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# GREEN BIOSYNTHESIS OF SILVER NANOPARTICLES (AgNPs) FROM *Vitex negundo* PLANT EXTRACT AND ITS PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ASSESSMENT NEXT TO PATHOGENIC MICROBES

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**ABSTRACT**

In the present study, green synthesis of silver-nanoparticle (AgNPs) is demonstrated using plant extract of *Vitex negundo*. Plant extract through six different solvents, including petroleum ether, benzene, chloroform, acetone, methanol, and water, was prepared and further investigated for its antimicrobial and antifungal activities using different bacterial and fungal strains. The phytochemical analysis was performed, where saponins, tannins, steroids, flavonoids and glycosides were detected in acetone, chloroform and methanolic extract. Subsequent analysis of synthesized AgNPs through dynamic light scattering suggested that particle sizes were 10-300 nm in size. The study indicated that the chloroform, acetone, and methanol extracts of *Vitex negundo* showed good inhibitory activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* based on the zone of inhibition. The antimicrobial activity of the synthesized AgNPs suggested that it can inhibit the growth of both gram +ve and gram -ve microorganisms. The MIC value of AgNPs of methanolic extract of V. *negundo* detected was 0.078 mg/mL, which was relatively lower than that of the MIC value of its crude extract (1.25mg/mL). The observed MIC values concluded that the synthesized AgNPs had better antimicrobial activity and could be necessary for various applications, including medicine, biology, and industry.

**Keywords:** *Vitex negundo; Green synthesis;* Silver-nanoparticle; antimicrobial; antifungal activities

## INTRODUCTION

Natural products have been considered a vital source of drugs for curing several ailments of humankind since ancient times. About half of the medicinal drugs are derived from natural sources (**Tewari *et al*., 2019**). Drugs of plant origin have served as the foundation for a significant fraction of the current pharmacopoeia (**Jugran *et al*., 2021**). Medicinal plants are gaining popularity over synthetic drugs, and extensive R&D work utilizes these medicinal plants' properties **(Dehelean *et al*., 2021; Tewari *et al*., 2019**). In the recent past, there has been a tremendous increase in plant-based health products in developing and developed countries resulting in the exponential growth of production and trade of herbal products globally **(Cordell, 2011; Tabassum *et al*., 2021**).

The annual global trade of plant-derived and plant-originated products around the U.S. is $62 billion, with surprisingly very little involvement from India at just about $ 1 billion (**Dubey *et al*., 2004**). European Union leads the market with a 45 per cent share [$ 28 billion], followed by North America, which makes up 11 per cent [$ 6.9 billion] of the total market (**Sammons *et al*., 2016**). ASEAN countries contribute 19% ($10.8 billion) of the total global herbal market share (**Bhuyar *et al*., 2021**). Despite having a minor stake in the herbal pharma market, India has an excellent standing worldwide. India is one of the biodiversity-rich countries in the world, with about 46,000 recorded plant species, 16 different agro-climatic zones, 10 vegetative zones and 15 biotic provinces, 15,000–18,000 flowering plants, 23,000 fungi, 2500 algae, 1600 lichens, 1800 bryophytes and 30 million micro-organisms. Indian herbal medicinal industry is growing fast with annual turnover of about 2,300 crores as against the pharmaceutical industry's turnover of Rs. 14,500 crores **(Kala, 2006**).

Recently there has been a shift from synthetic to herbal medicine, *i.e.* 'Return to Nature' **(Kiełczykowska & Musik, 2020; Zaid *et al*., 2019**). Since nature has bestowed our country with an enormous wealth of medicinal plants, it is often referred to as the Medicinal Garden of the world. India has a unique position in the world, where several recognized indigenous systems of medicine, viz., Ayurveda, Siddha, Unani, Homeopathy, Yoga and Naturopathy, are successfully utilized for the healthcare of people (**Sharma *et al*., 2008; Tabassum *et al*., 2021**). The herbal drugs are pretty popular among rural and urban communities of India and are still being used since these are considered safe. Due to the growing recognition that natural products are non-toxic, have more minor side effects, and are readily available at affordable prices, the demand for herbal-based medicines, health products, pharmaceuticals, food supplements, cosmetics, etc., is increasing in both developing and developed countries (**Tabassum *et al*., 2022**).

Indian Himalayan Region (IHR) is well known for its medicinal plant wealth. The geographical features, altitude, ecology, topography and climatic conditions are primarily responsible for making the Himalayan region a rich repository of biodiversity, especially in plant life (**Chandra, 2012**). Due to the specific climatic and geophysical conditions, temperate and alpine plants of the Himalayan region offer greater possibilities of having novel molecules and even the most considerable quantities of bioactive compounds (**Samant & Pant, 2006**). High altitude herbal medicines offer therapeutics for many disorders like memory loss, osteoporosis, and immune and age-related property, mainly for which no modern treatments are available. As the high-altitude plants are growing in stressful situations and exposed to high U.V. radiation, they also have immense potential in biological radioprotection (**Mehta *et al*., 2020**).

