UNIVERSITI PUTRA MALAYSIA

EXTRACTION EFFICIENCY, PHYSICOCHEMICAL PROPERTIES AND HYPERCHOLESTEROLEMIA-LOWERING CAPABILITY OF VIRGIN AVOCADO OIL

TAN CHIN XUAN

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BERILMU BERBAKTI

EXTRACTION EFFICIENCY, PHYSICOCHEMICAL PROPERTIES AND HYPERCHOLESTEROLEMIA-LOWERING CAPABILITY OF VIRGIN AVOCADO OIL

By

TAN CHIN XUAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Chairman: Professor Hasanah Mohd Ghazali, PhD
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Virgin avocado oil (VAO) has been touted as functional oil due to its high nutritional value and bioactive components. This study aimed to investigate the extraction efficiency and physicochemical properties of VAO extracted using green techniques [ultrasound-assisted aqueous extraction (UAAE) and subcritical CO_2 extraction (SCO_2)] as well as the hypercholesterolemia-lowering potential of VAO intake. The optimum UAAE parameters to produce the highest recovery of low free fatty acids VAO were 6 mL/g of water-to-powder ratio, 30 min of sonication time and 35ºC of sonication temperature. Oil yield obtained from solvent extraction (20.79%) was significantly greater (p<0.05) than SCO_2 (16.97%) and UAAE (15.13%). Regardless of the extraction methods used, the major fatty acids in avocado oil were oleic (40.73-42.32%), palmitic (28.12-34.48%) and linoleic (15.52-18.88%) acids whereas the major triacylglycerols in avocado oil were palmitoyl-dioleoyl-glycerol (22.48-23.01%) and palmitoyl-oleoyl-linoleoyl-glycerol (17.64-18.23%). Avocado oil extracted using SCO_2 exhibited the strongest antioxidant capacity and contained two to four times greater levels of α- and γ-tocopherols than UAAE and solvent extraction. The hypercholesterolemia-lowering capability of VAO was assessed using diet-induced hypercholesterolemia rats. This *in vivo* study comprised five groups of rats, namely, normal diet control, high-cholesterol diet (HCD) control, HCD administrated with 450 mg/kg body weight VAO (HL), HCD administrated with 900 mg/kg body weight VAO (HH) and HCD administrated with 10 mg/kg body weight simvastatin (HS). High-density lipoprotein cholesterol level was significantly increased (p<0.05; 45% for HL, 77% for HH and 23% for HS) and the total triacylglycerols level was significantly reduced (p<0.05; 42% for HL, 58% for HH and 52% for HS) in the VAO- and simvastatin-treated rats when compared with their respective baseline results. On the other hand, low-density lipoprotein cholesterol was significantly reduced (p<0.05) in the HH (31%) and HS (49%) when compared with their respective baseline results. Results of the metabolomics study revealed that VAO intake could partially recover the metabolism dysfunction induced by hypercholesterolemia mainly via lipid, energy,

amino acid and gut microbiota metabolism. In conclusion, VAO has the potential to be utilized as functional oil to lowering hypercholesterolemia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KECEKAPAN PENGESTRAKAN, SIFAT FIZILO-KIMIA DAN KEUPAYAAN MENURUNKAN HIPERKOLESTEROLEMIA MINYAK ALPUKAT DARA

