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Chemical composition and antimicrobial activity of *Meliola toddaliae* infected leaf oil of *Pamburus missionis*

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Pamburus missionis (Wight) Swingle, Rutaceae, is an essential oil bearing tree. We observed severe fungal infection on the leaves of *P. missionis*. This infection on its leaves was due to the fungus, *Meliola toddaliae* Doidge. Here we report the chemical variation and antifungal activity of essential oils isolated by hydrodistillation from the fungal infected and uninfected leaves of *P. missionis*. These oils were analyzed by gas chromatography-mass spectroscopy. β-Pinene and β-phellandrene were the major constituents in both these oils. Monoterpenes constituted 96% and their profiles were very similar in these leaf oils, whereas sesquiterpenes in these oils were only less than 4%. Antimicrobial analysis on these leaf oils against Gram-positive, Gram-negative bacteria and fungi *Candida albicans* and *C. glabrata* were carried out by the disc diffusion technique. This showed the absence of inhibition zones for both these oils against *Candida albicans* and *C. glabrata*. The absence of antifungal metabolites in the infected and uninfected leaf oils supported the continued growth of *M. toddaliae* as a 'parasitic symbiont' on the leaves of *P. missionis*.

Key words : *Pamburus missionis*, Rutaceae, *Meliola toddaliae*, essential oil, GC-MS, β-pinene, β-phellandrene, antimicrobial activity

INTRODUCTION

Essential oils from plants are a promising source of natural antimicrobial agents. Rutaceae is one of the plant families bearing essential oils and it comprises of over 1,700 species distributed in about 160 genera. Rutaceae plants are most abundant in the tropical regions (Mabberley, 1990). They are trees, shrubs, woody climbers or rarely herbs. They are of great economic importance as the source of *Citrus* fruits such as the citrons, lemons, limes and oranges. Flavonoids, vitamin C, carotenoids, coumarins, limonoids and volatile oils have been reported as the major metabolites from *Citrus* plants (Moriguchi *et al.*, 2003). *Citrus* flavonoids have antioxidant, anti-cancer, antiviral, anti-inflammatory and cholesterol-lowering ability (Manthey *et al.*, 2001). *Citrus* fruits and vegetables containing the carotenoid, lycopene, have been found to reduce significantly prostate and mammary cancer risk (Jung Park and Pezzuto, 2002; Stahl and Sies, 1996). *Citrus* leaves are used for flavouring foods and for medicinal infusions. Essential oils are extracted from their leaves and fruits and used in flavour products (Huang and Pu, 2000; Buccelato, 2002).

The genus *Pamburus* in the Rutaceae family is closely related to the genus *Citrus* (Anonymous, 1916). *Pamburus missionis* (Wight) Swingle is an evergreen tree of about 12 m height and it occurs both in the Eastern and Southern Western Ghats of Peninsular India. A previous phytochemical study has resulted in the isolation of various coumarins and an indole alkaloid from *P. missionis* from Sri Lanka (Kumar *et al.*, 1994). There is a single *P. missionis* tree growing in the campus of Tropical Botanic Garden and Research Institute located at Palode, Thiruvananthapuram in Kerala and most of its leaves are severely infected by the fungus,

Meliola toddaliae Doidge. This fungus has been first described on *Vepris (Toddalia) lanceolata* and subsequently on *Teclea natalensis*, *Fagara capensis* and *F. davii* from South Africa (Hansford, 1961). From India, this fungus was first reported on the above *P. missionis* tree (Hosagoudar and Pandurangan, 1994). Since then, the infection on this plant is being continuously observed. In this study, we report the chemical composition and antimicrobial activity of essential oils from the infected and uninfected leaves of *P. missionis*. There are no previous reports on essential oils from *P. missionis*.

MATERIALS AND METHODS

Plant materials and distillation of essential oils

Fresh leaves from the uninfected and infected twigs (Fig. I) of the above *P. missionis* tree were collected on 16th September 2004. The voucher specimen, No. 51853, was kept at the Herbarium of Tropical Botanic Garden and Research Institute. About 505 g of uninfected and 400 g of infected leaves were separately hydrodistilled in a Clevenger-type apparatus for 4 h. These leaf oils were colourless with a peculiar smell. The oil yields were 0.2% and 0.25% from uninfected and infected leaves, respectively.

