DETAIL END OF THE PROJECT REPORT

BIOACTIVITY -GUIDED ISOLATION OF ANTIMICROBIAL AGENT FROM COLEUS AMBOINICUS LOUR (TORBANGUN)

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Abstract

The leaves of Coleus amboinicus from Indonesia (CAL-I) and from Malaysia, *Coleus aromaticus* (CAT-M), *Pogostemon cablin* (PC-M), *Coleus blumei*–red leaves (CBR-M), *Coleus amboinicus*–(CAL-M) *Coleus blumei* –purple leaves (CBP-M) were collected from different localities, freeze dried and extracted with aqueous methanol. The biological activity in vitro, especially in relation to total phenolic & flavonoid contents, antioxidant and antimicrobial activities were studied. Total phenolic content was determined according to the Folin-Ciocalteu method whilst antioxidant activity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The antimicrobial activity of the extract was determined by making use of macro dilution and disc diffusion methods on two gram positive bacteria, two gram negative bacteria and on fungus as well. Furthermore, the toxicity was also assessed for the extracts by performing acute toxicity test. The phenolic content among the six Lamiaceae leaves extract showed significant difference (p<0.05) in result ranging from 55.21 - 95.17 mg GAE/g of dried samples. *Pogostemon cablin* (PC-M) had the highest content of phenolic followed by *C. blumei* (CBP-M). There were significant differences (p<0.05) of IC_{50} value of six *Lamiaceae* leaves extract ranging from 10.5 – 34.1 µg/ml. Among the species studied, *Coleus amboinicus* (CAL-M) and *Pogostemon cablin* (PC-M) showed higher antioxidant activity compared to the other leaves extracts. All leaves extracts showed activity at least against one strain of bacteria and result showed significant difference (p<0.05) between activities on the microorganism studied. On the contrary, all of the leaves extracts were not effective against *C. albicans.* The minimum inhibitory concentration (MIC) of all leaves extracts ranged from 1.0–2.0 mg/ml in inhibiting the growth of S*. aureus*, *E. Coli*, P. *aeruginosa* and *B.subtilis.* The acute toxicity test using *C. Blumei* leaves extract showed that there was no mortality of animals recorded at the highest dose of 5000 mg/kg body weight. This study shows that the extracts can be used as antioxidant and antimicrobial agent without having the toxic effect.

Key words: *Coleus***,** biological activity, antioxidant and antimicrobial

INTRODUCTION TO THE PROJECT:

In Malaysia traditional medicines are still in use and are practiced in the community for treatment of disease and maintaining health. There are more than 14,500 species of flowering plants and approximately 1,200 of these plants are reported to have medicinal properties (Koshy et al., 2009). However, currently only 200 species are used in the preparation of various traditional medicines. Well known Malaysian medicinal plants include Kacip Fatimah (*Labisia pothoina*), Tongkat ali (*Eurycoma longifolia*), Misai kucing (*Orthosiphon stamineus*), Pegaga (*Centella asiatica*) and Mengkudu (*Morinda citrifolia*) which are used to produce herbal products in various forms such as pills, powders, syrups, tea bags, coffee, tablets and tonics. These herbs are available in Malaysian markets.

According to WHO, the use of traditional medicine is rapidly increasing. In Africa up to 80% of population use traditional medicine to help meet their health care needs. In Asia and Latin America, the communities use traditional medicine due to the history and cultural believes. The use of Complementary and alternative medicine is also becoming more popular, in

Australia, Canada USA, Belgium and France (WHO, 2005). *Coleus blumei,* a herb has not yet been studied for its potential medicinal efficacy. This herb can be found in several parts of South East Asia including Malaysia, Indonesia and Vietnam. Currently, this plant is only used as an ornamental plant because of having attractive leaves shape and colour. This plant is used in some traditional preparations by certain communities in Indonesia and Malaysia. The leaves are used to treat wound, cough and breast milk stimulus in lactating mothers (Damanik, et al., 2006). However, plants from the *Lamiaceae* family are most widely used in medicinal preparations compared to other plants families. A study by Gulhan et al., (2003) shows that members from this family contain high phenol, antioxidant agents and antimicrobial agents. As a members of the mint plant family, coleuses are close related to peppermint, spearmint (a flavour used mainly in chewing gums), salvia (genus of plants in the mint family), basil (culinary herb), thyme (culinary and medicinal herb), oregano (perennial spice), and swedish (herbal treatment) (Consolacion et al., 2001). Therefore, this was an effort to study the effect of *Coleus blumei* (two species), *Coleus amboinicus* (two species), *Coleus aromaticus* and *Pagostemon cablin* from *Lamiaceae* family the biological activity in vitro in relation to total phenolic & flavonoid contents, antioxidants and antimicrobial activities.

Objectives:

The aim and objective of this research was to evaluate *Lamiaceae* plants leaves extracts for total phenolic and flavonoid contents, antioxidant activities, antimicrobial activities and toxicity etc.

1. To determine the total phenolic and flavonoid contents of the leaves extracts of selected *Lamiaceae* leaves extracts.
2. To determine anti oxidative activity of selected *Lamiaceae* leaves extracts.
3. To determine antimicrobial activity of selected *Lamiaceae* leaves extracts against *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans.*
4. To assess the toxicity of *Coleus blumei* (ati-ati leaves) extracts in experimental mice.
5. To compare the phenolic contents, antioxidant activities and antimicrobial activity between *Coleus blumei* (Ati-Ati leaves) extract with other *Lamiaceae* species.

SIGNIFICANCE OF STUDY:

Natural products consumed as a food or used in alternative medicine causes no side effects in the individuals. Therefore, there is a growing interest in preventive health care and efforts are made to accomplish this through the extraction of plants materials and evaluation for medicinal uses. The use of plant extract as an alternative medicine plant still increases (Demiray et al., 2009). There is great potential for the *Lamiaceae* use as an alternative, in medicine, food industry and cosmetics. Therefore, in this study few species of the *Lamiaceae* family which are traditionally used but have not been studied were assessed for the assessment of phenolic and flavonoid contents, antioxidant activity, antimicrobial activity and toxicity etc. These are considered to be important for the evaluation of plant extract to be used in alternative medicine, food or cosmetic.

RESEARCH HYPOTHESES:

1) Selected *Lamiaceae* plant leaves extract contain higher total phenolic compound and flavonoids.
2) Selected *Lamiaceae* plant leaves extract has high antioxidant activity.
3) There is significant antimicrobial activity among *Lamiaceae* leaf extracts against selected microorganism (Gram-positive bacteria, Gram-negative bacteria and fungus).

BACKGROUND TO THE PROJECT:

There is continued search for medicinal plants or herbs around the world to make better use of the resources available without putting ourselves to the synthetic drugs treatment. Studies have been conducted in different parts of the world to understand their properties, safety and efficacy for natural therapies. According to WHO (2005), medicinal plants would be the best source to obtain a variety of natural therapies. About 80% of individuals from developed countries are still using traditional medicine and the demand for herbal and natural products based pharmaceuticals is increasing. Certain plants components are for example of phenolic compounds in plants are important for normal growth of plant and also important for human health (Gordana et al., 2007). Similarly, antioxidants play important roles in human health

including prevention of oxidative damages and reduce risks of chronic disease (Demiray et al., 2009; Dimitrios, 2006; Muktar et al., 1994; Stanner et al., 2004). The balance between an individual's intake of antioxidants and exposure to free radicals is considered crucial for health (Holford, 1997). In numbers plants contain numerous phenolic compounds, flavonoids and antioxidant activity (Gazzani et al., 1998; Vinson et al., 1998). Recently, studies on tea provided evidence that green tea contains catechins which an ant oxidative property and have effect on angiogenesis, (Demeule et al., 2000). The use of plants and herbs as antioxidants in processed foods is becoming of increasing importance in the food industry as an alternative to synthetic antioxidants (Charalampos et al., 2008; Sari et al., 2007; Souri et al., 2008). The use of plants compounds for pharmaceutical purposes has also gradually increased. Therefore, such plants are considered important to be investigated to understand their properties, safety and efficacy. Plants products have been used as antimicrobial traits, which are because of the active components synthesized in plants in the secondary metabolism for example, the phenolic compounds which are part of the essential oils (Gislene et al., 2000). Plants from *Lamiaceae* family have been shown to possess antimicrobial activity. Plant from this family, such as *Coleus forkohlii*, *C. amboinicus, C. aromaticus, C. blumei* and others possibly have antimicrobial activity. The leaves of these plants are traditionally used for treatment of severe bronchitis, asthma, diarrhea, epilepsy, renal and vesicle calculi and fever (Kumar et al., 2007). The *Coleus species* are used in the Asian traditional medicine to treat angina (chest pain), asthma, bronchitis, epilepsy, insomnia (sleeping difficulties), painful urination, skin rashes and a wide range of digestive problems. These studies have reported that a number/concentration of phenolic components/contents is fairly higher. Therefore capacity to act as antioxidants and antimicrobial agents is presumably also higher.

