ISOLATION OF CHAVIBETOL AND HYDROXYCHAVICOL AND SELECTED ANTI-OBESITY STUDIES OF STANDARDIZED *PIPER BETLE* LINN. LEAF EXTRACTS

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LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrscopy
ACAT	acyl CoA:cholesterol acyl transferase
ALT	Alanine transaminase
As	Arsenic
AST	Aspartate aminotransferase
ATR	Attenuated total reflection
BMI	Body mass index
Cd	Cadmium
cfu	Colony-forming unit
$CDCL_3$	Deuterated chloroform
$CHCl_3$	Chloroform
CHV	Chavibetol
cm	centimeter
dl	deciliter
DMSO	Dimethyl sulfoxide
GC-MS	Gas chromatography-mass spectrometry
FC	Sub-fraction of chavibetol
$FeCl_3$	Ferric chloride
FH	Sub-fraction of hydroxychavicol
FT-IR	Fourier Transform Infrared spectroscopy
g	Gram
GAE	Gallic acid equivalent
HC	Hydroxychavicol
Hg	Mercury

Hz	Hertz
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
HNO_3	Nitric acid
HDL	High-density lipoprotein
HFD	High fat diet
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
IC_{50}	Half maximal inhibitory concentration
ID	Internal diameter
J	Coupling constant
kg	Kilogram
KOH	Potassium hydroxide
L	Liter
LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
Na_2CO_3	Sodium carbonate
NH_4OH	Ammonia
nm	Nanometer
m	Meter
mg	Milligram
MHz	Mega Hertz
min	Minutes
mL	Milliliter
mm	Millimeter
NaCl	Sodium chloride

NaOH	Sodium hydroxide
NMR	Nuclear Magnetic Resonance
Pb	Plumbum
p-NPB	*p*-nitrophenyl butyrate
ppm	Part per million
R_t	Retention time
RSD	Relative standard deviation
RVSEB	Rappaport Vassiliadis Salmonella Enrichment Broth
SCD	Soybean-Casein Digest agar
SD	Standard deviation
SDA	Sabouraud Dextrose agar
SE	Ethanol extract of *Piper betle*
SEW	50% ethanol extract of *Piper betle*
SW	Water extract of *Piper betle*
TC	Total cholesterol
TG	Total triglyceride
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UV	Ultraviolet
V	Volt
WHO	World Health Organization
w/w	Weight over weight
µg	Microgram
µl	Microliter
^oC	Celsius degree
%	Percent

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PENGASINGAN KAVIBETOL DAN HIDROKSIKAVIKOL SERTA KAJIAN ANTI-OBESITI TERPILIH UNTUK EKSTRAK TERPIAWAI DAUN *PIPER BETLE* LINN.

ABSTRAK

Piper betle L. telah digunakan secara meluas dalam perubatan herba tradisional di Asia. Walau bagaimanapun, tumbuhan ini masih kekurangan data analisis terutama berkaitan kualiti, keselamatan dan keberkesanannya. Kajian ini telah dijalankan untuk menyediakan maklumat mengenai pemiawaian daun *Piper betle*. Kajian ini dibahagikan kepada tiga bahagian: kualiti dan keselamatan bahan-bahan mentah daun *Piper betle*, piawaian ekstrak *Piper betle* menggunakan sebatian terasing dan anti-obesiti. Daun kering *Piper betle* ditentukan untuk kandungan logam berat dan kandungan ujian had mikrob (MLT). Tumbuhan ini didapati berada dalam lingkungan had yang ditetapkan bagi empat logam berat Cd (0.3ppm), Hg (0.5 ppm), Pb (10 ppm) and As (5.0 ppm) dan ia bebas dari pencemaran mikrob. Pemeriksaan fitokimia awal bagi ekstrak etanol, ekstrak 50% etanol dan ekstrak air menunjukkan kehadiran sebatian terpenoid, flavonoid, fenol, sterol, koumarin dan saponin. Dua sebatian iaitu kavibetol (CHV) dan hidroksikavikol (HC) telah diasingkan dan dikenalpasti melalui data UV, FTIR, TLC, HPLC, GC-MS dan NMR. Profil kimia yang menggunakan teknik spektroskopi dan kromatografi seperti UV, FTIR, HPTLC dan GC-MS digunakan dalam kajian pemiawaian. Manakala, kavibetol dan hidroksikavikol dijadikan sebagai penanda kimia untuk ujian analisis kualitatif dan kuantitatif. Satu sistem isokratik HPLC telah dibangunkan dan disahkan bagi penentuan kuantifikasi kavibetol dan hidroksikavikol untuk kesemua ekstrak *Piper betle*. Analisis HPLC terhadap ketiga-tiga ekstrak *Piper betle* mempamerkan

pelbagai kepekatan yang berbeza untuk kavibetol (0.0219 – 0.1020 μg/mL) dan hidroksikavikol (0.1371 – 0.3424 μg/mL). Kesemua ekstrak juga ditentukan untuk penilaian jumlah kandungan flavonoid, fenolik, glikosaponin, polisakarida dan protein. Ekstrak etanol *Piper betle* didapati mempunyai jumlah kandungan yang tertinggi untuk flavonoid (125.21 μg/mL), fenolik (361.26 μg/mL) dan protein (7.45%), manakala ekstrak 50% etanol telah menunjukkan jumlah kandungan yang tertinggi bagi glikosaponin (35.36%) dan polisakarida (46.44%). Ekstrak etanol, ekstrak 50% etanol dan ekstrak air daun *Piper betle* telah disaring untuk aktiviti anti-lipase dengan menggunakan lipase pankreas khinzir (PPL) dan 2,4-dinitrofenil butirat (*p*-NPB) sebagai enzim dan substrat tiruan. Ekstrak 50% etanol *Piper betle* menunjukkan aktiviti anti-lipase. Aktiviti anti-obesiti dan anti-hiperlipidemik dalam ekstrak 50% etanol *Piper betle* telah diperiksa menggunakan tikus diet lemak tinggi (HFD) yang menyebabkan hiperlipidemik sebagai model. Data menunjukkan ekstrak 50% etanol *Piper betle* adalah signifikan ($P<0.05$ dan $P<0.01$) menurunkan peratusan jumlah peningkatan berat badan dan indeks jisim badan (BMI). Penemuan juga menunjukkan ekstrak ini secara nyata ($P<0.01$) menurunkan paras jumlah kolesterol (TC) dan lipoprotein ketumpatan rendah (LDL) serta meningkatkan paras lipoprotein ketumpatan tinggi (HDL). Keputusan kajian mendapati bahawa kaedah HPLC yang dibangunkan boleh diaplikasikan untuk pemiawaian ekstrak daun *Piper betle*. Manakala, ekstrak 50% etanol telah menunjukkan keputusan yang positif sebagai agen anti-lipase, anti-obesiti and anti-hiperlipidemik.

