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Chemical Profile and Biological Activities of Essential Oil from Flowers of *Artemisia dubia* Wall. ex Bess. Growing Wild in Western Himalaya, India

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The flowers of *Artemisia dubia* wall ex Bess., on hydrodistillation provided a refreshing violet-blue viscous essential oil with sweet woody odour. The oil was found to be a complex mixture of monoterpenes, sesquiterpenes and their esters. A total of 67 chemical constituents comprising 79.43 % of the oil were characterized with the help of gas chromatography and mass spectrometry (GC/MS). Major chemical constituents of the oil were characterized as nerylisovalerate (9.79 %), 1,8-cineole (8.32 %), neryl-2-methyl-butanoate (7.32 %), chamazulene (5.92 %), linalool (4.15 %), camphor (4.10 %), germacrene D (4.04 %), nerol (3.37 %), linalyl propionate (3.32 %). The investigations performed on the flower essential oil of *A. dubia* allowed the distinction of this plant growing in the temperate Kashmir region of western Himalayas from the same plant with different varieties growing in different parts of the world. The essential oil was evaluated for its antifungal activity against *Candida* species and was found to be active against the tested strains with more sensitivity against *C. paropsilosis* and *C. krusei* strains. The antioxidant activity evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and thiobarbituric acid reactive substances (TBARS) assay evidenced that the essential oil has moderate antioxidant activity. The antiproliferative ability of the oil was evaluated by MTT assay against the two cell lines A549 (human lung adenocarcinoma epithelial cells) and HCT-116 (human colon cancer cells). The essential oil effectively inhibits the growth of A549 and HCT-116 cancer cells at 62.5 and 31.25 µg/mL concentration, respectively.

Keywords: *Artemisia dubia*, Essential oil, Monoterpenes, Sesquiterpenes, Antioxidant, Antifungal.

INTRODUCTION

Artemisia is a large, diverse genus of plants with about 500 species belonging to the daisy (or Asteraceae) family [1]. The genus Artemisia comprises shrubs and herbs, which are known for the powerful chemical constituents in their essential oils [2]. The different species of Artemisia grow in temperate climates of the world, usually in dry or semiarid habitats [3]. Some Artemisia species are known as important medicinal plants. It has been reported that a large number of structurally diverse and biologically active sesquiterpenoids, such as guaianolides, eudesmanolides, germacranolides, tricyclic sesquiterpenes and cadinane derivatives, have been identified from the *Artemisia* species [4,5]. The herbs have a characteristic flavour and smell due to the presence of mono and sesquiterpenes and are used worldwide as a stomachic, tonic, in anti-inflammatory tinctures and flavouring beverages. *Artemisia dubia* is used as traditional medicine in Magar of Bukini, Baglung, western Nepal for stomachic, purgative, hysteria, asthma, skin diseases like scabies and treatment of ulcers [6]. The medicinal plants from the genus *Artemisia* are currently under phytochemical research focus due to their diverse biological activities, chemical diversity and essential oil production. The essential oils generally have a wide spectrum of biological activity due to the presence of a large number of active secondary metabolites. As part of a program to assess systematically the chemical and biological diversity of several medicinal plants of western Himalaya, we undertook the investigation of the essential oil composition,

antifungal, antioxidant and anticancer activities of the flower essential oil of *A. dubia* growing wild in western Himalayas.

EXPERIMENTAL

The fresh aerial parts of *Artemisia dubia* were collected on 16th August 2017, at the flowering stage from hilly slopes at Naranag-Dumail, Kashmir, India (34º 22′ 21.38″ N, 74º 59′ 46.87″E, altitude 2398 m). The plant was identified and authenticated by the taxonomist at Center for Biodiversity and Taxonomy, University of Kashmir, Kashmir, India and the voucher specimen (2633 KASH) has been deposited at the herbarium of University of Kashmir, Kashmir, India.

