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J. Sci. Trans. Environ. Technov. 2017, 11(2) : 69-72

Scientific Transactions in Environment and Technovation

Effect of *Vites negundo L* on opportunistic fungal pathogens associated with HIV

https://doi.org/10.56343/STET.116.011.002.002
http://stetjournals.com

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Abstract

Various medicinal plants are extensively used as Ayurvedic Medicine for over many years. They are extensively used as Indian traditional medicine to cure various human ailments. The World pandemic of AIDS has been with us for more than twenty five years and shows no signs of abatement. The incidence of fungal infections is increasing because of raising number of immunocompromised patients widespread use of broad spectrum of antibiotics. Medicinal plants constitute a very important natural resource used by indigenous medicinal systems for the last 300 years. Antimicrobial activity of ethyl acetate, diethyl ether and methanol extracts of Vitex negundo was investigated against HIV associated opportunistic fungal pathogens. The plant extracts showed better inhibitory activity against the tested organism. Ethylacetate extracts produced much better activity when compared with methanolic and diethylether extracts. Phytochemical screening of the plant revealed the presence of various compounds, and the results are discussed.

Key words: AIDS, immunocompromised patients, *Vitex negundo,* HIV patients.

Received : March 2017 Revised and Accepted : November 2017

INTRODUCTION

The breakdown of body immune system is the hallmark of HIV infection. Infections which are rarely seen in those with normal immune systems are deadly to those with HIV. This makes AIDS patients susceptible to a variety of opportunistic infections. People with HIV can get many infections called opportunistic infections. OIs are caused by various pathogenic microorganisms such as bacteria, fungi, virus and parasites (Hirschtick *et al*., 1995). Infections with opportunistic pathogens have been one of the hallmarks of the AIDS since the beginning of the epidemic. Diseases caused by bacteria and fungi are responsible for higher death rates significant proportion is found among HIV population (Graden *et al*., 1992).

Fungal pathogens can lead to many of the complications seen in advanced HIV disease and are commonly identified in HIV infected population with decreased immune function. While infections with these organisms can be fatal, appropriate identification and management of the condition can result in reduced mortality and the opportunity for effective management of HIV disease with highly active antiretroviral therapy (HAART). The common genera of fungal organisms affecting individuals with AIDS include *Aspergillus, Blastomyces, Candida, Coccidioides, Cryptococcus, Histoplasma, Penicillium* and *Tinea*.

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P - ISSN 0973 - 9157
E - ISSN 2393 - 9249

Plants have a great potential for producing new drugs for human benefit. There is a continuous development of resistant strains which pose the need for search and development of new drug to cure diseases (Silver, 1993). Systematic screening of them may result in the discovery of novel effective antimicrobial compounds (Tomoko *et al*., 2002). According to a report of WHO, more than 80% of world's populations depend on traditional medicine for their primary health care needs. Plant-derived substances have recently become great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube *et al*., 2008). Medicinal plants are finding use as pharmaceuticals, nutraceuticals, cosmetics and food supplements. Even as traditional source of medicines and they continue to play pivotal rule (Swathi Sharma *et al*., 2010).

Vitex negundo Linn.

Vitex negundo Linn. a large aromatic shrub with bluish purple flower widely prevalent in north, western Himalayan region, has been used for various medicinal purposes in the Ayurvedic and unanic systems of medicine. It is commonly known as Nirgundi. It is a large, aromatic shrub, sometimes a small slender tree found throughout India. It contains various chemical classes such as alkaloids, tannins, flavonoids and carbohydrates. The extracts of leaves have antifungal activity against *Aspergillus* and *Candida*

albicans. Analgesic and anti-inflammatory actions of *V-negundo* seeds and fruits have been reviewed thoroughly (Nahrstedt *et al*., 1982).

Systematic position of *Vitex negundo* L.

Kingdom	:	Plantae
Order	:	Lamiales
Family	:	Verbenaceae
Genus	:	Vitex
Species	:	negundo
Common Name	:	Nirgund

MATERIALS AND METHODS

Fresh leaves of *V-negundo* were collected and identified. The plant materials were cleaned, shade dried and powdered. Fresh plant materials were washed with tap water, air dried, homogenized to a fine powder and stored in air-tight bottles.

Extraction

Two hundred and fifty gram of the powder was soaked in 1.25-1.5 L of 95% ethanol for 5 days at room temperature. The mixture was mixed daily for regular infusion. After a five day period, the extract was filtered by using Whatman filter paper No.1. The filtrate was dried by using rotary evaporator at 60^{0}C. The dried extract was stored in sterile glass bottles at -20^{0}C for further use.

