

Research Article

Received on: 08-04-2017
Accepted on: 12-04-2017
Published on: 15-04-2017

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Cajanus Cajan Mediated Gold Nanoparticle Synthesis, Characterization, Antimicrobial efficacy and its Antifouling Application on Metal Coupons

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ABSTRACT

The present study is focused on the biosynthesis of AuNPs using Leaf extract of Cajanus cajan and its characterization through UV-Vis Spectrophotometry, Scanning Electron Microscopy (SEM), Energy Dispersive Spectroscopy (EDS), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), DLS – Size Distribution and Zeta potential and biofilm accumulation analysis by Epi – Florescence Microscopy. The ability of the green synthesized Gold Nanoparticles to control/prevent the biofilm forming bacterial communities (which were considered as primary colonizers) was analysed by conducting the antimicrofouling studies. The Gold Nanoparticles were found to control the growth and survival of biofilm forming bacteria effectively on SS304 metal coupons. The present work gives scope for the possible development of formulations containing AuNps as effective antifouling agent that could prevent microfouling, thereby preventing marine biofouling.

Key-words: Cajanus cajan, Gold Nanoparticle, biosynthesis, metal coupons, Microfouling

Cite this article as:

Vidhya Ananthi T, Ahmed John S Cajanus Cajan Mediated Gold Nanoparticle Synthesis, Characterization, Antimicrobial efficacy and its Antifouling Application on Metal Coupons, Asian Journal of Pharmaceutical Technology & Innovation, 05 (23); 41-54, 2017. www.asianpharmtech.com

1.0 Introduction

Some plants can absorb and accumulate metals from water and soil in which they are grown. These are named as 'hyper-accumulators'¹. Alfalfa can accumulate gold and store it in its leaf and stem biomass as discrete nanoparticles of pure metal. In recent years, several plants have been successfully used and reported for efficient and rapid extracellular synthesis of silver, copper and gold nanoparticles.

Several plants and plant products have been successfully used for efficient and rapid extracellular synthesis of silver and gold nanoparticles. The synthesis methods using organisms, both unicellular and multicellular like yeast, fungi and bacteria which were able to synthesize inorganic materials either extracellularly or intracellularly².

Metallic nanoparticles are presently applied in different fields such as electronics, biotechnology, chemical and biological sensors, DNA labelling, drug delivery, cosmetics, coatings and packaging^{3,4}. There have been impressive developments in the field of nanotechnology in the recent past years, with numerous methodologies formulated to synthesize nanoparticles of particular size and also of shape depending on specific requirement.

Marine biofouling is one of the major problems encountered on the man-made objects in the marine environment. Biofouling has been defined as the undesirable accumulation of microorganisms, algae, diatoms, plants, and animals on wetted surfaces. The establishment of fouling community takes place in several stages. Initially, any submerged surface gets coated by a conditioning film consisting of organic and inorganic molecules. The subsequent onset of macrofouling may be preceded by the formation of bacterial biofilms (bacterial fouling) and such a biofilm may have a deleterious effect on the ability of the surface to remain free from larger fouling organisms.

Use of chemical antifouling agents is one of the common and easy approaches to control fouling caused by micro - and macrofoulers. However, many antimicrobial materials are less effective on microorganisms in biofilms compared to their planktonic counterparts. Therefore, high concentrations of chemical antifoulants are required for the effective control of fouling resulting in harmful secondary effects. Future research on the antifouling strategies may target the formation of the conditioning layer as a way to prevent subsequent colonization of the surface. An effective antifoulant should inhibit biofouling in all the materials used in the marine atmosphere. Hence, it is essential to study the effect of antifouling product against microbial consortia from different materials.

The biofilm consortia from material of SS304 are isolated and the effect of AuNPs on the same has been reported in this study. Further, all the work done so far has concentrated on the antibacterial activity of biogenic AuNPs against pure cultures of biofilm forming bacteria. Therefore, for effective control of both micro- and macro-fouling, the antifoulant should have a broad spectrum of anti-microfouling property. This report is a novel approach which carries the application of biogenic AuNPs to field testing in natural seawater.

2.0 Materials and Methods

2.1 Plant Material

Fresh leaves of *Cajanus cajan* was collected from Tiruchirappalli district of Tamil Nadu during the period of October - November 2015 and washed thoroughly thrice with distilled water, shade-dried up to 5 days and prepared fine powder by grinding. The fine powder of the plant material was sterilized at 121°C for 15 min and weighed. Sterilized fine powder, 20 g each was taken, mixed with 200 ml of Milli Q water and kept in boiling water bath at 60°C for 10 min. The extracts were filtered with Whatman 1 filter paper and the filtered extracts were stored in a refrigerator at 4°C for further studies to avoid microbial contamination.

2.2 Synthesis of gold nanoparticles

Biosynthesis of gold nanoparticles, gold chloride prepared at the concentration of 10⁻³ M with pre-sterilized Milli Q water. A quantity of 10 ml plant extract was mixed with 90 ml of 10⁻³ M gold chloride for the synthesis of gold nanoparticles. Gold chloride has taken in similar quantities without adding plant extracts to main respective controls. The saline bottles were tightly covered with aluminium foil in order to avoid photo reduction of gold ions, incubated at room temperature under dark condition and observations were recorded.

2.3 Characterization of Gold Nanoparticles

2.3.1 UV-vis analysis

The optical property of AuNPs was determined by UV-Vis spectrophotometer (Perkin-Elmer, Lamda 35, Germany). After the addition of HAuCl₄ to the plant extract, the spectrums were taken in different time intervals up to 24hrs between 450 nm to 540 nm. Then the spectrum was taken after 24hrs of HAuCl₄ addition.

2.3.2 SEM analysis

The morphological features of synthesized gold nanoparticles from *Cajanus cajan* plant extract were studied by Scanning Electron Microscope (JSM-6480 LV). After 24Hrs of the addition of H₂AuCl₄ the SEM slides were prepared by making a smear of the solutions on slides. A thin layer of platinum was coated to make the samples conductive. Then the samples were characterized in the SEM at an accelerating voltage of 20 KV.

