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Green Synthesis and Characterisation of Gold Nanoparticles Using Averrhoa Bilimbi Leaf and Its Anti-Inflammatory and Anti-Microbial Activity

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Abstract: Green synthesis of gold nanoparticles is a method which employs an environment friendly synthesis of nanoparticles with plant extracts and has gained importance over the recent years due to its reduced toxicity. The aim of the present study was to synthesize gold nanoparticles using *Averrhoa bilimbi* leaf extract and to evaluate its anti-inflammatory activity and anti-microbial activity after characterisation using Transmission Electron Microscopy (TEM). The gold nanoparticles were synthesised using *Averrhoa bilimbi* leaf extract. The synthesised gold nanoparticles were confirmed by using UV-Visible spectroscopy. The morphology of the *Averrhoa bilimbi* mediated gold nanoparticles was analysed using TEM. The green synthesised gold nanoparticles were further evaluated for its anti-inflammatory and anti-microbial activities . Visual observation showed colour change from golden yellow to dark red indicating the formation of spherical gold nanoparticles of average size ranging between 4-40nm. UV-Visible spectroscopy showed a peak between 500-550 nm at 14thh which indicated the presence of gold nanoparticles. The nanoparticles showed potent anti-inflammatory activity at minimal concentration when compared to diclofenac sodium and potent antimicrobial activity against *C. albicans* in comparison with *E. faecalis* and *S. mutans*. The present study, conclude that the green synthesis of gold nanoparticles is an effective, easy, affordable way to synthesize gold nanoparticles of high reaction rate for various biological purposes.

Keywords: Green synthesis, gold nanoparticles, *Averrhoa bilimbi*, anti-inflammatory, antimicrobial activity

INTRODUCTION

Nanotechnology is an interdisciplinary branch including physics, biology, chemistry, medicine and material science for which nanoparticles are considered to be the building blocks. (Rajeshkumar and Bharath, 2017; Santhoshkumar, Rajeshkumar and Kumar, 2017) Nano in Greek means Dwarf. Nanoparticles are small particles ranging from size of 1-100nm but with a maximum surface area. They have different shapes and show different characterization features like optical, catalytic, magnetic, chemical sensing, electrical conductivity, biomedical applications, antimicrobial activity and Surface enhanced Raman scattering (SERS). In the recent era, synthesis of nanoparticles especially from noble metals have increased interests with main focus on synthesis, approach, manipulation and characterization of nanoparticles. Among the noble metals used for nanoparticle synthesis, there is increased emphasis on gold and silver nanoparticles. (Rajeshkumar, 2016b; Menon *et al.*, 2018) These nanoparticles are synthesised using physical and chemical means which are hazardous to the environment and thereby preclude their applications in various fields of sciences. (Rajeshkumar and Naik, 2018) Hence a newer method called “Green synthesis” was devised. Green synthesis of nanoparticles is an eco-friendly, easily available, cheap method which employs the use of amino acids, aromatic amines, glycerol, phenolic acids, plant extracts, algae, fungi and virus. Phytochemicals like polyphenols, terpenoids and biomolecules present in the plant extracts act as reducing as well as stabilizing agents for plant mediated synthesis of nanoparticles.(Rajeshkumar and Bharath, 2017) This field of Nano-biotechnology helps in eliminating the toxic, hazardous and energy intensive routes of nanoparticle synthesis. However this field is still under improvement due to lack of innovative and modern methods in the industrial level. Hence there is a need to develop a simple, innovative, eco-friendly and cost effective mode of nanoparticles synthesis. (Agarwal, Menon and Kumar, 2018) Gold nanoparticles are used over other metal nanoparticles due to their advantages of smaller size, higher surface area to volume ratio, biocompatibility and non-cytotoxicity, magnetic, chemical and electronic characteristics. (Rajeshkumar *et al.*, 2013; Rajeshkumar, 2016a) Gold nanoparticles can be used as biosensors, carriers of biomolecules for target drug deliveries and to study cancer cells through optical scattering. Gold nanoparticles range from 2 to 100 nm but efficient cellular uptake is shown by particles of size 20-50 nm. Surface Plasmon resonance of nanoparticles during synthesis show colour change from light yellow to ruby red. Synthesis of nanoparticles is done through both physical and chemical methods. Physical methods include deposition-precipitation method, electro synthesis, ultrasonic spray pyrolysis and molecular cage using template synthesis. However nanoparticles synthesised by these methods were found to be costly, toxic and not suitable for biological applications. Since the biocompatibility of the synthesised nanoparticles have gained attention, a newer method called "green synthesis" is used which employs non-toxic, biocompatibility and renewable materials for nanoparticles synthesis. (Malarkodi, Rajeshkumar and Annadurai, 2017; Ponnanikajamideen *et al.*, 2019)