Extraction: The flowers were separated from the other parts of the plant. The essential oil from the flowers of *A. dubia* was obtained from 100 g of fresh flowers by extraction with hydrodistillation for 4 h using a Clevenger type apparatus. The hydrodistillations were repeated three times. The samples afforded blue violet viscous oil with the characteristic sweet woody aroma. The oil was dried over anhydrous Na_2SO_4 and stored at low temperature in a refrigerator for further analysis. The essential oil yield was reported on the fresh weight basis of flowers.

Essential oil analysis: The constituents of essential oil were detected by GC-MS analysis. The analysis was carried out on a gas chromatograph GCMS-QP2010 Plus with flame ionization detector (FID), using a fused-silica capillary column Rtx-5 (30 m × 0.32 mm; 0.25 mm film thickness) coated with 5 % diphenyl and 95 % polysiloxane. Oven temperature was programmed from 50-250 ºC at 3 ºC /min. Injector temperature (260 ºC); detector temperature (270 ºC). Helium was used as carrier gas (1.21 mL/min) with a linear velocity 39.9 cm/s, split ratio 110:0. Mass spectra:electron impact (EI) positive mode. Ion source temperature; 230 ºC, interface temperature; 270 ºC, Ionisation energy; 70 eV. Mass scan range was *m/z* 40-650 range.

Identification of the oil components was done by matching GC-MS mass spectral fragmentation pattern with those of MS library search (NIST 05 and Wiley) and comparing it with the literature data [7]. The relative percentage area of each constituent was calculated by comparing its average peak area with the total area without correction for response factors.

DPPH antioxidant activity: The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity was evaluated by the method described by Brand-Williams *et al*. [8]. Briefly, a solution of DPPH (0.005 %) was prepared in methanol and 1.95 mL of the solution was mixed with 50 µL of the different concentrations of essential oils (10-100 µg/mL). Ascorbic acid and butylated hydroxytoluene (BHT) were used as positive controls. The solutions were incubated for 1 h in dark and the absorbance was measured at a wavelength of 517 nm using a spectrophotometer (Lasany, India). The decrease in absorbance caused in the solution was taken as an increase in radical scavenging activity. The control solution contained 1.95 mL DPPH mixed with 50 µL of methanol. The percentage inhibition of the free radical activity was calculated as follows:

$$\text{Inhibition (\%)} = \frac{A_c - A_t}{A_c} \times 100$$

where A_c is the absorbance of the control and A_t that of test solution, respectively. The concentration of each sample required to cause 50 % inhibition of free radicals (IC_{50} value) were calculated.

TBARS assay: Thiobarbituric acid reactive substances (TBARS) assay was performed as per the method of Ohkawa *et al*. [9] with slight modifications. Briefly, to a mixture of 2.8 mM deoxyribose, 0.1 mM ferric chloride, 0.1 mM EDTA, 0.1 mM ascorbic acid, 1 mM H_2O_2, 20 mM KH_2PO_4 (pH 7.4) and 50 µL of different concentrations (10-100 µg/mL) of essential oil of *Artemisia dubia* in a final volume of 1 mL were incubated at 35 ºC for 1 h. Afterwards, 1 mL of 1 % solutions of thiobarbituric acid and 2.8 % (w/v) trichloroethanoic acid were added and heated at 50 ºC for 30 min. The TBARS was measured at a wavelength of 532 nm using spectrophotometer. The results were taken in terms of percentage inhibition of deoxyribose oxidation and were calculated as according to the following equation:

$$\text{Inhibition (\%)} = \frac{A_c - A_t}{A_c} \times 100$$

where A_c is the absorbance of control reaction (containing all reagents except the essential oil) and A_t is the absorbance of essential oil/known antioxidant (BHT and ascorbic acid).