2.3.3. Energy Dispersive Spectroscopy (EDS)

In this research work, Joel JSM-6480 LV SEM machine was used to characterize the mean particle size and morphology of nanoparticles. Compositional analysis on the sample was carried out by the energy dispersive X-ray spectroscopy (EDS) attached with the SEM. The EDS analysis of Au sample was done by the SEM (JEOLJSM 5800) machine. The EDS normally reveals the presence of phases.

2.3.4 X-ray diffraction method

The phase evolution of calcined powder as well as that of sintered samples was studied by X-ray diffraction technique (Philips PAN analytical, The Netherlands) using Cu radiation. The generator voltage and current was set at 40 KV and 30 mA respectively. The Au sample was scanned in the range 10.0000 - 90.0000o in continuous scan mode. The scan rate was 0.60/sec.

2.3.5 FTIR analysis

The chemical composition of the synthesized Gold nanoparticles was studied by using FTIR spectrometer (perkin-Elmer LS-55- Luminescence spectrometer). The solutions were dried at 75o C and the dried powders were characterized in the range 4000–400 cm⁻¹ using KBr pellet method.

2.3.6 DLS & Zeta potential analysis

Dynamic light scattering (DLS) which is based on the laser diffraction method with multiple scattering techniques was employed to study the average particle size of gold nanoparticles. The prepared sample was dispersed in deionized water followed by ultra-sonication. Then solution was filtered and centrifuged for 15 min. at 25°C with 5000 rpm and the supernatant was collected. The supernatant was diluted for 4 to 5 times and then the particles distribution in liquid was studied in a computer controlled particle size analyser (ZETA sizer Nanoseries, Malvern instrument Nano Zs).

2.4 Screening of antimicrobial activity

2.4.1 Collection of seawater

The 10 L of sea water sample (Single random sampling) was collected from 0 – 20 cm below the surface at Nagapattinam beach area. Physicochemical parameters i.e., pH, electrical conductivity (EC), total dissolved solids (TDS), and salinity were measured using a field kit (Thermo Orion 5-Star pH Multi-Meter) at the site ¹⁸. The sample was kept in iceboxes and processed within 10 h of collection. The total viable count (TVC) of bacterial strains were enumerated from the collected sea water samples.

2.4.2 Preparation and processing of metal coupons

The stainless steel (SS) 304 coupons were used in this study. The coupons (1" x 3" dimension) were pre-processed and pickled in Clarke's solution as per the ASTM recommendation (1995).

2.4.3. Isolation of biofilm organisms

The coupons were tight with strong thread and immersed in seawater (wide mouth) container (water depth of 1 meter below) at one week. After the incubation period, the biofilm layer was appeared on the surface of SS 304 coupons and were picked by using of sterile cotton swab and inoculated in to the sterile Milli-Q water. Then, the biofilming water sample was subjected in to the serial dilution and pure culture techniques. A total of 10 different bacterial strains were isolated from the nutrient agar plates and were stored in refrigerator for further uses.

2.4.4 Antimicrobial activity

The antimicrobial activity were analysed by standard disc diffusion method ⁶. The biofilm isolates were challenged against two different concentrations of Au-NPs. In this study, a sterile cotton swab was used to

inoculate the biofilm bacterial isolates on the surface of MHA agar plates ⁷. The 20 and 40 µL of sample coated disc were placed in agar plates, separately. For positive and negative control study, the standard antibiotic disk and sterile triple distilled water was used ⁸. The plates were incubated at 37±1°C for 24–48 h for bacterial culture. After incubation, the zone of inhibition was measured with ruler. All the trial was performed thrice and mean values were presented.

2.5 Screening of Antifouling activity

2.5.1 Isolation and Enumeration of biofilm bacteria

After one week of immersion, the developed biofilms on SS304 coupon was swab-removed using sterile cotton swabs and immediately transferred to culture tubes containing sterilized aged seawater (ASW). From the mixture (biofilm isolates + sterile aged seawater), bacterial strains were isolated and enumerated by pure culture technique (spread plating method - 0.1 ml) on selective medium plates with appropriate dilutions (10⁻¹, 10⁻², 10⁻³ and etc.) ¹⁸. The number of visible colonies were counted after 48 hrs of incubation in the order of magnitude above 10² CFUs cm⁻² (CFUs - Colony Forming Units). Nutrient and selective media plates were prepared with the addition of sterilized ASW. Average bacterial counts of the replicates were noted and the mean values were recorded. Partial identification of biofilm bacteria were done based on the results of morphologically dissimilar and distinct colonies. These were randomly selected and inoculated into rapid microbial limit test kits (Hi-media Laboratories Limited, India) for the identification of the bacterial strains ⁵.

2.5.2 Biofilm adherence/ accumulation analysis by Epi- fluorescence microscopy study

Biofilm coupons were first gently rinsed in sterilized ASW, fixed in 3% glutaraldehyde for 1 hour. The dye acridine orange (0.01 mg ml⁻¹ of sterile deionised water) was added directly on top of the biofilm on tested coupons and the coupons were incubated in the dark at room temperature for 15 minutes. The samples were observed under UV excitation using filter type C-FL Epi-FL and filter block type B-2A. Randomly chosen fields were photographed. Dye (acridine orange) taken up by the biofilm sample was visualized under 40X (0.60) objective and processed by Evolution MP Camera. The images were captured and saved to a computer using Q-Capture software.

2.5.3 AuNPs + Paint coating coupon preparation

The 50 ml of commercially available paint ('Asian paints' – Apcomin zinc chrome (AZC) primer yellow) was mixed with 5 g of lyophilized AuNPs. The AZC with AuNPs mixture was applied on the SS304 coupons (1" x 3" dimension) by a dip coating method which was then allowed to dry in the shade for one day. This process was carried out thrice and without AuNPs sample (AZC paint only) was taken as a control study.

