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ANTIMICROBIAL ACTIVITY OF LEAVES OF *VITEX NEGUNDO* AGAINST PATHOGENIC ORGANISMS COMPARED WITH CONTROL DRUG

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ABSTRACT

The crude extract from the leaves of Vitex negundo traditionally used in Indian system of medicines were screened against Escerichia coli NCIM 2931, Salmonella typhi MTCC 734, Salmonella typhimurium MTCC 98, Klebsiella pneumoniae MTCC432, Proteus vulgaris NCIM2857, Proteus mirabilis MTCC425, Pseudomonas aeruginosa NCIM5029, Staphylococcus aureus MTCC 96, Staphylococcus epidermis MTCC 435, Bacillus cereus NCIM2155, Bacillus subtilis NCIM 2063 and Bacillus megaterium NCIM 2087 by using agar well diffusion method. Vitex negundo crude extract showed significant activity against organisms. Zone of inhibition of the extract compared with the standard antibiotics.

KEYWORDS: Solvent extracts, Antibacterial activity, Agar well diffusion method.

INTRODUCTION

Plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines (Stuffness and Douros, 1982). Higher plants as sources of medicinal compounds have continued to play a dominant role in the maintenance of human health care since ancient times. Over 50% of all modern clinical drugs are of natural product origin and natural product play a vital role in modern drug development in the pharmaceutical industry (Baker *et al.*, 1995). Plants with possible antimicrobial activity should be tested against an appropriate microbial model to confirm the activity and to ascertain the parameters associated with it. The effects of plant extract on bacteria have been studied by a very large number of researches in different parts of the world (Ates and Erdogrul, 2003). Much work has been done on ethnomedicinal plants in India (Negi *et al.*, 1993). Interest in a large number of traditional natural products has increased (Taylor *et al.*, 1996). It has been suggested that aqueous and Ethanolic extract from plants used in allopathic medicine are potential sources of antiviral, Anti tumural and antimicrobial agents (Chung *et al.*, 1995). The selection of the crude plants extract for screening programmes has the potential of being more successful in initial steps than the screening of pure compounds isolated from natural products (Kusumoto *et al.*, 1995). Here is continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of actions because there has been alarming increase in the incidence of new and re-emerging infectious diseases.

MATERIALS AND METHODS

Selection of medicinal plant for this study
Vitex negundo
Family: Lamiaceae
Parts used: Leaf

Traditional uses: *Vitex negundo* relieves muscle aches and joint pains. The Ayurvedic and Unani Pharmacopoeia of India has documented the use of the leaf, seed and the root to treat excessive vaginal discharge, edema, skin diseases, pruritus, helminthiasis, rheumatism and puerperal fever (Jabeen et al., 2015).

Chemical constituents: Leaves contain Hydroxy-3, 6, 7, 3, 4- pentamethoxyflavone (Banerji, 1969); hydroxybenzoyl mussaenosidic acid (Sehgal,1982,1983); trimethoxyflavanone; (Achari, 1984); viridiflorol; β-caryophyllene; sabinene; 4-terpineol; gamma-terpinene; caryophyllene oxide; 1-oceten-3- ol; globulol (Singh,V. 1999); betulinic acid; ursolic acid; n-hentriacontanol; β-sitosterol; p-hydroxybenzoic acid (Chandramu,2003) protocatechuic acid; oleanolic acid; flavonoids (Surveswaran,2007) angusid; casticin; vitamin-C; nishindine; gluco-nonitol; phydroxybenzoic acid; sitosterol (Khare,2004). The seeds contain 3β -acetoxyolean-12-en-27-oic acid; 2α, 3α-dihydroxyoleana-5,12-dien- 28-oic acid; 2β,3α diacetoxyoleana-5,12-dien-28-oic acid; 2α, 3β-diacetoxy-18- hydroxyoleana-5,12-dien-28-oic acid (Chawla,1992) vitedoin-A; vitedoin-B; a phenylnaphthalene-type lignan alkaloid, vitedoamine-A; five other lignan derivatives (Ono, 2004); 6-hydroxy-4-

