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Original Article

Chemical composition, anti-dermatophytic activity, antioxidant and total phenolic content within the leaves essential oil of *Vitex trifolia*

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Abstract

This study was carried out to determine the contents of total phenol as well as antioxidant activity, the chemical composition and anti-dermatophytic activity of the *Vitex trifolia* (Family: Lamiaceae) essential oil of the leaves. Hydro-distillation of the *V. trifolia* was carried out using Clavenger Vendor to obtain the volatile oil. Total phenolic content and antioxidant activity were determined using the Folin-Ciocalteau reagent and DPPH radical scavenging assay test by spectrophotometric methods. Gas chromatography-mass spectrometric (GC-MS) analyses (BP - 1 chrompack capillary column) of the essential oil was performed and its composition determined. The *in-vitro* anti-dermatophytic activity was evaluated both by agar diffusion and microbroth dilution methods against *Candida albicans* ATCC 90029, *C. albicans* ATCC 1162, *C. albicans* ATCC Y-9-19, *C. parapsilosis* ATCC 20019, *C. glabrata* ATTC 91030, *C. krusei* ATCC 6258, *C. krusei* ATCC 71061-1113 and *C. kefyr* ATCC 1110. The fresh leaves were yielded 0.21% of yellowish colour essential oil. By GC-MS analysis of the essential oil, 50 constituents were identified. 1R-α-pinene, Ocimene, 3-carene, Toluene, p-ethyl-, Eucalyptol and 3-methoxy-5-methylphenol were the major constituents. The *V. trifolia* contained appreciable levels of amount of total phenolic content (74.66µg GAE/100mg) of the fresh weight and the DPPH free radical scavenging activity (the IC_{50} = 5.2 µg/ml). The anti-dermatophytic activity showed that essential oil had some antifungal activity on the tested *Candida* in the concentration 500 µg/ml. The highest antifungal activity was *C. krusei* ATCC 6258 and *C. kefyr* ATCC 1110 (zone diameter 27.97±0.4 mm and 23.88±0.8 mm) respectively and *C. krusei ATCC* 6258 (MIC = 1.953125 µl/ml).

Keywords: *Vitex trifolia,* Essential oils, GC-MS, Total phenolic content, Antioxidant activity, Anti-dermatophytic activity

Introduction

In recent years, using traditional medicinal knowledge in drug discovery seems so promising that even large pharmaceutical companies have begun to show interest in this field.[1,2] The genus *Vitex* includes approximately 270 known species of trees and shrubs in tropical and sub-tropical regions, although a few species are also found in temperate zones.[3]

Vitex trifolia (Lamiaceae) is a stout, aromatic shrub or a small tree grown wild which is traditionally used by the tribes and native medical practitioners for the treatment of various ailments including liver disorders, tumours, rheumatic pains, inflammation, sprains, fever and used in the treatment of tuberculosis.[4] Literature review reveals that the plant *V. trifolia* possess larvicidal, wound healing, anti-HIV, anticancer, trypanocidal, antimicrobial, antipyretic activities, hair tonic, analgesic, anticonvulsant and sedative, cytotoxic, antiemetic, expectorant, nervine, cephalic, emmenagogue and tonic and beneficial in thirst.[5-19] Vitexicarpin, a flavonoid was isolated from *V. trifolia* Linn., which induces apoptosis in K562 cells via mitochondria – controlled apoptotic pathway.[20] Four new hallimane type diterpenes, vitetrifolins D – G, were isolated from the fruits of *V. trifolia*.[21] The essential oils of *V. trifolia* have been shown to having insecticidal activity[22] headaches and colds, alleviation of fever, pain relief, sedation, anti-inflammatory treatment, etc.

The main objective of this study is to search medicinal value by determining the contents of total phenol as well as antioxidant activity, the chemical composition and anti-dermatophytic activity of the essential oil of the leaves of *V. trifolia* for the development of new antimicrobial agents and/or standardized phytomedicines where used in the Manipuri traditional medicine for the treatment skin diseases.

