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Original Research

Antibacterial potential of *Saussurea obvallata* petroleum ether extract: A spiritually revered medicinal plant

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Abstract: Uttarakhand Himalayan region holds Asteraceae or Compositae as the largest family of flowering, medicinal and aromatic plants. Species belonging to this family rises from low altitude to the alpine region. Among Asteraceae, *Saussurea obvallata* (DC.) Edgew. is widely used in several indigenous systems of medicine. Flowers, leaves and rhizomes of *S. obvallata* are used for several traditional, religious, therapeutic and ornamental purposes. Aim of this study was to determine the chemical composition and antibacterial efficacy of petroleum ether extract (PEE) of *S. obvallata*. Gas chromatography-mass spectrometry (GC-MS) analysis was used for identifying phytochemicals present in the plant extract. Furthermore, the PEE was assessed for *in-vitro* antibacterial activity against selected Gram positive and negative strains. Structure of squalene and α-linolenic acid methyl ester were identified in PEE by GC-MS analysis, by comparing the results obtained with NIST library and literature reports. PEE exhibited significant activity against *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Bacillus subtilis* with IC_{50} value of 87.2 ± 1.6, 98.4 ± 1.1 and 90.2 ± 1.8 µg/ml, respectively. These results showed that squalene and α-linolenic acid derivative identified in *S. obvallata* may be responsible for the observed antibacterial activity. To the best of our knowledge, this is the first report focused on the chemical composition and antibacterial activity of *S. obvallata*.

Key words: Ethnoreligious plants; Squalene; α-Linolenic acid; GC-MS; MIC.

Introduction

In recent years, numerous microorganisms are responsible for drug-resistant infections, and so, alternative chemotherapeutic options are mandatory and have been the focus of many researchers through the world (1-7). Medicinal plant therapy has been shown to be useful for treatment of many human and animal diseases (4, 6, 8-18). However, although plants have been greatly utilized in traditional healing systems, only in a few cases have their curative potentials in human diseases been proven (19-23).

Saussurea (Asteraceae) is an important genus containing approximately 410 species which are widely distributed throughout the world. Species belonging to the *Saussurea* genus are able to grow under different conditions from temperate to arctic climates. It has been reported as *Saussurea* spp. were found in Asia, Europe and North America, though the highest number of species belonging to the genus was found in the Himalayas and in Central Asia. These plants possess medicinal properties and, consequently, economic value; moreover, they are also used for religious ceremonies. In particular, *Saussurea* spp. plants represent a rich source of food, flavouring products, rubber, oil, insecticides, dyes, etc. and many species are even grown as ornamental plants (24).

Phytochemical and bioactivity studies carried out on several species of *Saussurea* throughout the world have revealed the presence of remarkable bioactive secondary metabolites such as phenolics, flavonoids, lignans, sesquiterpenes and lactones (25-28) with antioxidant (29), anti-inflammatory (30), anticancer (31) and hepatoprotective activities (32, 33).

Saussurea obvallata (DC.) Edgew (Brahma Kamal) is one of the Uttarakhand (India) most important flowering species, where it is recognised as state symbol and it is found in several regions as Kedarnath, the Valley

of Flowers, Tungnath and Hemkund Sahib. This specie is widespread around the entire Himalayan province, and it grows normally at 3000-4800 m. a.s.l. *S. obvallata* was also found in other Asian countries like China, Nepal and Pakistan. This species grows up to 5-10 cm height, its flowers bloom in July-August and are easily recognised for their purple colour, generally hidden from light green papery bracts that are crucial for their survival during the coldest days in the mountain areas (34). *S. obvallata* is traditionally used for the treatment of paralysis, cerebral ischemia, wounds, cuts, bruises, liver disorders, bone-ache, cough, intestinal and urinal problems (33, 35-37).

The present study deals with the characterization of secondary metabolites and antibacterial activity of *S. obvallata* petroleum ether extract (PEE), as a potential source of novel antibacterial agents.