Plants are known for their biological activities.[(Ashwini and Anitha, 2017; Nagar and Devra, 2018; Aneesa, Anitha and Varghese, 2019; Gayathri *et al.*, 2019; Kandhan *et al.*, 2019; Keerthiga *et al.*, 2019; Pranati *et al.*, 2019; Nivesh Krishna, Roy and Ezhilarasan, 2020; Preety *et al.*, 2020) Several studies have been done to reveal the role of plants and their extracts for biosynthesis of nanoparticles. Plant sources such as *Azadirachta indica, Ginger oleoresin, rosemary oleoresin, nutmeg oleoresin, Gymnema sylvestre and* stem extract of *Dalbergia coromandeliana* were used effectively for the synthesis of metallic nanoparticles. (Ashwini and Anitha, 2017; Nagar and Devra, 2018; Aneesa, Anitha and Varghese, 2019; Gayathri *et al.*, 2019; Kandhan *et al.*, 2019; Keerthiga *et al.*, 2019; Pranati *et al.*, 2019; Nivesh Krishna, Roy and Ezhilarasan, 2020; Preety *et al.*, 2020)

Gold nanoparticles synthesised using brown algae *Cystoseira baccata* were found to be spherical, stable polycrystals with mean diameter of 8.2 +/- 2.2 nm and showed stronger cytotoxic effect against HT-29 than $CacO_2$ while lacking toxicity on PCS-201-010. (González-Ballesteros *et al.*, 2017) Halder et. Al synthesised monodisperse, crystalline gold nanoparticles of size 7.86 nm with a peak at 531 nm from gallic acid which prevented viral attachment and penetration into vero cells. These were effective with EC_{50} of 32.3 µm in HSV-1 cells and 38.6 µm in HSV-2 cells. (Halder *et al.*, 2018) Gum Ghatti synthesis of gold nanoparticles at 530 nm by Box Behnken design produced spherical particles *with* a negative zeta potential of 21.3 VV. (Alam *et al.*, 2017) Ahmad synthesised Gold nanoparticles at 35-75 mm using aqueous *Elaeis guineensis* leaves extracted at room temperature. This produced well scattered spherical nanoparticles with a high negative zeta potential value of 14.7+/-4.68 mV. (Ahmad *et al.*, 2018)

Based on these previous studies, this study aims at using the plant extract of *Averrhoa bilimbi* (*A.bilimbi*) to synthesis gold nanoparticles. *A.bilimbi* is a fruit bearing tree of the genus *Averrhoa* and family *Oxalidaceae* seen mainly in south Asian countries. (Yasmin, Ramesh and Rajeshkumar, 2014) Phytochemical components of *A.bilimbi* leaves include alkaloids, tannins, saponins, flavonoids, cardiac glycosides, glycosides, triterpenes, phenols and carbohydrates. (Cabrera *et al.*, 2017) The plant is used for various medicinal purposes like itching, swelling, rheumatism, mumps, cold, cough and other inflammatory conditions. Pharmacological investigations by various researches showed activities such as anti-diabetic, anti-hypertensive, anti-thrombotic, hypolipidemic, hepatoprotective, cytotoxic, antimicrobial, wound healing, antihelminthic and anti-oxidant. (Roy, Geetha and Lakshmi, 2011)Due to its medicinal properties this plant was chosen for the synthesis of gold nanoparticles and to check its anti-inflammatory activity and anti-microbial activity. Our team has rich experience in research and we have collaborated with numerous authors over various topics in the past decade (Deogade, Gupta and Ariga, 2018; Ezhilarasan, 2018; Ezhilarasan, Sokal and Najimi, 2018; Jeevanandan and Govindaraju, 2018; J *et al.*, 2018; Menon *et al.*, 2018; Prabakar *et al.*, 2018; Rajeshkumar *et al.*, 2018, 2019; Vishnu Prasad *et al.*, 2018; Wahab *et al.*, 2018; Dua *et al.*, 2019; Duraisamy *et al.*, 2019; Ezhilarasan, Apoorva and Ashok Vardhan, 2019; Gheena and Ezhilarasan, 2019; Malli Sureshbabu *et al.*, 2019; Mehta *et al.*, 2019; Panchal, Jeevanandan and Subramanian, 2019; Rajendran *et al.*, 2019; Ramakrishnan, Dhanalakshmi and Subramanian, 2019; Sharma *et al.*, 2019; Varghese, Ramesh and Veeraiyan, 2019; Gomathi *et al.*, 2020; Samuel, Acharya and Rao, 2020)

