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Research Article

Sesquiterpene lactones of *Saussurea lappa* (Decne.) Sch.Bip. and comparative antimicrobial activity of its root oil and extracts

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Abstract

Dihydrodehydrocostuslactone and dehydrocostuslactone were isolated from the roots of *Saussurea lappa* syn. *Saussurea costus* as two major sesquiterpene lactones**.** The structures of the isolates were determined using their MS and NMR (^{1}H, ^{13}C) data. GC-MS of root oil and extracts (diethyl ether and hexane) showed about three dozen constituents, of which 33 compounds were identified. The oil/ extract was dominated by the presence of dehydrocostuslactone and/ or dihydrodehydrocostuslactone besides 10-epi-γ-eudesmol and 1,8-cineol as other constituents. The root oil, ether and hexane extracts were tested for antimicrobial activity against five bacterial species and two fungi. The extracts and the root oil showed moderate activity in controlling *A. hydrophila*, *B. subtilis*, *S. candidus* and *E. coli*.

1. Introduction

Saussurea lappa, an important medicinal plant locally known as 'Kuth', is a tall robust perennial alpine Himalayan herb indigenously used for the treatment of asthma, inflammation, ulcers and stomach problems [1, 2]. Pharmaceutical industries need new and improved medicinal agents, especially in view of the increasing incidence of antibiotic resistance towards various pathogenic microbes. One of the areas which is of considerable interest is activity of plant extracts and natural essential oils. Also, the increasing consumer demand for effective and safe natural products need qualitative and quantitative database on new plant constituents and extracts. Sesquiterpene lactones of *Saussurea lappa* constituents have been reported to possess antitrypanosomal activity [3] and hepato-protective, antiparasitic, Central Nervous System (CNS) depressant, anti-ulcer, anti-cancer activity [2,4-7]. It has been used as a tonic, spasmodic, in cough, cholera and skin diseases [8]. The oil shows antiseptic and disinfectant properties. It is cardiac stimulant, carminative, expectorant and diuretic. The roots also possess analgesic, anthelmintic and emmenagogic properties, stimulate the brain and cure blood diseases and liver and kidney disorders [9]. Root extract of *Saussurea lappa* is also prescribed effective in acute typhus fever, rheumatism, nervous disorders, irregular menstruation, heart disease, to improve complexion, as a hair wash to kill lice and to turn grey hair to black [10,11]. Due to the wide application in the field of

medicine *Saussurea lappa* has been investigated for its chemical constituents and biological activity [3, 4,12-15]. This communication reports isolation and characterization of two sesquiterpene lactones viz., dihydrodehydrocostuslactone **(1)** and dehydrocostuslactone **(2),** major constituents from the roots of *Saussurea lappa* using NMR spectral data (Table 1). The ^{1}H and ^{13}C NMR data are in good agreement with literature reports [16-19]. The GC and GC-MS of the essential oil and solvent (ether and hexane) extracts were carried out to determine compositional differences (Table 2) and also evaluated for their antimicrobial activity to find potential as antibiotic agents.

2. Materials and methods

2.1 Plant Material

The fresh roots sample (1250 g) of *Saussurea lappa* were collected from Dronagiri village of Chamoli district (Uttarakhand) in the month of September. The sample, identified by Botanical Survey of India, Dehradun (Voucher No.: Phyto/ CSM/ KTH/2012) was deposited in the Phytochemistry Research Laboratory Kumaun University, Nainital for future reference.

2.2 Extraction of Oil and Preparation of Extract

The plant materials (400 g) were subjected to steam distillation. The distillates were saturated with NaCl and extracted with n-hexane and dichloromethane. The organic phase was dried over anhydrous sodium sulphate and the solvents were distilled off. Plant material (800 g) was air dried, powdered and soaked with HPLC grade ether and hexane separately (400 g each). The extracts were concentrated. The essential oil was extracted by hydro distillation following the method reported previously [22]. Major compounds were isolated by fractionation of the essential oil on silica gel CC (230-400 mesh, Merck, 600 × 25 cm column) packed with hexane, using Et_2O-hexane as mobile phase with gradually increasing the amount of ether (2-20%).