Materials and Methods

Plant material

The plant material (*Saussurea obvallata* (DC.) Edgew.) was collected from the forest of Kedarnath valley (30.73 N latitude and 79.06 E longitude), Uttarakhand (India) in August 2015. The plant was identified and authenticated from Department of Botany, H.N.B Garhwal University, Srinagar, Uttarakhand, India. A voucher specimen (No. Pharma. Chem./ 113) of the plant was deposited in the Herbarium of Pharmaceutical Chemistry Department of same University. The whole plant material (879g) of *S. obvallata* was exposed to shade drying for about 4 weeks. The dehydrated plant material was crushed to produce powder (263g) then passed through the sieve mesh size 40 and kept, in an air tight container, for further examination.

Extraction and isolation

Shade dried plant material (200g) was subjected to solvent extraction using soxhlet apparatus for 24h continuously which yielded (7.98%) extract. The solvent used for extraction was petroleum ether. Crude extract (10 g) of *S. obvallata* was subjected to column chromatography. The fractions were eluted initially in benzene (100%) and then in benzene: ethyl acetate (8:2). Based on TLC profile identical fractions were combined and named as B1 (isolated from 100% benzene) and B2 (isolated from benzene:ethyl acetate 8:2). Both eluted fractions were subjected to gas chromatography coupled to mass spectrometry (GC-MS) analysis.

Gas chromatography-mass spectrometry analysis

A Thermo Scientific 1310 GC (Mumbai, India) interfaced with a TSQ-8000 Triple Quadrapol-MS detector was used to generate data. A TG-5MS (0.25 mm i.d., 0.25 µm film) column (Mumbai, India) was used for high-resolution capillary gas chromatography. Oven temperature was programmed from 50 to 300 °C at 20 °C/min, and helium was used as the carrier gas. All other chemicals were of analytical grade and provided by Sigma Chemicals Co. (Mumbai, India).

Identification of chemical compounds in petroleum ether fractions were carried out on GC-MS. Structural assignments were based on analysis of fragmentation pattern of mass spectra, direct comparison of mass spectral data with profiles in the National Institute of Standards and Technology library. Dried samples from petroleum ether fractions of *Saussurea obvallata* were separately diluted with respective solvents, filtered through 0.2 µm sterile syringe filters, and 1 µL of each fraction solution injected into gas chromatograph and analysed with triple quadruple mass spectrometric detector.

Antibacterial screening

All bacterial strains [*Escherichia coli* (*NCFT/BC* 5678.12)*, Salmonella typhi* (*NCFT/BC* 5678.13), *Pseudomonas aeruginosa* (*NCFT/BC* 5678.15), *Bacillus cereus* (*NCFT/BC* 5678.16)*, Bacillus subtilis* (*NCFT/BC* 5678.17) and *Staphylococcus aureus* (*NCFT/BC* 5678.18)] were grown and kept on Muller Hinton agar (Hi media, India) media at 37 ˚C (pH 7.3 ± 0.2).

The strains were sub-cultured overnight in Muller Hinton broth that was further adjusted to attain a turbidity comparable to McFarland (0.5) standard when required (38). Concentrations of 1.0, 2.0 and 3.0 mg/disk of the PEE were used for antibacterial assay, determined by disk diffusion method (National Committee for Clinical Laboratory Standards, NCCLS) (39). Test microorganisms were taken from the broth cultures with inoculating loop and shifted to the test tubes containing 5.0 ml of sterile distilled water. Inoculums were added until the turbidity was equal to 0.5 McFarland standards. Then, cotton swab was used to immunize the test tube suspension onto the surface of Muller Hinton agar plate and the consistently swabbed plates were then dried. On the dry inoculated surfaces, prepared disks were placed. Sterilized Whatman paper disks (6 mm in diameter) were prepared by placing 0.5 ml of different PEE concentrations on disks (1.0, 2.0 and 3.0 mg/disk) and allowing the disks to dry at 40 ˚C after each application. Disks containing PEE were placed with blunt-nosed thumb forceps on the immunized plates at equidistance in a circle. These plates were kept for 4-6 hrs at a low temperature (< 8 ˚C) to allow the diffusion of the extract from the disk into the medium. The same was done for the negative control. Reference antibiotic (Kanamycin) disks (positive control) were purchased as ready disks (30 µg/disk, Hi-media, India). The plates were then incubated at 37 ˚C for 24 h. The experiment was carried out in triplicate. Antibacterial activity was determined by the measurement of the inhibition zone diameter (mm) around each test organism.