Oleh

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Minyak alpukat dara (VAO) telah dianggap sebagai minyak berfungsi kerana mempunyai nilai nutrisi dan komponen bioaktif yang tinggi. Tujuan kajian ini adalah untuk menilai keberkesanan pengekstrakan dan menentukan ciri-ciri fiziko-kimia VAO yang diekstrak dengan kaedah-kaedah hijau [pengekstrakan air dengan bantuan ultrasonik (UAAE) dan pengekstrakan karbon dioksida subgenting (SCO_2)] serta potensi pengambilan VAO dalam menurunkan hiperkolesterolemia. Keadaan optimum parameter UAAE bagi memperolehi paling banyak VAO yang rendah dengan asid lemak bebas adalah nisbah air kepada serbuk 6 mL/g, masa sonifikasi 30 min dan suhu sonfikasi 35ºC. Penghasilan minyak yang diperolehi daripada pengekstrakan pelarut (20.79%) adalah signifikan lebih banyak (p<0.05) berbanding dengan SCO_2 (16.97%) dan UAAE (15.13%). Tanpa mengambil kira kaedah-kaedah pengekstrakan yang digunakan, asid lemak majoriti dalam minyak alpukat adalah asid oleik (40.73-42.32%), asid palmitik (28.12-34.48%) dan asid linoleik (15.52-18.88%) manakala triasilgliserol majoriti dalam minyak alpukat adalah palmitoyl-dioleoyl gliserol (22.48-23.01%) dan palmitoyl-oleoyl-linoleoyl gliserol (17.64-18.23%). Minyak alpukat yang diekstrak dengan SCO_2 mengandungi kapasiti antioksidan yang paling kuat dan dua hingga empat kali lebih banyak α- dan γ-tocoferol berbanding dengan UAAE dan pengekstrakan pelarut. Keupayaan menurunkan hiperkolesterolemia VAO telah dinilai menggunakan tikus-tikus yang diaruh hiperkolesterolemia. Kajian *in vivo* ini menglibatkan lima kumpulan tikus, iaitu kawalan diet normal, kawalan diet kolesterol tinggi, diet kolesterol tinggi [HCD] diberikan dengan 450 mg/kg berat badan VAO (HL), HCD diberikan dengan 900 mg/kg berat badan VAO (HH) dan HCD diberikan dengan 10 mg/kg berat badan simvastatin (HS). Tahap kolesterol lipoprotein berkepadatan tinggi meningkat secara signifikan (p<0.05; 45% untuk HL, 77% untuk HH dan 23% untuk HS) dan tahap triasilgliserol berkurang secara signifikan (p<0.05; 42% untuk HL, 58% untuk HH dan 52% untuk HS) pada tikus yang dirawat dengan VAO dan simvastatin apabila dibandingkan dengan nilai baseline masing-masing. Selain itu, tahap kolesterol lipoprotein berkepadatan rendah berkurang secara signifikan ($p<0.05$) dalam HH (31%) dan HS (49%) apabila dibandingkan dengan nilai baseline masing-masing. Hasil kajian metabolomik menunjukkan pengambilan VAO mampu memulihkan sebahagian disfungsi metabolisme yang disebabkan oleh hiperkolesterolemia khasnya melalui metabolisme yang berkaitan dengan lipid, tenaga, asid amino dan microbiota usus. Kesimpulannya, VAO mempunyai potensi untuk digunakan sebagai minyak berfungsi untuk menurunkkan hiperkolesterolemia.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ABTS	2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
ACAT	Acyl-CoA cholesterol acyltransferase
AI	Atherogenic index
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists' Society
AST	Aspartate aminotransferase
ASU	Avocado and soybean unsaponifiables
BBA	β-Carotene bleaching assay
BMI	Body mass index
BMP	Bone morphogenetic protein-2
BSO	Bamboo shoot oil
CV-ANOVA	Analysis of variance of the cross-validated residuals
CO_2	Carbon dioxide
C16:0	Palmitic acid
C16:1	Palmitoleic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
DSC	Differential scanning colorimeter
FAME	Fatty acid methyl ester
FFA	Free fatty acids
FID	Flame ionization detector
FRAP	Ferric-reducing antioxidant power
GAE	Gallic acid equivalent
GC-MS	Gas chromatography-mass spectrometry
3-HB	3-Hydroxybutyrate
HC	High-cholesterol diet control
HCD	High-cholesterol diet
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HH	High-cholesterol diet administrated with 900 mg/kg body weight/day virgin avocado oil
HL	High-cholesterol diet administrated with 450 mg/kg body weight/day virgin avocado oil
HS	High-cholesterol diet administrated with 10 mg/kg body weight/day simvastatin
HMG-CoA	3-Hydroxy-3-metylglutaryl coenzyme A
HTR	High-density lipoprotein cholesterol to total cholesterol ratio
IACUC	Institutional Animal Care and Use Committee
IDL	Intermediate-density lipoprotein
IL-1β	Interleukin-1 beta
KOH	Potassium hydroxide
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein

LDL-C	Low-density lipoprotein cholesterol
LLL	Trilinolein
LLO	Dilinoleoyl-oleoyl-glycerol
LOO	Linoleoyl-dioleoyl-glycerol
MS	Mass spectrometry
MUFA	Total monounsaturated fatty acids
NC	Normal diet control
nCEH	Neutral cholesterol ester hydrolases
NMR	Nuclear magnetic resonance
NOX4	Nicotinamide adenine dinucleotide phosphate oxidase 4
NR	No requirement
OOO	Triolein
PAG	Phenylacetylglycine
PCA	Principle component analysis
PLL	Palmitoyl-dilinoleoyl-glycerol
PLPo	Palmitoyl-linoleoyl-palmitoleoyl-glycerol
PLLn	Palmitoyl-linoleoyl-linolenoyl-glycerol
PLP	Palmitoyl-linoleoyl-palmitoyl-glycerol
PLS-DA	Partial least squares-discriminant analysis
POL	Palmitoyl-oleoyl-linoleoyl-glycerol
POO	Palmitoyl-dioleoyl-glycerol
POP	Palmitoyl-oleoyl-palmitoyl-glycerol
POS	Palmitoyl-oleoyl-stearoyl-glycerol
PPLn	Dipalmitoyl-linolenoyl-glycerol
PPP	Tripalmitin
PPPo	Dipalmitoyl-palmitoleoyl-glycerol
PUFA	Total polyunsaturated fatty acids
RCT	Reverse cholesterol transport
RSM	Response surface methodology
SCO_2	Subcritical CO_2 extraction
SFA	Total saturated fatty acids
SFAO	Semisolid formulation of avocado oil
SFI	Solid fat index
SOO	Stearoyl-dioleoyl-glycerol
SREBP	Sterol-regulatory element binding proteins family
STZ	Streptozotocin
TAG	Triacylglycerol
TC	Total cholesterol
TCA	Tricarboxylic cycle
TCC	Total carotenoid content
TE	Trolox
TEAC	Trolox equivalent antioxidant capacity
TG	Total triacylglycerols
TGF	Transforming growth factor
TLC	Thin-layer chromatography
TMAO	Trimethylamine-N-oxide
TOTOX	Total oxidation
TPC	Total phenolic content
TPTZ	2, 4, 6-Tris (2-pyridyl)-s-triazine
TSP	3-(Trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt

UAE	Ultrasound-assisted extraction
UAAE	Ultrasound-assisted aqueous extraction
UK	United Kingdom
US	United States
USA	United States of America
VAO	Virgin avocado oil
VBAO	Vitamin B_{12} cream containing avocado oil
VIP	Variable importance in projection
VLDL	Very low-density lipoprotein
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Background of the Study

The fruit of *Persea americana* Mill., commonly known as avocado, is nutritious, buttery and contains a high amount of lipids and essential minerals like potassium, phosphorus and magnesium (Dreher & Davenport, 2013). The avocado tree is an evergreen dicotyledonous plant grown in tropical or subtropical climates. Between 1993 to 2013, the average global per capita consumption of avocado fruit increased from 0.36 kg in 1993 to 0.66 per kg in 2013, equivalent to a growth rate of 87% per year (Paz-Vega, 2015). Fresh avocado fruit contributes a large market worldwide, alongside with its use in the oil, cosmetic and food processing industries (Mostert, 2007).