Materials and Methods

Plant material and extraction

The plant was collected from Imphal, Manipur and was identified by Botanical Survey of India, Kolkata

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and the herbarium voucher sample is deposited in the Departmental herbarium. The leaf was extracted for its essential oil using hydro-distillation.[23] Two kilograms of fresh leaves were soaked in 5 litres of water and boiled for 10 h in the Clevenger Vendor. The collected oil was stored in the dark at room temperature. The yield of the essential oil was calculated as under:

Yield (%) = weight of the essential oil/weight of *V. trifolia* × 100

Determination of total phenolic contents

The total phenolic content was assayed using the Folin-Ciocalteu reagent.[24] An aliquot (0.125 ml) of a suitable diluted acetone sample was added to 0.5 ml of deionized water and 0.125 ml of the Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before adding 1.25 ml of 7% sodium carbonate solution. The solution was then adjusted with deionized water to a final volume of 3 ml and mixed thoroughly. After incubation for 90 min at 23 °C, the absorbance versus prepared blank was read at 725 nm. Total phenolic content of essential oil was expressed as micrograms of gallic acid equivalents per 100 gram of fresh weight (µg of GAE/100 mg of FW) through the calibration curve with gallic acid.

DPPH radical scavenging assay

The scavenging activity of essential oil on DPPH was determined.[25] A total of 1 ml of essential oil prepared in 80% acetone was added to 0.5 ml of a 0.2 mmol/DPPH methanolic solution. The mixture was shaken vigorously and kept at room temperature for 30 min. The absorbance of the resulting solution was then measured at 517 nm after 30 min. The antiradical activity was expressed as IC_{50} (µg/ml), the concentration required to cause 50% DPPH inhibition. Ascorbic acid was used as a positive control. The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH Scavenging capacity (%) = 100 – [(absorbance of essential oil – absorbance of blank) × 100/absorbance of control]

GC-MS analysis

The GC-MS analysis was performed on Shimadzu GC-MS QP 5000 with GC17A GC system fitted with BP - 1 chrompack capillary column (30 m × 0.25 mm, film thickness 0.2 µm). Temperature programming: 50 °C–300 °C at 20 °C/min. Carrier gas used was He at 55 kPa pressure,1ml/min flow rate. Electron impact mode of ionization with ionization energy 70 eV and ion source temperature 170 °C. Sample (5 µl) was injected at 120 °C and split ratio was 50:1.

Identification of components

Peaks were identified by comparison of relative GC retention times with standards from literature, retention indices on BP - 1 column,[26,27] peak enrichment on co-injection with authentic standard wherever possible and comparison of mass spectra with literature data.[28,29]

Anti-dermatophytic activity assay

Yeast strains used

The activity of the essential oil was tested against American Type Culture Collection (ATCC) yeast strains including: *Candida albicans* ATCC 90029, *C. albicans* ATCC 1162, *C. albicans* ATCC Y-9-19, *C. glabrata* ATTC 91030, *C. parapsilosis* ATCC 20019, *C. krusei* ATCC 6258, *C. krusei* ATCC 71061-1113, *C. kefyr* ATCC 1110 are subcultured onto Sabouraud Dextrose Agar (Hi-Media) and incubated at 32±2 °C for 24 h.

Antimicrobial susceptibility testing

The antifungal activity tests were performed using agar diffusion and microbroth dilution methods according to the guidelines of the Clinical and Laboratory Standard Institute (CLSI) (formerly, National Committee for Clinical and Laboratory Standards)[30] (M-27-A2) for yeasts.

Agar Diffusion Test

Agar diffusion was carried out with RPMI-1640 agar supplemented with glucose 2%. The inoculum was prepared using 24 h plate cultures of *Candida* species. The colonies were suspended in 0.9% saline and the turbidity was compared with the 0.5 McFarland standards, to produce a yeast suspension of 1×10^6 to 5×10^6 cells/ml. The suspension was loaded on a sterile cotton swab that was rotated several times and press firmly against the inside wall of the tube to remove excess inoculum from the swab. The dried surface of a RPMI agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated two more times. Wells were bored into the agar media using a sterile 6 mm cork borer. The essential oil was prepared in 100% dimethylsulfoxide (DMSO) (Sigma) as stock solution 1 ml/ml and was tested at the concentration 500 µl/ml and 250 µl/ml with a final volume of 50 µl in that wells. The plates were incubated at 32±2 °C for 24 h. The zone diameter was read to the nearest whole millimetre at the point at which there is a sharp reduction in growth occurs. DMSO 100% was used as negative growth control whereas Amphotericin-B (Sigma) was used as positive control against tested yeasts at a concentration 16 µg/ml.

