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Short Communication

Comparative Study on Antimicrobial Activity of *Vitex negundo* var. *negundo* and *Vitex negundo* var. *purpurascens*

Prashith Kekuda TR[1], Vivek MN[1], Yashoda Kambar[1], Manasa M[1*], Raghavendra HL[2]

[1]Post Graduate Department of Studies and Research in Microbiology, Sahyadri Science College (Autonomous), Kuvempu University, Shivamogga-577203, Karnataka, India

[2]College of Medical and Health Sciences, Wollega University, Post Box No: 395, Nekemte, Ethiopia

Abstract

The present study was conducted with an aim of determining antimicrobial activity of *Vitex negundo* var. *negundo* (Vnvn) and *Vitex negundo* var. *purpurascens* (Vnvp).The powdered leaf materials of both varieties were extracted using methanol in soxhlet assembly. The content of total phenolics and flavonoids were estimated by Folin-Ciocalteau reagent and Aluminium chloride colorimetric estimation method respectively. Antibacterial activity of extracts was determined against five drug resistant urinary tract pathogens by agar well diffusion assay. Poisoned food technique was performed to determine antifungal effect of extracts. The extracts caused concentration dependent inhibition of urinary tract isolates. Marked antibacterial effect was shown by extract of Vnvp. Among bacteria, *Staphylococcus aureus* and *Klebsiella pneumoniae* were inhibited to high and least extent respectively by extracts. The extracts were effective in inhibiting test fungi as revealed by reduction in mycelial growth in plates poisoned with extracts. Here also, high inhibitory activity was observed in case of extract of Vnvp. Among fungi, *Helminthosporium* sp., *Alternaria* sp., and *C. capsici* displayed similar susceptibility to both extracts at concentration 1mg/ml. *Aspergillus flavus* was inhibited to least extent by extracts. Phytoconstituents *viz*., tannins, alkaloids, flavonoids, saponins, steroids and glycosides were detected in extract of both Vnvn and Vnvp. The total phenolic and flavonoid contents were high in extract of Vnvp. The extracts were effective against bacteria and fungi. The presence of high phenolic and flavonoid content could be ascribed to the marked inhibitory activities of the extract of Vnvp.

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***Corresponding Author:**

Manasa M

E-mail:
mansisgr@gmail.com

INTRODUCTION

The genus *Vitex* (family Verbenaceae) comprises of large shrubs or small trees distributed throughout the world. *Vitex negundo* Linn. is commonly distributed on roadsides and the banks of streams. It is called Lakki in Kannada. It is a large, silvery-tomentose shrub or small tree with bluish purple flowers in terminal panicles with short cymose branches. The plant is used in Ayurveda and is traditionally used as medicine in many part of the world. The leaves are considered as tonic, vermifuge and are given along with long pepper in caterrhal fever (Vishwanathan and Basavaraju, 2010; Kekuda *et al*., 2013; Rani and Sharma, 2013). It has been experimentally shown that *V. negundo* possess a wide range of biological activities such as antimalarial (Nguyen-Pouplin *et al*., 2007), anthelmintic (Merekar *et al*., 2011), wound healing (Roosewelt *et al*., 2011), antipyretic (RaamaMurthy *et al*., 2010), anti-inflammatory (Dharmasiri *et al*., 2003), analgesic (Dharmasiri *et al*., 2003), antioxidant (Raghavendra *et al*., 2010; Kekuda *et al*., 2013), antimicrobial (Sharma *et al*., 2011; Kekuda *et al*., 2013), hepatoprotective (Tendon *et al*., 2008), anti-microfilarial (Sahare *et al*., 2008), mosquito repellant (Hebbalkar *et al*., 1992), cytotoxic (Kekuda *et al*., 2013), anxiolytic (Adnaik *et al*., 2009), Snake venom neutralizing (Alami and Gomes, 2003), antiandrogenic (Bhargava, 1989), immunostimulatory (Singh *et al*., 2005) and CNS depressant activity (Gupta *et al*., 1999).The two varieties in *V. negundo* are *V. negundo* var. *negundo* and *V. negundo* var. *purpurascens*. In *V. negundo* var. *negundo* (locally called bili lakki), the lower surface of the leaflets is grey-pubescent and style is white. In case of *V. negundo* var. *purpurascens* (locally called kari lakki), the lower surface is purple in color. It also differs from *V. negundo* var. *negundo* in having deep purple corolla and purple stamina filaments and style (Manilal and Sivarajan, 1982). It has been reported that the extract of *V. negundo* var. *purpurascens* exhibit marked antibacterial, cytotoxic and antioxidant activity when compared *V. negundo* var. *negundo*. A correlation was observed between the observed activity and higher content of total phenolics and total flavonoids in *V. negundo* var. *purpurascens* (Kekuda *et al*., 2013). In the present study, we evaluated antibacterial and antifungal effect of extract of these two varieties of *V. negundo* against drug resistant urinary

