Original Article

Antimicrobial and Cytotoxic Activity of *Ocimum tenuiflorum* and *Stevia rebaudiana*-Mediated Silver Nanoparticles – An *In vitro* Study

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Abstract

Background: Silver nanoparticles (AgNPs) are the nanoparticles of silver between 1 nm and 100 nm in size. In this study, AgNPs were extracted from *Ocimum tenuiflorum* and *Stevia rebaudiana* which is a medicinal plant of Indian origin, worshipped by the Hindus and used in Ayurvedic medicine since ancient times. **Aim:** The aim of the study was to assess the antimicrobial and cytotoxic effect of AgNPs reinforced with the herb *O. tenuiflorum* and *S. rebaudiana* against oral pathogens. **Materials and Methods:** In this *in vitro* study, the organisms used were *Streptococcus mutans, Staphylococcus aureus, Lactobacillus* sp., and *Candida albicans*. Agar well-diffusion method was used to assess the antimicrobial efficacy of the nanoparticles at 25 µL, 50 µL, and 100 µL. To assess the cytotoxic effect, brine shrimp lethality assay was used. **Results:** Zone of inhibition was found to be highest at 100 µL against *S. mutans, S. aureus, Lactobacillus* sp., and *C. albicans*. The cytotoxic activity at 5 µL and 10 µL was 0%. The maximum cytotoxicity was seen at 80 µL where 30% of the Nauplii's died. **Conclusion:** The findings from this study suggest that AgNPs reinforced with *O. tenuiflorum* and *S. rebaudiana* extracts has the potential as an antimicrobial agent and has less cytotoxic effect on brime shrimp and can be used as an alternative to commercially available antimicrobial agents.

Keywords: *Anti-microbial, Ocimum tenuiflorum, oral pathogens, silver nanoparticles and Stevia rebaudiana*

Introduction

Dental caries is a disease of complex etiology. Microorganisms play a crucial role within the etiology of dental caries.[1] Reducing their levels within the oral cavity would offer an additional rationale for the prevention of cavity. The onus of the dentist to come up with robust, innovative, effective, feasible, and new strategies to manage the disease. One such strategy would be to verify the huge wealth of medicinal plants. Supportive periodontal therapy to maintain the improved periodontal health after active periodontal therapy has been shown to reduce the risk of tooth loss. Oral hygiene instructions for patients are usually effective among motivated patients.[2] People on all continents have long applied poultices and imbibed infusions of many indigenous plants dating back to the prehistoric eras. Currently, of the one-quarter to at least one half of all pharmaceuticals dispensed in the United States having plant origins, only a few are intended to be used as antimicrobials.[3]

Tulsi, scientifically referred to as *Ocimum sanctum*, is a time-tested premier medicinal herb. It is a plant of Indian origin, worshipped by the Hindus and utilized in Ayurvedic medicine since ancient times. Hence, it is also termed as the "queen of herbs" or "the mother medicine of nature". It is an herb that is bestowed with enormous antimicrobial substances and is used to treat a variety of illnesses ranging from diabetes mellitus, arthritis, bronchitis, skin diseases, etc.[4-6] Recent studies have additionally exhibited significant anticancer properties of *O. sanctum*.[7]

Literature review reveals that the antimicrobial property of Tulsi has been tested against a variety of microorganisms such as *Candida albicans, Staphylococcus aureus*, enteric pathogens, *Klebsiella, Escherichia coli,* and Proteus.[8] It has also demonstrated antigonorrheal efficacy against multiresistant strains of Neisseria

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gonorrhea and clinical isolates of beta lactamase-producing methicillin-resistant *S. aureus*.[9,10]

Nanomaterials usually refer to tiny solid particles with a diameter of 1–100 nm. Nanomaterials are promising in antibacterial therapies due to their enhanced and unique physicochemical properties such as ultra-small sizes, large surface-area-to-mass ratio, and increased chemical reactivity. Moreover, NPs combined with polymers or coated onto biomaterial surfaces were found to exhibit superior antimicrobial properties in the oral cavity.[11]