Minimum inhibitory concentration (MIC) was determined by the micro-dilution technique using serially diluted (2 folds) PEE according to NCCLS-2002. It was determined by the dilution of *S. obvallata* PEE to obtain different concentrations (0.0-25, 0.0-50, 0.0-75, 0.0-100, 0.0-125 and 0.0-150 g/ml). The equal volume of each extract and nutrient broth were mixed in a test tube. Specifically, 0.1 ml of standardized inoculum (1-2×10^7 cfu/ml) was added in each tube. The tubes were incubated aerobically at 37 ˚C for 18-24 hrs. Control tubes were maintained for each test batch. These included antibiotic control (tube containing extract and growth media without inoculum) and organism control (tube containing the growth medium, saline and the inoculum). The lowest concentration (the highest dilution) of the extract that produced no visible bacterial growth

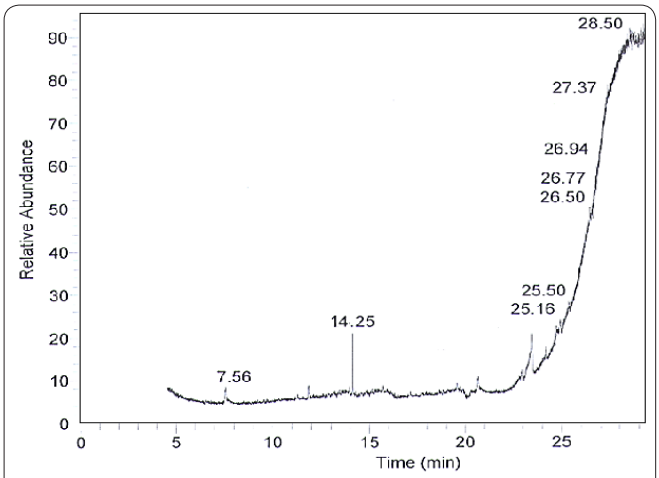

Figure 1. Total ion chromatogram of B1 fraction of *Saussurea obvallata* (DC.) Edgew with major peak at retention times (tR) 14.25 min.

(no turbidity) when compared with the control tubes were regarded as MIC.

Statistical analysis

All tests were performed in triplicate and MIC is presented as the mean ± standard deviation (S.D.) by using SPSS-16 software.

Results and Discussion

GC-MS analysis

Total ion chromatogram of B1 fraction was acquired at the condition reported below. It revealed 9 peaks showing the following retention times (tR): 07.56 min, 14.25 min, 25.16 min, 25.50 min, 26.50 min, 26.77 min, 26.94 min, 27.37 min and 28.50 min (Figure 1). Chromatographic analysis of the main peak (tR 14.25 min) revealed a molecular ion at *m/z* 410 (M^+) that can be proposed for a molecular formula $C_{30}H_{50}$. Indicative ions were also revealed at *m/z* 367(M^+-C_3H_7), 341(M^+-C_5H_9) and 69 (base ion). Other substantial fragment ions were detected at *m/z* 81, 95, 107, 121,136. Ion at *m/z* 341 was followed by the loss of ion bearing a mass of 69; however, at *m/z* 367, a 43-mass propyl fragment was eliminated. Fragment ion at *m/z* 83 agrees to the loss of ion ($C_{24}H_{39}$) of 327 mass. Ion peak at *m/z* 121 showed the loss of $C_{21}H_{37}$. In general, this mass ionization pattern indicates a 410 molecular mass compound of $C_{30}H_{50}$ formula, putatively squalene (Figure 2). This fragmentation pattern is confirmed by NIST library.

