Article

Synthesis, Characterization and Anti-microbial Activity of *Citrus limon* Mediated Nanoparticles

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Abstract: Previously the nanoparticles were synthesized by chemical methods which were costly and toxic to bio-systems. Plant extracts provides simpler, eco-friendly and cost efficient method for synthesizing nanoparticles. Lemon peel extract (LPE) was used to synthesize silver nanoparticles (AgNPs) which were evaluated for their antimicrobial effects after optimizing the pH of extract and concentration of both extract and synthesized AgNPs. The characterization of synthesized AgNPs was carried out using Ultraviolet-Visible (UV-Vis) Spectrophotometer, Fourier transform infrared spectroscopy (FTIR), and X-ray diffraction (XRD). Well diffusion method was used to determine the antimicrobial activities of synthesized AgNPs. The presence of phenols and proteins was assumed to reduce the Ag^{+} ion into silver nanoparticles. The characteristic surface plasmon resonance frequency was observed at 405-425 nm for all varying condition of silver nanoparticles synthesis. Furthermore, results revealed that the synthesized AgNPs remains stable upto 75 days. The average particle size was 2-5 nm, calculated with the help of scherrer's equation by using XRD data. LPE mediated AgNPs (200 µg/ml) showed significant antimicrobial activity, compared to commercially available nanoparticles while LPE (50 mg/ml) showed no effect. LPE mediated AgNPs might get attention of pharmacists in order to design medicines against different diseases including the infections of bacteria.

Keywords: Nanoparticles; biological; stability; antimicrobial activity

Introduction

The word "nano" originates from Greek, meaning "dwarf" and it is equivalent to one billion parts of a meter [1]. The submicron size particles having diameter in the range of 1-100 nm are termed as nanoparticles (NPs), showed special interest owing to vast applications in different fields including chemistry, photography, and especially in medicine [2,3]. There are two kinds of NPs, first one are synthesized by human beings intentionally, often termed as "engineered" NPs, and second one are a by-product or naturally occurring or wastes, normally considered as "free" NPs [4]. Metal

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nanoparticles have gained special importance because of its smile size, increase surface area, surface Plasmon property (SPR), good electrical and optical properties and many more [5].

The metal nanoparticles can be synthesized by using bottom-up or top-down approach both of which further comprise the chemical, physical and biological method [6,7]. The chemical and physical methods employed for the synthesis of NPs are excessively costly, furthermore including lethal, perilous chemicals such as ammonia (NH_3), sodium boro-hydrate, ammonium formate, sodium citrate, aniline and hydrazine that can directly damage the living organisms [8]. The chemical and physical methods employed for the synthesis of NPs are excessively costly, furthermore including lethal, perilous chemicals that can directly damage the living organisms [9]. Because of the limitations of the physical and chemical methods, biological method attracted its attention in the field of research. Jose-Yacaman was the first who tried and succeeded in the synthesis of silver and gold NPs using biological methods [10]. In green chemistry metal NPs can be synthesized by using different plants and bio organisms including fungus, yeast and bacteria [11]. NPs synthesized using microbes are not viable industrially because it involve highly aseptic environment which is difficuit to maintain while the phyto-mediated synthesis of NPs is simple efficient and eco-friendly method [12]. Numerous reducing agents are present in the plants naturally including phenols, flavonoids, ascorbic acid which helped in reducing and stabilizing the metal ions. For green synthesis of AgNPs fruit peel extracts can also be used because of the presence of various reducing agents in fruits peel [13]. *Citrus limon* (lemon) is a medicinal plant used in the cure of massive diversity of disorders including gastrointestinal disorders, rheumatism, headache and nervousness [14]. Lemon is rich in citric acid and ascorbic acid while flavonoids and polyphenols are also found in its peel which are important reducing agents thus used as bio reductive synthesis of metal NPs. *Citrus limon* have a broad continuum of biological activities including anticancer, antidiabetic, antioxidant, anti-inflammatory, antifungal, and antiviral activities [15]. The aim of present work was to synthesize lemon peel extract (LPE) mediated AgNPs and their characterization. Furthermore, the antimicrobial activity of LPE mediated AgNPs was evaluated by well diffusion method.

2. Materials and Methods

1.1. Preparation of Lemon Peel Extract

For the preparation of lemon peel extract (LPE), *Citrus limon* peel was obtained, washed thoroughly with distilled water to remove all the dust particles, incised in small pieces and 3 g of these pieces was shaked for 2 hours in 100 ml distilled water. After shaking the mixture was boiled for about 10 minutes and then filtered by using Whatman No. 1 filter paper. The resultant LPE was stored at about 4 °C for the preparation of AgNPs [16,17].

