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Study of Potential Anticancer, Antibacterial, Antioxidant and Photocatalytic Activities of Microwave Assisted Gold Nanoparticles using *Limonia acidissima* Fruit Pulp Extract

J. KALPANA[1,2,*], MD. MOULANA KAREEM[3] and G. VIJAYA LAKSHMI[2]

[1]Department of Chemistry, KVR Government Degree College (W) (A), Kurnool-518004, India
[2]Department of Chemistry, Telangana Mahila University, Koti, OU, Hyderabad-500095, India
[3]Department of Chemistry, Government Degree College, Manugur-507117, India

*Corresponding author: E-mail: kalpu.chem@gmail.com

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In this work, synthesis of gold nanoparticles (AuNPs) by microwave irradiation of *Limonia acidissima* fruit pulp is described. The optical, structural and morphological properties were evaluated for the synthesized nanoparticles. The UV-vis spectra of the formed AuNPs, the peak at 525 nm indicates the reduction of Au^{3+} ions into metal nanopartciles. The XRD analysis of AuNPs revealed their FCC structure and by looking at the TEM images, it can be seen that the prepared AuNPs had an average size of 11 ± 2 nm. Analysis of FTIR spectra was used to determine the potential functional groups in the extracts that bring out the reduction process in the synthesis of AuNPs. Furthermore, both Gram-positive and Gram-negative bacteria were used to test the antibacterial efficacy of the green-synthesized AuNPs. The synthesized AuNPs showed highly effective antioxidant and anticancer activity. Also, the synthesized AuNPs showed the excellent catalytic activity for the reduction of 4-nitrophenol into 4-aminophenol in the presence of $NaBH_4$.

Keywords: Green approach, Gold nanoparticles, Surface plasmon resonance, 4-Nitrophenol reduction, Biological activities.

INTRODUCTION

Noble metal nanostructures (like Au, Ag and Pt) are thought to be one of the cleverest types of nanomaterials because of their unique physical and chemical properties [1,2]. Gold nanoparticles (AuNPs) are the most stable nanostructure because they can be formed into a wide variety of shapes and their optical properties are adaptable [3]. The AuNPs have been proven to be a powerful catalyst for many reactions, such as the reduction of organic dyes and the reduction of nitro groups, *etc.* [4,5]. The surface-to-volume ratios and morphology are significant factors in the excellent catalytic activity of AuNPs [6]. In previous studies, it has been shown that immobilizing AuNPs on a suitable, non-toxic and recoverable support prevents their agglomeration and retains and enhances their catalytic and operational activities [7]. Natural and manmade materials such as polymers, clays, metal oxides, metal organic frameworks, and carbon compounds are all viable options for AuNP support [8,9]. The nature of the support and the interactions between the AuNPs support a crucial component in the construction of AuNP catalysts [10].

Recent research has demonstrated that the green-routed nanoparticles are eco-friendly, cost-effective and easily synthesized. There has been considerable effort put into developing environmental friendly nanoparticle synthesis protocols. The synthesis of AuNPs can be performed in a variety of ways, including through chemical, physical and biological methods [11,12]. Since biological methods are low-cost and use non-hazardous phytochemicals, they have an advantage over the chemical and physical methods. Biochemical nanoparticle synthesis is often done with microorganisms, plants and viruses using a green route [13-16]. In order to synthesize metallic nanoparticles, plant parts such as leaves, fruit peels, roots, seeds and flowers are used [17,18]. A conventional method of synthesizing AuNPs involves toxic reducing agents like trisodium citrate, sodium borohydride and dimethyl formamide, which can have a negative impact on the environment [19,20]. As a result, green methodology has become widely accepted as a means of overcoming the limitations mentioned above. Plants like *Aloe vera barbadensis*, *Nyctanthes arbortristis*, *Limonia acidissima* and *Curcuma longa* (rich in antioxidants, anti-

inflammatory properties) are directly regulated, controlled, cleaned up and remediated by nanoparticles, which contribute to improving environmental friendliness [21-23].

Limonia acidissima (LA) contains numerous bioactive compounds throughout all of its segments, including leaves, stems, bark and twigs. Plant, bacterial, fungal and algal-based nanoparticles are currently being synthesized with cost-effective, eco-friendly and quick methods [22,23]. Nanotechnology was used for fabric finishing. The biosynthesized nanoparticles from plants are coated on cotton and mixed cotton fabrics due to their high active surface, which acts as a medication against pathogens seen in infection.

