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Research Article

CHARACTERIZATION AND EVALUATION OF ANTIDERMATOPHYTIC ACTIVITY OF THE ESSENTIAL OIL FROM *ARTEMISIA NILAGIRICA* LEAVES GROWING WILD IN NILGIRIS

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ABSTRACT

The chemical composition of the essential oils of *Artemisia nilagirica* growing wild in Nilgiris has been studied. The essential oils from the leaves of *Artemisia nilagirica* obtained through steam distillation were characterized for its organoleptic properties and physicochemical constants. Freshly extracted essential oil was pale yellow or slightly greenish, soluble in common organic solvents and very slightly soluble in water. Its specific gravity was 0.9071; with 1.442 as refractive index; -21.31 as optical activity and chemical constants (in % w/v) viz, acid value 2.2, ester value of 151.86, aldehyde content 76, alcohol content 40.91 and phenolic content 15. The volatile constituents of essential oils were investigated by GC-MS. Twenty nine constituents were identified. The minimum inhibitory concentration (MIC) of the oil was determined using dilution method and antidermatophytic activity was tested by agar diffusion. The oil was found to have significant antidermatophytic activity against dermatophytes such as *Trichophyton rubrum* (ATCC 2818810), *Microsporum canis* (ATCC 118325) and *Microsporum gypseum* (ATCC 241025) with MIC values of: 500 µg/mL, 125 µg/mL and 62.50 µg/mL and the zones of growth inhibition were: 26 mm, 28 mm and 29 mm respectively.

Keywords: Essential oil, Antidermatophytic activity, *Artemisia nilagirica*, Nilgiris

INTRODUCTION

Artemisia is one of the diverse genera of Asteraceae family consisting of more than 800 species which are widespread over the world, with many important medicinally valuable essential oils and secondary metabolites. Essential oils of *Artemisia* spp. have been widely used for a variety of medicinal purposes for many years. *Artemisia nilagirica* (Clarke) pamp commonly called Indian worm wood, is widely found in the hilly areas of India. *A. nilagirica* has been reported to exhibit insecticidal activities[1]. Various species of *Artemisia* have been characterized for their biological activities. It is considered to produce most medicinally important secondary metabolites[2,3]. Several interesting studies using *Artemisia* spp. showed a series of antimicrobial and antioxidant activities[4-8]. The qualitative determination of various secondary metabolites like flavonoids, terpenoids, saponins and polysaccharides of *Artemisia* spp. were detected by HPLC, GC-MS and NMR[9]. Few considerable secondary metabolites were successfully isolated and used in food industry as an alternative to synthetic antimicrobials[10,11]. Furthermore, extracts of *Artemisia* spp. were used as natural pesticide and also in the treatment of few human diseases[12-15]. Essential oils make a major contribution into the plant's biological activity as well. For that reason the chemical composition of worm wood oils has been investigated in several studies.

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in immune compromised patients in developing countries. Although a large number of antimicrobial agents have been discovered, pathogenic microorganisms are constantly developing resistance to these agents[16]. There has been an increased interest in looking at antimicrobial properties of extracts from aromatic plants particularly essential oils. Essential oils have been shown to possess antibacterial, antifungal, antiviral insecticidal, antioxidant properties and traditionally used for treatment of infections and diseases all over the world for centuries[17]. In recent years there has been extensive research to explore and determine the antimicrobial activity of essential oils. The aim of the present study was to explore the chemical composition of *A. nilagirica* essential oils from Nilgiris and to determine its antidermatophytic activities.

Plant materials

Fresh leaves of *Artemisia nilagirica* were collected from Nilgiri district, Tamil Nadu, India and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Center, (PARC) Tambaram, Chennai. A voucher specimen (138) has been deposited in the herbarium of the School of pharmaceutical sciences, Vels University.

Recovery of the essential oils

The collected leaves were washed, dried between filter papers and air-dried. The leaves were cut into small pieces for extraction using steam distillation for 3 h, using Clavenger-type apparatus. The distilled oils were dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4°C until the analysis and test.

GC-MS analysis

The isolated volatile oil was analysed using GC/MS (JEOL GC mate) with two fused silica capillary column DB-5 (30µm, 0.25 mm i.d, film thickness 0.25 µm) and a flame ionization detector (FID) which was operated in EI mode at 70 eV. Injector and detector temperatures were set at 220°C and 250°C, respectively. One microliter essential oil solution in hexane was injected and analyzed with the column held initially at 60°C for 2 min and then increased by 3°C/min up to 300°C. Helium was employed as carrier gas (1 ml/min).

