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COMPARATIVE EVALUATION OF ANALGESIC, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF FIVE SELECTED MEDICINAL PLANTS

by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	: Percent sign
°C	: Degree celcius
β	: Beta
δ	: Delta
γ	: Gamma
κ	: Kappa
µ	: Micro
µ	: Mu
Aδ	: A-delta
ATCC	: American type culture collection
ANOVA	: Analysis of variance
BHT	: Butylated hydroxytoluene
CD_3OD	: Deuterated methanol
CNS	: Central nervous system
CFU	: Colony forming unit
cm	: Centimeter
cm^{-1}	: Recripocal centimeter (units of wavenumber)
cm/s	: Centimeter per second
COX	: Cyclooxygenase
% CV	: Percentage coefficient of variation
dd	: Doublet of doublet
DMSO	: Dimethyl sulphoxide
DPPH	: 2,2-diphenyl-1-picryl-hydrazyl
ESI	: Electrospray ionisation
FCR	: Folin-Ciocalteu reagent
$FeCl_3$	: Ferric chloride
FRAP	: Ferric reducing antioxidant power
FTIR	: Fourier Transform Infrared Red
GC-MS	: Gas Chromatography-Mass Spectroscopy
hrs	: Hours
HAT	: Hydrogen atom transfer

HCl	: Hydrochloric acid
HPLC	: High Performance Liquid Chromatography
HPTLC	: High Performance Thin Layer Chromatography
IASP	: International Association for the Study of Pain
ICH	: International Conference on Harmonisation
i.p.	: Intraperitoneal
KBr	: Potassium bromide
kHz	: Kilo Hertz
LC-MS	: Liquid Chromatography Mass Spectrometry
LOD	: Limit of detection
LOQ	: Limit of quantification
MIC	: Minimum inhibitory concentration
min	: Minutes
mg CAE/g	: Milligram catechin equivalents in 1 gram of sample
mg GAE/g	: Milligram gallic acid equivalents in 1 gram of sample
mg/kg	: Dose (weight of test substance in milligrams per unit weight of test animal)
mg/mL	: Concentration (weight of test substance in milligrams per volume of test concentration)
MHA	: Mueller–Hinton agar
mL	: Milliliter
mL/min	: Gas flow rate millimeter per minute
mM	: Millimolar
N	: Sample size
ng	: Nanogram
NIST	: National Institute of Standard and Technology
nm	: Nanometer
NMR	: Nuclear Magnetic Resonance
NSAIDs	: Non-steroidal anti-inflammatory drugs
PAG	: Peri-aqueductal gray
PDA	: Photodiode array detector
p-INT	: *p*-Iodonitrotetrazolium chloride
p.o.	: Oral administration

Q-TOF	: Quadrupole – Time of flight
R_f	: Retention factor
RNS	: Reactive nitrogen species
ROS	: Reactive oxygen species
RSD	: Relative standard deviation
R_T	: Retention time
s	: Seconds
s.c.	: Subcutaneous
SD	: Standard deviation
SEM	: Standard Error Mean
SPSS	: Statistical Package for the Social Sciences
TLC	: Thin Layer Chromatography
TPTZ	: 2,4,6-tris(2-pyridyl)-s-triazine
µg	: Microgram
µg/mL	: Microgram of plant extract per milliliter of assay mixture
µL	: Microliter
µm	: Micrometer
UPLC	: Ultra Performance Liquid Chromatography
USFDA	: United States Food and Drug Administration
UV-vis	: Ultraviolet – visible
UV-254nm	: Ultraviolet at 254 nm wavelength
UV-365nm	: Ultraviolet at 365 nm wavelength
VCEAC	: Vitamin C equivalent antioxidant capacity
v/v	: Volume per volume
W	: Watt
WHO	: World Health Organization
ZOI	: Zone of inhibition

LIST OF PUBLICATIONS AND CONFERENCES

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PERBANDINGAN AKTIVITI ANALGESIK, ANTIOKSIDA DAN ANTIMIKROBIAL ANTARA LIMA TUMBUHAN PERUBATAN TERPILIH

ABSTRAK

Tumbuh-tumbuhan yang mempunyai nilai-nilai perubatan selalunya mengandungi sebatian-sebatian bioaktif yang telah menyumbangkan kepada kewujudan beberapa sedian-sedian farmaseutikal terkini. Memandangkan kepada hal ini, kajian ini telah dijalankan untuk menilai aktiviti antioksida, analgesik and antimikrob tumbuh-tumbuhan tempatan yang digunakan secara tradisional seperti *Terminalia catappa*, *Piper betle*, *Bauhinia purpurea*, *Cinnamomum iners* dan *Callicarpa furfuracea*. Dalam kajian ini, teknik pengekstrakan sonikasi, maserasi dan Soxhlet telah digunakan untuk mendapat ekstrak tumbuhan masing-masing. Penentuan aktiviti *in vitro* antioksida dan antimikrob mendedahkan kehadiran aktiviti biologi dan kandungan polifenolik yang signifikan dalam ekstrak yang diperolehi melalui kaedah-kaedah sonikasi diikuti dengan maserasi dan pengesktrakan Soxhlet.

Antara ekstrak-ekstrak tumbuhan yang dikaji untuk aktiviti antioksida, ekstrak *T. catappa* menunjukkan aktiviti antioksida yang luar biasa dan diikuti oleh ekstrak *P. betle, B. purpurea, C. iners* dan *C. furfuracea.* Aktiviti antioksida yang dipaparkan oleh ekstrak *T. catappa* adalah lebih tinggi secara signifikan daripada ekstrak biji anggur komersial dan antioksida sintetik butil-hidroksitoluena. Di samping itu, esktrak *T. catappa* juga mendedahkan aktiviti antimikrob yang sederhana terhadap kedua-dua jenis bakteria Gram-positif dan Gram-negatif diikuti oleh ekstrak *B. purpurea, P. betle* dan *C. iners*. Ekstrak soxhlet etanolik daun, batang dan akar *C. furfuracea* memiliki kandungan polifenolik, aktiviti antioksida dan

antimikrob yang paling rendah apabila dibandingkan dengan ekstrak-ekstrak dari lain-lain tumbuhan. Ini merupakan kajian yang julung kali dijalankan untuk menyiasat aktiviti biologi *C. furfuracea*.