2.3 GC and GC-MS Analysis:

The oils were analyzed by using a Nucon 5765 gas chromatograph (Rtx-5 column, 30 m × 0.32 mm, FID), split ratio 1:48, N_2 flow of 4 kg/cm^2 and on Thermo Quest Trace GC 2000 interfaced with MAT Polaris Q Ion Trap Mass spectrometer fitted with a Rtx-5 (Restek Corp.) fused silica capillary column (30 m x 0.25 mm; 0.25 μm film coating). Analyses of essential oil and extracts were performed by following the method discussed by *Mathela et al.* [22]

2.4 Isolation and Identification of Constituents:

The essential oil was fractionated by column chromatography (CC), on silica gel CC (230-400 mesh, Merk, 600 x 25 cm column) packed with hexane, and eluted with hexane followed by gradient elution by Et_2O/hexane (1-20%). The identification was done on the basis of Linear Retention Index (LRI), determined with reference to homologous series of *n*-alkanes (C_9-C_{24}, Polyscience Corp., Niles IL under identical experimental condition), co-injection with standard (Sigma and Aldrich), MS Library search (NIST version 2.1 and Wiley registry of mass spectral data 7th edition), by comparing with the MS literature data [23] and by NMR (^{1}H, ^{13}C NMR) of major isolates. The relative amounts of individual components were calculated were based on GC peak area (FID response) without using correction factor.

2.5 Microbial Culture

The *in vitro* antibacterial activity of the essential oils was evaluated against a total of five bacteria including two Gram-positive *Micrococcus luteus* (MTCC-106) & *Bacillus subtilis* (MTCC-441) and three Gram-negative bacteria *Pseudomonas aeruginosa* (MTCC-424), *Aeromonas hydrophila subspecies hydrophila* (MTCC-646) & *Escherichia coli* (MTCC-443). The antifungal activity of the oils was performed against *Streptomyces candidus subspecies azaticus* (MTCC-703) and *Candida albicans* (MTCC-227). The test strains were purchased from the Institute of Microbial Technology (IMTECH), Chandigarh, India. MTCC (Microbial Technology Culture Collection) numbers represents the standard strain numbers assigned to these microorganisms. The bacteria were maintained on nutrient broth (NB) at 37°C and fungus was maintained on Malt yeast agar at 28°C. The Gram-positive bacteria (*Micrococcus luteus, Bacillus subtilis*) and Gram-negative bacteria (*Pseudomonas aeruginosa, Aeromonas hydrophila* subspecies *hydrophila, Escherichia coli*) were pre-cultured in nutrient broth overnight in a rotary shaker at 37°C, centrifuged and pellet was suspended in double distilled water while the fungus strains were pre-cultured in malt yeast broth at 28°C.

2.6 Determination of zone of inhibition

The antimicrobial activity of the essential oils was investigated by the well diffusion method using 24–48 h grown strains reseeded on nutrient broth (NB,

bacterial strains) and potato dextrose agar (PDA, fungal strains) [21]. The cultures were adjusted to 1×10^6 CFU/mL with sterile water. 100 µL of the suspensions were spread over Muller Hinton-agar (MHA) plates and potato dextrose-agar (PDA) plates to obtain uniform microbial growth. The wells (3.0 mm in diameter) were prepared with the help of sterile borer and filled with 20 µL of the test sample. The petri dishes were kept at 4°C for 2 h. The plates were incubated at 37°C (24 h) and at 30°C (48 h) for bacterial and fungal strains, respectively. The diameter of the inhibition zones (mean values) were measured in millimeter and considered as the zone of inhibition (ZOI). All experiments were performed in triplicate.