THE LAMIACEAE PLANTS FAMILY:

The *Lamiaceae* (syn. *Labiatae*) family is one of the largest plant family shrubs and herbs, found all over the world. The *Lamiaceae* is also called 'mint family' includes herbs (eg: Basil, Mint, Sage and Thyme), ornamental plants (*Coleus, Leonotis*) and weeds (Henbit, Ground Ivy and Self-Heal). It consists of more than 200 genus and 3500 species. Many species in the *Lamiaceae* have a long history of use in culinary spices and folklore medicine, including lavender, mint, oregano, basil and thyme. The use of medicinal plants in the treatment of

diseases from *Lamiaceae* species in Malaysia are compiled in theThe importance of many family members plants to the culinary and essential oil industries has been commercially explored for more than 75 years (Ghalem et al., 2008; Lawrence, 1992) as indicated in the Table 1.

Table 1. Some Medicinal plants from *Lamiaceae* species in Malaysia

Species	Local Name	Claimed Properties
Hyptis breyipes	Sawi enggang, sawi hutan, ati-ati puteh	Cuts, worms
Ocimum sanctum	Selasih	Diuretic, anti-pyretic
Orthosiphon aristatus	Misai kucing	Reduces blood presure, anti-diabetic
Pogostemon cablin	Pokok nilam	Boils, headaches

BOTANY OF THE LAMIACEAE:

The *Lamiaceae* is one of the most readily recognized families of flowering plants. Members of the family *Lamiaceae* are herbaceous, less often shrubs or rarely tree with stems and branches usually four-angled. The leaves are simple and not divided, in pairs up the stem, each pair at right angles to the last, and they are frequently hairy or with scent glands and emitting minty smell when crushed. Their flowers have two lips, one more protruding than the other (labia is the Latin for 'lip') give this plant family its original name of *Labiatae.* Generally, the upper lip has two lobes and forms a hood over the lower lip, and the lower lip consists of three lobes which form a landing platform for pollinating insects.

Botanical Classification of plants used in the project

Common Names: Ati-ati
Scientific Name: *Coleus blumei* (*Solenostemon scutellarioides*)
Family : *Lamiaceae* (*Labiatae*)
Genus : *Coleus* or *Solenostemon*
Part used: Leaves

Common Names: Ati-ati Merah
Scientific Name: *Coleus blumei* (*Coleus artopurpureus* and *Solenostemon scutellarioides*)
Family: *Lamiaceae* (*Labiatae*)
Genus: *Coleus* or *Solenostemon*
Part used: Leave

Common Names: Bangun-bangun (Malaysia) Torbangun Indonesia)
Scientific Name: *Coleus amboinicus Plectranthus amboinicus (Lour.) Spreng*
Family : *Lamiaceae* (*Labiatae*)
Genus : *Coleus* or *Plectranthus*
Part used : Leaves

Common Names: Jemuju, Bangun-bangun (Malaysia)
Scientific Name: *Coleus aromaticus*
Family : *Lamiaceae (Labiatae).*
Genus : *Coleus* or *Plectranthus*
Part used : Leaves

Common Names: Nilam (Malaysia)
Scientific Name: *Pogostemon cablin*
Family : *Lamiaceae (Labiatae).*
Genus : *Pogostemon*
Part used : Leaves

Uses of Lamiaceae plants:

Members of the family *Lamiaceae* possess great pharmacological and commercial values. Many of the species are used in traditional and modern medicine. There are different uses of the family members in traditional ways in different parts of the world. Members of the family can group to their uses into three main categories;

1) medicinal; 2) ornamental and 3) aromatic plants which are used as culinary herbs, vegetables and in the perfume industry (Farzaneh et al., 2005).

Medicinal Lamiaceae plants:

Orthosiphon Stamineus Benth also known Misai kucing from *Lamiaceae* has been used as a medicinal herbs for many centries in South East Asia (Malaysia, Indonesia and Thailand) for treating kidney ailments and bladder related diseases (Sahib et al., 2009). In Iran, 18% of the species are used for medicinal purposes. Leaves are the most widely used plant parts (Farzaneh et al., 2005). Essential oils of various members possess biological activities. Hori (1999) investigated the essential oils of ten *Lamiaceae* plants and found that it possesses the inhibitory and insecticidal activities.

Culinary herbs:

Lamiaceae also known for their culinary properties like oregano, rosemary, sage and thyme used as seasonings in the Mediterranean region, and especially oregano. United State Food and Drug Administration give Generally Recognized as Safe (GRAS) status to Oregano, rosemary, sage and thyme. Therefore, these are generally recognized as safe for human consumption without limitations on intake (U.S. Food and Drug Administration, 2008).

Lamiaceae are best known for their essential oils common to many members of the family. Many biologically active essential oils have been isolated from various members of this family (Farzaneh et al., 2005). The essential oils of Lamiaceous plant from mint like menthol, is being used in a variety of food and medicinal products.

Ornamental:

Many species of the *Lamiaceae* are attractive to grow in the garden, for example, the genus *Coleus. Coleus* is grown as an ornamental in various parts of the world. These plants are natives of Indonesia and Africa. *Coleus blumei*, which has nettle-like, bronze-coloured leaves. The colours of their foliage range through yellow, red, crimson, and pink (Chung et al., 2008).

Coleus blumei:

Coleus blumei also known as *Solenostemon scutellarioides* and *t*he local Malay name is 'Ati-ati' plant with colourful foliage. Varieties of *Coleus blumei*, from Java, are well-known house and garden plants up to one meter (three feet) tall. They have square stems and two-lipped

flowers, which in *Coleus blumei* are small, blue, and in spikes. The coleus is a brightly coloured shrub with square stems and leaves heart-shaped to deeply fringe. The foliage can be of any combination of yellow, pink, orange, red, or green. *Coleus blumei* is just one of over 150 species of *Coleus*. *Coleus blumei* (*Lamiaceae*) is an ornamental plant, growing all over the world in an enormous number of different cultivars that vary in colour *Coleus blumei* so interesting is the incredible foliage, with arrays of colour combinations unmatched by other species and shape of the leaves (Karen, 2007). *Coleus blumei* has an interesting ability to change its leaves colour depending on the intensity of the sunlight. In fact, on direct sunlight it will flame with beautiful burgundy-red colour when less intensity of the sunlight the leaves become greener. Since it was introduced to the horticultural world in 1825, it has always been popular especially as a garden plant. *Coleus blumei* is one of only a few species with members that can thrive in sun or shade. The male portions of coleus flowers, the stamens, are fused into a tube or sheath. Blumei - named for Karl Ludwig Blume (1796-1862), a Dutch writer who wrote about plants from Java. As explain before, there are many varieties of *Coleus blumei*. In Malaysia *Coleus blumei* was known as 'Pokok Ati-ati' because the shape of the leaves like (heart-shaped) only two selected *Coleus blumei* hybrid were studies. The first species known as the 'pokok ati-ati' with purple leaves and another known as 'pokok ati-ati tree with red leaves. Both species are widely used for medicinal purposes.

The uses of Coleus blumei:

Coleus species are famous for ornamental purposes, but it is also used for medicinal purposes in some communities. In Malaysia it has been used for the treatment of various diseased condition such as high blood pressure, cuts and wounds to stop bleeding (Ong & Nordiana, 1999; Ong & Norzalina, 1999 and Natasa et al., 2002).

The uses of Coleus amboinicus:

Coleus amboinicus is used for the treatment of cough, colds and fever (Rout et al., 2010). It is also used for the treatment of kidney and bladder stones, bone fractures, headaches, asthma, bronchitis, indigestion, diarrhea, dysentery insect bite, fever and cholera (Kumar et al., 2008). Bataknese lactating women traditionally consuming Torbangun (*Coleus amboinicus* Lour (CAL-I) leaves traditionally in the first month after giving birth to stimulate breast milk production (Damanik et al., 2006).

