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RAPID BIOSYNTHESIS OF ANTIMICROBIAL SILVER AND GOLD NANOPARTICLES BY *IN VITRO* CALLUS AND LEAF EXTRACTS FROM *LYCOPERSICON ESCULENTUM* MILL.

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ABSTRACT

The present work investigated the rapid biosynthesis of silver and gold nanoparticles by *in vitro* callus and leaf extracts derived from *Lycopersicon esculentum* Mill. The tissue culture-derived callus and leaf were able to produce both silver and gold nanoparticles, as confirmed by visual observation and UV-Vis spectroscopy. Appearance of brown colour and ruby colour indicated the synthesis of silver and gold nanoparticles in the reaction mixture. The callus extract could be able to produce silver and gold nanoparticles rapidly, compared to leaf extract. The silver and gold nanoparticles were found to be spherical in shape with variable size ranging from 30 to 40 nm, as evident by SEM and DLS. The nanoparticles appeared to be associated with some chemical compounds which possess hydroxyl and carbonyl groups, confirmed by FTIR. This work highlighted the possibility of using *Lycopersicon esculentum*-derived callus extract for rapid and clean synthesis of silver and gold nanoparticles.

KEYWORDS: ***Lycopersicon esculentum*, callus extract, silver and gold nanoparticles**

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INTRODUCTION

Nano-biotechnology plays a vital role in the production of various types of nanoparticles. Biosynthesis of nanoparticles can be achieved using either microorganisms or plant extracts [1-8]. Gold and silver nanoparticles are presently under intensive study for applications in optoelectronic devices, ultrasensitive chemical sensors, and biological sensors and as catalysts. Only a very few studies are available on the use of tissue culture derived callus for the synthesis of silver nanoparticles that too confined to synthesis of silver nanoparticles [6, 9-11]. Therefore, the present study was undertaken on the biosynthesis of gold and silver nanoparticles by using callus and leaf extracts derived from *Lycopersicon esculentum* Mill.

MATERIALS AND METHODS

(i) Chemicals

Tetra chloro auric acid ($HAuCl_4 \cdot 4H_2O$, 99.99%; Aldrich brand) and silver nitrate (Merck brand) were used. All the glassware used were cleaned with ultra pure water.

(ii) Collection of plant materials

The fresh fruit of *Lycopersicon esculentum* Mill, was obtained from the botanical garden of Parangi pettai, India.

(iii) Surface sterilization of seeds of Lycopersicon esculentum Mill

Seeds were washed with tween 20 for 15 min followed by running tap water for 15 min. Under laminar flow, seeds were disinfected with 0.1% mercuric chloride solution, and then rinsed 4-5 times with sterile distilled water for the complete removal of surface contaminants.

(iv) Induction of in vitro callus of Lycopersicon esculentum seeds

Induction of callus was achieved from seeds of *L.esculetum* on MS media. The MS media was incorporated with 2,4,-dichloro phenoxy acetic acid (2 ppm). The pH of the medium was adjusted to 5.8–6. The medium was gelled with 8 g of agar/l and distributed in screw cap tubes, autoclaved at 120 °C for 15 min at 15 lbs. The surface sterilized seeds were inoculated on the MS medium and kept under fluorescent illumination of about 3000×lux and maintained at 25°C. The proliferation of the callus was found within 2 weeks and it was harvested. This was repeated several times to obtain a mass of calli.

(v) In vitro regeneration of Lycopersicon esculentum plant

Similarly, after surface sterilization, the seeds were inoculated on MS (Murashige and Skoog, 1962) media supplemented with benzyl amino purine (0.5 pm) and later transferred to condition with a 16 h photoperiod at 25°C ± 2. It was noticed that seeds started growing in dark and then they were transferred to light. It was observed that germination was possible after 10-12 days of culture. After the shoot initiation, the explants were transferred to root initiation medium supplemented with naphthalene acetic acid (0.5 ppm).

