

ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED USING *SYZYGIUM AROMATICUM*, *CINNAMONUM TAMALA*, *CINNAMONUM CASSIA* PLANT EXTRACT

ABSTRACT

Syzygium aromaticum (Clove), *Cinnamomum tamala* (Bay leaf), *Cinnamomum cassia* (Cinnamon bark) are well known plants in India. All three plants are a rich source of secondary metabolites that find use as antimicrobial agent, in pharmaceutical industry, cosmetics, food and agriculture industry. In regards of antimicrobial activity, green silver nanoparticles were synthesised by using these plants aqueous extract (25% w/v). For silver nanoparticle synthesis different concentration of plant extract were mixed with AgNO₃ solution and exposed to sunlight and estimated by the UV-Visible spectrophotometer. Powdered silver nanoparticles (AgNPs) were dissolved in autoclaved distilled water at different concentration (20mg/ml, 10mg/ml, 5mg/ml, and 2.5mg/ml) and performed antimicrobial activity through agar well diffusion method. Silver nanoparticles of all three plants were showed antimicrobial activity against human bacterial pathogen *Escherichia coli* and *Bacillus subtilis*. The inhibition zone increased with increasing concentration of all three plants (*S. aromaticum*, *C. tamala* and *C. cassia*) AgNPs, so maximum inhibition zone was observed at 20mg/ml for both pathogens. At higher concentration, the inhibition zone of *E. coli* were 2.25 ±0.05, 1.7 ±0.1 and 1.85 ±0.05 cm in presence of *S. aromaticum*, *C. tamala* and *C. cassia* AgNPs respectively. Although, The presence of phytochemical terpenoids, tannin and glycosides are was confirmed by the chemical reagent test. In modern era, the nanoparticle based medicine is explored in pharmaceutical and medical science. Therefore this green synthesis of silver nanoparticle can be explored in pharmaceutical science.

KEYWORD: *Syzygium aromaticum*, *Cinnamomum tamala*, *Cinnamomum cassia*, *Escherichia coli* and *Bacillus subtilis*

1. INTRODUCTION

Nowadays nanotechnology is the most valuable field in modern science. It has great significance to create different applications in various fields of science. In nanotechnology, nanoparticles size range between 1-100nm and this range can goes up to 1-1000nm in size. Nanoparticles have been used in medicine, human health care, textiles, food packaging and cosmetics industry [1-2]. Among all metal nanoparticles, silver nanoparticles have caught the attention of researchers due to their special characteristics like antimicrobial activity, catalytic activity, chemical stability and electrical conductivity [3].

The efficiency of silver nanoparticles has been used to deliver drugs which is made it feasible in the field of medical science. Silver nanoparticles have been used ~~to~~ to resist infections, showing antibacterial and antifungal activity [4-5]. Silver nanoparticles are nontoxic to the immune system, reproductive system and cardiovascular system and not considered to be carcinogenic [6]. Silver ion (Ag⁺) based compounds are relatively toxic to the microorganisms. Silver ions destroy the peptidoglycan layer of the bacterial cell wall, inhibit the bacterial growth and shatter the bacterial metabolism when they interact with macromolecules which are present in the bacterial cell wall like DNA and protein. Silver ion binds with DNA and inhibits the replication in bacteria [7].

Nanoparticles are synthesized by using different approaches like physical, chemical and biological approaches. Chemical mode of synthesis requires short period of time and large amount of nanoparticles. Chemicals which are used for nanoparticles synthesis are highly toxic and non-eco-friendly. So, biological synthesis of nanoparticles could be regarded as a “green nanotechnology” [8]. For biological synthesis some microorganisms (Bacteria, fungi and yeast) have potential to accumulate and detoxify heavy metals due to various reductase enzymes, which leads to reduction of metal salts into metal nanoparticles [9]. Moreover, plant derived nanoparticle synthesis are explored very well because of high demand for nanoparticles in the biomedical and environmental areas. Many medicinal and non-medicinal plants leaves, fruits, stems, roots, and their extracts, have been used for the synthesis of metal nanoparticles [10]. The exact mechanism for plant-mediated synthetic of nanoparticles is still not yet clear but proposed proteins, amino acids, organic acid, vitamins, and secondary metabolites have significant roles in metal salt reduction and nanoparticle synthesis [11].

