EXTRACTION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM *Anisophyllea disticha* USING SUPERCRITICAL FLUID EXTRACTION

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master of Science (Biotechnology)

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FEBRUARY 2020

ABSTRACT

Anisophyllea disticha is one of the underexplored species from genus *Anisophyllea* despite its high utilization by the folklore. Till today conventional extraction methods are generally employed to obtain bioactive compounds from the plant; while often introduces contaminants that must be removed later. Supercritical fluid extraction (SFE) offers rapid, selective, environmental-friendly and green chemical processed products. In this study, *A. disticha* was extracted using soxhlet and the highest total yield was obtained by leaf followed by root and stem extracts with average values of 23.97 ± 0.19, 9.57 ± 1.16 and 8.75 ± 1.54%, respectively. Comparatively, total yield that ranged between 0.65-4.14% was observed by stem extracted from SFE. In phytochemical screening, steroids and flavonoids were detected in leaf while stem and root gave positive tests for steroids, terpenoids, flavonoids and saponins. Subsequent antimicrobial capability test using disc diffusion assay revealed positive growth inhibitory activity, but considered weak, for all extracts against gram-positive bacteria namely *Staphylococcus epidermidis* and *Staphylococcus aureus* with leaf extract displayed the highest zone of inhibition with values of 10.67 ± 0.58 and 8.67 ± 0.58 mm, respectively. On the other hand, at sample concentration of 3 mg/mL, stem showed the highest amount of total phenolic content (TPC) followed by root and leaf with mean value of 27.73 ± 4.10, 19.50 ± 0.87 and 12.19 ± 1.77 mg GAE/g extract, respectively. The similar trend was also observed in antioxidant activities where the highest scavenging activity (66.20 ± 11.74%) and reducing power (203.63 ± 16.03 mg Fe(II)/g extract) were found in stem at sample concentration of 0.6 mg/mL. Stem was chosen and subsequently extracted using SFE and further analyzed. Box-Behnken Design (BBD) was used to optimize and examine the effect of independent variables of SFE such as temperature (50-70˚C), pressure (20-30 MPa) and concentration of co-solvent (10-20%) on TPC and antioxidant activities of *A. disticha*. The model suggested that the optimal conditions of independent variables to be 50˚C, 21 MPa and 20% with predicted TPC of 77.06 mg GAE/g extract, scavenging activity of 109.51% and reducing power of 585.36 mg Fe(II)/g extract. Reverse-phase high performance liquid chromatography (RP-HPLC) was developed to identify and quantify phenolic compounds (gallic acid, *p*-coumaric, ferulic acid and quercetin) presented in *A. disticha*. SFE extracts that demonstrated the highest responses (SFE 7) was chosen and used to compare with those from soxhlet. The results showed that the extract from SFE possessed remarkable TPC, antioxidant activities and concentration of phenolic compounds identified by HPLC, indicating its superior recovery of compounds. Further study on *A. disticha* will be of great medicinal importance to treat diseases associated with bacteria and oxidative stress.