Gas chromatography-Mass spectroscopy analyses

Gas chromatography-Mass spectroscopy (GC-MS) analyses of these leaf oils were performed by splitless injection of 1.0 µl of each oil on a Hewlett Packard 6890 gas chromatograph fitted with an HP-5 MS cross-linked 5% PH ME siloxane, 30 m x 0.32 mm x 0.25 µm capillary column, coupled with a model 5973 mass detector. GC-MS operation conditions: injector temperature - 220°C; transfer line - 240°C; oven temperature programme - 60° to 243°C (3°C/min); carrier gas - He at 1.4 ml/min. Mass spectra: Electron Impact (EI^+) mode 70 eV, ion source temperature 240°C. Individual components in Table 1 were identified by Wiley 275.L database matching and by comparison of mass spectra with published data (Adams, 2001). Relative percentages of individual components in these oils (Table 1) were obtained from the peak area-per cent report of volatiles from GC-MS data. Relative retention indices of constituents in Table 1 were determined on the PH ME siloxane capillary column using n-alkanes as standards (van den Dool and Kratz, 1963; Adams, 2001).

Identification of fungus

The fungal infection on the leaves of *P. missionis* was identified as of *M. toddaliae* by Dr. V. B. Hosagoudar, one of the authors (Hansford, 1961; Hosagoudar and Pandurangan, 1994; Hosagoudar, 1996). *M. toddaliae* colonies are amphigenous, dense, velvety, up to 4 mm in diameter, confluent, easily detachable from the host leaves. Hyphae straight, branching mostly opposite at acute to wide angles, very closely reticulate and form solid mycelial mat, cells 15-18.5 × 6-8 µm. Appressoria opposite, straight to slightly curved, antrorse to subantrorse, 15-22 µm long; stalk cells cylindrical to cuneate, 3-9.5 µm long; head cells ovate, globose, oblong to cylindrical, entire to angular, 12-15.5 × 6-9.5 µm. Mycelial setae numerous, simple, straight, acute to obtuse at the tip, up to 572 µm long. Perithecia closely scattered, verrucose, up to 310 µm in diam.; ascospores obovoidal, 4-septate, slightly constricted at the septa, central cell slightly larger, 46-50 × 18-22 µm.

Materials examined: On the leaves of *Pamburus missionis* (Wight) Swingle (Rutaceae), Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, Kerala, November 20, 1992, A. G. Pandurangan HCIO 40890; Dec. 4, 1996, V. B. Hosagoudar, HCIO 42432, TBGT 105; Jan. 19, 2001, M. Kamarudeen HCIO 44063, TBGT 508; Mathew Dan HCIO 44516, TBGT 802. Figure I (3-8) shows the growth stages of *M. toddaliae*.

Bacterial and fungal strains

Gram-positive bacteria, *Staphylococcus aureus* (MTCC 96), *Bacillus cereus* (MTCC 430), *B. subtilis* (MTCC 441) and Gram-negative bacteria, *Serratia marcescens* (MTCC 97), *Pseudomonas fluorescens* (MTCC 103), *P. aerugenosa* (MTCC 741), *Klebsiella pneumoniae* (MTCC 109), *Proteus vulgaris* (MTCC 426), *Escherichia coli* (MTCC 443), *Salmonella typhi* (MTCC 733) and the fungi,

Candida albicans (MTCC 227), *C. albicans* (MTCC 1637), *C. albicans* (MTCC 3017), *C. glabrata* (MTCC 3019) were obtained as Microbial Type Culture Collection (MTCC) from the Institute of Microbial Technology, Chandigarh, India.