Nowadays, such plants are a new face in the green synthesis of Ag-nanoparticle and have been reported by various researchers (**Abd Aziz *et al*., 2018**). Due to the presence of different aromatic flavonoids, alkaloids, phenols, steroids etc., they are responsible for the intrinsic pharmacological properties of these high-altitude medicinal plants. *Vitex negundo* is high altitude herb and is less known for its industry and biotechnological applications (**Tandon, 2005**). Therefore, In the present study, we prepared plant extract *Vitex negundo* using six solvents such as petroleum ether, benzene, chloroform, acetone, methanol, and water. Further, the plant extract has tested its antimicrobial and antifungal activities using different bacterial and fungal strains. Later, Phytochemical analysis is performed to investigate the constitution of the plant extract. Silver nanoparticles (Ag-NPs) are synthesized and tested for their antimicrobial activity.

## MATERIAL AND METHODS

### Plant and microbes' collection

Plant material, i.e., roots in case of *leaves V. Negundo*were collected from (Sujanpur, Tihra area of Hamirpur, Himachal Pradesh, India) Wild, in bulk and brought to the laboratory in paper and polyethene bags for further processing. The plant materials were cleaned, dipped in sterile water, dried under shade and ground

to powder before storing in a glass jar until other use (figure 1) (**Samant & Pant, 2006**). The bacterial cultures used for antimicrobial and antifungal activities were gifted from the Department of Microbiology, Indira Gandhi Medical College & Hospital (IGMC), Shimla, Himachal Pradesh. At the same time, the fungal cultures used for antifungal activities have been taken from the Department of Biotechnology, Himachal Pradesh University Shimla, Himachal Pradesh. All the cultures were sub-cultured and stored on agar plates for further experimentation.

**Figure 1** Dried leaves of *V. negundo* and its powdery form (inset)

**Table 1** Used microbes with corresponding antibiotics in the present study

| Microorganism | Antibiotics used |
|---|---|
| Escherichia coli | Tetracycline |
| Klebsiella pneumoniae | Ciprofloxacin |
| Salmonella typhi | Chloramphenicol |
| Staphylococcus aureus | Gentamycin |
| Bacillus subtilis | Tetracycline |
| Pseudomonas aeruginosa | Gentamycin |

**Preparation of plant extract**

The plant extract was prepared using six different solvents (petroleum ether, benzene, chloroform, acetone, methanol, and water) based on their increasing polarity. The plant leaves dried, and powdered material stored in an airtight container was used for solvent extraction. As mentioned above, successful extraction in the mixed solvents was performed using the cold percolation method. In brief, 10g of dried powdered sample *V. negundo* was added to 100 ml of desired solvent (petroleum ether) in the ratio of 1:10 in a 250ml flask and kept on a rotary shaker (150 rpm) overnight at 35ºC (**Mehta *et al*., 2020**). Further, the extract obtained was filtered using Whatman No.1 filter paper and air-dried at room temperature until all solvent from the Petri plate had become extinct. Pure plant extract thus obtained was stored at 4oC in the refrigerator until the stock solution was prepared with DMSO. The residual plant material left after petroleum ether extraction was again used for successive extractions with chloroform, benzene, acetone, methanol, and water, following the method described for petroleum ether (**Mehta *et al*., 2020**).

**Determination of antimicrobial and antifungal activity**

Antimicrobial (antibacterial and antifungal) activities were determined by both disc diffusion methods (**Balouiri *et al*., 2016**)and well agar diffusion (**Chaman *et al*., 2013**), as described in the literature. The minimum inhibitory concentrations (MIC) of the plant extracts required to inhibit the growth of microorganisms in each case were checked using the resazurin dye indicator (**Andrews, 2001**). Resazurin is an oxidation-reduction indicator used to evaluate cell growth, particularly in various cytotoxicity assays. It is a purple non-fluorescent, and non-toxic dye that turns pink and fluorescent when reduced to resorufin by oxidation-reduction within viable cells. Resorufin is further reduced to hydro resorufin (which is colourless).

**Minimum inhibitory concentration analysis**

In brief, under aseptic conditions, 96 well microtiter plates (Himedia/ Tarson) were used for Resazurin-based Microtiter Dilution Assay (**Andrews, 2001**). In brief, 10 mg of extract was dissolved in 1 ml DMSO. The first row of microtiter plates was filled with 100 µl of test materials. All the wells of microtiter plates were filled with 100 µl of nutrient broth. Two-fold serial dilution (throughout the column) was achieved by transferring 100 µl test material from the first row to the subsequent wells in the next row of the same column so that each well has 100 µl of test material in serially descending concentrations. 10 µl of resazurin solution as an indicator was added to each well, and after that, 10 µl of standardized inoculums of each test organism was introduced into the mixture. Each microtitre plate had a set of 2 controls: (a) a column with streptomycin as the positive control and (b) a column with all solutions except the test extract. The plates were incubated at 37°C for 24 h, and the colour change of the well was observed visually. Any colour change followed from purple to pink or colourless was positive. The lowest concentration of plant extract at which colour change occurred was recorded as the MIC value.

**Phytochemical analysis**

For the phytochemical analysis of plant extract, the various assay has been performed, including Barford's test (**Panchal & Parvez, 2019**), Fehling's test (**Yadav & Agarwala, 2011**), Liebermann-Burchard Test (**Racadio *et al*., 2008**), Shinoda's Test (**Usman *et al*., 2009**), terpenoids test, Saponins, alkaloids test following the kinds of literature. Besides, a thin layer chromatography test colour was positive, indicating alkaloids and flavonoids in plant extract (**Hassan *et al*., 2017**).

