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Full Length Research Paper

Gas chromatography-mass spectrometric studies of essential oil of *Pinus roxburghaii* stems and their antibacterial and antifungal activities

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Essential oil of *Pinus roxburghaii* stems was analyzed by GC MS and their antibacterial activity was studied. Seventeen components out of fifty-two were identified in the oil. Major component in essential oil was α- pinene (41.9%) followed by 3-carene (16.3%), caryophyllene (12.3%), p-cymene (1.9%), Terpinenol (1.8%), Limonene (1.7%), Borneol acetate(1.1%), Caryophyllene oxide (1.0%),camphene (0.9%),Tepinyl acetate (0.8%), β-Phallenderene (0.7%), farnesene (0.6%),o-cymene (0.4%), Butanoic acid**,** 3-methyl**-,** 2-phenylethyl ester **(0.3%),1-terpinen-4-ol (0.2%), Farnesyle acetate (0.2%) and γ-terpinene (0.2%). Antibacterial activity of stem essential oil was observed against *Staphylococcus aureus* and *Bacillus subtilis* while no activity was observed against *E. coli* and *Enterobacter aerogenes.* Similarly, antifungal activity of *Pinus roxburghaii* essential oil was also found to be active against *Aspergillus terrus, Aspergillus flavus, Aspergillus candidus, Aspergillus vessicolor, Aspergillus niger* and *Trichoderma viride.***

Key words: *Pinus roxburgahii*, antibacterial activity, antifungal activity, α-pinene, caryophyllene.

INTRODUCTION

Pinus roxburghaii is locally known as “chir” in Pakistan and it belongs to genus *Pinus* and family Pinaceae (Sidiqui et al., 1999). This genus has the largest naturally occurring conifers which comprises of about 250 species spreading world wide (Janina, 2007 and Oluwadayo and Olakunle, 2008). Five species of Pinaceae including *P. roxburghaii* are found in Pakistan covering the total area of 1928 thousand hectares and spread over the range-lands of North West Frontier, Balochistan and Punjab provinces of Pakistan (Nasir and Nisir, 1987, Siddiqui, 1991). *P. roxburghaii* grows in the region of forests of 1200-1850 m altitudes and with mean coldest month temperatures of 5 - 15°C (Sidiqui et al., 1999). It is a tall ever green 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 yield (Sehgal et al., 1995). 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 oil while its wood is the major source of turpentine oil (Vallejo et al., 1994, Asta et al., 2006).

Essential oils have been used for thousands of years to promote well being and health of human being (Thomas, 2002 and Blunt, 2003). For health and economic considerations, 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 bacteria (Soliman and Badeea, 2002). It is also tapped commercially for resin. On distillation, the resin yieds an essential oil, commonly known as turpentine, and non-volatile rosin. The proportion of rosin and turpentine oil in Chir Pine is 75 and 22% respectively with 3% losses. The turpentine is chiefly used as a solvent in pharmaceutical preparations, perfume industry, in manufacture of synthetic pine oil, disinfectants, insecticides and denaturants. It is one of the most important basic raw materials for the synthesis of terpene chemicals which are used in a wide variety of industries such as adhesives, paper and rubber, etc.

Essential oils are chemically very diverse in their effect and cause different actions unlike synthetic chemicals

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which basically have one action. There is a little information on antibacterial and antifungal activities of essential oils extracted from coniferous trees (Hong et al., 2004).

Antifungal activity of essential oil of *Pinacea* species *P. densiflora and P. koraiensis* (Krauze et al., 2002; Eui et al., 2004 and Motiejunaite and Peciulyte, 2004), *P. sylvestris* (Vina and Carl, 2007) were investigated. Similarly antibacterial activity of *pinaceae* species *P. densiflora and P. koraiensis* (Eui et al., 2004 and Pradeep et al., 2006) had been reported. Chemical constituents of *P. caribeae* (Vallejo et al., 1994) *P. sylvestris* (Janina, 2007), *P. heldrichii* (Asta et al., 2006) yellow pine (Vina and Carl, 2007) and *P. densiflora* and *P. koraiensis* (Eui et al., 2004) were studied.

The aim of the present investigation was to determine the chemical constituents and antimicrobial activity of *P. roxburghaii* stem essential oils found in Pakistan.

METHODS

Plant material and extraction of essential oil

P. roxburghaii stems (250 gm) were collected from the planes of Lahore district of Punjab province. Essential oil was obtained through hydro-distillation using dean stark apparatus for eight hours. Oil obtained was rectified with diethyl ether and dried over anhydrous sodium sulphate. Pale yellow with characteristic smell was obtained and stored. Chemical composition of the essential oil was determined through GC-MS.