Plants give a best platform for nanoparticle synthesis because they are free from toxic compounds and they are very eco-friendly. Plants several biomolecules and those contain different functional groups which aid the reduction of silver ion and help in the formation of silver nanoparticles. *Syzigium aromaticum* shows ample sources of phenolic compounds like phenolic acids (gallic acid), flavonol glucosides, phenolic volatile oils (eugenol, acetyl eugenol) and tannins [12]. Recently, thyme oil based silver particle has been reported and showed the antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* [13]. Green synthesis of silver nanoparticles from *S. aromaticum* plant extract have been reported and showed antimicrobial activity [14-15].

In *Cinnamomum tamala* phytochemicals, tannins, alkaloids, flavonoids and terpenoids are reported as major phytochemical constituent and showed pharmaceutical value [16]. Recently, Nahar et al., 2020 reported the green synthesis of silver from *C. tamala* leaf extract and showed antibacterial activity on Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria [17]. However, *Cinnamomum cassia* bark is rich source of lignin, terpenoids, flavonoids, phenylpropanoids, alkaloids and steroids photochemical [18]. Including *C. cassia* other species, *C. zeylanicum*, *C. tamala*, and *C. wilsonii*, are famous herbs and their bark used

for treating cardiovascular, chronic gastrointestinal, and inflammatory diseases [19]. These three plant species are showing great effectiveness for pharmaceutical industry, cosmetics, food and agriculture industries as well as showing great antibacterial and antifungal activity [19-20].

Therefore, the nanoparticle of these plants may be part of nanomedicine as antimicrobial agents. The main purpose of this research is to synthesize silver nanoparticle by using *Syzygium aromaticum* (Clove), *Cinnamomum tamala* (Bay leaf), *Cinnamomum cassia* (Cinnamon bark) extracts and to check their antibacterial effect.

2. MATERIALS AND METHODS

Plant material and chemicals

Syzygium aromaticum (Clove), *Cinnamomum tamala* (Bay leaf), *Cinnamomum cassia* (Cinnamon bark) were purchased from local market, Calcutta, (West Bengal) India. Silver nitrate and antibiotics (Streptomycin) were obtained from SD Fine Chemical Ltd India. All the reagents used in this study were analytical grade.

Preparation of plant extract

The plant samples were cleaned with distilled water to remove dust particles followed by air drying at room temperature (25°C). Bay leaves, Clove and Cinnamon bark were separately crushed to make powder form using a grinder mixer. Five gram from each sample was added into 20ml of distilled water and heated for 30 minutes at 80°C. Then these three plant extracts were centrifuged for 5 min at 5000 rpm followed by filtering using a whatman filter paper grade 1. These extracts were stored at refrigerator (4°C) for further use.

Qualitative Phytochemical tests:

The qualitative analyses of phytochemical in the plants extract were performed through chemical test as previously described [21-22].

Test for tannins: Few drops of FeCl₃ (0.1%) were added to the three different plant extracts to observe blue-black or brownish green colour [21].

Test for glycosides: 2ml of plant extract was dissolved in 2ml water and 1ml of NaOH solution was added. Appearance of yellow colour indicates the presence of glycosides [22].

Test for terpenoids by the Salkowski Test: 5ml of plant aqueous extract was mixed with 2ml of chloroform and then 3ml of conc. H₂SO₄ was added carefully. Appearance of reddish brown colour at the interface indicates the presence of terpenoids [21].

