Natural Product Sciences
13(4) : 279-282 (2007)

Chemical Composition and Antimicrobial Activity of Cone Volatile oil of *Cupressus macrocarpa* Hartwig from Nilgiris, India

S.Manimaran[1], S.Themozhil[1], M.J.Nanjan[1], and B. Suresh[2]

[1]*Dept of Phytopharmacy and Phytomedicine, TIFAC CORE in Herbal Drugs*
[2]*Department of Pharmacology*
J.S.S College of Pharmacy, Ootacamund, Tamil Nadu, India

Abstract – The aim of the present study was to investigate the various chemical components present in the cone volatile oil of *Cupressus macrocarpa* and also determine its antimicrobial activity. Totally 13 compounds were identified with 99.99% by GC-MS analysis. The major compounds identified were terpinene-4-ol (19.42%), dinopol (15.63%), α-pinene (13.58%), and β-pinene (12.16%). The antimicrobial activity was carried out for the oil and a 2% cream formulation using cup plate method by measuring the zone of inhibition. The gram positive organisms used were *Bacillus subtilis, Staphylococcus aureus, Bacillus megaterium,* and *Bacillus cogulans.* The gram negative organisms used were *Escherichia coli, Kleibseilla pneumonia, Pseudomonas aeruginosa* and *Salmonella typhi. In vitro* antifungal studies were also carried out by using organisms, *Candida albicans, Aspergillus flavus, Trichoderma lignorum and Cryptococcus neoformans.* The standard drugs used were penicillin (100 μg/mL), gentamycin (100 μg/mL) and griseofulvin (100 μg/mL) for gram positive bacteria, gram negative bacteria and fungi respectively. Both oil and cream formulation showed good activity against fungi than bacteria. This study is being reported for the first time on cone volatile oil of this plant.
Keywords – Volatile oil, antimicrobial, terpinene-4-ol, dinopol, α and β-pinene, *Cupressus macrocarpa*.

Introduction

The word *Cupressus* is taken from the Greek Kuparissos. It is the ancient name of Cypress consisting of nearly 12 species of *Cupressus* (Ambasta, 1986). The common name of *Cupressus macrocaepa* is Monterey cypress, belonging to the family, Cupressaceae. It is an evergreen tree upto 23 m tall, branches are horizontal. Cones are subglobose, 2.0 - 2.5 × 1 - 1.5 cm brown when ripe, each with a central crescent shaped process, seeds 16 - 20 per scale, 1 - 2 mm long narrowly winged tubercles (Henry, 1985; Matthew, 1969). The volatile oil obtained from cones of *C. macrocarpa* having anti-inflammatory activity both in carrageenen and polyvinyl pyrrolidine induced rat paw edema models and also reported to have antimicrobial activity by two fold serial dilution method (Dhanabal, 1999). The essential oil of the leaves is used against rheumatism, whooping cough, and styptic problems (Duke, 2004). The biflavones, viz. amentoflavone, cupressuflavone and sequoiaflavone were reported in leaves of *C.macrocarpa* (Abdul Qasim, 1985). The chemical analysis of leaf essential oil of *C.macrocarpa* reported to contains more amounts of monoterpenes than sesqui and diterpenes (Zavarin, 1971).

The present study is to investigate the chemical composition of volatile oil from the cones of *Cupressus macrocarpa* and to evaluate the antimicrobial activity for the oil and its cream formulation.

Experimental

Plant material – The fresh cones of *Cupressus macrocarpa* were collected from Government Botanical Garden Ootacamund, Nilgiris at an altitude of 2000 m during the summer season (May 2005). It was identified and authenticated from Botanical Survey of India (BSI), Coimbatore. The voucher specimen was deposited in the Department of phytopharmacy and phytomedicine, TIFAC CORE in Herbal Drugs, JSS College of Pharmacy, Ootacamund, Nilgiris.

Extraction of volatile oil – The collected fresh cones were subjected to hydrodistillation for the extraction of essential oil for 3 h using Clevenger type apparatus. The distilled volatile oil was dried over anhydrous sodium

*Author for correspondence
Fax: +91-423-2442937; E-mail: manischitra@rediffmail.com

sulphate, filtered and stored at +4°C for further testing (Donata, 2005). The percentage yield of essential oil was found to be 1.60% (v/w).