tract pathogens and fungi (from sorghum seeds and chilli).

MATERIALS AND METHODS

Collection and Identification of Plant Materials

The plant materials of *V. negundo* var. *negundo* (Vnvn) and *V. negundo* var. *purpurascens* (Vnvp) were collected in the month of December 2013 at a village called Sulakodu, Shivamogga (District), Karnataka and authenticated by Dr. K.S. Vinayaka, Department of Botany, Kumadvathi First Grade College, Shimoga Road, Shikaripura, Karnataka. The voucher specimens were deposited in the department herbaria for future reference.

Extraction

The leaves were separated, washed well to remove any extraneous matter, dried under shade and powdered using a blender. About 25g of each leaf material was extracted using methanol in a Soxhlet apparatus. The methanol extracts were filtered through Whatman No. 1 and concentrated in vacuum under reduced pressure and dried in the desiccator (Kekuda *et al*., 2013).

Phytochemical Analysis

Extract of Vnvn and Vnvp were subjected to phytochemical screening in order to identify phytoconstituents *viz*., alkaloids, flavonoids, terpenoids, tannins, saponins, glycosides and sterols(Kekuda *et al*., 2012).

Estimation of Total Phenolic Content (TPC)

The TPC of extract of Vnvn and Vnvp was estimated by Folin-Ciocalteau reagent (FCR) method. A dilute concentration of extract (0.5 ml) was mixed with 0.5 ml of FC reagent (1:1) and 4ml of sodium carbonate (1M) and left for 15 minutes. The absorbance was measured at 765nm in a UV-Vis spectrophotometer (Elco-SL159). A standard curve was plotted using different concentrations of Gallic acid (reference standard, 0-1000 µg/ml) and the TPC of extracts was expressed as µg Gallic acid equivalents (GAE) from the graph (Junaid *et al*., 2013).

Estimation of Total Flavonoid Content (TFC)

Aluminium chloride colorimetric method was followed to estimate total flavonoid content (TFC). A dilute concentration of extract (0.5ml) was mixed with 0.5ml of methanol, 4ml of water, 0.3ml of $NaNO_2$ (5%) and incubated at room temperature for 5 minutes. 0.3ml of $AlCl_3$ (10%) was added to each tube and incubated at room temperature for 6 minutes. 2ml of NaOH (1M) and 2.4ml of distilled water were added and the absorbance was measured against blank (without extract) at 510nm using UV-Vis spectrophotometer (Elco-SL159). A calibration curve was constructed using different concentrations of Catechin (reference standard, 0-120 µg/ml) and the TFC of extracts was expressed as µg Catechin equivalents (CE) from the graph (Kekuda *et al*., 2013).

Antibacterial activity of extracts of Vnvn and Vnvp

A total of 5 isolates from urinary tract *viz*., *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* were tested for their susceptibility to extract of Vnvn and Vnvp. These clinical isolates were multidrug resistant (Manasa *et al*., 2013a). Agar well diffusion assay was employed to determine antibacterial activity of extracts. The clinical isolates were seeded into sterile Nutrient broth (HiMedia, Mumbai) tubes and incubated for 24 hours at 37^{o}C. The broth cultures thus obtained were then swabbed aseptically on sterile Nutrient agar (HiMedia, Mumbai) plates using sterile cotton swabs. Wells (8mm diameter) were punched in the plates using sterile cork borer. 100µl of leaf extracts (10 and 20mg/ml of 25% dimethyl sulfoxide [DMSO]), standard antibiotic (Chloramphenicol, 1mg/ml) and DMSO (25%, in sterile water) were transferred into labeled wells. The plates were incubated for 24 hours at 37^{0}C and the zone of inhibition was measured (Kekuda *et al*., 2013).

