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ANTIBACTERIAL ACTIVITIES OF GOLD NANOPARTICLES SYNTHESIZED BY *CITRUS LIMONUM* FRUIT EXTRACT

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Abstract

In this study gold nanoparticles were synthesized successfully in a different way by using *citrus limon* fruit extract and auric chloride. The phytochemistry of the *citrus limon* fruit extract was performed on GC-MS. The gold nanoparticles were characterized by X-ray Powder Diffraction Method (XRD), UV visible spectroscopy, Fourier Transformation Infrared Transformation (FTIR), and Scanning Electron Microscopy (SEM), coupled with EDX. The UV absorption peak at 550 nm was interpreted as that of gold nanoparticles. The XRD results indicated that gold nanoparticles were crystalline. The X-ray peak broadening showed the crystallite size of the order of ~ 32 ± 8 nm. The FT-IR, SEM, and EDX results also confirmed the formation of gold nanoparticles. The synthesized gold particles were studied with pathogens for antimicrobial activities. They showed large inhibitory zones compared to the standard antibiotic against foodborne pathogens such as *Klebsiella pneumonia* and *Listeria monocytogenes*.

Key words: *Citrus Limon* fruit extract; $AuCl_3$; Green synthesis; Gold nanoparticles; Antibacterial activity.

Introduction

Phytochemicals and some natural compounds are used for the reduction of metal ions into metal nanoparticles (Azam *et al*., 2012). Nanotechnology deals with the technology of producing nanoparticles in various sizes and shapes (Oves, *et al*., 2013; Sau & Rogach, 2010). Nanoparticles possess unique properties which have fascinated the scientific world in exploring new applications in various fields of science and engineering (Vilchis-Nestor *et al*., 2008). Industrial-scale production of nanoparticles by chemical methods is not only energy sensitive but also detrimental to an environment which makes them expansive (Sau & Rogach, 2010; Vilchis-Nestor, *et al*., 2008; Prakash, *et al*., 2013). Various metallic nanoparticles also have potential applications in biomedical sciences such as cancer treatment, antibacterial agents, DNA analysis, gene therapy, targeted drug delivery carriers, molecular imaging, catalysts, biosensors, and separation sciences, etc. (Alivisatos, 2004; Salata, 2004). Efforts are being made to develop green synthesis methods and technologies to make nanoparticles non-toxic and eco-friendly (Lakshmipathy *et al*., 2013). Biosynthesis of metallic nanoparticles using extracts of plants have been recommended as an alternative for the chemical methods (Bhattacharya and Gupta, 2005; Mohanpuria, *et al*., 2008; Murawala, *et al*., 2009; Wang *et al*., 2009) and for applications in biomedicine (Schulz-Dobrick, *et al*., 2005; Leonard, *et al*., 2011) due to their nontoxicity, unique dimensions and potential to deliver controlled drug dose (Datar & Cote, 2010). Moreover, nanoparticles may surpass physiological barriers for delivering the drug at targeted areas e.g. intracellular compartments (Vasir *et al*., 2005; Kannan *et al*., 2006; Wangoo *et al*., 2008; Justin *et al*., 2012). For this purpose, gold nanoparticles have been synthesized by various plant extracts such as *Medicago sativa* (Kumar & Yadav 2009), *Chilopsis linearis* (Mirkin *et al*., 1996), *Pelargonium graveolens* (Shankar *et al*., 2003), *Humulus lupulus* (Arya, 2010), *Cymbopogon flexuosus* (Iravani, 2011), *Avena sativa* (Armendariz *et al*., 2004), *Cicer arietinum* (Spielman-Sun, *et al*., 2017), *Hibiscus rosasinensis* (Philip, 2010), *Triticum aestivum* (Herizchi *et al*., 2016), *Murraya koenigii* (Dinesh *et al*., 2015), *Aloe vera* (Grzelczak *et al*., 2010), *Brassica juncea* (Siddiqi and Husen, 2016), *Emblica Officinalis* (Ankamwar *et al*., 2017), *Azadirachta indica (*Ahmed *et al*., 2016) and *Cinnamomum camphora (*Herizchi *et al*., 2016). Citrus fruit one of the main fruit crops grown throughout the world, belongs to the family of Rutaceae (Okwi & Emenike, 1996). Leaf and shoot extract of *Citrus lemon* is also used for the gold nanoparticles synthesis as its leaf and shoot extract possess high reducing potential (Prathna *et al*., 2011). Gold nanoparticles have high antimicrobial potential against foodborne pathogens and may be used in several nanomedicines and nano-food packing to prevent a microbial attack on food (products) (Chen *et al*., 2008). This study aims to synthesize gold nanoparticles using auric acid by *Citrus limon* fruit extracts which has high reducing and capping abilities for the conversion of auric chloride ions into Au metal nanoparticles. It is a simple, low-cost, synthesis of nanoparticles. The antimicrobial properties of these nanoparticles against foodborne pathogens will also be studied.

