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Antimicrobial effect of Triphala mediated gold nanoparticles and its based indigenous mouthrinse against common oral pathogens: An in vitro study

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***Abstract*---**The aim of the study was to synthesize gold nanoparticles (AuNP's) by using triphala plant extract and to evaluate antimicrobial activity of gold nanoparticles and its based mouthwash. This study used a triphala extract for nanoparticle synthesis in combination with gold chloride (AuCl2). The AuNP's were characterized for shape and size using (Transmission electron microscopy) TEM analyses and UV vis spectrophotometry. The gold nanoparticle-based mouthwash (10ml) was prepared using 600 µL nanoparticles. The antimicrobial activity was assessed using agar gel diffusion method. To determine the antimicrobial activity of AuNp's, the inhibition zones were measured for: Enterococcus faecalis, Streptococcus mutans, Staphylococcus aureus and Candida Albicans at 25µL, 50µL ,100µL concentration and at 25µL ,50µL, 100 µL concentration for mouthwash. AuNP's showed excellent antimicrobial activity against C. Albicans, S. mutans as compared to S. aureus, E. faecalis which showed good antimicrobial activity with phenomenal activity at 100 µL concentration. Mouthwash showed significant activity against S.

aureus and good activity against, E. faecalis, C. Albicans and S. mutans with highest activity at 100 µL. At concentrations of 25 µL, 50 µL, moderate antimicrobial potential was elicited by AuNP's and mouthwash. Significant antimicrobial activity was elicited by AuNP's against commonly present oral microorganisms.

***Keywords*---**nanoparticles, triphala, green chemistry, mouthwash, antimicrobial.

Introduction

Nanoparticles (NPs) [gold (Au), silver (Ag), ruthenium (Ru), palladium (Pd), etc.] have gained high appeal and insistence from researchers in all fields. Various applications of NP's are potential antimicrobial, anticancer, antioxidant agents, drug and gene delivery agents etc[1]. Gold nanoparticles (AuNP's) are superior to others owing to their reduced toxicity, good biocompatibility, successful use in drug and gene delivery. Due to the ultra-small size, it is barely toxic and has a high surface to volume ratio. Thus, interactions are confined to the surface resulting in accelerated absorption[2] and the drug loading to be relatively high. The large surface area and high charge density of NPs enable them to interact with the negatively-charged surface of bacterial cells to a sizeable extent resulting in enhanced antimicrobial activity[3] Few studies have elaborated on the antimicrobial potential of Gold NP's[4]. Gold NPs exert their antibacterial activities mainly by three mechanisms of (a) interacting with peptidoglycan cell wall and membrane and causing cell lysis; (b) interacting with bacterial proteins and deranging protein synthesis (c) interacting with bacterial DNA and preventing replication of DNA[5].

Inspite of the wide availability of the customary methods such as electrochemical, photochemical, sonochemical, etc. methods for NP production , there is a need for more environmentally friendly methods. As the above methods use toxic compounds as reducing/capping agents for reduction of ions[6]. Green synthesis gained popularity pertaining to usage of harmless plant extracts, nontoxic phytochemicals as reducing agents , thus alleviating the use of toxic agents. Triphala extract is an Ayurvedic medicine which is a combination of *Terminalia chebula, Terminalia bellerica, and Embelica officinalis* in equal proportions. Triphala is also known to possess stress-reducing potential, antidiabetic, antimicrobial, antineoplastic, anti-inflammatory, and antioxidant activities.[7]. Numerous studies have proved triphala as a good antimicrobial agent in toothpaste, mouthwash and other oral formulations[8–15]. Hence, in this study we selected triphala in combination with gold chloride for nanoparticle synthesis.Various studies have reported on the antimicrobial activity of AuNP synthesized using herbal extracts.[16–21]