ISOLATION OF CHAVIBETOL AND HYDROXYCHAVICOL AND SELECTED ANTI-OBESITY STUDIES OF STANDARDIZED *PIPER BETLE* LINN. LEAF EXTRACTS

ABSTRACT

Piper betle L. has been extensively used in traditional herbal remedies in Asia. Nevertheless, this plant still lacks of data especially on their quality, safety and efficacy. This study has been conducted to provide information on the standardization of *Piper betle* leaves. The study was divided into three parts: quality and safety of raw materials of *Piper betle* leaves, standardization of *Piper betle* extracts using isolation compounds and anti-obesity studies. Dry powdered leaves of *Piper betle* were determined to evaluate the content of heavy metals and the microbial limit test (MLT). This plant was found to be in the range of the acceptable limit of four main heavy metals Cd (0.3ppm), Hg (0.5 ppm), Pb (10 ppm) and As (5.0 ppm) and free from microbial contamination. The preliminary phytochemical screening using TLC on the ethanol, 50% ethanol and water extracts showed the presence of terpenoids, flavonoids, phenols, sterols, coumarins and saponins. Two compounds namely chavibetol (CHV) and hydroxychavicol (HC) were isolated and identified by UV, FTIR, HPTLC, HPLC, GC-MS and NMR data. The chemical profile using spectroscopic and chromatographic techniques such as UV, FTIR, HPTLC and GC-MS were used in the standardization. Chavibetol and hydroxychavicol were carried out as markers to examine the qualitative and quantitative analysis. An isocratic HPLC system has been developed and validated for the quantification of chavibetol and hydroxychavicol of *Piper betle* extracts. The HPLC analysis of three different extracts of *Piper betle* exhibited varying

concentration of chavibetol (0.0219 - 0.1020 μg/mL) and hydroxychavicol (0.1371 - 0.3424 μg/mL). The extracts also were determined in the evaluation of total flavonoids, total phenolics, total glycosaponins, total polysaccharides and total proteins. An ethanol extract of *Piper betle* was found to be the highest content of flavonoids (125.21 μg/mL), phenolics (361.26 μg/mL) and proteins (7.45%), while the 50% ethanol extract have demonstrated the highest amount of glycosaponins (35.36%) and polysaccharide (46.44%). Ethanol, 50% ethanol and water extracts of *Piper betle* leaf were screened for their anti-lipase activity using porcine pancreatic lipase (PPL) and 2,4-dinitrophenyl butyrate (*p*-NPB) as an enzyme and artificial substrate. The 50% ethanol extract of *Piper betle* indicated anti-lipase activity. The anti-obesity and anti-hyperlipidemic activities of 50% ethanol extract of *Piper betle* were examined using high fat diet (HFD) induced hyperlipidemic rats as a model. The data demonstrated 50% ethanol extract of *Piper betle* significantly ($P<0.05$ and $P<0.01$, respectively) lowered the percentage of total body weight increased and body mass index (BMI). The findings also exhibited this extract was significantly ($P<0.01$) decreased the level of total cholesterol (TC) and low density lipoprotein (LDL) while indicating an increased level of high density lipoprotein (HDL). Results of the study indicated that the developed HPLC methods can be applied for standardizations of *Piper betle* extracts. While, 50% ethanol extract has shown positive result as an anti-lipase, anti-obesity and anti-hyperlipidemic agent.

CHAPTER 1

INTRODUCTION

1.1 General

Herbal medicines have been utilized since ancient times to improve the world health as medicines which contained a rich source of therapeutic agents. Although there is no doubt that modern medicine has been highly effective in the prevention and treatment of many illnesses, herbal medicines still useful in allowing more people to live in healthy. Nearly 80% of world population (African, Asian and Indian) uses herbs and traditional medicines as their primary healthcare (Sahoo *et al.*, 2010).

A previous study has reported that almost 40% of 2000 varieties of diseases can now be treated with traditional medications and these had dramatically increased the life span of the individual (Abbot *et al.*, 1996). In Germany, herbal preparations have reached widespread acceptability as therapeutic agents for common cold, flu, headache, stomach ulcer, bronchitis, insomnia, digestive and skin diseases (Calixto, 2000).

Based on WHO definition, herbal medicines are generally characterized into four types which are known herbs, herbal materials, herbal preparations and finished herbal products that contain as active ingredients parts of plants, or other plant materials, or combinations. Herbs are crude plant material such as leaves, flowers, fruit, seed, stems, wood, bark, roots, rhizomes or other plant parts, which may be entire, fragmented or powdered. Herbal materials are well known in addition of herbs, fresh juices, gums, fixed oils, essential oils, resins and dry powders of herbs. While, herbal preparations represent the extracts, tinctures and fatty oils of herbal materials. Finished herbal

products are developed and created drugs by the herbal preparations made from one or more herbs which may contain excipients in addition to the active ingredients.