Gas chromatographs –mass spectrometery system

Agilant 5973 - 6890 gas chromatographs–mass spectrometery 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 (30 m, 0.25 mm, 0.25 um) 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 min. Sample (2ul) 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 their identification was performed by matching their retention indices and mass spectra with those obtained from authentic samples and the NIST library.

Antibacterial and antifungal 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 sub-cultured thrice onto fresh nutrient agar media to obtain a more vigorous population. The stock cultures were incubated at 37°C for 24 h.

Well diffusion method was performed for the assessment of antibacterial activity according to National Committee for Clinical Laboratory Standards (NCCLS) (National Committee for Clinical Laboratory Standards, 1993). Wells were made in the pre-inoculated culture media. Oil volume used was 10.7, 21.4, 42.8 and 64.2 µl for each sample and incubated at 37°C for 24 h. The diameter of zone of inhibition (mm) around the well was measured. The values shown (Table 2) are the means of tests performed in triplicate.

Pure cultures of fungal species (*Aspergillus terrus, Aspergillus flavus, Aspergillus candidus, Aspergillus vessicolor, Aspergillus niger* and *Trichoderma viride*) were obtained from the PCSIR laboratories complex, Lahore. Disc diffusion method as described by Shin and Kang (2003) was performed with slight modification to determine the antifungal activity of pine stem essential oil. 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 (5 mm diameter) of known strength were placed on the culture plates. Four concentrations of oils (5, 10, 15 and 20 µl) were used in the present study. 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 3) are the means of tests performed in triplicate.

RESULTS AND DISCUSSION

The essential oil of *pinus roxburghaii* stems was obtained through hydrodistillation in the yield of 0.24%. The oil percentage was greater than Oluwadayo and Olakunle (2008) 0.02% (3) and less than Asta et al. (2006) which was 0.25 - 0.49% The chemical composition of the essential oil was determined by GC MS. The data shown in Table 1 indicates the maximum percentage of constituent was α-pinene (41.9%) in pine stem essential oil. This constituent has been the necessary component in pinus species (Asta et al., 2006 and Ansari et al., 2005).

Other monoterpene hydrocarbon like camphene (0.9%), 3-carene (16.3%), o-cymene (0.4%), limonene (1.7%), phellandrene (0.7%), terpinene (0.2%), p-cymene (1.9%) were also present in stem essential oil. Oxygenated monoterpenes terpinenol (1.8%), terpinyle acetate (0.8%), 1-terpinen-4-ol (0.2%) and borneol acetate (1.1%) were also present in the oils of stem.

Sesquiterpenes caryophyllene (12.3%) and farnesene (0.6%) were present. These were reported in pinus species by oluwadayo and olakunle (2008) and Vallejo et al (1994). Oxygenated sesquiterpenes caryophyllene oxide (1.0%) and farnesyl acetate (0.2%) were also present in the oils; it was in accordance to the Eui et al (2004). The difference in percentage of oil and composition may be due factors such as time of collection, genetic, geographic and climatic conditions (oluwadayo and olakunle, 2008). Phellandrene which is present in 0.7% is an important constituent in fragrances because of its pleasing aroma. So it can be purified and may be a better source of perfumery chemicals.

The results of antibacterial activity of essential oils of chir were investigated by well method as summarized in Table 2. *E. coli and Enterobacter aerogenes* showed high resistance against all concentrations of oil. The activity of *Salmonella typhi* was slightly inhibited only on higher volume (64.2 µl) of pine wood oil. These results

Table 1. Chemical composition of essential oil stems of *Pinus roxburghaii*

Sr. No.	Retention time (Minutes)	Component	% age (w/v)
01	4.687	α-pinene	41.9
02	4.904	Camphene	0.9
03	5.797	3-Carene	16.3
04	5.928	o-Cymene	0.4
05	6.020	Limonene	1.7
06	6.054	β-Phellandrene	0.7
07	6.392	γ-Terpinene	0.2
08	6.827	p-Cymene	1.9
09	8.246	1-Terpinen-4-ol	0.2
10	8.492	α –Terpinenol	1.8
11	9.716	Borneol acetate	1.1
12	10.580	Terpinyl acetate	0.8
13	10.735	Farnesene	0.6
14	12.102	Caryophyllene	12.3
15	13.390	Butanoic acid,3-methyl-,2-phenylethylester	0.3
16	16.108	Caryophyllene oxide	1.0
17	20.617	Farnesyl acetate	0.2

Table 2. Antibacterial activity of essential oils of stems of *Pinus roxburghaii* diameter zone (mean ± s.e.) of inhibition (mm).

