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In vitro antioxidant, antimicrobial and cytotoxic activities and green biosynthesis of silver & gold nanoparticles using *Callistemon citrinus* leaf extract

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ABSTRACT

The current study was aimed to synthesis and characterization of silver (AgNPs) & gold (AuNPs) nanoparticles using *Callistemon citrinus* leaf extract, and to evaluate their *in vitro* antioxidant, antimicrobial and cytotoxic activities as well as their total phenolic content (TPC). Silver and gold nanoparticles were synthesized and characterized via UV-vis absorbance spectroscopy, transmission electron microscopy (TEM), and X-ray diffraction (XRD) analyses. The antioxidant activity was evaluated using dot-blot and DPPH staining, and via phosphomolybdenum assays. Also, the *in vitro* antimicrobial activity was evaluated via disc agar plate method. The cytotoxic activity was evaluated via brine shrimp lethality test (BSLT), and TPC was estimated via Folin-Ciocalteu's assay. The transmission electron microscopy (TEM) analysis showed that the sizes of the synthesized AgNps were ranged from 8 to 14 nm with maximum UV/vis absorbance at 450nm. Also, the synthesized AuNPs exhibited an average size of 5.8 to 8.84 nm with maximum UV/vis absorbance at 535nm. Moreover, the results revealed that TPC of the tested extracts was ranged from 548.85 to 123.30 mg gallic acid equivalent (GAE)/g dry extract. The total antioxidant capacity (TAC) was ranged from 643.90 to 147.96 mg ascorbic acid equivalent/g dry extract. Furthermore, there is a promising antimicrobial activity against four strains with inhibition zones were ranged from 8.5 to 15.5 mm, Penicillin G was used as positive control at concentration of 100 µg/disc. In terms of LC_{50} the *n*-butanol extract (63.09 µg/ml) was the most potent cytotoxic, followed by EtOAc (100.0 µg/ml). In, conclusion the leaves of *Callistemon citrinus* showed a noticeable antioxidant, antimicrobial & cytotoxic activities and the ability to produce AgNPs and AuNPs.

INTRODUCTION

Nanotechnology is an interesting field concerned by the green biosynthesis of nanoparticles (Mc Donell *et al.*, 1999; Govindaraju and Tamilselvan, 2010). The green biosynthesis of metal nanoparticles using medicinal plants extracts is a vital issue of research due to their importance in the different fields especially drug delivery (Chaudhuri *et al.*, 2016).

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Numerous reports are available on the use of medicinal plants in the green biosynthesis of nanoparticles especially silver AgNPs and gold AuNPs. Moreover, most plants usually contain polyphenolic secondary metabolites *viz.*, phenolic acids, flavonoids, and anthraquinones which act as strong reducing agents useful in the synthesis of nanoparticles (Siemieniec and Kruk, 2013). During the biosynthesis of silver and gold nanoparticles such polyphenolic compounds undergo keto-enol form conversion, which led to the reduction of $AuCl_4^-$ ion to Au^0 metal, and similarly the conversion of Ag^+ ion to Ag^0 metal (Siemieniec and Kruk, 2013; Abdel-Aziz *et al.*, 2014).

Callistemon is a genus of 34 species of shrubs in the family of Myrtaceae, all of which are endemic to Australia. It is sometimes considered as synonym of *Melaleuca* (Goyal *et al.*, 2012; Simon*et al.*, 2014). Phytochemical studies of different *callistemon* species revealed the presence of different monoterpenes, sesquiterpenes flavonoids (Chowdhury *et al.*, 2012; Haque *et al.*, 2013). Moreover, phytochemical explorations of members of this genus resulted in the identification of C-methyl flavonoids, triterpenoids, and phloroglucinol derivatives (Varma and Parthasarathy, 1975; Huq and Misra, 1997; Wollenweber *et al.*, 2000). *Callistemon citrinus* (Family Myrtaceae) known as the bottle brush is an ornamental tree (Ali *et al.*, 2011). It is native to South-Eastern Australia, and spread throughout the world as an ornamental plant in countries with mild climates (Haque *et al.*, 2012).The plant was reported to possess antioxidant Jamzad *et al.*, 2014), antimicrobial (Cock, 2012; Blesson *et al.*, 2014), and cytotoxic activities (Ali *et al.*, 2011).Therefore, the aims of this study were to use the leaves of *Callistemon citrinus* for the biosynthesis of silver (AgNPs) and gold (AuNPs) nanoparticles and to evaluate the antioxidant, cytotoxic, and antimicrobial activities of different fractions of *Callistemon citrinus* growing in Egypt.