The last few years the use of herbal medicines has increased highly in the developed world. World Health Organization (2000) reported the turnover of herbal medicines have been reported highly demanding sources for pharmaceutical manufacturing especially in China and Malaysia which indicate 33.1% share of the pharmaceutical market in 1995 and about 1 billion Malaysia ringgit annually, respectively.

In India many herbs and herbal extracts are used as over-the-counter products, ethical and classical formulations indigenous system of medicine (Ayurveda, Siddha, Unani, Homeopathy, Yoga and Naturopathy) and their sales are around US$ 1 billion in the local and the export of plant-based crude drugs is around US$ 80 million (Kamboj, 2000). It is interesting to note that the turnover of herbal medicines in USA and Europe as nutraceuticals (health foods) are estimated at about US$ 80–250 billion (Brower, 1998).

1.2 Justification of the research

Currently, Sengupta *et al*. (2012) reported an herbal formulation LI10903F containing 2:3 combination of *Piper betle* leaf aqueous-alcohol extract and Dolichos biflorus seed alcohol extract has indicated antiobesity activity. Whereas, methanolic extract of *Piper betle* exhibited a significant anti-hyperlipidemic activity (Thirumalai *et al.,* 2014). However its primary and secondary metabolites responsible for the observed effects has not yet been shown by this plant. Based on this information above, the present study is aimed at evaluating the anti-obesity and anti-hyperlipidemic activities of the 50% ethanol extract of *Piper betle* on high fat diet induced-hyperlipidemic rats.

1.3 Objectives of the research

i. To assess quality and safety of raw materials of *Piper betle* leaves

ii. To isolate chemical markers and standardize *Piper betle* leaf extracts based on the markers.

iii. To investigate the *in vitro* and *in vivo* anti-obesity studies on standardized *Piper betle* extracts.

1.4 *Piper betle* Linn.

Piper betle L. also known as betel vine (English) and sireh (Malay). It is a medicinal plant belongs to the family Piperaceae.

1.4.1 Plant profile

Kingdom	:	Plantae
Division	:	Magnoliphyta
Class	:	Magnolipsida
Order	:	Piperales
Family	:	Piperaceae
Genus	:	*Piper*
Species	:	*Piper betle*
Synonyms	:	*Chavica auriculata* Miq, *Arthanthe hixagona*
Common name	:	Sireh (Malaysia), Paan (Hindi), Betel vine (English), Plue (Thailand), Ikmo (Philippine)

(Kushagra *et al.*, 2011)

Figure 1.1 Pictures of *Piper betle* leaves

1.4.2 Plant Morphology

Piper betle L. is a shade loving climber, semi woody stem and can climb up trees as tall as 10 -15 m height or other supporting materials (Kumar, 1999). The stems are slender, twining, rounded and rooted (3 mm diameter, 2.5 - 4.0 cm long internodes) (Kushagra *et al.*, 2011). The leaves are smooth glossy dark green, perennial creeper and spiral. The petiole is 5.5 – 6.5 cm long. The base of blade is slightly heart shaped and the apex is acuminate (12 -18 cm long and 9 -11 cm wide) and has 5 - 7 veins arising from the base. It has a pungent aromatic smell, stimulant and carminative taste. The female flowers are long with single pendulous ovary while male flowers are dense with two stamens. *Piper betle* fruits are fleshy and orange berries (3 mm in diameter) and rarely produced (Periyanayagam *et al.*, 2012).

1.4.3 Plant habitat and distribution

Piper betle L. (Piperaceae), extensively cultivates in tropical countries such as Sri Lanka, India, Indonesia, Malaysia, Thailand, Taiwan, Philippine Islands, South and East Africa (Arambewela *et al.,* 2005a; Kushagra et *al.,* 2011). Studies have reported about 100 varieties of *Piper betle* are obtained around the world and it is originally derived from Malaysia. However, the plant is much more famous in India and also has 70 varieties found including in India and West Bengal (Maity, 1989; Samanta, 1994; Guha, 2006). The plant grows best under tropical forest conditions which have mild temperature, high humidity and well drained loamy and sandy loam soils (Rafiqul Haider *et al.,* 2013).

1.4.4 Traditional Usage

Piper betle L. is a great herbal and has been attributed with therapeutic properties among previous community and medicines in Asia. Practically, in India, East Indies and Philippines, the betel leaves were chewed together with slaked lime paste and betel nut as stimulant masticatory, breath refresher, natural tonic, preservative of the teeth, tonic for brain, appetite improver, treatment for diarrhoea and digestive (Dasgupta & De, 2004). In Filipino folks, the greased leaves with sesame oil were applied to the abdomens of children suffering from flatulence and as a poultice for boils. The crushed fresh leaves can be used as an antiseptic for cuts and wounds (Guha, 2006). Choudhary and Kale (2002) and Rai *et al.,* (2011) reported the application of the leaves to the chest, breast and friction of the spinal column have many beneficial to cure asthma, arrest lactation and treat colds. In Malaysia, the leaves are also used as anti-malaria medicine in some rural populations (Al-Adhroey *et al.,* 2010). In India traditional system medicine, the warm leaves of *Piper betle* are applied on the swellings of throat

and ear (Choudhary & Kale, 2002). Jayaweera (1982) reported the juice of betel leaves was used as cough medicine, eye drops for the night blindness adults, catarrh and diphtheria. The leaves of the plant was applied to purulent ulcers and orally given for gastric and lung disorders.

1.4.5 Review of biological and pharmacological activities

Anti-bacterial activity

Nalina and Rahim (2007) investigated the anti-bacterial activity of the crude water extract of *Piper betle* leaves on *Streptococcus mutans*. The anti-bacterial effects were aimed on the ultra structure and acid producing properties of *S. mutans*. By using transmission electron microscopy (TEM) and PH drop assay for determine the ultrastructure and acid producing properties of *Streptococcus mutans*, they obtained the changes of the ultra structure of *Streptococcus mutans* was affected at higher concentration (2 mg/mL) and the increment of the concentration of crude water extract at 1, 2, 5 and 10 mg/ml have also reduced the rate of acid production from 93.53 to 23.56%. The researchers found the hydroxychavicol, fatty acids (stearic acid and palmitic acid) and hydroxy fatty acid esters (hydroxyl esters of stearic, palmitic and myristic acids) were the main components in the crude water extract and has potential in anti-bacterial activities.