Collection of Biosamples

250 biosamples were collected from clinically suspected HIV patients of various Hospitals, Medical Colleges and Social Welfare Centres of Thanjavur and Trichy districts. All the patients were thoroughly evaluated by detailed history, clinical examination and biochemical parameters including CD4 count. All the strains were confirmed by cultural characteristics and maintained in slants for further use.

Phytochemical screening

Qualitative Chemical tests were carried out on the methanolic extract of the powdered specimens using standard procedures to identify the phytoconstituents as described by Trease and Evans, 1989; Sofowara, 1993.

Antifungal Sensitivity Tests (Bauer *et al*., 1966)

Saborauds agar medium was prepared and autoclaved at 121^{0}C under 15 psi pressures for 30 minutes. After cooling to about 65^{0}C, 25ml of the medium poured in Petri dishes. The plates kept at room temperature for solidification and stored at 4^{0}C until further use. Inoculum containing 108 cells/ml were spread over the medium. Antifungal activity was carried out by using hole-plate diffusion method (Bauer *et al.,* 1996; Begue and Kline (1972). The dried plant extracts were dissolved in respective solvent to final extract amounts of 10 to100mg/ml. Each hole of about 6mm diameter in each plate was filled with 10,50 and 100µl of plant extract separately. The inoculated agar plates were incubated at 37^{0}C for 24 hours. After the incubation period, the diameter of inhibition zone to each hole was measured in millimeter.

Thin layer chromatography (TLC)

TLC and bioautography (Oksana et al., 2007) were made to separate the antimicrobial principles. The crude leaf extracts showed high antifungal activity was subjected to TLC. The solvent used as 5 and 10% methanol in chloroform. 1 mg/ml concentration of the effective plant extract was spotted on the TLC plates and dried. They were then run with both ultraviolet and iodine chamber. Thin layer chromatography was performed on Merck TLC F254 plates, with chloroform: Methanol (95:5) as mobile phase. Developed chromatography plates of crude extract was dried overnight, sprayed with a suspension of actively growing cells of bacteria and fungi and incubated at 37 and 24°C respectively in a chamber at 100% relative humidity for 18 hrs. Plates were sprayed with MTT-3 (4,5-Dimethyl thiazol-2.5- Diphenyl tetrazolium Bromide) (5 mg/ml). Clear zones on the chromatogram indicated inhibition of growth after incubating for hours at 37°C.The separated components were visualized under visible and ultraviolet light (254 and 360 nm). The Retention Factor (RF) value was calculated by using the following formula.

$$RF = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

RESULTS AND DISCUSSION

Eight types of fungal pathogens were identified. They were *Aspergillus*, *Blastomyces*, *Candida*, *Cryptococcus*, *Fusarium*, *Histoplasma* and *Penicillium*. The predominant isolates include *C. albicans*, *A. niger*, *A. flavus, H. capsulatum*, *P. marneffei*, *C. neoformans, F. moniliforme*, *A. terreus, B. dermatidis* and *F. solani* (Table 1). Table 2 showed the qualitative screening of *V. negundo* Linn. The results of the phytochemical analysis (Table 2) showed that the methanolic extract of medicinal plant extracts showed that *V. negundo* showed positive results for saponins, flavonoids, glycosides, phenolic compounds, fixed oils and fats, alkaloids and protein and aminoacids. The effective bioactive compound of *V. negundo* was noted to be Acalyphin, a cyanogenic glucoside. There is no previous study conducted evaluating the anti-fungal property of *V. negundo*. Oksana *et al*. (2007) reported that flavonoids (quercetin, kaempferol, isorhamnetin, isoquercitrin), phenolic derivatives (gallicin, gallic,

Table.1. Isolated fungal pathogens of HIV+ Patients

S. No.	Fungi	Identified predominant spp.	Number of fungi isolated	Percentage
1	*Aspergillus* spp.	*A. flavus*	8	7.84
		A. niger	18	17.65
		A. terreus	2	1.96
2	*Blastomyces* spp.	*B. dermatidis*	2	1.96
3	*Candida spp.,*	*C. albicans*	48	47.06
4	*Cryptococcus* spp.	*C. neoformans*	4	3.92
5	*Fusarium* spp.	*F. moniliforme*	3	2.04
		F. solani	2	1.96
6	*Histoplasma* spp.	*H. capsulatum*	9	8.82
7	*Penicillium* spp.	*P. marneffei*	6	5.88

