g per 100 mL. In addition, a greater yield of TFC was obtained from water, 70 vol. % water–ethanol mixture and ethanol extracts (Fig. 1B). The TFC of the *E. robusta* aqueous extract was not statistically different from that of the 70 vol. % water–ethanol mixture and ethanol extracts ($p > 0.05$). The proanthocyanidins content was also observed to be greater in water, 70 vol. % water–ethanol mixture, ethanol, and acetone than in the acetonitrile and ethyl acetate extracts (Fig. 1C). The difference between the yields of proanthcyanidins was not statistically significant among the water, 70 vol. % water–ethanol mixture, ethanol, and acetone extracts ($p > 0.05$).

For the antioxidant activity, as determined by the ABTS, DPPH, and CUPRAC assays, water, 70 vol. % water–ethanol mixture, ethanol, and acetone extracts exhibited greater overall activity than acetonitrile and ethyl acetate (Figs. 2A–2C). Alothman et al. (2009) previously established that aqueous and 70 vol. %

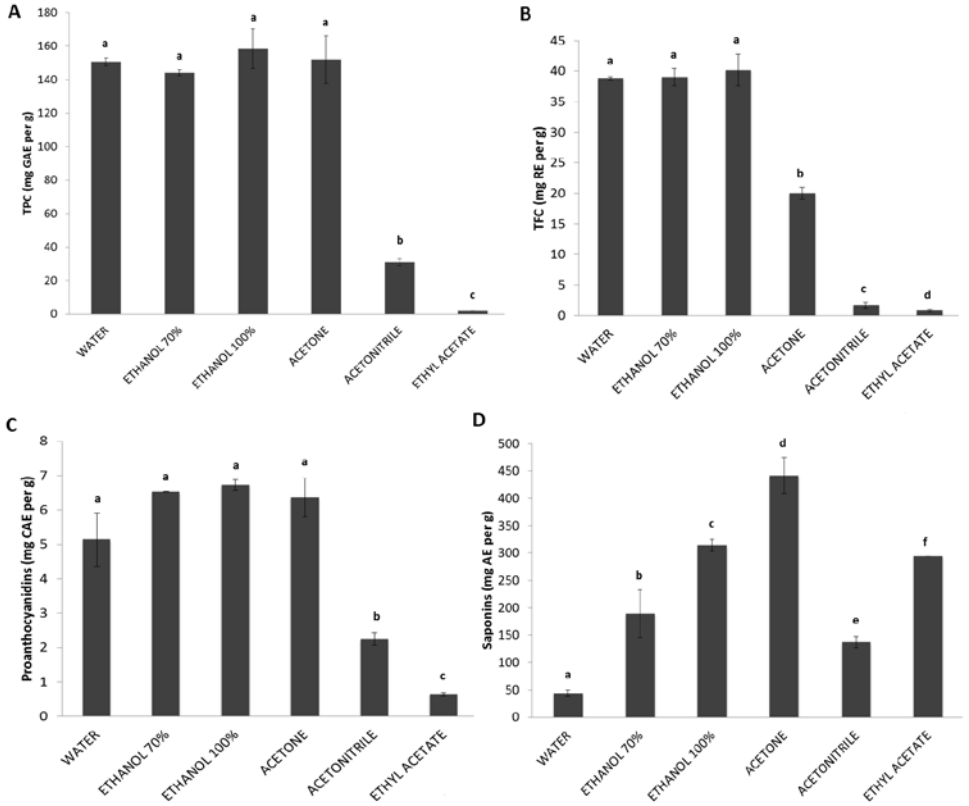

Fig. 1. Conventional extraction of TPC (A), TFC (B), proanthocyanidins (C), and saponins (D) from *E. robusta* leaves using different solvents ($n = 3$).

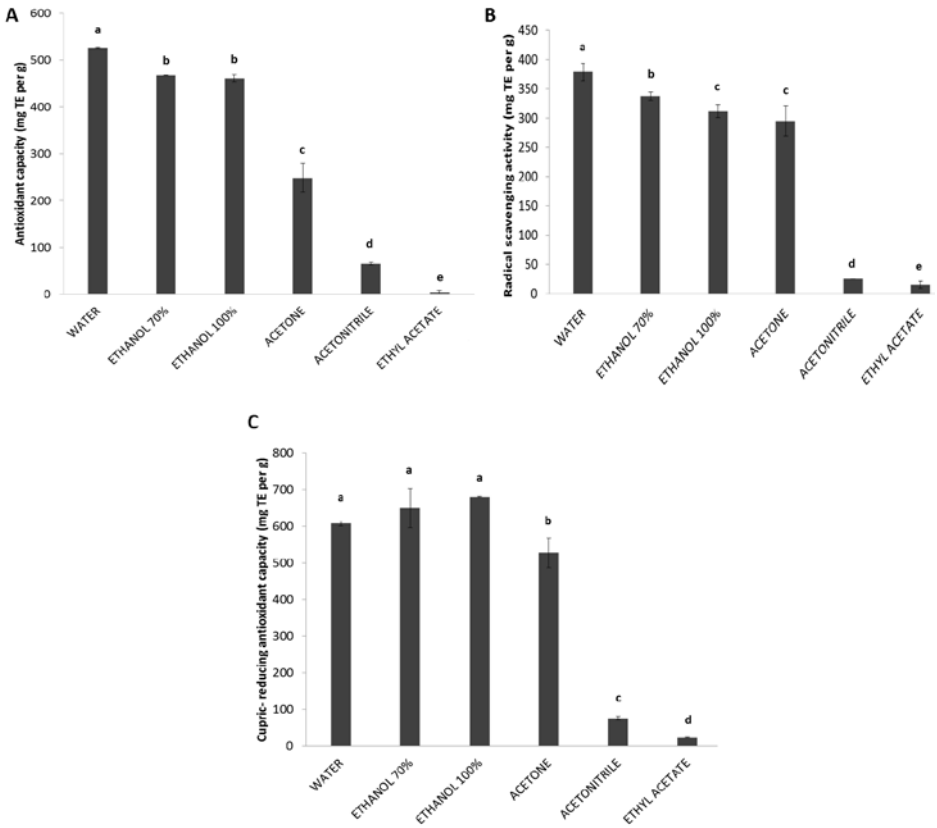

Fig. 2. Total antioxidant capacity (A), DPPH free-radical scavenging activity (B), and cupric-reducing antioxidant capacity (C) of *E. robusta* leaf extracts using different extraction solvents ($n = 3$).

water–ethanol mixture extracts of *Psidium guajava* L. exhibited similar DPPH values with no statistically significant difference. However, in the present study, the aqueous extract of *E. robusta* exhibited significantly higher antioxidant activity than the 70 vol. % water–ethanol mixture, ethanol, and acetone extracts and the difference was statistically significant ($p < 0.05$) in the ABTS and DPPH assays (Figs. 2A and 2B). On the other hand, the CUPRAC assay revealed that the difference between the antioxidant activity of the water, 70 vol. % water–ethanol mixture, ethanol, and acetone extracts of *E. robusta* was not statistically significant ($p > 0.05$). Accordingly, the cupric ion-reducing antioxidant capacity of the aqueous extract was at a similar level to the other three extracts (Fig. 2C). Vázquez et al. (2008) found that a combination of water and alcohol, when used as the solvent system, could yield higher antioxidant values in *E. globulus* bark extracts using the FRAP assay. In the present study, similar observations were made using the CUPRAC assay. It was noted that the 70 vol. % water–ethanol mixture extract exhibited a similar cupric ion-reducing antioxidant capacity to that of the aqueous, 100 % ethanol, and acetone extracts of *E. robusta*. This might be due to differences between the samples and the fact that the CUPRAC and FRAP assays work on different principles. The origin of the plant sample can have more influence than the polarity of the solvents on the antioxidant activity of the extracts (Koleva et al., 2002). The ABTS, DPPH, and CUPRAC assays displayed the same trends in the present study, as validated by the significant correlations between the ABTS, DPPH, and CUPRAC values. The correlation values (R^2) were 0.90, 0.89, and 0.95 for ABTS/DPPH, ABTS/CUPRAC, and DPPH/CUPRAC, respectively. Fu et al. (2010) proposed that the phenolic compounds present in *E. robusta* were significantly correlated to the antioxidant activity of the extracts. Similarly, positive correlation values were observed between TPC and ABTS ($R^2 = 0.9602$) and TPC and DPPH ($R^2 = 0.9666$) in *Sorbus torminalis* by Olszewska (2011). Saeed et al. (2012) also reported significant R^2 values of 0.7020 and 0.6777 for TPC/DPPH and TPC/ABTS, respectively, in *Torilis leptophylla*. The present data also revealed a positive correlation between the TPC and antioxidant capacity of *E. robusta* endorsing the previous finding. TPC showed R^2 values of 0.81, 0.94, and 0.96 with ABTS, DPPH, and CUPRAC, respectively. These high correlation values confirm that phenolic compounds are the major contributors to the antioxidant capacity of *E. robusta*.

The aqueous extract of *E. robusta* possessed the lowest amount of saponins, whereas acetone proved to be the best solvent for saponin extraction (Fig. 1D). A significantly higher saponin yield of (441.85 ± 32.88) mg of AE per g was obtained from acetone extracts ($p < 0.05$) followed by ethanol and ethyl acetate extracts. Solubility and effective diffusion are the two main factors that determine the efficiency of the maceration process (Cheok et al., 2014). In general, polar compounds are soluble in polar solvents and non-polar compounds are soluble in non-polar solvents. The mass transfer of solutes from the plant material to the extraction solvent leads to an effective diffusion (Cheok et al., 2014). Other factors that affect the extraction efficiency are: weakening of the solute–matrix interaction and swelling of the plant materials (Zuorro et al., 2014). Even though saponins are highly polar compounds, the extraction yield can be influenced by their structural variations, number, arrangement, and orientation of the sugar units and the number and types of sugar chains attached to the aglycone moiety (Majinda, 2012). As expected from saponins belonging to a different family of compounds, no correlation was observed between the saponin content and TPC of *E. robusta* extracts. Furthermore, the ABTS, DPPH, and CUPRAC values of the *E. robusta* extracts were also not correlated with the saponin content. It was previously reported that ethanol, methanol, acetone, and ethyl acetate are suitable solvents for the extraction of saponins from plants such as *Salicornia herbacea*, *Momordica charantia*, *Allium ampeloprasum*, and *Polygonatum odoratum* (Cheok et al., 2014; Habicht et al., 2011; Zhao et al., 2014). A parallel observation was made in the present study indicating that a greater yield of saponins can be obtained using acetone, ethanol and ethyl acetate as solvent systems for *E. robusta* extracts.

Implementation of optimal solvent on other Eucalyptus species samples for extraction of TPC, TFC, antioxidants, proanthocyanidins, and saponins

From the results, water was selected as the most suitable solvent for extracting TPC, TFC, and antioxidants from *E. robusta* leaves, water being the most environmentally friendly, non-toxic, economical, and accessible polar extraction solvent (Bhuyan et al., 2015). Due to the higher health risks associated with organic solvents, aqueous solvents should be used for extraction whenever possible (Vázquez et al., 2008). Various other studies have reported the use of water for the extraction of phenolic compounds from *E. globulus*, *E. grandis*, *E. urograndis*, and *E. maidenii* bark (Santos et al., 2011, 2012; Vázquez et al., 2008). As such, water was used for the extraction of TPC, TFC, antioxidants, proanthocyanidins, and saponins from *E. saligna*, *E. microcorys*, and *E. globulus* and the respective extracts were compared with those of *E. robusta*. In addition, the *E. microcorys* fruit was included in this study with the results displayed in Table 1.

Table 1. Yields of TPC, TFC, proanthocyanidins, and antioxidants from different species of *Eucalyptus* using conventional extraction with water ($n = 3$)

	E. robusta	*E. saligna*	*E. microcorys*		*E. globulus*
			Leaves	Fruits	
TPC (mg of GAE per g)	150.60 ± 2.47^a	17.58 ± 1.13^b	92.50 ± 3.10^c	29.85 ± 1.41^d	58.37 ± 3.19^e
TFC (mg of RE per g)	38.83 ± 0.23^a	16.33 ± 2.35^b	30.33 ± 0.47^c	5.5 ± 0.70^d	27.16 ± 2.12^c
Proanthocyanidins (mg of CAE per g)	5.14 ± 0.77^a	4.8 ± 0.12^a	1.55 ± 0.03^b	0.18 ± 0.03^c	4.32 ± 0.16^a
Saponins (mg of AE per g)	44 ± 5.9^a	63.75 ± 0.72^b	206.88 ± 15.49^c	42.70 ± 1.15^a	167.93 ± 11.04^d
TAC (mg of TE per g)	525.67 ± 1.99^a	40.56 ± 4.15^b	186.98 ± 7.08^c	72.16 ± 2.91^d	137.23 ± 0.41^e
DPPH (mg of TE per g)	378.61 ± 14.72^a	44.97 ± 5.98^b	161.95 ± 4.01^c	67.85 ± 5.69^d	89.39 ± 3.22^e
CUPRAC (mg of TE per g)	607.43 ± 6.69^a	86.29 ± 2.06^b	255.69 ± 1.34^c	112.84 ± 10.90^d	160.52 ± 22.09^e

All the values are means ± standard deviations and those in the same row not sharing the same superscript letter are significantly different from each other ($p < 0.05$).

The aqueous extract of *E. robusta* exhibited significantly higher levels of TPC than the *E. saligna*, *E. microcorys* (leaf and fruit), and *E. globulus* extracts ($p < 0.05$; Table 1). Al-Sayed et al. (2012) reported that an *E. gomphocephala* extract contained (389.05 ± 8.64) mg of GAE per g of dry mass of phenolic compounds. Similarly, Almeida et al. (2009) found that phenolic compounds comprised approximately 31 % of an *E. globulus* leaf extract. In a previous study, we reported that an aqueous *E. robusta* extract contained (58.40 ± 1.03) mg of GAE per g of dry mass of total phenolics when microwave-assisted extraction (MAE) was used (Bhuyan et al., 2015). However, the current study revealed that (150.60 ± 2.47) mg of GAE per g of dry mass of TPC could be obtained from aqueous *E. robusta* extracts using conventional extraction. The greater efficiency achieved by conventional extraction can be attributed to the heat sensitivity of the phenolic compounds present in *E. robusta*. Liazid et al. (2007) also previously reported that a higher temperature of 175 ℃ could degrade most of the phenolic compounds such as gallic acid, gentisic acid, caffeic acid, catechin, epicatechin, myricetin, and kaempferol. In the present study, a temperature of 85 ℃ (< boiling point of water) was used for conventional extraction, hence the thermal degradation of phenolic compounds was minimised, which was not the case for MAE.

The *E. robusta* aqueous extract was also found to possess the highest amount of TFC ($p < 0.05$), followed by *E. microcorys* (leaf) and *E. globulus* (Table 1). The difference between the TFC values of *E. microcorys* (leaf) and *E. globulus* extracts was statistically insignificant ($p > 0.05$), as revealed by the independent samples *t*-test. The *E. microcorys* (fruit) and *E. saligna* aqueous extracts exhibited the lowest TFC values. The TFC values showed a similar trend to that of TPC, which is not surprising as flavonoids comprise a large group of polyphenolic compounds in plants (Yao et al., 2004). As reported previously, (19.15 ± 1.06) mg of RE per g of dry mass of total flavonoids can be obtained from *E. robusta* using water and MAE with 3 min of irradiation (Bhuyan et al., 2015). However, in the present study, a two-fold increase in the TFC yield ((38.83 ± 0.23) mg of RE per g) was observed using water and conventional extraction for 15 min. In general, flavonoids and other phenolic compounds are susceptible to high temperatures (Ross et al., 2011). In MAE, it was not possible to regulate the temperature but, in the case of conventional extraction, the temperature was maintained at 85 ℃, which would inevitably lead to a higher yield of TPC and TFC.

E. robusta, *E. saligna*, and *E. globulus* aqueous extracts were found to have statistically similar proanthocyanidin contents ($p > 0.05$; Table 1). The aqueous extracts of the *E. microcorys* leaf and fruit had the lowest proanthocyanidin contents out of the extracts tested. Cadahía et al. (1997) noted that the aqueous methanol extract of *E. globulus* contained 2.15–3.50 mg of CAE per g of dry mass of proanthocyanidins. In the present study, a similar yield of proanthocyanidins ((4.32 ± 0.16) mg of CAE per g) in the aqueous *E. globulus* extract was also observed.

In the case of antioxidant capacity, the aqueous *E. robusta* extract displayed significantly higher values ($p < 0.05$) than the other extracts for all three antioxidant assays (Table 1). Overall, the antioxidant activity of the tested extracts decreased in the following order: *E. robusta, E. microcorys* (leaf), *E. globulus, E. microcorys* (fruit), *E. saligna.* As stated earlier, the antioxidant activity of the extracts is significantly influenced by the total content of phenolic compounds present, hence high correlation values were demonstrated between the TPC and antioxidant activity of the *Eucalyptus* extracts. R^2 values of 0.92, 0.93, and 0.93 were found between TPC and ABTS, TPC and DPPH, and TPC and CUPRAC, respectively (Table 2). Parallel observations were made for *E. grandis*, *E. urograndis*, and *E. maidenii* bark extracts (Santos et al., 2012). Free-radicals play an important role in many diseases, such as cardiovascular disease, cancer, neurodegenerative disease, and diabetes. Therefore, antioxidants, such as flavonoids and other phenolics, have attracted

Table 2. Correlation of TPC and saponins with antioxidant capacity of extracts

	R^2 value		
	Antioxidant capacity		
	TAC	DPPH	CUPRAC
TPC	0.92	0.93	0.93
Saponins	0.03	0.02	0.03

attention in recent years as potential agents for preventing and treating oxidative stress-related diseases (Fu et al., 2010; Gharekhani et al., 2012). The antioxidant activity of phenolics is primarily attributed to their redox properties that enable them to act as singlet oxygen quenchers, reducing agents, and hydrogen donors (Gharekhani et al., 2012).

Interestingly, *E. microcorys* (leaf) had a significantly higher saponin content ((206.88 ± 15.49) mg of AE per g) than the other *Eucalyptus* extracts tested ($p < 0.05$) (Table 1). *E. globulus* was found to possess (167.93 ± 11.04) mg of AE per g of dry mass of saponins followed by *E. saligna* (63.75 ± 0.72 mg of AE per g). *E. robusta* and *E. microcorys* (fruit) aqueous extracts contained the lowest amounts of saponins out of the species tested. Puttaswamy et al. (2014) established that (68 ± 0.8) mg per g of saponins could be extracted from *E. tereticornis* bark using water. However, in the present study, *E. microcorys* leaf aqueous extract was found to contain a higher amount of saponins than the *E. tereticornis* bark aqueous extract previously described. In addition, no significant correlation ($R^2 = 0.0063$) was observed between the saponin content and TPC of the extracts tested. The saponin content was also not significantly correlated with the antioxidant activity of the extracts (Table 2). Although saponins are known to possess antioxidant properties (Cheok et al., 2014), in the present study, the antioxidant activity was primarily attributed to the TPC of the extracts.

Conclusions

Water was demonstrated as the most suitable solvent for the extraction of TPC, TFC, proanthocyanidins, and antioxidants from *E. robusta* using conventional extraction. By contrast, the *E. robusta* acetone extract was proved to contain the highest amount of saponins. As water is the safest, most environmentally friendly, economical, and accessible of all the solvents tested, it was further selected to extract TPC, TFC, proanthocyanidins, antioxidants, and saponins from other species of *Eucalyptus* such as *E. saligna*, *E. microcorys* (both fruit and leaf), and *E. globulus*. This study revealed that the aqueous extract of *E. robusta* had the highest TPC, TFC, and antioxidant contents, whereas the proanthocyanidins content was on the same level as with *E. saligna* and *E. globulus*. By contrast, aqueous *E. microcorys* (leaf) extract had a significantly higher saponin content than the other *Eucalyptus* extracts tested. These findings demonstrate the potential of *Eucalyptus* species as a source of natural bioactive compounds such as polyphenolics, including flavonoids and proanthocyanidins, antioxidants, and saponins. The study also indicates the efficiency of conventional extraction over modern techniques such as MAE. Further studies are required to isolate and characterise the bioactive compounds present in *Eucalyptus* for their prospective use in the food, pharmaceutical, and nutraceutical industries.

Acknowledgements. The authors wish to acknowledge the funding support received from the following: Ramaciotti Foundation (ES2012/0104), and the University of Newcastle. The authors also wish to express their gratitude to Brad Potts and Paul Tilyard from the School of Biological Science, University of Tasmania, Australia for providing the E. globulus sample and for their valuable comments on the manuscript.

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CHAPTER 3

Evaluation and screening of eucalypt crude extracts for antiproliferative properties

3.1 Introduction

A number of *in vitro* and *in vivo* studies have exploited the anticancer properties of eucalypts mostly belonging to the genus *Eucalyptus* (Al-Fatimi et al., 2005; Benyahia et al., 2005; Ito et al., 2000; Mota et al., 2012; Soliman et al., 2014; Takasaki et al., 1995; Takasaki et al., 2000; Vuong et al., 2015a). In addition, eucalypt research is fundamentally focused on essential oils and their volatile compounds from a limited number of eucalypts rather than crude extracts. Vuong et al. (2015b) were the first to report the cytotoxicity of *E. robusta* aqueous crude extract against MIA PaCa-2 and ASPC-1 pancreatic cancer cells. As a consequence, there remains an unexploited resource for development of new anticancer agents specific to PC. Therefore, this aim was proposed to conduct in the form of two studies to evaluate the cytotoxicity of crude extracts from a total of eight species representing all three genera of eucalypts: *Angophora*, *Corymbia* and *Eucalyptus* against pancreatic adenocarcinoma cells. This approach distinguished the active crude extracts from the inactive ones and aided subsequent bioassay-guided fractionation studies.

3.2 Results and Discussion

The overall experimental design of the two studies is shown in Figure 2. The results obtained from the studies were published in the form of following two Research Papers:

Research Paper 4: **Deep Jyoti Bhuyan**, Quan V. Vuong, Danielle R. Bond, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. Exploring the least studied Australian eucalypt genera: *Corymbia* and *Angophora* for phytochemicals with anticancer activity against pancreatic malignancies. **Chemistry & Biodiversity (2017).** DOI: 10.1002/cbdv.201600291

Research Paper 5: **Deep Jyoti Bhuyan**, Jennette Sakoff, Danielle Bond, Melanie Predebon, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. *In vitro* anticancer properties of selected *Eucalyptus* species. **In Vitro Cellular & Developmental Biology – Animal (2017).** DOI: 10.1007/s11626-017-0149-y

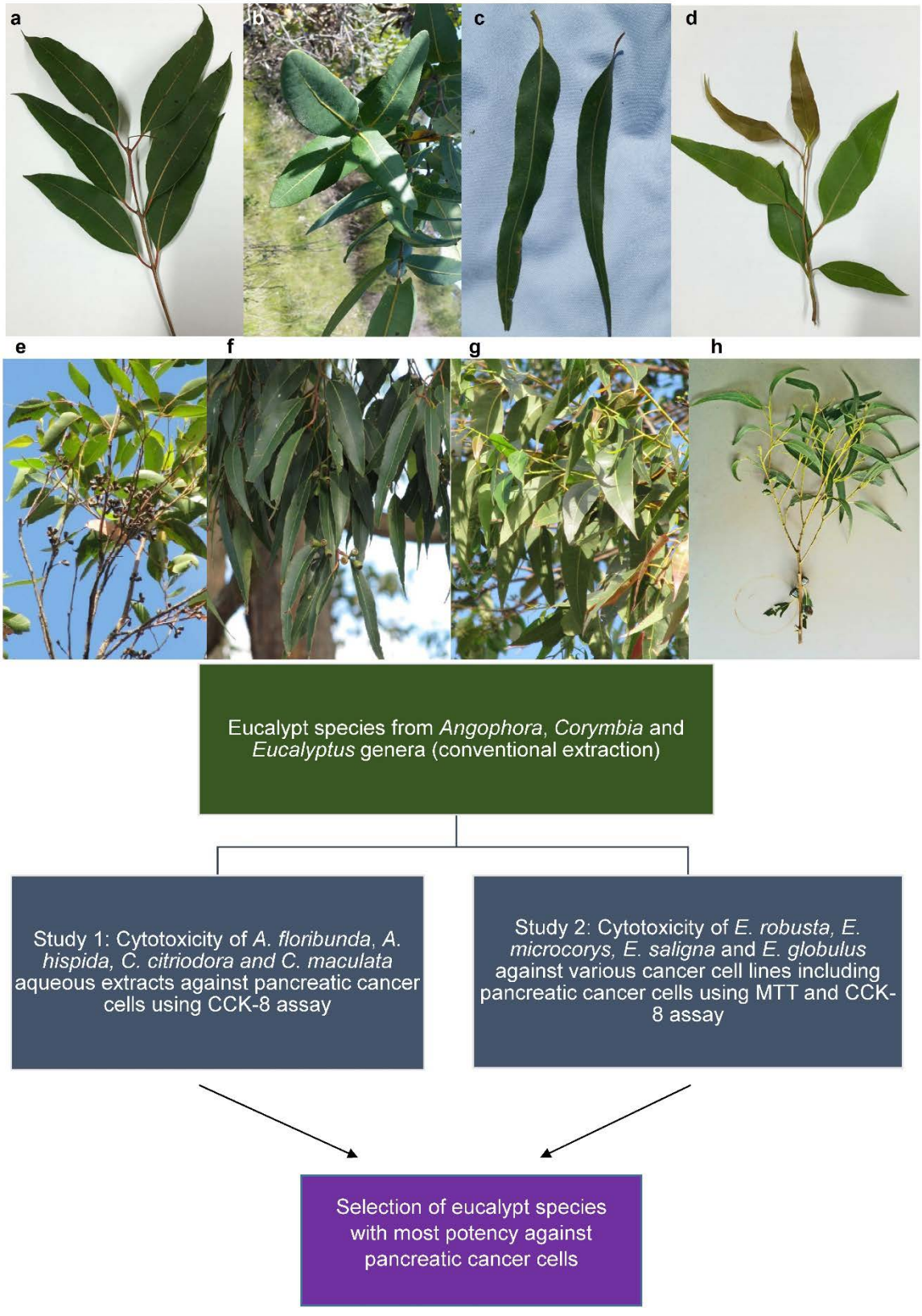

Figure 2: The overall experimental design of the two studies assessing the cytotoxicity of different extracts from *Angophora, Corymbia* and *Eucalyptus* species: a) *A.floribunda*, b) *A. hispida,* c) *C. citriodora*, d) *C. maculata*, e) *E. robusta*, f) *E. microcorys*, g) *E. saligna* and h) *E. globulus* against cancer cells.

3.3 Conclusions

Figures 3 and 4 summarise the key findings of the two studies. The aqueous extracts of *A. floribunda*, *A. hispida, E. microcorys* and the ethanolic extract *E. microcorys* exhibited statistically similar IC_{50} values in the CCK-8 assay against MIA PaCa-2 cells (Figure 3). Interestingly, both the tested *Corymbia* species (*C. citriodora* and *C. maculata*) showed low cytotoxicity against PC cells. Moreover, *E. microcorys* extracts were also found to exert more than 80% cell growth inhibition against glioblastoma, neuroblastoma and lung cancer cells at 100 µg/mL in the MTT assay. Similarly, in MIA PaCa-2 cells, the aqueous *E. microcorys* leaf and fruit extracts displayed significantly greater cell growth inhibition ($p > 0.05$) at both 100 and 50 µg/mL in the CCK-8 assay (Figure 4). Therefore, the aqueous *E. microcorys* leaf extract was selected for further investigation due to the easier availability of leaves compared to the seasonal fruits.

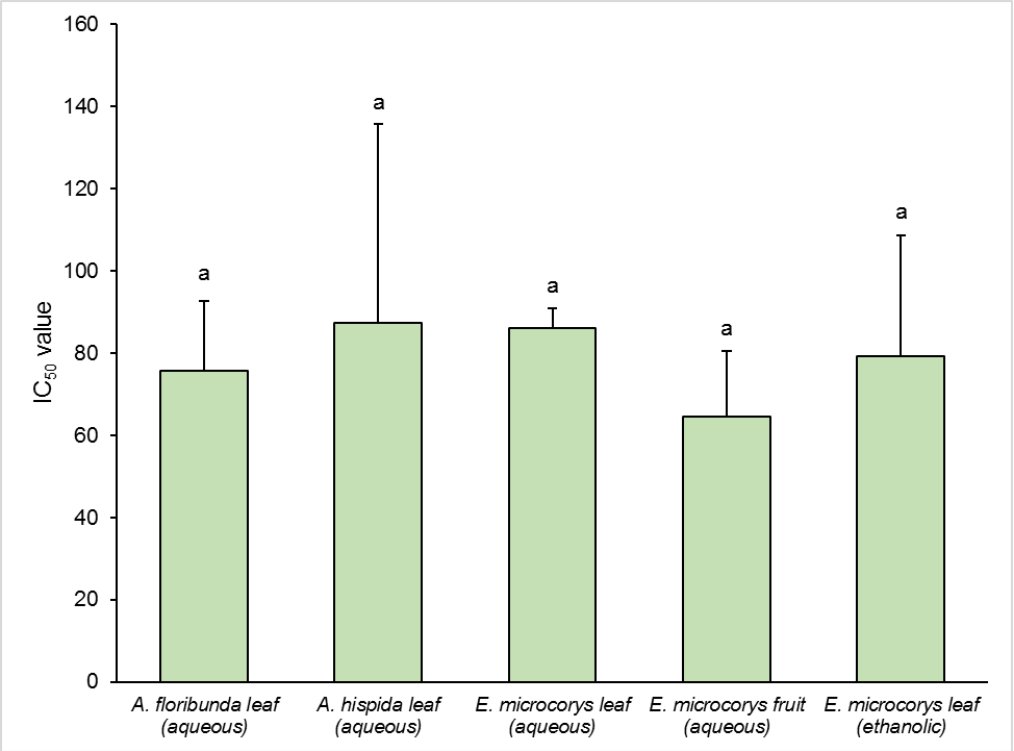

Figure 3: IC_{50} values of *Angophora* and *Eucalyptus* species as reported in Research Papers 4 and 5 against MIA PaCa-2 cells. All values are means ± SD (n =3) and bars sharing the same letter are statistically similar to each other ($p > 0.05$).

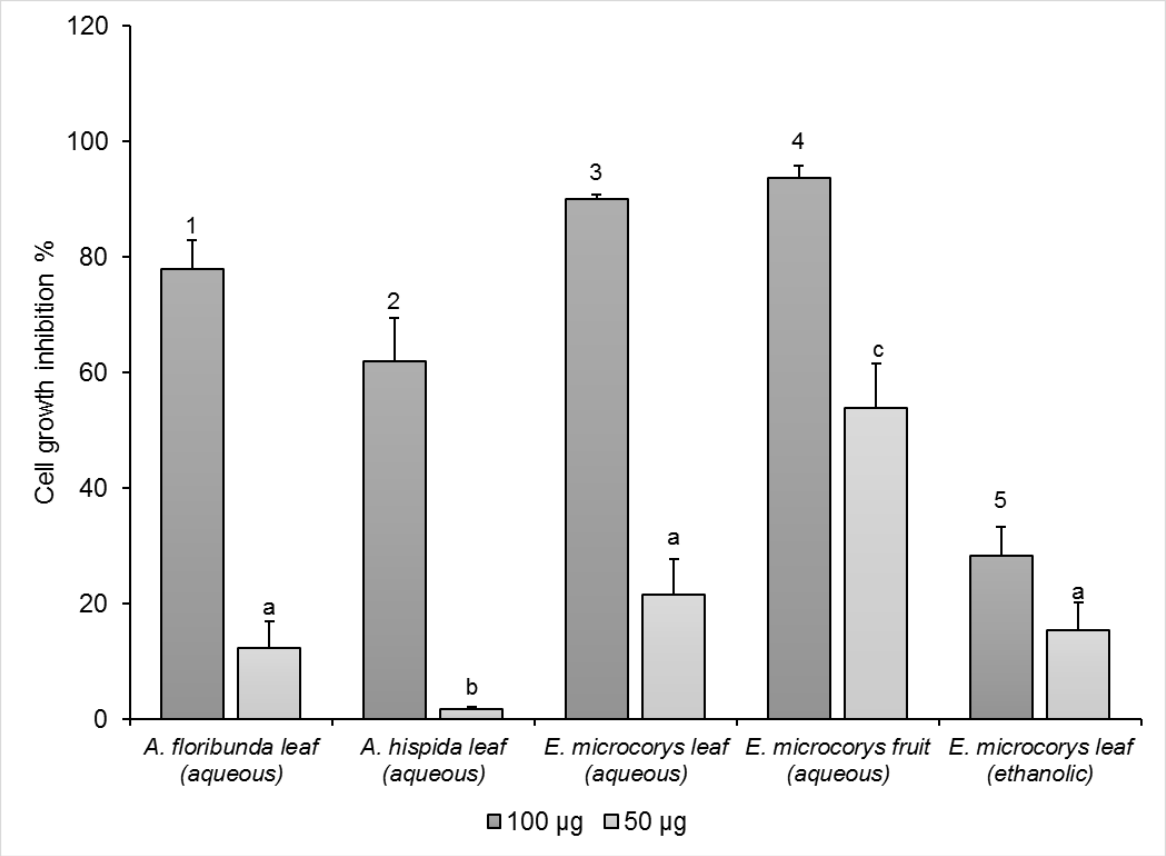

Figure 4: Cell growth inhibition % values of *Angophora* and *Eucalyptus* species as reported in Research Papers 4 and 5 against MIA PaCa-2 cells. All values are means ± SD (n = 6) and bars not sharing the same letter or number are statistically different from each other ($p < 0.05$) at 100 and 50 µg/mL, respectively.

CHEMISTRY & BIODIVERSITY

Exploring the Least Studied Australian Eucalypt Genera: Corymbia and Angophora for Phytochemicals with Anticancer Activity against Pancreatic Malignancies

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While the pharmacological and toxicological properties of eucalypts are well known in indigenous Australian medicinal practice, investigations of the bioactivity of eucalypt extracts against high mortality diseases such as pancreatic cancer in Western medicine have to date been limited, particularly amongst the genera *Corymbia* and *Angophora*. Four *Angophora* and *Corymbia* species were evaluated for their phytochemical profile and efficacy against both primary and secondary pancreatic cancer cell lines. The aqueous leaf extract of *Angophora hispida* exhibited statistically higher total phenolic content (107.85 ± 1.46 mg of gallic acid equiv. per g) and total flavonoid content (57.96 ± 1.93 mg rutin equiv. per g) and antioxidant capacity compared to the other tested eucalypts ($P < 0.05$). Both *A. hispida* and *A. floribunda* aqueous extracts showed statistically similar saponin contents. *Angophora floribunda* extract exerted significantly greater cell growth inhibition of 77.91 ± 4.93% followed by *A. hispida* with 62.04 ± 7.47% ($P < 0.05$) at 100 μg/ml in MIA PaCa-2 cells with IC_{50} values of 75.58 and 87.28 μg/ml, respectively. More studies are required to isolate and identify the bioactive compounds from these two *Angophora* species and to determine their mode of action against pancreatic malignancies.

Keywords: Eucalypts, Corymbia, Angophora, Anticancer, Pancreatic cancer.

Introduction

Eucalypts are one of the world's most important plants, with about twenty *eucalypt* species being exploited commercially worldwide for their medicinal and pharmaceutical properties as well as for food additives.[1][2] The classification of the eucalypts, especially recognition of the genus *Corymbia* has generated considerable controversy.[3][4] Molecular (nuclear DNA and plastid DNA) and morphological research have indicated that *Angophora* and *Corymbia* together are a distinct lineage, separate from *Eucalyptus*.[3][5][6] Therefore, they are considered a separate genus from *Eucalyptus* in the most recent comprehensive electronic flora treatment[5] and now eucalypts are divided into three different genera: *Eucalyptus, Corymbia*, and *Angophora*.

Corymbia has a largely tropical and arid zone distribution in Australia and New Guinea and comprises about 115 species whereas, the genus *Angophora* consists of 9 species, all confined to mainland eastern Australia.[4][5] The current literature contains numerous studies profiling the wide ranging bioactivity (including anticancer properties) of the genus *Eucalyptus*, comparatively little attention has been paid to the other two eucalypt genera: *Corymbia* and *Angophora*. Only a few studies have been conducted thus far that demonstrate the potential anticancer activity of leaf extract, resin and compounds isolated from *C. citriodora*.[7 – 11]

Traditionally, hot water extracts of dried leaves of *C. citriodora* are used as remedies in Chinese folk medicine for the symptoms of respiratory infections, such as cold, flu, and sinus congestion because of its anti-inflammatory, analgesic and antipyretic properties.[12] The essential oils derived from *C. citriodora* are also used for medicinal and pharmaceutical purposes and have been shown to possess broad spectrum antifungal activity.[12] *C. citriodora* is also used to treat nausea, vomiting, abdominal pain, indigestion, irritable bowel and bloating and is extensively used in aromatherapy.[13] Similarly, *C. maculata* has been shown to possess antimicrobial, antioxidant and hepatoprotective effects in some studies.[14][15] Juice extracted from the leaves of *C. maculata* is used to treat

rheumatism and paralysis by the Khasi traditional healers and villagers in Meghalaya, India.[16] Traditional Australian bush medicine employs gum derived from the bark of *C. maculata* and *A. costata* for treating bladder infections and diarrhoea, respectively.[17] However, the current literature has no information in regards to the medicinal and therapeutic properties of other *Angophora* species such as *A. hispida* and *A. floribunda,* let alone their anticancer activity.

Pancreatic cancer has been a daunting challenge for oncologists. It has a fatal prognosis due to its late diagnosis and high resistance to chemotherapy and radiation therapies.[18][19] According to the Australian Cancer Research Foundation,[20] pancreatic cancer has the highest mortality rate of any cancer, with approximately 2500 new Australian patients diagnosed with the disease annually. In 2010, pancreatic cancer was the 5th most common cause of cancer death in Australia. The incidence and mortality statistics of pancreatic cancer are similar throughout the world.[21]

There is little direct scientific evidence linking the prospective application of eucalypt-derived bioactive compounds in the management of pancreatic cancer. In this study, four eucalypt species (*A. hispida, A. floribunda, C. citriodora*, and *C. maculata*) belonging to the two least explored genera (*Angophora* and *Corymbia*) were studied and their phytochemical profile and anticancer activity against both primary and secondary pancreatic adenocarcinoma cells evaluated.

Results and Discussions

Extraction Yields and Phytochemical Content of Selected Eucalypts

Extraction yields (g/100 g of dry weight) of the different species of eucalypts are shown in the *Table 1*. *Angophora hispida* exhibited a significantly higher extraction yield in comparison to other tested eucalypts ($P < 0.05$). The extraction yields of all the extracts were significantly different from each other ($P < 0.05$). Availability of extractable components and sample type are some of the key factors that influence the extraction yield of a sample.[22]

Table 1 also depicts the yields of phytochemicals and antioxidants from the tested eucalypt species. Plant phenolics have been widely liked with anticancer properties in the literature,[23 – 26] therefore, this study analysed the levels of TPC, TFC, and proanthocyanidins in the four tested eucalypt species and the results are shown in *Table 1*. The yield of TPC was significantly greater in *A. hispida* aqueous extract followed by *C. citriodora* compared to other extracts ($P < 0.05$) (*Table 1*). *Angophora floribunda* and *C. maculata* had statistically similar TPC ($P > 0.05$). *Angophora hispida* also exhibited statistically higher extraction yield of TFC ($P < 0.05$) followed by *A. floribunda*. An earlier study by *Takahashi et al.*[14] isolated three flavonoids: 2′,6′-dihydroxy-3′-methyl-4′-methoxy-dihydrochalcone, eucalyptin, and 8-desmethyl-eucalyptin were isolated from *C. maculata* hexane/AcOEt extract. Despite this, in our study, aqueous *C. maculata* had the lowest TFC (14.81 ± 0.53 mg GAE/g) among the tested species ($P < 0.05$), by a considerable margin. We have previously reported that the aqueous extract of *E. robusta* leaves had a significantly higher TPC and TFC (150.60 ± 2.47 mg GAE)/g and 38.83 ± 0.23 mg RE/g, respectively) in comparison to that of *E. saligna, E. microcorys,* and *E. globulus* when conventional extraction was employed.[27] In the present study, TPC of aqueous *A. hispida* extract (107.85 ± 1.46 mg GAE/g) was found to be lower than that of previously reported *E. robusta* aqueous extract, however, the TFC was significantly higher.

Statistically similar proanthocyanidin contents were observed in *A. hispida* and *C. citriodora* extracts

Table 1. Extraction yield (g/100 g of dry weight) and yields of TPC, TFC, proanthocyanidins, and antioxidants from different species of eucalypts using conventional extraction with water ($n = 3$)

	Angophora hispida	*Angophora floribunda*	*Corymbia citriodora*	*Corymbia maculata*
Extraction yield	12.15 ± 0.35^{a}	10.42 ± 0.12^{b}	9.13 ± 0.54^{c}	7.36 ± 0.43^{d}
TPC (mg GAE/g)	107.85 ± 1.46^{a}	47.81 ± 0.58^{b}	57.02 ± 0.92^{c}	47.60 ± 1.43^{b}
TFC (mg RE/g)	57.96 ± 1.93^{a}	44.61 ± 0.63^{b}	21.61 ± 0.84^{c}	14.81 ± 0.53^{d}
Proanthocyanidins (mg CAE/g)	20.70 ± 2.62^{a}	59.56 ± 1.87^{b}	18.00 ± 0.34^{a}	7.35 ± 0.37^{c}
Saponins (mg AE/g)	401.13 ± 42.38^{a}	386.53 ± 45.77^{a}	333.00 ± 44.69^{a,b}	276.00 ± 14.14^{b}
TAC (mg TE/g)	98.81 ± 0.15^{a}	70.51 ± 1.52^{b}	87.85 ± 2.68^{c}	80.37 ± 2.64^{d}
DPPH (mg TE/g)	118.78 ± 0.82^{a}	60.98 ± 0.86^{b}	82.58 ± 0.76^{c}	66.16 ± 1.84^{d}
CUPRAC (mg TE/g)	186.56 ± 6.03^{a}	93.25 ± 2.47^{b}	126.88 ± 3.16^{c}	103.54 ± 2.08^{d}

All the values are means ± standard deviations and those in the same row not sharing the same superscript letter are significantly different from each other ($P < 0.05$).

($P > 0.05$). *Angophora floribunda* aqueous extract exhibited the greatest proanthocyanidin content among the tested eucalypts (59.56 ± 1.87 mg CAE/g) and was also significantly higher than the previously reported *E. robusta* aqueous extract.[27] The lowest proanthocyanidin content was obtained from *C. maculata* extract among the tested species ($P < 0.05$).

Similar to plant phenolics, saponins have also been reported to have anticancer properties in a number of studies.[28 – 31] This study further explored the levels of saponins in the tested eucalypt species. One way ANOVA suggested that there was no significant difference among the *A. hispida*, *A. floribunda* and *C. citriodora* aqueous extracts in terms of their saponin content ($P > 0.05$; *Table 1*). However, the saponin content of *C. maculata* was considerably different from that of *A. hispida* and *A. floribunda* ($P < 0.05$) but not from *C. citriodora* ($P > 0.05$). In the present study, all the tested eucalypts showed a higher saponin content compared to *E. microcorys* extract reported earlier.[27]

In the case of antioxidant activity, *A. hispida* extracts displayed greater antioxidant capacity in all the three assays, with values 98.81 ± 0.15, 118.78 ± 0.82, and 186.56 ± 6.03 mg TE/g for TAC, DPPH and CUPRAC, respectively, followed by *C. citriodora* (*Table 1*). However, these antioxidant values were lower than the values previously reported for *E. robusta* and *E. microcorys* aqueous leaf extracts, despite the use of identical extraction techniques and experimental parameters.[27] *A. floribunda* had the lowest antioxidant content among the tested eucalypts as revealed by TAC, DPPH and CUPRAC assays ($P < 0.05$). The antioxidant capacity of *C. maculata* extract was higher than *A. floribunda* but lower than *A. hispida* and *C. citriodora* extracts ($P < 0.05$; *Table 1*).

The findings clearly demonstrated that the phytochemical content and antioxidant capacity vary significantly between species and genera of eucalypts. *Angophora* and *Corymbia* genera are considerably different from previously studied species of *Eucalyptus* in terms of their phenolic, saponin and antioxidant contents.

Growth Inhibition of Crude Eucalypt Extracts on Primary and Secondary Pancreatic Cancer Cell Lines Using CCK-8 Assay

The anticancer activity of the eucalypt extracts was assessed against primary and secondary pancreatic cancer cell lines as well as on normal HPDE cells. *Table 2* shows cell growth inhibition (%) on four different cell lines in response to exposure to 100 and 50 μg/ml eucalypt extracts. In the case of MIA PaCa-2 cells, *A. floribunda* extract at 100 μg/ml exhibited significantly greater cell growth inhibition (77.91 ± 4.93%) followed by *A. hispida* with 62.04 ± 7.47% ($P < 0.05$). *Bhagat et al.*[10] earlier tested the growth inhibitory properties of *C. citriodora* aqueous leaf extract against ovary, prostate and lung cancer cells and observed at least 70% growth inhibition at a concentration of 100 μg/ml. However, in our study, both *C. citriodora* and *C. maculata* showed lower cell growth inhibition values at this same concentration with values of 8.21 ± 3.47 and 24.50 ± 7.94%, respectively, observed. Other studies have established the cytotoxic effects of resin derived from *C. citriodora* on melanoma B16F10 and human hepatoma HepG2 cells.[8][9] Discrepancies in the reported activities of extracts from the same species can be attributed to both differences in the cell lines used and to their genotype and phenotype. The variations in mutation profile of the cell lines also significantly influence the efficacy of any extract or drug action. Additionally, factors such as climate, growing location, solvent, technique and time used for extraction also have a major impact on the overall efficacy of the extracts. The difference between the cell growth inhibitory activity of *A. floribunda* and *C. maculata* extracts was not statistically significant ($P > 0.05$, *Table 2*) at 50 μg/ml. Moreover, *A. hispida* extract exerted the lowest growth inhibition of MIA PaCa-2 cells at 50 μg/ml (*Table 2*). Gemcitabine at 50 nM inhibited MIA PaCa-2 cell growth by 88.77 ± 0.96% which was significantly higher than the tested eucalypt extracts at both 100 and 50 μg/ml. However, the *A. floribunda* extract at 100 μg/ml exhibited slightly lower cell growth inhibitory activity against MIA PaCa-2 cell in comparison to gemcitabine (*Table 2*).

For the BxPC-3 cell line, low growth inhibition values were observed for all the tested extracts (*Table 2*). *Angophora hispida, C. citriodora*, and *C. maculata* extracts showed statistically similar cell growth inhibition values with at least 12.40 ± 8.66% ($P > 0.05$) at 100 μg/ml. Additionally, one way ANOVA suggested that there is no significant difference between *A. floribunda* and *C. maculata* extracts in terms of their growth inhibition of BxPC-3 cells at 100 μg/ml (*Table 2*). All four eucalypt extracts also displayed low cell growth inhibition (0.52 ± 0.22 – 12.90 ± 9.24%) at 50 μg/ml. The BxPC-3 cell growth inhibition values of all the extracts both at 50 and 100 μg/ml were significantly different to that of gemcitabine ($P < 0.05$) (*Table 2*).

Significantly lower cell growth inhibition values (< 20%) were shown by all four eucalypt extracts at

Table 2. Cell Growth Inhibition ([%]) in response to 100 and 50 μg/ml of eucalypt extracts across various pancreatic cell lines ($n = 6$) using CCK-8 assay. Higher values indicate greater growth inhibition

	MIA PaCa-2		BxPC-3		CFPAC-1		HPDE	
	100 μg/ml	50 μg/ml	100 μg/ml	50 μg/ml	100 μg/ml	50 μg/ml	100 μg/ml	50 μg/ml
Angophora hispida	$62.04 \pm 7.47_a^{ii}$	$1.69 \pm 0.43_a^{ii}$	$15.74 \pm 5.50_a^{ii}$	$6.70 \pm 1.70_a^{ii}$	$13.33 \pm 7.41_a^{ii}$	$9.70 \pm 3.20_a^{ii}$	$95.14 \pm 0.17_a^{i}$	$94.05 \pm 0.80_a^{i}$
Angophora floribunda	$77.91 \pm 4.93_b^{ii}$	$12.33 \pm 4.56_b^{ii}$	$4.72 \pm 0.46_b^{ii}$	$4.18 \pm 0.14_a^{ii}$	$12.20 \pm 5.43_a^{ii}$	$6.25 \pm 1.51_a^{ii}$	$91.45 \pm 2.95_a^{i}$	$54.90 \pm 4.04_b^{ii}$
Corymbia citriodora	$8.21 \pm 3.47_c^{ii}$	$6.15 \pm 0.80_c^{ii}$	$13.24 \pm 6.30_{ac}^{ii}$	$12.90 \pm 9.24_a^{ii}$	$18.82 \pm 4.81_a^{ii}$	$10.20 \pm 4.02_a^{ii}$	$87.14 \pm 3.00_b^{ii}$	$19.32 \pm 5.01_c^{ii}$
Corymbia maculata	$24.50 \pm 7.94_d^{ii}$	$9.77 \pm 3.76_{bc}^{ii}$	$12.40 \pm 8.66_{abc}^{ii}$	$0.52 \pm 0.22_b^{ii}$	$12.41 \pm 2.21_a^{ii}$	$8.99 \pm 3.60_a^{ii}$	$45.62 \pm 8.11_c^{ii}$	$32.06 \pm 4.13_d^{ii}$
Gemcitabine (50 nM)	88.77 ± 0.96^{i}		71.61 ± 4.39^{i}		86.42 ± 0.97^{i}		95.88 ± 0.10^{i}	

All the values are means ± standard deviations. [a,b,c] Values in the same column not having the same subscript letter are significantly different ($P < 0.05$) from each other. [i,ii,iii] Values in the same column not having the same superscript roman numeral are significantly different ($P < 0.05$) from the corresponding values obtained from gemcitabine (50 nM) as standard for the respective cell lines.

both the concentrations in CFPAC-1 cells in comparison to that of gemcitabine ($P < 0.05$, *Table 2*). Both at 50 and 100 μg/ml, the difference among the extracts was not significant as revealed by one way ANOVA ($P > 0.05$, *Table 2*). However, these values were significantly different from the inhibition value shown by gemcitabine at 50 nM.

Angophora hispida extract at both 50 and 100 μg/ml and *A. floribunda* extract at 100 μg/ml exhibited statistically similar values (> 90% cell growth inhibition) in comparison to gemcitabine at 50 nM in HPDE cells ($P > 0.05$, Table *2*). *C. maculata* extract was found to have the significantly lowest growth inhibition value (45.62 ± 8.11%) compared with the other three extracts at 100 μg/ml and gemcitabine ($P < 0.05$, *Table 2*). Whereas, at 50 μg/ml concentration, *C. citriodora* extract showed the lowest growth inhibition value (19. 32 ± 5.01%) even in comparison to gemcitabine at 50 nM in HPDE cell line ($P < 0.05$, *Table 2*).

As all four eucalypt extracts exhibited < 20% growth inhibition compared to the vehicle control in BxPC-3 and CFPAC-1 cells, even at the highest concentration of 100 μg/ml, only MIA PaCa-2 and HPDE cell lines were further evaluated for the determination of IC_{50} values. *Table 3* represents the IC_{50} values of the eucalypt extracts. *Angophora floribunda* extract showed the lowest IC_{50} value of 75.58 μg/ml among the four extracts followed by *A. hispida* (87.28 μg/ml) in MIA PaCa-2 cell line. GraphPad Prism software was unable to calculate the IC_{50} value of *C. citriodora* as the log[inhibitor] *vs.* response-variable slope did not converge (NC, *Table 3*). This was due to the absorbance values obtained from different concentrations of *C. citriodora* extract against MIA PaCa-2 cells in CCK-8 assay differing significantly from the ideal four-parameter dose-response curve employed by GraphPad Prism software. A very high IC_{50} value (*ca.* 462.90 μg/ml) was exhibited by the *C. maculata* extract in MIA PaCa-2 cells. The lower IC_{50} values exhibited by the *A. hispida* and *A. floribunda* extracts perhaps can be explained by the higher flavonoid ($P < 0.05$) and saponin (although, $P > 0.05$) contents of the extracts in comparison to *C. citriodora* and *C. maculata.* As previous studies have demonstrated the synergistic cytotoxic and anti-inflammatory effects of flavonoids and saponins derived from different plant samples,[32][33] the likelihood of a synergistic effect of flavonoids and saponins on the MIA PaCa-2 cell viability in our study cannot be ruled out. Further investigation is certainly required to purify and identify these compounds from the crude extracts and to elucidate their precise mechanisms of action.

Table 3. IC_{50} ([μg/ml]) (concentration that inhibits cell growth by 50%) value of the eucalypt extracts using CCK-8 assay. Lower value indicates greater growth inhibition efficiency

	MIA PaCa-2	HPDE
Angophora hispida	87.28	38.69
Angophora floribunda	75.58	68.22
Corymbia citriodora	NC	83.73
Corymbia maculata	~462.90	~62.90

NC, not converged.

In HPDE cells, *A. hispida* extract was found to have the lowest IC_{50} value (38.69 μg/ml) followed by *C. maculata* (~62.90 μg/ml) (*Table 3*). The *A. floribunda* extract with lowest IC_{50} value against MIA PaCa-2 cell line, exhibited an IC_{50} value of 68.22 μg/ml against HPDE cells.

Conclusion

The yields of the eucalypt extracts assessed were significantly different from each other with *A. hispida* producing the highest extraction yield ($P < 0.05$). *Angophora hispida* also exhibited statistically higher extraction yields of TPC and TFC ($P < 0.05$) compared to the other eucalypt species tested. The highest proanthocyanidin content of 59.56 ± 1.87 mg CAE/g was obtained from *A. floribunda*. However, both *A. hispida* and *A. floribunda* aqueous extracts showed high and statistically similar saponin contents. Greater antioxidant capacity was also observed in *A. hispida* aqueous extract in all three antioxidant assays ($P < 0.05$) followed by *C. citriodora*. The CCK-8 assay revealed that the aqueous *A. floribunda* extract exerted the significantly greatest cell growth inhibition with 77.91 ± 4.93% followed by *A. hispida* with 62.04 ± 7.47% ($P < 0.05$) at 100 μg/ml against MIA PaCa-2 cell line. On the contrary, both *C. citriodora* and *C. maculata* showed lower cell growth inhibition values of 8.21 ± 3.47 and 24.50 ± 7.94%, respectively at 100 μg/ml in MIA PaCa-2 cells. In the case of BxPC-3 and CFPAC-1 cells, all the four eucalypt extracts exhibited < 20% growth inhibition in comparison to the vehicle control both at 50 and 100 μg/ml concentrations. However, *A. hispida* (at 50 and 100 μg/ml) and *A. floribunda* (at 100 μg/ml) inhibited the growth of normal HPDE cells by > 90% with statistically similar values in comparison to gemcitabine at 50 nM ($P > 0.05$). The IC_{50} values of the aqueous *A. hispida* and *A. floribunda* extracts were found to be 87.28 and 75.58 μg/ml, respectively in MIA PaCa-2 cell line. These values are quite promising as these extracts are still in their crude forms and they are likely to display higher IC_{50} values once purified further. Studies to isolate and identify individual compounds from these crude extracts and re-evaluation are necessary to identify their precise mechanism of action against pancreatic cancer cell lines. After purification, the individual active compounds derived from the crude extracts of *A. hispida* and *A. floribunda* will potentially yield even lower IC_{50} values similar to the standard chemotherapeutic drugs used for the treatment of pancreatic cancer.

Experimental Section

Plant Materials

Fresh leaves of *C. maculata* and *A. floribunda* were collected on 10th November, 2015 from Ourimbah, Central Coast, NSW, Australia (33°21′22″ S 151°22′47″ E and 33°21′37″ S 151° 20′ 20″ E, resp.). *C. citriodora* and *A. hispida* fresh leaves were collected from Gosford, Central Coast, NSW, Australia (33°25′49″ S 151°19′43″ E and 33°25′50″ S 151°17′15″ E, resp.) on 3rd November, 2014. The collected eucalypts are shown in *Fig. 1*. The plants were authenticated by one of the authors (*A. C. C.*) and voucher specimens deposited with the Don McNair Herbarium, The University of Newcastle (Accession numbers - *A. hispida*: 10495, *A. floribunda:* 10498, *C. citriodora:* 10503, *C. maculata:* 10501). The leaves were then transferred to the laboratory and stored at −20 °C to avoid potential degradation of phytochemicals. The leaves were dried at 70 °C for 5 h to constant mass using a dry air oven,[4] then ground to a fine powder using in a commercial grade blender (*Rio*™ Commercial Bar Blender, *Hamilton Beach*), sieved (≤ 1 mm) using a 1 mm EFL 2000 stainless steel mesh sieve (*Endecotts Ltd.*, London, England) then packed in a sealed container and stored at −20 °C until required.

Extraction of Plant Materials for Preparation of Powdered Crude Extracts

Aqueous extracts were prepared as follows. 5 g of powdered leaf sample was mixed with 100 ml of distilled water in a 250 ml conical flask. The flask was then sealed with a bung covered in aluminium foil and heated at 85 °C for 15 min. in a shaker water bath (*Ratek instruments Pty Ltd.*, Victoria, Australia). The extracts were then cooled on ice to room temperature and then filtered using *Whatman*® No.1 filter paper. The filtrate was then concentrated to one-third initial volume using a rotary

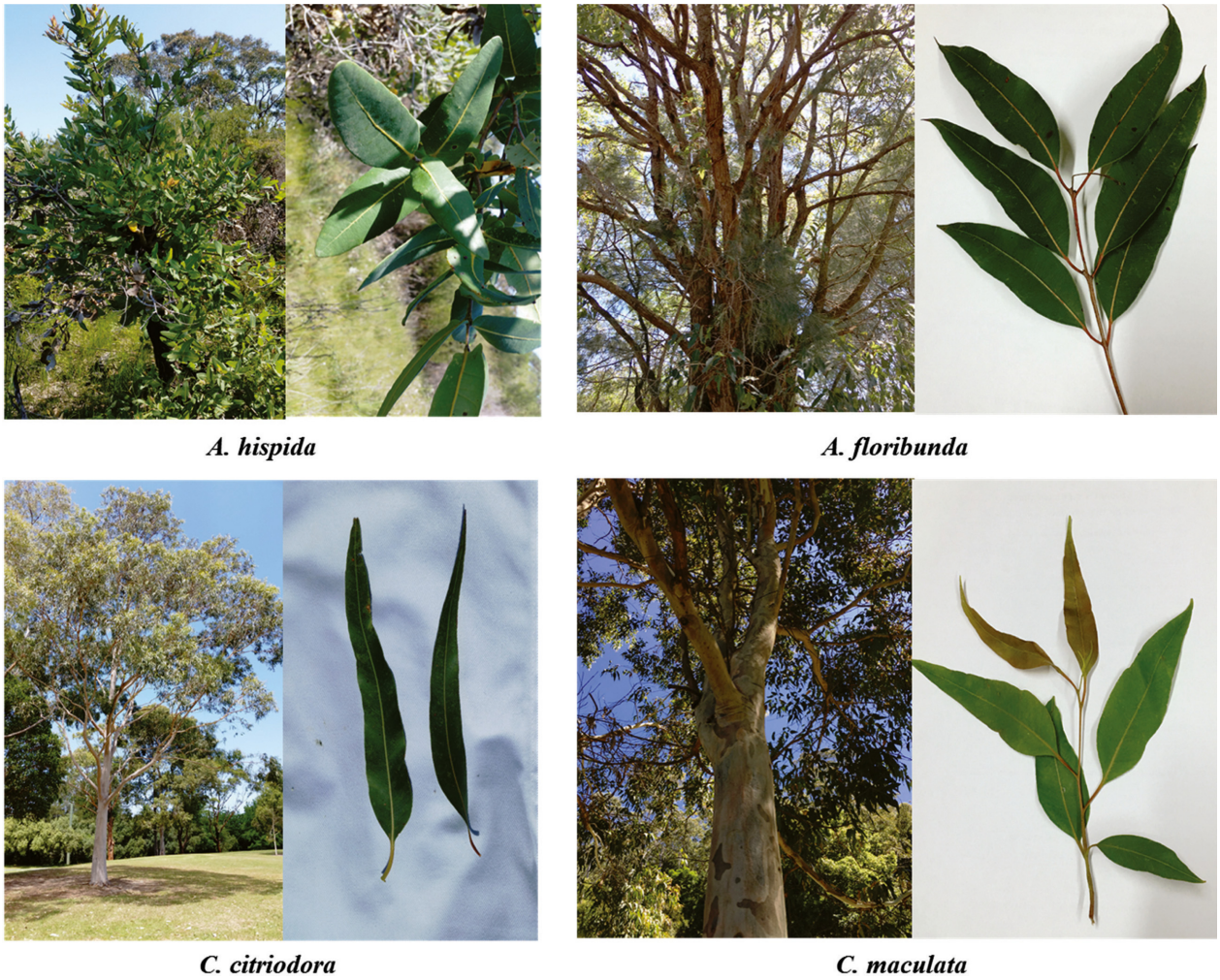

Figure 1. *Angophora hispida*, *Angophora floribunda*, *Corymbia citriodora,* and *Corymbia maculata*.

evaporator (*Buchi Rotavapor B-480*, *Buchi Australia*, Noble Park, Vic., Australia), then transferred to 15 ml *Corning* centrifuge tubes, sealed, immersed in liquid nitrogen and freeze-dried for 48 h using a freeze dryer (*Thomas Australia Pvt., Ltd.*, Seven Hills, NSW, Australia) at a drying chamber pressure of 2×10^{-1} mbar and cryo-temperature of -50 °C. The extraction yields were determined based on the mass of extract (g) obtained from mass of dried leaves (100 g).

Determination of Total Phenolic Content (TPC)

Colorimetric assessment of TPC was carried out according to the method described by *Škerget et al.*[34] Extracts were diluted up to 40× to fit within the optimal absorbance range (0.1 – 1.0). Gallic acid was used as the standard, with the results expressed as mg of gallic acid equiv. per g of dry weight (mg GAE/g).

Determination of Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was determined as per the method described by *Tan et al.*[35] TFC was expressed as mg of rutin equiv. (RE) per g of dry weight (mg RE/g) using rutin as the standard.

Determination of Proanthocyanidin Content

Proanthocyanidin content of the extract was determined using the vanillin-HCl method as described by *Broadhurst and Jones*.[36] The concentration of proanthocyanidins was expressed as mg of catechin equiv. (CAE) per g of dry weight (mg CAE/g).

Determination of Antioxidant Capacity

ABTS Total Antioxidant Capacity (TAC). TAC was measured using ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assay as

described by *Bhuyan et al.*[37] The extracts were diluted up to 40X. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard to construct the calibration curve. Results were expressed in mg trolox equiv. (TE) per g of dry weight (mg TE/g).

Free Radical Scavenging Capacity. Free radical scavenging activity of the extracts was analysed by the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay as described by *Bhuyan et al.*[37] Results were expressed in mg trolox equiv. (TE) per g of dry weight (mg TE/g).

Cupric Reducing Antioxidant Capacity (CUPRAC). The cupric ion chelating capacity of the extracts was determined by the CUPRAC assay as described by *Apak et al.*[38] Trolox was used as the calibration standard, with results expressed in mg of trolox equiv. (TE) per g of dry weight (mg TE/g).

Determination of Saponin Content

The method described by *Hiai et al.*[39] was used to determine the saponin content of the extracts. Aescin was used as the standard for the calibration curve, with results expressed as mg of aescin equiv. per g of dry weight (mg AE/g).

Cell Culture

Pancreatic cancer cell lines were obtained from the American Type Culture Collection (ATCC: Manassas, VA, USA). HPDE cells were originally from lab of Dr. *M. Tsao* (MD, FRCPC, University Health Network, Toronto, ON, Canada).[40] *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 to culture the MIA PaCa-2 cells. RPMI-1640 supplemented with 10% FBS and L-glutamine (100 μg/ml) was used to culture the BxPC-3 cells. *Iscove*'s Modified *Dulbecco*'s Media (IMDM) supplemented with 10% FBS and L-glutamine (100 μg/ml) was used for CFPAC-1 cells. For HPDE cells, Keratinocyte Serum-free Media (KSFM) supplemented with human Recombinant Epidermal Growth Factor (rEGF) and Bovine Pituitary Extract (BPE) was used.

Determination of Pancreatic Cancer Cell Viability

Anticancer activity of the eucalypt extracts was assessed against two primary pancreatic cancer cell lines (MIA PaCa-2 and BxPC-3), and one secondary pancreatic cancer cell line derived from a liver metastasis site (CFPAC-1). In addition, an immortalised normal human pancreatic ductal epithelial cell line (HPDE) was used to assess the cytotoxicity of the extracts. *Dojindo Cell Counting Kit-8* (CCK-8, *Dojindo Molecular Technologies, Inc.*, Maryland, USA) was used to determine the cell viability. CCK-8 assay is a colorimetric assessment that employs WST-8 salt (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) to determine the number of viable cells. The dehydrogenase activities in viable cells reduce the WST-8, which in turn forms a water soluble yellow-colour formazan dye. The number of viable cells is directly proportional to the amount of formazan dye produced.

In a 96 well plate, 200 μl of suspended cells were seeded at 3×10^3 cells per well for MIA PaCa-2 and 7×10^3 cells per well for BxPC-3, CFPAC-1 and HPDE, and incubated at 37 °C for 24 h to adhere. The cells were then treated with different concentrations of crude extracts, gemcitabine (50 nM) and vehicle control (0.5% DMSO). After 72 h, 10 μl of CCK-8 solution was added and incubated for 1.5 h at 37 °C in the presence of 5% CO_2. Using a microplate spectrophotometer (*BIORAD Benchmark Plus*™), the absorbance was measured at 450 nm and cell viability was determined as a percentage of control. The absorbance of the wells was also measured at 650 nm as a reference to eliminate the background that occurs due to turbidity and this value was subtracted from the absorbance of the same well measured at 450 nm. All experiments were performed in replicates of 6.