As reducing and stabilizing agent, the fruit pulp of *Limonia acidissima* was used in this study to synthesize gold nanoparticles (AuNPs) that are simple, inexpensive and environmentally friendly. Fruit pulp from *Limonia acidissima* contains a variety of phytochemicals that can be utilized to prepare nanoparticles. UV-Vis spectra, XRD, FTIR and TEM have all confirmed AuNPs synthesis. Green synthesized AuNPs were examined for their antibacterial, antioxidant and anticancer activities as well as investigated for their catalytic activity for reducing 4-nitrophenol to 4-amino-phenol.

EXPERIMENTAL

Gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$, ≥ 99.9% purity) was obtained from Sigma-Aldrich, USA. 4-Nitrophenol (purity: 99.0%) was provided by Merck Chemical Co., Inc., India. Sodium borohydride ($NaBH_4$) and all other chemicals were purchased from S.D. Fine Pure Chemical Co., Ltd., India.

General procedure

Preparation of *Limonia acidissima* fruit pulp extract: The *Limonia acidissima* fruit was purchased from a local market in Kurnool, India. Removed the pulp from the fruit, washed it thoroughly in double distilled water, dried it in the shade and powdered. Stirred 1 g of the extract at 60 ºC for 40 min over a hot plate. Later, the flask was incubated over a magnetic stirrer for 12 h at 25 ºC. Later, the extract was filtered using Whatman no. 1 filter paper. Aqueous *Limonia acidissima* fruit pulp extract was preserved for further use in the refrigerator at 4 ºC.

Preparation of AuNPs: *Limonia acidissima* fruit pulp extract capped AuNPs were synthesized using $HAuCl_4 \cdot 3H_2O$ solution and *Limonia acidissima* fruit pulp extract. Typical procedures involve mixing 6 mL of *Limonia acidissima* fruit pulp extract (0.5%) with 1 mL of 1 mM aqueous $HAuCl_4$ solution at room temperature. A microwave was used to irradiate the reaction mixture for 2 min at 660 watts. When AuNPs are formed, the colour of the resulting solution changes from light yellow to red.

Characterization: The formation of nanoparticles was confirmed by UV-visible spectroscopy. By using TEM, the prepared nanoparticles were characterized in terms of their shape and structure. The XRD was used to determine the crystal shape. A UV-Vis spectrophotometer (Shimazdu UV-3600, India) was used. By using Fourier-transform infrared spectroscopy (FT-IR, Shimadzu-8400), the functional groups present in the synthesized LA-AuNPs were analyzed in the range 4000-400 cm^{-1} using Kbr dics. The XRD was carried out using Rigaku Mini Flex. By using transmission electron microscopy (1200EX, JEOL Ltd., Japan) analysis, the size and morphology of prepared AuNPs were determined.

Catalytic reduction of 4-nitrophenol: In typical experiments, 1.5 mL of 4-nitrophenol (0.3 mM) was mixed with 1.2 mL of freshly prepared $NaBH_4$ (13 mM) aqueous solution at room temperature. 4-Nitrophenol was converted to nitrophenolate anion during this process, after which, catalyst (LA-AuNPs) 100 µL was added. The UV-vis spectroscopic analysis was employed at different time intervals to observe the reaction progress and noted the absorbances between 200 and 600 nm.

Antibacterial activity: Using the agar well diffusion method, the antibacterial activity of the green-synthesized AuNPs against the bacteria (*S. aureus* and *E. coli*) was evaluated. A nutrient agar medium, Petri plates, a bacterial incubator and other routine lab equipment were used to analyze the samples. An active bacterial culture was prepared by incubating one colony for 8-12 h at 37 ºC in 150 mL conical flask containing 50 mL nutrient broth medium. For MIC assay, the samples were dissolved in DMSO and made into aliquots of different concentrations. Every pathogenic bacterium was tested in triplicate. By measuring the zone of inhibition formed around the disc, the antibacterial effect could be determined.

Antioxidant activity: The DPPH method was used to analyze the antioxidant activity. The DMSO and 2.56 mL of DPPH (0.17 mM) solution are added to different volumes (10, 20, 30, 40, 50 and 60 µL) of AuNPs. Shaking vigorously and keeping the mixture at room temperature for 40 min. At 517 nm, absorbance measurements were conducted on DPPH and ascorbic acid blanks. The scavenging activity of free radicals was calculated using the following equation:

$$\text{DPPH scavenging effect } (\%) = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 and A_t is the absorbance of the control and sample respectively.

