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In vitro antimicrobial activity and GCMS analysis of essential oil of *Artemisia maritima* (Linn.) from Lahaul & Spiti (Cold Desert) region of North-Indian higher altitude Himalayas

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The essential oil analysis of *Artemisia maritima* (Linn.) has been done for the first time from Keylong of Lahaul & Spiti region of Himalayas. The extraction yield of *A. maritima* oil was 0.33% and representing 94.17% of the composition of oil. The major constituents of the oil were as follow: Eucalyptol (23.2%); Camphor (20.7%); Borneol (13.7%); Bornyl acetate (13.2%) and Germacrene-D (2.0%). The present study describes the phytochemical profile and antimicrobial activity of essential oil of *A. maritima*. Essential oil showed maximum zone of inhibition and minimal inhibition concentration against *Bacillus subtilis* (MTCC-2451) and *Pseudomonas fluorescence* (MTCC-664) bacterial strains.

Keyword: *Artemisia maritima* (Linn.); Essential oil, Anti-microbial, Minimal inhibition concentration, Keylong; Lahaul & Spiti (Cold Desert).

1. Introduction

The genus *Artemisia* known as "wormwoods" is one of the largest of herbs in the family Asteraceae, consisting of more than 800 species widely distributed throughout the world, especially, in South-West of Asia and Central Europe [1, 2]. Out of 34 species of genus *Artemisia* known from India, 15 species have been documented in the flora of Lahaul & Spiti [3]. Several *Artemisia* species have been found to grow above 8000 ft., used for various purposes such as flavourings, fragrances, rodents, mite repellents and as folk medicine for anti-spasmodic, anti-pyretic, anti-inflammatory and abortifacient activities [4, 5, 6]. The essential oils of various species of the genus are used in soaps, detergents, cosmetics, perfumes, as aromatherapy claims and also as a purgative, to treat ear ache and fever [7]. Thus, the genus *Artemisia* has always been of great interest to botanical, pharmaceutical and food industries [8]. Due to their multipurpose uses, chemical analyses of oils from different *Artemisia* species have been done by many workers [9, 10, 11, 12, 13, 14, 15]. In few research works α-thujone is reported as a major constituent in oil of *A. maritima* [16]. The principle component of *A. pallens* oil i.e. davanone is also reported along with hydroxydavanone in *A. maritima* [17]. In recent year, studies on the chemical composition and possible mechanisms underlying the antispasmodic and brochodilatory activities of the essential oil of *Artemisia maritima* L. has also been reported [18]. In spite of many studies on the genus *Artemisia* [19], there are still many problems in systematic interpretations because of lot of intraspecific chromosomal and structural complexities. Further, the composition of essential oil in the population of the species

from Keylong of Lahaul & Spiti is not known. There are a number of literature reports on the antimicrobial activity of the essential oils of many *Artemisia* species: *A. absinthium*, *A. dracunculus*, *A. herba-alba*, *A. afra*, *A. nilagirica*, *A. princeps* [20, 21]. But there have been no reports on the antimicrobial activity of the essential oil of *A. maritima* from this study area till now.

As a part of our investigation on aromatic medicinal plants, the aim of this work is to provide more information on the composition of essential oil obtained from *A. maritima* and with anti-microbial activity from a naturally grown species, collected from Keylong (3350 m) of Lahaul & Spiti (Cold Desert) region of North Indian higher altitude Himalayas. Thus, it is the first record of composition of essential oil with anti-microbial activity of *A. maritima* from this study area of "Cold Desert."

2. Experimental

2.1. Plant Material

Aerial parts of *A. maritima* at full flowering and fruiting stage were collected from Keylong, altitude range of (3350 m) in the month of July, 2007. Plants specimens were identified by the Botanical Survey of India (B.S.I., Northern Circle), Dehradoon and specimens were deposited in the Herbarium, Department of Botany, Punjabi University, Patiala (Punjab), India (Table 1).

Table 1: Collection details and essential oil yield of *A. maritima* Linn. from study area of North Indian higher altitude of Himachal Pradesh, Himalayas.