1.2. Synthesis of Silver nanoparticles

In this study the factorial design method "one factor at a time" was employed, in which one factor can be varied at a time while all other parameters remain the same [18,19]. For the synthesis of AgNPs 30 ml of freshly prepared 1 mM $AgNO_3$ (Germiston chemical) solution was added to 5 ml of LPE and then boiled for 30 minutes. In order to optimize the conditions for maximum yield of AgNPs, the pH, LPE volume at different times and concentrations of $AgNO_3$ was varied. The pH of 5

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ml LPE was adjusted as 6, 7, 8, 9, and 10 separately then $AgNO_3$ solution (30 ml) was added and boiled for 30 minutes. Similarly in next step, 1, 3, 5 and 7 ml of LPE was mixed with $AgNO_3$ solution (30 ml) and boiled. In the 3rd and last step the $AgNO_3$ concentration was altered i.e. 0.5, 1, 2, 3 mM $AgNO_3$ was used and added to 5 ml and 7 ml of extract separately and boiled. Stability of synthesized AgNPs was checked at different days after the reaction and UV-Vis spectrophotometer was used to analyze the synthesis of AgNPs.

1.3. Purification and drying of Silver nanoparticles

The AgNPs solution was centrifuged at a speed of 12000 rpm for 30 minutes at 4 °C. Process of centrifugation was repeated two times again, firstly by immersing the resultant pallet in de-ionized water followed by re-immersion of washed pallet of AgNPs in methanol while all other conditions kept the same [20]. Alcohol was evaporated and dried powdered AgNPs was collected for further analysis [21].

1.4. Characterization of silver nanoparticles

The synthesized AgNPs was firstly characterized by UV-vis spectrophotometry (T90+ UV/Vis Spectrometer.PG Instrument LTd) which was recorded in the range of 300 nm to 600 nm [22]. The dried AgNPs and LPE were subjected to FTIR (Agilant Microlab, Carry 630 FTIR), and XRD (Philips PW 3710/3020) [23].

1.5. Antimicrobial assay

The synthesized AgNPs were screened against one gram-positive (*Streptococcus mutans*), four gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii* and *Proteus mirabilis*) one fungus (*Candida albicans*) species. Antimicrobial activity was assessed by agar well diffusion method [24]. Test microbes were cultivated in Mueller Hinton Broth medium overnight (37 °C). Then, Mueller Hinton Agar plates were swabbed (sterile cotton swabs) with 100 µl of test microbial culture. Using the sterile cork borer, the well (6 mm) was made into the each plate. The plates were incubated at 37 °C for about 16–24 hours and after the incubation period, diameter of the inhibition zones of each well was measured.

3. Results

1.6. UV-Vis spectrophotometric analysis

The bio-reduction of Ag^+ ions in aqueous extract of *Citrus limon* was characterized using UV-Vis spectroscopy. The strong surface plasmon frequency was observed in the range of 405 to 425 nm and sharp peaks were observed at 410, 415, 420 and 405 nm for the AgNPs at pH 6, 7, 8, 9 and 10 respectively (Figure 1). At pH 8, 9 different volumes of LPE were also used and peak was examined just after the reaction. At pH 8 the absorption of AgNPs was increased gradually for 1, 3 and 5 ml of LPE but decreased for 7 ml of extract while at pH 9 the absorbance was increased sharply at 3 ml and almost remains same for 5 and 7 ml of extract (Figure 2). In order to check this abnormal behavior of 5 ml and 7 ml extract we changed the reaction conditions i.e. different concentrations of $AgNO_3$ solution was used at pH 8 and 9. At pH-8, using 5 ml and 7 ml of extract, the trend of AgNPs

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synthesis was normally goes on increasing as the concentration of $AgNO_3$ increases while at pH 9 the trend was irregular for 3 mM $AgNO_3$ solution. Initially AgNPs yield was increasing upto the 2 mM concentration of $AgNO_3$ but yield was low at 3 mM $AgNO_3$ (Figure 3 and 4). The stability of AgNPs was also checked upto the 75 days and it showed the gradual increase in the absorption (Figure 5).

