



Antibacterial, brine shrimp lethality assay and GC-MS analysis of *Juniperus recurva*

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Abstract

The composition of essential oil of leaves of *Juniperus recurva* collected through hydro distillation were determined by GC-MS system and showed the presence of 21 different compounds. The most abundant were β -Phellandrene (23.03%), D-Limonene (10.12%), Elemol (7.77%), Cyclofenchene (7.28%), β -Eudesmol (7.13%) and Terpinen-4-ol (7.25%). Also, the dried and powdered leaves were subjected to extraction with methanol. Antibacterial activity of the essential oil and methanolic extract of *J. recurva* was studied. Oil and extract exhibited moderate antibacterial activity. The LD₅₀ of the sample was found to be 130.4 μ g/mL in Brine shrimp lethality assay.

Keywords: *Juniperus recurva*, essential oil, GC-MS, activity

Introduction

The *Juniperus recurva* belongs to Cupressaceae family. It is commonly called Drooping juniper and called as Dhupi in Nepali. Plant is mainly spreading shrub distributed from Afghanistan eastward through the Himalayas to southeast China. Plant is about 10m tall with brown stem. Leaves are awl-shaped. Fruits are purplish-brown to black and ovoid [1, 2]. There are around 75 species of *Juniperus*, and is a very diverse genus ranging in habitat from sea level to above timberline [3].

Essential oil of Dhupi contain α and β -cedrene, thujopsene, cuparene, cedrol, widdrol. Major component of leaves oil are δ -3-carene, α -thujene, myrcene, α -terpinene, cymene, γ -terpinene, linalool, terpinen-4-ol, α -murolene, γ -cadinene, δ -cadinene, β -oplophenone and cadinol isomers [2, 3].

J. recurva has been used as insecticide by the natives in Nepal [4]. The local people use *J. recurva* to treat fever, headache, coughs, and colds. In the Nubra River valley of northern Jammu and Kashmir, the people use leaves of *J. recurva* to lower fever in children [3]. Dhupi is used to treat diarrhea, abdominal pain, diseases of spleen, ascites, tumors, piles, bronchitis, indigestion, constipation, vaginal discharges, stomachic, aphrodisiac, styptic. Essential oil is emmenagogue, abortifacient, tonic, anthelmintic, good for toothache and piles [1].

Experimental

Collection of Plant Materials

The plant material was collected from Solukhumbu, Nepal. The plants were identified by Department of Botany, Amrit Campus, Lainchour, Kathmandu.

Preparation of Plant Extract

The clean and dried leaves were grinded to powder and further proceeded via cold percolation process for 7 days for three times with 2.5 liters methanol. The methanol extract was concentrated by evaporation on rotavapour. Plants extracts

were stored at 4°C.

Extraction of Essential Oil

The mature leaves of *Juniperus recurva* were crushed for hydro distillation and subjected to a Clevenger apparatus for three hours. By this process about 4 ml of yellow coloured essential oils were collected and stored in a sealed glass vials at low temperature (0-4°C) prior to analysis.

GC-MS Analysis

The essential oils sample of *J. recurva* was subjected to GC-MS analysis. GC-MS analysis was performed on a gas chromatography mass spectrometer GCMS-QP2010 under the following condition: injection volume 1 μ L with split ratio 1:50; Helium as a carrier gas with a Rtx-5MS column of dimension 30m \times 0.25mm \times 0.25 μ m, temperature programmed at 40, 200 and 280°C with a hold time of 2.0, 3.0 and 4.0 min identification was accompanied by comparison of MS with those reported in NIST 05 and FFNSCI.3 libraries. It was performed in Department of Food Technology and Quality Control, Nepal Government, Babarmahal, Kathmandu, Nepal.