Patients undergoing orthodontic treatment encounter difficulties in removing plaque effectively from tooth surfaces. White spot lesions are initial demineralized areas seen around brackets in orthodontic patients due to the process of etching.[22] Because of the complexity of the orthodontic brackets and the presence of white spot lesions, it serves as a breeding ground for bacterial

adhesion and plaque accumulation. By using mechanical methods like tooth brushing patients cannot remove plaque effectively hence there is a need to resort to chemical methods like mouthrinses for plaque control in subjects undergoing orthodontic treatment.[23] Chlorhexidine has proven to be an effective antimicrobial oral rinse against various oral pathogens like *Streptococcus* sp., *Lactobacillus* sp., *Staphylococcus* sp., *Candida* sp.[24] It is known to be the best antimicrobial agent in reducing plaque accumulation. Chlorhexidine is a cationic bisbiguanide and has been a gold standard among all oral rinses.[25,26] This is mainly due to its high substantivity and broad antibacterial spectrum.[27] But it has certain shortcomings like brown enamel staining, dryness, and burning sensation, development of oral mucosal lesions and increased calculus accumulation. Staining of tongue, alteration of taste sensation, cytotoxic to periodontal ligament (PDL) cells[28] are other common side effects which limit its prolonged use. All of these disadvantages necessitate limiting its long-term use. A study by Balalakshmi *et al* stated chlorhexidine as a contraindication in immunocompromised patients and those having mucositis.[29] Hence there is a need for herbal substitutes over synthetic antibiotic based oral rinses.[30] Nanoparticle based oral rinses have been reported in literature and some studies have proved nanoparticle oral rinses to be as effective and are comparable to Chlorhexidine oral rinse. As good antimicrobial activity of Triphala extract and AuNp's has been reported in literature, the objective of this study was 1) to combine their antimicrobial properties by using triphala extract for green synthesis of AuNP and assess the antimicrobial activity against the most common oral pathogens 2) to prepare an oral rinse from the Triphala plant mediated gold nanoparticles and assess its antimicrobial activity.

Materials and Methods

Preparation of Plant Extract

Freshly collected leaf extract of the triphala plant was washed with distilled water several times and then dried in an incubator. It was then grounded into coarse particles by means of a mortar. 0.7 grams of coarse powder was mixed homogeneously with 70 ml distilled water. This solution was heated at 50 - 60 °C until the solution was boiled in a heating mantle. Purification of solution was done by filtration using a Whatman filter paper no.1. Residue collected in the filter paper was discarded and the supernatant was collected in a conical flask.

Preparation of Gold Nanoparticles: (Figure 1)

The synthesis of gold nanoparticles was simply obtained by the reduction of gold chloride solution. Triphala plant extract was used as a reducing or capping agent. In the procedure of nanoparticle synthesis, 10 mL gold chloride solution was mixed with the plant extract and kept overnight on an orbital shaker for homogenous mixing of all particles. The reaction mixture was stirred continuously on a magnetic stirrer till colour change in the mixture was observed. At hourly intervals the synthesis of nanoparticles was monitored by UV – vis spectroscopic analysis. Colour change of gold nanoparticles indicated its formation at a certain wavelength that was measured on a UV – vis spectrophotometer. Post spectroscopic analysis, the mixture was collected in 5

test tubes and AuNP's were separated from solution by centrifugation for 20 minutes.

Characterization of synthesized AuNP's: (Figure 2)

The UV-vis absorption peak of the synthesized AuNP's was recorded using UV -Vis Spectroscopy. The scanning range of the samples was between 350 to 660nm. UV- vis spectroscopic analysis relies on the reduction of gold salt solution to gold NP's. The visual change in the colour will indicate the formation of NP's. The size and shape of the nanoparticles were obtained using Transmission electron microscopic analysis (TEM).

Antimicrobial activity of AuNPs: (Figure 3 and table 1)

The antimicrobial activities of the synthesized AuNP's and its indigenous oral rinse were tested against *S. aureus, S. mutans, E. faecalis and C. Albicans.* The antibacterial activity was determined using agar well diffusion method. The different concentrations of the AuNP's (25 µL, 50 µL ,100 µL) were added to the wells made on nutrient agar plates. Agar plates were incubated at 37 ºC for 24 hours. After 24 hours, the inhibition zone was measured using a Vernier calliper to ascertain the extent of antimicrobial activity.

