ESSENTIAL OIL OF *PIPER BETLE* AND DERIVATIVES OF EUGENOL

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Abstract

The essential oil of *Piper betle* and the chemistry of its major compound have been investigated. The essential oil was obtained by hydrodistillation technique while the chemical compositions of the oil were determined by gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS) and Kovats Indices. The yield of essential oil obtained from this plant was 0.64%. The compounds identified in the *P. betle* oil were six phenylpropanoids (40.38 %), three monoterpenes (0.15 %), and twenty one sesquiterpenes (38.95 %). Phenylpropanoids were the most dominant group in the oil of *P. betle* leaf with eugenol (32.64 %) being the main component. Eugenol was then seperated from the oil by column chromatography and analyzed spectroscopically. The synthetic eugenol was subjected to methylation and acetylation to form methyl eugenol and eugenol acetate, respectively. The oil, eugenol and its derivatives were screened for antibacterial and antioxidant activities. For the antibacterial activity, only essential oil showed positive result at concentration of 900 ppm. While for the antioxidant activity, essential oil at IC_{50} = 16.83 µg/mL and eugenol at IC_{50} = 3.03 µg/mL, proved that both samples showed positive results on antioxidant assay.

Key Words: *Piperaceae, Piper betle, eugenol, antioxidant, antibacterial*

INTRODUCTION

The Piperaceae or also known as the pepper family is a large family of flowering plants consisting of 1,920 currently accepted species in 13 genera. The vast majority of peppers can be found within the two main genera which are *Piper* and *Peperomia*. Piperaceae species have been placed among the basal angiosperm and are adapted to a variety of habitats including moist forests, secondary vegetation and dry high lands [1].This study was conducted on *P. betle* which is one of the members of Piperaceae family. *P. betle* is blessed as evergreen and perennial plant. Betle leaf has been described from ancient times as an aromatic, stimulo-carminative, astringent and aphrodisiac. The leaves are chewed with betel nut to improve the taste and to prevent halitosis [2]. The root have been used for treatment of cough, bronchial asthma, rheumatism, stomachalgia, edema of pregnancy and as contraceptive [3]. Consequently, the aim of this study was to evaluate the chemical compositions of the essential oil in the leaf and the chemistry of its major compound.

EXPERIMENTAL

Source of Sample

The sample of *P. betle* leaves were collected from Felcra Sungai Ara at Kota Tinggi. The leaves were washed and cut into small pieces.

Extraction and Analysis of Essential Oil

The fresh leaves (355 g) were placed in a round bottom flask (5 L) and covered with distilled water. The flask was equipped with a Dean Stark apparatus and water condenser and hydrodistilled for 10 hours. The mixture of oil and water in the Dean Stark was run off, extracted with Et_2O, dried over anhydrous $MgSO_4$ and filtered. The filtrate was left at room temperature for a few hours to evaporate the solvent. Evaporation of the solvent yielded *P. betle* oil (2.28 g, 0.64%) as a yellow liquid with pungent smell. The essential oil was analysed using GC chromatogram of the oil. Identification of the constituents was performed on the basis of their Kovats indices, which were calculated in relation to a standard hydrocarbon (C6-C26) and compared with those given in the literatures [4].

Isolation of Major Constituent

The essential oil (2.28 g, 0.64 %) was subjected to CC packed with SiO_2 (24.0 g). An eluent system consisted of a mixture of hexane and EtOAc of increasing polarity has afforded 135 fractions. Each fraction was monitored by TLC with hexane:EtOAc (90:10) as the developing solvent. Fractions with the same TLC

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profiles were combined to give three new combined fractions. The fractions were labeled as F1 (0.047 g), F2 (0.196 g), and F3 (0.027 g). Fraction F3 was concentrated to yield eugenol **(1)** (0.027g, 1.20 %) as a yellow liquid; R_f 0.61 in (hexane:EtOAc/90:10).); IR (ATR) ν_{max} cm^{-1}: 3516 (OH), 3077 (C-H sp^2), 2938 (C-H sp^3), 1638 (C=C olefinic), 1612 and 1431 (C=C aromatic), and 1264 (C-O); ^{1}H NMR δ ($CDCl_3$): 3.39 (2H, d, *J=6.8* Hz, H-1'), 3.91 (3H, s, OCH_3), 5.18 (2H, m, H-3'), 5.85 (1H, s, OH), 6.04 (1H, m, H-2'), 6.76 (1H, dd, *J=8.4* Hz and *J=2.0* Hz, H-5), 6.94 (1H, d, *J=8.4* Hz, H-6), 6.95 (1H, d, *J=2.0* Hz, H-3); ^{13}C NMR δ ($CDCl_3$): 39.9 (C-1'), 55.9 (OCH_3), 111.1 (C-3), 114.3 (C-6), 115.6 (C-3'), 121.2 (C-5), 131.9 (C-4), 137.9 (C-2'), 143.9 (C-1) and 146.5 (C-2).