MATERIALS AND METHODS

Preparation Of Plant Extract

The 1 g leaf powder of *A.bilimbi* was added to 50 mL distilled water and boiled at 50^0 C. The boiled extract was filtered using Whatmann No.1 filter paper. The filtered solution was used for nanoparticles synthesis.

Synthesis of Gold Nanoparticles

Gold nanoparticles were synthesised using 10 mL of prepared plant extract added to 90 mL of gold chloride solution and this was placed in an orbital shaker. The readings were recorded using a double beam UV-Visible spectrophotometer at a wavelength of 400-650nm for every hour in order to record the maximum peak at the exact time of synthesis. At the end of the synthesis process the solution was centrifuged at 7500 rpm for 10mins and a concentrated solution of gold nanoparticles was obtained. The pellet was dried at 70º C for 4 h from which the powdered nanoparticles were collected. (Ahmad *et al.*, 2018)

Characterization
The size and shape characterization of the prepared gold nanoparticles was done using TEM.

Anti-inflammatory Activity
The obtained gold nanoparticle was used to check the anti-inflammatory activity. Bovine serum albumin (BSA) was prepared by adding 1%BSA in distilled water. The anti-inflammatory activity was checked by adding various concentrations of gold nanoparticles to 2 mL of BSA. These were then compared to various concentrations of diclofenac sodium added to 2mL BSA used as a standard. The control used was dimethyl sulfoxide added to 2 mL BSA. All the samples were placed in a water bath at 50^{o}C for 10mins and the readings were taken using a spectrophotometer. (Jain, Anitha and Rajeshkumar, 2019)

Antimicrobial activity
The antimicrobial activity of gold nanoparticles were tested against *S. mutans, E. faecalis and C. albicans* by standard well diffusion technique using the respective medium. Three agar plates were sterilized and then seeded with freshly prepared samples of pathogens in each plate respectively. Wells were prepared with the help of a sterile stainless cork borer and each plate consisted of four agar wells of 6 mm in diameter. The wells were labelled and loaded with the gold nanoparticles with concentrations of 25 µL, 50 µL, 100 µL and control respectively. The plates were then incubated in a dark chamber at 37^{0} C for 24 h for antibacterial activity and 48 hrs for antifungal activity.The zone of inhibition was recorded and tabulated. (Vignesh *et al.*, 2019)

RESULTS AND DISCUSSION
Visual Observation
Visual observation of the solution containing gold chloride and plant extract placed in an orbital shake at room temperature showed significant colour change from golden yellow to light red in one hour. There was progressive change in colour with a resultant colour of dark red after 5 h. This colour change indicated the synthesis of gold nanoparticles from the plant extract. The formation of brick red colour indicates gold nanoparticles synthesis. (fig.1)

UV-Visible Spectroscopy
UV-Visible spectroscopy showed a Surface Plasma Resonance (SPR) peak ranging between 500-550nm during the 2nd h with an absorbance ranging between 510-580 nm which gradually increased indicating the formation of gold nanoparticles. However a peak was seen between 500-550nm with maximum absorbance during the 14th h indicating the presence of gold nanoparticles. (fig.2)

