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Comparative GC-MS analysis, antioxidant, antibacterial and anticancer activity of essential oil of *pinus wallichaina* from Kashmir, India

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ABSTRACT

To evaluate chemical composition, antibacterial, antioxidant and anticancer activity of *Pinus wallichiana* essential oil. The oil was extracted by hydro-distillation which was analysed through GC-MS. The antibacterial activity was evaluated by agar well diffusion method and antioxidant activity was evaluated through DPPH assay while as anticancer activity was evaluated through MTT method. Beta-pinene and alpha-pinene were the major constituents present in the oil. This oil showed significant antibacterial, antioxidant and anticancer activity

Introduction

Reactive oxygen species (ROS) comprise various forms of activated oxygen including superoxide radical (o_2^-), hydroxide radical ($^{\cdot}oH$), hydrogen peroxide (H_2O_2), nitric oxide($^{\cdot}NO$) and peroxynitrite ($ONOO^-$) which often are generated as by products of biological reactions or from exogenous factors (Pan et al.., 2008). In healthy individuals ROS production is continuously balanced by natural anti-oxidative defence systems. Oxidative stress is a process where the physiological balance between pro-oxidants and antioxidants is disrupted in favour of the former leading to potential damage to the organism (Halliwell and Gutteridge, 1990). Essential oils and their components are gaining interest because of their relatively safe status, their widespread acceptance by consumers and their explortation for potential multipurpose functional use (Ormancey, Sisali, and Contiere, 2001). The auto-oxidation of lipids as well as the enzymatic oxidation of fats, oils and fat containing food during storage and processing are responsible for rancidity and deterioration of food quality. To overcome these problems essential oils are extensively used in food industry (Karanika, Komaitis and Aggelis, 2001).

The indiscriminate use of antimicrobial agents has resulted in the emergence of a number of drug resistant bacteria. To overcome the increasing resistance of pathogenic microbes, more resistant antimicrobial agents with novel modes of action must be developed. Medicinal plants used in traditional medicines to treat infectious diseases seems to be an abundant source of new bioactive secondary metabolite. Therefore in the last few years, a variety of medicinal plants and plant extracts have been secreened for their antimicrobial activity[Cantrell CL, Fishcher NH, Urbatsch L,Guire MS, and Franzblau SG, 1985, Cowann MM, 1999].

Essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties [Burt SA, 2004: Kordali S, Kottan R, Mavi A, Yildrim A, 2005]. Some oils have been used in cancer treatment [SylvestreM. Pchette A, Longtin A, Nagau f, 2006]. Essential oils are a rich source of biologically active compounds. There has been an increased interest in looking antimicrobial properties of extracts from aromatic plants particularly essential oils [Mihau G, Valentin A, Benoit F, Mallie M, 1997]. Medicinal and aromatic plants and their essences are rich in antibacterial compounds which could be an alternative way to combat bacterial diseases even against some bacteria which are becoming resistant to certain synthetic medicines [Meera P, Dora PA and Sameut JK, 1999; Ahmad I, Mehmood Z and Mehmood I, 1998; Aswal BS, Goel AK and Patneik GK, 1996].

Pinus wallichaina (blue pine/kail), the finest pine of north western Himalayan region is well known for its commercial and ecological importance. It has widespread distribution in Indian Himalaya, Afghanistan, Bhutan, Pakistan and Nepal (Chaturvedi and Panday 2001). The extensive literature survey revealed the number of phenolic compounds have been isolated from the bark and leaves of *Pinus wallichiana* (Willfor et al. 2009; Naeem et al. 2010). Chemical composition; antiproliferative and radical scavenging activities of *Pinus wallichaina* oil is reported (Dar et al. 2013).

The significance of present study lies in the fact that a number of synthetic phenolic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ) are commonly added to food lipids to stabilize them against oxidation, but their use has been questioned because of their suspected toxic properties. Therefore search for natural antioxidants has received much attention, and efforts have been made to identify natural compounds that can act as suitable antioxidants to replace the synthetic ones (Pan et al., 2008).The objective of present study is the biochemical secreening of essential oil of *Pinus wallichiana* growing in Kashmir (India). There is no literature documenting antibacterial and anticancer activity against such cancer cell lines.