Materials and Methods

Citrus limon fruit of medium size was collected from the botanical garden of Quaid-i-Azam University (Islamabad, Pakistan) and the voucher specimen was deposited in the herbarium of the Plant Sciences Department (ISL). Fruit of *Citrus limon* was squeezed to collect fruit juice which was filtered using Watts man filter paper to remove impurities. The 40 mL (0.002 molars) solution of $AuCl_3$ was mixed with the 10 mL lemon extract. The change of color indicated the completion of reduction process. The Gas Chromatograph (model GC-6890N) attached with Mass Spectrometer (model MS-5973) and Mass Selective Detector (MSD) was used for the phytochemical analysis of the extracts of *Citrus lemon.* The Fourier Transform Infrared spectrophotometer FTIR (model Bruker Vector 22) was used for functional group analysis of the extracts and gold nanoparticles. The UV Visible Spectrophotometer (Model SL164) with double beam was used for the characterization of gold nanoparticles. The crystalline structure was determined by an PanAnalytical X-Pert Pro X-ray powder diffractometer with a CuKα source and Ni filter, was used at COMSATS University Islamabad. The grain morphology of the gold particles was observed using high-resolution scanning electron microscopy.

The bacterial isolates, *Klebsiella pneumonia,* and *Listeria monocytogenes* were sub-cultured in nutrient broth and placed at 37^{o}C for 24 hours for the antibacterial test. Ampicillin (50mg/ mL) was used as a positive control for bacterial strain tests. Sterilized distilled water was used as the negative control.

Gold nanoparticles synthesized by *Citrus limon* fruit extract were tested for antibacterial activity by well diffusion method (Irobi, *et al*., 1994) against *Klebsiella pneumonia* and *Listeria monocytogenes.* Twenty-four hours of fresh cultures of the bacterial pathogens were prepared on agar plates and 100µl of inoculums were used for the antibacterial assay. Sterile distilled water to dissolve gold nanoparticles, AuNPs. Two wells with a 5 mm diameter size were made on each plate and 50µl AuNPs solution was loaded in each well. The plates were then placed at 37°C for 24 hours and the zones of inhibition were measured.

Results

The phytochemical compounds identified by GC-MS analysis (see Table 1) are: Ethanol, Ethyl Alcohol, Methylene Chloride, Trichloromethane, Chloroform, Glycerol, 1, 2-diacetate, Ascorbic.

Acid, 2,6-dihexanedecanoate, Ascorbic Acid, Octadec-9-enoic Acid, Octadeconic Acid, Stearic Acid. Phytochemical analysis indicated that *Citrus limon* had synergistic potentials of flavonoids, polyphenols, catechins, and various phytochemicals which converts $AuCl_3$ into AuNPs (Dhanavade *et al*., 2011). Chromatogram of GC-MS (Fig. 1).

