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Evaluation of the Antibacterial Potential, Preliminary Phytochemical Screening of Medicinal Plant against Plant Pathogen

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ABSTRACT

Vitex negundo L. belongs to family Verbenaceae is a hardy plant, flourishing mainly in the Indian subcontinent. All parts of the plant, from root to fruit, possess a multitude of phytochemical secondary metabolites which impart an unprecedented variety of medicinal uses to the plant. In the present study, the dried leaves of the *Vitex negundo* were powdered and extracted using cow urine extract, aqueous, hexane, chloroform, ethyl acetate and alcohol. The extracts were screened for antibacterial activity against *Xanthomonas campestris* pv *citri*. All the extracts and fractions were effective and showed 8 to 16 mm zone of inhibition. The chloroform extract (14 mm) and cow urine extract (16 mm) showed excellent antibacterial activity. The MIC values were determined by agar dilution method. Results of hexane and chloroform extracts had lowest MIC value 0.4 μg mL^{-1}. Aqueous ethyl acetate and cow urine extract had 1.85 μg mL^{-1}, respectively. Alcohol extracts had highest MIC value 5.55 μg mL^{-1}. Preliminary qualitative phytochemical analysis showed the presence of alkaloids, steroids, flavones, saponins, tannins, sugar, terpinoids, coumarins, phenols, phlobotanins, phytosterol and quantitative estimation evaluated that cow urine extract of *V. negundo* possess highest flavonoids (1.25 mg kg^{-1}) among which alkaloids, tannins, terpinoids contents were low. Aqueous and chloroform extracts had very low quantity of the phytochemicals. The main aim of this product development is to provide employment to the rural youth and to use safe disinfectant of cleaning floors etc.

Key words: Antibacterial activity, citrus canker, phytochemical analysis, Verbenaceae, *Xanthomonas*

INTRODUCTION

Vitex negundo Linn., (Verbenaceae), commonly known as Nirgundi, is already in clinical use in several traditional systems of medicine including Ayurveda, Unani and Siddha for management of pain, headache, inflammation, leucoderma, enlargement of the spleen, rheumatoid arthritis, gonerrhoea, bronchitis, fever, cold and cough, lactagogue and emmenagogue as juice, decoction and also as vapor (Panday and Chunekar, 1998; Sabnis, 2006; Anisuzzaman *et al*., 2007). It contains fragrant, volatile oil and resins with several reported phytomolecules e.g., nishindaside, negundoside (irridoid glycoside) and artemetin (Dutta *et al*., 1983; Banerji *et al*., 1969). Besides, several alkaloids, glycosides, flavonoids, reducing sugars, sterols, resin and tannins have also been reported (Chopra *et al*., 1958).

Citrus is a common term and genus of flowering plants in the family Rutaceae, originating in tropical and subtropical southeast regions of the world. Citrus fruits are notable for their fragrance, partly due to flavonoids and limonoids (which in turn are terpenes) contained in the rind and most are juice-laden. The juice contains a high quantity of citric acid giving them their characteristic sharp flavour. They are also good sources of vitamin C. Citrus juice also has medical uses (Andrews, 1961). Citrus canker is one of the most feared of citrus diseases, affecting all types of important citrus crops. The disease causes extensive damage to citrus plants and severity of this infection varies with different species and varieties and the prevailing climatic conditions. The disease is endemic in India, Japan and other South-East Asian countries. In India, citrus occupies third position among fruits after mango and banana and canker is one of the major constraints of its cultivation. Citrus canker was first reported from Punjab (Luthra and Sattar, 1942; Bedi, 1961). Intensive research on citrus canker is being carried out throughout the world which has been reviewed by Rossetti (1977), Civerolo (1981, 1984), Chand and Pal (1982), Stall and Civerolo (1991) and Goto (1992).

The causative agent *Xanthomonas campestris* pv *citri* is currently controlled by chemicals such as bordeaux mixture, copper oxychloride, mixture of sodiumdenate, perenox, ultra sulphur, copper sulphate, blitox, nickel chloride, streptomycin used sulphode are used nowadays to control. The synthetic pesticides have resigned supreme as the principal pest and disease control agents during the past five decades. It is esteemed that over five millions tones of pesticides have been used worldwide. The excessive use of antibiotic and chemical lead to the development of acquired resistance by the bacterial pathogens, environmental hazards and killing of non-target beneficial organism.