Experimental

Plant material

The plant material of Pinus wallichaina was obtained from Uri Baramulla, Kashmir. The plant material was identified in the department of taxonomy University of Kashmir, Srinagar under accession no. 1911.

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Chemicals

DPPH (2,2-diphenyl-1-picrylhydrazyl) radicalwas purchased from Sigma-Aldrich, Madrid, Spain. Dimethyl sulphoxide (DMSO), anhydrous sodium sulphate and all other reagents were of analytical grade (SISCO, Mumbai, India).

Essential oil extraction

The fresh needles and stem of the plant were finely chopped and then subjected to hydro-distillation separately in a clevenger like apparatus for three hours. The oil obtained was dried over anhydrous sodium sulphate and stored at 4^0c in sealed vials un-till analysis. The percentage yield of stem oil was found to be higher than needle oil.

GC-MS analysis

GC–MS analysis was carried on a Varian Gas Chromatograph series 3800 fitted with a VF-5 ms fused silica capillary column (60 m × 0.25 mm, film thickness 0.25 µm) coupled with a 4000 series mass detector under the following conditions: injection volume 0.5 µl with split ratio 1:60, helium as carrier gas at 1.0 ml/min constant flow mode, injector temperature 230 ^{0} C, oven temperature was programmed from 60 to 280 ^{0} C at 3 ^{0} C/min. Mass spectra: electron impact (EI+) mode, 70 eV and ion source temperature 250^{0}C. Mass spectra were recorded over 50–500 a.m.u range.

Test organism

The antibacterial activity of the essential oil of *pinus wallichiana* were tested against a panel of six bacterial strains obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Four strains of gram-negative bacteria [Pseudomonas. aeruginosa (MTCC-1688), Klebsiella pneumoniae (MTCC-19), Escherichia coli (MTCC-443) Proteus vulgaris (MTCC-1771)] and two strains of gram-positive bacteria [Bacillus subtilis (MTCC-441) and Staphylococcus aureus (MTCC-96)] were used. The cultures of bacteria were maintained in their appropraite agar slants at 4^0c throughout the study and used as stock cultures.

Antibacterial assay

The antibacterial susceptibility tests were carried out using agar well diffusion assay (Janssen, Scheffer and Baerheim Svendson, 1987) with some modification. Briefly the overnighit cultures of the indicator strains of bacteria were added to 20 ml of liquid nutrient agar(LNA). The contents of tubes were transferred to petri plates. After 10 minutes of solidification of the agar petri plates at room temperature, the wells punched on the plates were filled with 20 µl of essential oil, using a capillary micropipette. The incubation was carried out for 18 hours at 37^{o}C for bacteria. After the incubation period, the antimicrobial activity was evaluated by measuring the width of zones of inhibition, using a vernier scale against indicator strains of test organisms. Streptomycin sulphate (1000mg/l) was used as a positive control for bacteria.

2.7 Determination of minimum inhibitory concentration (MIC).

The antibacterial susceptibility tests were carried out using the agar well dilution method recommended by the National Committee for Clinical Laboratory Standards [Prudent D, Perineau F, Bessiere JM, Michel GM, Baccou JC, 1995] wth some modification. First Muller Hinton medium was prepared and 0.5% of tween-20 was dissolved per 100 ml of agar medium in order to enhance oil solubility. 20 ml aliquot was transferred in to each boiling tube. After this, sterilization of boiling tubes was carried out in autoclave. A series of two fold dilution of oil ranging from 0.2-25.6 mg/ml, was prepared in Mueller Hinton agar at 48^0c. Plates were dried at room temperature for thirty minutes prior to inoculation with 3 ul aliquots of culture containing approximately 10^5 CFU/ml of each organism. Inoculated plates were incubated at 37^0C for 18 hours and the MIC was determined. Experiments were carried out in triplicate. Inhibition of bacterial growth in the plates containing test oil was judged by comparison with growth, in blank control plate.

