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Components from Sri Lankan *Piper betle* L. leaf oil and their analogues showing toxicity against the housefly, *Musca domestica*

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ABSTRACT: The essential oil extracted from *Piper betle* L. leaf using pilot plant steam distillation was tested against the adult housefly, *Musca domestica*, for insecticidal activity. LC_{50} values at the end of 24 and 48 h exposure periods were 10.3 and 8.7 mg/dm^3, respectively. Ceylon citronella oil (*Cymbopogon nardus*) used as a standard showed LC_{50}s of 26.5 and 24.2 mg/dm^3 for the same exposure periods. Bioassay-guided fractionation of *P. betle* leaf oil revealed safrole and eugenol as the active principles against *M. domestica*, safrole showing LC_{50} values of 4.8 and 4.7 mg/dm^3, and eugenol 7.3 and 6.2 mg/dm^3 for the 24 and 48 h exposure periods, respectively, while citronellal (synthetic standard) showed equal LC_{50} values of 14.3 mg/dm^3 for the same exposure periods. Using safrole as the starting compound, eight analogues were prepared to study structure–activity relationships. Among the eight analogues, dihydrosafrole gave almost equal mortality at LC_{50} 4.7 mg/dm^3 as that of the parent compound safrole after 24 and 48 h exposure, but isosafrole was twice as active as safrole, showing LC_{50} values of 2.3 and 2.2 mg/dm^3 for the 24 and 48 h exposure periods. Our GC–MS studies on Sri Lankan *P. betle* leaf oil show that it contains safrole (52.7%), allylpyrocatechol diacetate (15.4%), eugenol (6.4%) and eugenyl acetate (5.8%) as the major components. Here we also present the GC–MS profile of fractions of Sri Lankan *P. betle* leaf oil. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS: *Piper betle* L.; leaf essential oil composition; *Musca domestica*; safrole; isosafrole; dihydrosafrole; insecticidal activity; LC_{50}; ^{1}H-NMR; ^{13}C-NMR.

Introduction

Plants and products derived from them are frequently used for insect control by humans. Currently there is an increasing trend to use plant-derived products in pest management. The plant derivatives may offer a safe alternative to synthetic pesticides such as organophosphates and carbamates. Among botanicals, plant-derived essential oils play a diverse role in pest management, showing antifungal, antimicrobial, cytostatic and insecticidal properties.[1] The genus *Piper* belongs to the family Piperaceae and has over 700 species distributed worldwide, with some *Piper* spp. reported to have insecticidal properties, such as *P. brachystachyu*, *P. guineense* and *P. falconeri*. *P. acutisleginum* shows insecticidal activity against *Musca domestica* Linnaeus (the housefly) and *Aedes aegyptii* (mosquito), while *P. aduncum* and *P. hispidum* are insect repellents.[2]

P. betle is widely cultivated in tropical countries such as Sri Lanka, India, Malaysia and The Philippines. People commonly use the leaves for chewing, either alone or with other plant materials including the areca nut, *Areca catechu* L.[3] *P. betle* is also reported to possess antifungal, antiseptic and anthelmintic properties, to serve as a contraceptive for humans and to possess antihypertensive properties.[2–3]

In this work we studied the insecticidal properties of Sri Lankan *P. betle* leaf oil. We report here the chemical composition of this oil and its chromatographic fractions, as determined by gas chromatography–mass spectrometry. Compounds of *P. betle* oil responsible for insecticidal activity against *M. domestica* were identified. We also provide structure–activity relationships of analogues of safrole, the most active compound isolated from the oil.

Materials and Methods

Biological

Adult houseflies, *M. domestica* (WHO strain), were used for these studies. Housefly maggots were obtained from a laboratory culture at Novartis Santé Animale S.A., St. Aubin, Switzerland, and maintained at 30 °C, 80% RH until eclosion. The emerged adults were fed on casein and sucrose until they were used for the experiments.

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Pilot Plant Steam Distillation of *P. betle* L. Leaf Oil

Fresh leaves (10 kg) were collected at Kottawa, District of Colombo, Sri Lanka, air-dried for 2 days and subjected to pilot plant steam distillation for 4 h (yield 40 ml, 0.40 v/w). The *P. betle* leaf oil, together with Ceylon citronella oil (positive control), was tested for insecticidal activity against *M. domestica* as described below.

Bioassay

Active houseflies (*M. domestica*), 3–4 days old, were used for tests with all the treatments. The experiments were carried out at 22 °C, 45–55% RH, under a 10:14 h light:dark (L:D) regimen. Serial dilutions of the oil/fractions were prepared in acetone. Acetone alone was used in controls. Each concentration was tested with five flies in six replicates. Aliquots of 0.5 ml were spread on Whatman filter paper disks (7 cm diameter) and the solvent was allowed to evaporate for about 10 min under the fume hood. After evaporation, filter papers were placed in aluminium plates (7 cm diameter, 3 cm high). Casein and sucrose were kept in a small dish (2 cm diameter, 0.5 cm high) and placed on the filter paper for the flies to feed. Flies were immobilized by holding them in a cold room (4–5 °C) for 3–4 min and then placed on the treated filter paper and covered with an upturned plastic cup (interior volume 0.25 dm^3, 8.8 cm high, 5 cm diameter at base and 7 cm at top) to prevent escape. A cotton plug in a small hole in the base of the cup was moistened to maintain the humidity level. Mortality was counted after 24 and 48 h exposure periods.

Since Sri Lankan *P. betle* leaf oil is more toxic to houseflies than Ceylon citronella oil, this *P. betle* leaf oil was subjected to bioassay-guided chromatographic fractionation in order to isolate and identify the active components.

