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Evaluation of Antimicrobial Activity of Synthesized Silver Nanoparticles using *Phyllanthus amarus* and *Tinospora cordifolia* Medicinal Plants

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Abstract

The main aim of this study is to evaluate the comparison of antimicrobial efficacy of silver nanoparticles synthesized from aqueous plant extracts of *Phyllanthus amarus* and *Tinospora cordifolia*. The synthesized silver nanoparticles were characterzed by UV-VIS spectroscopy, Fourier Transform Infra-Red spectroscopy, Transmission Electron Microscopy, Dynamic Light Scattering and zeta potential. The antimicrobial activity of synthesized silver nanoparticles was compared with their respective plant extracts by agar well diffussion method and minimum inhibitory concentration was also calculated. The zone of inhibition varied in range of 12 ± 1 to17 ± 0.58 mm with 100 µg/ml silver nanoparticles concentration, while acetonic, methanolic and aqueous extracts of respective plants does not showed any activity even at 1 mg/ml i.e. 10 times more than that of silver nanoparticles. MIC of silver nanoparticles was found to be in a range of 6.25-25 µg/ml against all tested microbes. The antimicrobial activity of synthesized silver nanoparticles was higher than that of the standard drug i.e. streptomycin (for bacteria) and ketoconazole (for fungus). The synthesized nanoparticles of *P. amarus* and *T. cordifolia* have shown good antimicrobial efficacy as compared to plant extracts and may prove to be better antimicrobial agent against wide range of microbes.

Keywords: *Phyllanthus amarus; Tinospora cordifolia*; Silver nanoparticles; Plant extracts; Comparative; Antimicrobial

Introduction

A large number of plants are being used in medicine for therapeutic and prophylactic purposes. The beneficial medicinal effects of plant products typically result from the combinations of secondary metabolites present in the plants. The therapeutic properties of medicinal plants are attributed owing to the presence of active substances such as alkaloids, flavonoids, glycosides, vitamins, tannins, and coumarins [1]. These affect the body of human beings, interact with the pathogens and interrupt their growth at different stages of development and make the body disease free.

Silver has long been recognized as having inhibitory effect on microbes present in medical and industrial process [2]. The most important application of silver and silver nanoparticles is in medical industry such as topical ointments to prevent infection against burn and open wounds [3]. Currently, there is a growing demand for the devising of environmentally agreeable protocols for the synthesis of nanomaterials that would avoid the hazardous by-products associated with current physicochemical processes [4,5].

Biological methods of synthesis have smooth way for the "greener synthesis" of nanoparticles and they offer enhanced manipulation and control over crystal growth and their stabilization. This has aggravated an upsurge in research on the synthesis routes that allow superior control of shape and size for various nanotechnological applications. The use of environmentally benign materials like plant extract for the synthesis of silver nanoparticles offer numerous benefits of ecofriendliness and compatibility for pharmaceutical and other biomedical applications [6]. Because of such a wide range of applications, numerous methods concerning the fabrication of silver nanoparticles, as well as various silver based compounds containing ionic silver or metallic silver have been developed. The antibacterial activities of silver nanoparticles are related to their size, with the smaller particles having higher activities on the basis of equivalent silver mass content.

The present work was carried out in response to the significance of biological synthesis of nanoparticles and the implications of their use in controlling pathogenic microbes. AgNPs were synthesized by utilizing two medicinally important plants, *Phyllanthus amarus* and *Tinospora cordifolia.* A comparative analysis of antimicrobial efficacy of AgNPs from these two plants with their different plant extracts were evaluated against nine different bacterial and two fungal ATCC strains.

Material and Methods

Preparation of the extract

The whole plant of *P. amarus* and *T. cordifolia* was collected locally from Herbal Garden, M.D. University, Rohtak, Haryana, India. The plants were thoroughly washed in distilled water, cut into fine pieces. 10 g of fresh plant material was boiled into 100 ml sterile distilled water (in separate flasks) and filtered through Whatman`s No.1 filter paper. The acetonic, methanolic and aqueous extract was prepared by cold percolation method. The extracts were stored at 4˚C for further experiments.