Aktiviti analgesik secara *in vivo* bagi ekstrak-ekstrak *T. catappa*, *C. furfuracea* dan *C. iners* pula mendedahkan adanya aktiviti analgesik sentral dan periferi manakala ekstrak kulit pokok *C. iners* dan daun *C. furfuracea* pula hanya mempunyai aktiviti analgesik periferi. Pengasingan bioaktiviti berpandu bagi ekstrak-ekstrak *T. catappa* dan *P. betle* dengan menggunakan kaedah kromatografi turus dan kromatografi lapisan nipis preparatif telah membawa kepada pengasingan asid galik, (+) katecin, (-) epikatecin dan kuercetin dari *T. catappa* dan euginol, alilpirokatekol dan euginol asetat dari *P. betle*, masing-masing. Dalam kajian ini, (+) katecin dan (-) epikatecin telah dilaporkan untuk kali pertama dari daun *T. catappa*. Kaedah HPTLC dan UPLC telah dibangunkan dan disahkan juga buat kali pertama dengan menggunakan sebatian-sebatian terpencil masing-masing untuk *P. betle* dan *T. catappa*. Kaedah-kaedah yang baru dibangunkan adalah mudah, cepat, tepat, sensitif dan boleh diulangi. Secara keseluruhannya, kajian yang dijalankan di sini menyokong penggunaan tumbuh-tumbuhan tersebut dalam perubatan tradisional dan sebatian bioaktif yang diasingkan daripada tumbuh-tumbuhan terpilih juga mempunyai ciri-ciri sebagai agen antioksida, antimikrob dan analgesik. Capjari kromatografi yang dibangunkan boleh membantu industri herba untuk tujuan kawalan kualiti bagi sampel-sampel tumbuh-tumbuhan tersebut atau kerja-kerja penghasilan formulasi masing-masing.

EVALUATION OF ANALGESIC, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF FIVE SELECTED MEDICINAL PLANTS

ABSTRACT

Plants with medicinal values are known to possess bioactive compounds and contributed greatly to the existence of several currently available pharmaceuticals. In view of this the present study was conducted to evaluate the antioxidant, antinociceptive and antimicrobial activities of traditionally used local plants like *Terminalia catappa, Piper betle, Bauhinia purpurea, Cinnamomum iners* and *Callicarpa furfuracea*. In this study sonication, maceration and soxhlet extraction techniques were used to obtain their respective plant extracts. The *in vitro* antioxidant, antimicrobial activity and polyphenolic content determination revealed the presence of significant biological activities and phenolic content in the extract obtained by sonication followed by maceration and soxhlet extraction.

Among the plant extracts studied for antioxidant activity, *T. catappa* extract showed remarkable antioxidant activity followed by *P. betle, B. purpurea, C. iners* and *C. furfuracea* extracts. The antioxidant activity displayed by *T. catappa* extract was significantly higher than commercially available grape seed extract and synthetic antioxidant butylated hydroxytoluene. In addition, *T. catappa* extract also revealed marked antimicrobial activity against both Gram-positive and Gram-negative bacterial strains followed by *B. purpurea, P. betle* and *C. iners*. Soxhlet ethanolic extract of *C. furfuracea* leaf, stem and root possessed least polyphenolic content, antioxidant and antimicrobial activities when compared with other plant extracts. This is the first ever study regarding the biological investigations of *C. furfuracea*.

In vivo antinociceptive activity of *T. catappa*, *C. furfuracea* and *C. iners* revealed central and peripheral antinociceptive activity with *T. catappa* extract and only peripheral antinociceptive activity with *C. iners* bark and *C. furfuracea* leaf extracts. Bioactivity guided isolation of *T. catappa* and *P. betle* leaf extract using column chromatography and preparative thin layer chromatography lead to the isolation of gallic acid, (+)-catechin, (-)-epicatechin and quercetin from *T. catappa* and eugenol, allylpyrocatechol and eugenol acetate from *P. betle*, respectively. (+)-Catechin and (-)-epicatechin were reported for the first time from *T. catappa* leaf in this study. HPTLC and UPLC methods were developed and validated for the first time using the isolated compounds for *P. betle* and *T. catappa*, respectively. These newly developed methods were simple, rapid, accurate, sensitive and reproducible. On the whole, the work undertaken herein supported the folk medicinal use of the selected medicinal plants and the bioactive compounds isolated from selected plants are responsible for the significant antioxidant, antimicrobial and antinociceptive effects. The chromatographic fingerprint developed could assist the herbal industry for the quality control of these plant samples or their respective formulations.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 RESEARCH BACKGROUND

Plants have been provided mankind with various herbal remedies in the treatment of a wide range of diseases since antiquity and still continue to contribute as potential therapeutical agents in primary health care in developing countries (Tshikalange et al., 2005; Newman and Cragg, 2012). Even with the advancement of vibrant technologies in modern medicine, the World Health Organisation (WHO) estimated around 75-80% of the world population still rely mainly on traditional medicine as a source of therapy (Gurib-Fakim, 2006; Sahoo et al., 2010; WHO, 2008). Around 20,000 medicinal plants used in different parts of the world was listed by WHO and several other estimation indicates that it may be around 35,000 to 70,000 (Lewington, 1993; Bhattarai and Karki, 2004). Among these plants only a small percentage have been investigated for their phytochemicals and of which the fractions as well isolated compounds submitted for pharmacological screening is even smaller (Calixto, 2000; Sarker et al., 2005).

The plant derived bioactive compounds are used either directly as therapeutic agents or serve as biologically active lead molecules which inspires in the synthesis of numerous structural analogs. These bioactive compounds also served to reveal several mechanisms underlying human diseases (Pan et al., 2009). It has been documented that around 74% of pharmaceuticals used today are derived from the plants on the basis of their ethnobotanical use (Calixto, 2000; Verpoorte et al., 2005; Ncube et al., 2008). Thus, ethnopharmacology is considered as the origin of most of the medicine and natural products are the most important source of drugs (Cos et al., 2006). Still numerous traditionally used unexplored medicinal plants have been

screened for various biological activities to provide the scientific evidence and many of the times it leads to the identification of numerous bioactive compounds of therapeutic importance (Ncube et al., 2012).

