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Bioautography Guided Isolation and Characterization of Antimicrobial Compounds of *Picea smithiana*

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Abstract

The present study targeted to explore the antimicrobial constituents of *Picea smithiana*. It's essential oil was analyzed by GC-MS and result showed that it has rich content of monoterpene compounds, and α-pinene (38.82%), β-pinene (7.41%), camphene (7.75%), Beta phellandrene (6.35%), a-bisabolol (5.60%), L-bornyl acetate (3.86%), Limonene (3.80%) and α-salinene (3.30%) were the major components. The results showed that the essential oil exhibited good antibacterial activity against *Micrococcus luteus, Bacillus subtilis* and *Pseudomonas alcaligenes* and moderate activity against *Alcalygens denitrificans, Campylobacter coli, Enterococcus fecalis* and *Pseudomonas aeruginosa* with zone of inhibition ranging from 6-15mm and significant antifungal activity against *Bipolaris specifera* and *Curvularia lunata* with IC_{50} value 2.29mg/ml and 2.87mg/ml (w/v) respectively. Whereas, both leaf and bark extracts showed comparatively lower antimicrobial activity against most of the test organisms. Two antimicrobial compounds were isolated from *Picea smithiana* essential oil by using bioautography, preparative TLC methods and identified as alpha-selinene and alpha-bisabolol and GC-MS analysis.

INTRODUCTION

Plants used in traditional medicine are effective in treating various diseases caused by oxidative stress and microbial infections. Most of the therapeutic properties of medicinal plants are attributed to their biologically active compounds like phenols, flavonoids, terpenoids, saponins etc. which are considered to be potential metabolites [1,2]. Researchers are interested in biologically active compounds from natural sources for the elimination of human and plant pathogens because of the resistance that microorganisms have built against synthetic antimicrobial agents [3]. Moreover, synthetic antibiotics are sometimes associated with adverse effects like hypersensitivity, immune suppression etc [4]. So, there has been growing interest in the research concerning alternative bioactive compounds of natural origin that are relatively less toxic to animals and environment [5].

Picea smithiana (Wall.) Boiss (Family: Pinaceae) is an evergreen tree commonly called Morinda Spruce, usually found in the Himalayan range at an altitude of 2400-3600 m in association with silver fir and deodar. The plant also has some commercial application like timber, paper, and food additives. Its edible parts like, Young male and female cones used as a flavoring agent. Dried bark of this plant is used as a thickener in soups or added to cereals when making bread. Moreover, it's essential oil also being used in room spray, deodorants [6]. Plants belonging to Pinaceae family, like *Pinus roxburgii, Pinus wallichaina* and *Cedrus deodara* etc. have abundant commercial uses and have also been scientifically explored for various biological activities. Essential oil of other species like *Picea abiesis* is used in the treatment of catarrhal diseases of children by inhalation with hot water and for rheumatic and neuralgic [7]. However, limited scientific reports on *Picea smithiana* are available to support its biological activity and its active components. Therefore, biochemical exploration of this plant is required, as there are possibilities of presence of potential bioactivity and bioactive compounds.

The present study aims to explore the antibacterial and antifungal properties of the essential oil and extracts of *Picea smithiana* and isolation and characterization of active compounds from its essential oil.

MATERIAL AND METHODS

Plant material and Isolation of essential oil

Plant material was collected from Seoj dhar region of Bhaderwah, Jammu and Kashmir. Identification of the plant was done by Dr. Harish Datt, Assistant Professor and taxonomist of Department of Botany, University of Jammu, Jammu and a voucher specimen has been deposited in the herberium of the department of botany, University of Jammu (Accession no: 14613). Fresh leaves of *Picea smithiana* were subjected to hydro-distillation for 4h in a Clevenger type apparatus for isolation of essential oil. The extracted oil was dried over anhydrous sodium sulphate and stored at low temperature.

Preparation of extracts

Some plant material (leaf and bark separately) was shade dried and powdered in an electronic grinder. Three types of extracts were prepared in three different solvents *viz.,* chloroform, methanol and water. 100g of dried plant material was extracted thrice for each solvent. Resulting extracts were pooled, filtered and the volume was reduced to 50 ml using rotary vacuum evaporator and finally lyophilized to dried powder.