Antibacterial activity

The antibacterial activities of these leaf oils against the above Gram-positive and Gram-negative bacteria were tested by the disc diffusion technique (Berghe and Vlietinck, 1991; Cappuccino and Sherman, 1998). The culture medium used for bacteria was Mueller-Hinton agar medium at pH 7.2. Agar medium was poured into the plates to uniform depth of 5 mm and allowed to solidify. Then the microbial suspensions were streaked over the surface of media using a sterile cotton swab to ensure the confluent growth of the organism. Aliquots of the oil were diluted with two volumes of dimethyl sulfoxide. Whatman No. 1 filter paper discs of 6 mm diameter were impregnated with 10 µl each of the diluted oil. These discs were then aseptically applied to the surface of the agar plates at well-spaced intervals. These plates were incubated at 37°C for 24 h and observed growth inhibition zones were measured. Control discs impregnated with 10 µl each of the inert solvent DMSO and streptomycin at 2 µg/disc, positive control for bacteria, were used alongside the test discs in each experiment (Table 2).

Antifungal activity

The antifungal activities of these oils were tested by the disc agar diffusion method as above. *Candida albicans* and *C. glabrata* strains were cultured in modified Sabouraud's agar. Oil dilution was 1:2 in DMSO, control discs impregnated with 10 µl of DMSO and fluconazole at 2 µg/disc were used alongside the test discs in each experiment (Table 2).

RESULTS

In this study, the fungal infection on the leaves of *P. missionis* was confirmed as of *M. toddaliae*. Volatile oils hydrodistilled from the uninfected and *M. toddaliae* infected leaves of *P. missionis* were analyzed by gas chromatography-mass spectroscopy and oil yields obtained by hydrodistillation were 0.2% and 0.25% respectively. About 22 (100%) and 26 (100%) constituents were identified from the uninfected and infected leaf oils respectively (Table 1). β-Pinene (43.4%) and β-phellandrene (45.3%) were the major constituents in the uninfected leaf oil. The respective percentages of these two constituents in the infected oil were 40.6% and 49.5%. The sesquiterpene percentages in these oils were less than 4%. The mono- and sesquiterpene profiles in the infected and uninfected leaf oils were nearly the same (Table 1).

Table 1 : Chemical composition of essential oils from the uninfected and infected leaves of *Pamburus missionis* determined by GC-MS

Constituent	Relative retention index	Uninfected leaf oil (%)	Infected leaf oil (%)
Camphene	953	0.2	0.2
β-Pinene	979	43.4	40.6
Myrcene	988	1.5	1.6
α-Phellandrene	1002	1.9	1.8
α-Terpinene	1018	0.5	0.4
β-Phellandrene	1027	45.3	49.5
δ-3-Carene	1031	-	0.1
(E)-β-Ocimene	1046	0.1	0.1
γ-Terpinene	1058	0.8	0.6
cis-Sabinene hydrate	1070	-	0.1
Terpinolene	1085	0.3	0.2
trans-Sabinene hydrate	1098	-	t
Linalool	1097	0.2	0.2
cis-p-2-Menthen-1-ol	1116	0.1	0.1
Terpinen-1-ol	1134	t	-
Terpinen-4-ol	1177	1.5	1.0
α-Terpineol	1187	0.5	0.3
β-Caryophyllene	1423	2.1	1.5
α-Humulene	1447	0.2	0.2
γ-Selinene	1465	0.1	t
β-Selinene	1478	0.6	0.4
Valencene	1486	t	t
α-Selinene	1490	0.5	0.3
Germacrene A	1509	t	t
7-*epi*-α-Selinene	1514	-	t
Caryophyllene oxide	1585	0.2	1.0
Torreyol	1652	-	t
Monoterpene hydrocarbons		94.0	95.1
Oxygenated monoterpenes		2.3	1.7
Sesquiterpene hydrocarbons		3.5	2.4
Oxygenated sesquiterpenes		0.2	1.0
Number of constituents identified		22	26
Percentage of constituents identified		100%	100%

t = trace < 0.1%

Table 2 : Antibacterial and antifungal activities of the uninfected and infected leaf oils of *Pamburus missionis* tested by the disc diffusion technique.

