BIOLOGICAL ACTIVITIES OF BUTTERFLY PEA AND FAH TALAI JONE EXTRACTS AND THEIR APPLICATION FOR DEVELOPMENT OF NEW SUPPLEMENTARY PRODUCT

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DOCTOR OF PHILOSOPHY IN APPLIED CHEMISTRY

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TIPPARAT SAEJUNG

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THIS DISSERTATION HAS BEEN APPROVED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN APPLIED CHEMISTRY

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ชื่อเรื่อง ฤทธิ์ทางชีวภาพของสารสกัดอัญชันและสารสกัดฟ้าทะลายโจรและการประยุกต์สำหรับพัฒนาผลิตภัณฑ์เสริมอาหารชนิดใหม่

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บทคัดย่อ

ดอกอัญชันเป็นพืชในตระกูล Fabaceae สารสกัดจากดอกอัญชันมีการศึกษาว่ามีฤทธิ์ทางชีวภาพที่หลากหลาย เช่น ฤทธิ์ลดน้ำตาลในเลือด ฤทธิ์ยับยั้งเชื้อจุลินทรีย์ ฤทธิ์ยับยั้งอนุมูลอิสระ และฤทธิ์ต้านการอักเสบ ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อเตรียมสารสกัดจากดอกอัญชัน ทดสอบสมบัติเคมีของสารสกัดและตั้งตำรับผลิตภัณฑ์เสริมอาหารที่มีสารสกัดจากดอกอัญชันเป็นองค์ประกอบ ดอกอัญชันสดถูกสกัดโดยการสกัดด้วยตัวทำละลายสามวิธี ได้แก่ การแช่หมัก การรีฟลักซ์ และการใช้คลื่นไมโครเวฟช่วยสกัด โดยใช้ตัวทำละลายสองชนิด (น้ำกลั่น และเอทานอล) การหาค่าที่เหมาะสมของแต่ละวิธีการสกัดถูกตรวจวิเคราะห์เพื่อประเมินสภาวะที่ดีที่สุดในการเตรียมสารสกัดดอกอัญชัน ตัวอย่างเช่น ชนิดของตัวทำละลาย อัตราส่วนของแข็งต่อของเหลว เวลาในการสกัด และกำลังไฟฟ้าของไมโครเวฟ ผลการทดลองพบว่าวิธีการสกัดที่ดีที่สุดสำหรับการเตรียมสารสกัดดอกอัญชันคือการแช่หมักเป็นเวลา 7 วัน โดยการใช้น้ำกลั่นเป็นตัวทำละลาย ที่อัตราส่วนพืชต่อตัวทำละลาย 1:20 โดยน้ำหนักต่อปริมาตร สารสกัดดอกอัญชันจากสภาวะนี้แสดงฤทธิ์ต้านอนุมูลอิสระ ปริมาณฟีนอลิกรวมและปริมาณแอนโทรไซยานินที่ 66.94 ± 0.27 มิลลิกรัมสมมูลโทรล็อคต่อกรัมสารสกัด 69.72 ± 0.71 มิลลิกรัมสมมูลกรดแกลลิกต่อกรัมสารสกัด และ 8.18 ± 0.38 มิลลิกรัมสมมูลไซยานิดีน-3-กลูโคไซด์ต่อกรัมสารสกัด ตามลำดับ อย่างไรก็ตามฟ้าทะลายโจรถูกสกัดด้วยน้ำกลั่นเพื่อให้ได้รับสารสกัดจากฟ้าทะลายโจร หลังจากนั้นถูกวิเคราะห์องค์ประกอบในการออกฤทธิ์ตัวหนึ่ง (แอนโดรกราโฟไลด์) และใช้เพื่อตั้งตำรับต้นแบบผลิตภัณฑ์เสริมอาหารในรูปแบบเม็ด (500 มิลลิกรัม) ร่วมกับสารสกัดดอกอัญชันในอัตราส่วนสารสกัดผสมที่แตกต่าง ผลิตภัณฑ์ถูกตรวจวิเคราะห์ปริมาณฟีนอลิกทั้งหมด ฤทธิ์ต้านอนุมูลอิสระ แอนโทไซยานิน และแอนโดรกราโฟไลด์ ผลการทดสอบพบว่าที่อัตราส่วนผสมของสารสกัดดอกอัญชันและฟ้าทะลายโจร 1:1 โดยน้ำหนัก ในรูปแบบเม็ดผลิตภัณฑ์ แสดงค่าปริมาณฟีนอลิกรวม (36.42 มิลลิกรัมสมมูลกรดแกลลิค/500 มิลลิกรัมเม็ดผลิตภัณฑ์) ฤทธิ์ต้านอนุมูลอิสระ (40.02 มิลลิกรัมสมมูลโทรล็อค/500 มิลลิกรัมเม็ดผลิตภัณฑ์) แอนโทไซยานิน (1.53 มิลลิกรัม/เม็ดผลิตภัณฑ์) และมีปริมาณแอนโดรกราโฟไลด์ (10.84 มิลลิกรัม/

เม็ดผลิตภัณฑ์) ผลิตภัณฑ์ต้นแบบของตำรับระหว่างสารสกัดดอกอัญชันและสารสกัดฟ้าทะลายโจร แสดงฤทธิ์ทางชีวภาพที่แตกต่างจากสารสกัดแต่ละชนิดอย่างเดียว แสดงให้เห็นว่าการผสมผสานของสารสกัดจากสมุนไพรอาจช่วยเสริมฤทธิ์ทางชีวภาพและสามารถพัฒนาเป็นผลิตภัณฑ์สมุนไพรชนิดใหม่ได้

คำสำคัญ : สารสกัดอัญชัน, แอนโทรไซยานิน, สารต้านอนุมูลอิสระ, ฟีนอลิกรวม, แอนโดรกราโฟไลด์, ผลิตภัณฑ์เสริมอาหาร

Title	BIOLOGICAL ACTIVITIES OF BUTTERFLY PEA AND FAH TALAI JONE EXTRACTS AND THEIR APPLICATION FOR DEVELOPMENT OF NEW SUPPLEMENTARY PRODUCT
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Degree	Doctor of Philosophy in Applied Chemistry
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ABSTRACT

Butterfly pea (*Clitoria ternatea*) is a plant belonging to the Fabaceae family. *C. ternatea* extract or called BP extract has been reported the various bioactivities including hypoglycemic, antimicrobial activity, antioxidant activity and anti-inflammatory activity. The purpose of this study is to prepare *C. ternatea* extract, study the chemical properties and formulate the supplementary product containing the *C. ternatea* extract. The fresh flower of *C. ternatea* was extracted using three solvent extraction methods including maceration, reflux, and microwave assisted extraction with two solvents (distilled water and ethanol). The optimization of each extraction method was determined to evaluate the best condition for preparing the BP extract for example type of solvent, the ratio of solid to solvent (w/v), extraction time, and microwave power. The result showed that the best extraction method for preparing the BP extract was maceration for 7 days by using distilled water as solvent and the ratio of solid to solvent at 1:20 w/v. BP extract from this condition exhibited the antioxidant activity, total phenolic contents and anthocyanin contents at 66.94 ± 0.27 mg Trolox equivalent per g extract, 69.72 ± 0.71mg GAE per g extract and 8.18 ± 0.38 mg cyanidine-3-glucoside equivalence per g extract, respectively. Moreover, Fah Talai Jone (*Andrographis paniculate*) was extracted using distilled water to obtain fah talai jone (FTJ) extract then analyzed the one of active components of FTJ extract (andrographolide) and used it to formulate the prototype of supplementary product in tableting (500 mg) with BP extract in various ratios of mixing extracts. The products

were evaluated the total phenolic contents, antioxidant activity, total anthocyanin and andrographolide. The results showed that the mixture of BP extract and FTJ extract at 1:1 w/w in tableting had the total phenolic contents (36.42 mg gallic acid equivalent/500 mg of tablet), antioxidant activity (40.02mg trolox equivalent/500 mg of tablet), anthocyanin content (1.53 mg/500 mg of tablet) and andrographolide 10.84 mg/500 mg of tablet. The prototype product of this formulation between BP extract and FTJ extract exhibited the biological activities difference from only each extract. It can be revealed that the combination of herbal extracts might be enhanced the biological activities and can be developed as a new supplementary product.

Keywords : Butterfly pea extract, Anthocyanin, Antioxidant, Total phenolic, Andrographolide, Supplementary product

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CHAPTER 1

INTRODUCTION

Medicinal plants are source of secondary metabolites that possess biological activities. Secondary plant constituents or secondary metabolites are responsible for many biological or pharmacological activities such as phenolics, alkaloids, terpenoids, saponins, flavonoids, tannins, and glycosides etc. Nowadays, medicinal plants and herbal plants become more popular in many topics of health care as natural products due to their bioactive compounds. The herbal plants can be used as natural options for food ingredients and helpful influences on human health based on their vital functions on pharmacological against difference human diseases. However, the extraction processes play a vital role on quality and therapeutic efficacy of herbal plants. In general, there are commonly used methods in the extraction of herbal plants such as maceration, percolation, Soxhlet extraction, microwave-assisted extraction, ultrasound-assisted extraction etc. This study aimed to assess various solvents of extractions, methods of extraction, and identification of bioactive compounds in herbal plants. In addition, herbal extracts, extracted using non-chemical extraction processes or less toxic organic solvents, will be formulated the potential supplement in the pharmaceutical and food industry.

Butterfly pea (*Clitoria ternatea*), called BP, is a plant belonging to the Fabaceae family which can be grown in all regions of Thailand. The butterfly pea flower contains anthocyanins which have good free radical scavenging activity and potential as antioxidants. In addition, BP extract comprises many bioactive substances such as anthocyanins, phenolics, flavonoids, glucosides, and other secondary metabolites. These bioactive compounds exhibit various biological or pharmacological activities. BP extract showed many pharmacological effects including hypoglycemic, anti-inflammatory, anti-diabetic, anti-microbial, and antioxidant activities etc.

Fah Talai Jone (*Andrographis paniculate*), called FTJ, has been used for the treatment of fever and many infectious diseases. The plant is native to tropical and sub-tropical regions of India and used as a pain killer, anti-inflammatory, antibacterial

and anti-viral agent etc. In this work, FTJ was prepared the FTJ extract and then formulated the supplementary product (Srivastava and Akhila, 2010) with BP extract.

1.1 Butterfly pea (Clitoria ternatea)

Butterfly pea (BP) (Figure 1) is a plant in species belonging of Fabaceae family. In Thailand, BP flowers are usually used as food drinks and as a natural food colorant and healthy beverages. Butterfly pea flower extract can be used as a natural blue colorant which containing various polyphenols such as flavonoid, anthocyanins and other compounds (Pasukamonset et al., 2016). Polyphenols play a vital function on pharmacological against difference human diseases such as allergies, cancer, cardiovascular disease (Heng et al., 2017). Moreover, the researchers reported that BP is promising medicinal plant with wide range of pharmacological activities including antioxidant, anti-inflammatory, antidiabetic, hypolipidemic, anticancer, antimicrobial, analgesic, antipyretic, anti-asthmatic activities, and anti-platelet-aggregation properties (Phrueksanan et al., 2014; Vasisht et al., 2016) which could be utilized in several medical applications because of its effectiveness and safety. BP flowers contain high amount of anthocyanins. Anthocyanins which present in edible fruits, vegetables and flowers are an important class of water-soluble pigments belonging to the flavonoid family.

Figure 1 Flower of butterfly pea

The chemical structure of common anthocyanins was presented in Figure 2 and Table 1, respectively (Fang, 2015). Anthocyanin is one of the main chemical constituents which is responsible for therapeutics of the plant. Anthocyanin pigments are responsible red to purple to blue colors. The different flower colors are mainly

due to the chemical structure of the different anthocyanins or anthocyanidins in the BP flower. Delphinidin is the main anthocyanin responsible for the deep blue to purple color in this BP flower (Askar et al., 2015a) .

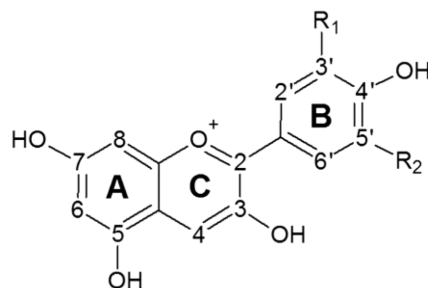

Figure 2 Chemical structure of common anthocyanins (Fang, 2015)

Table 1 The name of common anthocyanins

Chemical	R1	R2
Pelargonidin (Pg)	H	H
Cyanidin (Cy)	OH	H
Delphinidin (De)	OH	OH
Peonidin (Pn)	OCH_3	H
Petunidin (Pt)	OH	OCH_3
Malvidin (Ma)	OCH_3	OCH_3

1.2 Fah Talai Jone (Andrographis paniculate)

Fah Talai Jone (FTJ) is native to India and Sri Lanka and widely found in Southern and Southeastern Asia, including China, Thailand, Bangladesh, Indonesia, Malaysia, Myanmar, and Philippines. The aerial and flowers of FTG were shown in Figure 3. FTJ is a medicinal plant that belongs to the *Acanthaceae* family and is commonly known as king of the bitter. It has been experimentally verified to have a wide range of bioactive properties such as anti-retroviral, anti-oxidative, anticancer, antimalarial, anti-inflammatory, antibacterial, antifungal, antihypertension, and antidiabetic etc.

Figure 3 *Andrographis paniculata*

A. paniculata has been used to treat fever, herpes, sore throat, upper respiratory infections, and other chronic and infectious diseases. According reported have more than 80 compounds from this plant. Andrographolide and neoandrographolide (Figure 4) are major bioactive components of *A. paniculate* that contribute to most of its bitterness and bioactivities. These bioactive components have been identified as the main chemical constituents which are responsible for the therapeutics of this plant. Particularly, the leaves of this plant were found to contain the highest amount of andrographolide, followed by stem, root, and lastly seed (Lee et al., 2018a; Srivastava and Akhila, 2010).

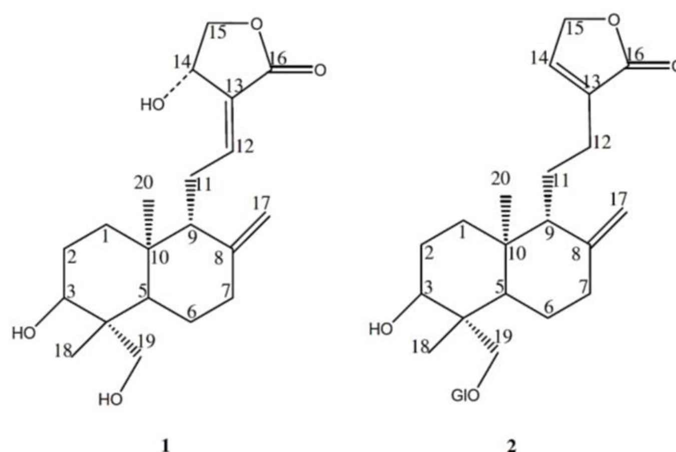

Figure 4 Structures of andrographolide (1) and neoandrographolide (2).