Bioassay-guided Fractionation of *P. betle* L. Leaf Oil

The oil (25.2 g) obtained from pilot plant distillation was subjected to flash chromatography (FC), using silica gel (Fluka, mesh size 230–400) as the stationary phase and hexane, toluene and ethyl acetate as the eluting solvents. The solvents were used in increasing order of polarity, as follows: hexane, 800 ml; hexane:toluene, 9:1, 500 ml; hexane:toluene, 8:2, 500 ml; hexane:toluene, 4:6 500 ml; hexane:toluene, 2:8, 500 ml; toluene, 500 ml; toluene:ethyl acetate, 97:3, 500 ml; toluene:ethyl acetate, 9:1, 500 ml; toluene:ethyl acetate, 8:2, 500 ml; and ethyl acetate, 500 ml. Fractions (~50 ml each) were collected and analysed by silica gel thin-layer chromatography (TLC). Fractions with the same R_f value were combined; the solvents were evaporated and eight major fractions were obtained: Their code names and weights were as follows: PBL/1, 2.2 g; PBL/2, 5.6 g; PBL/3, 8.9 g; PBL/4, 0.2 g; PBL/5, 2.5 g; PBL/6, 0.82 g; PBL/7, 1.26 g; and PBL/8, 1.08 g. All of these fractions were tested for insecticidal activity. Those fractions that showed activity were further fractionated by FC and the subfractions were also tested for activity.

^{1}H- and ^{13}C-NMR

Either a Varian 200 MHz (4.7 Tesla magnet) or a Bruker 400 MHz (9.4 Tesla magnet) spectrometer was used for ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectroscopy. ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectroscopy was carried out on the same Bruker spectrometer. $CDCl_3$ solutions were used for both ^{1}H-NMR and ^{13}C-NMR. Chemical shifts are given in p.p.m. units relative to $CHCl_3$ set to 7.26 (^{1}H-NMR) and 77.0 (^{13}C-NMR) (multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad). The identity of compounds was established by comparison of spectral data.

GC–MS

Gas chromatography–mass spectrometry (GC–MS) analysis was performed on Varian 3400-Saturn 3 and Trace GC-Polaris Q instruments (EI 70 eV), equipped with an analyser quadrupole ion trap (QIT), with helium as the carrier gas. GC analyses were performed on Varian model 3400 and Polaris Q instruments for essential oil analysis.

The separation was achieved on a ZB-5 capillary column (Phenomenex, USA, stationary phase 5% diphenyl:95% dimethyl polysiloxane, 30 m × 0.25 mm i.d., 0.25 μm film thickness); injector temperature, 220 °C; transfer line temperature, 240 °C; column temperature was held at 60 °C for 5 min, then programmed to 220 °C at 4 °C/min, then held at 220 °C for 20 min.

Isolation of Active Principles and Preparation of their Analogues

Isolation of 3-(3′,4′-methylenedioxyphenyl)-prop-1-ene (safrole) **1**

Fraction PBL/3, (8.0 g) was subjected to FC using toluene:hexane (1:5) as the eluent. This provided safrole **1** (7.8 g) as a light yellow oil and the main constituent of this fraction. ^{1}H-NMR and ^{13}C-NMR data were compared with those reported for safrole[4] in the literature.

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^{1}H-NMR (400 MHz) δ: 6.78 (1H, d, J = 7.9 Hz, 5′-H), 6.72 (1H, d, J = 1.4 Hz, 2′-H), 6.67 (1H, dd, J = 7.9, 1.7 Hz, 6′-H), 5.96 (1H, m, 2-H), 5.95 (2H, s, O-CH_2-O), 5.10 (2H, m, 1-H), 3.34 (2H, d, J = 6.7 Hz, 3-H). ^{13}C-NMR (100 MHz) δ: 148.04 (C-3′), 146.23 (C-4′), 138.02 (C-2), 134.28 (C-1′), 121.72 (C-6′), 116.20 (C-1), 109.52 (C-2′), 108.58 (C-5′), 101.21 (O-CH_2-O), 40.33 (C-3).

Isolation of 3-(4′-hydroxy-3′-methoxyphenyl)-prop-1-ene (eugenol) **10**

Fraction PBL/5 (2.4 g) was subjected to FC with EtOAC:hexane (3:1) to obtain eugenol **10** (2.100 g) as a pale yellow oil and as the primary constituent of the fraction. Data were compared with those reported for ^{1}H-NMR[5] and ^{13}C-NMR[6]. ^{1}H-NMR (200 MHz) δ: 6.83 (1H, d, J = 8.8 Hz, 5′-H), 6.66 (1H, dd, J = 8.4, 1.8 Hz, 6′-H), 6.65 (1H, d, J = 1.8 Hz, 2′-H), 5.94 (1H, m, 2-H), 5.50 (1H, br.s, D_2O exchangeable, OH), 5.05 (2H, m, 1-H), 3.86 (3H, s, OCH_3), 3.30 (2H, dt, J = 6.6, 1.5 Hz, 3-H). ^{13}C-NMR (100 MHz) δ: 146.87 (C-3′), 144.31 (C-4′), 138.26 (C-2), 132.34 (C-1′), 121.60 (C-6′), 115.44 (C-1), 114.70 (C-5′), 111.54 (C-2′), 56.27 (OCH_3), 40.32 (C-3).

Preparation of analogues from safrole, **1**

As safrole **1** has an allylic moiety attached to the aromatic ring, eight analogues, **2–9**, were prepared via modification of the allylic moiety (Figure 1). These analogues were then tested to evaluate structure–activity relationships. Since safrole **1** also has a methylenedioxy moiety, allyl benzene **11** was also tested to observe whether this moiety is essential for insecticidal activity.