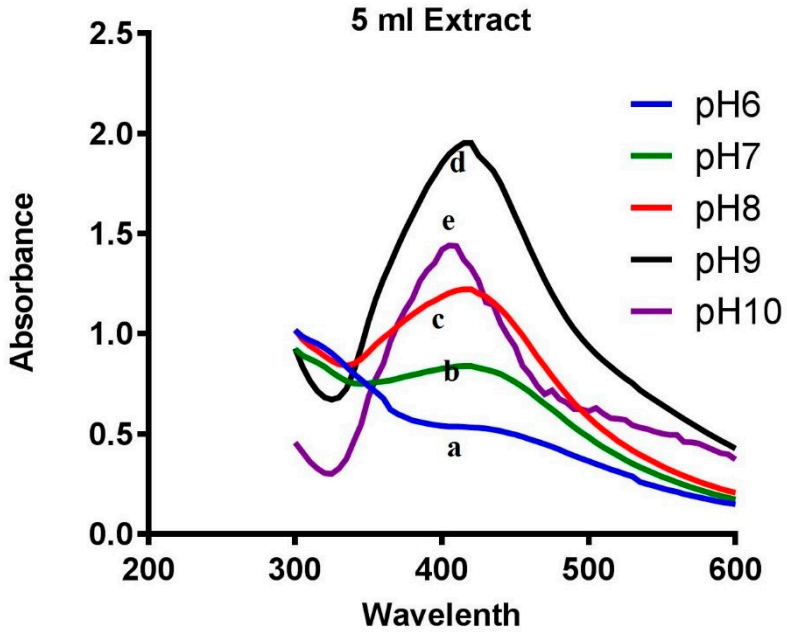

Figure 1. UV-Vis spectra of LPE mediated AgNPs fabricated at pH (6-10). Peak a, b, c, d and e show AgNPs at pH 6, 7, 8, 9 and 10 respectively. Peak (e) was obtained after the three times dilution of resulting solution of LPE mediated AgNPs

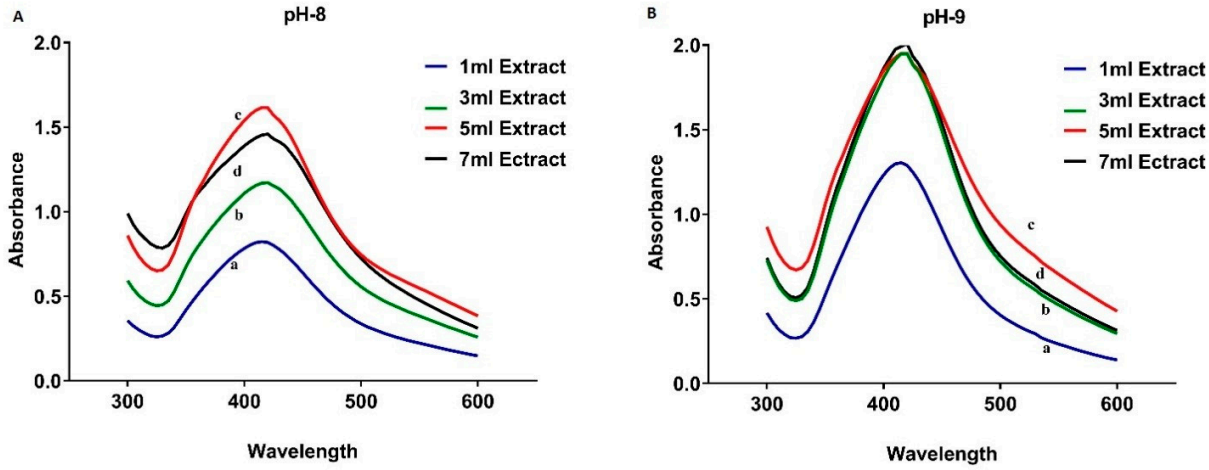

Figure 2. UV-Vis spectra obtained at pH 8 and 9 using different volumes of LPE. The graphs Show the absorption peaks of LPE mediated AgNPs by using different volume of LPE at (A) pH 8 and (B) pH 9