Form the ancient period cow's urine has been used as a medicine. The cow urine therapy is capable of curing several curable and incurable diseases. Cow urine distillate in a specific amount is scientifically proven to enhance the anti-microbial effects of antibiotic and antifungal agents. The invention relates to a novel use of cow urine as activity enhancer and availability facilitator for bioactive molecules, including anti-infective agents. The invention has direct implication in drastically reducing the dosage of antibiotics, drugs and anti-infective agent while increasing the efficiency of absorption of bio-active molecules, thereby reducing the cost of treatment and also the side-effects due to toxicity.

Vitex negundo Linn., plant leaves were soaked in cow urine and assayed for their antibacterial activity against the pathogen *Xanthomonas campestris* pv *citri*. In such a scenario, herbs (botanicals) and bio-pesticides have emerged as viable alternatives endowed with potential bactericidal, fungicidal and viridical properties (Urdangarin *et al*., 1999). In the present study, through scientific knowledge, various methods have been developed to test, isolate and characterize bioactive compounds from *Vitex negundo* Linn. In addition to that, it has been tested whether the formulation is having any nutrient or fertilizer activity.

MATERIALS AND METHODS

Isolation of microorganisms: Citrus canker infected leaves were collected from the Department of Plant Pathology, Agriculture College and Research Institute, Ramji Nagar, Trichy, in zip lock cover and transported to the laboratory within 2 h. Leaves were surface sterilized with sterile distilled water followed by 0.1% mercuric chloride and then rinsed with distilled water thrice. Cankered area alone was taken out and macerated into a smooth paste. A loopful of culture was transferred into nutrient agar (Himedia) and *Xanthomonas* selective medium (Himedia) and incubated at 37°C for 24 h. Physiological and biochemical screening were used to identify the isolate.

Collection of plant meterial:*Vitex negundo* plant leaves were collected from Srimad Andavan Arts and Science College, herbal garden located at Thiruvanai koil, Trichy, Tamil Nadu, India and authenticated by herbal division. Plant leaves were washed thoroughly three times with running tap water and once with sterile distilled water and then dried under shade. Fresh and shade dried leaves were used for the study.

Preparation of extracts

Cow urine extraction: The 3 kg of plant leaves were surface sterilized with sterile distilled water and cut into small pieces and placed in an earthen pot separately with 10 L of cow urine which was sufficient to sink all the leaves. The pot was kept in a pit dug in the soil incubated for 1-20 days. At the end of every 24 h the extracts were taken out and condensed at 40°C into a paste and stored at 4°C for further use.

Aqueous extraction: The 100 g of shade dried leaves were coarsely powdered and added with 300 mL of sterile distilled water and boiled for 30 min. Filtered through three layered muslin cloth and condensed in to solid form at 40°C using hot air oven.

Organic solvent extraction: The 100 g of shade dried leaves were coarsely powdered and added with 300 mL of organic solvent viz., hexane, chloroform, ethyl acetate, alcohol based on increasing polarity. Duration of incubation was 3 days at each solvent. The extracts so collected were evaporated on a water bath at atmospheric pressure and the solvents were completely removed in *vacou* and the remaining matter was quantified.

Anti bacterial assay: Antibacterial activity of organic fractions and cow urine extract of *Vitex negundo* leaves were assayed using the well diffusion method (Perez *et al.*, 1990). Appropriate quantity of extracts were dissolved in Di Methyl Sulfoxide (DMSO) and sterilized by using Sartorious syringe filter of pore size 0.22 μM (stock solution (0.04 g 1 mL^{-1})). Sterile Petri plates containing 20 mL of nutrient agar medium were seeded with 0.01 mL of 18 h old test bacterial strain isolate. Cow urine extracts and organic fractions were added at different concentration 400, 600, 1200 and 1800 μg were added into 6 mm diameter well. Incubation was made at 37°C for 24 h. The assessment of antibacterial activity was based on the measurement of diameter of the inhibition zone formed around the well, using Himedia scale. Streptomycin sulphates (30 μg) and DMSO (15 μg) were served as a negative control, respectively.

Determination of Minimum Inhibitory Concentration (MIC): Agar dilution method was used to find out minimal inhibitory concentration. Nutrient agar was prepared, sterilized and kept ready in molten condition. The 20 mL of the molten media was taken and was mixed with known concentration of different extracts/fractions and were added in different tubes. This mixture was swirled carefully for complete mixing of extract and media and poured onto the plate. After getting solidified it was inoculated at 37°C for 24 h. After incubation the tubes were then examined for microbial growth by observing for turbidity.

Qualitative and quantitative estimation of phytochemicals: All the extracts were analyzed to qualitative and quantitative estimation of phytochemical screening to detect the presence of secondary metabolites as per the standard methods (Harborne, 1984).

