Antifungal Activity of the Bark and Leaf Oils of *Cinnamomum verum* J.S. Presl. Alone and in Combination against Various Fungi

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ABSTRAK

Minyak daun dan kulit Cinnamomum verum J.S. Presl. telah dikaji aktiviti antifungal mereka ke atas 6 dermatofit (Trichophyton rubrum, T. mentagrophytes, T. tonsurans, Microsporum canis, M. gypseum, M. audouini), satu fungus filamentus (Aspergillus fumigatus) dan 5 jenis yis (Candida albicans, Ca. glabrata, Ca. tropicalis, Ca. parapsilosis dan Crytococcus neoformans) dengan menggunakan kaedah pencairan mikro kaldu. Aktiviti antifungal 4 sebatian piawai (sinamaldehid, eugenol, linalol dan α-terpineol) yang merupakan komponen utama dalam minyak-minyak ini telah dikaji dalam usaha untuk mengkorelasi efikasi minyak dengan komponen tersebut. Kesan kombinasi antifungal minyak pati terhadap M. canis, M. gypseum dan Cr. neoformans telah dikaji dengan assai checkerboard. Isobologram disediakan dan indeks Fractional Inhibitory Concentrations (FICI) dikira untuk menentukan kesan kombinasi antara minyak pati. Komposisi kimia minyak pati dianalisis dengan kromatografi gas (KG) dan kromatografi gas spektroskopi jisim (KG-SJ). Minyak-minyak pati ini menunjukkan aktiviti yang kuat ke atas kesemua fungus dengan nilai Minimum Inhibition Concentration (MIC) dari 0.04 hingga 0.31 mg/ml. Sinamaldehid sebagai komponen terbanyak dalam minyak kulit C. verum menunjukkan aktiviti yang terkuat ke atas semua fungus yang dikaji. Berdasarkan hasil assai ke atas sampel piawai, aras sinamaldehid dan eugenol yang tinggi dalam minyak pati dan percampuran dengan komponen minor mungkin bertanggungjawab ke atas aktiviti antifungal yang kuat. Kesan antifungal campuran minyak daun dan kulit C. verum didapati tidak sinergistik terhadap fungus kajian. Walau bagaimanapun kesannya adalah additif ke atas M. gypseum dan antagonistik ke atas Cr. neoformans dan M. canis.

Kata kunci: *Cinnamomum verum, minyak pati, aktiviti antifungal, dermatofit, yis, sinamaldehid, eugenol*

ABSTRACT

The leaf and bark oils of Cinnamomum verum J.S. Presl. were examined for their antifungal activity against 6 dermatophytes (Trichophyton rubrum, T. mentagrophytes, T. tonsurans, Microsporum canis, M. gypseum and M. audouini), one filamentous fungi (Aspergillus fumigatus) and 5 strains of yeasts (Candida albicans, Ca. glabrata, Ca. tropicalis, Ca. parapsilosis and Crytococcus neoformans) by using the broth microdilution method. The antifungal activities of 4 standard compounds (cinnamaldehyde, eugenol, linalool and α-terpineol) which were major constituents in the oils were also investigated in an effort to correlate the effectiveness of the oils with those of the components of the oils. The combined antifungal effect of the oils against M. canis, M. gypseum and Cr. neoformans was investigated by the checkerboard assay. Isobolograms were constructed and Fractional Inhibitory Concentrations Index (FICI) were calculated to determine the combination effects between the oils. The chemical composition of the oils was analyzed by gas chromatography (GC) and gas

chromatography- mass spectrometry (GC-MS). The oils showed strong activity against all the tested fungi with Minimum Inhibition Concentration (MIC) values ranging from 0.04 to 0.31 mg/ml. Cinnamaldehyde which was the most abundant component of the bark oil of C. verum showed the strongest activity against all the fungi studied. Based on the results of the assay on standard samples, it may be that the high levels of cinnamaldehyde and eugenol in the oils and in combination with the minor components could be responsible for the high antifungal activity of the oils. The antifungal effect of the leaf and bark oils of C. verum in combination against the tested fungi was not synergistic. However, the effect was additive against M. gypseum and antagonistic against Cr. neoformans and M. canis.