(4-hydroxy-3- methoxy-phenyl)-3-hydroxymethyl-7-methoxy-3, 4- dihydro-2-naphthaldehyde; β-sitosterol; p-hydroxybenzoic acid; 5-oxyisophthalic acid; ntritriacontane, n-hentriacontane; n-pentatriacontane; n-nonacosane (Khare,2004; Vishwanathan & Basavaraju, 2010). Roots contain 2β, 3α-diacetoxyoleana-5,12-dien-28-oic acid; 2α,3α-dihydroxyoleana-5,12- dien-28-oic acid; 2α,3β -diacetoxy-18-hydroxyoleana-5,12-dien-28-oic acid; vitexin and isovitexin (Srinivas,2001); negundin-A; negundin-B; (+)-diasyringaresinol; (+)-lyoniresinol; vitrofolal-E and vitrofolal-F (Azhar-Ul, 2004); acetyl oleanolic acid; sitosterol; 3-formyl-4.5-dimethyl-8- oxo-5H-6,7-dihydronaphtho (2,3-b)furan (Vishnoi,1983). Essential oil of *Vitex negundo* contains δ-guaiene; guaia-3,7-dienecaryophyllene epoxide; ethyl-hexadecenoate; α-selinene; germacren-4-ol; caryophyllene epoxide; (E)-nerolidol; β- selinene; α-cedrene; germacrene D; hexadecanoic acid; p-cymene and valencene. (Khokra, 2008).

Identification and Preservation of Plant materials

Fresh plant leaves were collected from the Nagpur area of India. The taxonomic identities of this plant was determined by the expertise of the Post Graduate Department of Botany of Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur. Specimen was labeled, numbered and noted with date of collection, the locally and their medicinal uses and their approximate dosages of administration were recorded. Plant leaves were washed with 70% alcohol and then rinsed with sterilized distilled water, air dried and stored in airtight bottles at 4^0C for further use.

Preparation of crude extract (Fresh juice)

Vitex negundo plant leaves were collected from around Nagpur region in the month of August-September. Leaves were cleaned under running potable water and cut into pieces and grounded in pestle and mortar (made up of dolerite stone) till homogenized mass was obtained. Homogenized mass was squeezed in 400 mesh nylon cloth (pore size 37 micron) to obtain crude extract. Crude extract was kept in sterilized glass bottle. All crude extract were prepared fresh and used before 2 hours.

Crude Extraction

Aqueous extraction: Ten grams of dried powder was extracted in 100 ml distilled water for 6h, at slow heat. Every 2h, it was filtered through 8 layers of muslin cloth and centrifuged at 5000RPM for 15 min. The supernatant was collected. This process was repeated twice and after 6h, the supernatant was concentrated to make the final volume one-fourth of the original volume (Shahidi Bonjar G.H. 2004). It was then autoclaved at 121^0C and 15 lbs pressure and then stored at 4^0C.

Solvent extraction: Ten grams of dried powder was extracted with 100 ml of each solvent (acetone, chloroform, methanol and petroleum ether) and flasks were kept on a rotary shaker at 190-220 rpm for 24h. Thereafter, it was filtered through 8 layers of muslin cloth and centrifuged at 5000RPM for 15 min. The supernatant was collected and the solvent was evaporated to make the final volume one-fourth of the original volume (Shahidi Bonjar, G.H. 2004). It was stored at 4^0C in airtight bottles for further studies.

Bacterial cultures: The microbial strains are identified strains and were procured from the National Chemical Laboratory (NCL), Pune, India. The studied bacterial strains were *Bacillus cereus* NCIM2155, *Bacillus subtilis* NCIM2063, *Bacillus megaterium* NCIM2087, *Escherichia coli* NCIM2931, *Proteus vulgaris* NCIM2857 and *Pseudomonas aeruginosa* NCIM5029. *Staphylococcus aureus* MTCC96, *Staphylococcus epidermis* MTCC 435, *Salmonella typhi* MTCC 734, *Salmonella typhimurium* MTCC 98, *Klebsiella pneumoniae*, MTCC432, *Proteus mirabilis* MTCC425, these strains were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. They were sub-culured on nutrient agar for every 15 days and maintained on nutrient agar slants at 4^0C, fresh inoculums were taken for test.

Media

Hi -Sensitivity test broth (M 486) and Hi-sensitivity test agar (M 485) were procured from Hi-media Mumbai, India. The media were prepared according to the instructions given (Tumane P.M.et al.2000).

Screening for the antimicrobial potential of the plant leaves extracts

The antimicrobial activity of different solvent extracts was evaluated by agar well diffusion (Perez C, et.al. 1990 & Parekh, J, et al.2007) using Hi-sensitivity test agar (M 485).

Preparation of inoculum – A loop full of culture was inoculated from the stock slant culture in 5 ml of Hi-sensitivity test broth and broth was incubated at 35 ± 0.5^0C in incubator for 18-20 hours. After incubation a loop full of actively growing culture was inoculated into 10 ml of Hi-sensitivity broth. Broth was incubated at 35 ± 0.5^0C for 6-8 hours. This culture was used for the inoculation of Hi-sensitivity test agar plates.