MATERIALS AND METHODS

Plant Materials

Leaves of *Callistemon citrinus* L. (Family Myrtaceae) were collected from Zoo Garden, Giza, Egypt in May 2014. The plant was identified by Dr. Threase Labib Consultant of Plant Taxonomy at the Ministry of Agriculture; formerly the Head of Taxonomist Specialists-El-Orman Botanical Garden, Giza, Egypt, a voucher specimen (No.C1/4/1) was kept at the herbarium of the garden.

Chemicals and Equipments

All solvents and reagents used were of analytical grade. 2,2'-diphenyl-1-picraylhydrazyl (DPPH) free radical, and Folin-Ciocalteu's reagent (FCR) was purchased from (Sigma-Aldrich Co.). Gold chloride ($AuCl_3$), silver nitrate ($AgNO_3$), sodium carbonate, sodium phosphate, ammonium molybdate, ascorbic acid, and gallic acid were purchased from (Merck Chemical Co.). Thin layer chromatography (TLC) was performed over pre-coated silica plates (GF254, Merck). The absorbance measurements for the total phenolic content and antioxidant activity were recorded using the UV-Vis spectrophotometer Spectronic 601 (Milton Roy, USA).

Extraction and Fractionation

The dry powdered leaves of *Callistemon citrinus* (150 g), were soaked in (1500 ml) of 85% methanol for one week at room temperature with shaking day by day followed by filtration and again extraction for four times. The organic solvent was removed in vacuo using rotatory evaporator. The 85% methanolic crude extract (50 g) was defatted by washing several times with petroleum ether (60-80 °C). Forty five grams of the defatted methanol extract was undergoes fractionation using organic solvents i.e., CH_2Cl_2, EtOAc, and n-BuOH (3 x 100 ml for each solvent). The yield of each fraction was determined and kept in dark for further analysis.

Determination of Total Phenolic Content (TPC)

The total phenolic content was determined using Folin-Ciocalteu's reagent according to the reported methods (Kumar*et al.*, 2008; El-Sayed *et al.*, 2009).

Antioxidant Assays

Rapid Screening of Antioxidant by Dot-blot and DPPH Staining

The antioxidant by dot-blot and DPPH staining was qualitatively estimated according to reported method (Ghareeb *et al*., 2014; Shoeb *et al*., 2014).

Determination of Total Antioxidant Capacity (TAC)

The antioxidant activity was determined according to phosphomolybdenum assay (El-Sayed *et al.*, 2010; Ghareeb *et al.*, 2013).

Antimicrobial Activity

The antimicrobial activity was evaluated via disc agar plate method according to the reported method (Bauer *et al*., 1966; Ghareeb *et al*., 2015a).

Cytotoxic Activity Using Brine Shrimp Lethality Test (BSLT)

The preliminary cytotoxic activity of all tested extracts of *C. citrinus* was evaluated according to the reported procedure (Ipsen and Feigi, 1970; Ghareeb *et al*., 2015b).

Preparation of Plant Leaf Extract for Biosynthesis of Nanoparticles

The fresh green leaves of *C. citrinus* were thoroughly washed with distilled water to remove of any odd materials especially soil and dust. Twenty gram of clean leaves were boiled in 50ml distilled water in Erlenmeyer flask of 500-ml volume for 30min and the leaf debris were removed by filtration through Whatman filter paper (No. 1),the extract was evaporated up to 25ml (Abdel-Aziz *et al.*, 2014).

Biosynthesis of Silver Nanoparticles (AgNPs)

Fifty milliliter of 5mM silver nitrate solution ($AgNO_3$) were prepared in stopper conical flask and 0.25ml of the formerly prepared *C. citrinus* leaf extract were added and left at room temperature for 12h and the produced reddish brown colour indicate the biosynthesis of silver nanoparticles (AgNPs) (Abdel-Aziz *et al.*, 2014).

Biosynthesis of Gold Nanoparticles (AuNPs)

Fifty milliliter of 5mM gold chloride solution ($HAuCl.3H_2O$) were prepared in stopper conical flask and 0.5ml of

the previously prepared *C. citrinus*leaf extract were added and left at room temperature for 12h and the produced purple- reddish colour indicate the biosynthesis of gold nanoparticles (AuNPs)(Abdel-Aziz *et al.*, 2014).

Characterization of AgNPs and AuNPs Nanoparticles

UV-vis Absorbance Spectroscopy Analysis

The bio-reduction of silver nitrate ($AgNO_3$) to AgNPs and gold chloride to AuNPs was monitored periodically by UV-vis spectroscopy (Shimazu2401PC) after dilution of the samples with deionized water (Raut*et al.,* 2009). A UV-vis spectrograph of AgNPs and AuNPs was recorded by using a quartz cuvette with water as reference. The UV-vis spectrometric readings were recorded at a scanning speed of 190-900 nm (Leela and Vivekanandan, 2008).