Isosafrole, (E)-3-(3′,4′-methylenedioxyphenyl)-prop-2-ene **2** (1.63 g, yield 82%), was prepared from safrole **1** (2.000 g) using the method of Thach *et al.*[7] ^{1}H-NMR (200 MHz) δ: 6.87-6.72 (3H, m, Ar-H), 6.30 (1H, dd, J = 15.8, 1.8 Hz, 3-H), 6.03 (1H, m, 2-H), 5.91 (2H, s, O-CH_2-O), 1.83 (3H, dd, J = 6.2, 1.5 Hz, 1-H). ^{13}C-NMR (100 MHz) δ: 148.3 (C-3′), 146.9 (C-4′), 132.9 (C-1′), 130.9 (C-3), 124.4 (C-2), 120.5 (C-6′), 108.6 (C-2′), 105.7 (C-5′), 101.3 (O-CH_2-O), 18.8 (C-1).

Dihydrosafrole,3-(3′,4′-methylenedioxyphenyl)-propane **3** (450 mg, yield 90%) was prepared from safrole **1** (500 mg) according to the method described by Narisada *et al.*[8] ^{1}H-NMR (200 MHz) δ: 6.71 (1H, d, J = 7.5 Hz, 5′-H), 6.66 (1H, d, J = 1.1 Hz, 2′-H), 6.60 (1H, dd, J = 7.8, 1.7 Hz, 6′-H), 5.90 (2H, s, O-CH_2-O), 2.49 (2H, t, J = 7.6 Hz, 3-H), 1.58 (2H, m, 2-H), 0.91 (3H, t, 1-H). ^{13}C-NMR (100 MHz) δ: 147.85 (C-3′), 145.82 (C-4′), 136.99 (C-1′), 121.52 (C-6′), 109.32 (C-2′), 108.41 (C-5′), 101.09 (O-CH_2-O), 38.20 (C-3), 25.24 (C-2), 14.12 (C-1).

3-(3′,4′-methylenedioxyphenyl)-prop-2-ol **5** (568 mg, yield 91%) was prepared from safrole **1** (622 mg) using the method described by Barreiro *et al.*[9] ^{1}H-NMR (200 MHz) δ: 6.70 (1H, d, J = 7.7 Hz, 5′-H), 6.66 (1H, d, J = 1.5 Hz, 2′-H), 6.60 (1H, dd, J = 7.7, 1.8 Hz, 6′-H), 5.86 (2H, s, O-CH2-O), 3.89 (1H, m, 2-H), 2.59 (2H, m, 3-H), 2.20 (1H, br.s, D_2O exchangeable, OH), 1.16 (3H, d, J = 6.2, 1-H). ^{13}C-NMR (100 MHz) δ: 148.16 (C-3′), 146.61 (C-4′), 132.61 (C-1′), 122.67 (C-6′), 110.07 (C-2′), 108.72 (C-5′), 101.29 (O-CH_2-O), 69.32 (C-2), 45.82 (C-3), 23.11 (C-1).

3-(3′,4′-methylenedioxyphenyl)-prop-2-one **4** (160 mg, yield 80%) was prepared from 3-(3′,4′-methylenedioxyphenyl)-prop-2-ol **5** (200 mg) using the method of Barreiro *et al.*[10] ^{1}H-NMR (200 MHz) δ: 6.75 (1H, d, J = 8.1 Hz, 5′-H), 6.65 (1H, d, J = 1.5 Hz, 2′-H), 6.61 (1H, dd, J = 7.8, 1.5 Hz, 6′-H), 5.92 (2H, s, O-CH_2-O), 3.58 (2H, s, 3-H), 2.12 (3H, s, 1-H). ^{13}C-NMR (100 MHz) δ: 206.99 (C-2), 148.31 (C-3′), 147.09 (C-4′), 128.22 (C-1′), 122.92 (C-6′), 110.16 (C-2′), 108.88 (C-5′), 101.47 (O-CH_2-O), 50.94 (C-3), 29.54 (C-1).

3-(3′,4′-methylenedioxyphenyl)-prop-1-ol **6** (1.37 g, yield 75%) was prepared from safrole **1** (1.820 g) according to the method of Gautam *et al.*[11] ^{1}H-NMR (200 MHz) δ: 6.71 (1H, d, J = 8.1 Hz, 5′-H), 6.67 (1H, d, J = 1.5 Hz, 2′-H), 6.62 (1H, dd, J = 7.7, 1.8 Hz, 6′-H), 5.90 (2H, s, O-CH_2-O), 3.64 (2H, t, J = 6.4, 1-H), 2.61 (2H, t, J = 7.7 Hz, 3-H), 1.82 (2H, m, 2-H), 1.48 (1H, br.s, D_2O exchangeable, OH). ^{13}C-NMR (100 MHz) δ: 147.98 (C-3′), 146.03 (C-4′), 136.06 (C-1′), 121.52 (C-6′), 109.29 (C-2′), 108.57 (C-5′), 101.17 (O-CH_2-O), 62.48 (C-1), 34.82 (C-2), 32.20 (C-3).

3-(3′,4′-methylenedioxyphenyl)-prop-1-al **7** (191 mg, yield 96%) was prepared from 3-(3′,4′-methylenedioxyphenyl)-prop-1-ol **6** (200 mg) using the method of Barreiro *et al.*[10] ^{1}H-NMR (200 MHz) δ: 9.78 (1H, t, J = 1.4 Hz, 1-H), 6.71 (1H, d, J = 7.7 Hz, 5′-H), 6.66 (1H, d, J = 1.8 Hz, 2′-H), 6.61 (1H, dd, J = 7.8, 1.7 Hz, 6′-H), 5.90 (2H, s, O-CH_2-O), 2.86 (2H, m, 3-H), 2.71 (2H, m, 2-H). ^{13}C-NMR (100 MHz) δ: 201.99 (C-1), 148.15 (C-3′), 146.39 (C-4′), 134.51 (C-1′), 121.48 (C-6′), 109.17 (C-2′), 108.72 (C-5′), 101.30 (O-CH_2-O), 45.95 (C-2), 28.28 (C-3).