The uses of Coleus aromaticus:

It is used in traditional medicines (Ayurveda (traditional medicine native to the Indian), homeopathy, Unani and Siddha (South Indian Medicines)). The Malay community use the juice or decoction (extraction by boiling) for pains relief around stomach and heart. The Chinese community of Peninsula use the juice with sugar for children cough treatment (Garcia et al., 1973; Lukhoba et al., 2006). The aromatic leaves are used for flavouring meat, soups, and fish and are eaten as a vegetable in most tropical countries and Africa. In India (south), externally it is applied for burns and insect bites, internally as a carminative and also to control asthma. Daily chewing of a leaf is recommended for all age groups especially during winter (Kiruba et al., 2006; Pritima et al., 2008). The Philippines externally apply macerated (soft by soaking) fresh leaves around burns or bruised and applied locally to the bites of centipedes and scorpions. They are also applied to the temples (side of the head behind the eyes) and forehead for headache (Leipzig, 1995).

The uses of Pogostemon cablin:

Pogostemon cablin is used in traditionally herbal medicine not only for the treatment common cold, nausea and diarrhea but also headache and fever. The volatile oil of *Pogostemon cablin* is used in cosmetics and oral hygiene products, such as scent perfumes and flavoured toothpaste (Tsung et al., 2009). It is also antidepressant, anti-inflammatory agent, antiseptic, aphrodisiac (sexual desire enhancer), astringent (chemical compound that tends to shrink or constrict body tissues), carminative, diuretic, febrifuge (A medication that reduces fever), fungicide, insecticide, sedative and tonic. (Supawan et al., 2006). *Pogostemon cablin* is a well-known herb in Korean traditional medicine. It has effects on regulating vital energy and eliminating phlegm (mucus) and is a typical used in treatments of, syncope and cerebral stroke (apoplexy) (Hyung et al., 2007).

PHYTOCHEMICALS IN PLANTS:

Phytochemicals are non-nutritive natural bioactive compounds found in plants which interact with nutrients and dietary fibre to protect or prevent diseases. Plants produce these micronutrients and variety of biologically active secondary metabolites which provide colour, flavour and natural toxicants to pests and sometimes humans. There are more than thousand known phytochemical of various plants. It is estimated that more than 25,000 terpenoids, 12,000 alkaloids and 8,000 phenolics have been identified in plants (Lampe, 2003). Recent study demonstrated that the bioactive non-nutrient compounds in fruit, vegetables and plants

have been linked to a reduced risk of major chronic diseases (Liu, 2003). The contribution of the phytochemicals effects in affect human health because of the important phytochemicals phenolic and its derivatives. The phytochemicals effectiveness in different parameters is shown in the Table 2 and 3.

Table 2 Different action of phytochemicals

Phytochemical action	Active compound	Source
Antioxidant activity	Polyphenol flavonoid	tea, fruit and vegetable
Hormonal action	Isoflavan	soy milk,
Stimulation of enzymes	Indoles	Cabbages
Interference with DNA replication	Saponins	Beans
Anti-bacterial effect	Allicin	Garlic
Physical action	Phytochemicals bind physically to cell walls thereby preventing the adhesion of pathogens to human cell walls.	

Table 3 Flavonoid subclasses, their chemical characteristics, name of prominent food Flavonoids and typical food sources

Flavonoid subclass	C ring unsaturation	C ring functional group	Prominent food flavonoids	Typical rich Food sources
Flavanol	none	3-hydroxy 3-O-gallate	(+)-Cathechin (+)-Gallocatechin (-)-Epicatechin-3-gallate	Teas, red grapes red wine, cocoa beverages and chocolate
Flavanones	none	4-Oxo	Eriodictyol Hesperetin Naringenim	Citrus foods (orange, lemon etc)
Flavones	2-3 Double bond	4-Oxo	Apigenin Luteolin	Green leafy spice e.g Parsley
Isoflavones	2-3 Double bond	4-Oxo	Daidzein Genistein Glycitein	Soybeans, soy foods and legumes
Flavonols	2-3 Double bond	3-Hydroxy, 4-Oxo	Isorhamnetin Kaempferol Myricetin Quercetin	Apple, tomato, cherry, Broccoli, yellow onion etc
Anthocyanidins	1-2-3-4 Double bonds	3-Hydroxy	Cyanidin Delphinidin Petunidin	Red, purple, blue berries, plum, strawberry etc

Source: Parul et al., (2007).

METHODOLOGY

Acquiring of Plants Leaves

This study involved six plants from *Lamiaceae* family. The leaves of *Coleus amboinicus* (CAL-I) and Jakarta (Indonesia), *Coleus aromaticus* (CAT-M), *Pogostemon cablin* (PC-M), *Coleus blumei* –red leaves (CBR-M), *Coleus amboinicus*– (CAL-M) *Coleus blumei* –purple leaves (CBP-M) from Kuantan (Malaysia). The list of the plants and methodology is indicated in the in Table 4 and flow chart 1 respectively. The abbreviation assigned here to the plants will be used in rest of the project report instead of full name.

Table 4 Characteristics of the plants extracts

Scientific name		Common name	Place of collection
Coleus amboinicus	CAL-I*	Torbangun	Jakarta
Coleus aromaticus	CAT-M**	Jemuju, Bangun-bangun	Kuantan
Pogostemon cablin	PC-M**	Nilam	Kuantan
Coleus blumei - purple leaves	CBR-M**	Ati-ati	Kuantan
Coleus blumei - red leaves	CAL-M**	Ati-ati merah	Kuantan
Coleus amboinicus	CBP-M**	Bangun-bangun	Kuantan

*Indonesia, **Malaysia

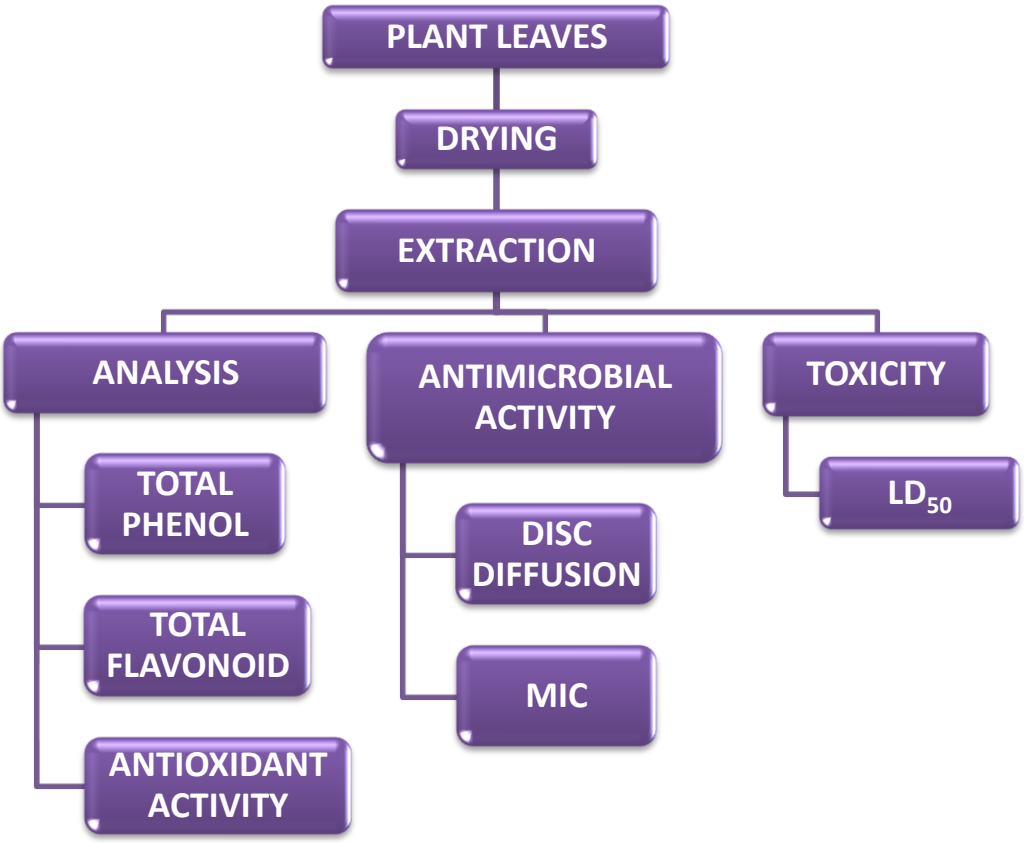


Figure 1 Flow Chart of the Methodology

Preparation of Plants Leaves

Fresh leaves were cleaned and rinsed with distilled water to remove all contaminates (dirt and soil). The leaves were rinsing and freeze-dried using a freeze dryer (Biotron Cleanvac 12). Freeze drying was performed for four days. The freeze dried leaves were ground and sieved to 0.45 µm using Micromill (Bel. Art Product). The ground sample was then stored in screw capped glass jar at 4oC until further analysis.