2.7 Determination of the minimum inhibitory concentration (MIC)

To determine the minimum inhibitory concentration (*MIC*) of the potent samples a series of dilutions of each potent sample ranging from 5-50 µL mL^{-1} were prepared In the agar-well diffusion technique [20], dilutions of the essential oils were prepared by diluting oil with DMSO to achieve a decreasing concentration range from 50 to 5 µL/mL using 100 µl of a suspension containing 1×10^6 CFU/ml of bacteria spread on Muller Hinton-agar (MHA) plates, whereas the fungal strains were reseeded on Potato dextrose-agar (PDA) plates. The wells were filled with 20 µl of essential oil solutions in the inoculated nutrient/potato dextrose agar plates. The bacterial plates were incubated at 37±2°C for 24–72 h., while fungal cultures were incubated at 30±2°C for 48 h. The *MIC* was defined as the lowest concentration of the oil inhibiting the visible growth of each bacterium on the agar plate so the least concentration of each essential oil showing a clear zone of inhibition was taken as the *MIC*. DMSO was used as the negative control. Nalidixic acid and Amikacin were used as positive controls for bacteria and fungi, respectively. Each test was replicated three times.

2.8 Statistical Analysis

Samples were analyzed individually in triplicate for its antimicrobial activities and data were analyzed by ANOVA statistical software. All data were expressed as mean ± standard deviation of triplicate measurements and $p<0.001$ of the difference mean was considered to be significant.

3 Results and discussion

3.1 Chemical Composition

Root oil, ether and hexane extracts of *Saussurea lappa* root possess largely the same major compounds but in different concentrations. Root essential oil was found to contain higher concentration of oxygenated sesquiterpenoids (91.3%), as compared to ether extract (76.6%) and hexane extract (76.9%). Root oil of *Saussurea lappa* contained 20.4% dihydrodehydrocostus lactone (*Supplementary Fig. S1 & S3*) and 68.8% dehydrocostuslactone (*Supplementary Fig. S2 & S4*) while ether the extract was found to contain 18.2% dihydrodehydrocostus lactone and 44.6% dehydrocostuslactone. The hexane extract contained 1.6% dihydrodehydrocostus lactone and 66.4% dehydrocostuslactone as major constituents (Table 2). The previous literature reports list the presence of dehydrocostuslactone only in the root oil of *Saussurea lappa* collected from Chamoli (Uttarakhand), India [20] but our investigations reveal dihydrodehydrocostuslactone and dehydrocostuslactone both as major constituents together in the root oil and extracts from the roots of *Saussurea lappa* (Fig 1).

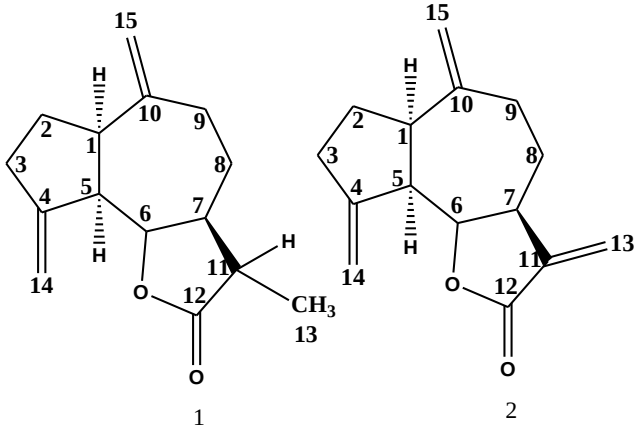


Figure 1. Sesquiterpene lactones isolated from the roots of *Saussurea lappa*

3.2 Antimicrobial Activity of Essential Oil and Ether and Hexane Extracts

The *in vitro* antibacterial activity of essential oil, organic extracts of *Saussurea lappa* against the was qualitatively assessed by the presence or absence of inhibition zones. The oil exhibited a potent inhibitory effect against *A. hydrophila, P. aeruginosa, E. coli, C. albicans and S. candidus* with diameter of inhibition