Statistical Analyses

SPSS statistical software (version 16.0, Chicago, IL, USA) was used to perform independent samples *t*-test, one-way ANOVA, and the LSD post-hoc to compare the means. All analyses were carried out at least in triplicate and the results were expressed as means ± standard deviations. At $P < 0.05$, the differences between the mean values in the experiments were taken to be statistically significant. The IC_{50} (concentration that inhibits cell growth by 50%) values were calculated by curve fitting the absorbance (viability) *vs.* log [concentration of treatment], using GraphPad Prism software (version 6.0b, San Diego, CA, USA).

Acknowledgements

We acknowledge the following funding support: *Ramaciotti Foundation* (ES2012/0104); the *Hunter*

Cancer Research Alliance (HCRA); the *Hunter Medical Research Institute* (HMRI) and the *University of Newcastle, Australia*.

Conflict of Interest

The authors declare no conflict of interest.

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Received August 15, 2016
Accepted November 16, 2016

In vitro anticancer properties of selected *Eucalyptus* species

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Definitive version published: Bhuyan, Deep Jyoti; Sakoff, Jennette; Bond, Danielle R.; Predebon, Melanie; Vuong, Quan V.; Chalmers, Anita C.; van Altena, Ian A.; Bowyer, Michael C.; Scarlett, Christopher J. "In vitro anticancer properties of selected Eucalyptus species". Published in In Vitro Cellular & Developmental Biology - Animal Vol. 53, Issue 7, p. 604-615 (2017)
Available from: http://dx.doi.org/10.1007/s11626-017-0149-y

Abstract

In spite of the recent advancements in oncology, the overall survival rate for pancreatic cancer has not improved over the last five decades. Eucalypts have been linked with cytotoxic and anticancer properties in various studies, however, there is very little scientific evidence that supports the direct role of eucalypts in the treatment of pancreatic cancer. This study assessed the anticancer properties of aqueous and ethanolic extracts of four *Eucalyptus* species using an MTT assay. The most promising extracts were further evaluated using a CCK-8 assay. Apoptotic studies were performed using a caspase 3/7 assay in MIA PaCa-2 cells. The aqueous extract of *E. microcorys* leaf and ethanolic extract of *E. microcorys* fruit inhibited the growth of glioblastoma, neuroblastoma, lung and pancreatic cancer cells by more than 80% at 100 µg/mL. The *E. microcorys* and *E. saligna* extracts showed lower GI_{50} values than the ethanolic *E. robusta* extract in MIA PaCa-2 cells. Aqueous *E. microcorys* leaf and fruit extracts at 100 µg/mL exerted significantly higher cell growth inhibition in MIA PaCa-2 cells than other extracts ($p < 0.05$). Statistically similar IC_{50} values ($p > 0.05$) were observed in aqueous *E. microcorys* leaf (86.05 ± 4.75µg/mL) and fruit (64.66 ± 15.97 µg/mL), and ethanolic *E. microcorys* leaf (79.30 ± 29.45 µg/mL) extracts in MIA PaCa-2 cells using the CCK-8 assay. Caspase 3/7 mediated apoptosis and morphological changes of cells were also witnessed in MIA PaCa-2 cells after 24 h of treatment with the extracts. This study highlighted the significance of *E. microcorys* as an important source of phytochemicals with efficacy against pancreatic cancer cells. Further studies are warranted to purify and structurally identify individual compounds and elucidate their mechanisms of action for the development of more potent and specific chemotherapeutic agents for pancreatic cancer.

Keywords: Eucalyptus, anticancer, pancreatic cancer, MTT, CCK-8, caspase 3/7

Introduction

Pancreatic adenocarcinoma, otherwise known as pancreatic cancer comes under the category of gastrointestinal cancers and is one of the most devastating forms of human cancers in the world (Bailey *et al.* 2016; de Sousa Cavalcante and Monteiro 2014; Pourhoseingholi *et al.* 2015). It is the 13th most common type of cancer worldwide (Jiao and Li 2010) with a median survival timeline of less than 5 months after diagnosis and a five-year survival rate of less than 5%, even after treatment (de Sousa Cavalcante and Monteiro 2014). Poor prognosis and few effective therapies make it the 8th major form of cancer-related death, resulting in more than 227,000 deaths annually worldwide (Biankin *et al.* 2012; Ghaneh *et al.* 2010; Jiao and Li 2010). Concerted attempts over the past 15 years to improve the survival rate of patients diagnosed with pancreatic cancer have, in large parts been unsuccessful, and have lagged well behind advancements in the treatment of other cancers (Okines *et al.* 2010; Vaccaro *et al.* 2012).

Natural sources such as plants, marine invertebrates and microorganisms have been extensively studied and utilised for their anticancer properties in traditional as well as modern medicine. Bioactive compounds derived from natural sources comprise more than 60% of the anticancer drugs commercially available today (Jimeno *et al.* 2004). Approximately, 50-60% of cancer patients in the United States use products isolated from different plant components, either alone or in conjunction with mainstream chemotherapy and/or radiation therapies (Wang *et al.* 2012). In respect to the management of pancreatic cancer, various natural and semi-synthetic compounds, including gemcitabine, the most commonly used pancreatic cancer drug, have been identified and characterized.

The antitumour and cytotoxic properties of *Eucalyptus* extracts and isolated individual phytochemicals contained therein have been reported in various studies (Al-Fatimi *et al.*

2005; Benyahia *et al.* 2005; Ito *et al.* 2000; Mota *et al.* 2012; Soliman *et al.* 2014; Takasaki *et al.* 2000; Takasaki *et al.* 1995; Vuong *et al.* 2015a). While, a number of reports suggested the anticancer properties of eucalypt extracts and essential oils, there is very little scientific evidence that emphasizes a direct role of eucalypts in the management of pancreatic cancer. Phenolic compounds such as gallic acid, quercetin, narigenin, myricetin, apigenin and epigallocatechin-3-gallate derived from other natural sources have been shown to exhibit anticancer activity against pancreatic cancer both *in vitro* and *in vivo* (Liu *et al.* 2012; Lou *et al.* 2012; Lyn-Cook *et al.* 1999; Melstrom *et al.* 2011; Phillips *et al.* 2011; Qanungo *et al.* 2005; Shankar *et al.* 2008; Zhou *et al.* 2010). These particular compounds are also found extensively in eucalypts, highlighting the potential of *Eucalyptus* as a source of novel therapeutic agents for the treatment of pancreatic cancer. The present study was designed to assess the anticancer activity of crude aqueous and ethanolic extracts of four different *Eucalyptus* species: *E. robusta, E. microcorys, E. saligna*, and *E. globulus*. This study was divided into two parts; with, the extracts initially evaluated for cell growth inhibition activity across a panel of cancer cell lines. The most effective crude extracts were then subsequently evaluated for their efficacy specifically against pancreatic cancer cells using both primary and secondary pancreatic cancer cell lines.

Materials and methods

Plant materials

Fresh leaves of *E. robusta, E. microcorys* and *E. saligna* were collected on 2nd April, 2014 from cultivated plants located at Ourimbah, Central Coast, NSW, Australia (latitude of 33.4° S, longitude of 151.4° E). *E. globulus* fresh leaves were collected from Tinderbox, Tasmania, Australia (latitude 43° S, longitude 147.2° E) on 6th May, 2014. The collected *Eucalyptus*

species are shown in Figure 1. The plants were authenticated by the author (A.C.C.) and voucher specimens deposited at the Don McNair Herbarium (Accession numbers- *E. robusta*: 10492, *E. saligna:* 10504, *E. microcorys:* 10499, *E. globulus:* 10505). The leaves were then immediately transferred to the laboratory and stored at -20° C to avoid potential degradation of the phytochemical profile. Using a dry air oven, the leaves were dried at 70° C for 5 h to constant weight. The leaves were then ground to a fine powder using a commercial grade blender (Rio™ Commercial Bar Blender, Hamilton Beach), then sieved (≤1 mm) using a 1 mm EFL 2000 stainless steel mesh sieve (Endecotts Ltd., London, England) and packed in a sealed container and stored at -20° C until required.

Extraction of plant materials for preparation of crude extract powder

Conventional extraction was carried out using water and 70% ethanol (Bhuyan *et al* 2016; Bhuyan *et al* 2017). For aqueous extracts, 5 g of powdered leaf sample was mixed with 100 mL of distilled water and heated at 85° C for 15 min. In the case of ethanolic extracts, 5 g of powdered sample was mixed with 100 mL of 70% ethanol and macerated at room temperature for 72 h. Conical flasks (250 mL) sealed with a stopper and covered with aluminium foil were used for extraction in a shaking water bath (Ratek instruments Pty Ltd., Boronia, VIC, Australia). Aqueous extracts were immediately cooled on ice to room temperature (RT). The extracts were then filtered using Whatman® No.1 filter paper. The extraction yields were determined based on the extract (g) obtained from dried leaves (100 g). The filtrate was then concentrated using a rotary evaporator (Buchi Rotavapor B-480, Buchi Australia, Noble Park, VIC, Australia) at 55° C with reduced pressure to one third of the initial volume. Concentrated extracts were then transferred to 15 mL Corning centrifuge tubes, sealed and immersed in liquid nitrogen and then freeze-dried for 48 h using a freeze

dryer (Thomas Australia Pvt., Ltd., Seven Hills, NSW, Australia) with drying chamber pressure of 2×10^{-1} mbar and cryo-temperature of −50° C.

Assessment of growth inhibition of crude Eucalyptus extracts on cancer cell lines

Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cytotoxicity of the crude *Eucalyptus* extracts was screened using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Deane *et al.* 2013) to assess cell growth inhibition across a panel of cancer cell lines. The cell line panel consisted of HT29 (colon); U87, SJ-G2, SMA (glioblastoma); MCF-7 (breast); A2780 (ovarian); H460 (lung); A431 (skin); Du145 (prostate); BE2-C (neuroblastoma); and MIA PaCa-2 (pancreas) together with one non-tumour derived normal breast cell line (MCF10A). Briefly, all cancer cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum, 50IU/mL penicillin, 50 µg/mL streptomycin and 2mM L-glutamine. The MCF10A cells were cultured in DMEM:F12 (1:1) cell culture media, 5% heat inactivated horse serum, supplemented with penicillin (50 IU/mL), streptomycin (50 µg/mL), 20mM Hepes, L-glutamine (2mM), epidermal growth factor (20 ng/mL), hydrocortisone (500 ng/mL), cholera toxin (100 ng/mL), and insulin (10 µg/mL). Cells were plated in triplicate in DMEM (100 µL) in a 96 well plate, at densities of 2500 – 4000 cells per well optimized to achieve logarithmic growth after 24 hours. The following day, 100 µL of media with or without (control) the crude extracts was added to each well to give the final concentration of 100 µg/mL. The cytotoxicity of the crude extracts was measured after 72 h of incubation using the MTT assay (Deane *et al.* 2013). The absorbance was read at 540 nm to determine growth inhibition based on the difference in the optical density values on day 0 versus those at the end of extract treatment. Cell growth inhibition as a percentage was determined, where a value of 100% is indicative of total

growth inhibition. A dose response curve (100 – 0.05 µg/mL) was also produced for extracts that showed promising activity, from which a GI_{50} value was obtained representing the extract concentrations that inhibited 50% cell growth.

Determination of pancreatic cancer cell viability

To validate the anticancer activity of the selected *Eucalyptus* extracts that showed promising activity against the MIA PaCa-2 cell line in the MTT assay, they were further evaluated against two primary pancreatic cancer cell lines (MIA PaCa-2 and BxPC-3), and one secondary pancreatic cancer cell line (CFPAC-1), derived from a liver metastasis site using the Dojindo Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, INC., MD, USA). Additionally, extracts were assessed on an immortalised normal human pancreatic ductal epithelial cell line (HPDE). Pancreatic cancer cell lines were obtained from the American Type Culture Collection (ATCC: Manassas, VA, USA). HPDE cells were originally from lab of Dr. M. Tsao (MD, FRCPC, University Health Network, Toronto, ON, Canada) (Furukawa *et al.* 1996).

Cell culture

MIA PaCa-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2.5% horse serum and L-Glutamine (100 µg/mL). BxPC-3 cells were cultured in RPMI-1640 supplemented with 10% FBS and L-Glutamine (100µg/mL). CFPAC-1 cells were cultured in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10% FBS and L-Glutamine (100 µg/mL). HPDE cells were cultured in Keratinocyte Serum-free Media (KSFM) supplemented with human Recombinant Epidermal Growth Factor (rEGF) and Bovine Pituitary Extract (BPE).

Cell viability

Cell viability was determined using the CCK-8 assay. CCK-8 assay is a sensitive colorimetric method for the determination of the number of viable cells using WST-8 salt (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium). WST-8 is reduced by dehydrogenase activities in viable cells and forms a water soluble yellow-color formazan dye. The amount of formazan dye produced is directly proportional to the number of viable cells. This method is more sensitive than other tetrazolium salts such as MTT, XTT, MTS or WST-1.

200 µL of suspended cells were seeded into a 96 well plate at 3 x 10^3 cells per well for MIA PaCa-2 and 7 x 10^3 cells per well for BxPC-3, CFPAC-1 and HPDE and allowed to adhere for 24 h. The cells were then treated with 100 µg/mL of crude extracts, gemcitabine (50 nM), or vehicle control (0.5% DMSO). After 72 h, 10 µL of CCK-8 solution was added and incubated at 37 °C for 90 min. The absorbance was measured at 450 nm using a microplate reader (BIORAD Benchmark Plus™, Bio-Rad Laboratories Pty Ltd., Gladesville, NSW, Australia) and cell viability was determined as a percentage of control. To eliminate the background that occurs due to turbidity, the absorbance of the wells was also measured at 650 nm as a reference and this value was subtracted from the absorbance of the same well measured at 450 nm. All experiments were performed in replicates of 6. The IC_{50} (concentration that inhibits cell growth by 50%) values were calculated by curve fitting the absorbance (viability) *vs.* log [concentration of treatment], using GraphPad Prism software version 6.0b (San Diego, CA, USA).

Muse cytofluorimetric analysis for evaluation of apoptosis

The MIA PaCa-2 cells were treated with 100 µg/mL of the selected extracts for 24 h. Gemcitabine (1µM/mL) and DMSO (0.5%) were used as positive and negative controls, respectively. Using the Muse caspase 3/7 (cat #MCH100108, Merck Millipore, Billerica,

MA, USA) assay, cells were analysed for the presence of early and late apoptosis and cell death as per the manufacturer's instructions. The Muse Cell Analyzer (Merck Millipore, Billerica, MA, USA) was used to analyse the cell counts. For detection of dead cells, a marker 7-aminoactinomycin D (7-AAD) was also included in this assay as an indicator of cell membrane structural integrity. This assay distinguishes four populations of cells: Live cells: Caspase-3/7(–) and 7-AAD(–), Apoptotic cells exhibiting Caspase-3/7 activity: Caspase-3/7 (+) and 7-AAD(–), Late Apoptotic/Dead cells: Caspase-3/7(+) and 7-AAD(+)and Necrotic cells: Caspase-3/7(–) and 7-AAD(+).

Statistical analysis

All analyses were carried out at least in triplicate. Independent samples *t*-test, one-way ANOVA, and the LSD post-hoc test were performed using SPSS statistical software (version 16.0, Chicago, IL, USA) to compare the means. The results were expressed as means ± standard deviations. At $p < 0.05$, the differences between the mean values in the experiments were taken to be statistically significant.

Results

Extraction yield of selected Eucalyptus species

Extraction yields (g/100 g of DW) of the different species of *Eucalyptus* for each solvent system are shown in the Table 1. The results indicated that the extraction yields with water were significantly higher in the case of *E. robusta*, *E. saligna* and *E. globulus* in comparison to that of 70% ethanol ($p < 0.05$). In the case of *E. microcorys* leaf and fruit extracts, the difference between the extraction yields with water and 70% ethanol was not statistically significant ($p > 0.05$).

Growth inhibition of crude Eucalyptus extracts on cancer cell lines using MTT assay

Growth inhibition values of the aqueous and ethanolic extracts of *Eucalyptus* species on cancer cell lines were calculated using the MTT assay (Table 2). Overall, the aqueous and ethanolic extracts from both leaf and fruit of *E. microcorys* and ethanolic extract of *E. robusta* exhibited more than 80% cell growth inhibition in glioblastoma, neuroblastoma and lung cancer cell lines at 100 µg/mL. In addition, aqueous *E. microcorys* leaf and 70% ethanolic extracts of *E. microcorys* fruit and *E. saligna* inhibited the growth of the pancreatic cancer cell line (MIA PaCa-2) by at least 80% at 100 µg/mL. In the case of skin cancer and normal breast cell lines, both the aqueous and ethanolic extracts of *E. microcorys* fruit at 100 µg/mL concentration showed more than 80% growth inhibition. The ethanolic extract of *E. saligna* resulted in more than 80% inhibition in pancreatic, colon, ovarian and skin cancer cell lines (Table 2). The aqueous and 70% ethanolic extracts of *E. globulus* displayed low to moderate activity (11 ± 5 - 77 ± 4% growth inhibition respectively) against the tested cancer cell lines. *E. robusta* (70% ethanol), *E. microcorys* fruit and leaf (water and 70% ethanol) and *E. saligna* (70% ethanol) extracts were selected for further investigation to assess the GI_{50} values against the cancer cell lines. Table 3 displays the GI_{50} values obtained from the selected *Eucalyptus* extracts and gemcitabine. The aqueous and ethanolic extracts of both *E. microcorys* leaf and fruit and the 70% ethanolic extract of *E.saligna* exhibited GI_{50} values of 68 ± 2.3, 76 ± 3, 63 ± 3.8, 73 ± 4 and 53 ± 3 µg/mL, respectively, in the MIA PaCa-2 pancreatic cancer cell line. In the case of the ovarian cancer cell line, all the extracts showed low to moderate activity with GI_{50} values ranging from 33 µg/mL (*E. saligna* ethanolic extract) to 80 ± 5 µg/mL (*E. robusta* ethanolic extract). Additionally, it was found that GI_{50} values were particularly higher (> 100 µg/mL) in the prostate cancer cell line for all the extracts. Similarly, in the breast cancer cell line (MCF-7), ethanolic extracts of *E. robusta*,

E.microcorys leaf and fruit and aqueous extract of *E.microcorys* leaf displayed a higher (> 100 µg/mL) GI_{50} values. Gemcitabine exhibited GI_{50} values ranging from of 1.4 ± 1.0 nM (ovarian cancer cell line) to 14 ± 5.0 nM (pancreatic cancer cell line) (Table 3).

Cell viability of primary and secondary pancreatic cancer cell lines using CCK-8 assay

The relatively lower GI_{50} values obtained from the aqueous and ethanolic extracts of *E. microcorys* leaf and fruit and ethanolic extract of *E. saligna* using the MTT assay might indicate the presence of solitary or more than one compound in the extracts with specific activity against the pancreatic cancer cell line (MIA PaCa-2). Hence, the aqueous *E. microcorys* leaf and fruit, and ethanolic *E. microcorys* leaf, and *E. saligna* extracts were further examined for their activity against other primary and secondary pancreatic cancer cell lines using the more sensitive CCK-8 assay. Between the two *E. microcorys* fruit extracts, only the aqueous extract *E. microcorys* fruit was considered for further examination due to limited availability of the fruit. Table 4 shows the cell growth inhibition (%) values in response to 100 µg/mL and 50 µg/mL of selected extracts across three different pancreatic cancer, and a normal pancreatic cell lines. Values obtained from extracts at a particular concentration were compared among each other (Table 4). In MIA PaCa-2 cells, statistically significant differences among the *E. microcorys* leaf (aqueous and ethanolic) and *E. microcorys* fruit (aqueous) extracts was observed at 100 µg/mL ($p < 0.05$) as indicated by the one-way ANOVA. Both the aqueous extracts of *E. microcorys* leaf and fruit inhibited the growth of MIA PaCa-2 cells by more than 80%. All three *E. microcorys* extracts (50 µg/mL) low to moderately inhibited (13.65 ± 5.43 – 53.82 ± 7.80%) the cell growth of MIA PaCa-2 cells. The aqueous extract of the *E. microcorys* fruit exhibited significantly greater cell growth inhibition value than the other extracts at both 100 and 50 µg/mL ($p < 0.05$). *E.*

saligna (70% ethanol) extract displayed moderate to low activity with < 50% growth inhibition at both 100 and 50 µg/mL in MIA PaCa-2 cells (Table 4).

In the BxPC-3 cell line, the selected extracts showed < 30 % cell growth inhibition mean values both at 100 and 50 µg/mL (Table 4). Aqueous and ethanolic extracts of *E. microcorys* leaf and ethanolic extract of *E. saligna* showed statistically similar cell growth inhibition values at 100 µg/mL concentration in BxPC-3 cells, as revealed by one-way ANOVA (p > 0.05). Gemcitabine (50 nM) inhibited the growth of BxPC-3 cells by 71.30 ± 2.40% (Table 4). All the tested extracts at both 100 and 50 µg/mL displayed < 15% growth inhibition in CFPAC-1 cells (Table 4). Moreover, there was no significant difference among the extracts in regards to their CFPAC-1 cell growth inhibition activity at 100 µg/mL (p > 0.05). Growth inhibition of > 90% was observed in the normal HPDE cells when they were treated with gemcitabine (50nM) and the four *Eucalyptus* extracts (100 and 50 µg/mL) (Table 4).

As the four *Eucalyptus* extracts exhibited < 50% growth inhibition in comparison to the vehicle control in BxPC-3 and CFPAC-1 cell lines, even at the higher concentration of 100 µg/mL, only MIA PaCa-2 and HPDE cell lines were further investigated for the determination of IC_{50} values. Table 5 represents the IC_{50} values of the selected *Eucalyptus* extracts. One-way ANOVA suggested that there was no statistical difference among the IC_{50} values obtained for aqueous extracts of *E. microcorys* leaf and fruit, and ethanolic *E. microcorys* leaf extracts in the MIA PaCa-2 cell line (p > 0.05, Table 5). The ethanolic extract of *E. saligna* displayed a significantly greater IC_{50} value (115.52 ± 5.36 µg/mL) in comparison to the other tested extracts in the MIA PaCa-2 cell line (p < 0.05, Table 5). Interestingly, the IC_{50} value of ethanolic *E. microcorys* leaf extract as ambiguous with wide standard deviations (Table 5). In the case of the normal HPDE cells, IC_{50} values < 20 µg/mL were obtained for all four extracts with ethanolic *E. microcorys* leaf extract being the lowest at 9.06 ± 2.12 µg/mL (Table 5).

Due to their significantly lower IC_{50} values compared to the *E. saligna* extract (70% ethanol) in the MIA PaCa-2 cell line, the aqueous extracts of *E. microcorys* leaf and fruit, and ethanolic extract of *E. microcorys* leaf were further studied for the induction of caspase 3/7 mediated apoptosis by using Muse caspase 3/7 assay. After 24 h of treatment, all extracts activated caspase 3/7 mediated apoptosis in the MIA PaCa-2 cell line (Figure 2). Treatment stimulated a net increase in the percentage of total apoptotic cells in comparison to the negative control (0.5% DMSO). The percentages of total apoptotic and live cells displayed by aqueous extracts of *E. microcorys* leaf and fruit were comparable to that of the positive control (gemcitabine). More than 12% of cells undergoing caspase 3/7 mediated apoptosis were detected after 24 h of treatment with the aqueous extracts of *E. microcorys* leaf and fruit, and gemcitabine (Figure 2). Morphological changes of the MIA PaCa-2 cells after 24 h of treatment with the selected extracts were also observed (Figure 3). Treated cells lost their characteristic elongated shape and became more circular. Membrane blebbing as a result of apoptosis was also observed (Figure 3).

Discussion

A greater extraction yield is often desired from a species or extraction method for further testing and utilisation of the extracts. The difference in the extraction yields can be attributed to the difference between the availability of extractable components, sample type, extraction solvent and technique implemented (Sultana *et al.* 2009). Our observations were in agreement with the previous findings of Sultana *et al.* (2009) who reported that a greater extraction yield can be achieved with extraction under reflux regardless of plant material and solvent used. We found that three out of four *Eucalyptus* species tested in our study showed significantly

higher extraction yield under reflux with water in comparison to the maceration with 70% ethanol.

The MTT assay was implemented to assess cell growth inhibition by the extracts across a panel of cancer cells as it is routinely used for high-throughput drug screening, and the correlation between the MTT assay and cell counting ($r > 0.98$) is well established (Hussain *et al.,* 1993). Our findings suggested that the anticancer activity of aqueous *E. robusta* freeze-dried extract against glioblastoma, neuroblastoma, breast, ovarian, lung and skin cancer cell lines is not different to that of aqueous *E. robusta* spray-dried extract previously demonstrated by Vuong *et al.* (2015b). The authors reported an 86% reduction in cell viability of MIA PaCa-2 cell line using 100 µg/mL of aqueous *E. robusta* spray-dried extract. Our results revealed a somewhat comparable value of 79 ± 2% growth inhibition in MIA PaCa-2 cells for a 100 µg/mL of aqueous *E. robusta* freeze-dried extract. Mota *et al.* (2012) reported the significant antiproliferative effects of ethanolic *E. globulus* bark extract in MDA-MB-231 human breast adenocarcinoma cells. In our study, aqueous and ethanolic extracts of *E. globulus* leaf inhibited the MCF-7 breast cancer cells only by 41 ± 3% and 22 ± 2%, respectively. The relatively lower activity of the extracts against breast cancer cells in contrast to the previous study can be explained by the differences in breast cancer cell lines and plant materials used in both the studies. Vuong *et al.* (2015b) reported the GI_{50} values of aqueous *E. robusta* spray-dried extract in glioblastoma, neuroblastoma, breast, ovarian, lung and skin cancer cell lines which are similar to the GI_{50} values of ethanolic *E. robusta* extract obtained from MTT assay in our study.

E. microcorys and *E. saligna* extracts exhibited < 30% growth inhibition in BxPC-3 and CFPAC-1 cell lines even at high concentration in the present study. The lower efficacy of the same extracts in BxPC-3 and CFPAC-1 cells than MIA PaCa-2 can be attributed to the variations in genetic profiles of the cell lines. For instance, *KRAS* (Kirsten rat sarcoma viral

oncogene homolog), *TP53* (encoding the p53 protein), *CDKN2A*, and *SMAD4* are the four most common types of mutations found in these pancreatic cancer cell lines, however, their mechanisms of occurrence vary among the cell lines (Deer *et al.* 2010).

The GI_{50} values obtained from MTT assay were compared with the IC_{50} values from CCK-8 assay for the aqueous *E. microcorys* leaf and fruit extracts and ethanolic *E. microcorys* leaf and *E. saligna* extracts against MIA PaCa-2 and HPDE cell lines. The IC_{50} value from a typical dose response curve is similar to the GI_{50} value provided the drug inhibits the growth of the cells completely (Paraiso *et al.* 2012). IC_{50} values between 10 - 50 and 50 - 100 µg/mL represent strong and moderate cytotoxicity, respectively (Doll-Boscardin *et al.*, 2012). We have previously established that aqueous *E. microcorys* leaf and fruit extracts possessed significantly higher total phenolic contents than aqueous extract of *E. saligna* (Bhuyan *et al.* 2016). It was also demonstrated that aqueous *E. microcorys* leaf extract had significantly higher saponin content compared to the aqueous *E. saligna* extract (Bhuyan *et al.* 2016). Plant phenolics and saponins have been reported to possess cytotoxicity and anticancer properties in a number of studies (Man *et al.* 2010; Podolak *et al.* 2010; Wahle *et al.* 2010). In addition, the synergistic cytotoxic and anti-inflammatory effects of flavonoids and saponins have also been reported (Puangpraphant and de Mejia 2009; Chávez-Santoscoy *et al.* 2016). The significantly lower IC_{50} value attained from the tested *E. microcorys* extracts in MIA PaCa-2 cells is potentially due to the presence of specific phenolic compounds and saponins. However, the IC_{50} value of 70% ethanolic extract of *E. microcorys* against MIA PaCa-2 cells was calculated with wide standard deviations. It could be attributed to the significantly lower efficacy (< 30%, $p < 0.05$) of ethanolic *E. microcorys* extract against MIA PaCa-2 cells even at a higher concentration (100 µg/mL).

The discrepancies in the percentage of cell growth inhibition, GI_{50} and IC_{50} values of tested *Eucalyptus* extracts obtained from MTT and CCK-8 assays can be explained by the technical

differences in the calculation of the values and the mechanisms of the assays. The IC_{50} value does not take into account the initial cell population unlike GI_{50} (Tong 2010). Moreover, the MTT assay primarily measures only the mitochondrial dehydrogenase activity of the cells (Ishiyama *et al.* 1996; Maioli *et al.* 2009), whereas, CCK-8 involves most of the dehydrogenase in a cell. Also, the formazan produced in CCK-8 assay is water soluble in nature and does not form crystals like MTT (Tominaga *et al.* 1999). The formazan crystals formed in MTT assay damage cells by puncturing membranes during exocytosis (Riss *et al.* 2004; Tominaga *et al.* 1999). Previous reports also suggested the unreliability of MTT assay when working with plant extracts, phenolic compounds and antioxidants as they can interfere with the assay by reducing the tetrazolium salt even in the absence of cells (Bruggisser *et al.* 2002; Maioli *et al.* 2009; Peng *et al.* 2005; Shoemaker *et al.* 2004; Wisman *et al.* 2008). Factors such as pH and glucose supply in the cell culture media can also affect the reduction of tetrazolium salt in MTT assay (Marshall *et al.* 1995; Sims and Plattner 2009). Altered cell metabolism due to some drugs can lead to changes in mitochondrial activity, which in turn influences the MTT assay by decreasing the reduction of tetrazolium salt (Hayon *et al.* 2003; Sims and Plattner 2009). Another important factor that can explain the discrepancies between the results obtained from MTT and CCK-8 assays in the present study is the difference in cell passage numbers used. Gene expression, morphology and cell development can be affected by the long-term subculturing (Deer *et al.* 2010) and can result in drug resistance and reduction in apoptosis (Ajabnoor *et al.* 2012; Pronsato *et al.* 2013).

The process of programmed cell death, apoptosis is downregulated in cancer (Shoja *et al.* 2015), therefore, induction of apoptosis by a drug or an extract is essential for reducing the viability of cancer cells. Activation of caspase 3/7 is considered a hallmark of apoptosis as these two effector caspases act downstream in the apoptotic pathway (Cullen and Martin 2009; Shoja *et al.* 2015). Activation of caspase 3/7 was observed in MIA PaCa-2 cell line by

the *E. microcorys* extracts after 24 h of treatment. Morphological changes such as cell shrinkage, rounding and plasma membrane blebbing due to caspase mediated apoptosis have also been reported in numerous studies (Coleman *et al.* 2001; Sebbagh *et al.* 2001; Ebrahimi Nigjeh *et al.* 2013). We also observed cell shrinkage, rounding and membrane blebbing of MIA PaCa-2 cells when treated with *E. microcorys* extracts for 24 h.

A number of studies in the literature have reported the IC_{50} values of extracts, essential oils and compounds isolated from other *Eucalyptus* species such as *E. camaldulensis, E. torquate, E. sideroxylon, E. cladocalyx* and *E. benthamii* against various cancer cell lines including human breast adenocarcinoma, human bladder carcinoma, human promyelocytic leukemia cells, Jurkat, HeLa and ovarian cancer cells (Al-Fatimi *et al.* 2005; Ashour 2008; Benyahia *et al.* 2005; Doll-Boscardin *et al.* 2012; Hrubik *et al.* 2012; Singab *et al.* 2011; Topçu *et al.* 2011). However, there is very little information in the literature about the activity of *E. microcorys* extract against pancreatic cancer cells.

Conclusion

Three out of four *Eucalyptus* species showed significantly higher extraction yields under reflux with water compared to the maceration with 70% ethanol at RT. The MTT assay revealed that the aqueous extract of *E. microcorys* leaf and ethanolic extract of *E. microcorys* fruit inhibited the cell growth of glioblastoma, neuroblastoma, lung and pancreatic cancer cells by more than 80% at 100 µg/mL. The selected *E. microcorys* leaf and fruit and *E. saligna* (70% ethanol) extracts displayed lower GI_{50} values than *E. robusta* (70% ethanol) extract in MIA PaCa-2 pancreatic cancer cell line in MTT assay. The CCK-8 assay further revealed that the aqueous *E. microcorys* leaf and fruit extracts at 100 µg/mL concentration exerted significantly higher cell growth inhibition (89.96 ± 0.91 and 93.75 ± 2.01%,

respectively) in MIA PaCa-2 cells compared to other tested extracts ($p < 0.05$). By contrast, < 30% growth inhibition was observed in BxPC-3 and CFPAC-1 cells by four tested *Eucalyptus* extracts at both 100 and 50 µg/mL concentrations. The IC_{50} values of aqueous *E. microcorys* leaf (86.05 ± 4.75µg/mL) and fruit (64.66 ± 15.97 µg/mL) and ethanolic *E. microcorys* leaf (~79.30 ± 29.45) extracts were found to be statistically similar ($p > 0.05$) in MIA PaCa-2 cells using CCK-8 assay. Our findings in regards to the GI_{50} and IC_{50} values of the crude extracts are particularly promising as crude extracts are mixtures of multiple components comprising both active and inactive compounds and hence higher doses are often required to observe an activity in comparison to standard chemotherapeutic drugs. Nevertheless, further investigations are warranted to isolate and identify more refined and solitary compounds from these extracts for achieving effective anticancer activity against pancreatic cancer cells, even with lower doses. Future studies to elucidate the mechanisms of action of the purified compounds from the *Eucalyptus* species are also crucial to develop therapeutic agents with greater potency and specificity against pancreatic cancer.

Acknowledgements

We acknowledge the following funding support: Ramaciotti Foundation (ES2012/0104); the Hunter Cancer Research Alliance (HCRA); the Hunter Medical Research Institute (HMRI) and the University of Newcastle. We would also like to express our gratitude to Brad Potts and Paul Tilyard from the School of Biological Science, University of Tasmania, Australia for providing the *E. globulus* sample.

Conflict of interest

The authors declare no conflict of interest.

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Figure caption

Figure 1: *E. robusta* (a), *E. microcorys* (b), *E. saligna* (c) and *E. globulus* (d).

Figure 2: Effect of control (0.5% DMSO) *(a)*, Gemcitabine-1 µM/mL*(b)*, *E. microcorys* leaf (water)- 100 µg/mL *(c)*, *E. microcorys* leaf (70% Ethanol)- 100 µg/mL *(d)* and *E. microcorys* fruit (water)- 100 µg/mL *(e)* on the apoptosis profile of MIA PaCa-2 cells studied by Muse Caspase 3/7 assay using Muse cytofluorimetric analysis.

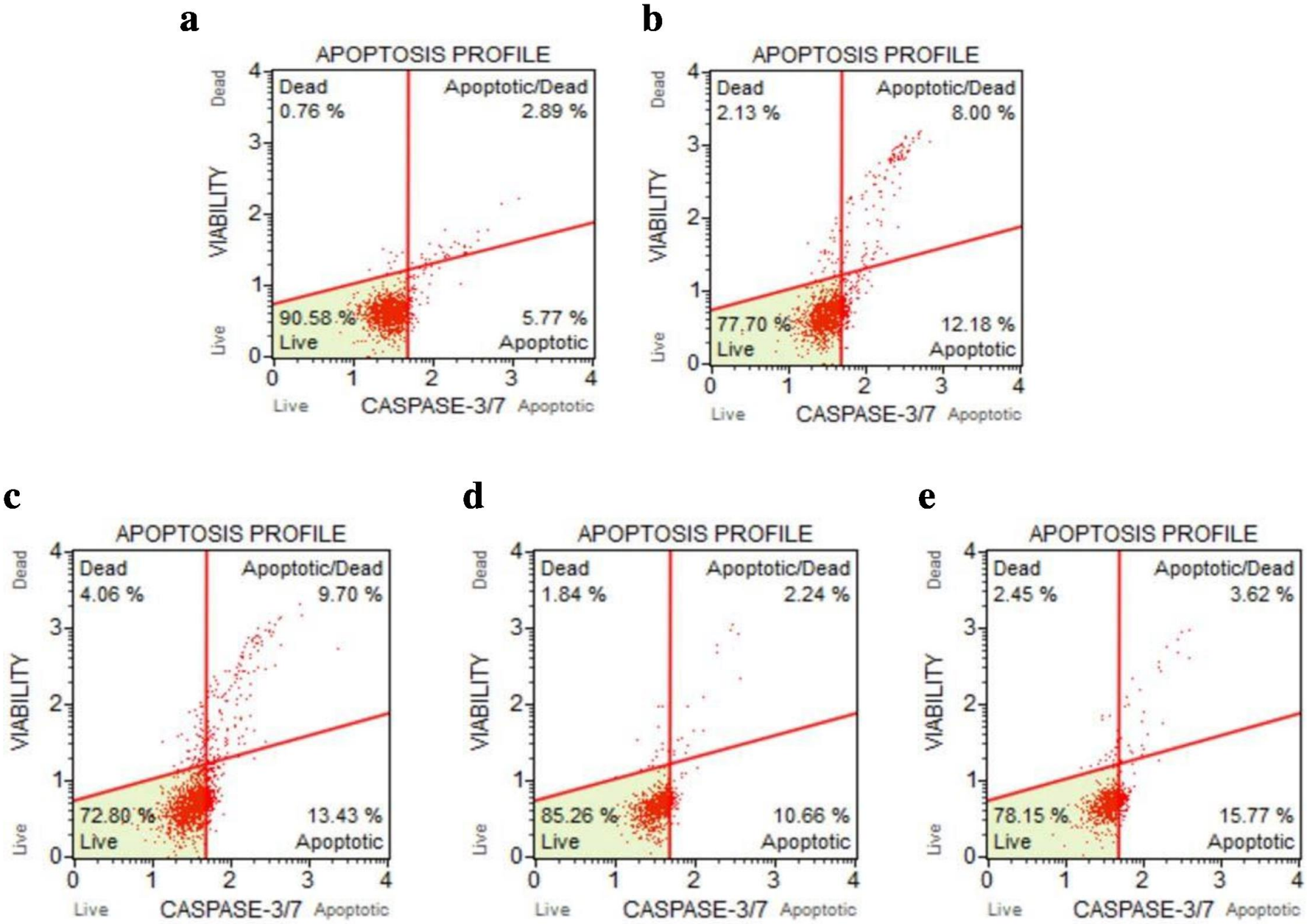
Figure 3: Morphological changes of MIA PaCa-2 cells after treatment with gemcitabine *(c)*, *E. microcorys* leaf (water) *(d)*, *E. microcorys* leaf (70% ethanol) *(e)* and *E. microcorys* fruit (water) *(f)* for 24 h. Arrows indicate rounded cells and membrane blebbing at 40x magnification. No morphological changes between before *(a)* and after *(b)* treatment with 0.5% DMSO for 24 h were observed in the control.

a

c

b

d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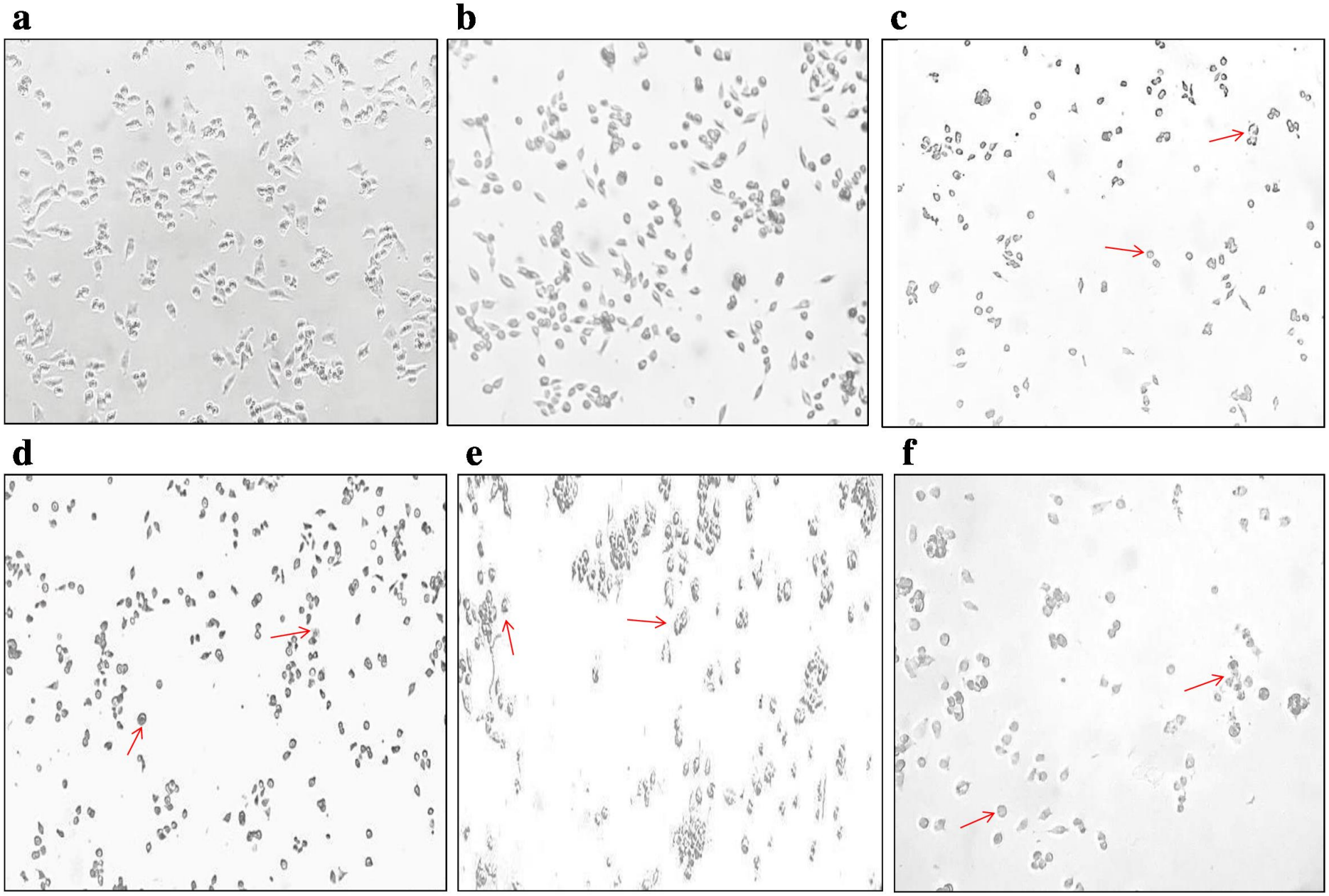
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Table 1. Extraction yields of different species of *Eucalyptus* (n=3) using conventional extraction with water and 70% Ethanol.

	Extraction yield (g/100 g of DW)	
	Water	70% ethanol
E. robusta	12.75 ± 0.22^{a}	10.97 ± 0.43^{b}
E. microcorys (leaf)	14.75 ± 1.85^{a}	13.15 ± 0.28^{a}
E. microcorys (fruit)	6.26 ± 0.78^{a}	5.44 ± 0.10^{a}
E. saligna	7.55 ± 0.27^{a}	6.42 ± 0.03^{b}
E. globulus	10.08 ± 0.77^{a}	8.74 ± 0.10^{b}

All the values are means ± standard deviations and those in the same row not sharing the same superscript letter are significantly different from each other ($p < 0.05$).

Table 2. Cell Growth Inhibition (%) in response to 100 µg/mL of extracts across various cancer cell lines (n = 3) using MTT assay. Higher values indicate greater growth inhibition.

		E. robusta		*E. microcorys*				*E. saligna*		*E. globulus*	
				Leaf		Fruit					
		Water	70% Ethanol	Water	70% Ethanol	Water	70% Ethanol	Water	70% Ethanol	Water	70% Ethanol
MIA PaCa-2	Pancreas	79 ± 2	65 ± 5	82 ± 2	67 ± 1	73 ± 2	80 ± 4	79 ± 4	84 ± 2	77 ± 4	20 ± 1
HT29	Colon	40 ± 6	65 ± 9	60 ± 6	72 ± 4	64 ± 4	78 ± 5	21 ± 3	89 ± 1	20 ± 3	26 ± 3
U87	Glioblastoma	34 ± 5	2 ± 3	36 ± 4	32 ± 5	42 ± 3	20 ± 5	35 ± 4	8 ± 4	35 ± 2	11 ± 5
SJ-G2	Glioblastoma	65 ± 9	85 ± 10	90 ± 7	95 ± 9	97 ± 4	103 ± 4	39 ± 8	47 ± 4	38 ± 7	27 ± 8
SMA	Glioblastoma (Murine)	52 ± 18	30 ± 13	56 ± 8	43 ± 11	59 ± 8	51 ± 8	45 ± 14	48 ± 9	40 ± 8	27 ± 14
MCF-7	Breast	39 ± 4	44 ± 4	43 ± 2	41 ± 6	56 ± 3	51 ± 4	53 ± 5	70 ± 3	41 ± 3	22 ± 2
A2780	Ovarian	63 ± 4	73 ± 4	75 ± 5	77 ± 4	86 ± 3	79 ± 4	64 ± 7	96 ± 0	56 ± 8	44 ± 1
H460	Lung	82 ± 14	85 ± 5	93 ± 2	95 ± 2	96 ± 2	98 ± 1	37 ± 13	64 ± 3	39 ± 12	46 ± 10
A431	Skin	57 ± 16	71 ± 9	75 ± 7	81 ± 6	87 ± 4	89 ± 4	39 ± 14	86 ± 2	48 ± 10	37 ± 8
Du145	Prostate	33 ± 3	35 ± 3	30 ± 3	22 ± 3	42 ± 4	33 ± 4	52 ± 8	49 ± 6	37 ± 13	14 ± 4
BE2-C	Neuroblastoma	68 ± 8	87 ± 9	90 ± 7	92 ± 8	97 ± 5	100 ± 4	43 ± 6	59 ± 2	44 ± 7	40 ± 4
MCF10A	Breast (Normal)	66 ± 6	67 ± 6	78 ± 4	79 ± 4	83 ± 3	84 ± 3	58 ± 7	44 ± 1	66 ± 6	53 ± 8

Table 3**.** Growth inhibition values (GI_{50}, concentration that inhibits cell growth by 50%) of the *Eucalyptus* (µg/ml) extracts and gemcitabine (nM) using the MTT assay. Lower value indicates greater growth inhibition efficiency.

		E. robusta	*E. microcorys*				*E. saligna*	Gemcitabine
			Leaf		Fruit			
		70% Ethanol	Water	70% Ethanol	Water	70% Ethanol	70% Ethanol	nM
MIA PaCa-2	Pancreas	94 ± 6	68 ± 2.3	76 ± 3.0	63 ± 3.8	73 ± 4	53 ± 3	14 ± 5.0
HT29	Colon	103 ± 13	95 ± 9	79 ± 6	85 ± 7	78 ± 6	53 ± 2	14 ± 3.0
U87	Glioblastoma	>200	146 ± 16	156 ± 39	133 ± 25	198 ± 37	177 ± 9	2.8 ± 0.0
SJ-G2	Glioblastoma	82 ± 11	69 ± 3	72 ± 9	62 ± 1	63 ± 3	103 ± 10	1.9 ± 0.0
SMA	Glioblastoma (Murine)	123 ± 15	81 ± 18	104 ± 18	79 ± 16	100 ± 13	101 ± 16	2.9 ± 0.0
MCF-7	Breast	122 ± 3	115 ± 7	112 ± 19	98 ± 10	105 ± 10	77 ± 5	3.6 ± 1.0
A2780	Ovarian	80 ± 5	67 ± 4	67 ± 6	52 ± 2	70 ± 5	33 ± 0	1.4 ± 1.0
H460	Lung	78 ± 3	72 ± 1	73 ± 2	65 ± 3	69 ± 2	90 ± 3	10 ± 3.0
A431	Skin	92 ± 11	84 ± 7	77 ± 7	68 ± 7	74 ± 8	47 ± 7	5.8 ± 2.0
Du145	Prostate	146 ± 14	162 ± 21	175 ± 15	151 ± 13	158 ± 19	119 ± 10	2.1 ± 0.0
BE2-C	Neuroblastoma	74 ± 9	68 ± 4	73 ± 10	61 ± 4	61 ± 3	81 ± 10	6.0 ± 1.0
MCF10A	Breast (Normal)	91 ± 6	75 ± 2	77 ± 6	69 ± 5	70 ± 3	106 ± 2	3.0 ± 0.0

Table 4. Cell Growth inhibition (%) in response to 100 µg/mL and 50 µg/mL of extracts and 50nM of gemcitabine across various pancreatic cell lines (n = 6) using CCK-8 assay. Higher values indicate greater growth inhibition.

			E. microcorys			*E. saligna*	Gemcitabine
			Leaf		Fruit		
		µg/mL	Water	70% Ethanol	Water	70% Ethanol	50nM
MIA PaCa-2	Pancreas	100	$89.96 \pm 0.91_{a}$	$28.30 \pm 5.04_{b}$	$93.75 \pm 2.01_{c}$	$45.42 \pm 2.67_{d}$	86.34 ± 0.60
		50	$21.61 \pm 6.02_{a}$	$15.48 \pm 4.62_{ab}$	$53.82 \pm 7.80_{c}$	$13.65 \pm 5.43_{b}$	
BxPC-3	Pancreas	100	$26.06 \pm 11.60_{a}$	$17.54 \pm 12.28_{a}$	$13.25 \pm 5.50_{b}$	$17.47 \pm 5.32_{a}$	71.30 ± 2.40
		50	$16.74 \pm 11.78_{a}$	$15.25 \pm 13.00_{a}$	$10.53 \pm 6.10_{a}$	$10.23 \pm 4.54_{a}$	
CFPAC-1	Pancreas	100	$12.68 \pm 4.90_{a}$	$11.67 \pm 4.65_{a}$	$14.66 \pm 3.03_{a}$	$10.42 \pm 3.45_{a}$	80.13 ± 2.10
		50	$8.94 \pm 2.50_{a}$	$8.59 \pm 4.52_{ab}$	$6.45 \pm 4.48_{b}$	$6.76 \pm 3.21_{b}$	
HPDE	Pancreas (Normal)	100	$93.91 \pm 0.34_{a}$	$94.32 \pm 0.14_{a}$	$95.76 \pm 0.19_{b}$	$93.45 \pm 0.76_{c}$	96.06 ± 0.08
		50	$92.34 \pm 1.43_{ab}$	$93.91 \pm 0.43_{a}$	$95.12 \pm 0.18_{c}$	$90.52 \pm 1.32_{b}$	

All the values are means ± standard deviations.

$_{a,b,c}$ Values in the same row not having the same subscript letter are significantly different ($p < 0.05$) from each other.

Table 5. IC_{50} values (µg/ml) (concentration that inhibits cell growth by 50%) of the selected *Eucalyptus* extracts using CCK8 assay. Lower value indicates greater growth inhibition efficiency.

		E. microcorys			*E. saligna*
		Leaf		Fruit	
		Water	70% Ethanol	Water	70% Ethanol
MIA PaCa-2	Pancreas	$86.05 \pm 4.75_a$	$\sim 79.30 \pm 29.45_a$	$64.66 \pm 15.97_a$	$115.52 \pm 5.36_b$
HPDE	Pancreas (Normal)	$16.25 \pm 1.34_a$	$9.06 \pm 2.12_b$	$15.96 \pm 1.87_a$	$12.43 \pm 3.43_{ab}$

All the values are means ± standard deviations.
$_{a,b,c}$ Values in the same row not having the same subscript letter are significantly different ($p < 0.05$) from each other.

CHAPTER 4

Evaluation of aqueous *E. microcorys* extract for antimicrobial properties

4.1 Introduction

Eucalypts contain abundant volatile and non-volatile compounds with a wide range of biological activities. Several studies have also reported the correlation between anticancer and antimicrobial properties of natural products (da Costa Sarmento et al., 2015; Felício et al., 2017; Gaspar et al., 2013) which may be due to their similar molecular mechanisms of action against microbial pathogens and cancer cells. Eucalypts are also used as traditional bush medicine to treat numerous bacterial and fungal infections by the indigenous people of Australia (Gilles et al., 2010). The scientific literature is also quite familiar with the antimicrobial activity of essential oils from eucalypts. However, the antimicrobial properties of eucalypt water based crude extracts are relatively unknown. Furthermore, *E. microcorys*, one of the least exploited species of eucalypt, has not been investigated in terms of its antimicrobial activity. As the development of multi-drug resistant pathogenic strains is currently one of the biggest concerns in medical science and plant phenolic compounds and antioxidants have shown potential in treating various microbial infections (Estevinho et al., 2008; Guil-Guerrero et al., 2016; Stojkovic et al., 2013; Süzgeç-Selçuk and Birteksöz, 2011), this aim was proposed to explore the possible antibacterial and antifungal properties of *E. microcorys* crude water extract.

4.2 Results and Discussion

The overall design of the study is shown in Figure 5. The results obtained from these studies were published in the form of following Research Paper:

Research Paper 6: **Deep Jyoti Bhuyan**, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, and Christopher J. Scarlett. Phytochemical, antibacterial and antifungal properties of an aqueous extract of *Eucalyptus microcorys* leaves. **South African Journal of Botany (2017),** 112: 180-185. DOI: 10.1016/j.sajb.2017.05.030

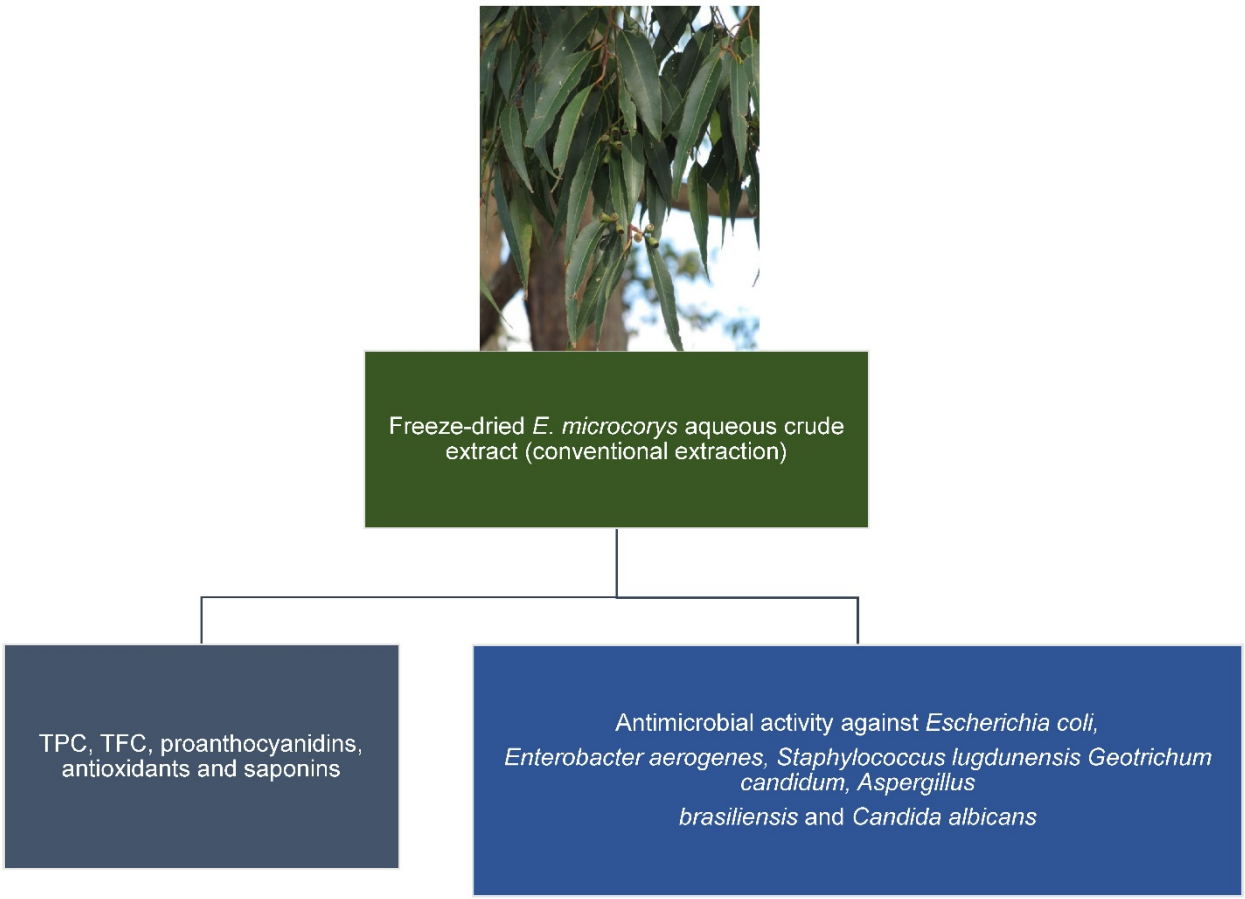

Figure 5: Overall design of the study assessing phytochemical and antimicrobial properties of freeze-dried *E. microcorys* aqueous crude extract.

4.3 Conclusions

The findings of the study emphasised the importance of *E. microcorys* crude aqueous extract as a source of antioxidant, antifungal and antibacterial agents and its potential future applications in the food, nutraceutical and pharmaceutical industries. In addition, it underlined the necessity of further studies to isolate and characterise bioactive compounds and determine their antimicrobial molecular mechanisms of action.

South African Journal of Botany 112 (2017) 180–185

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Phytochemical, antibacterial and antifungal properties of an aqueous extract of *Eucalyptus microcorys* leaves

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ARTICLE INFO

Article history:
Received 27 January 2017
Received in revised form 27 April 2017
Accepted 22 May 2017
Available online xxxx

Edited by S Van Vuuren

Keywords:
Eucalyptus microcorys
Phytochemical
Antimicrobial
Antioxidants
Phenolic compound

ABSTRACT

Australia is home to over 800 different species of *Eucalyptus* and traditionally, many *Eucalyptus* species have been utilised to heal wounds and treat fungal infections by the Indigenous people of Australia. In view of this, our study was designed to investigate the phytochemical, antibacterial and antifungal properties of crude aqueous extract of *E. microcorys* leaves. The freeze-dried powdered extract was prepared and the phytochemical profile was studied by analysing the total phenolic content (TPC), total flavonoid content (TFC), proanthocyanidins, antioxidants and saponins. The TPC, TFC and proanthocyanidin values found were: 501.76 ± 14.47 mg of gallic acid equivalents per g, 61.53 ± 0.83 mg of rutin equivalents per g and 10.76 ± 0.89 mg of catechin equivalents per g, respectively. The antioxidant values expressed in mg trolox equivalents per g of extract (mg TE/g) were: ABTS = 1073.13 ± 10.73 mg TE/g, DPPH = 1035.44 ± 65.54 mg TE/g and CUPRAC = 1524.30 ± 66.43 mg TE/g. The powdered extract was also evaluated for activity against three pathogenic bacterial strains (*Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus lugdunensis*); and three fungal strains (*Geotrichum candidum*, *Aspergillus brasiliensis* and *Candida albicans*) using the disc diffusion method and 96 well plate-based method with resazurin dye. The extract exhibited clear zones of inhibition against the tested bacteria and fungi. Minimum inhibitory concentration (MIC) values were demonstrated to be: *A. brasiliensis* = 2.44 μg/mL, *G. candidum* = 4.88 μg/mL, *S. lugdunensis* = 78 μg/mL, *E. coli* = 156.25 μg/mL, *E. aerogenes* = 312.5 μg/mL and *C. albicans* = 1250 μg/mL. These results reveal the significant potential of *E. microcorys* as a source of phenolics, antioxidants and antimicrobial agents and also highlight the necessity of further purification and characterisation of solitary bioactive compounds for their prospective applications in food, nutraceutical and pharmaceutical industries.

1. Introduction

Over 800 different species of eucalypts are indigenous to Australia (Vuong et al., 2015a). They are extensively planted for wood products and considered as one of the most important plants in the world (Gilles et al., 2010; Topçu et al., 2011; Gharekhani et al., 2012; Gupta et al., 2013). Eucalypts are a storehouse of both volatile and non-volatile compounds with a wide spectrum of biological activities. However, the volatile compounds found in the essential oils from eucalypts have so far, mostly been exploited for their application in the pharmaceutical and food industries. The Indigenous people of Australia have used eucalypts as traditional bush medicine to heal wounds and treat fungal infections since time immemorial (Gilles et al., 2010). Even though, eucalypts are native to Australia, they have been introduced throughout the tropics and subtropics including the Africa, Americas, Europe, the Mediterranean basin, the middle east, China and the Indian subcontinent for production of timber and paper (Gharekhani et al., 2012; Gupta et al., 2013). A number of more recent scientific studies have reported on the compounds present in the essential oils from eucalypts and their role as antimicrobials (Cimanga et al., 2002; Vilela et al., 2009; Gilles et al., 2010; Mulyaningsih et al., 2011; Fratini et al., 2014). However, there is little information available emphasising the potential antimicrobial properties of crude extracts from eucalypts. In addition, *Eucalyptus microcorys* is one of the least exploited *Eucalyptus* species in terms of its phytochemical content and biological activity.

Development of multi-drug resistance pathogenic strains is one of the biggest concerns in today's world as it has adverse effects on the efficacy of medical and pharmaceutical treatments, as well as agricultural and food industries. Various factors such as overuse, inappropriate prescription, extensive agricultural use of antibiotics and availability of very few new antibiotics contribute to the development of drug resistance in microorganisms (Ventola, 2015). Hence, there is an urgent need to look for novel antimicrobials, especially from nature. Plant phenolics and antioxidants have been extensively reported in the scientific literature for their antimicrobial potential (Davidson and Branden, 1981;

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http://dx.doi.org/10.1016/j.sajb.2017.05.030
0254-6299/

Tomás-Barberán et al., 1990; Nicholson and Hammerschmidt, 1992; Cowan, 1999; Zhu et al., 2004; Proestos et al., 2005; Estevinho et al., 2008; Süzgeç-Selçuk and Birteksöz, 2011; Stojkovic et al., 2013; Gyawali and Ibrahim, 2014; Guil-Guerrero et al., 2016). Therefore, the present study evaluated the phytochemical, antioxidant, antibacterial and antifungal properties of the aqueous crude extract from one of the least explored *Eucalyptus* species: *E. microcorys*.

2. Materials and methods

2.1. Plant materials

Fresh leaves of *E. microcorys* were collected from cultivated plants located at Ourimbah, Central Coast, NSW, Australia (latitude of 33.4° S, longitude of 151.4° E) on 2nd April, 2014. One of the authors (A.C.C.) authenticated the plant and a voucher specimen was deposited at the Don McNair Herbarium, The University of Newcastle (Accession number - 10499). The leaves were immediately transferred to the laboratory after collection and stored at −20 °C to avoid potential degradation of the phytochemicals. The leaves were then dried at 70 °C using a dry air oven for 5 h to constant weight (Vuong et al., 2015b). With the help of a commercial grade blender (Rio™ Commercial Bar Blender, Hamilton Beach), the leaves were then ground to a fine powder then sieved (≤1 mm) using a 1 mm EFL 2000 stainless steel mesh sieve (Endecotts Ltd., London, England) and packed in a sealed container and stored at −20 °C until required.

2.2. Extraction and preparation of crude extract powder

The solvent of choice in this study was water as it is the safest, cheapest and the most environmentally friendly solvent available and we have previously demonstrated its efficiency in terms of extracting phenolic compounds and antioxidants from *Eucalyptus* species using both conventional and modern techniques (Bhuyan et al., 2015, 2016, 2017). Conventional extraction using distilled water was carried out as follows. 5 g of powdered leaf sample was mixed with 100 mL of distilled water and heated at 85 °C for 15 min in a shaking water bath (Ratek instruments Pty Ltd., Boronia, VIC, Australia). The extract was immediately cooled on ice to room temperature (RT) and then filtered using Whatman® No.1 filter paper. Using a rotary evaporator (Buchi Rotavapor B-480, Buchi Australia, Noble Park, VIC, Australia) at 55 °C with reduced pressure, the filtrate was concentrated to one third of the initial volume. The concentrated extract was then immersed in liquid nitrogen and freeze-dried for 48 h in a freeze dryer (Thomas Australia Pvt., Ltd., Seven Hills, NSW, Australia) with drying chamber pressure of 2×10^{-1} mbar and cryo-temperature of −50 °C. The extract was resuspended in 5% dimethyl sulphoxide (DMSO) for performing the *in vitro* antimicrobial assays (Chandrasekaran and Venkatesalu, 2004).

2.3. Determination of total phenolic content (TPC)

TPC was measured according to the method described by Škerget et al. (2005). The extract was diluted up to 40 times to fit within the optimal absorbance range (0.1–1.0). The result was expressed as mg of gallic acid equivalents per g of dry weight (mg GAE/g) with gallic acid as a standard.

2.4. Determination of total flavonoid content (TFC)

Colorimetric assessment of TFC of the extract was performed as per the method described by Tan et al. (2014). Rutin was used as standard and TFC was expressed as mg of rutin equivalents per g of dry weight (mg RE/g).

2.5. Determination of proanthocyanidin content

The vanillin-HCl method as described by Broadhurst and Jones (1978) was employed to determine the proanthocyanidin content of the extract. Catechin was used as standard and the proanthocyanidin content was expressed as mg of catechin equivalents per g of dry weight (mg CAE/g).

2.6. Determination of antioxidant capacity

2.6.1. ABTS total antioxidant capacity (TAC)

The ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assay as described by Bhuyan et al. (2015) was used to measure the TAC of the extract. To construct the calibration curve, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard and the result was expressed in mg trolox equivalents per g of dry weight (mg TE/g).

2.6.2. Free radical scavenging capacity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay as described by Bhuyan et al. (2015) was employed to measure the free radical scavenging activity of the extract and the result was expressed in mg trolox equivalents per g of dry weight (mg TE/g).

2.6.3. Cupric reducing antioxidant capacity (CUPRAC)

The CUPRAC assay as described by Apak et al. (2004) was implemented to determine the cupric ion chelating capacity of the extract and the result was expressed in mg of trolox equivalents per g of dry weight (mg TE/g) with trolox as the calibration standard. Ascorbic acid was also included in all three antioxidant assays to make comparisons with the extract.

2.7. Determination of saponin content

To determine the saponin content of the extract, the method described by Hiai et al. (1976) was used. Aescin was used as standard with the result expressed as mg of aescin equivalents per g of dry weight (mg AE/g).

