

RESEARCH ARTICLE

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***In-vitro* Cytotoxicity Evaluation of Green Synthesized Gold Nanoparticles and Its Indigenous Mouthwash**

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Abstract

The aim of this study was to evaluate the cytotoxicity of the prepared Gold Nanoparticles from *Triphala* Plant extract and of the indigenous mouthwash prepared from these *Triphala* based gold nanoparticles (AuNP's) using *Artemia salina* test. Its objective was to assess whether the prepared mouthwash was safe and biocompatible to use as an alternative to commercial mouthwashes in orthodontic patients to reduce the plaque load around brackets and bands as an adjuvant to tooth brushing. 15 g of brine shrimps was hatched in salt solution after incubating them for 24 h. Prepared AuNP's at concentrations 0 μ L (microliter), 5 μ L, 10 μ L, 20 μ L, 30 μ L, 50 μ L were added using a micropipette in one of the microtiter plates and mouthwash prepared from these AuNP's were added at concentrations 0 μ L, 10 μ L, 20 μ L, 30 μ L, 40 μ L, 50 μ L in another microtiter plate. 10 Hatched *nauplii* (brine shrimps) were added in each of these microtiter plates and incubated for 24 h. All *nauplii* were alive at the end of 24 h in the microtiter plate containing AuNP's. At concentrations 0 μ L, 10 μ L, 20 μ L, 30 μ L *nauplii* were alive and at 40 μ L, 50 μ L concentrations no *nauplii* were alive at the end of 24 - h in the microtiter plate containing mouthwash. *Triphala* mediated AuNP's showed no toxic effect on the brine shrimps. AuNP's based mouthwash showed excellent compatibility at lower concentrations. Mild toxic effects were elicited at higher concentrations. Hence, AuNP's could be used to prepare mouthwashes at 20- 30 μ L concentrations.

Keywords: *A. nauplii*, Brine shrimps, plaque producing organisms

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INTRODUCTION

Nanotechnology is the art of using nanoscience to design nanomaterials and nanoparticles. Nanoparticles (NP's) are particles having size ranging from 1-100nm. Various NP's that are being experimented in the field of medicine are gold, silver, ruthenium, iridium, palladium etc. Of all, gold nanoparticles (AuNP's) have superior properties as gold is inert, have better compatibility with animal and plant cells and is nontoxic¹.

In recent times, NP's have gained popularity in the field of dentistry as well². NP's have been used for various applications like site specific drug delivery, as sensors, anticancer drugs, antioxidants, larvicides, Nanofluids, antimicrobial agents in dentistry³, as mouthwashes⁴ and for treatment of white spot lesions during orthodontic treatment², etc. Various physical and chemical methods used to produce NPs are expensive. Toxic reducing and capping agents are employed in these methods. Therefore, green synthesis of NP's has gained ground owing to its cost effectiveness and environmental compatibility⁵. NP's carrying drugs have extended half-life period, longer circulation time, and carry higher concentration of drugs⁶. Unlike Bulk metals, NP's possess good optical properties as the motions of electrons are restrained owing to the reduced size. Due to the ultra-small size, they are less toxic and have a high surface to volume ratio. Hence, interactions take place at the surface resulting in faster absorption⁷ and relatively high the drug loading⁶. The large surface area, high charge density of NPs enables them to interact with the negatively-charged surface of bacterial cells to a greater extent resulting in enhanced antimicrobial activity⁸.

NP's influence biological systems in multiple ways. Size of the NP's determine their cellular uptake, endocytosis, cytotoxicity, bio distribution, and clearance pathway. Bio-nano interface reactions affect the size and surface properties of the NPs after binding with proteins, glycans, ions and hence⁹ alter the uptake pathways (nonspecific and receptor-mediated). Conversely, bio-nano interactions may cause some change in the protein composition, the distribution of the biological molecules in the organism, and may constitute or show some hidden immune epitopes.

Improved understanding of nanotoxicity will help to avoid its unfavourable effects¹⁰.

