ANTIOXIDANT ACTIVITY OF PLANT AND HONEY EXTRACT AND EFFECT OF EXTRACTION PROCESS PARAMETER

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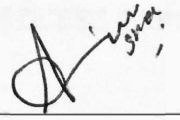

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ABSTRACT

The identification of antioxidant compounds and their different activities from the single natural product is very common practice but the combination of two natural products and identification of their activity was very rare. Scholars have acknowledged that activities of antioxidant compounds were enhanced by the combination of natural products but there is lack of significant scientific reports about the combination of natural products. Therefore, it is necessary to assess their activity by combining different natural products. So, the foremost objective of this research is to identify the antioxidant compounds from medicinal plants namely *Rhizophora apiculata (R. apiculata), Clinacanthus nutans (C. nutans), Carica papaya (C. papaya*) and stingless bee honey (SBH) extract separately by HPLC, and to assess the antioxidant activity of these medicinal plants individually and with the combination of SBH by UV spectrophotometer and quite important to examine the effect of extraction condition (extraction temperature and extraction time) of combination of above plants and SBH. For this purpose, the response surface methodology (RSM) software with the central composite design (CCD) was employed and identified the relationship between the two process variables and their response variable (antioxidant activity by UV spectrophotometer and the identification of quercetin by HPLC). According to the results, gallic acid, rutin and quercetin were present in all extracts. However, the antioxidant compound, ascorbic acid was not identified in the extract of *R. apiculata* and *C. nutans* but found in the *C. papaya* leaves and SBH extract. Whereas, *R. apiculata, C. nutans* leaves and SBH extract have kaempferol but this compound was not identified in the leaf extract of *C. papaya*. In addition, the antioxidant activity of selected plants individually and with the combination of SBH was assessed. Based on the obtained results of this research, it has been concluded that the antioxidant activity of plants was enhanced with a combination of SBH and they exhibited low IC_{50} values. The combination of *R. apiculata* and SBH had lowest IC_{50} value which means that this combination had the highest antioxidant activity as compared to the other two combinations. Furthermore, the validation of the RSM model coefficients was performed by the analysis of the variance (ANOVA). A high R^2 coefficient and low p-values ensure a satisfactory adjustment of the quadratic model to the experimental data. Moreover, the antioxidant activity and % yield of quercetin was increased with the increase in time and temperature but gradually reduced when the duration extraction time and temperature were extended above 40min and 60 °C. Based on the outcomes of these results, it has been concluded that the *C. nutans, R. apiculata* and *C. papaya* leaves and SBH extract have an antioxidant compound and the combination of SBH with the medicinal plants increases the antioxidant activity of plants.

Key words: Stingless bee honey, *Clinacanthus nutans, Rhizophora apiculata, Carica papaya,* Antioxidant compounds, HPLC, UV Spectrophotometer, Response surface methodology.