2.8. Antimicrobial assays

2.8.1. Microbial culture

Three bacterial strains: *Escherichia coli* (ATCC 10536), *Enterobacter aerogenes* (ATCC 13048) and *Staphylococcus lugdunensis* (ATCC 700328), two pathogenic fungal strains: *Geotrichum candidum* (ATCC 34614), *Aspergillus brasiliensis* (formerly known as *Aspergillus niger*) (ATCC 16404) and one yeast: *Candida albicans* (ATCC 10231) were used in this study. The microbial cultures were obtained from Thermo Scientific™ Oxoid™ in the form of Culti-Loops®. The stock bacterial and fungal cultures were maintained on nutrient agar medium and Sabouraud dextrose agar (SDA) medium, respectively at 4 °C.

2.8.2. Preparation of Inocula

Inoculums were prepared as per the method described by Chandrasekaran and Venkatesalu (2004) with some modification. Briefly, selected bacteria and yeast were grown for 24 h in nutrient broth (NB) at 37 °C and SDA at 28 °C, respectively and then mixed with sterile physiological saline and the turbidity was adjusted at a Mac Farland turbidity standard of 0.5 (10^6 colony forming units (CFU) per mL). The fungal isolates were subcultured on SDA and incubated at 28 °C for 7–14 days. The growth was aseptically macerated thoroughly in sterile distilled water and the absorbance of the fungal suspension was adjusted to 0.60 at 450 nm.

2.8.3. Disc diffusion bioassay

The disc diffusion method was employed to determine the zones of inhibition exhibited by different concentrations of the extract against the bacterial and fungal pathogens as per the method described by Chandrasekaran and Venkatesalu (2004) with some modification. Thermo Scientific™ Oxoid™ Blank antimicrobial susceptibility Disks (6 mm) were impregnated with 20 μL of extract (500 μg/mL or 250 μg/mL) and allowed to dry at RT. A blank disc with 5% DMSO was set as negative control and commercial discs (Thermo Scientific™ Oxoid™) with ciprofloxacin (5 μg) and fluconazole (25 μg) were set as positive controls for the antibacterial and antifungal assays, respectively. Bacterial and fungal inoculums (0.1 mL) were spread on Mueller Hinton agar (MHA) and SDA, respectively and allowed to dry for 5 min. The discs impregnated with extracts, positive and negative controls were then placed onto the microbial lawns using sterile forceps. The bacterial plates were incubated at 37 °C while the fungal plates were incubated at 28 °C. After 24 h, the inhibition zones (zone diameter) were measured. Zone diameters greater than 6 mm indicated the presence of inhibition. Each set of assay was carried out in triplicate.

2.8.4. Determination of minimum inhibitory concentration (MIC)

In vitro antibacterial and antifungal activity were determined using NB and Sabouraud Dextrose liquid (SDL) medium, respectively. Bacterial and fungal cells were treated with different concentrations of the extract and minimum inhibitory concentration (MIC) values were determined using the 96 well microtiter plate-based method with resazurin dye as described by Sarker et al. (2007) with some modification. In 40 mL of sterile distilled water, 270 mg of resazurin powder was mixed using a vortex mixer to obtain a homogenous solution. Ciprofloxacin and fluconazole were used as positive controls for antibacterial and antifungal assays, respectively, while 5% DMSO was set as the negative control for both the assays. Briefly, 100 μL of extract (2500 μg/mL in 5% *v*/v DMSO) was pipetted into the first row of the plate. NB or SDL (50 μL) was added to all other wells. Serial dilutions were performed using a multichannel pipette so that each well had 50 μL of the test material in serially descending concentrations. To each well, 10 μL of resazurin and 10 μL of bacterial suspension were added. A final volume of 100 μL was achieved in each well using NB or SDL. The plates were then wrapped loosely with cling film to ensure that bacteria/fungi did not become dehydrated. Each plate had a set of controls: a column with a positive control (ciprofloxacin or fluconazole in serial dilution), a column with all solutions with the exception of the test extract, and a column with all solutions with the exception of the test organism (10 μL NB or SDL instead). The plates were incubated at 37 °C for 24 h, 28 °C for 48 h and 28 °C for 72 h for bacteria, yeast and mycelial fungi, respectively. Any colour change visible by eye from purple to pink or colourless was recorded as positive which indicated microbial growth inhibition. The MIC value was determined as the lowest concentration at which no colour change occurred.

2.9. Statistical analyses

To perform independent samples *t*-test, one-way ANOVA, and the LSD post-hoc to compare the means, SPSS statistical software (version 16.0, Chicago, IL, USA) was used. The results were expressed as means ± standard deviations with all analyses carried out in triplicate, at least. The differences between the mean values in the experiments were taken to be statistically significant at $p < 0.05$.

3. Results and discussions

3.1. Phytochemical content of E. microcorys aqueous freeze-dried extract

Yields of TPC, TFC, proanthocyanidins and saponins from *E. microcorys* crude powder using conventional extraction with water are shown in Table 1. The quantitative assays revealed that the crude powder of *E. microcorys* aqueous extract contains high amount of phenolics (501.76 ± 14.47 mg GAE/g) and saponins (900.90 ± 34.86 mg AE/g). The TFC and proanthocyanidin content of the powdered extract were found to be 61.53 ± 0.83 mg RE/g and 10.76 ± 0.90 mg CAE/g, respectively. A study by Vuong et al. (2015b) obtained TPC, TFC and proanthocyanidin values of 407.54 ± 23.99 mg GAE/g, 144.50 ± 18.33 mg CAE/g, 14.42 ± 2.40 mg CAE/g, respectively from aqueous *E. robusta* leaf using identical extraction parameters and technique. We have also previously reported that 92.50 ± 3.10 mg GAE/g of TPC, 30.33 ± 0.47 mg RE/g of TFC and 1.55 ± 0.03 mg CAE/g of proanthocyanidins can be obtained from liquid *E. microcorys* leaf extract using conventional extraction with water (Bhuyan et al., 2016). It was also established that liquid *E. microcorys* leaf aqueous extract had significantly higher saponin content (206.88 ± 15.49 mg AE/g of dry weight) in comparison to aqueous extracts of *E. robusta*, *E. saligna* and *E. globulus* (Bhuyan et al. 2016). Another study by Moore et al. (2004) used near infrared spectroscopy modified partial least-squares regression model and predicted that 338.4 mg quebracho equivalents/g dry mass of total phenolics can be obtained from the *E. microcorys* leaves.

Table 1
Yields of TPC, TFC, proanthocyanidins and saponins from *E. microcorys* aqueous freeze-dried extract using conventional extraction with water (n = 3).

TPC (mg GAE/g)	501.76 ± 14.47
TFC (mg RE/g)	61.53 ± 0.83
Proanthocyanidins (mg CAE/g)	10.76 ± 0.90
Saponins (mg AE/g)	900.90 ± 34.86

All the values are means ± standard deviations.

The ABTS, DPPH and CUPRAC assays revealed that the crude powder of *E. microcorys* aqueous extract also possesses high antioxidant activity. The values indicated by ABTS (1073.13 ± 10.73 mg TE/g) and CUPRAC (1524.30 ± 66.43 mg TE/g) assays for *E. microcorys* crude powder were significantly higher than the corresponding values obtained from spray dried *E. robusta* crude powder (ABTS = 832.8 ± 57.1 mg butylated hydroxytoluene (BHT)/g and CUPRAC = 715.7 ± 43.9 mg BHT/g) by Vuong et al. (2015b). However, the spray dried *E. robusta* crude powder had significantly higher free radical scavenging capacity (1403.9 ± 107.1 mg BHT/g) than our freeze-dried *E. microcorys* crude powder (1035.44 ± 65.54 mg TE/g) as displayed by DPPH assay. Nevertheless, it should be noted that this comparison between these two species of *Eucalyptus* in terms of their antioxidant capacity is somewhat problematic as Vuong et al. (2015b) expressed his values as BHT equivalents as opposed to trolox equivalents even though identical extraction parameters and technique were implemented in both the studies. BHT has been shown to possess greater DPPH and ABTS radical scavenging activity compared to trolox (Gulcin, 2007; Gulcin et al., 2010). Setting aside the difference in the standard compound for measurement, these difference in antioxidant capacity can be attributed to the variations in the phytochemical profiles of two different species of *Eucalyptus* and the use of different methods for drying the extracts to obtain powder (freeze drying *vs.* spray drying). We have also previously demonstrated that the liquid *E. microcorys* aqueous extract had a significantly higher antioxidant capacity as revealed by ABTS, DPPH and CUPRAC assays in comparison to crude aqueous extracts of *E. saligna* and *E. globulus* (Bhuyan et al., 2016). In this study, the TAC and free radical scavenging capacity of *E. microcorys* crude powder were quite comparable to ascorbic acid, though, there were statistical differences between the values ($p < 0.05$) (Fig. 1). In addition, the cupric reducing antioxidant capacity of the extract was marginally lower than the ascorbic acid ($p < 0.05$) (Fig. 1).

3.2. Antimicrobial activity of E. microcorys aqueous freeze-dried extract

Our results indicated that *E. microcorys* aqueous freeze-dried extract possessed significant antibacterial and antifungal activity against the tested bacterial and fungal strains (Table 2). The disc diffusion assay

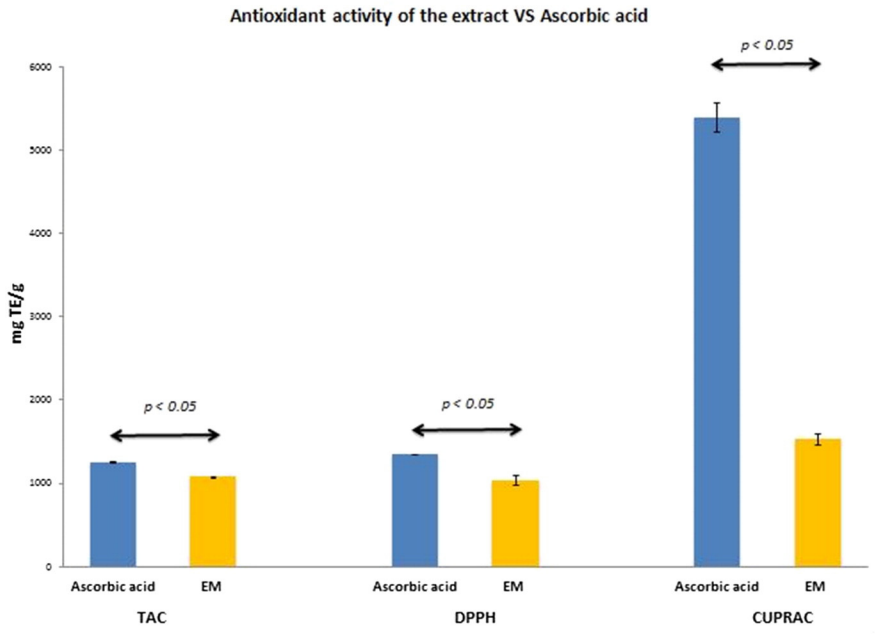

Fig. 1. Antioxidant activity of the *E. microcorys* (EM) aqueous freeze-dried extract *vs.* ascorbic acid.

revealed inhibition zones ranging from 18 ± 2.2 to 20 ± 1.0 mm against the 3 bacterial strains using 500 μg/mL of extract. Inhibition zones ranging from 14 ± 2.3 to 17 ± 0.5 mm were recorded against the same strains using 250 μg/mL of extract. *E. microcorys* extract exhibited the highest zones of inhibition against *S. lugdunensis* at both 500 μg/mL and 250 μg/mL among the tested bacterial strains. Moreover, the extract at 500 μg/mL displayed significantly greater antibacterial activity against *E. coli* and *S. lugdunensis* ($p < 0.05$) than at 250 μg/mL, but statistically similar activity with the ciprofloxacin (5 μg) ($p > 0.05$) (Table 2). The zones of inhibition against *E. aerogenes* displayed by both the concentrations of the extract were statistically similar to each other ($p > 0.05$). In the case of the fungal strains, inhibition zone diameters ranging from 7 ± 2.2 to 25 ± 1.4 mm were observed with the highest zone of inhibition against *A. brasiliensis* among the tested fungal strains at 500 μg/mL. *A. brasiliensis* also exhibited a higher zone of inhibition compared to other tested fungal strains at 250 μg/mL of extract. The extract at both the concentrations exhibited more significant activity against *A. brasiliensis* compared to fluconazole (25 μg) ($p < 0.05$). The inhibition zone exerted by 250 μg/mL of the extract against *G. candidum* was statistically similar to that of fluconazole ($p < 0.05$). The inhibition zones against the tested fungi were found to be directly proportional to the concentrations of the extract (Table 2). Parallel observations were also made by Chandrasekaran and Venkatesalu (2004) using aqueous and methanol extracts of *Syzygium jambolanum* seeds against different bacterial and fungal pathogens.

MIC values between 78 and 312.5 μg/mL were obtained for the *E. microcorys* aqueous freeze-dried extract against the bacterial strains while the range of 2.44–1250 μg/mL was observed against the fungal strains (Table 2). The tested *E. microcorys* extract exhibited the lowest MIC value of 2.44 μg/mL against *A. brasiliensis* followed by *G. candidum* (MIC = 4.88 μg/mL) and *S. lugdunensis* (MIC = 78 μg/mL). The MIC value of the extract against *C. albicans* was significantly larger in comparison to the other tested bacterial and fungal strains in this study (Table 2).

A number of reports have shown the antibacterial and antifungal properties of the essential oils from eucalypts such as *E. camaldulensis*, *E. tereticornis*, *E. alba*, *Corymbia citriodora* (synonym, *E. citriodora*), *E. deglupta*, *E. globulus*, *E. saligna*, *E. robusta* and *E. staigeriana* against *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, *S. chromogenes*, *S. warneri*, *S. xylosus*, *S. sciuri*, *A. flavus and A. parasiticus*

Table 2
Antibacterial and antifungal properties of *E. microcorys* aqueous freeze-dried extract.

Bacteria	Zone of inhibition (mm)			MIC (μg/mL)	
	E. microcorys extract		Ciprofloxacin	*E. microcorys* extract	Ciprofloxacin
	500 μg/mL	250 μg/mL	5 μg/disc		
Escherichia coli	19 ± 1.2[a]	15 ± 1.1[b]	20 ± 0.5[a]	156.25	1.56
Enterobacter aerogenes	18 ± 2.2[a]	14 ± 2.3[a]	24 ± 1.5[b]	312.50	0.78
Staphylococcus lugdunensis	20 ± 1.0[a]	17 ± 0.5[b]	22 ± 1.2[a]	78	0.39
Fungi	Zone of inhibition (mm)			MIC (μg/mL)	
	E. microcorys extract		Fluconazole	*E. microcorys* extract	Fluconazole
	500 μg/mL	250 μg/mL	25 μg/disc		
Geotrichum candidum	23 ± 1.5[a]	18 ± 0.5[b]	16 ± 1.5[b]	4.88	3.12
Aspergillus brasiliensis	25 ± 1.4[a]	19 ± 1.6[b]	14 ± 1.3[c]	2.44	12.5
Candida albicans	7 ± 2.2[a]	3 ± 1.5[b]	9 ± 0.6[a]	1250	100

Zone of inhibition values are means ± standard deviations.
[a,b,c] Values in the same row not having the same superscript letter are significantly different ($p < 0.05$) from each other.

(Cimanga et al., 2002; Vilela et al., 2009; Gilles et al., 2010; Mulyaningsih et al., 2011; Fratini et al., 2014). However, only a few reports exist that illustrate the antimicrobial properties *E. microcorys* essential oils, let alone its crude aqueous extract. A study by Kottearachchi et al. (2012) established that *E. microcorys* essential oil displayed significant inhibitory effect against the phyto-pathogenic fungi *Fusarium solani* and *Sclerotium rolfsii*. However, the ethanol extract of *E. microcorys* exhibited no efficacy against *S. rolfsii* and moderate efficacy against *F. solani*. Another interesting study by Arfao et al. (2013) evaluated whether the presence of *E. microcorys* extract in an aquatic microcosm, incubated under different temperatures, affects the growth of commensal and enteropathogenic *E. coli* strains. They concluded that the highest inhibition percentages were observed with most of the incubations temperatures at an extract concentration of 2%. This study supports our observation in verifying the inhibitory effect of *E. microcorys* aqueous extract against *E. coli*.

Numerous studies have highlighted the role of phenolics and saponins derived from plants as antibacterial agents against both gram-positive and gram-negative bacteria (Puupponen-Pimia et al., 2001; Hassan et al., 2010; Maddox et al., 2010; Cetin-Karaca and Newman, 2015), however, their precise mechanisms of action are still unknown. The antifungal properties of plant phenolics and saponins have also been reported widely against both human and plant fungal pathogens (Yang et al., 2006; Chapagain et al., 2007; Lambert et al., 2012; Teshima et al., 2013; Alves et al., 2014). The presence of hydroxyl (—OH) groups and the number and positions of double bonds in the structure have been linked with the antimicrobial and antioxidant activity of the phenolics (Cowan, 1999; Friedman et al., 2002; Gochev et al., 2010; Gyawali and Ibrahim, 2014). Gyawali and Ibrahim (2014) suggested that the —OH groups can interact with the bacterial cell membrane and disrupt it causing the leakage of cellular components and cell death. In addition, these groups have antioxidant activity and can modify microbial cell metabolism by binding to the active sites of key microbial enzymes (Cowan, 1999; Gyawali and Ibrahim, 2014). The antibacterial and antifungal properties of the *E. microcorys* aqueous freeze-dried extract are likely ascribed to the significant phenolic and saponin contents of the extract.

4. Conclusion

The aqueous crude extract of *E. microcorys* was found to be a good source of TPC, TFC, proanthocyanidins and saponins. It also exhibited prominent antioxidant activity in all the three antioxidant assays. Significant antibacterial and antifungal activity against the tested bacterial and fungal strains were also observed which is likely due to the presence of high amount phenolics and saponins in the extract. The MIC values are quite promising for a crude extract as it is a mixture of both active and inactive compounds. Lower MIC values, similar to the commercial antibacterial and antifungal agents, can potentially be achieved using the purified compounds from the extract. Further studies are warranted to isolate the solitary bioactive compounds present in the extract, to determine the major contributors to the antimicrobial activity and to elucidate their precise mechanisms of action. Nevertheless, the importance of *E. microcorys* crude extract as a potential source of antimicrobials and antioxidants should not be dismissed. This study highlights the plausible use of bioactive compounds derived from *E. microcorys* extract as biopreservatives, natural antioxidants, antibacterial and antifungal agents in the food, nutraceutical and pharmaceutical industries in the future.

Acknowledgements

The authors gratefully acknowledge the following funding support: Ramaciotti Foundations (ES2012/0104); the Hunter Cancer Research Alliance (HCRA); the Hunter Medical Research Institute (HMRI) and the University of Newcastle, Australia.

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CHAPTER 5

Bioassay guided fractionation of *E. microcorys* aqueous extract, tentative identification and molecular mechanisms of action of the most potent *E. microcorys* fraction

5.1 Introduction

Free radicals have been reported to have a positive correlation with cancer, cardiovascular and neurodegenerative diseases, diabetes and ageing (Rahman, 2007). Therefore, in recent years, a significant amount of research has been directed to understand the potential of antioxidants, such as flavonoids and other phenolics, in preventing and treating oxidative stress-related diseases including cancer (Fu et al., 2010; Gharekhani et al., 2012). Plant-derived flavonoids, sterols, lignanphenols, and various terpene-related compounds are potent natural antioxidants with many of these compounds not only having therapeutic values, but are also known for their cardioprotective, anticarcinogenic, antimutagenic and antimicrobial properties (Luis et al., 2014). Crude extracts are mixtures of multiple active and inactive components, which may or may not show efficacy against PC cell lines. It is not feasible to analyse and characterise the activity of each compound present in the crude extracts individually against cancer cell lines due to time constraints and associated costs. Therefore, this aim was proposed to further investigate the antipancreatic cancer activity of *E. microcorys* aqueous crude extracts on the basis of results obtained from the MTT and CCK-8 assays described in Chapter 3. Bioassay-guided fractionation of the extract using RP-HPLC was performed and cytotoxicity of each fraction was assessed using the CCK-8 assay against pancreatic cancer cell lines. This approach aided in delineating the key fraction with significant activity against pancreatic cancer cell lines. In addition, the antioxidant activity of the fractions was determined by the ABTS, DPPH and CUPRAC assays. The mechanisms of action of the most potent fraction was elucidated using the cell cycle, Annexin V and Cell Death and Western blot assays, to determine the extrinsic and intrinsic apoptotic pathways involved. Moreover, tentative identification of the compounds present in the active fraction was performed using HPLC-ESI/MS/MS.

5.2 Results and Discussion

The overall design of the study is shown in Figure 6. The results obtained from these studies were submitted in the form of following Research Paper to the peer reviewed journal ***Biomedicine and Pharmacotherapy*** (Review completed and ready for final decision).

Research Paper 7: **Deep Jyoti Bhuyan**, Quan V. Vuong, Danielle R. Bond, Anita C. Chalmers, Michael C. Bowyer, Christopher J. Scarlett. Eucalyptus microcorys leaf extract derived HPLC-fraction reduces viability of MIA PaCa-2 cells by inducing apoptosis and arresting cell cycle. Review completed **Biomedicine and Pharmacotherapy (2018).**

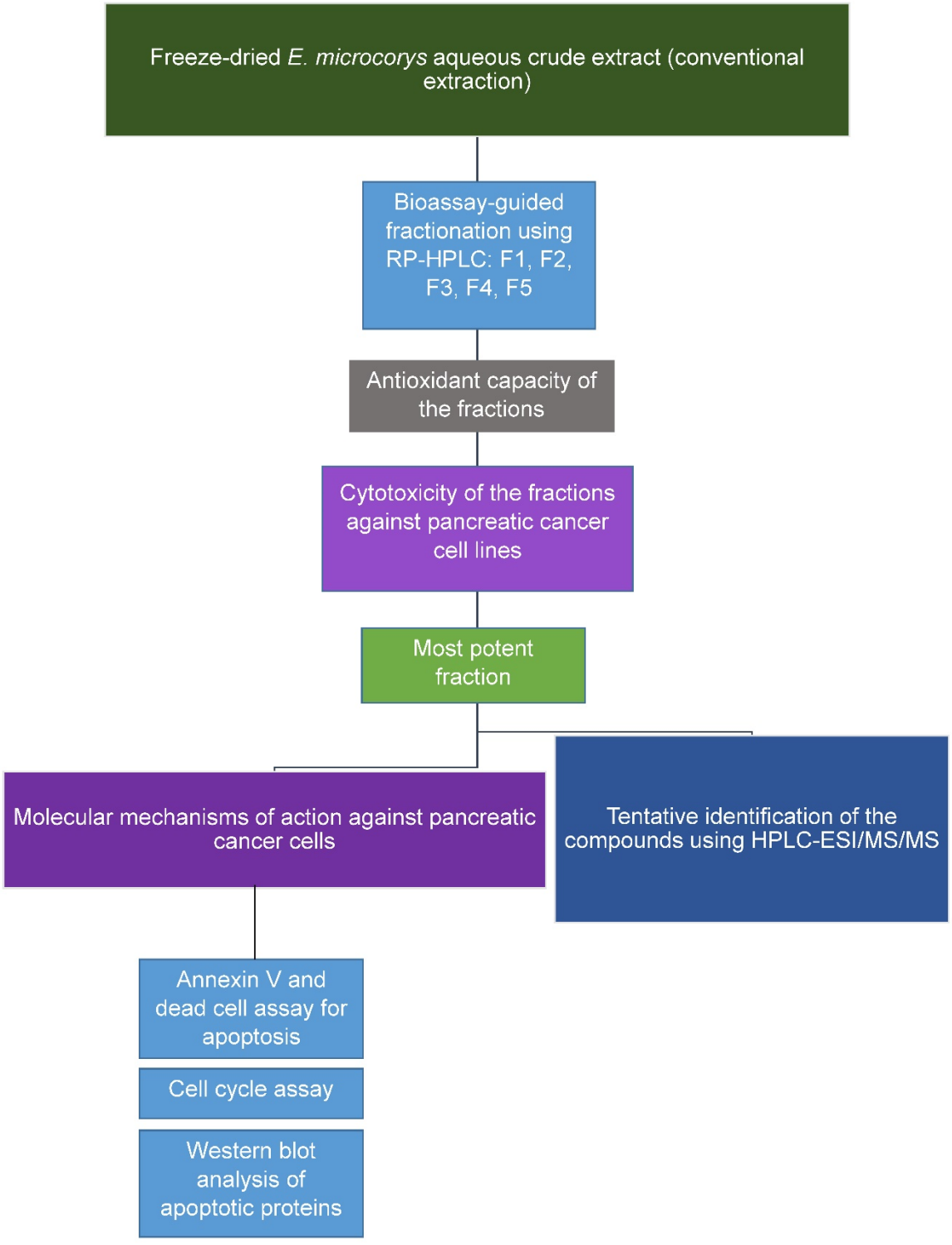

Figure 6: The overall design of the bioassay-guided fractionation study assessing the antioxidant capacity and cytotoxicity of fractions as well as the molecular mechanisms of action and tentative identification of compounds present in the most potent fraction of *E. microcorys* aqueous crude extract.

5.3 Conclusions

The findings of this study highlighted the significantly greater antioxidant and antiproliferative activity of fraction-1 (F1) derived from aqueous *E. microcorys* leaf extract. This fraction was also shown to induce apoptosis in MIA PaCa-2 cells and affected their cell cycle checkpoint. A significant impact on the key apoptotic proteins such as Bcl-2, Bak, Bax, cleaved PARP, procaspase-3 and cleaved caspase-3 in MIA PaCa-2 cells was also observed. This study also revealed that a combination of the F1 with gemcitabine was significantly more effective in inducing apoptosis and accumulating MIA PaCa-2 cells in the G2/M phase than F1 or gemcitabine alone, thus, indicating its potential implementation as a combination therapy with gemcitabine against PC. However, further *in vitro* and *in vivo* studies are required to understand the potential additive/synergistic interaction between gemcitabine and the compounds present in F1. In addition, HPLC-ESI/MS/MS revealed the similarities between the tentatively identified bioactive compounds present in the active fraction and phenolic compounds reported in the literature with antiproliferative activity against PC. This study also underlined the necessity of further examinations in order to characterise the active fraction for confirming the tentative identities by structural elucidation of the individual compounds.

Eucalyptus microcorys leaf extract derived HPLC-fraction reduces viability of MIA PaCa-2 cells by inducing apoptosis and arresting cell cycle

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List of Abbreviations

7-AAD	7-Aminoactinomycin D
ABTS	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
ANOVA	Analysis of variance
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated protein
Bcl-2	B-cell lymphoma 2
CCK-8	Cell counting kit-8
cdc2	Cell division cycle 2
cdc25	Cell division cycle 25
cdc25c	Cell division cycle 25 homolog c
CDKN2A	Cyclin-dependent kinase Inhibitor 2A
Chk-1	Checkpoint kinase-1
Chk-2	Checkpoint kinase-2
CUPRAC	Cupric reducing antioxidant capacity
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
HPDE	Human pancreatic ductal epithelial cell
HPLC	High performance/pressure liquid chromatography
HPLC-ESI/MS/MS	High performance/pressure liquid chromatography, electrospray ionisation, mass spectroscopy, mass spectroscopy
IC_{50}	The half-maximal inhibitory concentration
JNK	c-Jun N-terminal kinase
KRAS	Kirsten rat sarcoma
NA	Nutrient agar
p17	Protein 17
p53	Protein 53

PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PS	Phosphatidylserine
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase high performance/pressure liquid chromatography
TAC	Total antioxidant capacity
TBS	Tris-buffered saline
TBST	Tris-buffered saline + 0.1% Tween 20
TE	Trolox equivalent
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

Abstract

New therapeutic strategies such as the development of novel drugs and combinatorial therapies with existing chemotherapeutic agents are urgently needed to improve the clinical prognosis of pancreatic cancer. We have previously reported the antiproliferative properties of aqueous crude *Eucalyptus microcorys* extract against pancreatic cancer cell lines. In this study, bioassay-guided fractionation of the aqueous crude *E. microcorys* extract using RP-HPLC and subsequent assessment of the resultant fractions (F1 – F5) for their antioxidant activities and cytotoxicity against pancreatic cancer cell lines were performed. The molecular mechanisms associated with the cytotoxicity was characterised by studying the effects of the most potent fraction-1 (F1) on apoptosis and cell cycle profiles as well as its phytochemical constituents by LC-ESI/MS/MS. F1 displayed significantly greater antioxidant activity in three different assays ($p < 0.05$). Moreover, F1 exhibited significantly greater antiproliferative activity (IC_{50} = 93.11 ± 3.43 µg/mL) against MIA PaCa-2 cells compared to the other four fractions ($p < 0.05$). F1 induced apoptosis by regulating key apoptotic proteins- Bcl-2, Bak, Bax, cleaved PARP, procaspase-3 and cleaved caspase-3 in MIA PaCa-2 cells, suggesting the involvement of intrinsic mitochondrial apoptotic pathway and arrested cells at G2/M phase. A combination of gemcitabine and F1 exerted a greater effect on apoptosis and cell cycle arrest than F1 or gemcitabine alone ($p < 0.05$). LC-ESI/MS/MS revealed the tentative identities of phytochemicals present in F1 and their similarities with the phenolic compounds previously reported in *Eucalyptus* with antipancreatic cancer activity. Our study shows that the polyphenol and antioxidant-rich fraction of *E. microcorys* extract is a promising candidate for developing mono or combination therapies against pancreatic cancer.

Keywords: *Eucalyptus microcorys*, bioassay-guided fractionation, pancreatic cancer, apoptosis, cell cycle arrest, phenolic compounds

1. Introduction

Molecular heterogeneity, poor prognosis and few effective therapies make pancreatic cancer one of the most lethal malignancies. It is the seventh leading cause of cancer-related death worldwide [1]. According to GLOBOCAN 2012 estimates, 338,000 new cases were diagnosed in 2012, with the highest incidence in North America and Europe and the lowest in Africa and Asia [1, 2]. Pancreatic cancer has the highest mortality rate of any cancer with 3,364 new diagnoses were estimated in Australia in 2018 and its incidence and mortality statistics are similar throughout the world [3-5]. Only minor advances in the treatment of pancreatic cancer have been made over the last 15 years compared to other cancers [6, 7]. Pancreatic cancer patients experience an average five-year survival rate of about 7%, post-treatment due to its complex mutational landscape, few effective therapies with the emergence of resistance to chemotherapy and radiotherapy as well as poor prognosis [5]. It is also estimated that within a decade it will become the second cause of cancer death in the Western societies [5]. The Food and Drug Administration approved compound gemcitabine has been the drug of choice for pancreatic cancer over others such as 5-fluorouracil because of its overall clinical benefit and survival rate [6, 8]. In spite of this, gemcitabine is only minimally effective, as it improves patient survival by only a matter of weeks [9]. Interestingly, gemcitabine in conjunction with other drugs such as erlotinib and capecitabine, has been shown to be more efficacious than gemcitabine monotherapy [10, 11]. As drug discovery is a complex process that involves a large amount of time and resources, testing existing drugs in combinatorial therapies is a logical approach for improving overall efficacy.

Natural products research forms an integral part of cancer therapy drug development, with 74.9% of new anticancer drugs marketed between 1981 and 2010 directly derived from natural sources [12, 13]. A number of *in vitro* and *in vivo* studies have demonstrated the antipancreatic cancer properties of different plant derived phenolic compounds such as quercetin, myricetin, apigenin, naringenin, epigallocatechin-3-gallate, to name a few [5]. Vuong et al. highlighted the potential of bioactive compounds particularly from Australian native fruits in the treatment of pancreatic cancer [14]. Over 800 species of eucalypts are found in Australia and only a few

have been exploited by the pharmaceutical industry thus far [5]. Studies to date have illustrated the anticancer activity of only a limited number of eucalypts. Therefore, there remains an opportunity for natural product chemists to explore the chemical diversity of Australian eucalypts for development of new anticancer agents. We first reported the antipancreatic cancer properties of various species of Australian eucalypts [15-17]. *Eucalyptus microcorys,* commonly known as tallowwood, is one of the least exploited *Eucalyptus* species in terms of its phytochemical composition and bioactivity. We have recently demonstrated the phytochemical, antibacterial and antifungal properties of crude aqueous extract of *E. microcorys* leaves [18]. In addition, we have illustrated the antiproliferative activity of crude aqueous *E. microcorys* extract against MIA PaCa-2 pancreatic cancer cells and highlighted its significance as an important source of phytochemicals for pancreatic cancer therapy [17]. This study aimed to advance our previous research by establishing the antioxidant activity and antiproliferative effect of RP-HPLC fractions from the aqueous crude *E. microcorys* extract against both primary and secondary pancreatic cancer cell lines. Examinations to achieve a greater understanding of the phytochemical constituents and the molecular mechanisms of action of the most potent fraction were also undertaken.

2. Materials and methods

2.1. Plant materials

E. microcorys fresh leaves were collected from cultivated plants located at Ourimbah, Central Coast, NSW, Australia (33.4° S 151.4° E) on 2nd April 2014. The plant was identified by one of the authors (A.C.C.) and a voucher specimen deposited at the Don McNair Herbarium, The University of Newcastle (Accession number - 10499). After collection, the leaves were immediately transferred to the laboratory and stored at −20 °C to limit degradation of the phytochemical profile. Using a dry air oven (50 °C), the leaves were dried for 5 h to constant weight, then ground to a fine powder in a commercial grade blender (Rio™ Commercial Bar Blender, Hamilton Beach), sieved using a 1 mm EFL 2000 stainless steel mesh sieve

(Endecotts Ltd., London, England), packed in a sealed, airtight container and stored at -20 °C until tested.

2.2. Extraction and preparation of crude extract powder

Conventional aqueous extraction was carried out using the following method. Powdered leaf sample (5 g) was suspended in distilled water (100 mL) and heated at 85 °C for 15 min using a shaking water bath (Ratek instruments Pty Ltd., Boronia, VIC, Australia). The extract was then cooled on ice to room temperature and filtered through Whatman® No.1 filter paper. The filtrate was concentrated to one-third of its initial volume under reduced pressure (Buchi Rotavapor B-480, Buchi Australia, Noble Park, VIC, Australia) at a temperature of 45 °C. The concentrated extract was then freeze dried (Thomas Australia Pvt., Ltd., Seven Hills, NSW, Australia) for 48 h at a chamber pressure of 2×10^{-1} mbar and cryo-temperature of −50 °C.

2.3. RP-HPLC fractionation of E. microcorys extract

Fractionation of *E. microcorys* extract was performed using a Shimadzu LC-20AD HPLC system (Shimadzu, Rydalmere, NSW, Australia). The extract was dissolved in 70% ethanol and filtered through 0.45 µm Phenex syringe filters (Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia). An auto injector (SIL-10AP, Shimadzu, Rydalmere, NSW, Australia) was used to inject 500 µL of the extract onto a RP semi-prep Phenomenex Synergi 4U Polar-RP 80A column (250 mm x 10 mm) (Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia). The column temperature was maintained at 35 °C using a temperature controller (Phenomenex Therma Sphere TS 130, Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia). The mobile phase (flow rate = 3 mL/min) comprised two solvents: distilled water + 0.1% orthophosphoric acid (mobile phase A) and 100% acetonitrile (mobile phase B). A gradient elution schedule was implemented as follows: 0-10 min, 0% B; 10-30 min, 30% B; 30-45 min, 60% B; 45-50 min, 60% B; 50-60 min, 0% B. Phytochemicals were detected at wavelengths of 190 and 254 nm using a photodiode array detector (SPD-M20A, Shimadzu, Rydalmere, NSW, Australia). Based on retention time of the phytochemical profile, five major fractions (F1, F2, F3, F4 and F5) were obtained from the *E. microcorys* extract and collected

using an auto fraction collector (FRC-10A, Shimadzu, Rydalmere, NSW, Australia) as follows: F1: 5.25 − 8.60 min, F2: 29.70 − 37.70 min, F3: 41.50 – 43.00 min, F4: 50.30 – 52.70 min and F5: 54.00 – 58.40 min (Fig. 1). The collected fractions were evaporated using a rotary evaporator (40 °C), freeze-dried for 24 h (2×10^{-1} mbar, −50 °C), then stored at −20 °C until required. The yields of the fractions were also calculated (n = 10 runs, Fig. 1).

2.4. Determination of antioxidant capacity of E. microcorys fractions

2.4.1. TAC assay

The TAC of the fractions was determined using the ABTS assay as described by Bhuyan et al. [19]. Trolox was used as standard and the result was expressed in mg TE per g of dry weight (mg TE/g).

2.4.2. Free radical scavenging capacity

The free radical scavenging activity of the fractions was determined by the DPPH assay as described by Bhuyan et al. [20] and the result was expressed in mg TE per g of dry weight (mg TE/g).

2.4.3. CUPRAC assay

To determine the cupric ion chelating capacity of the fractions, the CUPRAC assay as described by Apak et al. [21] was implemented, with the result expressed as mg of TE per g of dry weight (mg TE/g), with trolox as the calibration standard.

2.5. Determination of pancreatic cancer cell viability of the fractions

E. microcorys fractions were evaluated against two primary pancreatic cancer cell lines (MIA PaCa-2 and BxPC-3), a secondary pancreatic cancer cell line (CFPAC-1) derived from a liver metastasis site and an immortalised normal HPDE using the CCK-8 assay as per the method described by Bhuyan et al. [16]. Pancreatic cancer cell lines were obtained from the American Type Culture Collection (ATCC: Manassas, VA, USA). The HPDE cells were originally from the lab of Dr. M. Tsao (MD, FRCPC, University Health Network, Toronto, ON, Canada) [22]. The cells were treated with different concentrations of the fractions (F1 - F5) and their combination (CF), gemcitabine (50 nM) and vehicle control (0.5% DMSO) for 72 h. All

experiments were performed in replicates of six. GraphPad Prism software, version 6.0b (San Diego, CA, USA) was used to calculate the IC_{50} values (concentration that inhibits cell growth by 50%) calculated by curve fitting the absorbance (viability) vs. log [concentration of treatment].

2.6. Flow cytometry for cell cycle analysis

Cytofluorimetric analysis of cell cycle distribution was analyzed using the Muse™ Cell Analyzer as per the manufacturer's instructions. Briefly, exponentially growing MIA PaCa-2 cells (6 x 10^5 cells/mL) were treated with 0.5% DMSO (control), gemcitabine: 50 nM, F1: 100 µg/mL, F1: 150 µg/mL and F1: 100 µg/mL + gemcitabine: 50 nM for 24 h. After treatment, cells were washed with PBS, trypsinised, harvested, resuspended in PBS and fixed with ice-cold 70% ethanol and stored at −20 °C for 24 h. Ethanol-fixed cells (200 µL) were then washed with PBS and stained with 200 µL of Muse™ Cell Cycle Reagent for 30 min and analysed using the Muse™ Cell Analyzer. In this assay, the percentage of cells in each cell cycle phase (G0/G1, S, and G2/M) was measured and discriminated by propidium iodide-based staining of DNA content.

2.7. Flow cytometry for evaluation of apoptosis

MIA PaCa-2 cells were treated with 0.5% DMSO (control), gemcitabine: 50 nM, F1: 100 µg/mL, F1: 150 µg/mL and F1: 100 µg/mL + gemcitabine: 50 nM for 24 h in a 6-well plate at 1.8 x 10^5 cells per well. Apoptosis profiles of the cells were studied by the Muse™ Annexin V & Dead Cell Kit (#MCH100105, Merck Millipore, Billerica, MA, USA) using the Muse™ Cell Analyzer as per the manufacturer's instructions. This assay uses Annexin V to detect PS on the external membrane of apoptotic cells and 7-AAD as a dead cell marker to indicate cell membrane structural integrity. It distinguishes four populations of cells: non-apoptotic cells: Annexin V (−) and 7-AAD (−), early apoptotic cells: Annexin V (+) and 7-AAD (−), late stage apoptotic and dead cells: Annexin V (+) and 7-AAD (+) and mostly nuclear debris: Annexin V (−) and 7-AAD (+).

2.8. Western blot analysis

After 48 h of treatment with 0.5% DMSO (control), F1: 100 µg/mL or F1: 150 µg/mL, MIA PaCa-2 cells were washed with PBS and lysed with 1% NP-40 lysis buffer (#FNN0021, Thermo Fisher Scientific, Scoresby, VIC, Australia) containing protease inhibitor (1:9) on ice. Adherent cells were dislodged from the surface by scratching with pipette tips, vortexed three times and centrifuged at 15,000 *g* for 15 min at 4 °C. The supernatant was collected and stored at –80 °C. The lysates were analysed for total protein by Micro BCA Protein Assay Kit (#23235, Thermo Fisher Scientific) as per the manufacturer's instructions. Protein samples (20 µL/well) at 15 µg/mL concentration were separated in NuPAGE™ 4-12% Bis-Tris Midi protein gels (#WG1402BOX, Thermo Fisher Scientific) followed by transferring to nitrocellulose membranes. Pre-stained protein molecular weight markers (#1610375, Precision Plus Protein™ Kaleidoscope™ pre-stained protein standards, Bio-Rad, CA, USA) were included in each gel. Membranes were blocked for 45 min at room temperature with 5% dry skim milk powder in 1X TBST and probed with various primary antibodies; such as anti-Bcl-2 (1:3000), anti-Bak (1:1000), anti-Bax (9.5:3000) (Merck Millipore) and an apoptosis Western blot cocktail containing antibodies for pro/p17-caspase 3, cleaved PARP1 (1:250) (#ab136812, Abcam, Cambridge, UK) for 2 h. To ascertain comparative expression and equal loading of the protein samples, anti-GAPDH (1:5000) (BioVision, Inc., CA, USA) for Bcl-2, Bak, Bax, cleaved PARP blots and muscle actin (1:100) (Abcam, Cambridge, UK) for procaspase-3 and cleaved caspase-3 blots were used as the loading controls. Following 1X TBST washes, membranes were incubated with anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase for 1 h. After washes with 1X TBST and 1X TBS, target proteins were detected using Luminata Classico Western HRP substrate (#WBLUC0500, Merck Millipore) and the band intensity was imaged and quantified by Amersham Imager 600 (GE Healthcare Life Sciences, MA, US).

2.9. LC/MS analysis

The fraction F1 was re-suspended in LC/MS grade acetonitrile before being diluted 1:100 prior to analysis by nanoflow RP Liquid Chromatography (Dionex Ultimate 3000 RSLCnano,

Thermo Fischer Scientific) coupled directly to a Q-Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fischer Scientific). Samples were loaded at 6 µL/min (2% acetonitrile, 0.1% trifluoroacetic acid in Water) for 10 minutes onto a 5 µm C^{18} nanoViper trap column (100 µm x 2 cm, Acclaim PepMap100, Thermo) for desalting and pre-concentration. Separation was then performed at 400 nL/min over an EASY-Spray PepMap column (3 µm C18, 75 µm x 15cm) utilising a gradient of 2-99% Buffer B (80% acetonitrile, 0.1% formic Acid) over 30 min, held at 99% Buffer B for 4 minutes before allowed to re-equilibrate at 2% buffer B for a further 16 min (60 min total run time per sample). Sub-fractionation of F1 was performed to make it more suitable for nanoflow LC-MS. F1 was resuspended in 1 mL of Ultrapure H_2O and vortexed thoroughly. The H_2O suspensions formed a precipitate which was pelleted by centrifugation and the supernatant was collected. Each pellet was then fully resuspended in 1 mL LC/MS grade acetonitrile. These two sub-fractions and F1 were then eluted directly into the EasySpray nano-ESI Ion Source (Thermo Fischer Scientific) for high resolution MS mass determination and MS/MS analysis of the top 8 ions in each MS scan. MS analysis scanned the mass range from 50-100 *m/z* in positive ion mode with resolution set to 70,000 at *m/z* 200, an AGC target of 1×10^6 and maximum injection time of 50 ms. Ubiquitous polysiloxane was utilised as a lock mass at *m/z* 445.12002. Top 8 MS/MS was performed on ions with intensities greater than 1.8×10^5 at a resolution of 35,000 with an AGC target of 2×10^5 and maximum injection time of 110 ms. Higher collisional dissociation normalised collision energy was stepped at 24 and 30 whilst dynamic exclusion time was set at 15 seconds. Nano-ESI source parameters were as follows: spray voltage 2000V, capillary temperature 200 °C, probe heater temperature 350 °C and S-lens RF 50. Thermo .raw MS Files were imported into both Compound Discoverer 2.0 platform (Thermo Fisher Scientific) for untargeted metabolomics workflows (retention time alignment, unknown compound detection, extraction ion chromatogram generation and integration, prediction of elemental composition and identification against ChemSpider databases or database searching of MS/MS fragment data against mzCloud). Manual peak detection, elemental composition and isotope pattern matching were also performed using CSI:FingerId and Thermo Xcalibur Qual Browser.

Compound Discoverer ChemSpider elemental composition algorithm was given the following constraints: Parent ion tolerance was set to 5 ppm, the following ions were allowed for MH^+ parent ion consideration ($[2M+ACN+H]^{+1}$; $[2M+ACN+Na]^{+1}$; $[2M+FA-H]^{-1}$; $[2M+H]^{+1}$; $[2M+K]^{+1}$; $[2M+Na]^{+1}$; $[2M+NH_4]^{+1}$; $[2M-H]^{-1}$; $[2M-H+HAc]^{-1}$; $[M+2H]^{+2}$; $[M+3H]^{+3}$; $[M+ACN+2H]^{+2}$; $[M+ACN+H]^{+1}$; $[M+ACN+Na]^{+1}$; $[M+Cl]^{-1}$; $[M+DMSO+H]^{+1}$; $[M+FA-H]^{-1}$; $[M+H]^{+1}$; $[M+H+K]^{+2}$; $[M+H+MeOH]^{+1}$; $[M+H+Na]^{+2}$; $[M+H+NH_4]^{+2}$; $[M+H-H_2O]^{+1}$; $[M+H-NH_3]^{+1}$; $[M+K]^{+1}$; $[M+Na]^{+1}$; $[M+NH_4]^{+1}$; $[M-2H]^{-2}$; $[M-2H+K]^{-1}$; $[M-H]^{-1}$; $[M-H+HAc]^{-1}$; $[M-H+TFA]^{-1}$; $[M-H-H_2O]^{-1}$). Unknown compound elemental composition was performed with a minimum element count for 1x Hydrogen and 1x Carbon and maximum element counts of C_{90}; H_{190}; Br_3; Cl_4; K_2; N_{10}; Na_2; O_{15}; P_3; S_5. Unknown compounds were grouped with a mass tolerance of 5 ppm and a retention time tolerance of 0.2 min. Compound Discoverer ChemSpider database searches were performed against the AraCyc; BioCyc; ChEMBL; Chemical Biology Department, Max Planck Institute of Molecular Physiology; ChemSpiderman; Natural Product Updates; Natural Products Discovery Institute; Nature Chemistry; NIST; NIST Spectra; Plant Metabolic Network; Planta Piloto de Química Fina. Universidad de Alcalá; PlantCyc and PubChem databases with 5 ppm mass tolerance. Whilst mzCloud MS/MS identification was attempted against all available compound classes (HighChem HighRes algorithm) with a precursor and fragment mass tolerance of 5 - 10 ppm.

2.10. Statistical analyses

All antioxidant, cell viability, flow cytometry and Western blot assays were carried out at least in triplicate. SPSS statistical software (version 16.0, Chicago, IL, USA) was used to perform one-way ANOVA, and the LSD post-hoc test to compare the means. The results were expressed as means ± standard deviations. The differences between the mean values in the experiments were taken to be statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Antioxidant activity of E. microcorys fractions

The antioxidant content values of F1 were: 548.32 ± 13.43, 653.28 ± 43.24 and 756.45 ± 57.87 mg TE/g for the TAC, DPPH and CUPRAC, respectively. F1 exhibited significantly greater antioxidant activity compared to the other four fractions as determined by the three assays ($p < 0.05$, Fig. 2). We have previously reported that aqueous freeze-dried crude extract of *E. microcorys* contains 1073.13 ± 10.73 (TAC), 1035.44 ± 65.54 (DPPH) and 1524.30 ± 66.43 (CUPRAC) mg TE/g of antioxidants [18]. The higher antioxidant content of the crude extract than its individual fractions is perhaps contibuted by the presence of higher amount as well as a wider range of antioxidant compounds. This also indicates the potential loss of antioxidants during the fractionation process. Similar observations were also made by Khled khoudja et al. [23] in crude extracts and their solvent fractions of *Calamintha clinopodium, Teucrium flavum* and *Thymus algeriensis.*

3.2. Cell growth inhibition of primary and secondary pancreatic cancer cell lines by the E. microcorys *fractions using CCK-8 assay*

The fractions obtained from the aqueous *E. microcorys* leaf extract were examined for their activity against both primary and secondary pancreatic cancer cell lines using the CCK-8 assay. To determine any loss of cytotoxicity occurring during fractionation of the extract, all five fractions were combined in appropriate proportions (CF, Table 1) based on their yields (Fig. 1) to produce an exact equivalent of the crude aqueous parent extract. Table 1 shows the cell growth inhibition (%) in response to 200 µg/mL, 100 µg/mL and 50 µg/mL of the individual fractions and CF across three different pancreatic cancer and normal pancreatic cell lines after 72 h of treatment. Values obtained from the fractions at a particular concentration were compared (Table 1). In MIA PaCa-2 cells, F1 at both 200 and 100 µg/mL exhibited significantly greater cell growth inhibition than the other four fractions ($p < 0.05$). However, the greatest inhibition of cell growth was observed in MIA PaCa-2 cells using CF (200, 100 and 50 µg/mL; $p < 0.05$). Both CF and the F1 inhibited the growth of MIA PaCa-2 cells by more than 95% at 200 µg/mL. At 100 and 50 µg/mL, CF exhibited 79.42 ± 2.31 and 36.34 ± 2.56% cell growth inhibition in MIA PaCa-2 cells, respectively. Only the IC_{50} value of F1 against MIA

PaCa-2 cells was calculated as it displayed more than 50% cell growth inhibition at 100 µg/mL, whereas, the other four fractions (F2 - F5) displayed < 30% growth inhibition (Table 1, $p < 0.05$). Comparatively, gemcitabine at 50 nM exerted 90.76 ± 1.27% growth inhibition in the MIA PaCa-2 cell line.

Statistically similar ($p > 0.05$) cell growth inhibition values against BxPC-3 cells were exhibited by CF and F1 at all three tested concentrations (Table 1). Moreover, their activity at 200 and 50 µg/mL were significantly greater ($p < 0.05$) than that of F2 – F5 (Table 1). However, all the fractions and CF inhibited the growth of BxPC-3 cells by < 50%, even at the highest tested concentration (200 µg/mL). Therefore, the fractions and CF were not considered for the determination of the IC_{50} values. Gemcitabine (50 nM) inhibited the growth of BxPC-3 cells by 75.53 ± 2.97% (Table 1).

In the case of CFPAC-1 cell line, the fractions and CF inhibited growth in the range of 1.99 ± 1.94 – 37.14 ± 1.37% in all three tested concentrations (Table 1). Treatment with CF showed a significantly greater cell growth inhibition value of 37.14 ± 1.37% at 200 µg/mL ($p < 0.05$) compared to the individual fractions. The IC_{50} values of the fractions were not calculated, as the individual fractions exhibited < 25% growth inhibition in CFPAC-1 cells, even at a higher concentration of 200 µg/mL (Table 1). Gemcitabine (50 nM), inhibited the growth of CFPAC-1 cells by 68.25 ± 1.95% (Table 1).

In HPDE cells, > 90% cell growth inhibition was observed with 200 µg/mL of individual fractions and CF and 50 nM of gemcitabine (Table 1). Moreover, F1 and CF inhibited HPDE cell growth in the range of 91.27 ± 0.63 – 98.45 ± 0.12% at all three tested concentrations (200, 100 and 50 µg/mL; Table 1). Gemcitabine at 50 nM, also inhibited HPDE cell growth by > 93%. In HPDE cells, F1 was found to have an IC_{50} value of 22.55 ± 4.84 µg/mL (Table 1).

Overall, the combination of fractions revealed greater antiproliferative effects on MIA PaCa-2, CFPAC-1 and HPDE cell lines than the individual fractions. These cell growth inhibition values are comparable to that of the aqueous *E. microcorys* crude extract reported recently [17]. The greater bioactivity of the parent extract when compared to its individual fractions, is not an unusual occurrence in bioassay-guided fractionation studies. Various studies have reported

diminished bioactivity after fractionation of crude extract [24, 25]. This inconsistency may be explained by the breakdown or alteration of active constituents by hydrolysis or esterification of acidic/alcoholic functional groups, UV radiation and oxidation during the fractionation process [26]. The phenomenon of pharmacodynamic synergy among the active constituents of an extract can also explain the discrepancies among the potency of individual fractions and their combination in the present study [27]. Synergistic effects of phytochemicals present in apples have been demonstrated by Barth et al. on colon cancer *in vivo* [28]. They suggested that cloudy apple juice is more effective than the individual apple polyphenols and fraction in a rat model of colon carcinogenesis. Similarly, the collective effects of apple polyphenols on the protein kinase c activity and the onset of apoptosis in human colon carcinoma cells were also observed by Kern et al. [29]. In pharmacokinetic synergy, substances with little or no activity assist the main active compound in a number of ways, including helping the active component engage the target by improving bioavailability, decreasing metabolism and excretion, complementing mechanisms of action, reversal of resistance, and modulating adverse effects [27]. Another credible explanation for the significantly lower activity of individual fractions in comparison to the parent extract would be the degradation of active components of an extract due to their separation from the protectants during fractionation [26]. Therefore, the widespread belief of achieving a better activity with purified compounds rather than the crude extracts is not always scientifically justified. In addition, the lower activity of the individual fractions and CF on BxPC-3 and CFPAC-1 compared to MIA PaCa-2 and HPDE cells can be ascribed to the variations in genetic profiles of the cell lines. The four most common mutations in pancreatic cancer- *KRAS*, *TP53*, *CDKN2A* and *SMAD4* vary among the cell lines. For instance, wild type *SMAD4* has been reported in MIA PaCa-2 cells, whereas, both BxPC-3 and CFPAC-1 carry a homozygous deletion in *SMAD4* [30]. Our previous study also showed variations in cytotoxicity of *E. microcorys* and *E. saligna* extracts against different primary and secondary pancreatic cancer cell lines [17].

3.3. Cell cycle analysis of MIA PaCa-2 cells after treatment

Investigations on the effect of F1 on cell cycle distribution revealed that when MIA PaCa-2 cells were treated with F1 at two different concentrations (100 and 150 µg/mL) and a combination of F1 (100 µg/mL) with gemcitabine (50 nM), an increase in cell population at the G2/M phase was evident, with a corresponding decrease of cells in the G0/G1 phase (Fig. 3). Fig. 3A-E shows the results of one experiment representative of all cell cycle experiments. After 24 hr of treatment, F1 (at both 100 and 150 µg/mL) and the F1/gemcitabine treatment led to significantly higher percentages of the cell population in G2/M phase compared to gemcitabine alone or the untreated control ($p < 0.05$, Fig. 3). F1 at both 100 and 150 µg/mL showed statistically similar accumulation of the G2/M population ($p > 0.05$). Interestingly, the combination of F1 with gemcitabine exhibited a greater effect on cell cycle arrest at G2/M phase than either F1 or gemcitabine alone ($p < 0.05$) (Fig. 3). Cancer cell growth inhibition can be triggered either by cell cycle arrest, or induction of apoptosis, or both [9]. Cell cycle arrest is considered a promising feature of chemotherapeutic drugs as halting any cell cycle event in the cancer cell leads to prevention of their growth and division [31, 32]. The ROS generation, JNK activation, increased phosphorylation of ataxia telangiectasia mutated, Chk-1, and Chk-2, and reduction in cdc25c levels, have been linked with the accumulation of cells in the G2/M phase [33]. Apigenin, a phenolic compound that is also commonly found in *Eucalyptus* species, has been shown to induce G2/M phase cell cycle arrest by reducing the levels of cyclin A, cyclin B, phosphorylated forms of cdc2 and cdc25 in four human pancreatic cancer cell lines: AsPC-1, CD18, MIA PaCa-2, and S2-013 [34]. Although p53 plays a key role in accumulating cells at G2/M phase, the G2/M arrest of MIA PaCa-2 pancreatic cancer cells by apigenin is attributed to a p53-independent pathway as these cells carry mutated p53 [34, 35]. The p53-independent pathways inhibit phosphorylation of cdc2 in response to DNA damage which in turn leads to G2/M arrest [35]. Therefore, the G2/M arrest of the MIA PaCa-2 cells by the polyphenol-rich F1 observed in our study is perhaps mediated by p53-independent pathway due to the presence of similar phenolic compounds in the fraction.

3.4. Apoptosis profile of MIA PaCa-2 cells after treatment

Due to its significantly greater cell growth inhibition against MIA PaCa-2 cells, F1 was further investigated at two different concentrations (100 and 150 µg/mL), and in combination with gemcitabine for the induction of apoptosis using the Muse™ Annexin V & Dead Cell Kit. After 24 h of treatment, all the treatments significantly decreased the number of live cells ($p < 0.001$, Fig. 4), exhibiting greater percentage of total apoptotic cells compared to the negative control (0.5% DMSO). F1 at both the concentrations, displayed significantly more total apoptotic cells compared to the positive control gemcitabine ($p < 0.001$). Our data also demonstrated that the combined treatment of F1 with gemcitabine led to a significantly greater induction of apoptosis than F1 or gemcitabine alone ($p < 0.001$, Fig. 4). The induction of apoptosis in MIA PaCa-2 cells by F1 is in line with our previous findings, that reported caspase 3/7-mediated apoptosis by the aqueous and ethanolic crude extracts of *E. microcorys* leaf [17]. Likewise, other eucalypt species such as *Corymbia citriodora* (previously known as *Eucalyptus citriodora*), *E. occidentalis* and *E. benthamii* and their bioactive compounds have been shown to induce apoptosis in different cancer cell lines [36-39]. Apoptosis is often mediated by caspases (which attack cytoplasmic and nuclear substrates), cleavage of DNA and alteration of plasma membrane phospholipid organization with PS externalization [40]. Necrosis causes potential tissue damage, while in apoptosis cells die without damaging neighbouring tissues [40, 41]. Therefore, apoptosis is often preferred to necrosis as the mode of cell death in anticancer research.

3.5. Western blot analysis of apoptotic proteins after treatment with F1

Changes in the expression levels of proteins associated with apoptosis: Bcl-2, Bak, Bax, cleaved PARP, procaspase-3 and cleaved caspase-3 in MIA PaCa-2 cells were analysed using Western blot after 48 h of treatment with F1: 100 µg/mL (F1-100) and F1: 150 µg/mL (F1-150) in comparison to the control. Exposure of cells to F1 at 150 µg/mL significantly decreased the expression levels of antiapoptotic protein Bcl-2 compared to the control ($p < 0.05$, Fig. 5). Furthermore, F1 at 150 µg/mL significantly increased the expression level of the proapoptotic proteins- Bak and Bax, compared to the control ($p < 0.05$, Fig. 5). In the case of

Bax expression, a statistically significant difference was observed between F1 at 100 and 150 µg/mL ($p < 0.05$, Fig. 5). Similarly, F1 at 150 µg/mL exhibited significantly greater Bax/Bcl-2 ratio (3.58 ± 1.19, $p < 0.05$) compared to the control (0.55 ± 0.15) and F1 at 100 µg/mL (1.02 ± 0.61). No statistical difference was observed between the Bax/Bcl-2 ratios of the control and F1 at 100 µg/mL ($p > 0.05$). The antiapoptotic Bcl-2 and the pore-forming Bax and Bak proteins are important components of the mitochondrial pathway (intrinsic) of apoptosis [42, 43]. An enhancement in the levels of Bcl-2 and depletion in Bak and Bax levels are frequently observed in various cancers [44]. Therefore, the chemotherapeutic effect of a drug is often measured by its regulation of Bcl-2, Bak and Bax expressions. Degradation of Bcl-2 due to chemotherapeutic drugs has been linked with caspase-dependent cleavage, ubiquitin-directed degradation or alterations of mRNA levels [45].

We also investigated the cleavage of caspase-3 and PARP to explore the mechanism of F1-induced apoptosis and the involvement of caspases. The MIA PaCa-2 cells treated with F1 showed a significant increase in the levels of apoptosis-specific 89 kDa PARP fragment (cleaved PARP) compared to the control ($p < 0.05$, Fig. 5). Cleaved PARP is generated by the active caspases from the full length PARP. In addition, we detected the procaspase-3 (32 kDa) in both control and treated cells. However, the active form of caspase-3 (p17 subunit) generated by cleavage of the procaspase-3 at Asp175 was only detected in the treated cells (Fig. 5) which validates our flow cytometry data of induction of apoptosis in MIA PaCa-2 cells by F1. In addition, cleavage of Bcl-2 by the activated caspases [45, 46] can also explain the down-regulation of Bcl-2 protein observed in this study. Therefore, our results support the premise that F1 triggers the intrinsic mitochondrial pathway of apoptosis. Parallel observations were also recently made in MIA PaCa-2 and PANC-1 cells by Kumar et al. using MDB5, a novel Hedgehog inhibitor [47].

3.6. Characterisation of compounds present in F1 by LC/MS analysis

Liquid chromatography coupled with mass spectrometry (LC/MS) has become a popular technique for screening and characterisation of both known and unknown compounds present

in plant extracts [48]. In untargeted metabolomics, LC/MS can detect thousands of compounds even in a small amount of a biological sample. Tandem MS (MS/MS) produces data by fragmenting the compound and recording the *m/z* of the fragments, which are particularly helpful in identifying unknown compounds. CSI:FingerID is a useful tool for searching molecular structure databases such as PubChem using tandem MS data of small molecules and it achieves significantly better results than existing state-of-the-art methods [49]. Therefore, we analysed the active fraction - F1 using LC-ESI/MS/MS coupled with various platforms including CSI:FingerID, Compound Discoverer 2.0 and Thermo Xcalibur Qual Browser to tentatively identify and quantify the compounds which could be responsible for the antiproliferative effect on the pancreatic cancer cells. In order to obtain more information about the molecular masses of the different compounds, the fragmentation of the predominant positive ions in the MS/MS mode was used. Tentative identification of major constituents of F1 and corresponding MS/MS fragmentation profiles are shown in Table 2. Most of these compounds, such as corylifolin, N,N-diethyl-2-phenylacetamide, 2,4-N-isobutylocta-2,4-dienamide, myrcen-8-ol and artemisinate, in their pure or derivative forms have been reported widely in the literature and listed on the PubChem database for their anticancer activity [31, 50-55, 56]. Potential *m/z* cloud identification of single MS/MS scan of compound 8 revealed a terpene-like compound with a molecular formula of $C_{10}H_{16}O$ (myrcen-8-ol; citral; α-pinene-2-oxide; pulegone). Compound 10 and 11 had similar retention time of 34.92 min which indicated that compound 10 was similar to 11 as it also showed a 387.181 peak evident in full MS. Similarly, compound 12 is perhaps compound 13 as it showed a 217.1433 peak in full MS (Supplementary Fig. 1). Fortes et al. [57] identified a new (microcoryn) and sixteen known phenolic compounds in the leaves of *E. microcorys.* Table 3 depicts the similarities of compounds found in our study in terms of their molecular weights with some of the previously identified phenolic compounds in *Eucalyptus*. Studies that have linked these compounds with antipancreatic cancer activity are also shown in Table 3. The anticancer properties of plant-derived phenolic antioxidants are quite well known in natural product research. Moreover, phenolic antioxidants such as flavone were reported to have protective properties against cell

DNA damage [58]. The antiproliferative effect exhibited by F1 in our study is possibly due to the presence of specific compounds in the active fraction which have been previously reported in the literature for their cytotoxicity against PC. However, this can only be confirmed by further isolation, identification and structural elucidation studies.

4. Conclusion and future direction

We investigated the antioxidant and antiproliferative activity of fractions from aqueous *E. microcorys* leaf extract using RP-HPLC against PC cells. F1 displayed significantly greater antioxidant activity in all three assays. In addition, the combination of all five fractions and F1 alone exhibited significantly greater antiproliferative activity against pancreatic cancer cells. This study also provides a reasonable mechanistic explanation of observed apoptosis induction by F1 with modulation of various key apoptotic proteins such as Bcl-2, Bak, Bax, cleaved PARP, procaspase-3 and cleaved caspase-3 in MIA PaCa-2 cells. The results are suggestive of the involvement of an intrinsic mitochondrial apoptotic pathway to explain the antiproliferative effects of F1. However, whether an extrinsic pathway (activation of caspase-8) is also involved in F1-induced apoptosis remains to be investigated. Furthermore, F1 also led to abrogation of cell cycle checkpoint by arresting MIA PaCa-2 cells in the G2/M phase. To evaluate the potential synergistic effects on apoptosis and cell cycle, a combination of gemcitabine and F1 was also tested and found to have a greater effect in both the assays compared to F1 or gemcitabine alone. However, more *in vitro* and *in vivo* studies are warranted to obtain a concrete understanding of the interactions among the individual components of F1 and gemcitabine for their potential use as a combination therapy against pancreatic cancer. Further LC/MS analysis of F1 in this study using an untargeted metabolomics approach provided putative identifications of its constituents and their similarities with the bioactive compounds reported in the literature in *Eucalyptus* with antipancreatic cancer activity. Nonetheless, further investigations using NMR are necessary to confirm the identity and absolute configuration of the active compounds present in this *E.*

microcorys fraction in order to develop mono or combinational therapeutic strategies against pancreatic cancer.

Acknowledgements

This work is supported by the following grants: Ramaciotti Foundation (ES2012/0104); the Hunter Cancer Research Alliance (HCRA); the Hunter Medical Research Institute (HMRI) and the University of Newcastle, Australia. We would also like to express our gratitude to Nathan Smith from the Analytical & Biomolecular Research Facility, Research and Innovation Division - Central Scientific Services at the University of Newcastle, Australia for his scientific and technical assistance in performing the LC/MS analysis of the sample.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Fig. 1. Reversed Phase HPLC fractionation of aqueous crude extract of *Eucalyptus microcorys*

Fig. 2. Antioxidant capacity of *Eucalyptus microcorys* Reversed Phase HPLC fractions

Fig. 3. Cell cycle analysis of MIA PaCa-2 cells as studied by the Muse™ Cell Analyzer using the Muse™ Cell Cycle Kit after 24 h of treatment. (A) Untreated control: 0.5% DMSO, (B) gemcitabine: 50 nM/mL, (C) Fraction 1: 100 µg/mL, (D) Fraction 1: 150 µg/mL and (E) Fraction 1: 100 µg/mL + gemcitabine: 50 nM/mL. Graphs (F) show the cell cycle percentages (n = 9; mean ± SD) of control and treated MIA PaCa-2 cells from three independent experiments. (***) indicates the values that are significantly different from the untreated control ($p < 0.001$).