In this in-vitro study, brine shrimp assay was used to evaluate the toxicity of prepared AuNP's from triphala extract. It also studied the cytotoxicity of the mouthwash prepared from it. Brine shrimp assay was first proposed by Michael et al. 1956, then developed by Vanhaecke et al. 1981. It has had its applications in detecting fungal toxins, heavy metals, plant extract toxicity, cytotoxicity testing of dental materials¹¹. *Artemia (A.nauplii)* is a genus of aquatic crustaceans, a zooplankton, known as brine shrimps. These species have small size, short life span, large offspring production, high adaptability to hypersaline environments at various temperatures. Various nanotoxicology conducted on animals proved to be time consuming due to the tedious procedure of obtaining ethical clearance. To overcome this, preference is given to in-vitro studies for evaluating toxicity. Hence, *A.nauplii* have been used as invertebrate models in nanotoxicology^{1,2,3}.

In this present study, the green synthesis method was employed to fabricate AuNP's using Triphala extract (*Terminalia chebula*, *Terminalia bellerica*, and *Embelica officinalis*). AuNP's were characterized by UV-Vis spectroscopic method which showed SPR (surface plasmon resonance peak) at 520nm, TEM (Transmission electron Microscope) analysis which revealed the size of AuNP's to be 12-35nm, spherical in shape. Mouthwash was prepared from these Triphala mediated AuNP's and antimicrobial effect of these AuNP's and its based mouthwash was evaluated against most commonly found oral pathogens in oral cavity and in orthodontic patients. Antimicrobial effect was tested against *Enterococcus faecalis*, *Streptococcus mutans*, *Staphylococcus aureus* and *Candida Albicans* at various concentrations. These AUNP's and its mouthwash showed good antibacterial activity against these pathogens. For this reason, prior to use in orthodontic patient's biocompatibility was evaluated using brine shrimp assay.

According to a study done by Anderson, it has been proved that chlorhexidine is effective in maintaining orthodontic hygiene due to its excellent antibacterial activity, thus causing a reduction in the gingival problems. But in the same

study, they mentioned shortcomings like burning sensation, staining, discolouration of tongue and composite restorations¹³. Another research by Balalakshmi et al stated that it is a contraindicated to use it in patients who are immunocompromised and have mucositis as there is presence of alcohol in 0.2% chlorhexidine mouthwashes³.

NP based mouthwash has been known to overcome certain disadvantages of commercial conventional mouthwashes. In a study by Norouzzadeh Helali et al metal oxide nanoparticles like Ag/ZnO or ZnO were checked for the antimicrobial activity against *S. mutans* and compared with 0.2% chlorhexidine mouthwash. The cytotoxicity of the ZnO and Ag/ZnO nanoparticles was evaluated in the A549 cell line. The results proved that no significant ill-effect is caused to the cells up to 0.1 mg/ml of ZnO and Ag/ZnO NP's. It was concluded that Ag/ZnO NP based mouthwash is an effective antimicrobial agent against *S. mutans* and could be considered as an alternative to 0.2% chlorhexidine¹⁴.

Thus, the objective of this study was to evaluate the biocompatibility of the prepared AuNP's and the mouthwash which could be used as an alternative to commercially available mouthwashes.

MATERIALS AND METHODS

Synthesis and antimicrobial evaluation of AuNP's and its based mouthwash

Owing to the good antimicrobial property possessed by Triphala plant extract and gold nanoparticles, the combination was selected to produce AuNP's and AuNP based mouthwash. Triphala extract powder was crushed into coarse powder and mixed with Gold chloride solution. The mixture was heated at 50 degrees and was homogenously mixed on an orbital shaker. The colour was pale yellow and it turned purple, this indicated the formation of AuNP's. This formation of AuNP's was confirmed using spectroscopic analysis which showed a peak of 520 nm corresponding to the surface plasmon resonance of AuNPs. Characterization using Transmission Electron Microscopy revealed spherical AuNP's with a size in the range of 18-35 nm. The antimicrobial property for AuNP's was then assessed using Zone of inhibition test. *C. albicans* exhibited excellent antimicrobial activity followed by *S. mutans*.