2.5.4 In-vitro antifouling activity

A bacterial strain (Marine isolate 1) was isolated from the seawater which is dominant in the nutrient agar plate (which was prepared by adding aged seawater) as well as SS304 coupon. So, it was selected for further studies. The 100 µl of biofilm pure culture (Marine isolate 1 = 18 h) was transferred to a conical flask containing 50 ml of nutrient broth. The AuNPs + paint coated coupon was immersed in cultured broth medium. The conical flasks were kept on orbital shakers at 150 rpm for 24 hrs at room temperature. Simultaneously, a control was run with paint coated coupon alone. All assays were performed in triplicate. In both studies (with and without AuNPs), the bacterial densities were enumerated by pure culture spread plate technique.

The 100 µl of biofilm pure culture (18 h) was transferred to a conical flask containing 100 ml of nutrient broth with 2 mg AuNPs and none for the control. The SS304 coupon was immersed in cultured broth medium and was kept in orbital shakers at 150 rpm for 24 hrs at room temperature. All assays were performed in triplicate. Simultaneously, a control was run without adding the AuNPs to the broth medium. In both studies (with and without adding AuNPs), the bacterial densities were enumerated by pure culture spread plate technique.

2.6 Mortality bioassay of *Artemia marina*

Brine shrimp *Artemia marina* was raised from the cysts in seawater at 29 °C and it was kept for 2 days before starting the bioassay. A light source was provided to attract the free-swimming *nauplii* and the *nauplii* were transferred to the experimental bioassay. AuNPs were dissolved in the sterile seawater and the final concentration of the samples became 10, 25, 50, 100 and 200 µg ml⁻¹, respectively. Approximately 30 *nauplii* were transferred to each test tube which had 5 ml of five different concentrations of seawater + AuNPs mixtures separately. The test tubes were incubated at 29 °C for 24 - 48 hrs ⁵. After incubation the test tubes

were observed using magnifying hand lenses. The number of survivors, dead and immobile *nauplii* in each test tube were counted and noted. The assays were performed in triplicate and the mean results were present.

The percentage mortality was calculated by following the formula:

$$\% \text{ Mortality} = \frac{\text{Number of dead } Artemia \text{ nauplii}}{\text{Initial number of live } Artemia \text{ nauplii}} \times 100.$$

3.0 Result and Discussion

3.1 Biological reduction of gold salt

Reduction of gold salt into gold nanoparticles during exposure to plant extracts was observed as a result of the colour change (Plate 1). The colour change is due to the Surface Plasmon Resonance (SPR) phenomenon.

3.2 UV-Vis spectrophotometer analysis

The metal nanoparticles have free electrons, which give the SPR absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with light wave. The sharp bands of gold nanoparticles were observed around 540 nm in case of *Cajanus cajan*. From different literatures it was found that the gold nanoparticles show SPR peak at around 540 nm. From our studies we found the SPR peak for *Cajanus cajan* at 540 nm.

It is confirmed that *C. cajan* leaf extract has more potential to reduce Au ions into Au nanoparticles, which lead us for further research on synthesis of gold nanoparticles from *Cajanus cajan* leaf extracts. The intensity of absorption peak increases with increasing time period. This characteristic colour variation is due to the excitation of the SPR in the metal nanoparticles. The reduction of the metal ions occurs fairly rapidly; more than 90% of reduction of Au⁺ ions is complete within 2 hrs after addition of the metal ions to the plant extract. The metal particles were observed to be stable in solution even 4 weeks after their synthesis. By stability, we mean that there was no observable variation in the optical properties of the nanoparticles solutions with time.

On the behalf of the UV-Vis data it was cleared that reduces metal ions. So the further characterizations were carried out with *Cajanus cajan* (Figure.1). The UV-Vis absorption spectroscopy is one of the main techniques followed to examine size and shape of the nanoparticles in the aqueous suspensions (Wiley et al. 2006). Huang et al. (2007) reported formation of gold nanoparticles when constant aqueous H₂AuCl₄ at 50 ml, 1 mM with 0.1 g biomass produced gold nanoparticles as indicated by sharp absorbance at around 540 nm in *Cinnamomum camphora*.

3.3 SEM Analysis of AuNPs

SEM provided further insight into the morphology and size details of the gold nanoparticles. Comparison of experimental results showed that the diameters of prepared nanoparticles in the solution have sizes several Nano meters i.e. between 1-100 nm. The size was more than the desired size as a result of the proteins which were bound in the surface of the nanoparticles (Figure 2).

3.4. EDS Analysis of AuNPs

EDS revealed the presence of pure gold nanoparticles in higher percentages. Gold peak is higher than other peak. The EDS reading proved that the required phase of gold (Au) is present in the sample. This is probably due to the presence of substrate over which the NP sample was held during SEM microscopy

3.5. X-ray diffraction (XRD) analysis of AuNPs

The *Cajanus cajan* leaf extract-mediated synthesized Au nanostructure was confirmed by the characteristic peaks observed in the XRD image which was shown in Figure 3. The XRD result shows four distinct diffraction peaks at 38.12°, 44.27°, 64.27° and 76.23°, which are indexed for the planes (111), (200), (220) and (311) respectively of the face centered cubic Au. Other peaks were also observed along with the main peaks. This may be due to the crude nature of the extracts containing other metabolites and salts.

3.6. FTIR analysis of AuNPs

FTIR measurement was carried out to study the interaction of the nanoparticles and to identify the possible biomolecules in *Cajanus cajan* leaf extract responsible for capping leading to efficient stabilization of the NPs. The intense IR bands (Figure 4) are observed at 3436, 2367, 2075, 1635 and 684 cm⁻¹. The bands

observed at 3436 are assigned to the stretching vibrations of Secondary amine (N-H asymmetric stretching). The broad band at 2367 cm^{-1} is due to Tertiary amine salt ($-\text{NH}^+$ stretching) modes. The absorption bands located at 2075, 1635 and 684 may be attributed to $\text{RCH}=\text{N}=\text{N}$, O- NO_2 Stretching asymm and C-S stretching modes, respectively. The bonds or functional group such indicates that's. Therefore, it may be assumed that water soluble compounds such as flavonoids, gold nanoparticles synthesized using the *Cajanus cajan* leaf extract surrounded by some proteins and metabolites such as flavonoids which are capping ligands of the nanoparticles.

