BIOACTIVE PROPERTIES AND PHYTOCHEMICAL ANALYSIS OF *Rubus fraxinifolius* Poir (ROGIMOT).

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THESIS SUBMITTED IN FULFILLMENT FOR DEGREE OF MASTER OF SCIENCE

FACULTY OF SCIENCE AND NATURAL RESOURCES
UNIVERSITI MALAYSIA SABAH

2018

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS THESIS

JUDUL: **BIOACTIVE PROPERTIES AND PHYTOCHEMICAL ANALYSIS OF *Rubus fraxinifolius* Pior (ROGIMOT)**

IJAZAH: **SARJANA SAINS (BIOTEKNOLOGI)**

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, citations, equations, summaries and references, which have been fully acknowledged.

17 August 2018

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ACKNOWLEDGMENT

In the name of Allah who is most kind and merciful.
I thank God for all the opportunity, strength, trials and courage that have been showered on me to complete not only my thesis but also the whole process of obtaining my Master of Science Degree. I have learned so much from this process not only in academic but also from a personal perspective.

I wish to express my deepest gratitude to my supervisor, Prof. Dr. Jualang Azlan Gansau and Ms. Azlinah Matawali for their encouragement in this work. Their endless support, priceless guidance, valuable advice and constructive criticism have been precious during these three years

I am also grateful to Centre for Research and Innovation, University Malaysia Sabah and Seaweed Research Unit for use of their laboratory facilities. This thesis also would not be successful without the assistance of staffs from the Faculty Science and Natural resources and Institute for Tropical Biology and Conservation. I would like to thank the faculty and institute for their help and support, especially in sample collection and sample identification process.

My heartfelt gratitude is extended to my family especially to my parents, have always provided me with positive encouragements, valuable suggestions and warm spirit to complete this thesis. Special thanks to them for their endless love and support. I would also like to extend my gratitude to my friends for their help, support, and understanding for these two years. I am grateful for their constant support and help.

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21 March 2018

ABSTRACT

Rubus fraxinifolius, locally known as Rogimot, is an underutilized edible fruit that can be found distributed from low tropic to subtropics region. The aims of the present study were to determine the antioxidant activities, phytochemicals content, anticholinesterase and antibacterial properties in different parts (i.e., fruit, stem, and leaves) of the plants. Antioxidants were analysed using Ferric Reducing Antioxidant Power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. All samples were freeze-dried and extracted using five different solvents (dH_2O, absolute ethanol, 80% (v/v) ethanol, absolute methanol, and 80% (v/v) methanol). An antioxidant test showed that 80 (%) methanol crude extract display a higher antioxidant value compares to the other extract. The extract of 80% methanol is then subjected to phytochemicals analysis, where the result of total phenolic and total flavonoid content is found higher in the leaves part with the values of 56.32±0.05 mg GAE/g and 31.36±1.05 mg CE/g, respectively. Meanwhile, fruit part showed the highest total anthocyanin and total carotenoid content with values of $22.27 \pm 1.28 \times 10^{-14}$mg C-3-GE/ g and 10.02±0.22 mg BC/g, respectively. For AChE inhibition activity, enzyme inhibition method is used with galanthamine as a positive control. The result of 80% (v/v) methanol extract of fruit, stem, and leaves showed a dependent inhibition activity against the AChE enzyme, where leaves part showed the highest ACh inhibition activity (63.48±0.48%) when tested at 250 µg/ml, followed by stem and fruit part. The leaves part is chosen for phytochemical analysis and were extracted with liquid-liquid extraction before undergoes fractionation by column chromatography. A total of 10 fractions were screened for their antioxidant, anticholinesterase, and antimicrobial activity. Nevertheless, fraction 5 and fraction 6 showed the highest antioxidant, AChE inhibition, and antibacterial activity. Therefore, both fractions are subjected to GC/MS analysis. The result on GC/MS analysis of both fractions revealed a total of 5 major compounds and among the major compounds that had been identified from both fraction is a phenol group compound, i.e phenol, phenol, 2,4-bis(1,1-dimethylethyl)- and a fatty acid group, i.e hexadecanoic acid, which is widely known for their bioactivity. As a conclusion, *R. fraxinifolius* leaves potentially provide protection against oxidation, antimicrobial, and having a potential as an acetylcholinesterase inhibitor.