1.3 Phytochemicals

Phytochemicals refer to all plant derived chemical compounds. Hence, macronutrients, carbohydrates (including dietary fiber), lipids and proteins, could be classified as phytochemicals. However, the phytochemicals refer to small non-essential nutrients with effecting the health-promoting actions. Phytochemicals can be divided into six broad classes including, alkaloids, organosulfurs, phenolics, carbohydrates, non-nutritive proteins, and lipids (Figure 5) (Bolling et al., 2011).

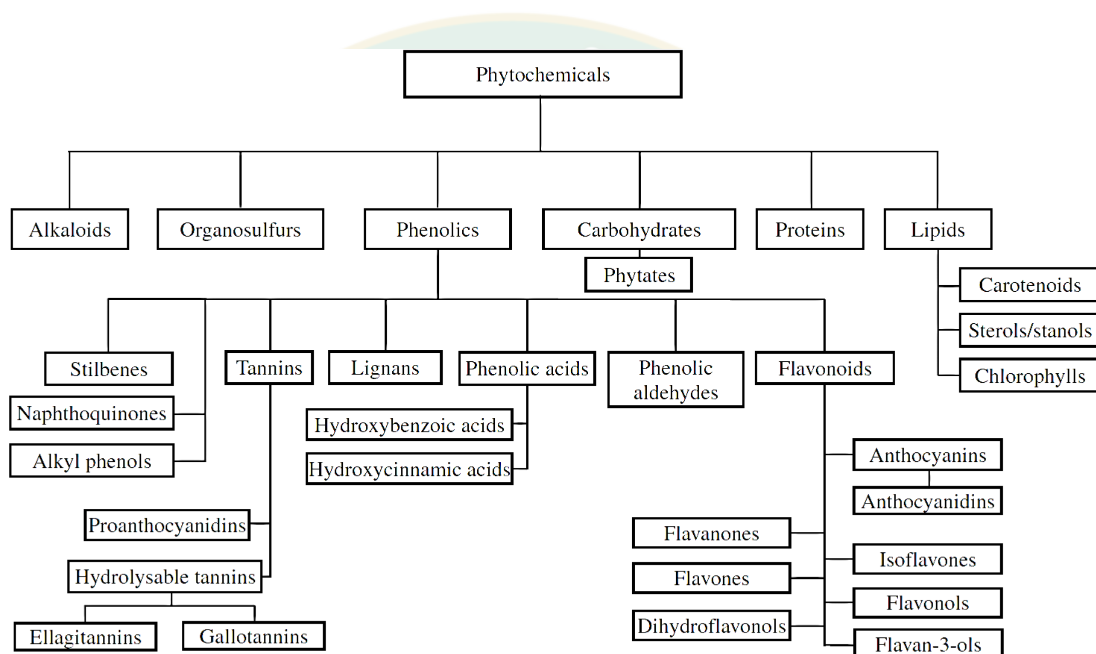

Figure 5 Classification of phytochemicals classes

Plant phenols are among the most abundant and widely represented class of existing plant natural products (Martín et al., 2017). In general, polyphenols or phenolic compounds belong to two main classes including, flavonoids and phenolic acids. Polyphenols are increasingly being recognized by their nutritional value since they may help reduce the risk of chronic diseases. In general, they have positive effects on health because of their free radical scavenging capacities, increasing the antioxidant activities and preventing the cellular oxidation. The capacity of phenolic compounds to trap free radicals depends upon their structures, in particular of the

hydrogen atoms of the aromatic groups that can be transferred to the free radicals and of the capacity of the aromatic compounds to cope with the uncoupling of electrons as a result of the surrounding displacement of the electron-π system. Several thousand polyphenols have been characterized in plants and several hundreds of them are found in food plants. Dietary polyphenols differ widely in their physicochemical properties, bioavailability, biological properties, and health effects. About 1 g of polyphenols per day is commonly ingested with foods, which is significant when compared with the estimated daily consumption of other phytonutrients such as carotenoids, vitamin E, and vitamin C, estimated at 5, 12, and 90 mg/d, respectively. Polyphenols are therefore the most abundant antioxidants in the diet, about 10 times higher than the intake of vitamin C and 100 times that of vitamin E (Bolling et al., 2011; Bueno et al., 2012; Martín et al., 2017).

1.4 Extraction Method

Extraction is an important process in the recovery of bioactive substance such as phenolic compounds from plant matrix (Alara et al., 2018; Chua et al., 2016). Many conventional extraction methods such as maceration, soxhlet, reflux extraction have been used for several years to extract the substances from fresh and also dried plants. Moreover, several non-conventional extraction methods such as ultrasound assisted extraction, microwave assisted extraction and supercritical fluid extraction have been developed in order to keep the bioactive substances and increase the efficiency of extract yields. In this research, maceration, reflux and microwave assisted extraction methods were used to prepare the plant extracts.

1.4.1 Reflux Extraction

Reflux extraction, one of the traditional solvent extraction methods, are often used for the extraction of different biologically active compounds (Ji et al., 2020; Zhang et al., 2018). Reflux extraction is a solid–liquid extraction process at a constant temperature with repeatable solvent evaporation and condensation for a particular period without the loss of solvent. The system is widely used in herbal industries as it is efficient, easy to operate and cost effective (Chua et al., 2016).

Reflux extraction is more efficient than percolation or maceration because it requires less extraction time and solvent.

1.4.2 Microwave Assisted Extraction

Microwaves are electromagnetic energy. This energy is transmitted as waves, which penetrates biomaterials and interact with molecules to generate heat. Microwaves interact selectively with the free water molecules present, leading to localized heating. Thus, temperature increases rapidly near or above the boiling point of water. There is rapid expansion leading to the rupture of their walls. Rapid heating is the key advantage of microwaves. Microwave Assisted Extraction (MAE) has shown to reduce extraction time and minimizing environmental impact by emitting lesser CO_2 and requiring only a small fraction of the energy used in conventional extraction methods. This method considerably enhances the efficiency of the extraction. Moreover, microwave extraction is relatively cost-effective. In addition, the short exposure time to microwaves, preserves most compounds from degradation reactions (Nn, 2015; Silva et al., 2018; Wang et al., 2007).

1.4.3 Maceration

Maceration extraction is a conventional method frequently used in the extraction of bioactive compounds. The procedure consists in stirring the sample in a solvent for a certain period. It is a simple technique, but very often requires long time periods (Albuquerque et al., 2017; Nn, 2015). Maceration is one of the widely used in medicinal plants extractions. It involves soaking plant materials (coarse or powdered) in a stoppered container with a solvent and allowed to stand at room temperature for a period of minimum 3 days with frequent agitation. The processes intend to soften and break the plant's cell wall to release the soluble phytochemicals. After 3 days, the mixture is pressed or strained by filtration.

1.5 Natural health products

Natural health products encompass products with a history of human use that are presented in therapeutic dose forms and contain ingredients such as vitamins

and minerals or other substances derived from nature. Such products are often also referred to under a variety of alternative umbrella terms such as nutraceuticals or alternative, traditional, or complementary medicines. Herbal essences and remedies are examples of types of natural health products that have a long history of traditional use. Natural health products are used mainly to relieve symptoms of minor, self-limiting conditions and to maintain good health and wellbeing. They are presented in a wide variety of dose forms, such as tablets, capsules, tinctures, solutions, creams, lotions, ointments and drops (Shan et al., 2007).

1.6 Tableting

Tableting is an important production process in various industries, such as the food, chemical and pharmaceutical industry. The prediction of mechanical and application-oriented tablet properties such as tensile strength and active pharmaceutical ingredient (API) distribution, based on raw material properties and process parameters is still very difficult and hardly possible. Therefore, the formulation and process development are still mainly empirical. The mechanical and application-oriented tablet properties are primarily determined by the microstructure of the tablet, which is in turn affected by various material, formulation, and process parameters, such as particle size and shape, deformation behavior, composition of the powder mixture and compaction stress (Schomberg et al., 2021).

Pharmaceutical tablets comprise a mixture of powder form components where all of them contribute to the final properties of the product. The tableting process can be divided into three stages. First, the powder is filled into the die cavity. Secondly, a compaction process takes place which involves compression and decompression of the powder bed. Finally, the compacted powder is ejected from the die in the form of a tablet after the required height is obtained. The tablets must be strong enough to sustain any possible load after the compaction process such as film coating, packing, and handling. It is agreed that the properties of the tablet such as mechanical strength or disintegration depend significantly upon the powder behavior during all three stages of the process. Therefore, it is very important to

understand the mechanical behavior of the powder during each stage for successful formulation processing.

To produce quality tablets, many studies have been carried out to investigate the compaction properties (compressibility and compatibility) of different types of excipients such as microcrystalline cellulose and lactose. Compressibility refers to the ability of the powder to change in volume when subjected to pressure whereas compatibility is the ability of powders to convert from small particles into coherent solid dosage and are widely used approaches in studying the compressibility of pharmaceutical powders (Ahmat et al., 2011).

1.7 Bioactive

Medicinal plants are the most important source of folk medicine for most of the the world's population. World health organization (WHO) estimates that 80% of world population relies on herbal medicines for primary health care. Several plant products have been identified through phytochemistry and the extract of their different plant parts are useful in curing various diseases without side effects. Plants contain lot of phytochemicals like alkaloids, tannins, flavonoids, terpenes, fatty acids, amino acids, saponins, glycosides and sterols that have disease preventive properties (Samejo et al., 2013).

1.7.1 Total Phenolic content

Phenol is an aromatic hydrocarbon containing a hydroxyl group (OH) attached to the benzene ring; it is a basic structural unit for a variety of synthetic organic compounds. The chemical structures of phenol and some commonly studied phenolic compounds are given in Figure 6. As an organic substance, phenol is soluble in most organic solvents and it is slightly soluble in water at room temperature, but entirely water soluble above 68°C. Because of the aromatic structure of phenol, it is resistant to natural biodegradation and phenolic compounds have been reported to have high stability due to the difficulty of cleaving the benzene ring (Al-Khalid and El-Naas, 2012).

Phenolic compounds are commonly found in both edible and nonedible plants, and they have been reported to have multiple biological effects, including antioxidant activity. Crude extracts of fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers, and consumers as the trend of the future is moving toward functional food with specific health effects.

Phenolic compounds are potential sources of antioxidant compounds have been searched in several types of plant materials such as vegetables, fruits, leaves, oilseeds, cereal crops, barks and roots, spices and herbs, and crude plant drugs. Flavonoids and other plant phenolics, such as phenolic acids, stilbenes, tannins, lignans, and lignin, are especially common in leaves, flowering tissues, and woody parts such as stems. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Kähkönen et al., 1999).

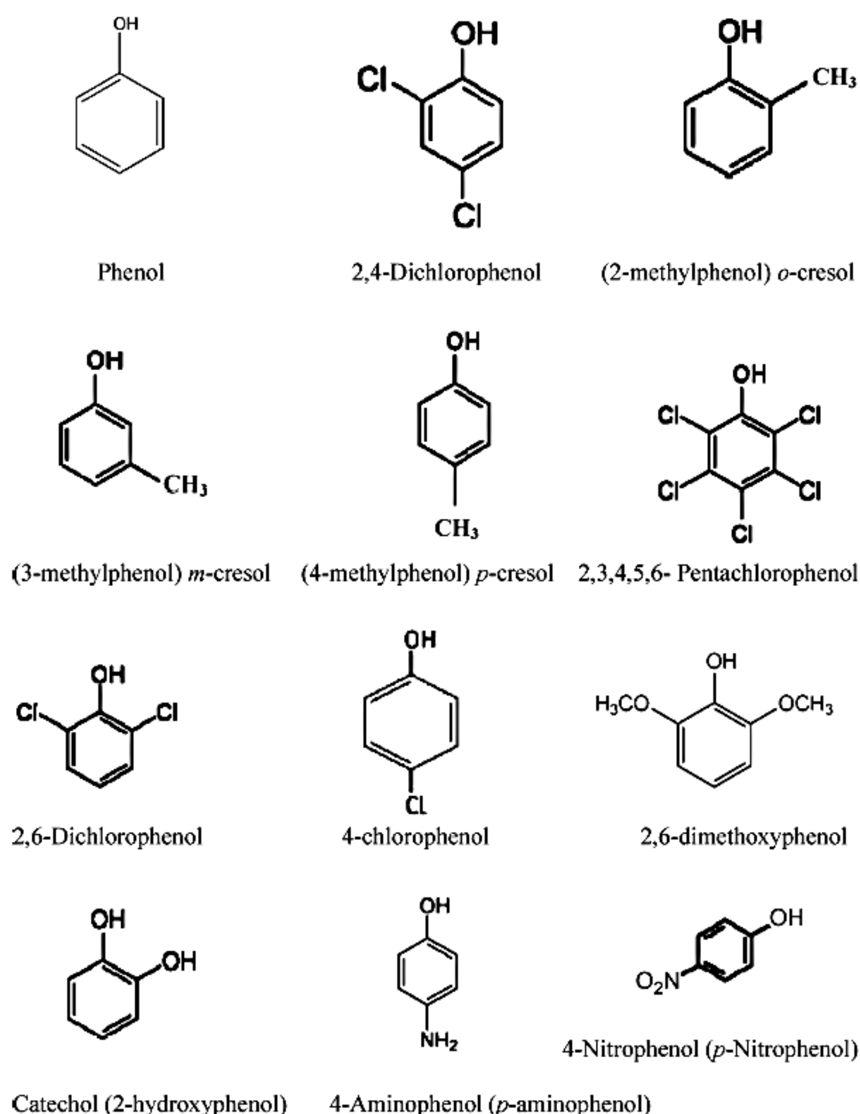

Figure 6 Chemical structure of common phenolic compounds.

The total phenolic content in the extracts was determined using Folin-Ciocalteu reagent method. The extracts 0.4 mL at a concentration of 1000 mg/L was mixed with 2 mL of Folin-Ciocalteu reagent 10% v/v. Then, 1.6 mL of 7.5% w/v Na_2CO_3 solution was thoroughly mixed. The mixture was left for 60 min and absorbance was measured at 765 nm using a UV–vis Spectrophotometer. Thereafter, the concentration of TPC in the plant extract was calculated from the gallic acid standard calibration curve ranging from 0 to 100 mg/L (Alara et al., 2018).

1.7.2 Antioxidant

Oxidative stress is a commonly used term to describe a disturbance in the balance between the production of free radicals that, upon accumulation, lead to the cellular breakdown of critical macromolecules and the status of *in vivo* antioxidant defense mechanisms that are required to combat the reactive nature of these molecules. Reactive oxygen species (ROS), for example, hydroxyl and superoxide anion radicals and hydrogen peroxide, are inactivated by enzymatic antioxidant systems, or peroxidases (e.g., salivary peroxidases and micro-peroxidases). The diet has a major role in contributing to both the source of free radicals, as well as combating the reactive nature of free radicals. The latter is accomplished by supplying specific substrates (e.g., glutathione) for PODs or, alternatively, by directly inactivating them. In general, different assays have been used to assess and compare the antioxidant activity according to the presence of a specific ROS in question (Liang and Kitts, 2014).