TEM Analysis

The suspensions containing AgNPs and AuNPs synthesized by *C. citrinus* leaf extract were sampled by TEM analysis using JEOL model 1200 EX electron microscope. TEM samples were prepared by placing a drop of the suspension of AgNP or AuNPs solutions on carbon-coated copper grids and allowing water to evaporate. The samples on the grids were allowed to dry for 4 min. The shape and size of nanoparticles from *C. citrinus* were determined from TEM micrographs (Elavazhagan and Arunachalam, 2011).

X-ray Diffraction (XRD)

Measurements XRD of the *C. citrinus*reduced silver nanoparticles or gold nanoparticles were carried out on drop-coated films of the respective solutions onto glass substrates by a Phillips PW 1830 instrument operating at a voltage of 40 kV with Cu Kx radiation(Abdel-Aziz *et al*., 2014).

Statistical Analysis

All data were presented as mean ± S.D. of triplicates (*n*=3) according to Annegowda *et al*. 2010 using SPSS 13.0 program (SPSS Inc. USA) (Annegowda *et al.*, 2010).

RESULTS AND DISCUSSION

Total Phenolic Content (TPC)

In the Folin-Ciocalteu's assay, the *n*-BuOH extract was found to be the most polyphenolic enriched extract of TPC (537.65 mg GAE/g dry extract) compared to that of EtOAc, defatted 85% MeOH, and aqueous extracts (442.20, 411.11, 315.50mg GAE/g dry extract, respectively), however the rest of tested extracts showed low content of phenolic compounds 117.80 and 67.75mg GAE/g dry extract for CH_2Cl_2 and pet. ether, respectively (Table 1).

Moreover, limited data is available on the previous investigations on the TPC of different parts of *C. citrinus.* Abdelhady *et al*. (2011) reported that the total phenolic content of 80% ethanolic extracts from leaves of three Callistemon species, namely *C. lanceolatus*, *C. comboynensis*, and *C. viridiflorous* was 104, 95.8, and 79 mg g^{-1}, respectively (Abdelhady *et al.*, 2011).

Table 1: Total extractable content, total phenolic content, and total antioxidant capacity of defatted 85% methanolic extract of *C. citrinus* as well as its sub-fractions.

Sample	Yield % (TEC)[1]	Total phenolic (mg gallic acid equivalent/ g extract)[2]	Total antioxidant capacity (mg AAE /g ext.)[3]
Defatted85% MeOH	20.55	411.11± 1.24	492.67 ± 1.55
Pet. ether	2.62	67.75 ± 1.32	110.56 ± 0.85
CH_2Cl_2	3.68	117.80 ± 1.56	175.58 ± 0.90
EtOAc	1.44	442.20 ± 0.98	510.56 ± 1.15
n-BuOH	2.04	537.65 ± 1.30	804.87 ± 1.20
H_2O	1.54	315.50 ± 0.68	252.02 ± 0.75

Results are expressed as mean values ± standard deviation (*n* = 3).
[1]TEC (total extractable content).
[2]TPC (total phenolic content) values are expressed as mg gallic acid equivalent/g extract (mg GAE/g ext.).
[3]Total antioxidant capacity values are expressed as mg ascorbic acid equivalent/g extract (mg AAE/g ext.).

Antioxidant Activities

The antioxidant activity of different parts of *C. citrinus*is well known (Jamzad *et al*., 2014; Puranik *et al.*, 2014). The qualitative antioxdant results revealed that most tested fractions showed promising activity which was confirmed via the appearance of white zones upon the dark purple background. Among them, the *n*-BuOH fraction showed the potent activity followed by the defatted 85% MeOH, 85% MeOH, EtOAc, and H_2O in comparison of two standards quercetin and ascorbic acid (Figure 1).

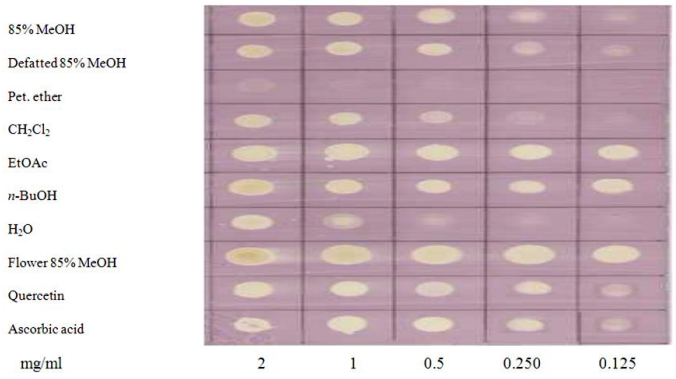

Fig. 1: Dot-blot qualitative antioxidant assay of different fractions of *C. citrinus* on silica sheet stained with DPPH˙ solution in methanol.

