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RESEARCH ARTICLE

Chemical Profiling and Antifungal Activity of Volatile Oil of *Cupressus torulosa* against Pathogenic Fungi

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ABSTRACT

The aim of the present study was to investigate the chemical constituents present in the volatile oil of the *Cupressus torulosa* (*Cupressaceae*) as well as the antifungal activity. The essential oil analyzed by GC and GC-MS was characterized by the presence of α- Pinene (31.99 %), Sabinene (19.23 %) and DL-Limonene (9.06 %) as major constiuents. The antifungal activity of the oil was determined at different dilutions i.e. neat, 1:2, 1:4 and 1:8 by disc diffusion method against different fungal pathogens shows the maximum activity against *Trichophyton rubrum* and *Trichophyton mentagrophytes* while the minimum against *Microsporum canis*. The MIC value was reported 0.5µl/ml against *Trichophyton rubrum* and *Trichophyton mentagrophytes* through two fold serial dilution method. So, these result suggest that the the oil of *Cupressus torulosa* have potent antifungal activity and used in preparation of antiseptic product.

Keywords: *Cupressus torulosa*, screening, MIC, GC-MS, antifungal activity

INTRODUCTION

Medicinal plants are good source of biologically active secondary metabolites which have many therapeutic properties. About 80% populations of developing countries used traditional medicines derived from medicinal plants. Essential oils components obtained from aromatic plants have been successively investigated through out the world for their antibacterial, antioxidant, antifungal [4], anti-inflammatory, analgesic properties. These essential oil and their components are of great interest because of its safe status and multi-purpose uses [20, 28]. The genus *Cupressus* (cupressaceae), comprising twelve species which is distributed in North America, the Mediterranean and subtropical region of Asia at high altitudes [24] and also distributed in Mexico (*C. arizonica* Greene, *C. lusitanica* Mill.), Tibet (*C. cashmeriana*), China (*C. funebris*), California (*C. goveniana*), Bhutan (*C. torulosa*) but it also found commonly in Himachal Pradesh districts Solan (*C. lusitanica*) and Manali (*C. arizonica*,), Uttarakhand districts Nainital, Ranikhet, Chomoli, Kathi, Pauri, (*C. torulosa*) and also distributed above 2400 m of Western Himalayas. Cupressus genus are mainly used as diuretic, stimulant, anti-inflammatory and antiseptic for common cold and wound healing in folk medicines[8, 11, 26]. The chemical analysis of essential oil of *Cupressus torulosa* contains mono- and di-terpenes [5], and these essential oils showing the antibacterial and antifungal activity against *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Microspora, Trycophyton ruberum* and *Trycophyton mentagrophytes* was also reported. The oil of *C. lusitanica* used in the treatment of rheumatism, whooping cough and styptic problems [9], and also showed antidermatophytic activity**,** while the ethanolic extract of *C. lusitanica* demonstrated cytotoxicity against cancerous cell line [13]. Similarly, antibacterial and antifungal activity from oil of *C. arizonica* and *C. torulosa* [3, 23] and the larvicidal activity form oil of *C.arizonica* [19] was also reported.

This study is to find out the chemical composition of essential oil of *C. torulosa* and to screen the antifungal activity against different pathogenic fungi. Nowadays, skin infections caused by dermatophytes like *Microsporum* sp., *Trycophytes* sp. or other fungal pathogen become serious problems in worldwide and also limited number of

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drugs available against them. So, an effort has been made to promote the novel antidermatophytic agent from oil of *Cupressus torulosa* to overcome these problems.

MATERIALS AND METHODS

Plant Material

The leaves of *C. torulosa* were collected from Joshimath (Uttarakhand). The plant specimens were dully identified and deposited in the herbarium of Centre for Aromatic Plants (CAP), Selaqui, Dehradun (Acc No. CAP -93).

Oil isolation

The essential oil from shade dried leaves of *C. torulosa* (200g of sample) was extracted by hydrodistillation for 4 hours using Clevenger apparatus. The oil obtained were dried over anhydrous Na_2SO_4 and kept in in a sealed glass vial at 4^{o}C prior to analysis.

Gas chromatography (GC)

GC analysis of essential oil of *C. torulosa* was carried out by Agilent (model 6890 N) gas chromatography equipped with flame ionization Detector (FID) using N_2 as carrier gas.The column was HP-5 fused silica capillary column (30 m × 0.32 mm, 0.25 μm flim thickness) and temperature program was used as follows: initial temperature of 60°C (hold: 2 min) programmed at a rate of 3°C/min to a final temperature of 220°C (hold 5 min). Temperature of the injector and FID were maintained at 210°C and 250°C respectively.The injection volumes was 0.2 μL.