GC-MS analysis of essential oil

Chemical composition of *Picea smithiana* essential oil was analyzed at Indian Institute of Integrative Medicine (CSIR, India), Canal Road, Jammu, India. System used for analysis is GC-MS 4000 (Varian, USA) with a Varian CP-SIL 8CB column (30m×0.32mm i.d., 1µm film thickness). Injector temperature was 230°C. Oven temperature program used was holding at 60°C for 5min, heating to 250°C at 3°C/min and keeping the temperature constant at 250°C for 10min. Helium as a carrier gas used at a constant flow of 1.0 ml/min and an injection volume used was 0.20µl. 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 NIST05 (version 2.0) library [8].

Antibacterial assay

Screening of the essential oil/extracts for antibacterial activity was carried out by agar well diffusion assay against five Gram positive *viz., Bacillus subtilis* MTCC2389*, Bacillus cereus* MTCC430*, Staphylococcus aureus* MTCC7443, *Enterococcus faecalis* MTCC439, *Micrococcus luteus* MTCC4821, and five Gram negative strains *Pseudomonas aeruginosa* MTCC2642, *Pseudomonas alcaligenes* MTCC493, *Campylobacter coli* MTCC1126, *Escherischia coli* MTCC212, *Alcaligenes denitrificans* MTCC299. Sterilized nutrient agar (20ml) was inoculated with 100µl bacterial suspension (10^8 CFU/ml) and poured into a sterilized petri plate. Plates were allowed to solidify and a well of 6mm was aseptically bored into the agar plate by using a cork borer. Essential oil (20µl) or extract (2mg) dissolved in DMSO was added into each well. Finally the plates were kept for incubation at 37ºC for 24h. Chloramphenicol (10µg) was used as positive reference.

Antifungal assay

The antifungal activity of essential oils and extracts was determined by Poisoned food technique against three pathogenic fungal strains *viz., Alternaria alternata, Curvularia lunata*, and *Bipolaris specifera*. Test essential oil or extract was added to the sterilized potato dextrose agar in 9cm petri plate. Plates containing different concentration of essential oil or extract were inoculated with test fungal culture (5mm bit) upside down. Inoculated plates were incubated at 26ºC. Hyphal growth was measured at every 24h interval till 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 [9].

Fungal growth inhibition (%) = $(D_a - D_b / D_a) \times 100$

D_a: Diameter of fungal growth in control plate; D_b: Diameter of fungal growth in test plate

Screening of antimicrobial acitvity By TLC-Bioautography method

Components of the essential oil were separated on TLC plate (silica gel 60 F_{254}) using hexane:ethyl acetate (8:2) solvent system and separation of analytes was checked by visualized under UV light (365 and 254 nm) or by spraying with vanillin/sulphuric acid spray reagent.

To screen the antibacterial activity of the components of essential oil, direct bioautography was performed [10]. Components of the essential oil were separated on TLC plate and dried to remove the residual solvent. *Bacillus subtilis* as test organism was then allowed to grow on the TLC plate and incubated at 37ºC for 24 h in humid conditions. After incubation plate was sprayed with 2mg/ml solution of INT (Iodo-nitro tetrazolium). One of the replication of plate was developed with vanilin/sulphuric acid spray reagent. Clear zones on chromatogram shows inhibition of growth.

For antifungal activity of the essential oil components, inoculum spray solution containing conidia of test fungal strain was prepared in potato dextrose broth. TLC plate was developed as mentioned previously. The plate was slightly sprayed with the inoculum spray solution until the plate appeared damp in colour and incubated in dark moist chamber for 4 days at 25ºC. Fungal growth inhibition zones appeared against the dark background [11].

Isolation and identification of antimicrobial constituents

Once the spots (corresponding to the zones of inhibition) on TLC plate had been identified, Preparative TLC was performed by loading the essential oil onto a silica gel 60 F_{254} coated glass plate (20 × 20 cm, 500 m thickness) and developed in *n*-hexane/ethyl acetate (8:2, v/v) solvent system [12]. The separated compounds were visualized under UV light (365 and 254 nm) or by spraying with vanillin/sulphuric acid spray reagent. The isolation was carried out by scrapping off the detected zones corresponding to the antimicrobial constituents and transferring them into percolator. Finally, the constituents were eluted from silica gel by dichloromethane.

The isolated fractions of the essential oil were analyzed by GC-MS and identification of the components was based on

comparison of their mass spectra with those of NIST05 (version 2.0) library.

