PRELIMINARY SCREENING ON ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF *Centrosema pubescens* LEAVES

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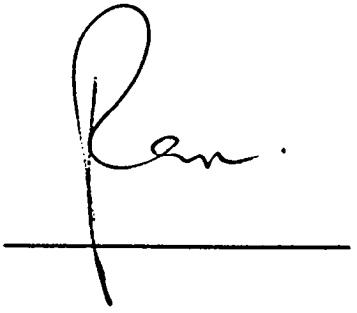

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ABSTRACT

Folkfore remedies has been traditionally used to cure many diseases. Since time immemorial, man has used various part of plants in the treatment and prevention of many ailments. These medicines are cheap and easy to obtain. *Centrosema pubescens* is a common type of legume that has been planted widely as cover crop in palm oil plantation. However the use of these crops in ethnomedicine are less known and undocumented. The study of *C. pubescens* for its medicinal value may have a great impact in ethnomedicine and potential to be developed into new medicine. Therefore this project aims to investigate the phytochemical constituents of *C. pubescens* and its bioactive properties. The bioactive compounds in *C. pubescens* leaves were succesfully extracted using four different solvents which are water, methanol, acetone and hexane. Among the four solvents water has the highest extraction yield (7.65%). Qualitative phytochemicals analysis shown that methanolic extract contains many phytochemical constituents among the four extracts. Methanol extract was found to contain many phytochemicals constituents which are saponin, flavonoid, tannin, steroid, terpenoid, cardiac glycoside and phenolic compounds. Proximate analysis revealed total phenolic compound is higher in water extract (254.14 mg GAE/g DW) and methanolic extract has the highest of total flavonoid content (89.47 mg QE/g DW). Antioxidant activity of the extract was determined using DPPH free radical scavenging method and gallic acid as the reference antioxidant. Lower IC_{50} value indicate that the extract is a good antioxidant agent. Methanolic extract of plant leaf has the lowest IC_{50} value (4.673mg mL^{-1})and shows the best antioxidant potential in this project. Antibacterial analysis revealed that only methanol and acetone extracts have antibacterial properties against the tested microorganism (*Streptococcus pneumonia* and *Pseudomonas aeruginosa*). The MIC values of both extract were also determined as 60 µg. Both methanolic and acetone extracts have bacteriostatic effect against *P. aeruginosa* and partial bactericidal effect against *S. pneumonia*.

ABSTRAK

Perubatan tradisional telah digunakan untuk menyembuhkan pelbagai penyakit. Sejak zaman dahulu lagi, manusia telah menggunakan pelbagai bahagian tumbuhan untuk merawat dan mencegah pelbagai penyakit. Ubat ini juga murah dan senang didapati. *Centrosema pubescens* ialah sejenis tanaman kekacang yang selalu ditanam secara meluas sebagai penutup bumi di ladang kelapa sawit. Bagaimanapun pengunaan tumbuhan ini dalam etnoperubatan adalah kurang diketahui dan direkodkan. Kajian mengenai *C. pubescens* dalam perubatan mungkin mempunyai impak besar dalam etnoperubatan dan berpotensi untuk membuat ubat baru sekaligus memajukan bidang farmasi negara ini. Oleh itu matlamat projek ini adalah untuk menyiasat kandungan fitokimia *C. pubescens* dan sifat bioaktifnya. Kompoun bioaktifnya telah berjaya diekstrak menggunakan empat pelarut berbeza iaitu air, metanol, aseton dan heksana. Daripada keempat-empat pelarut, air mempunyai hasil ekstrak yang tinggi (7.65%). Analisa kualitatif fitokimia yang dijalankan menunjukan ekstrak methanol mempunyai jumlah fitokimia yang tinggi di antara keempat-empat ekstrak tersebut. Ekstrak metanol mempunyai saponins, flavonoids, tannins, steroids, terpenoids cardiac glycosides dan kompoun phenolik. Analisa proksimat yang dijalankan mendapati jumlah phenolik kompoun yang tinggi di dalam ekstrak air (254.14 mg GAE/g DW) dan metanol mempunyai jumlah flavonoid yang tertinggi (89.47 mg QE/g DW). Aktiviti antioksida ekstrak telah ditentukan melalui kaedah DPPH merangkap radikal bebas, menggunakan asid galik sebagai rujukan antioksida. Rendah nilai IC_{50} menunjukan ekstrak adalah antioksida yang bagus. Ekstrak methanol mempunyai nilai IC_{50} yang rendah (4.673mg mL^{-1}) dan menunjukan potensi antioksida yang bagus dalam projek ini. Analisis antibakteria menunjukan hanya ekstrak methanol dan aseton mempunyai sifat antibakteria terhadap mikroorganisma yang diuji (*Streptococcus pneumonia* dan *Pseudomonas aeruginosa*). Nilai MIC kedua-dua ekstrak juga ditentukan sebagai 60 µg. Kedua-dua ekstrak juga mempunyai kesan bakteriostatik terhadap *P. aeruginosa* dan kesan baktericidal terhadap *S. pneumonia.*