Transmission Electron Microscope
The High-resolution transmission electron microscopy (HRTEM) characterisation of the biosynthesized gold nanoparticles was done to determine its morphological shape and particle size. TEM results showed clusters of gold nanoparticles which were almost spherical in shape with an average particle size ranging between 4-40nm. Most of the nanoparticles were spherical in shape. (Reddy *et al.*, 2019) (fig. 3)

Anti-inflammatory Activity
Gold nanoparticles were added in different concentrations to the BSA solution and its anti-inflammatory activity was compared to different concentrations of standard (diclofenac sodium in BSA). (fig. 4a) Percentage of inhibition of the test samples at concentrations 10µL, 20µL, 30µL, 40µL, 50µL were 79.6 %, 74.7 %, 70.7%, 66.1 % and 62.2 % respectively. (fig. 4b) Gold nanoparticles in the BSA solution showed higher percentage of inhibition at lower concentration when compared to diclofenac sodium in the BSA solution which was used as a standard. This showed that gold nanoparticles possess potent anti-inflammatory activity at minimal concentration when compared to the standard. (Ka *et al.*, 2019)

Antimicrobial Activity
The results showed potent antimicrobial activity against *C.albicans* when compared to *E. faecalis* and *S. mutans.* The zone of inhibition of *E. faecalis* at concentrations 25 µL, 50 µL, 100 µL and ab are 9mm, 12mm,15mm and 25mm respectively. The zone of inhibition of *S. mutans* at concentrations 25 µL, 50 µL, 100 µL and were 9mm, 10mm,15mm respectively and the standard showed a zone of inhibition of 25mm.(figure. 5,figure. 6,) The zone of inhibition of *C. albicans* at concentrations 25 µL, 50 µL, 100 µL was 16mm, 18mm, 20mm and the standard showed 15mm respectively (figure. 7). The zone of inhibition was seen to be proportional to the concentration of the nanoparticles and antimicrobial activity was maximum with increased concentration of nanoparticles. (Pranati *et al.*, 2019)

