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Green Synthesis of Silver Nanoparticles Using Fruit Peel Extract of Citrus Limon

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Abstract: ***Metal nanoparticles are being increasingly used in many sectors of economy, and there is a growing interest in ensuring biological and environmental safety of their production. Green synthesis of nanoparticles is considered an optimum approach due to it being inexpensive and eco-friendly. In the present study, after green synthesis of silver nanoparticles (AgNPs) from Citrus limonfruit peel extract, its antibacterial activity was studied against gram-negativeand gram-positive microorganisms. Antibacterial activity of lemon peel extract in methanol, ethanol and distilled water was studied too. The maximum activity was obtained against C. diptheriaeat 27 mm in ethanol extract; and for synergistic studies, the maximum activity was obtained against S. aureus in crude extract at 29 mm in methanol extract of lemon and kokum. Synthesized AgNPs were characterized by UV-visible spectroscopy, TEM and FTIR. UV-visible spectroscopy showed peaks in the range of 445 nm to 455 nm. TEM analysis showed the size of nanoparticles ranging from 20 nm to 50 nm. FTIR analysis showed presence of functional groups in the aqueous sample. Mainly C=C, C=O, C-O, N-H, C-H, C-I and C-Br groups were present. The AgNPs of Citrus limonfruit peel aqueous extract showed the maximum antibacterial activity against E. coli at 21 mm zone of inhibition at 45 minutes sunlight exposure followed by 24-hour room temperature incubation. This study encourages to use AgNPs as an effective antibacterial agent.***

Keywords: Green synthesis, Silver nanoparticles, *Citrus limon*, Antibacterial activity

1. Introduction

The ‘green’ environment friendly processes in biological and chemical technologies are becoming increasingly popular among researchers nowadays as they are much needed as a result of worldwide problems associated with environmental concerns. Green synthesis of nanoparticles provides advancement over other chemical methods as these are simple, one step, cost-effective, eco-friendly and often results in more stable materials without making use of any toxic or hazardous materials (1).

Silver is a nontoxic, safe inorganic antimicrobialagent that is capable of killing about 650 types ofdiseases causing microorganisms hence there is an increasing interest in silver nanoparticles on account of the antimicrobial properties that they display (2). And silver is one of the most commercialized nano-material with five hundred tons of silver nanoparticles production per year (3). Their unique characteristics have made them applicable in bio-molecular detection, catalysis, biosensors and medicine. And AgNPs are also known to have strong inhibitory and bactericidal effects along with the anti-fungal, anti-inflammatory and anti-angiogenesis activities (4).

Many studies in the past have reported about the use of plant extracts to synthesize AgNPs with significant antimicrobial activities (5),(6),(7). Research on fruit extract of Amla (*Emblica officinalis*), leaf extract of Neem (*Azadirachtaindica*), fruit extract of Kokam (*Garcinia indica*), and leaf extracts of Drumstick (*Moringa oleifera*) have provided more methods for nanoparticle synthesis (8),(9),(10),(11). Significant antibacterial activities with leaf, stem and seed extracts also can be applied in other fields (12),(13),(14).

Various techniques have been tried till date for the synthesis of silver nanoparticles like laser ablation, gamma irradiation, electron irradiation, chemical reduction, photo-reduction, photochemical methods, microwave processing, ion sputtering, chemical reduction, sol gel, and biological synthetic methods (4),(15). The chemical approaches for silver nanoparticle synthesis require use of toxic and harsh chemicals. This led to the need to come up with an approach which will be eco-friendly, financially viable and one which will not require use of hazardous chemicals and will not produce chemical waste.

Capping agents are widely used in nanoparticle synthesis; as they help in avoiding overgrowth and aggregation, and also to control the structural characteristics of the nanoparticle for uniform shape and size. Examples of capping agent are polyethylene glycol (PEG), ethylenediaminetetraacetic acid (EDTA), polyvinyl pyrrolidone (PVP) and polyvinyl alcohol (PVA). Plants or plant extracts contain secondary metabolites which act as natural capping agents for nanoparticle synthesis and are more advantageous over other processes as they eliminate the detailed process of culturing and maintaining of the cell, and thus can also be scaled up for large-scale nanoparticle synthesis easily (16),(17).