Bacteria/fungi (MTCC No.)	Diameter of zone of inhibition in mm excluding the diameter of the disc		
	Uninfected leaf oil	Infected leaf oil	Control
Gram-positive bacteria			
Staphylococcus aureus (96)	-	10	16
Bacillus cereus (430)	-	-	18
B. subtilis (441)	-	-	16
Gram-negative bacteria			
Serratia marcescens (97)	-	-	20
Pseudomonas fluorescens (103)	-	7	16
P. aerugenosa (741)	8	7	13
Klebsiella pneumoniae (109)	8	8	14
Proteus vulgaris (426)	-	-	9
Escherichia coli (443)	-	-	15
Salmonella typhi (733)	-	-	16
Fungi			
Candida albicans (227)	-	-	8
C. albicans (1637)	-	-	10
C. albicans (3017)	-	-	16
C. glabrata (3019)	-	-	12

Control - Streptomycin at 2 µg/disc for bacteria and Fluconazole at 2 µg/disc for fungi; oil dilution - 1:2 in DMSO; Experiments were done in triplicate and results are mean values.

Antifungal screening of both uninfected and infected leaf oils by disc diffusion technique revealed no activity against *C. albicans* and *C. glabrata* (Table 2). Similar results, no growth inhibition zones, were observed on a repetitive second batch of experiments with these oils. But inhibition zones were observed for fluconazole, positive antifungal control, in all these experiments. On antibacterial screening, only very low activity against the two Gram-negative bacteria, *Klebsiella pneumoniae* and *Pseudomonas aerugenosa*, was observed for these oils (Table 2).

DISCUSSION

The leaves of the *P. missionis* described in this study are severely infected by the fungus *M. toddaliae* belonging to the family *Meliolaceae*. Fungi in this family are strictly 'obligate biotrophs' (Hansford, 1961; Hosagoudar, 1996). They are host specific and have a very narrow host range. These fungi need to be associated with the living cells of their host plant for their growth and reproduction. Also, they can derive their nutrients only from the living cells of the host. More than 90% of Meliolaceous fungi are not pathogenic to their hosts. They establish a symbiotic relationship with their host plants and so they are termed as 'parasitic symbionts'.

In most cases, plants defend themselves against microbial attack by the generation of 'constitutive' and 'inducible' metabolites such as cell-wall polymers, proteins, peptides and secondary metabolites. Volatile secondary metabolites, either preformed or induced, play a significant role in plant microbial defense (Thomma *et al.*, 1998; Holopainen, 2004). The monoterpene, limonene, is one of the most abundant volatile oil components in nature. Its enantiomer d-limonene is a major component in most essential oils from lemons and oranges in the genus *Citrus*. Its most significant biological activity is inhibition of microbial growth, especially fungal growth. d-Limonene has also been found as the most frequent constituent in essential oils showing high antifungal activity (Wilson *et al.*, 1997; Duetz *et al.*, 2003; Buccelato, 2002). This antifungal constituent found in oils in the related genus *Citrus* has been found to be absent in the leaf oils of *P. missionis* (Table 1).

The antimicrobial screening results of the oil from uninfected leaves of *P. missionis* show the absence of 'preformed' fungal or bacterial resistance in the oil (Table 2). The infected leaf oil also do not show inhibition zones on antifungal screening. This is consistent with the very similar chemical profiles of the uninfected and infected leaf oils. These results show that *P. missionis* is not producing any new antifungal volatile terpenoids upon infection by *M. toddaliae*.

In conclusion, this study on the chemical composition, antifungal and antibacterial activities of the uninfect and infected leaf oils of *P. missionis* revealed the absence of 'constitutive' and 'induced' defense against *M. toddaliae* infection in *P. missionis*. This justifies the continued growth of *M. toddaliae* as a 'parasitic symbiont' on the leaves of *P. missionis*, as observed by us.