Metal and organic NPs have been applied in the several areas of dentistry because of their broad-spectrum bactericidal properties.[12] Smaller NPs could release their corresponding ions more to get a far better antibacterial effect. Many sorts of research focused on the antibacterial properties of NPs and showed that NPs possessed superior antibacterial activity in bacteria of drug resistance.[13-15] Melatonin as a topical and systemic formulation could be a potential host modulatory agent for periodontitis management.[16]

Conventionally, silver is an inorganic metal, exercised as antimicrobial agents for the treatment of several diseases such as Neisseria gonorrhea. Recently, silver has been extensively applied as silver nanoparticles (AgNPs) because it has a larger surface area than silver. The transmission electron microscopic analysis indicated the presence of AgNP inside the mitochondria and nucleus, implicating their direct involvement in the mitochondrial toxicity and DNA damage. A possible mechanism of toxicity is proposed which involves disruption of the mitochondrial respiratory chain by AgNP leading to the production of ROS and interruption of ATP synthesis, which in turn cause DNA damage. It is anticipated that DNA damage is augmented by deposition, followed by interactions of AgNP to the DNA leading to cell cycle arrest in the G2/M phase.[17] The efficacy of AgNPs synthesized from bio resources is a very effective antimicrobial agent, anticancer, photocatalytic, antioxidant, and mosquitocidal property have been proved.[18] Hence, the impetus for the study was the nonavailability of literature about the antimicrobial activity of Tulsi (*Ocimum tenuiflorum* and *Stevia rebaudiana*) mediated AgNP against caries-causing microorganisms such as *Streptococcus mutans, Streptococcus aureus, Lactobacillus,* and *C. albicans.*

Materials and Methods

Study design

In vitro study.

Ethical approval

Prior to start of the study, ethical clearance was obtained from the Institutional Ethics Committee (SRB reference No: IHEC/SDC/PHD-1902/21/82), Saveetha Dental College, SIMATS.

Collection and preparation of plant

Fresh powdered *O. tenuiflorum* and *S. rebaudiana* plant leaves were purchased from the market of South India, identified and authenticated by Botanist. The powder of *O. tenuiflorum* and *S. rebaudiana* is stored in an airtight container separately. 500 mg of *O. tenuiflorum and* 500 mg *S. rebaudiana* powder is diluted with 100 ml of distilled water and boiled for 20 min at 70°C–80°C using a heating mantle. The extract is filtered using Whatman No 1 filter paper and allowed to stand undisturbed for 20 min [Figure 1].

Preparation of silver nanoparticles extract

30 mM of silver nitrate is weighed and mixed with distilled water of 60 ml. The silver nitrate solution is mixed with 40 ml of filtered plant extract and was permitted to stand in a magnetic stirrer for 1 h at 380–420 rpm and kept in a shaker for intermixing of the particles to obtain green synthesis. UV spectrometers periodically monitored for every 24 h for 3 days for the reduction of silver nitrate to AgNPs. The color change was visually noted and photographed. Using Lark refrigerated centrifuge, the AgNPs solution is centrifuged at 8000 rpm for 10 min, and the pellet is collected and washed with distilled water twice. The final purified pellet is collected and dried at 100°C–150°C for 24 h, and finally, the nanoparticles powder is collected and stored in airtight Eppendorf tube [Figure 2].

Characterization of silver nanoparticles

The synthesized AgNPs solution is primarily characterized using ultraviolet (UV) Visible spectroscopy. 3 ml of the solution is taken in the cuvette and scanned using double beam UV-visible spectrophotometer from 300 to 650 nm wavelength. The results were recorded from the graphical analysis [Graph 1].