The preliminary identification of the constituents was based on the computer matching of mass spectral data of the components against the standard WILEY275 and NIST library spectra constituted from spectra of pure substances and components of the known essential oils, and literature MS data. They were confirmed by their GC retention time comparison with those of reference compounds and with those reported in the literature[18].

Physical analysis of the volatile Oil

Physical characterization of the isolated volatile oil consists of the description and determination of attributes.

Organoleptic properties

The volatile oil was placed in a transparent bottle over a white background and the color and clarity were observed; the characteristic odor was determined by sniffing; and to determine its characteristic feel to the touch, it was rubbed between fingers.

Solubility

The solubility of the volatile oil was determined by mixing increment volumes of the volatile oil in specified volumes of the following solvents: water, chloroform, alcohol, and petroleum ether.

Specific gravity

Specific gravity is an important criterion of the quality and purity of volatile oils. The actual weight or the tare of a vial or was determined accurately. The vial was filled with water and weighed. The procedure was repeated using the volatile oil in place of water. The specific gravity of the oil is expressed as the ratio of the weight of the volume of oil to that of an equal volume of pure water when both are determined at 25°C[19].

Specific rotation

Both the degree of rotation and its direction are important criteria of purity. The extent of optical activity of oil was determined by a polarimeter which measured the degree of rotation. The zero point of the polarimeter was adjusted and determined. The previously cleaned and dried polarimeter tube was filled with 10% alcoholic solution of the volatile oil. The analyzer was rotated until equal illumination of light of the two halves of the visual field is achieved[19].

The angles of rotation was determined and the specific rotation was calculated (USP 25, 2002).

$$[\alpha] = t/\lambda = 100\alpha/lcd$$

Where:

[α] = Specific rotation at λ, t = temperature, α = observed rotation in degrees, c = concentration, L = path length in dm, λ = wavelength of light used in nm, d = specific gravity of the oil

Refractive index

The test plate was attached to the refracting prism of the Abbe refractometer provided with the test plate, by moistening the test plate with the liquid and pressing it against the refractive prism. The light was focused on the test plate. The instrument was adjusted until the borderline of the critical angle coincides with the cross hairs in the telescope, and the reading of the refractive index was taken. The test plate was removed, cleaned, and 2-3 drops of the volatile oil was placed on the prism and the prism was clamped together firmly. The light source was fixed so that the light is reflected through the prisms and the instrument adjusted until the borderline between the light and dark halves of the field of view exactly coincides with the cross hairs of the telescope. The refractive index was then read. The values should be between 1.46 and 1.61 at 25°C[19]. The results were depicted in Table 2 and 3.

Chemical analysis

Chemically volatile oil is a mixture of several constituents such as hydrocarbons, alcohols, acids, ethers, esters, aldehydes and ketones. These constituents can be determined in terms of the saponification value, acid value, alcohol value, aldehyde content and phenolic content serves to detect adulteration and to establish the quality and purity of valued oils.

Acid value

For the determination of acid number, 1.5 gm of absolute oil was weighed accurately into a 250-mL saponification flask to which 15 ml of neutral 95% alcohol and 3 drops of 1% phenolphthalein solution was added. Titration of the free acid with a slandered 0.1 normal aqueous solution of NaOH was done by adding the alkali drop wise at a uniform rate. The contents of the flask were continuously agitated. The first appearance of red colour was considered as an end point and the acid value was calculated[20].

Ester content (Saponification Value)

About 1.5 to 2 grams of the oil, accurately weighed was placed in a 250-mL Erlenmeyer flask to which 10 mL of neutralized alcohol and 2 drops of phenolphthalein TS was added drop wise. Then 0.1N sodium hydroxide solution was added until a faint pink color appeared. After adding 25.0 mL of 0.5 N alcoholic potassium hydroxide, a reflux condenser was connected and was then heated on a boiling water bath for 1 hour. The mixture was allowed to cool, about 20 ml of water and 3 drops of phenolphthalein TS was then added and the excess alkali was titrated with 0.5 N hydrochloric acid[20]. A blank test was performed and the total ester was calculated using the following formula

$$\text{Ester value} = \frac{(\text{Titre value} - \text{blank}) \times 0.5\text{N Hcl} \times 56.11\text{mg/mEq}}{\text{Weight of sample}}$$

