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EVALUATION OF PHYTOCHEMICAL AND ANTIBACTERIAL EFFECT OF *V.NEGUNDO* AGAINST FOOT INFECTED CLINICAL PATHOGENS

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ABSTRACT

The aim of the present study was to evaluate the antibacterial effect of leaves of *Vitex negundo* against wound infecting pathogens. 75 patients satisfying criteria of diabetic foot infection randomly Selected and divided in to three age group (A,B,C). Clinical isolates were isolated from foot infection and identified based on biochemical characters. Their sensitivity pattern against *V.negundo* extract was performed by stroke method and the active fraction was identified by TLC. among the three groups, group B between 40-40 years showed maximum prevalence of gram positive *S.aureus* infection with 57.6 % methicilin resistant. Nearly 106 colonies were isolated and belongs to eight different genera namely *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Proteus mirabilis, Enterococcus faecalis, Enterobacter* sp, *Acinetobacter sp, Pseudomonas aeruginosa*. All these isolated strains were highly sensitive to hexane extract of *V.negundo* and the active fraction nature was identified as flavanoid.

KEYWORDS: *V.negundo*, Foot infection, Methicilin, Flavanoid, *S.aureus.*

1. INTRODUCTION

Diabetic foot infections (DFIs) typically begin in a wound, most often a neuropathic ulceration, while all wounds are colonized with microorganisms.[1] DFI is polymicrobial in nature. Earlier studies[2] have found *Staphylococcus aureus* as the main causative pathogen but recent investigation reported a predominance of Gram negative aerobes.[3] Infectious diseases and Antibiotic resistance of infectious agents has become a global concern.[4] The

increasing failure of chemotherapeutics coupled with antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of noel compounds for potential antimicrobial activity.[5] There is an urgent need to explore and discover new antimicrobial compounds with diverse chemical structures and novel mechanism of action for new and reemerging infectious diseases.[6] *Vitex negundo* (verbenaceae) commonly known as Nirkundi or Nallanocci. It is an aromatic large shrub or small tree about 3m in height and almost found throughout India. Different parts of Vitex negundo Linn., have been used in traditional Indian medicines as nervine sedative are of high value as constituents of ayurvedic preparations such as Vishagarbha thaila is widely used to treat rheumatism in India.[7] The essential oil of vitex negundo leaves showed significant antifungal activity against *Trichloroderma viride*, Fusarium Sp. *Collectrotrichum* and *Helminthosporium.*[8] Water extract of mature fresh leaves exhibited anti-inflammatory, analgesic and antihistamine properties.[9] Few studies have also been done on antimicrobial activity of *V. negundo* along with some other Indian medicinal plants. The aim of present study is to evaluate the antimicrobial activity of *Vitex negundo* L., extracts in order to use it as novel antimicrobial substances against important human pathogens.

MATERIALS AND METHOD

Sample Collection

The foot ulcer samples were collected in a sterile container from patients who had Type 2 diabetes and subjected to microbiological analyses. Sample collection (pus, wound exudates) had been undertaken from OPD of trichy local hospital, Tamilnadu, India.

Isolation and Identification of Pathogens

The swabs of samples were spread over the Nutrient agar and MacConkey agar. Then the plates were incubated for 24–48 hours at 37° C. Selective isolation was done by streaking the sample on Blood agar, Pseudomons differentiating agar, EMB agar, and Chrom agar plates. All the isolated colonies were identified using Gram staining, and Biochemical studies

Processing of Plants for Extract Preparation.[10]

About 50gm of dry sample powder was weighed and macerated with 500 ml of each solvent (Hexane, Ethyl acetate and Methanol) separately and kept overnight in shaker. The extract was collected after filtration using Whatman No.1 filter paper and evaporated below 40°C, which was used for further phytochemical analyses.

Phytochemical Analysis

Test for Carbohydrates: To 2ml of plant extract, 1ml of Molisch's reagent and few drops of concentrated sulphuric acid were added. Presence of purple or reddish color indicates the presence of carbohydrates.

Test for Tannins: To 1ml of plant extract, 2ml of 5% ferric chloride was added. Formation of dark blue or greenish black indicates the presence of tannins.

Test for Saponins: To 2ml of plant extract, 2ml of distilled water was added and shaken in a graduated cylinder for 15minutes lengthwise. Formation of 1cm layer of foam indicates the presence of saponins.

Test for Flavonoids: To 2ml of plant extract, 1ml of 2N sodium hydroxide was added. Presence of yellow color indicates the presence of flavonoids.