Table 1. ^{1}H-NMR and ^{13}C-NMR Data of compound **1** and compound **2**

Dihydrodehydrocostuslactone (1) (δ ppm)			Dehydrocostuslactone (2) (δ ppm)			Carbon No.
^{1}H-NMR	^{13}C-NMR	^{13}C-NMR (Reported [17]	^{1}H-NMR	^{13}C-NMR	^{13}C-NMR (Reported [17]	
2.88 m	47.0	46.9	2.92 m	47.6	47.5	1
1.87 m, 1.87m	30.2	30.0	1.91 m, 1.93 m	32.6	32.5	2
2.52 m, 2.52m	32.5	32.4	2.49 m	30.3	30.2	3
-	149.9	150.3	-	151.2	150.9	4
2.81 m	51.9	52.1	2.84 m	52.0	51.9	5
4.04 dd	85.2	85.1	3.96 dd	85.2	85.1	6
2.37 m	45.1	44.8	2.92 m	45.1	45.0	7
1.44 m, 1.83 m	30.1	28.7	1.46 m, 2.31 m	30.9	30.9	8
2.37 m, 2.08 m	37.6	37.4	2.49 m, 2.31 m	36.2	36.2	9
-	151.7	151.7	-	149.2	148.8	10
2.52 q	42.1	39.3	-	139.7	139.5	11
-	178.6	179.9	-	170.2	170.0	12
1.21 d, 1.21d	13.2	11.3	6.20 (d, J= 3.2 Hz) 5.48 (d, J= 3.2 Hz)	120.1	119.9	13
5.21 d, 5.06 dd	109.2	109.3	4.89 s, 4.77 s	109.5	109.4	14
4.89 s, 4.79 s	111.8	111.7	5.26 d, 5.04 d	112.6	112.4	15

Table 2. Essential oil constituents (%FID) of *Saussurea lappa*

Sl. No.	Compound	RI$_R$	RI	Root oil	Root ether extract	Root hexane extract
1.	*α*-Thujene	924	927	1.2	t	0.2
2.	*α*-Pinene	932	936	0.6	0.4	3.5
3.	Camphene	946	951	-	0.1	0.4
4.	*β*-Pinene	974	978	-	t	0.7
5.	*α*-Phellandrene	1002	1005	-	t	t
6.	*α*-Terpinene	1014	1019	t	-	t
7.	*p*-Cymene	1020	1022	t	-	5.8
8.	Limonene	1024	1025	t	2.5	t
9.	*β*- Phellandrene	1025	1025	-	-	t
10.	1,8 Cineol	1026	1026	t	3.2	5.4
11.	*(E)- β*-Ocimene	1032	1034	-	1.3	t
12.	*(Z)- β*-Ocimene	1044	1051	-	0.8	-
13.	Linalool	1095	1098	0.3	0.4	-
14.	Thymol	1289	1293	0.8	0.8	0.6
15.	Carvacrol	1298	1298	t	0.5	2.6
16.	*β*-Caryophyllene	1417	1418	-	0.1	1.2
17.	*α*-Humulene	1452	1454	-	0.4	
18.	*(E)- β*-Farnecene	1454	1459	-	-	t
19.	*β* –Chamigrene	1476	1481	-	0.3	t
20.	*ϒ*-Murrolene	1478	1484	-	0.1	t
21.	*ϒ*- Curcumene	1481	1486	-	0.1	t
22.	Germacrene D	1484	1487	-	2.1	t
23.	ar-Curcumene	1486	1488	-	1.0	t
24.	Valancene	1496	1494	-	t	t
25.	*δ*-Cadinene	1522	1524	0.2	-	t
26.	*β*-Bisabolene	1529	1535	0.5	t	t
27.	Germacrene D-4-ol	1574	1576	-	t	2.1
28.	Caryophyllene oxide	1582	1584	-	-	0.4
29.	Hummulene epoxide	1608	1608	-	-	0.3
30.	10-*epi*-*γ*-Eudesmol	1622	1626	2.1	13.2	5.7

Table 2. *(Continued)*

Sl. No.	Compound	RI_R	RI	Root oil	Root ether extract	Root hexane extract
31.	Cubenol	1645	1647	-	0.6	0.4
32.	**Dihydrodehydrocostus lactone**	**-**	**1882**	**20.4**	**18.2**	**1.6**
33.	**Dehydrocostuslactone**	**-**	**1934**	**68.8**	**44.6**	**66.4**
	Compound classes					
	Monoterpene hydrocarbons (%)			1.8	8.3	10.6
	Oxygenated monoterpenes (%)			1.1	1.7	8.6
	Sesquiterpene hydrocarbons (%)			0.7	4.1	1.2
	Oxygenated sesquiterpenes (%)			**91.3**	**76.6**	**76.9**
	Total identified (%)			**94.9**	**90.7**	**97.3**

t = trace (less than 0.1%), RI_R = Reported Retention Index, RI = Retention Index