(vi)Synthesis of silver and gold nanoparticles

Seed derived callus extract was prepared by grinding 20g of fresh callus, boiled for 5min and centrifuged at 3000rpm to get the callus extract. Similarly tissue culture derived leaf extract was prepared. Five milliliters of callus and leaf extracts was separately added to 45 ml of 10^{-3}M aqueous $AgNO_3$ solution and $HAuCl_4$. The reduction of silver and gold ions was monitored by measuring the absorbance of the reaction mixture in a range of wavelength from 300 to 600nm using UV–vis spectrophotometer (Elico, Chennai) to find the absorbance peak.

(vii) Scanning Electron microscopy (SEM)

The shape and size of the silver and gold nanoparticles were determined using JEOl-

Scanning Electron Microscope.JSM-5610 LV with an accelerating voltage of 20kv, at high vacuum mode and secondary electron image.

(viii) Dynamic light scattering pattern (DLS)

Dynamic light-scattering measurements were performed for analyzing the size groups of nanoparticles using a Nano ZS apparatus at 25ºC and started 2 min after the cuvette was placed in the DLS apparatus to allow the temperature to equilibrate. Measurements were carried out after 24 h of preparation of the suspensions

(ix) FTIR analysis

For FTIR analysis, 100 ml of nanoparticle solution was centrifuged at 5000 rpm for 10 min. The supernatant was again centrifuged at 10000 rpm for 60 min and the pellet was obtained. This was followed by redispersion of the pellet of silver and gold nanoparticles into 1 ml of deionized water. Thereafter, the purified suspension was freeze-dried to obtain dried powder. Finally, the dried nanoparticles were analyzed by FTIR

(x) Zeta Potential Measurement

The zeta potential measurements of lyophilized silver and gold nanoparticles were carried out using a Zetasizer Nano ZS (Malvern), Brookhaven Instruments Corporation. The pH values of silver and gold nanoparticles were measured prior to the zeta potential analysis.

(xi) Antimicrobial activity

The antimicrobial activity of silver and gold nanoparticles was done by disc-diffusion method. In this method 10-50µl of silver nanoparticle prepared from callus extract, was mixed in 1ml of distilled water and applied to sterile paper discs of 5mm diameter (Hi-Media,India). Similarly 10-50µl of gold nanoparticles of prepared from callus extracts was mixed in 1ml of distilled water and applied to sterile paper disc. The discs were then placed on Muller Hinton Agar swabbed with clinical strains of bacteria (Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebseilla oxytoca, Proteus vulgaris) and Potato Dextroase Agar with fungi (Alternaria alternata, Penicillium italicum, Fusarium equisetii and Candida albicans), at a concentration of 106 bacteria/ml for bacteria and 103 spore/ml for fungi. The plates were incubated at 37ºC for overnight. The zone of inhibition was measured in millimeter after the 24 h of incubation and recorded.

RESULTS

(i) Callus initiation and Plant regeneration

The media supplemented with 2, 4-dichloro phenoxy acetic acid (2 ppm) exhibited promising results in the production of callus. The mass of calli was obtained from the tomato seeds are shown in Fig.1.Similarly, the regenerated tomato plants are shown in Fig.2.

(ii) Visual observation of colour change

The change in colour of the reaction mixture was noted by visual observation (Fig.1). The callus and leaf extracts incubated with silver nitrate, at the beginning of the reaction showed yellow colour, and gradually increased in colour intensity to dark brown, with the increasing period of incubation. Similarly for gold nanoparticles, at the beginning of the reaction showed pink color, and gradually increased in colour intensity to ruby red colour (Fig.1). The colour of the reaction mixture changed to intense brown and ruby red after 2 hours of incubation. Control with callus or leaf extract alone without addition of substrate did not exhibit any change in colour.