Antibacterial Activity Assays

Antimicrobial assay of extracts and essential oil of plant was performed by agar well diffusion method in Muller Hilton Agar (MHA) and the minimum bactericidal concentration of those extract was determined by micro dilution method. All the strains of bacteria was cultured in Nutrient broth (NB) and incubated at 37 °C for 18 hours. After incubation each stain were diluted with sterile distilled water. The turbidity of dilution was compared with 0.5 McFarland standards (approximately 10⁸ CFU/ml). The suspensions were then diluted (1:100) in Muller Hilton Broth (MHB) to obtain 10⁶ CFU/ml. Prepared inoculums were incubated for 30 minutes at 37 °C prior to use.

Plant extracts (30 μ l) were loaded into the respective wells with the help of micropipette. The solvent (50% DMSO) was

tested for its activity as a control at the same time in the separate well. The Neomycin 20 µg/ml was used as a positive control. The plates were then left for half an hour with the lid closed so that extracts diffused to the media. The plates were incubated overnight at 37 °C. After proper incubation (18-24 hours) the plates were observed for the zone of inhibition around well which is suggested by clean zone without growth. The ZOI were measured with the help of the ruler and mean was recorded for the estimation of potency of antibacterial substance.

Determination of Minimum Bactericidal Concentration

The Minimum Bactericidal Concentration (MBC) was determined by micro dilution method. The methanol extracts were diluted by two fold to get series of concentrations from 0.048 to 25 mg/ml in freshly prepared sterile nutrient broth. 20µl of the microorganism suspension (correspond to 10⁶ CFU/ml) was added to each of the sample dilutions. These were incubated for 18 hours at 37°C and each tube content was subculture in fresh nutrient agar separately and minimum bactericidal concentration was determined that showed no growth at all.

Determination of the Minimum Inhibitory Concentration

The smallest amount of compounds required to kill or inhibit the growth of micro-organism *in vitro* can be determined by the dilution method. This amount is referred as minimum inhibitory concentration (MIC). It is a measure of potency which is expressed in terms of either µg or mg/ml. A stock solution of 25 mg/ml was prepared. This was serially diluted to obtain various ranges of concentrations between 25 mg/ml to 0.048 mg/ml.

Brine Shrimp Lethality Assay

Ten nauplii were exposed to each of different concentrations of the plant extract and number of survivors were calculated the percentage of death after 24 hours.

Result and Discussion

GC-MS Analysis

GC-MS analysis of essential oils of *J. recurva* shows the presence of 21 different compounds. The chemical compound identified in essential oils of the fruits of the *J. recurva*. plant are presented below:

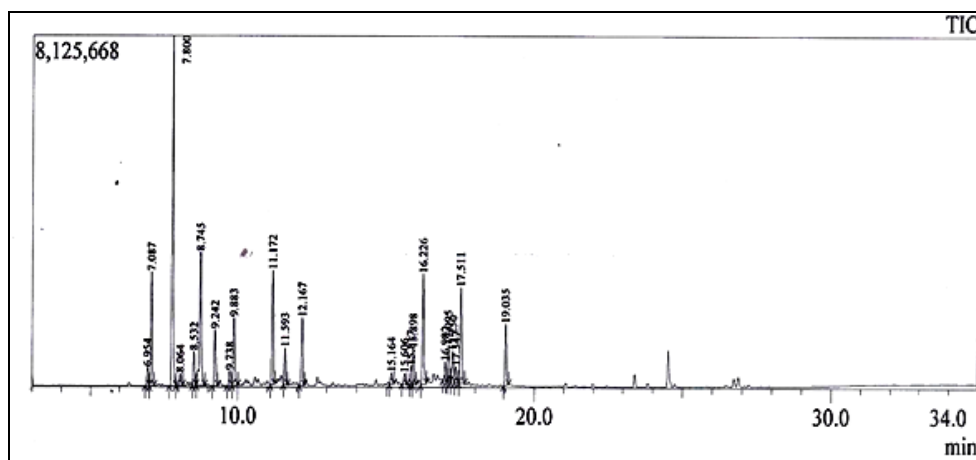


Fig 1: Chromatogram of essential oils of fruits of *J. recurva*.

The major constituents present in the essential oils sample were β -Phellandrene (23.03%), D-Limonene (10.12%), Elemol (7.77%), Cyclofenchene (7.28%), β -Eudesmol

(7.13%) and Terpinen-4-ol (7.25%). Constituents of essential oils of *J. recurva* are tabulated as follows.