3.7. DLS-Size Distribution

The dynamic light scattering (DLS) is a technique widely used for determining the size of colloidal nanoparticles. Distributions of the hydrodynamic diameters of the GNPs were measured by two different light scattering-based techniques. DLS measurements were performed at 90° with three repetitions of AuNPs. The results are presented in Figure 4. The intensity size distribution reveals a broad peak, indicating a potential aggregation of gold nanoparticles. The particle size distribution (PSD) of synthesized gold nanoparticles, it was found that Au nanoparticles size were in the range of 50-100nm. The highest fraction of AuNPs present in the solution was of 95 nm is very appropriate since it gives lowest average size of nanoparticles.

3.8. DLS- Zeta potential analysis of AuNPs

The Figure 5 shows the zeta potential (ζ) is a measure of the electrostatic potential on the surface of the nanoparticles and is related to the electrophoretic mobility and stability of the suspension of nanoparticles of the Nano gold. The measurements render a sharp and clean peak, whereas the deviation between the peaks is relatively small, which indicates that the measurements are indeed repeatable and consistent. The overall absorbance of Zeta Potential revealed the energetically insufficient instability.

3.9 Antimicrobial activity

The bioactive compounds from coastal regions may act as an effective antifouling agent and it also used for many beneficial purposes ^{5, 18}. In this study, the biofilm bacterial strains from the seawater samples were challenged against AuNPs. The test concentrations (20 and 40 $\mu\text{L}/\text{disc}$) produce zone on MHA plates. The AuNPs sample was most effective against BS 2 while smaller effect was noticed from BS 6 (Table 1). The bactericidal activity was dependent on the shape and size of the nanostructures and their concentrations ¹⁴. TOAg-NPs are known to bind with thiol groups of DNA and RNA and affect the protein biosynthesis of bacteria ¹⁶. Studies have demonstrated that silver ions interact with sulfhydryl (SH) groups of proteins as well as the bases of DNA leading either to respiratory inhibition or the unwinding of DNA ¹⁷.

In nanoparticles study, the higher (40 $\mu\text{L}/\text{disc}$) concentration got larger zone effect than the small (20 $\mu\text{L}/\text{disc}$) concentrations against certain microorganisms. All the microbial strains depict higher sensitivity to the higher concentration for the test sample ^{5, 18}. There is no antimicrobial activity in solution devoid of sample used as a vehicle control (sterile triple distilled water), reflecting that antimicrobial activity was directly related to the sample. A large EPS amount may create an anaerobic environment that in turn initiates corrosion processes. *Bacillus* sp., and *Pseudomonas* sp. are otherwise well adhering and biofilm forming bacterial strains in the marine environment ¹⁹. The cell wall of the gram-positive bacteria contain a thick layer of peptidoglycan, consisting of linear polysaccharide chains cross-linked by short peptides thus forming more rigid structure leading to difficult penetration of the Au-NPs compared to the gram-negative bacteria where the cell wall possesses thinner layer of peptidoglycan ²⁰. The banana peel extract mediated Au-NPs have shown efficient antimicrobial activity towards most of the tested fungal and bacterial cultures. The extracellular synthesis of Au and Ag nanoparticles by using the aqueous extract of *Solanum torvum* and these nanoparticles have shown strong free radical scavenging and antibacterial activity ^{21,22}.

3.10 Antifouling Screening

The different bacterial groups were enumerated from the sea water sample through using of pure culture techniques and the sample was added into the selective medium. After addition of sample into the selective medium plates, the plates were incubated at 37 $^\circ\text{C}$ for 24 – 48 h. After incubation, the colonies were counted from the selective medium plates and its mean value was recorded. The mean value of TVC, TC, TS and TV in sea water sample were 5.6 [$\times 10^5$], 4.2 [$\times 10^4$], 1.08 [$\times 10^4$] and 2.41 [$\times 10^4$] CFU/ml, respectively. On the other hand, the SS304 coupon was immersed into the sea water sample. After that the SS304 metal coupon was take

it out and the biofilm bacterial strains were removed by using sterile cotton swabs and was immersed into sterile water. The mean value of TVC, TC, TS and TV in SS304 metal coupon were 9.1×10^7 , 6.4×10^4 , 2.8×10^4 and 3.7×10^4 CFU/cm², respectively.

The AZC and AZC+AuNPs coated coupons were immersed in the nutrient broth which containing 100 μ l of biofilm pure culture (Marine isolate 1 = 18 h) separately, which was incubated in orbital shakers at 150 rpm for 24 hrs at room temperature. After incubation, the mean bacterial population on AZC and AZC+AuNPs coupons were 9.6×10^6 and 8.4×10^4 , respectively. This result indicated that AZC+AuNPs coated coupon prevent the bacterial adherence than the AZC coated coupon. Hence, it is confirmed that AuNPs may act as an alternative antifouling agent.

The 2 mg of AuNPs was added into the 100 ml of nutrient broth containing 100 μ l of biofilm pure culture (Marine isolate 1). The new SS304 metal coupon was immersed into the medium and incubated at orbital shaker with 150 rpm for 24 hrs at room temperature. The same method was used for control study without adding AuNPs. After incubation, the SS304 metal coupons were removed from both conical flask (control and experiment flask). The adhering biofilm bacterial groups were take it out from both SS304 metal coupon by using of sterile cotton swabs. The mean bacterial population in control and AuNPs treated metal coupons were 10.8×10^7 and 5.2×10^4 , respectively. This study indicated that the AuNPs effectively control the bacterial growth and it also prevent the attachment of bacterial population on metal coupons. In this study, the dead bacterial cultures may also attached on the metal coupons but it was not counted (It is performed total viable count only not total direct count). In seawater, bacterial biofilm formation was considered to be a prerequisite for fouling by larger organisms, such as algae and barnacles and the composition of the conditioning films was dependent on both the composition of the water and on the duration of immersion