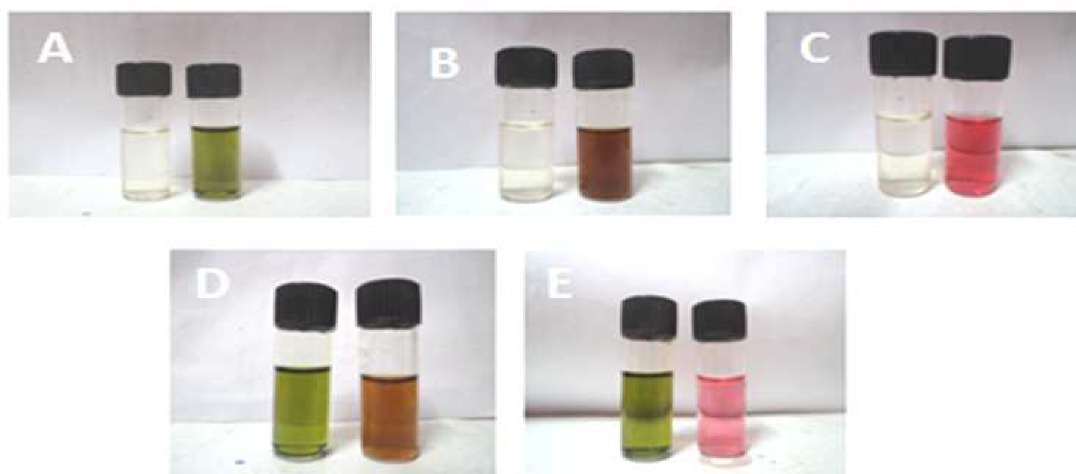

Figure. 1
Silver and gold nanoparticles synthesized from Lycopersicon esculentum Mill.

(A) In vitro callus and leaf extracts of *Lycopersicon esculentum* Mill.(B) Silver nanoparticles synthesized by callus extract (C) Gold nanoparticles synthesized by callus extract (D)Silver nanoparticles synthesized by leaf extract (E) Gold nanoparticles synthesized by leaf extract

(iii) Spectral observation of colour change

The absorbance peak of silver and gold nanoparticles synthesized by callus and leaf extract is depicted in fig.2. The peak of colour intensity was observed after 2 hours of incubation in the case of both the callus and leaf extracts. There was no significant change of colour intensity beyond 10 days of incubation. The highest colour intensity was recorded in callus extract.

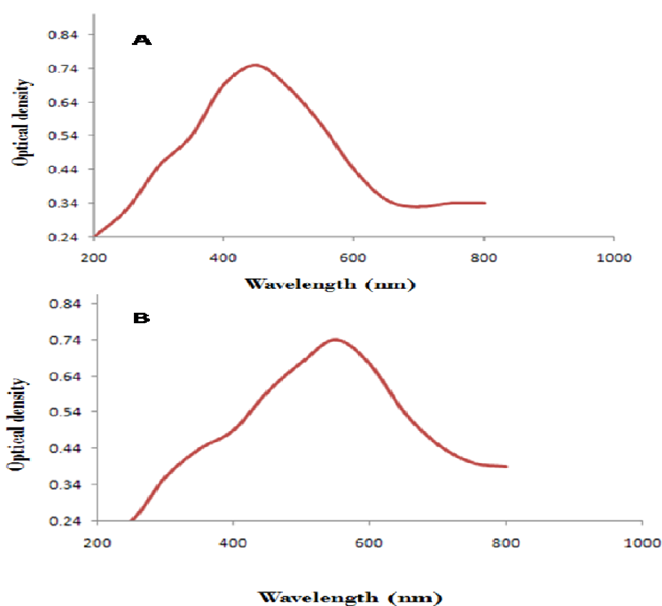

Figure.2.
UV-VIS spectra of nanoparticles synthesized by callus extract of Lycopersicon esculentum Mill. (A).Silver nanoparticles (B).Gold nanoparticles

(iv) SEM of silver and gold nanoparticles

The shape and size of silver and gold nanoparticles analyzed by SEM is depicted in Fig. 3. In general, the nanoparticles were spherical in shape with the size 33.6nm for silver nanoparticles and 36.7nm for gold nanoparticles. The DLS pattern also exhibited similar results. The silver and gold nanoparticles produced by callus and leaf extracts were more distinct and scattered in distribution.