Furthermore, in the phosphomolybdenum assaythe *n*-BuOHextract was the most potent antioxidant of TAC (804.87 ± 1.20mg ascorbic acid equivalent/g dry extract), followed by ethyl acetate, defatted 85% MeOH, aqueous extracts of TAC (510.56 ± 1.15, 492.67 ± 1.55, 252.02 ± 0.75, respectively), however the weak activity was recorded with the remaining fractions CH_2Cl_2 (175.58 ± 0.90), and pet. ether (110.56 ± 0.85) mg ascorbic acid equivalent/g dry extract)(Table 1). Puranik *et al*. (2014) reported that the antioxidant activity of the ethanol extract of *C. citrinus*

flower was assessed via DPPH assay, which showed an inhibition of free radical at a percentage of 56.04 ± 0.6 at lowest concentration 25 µg/ml, and 89.75 ± 0.8 at highest concentration 400 µg/ml (Puranik *et al.*, 2014). Also, Jamzad *et al*. (2014) have been investigated the antioxidant activity of the 80% methanol extract from the flowers, leaves and stems of *C. citrinus* using DPPH antioxidant assay, the highest antioxidant activity was observed for flowers (inhibition% = 93.32%), followed by leaves (inhibition % = 64.11%), and stems (inhibition % = 44.17%) (Jamzad *et al.*, 2014).

In Vitro Antimicrobial Activity

The different extracts of *C. citrinus* were tested for their *in vitro* antimicrobial activity against five pathogenic microbial strains including Gram +ve and Gram -ve bacteria and fungi *i.e.*, *Pseudomonas aeruginosa* with inhibition zones ranged from 18.0 to 7.5mm, *Staphylococcus aureus* with inhibition zones ranged from 17.5 to 7.5mm, Methicillin-resistant *Staphylococcus aureus* with inhibition zones ranged from 18.5 to 7.5mm,*Candida albicans* with inhibition zones ranged from 21.5 to 8.5mm,however85% MeOH of flower part only showed a moderate activity against *Aspergillus niger* with inhibition zone 9.5mm (Table 2). Blesson *et al*. (2014) reported on the antibacterial activity of the ethanolic leafextractof *C. citrinus* against *Staphylococcus aureus*, and the results revealed that it showed a significant antibacterial activity with inhibition zone diameter of 40 mm (Blesson *et al.*, 2014). The ethyl acetate and petroleum ether extracts of the stem bark of *C. citrinus* were subjected to screening for antimicrobial activity, these extracts showed moderate antimicrobial activity (9-10.5 mm) against Gram positive bacteria *i.e.*, *Bacillus subtilis, B. cereus, B. megaterium* & *Staphylococcus aureus*, (9-11 mm), and against Gram negative bacteria *i.e.*, *Vibrio parahaemolyticus, V. minicus*, *Echerichea coli*, *Salmonella typhi*, *S. para typhi*, *Shigella boydii*, *Sh. Dysenteriae*, *Pseudomonus sp.*, (9-10.5 mm), and against fungi *i.e.*,*Candida albicans* & *Aspergilus niger* at 300 µg/disc in comparison with kanamycin at 30 µg/disc (Haque *et al.*, 2012).The antimicrobial activity of the methanolic extract of *C. citrinus* leaf & flower was investigated against a panel of bacteria and fungi. The results revealed thatthe tested leaf extracts inhibited the growth of 43% and flower extracts inhibited the growth of 64% of the bacteria tested, respectively. Gram-positive bacteria (100% inhibited) were more susceptible to *C. citrinus* extracts than were Gram-negative bacteria (27% inhibited by leaf extracts; 55% inhibited by flower extracts) (Cock, 2012). Furthermore, the chloroform, ethanol, and aqueous extracts form the leaves of *C. citrinus* were subjected to screening their antimicrobial activities against Gram(+)ve and Gram(-) microorganisms *i.e.*, *Bacillus subtilis*, *Bacillus pumilis* and *Escherichia coli*, the chloroform extract exhibited moderate to significant activity against all the tested microbial strains, and the alcoholic extract exhibited moderate activity (Krishna *et al.*, 2012). The antibacterial potential of the petroleum ether, chloroform, and methanolic extracts of *C. citrinus* was examined against *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*, all the tested extracts showed good activity against *P. auregenosa* (12.3±0.44; 12.9±0.33 & 23±0.33), *E. coli* (11.3±0.00; 14.9±0.66 & 20.21±0.33), and *B. subtilis* (12±0.33; 15.5±0.33 & 25.09±0.33 at 100 mg/ml) all respectively for petroleum ether, chloroform, and methanolic extracts, and there is no any activity was recorded against the fungal strains (Shinde *et al.*, 2012).

These findings afford new outlooks for the use of the *C. citrinus* leaves as a good source of naturally occurring antimicrobial agents.