The FTIR spectrum of *Citrus limon* showed (Fig. 2a) the presence of functional groups (Table 2). The extract of *Citrus limon* revealed strong bands at 1229, 1316, 1721, 3000, and 3598 cm^{-1}. Strong bands at 1229 cm^{-1} were due to stretching of the C-OH of the secondary alcohols. The peak at 1316 cm^{-1} indicated stretching vibrations of C-N in the aromatic amines. Peak situated at 1721 cm^{-1} represented amide I and ascended due to carbonyl stretch vibrations in the amide associations of the proteins. Peaks at 3000 and 3598 cm^{-1} were due to the N-H stretching of the amide II and observed in both AuNPs and extract of *Citrus limon*, respectively. C-H groups asymmetric stretching is indicated by a weak band at 2854 cm^{-1}. FTIR spectral studies demonstrated a citric acid-containing O-H functional group that acts in the capping of nanoparticles. So, FT-IR results provided evidence that capping and reduction of AuNPs by biomolecules (present in the extract) of *Citrus limon.* This agreed with another study conducted by (Prathna *et al*., 2011). They also observed the presence of amide I in *Citrus limon* at 1624 cm^{-1}. The proteins may bond with the Au nanoparticles via free amine groups or carboxylate ions of the amino acid residues. The occurrence of the C-O stretching vibrations at 1721 cm^{-1} along with amide I band with a shift indicates the possibility that gold nanoparticles are bound to proteins and antioxidant molecules through free amine i.e. C-O and O-H groups, respectively. A convenient spectroscopic signature of the formation of gold nanoparticles as mentioned above was also indicated by the change of the color of the solution from white to violet. The absorption peak at 575 nm in the UV-spectra also revealed the formation of gold nanoparticles and its intensity increased with time (Fig. 3). The increase in the intensity of the peak may be directly proportional to the number of gold nanoparticles formed because of the reduction of gold ions with the reducing compounds present in the *Citrus limon* fruit extract.

Table 1. Phytochemicals of *Citrus limonum fruit extract.*

Sr. No.	Compound name	Molecular weight	Chemical formula
1.	Ethanol, Ethyl Alcohol	46	C_2H_6O
2.	Acetone,2-propanone	58	C_3H_6O
3.	Trichloromethane, chloroform	118	$CHCl_3$
4.	Alpha-Terpineol	154	$C_{10}H_{18}O$
5.	Nickel tetra carbonyl (Ni(CO)	170	C_4NiO_4
6.	Glycerol,1,2-diacetate	176	$C_7H_{12}O_5$
7.	Linalyl acetate	196	$C_{12}H_{22}O_2$
8.	Octadec-9-enoic Acid	282	$C_{18}H_{34}O_2$
9.	Octadeconic Acid, stearic Acid	284	$C_{18}H_{36}O_2$
10.	Ascorbic Acid, 2,6-dihexanedecanoate	652	$C_{38}H_{68}O_8$

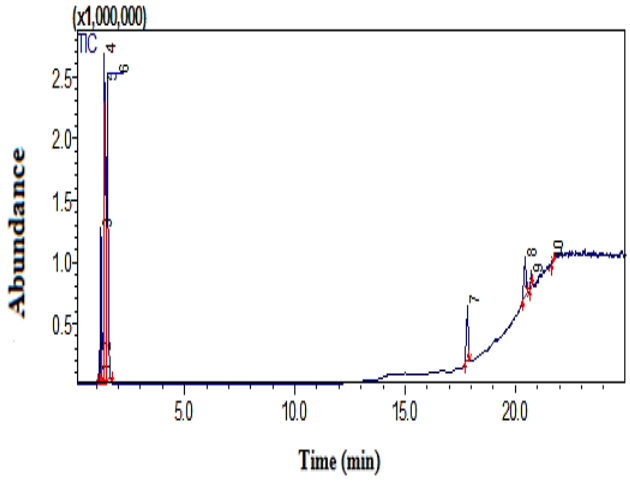

Fig. 1. GC-MS peaks of phytochemicals in *Citrus limon*.

