CHEMICAL STUDIES AND BIOLOGICAL ACTIVITIES OF EXTRACTIVES FROM *PIPER BETLE* LEAVES

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This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Sciences with Honours (Resource Chemistry)

Faculty of Resource Science and Technology
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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree or qualification of this or any other university or institution of higher learning.

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Isolation and Characterization of Compounds of Extractives from the Leaves of *Piper betle*

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ABSTRACT

Isolation of compounds and the biological activities of the extracts from the *Piper betle* leaves were carried out. The sample was extracted by using solvent extraction method using four different solvents with increasing polarity which are hexane, dichloromethane (DCM), ethyl acetate and methanol. Further fractionation and isolation by using chromatographic methods afforded a pure compound with molecular mass of 204.2 when subjected to GC-MS analysis, which is the compound amorphene. A semi pure compound was also obtained, giving one sharp peak when subjected to GC-FID analysis. Toxicity test revealed that the extractives of *Piper betle* leaves are not toxic against brine shrimp, *Artemia salina*, with LC_{50} greater than 100ppm. In antitermite toxicity test, DCM crude extract was found to be the most toxic, with 100.0% mortality after three days for three different concentrations. The antibacterial test showed that the DCM extract gave growth inhibition to the bacteria at 10.0% concentration. It was also found that the DCM crude extract is more toxic towards *Escherichia coli* as compared to *Staphylococcus aureus.*

Key words: *Piper betle*, solvent extraction, isolation, purification, toxicity test

ABSTRAK

Pemisahan sebatian and aktiviti biologi ke atas ektrak daripada daun sirih (Piper betle) telah dijalankan. Sampel tersebut diekstrak dengan menggunakan kaedah pengekstrakan pelarut menggunakan empat pelarut dengan kekutuban berbeza iaitu heksana, diklorometana (DCM), etil asetat dan metanol. Pemfraksian dan pemisahan yang lebih lanjut menggunakan kaedah-kaedah kromatografi telah mendapat suatu sebatian hampir tulen dengan jisim molekul 204.2 apabila dianalisis dengan GC-MS, iaitu sebatian amorfin. Suatu sebatian separa tulen juga diperoleh, memberikan satu puncak yang tajam apabila dianalisis dengan GC-FID. Ujian ketoksikan menunjukkan bahawa ekstrak daun sirih tidak toksik terhadap anak udang, Artemia salina, dengan LC_{50} melebihi 100ppm. Dalam ujian ketoksikan terhadap anai-anai, ekstrak DCM didapati paling toksik dengan 100.0% kematian selepas tiga hari bagi tiga kepekatan yang berbeza. Ujian antibakteria pula menunjukkan ekstrak DCM menghasilkan perencatan pertumbuhan bagi bacteria pada kepekatan 10.0%. Ia juga didapati bahawa ekstrak DCM adalah lebih toksik terhadap Escherichia coli berbanding Staphylococcus aureus.

Kata kekunci: *Piper betle, pengekstrakan pelarut, pemisahan, penulenan, ujian ketoksikan*

CHAPTER 1

INTRODUCTION

1.1 General Introduction

The genus *Piper* which belongs to the family Piperaceae contains about 700 species distributed in both hemispheres. They are widely distributed in the tropical and subtropical regions of the world. (Parmar *et al.*, 1997). The family Piperaceae contains about 1400 species distributed worldwide among its five genera which are *Piper*, *Peperomia*, *Lepianthes*, *Macropiper* and *Trianaeopiper* (de L. Moreira *et al.*, 2000).

Basically, the *Piper* are erect or climbing herbs and shrubs, or infrequently trees (Keng, 1983; Parmar *et al.*, 1997). Their leaves usually alternate or rarely opposite with unbranched spikes and their blades often pellucidly dolted. *Piper betle* Linn. which is locally known as 'sireh' is a perennial diocieous, semi-woody climber with stems strongly swollen at the nodes, papillose when young and soon entirely glabrous. Their leaves are simple, alternating and colour ranges from yellowish green to bright green (Arambewala *et al.*, 2005).