RESULTS

In the present study, plant powder was sequentially extracted with different solvents in increasing polarity order. Successive cold extractions of coarse powder of *Vitex negundo* leaves revealed that the extractive values in hexane and chloroform fractions are more (4%) compared to water (3%). Higher hexane and chloroform extractive values indicated the presence of non polar chemical constituents, while the aqueous extractive values revealed the presence of high polar constituents (Table 1).

The bactericidal effect of cow urine extracts of *Vitex negundo* was also showed encouraging results. Maximum zone was observed on 5th day extract (16 mm) (Fig. 1) followed by 1st, 3rd and 6th day. The 19th to 20th day extracts showed negative results. Variation of zone diameter was observed during the period of study. As the concentration of extract is increasing the zone of inhibition also increases (Table 2). The positive control streptomycin sulphate has shown 21.00 mm at 30 µg concentration. DMSO was used as negative control. The chloroform extract exhibits 14 mm (Fig. 2) and aqueous extract have shown 13 mm at 1600 µg. When the antibacterial activity of all the extract and fractions were compared, cow urine extracts was showed to be highly effective in controlling the pathogen (16 mm) followed by chloroform extract (14 mm), aqueous extract (13 mm), alcohol extract (12 mm) and hexane and ethyl acetate extract (11 mm) (Table 3, Fig. 3).

Table 1: Extraction value of *Vitex negundo* leaves in different organic solvents

Solvent	Volume of solvent added (mL)	Powder taken (g)	Incubation (days)	Volume of solvent collection (mL)	Wet weight (g)	Final dry weight (g)
Hexane	300	100.0	3	280	96.2	4.6
Chloroform	300	96.2	3	270	87.0	4.0
Ethyl acetate	300	87.0	3	260	83.0	3.5
Methanol	300	83.0	3	250	75.0	3.2
Alcohol	300	75.0	3	240	69.0	2.9
Water	300	69.0	3	250	60.0	2.5

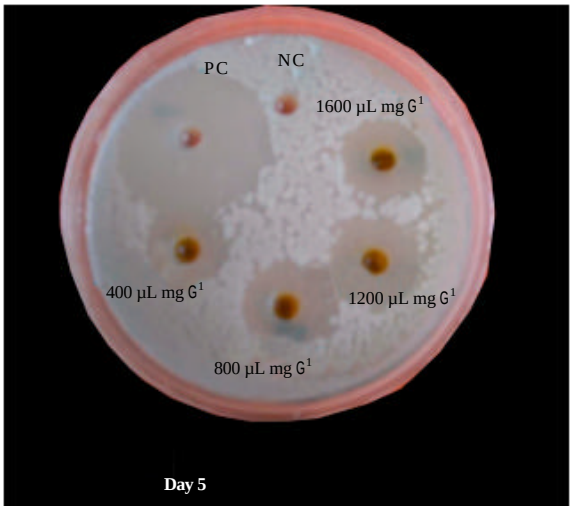

Fig. 1: Antibacterial activity of cow urine extract of *Vitex negundo* leaves against *Xanthomonas campestris* pv *citri*

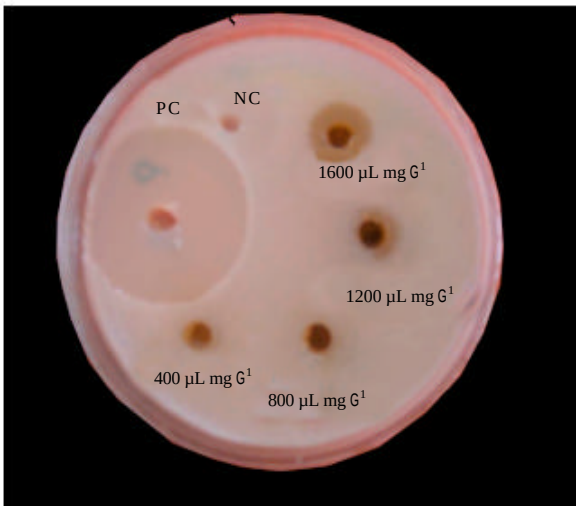

Fig. 2: Antibacterial activity of chloroform extract of *Vitex negundo* leaves against *Xanthomonas campestris* pv *citri*

Table 2: Antibacterial activity of cow urine extract of *Vitex negundo* leaves against *Xanthomonas campestris* pv *citri*