Antifungal activity: The antifungal activity of *Artemisia dubia* flower essential oil was evaluated by broth microdilution method as described by National Committee for Clinical Laboratory Standards [10] with slight modifications. The overnight grown fungal cultures were suspended in 0.9 % aqueous saline solutions to an optical density (OD) of 0.1 at 600 nm (OD_{600} = 0.1). 100 Fold dilutions of the cells (100 µL) were taken in a microtitre plate containing different concentrations of essential oil in 100 µL growth media. Media control and drug free growth control were also included in the assay and the plates were incubated for 48 h in dark at 35 ºC. The MIC_{80} which is defined as the 80 % inhibition of cells compared to growth control was determined by free eye visualization and microtitre plate reader (BioTek, USA) [11].

Cell culture and MTT assay: Human cancer cell lines (A549 and HCT-116) were purchased from National Centre for Cell Science (NCCS, Pune, India). The cells were grown in DMEM, supplemented with 10 % FBS and 1 % penicillin-streptomycin at 37 ºC in a humidified incubator containing 5 % CO_2. *in vitro* Anti-proliferative assay was performed by MTT assay to determine anticancer activity of the oil. For this purpose, the cell lines A549 and HCT-116 were seeded at 10^3 cells/well overnight. Next day, media was replaced with 200 µL of fresh medium before treatment with the oil. Cells were treated with different concentrations (15.6, 31.25, 62.5, 125, 250 and 500 µg/mL) of oil and dimethyl sulphoxide was used as an experimental control. After 24 h treatment, cell growth was evaluated by MTT assay. MTT solution of 50 µL (5 mg/mL of PBS) was added to each well and the plates were incubated for 3 h at 37 ºC in dark. The media was aspirated and 200 µL of MTT solvent (DMSO) was added to each well to solubilize the formazan crystals. The absorbances of plates were measured on ELISA reader (Benchmark, BioRad) at a wavelength of 570 nm. The sample was performed in triplicate and the experiment was repeated thrice.

RESULTS AND DISCUSSION

The essential oil from *Artemisia dubia* flowers was obtained with a 0.6 % (v/w) yield, based on hydrodistillations carried out three times. The chemical constituents identified in the flower essential oil of *A. dubia* from western Himalaya are recorded in Table-1 and the total ion chromatogram is shown in Fig. 1. The GC-MS analysis led to the identification of 67 chemical constituents, accounting for 79.43 % of essential oil. The major constituents (> 3 %) of essential oil were found to be nerylisovalerate (9.79 %), 1,8-cineole (8.32 %), neryl-2-methylbutanoate (7.32 %), chamazulene (5.92 %), linalool (4.15 %), camphor (4.10 %), germacrene D (4.04 %), nerol (3.37 %), linalyl propionate (3.32 %). Compared with the previous reports, presently analyzed oil from *Artemisia dubia* flowers show a variable composition from essential oils of the plant analyzed from China with the key compounds identified as 1,8 cineole (5.70 %), aromadendrene (94.12 %), 2-chloro-benzaldehyde (3.54 %) [12]. The *Artemisia dubia* from Nepal is reported to contain the major compounds as chrysanthenone (29 %), camphor (16.4 %) and verbenone (5.2 %) [13], while as the major constituents from Korean chemotype are identified as camphor (17.148 %), germacrene D (15.69 %), camphene (5.082 %) and β-thujone (6.569 %) [14]. In the present study, *Artemisia dubia* flower essential oil exhibits the qualitative and quantitative variation in chemical constituents with the same species analyzed previously with much disparateness from all the studied chemotypes.

TABLE-1
CHEMICAL COMPOSITION AND RELATIVE CONCENTRATIONS OF THE ESSENTIAL OIL OF *Artemisia dubia* Wall. Ex Bess.