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LIST OF SYMBOLS, UNITS & ABBREVATIONS

g	gram
mL	millilitre
v/v	volume by volume
w/v	v weight by volume
µL	microlitre
g L^{-1}	gram perlitre
µg L^{-1}	microgram perlitre
β	beta
kg ha^{-1}	kilogram per hectare
Mg ha^{-1}	Megagram per hectare
cm	centimeter
°C	degree Celcius
mm	milimiter
N	Nitrogen

CHAPTER 1

INTRODUCTION

1.1 Background of study

Healthcare is important in our daily life. We need to be healthy in order to work or do other things comfortably. The quote "health is wealth", defines health is so precious and need to be maintain carefully. People are more concern about their healthy lifestyle nowadays. They exercise regularly and eat balance diet to make sure their body are healthy. Some even buy expensive supplement at pharmacy to boost their immune system and to make sure their health is at the optimum stage. However, people will tend to fall sick easily due to many factors such as weather changes, infection pathogens and others. One of the reason they are prone to diseases because they have low immune system, expose to a unhealthy condition, poor lifestyle and others. There are several major health problems around the world where bacterial infections and cancer are common. When people have low immune system, pathogen can infect their body easily which result as infections. Similarly when people are exposed to an unhealthy condition such as exposure to ultraviolet light or continue with an unhealthy diet, may lead to many diseases. Cancer is mutation resulted from free radicals that damage the genetic material. These two health problems are the most commonly due to these modern lifestyle.

Bacteria is a living microorganism and some are pathogenic that can cause disease in a healthy human. They need to enter human host body first to initiate infection. They initiate infections through different routes such as gastrointestinal, respiratory, skin and many more. Skin infections are the most common bacterial infections in daily life. Skin is a barrier that limits invasion and growth of pathogenic bacteria. There are also good bacteria known as microflora grow on the skin. But when the skin is wounded it will be exposed to bacteria which can cause an infection. Example of a skin infection causing bacteria is *Staphylococcus aureus*, a gram positive bacteria. They cause skin infection such as Impetigo. Bacterial infections are usually treated by modern medicine such as antibiotics. Commonly used antibiotic is β-lactam antibiotic that kills wide range of bacteria. However, bacteria tend to adapt fast with the medicine effect and make the drug has no longer any effect to them. Recently, some of the pathogen has been reported to develop resistance to certain drugs, antibiotic and analgesic. For example they develop resistance to β-lactam antibiotic by producing β-lactamase that degraded the antibiotic to make it no longer effective on them (Goyal *et al.*, 2008).

Besides that, another health problem arise nowadays is cancer. Cancer cases increase gradually every year and it is estimated that 90-100 000 people in Malaysia living with cancer at one time. One of the most common cause of cancer in human is genetic mutation. Reactive oxygen species (ROS) cause damage of the genetic material and induce mutation. These will lead to tumor formation. There are two types of tumor which are benign and malignant tumor. Benign tumor does not posses metastasis ability and malignant tumor posses metastasis activity. Malignant tumor can spread to other parts of the body and they are called cancer. If cancer is not detected and treated at early stage the patient may die. Common treatment for cancer patient is chemotheraphy. However, chemotheraphy have many adverse side effects that can cause damage to the patient body (Tapas *et al.*, 2008).

The emergence of antibiotic resistance pathogens and difficulties in developing an effective cure for cancer indicates the need for alternative medicines that are effective and can overcome this problem. Folkfore remedies has been traditionally used to cure many diseases. Since time immemorial, man has used various part of plants in the treatment and prevention of many ailments. These medicine are also cheap and easy to obtain. World Health Organization has estimated that up to 80% of the world population rely on plants for their primary health care (WHO, 2001). A plant becomes a medicinal plant only when its biological activity has been ethnobotanically reported or scientifically established (Elujoba, 1997). More importantly, plants have been the main source of medicine for man before the advancement of Science and Technology (Schmelzer and Omino, 2003). Medicinal plants are further defined as plants that have at least one of their parts (leaves, stem, barks or roots) used for therapeutic purposes, plants provide a source of medicines, which are useful in treatment of various categories of human ailments and conditions. It has the potential to be processed into health product such as routine healthy drink, ointment and beauty products. Because of its remedies potential, parts of these crops need to be harvested and produced more so it can be commercialized. Since Malaysia is rich in its biodiversity, commercializing of potential medicinal plants can also improve our country economy and enhance the natural products industry (Sofowora, 1982).