Preparation of oral rinse: (Figure 2)

Gold nanoparticles made from triphala plant extract were used in the formulation of the oral rinse to combat plaque-producing microorganisms. For preparation of oral rinse, following procedure was employed: To 10 mL of distilled water ,600 µl of nanoparticle solution was added \(0.6 %)[8].Tripahla mediated AuNP solution was measured using a micropipette having attached disposable tips which were changed between transfers to prevent contamination and mixing of any solutions at any given point of time. To this solution 0.3 grams of sucrose, 0.005 grams of sodium benzoate were added, 0.01 grams of foaming agent sodium lauryl sulfate was mixed with the above solution and finally 0.1 ml of peppermint oil was added. This solution was vigorously mixed until a homogenous solution of the oral rinse was obtained. The following table summarizes the composition of the mouthwash.

MATERIALS	FUNCTIONS
10 ML DISTILLED WATER	SOLVENT
600 µL GOLD NANOPARTICLES	MAIN INGREDIENT
0.3 GRAMS SUCROSE	SWEETENING AGENT
0.005 GRAMS SODIUM BENZOATE	PRESERVATIVE
0.01 GRAMS SODIUM LAURYL SULPHATE	FOAMING AGENT
0.1 ML OF PEPPERMINT OIL	FLAVORING AGENT

Antimicrobial activity of oral rinse: (Figure 4 and table 2)

The antimicrobial activities of the AuNP indigenous oral rinse were tested against *S. aureus, S. mutans, E. faecalis and C. Albicans*. The antibacterial activity was determined by using agar well diffusion method. The different concentrations of oral rinse (25 µL ,50 µL, 100 µL) were added to the wells made on nutrient agar plates inoculated with the 4 microbes - *S.mutans, S.Aureus, E.Faecalis* and *C.albicans*.

Results

Synthesis of AuNPs

The Triphala extract when mixed with AuCl2 solution showed a rapid colour change from pale yellow to purple. This colour change indicated the formation of AuNP's (Figure 1).

UV-vis spectroscopy

The surface plasmon resonance (SPR) represents a peculiar phenomenon to noble metal NP's that contributes to intense electromagnetic fields on the particle surface, which in turn increases all radiative properties such as scattering and absorption[31]. Hence, in this study the formation of AuNP's was confirmed by UV–VIS spectroscopic analysis. There was a sharp peak at 520 nm which corresponded to the surface plasmon resonance (SPR) band of the AuNPs[32] (Figure 2).

Transmission electron microscope

TEM analysis revealed spherical shape of AuNPs with a size range from 12-35nm size (Figure 3) confirming the formation of Aunp's.

Antimicrobial activity of AuNP's

The inhibition zones for AuNP's at various concentrations for each of these organisms - *S. aureus, S. mutans, E. faecalis and C. Albicans* are depicted in the table. (Figure 3 and Table 1)

Antimicrobial activity of oral rinse

The inhibition zones at 25 µL ,50 µL, 100µL concentrations for each of these organisms - *S. aureus, S. mutans, E. faecalis and C. Albicans* are depicted in table 2 and Figure 4.

Discussion

In this study, triphala plant extract and gold chloride solution was used to make nanoparticles and oral rinse was prepared from these nanoparticles. TEM analyses revealed 12-35 nm spherical AuNP's and the absorption band at 520nm. For gold nanoparticles, the resonance of the most widely used spherical gold

nanoparticle with diameter of 13 nm occurs in the visible spectral region at around 500-550nm which is the origin of the brilliant red colour[33], thus confirming the formation of AuNPs The pale-yellow solution of the extract and AuCl turned violet at a wavelength of 520nm as measured using UV-vis spectroscopy which also serves as an indication of formation of AuNp's. Many other researches supported the above mentioned results.[34][16,18,19,35–39].