S. No.	Compounds	RT	Peak area (%)
1	*trans*-2-Hexenal	5.311	0.08
2	*cis*-3-Hexen-1-ol	5.414	0.21
3	Hexan-1-ol	5.815	0.43
4	Heptanal	6.776	0.03
5	α-Pinene	7.888	1.31
6	Camphene	8.881	0.24
7	Sabinene	9.414	1.43
8	β-Pinene	9.579	0.44
9	1-Octen-3-ol	9.877	0.49
10	Myrcene	10.156	2.80
11	α-Phellandrene	10.793	0.67
12	α-Terpinene	11.249	0.28
13	*p*-Cymene	11.634	0.86
14	1,8-Cineole	12.013	8.32
15	*(Z)*-β-Ocimene	12.139	0.10
16	*(E)*-β-Ocimene	12.592	0.09
17	γ-Terpinene	13.092	0.75
18	*cis*-β-Terpeneol	13.676	0.27
19	Terpinolene	14.300	0.16
20	Linalool	15.208	4.15
21	*cis-p*-Menth-2-en-1-ol	16.200	0.10
22	Geijeren	16.857	0.22
23	Camphor	17.187	4.10
24	Neroloxide	17.410	0.10
25	Borneol	18.432	1.95
26	Terpinen-4-ol	18.800	1.64
27	Linalyl propionate	19.576	3.32
28	Decanal	19.954	0.12
29	Nerol	20.986	3.37
30	Hexyl-2-methylbutanoate	21.297	0.05
31	Neral	21.411	0.10
32	Geraniol	22.085	0.61
33	Geranial	22.755	0.12
34	*cis*-3-Hexenyltiglate	25.172	0.06
35	Bornyl acetate	25.506	0.17
36	α-Terpinyl acetate	26.184	0.08
37	Eugenol	26.505	0.08
38	α-Copaene	27.392	0.19
39	Geranyl acetate	27.625	0.40
40	β-Elemene	28.049	1.10
41	*(Z)*-Jasmone	28.191	0.47
42	*(E)*-β-Caryophyllene	29.271	1.88
43	β-Copaene	29.670	0.15
44	*trans*-α-Bergamotene	29.855	0.13
45	Isogermacrene-D	30.050	0.01
46	*(E)*-β-Farnescene	30.774	1.38
47	Dehydro-sesquicineole	31.277	0.20
48	Geranylpropanoate	31.432	0.08
49	Germacrene-D	31.889	4.04
50	β-Selinene	32.131	0.06
51	*(Z,E)*-α-Farnescene	32.317	0.37
52	Bicyclogermacrene	32.413	0.81
53	(*E,E*)-α-Farnesene	32.912	1.99
54	δ-Cardinene	33.358	0.29
55	*(E)*-Nerolidol	35.218	0.27
56	Neryl-2-methylbutanoate	35.606	7.32
57	Nerylisovalerate	35.998	9.79
58	Geranyl-2-methylbutyrate	36.524	0.45
59	Geranylisovalerate	37.598	0.17
60	Isospathulenol	37.842	0.15
61	τ-Cardinol	38.200	0.42
62	*neo*-Intermedeol	39.14	2.17
63	α-Bisabolol	39.926	1.46
64	Chamazulene	41.512	5.92
65	β-Costol	42.779	0.10
66	Farnesyl acetate	45.006	0.41
67	*n*-Eicosane	58.585	0.21
	Total identified (%)		**79.43**
	Grouped constituents		
	Esters		22.30
	Oxygenated monoterpenes		25.03
	Sesquiterpene hydrocarbons		10.14
	Monoterpene hydrocarbons		9.13
	Oxygenated sesquiterpenes		4.77
	Others		8.06

Antioxidant activity: The antioxidant activity evaluation of essential oils and plant extracts cannot rely on a lone method as no single method is absolute. Each method is based on any one feature of antioxidant activity like the ability to scavenge the free radicals, ability to inhibit lipid peroxidation, chelation ability with transition metal ions, *etc*. In the present study, evaluation of antioxidant activity of *A. dubia* essential oil from flowers was carried out through two different assays, the DPPH assay and TBARS assay.