Over 50% of all modern clinical drugs are of natural product origin (Stuffness and Douros, 1982) and natural products play an important role in drug development programs of the pharmaceutical industry (Baker *et al*, 1995). Iwu *et al.,* (1999) reported that the primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatement. The use of traditional medicine and medicinal plants in most developing countries as therapeutic agents for the maintenance of good health has been widely observed. Medicinal plants such as herbs, contain phytochemicals that is produce by plant from their primary or secondary process of which enable them to posses bioactive properties. These bioactive properties contain high antioxidant and antimicrobial activities, enable them to be used as remedies to cure various kind of

diseases. Traditionally, people have use diferrent parts of the medicinal plant for remedies and proven to be very promising. For example, the flower of *Pureraria javanica* is use traditionally by the Chinese community as medicine to reduce reaction due to alcoholic consumption.

Leguminous crops have been planted in plantation as a cover crop. *Centrosema pubescens* is a common type of legume that has been planted widely as cover crop in palm oil plantation. The growth of the crops is fast and make it abundant in the estates. Since it is abundant it maybe under utilize if only use as cover crops in plantation without any other usage. Other than acting as a cover crops they also help in nitrogen fixation of the soil and improve soil fertility. However the use of these crops in ethnomedicine are less known and undocumented. The study of *C. pubescens* for its medicinal value may have a great impact in ethnomedicine and potential to be developed into new medicine for advanced in pharmaceutical field for the country. Therefore this project aim to further investigate the potential of this cover crop.

1.2 Objectives

1. To estimate the amount of phytochemicals present in leaves extract of *Centrosema pubescens.*
2. To determine the antioxidant value of leaves extract of *Centrosema pubescens.*
3. To determine the antimicrobial activity of leaves extract of *Centrosema pubescens* against some common pathogens such as *Streptococcus pneumonia* and *Pseudomonas aeruginosa.*

CHAPTER 2

LITERATURE REVIEW

2.1 *Centrocema pubescens*

Centrosema pubescens is a vigorous, creeping, and climbing perennial forage. It propagated by seed and the seed rate required for planting is about 5kg ha^{-1}. *C. pubescens* is usually known as Centro in short or the other commonly known name is butterfly pea as their flower has a butterfly like-shaped. *C. pubescens* is a type of legume that belongs to the family of leguminose (*Fabaceae*) (Heath *et al.*, 2003). Legume is the third largest family of the flowering plants after *Asteraceae* and *Orchidaceae* with 1800 species and about 650 genera (Polhill and Raven, 1981). The word "legume" is a term derived from French word which has a wider meaning and refers to any kind of vegetables (Charina, 2002). Centro originated from Central America and is now one of the most widely distributed legumes in the humid tropics such as Asia. Pure stand of butterfly pea yields up to 12 Mg ha^{-1} and seed yield may vary from 200 to 500 kg ha^{-1} (Teitzel and Peng, 1997).

C. pubescens has trifoliate, shining bright green and slightly hairy leaves. Leaf size is 1-7cm long and 0.5-4.5cm wide and the petiole can grow up to 5.5cm. Their leaflets are elliptic or ovate-elliptic obtuse or shortly obtusely acuminate and 1-7 cm by 0.5-4.5 cm in size. Stems are long, twining and vigorous growing, rooting moderately at the nodes and capable of extended to 14 feet. Their stem do not become woody for at least 18 months. Runners have a tendency to root at the nodes if soil moisture is high. Flowers are pale mauve in colour with purple lines in the centre, borne in axillary racemes with 3-5 flowers per raceme. The flower of Centro has a butterfly like-shaped. Pods are linear, 4-17cm x 6-7 mm in size, flattened with prominent margins, straight or slightly twisted, acumate and dark brown when ripe, containing up to 20 seeds. (Teizel and Chen, 1992 ; Reynolds, 1995 ; Skerman *et al.*, 1998).