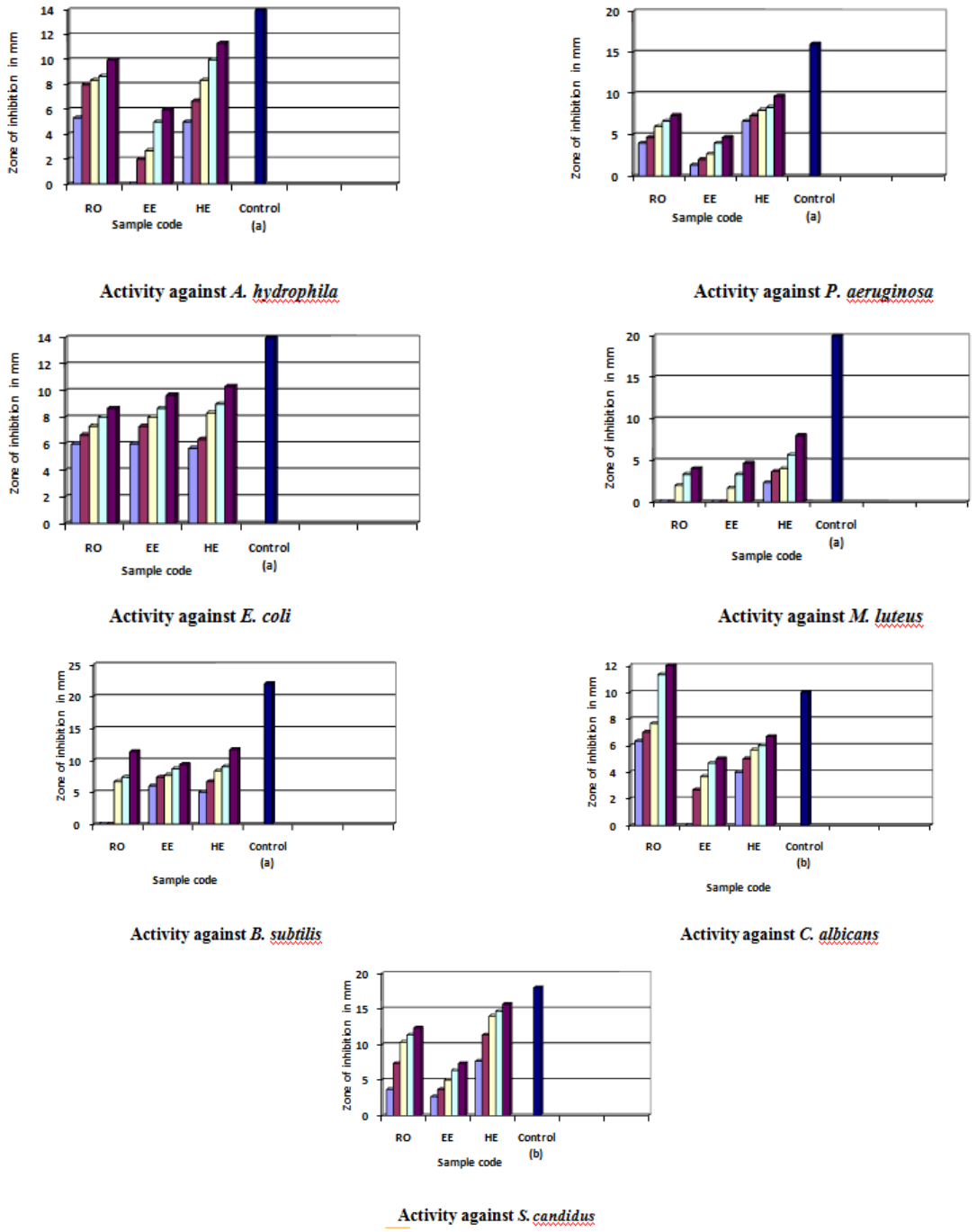
Table 3: Antibacterial and antifungal screening of the oil, hexane and ether extracts of *Saussurea lappa*