Fig. 4. Apoptosis profiles of MIA PaCa-2 cells as studied by the Muse™ Annexin V & Dead Cell Kit using the Muse™ Cell Analyzer after 24 h of treatment. (A) Control: 0.5% DMSO, (B) gemcitabine: 50 nM/mL, (C) Fraction 1: 100 µg/mL, (D) Fraction 1: 150 µg/mL and (E) Fraction 1: 100 µg/mL + gemcitabine: 50 nM. Graphs (F) show the live, total apoptotic and dead cell percentages (n = 9; mean ± SD) of control and treated MIA PaCa-2 cells from three independent experiments. Statistically significant difference was observed between the untreated control and treatments ($p < 0.05$). (**) and (***) indicate the values that are $p > 0.001$ and $p < 0.001$, respectively.

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Table legends

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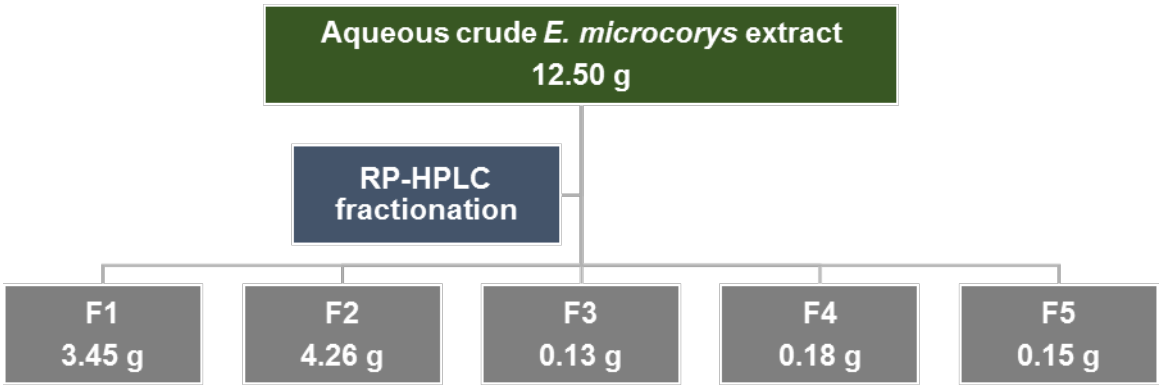


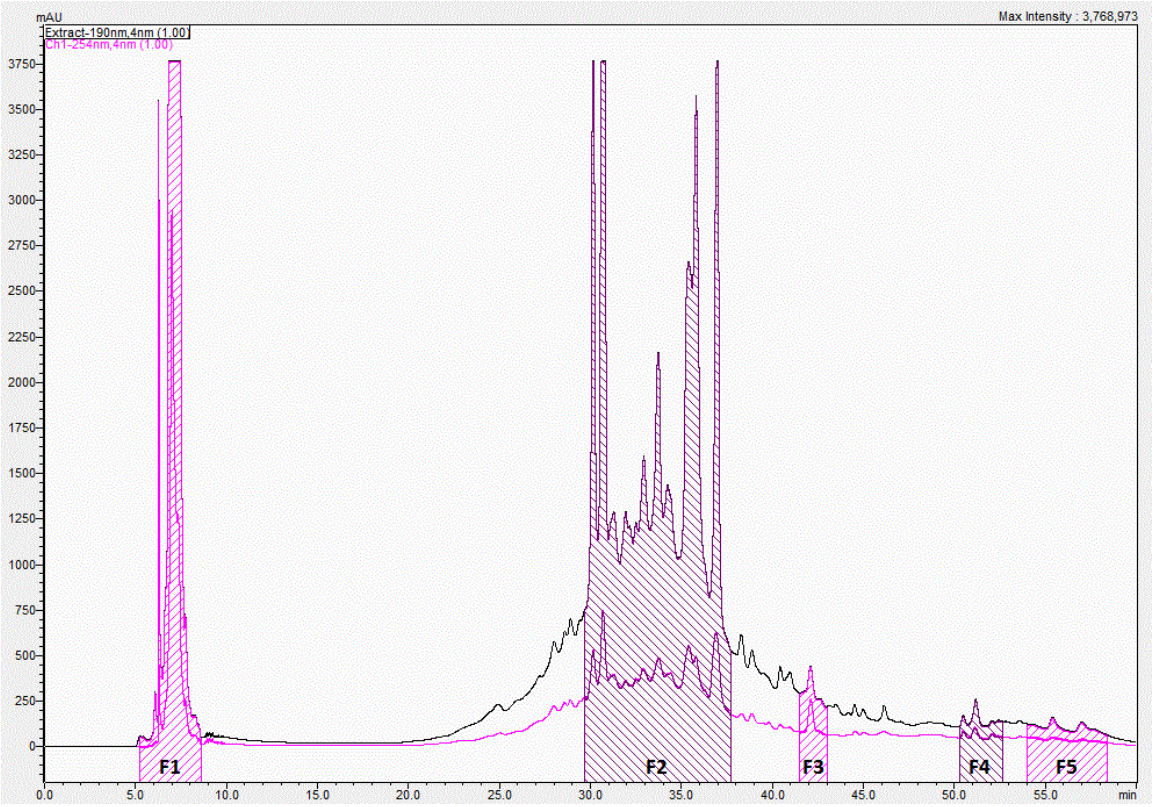
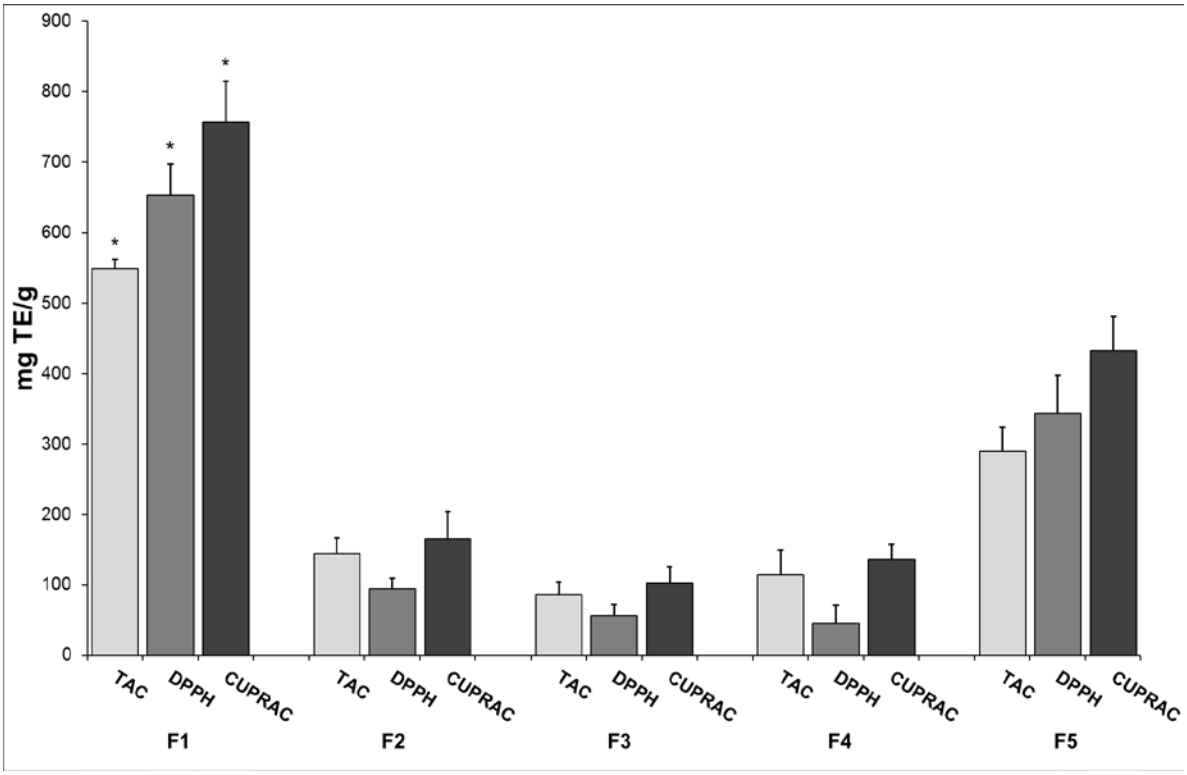


Fig. 1 Reversed Phase HPLC fractionation of aqueous crude extract of *Eucalyptus microcorys*



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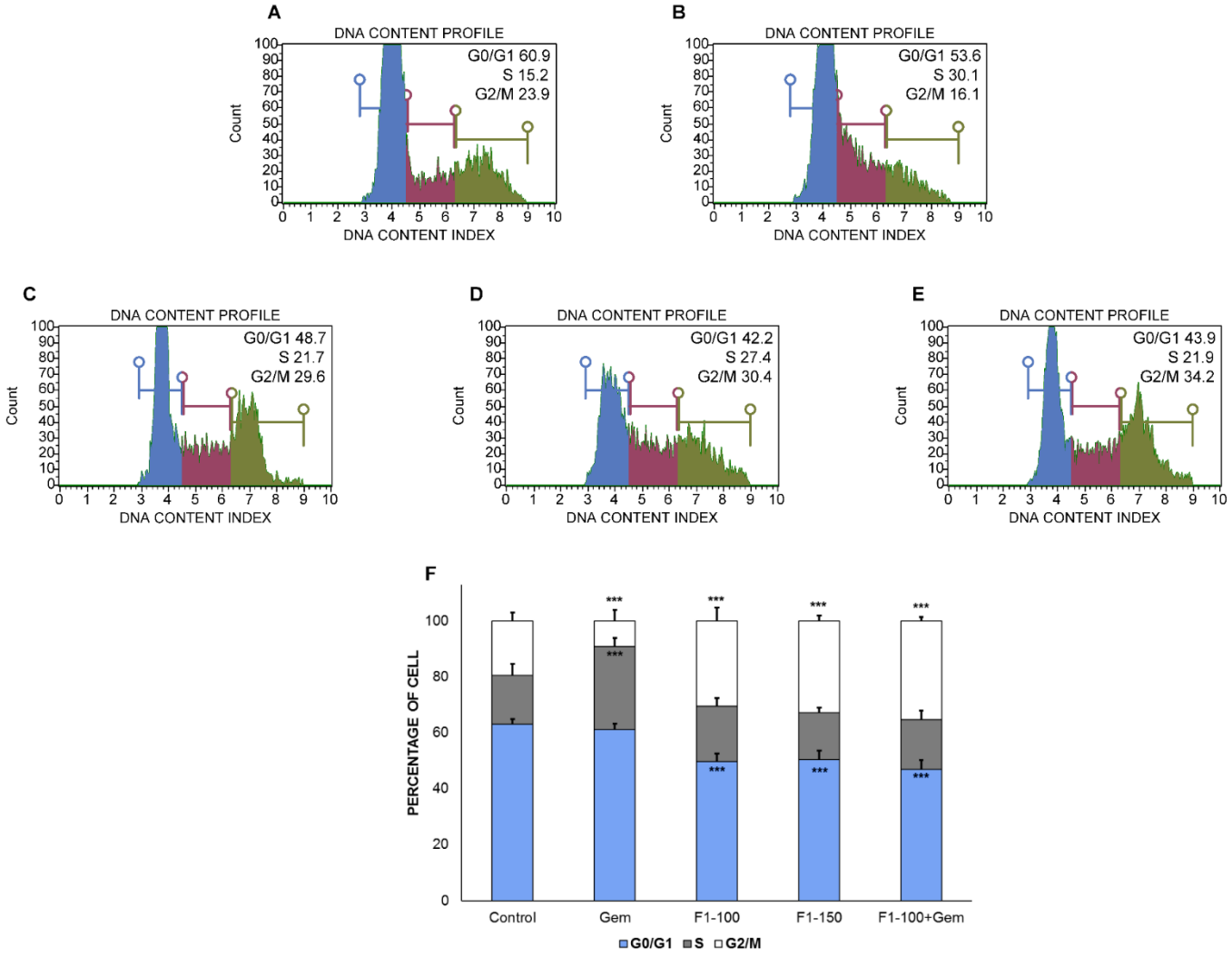

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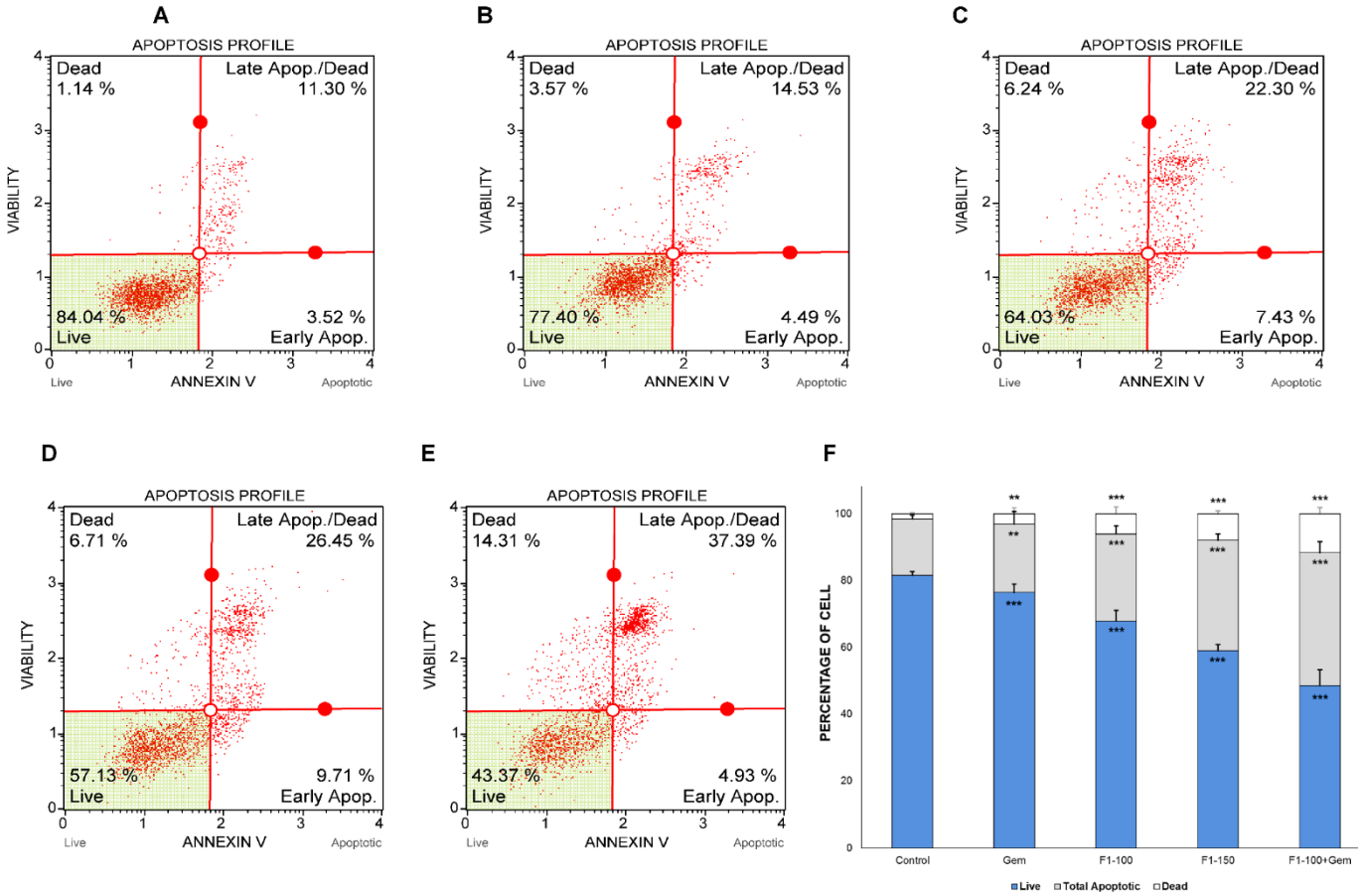

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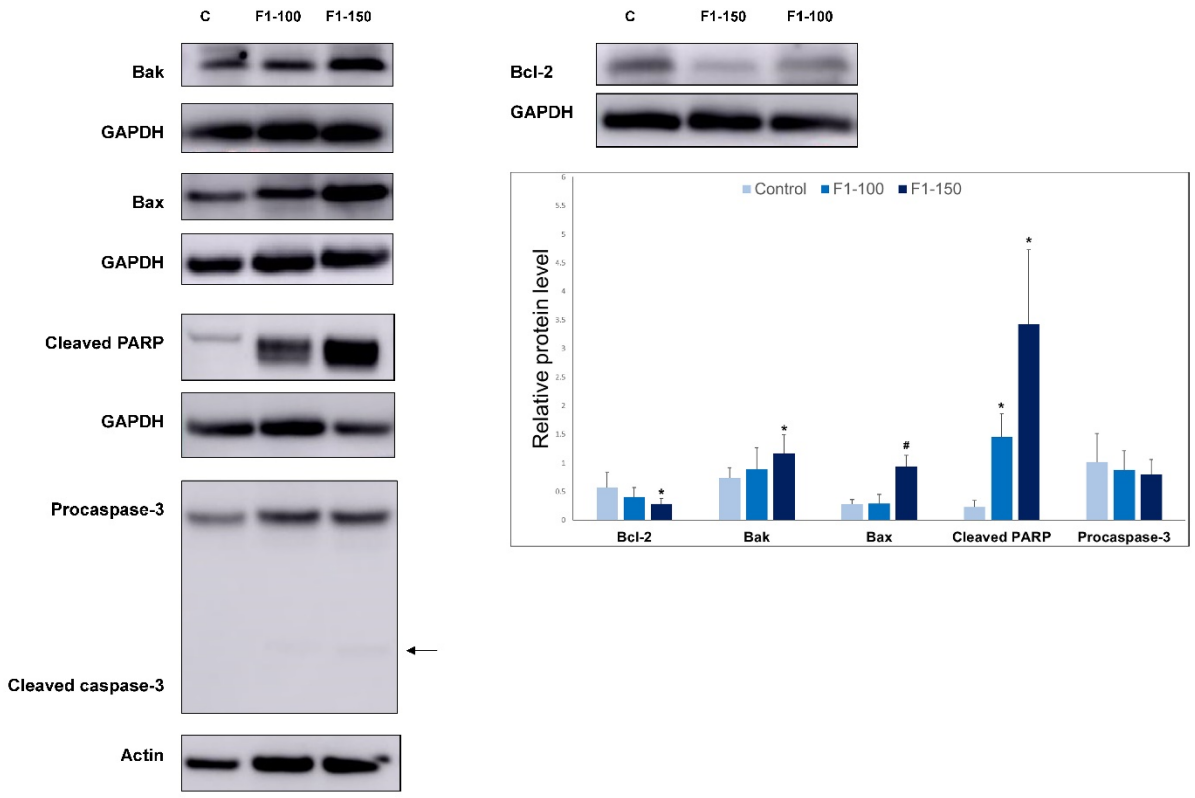

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Cell line	Type	µg/mL	F1	F2	F3	F4	F5	CF*	Gemcitabine (50nM)
MIA PaCa-2	Pancreas	200	$95.13 \pm 0.06_a$	$32.23 \pm 15.50_b$	$14.90 \pm 3.94_c$	$18.30 \pm 11.76_{bc}$	$55.32 \pm 4.08_d$	$98.41 \pm 0.13_e$	
		100	$58.34 \pm 1.58_a$	$28.00 \pm 4.66_b$	$3.08 \pm 2.63_c$	$14.58 \pm 9.75_d$	$13.96 \pm 6.75_d$	$79.42 \pm 2.31_e$	90.76 ± 1.27
		50	$9.93 \pm 0.77_a$ $IC_{50} = 93.11 \pm 3.43$ µg/mL	$13.37 \pm 8.15_{ab}$	$2.83 \pm 2.48_c$	$7.83 \pm 4.94_{acd}$	$11.96 \pm 2.08_{abd}$	$36.34 \pm 2.56_e$	
BxPC-3	Pancreas	200	$45.03 \pm 9.41_a$	$30.60 \pm 9.63_b$	$25.03 \pm 4.30_b$	$6.41 \pm 4.40_c$	$24.60 \pm 5.52_b$	$49.43 \pm 3.56_a$	
		100	$29.35 \pm 10.53_a$	$22.25 \pm 12.07_a$	$11.91 \pm 4.56_b$	$0.81 \pm 0.68_c$	$4.75 \pm 3.57_{bc}$	$30.89 \pm 2.43_a$	75.53 ± 2.97
		50	$18.80 \pm 5.20_a$	$10.35 \pm 9.11_b$	$4.60 \pm 4.37_b$	$0.66 \pm 0.54_c$	$2.20 \pm 1.41_b$	$20.14 \pm 1.60_a$	
CFPAC-1	Pancreas	200	$22.71 \pm 4.94_a$	$24.77 \pm 10.36_{ab}$	$16.14 \pm 5.53_a$	$7.62 \pm 3.20_c$	$4.37 \pm 2.60_c$	$37.14 \pm 1.37_d$	
		100	$9.73 \pm 2.00_a$	$10.21 \pm 7.51_{ab}$	$11.88 \pm 5.01_{ab}$	$5.79 \pm 5.10_{abc}$	$2.74 \pm 1.93_c$	$19.23 \pm 1.45_d$	68.25 ± 1.95
		50	$7.06 \pm 4.02_a$	$5.80 \pm 4.85_{ab}$	$10.90 \pm 4.86_a$	$4.52 \pm 2.91_{ab}$	$1.99 \pm 1.94_b$	$12.34 \pm 2.64_a$	
HPDE	Pancreas (Normal)	200	$94.02 \pm 0.25_a$	$91.00 \pm 0.90_b$	$92.60 \pm 0.38_c$	$91.70 \pm 0.76_d$	$98.45 \pm 0.12_e$	$98.45 \pm 0.12_e$	
		100	$93.80 \pm 0.32_a$	$87.00 \pm 1.70_b$	$64.63 \pm 8.70_c$	$93.23 \pm 0.74_a$	$95.53 \pm 0.52_d$	$95.53 \pm 0.52_d$	93.96 ± 0.24
		50	$93.07 \pm 0.26_a$ $IC_{50} = 22.55 \pm 4.84$ µg/mL	$30.53 \pm 6.67_b$	$7.17 \pm 2.55_c$	$93.40 \pm 0.35_a$	$91.27 \pm 0.63_d$	$91.27 \pm 0.63_d$	

All cell growth inhibition (%) values are means ± standard deviations. (*) Combination of fractions F1 – F5.

$_{a,b,c,d,e}$ Values in the same row not having the same subscript letter are significantly different ($p < 0.05$) from each other.

Table 2. Tentative identification of major constituents of fraction-1 of *E. microcorys* and corresponding MS/MS fragmentation profiles

N	t^R (min)	$(M+2H)^{2+}$	MH+	MW	Main MS^2 fragments	Predicted formula [a]	Tentative Identification (ID) [b]
1	26.64	340.25929	679.51123	678.50329	-78.04, -40.03, -26.01, 0, 13.97, 36.00, 56.02, 92.062, 106.07, 124.08, 142.09, 152.08, 170.09, 188.10, 206.18	$C_{11}H_{25}O_3$ (100% match)	No ID
2	26.69	396.8014	792.5956	791.5879	96.08, 114.091, 209.16, 277.10, 322.24, 387.79, 435.33, 566.42 , 679.51	$C_{32}H_{62}N_{12}O_4$ (15.67% match)	No ID
3	27.23	453.34303	905.67901	904.67107	114.09, 228.16, 245.19, 341.24, 444.34, 565.44, 679.51	$C_{36}H_{66}N_6O_6$ (83.31% match)	No ID
4	30.26	--	203.12779	202.11985	55.05, 67.05, 83.09, 93.07, 97.10, 107.09, 111.08,, 125.10, 135.08, 143.11, 153.09, 157.12, 171.10, 185.12 203.13	$C_{10}H_{18}O_4$ (100% match)	Monomethyl azelate; Dimethyl suberate
5	30.93	--	189.14855	188.14061	55.05, 59.05, 67.05, 69.07, 81.07, 83.09, 95.09, 97.10, 107.09, 111.04, 121.10, 125.10, 129.05, 139.11, 143.07 149.02, 157.09, 161.08, 175.10, 189.13	$C_{13}H_{16}O$ (100% match)	2-Benzylidenehexanal; Corylifolin; Benzoylcyclohexane
6	32.23	--	192.13836	191.13042	59.05, 100.08, 119.05, 133.10, 151.11, 164.10, 173.99, 192.14	$C_{12}H_{17}NO$ (100% match)	Delphene; N,N-Diethyl-2-phenylacetamide
7	32.68	--	196.16965	195.16171	159.065, 185.12, 196.17, 217.11, 236.11, 279.16, 327.01, 376.34, 419.31	$C_{12}H_{21}NO$ (100% match)	(2E,4E)-N-isobutylocta-2,4-dienamide
8	34.11	--	153.12739	152.1201	59.05, 81.07, 69.07, 93.07, 107.09, 109.10, 111.08, 135.12, 153.13	$C_{10}H_{16}O$ (100% match)	Myrcen-8-ol; Citral; α-Pinene-2-oxide; Pulegone
9	34.75	--	223.09649	222.08855	149.02, 177.05, 205.16, 225.043	$C_{12}H_{14}O_4$ (100% match)	3,5-Diphenyl-1,2,4-oxadiazole
10	34.92	--	404.20654	387.17987	69.034, 93.070, 105.07, 121.06, 129.05, 147.06, 267.12, 387.18	$C_{22}H_{26}O_6$ (99.6% match)	No ID
11	34.92	--	387.17992	386.17198	95.05, 119.05, 131.07, 267.12, 387.14	$C_{21}H_{22}O_7$ (96.09% match)	Peucenidin; Edultin, Isosamidin
12	35.61	--	234.16978	217.14334	97.10, 125.09, 135.08, 153.09, 203.13, 217.11, 235.17	$C_{15}H_{22}O_2$ (100% match)	No ID
13	35.61	--	217.14334	216.1354	69.07, 89.06, 101.06, 111.04, 129.05, 155.07, 173.08, 199.09, 217.11	$C_{10}H_{16}O_5$ (100% match)	Tuckolide, Decarestrictine O

t^R= Retention time, [a,b] Tentative identifications were achieved t^R alignment, unknown compound detection, extraction ion chromatogram generation and integration, prediction of elemental composition and identification against ChemSpider databases or database searching of MS/MS fragment data against mzCloud). Manual peak detection, elemental composition and isotope pattern matching were also performed using CSI:FingerId and Thermo Xcalibur Qual Browser.

Table 3. The similarity between the molecular weights (MW) of fraction-1 constituents and the monoisotopic masses of previously reported phenolic compounds in Eucalyptus species including E. microcorys. Antipancreatic cancer activity of these phenolic compounds reported in the literature.

Phenolic compounds previously reported in eucalypts	Monoisotopic mass (g/mol)	Reported in	References	MW observed in this study (g/mol)	Antipancreatic cancer activity
Kaempferol	286.047	*E. microcorys*	[57]	286.21441	[59, 60]
Quercetin	302.043	*E. microcorys*		302.2456, 302.24587	[61 - 66]
Ellagic Acid	302.006	*E. microcorys*		302.20914	[67 - 69]
3-O-galloyl-beta-d-glucose	332.074	*E. microcorys*		332.25627, 332.25625	--
4,6-hexahydroxydiphenoyl-β-D-glucose	482.070	*E. microcorys*		482.34502	--
Gemin D	634.081	*E. microcorys*		634.37766	
1,2,3,4,6-penta-O-galloyl-β-D-glucose	940.118	*E. microcorys*		940.66478	--
Apigenin	270.053	*E. globulus*	[70 - 72]	270.18311	[34, 73 – 77]
Naringenin	272.068	*E. camaldulensis, E. rudis, E. globulus*	[70, 71, 78 - 80]	272.19876	[81]
Epicatechin	290.079	*E. grandis, E. urograndis*[a], *E. globulus*	[82, 83]	290.92827	[84 - 86]
Myricetin	318.037	*E. rostrata*	[87]	318.2193	[88]

[a] *E. urograndis = Eucalyptus grandis x E. urophylla* hybrid

CHAPTER 6

Conclusions and future directions

6.1 Conclusions

At the inception of this PhD research project, there were many unknown aspects related to the phytochemical profile and bioactivity of Australian eucalypts. While significant research efforts have focused on the characterisation of the phytochemical profile and activity of eucalypt essential oils, information pertaining to the development and optimisation of methods to extract more structurally complex, non-volatile bioactives using environmentally friendly solvents like water, and the efficacy of these extracts and compounds against PC was absent from the literature. Therefore, the major aims of this thesis were to optimise the extraction techniques and parameters for preparing crude extracts rich in phenolic compounds and antioxidants from eight different species of Australian eucalypts; to investigate the antiproliferative activity of crude extracts against PC cells; and to explore the bioactive compounds present in the extracts for their potential use as future novel antipancreatic cancer drugs. In this project, we wanted to focus on the phenolic and antioxidant contents of eucalypts and their potential relevance in the development of therapeutic strategies against PC.

The seven studies presented in this thesis were devised to address various research goals including determining optimal extraction conditions to prepare phenolic and antioxidant rich crude extracts from eucalypt biomass, investigating their cytotoxic activity against PC cell lines and assessing the antimicrobial activity of the extracts. The three studies presented in Chapter 2 were dedicated to the optimisation of various parameters for extracting phenolic compounds and antioxidants from different *Eucalyptus* species using conventional as well as advanced and green extraction techniques such as MAE and UAE. These studies demonstrated that conventional extraction using water was the most efficient and easiest method for extracting phenolics and antioxidants from *Eucalyptus* species. The different temperatures for drying the eucalypt leaves and the preparation of extracts were based on the previously optimised methods for total phenolic extraction from *E. robusta* (Vuong et al., 2015b). However, the potential detrimental effects of high temperature on the bioactive compounds present in eucalypt species cannot be ruled out.

The fourth and fifth studies in Chapter 3 distinguished the phenolic-rich crude extracts from eight different species representing the three main genera of eucalypts based on their growth inhibition activity against PC cell lines. The results of these studies demonstrated that *A. floribunda*, *A. hispida,* and *E. microcorys* crude extracts were the most potent among the tested species and exhibited statistically similar antiproliferative activity against MIA PaCa-2 cells. Furthermore, *E. microcorys* extracts exerted significantly more cytotoxicity against glioblastoma, neuroblastoma and lung cancer cells compared to the other extracts. Due to the pronounced cytotoxicity against a greater number of cancer cell lines (including MIA PaCa-2), the aqueous *E. microcorys* extract was selected as the candidate for further investigation. To the best of our knowledge, these were the first studies to provide comprehensive information regarding the *in vitro* antiproliferative activity of water-based extracts derived from all three genera of eucalypt against PC cells. Previous studies on eucalypts were mostly directed at investigations of the bioactivity of their essential oils and volatile compounds (Bardaweel et al., 2014; He et al., 2015; Islam et al., 2015; Pereira, 2013; Topçu et al., 2011; Tyagi and Malik, 2011; Vilela et al., 2009; Zhang et al., 2014; Zhi Long et al., 2011; Zhou et al., 2003) with just one study reporting the cytotoxicity of *E. robusta* aqueous crude extract against PC cells (Vuong et al., 2015b).

The antioxidant, antifungal and antibacterial properties of the aqueous *E. microcorys* crude extract were the highlights of the sixth study presented in Chapter 4. The prominent antimicrobial effects imparted by the extract may be ascribed to the high amount of phenolics and saponins present in the extract. The findings of this study provided the basis for potential future application of *E. microcorys* aqueous extract as the source of antioxidant and antimicrobial agents in the food, nutraceutical and pharmaceutical industries. However, further refinement studies are required to isolate and characterise bioactive compounds and determine their mode of action against the fungal and bacterial pathogens.

The seventh study presented in Chapter 5 describes the bioassay-guided fractionation of aqueous *E. microcorys* leaf extract by semi-preparative chromatography to delineate the polyphenol-rich fractions based on their bioactivity against PC cell lines as well as their

antioxidant properties. In this study, the extract was divided into five reversed-phase HPLC fractions, of which, the first fraction exhibited significantly greater antioxidant and antiproliferative activity, especially against the MIA PaCa-2 PC cells. This pronounced antiproliferative activity was linked with the induction of apoptosis and abrogation of cell cycle checkpoint in MIA PaCa-2 cells. Western blot analysis also revealed that the antiapoptotic protein Bcl-2 and proapoptotic proteins Bak and Bax as well as cleaved PARP, cleaved caspase and procaspase-3 in MIA PaCa-2 cells were significantly impacted after the treatment with the active fraction. Interestingly, a significant increase in apoptosis and accumulation of MIA PaCa-2 cells in G2/M phase occurred when cells were treated with a combination of active fraction (F1) and gemcitabine when compared to individual treatments. This could be explained by a potential additive/synergistic effect between gemcitabine and the bioactive constituents of the fraction on the PC cells. Due to the emergence of drug resistance, the combination of gemcitabine with phenolic compounds has been demonstrated to be a more effective therapeutic approach than gemcitabine alone (Lee et al., 2008; Pandita et al., 2014; Yoshida et al., 2017). For instance, apigenin, a phenolic flavonoid also reported from eucalypts, was shown to induce more growth inhibition and apoptosis both *in vitro* (MiaPaCa-2 and AsPC-1 pancreatic cancer cells) and *in vivo* (MiaPaCa-2 subcutaneous xenograft model) by down-regulating NF-*κ*B activity with suppression of Akt activation when combined with gemcitabine (Lee et al., 2008). Similarly, curcumin, another polyphenol, was found to sensitize chemoresistant PC cells to gemcitabine and reduced the tumour growth by attenuating Polycomb Repressive Complex 2 subunit Enhancer of Zeste Homolog-2, and the lncRNA PVT1 expression (Yoshida et al., 2017). For exploring the possibility of developing combinational strategies against PC, further studies are warranted to understand the molecular interaction of gemcitabine with the compounds present in the polyphenol-rich fraction of *E. microcorys* aqueous extract.

Untargeted metabolomics in natural product research enables the systematic study of complex mixtures of plant extracts facilitating the correlation between the specific compounds and the observed bioactivity (Nguyen, 2016). Therefore, untargeted metabolomics using LC-

MS was implemented in our study and tentative identities of the phytochemical constituents of the active fraction were obtained. These results revealed polyphenolics as the major class of compounds in the fraction, of which several have previously been described to possess antipancreatic cancer activity. Interestingly, most of these compounds have also formerly been reported in *Eucalyptus* species including *E. microcorys*. Untargeted metabolomics of extracts has the problem of providing numerous compound identities for a single mass when database searching and matching based on accurate mass data is performed (Nguyen, 2016; Shyur et al., 2013). Our study also faced this common challenge upon direct comparison of chromatographic and tandem mass spectral data, as multiple "matches" were obtained from the databases for each mass and its corresponding MS^2 fragments. Isolation of a solitary compound responsible for its cytotoxicity against PC cells was not achieved in this study due to the phytochemical complexity of the fraction *i.e.* a number of compounds exerted potential additive/synergistic activity against the cells rather than a single compound alone. This also explains why a combination of all five fractions from *E. microcorys* displayed greater antiproliferative effects on MIA PaCa-2, CFPAC-1 and HPDE cell lines than the individual fractions in this study. The complexity of metabolites and their varied actions impart some of the biggest challenges in natural product research in terms of isolating individual compounds from extracts (Hegeman, 2010; Nguyen, 2016; Shyur et al., 2013; Wolfender et al., 2015). Therefore, confirming the tentative identities using reference compounds and elucidating the structures of individual compounds in the active fraction with NMR should be one of the future directions of this research.

6.2 Future directions

The aim of the work embodied by this thesis was to investigate the relevance of Australian eucalypts as the source of novel bioactive compounds for developing therapeutic strategies against PC. Our studies have shown that eucalypts are indeed promising sources of phytochemicals with antipancreatic, antioxidant and antimicrobial properties. The finding of

these studies have emphasized that this area warrants further investigations and has produced several interesting ideas for future research.

1. This thesis investigated the cytotoxicity of ethanolic and aqueous extracts of *Eucalyptus* species, which predominantly contain polar and medium polar non-volatile compounds against different types of cancer cells, including PC. Therefore, it would be of interest to study the eucalypt extracts prepared with non-polar solvents like hexane and chloroform in terms of their cytotoxicity on cancerous and normal cell lines. However, concerns related to the toxicity of these solvents on the cells should also be taken into account. Essential oils derived from eucalypts containing different volatile compounds can also be assessed against different PC cell lines. In addition, studies evaluating the antiproliferative activity of extracts prepared from other parts of eucalypt plants including bark, resin and fruit would provide a deeper understanding about the potential of eucalypts as sources of novel anticancer agents. Seasonal variations of eucalypt phytochemical constituents and their corresponding effect on cytotoxicity would also be an interesting and important aspect to examine.
2. Our studies have found *A. floribunda, A. hispida, E. microcorys* crude extracts to be equally effective against PC cells. However, out of the three only *E. microcorys* crude extracts were investigated against other cancer cells due to time constraints, and it would be worthwhile to evaluate the two *Angophora* species against a panel of cancer cells. Furthermore, studies deciphering the molecular mechanisms of actions driving the effect in glioblastoma, neuroblastoma and lung cancer cells by *E. microcorys* crude extracts could also be performed.
3. Studies to examine the effect of the *E. microcorys* active fraction, and its bioactive compounds, on the key drivers of PC including COX-2, KRAS, ALDH1, HIF-1α, GLUT-1, VEGF, glycogen synthase kinase-3β, geminin, focal adhesion kinase, vimentin, Twist 2 and proteins involved in the PI3K/Akt and TGF-β1/Smad3 signalling pathways should also be carried out. Synergistic effects of the polyphenol-rich eucalypt fraction and

gemcitabine on cyclins and CDKs, Rb, phospho-Rb, p21 and p27 should be further investigated to better understand the mechanisms of cell cycle abrogation in MIA PaCa-2 cells. Moreover, how this combination impacts the cell cycle and p53 levels in other PC cell lines carrying wild type *TP53* such as the SW1990 cells and the involvement of extrinsic pathways (activation of caspase-8) of apoptosis should also be considered for future research.

4. Further efforts should be made to conduct *in vivo* studies in order to determine whether similar effects are observed in xenograft animal models as well as to assess the bioavailability and toxicity of the polyphenol-rich eucalypt fraction.
5. Finally, the active fraction could be subjected to structural elucidation for confirming the tentative identities of its phytochemical constituents by using NMR and X-ray crystallography. Characterizing the whole *E. microcorys* aqueous crude extract to formulate a standard preparation containing all its active ingredients and subjecting it to more *in vitro* and *in vivo* experiments may reveal insights into the mechanisms involved in their synergistic effects against PC.

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Appendices

Appendix 1: Additional publications

Book chapter

Deep Jyoti Bhuyan and Amrita Basu. Phenolic compounds: Potential health benefits and toxicity. *In: Utilization of bioactive compounds from agricultural and food waste.* Edited by Quan V. Vuong. ISBN: 9781498741316. **CRC Press, Taylor & Francis Group (2017).**

Additional Research paper 1

Quan V. Vuong, Chloe D. Goldsmith, Trung Thanh Dang, Van Tang Nguyen, **Deep Jyoti Bhuyan**, Elham Sadeqzadeh, Christopher J Scarlett, Michael C Bowyer: Optimisation of Ultrasound-Assisted Extraction Conditions for Phenolic Content and Antioxidant Capacity from *Euphorbia tirucalli* Using Response Surface Methodology. **Antioxidants (2014),** 3(3): 604-617.

Antioxidants **2014**, *3*, 604-617; doi:10.3390/antiox3030604

OPEN ACCESS

antioxidants

ISSN 2076-3921

www.mdpi.com/journal/antioxidants

Article

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

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Received: 8 July 2014; in revised form: 20 August 2014 / Accepted: 25 August 2014 / Published: 17 September 2014

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.

Keywords: antioxidant; *Euphorbia tirucalli*; ultrasonic-assisted extraction; optimization; phenolic compounds; response surface methodology

1. Introduction

Euphorbia tirucalli (*E. tirucalli*) is native to Madagascar and Africa, but it is now widespread around the world because of its tolerance to a wide range of climatic conditions [1,2]. *E. tirucalli* has been used as folk medicines 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 [3–5]. Recently, it has been linked to other benefits including hepatoprotective, antimicrobial, antioxidant, insecticidal, larvicidal, molluscicide and antiarthritic activities [6], which have resulted in significant scientific interest in the phytochemical profile of the plant [1].

Phenolic and terpenoid compounds have been identified in the phytochemical profile of *E. tirucalli*, and extracts have been shown to possess potent antioxidant properties [7,8]. Phenolic compounds are major bioactive compounds in medicinal plants and have been reported as powerful antioxidants and health promoters. The phenolic profile and antioxidant properties of *E. tirucalli* have been reported in a previous study, which found that acetone extracts yielded a higher concentration of phenolics than more polar solvent systems (80% aqueous methanol) [8]. However, optimal extraction conditions were not investigated and further study is required to fully characterise the phenolic profile.

Ultrasonic-assisted extraction (UAE) has been shown to be a fast and effective technique for extracting phytochemicals from plant materials that is easily up-scaleable [9]. Ultrasonic parameters such as temperature, time and power have been reported to exert significant impact on the extraction yield of plant phytochemicals and antioxidants [10–12]. A mixture of ethyl acetate: ethanol (4:1 v/v) was reported to efficiently extract triterpenoids from the root of *Euphorbia pekinensis* Rupr [13]. Therefore, this solvent mixture was used in this study and it was hypothesised that UAE temperature, time and power had significant effects on extraction efficiency of total phenolic compounds (TPC) and total antioxidant capacity (TAC) of the extracts from *E. tirucalli* leaf and the optimal extraction conditions could be established using response surface methodology (RSM).

The current study aimed to optimise the UAE parameters of temperature, time and power for the extraction of phenolics from *E. tirucalli*. These optimal conditions can be applied for further isolation and purification of phenolic compounds from *E. tirucalli*.

2. Experimental Section

2.1. Plant Materials

The leaf of the *Euphorbia tirucalli* tree (phylloclades) was collected on July 16, 2013 from a property located in Saratoga, NSW, Australia (33.47° S, 151.35° E). The leaf was then immediately transferred to the laboratory and stored at −20 °C to minimise phenolic degradation. Before commencing experiments, the leaf was immersed in liquid nitrogen, then particulated using a commercial blender. The fresh ground leaf was then stored at −20 °C until required.

2.2. Ultrasound-Assisted Extraction (UAE)

A mixture of ethyl acetate: ethanol 4:1 (v/v) was utilized as the solvent system for optimisation of UAE conditions and was applied at a solvent-to-sample ratio of 100:32 mL/g of fresh leaf weight. The ground *E. tirucalli* leaf was placed in the extraction chamber, and filled with extraction solvent. The extraction chamber was completely immersed into an ultrasonic bath (Soniclean, 220 V, 50 Hz and 250 W, Soniclean Pty Ltd., Thebarton, Australia) with pre-set conditions for temperature, time and power as designed by response surface methodology software. When the ultrasonic extraction was completed, the extracts were immediately cooled on ice to room temperature, filtered using a 5 mL syringe fitted with a 0.45 μm cellulose syringe filter (Phenomenex Australia Pty. Ltd., Lane Cove, Australia) and diluted to the required volume for quantitative analysis.

2.3. Response Surface Methodology (RSM)

A response surface methodology (RSM) approach with a Box-Behnken design was employed to design experimental conditions to investigate the influence of the three independent ultrasonic parameters: temperature (30, 45, and 60 °C), time (30, 45, and 60 min) and power (60%, 80%, and 100% or 150, 200, and 250 W).

The independent variables and their code variable levels are shown in Table 1. To express the level of total phenolic compounds (TPC) and total antioxidant capacity (TAC) as a function of the independent variables, a second-order polynomial equation was used as follows [14]:

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \sum_{i=1}^{k} \beta_{ii} X_i^2 \quad (1)$$

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

The three independent ultrasonic parameters were assigned as; X_1 (temperature, °C), X_2 (time, min.) and X_3 (power, %). Thus, the function containing these three independent variables is expressed as follows:

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

2.4. Determination of Total Phenolic Content (TPC)

The extract was diluted 20× to fit within the optimal absorbance range for colorimetric assessment of total phenolic compounds (TPC), which was determined according to the method of Vuong *et al*. [15]. Gallic acid was used as the standard for the construction of a calibration curve, with the results expressed as mg of gallic acid equivalents per gram of fresh weight (FW) (mg GAE/g).

Table 1. Box-Behnken design and observed responses

Run	Ultrasonic Conditions			Experimental Values (n = 3)			
					Antioxidant Capacity		
	Temperature (°C)	Time (min)	Power (%) *	TPC (mg GAE/g)	ABTS (%)	DPPH (%)	CUPRAC (mM TE/g)
1	30	30	80	2.99	42.81	17.80	37.07
2	30	60	60	2.05	56.31	21.94	42.74
3	30	60	100	2.40	50.20	19.22	41.01
4	30	90	80	2.34	52.59	18.12	36.61
5	45	30	60	2.20	74.88	25.17	56.66
6	45	30	100	2.99	74.01	27.32	67.63
7	45	60	80	2.71	66.07	30.25	55.67
8	45	60	80	3.11	46.91	18.58	38.56
9	45	60	80	3.51	74.52	25.17	66.72
10	45	90	60	2.63	64.23	23.43	46.81
11	45	90	100	3.34	74.27	30.28	67.00
12	60	30	80	2.05	74.52	20.90	71.70
13	60	60	60	2.39	53.82	47.46	51.95
14	60	60	100	2.83	69.13	43.27	57.17
15	60	90	80	3.12	62.22	39.66	48.13

* 60%, 80% and 100% power were equivalent to 150, 200 and 250 W.

2.5. Determination of Antioxidant Capacity

ABTS total antioxidant capacity: The extract was diluted 40× to fit within the optimal absorbance range for colorimetric assessment. Total antioxidant capacity (TAC) was measured using 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay as described by Thaipong *et al*. [16]. Results were expressed as percentage of inhibition and were calculated using the formula:

$$\text{TAC (\%)} = (Abs_{control} - Abs_{sample}) \times 100/Abs_{control} \quad (3)$$

where $Abs_{control}$ = control absorbance and Abs_{sample} = sample absorbance.

Free radical scavenging capacity: The extract was diluted 40× and analyzed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as described by Vuong *et al*. [15] with results expressed as percentage of inhibition, calculated according to Equation 3.

Cupric reducing antioxidant capacity (CUPRAC): The extract was diluted 40× and its iron chelating capacity analyzed using cupric ion reducing antioxidant capacity (CUPRAC) assay as described by Apak *et al*. [17]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the calibration standard, with results expressed as mM of trolox equivalents per g of fresh weight (mM TE/g).

2.6. Statistical Analysis

RSM experimental design and analysis were conducted using JMP software (Version 11, SAS, Cary, NC, USA). The software was also used to establish the model equation to graph the 3D- and 2D-contour plots of variable responses, and to predict optimum values for the three response variables. The Student's *T*-test (conducted using the SPSS statistical software version 20, IBM, Armonk, NY, USA) was used for

comparison of the means analysis. Differences between the mean levels in the different experiments were taken to be statistically significant at $p < 0.05$.

3. Results and Discussion

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

It is necessary to test the reliability of the RSM mathematical model in predicting optimal variances and accurately representing the real interrelationships between the selected parameters. Therefore, fitting of the models for total phenolic content and antioxidant capacity of the euphorbia extracts was undertaken. Results of analysis of variances of the Box-Behnken design are shown in Figure 1 and Table 2. Figure 1 revealed a correlation between the predicted and experimental values, while Table 2 presents the summary of variance analysis.

Table 2. Analysis of variance for determination of model fitting.

Sources of Variation	TPC	Antioxidant Capacity		
		ABTS	DPPH	CUPRAC
Lack of fit	0.77	0.0007 *	0.13	0.0025 *
R^2	0.83	0.84	0.88	0.87
Adjusted R^2	0.53	0.54	0.67	0.65
PRESS	3.73	4661.69	2121.07	4042.87
F Ratio of Model	2.73	2.81	4.23	3.88
***p* of Model > F**	0.14	0.13	0.06	0.07

* Significantly difference with $p < 0.05$.

Figure 1A showed that the model TPC outputs did not differ significantly from the experimental values ($p > 0.05$). Furthermore, the coefficient of determination (R^2) of the model was 0.83, indicating that 83% of the experimental data can be predictively matched against the model data for TPC. Table 2 also showed that the p value for "lack of fit" was 0.77, indicating that the lack of fit was not significant ($p > 0.05$). In addition, the Predicted Residual Sum of Square (PRESS) for the model (a measure of how well the predictive model fits each point in the design) [12], was 3.73 and the F value of the model was 2.73, further revealing that the mathematical model was adequate for prediction of TPC and was fitted to the following second-order polynomial formula:

$$Y_{TPC} = 3.1067 + 0.0754X_1 + 0.1503X_2 + 0.2863X_3 + 0.4284X_1X_2 + 0.0224X_1X_3 - 0.0157X_2X_3 - 0.4273X_1^2 - 0.0556X_2^2 - 0.2621X_3^2 \quad (4)$$

Fitting of the models for three different antioxidant properties including total antioxidant capacity (ABTS), DPPH free radical scavenging capacity and cupric reducing antioxidant capacity (CUPRAC) were also tested. The results (Figure 1B–D) revealed p values for ABTS, DPPH and CUPRAC of 0.13, 0.06 and 0.07, respectively, indicating that there was no significant difference between the predicted values and experimental values ($p > 0.05$). Coefficient of determination (R^2) for the ABTS, DPPH and CUPRAC models (Table 2) were determined to be 0.84, 0.88 and 0.87, respectively, revealing a close correlation between the predicted values and experimental values, with at least 84% of data matching.

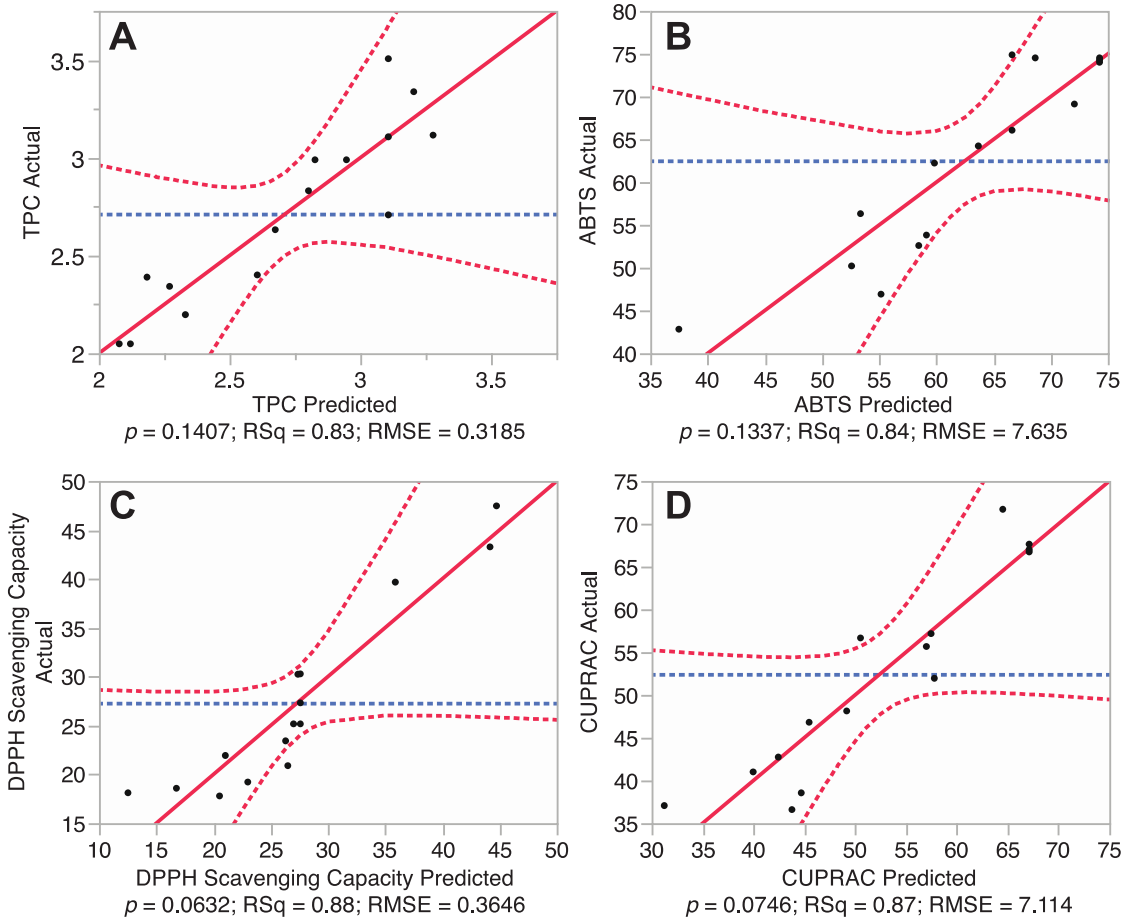
PRESS values (4661, 2121 and 4042) and F values (2.81, 4.23 and 3.88) revealed that these mathematical models were reliable predictors of antioxidant capacity and could be fitted to the following second-order polynomial formulas:

$$Y_{ABTS} = 74.27 + 7.22X_1 + 2.13X_2 + 3.59X_3 - 0.42X_1X_2 - 3.95X_1X_3 - 2.13X_2X_3 - 10.94X_1^2 - 0.19X_2^2 - 11.06X_3^2 \quad (5)$$

$$Y_{ABTS} = 74.27 + 7.22X_1 + 2.13X_2 + 3.59X_3 - 0.42X_1X_2 - 3.95X_1X_3 - 2.13X_2X_3 - 10.94X_1^2 - 0.19X_2^2 - 11.06X_3^2 \quad (6)$$

$$Y_{ABTS} = 74.27 + 7.22X_1 + 2.13X_2 + 3.59X_3 - 0.42X_1X_2 - 3.95X_1X_3 - 2.13X_2X_3 - 10.94X_1^2 - 0.19X_2^2 - 11.06X_3^2 \quad (7)$$

Figure 1. Correlations between predicted and experimental total phenolic content and antioxidant capacity. (**A**) Phenolic content; (**B**) 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) antioxidant capacity; (**C**) 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging capacity; and (**D**) Cupric reducing antioxidant power (CUPRAC).

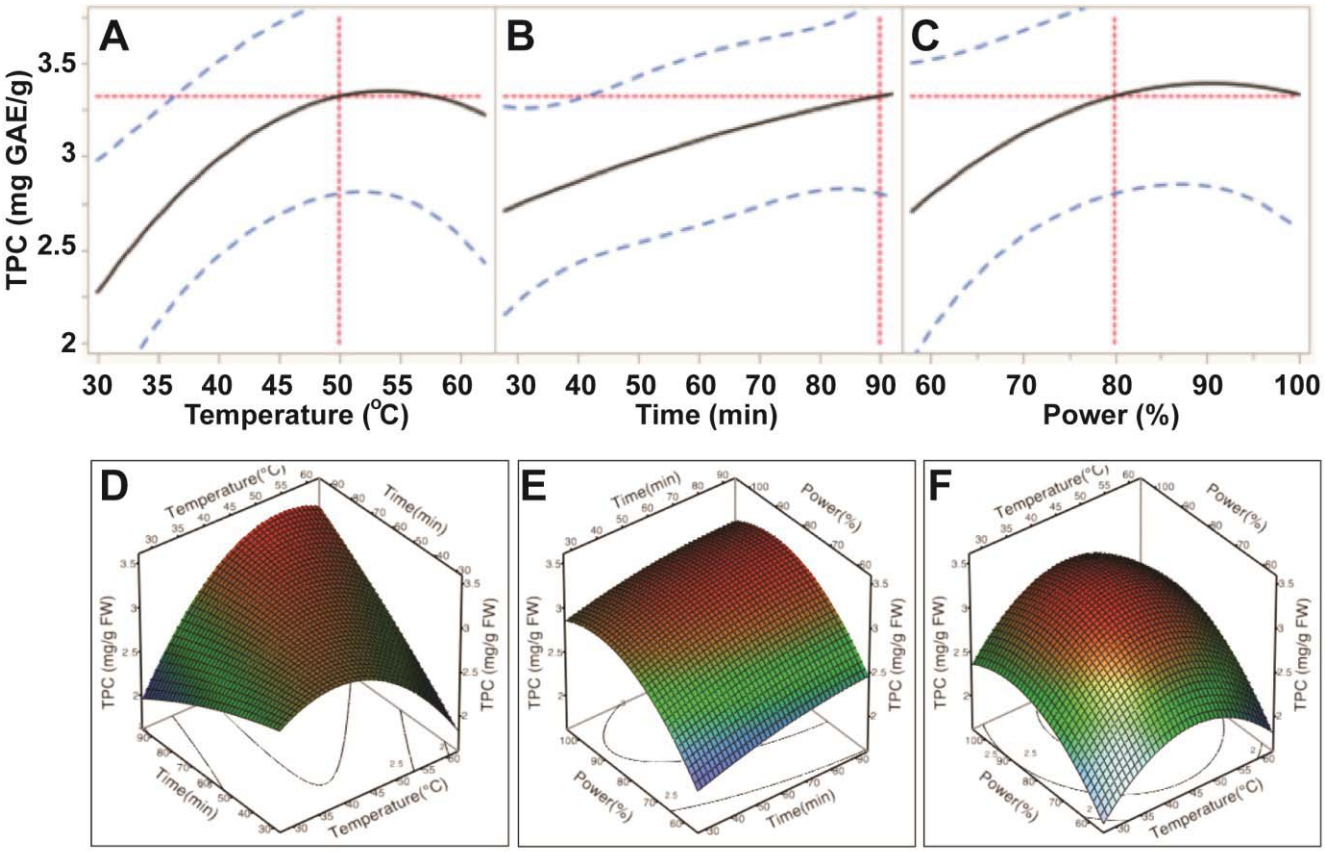


3.2. Effects of Ultrasonic Variables on Total Phenolic Content of E. tirucalli Extracts

Our data showed that the extraction efficiency of TPC had a positive correlation to the three experimental variables; bath temperature (30–50 °C), sonication time (30–90 min.) and sonication power (60%–80%; 150–200 W). Levels of TPC increased steadily when ultrasonic temperature increased from

30 °C to 50 °C; however, the levels of TPC decreased when temperature exceeded 55 °C (Figure 2). Levels of TPC also increased when ultrasonic time increased to 90 min. However, both ultrasonic temperature and time did not significantly affect levels of TPC ($p > 0.05$; Table 3), indicating that these two factors had the least impact on extraction efficiency of TPC. This demonstrated that ultrasonic power was the only parameter to significantly influence the extraction efficiency of TPC from *E. tirucalli* leaf ($p < 0.05$; Table 3). Levels of TPC plateaued when ultrasonic power exceeded 200 W (80%) (Figure 2). Therefore, the maximum TPC could be obtained at ultrasonic-assisted extraction conditions of 50 °C, 90 min and 200 W.

Figure 2. Impact of ultrasonic temperature (30–50 °C), time (30–90 min) and power (60%–100% or 150–250 W) on total phenolic compounds. The 2D impact of temperature, time and power were expressed in Figure 2A–C; while their 3D effects were shown in Figure 2D–F.



The impact of ultrasonic parameters on profile of the TPC extraction curve was similar to findings in previous studies examining seed cake extracts [10], olive pomace [12] and procyanidins removal from *Larix gmelinii* bark [18], which reported that the general application of higher temperatures, longer extraction times and/or higher sonication power increased TPC yields. The rising profile may be explained by two distinct processes; namely, a fast extraction of phenolics from cells close to the surface of the plant material, which are quickly solubilised, and slower diffusion and osmosis-based processes (known as "slow extraction") involving the liberation of more deeply embedded compounds [12].

3.3. Effects of Ultrasonic Conditions on Antioxidant Capacity of E. tirucalli

As the antioxidant activities of a sample could vary depending on the efficiency of antioxidant assays used [16], the impact of UAE conditions on antioxidant capacity of *E. tirucalli* was determined using three antioxidant assays; ABTS, DPPH and CUPRAC. Analysis of the ABTS assays of the extracts revealed that antioxidant capacity was influenced by all three ultrasound bath variables, however, only ultrasonic temperature significantly affected antioxidant capacity ($p < 0.05$; Table 3). These data indicated that ultrasonic temperature was the major factor influencing ABTS antioxidant capacity, whereas, ultrasonic time and power had least impact on ABTS antioxidant capacity.

Table 3. Analysis of variance for the experimental results.

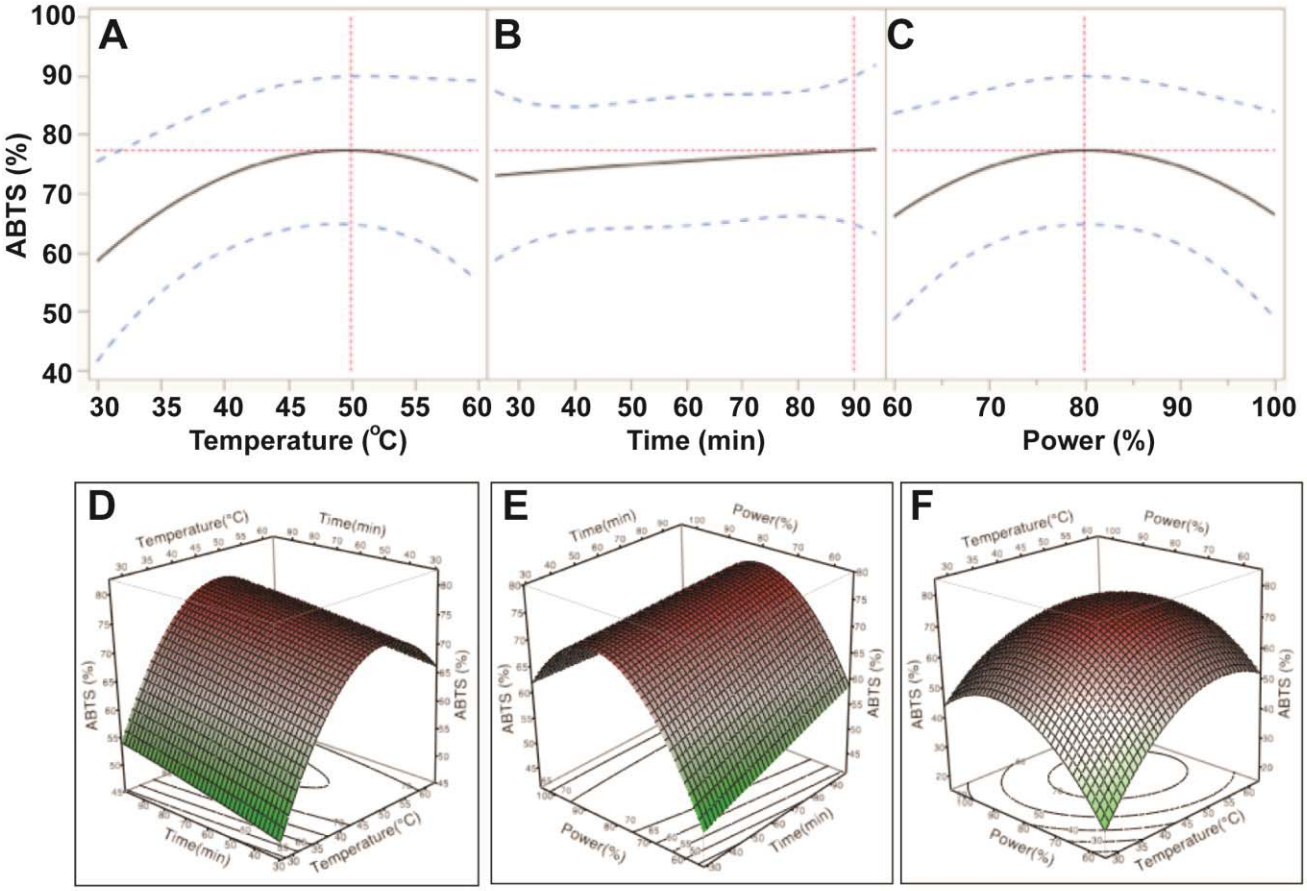
Parameter	DF	TPC		Antioxidant Capacity					
				ABTS		DPPH		CUPRAC	
		F	*p* > *F*	*F*	*p* > *F*	*F*	*p* > *F*	*F*	*p* > *F*
β_0	1	16.90	<0.01 *	16.85	<0.01 *	8.91	<0.01 *	16.34	<0.01 *
β_1	1	0.45	0.53	7.16	0.04 *	23.92	0.00 *	12.63	0.02 *
β_2	1	1.78	0.24	0.62	0.47	1.46	0.28	0.32	0.59
β_3	1	6.47	0.05 *	1.78	0.24	2.22	0.20	2.98	0.15
β_{12}	1	7.24	0.04 *	0.01	0.92	5.96	0.06	0.35	0.58
β_{13}	1	0.02	0.89	1.07	0.35	0.35	0.58	0.00	0.99
β_{23}	1	0.02	0.90	0.31	0.60	0.86	0.40	0.26	0.63
β_{11}	1	6.65	0.05 *	7.59	0.04 *	0.85	0.40	7.26	0.04 *
β_{22}	1	0.11	0.75	0.00	0.96	2.17	0.20	1.89	0.23
β_{33}	1	2.50	0.17	7.75	0.04 *	0.10	0.77	11.59	0.02 *

* Significantly different at $p < 0.05$; β_0: Intercept; β_1, β_2, and β_3: Linear regression coefficients for temperature, time and power; β_{12}, β_{13}, and β_{23}: Regression coefficients for interaction between temperature × time, temperature × power and time × power; β_{11}, β_{22}, and β_{33}: Quadratic regression coefficients for temperature × temperature, time × time and power × power.