Test for Alkaloids: To 2ml of plant extract, 2ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.

Test for Quinones: To 1ml of extract, 1ml of concentrated sulphuric acid was added. Formation of red color indicates presence of quinones.

Test for Glycosides: To 2ml of plant extract, 3ml of choloroform and 10% ammonia solution was added. Formation of pink color indicates presence of glycosides.

Test for Cardiac Glycosides: To 0.5ml of extract, 2ml of glacial acetic acid and few drops of 5% ferric chloride were added. This was under layered with 1 ml of concentrated sulphuric acid. Formation of brown ring at the interface indicates presence of cardiac glycosides.

Test for Terpenoids: To 0.5ml of extract, 2ml of chloroform was added and concentrated sulphuric acid was added carefully. Formation of red brown color at the interface indicates presence of terpenoids.

Test for Triterpenoids: To 1.5ml of extract, 1ml of Libemann –Buchard Reagent (aectic anhydride+concentrated sulphuric acid) was added. Formation of blue green color indicates presence of triterpenoids.

Test for Phenols: To 1ml of the extract, 2ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green color indicates presence of phenols.
Test for Coumarins: To 1 ml of extract, 1ml of 10% NaOH was added. Formation of yellow color indicates presence of coumarins.

Steroids and Phytosteroids: To 1ml of plant extract equal volume of chloroform is added and subjected with few drops of concentrated sulphuric acid appearance of brown ring indicates the presence of steroids and appearance of bluish brown ring indicates the presence of phytosteroids.

Phlobatannins: To 1ml of plant extract few drops of 2% HCL was added appearance of red color precipitate indicates the presence of phlobatannins.

Anthraquinone: To 1ml of plant extract few drops of 10% ammonia solution was added, appearance pink color precipitate indicates the presence of anthraquinones.

Antibacterial Activity of *V.Negundo* Against Clinical Pathogens[11]

Well dried finely powdered root and leaf extracts of were concentrated by evaporation. The crude extracts were assayed against clinical pathogens isolated from wound sample by stroke method. 100 µl of *V.negundo* extracts (1 mg/ml) were loaded on sterile disc and were tested against test pathogens. The test pathogens were swabbed on Mueller Hinton agar plates and the sample disc were placed on the center of plates along with a positive control amoxicillin-clavulanic acid and a negative control Ethyl acetate. All the plates were replicated three times and the zone of inhibition of calculated by the mean values.

Bio Assay of TLC Fractions

The TLC plates were prepared and developed with methanol: chloroform (19:1) as the developing solvent. Two sets of TLC plates were prepared One set was used as the reference chromatogram for flavanoid detection and other set was used for Bioautography. 24 h freshly grown bacterial culture were swabbed over Mueller Hinton Agar plates. The TLC bioautographic plate was kept over the surface of inoculated plates and was incubated at 37^{o}C for 24 hours. After incubation period MTT 3-4-5- Dimethyl Thiazole 2 yl -2, 5 diphenyl tetrasolium bromide solution was sprayed over the slide at a concentration of 1 mg/ml.

RESULT AND DISCUSSION

Frequency of Isolated Pathogens from Wound Sample

Of the total 75 samples of diabetic foot patients studied 60 % were males and 40 % were females. The data in Table 1 shows the profile of the pathogens isolated and most frequently isolated at the age of 40-50. A total of 106 pathogens were isolated with an average of 1.85 organisms per patient. The frequency of these isolates were 35.5>13.5>10.5>9>8.2>6>5>3 (Table 1). Among the isolates, *S.aureus* (35.5 %) was most frequently isolated followed by *E.coli* (13.5 %). Less frequently isolated pathogens were identified as *Acinetobacter sp*. The maximum prevalence of Gram positive was found in age of 40–50. Similarly sample collected between 50 and 60 showed maximum prevalence of Gram negative bacteria. Of these 59 *S.aureus* 34 (57.6%) were found to be methicillin resistant *Staphylococcus aureus.* This finding agrees with the study carried out by Pittet et al.[12] The predominance of *S. aureus* is in agreement with the results also reported in most of the studies.[13] This difference could be explained by different types of infections as most mild infections are caused by aerobic Gram-positive cocci such as *S. aureus* and streptococci. Deeper, limb threatening infections are usually polymicrobial and caused by aerobic Gram-positive cocci, Gram-negative bacilli like *Escherichia coli*, *Klebsiella* species, and *Proteus* species.[14]