Antioxidant compounds play a vital role in human physiology. They prevent the oxidation of biomolecules by scavenging free radicals produced during physiochemical processes and/or as a result of several pathological states. A balance between the reactive oxygen species (free radicals) and antioxidants is essential for proper physiological conditions. Excessive free radicals cause oxidative stress which can lead to several human diseases. Therefore, synthesis of the effective antioxidants is crucial in managing the oxidative stress. Biotransformation has evolved as an effective technique for the production of structurally diverse molecules with a wide range of biological activities. This methodology surpasses the conventional chemical synthesis due to the fact that enzymes, being specific in nature, catalyze reactions affording products with excellent regio and stereoselectivities. Structural transformation of various classes of compounds such as alkaloids, steroids, flavonoids, and terpenes has been carried out through this technique. Several bioactive molecules, especially those having antioxidant potential have also been synthesized by using different biotransformation techniques and enzymes. Hydroxylated, glycosylated, and acylated derivatives of phenols, flavonoids, cinnamates, and other molecules have proven abilities as potential antioxidants. A

critical review of the biotransformation of these compounds into potent antioxidant metabolites is presented here. Antioxidant molecules scavenge the reactive oxygen species (ROS) and free radicals in the biological system and thus can prevent the diseases/biological complications prevailing because of free radicals, by preventing the adverse oxidation of proteins, lipids, and DNA in the human body. The ROS are usually released in the cells through a process known as the respiratory burst.

Aromatic compounds are abundant in nature and their biosynthesis is well established. Aromatic compounds belonging to the sub-classes; lignans, coumarins, flavones, flavonols, and cinnamic acid derivatives have been amply isolated from plants and have proven abilities of scavenging free radicals. Figure 7 shows a proposed mechanism of how these compounds hunt the free radicals. Following is an insight into how several classes of these aromatic compounds has been structurally modified, using biocatalytic techniques, into compounds having more significant antioxidant potential (Zafar et al., 2016).

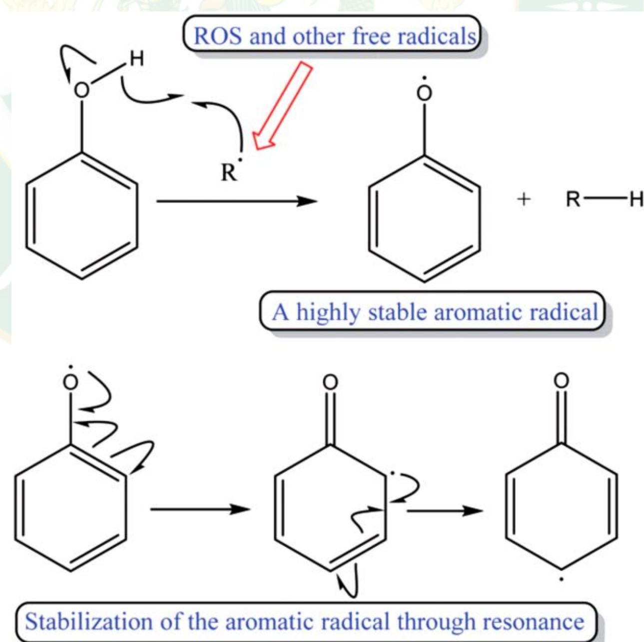

Figure 7 Free radical scavenging mechanism in aromatic compounds (Zafar et al., 2016).

The presence of antioxidant compounds to the disappearance of radical chromogens, and the activity in doing so is calculated from the disappearance of color or absorbance readings generated from a specific UV spectra. Examples of widely used chromogens that have received considerable use in chemical methods of antioxidant detection are the stable free radicals, DPPH and ABTS$^{\bullet +}$, in the DPPH and ABTS assay, respectively. In the DPPH assay, an odd electron displays a strong absorption band at a wavelength of 519 nm, which loses absorption once the odd electron is paired off by a hydrogen or electron-donating antioxidant (Figure 8). Other chemical assays use unstable free radicals, such as peroxyl radical (ROO^{-}), superoxide radical anion (O^{2-}) and hydroxyl radical (HO^{-}) as examples of ROS, generated at fixed rates over time in chemical reactions that attempt to mimic a physiological mechanism in vivo. In these examples, the oxygen radical antioxidant capacity (ORAC), hydroxyl radical scavenging assay and superoxide radical scavenging capacity assay have all been used, respectively (Alara et al., 2018; Darvishi et al., 2019; Liang and Kitts, 2014).

Figure 8 Reaction mechanism of DPPH with antioxidant (Ji et al., 2020)

1.7.3 Total anthocyanin

Anthocyanin pigments are important to food quality because of their contribution to color and appearance. There is increasing interest in the anthocyanin

content of foods and nutraceuticals because of possible health benefits. Anthocyanin pigment content can also be a useful criterion in quality control and purchase specifications of fruit juices, nutraceuticals, and natural colorants. An AOAC method is needed to rapidly and precisely determine total monomeric anthocyanin content. Our goal was to design, organize, and conduct a collaborative study to validate the pH differential method as an AOAC method. Anthocyanins are responsible for the red, purple, and blue hues present in fruits, vegetables, and grains. There are 6 common anthocyanidins (pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin), whose structures can vary by glycosidic substitution at the 3 and 5 positions. Additional variations occur by acylation of the sugar groups with organic acids. The pH differential method has been used extensively by food technologists and horticulturists to assess the quality of fresh and processed fruits and vegetables. The method can be used for the determination of total monomeric anthocyanin content, based on the structural change of the anthocyanin chromophore between pH 1.0 and 4.5 (Figure 9).

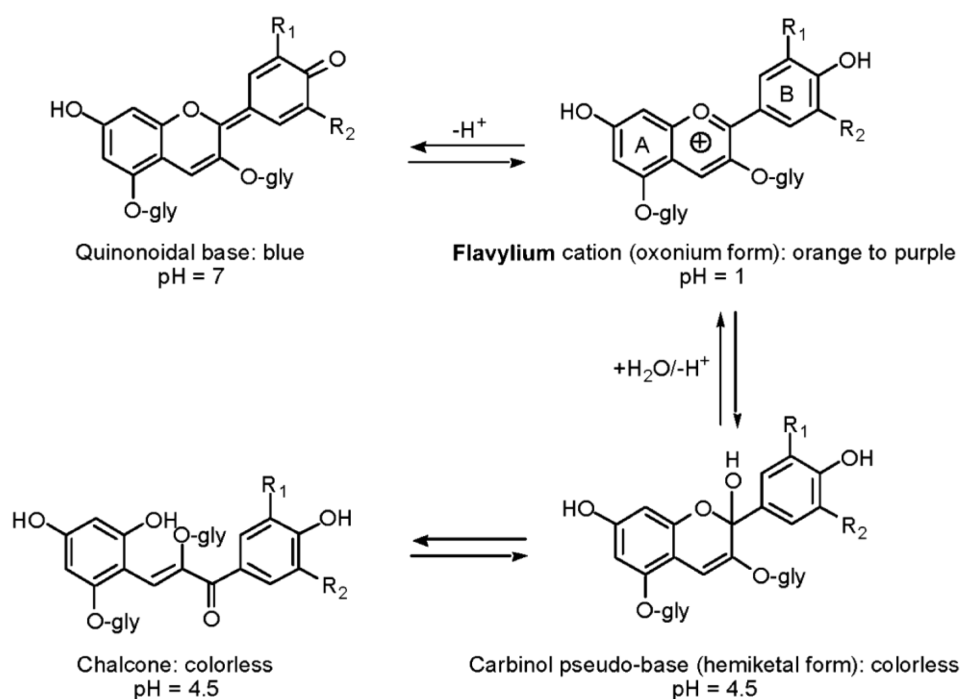

Figure 9 Predominant anthocyanin structural forms present at different pH levels.

Monomeric anthocyanins undergo a reversible structural transformation as a function of pH (colored oxonium form at pH 1.0 and colorless hemiketal form at pH 4.5, Figure 9). Thus, the difference in absorbance at the λvis-max (520 nm) of the pigment is proportional to the concentration of pigment. Figure 10 shows the spectra of huckleberry anthocyanins in buffers at pH 1.0 and 4.5. Degraded anthocyanins in the polymeric form are resistant to color change with change in pH. Therefore, polymerized anthocyanin pigments are not measured by this method because they absorb both at pH 4.5 and 1.0. Absorbance should be measured at the λvis-max of the pigment solution, and the pigment content should be calculated by using the molecular weight (MW) (Lee et al., 2005).

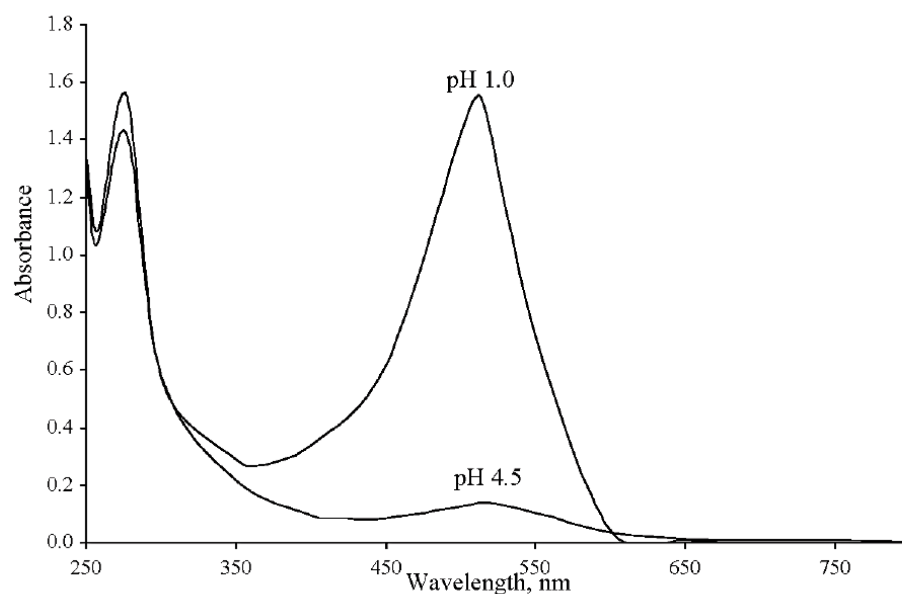

Figure 10 Spectral characteristics of anthocyanin in buffers at pH 1.0 and 4.5 (Lee et al., 2005).

Total anthocyanins can estimated by a pH differential method (Giusti and Wrolstad, 2001). Absorbance was measured at 510 nm and at 700 nm. Samples were diluted with buffer at pH 1.0 (0.025 mM KCl) and left at rest for 15 min before measurements. Then diluted sample with the buffer pH 4.5 (0.4 mM CH_3COONa) were ready for measurement after 15 min. The pure buffer solution was used as reference sample in the spectrophotometer. The absorbance shift was calculated as:

$$A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5} \quad (1)$$

$$\text{Total anthocyanin} = \frac{A \times Mw \times DF \times 1000}{\varepsilon \times l} \quad (2)$$

When Mw is 449.2 $g.mol^{-1}$ for cyaniding-3-glucoside

ε is 26,000 molar extinction for cyanidin-3-glucoside

l is path length 1 cm

1.8 Research Objectives

1.8.1 To prepare the butterfly pea extract using different extraction methods.

1.8.2 To formulate supplementary products containing the butterfly pea extract.

1.8.3 To analyze the bioactivities of butterfly pea extract and supplementary products.

1.9 Expectation

1.9.1 The best extraction method for preparing the butterfly pea extract and its bioactivities.

1.9.2 The new formulation of supplementary product containing butterfly pea extract.

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CHAPTER 2
LITERATURE REVIEW

2.1 Butterfly pea

Nowadays, the medicinal plants are used as alternate herbal medicines or supplementary products to improve health which are observed from an increase in the evidence reports on the benefits of medicinal plants (Britton et al., 2006; Gratus et al., 2009). Butterfly pea (BP) or called blue pea is a common name of *C. ternatea* Linn. which is a plant species in the *Fabaceae* family. This plant is grown and distributed in Asia and Africa. The phytochemical screening of BP from different plant parts; leaf, flower, root, seed, has been reported and showed that BP extract composes of various secondary metabolites such as tannins, anthocyanins, flavonoid glycosides, triterpenoids, saponins, tannins, phenols, anthraquinone, and cardiac glycosides. Chemical compounds have been identified including Kaempferol, Quercetin, Myriceti, Taxaxerol, Tannic acid, 3-monoglucoside, β-Sitosterol, Delphinidin-3,5-diglucoside, Hexacosanol, *p*-hydroxycinnamic acid, Malvidin-3β-glucoside etc. Additionally, BP has been gained more interest because of its biological and pharmacological activities. Various pharmacological actions of *C. ternatea* have been reported such as memory enhancement, enhancement of acetylcholine content (Rai et al., 2001; Taranalli and Cheeramkuzhy, 2000), anti-stress, antidepressant, antioxidant, anti-inflammatory (Devi et al., 2003), anticancer, anti-diabetic (Sharma and Majumder, 1990), hypolipidemic, tranquilizing effects (Solanki and Jain, 2010), analgesic, and antipyretic (Gollen et al., 2018). The reported activities of *C. ternatea* make it a potential source of drug molecules for treatment of various ailments. Interestingly, *C. ternatea* has been used since long as memory enhancing and anxiolytic agent in Ayurvedic system of medicine. Therefore, it can be evaluated clinically for the efficacy and safety of *C. ternatea* extracts in various types of dementia. Additionally, *C. ternatea* extract and drug (lisinopril) have been reported the prevention of the L-NAME-induced development of hypertension which are associated with the alleviation of cardiovascular dysfunction in rats. These effects

were associated with the suppression of renin–angiotensin system (RAS) activation, oxidative stress, and inflammation (Maneesai et al., 2021). Additionally, (Raghu et al., 2017). has been reported the age dependent neuroprotective effects of one of Indian traditional medicinal systems in Ayurveda (medhya rasayana) prepared from *C. ternatea* Linn. in stress induced rat brain. These findings suggest autophagy directed pathways by medhya rasayana prepared from *C. ternatea* protects the brain cells from stress induced injury and also significantly increases episodic memory in rats (Raghu et al., 2017). In Thailand, extracts of *C. ternatea* flowers are used as an ingredient of cosmetics, beverage and food industries because the chemical composition of the *C. ternatea* flowers suggest that they may have antioxidant activity. The aqueous and ethanol extracts of *C. ternatea* have been evalusted and found that the aqueous extracts of *C. ternatea* were shown to have stronger antioxidant activity than ethanol extracts (Kamkaen and Wilkinson, 2009). For anti-inflammatory and analgesic activity of *C. ternatea*, petroleum ether extract from the flowers of *C. ternatea* has been studied in animal model using the dose level of extract (200 and 400 mg/kg body weight). The results showed that this extract exhibited significant anti- inflammatory activity at both 200 and 400 mg/kg body weight. In addition, the methanol extract of *C. ternatea* represented a significant antipyretic activity (Lijon, 2017). Anti-allergy and anti-tussive activity of ethanolic extract of *C. ternatea* flowers in experimental animals were evaluated and found that this ethanolic extract could be considered as a potential therapeutic alternative in the management of allergy-induced asthma because this extract at 400 mg/kg ameliorates the sulfur dioxide- and citric acid-induced cough in experimental animals. Additionally, this extract at 400 mg/kg attenuates the inflammation in carrageenan and acetic acid induced inflammation in rats (Singh et al., 2018). Microencapsulation of phenolic extracts of *C. ternatea* petal flower extract through extrusion method of alginate with calcium chloride ($CaCl_2$) was studied to improve the stability as well as the biological activity of polyphenols in plant extracts. The results demonstrated that the microencapsulation of *C. ternatea* significantly retains higher polyphenols and improves antioxidant capacity, pancreatic **α**-amylase inhibitory activity, and bile acid

binding after the gastrointestinal digestion (Pasukamonset et al., 2016). Moreover, there have been reported the protection of *C. ternatea* flower petal extract against *in vitro* 2,2′ -azobis-2-methyl-propanimidamide dihydrochloride (AAPH) induced hemolysis and oxidative damage in canine erythrocytes. The experimental evidence indicates that *C. ternatea* flower petal is a rich source of anthocyanins that manifest DPPH and peroxyl radical scavenging activity. *C. ternatea* flower petal can effectively protect AAPH-induced hemolysis and oxidative damage oxidation in erythrocytes (Phrueksanan et al., 2014)

