GC-MS-BASED METABOLITE PROFILING OF *SALACCA ZALACCA* FLESH EXTRACTS OBTAINED BY DIFFERENT EXTRACTION METHODS AND THEIR INHIBITORY ACTIVITY ON ALPHA-GLUCOSIDASE

BY

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A thesis submitted in fulfillment of the requirement for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Chemistry)

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ABSTRACT

Present study was performed to explore the potential of *Salacca zalacca* flesh extracts as α-glucosidase inhibitor; which is one of the anti-diabetic agents used to manage the disease. This study aimed to obtain extracts of *Salacca zalacca* flesh by using three extraction methods – Soxhlet extraction (SE), supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE). All extracts obtained were then subjected to GC-MS-based metabolite profiling and their *in vitro* α-glucosidase inhibitory assay. Different extraction methods were employed in this study to determine the most appropriate method and extraction condition in drawing out desired bioactive compounds from the plant sample. The results showed the highest extraction yield was obtained by 50% ethanol extract of SE (73.2 ± 4.4%), whereas SFE_1 showed the lowest yield (0.42 ± 0.08%). Besides, overall extraction yield suggested that both factors – extraction method and solvent type had significantly influenced the outcome. Following that, all extracts were evaluated for *in vitro* α-glucosidase inhibitory activity, measured by their IC_{50} values in comparison to that of quercetin, the positive control (IC_{50} = 2.7 ± 0.7 μg/mL). The lowest α-glucosidase inhibitory activity was indicated by water extract of SE (IC_{50} = 724.3 ± 13.15 μg/mL) and the highest activity was demonstrated by 60% ethanol extract of UAE (IC_{50} = 16.3 ± 1.3 μg/mL). The *in vitro* assay of α-glucosidase inhibitory activity was performed in microtiter plates, which are well acknowledged as fast, robust, cost-effective and reproducible method. Subsequently, all extracts including active and inactive ones were analysed by GC-MS to identify metabolites that may possess the potential as α-glucosidase inhibitor. Carbohydrates, fatty acids, organic acids, phenolic acids, sterols and alkane-based compounds were identified from active UAE and SFE extracts. The list of identified metabolites was compared with the previously reported metabolites with significant α-glucosidase inhibitory activity such as citric acid, palmitic acid, stearic acid, linoleic acid, oleic acid, 9-octadecenoic acid, gallic acid, stigmasterol and β-sitosterol. Hence, the reported activity against α-glucosidase enzyme might be attributable to the presence of these metabolites. As a conclusion, this study explored the key potential of *S. zalacca* flesh extract as α-glucosidase inhibitor.

