Bioactive potential of *Cedrus deodara* (Roxb.) Loud essential oil (bark) against *Curvularia lunata* and molecular docking studies

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Abstract

A focus on evaluation of in vitro antifungal activity of essential oil of *Cedrus deodara* (bark), against fungus *Curvularia lunata* was studied. In this study, the essential oil was extracted via hydro distillation method, further chemical compositions were identified by GC–MS and antifungal potential was assessed against three pathogenic fungal strains viz., *Alternaria alternata*, *Curvularia lunata* and *Bipolaris spicifera* through poison food technique. The essential oil showed strong antifungal potential against *Curvularia lunata*, further leading to isolation of antifungal fraction using TLC bio-autography against *Curvularia lunata*, the identification revealed the presence of ten molecules in antifungal fraction identified through GC–MS. Additionally, molecular docking study was applied to validate antifungal activity and results predicts strong binding affinity of isolated antifungal molecules with an important virulence factor of *Curvularia lunata*, a melanin biosynthetic enzyme trihydroxy naphthalene reductase (PDB ID-1YBV) confirming its antifungal potential. Thus, obtained results suggested the importance of essential oil from *Cedrus deodara* (bark) as a beneficial native source of the compounds with antifungal properties.

Keywords Antifungal activity · *Cedrus deodara* · Essential oil · Molecular docking

Abbreviations

EO Essential oil
CDEO *Cedrus deodara* essential oil
GC–MS Gas chromatography and mass spectrometry
TLC Thin layer chromatography

1 Introduction

Fungal pathogens are responsible for extensive damage to crops resulting in significant economic losses across the world [1]. Even though synthetic fungicides had been effectively used to control infections, the exponential increase in use of fungicides resulted in the development of fungicide tolerant pathogen strains, environmental

pollution as well as residual toxicity in the food chain recommended above safe limits [2]. These limitations imposed restrictions over the usage of synthetic fungicides, leading to promote new strategies that could support the regulations on plant protection products regulated by “The European Parliament” under the regulation (91/414/EC) and Environmental Protection Agency (EPA), they even suggested natural product as an alternative to control pathogens, as it is considered not to present side effects, eco-safe, economic and has an extensive public acceptance [3, 4]. Among natural antimicrobial products, plant-derived secondary metabolites like essential oils (EOs) are considered to have more potential to suppress the growth of plant pathogens and reduce disease development, without any

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side effects to the environment while being convenient as integrated pest management [5].

Essential oils are aromatic, complex, volatile, oily liquids obtained from different parts of plants. These are the mixtures of over 20 groups of chemical compounds, such as mono- and sesquiterpenes (terpenes, aliphatic aldehydes, alcohols, and esters) and nonvolatile components (hydrocarbons, fatty acids, sterols, carotenoids, waxes, coumarins, and flavonoids) produced by aromatic plants as secondary metabolites [6]. The composition of essential oil depends on several factors, including species, part of the plant, geographic region, agriculture method, and extraction procedures [7]. Various studies have attributed different biological activities to EOs such as antiviral, antiangiogenic, antispasmodic, carminative, analgesic, healing, expectorant, antiseptic, respiratory tract, antimicrobial, anti-inflammatory activities etc. [8, 9] and factors determining the activity of essential oils are mostly composition, functional groups present in active components and their synergistic interactions [10, 11].

Cedrus deodara (Roxb. ex D. Don) is a conifer that grows at altitudes of 1200–3000 m in the Himalayan regions of India, Pakistan, and Nepal. The plant is an evergreen tree belonging to the family Pinaceae and forms widespread forest along the Himalayan Mountain. The plant is traditionally used by people for thatching, sheltering, making furniture, as fuel wood and is also used extensively for medicinal purpose by the local people such as for treatment of fever, intestinal parasite, sinusitis, diarrhea, dysentery, rheumatism, diabetes, cancer, ulcer, antifungal, tuberculous glands inflammation, as insecticidal and larvicidal agent against stored pests and houseflies [12–14]. The wood oil is used for the treatment of arthritis, joints pain, cracks, body ache, skin rashes, external ulcers and itching, while bark oil is used to cure itching, dermatitis and relief from stomach worms [15, 16]. However, the potential of bark essential oil of *Cedrus deodara* was seemed to be least explored. Thus, the present study was focused on the antifungal properties of bark essential oil of *Cedrus deodara* against plant pathogens, as a natural fungicide.