Although many medicinal plants are screened for various pharmacological activities leading to isolation of bioactive compounds, still numerous available medicinal plants are not yet explored scientifically for their novel pharmacological activities (Hamburger and Hostettmann, 1991). This might be due to their chemical complexity, poor reproducibility, existence of synergetic pharmacological activity between the various components present in the plant extract as well as isolation and structure elucidation process is time consuming and laborious (Harvey, 2002). Hence, most of the natural product research involves either preliminary screening of herbal material for biological activity or the random isolation of phytoconstituents with very few studies reported regarding the bioactivity guided isolation. This has hindered the natural product associated drug discovery (Hamburger and Hostettmann, 1991).

Public interest regarding the use of herbal medicine has increased markedly in the past decades due to the belief of people that herbal drugs are purely natural, free from side effects, time tested efficacy and cheaper when compared to synthetic medicines (Calixto, 2000). This renewed interest in the herbal medicine leads to an increase in their demand. In order to ensure the claimed health effect and for the sake of profit, some of the herbal formulations are adulterated with illegal synthetic drugs or substituted with substandard plant species (Chen et al., 2009, Li et al., 2010). This is resulted in WHO to issue several guidelines to ensure safety, quality and efficacy of herbal medicines (WHO, 2004). WHO accepted either spectroscopic or chromatographic fingerprint analysis as a quality control method for the assessment of natural products (Tistaert et al., 2011). Hence, various HPLC, LC-MS, GC-MS

and HPTLC methods were developed for both analyses of the phytoconstituents and to provide the respective chromatographic fingerprints (Lu et al., 2007; Chen et al., 2009).

However, the strategy followed in the natural product research includes the preparation of an extract, preliminary screening for wide biological activities on the basis of traditional use, bioactivity guided fractionation, isolation, structural elucidation and the development of chromatographic or spectroscopic fingerprints which could serve as quality control tool for that herb or its product (Hostettmann et al., 2000). Normally bioactivity guided isolation avoids the time consuming and costly isolation of trivial bioactive natural products (Robards, 2003).

Discovery of novel analgesic, antimicrobial and antioxidant compounds from natural products is a broad area of research. This is mainly due to the severe adverse effects associated with the use of currently available analgesic agents and also the existence of microbial resistance to the existing traditional antibiotics (Wolfe et al., 1999; Tapiero et al., 2002a; Naz et al., 2007). Several phenolic antioxidants were reported to exert the potential analgesic and antimicrobial activities through their antioxidative mechanism along with many other health promoting benefits (Mirzoeva et al., 1997; Caturla et al., 2003; Martínez et al., 2009; Mothana et al., 2012). Hence, research related to the discovery of natural antioxidants, antinociceptives and antimicrobial is gaining more importance.

South East Asian countries are enriched with flora and fauna with inexhaustible traditionally used medicine. Huge number of unexplored traditional plant species without scientific evidences are reported from Malaysia. Most of these plants are traditionally employed to treat wide number of diseases (Burkill, 2002; Wiart et al., 2004; Husen et al., 2004). Thus, research on these traditional plants

provides the scientific evidence for the reputed traditional efficacy and helps in producing large number of hits (Lee and Houghton, 2005).

The main goal of the present research work was to identify novel analgesic compounds from medicinal plants available in Malaysia. Hence, in the present study five plants namely *Terminalia catappa*, *Cinnamomum iners*, *Callicarpa furfuracea*, *Bauhinia purpurea* and *Piper betle* were selected on the basis of the literature survey and various traditional uses in Malaysia as well as globally. In addition to the analgesic activity, these plants were also studied for antioxidant and antimicrobial activities as these are pharmacological events that accompany pain when there is bacterial infection. To further understand the chemical constituents responsible for the biological activity, a bioassay guided method will be carried out to isolate compounds along with the development of chromatographic analytical methods for evaluation of quantity of these substances in various plant extracts.

1.2 LITERATURE REVIEW

1.2.1 Literature review of selected plants

1.2.1.1 *Terminalia catappa* Linn

T. catappa L is a Combretaceous plant which grows commonly in tropical and subtropical countries. The family *Combretaceae* comprises of about 200 species of *Terminalia* most of them are widely used for medicinal purposes (McGaw et al., 2001). In Malaysia, *T. catappa* is known with the local name of Katapang (Burkill, 2002). The leaf, bark and fruit of this plant have long been used in folk medicine for antidiarrheic, antipyretic, dermatitis (Nair and Chanda, 2008), hepatitis (Tang et al., 2006), severe pain (headache) (Ratnasooriya et al., 2002) and haemostatic purposes in Taiwan, India, Phillippines, Malaysia and Indonesia (Lin, 1992; Lin et al., 2001).

Tanaka et al. (1986) reported the presence of number of hydrolysable tannins such as punicalagin, punicalin, chebulagic acid, corilagin, geranin, terflavins A and B, tergallagin, gratin B from the leaves of *T. catappa*. Various flavonoids like apigenin 6-*C*-(2″-*O*-galloyl)-β-*D*-glucopyranoside, apigenin 8-*C*-(2″-*O*-galloyl)-β-*D*-glucopyranoside, isovitexin, vitexin, isoorientin and rutin (Lin et al., 2000) and triterpenoids such as ursolic acid and 2α, 3β, 23-trihydroxyurs-12-en-28 oic acid (Fan et al., 2004) were isolated from the leaves of *T. catappa*. GC-MS analysis of red fallen leaves shown the presence of six phenolic compounds such as *p*-hydroxybenzoic acid, gallic acid, 4-hydroxyphenylpropionic acid, *m*-coumaric acid, 3,4-dihydroxybenzoic acid and *p*-coumaric acid (Chyau et al., 2006). Kinoshita et al. (2007) isolated and identified corilagin and chebulagic acid as the two potent antioxidants from the leaves of *T. catappa* and quantified their amount in hot water and 50% ethanolic extract using Nova-Pak C_{18} Radial-Pak HPLC column.