2.2 Anthocyanins

Anthocyanins are pigments which perform the attractive purple, red or blue colors of many fruits, flowers, and vegetables. Water soluble anthocyanins have received great interests in research because they have antioxidant properties and health benefits such as enhancement of sight acuteness, antioxidant capacity, controlling type II diabetes, reducing coronary heart disease and prevention of cancer (Askar et al., 2015b). However, anthocyanin is relatively unstable and often undergoes degradation during processing and storage. In general, anthocyanins can be divided into three groups based on the types of aglycones of anthocyanins. Three groups of anthocyanins compose of cyanidin glycoside, peonidin glycoside and pelargonidin glycoside. Fruits or plants which contain higher cyanidin contents are more likely to produce an anti-inflammatory effect. This is consistent with the hypothesis that one or more stable phenolic acid metabolites contribute to the anti-inflammatory effects of anthocyanin-rich fruits (Fang, 2015). Recently, identification of anthocyanin compounds in butterfly pea flowers extract by ultra-performance liquid chromatography/ultraviolet coupled to mass spectrometry have been reported. The results revealed that five anthocyanins were identified in the butterfly pea flower extract. These were delphinidin-3-(6″-*p*-coumaroyl)-rutinoside, cyanidin 3-(6″-p-coumaroyl)-rutinoside, delphinidin-3-(*p*-coumaroyl) glucose in both *cis*- and *trans*-isomers, cyanidin-3-(*p*-coumaroyl-glucoside) and delphinidin-3-pyranoside. Among all compounds, it was determined that cyanidin-3-(*p*-coumaroyl-glucoside) was the most abundant anthocyanin, followed by cyanidin 3-(6″-*p*-coumaroyl)-rutinoside,

delphinidin-3-(*p*-coumaroyl-glucoside), delphinidin-3-(6″-*p*-coumaroyl)-rutinoside and delphinidin-3-pyranoside, respectively (Thuy et al., 2021). In addition, anthocyanin in butterfly pea flower extract may be increased by co-pigmentation with catechin. An increase in the co-pigment ratio of catechin: anthocyanins by weight significantly retarded the degradation anthocyanins at high temperature such as at 90° C. In addition, co-pigmentation also intensified the color of butterfly pea extract. The highest anthocyanin stability was obtained at co-pigment ratio of 100:1 w/w (Charurungsipong et al., 2020).

2.3 Andrographis paniculata

A. paniculata, known as "king of bitters", which belongs to the Acanthaceae family. *A. paniculata* is currently used in traditional medicine to treat several infectious sicknesses such as common cold, diarrhea, and fever. A major bioactive component of *A. paniculata* is a diterpene lactone called andrographolide. Nowadays, *A. paniculate* extract and andrographolide have been reported the effective of anti-SARS-CoV-2 activity by using a legitimate model of infected human lung epithelial cell, Calu-3. The results showed that postinfection treatment of *A. paniculata* and andrographolide in SARS-CoV-2-infected Calu-3 cells significantly inhibited the production of infectious virions with an IC_{50} of 0.036 μg/mL and 0.034 μM, respectively, as determined by the plaque assay. Moreover, cytotoxic profiles of *A. paniculate* extract and andrographolide over five major human organs, including lung, brain, liver, kidney, and intestine, were achieved by the five cell line representatives, and found both *A. paniculate* extract and andrographolide have been demonstrated the favorable cytotoxicity profiles (Sa-ngiamsuntorn et al., 2021). The biosynthetic route to andrographolide was studied using ^{13}C NMR spectra, including [1-^{13}C]acetate, [2-^{13}C] acetate and [1,6-$^{13}C_2$]glucose and suggested that deoxyxylulose pathway (DXP) is the major biosynthetic pathway to this diterpene because the peak enrichment of eight carbon atoms were presented in the ^{13}C NMR spectra of andrographolide (Sa-ngiamsuntorn et al., 2021). In 2018 Sin Yee Lee et.al. have been reported the improving aqueous solubility of andrographolide through particle engineering using Solution Enhanced Dispersion by Supercritical Fluids (SEDS)

approach and can be concluded that *A. paniculata* powder precipitated from CO_2-acetone system at 150 bar, 40 °C was found to be large, irregularly shaped, less crystalline with the highest andrographolide aqueous solubility (Lee et al., 2018b). In 2017, Aishwarya Balap et.al. have been reported the pharmacokinetic and pharmacodynamic (anti-arthritic) herb-drug interactions of *A. paniculata* Nees extract and pure andrographolide with naproxen after oral co-administration in wistar rats. The results suggested that naproxen (10 mg/kg) alone and naproxen+pure andrographolide (10+60 mg/kg) groups exhibited significant synergistic anti-arthritic activity (Balap et al., 2017). Additionally, a number of databases were used to search for the cytotoxic/anticancer effects of andrographolide in pre-clinical and clinical studies. These systematic reviews are concluded that andrographolide a diterpene lactone from *A. paniculata* can be one of the potential agents in the treatment of cancer because andrographolide was found to exhibit the cytotoxic/anticancer effects on almost all types of cell lines with the mechanisms involving anti-inflammatory, immune system mediated effects, oxidative stress, cell cycle arrest etc. (Islam et al., 2018). In 2018 Yandi Syukri et.al have been prepared and characterized the loaded Self-Nano Emulsifying Drug Delivery System (SNEDDS) to enhance the oral dissolution and bioavailability of andrographolide isolated from *A. paniculate* using the formulation containing Capryol-90, Tween 20 and PEG 400 (20: 70: 10 and 20: 60: 20 w/w). The results suggested that SNEDDS formulation could be improved the dissolution and the bioavailability of andrographolide (Syukri et al., 2018).

2.4 Extraction

The selection of method for extracting the herb and extracts should be evaluated depending on the suitability of samples and objectives needed to be achieved. For *C. ternatea*, it has been reported that both conventional and non-conventional extraction methods can be used to prepare the *C. ternatea* extracts from each part of *C. ternatea*. Most studies on *C. ternatea* extracts have been shown the utilization of *C. ternatea* flowers in various features in extractions such as air/oven-dried, fresh flowers, dried flowers, or grounded/powdered flowers.

Conventional extraction methods usually employ the different solvents both polar and non-polar solvents with heat and/or mixing such as soxhlet extraction, maceration and hydrodistillation etc. The effective of conventional extraction methods are costly but they require long extraction time and must separate the solvents after finishing the extraction time. Extraction studies on *C. ternatea* flower have been reported the utilizing various solvent mixtures. The conventional extraction methods usually involve the use of aqueous solvent mixtures with alcohol (ethanol or methanol) with heat and/or mixing to prepare *C. ternatea* extracts. Moreover, several studies investigated on the optimal solvent and/or extraction parameters. Additionally, the phytochemical contents of the *C. ternatea* flower were correlated with polarity of solvents and depending on polarity of compounds. The hydrophilic (methanol) and hydrophobic (ethyl acetate and hexane) extractions contained different phytochemical contents. The anthocyanins, kaempferol and quercetin glycosides can be found in hydrophilic extraction while fatty acids, phytosterols and tocopherols were found in hydrophobic extraction. In general, phytochemical contents have been isolated from *C. ternatea* flower such as anthocyanins, phenolics, flavonoids, fatty acids, phytosterols and α-tocopherol using different solvents for example 40-95% ethanol, methanol, water, ethyl acetate : hexane (1:1 v/v) etc. (Jeyaraj et al., 2021). However, many non-conventional extraction methods are beneficial in determining extraction efficiency of various phytochemicals of *C. ternatea* flower for example ultrasound assisted extraction, pulsed-electric field, pressurized liquid and microwave assisted extraction. These non-conventional extraction methods are called green extraction techniques which are more effective than the conventional extraction method for extraction of phenolics and anthocyanins. Other studies have revealed that non-conventional extraction methods were more effective than the conventional extraction method for extraction of various phytochemicals which required shorter extraction time and were also useful in preventing oxidation of compounds (Caldas et al., 2018).

CHAPTER 3

EXPERIMENTAL

3.1 Material, Chemicals and instruments

3.1.1 Material and chemical

The material and chemical for used in this research show in table 2

Table 2 The material and chemical

Number	Material/Chemical	Grade	Company	County
1	Butterfly pea flower	-	-	Thailand
2	*Andrographis paniculate*	-	-	Thailand
3	Ethanol	95%	RCl Labscan	-
4	Gallic acid	AR	Merck	Germany
5	Distilled water	-	-	Thailand
6	Sodium carbonate	AR	QReC	New Zealand
7	Folin-Ciocalteu, VWR	AR	VWR	-
8	DPPH (2,2- diphenyl-1-picrylhdrazyl)	AR	ALDRICH	Germany
9	Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chromane-2-carboxylic acid)	AR	ALDRICH	Germany
10	Potassium chloride	AR	Merck,	Germany
11	Sodium acetate	AR	Merck,	Germany
12	Hydrochloric acid	-	UNIVAR	Canada
13	Andrographolide	AR	Merck,	Germany
14	Acetic acid	AR	Merck,	Germany
15	Acetonitrile	AR	Merck,	Germany

3.1.2 Apparatus and instrument

The apparatus and instrument for used in this research show in table 3

Table 3 The apparatus and instrument

Number	Apparatus / Instrument	Company	Country
1	Round bottom	SCHOTT DURUN	Germany
2	Condenser	-	-
3	Heating mantle	QTOPO	Canada
4	Beaker	PYREX	USA
5	funnel	SCHOTT DURUN	Germany
6	Stand camp	-	-
7	Filter paper	Whatman	-
8	Volumetric flask	SCHOTT DURUN	Germany
9	Measuring cylinder	SCHOTT DURUN	Germany
10	Balance	METTLER	Switzerland
11	Specular	-	-
12	Mortar and pestle	WALLER CHEMICAL	Thailand
13	desiccator	-	-
14	Hydraulic Press	-	-
15	UV-visible	HITACHI	Japan
16	Evaporator	BUCHI	Switzerland
17	Freeze dry	Labconco	USA
18	HPLC	PerkinElmer	USA
19	Micro pipette	NICHIRYO	Japan

3.2 Preparation of butterfly pea extract

In this work, the fresh butterfly pea (BP) flowers were extracted using three different extraction methods including maceration extraction, reflux extraction and microwave assisted extraction, respectively.

3.2.1 Maceration extraction

Fresh flower of butterfly pea (20 g) was placed in a bottom flask and mixed with an extraction solution (ethanol /or distilled water). The extraction was performed at a solid to liquid ratio of 1:10, 1:20 and 1:30 (w/v) in a maceration system for 3-7 day. All the experiments were performed in triplicate. After the maceration, the samples were filtered, and concentrated using a rotary evaporator at approximately 60 °C after evaporation the sample was freeze dried to obtain the BP extract, weighed and stored prior to further analysis (Ben Attia et al., 2018).

3.2.2 Reflux extraction

Fresh flower of butterfly pea (20 g) was placed in a round bottom flask and mixed with an extraction solution (ethanol /or distilled water). The extraction was performed at a solid to liquid ratio of 1:10, 1:20 and 1:30 (w/v) in a reflux system for 1-3 hours. All the experiments were performed in triplicate. After the reflux extraction, the samples were filtered, and concentrated using a rotary evaporator at approximately 60 °C after evaporation the sample was freeze dried to obtain the BP extract, weighed and stored prior to further analysis (Che Sulaiman et al., 2017).

3.2.3 Microwave assisted extraction

Fresh flower of butterfly pea (20 g) was placed in a bottom flask and mixed with an extraction solution (ethanol /or distilled water). The extraction was performed at a solid to liquid ratio of 1:10, 1:20 and 1:30 (w/v), microwave power of 300, 450 and 600 watt for 5-10 mins in a microwave system. All the experiments were performed in triplicate. After the microwave assisted extraction, the samples were filtered, and concentrated using a rotary evaporator at approximately 60 °C after evaporation the sample was freeze dried to obtain the BP extract, weighed and stored prior to further analysis (Ani et al., 2012).

3.2.4 Characterization of BP extract

The powder of BP extract obtain from optimized condition was prepared in the solution to characterized the maximum absorption (λ_{max}) by using UV-Visible and

The powder of BP extract was determined the functional group by using FTIR respectively.

3.3 Preparation of Fah Talai Jone (FTJ) or *A. paniculata* extract

3.3.1 Maceration extraction

was placed in a bottom flask and mixed with an extraction solution (ethanol /or distilled water). The extraction was performed at a solid to liquid ratio of 1:20 (w/v) in a maceration system for 7 day. All the experiments were performed in triplicate. After the maceration, the samples were filtered, and concentrated using a rotary evaporator at approximately 60 °C after evaporation the sample was freeze dried to obtain the FTJ extract, weighed and stored prior to further analysis.

3.4 Tableting supplementary product

The prototype of supplementary natural product was prepared from the FTJ and BP extracts in tableting. Tableting supplementary product in 500 mg per tablet were formulated by varying amount of FTJ extract at 0-30% and BP extract at 30% using lactose as binder. The mixture extracts and binder were mixed by using mortar and tableted with hydraulic press at 5 psi.

3.5 Analysis of total phenolic contents

The total phenolic contents in all extracts and prototype of supplementary natural product were determined using Folin-Ciocalteu reagent method (Alara et al., 2018). The samples (0.4 mL) at a concentration of 1000 ppm was mixed with 2 mL of 10% Folin-Ciocalteu reagent. Then, 1.6 mL of 7.5% Na_2CO_3 solution was thoroughly mixed with the mixture after 5 min. The mixture was left for 30 min. The absorbance of mixture was measured at 765 nm using a UV–vis Spectrophotometer. The concentration of total phenolic contents in the samples were calculated from the gallic acid standard calibration curve (ranging from 0 to 100 mg/L) with equation of line $y=0.0127x + 0.0309$, $R^2=0.9991$ (where y is the absorbance at 765 nm and x is the sample concentration from

the calibration curve), The total phenolic contents of samples were reported as gallic acid equivalent in milligram per gram extract.

3.6 Analysis of antioxidant activity

The antioxidant activities of extracts and prototype of supplementary natural product were verified using DPPH assay (Alara et al., 2018). A 2000 µL of 0.1mM DPPH was added to 200 µL of 1000 ppm of sample. The percent inhibition of sample was calculated from the trolox standard calibration curve (ranging from 0 to 100 mg/L) with equation of line $y = 0.8127x + 0.3934$, $R^2 = 0.9975$. An absorbance of the mixture was recorded at 517 nm after incubation for 30 mins in the dark at room temperature. The ability of the sample to scavenge DPPH radical was calculated as following equation:

$$\text{Percentage of DPPH inhibition capacity} = \frac{(A_{control} - A_{sample}) \times 100}{A_{control}} \quad (3)$$

when $A_{control}$ represents absorbance measure for DPPH solution

A_{sample} represents absorbance of the mixture of extract and DPPH solution.