Table 3 indicates that the magnitude of the effect of the ultrasonic variables for ABTS antioxidant capacity was: temperature > power > time. ABTS antioxidant capacity increased when increasing the ultrasonic temperature from 30 °C to 50 °C and power from 150 W to 200 W. However, antioxidant capacity likely decreased when ultrasonic temperatures and ultrasonic power exceeded 50 °C and 200 W, respectively (Figure 3). Results from the ABTS assay revealed that the optimal conditions for

maximum extraction of TPC could result in the highest antioxidant capacity. These findings can be explained by the high correlation between TPC and antioxidant capacity [15].

Figure 3. Impact of ultrasonic temperature (30–50 °C), time (30–90 min) and power (60%–100% or 150–250 W) on ABTS antioxidant capacity. The 2D impact of temperature, time and power were expressed in Figure 3A–C; while their 3D effects were shown in Figure 3D–F.

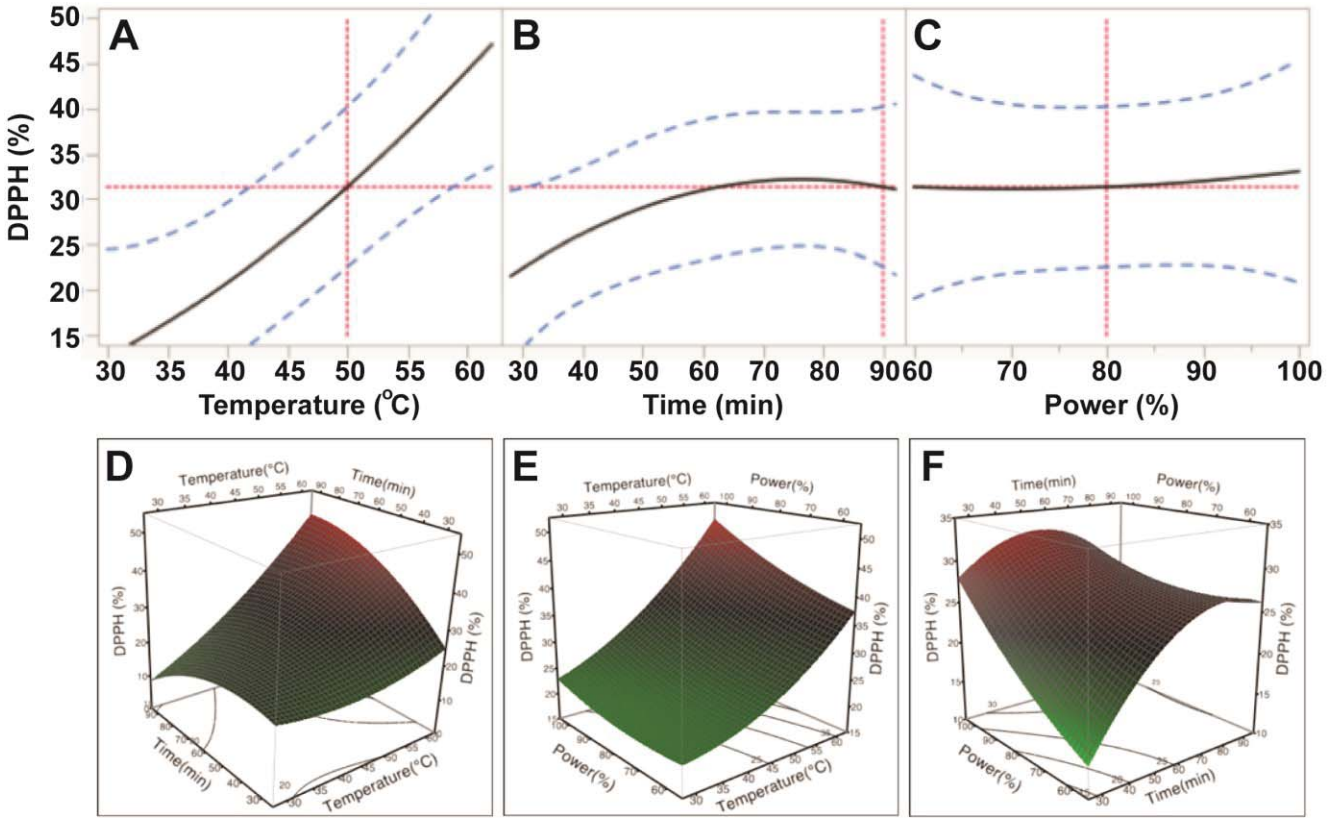


Data from the DPPH assay also indicated that free radical scavenging capacity of *E. tirucalli* extracts varied with the application of different ultrasonic temperature, time and power conditions; however, only temperature was reported to significantly affect free radical scavenging capacity ($p < 0.05$; Table 3). Based on the p values, the order of the influence was also found to be similar to ABTS: temperature > power > time. Free radical scavenging capacity of *E. tirucalli* extract increased steadily when ultrasonic temperature increased from 30 °C to 60 °C; whereas, its free radical scavenging capacity increased slightly with increasing power (150–250 W) and time (30–80 min; Figure 4). These data revealed that the highest DPPH free radical scavenging capacity of the extract was obtained at extraction conditions of 60 °C, 80 min and 250 W. At the optimal conditions for TPC (50 °C, 90 min and 200 W), only 66.5% of the maximum DPPH antioxidant value could be obtained.

The results from the CUPRAC assay further confirmed that ultrasonic temperature had a significant impact on cupric reducing antioxidant capacity (CUPRAC) of the antioxidant capacity ($p < 0.05$). Ultrasonic time and power affected, but not significantly, the CUPRAC of the *E. tirucalli* extract ($p > 0.05$; Table 3). Based on the p values, the order of the effects was similar to those of ABTS and

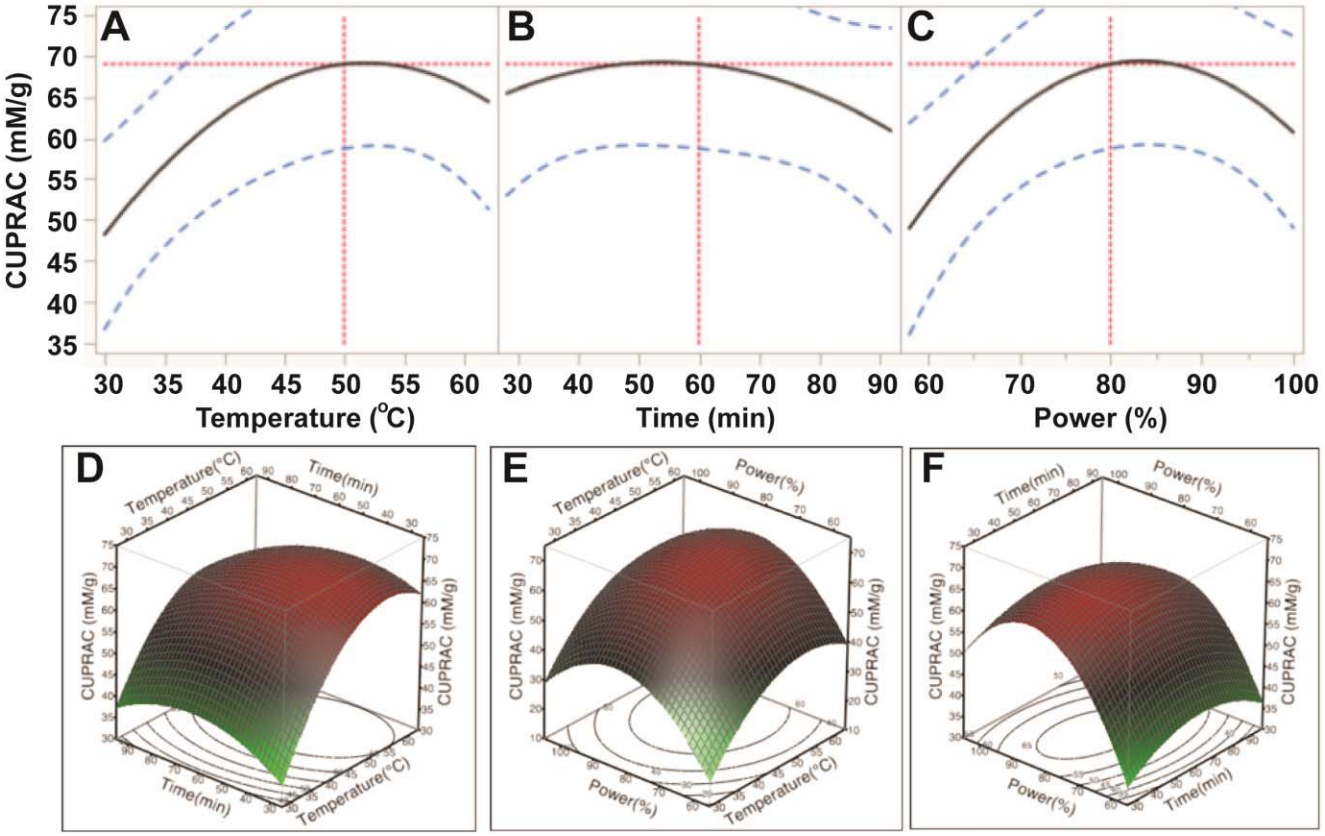
DPPH assays (temperature > power > time; Table 3). CUPRAC was observed to increase when increasing temperature from 30 °C to 50 °C, time from 30 min to 60 min and power from 150 W to 200 W; however, when ultrasonic temperature, time and power exceeded 50, 60 and 200 W, respectively, the CUPRAC of *E. tirucalli* extract decreased (Figure 5). These findings further confirmed that optimal extraction conditions for TPC could obtain maximum cupric reducing antioxidant capacity from *E. tirucalli* leaf.

Figure 4. Impact of ultrasonic temperature (30–50 °C), time (30–90 min) and power (60%–100% or 150–250 W) on DPPH free radical scavenging capacity. The 2D impact of temperature, time and power were expressed in Figure 4A–C; while their 3D effects were shown in Figure 4D–F.



The current findings showed that ultrasonic temperature, time and power affected the antioxidant capacity of the *E. tirucalli* extract; however temperature had the highest impact, followed by power, while time had the least impact on antioxidant capacity of the *E. tirucalli* extract. Sahin *et al*. [12] also found that ultrasonic temperature and time affected antioxidant capacity of an *Artemisia absinthium* extract and generally, antioxidant capacity increased when the time increased, but decreased when temperature exceeded 50 °C. Teh *et al*. [10] also reported that ultrasonic temperature and time influenced antioxidant capacity of extract from defatted hemp, flax and canola seed cakes. The impact of ultrasonic conditions on antioxidant capacity can be explained by the influence on the total phenolic compounds, which were found to contribute significantly to the antioxidant capacity of plant extracts [19–21].

Figure 5. Impact of ultrasonic temperature (30–50 °C), time (30–90 min) and power (60%–100% or 150–250 W) on cupric reducing antioxidant capacity. The 2D impact of temperature, time and power were expressed in Figure 5A–C; while their 3D effects were shown in Figure 5D–F.



3.4. Optimisation of Ultrasonic Extraction Conditions for Total Phenolic Content and Antioxidant Capacity of E. tirucalli

Previous epidemiological studies have established links between the consumption of foods containing high concentrations of phenolic and antioxidant compounds and a lower incidence of cardiovascular diseases and certain types of cancer [22–24]. An understanding of the factors affecting the extraction efficiency of phenolics and/or antioxidant compounds from plant sources is therefore important.

Based on the predictive models and values shown in Figures 2–5, the optimum UAE conditions for the extraction of total phenolics, ABTS and CUPRAC from *E. tirucalli* leaf were determined to be: temperature = 50 °C, time = 90 min and power = 200 W; whereas, the optimum conditions for DPPH free radical scavenging capacity were: temperature = 60 °C, time = 80 min and power = 250 W. Of note, extraction under optimum conditions for TPC, ABTS and CUPRAC could obtain 66.5% of the maximum DPPH antioxidant value under its optimum conditions (60 °C, 80 min and 250 W). Therefore, the conditions of 50 °C, 90 min and 200 W were selected as optimal conditions for extraction of TPC and enhanced antioxidant capacity.

To validate the optimum conditions predicted by the models, *E. tirucalli* leaf was extracted under UAE conditions of 50 °C, 90 min and 200 W and the results showed that the predicted values of TPC

and three antioxidant assays were similar to those of the experimental values ($p > 0.05$; Table 4). Therefore, these conditions were suggested for use to extract TPC and antioxidants from *E. tirucalli* leaf for further isolation and utilisation. In addition, these findings further confirmed the appropriateness of the models used for optimising the extraction conditions using UAE, and also revealed that response surface methodology was an effective technique for designing and optimising the extraction conditions.

Table 4. Validation of the predicted values for total phenolic content (TPC) and antioxidant capacity.

Variables	Values of TPC and Antioxidant Capacity	
	Predicted	**Experimental (n = 4)**
TPC (mg GAE/g FW)	3.32 ± 0.74 [a]	2.93 ± 0.14 [a]
ABTS (%)	77.26 ± 17.73 [a]	71.50 ± 1.06 [a]
DPPH (%)	31.33 ± 12.47 [a]	35.24 ± 0.66 [a]
CUPRAC (mM TAE/g FW)	61.77 ± 16.53 [a]	54.03 ± 3.53 [a]

All the values are means ± standard deviations and those in the same row not sharing the same superscript letter are significantly different from each other ($p < 0.05$).

4. Conclusions

As hypothesised, ultrasonic temperature, time and power had effects on extraction efficiency of total phenolic content and antioxidant capacity of the extracts from *E. tirucalli* leaf; however, the effects varied. Ultrasonic power had the highest impact on TPC; whereas, temperature had the strongest influence on antioxidant capacity. Levels of TPC and antioxidant capacity increased when ultrasonic temperature, time and power increased to 50 °C, 90 min and 200 W, respectively. Therefore, the optimum ultrasonic-assisted extraction conditions for TPC and antioxidant capacity from *E. tirucalli* leaf were: temperature of 50 °C, time of 90 min and power of 200 W. These conditions can be applied for further isolation and purification of phenolic compounds from the *E. tirucalli* leaf.

Acknowledgments

The authors would like to acknowledge the following funding support: Ramaciotti Foundation (ES2012/0104); Cancer Australia and Cure Cancer Australia Foundation (1033781). The authors also kindly thank the Vietnamese Government through the Vietnam International Education Development - Ministry of Education and Training (Project 911) and the University of Newcastle for awarding a VIED-TUIT scholarship to VTN and TTD.

Author Contributions

Quan V. Vuong, Chloe D. Goldsmith, Trung Thanh Dang, Van Tang Nguyen, Deep Jyoti Bhuyan and Christopher J. Scarlett designed the study. Quan V. Vuong, Chloe D. Goldsmith, Elham Sadeqzadeh, Christopher J. Scarlett and Michael C. Bowyer analysed and interpreted the results. All Authors prepared and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Additional Research paper 2

Quan V. Vuong, Van Tang Nguyen, Thanh Trung Dang, **Deep Jyoti Bhuyan**, Chloe D. Goldsmith, Elham Sadeqzadeh, Christopher J. Scarlett, Michael C. Bowyer. Optimization of ultrasound-assisted extraction conditions for euphol from the medicinal plant, *Euphorbia tirucalli*, using response surface methodology. **Industrial Crops and Products (2015),** 63: 197–202.

Industrial Crops and Products 63 (2015) 197–202

Contents lists available at ScienceDirect

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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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ARTICLE INFO

Article history:
Received 22 June 2014
Received in revised form 25 August 2014
Accepted 28 September 2014
Available online 16 October 2014

Keywords:
Euphol
Euphorbia tirucalli
Ultrasonic-assisted extraction
Optimization
Response surface methodology

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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http://dx.doi.org/10.1016/j.indcrop.2014.09.057
0926-6690/

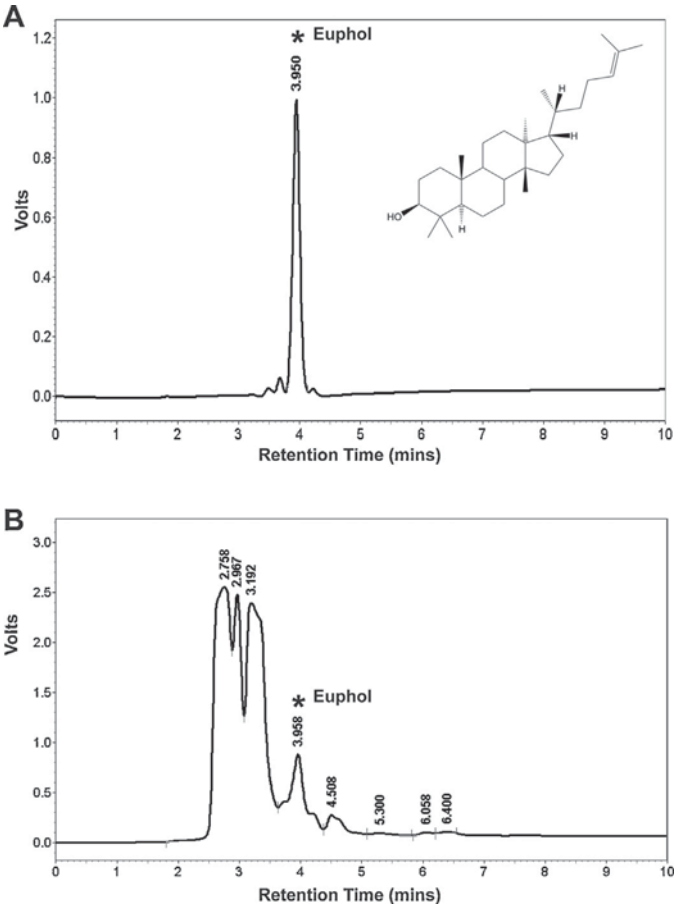

Fig. 1. HPLC chromatogram of euphol standard and euphol structure (A) and the extract *E. tirucalli* leaf (B).

(Wang et al., 2011), thus contributes to the optimization of euphol extraction from *E. tirucalli*.

Ultrasonic extraction has gained increasing popularity as method to be used both in conjunction with, or in place of, traditional extraction techniques because of its efficient energetics and reduced extraction times (Mierzwa et al., 1997). Ultrasound assisted extraction (UAE) is now well established as a technique for the extraction of low molecular weight compounds from plant sources (Hromadkova et al., 2002), and has been found to be more effective in extracting phytochemicals from plants and is easily adapted to the industrial scale (Vinatoru, 2001).

This study aimed to determine the most effective extraction solvents and then utilize RSM to develop optimal conditions for the extraction euphol from *E. tirucalli* by ultrasonic-assisted extraction as a useful engineering tool for commercial applications to prepare euphol from *E. tirucalli* for utilization in pharmaceuticals.

2. Materials and methods

2.1. Plant materials

The phylloclades of a *E. tirucalli* tree were collected on July 16 from a property located in Saratoga, NSW, Australia (33.47°S, 151.35°E) and immediately transferred to the laboratory and stored at −20 °C. Phylloclade samples were then freeze dried in liquid nitrogen, ground to consistent particle size using a commercial blender, then stored at −20 °C until required.

2.2. Extraction of euphol using ultrasound-assisted extraction (UAE)

To test the impact of solvents on extraction efficiency of euphol, ground fresh leaf was extracted in different solvents including ethanol, acetonitrile:ethanol (4:1, v/v), acetone:ethanol (4:1, v/v), ethyl acetate:ethanol (4:1, v/v), or hexane:ethanol (4:1, v/v) at a solvent-to-sample ratio of 100:32 mL/g. The extraction was conducted using a tunable ultrasonic bath (Soniclean, 220 V, 50 Hz and 250W, Soniclean Pty Ltd, Australia) under ultrasonic conditions of room temperature, 60 min and power of 150 W.

To optimize UAE conditions, the most effective solvent (ethyl acetate:ethanol (4:1, v/v)) was used for extraction of fresh leaf at a solvent-to-sample ratio of 100:32 mL/g. UAE was conducted using a tunable ultrasonic bath (Soniclean, 220 V, 50 Hz and 250 W, Soniclean Pty Ltd, Australia) to be set at different conditions as designated by RSM design. An external digital thermometer was also used to measure the temperature of the ultrasonic bath, while tap water was used to cool the water to the required temperature if the ultrasonic bath exceeded the designated temperature due to ultrasonic energy.

2.3. Response surface methodology design

Response surface methodology (RSM) with a Box–Behnken design was employed to design the experiment and investigate the influence of the three independent ultrasonic parameters: power (60%, 80%, 100% or 150, 200, 250 W), temperature (30, 45, 60 °C), and time (30, 60, 90 min). After extraction, the extracts were then immediately cooled to room temperature (ice bath), then filtered through a 5 mL syringe fitted with a 0.45 μm cellulose syringe filter (Phenomenex Australia Pty. Ltd, NSW, Australia), then transferred into brown glass HPLC vials for HPLC analysis.

The independent variables and their code variable levels are shown in Table 1. To express the level of euphol as a function of the independent variables, a second-order polynomial equation (Eq. (1)) was used as follows (Vuong et al., 2011):

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \sum_{i=1}^{k} \beta_{ii} X_i^2 \quad (1)$$

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

In this study, the three independent ultrasonic parameters: X_1 (ultrasonic temperature, °C), X_2 (ultrasonic time, min) and X_3 (ultrasonic power, %) were applied (Table 2), with euphol extraction efficiency expressed as the following polynomial (Eq. (2)):

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

2.4. Determination of euphol

Euphol standard was prepared from the latex of *E. tirucalli* in our laboratory using a Shimadzu HPLC system fitted with a semi-prep Luna C_{18} reversed-phase column (Phenomenex, Australia) maintained at 35 °C and coupled to auto fraction collector (Shimadzu Australia, Rydalmere, NSW, Australia). The sample was detected using UV detector set at 210 nm as per the work of Vuong et al. (2012). A representative chromatogram of euphol standard is shown in Fig. 1A. Quantitative analysis of euphol content in different filtered sample extracts was then undertaken using

Table 1
Box–Behnken design and observed responses of euphol extracted from *Euphorbia tirucalli* phylloclades using UAE.

Run	Pattern[a]	UAE temperatures (°C)	UAE time (min)	UAE power (%)[b]	Euphol (mg/g)	
					Experimental values[c]	Predicted values
1	+−0	60	30	80	3.74	3.40
2	0++	45	90	100	3.62	3.33
3	000	45	60	80	3.57	3.57
4	0−+	45	30	100	2.85	3.03
5	+0+	60	60	100	3.29	3.45
6	000	60	90	80	3.92	4.04
7	0−−	45	30	60	2.43	2.71
8	000	45	60	80	3.57	3.57
9	−0−	30	60	60	2.03	1.87
10	0+−	45	90	60	3.81	3.63
11	−−0	30	30	80	1.97	1.85
12	+0−	60	60	60	4.14	4.20
13	−0+	30	60	100	2.69	2.63
14	−+0	30	90	80	2.09	2.43
15	000	45	60	80	3.57	3.57

[a] Pattern −, 0, + are the minimum values, center points, and maximum values, respectively, of UAE temperature, time and power in the tested ranges.
[b] Corresponding values of power were 250 W for 100%; 200 W for 80%; and 150 W for 60%.
[c] Values were obtained from triplicated experiments and expressed as mg/g FW.

a reversed-phase analytical column (250 mm × 4.6 mm Synergi 4 mm Fusion-RP 80A column, Phenomenex Australia) maintained at 35 °C with peak detection set at 210 nm.

The mobile phase (flow rate = 1 mL/min) consisted of two feed solvents; methanol (A) and acetonitrile (B). A gradient elution schedule was used as follows: 100% A for the first 5 min.; followed by a linear gradient changed to 50% A and 50% B (5–25 min.); then finally, a linear gradient changed back to 100% A (25–30 min.). An auto-injector was used to inject 20 μL of all sample extracts onto the HPLC column. A representative chromatogram of euphol in the *E. tirucalli* leaf is shown in Fig. 1B. The content of euphol was calculated based on the standard curve and expressed as mg of euphol per gram of fresh weight (FW).

2.5. Determination of phenolic content and antioxidant capacity

Total phenolic content (TPC) of the extract prepared under optimal conditions was determined using a method described by Vuong et al. (2013). Gallic acid was used as the standard for the construction of a calibration curve, with the results expressed as mg of gallic acid equivalents per gram of fresh weight (FW) (mg GAE/g FW).

Three different antioxidant assays described in previous studies were employed to test antioxidant properties of the extract under optimal conditions, including ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) (Thaipong et al., 2006), DPPH (1,1-diphenyl-2-picrylhydrazyl) assay (Vuong et al., 2013) and FRAP (ferric reducing antioxidant power) assay (Thaipong et al., 2006). Values of ABTS and DPPH were expressed as percentage of inhibition and value of FRAP was expressed as Trolox equivalents per gram of fresh weight (TE/g FW).

2.6. Statistical analysis

RSM experimental design and analysis were conducted using JMP software (Version 11). The software was also used to establish the model equation, to graph the 3-D plot, 2-D contour of the response and to predict the optimum values for the three response variables. The student's *T*-test was conducted using the SPSS statistical software (Version 20) for comparison of sample means. Differences between the mean euphol concentrations in the different samples were taken to be statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Impact of solvents on extraction efficiency of euphol

Previous studies indicated that different solvents could lead to different extraction efficiencies of bioactive compounds (Vuong et al., 2013; Zhang et al., 2013). Therefore, this study determined

Table 2
Estimated regression coefficients for the quadratic polynomial model and the analysis of variance for the experimental results.

Parameter[a]	Predicted coefficients	Standard error	DF[b]	Sum of squares	*F* value	Prob > *F*
Intercept				Model	7.283	0.0208*
β_0	3.568	0.19	1			
β_1	0.789	0.12	1	4.986	45.606	0.001*
β_2	0.306	0.12	1	0.750	6.862	0.047*
β_3	0.004	0.12	1	0.000	0.001	0.972
β_{12}	0.014	0.17	1	0.001	0.007	0.935
β_{13}	−0.375	0.17	1	0.565	5.165	0.072
β_{23}	−0.154	0.17	1	0.094	0.864	0.395
β_{11}	−0.388	0.17	1	0.557	5.091	0.074
β_{22}	−0.248	0.17	1	0.228	2.086	0.208
β_{33}	−0.144	0.17	1	0.076	0.698	0.442
Lack of fit			3	.	.	.
Pure error				.		
R^2	0.929		Adjusted R^2	0.801		
PRESS	8.746		RMSE	0.33		

[a] Coefficients refer to the general model.
[b] Degree of freedom.
* Significant different at $p < 0.05$.

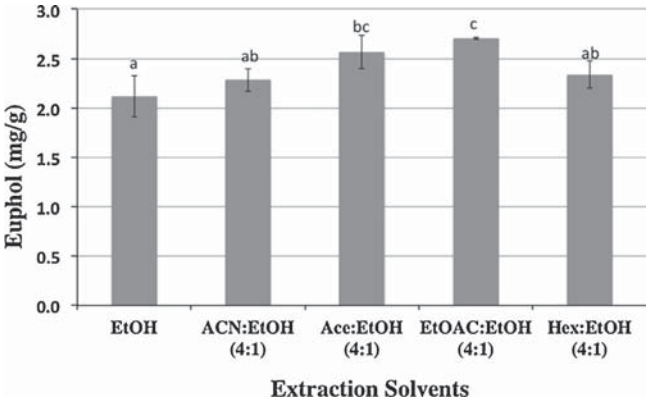

Fig. 2. Impact of extraction solvents on extraction efficiency of euphol from *E. tirucalli* phylloclades. Values (mean ± SD, $n = 3$) not sharing a letter (on top of the columns) are significantly different at $p < 0.05$.

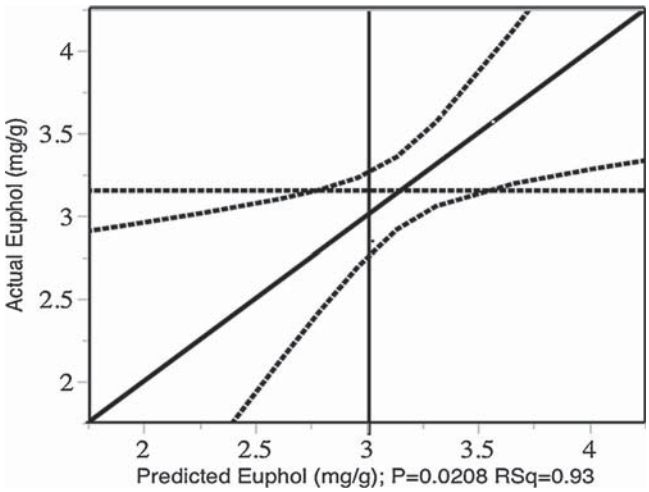

Fig. 3. Correlation between the predicted and actual levels of euphol.

the impact of five different extraction solvents with various polarity indexes to identify the most effective solvent for further optimization using response surface methodology. The results (Fig. 2) showed that different extraction solvents significantly affected the extraction efficiency of euphol. Extraction efficiency of euphol was low when using ethanol alone; however, extraction efficiency was significantly improved when it was used in combination with other solvents of lower polarity at the ratio of 1:4 (v/v). A solvent mixture of ethyl acetate:ethanol (4:1, v/v) was found to be the most effective solvent for extraction of euphol from *E. tirucalli* leaf. The current findings were in agreement with a previous study (Zhang et al., 2013), which reported that a mixture of ethyl acetate:ethanol (4:1 v/v) had higher extraction efficiency than methanol when extracting euphol from root of *Euphorbia pekinensis* Rupr; and these differences could be explained by the variation of solvent polarities (Vuong et al., 2013). Therefore, solvent of ethyl acetate:ethanol (4:1, v/v) was used for further optimization process.

3.2. Fitting the model for the prediction of euphol

Response surface methodology (RSM) is an effective statistical procedure using a minimum set of experiments for determination of the coefficients of a mathematical model and optimization of the conditions (Yemiş and Mazza, 2012). However, it is important to test the appropriateness of the RSM mathematical model for predicting the optimal variances and adequately representing the real relationship between the selected parameters. The analysis of variances for the experimental results of the Box–Behnken design (Fig. 3) showed that the coefficient of determination (R^2) of the model was 0.93, suggesting that 93% of the actual levels can be matched with the model-predicted levels of euphol (Li et al., 2011). The analyzed results (Table 2) also showed that root mean square error (RMSE), which is used to estimate the standard deviation of the random error, was 0.33, further confirming a linear correlation exists between the predicted levels and actual levels of euphol present in the samples analyzed.

Results of analysis of the actual experiments (Table 2) revealed that the Predicted Residual Sum of Squares (PRESS) for the model – a measure of how a chosen model fits each point in the design (Zhang et al., 2009) was 8.746, indicating a good measure. In addition, the p and F-values of the model were 0.02 and 7.283, respectively, indicating that the model predictions were significant (Zhang et al., 2009). Moreover, the Lack of Fit report was not generated as a consequence of the analysis of the experimental data, implying that the model was well fitted as it was impossible to assess lack of fit because there were as many estimated parameters as there were observations (SAS-Institute, 2014). Therefore, the results revealed that the mathematical model was adequate for the prediction of euphol and was fitted to the following second-order polynomial formula (Eq. (3)):

$$Y_{\text{Euphol}} = 3.568 + 0.789X_1 + 0.306X_2 + 0.004X_3 + 0.014X_1X_2 - 0.038X_1X_3 - 0.154X_2X_3 - 0.388X_1^2 - 0.248X_2^2 - 0.144X_3^2 \quad (3)$$

3.3. Effect of extraction parameters on the UAE performance

The impacts of three UAE parameters on extraction efficiency of euphol were investigated. Results (Fig. 4A) showed that UAE temperature was a significant parameter, with rising UAE temperature correlating to a higher extraction efficiency. At fixed conditions of 75 min and UAE power at 60%, euphol extraction increased from 1.7 mg/g to 4.2 mg/g across a temperatures ranging between 30 and 60 °C.

Table 2 shows a p value of 0.001 for UAE temperature, further confirming the significance of temperature on extraction efficiency. The findings are in agreement with previously studies assessing the effectiveness of UAE. Teh and Birch (2014) found that UAE temperature significantly affected the yield of phenolic compounds from the seed cake extracts, while Xu and Pan (2013) reported a similar influence in the extraction of all-trans-lycopene from red grapefruit.

The general application of higher UAE temperatures in these studies resulted in higher yields of bioactive compounds and these observations may be explained by the higher system energies could increase the solubility of target compounds, and consequentely improve their liberation from the sample matrix by destroying the integrity of connective and structural tissues (Teh and Birch, 2014).

Table 2 also shows that UAE time significantly affected the extraction efficiency of euphol ($p = 0.047$). Under the fixed conditions of UEA temperature (60 °C) and power (60%), yields of euphol were shown to increase from 3.2 mg/g to 4.2 mg/g for UEA times between 30 and75 min. Beyond 75 min, plateauing of the yield was observed (Fig. 4A), indicating that prolonged sonication did not result in further improvements in extraction efficiency. Similar findings were reported in previous studies relating to the extraction of total phenolic compounds from olive leaf (Şahin and Şamlı, 2013) and procyanidins from *Larix gmelinii* bark (Sun et al., 2013).

The rising profile of the extraction curves for temperature and time may be considered to be the result of two distinct process; namely a fast extraction of euphol from cells close to the surface of

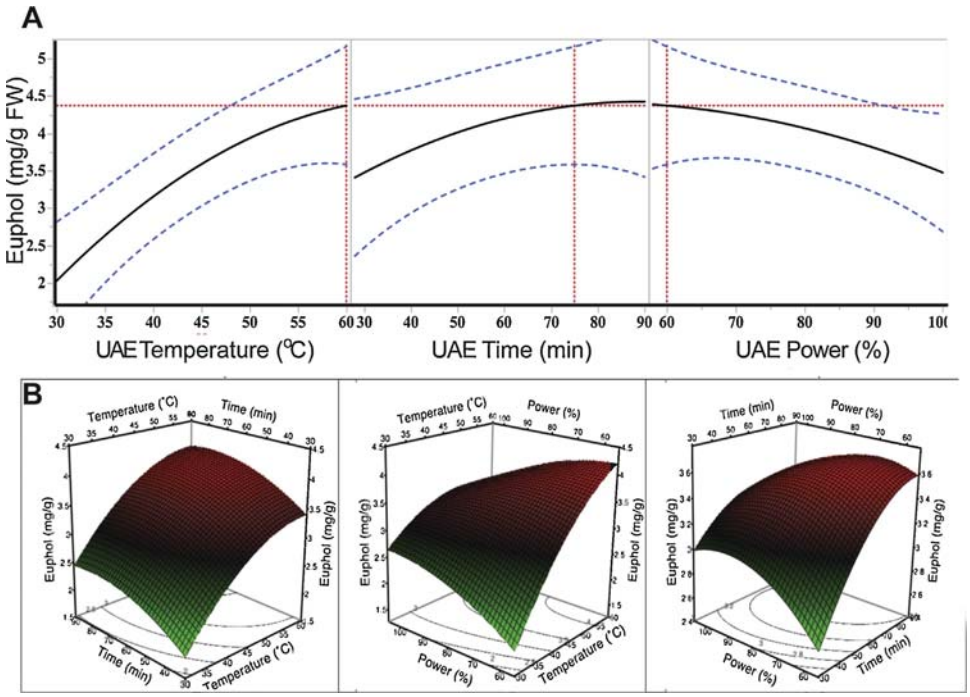

Fig. 4. Impact of ultrasonic temperature, time and power on the extraction efficiency of euphol (A); and the 3D response surface profilers and 2D contour plots of euphol as affected by ultrasonic temperature, time and power (B).

the plant material, which is quickly solubilized, (a process known as "washing"), and a second, slower, diffusion and osmosis-based processes (known as "slow extraction") involving the liberation of more deeply embedded euphol (Şahin and Şamlı, 2013).

Table 2 showed that extraction efficiency was not significantly affected by UAE power either alone or in combination with changes in temperature or solvent ratio change. Somewhat unexpectedly, Fig. 4A showed that under fixed conditions of temperature (60 °C) and time (75 min), euphol decreased from 4.2 mg/g to 3.4 mg/g as UAE power was increased from 60% to 100% (150–250 W). Our findings contrast with results presented in previous studies, which found a positive correlation between power rise and extraction efficiency for the liberation of both procyanidins from *L. gmelinii* bark (Sun et al., 2013) and anthocyanins and polyphenols from *Nephelium lappaceum* L. fruit peel (Prakash Maran et al., 2013). We tentatively apportion our findings to destruction of the steroidal skeleton as a result of the prolonged exposure to ultrasonic waves. A recent report probing the application of ultrasound in the destruction of contaminant steroids in ground- and wastewater identified significant degradation (60–98%) of steroids such as estrone, estriol, equilin, 17-dihydroequilin, and norgestrel following sonication at powers ranging between 0.6 and 4 kW for prolonged time periods (40–60 min) (Suri et al., 2007). While our experiments were at a lower power range, time periods were similar, suggesting the potential for a similar outcome. Attempts to examine the HPLC traces of the high power extracts failed to clearly identify the presence of degradation products due to the complexity of the extract profile.

3.4. Optimization of ultrasonic conditions for extraction of euphol, and determination of phenolic content and antioxidant capacity of the extract

Based on the regression coefficients, the scale of impact of the ultrasonic parameters on the euphol extraction efficiency (from the largest to the smallest) could be listed as follows: (i) ultrasonic temperature, (ii) ultrasonic time and (iii) ultrasonic power.

Table 3
Bioactive compounds and antioxidant capacity of *E. tirucalli* leaf extract prepared under optimal UAE conditions.

Bioactive compounds/antioxidant assays	Content/capacity
Euphol (mg/g FW)	4.1 ± 0.1
Phenolic content (mgGAE/g FW)	2.5 ± 0.8
ABTS (%)	61.7 ± 18.2
DPPH (%)	40.5 ± 12.8
FRAP (mM TE/g FW)	47.0 ± 17.0

The values are the mean ± standard deviations ($n = 4$).

Specific interrelationships between the respective parameters were visually illustrated in 3D profiles shown in Fig. 4B. The R^2 of 0.93 (Table 2) indicated that a total of 93% of the variation in the extraction efficiency of euphol could be explained while only 7% of the variation could not be explained by the prediction profilers. Therefore, the optimal ultrasonic conditions for maximising the extraction efficiency of euphol were deduced as: UAE temperature of 60 °C, UAE time of 75 min and UAE power of 150 W (60% power).

Under optimal ultrasonic conditions, the modeling predicted a euphol yield of 4.37 ± 0.79 mg/g of fresh euphorbia leaf. To validate this prediction, euphorbia leaf was extracted under optimal conditions ($n = 4$). The results (Table 3) revealed an average experimental yield of 4.1 ± 0.1 mg/g, which was found to be within experimental tolerances of the predicted value ($p > 0.05$), thereby validating the model.

Additionally, phenolic content and antioxidant capacity of the *E. tirucalli* leaf extract under optimal conditions were also investigated. The results (Table 3) showed that this extract also contained 2.5 mg/g FW of phenolic content. In comparison with other materials, this extract contained higher phenolic content than that of *Piper betel* (2.12 mg/g FW) and *Polyalthia longifolia* (2.44 mg/g FW) (Kaur and Mondal, 2014) and loquat (*Eriobotrya japonica* (Thunb.) (0.129–0.578 mg/g FW) (Polat et al., 2010). Of note, these conditions were not optimal for maximum level of phenolic content; therefore, it is worthy to optimize extraction of phenolic content in

future study for further isolation of phenolic compounds. In addition, Results (Table 3) also showed that percentage of inhibition from ABTS and DPPH assays were 62% and 40%, respectively. Ferric reducing antioxidant power was found to be 47 mM TE/g FW. The DPPH value was comparable to those of *Citrus aurantifolia* and *P. betel* (40 and 35%, respectively) (Kaur and Mondal, 2014) and the FRAP value was higher than that of fresh date (*Phoenix dactylifera* L.) (11.7–20.6 mM TE/g FW) (Al-Farsi et al., 2005). These data indicated that this *E. tirucalli* leaf extract exhibited potent antioxidant capacity, and future studies are required to identify the major contributors of the antioxidant capacity.

4. Conclusions

The solvents and ultrasonic parameters including temperature, time and power were found to significantly affect the extraction efficiency of euphol from *E. tirucalli* leaf. The solvent ratio of ethyl acetate:ethanol (4:1, v/v) was the most effective for the extraction of euphol. Utrasonic temperature and time had a positive impact on euphol yield; whereas, ultrasonic power had a negative effect. The optimal ultrasonic conditions for extraction of euphol were as follows: temperature of 60 °C, time of 75 min and power of 150 W. Under these optimal conditions, the extract also contained high levels of phenolic compounds and possessed potent antioxidant capacity. These ultrasonic extraction conditions can be scaled up to pilot and then industrial scale for further isolation and potential utilization of euphol in the pharmaceutical industry.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors would acknowledge the following funding support: Ramaciotti Foundation (ES2012/0104); Cancer Australia and Cure Cancer Australia Foundation (1033781). The authors also kindly thank the Vietnamese Government through the Vietnam International Education Development - Ministry of Education and Training (Project 911) and the University of Newcastle for awarding a VIED-TUIT scholarship to VTN and DTT.

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Additional Review paper

Quan V. Vuong, Anita C. Chalmers, **Deep Jyoti Bhuyan**, Michael C. Bowyer, Christopher J. Scarlett. Botanical, Phytochemical, and Anticancer Properties of the *Eucalyptus* Species. **Chemistry & Biodiversity (2015),** 12(6): 907–924.

REVIEW

Botanical, Phytochemical, and Anticancer Properties of the *Eucalyptus* Species

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The genus *Eucalyptus* (Myrtaceae) is mainly native to Australia; however, some species are now distributed globally. *Eucalyptus* has been used in indigenous Australian medicines for the treatment of a range of aliments including colds, flu, fever, muscular aches, sores, internal pains, and inflammation. *Eucalyptus* oils containing volatile compounds have been widely used in the pharmaceutical and cosmetics industries for a multitude of purposes. In addition, *Eucalyptus* extracts containing nonvolatile compounds are also an important source of key bioactive compounds, and several studies have linked *Eucalyptus* extracts with anticancer properties. With the increasing research interest in *Eucalyptus* and its health properties, this review briefly outlines the botanical features of *Eucalyptus*, discusses its traditional use as medicine, and comprehensively reviews its phytochemical and anticancer properties and, finally, proposes trends for future studies.

Contents

1. Introduction. – The genus *Eucalyptus* belongs to the Myrtaceae family (*Fig. 1*). With a few exceptions, the *ca.* 800 *Eucalyptus* species identified are native to Australia, although they are now distributed around the world [1]. Within Australia,

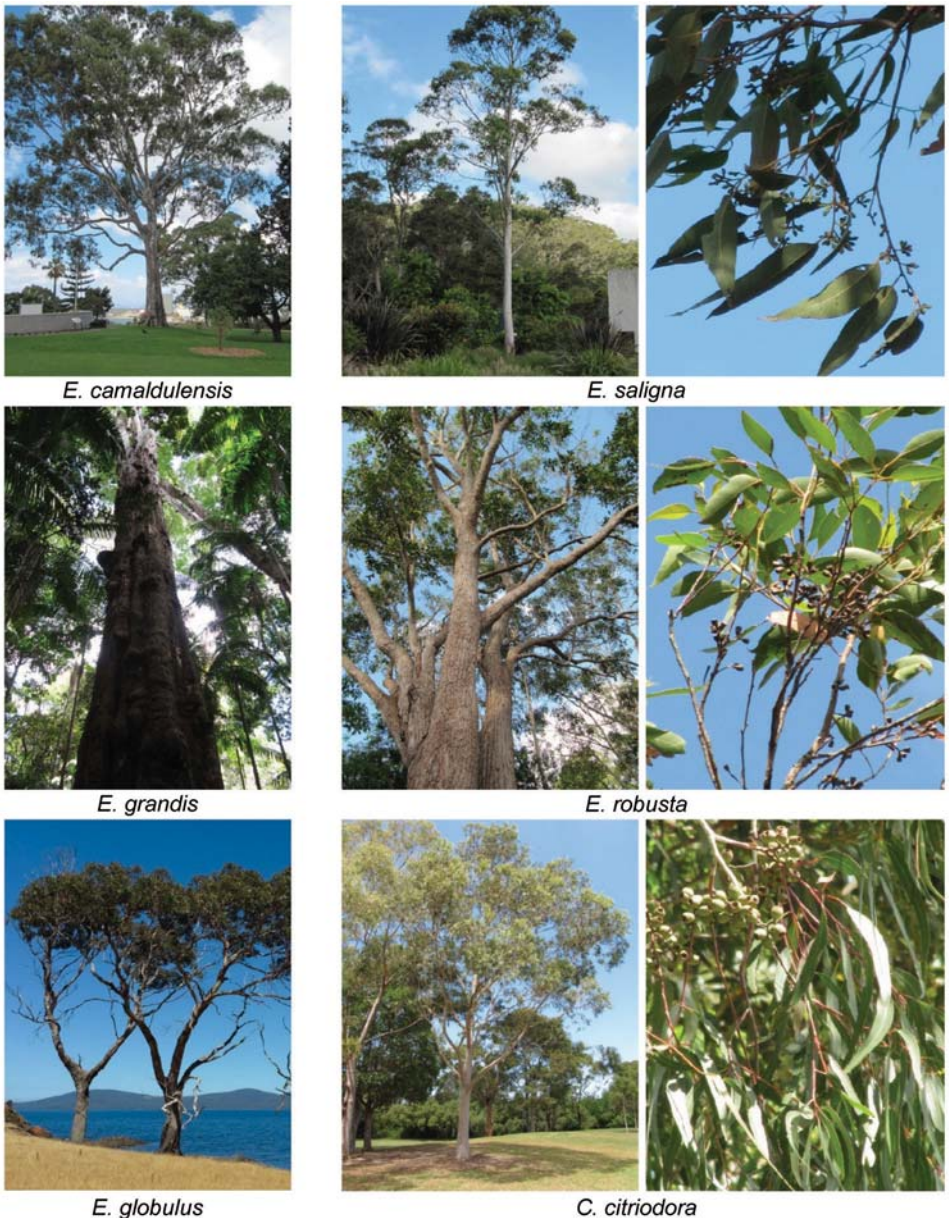

Fig. 1. *Selected common* Eucalyptus *species*

Eucalyptus is mainly cultivated for timber and paper production [1][2]. Different parts of the plant such as leaves, bark, and stems have been used to produce essential oils, which contain a variety of volatile compounds that have been widely utilized in the pharmaceutical and cosmetics industries [1]. Numerous nonvolatile compounds

including triterpenoids, flavonoids, and tannins have also been identified [3] and are associated with a range of health benefits, including cancer prevention [4][5].

Eucalyptus is used in numerous herbal preparations to treat a range of ailments, including colds, flu, fever, aching, sore, internal pain, and inflammation [3][6]. The effectiveness of these formulations has, however, yet to be supported by experimental or clinical data. The widespread use of *Eucalyptus* within indigenous communities does nevertheless suggest the presence of key bioactive compounds having the potential to impart health benefits. While *Eucalyptus* is known to be a rich resource of antioxidants such as flavonoids, phloroglucinol derivatives, and tannins [3], studies to date have identified only a limited number of bioactive compounds.

Cancer remains a burdensome disease for many countries around the world, particularly in first world economies, where ageing populations dominate the population profile. Despite some success, the development of effective therapeutic agents remains elusive for many forms of cancer [7].

In the last 30 years, *ca*. 45% of all new anticancer drugs have been derived directly or indirectly from plant-based natural products [8]. While computer-aided drug design has made significant inroads as a tool in drug discovery, the screening of plant extracts remains a useful and effective strategy for the development of new, effective anticancer therapies [9].

Preliminary studies of the extracts of several *Eucalyptus* species such as *E. globulus*, *E. camaldulensis*, *E. citriodora*, *E. maidenii*, and *E. torquata* allowed to identify anticancer activity against a range of cancer-cell lines both *in vitro* and *in vivo*. These studies comprised only a tiny fraction of the more than 750 identified *Eucalyptus* species; thus, there is an enormous untapped potential for the discovery of new anticancer compounds from these plants. In this review, the genus *Eucalyptus* is briefly described and the traditional medicinal uses of *Eucalyptus* are highlighted, to emphasize their potential as herbal therapies. The bioactive compounds that have been identified in *Eucalyptus* species to date are discussed, the techniques associated with the extraction and isolation of *Eucalyptus* phytochemicals are summarized, and possibilities for a yield improvement are suggested. Finally, links between *Eucalyptus* extracts and anticancer activity are reviewed, and directions for future studies are proposed.

2. Botany. – The natural distribution of the genus *Eucalyptus* (Myrtaceae) is predominantly confined to the Southern Hemisphere, ranging from 9°N to 44°S in latitude [10]. The genus includes more than 750 species, the majority of which are endemic to Australia, with a small number of species found in the neighboring South East Asian countries such as Indonesia, the Philippines, Timor, and Papua New Guinea [10][11]. *Eucalyptus* species possess diverse physical structure, occurring as trees, mallees (*i.e.*, multi-stemmed dwarf forms), or shrubs. Individuals of some species can reach 400–500 years in age [10].

The distribution and dominance of *Eucalyptus* within Australia means that the genus is of great ecological importance, providing essential food and habitat resources for a diverse range of fauna. Within Australia, *Eucalyptus* dominate the natural forests and woodlands of non-arid climates, but are notably absent from rainforests [12]. The mallee form covers vast areas in the drier regions of southern Australia, but *Eucalyptus*

is largely absent from the arid inland zones of the continent [12]. The genus is economically important, providing hardwood timber, nectar resources for honey, shelter in pastoral regions, fuel, and essential oils [12].

The genus *Eucalyptus* was named in 1788 by the French botanist *Charles-Louis L'Héritier de Brutelle* [12]. *Eucalyptus* means 'well covered' and refers to the operculum (or calyptra), a distinguishing characteristic of the genus that completely covers and protects the many stamens and the single style of eucalyptus flowers in buds [13].

Although hybridization of reproductively compatible *Eucalyptus* species is relatively restricted (occurring in 15% of geographically adjacent pairs), the resulting intermediate forms can make the species identification difficult [13]. The genus has a long and controversial taxonomic history [11][12], with some species remaining undescribed [13]. There is general agreement about what constitutes the group broadly known as 'eucalypts', but there is controversy around different generic level classifications and especially the recognition of the genus *Corymbia* [11].

Eucalypts have been traditionally classified into one of two genera, *i.e.*, *Angophora* Cav. and *Eucalyptus* L'Hér. [13]. The debate over the reclassification of a group of *Eucalyptus* species, colloquially known as ghost-gums and bloodwoods, to the genus *Corymbia* is well documented [10][14]. However, a number of molecular studies now provide convincing evidence to support both *Angophora* and *Corymbia* being recognized as separate genera [11].

The genus *Angophora* consists of nine species, all confined to mainland eastern Australia [11]. There are 113 species of *Corymbia* K.D.Hill & L.A.S.Johnson, all of which occur in either Australia or New Guinea [11]. Four other small genera of rainforest trees are also included in the eucalypt group [11], but are only represented by one or two species.

The practical implications for phytochemical research is that different interpretations of the *Eucalyptus* taxonomy may result in the names of *Corymbia* and *Eucalyptus* being inconsistently applied in the literature, leading to confusion amongst researchers and scholars. Further, it is important to note that the close evolutionary relationship between the sister eucalypt taxa of *Angophora*, *Corymbia*, and *Eucalyptus* means that they most likely have a similar phytochemical profile.

3. Ethnopharmacology. – In Australia and some other parts of the world, *Eucalyptus* has historically been used to treat a variety of ailments. Although there is no strong scientific evidence to elucidate the mechanism of action in many of these cases, many treatments are still considered to be effective, finding favor to the present, suggesting that *Eucalyptus* may contain health-promoting bioactive compounds.

There is no information in the current literature highlighting the use of *Eucalyptus* extracts as a traditional cancer treatment. However, the use of extracts to treat a range of ailments does suggest clinical potential and is further supported by recent *in vitro* and *in vivo* research findings.

3.1. *Flu, Colds, and Fever*. *Eucalyptus* leaves and inner bark are traditionally brewed in water and the decoctions used as a wash to treat colds, flu, and fever. Small amounts of decoction can also be ingested to further aid treatment [15][16]. Smoke generated by placing red or young *Eucalyptus* leaves in a pit over hot coals is also used to relieve

cold, flu, and fever. The user stands, sits, or leans over the smoke source inhaling the smoke. This treatment is considered to be very effective and is still commonly used today in lieu of western medicines [6].

3.2. *Aching, Sore, Internal Pain, and Headache. Eucalyptus* extracts from leaves, stem, and bark have been traditionally employed to alleviate aching, internal pain, and headache. The strained liquids are also used as an antiseptic wash to sterilize sores, cuts, and any skin infections [15]. Extracts are also used as a warm body wash to relieve internal pain associated with influenza, fever, or rheumatism and to reduce the effects of chronic joint pain of the hips, knees, and ankles. It is also effective for relieving chest pain [6]. The strained liquid from leaves can also be taken internally in small quantity, to alleviate nasal congestion and headache [15].

Juice extracted from freshly crushed young *Eucalyptus* leaves can be applied directly to infected skin, to heal sores and cuts [15]. In addition, an astringent viscous exudate (resin), known as *Eucalyptus* kino, found on the trunk and branches of many *Eucalyptus* trees after pathological or mechanical wounding of the wood, is also used for the treatment of sores and cuts. Kino can be applied directly onto infected skin lesions and rubbed gently or dissolved in cold or hot water and used as a wash on cuts and open sores. A fine powder of ground, crystalline kino can also be applied directly on open sores as a healing agent [17].

3.3. *Toothache and Oral Infection*. *Eucalyptus* has been used as a traditional treatment for toothache and oral infection. An aqueous extract prepared by infusing fresh inner bark stripped from trees is commonly used as a mouthwash for mouth sores and tongue inflammation [6]. Solutions prepared by mixing ground kino and water have also been utilized as a general tonic for rinsing the mouth to alleviate the symptoms of toothache. Kino can also be plugged directly into a tooth cavity to act as an analgesic [18], and it is used as tooth cleanser [17].

3.4. *Childbirth*. A number of indigenous communities in Australia have employed *Eucalyptus* leaves as a relaxant during childbirth, with fresh-cut leaves and stems placed on a bed of hot coals. The smoke and vapors released in this process are then inhaled by the mother and newborn [6]. Moreover, extracts from freshly macerated leaves have been rubbed over the mother's breasts to stimulate the milk release. The extracts have also been fed to the newborn to treat thrush of the mouth, when required [6].

3.5. *Other Ailments. Eucalyptus* leaves can be used to relieve the symptoms of chest and respiratory-tract infection by inhaling the vapors from an infusion of freshly picked leaves dispersed in boiling water [19]. Leaves have also been used to alleviate joint and muscle pain by heating them over a small flame and then holding them in contact with the affected body part [15].

Kino mixed with campfire ash is documented as being applied to wounds during male initiation ceremonies, to accentuate the decorative scarring on the chest and arms. Small amounts of the resin boiled in water have also been used to relieve eye soreness by splashing the resulting infusion around the eyes [6][18].

4. Phytochemicals: Volatile and Nonvolatile Compounds in *Eucalyptus*. – 4.1. *Volatile Compounds in* Eucalyptus. *Eucalyptus* contains high levels of volatile organic compounds (VOC), which comprise its essential-oil profile. Both the bark and leaves of *Eucalyptus* have been utilized for extracting essential oil by steam distillation. The

oils contain various VOC, many of which possess antiseptic properties, making them a valuable element in both perfumery and medicinal preparations [1]. The major component found in *Eucalyptus* oil is the monoterpene ether 1,8-cineole (**1**), otherwise known as eucalyptol (*Fig. 2*), which accounts for more than 70% of the oil mass and is responsible for its camphor-like smell. It has been widely applied in the pharmacopoeias of many countries, such as Britain, France, Germany, Belgium, Netherlands, USA, Australia, Japan, and China [1]. The biological activity of 1,8-cineole has been linked to antibacterial, anti-inflammatory, and anticancer effects [20].

Other principal constituents present in *Eucalyptus* oil (*Fig. 2*) include (−)-limonene (**2**) and (+)-α-terpineol (**3**), which are derived from the menth-1-en-8-yl cation, the same biogenetic precursor from which cineole is derived [21].

The VOC present in *Eucalyptus* oil have been extensively profiled using GC and GC/MS analyses. GC/MS Analysis is a convenient and useful analytical tool, as individual compounds separated on the GC column can be immediately identified by comparing the resulting mass spectra against an *in silico* reference library [21].

The extraction yields of essential oils and VOC, especially of 1,8-cineole, were found to be dependent upon the eucalypt source, with oil contents varying widely between species. Indeed, *E. bakeri*, *E. kochii*, *E. camaldulensis*, *E. sparsa*, and *E. polybractea* showed higher levels of essential oil than *E. globulus*, *E. sturgissiana*, and *E. smithii*. Within species, 1,8-cineole levels were shown to be present in lower

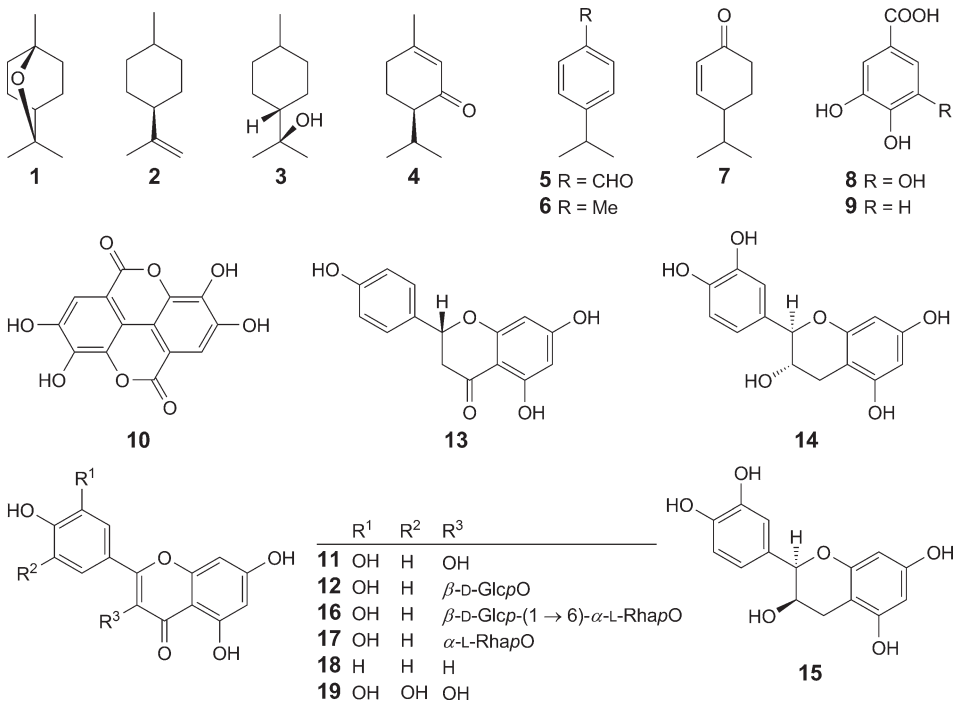

Fig. 2. *Chemical structures of major volatile and non-volatile compounds isolated from* Eucalyptus *species*

concentration in *E. globulus* relative to *E. polybractea* and some other species. Somewhat paradoxically, however, *E. globulus* was recognized as a major source of 1,8-cineole, because of its worldwide availability for use in wood and pulp production [21]. The concentration of other VOC such as compound **3**, D-piperitone (**4**), and cuminal (**5**; *Fig. 2*) also varied between *Eucalyptus* species [22].

Harvesting time, seasonal factors (sunlight, temperature), and habitat conditions (water availability, nutrient levels) all affected the oil composition and yield [23][24]. For example, *Eucalyptus* species grown in Western Australia contained higher concentrations of VOC than similar species growing in Eastern Australia [25]. Oil extracted from *E. camaldulensis* trees grown in Kenya contained higher concentrations of 1,8-cineole compared with plants of the same species growing in Ethiopia. In contrast, the Ethiopian plantations were reported to contain high levels of the terpene derivatives *p*-cymene (**6**) and cryptone (**7**; *Fig. 2*) [26].

As with the extraction of oils from other plants, the extraction yields of *Eucalyptus* oil and VOC were found to be affected by variables such as extraction temperature, time, agitation rates, solvent-to-sample ratios, and the physicochemical properties of the extraction solvent [27]. Steam distillation is the primary commercial extraction method for obtaining *Eucalyptus* essential oils [28]. Other methods utilized include hydrodistillation, solvent extraction, *Soxhlet* extraction, and thermal desorption [29][30]. However, these methods are generally considered to be low yielding, more time consuming, and resulting in the production of an inferior quality product, due to the degradation of key components, including unsaturated hydrocarbons and esters. More recently, research has explored the use of more passive technologies such as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), and ultrasonic-assisted extraction (UAE), in the hope of improving the quality and quantity of oil recovered. These protocols, however, require high capital outlay in comparison to steam distillation and are often limited in the quantity of material that they can process in a single event [29][31].

Eucalyptus VOC not only contribute to fragrance, but also exhibit pharmaceutical properties such as stimulation of the mucous-secreting cells in the nose, throat, and lungs and antiseptic effects [32]. As a consequence, *Eucalyptus* oil is utilized in numerous commercial preparations, encompassing multi-purpose applications including insect repellents, aromatherapy, personal hygiene products, oral health care, cleaning products, and medicines [32]. It is therefore important to consider all factors affecting the extraction yield of essential oil and VOC and their impact on quantity, quality, and cost-effectiveness in producing oil for commercial purposes.

4.2. *Nonvolatile Compounds in* Eucalyptus. Phenolic compounds have been reported as the major nonvolatile compounds in *Eucalyptus*, and they have been found as major contributors to the antioxidant activities of *Eucalyptus* extracts (*Table 1*). The total phenolic content (TPC) is a commonly estimated antioxidant property, determined using the *Folin–Ciocalteu* reagent assay, while the general antioxidant capacity (encompassing a broader range of antioxidant compounds) is estimated using a range of chemical techniques including the DPPH (2,2-diphenyl-1-picrylhydrazyl)-radical scavenging, ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-radical cation scavenging, FRAP (ferric reducing ability of plasma), deoxyribose, and superoxide anion scavenging assays [38]. Phenolic compounds have

Table 1. *Total Phenolic Content and Antioxidant Properties of* Eucalyptus *Species*

Species (plant part)	Extraction conditions[a])	Total phenolic content [mg GAE/g][b])	Antioxidant capacity[c])	Reference
E. globulus (bark)	EtOH/H_2O 50 : 50 (*v*/*v*)	22.30	FRAP: 2146 nmol AAE/mg	[33]
E. robusta (fruit)	Tetrahydrofuran, then MeOH/AcOH/H_2O 50 : 3.7 : 46.3 (*v*/*v*)	54.80	FRAP: 502 μmol Fe(II)/g	[34]
E. globulus (bark)	52% EtOH, 264 min, 82.5°	30.40	FRAP: 2.16 mmol AAE/g	[35]
E. globulus (bark)	H_2O, 60 min, 100°	25.80	FRAP: 0.119 mmol AAE/g	[36]
E. globulus (leaf)	H_2O, 10 min, 40°	311.00[d])	DPPH: IC_{50} = 12.00 μg/ml	[37]
E. gomphocephala (leaf)	70% aq. acetone	389.05[d])	DPPH: IC_{50} = 15.42 μg/ml	[38]
E. globulus (bark)	SFE: 70°, 20% EtOH, 10 g of CO_2/min	57.22	DPPH: 49.74 mg AAE/g	[39]
E. camaldulensis (leaf)	MAE: 50% EtOH, 20 : 1 ml/g, 5 min, 600 W	77.00	ND	[40]
	UAE: 50% EtOH, 20 : 1 ml/g, 30–40°, 1 h	82.00	ND	
	CE: 50% EtOH, 20 : 1 ml/g, 25°, 24 h	82.00	ND	

[a]) SFE: Supercritical fluid extraction; MAE: microwave-assisted extraction; UAE: ultrasound-assisted extraction; CE: conventional extraction. [b]) Total phenolic content given in mg gallic acid equivalent/g of dried extract. [c]) FRAP: Ferric reducing ability of plasma; DPPH: DPPH-radical scavenging activity; AAE: ascorbic acid equivalent; ND: no activity detected. [d]) Given in mg/g of dried extract.

been reported to be abundant in *Eucalyptus* extracts, comprising *ca.* 39% of the extracts prepared from *E. gomphocephala* leaves [38] and 31% of the extract prepared from *E. globulus* leaves [37]. *Eucalyptus* extracts therefore represent a potentially important source of phenolics for value adding to food and pharmaceutical products.

More than 20 individual nonvolatile compounds have been isolated from various *Eucalyptus* species (*Table 2*) using GC and HPLC techniques, either alone or in combination with an auxiliary spectroscopic technique such as MS or NMR. The phenolic compounds shown in *Fig. 2*, such as the phenolic acids gallic acid (**8**), protocatechuic acid (**9**), and ellagic acid (**10**), have been identified in *Eucalyptus* extracts [36][38], together with other phenolic compounds such as quercetin (**11**), quercetin 3-glucoside (=quercetin 3-*O*-*β*-D-glucopyranoside, **12**), naringenin (**13**), catechin (**14**), epicatechin (**15**), rutin (**16**), quercitrin (=quercetin 3-*O*-*α*-L-rhamnopyranoside, **17**), apigenin (**18**), and myricetin (**19**) [36][38]. Moreover, monoterpenes, cyanogenic glycosides, and the triterpene cladocalol have also been identified [2]. Many of these compounds are associated with health benefits including strengthening of the immune system, reducing the risk of diabetes, obesity, and cardiovascular diseases [59–62].

Links between nonvolatile bioactive compounds isolated from *Eucalyptus* species and anticancer activity have been reported (*Table 3*); however, the number of studies undertaken to date is limited. This fact, together with the relatively small number of phytochemical studies undertaken on eucalypt species, suggests that untapped potential remains for the further characterization of bioactive compounds from species of this genus.