The antimicrobial activity of AuNP's was assessed using nutrient agar well diffusion method against the most common disease producing oral pathogens like *S. aureus, S. mutans, E. faecalis and C. Albicans.* In this method, Zone of inhibition test, also called as Kirby-Bauer Test, a qualitative method was used clinically to measure antibacterial activity of the AuNP. The size of the zone of inhibition has relation to the amount of antimicrobial activity present in the sample or product – antimicrobial potency depends on the size of the zone. AuNP's showed significant antimicrobial activity against *C. Albicans, S. mutans* as compared to S. *aureus and E. faecalis, which* showed good antimicrobial activity with best activity at 100 µL concentration. At concentrations of 25 µL, 50 µL,as well, good antimicrobial potential was elicited by AuNP's Thus, at low concentrations and small particle sizes effective antimicrobial activity has been observed. Various studies are conducted on assessment of antimicrobial activity of AuNP using herbal extracts[32,38][16–21].

Antimicrobial activity of AuNP's:

The antibacterial activity was evaluated using the Zone of inhibition test. The size of the zone of inhibition is proportional to the amount of antimicrobial activity present in the sample or product – antimicrobial potency is proportional to zone size. According to Johnson et al, bacteria with an inhibition zone of 16mm have good antimicrobial effect, an inhibition zone of 11-15mm have moderate antibacterial effect and bacteria with an inhibition zone of 10mm have poor antimicrobial effect.[40]The results of our study showed that Triphala mediated AuNP's had greatest antimicrobial activity against C.Albicans which revealed 13 mm, 14.2mm, 16 mm inhibition zones at 25µL, 50µl, 100 µL respectively followed by S.Mutans showing inhibition zones of 11.75 mm, 12.6mm and 14.9 mm at 25µL, 50µl, 100 µL respectively. Inhibition zones in the range of 10.5- 11.2 mm were present for *E. Coli* and *S. Aureus* indicating intermediate antimicrobial potential of Triphala mediated AuNPs. The results of numerous researches which were consistent with the results of our studies are described in the following paragraph.

Khan et al revealed good antimicrobial activity of C. *inerme* plant extract synthesized AuNP's against *Staphylococcus aureus* and *Escherichia coli* at 14µg/mL and 17 µg/Ml respectively[18] . Hamelian et al used Thyme manufactured AuNP's in their study proving great antimicrobial activity against *E. coli and Staphylococcus aureus* at concentration of 31µg/ml AuNP's with the formation of 23mm and 22 mm of inhibition zones respectively[37]. Another study by Sadeghi et al revealed that AuNP's can be considered as good antimicrobial agents against *Staphylococcus aureus[19].* Mohan Kumar et al concluded better antimicrobial activity of *Terminalia chebula* mediated AuNP's against gram positive *Staphylococcus aureus* than gram negative *Escherichia coli at 25*µL and

50µL[38]. AlSaqr et al used MIC and MBC for testing and concluded that *Benincasa hispida* mediated AuNP's revealed good antimicrobial potential[34].

The results of the studies conducted by Aljabali et al and Hernández-Sierra et al observed to be inconsistent with the ones obtained in our study. Aljabali et al tested the antimicrobial activity of *Ziziphus Zizyphus* synthesized AuNP's against *E. coli* and *C. Albicans*. The results revealed that at 5mg/ml concentration of AuNPs, an inhibition zone of 0.5mm for *E. coli* and 0.3 mm for C. Albicans was observed. This indicated that AuNP's did not possess antimicrobial or antifungal activity. The same study revealed excellent antibacterial activity of gold ions against both organisms indicating the antimicrobial activity to be as a result of the presence of gold ions[16]. Hernández-Sierra et al evaluated the antimicrobial potential using MIC and MBC and indicated that AuNP at concentration of 197 µL showed presence of moderate antimicrobial activity against *Streptococcus Mutans[21]*

In the present study, Gold nanoparticle based oral rinse showed potent antimicrobial activity against *S. aureus* followed by good activity against *E. faecalis*, *C. Albicans* and *S. mutans* with the highest activity at 150 µL. At concentrations of 50 µL, 100 µL too, good antimicrobial potential was elicited by AuNP's and oral rinse. Thus, at low concentrations and small particle sizes effective antimicrobial activity has been observed. A substantial body of evidence have reported the effect of Triphala oral rinse to reduce gingival inflammation, bacterial and plaque load. Triphala-based oral rinses have been shown to have lesser negative effects.[41] It is composed of : T. chebula, T. bellirica, and E. officinalis. Although all three components have been evaluated separately in the literature, the greatest antimicrobial efficacy is demonstrated when all three are used together. Triphala oral rinses have been known to reduce 65 % of the *S.Mutans* count[42–44] Tannic acid is a significant component of the triphala plant . Tannic acid contains phenolic groups, which have the unique property of binding with mucosa and teeth in the oral cavity, thereby extending the extract's action.[45]