Anticancer activity: In three separate experiments, the MTT assay was used to test the viability of cells that had been exposed to six different concentrations of compounds in triplicate. The MTT assay was used to evaluate cell viability in triplicate in three independent experiments. The viability of the cells was tested by trypsinizing them and performing the tryphan blue assay. The cells were counted using a hemocytometer. Incubation was performed overnight at 37 ºC with 5.0×10^3 cells/well in 96 well plates containing 100 µL media. The old media should be removed after 48 h and the fresh media containing different concentrations of the test compound should be added to the wells. The fresh media should be added to each well after discarding the AuNPs and incubated for 3 h at 37 ºC in MTT solution (0.5 mg/mL). After incubation, the cells with metabolically active mitochondria reduce the MTT salt into chromophore formazan crystals, which precipitated. The DMSO-soluble crystals were measured optically at 570 nm using a microplate reader. To calculate the growth inhibition percentage, following formula was applied as follows:

$$\text{Inhibition (\%)} = \frac{100\ (\text{Control} - \text{Treatment})}{\text{Control}}$$

RESULTS AND DISCUSSION

UV-Vis studies: The formation of AuNPs in aqueous solution was investigated using a UV-Vis spectrophotometer. By reducing Au^{3+} to zero valent Au with *L. acidissima* fruit pulp extract, the reaction mixture changed colour from yellow to red, indicating the formation of AuNPs. Gold surface plasmon resonance is characterized by absorbance peaks between 520-550 nm.

To improve the size and quality of nanoparticles, the concentration of *L. acidissima* fruit pulp extract, the concentration of $HAuCl_4$, the pH and the time of the reaction were all optimized. *Limonia acidissima* fruit pulp extract (0.1-0.5%) solutions used to produce the LA-AuNPs varied in concentration (Fig. 1), retaining a fixed concentration of 1 mM of $HAuCl_4$, extract pH-11 and maintaining a constant MWI duration of 120 s, the influence of *Limonia acidissima* fruit pulp extract concentration in the synthesis of LA-AuNPs was examined. According to Fig. 1, when the concentration of *L. acidissima* fruit pulp extract solution increases, the absorption band intensity increases, *i.e.* the nanoparticle formation also increases.

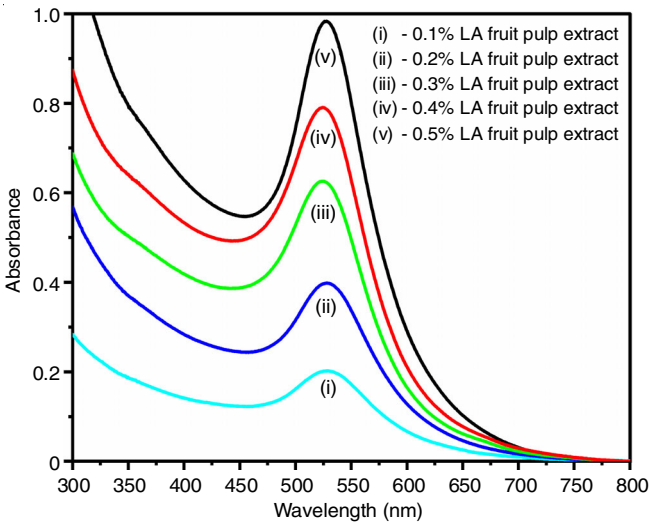

Fig. 1. UV-Vis absorption spectra of AuNPs synthesized at different concentrations of *Limonia acidissima* fruit pulp extract

In Fig. 2, a similar experiment was done by changing the concentration of $HAuCl_4$ (0.2-1.0 mM) while keeping the concentration of *L. acidissima* fruit pulp extract solution (0.5%) constant, at pH-11 extraction and a MWI duration of 120 s. When the concentration of $HAuCl_4$ increase, the absorbance intensity also increases *i.e.* there are more LA-AuNPs in the solution when the concentration of $HAuCl_4$ increased.

Fig. 3 displays the AuNPs absorption spectra obtained at various MW irradiation (40-120 s), while remaining parameters were constant. The LA-AuNPs production is significantly influenced by the duration of the microwave irradiation. A weak and wide SPR band that indicates poor conversion of Au^{3+} ions into AuNPs was seen when the irradiation period for 40 s. The SPR peak intensity of AuNPs increased significantly when the

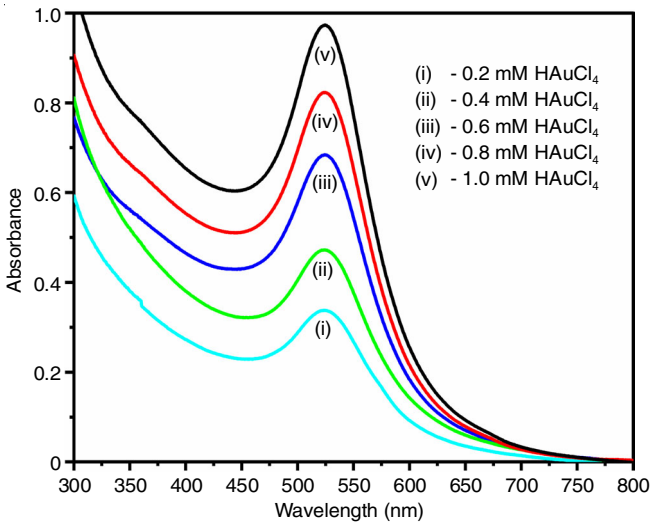

Fig. 2. UV-Vis absorption spectra of AuNPs synthesized at different $HAuCl_4$ concentrations