Figure 1.1.1 The *Piper betle* leaves

The *Piper* species have high commercial, economical and medicinal importance. They are used medicinally in various manners. Economically the Piperaceae is important for the pepper in the worldwide market. The ripened fruit of *Piper nigrum* is the source of white pepper, while the unripe fruit of the same species is the source of black pepper (Parmar *et al.*, 1997). In addition, many of the *Piper* species enjoys folklore uses as traditional medicine (Taufiq-Ur-Rahman *et al.*, 2005).

Piper betle which is the native of this part of the world, was domesticated in comparatively early times. It provides the fresh pepper leaves that are chewed along with lime and slices of betel nuts. Race, age of plant, exposure to light and even the position of the leaf on the shoot are all factors in determining the excellence of the leaf for chewing. The practice of chewing this pepper leaf is said to confer on the person some protection against parasitic worms (Hoi-Sen, 1990).

The phytochemistry investigations of *Piper* species have led to the isolation of several classes of physiologically active compounds such as alkaloids, amides, pyrones, dihydrochalcones, flavonoids, phenylpropanoids, lignans and neolignans (Parmar *et al.*, 1997; Rukachaisirikul *et al.*, 2004).

This project is of interest due to the valuable and recognized medical properties possessed by this species. This study will be conducted in order to isolate and characterize the bioactive compounds from the extracts of the leaves from *Piper betle.* The toxicity of the compounds will be evaluated. This study is meant to extend the researches that have been done previously on this species. By the end of this study, certain bioactive compounds will be isolated and characterized.

1.2 Objectives of Study

The objectives of this study are:

i. To extract the extractives from *Piper betle* by using solvent extraction method.
ii. To isolate, purify and characterize the chemical constituents in the extractives.
iii. To carry out toxicity tests to determine the biological activities of the *Piper betle* leaves.

CHAPTER 2

LITERATURE REVIEW

2.1 *Piper* species

The family Piperaceae belongs to the order Piperales (Hsuan Keng, 1983), and principally comprises of five genera, which are *Piper*, *Peperomia*, *Lepianthes*, *Macropiper* and *Trianaeopiper*, and about 1400 species distributed worldwide (de L. Moreira *et al.*, 2000). The genus *Piper* has over 700 species distributed in both hemispheres. They are erect or scandent herbs, shrubs or infrequently trees (Parmar *et al.*, 1997).

The phytochemical studies carried out thus far on the *Piper* species yielded the classes of bioactive compounds such as amides, pyrones, flavonoids, alkaloids, dihydrochalcones, lignans, neolignans and phenylpropanoids (Parmar *et al.*, 1997).

2.2 Traditional uses

The root of *Piper methysticum*, which is also known as the kava shrub, is the source of perhaps the most important traditional beverage for many South Pacific Island people which can give a relaxing effect (Parmar *et al.*, 1997; Dragull *et al.*, 2003). Due to its anxiolytic properties it has become a popular remedy in Europe and North America. It has become an important economic crop throughout the South Pacific when its lipophilic extracts are used in pharmaceutical industry to produce dietary supplements. However, the

products were subsequently banned in Germany and several other countries when cases of liver damage are reported since 1998 in European countries due to medicinal usage (Dragull *et al.*, 2003).

The antimalarial activity of *Piper cumanense* fruits and leaves and *Piper holtonii* aerial parts which have been traditionally used in Colombia to treat malaria symptoms was reported. They were active against *Plasmodium falciparum* in vitro but inactive in the vivo model. *Piper cumanense* fruits and leaves were found to be toxic at 250 mg/kg. The extract of fruits was four times less active than that of leaves in the ferriprotoporphyrin biomineralization inhibition test (FBIT) (Garavito *et al.*, 2006).

