

GC-MS studies of needles essential oils of *Pinus roxburghii* and their antimicrobial activity

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Abstract: Essential oils of *Pinus roxburghii* needle were analyzed by GC-MS and their antimicrobial activity was studied. Nine components out of forty one were identified from their fragmentation pattern. Major components in essential oils of needles were α -pinene (29.3%), followed by caryophyllene (21.9%), 3-carene (14.2%), (10.5%), α -terpineol (4.5%), caryophyllene oxide (3.1%), borneol acetate (2.2%), α -longipinene (1.2%), β -myrcene (1.1%) and terpinyl acetate (1.0%). Antibacterial activity of essential oil of the needles indicate that this oil showed maximum activity against *Staphylococcus aureus* and *Bacillus subtilis*, while no activity was observed against *Escherichia coli*, *Salmonella typhi* and *Enterobacter aerogenes*. Moreover, there was a significant and dose dependent inhibition of the growth of the fungi tested in the present study.

Keywords: *Pinus roxburghii*, α -pinene, caryophyllene, antibacterial activity, antifungal activity.

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INTRODUCTION

Pinus roxburghii, locally known as “chir” in Pakistan, belongs to genus *Pinus* and family *Pinaceae*¹. This genus has the largest naturally occurring conifers comprising of about 250 species spreading worldwide^{2,3}. Five species of *Pinaceae* including *Pinus roxburghii* are found in Pakistan covering the total area of 1928 thousand hectares and spread over the rangelands of North West Frontier, Balochistan and Punjab provinces of Pakistan^{4,5}. *Pinus roxburghii* grows in the region of forests of 1200-1850 m altitude and with mean coldest month temperatures of 5-15°C¹. It is a tall evergreen tree having 14-16 cm long needles. It is one of the commercially important species and is well known for its timber, paper pulp and resin yield⁶. Pine needles are among the non-wood material and are abundantly available in Pakistan. These needles and stems are rich in vitamin C, tannins, alkaloids and essential oils, while its wood is the major source of turpentine oil^{7,8}.

Essential oils have been used for thousands of years to promote well being and health^{9,10}. Research has been carried out to find some essential oils that could safely be used as a substitute for fungicides and bactericides to partially or completely inhibit the growth of fungi and bacteria¹¹. Essential oils are chemically very diverse in their effects and cause different actions unlike synthetic chemicals which basically have one action¹².

Antifungal activities of essential oil of *Pinaceae* species was investigated by Krauze¹³, Eui¹⁴ (*P. densiflora* and *P. koraiensis*), Motiejunaite and Peculyte¹⁵ (*P. sylvestris*), and Vina and Carol¹⁶. Similarly antibacterial activity of *Pinaceae* species had been reported by Eui et al.¹⁴ (*P. densiflora* and *P. koraiensis*) and Parihar et al¹⁷ (*P. roxburghii*).

Chemical constituents of *Pinus caribaea*⁷, *Pinus sylvestris*², yellow pine¹⁶ and *Pinus densiflora* and *Pinus koraiensis*¹⁴ were studied.

The aim of present investigation was to determine the chemical constituents, antifungal and antibacterial activity of *Pinus roxburghii* needle essential oils found in Pakistan.

MATERIALS AND METHODS

Pinus roxburghii needles were collected from the planes of Lahore district of Punjab province. Needles were separated from the wood and dried under shade for 10 days. Essential oil was obtained through hydro-distillation using deanstark apparatus for eight hours. Oil obtained was rectified and dried over anhydrous sodium sulphate. Chemical composition of the essential oil was determined through GC-MS.