3.11 Mortality bioassay of *Artemia marina*

The mortality bioassay of *Artemia* nauplii was performed with five different concentrations of AuNPs against 30 *Artemia* sp. with two different time interval such as 24 h and 48 h. The mechanism of this study is to accumulate the AuNPs in the *Artemia* nauplii gut region and it cause the death within short time duration. In control study, the mortality rate was observed in both 24 h (10 %) and 48 h (23.3 %) incubation which is negligible. Similar study as made by Muthukumar and his co-workers (2015) on Tuticorin sea water. At 10 μ g ml⁻¹ concentration, the mortality rate was 16.6 % and 40 % in 24 h and 48 h interval, respectively. The increasing concentration of AuNPs and the long-time duration causes the death, which denoted that concentration and time duration play an important role in the mortality assay of *Artemia* nauplii against AuNPs. At 25 μ g ml⁻¹ in 24 h duration, the mortality was 30.0 % and as the concentration increases from 50, 100 and 200 μ g ml⁻¹ it reached about 40.0 %, 46.6 % and 60.0 %. After 48 h the mortality rate was gradually increased when compared to the 24 h incubation. At 10 μ g ml⁻¹ in 48 h exposure, the mortality was 40.0 % and as the concentration increases from 25, 50, 100 and 200 μ g ml⁻¹ it reached about 60.0 %, 80.0 %, 86.6 % and 93.3 %, respectively. The AZC + AuNPs paint mixture prevented biofouling to a large extent on the test coupons when compared to commercial AZC painted coupons due to the antimicrobial activity. Hence, it is confirmed that AuNPs may act as an alternative antifouling agent.

Table 1. Antibacterial activity of AuNps against Biofilm Bacteria

S.No	Test Microorganisms	AuNPs		Positive Control
		20 μ L/disc	40 μ L/disc	10 mcg
1.	Biofilm isolate 1 (BS 1)	10	11	12
2.	Biofilm isolate 2 (BS 2)	14	16	16
3.	Biofilm isolate 3 (BS 3)	12	13	24
4.	Biofilm isolate 4 (BS 4)	11	13	18
5.	Biofilm isolate 5 (BS 5)	12	14	26
6.	Biofilm isolate 6 (BS 6)	8	9	14
7.	Biofilm isolate 7 (BS 7)	13	15	14
8.	Biofilm isolate 8 (BS 8)	10	11	20
9.	Biofilm isolate 9 (BS 9)	12	13	14
10.	Biofilm isolate 10 (BS 10)	9	11	18

Table 2. Chemical composition of the tested alloys

Coupon Name	Composition															
	Fe	Cr	Ni	Mo	Cu	Mn	C	S	N	Si	P	Zn	O	Na	Ca	Others
SS304	Ba*	18.28	8.23	0.17	0.19	0.48	0.047	0.011	0.076	0.49	0.019	---	--	---	---	Co - 0.14

SS304 – Stainless Steel 304, Ba* - Balance

[(--- Not present), **Fe**- Iron; **Cr**-Chromium; **Ni**- Nickel; **Mo**- Molybdenum; **Cu**- Copper; **Mn**- Manganese; **C**- Carbon; **S**- Sulfur; **N**-Nitrogen; **Si**- Silicon; **P**- Phosphorus; **Zn**- Zinc; **O**-Oxygen; **Na**-Sodium; **Ca**- Calcium; **Mg**- Magnesium; **Al**-Aluminum].

Table 3. Pickling process of alloy coupons before immersion

S.No	Marine alloys	Preparation of metal coupons						
		Pickled	Polished on motor wheels	Fineness of metallographic emery paper	Mirror finish on alumina slurries (1, 0.1 & 0.3 µm)	Wash with soap solution	Degreased with acetone	Rinsed in ethanol
1	Glass (GL)	-	-	-	-	+	+	+

+ = Processing; - = Not processing

Table 4. Details of specific culture media used for quantitative bacterial analysis

S.No	Bacterial population	Culture Medium	Colonies nature	Incubation
1.	Total Viable Count (TVC)	Nutrient Agar	All colonies counted	37 °C ± 1°C for 24 to 48 h
2.	Total Coliforms (TC)	MacConkey Agar	All colonies counted	37 °C ± 1°C for 24 to 48 h
3.	Total Streptococci (TS)	M Enterococcus Agar	All colonies counted	37 °C ± 1°C for 24 to 48 h
4.	Total Vibrios (TV)	TCBS Agar	All colonies counted	37 °C ± 1°C for 24 to 48 h

Table 5. Bacterial population in raw seawater and SS304 coupons

S.No	Parameters	Bacterial population in seawater (CFU/mL)	Bacterial population in glass coupon (CFU/cm ²)
1.	Total Viable Count (TVC)	5.6 [$\times 10^5$]	9.1 [$\times 10^7$]
2.	Total Coliforms (TC)	4.2 [$\times 10^4$]	6.4 [$\times 10^4$]
3.	Total Streptococci (TS)	1.08 [$\times 10^4$]	2.8 [$\times 10^4$]
4.	Total Vibrios (TV)	2.41 [$\times 10^4$]	3.7 [$\times 10^4$]

Table 6. Bacterial population on AZC coupon alone and AuNPs + AZC paint coated coupon

S.No	Parameters	Bacterial population in AZC coupon (CFU/cm ²)	Bacterial population in AZC + AuNPs coupon (CFU/cm ²)
1.	Marine isolate 1	9.6 [$\times 10^6$]	8.4 [$\times 10^4$]