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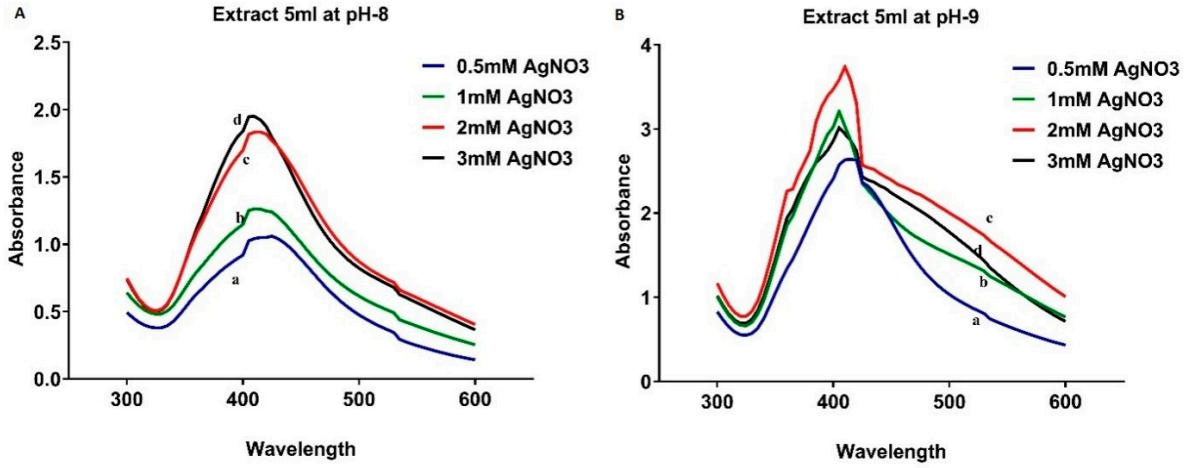

Figure 3. UV-Vis absorption spectra of LPE mediated AgNPs obtained at pH 8 and 9 using 5 ml extract with varying concentration of $AgNO_3$ (0.5, 1, 2 and 3 mM). Graphs show the absorption peaks at (A) pH 8 and (B) pH 9

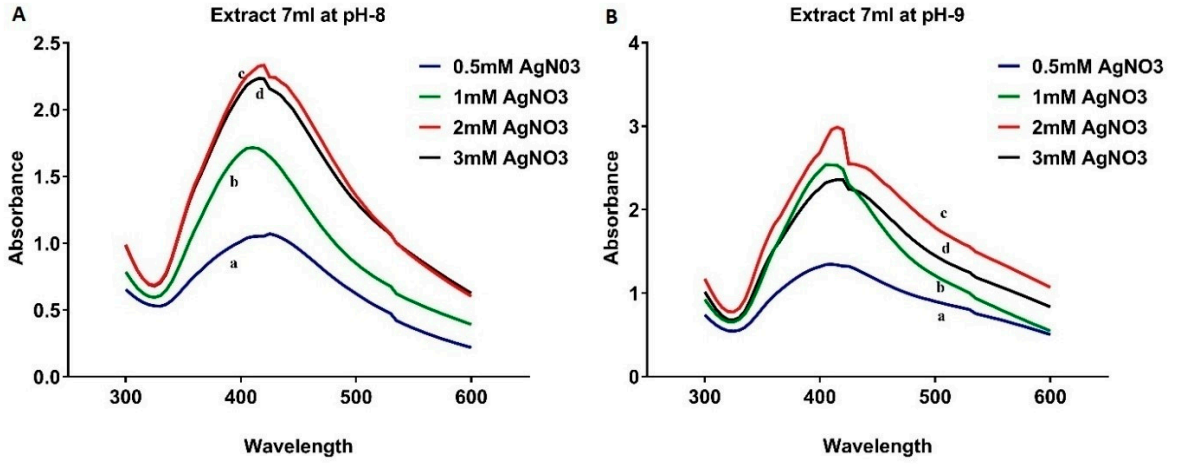

Figure 4. UV-Vis absorption spectra of LPE mediated AgNPs obtained at pH 8 and 9 using 7 ml extract with varying concentration of $AgNO_3$ (0.5, 1, 2 and 3 mM). Graphs show the absorption peaks at (A) pH 8 and (B) pH 9

