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Biogenic Silver Nanoparticles from Leaves Extract of *Vitex negundo* and their Potential Control of Biofilm, Betalactamse and ESBL Producing Bacteria Isolated from Different Food Samples

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A B S T R A C T

The intelligence of revamping metals into their nanoparticles and their application in the biological field made nanotechnology evolve globally. It is one of the modern, contemporary, eco-friendly, non-toxic and economical method that fascinates the researchers around the world. Pathogens were isolated from discrete food samples. They were tested for multi drug resistance, biofilm and betalactamase production. *Vitex negundo* leaves were shade dried and extract was prepared using ethanol and acetone as solvents. Followed by the phytochemical studies the antibacterial activities of the plant extracts were studied. The best extract was selected for silver nanoparticle synthesis and it was characterized by by UV-Vis spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy with Energy Dispersive X-Ray (EDX) analysis and Transmission electron microscopy (TEM) imaging and Energy Dispersive X-Ray (EDX) analysis. The antibacterial activity was examined by agar well diffusion method. In this study we have identified that the silver nanoparticles of ethanol extract of *Vitex negundu* showed the potent anti-microbial activity against biofilm and betalactamse producing bacterial pathogens. The plant has to be further exploited for its strong antimicrobial activity and it can be used as the alternative for the antibiotics against such virulent pathogens.

***Key words*:** *Vitex negundo,* ESBL, Biofilm, Antimicrobial activity, Phytochemicals, Silver nanoparticles

Nanoparticles extend bizarre communication with the biomolecules that include both the surface and inside the cell and these are very small, when compared to human cells they are 100 to 10,000 times smaller in size. They are applied in diverse applications in biology, physics, pharmaceuticals and many more due to their small size and are comparable to the cellular components that facilitate them in the entry of living cells by endocytosis mechanisms [1]. One of the deliberate public health issues is the antimicrobial resistance and bacteria isolated from food exhibit immense resistance to these agents [2]. In food industries biofilms are considered as one of the influential objections due to their accumulation on utensils, surface and products which is also troublesome to evacuate [3]. The endurance of bacteria in stressful habitats like food processing plants, slaughter-houses etc. is endorsed by biofilm production. The superlative resistance to disinfectants and antimicrobials are further boomed by the formation of biofilm [4]. In bacteria the resistance to antibiotics can also be achieved by the production of β-lactamase enzymes which break the β-lactam ring present in the antibiotic [5].

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To culminate this hitch, researches are consistently centralizing on natural products to flourish enhanced remedy against multidrug resistant bacterial strains [6]. Plants produce immense diversity of secondary metabolites. A conventional theory says that extracts from plants that exhibit target sites are highly effective than the antibiotics that use plants against these drug resistant bacterial pathogens [7]. The presence of saponins, tannins, essential oils, flavanoids and phenolic compounds in plants makes them potential antimicrobial agent [8]. Plants afford an excellent source for the synthesis of nanoparticles since it does not contain any toxic chemicals and also acts as natural capping agent [9]. Globally herbal medicines are used as a traditional practice. Herbs contribute to most of the pharmaceutical products as drugs that are in use now. Around 80% of human population in the world use plants for treatment of many diseases. The presence of secondary metabolites in plants is the major reason for its antibiotic activity [8].

Vitex negundo generally named as “chaste tree”, is an Ethno botanically significant plant that possess colossal medicinal properties. A manifold number of biologically active compounds have been extricated from seeds, leaves and roots in the form of flavonoids, volatile oils, iridoids, lignans, steroids and terpenes. Such bioactive compounds are responsible for the antioxidant, antimicrobial, anticancer, anti-inflammatory and antidiabetic activities of the plant [10]. Gonzalo *et al*. [11] reported the antimicrobial effect nanoparticles incorporated

with the extract of *Vitex negundo* against *Escherichia coli* and *Staphyylococcus aureus*. They identified the inhibition of both the bacteria by agar diffusion assays. The current study is aimed to study the antibacterial activity of *Vitex negundo* leaves extracts against the food borne bacterial pathogens. The aspiration of the work is amalgamation of silver nanoparticles of *Vitex negundo* leaves extract and to scrutinize its endurance and control of pathogenic bacteria isolated from food samples.