Pine stem oil (µl)	*E. coli*	*B. subtilis*	*S. typhi*	*E. aerogenes*	*S. aureus*
10.7	-	-	-	-	-
21.4	-	8.5 ± 0.353	-	-	9.5 ± 0.565
42.8	-	19.5 ± 0.989	-	-	21 ± 1.131
64.2	-	31 ± 0.848	10 ± 0.707	-	33 ± 0.424

Resistant, full growth of microbe.

Table 3. Antifungal activity of essential oils of stems of *Pinus roxburghaii*.

Pine stem oil (µl)	*A. terrus*	*A. flavus*	*A. candidus*	*A. vessicolor*	*T. viride*	*A. niger*
5	18 ± 0.141	12 ± 1.414	-	-	0.00	-
10	20 ± 0.283	20.5 ± 0.707	-	-	8.5 ± 1.414	-
15	26 ± 1.414	27.5 ± 0.707	-	-	12.5 ± 0.707	-
20	38.5 ± 2.121	29 ± 1.414	-	-	19 ± 1.061	-

Diameter zone (mean± s.e.) of inhibition (mm) sensitive, no growth was observed.

contradicts the findings of Parihar et al. (Parihar at al., 2006) who observed that leaf extract of *P. roxburghii* inhibit the growth of *E. coli* and *Salmonella sp.* but their results showed similarity with present findings in case of stem extracts of *P. roxburghii*.

Highest diameters of zone of inhibition were recorded in case of *B. subtilis* and *S. aureus* on higher concentra-tion (64.2 µl) of oil but as the concentration of oil decreased, diameters of zone of inhibition of both microbes also decreased. Similarly, *S. typhi* was resistant on lower concentration of stem essential oil and sensitive on higher concentration. Results clearly indicated that the microbial activity is concentration dependent. Similar findings have been reported by Hong et al. (2004) and Pradeep et al. (2006) in the *Pinus* species. They reported negative activity against *E. coli* and *Candida albicans* while active against *Staphylococcus aureus* and *Salmonella typhimurium.*

Results as shown in Table 3 clearly indicate that growth of fungal strains (*Aspergillus terrus, Aspergillus flavus and Trichoderma viride)* was dose dependent and there is linear relationship between volume of essential oil and

zone of inhibition. The highest zone (38.5 ± 2.121) was observed in case of *A. terrus* at the highest concentration of pine stem oil (20 µl). While 29 ± 1.414 and 19 ± 1.061 zones were recorded in case of *A. flavus* and *Trichoderma viride* respectively. Motiejunaite and Peciulyte (2004) also observed the growth inhibition of *T. viride* after 7-day incubation effect of vapor of pine needles. 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 18 ± 0.141 and 12 ± 1.414 respectively. An interesting feature was observed while using the pine stem oil in case of *Aspergillus candidus, A. niger* and *Aspergillus vessicolor*. These three strains are highly sensitive to all concentrations of pine stem oil used in the present study. Similar results were obtained in case of *A. niger* by Motiejunaite and Peciulyte (2004) who studied the inihibition of *A. niger* during all period of their investigation when treated with pine needles oil.

Present study indicated that essential oil of *P. roxburgahii* stems contain active components which showed greatest activity against certain tested fungi and bacteria. Similar findings have been reported by Hong et al. (2004) who also stated that the antibacterial and antifungal activities of various components of the coniferous trees essential oils.

Conclusion

It can be concluded that Seventeen components out of fifty-two were identified in the oil. Major component in essential oil was α- pinene, followed by 3-carene, caryophyllene p-cymene, Terpinenol, Limonene, Borneol acetate, Caryophyllene oxide , camphene, Tepinyl acetate, β-Phallenderene, farnesene, o-cymene, Butanoic acid, 3-methyl-, 2-phenylethyl ester ,1-terpinen-4-ol, Farnesyle acetate, and γ-terpinene. Terpenes of stem essential oil of pine are active against *Staphylococcus aureus*, *Bacillus subtilis, Aspergillus terrus, Aspergillus flavus, Aspergillus candidus, Aspergillus vessicolor, Aspergillus niger* and *Trichoderma viride.* As this oil significantly inhibited the growth of certain bacteria and fungi tested in the present experiments. However further studies for LD_{50} and toxicology are required to prove its fitness for human consumption.

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