Extraction of freeze dried leaves sample

The ground sample (500 mg) was extracted by suspended in 50 ml of (40: 60) aqueous methanol (v/v). The aqueous methanol containing sample was heated and shaked at 60 ^{o}C for 24 hours. The extracts were filtered through fast filter paper (Whatman No 3) (Whatman International Limited) into 100 ml volumetric flask, rinsed with aqueous methanol and the volume was made up to 100 ml.

DETERMINATION OF TOTAL PHENOL CONTENT:

Principle of the Method:

Total phenolic contents were determined by the Folin–Ciocalteau method (Singleton et al., 1965), based on complex formation of molybdenum-tungsten blue. The samples were allowed to react with Folin-Ciocalteu's reagent and sodium carbonate solution. The phenolic contents were estimated using a standard curve of gallic acid and expressed as mg of gallic acid equivalents (GAE) / g dried weight of sample (DW).

Sample Analysis:

One ml of the extract was pipetted into a 50 ml volumetric flask and made up to 50 ml with aqueous methanol. Aliquot (1 ml) of each of the extract was mixed with one ml Folin-Ciocalteu reagent in a test tube that contained nine ml of distilled water. Sample was shaken using vortex mixer and added one ml sodium carbonate solution (20% m/v anhydrous sodium carbonate in distilled water). Sample was shake again and left to stand for 30 minutes to obtain maximum colour development. The absorbance was measured at 760 nm using UV-Vis spectrophotometer (Shimadzu UV-1650PC spectrophotometer with 10 mm path length cell). Aqueous methanol was used as a blank. Pure gallic acid was used as the standard reference compound. A calibration curve of gallic acid (range 0–20 µg/ml) was prepared and the results determined from regression equation of the calibration curve were expressed as mg of gallic acid equivalents (GAE)/g sample on dry weight basis.

The contents of phenolic compounds in the sample were reported as of gallic acid equivalents per g dried sample. Total phenolic content of each sample was calculated as:

$$\text{Phenol content GAE mg/g} = \frac{[\,(\text{Slope} * \text{absorbance}) + c\,]}{\text{Sample Concentration}}$$

Where: GAE is gallic acid equivalents

c is the y-intercept

DETERMINATION OF TOTAL FLAVONOID CONTENT:

Principle of the Method:

The total flavonoid content of the plant extract was estimated according to Aluminium chloride method. Quercetin was used as reference compound to produce the standard curve and expressed as mg of Quercetin Equivalents (QE)/100 mg of plant extract.

Sample Analysis:

For the determination of total flavonoid content in the plant extract, 2ml of extract solution were pipetted into 25 ml volumetric flask containing 1.0 ml of aqueous aluminium trichloride ($AlCl_3$) solution at 2.5% and the volume made up with methanol. After 30 min the absorbance was read at 425 nm against a blank sample.

A standard curve was built with quercetin reference solutions. Aliquots ranging from 2 to 6 ml of standard quercetin ethanolic solution (50µg/ml) were pipetted into 25 ml volumetric flasks containing 1.0 ml of aqueous aluminium trichloride ($AlCl_3$) solution at 2.5% (w/v) and the volume made up with methanol. The blank was prepared by diluting 1ml of aluminium trichloride ($AlCl_3$) solution in a 25ml volumetric flask with methanol. After 30 min, the absorbance measured at 425 nm.

DETERMINATION OF ANTIOXIDANT ACTIVITY:

Principle of the method

Radical scavenging activity of extracts was measured by modified method of Brand-Williams, et al., (1995). This method has been developed to determine the antioxidant activity of foods which utilizes the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. The odd electron in the DPPH free radical (purple colour) gives a maximum absorption at 517nm. When the odd electron of DPPH radical makes pair with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H, the colour turns from purple to yellow. The resulting discolorization is stoichiometric with respect to number of electrons captured.

Sample Analysis:

Aqueous methanol leaves extract (5 mg/ml) were diluted to final concentrations of 3, 7, 13 and 20 µg/ml in methanol. The DPPH solution with a concentration of a 0.004% (w/v) solution of DPPH in methanol was prepared. Three ml of this solution was mixed with one ml extract solution in a test tube. Each mixture was then shaken vigorously and held for 30 min at room temperature in the dark. Discoloration was measured at 517 nm after incubation for 30 min by using a spectrophotometer as described by (Zakaria et al., 2008). The actual decrease in absorption induced by the test compounds was compared with the positive controls. The

inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

Inhibition % = [(Abs_{t0} - Abs_{t30min}) / Abs_{t0}] x 100

Where:

$Abs_{t0min:}$ the absorbance of DPPH at time zero.

Abs_{t30min}: the absorbance of DPPH after the 30 minutes of incubation.

Ascorbic acid was used for comparison or as positive controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals. The experiment was carried out in triplicate. A plot of absorbance vs. concentration was made to establish the standard curve and the regression equations were used to calculate the IC_{50} value (Zakaria et al., 2008).

Absorption of blank sample containing the same amount of methanol and DPPH• solution was prepared and measured. The inhibition percentage determined from the absorbance of DPPH was compared between with each concentration of the sample extract solution added.

ANTIMICROBIAL ACTIVITY OF THE PLANT EXTRACT:

Principle of the method:

Bacteria test organism was maintained on stock culture agar inoculated in nutrient broth. The broth seeds were inoculated at 37^{o}C for 24 hours. The antibacterial assay was carried out by disc diffusion technique. Disc was impregnated with extracts, dried and then placed on the inoculated agar. The clear zone of inhibition around the disc was measured in millimeters.

Type of Microorganism

The methanolic extracts of the leaves were tested for their antimicrobial activity for the following microorganisms:

1. Gram Positive bacteria :
    a. *Bacillus subtilis*
    b. *Staphylococcus aureus*
2. Gram Negative bacteria:
    a. *Escherichia coli*

b. *Pseudomonas aruginosa*

3. Fungi :

a. Candida albicans

These microorganisms were obtained from the culture collection at the Biotechnology Laboratory, Kulliyyah of Science, International Islamic University Malaysia (IIUM), Kuantan.

Preparation Mc Farland standard and inoculum

McFarland 0.5 turbidity standards were prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$) solution to 99.5 ml of 1% (v/v) sulfuric acid. The accuracy of the density of a prepared McFarland standard was checked by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standard, the absorbance at a wavelength of 625 nm should be 0.08 - 0.1 A. The McFarland standard was used to adjust the turbidity of the inoculums for the susceptibility test.

At least three to five well-isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4 to 5 ml tryptic soy broth. The broth culture was incubated at 35°C until it achieved or exceeded the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours). The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standard. To perform this step properly, visually compared the inoculums tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines. This resulted in a suspension containing approximately 1 - 2 x 10^8 CFU/ml for microorganism (Handa et al., 2006).

Preparation of Disc:

Dried ground leaves were weighed, macerated with 60% methanol for 24 hours at 60°C and then filtered. The filtrate was evaporated to a thick filtrate at 60°C then the extracts were reconstituted with 60% methanol. The concentration brought to be equivalent to 100mg of dried leaves extracts / ml.

The discs (diameter, 6mm) were impregnated with 20 µl of extract, corresponding to 2mg of dried leaves per discs and allowed to dry under laminar flow cabinet. Disks impregnated with 20 ul of aqueous methanol and sterilized water served as negative controls. The discs stored at 4 ^{o}C until use (Wei, et al., 2008)

Disc Diffusion Method:

Screening of leaf extracts for antimicrobial activity was performed by the disk diffusion method. It was performed using an 18 h culture (approximately 10^5CFU/ml at 37°C in 10 ml of Mueller Hinton Broth). Five hundred micro-liters of the suspensions were spread over the plates containing Mueller-Hinton agar using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. The discs (contained crude extract) placed on the inoculated agar. A standard disc contained chloramphenicol (25µg/disc) was used as positive reference control whereas blank disc was used as negative controls.

All petridishes were sealed with sterile parafilm to avoid eventual evaporation of the samples. The plates were left for 30 min at room temperature to allow the diffusion of extract, and then incubated at 37^{o}C for 24 h (18 h was fixed as the maximum since there was no change in the inhibition up to 24 h for bacteria whereas the incubation of 30^{o}C for 24-48 h was used for yeast and fungi. The results were recorded by measuring the zones of growth inhibition surrounding the disc. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. The antibacterial activity was interpreted from the size of the diameter from the clear zones surrounding the discs. After incubation, the diameter of inhibition zone was measured in millimeters. An inhibition zone of 14 mm or greater (including diameter of the disc) was considered as high antibacterial activity. Diameter of inhibition zones were measured manually in triplicate (Nagshetty et al., 2010; Ramzi et al., 2005).