T. catappa leaf has been reported to possess wide biological activities which include antioxidative, hepatoprotective, antimicrobial, antidiabetic, anti-inflammatory and anti-HIV reverse transcriptase activity. Kinoshita et al. (2007) evaluated antioxidant and hepatoprotective activity of *T. catappa* extract and its phytoconstituent corilagin (1 mg/kg, i.p.) against galactosamine induced hepatotoxicity and reported that the potent hepatoprotective activity of the *T. catappa* extract and corilagin is through the antioxidant mechanism. Two more phytoconstituents of *T. catappa*, punicalagin and punicalin (5 mg/kg, i.p.) displayed hepatoprotective activity against acetaminophen induced liver damage in rats (Lin et al., 2001). Fan et al. (2004) conducted bioactivity guided isolation of anti-inflammatory constituents from the ethanolic extract of *T. catappa* leaf and identified two triterpenic acids as the bioactive constituents.

Aqueous leaf extract of *T. catappa* (46 mg/kg, p.o.) demonstrated significant antidiabetic activity with pancreatic cell regeneration in alloxan-induced diabetic rats (Ahmed et al., 2005). The macerated juice of *T. catappa* tender leaves with the oral dose of 5, 10 and 15 mL/kg body weight exhibited dose dependent analgesic activity in hot plate test and in early phase of formalin test and devoid of analgesic activity in tail flick test (Ratnasooriya et al., 2002). Nair and Chanda (2008) reported the good antibacterial activity of *T. catappa* leaf aqueous and methanolic extract against *S. aureus*, *B. cereus* and *K. pneumoniae*. On the other hand, Goun et al. (2003) showed the potent antifungal activity of *T. catappa* aerial part methanolic and methylene chloride extracts (100 mg/kg). Chen et al. (2000a) demonstrated the protective effect of aqueous extract of *T. catappa* leaf and punicalagin against bleomycin induced genotoxicity in Chinese hamster ovary cells. Various solvent extracts of *T. catappa* leaf were studied for their antioxidant activity by (Chyau et al., 2002) and the results displayed that methanolic extract possess significant antioxidant activity in DPPH assay, reducing power assay and metal chelating assay. Mau et al. (2003) reported the potent potential inhibition of lipid peroxidation by the supercritical fluid *T. catappa* leaf extracts.

1.2.1.2 *Piper betle* Linn

P. betle L is traditionally as well as economically important plant belongs to the family *Piperaceae* and widely distributed in India, Sri Lanka, Malaysia, Indonesia, Thailand, China, Philippines and subtropical countries. In Malaysia, *P. betle* is known with a local name of Sireh (Burkill, 2002). Since ancient time, *P. betle* not only used traditionally for chewing purpose along with areca nut, clove and cardamom but also as a source of medicine. Traditionally *P. betle* leaves were used

to treat cold, cough and also as a carminative, stimulant, digestive (Rawat et al., 1989), antiseptic for cuts and wounds (Rimando et al., 1986), tonic, hepatoprotective (Nair and Chanda, 2008), anthelmintic, fungal (Evans et al., 1984) and to treat earache as well as ear and throat swelling (Choudhary and Kale, 2002).

Ramji et al. (2002) isolated and identified phenolic constituents such as eugenol, eugenol acetate, allylpyrocatechol (APC), APC monoacetate, from leaves of *P. betle.* In addition to these constituents, Rimando et al. (1986) also isolated and identified methyl eugenol, APC diacetate and safrole along with some terpenes such as camphene, α-pinene, 1.8-cineole and caryophyllene from the leaves of Philippine *P. betle* leaves. Zeng et al. (1997) identified piperbetol, methylpiperbetol, piperol A and B from the *P. betle* and reported their potential selective inhibition of platelet aggregation induced by platelet activating factor. Other essential oils isolated includes, p-cymene, α-terpineol, terpinyl acetate, caryophyllenes (Sharma et al., 1983), 1,8 cineole, α and β-pinene, α-terpinene, α-terpinene, β-myrcene, camphene (Rawat et al., 1989, Jantan et al., 1994). Arambewela et al. (2005b) reported the variation in the phenolic constituents and volatile oils in the leaves of different stages of maturation as well as among the leaves of different cultivars and in the different parts of *P. betle* plant.

Pin et al. (2010) developed RP-HPLC method for the quantification of eugenol and hydroxychavicol and to develop a chemical profile on a Phenomenex Luna C18 100A column. Recently, Maity et al. (2011) developed and validated a new RP-HPLC method for the simultaneous quantification of hydroxychavicol and chlorogenic acid from the leaves of *P. betle*. Earlier, Rathee et al. (2006) developed and employed a HPTLC method for the quantification of chavibetol and APC from

the extracts of *P. betle* with the mobile phase consisting of ethyl acetate and hexane (2:8) on silica gel 60 GF_{254} plates using Camag HPTLC instrument.

In vitro antioxidant activity of aqueous and ethanolic extract of three varities of *P. betle* revealed potential antioxidant activity in various antioxidant model as reported by Dasgupta and De (2004) and Arambewela et al. (2006). Choudhary and Kale (2002) conducted the *in vitro* and *in vivo* antioxidant activity of 50% hydroalcoholic extract of *P. betle* and reported the potent inhibition of radiation induced lipid peroxidation along with the increase in the amount of superoxide dismutase and glutathione peroxidase antioxidant enzymes. Rathee et al. (2006) isolated, identified and evaluated the antioxidant activities of chavibetol and APC from ethanolic extract of *P. betel* leaf. They reported the significant inhibition of ferrous induced lipid peroxidation of liposomes and rat brain homogenates by APC when compared to chavibetol. Hydroxychavicol isolated from *P. betle* through bioactivity guided isolation exhibited potent xanthine oxidase inhibitor activity and the activity was higher than the standard allopurinol (Murata et al., 2009).

Oral administration of *P. betle* leaf suspension (75 and 150 mg/kg) to the streptozotocin induced diabetic rats resulted in marked reduction of oxidative stress induced thiobarbituric acid reactive substance and significantly increased the amount of glutathione, glutathione peroxidase, catalase and superoxide dismutase (Santhakumari et al., 2003). Saravanan et al. (2006) and Young et al. (2007) reported the hepatoprotective effect of *P. betle* leaf extract administered orally against carbon tetrachloride-induced liver fibrosis in rats through significantly increasing the antioxidant enzymes and decreasing the liver ezymes (AST and ALT) .