خلاصة البحث

إن عائلة نبات *Anisophyllea disticha* هي واحدة من الأنواع غير المستكشفة من جنس *Anisophyllea* وتستخدام كثيرا من قبل التراث الشعبي حتى اليوم. يتم استخدام الطرق التقليدية عمومًا للحصول على مركبات نشطة بيولوجيًا من هذا النوع من النبات؛ بينما يقدم في كثير من الأحيان الملوثات التي يجب إزالتها في وقت لاحق. يوفر استخراج السوائل فوق الحرجة (SFE) ومنتجات كيميائية انتقائية وصديقة للبيئة والمنتجات المصنعة الكيميائية الخضراء. في هذه الدراسة، تم استخراج *A. disticha* باستخدام soxhlet وتم الحصول على أعلى انتاجية إجمالية بواسطة ورقة تليها مقتطفات الجذر والساق مع متوسط قيم 23.97 ± 0.19 و 9.57 ± 1.16 و 8.75 ± 1.54 ٪، على التوالي. نسبيا بالفرق، لوحظت العائد الكلي الذي تراوحت بين 0.65–4.14 ٪ عن طريق الجذع المستخرج من SFE. في الفحص الكيميائي النباتي، تم الكشف عن المنشطات والفلافونويد في ورقة بينما أعطت الجذع والجذر اختبارات إيجابية للستيرويدات واليربينويدات والفلافونيدات والسابونين. كشف اختبار القدرة الفاعلة لمضادات الميكروبات باستخدام اختبار نشر القرص عن نشاط تثبيط إيجابي للنمو، ولكنه يعتبر ضعيفًا، بالنسبة لجميع المستخلصات ضد البكتيريا الموجبة للجرام وهي المكورات العنقودية الذهبية والمكورات العنقودية الذهبية مع مستخلص الأوراق، حيث أظهرت أعلى منطقة تثبيط مع قيم 10.67 ± 0.58 و 8.67 ± 0.58 مم، على التوالي. من ناحية أخرى، في تركيز عينة من 3 ملغ / مل، أظهر الجذع أعلى كمية من محتوى الفينول الكلي (TPC) يليه الجذر والورقة بقيمة متوسطة تبلغ 27.73 ± 4.10 ، 19.50 ± 0.87 و 12.19 ± 1.77 ملغ GAE / المستخرج، على التوالي. ولوحظ أيضًا اتجاه مشابه في أنشطة مضادات الأكسدة حيث تم العثور على أعلى نشاط مسح (66.20 ± 11.74٪) وقدرة منخفضة (203.63 ± 16.03 ملغ Fe (II) / المستخرج) في جذع بتركيز العينة 0.6 ملغ / مل. وقد تم اختيار الجذعية واستخراجها في وقت لاحق باستخدام SFE وتحليلها. تم استخدام Box–Behnken Design (BBD) لتحسين ودراسة تأثير المتغيرات المستقلة لـ SFE مثل درجة الحرارة (50–70 درجة مئوية) والضغط (20–30 ميجا باسكال) وتركيز المذيب المشترك (٪10–20) على TPC وأنشطة مضادات الأكسدة من *A. disticha*. النموذج المقترح أن تكون الظروف المثلى للمتغيرات المستقلة 50 درجة مئوية و 21 ميجا باسكال و 20٪ مع TPC المتنبأ به لمستخلص 77.06 GAE / g ملغ، ونشاط الكسح بنسبة 109.51٪ وتقليل القدرة بمقدار 585.36 ملغ Fe (II) / g المستخرج. تم تطوير تحليل كروماتوجرافي سائل ذو أداء عكسي عالي (RP-HPLC) لتحديد وقياس المركبات الفينولية (حمض الغاليك، p- الكوماريك، حمض الفيروليك وكيرستين) المقدم في *A. disticha*. تم اختيار مقتطفات SFE التي أظهرت أعلى الاستجابات (7SFE) واستخدمت للمقارنة مع تلك الموجودة في soxhlet. أظهرت النتائج أن المستخلص من SFE يمتلك TPC، أنشطة مضادات الأكسدة بشكل ملحوظ وتركيز المركبات الفينولية المحددة بواسطة HPLC، مما يشير إلى استعادته الفائقة للمركبات. سيكون إجراء مزيد من الدراسة على *A. disticha* ذو أهمية طبية كبيرة لعلاج الأمراض المرتبطة بالبكتيريا والإجهاد التأكسدي.

APPROVAL PAGE

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DECLARATION

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ACKNOWLEDGEMENTS

All praises belong to Allah, the Most Gracious and the Most Merciful, for the strength, patience as well as good health given to me in completing this master project successfully.

I wish to express my appreciation and thanks to my main supervisor, Asst. Prof. Dr. Sahena Ferdosh for all the knowledge, guidance and support from the beginning till the end of this project. My appreciation also goes to my co-supervisors, Prof. Dr. Md. Zaidul Islam Sarker and Asst. Prof. Dr. Rozilawati Shahari for suggestion, guidance and encouragement directly or indirectly.

My honor appreciation goes to all staffs form Kulliyyah of Science, especially Brother Muzammil and Brother Mizan for their time, help and support for this project.

It is my utmost pleasure to dedicate this work to my dear parents, husband, son and my family, who granted me the gift of their unwavering belief in my ability to accomplish this goal, endless dua', continuous support, encouragement and love.

Special thanks to my postgraduate friends especially Fatin Nazirah, Marhamah, Syazwani, Anis Fadhlina, Hassan and Munzir for their assistance, support and motivation. Finally, to all individuals who I have not been able to mention their names, which were involved directly or indirectly in any respect towards the completion of this project, may Allah bless all of us with His mercy and compassion, here and hereafter.