Green synthesis of nanoparticles is a biocompatible method which was used to reduce the toxicity from the synthesis of nanoparticles with other physical and chemical methods. This is due to the reducing property of the plant extract on gold chloride solution which helps in synthesising nanoparticles with similar efficacy and reduced toxicity which was confirmed by cytotoxicity tests. The anti-inflammatory activity of the synthesised nanoparticles were equivalent to those when compared to diclofenac sodium showing that it's a potent anti-inflammatory agent. The nanoparticles formation was confirmed with a colour change from light yellow to red seen in the synthesis of gold nanoparticles from *A.bilimbi* extract. This was in accordance with the colour change seen by Zha *et al* who synthesised crystalline, global shaped gold nanoparticles of size 10nm-40nm with an absorbance peak at 544.5nm from leaf extract of *Ginkgo biloba.* (Zha *et al.*, 2017)
Several studies on synthesis of gold nanoparticles showed similar results to this study where the SPR showed a peak ranging between 500-550nm. Balalakshmi et al synthesised spherical gold nanoparticles with mean diameter of 25nm using *Sphaeranthus indicus* leaf extract and obtained a similar SPR peak at 531nm. These nanoparticles showed no toxicity on plant cells and aquatic invertebrates, no mortality on *Artemia nauplii* but was seen to promote mitotic division in *Allium Cepa* root tips. (Balalakshmi *et al.*, 2017) Gold nanoparticles synthesised using 5,7 dihydroxy-6-methoxy-3,4 methylenedioxy isoflavone obtained from the roots of *Dalbergiacor mandeliana* produced spherical, monodispersed nanoparticles of size 10.5nm with a crystalline lattice structure and SPR peak at 532nm. These nanoparticle reductions were catalysed by first order kinetics and were seen to be stable for more than 5 months. (Umamaheswari, Lakshmanan and Nagarajan, 2018) Hamelian et al synthesised of gold nanoparticles using Thyme at room temperature and found presence of particles with average size of 35nm showing peak at 530nm. These nanoparticles showed antibacterial activity on gram positive and gram negative bacteria and also showed increased antioxidant property when compared with standard antioxidant (butylated hydroxytoluene). (Hamelian, Varmira and Veisi, 2018) Lymph node targeted mannan capped gold nanoparticles synthesised were spherical in shape with an average diameter of 9.18+/-0.71nm and SPR peak at 522nm. These nanoparticles can be used as biologically targeted contrast agents for CT imaging. (Uthaman *et al.*, 2018) Upon analysis the synthesised nanoparticles showed potent anti-inflammatory activity in-vitro. This is in accordance with the study results of Singh et al which stated that the green synthesis of spherical silver nanoparticles and monodispersed hexagonal gold nanoparticles with fruit extract of *Prunus serrulata* showed reduced expression of inflammatory mediators. (Singh *et al.*, 2018) The silver nanoparticles synthesised using plant extract showed very good anti-inflammatory activity. (Roy, Rajeshkumar and Lakshmi, 2019) Study by Baharara *et al* showed that silver nanoparticles synthesised with *Salvia officinalis* extract possess anti-oxidant and anti-inflammatory activity. (Asadi-Samani *et al.*, 2017) Synthetic peptide-gold nanoparticle hybrid was also seen to be efficient in acute lung injury due its anti-inflammatory potential. (Gao *et al.*, 2019) The zone of inhibition formed around *C. albicans* was marginally more followed by *E. faecalis* and *S. mutans* and its size increases with increase in concentration. This shows that the nanoparticles show potent antimicrobial activity against these micro-organisms at a concentration of 100µL but there was potent antimicrobial activity against *C. albicans* even at a minimum concentration of 25µL. (Gautam *et al.*, 2011) This is due to the lower resistance of *C. albicans* against nanoparticles when compared to conventional antimicrobials. The antimicrobial efficacy of the current study is in accordance with the results obtained from other studies. However a few minor discrepancies are seen with reference to the concentration effectiveness. These may be due to various in-vitro conditions like temperature, absence of proper oral environmental conditions like blood, saliva and oxidation-reduction reactions. Hence it is necessary to conduct further evaluations in vivo to assess the efficacy of the nanoparticles in the oral environment. (Charannya *et al.*, 2018)
The therapeutic potential of plants as antimicrobial agents is well explored and the utilization of plant products for various synthesis for eco-friendly nanoparticles are recent trends. *A.bilimbi* is a well-studied plant for its various pharmacological actions and hence, in this study *A.bilimbi* leaf extract was used for the synthesis of gold nanoparticles and evaluated for its antimicrobial and anti-inflammatory activity. Further study is extended to incorporate it in oral toothpaste or gel as it has both antimicrobial and anti-inflammatory activity after due toxicity studies. (Roy, Geetha and Lakshmi, 2011)Our institution is passionate about high quality evidence based research and has excelled in various fields ((Pc, Marimuthu and Devadoss, 2018; Ramesh *et al.*, 2018; Vijayashree Priyadharsini, Smiline Girija and Paramasivam, 2018; Ezhilarasan, Apoorva and Ashok Vardhan, 2019; Ramadurai *et al.*, 2019; Sridharan *et al.*, 2019; Vijayashree Priyadharsini, 2019; Chandrasekar *et al.*, 2020; Mathew *et al.*, 2020; R *et al.*, 2020; Samuel, 2021)

CONCLUSION

This study on green synthesis of gold nanoparticles was a simple yet eco-friendly approach with *A.bilimbi* leaf extract. The nanoparticles synthesised were spherical in shape and size was between 4-40nm . The A.bilimbi mediated gold nanoparticles showed potent anti-inflammatory activity at minimal concentration. Based on the present results the gold nanoparticles can be used for its applications such as antiinflammatory and antimicrobial activity .

