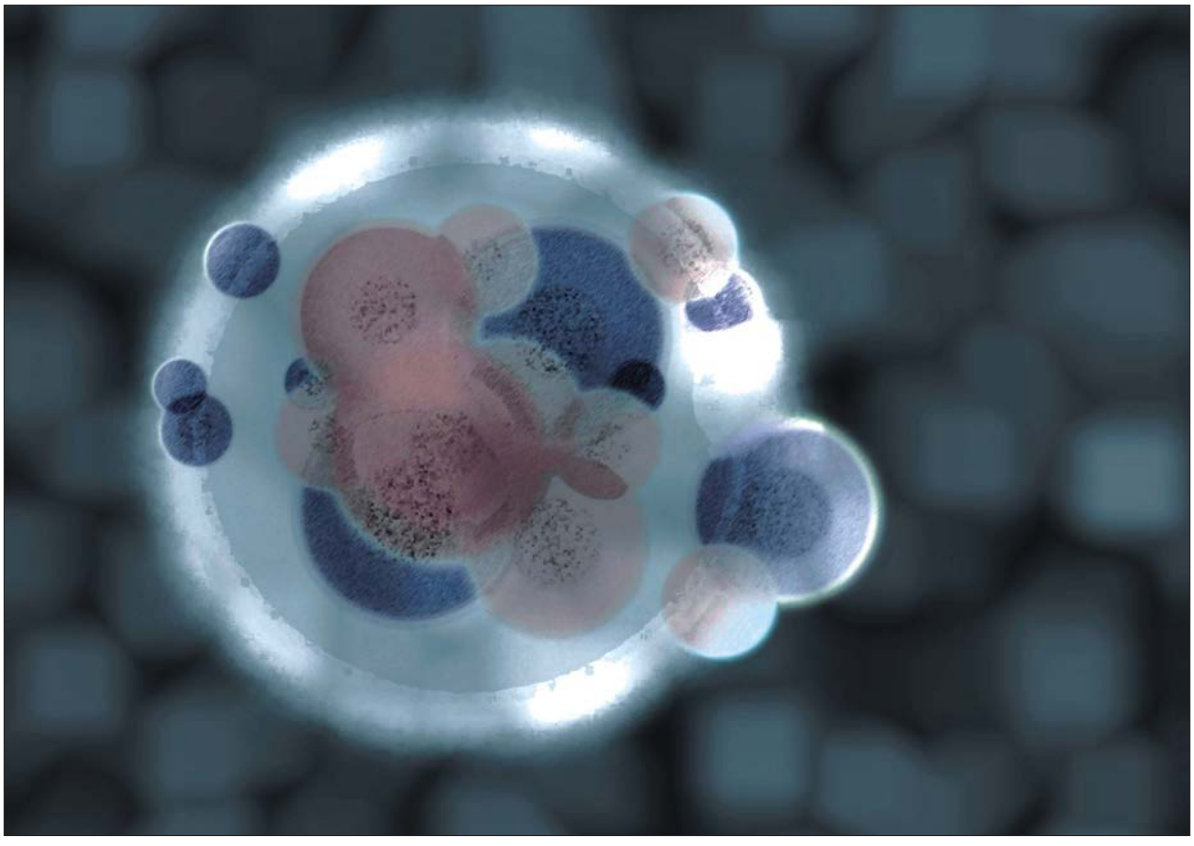

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Antimicrobial Activity of *Cinnamomum impressicostatum* and *C. pubescens* and Bioassay-Guided Isolation of Bioactive (E)-Methyl Cimamate