Key words: Cinnamomum verum, essential oils, antifungal activity, dermatophytes, yeasts, cinnamaldehyde, eugenol

INTRODUCTION

Increased fungal infections, toxicity of some antifungal agents and their interaction with other drugs, and development of resistance of some species of fungi have led many studies to search for new antifungal agents (Shahi et al. 1999; Dismukes 2000). Plants are a potential source for this purpose as they produce a variety of low molecular weight metabolites which contribute in protecting them from microbial infections (Clark & Walker 2000; Harborne 1999). Antifungal properties of a large number of essential oils and their constituents against various species of fungi have been reported (Hammer 2003; Suresh et. al. 1997; Ibrahim et. al. 2003; Cimanga et. al. 2002; Guynot 2003). However, many essential oils are only fungistatic and their activity against human pathogenic fungi is generally milder than the commercial synthetic antifungal drugs (Shin & Kang 2003).

The combined antifungal effect of different essential oils has been evaluated recently for potential synergistic effect. The synergistic antifungal activity of tea tree and lavender oils against *Trichophyton* species has been demonstrated (Cassella et al. 2002). The combination of essential oils with synthetic agents to improve efficacy besides reducing toxicity and development of resistance has been widely investigated. Santolina oil exhibited synergistic effect with clotrimazole against *Candida albicans* (Suresh et al. 1997). *Agastache rugosa* oil and its main component, estragole, enhanced the antifungal activity of ketoconazole against *Blastoschyzomyces capitatus* (Shin & Kang 2003). The essential oil of *Pelargonium graveolens* and its main components showed synergistic activity with ketoconazole against *Trycophyton* spp. (Shin & Lim 2004).

Cinnamomum verum J.P. Presl. (Family: Lauraceae) is well known since ancient time for its fragrance and medicinal value. Its bark is commercially traded as cinnamon. *C. verum* is indigenous in Sri Lanka and is also found in south India and is cultivated in Malaysia (Jain 1983; Jaganath & Ng 2000). The antifungal activities of *C. verum* and its main component cinnamaldehyde against various fungi have been evaluated (Mahmoud 1994; Hicham et. al. 1999; Ranasinghe et al. 2002). However, the antifungal combination effects of the leaf and bark essential oils of *C. verum* against dermatophytes and yeasts have not been reported until now.

In this study the antifungal activity of the bark and leaf oils of *C. verum* J.S. Presl. and their main components, cinnamaldehyde, eugenol, linalool and α-terpineol was evaluated against six dermatophytes (*Trichophyton mentagrophytes, Tr. tonsurans, Tr. rubrum, Microsporum canis, Mc. gypseum* and *Mc. audouinii*), five isolates of yeast-like

fungi (*Candida albicans, Ca. glabrata, Ca. parapsilosis, Ca. tropicalis and Cryptococcus neoformans*) and one filamentous fungi (*Aspergillus fumigatus*), in an effort to correlate the effectiveness of the oils with those of the components of the oils. The chemical composition of the essential oils was analysed by GC and GC-MS. The antifungal effect of the oils in combination against *M. canis*, *M. gypseum* and *Cr. neoformans* was also evaluated.

MATERIALS AND METHODS

PLANT MATERIAL

The ground leaves and bark of *C. verum* were collected from the Forest Research Institute of Malaysia (FRIM), Kepong, in June 2003. The voucher specimens of the plants were identified and deposited in the herbarium of the institute. The plant materials were air-dried, comminuted and 150 g of each sample was hydro-distilled in Clevenger-type apparatus for 8 h. The oily layers obtained were separated and dried over anhydrous magnesium sulfate. The yields were averaged over three experiments and calculated based on dry weight of the plant materials.