Cytotoxic Activity via BSLT

In the BSLT, different extracts of *C. citrinus* were tested as preliminary cytotoxic agent using *Artemia salina* Leach eggs (Meyer *et al*., 1982), and the results in (Table 3 and Figure 2) revealed that the *n*-BuOH extract was the strongest cytotoxic with LC_{50}= 63.09 µg/ml, followed by 85% methanol (LC_{50}= 125.89), defatted 85% methanol (LC_{50}= 158.48), EtOAc (LC_{50}= 100.0), and H_2O (LC_{50}= 398.10) µg/ ml. On the other hand the lowest cytotoxic effect was recorded with CH_2Cl_2 (LC_{50}= 398.10), pet. ether (LC_{50}= 501.18), compared with the 85% methanol of flower part (LC_{50}= 79.43) µg/ ml. Ali *et al*. (2011) have been evaluated the brine shrimp cytotoxicity of fruits of *C. citrinus*, and the results revealed that the EC_{50} value was 65.5 ± 7.28 (60.8- 69.4, *n*=4) mg/ml, which imply that the plant may be a source of cytotoxic agents and therefore, may be a source of anticancer constituents (Ali *et al.*, 2011).

Furthermore, the petroleum ether, ethyl acetate, and methanol extracts of the stem bark of *C.citrinus* were subjected to screenings for BSLT, and the results revealed that the methanol extract showed potent cytotoxicity with LC_{50}value of 11.27 µg/ml (Haque *et al.*, 2012).

Table 2: Antimicrobial activity of the defatted 85% methanolic extract of *C. citrinus* as well as its derived sub-fractions.

Sample	Clear Inhibition zone (ϕmm)				
	Staphylococcus aureus	***MRSA***	***Pseudomonas aeruginosa***	***Candida albicans***	***Aspergillus niger***
85% MeOH	14.5 ± 0.70	15.5 ± 0.70	16.5 ± 0.70	18.5 ± 0.70	-
Defatted 85% MeOH	8.5 ± 0.70	9.5 ± 0.70	13.5 ± 0.70	13.5 ± 0.70	-
Pet. ether	17.5 ± 0.70	18.5 ± 0.70	18.0 ± 1.41	21.5 ± 0.70	-
CH_2Cl_2	10.5 ± 0.70	7.5 ± 0.70	9.5 ± 0.70	10.0 ± 1.41	-
EtOAc	7.5 ± 0.70	8.5 ± 0.70	12.0 ± 1.41	14.5 ± 0.70	-
n-Butanol	9.5 ± 0.70	10.5 ± 0.70	13.5 ± 0.70	12.0 ± 1.41	-
H_2O	8.5 ± 0.70	9.5 ± 0.70	7.5 ± 0.70	8.5 ± 0.70	-
85% MeOH Flower	13.0 ± 1.41	10.5 ± 0.70	13.5 ± 0.70	14.0 ± 1.41	9.5 ± 0.70
Penicillin G	27.5 ± 0.85	28 ± 0.95	20 ± 1.15	25 ± 1.20	-

The results of samples against Staphylococcus aureus (G+ve bacteria); Methicillin-resistant Staphylococcus aureus (MRSA), Pseudomonas aeruginosa; Candida albicans (yeast); Aspergillus niger (fungus); (-); inactive. Penicillin G as positive control.

Table 3: Cytotoxic activity of defatted 85% methanol extract of *C. citrinus* as well as its derived sub-fractions.

Sample	LC_{50}± SE	(CI)
85% Methanol	125.89 ± 6.64	(139.17 – 112.61)
Defatted 85% Methanol	158.48 ±5.24	(168.96 – 148)
Petroleum ether	501.18 ± 10.33	(521.84 – 480.52)
CH_2Cl_2	398.10 ± 9.10	(416.30 – 379.9)
Ethyl acetate	100.0 ± 5.14	(110.28 – 89.72)
n-BuOH	63.09 ± 4.50	(72.09 – 54.09)
H_2O	398.10 ± 8.52	(415.14 – 381.06)
85% Methanol Flower	79.43 ± 5.11	(89.65 – 69.21)

Means ± standard error.
95% confidence limits in parentheses.