In the Malay and Indonesian Archipelago, the leaves and roots of *Piper sarmentosum* are used for the treatment of toothache, fungoid dermatitis on the feet, coughing asthma and pleurisy. In Thailand, this plant and its fruits are used as an expectorant. As the *Piper cumanense* and *Piper holtonii*, this plant also showed considerable antiplasmodial activity against *Plasmodium falciparum* and *Plasmodium berghei* parasites (Rukachaisirikul *et al.*, 2004).

The *Piper longum* has been used in an ayurvedic contraception in India since ancient times. It is combined with *Embelia ribes* and borax in equal proportion to produce pippaliyadi yoga which gives the contraceptive potential (Balasinor *et al.*, 2007). The present study by Balasinor *et al.* (2006) suggests that in utero exposure to pippaliyadi yoga does not have any adverse effect on the postnatal development and reproductive performance of the F_1 progeny. Besides this species, *Piper nigrum* is also used extensively in the Ayurvedic system of medicine. The ayurvedic preparation traditionally used the aqueous extract of

black pepper (Srinivas and Rao, 1999). The fruits of *Piper nigrum* are also used as a condiment and also as stimulant, rubafacient and disinfectant when applied externally (Martins *et al.*, 1998).

In Asian countries the leaves of *Piper betle* are used for chewing and are credited with many medicinal properties such as digestive, stimulative, carminative and aphrodisiac (Arambewala *et al.*, 2005).

The leaves of *Piper capense* whose vernacular name in S.Tomé "Fiá Boba Piquina" are used as stomachic and carminative in indigestion, flatulence and colic, and is also said to cause sweating and sleepiness. The leaves of *Piper guineense*, which is also known as 'Pó Pimenta' and 'Ashanti pepper' in other countries, are widely used as an antibacterial, especially to heal wounds. The leaves of *Piper umbellatum* which is also known as 'Fiá Boba d'Obô' are used to heal wounds and to reduce swellings and skin irritations (Martins *et al.*, 1998).

Piper aduncum is widely used in folk medicine to treat trachoma, vagnitis and stomach aches (de L. Moreira *et al*., 1998a). It is also used as remedy for stomach aches and as insect repellent (Baldoqui *et al.*, 1999). *Piper dilatatum* has been studied since it is used by the Kuna Indian of Panama as a constituent of a mixture of plants applied as a tonic bath for various afflictions (Terreaux *et al.*, 1998).

Piper marginatum which is popularly known as 'malvaisco' is used in the Brazilian state of Paraiba as a food flavouring agent (seeds) and also as an antidote for snakebites (root) (de O.Santos *et al.*, 1998; de Oliveira Santos and de Oliveira Chaves, 1999a, b). It is

commonly used in the treatment of liver diseases and as a spasmolotic by the Amazonian aborigines (de Oliveira Santos and de Oliveira Chaves, 1999). *Piper tuberculatum* is also used in Paraiba, Brazil as a sedative and as an antidote for snakebites and the fruits are used for toothache (de Araújo Júnior *et al.*, 1999; Felipe *et al.*, 2007). In other communities, the fruits are used as food spice with beans (Felipe *et al.*, 2007).

Piper gaudichaudianum is the species more distributed in the Brazilian Atlantic forest, from the Northeast to the South of Brazil. This species also reaches Argentina and Paraguay. They are known by Pariparoba, Paripaioba, Muta, Iaborandi or Jaborandi. Their leaves are used traditionally in popular medicine to relief toothache and also as antiinflammatory (Péres *et al.*, 2006).

Piper chaba which is available in various parts of India and Malay Islands also enjoys vast folklore uses as traditional medicine. The stem is used to alley post-delivery pain in mothers and useful in rheumatic pains and diarrhoea. The root is alexiteric, useful in treating asthma and bronchitis. The fruit has carminative and stimulant properties, and is used in haemorrhoidal affections. The fruit is useful in asthma, bronchitis, fever, inflammation, piles, pain in the abdomen and the anus (Taufiq-Ur-Rahman *et al.*, 2005).