**Green synthesis of silver nanoparticles**

Green synthesis of silver nanoparticles has been performed following the method described by Srikal et al. (**Srikar *et al*., 2016)**. In brief, a 1mM aqueous solution of silver nitrate ($AgNO_3$) was prepared and used to synthesize silver nanoparticles. In brief, 10ml of plant extract was added to 90ml of the aqueous solution of 1mM silver nitrate (for reduction into Ag+ ions) and incubated overnight at room temperature in the dark. U.V. Spectroscopy monitored the completion of the reaction. The synthesized silver nanoparticles were separated by centrifugation at 15,000 rpm for 20 mins. Dispersion pellets repeated the process in water to obtain coloured supernatant solutions. The pellet was lyophilized, and the sample was stored at -4°C for further use.

**Characterization of Nanoparticles**

Synthesized silver nanoparticles were further characterized by using a UV-visible spectrophotometer, and the particle size was determined by the Dynamic Light Scattering (DLS) technique by measuring the random changes in the intensity of light scattered from a suspension or solution following the method described by **Lim *et al.* (2013**).

**RESULTS AND DISCUSSION**

To check the antimicrobial activity of *Vitex negundo*, the extracts were prepared using different solvents by cold percolation method. Their antimicrobial activities were reviewed by the agar healthy diffusion method. These extracts' minimum inhibitory concentration (MIC) against the pathogenic strains was performed using a resazurin dye indicator. The extracts showing the best results were further used to synthesize nanoparticles. Furthermore, these nanoparticles were analysed qualitatively and quantitatively to be used for future healthcare applications.

**Antimicrobial assay of *V. negundo* plant extract**

The effectiveness of various solvent extracts of *Vitex negundo*, i.e., petroleum ether, benzene, chloroform, acetone, methanol and aqueous extracts, were studied against multiple pathogenic bacterial strains,*i.e. Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Shigella flexneri* and *Escherichia coli* and fungal strains viz *Aspergillus*, *Alternaria alternate* and *Rhizokatonia solani*. The antibacterial assay was performed by using the healthy diffusion method. Different concentrations ranged from 10-40µl of stock solution (100 mg/ml) of each plant extract were separately poured in a 6 mm diameter well to get the final extract concentration in each reasonable range from 1mg-4mg. The result of plates incubated at 37ºC for 24 hrs to observe the effect of different plant extracts was followed by visualizing the zone of inhibition (clearing zone) in each case. In the observed result, chloroform, acetone, and methanol extracts of *Vitex negundo* showed good inhibitory activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* based on the zone of inhibition. In contrast, Petroleum ether, benzene and aqueous extract did not show any inhibitory effect against the pathogenic strains used, table 2. The solvents showing positive results were selected for further experimentation.

**Table 2** Effect of various extracts of *Vitex negundo* against pathogenic microbes.

| Bacterial strains | Extracts of *V. negundo* | | | | | |
|---|---|---|---|---|---|---|
| | Petroleum ether | Benzene | Chloroform | Acetone | Methanol | Aqueous |
| *E. coli* | - | - | - | - | - | - |
| *S. typhi* | - | - | - | - | - | - |
| *S. aureus* | - | - | + | + | + | - |
| *B. subtilis* | - | - | + | + | - | - |
| *P. aeruginosa* | - | - | - | + | - | - |
| *K. pneumoniae* | - | - | - | - | - | - |
| *S. flexneri* | - | - | - | - | - | - |
| Fungal strains | | | | | | |
| *Rhizokatoniasolani* | - | - | - | - | - | - |
| *Aspergillus* | - | - | - | - | - | + |
| *A. alternata* | - | - | - | - | - | - |

Note: (-) = no zone of inhibition, (+) = shows zone of inhibition

**Effect of Different conc. of various extracts of *V. negundo* on pathogenic microbes**

*Chloroform Extract*

The results of different concentrations of chloroform extract on *S. aureus* and *B. subtilis* following the agar well diffusion method have been presented in table 3 and figure 2 (a). A detailed evaluation of the result showed that both *Staphylococcus aureus* and *Bacillus subtilis* exhibit a maximum zone of inhibition (13mm) at a 4mg/ml concentration of *V. negundo* extract (figure 5.1.1a, 5.1.1b). Gentamycin was used as a positive control against *Staphylococcus aureus* and tetracycline against *Bacillus subtilis*, while DMSO was used as -ve control. As shown in table 5.1.1(A), a comparison of results revealed that both plant extracts were effective against the pathogenic bacterial isolates even at lower conc. (10µl/1mg). Further, at higher conc. Of these plant extracts (40 µl/4mg), the inhibition was quite high,i.e., up to 13mm, because the extract used was crude in comparison to the purified positive control,i.e., Gentamycin (21mm) and tetracycline (18mm).