Total ion chromatogram of B2 fraction evidenced 5 peaks at different tR: 06.08 min, 11.17 min, 23.00 min, 24.56 min and 25.12 min (Figure 3). Compound identification, in B2 fraction at tR 11.17 min, was determined by observing the indicative ion peaks at *m/z* 79, 93, 173, 236 and 292, and ion at *m/z* 79 was the base peak. Peak at *m/z* 108 is an omega (ω) ion which is representative for methyl ester of polyunsaturated fatty acids with an n-3 terminal double bond. Fragmentation ion at *m/z* 236 is an alpha (α) ion defining Δ9,12 carbons as the spot of first double-bond. This ion is designed by a cleavage at the carboxyl end of unsaturated fatty acid to yield a fragment comprising 2 double bonds and a second methylene group (minus a proton). The molecular ion peak at *m/z* 292 composed with (M^+-31), for the loss of methoxyl group elements and a hydrogen atom, was indicative of polyenoic fatty acid with methylene-interrupted unsaturation. These diverse ions were typical for an n-3 homo-allylic unsaturated fatty acid of molecular formula $C_{19}H_{32}O_2$, identified as α-linolenic acid methyl ester (Figure 4). This result was in agreement with the NIST library.

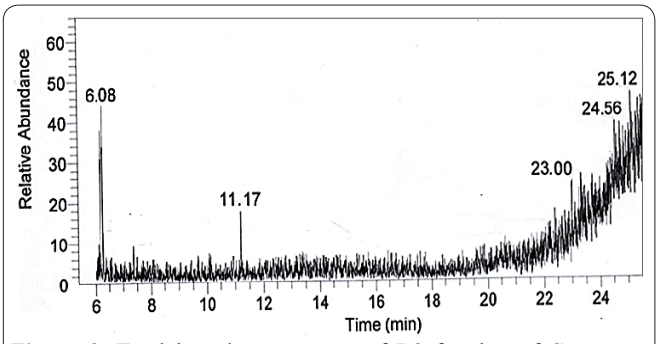

Figure 3. Total ion chromatogram of B2 fraction of *Saussurea obvallata* (DC.) Edgew with major peak at retention times (tR) 11.17 min.

Antibacterial activity

Antibacterial activity of *S. obvallata* PEE is showed in Table 1, in comparison with the antibiotic kanamycin. The mean zone of inhibition produced by the reference was between 17 and 26 mm which was higher than that (7.0-15.2 mm) produced by the extract. The extract at the three different concentrations 1.0, 2.0 and 3.0 mg/disk showed significant ($P < 0.05$) zone of inhibitions against Gram-positive *B. subtilis* (9, 11 and 13 mm, respectively), *B. cereus* (9.6, 12.8 and 15 mm, respectively) and *S. aureus* (10, 13.4 and 15.2 mm, respectively), and Gram-negative *P. aeruginosa* (7, 8.1 and 8.6 mm, respectively), *S. typhi* (8.7, 10.4 and 12.3 mm, respectively) and *E. coli* (9.8, 12.2 and 14 mm, respectively).

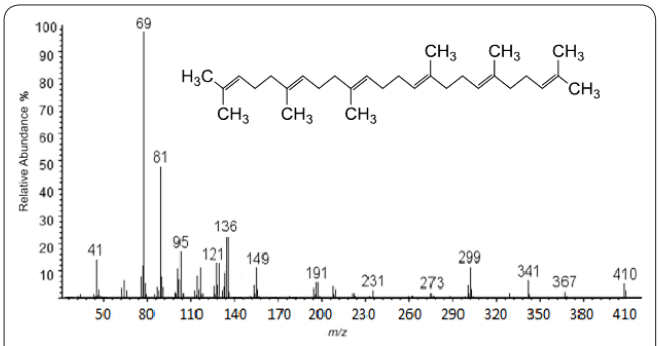

Figure 2. Chemical structure of squalene present in B1 fraction of *Saussurea obvallata* (DC.) Edgew.

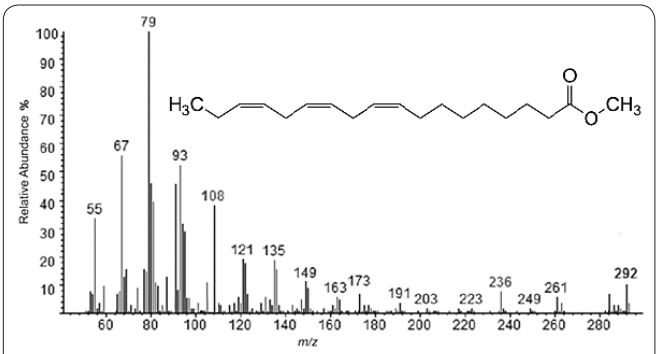

Figure 4. Chemical structure of α-linolenic acid methyl ester present in B2 fraction of *Saussurea obvallata* (DC.) Edgew.

