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Phytochemical Screening of Vitex Negundo Leaf Extracts and Its Role in Green Synthesis and Antimicrobial Properties of Silver Nanoparticle

Adhya K.M., Anu Radhakrishnan, Bhuvaneswary M. G., Gayathri T. H.*

Research and Post Graduate Department of Chemistry, Sree Narayana Mangalam (S.N.M.) College, Maliankara, Ernakulam, Kerala, India -683516

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KEYWORDS

Vitex negundo leaf extracts, Phytochemical screening, Human pathogenic bacteria, Antibacterial activity, Green synthesis, Silver nanoparticles, Disc diffusion method

ABSTRACT:

Introduction: The rise in drug resistance shown by human pathogenic bacteria become a clinical crisis from all over the world. In order to control the situation, it is essential to develop alternative drugs for the treatment of infectious diseases. Recent advancements in the field of green synthesis have enabled the development of alternative drugs.

Objectives: The work described in this paper details the phytochemical screening and biological investigation of Vitex negundo leaf extracts, which is mentioned in Ayurveda as a potential medicine to cure several diseases.

Methods: Acetone, ethanol, and aqueous extracts of Vitex negundo leaves were prepared using the Soxhlet apparatus. Preliminary phytochemical screening was done on aqueous leaf extract using qualitative phytochemical screening methods. The antibacterial activity of water, acetone, and alcohol extracts was tested against human pathogenic bacteria such as Staphylococcus aureus, Escherichia coli, and Klebsiella pneumonia by adopting a disc diffusion method.

Results: Phytochemical screening of the aqueous extract of Vitex negundo leaves reveals the presence of phytochemical constituents like carbohydrates, alkaloids, tannins, saponins, and flavonoids. Biological investigation revealed that Staphylococcus aureus was highly inhibited by water extract of vitex negundo and the acetone extract shows the greatest inhibitory action against K. pneumoniae. Biologically synthesized silver nanoparticles also exhibit greater inhibitory action against Staphylococcus aureus.

Conclusions: The results suggested that the leaf extract of Vitex negundo and the silver particle prepared from the leaf extract indeed possessed significant antimicrobial activity against all the bacteria tested but in both cases, the effect was greater towards S aureus.

1. Introduction

Plants have always played a vital role in the medical field. From earlier times, plants have been the core of traditional medicines and even now many drugs are derived from plants[1]. In India, traditional systems of medicine like Ayurveda, Yunani, and Siddha have been using herbs for treatment[2]. They use herbs to treat various health problems like asthma, tuberculosis, diabetes mellitus, cancer, heart diseases, hypertension, etc. Plants containing phytochemicals such as alkaloids, terpenes, polyphenols, flavonoids, etc. have been used to cure illness and promote good health[3]. These phytochemicals are mainly stored in the leaves and flowers of plants[4]. The medicinal plants seen in India like G. Sylvestre have anti-diabetic, anti-inflammatory, and anti-microbial properties and help to decrease LDL cholesterol and triglycerides, and T.populnea is used as an analgesic, immune-modulatory, and neuroprotective. Herbs like S. Auriculata are effective against various medical conditions such as diabetes, asthma, cancer, leprosy, etc[1]. The aqueous extracts of Anethum graveolens, Elettaria cardamomum, Foeniculum vulgare, Trachyspermum ammi, and Viola odorata have better antibacterial activities than the standard antibiotics available in the market[5]. So, herbs are one of the core materials of both ancient and advanced medical systems. They are receiving global interest because of their better compatibility, adaptability, and lack of side effects[6]. So, herbs are one of the core materials of both ancient and advanced medical systems.

Vitex negundo L. a member of the Lamiaceae family (formerly classified under Verbenaceae family)[7],

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also known as five leave chase tree (Nirgundi in India)[8], is a herb widely used in traditional medicine in Bangladesh, India, China, Indonesia, Nepal, Pakistan, Philippines, and Sri Lanka[9]. It is a slender tree, reaching up to 3-4.5 m in height which grows in wastelands, especially in hotter zones[10]. Even though all parts of the plants are used in medicine, their leaves and bark extracts are the most effective. The leaves are potent in treating leucoderma, eye diseases, toothache, skin ulcers, rheumatoid arthritis, spleen enlargement, gonorrhea, and bronchitis[11]. It also shows antioxidant[11,12], anti-inflammatory[12,13], anti-histaminic, antipyretic[14], antifungal[15], insect repellant[16], and anti-bacterial properties[17]. It has been reported that many phytochemicals like volatile oils[18], flavonoids[19,20], lignans, terpenes, and steroids are present in different parts of Vitex negundo. According to Balraj Singh et al. Vitex negundo has the potential to modulate cellular events like apoptosis, cell cycle, motility of sperm, polycyclic ovary disease[21], and menstrual cycle[10]. The negundoside isolated from the leaves of Vitex negundo L. has shown neuroprotective properties against cerebral ischemia. So, Vitex negundo L. is a potent herb that has been widely used in traditional medicine, whose more phytochemical properties are yet to be discovered.