Chemical Modification of Eugenol (1)

Methylation

Eugenol **(1)** (0.10 g, 0.61 mmol) was mixed with CH_3I (0.15 g, 24.1 mmol) and K_2CO_3 (2.0 g) in acetone (10 mL) and refluxed for 10 h. The reaction mixture was then filtered and concentrated. The concentrated product was extracted with EtOAc (3×10 mL). The combined EtOAc extracts was dried over anhydrous $MgSO_4$, filtered and evaporated to dryness to give methyl eugenol as a yellow liquid (0.054 g, 53.8%) and R_f 0.60 (hexane:EtOAc/90:10). IR (ATR) ν_{max} cm^{-1}: 3075 (C- H sp^2), 2936 (C-H sp^3), 1638 (C=C olefinic), 1511 and 1463 (C=C aromatic), 1260 (C-O); ^{1}H NMR δ ($CDCl_3$): 3.36 (2H, d, *J*=6.8 Hz, H-1'), 3.88 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 5.05 (2H, m, H-3'), 5.98 (1H, m, H-2'), 6.72 (1H, s, H-3), 6.83 (1H, d, *J*=8.0 Hz, H-5), 6.87 (1H, d, *J*=8.8 Hz, H-6). ^{13}C NMR δ ($CDCl_3$): 39.9 (C-1'), 55.8 (OCH_3), 55.9 (OCH_3), 111.3 (C-3), 111.9 (C-6), 114.2 (C-3'), 121.2 (C-5), 132.6 (C-4), 137.8 (C-2'), 143.9 (C-1) and 146.4 (C-2).

Acetylation

Eugenol **(1)** (0.1042 g, 0.61 mmol), acetic anhydride (0.1299 g, 1.24 mmol) and pyridine (0.1356 g, 0.062 mmol) were stirred overnight. The organic layer was extracted with CH_2Cl_2 (3x5 mL) and dried over anhydrous $MgSO_4$. The organic layer was then evaporated to dryness to give eugenol acetate **(3)** (0.0913 g, 87.6%) as a yellow liquid and R_f 0.62 (hexane:EtOAc/90:10). IR (ATR) ν_{max} cm^{-1}: 3077 (C-H sp^2), 2939 (C-H sp^3), 1762 (C=O), 1638 (C=C olefinic), 1604 and 1508 (C=C aromatic), 1267 (C-O); 1H NMR δ ($CDCl_3$): 2.32 (3H, s, CH_3COO), 3.39 (2H, d, *J*=6.3 Hz, H-1'), 3.83 (3H, s, OCH_3), 5.14 (2H, m, H-3'), 5.97 (1H, m, H-2'), 6.79 (1H, dd, *J*=8.4, and *J=2.4,* H-5), 6.81 (1H, d, *J*=2.4 Hz, H-3), 6.97 (1H, d, *J*=8.4 Hz, H-6). ^{13}C NMR δ ($CDCl_3$): 20.7 (CH_3), 40.1 (C-1'), 55.8 (OCH_3), 112.7 (C-3), 116.2 (C-3'), 120.7 (C-5), 122.5 (C-6), 137.0 (C-4), 137.9 (C-2'), 139.0 (C-1), 150.8 (C-2) and 169.3 (C=O).

Antibacterial Test

The tested microorganisms were Gram-positive and Gram-negative bacteria namely *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATCC 9027. The essential oil, eugenol **(1)**, methyl eugenol **(2)**, and eugenol acetate **(3)** were tested for antibacterial activity. Each sample was dissolved in DMSO to make 1.8 mg/mL stock sample. Disc of Streptomycin sulphate was used as the positive control while DMSO as the negative control. The McFarland reference was prepared by adding sulphuric acid (1% into broth nutrient) and hydrated barium chloride (1% into broth nutrient) (0.05 mL) in a covered vial and left at room temperature. The antibacterial tests were carried out through MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) method. The nutrient agar (20 g) and nutrient broth (8 g) were prepared by dissolved it with 1000 ml of distilled water. Then, the prepared solution were autoclaved for 2 hours 30 minutes at 50°C. The nutrient agar solution (5 mL) was pipetted into the agar plates. The agar plates were then kept in the refrigerator.