In the present study, AgNPs were synthesized using fruit peel extracts of Citrus limon which is a species of small evergreen tree in the flowering plant family Rutaceae, native to Asia. Lemons are a rich source of vitamin C and contain numerous phytochemicals, including polyphenols, terpenes, and tannins. Apart from its culinary use, it is cultivated mainly for its alkaloids, which are found to have anticancer activities and antimicrobial potential in crude extracts of different parts of the plant like leaves, stem, root and flower, which show considerable activity against clinically significant bacterial strains. The peel of Citrus limon is a rich source of flavonoid glycosides, coumarins, β sitosterol, glycosides and volatile oils (18). Studies have shown that the peel of lemon is not only an astringent but also is a good

antimicrobial agent (19). The citrus peels are rich in nutrients and phytochemicals because of which they can be used as drugs or even as food supplements (20).

2. Materials and Methods

A) Extract preparation

For preparation of aqueous extract 10 gm of lemon peel powder was added to 100 ml of distilled water and boiled for 10 minutes. The mixture was filtered with Whatman filter paper No. 1 and boiled further on boiling water bath for two hours to evaporate excess water. Methanol and ethanol extracts were prepared by adding 5 gm lemon peel powder to 50 ml of Methanol (96%) and Ethanol (96%) respectively. The mixtures were kept on shaker for 24 hours. The mixture was then filtered with muslin cloth and boiled on boiling water bath for two hours to evaporate excess alcohol. Crude extract was prepared by adding 1 gm lemon peel extract powder in 5 ml distilled water. All the extracts, once prepared, were stored at 4°Celsius until further use.

B) Antimicrobial activity of plant extract

Antimicrobial activity was checked by using well diffusion method. The wells were bored by using a 6 mm cork borer. 20 µl sample of the lemon peel extract was added in each well and the plates were incubated at 37°Celsius and were observed after 24 hours against selected microorganisms. The antimicrobial activity of crude, methanol, ethanol and aqueous extract of lemon peel was observed against 5 gram-negative (*Escherichia coli*, *Klebsiella pneumonia, Salmonella typhi, Pseudomonas aeruginosa and Shigella*) and 5 gram-positive (*Staphylococcus aureus*, *Bacillus subtilis, Streptococcus pyogenes, Candida B, and Cornybacteriadiptheriae*) microorganisms. After 24 hours, zone of inhibition was measured.

C) Phytochemical analysis

Phytochemicals are the biologically active compounds present in the plants. Studies have shown the presence of saponin, tannin, flavonoids, and steroid in lemon seed and in lemon peel (33). The antimicrobial activity shown by the plant is usually due to the presence of phytochemicals (21).A fraction of the extract was subjected to phytochemical analysis as described in literature. Tests were performed to detect the presence of saponins (21), flavonoids (21)(23), tannins (22)(25), phenols (22)(25), steroids (23), proteins (22), reducing sugar (23), terpenoid (23), fixed oil (25).

D) Antioxidant analysis

DPPH assay was carried out for studying the antioxidant activity of the lemon peel aqueous extract only as the other extracts were far too viscous. 5.91 mg/50 ml of DPPH was prepared and used as control. The absorbance of the control and the samples was recorded at 517 nm.

E) Synthesis of Silver nanoparticles

1.698 grams of AgNO3 powder was dissolved in 1000 ml of distilled water to make 10^{-2} M concentration of AgNO3. 1.5 ml of extract was added to 20 ml of 10^{-2} M concentration of AgNO3. The tubes were exposed to direct sunlight for 15, 30, 45 and 60 minutes. Colour changed from light yellow to brown. Then the tubes were kept for incubation at Room Temperature for 24 hours. After 24 hour room incubation, the O.D. was checked at spectrum of 200 nm to 900 nm for confirming the silver nanoparticle synthesis using UV-Visible Spectrophotometer (Shimadzu UV-1601).