As an Oral care agent and anti-microbial activity

Ramji *et al.* (2002) reported the ether fraction of methanol extract of *Piper betle* leaves could be used in prevention of bad breath (halitolisis) which is caused by the microbial degradation of proteins such as methylmercaptan, hydrogen sulfide and volatile fatty acids. They also found the present of allylpyrocathecol (APC) as the active compound

in the ether fraction exhibited an anti-microbial activity in the minimum inhibitory concentration (MIC) assays, biofilm assay and volatite sulfur compounds (VSC) assay.

Anti-histaminic activity

Hajare *et al.* (2011) studied the anti-histaminic activity of essential oil and ethanol extract of *Piper betle* L.leaves in guinea pig. The experiment was tested on the histamine induced bronchoconstriction in guinea pig and isolated ileum and tracheal chain of guinea pig. Result indicated the groups at dose 100 and 200 mg/kg for both essential oil and ethanol extract were significantly prevented the Guinea pigs from histamine-induced bronchospasm. In the meantime, there was an inhibition of histamine-induced contraction in the isolated ileum and tracheal chain assays after the essential oil and ethanol extract (100 μg/mL) were treated. Therefore, the study can be concluded that the essential oil and ethanol extract of *Piper betle* L. was possessed as anti-histaminic activity.

Anti-inflammatory activities

Pin *et al.* (2010) reported the anti-oxidant and anti-inflammatory activities extracts of *Piper betle* leaves from water, ethanol, ethyl acetate and hexane. The study of anti-oxidant utilized two *in vitro* assay such as xanthine/xanthine oxidase superoxide scavenging assay (SOD assay) and 1,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay (DPPH assay) while the anti-inflammatory assays used were hyaluronidase (HYA), xanthine oxidase (XOD) and lipoxygenase (LOX) inhibition assays. The results indicated that all the extracts were highly active in both antioxidant assays and also exhibited significant inhibition in XOD and LOX assays. The bioactivity of the extracts was related to hydroxychavicol and eugenol compounds.

Anti-diabetic activity

Arambewela *et al.* (2005b) reported the hot water extract (HWE) and cold ethanolic extract (CEE) of *Piper betle* leaves possessed therapeutic potential as anti-diabetic agent. In light of this, both extracts were evaluated in normoglycaemic and strepozotocin (STZ)-induced diabetic rats. Treatment with HWE and CEE provided significantly reduced the blood glucose level in different doses and lowered the external glucose load. Moreover, HWE and CEE treated rats were found no signs of toxicity, hepatotoxicity and renotoxicity. Thus, it can be concluded that *Piper betle* HWE and CEE are non toxic and scientifically showed anti-diabetic activity.

Anti-ulcer activity

Vyawahare *et al.* (2010) have studied the anti-ulcer activity of hydroalcoholic extract *Piper betle* L. leaves (HEPB) in winstar rats. The rats were divided in three model experiments where are induced with gastric ulcers of HCl-ethanol, acute stress and pylorus ligation. All of the models have received three doses (50, 100 and 200 mg/kg) and sucralfate and omeprazole were used as a standard drug. In the HCl-ethanol and acute stress induced ulcers, there were a good percentage of ulcer inhibitions at dose 100 mg/kg (40.23 and 18.64%, respectively). While at 200 mg/kg dose, the pylorus ligated rats exhibited highly ulcer inhibition (50.84%) and it also could lowered the volume and increased the pH of gastric fluids. Therefore, the study proved HEPB possessed overt anti-ulcer activity.

Anti-malarial activity

Al-Adhroey *et al.* (2011) found the anti-malarial activity of methanol leaf extract of *Piper betle* L. against *Plasmodium berghei* (NK65). In brief, all the mice were

introduced and established with malarial infections (1×10^6 of *Plasmodium berghei*) by the intraperitoneal (i.p.) administration. The infected mice were then administrated orally with four doses of methanol extract (50, 100, 200, 400 mg/kg) towards to the suppressive, curative and prophylactic anti-malarial activity models. Results were compared with the standard drugs (pyrimethamine 1.2 mg/kg or chloroquine 20 mg/kg). Evaluation of the suppressive model significantly showed the reduction of the parasitaemia of the infected mice at the 200 and 400 mg/kg doses ($P<0.01$) whereas curative and prophylactic models exhibited long life expectancy when compared to the control and also showed significant findings ($P<0.05$) to all doses. Thereafter, the methanol extract of *Piper betle* L. was found to be a strong anti-oxidant agent. This can be suggested that the potent anti-malarial activity of the methanol extract *Piper betle* L. may be due to the presence of anti-oxidant activity in this plant.

Anti-fungal activity

Widodo and Sukmawanti (2010) reported that the cream of 10% *Piper betle* L. leaves ethanol extract to possess anti-fungal activity against *Candida albicans*. Study was began by grouping all the white female rabbits into 5 groups (1; treatment with ketonazole 2% cream, 2; treatment with a base cream, 3-5; treatment with 2.5, 5.0 and 10.0% w/v of *Piper betle* L. var *rubrum* cream. All the rabbit's vaginas were let infected with *Candida albicans* for 48 hours before the cream was applied twice a day. After twelve days treatment, macroscopic and microscopic data have indicated that the 10.0% w/v of *Piper betle* L. cream was comparable with the group 1. This was proven when the healing time of the 10.0% w/v of *Piper betle* L. var *rubrum* cream was the same (day 7) with the ketonazole's group. On the other hand, the number colonies of

Candida albicans also depleted on the day 8. Findings showed the cream of 10% *Piper betle* L. leaves ethanol extract may be useful in the treatment of vagina infection.