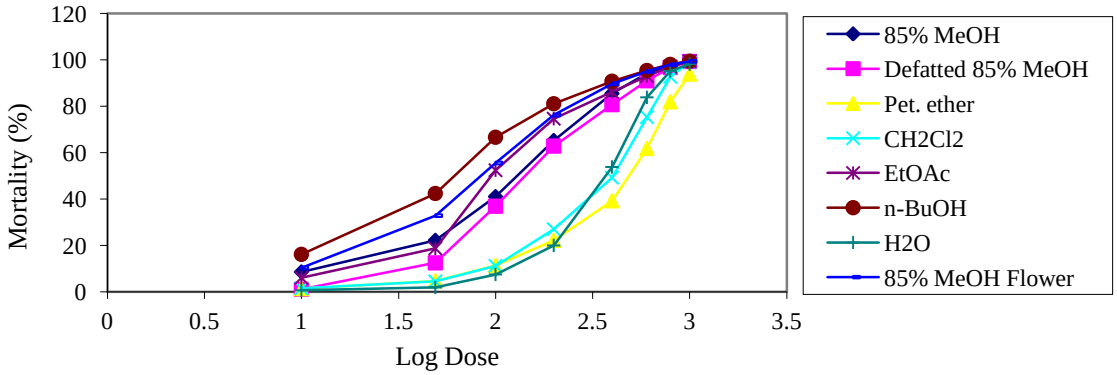

Fig. 2: Estimation of LC_{50} by plot of percent mortality of brine shrimp larvae against different dosage of different extracts of *C. citrinus*.

Biosynthesis of Nanoparticles

The chemical and physical techniques used in synthesis process of nanoparticles have some disadvantages including highly cost, highly energy consumption, and environmental risks. Therefore, scientists try to use alternative eco-friendly biological methods to overcome such disadvantages via using fungus, plant extracts, microorganisms, and enzymes (Willner *et al.*, 2006; Jha *et al.*, 2009; Mittal *et al.*, 2013; Chaudhuri *et al.*, 2016).

Biosynthesis of Silver Nanoparticles (AgNPs)

Silver, is a very common and well-known metal, has gained value due to its wide range of applications. Among the nanoparticles, silver nanoparticles have several vital applications especially in the biomedical field (Pal *et al.*, 2007; Christopher *et al.*,2015). In the current study, when *C. citrinus* leaf extract was added to an aqueous solution of silver nitrate (5mM), the colour was changed to brownish colour due to the reduction of silver ions to metallic silver (Figure 3). By measuring the UV/vis absorbance of the produced solution, it has been found the appearance of peak at about 450 nm (Figure 4). Chaudhuri *et al.* (2016) reported the green synthesis of silver nanoparticles (AgNPs) using aqueous extract of Roheda (*Tecomella undulata*) at 60 °C and the colour was changed from pale yellow to light brownish colloidal solution, the UV-vis analysis of the synthesized nanoparticles were in the range of 410-450nm indicating the formation of AgNPs with particles size from 5.85 nm and 77.48 nm, and atomic force microscopy indicated that height of the particle ranges from 3-18 nm (Chaudhuri *et al.*, 2016).During the synthesis of silver nanoparticles using *Annona reticulata* the color of the reaction mixture, after 20 min, at room temperature, changed to dark brown, indicating the formation of AgNPs (Sivakumar and Vidyasagar, 2014). Moreover, Ahmad*et al.* (2010) reported that the leaf extract of sacred basil was used to synthesize spherical silver nanoparticles (AgNPs) of a diameter of around 10 nm (Ahmad *et al.*, 2010). Our results revealed that a change in colour has been achieved from colourless to yellowish brown to reddish brown to colloidal brown indicating AgNPs formation. The UV/vis maximum of the produced AgNPs has been detected in the range 425 to 475nm due to surface plasma resonance. Transmission electron microscopy was used to determine the microstructure of silver nanoparticles synthesized by using the leaf extract of *C. citrinus* and it has been found that AgNPs sizes of 8-14nm were produced (Figure 5). Also, Figure 6 showing the characteristic peaks of metallic Ag located at 37.8°, 43.3°and 63.5° corresponding to the crystallographic planes (1 1 1), (0 0 2), and (0 2 2) of silver, respectively, establishes a characteristic of crystalline metallic Ag phase. Based on the line width of the peak from crystalline plane (1 1 1), crystallite sizes were found to be around 20 nm for Ag. Several investigations studied the XRD of the plant leaf extract biosynthesized AgNPs have been done to study the production of metallic silver (in nano state) and their purity (Rashidipourand Heydari, 2014; Roy *et al.*, 2015).

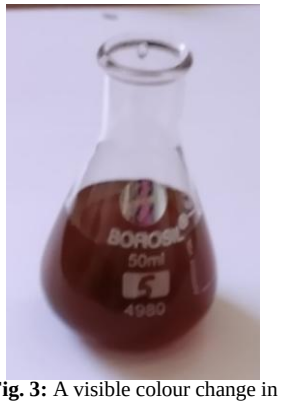

Fig. 3: A visible colour change in color during silver nanoparticle formation.