Antimicrobial activity

Media preparation

One hundred mL of Mueller Hinton agar for *S. mutans, S. aureus, Lactobacillus* sp., and SDA agar for *C. albicans*

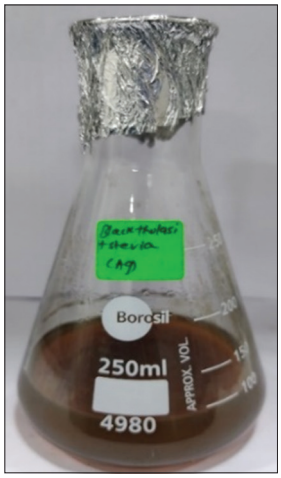


Figure 1: Plant extract (*Ocimum tenuiflorum* and *Stevia rebaudiana*)

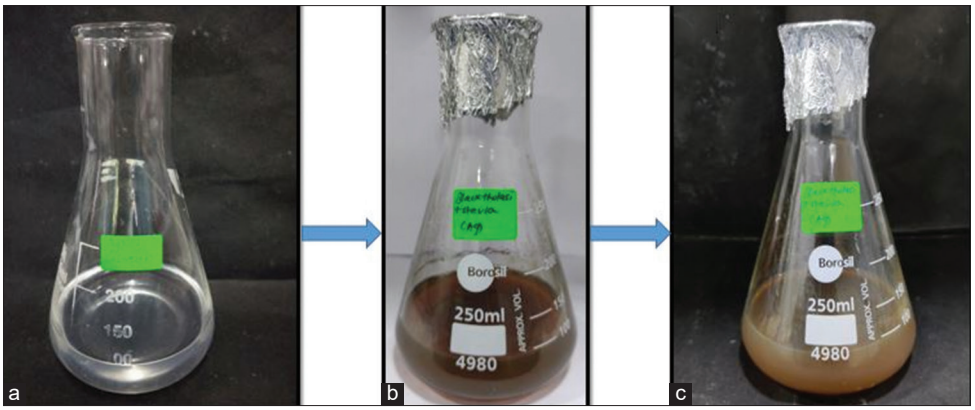
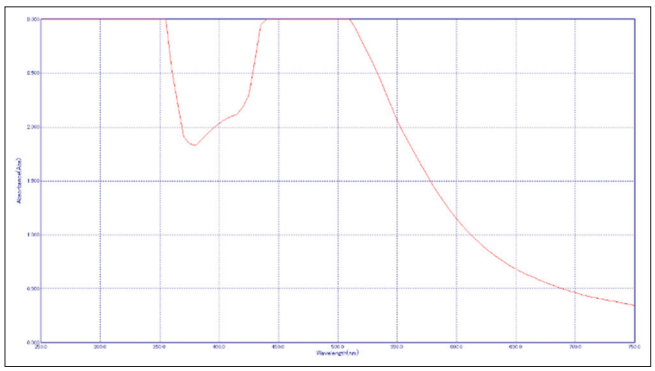


Figure 2: (a) silver nitrate extract; (b) Plant extract with silver nitrate (day 1 color change; (c) Day 3 color change



Graph 1: Spectroscopic analysis of silver nanoparticles

and was prepared, sterilized, and poured onto the Petri plates. The plates were allowed for solidification. Agar well-diffusion method was used to assess the antimicrobial efficacy [Figures 3 and 4].

Swabbing

After solidification, the respective plates were swabbed with the oral pathogens- *S. mutans*, *S. aureus, Lactobacillus* sp., and *C. albicans.*

Well formation

After swabbing, three wells on each plate were formed using a gel puncher. To those three wells, the obtained AgNPs solution was loaded in the concentration range of 25 µL, 50 µL, and 100 µL. The plates were then incubated at 37°C for 24 h and after incubation, the zone of inhibition was measured and calculated.

Cytotoxic effects

Setup preparation

The artemia tank was filled with 6 l of distilled water. To that 50 g of iodine free salt was added and mixed well using a spatula. Two capsules containing 15 g of Brine Shrimp eggs were added to the tank and left undisturbed for 5 min for proper soaking in salt water. After that, airline tip was placed inside the artemia tank and the aeration level was increased to the maximum level. After 24 h of incubation, the nauplii's hatch out from the brine shrimp eggs and observed using stereomicroscope [Figure 5].