ABSTRAK

SIFAT BIOAKTIVITI DAN ANALISIS FITOKIMIA *Rubus fraxinifolius* Pior (Rogimot)

Rubus fraxinifolius atau lebih dikenali sebagai Rogimot di kalangan masyarakat tempatan merupakan buah yang boleh dimakan dan ianya boleh ditemui di sekitar kawasan tropika rendah hingga ke subtropika. Kajian ini dilakukan bagi menentukan kebolehan bahagian buah, batang, dan daun tumbuhan ini sebagai antioksidan, kebolehan merencat enzim asetilkolin, jumlah kandungan fitiokimia, dan sebagai antimikrob. Aktiviti antioksidan ditentukan menggunakan kaedah kuasa penurunan ion ferik (FRAP), ujian penghapusan radikal bebas DPPH dan ujian penghapusan radikal bebas ABTS. Bahagian buah, batang dan daun yang telah diasingkan kemudiannya dikeringkan menggunakan pengering beku dan di ekstrak menggunakan lima pelarut yang berbeza (iaitu dH_2O, 100% etanol, 80% (v/v) etanol, 100% metanol, dan 80% (v/v) metanol). Berdasarkan keputusan ujian antioksidan, ekstrak menggunakan pelarut 80% (v/v) metanol menunjukkan keputusan aktiviti antioksidan yang lebih tinggi berbanding ekstrak larutan yang lain. Keputusan ujian fitokimia daripada ekstrak 80% (v/v) metanol menunjukkan bahawa bahagian daun mengandungi kandungan fenolik (56.32±0.05mg GAE/g) dan flavonoid (31.36±1.05mg CE/g) yang lebih tinggi berbanding bahagian batang dan buah. Manakala, jumlah kandungan antosianin dan karotenoid adalah lebih tinggi pada bahagian buah dengan nilai masing-masing iaitu $22.27 \pm 1.28 \times 10^{-14}$mg C-3-GE/g dan 10.02±0.22mg BC/g. Bagi aktiviti perencatan enzim asetilkolinesterase, galantamin digunakan sebagai kawalan positif. Semua bahagian tumbuhan yang diuji menunjukkan aktiviti perencatan terhadap enzim asetilkolinesterase, di mana bahagian daun menunjukkan aktiviti perencatan tertinggi (63.48±0.48%), apabila diuji pada kepekatan 250 µg/ml. Bahagian daun dipilih untuk melalui analisis fitokimia dan ianya diekstrak dengan pengekstrakan cecair-cecair sebelum dipisahkan dengan kromatografi turus. Kesemua 10 fraksi yang dikumpul daripada kromatografi turus, diuji semula terhadap ujian aktiviti antioksidan, perencatan enzim asetilkolin dan aktiviti antimikrob. Keputusan ketiga-tiga ujian terhadap 10 fraksi tersebut menunjukkan bahawa fraksi 5 dan 6 menunjukkan aktiviti antioksida, perencatan enzim asetilkolin dan antimikrob tertinggi dan kedua-dua fraksi tersebut dipilih sebagai subjek analisis fitokimia menggunakan GC/MS. Hasil daripada analisis GC/MS pada kedua-dua fraksi menunjukkan kehadiran 5 sebatian utama dan antara sebatian utama yang telah dikenalpasti adalah daripada kumpulan fenol iaitu "phenol, 2,4-bis (1,1-dimetilethyl)-" dan asid lemak iaitu asid heksadekanoik. Sebagai kesimpulan, daun R. fraxinifolius berpotensi memberikan perlindungan dan mengelak proses pengoksidaan, antimikrobial, dan berupaya menghentikan kesan enzim asetilkolinesterase.

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LIST OF SYMBOLS

B	Beta
°C	Degree Celsius
rpm	Revolution per minutes
±	Plus minus
%	Percentage
(v/v)	Volume per volume
ml	Milliliter
µg	Microgram
µl	Microliter
µg/ml	Microgram per milliliter
mg/ml	Milligram per milliliter
µM	Microgram
nm	Nanometer
xg	X gravitational forces
g	Gram
µg	Microgram
µM	Micromolar
mM	Millimolar
min	Minutes
g/l	Gram per litre
U/ml	Unit per milliliter
CFU/ml	Colony-forming unit per milliliter
mm	Millimeter
°C/min	Degree Celsius per minunte
~	Approximately equal
≤	Less than or equal to
>	Greater than
<	Less than
=	Equal