C. pubescens prefer rainfall of 1500mm or more per year, but can grow in areas receiving between 750-1000mm, however, where rainfall exceeds 2500mm it may attack by leafspot. It has a good root system and can withstand a long dry season, but is not adapted in areas receiving less than 1020mm of rainfall. The optimum temperature for growth is between 20-30°C. Centro are able to survive 3-4 month dry period, growth in the dry season can be improved with irrigation. However the cooler temperatures associated with the dry-season may inhibit germination and growth. It has the ability to grow on a wide range of soils, with an optimum pH range between 4.9 - 5.5 and textures ranging from sandy loams to clay and moderately tolerant a poorly drained condition (Teizel and Chen, 1992 ; Reynolds, 1995 ; Skerman *et al.*, 1998). Centro grows well on fertile soils and on poor soils, responds to phosphorus, molybdenum and possibly magnesium fertilization. Initial establishment is rather slow but subsequent growth is rapid. It can grow suitably with other pasture grasses depending on nutrients availability. It climbs on anything it encounters and can be seen growing up on trees. It can withstand heavy grazing (Steel and Humpreys, 1974 ; Eng *et al.*, 1978 ; Skerman *et al.*, 1988).

C. pubescens is a common type legume widely planted in plantation as cover crop in rubber and oil palm plantation in Malaysia (Baligar *et al.*, 2007). Cover crops are planted for the purpose of benefiting the soil and other crops. The plantation industry in Malaysia including, cocoa, oil palm rubber and others has been practicing "green manure" agriculture. For example, planting of tree crops on terraces, use of slit pits and planting of ground cover crop to conserve soil and water. Cover crop are usually planted to prevent soil erosion by wind and water, conserve soil moisture, smother or suppress weeds, improve soil fertility and control pests and disease. When planted to reduce nutrient leaching following a main crop, they are also called catch crops. The choice of a cover crop depends on some intrinsic factor including the ability of rapid establishment, reduced competition for moisture and nutrients with the main crop, good foliage cover even during non-croopping periods and efficient production of rapidly recycling biomass. It also acts as a natural fire and weed control agent (Fongod *et al.*, 2010).

C. pubescens has the ability to fix atmospheric nitrogen with their root nodules. It provides organic fertilizer that improves the soil fertility and moisture conditions. Symbiotic system of nitrogen fixation has long been recognized to be an important source of nitrogen for agricultural crops. In fact, it has been estimated that of the 10 tons of nitrogen fixed annually, the largest portion comes from symbiotic sources, especially from the nodules of leguminous plants growing in natural association and in agriculture (Donald, 1960). One of the ways in which nitrogen enters the soil is through the decay of the legume root nodules. This is usually happens when the carbohydrate supply to the nodule is restricted, resulting in the sloughing off of the nodule tissues (Butler and Bathurst, 1956). Other factors which enhance decomposition of legume root nodules and therefore accelerate nitrogen release into the soil include fruting, extremes of soil temperature or soil moisture content (Wilson, 1931), defoliation (Wilson, 1942) and pronounced shedding (Sg and Trumble, 1939). Others routes of nitrogen entry into the soil include excretion of nitrogenous compounds by legume roots and nodules (Butler and Bathurst, 1956), in association with grass pastures, the sloughing-off and decay of legume root tissues, leaching of nitrogenous compounds from legume leaves by

rain water, decay of legume leaves and decapitated petioles (Butler and Bathurst, 1956). Most of the nitrogen in decaying leaves and petioles however has been remobilized by the plant during the processes of senescence. Therefore only a minor amount of nitrogen is released to the soil by this mean (Williams, 1954).

Apart from its use as cover crops *C. pubescens* may have other added values and may be use in many beneficial ways. In the Philipphines, it is by far the most successful among the pastures legumes. Excellent centro-based pastures are found in coastal areas in Davao, Bukidnon. It can grow in mixture of grasses for better forage nutrition. Poultry plays significant role in the provision of animal protein required by man to meet his daily protein intake. *C. pubescens* has been as leaf meals as poultry diets for livestock. Nworgu *et al.*, (2004) recommended that 2.5% centro for broiler starter and finisher. The importance of legume leaf meals in poultry has been recognized by farmers because of their relatively high content of protein, some minerals and vitamins (Topps, 1992 ; Nworgu, 2004). Leaf meals supplements have been included into the diets of poultry as a mean of reducing high cost of conventional protein sources and to improve profit margin (Topps, 1992 ; D'Mello, 1995 ; Odunsi *et al.*, 1995 ; Nworgu and Fapohuncia, 2002). Green matter yield of *C. pubescens* varied from 13.5 to 40 t/ha/year. Nworgu and Ajayi (2005) reported that biomass and dry matter yield of *C. pubescens* are 7.34-7.56 and 3.75-3.78 t/ha/year respectively. The proximate composition of *C. pubescens* leaf meal (CLM) are presented in Table 1. Centro leaf meal is rich in crude protein (22.45%), phosphorus (0.53%), calcium (0.80%), potassium (0.72%), and magnesium (0.30%).

Table 1.0 : Proximate chemical composition of *Centrosema pubescens* leaf meals for poultry (Nworgu *et al.*, 2007).