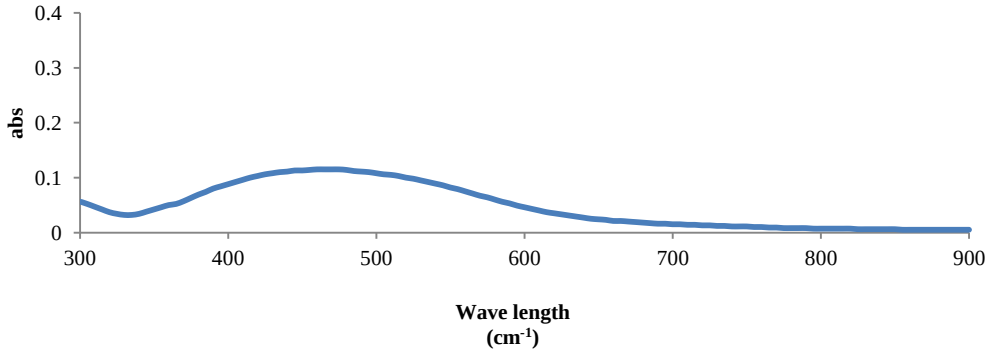

Fig. 4: UV-vis absorption spectrum of silver nanoparticles biosynthesized by *C. citrinus* leaf extract.

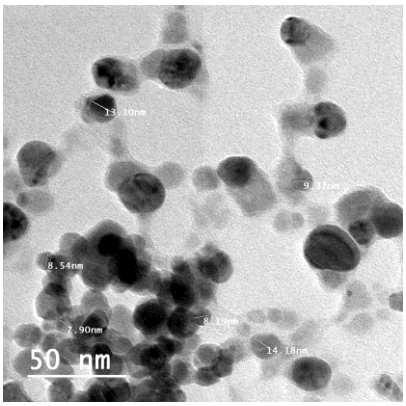

Fig 5: TEM micrographs of silver nanoparticles solution formed by incubation of $AgNO_3$ solution containing *C. citrinus* leaf extract.

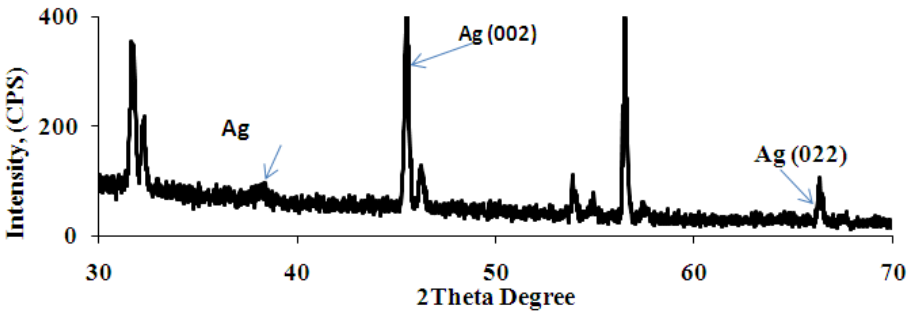

Fig. 6: X-ray diffraction (XRD) pattern of silver nanoparticles biosynthesized by *C. citrinus* leaf extract.

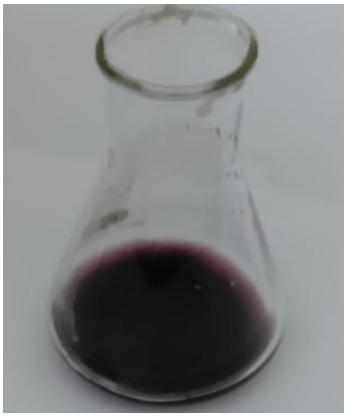

Fig. 7: A visible colour change in color during gold nanoparticle formation.

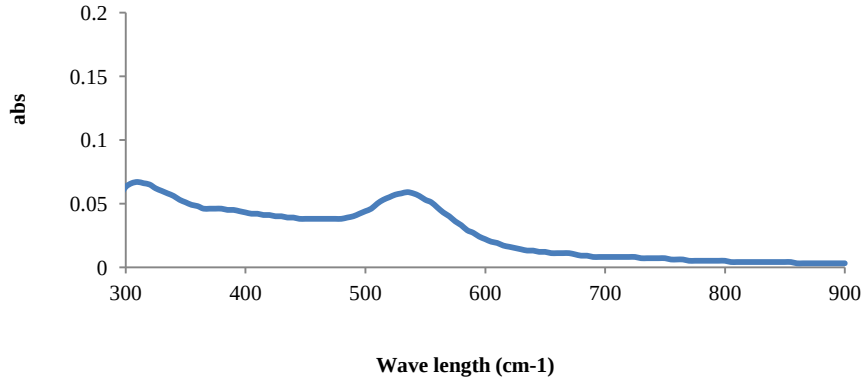

Fig. 8: UV-vis absorption spectrum of gold nanoparticles biosynthesized by *C. citrinus* leaf extract.