2.3 Phytochemical studies and biological activities

Due to their medicinal, economic and ecological importance, a number of the *Piper* species have been phytochemically investigated yielding several classes of compounds (Martins *et al.*, 2003).

Experimentally, leaves of *Piper betle* were shown to possess antimicrobial, gastroprotective, wound healing, hepatoprotective, antioxidant, antifertility on male rats and antimotility effects on washed human spermatozoa (Arambewala *et al.*, 2005). The essential oil and leaf extracts of *Piper betle* possess activity against several Gram-posive and Gram-negative bacteria. These include *Bacillus subtilis*, *Bacillus megaterium*, *Diplococcus pneumoniae*, *Escherichia coli*, *Erwinia carotovora*, *Micrococcus pyogenes*, *Proteus vulgaris*, *Pseudomonas solanaoearum*, *Salmonella typhosa*, *Sarcina lutea*, *Shigella dysentriae*, *Streptococcus pyogens* and *Vibrio comma*. Antiseptic activity is probably due to chavicol. Essential oil and leaf extracts also show antifungal activity against *Aspergillus niger*, *Aspergillus oryzae*, *Curvularia lunata* and *Fusarium oxyporum* (Duke, 1985).

Antidiabetic activity of *Piper betle* was tested in normoglycaemic and strepozotocin (STZ)-induced diabetic rates using oral administration of hot water extract and cold ethanolic extract of the betel leaves. In normoglycaemic rats, both extracts significantly lowered the blood glucose level in a dose-dependent manner. In glucose tolerance test, both extracts markedly reduce the external glucose load. This shows that the *Piper betle* has strong antidiabetic activity. In addition, the toxicity of the extracts was also tested

using chronic administration. Both extracts were found to be non-toxic and well tolerated (Arambewala *et al.*, 2005).

The purification of the extract from leaves of *Piper lhotzkyanum* by chromatographic methods has led to the isolation of a new chromene, the lhotzchromene **(1)**. Two known phenylated benzoic acid derivatives isomers, the (E) and (Z) of 4-hydroxy-3-(3',7'-dimethyl-1'-oxo)-2',6'-octadienylbenzoic acid **(2 and 3)** were found. These isomers have proviously been isolated from leaves of *Piper murrayamum*. This research also isolated a mixture of hydroxylated sesquiterpenes, which includes spathulenol **(4)**, guaiol **(5)**, epi-γ-eudesmol, hinesol **(6)**, β-eudesmol and acyclic diterpene phytol. The isolated substances were identified using spectroscopic analysis. The mixture were analysed by GC-MS and the substances were identified by comparison of the retention indices (RI) and mass spectra with literature records. ^{1}H NMR and IR analyses were also used to confirm the major substances in this mixture (de L. Moreira *et al.*, 1998b).

The column chromatography of the dichloromethane fraction obtained from the methanolic extract of the leaves of *Piper lhotzkyanum* yielded for the first time in the family Piperaceae the compounds 5-hydroxy-7-methoxy-8-C-β-glucosylfavone (C-glucosylfavone) **(7)**, sakuranetin **(8)** and methyl-4-methoxydihydroferulate **(9)**. The mixture of methyl-4-methoxydihydroferulate with the C6-C3 derivatives 4-methoxydihydroferulic acid, ethyl 4-methoxydihydroferulate and methyl ferulate was also isolated. The isolated compounds were identified using spectroscopic analysis which are 1D and 2D ^{1}H and ^{13}C-NMR, and also UV. The mixture was analysed by GC/MS and the substances were identified by comparison of their mass spectra with literature data and by analysis of their mass fragmentation patterns (de L. Moreira *et al*., 2000).