Test for cardiac glycosides by the Keller–Kiliani Test: 5ml of plant extract was mixed with 2ml of glacial acetic acid followed by a drop of FeCl_3 solution and followed by an addition of 1ml conc. H_2SO_4 . The appearance of a brown ring at the interface indicates the presence of cardenolides [21].

Synthesis of silver nanoparticles (AgNPs)

1mM silver nitrate (AgNO_3) solution was prepared in distilled water. 20 ml of AgNO_3 (1mM)solution was separately added with 0.5, 1.0, 2.0 and 4.0 ml of aqueous extracts previously prepared. Thereafter, solutions were kept in sunlight for 2minutes for reaction. After sunlight incubation the colour was changed. The solutions were centrifuged at 10,000 rpm for 20 min to collect nanoparticles and further kept in hot air oven at 60°C . Next day pellet was extracted as dried form and kept in an eppendorf tube for further experiments.

Characteristics of silver nanoparticles synthesized byUV-Vis spectroscopy

After exposure to sun light, the syntheses of AgNPs in solution were characterized by using a UV-visible spectrophotometer. The synthesis of silver nanoparticles was checked by recording the UV-visible spectra of solutions between 300–600 nm.

Agar well diffusion assay for antimicrobial activity of AgNPs

Antimicrobial activity of *S. aromaticum*(Clove), *C. tamala* (Bay leaf), *C. cassia* (Cinnamon bark) water extract and their AgNPs were tested on two isolated human bacteria pathogens *Bacillus subtilis*, and *Escherichia coli*. Antimicrobial activity was conducted through agar well diffusion method [23]. The bacterial organisms were grown in the nutrient broth overnight to attain the colony-forming unit (CFU) of $\sim 10^6$ per/ml. 100 μl of each bacteria culture was spread on the Luria–Bertani agar plates. Dried AgNPs, dissolved in autoclaved distilled water to make 20mg/ml, 10mg/ml, 5mg/ml, and 2.5mg/ml. Agar wells (6 mm diameter) were made with the help of sterilized cork borer and loaded different concentration of AgNPs, AgNO_3 solution (1mM as control) and Streptomycin (0.01mg/ml). From each concentration, 20 μl were added to well and plates were incubated for 24 h at 37°C , and diameters of zone of inhibition were recorded in centimetres.

Statistical Analysis

Graph Pad prism, version 5.01 (GraphPad Software, San Diego, CA) was used for calculation of one-way ANOVA analysis with a Dunnett multiple test. The statistical significance of differences between aqueous extract and AgNPs sample was tested at different p values.

3. RESULTS AND DISCUSSION

Detection of phytochemicals by colour test

Existence of secondary metabolites in any plant samples are mainly used for medicinal activities. They are mainly phenolic compounds, alkaloids, tannins, carbohydrates, glycosides, terpenoids, flavanoids, steroids, etc. which are found all over the plant kingdom. Here, the presence of tannin, glycosides and terpenoids **was noticed** in all three plant extract as shown in table 1. However, Cardiac glycosides, a digitalis compound was absent in all three plant extract. Color appearance of the respective compound test has been shown in figure 1. Previously, the presence of tannin, glycosides and terpenoids have been reported in *S. aromaticum* and played role as pharmacological value in various **traditional** medicines [24]. *Cinnamomum* species retains all three compounds and their extracts have been used **for their** immunomodulatory, anti-inflammatory, antimicrobial, antioxidant, and anticancer **activities** [25].

