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Full Length Research Paper

Antioxidant and antimicrobial activities of essential oil of *Skimmea laureola* growing wild in the State of Jammu and Kashmir

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Fresh plant material of *Skimmea laureola* growing wild in the state of Azad Jammu and Kashmir was collected and subjected to Clevenger-type hydro distillation apparatus for essential oil extraction. The essential oils have been analyzed by gas chromatography mass spectrometry (GC-MS). Twenty components were identified, which yields about 96% of the total oils. The major components of the oils were linalalyl butyrate (35%), geraneol (9.5%), nerol (8.8%), citrolenol (7.7%) and ά-phallendrene (6.5%). Essential oil also exhibited good 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) radical scavenging activity showing 93.1% of inhibition and inhibition of peroxidation 45.02%. The essential oil of *S. laureola* showed appreciable antimicrobial activity against battery of selected species of bacteria and fungi, assessed by disc diffusion and measurement of minimum inhibition by micro dilution method. The plant was selected because of their reported medicinal importance in the indigenous system of medicines from ancient times and is abundantly available in various parts of Pakistan and particularly in State of Jammu and Kashmir.

Key words: *Skimmea laureola,* essential oils composition, *in vitro* antimicrobial and antioxidant activity.

INTRODUCTION

Skimmia laureola Hook. (Rutaceae) is known as an aromatic gregarious evergreen shrub, 0.9 to 2.4 m high, distributed throughout the temperate Himalayas from Kashmir eastwards at an altitudes of 1800 to 3000 m. The leaves of this plant are used for the treatment of smallpox. *Skimmia* species contain triterpenoids that are generally of the lupane type (Rahman et al., 1998, Shah et al., 2003) reported the volatile oil of *S. laureola* from the region of Gulmarg Jammu and Kashmir in different seasons shows the concentration of linalyl acetate and linalool vary from 37 to 64 and 4 to 28%, respectively (Nighat et al., 2009) reported a new triterpene from the aerial parts of *Skimmia laureola* including fourteen known compounds. The structures of the compounds were identified by spectroscopic analysis.

Goel et al. (1989) have reported that the linalyl acetate is a rich source of *S. laureola.* The volatile oil extracted from the leaves of this plant from Uttarkashi Forest Division showed the presence of linalyl acetate 39%, linalool 4.44%, citronellol 10.86%, geraniol 7.71% and nerol 8.88% and remaining 29% portion of the oil included unidentified constituents. *S. laureola* is the potential source of linalyl acetate and other components of the oil have also been used in perfume industry.

Volatile oil from the leaves and bark of *S. laureola* showed no difference in chemical constituents but showed significant differences in the quantity. Essentialoils from leaf and stem material were 1.99 and 1.58%, respectively. The leaves contained 6.1% monoterpenoids, 28.7% monoterpene alcohols, 43.9% acyclic monoterpene esters, 5.9% acyclic carbonyls,

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4.61% aromatic aldehydes, 4% coumarins and 6.76% other compounds which included 0.25% cadinene. The essential oil of the bark contained 10.53, 27, 39.4, 6.3, 4.9, 4, and 6.87% (2.69% cadinene), respectively of the same components (Razdan and Koul, 1978, Aziz et al., 2010).

Present research work has been carried out on *S. laureola* after intensive review of literature. It has been investigated that no detailed work on its biological activities was reported earlier. So we plan this piece of work on this plant to explore the hidden flora to exploit the new source of aromatic compounds identified by gas chromatography mass spectrometry (GC-MS). This piece of work is being appears to be the first report in literature on the antioxidant and antimicrobial activities of essential oils of *S. laureola* at high altitude of State of Azad Jammu and Kashmir.

MATERIALS AND METHODS

Collection of plant material

S. laureola has been collected from Peer Chanasi at High altitude of the State of Azad Jammu and Kashmir. Collection of plant material has been made according to the plan of research work. The plant was identified and authenticated by the plant taxonomist at the Department of Botany, University of Azad Jammu and Kashmir, Muzaffarabad.

Hydrodistillation method

Air-dried plant material of *Skimmea laureola* was subjected to hydro-distillation for 3 h, using a Clevenger-type apparatus as recommended by British Pharmacopeia (1988). Briefly, the plant was immersed in water and heated to boiling, after which the essential oil was evaporated together with water vapors and finally collected in a condenser. The distillate was isolated and dried over anhydrous sodium sulfate (Irshad et al., 2011; Fakhar et al., 2005).