Antioxidant activity

DPPH free radical-scavenging activity

DPPH free radical scavenging activity was evaluated by measuring the scavenging activity of the essential oil on stable 2,2-diphenyl- 1-picryl hydrazyl radical. A 0.5 mM solution of DPPH in methanol was prepared and a stock solution of oil sample (1 mg/ml) in methanol was prepared. Various concentrations (20–100 µg/ml) were added to 1ml (0.5 mM DPPH) and final volume was made to 3 ml with methanol. The mixture was shaken thoroughly and kept standing at room temperature for 10 min. Then, the absorbance of the mixture was measured at 517 nm on a spectrophotometer. A decrease in the absorbance indicates an increase in DPPH-radical scavenging activity.

The percentage inhibition was calculated by the following equation:

DPPH radical scavenging= [(Ac- As)/Ac)×100]

Where, Ac is the absorbance of the control and As is the absorbance of the sample L-ascorbic acid served as positive control.

Cytotoxic assessment

Anticancer activity

Human cell lines and culture

Cytotoxic assay was carried out by MTT (3-(4,5-Dimethylthiazol-2-yl)- 2,5 diphenyltetrazolium bromide, a tetrazole) protocol in order to evaluate the anti proliferative effect of oil and its constituents. For this purpose, a sufficient number of exponentially growing cells were used to avoid confluence of the culture during the treatment. The cell lines A549, C6, T47D, MCF, and TH-1 were seeded at 10^4 cells/well and allowed to adhere for 12 h.

Cytotoxicity assay

In order to evaluate the optimum concentration at which the oils inhibited the cell proliferation in all the five cell lines, cells were treated with the oils at a concentration of 100 µg/ml. DMSO was used as a solvent for the dilution of oil, which was also used as an experimental control. Mitomycin-C was used as positive controls at a concentration of 1×10^{-5} µg/ml. After 48 h treatment, cell growth was evaluated by MTT assay (Alley et al. 1986, 1988). MTT solution of 50 µl (5 mg/ml of PBS) was added to each well and the plates were incubated for 3 h at 37^{o}C in dark. The media was aspirated and 150 µl of MTT solvent (4 mM HCl, 0.1% Nondet P-40, all in isopropanol) was added to each well to solubilize the formazan crystals. The absorbances of plates were measured on ELISA reader (Benchmark, BioRad) at a wavelength of 570 nm. Each sample was performed in triplicate, and the entire experiment was repeated thrice.

Results and discussions

Chemical composition

The different essential oil constituents of the leaf and stem of *Pinus wallichaina* are shown in table-1, in order of their elution from RTX-5 column. GC-MS analysis led to the identification of 13 components in the leaf and 12 components in the stem essential oil. The total ion chromatogram (TIC) of the two essential oils are shown in fig-1 and fig-2.