Arambewela et al. (2005b) conducted the antidiabetic activity of *P. betle* leaf aqueous and ethanolic extract (100, 200 and 300 mg/kg, p.o.) against streptozotocin-

induced diabetes in rats and reported the significant and dose dependent decrease in the blood glucose level. *P. betle* leaf extracts exhibited potent anti-inflammatory activity by inhibiting the xanthine oxidase, lipoxygenae and hyaluronidase enzymes (Pin et al., 2010). Arambewela et al. (2005c) studied the antinociceptive activity of ethanolic extract of *P. betle* (125, 200, 300 and 500 mg/kg, p.o.) in rats and results of this study revealed the central analgesic activity as they have shown marked activity in hot plate and formalin test. Various phenolic constituents isolated from *P. betle* leaves were reported to possess antifungal, nematocidal and antibacterial activity (Evans et al., 1984, Nair and Chanda, 2008, Sharma et al., 2009b, Ali et al., 2010). Most of the pharmacological activities like anti-ulcer, hepatoprotective, anti-carcinogenic, antiseptic and modulation of platelet aggregation possessed by *P. betle* leaf is predominately through the antioxidant mechanism (Jeng et al., 2002, Majumdar et al., 2002, Young et al., 2007).

1.2.1.3 *Bauhinia purpurea* Linn

B. purpurea L. is a medium sized deciduous flowering tree belongs to the family *Leguminosae*, commonly found in Southeast Asia (Kumar and Chandrashekar, 2011). *B. purpurea* is well known Malaysia with a local name of Tapak kuda (Burkill, 2002). Different parts of this plant have been used in traditional medicine to cure a host of illness such as body pain, rheumatism, fever, dropsy, ulcer, stomach tumour, skin diseases, septicemia and diarrhoea (Asolker et al., 1992; Kirthikar and Basu, 2001). The decoction prepared from the flower of this plant is used as a laxative and anthelmentic (Wassel et al., 1986). Root is used as a carminative and the decoction prepared from the stem bark is used to treat asthma, respiratory disorders and also as an anti-inflammatory agent (Kumar and Chandrashekar, 2011).

Various classes of phytoconstituents are identified from different part of *B. purpurea*. Yadava and Tripathi (2000) isolated a flavone glycoside 5,6-dihydroxy-7-methoxyflavone 6-O-b-D-xylopyranoside from ethanolic extract of *B. purpurea* stem. Several foliar flavonoids such as kaempferol, quercetin and isorhamnetin are isolated from the 80% methanolic leaf extract of *B. purpurea* (Salatino et al., 1999). From the whole plant as well as root of *B. purpurea*, Pettit et al. (2006) isolated and identified new oxepins such as bauhiniastatins 1, 2, 3 and pacharin and these constituents exerted potential growth inhibition against human cancer cell lines. Boonphong et al. (2007) reported eleven new compounds such as bauhinoxepin C-J, bauhinobenzofurin A, bauhinol E, bauhispororin A along with two flavones from the dichloromethane root extract of *B. purpurea*. Three glycerol derivatives, 2,3-dihydroxypropyl oleate, 2,3-dihydroxypropyl linoleate, 2,3-dihydroxypropyl 16-hydroxyhexadeconate along with a novel flavonone glycoside 6-butyl-3-hydroxyflavone, 6-(3”-oxobutyl)-taxifolin were isolated and identified by Kuo et al. (1998) from the methanolic extract of *B. purpurea* heartwood. Ragasa et al. (2004) reported phytol, fatty esters, leutin and *β*-sitosterol from the leaves of *B. purpurea*.

Aqueous alcoholic extract of *B. purpurea* bark powder at a dose of 2.5 mg/kg (p.o.) inhibited the metformin-induced hypothryroidism in type 2 diabetic mice (Jatwa and Kar, 2009). Earlier, Panda et al. (2003) also reported the increase in serum T_3 and T_4 by ethanolic extract of *B. purpurea* bark (5 mg/kg, p.o.) and hepatoprotective activity by decreasing lipid peroxidation as well as by increasing antioxidative enzymes such as catalase and superoxide dismutase. Recently, Zakaria et al. (2011) studied the anti-ulcer activity of aqueous extract of *B. purpurea* leaf (500 and 1000 mg/kg, p.o.) against absolute ethanol-induced gastric ulcer model and in SD rats. The outcome of the study showed that *B. purpurea* extract at the tested

doses significantly reduced the gastric lesion and total ulcer area and they concluded that it could be due to the presence of saponins or polyphenols in the plant extract. Muralikrishna et al. (2008) reported the significant hypoglycaemic activity of *B. purpurea* stem ethanolic extract and its purified fraction (100 mg/kg, i.p.) against alloxan-induced diabetes in rats. Boonphong et al. (2007) reported the potent antimycobacterial activity of the components isolated from the root extract of *B. purpurea* along with antimalarial activity and potent anti-inflammatory activity.

Earlier, various *in vivo* studies such as antinociceptive, anti-inflammatory and antipyretic activities were conducted by Zakaria (2007) using various doses of aqueous extract (6, 30 and 60 mg/kg, s.c.) of *B. purpurea* leaf. They reported the significant central and peripheral antinociceptive activity as revealed from both phases of formalin test and in hot plate test also with anti-inflammatory and antipyretic activities. Further, Zakaria et al. (2009) recently reported significant central and peripheral antinociceptive activity as well as anti-inflammatory activity of *B. purpurea* leaf chloroform extract at a dose of 20, 100 and 200 mg/kg (p.o.). Shreedhara et al. (2009) also reported the potential central and peripheral analgesic activities of ethanolic extract of *B. purpurea* stem bark at a dose of 100 mg/kg (i.p.) along with anti-inflammatory activity and the found activity was comparable with the corresponding standard drugs.

The aqueous and methanolic extract of *B. purpurea* leaf exhibited concentration dependent superoxide anion scavenging activity and moderate DPPH radical scavenging activity along with potential inhibition of the proliferation of various cancer cells (Zakaria, 2007; Zakaria et al., 2011). Lakshmi et al. (2009) reported the nephroprotective activity of both leaf and pod ethanol extract of *B. purpurea* (300 mg/kg, p. o.) against gentamicin-induced nephrotoxicity in rats.

Terminalia catappa

Piper betle

Bauhinia purpurea

Cinnamomum iners

Callicarpa furfuracea

Figure 1.1 Photographs of medicinal plants selected for the study.