Agilent 5973-6890 gas chromatograph–mass spectrometry system, operating in EI mode at 70 ev equipped with a split-splitless injector was used. Helium was used as a carrier gas at the flow rate of 1ml/min, while HP-5MS (30m, 0.25mm, 0.25um) capillary column was used. The initial temperature was programmed at 50-100 °C at the rate of 5 °C/min and then 100-250 °C at the rate of 3°C/min followed by a constant temperature at 260°C for a period of 20 minutes. Sample (2 μ l) was injected to the column programmed at 200 °C and resolution of components was attained. Identification of individual components was carried out by comparison of their relative retention time with those of authentic samples (Supleco; Bellefonte, USA) by co-elution and MS analysis. For the components like terpenes and aliphatic compounds, the reference samples were not available. Therefore, their identification was performed by matching their retention indices and

mass spectra with those obtained from authentic samples and the NIST library.

Bacterial pathogens and tests for antibacterial activity

Pure cultures of bacterial species (*Escherichia coli*, *Bacillus subtilis*, *Salmonella typhi*, *Enterobacter aerogenes*, and *Staphylococcus aureus*) obtained from PCSIR laboratories complex, Lahore and from pathological laboratories of local hospitals, were used in the present study. Prior to inoculation, bacterial strains were subcultured thrice on fresh nutrient agar media to obtain a more vigorous population. The stock cultures were incubated at 37°C for 24h. Well/cup plate method was performed for the assessment of antibacterial activity in which wells were made in the preinoculated culture media. Oil volumes used were 10.7, 21.4, 42.8 and 64.2µl for each sample and incubated at 37°C for 24h. The diameter of zone of inhibition (mm) around the well was measured. The values shown (Table 1) are the means of tests performed in triplicate.

Fungal pathogens and tests for antifungal activity

Pure cultures of fungal species (*Aspergillus terreus*, *Aspergillus flavus*, *Aspergillus candidus*, *Aspergillus versicolor*, *Aspergillus niger* and *Trichoderma viride*) were obtained from the PCSIR laboratories complex, Lahore. Disc diffusion method¹⁸ was performed with slight modification to determine the antifungal activity of pine stem essential oils. Fungal broth culture aliquots were added to potato dextrose agar medium and distributed uniformly. Whatman #1 filter paper was used for making discs. Oils impregnated sets of discs (5mm diameter) of known strength were placed on the culture plates. Four volumes of oils (5, 10, 15 and 20µl) were used. The diameter of zone of inhibition (mm) around the disc was measured after cultivation at 24-28 °C for 2 days. The values shown (Table 2) are the means of tests performed in triplicate.

RESULTS AND DISCUSSION

The essential oils of *Pinus roxburghii* needles were obtained through hydrodistillation in the yield of 0.11%. The chemical composition of the essential oils was determined by GC-MS. Table 3 shows that the maximum percentage (29.3%) of constituent was that of α-pinene. This constituent has been the necessary component in pinus species^{8,19}. Other monoterpene hydrocarbons like 3-carene (14.2%) and β-myrcene (1.1%) were also identified. Oxygenated monoterpenes α-terpineol (4.5%), terpinyle acetate (2.2%) and borneol acetate were also found. Longpinene (1.2%) and cryophyllene (21.9%), sesquiterpenes, and oxygenated sesquiterpene caryophyllene oxide were also found in the oils found the present study have also been reported in pinus species^{7,14,20}.

The antibacterial activity of essential oils of chir needles is summarized in Table 2. *Escherichia coli*, *Salmonella typhi* and *Enterobacter aerogenes* showed high resistance against the oil tested. The leaf extract of *Pinus roxburghii*, however, inhibits the growth of *E.coli* and *Salmonella typhi*¹⁷.

The volumes of oil (21.4 µl and 42.8 µl) showed moderate activity against *Bacillus subtilis* showing diameter of zones of inhibition 12.12±0.121mm and 22.89±0.414mm respectively while highest zone of inhibition (34.11±0.707 mm) was observed on the oil volume of 64.2 µl (Table 1). Significant inhibition of the growth of *Staphylococcus aureus* was observed with all the volumes.

The diameter of the zones of inhibition increases as volume of oil increases (Table 2). The diameter of zone (10.9±0.707mm) was observed on 10.7µl while highest inhibitory effect (41.25±0.121mm) was found with 64.2µl of oil. Similarly, 21.4µl and 42.8µl of oil also exhibit significant inhibition of the growth of *Staphylococcus aureus* (Table 2).