3-(3′,4′-methylenedioxyphenyl)-1,2-epoxy-propane **8** was prepared as follows. *Meta* chloroperbenzoic acid (3.730 g) was added to a cold solution of safrole **1** (815 mg) in $CHCl_3$ (25 ml) and the mixture was left at room temperature for 3 h. The solution was then washed successively with H_2O (75 ml), 5% $NaHCO_3$ (50 ml) and saturated brine (2 × 25 ml), dried over Na_2CO_3 and evaporation, followed by flash chromatography on silica gel with EtOAc:toluene (1:19), gave 3-(3′,4′-methylenedioxyphenyl)1,2-epoxy-propane **8** (642 mg, 79%). ^{1}H-NMR (400 MHz) δ: 6.78 (1H, d, J = 7.9 Hz, 5′-H), 6.77 (1H, d, J = 1.4 Hz, 2′-H), 6.71 (1H, dd, J = 7.6, 1.4 Hz, 6′-H), 5.96 (2H, s, O-CH_2-O), 3.13 (1H, m, 2-H), 2.87–2.75 (3H, m, 3-H and 1-H), 2.55 (1H, m, 1-H). ^{13}C-NMR (100 MHz) δ: 148.09 (C-3′), 146.71 (C-4′),

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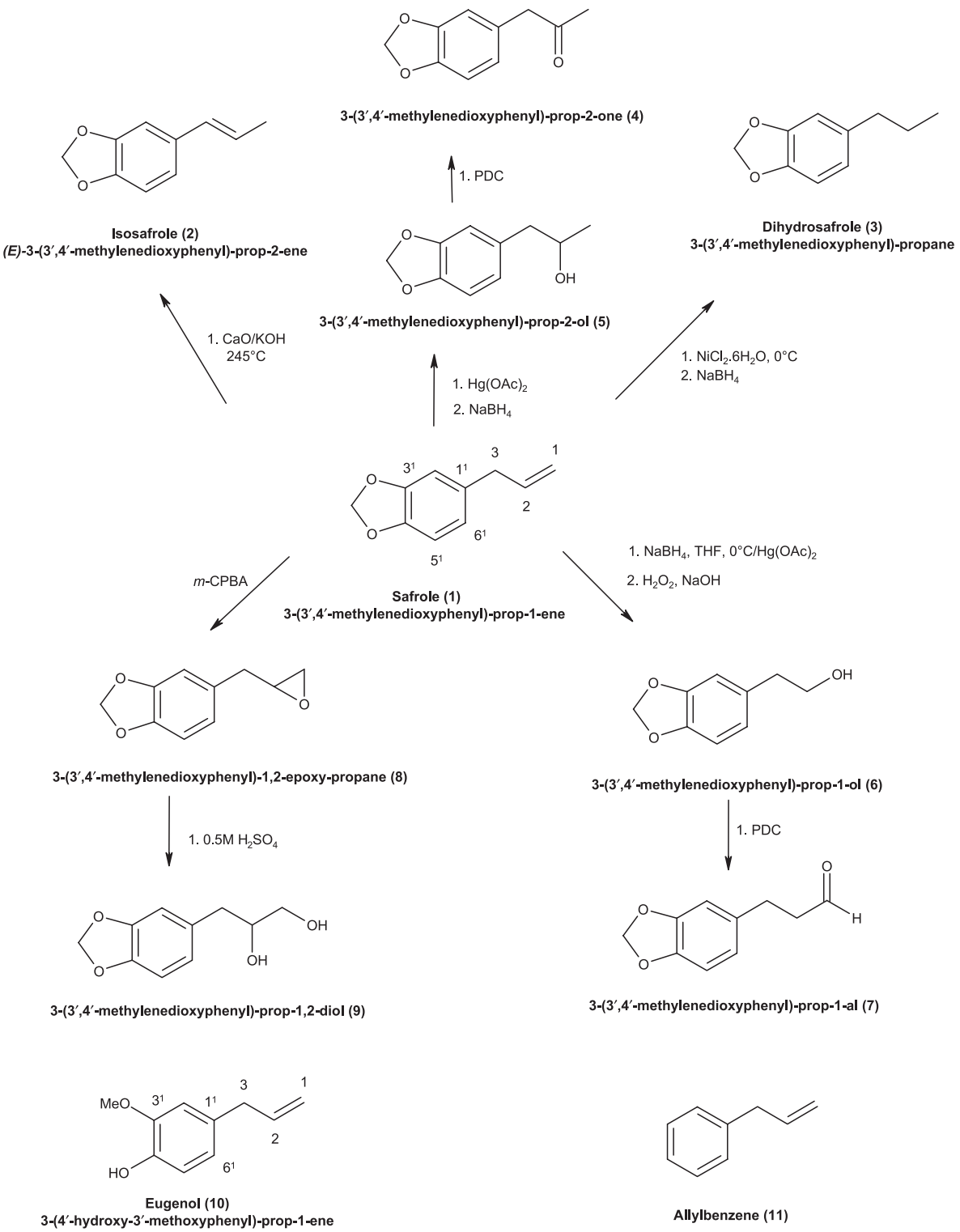

Figure 1. Analogues of safrole.

131.23 (C-1′), 122.29 (C-6′), 109.87 (C-2′), 108.69 (C-5′), 101.31 (O-CH_2-O), 52.98 (C-2), 47.23 (C-1), 38.82 (C-3).