RESULTS AND DISCUSSION

Hydro-distillation of fresh needles of *Picea smithiana* yielded 0.9% pale yellow colored essential oil. Chemical composition of the essential oil was analyzed by Gas Chromatography and Mass Spectrometry (GC-MS). Analysis showed the presence of twenty seven (27) components accounting for 99% of total components of the essential oil. The list of identified compounds has been given in Table (1). α-pinene (38.82%), β-pinene (7.41%), camphene (7.75%), β-phellandrene (6.35%), α-bisabolol (5.60%), L-bornyl acetate (3.86%), limonene (3.80%) and α-salinene (3.30%) were the major components found in the oil. The oil contained eleven monoterpenes (74.37%); five oxygenated monoterpenes (5.72%); and eleven sesquiterpenes compounds (19.48%). In general, the oil is rich in monoterpenes and with α-pinene as the major component. Chemical composition of the essential oil of *Picea smithiana* growing in Gulmarg region of J&K, India, has also been reported by other researcher [13]. Their report is in agreement that the essential oil is rich in monoterpenes (85.5%), although they observed good content of δ-3-carene (26%) and limonene (25%), and less amount of α-pinene (6.6%) in essential oil of *Picea smithiana* from Kashmir. Sharma et al. [14], also analyzed the chemical composition of *P. smithiana* essential oil and identified Seventeen (17) components by GC and GC-MS. Their analysis also shows the richness of monoterpenes components (66.0–86.8%), oxygenated monoterpenes (0–0.6%), sesquiterpene hydrocarbons (12.0–16.1%) and oxygenated sesquiterpenes (3.5–6.6%) in *P. smithaiana* essential oil. Major components of *P. smithiana* essential oil reported by Sharma et al., and those presented in current studies, does not very much in terms of percentage (Table 1).

Table 1: Chemical composition of *Picea smithiana* essential oil with their respective amount.

S. no. (%)	Name of the compound	Nature of the compound	Amount
1	Santene	Monoterpene	1.91
2	Alpha pinene	Monoterpene	38.82
3	Camphene	monoterpene	7.75
4	Beta pinene	monoterpene	7.41
5	Alpha-phellandrene	monoterpene	3.15
6	Alpha terpinene	monoterpene	0.5
7	Para cymene	monoterpene	0.68
8	Limonene	monoterpene	3.8
9	Beta phellandrene	monoterpene	6.35
10	Terpinolene	monoterpene	0.83
11	5-isopropyl-2-methylbicyclo[3.1.0] hexane-2, 3- diol	monoterpene	3.17
12	Alpha terpineol	Oxygenated monoterpene	0.48
13	p-menth-2-en-1-ol	Oxygenated monoterpene	2.75
14	Trans-piperitol	Oxygenated monoterpene	1.4
15	Geranyl formate	Oxygenated monoterpene	0.72
16	Piperitone	Oxygenated monoterpene	0.37
17	L-bornyl acetate	sesquiterpene	3.86
18	Beta caryophyllene	sesquiterpene	1.67
19	Alpha caryophyllene	sesquiterpene	1.16
20	c-murolene	sesquiterpene	0.58
21	Germacrene D	sesquiterpene	1.19
22	Delta elemene	sesquiterpene	0.66
23	Beta bisabolene	Sesquiterpene	0.53
24	Delta cadinene	Sesquiterpene	0.85
25	Alpha gurjunene	Sesquiterpene	0.08
26	Alpha selinene	Sesquiterpene	3.3
27	Alpha bisabolol	Sesquiterpene	5.6
Monoterpenes = 74.37; Oxygenated monoterpenes = 5.72; Sesquiterpenes = 19.48			

The results of antibacterial activity assay of the essential oil and different extracts have been shown in Table (2). The test components were tested against five Gram positive and five Gram negative bacteria. Essential oil strongly inhibited the growth of *Micrococcus luteus, Bacillus subtilis, Pseudomonas alcaligenes* and *Alcaligenes denitrificans* (zone of inhibition more than 10mm) and moderate activity against *Campylobacter coli, Enterococcus fecalis, Pseudomonas aeruginosa, Bacillus cereus* and *staphylococcus aureus*. Only *Escherichia coli* has not shown sensitivity to essential oil. Among the three extracts, only chloroform extract of leaf showed moderate activity (4-8mm) against most of the test bacteria except *Bacillus cereus* and *Pseudomonas aeruginosa,* whereas, methanolic leaf extract proved sensitive only against *Staphylococcus aureus, Alcaligenes denitrificans, Pseudomonas alcaligenes* and *Campylobacter coli*. Aqueous extract of leaf and bark showed negligible antibacterial activity. Sati and Joshi [15] investigated the antibacterial activity of different extracts of *Picea smithiana* growing in Nanital, Kumaun. Other species of *Picea* have also been investigated for antibacterial activity. Canillac and Mourey [16] observed effective inhibitory activity of *Picea excelsa* essential oil against different strains of *Listeria ivanovii*, *Listeria monocytogenes* and *Staphylococcus aureus* (Table 2).