Species name	Place of collection/Accession number (PUN*)	Altitude of study area (m)	Month & year of collection	Oil yield (%)
Artemisia maritima Linn.	Keylong (H.P.) Himalayas (PUN*)-52014	3350	July, 2007	0.33

*PUN: Abbreviation for Herbarium, Department of Botany, Punjabi University Patiala as indicated in Index Herbariorum.

2.2. Oil Distillation

One hundred grams fresh sample of aerial parts of leaves and fine stems were separated and grounded, then immersed in water in a round bottom flask and hydrodistilled for 3 h in a full glass Clevenger-type apparatus as recommended by British Pharmacopoeia giving transparent light yellow oil. To improve the recovery and analysis, the essential oil was dried over anhydrous sodium sulphate (Merck) until the last traces of water were removed and then stored in a dark glass bottle at 4 ºC prior to GC-MS analysis [22].

2.3. Gas Chromatography-Mass-Spectrometry

GCMS (70ev) data were measured on GCMS (QP 2010 series Shimadzu, Tokyo, Japan) equipped with AOC 20i autosampler and BP20 Capillary column (SGC International Ringwood, Australia) of 30 m length, 0.25 mm i.d. and 0.25 µm film thickness. Temperature was programmed from 70-220 ºC at a rate of 4 ºC/min, held isothermally at 70 ºC and 220 ºC for 4 and 5 min, respectively. Mass spectrometer source temperature, 200 ºC; interface temperature, 220 ºC; injector temperature, 220 ºC. Sample injection volume 2 µL (diluted 5 µL oil in 2 mL dichloromethane, HPLC grade); split ratio, 1:50 and mass scan, 50-600 amu. Helium was used as a carrier gas with 1.1 mL/min flow rate.

2.4. Identification of Components:

The retention index was calculated for all volatile constituents using a homologous series of *n*-alkanes. The components of oil were identified by matching their mass-spectra with those stored in the computer library such as Wiley, New York mass spectral (MS) library, National Institute of Standards and Technology (NIST) [23] and our own library and their retention indices (RI) either with authentic compounds or with published data in the literature [24, 25, 26] based on retention indices of components on same phases of polar columns such as: BP-20, CW-20M, HP-20M and Supelcowax-10.

2.5. Anti-microbial Activity

2.5.1. Microbial Strains: The microorganism strains used in the agar well diffusion method

were supplied by the Institute of Microbial Technology, Chandigarh, India. Gram-positive bacteria: *Bacillus subtilis* (MTCC-2451), *Staphylococcus aureus* (MTCC-740), *Staphylococcus epidermis* (MTCC-435), Gram-negative bacteria: *Escherichia coli* (MTCC-443), *Salmonella typhimurium* (MTCC-1251), *Pseudomonas fluorescence* (MTCC-664) and *Acinetobacter calcoaceticus* (MTCC-127).

2.5.2. Anti-microbial Screening:

In vitro antibacterial activity of the *A. maritima* essential oil was studied against seven bacterial strains by the agar well diffusion method as described by Perez and co-workers with certain modifications [27]. Nutrient agar (Hi Media, India) was used as the bacteriological medium. The antibacterial activity of essential oils was taken at (5, 10, 20, 40 and 80 µL/well). The nutrient agar was melted and cooled to 48-50 ^{o}C and a standardized inoculum of 1×10^{6} CFU/mL, (0.5 McFarland) was then added aseptically to the molten agar and poured into sterile petri dishes to give a solid plate. Wells were prepared in the seeded agar plates. The test compound was introduced in the well (8.5 mm). The plates were incubated overnight at 37 ^{o}C. The antimicrobial spectrum of the oils was determined for the bacterial species in terms of zone sizes around each well. The diameters of zone of inhibition produced by the agent were compared with those produced by the commercial control antibiotics, 20 µL each of amoxicillin and ciprofloxacin (5 mg/mL of autoclaved distilled water). These are commonly used antibiotics to treat infections caused by several Gram-positive and Gram-negative bacteria. For each bacterial strain positive controls were maintained. The experiment was performed three times to minimize the error and the mean values are presented.