Anti-fertility activity

Sarkar *et al.* (2000) revealed the effectiveness of *Piper betle* leaf-stalk ethanol extract as an anti-fertility agent on Swiss albino male mice. Two groups (40 mice/group) were fed orally with 500 mg/kg of the ethanol extract for the first 30 days and only one group was continued with 1000 mg/kg for next 30 days. Olive oil (0.5 mL) was given as a placebo for both groups. After administration, half of the treated mice from each group were sacrificed for autopsy testing. Another half were allowed to recover from the extract for 30 days and 60 days before the fertility test was studied and the mice were sacrificed. The treated mice (60 days) showed a highly significant reduction in the reproductive organs weight, sperm count and motility ($p<0.05$) and fertility (0%). While, the recovered mice were observed to possess *vice versa* results if compared with the treated mice. In the light of this, the authors have recognized that the prolonged consumption of *Piper betle* leaf-stalk ethanol extract in high dose provided better anti-fertility potential on the male mice.

Anti-oxidant activity

Dasgupta and De (2004) studied the anti-oxidant activity of water extract of three local varieties of *Piper betle* leaves which are recognized as Kauri, Ghanagete and Bagerhati. Hydroxyl radical scavenging activity, superoxide radical scavenging activity in a NBT system, DPPH radical scavenging activity and inhibition of lipid peroxidation induced by $FeSO_4$ in egg yolk methods were employed to evaluate this study. Moreover, acid ascorbic, gallic acid and tea leaves (CTC HGH) were used as a

comparison for determination of total anti-oxidant activity. All the *in vitro* systems have showed that the water extract of each variety able to suppress the lipid peroxidation with different data of anti-oxidant activity. From the results it was suggested that the highest anti-oxidant activity was found in Kauri and followed by Ghanagete and Bagerhati. All the varieties are considered as a free radical inhibitor.

Larvicidal, anti-oxidant and anti-microbial activities

Row and Ho (2009) concluded that the essential oil potentially indicated favorable results in the mosquito larvicidal activity, anti-oxidant activity, anti-microbial activity and tyrosinase inhibition compared to methanol and water extracts of *Piper betle* leaves. In the mosquito larvicidal assay, the essential oil was the extract showed significant LD_{50} value after 2 hours and 24 hours treatment (86 and 48 ppm) and it may be caused by the presence of eugenol in the oil. The essential oil inhibited the activity of all the organisms (*C. albicans, S. aureus, M. pachydermatis, E. coli*) after using agar disk diffusion method and it also exhibited strong antioxidant activity when DPPH and reducing power assay were done. Moreover, a good IC_{50} value of the essential oil (126 ppm) once again believed that this oil could be considered as a tyrosinase inhibitor.

1.2.6 Review of chemical constituents

Main constituents of this plant are phenols and terpenes (Bajpai *et al.*, 2010; Rimando *et al.,* 1986). The characteristic of *Piper betle* is due to phenolic compounds. More than 53 compounds have been identified from the essential oil and 35 compounds from water, ethanol, hexane, benzene and petroleum ether extracts, respectively. The most active compound of *Piper betle* oil which is obtained from the leaves are chavibetol, safrole, eugenol, eugenyl acetate, chavicol, γ-muurolene, β-caryophyllene, γ-cadinene, α-humulene, cedrol, α–cadinene, phytol, ε-muurolene, α-bergamotene, 4-Allyl-1.2-diacetoxybenzene and methyl chavicol (Jantan *et al.,* 1994; Sugumaran *et al.*, 2011). While, the aqueous extract of the *Piper betle* leaf reported to yield hydroxychavicol, stearic acids and palmitic acids (Nalina & Rahim, 2007).

In this study, chavibetol (CHV) and hydroxychavicol (HC) are important phytochemicals found in betel leaves. Chavibetol is a bioactive compound of the phenylpropanoid class. It is the main compound of the essential oil from the leaves of the *Piper betle* plant. Chavibetol is an isomer of eugenol with a pungent odor. This compound only found in Philiphine sample, Malaysian sample and Taiwan sample (Rimando *et al.,* 1986; Jantan *et al*., 1994; Row & Ho, 2009). Chavibetol is reported to contribute to many bioactivities in betel leaves such as antioxidant, antileishmanial and radioprotective (Rathee *et al.* 2006; Sarkar et al., 2008; Bhattacharya *et al*., 2005) Hydroxychavicol is a most important compound present in water extract of the *Piper betle* leaf which reported to possess antimutagenic , anticarcinogenic and antifungal activities (Amonkar *et al.,* 1986; Amonkar *et al.,* 1989; Ali *et al*., 2010). Beside this, previous finding reported that the isolated of hydroxychavicol from the chloroform extraction of aqueous extract of *Piper betle* leaves exhibited potent antimicrobial, antioxidant, anti-inflammatory effects and it is also useful as an oral care agent

(Sharma *et al.,* 2009). Hydroxychavicol exhibited significant inhibition of superoxide anion and elastase released by human neutrophils, in response to fMLP/CB (Lin *et al.*, 2013). The chemical constituents identified in this plant based on the regions are summarized in Table 1.1 and structures of 86 some chemical constituents are given in Figure 1.2.

Table 1.1 Chemical constituents of *Piper betle*.