NPs have been used as remineralizing agents extensively. AuNPs have been compared with silver nanoparticles by using them in toothpastes and were more effective against gram positive bacteria.[46] AuNPs reduce plaque load considerably owing to their antibacterial action[47].No study has been done on AuNP oral rinses. This is the first study in which AuNP has been used in oral rinses. But other nanoparticles have been used to produce oral rinses. Nanoparticle based oral rinse has been known to overcome certain disadvantages of conventional oral rinses. Kachoei *et al* concluded that Ag/ZnO NP based oral rinse was an effective antimicrobial agent against *S. mutans* and can be considered as a good alternative to 0.2% chlorhexidine. Abadi MF *et al* used silver NP to make alcohol free oral rinse to prevent oral cavity infections in immunocompromised oncologic patients, which showed to have high antimicrobial effect against *S. mutans, E. coli, P aeruginosa C. Albicans*.[48] Barma et al proved that silica based NP oral rinse was effective against *S.Aureus* and *E.Faecalis*.[49] As the nanoTiO2-containing oral rinse was found to be an effective antimicrobial agent, it can be used as an alternative to chlorhexidine or sodium fluoride mouthrinses in the oral cavity. Al- Sharani showed comparable

antimicrobial activity of nanosilver and chlorhexidine oral rinse.[50] Thus, the rationale of this study was to prepare a nanoparticle based oral rinse in an attempt to overcome the above shortcomings of commercially available oral rinses. Because AuNP oral rinses have not been studied, additional in-vitro and in-vivo studies are required to confirm their efficacy.

Strengths

Most common plaque producing organisms were tested in this study. oral rinse was devoid of alcohol, had low concentration of nanoparticles, small particle size, and has low toxicity in comparison with commercial oral rinse. It is a good alternative in patients having sensitive or inflamed mucosa, which is observed in orthodontic patients, owing to absence of alcohol.

Limitations

This was an in-vitro study and further in-vivo experiments need to be carried out. Cost of gold nanoparticles to prepare the oral rinse should be taken into account.Further studies need to be carried out to check for the biocompatibility, shelf life of oral rinse as this study lacked to gather the data. Clinical relevance: The results of this study prove that AuNPs could be incorporated in oral formulations like toothpastes, mouthwashes, adhesives, coated on orthodontic brackets. Etc due to their good antimicrobial potential.

Conclusion

Thus, AuNP's and its based oral rinse elicited good antibacterial effects against most common plaque producing microorganisms and this study forms the first of its kind. Further studies need to be conducted on a larger scale to elucidate its effects This could be of use in the field of dentistry by incorporating AuNPs in various oral formulations to control bacterial activity and as anti-plaque agents.

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Figures

Figure 1: Preparation of gold nanoparticles and oral rinse and visual observation: Color change was observed from pale yellow to violet indicating formation of nanoparticles. To the extreme right oral rinse is depicted

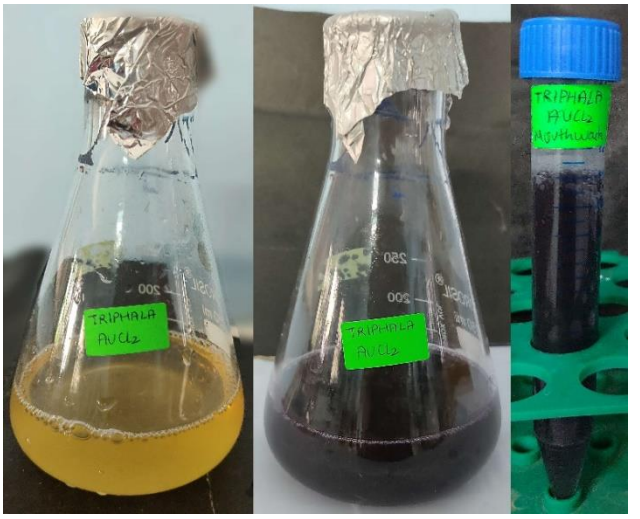

Figure 2 Left : UV-vis spectroscopy :SPR peak at 520nm ; Right:TEM analysis , 12-35nm spherical nanoparticle