2 Materials and methods

2.1 Plant material and essential oil extraction

The bark of *Cedrus deodara* was collected from the Bhaderwah region of Jammu and Kashmir, India (latitude 32.983025 and longitude 75.710983 and coordinates with 32° 58' 58.8900" N and 75° 42' 39.5388" E). Identification of the plant was done by a taxonomist of the Department of Botany, University of Jammu, Jammu and a voucher specimen has been deposited in the herbarium of the

department of botany, University of Jammu (Accession no: 15916 dated 7-08-2018). The *Cedrus deodara* bark was hydro-distilled at atmospheric pressure for 3 h, using a Clevenger-type apparatus. The essential oil was collected, stored and preserved in the dark at 4–10 °C for further analysis [17].

2.2 Characterization of essential oil by GC–MS

The chemical composition of *Cedrus deodara* (bark) essential oil was analyzed at the Indian Institute of Integrative Medicine (CSIR, India), Canal Road, Jammu, India by using GC–MS 4000 (Varian, USA) with a Varian CP-SIL 8CB column (30 m × 0.32 mm i.d.,) of 1 µm film thickness. An injector temperature was maintained at 230 °C. Oven temperature program used was holding at 60 °C for 5 min, further heating to 250 °C at 3 °C/min and maintaining the constant temperature at 250 °C for 10 min. Helium was used as a carrier gas with a constant flow of 1.0 ml/min with 0.20 µl injection volume. The Mass spectrometer scan parameters included electron impact ionization voltage of 70 eV, a mass range of 40–500 m/z. The essential oil components were characterized by comparing their mass spectra with those of the NIST05 (version 2.0) library [18].

2.3 Antifungal assay

The antifungal activity of essential oil was determined by poisoned food technique against three pathogenic fungal strains viz., *Alternaria alternata*, *Curvularia lunata*, and *Bipolaris spicifera*. Test essential oil was added to the sterilized potato dextrose agar and twenty milliliters of the medium was dispensed in the 9 cm Petri plate, with enough care taken to avoid trapping of air bubbles. Plates containing different concentrations of essential oil were inoculated with test fungal culture (5 mm bit) upside down. Inoculated plates were incubated at 26 °C. Hyphal growth was measured at every 24 h interval until the growth of test fungus in the control plate reached the edge of the plate. The experiment was conducted in triplicates and the results were expressed as average [19]. The antifungal index was calculated using the below formula:

$$\text{Antifungal index (\%)} = (D_a - D_b/D_a) \times 100$$

2.4 Fractionation of essential oil components by thin-layer chromatography

Components of the essential oil were separated on TLC plate (silica gel 60 F₂₅₄) using hexane: ethyl acetate or dichloromethane solvent (8:2) solvent system, further separation of analytes were checked by visualizing under

UV light (365 and 254 nm) or by spraying with vanillin/sulphuric acid spray reagent [20]. *R_f* values of the spots were calculated by the formula:

$$R.f. = \frac{\text{Distance moved by the spot}}{\text{Distance moved by the solvent front}}$$

2.5 Screening of antifungal activity by TLC-bioautography method

The antifungal activity of the essential oil components was screened by a direct bioautography method. In which, components of the essential oil were separated on the TLC plate and were dried to remove the residual solvent. Inoculum spray solution containing conidia of *Curvularia lunata*, fungal strain was prepared in potato dextrose broth. The plate was lightly sprayed with the inoculum spray solution until it appeared damp in color. Later, incubated in a dark moist chamber for 4 days at 26 °C. The incubation plate was sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma®) (INT). One of the replications of plate was developed with vanillin/sulphuric acid spray reagent. Clear zones on chromatograms show inhibition of growth. The fungal growth inhibition regions appeared with dark background [19]. White bands indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested organisms.