خلاصة البحث

تم إجراء هذه الدراسة لاستكشاف إمكانية مستخلص لب ثمرة السالاكا زالاكا (Salacca zalacca) كمثبط لإنزيم ألفا جلوكوسيديس (α-glucosidase). وهو أحد العوامل المضادة للسكري المستهدفة في علاج المرض. هدفت هذه الدراسة إلى الحصول على مستخلص لب ثمرة السالاكا زالاكا باستخدام ثلاث طرق للاستخلاص، وهي طريقة السوكسليت، وطريقة استخلاص السوائل فوق الحرجة، والاستخلاص بمساعدة الموجات فوق الصوتية. خضعت جميع المستخلصات التي تم الحصول عليها للتوصيف الميتابولومي باستعمال الكروماتوغرافيا الغازية الكتلية الطيفية (GC-MS) والتحقق من فعاليتها كمثبط لإنزيم ألفا جلوكوسيديس. تم استخدام طرق استخلاص مختلفة في هذه الدراسة لتحديد الطريقة والحالات الأنسب لاستخلاص المركبات النشطة بيولوجيا المرغوبة من عينة النبات. تم الحصول على أعلى ناتج للمستخلصات بالإيثانول 50٪ بالسوكسليت (73.2±4.4٪)، بينما أظهرت طريقة استخلاص السوائل فوق الحرجة أقل إنتاجية (0.42±0.08٪)، وأشار المحصول الكلي إلى أن عاملي طريقة الاستخراج ونوع المذيب قد أثرا بشكل كبير على الإنتاجية. بعدها تم تقييم جميع المستخلصات للتحقق من النشاط المثبطة لإنزيم ألفا جلوكوسيديس في المختبر وذلك بقياس قيم IC_{50} مقارنة مع الكويرسيتين كضابط إيجابي(IC_{50}= 2.7±0.7 ميكروغرام/مل). أظهرت المستخلص المائي باستعمال السوكسليت أقل تثبيط لانزيم ألفا جلوكوسيديس (IC_{50}=724.3±13.15 ميكروغرام/مل) وأما أعلى نسبة تثبيط فكانت بالإيثانول 60% بطريقة استخلاص السوائل فوق الحرجة (IC_{50}=16.3±1.3 ميكروغرام/مل). تم إجراء فحوص تثبيط إنزيم ألفا جلوكوسيديس خارج الجسم الحي على أطباق معايرة دقيقة المعروفة بسرعتها، وفعاليتها من حيث التكلفة، وقابليتها للتكرار. تم لاحقا تحليل جميع المستخلصات بما في ذلك النشطة وغير النشطة بواسطة GC-MS لتحديد المستقلبات القادرة على تثبيط إنزيم ألفا جلوكوسيديس. تم العثور على كربوهيدرات، أحماض دهنية، أحماض عضوية، أحماض فينولية، ستيرولات، ومركبات مبنية على الألكانات في مستخلصات طريقة السوكسليت والاستخلاص السائل فوق الحرج النشطة. تمت مقارنة قائمة المستقلبات المحددة مع المستقلبات النشطة ضد إنزيم الألفا جلوكوسيديس المكتشفة سابقا مثل حامض الستريك، وحمض اللينوليك وحمض الأوليك، وحمض الأوكسيتاديكويك-9، وحمض الغاليك، والستيجماسترول، والبيتسوستيرول. وبالتالي فإنه يمكن أن يعود النشاط المبلغ عنه ضد إنزيم الألفا جلوكوسيديس إلى وجود هذه المستقلبات. في الختام فقد استكشفت هذه الدراسة الإمكانية الرئيسية لمستخلص لب ثمرة السالاكا زالاكا كمثبط لإنزيم ألفا جلوكوسيديس.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Chemistry).

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LIST OF ABBREVIATIONS

SE	soxhlet extraction
SFE	supercritical fluid extraction
UAE	ultrasound-asisted extraction
IC_{50}	the concentration required to inhibit the response at 50%
SE_Water	water extract of soxhlet extraction
SE_50% Ethanol	50% ethanol extract of soxhlet extraction
SE_100% Ethanol	100% ethanol extract of soxhlet extraction
UAE_Water	water extract of ultrasound-assisted extraction
UAE_20% Ethanol	20% ethanol extract of ultrasound-assisted extraction
UAE_40% Ethanol	40% ethanol extract of ultrasound-assisted extraction
UAE_60% Ethanol	60% ethanol extract of ultrasound-assisted extraction
UAE_80% Ethanol	80% ethanol extract of ultrasound-assisted extraction
UAE_100% Ethanol	100% ethanol extract of ultrasound-assisted extraction
UAE_Hexane	hexane extract of ultrasound-assisted extraction
SFE_1	the first condition used in SFE (absence of co-solvent, 50 °C)
SFE_2	the second condition used in SFE (10% ethanol as co-solvent, 50 °C)
SFE_3	the third condition used in SFE (10% ethanol as co-solvent, 60 °C)

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Primary metabolites are produced by plants to carry out their basic life functions such as cell division, cell differentiation and respiration. Following that, secondary metabolites are generated as by-products of the primary metabolites' metabolism, which are mainly under biotic and abiotic stress. In addition, their types and abundance are predominantly determined by natural selection throughout the evolution process. Generally, these metabolites, which are also known as phytochemicals are very important in maintaining the plants viability and survival through their favorable biological activities. On that account, plant secondary metabolites have been exploited extensively in traditional and complementary medicines field as well as food additives and industrial raw materials (Benderoth et al., 2006).

The presence of plants' secondary metabolites has certainly led to a diverse research topic, including plants metabolite profiling. Metabolite analysis is crucial to understand the detail process of plant metabolism and their biochemical function. These analyses may suggest or provide a presumed connection between genotype and phenotype properties of plants and thus, elucidating the regulation mechanism at metabolic level (Fiehn et al., 2000). The concept of metabolite profiling has been immensely explored by previous studies to analyse the chemical constituents available in selected plant extracts by using an analytical tool (Roessner et al., 2001; Fiehn, 2002; Fernie et al., 2004; Trethewey, 2004). In this study, gas chromatography-mass

spectrometry (GC-MS) was utilized to carry out the analysis in order to establish the metabolite profiles for the studied plant extracts; *Salacca zalacca*.