ABSTRAK

AKTIVITI ANTIOKSIDAN TUMBUHAN DAN EKSTRAK MADU SERTA KESAN DARIPADA PARAMETER PROSES PENGEKSTRAKAN

Pengenalpastian sebatian bioaktif dan aktiviti berbeza dari produk semulajadi tunggal adalah amalan yang sangat biasa tetapi gabungan dua produk semulajadi dan pengenalan aktiviti mereka sangat jarang berlaku. Para ilmuwan telah mengakui bahawa aktiviti-aktiviti sebatian bioaktif dipertingkatkan dengan gabungan produk semulajadi tetapi tidak ada laporan saintifik yang ketara mengenai kesan sinergi gabungan produk semulajadi. Oleh itu, adalah perlu untuk menilai kesan sinergi daripada sebatian bioaktif dan aktiviti mereka dengan menggabungkan produk semula jadi yang berbeza. Oleh itu, objektif utama penyelidikan ini adalah untuk mengenal pasti sebatian antioksidan dari tumbuhan ubat iaitu Rhizophora apiculata (R. apiculata), Nutacinaceae (C. nutans), Carica papaya (C. papaya) dan madu lebah (SBH) oleh HPLC, dan menilai aktiviti antioksidan tumbuhan ubatan ini dengan gabungan spektrofotometer UV oleh SBH. Terdapat kekurangan kajian terhadap aktiviti antioksidan tumbuhan ubatan dengan kombinasi SBH, jadi sangat penting untuk mengkaji kesan keadaan pengekstrakan (suhu pengekstrakan dan waktu pengekstrakan) kombinasi tumbuhan di atas bersama SBH. Untuk tujuan ini, perisian 'response surface methodology' (RSM) dengan 'central composite design' (CCD) digunakan dan mengenal pasti hubungan antara kedua-dua pemboleh ubah proses dan pemboleh ubah tindak balas mereka (aktiviti anti-oksidan oleh spektrofotometer UV dan pengenalan quercetin oleh HPLC). Mengikut keputusan, asid gallik, rutin dan quercetin hadir dalam semua ekstrak. Walau bagaimanapun, sebatian antioksidan, asid askorbik tidak dikenal pasti dalam ekstrak R. apiculata dan C. nutans tetapi terdapat dalam daun C. papaya dan ekstrak SBH. Sedangkan, R. apiculata, C. nutans daun dan ekstrak SBH mempunyai kaempferol tetapi sebatian ini tidak dikenalpasti dalam ekstrak daun C. papaya. Di samping itu, aktiviti antioksidan tumbuhan terpilih dengan kombinasi SBH dinilai. Berdasarkan hasil kajian ini dan literasi yang ada, telah disimpulkan bahwa aktiviti antioksidan tumbuhan ditingkatkan dengan gabungan SBH dan mereka memperlihatkan nilai IC50 yang rendah. Gabungan R. apiculata dan SBH mempunyai nilai IC50 terendah yang bermaksud gabungan ini mempunyai aktiviti antioksidan tertinggi berbanding dengan dua kombinasi yang lain. Selain itu, pengesahan pekali model RSM dilakukan dengan analisis varians (ANOVA). Pekali R2 yang tinggi dan nilai p yang rendah memastikan pelarasan memuaskan model kuadratik kepada data eksperimen. Selain itu, aktiviti antioksidan dan hasil quercetin meningkat dengan peningkatan masa dan suhu tetapi secara beransur-ansur dikurangkan apabila masa dan suhu pengekstrakan masa dilanjutkan melebihi 40min dan 60 oC. Berdasarkan keputusan kajian ini, telah disimpulkan bahawa C. nutans, R. apiculata dan daun C. papaya dan ekstrak SBH mempunyai sebatian antioksidan dan gabungan SBH dengan tumbuhan ubatan meningkatkan aktiviti antioksidan tumbuhan.

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LIST OF ABBREVIATIONS

KH	-	Kelulut honey
EFN	-	Extrafloral nectaries
R. apiculata	-	*Rhizophora apiculata*
C. nutans	-	*Clinacanthus nutans*
C. papaya	-	*Carica papaya*
SBH	-	Stingless bee honey
OS	-	Oxidative stress
UAE	-	Ultrasound assistant extraction
DPPH	-	2,2-diphenyl-1-picrylhydrazil
HPLC	-	High pressure liquid chromatography
RSM	-	Response surface method
CCD	-	Central composite design
PCA	-	Protocatechuic acid
ANOVA	-	Analysis of Variance

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CHAPTER 1

INTRODUCTION

1.1 Background

The global development in medical research focuses on establishing relations between two essential natural medicinal products for the health of humans: honey and medicinal plants. The term "natural" is described as something that exists or is manufactured naturally and is not synthetic or artificial. Normally, the sources of natural products are animals, microbes and plants (Fong, 2015).

Chemical compounds are produced by plants and these chemicals are used for defense against various herbivores. The plant chemicals are classified as either primary or secondary metabolites. The compounds of secondary metabolites are biosynthesized from compounds of the primary metabolites and their locations of storage in the plants differ in families or in a species of specific taxonomic groups. There are many phytochemicals, such as alkaloids, glycosides, polyphenols and terpenes. Plants produce these phytochemicals for defense against various herbivores. These compounds are commonly used as medicine in control of various diseases. Phytochemicals are present in all parts of plants, including the leaves, seeds, flowers, stems, fruit and nectar. These phytochemicals are transferred into honey from the nectar of plants (Yamani, 2015).

The food of bees consists of the abundant products of plants (nectar and pollen) that transfer amino acids, sugars, lipids, minerals and vitamins. In addition to these micro- and macronutrients, pollen and nectar are sources of various phytochemicals which contain phenolics, terpenoids and alkaloids (Erler and Moritz, 2015).

Normally, bees collect the nectar from the flowers, but actually, the nectar starts in the leaves. Plants use the energy in sunlight to make sugar from carbon dioxide and water, the process that is called photosynthesis. Most of the sugar is made in the leaves and from the leaves, this sugar travels through the plant's conducting tissues to the other parts of the plant like the stems, flowers and roots. These plant parts then remove the sugar from the conductive tissues and use it to fuel all their metabolic processes (Sartell, 2017). So, the researchers consider that honey also has phytochemicals.