3-(3′,4′-methylenedioxyphenyl)-prop-1,2-diol **9** was prepared as follows. 3-(3′,4′-methylene-dioxy phenyl)-1,2-epoxy propane **8** (200 mg) in aq. THF (15 ml) was stirred with 0.5 M H_2SO_4 (3 ml) at room temperature for 1 h. Work-up followed by FC with EtOAc:toluene (4:1) gave 3-(3′,4′-methylenedioxyphenyl)-prop-1,2-diol **9** (160 mg, 80%). ^{1}H-NMR (200 MHz) δ: 6.73 (1H, d, J = 8.1 Hz, 5-H′), 6.69 (1H, d, J = 1.5 Hz, 2-H′), 6.62 (1H, dd, J = 8.1, 1.7 Hz, 6-H′), 5.90 (2H, s, O-CH_2-O),

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3.85 (1H, m, 2-H), 3.79-3.41 (2H, m, 1-H), 2.74-2.56 (2H, m, 3-H), 2.60 (2OH, br.s, D_2O exchangeable, 1-OH and 2-OH). ^{13}C-NMR (100 MHz) δ: 147.67 (C-3′), 146.14 (C-4′), 131.39 (C-1′), 122.14 (C-6′), 109.57 (C-2′), 108.26 (C-5′), 100.84 (O-CH_2-O), 73.06 (C-2), 65.79 (C-1), 39.30 (C-3).

Allylbenzene **11** (~97% GC pure) and *citronellal* (~98% GC pure) were purchased from Fluka, Switzerland.

Standards

Ceylon citronella oil was used as a positive control for the bioassay of *P. betle* oil, whereas citronellal (synthetic) was used in the bioassays involving isolated compounds and newly prepared analogues.

Statistical analysis

The mortality was corrected according to the following equation: $(a - b)100/a$, where a and b are numbers of surviving adult flies in the control and test experiments, respectively.

Since all the essential oils, fractions and the compounds used in the experiment were volatiles, the concentrations of these test substances are estimated here in mg/dm^3 assuming these substances to have been fully evaporated within the cup (volume of the plastic cup, 0.25 dm^3; the amounts of the oils, fractions or compounds placed on the filter paper are in mg).

LC_{50} values were determined using the Environmental Protection Agency (EPA) probit analysis program, version 1.5. Comparisons between treatments were made by one-way ANOVA, after log-transforming of the LC_{50} values, and ranked by Duncan's multiple range test (DMRT).

Results

P. betle L. leaf oil showed lower LC_{50} values of 10.3 and 8.7 mg/dm^3 after 24 and 48 h periods, respectively, compared to Ceylon citronella with LC_{50} values of 26.5 and 24.2 mg/dm^3 for the same exposure periods (Table 1). Among the eight fractions of *P. betle* leaf oil, fractions PBL/3 and PBL/5 showed even lower LC_{50} values of 5.3 and 8.5 mg/dm^3 after 24 h exposure, respectively. Fractions PBL/2 and PBL/6 also showed low LC_{50} values of 8.8 and 9.9 mg/dm^3 for the same exposure period (Table 2). Bioassay-guided fractions of PBL/3 and PBL/5 yielded safrole and eugenol as the active components, with LC_{50} values of 4.8 and 4.7 mg/dm^3 and 7.3 and 6.2 mg/dm^3 after 24 and 48 h exposure periods, respectively (Table 3).

The analogues of safrole showed greater toxicity than safrole itself. Analogue **2** (isosafrole) had the highest toxicity at 24 h (LC_{50} = 2.3 mg/dm^3) and was significantly higher than all the other compounds tested. The activity of safrole **1** (LC_{50} = 4.8 mg/dm^3), dihydrosafrole **3** (LC_{50} = 4.7 mg/dm^3) and eugenol **10** (LC_{50} = 7.3 mg/dm^3) were not significantly different from each other after 24 h exposure, but these compounds were significantly different from analogue 3-(3′,4′-methylenedio-xyphenyl)-prop-2-one **4** (LC_{50} of 37.4 mg/dm^3) and citronellal (LC_{50} of 14.3 mg/dm^3) for the same exposure period. Compound **4** showed the lowest toxicity. Compounds

Table 1. LC_{50} values of *P. betle* leaf and Ceylon citronella oils for *M. domestica*

Name of oil	LC_{50} (mg/dm^3)*	
	24 h	48 h
Piper betle leaf	10.3[b]	8.7[b]
Ceylon Citronella[#]	26.5[a]	24.2[a]

[#] Ceylon citronella oil was used as a positive control.
* LC_{50} values not followed by the same letters in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.
The highest dose tested was 20.15 mg (i.e. 80.6 mg/dm^3).

Table 2. LC_{50} values of *P. betle* leaf oil fractions for *M. domestica*

Fraction No.	Active or inactive	LC_{50} (mg/dm^3)	
		24 h	48 h
PBL/1	Very mild activity	23.5	23.5
PBL/2	Good activity	8.8	8.8
PBL/3	High activity	5.3	5.3
PBL/4	Inactive	—	—
PBL/5	Good activity	8.5	8.5
PBL/6	Mild activity	9.9	9.5
PBL/7	Inactive	—	—
PBL/8	Inactive	—	—

The highest dose tested was 20.15 mg (i.e. 80.6 mg/dm^3).

Table 3. LC_{50} values of eugenol, safrole and its analogues for *M. domestica*

Compound	Active or inactive	LC_{50} (mg/dm^3)*	
		24 h	48 h
Eugenol **10**	Active	7.3[c]	6.2[c]
Safrole **1**	Active	4.8[c]	4.7[c]
Isosafrole **2**	Active	2.3[d]	2.2[d]
Dihydrosafrole **3**	Active	4.7[c]	4.7[c]
4	Active	37.4[a]	29.8[a]
5	Inactive	—	—
6	Inactive	—	—
7	Inactive	—	—
8	Inactive	—	—
9	Inactive	—	—
Allylbenzene **11**	Inactive	—	—
Citronellal[#]	Active	14.3[b]	14.3[b]

[#] Citronellal was used as a positive control.
* LC_{50} values followed by same letters within a column are not significantly different ($p < 0.05$) Duncan's multiple range test.
The highest dose tested was 20.15 mg (i.e. 80.6 mg/dm^3).