Piper hispidum and *Piper tuberculatum* accumulate amides bearing isobutyl, pyrrolidine, dihydropyridone and piperidine moieties. The isolation and characterization of several representatives were performed by chromatographic techniques and by analysis of spectroscopic data. This included two unreported amides, the (3Z,5Z)-N-isobutyl-8-(3',4'-methylenedioxyphenyl)-heptadienamide **(10)** isolated from stems of *Piper hispidum* and 8(Z)-N-(12,13,14-trimethoxycinnamoyl)-Δ^3-pyridin-2-one **(11)** isolated from seeds of *Piper tuberculatum.* The antifungal activity of the amides was evaluated by using direct bioautography agains *Cladosporium sphaerospermum* (Novickiene *et al.*, 2000).

Piplartine **(12)**, a bioactive compound isolated from *Piper tuberculatum* showed a potent anxiolytic activity when tested on mice. The effect of this amide alkaloid was comparable to that of diazepam, an anti-anxiety agents (Felipe *et al.*, 2007). The compound cephranone B has also been reported for the first time for *Piper tuberculatum* (Mundina *et al*., 2001).

Various types of piperidine and piperidine alkaloids occurs in *Piper nigrum* (Parmar *et al.*, 1997), the most important being piperine **(13)**, known to possess a variety of chemical properties such as analgesic, antifeedant activities and antipyretic (Srinivas and Rao, 1999). The petrol extract of the berries of *Piper nigrum* yielded a new pyrrolidine alkamide, isopiperolein B **(14)**. The structure was established as 1-[(E)-10-(3,4-methylenedioxyphenyl)-dec-9-enoyl]pyrrolidine based on degradative and spectroscopic evidence (Srinivas and Rao, 1999). A bioguided fractionation of the petroleum ether extract of the berries of *Piper nigrum* afforded 2E,4E,8Z-N-isobutyleicosatrienamide, pellitorine **(15)**, trachyone, pergumidiene and isopiperolein B. Trachyone and pergumidiene were isolated for the first time from this plant. All the isolated compounds

were proven active against *Bacillus subtilis*, *Bacillus sphaericus* and *Staphylococcus aureus* among Gram positive bacteria. Among Gram negative bacterial strains, they were active against *Klebsiella nerogenes* and *Chromobacterium violaceum* (Reddy *et al.*, 2004).

Phytochemical investigation on stems and fruits extract of *Piper aduncum* led to the identification of a new chromene, methyl 2,2-dimethyl-8-(3-methyl-2-butenyl)-2H-chromene-6-carboxylate **(16)** in addition to eupatoriochromene, monoterpenes and sesquiterpenes. The investigation also identified from this plant the compounds 5-hydroxy-7-methoxyflavone, 2',6'-dihydroxy-4'-methoxychalcone, 7-hydroxy-5-methoxyhihydroflavone, 2'-hydroxy-4',6'-dimethoxydihydrochalcone, 2',6'-dihdroxy-4'methoxydihydrochalcone, 2',4-dihydroxy-4',6',3-trimethoxydihydrochalcone, 2',4-dihydroxy-4',6'-dimethoxydihydrochalcone and a mixture of sitosterol and stigmasterol (de L. Moreira *et al*., 1998a).

The investigation on *Piper aduncum* and *Piper hispidum* resulted in the isolation of the compounds prenylated benzoic acid and pyrrolidine amides. These were identified as cytotoxic and antifungal compounds, respectively (Martins *et al*., 2000).

The fractionation of CH_2-Cl_2- soluble part of MeOH extract of the leaves of *Piper aduncum* afforded the compounds nerolidol **(17)**, 2',6'-dihydroxy-4'-methoxydihydrochalcone, methyl-2,2-dimethyl-8-(3'-methyl-2'-butenyl)-2H-1-chromene-6-carboxylate, methyl-2,2-dimethyl-2H-1-chromene-6-carboxylate and methyl-8-hydroxy-2,2-dimethyl-2H-1-chromene-6-carboxylate. 2 new natural products were also isolated, the 2,2-dimethyl-2H-1-chromene-6-carboxylic acid **(18)** and 3-(3',7'-dimethyl-2',6'-octadienyl)-4-methoxybenzoic acid **(19)**. The structures of the isolates were established on

the basis of spectroscopic data analysis including ^{1}H, ^{13}C NMR and electrospray mass spectrum (ES-MS). The isolated compounds were tested against mutant strains of *Saccharomyces cerevisiae* for their DNA-damaging activity investigation (Baldoqui *et al.*, 1999).