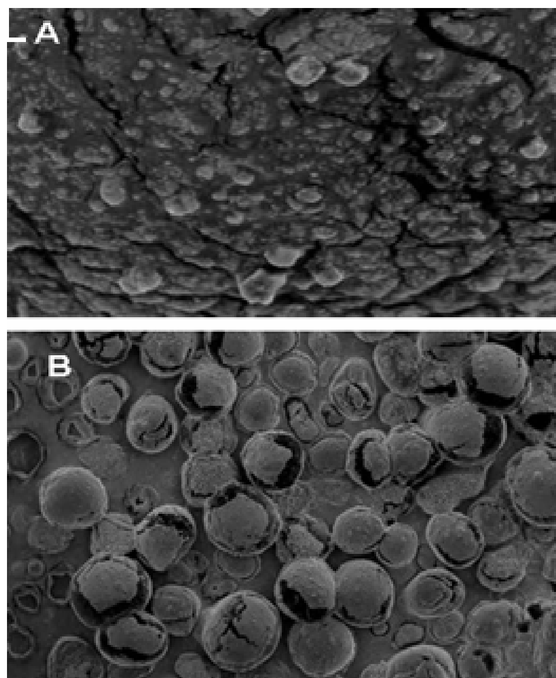

Figure 3
SEM photographs of silver and gold nanoparticles synthesized by callus extract of Lycopersicon esculentum Mill.(A)Silver nanoparticles (B) Gold nanoparticles

(v) FTIR Spectra

FTIR spectrum was used for confirming presence of chemical groups other than silver and gold nanoparticles. The peaks at above 3400 revealed the strong OH group, representing the aromatic nature of alcohol (phenols). Absorbance bands range at 1600-1400 cm^{-1} were assigned to the stretching vibrations of primary and secondary amines, respectively (Fig. 4 a and b).The sharp peaks at above 1111-600 indicating the presence of strong C=O and –C-OH stretching. The silver and gold nanoparticles appeared to be associated with some chemical compounds which possess hydroxyl and carbonyl groups. The compounds are likely to be phenols and their derivatives. The result revealed that the capping ligand of the silver and gold nanoparticles may be an aromatic compound or amines.

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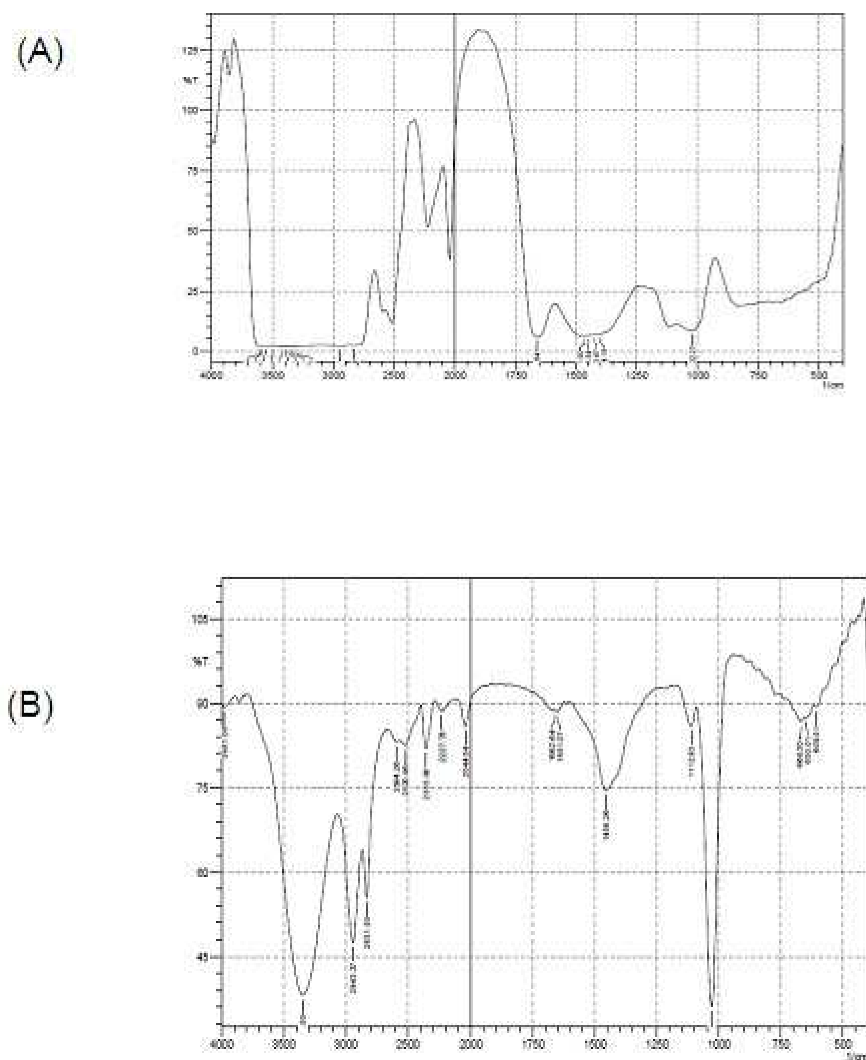