3.7 Analysis of anthocyanin content

The anthocyanin content of BP extracts and prototype of supplementary natural product were verified using pH differential method (Lee et al., 2019). The 1000 ppm of sample was diluted with 0.025 M of potassium chloride buffer pH 1. The mixture was left for 30 min and absorbance was measured at 540 and 700 nm using a UV–vis Spectrophotometer. The 1000 ppm of sample was diluted with 0.4 M of sodium acetate buffer, pH 4.5 and the mixture was left for 30 min. The absorbance was measured at 540 and 700 nm using a UV–vis Spectrophotometer. The total anthocyanin pigment concentration of sample was calculated using the following equation:

$$\text{Total anthocyanin} = \frac{[(Abs_{540} - Abs_{700})pH_1 - (Abs_{540} - Abs_{700})pH_{4.5}] * MW * DF * 1000}{\varepsilon * 1} \quad (4)$$

MW is the molecular weight of cyaniding-3-glucoside = 449.2 g/mol

DF is the dilution factor (for example, if a 0.2 ml sample is diluted to 3 ml, DF = 15)

ε is the molar absorptivity of cyaniding-3-glucoside = 26,900 molar extinction in L/mol/cm

3.8 Analysis of andrographolide in FTJ extract

A PerkinElmer HPLC (model FLEXAR™ LC Systems A) with photodiode array detector were used for analyzing the andrographolide in FTJ extract. HPLC instrument was used for the chromatographic separation using C 18 column (250 nm x 4.6 nm). Isocratic elution was carried out with 0.5% acetic acid in water and acetonitrile (35:65 v/v of ratio) at a flow rate 1.5 mL/min. The detection was performed with a photodiode array at 223 nm wavelength. The peak areas of andrographolide in FTJ extract were evaluated compare with the peak areas of standard andrographolide.

CHAPTER 4
RESULTS AND DISCUSSION

The purpose of the present work was to apply conventional extraction methods including maceration, reflux and microwave assisted extraction for preparing the butterfly pea extracts (BP) from fresh butterfly pea flowers using two solvents of distilled water and 95% ethanol. The effects of various factors involved the solvent types, extraction time, and solid (material) to liquid (solvent) ratios on the extraction were investigated. Each extract was evaluated the percentage yield and the biological activities such as total phenolic contents, antioxidant activity and total anthocyanin contents. The BP extract was obtained from each extraction methods shown in table 4-11, respectively

4.1 Maceration

4.1.1 The effect of ratio of solid/liquid

The maceration extraction was used to study the factors of solid (butterfly pea flowers) /liquid (solvents) ratios to the percentage yields and biological activities including total phenolic contents, antioxidant activity and total anthocyanin contents. Two solvents (distilled water and 95% ethanol) were chosen for macerating the butterfly pea flowers and performed all experiments for 5 days (Ferioli et al., 2020). The results of percentage yields and various biological activities were reported in table 4.

These results will discuss the different parameters of maceration available for BP extraction from fresh butterfly pea flowers. The main parameters involved (solvent types, ratios of solid/liquid). The highest extraction yields were recorded by distilled water for maceration when using ratios of solid/liquid at 1:20 (w/v). Moreover, the total phenolic contents, antioxidant activity and total anthocyanin contents obtained by maceration had the highest values, and distilled water using ratios of solid/liquid at 1:20 (w/v) was the best solvent for the extraction of BP secondary metabolites. Therefore, maceration was suitable for BP extractions, with distilled water being the best solvents and used the ratios of solid/liquid at 1:20

(w/v) for prepared BP extracts. Subsequently, the effect of ratios of solid/liquid and type of solvents were evaluated and used these results to study the effect of extraction time with BP extraction.

4.1.2 The effect of extraction time

In this study, the solvent types and extraction times applied were factors that significantly determined the efficiency of BP extraction to obtain bioactive compounds or secondary metabolites, showing a different impact on percentage yields and other biological activities studied. The extraction time is one of the parameters play an important role to BP extraction for maceration. In this study, the designed experiments for macerating for 3, 5, and 7 days using two solvents and the ratios of solid/liquid at 1:20 (w/v) were also discussed in detail, providing insight into their biological activities and percentage yields (Ferioli et al., 2020). The results of percentage yields and all biological activities were presented in table 5

In this work, distilled water, and ethanol 95% (v/v) were used as solvents for maceration for 3, 5, and 7 days. The extraction yield and all biological activities raised with increasing of extraction times where were found these results in both solvents. In general, distilled water was not used in maceration because of microbial growth contamination during the processes, which took a relatively long time. Thus, to solve this problem, all macerated flowers were kept in refrigerators at 4 °C. The results revealed that maceration using the ratios of solid/liquid at 1:20 (w/v) with distilled water for 7 days provided the highest yield of BP extract (7.04 % dry weight) and highest contents of total phenolics, anthocyanin and antioxidant activity. The medium yield and biological activities were found when macerating with water for 5 days. These results also suggest that the percentage yields and biological activities in BP extraction for maceration were better extracted by distilled water than ethanol.

From the results of maceration, it can be concluded that the BP extraction yields, and their biological activities were significantly affected by various parameters for example the ratios of solid/liquid, type of solvents, extraction times. The excellent condition for maceration was carried out using distilled water as solvent for

7 days at 4 °C, and the ratios of solid/liquid at 1:20 (w/v) which affording the highest yield and biological activities.

Table 4 The effect of ratio of solid/liquid to the percentage yields, total phenolic contents, antioxidant activity and total anthocyanin contents from maceration.

Solvents	Ratio of solid/liquid (w/v)	Percentage yields (%)	Total phenolic contents (mg GAE/g extract)	Antioxidant activity (mg Trolox/g extract)	Total anthocyanin contents (mg/g extract)
Distilled water	1:10	3.44 ± 0.17	58.96 ± 1.72	56.45 ± 0.15	6.30 ± 0.03
	1:20	5.07 ± 0.21	67.80 ± 0.67	64.80 ± 0.79	7.35 ± 0.03
	1:30	3.83 ± 0.37	65.81 ± 0.71	62.94 ± 0.27	7.35 ± 0.03
Ethanol	1:10	3.12 ± 0.07	47.78 ± 1.16	44.97 ± 0.72	4.95 ± 0.04
	1:20	3.54 ± 0.08	54.73 ± 1.36	48.82 ± 0.68	5.10 ± 0.03
	1:30	3.52 ± 0.27	52.95 ± 0.75	48.15 ± 1.06	5.10 ± 0.01

Table 5 The effect of extraction time to the percentage yields, total phenolic contents, antioxidant activity and total anthocyanin contents from maceration.

Solvents	Extraction time (days)	Percentage yields (%)	Total phenolic contents (mg GAE/g extract)	Antioxidant activity (mg Trolox/g extract)	Total anthocyanin contents (mg/g extract)
Distilled water	3	2.61 ± 0.19	59.72 ± 1.12	56.45 ± 0.15	6.26 ± 0.50
	5	5.07 ± 0.21	67.80 ± 0.67	64.80 ± 0.79	7.35 ± 0.03
	7	7.04 ± 0.92	69.72 ± 0.71	66.94 ± 0.27	8.18 ± 0.38
Ethanol	3	1.99 ± 0.38	48.70 ± 2.52	44.97 ± 0.72	4.93 ± 0.38
	5	3.54 ± 0.08	54.73 ± 1.36	48.82 ± 0.68	5.10 ± 0.03
	7	4.52 ± 0.45	57.97 ± 1.38	51.15 ± 1.06	6.01 ± 0.50

4.2 Reflux extraction

4.2.1 The effect of ratio of solid/liquid

This study investigated the optimization of BP extraction using reflux extraction. The ratios between sample quantity and solvent, as well as type of solvents, are also factors that should be taken into consideration to maximize extraction yield, pharmacological and biological properties. For this experiment, the effect of ratio of solid/liquid to the percentage yield, total phenolic contents, antioxidant activity and total anthocyanin contents from reflux extraction were determined. The optimization of reflux extraction for 1 hour (Alonso-Carrillo et al., 2017) was carried out using the parameters of solvent types (distilled water and ethanol) and ratios of solid/liquid at 1:10-1:30 w/v by keeping the temperature at a minimum level (80 °C). All results were shown in table 6.

As shown in table 6, two type of solvents and three ratios of solid/liquid in the ranges of 1:10-1:30 w/v were investigated. At a constant extraction time for 1 hour, the extraction yield was found to be the highest under condition of ratios of solid/liquid at 1:20 w/v using distilled water as solvent by keeping the temperature at a minimum level (80 °C). Similar results of the highest total phenolic contents, antioxidant activity and anthocyanin contents were observed. It revealed that these biological activities were affected by the ratios of solid/liquid and type of solvent when conducted at the same temperature. These results also suggest that the percentage yields and biological activities in BP extraction using maceration were better extracted by distilled water than ethanol. In addition, the effect of extraction time for reflux extraction was studied by using the ratios of solid/liquid at 1:20 w/v and two type of solvents and investigated the biological activities of all BP extracts.

4.2.2 Effect of extraction time

Optimized conditions of extraction time for the maximum extraction yields, total phenolic contents, antioxidant activity and total anthocyanin were determined when using distilled water and ethanol as solvents. The fixed parameter of solid/liquid ratio at 1:20 w/v and temperature of 80 °C was chosen to study and

varied the extraction time for 1, 2, 3 and 4 hours, respectively. The results of percentage yields, total phenolic contents, antioxidant activity and total anthocyanin contents from reflux extraction were presented in table 7.

The results showed that reflux extraction with both distilled water and ethanol provided the highest yield of BP extracts, total phenolic contents, antioxidant activity and total anthocyanin contents when increasing in extraction time from 1 to 4 hours. The highest yield was approximately 10.66% at a solid/liquid ratio at 1:20 w/v and an extraction time of 3 hours for reflux extraction using distilled water. Moreover, this condition had the highest effect on the total phenolic contents, antioxidant activity and total anthocyanin contents. The extraction time is one of the parameters that plays an important role in the extraction of bioactive compounds. In general, an increasing in extraction time is reported to improve the overall extraction because of the enhancement of mass transfer and diffusivity of solvent into the plants (Anisa and Morad, 2014). Moreover, the type of solvents plays a role on the process of extraction of the bioactive compounds from plant material via diffusion of bioactive compounds through the cell wall. From the results, it indicated that distilled water was the best solvent for reflux extraction.

From the results of reflux extraction, it can be concluded that the BP extraction yields, and their biological activities were significantly affected by various factors such as the ratios of solid/liquid, type of solvents, and extraction times. The excellent condition for reflux extraction was performed using distilled water as solvent for 3 hours at 80 °C, and the ratios of solid/liquid at 1:20 (w/v) which affording the highest yield of BP extract and biological activities.

Table 6 The effect of ratio of solid/liquid to the percentage yields, total phenolic contents, antioxidant activity and total anthocyanin contents from reflux extraction.

Solvents	Ratio of solid /liquid (w/v)	Percentage yields (%)	Total phenolic contents (mg GAE/g extract)	Antioxidant activity (mg Trolox/g extract)	Total anthocyanin contents (mg/g extract)
Distilled water	1:10	7.73 ± 0.98	56.20 ± 1.47	47.67 ± 0.15	5.93 ± 1.01
	1:20	8.45 ± 0.91	61.84 ± 1.92	56.05 ± 0.15	6.76 ± 0.25
	1:30	8.12 ± 1.08	58.30 ± 1.03	51.49 ± 0.13	6.26 ± 0.66
Ethanol	1:10	3.16 ± 0.90	47.28 ± 1.02	45.63 ± 0.23	4.76 ± 0.50
	1:20	4.67 ± 0.33	49.35 ± 0.73	47.71 ± 0.72	5.43 ± 0.30
	1:30	3.98 ± 0.42	48.59 ± 1.64	45.97 ± 0.91	4.93 ± 0.52

Table 7 The effect of extraction time to the percentage yields, total phenolic contents, antioxidant activity and total anthocyanin contents from reflux extraction.

Solvents	Extraction time (hours)	Percentage yields (%)	Total phenolic contents (mg GAE/g extract)	Antioxidant activity (mg Trolox/g extract)	Total anthocyanin contents (mg/g extract)
Distilled water	1	8.45 ± 0.91	61.84 ± 1.92	56.05 ± 0.15	6.76 ± 0.25
	2	10.10 ± 0.21	63.66 ± 0.69	61.78 ± 0.79	7.35 ± 0.63
	3	10.66 ± 0.42	64.91 ± 0.32	63.90 ± 1.29	7.60 ± 1.04
	4	10.21± 0.65	62.02± 0.42	60.56± 0.42	7.11± 0.42
Ethanol	1	4.67 ± 0.33	49.35 ± 0.73	47.71 ± 0.72	5.43 ± 0.30
	2	6.16 ± 0.60	52.21 ± 0.49	50.75 ± 0.68	5.76 ± 0.90
	3	6.81 ± 0.19	54.44 ± 0.18	53.04 ± 1.06	6.10 ± 0.38
	4	6.01 ± 0.19	51.64 ± 0.18	50.95 ± 1.06	5.82 ± 0.38

4.3 Microwave assisted extraction

The process efficiency of microwave assisted extraction depends on many factors such as extraction time, extraction temperature, solvent composition, and solvent to sample ratio. In this work, BP extracts were performed using microwave assisted extraction. Three parameters namely solid/liquid ratio, microwave power, and extraction time were investigated using distilled water or ethanol as solvent.

4.3.1 Effect of ratio of solid/liquid

The focus of the current study was to optimize an analytical extraction for microwave assisted extraction to determine the percentage yield of BP extracts and their biological activities. In this topic, the effect of ratios of solid/liquid (1:10 to 1:30 w/v) was evaluated using distilled water and ethanol as solvent and fixed the extraction time (5 mins) and microwave power at 450 W. The results of yields and all biological activities were shown in table 8.

The optimum extraction yields for the studied active compounds in BP can be achieved by applying 5 mins of extraction time and microwave power at 450 W using distilled water as the extraction solvent with a 1:20 solid to liquid ratio. Additionally, the higher solid to liquid ratio should allow for increasing efficiency of extraction methods because of diffusion rate and it exhibited the highest total phenolic contents, antioxidant activity and total anthocyanin contents when using distilled water as solvent for BP extraction. It can be concluded that the solubility of the compounds was affected by the interactions of the compounds with the extraction solvent. The increase of extraction yields with the increase of solvent to solid ratio is consistent with mass transfer principles. Smaller volumes of solvent can lead to incomplete target extraction while larger volumes can make the extraction procedure becomes complex. Therefore, suitable solvent to solid ratio is preferred to achieve higher extraction yields (Mohamad et al., 2012). From this result, the ratio of solid to solvent at 1:20 w/v was chosen for further study in the factor of microwave power.

4.3.2 Effect of microwave power

To achieve an efficient microwave assisted extraction for BP extraction, microwave powers (300, 450 and 600 W) were performed by studying in two solvents of distilled water and ethanol. The extraction yields and various biological activities were evaluated by running extractions at the optimum microwave assisted extraction condition for 5 min and 1:20 solid to liquid ratio. The results were presented in table 9.