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1–4 and **10** and citronellal ranked the same at 48 h as for the 24 h exposure. Other analogues and allylbenzene **11** showed no mortality at the highest dose tested, i.e. 80.6 mg/dm^3, even at 48 h exposure (Table 3).

GC–MS analysis revealed that safrole (52.7%) was the major component present in the pilot plant distilled oil, followed by allylpyrocatechol diacetate (15.4%), eugenol (6.4%) and eugenyl acetate (5.8%). Fractionation of this oil using hexane, toluene and ethyl acetate in increasing polarity revealed that some of the compounds present in the oil as minor compounds could be enriched by fractionation. Fraction 1 showed sabinene (12.1%), α-selinene (8.2%), β-selinene (6.8%) and β-caryophyllene (6.4%) as the major components. Fraction 2 yielded safrole (34.3%), α-humulene (9.6%), sabinene (7.1%) and germacrene B (6.5%) as the major components. Fraction 3 yielded safrole at 98.7% purity, whereas fraction 4 yielded *n*-eicosane (80.0%) and *n*-docosane (8.2%) at high concentrations. Fraction 5 yielded 91.8% eugenol. Fraction 6 was rich in methyl eugenol (31.6%), eugenyl acetate (31.6%) and eugenol (22.1%). Fraction 7 yielded allylpyrocatechol diacetate (26.5%) as the major component, followed by eugenyl acetate (18.4%), terpinene-4-ol (17.5%) and methyl eugenol (6.9%). Fraction 8 yielded allylpyrocatechol monoacetate (23.0%), allylpyrocatechol diacetate (13.2%), α-terpineol (9.6%) and α-cadinol (6.4%) as the major components. Table 4 illustrates the chemical composition of the *P. betle* leaf oil and its fractions.

Discussion

The present study demonstrates that the essential oil of Sri Lankan *P. betle* leaf exhibits insecticidal activity on *M. domestica* adults. Citronella oil is used as an insecticidal agent against the adult angoumois grain moth, *Sitotroga cerealella* (Olivier)[12] and citronellal, which is one of the active constituents of citronella oil, is also insecticidal to adult *M. domestica* and the red flour beetle, *Tribolium castaneuma* (Herbst), and larvicidal to the southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber.[13]

In this study, *P. betle* leaf oil was shown to be more promising as an insecticide than Ceylon citronella oil, at 2.6 and 2.8 times more toxic at 24 and 48 h exposure periods to housefly adults. Since safrole (52.7%) and eugenol (6.4%) are both abundant in the oil and both show insecticidal activity on their own, they may account for the insecticidal activity of *P. betle* leaf oil. Safrole proved almost three times more toxic than citronellal (positive control) for both the 24 and 48 h exposure periods, while eugenol proved almost two and 2.3 times more toxic than citronellal for the same exposure periods, respectively. The lower LC_{50} values of fraction PBL/2 can also be attributed to its major component, safrole (34.3%), and the low LC_{50} values for fraction PBL/6 are probably due to presence of eugenol (22.1%) as one of the major components (Tables 1–4). Among the laboratory-prepared safrole analogues, isosafrole, dihydrosafrole and compound **4** showed insecticidal properties.

The studies on structure–activity relationships of analogues revealed isosafrole to be more active than safrole. This was also demonstrated by Huang *et al.*,[14] where isosafrole was found to be more toxic than safrole against adults of the maize weevil, *Sitophilus zeamais*, and *Tribolium castaneum*. Their conclusions are in agreement with ours on housefly adults. However, our studies have also shown dihydrosafrole and compound **4** as toxic products. The major structural difference between safrole and allylbenzene **11** is the presence of two additional oxygen atoms in the phenylpropanoid skeleton of safrole. Since the allylbenzene was not active at the highest dose tested, the presence of these two oxygen atoms in the phenyl propanoid skeleton is essential for the activity. Eugenol also has two oxygen atoms in its phenyl propanoid skeleton and is toxic to *M. domestica*, further confirming the necessity of two oxygen atoms for activity.

The mode of action of many insecticides is due to their interference with the functioning of the nervous system of insects. The primary toxic action of organophosphates and carbamates involves cholinesterase. In our study, the insecticidal action on *M. domestica* of isosafrole, safrole, dihydrosafrole and eugenol might be due to interference with the nervous system of the insect.

In the present study, we found safrole (52.7%), allylpyrocatechol diacetate (15.4%), eugenol (6.4%) and eugenyl acetate (5.8%) as the major components of the Sri Lankan *P. betle* leaf oil. In contrast, studies of Philippine, Indian, Vietnamese and Malayasian *P. betle* have indicated wide variation in the oil constituents. Published data on Philippine *P. betle* leaf oil show that it contains phenolic compounds, viz. chavibetol (53.1%) and chavibetol acetate (15.5%), as the major components. Other phenolic compounds of *P. betle* leaf oil included allylpyrocatechol monoacetate, allylpyrocatechol diacetate, eugenol, methyl eugenol, safrole and terpenes, viz. camphene, β-caryophyllene, 1,8-cineole, *p*-cymene, limonene, α-pinene and β-pinene.[15] The essential oil of *P. betle* leaves from southern India contain safrole (39.9%), eugenol (9.0%), allo-pyrocatechol monoacetate (8.5%) and terpinen-4-ol (6.3%) as the major constituents.[16] Sharma *et al.* reported eugenol at 82.2% and 90.5% and methyl eugenol at 6.9% and 4.1%, respectively, as the major components in *P. betle* cultivars originating from Desi Bangla and Ramtek Bangla, India, with *p*-cymene, α-terpineol and terpinyl acetate as minor components.[17] They also reported terpenyl acetate at 44.93% and 45.9% and eugenol at 26.65% and 28.29% in the essential oil of *P. betle* leaves from cultivars of Desi Desawari and Mahoba Desawari, India, respectively.[18] It is also reported that high percentages of eugenol, at 13.90%, 33.22%, 20.47%, 63.56% and