Synthesis of Silver nanoparticles from plant extract

The aqueous solution of 1 mM silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles. 15 ml of aqueous plant extract was added into 200 ml of aqueous solution of 1 mM silver nitrate for reduction into Ag^+ ions and kept for 15-20 minutes at 60-70°C. This aqueous extract acts as reducing and stabilizing agent for 1 mM of $AgNO_3$.

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Characterization of AgNPs

The synthesized AgNPs from *P. amarus* were further characterized by five techniques:

UV-VIS Spectroscopy: The AgNPs were characterized in a Shimadzu UV-VIS Spectrophotometer. The scanning range for the samples was 300-700 nm. The double distilled water used as a blank reference.

Fourier Transform Infra-red Spectroscopy: (FTIR) To remove any free biomass residue or compound that is not the capping ligand of the nanoparticles, after complete reduction, silver nanoparticles were concentrated by repeated centrifugation (3 times) of the reaction mixture at 15,000 rpm for 20 min. The supernatant was replaced by distilled water each time. Thereafter, the purified suspension was freeze dried to obtain dried powder. Finally, the dried nanoparticles were analyzed by ALPHA FT-IR Spectrometer (from Bruker, Germany) for the detection of different functional groups by showing peaks from the region of 4000 cm^{-1} to 500 cm^{-1}.

Transmission Electron Microscopy (TEM): The shape and size of AgNPs was determined by transmission electron microscopy. A drop (2 ul) of water that dissolved synthesized nanoparticles was placed on a copper grid. The images were obtained with a Tecnai, Twin 200 KV (FEI, Netherlands) at a bias voltage of 200 kV used to analyze samples.

Dynamic Light Scattering (DLS) and Zeta potential: The size distribution or average size of the synthesized AgNPs were determined by dynamic light scattering (DLS) and zeta potential measurements were carried out using DLS (Malvern). For DLS analysis the samples were diluted 10 folds using 0.15 M PBS (pH 7.4) and the measurements were taken in the range between 0.1 and 10,000 nm.

Antimicrobial assay

Preparation of test samples: Test samples of the AgNPs and plant extracts were prepared in DMSO (Dimethyl Sulfoxide) i.e.100 µg/ml and 1 mg/ml respectively. Plant extracts includes acetonic, methanolic and aqueous extract.

Tested microorganisms: The reference bacterial and fungal ATCC strains were used for antimicrobial studies i.e. *Staphylococcus aureus* ATCC 259323, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 43071, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* ATCC 13311, *Klebsiella pneumonia* ATCC 700603, *Shigella flexneri* ATCC 12022, *Serratia marcescens* ATCC 27137, *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404.

Antimicrobial bioassay: The antimicrobial activities of the extracts were determined by agar well diffusion assay [7]. Streptomycin for bacteria (10 µg) and ketoconazole (10 µg) for fungus were used as positive controls and DMSO was used as a negative control. Finally, the petridishes were incubated for 24 hours at 37°C in case of bacteria and 48 hours at 37°C in case of fungus. The diameter of zone of inhibition as indicated by clear area which was devoid of growth of microbes was measured.

Minimum inhibitory concentration method (MIC)

MIC was calculated for AgNPs against all reference strains. This method is based on a micro broth dilution method in 96 multi-well microtitre plates with slight modifications [8]. Indicator resazurin of purple color reduced in the presence of living bacteria. Colour change from purple to pink or to colorless. In the absence of living organism the colour of the indicator were remain purple. The lowest concentration at which colour change occurred was taken as MIC.

Results

In present work, we have synthesized the AgNPs from aqueous extracts of *P. amarus* and *T. cordifolia*. Further we evaluated the comparison of antibacterial activity of AgNPs with plant extracts against reference ATCC bacterial and fungal strains.

Synthesis of AgNPs by green synthesis process

The green synthesis of silver nanoparticles through plant extracts were carried out. On mixing the aqueous plant extract of *P. amarus* and *T. cordifolia* with silver nitrate solution (1 mM), a change in the colour from pale yellow to dark brown was observed for *P. amarus* and *T. cordifolia* (Figure 1).