One of the most important parameters in the extraction of active compounds from plant is the selection of the most appropriate solvent. In general, Temperature is a factor that is interrelated with microwave power that controls the quantity of energy converted to heat in the dielectric material (Lovrić et al., 2017). For this experiments, distilled water was the best solvent for preparing the BP extract using microwave power at 450 W for 5 min because it gave the highest yield, and all investigated biological activities. High microwave power at 600 W which referred to high temperature was not selected properly because it can also lead to degradation and thus decrease the extraction yield and biological activities which found this effect when using distilled water. Moreover, the effect of extraction time for microwave assisted extraction was studied by using the ratios of solid/liquid at 1:20 w/v, microwave power at 450 W and two type of solvents and investigated the biological activities of all BP extracts.

4.3.3 Effect of extraction time when using two solvents

Selecting a proper extraction time is very important to enable completion of the extraction but also preservation and stability of phytochemicals in plants. The stability of compounds at high temperatures can be related to the short time of extraction and may be affected to the amounts of biological activities. In this work, the effect of extraction time was studied for microwave assisted extraction. The percentage yield, total phenolic contents, antioxidant activity and total anthocyanin contents of BP extract in two type of solvents were observed using various extraction times for 5, 10 and 15 min and fixed the ratios of solid/liquid at 1:20 w/v, and microwave power at 450 W. The results were presented in table 10.

In this study, the extraction time was set to 5, 10 and 15 min for microwave assisted extraction. The time of extraction had influence on percentage yield and the biological activities. The highest amounts of total phenolic contents, antioxidant activity and total anthocyanin contents were obtained in the 10 min for distilled water and 15 min for ethanol. However, the percentage yields and other biological activities when using distilled water as solvent for BP extraction gave higher value than using ethanol as solvent. On the other hand, extending the extraction time from 10 to 15 min did not seem to have a significant effect on the amounts of yields and all biological activities when using distilled water as solvent. Thus, shorter extraction time is favorable, as it reduces the risk of decomposition and oxidation of active compounds, other phytochemicals, and energy consumption (Tatke and Jaiswal, 2011).

This research was undertaken to investigate the influence of extraction parameters during three solvent extraction methods including maceration, reflux extraction and microwave-assisted extraction on the percentage yields as well as to evaluate the total phenolic contents, antioxidant activity, and total anthocyanin contents of fresh BP flowers. The optimization of each extraction method was summarized in table 11. One of the extraction methods will be chosen to prepare the BP extract and formulating the supplementary product in tableting with herb extract.

From the results in table 11, the obtained results showed that distilled water was more appropriate solvent for extraction of active compounds than ethanol for maceration at the ratio of solid/solvent 1:20 w/v because the amount of total phenolic contents, antioxidant activity, and total anthocyanin contents of BP extracts were higher in this extraction method. Although, BP extract which prepared using microwave assisted extract and distilled water as solvent gave the lowest extraction time and higher yield, all biological activities of BP extract from this extraction technique were decreased due to the degradation of compounds. These results can be suggested that bioactive substances can decompose by temperature, since most bioactive compounds are sensitive to elevated temperature, keeping them for a

longer period would lead to the thermal decomposition of the bioactive compounds (Mohamad et al., 2012).

Table 8 The effect of ratio of solid/liquid to the percentage yields, total phenolic contents, antioxidant activity and total anthocyanin contents from microwave assisted extraction.

Solvents	Ratio of solid/liquid (w/v)	Percentage yields (%)	Total phenolic contents (mg GAE/g extract)	Antioxidant activity (mg Trolox/g extract)	Total anthocyanin contents (mg/g extract)
Distilled water	1:10	7.74 ± 0.14	60.90 ± 1.26	56.98 ± 1.22	4.68 ± 0.39
	1:20	8.34 ± 1.09	64.99 ± 0.70	63.28 ± 0.43	5.18 ± 0.29
	1:30	8.28 ± 0.27	64.08 ± 0.93	62.91 ± 0.31	4.76 ± 1.09
Ethanol	1:10	2.05 ± 0.56	47.15 ± 1.89	44.29 ± 0.38	3.76 ± 0.66
	1:20	5.73 ± 0.07	48.72 ± 1.95	46.46 ± 0.20	4.51 ± 0.75
	1:30	5.01 ± 1.43	48.22 ± 0.42	46.82 ± 1.32	4.43 ± 0.15

Table 9 The effect of microwave power to the percentage yields, total phenolic contents, antioxidant activity and total anthocyanin contents from microwave assisted extraction.

Solvents	Power (W)	Percentage yields (%)	Total phenolic contents (mg GAE/g extract)	Antioxidant activity (mg Trolox /g extract)	Total anthocyanin contents (mg/g extract)
Distilled water	300	6.93 ± 1.10	59.04 ± 2.10	56.73 ± 1.19	3.41 ± 1.84
	450	8.28 ± 0.27	64.08 ± 0.94	62.91 ± 0.31	5.18 ± 0.29
	600	8.12 ± 0.94	63.26 ± 1.85	61.73 ± 0.50	4.77 ± 0.24
Ethanol	300	3.94 ± 1.54	47.17 ± 0.73	43.64 ± 0.16	2.01 ± 1.13
	450	5.73 ± 0.07	48.72 ± 0.49	46.46 ± 0.20	4.51 ± 0.75
	600	5.78 ± 0.19	48.93 ± 0.16	46.18 ± 0.59	4.34 ± 1.01

Table 10 The effect of extraction time (5, 10, 15 min) to the percentage yields, total phenolic contents, antioxidant activity and total anthocyanin contents from microwave assisted extraction.

Solvents	Time (min)	Percentage yields (%)	Total phenolic contents (mg GAE/g extract)	Antioxidant activity (mg Trolox /g extract)	Total anthocyanin contents (mg/g extract)
Distilled water	5	8.28 ± 0.27	64.08 ± 0.94	62.91 ± 0.31	5.18 ± 0.29
	10	11.57 ± 1.26	65.70 ± 0.52	64.80 ± 0.72	6.35 ± 0.95
	15	10.00 ± 0.94	63.87 ± 0.52	62.66 ± 0.33	5.93 ± 0.77
Ethanol	5	5.73 ± 0.07	48.72 ± 0.49	46.46 ± 0.20	4.51 ± 0.75
	10	7.01 ± 0.07	53.55 ± 0.79	50.81 ± 1.06	4.59 ± 0.38
	15	7.20 ± 0.29	53.68 ± 0.91	50.93 ± 0.42	4.93 ± 0.38

Table 11 The optimization of each extraction method effect on the percentage yields, total phenolic contents, antioxidant activity, and total anthocyanin contents of fresh BP flowers.

Methods/ Solvents	Ratio solid/liquid (w/v)	Time	Yield (%)	Total phenolic contents (mg GAE/g extract)	Antioxidant activity (mg Trolox /g extract)	Total anthocyanin contents (mg/g extract)
Reflux/ distilled water	1:20	3 hr.	10.66	64.91	63.90	7.60
Maceration/ distilled water	1:20	7 days	7.04	69.72	66.94	8.18
MAE/ distilled water	1:20	10 min	11.57	65.70	64.80	6.35

4.4 The Characterization of BP extract

The powder of BP extract (Figure 11) were characterized the maximum absorption (λ_{max}) and functional group by using UV-Visible and FTIR respectively.

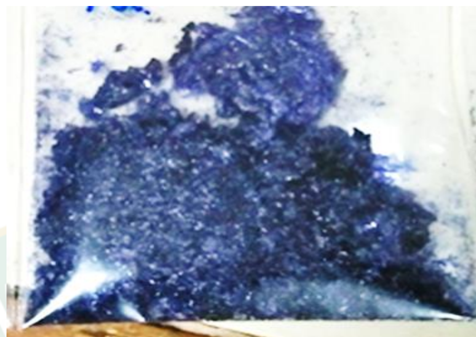


Figure 11 The powder of BP extract

The UV-Vis spectrum of BP extract show the wavelength of maximum absorption (λ_{max}) at 272, 298 and 640 nm this spectrum show in Figure 12. Compared to the UV-Vis spectrum of blue CTAE in distilled water exhibited wavelength of maximum absorption (λ_{max}) at 268, 295, 574 and 619 nm and a shoulder at 540 nm. The UV absorption bands at 268 and 295 nm correspond to Band II of the A-ring benzyol system of anthocyanin and also phenolic compounds in the extract while the visible absorption bands correspond to Band I of the B-ring of hydroxyl cinnamoyl system of anthocyanin. The shoulder at 352 nm may indicate the presence of flavonol glycosides which was found to exist in the extract of Clitoria ternatea (Kazuma et al., 2003; Lee and Abdullah).

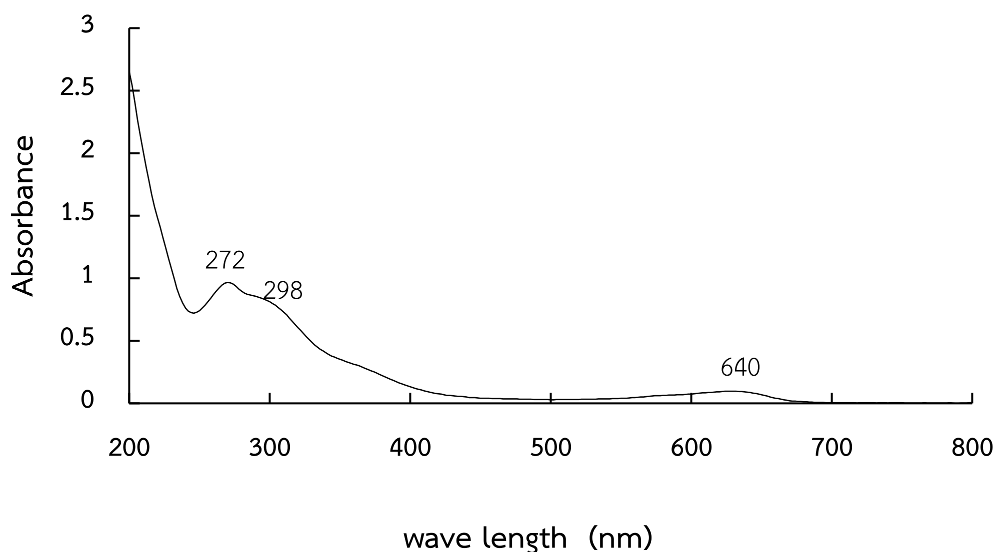


Figure 12 UV-Vis spectrum of BP extract

The FTIR spectra of BP extract are shown in Figures 13 and Table 12. A peak between 3275 cm^{-1} was indexed to the OH group. Peaks at 1034 cm^{-1} were attributed to C–O group. Peaks at 1602 cm^{-1} with slightly different transmittance intensities were attributed to the C=O. The spectral region within 1500–2000 cm^{-1} indicated the infrared absorption of C=C. Compared to the FTIR spectra of the C. ternatea flowers functional group positions. A peak between 3000 cm^{-1} and 3700 cm^{-1} was indexed to the OH group. Peaks at 1074 and 1105 cm^{-1} were attributed to C–O group. Peaks at 1634 and 1645 cm^{-1} with slightly different transmittance intensities were attributed to the C=O and H–C–H groups respectively. The spectral region within 1500–2000 cm−1 indicated the infrared absorption of C=C (Ludin et al., 2018).

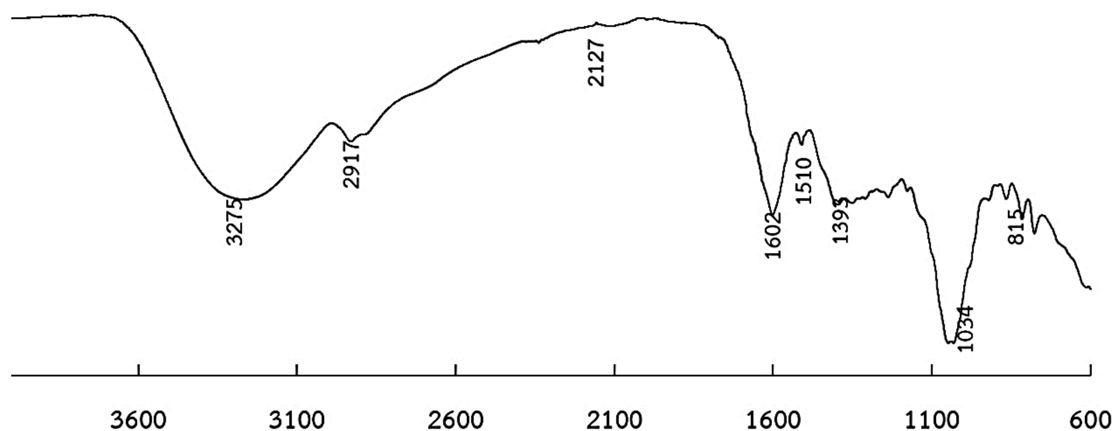


Figure 13 FTIR spectra of BP extract.

Table 12 FTIR spectra of BP extract

Wave number (cm^{-1})	Functional group
3275	O-H stretching
2917	C-H stretching
1602	C=O stretching
1510	C=C stretching
1034	C-O stretching

4.5 *Andrographis paniculata* extract (FTJ extract)

FTJ extract was prepared using distilled water as solvent for maceration. The ratio of the solid to solvent at 1:20 w/v and extraction time for 7 days was performed to obtain the FTJ extract (Rajani et al., 2000). This extract was investigated the percentage yield, total phenolic content, antioxidant activity and andrographolide content. The results were presented in Table 13. Moreover, the andrographolide content in FTJ extract was studied and calculated by compared with andrographolide standard using HPLC.

Table 13 The active compound from FTJ extract.

Experiments	Value (unit)
Percentage yield	18.52 %
Total phenolic contents	75.02 mg GAE/g extract
Antioxidant activity	80.54 mg trolox/g extract
Andrographolide content	47.75 mg/g extract

paniculate extract or FTJ extract was obtained using distilled water as solvent for maceration. The percentage yield of FTJ extract was 18.52%. The Andrographolide has been identified as the main chemical constituents which are responsible for the therapeutics of the plant. Particularly, the leaves of *A. paniculate* were found to contain the highest amount of andrographolide, followed by stem, root, and lastly seed (Lee et al., 2018b; Srivastava and Akhila, 2010). For this FTJ extract gave the andrographolide content at 47.75 mg/g extract from analyzing with HPLC and calculated using HPLC chromatogram of andrographolide standard as shown in Figure 14 Total phenolic contents and antioxidant activity have been described in Table 13 and corresponding to the previous research (Rafat et al., 2010). This FJT extract was used as one of the active compounds in formulating of supplementary product containing BP extract.