GC-MS analysis of oil – The GC-MS analysis of the essential oil was performed using Hewlett-Packard (HP) (Kyoto, Japan) with ZP-5 column (20 m × 0.25 mm). Helium was the carrier gas at a flow rate of 1 mL / min. The oven temperature was programmed from 70°C to 260°C at 10°C/min. Injector temperature was 250°C and the interface temperature was kept at 300°C. Identification of essential oil compounds was based on comparison of their relative retention times and mass spectra. The results were also confirmed by computer matching of mass spectra with Wiley 139 library data of the GC-MS system. The relative percentage of the essential oil constituents were calculated from GC peak areas.

Formulation of the essential oil – The distilled essential oil was formulated in the form of cream by using a suitable base (oil in water type: O/W) listed in the Table 1. Aqueous cream was just as effective as the test agents because of its easy penetration through skin (Muller *et al*., 2003). The stearic acid, cetosteryl alcohol, liquid paraffin and petroleum jelly were melted on a steam bath at 75°C. The remaining ingredients were dissolved in water and boiled at 75°C. The aqueous solution was than added to the above oily phase with agitation. The potassium hydroxide reacts with a portion of stearic acid to form potassium stearate, which emulsifies the un-reacted stearic acid as dispersed phase. Excessive agitation was avoided so that air is not entrapped within the cream. 2 mL of volatile oil was mixed finally to the above prepared cream base with uniform stirring using mechanical stirrer. Glycerin was added finally and mixed. The formulated cream was filled in a suitable plastic container.

Antimicrobial Screening of the oil and the cream formulation – The cylinder plate assay of drug potency is based on measurement of the diameter of zone of microbial growth inhibition surrounding the cylinder (cups) containing various dilutions of test compounds (Cappucrino and Sharman, 1992). Gram positive organisms used were; *Bacillus coagulans, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus,* and gram negative organisms used were; *Escherichia coli, Kleibseilla pneumonia, Pseudomonas aeruginosa,* and *Salmonella typhii.* The fungal strains used were; *Aspergillus flavus, Candida albicans, Trichoderma lignorium* and *Cryptococcus neoformans.* All the microorganisms were procured from National Collection of Industrial Microorganisms (NCIM), Pune and Dermatology department of Calicut Medical College, Calicut, Kerala.

The petri dishes were cleaned, dried and sterilized. Then they were filled with nutrient agar and sabouraud's dextrose agar (SDA) medium for bacteria and fungi respectively. After solidifying, the plates were inoculated with the organisms.

Three holes were made in to each inoculated plates by means of stainless steel sterile borer with a height of 10 mm and internal diameter of 6 - 8 mm. The essential oil 0.1 mL, 200 mg of cream and cream base was added to the hole under aseptic condition (Hailu, 2004). Then the plates were kept in refrigerator for two hours for diffusion. All the plates were incubated at 37 ± 1°C for 24 hours and 28 ± 1°C for 48 hours for bacteria and fungi respectively. The standard drugs used were penicillin, gentamycin and griseovulvin for gram positive, gram negative bacteria and fungi respectively with the concentration of 100 μg/mL. The zone of inhibition was measured by the antibiotic zone reader.

Results and Discussion

Totally 13 compounds were identified with 99.99% by GC-MS analysis. Out of 99.99%, the monoterpene, sesquiterpene and diterpene were identified with 56.83, 22.95 and 20.21% respectively. The major compounds identified were terpinel-4-ol (19.42%), dinopol (15.63%), α-pinene (13.58%), and β-pinene (12.16%), remaining are minor components. The name of the compounds, retention times with percentage are given in Table 2.

Significant activity was observed against gram positive bacteria than gram negative bacteria, particularly against *B.coagulans, B. megaterium* and *S.subtilis.* The oil and 2% cream formulation showed better activity against all fungal strains when compared to that of bacteria and also showed equipotent activity against standard drug, griseofulvin. The results of antimicrobial activity are given in Table 3.