2.6 Isolation and identification of antifungal constituents

The TLC plates with a silica gel, 60 F₂₅₄ coated glass plate (20 × 20 cm, 500 µm thickness) were loaded with the essential oil and eluted with *n*-hexane/ethyl acetate 8:2, v/v) solvent. The inhibition regions were identified as spots on the TLC plate. Later, separation of compounds has been visualized under UV light (365 and 254 nm) and also by spraying with sulphuric acid spray reagent. The antimicrobial constituents were detected and were isolated by scraping off the detected region and shifted to the percolator. Finally, these constituents were eluted with silica gel, column chromatography method with dichloromethane solvent [21]. The isolated fractions of the essential oil were analyzed by GC–MS and identification of the components was based on a comparison of their mass spectra with those of the NIST05 (version 2.0) library.

2.7 Docking analysis

The binding sites, binding affinity, and interactions studies of the isolated antifungal compounds from *Cedrus*

deodara (Bark) essential oil were done with virulence protein tri-hydronaphthalene reductase (ID-1YBV) of *Curvularia lunata* by docking studies. The 3D structure of the protein with 4A resolution was considered and extracted from <https://www.rcsb.org/> and with Biovia the 3D structures of the 10 compounds were designed. The molecular docking studies were performed using GEMiDOCK software and to visualize the interactions generated from the docking studies the Molegro viewer tool was used [22, 23].

3 Results and discussion

3.1 Essential oil characterization

The yellow-colored essential oil was obtained from *Cedrus deodara*, bark with a 0.50% (v/w) yield. The characterization of the essential oil through gas chromatography and mass spectrometry (GC–MS) revealed the presence of thirty (30) components accounting for 99% of total components present in essential oil. Mostly it constitutes the high amounts of oxygen-containing monoterpenes, 44.67% was monoterpenes, 18.88% diterpenes, and 36.43% sesquiterpenes as well as abundance of α-pinene (24.28%), cis-Ocimene (14.11%), cembrene (6.69%), α-Copaene (6.56%), α-terpineol (3.73%) & verticellol (3.03%) respectively (Table 1 and Fig. 1). The previous study reported several compounds mainly α-himacholone, β himacholone, α-pinene, β-pinene, myrcene, himachalene, cis-atlantone, α-atlantone from wood oil [24, 25], further, borneol, bornyl acetate, limonene, camphene, α-pinene were also reported from pine needle oil of *Cedrus deodara* belonging to different region and places [26]. The variation in essential oil chemotypes was not always concordant with the bioclimatic zones and observed to be rather linked with the geographic location as well as various local selective forces acting on the chemotype diversity such as abiotic (topography, moisture, temperature, and edaphic factors) and/or biotic selective factors (associated fauna and flora), genotypic variability, etc. contribute to the emergence of different chemical profiles [22, 27, 28].

3.2 Antifungal activity

Fungi are the leading plant pathogens that are responsible for several plant diseases [29]. Among many plant pathogens, *Curvularia lunata*, *Alternaria alternata* and *Bipolaris spicifera* are responsible for many plant diseases such as leaf spot, Southern corn leaf blight, northern corn leaf blight, corn smut, etc. [30–32]. In this study, essential oil of *Cedrus deodara* bark (CDEO) was explored against fungal pathogens *Curvularia lunata*, *Alternaria alternata* and *Bipolaris spicifera* by poisoned food technique. As depicted

Table 1 Major constituents of fraction of *Cedrus deodara* bark essential oil (CDEO) as determined by GC–MS

