UNIVERSITI PUTRA MALAYSIA

EXTRACTION, FRACTIONATION AND CHARACTERIZATION OF DURIAN (Durio zibethenius M.) LEAF EXTRACT AS POTENTIAL SOURCE OF SQUALENE AND ITS APPLICATION IN GELATIN-BASED FILM

KAM WAI YEE

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By

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Chair : Associate Professor Seyed Hamed Mirhosseini, PhD
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The utilization of extract from durian leaf by traditional practioners has indicated the presence of phytochemical compounds with anti-inflammatory as well as antioxidant properties. However, there is no updated scientific reports on phytochemical compound recovery from durian leaf via extraction methods. The main objective of this study was to investigate the most efficient extraction method on durian leaf, proceeded by recovery of high squalene content and antioxidant activity via fractionation of the crude leaf extract. Two extraction methods that were applied in this study were ultrasound-assisted extraction (UAE) and accelerated solvent extraction (ASE). The effectiveness of both extraction methods were investigated on the yield of crude leaf extract, DPPH free radical scavenging activity, ferric reducing antioxidant power (FRAP), total phenolic content (TPC), total flavonoid content (TFC) and squalene content. The results revealed that UAE was more efficient than ASE in obtaining natural extract from durian leaf waste with optimum antioxidant activity and squalene content. In addition, UAE was reported to require shorter processing time and less energy required at lower temperature condition. Results from the optimization process revealed that the optimum condition for UAE was 5 min extraction time under continuous mode with the amplitude of 66% (power intensity 261 W/cm2), using 100% hexane at the solvent-sample ratio of 13:1. Under this optimum condition, a crude yield extract of 6.63% was obtained, and possessing antioxidant activity at 6.63 mg/mL TE per 100 mg DW in DPPH assay, 55.96 mg/mL TE per 100 mg DW in FRAP assay, 69.61 g GAE / 100 g DW in TPC and 1210.8 mg QE / 100 mg DW in TFC. Squalene content was reported at 20.56 %. The crude extract sample from UAEôs optimum condition was preceded for further fractionation. The objective was to achieve fractions that were high in antioxidant activity and/or squalene content. Four fractions were obtained from this experiment and labeled as following : óAô contained the highest

amount of squalene (62.72 mg in 100 mg DW), fraction B contained the highest total phenolic content value (18.22 g GAE / 100 g DW), fraction C contained the highest DPPH radical scavenging activity (5.82 mg/mL TE / 100 mg DW) and fraction D contained highest FRAP value (108.15 mg/mL TE / 100 mg DW) and total flavonoid content (527.27 mg QE / 100 mg DW). This experiment revealed that the non-modified (both physical and chemical) alumina was efficient in the process of recovering squalene from the crude extract. n-hexane was the most efficient eluent for squalene recovery during fractionation. Fractions with high antioxidant activity were efficiently recovered by alumina and silica gel with modification using 10% $AgNO_3$ and 10% NaCl impregnation respectively. Chloroform had efficiently recovered fraction with high antioxidant activity when using non-modified adsorbent material. The four fractions were subsequently incorporated into a gelatin-based packaging film at two usage levels (0.2% and 0.5%). All film samples were tested and compared on physicochemical properties against a negative and a positive control. The present study shows that the incorporation of fraction D from the crude leaf extract was able to enhance the radical scavenging activity in gelatin-based film. Results from the water vapor permeability test revealed that the film samples with added leaf extract fractions were not significantly ($p > 0.05$) improved compared to the positive control. In addition to the physicochemical tests, the potential use of the film samples were also tested on their final application use as a packaging film for minimally processed durian fruit pulp. All durian fruit pulps wrapped in the gelatin film samples were evaluated on physical changes after storage under refrigerated condition for 4 weeks. The finding of the study revealed that gelatin film with added leaf extracts were not able to control weight loss and color changes of the fruit during storage. Sensory evaluation was carried out by trained panel and the results revealed that the overall acceptance of the durian fruit after storage for three weeks under refrigerated condition were not significantly ($p > 0.05$) affected by the addition of leaf extract in gelatin film samples. In general, film samples with added leaf extract fractions that were high in antioxidant activity demonstrated improvement in physicochemical properties especially in DPPH radical scavenging activity. Finally, all gelatin film samples with added leaf extract did not bring prominent impact towards the physical changes in durian fruit pulp for the shelf-life extension. This study had revealed that UAE is more appropriate for obtaining a crude extract with maximum antioxidant activity and squalene content from durian leaf waste. Physicochemical properties of gelatin films were significantly improved in antioxidant activity after incorporated with leaf extract. The film samples with added leaf extract also resulted in negligible physical and sensory changes to the durian fruit pulp during storage..