Nanoparticles accomplished a vital role in bringing up revolutionary changes in modern medicine. From drug delivery to sensors and nano-bots, applications of nanoparticles in the medical field are still under development. Particles having dimensions in the range of 1-100 nm, showing unusual physical, chemical, and biological properties from their bulk counterparts are defined as nanoparticles. Nanoparticles of silver have been widely used due to their distinctive biological activity. Their anti-microbial activity makes them suitable to be used in medicine in drug delivery, bio-labeling, coating medical devices, etc[22]. Silver nanoparticles are widely used for antimicrobial and anticancer therapy[23], as anti-diabetic agents[24], and as biosensors[25]. Currently, plant-mediated green synthesis of nanoparticles is acquiring greater attention over conventional chemical and physical methods, as they are highly eco-friendly, cost-effective, less toxic, and readily available[26]. Biosynthetic methods can produce large quantities of nanoparticles that have a well-defined size and morphology than those obtained from physicochemical methods[27].

2. Objectives

Vitex negundo has been indicated in traditional medical transcripts as a useful medicine to treat various diseases. So, this study aims to do a phytochemical screening of aqueous extract of Vitex negundo leaves to find out the presence of various phytochemicals responsible to these medicinal properties.

This study also intent to prepare different extracts of Vitex negundo leaves, to compare their antibacterial properties against some common human pathogenic bacteria like Staphylococcus aureus, Escherichia-coli and Klebsiella pneumonia by adopting a disc diffusion method. Since silver nanoparticles have always shown significant antimicrobial capabilities, this work also hopes to compare the antibacterial activity of silver nanoparticles of Vitex negundo leaf extracts synthesised using green methods.

3. Methods

3.1. Materials:

The following reagents were used in this study: Silver nitrate ($AgNO_3$), Acetone, Ethanol, Sulphuric acid, Nitric acid, Hydrochloric acid, Sodium hydroxide, Picric acid, Chloroform, and Ferric chloride. All the chemicals and reagents used were of analytical grade and were used without further purification. Double distilled water was used for the preparation of solutions and other purposes. The stock cultures of Staphylococcus aureus, Escherichia coli, and Klebsiella pneumonia were obtained from the Department of Botany, U.C College, Aluva. The stock cultures were subcultured and maintained in nutrient broth at 4ºC. Fresh plant leaves of Vitex negundo were collected from local areas of Moothakunnam village, Kerala.

3.2. Methods:

3.2.1. Soxhlet extraction process:

The fresh leaves of Vitex negundo were cleaned and dried under shade for about four days. The dried leaves are then powdered and used for the extraction process. The extracts are prepared using acetone, ethanol, and water with the Soxhlet apparatus.

3.2.2 Phytochemical analysis:

The preliminary phytochemical screening was done with the aqueous leaf extract. The presence or absence of

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phytochemicals in the extract was tested using the qualitative phytochemical screening methods as shown in Table 1.

Table 1. Qualitative phytochemical screening of Vitex negundo

Phytochemicals	Test
Carbohydrate	• Molisch's Test: Two drops of Molisch's reagent were added to 2 ml of the leaf extract. Conc.H_2SO_4 was added along the sides of the test tube. The appearance of a reddish violet ring at the junction of the two solutions indicates the presence of carbohydrates • Fehling's Test: 1 ml of the leaf extract was added to a mixture of about 2 ml of Fehling's solution A and B. Formation of orange or red precipitate confirms the presence of carbohydrates. • Benedict's test: The plant extract is added to 5 ml of Benedict's reagent and boiled. An orange precipitate indicates a positive test for carbohydrates.
Proteins	• Biuret Test: 3 ml of plant extract is mixed with an equal volume of Biuret reagent. The presence of protein induces a violet color. • Nitric acid Test: The leaf extract is added slowly along the sides to a test tube containing 3 ml of Conc. nitric acid. A dark ring formed at the junction of two solutions confirms the presence of protein.
Flavonoids	• Alkaline reagent test: 1 ml of the leaf extract is treated with 3 ml of NaOH. Formation of a yellow precipitate which disappears on the addition of dil. HCl confirms the presence of flavonoids.
Tannins	• Ferric chloride test: 1 ml of the sample is treated with $FeCl_3$ solution. A brownish-green or blue-black coloration indicates the presence of tannins.
Saponins	• Froth test: 1 ml of plant extract is shaken with 2 ml of distilled water. Frothing indicates the presence of saponins.
Terpenoids	• Salkowski test: 1 ml of the extract is mixed with 2 ml of chloroform. Con.H_2SO_4 is added carefully to form a layer. Reddish brown coloration at the junction confirms the presence of terpenoids.
Alkaloids	• Hagera's test: 1 ml of leaf extract is treated with 3 ml of saturated