S. aureus and *E. coli* also showed intermediate antimicrobial potential. AuNP based mouthwash was prepared using 600 µL AuNP's and all basic ingredients of mouthwash. The mouthwash also depicted good antimicrobial activity against all pathogens.

Chemicals used: 1) AuNP's prepared from triphala plant extract. 2) mouthwash prepared from triphala mediated AuNP's. 3) *A. nauplii* eggs.

Hatching the brine shrimp

27g of table salt was weighed and added to 3 litres of distilled water in a cylindrical jar and thoroughly stirred. For good aeration, an air pump was placed into the bottom of the jar. 15 g of brine shrimp eggs were added at the top-level water of the jar and mixed. A light bulb (60–100-Watt bulb) was placed a few inches from the jar. After 20-24 h of incubation period, *nauplii* hatched. Hatched *nauplii* was separated from the empty egg by turning off air and switching off the lamp. This ensured that the empty eggs were floating on top and the brine shrimp were concentrated at the bottom of the water column.

Microscopic evaluation of the brine shrimps

Two brine shrimp eggs were inoculated on a clean slide with 2 drops of distilled water. They were observed under a light microscope at 40X and 10X magnification. (Fig. 3) Shrimps had an unsegmented body and a single eye, about 22 mm long.

Toxicity testing on the *Artemia nauplii*: (Fig. 2)

At the end of 24 h, wells of the microtiter plates were inoculated with AuNP's of 0µL, 5µL, 10µL, 20µL, 30µL, 50µL concentrations. Mouthwash at concentrations 0µL, 10µL, 20µL, 30µL, 40µL, 50µL was added in another microtiter plate. In one of the wells in both plates, a sterile salt solution was used as control. *A. nauplii* were collected in a petri dish from the cylindrical jar. 10 hatched *nauplii* were collected using a dropper and added in each well in both plates. They were counted using a magnifying glass. This was cross checked by another researcher to avoid any error. At the end of 24 h, *nauplii* in each well in each of the microtiter plates were counted.

RESULTS AND DISCUSSION

At the end of 24 h, the microtiter plate which was inoculated with AuNP's alone showed all *A. nauplii* to be alive in each well and in the

well with the control group. In the microtiter plate containing mouthwash 10 *nauplii* were alive at 0 μ L, 8 *nauplii* at 10 μ L, 7 *nauplii* at 20 μ L, 3 *nauplii* at 30 μ L, 0 *nauplii* at 40 μ L and 50 μ L.

The cytotoxic effect of AuNP's on *A. nauplii* revealed that all brine shrimps are alive at the end of 24 h which indicate no cytotoxic effect of AuNP's on the brine shrimps. The cytotoxic

effect of AuNP based indigenous mouthwash on *A. nauplii* revealed that all brine shrimps are alive at the end of 24 h at low concentrations which indicate no cytotoxic effect of AuNP's on the brine shrimps. At higher concentrations, the brine shrimps are not alive indicating the toxic effect of the mouthwash on brine shrimps at higher concentrations.

Mortality rate was calculated as follows

$$\text{Mortality (\%)} = (\text{Number of dead } nauplii / \text{Total number of } A. nauplii) \times 100$$

Mortality rate for AuNP's was 0% and for AuNP based mouthwash was 46.6%.

In this current study, triphala plant extract mediated AuNP's and mouthwash (Figure 1) showed good antimicrobial activity against *Enterococcus faecalis*, *Streptococcus mutans*, *Staphylococcus aureus* and *Candida Albicans*. Therefore, toxicity of AuNP's was evaluated to so AuNP's could be used for oral applications for bacterial load reduction. NP based mouthwashes do not possess the disadvantages of commercially used ones. They have been known to have

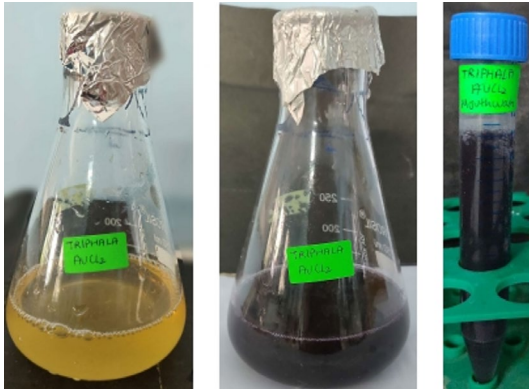


Fig. 1. Triphala plant mediated AuNP's and its based mouthwash. It represents color change from pale yellow to violet indicating formation of Gold nanoparticles from which the mouthwash is prepared.