Vitex negundo cow urine extract (days)	Conc. of extract (µg)/zone of inhibition (mm)					
	+ve	-ve	400	600	1200	1600
1	21	-	10	10	11	13
2	21	-	10	10	10	11
3	21	-	10	10	10	13
4	21	-	10	10	11	12
5	21	-	11	14	15	16
6	21	-	11	12	13	13
7	21	-	10	11	11	12
8	21	-	10	11	11	12
9	21	-	10	10	11	11
10	21	-	11	11	11	12
11	21	-	10	10	10	11
12	21	-	10	10	11	11
13	21	-	8	9	9	10
14	21	-	8	8	9	10
15	21	-	-	-	9	11
16	21	-	-	-	10	11
17	21	-	-	-	10	10
18	21	-	-	-	9	10
19	21	-	-	-	-	-
20	21	-	-	-	-	-

A result of minimum inhibitory concentration was 1.85 µg mL^{-1} for cow urine extract, ethyl acetate, aqueous and 0.4 µg mL^{-1} for hexane, chloroform and 5.55 µg mL^{-1} for alcohol fraction (Table 4).

Preliminary qualitative phytochemical analysis of *Vitex negundo* leaves are performed for both cow urine and organic fraction. Secondary metabolites are found in all the 20 days extracts and

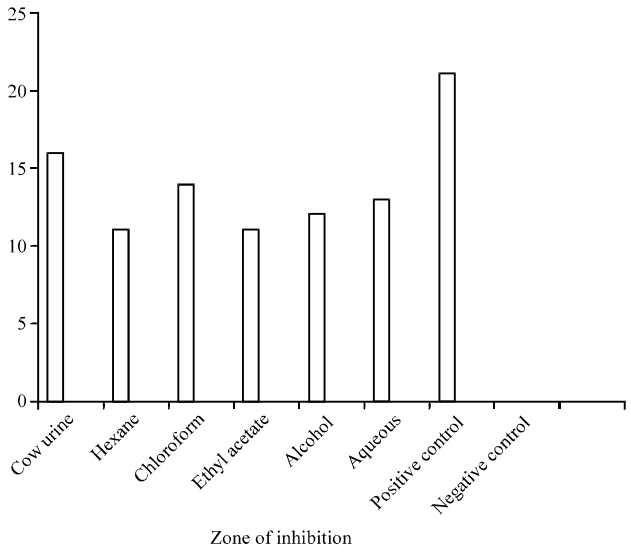

Fig. 3: Antibacterial effect of organic fraction and cow urine extract at 1600 µg mL^{-1}

Table 3: Antibacterial activity of organic fraction of *Vitex negundo* leaves against *Xanthomonas campestris* pv *citri*

Vitex negundo fractions	Conc. of extract (µg)/zone of inhibition(mm)					
	+ve	-ve	400	600	1200	1600
Hexane	21	-	9	10	10	11
Chloroform	21	-	10	11	12	14
Ethyl acetate	21	-	9	10	10	11
Alcohol	21	-	9	10	11	12
Aqueous	21	-	10	11	12	13

Table 4: Minimum inhibitory concentration of *Vitex negundo*

Extracts	Conc. of extracts (µg mL^{-1})
Hexane	0.40
Chloroform	0.40
Ethyl acetate	1.85
Alcohol	5.55
Aqueous	1.85
Cow urine extract	1.85

showed the presence of flavones, saponins, tannins, alkaloids, sugar, terpinoids, coumarins, phenols, phlobotanins, phytosterol (Table 5). The chloroform fraction contains flavones, coumarins, phenols, phlobotanins, phytosterol. Even though aqueous extract showed positive for alkaloid, quinine, steroids (Table 6).

Results of quantitative estimation revealed that cow urine extract of *V. negundo* possess total alkaloids (0.74 mg kg^{-1}), flavonoids (1.25 mg kg^{-1}), tannins (0.25 mg kg^{-1}), terpinoids (0.08 mg kg^{-1}) among which flavonoid content was high. Chloroform extract had alkaloids (0.07 mg kg^{-1}), flavonoids (0.98 mg kg^{-1}), tannins (0.05 mg kg^{-1}), terpinoids (0.04 mg kg^{-1}). The cow urine extract of *Vitex negundo* showed higher quantity of flavonoids. This extract which gives maximum antibacterial activity was due to flavonoid.