Table. 2. Qualitative phytochemical screening of medicinal *Vitex negundo* Linn

S. No.	Phytochemicals	Result	Observation
1	Tannins	+	Appearance of brownish green colour
2	Saponin	+	Appearance of foam
3	Flavanoids	+	Appearance of yellow colour
4	Terpenoids	+	Colour change was observed
5	Glycosides	-	No ring formation
6	**Phytosterols**	+	An array of colour changes was observed.
	Libermann Burchard's test		
7	**Phenolic**		
	i) Ferric Chloride	-	No colour changes observed.
	ii)G elatin	+	Precipitate formed
8	**Alkaloids**		
	i) Mayer's test	-	No precipitate formed.
	ii) Wagner's test	+	Reddish brown precipitate observed.
	iii) Hagner's test	-	No precipitate formed
9	**Carbohydrates**		
	i) Molisch's test	+	Violet ring formed
	ii)Fehling test	-	No precipitate formed
	iii)Benedict's test	-	No precipitate formed.

+ = Present - Absent

syringic, and caffeic acids), and coumarin (scopoletin) have potent anti-fungal activity against *Microsporum* spp. and *Trichophyton* spp. (Oksana *et al.,* 2007). The ethylacetate, methanol and diethyl ether extracts of *V. negundo* showed variable inhibition zones. The results were statistically substantiated with one-way ANOVA(Table 3). The 'F' values of all extracts were significant at 5% level. It showed that the ethylacetate extracts all plants were used for controlling the fungal pathogens. Fungal infections are among some of the most common opportunistic infections that occur in HIV patients. Most people have been exposed to the disease causing fungi because they are everywhere. However, infections only occur in individuals who have weakened immune systems that cannot prevent the fungus from growing. Invasive fungal infections are most common opportunistic infections associated with significant morbitity and mortality for patients with HIV infection, and the risk of invasive fungal infections varies with host immunity as well as environmental exposure (Huang et al., 2007).

Table. 3. One-way ANOVA for various extracts of *Vitex negundo* with respect to fungal pathogens

Name of the extract used	Groups	Sum of Squares	Df	Mean Square	'F' value	5% limit
Ethyl acetate	Between Groups	526.55	3	175.52	5.388	S
	Within Groups	521.2	16	32.575		
	Total	**1047.75**	**19**			
Diethyl ether	Between Groups	27.6	3	9.2	0.676	NS
	Within Groups	217.6	16	13.6		
	Total	**245.2**	**19**			
Methanol	Between Groups	162.55	3	54.183	1.378	NS
	Within Groups	629.2	16	39.325		
	Total	**791.75**	**19**			

Df – Degree of freedom

The diethyl ether extracts of herbal plants showed different zones of inhibition on the fungal pathogens. The 'F' values of *V. negundo* and *A. calamus* only significant, others were non-significant at 5% level. It reveals the plant extracts showed non-significant result were only moderate to control the pathogens when compared with the extracts shown significance result (Table 3). The methanol extracts of herbal plants showed different zones of inhibition on the pathogens. The results substantiated with ANOVA showed the

'F' value of *V. negundo* only non-significant at 5% level. Other plants were significant to control the fungal pathogens very well (Table 3). Gangadevi *et al., (2008)* reported that ethyl acetate extract of leaves and roots of *Vitex negundo* recorded higher activity than the methanol and hexane earlier reported that the endophytic fungi isolated from the leaves of *V. negundo* elicited promising antibacterial activity against the three human pathogenic bacteria. Crude ethanolic extract of fruits of V. negundo L, showed excellent results against F. solani showed (90%) (Mahmud *et al*., 2009). Methanolic extracts of medicinal plants selected based on their reputation in Ayurvedic and Indian traditional system of medicine were analyzed for their antifungal properties (Gangadevi et al., 2008).

TLC and Bioautography of *V. negundo*

Six lanes namely A, B, C, D, E, F and G indicated the separation of various components in the extract. Growth inhibition zones, indicated in lane G, showed the presence of active fungicidal components, well separated from each other, in at least one major and six minor positions of bioautogram. Rf values for *V. negundo* detected by TLC and bioautography were found to be 0.16, 0.26, 0.39, 0.5, 0.11, 0.78 and 0.97. Therefore, suggesting thereby the presence of antimicrobial active substances in the specific bands.

CONCLUSION

To conclude that, this study would definitely create awareness among the HIV positive patients for taking control or preventive measures based on herbal plants against the common opportunistic bacterial and fungal infections causing pathogens. However, due to changing pattern of infections depending upon the degree of immunosuppression, constant monitoring of infections in HIV positive population is important for better management and to improve the quality of life of such patients. Alternative to antibiotics, the patients should be recommended to take the herbal based medicines particularly *V. negundo* surely minimise the fungal related OI and increase the life span of HIV patients.

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