2.5.3. Minimal Inhibition Concentration

The essential oils that exhibited considerable activity were diluted with nutrient broth (1:1) in a series of seven sets of three test tubes for different microorganisms [28]. An aliquot of 1mL of the bacterial suspension ($1x10^{6}$) was inoculated into each tube. The control tubes were inoculated with same quantity of sterile distilled water and 75% ethanol. All tubes were incubated at 37 ^{o}C for 24 hrs. The lowest concentration that did not permit any visible growth when compared with the control was considered as the minimum inhibitory concentration. The contents of all tubes that showed no visible growth were cultured on nutrient agar, incubated at 37 ^{o}C for 24 hrs. The minimum bactericidal concentration was considered as the lowest concentration that could not produce a single bacterial colony.

3. Results and Discussion:

The volatile oil from aerial parts of *A. maritima* was obtained by conventional hydro distillation, which gave pale yellow oil. The extraction yield for the essential oil was 0.33%. By gas chromatography mass spectroscopy (GC-MS) analysis the components of the essential oil were identified. The essential oil analysis led to the identification of 30 constituents, representing 94.17% of the composition of oil. The GCMS chromatograph showing different peaks of the essential oil constituents (Figure 1). The major constituents reported from essential oil of *A. maritima* were: Eucalyptol (23.2%); Camphor (20.7%); Borneol (13.7%); Bornyl acetate (13.2%); *cis*-3-Hexenyl isobutyrate (2.8%); Terpene-4-ol (2.6%); and Germacrene-D (2.0%), along with that, some important constituents with low percentage such as: Dehydro1,8-cineol (1.5%); α-Phellandrene (1.1%); β-Thujone (0.4%); Carvone (1.1%); Sabinyl acetate (1.4%); and Myrtenal (1.0%) etc. were also identified. The sample of oil was reported to contain oxygenated mono-terpene with high percentage, such as: Camphor (20.69%), Borneol (13.71%) etc., which contributed special odour to this oil sample. The components of the volatile oil and the percentage of each constituent are shown in Table 2.

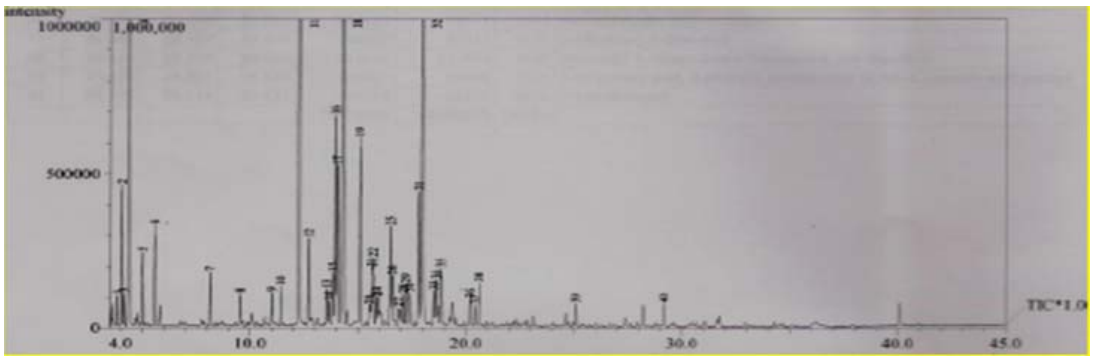

Fig 1: GCMS Chromatogram of *A. maritima* (Linn.) from Keylong (3350 m).

Table 2: Volatile oil composition of *A. maritima* (Linn.) from Keylong (3350 m).