Antifungal Activity of Extract of Vnvn and Vnvp

Four fungi were tested for their susceptibility to extracts. *Colletotrichum capsici* was isolated in our previous study from anthracnose of chilli (Kambar *et al*., 2013). Fungi *viz*., *Aspergillus flavus*, *Helminthosporium* sp., *Alternaria* sp. were isolated from sorghum seeds by standard blotter technique (Panchal and Dhale, 2011). The fungi were identified on the basis of cultural and microscopic characteristics (Barnett and Hunter, 1998). The antifungal effect of extract of Vnvn and Vnvp was evaluated by Poisoned food technique (Kambar *et al*., 2013). Potato dextrose agar (PDA) was poisoned with leaf extracts (0.5 and 1%), sterilized by autoclaving, poured into sterile petri plates and allowed to solidify. The spore suspension of test fungi were inoculated by point inoculation on poisoned PDA plates and incubated for 5days at 28^{o}C. Colony diameters in mutual perpendicular directions were measured on 5th day. Antifungal effect of leaf extracts was recorded in terms of inhibition of mycelial growth (%) and calculated using the formula:

Inhibition of mycelia growth (%) = ([C-T]/C) x 100,

where C is average diameter of colony in control plates and T is average diameter of colony in poisoned plates.

Statistical Analysis

The experiment was conducted in triplicate. Results are represented as Mean±Standard deviation (SD).

RESULTS

Preliminary phytochemical analysis showed the presence of tannins, alkaloids, flavonoids, saponins, steroids and glycosides in both Vnvn and Vnvp. The content of both phenolics and flavonoid contents were high in extract of Vnvp (Table 1).

Table 1: Content of total phenolics and flavonoids in the extract of Vnvn and Vnvp.

Extract	TPC (µg GAE/mg)	TFC (µg CE/mg)
Vnvn	241.68±0.5	22.15±0.2
Vnvp	265.13±0.1	26.33±0.1

The result of antibacterial activity of extract of Vnvn and Vnvp is shown in Table 2 and Figure 1. The extracts displayed concentration dependent inhibition and were found inhibitory against all isolates at concentration 20mg/ml. Among extracts, marked antibacterial effect was shown by extract of Vnvp. Among bacteria, *S. aureus* and *K. pneumoniae* were inhibited to high and least extent respectively. Both the extracts were not inhibitory against *K. pneumoniae* at extract concentration of 10mg/ml.

Table 2: Inhibitory activity of extract of Vnvn and Vnvp against clinical isolates.

Test Bacteria	Zone of inhibition in cm (Mean±SD)					
	Vnvn		Vnvp		Standard	DMSO
	10mg/ml	20mg/ml	10mg/ml	20mg/ml		
S. aureus	1.5±0.1	1.8±0.1	1.6±0.1	1.9±0.2	3.1±0.1	0.0±0.0
E. faecalis	1.2±0.0	1.5±0.1	1.3±0.1	1.6±0.1	2.8±0.0	0.0±0.0
P. aeruginosa	1.0±0.0	1.4±0.1	1.0±0.0	1.4±0.1	2.3±0.1	0.0±0.0
K. pneumoniae	0.0±0.0	1.0±0.0	0.0±0.0	1.0±0.0	2.1±0.2	0.0±0.0
E. coli	0.8±0.0	1.4±0.1	1.0±0.0	1.5±0.1	2.3±0.1	0.0±0.0

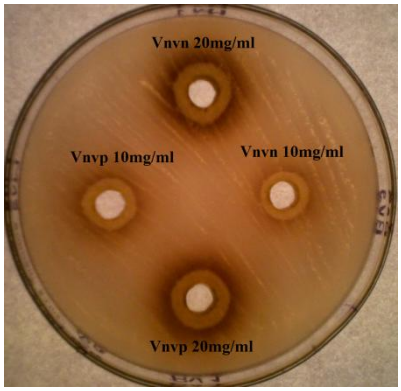

Figure 1: Inhibition of *E. faecalis* by extract of Vnvn and Vnvp.