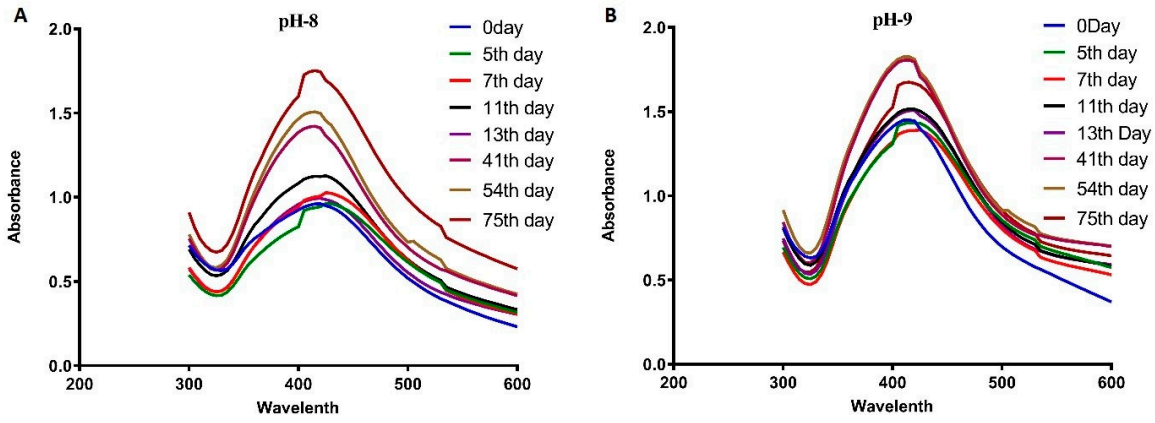

Figure 5. UV-Vis spectra obtained at different days in order to check the stability while the other condition remained constant like volume of LPE (5 ml), concentration of $AgNO_3$ (1 mM) at (A) pH 8 and (B) pH 9.

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1.7. FTIR analysis and XRD analysis

Fourier transform infrared spectroscopy (FTIR) analysis is used to determine the possible functional groups present on the surface of AgNPs that might be involved in capping and stabilizing the AgNPs. LPE and AgNPs showed the total 9 peaks with characteristic absorption at 3299, 1002, 1408, 1101,1037, 821, 807, 777, 987 cm-1 and 2917, 2851, 2342, 2355, 2112, 2124, 2120, 1933, 988 cm^{-1} respectively (Figure 6).