Table 1. Antibacterial activity of the petroleum ether extract of *Saussurea obvallata* (DC.) Edgew.

Bacterial Strains	MTCC	Type	Solvent Extract			Kanamycin
			ZOI* (mm)			MIC** (µg/ml)	ZOI* 30 µg/disk
			(1 mg/disk)	(2 mg/disk)	(3 mg/disk)		
Escherichia coli	1610	Gram negative	9.8	12.2	14	98.4 ± 1.1	24
Salmonella typhi	3231	Gram negative	8.7	10.4	12.3	>100	26
Pseudomonas aeruginosa	1934	Gram negative	7.0	8.1	8.6	>100	17
Bacillus cereus	0430	Gram positive	9.6	12.8	15	90.2 ± 1.8	24
Bacillus subtilis	0121	Gram positive	9.0	11	13	>100	21
Staphylococcus aureus	0902	Gram positive	10	13.4	15.2	87.2 ± 1.6	22

*ZOI=Zone of inhibition, **MIC= Minimum inhibitory concentration.

Therefore, the extract exhibited the highest zone of inhibition against the Gram-positive *S. aureus* (15.2 mm) at 3.0 mg/disk, whereas the Gram-negative *P. aeruginosa* was the less sensitive bacterial strain.

Minimum inhibitory concentration

The MIC of *S. obvallata* leaf PEE was measured against different bacterial strains, and it ranged from 87.2 to > 100 µg/mL. In particular MIC values were slightly lower for Gram-positive bacteria (start at 87.2) compared with Gram-negative bacteria (start at 98.4). *S. obvallata* PEE showed promising MIC values when tested *vs.* Gram-positive *B. cereus*, *B. subtilis* and *S. aureus* and Gram-negative *E. coli*, *P. aeruginosa* and *S. typhi* bacteria.

In the present study, the GC-MS analysis of the fractions isolated from the petroleum ether extract of *S. obvallata* showed the presence of two well-known compounds *i.e*. squalene and α-linolenic acid methyl ester. Squalene, represented by a peak at 14.26 min in the B1 fraction of PEE, is a triterpene isoprenoid comprising 6 double bonds. Owing to double bond structure squalene acts as an antibiotic agent (40) and has an important biological role as precursor of steroids. It has been demonstrated that squalene exerts a cancer chemopreventive activity, inhibits biotransformation of procarcinogens and acts as a potent cytoprotective agent, protecting non-target cell against chemotherapeutic toxicity (41-45). α-Linolenic acid methyl ester, an aliphatic acid ester, is known for its ability in inhibiting the proliferation of both ER-positive and ER-negative breast cancer cells (46). It has been also shown to exert antiangiogenic effects in colorectal cancer and in HUVEC cells (47).

Sun et al. (48) tested the longer chain fatty acids, capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1, n-9), palmitoleic acid (C16:1, n-9) and linolenic acid (C18: 3, n-9,12,15) against *Helicobacter pylori* over the concentration range of 0.1-10 mM. The most effective fatty acids were lauric acid and palmitoleic acid with 61 mM MBCs and linolenic acid (MBC = 60.5 mM). According to Choi et al. (49), long chain unsaturated fatty acids including oleic acid, linoleic acid and linolenic acids has powerful antibacterial effect than the long chain saturated fatty acids such as palmitic acid and stearic acid.

S. obvallata is a medicinal plant used for a number of infectious diseases. The present study has revealed that *S. obvallata* is a significant source of fatty acids and terpenoids like α-linolenic acid methyl ester and squalene, respectively. Therefore, this plant can also be utilized as a dietary supplement of these bioactives. However, further studies are needed in order to identify other phytochemicals from different *S. obvallata* extracts, and to investigate their bioactivities as well as their possible mechanisms of action.

Conflicts of interest

Authors declare no conflict of interest.

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