Antioxidant Activity

The essential oil, eugenol **(1)**, methyl eugenol **(2)** and eugenol acetate **(3)** were measured for antioxidant activity by observing the change of purple colour solution of DPPH (diphenylpicrylhydrazyl). The sample solution was prepared by dissolving each sample (1 mg) in methanol (1 mL) to produce a concentration of 1000

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ppm. The dilution from the stock solution were prepared in concentration of 100 ppm, 80 ppm, 60 ppm, 40 ppm, 20 ppm and 10 ppm. The purple colour solution of DPPH was prepared by dissolving DPPH (4 mg) in methanol (100 mL). The sample solution (100 µL) was added to the DPPH solution (100 µL) in a sample well. Sample which bleached the purple colour to yellow was considered active as DPPH radical scavenging.

RESULTS AND DISCUSSION

The Chemical Composition of Piper betle Oil

The GC and GC-MS analysis of this oil have been yielded thirty constituents. The compounds identified in the *P. betle* oil were six phenylpropanoids (40.38 %), three monoterpenes (0.15 %), and twenty one sesquiterpenes (38.95 %). Phenylpropanoid was the most dominant group in the oil of *P. betle* leaf with eugenol **(1)** (32.64 %) being the main component. Sesquiterpenes such as elemol (24.92 %), α-cadinol (4.97 %), γ-elemene (1.60 %), and Germacene D (2.43 %) were the most abundant constituents in *P. betle* leaf oil. While for monoterpenes, the compounds present in the oil were camphene (0.04 %), *cis*-ocimene (0.03 %), and linalool (0.08 %).

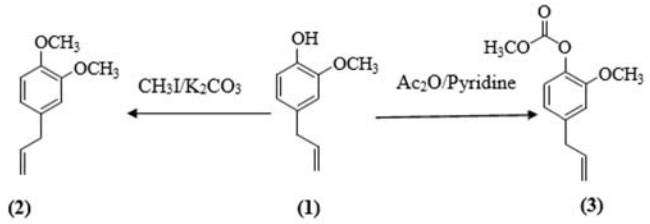
Chemical Constituents of Piper betle Leaf Oil

Eugenol **(1)** was isolated as a yellow liquid with a strong smell of clove. The TLC analysis displayed a single yellow spot with R_f value 0.61 in hexane:EtOAc (90:10). The IR spectrum showed a strong broad band at 3516 cm^{-1}, indicating the presence of hydroxyl group which was confirmed by an absorption band at 1264 cm^{-1} attributed to C-O. The bands at 1612 cm^{-1} and 1431 cm^{-1} were assigned as C=C of aromatic stretching, while the C=C olefinic stretching was represented by a band at 1638 cm^{-1}.

The ^{1}H NMR spectrum displayed two singlets at δ 3.91 and δ 5.85 corresponding to methoxyl and hydroxyl groups, respectively. The presence of an allylic side chain was confirmed by the signal resonance at δ 3.39 (2H, d, *J=6.8* Hz), 5.18 (2H, m) and 6.04 (1H, m) corresponding to H-1', H-3' and H-2', respectively. The ^{1}H NMR spectrum also showed a doublet at δ 6.96, *J=2.0* Hz attributed to aromatic proton H-3 while two mutual coupling aromatic proton of H-5 and H-6 were represented each by signal at δ 6.76 (dd, *J=8.4* Hz and *J=2.0* Hz), and δ 6.94 (d, *J=8.4* Hz). The ^{13}C NMR spectrum showed the presence of 10 carbons with a methoxyl carbon at δ 55.9, and the aromatic carbons (C-3, C-5 and C-6) resonated at δ 111.1, δ 114.3 and δ 121.2, respectively. The three allylic carbons resonated at δ 39.9 (C-1'), δ 137.9 (C-2') and δ 115.6 (C-3'). The quaternary carbons, C-1, C-2 and C-4 were observed at δ 143.9, δ 146.5 and δ 131.9, respectively.

Chemistry of Eugenol (1)

Eugenol **(1)** was subjected to methylation, and acetylation reaction which resulted in methyl eugenol **(2)**, and eugenol acetate **(3)**.



Methyl Eugenol (2)

Methylation of eugenol with CH3I in the presence of K2CO3 as base yielded methyl eugenol as a yellow liquid (0.054 g, 53.8%) %) and R_f 0.60 (hexane:EtOAc/90:10). The mild base, K2CO3 abstracted the hydrogen of the hydroxyl group to form the phenoxide ion which undergo SN2 reaction with methyl iodide to form **(2).** The IR spectrum of **(2)** exhibited the disappearance of OH absorption band at 3516 cm^{-1}. The ^{1}H NMR spectrum confirmed the formation of compound **(2)** by exhibiting two singlet signals at δ 3.88 and δ 3.89 corresponding to two methoxyl groups. In addition to this, the aromatic protons signals were observed at δ 6.72 (1H, s, H-3), 6.83 (1H, d, *J*=8.0 Hz, H-5) and 6.87 (1H, d, *J*=8.8 Hz, H-6). The ^{13}C NMR spectrum further

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confirmed the success of methylation reaction by showing two signals at δ 55.8 and 55.9 attributed to two methoxyl groups.