S. No.	Compound name	Nature of compound	Percentage (%)
1	alpha pinene	Monoterpene	24.28
2	Cisocimene	Monoterpene	14.11
3	Limonene	Monoterpene	1.73
4	Bicyclo[2.2.1]heptane-2,5-diol, 1,7,7-trimethyl-, (2-endo,5-exo)	Monoterpene	0.82
5	alpha terpineol	Monoterpene	3.73
6	alpha longipinene	Sesquiterpene	2.50
7	alpha-Copaene	Sesquiterpene	6.56
8	alpha ylangene	Sesquiterpene	1.00
9	Longifolene	Sesquiterpene	8.28
10	Caryophyllene	Sesquiterpene	2.28
11	alpha-Humulene	Sesquiterpene	0.64
12	alpha-Muurolene	Sesquiterpene	1.12
13	e-Cadinene	Sesquiterpene	0.59
14	1H-Naphtho[2,1-b]pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-, [3R(3a,4aa,6aa,10aa,10ba)]-	Sesquiterpene	2.99
15	Iso-Velleral	Sesquiterpene	1.73
16	Epjuvabione	Sesquiterpenes	2.22
17	Propanoic acid, 2-methyl-, (dodecahydro-6a-hydroxy-9a-methyl-3-methylene-2,9-dioxoazuleno[4,5-b]furan-6-yl) methyl ester, [3aS-(3aa,6a,6aa,9aa,9ba)]-	Sesquiterpenes	0.61
18	Verticellol	Sesquiterpene	3.03
19	alpha gurjunene	Sesquiterpene	1.76
20	alpha ionone	Sesquiterpene	1.12
21	Cambrene	Diterpene	6.69
22	CembreneA	Diterpene	0.89
23	Kaurene	Diterpene	1.13
24	Isopimaral	Diterpene	2.13
25	Dehydroabietal	Diterpene	0.81
26	4-epi-abietal	Diterpene	1.43
27	Abietal	22	2.42
28	Iso pimaradien-3-ol	Diterpene	0.89
29	Glycocholic acid	Diterpene	0.63
30	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandro-8-en-17-yl)-	Diterpene	1.86

Fig. 1 GC–MS chromatogram of *Cedrus deodara* essential oil (bark) CDEO

in Fig. 2 all the pathogens were susceptible to essential oil of *Cedrus deodara* bark (CDEO) and formed different zones of inhibition showing its antifungal effect. Among all the test fungal pathogens essential oil was more sensitive against *Curvularia lunata* (IC_{50} value of $2.22 \pm 0.13 \mu\text{l/ml}$) followed by *Alternaria alternata* ($3.71 \pm 0.12 \mu\text{l/ml}$) and least antifungal effect was observed against *Bipolaris spicifera* ($4.8 \pm 0.34 \mu\text{l/ml}$), here dimethyl sulphoxide and chloramphenicol were used as negative and positive controls. The susceptibility of fungi to essential oil was based on growth inhibition percentage and it varied according to fungi strains and concentrations. Earlier also antifungal activity against *Aspergillus fumigatus*, *Aspergillus niger* and *Geotrichum candidum* were reported from leaves and root oil of *C. deodara* [33, 34]. The antifungal activity of *C. deodara* oils may be mainly attributed due to its richness of monoterpenes, whereas wide variety of essential oils are known to possess antifungal properties and in many cases, this activity is due to the presence of active constituents, such as monoterpenes, sesquiterpenes, and related

alcohols, hydrocarbons, and phenols [35]. Additionally, monoterpenes diffuse into pathogens and damage cell membrane structures resulting in its death [36, 37].