S. No.	Constituents	RI[a]	RA[b] (%)
1.	dl-Limonene	1154	0.41
2.	Eucalyptol	1206	23.23
3.	α-Phellandrene	1216	1.15
4.	Dehydro 1,8-cineole	1228	1.54
5.	γ-Terpinene	1231	0.75
6.	α-Terpinolene	1287	0.29
7.	*t*-Sabinene hydrate	1364	0.37
8.	*cis*-3-Hexenyl isobutyrate	1377	2.79
9.	β-Thujone	1433	0.37
10.	Camphor	1467	20.69
11.	Copaene	1487	0.51
12.	L-Linalool	1507	0.30
13.	*cis*-Sabinene hydrate	1520	0.45
14.	*cis*-Chrysanthenyl acetate	1568	2.50
15.	Bornyl acetate	1573	13.23
16.	Terpene-4-ol	1600	2.60
17.	Myrtenal	1632	0.95
18.	*cis*-Verbenol	1645	0.50
19.	Isopinocarveol	1650	0.67
20.	Borneol	1653	13.71
21.	δ-Terpineol	1655	0.57
22.	α-Terpineol	1661	0.26
23.	Sabinyl acetate	1665	1.37
24.	Germacrene-D	1705	2.02
25.	p-Mentha-1,5-dien-8-ol	1708	0.21
26.	Carvone	1715	1.13
27.	Bicyclogermacrene	1727	0.46
28.	Myrtenol	1733	0.59
29.	Methyl cinnamate	2051	0.29
30.	Spathulenol	2061	0.26

RI[a] : Actual retention indices of components on same phases of polar columns (BP-20, CW-20M, HP-20M and Supelcowax-10), RA[b] : Percentage of components.

In few previous studies of *A. maritima* oil, cineol, thujone and monoterpenes were reported to be the major constituents, along with α-thujone (63.25%); sabinene (7.83%); 1, 8-cineole (6.54%) and Germacrene-D (2.22%) [29]. Another report on populations of *A. maritima* indicated the

percentage of 1, 8-cineole and chrysanthenone, which was found to be 23.8, 37.3, 44.22% and 17.54, 38.1, 0.80% respectively, from higher altitude Himalayas [30]. Therefore, it is interesting to find such a high percentage of these major constituents: Eucalyptol (23.2%) which is the main component of the essential oil of *Eucalyptus* leaf (*Eucalyptus globulus*) effective in reducing inflammations pain and promoting leukaemia cell death; Camphor (20.7%); Borneol (13.7%); Bornyl acetate (13.2%), first time in essential oil of the species in the population from Keylong of Lahaul & Spiti (Cold Desert) region of North Indian higher altitude Himalayas.

Antimicrobial activity showed that, the inhibition zones were found increased considerably when the concentration rate increased. Therefore it can be said that quantity of the oil was important for inhibition effect. Among all gram positive bacterial growths, the maximum zone of inhibition was recorded against *Bacillus subtilis* (MTCC-2451) i.e. 37 mm, followed by *Staphylococcus epidermis* (MTCC-435) i.e 29mm and 17 mm zone of inhibition against *Staphylococcus aureus* (MTCC-740). On the other hand four different gram negative bacterial strains were used and among all microorganisms *Pseudomonas fluorescence* (MTCC-664) showed maximum zone of inhibition i.e. 35mm, followed by *Acinetobacter calcoaceticus* (MTCC- 127) i.e. 20 mm (Figures 2. & 3.). The minimum zone of inhibition was recorded against the *Escherichia coli* (MTCC-443) strain i.e. 2.5mm (Table 3.). The minimal inhibition concentration (MIC) was 1.2 µL recorded in gram negative strain *Pseudomonas fluorescence* (MTCC-664) (Figure 4.) followed by a gram positive strain *Bacillus subtilis* (MTCC-2451), showed 2µL of minimal inhibition concentration (MIC) (Table 4.). Various *Artemisia* essential oils as well as the major components found in *A. douglasiana* leaf essential oil have previously shown antimicrobial activity.