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Abstract: The air dried barks of *Cinnamomum impressicostatum* and *C. pubescens* were separately and sequentially extracted with hexane, chloroform and methanol and assayed against five bacteria and four fungi by disc diffusion method. The hexane and chloroform extracts of the plants strongly suppressed the growth of the four fungi but range from moderate to weak towards bacteria. The methanol extracts only displayed weak activity on some of these organisms. Based on these results, further work was carried on bioassay-guided isolation of active extracts of *C. impressicostatum* and *C. pubescens* and on both occasions the active component was identified as (E)-methyl cinnamate. Other compounds were also isolated and identified as safrole, (E)-piperonylprop-2-enal, β-sitosterol, (E)-piperonylprop-2-enol and cinnamic acid. The structures of the compounds were established by spectroscopic method and comparison with literatures. (E)-methyl cinnamate exhibited strong growth inhibition towards four of the fungi *Saccharomyces cerevisiae*, *Candida lipolytica*, *C. albicans*, *Microsporum canis* with inhibition zone ranging from 24.5 to 19.8 mm. The compound is also capable of suppressing the growth of bacteria *Bacillus cereus*. It is proposed that the excellent antifungal activities of the hexane and chloroform extracts of *C. impressicostatum* and *C. pubescens* correlate with the presence of (E)-methyl cinnamate as the major component.

Key words: *Cinnamomum impressicostatum*, *Cinnamomum pubescens*, bioassay-guided, antimicrobial, (E)-methyl cinnamate

INTRODUCTION

The genus *Cinnamomum* comprises between 250-300 species occurring mostly in tropical Asia and Pacific. About 25 species have been recognized in Peninsular Malaysia and locally the plant is known as medang or medang teja (Kochummen, 1989). It is an evergreen or deciduous shrub or small to large tree which can grow up to 50 m tall. The ethnobotanical uses of *Cinnamomum* in Malaysia have been reported previously (Perry, 1980; Burkhill, 1966). In traditional medicine preparation, the plant has been widely used for the treatment of spasmodic and chronic diarrhea. The roots and leaves were also administered to women as a postpartum medicine and the poultice for the treatment of rheumatism.

Early chemical studies on the *Cinnamomum* species were primarily focused on the identification of their essential oils constituents (Brophy *et al*., 2001; Chalchat and Valade, 2000; Jantan and Goh, 1992). Some of the oils have been reported to be good source of antifungal and antibacterial compounds (Mastura *et al*., 1999; Nor Azah *et al*., 2002). The oils are widely known as sources of aroma chemicals and used in food preparation, pharmaceutical products, cosmetics, toiletries and detergents. Previous phytochemical works on the *Cinnamomum* species revealed the presence of a number of aromatic compounds such as flavanols, phenyl propanoids, lignans, terpenoids, alkaloids and proanthocyanadins (Mukherjee *et al*., 1994; Wu *et al*., 1994). During the past two decades, incidences of bacterial and fungal infections in patients suffering from AIDS, pneumonia and those undergoing chemotherapy and surgery has increased tremendously (Zacchino *et al*., 1999). Fungal infections are caused by dermatophytes and pathogenic fungi such as *Candida albicans* and frequently very difficult to eradicate. Development of resistance of pathogens to many antibiotics in use today is a strong reminder that new anti-infective agents are needed to combat infection. In this study six extracts of *C. impressicostatum* and *C. pubescens* and were evaluated for their antimicrobial activity against five bacteria and four fungi species by disc diffusion method. The active extracts of the plants were further investigated to identify the active components by bioassay-guided isolation work and evaluate their antibacterial and antifungal properties. Previous study on the plants has concentrated mainly of the essential oils and their antifungal activity (Jantan *et al*., 1993). Both plants species are small trees which can grow to just 7 m tall and *C. pubescens* is endemic in Peninsular Malaysia.

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MATERIALS AND METHODS

General experimental procedures: Melting points were measured on a Kofler hot stage apparatus and are uncorrected. The IR spectra were recorded using KBr discs on Perkin Elmer FTIR spectrophotometer model 1275X. The UV spectrum was recorded on a Shimadzu UV 160A spectrophotometer in MeOH. The ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a JOEL FTNMR 400 MHz spectrometer operating at 400 and 100 MHz, respectively. Chemical shifts are shown in δ values (ppm) with tetramethylsilane as an internal standard. Qualitative analysis by GC and GC/MS were performed on Shimadzu GC and Hewlett-Packard GCMSD apparatus with SE-30 capillary column.