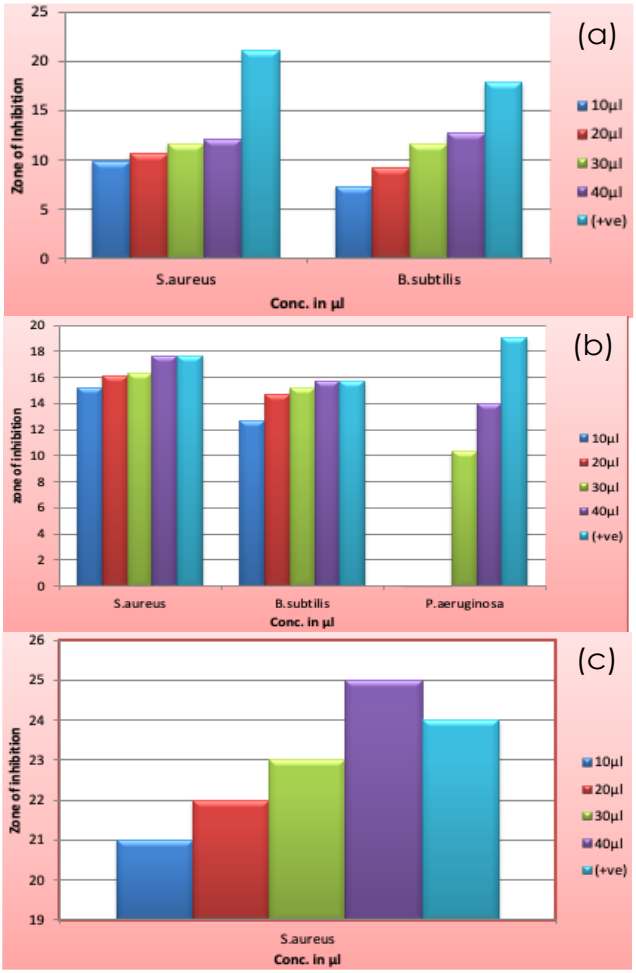


**Figure 2** (a) Antimicrobial activity of chloroform extract of *V. negundo* against selected bacteria. (b) Antimicrobial activity of acetone extract of *Vitex negundo* against selected bacteria. (c) Antimicrobial activity of methanol extract of *Vitex negundo* against selected bacteria.

*Acetone Extract*

The results of different concentrations of acetone extract of *Vitex negundo* by agar well diffusion method have been presented in table 4 and figure 2(b). When used at a 4mg/ml concentration, the acetone extract showed the highest activity against *Staphylococcus aureus* with the zone of inhibition of 17.66 mm, followed by *B. subtilis* (16mm) and least against *P. aeruginosa* (14mm). The positive control Gentamycin showed a 21mm zone of inhibition in the case of *S. aureus and* 19mm in the case of *P. aeruginosa*. In comparison, tetracycline showed an 18 mm zone of inhibition against *B. subtilis*. The results on the effect of various conc. Of acetone extract of *V. negundo* against selected pathogenic isolates revealed good efficacy of three extracts at all concentrations ranging from 10-40 µl. Even at higher conc. of 4mg, the inhibition was quite comparable i.e., 15,16,18mm (figure 5.1.1c-e).

**Table 3** Antibacterial activity of chloroform extract of *V. negundo*

| Microbial isolates | Chloroform extract (µl) | Zone of inhibition (mm)* | | | Mean ± S.D. |
|---|---|---|---|---|---|
| | | $P_1$ | $P_2$ | $P_3$ | |
| *Staphylococcus aureus* | 10 | 10 | 10 | 9.5 | 9.83 ± 0.288 |
| | 20 | 11 | 11 | 10 | 10.66 ± 0.57 |
| | 30 | 12.5 | 12 | 10.5 | 11.66 ± 1 |
| | 40 | 13 | 12 | 11 | 12 ± 1 |
| | +ve Control (Gentamycin) | 21 | 22 | 20 | 21 ± 1 |
| *Bacillus subtilis* | 10 | 8 | 7 | 7 | 7.33 ± 0.57 |
| | 20 | 10 | 9.5 | 8 | 9.16 ± 1 |
| | 30 | 12 | 11 | 12 | 11.66 ± 0.57 |
| | 40 | 13 | 13 | 12 | 12.66 ± 0.57 |
| | +ve Control (Tetracyclin) | 18 | 17.5 | 18 | 17.8 ± 0.288 |

**All results in triplicates*

**Table 4** Antibacterial activity of acetone extract of *V. negundo*.

| Microbial isolates | Acetone extract (µl) | Zone of inhibition (mm)* | | | Mean ± S.D. |
|---|---|---|---|---|---|
| | | $P_1$ | $P_2$ | $P_3$ | |
| *Staphylococcus aureus* | 10 | 15 | 15.5 | 15 | 15.16 ± 0.28 |
| | 20 | 17 | 15 | 16.5 | 16.16 ± 1 |
| | 30 | 16 | 16 | 17 | 16.33 ± 0.57 |
| | 40 | 18 | 17 | 18 | 17.66 ± 0.57 |
| | +ve Control (Gentamycin) | 21 | 21 | 21 | 21 ± 0 |
| *Bacillus subtilis* | 10 | 13 | 12 | 13 | 12.66 ± 0.57 |
| | 20 | 15 | 14 | 15 | 14.66 ± 0.57 |
| | 30 | 15 | 15 | 15.5 | 15.16 ± 0.28 |
| | 40 | 16 | 15 | 16 | 15.66 ± 0.57 |
| | +ve Control (Tetracycline) | 24 | 24 | 24 | 24 ± 0 |
| *Pseudomonas aeruginosa* | 10) | - | - | - | |
| | 20 | - | - | - | |
| | 30 | 11 | 10 | 10 | 10.33 ± 0.57 |
| | 40 | 14 | 15 | 13 | 14 ± 1 |
| | +ve Control (Gentamycin) | 19 | 19 | 19 | 19 ± 0 |
| | -ve Control (DMSO) | - | - | - | - |