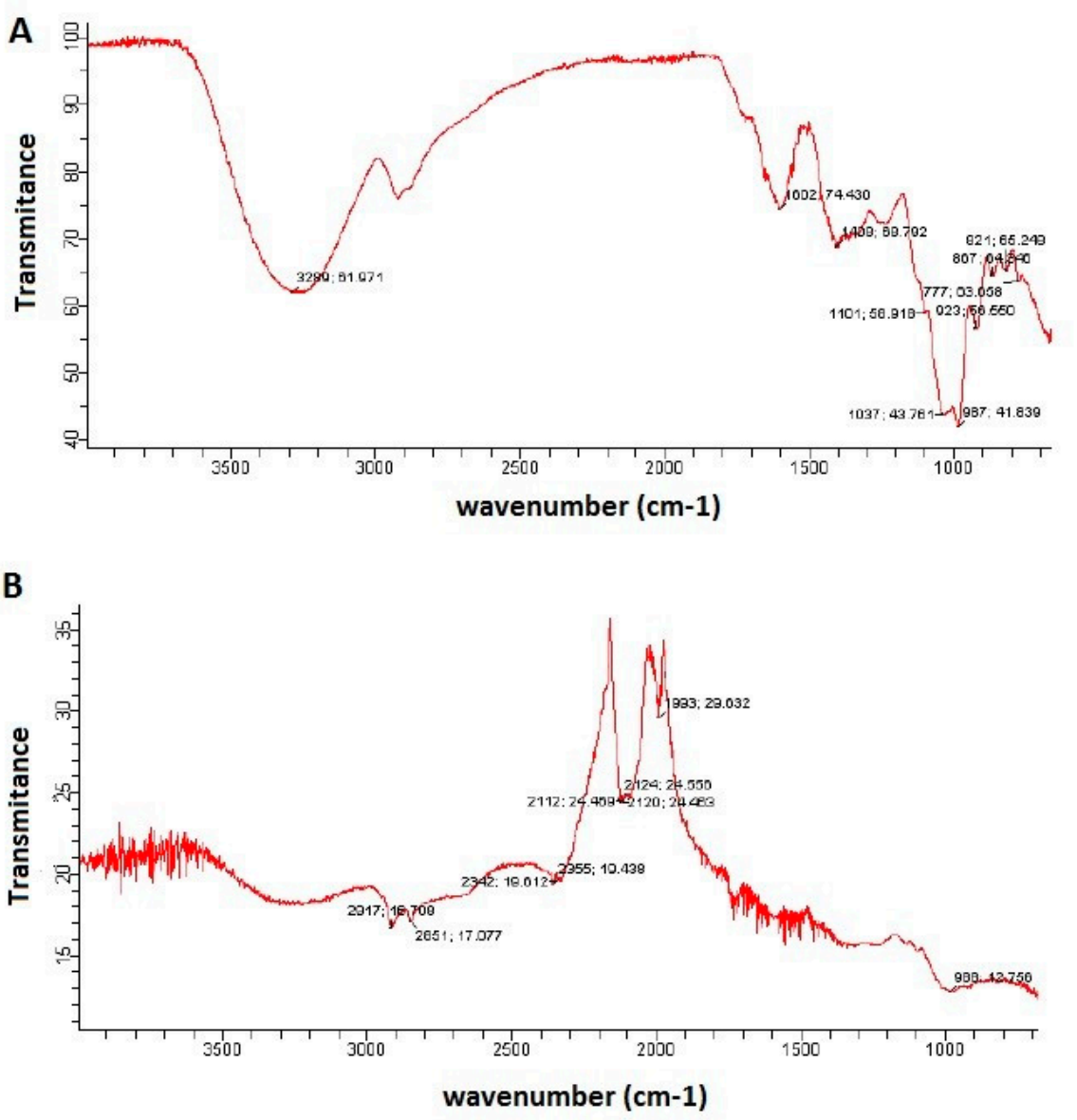

Figure 6. FTIR transmittance spectra. (A) spectra of LPE, and (B) spectra LPE mediated AgNPs.

The peaks were related to specific functional group including alcohol, alkane, alkyne, aromatic, amine ether, ester, alkyl halide and alkene (Table 1). The XRD data (Figure 7) was then used to calculate the value of d (light diffraction from particles) with help of the bragg's equation (Table 2) and particles size of LPE mediated AgNPs by using Scherrer's equation. The calculated particles size of LPE mediated AgNPs was approximately equal to 2 and 5 nm (Table 3).

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Table 1. Peaks obtained from FTIR analysis and their corresponding functional groups. (S=Stretching, B=bending, Br= broad, *M=medium, W=weak, St=strong)*

Wavenumber (cm^{-1})	Functional groups	Vibrations	Intensity
3299	O—H (alcohol), N—H	S	Br
2917, 2851	C—H (SP^3, alkane)	S	M
2112, 2124, 2120	—C≡C—	S	W
1408	C=C (Aromatic)	B	St
1101	C—N (Amine)	S	M
1037, 1002	C—O (May be ether, ester) C—F (Alkyl halide)	S	W
821, 807, 777, 987, 88	=C—H (Alkene)	B	W

Table 2. Peaks obtained from XRD analysis and further description about shape using bragg's equation ($2d\sin\theta=n\lambda$).

Peak number	Two Theta (2θ)	D	I	I/Ix 100 (%)
1.	8.331	10.605	9	16.071
2.	12.496	7.078	1.6	28.57
3.	18.328	4.836	5.6	100
4.	24.437	3.639	0.8	14.28
5.	27.492	3.247	0.9	16.07
6.	44.709	2.025	1.3	23.21
7.	57.762	1.595	1.7	30.35

Table 3. Size of AgNPs calculated using Scherrer's equation.

Sr. #	B	Θ	βCosθ	$D=\frac{0.9\lambda}{\beta Cos\theta}$ (nm)
1.	0.305	4.165	0.305	5
2.	0.277	6.248	0.275	5
3.	0.833	9.164	0.822	2
4.	0.249	12.289	0.244	5
5.	0.277	13.746	0.269	5
6.	0.805	22.355	0.745	2
7.	0.775	28.881	0.679	2

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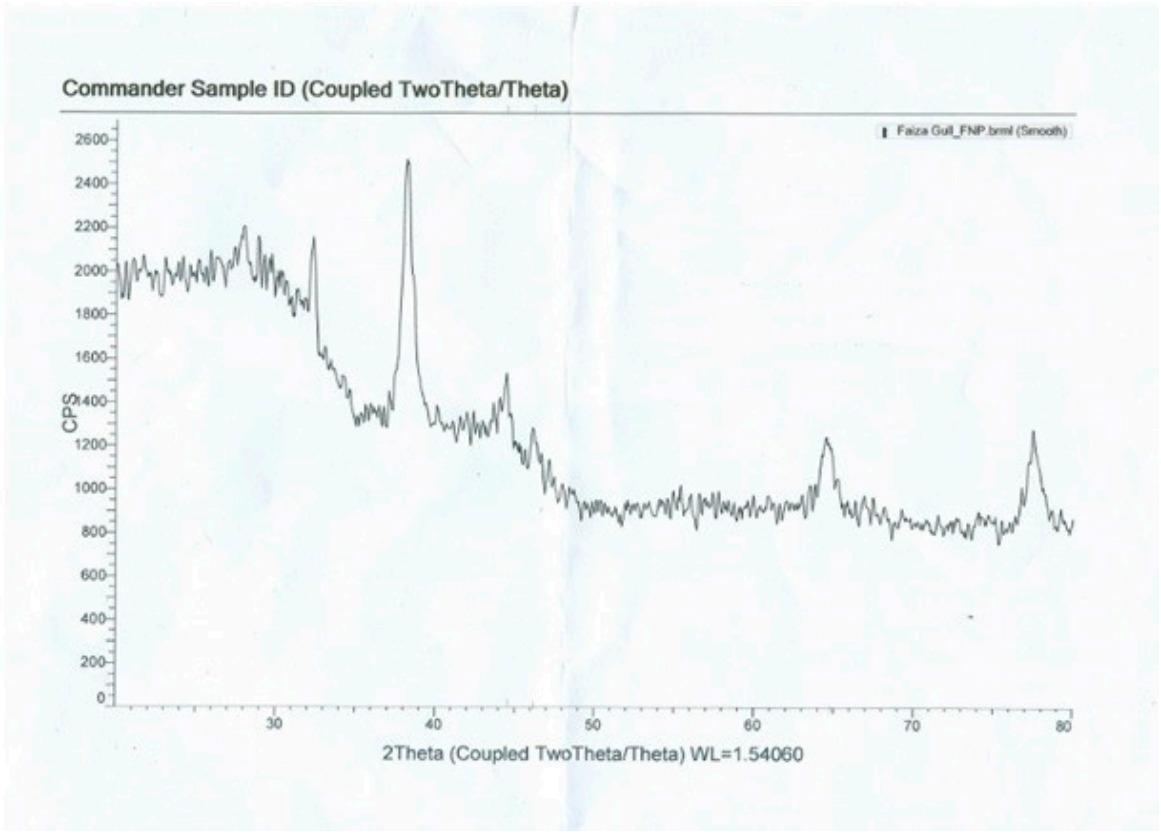