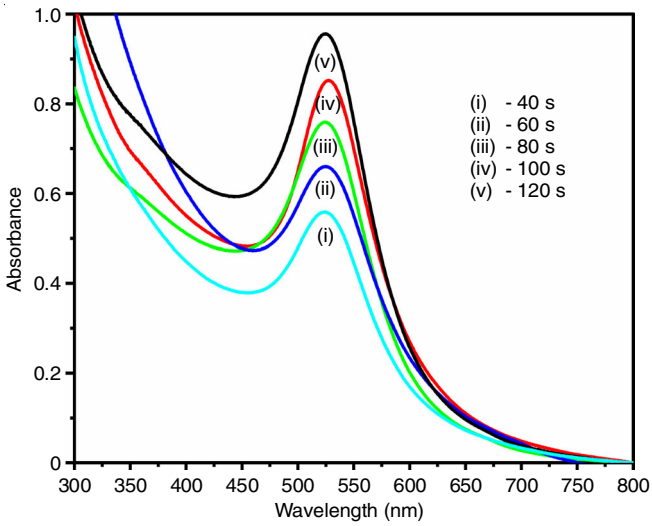

Fig. 3. UV-Vis absorption spectra of AuNPs synthesized at varying the MWI time

irradiation period was gradually extended to 120 s. This means that throughout this time, a significant number of Au^{3+} ions were efficiently converted to AuNPs and encapsulated by *L. acidissima* fruit pulp extract molecules.

Effect of pH: Under constant conditions (1 mM of $HAuCl_4$, 0.5% *L. acidissima* fruit pulp extract and maintaining a constant MWI duration of 120 s), the impact of varied pH on AuNPs formation was studied. In Fig. 4, the UV-vis spectra of AuNPs produced at different pH levels are shown. Also, when the pH value increases, the peak intensity increases gradually. The optimum pH for AuNPs formation was observed at pH-11 using the UV-visible analysis. Therefore, 1 mM $HAuCl_4$, 0.5% *L. acidissima* fruit pulp extract and a constant MWI duration of 120 s were used to biosynthesize AuNPs and pH 11.

FT-IR studies: The FT-IR spectra of *L. acidissima* fruit pulp extract and AuNPs prepared from *Limonia acidissima* fruit pulp is shown in Fig. 5. There are different bands in FT-IR that correspond to different functional groups. The peaks at 3428 and 2918 cm^{-1} correspond to the stretching bands of

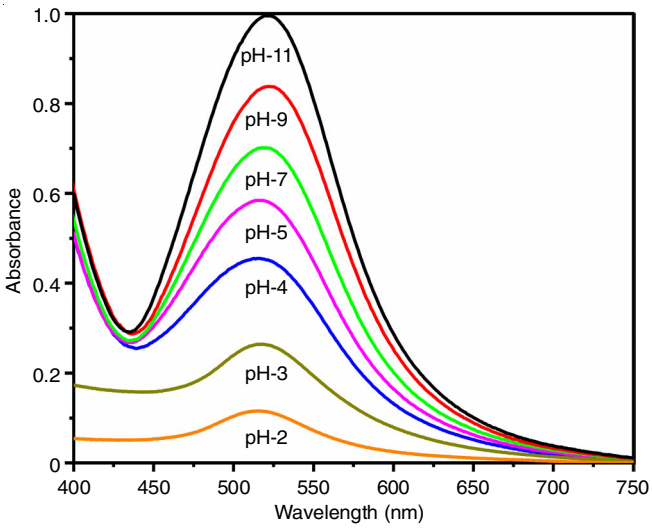

Fig. 4. UV-visible absorption spectra of *Limonia acidissima* fruit pulp extract-AuNPs with varying the pH of the solution

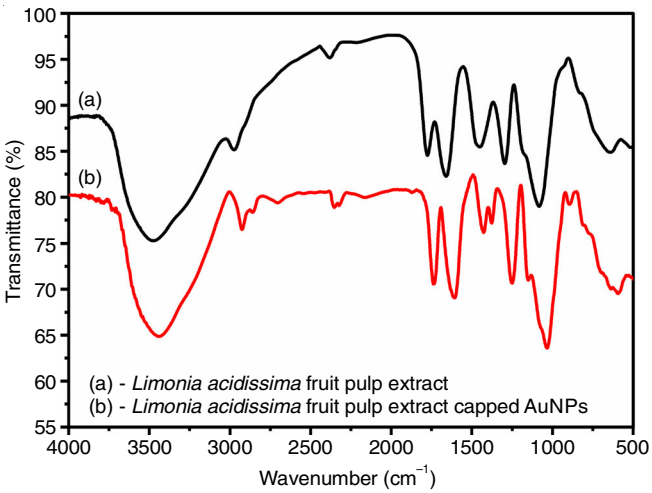

Fig. 5. FTIR spectra of *Limonia acidissima* fruit pulp extract and *Limonia acidissima* fruit pulp extract-AuNPs