*All results in triplicates

*Methanolic Extract*

The inhibitory effect of methanolic extract of *V. negundo* has been presented in table 5 and figure 2 (c). The zones of inhibition were 21 mm, 22 mm, 23 mm and 25 mm, respectively, at 10 µl, 20 µl, 30 µl and 40 µl concentrations. A maximum

zone of inhibition, *i.e.*, 25mm, was observed using 40 µl of the methanolic extract on *Staphylococcus aureus,* which was even higher than the positive control. As shown in table 5 and figure 3, a quick look at all results revealed that the methanolic extract of *V. negundo* constituents showed an excellent inhibitory effect against *S. aureus* even at 10 µl conc. (21mm). The more effectiveness of the plant extract than positive control speaks for its increased efficacy against pathogens, even in crude form. The methanolic extract showed a perfect inhibitory effect against the pathogenic isolates at all concentrations.

**Table 5** Antibacterial activity of methanol extract of *V. negundo.*

| Microbial isolates | The methanol extract (µl) | Zone of inhibition(mm)* | | | Mean ± S.D. |
|---|---|---|---|---|---|
| | | $P_1$ | $P_2$ | $P_3$ | |
| *Staphylococcus aureus* | 10 | 22 | 20 | 21 | 21 ± 1 |
| | 20 | 23 | 21 | 22 | 22 ± 1 |
| | 30 | 24 | 23 | 23 | 23.33 ± 0.57 |
| | 40 | 26 | 25 | 25 | 25.33 ± 0.57 |
| | +ve Control | 24 | 24 | 24 | 24 ± 0 |

**All results in triplicates*

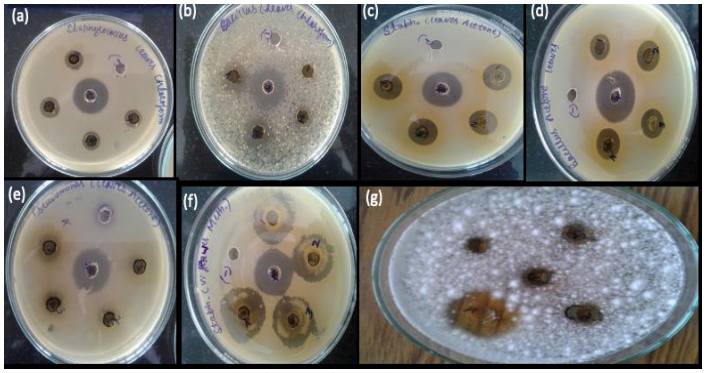


**Figure 3 (**a) Inhibitory zone of chloroform extract on *S. aureus* (b)Inhibitory zone of chloroform extract on *B. subtilis* (c) Inhibitory zone of acetone extract on *S. aureus* (d) Inhibitory zone of acetone extract on *B. subtilis (e)* Inhibitory zone of acetone extract on *P. aeruginosa (f)* Inhibitory zone of methanol extract on *S. aureus (g)* Inhibitory zone of *V. negundo* extract on *A. niger.*

**Phytochemical analysis of *V. Negundo*plant extracts**

Based on the encouraging results obtained with various solvent extract *V.negundo,* efforts were made to analyze the plant extract further, as depicted in table 6. The qualitative phytochemical investigation was performed to test the presence of carbohydrates, alkaloids, tannins, glycosides, steroids, terpenoids, saponins, flavonoids, and soluble starch reported to help inhibit pathogenic microbes. The phytochemical analysis of the plant extracts (Meth, Chl, Act.) of *V. negundo* suggested that *V. negundo* extracts showed the presence of tannins, glycosides, steroids, saponins and flavonoids, figure 3.

**Table 6** Phytochemical analysis of *Vitex negundo.*

| Compounds | Chloroform extract | Acetone extract | Methanol extract |
|---|---|---|---|
| Carbohydrates | - | - | - |
| Alkaloids | - | - | - |
| Tannins | + | + | - |
| Glycosides | - | - | + |
| Steroids | + | + | - |
| Terpenoids | - | - | - |
| Saponins | + | + | + |
| Flavonoids | - | + | + |
| Soluble starch | - | - | - |

Steroidal compounds appeared blue or green in colour or a mixture of the two shades. figure 3(a), alkaloids were confirmed with the appearance of reddish-brown coloured precipitates establishing the presence of alkaloids, figure 3(b), saponins were tested using foam test with the formation of foam, figure 3(c) and Flavonoids were confirmed with appearance orange or red colouration using Shinoda's test, figure 3(d).