Six well enzyme-linked immunosorbent assay plates were taken and to each plate 6–8 ml of saltwater was added. AgNP reinforced with *O. tenuiflorum* and *S. rebaudiana* was loaded in the concentration range of 5 µl, 10 µl, 15 µl, 30 µl, and 50 µl. To that 10 nauplii's [Figure 6] were added in each test tubes. A control plate was prepared by adding 3 ml of artificial sea water with 10 nauplii. The plates were kept for incubation for 24 h. After incubation, the live and dead nauplii's were counted and percentage death was calculated.

$$\text{Percentage death} = \frac{\text{Number of dead nauplii}}{\text{Number of dead nauplii} - \text{number of live nauplii}} \times 100$$

Results

The diameter of the inhibition zone for all tested concentrations of AgNP achieved for bacterial strains is presented in Table 1. Figure 3 depicts the antimicrobial activity of AgNPs reinforced with *O. tenuiflorum* and *S. rebaudiana* extract against *S. mutans* and *S. aureus*. For *S. mutans,* the zone of inhibition was found to be 10 mm, 13 mm, and 15 mm for concentration 25 µL, 50 µL, and 100 µL, respectively. Zone of inhibition against *S. aureus* at 25 µL was 9 mm, at 50 µL was 10 mm and at 100 µL was 15 mm. Figure 4 depicts the antimicrobial activity of AgNPs reinforced with *O. tenuiflorum* and *S. rebaudiana* extract against *C. albicans* and *Lactobacillus*. Zone of inhibition against *Lactobacillus* sp. at 25 µL was 9 mm, 50 µL was 9 mm, and at 100 µL was 11 mm. Zone of inhibition against *C. albicans* at 25 µL was 10 mm, 50 µL was 13 mm, and at 100 µL was 15 mm. Hence, it was seen that as the concentration of the AgNPs reinforced with *O. tenuiflorum* and *S. rebaudiana* extract increased, the antimicrobial activity increased.

Table 2 depicts the cytotoxicity of AgNPs reinforced with *O. tenuiflorum* and *S. rebaudiana*. At 5 µl and 10 µl concentration, there was 0% of death of nauplii, 20 µl

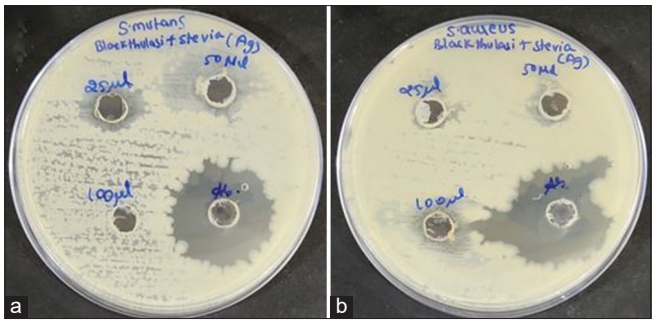


Figure 3: Antibacterial activity of silver nanoparticles reinforced with *Ocimum tenuiflorum* and *Stevia rebaudiana* extract against *Streptococcus mutans*.(a) and *Streptococcus aureus*. (b) Against *S. mutans* the zone of inhibition at 25 µl, 50 µl, 100 µl was 10 mm, 13 mm, 15 mm respectively. Against *S. aureus* the zone of inhibition at 25 µl, 50 µl, 100 µl was 9mm, 10mm, 15mm respectively

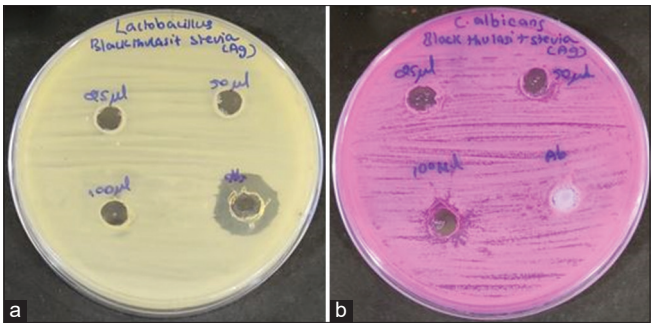


Figure 4: Antibacterial activity of silver nanoparticles reinforced with *Ocimum tenuiflorum* and *Stevia rebaudiana* extract against *Candida albicans*. (a) and *Lactobacillus*.(b) Against *C. albicans* the zone of inhibition at 25 µl, 50 µl, 100 µl was 10 mm, 13 mm, 15 mm respectively. Against *Lactobacillus sp* the zone of inhibition at 25 µl, 50 µl, 100 µl was 9 mm, 9 mm, 11 mm respectively