Alcohol value

About 5 ml of the dried oil was taken in a round bottom flask and 5ml of acetic anhydride and 1.0g fused sodium acetate was added. The mixture was refluxed on the sand bath for 3 hr, cooled and some water was added and warmed on the water bath for 1-2 hr. The content was transferred in to a separation funnel and the oil was washed free of acidic impurities, dried over anhydrous Na_2SO_4. Then the procedure was followed as for ester value. Alcohol value was calculated from the expression

$$\text{Alcohol Value} = \frac{56.1 \times R \times \text{Normality}}{\text{Wt .of oil in g}}$$

Aldehyde content

About 1g of oil was weighed accurately; 5ml of benzene, 3.5 percent alcoholic hydroxylamine hydrochloride was added and made up to 100 ml of water. The liberated hydrochloric acid was titrated against 0.5 N alcoholic potassium hydroxide using methyl orange as indicator until the yellow colour of the lower layer remains unchanged after 2 minutes of vigorous shaking.

Phenolic content

The content of total phenolics in the essential oil of *Artemisia nilagirica* leaf was determined by using Folin –Denis reagent. About 1gram of the oil was heated in an ultrasonic wave bath with 80 ml of aqueous ethanol solution (70%v /v) for 2 hrs. After cooling the volume of the solution was adjusted to 100 ml. The final solution was centrifuged prior to the colorimetric determination. Tannic acid (10-110 µg/ml) dissolved in 100 ml of aqueous ethanol solution (70%v/v) respectively was used as standard solution. About 10 ml of Folin-Denis reagent was added to 1ml of sample solution and 1ml of standard solution. After reacting for 3min, 10 ml of 35% sodium carbonate solution was added and the test solution was dilute to 100 ml with water after 45 min. An aliquot was centrifuged for 5 min. The absorption coefficient of the supernatant liquid was measured at 745 nm. The total phenolic content of the extract was calculated using the mean regression coefficient from the standard[21].

The results were presented in Table 4.

Evaluation of antidermatpohytic activity

The dermatpohytic activity of the volatile oil was evaluated by agar diffusion method using a paper disc.

Microorganisms

The standard strains were procured from the American type culture collection (ATCC), Rockville, USA, and the pathological strains were procured from the department of dermatology, Sri Ramachandra Medical College and Research Institute, Porur, Tamilnadu, India.

Fungi:

Trichophyton rubrum ATCC 2818810

Microsporum canis ATCC 118325

Microsporum gypseum ATCC 241025

Agar Diffusion method

The sabouraud dextrose agar medium was sterilized at 121°C for 15 minutes and allowed to cool, transferred to petri dishes[22]. The plates were incubated within 15 minutes after inoculating the organisms and the plate was divided into sections according to the number of antibiotic test and control solutions to be used. The sterilized (autoclaved at 120°C for 30 mins) medium (40-50°C) was inoculated (1 ml/100 ml of medium) with the suspension of the microorganism. A disc containing only DMSO was used as the negative control. The sterile filter paper discs (6-mm diameter) impregnated with the volatile oil (50, 100 and 150 µg/disc) were placed on the solidified medium. The plates were pre incubated for 1 h at room temperature and incubated at 48 h. Terbinafine (20 µg /disc) was used as standard. After the incubation period, the zone of inhibition was measured with a calliper. Experiments were performed in triplicate, and the results are presented as the mean values of the diameters of the inhibitory zones from three runs. The observed zones of inhibition are presented in Table 5.

Determination of the MIC by dilution method

Thirteen screw-capped test tubes (13mm x 100 mm) were sterilized and numbered individually. One mL of Sabouraud glucose broth was introduced into tubes 2 to 11. To tube 12, 2.0 mL of Sabouraud broth was introduced; 1 mL of the volatile oil was pipetted into tube 1 and 2 and capped, it was vortexed for 5 seconds; 1.0 mL was withdrawn from the contents of tube 2 and transferred to tube 3, after capping the tube and mixing by shaking the contents, 1.0 mL from the contents of tube 3 was withdrawn and transferred to tube 4, the tube was capped, shaken and mixed well. This process was continued until 1.0 mL was withdrawn from tube 9 and subsequently added to tube 10, capped and shaken. One mL of the diluted inoculum was introduced into tubes 1 to 11 and to tube 13. To tube 13, 1.0 mL of the antibiotic standard was added. The tubes were incubated at 35oC for 18 to 24 hours. After incubation the tubes were examined for fungal growth. This can be visible as turbidity in the tube or as whitish pellet at the bottom of the tube. The tube with the lowest concentration of the volatile oil at which no growth or turbidity is observed was reported as the MIC against the organism[23].