FUNGAL STRAINS USED

The following fungi were obtained from the Institute for Medical Research, Kuala Lumpur; 6 dermatophytes i.e. *Trichophyton mentagrophytes* (clinical isolate), *Tr. tonsurans* (T14 -Australian QC), *Tr. rubrum* (T28 -Australian QC), *Microsporum canis* (M17), *M. gypseum* (M141), *M. audouini* (M142) and one filamentous fungi (*Aspergillus fumigatus* [A31]); 5 isolates of yeast-like fungi i.e. *Candida albicans* (ATCC 10231) , *Ca. glabrata* (ATCC 1300), *Ca. parapsilosis* (ATCC 200219), *Ca. tropicalis* (ATCC 7110) and *Cryptococcus neoformans* (C6185). The fungi were maintained on potato dextrose agar (PDA) plates (Merck, Germany) at 30°C for dermatophytes and *Aspergillus fumigatus* and at 35°C for yeasts. The fungal suspension of each organism was prepared in normal saline (0.85%) to produce a cell suspension containing 1-5 x 10^6 cells or conidia per ml (CFU/ml) with turbidity comparable to that of McFarland standard tube No. 5 (NCCLS 1997). The inoculum size was determined spectrophotometrically at 530 nm and further confirmed by using Neubauer Counting Chamber (Pfaller et al. 1988). Each fungal suspension was then diluted with M-3 broth to obtain 1-5 x 10^4 CFU/ml for dermatophytes and *Aspergillus fumigatus* and 1-5 x 10^3 CFU/ml for yeasts.

ESSENTIAL OIL ANALYSIS

The oils were analyzed on a Shimadzu GC 2000 chromatograph equipped with a FID detector using a DB-5 capillary column (25 m x 0.25 mm, 0.25 μm film thickness). The operation parameters were: nitrogen as carrier gas at 50 cm/s, injector and detector temperatures were maintained at 250°C. The column was programmed initially at 75°C for 10 min, then 3°C /min to 210°C and held for 1 min. The oils were also examined using a DB-1 stationary phase column (25 m x 0.25 mm, 0.25 μm film thickness) programmed from 60°C for 10 min, then 3°C/min to 180°C and held for 10 min. Peak areas and retention times were measured by electronic integration. The relative amounts of individual components are based on peak areas obtained, without FID response factor correction. Temperature program linear retention indices of the compounds were also determined relative to *n*-alkanes (Kovats 1965). The oils were also analyzed by GC-MS

with a Hewlett-Packard GC-MSD 5890 series 2 mass spectrometer (70eV direct inlet) on a BPX5 column (30 m x 0.25 mm, 0.25 µm film thickness) initially at 75°C for 10 min, then 3°C/min to 210°C and held for 1 min with helium as carrier gas. The constituents were identified by comparison of their retention indices with literature values and their mass spectral data with those from the Wiley mass spectral database, and in some cases by co-chromatography on the different columns with authentic samples (Adams 1989; McLafferty & Staufer 1989; Davies 1990).

DETERMINATION OF MINIMUM INHIBITION CONCENTRATION (MIC)

