Volatile constituents of *Pinus roxburghii* from Nepal

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ABSTRACT

Background: *Pinus roxburghii* Sarg. Is one of 3 species of pine found in Nepal, the oil of which is traditionally used to treat cuts, wounds, boils, and blisters. **Objective:** To obtain, analyze, and examine the anti-microbial and cytotoxic activities of the essential oils of *P. roxburghii*. **Materials and Methods:** Three plant parts (cone, needle, and bark) of *Pinus roxburghii* were collected in Biratnagar, Nepal. The essential oils were obtained by hydrodistillation, and the chemical compositions were determined by GC-MS. The needle and cone essential oils were screened for anti-microbial activity against *Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, and *Aspergillus niger;* brine shrimp (*Artemia salina*) lethality; and *in-vitro* cytotoxicity against MCF-7 cells. **Results:** GC-MS analysis for the cone oil revealed 81 compounds with 78 components being identified (95.5% of the oil) while 98.3% of needle oil was identified to contain 68 components and 98.6% of the bark oil (38 components) was identified. The 3 essential oils were dominated by sesquiterpenes, particularly (*E*)-caryophyllene (26.8%-34.5%) and α-humulene (5.0%-7.3%) as well as monoterpene alcohols terpinen-4-ol (4.1%-30.1%) and α-terpineol(2.8%-5.0%). The monoterpene δ-3-carene was present only in needle and cone essential oils (2.3% and 6.8%, respectively). Bio-activity assays of the cone essential oil of *P. roxburghii* showed remarkable cytotoxic activity (100% killing of MCF-7 cells at 100 μg/mL) along with notable brine shrimp lethality (LC_{50} = 11.8 μg/mL). The cone essential oil did not show anti-bacterial activity, but it did exhibit anti-fungal activity against *Aspergillus niger* (MIC = 39 μg/mL). **Conclusion:** The bioactivity of *P. roxburghii* essential oil is consistent with its traditional medicinal use.

Key words: Anti-fungal, brine shrimp lethality,cytotoxicity, essential oil composition, α-humulene

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INTRODUCTION

Pinus roxburghii Sarg. is a species of several evergreen trees belonging to the Pinaceae and is native to the Himalayas and distributed throughout Pakistan, India, Nepal, and Bhutan.[1] With around 105 species found worldwide,[2] only 3 *Pinus* species (*P. roxburghii, P. wallichiana,* and *P. wallichiana* var. *manangensis*) are found in Nepal.[3] *P. roxburghii* is a large tree attaining up to 28-55 m in height with a trunk diameter reaching up to 2 m. The cones of *P. roxburghii* are ovoid conic and usually open up to 20 cm to release the seeds.[4] *P. roxburghii* oil has been traditionally used to treat cuts, wounds, boils, and blisters.[5] In addition, phytochemical screening of *Pinus* needles and stems have found abundant amounts of vitamin C, tannins, and alkaloids while the stem has been primarily used as a source of turpentine oil.[6,7]

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Microbiological activity research into the essential oil of *P. roxburghii* has shown significant anti-fungal activity[8] while alcoholic extract of the needle, stem, and cones are reported to exhibit strong anti-bacterial activity.[9] Investigation into the cytotoxicity activity of twig essential oil has been also reported to show activity against human cancer cells and Ehlirch ascites carcinoma cells.[10] Furthermore, mosquito repellent and larvicidal activities[11] as well as allelopathic activities have been reported from India.[12] Essential oil compositions of needle[13] and stem[8] from Pakistan as well as twig,[10] needle, and cone[14] from Egypt have been previously reported. To our knowledge, this is the first report of the essential oil compositions of *P. roxburghii* from Nepal.

