Extraction of Bioactives and Oil from Gac Seeds

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M. Sc. In Food Science and Technology

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

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STATEMENT OF ORIGINALITY

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

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THESIS BY PUBLICATION

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

Anh Van Le

Date: 17/12/2018

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LIST OF PUBLICATIONS INCLUDED AS PART OF THE THESIS

Paper I. Anh V. Le , Sophie E. Parks, Minh H. Nguyen and Paul D. Roach (2017).

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

Published in *Advance Journal of Food Science and Technology*, 13(4), 170-177, doi: 10.19026/ajfst.13.4444.

Paper II. Anh V. Le , Sophie E. Parks, Minh H. Nguyen and Paul D. Roach.

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

Published in *Technologies*, 6(4), 94; doi:10.3390/technologies6040094.

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

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

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

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

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

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STATEMENT OF CONTRIBUTION BY OTHERS

To whom it may concern,

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

Regards,

Doctor
Paul D. Roach

A/Professor
Minh H. Nguyen

Doctor
Sophie E. Parks

Doctor
Tien T. Huynh

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This project was led by Anh V. Le. She conducted all data collection and all analyses, and was primarily responsible for manuscript preparation. Numerically, the contribution from the authors were: Anh Le, 60%; Paul Roach, 20%; Sophie Parks and Minh Nguyen, 10% each.

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This project was led by Anh V. Le. She conducted most data collection and analyses, and she was primarily responsible for manuscript preparation. Tien Huynh conducted the analysis and data collection for the anticancer potential. Numerically, the contribution from the authors were: Anh Le, 50%; Tien Huynh, 20%; Paul Roach, 20%; Sophie Parks and Minh Nguyen, 5% each.

LIST OF ABBREVIATIONS AND UNITS OF MEASUREMENT

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

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

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

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ABSTRACT

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

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

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

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

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

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

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

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

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

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

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

SYNOPSIS

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

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

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

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

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

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