The antifungal activity of the essential oils and the essential oil standards (cinnamaldehyde, eugenol, linalool and α-terpineol) (Sigma, USA) were determined by the broth microdilution method according to NCCLS (1997) and Hammer et al. (2002) with a slight modification. The experiments were carried out in a class 2 laminar flow cabinet. Sterility conditions were maintained throughout the experiments. Serial dilutions of the essential oil solutions and the 4 essential oil standards were placed in eppendorf tubes labeled A to H. Tube A was filled with 100 µl of essential oil stock solution (500 mg/ml, DMSO). Only 50 µl of the stock solution in tube A was transferred to tube B and diluted with 50 µl of DMSO. The procedure was repeated for solutions in tube B to H. Each tube was diluted with M-3 broth to obtain concentrations ranging from 10.0 – 0.08 mg/ml. 100 µl from each tube was then transferred into 96-well microtitre plates. Each well was then filled with 100 µl fungal suspension to obtain serial dilution of the test materials (5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08 and 0.04 mg/ml). The mixtures were mixed thoroughly and incubated at 35°C overnight for the yeasts and *Aspergillus fumigatus* and for 4 days at 30°C for the dermatophytes. The final inoculum size for fungi was 2.5 x 10^4 CFU/ml for dematophytes and *A. fumigatus* and 2.5 x 10^3 for yeasts. Amphotericin B (100 µg/ml) was used as a positive control , 1% DMSO served as a negative control, M-3 broth as sterility control and M-3 with fungal suspension as growth control. Turbidity was taken as an indication of growth and the lowest concentration at which it remained clear after macroscopic evaluation was recorded as the minimum inhibitory concentration (MIC). The MIC value was recorded as the mean concentration of duplicates. MIC value of <1.0 mg/ml was considered as strong, while values between 1.0 to 4.9 mg/ml and ≥ 5 mg/ml were categorized as moderate and weak, respectively.

CHECKERBOARD MICROTITRE TEST

In vitro antifungal combination assay was performed to investigate the combined effect of the leaf and bark oils of *C. verum* against *Cr. neoformans, M. gypseum* and *M. canis* by using the checkerboard technique, as described by Krogstad and Moellering (1986) and Davidson and Parish (1989). The assay involved multiple dilutions of the two oils in concentrations equal to, above, and below their MIC values for the fungi being tested. The concentrations tested for each oil ranged from 4 to 5 dilutions below the MIC to twice the MIC, using two-fold dilutions. Seven serial two-fold dilutions of the leaf and bark oils were prepared in DMSO as described in the broth microdilution procedure and then diluted with M-3 broth to obtain a series of dilutions at concentrations 4 times higher than its final concentrations in the reaction mixtures. Fifty microlitre aliquots of each bark oil solution (19.5 to 1250 µg/ml) was dispensed into the wells vertically down the 96-well microtitre plate and 50 µl aliquots of each leaf oil solution (19.50 to 1250 µg/ml) was dispensed horizontally. A 100 µl suspension (1-5 x 10^4 CFU/ml) of *Cr. neoformans* or *Mc. gypseum* or *Mc. canis* was added into each well. The final concentrations of each oil

in the reaction mixtures ranged from 4.80 to 312 µg/ml. The result was that each square in the checkerboard (well) contained a series of combination of the two oils being tested.

To assess whether synergistic antifungal activity occurred between the two sample solutions, two methods were used as described by Warnock (1989). The first method was by plotting an isobologram with axis representing the concentrations of the two oils. The lowest concentration of combined sample showing fungal growth inhibition was plotted. The combination effect of the mixture is considered synergistic if the line is shifted to the left, antagonistic if it is shifted to the right and additive if the line is straight. The second method was by calculating the fractional inhibitory concentrations (FIC) which is the concentration of each sample necessary to inhibit growth in a given row or column divided by the MIC value of the sample alone against the test organism. The FIC index was obtained by adding the FIC value of each sample and interpreted as follows: synergistic effect if it was < 1, additive if it was = 1 and as antagonistic if it was > 1 (Meadowas et al. 2002).

RESULTS AND DISCUSSION

Water distillation of the leaf and bark oils of *C. verum* gave the following yields: leaf oil (150 g, 5.5%) and bark oil (150 g, 1.5%). The list of constituents identified in the leaf and bark oils is shown in order of elution on a DB-5 type column in Table 1. The leaf oil of *C. verum* could be a natural source of eugenol as it contained 90.2% of the compound. Except for β-caryophyllene (2.0%), the other compounds were present in minor amounts (< 0.5%) in the oil. The most abundant compound in the bark oil was cinnamaldehyde (50.4%). Other compounds present in the bark oil in appreciable amounts were β-caryophyllene (6.4%), β-phellandrene (6.6%), β-cymene (4.9%), α-copaene (4.5%), linalool (3.8%), tetradecanal (2.9%), eugenol (1.8%) and α-terpineol (1.1%).