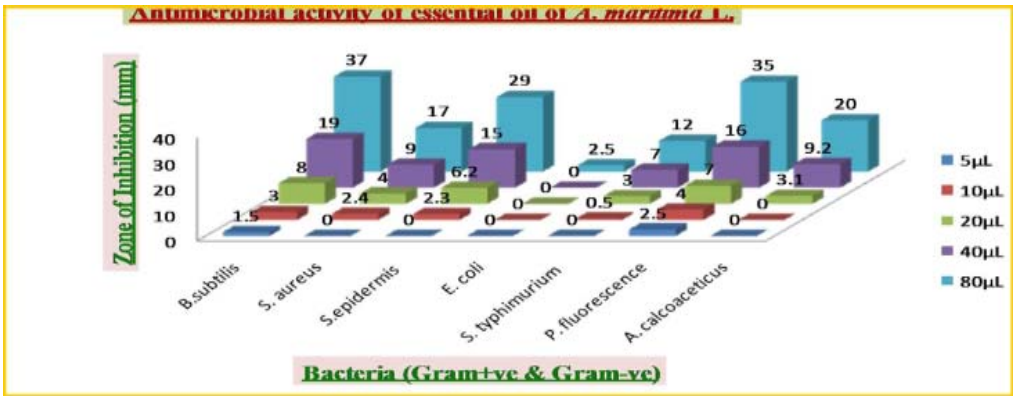

Fig 2: Bar diagram showing antimicrobial activity of essential oil against bacterial strains.

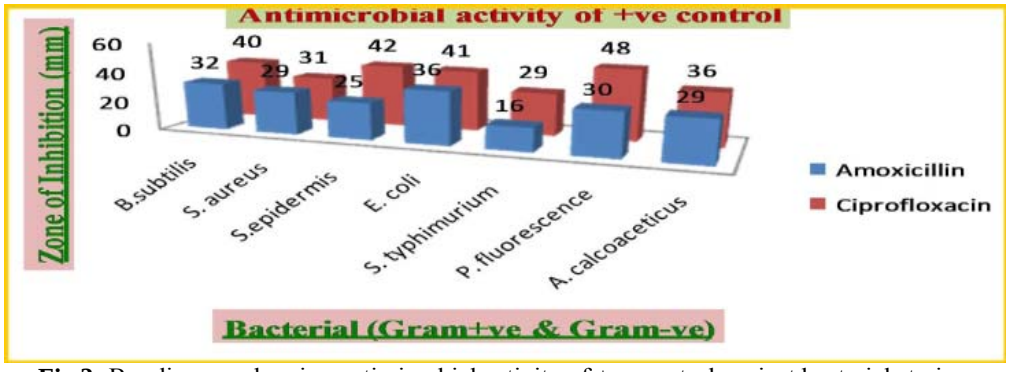

Fig 3: Bar diagram showing antimicrobial activity of +ve control against bacterial strains.

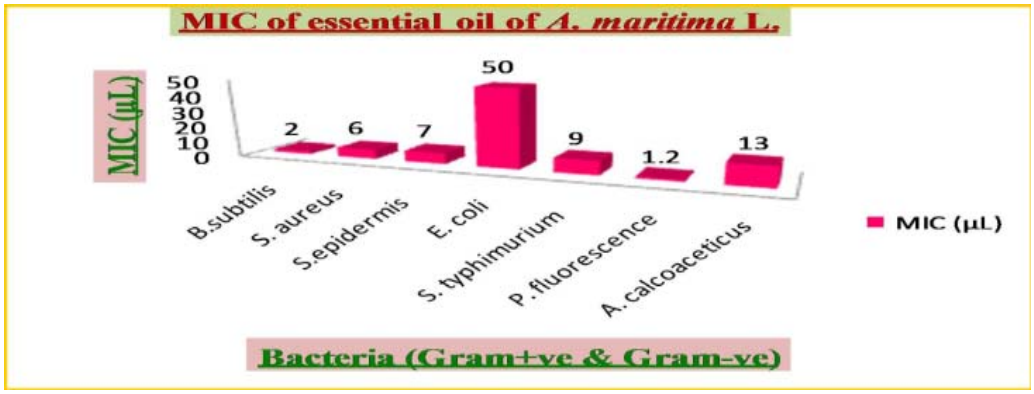

Fig 4: Bar diagram showing minimum inhibitory concentration against bacterial strains.

Table 3: Antimicrobial activity of essential oil of *A. maritima* Linn. against gram positive and gram negative bacterial strains.