The extraction efficiency of bioactive compounds from source materials can be influenced by a variety of factors including the plant species, growing location, seasonality, sample preparation method, and extraction conditions [70]. As with the extraction of essential oils from *Eucalyptus*, both conventional and advanced techniques have been applied to capture bioactive compounds from *Eucalyptus* (*Table 2*). The advanced extraction techniques SFE, MAE, and UAE have all proven effective in extracting high levels of bioactive compounds [39][40][54][71]. Information on the effect of the aforementioned variables on the yield of nonvolatile compounds remains limited and requires further study. The optimization of the preparation methods, including drying techniques, drying times, storage methods, and sample-preparation techniques, needs to be fully investigated to establish quality control parameters for the efficient extraction of bioactive compounds.

5. Anticancer Activities of *Eucalyptus* Extracts. – Limited information has been published on the link between *Eucalyptus* oils or extracts and cancer in human clinical trials; however, numerous *in vitro* and several *in vivo* studies have been conducted to assess the anticancer properties of extracts obtained from different plant parts of *Eucalyptus* (*Table 4*). Antiproliferative and cytotoxic studies with *Eucalyptus* oils and extracts have been undertaken, revealing positive effects against several cancer-cell lines. Essential oils extracted from *E. globulus*, *E. torquata*, *E. sideroxylon*, and *E. benthamii* have shown to inhibit the nuclear translocation of NF-*κ* B induced by LPS in leukemic monotype THP-1 cells. These same extracts also exhibited cytotoxic effects on the human breast adenocarcinoma cell line (MCF7), Jurkat cells (J774A.1), and

Table 2. *Extraction and Identification of Volatile and Nonvolatile Compounds Derived from* Eucalyptus *Species*

Species (plant part)	Extraction method[a])	Identification method	Volatile and nonvolatile compounds	Reference
E. globulus (bark)	SFE: 70°, 20% EtOH, 10 g of CO_2/min	HPLC-UV	Gallic acid (**8**), protocatechuic acid (**9**), ellagic acid (**10**), quercetin (**11**), naringenin (**13**)	[39]
E. globulus (bark)	SFE: 160 bar, 40°, 8% EtOH and *Soxhlet* extraction with CH_2Cl_2	GC/MS	β-Sitosterol, β-amyrin, betulonic acid, oleanolic acid, betulinic acid, ursolic acid, 3-acetyloleanolic acid, 3-acetylursolic acid	[41]
E. globulus (fruit)	95% EtOH	^{1}H-NMR	Eucalyptals D and E	[42]
E. globulus (bark)	52% EtOH, 264 min, 82.5°	GC/MS	Galacturonic acid, 4-*O*-methylglucuronic acid, **8**	[43]
E. globulus (bark)	H_2O, 60 min, 100°	HPLC	Catechin (**14**), epicatechin (**15**), **10**, quercitrin (**17**), isorhamnetin	[36]
E. globulus (leaf)	H_2O, 10 min, 40°	HPLC	Chlorogenic acid, **10**, rutin (**16**), **17**	[37]
E. globulus (leaf)	MAE: 60 sec, 1000 W, 10:1 ml/g	GC	1,8-Cineole (**1**), α-pinene, camphene, *p*-cymene (**6**), limonene (**2**), γ -terpinene	[44]
E. gomphocephala (leaf)	70% aq. acetone	LC-PDA/ESI-MS/MS	Chlorogenic acid, cypellocarpin B, ellagic acid hexoside, apigenin glucuronide, **8**, globuluside, myricetin hexoside, quercetin hexoside, galloyl cypellocarpin B, **14** or **15**	[38]
E. camaldulensis (fruit)	Direct thermal desorption	GC/MS	Aromadendrene, **1**, γ-gurjunene, terpinolene, spathulenol, α-pinene, ledene, longifolene	[45]
E. citriodora[b]) (leaf)	Steam distillation and solvent extraction (Et_2O)	GC/MS	Citronellal, citronellol, citronellyl acetate, *cis*-*p*-menthane-3,8-diol, *trans*-*p*-menthane-3,8-diol	[46]
E. citriodora[b]) (leaf)	Microwave-assisted HS-SPME	GC-FID and GC/MS	β-Pinene, eucalyptol, γ-terpinene	[47]
E. citriodora[b]) (leaf)	Hydrodistillation	GC/MS	Citronellal, isopulegol, 3,8-terpinolhydrate, citronellol, citronellic acid	[48]
E. citriodora[b]) (leaf)	Hydrodistillation and SFE	GC/MS	Citronellal, citronellol, isopulegol, α-terpinene, neoisopulegol, *p*-mentha-1(7),8-diene, *p*-mentha-3,8-diene, sabinene, α-phellandrene	[49]
E. dunnii, *E. citriodora*[b]), and *E. saligna* (leaf)	HS-SPME	GC/ITMS	(3*E*)-4,8-dimethylnona-1,3,7-triene, (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene, (*E*,*E*)-α-farnesene, (*E*,*E*,*E*)-3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene, β-caryophyllene, α-humulene, germacrene D, β-cubebene	[50]

Table 2 (cont.)

Species (plant part)	Extraction method[a])	Identification method	Volatile and nonvolatile compounds	Reference
E. dunnii, *E. citriodora*[b]), and *E. saligna* (leaf)	HS-SPME	GC/ITMS	(*E*)-β-Ocimene, β-caryophyllene, α-pinene, **6**, **2**, **1**, campholenal, **3**, γ-terpinene, campholenal, *trans*-carveol, rose oxide	[51]
E. jensenii (leaf)	Acetone	RP-HPLC	Quercetin 3-glucoside (**12**), sideroxylin, 4-*O*-demethyl miniatone, grandinol, jensenone, miniatone, euglobals G1, G2, G3, and G4	[52]
E. hybrida (leaf)	MAE with organic solvents	HPTLC	**8**	[53]
E. hybrida (leaf)	MAE with organic solvents	HPLC	Ursolic acid	[54]
E. spathulata, *E. salubris*, *E. brockwayi*, and *E. dundasii* (leaf)	Steam distillation: aqueous volatile fractions (AVFs)	GC/MS	**1**, pinocarvone, *trans*-pinocarveol, **3**, globulol, isomenthol	[55]
E. cinerea and *E. camaldulensis* (leaf)	Hydrodistillation and SFE	GC/MS	**1**, *p*-menth-1-en-8-ol, terpinen-4-ol, α-pinene, 8,14-cedranoxide, **6**, **2**, myrtanal, caryophyllene alcohol	[56]
E. camaldulensis (leaf)	SFE	HPLC	**8**, **10**, 5-hydroxy-7,4′-dimethoxyflavone, 5-hydroxy-7,4′-dimethoxy-8-methylflavone	[57]
E. camaldulensis and *E. radiate* (leaf)	Hydrodistillation and SFE	GC/MS	**1**, α-pinene, β-pinene, **6**, terpinen-4-ol, **3**, globulol	[58]

[a]) SFE: Supercritical fluid extraction; MAE: microwave-assisted extraction; HS-SPME: headspace solid-phase microextraction. [b]) The currently accepted name of *Eucalyptus citriodora* is *Corymbia citriodora*.

Table 3. *Selected Nonvolatile Bioactive Compounds Isolated from* Eucalyptus *Species and Their Link with Anticancer Activities*

Compounds	Anticancer activity and/or mechanism of action	Reference
Globulusin A and eucaglobulin	Concentration-dependent suppression of inflammatory cytokine production, tumor-necrosis factor-α, and interleukin-1β in cultured human myeloma THP-1 cells co-stimulated with phorbol myristate acetate; inhibition of melanogenesis in cultured murine melanoma B16F1 cells without any significant cytotoxicity	[2]
Euglobal III, Ib, IIa, Ic, Ia1, and Ia2	Strong antitumor-promoting activity *in vitro* on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced *Epstein–Barr* virus early antigen (EBV-EA); the activity of euglobal III was higher than those of euglobals Ib, IIa, Ic, Ia1, and Ia2	[63]
Euglobal-G1, -G5, -Am-2, and -III	Significant inhibitory effects on *Epstein–Barr* virus (EBV) activation induced by the tumor promoter TPA; euglobal-G1 and -III also exhibited strong inhibition of the effect of the cell cycle induced by TPA and showed remarkable antitumor-promoting effects on mouse skin tumors in an *in vivo* two-stage carcinogenesis test	[64]
Euglobal-1	Remarkable inhibitory effect on mouse skin tumors induced by 7,12-dimethylbenz[*a*]-anthracene (DMBA) and promoted by fumonisin-B1 (mycotoxin produced by *Fusarium moniliforme*) in a two-stage carcinogenesis test; potent antitumor-promoting activity on mouse pulmonary tumors in a two-stage carcinogenesis test using 4-nitroquinoline-*N*-oxide (4-NQO) as initiator and glycerol as promoter	[4]
Luteolin, apigenin (**18**), and quercetin (**11**)	Inhibition of glycogen synthase kinase 3β (GSK-3β), leading to decreased cancer cell proliferation and survival by abrogating the nuclear factor κB (NFκB) activity and, hence, suppression of the growth of pancreatic tumors	[65]
3β-Acetoxy-urs-11,13(18)-dien-28-oic acid, 3β-hydroxy-urs-11-en-28,13β-olide, 3β-acetoxy-urs-11-en-28,13β-olide; 3-acetyl-betulinic acid; oleanolic acid; ursolic acid, and β-amyrin acetate	Moderate activity (IC_{50} = 9.5–38.5 mg/ml) against A2780 human ovarian cancer cell lines; ursolic and oleanolic acids exhibited the highest activity with IC_{50} values of 9.5 and 10.7 mg/ml, respectively	[66]
Cladocalol (17β-(formyloxy)-28-*nor*-urs-12-ene-3β-ol), 28-*nor*-urs-12-ene-3β,17β-diol, and 3β-(acetyloxy)-17β-(formyloxy)-28-*nor*-urs-12-ene	Cytotoxic activity on HL-60 human promyelocytic leukemia cells as revealed by the MTT assay	[67]
	Cytotoxic effect against Jurkat and J774A.1 cell lines, similar to that observed for volatile oils	[68]
Cypellocarpins A, B, and C, and a related chromene glucoside	Potent *in vitro* antitumor-promoting activity in a short-term bioassay evaluating the inhibitory effect on EBV-EA activation induced by TPA; suppression of *in vivo* two-stage carcinogenesis induced with NO and TPA on mouse skin	[69]

Table 4. *Anticancer Activities of Essential Oils, Extracts, and Isolated Compounds Obtained from Various* Eucalyptus *Species*

Species (plant part)	Test sample (oil, extract, or compound)	*In vitro* and *in vivo* test systems (cells, organs, tumors)	Effects and/or related mechanisms	Reference
E. citriodora[a]) (leaf)	Organic-solvent and H_2O crude extracts	*In vitro:* colon (SW-620), liver (HEP-2), ovary (OVCAR-5), prostate (PC-3), cervix (HeLa), neuroblastoma (IMR-32), and lung (HOP-62) cell lines; *in vivo: Ehrlich* ascites carcinoma (EAC)	Growth inhibition and suppression	[27]
E. globulus (bark)	EtOH crude extract	MDA MB-231 Human breast adenocarcinoma cells	Antiproliferative effects	[35]
E. globulus (fruit)	Aromadendrene, cadinene, and a spirosesquiterpene	Human BGC-823 and KE-97 gastric, Huh-7 hepatocarcinoma, and Jurkat T lymphoma cancer cell lines	Cytotoxic effects	[42]
E. globulus (leaf)	Essential oil	THP-1 Leukemic monocyte cells	Inhibition of nuclear translocation of NFκB induced by LPS	[72]
E. maidenii[b]) (branches)	Resveratrol, piceatannol, gallic acid (**8**), and macrocapal G	Breast cancer (MCF-7), hepatocellular carcinoma (SMMC-7721), human myeloid leukemia (HL-60), colon cancer (SW480), and lung cancer (A-549) cells	Moderate inhibitory effects on HL-60 cells; moderate inhibitory effect on SMMC-7721 cells observed only for macrocapal G; no effect on other cell lines	[5]
E. camaldulensis (leaf)	Petroleum ether (PE) extract	*In vivo*: *Ehrlich* ascites carcinoma (EAC)	Reduced tumor-growth rate and enhanced the life span of EAC bearing mice. Toxic effect of the extract is minimum and mostly reversible with time.	[73]
E. camaldulensis (leaf)	AcOEt and BuOH extracts	MCF 7 and MDA-MB-231 Human breast adenocarcinoma cells	Cytotoxic effects (IC_{50} = 5–41 μg/ml)	[74]
E. camaldulensis (leaf)	Aqueous acetone extract	MCF-7 Human breast adenocarcinoma cells	Growth-inhibitory effect (IC_{50} = 36.5 μg/ml)	[75]
E. camaldulensis (leaf and fruit)	MeOH, hexane, and CH_2Cl_2 extract	A2780 Ovarian cancer cells	Antiproliferative effects; fruit extracts were more potent than leaf extract	[66]

Table 4 (cont.)

Species (plant part)	Test sample (oil, extract, or compound)	*In vitro* and *in vivo* test systems (cells, organs, tumors)	Effects and/or related mechanisms	Reference
			(IC_{50} = 17.5 and 17.2 *vs.* 19.3 μg/ml, resp.)	
E. camaldulensis (resin)	MeOH extract	ECV-304 Human bladder carcinoma cell line with endothelial properties	Cytotoxic effect (IC_{50} = 20.7 μg/ml)	[76]
E. torquata (stem and leaf)	Essential oils	MCF 7 Human breast adenocarcinoma cells	Cytotoxic effects: stem extract (IC_{50} = 1.34 μg/ml) > leaf extract (IC_{50} = 5.22 μg/ml)	[77]
E. sideroxylon (leaf)	Essential oils	MCF 7 Human breast adenocarcinoma cells	Cytotoxic effects (IC_{50} = 6.76 μg/ml)	[77]
E. cladocalyx (leaf)	Cladocalol (17β-(formyloxy)-28-*nor*-urs-12-ene-3β-ol), 28-*nor*-urs-12-ene-3β,17β-diol, and 3β-(acetyloxy)-17β-(formyloxy)-28-*nor*-urs-12-ene	HL-60 Human promyelocytic leukemia cells	Cytotoxic effects (IC_{50} = 42, 51, and 83 μM)	[67]
E. benthamii (leaf)	Essential oils, α-pinene, terpinen-4-ol, and γ-terpinene	Jurkat, J774A.1, and HeLa cells	Cytotoxic effects (essential oils > α-pinene and γ-terpinene); cytotoxic activity probably involved cell death by apoptosis	[68]
E. cypellocarpa (leaf)	Cypellocarpins A, B, and C, and a related chromene glucoside	*In vitro*: Raji cells; *in vivo*: mouse skin tumor	30% Reduction of tumor-carrying mice after 10 weeks at a concentration of 50 mol ratio of 12-*O*-tetradecanoylphorbol-13-acetate relative to control	[69]
E. grandis (leaf)	Euglobal-G1	Mouse skin and mouse pulmonary tumors	Inhibition of the two-stage carcinogenesis	[4]

[a]) The currently accepted name of *Eucalyptus citriodora* is *Corymbia citriodora*. [b]) The currently accepted name of *Eucalyptus maidenii* is *Eucalyptus globulus* subsp. *maidenii*.

HeLa cell lines [68][72][77]. The level of cytotoxic activity differed, depending on the part of the plant from which the extract was sourced [77]. Therefore, examination of extracts from all parts of *Eucalyptus* plants is necessary, to effectively screen for bioactive compounds.

Both aqueous and organic solvent crude extracts from different *Eucalyptus* species have been reported to exhibit cytotoxic and antiproliferative effects *in vitro*. Extracts from *E. citriodora*, *E. globulus*, *E. maidenii*, and *E. camaldulensis* were found to inhibit the growth of colon (SW-620, SW480), liver (HEP-2), ovary (OVCAR-5, A2780), prostate (PC-3), cervix (HeLa), neuroblastoma (IMR-32), lung (HOP-62, A-549), breast (MCF7, MDA-MB-231), and gastric (BGC-823, KE-97) cell lines [27][35][73]. Crude *E. citriodora* extracts were also shown to suppress the growth of *Ehrlich* ascites carcinoma [27]. It is of interest to note that the cytotoxic and antiproliferative effects of the extracts also varied according to both the extraction method and extracting solvent used. For example, AcOEt extracts were found to exhibit higher inhibition on cancer cell lines than extracts with other organic solvents such as MeOH and EtOH [27]. These differences in inhibition can be explained by the relative compatibilities between the solvent and the physicochemical properties of the bioactive compounds. Optimization of the extraction conditions (including sequential extraction using solvents of varying polarity) is important to ensure the complete capture of potential bioactive compounds.

Several individual volatile compounds such as 1,8-cineole, α-pinene, terpinen-4-ol, and γ-terpinene have been isolated and tested against several cancer cell lines, showing favorable results in *in vitro* studies [20][68]. In addition, several nonvolatile compounds such as euglobal-G1 isolated from *E. grandis* and resveratrol, piceatannol, and macrocapal G isolated from *E. maidenii* have shown to inhibit the growth of several cancer cell lines *in vitro* [4][5]. Other nonvolatile compounds such as cypellocarpins and chromene glucosides (isolated from *E. cypellocarpa*) were found to reduce the tumor growth *in vivo* [69].

To fully explore *Eucalyptus* for potential health benefits (especially for anticancer properties), a clear pathway for future research is required. Future studies need to screen key volatile and nonvolatile compounds from various *Eucalyptus* species and then develop the optimum extraction and isolation conditions to prepare the crude extracts with high contents of bioactive compounds. To achieve maximum efficiency, the crude extracts must be tested for their anticancer properties, to formally identify the most potent extracts, which can then be purified to isolate and identify individual compounds, to eventually elucidate their anticancer mechanisms. These compounds will then serve as the starting point for synthetic studies to develop analogues exhibiting greater potency and specificity as therapeutic agents.

6. Conclusions. – The available preliminary data from the literature has revealed that, although studies are limited on several *Eucalyptus* species so far, the genus *Eucalyptus* is an abundant source of phytochemicals that possess potent antioxidant capacity and exhibit antiproliferative and cytotoxic effects against different types of cancer cells. With numerous species having not yet been studied, the opportunities of screening to find new key bioactive components for the development of anticancer agents are enormous. Thus, future studies are needed to screen, extract, purify, and

identify the key bioactive compounds from plants of the very promising genus *Eucalyptus* and then test their anticancer properties *in vitro*, *in vivo*, and in clinical trials, to develop novel therapeutic agents for the prevention and/or treatment of cancers.

We acknowledge the funding support from the *Ramaciotti Foundation* (ES2012/0104), the *Hunter Cancer Research Alliance* (HCRA), the Hunter Medical Research Institute (HMRI), and the University of Newcastle.

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Received September 3, 2014

Additional Research paper 3

Bahareh Saberi, Rahul Thakur, **Deep Jyoti Bhuyan**, Quan V. Vuong, Suwimol Chockchaisawasdee, John B. Golding, Christopher J. Scarlett, Costas E. Stathopoulos. Development of edible blend films with good mechanical and barrier properties from pea starch and guar gum. **Starch/Stärke (2017),** DOI: 10.1002/star.201600227

RESEARCH ARTICLE

Development of edible blend films with good mechanical and barrier properties from pea starch and guar gum

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The individual and interactive impacts of guar gum and glycerol on the pea-starch-based edible film characteristics were examined using three factors with three-level Box–Behnken response surface design (BBD). The results showed that density and elongation at break were only significantly ($p < 0.05$) affected by pea starch and guar gum in a positive linear fashion. The quadratic regression coefficient of pea starch showed a significant effect ($p < 0.05$) on thickness, density, puncture force, water vapor permeability, and tensile strength, while tensile strength and Young's modulus were affected by the quadratic regression coefficient of glycerol and guar gum, respectively. The results were analyzed using Pareto analysis of variance (ANOVA) and the developed predictive equations for each response variable presented reliable and satisfactory fit with high coefficient of determination (R^2) values ($\geq$0.96). The optimized conditions with the goal of maximizing mechanical properties and minimizing water vapor permeability were 2.5 g pea starch, 0.3 g guar gum, and 25% w/w glycerol based on the dry film matter in 100 mL of distilled water.

Received: July 15, 2016
Revised: August 9, 2016
Accepted: August 9, 2016

Keywords:
Edible films / Guar gum / Mechanical properties / Pea starch / Response surface methodology

1 Introduction

The application of biodegradable resources with characteristics that ensure food safety along with decreasing the environmental impacts has gained a significant amount of interest worldwide [1]. Contrary to synthetic polymers, materials made from polysaccharides are commonly eco-friendly because they can undergo disintegration without environmentally damaging remainders [2]. As polysaccharides are mostly available in nature and are known as structural substances, they have been regarded as proper alternatives for biodegradable films [3].

Starch has the capability to be used for edible films production because it can produce tasteless, odorless, and transparent films with similar properties to synthetic polymers [4]. However, starch edible films generally show poor mechanical strength and high moisture sensitivity [5]. To overcome these drawbacks, studies on different additives, sources of starch, various modifications, and process factors have been conducted [6–9]. The physical and functional characteristics of starch films can be improved by combining with other biopolymers, hydrophobic materials, and antioxidant/antimicrobial compounds [10–13]. The blending of starch and other hydrocolloids has been shown to modify the mechanical properties of the resultant film [9], which

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Abbreviations: PD, puncture deformation; **RSM,** response surface methodology; **WVP,** water vapor permeability

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depends on the compatibility/incompatibility of binary polymeric blends [9, 14]. Moreover, incorporation of hydrocolloids in starch edible films results in increasing viscosity of starch systems [9, 15] and decreasing their retrogradation rate [16]. Interactions between starch and other hydrocolloids are determined by their molecular weight, chemical structures, conformations, and hydration behaviors [14].

Recent studies have investigated the effect of the incorporation of hydrocolloids on physical and mechanical properties of different starch films. It has been indicated that the incorporation of the xanthan gum (0–0.1% w/w) to cassava starch (3–5%) did not show a considerable impact on either the mechanical properties or on the water absorption kinetics of films [17]. Bangyekan et al. [18] reported that the water vapor permeability (WVP) of cassava starch films was reduced with increasing chitosan concentration and increased with increasing in glycerol content. Lafargue et al. [19] studied the combination of hydroxypropylated pea starch with κ-carrageenan to make edible films. They showed an improvement in film drying and formation, without any changes in its properties. Chillo et al. [20] observed that the mechanical properties of tapioca starch films improved by incorporation of chitosan. While, WVP and mechanical strength and elongation of tapioca starch films increased by addition of hsian-tsao leaf gum [21]. da Matta et al. [22] found that the addition of xanthan gum to the green pea starch with high content of amylose (*cv. Utrillo*), had no effect on mechanical properties of the films. The association of agar to maize starch films increased the transparency, thickness, and WVP of the obtained films [4].

We have previously demonstrated that incorporation of guar gum and pea starch resulted in biocomposite edible films with improved physical and optical properties [23]. However, to the best of our knowledge, there is no specific study on the effect of blending guar gum on mechanical and barrier properties of pea starch edible films. Guar gum is derived from the endosperm of an annual legume plant *Cyamopsis tetragonoloba*, which is grown mainly in India and Pakistan and to a smaller extent in Australia, Africa, and United States, is a type of galactomannan [24]. Seed galactomannans from various legume plants have some common structural properties, though they vary noticeably in their molecular weight, ratio of the component sugars (mannose-to-galactose ratio, or M:G), the specific location of single galactose residues on the linear mannose backbone in their molecule, and their functional characteristics [25]. There are a number of galactomannans used industrially, namely tara gum with a M/G ratio of 3:1, locust bean gum with an M/G ratio of 4:1 [26, 27], and fenugreek gum with an M/G ratio of 1:1. According to these ratios, guar gum is most similar to fenugreek gum. Guar gum is a linear galactomannan, the molecule of which is composed of a $\beta(1 \rightarrow 4)$-linked mannopyranose backbone, with several its branch points (grafts) from the C-6 position of mannopyranose, linked by $\alpha(1 \rightarrow 6)$ bond to a single D-galactopyranose sugar [28]. It has many applications owing to low cost and many distinctive characteristics including biodegradability, biocompatibility, and non-toxicity [24]. Owing to long polymeric chain, high molecular weight, and wide availability of guar gum, it is a potential alternative for the development of a renewable source-based biodegradable packaging material as in comparison with other biopolymers [29]. Very few reports exist on guar gum-based biodegradable packaging films [23, 29–32].

Therefore, the aim of the present work was to develop a biocomposite edible film by combining pea starch and guar gum and assessing the effect of different ratios of these polysaccharides on barrier and mechanical properties of the film using response surface methodology (RSM) analysis.

2 Materials and methods

2.1 Materials

Canadian non-GMO yellow pea starch with 13.2% moisture, 0.2% protein, 0.5% fat, 0.3% ash, and $36.25 \pm 0.32\%$ amylose, was used in all experiments (supplied by Yantai Shuangta Food Co., Jinling Town, P.R. China). Guar gum (E-412) was purchased from The Melbourne Food Ingredient Depot, Brunswick East, Melbourne, Australia. All other chemicals were purchased from Merck Millipore Pty. Ltd., Victoria, Australia.

2.2 Films preparation

The film-forming solution was prepared by dissolving pea starch (2–3 g) and guar gum (0.1–0.5 g) in 100 mL degassed deionized water with gentle heating (about 40°C) and magnetic stirring, followed by addition of 15, 25, and 35% w/w glycerol based on the dry weight of film matter. The dispersion was then heated at 90 °C for 20 min with gentle magnet stirring to allow complete gelatinization of the starch. After gelatinization, the film solution was cooled to room temperature with gentle magnetic stirring for 1 h to reduce air bubbles. All the films were prepared by casting method where 20 g of filmogenic suspensions were poured onto Petri dishes (10 cm in diameter). Films were formed by drying at 40°C in an oven until reaching constant weight (about 24 h). The prepared films were peeled-off from Petri dishes and equilibrated at 25°C, 65% relative humidity for 72 h prior to further examination [33].

2.3 Film thickness and density

A digital micrometer (Mitutoyo Corp., Code No. 543-551-1, Model ID-F125, Japan) was used to determine the thickness (THI) of the films. Ten measurements were randomly taken at different locations for each specimen and the mean value

was reported and used in the calculations of the mechanical properties and WVP. Film density (D) was evaluated by dividing the film weight by the film volume, where the film volume was calculated by multiplying the film area by the thickness [34].

2.4 Water vapor permeability

The water vapor transmission of the films was determined gravimetrically according to the ASTM E96 procedure [35] with a 75% RH gradient at 25°C. Permeation cells containing anhydrous $CaCl_2$ (0% RH) were sealed by the test film (0.7065 mm^2 film area) using melted paraffin (leaving an air gap of 1 cm between the film and the desiccant). To keep a 75% RH gradient across the film, a saturated NaCl solution (75% RH) was used in the desiccators. The RH inside the cell was always lower than outside, and water vapor transport was determined using the weight gain of the cell at a steady state of transfer. Changes in the weight of the cell were recorded to the nearest 0.0001 g and plotted as a function of time. The slope of each line was evaluated by linear regression ($r^2 > 0.99$), and the water vapor transmission rate was calculated through the slope of the straight line (g/s) divided by the test area (m^2). All values for water vapor transmission rate (WVTR) were corrected for air-gap distance between the calcium chloride and the film surface according to the equations of Gennadios et al. [36]:

$$\mathrm{WVTR} = \frac{\text{slope}}{\text{film area}} = \frac{\Delta m}{\Delta t \times A} \tag{1}$$

After the permeation tests, the film thickness was measured and WVP ($g\,Pa^{-1}\,s^{-1}\,m^{-1}$) was calculated as:

$$\mathrm{WVP} = \frac{\Delta m}{A\Delta t}\frac{X}{\Delta P} \tag{2}$$

where $\Delta m/\Delta t$ is the weight of moisture gain per unit of time (g/s), X is the average film thickness (mm), A is the area of the exposed film surface (m^2), and ΔP is the water vapor pressure difference between the two sides of the film (Pa). WVP was measured for three replicated samples for each type of film [37].

2.5 Mechanical tensile test

The mechanical properties of the films were determined using a Texture Analyzer (LLOYD Instrument Ltd., Fareham, UK) according to ASTM standard method D882-00 [38]. Eight film specimens ($40 \times 15\ mm^2$) of each formulation were used for mechanical tests and fixed between the grips of the machine. The maximum load (N) and extension (mm) curves were recorded to calculate tensile strength (TS), percent elongation at break (%E), and Young's modulus (YM) at break of the films using a tensile test at crosshead speed of 1 mm/s and initial grip distance 40 mm [37].

2.6 Mechanical puncture test

The puncture test was performed to realize the mechanical resistibility of films under sharp stress by using a Texture Analyzer (LLOYD Instrument LTD, Fareham, UK). Films were cut into a 4 cm-diameter disk and fixed in an annular ring clamp (3 cm diameter). A spherical probe of 1.0 mm diameter was moved vertically to the film surface at a constant speed of 1 mm/s until the probe passed through the film, and force–deformation curves were plotted. Force (N) and deformation (mm) values at the puncture point were then recorded to represent the puncture force (PF) (N) and deformation (PD) (mm) of the films. For each sample, eight replicates were performed [37].

2.7 Experimental design

The effect of process parameters (pea starch (X1): 2–3 g, glycerol (X2): 15–35%, and guar gum (X3): 0.1–0.5 g) on film properties (Table 1) was studied by applying a three-level-three-factor, Box–Behnken response surface design (BBD) with five central point replicates. The preliminary single factor tests were performed to select the optimum levels of the independent variables (data are not shown). All experimental runs are listed in Table 2. The experimental data obtained for the 17 experimental runs were fitted to the following second-order polynomial model:

$$Y = \beta_0 + \sum_{i=1}^{k}\beta_i X_i + \sum_{i=1}^{k-1}\sum_{j=2}^{k}\beta_{ij} X_i X_j + \sum_{i=1}^{k}\beta_{ii} X_i^2 + e_i \tag{3}$$

where various X_i values are independent variables affecting the responses Y; β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, interaction coefficients of linear, quadratic, and the second-order terms, respectively, and k is the number of variables [39].

2.8 Statistical analysis

The model equations, the 3D- and 2D-contour plots of variable responses and the optimum values for the three independent variables were established by JMP software (Version 11, SAS, Cary, NC, USA). The lack of fit and the

Table 1. Independent variables and their code variable levels used for the Box–Behnken design

	Independent variables		
Coded variable levels	Pea starch (g)	Glycerol (% w/w)	Guar gum (g)
+1	2	15	0.1
0	2.5	25	0.3
−1	3	35	0.5

Table 2. Box–Behnken experimental design with process variables (un-coded) and observed responses

Run	Starch (g)	Glycerol (%w/w)	Guar gum (g)	THI (mm)	$D \times 10^{-3}$ ($g\,mm^{-3}$)	WVP $\times 10^{-10}$ ($gPa^{-1}\,s^{-1}\,m^{-1}$)	TS (MPa)	E (%)	YM (MPa)	PF (N)	PD (mm)
1	2	25	0.1	0.110	1.225	6.702	6.605	25.372	26.049	6.935	9.927
2	2	15	0.3	0.119	1.229	8.161	12.863	19.216	68.305	8.129	7.459
3	2	35	0.3	0.122	1.281	9.367	8.449	21.218	40.172	7.881	8.870
4	2	25	0.5	0.128	1.366	10.673	15.999	13.525	119.356	11.842	6.175
5	2.5	15	0.1	0.115	1.680	11.368	23.417	18.033	130.278	15.479	7.426
6	2.5	35	0.1	0.121	1.708	12.721	19.538	20.134	98.136	13.812	8.842
7	2.5	25	0.3	0.131	1.720	13.674	26.695	15.155	177.636	18.949	6.776
8	2.5	25	0.3	0.129	1.740	13.851	27.240	13.226	170.960	21.049	5.878
9	2.5	25	0.3	0.129	1.680	13.971	24.698	14.281	172.945	18.093	7.661
10	2.5	25	0.3	0.128	1.700	14.173	28.057	17.353	161.682	18.705	6.788
11	2.5	25	0.3	0.130	1.760	14.046	26.783	15.759	169.959	20.648	6.594
12	2.5	15	0.5	0.136	1.775	14.875	29.496	9.698	313.304	25.165	4.288
13	2.5	35	0.5	0.143	1.850	16.935	25.809	13.112	198.307	21.127	6.098
14	3	25	0.1	0.147	1.882	20.218	30.988	14.552	231.499	28.042	6.820
15	3	15	0.3	0.152	1.997	22.816	32.757	12.052	276.732	33.973	3.820
16	3	35	0.3	0.157	2.001	24.321	30.517	12.842	251.020	30.643	5.339
17	3	25	0.5	0.162	2.032	27.295	34.101	8.364	415.998	37.708	2.655

THI, thickness; D, density; WVP, water vapor permeability; TS, tensile strength; E, elongation at break; YM, Young's modulus; PF, puncture force; PD, puncture deformation.

coefficient of determination (R^2) confirmed the adequacy of the RSM second-order polynomial model. Statistical software of Statistical Package for Social Science 16 (SPSS, Inc., Upper Saddle River, NJ, USA) was used to separate the means analysis by independent samples t-test. The differences between the mean values in the achieved experiments were taken to be statistically significant at $p < 0.05$.

3 Results and discussion

3.1 Fitting the response surface methodology model

According to the Box–Behnken experimental design, fitting the models was performed in this study to decide the reliability of the RSM mathematical model in representing the actual interrelationships between the independent variables (starch, glycerol, and guar gum) and the dependent variables (THI, D, WVP, TS, E, YM, PF, and PD) of the pea starch-based biodegradable edible films. The results of analysis of variance of the Box–Behnken design are given in Table 3 and Fig. 1.

Fitting the model for thickness and density showed that R^2 value of the models was 0.99. The p-value for lack of fit, PRESS (predicted residual sum of square), F- and p-value of the model for thickness were 0.09, 0.0003, 122.95, and <0.0001, respectively, and for density were 0.38, 0.07, 104.32, and <0.0001, respectively. The results verified the reliability of the model in predicting thickness and density of films.

The RSM mathematical models for WVP were also calculated. The results (Fig. 1C) revealed p values for WVP of <0.0001. Coefficient of determination (R^2) for the WVP model (Table 3) was estimated to be 1.00, further specifying a close correlation between the predicted values and experimental values. Lack of fit value (0.066), PRESS value (23.91) and F-value (247.35) showed that the mathematical model was successful predictor of WVP properties of the pea starch edible films.

Table 3. Analysis of variance for determination of model fitting[a)]

Sources of variation	THI (mm)	$D \times 10^{-3}$ ($g\,m^{-3}$)	WVP $\times 10^{-10}$ ($gPa^{-1}\,s^{-1}\,m^{-1}$)	TS (MPa)	E (%)	YM (MPa)	PF (N)	PD (mm)
Lack of fit	0.096	0.377	0.066	0.992	0.855	0.088	0.806	0.721
R^2	0.993	0.992	0.997	0.994	0.961	0.987	0.994	0.96
Adjusted R^2	0.986	0.983	0.993	0.987	0.911	0.97	0.99	0.909
PRESS	0.0003	0.071	23.91	11.866	44.688	31394.02	36.514	11.576
F ratio of model	122.95	104.32	247.35	134.98	19.2	58.91	122.149	18.849
p of model > F	<0.0001[a)]	<0.0001[a)]	<0.0001[a)]	<0.0001[a)]	0.0004[a)]	<0.0001[a)]	<0.0001[a)]	0.0004[a)]

a) Significant difference with $p < 0.05$.

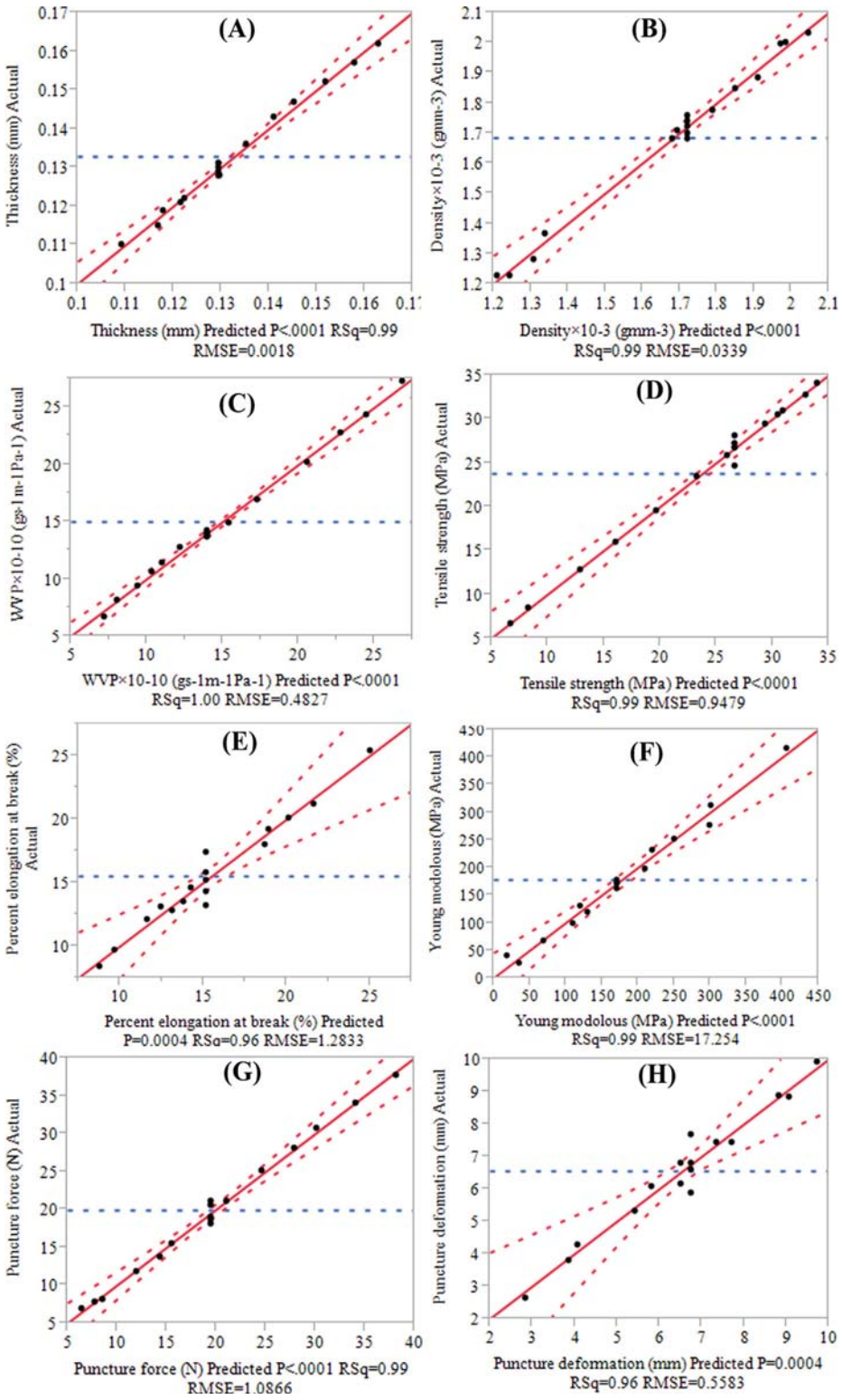

Figure 1. Correlations between predicted and experimental thickness (A), density (B), water vapor permeability (C), tensile strength (D), percent elongation at break (E). Young's modulus (F), puncture force (G), and puncture deformation (H).

In a similar manner, the results for mechanical properties: TS, %E, and YM also confirmed the competency of the model. The R^2 values of TS, %E, and YM were 0.99, 0.96, and 0.99, respectively (Fig. 1D–F). The p values for lack of fit for all tensile mechanical parameters were found insignificant ($p > 0.05$, Table 3). The p values (0.99, 0.86, and 0.008, respectively), F values (134.98, 19.20, and 58.91, respectively), and PRESS values (11.87, 44.69, and

31394.02, respectively) of the model also supported the efficiency of these models in correctly evaluating the mechanical properties of pea starch edible films.

The RSM mathematical models for PF and PD were also calculated. The results (Fig. 1G and H) revealed p values for PF and PD of <0.0001 and 0.0004, respectively. Coefficient of determination (R^2) for the PF and PD models (Table 3) were estimated to be 0.99 and 0.96, respectively, further specifying a close correlation between the predicted values and experimental values. PRESS values (36.51 and 11.58) and F values (122.15 and 18.85) showed that these mathematical models were successful predictors of puncture mechanical properties of the pea starch edible films.

The higher model F values with low probability values ($p < 0.0001$) for all responses obviously specified that the established models were significant to predict physical and mechanical characteristics of the films [4].

Empirical models were developed by applying multiple regression analysis on the experimental data obtained from BBD to represent an accurate correlation between independent variables and responses. The predicted responses expressed in terms of coded factors as follows:

$$\mathrm{THI} = 0.13 + 0.02x_1 - 0.003x_2 + 0.10x_3 - 0.0005x_1x_2 - 0.0008x_1x_3 - 0.0003x_2x_3 + 0.008x_1^2 + 0.00005x_2^2 - 0.0007x_3^2$$

$$D = 1.72 + 0.35x_1 - 0.02x_2 + 0.07x_3 - 0.012x_1x_2 + 0.002x_1x_3 - 0.011x_2x_3 - 0.11x_1^2 + 0.017x_2^2 + 0.02x_3^2$$

$$\mathrm{WVP} = 13.94 + 7.47x_1 + 0.77x_2 + 2.35x_3 + 0.08x_1x_2 + 0.78x_1x_3 + 0.17x_2x_3 + 2.24x_1^2 - 0.008x_2^2 + 0.04x_3^2$$

$$\mathrm{TS} = 26.69 + 10.56x_1 - 1.78x_2 + 3.11x_3 + 0.54x_1x_2 - 1.57x_1x_3 + 0.05x_2x_3 - 4.09x_1^2 - 1.45x_2^2 - 0.68x_3^2$$

$$E = 15.15 - 3.94x_1 + 1.04x_2 - 4.17x_3 - 0.30x_1x_2 + 1.41x_1x_3 + 0.33x_2x_3 + 0.69x_1^2 + 0.48x_2^2 - 0.39x_3^2$$

$$\mathrm{YM} = 170.63 - 115.17x_1 - 25.12x_2 + 70.13x_3 + 0.61x_1x_2 + 22.80x_1x_3 - 20.71x_2x_3 + 0.82x_1^2 - 12.40x_2^2 + 26.77x_3^2$$

$$\mathrm{PF} = 19.489 + 11.947x_1 - 1.160x_2 + 3.947x_3 - 0.770x_1x_2 + 1.190x_1x_3 - 0.593x_2x_3 + 1.452x_1^2 - 0.784x_2^2 + 0.191x_3^2$$

$$\mathrm{PD} = 6.740 - 1.725x_1 - 0.769x_2 - 1.725x_3 + 0.027x_1x_2 - 0.103x_1x_3 + 0.098x_2x_3 - 0.318x_1^2 - 0.049x_2^2 - 0.027x_3^2$$

3.2 The effect of independent variables on thickness

Controlling of thickness is important for the barrier and mechanical characteristics of the ultimate film because this parameter can cause differences in the film structure by affecting the drying kinetics [40]. The data showed that the thickness of films had a positive correlation to the three experimental variables. Starch, glycerol, and guar gum were all shown to have a significant influence on the film thickness. It was found that thickness of film was increased from 0.110 to 0.162 mm, owing to the increasing of starch, glycerol, and guar content in the film forming solution. The dry matter of solutions increased because of the differences in film-forming solution formulations, which resulted in the differences in thickness of the films [4]. The increasing of thickness by increasing amount of starch and guar gum is attributed to the development of inter-molecular hydrogen bonds between guar gum and starch, the content of dry matter and also interaction between polysaccharides [4]. The higher glycerol content, the more moisture absorbs resulting in increasing the thickness of the film because of swelling process [41]. The similar behavior has been reported by Jouki et al. [42] and Ahmadi et al. [43]. The thickness increased slightly when glycerol amount was increased in film forming solution (Fig. 2A–C). The incorporation of glycerol enhances the opportunity of interaction between the film polymers and glycerol and produces thicker films [4]. The interaction between independent variables starch × glycerol, starch × guar gum, and glycerol × guar gum had no significant influence on the thickness ($p > 0.05$, Table 4).

3.3 The effect of independent variables on density

The analysis of variance indicated that starch and guar gum level significantly ($p < 0.05$) affected the density of films (Table 4). The density of films as function of process variables are depicted in Fig. 2(D–F). Guar gum forms strong intermolecular interactions with pea starch and creates a compressed structure. Consequently, increase in guar gum and starch proportion with incorporation of glycerol causes increasing in film density [40]. Difference in density value is affected by molecular weight, composition, and interaction components of the polymeric film structure [44].

The values of density obtained in this work was almost similar to that reported for cassava starch films [45], for cassava starch films and cassava starch-wheat bran composites [46] and for banana starch film [44].

3.4 The effect of independent variables on WVP

Because of the role of water in deteriorative reactions in foods, moisture transfer between the food and surrounding atmosphere should be restricted [47]. Therefore, the WVP is extensively studied as the most important characteristic of

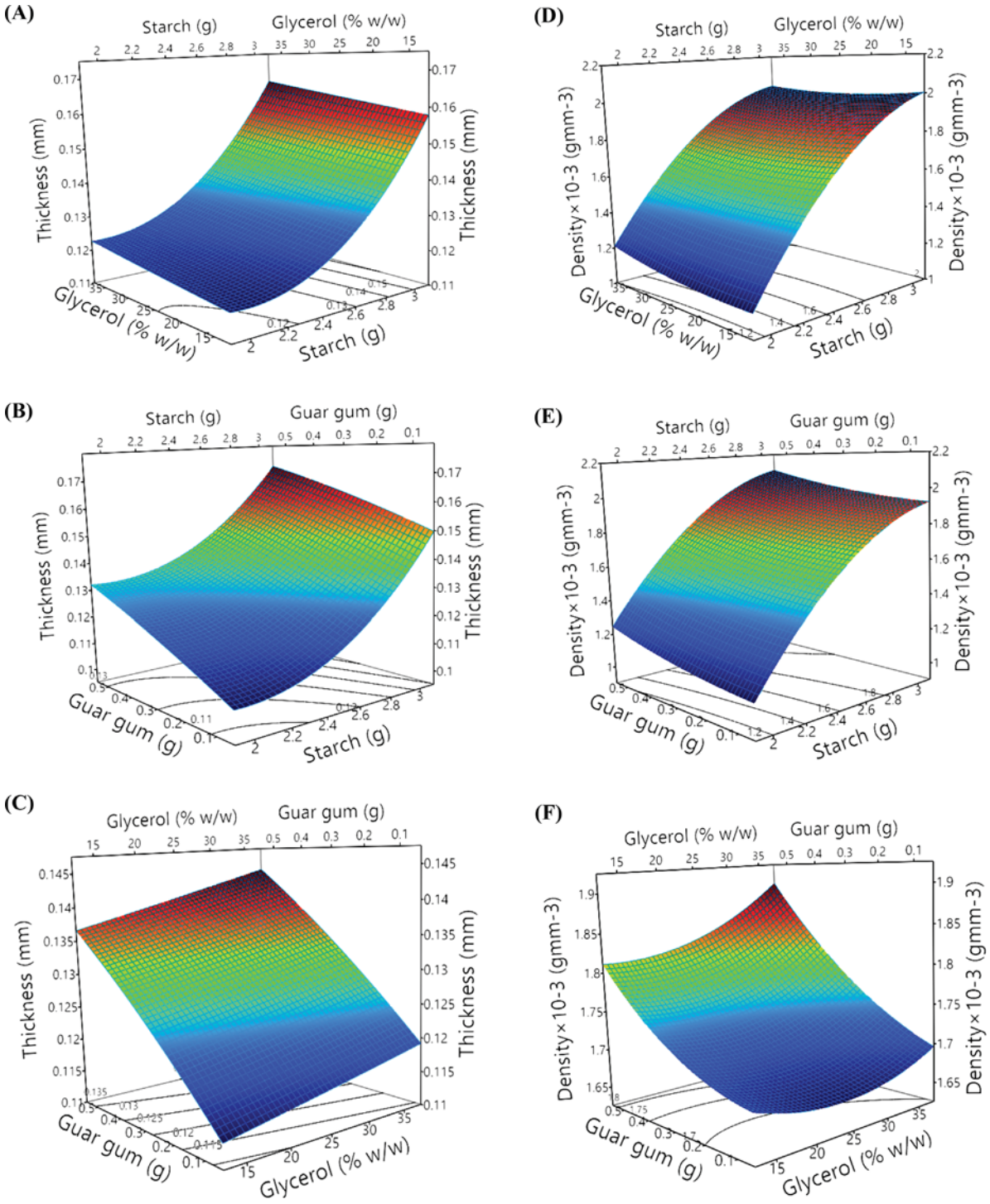

Figure 2. Response surface plots showing the interaction effects of process variables on thickness (A–C) and density (D–F).

edible films. The film structure, plasticizer, RH gradient, and temperature of the environment affect this property [48]. All independent variables found to exert significant impact on the WVP ($p < 0.05$, Table 4). Interaction between factors starch × guar gum was also shown to influence the WVP ($p < 0.05$, Table 4). The water vapor permeation through films is enhanced with the increase in concentration of guar gum and starch (Fig. 3A–C). This behavior could be owing to their hydrophilicity nature and accessible hydroxyl groups which are capable to interact with water by hydrogen bonds. Thus, water molecules are more freely absorbed into the surface of the films (i.e., higher solubility) and permeate

Table 4. Analysis of variance for the experimental results[a)]

		THI		*D*		WVP		TS		*E*		YM		PF		PD	
P	DF	Estimate	Prob > |*t*|	Estimate	Prob > |*t*|	Estimate	Prob > |*t*|	Estimate	Prob > |*t*|	Estimate	Prob > |*t*|	Estimate	Prob > |*t*|	Estimate	Prob > |*t*|	Estimate	Prob > |*t*|
β_0	1	0.13	<0.0001[a)]	1.72	<0.0001[a)]	13.94	<0.0001[a)]	6.740	<0.0001[a)]	170.64	<0.0001[a)]	26.69	<0.0001[a)]	15.15	<0.0001[a)]	170.64	<0.0001[a)]
β_1	1	0.02	<0.0001[a)]	0.35	<0.0001[a)]	7.47	<0.0001[a)]	−1.725	<0.0001[a)]	115.17	<0.0001[a)]	10.56	<0.0001[a)]	−3.94	<0.0001[a)]	115.17	<0.0001[a)]
β_2	1	0.003	0.0041[a)]	0.02	0.1412	0.77	0.0028[a)]	0.769	0.0059[a)]	−25.12	0.0059[a)]	−1.78	0.011[a)]	1.04	0.0559	−25.12	0.0045[a)]
β_3	1	0.01	<0.0001[a)]	0.07	0.0009[a)]	2.35	<0.0001[a)]	−1.725	<0.0001[a)]	70.12	<0.0001[a)]	3.11	<0.0001[a)]	−4.17	<0.0001[a)]	70.12	<0.0001[a)]
β_{12}	1	0.0005	0.5899	-0.01	0.5018	0.08	0.7613	0.027	0.9253	0.61	0.9253	0.54	0.2891	−0.30	0.6511	0.61	0.9460
β_{13}	1	−0.0007	0.4250	0.002	0.8981	0.78	0.0147[a)]	−0.103	0.7226	22.80	0.7226	1.57	0.0129[a)]	1.41	0.0633	22.80	0.0333[a)]
β_{23}	1	0.0003	0.7858	0.01	0.5105	0.17	0.4943	0.099	0.7345	−20.71	0.7345	0.05	0.9222	0.33	0.6247	−20.71	0.0447[a)]
β_{11}	1	0.0081	<0.0001[a)]	−0.11	0.0003[a)]	2.24	<0.0001[a)]	−0.318	0.2801	0.82	0.2801	4.09	<0.0001[a)]	0.69	0.3044	0.82	0.9250
β_{22}	1	0.00005	0.9554	0.02	0.3377	-0.01	0.9743	−0.049	0.8616	−12.40	0.8616	−1.45	0.0162[a)]	0.48	0.4642	−12.40	0.1838
β_{33}	1	-0.0007	0.4440	0.02	0.3581	0.04	0.8565	−0.027	0.9237	26.77	0.9237	-0.68	0.1865	−0.39	0.5481	26.77	0.0154[a)]

P, parameter; β_0, intercept; β_1, β_2, and β_3, linear regression coefficients for pea starch, glycerol and guar gum; β_{12}, β_{13}, and β_{23}, regression coefficients for interaction between starch × glycerol, starch × guar gum, and glycerol × guar gum; β_{11}, β_{22}, and β_{33}, quadratic regression coefficients for starch × starch, glycerol × glycerol, guar gum × guar gum.
a) Significantly different at $p < 0.05$.

through the film structure more simply (i.e., higher diffusivity) bringing about the increase in WVP [37]. The addition of glycerol can make the film less compact by increasing the molecular mobility and enhancing free volume in the film network [49] and can increase the sorption of water. Prakash Maran et al. [4] also observed a similar trend in maize starch and agar blend films. Maran et al. [40] documented that tapioca starch films exhibited an increase in WVP with the increase of agar concentration.

3.5 The effect of independent variables on tensile strength (TS)

Table 4 shows that starch and guar gum have significant positive linear effects on TS, whereas, glycerol and its quadratic interaction have significant negative effects. The TS increased with increasing of starch and guar gum concentration, so the maximum TS was obtained with maximum value of starch and guar (Fig. 3D–F). This is associated with the development of intermolecular hydrogen bonds between guar and starch and also the cohesive molecular structure of the films which improved the tensile strength accordingly [50]. Other studies have reported that the addition of hydrocolloids increases the film mechanical strength [22, 40, 50–53]. The organized crystalline structure of the starch molecules is disordered by the gelatinization process that happens during film preparation, leading to the exposure of –OH groups that rapidly establish hydrogen bonds with the guar gum [54]. The maximum TS ranged from 6.61 MPa for film containing 2 g of starch, 0.1 g of guar, and 25% glycerol to 34.10 MPa for film with 3 g of starch, 0.5 g of guar, and 25% glycerol. In the central point, the value was 26.70 MPa. The maximum TS of pea starch film with 70% glycerol conditioned at 23°C and 50% RH ranged from 2.65 to 4.32 MPa [55]. Zhang and Han [56] reported maximum TS of 5.8 MPa for film obtained from 3% yellow pea starch and 20/50 w/w glycerol conditioned at 25°C and 50% RH for 72 h. However, yellow pea starch films containing 3% starch and 40/60 w/w glycerol conditioned for 48 h at 50% RH and 25°C showed maximum TS of 2.3 MPa [57]. The observations regarding the effect of the glycerol content on the maximum TS are consistent with those found in the literature. It has been documented that the incorporation of glycerol decreases the TS by interrupting direct interactions of the film-forming polymer [56, 58] resulting in decreasing the cohesiveness of the film network [22].

3.6 The effect of independent variables on elongation at break (*E*)

As can be inferred from the Table 4, *E* values were negatively affected by the linear terms of guar gum and starch contents. Figure 4(A–C) represents the three-dimensional surface

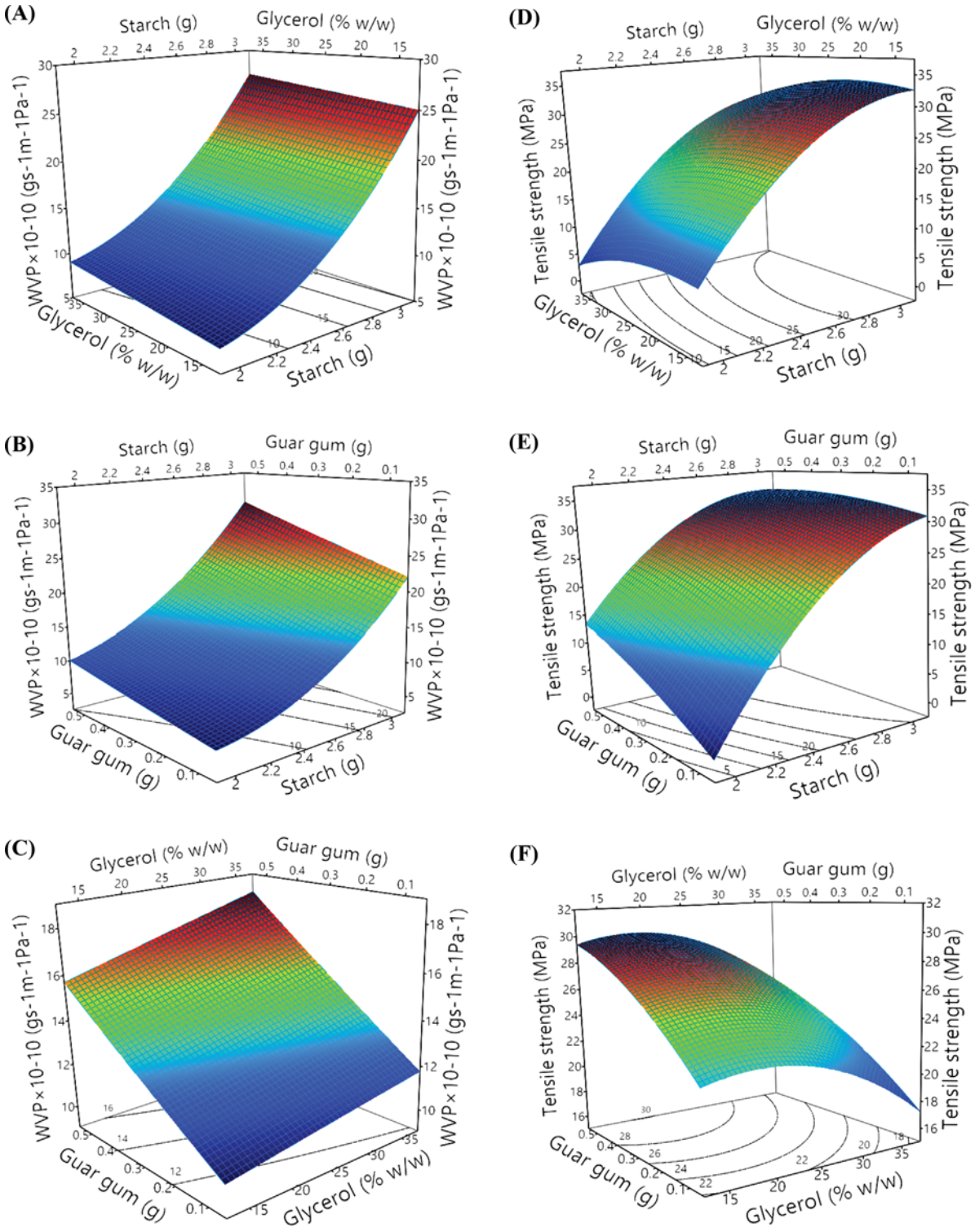

Figure 3. Response surface plots showing the interaction effects of process variables on WVP (A–C) and tensile strength (D–F).

response plot of *E* as a function of the two independent variables calculated in this study. The highest *E* values of the composite film were obtained at the high concentration of glycerol and at the low concentration of guar gum. The synergism interaction between the guar gum and the pea starch influences the film elongation at break [59], inhibiting amylose-amylose interactions [60]. The more unfolded network, the weaker interaction forces and lower elongation

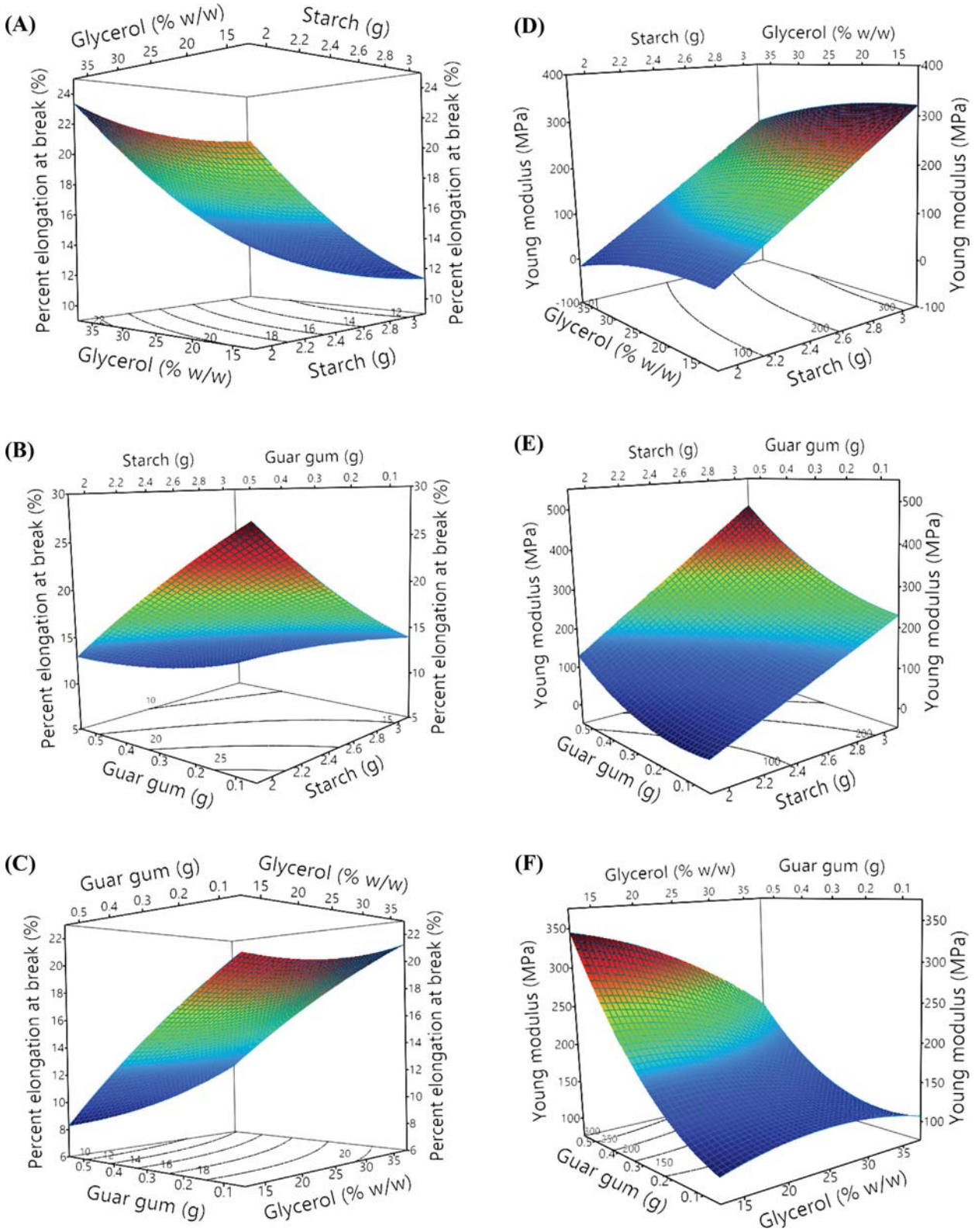

Figure 4. Response surface plots showing the interaction effects of process variables on percent elongation at break (A–C) and Young's modulus (D–F).

at break performance will be [9]. The reduction of *E* may be explained by a binary hydrocolloid phase separation as a result of weakening gel structure by addition of guar gum to pea starch dispersion [9]. High temperature and shear force during the preparation of the starch films destroy the granular and crystalline structure of starch, promoting the presence of plasticizer into the matrix [50]. The development of hydrogen bonds between the hydroxyl groups of biopolymers and glycerol could reduce the links between nearby biopolymer chains, leading to improving flexibility of these chains and free volume between the nearby starch chains [8, 49]. Similar results were observed by Bourtoom and Chinnan [54], Prakash Maran et al. [50], and da Matta et al. [22] for rice starch–chitosan, maize starch–agar, and pea starch–xanthan composite films.

3.7 The effect of independent variables on Young's modulus (YM)

The YM signifies the stiffness of the film; a greater value attributes to a more stiff material [52]. The glycerol concentration had a negative effect on the YM values. On the other hand, the YM values were positively affected by the individual term of starch and guar gum, the interaction term of starch-guar gum, the quadratic term of guar gum, and negatively affected by the interaction guar gum-glycerol (Table 4). Subsequently, the YM values increased with the increase of the guar gum and with the decrease of the glycerol, which is in agreement with results that have been reported in the literature [9, 50, 52, 61, 62]. The pea starch films become more cohesive at higher guar gum proportions, causing greater resistance and higher Young's modulus. Increasing glycerol molecules cause reduction in Young's modulus of the films by disordering polymer alignment, increasing free volume and decreasing structural orientation in the polymeric system.

3.8 The effect of independent variables on puncture mechanical properties

Suitable mechanical strength and flexibility are commonly necessary for a packaging film to endure external stress and to preserve its stability along with barrier characteristics during applications in packaging [31]. The linear regression coefficients and their statistical significance are presented in Table 4. All the three independent process variables had significant effect on the puncture force (PF) ($p < 0.05$). The PF increased steadily when starch and guar gum increased; however, the levels of PF decreased when glycerol increased from 15 to 35% (Fig. 5A–C), indicating the reinforcing influence of guar gum and the plasticizing effect of glycerol. These phenomena considerably depend on the distribution and quantity of inter- and intramolecular associations [63]. These interactions were increased by enhancing the number of guar gum molecules, which consequently, imparted a stronger character to the film [62]. During gelatinization process, the organized structures of pea starch molecules are demolished, causing the exposure of OH groups to easily form hydrogen bonds with guar gum due to the chemical similarity of both polysaccharides [54]. On the other hand, the PF of composite films increased because of increasing the regions of the three-dimensional network structure formed by interaction between guar and pea starch chains [64].

The incorporation of glycerol noticeably decreased the PF. Glycerol is a relatively small hydrophilic molecule and could penetrate between starch chains and form hydrogen bonds with hydroxyl groups of starch. The direct interactions and the adjacency between starch chains were reduced by incorporation of glycerol in to the network [50]. Accordingly, under stress, movements of starch chains were accelerated and reduced the PF.

It can be noted that the linear coefficient of glycerol was positive, while the linear coefficients of pea starch and guar gum were negative for puncture deformation (PD) response. Interaction and quadratic regression coefficients did not show a significant impact on PD. Figure 5D–F demonstrates the contour plot of PD. It can be seen that an increasing proportion of glycerol, contributed to the increase of PD, while PD decreased as the pea starch and guar gum proportions simultaneously increased. Possibly, interaction between gum and starch chains through hydrogen bonds developed a more resistant network with lower flexibility behavior, making impossible the movement of polymeric chains [61]. A similar trend was observed by Prakash Maran et al. [50] who reported a significant and negative effect of the agar on PD of maize starch-based edible films.