Antifungal activity of the extracts and essential oil of *Picea smithiana* was determined by poisoned food technique. The results of antifungal activity of the *Picea smithiana* are mentioned in Table (3). Essential oil possesses potential antifungal activity against *Bipolaris specifera* and *Curvularia lunata* with IC_{50} value 2.29mg/ml and 2.87mg/ml (w/v) respectively. Methanol extract of *Picea smithiana* showed poor antifungal activity against *Bipolaris specifera* and *Alternaria alternata* with IC_{50} values 3.9mg/ml and 4.3mg/ml respectively. Chloroform and aqueous extracts did not inhibit the growth of any of the three fungal strains (Table 3).

Antibacterial activity of essential oil was also determined on TLC plate by direct bio-autography technique using *Bacillus subtilis* as test organism. Two clear zones (zones of inhibitions) against blue background were observed on bacterial bio-

Table 2: Antibacterial activity of the essential oil and extracts of *Picea smithiana.*

Bacterial strain	Zone of inhibition (in mm)							
	E.oil	LME	LAE	LCE	BME	BAE	BCE	Control
B. subtilis	14±0.6	06±0.2	7±0.3	6±0.3	5±0.2	-	7±0.2	20±0.9
S. aureus	06±0.2	5±0.2	-	7±0.3	-	-	-	11±0.4
E. coli	-	-	-	4±0.2	-	-	-	7±0.3
E. fecalis	9±0.4	-	-	5±0.2	-	-	4±0.2	11±0.5
B. cereus	7±0.3	-	-	-	-	-	-	15±0.4
M. luteus	15±0.5	-	-	5±0.2	6±0.3	4±0.2	-	28±1.3
A. denitrificans	10±0.4	7±0.3	-	8±0.3	-	-	-	17±0.7
P. aeroginosa	9±0.3	-	-	-	-	-	-	20±0.8
P. alcalygens	12±0.6	9±0.4	-	5±0.2	-	-	-	15±0.6
C. coli	9.5±0.3	5±0.2	-	6±0.3	-	-	0	11±0.5

Data presented as mean ± standard deviation; E.oil= essential oil; LME: Leaf Methanol Extract; LAE: Leaf Aqueous Extract; LCE: Leaf Chloroform Extract; BME: Bark Methanol Extract; BAE: Bark Aqueous Extract; BCE: Bark Chloroform Extract.

Table 3: Antifungal activity of the essential oil and extracts of *Picea smithiana.*

Fungal strain	Antifungal activity (IC_{50} values) mg/ml						
	E.oil	LCE	LME	LAE	BCE	BME	BAE
A. alternata	4.08± 0.2	-	4.3±0.15	-	-	-	-
C. lunata	2.87±0.12	-	-	-	-	-	-
B. specifera	2.29±0.11	-	3.9±0.13	-	-	-	-

Data presented as mean ± standard deviation; LME=Leaf methanol extract; LAE=Leaf aqueous extract; LCE=Leaf chloroform extract; BME=Bark Methanol extract; BAE=Bark aqueous extract; BCE=Bark chloroform extract.

autography plate at R_f values 0.72 and 0.79. The clear zones demonstrated the presence of two antibacterial compounds/ metabolites in the essential oil. Similarly, direct bioautography was performed to analyze the antifungal activity using *Alternaria alternata* as test organism. Zones of inhibition on fungal bio-autography plate were observed as clear zones against dark background, and found at the same position as in case of bacterial bio-autography (at R_f values 0.72 and 0.79). Appearance of clear zones clearly indicated the presence of two active compounds present in essential oil possess both antibacterial and antifungal activity and marked as P_1 and P_2 (Figure 1).