Gas-Chromatography and Mass-Spectrometry (GC-MS)

GC-MS analyses of the oils was performed with a Perkin Elmer Claurs 500 gas chromatography equipped with a split/splitless injector (split ratio 50:1) data handling system. The column was Rtx-5 capillary columns (60 m × 0.32 mm, 0.25 μm film thickness). Helium (He) was the carrier gas at a flow rate 1.0 mL/min. The GC was interfaced with (Perkin Elmer Clarus 500) mass detector operating in the EI positive mode. The mass spectra were generally recorded over m/z 40-500 amu that revealed the total ion current (TIC) chromatograms. Temperature program was used as the same as described above for GC analysis. The temperature of the injector, transfer line and ion source was maintained at 210°C, 210°C and 200°C respectively. The identification of compound was performed by MS library search with Wiley and NIST and compare with MS literature search [2, 6]. (Table 1)

Test microorganisms

To check the antifungal activity from oil of *C. torulosa* against plant pathogenic fungi like *Aspergillus niger, Aspergillus terreus, Candida* sp.(two species), *Trichophyton rubrum, Trichophyton mentagrophytes Microsporum audouinii, Microsporum canis, Pencillium crysogensum, Pencillium expansum* and *Pencillium griseofulvum* All test were performed by Dolphin Institute of Biomedical and Natural sciences Dheradun.

Screening of essential oil of *Cupressus torulosa*

Preperation of inoculumn

Durning the preparation of inoculumn the fungal culture was inoculated in Sabouaud's Dextrose Broth. The inoculum was standardized by adjusting the turbudity of culture to McFarland 0.5 standard (~ 10^6 cfu/ml) with sterile broth or by further incubation [16].

Determination of the antifungal activity

The antimicrobial activity of oil obtained from *C. torulosa* was determined by using disc diffusion method. 100 µl of fungal suspension was spread over plate and sterile whatman filter paper discs were soaked in 10 µl of different dilution of oils i.e. 1:2, 1:4 and 1:8 with 20% DMSO and DMSO was used as control. The standard antifungal amphotericin B (10µg/disc) used as antibiotics and the plates were incubated at 28°C for 3 days [25]. After incubation the antifungal activity was evaluated by measuring zone of inhibition. Each experiment was carried out in triplicate. The results have been shown in (Table 2)

Determination of minimum inhibitory concentration

The Minimum Inhibitory Concentrations of pathogenic fungi were determined using two fold serial dilution method [12].

Culture preparation

The test fungal pathogen was inoculated into sterile Sabouaud's Dextrose Broth. The final concentration of the inoculums was adjusted to McFarland 0.5 standard. In two fold serial dilution method, 32µl of essential oil was mixed with test tube containing 1.968 ml of sterile Sabouaud's Dextrose Broth to produce the concentration of 32 µl/2ml (1st tube) i.e the final concentration become 16 µl/ml, from the 1st tube (32 µl/ml) 1 ml was transferred to second test tube containing 1 ml of sterile Sabouaud's Dextrose Broth to obtained 16 µl/2ml

(2nd tube) i.e the final concentration become 8 µl/ml. Similarly the oil was serially diluted in two-fold manner to prepare different concentration extract ranging from 0.0078-16µl/ml. Finally 1ml from last dilution was discarded to keep the volume constant in all tubes. Each test tube of different concentration were then inoculated with test pathogen and incubated at 28 ^{o}C for 48 h. In each test set one tube without oil and another sterile Sabouaud's Dextrose Broth was used as positive and negative control. After incubation the lowest concentration in which no visible is growth observed i.e. no turbidity was determined the MIC of that compound.

RESULTS AND DISCUSSION

The chemical composition of essential oil of *C. torulosa* were studied and the major constituent present in the oil are α- Pinene (31.99), Sabinene (19.23), β- Myrcene (4.56), δ-3- Carene (6.52), α-Terpinene (2.21), DL- Limonene (9.06), γ-Terpinene (3.20), α- Terpinolene (2.58), 4-Terpineol (2.72), Bornyl acetate (2.94), β-Cubebene (3.05) etc. (**Table 1**) Similarly, maximum percentage of α- Pinene 34.26 %, 32.0 % and 30.30% from Kalsi Dehradun, Joshimath Chamoli and Jehrikhal Pauri respectively[15] while the maximum α- Pinene 17.76% from female branch and Sabinene 14.33% from female cone of *C. torulosa* D.Don [22] and α- Pinene 25.8 % and Sabinene 22.30 % from leaf oil of *C. torulosa*[17] was also reported.