Phytochemical test	Observation	Inference		
		<i>S. aromaticum</i>	<i>C. tamala</i>	<i>C. cassia</i>
Tannins	Blueish-black colour	++++	++++	+++
Glycosides	Yellow colour	++++	+++	++++
Terpenoids	Reddish brown colour	++++	++++	++++
Cardiac glycosides	Brown ring	-	-	-

Table 1. Phytochemicals tests for *S. aromaticum*, *C. tamala*, and *C. cassia* plant extract

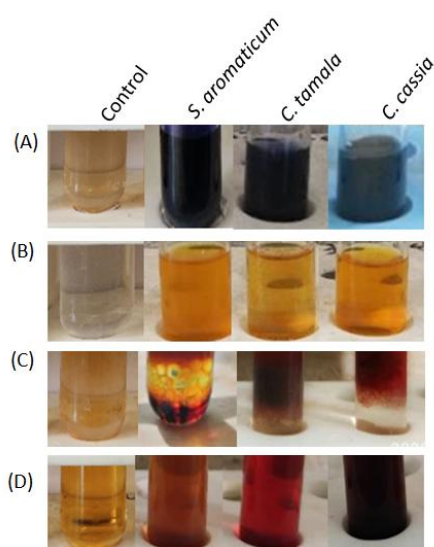


Figure 1. Phytochemical test of *S. aromaticum*, *C. tamala*, and *C. cassia* plant extract (A) tannins (B) glycosides (C) terpenoids (D) cardiac glycosides.

Synthesis of silver nanoparticles using plant extract

Till now much research work has been published on green synthesis of nanoparticles using plant extracts. Biosynthesis of silver nanoparticles of *S. aromaticum* (clove) has been reported as, significant antimicrobial and cytotoxic activity [15, 26]. In this study, the aqueous extract of *S. aromaticum* (Clove), *C. tamala* (Bay leaf), *C. cassia* (Cinnamon bark) were tested for synthesis of AgNPs. Aqueous extract of three plants were separately added to AgNO₃ solution and the changes in colour were observed after sunlight exposure (Figure 2). Different amounts of plant extract (0.5 ml, 1.0 ml, 2.0 ml and 4.0 ml) were added to silver nitrate solution which was tested for absorbance. The change in colour of silver nitrate revealed the formation of AgNPs in solution by the reduction of silver ions to silver nanoparticle [17]. Indeed aqueous extract of these plants contains the several phytochemical that lead to the reduction of silver ions and results into formation for AgNPs. Recently reported, phytochemical chatequin, epichatequin and gallic acid in *Accasellowiana* extract showed the AgNPs synthesis through reduction of silver ions (Ag⁺) to silver nanoparticle (Ag⁰) [2]. In present study, maximum colour changes and spectrophotometer absorption were observed in 4ml extract added silver nitrate solution as shown in figure 2.

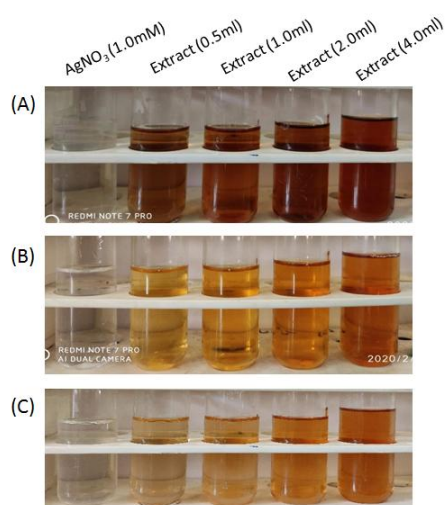


Figure 2. Silver nanoparticles synthesis from aqueous extract of (A) *S. aromaticum*, (B) *C. tamala* (C) *C. cassia* under sunlight exposure.

A research article was published on *C. tamala* AgNPs which was synthesised at 70 °C temperature for 30 min and shown antibacterial activity [27]. Similarly, Silver nanoparticles were synthesized using aqueous extract of cinnamon berks using conventional heating, microwave heating method and proved their role as anti-bacterial activity [28]. Silver nanoparticles from other medicinal plants extract also have been reported and tested for their antimicrobial activity [29-30].