Table 1. Composition of cream base

Ingredients	Composition (in % w/w)
Stearic acid	16.00
Potassium hydroxide	00.50
Cetosteryl alcohol	05.00
Liquid paraffin	03.50
Petroleum jelly	03.50
Methyl paraben sodium	00.16
Propyl paraben sodium	00.04
Glycerin	07.00
Purified water	64.30

Table 2. GC-MS Analysis of essential oil

R.Time	Name of the Compound		Percentage
1.067	Ocimene	M	00.01
2.325	Terpinene-4-ol	M	19.42
3.525	α-Pinene	M	13.58
4.158	β - Pinene	M	12.16
5.908	Terpinoline	M	00.34
6.967	β-Phellandrene	M	11.32
8.542	Cis-Muurola-3,5-diene	S	09.14
11.033	β-Cadinene	S	08.88
12.092	10-epi cubenol	S	02.92
13.475	Muurol-5-en-4-ol	S	02.01
16.075	Dinopol	D	15.63
17.350	5-alpha Pregnan-15-one	D	02.18
18.742	Phyllocladene	D	02.40
		Total	99.99%

M-Monoterpine: 56.83%, S-Sesqiterpine: 22.95%, D-Diterpine: 20.21%

C.macrocarpa essential oil here investigated, 13 compounds were identified. These represented 99.99% of the total compounds detected. The oil is mainly composed of monoterpenes (56.83%) with being the predominant constituents of terpine-4-ol (19.42%), α-pinene (13.58%), and β-pinene (12.16%). The sesquiterpines accounted for 22.95% of the oil, the main component being β-cadinene (8.88%). The diterpine amounted to 20.21%, the main componenet being dinopol (15.63%). The unidentified compounds were 0.01%.

The leaf essential oil was reported to contain more amounts of monoterpenes compared to sesqui and diterpenes (Zavarin, 1971). The present investigation also indicated to contain more content of monoterpenes from cone volatile oil. Both of the leaf and cone volatile oil contains major amount of monoterpenes than sesqui and diterpenes, but the individual components are different.

The essential oil obtained from this plant and its cream formulations exhibited antibacterial and antifungal activities. These activities may be attributed to the presences of above mentioned compounds of mono, sesqui and diterpenes (Magiatis, 1999; Tzakou, 2001; Oumzil, 2002). These chemical components exert their toxic effects against these microorganisms through the disruption of bacteria or fungal membrane integrity and also increase the membrane permeability in yeast cells and isolated mitochondria (Uribe, 1985; Knoblock, 1988). This is strongly supported by the study on the effects of different essential oil components on outer membrane permeability in gram negative bacteria (Helander, 1998).

Table 3. Antimicrobial activity of essential oil and cream formulation

Name of the Microorganisms	Zone of Inhibition (in mm)		
Gram positive bacterial organisms	Penicillin	Essential oil	2% Cream formulation
B. coagulans (NCIM No-2313)	18	15	11
B. megaterium (NCIM No-2187)	20	17	12
B. subtilis (NCIM No-2063)	15	14	10
S. aureus (NCIM No-2079)	26	15	11
Gram negative bacterial organisms	Gentamycin	Essential oil	2% Cream formulation
E. coli (NCIM No-2345)	25	14	9
K. pnemonia (NCIM No-2239)	24	11	8
P. aeruginosa (NCIM No-2200)	20	12	9
S. typhii (NCIM No-2080)	28	14	10
Fungal organisms	Griseofulvin	Essential oil	2% Cream formulation
A. flavus (NCIM No-0535)	18	16	14
C. albicans (NCIM No-3471)	19	16	13
C. neoformans (Procured from CMC)	18	17	15
T. lignorum (Procured from CMC)	17	16	14

Volatile compounds, such as caryophyllene, α-terpineol, α-pinene, β-pinene, α-humulene and á-cadinol, are likely to be the precursors of the complex menthols or resins which have been claimed to also contain the antibacterial and antifungal properties (Filippowiez, 2003). In our present study found that both oil and 2% cream formulation showed better activity against fungi than bacteria. This may be presence of more amounts of monoterpenes (56.83%) with high percentage of terpine-4-ol (19.42%), α-pinene (13.58%), and β-pinene.

From this study it was observed that the product developed in the form of a cream formulation (2%) from cone essential oil of *Cupressus macrocarpa* can be used as antimicrobial cream to cure the skin diseases. Further clinical studies are to be conducted to confirm the activities by human volunteers.

Acknowledgement

The authors are thankful to TIFAC CORE in Herbal Drugs and the Management of JSS Mahavidyapeetha for providing necessary facilities to carryout the research work.

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(Accepted September 13, 2007)