The chemistry of *Piper methysticum* has been widely studied with more than 40 compounds from the classes kavapyrones, alkaloids, steroids, chalcones, long chain fatty acids and alcohol have been isolated and identified (Parmar *et al*., 1997). Chromatographic separation of the methylene chloride extract of *Piper methysticum* roots yielded fourteen compounds. Preliminary spectroscopic analysis by ^{1}H and ^{13}C NMR indicated that these fourteen compounds consisted of nine kavalactones, three chalcones, 3,4-methylenedioxycinnamylideneacetone and stigmasterol. This is the first report of kavalactone, 11-methoxy-5,6-dihydroangonin **(20)** (Dharmaratne *et al.*, 2002). The other thirteen compounds were previously reported for *Piper methysticum* (Parmar *et al.*, 1997). Kavalactones have been recognized as the constituents responsible for the reported biological activities in *Piper methysticum* (Dharmaratne *et al.*, 2002). The presence of alkaloids in relatively high concentration in the aerial parts of *Piper methysticum* was also reported. Two new piperidine alkaloids, 3α-4α-epoxy-5β-pipermethystine **(21)** and awaine **(22)** were isolated and identified in the stem peelings and unopen young leaves, respectively (Dragull *et al.*, 2003).

The investigation of the stem bark of *Piper chaba* revealed the presence of lignans and alkaloids such as piperamin 2,4-decadienoic acid piperidide, kasunokinin and pellitorine. The presence of alkamides such as piperine, slyvatine, piplartine, piperlonguminine and β-sitosterol **(23)** were also reported. Studies have shown that the extract from the fruit of this

plant and the isolated alkamides were protective against ethanol and indornethacin induced gastric lesions in rats. Chabamide, a novel piperine dimer has also been identified in the stem bark. The fruit oil of the plant showed the presence of β-caryophyllene **(24)**, caryophyllene oxide **(25)**, a few monoterpene hydrocarbons, a moderate content of sesquiterpenes and high amount of aliphatic hydrocarbons. The crude extract of this plant was found to possess antibacterial activity (Taufiq-Ur-Rahman *et al.*, 2005).

The essential oils from the leaves and spikes of *Piper lanceaefolium* obtained by hydrodistillation were analysed by GC-FID, GC-MS and ^{13}C NMR methods. The essential oil from leaves was characterized by high amount by sesquiterpene hydrocarbons (42.8%), especially β-carophyllene (20.6%) and germacene D (12.5%). The essential oil from leaves also contains phenylpropanoids, of which elemicin **(26)** and parsley apiol **(27)** were the major ones. The volatile oil from spikes showed α- and β-pinene and the phenylpropanoids elemicin and parsley apiol (Mundina *et al.*, 2001).

The isolation of 3-farnesyl-2-hydroxybenzoic acid **(28)** from *Piper multiplinervium* was also reported. Antimicrobial screening using MeOH extract of *Piper mutiplinervium* leaf has showed activity against *Helicobacter pylori* which causes chronic gastritis and peptic ulcers (Rüegg *et al*., 2006).

The isolation of chemical constituents from the fruits of *Piper sarmentosum* has resulted in antituberculosis and antiplasmodial activities on some isolates. Antiplasmodial activity was evaluated against the parasite *Plasmodium falciparum* which was cultured continuously, while the antituberculosis activity was assessed against *Mycobacterium tuberculosis* H37Ra strain. The result showed that sarmentine **(29)** and 1-piperttyl pyrrolidine **(30)**