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	picric acid solution. Yellow precipitate indicates the presence of alkaloids.

3.2.3. Synthesis of Silver nanoparticles

About 20 g of cleaned and air-dried leaves of Vitex negundo were finely chopped, added to 100 ml of deionized water, and stirred for 20 minutes at 60ºC. The boiled leaf extract was cooled and filtered, giving a transparent yellow color leaf broth. In the green synthesis of Silver nanoparticles (AgNPs), 5 ml of leaf broth was mixed with 45 ml of 0.01M aqueous solution of $AgNO_3$ (99.99%) and allowed to react at ambient conditions. A color change from yellow to dark brown was observed after optimized reaction conditions indicating the formation of silver nanoparticles. The reduction of silver ions to nanoparticles was confirmed by the UV-Vis spectrum of the solution. The AgNP solution was centrifuged and the excess liquid was removed by evaporation, yielding black-colored silver nanopowder.

3.2.4. Characterization

The synthesis of silver nanoparticles and reduction of silver ions was confirmed by a UV-Vis spectrometer. The absorption maxima of the aqueous sample were scanned 2 hours after the reaction, by UV-Vis spectrophotometer in the wavelength range of 300-800 nm on a Beckman Du-50 spectrometer.

3.2.5. Antibacterial study

The antibacterial activity was studied using a gram-positive (*Staphylococcus aureus)* and two gram-negative (*Escherichia coli* and *Klebsiella pneumonia)* bacteria. The antibacterial activity of the leaf extracts and the synthesized AgNPs were tested against these selected pathogenic microorganisms using the disc diffusion method.

The lawn cultures of test organisms were made on nutrient agar plates under hygienic conditions using a sterile cotton swab. The AgNPs and crude extract (25 µg, 50µg, and 100µg) were impregnated on sterile discs (6 mm diameter). The discs soaked in *Ciprofloxacin* (positive control) and in the test solutions were then placed on the nutrient agar surface. The petri dishes were incubated at 27ºC for 18-24 hours. The inhibition zones of AgNPs and leaf extracts against different pathogenic bacteria were measured in millimeters.

3.2.6. Determination of Activity Index

The activity index of the crude plant extracts can be calculated as;

$$Activity\ index\ (AI) = \frac{Zone\ of\ inhibition\ of\ the\ extract}{Zone\ of\ inhibition\ obtained\ for\ Std.\ antibiotic\ drug}$$

4. Results

4.1. Solvent Extraction

The percentage yield of extracts obtained with different solvents using the Soxhlet extraction technique was calculated by the given formula and the results are tabulated in Table 2.

$$\text{Yield (\%)} = \frac{weight\ of\ extract}{weight\ of\ sample} \times 100$$

Table 2. The percentage yield of solvent extraction using Soxhlet apparatus

Solvent	Yield (%)
Acetone	10.06
Ethanol	10.94
Water	11.665

4.2. Phytochemical Analysis

The present study reveals the presence of phytochemical constituents like carbohydrates, alkaloids, tannins, saponins, and flavonoids in the water extract of Vitex negundo. The results are tabulated in Table 3.

Table 3. Results of Phytochemical analysis

PHYTOCHEMICAL CONSTITUENTS	STATUS
Carbohydrate	+
Proteins	-
Alkaloid	+
Tannins	+
Triterpenoids	-

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Saponins	**+**
Flavonoids	**+**

Note :-' + ' Present ' – ' Absent

4.3. Synthesis of silver nanoparticles

During the reaction of aqueous leaf extract of V. negundo with silver nitrate, a visible color change was observed from transparent to dark brown, indicating the formation of AgNPs. Figure.1 displays the reaction mixture of the leaf extract and $AgNO_3$ as a function of time.