Figure. 4
FTIR spectrum of silver and gold nanoparticles synthesized by callus extract of ~~Lycoprsicon~~ Lycopersicon esculentum Mill. (A) Silver nanoparticles (B) Gold nanoparticles

(vi) Zeta Potential Measurement

The negative zeta potential values of nanoparticles synthesized from callus and leaf extracts were -52.10 mV and -28.9mV respectively for silver nanoparticles and -59.8 mV and 31..32 mV for gold nanoparticles for providing the necessary repulsive forces for the particles to remain stable in solution. Silver nanoparticles synthesized from callus extract exhibited good stability with the mV value -52.10 and gold nanoparticles exhibited very excellent stability with the negative mV value -59.8.

(vii) Antimicrobial activity

Antimicrobial activities of silver and gold nanoparticles synthesized by callus extracts from *L. esculentum* are shown in Tables 1-4. The gold nanoparticles showed higher antimicrobial activities than silver nanoparticles against all the test organisms. In common, antibacterial activity was more pronounced than antifungal activity. Regarding antibacterial activity, the highest inhibition zone of 23.25 mm diameter was formed against Staphylococcus aureus by the gold nanoparticles synthesized by callus extract, and the lowest of 12.22mm was produced

against Pseudomonos aeruginosa by silver nanoparticles synthesized by callus extract (Table 1 and 3). Concerning antifungal activity, the highest inhibition zone of 14mm diameter was formed against Penicillium species by the gold nanoparticles synthesized by callus extract added with PVA and the lowest of 9.3 mm was produced against Fusarium equisetii by gold nanoparticles synthesized by callus extract (Table 3 and 4).

Table 1
Antibacterial activity of silver nanoparticles synthesized by callus extract of Lycopersicon esculentum Mill.

Silver(µl)	E.coli	P.aer	S.aur	K.oxy	P.vul	Species
10	14.925±0.26^{a}	12.225±0.12^{a}	17.900±0.21^{a}	15.850±0.26^{a}	16.150±0.18^{a}	**(F- 6.82: P-0.001)
20	15.250±0.26^{a}	13.825±0.12^{b}	17.700±0.21^{a}	16.500±0.26^{a}	16.075±0.18^{a}	**(F- 13.15: P-0.001)
30	16.100±0.26^{b}	14.525±0.12^{c}	18.750±0.21^{b}	16.500±0.26^{a}	17.450±0.18^{b}	**(F- 32.35: P-0.001)
40	16.450±0.26^{b}	15.525±0.12$_{d}$	19.600±0.21^{c}	19.250±0.26^{b}	18.400±0.18^{c}	**(F- 9.34: P-0.001)
50	16.575±0.26^{b}	16.250±0.12^{e}	21.950±0.21^{d}	20.525±0.26^{c}	19.425±0.18^{d}	**(F-23.65: P-0.001)
Silver concentration	** (F- 7.88: P-0.001)	** (F- 152.19: P-0.001)	** (F- 65.90: P-0.001)	** (F- 61.82: P-0.001)	** (F- 64.23: P-0.001)	

Values are mean of three replicates±standard deviation. Dissimilar alphabets given as superscript indicating significance.
(E.coli- Escherichia coli,P.aer- Pseudomonas aeruginosa, S.aur- Staphylococcus aureus, K.pne- Klebseilla oxytoca, P.vul-Proteus vulgaris)

Table 2
Antifungal activity of silver nanoparticles synthesized by callus extract of Lycopersicon esculentum Mill.