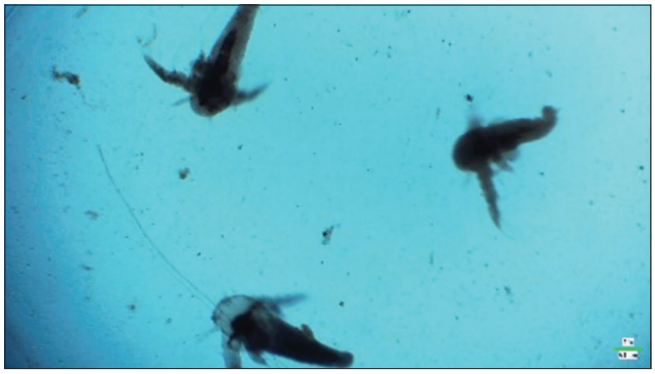


Figure 5: Microscopic picture of nauplii

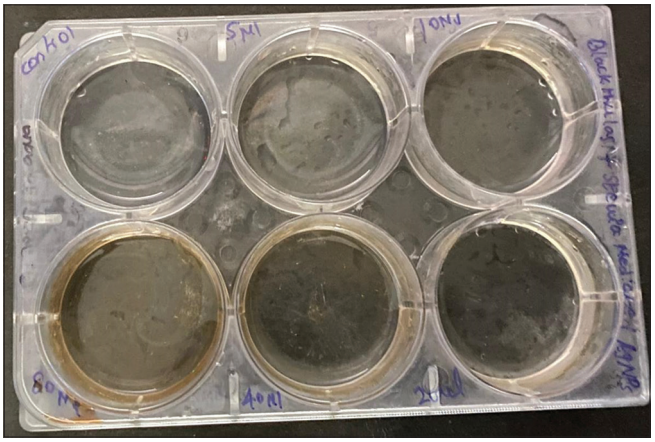


Figure 6: Nauplii in a 6 well ELISA plates. ELISA: Enzyme-linked immunosorbent assay

Table 1: Bacterial growth inhibition of biosynthesis silver nanoparticles based on disc diffusion method

AgNPs (µg/ml)	Zone of inhibition (mm)			
	Streptococcus mutans	*Staphylococcus aureus*	*Lactobacillus* spp.	*Candida albicans*
25	10	9	9	10
50	13	10	9	13
100	15	15	11	15
Ab	32	33	26	10

AgNPs: Silver nanoparticles

Table 2: Cytotoxicity activity of silver nanoparticle reinforced with *Ocimum tenuiflorum* and *Stevia rebaudiana* extract

Concentration (µL)	Initial number of nauplii	Viable nauplii	Death (%)
5	10	10	0
10	10	10	0
20	10	8	20
40	10	8	20
80	10	7	30
Control	10	10	0

concentration 20% of death, at 40 µl concentration 20% of death of nauplii, and at 80 µl concentration 30% of death of nauplii. By the above results, it is clearly understood that as the concentration increased the cytotoxicity of the nanoparticles increased.