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AChE	Acetylcholinesterase
ACh	Acetylcholine
AOC	Antioxidant Capacity
ArOH	Antioxidant
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BChE	Butrylcholinesterase
CAS	Catalytic anionic site
CNS	Central nervous system
dH_2O	Distilled Water
FRAP	Ferric Reducing Antioxidant Power
HAT	Hydrogen Atom Transfer
HNO_2	Nitrous acid
$HO^\bullet$	Hydroxyl
HOCl	Hypochlorous acid
H_2O_2	Hydrogen Peroxide
IP	Ionization Potential
BDE	Bond Dissociation Energy
LLE	Liquid-Liquid Extraction
$LOO^\bullet$	Lipid Peroxyl
LOOH	Lipid Peroxide
$NO^\bullet$	Nitric oxide
$NO_2^\bullet$	Nitric dioxide
N_2O_3	Dinitrogen Trioxide
PAS	Peripheral anionic site
ROS	Reactive Oxygen Species
$ROO^\bullet$	Peroxyl
RNS	Reactive Nitrogen Species
SET	Single Electron Transfer
TRAP	Total Radical Trapping Antioxidant Parameter
ORAC	Oxygen Radical Absorbance Capacity

$^{1}O_2$	Singlet Molecular Oxygen
O_3	Ozone
$ONOO^-$	Peroxynitrite
OPs	Organophosphorus
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

1.0 Research Background

High demands in fruit production are expected worldwide due to the health benefits of fruits and their products. Developing countries contribute to about 98% of the total production of tropical fruits in the world (FAO, 2003). Southeast Asia is known to possess a rich diversity of potent commercial fruit and among successfully marketed fruits are mango, papaya, and banana. In spite of this commercialized fruit, there are also several fruits that remain underutilized and only sold locally. In Borneo Island, there are about 200 species of edible fruits, which some has been used as ethnomedicine and was beneficial to health, however, they are still not fully utilized and commercialized (Wong *et al.,* 1994; Abu Bakar *et al.,* 2009).

More than hundreds of indigenous fruit can be found in small-scale orchards, even in a backyard and tropical rainforest in Borneo. However, many of this fruits are only available locally and consider underutilized as not much research on these plant. An underutilized fruit has not received much attention among consumer compared to commercial fruit. This could be due to the lack of scientific data and information on their bioactive potentials and nutritional values, lack of popularity among local communities, and lack of promotional campaigns (Ikram *et al.,* 2009). Recently, there are increasing interests on rare and underutilized fruit especially in developing countries to promote and develop a new generation of 'superfruit' which can be exported and commercialized globally (Abu Bakar and Fry, 2013).

In present times, various wild edible plant species are studied for their nutritional content as they are considered as economically and nutritional important (Ahmad *et al.,* 2015). Plants contain varieties of free radical scavenging molecules, such as vitamins, dietary glutathione, polyphenols, and endogenous metabolites. These natural metabolites make a potential and good antioxidant. Although the human body can produce several enzymes that help scavenge free radical, but antioxidant such as α-tocopherol (vitamin E), β-carotene and ascorbic acid (vitamin C) is only supplied through diet (Levine *et al.,* 1991; Lobo *et al.,* 2010). High consumption of vegetables and fruits are also associated with protection against various age-related diseases. Moreover, epidemiological studies shows that intake of fruit and vegetables is associated with the lower risk of all-cause mortality and reduced potential of cardiovascular diseases (Wang *et al.,* 2014), cancer mortality (Wu *et al.,* 2016; Aune *et al.,* 2017), age-related diseases (Jiang *et al.,* 2017).

Recently, research regarding on natural antioxidant has increased in various fields including food biology, food chemistry, cosmetic and other medical healthcare industries (Duda Chodak and Tarko, 2007; Nimmi and George, 2012; Li *et al.,* 2014). The increased interest, especially in aromatic plant, spices, and several potential medicinal plants as a natural antioxidant, are due to the public's awareness, perception that natural dietary antioxidant is safer than the synthetic analogues (Dogan *et al.,* 2010) and the safety consideration of the potentially harmful effect of synthetic antioxidant (Seifu *et al.,* 2012). Food industries had been promoting the benefits of antioxidant content in fruits and vegetables which lead to a growing interest on research to find a new source of natural antioxidant (Peschel *et al.,* 2006; Hidalgo and Almajano, 2017). It is proven scientifically in laboratory and epidemiology studies where a high intake of fruits and vegetable can protect and reduce a considerable number of chronic diseases and the protective evidence are associated with their antioxidant and phytochemicals compound (Jiang *et al.,* 2017; Abdelrehim *et al.,* 2018).