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CONFLICT OF INTEREST
None

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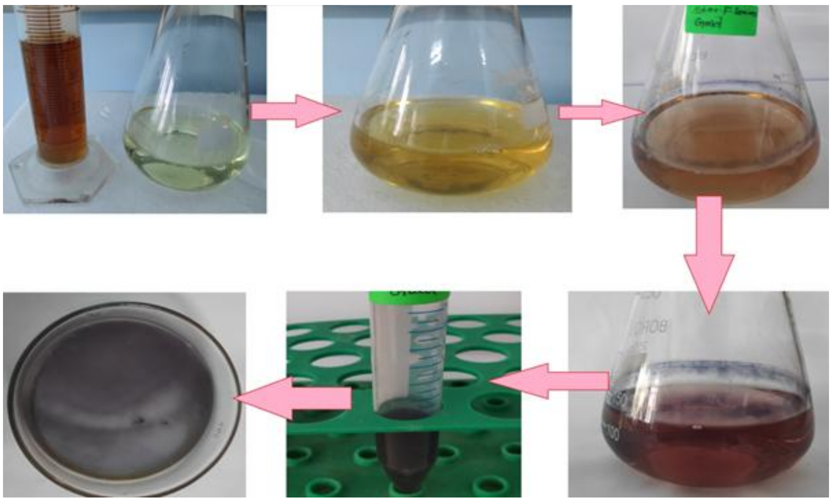

Fig. 1: Visual observation of A.bilimbi leaf mediated gold nanoparticles synthesis