MATERIALS AND METHODS

Plant material

The plant materials of *Pinus roxburghii* were collected from city of Biratnagar (26°28' N, 87°16' E, 72 m above sea level), in Morang district in Koshi Zone, in Nepal on 13 May 2011. The plant was identified by Tilak Gautam (Lecturer of Botany, MMAMC Campus, Tribhuvan University, Biratnagar, Nepal), and a voucher specimen (HN669) has been deposited in the herbarium of the Tribhuvan University, Post-Graduate Campus, Botany Department, Biratnagar.The fresh needle sample (100 g), was crushed and hydrodistilled using a Clevenger type apparatus for 4 h to give clear pale yellow essential oil (0.053 g), which was stored at 4ºC until analysis. Hydrodistillation of the fresh bark (100 g) gave 0.001 g clear pale yellow oil, while the fresh cone (100 g) gave 0.012 g pale yellow clear oil.

Gas chromatographic – mass spectral analysis

The essential oils of *P. roxburghii* were analyzed by GC-MS using an Agilent 6890 GC with Agilent 5973 mass selective detector [MSD, operated in the EI mode (electron energy=70 eV), scan range=45-400 amu, and scan rate=3.99 scans/sec], and an Agilent ChemStation data system. The GC column was an HP-5ms fused silica capillary with a (5% phenyl)-polymethylsiloxane stationary phase, film thickness of 0.25 μm, a length of 30 m, and an internal diameter of 0.25 mm. The carrier gas was helium with a column head pressure of 48.7 kPa and a flow rate of 1.0 mL/min. Injector temperature was 200°C, and detector temperature was 280°C. The GC oven temperature program was used as follows: 40°C initial temperature, hold for 10 min; increased at 3°C min to 200°C; increased 2°/min to 220°C. A 1% w/v solution of each sample in CH_2Cl_2 was prepared, and 1 μL was injected using a splitless injection technique.

Identification of the oil components was based on their retention indices determined by reference to a homologous series of *n*-alkanes and by comparison of their mass spectral fragmentation patterns with those reported in the literature[15] and stored on the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version C.00.01.080)]. The percentages of each component are reported as raw percentages based on total ion current without standardization. The essential oil compositions of *P. roxburghii* from Nepal are summarized in Table 1.

Anti-microbial screening

The essential oil was screened for anti-microbial activity against Gram-positive bacteria, *Bacillus cereus* (ATCC No. 14579), and *Staphylococcus aureus* (ATCC No. 29213); Gram-negative bacteria, *Pseudomonas aeruginosa*(ATCC No. 27853) and *Escherichia coli* (ATCC No. 10798). Minimum inhibitory concentrations (MICs) were determined using the microbroth dilution technique as described previously.[16] Dilutions of the crude extracts were prepared in cation-adjusted Mueller Hinton broth (CAMHB) beginning with 50 μL of 1% w/w solutions of crude extracts in DMSO plus 50 μL CAMHB. The extract solutions were serially diluted (1:1) in CAMHB in 96-well plates. Organisms at a concentration of approximately 1.5×10^8 colony-forming units (CFU)/mL were added to each well. Plates were incubated at 37°C for 24 hours; the final minimum inhibitory concentration (MIC) was determined as the lowest concentration without turbidity. Gentamicin was used as a positive antibiotic control; DMSO was used as a negative control. Anti-fungal activity against *Aspergillus niger* (ATCC No. 16888) was determined as above using YM broth inoculated with *A. niger* hyphal culture diluted to a McFarland turbidity of 1.0. Amphotericin B was the positive control.

Brine shrimp lethality assay

The brine shrimp (*Artemia salina*) lethality test was carried out using a modification of the procedure by McLaughlin as previously described.[16] *Artemia salina* eggs were hatched in a sea salt solution (Instant Ocean®, 38 g/L) with an incandescent light bulb as the heat source. After 48 hours, the newly hatched nauplii were counted using a micropipette and transferred to 20-mL vials. Nine vials each containing 10 *A. salina* nauplii in 10 mL of sea salt solution (same as the hatching solution) were prepared. Three vials were labeled as controls with first one containing no DMSO, another with 10 μL, and the last one with 100 μL DMSO. Three replicate vials contained 10 μL of 1% essential oil solution in DMSO, and the other 3 were prepared by adding 100 μL of 1% essential oil solution in DMSO. Surviving *A. salina* were counted after 24 hours.