Eugenol Acetate (3)

Treatment of eugenol **(1)** with acetic anhydride in pyridine has affored eugenol acetate as a yellow liquid (0.0913 g, 87.6%) and Rf 0.62 (hexane:EtOAc/90:10). The IR spectrum of **(3)** showed the presence of an absorption band at 1762 cm^{-1} corresponding to an acetyl function. The presence of the acetyl group was further supported by the ^{1}H NMR spectrum which showed a singlet at δ 2.32 attributed to three protons of an acetyl group. The dissappearance of hydroxyl signal at δ 5.85 for eugenol **(1)**, have proved that the hydroxyl has been subtitude by the acetyl group. The rest of the signals for protons were similar to eugenol **(1)**. The ^{13}C NMR spectrum displayed a signal at δ 169.3 corresponding to a carbonyl group. Based on these data, **(3)** was characterised as 4-allyl-2- methoxyphenyl acetate or eugenol acetate. The mechanism occurred when pyridine as a nucleophile attacked the carbonyl of acetic anhydride to form the acetate and pyridinium ions. The acetate ion which acted as a base, abstracted a proton from eugenol **(1)** to produce the phenoxide ion. The phenoxide ion attacked the pyridinium ion followed by elimination of pyridine to form eugenol acetate **(3)**.

Bioactivity studies of Piper betle

Antibacterial Test

Essential oil, eugenol **(1)**, methyl eugenol **(2)** and eugenol acetate **(3)** were examined for antibacterial activity using MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) as the quantitative assays. The essential oil was found to have weak antibacterial activity against *B.subtilis* and *E.coli* with a concentration of 900 ppm. On the other hand, eugenol **(1)** and its derivatives, methyl eugenol **(2)** and eugenol acetate **(3)** were inactive towards the tested bacteria. It might due to the synergistic effect of the components presence in the essential oil contributed to the activity.

Antioxidant Assay

The rapid screening for antioxidant activity was performed on the essential oil, eugenol and its derivatives. DPPH or 1,1-diphenyl-2-picrylhydrazyl radical solution which was purple in colour and have a strong absorption at 517 nm was used for this assay. Sample which able to change the purple to yellow colour of DPPH is claimed to have active radical scavenger of DPPH as the molar absorptivity of DPPH radical at 517 nm decreased due to the odd electron of DPPH radical becomes paired with hydroxylated benzene to form the reduced DPPH-H. As a result, essential oil and eugenol was proven to have an antioxidant activities which the changes of the purple solution were observed. From the **Table 1**, IC_{50} for the essential oil and eugenol were 16.83 µg/mL and 3.03 µg/mL respectively. Eugenol **(1)** had the lowest IC_{50} value which is 3.03 µg/mL compared to ascorbic acid with IC_{50} = 10.83 µg/mL. It showed that eugenol is a potent antioxidant compared to ascorbic acid and the *P.betle* oil.

Table 1 : IC_{50} values for the samples

Sample	IC_{50} (µg/mL)
Essential oil	16.83
Eugenol	3.03
Ascorbic acid	10.83

CONCLUSION

The hydrodistillation of fresh P. betle leaves afforded the essential oil (2.28 g, 0.64 %) as a yellow oil. The GC and GC-MS analysis of this oil have identified thirty constituents of six phenylpropanoids (40.38 %), three monoterpenes (0.15 %) and twenty one sesquiterpenes (38.95 %). A phenylpropanoid was found to be the main component in 32.64 % of eugenol (1). Camphene, cis-ocimene, and linalool were the compounds of

monoterpenes while elemol, α- cadinol, γ-elemene, and Germacene D represented the class of sesquiterpenes. Purification of the essential oil by column chromatography afforded the compound identified as eugenol (1). Methylation and acetylation of eugenol (1) produced methyl eugenol (2), and eugenol acetate (3) respectively. The essential oil, eugenol (1) and the derivatives were screened for antibacterial and antioxidant activity. For the antibacterial activity, only essential oil showed positive result at concentration of 900 ppm. While for the antioxidant activity, essential oil at IC50 = 16.83 µg/mL and eugenol at IC50 = 3.03 µg/mL, proved that both samples showed positive results on antioxidant assay.

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