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$$\text{Yield (\%)} = \frac{\text{Mass of extract}}{\text{Mass of sample}} \times 100$$

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$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} B_{jj} X_j^2 + \sum_{i} \sum_{<j=2}^{k} \beta_{ij} X_i X_j + e_i$$

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$$C = c \times \frac{V}{m}$$

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$$I\ (\%) = \frac{Ao - A}{Ao} \times 100$$

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$$C = c \times \frac{V}{m}$$

4.1 Second Order Polynomial Regression of Yield 66

$$Y = 2.03 + 0.038X_1 - 0.5437X_2 + 0.9500X_3 - 0.8675X_1X_2 + 0.0650X_1X_3$$
$$-0.5950X_2X_3 + 0.1554X_1^2 + 0.4254X_2^2 - 0.6521X_3^2$$

4.2 Second Order Polynomial Regression of TPC 70

$$Y = 25.29 - 11.72X_1 - 4.15X_2 + 14.17X_3 - 7.75X_1X_2 - 14.69X_1X_3$$
$$-8.44X_2X_3 + 4.49X_1^2 - 3.37X_2^2 + 4.99X_3^2$$

4.3 Second Order Polynomial Regression of Scavenging Activity 73

$$Y = 58.60 - 12.86X_1 - 9.92X_2 + 16.73X_3 - 1.85X_1X_2 - 13.47X_1X_3$$
$$-16.25X_2X_3 - 0.9021X_1^2 - 2.07X_2^2 - 8.87X_3^2$$

4.4 Second Order Polynomial Regression of Reducing Power 77

$$Y = 134.61 - 89.12X_1 - 49.72X_2 + 104.35X_3 - 49.11X_1X_2 - 107.31X_1X_3$$
$$-75.29X_2X_3 + 41.00X_1^2 + 12.46X_2^2 + 42.62X_3^2$$

LIST OF SYMBOLS

mg	milligram
g	gram
mL	mililitre
±	plus or minus
µg	microgram
%	percent
mm	millimeter
µL	microlitre
min	minute
˚C	degree Celsius
MPa	megapascal
cm	centimeter
kg	kilogram
ppm	parts per million

LIST OF ABBREVIATIONS

SFE	Supercritical fluid extraction
CO_2	Carbon dioxide
HPLC	High performance liquid chromatography
TPC	Total phenolic content
DPPH	2,2-diphenyl-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
BBD	Box-Behnken design
RSM	Response surface methodology

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Human beings rely upon traditional remedies from nature to enhance their health and to treat different ailments since they inhabited the earth. The history of the usage of natural medicines by traditional healers played important roles in modern drug discovery and development. Nature like plants, animals and microorganisms provide an outstanding diversity and tremendous potential of bioactive compounds. Plants are the vital sources of valuable biologically active agents with estimation of more than 25% of modern medicines contain at least one constituent derived from plants (Cock, 2012). Bioactive compounds from herbal products are produced as secondary metabolites and can be categorized into a few groups such as phenolic compounds, alkaloids, terpenes and terpenoids with potential in preventing allergic response, atherogenesis as well as being able to reduce inflammation. They also act as anti-microbial, antioxidant, anti-thrombotic and show cardioprotective and vasodilatory activity (Balasundram et al., 2006). It is commonly agreed that plant-derived medicines are safer than their synthetic counterparts, more effective, pose less side effects, easily available and relatively cheap (Shinde & Mulay, 2015). As reported by Paulsen (2010), however, it is estimated that only 10% of approximately 250 000 plant species from all over the world has been examined for the presence of phytochemicals. With countless plants yet to be investigated, especially from the untapped origins, expanding the exploration of nature for sources of new drug

candidates may offer inspiration for innovations in a variety of research fields like medicine, nutrition, and life sciences.

Anisophyllea disticha or generally known as *Raja Berangkat,* is a small treelet that is distributed in swamp and lowland forest throughout Peninsular Malaysia, Sabah, Sarawak, Sumatra, Brunei and Kalimantan. *A. disticha* is from Anisophylleaceae family that made up of two markedly different sizes of leaf blades arranged along the branches (Soepadmo & Wong, 1995). The leaves and stem are generally used by the folklore to treat diarrhea, dysentery as well as fever (Singh, 2016). In addition, Quattrocchi (2012) also stated the ability of leaves in healing jaundice, cuts and wounds while fruits are useful for poisoned stings by bees and hornets. Apart from that, roots of *A. disticha* serve in relieving tiredness and body aches, refreshing body, revitalizing birth canal, delaying ageing process as well as treating weakness in men and infertility in women (Chian, 2017; Ong, 2004; Quattrocchi, 2012; Suharjito, 2014).

