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GC–MS analysis of essential oil of *Pinus roxburghii* Sarg. (Chir pine) needles and evaluation of antibacterial and anti-proliferative properties

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The present study was carried out to analyse the biological properties and chemical composition by GC-MS analysis of the essential oil of the needles of *Pinus roxburghii*. Sixteen components were identified from their fragmentation pattern in the essential oil. Major component present in the essential oil belonged to monoterpene (84.38 %), oxygenated monoterpene (4.23 %) and Sesquiterpene (11.35 %) category. The antibacterial potential was analysed by paper disc diffusion assay against 5 gram-positive bacteria and 5 gram-negative bacteria. Oil showed a broad spectrum of activity by inhibiting all the 5 gram-positive bacteria and 2 gram-negative bacteria viz., *Escherichia coli* and *Campylobacter coli*. The antiproliferative property was measured against A-549 (lung), T98G (glioblastoma), PC-3 (prostate) and T47D (breast) human cancer cell lines by Sulphorhodamine-B assay. Here, the essential oil exhibited cytotoxic properties against human cancer cell lines with IC_{50} values of 161.30 µg/mL and 154.30 µg/mL for A549 and T98G respectively. The results suggest that active component of the essential oil of *Pinus roxburghii* possess antimicrobial and antiproliferative properties which may be useful for food preservation, pharmaceutical treatment and natural therapies.

Keywords: Antibacterial, Cytotoxic, Essential oil, Needles, *Pinus roxburghii*.

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Introduction

Essential oils obtained from many plants are the complex mixtures of odorous and volatile compounds existing in low concentrations. The essential oils can be obtained from many plants and have gained enormous popularity and scientific interest[1,2]. Various beneficial properties of the volatile oil are reported in the prevention/ treatment of cancer, cardiovascular diseases including atherosclerosis, thrombosis, as well as other bioactivities like antibacterial,

The plant *Pinus roxburghii* Sarg. belonging to the family Pinaceae is commonly known as 'Chir pine'. It is a tall evergreen tree having 14-16 cm long needles and reaching the heights of 30-50 m with a trunk diameter of up to 2 m. It is found at the height of 500 to 2500 m AMSL. It is recognised as commercially important species and is well known for its timber, paper pulp and resin yield. Traditionally, the plant is well known in the Indian system of medicine and Ayurveda. The plant is sweet, bitter, pungent, heating, oleaginous, intestinal antiseptic, antidyslipidemic and is used in diseases of the eye, ear, throat, blood and skin, bronchitis, diaphoresis, ulcer, inflammations and itching[8]. Gum is bitter and heating, oleaginous, purgative, carminative, emmenagogue, expectorant, aphrodisiac, fattening, diuretic, anthelmintic and analgesic and is used in diseases of vagina and uterus, eye, dyspepsia, ulcer, diaphoresis, scabies, zema, piles, diseases d spleen, gleets, ear discharge, toothache, lumbago and tuberculous glands[9,10].

The resin of Chir pine is applied to heel cracks, boils and to reduce eye swelling[11]. Wood is stimulant, diaphoretic and is used in cough, fainting and ulceration[9].

These needles and stems are rich in vitamin C, tannins, alkaloids and essential oils, while its wood is the major source of turpentine oil. The phytoconstituents friedelin, ceryl alcohol and β- sitosterol were isolated from the bark of *P. roxburghii*. Longifolene was isolated from leaves.

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Xylem resin has been reported to contain α-pinene, β-pinene, car-3-ene[12-14]. Various workers had reported hepatoprotective[15], antidyslipidemic, antioxidant[16], antifungal[17], antiinflammtory[31] activities from the plant.

In the present investigation, essential oil from *P. roxburghii* needles is explored for its potential against antimicrobial infection as well as for anti-proliferative effectively.

Materials and Methods

Sample collection

Fresh needles of *P. roxburghii* were collected from the campus of the University of the Jammu, (J&K) in the month of April 2012. The plant was identified by the taxonomist Dr Harish Dutt, Assistant Professor, Department of Botany, University of Jammu and was deposited in the herbarium with voucher specimen no 9774.

Extraction of essential oil

Needles of *P. roxburghii* were harvested from healthy, well-grown tree. Freshly harvested samples (500 g each) were subjected to hydrodistillation using a modified Clevenger-type glass apparatus for 4 h. The oil samples so obtained were dried over anhydrous sodium sulphate (Na_2SO_4) and stored at 4 ^oC until analysed.

Analysis by GC-MS

Analysis of the oil was carried out at CSIR-Indian Institute of Integrative Medicine (CSIR-IIIM), Jammu, India using GC-MS 4000 (Varian, USA) system with a varian CP-SIL 8CB column (30 m×0.32 mm i.d., 1 μm film thickness). The injector temperature was 230 °C. Oven temperature programme used was holding at 60 °C for 5 minutes, heating to 250 °C at 3 °C/min and keeping the temperature constant at 250 °C for 10 minutes. Helium was used as a carrier gas at a constant flow of 1.0 mL/min and an injection volume of 0.20 μL was employed. The MS scan parameters included electron impact ionization voltage of 70 eV, a mass range of 40–500 m/z. The identification of essential oil components was based on a comparison of their mass spectra with those of NIST05 (version 2.0) library.