Discussion

Natural products are precious and undiscovered sources of effective antimicrobials, with less amount of toxicity. Current researches in analytical technology, particularly those associated with phytotherapy, have introduced a new era of anti-plaque therapies and natural products.[19,20] In a study done by Patil *et al*., flow cytometry technique was used to assess the surface markers of these cells such as CD73, CD90, and CD105, CD34, CD45, and human leukocyte antigen – DR isotype (HLA-DR). Further, an MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay was performed on the cells after subjecting them to various concentrations of cordycepin and it was found that the dental pulp stem cells showed strong positive expression for CD73, CD90, and CD105 and faint expression of CD34, CD45, and HLA-DR. MTT assay revealed that 5 µM was the optimum concentration of cordycepin for all the assays.[21] Dental pulp-derived stem cells expressed CD73,

CD90, and CD105 and did not express CD34, CD45, and HLA-DR, which demonstrated that they were mesenchymal stem cells. The MTT assay revealed that various concentrations of taurine did not affect the cell viability of DPSCs.[22]

The chemical composition of Tulsi is highly complex, containing many nutrients and other biologically active compounds, the proportion of which may vary considerably between strains and even between the plants of the same field. Furthermore, the quantity of many of these constituents is affected by differing growing, harvesting, processing and storage conditions, which are not yet well understood.[23] Eugenol (l-hydroxy-2-methoxy-4-allylbenzene), the active constituent present in *O. sanctum*, perhaps is largely responsible for the therapeutic potential of Tulsi.[4]

AgNPs has properties such as optical and catalytic properties, which depend on the size and shape of the produced nanoparticles.[24] AgNPs have an antibacterial effect due to its activity of forming pits within the cell wall of the bacteria. Silver particles have the numerous reactions with a bacterial cell, that are interaction with cell wall causing lysis, preventing DNA replication and also disrupts bacterial protein synthesis. There is an evident increase in the cell permeability which will result in necrobiosis.[25] AgNPs have acquired substantial attention because of their potential applications in the medical field, especially in the production of biodegradable surgical sutures.[26]

Considering the liquid dilution method to seek out the minimum inhibition concentration of 25 nm, AgNP is present against *S. mutans* and average of 4.8 µg/mL is also responsible for the excellent antimicrobial activity of AgNPs.[27] The potential sources of AgNPs includes leaching of intact particles from consumer products, disposal of waste from industrial processes, intentional release into contaminated waters, and therefore, the natural formation of AgNPs in surface and groundwater.[28] Oxidative dissolution in experimental conditions (maximally 15% in 24 h) is the key to the toxicity of most Ag NPs, highlighting a critical role for dissolved silver complexed with thiols in the toxicity of all tested Ag NPs.[29]

In this study, the AgNPs that were formed showed a peak of 450 nm. The AgNPs had good antibacterial effect even at low concentrations that increased as the concentration was increased. Antifungal activity as present that was better at higher concentration. The toxicity results showed that in lower concentrations, all the nauplii survived, mild toxicity was seen in higher concentrations.

According to Yamani *et al*., 2016, bacteriostatic activity of Tulsi oil was high at levels of 2.25–2.5 µg/ml against *S. aureus*, including MRSA and *E. coli*, but less activity against *P. aeruginosa*.[30] Mishra P., Mishra S., reported good inhibition of both Gram-positive and Gram-negative species, indicated by a reduction in optical density.[31] The increased appearance of antimicrobial resistant strains to common antimicrobials has become a threat to human and animal health. In the search for novel antimicrobials, Stevia was found to be a promising candidate with high antimicrobial activity.[32,33]

Among the reports of antimicrobial activity of stevia leaf extracts on *Listeria spp*., our results show higher inhibition for all microbes than that reported by Belda-Galvis *et al*.[34] According to Theophilus *et al*., the antimicrobial properties of Stevia extracts go beyond growth inhibition of bacterial cultures; it was shown that alcohol-based extracts were effective in inhibiting the growth of *B. burgdoferi* biofilms and of persisters that were not susceptible to antibiotic treatment.[35] These results show the potential of Stevia plants to become a natural source of antimicrobials broadening its application spectra.

Conclusion

In conclusion, AgNPs synthesized using extracts from *O. tenuiflorum* and *S. rebaudiana* is an eco-friendly approach in developing antimicrobial agents and with nontoxic effect. It is one of the cost effective methods that may be very useful in future for nano-product development in biomedical applications.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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