The MIC values of the tested oils and the essential oil standards are shown in Table 2. Both oils showed strong activity (<0.04-0.31 mg/ml) against the fungi. Cinnamaldehyde and eugenol, main components of the bark and leaf oils, respectively, showed strong activity against the tested fungi with MICs ranging from (<0.04-0.63 mg/ml). However, linalool and α-terpineol showed moderate to strong activity (0.16-1.25 mg/ml) against the tested fungi. Based on the results of the antifungal assays on the essential oils and standard samples, a chemical composition-antifungal relationship analysis was determined. The results demonstrated that the strong antifungal activity of the bark and leaf oils of *C. verum* could be related to the high levels of cinnamaldehyde (50.4%) and eugenol (90.2%), respectively, although other constituents may also contribute to the activity of the oils (Table 1 & 2). Previous studies have also indicated that cinnamaldehyde present as major component was responsible for the strong antifungal activity of cinnamon oil (Hicham et al. 1999; Simic et al. 2004).

The combined antifungal effect of the oils against *M. canis, M. gypseum* and *Cr. neoformans* was investigated by the checkerboard assay. Isobolograms were constructed and FICI were calculated to determine the combination effects between the oils. Table 3 shows that there was no synergistic effect between the oils. Antagonistic effect was observed from the combined oils against *Cr. neoformans* and *M. canis* as the FICI was 2. The isobolograms of the combined oils against *Cr. neoformans* (Fig.1 a) and *M. canis* (Fig.1 b) also proved that there was antagonistic action between the combined oils, as the lines shifted to the right. However, the isobologram in Figure 1 c showed additive effect of the combined oils against *M. gypseum,* as indicated by the FICI of 1 (Table 3). The antifungal effect of the leaf and bark oils of *C. verum* in combination against the tested

fungi was not synergistic. However, further study is recommended to assess the potential synergistic effect of the *C. verum* oils with other oils or azole-type of antifungal agents such as ketoconazole and fluconazole.

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TABLE 1 Percentage composition of the essential oils of *Cinnamomum verum*

Compound	Retention Index	Leaf	Bark	Method of identification
Styrene	870	-	0.6	MS, RI
α-Thujene	931	0.1	0.3	MS, RI
α-Pinene	939	0.4	2.0	MS, RI, Co
Benzaldehyde	956	0.1	0.2	MS, RI, Co
Sabinene	963	-	t	MS, RI, Co
β-Pinene	984	0.1	0.3	MS, RI, Co
Myrcene	991	0.1	t	MS, RI, Co
α-Phellandrene	1009	0.3	2.8	MS, RI, Co
p-Cymene	1026	0.3	4.0	MS, RI, Co
β-Phellandrene	1036	0.5	8.0	MS, RI, Co
γ-Terpinene	1064	-	0.2	MS, RI, Co
Terpinolene	1089	t	0.4	MS, RI, Co
Linalool	1099	0.1	4.8	MS, RI, Co
Terpinen-4-ol	1178	t	0.9	MS, RI, Co
α-Terpineol	1189	t	1.6	MS, RI, Co
Cinnamaldehyde	1276	-	44.2	MS, RI, Co
Eugenol	1362	90.2	1.6	MS, RI, Co
Methyl(E)-cinnamate	1380	-	0.6	MS, RI, Co
α-Copaene	1395	-	4.8	MS, RI, Co
Methyl eugenol	1396	0.1	-	MS, RI, Co
β-Caryophyllene	1418	2.0	6.9	MS, RI, Co
trans-α-Bergamotene	1432	-	0.2	MS, RI
Aromadendrene	1437	-	0.1	MS, RI, Co
α-Humulene	1454	-	1.5	MS, RI, Co
γ-Muurolene	1477	-	0.3	MS, RI
β-Selinene	1485	0.4	-	MS, RI
δ-Cadinene	1525	t	0.7	MS, RI, Co
Globulol	1590	t	-	MS, RI
Tetradecanal	1611	-	2.8	MS, RI, Co
Benzyl benzoate	1768	-	0.3	MS, RI, Co

Percentages were obtained by peak-area normalization on column DB-5, all relative response factors being taken as one; t =trace; tentative identification for all compounds except for Co; MS = mass fragmentation; RI = retention index; Co = co-chromatography with authentic sample.