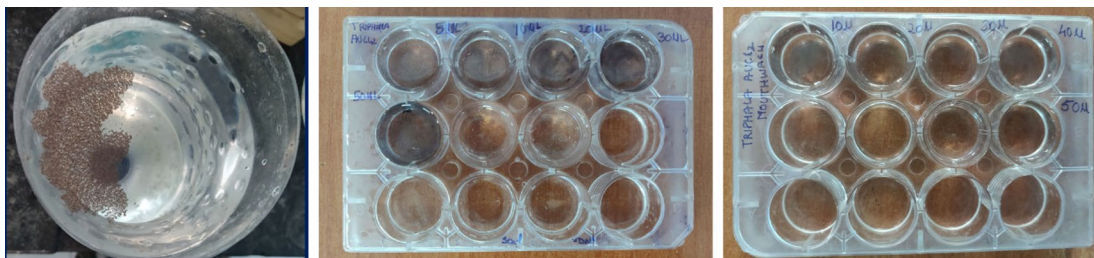


Fig. 2. Hatched brine shrimps (left), AuNP's with *A. nauplii* (centre) , Mouthwash with *A. nauplii* (right).

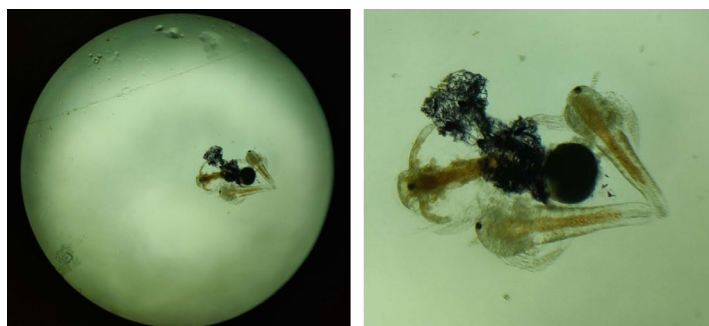


Fig. 3. Light microscope - *A. nauplii* : 40X (left) , 10X (right); represents the microscopic image of the brine shrimp attached to a gold nanoparticle.

comparable antimicrobial activity as the gold standard mouthwashes. In an attempt to use it as an adjunct to mechanical cleansing in orthodontic patients for reduction of plaque load and white spot lesions, its cytotoxic evaluation was carried out on brine shrimps¹⁴.

The oral cavity encounters a plethora of pathogens. Plaque biofilm- a major cause of caries, periodontitis and other dental diseases- is a complex community of various microorganisms that cause infections¹⁵. Many methods have been employed but the desired outcome is never achieved due to fast degradation and rapid release of antimicrobial agents resulting in low efficiency and safety concerns^{16,17}. NPs are tiny particles with a diameter of 1–100 nm. They are useful in antibacterial therapies because of improved characteristics like ultra-small sizes, large surface-area-to-mass ratio and increased chemical reactivity¹⁶. NPs may be able to come up with a new strategy for treatment of and prevention of dental infections¹⁸. The large surface area and high charge density of NPs help them to come in contact with the negatively-charged surface of bacterial cells to a greater extent resulting in enhanced antibacterial activity⁸. Metal and organic NPs have been of use in dentistry due to their broad-spectrum antimicrobial properties¹⁸.

Certain studies have proven that NP's are nontoxic within certain concentration ranges¹⁹⁻²⁵. Some other studies show that the low concentrations are nontoxic but larger concentrations may have some toxic effect. A few other researchers conclude that toxicity of NP's is dose dependent²⁶⁻²⁸. In conclusion, the toxicity of antimicrobial NPs is caused by multiple factors like dosage, types, distribution, duration of action, particle size, interaction with other compounds². Nanoparticles require special attention, as these compounds in this ultra-small size range have chemical properties that vary in comparison with those of their larger counterparts.