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Table 4. Chemical compositions of *Piper betle* L. leaf oil and its fractions

No.	Compound	Percentage (%, w/w)									Retention Index (RI)
		PBL Oil	PBL Fr. 1	PBL Fr. 2	PBL Fr. 3	PBL Fr. 4	PBL Fr. 5	PBL Fr. 6	PBL Fr. 7	PBL Fr. 8	
1.	α-Thujene	0.4	2.4	0.6	—	—	—	—	—	—	926
2	α-Pinene	0.5	3.6	0.4	—	—	—	—	—	—	933
3	Camphene	0.2	1.2	0.2	—	—	—	—	—	—	948
4	Sabinene	2.2	12.1	7.1	—	—	—	—	—	—	971
5	β-Pinene	0.1	0.3	trace	0.1	—	—	—	—	—	974
6	Myrcene	1.1	4.1	2.0	—	—	—	—	—	—	987
7	α-Phellandrene	0.1	0.7	0.2	—	—	—	—	—	—	1005
8	(*Z*)-3-Hexenyl acetate	—	—	—	—	—	1.2	—	—	—	1004
9	1,4-Cineole	—	—	—	—	—	—	0.6	—	—	1014
10	α-Terpinene	0.5	4.8	2.3	0.1	—	—	—	—	—	1013
11	*p*-Cymene	0.5	2.6	3.9	—	—	—	—	—	—	1025
12	β-Phellandrene	0.7	4.3	1.8	—	—	—	—	—	—	1029
13	Benzylalcohol	—	—	—	—	—	—	—	—	0.1	1031
14	1,8-Cineole	0.1	—	—	—	—	—	0.5	3.6	—	1034
15	(*Z*)-β-Ocimene	—	0.1	0.1	—	—	—	—	—	—	1041
16	(*E*)-β-Ocimene	trace	0.4	0.3	—	—	—	—	—	—	1054
17	γ-Terpinene	0.9	5.1	3.0	0.2	—	—	—	—	—	1065
18	*cis*-Sabinene hydrate	—	—	—	—	—	—	—	0.7	5.1	1070
19	Fenchone	—	—	—	—	—	—	—	5.9	5.6	1090
20	Terpinolene	0.2	1.8	1.1	trace	—	—	—	—	—	1097
21	2-Nonanone	—	—	—	—	—	—	0.5	—	—	1099
22	*trans*-Sabinene hydrate	trace	—	—	—	—	—	—	0.9	1.6	1106
23	Linalool	0.2	—	—	—	—	—	—	—	—	1111
24	*p*-Menta-1,3,8-triene	—	trace	trace	—	—	—	—	—	—	1115
25	*cis*-*p*-Menth-2-en-1-ol	—	—	—	—	—	—	—	—	3.6	1132
26	*cis*-Limonene oxide	—	—	—	—	—	trace	0.2	—	—	1140
27	Benzyl acetate	—	—	—	—	—	trace	—	—	—	1155
28	Terpinen-4-ol	2.2	—	—	—	—	—	—	17.5	1.9	1178
29	*m*-Cymen-8-ol	—	—	—	—	—	—	—	—	0.4	1180
30	α-Terpineol	0.1	—	—	—	—	—	—	0.1	9.6	1190
31	*cis*-Piperitol	trace	—	—	—	—	—	—	0.33	0.15	1193
32	n-Decanal	—	—	—	—	—	trace	—	—	—	1206
33	*trans*-Piperitol	trace	—	—	—	—	—	—	0.6	—	1208
34	*cis*-Sabinene hydrate acetate	—	—	—	—	—	trace	—	0.2	—	1223
35	*trans*-Sabinene hydrate acetate	—	—	—	—	—	1.08	—	—	—	1258
36	Chavicol	—	—	—	—	—	—	—	0.2	—	1259
37	2-Phenyl ethyl acetate	—	—	—	—	—	—	2.0	0.1	—	1264
38	Safrole	52.7	—	34.3	98.7	—	—	—	—	—	1296
39	*p*-Cymen-7-ol	—	—	—	—	—	—	—	—	0.10	1298
40	Thymol	—	—	—	—	—	trace	—	—	—	1301
41	2-Undecanone	—	—	—	—	—	—	1.9	—	—	1303
42	*iso*-Ascaridole	—	—	—	—	—	—	—	—	0.1	1305
43	*cis*-Piperitol acetate	—	—	—	—	—	trace	—	—	—	1332
44	5-Indanol	—	—	—	—	—	trace	—	—	—	1335
45	δ-Elemene	—	0.4	0.1	—	—	—	—	—	—	1339
46	α-Terpinyl acetate	trace	—	—	—	—	1.3	—	—	—	1348
47	α-Cubebene	0.1	0.9	0.1	—	—	—	—	—	—	1349
48	Eugenol	6.4	—	—	—	—	91.8	22.1	1.3	0.4	1361
49	n-Undecanol	—	—	—	—	—	—	—	—	0.1	1372
50	Isoledene	—	0.1	—	—	—	—	—	—	—	1373

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Table 4. (*Continued*)