Avocado oil is one of few plant oils not derived from the seed. Instead, it is extracted from the flesh of the fruit. Virgin avocado oil (VAO) is a specialty oil extracted via natural or mechanical approaches at low temperatures (<50ºC) and without undergoing refining (Woolf et al., 2008). Green extraction methods such as subcritical carbon dioxide extraction (SCO_2) and ultrasound-assisted aqueous extraction (UAAE) are recently proposed as new alternatives for the conventional solvent extraction in manufacturing edible oil (Chia et al., 2015; Khoei & Chekin, 2016). Both methods employ green solvents (carbon dioxide (CO_2) for SCO_2 and water for UAAE) to extract the oil from plant materials. The physical and chemical properties of plant oils are strongly influenced by the extraction conditions (e.g. solvents, temperatures and time) used in oil manufacturing process (Kiralan et al., 2014; Chia et al., 2015; Santana et al., 2015; da Silva et al., 2016). There is an increased interest in the development of oil extraction technologies that preserve the nutritional characteristics of avocado oil.

Plant oils are sources of phytosterols, tocols and unsaturated fatty acids. These components are able to modulate the metabolic processes, thereby reducing the risk of certain chronic diseases. Previously, avocado oil has been reported to possess pharmacologic and therapeutic effects on osteoarthritis treatment (Kucharz, 2003), blood pressure management (Salazar et al., 2005), wound healing (de Oliveira et al., 2013), body weight management (Furlan et al., 2017) and diabetes treatment (Ortiz-Avila et al., 2017) as it contains a variety of bioactive compounds. The extraction conditions used in producing VAO could preserve the most thermosensitive natural bioactive compounds present in the avocado pulp, which are crucial for its functional and health-promoting properties.

Hypercholesterolemia is a chronic disease characterized by elevated levels of cholesterol and low-density lipoprotein (LDL) cholesterol in the blood (Bunnoy et al., 2015; Cheong et al., 2018). It is a silent killer without showing obvious symptoms for prolonged periods. Pharmacological medicines such as statins (e.g. simvastatin, lovastatin, atorvastatin and rosuvastatin) and fibrates (e.g. fenofibrate, clofibrate, bezafibrate and ciprofibrate) are commonly used for the management of hypercholesterolemia, but long-term intake of these medicines may generate side effects such as nausea, myositis, diarrhea and abnormal liver function (Li et al., 2015). As an alternative to pharmacological medicines, the use of dietary bioactive compounds to control hypercholesterolemia has recently received a considerable amount of attention. For example, Ortiz-Avila et al. (2015) reported that supplementation of avocado oil normalized the total cholesterol (TC) level and reduced the total triacylglycerols (TG) level of streptozotocin-induced diabetic rats. Also, Furlan et al. (2017) showed that replacement of butter with avocado oil resulted in improved levels of TC, LDL-cholesterol and TG in overweight humans. These studies indicate the hypocholesterolemic potential of avocado oil in clinical application. However, hypocholesterolemic effect of VAO in diet-induced hypercholesterolemia rat model remains unknown. Due to the potential side effect of pharmacological medicines to treat hypercholesterolemia, it is worthwhile to investigate the hypercholesterolemia lowering-capability of VAO in diet-induced hypercholesterolemia rats.

Problem Statements

Plant oil rich in bioactive substances associated with health benefits is known as specialty oil (Gunstone, 2011). The exploration of specialty oil is of great interest to researchers over the last decade because of its potential application in functional oils, nutraceuticals and dietary supplements. As opposed to commodity oil, specialty oil is produced at low volumes (less than one million tons per year) and without undergoing refining (Gunstone, 2011; Sanders, 2016). Avocado oil is rich in bioactive components and the health benefits of solvent-extracted avocado oil consumption are well documented (Carvajal-Zarrabal et al., 2014; Toro-Carbot et al., 2015; Toro-Equihua et al., 2016; Furlan et al., 2017). However, it is important to choose an appropriate extraction method so that these compounds are preserved. Nowadays, the utilization of organic solvents such as hexane and/or petroleum ether in extraction of edible oils is under greater scrutiny due to increasing government restrictions and consumer concerns about the safety of using organic solvents in food processing (Yahia & Woolf, 2011). Green solvents such as CO_2 and water are an attractive alternative in place of conventional organic solvents.