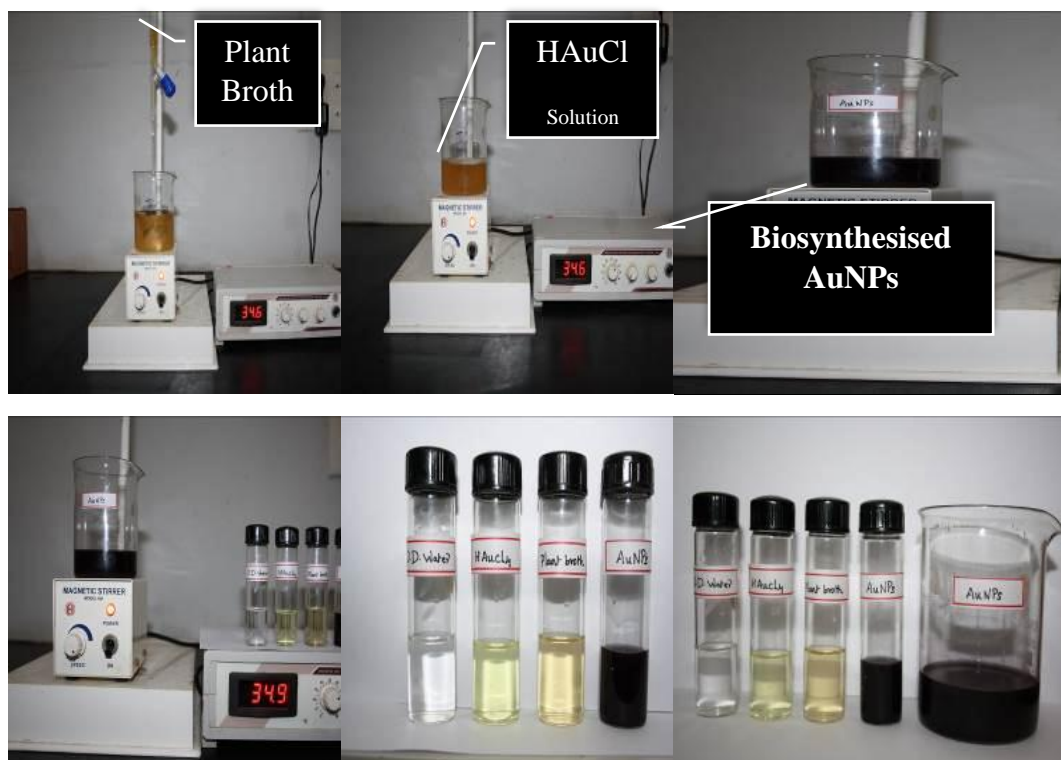
Table 7. Bacterial population on SS304 metal coupon in AuNPs added and without adding AuNPs medium

S.No	Parameters	Bacterial population on SS304 metal coupon at AuNPs added broth (CFU/cm ²)	Bacterial population in SS304 metal coupon at AuNPs not-added broth (CFU/cm ²)
1.	Marine isolate 1	5.2 [$\times 10^4$]	10.8 [$\times 10^7$]

Table 8. The mortality assay of Artemia nauplii (mortality rate) against AuNPs with different time (24 & 48 hours) interval - An in-vitro study

Parameter	AuNPs concentration ($\mu\text{g ml}^{-1}$)	Initial number of Artemia nauplii	Number of nauplii dead after 24 hours	% of mortality after 24 hours	Number of nauplii dead after 48 hours	% of mortality after 48 hours
Mortality rate after 24 and 48 hours	Control	30	3	10	7	23.3
	10	30	5	16.6	12	40
	25	30	9	30	18	60
	50	30	12	40	24	80
	100	30	14	46.6	26	86.6
	200	30	18	60	28	93.3