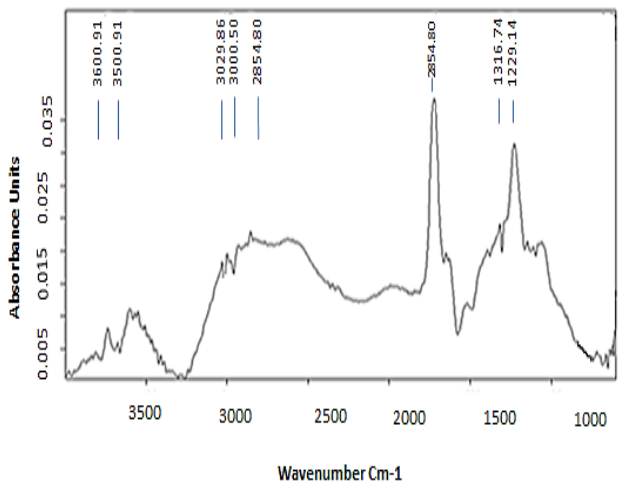

Fig. 2(a). FTIR spectra for functional groups obtained from Citrus limonum.

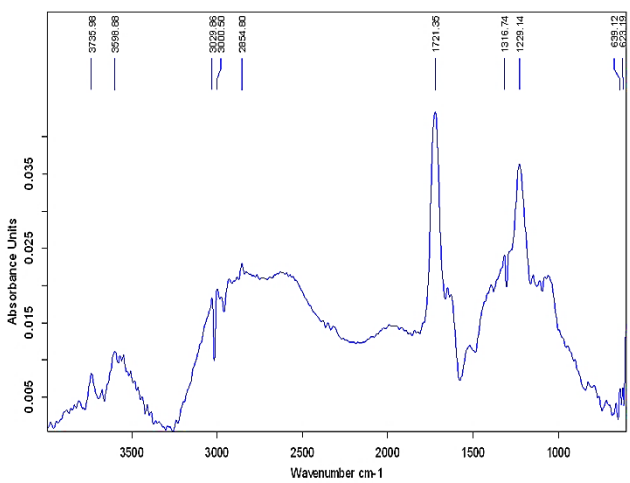

Fig. 2(b). FTIR spectra analysis for AuNPs.

The micrograph of gold nanoparticles taken from a scanning electron microscope depicts surface morphology including grain shape and size (Fig. 4). It was shown that gold nanoparticles did not show definite shape but they might be cylindrical in shape and are agglomerated at places The size of the gold particles was of the order of few nanometers. The elemental analysis of the grains was carried out by an energy dispersive spectrometer attached with the SEM. EDX revealed that small particles were identified as that of pure gold whereas, large grains were of $AuCl_3$. Table 3 presents results of EDX analysis indicating 90% gold. A similar result was reported by *Smitha et al*., 2009. The XRD pattern for synthesized gold nanoparticles was taken from 2θ values in the range of 20 to 70^o (Fig. 5). The peaks observed **i**n the XRD spectrum were located at 2θ values of 38.20^o, 44.36^o, and 64.62^o with d spacing 2.36, 2.04, 1.44 Angtram and indexed as (111), (200), (220), respectively (Table 4) according to the ICDD (International Centre for Diffraction Data) card No. 00-002-1095 which belonged to Au. XRD result correlates with those of *Molnár et al*., (2018).

Above mentioned results confirmed that Au nanoparticles were synthesized by the juice of Citrus limon. In the next section, we will discuss the antibacterial characteristics of these Au nanoparticles.

Table 3. EDX show weightage for Gold Nanoparticles.

No.	Element	Weight%	Error%
1.	PK	4.9	10
2.	FeK	4.7	12
3.	AuK	90.4	6.8

Discussion

Phytochemical analysis of filtered juice by GC-Ms analysis (Fig. 1), indicated that it contained, synergistic potentials of flavonoids, polyphenols, catechins, and various phytochemicals (Table 1) which could be helpful in the synthesis of Au nanoparticles (Dhanavade, *et al*., 2011). The FTIR spectrum of *Citrus limon* juice showed (Fig. 2a) the presence of functional groups (see Table 2). This analysis showed that juice contained O-H functional group which might act as a capping agent for nanoparticles. Thus, it is concluded that *Citrus limon* juice can be used for the capping and reduction of AuNPs by biomolecules.