Cytotoxicity screening

Human MCF-7 breast adenocarcinoma cells (ATCC No. HTB-22)[17] were grown in a 3% CO_2 environment at 37°C in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100,000 units penicillin and 10.0 mg streptomycin per liter of medium, 15 mM of Hepes, and buffered with 26.7 mM $NaHCO_3$, pH 7.35. Cells were plated into 96-well cell culture plates at 2.5×10^4 cells per well. The volume in each well was 100 μL. After 48 h, supernatant fluid was removed by suction and replaced with 100 μL growth medium containing 1.0 μL of DMSO solution of the essential oil (1% w/w in DMSO). This gave a final concentration of 100 μg/mL in each well. Solutions were added to wells in four replicates. Medium controls and DMSO controls (10 μL DMSO/mL) were

Table 1: Chemical compositions of *Pinus roxburghii* essential oils from Nepal

RI[a]	Compound	% Composition		
		Needle	Bark	Cone
853	(3*Z*)-Hexenol	0.2	---	---
934	α-Thujene	0.1	---	tr[b]
940	α-Pinene	0.4	---	0.2
953	Camphene	tr	---	---
973	Verbenene	---	---	tr
975	Sabinene	0.5	---	0.6
978	β-Pinene	---	---	0.4
991	Myrcene	0.3	---	0.3
994	Mesitylene	---	---	0.1
1004	α-Phellandrene	---	---	tr
1010	δ-3-Carene	2.3	---	6.8
1016	α-Terpinene	0.3	---	0.3
1021	1,2,4-Trimethylbenzene	---	---	tr
1024	*p*-Cymene	tr	---	0.4
1027	Limonene	0.7	---	---
1028	Limonene+β-Phellandrene	---	---	0.5
1031	1,8-Cineole	tr	---	0.1
1043	Phenylacetaldehyde	---	---	tr
1058	γ-Terpinene	0.5	---	0.6
1066	*cis*-Sabinene hydrate	0.1	---	tr
1071	*n*-Octanol	0.2	---	---
1088	Terpinolene	2.1	0.3	3.6
1097	*trans*-Sabinene hydrate	0.1	---	tr
1100	Linalool	1.6	6.4	0.1
1105	Nonanal	tr	---	---
1112	*endo*-Fenchol	0.1	---	---
1112	Phenylethyl alcohol	---	---	0.2
1120	*cis*-*p*-Menth-2-en-1-ol	0.9	---	0.2
1134	Terpinen-1-ol	tr	---	---
1134	*cis*-*p*-Mentha-2,8-dien-1-ol	---	---	tr
1138	*trans*-Pinocarveol	---	---	tr
1139	*trans*-*p*-Menth-2-en-1-ol	0.6	---	0.2
1144	*cis*-β-Terpineol	tr	---	---
1146	Camphene hydrate	tr	---	---
1147	Veratrole	---	---	tr
1160	α-Phellandrene-8-ol	---	---	2.0
1160	Pinocarvone	0.2	---	---
1164	Isoborneol	tr	0.2	---
1167	*p*-Mentha-1,5-dien-8-ol	tr	0.3	0.9
1178	Terpinen-4-ol	30.1	4.1	16.2
1181	*m*-Cymen-8-ol	0.1	0.5	1.3
1184	*p*-Cymen-8-ol	0.3	1.3	2.7
1186	Unidentified monoterpenoid	0.1	0.5	---
1190	α-Terpineol	5.0	4.9	2.8
1194	*cis*-Piperitol	0.2	---	---
1195	Myrtenol	---	---	tr
1197	*o*-Cumenol	0.1	---	---
1197	Estragole (=Methyl chavicol)	---	0.5	---
1206	*trans*-Piperitol	0.3	---	---
1206	Verbenone	---	---	0.2
1217	*trans*-Carveol	---	---	tr
1220	Coahuilensol methyl ether	---	---	0.2
1227	Citronellol	0.1	1.0	0.1
1238	Neral	---	1.3	---
1250	Car-3-en-2-one	---	---	0.3
1251	Geraniol	0.3	3.0	---
1252	Piperitone	---	---	0.3
1270	Geranial		2.1	---
1277	Pregeijerene B	---	---	0.1
1285	Isobornyl acetate	0.1	0.5	0.1
1305	Unidentified monoterpenoid	---	---	0.8

Contd...