Microdilution Test

Testing of the essential oil for antifungal activity was determined by the micro-broth dilution techniques. All the testing was done in triplicate. Stock solution was prepared by dissolving 1 ml in 1 ml (DMSO). The resulting 1ml/ml oil solution was diluted 9 times to obtain a concentration of 1.953125 µl/ml. To avoid bacterial contamination each solution was sterilized by filtration through a 0.22 µm Millipore-filter. Serial dilutions were made in RPMI 1640 with L-glutamine but without bicarbonate (GIBCO BRL). Aliquots of 100 µl of the diluted extracts were dispensed into the wells of flat-bottomed 96-well micro-titre plates (Tarson).

The inocula were adjusted turbid metrically to a

McFarland to give a final concentration of 0.5×10^3 CFU/ml in RPMI 1640 medium buffered to pH 7 with 0.165M morpholine-propanesulfonic acid (MOPS) (Alfa Aesar). A constant volume (100 µl) of the inoculum was added to each micro-dilution well containing 100 µl of the serial dilution of the oil extract to reach final concentration of 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.90625 and 1.953125 µl/ml respectively. The micro-titre plates were incubated at 32±2 °C for 24 h. DMSO 100% was used as negative growth control and Amphotericin-B was used as positive control against tested yeasts. It was considered that if the essential oil displayed a MIC equal or less than 100 µl/ml, the antifungal activity was strong; from 100 to 500 µl/ml the antifungal activity was moderate; over 500 µl/ml the antifungal activity was considered inactive.

Minimum inhibition concentrations (MICs) were determined after 24 h, after which readings were performed visually by comparing with growth in control wells and the essential oil blank, which consisted of uninoculated plates. MICs of the essential oil were defined as the lowest concentration of oil extract that visually showed no growth was less than 5% in comparison to that of growth control (MIC_0).

Statistical analysis

Sampling proceeds on three independent replications (n=3) for each analytical parameters. Results presented in tables were reported as means ± standard deviation (SD). Data were subjected to one-way analysis of variance ANOVA, and the significant difference between means was determined by Duncan's multiple range test. Differences at $p<0.05$ were considered statistically significant. Coefficients of determination (r^2) were calculated using Microsoft Excel 2010.

Results and Discussion

Chemical composition of the essential oils

The fresh leaves were subjected to hydro-distillation using a Clevenger Vendor apparatus, yielded 0.21% of yellowish colour essential oil. GC-MS analysis of the essential oil was shown in Table 1, 50 constituents was identified by comparing the retention time of authentic samples and comparing mass spectra with standard library (NIST) and comparing kovats index. 1R-α-pinene, Ocimene, 3-carene, Toluene, p-ethyl-, Eucalyptol and 3-methoxy-5-methylphenol were the major constituents in the oil ranging.

Chemical studies of leaves and twigs yield an essential oil of the *V. trifolia* were l-d-pinene casticin, luteolin, isoorientin, α-pinene, linalool, terpinyl acetate, β-caryophylline , caryophylline oxide, 5-methyl artemitin, β-sitosterol, vitetrifolins, dihydrosolidgenone abietatriene, vitetrifolin A, limonene, humulene oxide, α-humulemne, 20 hydroxycdysone, ecdysteroids, flavonoids, lignans, triterpenoids, iridoids, vitexin, β-sitosterols[7-9,13,31-41] whereas the acetone extract of the fruits of *Vitex trifolia* Linn isolated vitetrifolin A, B and C. Pulverized leaf of *V. agnus-castus* oil revealed the abundance β-pinene (20.0%),