Antibacterial activity

The antibacterial activity of the essential oil was performed by the disk diffusion method[18] against five gram-positive strains *viz*., *Micrococcus luteus* (MTCC- 106T), *Staphylococcus aureus* (MTCC-1144), *Enterococcus fecalis* (MTCC-439), *Bacillus subtillis* (MTCC-121T), *B. cereus* (MTCC-430) and five gram-negative bacteria *viz*., *Escherichia coli* (MTCC-40), *Campylobacter coli* (MTCC-1126), *Pseudomonas aeruginosa* (MTCC-647), *P. alcaligenes* (MTCC-493) and *Alcaligenes denitrificans* (MTCC-299). All the test microorganisms were purchased from Microbial Type Culture Collection Centre, IMTECH, Chandigarh, India and were maintained on nutrient agar slants.

Briefly, 0.1 mL of a suspension of the test microorganism (10^8 cells/mL) was spreaded on Mueller-Hinton agar plates. Sterile 6 mm disks, containing 10 μL of essential oil were placed on the microbial lawns. The plates were incubated at 37 ºC for 24 h. The diameters of the zones of inhibition were measured in mm. Triplicate tests were carried out in all experiments.

Antiproliferative potential

The cytotoxicity assay was performed as earlier discussed[19]. The protein-staining sulforhodamine B (SRB) assay was used for measurement of cell proliferation. The test is based on the estimation of cell number indirectly by providing a sensitive index of total cellular protein content which is linear to cell density. The cells were trypsinized, and seeded in 96-well plates at $5x10^4$ cells/mL for 24 h, further, cells were treated with serial dilutions of the samples to obtain different concentrations of essential oils. After 48 hours of exposure, 100 μL of ice-cold 40 % trichloroacetic acid (TCA) was added for 1 h at 4 ^oC and washed with distilled water. The TCA-fixed cells were stained for 30 minutes with 50 μL of 0.4 (w/v)% SRB in 1 % acetic acid. The plates were washed with 1 % HOAc and air-dried overnight. For the reading of the plate, the bound dye was solubilised with 100 μL of 10 mM tris base tris[hydroxymethyl] aminomethane). Cell survival was measured as the percentage absorbance compared to the untreated control. Vinblastine sulfate salt and Taxol were used as positive control.

Statistical analysis

All experiments were carried out in triplicate. The variance analyses were carried out using the SPSS 14.0 software package for Windows. Values of $p < 0.05$ were considered to be statistically significant.

Results

The chemical composition of the essential oil of *P. roxburghii* (needles) is shown in Table 1 and results revealed the presence of high amount of

Table 1 — The chemical constituents of essential oil of *Pinus roxburghii* (needles)

S. No	Compound	Amount (%)
1	α-thujene	1.67
2	α-pinene	15.44
3	β-Phellandrene	4.63
4	β-Pinene	2.46
5	α-Phellandrene	39.63
6	α-Terpinene	1.69
7	β-Cymene	2.62
8	Limonene	1.19
9	τ-Terpinene	3.51
10	α-Terpinolene	11.78
11	4-Terpinol	2.64
12	β-fenchol	0.37
13	Terpinyl acetate	1.23
14	Caryophyllene	6.43
15	α-Caryophyllene	4.33
16	β-Caryophyllene Oxide	0.60

α-phellandrene i.e., 39.03 % followed by α-pinene (15.44 %) and α-terpinolene (11.78 %). Other compounds such as caryophyllene, α-caryophyllene, β-phellandrene, τ-terpinene, β-pinene, α-terpinene, α-thujene, 4-terpinol, β-cymene, terpinyl acetate, limonene, β-caryophyllene oxide and β-fenchol were present in relatively small amount.

Antibacterial activity

The results of the antimicrobial activities of the essential oil indicated that the oil exhibited moderate to high antimicrobial activity. According to these results, essential oil of this species exhibited considerable antibacterial activity on tested bacteria by paper disk diffusion assay. The test was conducted against 5 gram-positive strain *viz*., *M. luteus* (MTCC-106T), *S. aureus* (MTCC-1144), *E. fecalis* (MTCC-439), *B. subtillis* (MTCC-121T), *B. cereus* (MTCC-430) and 5 gram-negative bacteria *viz*., *E. coli* (MTCC-40), *C. coli* (MTCC-1126), *P. aeruginosa* (MTCC-647), *P. alcaligenes* (MTCC-493) and *A. denitrificans* (MTCC-299). Essential oil of *P. roxburghii* was resistant against all the gram-positive bacteria tested in the study and maximum zone of inhibition was observed against *S. aureus* with 13 mm of zone of inhibition which was comparable to the positive control 20 µg of camptothecin used for the study (Table 2). When tested against 5 different gram-negative bacteria; essential oil showed activity only against 2 gram-negative bacteria with zone of inhibition of 22 mm against *E. coli* and 8 mm against *C. coli*. Hence, the essential oil of *P. roxburghii* was found to be more effective against gram-positive bacteria than gram-negative bacteria tested in the study.