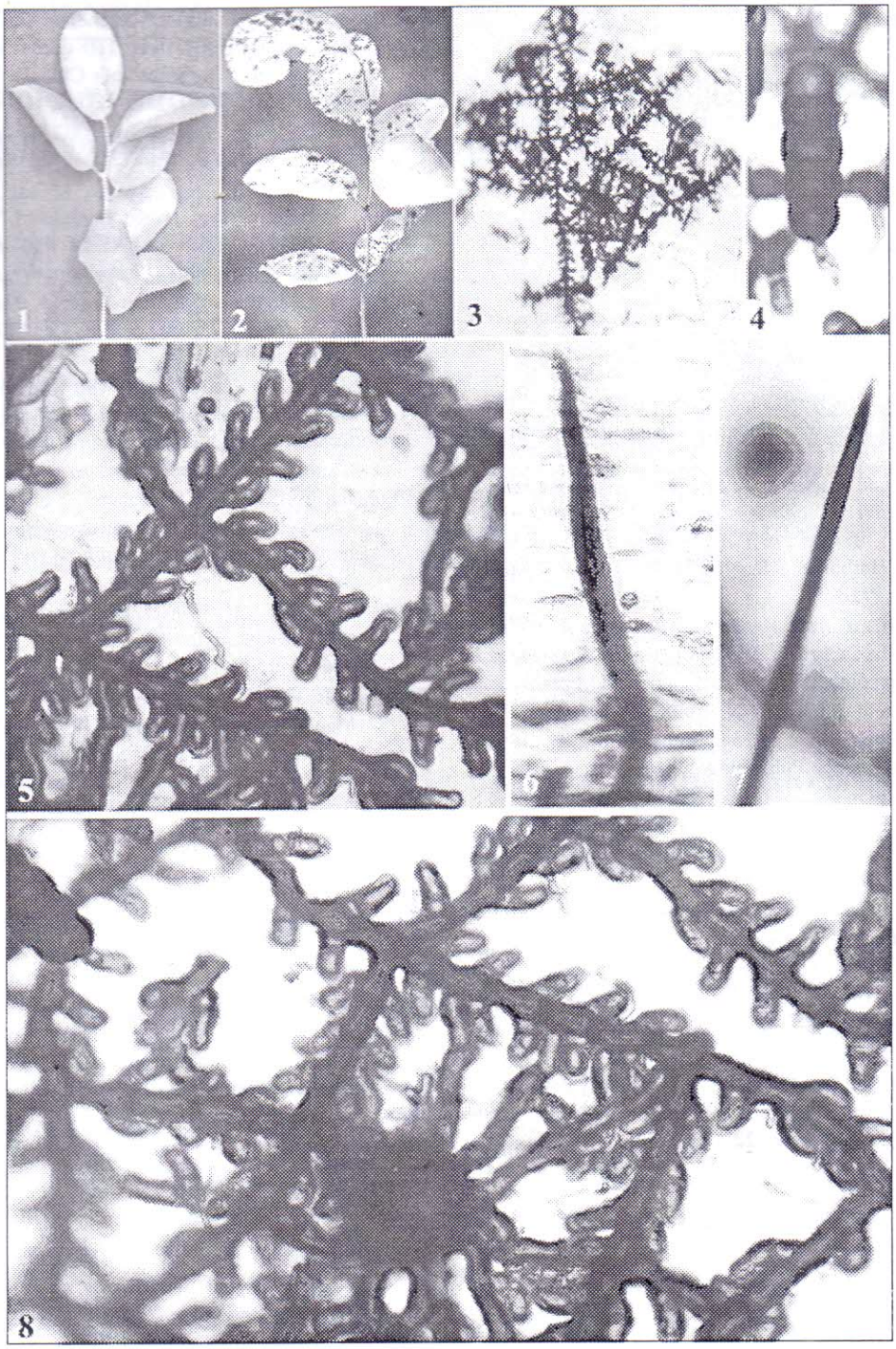

Fig. 1 : *Meliola toddaliae* Doidge on *Pamburus missionis* (Wight) Swingle. (1) Uninfected twig; (2) Infected twig; (3) Fungal colony; (4) Ascospore; (5) Mycelium with oppositely arranged appressoria; (6) Mycelial setae; (7) Apical portion of the mycelial setae; (8) Developing Perithecium in the colony.

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