The DPPH assay is based on the ability of stable DPPH radical to get reduced to DPPH-H in presence of hydrogen or electron donor which results in the colour change of solution from purple to yellow. In the TBARS assay, hydroxyl radicals are formed by Fe^{3+} ascorbate-EDTA-H_2O_2 system and attack deoxyribose which leads to the degradation of deoxyribose

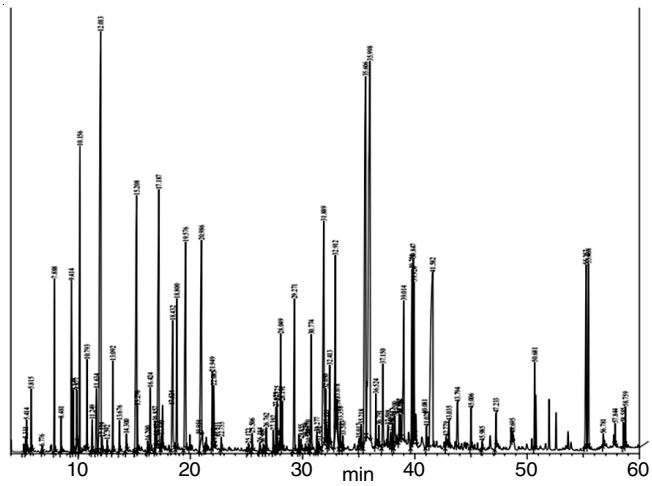

Fig. 1. Total ion chromatogram (TIC) of flower essential oil of *Artemisia dubia* from Kashmir Himalaya

and results in the formation of thiobarbituric acid reactive substances which form a pink chromogen when heated with TBA in acid solution [15]. *A. dubia* essential oil exhibited a moderate antioxidant activity that could be related to the small percentage of phenolic compounds. The antioxidant potential of essential oils was compared with the antioxidant activity of ascorbic acid and BHT at the same concentration and expressed in terms of IC_{50} (µg/mL), defined as the concentration of the antioxidant required to decrease the absorbance of the radical solution by 50 % of the initial absorbance. The antioxidant activity of the flower essential oil as evaluated by DPPH radical scavenging activity was found to be greater than evaluated by hydroxyl radical scavenging activity with IC_{50} values of 74.1 ± 1.2 µg/ml and 94.3 ± 0.8 µg/mL as calculated by DPPH and TBARS assay, respectively. The results of antioxidant activity are shown in Table-2. The antioxidant activity of essential oil can be correlated with the chemical profile of essential oil of *A. dubia* in light of the work done by different authors on essential oil constituents like 1,8 cineole, camphor, terpinene, linalool, β-pinene, *etc*. None of these essential oil constituents have been reported to show significant antioxidant activity [16-18].

TABLE-2
ANTIOXIDANT ACTIVITY OF FLOWER ESSENTIAL OIL OF *Artemisia dubia* EVALUATED BY DPPH AND TBARS ASSAY

Sample	IC_{50} value (µg/mL)	
	DPPH assay	TBARS assay
Ascorbic acid	39.6 ± 0.7	45.5 ± 1.2
BHT	46.0 ± 0.4	51.1 ± 0.3
Artemisia dubia essential oil	74.1 ± 1.2	94.1 ± 0.8

Antifungal activity: The antifungal activity of essential oil of *A. dubia* was evaluated against seven different *Candida* strains. The MIC_{80} values ranged from 1.007 to 0.252 µL/mL. The MIC_{80} values (Table-3) clearly depicted that essential oil of *A. dubia* showed better antifungal activity against *Candida propsilosis* and *Candida krusei* strains while the essential oil seemed to be least effective against *Candida dubliniensis* at the tested concentration. Rest of the *Candida* strains showed intermediate susceptibility towards the essential oil from *A. dubia*. Thus, the antifungal activity of *A. dubia* essential oil from flowers revealed its potentiality to serve as a natural source to control the growth of *Candida* species.