MATERIALS AND METHODS

Isolation and identification of bacteria [12]

A total of 13 samples (5 chicken and 8 fresh fruit juices – apple, pomegranate, orange and papaya) were possessed from the local shops of Namakkal area. All the samples were aseptically packed and transferred to laboratory within 2 hours. The samples were investigated within 24 hours. Chicken samples were crushed with phosphate buffer using a mortar and pestle whereas fruit juice samples were used as such. A loopfull of all the samples were inoculated in chromogenic agar, *Salmonella Shigella* agar which were properly labelled and incubated for 24 hours at 37°C. The identified colonies were then subjected for further studies.

Antimicrobial susceptibility testing

Kirby-Bauer method using Muller-Hinton agar plates was used for antimicrobial testing in accordance with Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines [13]. A range of 12 to 15 antibiotics was used to test the efficiency of the bacteria. Bacteria that exhibit more than 50% resistance were subjected for the biofilm study.

Determination of biofilm producing isolates [14]

The selected colonies were inoculated on sterile Brain heart infusion agar plates by single streak method and incubated for 24 hours at 37°C. Those colonies that formed black colour were adopted as positive and endured for betalactamse production study.

Screening of betalactamase producing isolates by iodic method [15]

A loop full of test bacteria inoculated in a penicillin solution was taken and inoculated in a solid media which was incubated for overnight and the resulting colony was suspended in penicillin solution to make a density of at least 10^4CFU/ml and left for 1 hour at room temperature. To these 2 drops of starch indicator followed by 1 drop of iodine reagent were added. The disappearance of blue colour indicates a positive result.

ESBL – Gene identification by PCR method

Hong *et al*. [16] procedure was used to screen the ESBL genes (SHV, TEM and CTX-M) on betalactamase positive isolates by a multiplex PCR. The amplified PCR products were subjected to electrophoresis at a 1.5% agarose gel in 1XTBE buffer. The 100bp molecular weight marker was used to measure the molecular weights of amplified products.

Collection of plant material and extraction [17]

In the present study, leaves of *Vitex negundo* have been used were collected from the Salem area, Tamil Nadu, India. The leaves sample was cleaned and air-dried and then powdered with grinder. The powder was extracted in a Soxhlet extractor successively with 200 ml of ethanol and Acetone until colourless extract was obtained at the top of the extractor. Each of the solvent extract was concentrated separately under reduced pressure. After complete solvent evaporation, each of these solvent extracts was weighed and subjected to further studies. Extracts were maintained at a temperature between 2 - 8°C for further studies.

Phytochemical screening of leaves extract

The presence of various phytochemical compounds like alkaloids, carbohydrates, flavonoids, phenols, saponins, quinols and proteins in the leaves extract of *Vitex negundo* was confirmed by using the methods of Solomon *et al.* [18] procedure.

Antibacterial activity of *Vitex negundo* [19]

Agar well diffusion method was employed to investigate the antibacterial activity of leaves extracts against 8 bacteria. The 24 hours old Nutrient broth cultures of test bacteria were swab inoculated on sterile Mueller- Hinton Agar plates and wells of 8 mm were created in the plates with the help of sterile cork-borer. The wells were labeled and filled with different concentration of bark extract, reference antibiotic (Chloramphenicol, 1 mg/ml of sterile distilled water) and DMSO. The plates were incubated in the upright position at 37°C for 24 hours and the zones of inhibition were measured.