Preparation of Hi-sensitivity test agar medium

Hi-sensitivity test agar medium was prepared as per instructions of manufacturer. Required amount of agar medium was melted and 25 ml of molten medium was distributed in test tubes (25x150 mm). Medium was autoclaved at 15 lb. for 20 min. After autoclaving, medium was maintained at $45\text{-}50^0$C in constant temperature water bath.

Inoculation of medium with test organism

0.5 ml of 6-8 hours old test organism is transferred to petridish of 100 mm size (Sterilized in oven at 180^0C for 1h.) using sterile micropipette. Hi-sensitivity test agar medium maintained at $45\text{-}50^0$C was poured and mixed

properly to ensure uniform distribution of organism with medium. Seeded plates are allowed to set at room temperature.

Preparation of agar well for fresh leaves juice

10 mm borer was used to prepare wells in agar. Four wells per plate at four equidistant corners were made. A 100 µl crude extract (fresh leaves juice) was transferred by micropipette per well. Plates were immediately kept at 4^0C in refrigerator for 1 hr. for the diffusion of extract and then shifted to 35 ± 0.5^0C in incubator. Zone of inhibition was measured after 24 hrs. of incubation by zone scale.

Preparation of agar wells for different solvent extracts

5 mm borer was used to prepare wells in agar. Four wells per plate at four equidistant cornrs were made.

A 50 ul solvent extract was transferred by micropipette per well. Plates were immediately kept at 4^0C in refrigerator for 1h. and then shifted to $35^0C\pm0.5^0$C in incubator. Zone of inhibition was measured after 24 hours. of incubation. For each bacterial strain, controls were maintained in which pure solvents were used instead of the extract. The control zones were subtracted from the test zones and the resulting zone diameter is obtained.

RESULTS AND DISCUSSION

Table 1: Results of antimicrobial activities of fresh leaves juice and solvent extracts of *Vitex negundo* leaves and compared with standard antibiotics.

Sr. No. Micro4organisms	Zone of inhibition in millimeter										
	Leaves extracts						Standard antibiotics				
	FJ	WE	AE	CE	ME	PE	Am^{30}	Cf^{30}	Co^{25}	G^{50}	T^{30}
1. *Escherichia coli*	26	---	---	---	---	---	32	29	24	17	22
2. *Salmonella typhi*	---	ND	ND	ND	ND	ND	38	28	25	18	19
3. *Salmonell typhimurium*	16	ND	ND	ND	ND	ND	32	22	24	17	17
4. *Klebsiella pneumoniae*	23	ND	ND	ND	ND	ND	15	16	19	15	12
5. *Proteus vulgaris*	---	---	---	---	11	---	---	23	31	20	24
6. *Proteus mirabilis*	---	ND	ND	ND	ND	ND	20	20	20	14	12
7. *Pseudomonas aureginosa*	---	---	---	---	---	---	14	36	---	34	22
8. *Staphylococcus aureus*	---	---	---	---	---	---	31	23	20	16	17
9. *Staphylococcus epidermis*	13	ND	ND	ND	ND	ND	36	27	15	26	21
10. *Bacillus cereus*	19	---	---	---	---	---	15	27	---	23	24
11. *Bacillus subtilis*	16	---	13	10	13	---	31	50	36	40	32
12. *Bacillus megaterium*	18	---	10	---	10	---	29	46	24	23	33

Key: FJ-Fresh juice of leaves; WE-Water extract; AE-Acetone extract; ME-Methanol extract; CE- Chloroform extract; PE-Petroleumether; Am^{30}-Amoxycillin; Cf^{30}-Ciprofloxacin; Co^{25}- Cotrimoxazole; G^{50}-Gentamicin; Tetracycline--T^{30} ND-Not determined; Negative.

Antibacterial activity of different solvent extracts of leaves of *Vitex negundo (VN*) zone of inhibition in millimetre (mm).

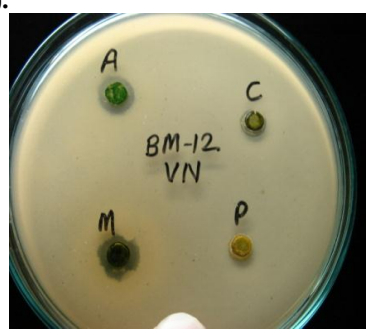

Figure- 1.
Activity against *Bacillus megaterium*
Acetone extract (A)--10 mm
Chloroform extract (C) ---
Methanol extract (M)--10 mm
Petroleum ether extract (P) ---