Biosynthesis of GoldNanoparticles (AuNPs)

In the current work, when *C. citrinus* leaf extract was added to gold salt ($HAuCl_4$), a change of colour form yellow to purple (violet) has been achieved due to the surface plasma resonance phenomenon (Figure7).

Spectrophotometric studies (UV/vis) revealed that the produced nanogold solution had maximum absorbance at 535nm as measured by Shimadzu 2401PC (Figure 8). *Eclipta prostate* leaf extract was used for the biological synthesis of gold nanoparticles (AuNPs) and the produced AuNPs exhibited a ruby-red colour and had maximum spectral absorbance at 534nm (Rajakumar *et al.*, 2016).Also, a violet color was originated as an evident of the formation of Au metal when Au ions were treated with *Elettaria cardamonum* (ELAICHI) aqueous extract (Pattayanak and Nayak, 2013).Our results also revealed that the formed AuNPs showed maximum absorbance at 540, 550 and 540nm according to the ratio of Au solution and plant extract. Transmission electron microscopy (TEM) measurements of the synthesized AuNPs exhibited an average size of 5.8 to 8.84nm (Figure 9). The structural properties of Au-NPs were investigated using the XRD technique. Figure 10 represented the Au NPs acquired in existence of $AuCl_4$-analogous diffraction peaks are allocated to metallic Au phase with the characteristic peaks at 38.4°, 44.5° and 64.3° attributed to the crystallographic planes (1 1 1), (2 0 0) and (2 2 0), respectively. Several XRD studies have been done to determine the purity and presence of AuNPs (Ismail *et al.*, 2014; Rao and Paria, 2014). Moreover, Chandran *et al*. (2006) reported that the leaf extract of *Aloe vera* was used in biosynthesis of triangular gold nanoparticles (AuNPs) (Chandran *et al.*, 2006). Singh *et al*. (2012) reported the synthesis of gold nanoparticles using *Cinnamomum camphora* and *Emblica officinalis* leaves with particles size of 55-80 nm and 15-25 nm respectively with triangular and spherical shapes (Singh *et al.*, 2012).

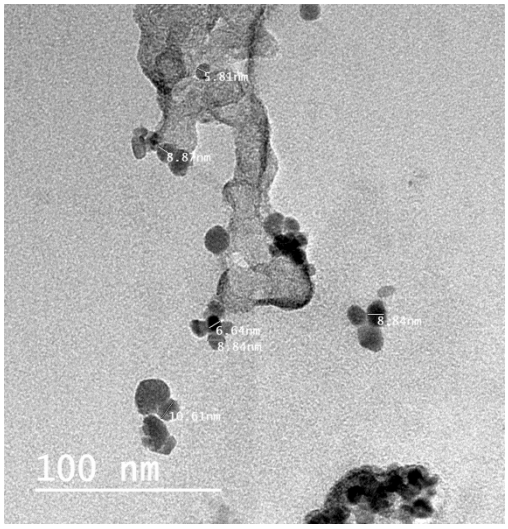

Fig. 9: TEM micrographs of gold nanoparticles solution formed by incubation of $HAuCl_4$ solution containing *C. citrinus* leaf extract.

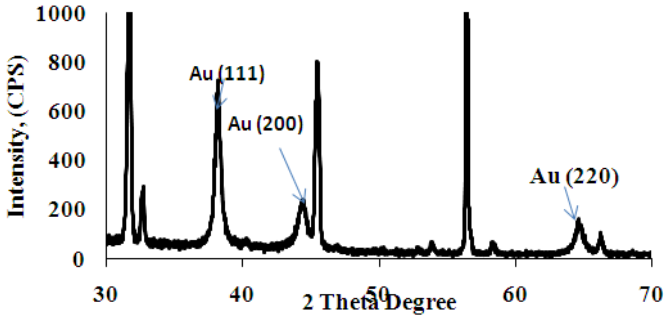

Fig. 10: X-ray diffraction (XRD) pattern of gold nanoparticles biosynthesized by *C. citrinus* leaf extract.

CONCLUSION

The current study revealed that silver and gold nanoparticles can be synthesized using *C. citrinus* leaves extract. The TEM analysis showed that the sizes of the synthesized AgNps and AuNPs were ranged from 8-14nm and 5.8-8.84nm respectively. Also, the most tested extracts of *C. citrinus* showed strong qualitative and quantitative antioxidant activities. Moreover, these fractions showed strong *in vitro* antimicrobial against four pathogenic microbial strains namely; *Staphylococcus aureus, MRSA, Pseudomonas aeruginosa*, and *Candida albicans*. Furthermore, the cytotoxic results showed LC_{50} values ranged from 63.09 to 501.18 µg/ ml. This finding provides an insight into the usage of the *C. citrinus* leaves as good source for the naturally occurring antioxidant, antimicrobial, and cytotoxic agents.

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