Gas chromatographic (GC) analysis

The essential oils were analyzed using a Perkin-Elmer gas chromatograph model 8700, equipped with flame ionization detector (FID) and HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). Injector and detector temperatures were set at 220 and 290℃, respectively.

Column oven temperature was programmed from 80 to 220℃ at the rate of 4℃ min^{-1}; initial and final temperatures were held for 3 and 10 min, respectively. Helium was used as a carrier gas with a flow of 1.5 ml min-1. A sample of 1.0 µl was injected, using slit mode (split ratio, 1:100). Quantification was completed by built-in data-handling software supplied by the manufacturer (Perkin-Elmer, Norwalk, CT, USA) of the gas chromatograph. The results (composition) were reported as a relative percentage of the total peak area.

Gas chromatography mass spectrometry (GC-MS) analysis

GC–MS analysis of the essential oils were performed using an Agilent Technologies (Little Falls, CA, USA) 6890 N Network gas chromatographic (GC) system, equipped with an Agilent Technologies 5975 inert XL mass selective detector and Agilent Technologies 7683B series auto-injector. Compounds were separated on an HP-5 MS capillary column (30 m× 0.25 mm i.d., film thickness 0.25 µm; Agilent Technologies). A sample of 1.0 µL was injected in the split mode with split ratio 1:100. For GC–MS detection, an electron ionization system, with ionization energy of 70 eV, was used. Column oven temperature programme was the same as in GC analysis. Helium was used as the carrier gas at a flow rate of 1.5 ml/min. Mass range was 50 to 550 m/z, while injector and MS transfer line temperatures were set at 220 and 290℃, respectively.

Compounds identification

The identification of the components was based on comparison of their mass spectra with those of NIST mass spectral library (Massada, 1976; Mass Spectral Library, 2002), and those described by Adam (2001), as well as on comparison of their retention indices either with those of authentic compounds or with literature values (Mimica-Dukic et al., 2003; Adam, 2001; Vagionas et al., 2007).

Antibacterial assay by disc diffusion method

Nutrient agar (Oxoid, UK) 28 g/L was suspended in distilled water and distributed homogenously and autoclaved. Inoculum (100 µl/100 ml) was added to the medium and poured in sterilized Petri-plates. After this, 6 mm wicks of paper were laid flat on growth medium containing 100 µl of essential oil. The petri plates were then incubated at 37ºC for 18 h, for the growth of bacteria. The essential oil extract having antibacterial activity, inhibited the bacterial growth and clear zones were formed. The zones of inhibition were measured in millimeters using zone reader (Abubakar, 2009).

Growth medium, culture and inoculum preparation

Pure cultures of the fungi were maintained on sabouraud dextrose agar (SDA) medium in slant add Petriplates that were pre-sterilized in hot air oven at 180ºC for 3 h. These cultured slants were incubated at 28ºC for 3 to 4 days for the multiplication of fungal strains.

Antifungal assay by disc diffusion method

The prepared sterilized growth medium was transferred to the sterilized petri-plates. The Petri-plates were then incubated at 28ºC for 48 h, for the growth of fungus. Small filter paper discs were laid flat on growth medium having fungal growth, and 100 µl of essential oil extract was applied on each disc. The petri-plates were again incubated. The essential oil extracts having antifungal activity exhibited clear zones around the discs. The zones of inhibition were measured in millimeters using zone reader (Irkin and Korukluoglu, 2009).

Minimum inhibitory concentrations (MIC) of essential oils

The MIC defined as the lowest concentration of compound that showed no increase in absorbance for all the replicates compared to the negative control. Minimum inhibitory concentration (MIC) of essential oil of *S. laureola* was checked on the selected fungal and

bacterial cultures using microtitre plate-based assay. Resazurin was used as an indicator of cell growth (Sarker et al., 2007).

Micro-dilution broth method

For calculation of minimum inhibitory concentration (MIC), which represents the concentration that completely inhibits the growth of microorganisms, a micro-dilution broth susceptibility assay was used, as reported by CLSI (2010). A series of dilutions were prepared in the range 0.01 to 72.0 mg/ml of the *S. laureola* essential oil in a 96-well microtiter plate, including one growth control (NB/SDB + Tween 80) and one sterility control (NB/SDB + Tween 80 + test oil). 160 µl NB and SDB for bacteria and fungi, respectively, were added onto the micro-plates with 20 µl of the tested solution. Then, 20 µl 5 × 105 CFU/ml of standard microorganism suspension was inoculated onto the microplates. The plates were incubated at 37℃ for 18 h for bacteri a and at 30℃ for 48 h for fungi. Amoxycillin was used as a reference compound for antibacterial and flumequine for antifungal activities. The growth was indicated by the presence of a white 'pellet' on the well bottom. The MIC was calculated as the highest dilution showing complete inhibition of the tested strains.