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

PENGEKSTRAKAN, PEMERINGKATAN DAN PENCIRIAN DAUN DURIAN (*Durio zibethenius* M.) SEBAGAI SUMBER SKUALENE SEMULAJADI DAN PENGGUNAANNYA DI DALAM FILEM GELATIN

Oleh

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Penggunaan jus daun durian pada zaman dahulu telah menunjukkan kehadiran bahan fitokimia yang berfungsi sebagai bahan anti-keradangan serta anti-pengoksidaan. Walau bagaimanapun, tiada laporan saintifik terkini mengenai kaedah pengekstrakan bahan fitokimia daripada daun durian. Objektif utama penyelidikan ini adalah mengkaji kaedah pengekstrakan daun durian yang paling berkesan diikuti dengan kaedah pemulihan semula yang tinggi kandungan skualene serta aktiviti pengoksidaan ekstrak daun. Dua kaedah pengekstrakan yang dikaji adalah pengekstrakan menerusi gelombang-ultra (UAE) dan pemecutan pelarut (ASE). Keberkesanan kaedah pengekstrakan ini dikaji dari segi hasilan ekstrak daun kasar, aktiviti anti-pengoksidaan dari segi pembuangan radikal bebas DPPH serta kuasa penurunan ferik (FRAP), jumlah kandungan fenolik (TPC), jumlah kandungan flavonoid (TFC) serta kandungan skualene. Hasil kajian menunjukkan bahawa UAE lebih berkesan daripada ASE dalam pemulihan ekstrak daripada daun durian yang mengandungi aktiviti anti-pengoksidaan serta kandungan skualene yang optimum. Di samping itu, UAE juga menggunakan masa pemprosesan yang lebih pendek dan kurang memerlukan tenaga suhu yang rendah. Hasil keputusan daripada pengoptimuman proses menunjukkan masa pengekstrakan pada keadaan operasi optimum untuk UAE adalah 5 min dengan amplitud 66% (intensiti kuasa 261 W/cm2), menggunakan 100% heksana dengan nisbah pelarut kepada sampel 13:1 . Pada keadaan operasi optimum ini, sebanyak 6.63% ekstrak kasar dihasilkan dan aktiviti anti-pengoksidaan DPPH dilaporkan sebanyak 6.63 mg/mL TE / 100 mg DW, FRAP adalah 55.96 mg/mL TE / 100 mg DW, TPC dilaporkan sebanyak 69.61 g GAE / 100 g DW dan TFC dilaporkan 1210.8 mg QE / 100 mg DW. Kandungan skualene dilaporkan sebanyak 20.56 mg di dalam 100 mg ekstrak. Hasil ekstrak kasar daripada UAE kemudian dilanjutkan ke proses pemeringkatan seterusnya. Objektif proses ini adalah untuk pemulihan aktiviti anti-pengoksidaan serta / atau kandungan skualene yang lebih tinggi. Sebanyak empat bahagian telah diperolehi daripada eksperimen. Setiap