The compounds correspond to the zones P_1 and P_2 at R_f values 0.72 and 0.79, were further concentrated and isolated by preparative TLC. After concentrating the isolates, both fractions were again tested for their potential activity by bacterial and fungal bioautography technique. After incubation prominent spots were observed at their respective positions (ie. R_f values 0.72 and 0.79). Afterwards both isolates of *Picea smithiana* essential oil were analyzed and identified by GC-MS. The two active constituents of *Picea smithiana* essential oil corresponding to P_1 and P_2 was analyzed by GC-MS and identified as α-selinene and α-bisabolol. To the best of our knowledge, this is the first report that unveiled the antimicrobial compounds of *Picea smithiana*. The amount of α-selinene and α-bisabolol in the crude essential oil has also been determined by GC-MS and was found to be 3.30% and 5.60% respectively. α-bisabolol also known as Levomenol, is a monocyclic sesquiterpene and one of the component of German Chamomile plant [17,18]. Sesquiterpenes constitute a group of secondary metabolites, and some of them

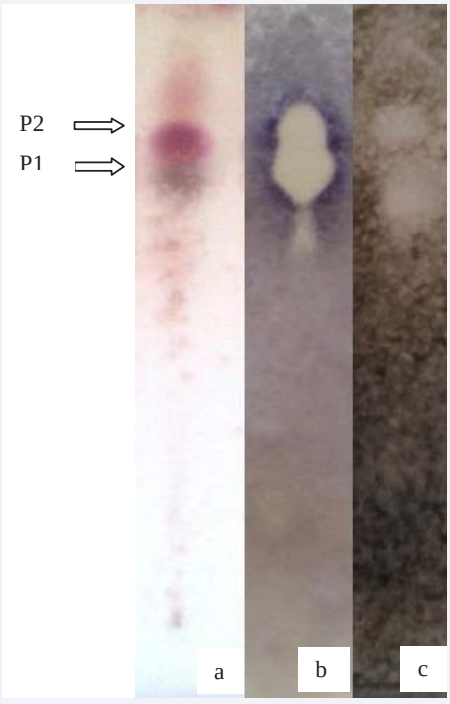

Figure 1 TLC of essential oil from *Picea smithiana* and *in situ* localization of antimicrobial components, (a) Detection of analytes of essential oil with vanillin/sulphuric acid reagent on TLC plate; (b & c) Visualization of antibacterial and antifungal compounds (P1 and P2) of *Picea smithiana* essential oil using bioautography.

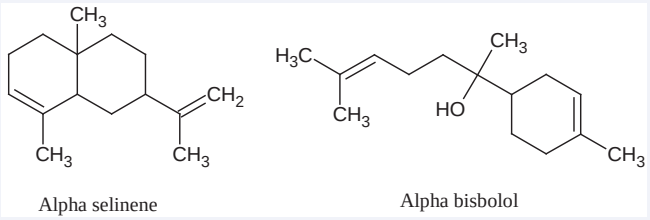

Figure 2 Chemical structure of the antimicrobial compounds isolated from *Picea smithiana* essential oil.

are stress compounds formed during injury or disease. They have properties like anti-inflammatory, anti-septic, analgesic and anti-allergic. U.S. Food and drug administration has also approved this compound as safe and already been used in skin care treatments as moisturizer, sunscreen, anti-aging and eye cream etc. [19]. α-bisabolol has also been used to enhance the percutaneous absorption of some other molecules [20]. Forrer and co-workers [21] also reported antimicrobial activity of α-bisabolol against *Solobacterium moorei*. α-Selinene is one of the major components of celery oil (*Apium graveolens*) and other plants like, ginger (*Zingiber officinale*). Shah and Dhar [13] have reported other biological activities of the essential oil of *Picea smithiana* growing in Gulmarg region of J&K, India. They reported that the essential oil has significant antioxidant and anti proliferative potential against different human cancer cell lines. Analysis of essential oils reported by Sharma et al. [14], also established that α-bisabolol is present in various gymnosperms including *Abies pindrow*, *Picea smithiana* and *Cedrus deodara* and its concentration varies around 3.5–6.6% in different sources (Figure 2).

CONCLUSION

The present study demonstrated the chemical composition and antimicrobial potential of essential oil and different extracts of *Picea smithiana* growing in Bhaderwah region of Jammu and Kashmir. Essential oil showed significant inhibitory activity against wide range of bacterial and fungal strains. Further it was investigated that α-selinene and α-bisabolol were the active compounds responsible for the antimicrobial activity of *Picea smithiana.*

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