Malaysia is a tropical country with a huge range of flora and fauna species. Malaysia's floras are well known with their potent medicinal properties and there are still a lot to be explored and discovered, including salak, which is scientifically known as *S. zalacca*. Gorinstein et al. (2009) has pointed out that *S. zalacca* was less acknowledged and less explored by researchers compared to other tropical fruits. *S. zalacca* is one of the palm tree species (family Arecaceae) native to South Sumatra and Southwest Java, Indonesia. Currently, it is widely planted in Southeast Asia region including Malaysia, Thailand and Philippines, as well as in other regions worldwide. It has been reported as a good source of carbohydrate, dietary fibre and antioxidants (Aralas et al., 2009). In addition, it was revealed that the flesh possessed strong antioxidant capacity due to its phenolic compounds constituent (Leong et al., 2002; Shui et al., 2005). As a matter of fact, a strong correlation between antioxidant and antihyperglycemic activity through the mechanisms of α-glucosidase and α-amylase inhibitory activity has been established (Manaharan et al., 2012). Besides, another strong correlation between total phenolic content (TPC) and α-glucosidase inhibitory activity of plant extracts has also been previously demonstrated (Silva Pinto et al., 2009; Sulaiman et al., 2013).

International Diabetes Federation (IDF) has reported that about 415 million people worldwide have diabetes, with 3.3 million cases are in Malaysia (International Diabetes Federation, 2017). α-glucosidase inhibitor (AGI) is one of standard treatments to manage the disease, which therapeutically works by suppressing the action of α-glucosidase enzyme to delay the glucose absorption into blood. Due to side effects

possessed by the synthetic α-glucosidase inhibitors (e.g. Acarbose), there is an increase demand to screen for potential α-glucosidase inhibitor from natural sources.

In this study, *S. zalacca* is chosen as the subject plant to be investigated for its α-glucosidase inhibitory potential. A study by Sahputra in 2008 has illustrated that the flesh and skin of *S. zalacca* originated from Balikpapan, Indonesia demonstrated a significant inhibitory activity against α-glucosidase enzyme. For this research, *S. zalacca* was acquired from a farm in Melaka, Malaysia.

1.2 STATEMENT OF THE PROBLEM

According to the World Health Organization (WHO), the number of people with diabetes has increased from 108 million in 1980 to 422 million in 2014 (WHO, 2017). In addition to that, the International Diabetes Federation (IDF) has reported that there were 3.3 million of diabetes cases in Malaysia for the year 2015 (International Diabetes Federation, 2017). This disorder has many dreadful complications including blindness, kidney failure, heart attacks, stroke and lower limb amputation (WHO, 2017). Therefore, it is very important for patients to thoroughly monitor their daily blood glucose levels and be compliant towards medications, including insulin injections and oral anti-diabetic agents, in order to keep blood sugar level controlled and thus, to be able to prevent these complications in the future.

α-glucosidase inhibitor is a group of anti-diabetic agent that decreases the absorption rate of carbohydrates in the small intestine by inhibiting the responsible enzyme, the α-glucosidase. The mechanism of this anti-diabetic agent is capable of reducing the postprandial glucose without causing hypoglycemia to patients. Besides, it possesses synergistic effects when is taken with other oral anti-diabetic and may also be combined with insulin (CPG Secretariat, 2015). In spite of that, α-glucosidase

inhibitors are well known for their nasty side effects, which has greatly influenced the patient's compliance towards the medication. Thus, there is a need to discover potent α-glucosidase inhibitor from natural origin, which may have better efficacy and safety profiles towards diabetic patients.

1.3 RESEARCH OBJECTIVES

1) To obtain extracts of *S. zalacca* flesh using conventional (Soxhlet extraction) and non-conventional extraction methods (supercritical fluid extraction and ultrasound-assisted extraction).
2) To determine the metabolite profiles of all *S. zalacca* flesh extracts using gas chromatography-mass spectrometry (GC-MS) as the analytical tool.
3) To determine the *in vitro* inhibitory activity of all prepared *S. zalacca* flesh extracts against α-glucosidase enzyme.

1.4 RESEARCH HYPOTHESES

1) Different extraction methods would generate different overall yields.
2) Different extraction methods would generate different metabolites and hence, different metabolite profiles.
3) *S. zalacca* flesh extracts contribute to the inhibitory activity against α-glucosidase enzyme.