Figure 7. XRD pattern of LPE mediated AgNPs.

1.8. Antimicrobial activity

Six species of microbes (*Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii, Proteus mirabilis, Streptococcus mutans* and *Candida albicans*) was tested against LPE mediated AgNPs. The zone of inhibition for LPE mediated AgNPs was 20, 19, 18, 17, 16 ,15 mm for *Pseudomonas aeruginosa, Streptococcus mutans, Escherichia coli, Proteus mirabilis, Acinetobacter baumannii* and *Candida albicans* respectively. The antimicrobial activity of was not shown by LPE even at 50 mg/ml concentration (Table 4).

Table 4. Antimicrobial activity of LPE mediated AgNPs by well diffusion assay.

Indicator microbes	Zone of inhibition (mm)		
	LPE (50 mg/ml)	LPE Meadiated AgNPs (200 µg/ml)	Commercial AgNPs (200 µg/ml)
Escherichia coli	-	18 ± 0.09	16.25± 0.32
Pseudomonas aeruginosa	-	20 ± 0.05	10.8 ± 0.04
Acinetobacter baumannii	-	16 ± 0.09	10.1 ± 0.72
Proteus mirabilis	-	17± 0.14	11.2 ± 0.72
Streptococcus mutans	-	19 ± 0.41	11 ± 0.071
Candida albicans	-	15 ± 0.07	9.1 ± 0.32

LPE stands for Lemon peel extract. Experiments repeated three times and data was shown as Mean ± SD.

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4. Discussion

Nanotechnology has gained a special attention as it can be beneficial to every field relating to the human health or the environment. Silver nanoparticles (AgNPs) has potential applications in medicines as well as in various useful products such as fabrics, cosmetics, water treatment, imaging, and in targeting drug delivery [25]. In present research, a new method was employed for synthesizing the potential AgNPs by using the peel extracts of medicinal plant *Citrus limon*. The change in color from light yellow to lighter dark brown was the indication of formation of AgNPs as described in previous researches [26]. The change of color was observed just after the 5 minute and it was continuously changing up-to the half an hour, after that the color change was stopped indicating the completion of reaction and this finding supported by the results of Aparajita Vermaand and his coworker [11]. In all conditions of varying lemon peel extract (LPE) volume, pH or $AgNO_3$ concentration, AgNPs synthesized from LPE showed the characteristic surface Plasmon resonance (SPR) spectra in the range of 405-425 nm, which might be due to conduction electrons coherent excitation or oscillation localized on AgNPs [27]. The appearance of single peak can be explained by the fact of well-dispersion in the solution having either no aggregation or the aggregation to such an extent that NPs size goes out of the range of spectrophotometric absorption [28]. It is expected that varying the pH may have influence on the ionization capacity of functional groups in LPE as well as on the oscillation of the conduction electrons [29]. The SPR peak was observed at 415, 420 nm for the LPE adjusted at pH-7, 8, and 9 showed the maximum absorption at 0.839, 1.222, 1.952 respectively, indicating that AgNPs yield increases as the pH increases. At pH-10, SPR peak was speculated at 405 nm on three times dilution indicating the smaller size NPs with maximum yield, concluding that higher alkalinity produces smaller size NPs [30]. The appearances of peak in the range of 420-430 nm usually coincided with particle size of 2-100 nm as reported by Ahmad. R Shahvardi and his colleagues [31,32]. The prepared AgNPs shape might be spherical, as the peaks were in the range of 410-450 nm which clues the formation of spherical shaped NPs, according to literature [33].