Test organisms: Nine test organisms were used in the study. The bacterial strains used were *Straphylococcus aureus*, *S. epidermidis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*. The four fungal strains used were yeast-like fungi *Candida albicans*, *C. lipolytica*, *Saccharomyces cerevisae* and *Microsporum canis*. *M. canis* was obtained from the American Type Culture Collection (ATCC) while the rest of the strains were obtained from Northern Regional Research Laboratories (NRRL) and College of Pharmacy, University of Iowa. The strains were cultured at the Animal Tissue Culture Laboratory, Universiti Putra Malaysia. The bacteria and fungi were maintained on nutrient agar and potato dextrose agar slants, respectively and stored at 4°C.

Disc diffusion assay: The antimicrobial activities of the samples were determined by the disc diffusion method (Bauer *et al*., 1966). Antibiotic discs of 6 mm diameter were prepared by impregnating them with 5 μL of each sample. The disc were then dried under sterile condition and then placed on the agar plate previously inoculated with the suspension of each microorganism to be tested. Streptomycin and nystatin were used as standard and methanol as negative control. The agar plates were then incubated for 24 h at 30°C and the antimicrobial activities were recorded my measuring the zone of inhibition around each disc. The experiments were carried out in triplicates and averaged.

Plant material and isolation of compounds: The barks of *C. impressicostatum* and *C. pubescens* were collected from Bukit Larut, Taiping and Gunung Berembun, Cameron Highlands, Perak in 1999, respectively. Voucher specimen were deposited at the Herbarium, Forest Research Institute Malaysia, Kepong, Malaysia.

Extraction of the plant samples: The air-dried plant samples were extracted exhaustively and separately with hexane, chloroform and methanol each over a period of two weeks. The extracts were then concentrated under reduced pressure and stored in freezer until required for analysis. The hexane, chloroform and methanol extraction of bark of *C. impressicostatum* (1092.00 g) gave 9.20, 14.34 and 37.55 g of dark viscous extracts after solvent removal, respectively (Table 1). While similar extraction on *C. pubescens* (1962.44 g) gave 15.25, 70.48 and 116.05 g of crude extracts, respectively.

Bioassay-guided isolation: These extracts were evaluated for antimicrobial activity by disc diffusion method. The hexane and chloroform extracts of *C. impressicostatum* and *C. pubescens* inhibited the growth of fungi strongly. Therefore, both the hexane and chloroform extracts were subjected to silica gel column chromatography separately. For the *C. pubescens* hexane extract, the column was eluted with gradient hexane and ethyl acetate and 83 fractions of 100 mL each were collected. Fractions showing similar TLC profiles were bulked to give F1 (fractions 1-7), F2 (7-15), F3 (15-19), F4 (19-26), F5 (26-34), F6 (34-41), F7 (42-45), F8 (46-51) F9 (51-55), F10 (55-66), F11 (67-73) and F12 (73-83). These fractions were tested for antimicrobial activity by disc diffusion method and the active fractions F2, F4, F5, F7, F8, F9 and F10 were determined (Table 2). Further chromatographic isolation of F4 and F5 yielded safrole (1, 28 mg) as yellowish viscous oil. The more active fractions (F8, F9 and F10) were further isolated by column chromatography. Both fractions F8 and F9 yielded (E)-methyl cinnamate (2, 60 mg) as white solid which on recrystallization gave white needles, m.p 36°C. Similarly, fraction F10 was further fractionated by silica gel chromatography eluted with hexane-ethyl acetate to afford 50 fractions. Fractions 22-32 and 34-40 were purified by preparative TLC (100% chloroform) to give another batch of (E)-methyl cinnamate