Synthesis of silver nanoparticles

Based on the phytochemical and antibacterial study nanoparticles were synthesized using ethanol extract with the help of silver nitrate. Around 0.5ml of ethanol leaf extract of *Vitex negundo* was added to 5ml of silver nitrate ($AgNO_3$) solution with te concentration of 2 mM. A colour change of dark brown to light brown indicates the formation of silver ions (AgNPs). These AgNPs were then subjected for antibacterial study using well diffusion method.

RESULTS AND DISCUSSION

Out of 13 food samples 56 different isolates of various pathogens (*E. coli* - 9, *Enterococcus sp* - 9, *Klebsiella sp* - 7, *Pseudomonas sp* - 4, *Proteus sp* – 2, *Staphylococcus sp* - 9, *Shigella sp* - 12 and *Salmonella sp* - 4) were isolated (Fig 1). This result is correlated with Khater *et al.* [20] who stated the presence of diverse bacterial genera in different food samples. Similarly, Balvindra and Neelam [21] stated that different bacteria especially *Salmonella* sp, *Klebsiella* sp, *Proteus* sp., *S. aureus* and *Pseudomonas* sp were predominantly seen in fruit juices is a dangerous condition for human population.

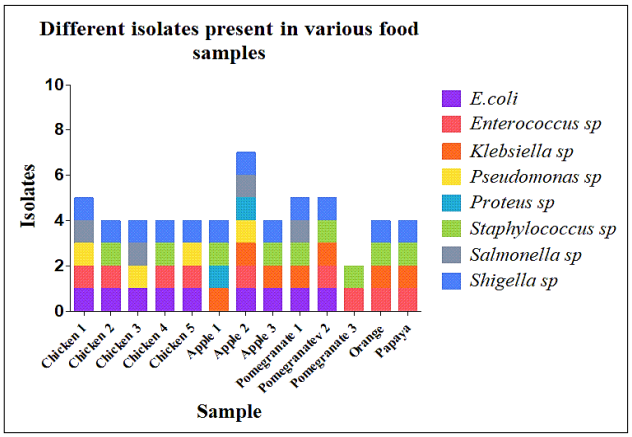


Fig 1 Isolation of bacterial pathogens from food sample

Among this, 32 isolates were exhibiting more than 50% resistance against the tested antibiotics. Likewise in a study performed by Moges *et al.* [22] revealed that the overall MDR

prevalence in tested 833 samples was about 85.8%. In developing countries one of the most serious issues is the evolution of bacteria that are resistance to current antibiotics. It has also been stated that there is a commendable increase in the bacteria that are becoming resistance to most of the antibiotics. Wedajo *et al.* [23] have mentioned that many bacteria are capable to establish resistance against antibiotics but the percentage of resistance may vary based on time, isolates etc.

Bacteria produce an extracellular polysaccharide matrix that helps in dodging host immune system there by helping resistance against antimicrobial agents which leads to chronic infections. Our results showed that out of 32 isolates 24 were positive for biofilm production. Sadik *et al.* [24] stated that more resistance to antimicrobial agents was seen in bacteria inside a biofilm when compared with the plantonic forms and hence those bacteria that are susceptible to antimicrobials may develop resistance after the formation of biofilm. One of the major causes of serious recurrent chronic infection is the biofilm formation. Biofilm formation acts as one of the prime factor responsible for the antimicrobial resistance [25].

In the treatment of antimicrobial resistant isolates β-lactam resistance plays an important role and it is considered as a global obstacle. ESBL producing strains are chiefly present in food animals. Many research carried out globally indicated the presence of ESBL producing isolates in contaminated food of plant and animal origin. This leads to severe illness to mankind and spoilage of food either. In current study out of 24 biofilm positive isolates 20 were identified to present betalactamase activity which was evidenced by the decolorization of iodine. This result is supported by the study made by Chinnam *et al.* [26] that isolated 72 isolates of betalactamase positive bacteria from the foods of animal origin.