Table 1: List of compounds in essential oils of *J. recurva*

S. No	Name of the compounds	Molecular Formula	Molecular Weight	Retention Time	Area %	Height %
1.	α -Phellandrene	C ₁₀ H ₁₆	136	6.954	1.16	1.25
2.	Cyclofenchene	C ₁₀ H ₁₆	136	7.087	7.28	7.77
3.	β -Phellandrene	C ₁₀ H ₁₆	136	7.800	23.03	23.83
4.	β -Myrcene	C ₁₀ H ₁₆	136	8.064	0.84	0.83
5.	2-Carene	C ₁₀ H ₁₆	136	8.532	2.13	2.33
6.	D-Limonene	C ₁₀ H ₁₆	136	8.745	10.12	9.09
7.	γ -Terpinene	C ₁₀ H ₁₆	136	9.242	3.57	3.85
8.	2-Carene	C ₁₀ H ₁₆	136	9.738	1.01	1.01
9.	Linalool	C ₁₀ H ₁₈ O	154	9.883	4.65	4.65
10.	Terpinen-4-ol	C ₁₀ H ₁₈ O	154	11.172	7.25	7.77
11.	Dihydrocarvone	C ₁₀ H ₁₆ O	152	11.593	2.37	2.50
12.	Carvone	C ₁₀ H ₁₄ O	150	12.167	4.76	4.63
13.	n-Dodecanol	C ₁₂ H ₂₆ O	186	15.164	0.90	0.76
14.	α -Amorphene	C ₁₅ H ₂₄	204	15.606	0.82	0.75

15.	γ -Cadinene	C ₁₅ H ₂₄	204	15.817	1.33	1.25
16.	β -Cadinene	C ₁₅ H ₂₄	204	15.898	2.38	2.47
17.	Elemol	C ₁₅ H ₂₆ O	222	16.226	7.77	7.55
18.	Cedrol	C ₁₅ H ₂₆ O	222	16.982	1.42	1.31
19.	Apiol	C ₁₂ H ₁₄ O ₄	222	17.095	2.45	2.45
20.	Guaiol	C ₁₅ H ₂₆ O	222	17.250	2.31	2.12
21.	δ -Cadinol	C ₁₅ H ₂₆ O	222	17.347	1.22	1.09
22.	β -Eudesmol	C ₁₅ H ₂₆ O	222	17.511	7.13	6.53
23.	Elemol	C ₁₅ H ₂₆ O	222	19.035	4.10	4.20
					100.00	100.00

Antibacterial Activity

Table 2: Antibacterial activity of the essential oils of the *J. recurva*.

Sample	MIC Values			MBC Values		
	SA	MRSA	KP	SA	MRSA	KP
Neomycin* (μ g/ml)	0.078	0.156	0.0195	1.25	5	0.156
Oil (mg/ml)	-	0.195	0.195	-	6.25	12.5
Extract (mg/ml)	1.56	0.78	0.048	12.5	25	6.25

*Control Antibiotics

SA= *Staphylococcus aureus* (ATCC) 25923, MRSA = Methicillin resistance *Staphylococcus aureus* (MRSA)

KP = *Klebsiella pneumoniae* (MDR)

Brine Shrimp Lethality Assay

The LD₅₀ of the sample was found to be 130.4 μ g/mL in Brine shrimp lethality assay.

Conclusion

The major constituents present in the essential oils sample were β -Phellandrene (23.03%), D-Limonene (10.12%), Elemol (7.77%), Cyclofenchene (7.28%), β -Eudesmol (7.13%) and Terpinen-4-ol (7.25%). Essential oils and extracts of *Juniperus recurva* showed antibacterial activity against *Staphylococcus aureus* (ATCC) 25923, Methicillin resistance *Staphylococcus aureus* (MRSA) and *Klebsiella pneumoniae* (MDR). The LD₅₀ of the sample was found to be 130.4 μ g/mL.

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