No.	Contituents	Locations	Parts	Extracts	References
1.	Safrole	India	Leaves	Essential oil	(Sugumaran *et al.*, 2011)
		Sri Lanka	Leaves, Stems, Stalks, Fruits, Roots	Essential oil	(Arambewela *et al.*, 2005a)
		Philippines	Leaves	Essential oil	(Rimando *et al.*, 1986)
2.	Eugenol	India	Leaves	Essential oil	(Sugumaran *et al.*, 2011)
		Sri Lanka	Leaves, Stems, Stalks, Fruits, Roots	Essential oil	(Arambewela *et al.*, 2005a)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
		Philippines	Leaves	Essential oil	(Rimando *et al.*, 1986)
		India	Stalks	Benzene	(Dwivedi *et al.*, 2010)
3.	Eugenol acetate	India	Leaves	Essential oil	(Sugumaran *et al.*, 2011)
		Malaysia	Leaves	Essential oil	(Jantan *et al.*, 1994)
4.	Chavibetol	Malaysia	Leaves	Essential oil	(Jantan *et al.*, 1994)
		Philippines	Leaves	Essential oil	(Rimando *et al.*,. 1986)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
5.	Chavicol	Malaysia	Leaves	Essential oil	(Jantan *et al.*, 1994)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)

6.	Chavibetol acetate	Philippines	Leaves	Essential oil	(Rimando *et al.*, 1986)
		Taiwan	Leaves	Essential oil	(Row and Ho, 2009)
		Sri Lanka	Leaves, Stems, Stalks, Fruits, Roots	Essential oil	(Arambewela *et al.*, 2005a)
7.	Allylpyrocatechol diacetate	Sri Lanka	Leaves, Stems, Fruits, Roots	Essential oil	(Arambewela *et al.*, 2005a)
		Philippines	Leaves	Essential oil	(Rimando *et al.*, 1986)
8.	γ-selinene	Sri Lanka	Stems, Stalks, Fruits, Roots	Essential oil	(Arambewela *et al.*, 2005a)
		India	Leaves	Essential oil	(Sugumaran *et al.*, 2011)
9.	4-terpineol	India	Leaves	Essential oil	(Sugumaran *et al.*, 2011)
		Sri Lanka	Leaves, Stems, Stalks, Fruits	Essential oil	(Arambewela *et al.*, 2005a)
10.	Germacrene D	India	Leaves	Essential oil	(Sugumaran *et al.*, 2011)
		Taiwan	Leaves	Essential oil	(Row and Ho, 2009)
11.	β – Phellandrene	India	Leaves	Essential oil	(Sugumaran *et al.*, 2011)
		Sri Lanka	Leaves, Fruits, Roots	Essential oil	(Arambewela *et al.*, 2005a)
12.	γ-muurolene	Malaysia	Leaves	Essential oil	(Jantan *et al.*, 1994)
		Sri Lanka	Leaves, Stems, Stalks, Fruits	Essential oil	(Arambewela *et al.*, 2005a)
13.	β-caryophyllene	Malaysia	Leaves	Essential oil	(Jantan *et al.*, 1994)

		Sri Lanka	Leaves, Stems, Stalks, Fruits, Roots	Essential oil	(Arambewela *et al.*, 2005a)
		Philippines	Leaves	Essential oil	(Rimando *et al.*, 1986)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
14.	α-humulene	Malaysia	Leaves	Essential oil	(Jantan *et al.*, 1994)
		Sri Lanka	Leaves, Stems, Stalks, Fruits	Essential oil	(Arambewela *et al.*, 2005a)
		India	Leaves	Essential oil	(Sugumaran *et al.*, 2011)
15.	α-bergamotene	Malaysia	Leaves	Essential oil	(Jantan *et al.*, 1994)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
16.	4-Allyl-1,2-diacetoxybenzene	Malaysia	Leaves	Essential oil	(Jantan *et al.*, 1994)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
17.	Campene	Philippines	Leaves	Essential oil	(Rimando *et al.*, 1986)
		Sri Lanka	Stems, Fruits, Roots	Essential oil	(Arambewela *et al.*, 2005a)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
18.	β –pinene	Philippines	Leaves	Essential oil	(Rimando *et al.*, 1986)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
19.	α –pinene	Philippines	Leaves, Stems, Fruits, Roots	Essential oil	(Rimando *et al.*, 1986)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
20.	α-terpineol	Sri Lanka	Roots	Essential oil	(Arambewela *et al.*, 2005a)

		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
21.	Isoeugenol	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
		India	Stalks	Benzene	(Dwivedi *et al.,* 2010)
22.	Chavicol acetate	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
23.	Phytol	Malaysia	Leaves	Essential oil	(Jantan *et al.,* 1994)
24.	Methyl chavicol	Malaysia	Leaves	Essential oil	(Jantan *et al.,* 1994)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
25.	Chavibetol methyl ether	Philippines	Leaves	Essential oil	(Rimando *et al.,* 1986)
26.	Sabinene	India	Leaves	Essential oil	(Sugumaran *et al.,* 2011)
27.	α –cadinene	Malaysia	Leaves	Essential oil	(Jantan *et al.,* 1994)
28.	Isoeugenol methyl ether	India	Leaves	Essential oil	(Sugumaran *et al.,* 2011)
29.	β – Elemene	India	Leaves	Essential oil	(Sugumaran *et al.,* 2011)
30.	Eudesma-4(14), 11-diene	India	Leaves	Essential oil	(Sugumaran *et al.,* 2011)
31.	γ-cadinene	Malaysia	Leaves	Essential oil	(Jantan *et al.,* 1994)
32.	Cedrol	Malaysia	Leaves	Essential oil	(Jantan *et al.,* 1994)
33.	α –limonene	Philippines	Leaves	Essential oil	(Rimando *et al.,* 1986)
34.	1,8-cineol	Philippines	Leaves, Roots	Essential oil	(Rimando *et al.,* 1986)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
35.	Allylpyrocatechol monoacetate	Philippines	Leaves	Essential oil	(Rimando *et al.,* 1986)
36.	*p*-cymene	Sri Lanka	Leaves, Fruits	Essential oil	(Arambewela *et al.,* 2005a)

