INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACY AND CHEMISTRY

Available online at www.ijrpc.com

Research Article

GC-MS ANALYSIS, ANTIBACTERIAL, ANTIOXIDANT AND ANTICANCER ACTIVITY OF ESSENTIAL OIL OF *PINUS ROXBURGHII* FROM KASHMIR, INDIA

Mahahpara Qadir and Wajaht. A. Shah*

Department of Chemistry, University of Kashmir, Hazratbal, Srinagar - 190 006, Jammu and Kashmir, India.

ABSTRACT

This work was carried out to evaluate chemical composition, antibacterial, antioxidant and anticancer activity of *Pinus roxburghii* 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. Alpha-pinene and beta-pinene were the major constituents present in the oil. This oil showed significant antibacterial and anticancer activity.

Keywords: *Pinus roxburghii* , GC-MS, antioxidant, antibacterial and anticancer activity.

INTRODUCTION

P. roxburghii sergeant (family : Pinacae) is commonly known as “chir pine” and has a long history of medicinal use. Pinus consists of 110-120 species that are distributed throughout temperate regions of the northern Hemisphere. *P. roxbhurghii* is a pine inhabitant to the Himalaya, the region extends from northern Pakistan (North West Frontier Province, Azad Kashmir), across northern india (Jammu and Kashmir, Punjab, Himachal Pradesh, Uttrakhand and Sikkim) and Nepal to Bhutan. In addition to being a Commercially important species in the Himalayan region, where it is known for its timber, paper, pulp, turpentine and resin yield. *P. roxbhurgii* also as many traditional medicinal uses (e.g., antiseptic, diuretic, diaphoretic, ionic, vermifuge and rube facient) as well as cultural uses (e.g., charcoal, pigment, herbicide, resin and wood)(Siddiqui MF, Ahmed M,Wahab M,Khan N, Khan MU, Nazim K,Hussain SS.2009). In ayurvedic medicine, P. roxbhurghii is prescribed as an intestinal antiseptic, antidyslipedemic, spasmolytic and antioxidant(Puri A, Srivastava AK, Singhal B, Mishra SK, Srivastava S, Lakshmi V, 2011) where as in other parts of its range, traditional medicinal uses include treating diseases of the eyes, ears, throat, blood and skin, bronchitis, diaphoresis, ulcer, inflammations and itching (Abassi AM, KhanMA, Ahmad M, Zaffar M, Jahan S, Sultana S, 2010). The chief chemical constituents of turpentine oil from *P. roxbhurgii* are alpha-pinene, beta- pinene, car-3-ene and longifolene (Smalech M, Sharma OP, Dobhal NP, 1976).

The literature survey revealed that the essential oil composition of needle and stem of Pinus roxburghii is reported from Pakistan. Needle oil consists of nine components in which major constituents are alpha-pinene (29.3%), Carryophyllene (21.9%) and 3-carene (14.2%). While as stem oil consists of 17 components in which major constituents are alpha-pinene (41.9%), 3-carene(16.3%) and carryophyllene (12.3%). The major components in both these oils are same, but they differ in their relative percentage. Both these oils were found to be active against S. aureus and B. Subtilis while as these oils were inactive against E.coli and Enterobacter aerogenes. Stem oil is also reported to exhibit antifungal activity against A. Terrus, A. Flavus, A. Candidus, A. Vessicolor, A. Niger and Trichoderma viride(Hassan A and Amjid I, 2009: Zaffar I, Fatima A, Khan SJ, RehmanZ, Mehmud S, 2010).Essential oil composition of twigs, needles and cones is also reported from Egypt(Islam W T, 2004: Islam W T, 2006).

Essential oil composition of cone, needle and bark of *Pinus roxburghii* are also reported from Nepal. The three essential oils were dominated by sesquiterpenes, particularly (E)-Carryophylene(26.8-34.5%) and alpha-humulene(5.0-7.3%) as well as monoterpene alcohols terpinen-4-ol(4.1-30.1%) and alpha-terpineol(2.8-5.0%). Bioactivity assays of cone essential oil showed remarkable cytotoxic activity(100% killing of MCF-7 cells at 100 ug/ml) along with notable Brine shrimp lethality(Lc_{50}=11.8ug/ml) and antifungal activity against A. Niger(MIC=39ug/Ml). Both the bark and needle oil showed in vitro cytotoxic activity against MCF-7 cells at 100ug/ml concentrations 70.9-1.4 and 100% kills respectively(P. Satyal, P. Paudel, J. Raut, A. Deo, Noura S, N Setzer, 2013).

2. MATERIALS AND METHODS

2.1 Plant material

The plant material of *Pinus roxburghii* was collected from University of Kashmir, Srinagar. The plant material was properly identified and the voucher specimen of *Pinus roxburghii* bearing specimen no.1912 was deposited at KASH herbarium in Centre of plant Taxonomy, University of Kashmir, Srinagar, J & K, India.

2.2 Essential oil isolation

The essential oil of the fresh fruits of *Pinus roxburghii* was obtained by hydrodistillation using a clevenger-type apparatus for three hours. The oil sample was dried over anhydrous sodium sulphate and kept in glass vials at – 4^{o}c prior to analysis.

2.3 Chemical composition

2.3.1. 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.