Plate 1. Step by step biosynthesis process of AuNPs



Control and AuNPs solutions

Figure 1. UV analysis of AuNPs

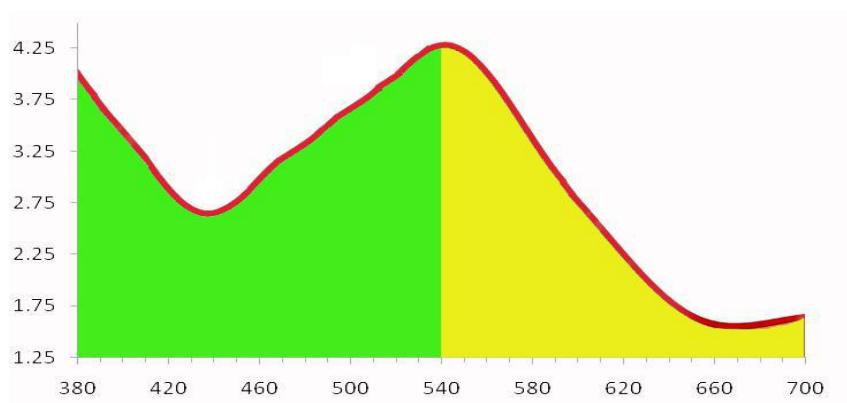


Figure 2. SEM image of AuNPs

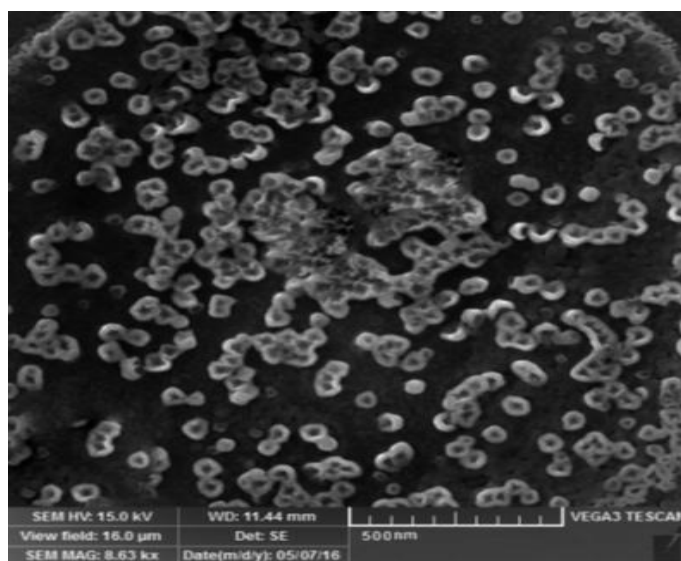


Figure 3. EDS Analysis of Au-NPs

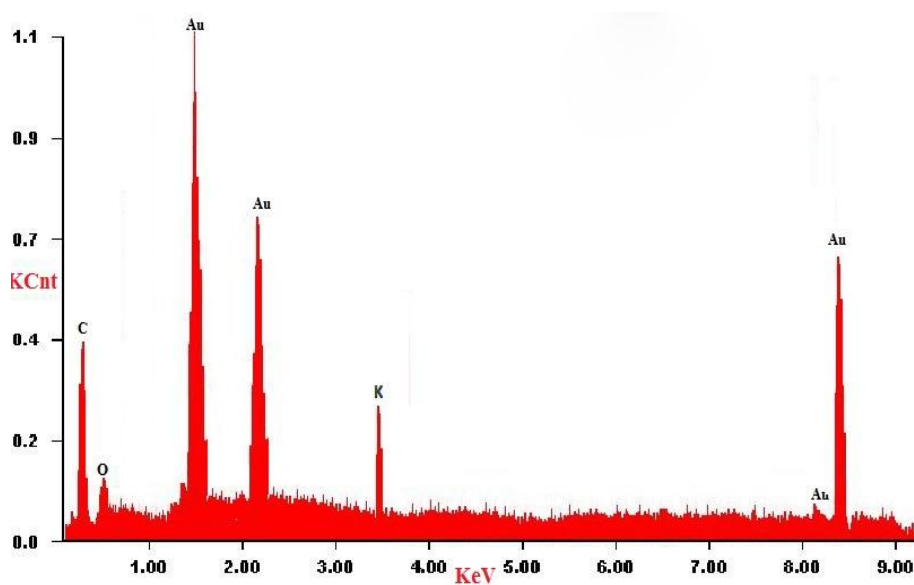


Figure 4. XRD pattern of AuNPs

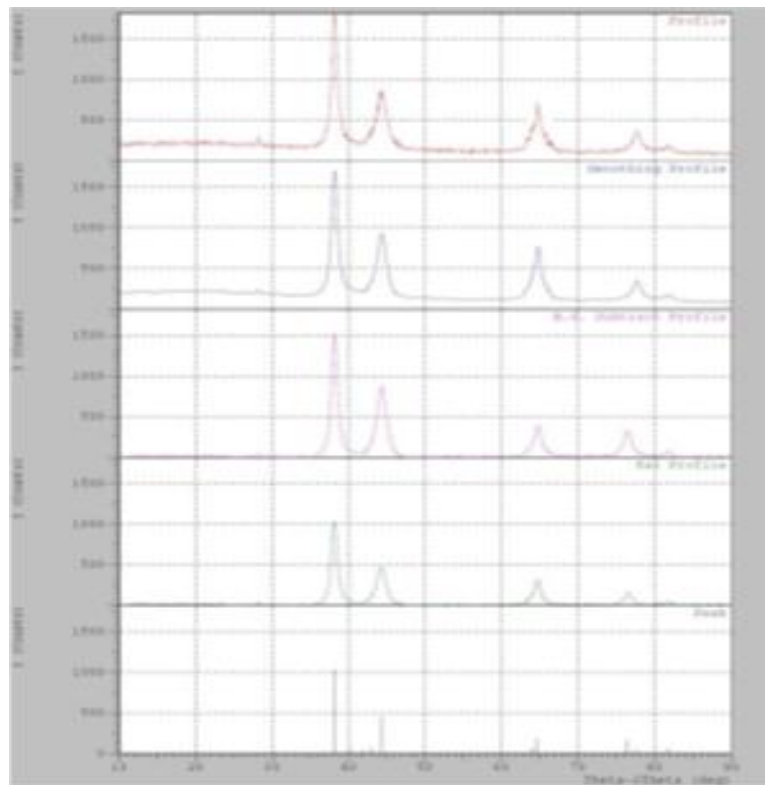


Figure 5. FTIR analysis of AuNPs

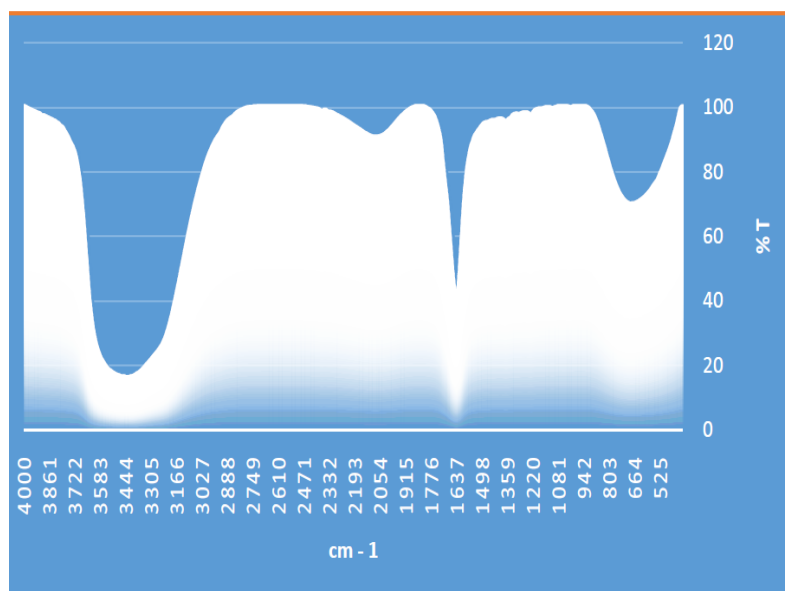


Figure 6. DLS-Size distribution of AuNPs

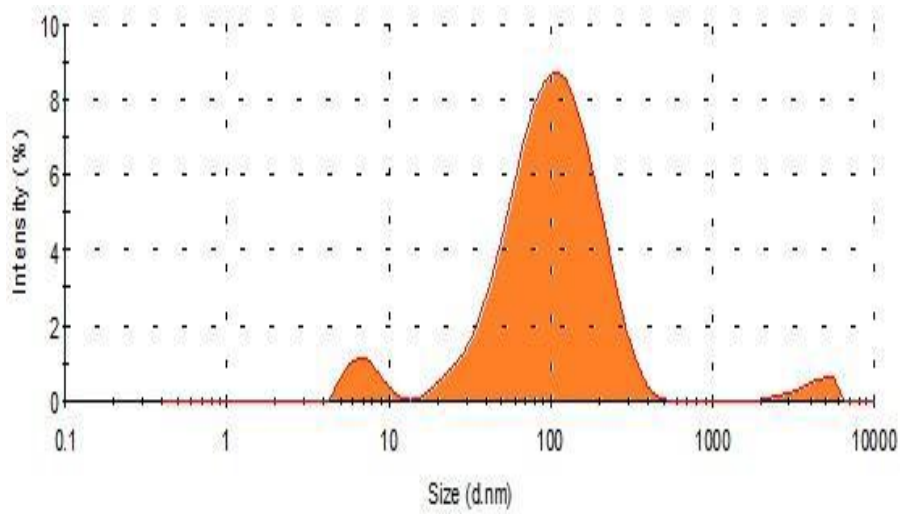


Figure 7. DLS-Zeta potential of AuNPs

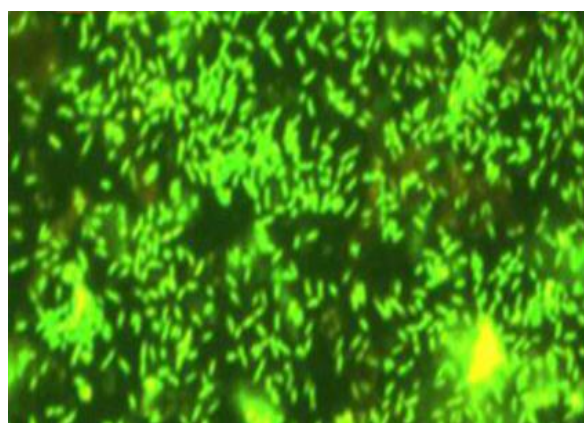
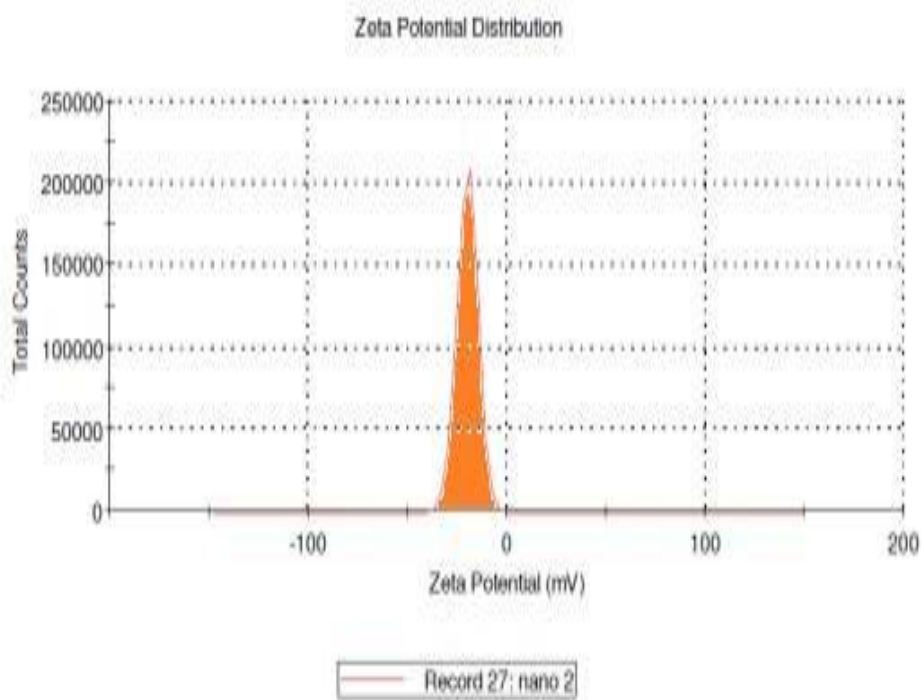
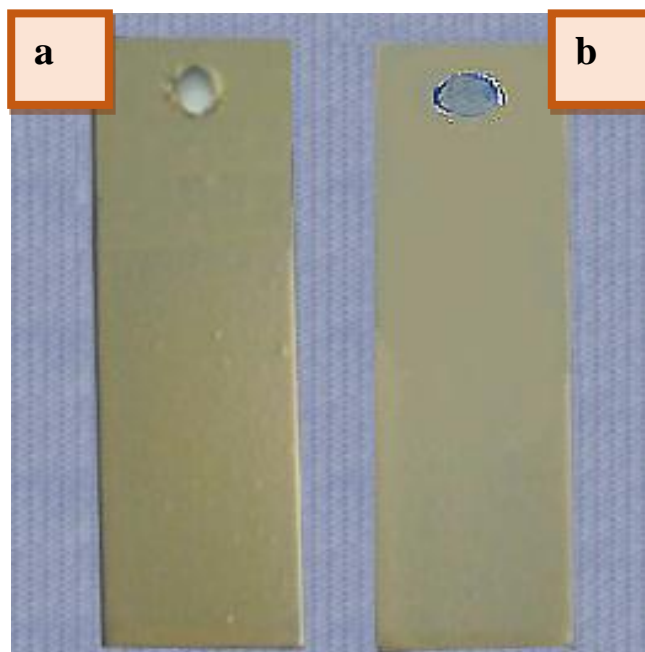


Plate 3. Bacterial biofilm formation on SS304 coupons

**Plate 4. a) AuNPs + AZC paint coated coupon
b) AZC paint (alone) coated coupon**



4.0 Conclusion

The biological reduction of aqueous gold ions by *Cajanus cajan* has been confirmed. This green approach towards the synthesis of gold nanoparticles has many advantages such as ease with which the process can be scaled up, economic viability, etc. The antibiofilm activity of AuNPs sample was most effective against BS 2 while smaller effect was noticed from BS 6. The present field study of phytigel and Apcomin zinc chrome paint coated with *Cajanus cajan* mediated Gold nanoparticles (AZC+AuNPs) prevented the biofouling organisms. Hence it proves as an alternative, eco-friendly, cost-effective, and non-toxic antifoulant against marine biofoulers. This study may encourage the development of novel broad-spectrum environmental friendly antifouling paints in the future. Particularly, since the quest for new antifouling technologies is increasing, biogenic silver nanoparticles may provide new path ways of green antifouling compounds.

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