Silver(µl)	A.alt	P.ita	F.equ	C.albi	Species
10	10.425 ±0.07^{a}	12.100±0.11^{a}	10.175±0.10^{a}	10.650±0.13^{a}	** (F- 9.07: P-0.001)
20	10.675±0.07^{b}	12.750±0.11^{b}	10.525±0.10^{b}	11.025±0.13^{a}	** (F- 3.89: P-0.001)
30	11.900±0.07^{c}	13.000±0.11^{b}	10.800±0.10^{b}	11.675±0.13^{b}	** (F- 7.89: P-0.001)
40	12.575±0.07^{d}	13.550±0.11^{c}	11.650±0.10^{c}	12.450±0.13^{c}	** (F- 3.9: P-0.001)
50	13.650±0.07^{e}	13.625±0.11^{c}	12.400±0.10^{d}	12.525±0.13^{c}	** (F- 6.7: P-0.001)
Silver concentration	** (F- 344.60: P-0.001)	** (F- 29.81: P-0.001)	** (F- 71.56: P-0.001)	** (F- 36.07: P-0.001)	** (F- 8.98: P-0.001)

Values are mean of three replicates±standard deviation. Dissimilar alphabets given as superscript indicating significance.
(A.alt- Alternaria alternata, P.ita-Penicillium italicum, F/ equ - Fusarium equisetii and C. albi - Candida albicans)

Table 3
Antibacterial activity of gold nanoparticles synthesized by callus extract of Lycopersicon esculentum Mill.

Gold(µl)	E.coli	P.aer	S.aur	K.oxy	P.vul	Species
10	15.125±0.14^{a}	14.175±0.10^{a}	20.250±0.11^{a}	18.150±0.09^{a}	17.250±0.05^{a}	**(F- 12.98: P-0.001)
20	16.100±0.14^{b}	14.950±0.10^{b}	21.375±0.11^{b}	19.050±0.09^{b}	17.325±0.05^{a}	**(F- 1.78: P-0.001)
30	16.725±0.14^{c}	15.200±0.10^{c}	21.925±0.11^{c}	19.175±0.09^{b}	17.550±0.05^{b}	**(F- 8.67: P-0.001)
40	18.150±0.14^{d}	16.250±0.10^{d}	22.675±0.11^{d}	19.800±0.09^{c}	18.325±0.05^{c}	**(F- 9.45: P-0.001)
50	20.425±0.14^{e}	17.475±0.10^{e}	23.250±0.11^{e}	20.150±0.09^{d}	19.175±0.05^{d}	**(F-9.23: P-0.001)
Gold concentration	** (F- 191.24: P-0.001)	** (F- 137.42: P-0.001)	** (F- 103.93: P-0.001)	** (F- 66.05: P-0.001)	** (F- 220.17: P-0.001)	

Values are mean of three replicates±standard deviation. Dissimilar alphabets given as superscript indicating significance.
(E.coli- Escherichia coli,P.aer- Pseudomonas aeruginosa, S.aur- Staphylococcus aureus, K.pne- Klebseilla oxytoca, P.vul-Proteus vulgaris)

Table 4
Antifungal activity of gold nanoparticles synthesized by callus extract of Lycopersicon esculentum Mill.

Gold(µl)	A.alt	P.ita	F.equ	C.albi	Species
10	10.750±0.11^{a}	11.500±0.09^{a}	9.375±0.10^{a}	10.625±0.11^{a}	** (F- 18.34: P-0.001)
20	10.975±0.11^{a}	11.925±0.09^{b}	9.950±0.10^{b}	10.925±0.11^{a}	** (F- 36.56: P-0.001)
30	11.625±0.11^{b}	12.375±0.09^{c}	10.200±0.10^{b}	11.500±0.11^{b}	** (F- 39. 98: P-0.001)
40	12.675±0.11^{c}	13.650±0.09^{d}	11.125±0.10^{c}	12.250±0.11^{c}	** (F- 54.91: P-0.001)
50	13.250±0.11^{d}	14.000Ë±0.09^{e}	13.450±0.10^{d}	12.800±0.11^{d}	** (F- 61.27: P-0.001)
Gold concentration	** (F- 96.88: P-0.001)	** (F- 121.49: P-0.001)	** (F- 234.48: P-0.001)	** (F- 62.61: P-0.001)	** (F- 81.98: P-0.001)

Values are mean of three replicates±standard deviation. Dissimilar alphabets given as superscript indicating significance.
(A.alt- Alternaria alternata, P.ita-Penicillium italicum, F/ equ - Fusarium equiseti and C. albi - Candida albicans)