UV-Visible AgNPs Synthesis

The primary characterization of AgNPs bioreduced with plants aqueous extract was conducted by UV-visible spectroscopy. The color changes indicate a possible formation of silver nanoparticles, and therefore, should be validated through spectrophotometer absorption. Change in colour by addition of plant extract demonstrates the reducing ability of the plant extract for synthesis of AgNPs. UV-visible spectra of the silver nanoparticle's colloidal solution synthesised using extract of the *S. aromaticum* (Clove), *C. tamala* (Bay leaf), *C. cassia* (Cinnamon bark) showed the concentration dependent need for silver nitrate reduction. Previously, *C. cassia*, *C. tamala* and *S. aromaticum* plant extract are used for reduction of Ag⁺ ion and their AgNPs synthesis validated through UV-Visible spectrophotometer [26-28]. In present study, the maximum colour development was observed in mixture containing 4ml of plant extract (Figure 1). Spectrophotometric absorbance of maximum colour developed plant extract of all three plants is shown in figure 3.

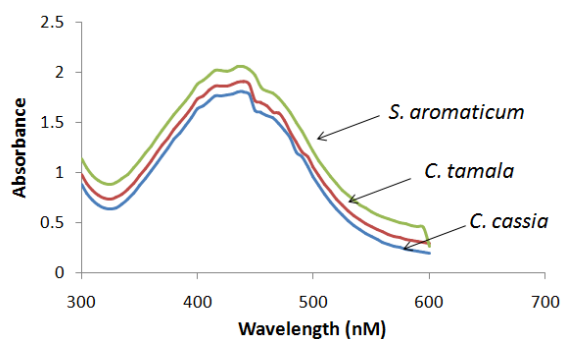


Figure 3. UV-Visible Spectrophotometer of *S. aromaticum*, *C. tamala* and *C. cassia* AgNPs

Antimicrobial activity of *S. aromaticum* AgNPs

Prior to checking antimicrobial activity of AgNPs, antimicrobial activity was tested against *E. coli* and *B. subtilis* (using the agar well diffusion method) of these three *S. aromaticum* (Clove), *C. tamala* (Bay leaf), *C. cassia* (Cinnamon bark) aqueous extract samples at different concentrations (2.5, 5, 10 and 20 mg/ml) (Data not shown here). Earlier, *S. aromaticum* oil as well as their extract has been showed antimicrobial activities against *Staphylococcus aureus*, *E. coli*, *B. subtilis* and *Pseudomonas aeruginosa* [31-32]. Singh et al., 2020 reported *C. cassia* plant extract as antimicrobial activity against *S. aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Streptococcus pyogenes*, *E. coli*, *Salmonella bongori* and *P. aeruginosa* [33]. Similarly, *C. tamala* plant extract also showed antimicrobial activities against *B. subtilis*, *B. atrophaeus*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. typhi*, *K. pneumonia*, *Agrobacterium tumifaciens*, and *Candida albicans* [16, 34]. However, we were looking antimicrobial activity of their AgNPs. Here, all three plant samples showed antibacterial activity against tested pathogen but maximum inhibition zone was reached at 20mg/ml concentration which is considered as reference.

