



Phytochemicals derived from Australian eucalypts as anticancer agents for pancreatic malignancies

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School of Environmental and Life Sciences

Faculty of Science

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Statement of Originality

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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

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To Whom It May Concern

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Other publications related to the thesis

Book Chapter

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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
4. 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
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

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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.

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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.

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

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.

Figure 3: IC₅₀ values of *Angophora* and *Eucalyptus* species as reported in the Research Papers 4 and 5 against MIA PaCa-2 cells. All values are means $\pm$ SD (n =3) and bars sharing the same letter are statistically similar to each other ($p > 0.05$).

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

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.

*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-3 β
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
IC₅₀	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-κB	Nuclear factor κ B
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