Table 3 reveals the antifungal effect of extract of Vnvn and Vnvp in terms of reduction in the colony diameter of test fungi in poisoned plates when compared to control plates. The extent of inhibition of test fungi (%) is depicted in Figure2. Both extracts were found to exhibit inhibition of mycelial growth of test fungi and the inhibitory effect was concentration dependent. Among extracts, high inhibitory activity was observed in case of extract of Vnvp when compared with extract of Vnvn. Among fungi, *Helminthosporium* sp., *Alternaria* sp., and *C. capsici* were inhibited to more or less similar extent by both the extracts at concentration 1mg/ml. *A. flavus* was least inhibited by leaf extracts.

Table 3: Colony diameter of test fungi in control and poisoned plates.

Test fungi	Colony diameter in cm (Mean±SD)				
	Control	Vnvn		Vnvp	
		0.5%	1%	0.5%	1%
A. flavus	3.1±0.2	2.6±0.1	2.4±0.0	2.6±0.0	2.2±0.1
Helminthosporium sp.	4.4±0.2	3.6±0.2	3.1±0.1	2.9±0.0	2.6±0.1
Alternaria sp.	2.3±0.1	1.9±0.1	1.6±0.1	1.8±0.1	1.4±0.0
C. capsici	2.5±0.1	2.1±0.0	1.8±0.1	1.8±0.0	1.5±0.1

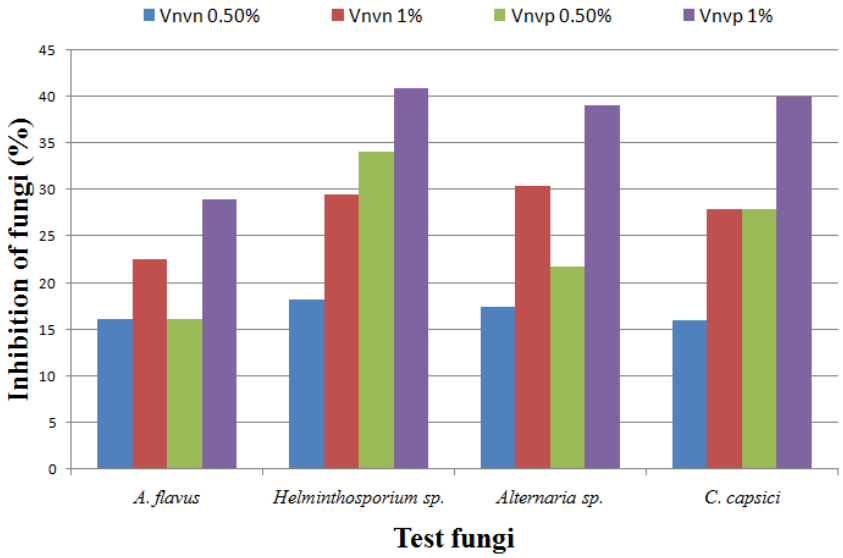

Figure 2: Inhibition of test fungi (%) by extract of Vnvn and Vnvp.

DISCUSSION

Urinary tract infections are one of the most common bacterial infections in community and in hospital settings. UTIs affect individuals of all ages of both sexes. The prevalence of UTIs are higher in females than in males. The bacteriology of UTIs may represent a single species or it may be polymicrobial. The most common bacteria causing UTIs are *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis*, *Enterobacter* species, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Among these, *E. coli* is the most common pathogen isolated from majority of UTIs including pediatric cases. Antibiotics are commonly prescribed in the treatment of UTIs. However, overuse and abuse of these antibiotics resulted in emergence of antibiotic resistant uropathogens. The high resistance rates to most commonly used antimicrobials are of special consideration (Hryniewicz *et al*., 2001; Kurtaran *et al*., 2010; Edlin *et al*., 2013; Manasa *et al*., 2013a). Plants have been exploited for medicinal purposes from ancient time all over the world. Plant extracts and the purified components of plants are shown to possess antimicrobial activity. Many plant species have been found to possess inhibitory activity against several uropathogens (Cowan, 1999; Peneira *et al*., 2004; Sahoo *et al*., 2008; Sharma *et al*., 2009; Dulger and Dulger, 2012; Manasa *et al*., 2013a).