1.5 SIGNIFICANCE OF THE STUDY

There are three main significance of this study. Firstly, the evaluation of inhibitory activity of *S. zalacca* flesh extracts against α-glucosidase enzyme will present

functional data for further studies concerning its anti-diabetic potential as well as its definite pharmacological mechanism.

Secondly, the establishment of metabolite profiling of *S. zalacca* flesh extracts will provide useful data and information for the future research and development (R&D) in medicinal plant and natural product specialty. It will contribute knowledge for the determination of quality control of a natural product.

Thirdly, this study will provide a new insight of *S. zalacca* flesh potential in steps to enhance its commercialization especially in Malaysia and Southeast Asia region. The economic and pharmaceutical values are anticipated to increase its incredible contribution to the Malaysian population.

CHAPTER TWO

LITERATURE SEARCH

2.1 BACKGROUND OF *SALACCA ZALACCA*

2.1.1 Origin, Botany, Morphology and Structure

Salak is scientifically known as *Salacca zalacca* (Gaertner) Voss. It has a number of synonym names, which include *Calamus salakka* Willd. ex Steud., *Calamus zalacca* Gaertn. basionym, *Salacca blumeana* Mart., *Salacca edulis* Reinw., *Salacca edulis* var. *amboinensis* Becc., *Salacca rumphii* Wall., nom. illeg., *S. zalacca* var. *amboinensis* (Becc.) Mogea, *Salakka edulis* Reinw. ex Blume (Lim, 2012). *S. zalacca* is a palm tree species, belonging to the Arecaceae family. Its common English names include edible-fruited salak palm, edible *salacca*, salak, salak palm, snake fruit, snake palm, snake-skinned fruit. Other than that, it has various different names across the globe, with the familiar ones are; Salak (Malaysia and Indonesia), Sala (Thailand), Ke Shi Sa La Ka Zong / She Pi Guo (China), Sarakka Yashi (Japan), Salaca (Spain) and Schlagenfrucht (Germany) (Lim, 2012).

Originally, *S. zalacca* is native to South Sumatra and Southwest Java, Indonesia. Nowadays, the plant has been introduced and planted in other parts of the world including Thailand, Malaysia, New Guinea, Philippines, Queensland, northern Australia, Ponape Island (Caroline Archipelago), China, Surinam, Spain and the Fiji Islands (Lim, 2012).

In Malaysia, three species of salak has been commercially grown including *S. glaberescens, S. edulis and S. sumatrana*. *S. glaberescens* is also widely known as local salak with nine clones (SJ15, SJ17, SJ34, SJ36, SJ39, SJ40, ST1, ST2, ST3) being reproduced and bred for agricultural purposes. On the other hand, the other two species; *S. edulis (S. zalacca) and S. sumatrana* were brought from Indonesia, and locally cultivated in Malaysia (Aralas et al., 2009).

Salak trees flourish well in humid tropical environment with an average annual rainfall of 1700 to 3000 mm. The fruits grow in clusters like palm and are also known as snake fruit due to its reddish-brown scaly skin (Afrianti Priyatno et al., 2012). *S. zalacca* trees have some distinctive features compared to other palm tree species – short stem (1-2 m high, 10-15 cm diameter), shallow roots, short internodes and pinnatipartite leaves (3-6 m long) (Lim, 2012). The dioecious fruits are produced after 3-4 years of planting. However, some salak varieties are able to self-pollinate as they are found to produce monoecious fruits, such as salak Bali. Conventionally, assisted pollination is performed by putting two mature male inflorescences in adjacent to a female inflorencence to enhance the quality of fruits (Supapvanich et al., 2011).

Peeling salak fruits for their flesh requires specific technique in order to avoid from cutting the fingers due to their outer scales. The best way is to pinch and pull the fruit tip to disclose the flesh. The standard salak fruit consists of a garlic-like clove that is made up of 1–3 individual flesh, irregular in size as illustrated in Figure 2.1.

Figure 2.1: *Salacca zalacca* flesh (Mybaliguide.com, 2015)

During the growth stage, several significant changes take place in terms of the fruit texture as well as its sugar, acids and minerals content. In addition, an increase of weight, edible portion percentage, sugar/acid ratio, vitamin C, starch and total sugar is experienced by the fruit during its ripening process. On the other hand, salak fruits encounter a decline in the percentage of peel to fruit ratio, water content, tannins and acids throughout the growth (Lestari et al., 2011).