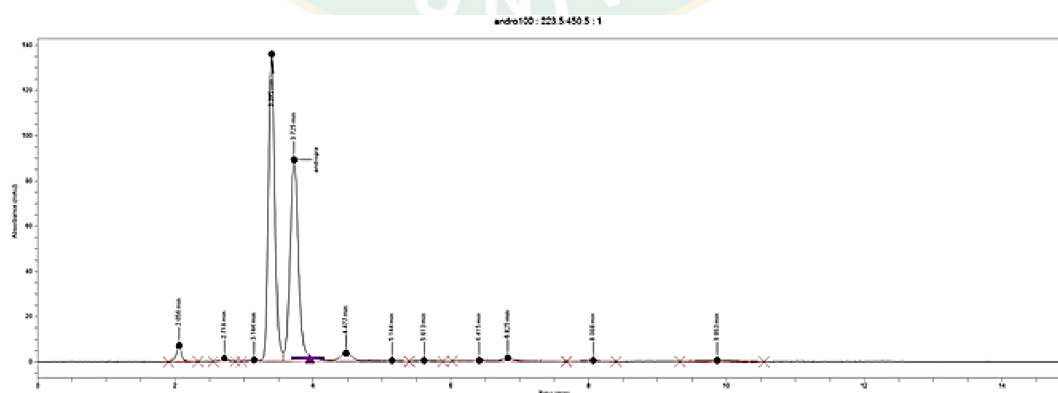


Figure 14 The chromatogram of andrographolide standard.

The UV-spectrum of *A. paniculata* extract (Figure 15) showed maximum absorbance at 217, 290 and 321 nm. The UV-spectrum of Andrographolide showed maximum absorbance at 321 nm (Pancham et al., 2019). Compared to the UV absorbance spectra of ethanolic leaves extract of *A. paniculata* were recorded in the range of 250-320 nm. The spectrum shows weak abosorption bands at 315-320 nm due to the nature of ketone, acetophenone, acrolein, quinoline and 2 nitro furan. The broad band spectrums at 312 nm and 278 nm indicate the presence of napthalene and acetophenone, ketones group. These groups confirm the presence of flavone & fistein types of flavonoids. The characterestics bands at 292 nm, 284 nm, 283 nm, 282 nm, 281 nm, 280 nm show the apperence of flavone & fistein inrespect of aldehyde, ketone, styrene, benzaldehyde, nitro benzene, benzene, 2 methyl-2-nitro propane groups. The spectrum bands at 289 nm, 288 nm express 3° amine, polyene (β-Carotain), quinoline, pyrrole along with the existence of quercetin and fistein types of flavonoids. There is a band at 270 nm reveals the presence of quercetin & anthocyanin flavonoids due to acetone, phenol, benzoic acid, quinoline, thiophene and octyl nitrate. The bands at 273 nm and 286 nm indicate alkene group (naphthalene). Another important group amide (protein) is showed by at 287 nm band. Carbon tetrachloride is disclosed by the band at 265 nm.The sharp bands at 300 nm, 285 nm, 262 nm, 256 nm are allowed for nitroso butane, amino group (aniline), toluene, anthracene group respectively (Sangeetha et al., 2014)

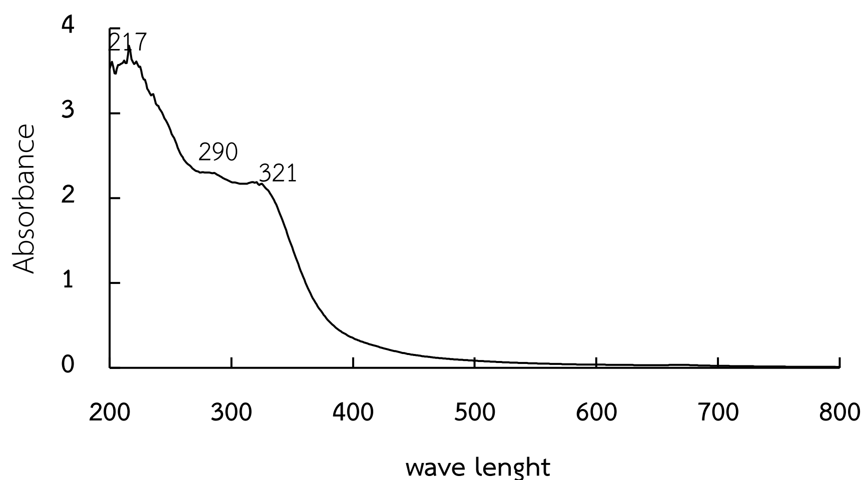


Figure 15 UV-spectrum of *A. paniculata* extract

The FTIR spectrum of *A. paniculata* extract (Figure 16) shows the peak at 584.73 cm^{-1} indicates the presence of alkyne, C-H bending vibrations and quercetin. The sharp peak at 778.55 cm^{-1} is due to aromatic substitution, gem distributed, olefinic groups. This peak again confirms the presence of quercetin. The sharp peak at 1055. 05 cm^{-1} allows the appearance of sulfur compound, S=O stretching vibrations. The FT-IR spectrum shows the peak at 1249. 79 cm^{-1} specifies the existence of C-N stretching, C-O stretching vibration and aliphatic amine, secondary alcohol functional group. The characteristic peaks at 2928.64 cm^{-1} presence of C-H stretching. The peak at 1705.97 cm^{-1} shows C=C stretching vibration, alkenes. There is a clear hump at 3372.58 cm^{-1} is corresponding to primary amides functional group and N-H stretching, O-H stretching vibrations. Compared to the FTIR spectrum of ethanolic extract of *A. paniculata* leaf (Sangeetha et al., 2014). The FTIR spectrum of andrographolide standard (Figure 17) (Singh et al., 2006) it was shown the important functional group similar to FTJ extract were obtain from this condition and the main functional group of FTJ extract was present in Table 14

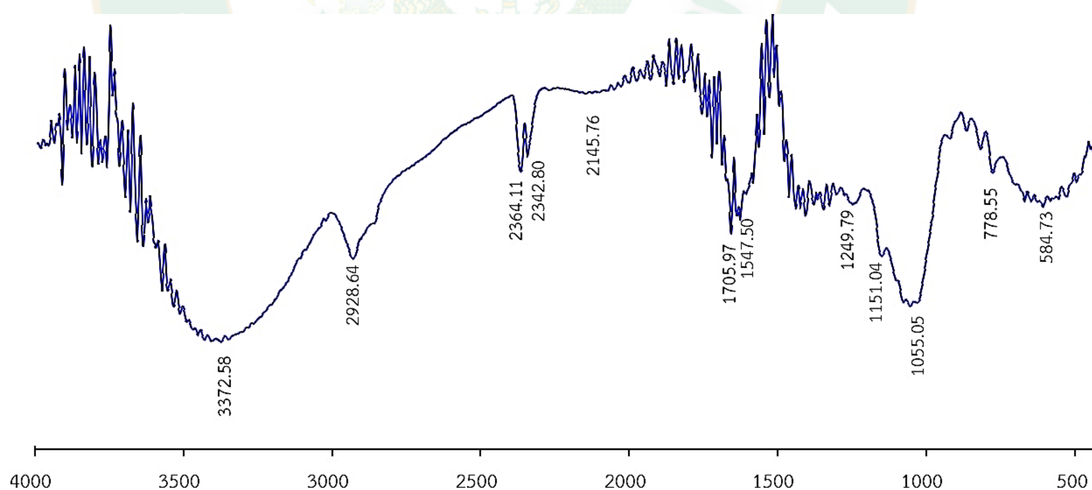


Figure 16 the FTIR spectrum of *A. paniculata* extract

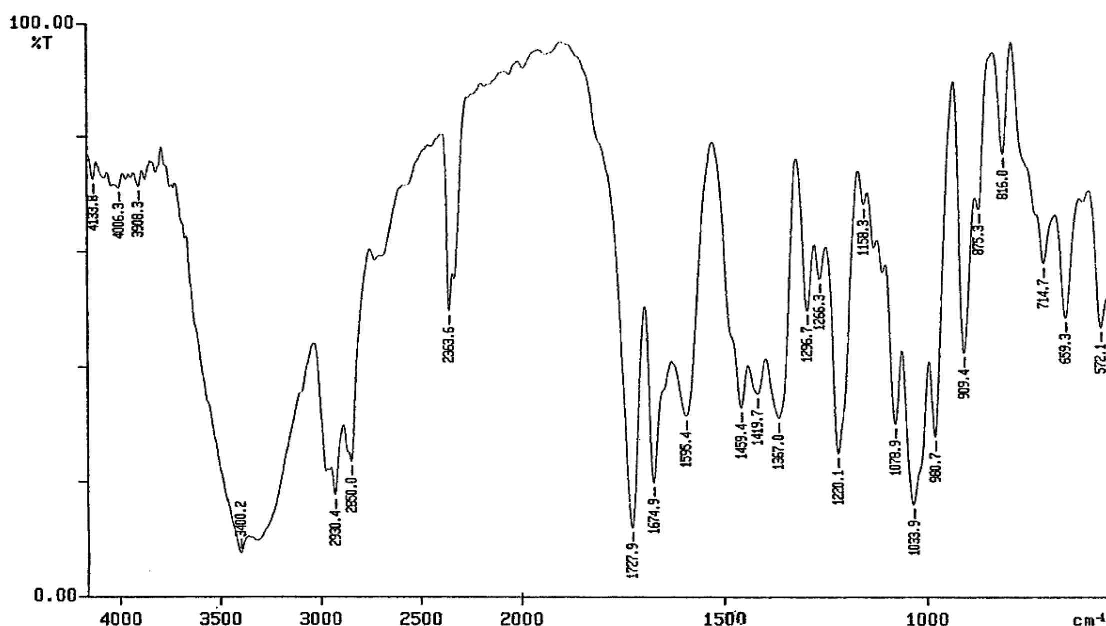


Figure 17 the FTIR spectrum of andrographolide standard

Table 14 The main functional group of FTJ extract

Wave number (cm^{-1})	Functional group
3372.58	O-H stretching
2928.64	C-H and C=O stretching
1705.97	C=C stretching
2364.11	C=C stretching
2342.80	C-O stretching

4.6 Tableting of supplementary product

Tableting supplementary product containing BP and FTJ extracts were performed in different contents using lactose as binder and prepared them approximately 500 mg per tablet (Figure 18). The mixture extracts power and binder were mixed by using mortar and pestle and tableting with hydraulic press at 5 psi and gave the supplementary product. In addition, the tablet of this product was analyzed the total phenolic contents, antioxidant activity, total anthocyanin contents and andrographolide contents, respectively. All results of formulations of BP and FTJ

extracts in tableting, active compounds contents and biological activities have been shown in Table 15.

Figure 18 tableting supplementary product

Table 15 The formulations of BP and FTJ extracts in tableting, active compounds contents and biological activities.

BP extract (%)	FTJ extract (%)	Total phenolic contents (mg GAE/tablet	Antioxidant activity (mg Trolox/tablet)	Total anthocyanin contents (mg/tablet)	Andrographolide content (mg/tablet)
30	0	12.45	15.69	1.52	0.00
30	10	17.69	19.96	1.41	3.64
30	20	22.98	25.42	1.57	7.91
30	30	36.42	40.02	1.53	10.84
0	30	21.52	23.05	0.00	10.84

For the formulation of supplementary product, BP extract was combined with FTJ (10%-30%). The results of total phenolic contents and antioxidant activity of products at 1:1 w/w of each extract were higher when increasing the FTJ contents as well as the andrographolide content except the total anthocyanin contents. It is interestingly, the mixing of two extracts exhibited the higher biological activities than only one extract. This result can suggest that the combination extracts in

supplementary product may be increased the biological activities such as total phenolic content and antioxidant activity. These results may be used as the examples of development of combination drugs or herbs which can be utilized in the pharmacy or studying about drug resistant in the future.

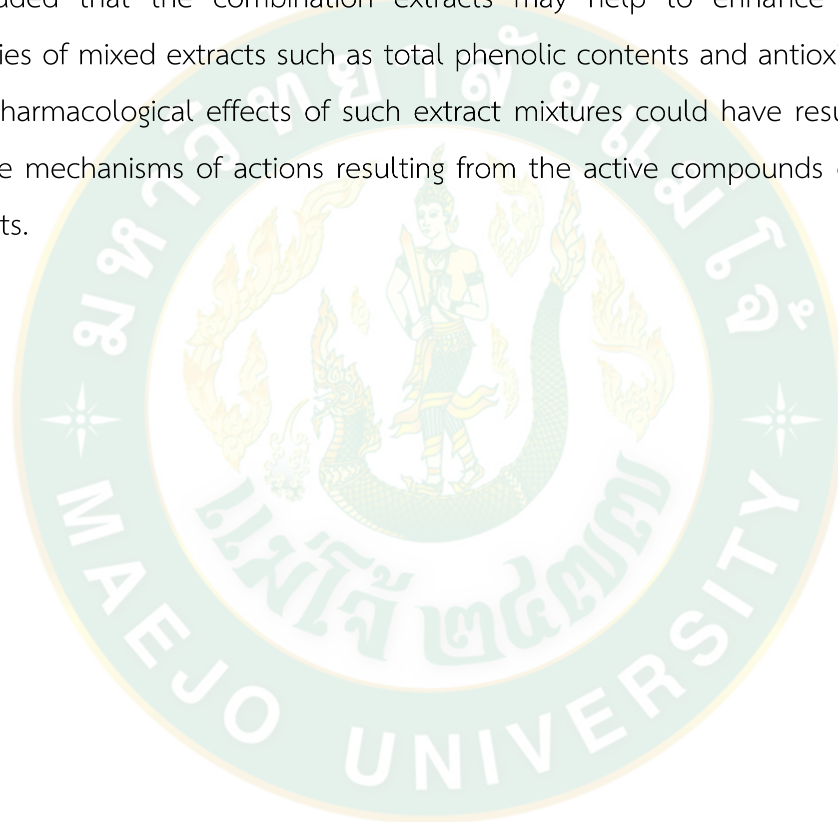
CHAPTER 5
CONCLUSION

C. ternatea extract or called BP extract was performed in this research. They have high antioxidant activities. The results revealed that the extraction time, temperature, microwave power and ratios of solid to solvent (w/v) affected amount of total phenolic contents and antioxidant activities of extracts. The best extraction conditions from three extraction methods composed of 1) maceration using ratio of solid to solvent at 1:20 w/v for 7 days, 2) reflux extraction using ratio of solid to solvent at 1:20 w/v for 3 hours and 3) microwave assisted extraction using ratio of solid to solvent at 1:20 w/v, microwave power at 450 W for 10 mins. The finding of the present study suggested that aqueous extract of *C. ternatea* flower using maceration at 1:20 w/v for 7 days could be used as a potential source of antioxidants and anthocyanins which should have great importance as pharmacological formulation.

A. paniculata extract or called FTJ extract was prepared by maceration using distilled water to obtain aqueous extract of *A. paniculata*. The best condition for extraction performed when using the ratio of solid to solvent at 1:20 w/v and extraction time for 7 days and gave the percentage yield of 18.52%. The biological activities of aqueous extract of *A. paniculata* were evaluated. The results showed that the total phenolic content and antioxidant activity were 75.02 mg GAE/g extract and 80.54 mg trolox equivalent/g extract, respectively. Moreover, andrographolide in aqueous extract of *A. paniculata* was determined in 47.75 mg per g extract when compared to chromatogram of standard andrographolide.