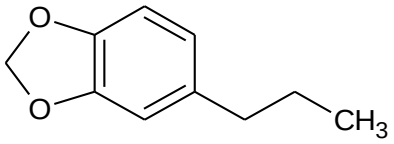
37.	β–cadinene	Sri Lanka	Leaves, Stems, Stalks, Fruits, Roots	Essential oil	(Arambewela *et al.*, 2005a)
38.	α-elemene	Sri Lanka	Stems, Stalks, Roots	Essential oil	(Arambewela *et al.*, 2005a)
39.	Limonene	Sri Lanka	Fruits, Roots	Essential oil	(Arambewela *et al.*, 2005a)
		Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
40.	α-myrcene	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
41.	Linalool	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
42.	Camphor	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
43.	β-isosafrole	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
44.	Thymol	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
45.	α-famesene	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
46.	α-muurolene	Taiwan	Leaves	Essential oil	(Row &Ho, 2009)
47.	α-bisabolene	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
48.	benzoic acid	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
49.	Ledol	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
50.	caryophyllene oxide	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
51.	α-bisabolol	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
52.	α-cadinol	Taiwan	Leaves	Essential oil	(Row & Ho,2009)
53.	Isoledene	Taiwan	Leaves	Essential oil	(Row & Ho, 2009)
54.	Hydroxychavicol	Malaysia	Leaves	Water	(Nalina & Rahim, 2007)
55.	Stearic acid (fatty acids)	Malaysia	Leaves	Water	(Nalina & Rahim, 2007)

		India	Roots	Petroleum ether	(Ghosh & Bhattacharya, 2005)
56.	Palmitic acid (fatty acids)	Malaysia	Leaves	Water	(Nalina & Rahim, 2007)
57.	Palmitic acid, 2,3-bishydroxypropyl ester	Malaysia	Leaves	Water	(Nalina & Rahim, 2007)
58.	Stearic acid, 2,3-bishydroxypropyl ester	Malaysia	Leaves	Water	(Nalina & Rahim, 2007)
59.	Myristic acid, 2,3-bishydroxypropyl ester	Malaysia	Leaves	Water	(Nalina & Rahim, 2007)
60.	Hexadecanamide	Malaysia	Leaves	Water	(Nalina & Rahim, 2007)
61	2-Monopalmitin	Malaysia	Leaves	Water	(Nalina & Rahim, 2007)
62.	Hexadecanoic acid, (Palmitic acid) methyl ester	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
63.	Dodecanic acid,ethyl ester	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
64.	Dodecanoic acid, methyl ester (methyl laurate)	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
65.	Tetradecanoic acid,methyl ester	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
66.	Octanoic acid,3-methyl butyl ester	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
67.	Octadecanoic acid, methyl ester (Stearic acid methyl ester)	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
68.	Methyl undecanote	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
69.	Tridecanoic acid,methyl ester	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
70.	3-methyl butyl decanoate	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)

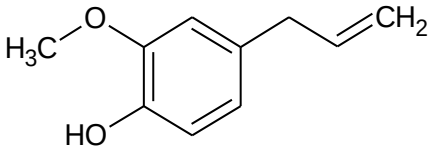
71.	Tetradecanoic acid, ethyl ester	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
72.	9-Heptadecanone	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
73.	Hexadecanoic acid, ethyl ester	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
74.	10-Nonadecanone	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
75.	2-methyl undecanol	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
76.	9-Heneicosanone	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
77.	4-methyl Hexadecane	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
78.	Hexadecane	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
79.	1-Dodecanol (lauryl alcohol)	India	Stalks	Hexane	(Dwivedi *et al.*, 2010)
80.	4-ethyl benzaldehyde	India	Stalks	Benzene	(Dwivedi *et al.*, 2010)
81.	1-methoxy-4 methylbenzene	India	Stalks	Benzene	(Dwivedi *et al.*, 2010)
82.	Tetradecanoic acid (Myristic acid)	India	Stalks	Benzene	(Dwivedi *et al.*, 2010)
83.	Aristololactam A-II	India	Roots	Ethanol	(Ghosh & Bhattacharya, 2005)
84.	4-allyl resorcinol	India	Roots	Ethanol	(Ghosh & Bhattacharya, 2005)
85.	stigmast-4-en-3,6-dione	India	Roots	Petroleum ether	(Ghosh & Bhattacharya, 2005)
86.	β-sitosterol	India	Roots	Petroleum ether	(Ghosh & Bhattacharya, 2005)

Number in column shows the number of the structure.

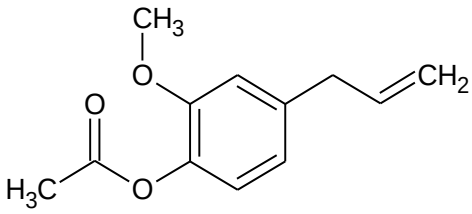
The main chemical constituents of *Piper betle*



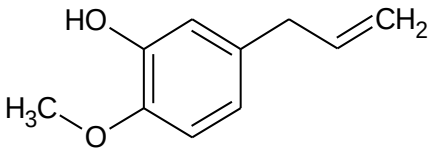
1. Safrole



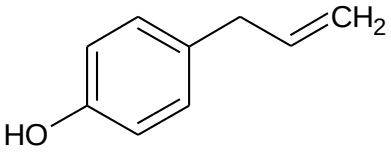
2. Eugenol



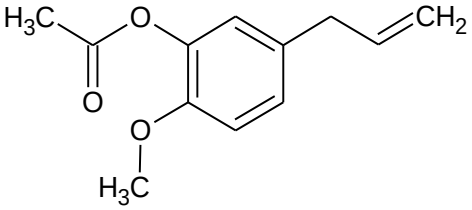
3. Eugenyl Acetate



4. Chavibetol (Isomer of Eugenol)

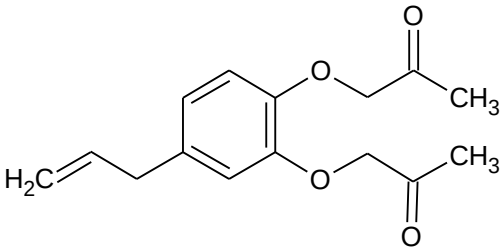


5. Chavicol

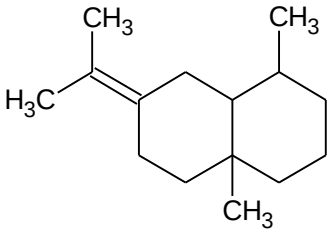


6. Chavibetol Acetate

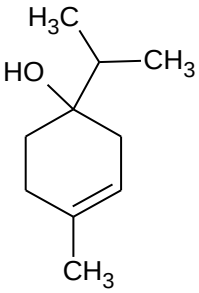
Figure 1.2 Structures of 86 chemical constituents of *Piper betle.*