**Thin layer chromatography analysis**

The effectiveness of the solvent extracts of *V. negundo* against various microbial pathogens and the presence of several phytochemicals (qualitative confirmation) in these extracts encouraged us to investigate and confirm the antimicrobial agent further. To this extent, thin layer chromatography was carried out with various solvent extracts (acetone and methanol) to ensure the presence of alkaloids and flavonoids. Results have been presented in table 7. Dragendroff's reagent, which gives orange-pink colour with alkaloids, was used to detect alkaloids. No orange-pink colour was developed in both acetone and methanolic extracts of *V. negundo.* For detection of flavonoids and saponins, an anisaldehyde reagent was used that gives pink and purple colour with flavonoids and black with saponins. Both pink, purple and black colours were observed in acetone and methanol extract of *V. negundo,* which confirmed the presence of both alkaloids and saponins in *V. negundo*.

**Table 7** Thin-layer chromatography for confirmation of alkaloids and flavonoids

| Plant extract | Alkaloids | Flavonoids | Rf value |
|---|---|---|---|
| V. negundo (Acetone) | - | + | 3.75 |
| V. negundo (Methanol) | - | + | 3.00 |

**Synthesis of silver nanoparticles**

Nanotechnology can modify and develop beneficial properties of silver nanoparticles (with the high surface area to volume ratio and unique chemical-physical properties), which apply against various pathogenic microbes. The biological method is more advantageous in comparison to chemical and physical processes. The experiment aimed to explore the effectiveness of the silver nanoparticle synthesized from *V. negundo* solvent extract (methanol) as a source of nanomedicine against various microbial diseases. This was also deliberated tried to test the efficacy of these nanoparticles and, on the other to justify the use of these plants in traditional medicine systems, including Ayurveda.

As the methanolic crude extracts show the highest inhibitory activity against some of the pathogenic microbes, silver nanoparticles were synthesized from the methanolic extracts of *V. negundo.* Silver nanoparticle solution is dark brown or dark reddish. The colour of the methanolic extracts of *V. negundo* was light yellow before the addition of $AgNO_3$. after its treatment with $AgNO_3$, its colour changed to dark brown (figure 4 a and b), which indicated the formation of AgNPs, which was later confirmed by UV-Visible Spectra Analysis and Dynamic light scattering analysis. This colour change is due to quantum confinement, nanoparticles' dependence on their optical properties.

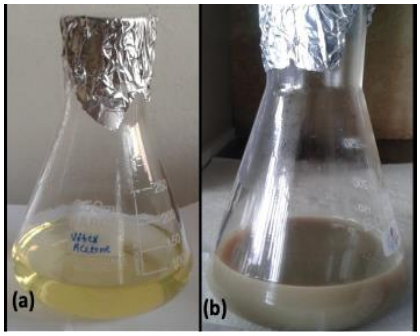


**Figure 4** Synthesis of silver nanoparticle of *V. negundo* (a)plant extract with $AgNO_3$ (b) synthesized nanoparticles.

**Characterization of synthesized silver nanoparticles**

*UV-visible spectra analysis of synthesized silver nanoparticles*

UV-Visible spectroscopy was used to confirm the formation of silver nanoparticles and their stability in an aqueous solution and to examine the size and shape of controlled nanoparticles in aqueous suspensions. Characteristic surface plasmon resonance bands for synthesized AgNPs were observed at 470nm in *V. negundo* figure 5 (a).

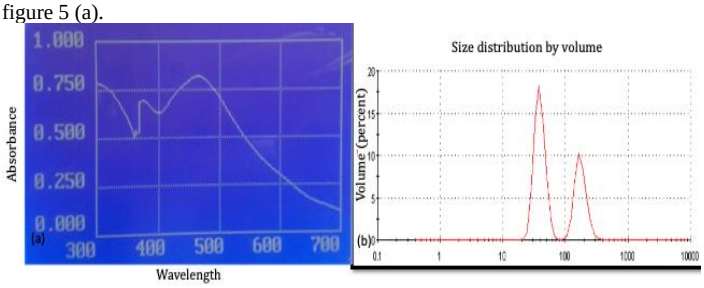


**Figure 5** (a) UV-Vis spectrum of the biologically synthesized silver nanoparticles by *Vitex negundo* (b) DLS graph showing the particle size of methanol extract of *V. negundo*

**Dynamic light scattering**

The dynamic light scattering (DLS) technique was used to determine the size of synthesized nanoparticles. DLS graph (figure 8 (b), table 8) of methanolic extract

of *V. negundo* revealed that the particle size was in the range of 10-300 nm as compared with literature (**Singh *et al.*, 2014**).

**Table 8** Size and volume percent of synthesized silver nanoparticles from different plant extract

| Plant Sample (Extract) | Size in nm | Volume % of particles |
|---|---|---|
| *V. negundo* (methanol) | 33.56 | 11.8 |
| | 13.34 | 85.5 |

**Antimicrobial activity of synthesized nanoparticles**

The 10mg/ml test samples of Ag-NPs were prepared in DMSO for antimicrobial assay. The antimicrobial activity of the synthesized Ag-NPs was tested against all the pathogenic microbial isolates tested earlier by the agar well diffusion method (**Bhuyar *et al.*, 2020a**). Nanoparticles of Methanolic extract *V. negundo* have shown the highest inhibitory activity against *Staphylococcus aureus,* figure 6.