3.3 Isolation and identification of antifungal constituents

As the essential oil was found to be more sensitive against fungal pathogen *Curvularia lunata* with minimum inhibitory concentration (IC_{50} value of $2.22 \pm 0.13 \mu\text{l/ml}$) thus, to isolate the antifungal compounds *Curvularia lunata* was considered as a test organism and by using direct TLC bio-autography technique antifungal constituents were separated. Bioautography is a microbial detection method hyphenated with planar chromatography techniques. It is based mainly on antimicrobial or antifungal properties of analysed substances, this technique links separation on an adsorbent layer with biological tests performed directly on it. Therefore, it is a very convenient method for searching plant constituents with biological activity such as antimicrobial. In this study, the phytocompounds present in the essential oil of *Cedrus deodara* bark were separated using TLC plate, which showed two clear zones (zones of inhibitions) against dark background at R_f values 0.34 (Fig. 3a). The appearance of clear zones indicated the presence of active compounds present in essential oil possessing significant antifungal activity (Fig. 3a), these were further concentrated and isolated by preparative TLC (Fig. 3b) and identified by GC-MS. The isolated fractions were further subjected to TLC and bio-autography to test the purity and activity. The antifungal fraction separated from *C. deodara* bark essential oil against *Curvularia lunata* on identification revealed to have mixture of ten compounds viz., cambrene, verticellol, isovelleral, epijuabione, cembrene A,

Fig. 2 IC_{50} value of *Cedrus deodara* essential oil (bark) CDEO against *Curvularia lunata*, *Alternaria alternata* and *Bipolaris spicifera*

Fig. 3 TLC of essential oil from *Cedrus deodara* essential oil (bark) and in situ localization of antifungal components, **a** detection of analytes of essential oil on TLC plate and visualization of antifungal compounds using Bioautography, **b** Preparative TLC for isolation of antifungal molecule

velleral, α -gurjunene, iso methyl ionone, sclareol, cyclooctasiloxane and hexadecamethyl respectively (Fig. 4 and Table 2). These results are in agreement with those previously reported for EOs of different *M. piperita* species [36]. Conceivably, the antifungal activity, as well as the synergistic effect of EO, may be attributed to the high percentage of oxygenated monoterpenes, which represent the major components of the CDEO. Earlier studies reported the antifungal potential of sesquiterpenes isolated from leaves of *C. deodara* against *Aspergillus flavus*, *A. niger*, *A. ochraceus*, *A. parasiticus*, and *A. sydowii* [38]. Similarly, our observation also highlights that bark essential oil of *C. deodara* that is rich in terpenes family molecules and may be responsible for antifungal properties especially due to the synergistic effect of constituents present in the antifungal fraction. It is also important to mention here that several in vitro studies have highlighted the potential synergistic effect of the essential oils and their components against various human and plant pathogens, accordingly several modes of action have also been put forward by which antibiotics and the essential oil hit multiple targets through physicochemical interactions while inhibiting antibacterial-resistance mechanisms [39, 40].

3.4 Molecular docking analysis

Additionally, docking analysis of the identified antifungal molecules of *Cedrus deodara* (bark) essential oil was done with antifungal protein tri-hydro naphthalene reductase (ID-1YBV) of *Curvularia lunata*, a facultative fungal pathogen causing many plant diseases such as *Curvularia* leaf spot in the maize leaf. To uncover the pathogenic mechanisms of diseases, most of the research has focused on identifying virulence factors, such as melanin or toxins

Table 2 Compounds identification by GC–MS analysis from antifungal fraction of *Cedrus deodara* bark essential oil (CDEO)

S. no	Compound name	Retention time	Area (%)
1	Cambrene	34.20	67.23
2	Verticellol	32.41	9.61
3	Iso-Velleral	31.48	8.20
4	Epjuvabione	31.60	4.46
5	Cembrene A	34.69	2.78
6	Velleral	32.03	2.37
7	α -gurjunene	32.98	1.77
8	Iso methyl ionone	33.20	1.55
9	Sclareol	33.43	1.08
10	Cyclooctasiloxane, hexadecamethyl	0.97	23.29