Table 1: The extracts of *C. impressicostatum* and *C. pubescens*

Plant species	Voucher No.	Sample weight (g)	Solvent used	Weight of extracts (g)
C. impressicostatum (bark)	FRI42962	1092	Hexane	9.2
			Chloroform	14.34
			Methanol	37.55
C. pubescens (bark)	FRI42965	1962.4	Hexane	15.25
			Chloroform	70.48
			Methanol	116.05

Table 2: Inhibition zone (in mm) of various samples *Cinnamomum pubescens* against bacteria and fungi

Samples	Part	Extract	Sa	Se	Bcr	Pa	Bce	Sc	Cl	Ca	Mc
C. impressicostatum	Bark	Hexane	11.1	10.2	9.3	8.5	7	-	15.3	20.3	18.5
		$CHCl_3$	13.8	15.3	10.5	10.7	11.6	11	16.6	18.3	23.4
		MeOH	10.8	8.8	11.1	9.8	10.5	-	-	-	-
C. Pubescens	Bark	Hexane	10.8	6.3	8.2	6	6.2	18	20.3	15	20.4
		$CHCl_3$	10.8	11.3	13.5	11.3	6.5	16.7	23.9	-	33.4
		MeOH	6.9	9.6	12.2	7.9	-	7.9	-	-	8.2
Sterptomycin			14.8	15.2	18.1	16.7	13.8	-	-	-	-
Nystation			-	-	-	-	-	11.5	21.4	18.8	21.3
Hexane extract											
F2			-	-	-	-	-	-	6.5	6.2	6.2
F4			-	-	-	-	-	-	6.5	6.7	6
F5			-	-	-	-	-	12.5	16.4	12.1	14.9
F7			6.7	-	12.8	-	-	7.8	7.8	9.1	8.3
F8			9.6	-	16.7	-	-	17.9	12	12.1	17
F9			10	-	19.4	-	-	19.1	15	16	19.1
F10			14.3	-	21.9	-	-	19.4	18.6	22.2	17.8
$CHCl_3$ extract											
F1-11			-	-	6.7	-	-	6	9.2	10.4	-
F12-20			6.5	6.1	19.3	7.9	9.8	23.9	20.2	24.4	8.3
F21-40			-	7.2	16.8	8.6	-	12.2	9	12.5	-
F41-60			-	-	6.1	-	-	6	8.3	-	-
F61-78			-	6.1	6.8	7.5	-	-	-	11.6	-
F79-88			-	-	-	-	-	-	-	-	
F89-130			-	8.6	-	9.6	-	9.7	-	0	-
Safrole (1)			-	-	-	-	-	5.8	9.5	9.2	-
(E)-methyl ciunamate (2)			7	6	18.8	-	-	22.6	21.1	24.5	19.8
Commercial (E)-methyl cinnamate (2)			7.7	6	17.5	-	6.5	15.2	17.7	14.8	14.6
(E)-piperonylprop-2-enal (3)			-	-	6	-	6	-	8.6	-	-
β-Sitosterol (4)			-	-	-	-	-	-	-	-	-
Streptomycin			13.4	16.8	16.3	27.4					
Nystatin							15	18.5	19.3	19.9	19.5

Sa: *Staphylococcns aurens*; Se: *S. epidermis*; Bcr: *Bacillns cerens*; Pa: *Pseudomonas aeruginosa*; Bce: *Burkholderia cepacia*; Sc: *Saccharomyces cerevisiae*; Cl: *Candida lipolytica*; Ca: *C. albicans*; Mc: *Microsporum canis*