Medicinal plants were shown to play a significant role in curing many diseases of ancient times. The plant kingdom is truly a goldmine of potential drug compounds. Several earlier reviews and research studies summarized that the products from natural sources have contributed significantly to the discovery of drugs and health benefits for people. Many older systems of medicine like Ayurveda, Chinese and others are mainly supported by natural products. Moreover, it is believed that natural products are less deadly than synthetic medicines because of their plant origins (Kaur, Stashenko, Kumar, Sahrawat and Talniya, 2018). Medicinal plants have been traditionally used in various parts of the world as traditional treatments for health maintenance. A medicinal plant named *Labisia pumila* (Kacip Fatimah) has been reported to protect osteoporotic bone in an estrogen-deficient rat model (Fathilah, Nazrun Shuid, Mohamed, Muhammad and Nirwana Soelaiman, 2012) and protects the cells of the skin from photo-aging which is caused by UVB radiation (Choi *et al.*, 2010). The other medicinal plant that is well known for its efficacy on erectile function improvement is the *Eurycoma longifolia* (Tongkat Ali) (Kotirum, Ismail and Chaiyakunapruk, 2015) while *Andrographis paniculata* (Hempedu bumi) was reported to have anti-cancer and anti-malarial activities (Banerjee *et al.*, 2017). Generally, the studies of pharmacological activities have confirmed the traditional use of *Ficus deltoidea* (mas cotek), mainly for fertility and maintenance of the female reproductive system (Salleh and Ahmad, 2013). *Ficus deltoidea* has also been reported to have anti-diabetic (Kalman, Schwartz, Feldman and Krieger, 2013), anti-inflammatory and anti-nociceptive, anti-melanogenic and anti-photo aging properties (Oh *et al.*, 2011), as well as anti-bacterial (Samah, Zaidi and Sule, 2012), wound healing (Abdulla, Ahmed, Abu-Luhoom and Muhanid, 2010), anti-cancer and cytotoxicity activities (Farsi *et al.*, 2013). *Catharanthus roseus* has a variety of medicinal properties, such as antibacterial, antifungal, antioxidant and antiviral, anticancer activity (Rao and Ahmed, 2014).

Like plants, honey is also used in the treatment of different diseases. The class of alternative drug treatment in which products of the honey bee are used, such as pollen, honey, propolis, bee venom and royal jelly, which is known as apitherapy (Cassileth, 2011). Like honeybees, stingless bees also belong to the Hymenoptera order under the family Apidae and subfamily of Apinae. However, stingless bees differ from honey bees wherein they are categorized in the tribe Meliponini. The tribe Meliponini can be further divided into two main genera, which are Trigona, the largest group and Melipona (Rahman, Das, Rajkumari, Saikia and Sharmah, 2015).

The honey of stingless bee, like many other types of honey, is a sugary liquid that has a superb taste and odor. Commonly, honey manufactured by a stingless bee is commonly known as "Kelulut" in Malaysia. Furthermore, "Kelulut" honey (KH) has higher contents of polyphenols and flavonoids in contrast to the honey produced by the Apis spp. (Maringgal, Hashim, Tawakkal, Mohamed and Shukor, 2019). A previous study demonstrated that KH is made of mainly water, carbohydrates, amino acids, minerals and vitamins. Another study reported that KH possesses distinctive and divergent phenolic and flavonoid composites that have been shown to have a vital function with regard to its anti-inflammatory, anti-bacterial and anti-oxidant activities of the Borneo (Sabah and Sarawak) SBH (Tuksitha, Chen, Chen, Wong and Peng, 2018). According to Rao and Co., the honey of the stingless bee is also used in the treatment of glaucoma and cataracts (Rao, Krishnan, Salleh and Gan, 2016). The honey of the stingless bee also has wound healing (Kakkar and Bais, 2014) and anti-cancer (Kakkar and Bais, 2014) properties.