O-H and aliphatic C-H, respectively. The peaks at a range of 1606 cm^{-1} for C=O and 1385 cm^{-1} for C=C stretching were also observed. The formation of nanoparticles may be attributed to the presence of C=O bonds in flavonoids (carboxylic acids and their derivatives) and O–H bonds in alcohol and phenol, which reduce their ions and proteins can bind AuNPs through free amine groups [24]. Thus, it becomes evident that biomolecules play an important role in reducing and stabilizing AuNPs.

Powder-XRD studies: The XRD patterns revealed the crystalline nature of AuNPs. Fig. 6 illustrates four significant peaks identified in XRD pattern 2θ at 37.07º, 44.25º, 64.97º and 77.80º, which correspond to AuNPs crystalline structure (111), (200), (220) and (311) indexes (JCPDS 04-0784). A cubic face-centered gold particle was identified in the sample and no peaks of crystallographic impurities were observed, which confirmed that the AuNPs were made of pure gold. Previous studies of AuNPs have found similar results [25].

TEM analysis: TEM images and histograms of AuNPs are shown in Fig. 7a-d. The images show that AuNPs have a

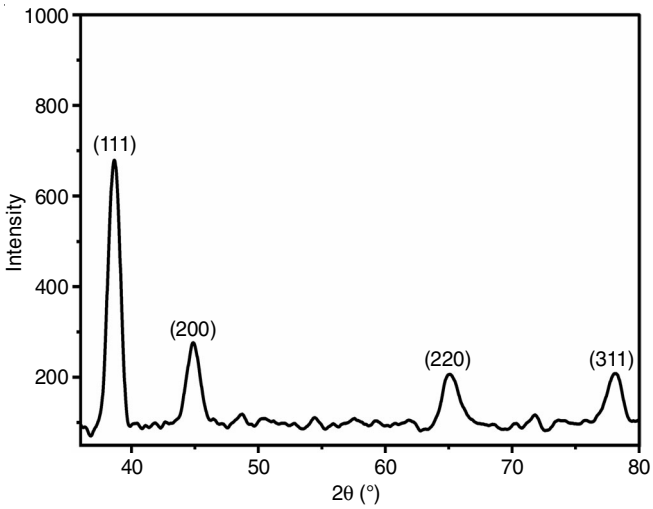

Fig. 6. Powder XRD spectra of *Limonia acidissima* fruit pulp extract-AuNPs

spherical shape, while the nanoparticles were highly homogeneous and well dispersed [26]. It was found that AuNPs had particle sizes smaller than 10 nm. It can be seen from the histogram that the synthesized AuNPs are narrowly distributed with spherical shapes (between 5 and 12 nm) having average particle diameter of 10 ± 2 nm.

Catalytic reduction of 4-nitrophenol: To evaluate the efficiency of the catalytic activity of synthesized AuNPs, an initial probe reaction was selected using the reduction of 4-nitrophenol in the presence of $NaBH_4$. As the reaction progressed, the UV-vis spectroscopy was thoroughly monitored. As depicted in Fig. 8, 4-nitrophenol shows absorption at 317 nm and 227 nm due to its n-π* and π-π* transitions, respectively. An aqueous 4-nitrophenol solution becomes dark yellow colour when $NaBH_4$ was added, resulting in the formation of 4-nitrophenolate ions. As a result, the absorption peak shifts from 317 nm to 400 nm (Fig. 8). After several hours of reaction with the nitrophenolate ion solution, its colour and 400 nm peak intensity did not change. Therefore, 4-nitrophenol cannot be reduced to 4-aminophenol by $NaBH_4$. When AuNPs catalyst was added, immediate reduction occurs, as evidenced by the fading and eventual bleaching of the solution colour. A UV-visible absorption spectrum was monitored every minute during the reduction reaction by 100 µL catalyst (Fig. 9a). Within 8 min, the reduction gets completed. A characteristic peak at 400 nm grew weaker as the reaction time grew. As time went on, an increased intensity peak attributed to 4-aminophenol formation appeared at 297 nm.