Table 5: Preliminary phytochemical analysis of cow urine extracts of *Vitex negundo*

Cow urine extract of *Vitex negundo*	Days of incubation																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Terpenoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Flavones	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Sugar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Alkaloid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Quinone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coumarin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Tannin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Saponin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenols	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Steroids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phlobotannins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Phytosterol	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Starch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 6: Preliminary phytochemical analysis of organic fraction of *Vitex negundo*

Test	Hexane	Chloroform	Alcohol	Ethyl acetate	Aqueous
Terpenoids	-	-	-	-	-
Flavones	+	+	+	+	-
Sugar	-	+	+	-	-
Alkaloid	-	-	-	-	+
Quinone	-	-	+	-	+
Coumarin	+	+	+	+	+
Tannin	-	+	+	+	-
Saponin	-	-	-	-	-
Glycoside	-	+	-	+	-
Phenols	+	+	+	+	+
Steroids	-	-	-	-	+
Phlobotannins	-	+	+	+	+
Phytosterol	+	+	+	+	+
Starch	-	-	-	-	-

DISCUSSION

There is a worldwide interest in searching for the safe and effective novel antibacterial compounds of plant origin for the control of plant pathogenic bacteria which is responsible for the great impact on the growth and productivity of agriculture crops. The use of plant extracts is found to be an effective way of controlling plant diseases compared to synthetic chemicals as plant extracts have several advantages over it (Opara and Wokocha, 2008). *Xanthomonas campestris* pv. *citri* causes canker disease in all types of important citrus crops. Millions of dollars are spent annually on prevention, quarantines, eradication programs and disease control in worldwide (Das, 2003). These disease controlled by chemical sprays with copper compounds, but available measures are not effective and one of the major limitations of using chemical control agents is the development of resistance in bacteria (Sigee, 1993).

Antibacterial properties of plant extracts against human pathogenic bacteria have been reported by several studies but only a few studies have been done on plant pathogens using plant

extracts. It was found that the polarity of the solvents seems to play an important role in the extraction of natural products which influences the antibacterial activity of the extracts (Dos Santos-Neto *et al*., 2006) and in a sequential extraction technique, chemical constituents are partially separated according to their polarity, the least polar components separates into the low polar solvents and this progressing through the separation of active components based on their polarity and the polarity of the solvent used (Chawla *et al*., 1992). This partial separation of active components may be an advantage to reduce the antagonistic effects of chemical constituents because the compounds present in crude mixturemay interfere with the action of the other (Azhar-ul-Haq *et al*., 2004). Razia *et al*. (2013) reported that the methanolic extract showed highest zone of inhibition (29.3 mm) at 100 µL was *P. mirabilis* and the chloroform extract showed a degree of growth inhibitions less compared to methanol extract. A novel pharmaceutical composition present in cow urine distillate has been patented. A recent study on cow urine distillate has been shown to effectively control both bacteria and fungi at 15 µL concentration (Khanuja *et al*., 2002). Cow urine distillate has immuno-modulatory activity in broiler chickens (Jojo *et al*., 2011). But recently reported that, fresh cow urine was more effective antimicrobial agent than its distillate this may be because fresh urine is more acidic in nature (Ahuja *et al*., 2012).

Previous phytochemical studies on *V. negundo* have afforded several types of compounds, such as volatile oils (Singh *et al*., 1999; Mallavarapu *et al*., 1994; Dayal and Singh, 2000; Taneja *et al*., 1979) lignans (Chawla *et al*., 1992; Azhar-ul-Haq *et al*., 2004) flavonoids (Achari *et al*., 1984; Banerji *et al*., 1988; Subramanian and Misra, 1979; Banerji *et al*., 1969, 1988; Misra and Subramanian, 1980) iridoids (Dutta *et al*., 1983; Sehgal *et al*., 1982, 1983) terpenes (triterpenes, diterpenes, sesquiterpenes) (Vishnoi *et al*., 1983; Chandramu *et al*., 2003; Chawla *et al*., 1991) and steroids (Mukherjee and Badruddoza, 1981). Dayrit and Lagurin reported the high amount of flavonoids in *V. negundo*. One of the features of secondary metabolism is to cope with and adapt to a continually changing environment relates to chemical diversification, with intra-population variation being inherent. Furthermore, tannins are also an important secondary metabolite, which has antibacterial, antifungal and antiviral activities.

CONCLUSION

From the analysis and results recorded above, it was concluded that cow urine extract was highly effective and stable in controlling the growth of bacteria because of some active compounds may be responsible for biotransformation of constituents due to cow urine treatment. The plant leaves with cow urine extract do not develop resistance in pathogens and do not affect the beneficial organisms. Hence this single traditional formulation could be act as bactericide, pesticide and insecticide. After a careful analysis of the literature, it has been identified that less work was done in the anti bacterial properties of medicinal plants, especially in combination with cow urine. If the botanical insecticides are effective against the bacterial plant pathogens, a dual role can be performed by the same formulation, which can reduce the workload, cost, above all the earth can be protected from hazardous chemicals.

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