1.1 Problem Statements

The natural or synthetic antioxidant has been added to vegetables, fruits, prepared food items, pharmaceuticals, and cosmetics in order to maintain their quality and increase their shelf life by inhibiting, slow or arrest the fermentation, microbial decomposition, acidification, and microbial contamination in this product (Anand and Sati, 2013). They are also has been used in various industries and fields including medical and food industry to prevent rancidification, owing to their wide availability and high performance (Anbudhasan *et al.,* 2014). Free radicals that are formed as by-products of many biochemicals can induce lipid peroxidation, which is the predominant cause of the destruction of vitamins, food decay, and rancidity during storage and transformation (St Angelo, 1996). An antioxidant can scavenge the harmful active free radical in cell and reduce potential mutation that can cause lipid alteration (Meroni and Raikso, 2018)

Synthetic antioxidants such as butylhydroxytoluene (BHT) and butyl hydroxyanisole (BHA) and propyl gallate are a synthetic phenolic antioxidant (Anbudhasan *et al.,* 2014). These compounds are been suspected cause of liver damage due to their toxicity as well as the carcinogenic effect (Barlow, 1990). As an example, synthetic antioxidants such as BHT, BHA, and TBHQ are used to overcome the problem on the stability of frying oil but a study by Gohari Ardabili *et al.* (2010) provided an evidence for the role of these synthetic antioxidants as carcinogens. Even though synthetic antioxidant help in protecting the quality of food, excess antioxidant added to food may produce toxicity or mutagenicity (Anbudhasan *et al.,* 2014).

Antioxidant from natural sources also has shown significant benefit in preventing a number of human diseases including neurological disease (Juliet and Sivakumar, 2017). Parkinson's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), memory loss, and depression are among of the neurological disease that has been investigated to be caused by oxidative stress (Si, 2001; Singh *et al.,* 2004; Christen, 2000; Butterfield, 2002). Protective effect of natural antioxidants are through to a variety of biological actions including modulation in the activity of a different enzyme, inactivation of free radicals, interaction with transition metals, and effect on gene expression and intracellular signalling pathways (Obrenovich *et al.,* 2010; Soobrattee *et al.,* 2006).

Various food advertisers have begun to take note and publicizing the fact on the protective evidence of natural antioxidant against various types of diseases (Anbudhasan *et al.,* 2014). Moreover, the adverse effect of synthetic antioxidant has caused an increase on a demand for natural antioxidant (Shah *et al.,* 2014). In addition to that, trends in pharmaceutical and healthcare industries together with a new concept of natural healthcare in a market create a demand of modern society (Li *et al.,* 2014). This triggered intense research on not only to the edible part of a plant such as fruit but also the underutilized part, such as stem, flower, root, bark, and leaves. Many research and studies devoted to many natural antioxidants such as ascorbic acid, tocotrienols, tocopherols, β-carotene on various endemic plants where these compounds can be used as phytoceuticals and nutraceuticals (Ali Hassan *et al.,* 2013).

1.2 Objectives

This research is carried out to investigate potency of an underutilized plant of Sabah that may have potential benefits especially this selected plant which is known as *Rubus fraxinifolius* (locally called as Rogimot). Part of the plant with high phytochemical content display a wide range of bioactivity could be recommended as possible part of disease preventing. Furthermore, a better understanding of the functional and nutraceutical potential of this plant will further contribute to conservation and enhancement of species to sustain the household income of local communities. Therefore, the objectives of this study are:

i. To determine the phytochemicals, antioxidants, and acetylcholinesterase inhibition properties of *Rubus fraxinifolius* Poir using different plant parts and extraction solvents.
ii. To fractionates and isolate the bioactive compounds from potential *Rubus fraxinifolius* Poir. extract and subsequently determine the antioxidant, acetylcholinesterase inhibition, and antimicrobial activity.