Extraction is the most vital first stage in the study of biologically active compounds in plant resources that lead to further separation, identification and characterization process. Consideration must be given to the suitability of the extraction methods chosen in order to prevent lost, distortion and destruction of potential chemical components (Sasidharan et al., 2011). Basically, methods of extraction can be divided into two, namely conventional and non-conventional techniques. The former is based on the extracting power of solvents used as well as the application of heat and/or mixing. Previous literature reviews are limited on the use of conventional methods in extracting phytoconstituents from genus *Anisophyllea* namely maceration, percolation and soxhlet (Dem'yanov et al., 1984; Kargbo et al., 2015; Khallouki et al., 2007; Khallouki et al., 2009; Onivogui et al., 2014; Onivogui et

al., 2015). However, as indicated by Azmir et al. (2013), these type of extraction methods are time consuming, involve high consumption of toxic solvents that require additional evaporation steps, costly, low extraction selectivity and are suitable for extracting thermo-labile compounds.

Drawbacks from conventional methods and increase demand for solvent-free products have encouraged the growing interest particularly in green and alternative non-conventional extraction technologies such as pressurized-liquid extraction, solid-phase extraction, supercritical fluid extraction, surfactant-mediated methods, microwave and ultrasound-assisted extraction (Easmin et al., 2015). In this study, supercritical fluid extraction (SFE) has been conducted for the first time to extract bioactive compounds from *A. disticha*. SFE has become a very attractive option as it offers several advantages: (a) application of supercritical carbon dioxide (CO_2) as extracting solvent which is easily available, cheap and nontoxic; (b) CO_2 can be perfectly detached from the final products as it vaporizes at ambient temperature and pressure, resulting in solvent-free extract; (c) high extraction efficiency and selectivity as its solvating power can be tuned by altering the process variables; and (d) reduced thermal degradation effect as extraction is conducted at a low temperature. However, CO_2 is non-polar solvent; hence adding small volume of food grade modifier like ethanol can significantly enhance the solubility of the extraction (Sheridan et al., 2012).

The process to separate, identify and characterize bioactive compounds has still remained a huge challenge as plant extracts consist of mixture of numerous types of phytochemicals with different polarities. A number of chromatographic techniques like thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have been used for

the qualitative and quantitative determination of chemical components in samples (Tian et al., 2013). Among numerous analytical methods, HPLC is gaining popularity due to its resolving power that ideally suited for rapid processing of multi component samples, versatile as well as robust. Generally, HPLC consists of solvent delivery pump, sample introduction device, analytical column, detector and recorder or printer. Combinations of HPLC with detectors such as diode array detection (DAD), evaporative light-scattering detection (ELSD), fluorescence detection (FD), mass spectrometry (MS) as well as ultraviolet (UV) are being employed to facilitate rapid and accurate detection of target compounds (Sasidharan et al., 2011). The overall achievement in identification of natural products is influenced by the processing of crude extract as well as the choice of solvent used.

1.2 STATEMENT OF THE PROBLEM

Many literature reviews on wide range of species from Anisophylleaeceae family have been documented. However, in spite of therapeutic values and high utilization of *A. disticha* in treating various ailments, presently, researches are extremely sparse regarding this plant. Therefore, screening of major phytochemical constituents and evaluation of biological activities of underexplored species like *A. disticha* are necessary to support and verify their traditional claim as well as to provide base line information for the scientists to carry on further study. Furthermore, extraction of bioactive compounds from genus *Anisophyllea* involves high utilization of organic solvents at high temperature, and their identification and characterization is still remain a big challenge as some bioactive compounds are labile to heat, air and light. Thus, there is an urgent and continuous need of exploration and development of more

efficient extraction methods for retrieval of high purity of targeted phytoconstituents from plant materials.

1.3 RESEARCH QUESTIONS

1. Do different parts of *A. disticha* extracted using soxhlet possess antimicrobial and antioxidant activities?
2. How do different process variables of SFE affect the recovery of phenolic compounds and antioxidant activities of the plant?
3. Which extraction techniques produce higher yield of phenolic compounds and antioxidant activities?

1.4 RESEARCH OBJECTIVES

1. To screen bioactive compounds and biological activities namely antimicrobial and antioxidant capabilities of leaf, stem and root of *A. disticha* using soxhlet extraction.
2. To investigate the effect of different process variables such as temperature, pressure and concentration of co-solvent on supercritical fluid extraction of *A. disticha* for maximum recovery of total phenolic contents and its antioxidant capacity in terms of scavenging and reducing activities.
3. To identify the phenolic compounds in the extracts of conventional soxhlet extraction and non-conventional SFE by HPLC.

1.5 RESEARCH HYPOTHESES

A. disticha possesses significant concentration of phenolic compounds as well as potent biological activities like antimicrobial and antioxidant capabilities. SFE can be utilized as an alternative extraction method for obtaining herbal extract with better bioactivity compared to traditional extraction methods.

1.6 OVERVIEW OF THE STUDY