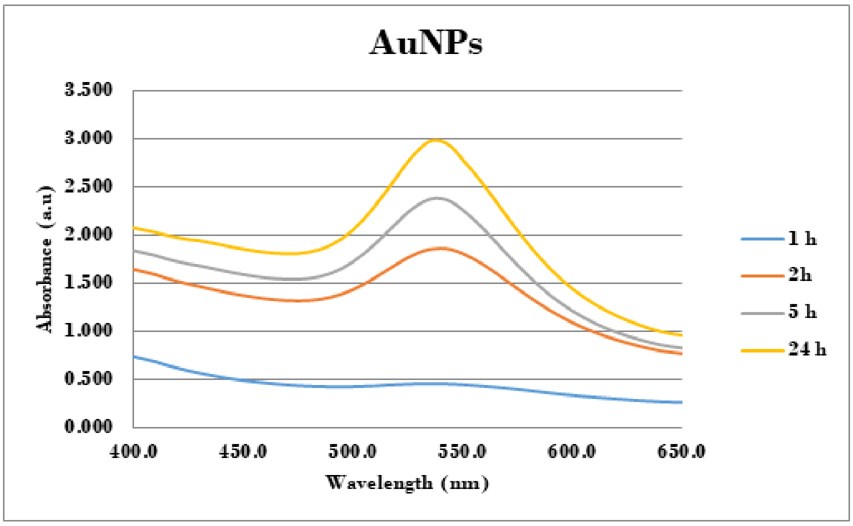

Fig. 2: UV-Visible spectroscopic analysis of A.bilimbi leaf mediated AuNPs

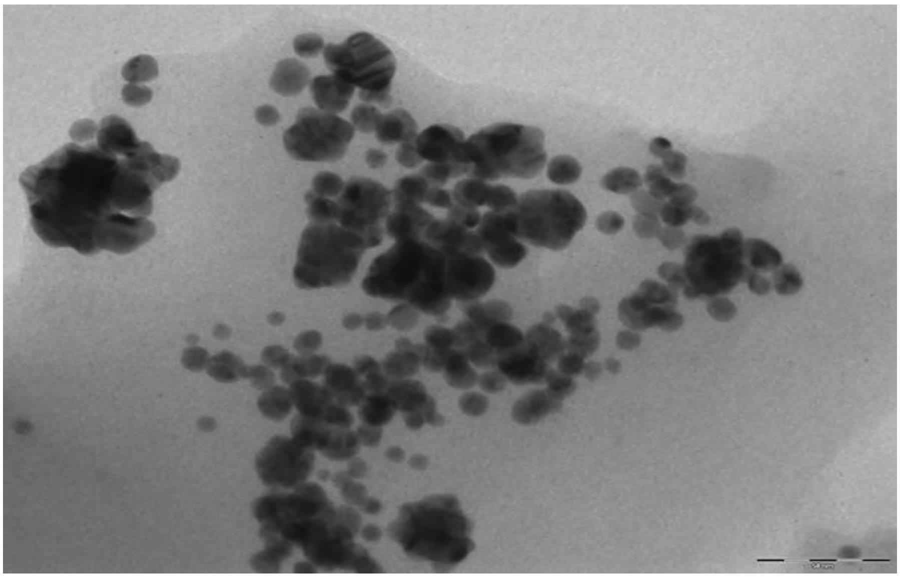

Fig. 3: TEM image of A.bilimbi leaf mediated Gold nanoparticles

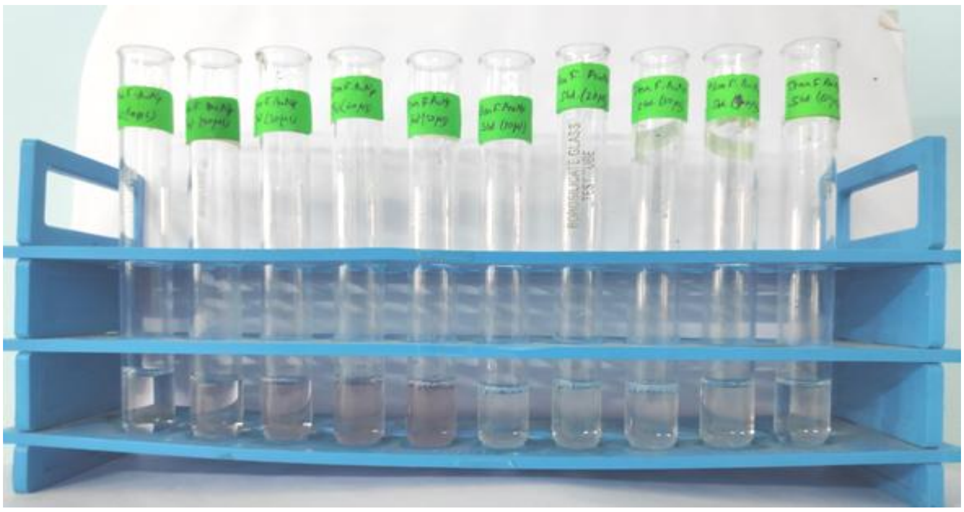

Fig. 4 a: Anti-inflammatory activity of A.bilimbi leaf mediated gold nanoparticles

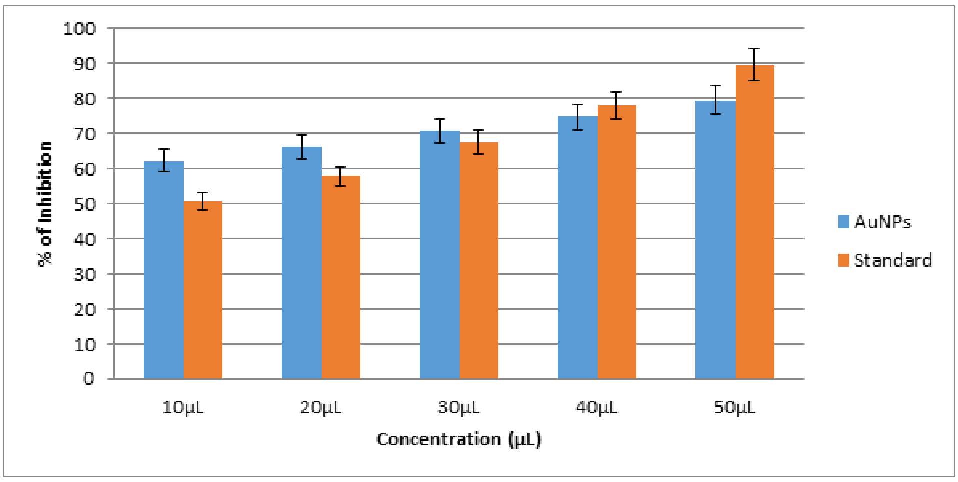

Fig. 4b.:Anti-inflammatory activity of A.bilimbi leaf mediated gold nanoparticles