A different concentration of the LPE was used to study the yield of the AgNPs. SPR peak fluctuate between 410-420 nm for the AgNPs synthesized at pH-8 and 9 having extract volume of 1 ml, 3 ml, and 5 ml. As the volume of LPE increased, the absorption also increased which is might be due to excessive production of AgNPs [34]. Using 7 ml extract, the absorption of AgNPs was low as compared to 5 ml of LPE volume which was possibly due to maximum saturation of reactants at 5 ml or it might be because of agglomeration of AgNPs [35]. Since spectrophotometer is sensitive to size therefore greater sized AgNPs excludes out of spectrophotometric absorption range.

The influence of different dilutions of $AgNO_3$ concentration on the synthesis of AgNPs and their plasmonic properties was studied which revealed that at pH 8 and 9 with LPE volume 5 ml and 7 ml, the characteristic absorption of AgNPs was increased with increasing the concentration of $AgNO_3$. Further increase in the concentration have no effect on AgNPs production as all the capping agent present in extract might be consumed by Ag^+. This hypothesis was confirmed by the several researches [32]. The LPE mediated AgNPs was stable particles as their absorbance increased upto 75 days at pH 8 while at pH 9 their absorption got constant at the 75 day which revealed that these AgNPs remains stable upto longer period of time.

The FTIR spectra of the finely dried LPE and AgNPs showed the band at 1101 and 3229 cm^{-1} might be due to the presence of –C–O functional groups of the polyphenols, polysaccharides, flavones and

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terpenoids. Peak at 3229 cm-1 was might be due to the occurrence of N-H group of amine or O-H group indicating the presence of alcohols and proteins. This band is diminished in the NPs indicating the change in functional groups or the occurrence of some kind of a reaction between the Ag^{+} with N-H or O-H group of protein or alcohols. This might be due the complex formation of Ag^{+} with aromatic ring as it can provide the pair of electron to the silver for bonding [36].

The D spacing and percentage intensity values can be compared with some standard values using referenced book in order to find out the shape of AgNPs. The size of the NPs was calculated using scherrer's equation written as:

$$D = \frac{K\lambda}{\beta Cos\theta}$$

Where D is particle size, β is FWHM (Full width at half maximum given in radians), and λ is wavelength (1.54060 A^{0}), K is Scherrer's constant (For spherical shaped NPs= 0.94) which depends on shape of NPs [30,33]. The average particle size calculated was approximately equal to 2 and 5 nm. It is noteworthy that the peak with maximum intensity (5.6 mm in intensity), or a peak having highest FWHM (3 mm equivalent to 0.833^{0}) denotes the smallest size (1.6 nm) NPs among all [12]. We found the size of LPE mediated AgNPs ranged between 2-5 nm.

Biosynthesized AgNPs showed the enhanced microbial activity as compared to the commercial AgNPs. The highest level of antimicrobial activity was recorded in *Pseudomonas aeruginosa* while the least bactericidal activity was noted in *Candida albicans* which was also higher from the standard AgNPs bactericidal activity. That might be due to some compounds or entities that have influence in enhancing the antimicrobial activity of the LPE mediated AgNPs.

5. Conclusions

Stable nanoparticles were synthesized by using LPE at different conditions such as extract volume, pH and varying concentration of $AgNO_3$. It was noticed that there was a direct relationship between the yield of AgNPs and other adjusting parameters like alkalinity, extract volume and $AgNO_3$ concentration. Higher pH (pH ≥ 10) leads to the aggregation of AgNPs while at pH ≤ 9, the particles showed mono-dispersivity in the solution. LPE have potent capping, reducing and stabilizing agents including proteins, phenols, terpenes, and alcohols which might be involved to synthesize stable AgNPs, approximately 5 nm in size. These particles showed potent activity against bacteria compared with LPE and purchased AgNPs synthesized by chemical method which predicted that LPE mediated AgNPs might have potential and can be used as medicines against the infections caused by these bacteria. Further research is required to uncover its effects on different diseases after unexposed their cytotoxic effects on vital organ and cells.

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Conflicts of Interest: The authors declare no conflict of interest.

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