ESBL producing organisms are identified in many food samples that harbour MDR isolates without any recognized risk factors. This made the importance in evaluating the ESBL producing isolates from food samples. Even though there are many methods molecular characteristics acts as the impressive method due to their decisive and meticulous results. In the current study betalactamse positive isolates were exposed to PCR amplification for ESBL genes of blaSHV, blaTEM and blaCTX-M. Surprisingly all the 20 isolates were possessing ESBL gene of which 8 different isolates (*E. coli*, *Klebsiella sp*, *Enterococcus sp*, *Pseudomonas sp*, *Proteus sp*, *Staphlococcus sp*, *Shigella sp* and *Salmonella sp*) were taken for plant assay. Our results are supported by Sivakumar *et al.* [27] study. He revealed the presence of 60.62% ESBL producing bacteria from raw food samples (Fig 2).

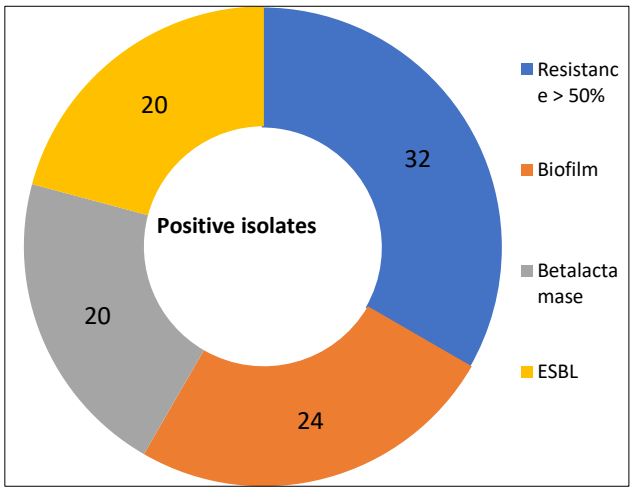


Fig 2 Prevalance of Antibiotic resistance, Biofilm, Betalactamase and ESBL in food isolates

There are many potential and precious bioactive components present in plants that are used for the production of many novel therapeutic agents. So, these researches will help in assisting a novel drug that can be used for human therapy. The phytochemical studies performed on ethanol and acetone extracts of *Vitex negundo* leaves divulged the presence of many biologically active compounds which are listed in (Table 1). It showed the presence of alkaloids, carbohydrates, flavanoids, phenols, tannins, proteins in both ethanol and acetone extracts, whereas terpenoids were present only in acetone extract. Pawar and Kamble [28] performed the phytochemical studies of *Vitex negundo* with 3 different solvents and showed the presence of most of the primary and secondary metabolites in acetone extract correlated with our result. Manju *et al.* [29] has stated the presence of flavanoids in ethanol and aqueous extracts of *Vitex negundu* leaves. These secondary metabolites that are present in plants possess wide biological activities [30].

Table 1 Phytochemical constituents of *Vitex negundo*

Constituents	Name of the test	Ethanol extract	Acetone extract
Alkaloids	Wagner's	+	+
Carbohydrates	Molisch's	+	+
Flavonoids	With NaOH	+	+
Phenols	Ferric chloride	+	+
Saponins	Foam test	-	-
Sterols	Braymer's test	-	-
Tannins	With HCl	+	+
Terpinoids	Salkowski test	-	+
Quinols	Salkowski test	-	-
Proteins	Millon's test	+	+