Characterization of AgNPs

The UV-VIS absorption spectra of the Ag NPs of *P. amarus* and *T. cordifolia* were shown in Figure 2. A peak specific for the synthesis of silver nanoparticles was obtained at 420-430 nm by UV-Visible spectroscope. FTIR analysis showed the presence of bands due to aldehydic C–H stretching (2,914 and 2,847 cm^{-1}), N–H bend (1,514 and 1,462 cm^{-1}) and C-O stretch (dialkyl) (1,018 cm^{-1}) (Figure 3a). FTIR analysis showed the presence of bands due to aldehydic C–H stretching (2,914 and 2,848 cm^{-1}), C-O stretch (1,634 cm^{-1}) arises from carbonyl group, C–O stretching (carboxylic acid) (1,361 cm^{-1} and1, 201 cm^{-1}) and C-O stretch (dialkyl) (1,019 cm^{-1}) (Figure 3b). TEM has revealed the size of silver nanoparticles 51 ± 28 nm and 53 ± 31

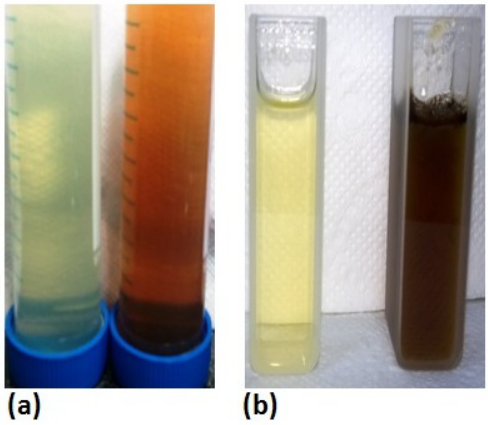

Figure 1: Figure showing colour change from pale yellow to dark brown indicating the synthesis of AgNPs of (a) *P. amarus* and (b) *T. cordifolia*.

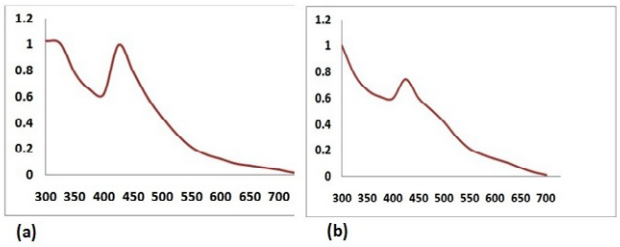

Figure 2: Figure showing Absorption Spectra of AgNPs of (a) *P. amarus* and (b) *T. cordifolia* in a range of 300-700 nm.

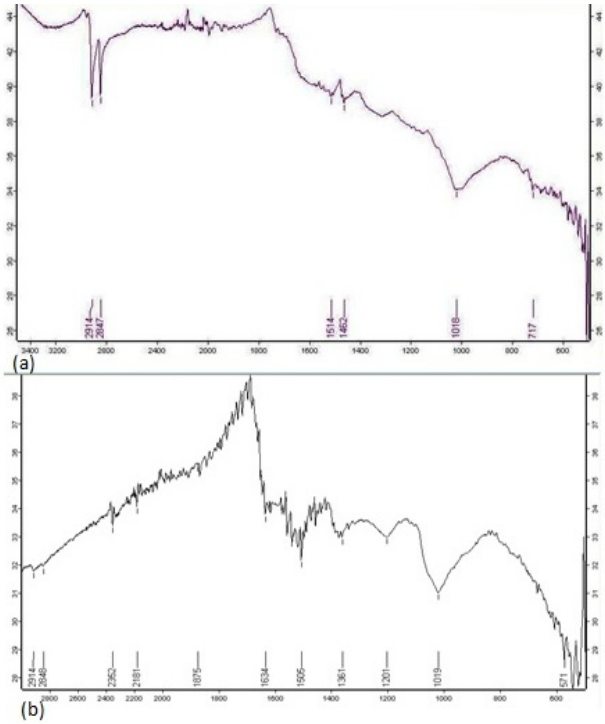

Figure 3: FTIR of AgNPs of (a) *P. amarus*and (b) *T. cordifolia*.