Minimum Inhibitory Concentrations (MIC) Method:

The MIC method using broth dilution method was applied on extracts that proved their high efficacy against test microorganisms by disc diffusion methods. The standard agar dilution protocol with doubling dilution was used. The extract was incorporated into nutrient broth at concentrations of 0.5 – 2.5 mg/ml. A control without the extract was also set up. 10*µl* each of the test organisms, previously diluted to give 10^{6} CFU/ml was used to inoculate the plates (see Appendix IV). These were incubated at 37 ^{O}C for 24 hours in the first instance, and for another 24 hours, before the results were recorded after observing for growth. The minimum inhibitory concentrations (MICs) of the extract for each test microorganism were regarded as the agar plate with the lowest concentrations without growth (Goran et al., 1988; Klančnik et al., 2010).

ACUTE TOXICITY ASSESSMENT IN ANIMALS:

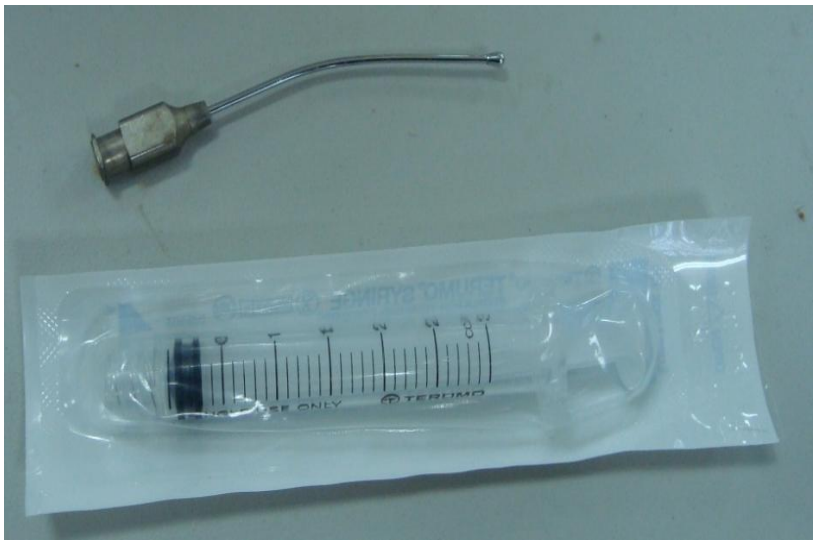
Albino Swiss mice of both sexes weighing between 25 – 30 g were used in this study. Thirty mices were randomly divided into six groups of five mices each for oral acute toxicity. The animals were housed in well ventilated room at temperature 24^{o}C, feed with standard) and water.

Determination of Median Lethal Dose (LD_{50})

The acute toxicity of the fraction was evaluated according to General Guideline for Methodologies on Research and Evaluation of Traditional Medicine (WHO, 2000) after single orally feeding of the extract.

Acute toxicity of the aqueous leaves extract of *Coleus blumei* was determined orally in mice. The first groups was given 2 ml of normal saline while groups 2 - 6 were given graded doses orally of 1000, 2000, 3000, 4000, 5000 mg/kg body weight of the extract respectively using CRV feeding needle (Kent Scientific Crop). The mice were observed for signs of acute toxicity like behavioral changes and death over 72h. The Acute toxicity (LD_{50}) of the extract was calculated using the arithmetic method of Karber as modified by (Aliu et al., 1982; Sarkiyayi et al., 2009). The Acute toxicity (LD_{50}) of the extract was calculated using the arithmetic method of Karber as modified by (Aliu et al., 1982; Sarkiyayi et al., 2009)

$$LD_{50} = \text{Least dose that killed all the animals} - \frac{\text{Sum of (Dose difference} \times \text{Mean dose)}}{\text{No. of animals/group}}$$



CRV feeding needle and syringe

Mice feed with extract using CRV feeding needle

STATISTICAL ANALYSIS:

Data were reported as mean and standard deviation for triplicate measurements. Analysis of variance and Tukey's honestly significant difference tests were conducted using SPSS software for Windows (Version Rel. 11.0.5., 1999, SPSS Inc., Chicago) to determine differences among means and the statistical significance was determined at 95% confidence interval.

RESULTS:

This study was conducted on the various species of *Lamiaceae* from Malaysia and Indonesia. The parameters studied includes, the determination of phenolic and flavonoid contents, antioxidant activity, anti-microbial activity and toxicity of the dried leaves extracts. The details of the results for the various listed parameters are indicated in the preceding sections;

Phenolic content of *Lamiaceae* dried leaves

As one of the important plant components, phenolic compounds were widely investigated in many medicinal plant (Terán et al., 2008). The observed content of total phenol in *Lamiaceae* leaves extract are given in Table 1. The amount of total phenolic content varied in dried leaves ranging from 55.21 - 95.17 mg GAE/g dried sample. There was significant difference (p<0.05) among the total phenol content for six *Lamiaceae* leaf extracts. The phenolic contents from PC-M and CBP-M extract were not statistically different from each other. However, the phenolic contents in the PC-M and CBP-M extracts were significantly (p<0.05) higher in

concentration than in the other four extracts. Among the extracts, CAL-I contained significantly lower concentration of total phenolics contents compared to all other extracts of *Lamiaceae*. The ranking of the *Lamiaceae* based on the highest to the lowest phenolic content in the extract of dried of leaves is as followed:

PC-M > CBP-M > CAT-M > CAL-M > CBR-M > CAL-I.

Table 1 Total phenol content in different species of dried *Lamiaceae* leaves.

Plants	Phenol content (mg GAE/g DW)
Coleus amboinicus– Indonesia (CAL-I)	55.21 ±3.20[d]
Coleus amboinicus – Malaysia (CAL-M)	86.27 ±4.67[c]
Coleus blumei –red leaves (CBR-M)	85.35 ±3.90[c]
Coleus aromaticus (CAT-M)	90.41 ±1.95[b]
Coleus blumei –purple leaves (CBP-M)	94.01 ±4.61[a]
Pogostemon cablin (PC-M)	95.17 ±2.82[a]

Data are mean ± standard deviation, n=18

Means with different superscripts are significantly different ($p<0.05$)

Flavonoid Content of Plant Extract:

The flavonoid contents determined in the dried *Lamiaceae* leaves extract are presented in Table 5. The amount of flavonoid in dried leaves ranged from 0.18 - 15.21 mg QE/g dried samples. There were significant differences ($p<0.05$) in flavonoid contents among the six *Lamiaceae* leaves extract. CBP-M extract contained significantly ($p<0.05$) high amount of flavonoid compared to other leaf extracts, while the extracts of CAL-I had significantly lowest flavonoid content. Briefly amounts of flavonoid was the highest in CBPM followed by CAL-M, CBR-M, PC-M, CAT-M and CAL-I.

Table 5 Total flavonoid content in the various species of *Lamiaceae* leaves.

Plants	Flavonoid content * (mg QE/g)
Coleus amboinicus– Indonesia (CAL-I)	0.178±.07^{e}
Coleus amboinicus– Malaysia (CAL-M)	14.08 ±0.20^{b}
Coleus aromaticus (CAT-M)	6.00 ±0.24^{d}
Pogostemon cablin (PC-M)	8.88 ±0.33^{c}
Coleus blumei –red leaves (CBR-M)	13.66 ±0.18^{b}
Coleus blumei –purple leaves (CBP-M)	15.21 ±0.28^{a}

*Data are mean ± standard deviation, n=18
Means with different superscripts are significantly different ($p<0.05$)

Antioxidant Activity of Lamiaceae leaves:

The IC50 of various *Lamiaceae* leaf extracts and the positive control ascorbic acid, is shown in the Table 6. The IC50 range from 10.5 – 34.1 μg/ml and there was a significant difference ($p<0.05$) in IC_{50} among the six *Lamiaceae* leaves extracts. The IC_{50} value of ascorbic acid was 2.48 μg/ml and the extract of plants PCM, CAL-M, CBP-M, CAT-M, CBR-M and CAL-I were 10.5, 12.5, 12.9 , 14.5, 15.9 and 34.1 μg/ml respectively (Table 6). These results indicate that CAL-M and PC-M had higher antioxidant activity compared to the other leaf extracts.