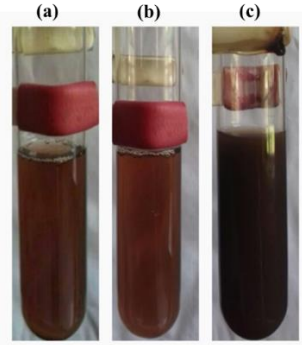

Figure 1: Images of synthesized AgNPs solution over several reaction times

(a) 10 min (b) 20 min (c) 70 min.

The formation of AgNPs was confirmed by a UV-Vis spectrometer. It is well known that silver nanoparticles exhibit a yellowish-brown color in water due to surface Plasmon vibration. Figure 2 shows the UV-Visible spectrum which is recorded after the completion of the reaction. Peak specific for the synthesis of silver nanoparticles was obtained at 440 nm. Various studies showed that silver nanoparticles absorb light in this region. The frequency and width of the surface Plasmon adsorption depend on the size and shape of the metal nanoparticles as well as the dielectric invariable of the metal itself and the surrounding medium. The intensity of the absorption peak at 440 nm was increased with the increasing time period of the aqueous component.

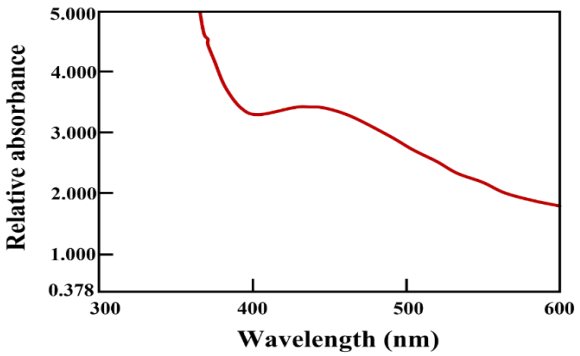

Figure.2 UV-Vis spectrum of AgNPs of Vitex negundo.

4.4. Pharmacological analysis

The study reveals the profound antimicrobial activity of different extracts of vitex negundo on bacteria S.aureus, E.coli, and K.pneumoniae (Figure.3). Water extract of vitex negundo shows the greatest inhibitory action, alcohol extract shows medium, and acetone extract show the minimum inhibitory action against S. aureus. The inhibitory action against E. coli is the highest for the aqueous extract, followed by alcohol and acetone extracts of vitex negundo. The antibacterial activity of vitex negundo against K.pneumoniae is found to be the highest in its acetone extract, followed by the alcohol and water extracts. The antibacterial activity of different extracts of Vitex negundo against various pathogenic bacteria is noted in the Table.4 and Figure.4 shows the comparison of the antibacterial activity of water, alcohol, and acetone extracts of Vitex negundo against S. aureus, E.coli, and K.pneumoniae.

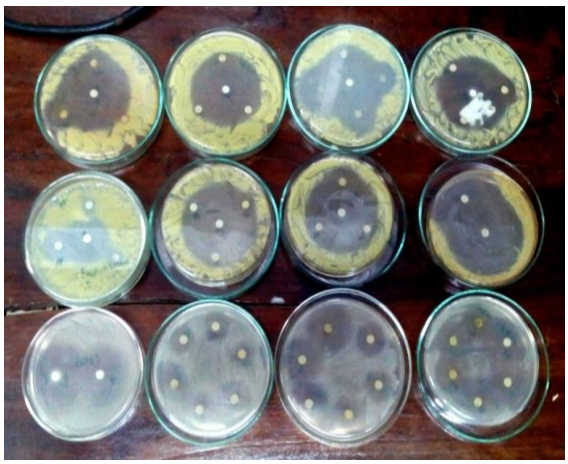

Figure.3 Antibacterial activity of different extracts of vitex negundo on bacteria S.aureus, E.coli, and K.pneumoniae

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Table.4 Antibacterial activity of Vitex negundo against human pathogenic bacteria										
Pathogenic bacteria	Standard	Water Extract			Alcohol Extract			Acetone Extract		
		T_2	T_3	T_4	T_2	T_3	T_4	T_2	T_3	T_4
S.aureus	41 ± 1	26.6 ± 1.15	20.6 ± 1.15	12.3 ± 0.57	21.3 ± 1.52	16.6 ± 0.57	13.6 ± 0.57	15.6 ± 0.57	10.6 ± 0.57	9.3 ± 0.57
E.coli	41 ± 1	24.3 ± 0.57	22 ± 2	17.6 ± 0.57	13 ± 0	12 ± 0	11 ± 0	9 ± 0	8 ± 0	7 ± 0
K. pneumonia	38 ± 1	21 ± 1	17.3 ± 0.57	16.6 ± 1.154	26.3 ± 0.577	20.3 ± 2.08	18.3 ± 1.52	31 ± 2	24.3 ± 0.577	19.6 ± 1.52

T_2, T_3, and T_4 represents the discs impregnated in 100µg, 50 µg, and 25 µg respectively.