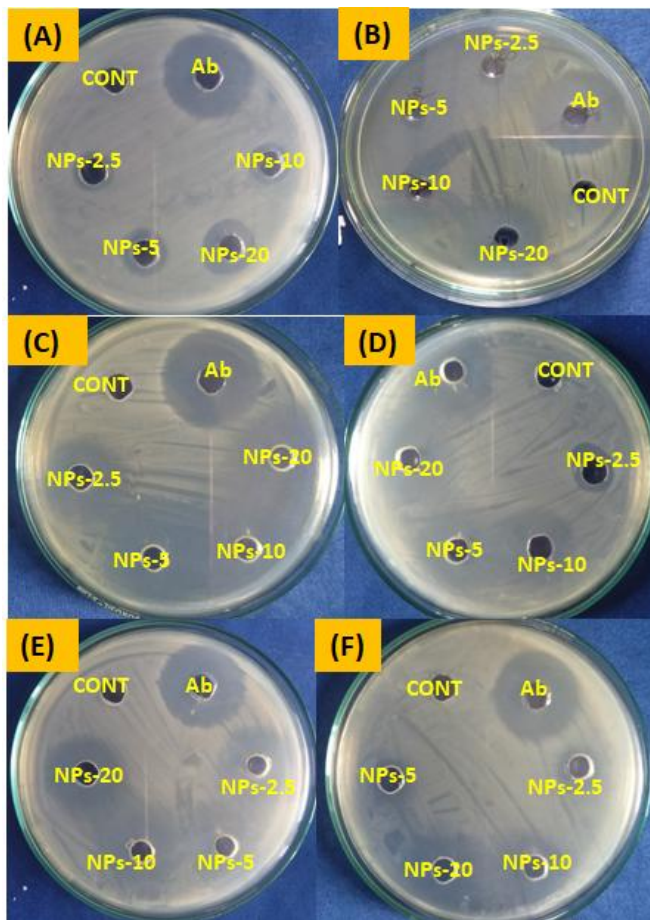
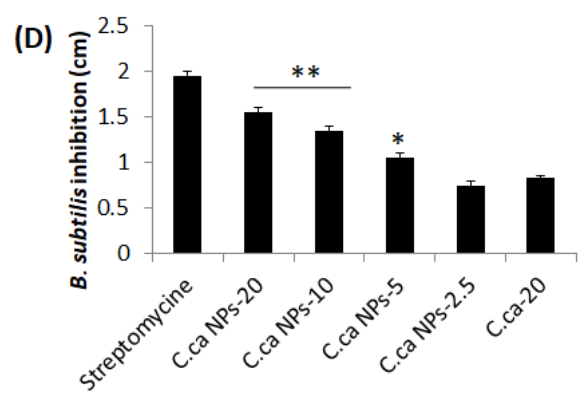
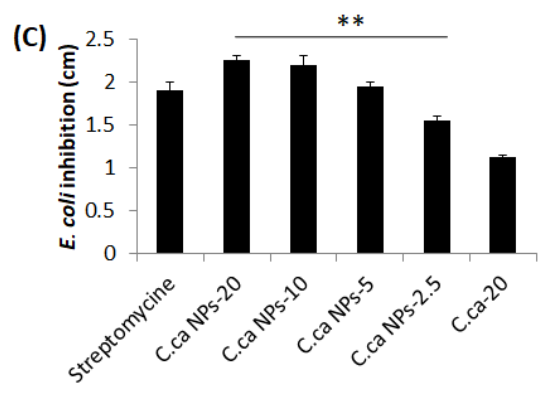
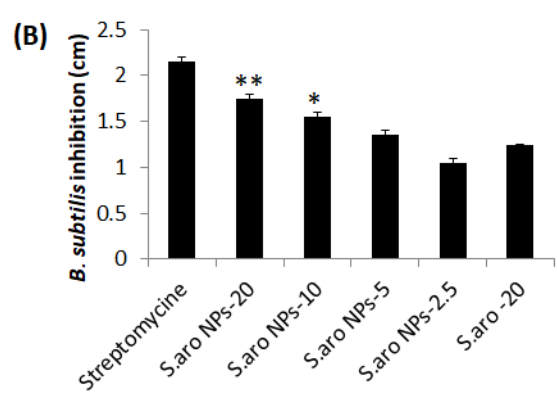
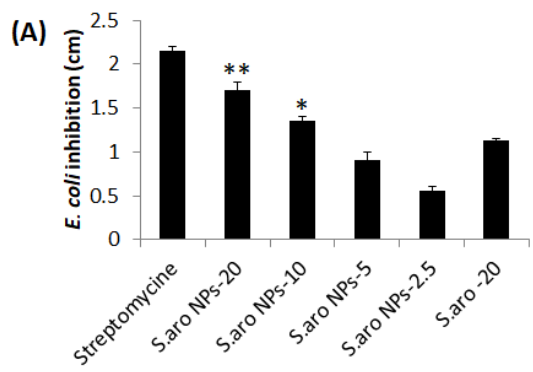