TABLE 2. Minimum inhibitory concentration (MIC)* of the essential oils of *Cinnamomum verum* and essential oil standards.

Fungi species	MIC* (mg/ml)					
	Le	Ba	Eu	Cin	Lin	Ter
Trichophyton rubrum	<0.04	0.08	0.16	<0.04	0.16	0.63
Trichophyton mentagrophytes	0.08	0.08	0.08	<0.04	0.31	0.31
Trichophyton tonsurans	0.08	<0.04	0.08	<0.04	0.31	0.31
Microsporum canis	<0.04	0.08	0.16	<0.04	0.63	0.13
Microsporum gypseum	0.08	0.08	0.08	<0.04	0.16	0.63
Microsporum audouinii	<0.04	<0.04	<0.04	<0.04	0.16	0.13
Aspergillus fumigatus	0.31	0.16	0.16	<0.04	0.16	1.78
Candida albicans	0.31	0.16	0.63	0.08	0.63	1.25
Candida glabrata	0.31	0.16	0.63	0.16	1.25	1.25
Candida tropicalis	0.31	0.16	0.63	0.08	0.63	1.25
Candida parapsilosis	0.31	0.08	0.31	0.08	0.31	1.25
Cryptococcus neoformans	0.31	0.16	0.63	<0.04	0.31	1.25

*Values are given as mean values (mg/ml) from duplicate experiments. Leaf=leaf oil, Bark=bark oil, Eu= eugenol, Cin= cinnamaldehyde, Lin= linalool, Ter= α-terpineol.

TABLE 3. Minimum inhibitory concentrations (MIC) (µg/ml) and fraction inhibitory indices (FICI) of the bark (B) and leaf (L) oils of *Cinnamomum verum* alone and in combination against several fungi.

			MIC (µg/ml)			
			Bark oil		Leaf oil	
Fungi species	Effect	FICI	alone	combined	alone	combined
Cyptococcus. neoformans	antagonist	2	156.0	156.0	312.0	312.0
Microsporum gypseum	additive	1	78.0	39.0	78.0	39.0
Microsporum canis	antagonist	2	78.0	78.0	156.0	156.0

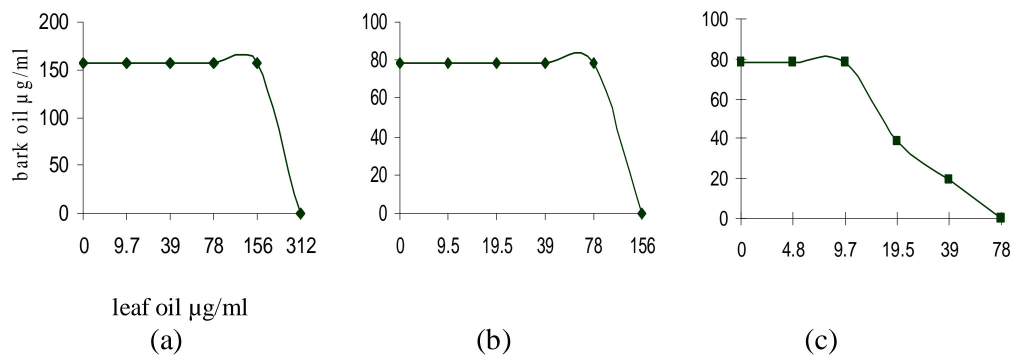

FIGURE 1. Isobolograms showing the antagonistic and additive effects of the leaf and bark oils of *Cinnamomum verum* against (a) *Cryptococcus neoformans*, (b)*Microsporum canis* and (c) *Microsporum gypseum.*