2.1.2 Commercial and Economic Value of Salak

In Indonesia, salak has been a massive contributor to local and export market for fruits. More than 30 varieties of salak are available in Indonesia, as the country was the native source of salak. The salak cultivars in Indonesia are distinguished and grouped by three aspects – cultivating location (e.g. Bali, Suwaru, Condet, Enrekang or Padang Sidempuan), taste (e.g. gula pasir, pondoh super, pondoh hitam, pondoh menggala or madu) and fruit colour (e.g. putih or gading). The total marketable salak per annum is approximately 420,000 tons, including both domestic and export market, covering fresh and processed form of salak. In addition to that, the demand from other countries like China, Japan, Europe and United States has escalated since the recognition and commercialization of salak Pondoh cultivar. All in all, Indonesia generates around 60–

70% (334,000 tons) of global salak market, with roughly 32,755 tons per year total exports (Dimyati et al., 2008).

As in Malaysia setting, the statistical data for fruit crop was published in 2016 by the Department of Agriculture, Putrajaya, Malaysia. Table 2.1 presented the overall data for major fruit crops in Malaysia describing their hectare age (Ha), production and value of production for the year 2016. From the table, it was determined that salak production was minor compared to other fruits, with only 895.8 Ha (0.47%) of planted area and 631.3 Ha (0.47%) of harvested area. Besides, salak also has recorded 4,131.6 Mt (2.5%) of total yearly production, that was valued around RM6,197.30 (0.15%). The average yield of salak was 6.5 Mt/Ha.

On the other hand, Table 2.2 displayed the statistics of overall salak production according to states in Malaysia. From the table, Pahang was observed to have the largest hectar age (291.8 Ha) and Kelantan had the largest harvested area (197.4 Ha). Total of hectar age and harvested area in Peninsular Malaysia was 757.8 Ha and 500.3 Ha respectively. As in East Malaysia, only Sabah state contributed to salak fruit production, with 138 Ha of hectar age and 131 Ha of harvested area across the state. With respect to the production aspect, Kelantan showed the highest production that weighed 1,316 Mt, valued around RM1,974.10. Besides, the production of salak in Sabah also recorded a massive amount, with approximately 1,098.1 Mt, leading to an estimated cost of RM1,647.20.

Table 2.1: Hectar age, production and value of production of major fruit crops in Malaysia in 2016 (Department of Agriculture, 2016)

Type of Fruits	Planted area (Ha)	Harvested area (Ha)	Percentage of harvested area (%)	Production (Mt)	Production Value (RM)	Average yield (Mt/Ha)	Potential Production (Mt/Ha)
Starfruit	872.2	542.3	62.2	8474.3	20,917.8	15.6	35.0
Papaya	4334.5	3980.4	91.8	65966.9	101,149.2	16.6	60.0
Cempedak	7584.6	4889.3	64.5	28929.2	72,322.9	5.9	15.2
Dokong	6645.8	4749.3	71.5	33832.5	45,673.8	7.1	11.9
Duku	5663.1	3515.7	62.1	23130.6	34,117.6	6.6	10.0
Durian	66037.5	43531.9	65.9	302645.8	1,972,242.1	7.0	13.2
Langsat	5138.0	3,257.9	63.4	30,688.4	69,048.9	9.4	5.4
Pomelo	1173.2	910.4	77.6	12,857.5	30,215.2	14.1	10.5
Mango	5816.4	3,107.1	53.4	17,429.7	57,518.0	5.6	6.5
Mangosteen	3830.6	2,460.9	64.2	21,587.8	73,398.4	8.8	22.0
Dragon fruit	695.3	512.6	73.7	4,401.5	13,644.7	8.6	10.0
Pineapple	13148.9	10,354.1	78.7	391,714.4	515,248.7	37.8	62.0
Jackfruit	5413.9	3,417.1	63.1	28,767.2	55,376.8	8.4	19.3
Banana	28036.4	22,293.8	79.5	309,507.6	541,638.4	13.9	24.0
Pulasan	319.9	205.1	64.1	998.7	2,746.4	4.9	8.3
Rambutan	15386.6	10,942.9	71.1	63,699.9	108,289.8	5.8	8.3
Salak	**895.8**	**631.3**	**70.5**	**4,131.6**	**6,197.3**	**6.5**	**8.7**
Watermelon	11986.8	11,058.2	92.3	192,909.8	244,995.4	17.4	30.0
Total	**189335.7**	**135,071.7**	**71.3**	**1,621,813.8**	**4,196,260.5**	**12.0**	**453.3**