Figure 4. Antimicrobial activity of various AgNPs concentration (A) *S. aromaticum*AgNPs on *E. coli* growth (B) *S. aromaticum*AgNPs on *B. subtilis* growth (C) *C. cassia* AgNPs on *E. coli* growth (D) *C. cassia* AgNPs on *B. subtilis* growth (E) *C. tamala*AgNPs on *E. coli* growth (F) *C. tamala*AgNPs on *B. subtilis* growth. NPs-20 (Ag-Nanoparticles- 20mg/ml), NPs-10 (Ag-Nanoparticles- 10mg/ml), NPs-5 (Ag-Nanoparticles- 5mg/ml), NPs-2.5 (Ag-Nanoparticles- 2.5mg/ml).

Antimicrobial activity of various concentration of *S. aromaticum*AgNPs against to *E. coli* and *B. subtilis* are shown in figure 4a,b. Inhibition zones were decreasing with decreasing the concentration of *S. aromaticum*AgNPs in case of both pathogens. Maximum inhibition zone for *E. coli* and *B. subtilis* were 1.7 and 1.75cm respectively, which was followed by positive control antibiotics inhibition zone (0.01mg/ml) (Figure 5a,b). Water extract of respective plant sample have shown antimicrobial activity but their AgNP have more inhibition potential [15]. Ajitha et al., (2019) have reported *S. aromaticum*AgNPs as antimicrobial against the fungal and bacterial pathogens [14]. Several other research published on green AgNPs as for the antibacterial against *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Azotobacter chroococcum* WR 9, and *Bacillus licheniformis* (MTCC 9555) [15,28,35].