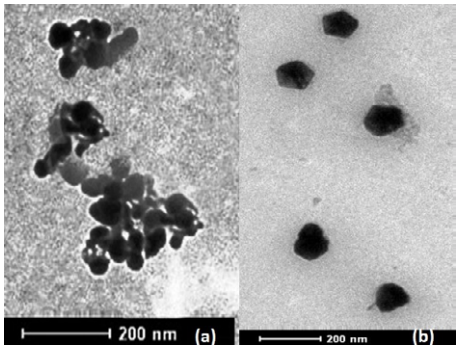

Figure 4: Image of TEM of AgNPs of (a) *P. amarus* and (b) *T. cordifolia*.

nm by *P. amarus* and *T. cordifolia* respectively. It gave a clear image of silver nanoparticles. The TEM image showing silver nanoparticles synthesized using plant extract confirmed the development of silver nanostructures by *P. amarus* and *T. cordifolia* (Figure 4). DLS and zeta potential graph of AgNPs of *P. amarus* and *T. cordifolia* have shown an average size of 33.7 nm 35.4 nm (Figure 5) respectively. Zeta potential graph shows that particles carry a charge of -18 mV and -11 mV in case of *P. amarus* and *T. cordifolia* respectively (Figure 6). Poly disparity index (PDI) was also calculated and found to be 0.46 for AgNPs of *P. amarus* and 0.48 for AgNPs for *T. cordifolia*.

Antimicrobial assay

Antimicrobial activity of AgNPs was studied against 9 different reference bacterial and 2 fungal strains.

Agar Well Diffusion test of AgNPs

The effect of different concentration i.e. 100 µg/ml of AgNPs, 1 mg/ml of plant extracts, against different bacteria and fungus were reported in case of *P. amarus* and *T. cordifolia* (Table 1). The zone of inhibition was found to be in the range of 12 ± 1 to 17 ± 0.58 mm. Maximum antibacterial activity was shown by AgNP extract of *T. cordifolia* with inhibition zone minimum of 17 ± 0.58 mm (at AgNPs concentration of 100 µg/ml) against *P. aeruginosa*. Whereas plant extracts has not been shown any antibacterial activity against any even at 1 mg/ml. Moreover, AgNPs have shown more antimicrobial activity than that of the standard drug i.e. streptomycin (for bacteria) and ketoconazole (for fungus). Also MIC in case of AgNPs was found to be 6.25-25 µg/ml (Figure 7).

Discussion

Synthesis and characterization of AgNPs

Synthesized AgNPs exhibit yellowish brown colour in aqueous

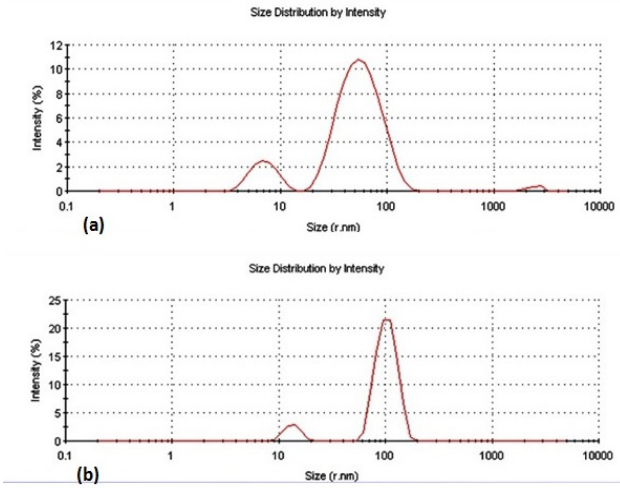

Figure 5: DLS of AgNPs of (a) *P. amarus* and (b) *T. cordifolia*.

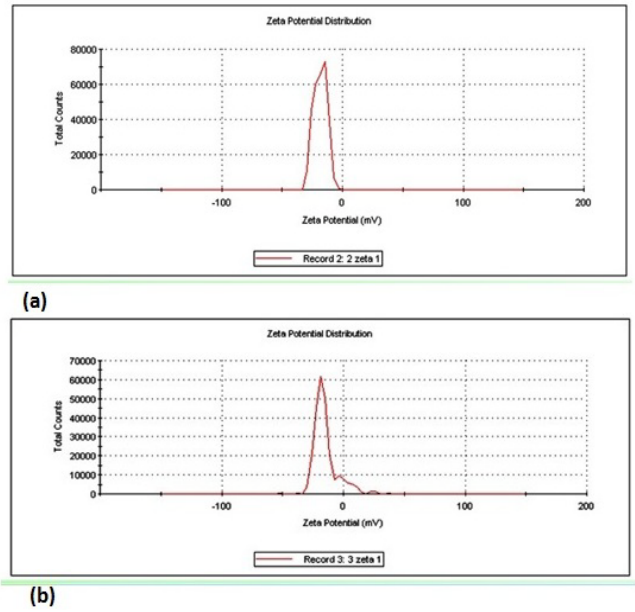

Figure 6: Zeta potential of AgNPs of (a) *P. amarus* and (b) *T. cordifolia*.