Filtered juice of *Citrus limon (10 ml)* is mixed with the 40 mL (0.002 molars) aqueous solution of $AuCl_3$ and overnight stirring in dark resulted change of color-less to violet color to violet color. The change of color and its intensity indicated the completion of the reduction process. The absorption peak at 575 nm in the UV-spectra revealed the formation of gold nanoparticles and its intensity increased with time (Fig. 3). The increase in the intensity of the peak may be directly proportional to the number of gold nanoparticles formed because of the reduction of gold ions with the reducing compounds present in the *Citrus limon* fruit extract. Thus, the formation of gold nanoparticles was confirmed by visual color changes (Prathna *et al*., 2011) and UV analysis. Our result matched with the finding of Padalia, *et al*., (2015) and Arokiyaraj *et al*., (2017).

The Au nanoparticles from the solution are removed by filtering. Its structure, grain shape, and morphology were determined by XRD and SEM, and EDX analysis. Theses analysis confirmed that nanoparticles are of pure Au but possess no definite shape but appears to be predominantly cylindrical and agglomerated at some places. The size of the gold particles was of the order of few nanometers. These results are in agreement with the work of Smitha *et al.*, (2009).

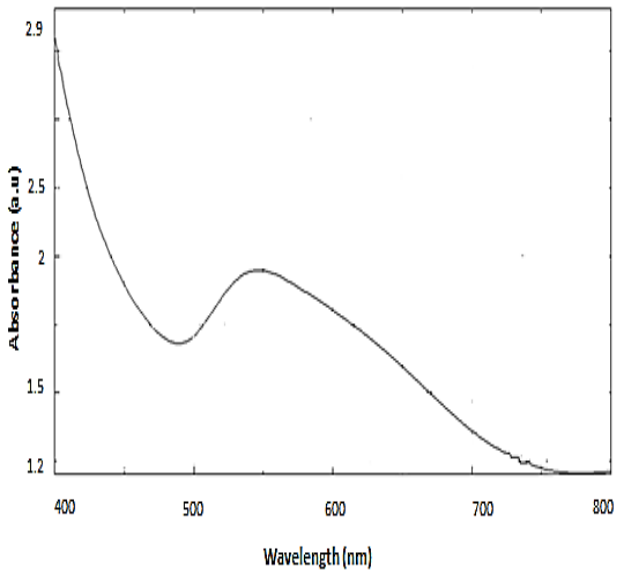

Fig. 3. UV- Visible spectrum show wavelength for Au NPs.

The antimicrobial activity of AuNPs synthesized by *Citrus limon* fruit extract was investigated against the most common human pathogens and multi-drug resistant bacteria *Listeria monocytogenes* and *Klebsiella pneumonia*. Lemon fruit extract and AuNPs (Table 5a) showed the maximum zone of inhibition of 17.1 mm and 16.05 mm against *Klebsiella pneumonia*. Lemon fruit extract and AuNPs (Table 5b) showed the maximum zone of inhibition of 15.1 mm and 28.05 mm against *Listeria monocytogenes*. Our results are consistent with Vijayan *et al.*, (2018). It can be concluded that AuNPs had enhanced antibacterial activity against *Listeria monocytogenes* as compared to *Klebsiella pneumonia as shown in* (Fig. 5). The FT-IR spectrum of *Citrus limon* fruit extract is shown in Figure 2b and identified functional groups in Table 2. The extract of *Citrus limon* fruit showed four distinct bands at 1229, 1316, 1721, 3000, and 3598 cm^{-1}. FTIR spectrum of *Citrus limon* extract mediated AuNPs (Fig. 2b) indicated the reduced intensity of broadband at 3598 cm^{-1} which may be due to the attachment of OH functional groups with AuNPs as compared to others.