**Figure 6** Inhibitory zone of methanolic Ag-NPs of *V. negundo* on *S. aureus.*

**Table 9** Antibacterial activity of *V. negundo*-based Ag-NPs against pathogenic isolates

| Bacterial isolates | Zone of inhibition (mm) |
|---|---|
| *P. aeruginosa* | 18mm |
| *B. subtilis* | - |
| *E. coli* | - |
| *S. aureus* | 21mm |
| *K. pnueumoniae* | 16mm |
| *S. flexneri* | 12mm |

**Comparison of antimicrobial activity of crude plant extracts and synthesized Ag-NPs through MIC**

10mg of each plant extract and synthesized Ag-NPs were separately dissolved in 1ml DMSO. It was subsequently diluted (2 folds), and further dilutions were made from this stock solution to obtain conc. 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.019mg/ml$^{-1}$. The test organism was introduced into the mixture separately using standard inoculum and incubated at 37°C for 24 hours. The lowest extract concentration that inhibited the test organisms was considered the minimum inhibitory concentration (MIC), table 9. The activity of Ag-NPs was tested against the pathogenic microbes (i.e., *S. aueus, E. coli* and *P. aeruginosa*), which were earlier found to be sensitive to crude methanolic plant extracts, table 10. It was clear that the silver nanoparticles were more potent antimicrobials than the standard and crude extracts. The MIC of Ag-NPs of methanolic extract of *V. negundo* was 0.078mg/ml, which was quite lower than the crude (1.25mg/ml).

**Table 10** MIC (10mg/ml) of acetone extract of *Vitex negundo and V. negundo* based *AgNPs against P. aeruginosa*.

| Concentration of plant extract (mg/ml) | *Vitex negundo* *P. aeruginosa* | |
|---|---|---|
| | *Acetone extract* | *Ag-NPs* |
| 10 | - | - |
| 5.0 | - | - |
| 2.5 | - | - |
| 1.25 | + | - |
| 0.625 | + | - |
| 0.312 | + | - |
| 0.156 | + | - |
| 0.078 | + | - |
| 0.039 | + | - |
| 0.019 | + | - |
| MIC (mg/ml) | 2.5 | 0.019 |

**Table 11** MIC of Ag-NPs of methanolic extracts of *V. negundo* against *S. aureus* in comparison to crude extract

| Plant extracts Microorganisms | B V. negundo | |
|---|---|---|
| | ME | Ag-NPs |
| *S. aureus* | 1.25 | 0.078 |

ME – Methanolic Extract, Ag-NPs Silver Nanoparticles

**DISCUSSION**

Due to their unique features and therapeutic promise in treating AIDS, hepatitis B, cancer, and Diabetes, silver nanoparticles have proven helpful as nanomedicine. Silver particles are also used as catalysts in chemical reactions in solar cells, biochemical sensors, and batteries (**Patil & Kumbhar, 2020**).In the present study, chloroform, acetone and methanol extracts of *V. negundo* showed good inhibitory activity against *Staphylococcus aureus, Pseudomonas aeruginosa*, *Bacillus subtilis* based on the zone of inhibition. The highest zone of inhibition was noticed against *Staphylococcus aureus* (21mm), followed by *Pseudomonas aeruginosa* (18mm) and *Klebsiella pneummoniae* (16mm). **Nagarsekar *et al.* (2010)** found the significant antibacterial activity of petroleum ether extract and steam distilled oil extract against Bacillus subtilis and *Staphylococcus aureus* (**Nagarsekar *et al.*, 2010**).