DISCUSSION

The media supplemented with 2,4,-dichloro phenoxy acetic acid (2 ppm) exhibited promising results in the production of callus. This results are comparable with Devi et al.,2008 [12].Similar work in callus initiation was done by early workers. [13-16] In the present study, the callus and leaf extract exhibited very rapid colour change when they were added with the substrates - silver nitrate or chloroauric acid. Around two hours, the colour of the solution was completely changed into brown and ruby colour (Fig.1). However, control without silver or gold ion did not show any change in colour of the extracts. Appearance of brown colour is an indication of silver nanoparticles, while ruby colour indicates the synthesis of gold nanoparticles in the reaction mixture. This is due to the excitation of surface Plasmon vibrations, typical of the silver and gold nanoparticles. Mono dispersity is an important characteristic feature of the nanoparticles and it is reported promising for silver and gold

nanoparticles 6. In the present study, the colour of the callus and leaf extracts changed to intense brown or ruby after 2 hours of incubation. The solution remained as hydrosol and there was no precipitation even after 10 days of incubation. This indicated that the particles were well dispersed in the solution and there was no aggregation of particles. The shape and size of silver and gold nanoparticles were analyzed by SEM is depicted in Fig. 3. In general, the nanoparticles were in spherical in shape with the size 33.6nm for silver nanoparticles and 36.7nm for gold nanoparticles which is also confirmed with DLS.

Different sizes of nanoparticles derived from tissue culture derived extracts have been recorded by early workers. Satyavani et al. (2011) [10] have recorded high size range (75-100nm) of the silver nanoparticles synthesized by *Citrullus colosynthis*, while Asmathunisha et al. (2010) [6] have registered low size range (5-20nm) of silver nanoparticles from callus and leaf extracts of *Sesuvium portulacastrum* L. Thus, the biosynthesis of nanoparticles varied with species. Among the extracts tested in the present study, the synthesis of silver and gold nanoparticles was high in callus extract (Figs.1).And this synthesized nanoparticles were exhibited good stability, it was confirmed by zeta potential measurement. In FTIR spectra of silver and gold nanoparticles exhibited prominent peaks at above 3400 revealed the strong OH group, representing the aromatic nature of alcohol (phenols). Absorbance bands range at 1600-1400 cm^{-1} were assigned to the stretching vibrations of primary and secondary amines, respectively (Fig. 4).The sharp peaks at above 1111-600 indicating the presence of strong C=O and –C-OH stretching . The silver and gold nanoparticles appeared to be associated with some chemical compounds which possess hydroxyl and carbonyl groups. The compounds are likely to be phenols and their derivatives. The result revealed that the capping ligand of the silver and gold nanoparticles may be an aromatic compound or amines. Polyphenols like tannic acids are the plant-derived compounds, which are efficient reducing agent in the synthesis of silver nanoparticles [17]. The coastal plants are generally rich in polyphenolic compounds [18]. Thus phenolic derivatives may determine the nanoparticle synthesis by the coastal plants (Asmathunisha 2010) [7] and however, the exact mechanism is yet to be elucidated. The antimicrobial activity in terms of inhibition zone significantly varied with test microbes and type of extracts. This differential antimicrobial activity of silver and gold nanoparticles can be accredited to their differential sizes and shape: the antimicrobial activity increases with decreasing size of nanoparticles. The present study recorded that the antibacterial activity was more pronounced than antifungal activity (Tables 1-4). This can be attributed to the fact at low concentrations of nanoparticles does not enter the fungal cells, but it is adsorbed onto the bacterial surface just as silver and gold tends to adsorb to other surfaces, thus silver and gold ions immobilize dehydrogenation because respiration occurs across the cell membrane in bacteria rather than across the mitochondrial membrane as in eukaryotic cells of fungi.

CONCLUSION

The result of the present study indicated the stem derived callus extract was more efficient in synthesis of the silver nanoparticles than regenerated leaf extract (Figs. 1). This may be due to mass of young cells with different ploidy in the callus which may be metabolically active to produce various types of chemicals responsible for the reduction of silver and gold ions.

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