bahagian masing-masing telah dilabelkan sebagai : ôAô mengandungi skualene tertinggi (62.72 %), ôBô mengandungi nilai TPC tertinggi (18.22 g GAE / 100 g DW), ôCô mengandungi nilai DPPH tertinggi (5.82 mg/mL TE / 100 mg DW) dan ôDô mengandungi nilai FRAP tertinggi (108.15 mg/mL TE / 100 mg DW) serta TFC tertinggi (527.27 mg QE / 100 mg DW). Eksperimen ini menunjukkan bahawa alumina yang tidak terubahsuai (secara kimia atau fizikal) adalah berkesan dalam penghasilan skualene daripada ekstrak kasar. Di samping itu, n-heksana merupakan eluen yang paling berkesan dalam menghasilkan skualene sewaktu proses pemeringkatan. Bahagian yang menunjukkan aktiviti anti-pengoksidaan tertinggi dilaporkan oleh alumina dan gel silika masing-masing diubahsuai menggunakan 10% AgNO3 and 10% NaCl. Kloroform dilaporkan berkesan dalam penghasilan aktiviti anti-pengoksidaan tertinggi apabila diguna bersama ajen penjerap yang tidak diubahsuai. Empat bahagian ekstrak yang diperolehi daripada proses pemeringkatan kemudian ditambahkan ke filem pembungkusan berasaskan gelatin pada dua tahap (0.2% dan 0.5%). Semua sampel filem telah diuji dan dibandingkan dengan sampel kawalan positif dan negatif masing-masing dari segi ciri-ciri fizikokimia. Hasil kajiselidik ini menunjukkan bahawa penggunaan bahagian ekstrak ôDô berupaya meningkatkan tahap aktiviti anti-pengoksidaan DPPH dalam filem berasaskan gelatin. Walau bagaimanapun, penggunaan ekstrak daun durian tidak menujukkan kesan yang signifikan ($p > 0.05$) dalam filem gelatin dari segi ketelapan wap air apabila dibanding dengan sampel kawalan positif. Selain daripada ujikaji fizikokimia, penggunaan filem gelatin sebagai pembungkus durian juga telah dikaji. Semua isi durian yang dibungkus dengan filem gelatin disimpan dalam peti-sejuk selama 4 minggu dan perubahaan fizikal durian dikaji. Hasil keputusan menunjukkan bahawa filem gelatin yang ditambah ekstrak daun tidak berupaya mengawal isi durian dari segi kehilangan berat serta perubahan warna sepanjang masa penyimpanan. Ujian sensori telah dijalankan dengan menggunakan panel terlatih. Hasil kajian menunjukan penerimaan keseluruhan isi durian tidak dipengaruhi secara signifikan ($p > 0.05$) oleh penambahan ekstrak dalam filem gelatin. Secara keseluruhannya, penggunaan ekstrak daun yang tinggi aktiviti anti-pengoksidaan telah meningkatkan keupayaan filem gelatin terutamanya dari segi aktiviti pembebasan radikal DPPH. Walau bagaimanapun, tiada hasil keputusan yang signifikan dari segi pemanjangan tempoh hayat penyimpanan isi durian. Kesimpulan daripada kaji-selidik ini ialah UAE diikuti dengan proses pemeringkatan merupakan kaedah pengekstrakan yng lebih sesuai untuk hasilan ekstrak yang tinggi kandungan skualene serta mengandungi aktivit anti-pengoksidaan yang tinggi daripada daun durian yang gugur. Ciri-ciri fizikokimia filem gelatin dipertingkatkan dengan signifikan dari segi aktiviti anti-pengoksidaan apabila ditambah ekstrak daun. Sampel filem gelatin ditambah dengan ekstrak daun menyebabkan perubahan fizikal dan deria yang minimum terhadap isi buah durian semasa penyimpanan.

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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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a*	Redness / greeness
ANOVA	Analysis of variance
$AgNO_3$	Silver nitrate
ASE	Accelerated solvent extraction
b*	Yellowness / blueness
CCD	Central composite design
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	dried weight
FRAP	Ferric reducing antioxidant power
g	gram
GAE	Gallic acid equivalent
GC-MS	Gas chromatography ï mass spectrometer
GC-FID	Gas chromatography ï flame ion detector
HCl	Hydrochloric Acid
kg	kilogram
L*	Lightness
NaCl	Sodium chloride
mg	Milligram
mg/ml	Milligram per mililiter
min	minute
ml	mililiter
mm	milimeter
mM	milimolar
mmol	milimole
R	Correlation coefficient
RSM	Response surface methodology
sec	second
Sq	Squalene
TFC	Total flavonoid content
TPC	Total phenolic content
UAE	Ultrasound-assisted extraction
v/v	Volume per volume
v/w	Volume per weight
W/cm^2	Watt / square centimeter
WVP	Water vapor permeability