Honey is made from nectar. Nectar is the currency and it is used by plants to pay insects and other animals to do their bidding. It costs plants nothing to photosynthesize this sweet medium of exchange using energy from the sunlight to rearrange the elements provided by carbon dioxide and water. Even so, flowers are not the only place where nectar serves as coinage in exchange for services. However, when nectar sources are deficient, bees collect sugars from any available source, such as honeydew, sweet-tasting juices from overripe fruits and plant exudates, and store them in the hive (Yamani, 2015). Most people are aware that flowers commonly produce nectar but people are rarely aware of the extrafloral nectaries (EFN). EFN is the nectar-producing glands actually apart from the flower. These glands can be found on petioles, leaf laminae, stipules, bracts, fruit,

pedicels, etc., and their shapes, secretions and sizes differ with the taxa of the plant (Mizell, 2004).

In this study, three medicinal plant leaves, *Rhizophora apiculata* (*R. apiculata*), *Clinacanthus nutans* (*C. nutans*) and *Carica papaya* (*C. papaya*) and stingless bee honey (SBH) were selected and their anti-oxidant compounds identified, and they were assessed for their antioxidant activities. It has been hypothesized that various chronic and degenerative diseases are caused by oxidative stress (OS). OS can lead to oxidative damage to large biomolecules, such as DNA, lipids and proteins and, subsequently, cause an increased risk of inflammatory diseases, cardiovascular disease, diabetes, cataracts, cancer, Alzheimer's disease and age-related functional degeneration (Attanayake and Jayatilaka, 2016). Anti-oxidant compounds are beneficial in the cure of these various diseases because anti-oxidant compounds diminish the oxidative stress in cells (Badhani, Sharma and Kakkar, 2015).

Nowadays, people rely on natural product treatments. Moreover, the honey of the stingless bee is becoming gradually more popular because of its potential part in contributing to the health of human beings. SBH is mainly a rich source of antioxidant compounds, which act as natural antioxidants. So, the novelty of this research, to assess the antioxidant activity individually and combination of selected medicinal plant with SBH and compared their results.

1.2 Problem Statement

There are various source of antioxidant compounds such natural and synthetic products. The sources of natural products are animals, microbes and plants in other hand synthetic products synthesized by employing different methodologies in the laboratory and these are the medicines which are made by human. Synthetic products give fast relive and people think that this treatment was very good but very few people aware that, synthetic products have various side effect and their effects are not long lasting. Natural products work slowly but results are more sustainable and last longer than chemical drugs. Natural products are safer to use, little to no side effects, no addiction, no poisoning of the body with chemicals and toxins and finally, they do not cost a fortune.

Recently, researcher focused on the natural products and find out that how to increase and faster their effect. Many people claim that the combination of different natural product give fast relive. Such as honey and turmeric useful for cough, good for digestive tract and it is grate for skin. The combination of honey and lemon tea help in burring fat and flush out toxin from the body. Milk and turmeric improve the healing of wounds but the lack of proper scientific reports.

Moreover, only a few studies have been undertaken on the combination of honey and the leaves of medicinal plants, such as Mboto et al.'s study on the anti-microbial activities and phytochemical properties of the combined effect of the extracts of the *Garcinia kola* and *Vernonia amygdalina* leaves and honey on a few main microorganisms. Their result suggests that the preparation exhibited significant in vitro anti-microbial activity (Mboto, Adegoke, Iwatt and Asikong, 2009). In 2013, another researcher studied the combined anti-bacterial activity of garlic and SBH extracts and the combination had greater activity than garlic and honey alone. The researcher suggested that a combination of garlic with SBH can work as an alternative natural anti-microbial remedy for the cure of pathogenic bacterial infections (Andualem, 2013).

As for the researcher's knowledge and literature review, there is a lack of studies of the medicinal plants leaves with the combination of stingless bee honey (SBH) to assess the antioxidant activities. In this research assess the antioxidant activity of three medicinal plant namely *R. apiculata, C. nutans* and *C. papaya* individually or with the combination of SBH. And observed that what happen on antioxidant activity when combine the plant with SBH. Moreover, during combination extraction of plant and SBH, how extraction process parameter affects the antioxidant activity.

1.3 Objectives of the Study

The overall goal of this research has been to identify the presence of antioxidant compounds in the extract of SBH and *R. apiculata, C. nutans* and *C. papaya* leaves along and assess the anti-oxidant activities of the individual and combined extracts of the SBH and the medicinal plant leaves.

1.3.1 Specific Objective

1 To detect the antioxidant compounds (gallic acid, rutin, ascorbic acid, quercetin and kaempferol) from the honey of stingless bee, *C. nutans, R. apiculata* and *C. papaya* leaf extracts using HPLC.

2 To assess the antioxidant activities of medicinal plants (*C. nutans, R. apiculata and C. papaya*), SBH and combination of medicinal plants with SBH.