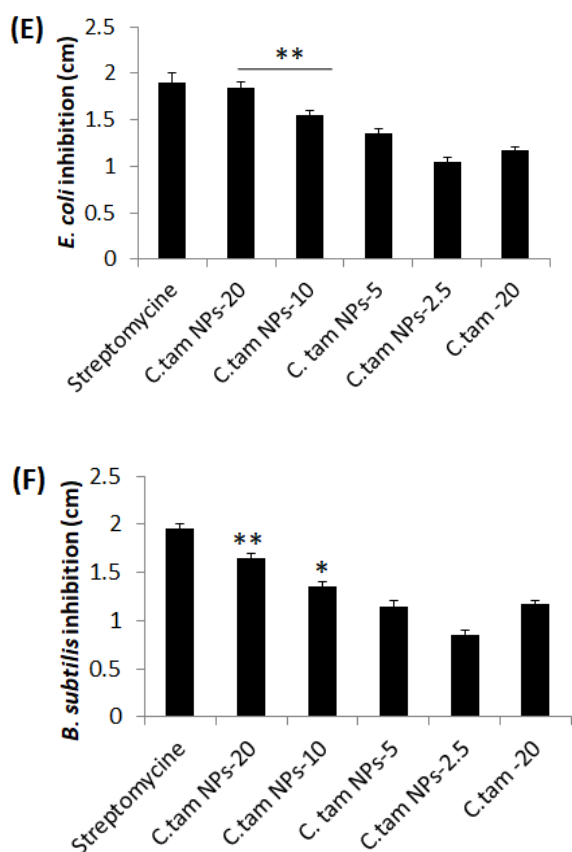


Figure 5. Antimicrobial activity of AgNPs at different concentration on (A) Inhibition zone of *E. coli* by *S. aromaticum* AgNPs (B) Inhibition zone of *B. subtilis* by *S. aromaticum* AgNPs (C) Inhibition zone of *E. coli* by *C. cassia* AgNPs (D) Inhibition zone of *B. subtilis* by *C. cassia* AgNPs (E) Inhibition zone of *E. coli* by *C. tamala* AgNPs (F) Inhibition zone of *B. subtilis* by *C. tamala* AgNPs. NPs-20 (Ag-Nanoparticles- 20mg/ml), NPs-10 (Ag-Nanoparticles- 10mg/ml), NPs-5 (Ag-Nanoparticles- 5mg/ml), NPs-2.5 (Ag-Nanoparticles- 2.5mg/ml) (*S.aro*-20; *S. aromaticum* water extract 20mg/ml, *C.ca*-20; *C. cassia* water extract 20mg/ml, *C.tam*-20; *C. tamala* water extract 20mg/ml). Asterisks indicate a significant difference from the water extract at p value $* < 0.1$, $** < 0.05$

Antimicrobial activity of *C. cassia* AgNPs

The antimicrobial activity of *C. cassia* AgNPs against *E. coli*, and *B. subtilis* are shown in figure 4 c & d, which is prominent over their aqueous extract. The inhibition zone of *E. coli*, at 10 and 20 mg/ml concentration of *C. cassia* AgNPs were substantially more efficient as inhibitors (2.25cm) than streptomycin antibiotics. However, *B. subtilis* was less sensitive and inhibition zone was lesser than positive control antibiotics (Figure 5c,d). Previously Abdalla et al., have proved the antibacterial activity of *C. cassia* AgNPs against *E. coli* [28]. In other experiments, after application of *C. cassia* AgNP substantially inhibited the growth of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus cereus* by 19 mm, 13 mm, 20 mm and 11 mm

respectively[35]. Therefore, AgNPs of this plant showed very prominent antimicrobial against both bacterial pathogens..

Antimicrobial activity of *C. tamala*AgNPs

Aqueous extract of *C. tamala* independently showed the antibacterial activity against above mentioned pathogens, but its AgNPs is another alternative. Naturally, this plant known for the antimicrobial activity, while formation of its nanoparticle is another area to be explored [16]. Dash et al.,(2020) recently reported the antimicrobial activity of *C. tamala*AgNPs against the multidrug-resistant bacterial strains such as *Escherichia coli* (EC-1), *Klebsiella pneumonia* (KP-1), and *Staphylococcus aureus*(SA-1) [27]. In present study also, *C. tamala*AgNPs showed antimicrobial activity against *E. coli* and *B. subtilis* bacterial pathogen but its antimicrobial activity is less than that of the antibiotic (Figure 4e,f). Maximum inhibition zone of *E. coli* was 1.85 and 1.55 cm at 20mg/ml and 10mg/ml concentration of *C. tamala*AgNPs, respectively (Figure 5e). However, in case of *B. subtilis*, maximum inhibition was 1.65 and 1.35 cm corresponding to 20mg/ml and 10mg/ml concentration of *C. tamala*AgNPs respectively (Figure 5f). In present study, *C. tamala*AgNPs was better inhibit for *E. coli* than *S. aromaticum* and approximately similar in case of *B. subtilis*. Among three selected plant, the *C. cassia*AgNPs was showed better antimicrobial activity than others.

4. CONCLUSION

All three plants *S. aromaticum* (Clove), *C. tamala* (Bay leaf), *C. cassia* (Cinnamon bark) pertains the tannin, terpenoids, and glycosides in their respective tissue. All three plants extract showed reducing potential of Ag^+ to Ag^0 formation and leads to formation of silver nanoparticles in solution. In present study also supported the antimicrobial activity of these plant extract but their AgNPs showed better inhibition zone than their respective aqueous extract. AgNPs of *S. aromaticum* and *C. tamala* showed better inhibition area at above 10 mg/ml concentration of AgNPs concentration than their respective water extract control. Interestingly, *C. cassia*AgNPs showed very well antimicrobial activity as compared with other two plants. In future, the AgNPs of these plants can be explored in antimicrobial formulation or other pharmaceutical application.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly products used in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of

knowledge. Also, the research was not funded by the producing company, rather it was funded by personal efforts of the authors.

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