Fig. 5: Antimicrobial activity of A.bilimbi leaf mediated gold nanoparticles against S. mutans,E. faecalis and C.albicans

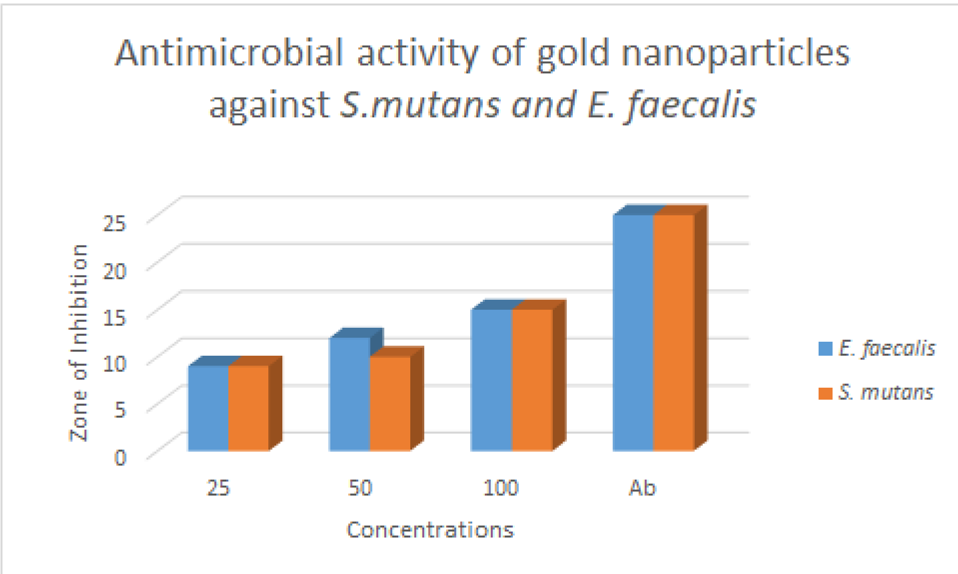

Fig. 6: Antibacterial activity of A.bilimbi leaf mediated gold nanoparticles against S. mutans and E. faecalis

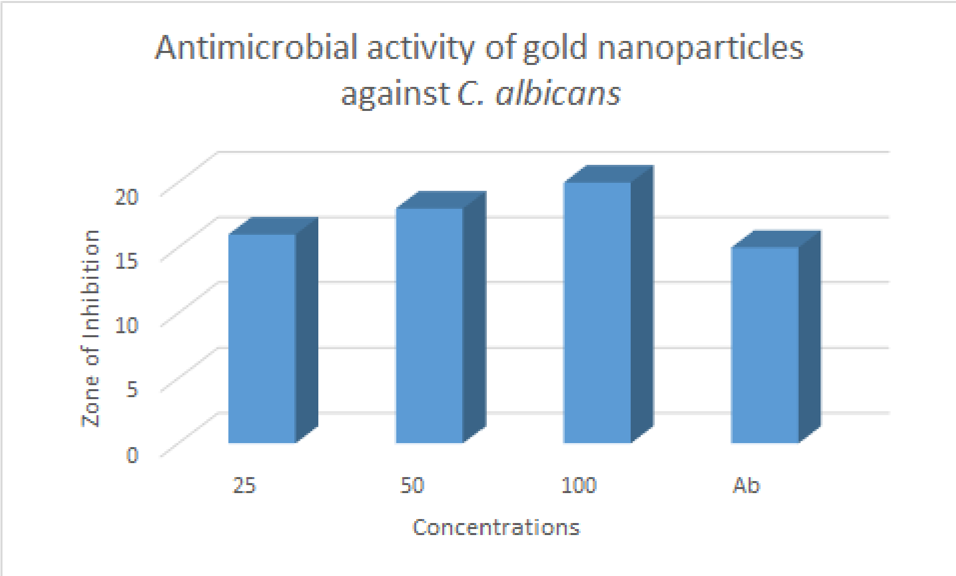

Fig.7: Antifungal activity of A.bilimbi leaf mediated gold nanoparticles against C. albicans