The results showed that at a 4mg/ml concentration of *V. negundo* extract, both *Staphylococcus aureus* and *Bacillus subtilis* exhibited maximum zone of inhibition (13mm), and plant extracts were effective against pathogenic bacterial isolates even at lower concentrations (10µl/1mg). Furthermore, the inhibition was relatively substantial at greater concentrations of these plant extracts (40 µl/4mg), i.e., up to 13mm, even though the extract employed was crude. The effects of various concentrations of acetone extract of *V. negundo* against selected pathogenic isolates revealed that all three extracts were effective at all concentrations ranging from 10 to 40µl. The inhibition was comparable even at greater concentrations of 4mg, i.e., 15,16,18mm. On *Staphylococcus aureus*, a maximum zone of inhibition of 25mm was reported using 40 µl of methanolic extract, higher than the positive control. Even at 10 µl conc., the methanolic extract of *V. negundo* components had a strong inhibitory effect against *S. aureus* (21mm). Even in crude form, the plant extract outperforms the positive control, indicating more efficiency against pathogens. Antimicrobial activity may be mediated by microbial DNA damage, protein synthesis suppression, or peptidoglycan destruction in the microbial cell wall (**Patil & Kumbhar, 2020**). *V. negundo* extracts showed the presence of tannins, glycosides, steroids, saponins and flavonoids. Nishindine, an alkaloid, flavonoids such as flavones, luteolin-7-glucoside, casticin, iridoid glycosides, an essential oil, and other elements such as vitamin C, carotene, gluco-nonital, benzoic acid, -sitosterol, and C-glycoside are found in the leaves (**Tandon, 2005**). Because the methanolic crude extracts of *V. negundo* have the most substantial inhibitory effect against various pathogenic bacteria, silver nanoparticles were produced from them. Zargar et al. made a methanolic extract of V.N. leaves in 2011(**Dubey *et al.*, 2009; Zargar *et al.*, 2011**). At 470nm, *V. negundo* showed characteristic surface plasmon resonance bands for produced Ag-nanoparticles. The mixture's hue changed from light yellow to dark brown after 24 hours of incubation. The colloidal solution has a distinctive brown colour because of the Ag-NPs' surface plasmon resonance (SPR) characteristics. The UV-Vis spectrophotometric measurement revealed an absorbance peak at 470 nm, which supported the synthesis of Ag-NPs using the leaf extract. Figure 4's right side demonstrates the transformation from light yellow to dark brown. The existence of spherical nanoparticles is indicated by the SPR peak at 470 nm that was detected by UV-Vis spectrophotometry (**Dubey *et al.*, 2009**). The size of produced nanoparticles was determined using the DLS technique. When comparing the particle size of the methanolic extract of *V. negundo* to literature, the DLS graph (figure 8 (b), table 8) revealed that the particle size was in the range of 10-300 nm (**Zargar *et al.*, 2011**). The form and size of as-synthesized silver nanoparticles were well dispersed in nature, with particle sizes ranging from 5 to 47 nm, as determined by TEM **(Kathireswari *et al.*, 2014**). SEM confirmed the particle size of as-synthesized silver nanoparticles in the 40-100 nm range (**Kathireswari *et al.*, 2014**). The most effective inhibitory action against *Staphylococcus aureus* was found in nanoparticles of Methanolic extract *V. negundo.* Ag-NPs were more effective antimicrobials than conventional and crude extracts. Several methods have explained Ag-NPs' bactericidal activity, but their exact mode of action is still unknown. According to several research, the attachment of Ag-NPs impairs the integrity of the bacterial cell membrane (**Parmar *et al.*, 2022**), which interferes with the cells' respiratory system and allows the nanoparticles to penetrate the cells (**Parmar *et al.*, 2022**). Small nanoparticles interact with bacteria more readily than large nanoparticles due to their larger collective surface area, which has a more substantial bactericidal impact (**Abass *et al.*, 2021**). The strength of nanoparticle-mediated antimicrobial activity also depends on the shape of the nanoparticles (**Bhuyar *et al.*, 2020a, b**). Gram-negative bacteria showed stronger resistance to the nanoparticles than gram-positive bacteria, perhaps owing to differences in cell wall architecture (**Abass *et al.*, 2021**). The form of the nanoparticles impacts how powerful they mediate the antibacterial activity. Due to variations in cell wall design, gram-negative bacteria exhibited greater resilience to the nanoparticles than gram-positive bacteria.

## CONCLUSION

Ag-NPs are synthesized using plant extract of *Vitex negundo* through a chemically reducing agent to reduce $Ag^{+}$ ions to Ag-nanoparticles. Six different solvents, including petroleum ether, benzene, chloroform, acetone, methanol, and water, were prepared and further investigated for their antimicrobial and antifungal activities using different bacterial and fungal strains. Besides, the phytochemical analysis is performed to examine the constituent of the plant extract. Phytochemical analysis of synthesized Ag-NPs through DLS suggested that the particle sizes were 10-300 nm. Results concluded that the Ag-NPs exhibit excellent inhibitory activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* based on the zone of inhibition. The antimicrobial activity of the synthesized Ag-NPs suggested that it can inhibit the growth of both gram +ve and gram -ve microorganisms. Besides, The MIC value of Ag-NPs of methanolic extract of *V. negundo* is observed to be 0.078 mg/mL, which was relatively lower than the crude (1.25mg/mL). These observations concluded that synthesized Ag-NPs had better antimicrobial activity and could be necessary for various applications, including medicine, biology, and industry.

### List of abbreviations

AgNp – Silver Nanoparticles
Conc. – Concentration
*V. negundo* - *Vitex negundo*
*P. aeruginosa* - *Pseudomonas aeruginosa*

**Declarations:** Not applicable.

**Ethics approval and Consent to participate:** Not applicable.

**Consent for publication (include appropriate statements)**

I, Prakash Bhuyar hereby declare that I participated in the study and in the development of the manuscript titled “Green biosynthesis of silver nanoparticles (AgNPs) from Vitex negundo plant extract and its phytochemical screening and antimicrobial assessment next to pathogenic microbes”. I have read the final version and give my consent for the article to be published in JBFMS.

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### Authors' contributions

Conceptualization: Sarita Dogra, Mamta Devi Sharma; Methodology: Sarita Dogra, Mamta Devi Sharma; Formal analysis and investigation: Sarita Dogra; Writing - original draft preparation: Sarita Dogra, Mamta Devi Sharma, Puranjan Mishra, Arvind Kumar Bhatt; Prakash Bhuyar; Writing - review and editing: Sarita Dogra, Mamta Devi Sharma, Prakash Bhuyar, Shabana Tabassum, Puranjan Mishra, Arvind Kumar Bhatt; Funding acquisition: Mamta Devi Sharma; Resources: Mamta Devi Sharma; Supervision: Mamta Devi Sharma.

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