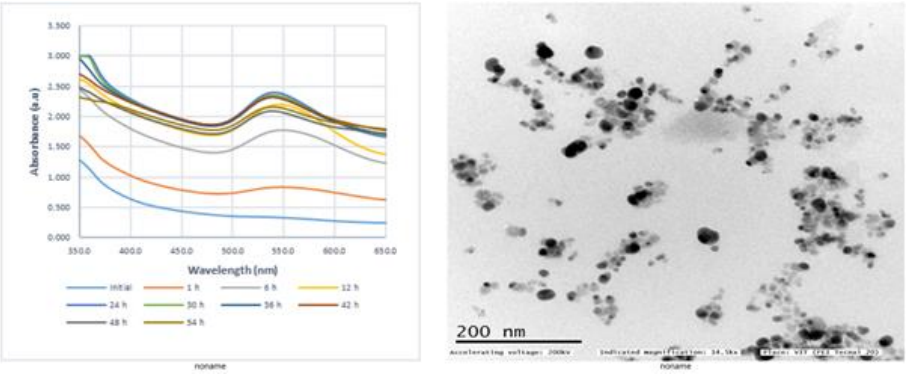

Figure 3: Inhibition zones for each organism: Antimicrobial activity of AuNP's was assessed

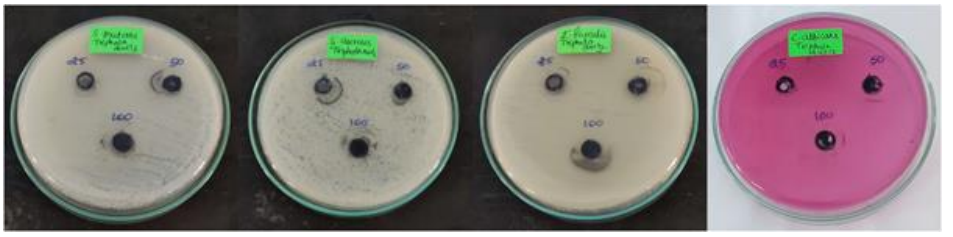

Figure 4: Inhibition zones for each organism

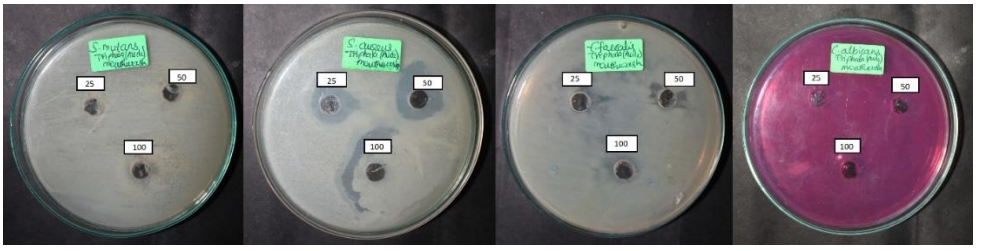

Table 1
Depicts the various zones of inhibition for the AuNP's at different concentrations of the nanoparticles for different oral pathogens

ORGANISMS	ZONE OF INHIBITION (mm) (AuNP's)		
CONCENTRATION(µL)	25	50	100
S. mutans	11.75	12.6	14.9

S. aureus	11	11.3	11.6
E. faecalis	10	10.9	11.2
C. Albicans	13	14.2	16

Table 2
Antimicrobial activity of oral rinse

ORGANISMS	ZONE OF INHIBITION (mm) (AuNP based oral rinse)		
CONCENTRATION(µL)	25	50	100
S. mutans	11.8	12.9	15.1
S. aureus	14	16	18
E. faecalis	12	12.6	13
C. Albicans	13.6	14.2	16