3.9 Optimization and validation of the models

The simultaneous optimization of the multiple responses was determined based on the desirability function, which simultaneously optimizes the requirement for each response in the design by developing a mixture of independent variables [40]. The aim of this study was to maximize mechanical properties and to minimize WVP. Hence, these responses were selected to examine the possibility of considering one formulation which optimizes the characteristics of studied edible films. The desirability function was calculated by applying maximum, minimum, and average values of these variables experimentally obtained in the Box–Behnken experimental design (Table 2). The optimum level of pea starch of 2.5 g, glycerol of 25%, and guar gum of 0.3 g with an overall desirability of 0.75 was obtained according to the methodology of desired function.

The experimental validation of the above mentioned optimal conditions was implemented to prove the reliability

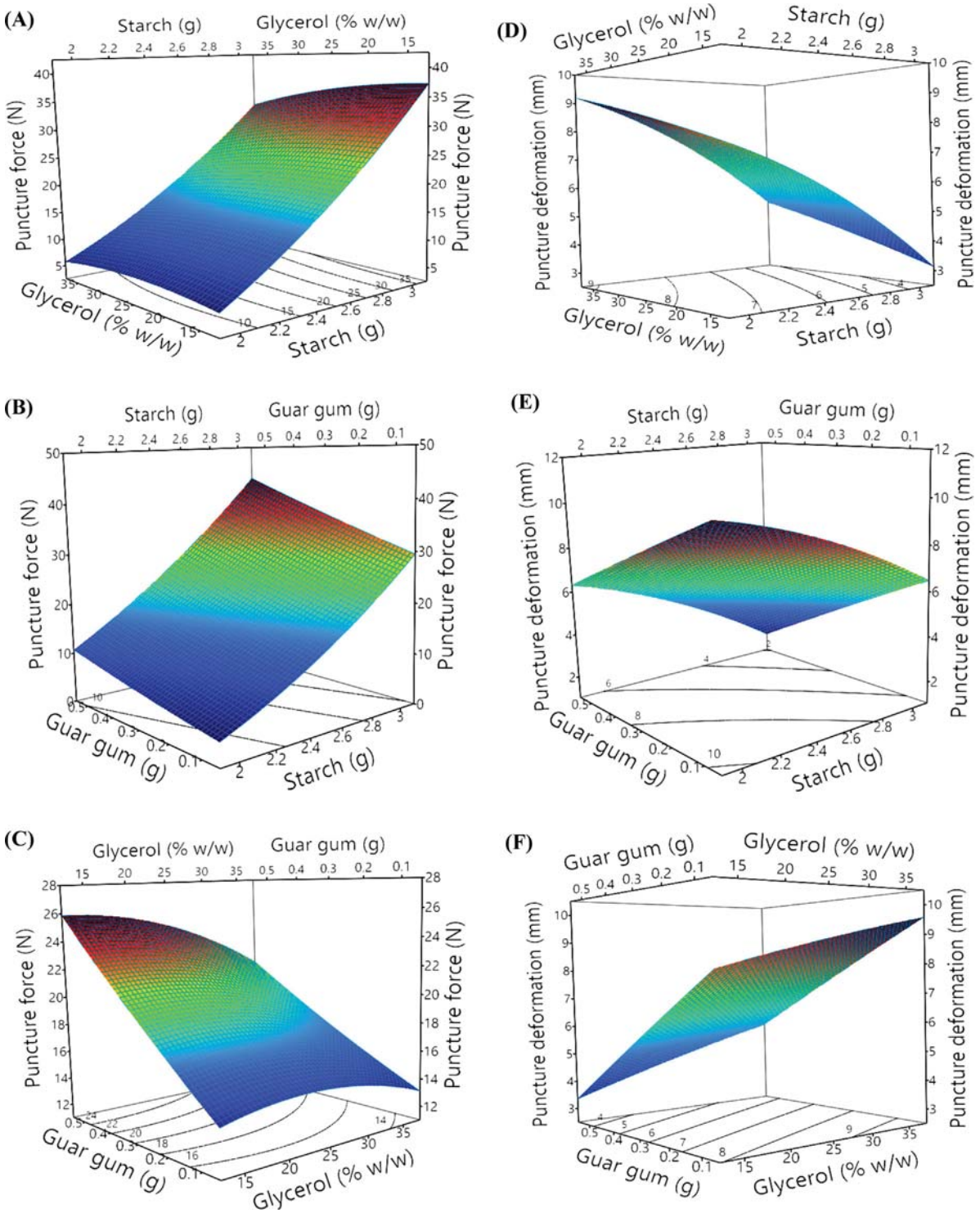

Figure 5. Response surface plots showing the interaction effects of process variables on puncture force value (A–C) and puncture deformation value (D–F).

of the models. The experimental results were compared with the predicted values of the responses with triplicate experiments (Table 5). The absolute residual error for the dependent variables ranged from 0.775 to 7.110%, representing adequacy of the methodology established for the optimization of the process conditions, and reliability of the surface responses obtained by the Box–Behnken experimental design [23].

Table 5. Results of experimental validation of the optimal conditions for the development of pea starch–guar gum edible film[a)]

Responses	Predicted value	Experimental value ($n = 3$)[b)]	Absolute residual error (%)[c)]
Thickness (mm)	0.130 ± 0.007^{a}	0.129 ± 0.042^{a}	0.775
Density $\times 10^{-3}$ ($g\,mm^{-3}$)	1.727 ± 0.638^{a}	1.712 ± 0.323^{a}	0.876
WVP $\times 10^{-10}$ ($g\,Pa^{-1}\,s^{-1}\,m^{-1}$)	14.085 ± 1.112^{a}	13.874 ± 1.107^{a}	1.521
Tensile strength (MPa)	26.901 ± 2.366^{a}	27.792 ± 2.204^{a}	3.206
Elongation at break (%)	15.077 ± 1.252^{a}	16.231 ± 1.732^{a}	7.110
Young's modulus (MPa)	172.910 ± 20.751^{a}	175.683 ± 14.163^{a}	1.578
Puncture force (N)	19.725 ± 2.469^{a}	20.315 ± 1.521^{a}	2.904
Puncture deformation (mm)	6.707 ± 0.505^{a}	7.100 ± 1.004^{a}	5.535

a)All the values are means ± standard deviations and those in the same row not sharing the same superscript letter are significantly different from each other ($p < 0.05$).
b)Values obtained at optimum conditions (pea starch 2.5 g; glycerol 25%; and guar gum 0.3 g).
c)Absolute Residual Error = [(experimental value − predicted value)/experimental value] × 100.

4 Conclusions

This study showed that the mechanical and barrier properties of pea starch edible films were improved by optimizing concentrations of pea starch (2–3 g), guar gum (0.1–0.5 g), and glycerol (15–35%) by using BBD. The results revealed that increasing starch and guar gum content favored formation of a more resistant polymeric structure with higher thickness, density, as well as WVP. Higher concentration of glycerol increased the flexibility and reduced the resistance and stiffness of the film. The optimal conditions for the production of a pea starch-guar gum edible film with good mechanical properties and low WVP were 2.5 g pea starch, 25% w/w glycerol and 0.3 g guar gum. Close correlation between the experimental and predicted values and improved mechanical and barrier characteristics of optimized film demonstrated the potential application of pea starch-guar gum composite films in food packaging industry. It should be noted that further research on the impact of interaction between pea starch and guar gum on film formation is suggested for better understanding of the utilization of the pea starch-guar gum film in food preservation and packaging.

This study was funded by the University of Newcastle, NSW Australia.

The authors have declared no conflict of interest.

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Additional Research paper 4

Thanh Trung Dang, **Deep Jyoti Bhuyan**, Danielle R. Bond, Michael C. Bowyer, Ian A. Van Altena & Christopher J. Scarlett. Fucoxanthin content, isolation and cytotoxic activity against pancreatic cancer from brown alga *Hormosira banksii* (Turner) Decaisne. Submitted to **Algal Research (2017).**

Fucoxanthin content, isolation and cytotoxic activity against pancreatic cancer from brown alga *Hormosira banksii* (Turner) Decaisne

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Abstract

Fucoxanthin content has recently gained increased attention due to its health benefits. This study aimed to isolate and purify fucoxanthin from the brown alga *Hormosira banksii* and the purified fucoxanthin was investigated for cytotoxic activity against pancreatic cell lines. The HPLC analysis showed that fucoxanthin content in the alga was determined to be 0.58 mg Fx.g^{-1} alga, while its purity was achieved at 92.3% via column chromatography. This pigment showed high anti-proliferative activity on both primary (MiaPaCa2) and secondary (BxPC3, CFPAC1) pancreatic cancer cell lines at concentrations of 100-200 µg.mL^{-1}. It provided rationale for future clinical use of fucoxanthin for the treatment of pancreatic cancer and investigations into the mechanism of action of this compound on apoptosis and cell cycle.

Keyword: Fucoxanthin content; *Hormosira banksii*; HPLC analysis; Pancreatic cancer.

1. Introduction

Marine algae are renewable and sustainable resources that contain biologically active metabolites that could be potentially exploited for the development of new drugs and healthy foods [1]. The natural components of algal extracts such as phenolic compounds, fucoxanthin, sulfated polysaccharides, terpenoids and other secondary metabolites were found to have a strong relationship with antioxidant capacity [2]. It has been demonstrated that these compounds are linked to the treatment of some chronic diseases, especially several types of cancers [3, 4]. Brown algae in particular, was found to have a higher antioxidant capacity than red or green algae [2]. However, it is not only phenolic compounds (phlorotannins), but also carotenoids from brown algae that have recently gained attention due to its potent applications in the food and pharmaceutical industries [5]. Fucoxanthin is one of the most abundant carotenoids of brown algae and estimated to be around 10% of total carotenoids found in nature [6]. The structure of fucoxanthin includes a usual allenic bond and 5, 6-monoepoxide in its molecule and the type of this compound found in brown algae was almost all *trans*-fucoxanthin (Figure 2) [7, 8]. Fucoxanthin has been isolated and purified from marine algae and its efficacy against several cancer cell lines has been previously studied [9-11].

Pancreatic cancer is one the leading causes of cancer death in Western countries due to the late onset of symptoms and diagnosis at an advanced stage. Pancreatic ductal adenocarcinoma (PDAC) is the most common pancreatic cancer type (95%) [12, 13], and currently, therapeutic options applicable to PDAC are still limited to surgery at the early stage, while chemotherapy and radiotherapy are applied for almost all PDAC patients [14]. However, chemotherapeutics using synthetic drugs such as gemcitabine (2-2-difluorodeoxycytidine) or the combination of 5-fluorouracil, leucovorin, oxaliplatin and irinotecan (FOLFIRINOX) treatment were found to not be effective due to their significant

toxicity and emerging drug resistance [12, 15]. With limited therapeutic options for several cancers, it is necessary to screen the anticancer activity of active compounds found in marine plants. The biologically active components within the algal extracts may be potentially useful for several cancers, including pancreatic cancer.

Based on our best knowledge, fucoxanthin content of brown alga *Hormosira banksii* (collected from the eastern coast of NSW, Australia) has yet to be investigated. The aims of our work were to isolate and purify fucoxanthin from this alga, with the anticancer activity of fucoxanthin then evaluated using several pancreatic cancer cell lines for potential applications in pharmaceutical field.

2. Materials and methods

2.1. Reagents

All solvents and chemicals such as n-hexane (95.0%), acetone (99.5%), methanol (99.8%), ethanol (99.5%) and acetonitrile (99.8%) used in the study were analytical grade (Sigma-Aldrich, Australia). A silica gel plate (20x20 cm, Kieselgel $60F_{254}$, 0.25 mm); Kieselgel $60GF_{254}$ powder for chromatography column (Merck KGaA, Darmstadt, Germany). The standard fucoxanthin (95% purity established by HPLC) for this work was purchased from Sigma-Aldrich, Castle Hill, NSW 2154, Australia.

2.2. Materials and sample preparation

The brown alga *H. banksii* was collected in March, 2016 from a rocky shore at Bateau Bay, NSW, Australia (Latitude of 33°22'55.2"S; longitude of 151°29'6"E). The sample was washed with seawater, and then washed thoroughly with freshwater and freeze dried for 48 h using a freeze dryer (Thomas Australia Pvt. Ltd., Seven Hills, NSW, Australia). The dried sample was pulverized, sieved (≤ 600 μm particle size) and stored at – 20 ˚C for further analysis. The extraction process was performed as previously described by Fung et al. [16] with some modifications. Briefly, the dried alga (10g) was extracted with ethanol (300 mL) and stirred using a magnetic bar for 8 h at room temperature. The process was repeated (n=3) till the samples became colourless. The combined extracts were filtered, concentrated using a rotary evaporator and freeze dried to obtain the crude extracts.

2.3. Isolation and purification of fucoxanthin

The process for isolating fucoxanthin from the extract was conducted as described by Kim et al. [17], with slight modifications. Fucoxanthin was isolated through a chromatography column using silica gel (Kieselgel $60GF_{254}$). A clean glass column (3.5x32 cm) initially was inserted with cotton wool at the bottom, and silica gel (30g) dissolved with the *n*-hexane was poured immediately into the column to avoid breakage or bubbles. The column was kept one

day for proper binding of silica. The extract (1g) dissolved in methanol was loaded in the column. Hexane (100%) was initially eluted to remove non-polar content such as chlorophyll, other carotenoids, than fucoxanthin. The next elution was conducted with the mixture of hexane: acetone (7:3 v/v) to obtain fucoxanthin. Finally, acetone was used to elute resident fucoxanthin. The fractions of acetone and a mixture of hexane:acetone were collected and concentrated using a rotary evaporator. The experiment was conducted in the dark room to avoid the possibility of oxidation/degradation of fucoxanthin by light.

2.4. Thin layer chromatography (TLC) and HPLC analysis

TLC for the extract and fucoxanthin was performed on a silica gel plate (20x20 cm, Kieselgel $60F_{254}$, 0.25 mm). The mixture of hexane and acetone with the ratio of 7:3 (v/v) was used as a mobile phase [17].

Fucoxanthin was determined using HPLC [18]. The HPLC system consisted of a LC-20AT pump system (Shimadzu) and a UV–Vis SPD-20A (Shimadzu) absorbance detector. Fucoxanthin was separated on a Luna 5 µm C18 column (250 x 4.6 mm) (Phenomenex, Australia). The mobile phase used was a mixture of methanol/acetonitrile (7/3 v/v) at a flow rate of 1 ml/min and the sample injection volume was 50 µl. The detection wavelength was set at 450 nm.

2.5. Cell culture

Human pancreatic cancer cells (two primary pancreatic cancer cell lines: MiaPaCa2, BxPC3 and one secondary pancreatic cancer cell line: CFPAC1) and Human pancreatic ductal epithelial cells (HPDE) were cultured at 37 °C, 5% CO_2 [19]. Keratinocyte Serum-free Media (K-SFM) with human Recombinant Epidermal Growth Factor (EGF) and Bovine Pituitary Extract (BPE) (10%) was used for HPDE. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2.5% horse serum and L-glutamine (100 $\mu g.mL^{-1}$) was used to culture MiaPaCa2 cells, while RPMI media with 10% FBS and L-

glutamine (100 µg.mL^{-1}) was applied for BxPC3 and CFPAC1 cells were cultured in IMDM media supplemented with 10% FBS and L-glutamine (100 µg.mL^{-1}).

2.6. Determination of cytotoxic activity of the purified fucoxanthin

Cytotoxic activity of purified fucoxanthin was determined using the Dojindo Cell Counting Kit-8 (CCK-8: Dojindo Molecular Technologies, Inc., Rockville, MD, USA) assay. Pancreatic cells were seeded into a 96-well plate at 3x10^3 cells per well (200 µL) for MiaCaPa2 and 7x10^3 cells for HPDE, BxPC3 and CFPAC1, and incubated at 37 °C with 5% CO_2 for 24 h. The cells were then treated with various concentrations (25-200 µg.mL^{-1}) of purified fucoxanthin, gemcitabine (50 nM) and 0.5% DMSO as vehicle control. After 72 h, 10 µL of CCK-8 solution was added before incubating at 37 °C with 5% CO_2 for 90 min. The absorbance was measured at 450 nm on a multi-well spectrophotometer (BIORAD Benchmark Plus^{TM}), and cell viability was determined as a percentage of control. Six repeats were performed for each concentration (n=6).

2.7. Statistical Analysis

A one-way ANOVA and LSD post-hoc test were employed (SPSS Statistical Software, Version 16) to analyse the differences between the independent samples. Differences between the mean levels of analyses were taken to be statistically significant at $P<0.05$. All experiments were conducted at least in triplicate and the results were performed as means ± standard deviations. The IC_{50} values (the concentration required to inhibit cell growth by 50%) were calculated by curve fitting the absorbance (viability) *vs.* log [concentration of treatment] using GraphPad Prism software (version 7.03).

3. Results and Discussion

3.1. Thin layer chromatography (TLC) for the extract

The chromatographic profile of the extract visualised under visible light, indicated the presence of 4 colourful bands (B1-B4) in the *H. banksii* ethanol extract on the TLC plate. The

light yellow (B1), grey (B2) and green band (B3) on the TLC plate could correspond to some pigments as β-carotene, pheophytin-like compounds and chlorophyll *a, b*. It has been reported that β- and α-carotene was usually detected on the top of the TLC, followed by xanthophylls and chlorophylls, while lutein/zeaxanthin was found at the bottom of the layer [20]. Moreover, the dark grey band is likely pheophytin-like compounds that appeared from the partial degradation process of chlorophyll *a* caused by the high temperature or the long-time process of the extraction [21]. The grey band is observed to be small and not clear. It could be that a small amount of pheophytin-like compounds were appeared because the sample was evaluated immediately after extraction. The fucoxanthin content (B4) with the colour of orange-yellow was separated to investigate the cytotoxic activity on pancreatic cancer cell lines.

3.2. Fucoxanthin content

Fucoxanthin and other pigments of brown algae are visualised on the TLC plate, while the quantitative data on fucoxanthin based on HPLC analysis are found to be of 0.58 mg $Fx.g^{-1}$ alga (dry weight). Fucoxanthin content was estimated by comparing between the areas of the peaks of the purified fucoxanthin and the purified fucoxanthin added with the standard commercial fucoxanthin (Figure 2). This finding was fitted to fucoxanthin content (0.61 mg $Fx.g^{-1}$ alga) that was measured by reading the absorbance of the different wavelengths through a UV-Visible spectrophotometer in our previous study [22].

Fucoxanthin was successfully isolated from the extract by a chromatography column using silica gel powder (Kieselgel $60GF_{254}$). The mobile phase used to separate the pigments was a mixture of hexane and acetone (7/3 v/v). The purity of fucoxanthin was found to be 92.3% through the chromatography column checked by HPLC analysis (Figure 2). The isolation and purification of fucoxanthin from extract *H.banksii* via a chromatography column was shown to have high purity at the first pass (>90% purity). In addition, the purification process of

fucoxanthin from the extract was simple due to the long distances between the pigments in the TLC plate using this mobile phase (Figure 1) and the mixture of hexane and acetone (7/3 v/v) was optimal for isolating fucoxathin compared to the other ratios of this mixture or other solvents such as dichloromethane, ethyl acetate, mixtures of dichloromethane, ethyl acetate with ethanol or methanol (data not shown). The results were supported by Jaswir et al. [23] who reported that the purity of fucoxanthin from alga *Padina australis* was 94.8% through the SiO_2 chromatography column (a mobile phase; n-hexane/acetone: 6/4 v/v) and up to 98.1% purity as the process was repeated using a HPLC column. The purity of fucoxanthin >90% was also found in two brown algae *Sargassum binderi* and *Sargassum duplicatum* using a silica column (Silica 60G, Merck, 0.040 - 0.063 mm) eluted with a cold mixture of n-hexane/acetone: 6/4 (v/v) and acetone [24]. Fucoxanthin content of *Himanthalia elongata* extract was isolated using a mixture of low polarity solvents (n-hexane, diethyl ether and chloroform), and then purified with preparative thin layer chromatography using chloroform/diethyl ether/n-hexane/acetic acid (10:3:1:1, v/v/v/v) as a mobile phase. The compounds of interest were collected carefully using a scalpel and the purity of fucoxanthin was found to be 97% based on HPLC analysis [25]. In addition, Jaswir et al. [7] outlined that fucoxanthin separated from two brown algae *Sargassum plagyophyllum* and *Turbinaria turbinata* by open column chromatography (silica gel: Kieselgel 60 silanisiert; 0,063–0,200 mm; Merck KGaA, Darmstadt, Germany) and a mobile phase of hexane: acetone = 6:4 (v/v) and acetone 100% was a type of all *trans*-fucoxanthin. However, from brown alga *Undaria pinnatifida*, fucoxanthin content was achieved at low purity with the first extract (43.5%) and second extract (60.8%) via column chromatography using a mobile phase of hexane: acetone = 6:4 (v/v) [11]. On the other hand, different solvents were applied to isolate fucoxanthin. Fucoxanthin was obtained from the chloroform fraction of the ethanol *Fucus evanescens* extract and via column chromatography using a mobile phase as the mixture of benzene and

hexane with ethyl acetate [10]. The ethyl acetate fraction from the methanol *Undaria pinnatifida* extract via preparative thin layer chromatography (mobile phase developed with chloroform/methanol/water; 65/25/4 (v/v/v), and then n-hexane/acetone; 6/4 (v/v) was successfully applied for purification of fucoxanthin) [26]. Therefore, the selection of the solvents and methods for isolation and pure level of fucoxanthin depended on the algal species, the different constituents and the proportion of them within the algae.

The HPLC chromatogram of the algal pigments from *H. banksii* is shown in Figure 2. The purified pigment (B4) was detected at the wavelength of 450 nm and the retention time was 3.95 min. This compound was confirmed to be fucoxanthin by TLC analysis (Figure 1), and by comparing the retention time between the purified pigment (Figure 2) and the purified pigment added to the standard commercial fucoxanthin (95% purity) (Figure 2).

Cytotoxic activity of the purified fucoxanthin

The anti-cancer activity of purified fucoxanthin was assessed against pancreatic cancer cell lines (primary and secondary cell lines) as well as on non-tumourigenic cells (HPDE) at serial concentrations (25-200 $\mu g.mL^{-1}$). The results showed that there was significant difference between high and low concentrations of the treatments ($P<0.05$). This suggests that purified fucoxanthin inhibited the growth of pancreatic cancer cells in a dose-dependent manner (Table 1). In the case of MiaPaCa2 cells, it was shown that there was a significant difference in cell growth inhibition (%) with fucoxanthin treatment at the different concentrations ($P<0.05$). High growth inhibition (>70%) of the cells was observed at concentrations $\geq$ 100 $\mu g.mL^{-1}$. Cell growth inhibition at 200 $\mu g.mL^{-1}$ (92.81%) was significantly higher than that of gemcitabine 50 nM (87.28%) ($P<0.05$). In addition, the difference in cell growth inhibition following treatment with gemcitabine and fucoxanthin at 150 $\mu g.mL^{-1}$ was not statistically significant ($P>0.05$). However, at the lowest concentration of the fucoxanthin (25 $\mu g.mL^{-1}$), the cytotoxic activity for the cells was only 7.66%. For the

BxPC3 cell line, no significant difference of growth inhibition was observed for the cells treated with fucoxanthin at 200, 150 μg.mL^{-1} and gemcitabine (*P>0.05*, Table 1). At the concentration of 100 μg.mL^{-1}, 55.93% of cell growth was inhibited, while the cytotoxicity to the cells was low (< 20%) at lower concentrations (≤ 50 μg.mL^{-1}). In regards to CFPAC1 cells, purified fucoxanthin at concentrations of 200, 150 and 100 μg.mL^{-1} inhibited 60.08, 47.7 and 30.91% of cell growth, respectively. These results were low compared to that of the standard (gemcitabine-50nM) with 92.42% of cell growth inhibition. In HPDE cells, it was found that fucoxanthin exerted a medium level of cytotoxicity of the non-tumourigenic pancreatic cells with 70.85 and 55.25% cells showing growth inhibition at concentrations of 150 and 100 μg.mL^{-1}, respectively. At low doses of fucoxanthin (25 and 50 μg.mL^{-1}), the non-tumourigenic cells displayed 39.29 and 21.34% growth inhibition, respectively, while high cytoxicity (89.67% growth inhibition) was observed with the standard; gemcitabine (50 nM). Purified fucoxanthin showed potential efficacy against the pancreatic cancer cell lines as evidenced by the low IC_{50} values (the concentration that inhibits the cell growth by 50%). The IC_{50} value of fucoxanthin for MiaCaPa2 was 67.47 μg.mL^{-1} (Table 3), whereas, in BxPC3 and CFPAC1 the IC_{50} values were 97.68 and 166.31 μg.mL^{-1}, respectively, and 71.81μg.mL^{-1} for non-tumourigenic HPDE cells.

Fucoxanthin and its metabolite; fucoxanthiol (metabolising by digestive enzymes of the gastrointestinal tract) was found to be efficacy against several types of cancers and other diseases and actions of fucoxanthiol were than that of fucoxanthin [27-29]. The mechanisms of action of these compounds are mediated through different signalling pathways, including the caspases, Bcl-2 proteins, MAPK, PI3K/Akt, JAK/STAT, AP-1, GADD45, and several other molecules that are involved in cell cycle arrest, apoptosis, anti-angiogenesis or inhibition of metastasis [28].

The selective cytotoxicity of fucoxanthin from algae against pancreatic cancer cell lines has been demonstrated by several previous studies. It was reported that both the *Ulva sp.* and *H. banksii* extract had selective cytotoxicity towards a pancreatic cancer cell line with no toxicity towards a normal murine cell line and the *H. banksii* ethyl acetate extract showed high cytotoxic activity (76-100%) on MiaCaPa2 cells at 100 $\mu g.mL^{-1}$ [30]. The cytotoxicity of the *Fucus vesiculosus* acetone extract (Fv1) was evaluated on pancreatic cancer cell lines (panc1; panc98; pancTU1 and Colo357). It showed that Fv1 inhibited strongly the growth of different tumour cell lines with the EC_{50} values (effective half maximal concentration) of Fv1 17.35 $\mu g.mL^{-1}$ for PancTU1; 17.5 $\mu g.mL^{-1}$ for Panc89; 19.23 $\mu g.mL^{-1}$ for Panc1 and 28.9 $\mu g.mL^{-1}$ for Colo357. Importantly, Fv1 showed low cytotoxic activity against non-malignant resting T cells [31].

Fucoxanthin from *H. banksii* extract showed significant inhibition of cell growth of pancreatic cancer cell lines compared to other extracts from plants. In a Davidson's plum extract (DP; *Davidsonia pruriens* F. Muell), no toxicity to the non-tumourigenic cells (HPDE) at concentrations $\leq$ 200 $\mu g.mL^{-1}$ was observed. However, it only showed efficacy against ASPC1 cells (40-60% growth inhibition) at high concentrations between 200 – 400 $\mu g.mL^{-1}$ [32]. Cytotoxic activity of Eucalypt extracts against pancreatic cell lines (MiaPaCa2; BxPC3 and CFPAC1) outlined by Bhuyan et al. [33] was low with almost all extracts, except for species *Angophora floribanda* and *Angophora hispida* with 77.91% and 62.04% growth inhibition at the concentration of 100 $\mu g.mL^{-1}$, with the two extracts above showing high toxicity to non-tumourigenic cells (the growth inhibition >90% for both *Angophora floribanda* and *Angophora hispida* at concentration of 100 $\mu g.mL^{-1}$).

Algae-derived fucoxanthin has been also shown to exert cytotoxicity against several types of cancer cell lines. Fucoxanthin purified from brown alga *Padina australis* showed low efficacy against human lung cancer (H1299) with an IC_{50} value 2.45 mM [23]. Purified

fucoxanthin from brown alga *Fucus evanescens* C Agardh, showed quite high anticancer activity (IC_{50} -114 μM) against SK-MEL-28 (ATCC HTB-72) human melanoma cell line [10]. Moreover, growth of the melanoma cell line (B16F10 cells) was inhibited by 87% upon 72 h exposure to 200 μM fucoxanthin isolated from brown alga *Ishige okamurae*. Morphological changes of B16F10 cells were also observed in which fucoxanthin induced apoptosis through the presence of apoptotic bodies and nuclear condensation using the Hoechst 33342 stain [34].

On the other hand, it is important to evaluate the toxicity of algae-derived fucoxanthin on non-tumourigenic cells. It was reported by Wang et al. [11] that in three non-tumourigenic cell lines, the HUVEC cell line was the most sensitive to fucoxanthin (IC_{50}= 4.42 μM) after 72 h treatment, while the growth of HDFB (IC_{50}= 21.05 μM) and HEK293 (IC_{50}= 13.48 μM) cells were less affected by fucoxanthin at concentrations lower than IC_{50} value. Lower doses of fucoxanthin (1.56 μM) could stimulate the growth of normal cells and growth inhibition was not found in HEK293 cells with fucoxanthin at 6.25 μM after 24 and 48 h of treatment. It has been shown that HUC-Fm (human male umbilical cord fibroblast) and MRC-5 (human normal embryonic lung fibroblast) cells were unaffected by the treatment with fucoxanthin at 5 μM, however, both normal and cancer cells have been shown to have growth suppression in a dose-dependent manner, particularly with 10 μM fucoxanthin [35].

From the findings, it has been demonstrated that fucoxanthin has high anti-proliferative activity on pancreatic cancer cell lines, while it has mild toxicity to normal cells. However, it was shown that fucoxanthin shows toxic effect on non-malignant cells in cell culture, but has no toxic effect *in vivo* [31]. Fucoxanthin was safe for mice fed with a single dose of 1000-2000 mg/kg, or repeated doses of 500-1000 mg/kg for 30 days. No fatalities nor abnormalities were found in fucoxanthin treated groups [36]. Therefore, our in vitro data suggest that fucoxanthin from *H.banksii* may present a promising compound for further

pharmaceutical applications. In further studies, combination of fucoxanthin and other compounds (gemcitabine, algal phenolics) will be evaluated for selective cytotoxicity against pancreatic cancer cell lines. *In vivo* experiments would be necessary to confirm the promising *in vitro* data and to evaluate potential side effects of the algal extracts. In addition, the mechanism of action of fucoxanthin on the pancreatic cancer cell lines will be investigated.

4. Conclusions

The isolation and purification of fucoxanthin from the brown alga *H.banksii* has been successfully demonstrated at the laboratory-scale. The purified pigment was confirmed to be fucoxanthin by comparing with a standard fucoxanthin through TLC and HPLC analysis. The fucoxanthin content in the alga was of 0.58 mg $Fx.g^{-1}$ alga and the purity of this compound was achieved 92.3% purity using a chromatography column. Fucoxanthin showed high anti-cancer activity on pancreatic cancer cell lines compared to the gemcitabine, and only showed a moderate level of toxicity against non-tumourigenic cells. The *H. banksii* extract-derived fucoxanthin has potential to be used as a treatment against pancreatic cancer and further applications in pharmaceutical fields, however its efficacy and mechanism of action needs to be elucidated by investigating its effect on apoptosis and the cell cycle of cancer cells.

Acknowledgements

The authors also kindly thank to University of Newcastle; the Vietnamese Government through the Ministry of Education and Training; the Ministry of Agriculture and Rural Development for awarding a VIED-TUIT scholarship to Thanh Trung DANG.

Conflict of interest

The authors declare no conflict of interest.

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Legend to Figures

Fig.1: TLC for the extract and purified fucoxanthin (PF) compared with the standard commercial fucoxanthin (Std). B1-B4 represent for the bands of the pigments

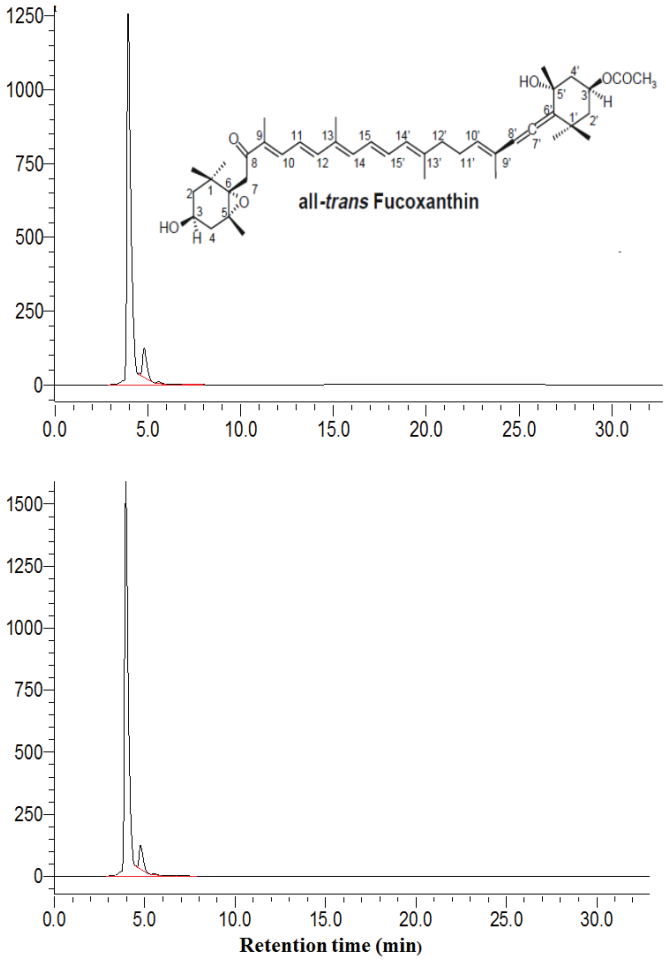

Fig. 2: HPLC chromatogram of the purified fucoxanthin (**top**) and purified fucoxanthin added to the standard fucoxanthin (**bottom**), and structure of all-*trans* Fucoxanthin.

Table 1. The cytotoxic activity (percentage of cancer cell growth inhibition; %) of the purified fucoxanthin at different concentrations in pancreatic cancer cell lines

Cell lines	Concentration of the fucoxanthin (µg.mL^{-1}) and gemcitabine (50 nM)					
	200	150	100	50	25	Gem.
HDPE	82.72±3.39^{a}	70.85±2.92^{b}	55.25±4.19^{c}	39.29±3.38^{d}	21.34±4.89^{e}	89.67±0.98^{f}
MiaCaPa2	92.81±1.72^{a}	84.5±2.52^{b}	75.9±2.95^{c}	31.73±3.78^{d}	7.66±4.65^{e}	87.28±1.59^{b}
BxPC3	71.71±5.81^{a}	70.3±4.53^{a}	55.93±4.97^{b}	14.62±2.09^{c}	6.74±2.92^{d}	75.03±1.82^{a}
CFPAC1	60.08±4.17^{a}	47.70±3.61^{b}	30.91±1.46^{c}	14.48±3.32^{d}	6.52±1.88^{e}	92.42±2.34^{f}

All values are means ± standard deviation (n=6) and those in the same row not sharing the same superscript letter are significantly different from the others ($P<0.05$).

Table 2. Value IC_{50} - The concentration of fucoxanthin purified from brown alga *H.banksii* that inhibits cell growth by 50% for normal and pancreatic cell lines.

Cell lines	IC_{50} Value (µg.mL^{-1})
HDPE	71.81
MiaCaPa2	67.47
BxPC3	97.68
CFPAC1	166.31

Author Contributions:

Thanh T. Dang participated in the experimental design and completion, as well as interpretation, and drafting the article. Deep J. Bhuyan, Danielle R. Bond, Michael C. Bowyer, Ian A. Van Altena participated in the experimental design and data interpretation. Christopher J. Scarlett participated in manuscript design and data interpretation. All authors read and approved the final version to be submitted.

Conference oral presentations/posters/proceedings

Conference poster

Elham Sadeqzadeh, Quan V. Vuong, Chloe D. Goldsmith, Van Tang Nguyen, **Deep J. Bhuyan**, Trung Thanh Dang, Anita C. Chalmers, Ian A. van Altena, Troy F. Gaston, Natalie Moltschaniwskyj, Michael C. Bowyer, Rick F. Thorne, Judith Weidenhofer, Phuong Thien Thuong, Nguyen Minh Khoi, Christopher J Scarlett. A natural product drug discovery pipeline for novel pancreatic cancer therapies: A new cancer research hub for the hunter region of NSW. Hunter Cancer Research Alliance Annual Symposium, NSW, Australia 12/2014. **Asia-Pacific Journal of Clinical Oncology (2014),** 10: 18-18.

A NATURAL PRODUCT DRUG DISCOVERY PIPELINE FOR NOVEL PANCREATIC CANCER THERAPIES: A NEW CANCER RESEARCH HUB FOR THE HUNTER REGION OF NSW

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Background: The diagnosis of pancreatic cancer (PC) has dire consequences as it presents late and is rapidly progressive. Due to the significant heterogeneity, PC is one of the most devastating of human cancers. Treatment options are limited to surgery and/or treatment with gemcitabine, and regardless of intervention, patient outcomes are modest at best. Novel approaches and new treatments are urgently required, and natural product-derived compounds, such as taxol/paclitaxel, irinotecan and gemcitabine provide justification for their continued investigation for novel drug discoveries.

Aims: New therapeutic interventions begin in the laboratory. At the University of Newcastle's Central Coast Campus, we are aiming to commission an Australian PC screening facility as part of a PC translational treatment pipeline. Our focus is to exploit the unique evolutionary adaptations that natural products have developed to identify biologically active compounds that target aberrant mechanisms driving pancreatic carcinogenesis.

Methods: Terrestrial and marine flora from Australia and Vietnam, as well as marine invertebrates from Australian temperate seas, are ethnobotanically selected for further investigation. Using optimised extraction chemistry techniques, we are extracting, isolating and identifying biologically active compounds and assessing their anti-PC activity in vitro.

Results: We have extracted numerous compounds (as isolated compounds and within crude extracts). To date we have assessed the activity of >50 natural product extracts (including fractions containing phenolic compounds, saponins and terpenoids) from 12 terrestrial flora historically used for their traditional medicine properties. We have also established a pipeline for the extraction of complex cytotoxic molecules from marine invertebrates. Significant efficacy at low dose concentrations against PC cell lines have been observed (single compounds <1 μM; crude extracts <200 μg/mL) while exhibiting only limited toxicity towards normal pancreatic cells.

Conclusions: This unique drug discovery pipeline utilises powerful bioactive natural chemical compounds from an unmatched and untapped natural products source as well as a unique resource of PC cell lines and expertise (cancer biology, natural product chemistry and chemical synthesis capability) to identify, develop and assess novel therapeutic agents for PC.

Translational research aspect: This study aligns with the T1 translational pipeline in that is assessing the in vitro potential of novel chemotherapeutic agents for pancreatic cancer.

Conference oral presentation

Deep Jyoti Bhuyan, Quan Van Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, Christopher J. Scarlett. Optimisation of microwave-assisted extraction parameters for total phenolic content from *Eucalyptus robusta* using response surface methodology. International Journal of Food Science and Technology Conference, Lincoln, New Zealand 02/2015.

Optimisation of microwave-assisted extraction parameters for total phenolic content from *Eucalyptus robusta* using response surface methodology

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Abstract: *Eucalyptus robusta* (*E. robusta*) has been shown to possess many pharmacological properties *in vitro* in addition to its significance in traditional medicine. This study utilised microwave-assisted extraction (MAE) to yield optimal total phenolic content (TPC) and total antioxidants from *E. robusta*. Response surface methodology (RSM) with a three-level-three-factor Box–Behnken design evaluated the effect of parameters associated with MAE such as irradiation time, power and sample-to-solvent ratio on the yields of TPC and total antioxidants using Folin-Ciocalteu and ABTS assays respectively. The accuracy and reliability of RSM as a tool for predicting the yield of TPC using MAE was validated (model, $p = 0.0014$; $R^2 = 0.97$; lack of fit, $p = 0.052$). TPC yield was highly affected by the sample-to-solvent ratio followed by power and irradiation time. An experimental yield of 58.40 ± 1.03 mg GAE/g was obtained for TPC and 64.6% of the maximum ABTS value was liberated under the optimal conditions- 3 min irradiation time, 50% (600 W) microwave power, 2 (g/100mL) sample-to-solvent ratio. It was concluded that MAE is a reliable and efficient method for extracting high yields of TPC from *E. robusta* with significant potential to be up-scaled for nutraceutical or pharmaceutical applications

Key words: *Eucalyptus robusta*; microwave-assisted extraction; optimisation; RSM; BBD

1. Introduction

The leaves and bark of *E. robusta* have been used to treat an array of ailments including fever, skin diseases, dysentery, malaria and bacterial diseases as a part of traditional medicine [1, 2]. Several phenolic compounds such as gallic acid, ellagic acid, quercetin, naringenin, catechin, epicatechin, rutin, apigenin, and myricetin have been isolated from *Eucalypt* extracts [3, 4]. In addition, recently it has been demonstrated that there is a positive correlation between phenolic contents and the antioxidant activities of *E. robusta* fruit extracts [5]. These studies highlighted the role of *E. robusta* as a prospective source of biologically active phenolic compounds. Optimal extraction conditions for polyphenolics of *E. robusta* have not yet been established. MAE is a novel and green extraction method with shortened extraction time and reduced solvent consumption and water is inarguably the safest, cheapest and most environmentally friendly and accessible polar extraction solvent [6 - 8]. Therefore, this study was undertaken to optimize the three MAE parameters of irradiation time, power and sample-to-solvent ratio for extracting maximal levels of TPC and TAC from *E. robusta* using RSM and water.

2. Materials and Methods

2.1. Plant materials

Fresh leaves of *E. robusta* were collected from Ourimbah, Central Coast, NSW, Australia. The leaves were then stored at -20°C. The leaves were dried at 70ºC for 5 h using a dry air oven, ground to a fine powder and then stored at -20°C until required.

2.2. Microwave-assisted extraction (MAE)

A household microwave equipped with inverter technology (1200 W, Frequency 2450 MHz, Sharp Carousel, Japan) was used. Parameter permutations as designed by RSM were implemented. The extraction was carried out in sealed vessels and no evaporation was observed.

2.3. Response Surface methodology (RSM)

JMP software (v11.0) was utilized for RSM experimental design and analysis. A three-level-three-factor, BBD was applied with three central point replicates for designing experimental conditions based on the results of preliminary single-factor-test [9] to elucidate the influence of the three primary independent microwave parameters: irradiation time (1 – 3 min), power (40 – 60% or 480W – 720W) and sample-to-solvent ratio 2 – 8 (g/100mL). Prior to irradiation, each suspension was pre-leached for 5 min. After irradiation, the extracts were immediately cooled to room temperature (ice bath), whereupon the solution volume was made up to 250 mL using distilled water and filtered using a 0.45 µm cellulose syringe filter (Phenomenex Australia Pty. Ltd, NSW, Australia) prior to further investigation. A second order polynomial equation was postulated for each experimental response Y, as follows [10]:

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

Where Y is the dependent variable (TPC and TAC) and various X_i values are independent variables affecting the responses Y; β_0, β_1, β_2, and β_3 are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively; and k is the number of variables.

2.4. Determination of total phenolic content (TPC) and total antioxidant capacity (TAC)

TPC was determined according to the method of Vuong et al. [11]. TAC was measured using ABTS assay as described by Thaipong et al. [12].

2.8. Statistical analysis

JMP software was used to graph the 3D- and 2D-contour plots of variable response, and to predict optimal values for the three response variables. All experiments were performed in triplicate and the results averaged. SPSS statistical software (v16.0) was utilised to compare the means analysis by independent samples t-test. At $p < 0.05$, the differences between the mean values were taken to be statistically significant.

3. Results and Discussion

3.1. Fitting the models

The analysis of variance of the Box-Behnken design for determining the model fit is illustrated in Table 1 and Fig. 1. In the case of TPC, the R^2 of the model was 0.97, signifying a 97% match between the predicted and experimental data. The *p*-value for lack of fit was estimated to be 0.052 further specifying that the lack of fit of the model was not significant ($p > 0.05$). Moreover, the Predicted Residual Sum of Squares (PRESS), F and *p* values of the model were estimated to be 329.01, 19.98 and 0.0014, respectively. Similarly for TAC, R^2, *p*, F and PRESS values were found to be 0.93, 0.0217, 7.13 and 3317.61, respectively, establishing the competency of the model in accurately predicting the antioxidant capacity of *E. robusta* extracts. The *p*-value for lack of fit for TAC was found insignificant ($p > 0.05$). By applying multiple regression analysis on the experimental data, a predicted response, *Y* for the TPC and TAC of the *E. robusta* extracts can be expressed by the following second-order polynomial equations:

$$Y_{TPC} = 29.78 + 2.22\ X_1 + 3.38\ X_2 - 3.52\ X_3 - 0.51\ X_1X_2 - 2.05\ X_1X_3 - 1.00\ X_2X_3 + 9.69\ X_1^2 + 2.98\ X_2^2 + 7.12\ X_3^2$$

$$Y_{ABTS} = 100.16 - 3.37\ X_1 + 0.18\ X_2 + 12.97\ X_3 + 0.05\ X_1X_2 - 6.76\ X_1X_3 - 0.27\ X_2X_3 - 0.93\ X_1^2 + 5.51\ X_2^2 - 24.29\ X_3^2$$

Table 1. Box-Behnken design and observed responses.

Run	Microwave conditions			Experimental values (n=3)	
	Irradiation Time (X_1)	Power (X_2)	Ratio (X_3)	**TPC**	**TAC**
	min*	%**	g:100mL	mg GAE/g	mg TE/g
1	1	40	5	37.97	104.85
2	1	50	8	42.05	97.81
3	1	50	2	43.76	65.45
4	1	60	5	45.42	104.72
5	2	50	5	30.05	91.15
6	2	60	8	38.32	98.01
7	2	50	5	29.08	104.53

8	2	40	2	39.45	64.19
9	2	50	5	30.21	104.79
10	2	40	8	33.18	97.82
11	2	60	2	48.59	65.49
12	3	60	5	45.91	104.75
13	3	40	5	40.53	104.64
14	3	50	2	55.26	65.56
15	3	50	8	45.33	70.88

***** Extraction time is 2X of irradiation time as 10 sec ON and 10 sec OFF strategy was implemented. ****** 40, 50 and 60% power were equivalent to 480 W, 600 W and 720 W respectively.

3.2. Effect of microwave independent variables on TPC of *E. robusta* extracts

The extraction yield of TPC within *E. robusta* extracts was significantly influenced by all the three independent microwave variables: irradiation time, power and sample-to-solvent ratio ($p < 0.05$). Similar observations were made in the extraction of TPC from prune (*Prunus domestica*) [13] and apple pomace [14] earlier. However, based on the *p*-values the impact on TPC can be summarized as sample-to-solvent ratio > power > time. An increase in sample-to-solvent ratio resulted in a decrease in TPC yield (Fig. 1). Parallel observations were made by earlier stating that decrease in solid-to-solvent ratio can increase TPC in the microwave-based extraction of *Melissa officinalis* [7, 8, 11]. Interaction between irradiation time x power, irradiation time x ratio and power x ratio had no significant impact on the extraction yield of TPC ($p > 0.05$). The negative impact of the sample-to-solvent ratio on the extraction yields can be explained by the inhibitory effect that a highly dense suspension possesses on the liberation of contents within a cell. As the sample-to-solvent ratio increases, suspension density increases, resulting in less effective solvation of the liberated cellular contents.

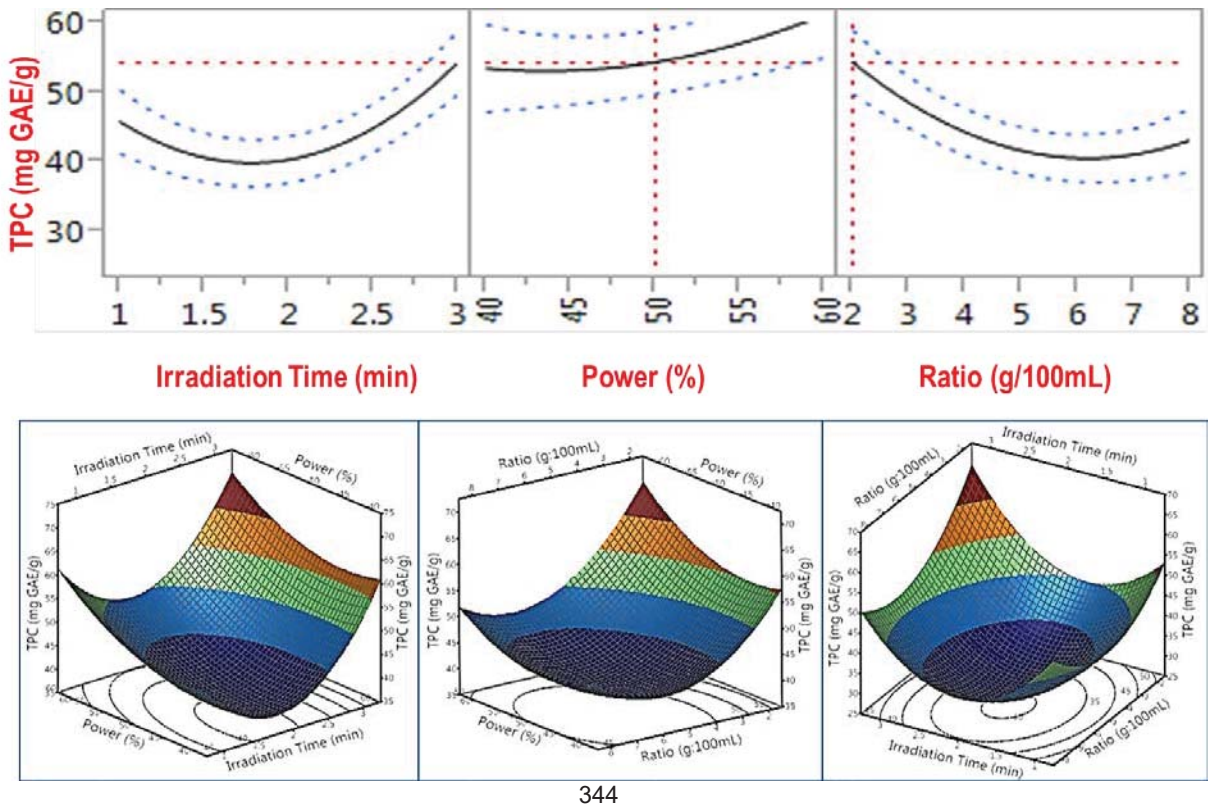
Figure 1: 2D and 3D impact of irradiation time, microwave power and sample-to-solvent ratio on TPC

3.5. Effect of microwave independent variables on TAC of *E. robusta* extracts

The only factor that influenced the TAC significantly was sample-to-solvent ratio ($p < 0.05$). Based on the *p*-values, the degree of effect of the microwave independent variables can be represented as following order: ratio > time > power. The interaction between time x power, irradiation time x ratio, and power x ratio had no noteworthy consequences on TAC. Parallel observations were made in star anise oil from *Illicium verum* Hook.f previously by Cai et al. [15].

3.6. Optimization and validation of the models

On the basis of the RSM predictive models and experimental values (Fig. 1), the ideal MAE conditions for the extraction of TPC were estimated to be: 3 min – 50% - 2 (g/100 mL); and 3 min – 40% - 5 (g/100 mL) for the TAC. Under the optimal extraction conditions for TPC, 64.6% of the maximum TAC value could be obtained under their respective ideal conditions. Hence, it was concluded that the optimal MAE conditions for extraction of TPC and TAC of *E. robusta* were as follows: 3 min – 50% - 2 (g/100 mL). Validation experiments were performed using these optimal conditions. Results showed that TPC and TAC levels of 58.40 ± 1.03 mg GAE/g and 74.95 ± 0.33 mg TE/g were obtained, respectively. TPC and TAC levels were not significantly different from the predicted values (54.40 ± 6.48 mg GAE/g and 65.33 ± 24.86 mg TE/g, respectively; $p > 0.05$). The close correlations between the predicted and



experimental values indicated the adequacy of the models to predict the optimal MAE conditions.

4. Conclusion

Box-Behnken Design was successfully implemented for optimizing the MAE parameters. RSM was established to be an appropriate, effective and reliable tool in evaluating the influence of three independent parameters for extractions of TPC from *E. robusta* and modelling and optimizing extraction conditions. Sample-to-solvent ratio had the greatest impact on the TPC and TAC yields of *E. robusta*. From the results obtained, it can be concluded that the optimal extraction conditions for TPC and TAC were: 3 min – 50% - 2 (g/100 mL). This study has the potential to be particularly valuable for industrial scale MAE of phytochemicals from *E. robusta* using water. The findings highlight the efficiency of MAE and its importance as an alternative method of extraction to the conventional procedures. This work also emphasises the need for further study to isolate and characterise the phytochemical profile of *E. robusta* leaves.

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Acknowledgements

We acknowledge the following funding support: Ramaciotti Foundation (ES2012/0104); Cancer Australia and Cure Cancer Australia Foundation (1033781); the Hunter Cancer Research Alliance (HCRA); the Hunter Medical Research Institute (HMRI) and the University of Newcastle.

Conference poster

Danielle Bond, Alexandra Turner, Rebecca Richmond, Elham Sadeqzadeh, Quan Vuong, **Deep Bhuyan**, Yusnita Rifai, Anita Chalmers, Ian van Altena, Troy Gaston, Michael Bowyer, Joshua Brzozowski, Helen Jankowski, Judith Weidenhofer, Jennette Sakoff, Phuong Thien Thuong, Do Thi Ha, Nguyen Minh Khoi, Christopher Scarlett. The search for novel treatment agents for pancreatic cancer: Tales from the land and sea. Hunter Cancer Research Alliance Annual Symposium, NSW, Australia 12/2015. **Asia-Pacific Journal of Clinical Oncology (2015),** 11 (S5), 6-19.

PP44

THE SEARCH FOR NOVEL TREATMENT AGENTS FOR PANCREATIC CANCER: TALES FROM THE LAND AND SEA

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Background: Pancreatic cancer (PC) is a disastrous disease with a dismal survival rate of only 5%. The current standard of care for PC patients consists of surgery and/or treatment with gemcitabine, a natural product-derived chemotherapeutic which has been shown to have a modest effect for some patients, however it is not a long-term curative treatment and results in many unwanted side effects. Natural therapies have the advantage of being relatively inexpensive to manufacture and are usually very tolerable and effective. Therefore, there is a strong need for novel, naturally occurring therapeutic options for PC.

Aim: To determine if crude extracts and/or purified compounds from various Australian and South East Asian flora and fauna (Vietnamese medicinal plants, Blueberry ash, bitter melon, Eucalyptus and marine invertebrates) exert anti-cancer activity *in vitro*, with an emphasis on pancreatic cancer.

Methods: Pancreatic cancer cell lines (BxPC3, MiaPaCa2 and CFPAC-1) and normal human pancreatic epithelial cells (HPDE) were treated with varying doses of crude extracts and purified compounds over a range of treatment times. Cell viability was assessed using CCK8 and MTT viability assays to determine the extent of anti-cancer activity due to growth inhibition and/or apoptosis.

Results: Significant growth inhibition of pancreatic cancer cells was observed with the purified compound pristimerin (from 2 Vietnamese medicinal plants), which displayed an IC_{50} of <2.7 uM in pancreatic cancer cell lines compared to 4.9 uM in normal HPDE cells. Crude extracts also showed significant selective efficacy at concentrations below 100 μg/mL; such as blueberry ash extract with IC_{50} values of 38.53 ug/mL and 57.34 ug/mL in BxPC3 pancreatic cancer cells and HPDE cells respectively; saponin enriched bitter melon extract IC_{50} values were 11.02 μg/mL and 54.60 μg/mL for MiaPaCa2 and HPDE cells, respectively; while specific Eucalypt crude extracts showed very low IC_{50} values (7-11 μg/mL in MiaPaCa2 cells). Further, 2 semi-purified compounds from a marine invertebrate had IC_{50} values <12.5 μg/mL for MiaPaCa-2 cells and >25 μg/mL for HPDE cells.

Conclusions: Purified compounds and crude extracts from plants and marine invertebrates show significant selective pancreatic cancer growth inhibition *in vitro* at concentrations with minimal effect on normal pancreatic cells, therefore showing promise as novel anti-pancreatic cancer therapies.

Translational research aspect: This is a T1 study as it is assessing ant-cancer activity of compounds *in vitro*, however it has the potential to move through the translational pipeline into T2 research in the future.

PP45

A BISPIDINONE ANALOGUE INDUCES AN APOPTOSIS-MEDIATED CYTOTOXIC EFFECT ON PANCREATIC CANCER CELLS *IN VITRO*

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Background: Pancreatic ductal adenocarcinoma (PDAC) is a disease with a very poor prognosis and limited therapeutic options. Current therapies and clinical trials have failed to improve survival outcomes. Due to the known complexity and heterogeneity of PDAC there are potential significant benefits in the continued discovery of novel targeted drug treatments, and bispidinone analogues have yet to be investigated as cytotoxic agents against pancreatic cancer cells.

Aim: This study investigated the cytotoxicity of bispidinone analogue **FD5006**, (2*S*,2*S*′)-1,1′-(9-oxo-1,5-diphenyl-3,7-diazabicyclo[3.3.1]nonane-3,7-diyl)bis(2-amino-2-(1*H*-indol-3-yl)ethanone).4HCl, against pancreatic cancer cell lines.

Methods: The cytotoxic effect of **FD5006** was assessed against 3 pancreatic cancer cell lines (MiaPaca-2, CFPAC-1, and BxPC-3). Viability was assessed using a CCK-8 colorimetric assay, and apoptotic cell death was confirmed using fluorescence microscopy, fluorescence/luminescence assays and flow cytometry.

Results: Initial viability screening results revealed significant cytotoxic activity from analogue **FD5006** treatment (concentration range 1 μM–100 μM) on all three cell lines compared to a no treatment control. A logarithmic dose-response analysis calculated IC_{50} values for MiaPaca-2, BxPC-3, and CFPAC-1 (16.9 μM, 23.7 μM, and 36.3 μM respectively). Cytotoxic treatment time-response (4 h, 24, and 48 h) from multiple viability assay replicates revealed a 24 h treatment time was sufficient to produce a cytotoxic effect on all cell lines in this study. Further investigations for markers of apoptotis on **FD5006** treated MiaPaca-2 pancreatic cancer cells revealed dose-dependent characteristic apoptotic morphological changes using light microscopy images, and fluorescent DAPI staining. Further fluorescence/luminescence assays and flow cytometry confirmed apoptotic cell death by findings of dose-dependent activated caspase-3/-7.

Conclusions: This study showed that the bispidinone analogue **FD5006** induced an apoptosis-mediated cytotoxic effect on MiaPaca-2 cell lines, and significant cytotoxicity on CFPAC-1 and BxPC-3 cell lines, in a dose- and time-dependent manner. This study paves the way for further investigations into the precise cellular mechanisms of action necessary for potential development into pre-clinical trials.

Translational research aspect: This study aligns with the T1 translational pipeline in that is assessing the *in vitro* potential of novel chemotherapeutic agents for pancreatic cancer.

Conference e-poster

Deep Jyoti Bhuyan, Quan V. Vuong, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, and Christopher J. Scarlett. Phytochemical, antibacterial and antifungal properties of an aqueous extract of *Eucalyptus microcorys* leaves. IFT16 (Institute of Food Technologists), Chicago, Illinois, USA 07/2016.

Phytochemical, antibacterial and antifungal properties of an aqueous extract of *Eucalyptus microcorys* leaves

Deep Jyoti Bhuyan[1,2], Quan V. Vuong[1,2], Anita C. Chalmers[2], Ian A. van Altena[2], Michael C. Bowyer[1,2], and Christopher J. Scarlett[1,2]

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Australia is home to about 800 different species of *Eucalyptus* and traditionally, many *Eucalyptus* species have been utilised to heal wounds and treat fungal infections by the Indigenous people of Australia. Research has mostly been carried out on *Eucalyptus* essential oils with less emphasis on crude aqueous extracts. Moreover, *Eucalyptus microcorys* is one of the least exploited species in terms of its chemical content and antimicrobial properties. In view of this, our study was designed to investigate the phytochemical, antibacterial and antifungal properties of crude aqueous extract of *E. microcorys* leaves. Freeze-dried powdered extract was prepared and the phytochemical profile was studied by analysing the total phenolic content (TPC), total flavonoid content (TFC), proanthocyanidins, antioxidants and saponins. The TPC, TFC and proanthocyanidin values were: 501.76 ± 14.47 mg of gallic acid equivalents per g, 61.53 ± 0.83 mg of rutin equivalents per g and 10.76 ± 0.89 mg of catechin equivalents per g, respectively. ABTS, DPPH and CUPRAC assays were employed to determine the antioxidant properties of the extract. The antioxidant values were expressed in mg trolox equivalents per g of extract (mg TE/g) and the values were: ABTS = 1073.13 ± 10.73 mg TE/g, DPPH = 1035.44 ± 65.54 mg TE/g and CUPRAC= 1524.30 ± 66.43 mg TE/g. These values were compared with ascorbic acid. The powdered extract was also evaluated for activity against 3 pathogenic bacterial (*Escherichia coli, Enterobacter aerogenes*, *Staphylococcus lugdunensis*); and 3 fungal strains (*Geotrichum candidum, Aspergillus niger* and *Candida albicans*) using the *96 well* microtiter *plate*-based *method with resazurin dye. The* extract *demonstrated significant efficacy with* minimum inhibitory concentration (MIC) values found to be: *A. niger* = 2.44 µg/ml, *G. candidum* = 4.88 µg/ml*, S. lugdunensis* = 78

µg/ml, *E. coli* = 156.25 µg/ml, *E. aerogenes* = 312.5 µg/ml and *C. albicans* = 1250 µg/ml. These results demonstrate the significant potential of *E. microcorys* as a source of phenolics, antioxidants and antimicrobial agents and also highlight the necessity of further purification and characterization of solitary bioactive compounds for their prospective applications in food, nutraceutical and pharmaceutical industries.

Keywords: *Eucalyptus microcorys*, phytochemical, antimicrobial

Conference poster

Deep Jyoti Bhuyan, Quan V. Vuong, Danielle R. Bond, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, and Christopher J. Scarlett. Aqueous *Eucalyptus microcorys* extract derived HPLC fractions with antioxidant and anti-pancreatic cancer activity. Australian Society of Medical Research Newcastle Satellite Scientific Meeting, Newcastle, NSW, Australia 06/2017.

Aqueous *Eucalyptus microcorys* extract derived HPLC fractions with antioxidant and anti-pancreatic cancer activity

Deep Jyoti Bhuyan [1,2], Quan V. Vuong [1,2], Anita C. Chalmers [2], Michael C. Bowyer [1,2], Christopher J. Scarlett [1,2]

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In Australia, pancreatic cancer (PC) has the highest mortality rate of any cancer with approximately 2500 new patients diagnosed every year. It has a poor prognosis due to its complexity and availability of very few effective therapies. More than 60% of the commercial anticancer drugs are derived from natural sources. Various natural and semi-synthetic drugs such as gemcitabine have been used in the treatment of PC. Although a number of reports suggested the anticancer properties of Australian eucalypts, there is very little scientific evidence emphasizing a direct role of eucalypts in the treatment of PC. The present study was designed to assess anti-pancreatic cancer and antioxidant activity of five HPLC fractions derived from the aqueous *Eucalyptus microcorys* extract. The cytotoxicity of the fractions against the MIA PaCa-2 pancreatic cancer and HPDE normal cell lines was elucidated using the Cell Counting Kit-8 assay. The Muse caspase 3/7 assay was applied to evaluate the apoptotic profile of the cancer cells. The antioxidant properties of the fractions were determined by ABTS, DPPH and CUPRAC assays. The fraction 1 (F1) at both 200 and 100 µg/mL exhibited significantly greater cell growth inhibition than the other four fractions ($p < 0.05$) with activation of caspase 3/7 mediated apoptosis in MIA PaCa-2 cells. F1 also displayed significantly greater antioxidant properties compared to the other four fractions in all three antioxidant assays ($p < 0.05$). These findings reveal the significance of the *E. microcorys* fractions as a potential source of phytochemicals with antioxidant and anti-pancreatic cancer activity.

Lay summary

Pancreatic cancer (PC) is one of the most devastating forms of human cancers in the world with no improvement of the overall survival rate over the last five decades. Australian eucalypts

have been linked with anticancer activity in a number of studies, but not specifically against PC. This study investigated the anti-pancreatic cancer and antioxidant activity of five fractions derived from *Eucalyptus microcorys* water extract. All five fractions showed potent anti-pancreatic cancer and antioxidant activity. Further studies are necessary to purify and identify specific compounds present in the fractions for developing more potent chemotherapeutic agents for pancreatic cancer.

Conference oral presentation

Deep Jyoti Bhuyan, Quan V. Vuong, Danielle R. Bond, Anita C. Chalmers, Ian A. van Altena, Michael C. Bowyer, and Christopher J. Scarlett. Aqueous *Angophora floribunda* extract as a source of phenolics and antioxidants with anti-pancreatic cancer and antimicrobial activity. International Conference on Scientific Frontiers in Natural Product Based Drugs 2017, Pharmacological Society of Singapore, NUS, Singapore 07/2017.