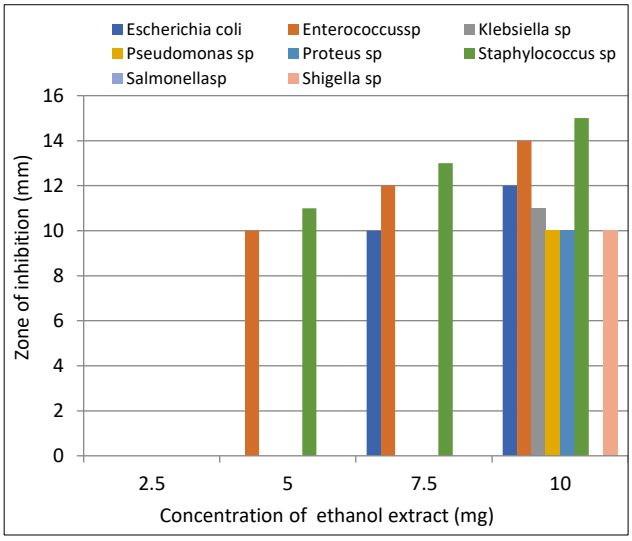


Fig 3 Antibacterial activity of ethanol extract of *Vitex negundo* leaves

Due to the presence of many phytochemical components that are the secondary metabolites of the plant makes it act as the defence mechanism against many microorganisms. On performing antimicrobial activity both the extracts were active against most of the 8 selected antimicrobial resistant, biofilm, betalactamase and ESBL producing food bacteria. In the case of ethanol highest zone of inhibition was found against *Stapylococcus* sp and no zone was seen against *Salmonella* sp. While using 10 mg concentration of ethanol extract all the 7 isolates except *Salmonella* were inhibited (Fig 3). Similar results were observed by Arjit Chaturvedi and Nag [31] who stated that *Vitex negundo* leaves and seeds exhibited maximum activity against all the tested microorganisms except *S.*

typhimurium. In case of acetone extract only 3 out of 7 (*Enterococcus sp*, *Pseudomonas sp* and *Staphylococcus* sp) were inhibited at same concentration (Fig 4).

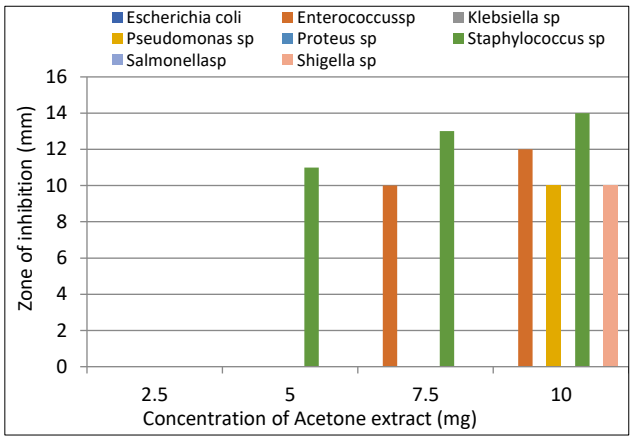
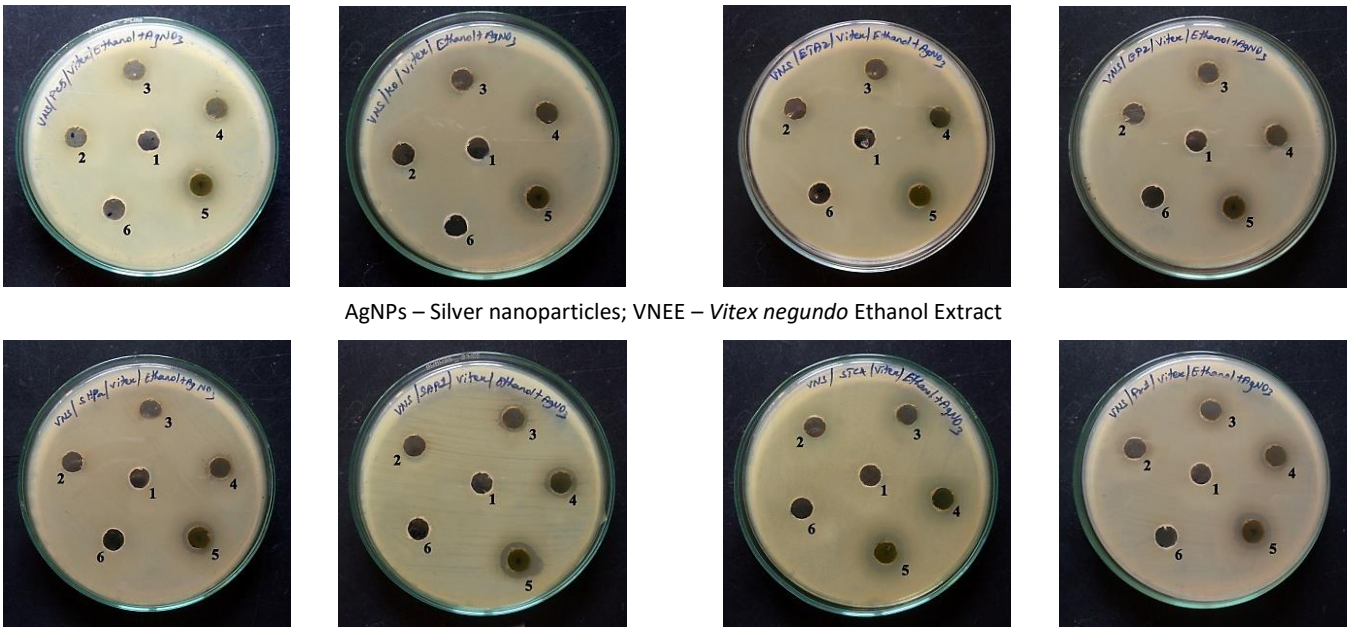