Sample Code	Concen-tration (µL/mL)	*A. hydrophila*	*P. aeruginosa*	*E. coli*	*M. luteus*	*B. subtilis*	*C. albicans*	*S. candidus*
RO	10	5.33 ± 1.15	4.00± 2.00	6.00 ± 0.00	NA	NA	6.33 ± 0.57	3.67 ± 0.57
	20	8.00 ± 2.00	4.67 ± 1.15	6.67 ± 1.15	NA	NA	7.00 ± 0.00	7.33 ± 1.15
	30	8.33 ± 1.15	6.00 ± 2.00	7.33 ± 1.15	2.00 ± 0.00	6.67 ± 1.15	7.66 ± 0.57	10.33 ± 0.57
	40	8.67 ± 1.15	6.67 ± 1.15	8.00 ± 0.00	3.33 ± 0.57	7.33 ± 1.15	11.33 ± 1.15	11.33 ± 1.15
	50	10.00 ±0.00	7.33 ± 1.15	8.67 ± 1.15	4.00 ± 1.15	11.33 ± 1.15	12.00 ± 0.00	12.33 ± 0.57
EE	10	NA	1.33 ± 0.57	6.00 ± 0.00	NA	6.00 ± 0.00	NA	2.66 ± 1.15
	20	2.00± 0.00	2.00 ± 0.00	7.33 ± 1.15	NA	7.33 ± 1.15	2.67 ± 1.15	3.67 ± 0.57
	30	2.67 ± 1.15	2.67 ± 1.15	8.00 ± 0.00	1.67 ± 1.15	7.67 ± 0.57	3.67 ± 0.57	5.00 ± 1.00
	40	5.00 ± 0.57	4.00 ± 0.00	8.67 ± 1.15	3.33 ± 1.15	8.67 ± 1.15	4.67 ± 0.57	6.33 ± 0.57
	50	6.00 ± 0.00	4.67 ± 1.15	9.67 ± 0.57	4.67 ± 1.15	9.33 ± 1.15	5.00 ± 0.00	7.33 ± 1.15
HE	10	5.00 ± 0.57	6.67 ± 1.15	5.67 ± 1.15	2.33 ± 1.15	5.00 ± 0.57	4.00 ± 0.00	7.67 ± 0.57
	20	6.67 ± 1.15	7.33 ± 1.15	6.33 ± 1.15	3.67 ± 1.15	6.67 ± 1.15	5.00 ± 1.00	11.33 ± 1.15
	30	8.33 ± 1.15	8.00 ± 0.00	8.33 ± 1.15	4.00 ± 1.15	8.33 ± 1.15	5.67 ± 0.57	14.00 ± 0.00
	40	10.00 ± 0.00	8.33 ± 1.15	9.00 ± 0.00	5.67 ± 1.15	9.00 ± 1.15	6.00 ± 0.00	14.67 ± 0.57
	50	11.33 ± 1.15	9.67 ± 1.15	10.33 ± 0.57	8.00 ± 0.00	11.67 ± 0.57	6.67 ± 1.15	15.67 ± 0.57
Positive control	30 µg	14 [a]	16 [a]	14 [a]	20 [a]	22 [a]	10[b]	18[b]

Positive Control: a = Nalidixic acid, b = Amikacin, Negative Control = DMSO, RO = *Saussurea lappa* root oil, EE = Ether extract of the roots of *Saussurea lappa* and HE = Hexane extract of the roots of *Saussurea lappa* , NA = Not active, Zone of Inhibition (ZOI) in mm ± Standard Deviation.