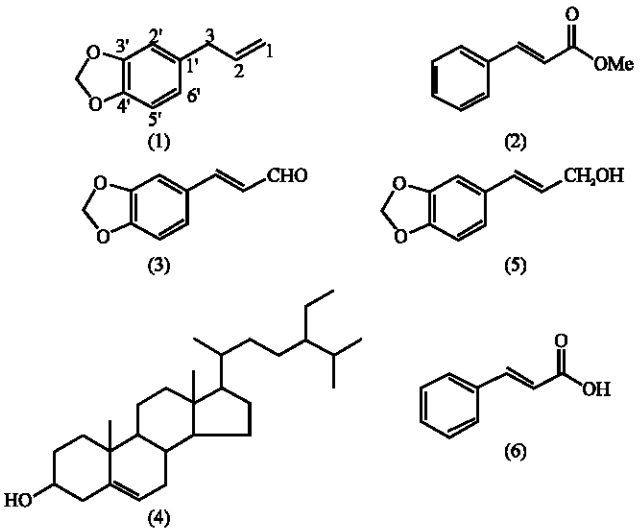

Fig. 1: The component, (1) safrole, (2) (E)-methyl cinnamate, (3) (E)-piperonylprop-2-enal, (4) β-sitosterol, (5) (E)-piperonylprop-2-enol and (6) cinnamic acid

(2, 72 mg) and (E)-piperonylprop-2-enal (3, 46 mg) as white solid. The later compound was recrystallized with chloroform to afford white shiny needle, m.p. 83-85°C (Parsons *et al*., 1993). β-Sitosterol (4, 55 mg) was obtained from fractions 42-48 as white needles (Fig. 1).

The chloroform extract of *C. pubescens* (10.0 g) was subjected to flash column chromatography using gradient mixture of hexane and ethyl acetate to give 130 fractions of 100 mL each. Based on TLC profiles, the fractions were bulked and selected fractions (F1-11, F12-20, F21-40,

F41-60, F 61-78, F79-88 F89-130) were subjected to disc diffusion assay. The more active fractions (F12-20 and F21-40 were repeatedly separated by column chromatography and preparative TLC and eluting with various mixture of hexane and ethyl acetate. Safrole (1, 15 mg), (E)-methyl cinnamate (2, 47 mg) and (E)-piperonylprop-2-enal (3, 21 mg) were obtained from fraction F12-20. From fraction F21-40, β-sitosterol (4, 39 mg) and (E)-piperonylprop-2-enol (5, 16 mg) were obtained as brownish oily liquid. The various UV, IR, MS, NMR spectral data of the compounds were obtained, analyzed and compared with reported data (detailed spectral data could be obtained from the author).

Bioassay-guided isolation work on the hexane extract of *C. impressicostatum* was also carried out with the isolation of similar antimicrobial (E)-methyl cinnamate (2) and three other components safrole (1), (E)-piperonylprop-2-enal (3) and cinnamic acid (6) (Fig. 1).

RESULTS

The growth of the four fungi species were strongly suppressed by the hexane extracts of *C. pubescens* with inhibition zone range from 15.0 to 20.4 mm. However, the extract only exhibited moderate to weak activity towards bacteria. Similar trend was observed for the chloroform extract. Both the hexane and chloroform extracts of *C. impressicostatum* were strongly active when tested against three of the fungi, *Candida lipolytica*, *C. albicans*, *Microsporum canis*, but moderately active and inactive towards *Saccharomyces cerevisiae*, respectively. Moderate inhibition activity was also observed for chloroform extract against the bacteria. By using bioassay-guided isolation technique, the hexane and chloroform extracts of extracts *C. pubescens* furnished six compounds with (E)-methyl cinnamate as the most active component and the structure of the compounds were elucidated by detail spectroscopic method and comparison with literature values. For the pure isolated (E)-methyl cinnamate, the compound displayed excellent activity against the tested fungi with inhibition zone range from 24.5 to 19.8 mm. (E)-Piperonylprop-2-enal (3) only gave very weak inhibition on the growth of two of the bacteria and one of the fungi. β-Sitosterol (4) failed to exhibit any activity to both microbes (Table 2).