S.No.	ATCC Reference Strains		Zone of Inhibition (in mm)				
		+ve control	AE (1mg/ml)	ME (1mg/ml)	AQE (1mg/ml)	SNE of *P. amarus* (100 µg/ml)	SNE of *T. cordifolia* (100 µg/ml)
1	*Enterococcus faecalis* ATCC 29212	13 ± 0.39	-	-	-	14 ± 0.13	15 ± 0.33
2	*Klebsiella pneumonia* ATCC 700603	12 ± 0.31	-	-	-	15 ± 1	16 ± 1
3	*Escherichia coli* ATCC 25922	11 ± 1	-	-	-	15 ± 0.58	14 ± 0.58
4	*Shigella flexneri* ATCC 12022	12 ± 0.58	-	-	-	15 ± 0.73	14 ± 0.33
5	*Salmonella typhi* ATCC 13311	13 ± 0.58	-	-	-	14 ± 0.87	13 ± 0.47
6	*Proteus mirabilis* ATCC 43071	11 ± 0.76	-	-		13 ± 1	13 ± 1
7	*Pseudomonas aeruginosa* ATCC 27853	10 ± 0.13	-	-	-	17 ± 0.11	17 ± 0.58
8	*Staphylococcus aureus* ATCC 259323	13 ± 0.77	-	-	-	14 ± 0.17	15 ± 1
9	*Serratia marcescens* ATCC 27137	10 ± 0.87	-	-	-	12 ± 1	15 ± 1
10	*Aspergillus niger* ATCC 16404	13 ± 0.51		-	-	15 ± 0.58	16 ± 0.58
11	*Candida albicans* ATCC 10231	12 ± 0.33	-	-	-	13 ± 0.58	14 ± 0.58

AE=Acetonic Extract, ME=Methanolic Extract, AQE=Aquous Extract, SNE=Silver nanoparticles Extract , +ve control taken as streptomycin in case of bacteria and ketoconazole in case of fungus.

Table 1: Results of antibacterial activity of different plant extracts and AgNPs of *P. amarus* and *T. cordifolia* against reference ATCC bacterial and fungal strains.

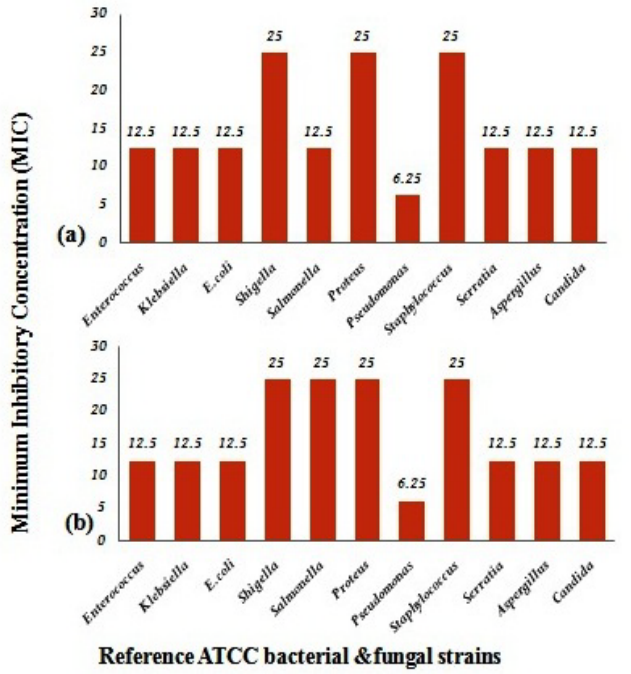

Figure 7: MIC of AgNPs of (a) *P. amarus* and (b) *T. cordifolia* against reference ATCC bacterial and fungal strains.