Table 1. Constituents' essential oil of *Vitex trifolia*

Peak	RT	%RA	Name
1	6.361	0.172	α - phellandrene
2	6.496	4.041	1R- α - pinene
3	6.786	0.039	Camphene
4	6.856	0.014	Cinnamyl carbanilate
5	7.186	6.879	Ocimene
6	7.271	1.241	3-carene
7	7.341	0.004	3-cycloheptenone
8	7.441	0.037	3,7,11-trimethyl-3-hydroxy-6, 10-dodecadien-1-yl acetate
9	7.626	0.005	3-octanol
10	7.706	0.005	β - phellandrene
11	7.922	0.066	1,3,8-P-menthatriene
12	8.057	1.951	Toluene, p-ethyl-
13	8.132	0.517	Limonene
14	8.227	6.133	Eucalyptol
15	8.597	0.141	γ - terpinene
16	8.842	0.019	α - terpinolene
17	9.042	0.023	α - terpinene
18	9.127	0.011	Camphenol
19	9.207	0.035	Nonadecane
20	9.267	0.016	3-p - menthene
21	9.342	0.011	α - fenchene
22	9.542	0.007	4-chlorooctane
23	9.592	0.005	Thujone
24	9.722	0.051	Campholenal
25	9.767	0.005	1-formyl-2,2-dimethyl-3-trans-(3-methyl-but-2-enyl)-6-methylidene-cyc
26	9.972	0.027	Myrtenyl acetate
27	10.037	0.024	1,3,8-p-menthatriene
28	10.302	0.005	3-methylselenomethylfuran
29	10.543	0.11	Arteannuic acid
30	10.598	0.005	α - thujenal
31	10.763	0.015	L- 4- terpineneol
32	10.793	0.024	(-) - myrtenol
33	11.068	0.006	Fenchyl acetate
34	11.388	0.004	Hydro cinnamaldehyde
35	11.558	0.004	(Z) - verbenol
36	12.018	0.062	2-methylbicyclo[4.3.0]non-1(6)-ene
37	12.088	0.067	Thujopsene
38	12.393	0.053	δ 3-p-menthene
39	12.844	2.185	3-methoxy-5-methylphenol
40	12.924	0.01	2-methyl-2-hydroxy-decalin-4A-carboxylic acid, 2,4A-lactone
41	13.404	0.008	(+) – γ - gurjunene
42	13.639	0.011	Humulen-(V1)
43	13.839	0.028	Isocaryophillene
44	14.079	0.025	β - humulene
45	14.199	0.004	(-)-aristolene
46	14.349	0.084	Longifolene – (V4)
47	15.485	0.035	Andrographolide
48	15.780	0.014	β - vatirenene
49	15.860	0.411	Caryophyllene
50	16.175	0.005	Icosapent

RT indicates the retention time on the column in minutes

RA indicates relative area

viridiflorol (9.8%), α-pinene (9.1%), cisocimene (8.4%), 1,8-cineole (6.7%), β-farnesene (5.4%), terpinen-4-ol (4.2%), α-terpineol (4.1%) and β-phellandrene (4.1%).[42]

Phytochemicals estimation and antioxidant activity

The amount of total phenolic content of *V. trifolia* was tested, and occurred in 74.66 µg GAE/100 mg with reference to gallic acid standard curve (y = 0.015x+0.03841, R^2 = 0.9811) (Figure 1). The methanol leaves extract of *V. trifolia* showed high antioxidant activities of phenol contents in the 44.6666 µg GAE/mg of fresh weight.[43] The total phenolic content is known to be bioactive compounds and all play a role for antioxidant and antifungal activities The DPPH free radical scavenging activity of essential oil of *V. trifolia* had been shown in Figure 1. The IC_{50} value of essential oil was recorded in 5.2 µg/ml. The methanolic and chloroform extracts of *V. trifolia* roots tested for its free radical scavenging and antioxidant property by using different *in vitro* models extract.[44] Essential oils are volatile and natural complex mixtures of compounds characterized by strong odours and formed by aromatic plants as secondary metabolites.[45] Phenolic and flavonoid compounds are important phytochemicals. Volatile compounds in essential oils from medicinal and aromatic plants have been known since ancient times to possess many biological activities, especially antibacterial, antifungal and antioxidant properties.[46-49] Our results indicate that presence of significant activity in essential oil making to interpret the promising health beneficial phytochemicals. Phenolic contents may significantly contribute to overall antioxidant activity and also to anti-dermatophytic activity. A positive correlation between total phenolic content and their antioxidant activity had showed.[43,50]

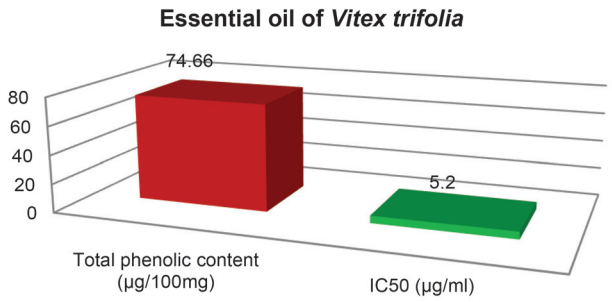

Figure 1. Showing the total phenolic content and antioxidant activity of essential oil of *Vitex trifolia*.