2.4 Antimicrobial assay

2.4.1. Microbial strains and culture media

The antibacterial activity of the essential oil of *Pinus roxburghii* were tested against a panel of six bacterial strains obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The bacterial strains used were Bacillus subtilis (MTCC-441), P. aeruginosa (MTCC-1688), S. aureus (MTCC 96), K. pneumonia (MTCC-19), E. coli (MTCC-443) and P. vulgaris (MTCC-1771). Bacterial strains were grown on nutrient agar plates at 37 ^{0}C and maintained on nutrient agar slants. Cell suspension of microorganisms in 0.9% NaCl was adjusted at 0.5 McFarland to obtain approximately 10^{6} cfu/ml.

2.4.2 Antimicrobial activity and determination of minimum inhibitory concentration (MIC)

The antibacterial susceptibility tests were carried out using the agar well diffusion assay/microdilution assay with 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 facilitate proper diffusion of agar in the oil. 20ml aliquot was transferred in to each boiling tube. After this sterilization of boiling tubes was carried out in autoclave. Temperature of tubes was regulated upto 38^{0}C and oil samples were added in the concentration range of 0.2-25.6mg/ml. The contents of the tube were transferred into plates which were kept under laminarflow and allowed to dry for 30 minutes. Finally bacteria were inoculated from fresh cultures into the broth and its turbidity was adjusted in the range of .08-0.13 at 625nm. Later 3µl of inoculums of each bacteria was added into the plates. Streptomycin sulphate (1000mg/l) was used as positive control for bacteria. The MIC of oil was determined by the microdilution method, recommended by the National Committee for Clinical Laboratory Standards (NCCLS) as described previously (Ashour et al., 2009). The oil was dissolved in dimethyl sulphoxide and added to the medium, and then diluted in order to obtain concentrations in the range of 0.25– 25.6 mg/ml. Inoculum suspension with a final concentration of 10^{6} cfu/ml was added to plate. The MIC was defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate any visible growth after incubation at 37 ^{0}C for 24h.

2.5 Antioxidant activity

2.5.1. 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.

2.6 Cytotoxic assessment

2.6.1. 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.

2.6.2. 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 37^0C 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.

3 RESULTS AND DISCUSSIONS

3.1 Chemical composition

The different essential oil constituents of the leaf and stem of *Pinus roxburghii* are shown in table-1, in order of their elution from RTX-5 column. GC-MS analysis led to the identification of 15 components essential oil.

The principle constituents of the oil were alpha-pinene (60.8%), beta-pinene (30.2%), limonene (3.1%), camphene (1.0%), l-beta-pinene (1.8%), beta-caryophyllene(1.1%)and alpha terpinol(1.6%).This showed a marked variation in the essential oil composition reported in this study than the previous one reported earlier. This difference in the essential oil composition may be due climatic, seasonal, geographical or genetic differences.

3.2 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. Essential oil of *Pinus roxburghii* showed significant antibacterial effect against the entire test microorganisms used for screening. This oil was mainly effective against P. vulgaris and E. coli with highest inhibition zone of 32 and 30mm respectively. 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 roxburghii* essential oil seem closer to reference antibiotic. The MIC value of P. vulgaris was found between 6.4-12.8 mg/ml. While as MIC of other tested bacteria was found within the range of 12.8 mg/ml. As can be clearly seen from the photographs (Figure 1) that no visible growth of any bacteria was found at this concentration.

3.3 Antioxidant activity

The radical scavenging activity of the essential oil of *P. roxburghii* oil was measured by the DPPH assay in-vitro.The DPPH radical scavenging assay is commonly employed in evaluating the ability of antioxidants to scavenge free 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, Alasavar and Liyana-Pathirana, 2007; Alasalwar et al., 2009). The radical scavenging activity of this essential oil was found to be negligible. 10% radical scavenging activity was found at a concentration of 100 microgram of the oil.

3.4 Cytotoxic activity

In order to understand the effect of *Pinus roxburghii* 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-3. 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 roxburghii* essential oil.

Table 1: The different essential oil constituents identified in the essential oils of *Pinus roxburghii* are given as under

S.No.	Compound	%age of oil
1	Alpha-thujene	0.2
2	Alpha-pinene	60.8
3	Camphene	1.0
4	Sabinene	0.0
5	Beta-pinene	30.2
6	L-beta-pinene	1.8
7	Limonene	0.9
8	Terpenolene	0.1
9	Trans-pinocarveol	0.1
10	4-Terpineol	0.0
11	Alpha-terpinol	1.6
12	L-bornyle-acetate	0.3
13	Beta-caryophyllene	1.1
14	Alpha-caryophylene	0.4
15	delta-cadinene	0.4

Table 2: In-vitro antibacterial activity of essential oil of *Pinus roxburghii* and refrence antibiotic determined with Agar well Diffusion Method

S.No.	Test bacteria	Zones of inhibition (in mm)	Zone of inhibition of antibiotic (in mm)	MIC (in mg/ml)
Gram-Positive Bacteria				
1	S. Aureus MTCC 96	22	18	12.8
2	B.subtilis MTCC 441	19	30	12.8
3	K.pneumoniae MTCC 19	22	17	12.8
Gram-Negative Bacteria				
4	E.coli MTCC 443	30	20	12.8
5	P.aeroginosa MTCC 1688	23	30	12.8
6	P.vulgaris MTCC 426	32	20	6.4-12.8

Table 3: In-vitro cancer activity of *Pinus roxburghii* essential oil

Cell-type	A549	C6	T47D	MCF	TH-1
DMSO	6	6	4.5	6.5	7
Mitomycin C	92	89	91	78	83
Oil	90	85	71	81	77

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