Hypercholesterolemia is a chronic disease inflicting the world population. Globally, elevated cholesterol is estimated to cause 29.7 million disability-adjusted life years and 2.6 million deaths (WHO, 2017). In Malaysia, the prevalence of hypercholesterolemia among adults aged 18 and above had been increasing at an alarming rate, from 32.6% in 2011 to 47.7% in 2015, with a percentage increment of 46.3% (Institute for Public Health, 2015). There is an increasing prevalence of hypercholesterolemia around the world despite the vigorous efforts done by various research teams and government sector. This asymptomatic disease is associated with the development of non-alcoholic fatty liver disease and atherosclerosis. Lately, people are seeking for plant-based alternative medicines for the management of chronic diseases due to concerns over the side effects rendered by prolonged administration of synthetic pharmaceutical medicines. The use of bioactive compounds as part of disease prevention and management has been highly accepted due to its vast health benefits besides the low cost and safe property. Many *in vitro* and *in vivo* studies have shown that bioactive compounds have substantial effects on the prevention of cellular damage and atherogenesis, with the capacity to serve as anti-inflammatory, anti-tumor and anti-allergy agents (Kaliora, Dedoussis & Schmidt, 2006; Parihar & Parihar, 2017). In the food industry, the utilization of natural dietary bioactive compounds is highly promoted due to the potential toxicity effect of synthetic antioxidants. Dietary bioactive compounds play an important role in scavenging free radicals as well as delaying or inhabitation cell proliferation and DNA damage that will result in cell death (Kaliora et al., 2006). Hence, there is a great need to identify natural food products that can offer protection against hypercholesterolemia.

Objectives

The objectives of this study were as follows:

1. To optimize the extraction conditions for ultrasound-assisted aqueous extraction (UAAE) of VAO
2. To determine the physicochemical properties of avocado oil extracted using optimized UAAE, subcritical CO_2 (SCO_2) and established solvent (hexane) methods
3. To determine the hypocholesterolemic and hepatoprotective effects as well as the therapeutic mechanism of VAO in diet-induced hypercholesterolemia rats

Significance of the Study

Green extraction techniques such as SCO_2 and UAAE are recently proposed as new alternatives to the conventional solvent method (Chia et al., 2015; Khoei & Chekin, 2016). Attention has been directed to the use of such methods for the extraction of specialty oils for their feasibility in pharmaceutical and food industries. The physical and chemical characteristics of specialty oils are greatly affected by the solvents, temperatures and time used in the oil manufacturing process (Kiralan et al., 2014; Chia et al., 2015; Santana et al., 2015; da Silva et al., 2016). Thus, one of the objectives in this study was to evaluate the extraction efficiencies and physicochemical properties of avocado oil extracted using green technologies with the conventional solvent method. Data generated may benefit the development of eco-friendly extraction techniques for VAO.

In Malaysia, the prevalence of hypercholesterolemia is expecting to rise steadily in the coming years. Recent studies have shown the hypocholesterolemic potential of avocado oil intake on streptozotocin-induced diabetic rats and overweight humans (Ortiz-Avila et al., 2015; Furlan et al., 2017), but the hypocholesterolemic effect of VAO in diet-induced hypercholesterolemia rat model remains unknown. It is also crucial to understand the therapeutic mechanism of VAO using metabolomics. Data obtained from this study can provide useful scientific evidence for future human application by the health professionals.

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