1.2.1.4 *Cinnamomum iners* Reinw

C. iners R is one of the 250 species from the genus *Cinnamomum* belongs to the family *Lauraceae*. It is a small to average height evergreen tree distributed commonly in India, Malaysia, China, Philippines, Thailand and Indonesia (Mustaffa et al., 2012). In Malaysia, it is known with the local name Medang teja (Burkill, 2002; Ong, 2008). Traditionally, various parts of this plant have been used as antidiarrhoeal, diuretic, carminative, laxative, anti-infective (Butkhup and Samappito, 2011), tonic for stomach, in the treatment of rheumatism (Nguyen et al., 2004) and to relieve headache and breathing problems (Mustaffa et al., 2011). Several essential oils such as linalool, caryophellene oxide, cardinol were identified from the leaves (Phutdhawong et al., 2007) and 1,8-cineole, α-terpineol, terpinen-4-ol, β-pinene and, caryophyllene oxide from the stem bark of *C. iners* (Baruah et al., 2001). Xanthorrhizol was isolated and identified as an antibacterial bioactive compound from the ethyl acetate fraction of methanolic extract of *C. iners* leaf against the methicillin resistant *Staphylococcus aureus* (Mustaffa et al., 2011). Moreover, with the synonym of wild cinnamon, the bark of this plant was used as a substitute for cinnamon in various parts of Malaysia and Thailand (Mustaffa et al., 2012).

Hydrolysed extract of *C. iners* rind exhibited good DPPH radical scavenging activity as well as antimicrobial activity as evaluated by disc diffusion method against *Streptococcus faecalis*, *E. coli*, *Salmonella typhi*, *Shigella dysenteriae* and *Proteus vulgaris* (Butkhup and Samappito, 2011). Essential oils from the leaves of *C. iners* exhibited potent antifungal activity against wood rotting fungi (Jantan and Ali, 1994). Essential oils obtained from leaves of *C. iners* by hydrodistillation and microwave assisted extraction displayed potential antioxidant activity in DPPH, ABTS and FRAP assays (Phutdhawong et al., 2007). Mustaffa et al. (2010b) also

reported the marked antioxidant activity of methanolic extract of *C. iners* leaf as obtained from the DPPH assay, reducing power and hydrogen peroxide assay and the found activity was higher than vitamin E. Methanolic extract of *C. iners* at a dose of 200 and 500 mg/kg (p.o.) exerted significant activity in the late phase of formalin assay but not in the early phase as well in hot plate and tail flick tests revealing its peripheral analgesic activity (Mustaffa et al., 2010b). Pang et al. (2009) reported the significant anticancer activity of both acetone and methanol extract of *C. iners* leaves by inhibiting the Mitogen activated protein Kinase Kinase (MKK1).

1.2.1.5 *Callicarpa furfuracea* Ridl

Callicarpa furfuracea belongs to the genus *Callicarpa* is a small shrub belongs to the family *Lamiaceae*. This plant is normally found distributed in Southern Thailand and Peninsular Malaysia (Leeratiwong et al., 2007). Traditionally aerial part of this plant is used in the treatment of pain and various infections.

1.2.2 Importance of ethnopharmacology

Ethnopharmacology is considered as the origin of most of medicine and thus natural products are the important source of drugs. Ethnopharmacology establish a link between the traditional knowledge and discovery of novel biological target (Harvey, 2002). The ethnomedical knowledge regarding the use of natural products as source of medicine by human has been accumulated and transferred from one generation to other since from thousands of years (Pan et al., 2009). Ethnopharmacology greatly helped in the discovery of various historically and therapeutically important drugs such as morphine, quinine, quinidine, digitoxin, ephedrine, cocaine, artemisinin, vincristine and taxol (Do and Bernard, 2006; Newman and Cragg, 2012). Hence,

natural products as a source of medicine contributed enormously in the development of potent and important therapeutic drugs which are currently used in modern medicine (Cragg et al., 1997; De Smet, 1997; Shu, 1998).

The success rate of obtaining bioactive compounds is 19.9% if the natural product research is conducted on the basis of ethnopharmacological information. On the other hand random screening resulted in obtaining only around 10.4% of bioactive compounds (Harvey, 2002). The lead molecules obtained from the natural products helped in many ways for the success of pharmaceutical industries. The high chemical diversity, biochemical specificity, greater number of chiral centres, higher number of oxygen atoms, higher number of solvated hydrogen-bond donors and acceptors, lower ratio of aromatic ring atoms to total heavy atom and broader octanol-water partition coefficient made natural products as favourable lead structures for the drug discovery (Koehn and Carter, 2005). Although natural products are considered as a rich source of pharmaceutically important lead molecules, they have been neglected in the past decades due to the difficulties in applying modern screening assays to them (Koehn and Carter, 2005). In brief, ethnopharmacology and phytochemistry in the past enormously have contributed and still contributing in the natural product drug discovery. The natural product lead discovery involves the selection of appropriate plant sample, extraction, fractionation, biological screening and isolation of one or more bioactive compounds.

1.2.3 Isolation of bioactive compounds

Normally plants selected for the natural product research should be collected from the field according to the ethnopharmacological knowledge (Do and Bernard, 2006). Authentication of the plant material is one of the important parameter considered

during standardisation as it ensures the correct identity of the plant. Once the plant is identified, the plant is either in the fresh form or dry form used for the extraction purpose. Normally, dry form is preferred over fresh form as it reduces the interference of water during solubility, isolation, while evaluating biological activity and prevent the microbial growth and degradation of bioactives by the enzymes as well as easy to handle (Romanik et al., 2007). In addition, even traditional herbal healers use the plants in dry form or as an aqueous extract (Baris et al., 2006). Various methods such as drying under direct sunlight, shade drying, oven drying and freeze drying are normally employed for drying purpose. However with the advantages such as decrease in the loss of bioactive constituents at lower drying temperature, exposure to air and radiations made freeze drying as an ideal method for drying plant materials (Brewer, 2011). Powdering of dried plant materials enhance the interfacial area of the plant material to the solvent, easy destruction of biological cell walls and hence expose the cellular components to the solvent. This will leads to an increased extraction of phytoconstituents from the plant material (Kim and Lee, 2001; Romanik et al., 2007).