The rate of the reaction can be monitored with spectrophotometry by monitoring the shift in the peak at 400 nm/min. A larger concentration of $NaBH_4$ (13 mM) was employed in this reaction than either 4-nitrophenol or catalyst (AuNPs). The kinetics of the reaction follow pseudo-first order as the concentration of $NaBH_4$ is constant. In the rate equation, $k = 1/t \ln [A_0]/[A_t]$, k represents the pseudo-first-order rate constant, t represents the reaction time, $[A_0]$ represents the initial absorbance of 4-nitrophenol and $[A_t]$ represents the absorbance of 4-nitrophenol at time t. From the plot of $\ln [A_0]/[A_t]$ *vs.* time (Fig. 9b), the rate constant value was found to be 0.663 min^{-1}.

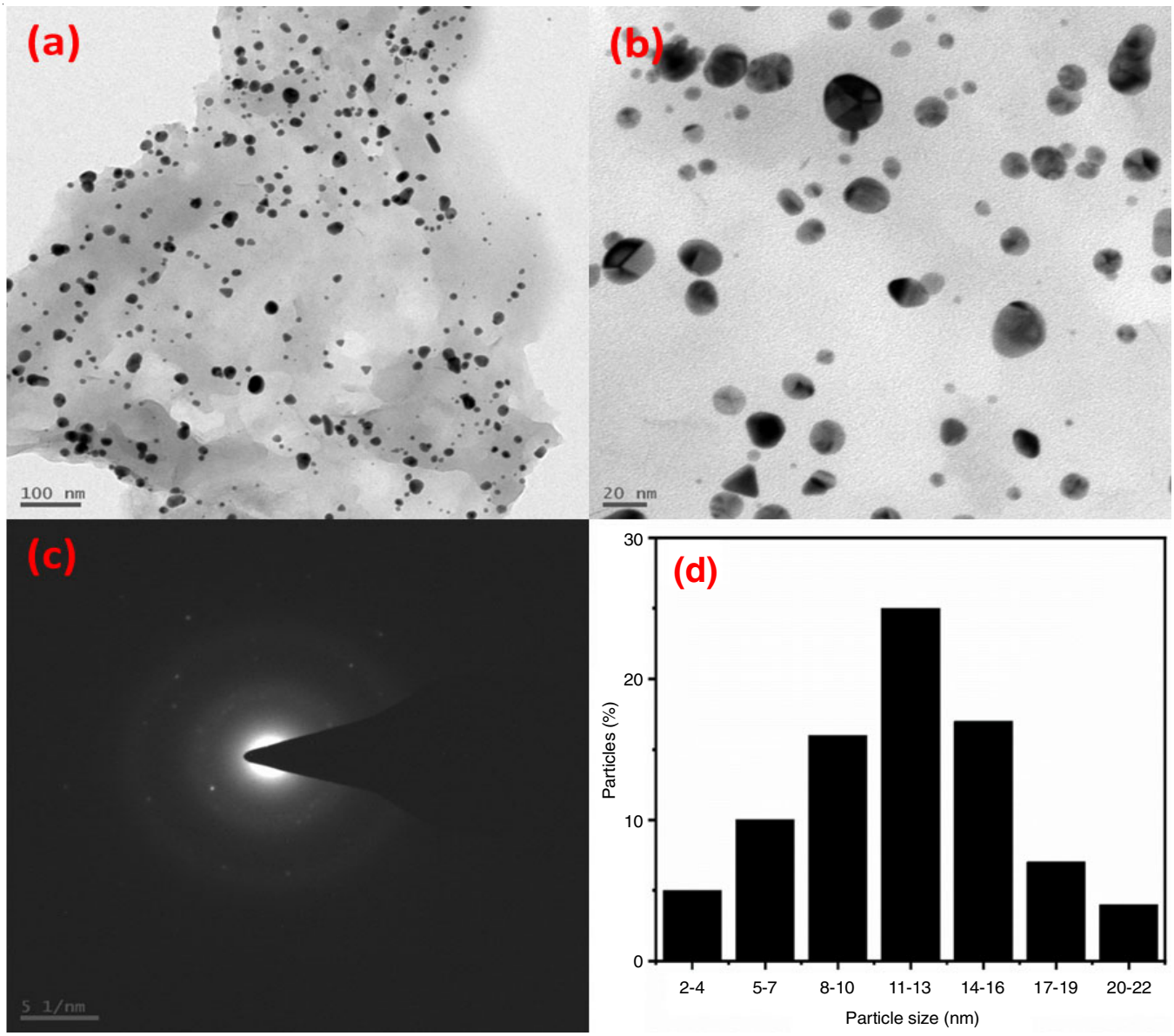

Fig. 7. TEM images of synthesized *Limonia acidissima* fruit pulp extract-AuNPs (a) 100 nm scale (b) 20 nm scale, (c) SEAD pattern and (d) histogram of size distribution of AuNPs