DISCUSSION

Both the hexane and chloroform extracts of barks of *C. impressicostatum* and *C. pubescens* were found to be active against all microbes tested especially towards fungi *S. cerevisiae*, *C. albicans*, *C. lipolytica* and *M. canis* by using disc diffusion method (Table 2). The activities are comparable with nystatin standard used. However, compared to standard streptomycin, the activities of the extracts against bacteria were much weaker. In the case of methanol extract, it is only selectively active and the quantum of activity is much lower. Having established the active extracts, the hexane and chloroform extracts of both plants were selected for further chromatographic isolation. Selected fractions from these were subjected to bioassay-guided isolation work. Further isolation of fractions F4 and F5 yielded safrole (1) as yellowish oil. Based on GC and GC-MS analysis, fractions F8, F9, F10, F12-20 and F21-40 were found to contain one major component. Bioassay-guided fractionation of fraction F8, F9 and F10 led to the isolation of four compounds (2, 3, 4), with compound (2) as the major component.

The spectral data of (1) revealed that the compound was safrole previously isolated from many plant species including *Cinamomum petrophilum*, *C. mollissimumand Piper hispidinervum* (Rocha and Lin, 1999). The compound was weakly active towards three of the fungi. The major component of the extract was compound (2) and the spectral data established the structure as (E)-methyl cinnamate (Bauer *et al*., 1990). The compound was isolated from both plant extracts and exhibited strong inhibition on the growth of all four fungi *S. cerevisiae*, *C. albicans*, *C. lipolytica* and *M. cani* with inhibition zone ranging from 24.5 to 19.8 mm. The compound also suppressed the growth of one of the bacteria (*Bacillus cereus*) with inhibition zone of 18.8 mm. These results are comparable with nystatin used as standard for antifungal property. The minimum inhibitory concentration of the compound using the broth microdilution method was also determined and it displayed MIC values of 1.25 μg μL^{-1} for all the fungi tested and 5.00 μg μL^{-1} for *B. cereus*. While we were preparing this manuscript, another article appeared which reported similar findings on the antimicrobial activity of commercial (E)-methyl cinnamate, but the test was carried out against *E. coli*, *B. subtilis*, *S. aureus* and *C. albicans* (Huang *et al*., 2009). In the current study, similar result was obtained towards *C. albicans*, but the compound was found to be weakly active against *S. aureus*. (E)-methyl cinnamate can be found it many other plant species and used in many application, as an ingredient in decorative cosmetics, fine fragrances, shampoos, toilet soaps and many other toiletries as well in non-cosmetic products (Rosas *et al*., 2005; Gupta, 1996; Bhatia *et al*., 2007). Various studies have been conducted to assess the toxicological and dermatological properties of the compound as an ingredient in fragrances (Belsito *et al*., 2007). The third

compound isolated was identified as (E)-piperonylprop-2-enal (3) by analyzing the spectral data acquired and compared with literature values. It was obtained as white needles with melting point of 83-85°C (Parsons *et al*., 1993, m.p. 84-85°C). The compound was only weakly capable of suppressing the growth of certain bacteria and fungi, while β-sitosterol (4) failed to exhibit any activity at all. β-Sitosterol (4) is widely distributed in many plant species and can be obtained quite easily from their extracts.

Chromatographic separation of most active fractions F12-20 and F21-40 from the chloroform extract afforded the same four compounds described above and a brownish oily liquid established as (E)-piperonylprop-2-enol (5). Similarly, the structure of the compound was elucidated by spectral data analysis. This is a reduced form of the above aldehyde (3). Due to insufficient amount of sample isolated, the growth inhibition property of (3) against the microbes was not performed. Bioassay-guided chromatographic separation work was also conducted on the hexane extract of the leaves of *C. pubescens* to afford (E)-methyl cinnamate (2) as the most active component together with safrole (1), (E)-piperonylprop-2-enal (3) and cinnamic acid (6). The occurrence of (E)-piperonylprop-2-enal (3) was reported for the first time in the genus *Cinnamomum* although it has been isolated and identified previously from *Brombya platynema*. The assay results showed that (E)-methyl cinnamate (2) contributed to the overall antifungal activity of the hexane and chloroform extracts.

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