Durning screening of oils of *Cupressus torulosa* was tested by disc diffusion method against different pathogenic fungal strains i.e. *A. niger, A. terreus, Candida* sp., (two species), *T. mentagrophytes, T. rubrum, M. audouinii, M. canis, P. crysogensum, P.expansum* and *P. griseofulvum*, the maximum zone of inhibition against *T. mentagrophytes* and *T. rubrum* with diameter 16 ± 0.78 and 16 ± 0.61 respectively, while minimum zone of inhibition against M. audouinii with diameter 12 ± 0.18 in presence of undiluted *C. torulosa* essential oil. Among all the fungal strains only six fungal strains i.e. *A. terreus, Candida* sp., *Candida* sp., *T. rubrum, T. mentagrophytes* and *M.canis* showed antifungal activity at 1:2, 1:4 and 1:8 dilutions. *M. audouinii* and *P. griseofulvum* showed inhibition only at 1: 2 dilutions and unable to shows any antifungal activity in other dilution. The standard antifungal amphotericin B showed inhibition in the range of 4-14 mm of zone of inhibition at concentration of 10µg/dics (**Table 2**). Amphotericin B was used as a positive control because it can complex with ergosterol in the fungal membranes, thereby compromising their barrier function to the point of causing leakage of cellular contents [21].

Table 1: Percentage components of *C. torulosa* oil

Components	R.I	Percentage
α- Thujene	930	1.62
α- Pinene	939	31.99
α- Fenchene	953	0.26
Camphene	954	1.06
Sabinene	975	19.23
β - Pinene	979	1.23
β- Myrcene	991	4.56
L-Phellandrene	1002	0.65
δ-3- Carene	1031	6.52
α- Terpinene	1017	2.21
p- Cymene	1025	0.42
DL- Limonene	1029	9.06
γ- Terpinene	1060	3.20
α- Terpinolene	1087	2.58
Cis-p-menth-2-en-ol	1118	0.53
Trans-p-menth-2-en-ol	1136	0.45
Camphor	1141	0.73
4-Terpineol	1177	2.72
α-Terpineol	1186	0.37
Bornyl acetate	1289	2.94
β- Terpinyl acetate	1349	0.44
α- Cubebene	1351	1.40
β- Cubebene	1387	3.05
Germacrene D	1485	0.83
δ- Cadinene	1523	0.40

Table 2: Screening of pathogenic fungi

Pathogenic Fungi	Zone of Inhibition (mm)					Amphotericin B 10µg
	Tween 80	Neat	1:2	1:4	1:8	
A. niger	-	14 ± 0.41	-	-	-	14
A. terreus	-	13 ± 0.44	10 ± 0.43	8 ± 0.37	6 ± 0.24	4
Candida sp.`	-	15 ± 0.65	13 ± 0.52	10 ± 0.38	10 ± 0.28	6
Candida sp.	-	14 ± 0.54	11 ± 0.80	8 ± 0.53	6 ± 0.46	6
T. mentagrophytes`	-	16 ± 0.78	12 ± 0.30	10 ± 0.44	8 ± 0.30	13
T. rubrum	-	16 ± 0.61	13 ± 0.63	9 ± 0.69	8 ± 0.28	14
M. audouinii	-	7 ± 0.38	3 ± 0.33	-	-	12
M. canis	-	12 ± 0.18	4 ± 0.26	12 ± 0.49	8 ± 0.26	14
P. crysogensum	-	12 ± 0.52	-	-	-	6
P. expansum	-	10 ± 0.38	-	-	-	5
P. greseofulvum	-	14 ± 0.41	5 ± 0.28	-	-	5

- Standard Amphotericin B (10µg/ disc)
- Zone of inhibition: Values are means of triplicate reading (Mean ± SD)

The oil of cinnamon and nutmeg showed inhibitory effect against *A.niger A. terreus* and *Penicillium* sp. [7] and the oil of lemon grass showed potent antifungal activity against *Candida albicans* [14] was also reported. The essential oil and ethanolic leaf extract of *Lonicera japonica* showed the potent antifungal activity against *Micrisporum canis* KCTC6348, *Trichophyton rubrum* KCTC 6345, 6352, 6375 and *Trichophyton mentagrophytes* KCTC 6077[1] similarly, the antifungal activity against *Trichophyton rubrum* and *Micrisporum canis* against leaves of *Inula viscose* and seed of *Ammi visnaga* [18] was also reported.

The MIC value of essential oil of *C. torulosa* was reported to be 0.5µl/ml against *Trichophyton rubrum* and *Trichophyton mentagrophytes* showing good activity. The MIC value of essential oil from leaves of *Plinia cerrocampanensis* at a concentration of 32 and 62.5 µg/ml against *Trichophyton rubrum* and *Trichophyton* mentagrophytes[27] another report from also found that MIC value 62.5 µg/ml from oil of aerial part of *Baccharis Grisebachii* against *Trichophyton rubrum* and *Trichophyton mentagrophytes* dermatophytes [10] also reported.

These results support the rational use of *C. torulosa* essential oil for the inhibition of dermatophyte growth. So, *C. torulosa* essential oil could lead to future developments involving it in health care product like in preparation of cream formulation and antiseptic against dermatophytes.

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