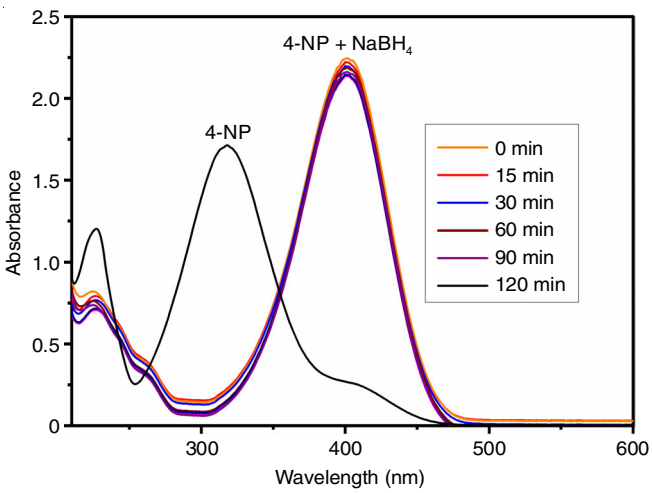

Fig. 8. UV-vis spectra of catalytic reduction of 4-NP and 4-NP + $NaBH_4$ (absence of catalyst)

Antibacterial activity: Agar well diffusion was used to test the antibacterial activity of *L. acidissima* fruit pulp extract-AuNPs against *S. aureus* and *E. coli*. In terms of zone of inhibition (ZOI), *L. acidissima* fruit pulp-AuNPs exhibited antibacterial efficacy against all tested species. The maximum and minimal inhibitory zones against *S. aureus* and *E. coli* were found to be 17.24 ± 0.35 and 12.56 ± 0.41 mm, respectively. The MIC of *L. acidissima* fruit pulp extract-AuNPs was determined. As a result of incubation, the growth of *S. aureus* and *E. coli* was visibly suppressed by treating with 10, 15, 20 and 25 µg/mL of *L. acidissima* fruit pulp extract-AuNPs and the maximum inhibition was observed at 25 µg/mL of *L. acidissima* fruit pulp extract-AuNPs. Table-1 shows the minimum inhibitory concentration (mm) of both bacteria.

Antioxidant activity: The antioxidant activity of the prepared LA-AuNPs was assessed using the DPPH test with ascorbic acid as reference. The AuNPs were evaluated for their

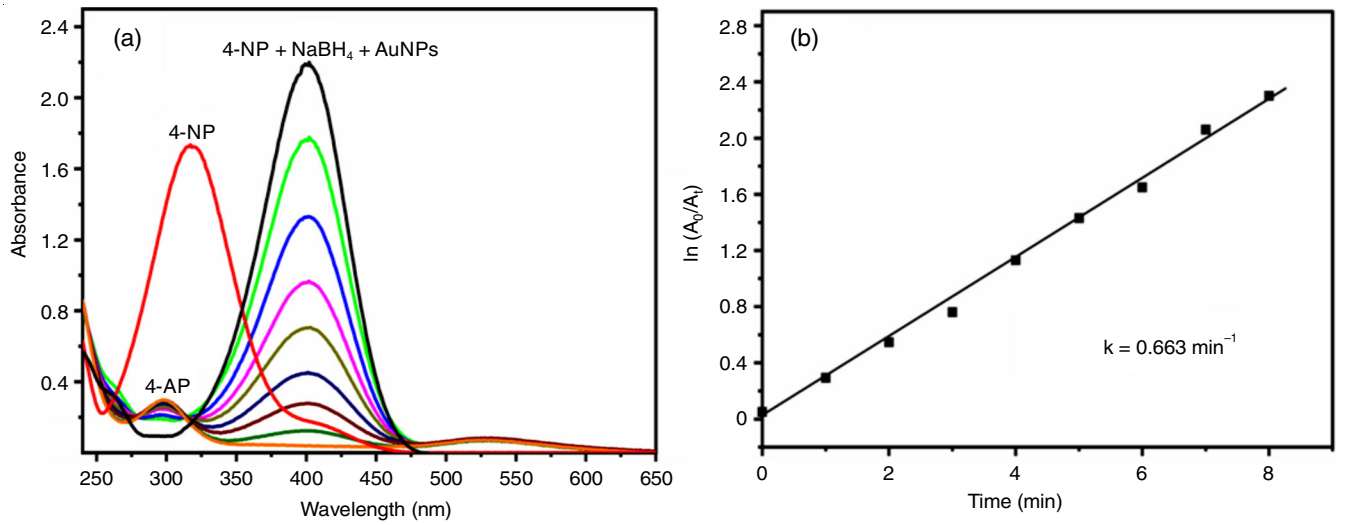

Fig. 9. (a) UV-Vis absorption spectra recorded during the reduction of 4-nitrophenol with $NaBH_4$ catalyzed by AuNPs (b) the plot of ln (A_0/A_t) *versus* time