Table 2. Functional groups in *Citrus limonum fruit extract.*

S. No.	Functional groups	Characteristic absorption range	Citrus *limonum*	Citrus *limonum* + Au nanoparticles	Vibration type
1.	O-H	3200-3600	3549	3599	Stretch, H-bonded
2.	=C-H	3010-3100	3030	3000	Stretch
3.	C-H	2850-3000	2855	2855	Stretch
4.	C=O	1700-1725	1716	1721	Stretch
5.	C=C	1620-1680	1650	_	Stretch
6.	C-N	1280-1335	1317	1316	Stretch
7.	C-O	1210-1310	1231	1229	Stretch
8.	Gold	-	-	639/623	Gold particles

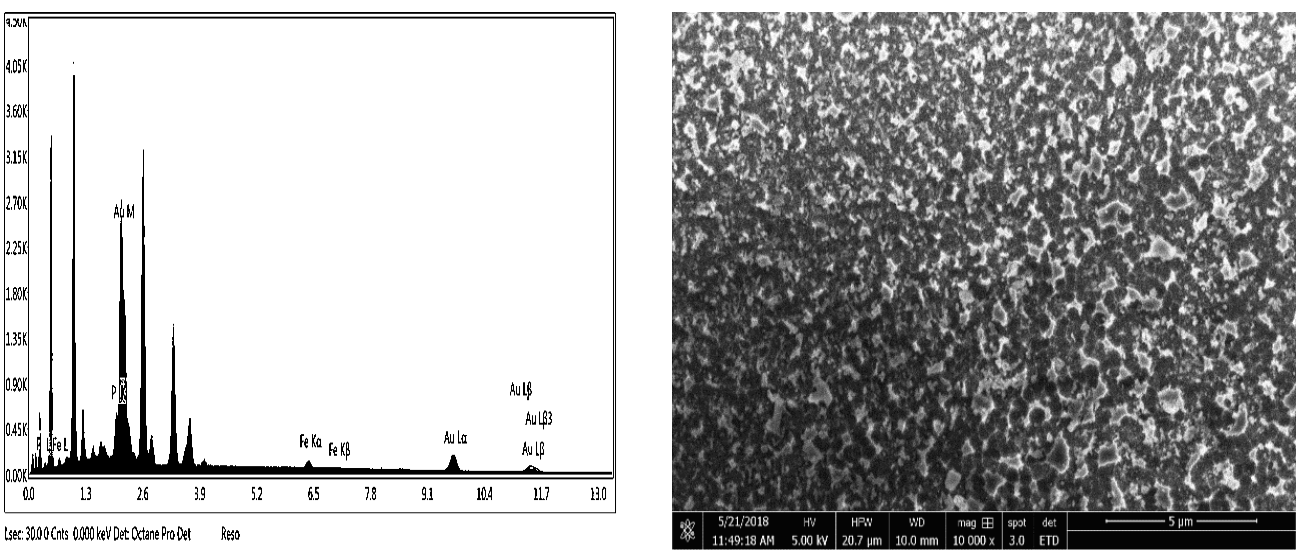

Fig. 4. SEM micrograph showing (a) - gold nanoparticles and their (b) - EDX spectrum.

Table 4. XRD data and crystallite size of Au Nanoparticles prepared by *Citrus limonum fruit extract.*

No.	Peak. pos. [°2Th]	D. spacing	FWHM	Intensity	Crystallite. size (nm)	hkl Au	hkl AuCl
1.	28.410	3.14	0.275	74	30	-	200
2.	38.204	2.36	0.354	100	31	111	-
3.	40.820	2.21	0.157	29	54	-	113
4.	44.360	2.04	0.354	32	24	200	-
5.	64.620	1.44	0.236	18	40	220	-

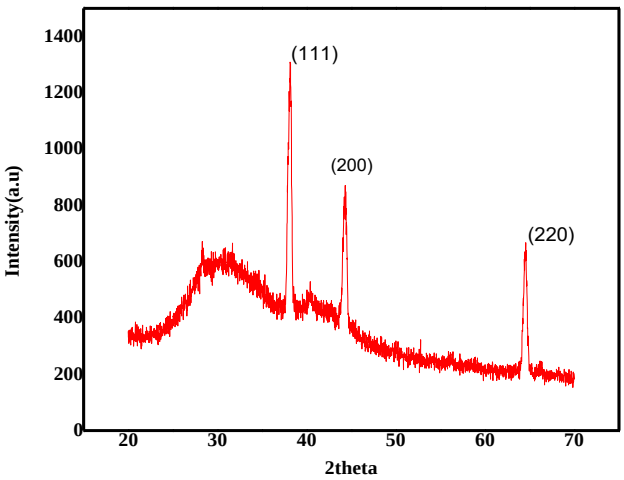

Fig. 5. XRD Spectrum indicate peaks for Au NPs.