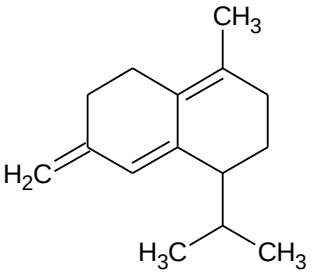
7. Allylpyrocatechol diacetate



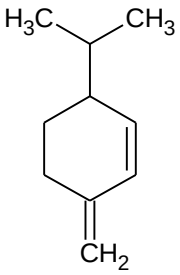
8. γ-Selinene



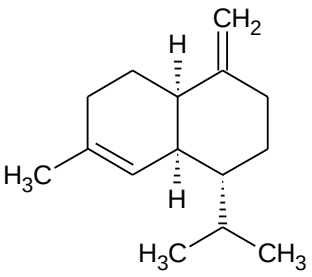
9. 4-Terpineol



10. Germacrene D

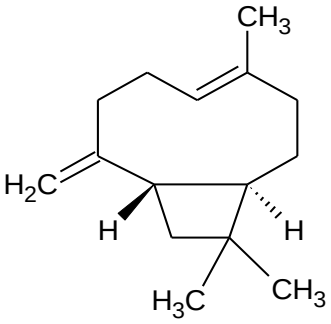


11. β– Phellandrene

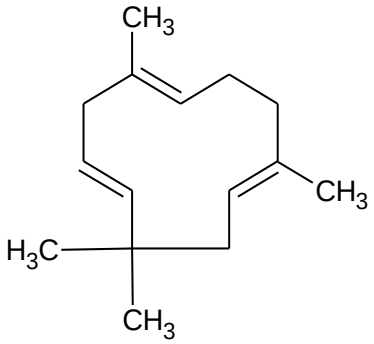


12. γ-Muurolene

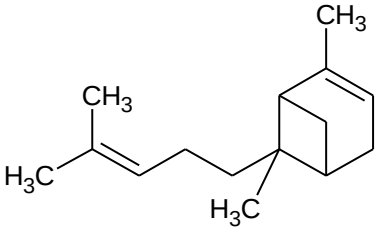
Figure 1.2 continued



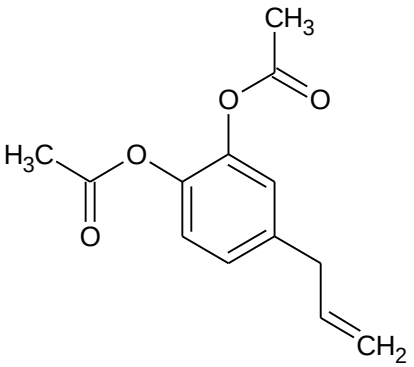
13. β–Caryophyllene



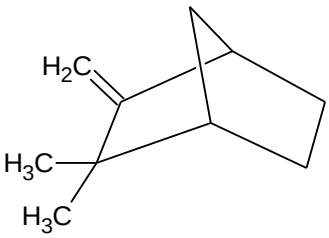
14. α-Humulene



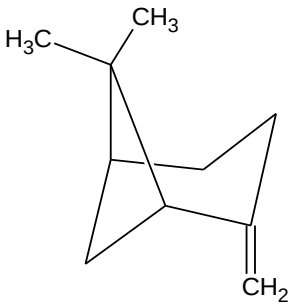
15. α-Bergamotene



16. 4-Allyl-1,2-diacetoxybenzene

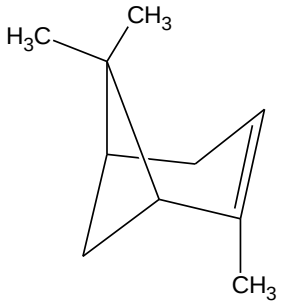


17. Camphene

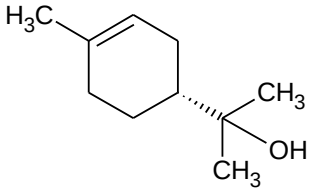


18**.** β-Pinene

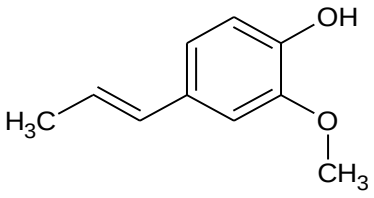
Figure 1.2 continued



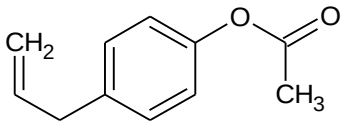
19. α-Pinene



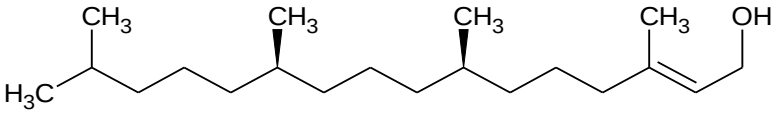
20. α-Terpineol



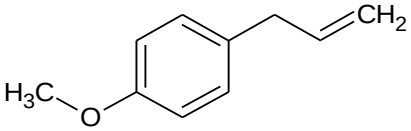
21. Isoeugenol



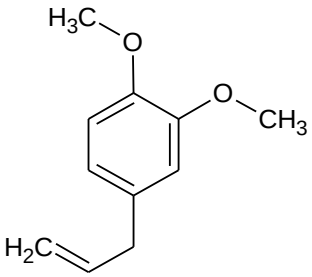
22. Chavicol acetate



23. Phytol



24. Methyl chavicol (Estragole)



25. Chavibetol methyl ether

Figure 1.2 continued