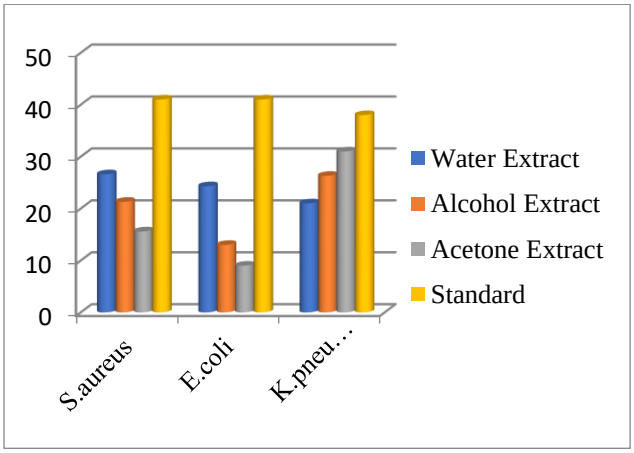

Figure 4 Comparison of antibacterial activity of different extracts of V. negundo against various human pathogenic bacteria.

This study shows that the synthesized silver nanoparticles have greater inhibition against S.aureus, medium against E.coli, and lesser against K.pneumoniae. Table 5 shows the antibacterial activity of synthesized silver nanoparticles against the selected bacteria and Figure.5 exhibits the comparison of the effect of AgNPs against different bacteria.

Table 5 Antibacterial activity of synthesized silver nanoparticles against human pathogenic bacteria			
Pathogenic Bacteria	Standard	Silver nanoparticle	
		T_3	T_4
S.aureus	35	20	13
E.coli	35	19.6	12.6
K. pneumoniae	37	18	10

T_3 and T_4 represent the discs impregnated in 50 µg, and 25 µg respectively.

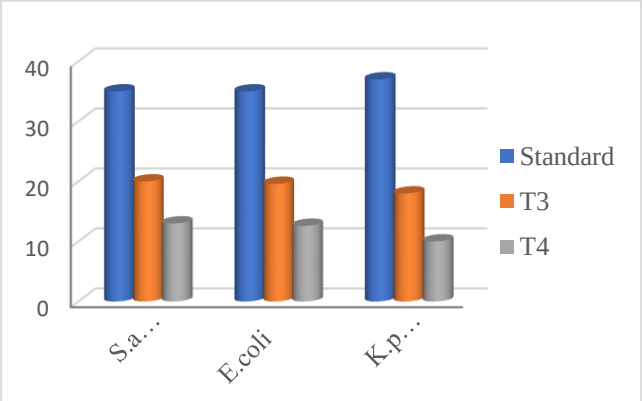

Figure.5 Comparison of antibacterial activity of synthesized AgNP's against different human pathogenic bacteria.

5. Discussion

Vitex Negundo is a traditionally known ayurvedic medicine which is used to treat several diseases. The outcome of the present study reveals the medicinal properties of Vitex Negundo. The phytochemical screening of aqueous extract of vitex negundo confirmed the presence of primary and secondary metabolites like carbohydrates, alkaloids, tannins, saponins, and flavonoids. The presence of these phytochemical constituents suggests that the Vitex negundo has remedial capability against several diseases. The antibacterial activity of water, acetone, and alcohol extracts was studied against human pathogenic bacteria like S. aureus, E. coli, and K. pneumoniae using the disc diffusion method. Aqueous extract of vitex negundo showed greater inhibitory action against S. aureus and E. coli whereas against K. pneumoniae the highest antibacterial activity was observed for acetone extract. Green synthesized silver nanoparticles from vitex negundo extract also exhibit antimicrobial activity. Silver nanoparticles have greater inhibition against S. aureus. The study unveils the scientific basis of the therapeutic uses of traditional medicinal plants. The obtained results provide support for the use of this plant in traditional medicine and its further investigation. Further studies are required to identify the responsible bioactive molecule and to isolate the compounds responsible for the activity. Our findings confirm the presence of some phytochemical compounds and the anti-microbial activity of vitex negundo.

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