Solvent plays an important role in the isolation of bioactive compounds as not all the bioactives are soluble in a single solvent. The solvent employed for the study should have low toxicity, easy of evaporation at low temperature, preservative ability and not complex or dissociate with the bioactive of the interest (Ncube et al., 2008). The amount of bioactive compounds extracted also depends on the type of solvent employed for extraction purpose (Sultana et al., 2009). The most commonly used solvents for the extraction of phytoconstituents from plant material are petroleum ether, dichloromethane, ethyl acetate, acetone, ethanol, methanol and their aqueous solvents (Stalikas, 2007). During the routine extraction of herbal material, ethanol is

normally employed rather than methanol in order to avoid the toxic properties of methanol. In addition, ethanol has high penetrating ability, safer to apply to food, environmental friendly and widely accepted solvent (Kim and Lee, 2001; Shi et al., 2005; Maisuthisakul et al., 2007). Evaporation of the solvent with the extracted material should be carried out under reduced pressure at low temperature in order to minimise the degradation of phytoconstituents.

1.2.4 Various natural product extraction techniques

Herbal extract is a concentrated preparation with liquid, solid or intermediate consistency (Srinivasan, 2006). Extraction is a critical step in the drug discovery which determines the amount of phytoconstituents in the final extracts as well as biological activities (Romanik et al., 2007). The main objective of extraction is to remove either completely or partially the possible phytoconstituents from solid plant material in which it exists naturally by transferring them into an extracting solvent (Xiao et al., 2009; Stalikas, 2010). The quality of the extract obtained mainly depends on the solvents employed, temperature used, and duration of extraction, sample-to-solvent volume ratio and mechanical agitation (Kim and Lee, 2001; Hayouni et al., 2007; Jacques et al., 2007). Extraction method used must extract and retain the potential bioactive without altering or destroying (Cos et al., 2006).

Various extraction methods such as soxhlet extraction, supercritical fluid extraction, microwave assisted extraction, accelerated solvent extraction, sonication along with traditional extraction methods like maceration, infusion, decoction and percolation were used to prepare the herbal extract (Srinivasan, 2006; Wang and Weller, 2006; Romanik et al., 2007). Extraction technique selected must not be only on the basis of extraction yield but on the time duration, cost and volume of solvent

(Romanik et al., 2007). Findings from various researchers revealed the variation in phytoconstituents as well as biological activities of the herbal material depends on the extraction technique employed (Grigonis et al., 2005; Bajer et al., 2007; Karakaya et al., 2011). In the following section the most commonly used extraction techniques which were used in this study are briefly reviewed.

1.2.4.1 Maceration

Maceration is one of the traditional extraction techniques used for the extraction of herbal material. In this method the ground plant material to be extracted will be placed in a stoppered conical container and soaked with the required amount of the solvent. The phytoconstituents are allowed to diffuse at room temperature in the dark for the required duration of time (3 to 10 days) along with occasional stirring. Maceration technique is very simple and cost effective compared to all other extraction techniques. However this method suffers from the disadvantages like longer duration of extraction and requirement of large volume of solvents (Sae-Yun et al., 2006).

1.2.4.2 Soxhlet extraction

Soxhlet extraction is also one of the classical techniques employed for the extraction of plant metabolites which are heat stable and has low volatility. In this method, the plant material filled in a thimble made up of cellulose is placed in a thimble holder. The vapours of the solvent from the distillation flask condense and fall into the thimble. When liquid in the siphon tube reaches the overflow level, the solute in the thimble holder unloads it back into the distillation flask. Again the solvent will distillate, vaporise and the vapours of fresh solvent pass through the plant material leaving the solute in the distillation flask (Luque de Castro and Garcia-Ayuso, 1988).

Main advantages of this technique are simple, economical, high extraction yield and every time fresh solvent comes in contact with the plant material. However, it suffers from various shortcomings such as longer extraction time, higher consumption of solvent and thermal decomposition of some phytoconstituents at the boiling point of the solvents employed (Wang and Weller, 2006; Romanik et al., 2007).

1.2.4.3 Sonication assisted extraction

Sonication assisted extraction allows efficient extraction of various phytoconstituents in a shorter interval of time as compared to conventional extraction methods (Paniwnyk et al., 2001). Various bioactive compounds such as flavonoids, steroids, terpenoids and volatile compounds are extracted using sonication extraction method (Romanik et al., 2007). In sonication method of extraction the required amount of grounded plant material is soaked in the selected solvent in a container followed by exposing the container to the ultrasonic waves. Sonication at a frequency of ultrasound in the kilo hertz (kHz) range significantly enhanced extraction yield in a short time interval. In this method, the extraction takes place through the cavitational phenomenon. Ultrasonic sound generates countless number of microscopic solvent bubbles which are then compressed and imploded at high pressure. This leads to formation of a powerful shock wave with the ability to penetrate effectively inside cell wall of the plant material and releasing them into the extraction solvent (Wang and Weller, 2006). During the sonication extraction, the temperature of the ultrasonic bath should be maintained at room temperature as the higher temperature may reduce the cavitational effect and cause the degradation of phytoconstituents (Kim and Lee, 2001).

1.2.5 Liquid-liquid extraction

Extracts of natural products obtained from any extraction technique consists of various phytoconstituents belongs different groups of secondary metabolites. The constituents other than the targeted compound/s interfere during the separation and isolation of phytoconstituents and prolong the time required for the isolation of bioactive compound/s. This interference can be reduced by liquid-liquid fractionation as it separates the constituents according to their polarity from extremely polar to medium polar to extremely non-polar (Cos et al., 2006; Romanik et al., 2007). In addition, fractionation of any natural product sample enhances the relative concentration of bioactive compound present in minor quantity in the extract providing an opportunity to uncover the novel bioactive compound (Robards, 2003, Koehn and Carter, 2005).

1.2.6 Separation and isolation of phytoconstituents

Isolation of any compound is conducted on the basis of either structure directed or on bioactivity directed. Bioactivity directed isolation method is employed only when the plant used for the study is on the basis of traditional use (Sarker et al., 2005). Chromatography is a common technique generally employed for the separation and isolation and identification of compounds from any complex mixtures. Various chromatography techniques such as preparative thin layer chromatography (TLC), paper chromatography, column chromatography and preparative high performance liquid chromatography (HPLC) along with electrophoresis are used for the separation of phytoconstituents from plant extracts or from liquid fractions (Tsao and Deng, 2004; Stalikas, 2007). The amount of bioactive compound isolated should be enough to elucidate the structure of the compound and to evaluate the targeted biological activity.