No.	Compound	Percentage (%, w/w)									Retention Index (RI)
		PBL Oil	PBL Fr. 1	PBL Fr. 2	PBL Fr. 3	PBL Fr. 4	PBL Fr. 5	PBL Fr. 6	PBL Fr. 7	PBL Fr. 8	
51	(*E*)-Isosafrole	—	—	—	0.2	—	—	—	—	—	1374
52	α-Copaene	0.5	3.6	0.3	—	—	—	—	—	—	1376
53	(*E*)-β-Damascenone	—	—	—	—	—	—	2.2	—	—	1378
54	β-Bourbonene	0.2	1.3	0.2	—	—	—	—	—	—	1381
55	β-Elemene	0.2	2.2	1.7	—	—	—	—	—	—	1386
56	Vanillin	—	—	—	—	—	—	—	—	0.1	1388
57	Methyl eugenol	0.7	—	—	—	—	—	31.6	6.9	—	1401
58	β-Caryophyllene	1.2	6.4	3.4	—	—	—	—	—	—	1413
59	(*E*)-α-Ionone	—	—	—	—	—	—	trace	—	—	1424
60	β-Gurjunene	0.1	1.3	0.2	—	—	—	—	—	—	1428
61	γ-Elemene	—	—	0.2	—	—	—	—	—	—	1430
62	Neryl acetone	—	—	—	—	—	—	trace	—	—	1432
63	Aromadendrene	—	0.4	0.1	—	—	—	—	—	—	1436
64	α-Humulene	0.8	2.6	9.6	0.1	—	—	—	—	—	1452
65	(*Z*)-Methyl isoeugenol	—	—	—	—	—	—	trace	—	—	1456
66	*cis*-Muurola-4(14)-5-diene	—	0.2	—	—	—	—	—	—	—	1472
67	γ-Muurolene	0.8	4.4	1.7	—	—	—	—	—	—	1477
68	Germacrene D	0.6	4.6	5.3	—	—	—	—	—	—	1482
69	β-Selinene	1.2	6.8	3.0	—	—	—	—	—	—	1487
70	*Epi*-Cubebol	—	—	—	—	—	—	—	0.4	—	1495
71	α-Selinene	1.4	8.2	4.6	—	—	—	—	—	—	1497
72	*trans*-β-Guaiene	0.3	—	1.9	—	—	—	—	—	—	1501
73	Cuparene	—	—	—	trace	—	—	—	—	—	1502
74	Germacrene A	—	trace	0.9	—	—	—	—	—	—	1507
75	Cubebol	—	—	—	—	—	—	—	0.4	—	1518
76	7-*epi*-α-Selinene	0.2	—	0.5	—	—	—	—	—	—	1519
77	δ-Cadinene	0.5	4.0	1.4	—	—	—	—	—	—	1521
78	Hydroxy chavicol	—	—	—	—	—	—	—	—	4.7	1522
79	Allylpyrocatechol monoacetate	—	—	—	—	—	—	—	—	23.0	1530
80	Eugenyl acetate	5.8	—	—	—	—	—	31.6	18.4	—	1531
81	Cadina-1,4-diene	—	0.3	0.1	—	—	—	—	—	—	1534
82	α-Cadinene	—	0.4	0.1	—	—	—	—	—	—	1538
83	Selina-3,7 (11)-diene	—	0.3	—	—	—	—	—	—	—	1546
84	Germacrene B	0.3	0.7	6.5	0.2	—	—	—	—	—	1557
85	(*E*)-Nerolidol	—	—	—	—	—	—	1.3	—	—	1560
86	Spathulenol	trace	—	—	—	—	—	—	1.2	—	1577
87	β-Caryophyllene oxide	—	—	—	—	—	—	—	1.1	—	1582
88	Globulol	trace	—	—	—	—	—	—	0.9	4.5	1583
89	Humulene epoxide II	—	—	—	—	—	—	—	0.5	—	1605
90	1-*epi*-Cubenol	trace	—	—	—	—	—	—	1.6	—	1626
91	Cubenol	—	—	—	—	—	—	—	trace	—	1642
92	Allylpyrocatechol diacetate	15.4	—	—	—	—	—	—	26.5	13.2	1647
93	α-Muurolol	—	—	—	—	—	—	—	—	4.9	1652
94	α-Cadinol	trace	—	—	—	—	—	—	—	6.4	1660
95	n-Eicosane	trace	—	—	—	80.0	—	—	—	—	1994
96	n-Docosane	trace	—	—	—	8.2	—	—	—	—	2193

PBL Oil – *Piper betle* leaf oil (pilot plant steam distillation)
PBL Fr. – *Piper betle* leaf oil (pilot plant steam distillation) fractions

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18.92%, occur in five cultivars of *P. betle* grown in India, named Sanchi, Kapoori, Desawari, Bangla and Meetha, respectively. Anethole (19.31%) and *cis*-caryophyllene (10.64%) were high in 'Meetha', eugenol acetate (18.68%) rich in 'Bangala' and isoeugenol (10.59%) abundant in 'Kapoori'. The major constituent in 'Desawari' and 'Sanchi' is reported to be 1,3-benzodioxole (5)-2-propenyl, with 45.34% and 22.75%, respectively. Among the other cultivars, 'Sanchi' is also reported to have stearaldehyde (2.69%), which is unique to this cultivar and absent from the other cultivars.[19] Essential oil from mature leaves of the *P. betle* cultivar Sagar Bangla grown in India has chavicol (47.81%) as the major constituent.[20] The essential oil obtained from the rhizomes of *P. betle* collected around the Hue area, Vietnam, was reported to have more than 40 compounds, of which the major ones were α-cadinol (26.2%), δ-cadinene (11.7%), and T-cadinol and T-muurolol (20.7%).[21] The essential oil of *P. betle* flowers contains mainly safrole (27.6%) and myrcene (26.4%), along with hydroxychavicol, eugenol, isoeugenol and methyl eugenol as minor components.[22] The chemical composition of the leaf oil of *P betle* collected at Masjid Tanah, Melaka, Malayasia, is reported to have chavibetol (69.0%), eugenyl acetate (8.3%) and chavicol (6.0%) as the major components.[23]

We believe the variation in the chemical composition of these *P. betle* oils to be due to differences in geographical conditions of growth. Insecticidal activity of the oils obtained from other countries may not to be the same as the Sri Lankan *P. betle* leaf oil reported in the present study.

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