The MIC values of volatile oil against the micro organisms were presented in the Table 6.

RESULTS AND DISCUSSIONS

Percentage yield of the volatile oil

The air dried leaves were subjected to steam distillation in order to extract the volatile oil. Two hundred fifty six (101) mL was obtained from about 20 kilograms of air dried leaves. The oil yields of *Artemisia nilagirica* obtained were 0.50% (w/w).

Chemical analysis of *Artemisia nilagirica* volatile oil

The *Artemisia nilagirica* volatile oil was analysed in Indian Institute of Technology (IIT), Chennai, Tamilnadu, using Gas Chromatography-Mass Spectrometry to identify the different constituents of the volatile oil. GC-MS analysis of the oil resulted in the identification of 29 components (Fig 1). List of compounds identified in the oil are presented in Table 2. The table shows the peaks of the sample with their corresponding retention times and their possible identities. The oils represent mainly a mixture of monoterpenes and sesquiterpenes.

Physical properties and constants of *Artemisia nilagirica* Volatile Oil

Organoleptic evaluation of the volatile oil was done and noted for color, odor, taste and feel to the touch. The solubility in different solvents such as water, ethyl alcohol, methanol, chloroform and petroleum ether were determined. Physical constants such as specific gravity, optical rotation and refractive index of the volatile oil were also determined.

Table 1 shows the physical properties of the volatile oil. Freshly extracted essential oil is pale yellow or slightly greenish, soluble in common organic solvents and very slightly soluble in water. It has a camphoraceous odour, pungent, bitter-sweet taste and greasy to touch.

Physical constants serve as a means of assessing the purity and quality of the volatile oil as well as for identification. The specific gravity, optical activity and refractive index were determined. Table 3 shows the values obtained from three determinations.

Table 1: Organoleptic evaluation of *Artemisia nilagirica* volatile oil

Organoleptic property	Description
Color	Pale yellow to slightly greenish
Odour	Fresh-camphoraceous
Taste	Pungent, Bitter-sweet
Characteristic feel	Greasy

Table 2: Chemical composition of *Artemisia nilagirica* volatile oil

S. No	Retention time (min)	Possible identity
1	22.03	1,8-cineol
2	18.27	bornyl acetate
3	17	terpinene-4-ol
4	16.16	Citronellal
5	15.28	α-campholene aldehyde
6	13.83	amyl alcohol
7	13.47	Limonene
8	13.03	γ-terpinene
9	12.82	2-methyl-6-methylene-oct-2-ene
10	12.39	geraniol
11	11.98	β-caryophyllene
12	11.55	β- terpineol
13	11.14	α-humulene
14	10.93	Sabinene
15	10.73	β-selinene
16	10. 52	E-citral
17	10.04	Z-citral
18	9.49	Camphene
19	9	α-cadinene
20	8.82	caryophyllene oxide
21	8.68	β- terpineol
22	7.47	α- thujone
23	7.31	Linalool oxide
24	7.17	γ-terpinene
25	6.9	β- Ocimene
26	6.62	limonene
27	5.37	α- pinene
28	4.96	β – Myrcene
29	4.14	β- pinene

Chemical constants of *Artemisia nilagirica* volatile oil

The constituents of volatile oil can be determined in terms of the saponification value, acid value, alcohol value, aldehyde content and phenolic content that serves to detect adulteration and to establish the quality and purity of valued oils. The values were presented in Table 4.

Anti-dermatophytic activity

Dermatophytosis is caused by fungi in the genera *Microsporum, Trichophyton* and *Epidermophyton* affect keratinous tissues of humans and of other vertebrates, causing superficial fungal infection[24]**.** A survey of literature reveals that there are many essential oils which possesses antifungal activity[25]. Agar-disc diffusion method was used to evaluate the antidermatophytic activity of the volatile oil. The diameter of the clear zone surrounding the well containing the volatile oil was measured. This indicated the extent of the inhibitory power of the *Artemisia nilagirica* oil against the test organisms. The volatile oil exhibited antidermatophytic activity against all the tested dermatophytes. The results of all volatile oil were comparable with that of the standard terbinafine. The measured zones of growth inhibition and MIC of *Trichophyton rubrum* (ATCC 2818810), *Microsporum canis* (ATCC 118325) and *Microsporum gypseum* (ATCC 241025) were shown in the Table 5 and Fig 2-4. Minimum inhibitory concentration is the lowest concentration that kills 99.9% of bacteria and fungi. The MIC of the volatile oil was determined by micro dilution method using two-fold dilution for the dilution series. Table 6 shows the results of the MIC of the test organisms.