Table 6 IC_{50} of *Lamiaceae* leaves based on the extracts free DPPH radical scavenging activity

Plants	IC_{50} * (µg/ml)
Ascorbic acid	2.48
Coleus amboinicus – Indonesia (CALI)	34.1 ±1.8^{e}
Pogostemon cablin (PCM)	10.5 ±0.22^{a}
Coleus amboinicus– Malaysia (CALM)	12.5 ±0.32^{b}
Coleus blumei –purple leaves (CBPM)	12.9 ±0.26^{bc}
Coleus aromaticus (CATM)	14.5 ±0.08^{cd}
Coleus blumei –red leaves (CBRM)	15.9 ±0.21^{d}

Data are mean ± standard deviation
Means with different superscript are significantly different at ($p<0.05$)

Minimum Inhibitory Concentration (MIC) of *Lamiaceae* leaf extracts

The MIC of six *Lamiaceae* plant extracts are shown in Tale 7. The lowest MIC means the highest antimicrobial activity whereas MIC higher than 25mg/ml were considered inactive (Paul et al., 2006). From the Table 7 it is evident that all plant extract showed minimum inhibitory concentration (MIC) and ranged from 1.0–2.0 mg/ml inhibiting the growth of S*. aureus*, *E. coli*, *P. aeruginosa* and *B.subtilis.* All the plant extracts do not inhibit the growth of *C. albicans* at concentration ranged 0.5-2.5 mg/ml. The MIC values indicated that extracts of all Lamiaceae *were* more potent against bacteria than against fungi. The aqueous methanol extract of *CALI* showed the lowest MIC (1.0 mg/ml) compared with other leaf extracts 2.0 mg/ml. Overall antimicrobial activity screening results is indicative of the potential of the *Lamiaceae* plant as effective medicaments in the treatment of microbial infection.

Disc diffusion of *Lamiaceae* leaf extracts

The results indicate that the growth of Pseudomonas *aeruginosa, Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus* were inhibited. All of the extracts were not effective to inhibit the growth of *Candida albicans.* The size of the zone inhibition was compared with the standardized zones of inhibition by antibiotic (Figure 2)

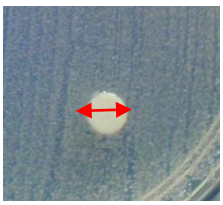
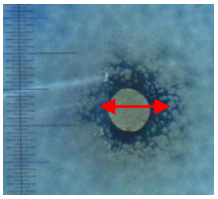
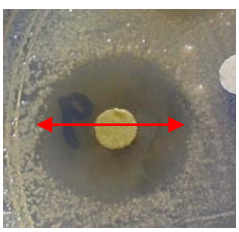
	zone diameter (mm)	Description
	≤ 12 mm Resistant	If the zone of inhibition is less than the standard, the organism is considered to be resistant
	13 - 17 mm Moderately susceptible	If a bacterial colony is somewhat susceptible to an antibiotic, then the zone of inhibition will measure in-between that of a susceptible and resistant colony
	≥18 mm Susceptible	If a bacterial colony is susceptible to an antibiotic, then a zone of inhibition will form around an antibiotic disk

Fig. 2 Classification for zone diameter interpretive with standards (Chloramphenicol)

Table 7 Minimum Inhibitory Concentration (MIC) Values of the 60% Methanol leaf extracts

Plant leaves Extracts	Conc. mg/ml	Presence/Absence of Growth				
		Bacteria strain				Fungus
		Pa	Ec	Sa	Bs	Ca
Coleus amboinicus – Indonesia (CALI)	2.5	-	-	-	-	+
	2.0	-	-	-	-	+
	1.5	-	-	-	-	+
	1.0	-	-	-	-	+
	0.5	+	+	+	+	+
Coleus aromaticus (CATM)	2.5	-	-	-	-	-
	2.0	-	-	-	-	-
	1.5	+	+	+	+	+
	1.0	+	+	+	+	+
	0.5	+	+	+	+	+
Pogostemon cablin (PCM)	2.5	-	-	-	-	+
	2.0	-	-	-	-	+
	1.5	+	+	+	+	+
	1.0	+	+	+	+	+
	0.5	+	+	+	+	+
Coleus blumei –purple leaves (CBPM)	2.5	-	-	-	-	+
	2.0	-	-	-	-	+
	1.5	+	+	+	+	+
	1.0	+	+	+	+	+
	0.5	+	+	+	+	+
Coleus blumei –red leaves (CBRM)	2.5	-	-	-	-	+
	2.0	-	-	-	-	+
	1.5	+	+	+	+	+
	1.0	+	+	+	+	+
	0.5	+	+	+	+	+
Coleus amboinicus – Malaysia (CALM)	2.5	-	-	-	-	+
	2.0	-	-	-	-	+
	1.5	+	+	+	+	+
	1.0	+	+	+	+	+
	0.5	+	+	+	+	+

(+) = presence of growth, (-) = absence of growth
Sa = S. aureus, Ec = E. coli, Pa = P. aeruginosa, Bs = B.subtilis, Ca = C. albicans

Antimicrobial Properties of CATM, PCM, CBPM, CBRM and CALM

The antimicrobial activity of dried leaves extract of CATM, PCM, CBPM, CBRM and CALM is indicated in 8. The dics absorbed extracts at a concentration of 2mg/disc are significant by different (p<0.05) among the zone inhibition of five microorganisms. Size of zone inhibition of *Staphylococcus aureus* (17.8-21.6 mm) was significantly (p<0.05) larger than for the other four microorganism *Bacillus subtilis* (7.3-9.5mm), *Pseudomonas aeruginosa* (6.5-8.8.0mm), *Escherichia coli* (7.3-9.3 mm) and *Candida albicans*. Result also show that CATM, PCM, CBPM, CBRM and CALM extract are more effective inhibiting *Staphylococcus aureus* compared to

other bacteria *Pseudomonas aeruginosa, Bacillus subtilis*. All extracts are more active inhibiting gram positive bacteria *Staphylococcus aureus* rather than gram negative bacteria *Pseudomonas aeruginosa, Bacillus subtilis.* However all the extracts are not effective in inhibiting the growth of fungus *Candida albicans.* The results also shows that all Lamiaceae leaves extracts from Malaysia show the same pattern of inhibition growth of microorganism , that only *Staphylococcus aureus is* susceptible to CATM, PCM, CBPM, CBRM and CALM extracts while other microorganisms (*Pseudomonas aeruginosa, Escherichia coli*, *Bacillus subtilis* and *Candida albicans*) are resistant to the extracts.

Table 8 Antibacterial activities of six plants extracts against five microorganism using the disc diffusion method

Plant Extracts (2mg/disc)	Zone of Inhibition (mm)				
	Gram negative bacteria		Gram positive bacteria		Fungi
	Pa	Ec	Sa	Bs	Ca
CALI	18.5±0.3^{a}	16.6±1.5^{a}	Na	13.0±0.8^{a}	N
CATM	7.3±0.5^{c}	7.3±0.5^{bc}	19.3±0.5^{ab}	7.5±0.0^{c}	N
PCM	10.0±1.3^{b}	8.3±1.5^{bc}	21.6±1.5^{a}	8.8±0.2^{bc}	N
CBPM	7.5±0.8^{c}	9.3±0.5^{b}	19.8±1.7^{ab}	8.1±0.2^{bc}	N
CBRM	6.5±0.0^{c}	6.6±0.2^{c}	17.8±0.2^{b}	7.5±1.7^{c}	N
CALM	9.6±1.0^{bc}	7.3±0.5^{bc}	19.8±0.2^{ab}	8.1±1.1^{bc}	N

Sa = *S. aureus*, Ec = *E. coli*, Pa = *P. aeruginosa*, Bs = *B.subtilis,* Ca = *C. albicans*,
Data are mean ± standard deviation, n=3 ; N = no activity.