Table 1: Antibacterial activity of essential oils of needle of *Pinus roxburghii*.

Pine needles oil (µl)	<i>E. coli</i>	<i>B. subtilis</i>	<i>S. typhi</i>	<i>Ent. aerogenes</i>	<i>Staph. aureus</i>
10.7	-	0.00	-	-	10.9 ±0.707
21.4	-	12.12±0.121	-	-	24.1±1.141
42.8	-	22.89±0.414	-	-	36.91±0.9
64.2	-	34.11±0.707	-	-	41.25±0.121

(-) Resistant, full growth of microbe, (Diameter zone (mean±SEM) of inhibition (mm)

Table 2: Antifungal activity of essential oils of needles of *Pinus roxburghii*

Concentration of Pine stem oil (µl)	<i>Aspergillus terreus</i>	<i>Aspergillus flavus</i>	<i>Aspergillus candidus</i>	<i>Aspergillus versicolor</i>	<i>Trichoderma viride</i>	<i>Aspergillus niger</i>
5	20±0.19	13±1.14	-	-	0.00	-
10	23±0.14	22.5±0.107	-	-	11.5±0.707	-
15	27±1.6	29.5±0.50	-	-	16.5±0.14	-
20	40.5±1.14	31±1.12	-	-	23±1.1	-

(-) Sensitive, no growth was observed, Diameter zone (mean±SEM) of inhibition (mm)

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Essential oils from coniferous species (*Pinus densiflora*, *Pinus koraiensis* and *Chamaecyparis obtuse*) have been shown to have mild antimicrobial properties including inhibition of gram positive (*Staphylococcus aureus*) and gram negative bacteria (*E.colli*, *Salmonella sp.*, *Klebsiella pneumoniae*)¹². Similar results were obtained in case of *A. niger* by Motiejunaite and Peciulyte¹⁵ who studied the inhibition of *A. niger* during all period of their investigation when treated with pine needles oil. Low concentration of stem oil (5 µl) did not inhibit the activity of *Trichoderma viride* but this low concentration inhibit the growth of *A. terrus* and *A. flavus* by forming the diameter of zone of inhibition 20 mm±0.19 and 13 mm±1.14, respectively.

The highest diameter of zone 23mm±1.1 was recorded when 20 µl concentration of oil was tested. Motiejunaite and Peciulyte¹⁵ also observed. The growth inhibition of *T. viride* after 7-day incubation effect of vapor of pine needles. While 10 and 15µl oil gave 11.5 mm±0.707 and 16.5 mm±0.14 diameter of zones, respectively.

The activities of *A. terrus* and *A. flavus* were dose dependent because as concentration of pine needle oil increases the diameter of zones of inhibition also increases in case of both strains. The highest diameter of zones of inhibition recorded were 40.5 mm±1.14 and 31 mm±1.12 on high concentration of oil 20 µl in *A. terrus* and *A. flavus* respectively (Table 3).

Table 3: Chemical composition of essential oil of *Pinus roxburghii* needles

No.	Retention time (minutes)	Component	%
1	6.037	α-pinene	29.3
2	6.958	β-Myrcene	1.1
3	7.336	3-Carene	14.2
4	7.674	Terpinyl acetate	1.0
5	10.369	α-Terpineol	4.5
6	11.656	Borneol acetate	2.2
7	12.686	α-longipinene	1.2
8	13.922	Caryophyllene	21.9
9	18.036	Caryophyllene oxide	3.1

It can be concluded from the present study that that the components (terpenes) of essential oil of *Pinus roxburghii* needles are highly active against microbes. As this oil significantly inhibited the growth of certain bacteria and fungi tested. Therefore, this oil can be used for the treatments of skin problems as well as infectious diseases. However, further studies for LD₅₀ and toxicology are required to prove its fitness for human consumption.

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