International Conference on Scientific Frontiers in Natural Product Based Drugs 2017, Pharmacological Society of Singapore, NUS, Singapore 07/2017

Aqueous *Angophora floribunda* extract as a source of phenolics and antioxidants with anti-pancreatic cancer and antimicrobial activity

Deep Jyoti BHUYAN[1,2], Quan V. VUONG[1,2], Danielle R. BOND[1,2], Anita C. CHALMERS[2], Michael C. BOWYER[1,2], Christopher J. SCARLETT[1,2]

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OBJECTIVE To elucidate the phytochemical, anticancer, antibacterial and antifungal properties of crude aqueous freeze-dried extract of *Angophora floribunda* leaves. **METHODS** The phytochemical profile was studied by analyzing the total phenolic content (TPC), total flavonoid content (TFC), proanthocyanidins and antioxidant properties of the extract. The cytotoxicity of the extract against the MIA PaCa-2 pancreatic cancer cell line was evaluated using the Dojindo Cell Counting Kit-8 assay (CCK-8). The Muse caspase 3/7 assay was applied to evaluate the apoptotic profile of the cancer cells. The antimicrobial activity of the extract was elucidated against 2 pathogenic bacterial strains (*Escherichia coli* and *Staphylococcus lugdunensis*) and 2 fungal strains (*Aspergillus brasiliensis* and *Candida albicans*) using the 96 well plate-based method with resazurin dye. **RESULTS** The TPC, TFC and proanthocyanidin values found were: 227.16 ± 14.62 mg of gallic acid equivalents per g, 91.42 ± 3.32 mg of rutin equivalents per g and 102.15 ± 4.57 mg of catechin equivalents per g, respectively. The antioxidant values expressed in mg trolox equivalents per g of extract (mg TE/g) were: ABTS = 657.53 ± 14.24 mg TE/g, DPPH = 758.21 ± 43.65 mg TE/g and CUPRAC= 834.52 ± 73.43 mg TE/g. The extract at 80 µg/mL inhibited the growth of MIA PaCa-2 cells by 59.71 ± 11.76%, with caspase 3/7 mediated apoptosis of cells observed after 24 h of treatment. In regards to the antimicrobial activity of the extract, minimum inhibitory concentration values were demonstrated to be: *A. brasiliensis* = 9.75 µg/mL, *S. lugdunensis*

= 156.25 µg/mL, *E. coli* = 312.5 µg/mL and *C. albicans* = 2500 µg/mL. **CONCLUSION** These findings reveal the significance of the *A. floribunda* aqueous extract as a potential source of phenolics and antioxidants with anti-pancreatic cancer, antibacterial and antifungal properties.

Key words: *Angophora floribunda*; anticancer; pancreatic cancer; CCK-8; caspase 3/7; antibacterial; antifungal

Acknowledgements: We acknowledge the following funding support: Ramaciotti Foundation (ES2012/0104); the Hunter Cancer Research Alliance (HCRA); the Hunter Medical Research Institute (HMRI) and the University of Newcastle, Australia.

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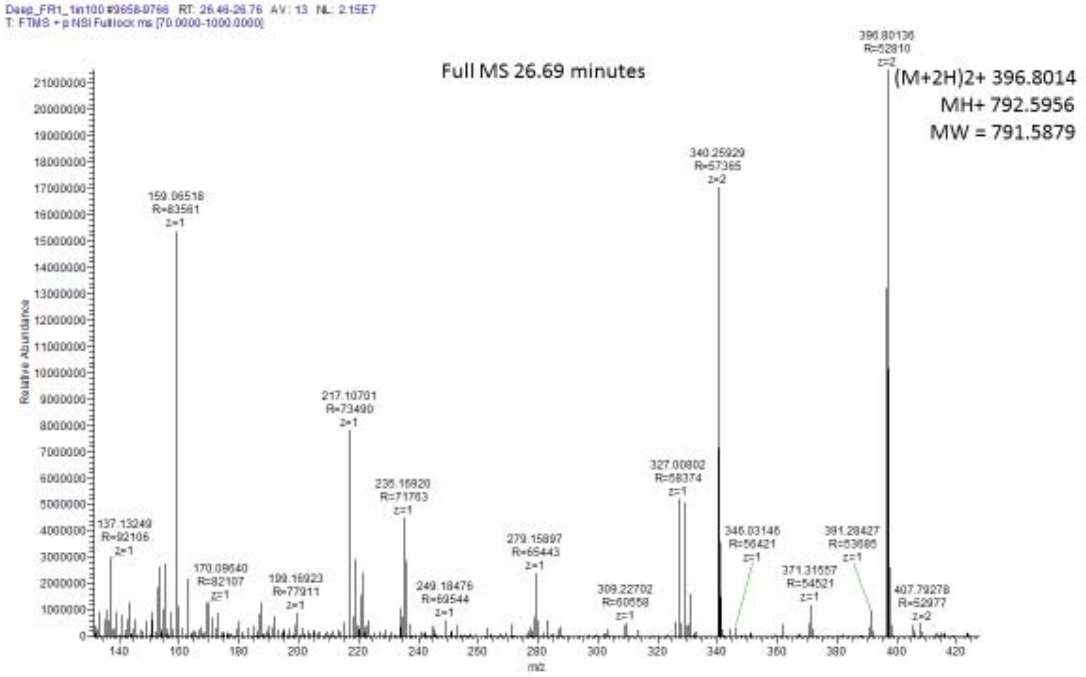
APPENDIX 2

Mass spectra (LC-ESI/MS/MS) of fraction 1 from *E. microcorys* aqueous extract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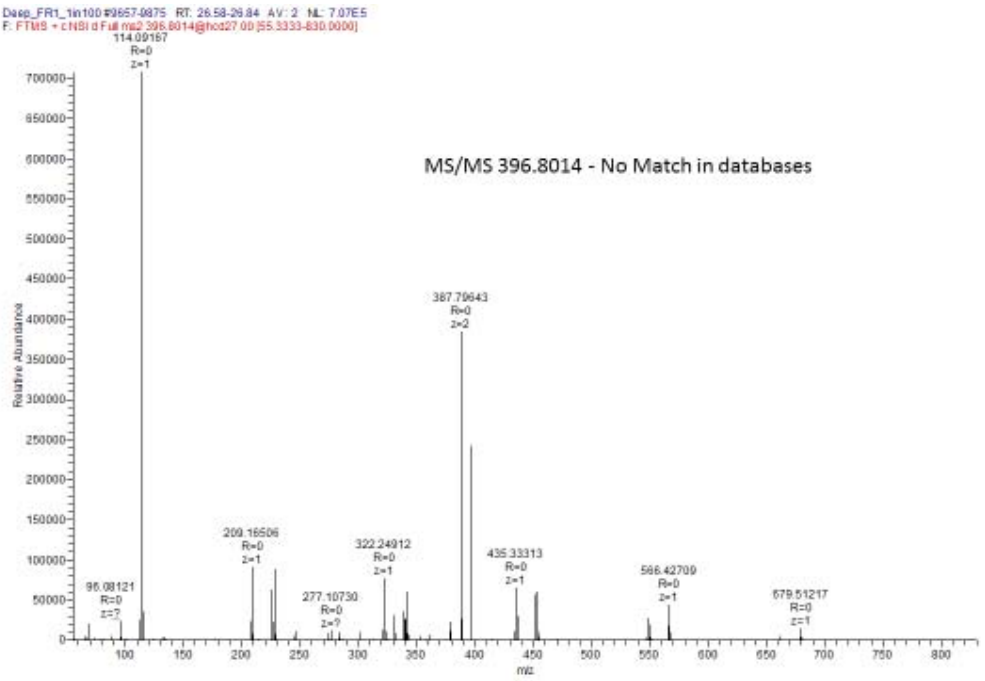
MH+ 792.5956
MW = 791.5879
Relative Abundance
m/z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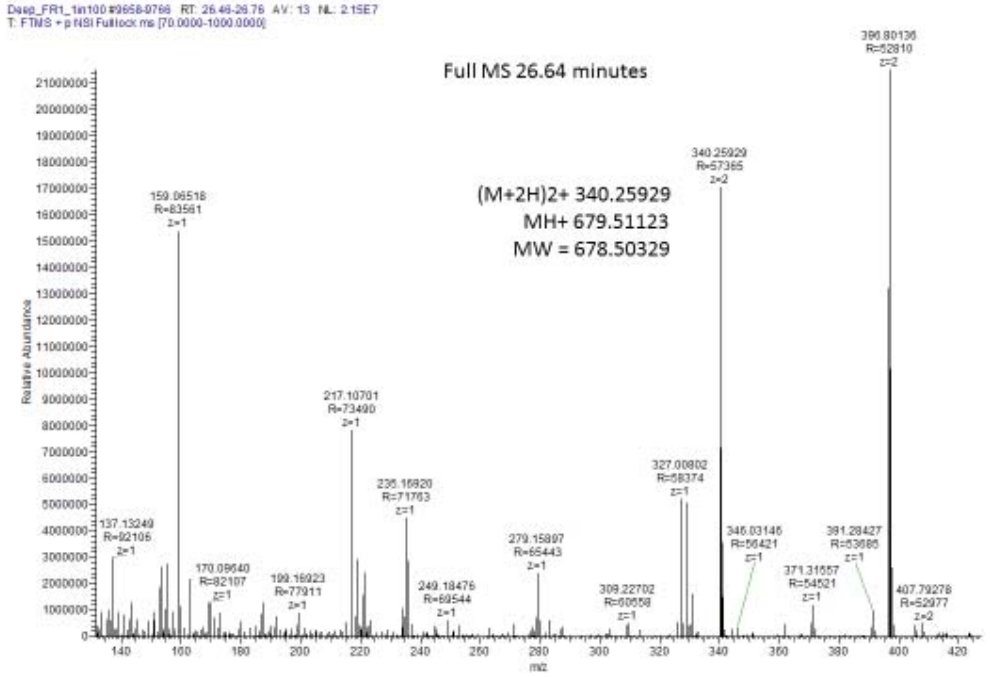
Relative Abundance
m/z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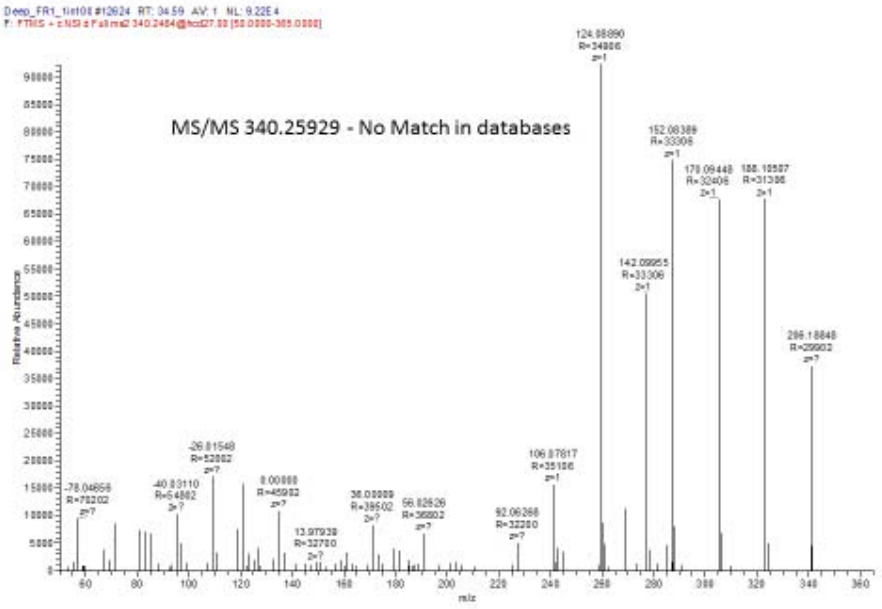
MW = 678.50329
Relative Abundance
m/z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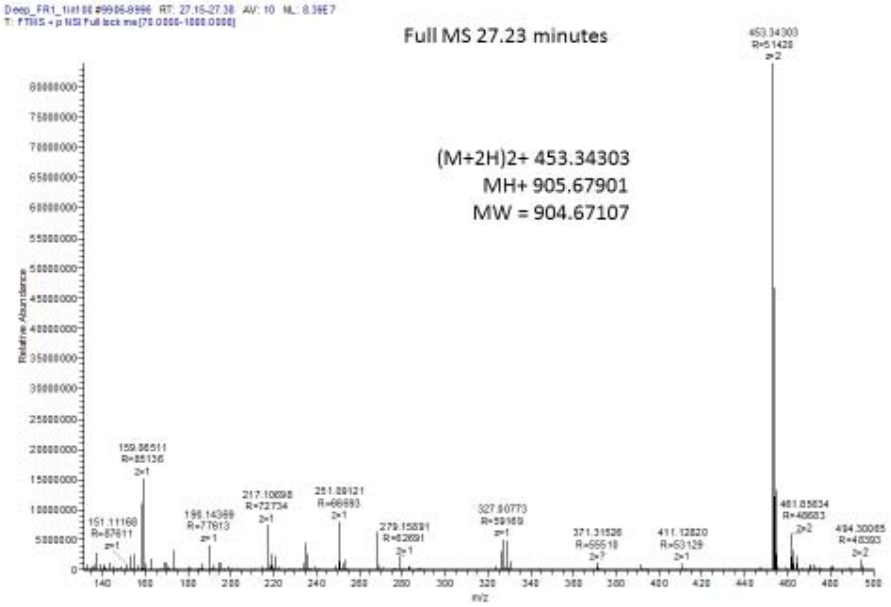
Relative Abundance
m/z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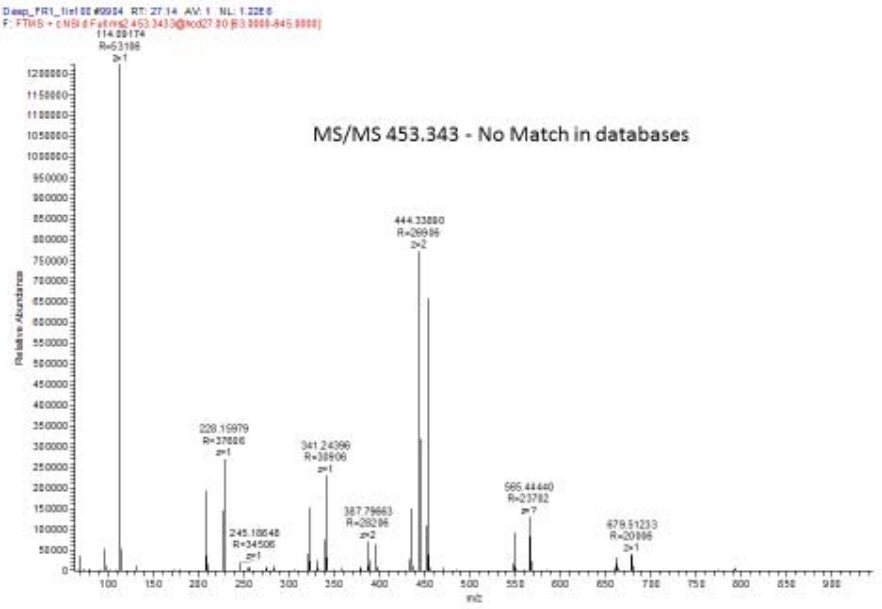
MW = 904.67107
Relative Abundance
m/z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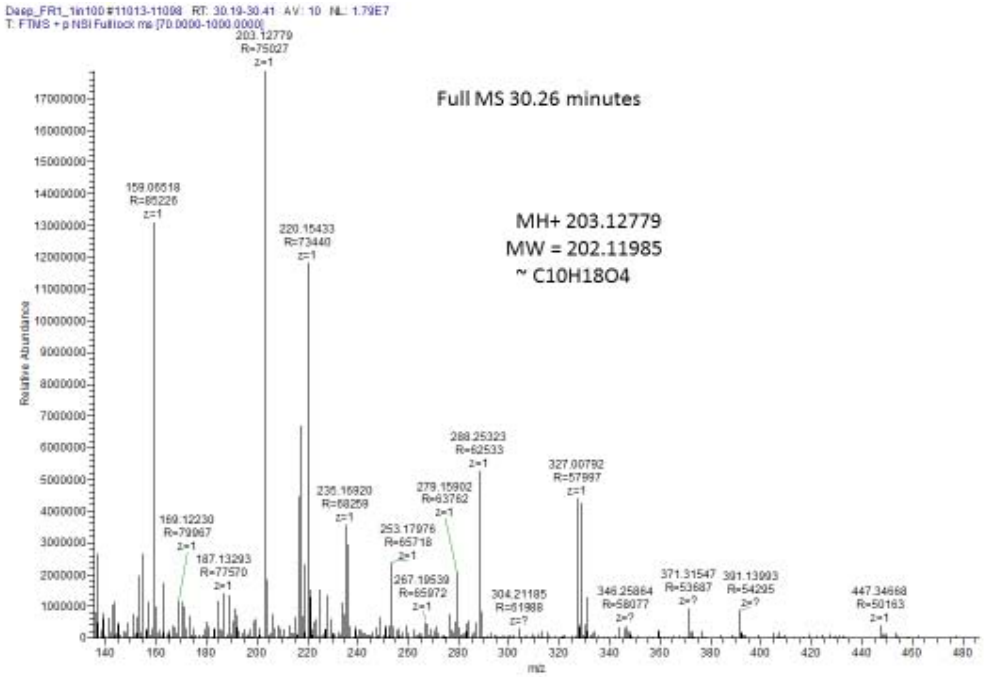
Relative Abundance
m/z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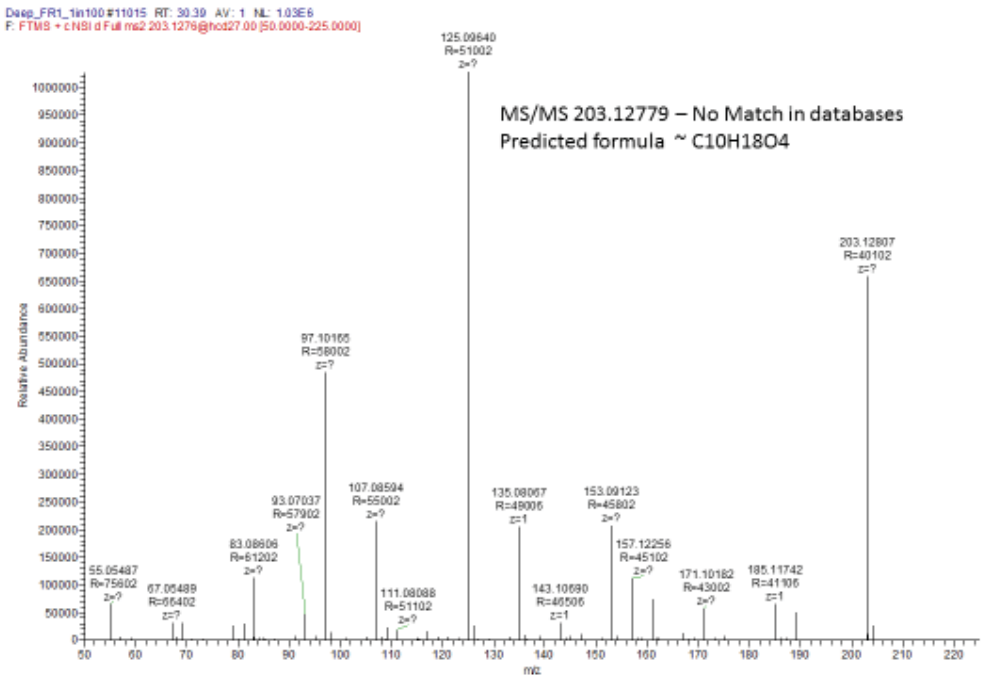
~ C10H18O4
Relative Abundance
m/z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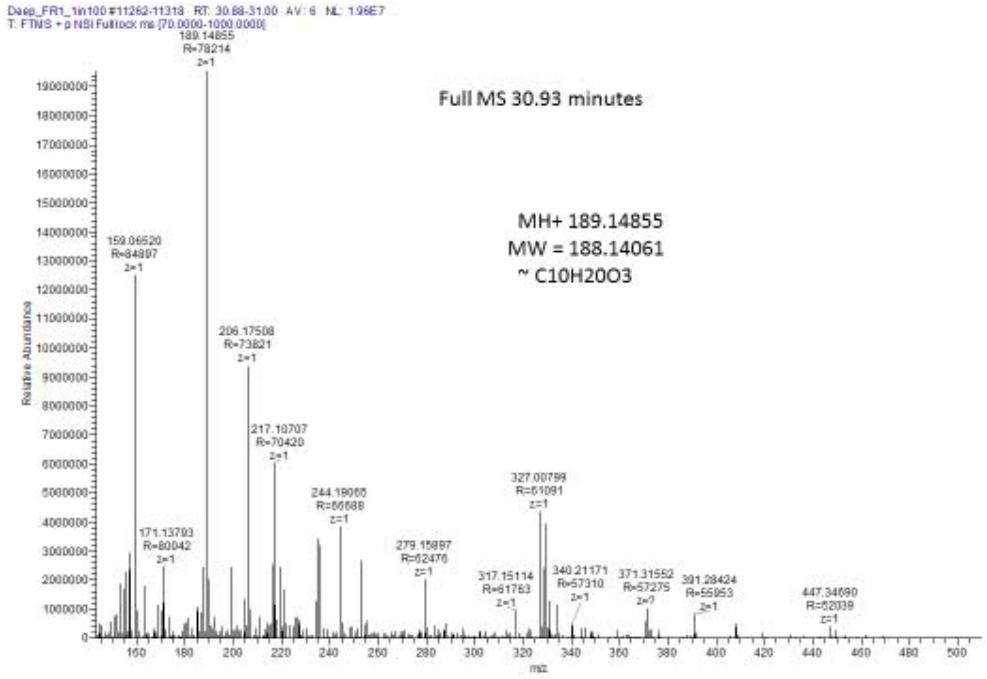
Predicted formula ~ C10H18O4
Relative Abundance
m/z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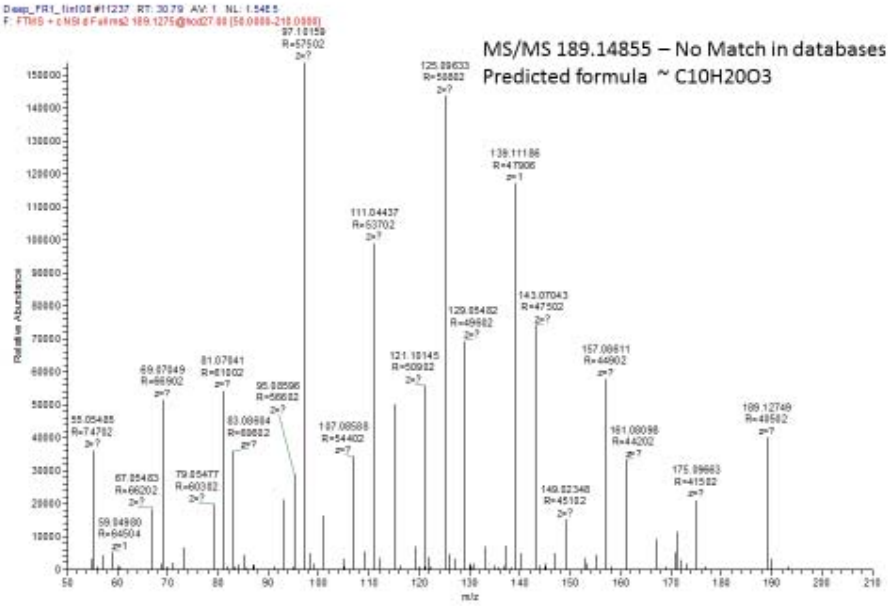
~ C10H20O3
Relative Abundance
m/z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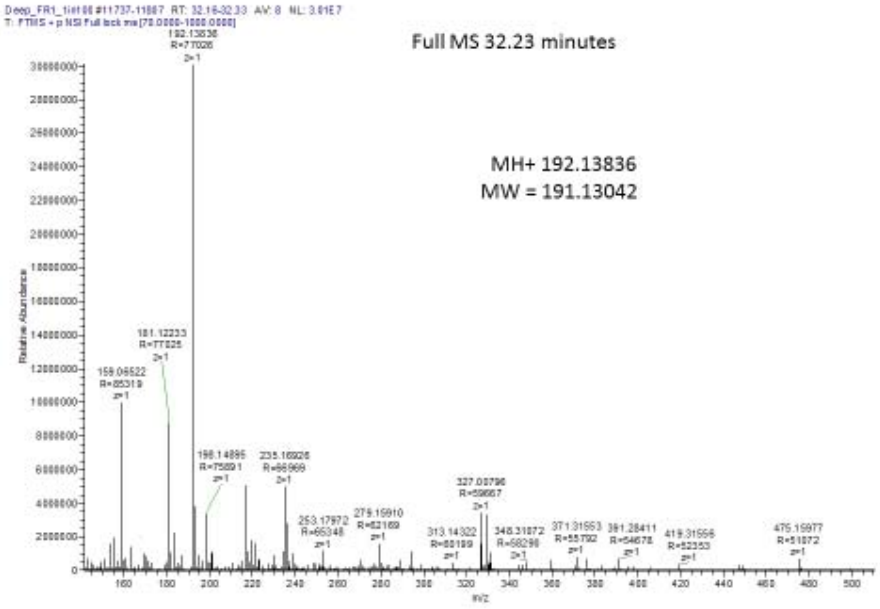
Relative Abundance
m/z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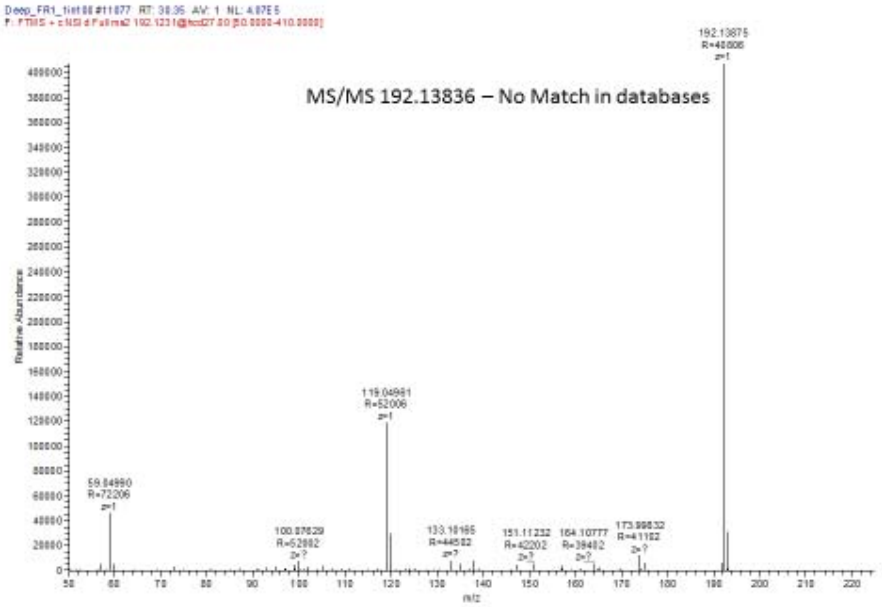
MW = 191.13042
Relative Abundance
m/z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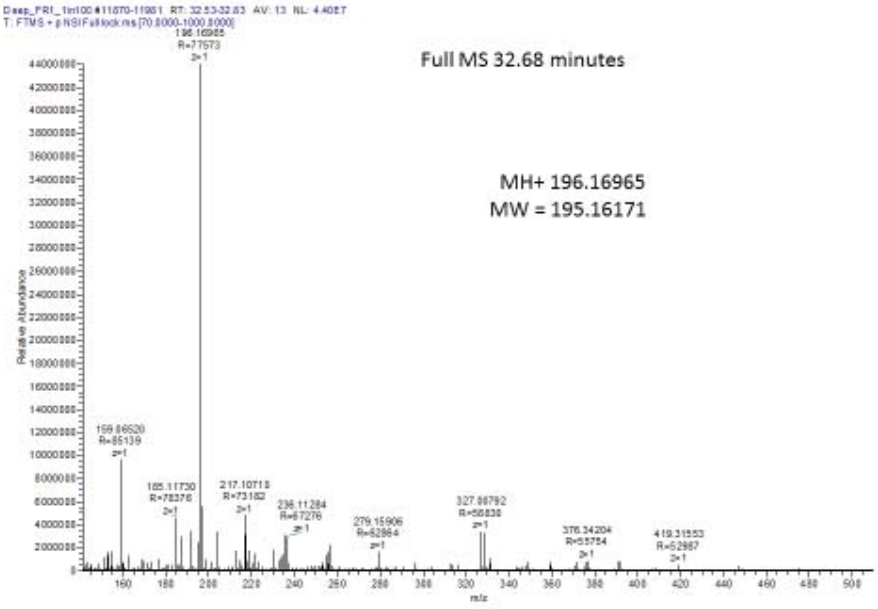
Relative Abundance
m/z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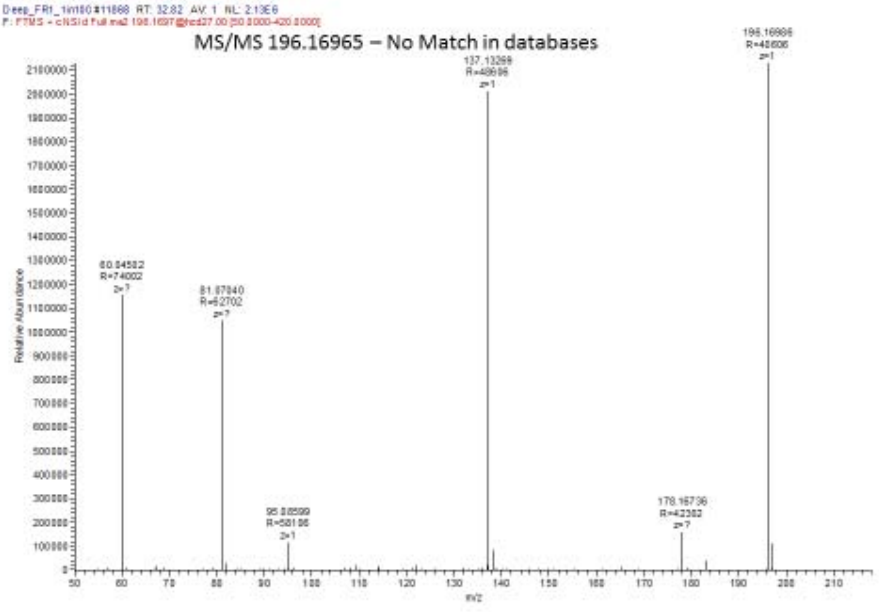
Relative Abundance
m/z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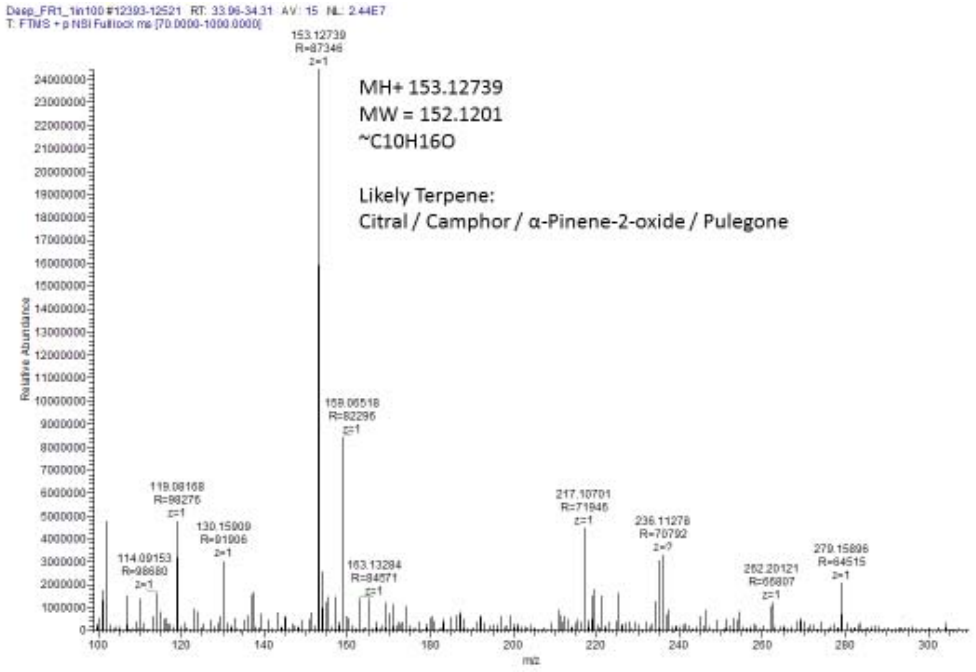
Relative Abundance
m/z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R=87346
z=1
MH+ 153.12739
MW = 152.1201
~C10H16O
Likely Terpene:
Citral / Camphor / α-Pinene-2-oxide / Pulegone
Relative Abundance
m/z
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R=82296
z=1
119.08168
R=98276
z=1
130.15909
R=91906
z=1
114.09153
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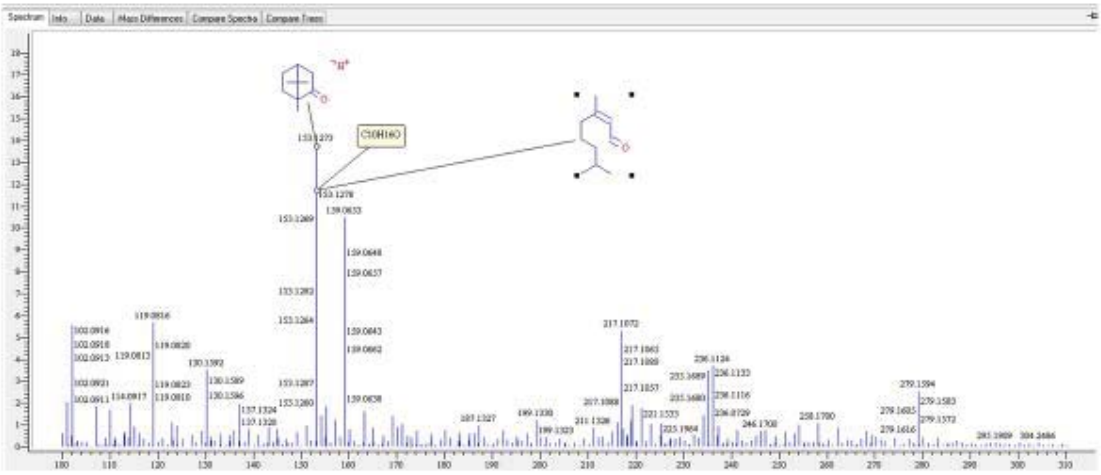
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Compare Spectra
Compare Trees
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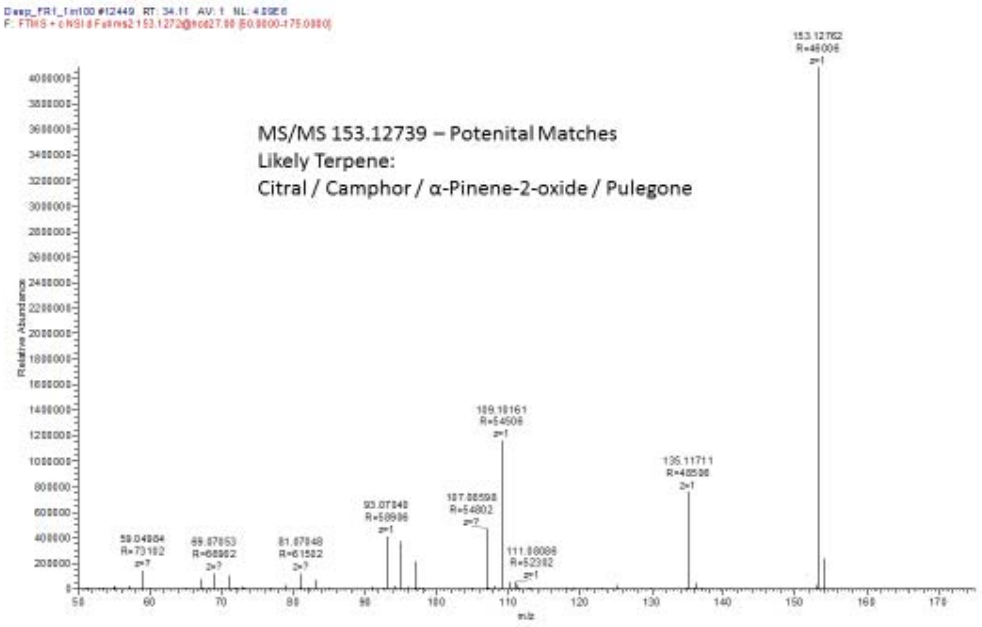
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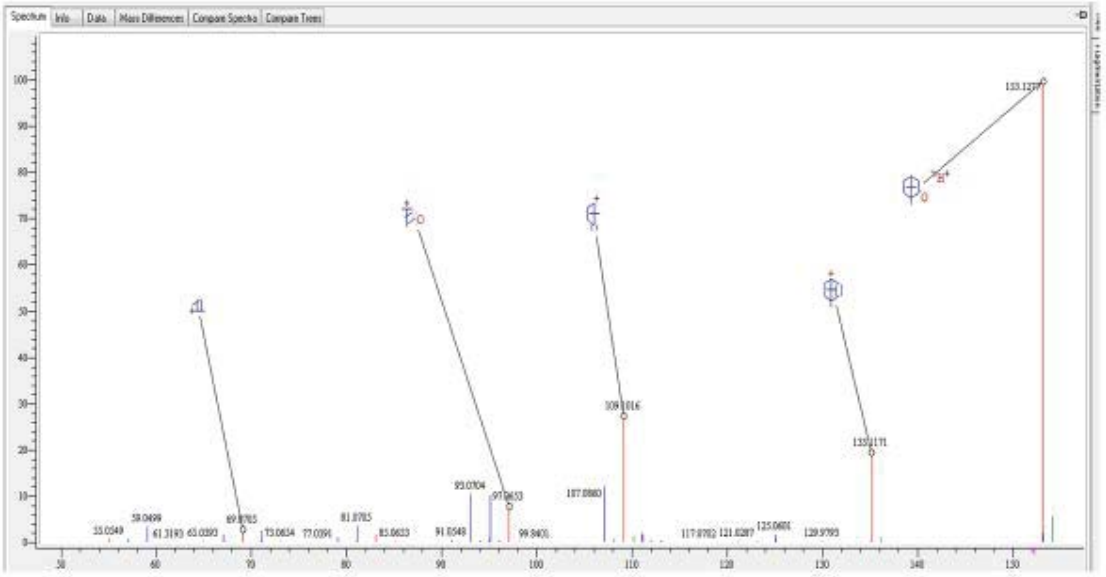
Likely Terpene:
Citral / Camphor / α-Pinene-2-oxide / Pulegone
Relative Abundance
m/z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Info
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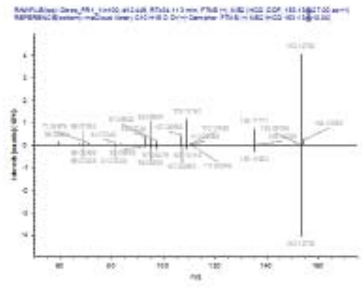
Compare Trees



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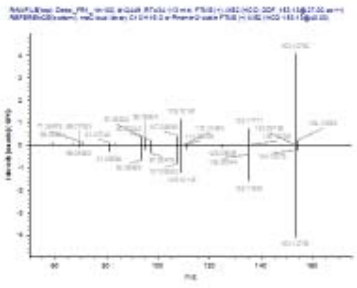

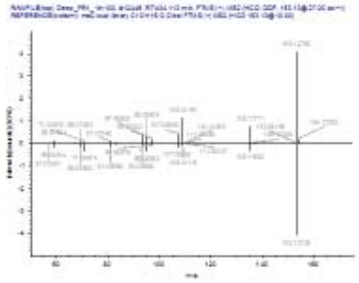

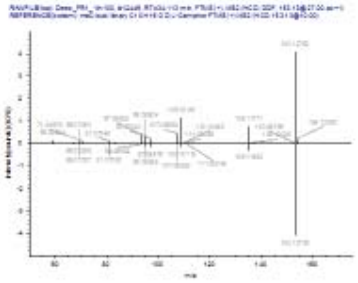

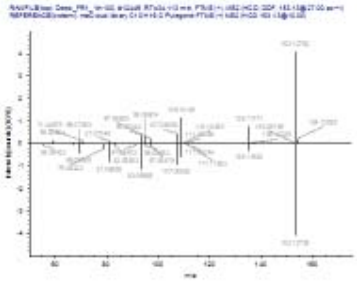

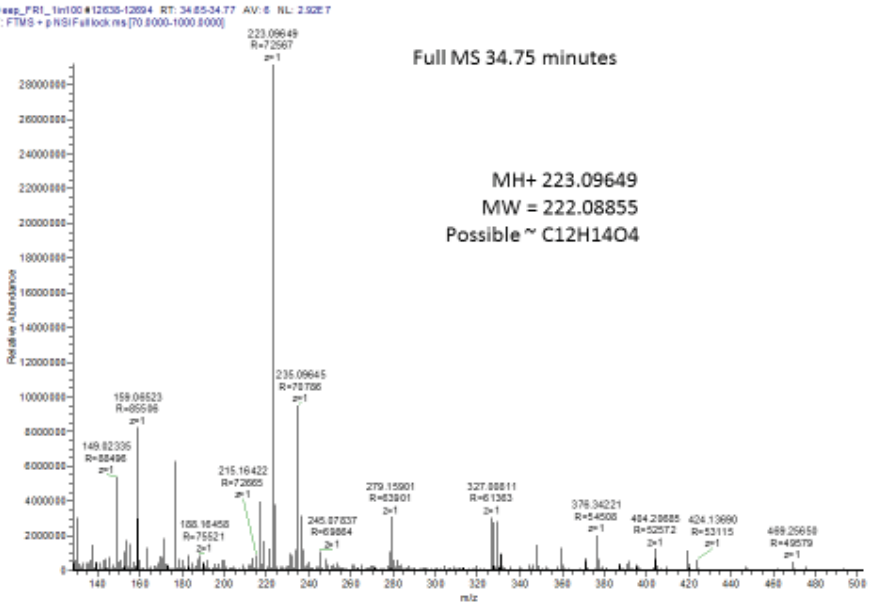
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MH+ 223.09649
MW = 222.08855
Possible ~ C12H14O4
223.09649
R=72567
z=1
235.09645
R=70706
z=1
159.08523
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z=1
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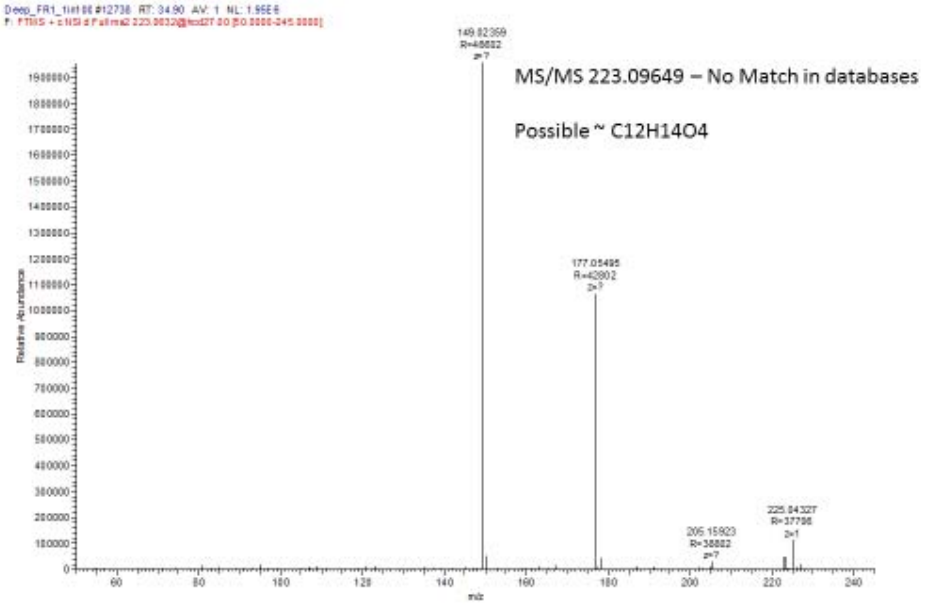
z=1
Relative Abundance
m/z


MS/MS 223.09649 – No Match in databases
Possible ~ C12H14O4
149.02359
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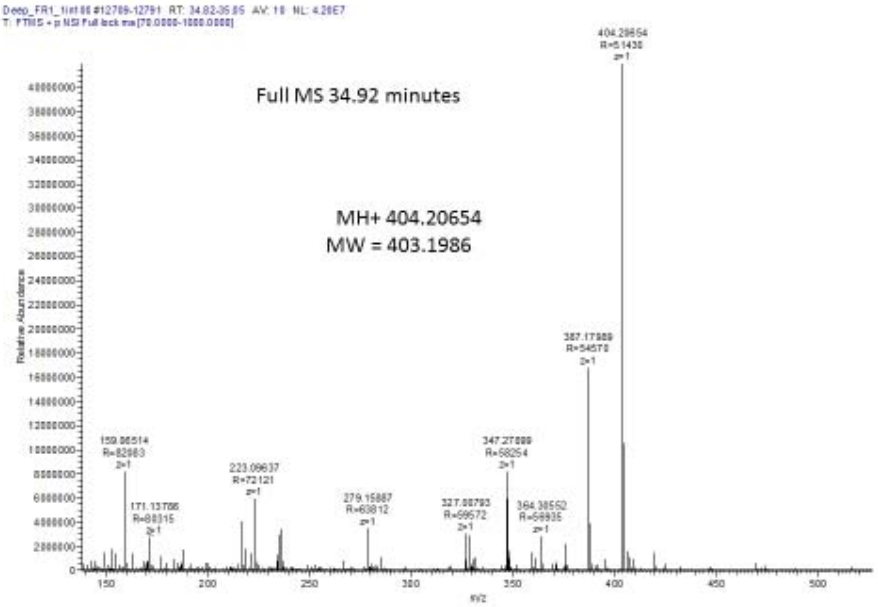
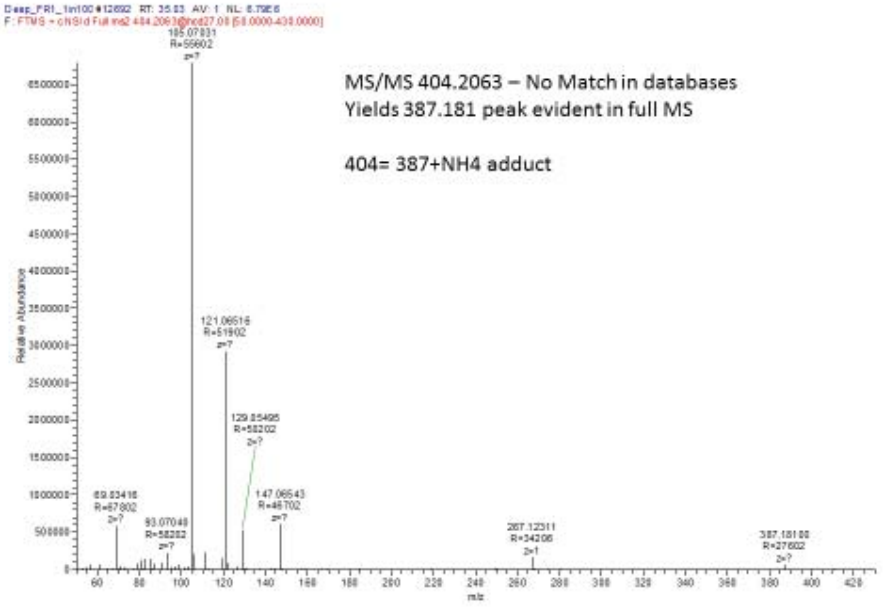
Relative Abundance
m/z


Full MS 34.92 minutes
MH+ 404.20654
MW = 403.1986
Relative Abundance
m/z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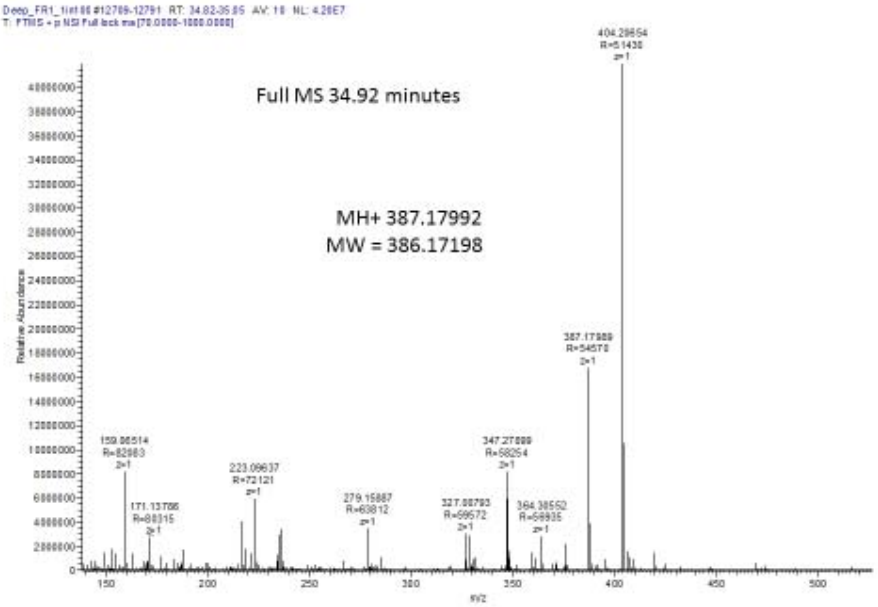
Yields 387.181 peak evident in full MS
404= 387+NH4 adduct
Relative Abundance
m/z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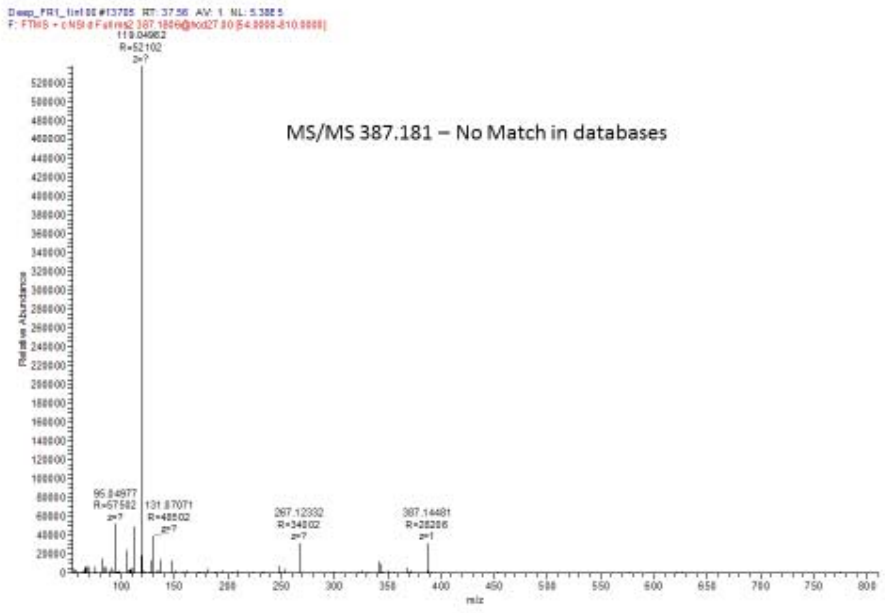
MH+ 387.17992
MW = 386.17198
Relative Abundance
m/z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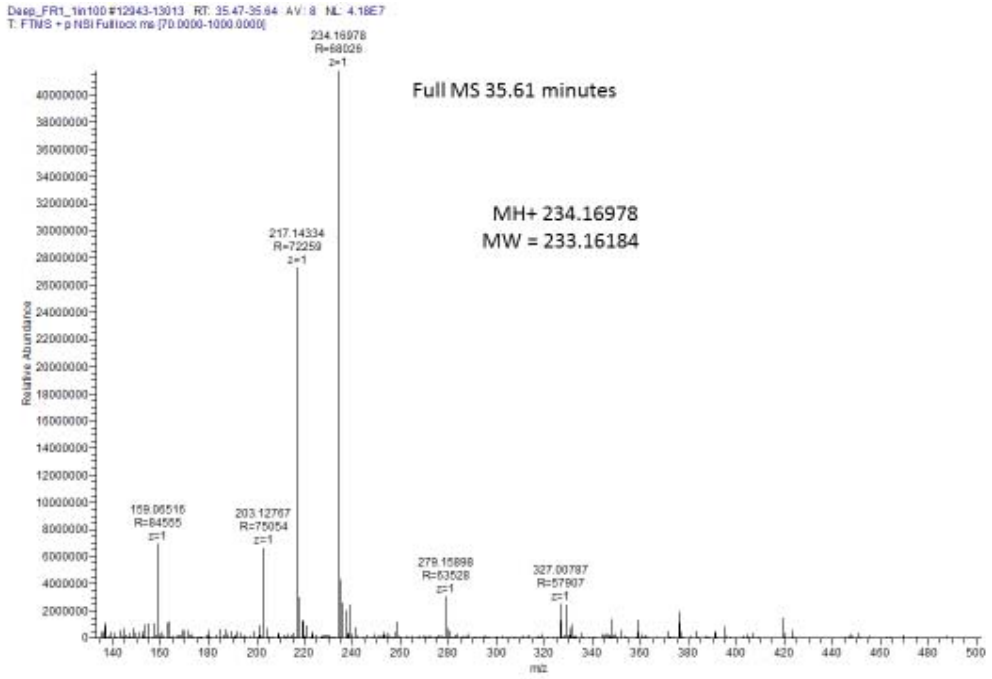
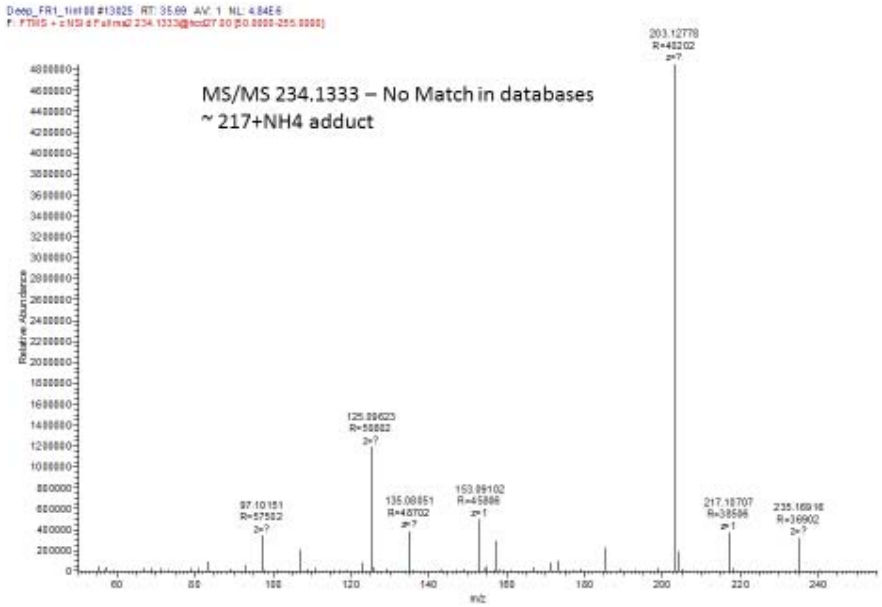
Relative Abundance
m/z


Full MS 35.61 minutes
MH+ 234.16978
MW = 233.16184
234.16978
217.14334
159.06516
203.12767
279.15898
327.00787
Relative Abundance
m/z


MS/MS 234.1333 – No Match in databases
~ 217+NH4 adduct
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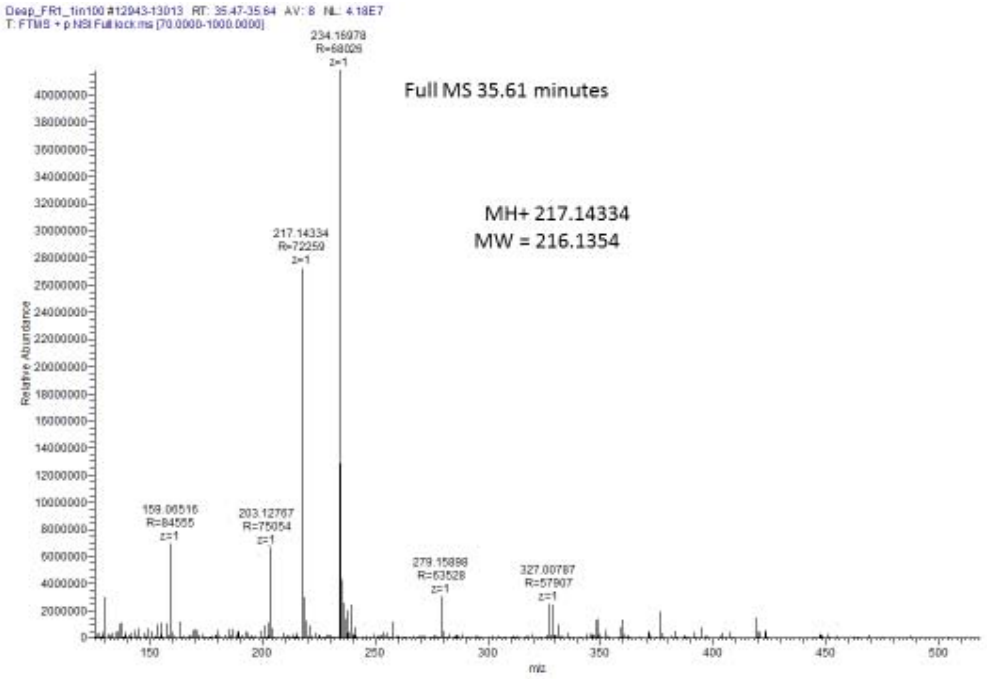
217.18707
235.16916
Relative Abundance
m/z


Full MS 35.61 minutes
MH+ 217.14334
MW = 216.1354
234.15978
R=68029
z=1
217.14334
R=72259
z=1
159.06516
R=84555
z=1
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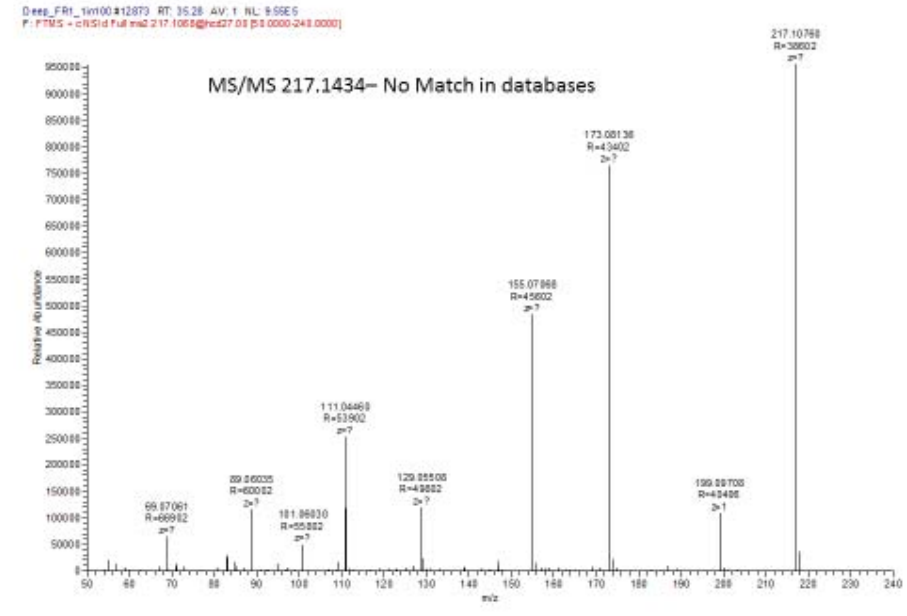
Relative Abundance
m/z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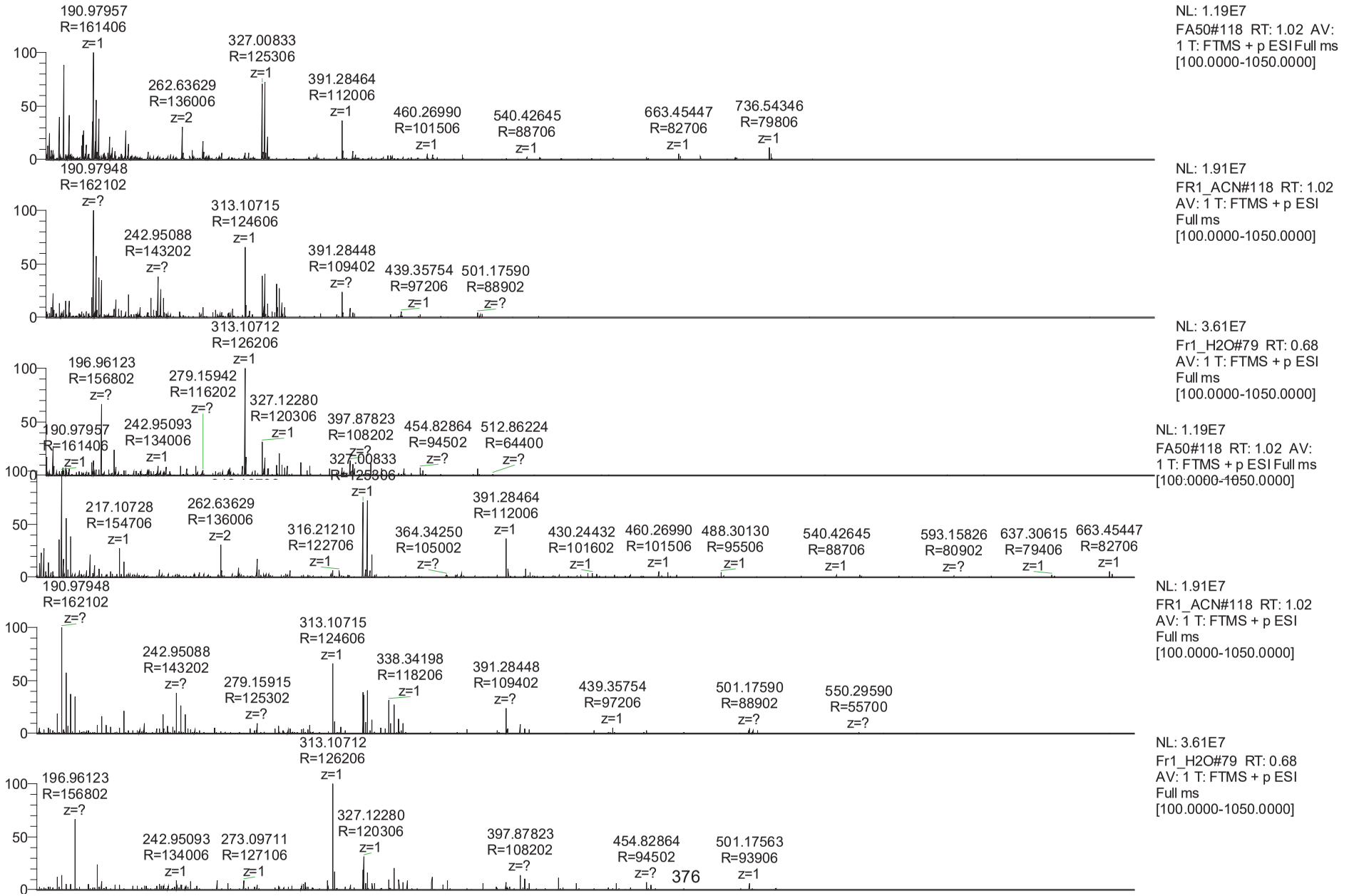
Relative Abundance
m/z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z=1
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663.45447
R=82706
z=1
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FA50#118 RT: 1.02 AV:
1 T: FTMS + p ESI Full ms
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AV: 1 T: FTMS + p ESI
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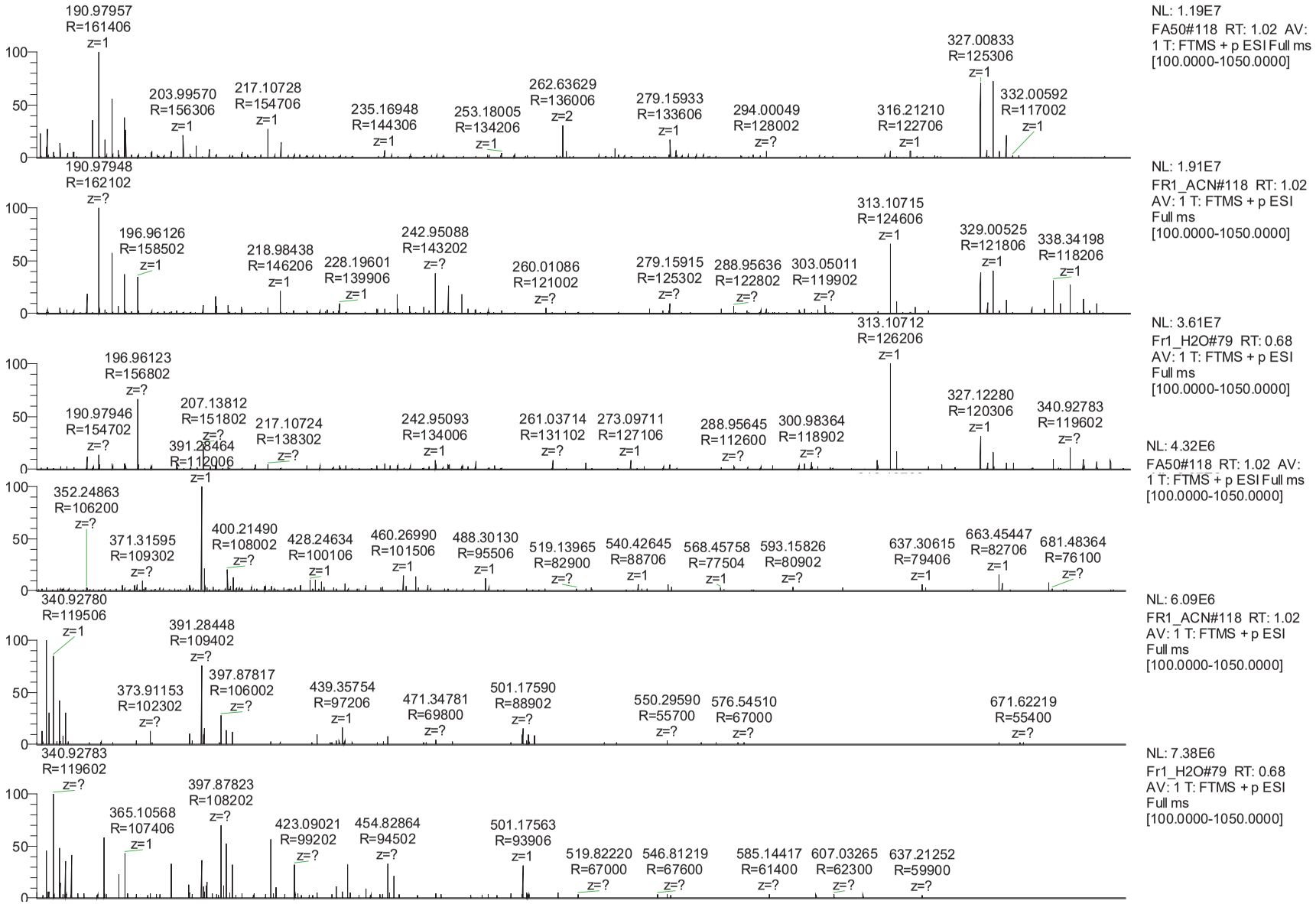
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