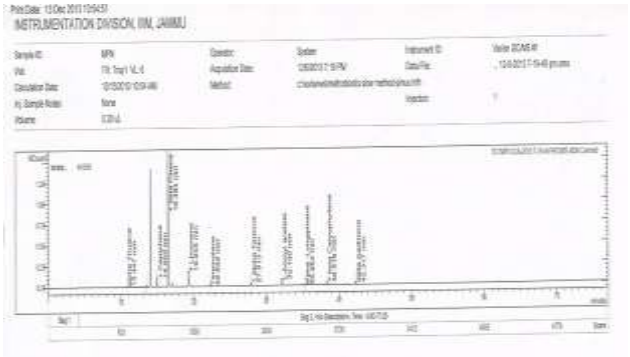


Fig-1: Total ion chromatogram of needle essential oil of *Pinus wallichaina*

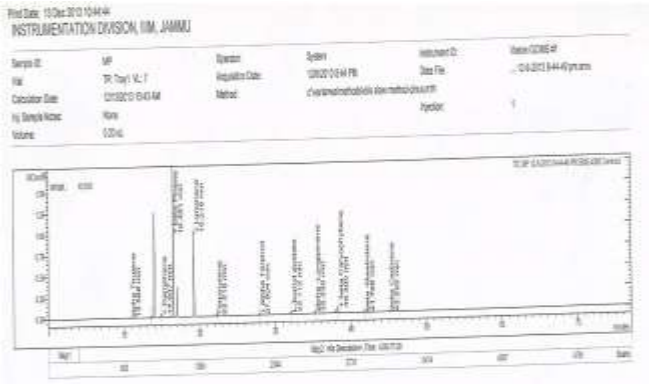


Fig-2: Total ion chromatogram of stem essential oil of *Pinus wallichaina*

The principle constituents of the leaf were beta-pinene (49.0%), alpha-pinene (40.6%), limonene (3.1%), camphene (2.4%), l-beta-pinene (1.3%) and l-bornyl-acetate (1.3%). The principle constituents of the stem essential oil were beta-pinene (45.9%), alpha-pinene(29.0%), limonene (14.9%) and l-beta-pinene (6.8%). This showed a marked variation in the essential oil composition of needles and stem. The chemical composition of needle oil reported in this study differs from the previous one reported earlier (Dar et al., 2013). This difference in the essential oil composition may be due climatic, seasonal, geographical or genetic differences.

Antibacterial activity

The in vitro antibacterial activity of essential oil was qualitatively and quantitatively assessed by the presence or absence of inhibition zones, zone diameters and minimum inhibitory concentration (MIC) values. Results from antimicrobial activity by agar well diffusion method are presented in table 2. The smaller zones of inhibition were obtained for stem and leaf oil. But greater zones of inhibition were obtained when stem and needle oil were used in combination. Thus whole plant essential oil of *Pinus wallichiana* showed significant antibacterial effect against the entire test microorganisms used for screening. This oil was mainly effective against S. aureus with highest inhibition zone of 30mm. Streptomycin sulphate was used as a positive control which showed inhibition zones between 20 -30 mm against different microorganisms tested. Therefore the antibacterial activity of *Pinus wallichiana* essential oil seem closer to reference antibiotic. The MIC of only whole plant oil was evaluated because greater zones of inhibition were obtained as already discussed. The MIC value of B. subtilis and P. vulgaris was found between 1.6-3.2 mg/ml. While as MIC of other four tested bacteria was found between 3.2-6.4 mg/ml. As can be clearly seen from the photographs (Figure 3) that no visible growth of any bacteria was found at this concentration. The antibacterial activity of this oil against all the tested bacteria is reported first time.

Antioxidant activity

The radical scavenging activity of the stem, needle and whole plant essential oil of *Pinus wallichaina* was measured by the DPPH assay in-vitro. The combination of stem and needle essential oil plant oil was found to be more potent antioxidant than needle or stem oil. The highest activity (47.19%) was found at a concentration of 100 µg/ml as is shown in the Fig-4. The Ascorbic acid which is used as a standard showed 78.7% radical scavenging activity at a conc. of 100 µg/ml. The DPPH radical scavenging assay is commonly employed in evaluating the ability of antioxidants to scavenge free radicals. This method has been used extensively to predict the antioxidant activity because of the relatively short time for analysis. The change in absorbance at 517 nm is used as a measure of scavenging effect of a particular sample for DPPH radicals. The more rapidly the absorbance decreases, the more potent the antioxidant activity of the sample in terms of its hydrogen atom-donating capacity (Shahidi, Alasalvar and Liyana-Pathirana, 2007; Alasavar et al., 2009).