Artemia is one of the most valuable test crustaceans used for marine ecotoxicity testing as it is widely used as a nutritious live food source to the larvae of a variety of marine organisms. This makes them the most convenient, least labour-intensive live food available for aquaculture and will be impacted greatly by the release of NP 's into aquatic systems because of their greater

interaction with the environment via nonselective filter feeding²⁹.

Initially, in-vivo animal studies were profoundly used for assessing toxicity. But animal rights activists from IACUC criticized this idea. Due to high costs and increased time in in-vivo studies, certain in-vitro methods were employed. These techniques were XTT assay, MTT assay, cell culture, the WST-1 assay, BrdU assay, fluorescence Microscopy and LDH¹¹. But these techniques were time consuming and expensive. In an attempt to obtain a rapid and cost-effective screening technique brine shrimp assay test was introduced and is being currently used in assessing the toxicity of nanoparticles³⁰. Many studies have used brine shrimps for evaluating biocompatibility³¹⁻³⁵.

Our study concludes that the AuNP's have no toxic effect on the brine shrimps. AuNP's based mouthwash showed a very mild toxic effect as the concentrations were raised to 40µL and 50µL. Gold nanoparticle-based mouthwash can be made using low concentrations of NP's.

Silver nanoparticles and ZnO based mouthwashes have been shown to have comparable antimicrobial properties like the most commonly used mouthwash in dentistry i.e., Chlorhexidine. Silver nanoparticle and ZnO based mouthwashes have been used effectively in Orthodontic patients^{29,14}. Along with good antimicrobial properties, silver nanoparticles have been known to have cytotoxic effect of human dermal fibroblasts at high concentrations²⁶. Sreenivasagan et al proved its cytotoxicity at 20 and 25 µL³⁶. A study reported by Cierech et concluded cytotoxic effect of Zinc oxide nanoparticles beyond a concentration of 20 µL³⁷. No literature is available on gold nanoparticle-based mouthwashes. Further studies need to be carried out on use of gold nanoparticle-based mouthwashes. There is a dire need that this Triphala plant mediated AuNP based mouthwash needs to be compared with other commercially available mouthwashes to evaluate its antimicrobial properties.

Limitations

There is no study reported on gold nanoparticle-based mouthwashes in dental patients. Therefore, more studies are needed in this field. AuNP based mouthwash needs to be compared with other nanoparticle-based mouthwashes and with other commercially

available mouthwashes. Antimicrobial activity against most commonly found *Lactobacillus* spp in orthodontic patients was not included in this study. Hence, this must be tested too. Cost of gold nanoparticles needs to be taken into account to make it affordable. Cytotoxicity testing was done at aquatic level. Further toxicity tests should be employed prior to employing its use in orthodontic patients.

CONCLUSION

This study has shown that AuNP's have no toxic effect on the brine shrimps upto 20 μ L concentration. There is a mild toxic effect elicited by the mouthwash at 30 μ L and toxicity increased as the concentration of the mouthwash is increased. Hence, it is a potential alternative to commercially available mouthwashes in orthodontic patients at 20-30 μ L concentration. AuNP based mouthwashes could be used as an adjuvant to mechanical cleansing like tooth brushing to reduce the bacterial load after testing it at higher levels. As other nanoparticles have reported harmful effects on the oral cavity with increasing concentrations, AuNP's should be studied more elaborately owing to their unique properties.

ABBREVIATIONS

AuNP's : Gold Nanoparticles; NPs : Nanoparticles; μ L : Microliter; ZnO: Zinc oxide; IACUC: Institutional Animal Care and Use Committees.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTIONS

All authors designed the experiment. LS and RK performed the experiments. LS, RK, SK analysed the data. LS wrote the manuscript. All the authors read and approved the manuscript.

FUNDING

None.

DATA AVAILABILITY

All the datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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