Effect of Phenol content and antioxidant activity to antimicrobial activity

Among the Malaysian leaf extracts, as shown in Table 9 in the total phenol content in PCM and CBPM are statistically significant (p<0.05) highest than CBRM and CALM, but the zone inhibition for growth of *Staphylococcus aureus* antimicrobial activity are not significant by different. This result shows that the total amount phenol content was not the factor can effected the growth of *Staphylococcus aureus.* If compared to CALI the lower total phenol content, CALI was more effective to inhibit the growth of three bacteria strain (*Pseudomonas aruginosa, Escherichia coli* and *Bacillus subtillis*

Table 9 Phenol content, Flavonoid content, Antioxidant activity (IC_{50}) and antimicrobial activity of six *Lamiaceae* plants extracts

Plant Extracts	Phenol content	Flavonoid content	Antioxidant activity	Antimicrobial activity [Zone of Inhibition (mm)]				
				Gram -ve bacteria		Gram +ve bacteria		Fungi
				Pa	Ec	Sa	Bs	Ca
CALI	55.21 ±3.20^{d}	0.178±.07^{e}	34.1 ±1.8^{e}	18.5±0.3^{a}	16.6±1.5^{a}	na	13.0±0.8^{a}	N
CATM	90.41 ±1.95^{b}	6.00 ±0.24^{d}	14.5 ±0.08^{cd}	7.3±0.5^{c}	7.3±0.5^{bc}	19.3±0.5^{ab}	7.5±0.0^{c}	N
PCM	95.17 ±2.82^{a}	8.88 ±0.33^{c}	10.5 ±0.22^{a}	10.0±1.3^{b}	8.3±1.5^{bc}	21.6±1.5^{a}	8.8±0.2^{bc}	N
CBPM	94.01 ±4.61^{a}	15.21 ±0.28^{a}	12.9 ±0.26^{bc}	7.5±0.8^{c}	9.3±0.5^{b}	19.8±1.7^{ab}	8.1±0.2^{bc}	N
CBRM	85.35 ±3.90^{c}	13.66 ±0.18^{b}	15.9 ±0.21^{d}	6.5±0.0^{c}	6.6±0.2^{c}	17.8±0.2^{b}	7.5±1.7^{c}	N
CALM	86.27 ±4.67^{c}	14.08 ±0.20^{b}	12.5 ±0.32^{b}	9.6±1.0^{bc}	7.3±0.5^{bc}	19.8±0.2^{ab}	8.1±1.1^{bc}	N

Sa = *S. aureus*, Ec = *E. coli*, Pa = *P. aeruginosa*, Bs = *B.subtilis,* Ca = *C. albicans*,
Data are mean ± standard deviation, n=3 ; N = no activity.

Acute toxicity studies of *Coleus blumei* (purple leaves):

Mortality is the main criteria in assessing the acute toxicity (LD_{50}) of any drug. The result of toxicity signs observed following administration of the extract and calculated as LD_{50} are presented in 10 and 11. The results of the oral acute toxicity shows that there was no mortality in the groups that received normal saline and the group that received 1000 - 5000 mg/kg body weight of *Coleus blumei* leaves extract after 24 hours. Therefore the LD_{50} value of *Coleus blumei* leaves extract was estimated to be above 5000 mg/kg body weight. There was no mortality recorded even at the highest dose level 5000 mg/kg. body weight observed for 24 h for toxicity signs and death, which proves that *Coleus blumei* plant extract have no significant toxic effect at least in mice. There was neither death recorded nor discernible gross pathological lesion seen in animals dosed with 1000 - 5000 mg/kg of the aqueous *Coleus blumei* plant extract.

Table 10 The mortality rate in mice given *Coleus blumei –purple leaves* (CBPM) extracts in various doses

Group	Dose ($mgkg^{-1}$ of extract)	Mice	
		Toxicity sign	Mortality rate
Control	Normal saline	NT	ND
1	1000	NT	ND
2	2000	NT	ND
3	3000	NT	ND
4	4000	NT	ND
5	5000	NT	ND

n=5

NT - No toxic sign noticed
ND - No death recorded

Table 11 Determination of acute toxicity (LD_{50}) of *Lamiaceae* species using Karber method

Dose ($mgkg^{-1}$)	Dose different	Mice		
		No of dead (n)	Mean dead	Dose x mean different dead
Control	0	0	0	0
1000	1000	0	0	0
2000	1000	0	0	0
3000	1000	0	0	0
4000	1000	0	0	0
5000	1000	0	0	0

n=5

Table 12 shows the mean body weights of the mice in the six groups before and after 7 days of treatment. The body weight gains in the six groups (control, group 1, group 2, group 3, group 4, and group 5). No significant clinical findings were noted in any of the group. Body weight did not change significantly in any treated group as compared to control group.

Table 12 The weight of mice after single intraperitoneal of *Coleus blumei* –purple (CBPM) *leaves* extract in various doses

Group	Body weight (g)					
	Day 0	Day 1	Day 2	Day 3	Day 6	Day 7
Control	33.68±3.36	33.92±0.95	34.57±4.09	35.79±3.96	34.66±4.48	35.11±4.54
1	30.20±1.94	30.21±2.80	31.06±0.80	31.08±1.01	30.17±1.63	32.00 ±0.51
2	32.52±4.16	33.33±4.51	34.26±4.30	34.00±6.28	37.22±9.68	37.94±10.26
3	33.20±2.84	33.37±5.68	34.26±7.06	33.57±4.09	33.20±4.03	34.64±4.06
4	30.78±1.67	31.43±0.95	34.18±1.78	32.24±0.95	32.52±0.78	34.11±1.05
5.	29.44±0.75	29.35±3.55	30.47±3.76	30.28±2.91	30.13±2.48	30.90±2.26

Data are mean ± standard deviation, n=5

DISCUSSION:

The present study revealed some interesting findings for the *Lamiaceae* species grown in this part of the world. The biological activity in vitro, especially in relation to total phenolic & flavonoid contents, antioxidant and antimicrobial activities were evaluated for the extracts of aforementioned plants of *Lamiaceae*.

Total Phenolic Contents:

Comparing with other *Lamiaceae* species most of the plant showed higher phenolic contents and this has been indicated in other studies that the extracts from these plants are rich in phenolic compounds (Kalliopi 2001, Devi &Yogyarti 2006 and Pourmorad 2006). The contents ranges from 24 ± 1.00 to 290 ± 5.00 mgGAE/g. These results show variable amount of phenolic compounds for each plant species examined. This might be related to the physiology of different specie having different metabolic pathway for producing the phytochemicals. As mentioned earlier that the total phenolic contents of all Malaysian *Lamiaceae* species (PCM, CBPM, CATM, CALM and CBRM) were significantly different ($p < 0.05$), even though the plants were grown in the same region/conditions. In a study report by Meral (2003) reports that phenolic contents of three spices grown in the same region of Turkey were also significantly different. Indeed, there seems to be a variety of factors involved those having effect on the phenolic compounds concentration in plants, such as the species, variety, cultivation time, region, weather conditions, ripeness, harvesting time, storage time and other unknown conditions (Arts et al., 2000; Coward et al., 1993; Lakenbrink et al., 2000; Reid et al., 2004). Results of this study indicate that *Lamiaceae* leaves extract from Malaysia have higher total phenolic contents compared to *Lamiaceae* plants leaves from Indonesia for the same species. . This can be explored further for the medicinal uses and preference to use the plants in different preparation over one another. The Folin-Ciocalteu assay is used to quantify the total concentration of phenolic hydroxyl groups present in the extract. The method does not indicate the particular phenolic compounds present in the extract. According to Hahn (1984) the Folin-Ciocalteu method is based on the reducing power of phenolic hydroxyl groups and is not very specific but detects total phenols with varying sensitivity. The method does not distinguish between different types of phenol compounds. The greater the amount of phenol hydroxyl groups, the greater the concentration of phenolic compounds detected by the assay. Flavonoids are polyphenolic plant secondary metabolites, synthesized by the polypropanoid pathway with phenylalanine as a starting molecule (Janićijević et al., 2007).

There were significant differences (p<0.05) in flavonoid contents among the six *Lamiaceae* plants leaves extracts examined. The CBP-M extracts contained significantly (p<0.05) higher amount of flavonoid compared to other leaves extracts, while the extracts of CAL-I had significantly lower flavonoid contents. Briefly amounts of flavonoid were the highest in CBP-M followed by CAL-M, CBR-M, PC-M, CAT-M and CAL-I. The flavonoid contents are affected due to the exposure to light which has considerable effect on most flavonoid contents (Kondakova et al., 2009; Lee., et al., 2006; Stefanovits-Bányai et al., 2003). Furthermore it is not clear that to what extent the concentration is affected by the procedure adopted for the measurement of the flavonoid contents. There would be losses in procedure therefore, has to be consideration in the interpretation of data. There are possibilities of underreporting the amount/concentration of the flavonoid.

Relationship between total phenol and flavonoid contents:

The correlation analysis between total phenolic and flavonoid contents in *Lamiaceae* leaves extracts revealed that there was a significant correlation being the correlation coefficient r = 0.59. The results suggest that the phenolic or flavonoid concentration is dependent on one another. Therefore, the suggestions that these two amounts of plant components are independent can be considered misleading. However, there are contradicting results reported on this aspect that the correlation between the phenol and flavonoid contents is positively dependent or not dependent at all. In a study reported by Kumar (2008) shows that phenol content of four selected Indian Medicinal Plants *(Camellia sinensis, Sesbania grandiflora, Thespesia populnea, Cassia auriculata)* independent to flavonoids contents. This shows that the concentration of phenol and flavonoid content are affecting one another. For example, Meral (2003) found a correlation between the phenol and flavonoid contents and the antioxidant activity as well. As mention earlier Folin-Ciocalteu method does not show the particular phenolic compounds present in the extract, this indicates that each plant species may contain different phytochemicals contents therefore in some cases lake of relationship is observed. However, the individual phenolic content was not estimated in the present study therefore the question remain unanswered.