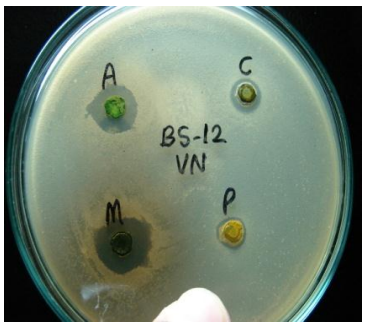

Figure-2.
Activity against *Bacillus subtilis*
Acetone extract (A)--13 mm
Chloroform extract (C)--10 mm
Methanol extract (M)--15 mm
Petroleum ether extract (P) ---

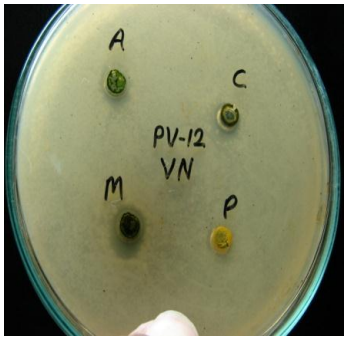

Figure-3.
Activity against *Proteus vulgaris*
Acetone extract (A)--10 mm
Chloroform extract (C)---
Methanol extract (M)--11 mm
Petroleum ether extract (P) ---

The extracts prepared from *Vitex negundo* leaves using different solvents showed varying degree of antimicrobial activity against organisms selected for the study. The fresh leaves juice was found to be active against only *Bacillus megaterium* (Fig-1), *Bacillus subtilis* (Fig-2), *Proteus vulgaris* (Fig-3) bacteria. When we compared the activity of aqueous extract with fresh leaves juice, the fresh leaves juice is more active. Acetone and methanol extracts are active against only *Bacillus megaterium* (Fig-1), *Bacillus subtilis* (Fig-2) and *Proteus vulgaris* (Fig-3) and only Chloroform extract active against *Bacillus subtilis* (Fig-2). All the organisms are susceptible to Ciprofloxacin- Cf,[30] Gentamicin-G,[50] and Tetracycline-T.[30] *Proteus vulgaris* is found to be resistant to Amoxycillin Am,[30] *Pseudomonas aerugenosa* and *Bacillus cereus* found to be resistant to Cotrimaxozole Co.[25]

The development of the modern antibiotics has vastly improved the treatment of cutaneous bacterial infections, particularly those caused by *S. aureus*. Therefore, today antibiotics can treat most bacteria causing skin diseases effectively. Nevertheless, unfortunately, the indiscriminate use of antibiotics in some parts of the world in both human and veterinary medicine has led to the emergence of resistant strains of bacteria. Thus, the rational use of antibiotics is of utmost importance. Since, over the last few years a large number of plant species have been evaluated for their antibacterial activity (Saranraj and Sivasakthi, 2014; Pandey and Khan, 2013; Agrawal *et. al*., 2013; Agrawal *et. al*., 2012a; Agrawal *et. al*., 2012b; De Boer *et. al*., 2005; Srinivasan *et. al*., 2001; Ahmad and Beg, 2001). This is why, the antibacterial therapy by medicinal plants will focus on a few problem areas from the point of view of a dermatologist and researchers.

Plants have provided a source of inspiration for novel drug compounds as plant-derived medicines have made significant contribution towards human health. There are number of naturally occurring compounds called secondary metabolites that possess plant protection properties. These compounds are effective against bacteria (Bravo *et. al.*, 1997) and showed the antimicrobial activity (Ragasa *et. al.*, 1999). According to Jeevan Ram *et. al*., (2004) infectious diseases, particularly skin and mucosal infections are common in most of the tribal inhabitants due to lack of sanitation, potable water and awareness of hygienic food habits. An important group of these skin pathogens was the fungi, among which dermatophytes and *Candida albicans*, besides certain pathogenic bacteria are the most frequent (Caceres *et. al*., 1993; Desta, 1993). The antimicrobial activity was expressed at varying degrees with the activity being all microbial strains and dose dependent. The various crude extracts of *V. negundo* showed significant activity against all the microbes tested. Similar, results of biological activity of *V. negundo* against bacterial strains were reported by (Agrawal *et. al*., 2012c, Zaidan *e.t al*., 2005) and (Perumal Samy *et. al*., 1998).

CONCLUSION

From our investigation of screening *Vitex negundo* plant species, the results obtained confirm the therapeutic potency of plants used in traditional medicine. *Vitex negundo* relieves muscle aches and joint pains. The Ayurvedic and Unani Pharmacopoeia of India has documented. The results of the present study support the folkloric usage of the studied plant and suggest that some of the plant extracts possesses compounds with antibacterial properties that can be used as antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens. The most active extracts can be subjected to isolation of the therapeutic antimicrobials and undergo further pharmacological evaluation. Screening of *Vitex negundo* leaves having natural organic compounds and identifying active agents is the need of the hour, because successful prediction of lead molecule and drug like properties at the onset of drug discovery will pay off later in drug development.

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