Phytochemical Analysis of *V.negundo*

The phytochemical analysis of different extract of *V.negundo* was summarized in table 2. Saponins, isoquinoline alkaloids, tropane alkaloids, reducing sugars, and lipids were present in all three extracts of *V.negundo*. Tannins, glycosides and phenols were reported only in methanolic extracts of *V.negundo*. Flavonoids were present in two extracts and found to be absent in chloroform extract. Steroids, terpenoids, phytosterols and triterpenoids were reported only in the chloroform extract. Test for phlobatannins answered negative for all the extracts of *V.negundo*. Most of these phytochemical constituents are potent bioactive compounds found in medicinal plant parts, which are precursors for the synthesis of useful drugs.[15]

Antimicrobial Study of *V.Negundo* Extracts

In the present experiment an attempt has been made to evaluate the antibacterial activity of different extracts against ten different clinical isolates. The ethyl acetate Hexane and methanolic crude extracts of the aerial part of *V.negundo* were screened against ten human pathogenic bacteria to check antibacterial activity. Of these three extracts hexane extract were

found to be effective and its Maximum zone of inhibition was 20.±1.32 mm against MRSA followed by 18±1.53 mm against *E.coli*. All values are expressed as means ± standard deviation and the results were presented in Table 3. All the test pathogens were highly sensitive to ethyl acetate extract and few were moderately sensitive to ethyl acetate extract. The bioautography of TLC fraction reveals that fraction with 0.38 R_f value showed positive antimicrobial activity against MRSA and it was detected as flavonoid in nature. Hexane is a suitable solvent for the extraction of Flavonoids. Previously, flavonoids have been reported to possess antimicrobial activity.[16] It has been reported that the antimicrobial activity of flavonoids is due to their ability to complex with extracellular and soluble protein and to complex with fungal cell.[17] Thin layer chromatography (TLC) was employed as a preliminary phytochemical screening technique by bioassay guided fractionation to detect active components.[18] Bioautography is a very convenient and simple test for plant extracts and pure substances, to assess their effects on both human and plant pathogenic microorganisms.[19]

Table 1. Isolation of bacteria from diabetic foot infections.

S. No	Bacteria	*N* (%)	Age Group		
			A(30-40)	B(40-50)	C(50-60)
1	*Staphylococcus aureus*	59 (35.5)	13	35	11
2	*Escherichia coli*	18 (13.5)	3	6	9
3	*Proteus vulgaris*	14 (10.5)	4	5	5
4	*Pseudomonas aeruginosa*	12 (9)	3	4	5
5	*Proteus mirabilis*	11 (8.27)	-	4	7
6	*Enterococcus faecalis*	8 (6)	2	-	6
7	*Enterobacter* sp.	7 (5.2)	1	2	4
8	*Acinetobacter sp*	4 (3)	-	-	4
	Total	133			

Table 2. Phytochemical analysis of *V.negundo*.

Phytochemical test	Inference		
	Hexane	Ethyl acetate	Methanol
Carbohydrates Molisch's test Fehling's test Iodine test	 + + -	 - + -	 - + -
Tannins test	-	-	+
Saponin test	-	+	+
Flavonoid test	-	+	+
Alkaloid test Wagner's Dragendorff's	 + -	 - -	 + -
Quinones	++	+	+
Glycosides test	-	-	+
Cardiac glycosides test	-	-	-
Terpenoids test	+	+	+
Triterpenoids	+	+	-
Phenols	-	-	+
Coumarins	-	+	+
Proteins	-	-	-
Steroids and Phytosteroids	+	+	-
Phlobatannins	-	-	-
Anthraquinones	-	-	-

Table 3. Antibacterial effect of extract of *V.negundo* against isolated test pathogens.

S. No	Pathogen	Zone of inhibition (mm in diameter) (M±SD)		
		ethyl acetate	hexane	Methanol
1	*S.aureus*	10.33±1.53	15±1.50	-
2	*E. coli*	11.33±1.69	18±1.53	11.33±1.53
3	MRSA	15.50±0.50	20.±1.32	-
4	*Acinetobacter sp*	15.00±1.00	15±1.50	13.67±1.53
5	*Pseudomonas aeruginosa*	-	16±1.50	-
6	*Enterobacter* Spp.	13.00±1.00	16±1.76	12.67±1.20
7	*Proteus mirabilis*	-	15±1.50	-
8	*Proteus vulgaris*	-	15±1.20	-
9	*Enterococcus Spp.*	-	16±1.23	-

CONCLUSION

On the basis of above study it is concluded that gram *S.aureus* is a most prevalent pathogen in foot infection and it was found that *V.negundo* flavanoids possess potent antibacterial activity against wound infecting pathogens.

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