It has been found that crude extract and components of *V. negundo* possess antibacterial activity. Supercritical fluid extract (Nagarsekar *et al*., 2010), essential oil (Singh *et al*., 2010) of leaves and flavonoid extract of leaves and seeds (Sharma *et al*., 2011) were shown to possess antibacterial activity. Leaf extract was found to inhibit clinical isolates recovered from HIV patients (Bharathi *et al*., 2011). Ethanol extract of leaves were shown to possess inhibitory activity against clinical pathogens (Renisheya *et al*., 2011). The ethanol extract of leaves were found to be inhibitory against antibiotic resistant and sensitive clinical isolates such as *S. aureus* and *E. faecalis* (Dubey and Padhy, 2012). Leaf, stem, root and flower extract exhibited antibacterial activity (Gautam and Kumar, 2012). In the present study, we determined antimicrobial activity of extract of Vnvn and Vnvp. The extract of Vnvp displayed marked inhibitory activity against bacteria and fungi tested. It was observed that Gram positive clinical isolates were found to be more susceptible to both the extracts. Similar result was obtained in an earlier study of Kekuda *et al*. (2013) where extract of Vnvp showed higher inhibition of Gram positive bacteria than Gram negative bacteria. The low susceptibility of Gram negative bacteria to extracts of Vnvn and Vnvp could be ascribed to their cell wall structure. Gram negative bacteria possess an outer membrane which forms an additional barrier for the entry of substances into the cells (Lodhia *et al*., 2009; Nalubega *et al*., 2011).

Plants suffer from a large number of diseases caused by bacteria, fungi, viruses and parasites. Among the pathogenic microorganisms, fungi are dominant. The fungal diseases results in death of plants as well as drastic reduction in the yield. These fungi, not only cause diseases in plants in fields, but also cause spoilage after harvest i.e., during storage (post harvest spoilage). The species of fungal genera such as *Alternaria*, *Curvularia*, *Helminthosporium*, *Drechslera*, *Fusarium*, *Pythium* etc., infect plants in fields. Fungi such as species of *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor* etc., cause deterioration of stored commodities. Besides, these fungi also produce toxins such as aflatoxins and others which produce various symptoms on consumption (Amadi and Adeniyi, 2009; Keller *et al*., 2012; Joshaghani *et al*., 2013; Suleiman and Omafe, 2013). Fungicides have been extensively used for control of plant pathogenic fungi. The extensive use of synthetic agents poses several ill effects such as fungicide residues in food commodities, environmental pollution and development of resistance in pathogenic fungi. Hence, search of eco-friendly methods for disease control is highly desirable. The use of microbial antagonists, plant based formulations etc., are among the best and alternate strategies for the control of plant pathogens (Mohana and Raveesha, 2007; Syed *et al*., 2012; Manasa *et al*., 2013b; Vivek *et al*., 2013; Rakesh *et al*., 2013). In the present study, the extract of Vnvn and Vnvp were shown to possess inhibitory activity against fungi isolated from chilli fruit and sorghum seeds. Similar to antibacterial activity, extract of Vnvp caused higher inhibition of test fungi than extract of Vnvn. It has been shown experimentally that *V. negundo* possess antifungal activity. The fruit extract was found to exhibit antifungal activity against *Fusarium solani* and *Microsporum canis* (Mahmud *et al*., 2009). The leaf and seed extract were found to exhibit inhibitory activity against *Candida albicans* and *Trichoderma viridae* (Sharma *et al*., 2011). Leaf extract was shown to cause dose dependent inhibition of *C. albicans* isolated from HIV subject (Bharathi *et al*., 2011). Extracts from different parts *viz*., leaf, stem, root and flower were shown to exhibit inhibitory activity against *A. flavus* and *C. albicans* (Gautam and Kumar, 2012).

CONCLUSION

The present study compared the efficacy of extract of Vnvn and Vnvp against antibiotic resistant urinary tract isolates and fungi from seeds and chilli. Extract of Vnvp exhibited marked inhibition of bacteria and fungi. The presence of high phenolic and flavonoid content could be ascribed to the marked inhibitory activities of the extract of Vnvp. These plants can be used against drug resistant uropathogens and field as well as storage fungi.

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