[41, 42]. In *Curvularia lunata*, melanin production plays a significant role in disease dissemination by accumulating melanin metabolites. Fungal melanins are formed by the oxidative polymerization of phenolic compounds that are essential to enhance the mechanical strength of the infection into the host plant epidermis through the formation of the highly specialized infection cell, known as the appressorium (melanized conidia), facilitating adhesion to the plant surface and penetration of the hypha into the host tissues, contributing to virulence in numerous types of plant diseases [43]. Melanin biosynthesis starts with a polyketide synthase using acetate as a substrate the hydroxynaphthalene reductase that reduces 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-tetra-HN) to scytalone and further dehydratase metabolizes scytalone to 1,3,8-trihydroxynaphthalene (1,3,8-tri-HN) [44, 45]. Thus, in this study docking studies were conducted

Fig. 4 GC–MS chromatograph of antifungal C_1 fraction isolated from *Cedrus deodara* essential oil (bark)

Fig. 5 In silico molecular docking of identified antifungal compounds from CDEO with tri-hydronaphthalene reductase (ID-1YBV) protein

to analyze the binding affinity of the antifungal compounds with protein tri-hydro naphthalene reductase (ID-1YBV) as indicated by various reports of its involvement with melanin biosynthetic pathway [41, 42]. All the antifungal compounds (1-10) showed active binding sites on tri-hydronaphthalene reductase (ID-1YBV) protein, whereas compound no 3, 6 and 9 (Table 2) showed the highest binding affinity towards the protein, suggesting these binding residues may have a predominant role in antifungal activity. Also, interaction studies revealed that all the identified compounds from series 1-10 enter the hydrophobic region of 1YBV and interact with Gly 210,

Ile 211, Thr 213, Try 178 having hydrogen bonding affinity with the ligand molecules. In addition, there are also steric interactions with Ile 211, Pro-208, Ile-41, Ser-164, Gly-210, Gly-163, Pro-208, Met-215, Tyr-216, Met-162, Ser-164, Thr-213, Met-215 (Fig. 5 and Table 3). It may be possible that these reduction steps of melanin biosynthesis were repressed by the compounds of essential oil leading to the inhibition of the DHN-melanin biosynthesis pathway and further its antifungal effect. Thus, the results obtained from *in silico* studies are in agreement with the *in vitro* antifungal studies of *Cedrus deodara* (bark) essential oil.

Table 3 Hydrogen and steric interactions binding residues of tri-hydronaphthalene reductase (ID-1YBV) protein of *Curvularia lunata* with identified antifungal compounds of CDEO as determined by molecular docking

S. no.	Compound name	H interactions	Steric interactions
1	Cambrene	Gly-210, Ile-211	Pro-208
2	Verticellol	Try-178, Lys-182, Gly -210,	Ile-211
3	Isovelleral	Arg-39, Gly-42, Asn-114, Try-178	Ile-41, Ser-164, Gly-210
4	Epiluvabione		Gly-210,Ile-211
5	Cembrene A	Ile-211	Ile-211,Pro-208
6	Velleral	Gly-210, Ile-211,Thr-213	Gly-163,Pro-208,Met-215,Tyr-216
7	α -gurjunene		Gly-163,Gly-210, Ile-211
8	Iso methyl ionone		Gly-210, Ile-211
9	Sclareol	Thr-213	Met-162,Ser-164,Gly-163,Pro-208,Gly-210,Thr-213, Met-215
10	Cyclooctasiloxane, hexadecamethyl	Thr-213	Gly-210,Ile-211

4 Conclusions

In this study, chemical composition and antifungal property of bark essential oil from *Cedrus deodara* were evaluated. The isolated antifungal molecules showed the antifungal property synergistically, offering possibilities for the treatment of fungal diseases caused by *Curvularia lunata* by inhibiting pentaketide melanin biosynthesis as confirmed by in vitro and molecular docking studies. Based on present results bark essential oil of *Cedrus deodar* and its components holds the therapeutic potential to establish itself as a natural antifungal agent in fungal disease management and could be suggested as an alternative to synthetic fungicides.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest, financial or otherwise.

Ethics approval and consent to participate Not applicable.

Human and animal rights No Animals/Humans were used for studies that are the basis of this research.

Consent for publication Not applicable.

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