CHAPTER 1

INTRODUCTION

Natural antioxidant has now become one of the major subjects proceeding with growing demand for natural food ingredients in the food industry. The safety concern about using synthetic antioxidants (such as butylatedhydroxytoluene and butylatedhydroxyanisole) has motivated the food industry to seek natural alternatives (Brewer, 2011). Plant extract is generally a good source of natural antioxidant. The plant-based extracts contain the active compounds (such as flavonoids, phenolic acids, carotenoids, and tocopherols) with a potential antioxidant activity. They can inhibit Fe^{3+}/AA induced oxidation, scavenge free radicals, and act as reductants (Khanduja & Bhardwaj, 2003; Ozsoy, Candoken, & Akev, 2009). Previous researchers reported different natural antioxidants in various plant leaves (Fu *et al.*, 2016; Nowak, Czyzowska, Efenberger, & Krala, 2016; Romero-García *et al.*, 2016; Tahir *et al.*, 2015). However, the quality, antioxidant activity and composition of the plant-based extract depend on the extraction type and condition. Squalene was reported to be possessing antioxidant effect (Conforti *et al.*, 2005). Plant-based squalene has been sourced extensively with the intention to replace animal origined squalene, which is commonly available from shark liver oil. During the preliminary investigation of this study, squalene was identified as a major compound in durian leaf waste when extracted using n-hexane.

Various extraction methods have been developed for the optimum recovery of extraction yield from plants. In this case where squalene extraction was of concerned, the commonly reported extraction method was supercritical fluid extraction (Kraujalis & Venskutonis, 2013; Suleiman, Baharin, Mirhosseini, Sarker, & Islam, 2012). To date, ultrasound-assisted extraction (UAE) and accelerated solvent extraction (ASE) are the modern extraction methods reported to be efficient for the recovery of phytochemical compounds via optimization (Kang, Kim, & Moon, 2016; Kazemi, Karim, Mirhosseini, & Hamid, 2016). Accelerated solvent extraction was specifically efficient for the extraction of non-polar compounds even when polar solvents along with high pressure are applied (Zaghdoudi *et al.*, 2015). This has become the advantage of this technique in extracting natural compounds of various polarities from the same plant materials in comparison to other modern techniques. However, limited report available on the recovery of squalene from plant materials using these two extraction methods.

Durian (*Durio zibethinus* M.) leaf is known used by traditional practitioners for therapeutic purposes. Tate (1999) reported a few findings on pharmacological properties and therapeutic effects of durian leaves. It was believed that decoction from durian leaves and root possessing anti-inflammatory effect, which helped to relief patient who was suffering from high fever. Phytochemical properties in durian have been reviewed by Ho and Bhat (2015), but mainly emphasizing durian fruit which doesn't directly reflect the leaf especially antioxidant capacity.

Hence, this study has been carried out to report on optimal extraction condition for crude extract from durian leaf waste using different modern solvent extraction methods, which are ultrasound-assisted extraction (UAE) and accelerated solvent extraction (ASE). The main goal and novelty of this study was to report the most efficient extraction technique on durian leaf, which ultimately recovered the highest antioxidant activity as well as squalene content from the leaf extract. At the final part of the study, the leaf extract was applied into gelatin-based film. This was to identify the potential use of the leaf extract as a functional ingredient in the gelatin-based film. Film samples with added leaf extracts were evaluated on antioxidant properties as well as functional use in protecting durian fruit pulp from physical changes during storage (4 weeks). Physcial changes in durian fruit pulp that were investigated including water loss, color and texture. Therefore the specific objectives of this research were as follow:

1. To optimize the ultrasound-assisted extraction (UAE) for durian leaf extract with the highest yield, antioxidant activity and squalene content.

2. To optimize the accelerated solvent extraction (ASE) for durian leaf extract with the highest yield, antioxidant activity and squalene content.

3. To investigate the effect of purification/fractionation condition on the yield and antioxidant activity of durian leaf extract

4. To examine the potential use of durian leaf extract as a functional ingredient in a gelatin-based film for controlling physical changes in durian fruit pulp during storage period.

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