TABLE-3
MIC_{80} VALUE OF ESSENTIAL OIL OF *A. dubia* AGAINST DIFFERENT *Candida* SPECIES

Strains	MIC_{80} values (µL/mL)
C. albicans (ATCC-24433)	1.007
C. glabrata (ATCC-2001)	1.007
C. parapsilosis (ATCC-90018)	0.252
C. krusei (ATCC-258)	0.252
C. tropicalis (ATCC-750)	1.007
C. kefyr (IL-130)	1.007
C. dubliniensis (ATCC-33)	1.259

The MIC method is more reliable in antimicrobial evaluation of essential oils than disc diffusion method. Since the essential oils are volatile and are more likely to be evaporated with the dispersing solvent besides their lipophilic character prevents them from diffusing through the agar media due to which agar well diffusion method is considered less suitable for antimicrobial testing [19].

A number of studies involving antifungal activities of the essential oils from different species of genus *Artemisia* have been already reported. The essential oil from *A. campestris* has been found to be effective against a number of fungal strains with *Fusarium graminearum* as the most sensitive strain [20]. In another study by Kordali *et al*. [21], essential oils of *A. santonicum*, *A. spicigera* and *A. absinthium* have been found to show fungitoxic effect on 38 agricultural fungi. The essential oil of *A. annua* has also been found to be effective in controlling the growth of some yeast species including *Candida albicans* [22-24], *Candida krusei* [25] and *Saccharomyces cerevisiae* [23]. To our best of knowledge, there exists no published work regarding the antifungal activity of *A. dubia*. Several components identified in *A. dubia* have been found to possess some anticandidal activity, such as α-terpineol, α-pinene, camphor and 1,8-cineole [26]. It has been proposed that the antifungal activity could be due to both major and minor components and the antimicrobial activity is regulated by the interaction between the minor and major constituents [27].

Anticancer activity: Anticancer activity of essential oil was determined by using anti-proliferative assay. Results of the anti-proliferative activity showed that essential oil effectively inhibits the growth of A549 and HCT-116 cancer cells at 62.5 and 31.25 µg/mL concentration, respectively. As shown in Fig. 2, it could be concluded that the oil treatment inhibits the growth of human cancer cell lines of varied tissue origin.

Conclusion

In conclusion, the essential oil isolated from the flowers of *Artemisia dubia* was found to be rich in aroma chemicals like terpene esters, oxygenated monoterpenes and sesquiterpenes with nerylisovalerate (9.79 %), 1,8-cineole (8.32 %), neryl-2-methylbutanoate (7.32 %), the three major chemical constituents. The essential oil shows antifungal activity against *Candida* species. The antioxidant activity was found to be moderate by DPPH and hydroxyl scavenging methods. *Artemisia dubia* has the potential to be used in control of fungal diseases and can find applications in the fragrance industry as the plant is rich in aroma and fragrance chemicals. The oil showed the strong

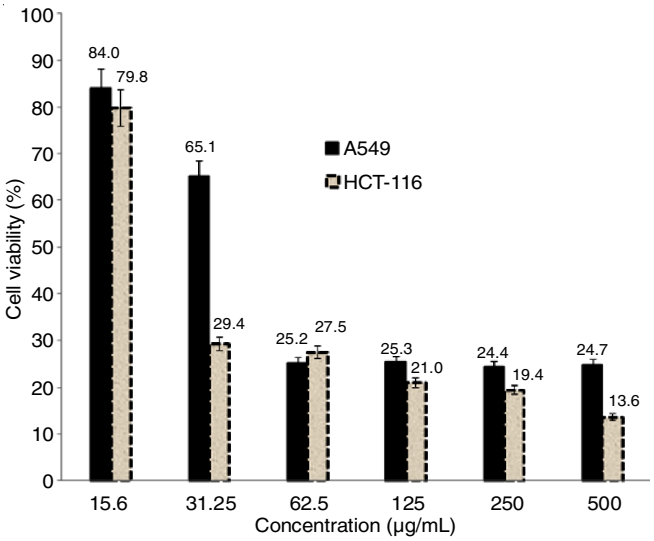

Fig. 2. Anticancer activity of essential oil of *Artemisia dubia* against A549 and HCT-116 human cancer cell lines

antiproliferative action against the A549 and HCT-116 human cancer cell lines.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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