solution due to excitation of surface plasmon vibrations in silver nanoparticles [9,10]. It was due to the reduction of Ag^+ which indicates the formation of AgNPs. The phytochemicals involved in reduction are mainly alkaloids like berberine, tinosporin etc. in *T. cordifolia* and lignans like phyllanthin, hypophyllanthin etc. in *P. amarus* along with other phytochemicals which are present in plant extracts. The UV-VIS spectra have shown a peak specific at 420-430 nm for synthesis of nanoparticles. It is well known that colloidal silver nanoparticles exhibit absorption at the wavelength from 390 to 420 nm due to Mie scattering [11]. Hence, the band at 420-430 nm can be attributed to the property of Mie scattering. This may not include the protecting agent, because the Mie scattering responds only to the silver metal [12]. The FTIR analysis strongly supported the capping behaviour of bio-reduced synthesized silver nanoparticles which in turn imparted the high stability of the synthesized silver nanoparticles. Dynamic light scattering (DLS) is a technique used to determine the size, size distribution profile and poly dispersity index of particles in a colloidal suspension. A PDI is a measurement for distribution of AgNPs from 0.001 to 0.5, if PDI greater than 0.5 values, it indicates the aggregation of particles. From this, it was clear that AgNPs synthesized from the *P. amarus* extracts does not aggregate at all. Zeta potential measures the potential stability of the particles in the colloidal suspension. Silver nanoparticles generally carry a negative charge. All silver nanoparticles synthesized from *P. amarus* and *T. cordifolia* showed negative charge and were stable at room temperature [13,14].

Antimicrobial efficacy

Some of the chemical antimicrobial agents are irritant and toxic, while plants are easily available, safe, and nontoxic in most cases, but do not have as effective as other chemical agents. Therefore, there is vital need and much interest in finding ways to formulate new types of safe and cost-effective biocidal materials [15]. Although, there are number of researchers which reported that *P. amarus* and *T. cordifolia* have shown antimicrobial activity against pathogenic microbial strains [16-20]. Whereas, the antimicrobial properties of silver compounds and silver ions had been historically recognized and applied in the wide range of applications [21]. Due to the growing need to develop environmentally benign technologies in material synthesis, biosynthesis of nanoparticles has received considerable attention [22]. Also environment friendly AgNPs have gained insight as an excellent antimicrobial agent due to its non-toxic effect on human cells in its low concentration and weaker ability to develop resistance towards silver ions [23,24]. On the other hand, plants have a broad variety of metabolites that can help in the reduction of silver ions, and are quicker than microbes in the synthesis of AgNPs, that is the major advantage of using plant extracts for AgNPs synthesis. The main mechanism considered for synthesis of silver nanoparticles is plant-assisted reduction due to phytochemicals like flavones, organic acids, and quinones are water-soluble phytochemicals that are responsible for the immediate reduction of the ions [25]. Therefore, in this study, we compared the antimicrobial properties of synthesized AgNPs (were found to be effective antimicrobial agents) with their different plant extracts. Our results showed that antimicrobial activity of AgNPs from both the plant were found to be significant against all bacterial and fungal ATCC strains while all plant extracts does not showed any activity if taken 10 times more than the amount of AgNPs. Further

when they are taken more than 20-30 times than that of AgNPs i.e. 2-3 mg/ml, then they showed some activity, still lower as compared to AgNPs. Also, antimicrobial activities shown by AgNPs were more than that of the standard drug i.e. streptomycin in case of bacteria and ketoconazole in case of fungus. Whereas, MIC of AgNPs was found to be in a range of 6.25-25 µg/ml against all microbes tested. Moreover the synthesized AgNPs enhances the therapeutic efficacy and folklore claim of both the plants when combining with silver.

Conclusion

Biosynthesis of silver nanoparticles was carried out using by using the aqueous extracts of medicinal plants with the bio-reduction of silver ions in short period and tested for their antimicrobial activity. The AgNPs of *P. amarus* and *T. cordifolia* have shown good antimicrobial efficacy and hence has a potential to be used as antimicrobial agent against wide range of microbes over conventional antibiotics.

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