Antioxidant potential of essential oil

2,2-diphenyl-1-picrylhydrazyl radical (DPPH) radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay was carried out spectrophotometrically as described by Tape et al. (2007). Aliquot (50 µl) of various concentrations (10 to 100 µg/ml) of the essential oil were added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm:

$$(\%) = 100 \times (A_{blank} - A_{sample}/A_{blank})$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from a graph plotting percentage inhibition against extract concentration.

Antioxidant activity determination in linoleic acid system

The antioxidant activities of *S. laureola* essential oil was determined in terms of measurement of percentage inhibition of peroxidation in the linoleic acid system, following the method described by Iqbal and Bhanger (2005) with some modifications. Essential oil and extracts (5 mg) were added to a solution mixture of linoleic acid (0.13 ml), 99.8% ethanol (10 ml) and 10 ml 0.2 M sodium phosphate buffer, pH 7. The total mixture was diluted to 25 ml with distilled water. The solution was incubated at 40℃ for 175 h. The extent of oxidation was measured by the peroxide value, using the colorimetric method as described by Yen et al. (2000).

RESULTS AND DISCUSSION

The plant material was collected in May 2008 from Peer Chinasi, District Muzaffarabad, State of Azad Jammu and Kashmir. The fresh material (1.0 Kg) was subjected to hydrodistillation for five hours. The sample afforded 7.0 ml (0.7%). The oil has been analyzed by GC-MS analysis.

Shah et al. (2003) reported the volatile oil of *S. laureola* from the region of Gulmarg Jammu and Kashmir in different seasons showed the concentration of linalyl acetate vary from 37 to 64% and linalool 4 to 28%, respectively.

Goel et al. (1989) have been reported the linalyl acetate as rich source of *S. laureola.* The volatile oil extracted from the leaves of this plant from Uttarkashi Forest Division showed the presence of linalyl acetate 39%, linalool 4.44%, citronellol 10.86%, geraniol 7.71% and nerol 8.88% and remaining 29% portion of the oil included unidentified constituents. Volatile oil from the leaves and bark of *S. laureola* showed no difference in chemical constituents but showed significant differences in the quantity. Razdan and Koul (1978) investigated the essentialoils from leaf and stem material were 1.99% and 1.58% respectively.The leaves contained 6.1% monoterpenoids, 28.7% monoterpene alcohols, 43.9% acyclic monoterpene esters, 5.9% acyclic carbonyls, 4.61% aromatic aldehydes, 4% coumarins and 6.76% other compounds which included 0.25% cadinene. The essential oil of the bark contained 10.53, 27, 39.4, 6.3, 4.9, 4, and 6.87% (2.69% cadinene), respectively.

The present work on the essential oils of *S. laureola* revealed the presence of 20 components, representing 96% of the oil. The major components of the oils were linalalyl butyrate (35%), Geraneol (9.5%), Nerol (8.8%), Citrolenol (7.7%) and ά-phallendrene (6.5%). From the literature review on this plant resulted that linalalyl butyrate is the major component instead of linalalyl acetate reported previously and is seems to be first time isolated from this plant. The constituents are shown in Table 1.

The antioxidant activity of the oil of *S. laureola* as assessed by *DPPH* radical scavenging assay and expressed in terms of 50% inhibition (IC_{50}) is given in Table 2. Free radical scavenging capacity of the essential oil was noted to be increased in a concentration dependent manner. The *S. laureola* essential oil exhibited appreciable free radical scavenging activity with IC_{50} - value 33.23 ± 0.46 µg/ml. When DPPH scavenging activity of essential oil was compared with synthetic antioxidant butylated hydroxytoluene (BHT), the oil provided weaker activity (Table 2). No earlier studies were reported in the literature regard to the DPPH radical scavenging capacity of *S. laureola*oil. Similarly, DPPH inhibition percentage of oil was recorded lower (93.1%) as compared to synthetic BHT (98.4%).

S. laureola essential oil exhibited 45.02% inhibition of peroxidation that was significantly ($p < 0.05$) lower than BHT (94.106). Table 2 shows the level of % inhibition of linoleic acid oxidation as exhibited by the essential oil of *S. laureola*. Linoleic acid is a polyunsaturated fatty acid, upon oxidation peroxides are formed which oxidize Fe^{2+} to Fe^{3+}, the later forms complex with SCN^{-}, concentration

Table 1. Chemical composition of the essential oil of *Skimmia laureola* by GC-MS.