***Artemisia maritima* Linn.**									
S. No.	**Nature of Bacteral Strains**	**Microorganisms (Bacterial Source Number)**	**Diameter of inhibition zone (mm) of essential oil concentration used for anti-microbial analysis (µL/well) (*n=3*)**					**Control +ve (*n=3*)**	
								Amoxicillin 5 mg/mL	**Ciprofloxacin 5 mg/ mL**
			5 µL	**10 µL**	**20 µL**	**40 µL**	**80 µL**	**20 µL**	**20 µL**
1.	Gram +ve	*Bacillus subtilis* (MTCC-2451)	1.2	3	8	19	37	32	40
2.		*Staphylococcus aureus* (MTCC-740)	N.A	2.5	4	9	17	29	31
3.		*Staphylococcus epidermis* (MTCC-435)	N.A	2.3	6.2	15	29	25	42
4.	Gram -ve	*Escherichia coli* (MTCC-443)	N.A	N.A	N.A	N.A	2.5	36	41
5.		*Salmonella typhimurium* (MTCC-1251)	N.A	0.5	3	7	12	16	29
6.		*Pseudomonas fluorescence* (MTCC-664)	2.5	4	7	16	35	30	48
7.		*Acinetobacter calcoaceticus* (MTCC-127)	N.A	N.A	3.1	9.2	20	29	36

All values are mean of triplicates (*n=3*); Gram +ve: gram positive; Gram –ve: Gram negative; N.A: No Activity.

Table 4: Minimal inhibition concentration (MIC) (µL) of essential oil of *A. maritima* L.

***Artemisia maritima* Linn.**			
S. No.	**Nature of Bacteria**	**Microorganisms (Bacterial Source Number)**	**MIC (µL)**
1.	Gram +ve	*Bacillus subtilis* (MTCC-2451)	2
2.		*Staphylococcus aureus* (MTCC-740)	6
3.		*Staphylococcus epidermis* (MTCC-435)	7
4.	Gram -ve	*Escherichia coli* (MTCC-443)	50
5.		*Salmonella typhimurium* (MTCC-1251)	9
6.		*Pseudomonas fluorescence* (MTCC-664)	1.2
7.		*Acinetobacter calcoaceticus* (MTCC-127)	13

MIC: Minimal Inhibition Concentration; Gram +ve: Gram positive; Gram –ve: Gram negative.

Thus, *A. caerulescens*, *A. mexicana*, *A. afra*, and *A. asiatica* have already been reported to exhibit antibacterial activity, and many are used as herbal medicines [31, 32, 33, 34]. In few research works on the leaf essential oils of *A. douglasiana* reported, that camphor as a main component and exhibit bacteriostatic activity [35], after this research work it was again reported that camphor act as one of the major active component in many antibacterial essential oil bearing medicinal plants [36]. Along with camphor, 1,8-Cineole, 4-terpineol and *endo*-borneol have also been previously reported to exhibit antibacterial and antifungal activity [37, 38, 39]. From the above results and discussions, it is concluded that GCMS essential oil analysis of *A. maritima* Linn. has been done first time from the study area of higher altitude Himachal Pradesh Himalayas. It is reported that some of the constituents are in higher percentage such as: Eucalyptol (23.2%); Camphor (20.7%); Borneol (13.7%); Bornyl acetate (13.2%) as compared to the previous work done by many researchers. During anti-microbial activity, it can be said that higher quantity of essential oil (80µL/well) is needed for maximum zone of inhibition against all the microorganisms. Essential oil showed maximum zone of inhibition and minimal inhibition concentration against *Bacillus subtilis* (MTCC-2451) and *Pseudomonas fluorescence* (MTCC-664) bacterial strains, which indicate that *A. maritima* Linn. essential oil has capacity to inhibit the growth of both gram positive and gram negative bacterial strains. Further, it can be concluded on the basis of previous studies on *Artemisia* genus and present results that *A. maritima* Linn. a higher altitude medicinal and aromatic plant act as an important anti-microbial agent against many gram positive and gram negative bacterial strains with higher percentage of some most important chemical constituents and was previously undescribed from this study area of "Cold Desert."

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