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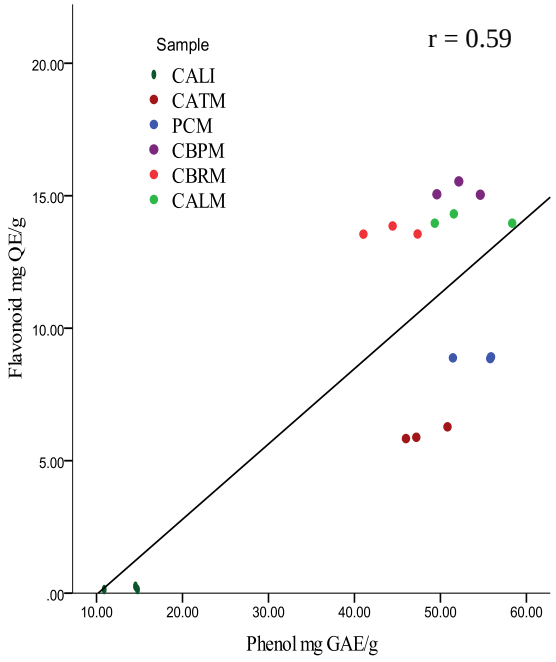

Fig 3. Correlation Analysis of Total phenolic and flavonoid content

Relationship between Phenol Content and Antioxidant Activity.

The correlation analysis revealed that there was strongest negative correlation between free radical scavenging activity (IC_{50}) with total phenolic contents being the correlation coefficient r = -0.94 is shown in Figure 4. This means that when the total phenolic contents level is higher, the IC_{50} is lower and results in higher level of Acid Equivalent Antioxidant Capacity (AEAC). This is due to the higher amount of polyphenolic constituents present in the plant leaves extract, which were capable of functioning as free radical scavengers. In other words it means that the higher the total phenol contents the higher is the antioxidant activity (Low IC_{50} value). Thus it appears that phenolic components of the *Lamiaceae* species contribute to a significant antioxidant capacity of the extracts.

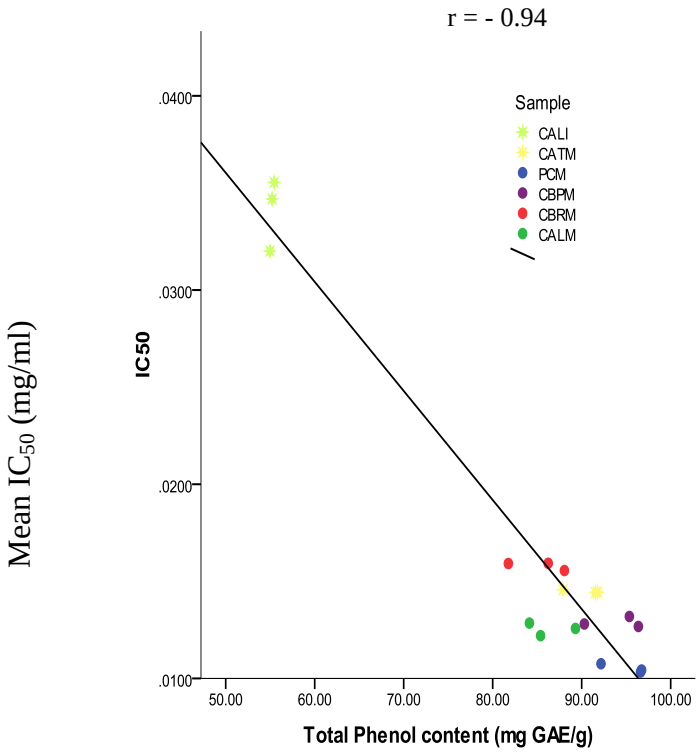

Fig4. Correlation between total phenol content and IC_{50}

Many herbal species, especially those belonging to the *Lamiaceae* family, possess strong antioxidant activity (Nakatani, 2000). However, it is difficult to attribute the antioxidant activity any of the components present in the extracts since it is a complex mixture of different chemical compounds. However, phenolic components generally exhibits maximum antioxidant activity (Mimica-Dukic et al., 2003, Surveswaran, 2007 and Hodzic, 2009). The scavenging effect of leaves extracts is not limited to phenolics contents but it may be also due the presence of other antioxidants for example secondary metabolites in the extracts such as volatile oils, carotenoids, and vitamins (Javanmardi et al., 2003). Apart from the free radical scavenging activity can also be due to other compounds such as betulinic acid, oleanolic acid and ursolic acid. Furthermore, *O.stamineus* also contains many staminane-type diterpenes as reported by Tezuka (2000). However, these were not studied in the present study. As mentioned before, that antioxidants are compounds which have the capability to protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite (aka peroxonitrite). An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage (Cheng et al., 2003). Oxidative stress has

been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases (Parkinson's and Alzheimer's). Therefore, this property of these plants can be medicinally as well as commercially exploited for the prevention of these listed diseases.

Anti Microbial effects of the plants leaves extracts:

In the present study, all Lamiaceae leaf extracts from Malaysia, had greater on the diameter of the inhibition zone for *S. aureus* than for *E. coli* strain, indicating that the gram-positive strain is more sensitive than the gram-negative strain. All leaves extracts from Malaysia having inhibitory effect on the gram positive bacteria (*Staphylococcus aureus)* compared to the gram negative bacteria (*Escherichia coli and Pseudomonas aeruginosa*). Generally, gram negative bacteria are more resistant to plants extract compared to gram positive bacteria (Basri et al., 2005). This may be due to the permeability barrier provided by the cell wall. The reason is that the density of the lipopolysaccharide layer in the outer surface of bacterial cell wall, which is greater in the gram-negative bacteria as compared to the gram-positive bacteria (Burn, 1988). It has been postulated that the antibacterial properties is of due to nucleotide damage with increase in spatial division and condensation of genetic material (Chakraborty et al., 2007). It is not surprising that there are differences in the antibacterial activities of the different extracts tested. This could be due to the difference in the phytochemical composition among them (Dikbas et al., 2009; Yagoub et al.). Results show that the quantity of phenol among different *Lamiaceae* species studied does not necessarily affect the ability to stop the microbial growth. Baydar (2004) report that the greater the amount of phenolic compound in the extract would imply the greater inhibitory effect of a particular extract on the test microorganism involved, provided the test microorganism is sensitive to the extract. Phenolic compounds are known to be synthesized by plants in response to microbial infection. It is therefore logical that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms (Cowan, 1999). Gordana (2007) report that the antimicrobial activity of investigated extracts depend not only on phenolic compounds. The presence of different secondary metabolites also contributes to the antimicrobial activity. All the plant extracts tested have traditional has been claimed for antibacterial activity and this finding is in line with their indication. This finding can form the basis for further studies to prepare an optimized preparation of the herbal extract to further evaluate them against a wider range of bacterial strains.

Toxicological effects of the plants leave extracts:

The present study indicates that the plants under investigations are safe for the consumption as reported in early studies. Therefore, the use of the *Lamiaceae* family is regarded safe for if consumed. Acute toxicity test also revealed that with LD_{50} (estimated to be >5000 mg/kg) shows no toxic sign and is categorized *as* non-poisonous and generally considered to be safe. This can be planted in house or kindergarten safely (Patel, 2010 and IWK, 2002). Similarly, feeding normal young rats orally with methanolic extracts of *Lamiaceae* species for any possible toxic effect however no mortality was recorded (Han, 2008).

Conclusion:

Lamiaceae plants extracts were found to be rich in phenolic contents therefore; it can be used as free radical scavengers. However qualitative and quantitative analysis of major individual phenolic may explain the relationships between total antioxidant capacity and total phenolic contents in the species. Furthermore, the results indicate that these Lamiaceae leaf extracts can be used as antioxidant and antimicrobial agent without having the toxic effect.

Future Plan of the research:

In future it is intended that extracts from *Lamiaceae* plants leaves will be studied in the following different direction;

1. Animals Studies Models
    a. Obese
    b. hyperlipidaemic
    c. and diabetic
2. Human Studies Models
    a. Obese
    b. hyperlipidaemic
    c. and diabetic

3. Publication of the current results

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