Fraction	*C. pubescens* Leaf Meal (% on DM basis)
Dry matter	88.88
Crude protein	22.45
Ether extract	3.00
Crude fibre	6.43
Ash	7.74
Nitrogen free extract	60.38
Gross energy ($kcaL^{-1}kg$)	4402
Phosphorus	0.53
Calcium	0.80
Potassium	0.72
Magnesium	0.30
Sodium	0.20

C. pubescens is a common folk medicines used in the treatment of dropsy. The chemical study of this plant reveal it consist of amino acid, flavonoid, isoflavonoid, cyclohexitol, an galactotosyl-pinitol, and isoflavone glycoside (Bernadete*et al.*, 2000). Several polysaccharide posses reticuloendothelial system potentiating activity which demonstrated phagocytosis enhancement and suggesting imunostimulatory properties (Bernadete *et al.*, 2000). The leaves of *C. pubescens* can be used for the treatement of wound healing and burn (Ekpo *et al.*, 2011). In Laos *C. pubescens* is commonly known as Thu lai and can help to cure snake and scorpion bites (Delang *et al.*, 2007). The seed pod are use for womb cleansing after delivery. The pod is prepare as peper soup and the soup is taken twice daily by the woman who has just delivered (Chima *et al.*, 2013).

2.2 Phytochemicals in plant

Therapeutic value of plants used in trado-medicine derives from the presence of phytochemical principles, which are found in parts of the plants (Ayodele, 2003). Medicinal plants contain biologically active chemical substances. These complex chemical substances of different compositions are found as secondary plant metabolites in these plants. In modern medicine, plants are used as sources of direct therapeutic agents, as models for new synthetic compounds and as a taxonomic marker for discovery of new compounds (Sofowora, 1993).

Phytochemicals are the individual chemicals from which the plants are made. Phytochemicals is simply a word that means plant chemicals. Plants have the ability to synthesize mixtures of structurally diverse bioactive compounds with multiple and mutually potential therapeutic effects. Phytochemicals are divided into two groups, which are primary and secondary metabolites (Fraenkel, 1959). Primary is directly involved in normal growth, development and reproduction, while secondary are organic compounds that are not directly involved in the normal growth, development and reproduction of organism. Secondary metabolites have both defensive role against herbivory, pathogen attack and inter-plant competition and an attractant role towards beneficial organism such as pollinators or symbionts (Kaufman *et al.*, 1999). Plant secondary products also have protective actions in relation to abiotic stress such as those associated with changes in temperature, water status, light levels, UV exposure and minerals nutrients. Furthermore recent work has indicated potential roles of secondary products at the cellular level of plant growth regulator, modulator of gene expression and in signal transduction (Kaufman *et al.*, 1999).

Primary metabolites are of major importance to plants while secondary metabolites are of medicinal value to man and these can be equally obtained from various anatomical structures of plants. Man has benefited from the presence of these

chemicals by exploting the plant products as sources of sustenance in a variety of ways. In addition these chemical principles vary in distribution within the plant parts, as well as their occurrence within plant species (Bako *et al.*, 2005). These are influenced mainly by cultivation period, season of collection and plant-to-plant variability in the medicinal content (Nalawade and Tsay, 2003). That is why phytochemicals screening of plants must be done constantly, even on the ones whose secondary metabolites are already known. Addo *et al.*, (2008) reported that the leaf is the plant organ most widely used in phytomedicine and herbal theraphy. Hundreds of phytochemicals are currently being studied. Many are believed to have a major positive impact on human health. Important plant secondary metabolites have been isolated over a period of time from natural sources

2.2.1. Alkaloid

Alkaloid are naturally occurring chemical compounds containing one or more basic nitrogen atoms usually in combination as part of a cyclic system (Figure 2.1) (Huffman, 2003). The term alkaloid is derived from Arabic word *al-qali* that refers to potassium carbonate-containing ashes from plant material. The name alkaloid is also derived from the word alkaline (Raaman, 2006). Alkaloid have an alkaline pH and bitter taste. The basicity of alkaloids depend on the lone pairs electrons on their nitrogen atoms. As organic bases, alkaloids form salts with mineral acids such as hydrochloric acid, sulfuric acid and organic acid such as tartaric acid or maleic acid. These salts are usually more water-soluble than their free base form. Many alkaloids can be purified from crude extracts by acid-base extraction method. They are colourless, often optically active substances, most are crystalline but fews are liquid at room temperature. The most common precursor of alkaloid is amino acid and the biosysnthesis of most alkaloids are more complex (Hussain *et al.*, 2011).

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