3 To investigate the effects of extraction process parameters on the antioxidant activity (% inhibition) and % yield of antioxidant compounds from the extract of *R. apiculata* and SBH, *C. nutans* and SBH, *C. papaya* and SBH.

1.4 Significance of the Study

It is a general belief of the people that everything that exists in natural products has valuable effects as compared to synthetic products. In the current era, with improvement in the health profession, the influence of diseases has also become greater than before. Numerous new drugs are being discovered for the cure of dangerous diseases but, on the other hand, these drugs are also causing various side effects that range from severe to minor in intensity. The remedies from natural products seem to be more effective than synthetic origin drugs. Nowadays, researchers are moving towards the combination of therapies rather than the single ones because the combination of natural products has increased the intensity of the treatment; and, because of their natural origin, there are no side effects or at the most only very minor ones. In contrast, this study provides scientific information regarding the antioxidant compounds and the antioxidant activity of the *R. apiculata, C. nutans* and *C. papaya* leaves in combination with SBH and this can be helpful for the treatment of inflammatory-related diseases.

1.5 Scope of the Research

To achieve the goal of the study, the work involved in this research was divided into 3 parts based on the specific objectives. Natural products have different types of antioxidant compounds. Before performing any qualitative and quantitative analysis it is necessary to extract these compounds from the raw material of the plants. In this study, for the

extraction of these compounds, the ultrasound assistant extraction (UAE) was used. After that, different tests were performed on these extracts. In the first part, the antioxidant compounds (gallic acid, rutin, ascorbic acid, quercetin and kaemferol) were identified in the extracts of the stingless bee honey and the *R. apiculata, C. nutans* and *C. papaya* leaves by using the HPLC to complete the objective number one. To achieve the second objective, the antioxidant activity was calculated based on the scavenging of the stable free radical 2,2-diphenyl-1-picrylhydrazil (DPPH) by the individually and combinations of the extracts of the *C. nutans, R. apiculata* and *C. papaya* leaves with the SBH by UV spectrophotometer. IC_{50} (effective concentration to scavenge 50% of the radical) values were used to determine the antioxidant potential of the extracts and compared the individual and combination results or with previous studies undertaken by different researchers. In the third part, the best extraction condition (time and temperature) for the antioxidant activity and identification of antioxidant compounds from the combinations of the extracts of the *C. nutans, R. apiculata* and *C. papaya* leaves with the SBH were investigated by the UV spectrophotometer and the HPLC. For this purpose, the response surface methodology (RSM) software with the Central composite design (CCD) was used to achieve objective number three.

1.6 Research Methodology

Systematic steps and the literature review of the work were carried out to accomplish the research objectives. Thus, the research methodology was carefully laid out as follows:

1.6.1 Literature Review

The literature review covered the important characteristics, phytochemistry and the pharmacological activities of *R. apiculata, C. nutan, C. papaya* leaves and SBH. The application and analysis of the statistical experimental design and the selection of the appropriate analytical and quantification methods for studying antioxidant compounds were also studied.

1.6.2 Selection of the Antioxidant Compounds

There is a list of antioxidant compound. For the detection of antioxidant compound in samples we must need standard to compare. Standards are very expensive that's why I have selected 5 antioxidant compounds (gallic acid, rutin, ascorbic acid, quercetin and kaempferol) which have strong antioxidant activity and easily available.

1.6.3 Selection of the Instruments:

In this study, a few instruments were selected according to the requirements of the research and their easy availability.

1.6.4 Selection of the Design of the Experiment.

The experimental setting was performed by using a CCD. The CCD is an experimental design that is used to achieve the best information about a process from a few numbers of experiments (Prakash Maran, Manikandan, Vigna Nivetha and Dinesh, 2017). The CCD was used in this research to determine the optimal conditions and study the effect of two variables' extraction times (20 – 60 min) and temperatures (40 – 80°C), and the extractions of two responses.

1.7 Thesis Organization

Chapter 1 started with a background overview of natural products (medicinal plants and honey). This chapter covered the importance of natural products; the problem statement, objectives, scope and significance of the study and the methodology are also carefully laid out accordingly.

Chapter 2 presents the literature review of the medicinal plants and SBH. The chapter also explains the basic terms in the experimental design applied for the experiment.

Chapter 3 presents the process of the research involving the identification of the antioxidant compounds by HPLC and the antioxidant activity through the DPPH test and the optimization by UV spectrophotometer and an HPLC.