Table 2 — Antibacterial activity of *Pinus roxburghii* (needles) essential oil

Essential oil of *Pinus roxburghii*	Zone of Inhibition (in mm)	Positive control Camptothecin (20 µg)
Gram positive		
Micrococcus leteus	10	26
Staphylococcus aureus	13	12
Enterococcus fecalis	6	18
Bacillus subtills	11	22
Bacillus cereus	6	14
Gram negative		
Escherichia coli	22	9
Campylobacter coli	8	15
Pseudomonas aeruginosa	-	21
Pesudomonas alcalygens	-	15
Alcalygens denitrificans	-	17

Table 3 — Cytotoxic activity of *Pinus roxburghii* (needles) essential oil

IC_{50} in µg/mL

Essential oil of *Pinus roxburghii*	Lung A-549	Glioblastoma T98G	Prostate PC-3	Breast T47D
	161.30±1.2**	154.30±1.1**	>200	>200

Data are given as the mean of at least three independent experiment values are mean±S.D. ** p <0.01 *vs.* control.

Antiproliferative activity

In order to understand the effects of *P. roxburghii* essential oil on human cancer cell lines, experiments were carried out using cultured A-549 (lung), T98G (glioblastoma), PC-3 (prostate), and T47D (breast) cell lines by Sulphorhodamine-B assay. Paclitaxel (for A-549), Mitomycin C (for Glioblastoma) and Adriamycin (for T98G & T47D) were used as positive control. All cell lines were submitted to increasing concentration of oil from 50 to 250 mg/mL for 48 hours, which reduced the viability of these cell lines. As shown in Table 3, IC_{50} of the lung cancer cell line was 161.30 µg/mL and glioblastoma cancer cell line was 154.30 µg/mL, whereas for prostate and breast cancer cell line showed higher IC_{50} values (Table 3).

Discussion

For centuries, essential oils have been used in aromatherapy and other medical purposes. Interest in essential oils has been revitalized in recent years with

the hope that oils with newer therapeutic activities might be discovered, as these essential oils are complex mixtures comprising many single compounds and each of these constituents contributes to the various biological effects. Hydrodistillation of fresh needles of *P. roxburghii* yielded pale yellow coloured oil with a pleasant smell and majority components of *P. roxburghii* (needle) essential oil are cyclic monoterpene viz., α-phellandrene (39.62 %), α-pinene (15.4 %), α-terpinolene (11.775 %) etc. The monoterpenes from plant essential oil are described for several biological activities including insecticidal, herbicidal, fungicidal, bactericidal properties etc[20-21,32].

The essential oil of *P.roxburghii* showed potential antimicrobial activity against both gram-positive as well as gram-negative bacteria and highlighted its potential for broad spectrum of the activity. It has frequently been reported that gram-positive bacteria are more sensitive to essential oil and their components than gram-negative bacteria[22]. This result may be explained by the presence of high content of α-phellandrene (39.62 %), α-pinene (15.4 %), α-terpinolene (11.775 %) present in the essential oil of *P. roxburghii* needles. Antibacterial and antifungal activities of these compounds have been reported in previous studies[23-25]. Similarly, the pinene type monoterpene hydrocarbon (α-pinene) is well known compound for antimicrobial properties but the activity of the essential oil varies with its concentration and kind of bacteria[26]. These differences in the susceptibility of the test organisms to essential oil could be attributed to the variation in the rate of the monoterpene penetration through cell wall and cell membrane structures. The ability of the essential oil to disrupt the permeability barrier of cell membrane structures and the accompanying loss of chemiosmotic control is the most likely source of its lethal action[24]. Based on these results, it is possible to conclude that the essential of *P. roxburghii* needles possess stronger and broader spectrum of antimicrobial activity.

The essential oil of *P. roxburghii* (needles) was also found effective against various human cancer cell lines *viz*., A-549 (lung), T98G (glioblastoma), PC-3 (prostate) and T47D (breast) cell lines, where the results were comparable to the positive control used. It may be suggested herein that complex mixture of mono and sesquiterpenes present in essential oil of *P. roxburghii* (needles) may accounts for the cytotoxic effect against human cancer cell lines when treated. Earlier reports suggest that α-pinene exhibits the antiproliferative activity[27] and synergistic effects of the active chemicals present along with other constituents of the essential oils may also have to be taken into consideration[28]. Another study conducted by Sikkema and co-worker indicated that the higher cytotoxicity of the essential oil may be attributed to the existence of highly hydrophobic components of a low molecular weight such compounds could easily cross and/or interact with the membrane to cause a loss of structural integrity, thus increasing the permeability of protons and ions that could result in cell death[29].

Conclusion

This study indicates that the essential oil isolated from needles of *P. roxburghii* was rich in cyclic monoterpene alpha-phellandrene and possesses promising *in vitro* antibacterial as well as anti-proliferative potential and can be explored for innovative therapeutic or preventive strategies in particular against infections or cancer that can be for great interest to both pharmaceutical and food industries.

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