In this present work, tablet of BP extract and FTJ extract were prepared and studied their biological activities. For BP tablet, it has been evaluated the total phenolic content (12.45 mg GAE/tablet), antioxidant activity (15.69 mg trolox equivalent/tablet) and total anthocyanin content 1.52 mg/tablet. For FTJ tablet, it has been determined the total phenolic content (21.52 mg GAE/tablet), antioxidant

activity (23.05 mg trolox equivalent/tablet) and andrographolide (10.84 mg/tablet). Moreover, the combination of two extracts involved BP and FTJ extracts were formulated the supplementary product in tableting using several formulas. The results revealed that the best condition of tableting using 30% BP extract and 30% FTJ extract per tablet had total phenolic content (36.42 mg GAE/tablet), antioxidant activity (4 0 . 0 2 mg trolox equivalent/tablet) and total anthocyanin content 1.53 mg/tablet and andrographolide (10.84 mg/tablet). From the results, it can be concluded that the combination extracts may help to enhance the biological activities of mixed extracts such as total phenolic contents and antioxidant activities. The pharmacological effects of such extract mixtures could have resulted from the diverse mechanisms of actions resulting from the active compounds of each herbal extracts.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APPENDIX

APPENDIX A

A.1 Weight of Butterfly pea flower extract

A.1.1 Weight of Butterfly pea flower extract from maceration

The maceration method was measurement the weight of extract from difference condition to calculate percent yield which show in table A1-A2

Table A1 percent yield from maceration at 1:10-1:30 w/v of solid to solvent for 5 days by using two solvents

Solvent	ratio	Weight BP (g)	Weight extract (g)		
			1	2	3
Water	1:10	20	0.6943	0.7175	0.6493
	1:20	20	0.9803	1.1084	0.9589
	1:30	20	0.8252	0.7578	0.7155
Ethanol	1.10	20	0.6221	0.6654	0.6389
	1.20	20	0.6826	0.6944	0.7500
	1.30	20	0.675	0.5635	0.8824

A.2 Calculate percentage yield

For example, calculation of percentage yield

If extracted the BP 20 g after the reaction was gave the BP extract 0.6943 g.

$$\text{percentage yield} = \frac{\text{g of extract} \times 100}{\text{g of sample}}$$

$$= \frac{0.6943 \times 100}{20}$$

$$= 3.4715\%$$

If state with BP 20 g and obtain BP extract 0.6943 g it was presented the percentage yield equal 3.4715%

APPENDIX B

B.1 Absorbance for analyze total phenolic of butterfly pea flower extract

B.1.1 Absorbance for analyze total phenolic from maceration

The difference condition of maceration method was measurement the absorbance at 765 nm for determine the total phenolic content which show in table B1-B2

Table B1 Absorbance of BP extract 1000 ppm from maceration at 1:10-1:30 w/v of solid to solvent for 5 days by using two solvents

Solvent	ratio	Weight BP (g)	Abs at 765 nm		
			1	2	3
Water	1:10	20	0.756	0.784	0.799
	1:20	20	0.884	0.891	0.901
	1:30	20	0.861	0.862	0.877
Ethanol	1.10	20	0.643	0.621	0.649
	1.20	20	0.706	0.736	0.736
	1.30	20	0.703	0.713	0.694

B.2 The calibration curve of gallic acid

The analysis of total phenolic content was used gallic acid for standard the calibration curve show in Figure B1.

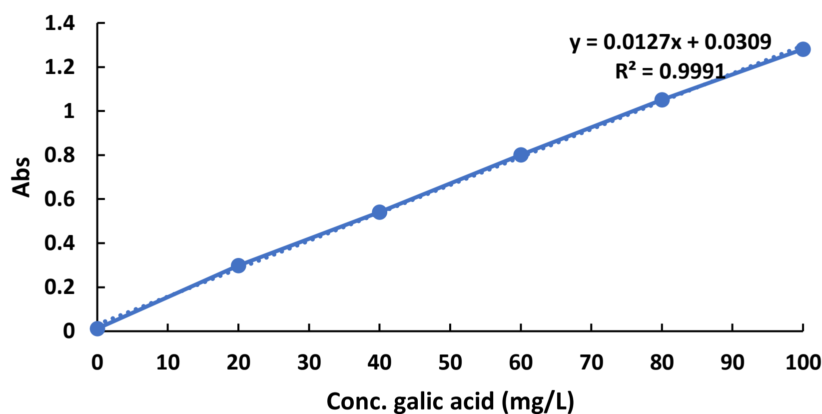


Figure. B1 the calibration curve of gallic acid

B.3 Calculation total phenolic content

The absorbance was measured to determine the total phenolic contents in both extracts separately using the formula,

$$C = X * V/m$$

where C = total phenolic content in mg/g in GAE (Gallic acid equivalent)

X = concentration of Gallic acid established from the calibration curve in mg/ml

V = volume of extract in ml

m = the weight of the plant extract in g

For example : The absorbance is 0.702

From $y = 0.0127x + 0.0309$

$$0.702 = 0.0127x + 0.0309$$

$$X = \frac{0.702 - 0.0309}{0.0127}$$

$$X = 52.84 \text{ mg/l}$$

From $C = X*V/m$

$$C = \frac{52.84 \text{ mg. L}^{-1} \times 1 \text{ L}}{1\text{g}}$$

$$C = 52.84 \text{ mg GAE/g extract}$$

Total phenolic contents is 52.84 mg GAE/g extract

APPENDIX C

C.1 Absorbance for analyze antioxidant activity of butterfly pea flower extract

C.1.1 Absorbance for analyze antioxidant activity from maceration

The difference condition of maceration method was measurement the absorbance at 517 nm for determine the antioxidant from DPPH inhibition which show in Table C1

Table C1 Absorbance of BP extract 1000 ppm from maceration at 1:10-1:30 w/v of solid to solvent for 5 days by using two solvents

Solvent	ratio	Abs control	Abs at 517 nm		
			1	2	3
Water	1:10	1.322	0.730	0.695	0.758
	1:20	1.322	0.635	0.629	0.629
	1:30	1.322	0.657	0.682	0.643
Ethanol	1.10	1.322	0.857	0.842	0.833
	1.20	1.322	0.775	0.819	0.814
	1.30	1.322	0.815	0.802	0.813

C.2 The calibration curve of Trolox

The analysis of total phenolic content was used gallic acid for standard the calibration curve show in Figuer C1.

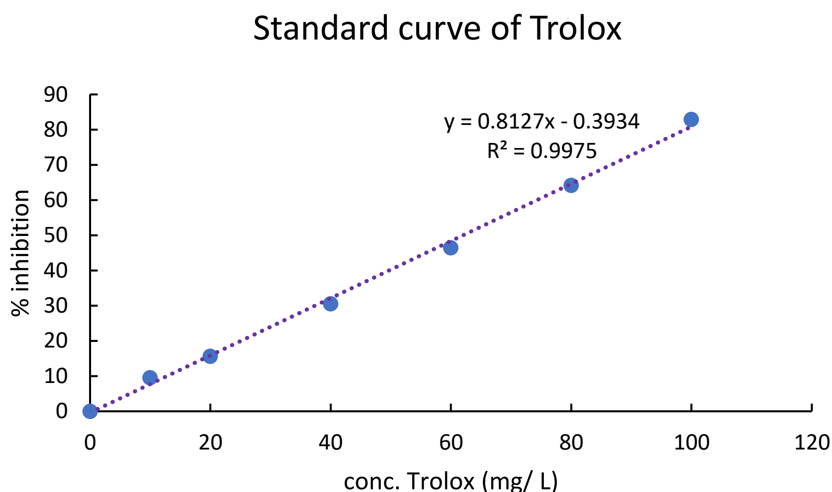


Figure. C1 the calibration curve of Trolox

C.3 Calculate total phenolic content

The absorbance was measured to determine the antioxidant activity from DPPH inhibition using the formula,

$$\text{Percentage of DPPH inhibition} = \frac{(A_{control} - A_{sample}) \times 100}{A_{control}} \quad (1)$$

when $A_{control}$ represents absorbance measure for DPPH solution

A_{sample} represents absorbance of the mixture of extract and DPPH solution.

For example : The absorbance of extract is 0.702

The absorbance of control is 1.322

From Percentage of DPPH inhibition $= \frac{(A_{control} - A_{sample}) \times 100}{A_{control}}$

$$\text{Percentage of DPPH inhibition} = \frac{(1.322 - 0.702) \times 100}{1.322}$$

$$\text{Percentage of DPPH inhibition} = 46.90$$

From

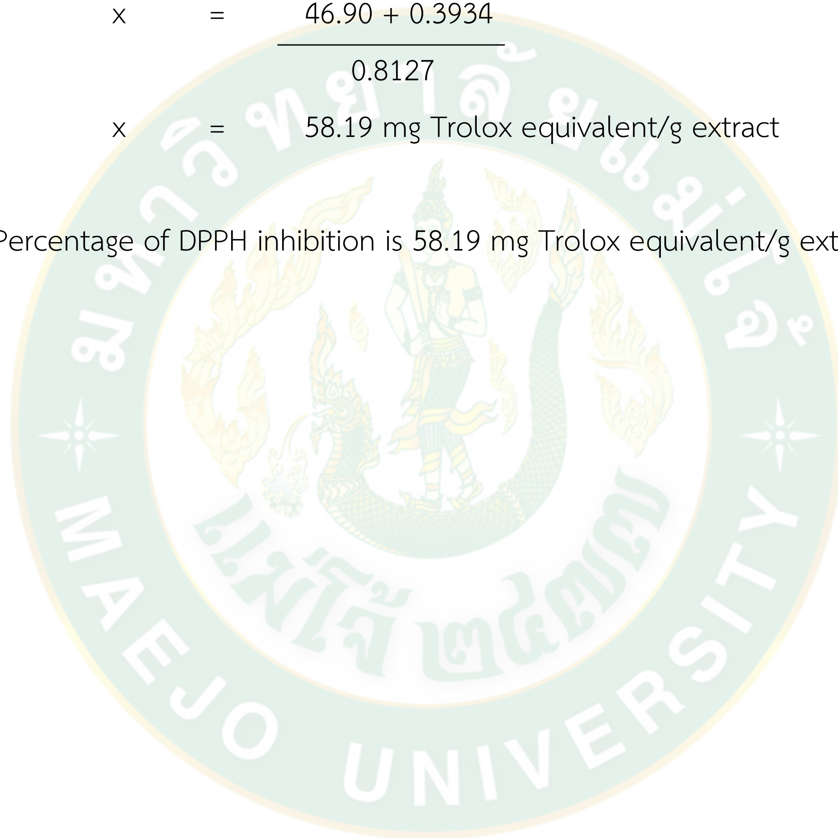
$$y = 0.8127x - 0.3934$$

$$46.90 = 0.8127x - 0.3934$$

$$x = \frac{46.90 + 0.3934}{0.8127}$$

$$x = 58.19 \text{ mg Trolox equivalent/g extract}$$

Percentage of DPPH inhibition is 58.19 mg Trolox equivalent/g extract



APPENDIX D

D.1 Absorbance for analyze total anthocyanin content of butterfly pea flower extract

D.1.1 Absorbance for analyze total anthocyanin content from maceration

The difference condition of maceration method was measurement the absorbance at 540 and 700 nm for determine the total anthocyanin content which show in table D1

Table D1 Absorbance of BP extract 1000 ppm from maceration at 1:10-1:30 w/v of solid to solvent for 5 days by using two solvents

Solvent	ratio	pH	Abs at 540 nm			Abs at 700 nm		
			1	2	3	1	2	3
Water	1:10	1.0	0.081	0.077	0.082	0.004	0.003	0.006
	1:20		0.101	0.102	0.102	0.006	0.005	0.005
	1:30		0.077	0.078	0.072	0.005	0.003	0.002
Ethanol	1.10		0.075	0.075	0.076	0.003	0.004	0.005
	1.20		0.081	0.078	0.082	0.006	0.005	0.006
	1.30		0.076	0.075	0.077	0.005	0.004	0.005
Water	1.10	4.5	0.056	0.056	0.058	0.006	0.006	0.006
	1.20		0.071	0.073	0.073	0.004	0.005	0.007
	1.30		0.049	0.051	0.046	0.006	0.004	0.003
Ethanol	1.10		0.058	0.06	0.054	0.005	0.007	0.006
	1.20		0.058	0.059	0.058	0.004	0.005	0.005
	1:30		0.057	0.054	0.055	0.004	0.004	0.004

D.2 Calculate total anthocyanin content

The absorbance of extract solution was measured at pH1 and pH 4.5 to determine the total anthocyanin content using the formula,

$A = (A_{540} - A_{700})_{pH\ 1.0} - (A_{540} - A_{700})_{pH\ 4.5}$

$\text{Total anthocyanin} = \dfrac{A \times Mw \times DF \times 1000}{\varepsilon \times l}$

When Mw is 449.2 $g.mol^{-1}$ for cyaniding-3-glucoside

ε is 26,000 molar extinction for cyanidin-3-glucoside

l is path length 1 cm

For example : The absorbance of extract pH 1.0 at 540 nm is 0.081

The absorbance of extract pH 1.0 at 700 nm is 0.004

The absorbance of extract pH 4.5 at 540 nm is 0.056

The absorbance of extract pH 4.5 at 700 nm is 0.006

From $A = (A_{540} - A_{700})_{pH\ 1.0} - (A_{540} - A_{700})_{pH\ 4.5}$

$A = (0.081 - 0.004) - (0.056 - 0.006)$

$A = 0.027$

From $\text{Total anthocyanin} = \dfrac{A \times Mw \times DF \times 1000}{\varepsilon \times l}$

$\text{Total anthocyanin} = \dfrac{0.027 \times 449.2 \times 15 \times 1000}{26000 \times 1}$

Total anthocyanin = 6.997 mg /g extract

Total anthocyanin is 6.997 mg /g extract

APPENDIX E

E.1 list of presentation

E 1.1 Publication

1. Tipparat Saejung, Julinta Don-In and Thitiphan Chimsook, Preparation of Ethanolic Butterfly Pea Extract Using Microwave Assisted Extraction and Loaded Nanostructured Lipid Carriers: Evaluation of Antioxidant Potential for Stabilization of Fish Oil. Key Engineering Materials. ISSN: 1662-9795, Vol. 873, pp 1-5

2. Julinta Don-In1,a , Tipparat Saejung2,b, Thitiphan Chimsook, Preparation Adlay and Black Sesame Seeds Extracts Using Osmotic Dehydration and Increasing the Stability of Extracts Using Encapsulation, Key Engineering Materials Submitted: 2020-05-04 ISSN: 1662-9795, Vol. 873, pp 7-12

E.1.2 Proceeding

1. Tipparat Saejung1, Julinta Don-in2 and Thitiphan Chimsook, Bioactivity of Clitoria Ternatea Extract from Different Extraction Methods, The 2nd Maejo-Engineo International Conference on Renewable Energy (MEICRE 2018), 107-115

2. Tipparat Saejung, Julinta Don-in and Thitiphan Chimsook, Bioactivities of Cordyceps militaris and formulation of health product containing Cordyceps militaris, The 2019 Pure and Applied Chemistry International Conference (PACCON 2019), 12-16

3. Tipparat Saejung and Thitiphan Chimsook, Preparation of Garlic Extract Containing Allicin using Different Extraction Methods, 10th International Science, Social science, Engineering and Energy Conference 20th -23rd November, 2019, Rajamangala University of Technology Isan Sakon Nakhon Campus, Thailand, 1-15

4. Julinta Don-in, Tipparat Saejung, and Thitiphan Chimsook, Antioxidant Activity, Total Phenolic Contents and Cytotoxicity of Coix Lacryma-Jobi Extracts From Different Extraction Methods, The 2nd Maejo-Engineo International Conference on Renewable Energy (MEICRE 2018), 116-126

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