S/N	Compounds	RI	Percentage age composition	Identification
1.	Myrecene	933	0.3	RI,MS,CO
2.	*α*-pinene	939	2.3	RI,MS, CO
3.	Sebenine	973	0.3	RI,MS,CO
4.	*α*-phellandrene	1009	6.5	RI,MS, CO
5.	3-carene	1011	4.6	RI,MS,CO
6.	Limonene	1032	0.5	RI,MS,CO
7.	*β*-Phellanderine	1033	1.4	RI,MS
8.	*Cis*-Ocemene	1041	3.0	RI,MS,CO
9.	*α*-tepeneol	1189	1.3	RI,MS,CO
10.	Nerol	1229	8.8	RI,MS,CO
11.	Citrolenol	1231	7.71	RI,MS
12.	Geraneol	1273	9.5	RI,MS
13.	Anthranilic acid	1337	0.76	RI,MS
14.	*α*-terpenyl acetate	1350	0.8	RI,MS
15.	Neryl acetate	1364	3.5	RI,MS,CO
16.	Geranyl acetate	1382	0.13	RI,MS
17.	Isocaryophyllene	1418	5.6	RI,MS
18.	Linalyl butyrate	1422	**35.0**	RI, MS,CO
19.	Elemol	1549	0.6	RI,MS
Total			96	

RI: Retention index on HP-5 MS column; CO: co-injection with authentic samples; MS: mass fragmentation; RT: retention time.

Table 2. Antioxidant activities of*Skimmealaureola*essential oil by DPPH radical scavenging and linoleic acid inhibition assay.

Assay method	Essential oil	BHT
DPPH inhibition percentage (%)	93.1	98.4
DPPH, IC_{50} (µg/mL)	33.23 ± 0.46	18.23 ± 0.78
Inhibition in linoleic acid system (%)	45.02 ±0 .76	94.04 ± 1.06

Values are mean ± standard deviation of three independent experiments. Different letters in superscript indicate significant differences within solvent.

of which is determined spectrophotometrically by measuring absorbance at 500 nm. Higher the absorbance, higher will be the concentration with control BHT (94.04%). Ahmad and Sultana**,** (2003) reported that crude ethanolic extract of *S. laureola* exhibited good antifungal activity against *Microsprum canis* and *Fusarium solani* at a concentration of 400 µg/mL. The growth of *M. canis* was inhibited in 67.7% by crude extract while the growth of standared fungicide Miconazole, Ketoconazole inhibited the growth of *M. canis* at a concentration of 72.10 and 62.25 µg /ml. respectively. The essential oils of *S. alureola* showed maximum antibacterial activity against *B. subtils, E. coli* and minimum activities against *S. aureus* and *P. multocida.*

The antifungal activities of the oil have been tested against the set four fungal strain: *Aspergillus niger, Aspergillus flavus, Rhizoctonia solani* and *Fusarium solani.* The sensitivity order of essential oil against the selected fungal strain is *F. solani*> *R. solani*> *A. niger.* The essential oils show maximum antifungal activity against *F. solani* and minimum against *A. niger.* No literature has been found for the antimicrobial activities of essential oils on *S. laureola* so for. So, we have reported the antifungal and antibacterial activity on the essential oils of this plant. The results have been shown in Table 3.

Conclusion

*S. laureola*is an important medicinal plant and available in various parts of Pakistan, particularly in State of Jammu and Kashmir. The essential oils of this plant were tested for biological activities like antioxidant, antimicrobial and antifungal. The essential oils show maximum antifungal activity against *F. solani*. This is the first report of

Table 3. Antimicrobial activities of essential oils of S*kimmea laureola.*

Medicinal plants	Concentration of sample	Bacterial strain			
		B. subtils	***S. aureus***	***E. coli***	***P. multocida***
		Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Skimma laureda	100 µl	12.1 ±1.60	11.6 ± 0.76	12.0 ± 1.32	11.5 ± 0.5
	MIC	145.6± 1.35	251.6.6± 1.36	231.5± 2.31	252.3± 2. 36
		Fungal strain			
		A. niger	***A. flavus***	***R. solani***	***F. solani***
	150 µl	13.16 ± 2.7	12.16 ± 3.7	13.66 ±3.42	15.83 ±3.65
	MIC	126.2 ± 1.29	124.2 ± 1.27	121.7 ± 1.18	107 ± 1. 17

The values presented in the table are the mean of the three independent observations with standard deviation.

antifungal and antimicrobial activities of this plant.

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