Table 1: Contd...

RIa	Compound	% Composition		
		Needle	Bark	Cone
1311	*p*-Vinylguaiacol	tr	---	---
1338	Linalool propanoate	0.4	1.2	1.1
1349	α-Terpinyl acetate	2.2	4.9	4.5
1353	Citronellyl acetate	tr	0.2	---
1356	Eugenol	---	11.4	---
1365	Neryl Acetate	---	---	0.1
1375	α-Copaene	0.1	---	tr
1385	Geranyl acetate	0.4	0.9	0.4
1392	β-Elemene	---	0.4	---
1404	Longifolene (=Junipene)	---	0.6	0.5
1406	Methyl eugenol	0.3	1.8	0.4
1421	(*E*)-Caryophyllene	31.7	34.5	26.8
1436	α-*trans*-Bergamotene	---	0.1	---
1454	α-Humulene	7.3	5.7	5.0
1458	(*E*)-β-Farnesene	0.2	0.3	0.1
1462	Precocene I (=6-Demethoxyageratochromene)	---	0.1	---
1466	(*E*)-Ethyl cinnamate	---	---	0.4
1474	*n*-Dodecanol	---	0.4	0.2
1478	γ-Muurolene	tr	---	tr
1481	α-Amorphene	---	0.2	---
1482	Germacrene-D	0.4	---	0.2
1488	Phenylethyl 2-methylbutanoate	0.1	---	---
1492	Phenylethyl 3-methylbutanoate	1.6	0.9	0.5
1501	α-Muurolene	0.2	---	0.1
1514	γ-Cadinene	---	0.4	---
1524	δ-Cadinene	0.3	0.1	0.1
1552	(*Z*)-Caryophyllene oxide	---	---	0.2
1557	Germacrene B	0.4	---	---
1565	(*E*)-Nerolidol	0.2	0.1	tr
1570	Caryolan-8-ol	tr	---	---
1583	(*E*)-Caryophyllene oxide	1.2	0.9	4.0
1596	Longiborneol (=Juniperol)	---	---	tr
1609	Humulene epoxide II	0.2	---	0.6
1623	(*Z*)-Asarone	---	0.5	---
1635	Caryophylla-4(12),8(13)-dien-5-ol	0.1	---	0.2
1641	τ-Cadinol	---	0.7	---
1642	τ-Muurolol	0.1	---	tr
1645	α-Muurolol (=Torreyol)	tr	---	tr
1654	α-Cadinol	0.1	---	tr
1669	14-Hydroxy-9-*epi*-(*E*)-caryophyllene	0.3	---	0.4
1722	(2*Z*,6*E*)-Farnesol	1.1	4.2	1.9
1843	(2*E*,6*E*)-Farnesyl acetate	0.2	1.4	0.8
1914	(5*E*,9*E*)-Farnesyl acetone	0.6	---	---
1924	Unidentified cembrene isomer	0.6	---	---
1938	Cembrene	0.2	---	---
1955	Hexadecanoic acid	---	---	0.3
2019	Abieta-8,12-diene	---	---	0.4
2052	Unidentified diterpenoid	0.6	---	
2057	Abietatriene	---	---	0.6
2082	Abieta-7,13-diene	---	---	1.4
2148	Abieta-8(14),13(15)-diene	---	---	0.6
2238	Unidentified diterpenoid	0.2	---	1.2
2271	Dehydroabietal	---	---	0.7
2302	4-*epi*-Abietal	---	---	1.1
2303	Methyl levopimarate	0.3	---	---
2367	Unidentified diterpenoid	---	---	0.8
	Total identified	98.3	98.6	95.5