1.2.7 Chromatographic separation of phytoconstituents

Thin layer chromatography is the simple, rapid and economical method normally employed in the discovery of drugs from natural products. TLC is one of the important methods to evaluate the botanical identity and for the standardisation herbs and their products (Poole, 2003). This method provides advantages of employing TLC plates coated with different adsorbents such as alumina, silica gel, polyamide, etc coated on either glass, aluminium or plastic sheets are used. The herbal extract to be evaluated is applied either in the band or spot form around 1 cm above from the lower edge of the plate and allowed to develop in a twin trough TLC chamber saturated with the mobile phase. Mobile phase consist of either single or mixture of solvents along with either acid or basic modifier were used for the better separation of compounds from the complex plant matrix. Compounds of different polarity were separated according to their affinity towards either mobile phase or stationary phase as the mobile phase pass through the adsorbent by capillary action. Compounds separated on TLC plates were then identified either by observing under UV light (254 or 365 nm) or by spraying with suitable detection reagents (Garcia et al., 1993).

TLC- bioautography is a simple and rapid chromatographic method which allows the separation of a complex herbal material and simultaneously helps to locate the bioactive compounds on the TLC plate (Marston, 2011). The major application of TLC-bioautography method is in the bioactivity guided isolation procedures. Various bioactive like antioxidants, antimicrobials, antidiabetic and acetylcholinesterase were isolated from natural resources by this method (Marston, 2011). In this method, compounds separated on TLC plate were sprayed with a particular reagent on the either side of plate covering the remaining portion to identify the target bioactive compound/s. The portion of where the target compound located is then scrapped and

removed carefully followed by desorbing from the adsorbent using appropriate solvent that dissolve the compound (Gu et al., 2009; Xiao et al., 2010). Though, this is one of the fastest methods it suffers from the drawbacks of restricted application in the pharmacological screening and the amount of bioactive separated will be in few milligrams.

In order to obtain the compounds in milligram or gram quantity for multiple utilisation, column chromatography and preparative HPLC is normally employed. With column chromatography use of wide number of adsorbents such as normal silica gel, octadecyl silca (ODS), alumina, polyamide, diaion HP-20, sephadex LH-20 etc were possible for the separation and isolation of compounds from a complex plant matrices. In column chromatography the extract from which compounds to be separated is loaded on the top of the adsorbent which is packed properly and conditioned with the appropriate mobile phase in an open glass column of required dimension. The column is then eluted with a single solvent or a mixture of solvent of increasing polarity either by normal gravity or by applying positive pressure at the inlet using nitrogen gas or by vacuum suction. Mixture of compounds was separated on the basis of their affinity towards mobile phase, stationary phase, hydrogen bonding, charge, molecular size and Van-der Waals force (Reid and Sarker, 2005; Zhang et al., 2012). TLC in combination with column chromatography is routinely used for the separation of phytoconstituents in appreciable quantity (Cserháti and Forgács, 2001; Fair and Kormos, 2008). The chemical profile of the column fractions were always monitored by TLC for the isolation of various bioactive compounds (Cakir et al., 2003). Preparative-HPLC method is also employed for the isolation of various compounds from natural products (Fang et al., 2005).

1.2.8 Identification and structure elucidation

The important objective of natural product research is to identify the structure of the isolated bioactive compound. Various spectroscopic methods available provide the structural information of the isolated compounds. If the isolated compound is known then the values of spectroscopic studies along with their physical properties like melting point and solubility are compared with the values reported for the particular known compound. If the isolated compound is unknown then spectroscopic information from UV-visible spectroscopy, fourier-transform infrared red spectroscopy (FTIR), mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy are used for the elucidation of the structure (Sarker et al., 2005).

1.2.8.1 Spectroscopic studies of isolated compounds

1.2.8.1. a) UV-visible and FTIR spectroscopy

The presence of number of conjugated double bonds and aromatic bonds in any compounds exhibits a higher or lower absorption in the UV or UV-visible region. In case of phenolic compounds, the absorption maxima are observed in the range of 200 to 290 nm with some exceptions (Stalikas, 2007). The absorption maxima increase with the substitution pattern and conjugation. Hence, different classes of compounds have their characteristic absorbance peaks which help in the identification of that particular group of compound or class of compounds (Sarker et al., 2005). FTIR also reported to be one of the valuable tools which contribute in the structure elucidation of both known as well as unknown compounds (Hazra et al., 2007). In case of FTIR, if the frequency of the dipole movement of any functional group of a molecule is similar to the applied IR frequency then they absorb the energy to give IR spectra. In FTIR spectroscopy, the extensively studied part of IR is the mid IR region of 4000 to

400 cm^{-1}. This region consists of two parts i.e. 4000 to 1300 cm^{-1} is functional group region where information regarding the functional groups such as –C=O, -C-H, -OH, -NH, -NH_2, -C=C, etc are observed. The region between1300 to 900 cm^{-1} is referred as fingerprint region which is unique for each molecule (Lampman et al., 2010).

1.2.8.1. b) Proton and carbon 13 NMR

High resolution NMR technique is an advanced spectroscopic technique which contributed in the elucidation of structure of various complicated molecules (Koehn and Carter, 2005). NMR reveals the information regarding the number and types of protons and carbon in a molecule along with their positions and relationship among these atoms. The information obtained from NMR about a molecule helps in the elucidation of structure of that molecule (Bross-Walch et al., 2005). There are two types of NMR experiments, one dimensional and two dimensional NMR techniques. Examples for one dimensional NMR techniques are proton NMR (^{1}H NMR) and carbon-13 NMR (^{13}C NMR) and two dimensional NMR techniques are correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), nuclear overhauser enhancement spectroscopy (NOESY), heteronuclear single quantum coherence spectroscopy (HSQC), heteronuclear multiple bond correlation spectroscopy (HMBC), etc (Koehn and Carter, 2005). The information obtained from ^{1}H NMR spectrum such as chemical shifts, spin-spin couplings and peak integrals provides the information regarding the relative position of various types of proton in a molecule. In the same way, spectrum of ^{13}C NMR offers information regarding the number and relative position of various carbon atoms in a molecule.