In vitro anti-dermatophytic activity

The effects of different concentrations of the *V. trifolia* essential oil and standard drug of antifungal activities using agar diffusion and micro-broth dilution assays were summarized in Table 2. The result showed that essential oil had some antifungal activity on the tested *Candida* in the concentration 500 µg/ml. It showed the highest antifungal activity was *C. krusei* ATCC 6258 and *C. kefyr* ATCC 1110 (zone diameter 27.97±0.4 mm and 23.88±0.8 mm) respectively (Table 2). This also showed that at higher concentration all the tested micro-organisms were not inhibited.

Table 2 showed the minimum inhibitory concentration (MIC) of the essential oil. *V. trifolia* (leaf) it's essential oil presented strong activity against the *Candida albicans* ATCC 90029, *C. albicans* ATCC 1162, *C. parapsilosis* ATCC 20019, *C. krusei* ATCC 6258, *C. krusei* ATCC 71061-1113, *C. kefyr* ATCC 1110 with MICs of 62.5, 7.8125, 31.25, 1.953125, <31.25 and 3.90625 µl/ml, respectively whereas *C. albicans* ATCC Y-9-19 and *C. glabrata* ATTC 91030 showed moderate activity. The best anti-fungal activity was shown by *C. krusei ATCC* 6258 (MIC = 1.953125 µl/ml).

The antifungal activity was compared with the standard antifungal, Amphotericin-B at 16 µg/ml. Similar study of screening natural plant extracts against different fungal pathogens was well recorded in literature. *V. trifolia* leaf extracts showed significant antibacterial activity and antifungal.[11,39,51-58]

Acceptance of medicines from plant origin as an alternative form of healthcare is increasing because they are serving as promising sources of novel antibiotic prototypes.[59] Some of the phytochemical compounds e.g. glycosides, saponin, tannin, flavonoids, trepenoid, alkaloid have variously been reported to have anti-microbial activity.[60,61]

Conclusion

The results obtained in this study potentially supported

Table 2. Anti-dermatophytic activity (zone of inhibition and MIC) of *Vitex trifolia* extracts compared with antibiotic 16 µg/ml Amphotericin-B

	Conc.	Inhibition zone (mm) of different crude extracts on agar well							
		CA1	CA2	CA3	CG	CP	CKR1	CKR2	CKE
Essential Oil (µl/ml)	500	-	11.67±0.24	-	-	11.57±0.42	27.97±0.4	12.17±0.34	23.88±0.8
	250	-	-	-	-	11±0.56	23.72±0.22	-	20.4±0.44
	MIC	62.5l	7.8125	250	>500	31.25	1.953125	<31.25	3.90625
Am	16	26.35±0.2	22.15±0.12	25.18±0.22	29.15±0.2	23.15±0.31	27.93±0.5	19.19±0.41	22.6±0.32
	MIC	0.25	<0.06	0.25	0.125	0.25	<0.06	0.25	0.06
DMSO	50µl	-	-	-	-	-	-	-	-

Means ± SD of triplicates where the values were not significant different at $P < 0.05$
Candida albicans ATCC 90029 (CA1); *C. albicans* ATCC 1162 (CA2); *C. albicans* ATCC Y-9-19 (CA3); *C. glabrata* ATTC 91030 (CG); *C. parapsilosis* ATCC 20019 (CP); *C. krusei* ATCC 6258 (CKR1); *C. krusei* ATCC 71061-1113 (CKR2); *C. kefyr* ATCC 1110 (CKE); – no activity; Amphotericin-B – Standard

that *V. trifolia* can be used as a leading factor in a wide range of activities. It shows significant source of natural antioxidants and as a new anti-dermatophytic agents to control superficial human fungal infections against *Candida* sps. Thus, we found biologically active compounds of pharmaceutical importance in the plant which can be explored for possible anti-fungal agents as this study provides preliminary scientific validation for the traditional medicinal use by indigenous people of Manipur.

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