[a]RI="Retention Index" on an HP-5ms column with respect to a homologous series of normal alkanes. [b]tr="trace" (i.e.,<0.05%)

used. Tingenone[18] was used as a positive control. After the addition of compounds, plates were incubated for 48 h at 37°C in 5% CO_2; medium was then removed by suction and 100 μL of fresh medium was added to each well. In order to establish percent kill rates, the MTT assay for cell viability was carried out.[19] After colorimetric readings were

recorded (using a Molecular Devices Spectra MAX Plus microplate reader, 570 nm), average absorbances, standard deviations, and percent kill ratios ($\%kill_{cmpd}/\%kill_{DMSO}$) were calculated.

RESULTS AND DISCUSSION

Needle, bark, and cone essential oils of *P. roxburghii* were obtained in 0.053%, 0.001%, and 0.012%, respectively. Chemical compositions of *P. roxburghii* cone, needle, and bark essential oils revealed a total of 117 components, of which 111 were identified. A total of 95.5% of the cone essential oil of *P. roxburghii* was identified with major components being (*E*)-caryophyllene (26.8%), terpinen-4-ol (16.2%), with smaller amounts of δ-3-carene (6.8%) and α-humulene (5.0%). Examination of the needle essential oil yielded 98.3% identified components, mostly comprised of (*E*)-caryophyllene (31.7%), terpinen-4-ol (30.1%), α-humulene (7.3%), and α-terpineol (5.0%). A total of 38 components were identified in the bark essential oil accounting for 98.6% of the composition. The major components were (*E*)-caryophyllene (34.5%) and eugenol (11.4%), along with linalool (6.4%), α-humulene (5.7%), α-terpineol (4.9%), and terpinen-4-ol (4.1%).

The *P. roxburghii* essential oil compositions in this present study are very different in comparison with previous reports from Egypt[10,14] and Pakistan.[8,13] The needle and cone essential oils from Egypt indicated a remarkably different chemotype led by δ-3-carene, comprising 26.3% and 45.87% of the needle and cone, respectively, as oppose to the Nepalese sample, which contained only 2.3% and 6.8% of needle and cone oil, respectively. In addition, the presence of α-pinene (29.3% in the needle[13] and 41.9% in the stem[8]) in essential oils from Pakistan is in stark contrast to the trace amount found in the *P. roxburghii* samples from Nepal. *P. roxburghii* needle and stem oils from Pakistan were also rich in δ-3-carene (14.2% and 16.3%, respectively) and (*E*)-caryophyllene (21.9% and 12.3%, respectively).

Both, the needle and cone essential oils of *P. roxburghii,* were screened for anti-microbial activity. Neither of the oils exhibited anti-bacterial activity (MIC ≥625 μg/mL against *B. cereus*, *S. aureus*, *P. aeruginosa*, and *E. coli)*. The major components (*E*)-caryophyllene and α-humulene had previously shown moderate anti-bacterial activity against *B. cereus* and *S. aureus*, while terpinen-4-ol was inactive.[20] Both the needle and cone oils, on the other hand, were notably anti-fungal against *A. niger* (MIC=156 and 39 μg/mL, respectively), and α-humulene was previously shown to be anti-fungal against *A. niger*.[20] Both the needle and bark oils also showed *in-vitro* cytotoxic activity against MCF-7 cells at 100 μg/mL concentrations (70.9 ± 1.4% and 100% kill, respectively). The cytotoxicities of these essential oils are likely due to the high concentrations of terpinen-4-ol, (*E*)-caryophyllene, and α-humulene, which have been shown to be cytotoxic on MCF-7 cells.[21] *P. roxburghii* cone oil was toxic to *Artemia salina* with LC_{50}=11.8 μg/mL. The biological activities of *P. roxburghii* oil in this study are consistent with previous literature reports[8,13] and are consistent with the traditional medicinal uses of this plant.

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