1.2.1 Research Justification

This research is important as nowadays as people start to consume and utilized more on natural pharmaceutical as sources or alternative sources of remedies compare to other chemical-based medicine. It is because synthetic pharmaceutical may contain effect to human bodies. This fruit is popular among the local community in some area of Sabah which covers Bundu Tuhan, Kundasang, Keningau, and Ranau. However, it remains unpopular among the people in other regions of Sabah. Previously, there are very rarely publications, studies, and scientific information regarding this selected underutilized plant especially for their antioxidant, acetylcholinesterase inhibition and antimicrobial properties, hence this study intends to provide scientific data on the potential of different parts of this selected species.

1.2.2 Hypothesis of Study

Rubus fraxinifolius have potential health benefits with phytochemical compounds that act as a natural antioxidant agent, good antimicrobial and acetylcholinesterase inhibitor properties.

CHAPTER 2

LITERATURE REVIEW

2.1 General Benefits of Fruits and Vegetables

Eating habit of consumers is changing with the 21^{st}-century lifestyle and there is a better understanding of the effect of food consumed on quality and health of life (Ancos *et al.,* 2015). Nowadays, there is increasing demand by consumers on fresh or processed fruits and vegetables, especially for canned, frozen or minimally processed products. Other than that, microbiologically safe and food that offers biological properties beyond nutritional factor also become an attraction among consumers. In line with the tendency to consume healthy, safe and free synthetic additive food, these have become the highest demand for a consumer (Ancos *et al.,* 2015). Moreover, numerous published report and data on ways of obtaining phytochemicals from fruits and vegetables lead to increasing of public awareness (Aguedo *et al.,* 2012; O'shea *et al.,* 2012; Wijngaard *et al.,* 2012; Yu and Ahmedna, 2013).

Many research also has described the positive association between high consumption of plant-based foods with protective and decrease of various other chronic diseases such as cardiovascular disease (Saiko *et al.,* 2008), Alzheimer's disease (Mani and Milind, 2007), cancer (Wang and Lewers, 2007), lower all-cause mortality in several populations (Heidemann *et al.,* 2008; Leenders *et al.,* 2013), reduce the risk of coronary heart disease, stroke, hypertension, obesity and many more (Ledoux *et al.,* 2011; Boeing *et al.,* 2012). Different varieties and species of fruits, vegetables, and grains have different phytochemicals profiles Chu *et al.,* 2002). Moyer *et al.* (2002) reported that dark coloured and highly pigmented coloured fruits have the highest values of phenolic, anthocyanin and antioxidant content compared to other plants generally for fruit and vegetables. As an example, different species and varieties of blackcurrant fruits such as *Ribes valdivianum*, *Ribes nigrum* cvs. Consort, and *Ribes odoratum* has a different total phenolic content (TPH/100g) with the value of 1790 ± 59, 1342 ± 28, and 958 ± 33; respectively (Moyer *et al.,* 2002). Research on 26 species of berries family such as *Ericaceae* genus *Vaccinium,* family *Grossulariaceae* genus *Ribis* and family *Rosaceae* genus *Rubus* also showed a high variation in total phenols content between the different genera and species (Jaime Guerrero *et al.,* 2010).

2.2 Genus *Rubus*

Rubus is a Latin name that refers to a holy bramble or blackberry (Zohary, 1972). *Rubus* is a member of raspberries which include other trailing brambles such as loganberry and blackberry. *Rubus* is also one of the most diverse genera in a plant kingdom, which contains about 500 species (Graham and Woodhead, 2011). The subgenera *Rubus* are divided into 12 section including *Allehgeniensis*, *Arugti, Flaggellares, Rubus, Ursini*, and/or *Verotrivialis* (Finn and Hancock, 2008).

2.2.1 Geographical Distribution of *Rubus*

Rubus plants can be found on all continents except Antarctica and it is distributed from low and tropic to subtropics region (Menzies, 2002; Yang and Pak, 2006). *Rubus* fruits are consumed globally because of their taste and due to the health benefits of it (Lee *et al.,* 2012). In Malesia region, there are about 46 species of *Rubus* are found (Kalkman, 1993). In Malaysia Borneo (Sabah), *Rubus* plant can be found in the highland area. Record by Corner and Beaman (1996), stated that there are more than 8 species of *Rubus* can be found in Mount Kinabalu, such as, *Rubus benguetensis, Rubus elongates,* and *Rubus rosifolius.*