TABLE-1
ANTIBACTERIAL ACTIVITY OF SYNTHESIZED AuNPs AT DIFFERENT CONCENTRATIONS AGAINST PATHOGENS

Minimum inhibitory concentration (mm)			
E. coli			
10 µL	15 µL	20 µL	25 µL
6 mm	8 mm	12 mm	14 mm
Staphylococcus sp.			
10 µL	15 µL	20 µL	25 µL
–	–	6 mm	10 mm

antioxidant activity by measuring the % suppression of DPPH radicals. Ascorbic acid (standard) and LA-AuNPs have been compared to determine the effectiveness of their inhibitory activity and it has been found that LA-AuNP concentrations increase DPPH radical inhibition (Fig. 10). A scavenging rate of 24.8, 50.3, 62.9, 67.2, 76.3 and 81.2% was demonstrated at 10, 20, 30, 40, 50 and 60 µL, respectively. In comparison to ascorbic acid, LA-AuNPs demonstrated the modest activity. The high quenching rate of DPPH radicals serves as evidence of exceptional antioxidation capabilities of the synthesized LA-AuNPs [27].

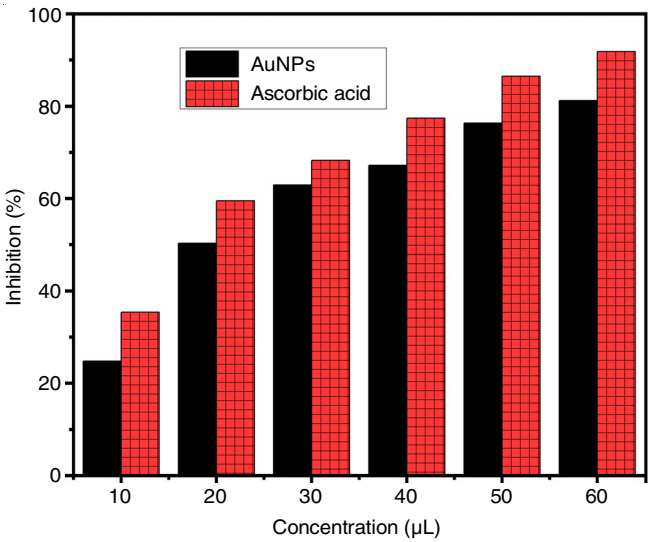

Fig. 10. Antioxidant activity of *Limonia acidissima* fruit pulp extract synthesized AuNPs with the effect of concentration from 10-60 µL with the comparison of standard (ascorbic acid)

Anticancer activity: Different concentrations of the green synthesized AuNPs, such as 20, 40, 60, 100 and 200 µg were used to examine the anticancer activity using Hela cells. The percentage of viability of the cancer cell lines (Hela) was dramatically decreased as the concentration of the synthesized AuNPs increased. Table-2 showed the Hela cell line proliferation inhibitory analysis data of the synthesized AuNPs.

TABLE-2
HeLa CELLS LINE INHIBITORY ACTIVITY OF SYNTHESIZED AuNPs

Conc. (µg)	Absorbance at 570 nm	Inhibition (%)	Viability (%)	IC_{50} (µg)
20	0.521	2.06	97.94	
40	0.506	4.88	95.12	
60	0.412	13.15	86.85	
100	0.389	27.75	72.25	116.2
200	0.351	37.66	62.34	
Untreated	0.552	0	100	
Blank	0	0	0	

Conclusion

In this study, an extract of *Limonia acidissima* fruit pulp is used to make AuNPs. The active compounds in the extract acted both as a reducing agent and a capping agent. The XRD, UV-vis, FT-IR and TEM analyses were performed on the green synthesized LA-AuNPs. According to the UV-vis spectrum, AuNPs have a characteristic peak at 543 nm,which was due to their surface resonance. Various concentrations of extract and $HAuCl_4$ were investigated, for the reaction time and pH of the solution. According to FT-IR analysis, *L. acidissima* fruit pulp extract contains the phytochemicals are responsible for stabilizing AuNPs and forming AuNPs. Based on the XRD pattern, AuNPs are highly crystalline in nautre and the particle size was found to be 11 ± 2 nm. With $NaBH_4$, *L. acidissima* fruit

pulp extract-AuNPs displayed outstanding catalytic activity for the reduction of 4-nitrophenol. The synthesized AuNPs showed the significant antibacterial activity against both the tested bacteria viz. *S. aureus* and *E. coli*. The prepared AuNPs exhibited excellent antioxidant and anticancer activities too.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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