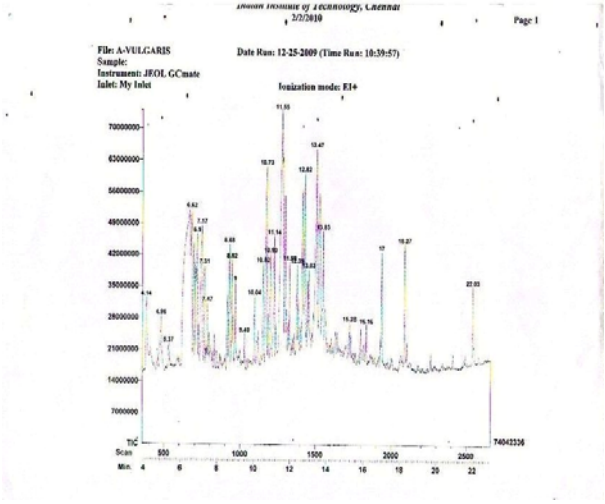

Fig. 1: GC-MS analysis of *Artemisia nilagirica* volatile oil

Table 3: Physical constants of the *Artemisia nilagirica* volatile oil at 25°C

S. No	Specific gravity	Optical activity	Refractive index
1	0.8786	- 13.25º	1.350
2	0.9164	-21.35 º	1.486
3	0.9265	- 29.35º	1.490
Average	0.9071	-21.31	1.442

Table 4: Chemical constants of the *Artemisia nilagirica* volatile oil

S. No	Parameters	Value (% w/v)
1	Acid value	2.2
2	Ester value	151.86
3	Alcohol content	40.91
4	Aldehyde content	76
5	Phenolic content	15 µg/ml

Table 5: Zone of Inhibition of *Artemisia nilagirica* volatile oil

S. No	Organism	Concentration (µg/ml)	Zone of inhibition (in mm)	
			Essential oil of *A. nilagirica*	Standard 20 µl/disc
1.	*Trichophyton*	50 µl	15 mm	26 mm
	Rubrum	100 µl	19 mm	
		150 µl	22 mm	
2.	*Microsporum*	50 µl	16 mm	28 mm
	Canis	100 µl	18 mm	
		150 µl	23 mm	
3.	*Microsporum*	50 µl	17 mm	29 mm
	Gypseum	100 µl	21 mm	
		150 µl	25 mm	

Table 6: Minimum inhibitory concentration (MIC) results of *Artemisia nilagirica* volatile oil for *T. rubrum*, *M. canis* and *M. gypseum*

Conc. (µg/mL) Dilution level	Growth of test organisms		
	T. rubrum	*M. canis*	*M. gypseum*
1000	-	-	-
500	-	-	-
250	+	-	-
125	+	-	-
62.50	+	+	-
31.30	+	+	+
15.60	+	+	+
7.80	+	+	+
3.90	+	+	+
1.95	+	+	+

The minimum inhibitory concentration of *T. rubrum* is 500 µg/mL, *M. canis* is 125 µg/mL and *M. gypseum* is 62.50 µg/mL

Some volatile oils containing plants are known to show qualitative and quantitative variations when they grow at different geographical location. So, the chemical composition of *Artemisia nilagirica* oil, which grows wild in Niligiris Dt, Tamilnadu were studied, which showed the presence of 29 components. The ability to assure the physical and chemical properties of an active pharmaceutical ingredient in a drug product is critical for regulatory approval and therapeutic success. Physical constants such as specific gravity, refractive index, optical rotation obtained confirms that the *Artemisia nilagirica* volatile oil is pure and possesses the characteristics of a volatile oil. The dilution method and the disc diffusion method/agar well diffusion are used to determine the antidermatophytic activity of the oil. Volatile oil exhibited high inhibitory potency against dermatophytes *Trichophyton rubrum*, *Microsporum canis* and *Microsporum gypseum* and showed maximum inhibition against *Microsporum gypseum.* Furthermore, the development of natural antimicrobials will help to decrease the negative effects of synthetic drugs.

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