zones ranging from of 3.67 ± 0.57 to 12.33 ± 0.57 mm, as shown in Table 3. Ether and hexane extract also revealed a great potential of antimicrobial activity against all microbes (Table 3). Hexane extract showed the strongest antifungal effect against *S. candidus* (inhibition zone 15.67 ± 0.57 mm). Ether extract displayed a moderate inhibitory effect against most of the microbes. In this study, the oil, ether and hexane extracts exhibited significant antimicrobial activity than that of positive control. The blind control did not inhibit the growth of the bacteria tested. Further, the results of antibacterial and antifungal activity of *Saussurea lappa*, investigated against different pathogens by Well Diffusion method, are presented in Table 3 (ZOI) and Table 4 (MIC). It was found in the present study that the root oil, ether extract and

hexane extract of *Saussurea lappa* exhibited maximum zone of inhibition against *E. coli* (9.67 ± 0.57 mm) and *S. candidus* (15.67 ± 0.57 mm), while minimum zone of inhibition was shown against *P. aeruginosa* (1.33 ± 0.57 mm) and *M. luteus* (2.33 ± 1.15) respectively, after 24 h and 48 h of incubation at 37^0C and 28^0C. All microbes were further tested at different concentrations to determine the minimum inhibitory concentration (MIC) values. The MIC value for root oil is 5 µL/mL



(RO = Root oil of *Saussurea lappa* , EE = Ether extract of the roots of *Saussurea lappa* , HE = Hexane extract of the roots of *Saussurea lappa* , Control: a = Nalidixic acid, b = Amikacin)

Figure 2. Antimicrobial activity of the roots of *Saussurea lappa* showing comparative zone of inhibition against different pathogens

Table 4: Antibacterial and Antifungal screening of the volatile constituents of *Saussurea lappa*

Sample Code	*A. hydrophila*	*P. aeruginosa*	*E. coli*	*M. luteus*	*B. subtilis*	*C. albicans*	*S. candidus*
RO	5	8	5	30	30	8	10
EE	20	10	8	30	8	20	10
HE	5	5	5	10	5	10	8

RO = *Saussurea lappa* root oil, EE = Ether extract of the roots of *Saussurea lappa* and HE = Hexane extract of the roots of *Saussurea lappam*, [Minimum inhibitory concentration (MIC) in µL/mL]

against *A. hydrophila* and *E. coli*, for ether extract is 8 µL/mL against *E. coli* and *B. subtilis* while for hexane extract the MIC value is 5 µL/mL against *A. hydrophila, P. aeruginosa, E. coli* and *B. subtilis*. (Table 3, 4 & Fig 2).

4 Conclusions

The hexane extract shows dominant presence of dehydrocostuslactone (66.4%) followed by 1,8-cineole (5.4%), p-cymene (5.8%) and 10-epi-γ-eudesmol while ether extract possessed lower content of dehydro- but more of dihydrodehydrocostus as compared to the hexane extract. The hydro distillation of roots of *Saussurea lappa* yields both compounds (dehydrocostuslactone and dihydrodehydrocostus lactone) in appreciable concentration. Thus, hexane as solvent may be preferred for selective extraction of dehydrocostuslactone while ether extraction appears more suitable for isolation of dihydrodehydrocostus as major compound. The essential oil and organic extracts in our study showed a great potential of antibacterial activity against *A. hydrophila*, *B. subtilis*, *S. candidus* and *E. coli*. Root oil and hexane extract showed higher activity in comparison with ether extract. This activity could be attributed to the presence of major components (dehydrocostuslactone) and/or other components present in the oil. Results of our study suggest the possibility of using the oil or organic extracts of roots of *Saussurea lappa* as natural antimicrobials in food or pharmaceutical industry because of their moderate antibacterial activities. Furthermore, detailed investigations on other bacteria and fungi may be carried out on isolated lactones to develop their different medicinal activity potential.

Authors' contributions

Conceptualization, CSM; methodology BSB and DS; formal analysis BSB and DS; plant material collection CSM and BSB; bioactivity experiments, AP; original draft preparation, BSB; reviewing and editing, CSM; all authors have agreed to the communicated version of manuscript.

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Conflicts of interest

No conflict of interest

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