Fig 4 Antibacterial activity of acetone extract of *Vitex negundo* leaves

The remaining 4 isolates (*Escherichia coli*, *Klebsiella sp*, *Proteus sp*, *Salmonella sp* and *Shigella sp*) were seemed to be with no zone of inhibition. Similarly, research made by Priyadarshini *et al*. [32] showed the specificity of ethanol extract of *Vitex negundo* leaves extract against the growth of the bacteria. The synthesized nanoparticles using ethanol extract of *Vitex negundo* leaves effectively controlled all the test pathogens. A clear zone of inhibition was examined against all the 8 isolated pathogens (Fig 5).

Similar results were given by Moideen *et al.* [33]. They said that the nanoparticles exhibited commendable bactericidal property. The cells main function of respiration and permeability may be affected by the attachment of silver nanoparticles in its surface. Particle binding to bacterial surface is directly proportional to the cell surface area. Few nanoparticles may also enter the cell and bind to DNA which leads to the changes in gene expression required for its metabolism. When the size of the particles is smaller it possesses larger surface area for interaction there by exhibiting high bactericidal effect.



AgNPs – Silver nanoparticles; VNEE – *Vitex negundo* Ethanol Extract

1- Ampicillin (10 mg), 2- 2.5 mg (AgNPs of VNEE), 3- 5 mg (AgNPs of VNEE), 4- 7.5 mg (AgNPs of VNEE), 5- 10 mg (AgNPs of VNEE), 6- Control ($AgNO_3$)

Fig 5 Antibacterial activity of silver nanoparticles of ethanol leaves extract of *Vitex negundo*

CONCLUSION

Hence from the above results it is confined that the ethanol and acetone extracts of *Vitex negundo* leaves exhibit potent antibacterial activity against virulent bacterial isolates. It has been evidenced that highest antimicrobial activity was seen in ethanol extract when compared with acetone extract. The antimicrobial property of the plant extract may be due to the presence of various phytochemical components that are the secondary metabolites of the plant. Accordingly further research is needed to figure out the particular component that is responsible for antimicrobial activity exulted by the plant. The outcome of the above research shows the wide spectrum antibacterial activity of *Vitex negundo* leaves against ESBL producing bacteria isolated from food. Hence this plant can be used as the alternative source in the control of multi drug resistant, ESBL producing bacteria. Green synthesis is the best solution for the control of drug resistant bacteria without many side effects.

Conflict of interest

The authors have no conflicts of interest.

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