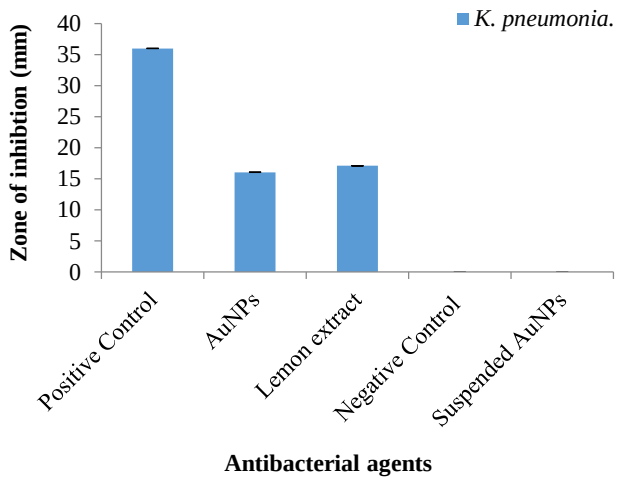

Fig. 6(a). Antibacterial activity of AuNPs against *K. pneumonia*.

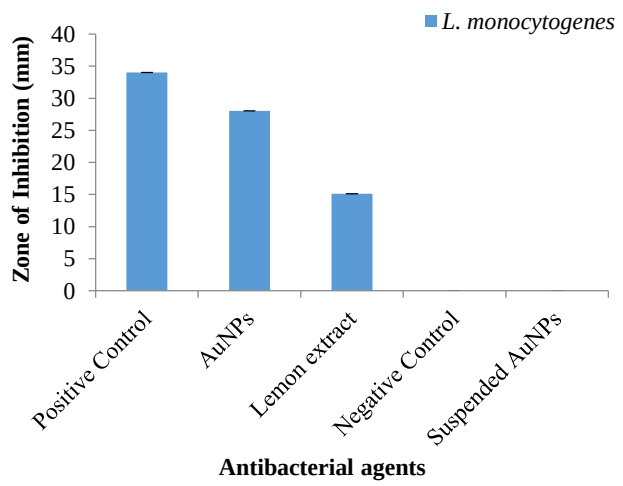

Fig. 6(b). Antibacterial activity of AuNPs against *L. monocytogenes*.

Thus, it is concluded that OH functional groups play a major role in antibacterial actions. The general antibacterial trend observed for the nanoparticles was AuNPs >lemon extract > suspended AuNPs. Thus, it is concluded that the antibacterial activity of suspended AuNPs has enhanced as compared to lemon extract and AuNPs.

The gram-positive bacteria showed higher antibacterial activity than the gram-negative bacteria. This may be due to the variance in the composition of the cell wall of these bacteria. The cell wall of gram-positive bacteria is negatively charged due to phosphate in their structure while the outer membrane of gram-negative bacteria imparts a very strong negative charge due to the presence of phospholipids and lipopolysaccharide (Prakash *et al*., 2013).

Conclusions

Gold nanoparticles were successfully synthesized using *Citrus limon* fruit extract and $AuCl_3$ solution. Phytochemical analysis and functional group analysis were also conducted with encouraging results. The gold nanoparticles were identified by UV spectroscopy and FT-IR analysis and confirmed by XRD investigations and SEM plus EDX analysis. The size of the gold nanoparticles was recorded at about 30 ± 6 nm. The antimicrobial potential of synthesized gold nanoparticles against *Listeria monocytogenes* and *Klebsiella pneumonia* showed considerable antimicrobial potential against these two strains. This study has shown that green synthesized AuNPs have great antimicrobial potential.

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