Fig-4: Antioxidant activity of essential oil of *P. wallichiana*

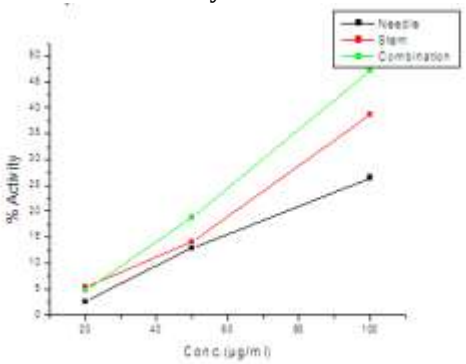


Table 1. The different essential oil constituents identified in the leaf and stem essential oils of *Pinus wallichaina* growing in Kashmir

S.No.	Compound	%age leaf	%age stem
1	Alpha-thujene	0.1	---
2	Alpha-pinene	40.6	29.0
3	Camphene	2.4	0.6
4	Sabinene	0.1	0.3
5	Beta-pinene	49.0	45.9
6	L-beta-pinene	1.3	6.8
7	Limonene	3.1	14.9
8	Terpenolene	0.2	0.2
9	Alpha-terpinol	0.5	---
10	L-bornyle-acetate	1.3	0.3
11	Alpha-longipinene	---	0.2
12	Longifolene	---	0.2
13	Beta-caryophyllene	0.3	0.6
14	Alpha-caryophylene	0.1	0.1
15	Tau-cadinene	0.2	---

Table-2: Invitro antibacterial activity of stem, needle and whole plant essential oil of *Pinus wallichaina* and reference antibiotic determined with agar well diffusion method.

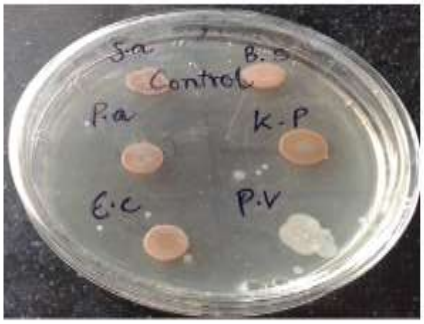
		Diameter of inhibition zone (mm)				
S. No.	Test bacteria	Needle	Stem	Whole plant	Antibiotic	MIC (mg/ml)
01	P. vulgaris MTCC 426	15	13	24	30	>1.6
02	B. subtilis MTCC 441	13	12	24	22	>1.6
03	K. pneumonia MTCC 19	-	-	23	18	>3.2
04	E. coli	-	-	25	25	>3.2
05	P. aeroginosa MTCC1688	12	11	25	17	>3.2
06	S. aureus MTCC 96	10	10	30	20	>3.2

Table-3: In-vitro antioxidant activity of stem, needle and their combination essential oil of *Pinus wallichiana.*

S. No.	Amount of oil	Needle	Stem	Whole plant
1	20	2.6	5.3	4.7
2	50	12.9	14.1	18.8
3	100	26.5	38.6	47.1

Table-4: In-vitro anticancer activity of *Pinus wallichiana* essential oil.

Tissue type		Lung	Gloima	Breast	Breast	Colon
Cell-line type		A549	C6	T47D	MCF	TH-1
Material	Conc(µg) .					
Mitomycin c	10^{-5}	92	89	91	78	83
Oil sample	100	51	43	49	50	40



Anticancer activity

In order to understand the effect of *Pinus wallichaina* essential oil on human cancer cell lines, experiment were carried using cultured A549 (lung), C6 (glioma), T47D (breast), MCF (breast) and TH-1(colon) cell lines by MTT assay as shown in the table-4. The oil was active against all the five cancer cell lines tested. The abundance of various components in the essential oil comprising a complex mixture of mono and sesquiterpenes, accounts for the cytotoxic activity of the *Pinus wallichaina* essential oil.

Conclusion

This essential oil showed a broad spectrum of antimicrobial effects against gram-positive and Gram-negative bacteria used. Also the antioxidant and cytotoxic properties of this essential oil will be of great interest to both pharmaceutical and food industries because of their possible use as natural additives to replace toxic synthetic food additives. Therefore, isolation, characterisation and activity evaluation of major constituents of the essential oil will be the research programme.

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