F) Antimicrobial activity of synthesized Silver nanoparticles

The protocol was same as that followed for the antimicrobial activity of the lemon peel extracts. 20 µl sample of the synthesized silver nanoparticles was added in each well and the plates were incubated at 37°Celsius and were observed after 24 hours. The antimicrobial activity of synthesized silver nanoparticles was observed against 5 gram-negative (*Escherichia coli*, Klebsiella pneumonia, Salmonella typhi, *Pseudomonas aeruginosa and Shigella*) and 5 gram-positive (*Staphylococcus aureus*, *Bacillus subtilis, Streptococcus pyogenes, Candida B, and Cornybacteriadiptheriae*) microorganisms. $AgNO_3$ and double distilled water (as control) were added in separate wells too and incubated at 37°Celsius for 24 hours and the antimicrobial activity was measured based on the inhibition zone around the well impregnated with plant extract and synthesized AgNPs.

G) Characterization of Silver nanoparticles

After synthesis of nanoparticles, the pellet was obtained of the nanoparticles for characterization. The steps involved the extracts to be centrifuged thrice at 10,000 rpm and 4°Celsius : first time for 1 hour, second time for 30 minutes and the third time for 15 minutes. Washings were given with distilled water in between. After the third wash, the colourless supernatant was discarded and the final pellet was re-suspended in 5 ml D/W and was refrigerated at 4 degree Celsius until further use for characterization purposes. UV spectra of synthesized nanoparticles were monitored as a function of time of reaction on a spectrophotometer (Shimadzu UV-1601) in 200–900 nm range operated at a resolution of 2 nm. 200 µl of sample was aliquoted and 800 µl of distilled water was added to it and the readings were taken. The FTIR (Fourier transform infra-red spectroscopy) was recorded in the range of 400–4000 cm^{-1} with sample in powdered form. The model of the FTIR instrument used for characterization was 3000 Hyperion Microscope with Vertex 80 FTIR System. FTIR characterization gave information about the functional groups which were binding to the nanoparticles. TEM (Transmission electron microscopy) analysis was done to visualize the shape as well as to measure the diameter of the bio-synthesized silver nanoparticles, at accelerated voltage of 120 kV. The machine model was CM 200. The sample was dispersed in distilled water. A drop of thin dispersion was placed on a "staining mat". Carbon coated copper grid was inserted into the drop with the coated side upwards. After about 45 minutes, the grid was removed and air dried. Then it was screened in Transmission Electron Microscope which showed size and shape of nanoparticles.

3. Results and Discussion

A) Antimicrobial activity of plant extract

Antimicrobial activity of the lemon peel extracts was obtained against 5 gram positive and 5 gram negative microorganisms. Maximum activity for methanol extract

was found in *Candida B and C. diptheriae*as 25 mm. Maximum activity for ethanol, crude and aqueous extract was found against *C. diptheriae*as 27 mm, 26 mm and 24 mm respectively. Zones obtained of *E. coli* results were in accordance with Chandrasekar S. *et al*. (29). The zones obtained against *Bacillus subtilis* and *E. coli* were in congruence to those obtained by Praphulla Rao *et al* (30). Aysha O.S. *et al* reported consonant work against *E. coli, K. pneumoniae* and *P. aeruginosa* (31). Synergistic antimicrobial activity was observed of lemon peel extracts with amla extracts, kokum peel extracts and orange peel extracts against 5 microorganisms. Protocol was same as that of antimicrobial activity of lemon peel extracts. Mamatha Pingili*et al* had carried out the synergistic antimicrobial activity of lemon and amla along with different combinations (32). Maximum synergistic antimicrobial activity of lemon and amla was obtained against *C. diptheriae*and *Candida B* at 25 mm and of lemon and kokum was obtained against *S. aureus* in methanol extract as 29 mm and that of lemon and orange against *C. diptheriae*in aqueous extract at 16 mm.

B) Phytochemical analysis

Phytochemical analysis has been carried out in the past of *Citrus limon.* Analysis has been performed to test the presence of steroid and reducing sugar (34), fixed oils (35), proteins and terpenoids (21), phenols (22), saponins and flavonoids (23) and tannins (32).

C) Antioxidant analysis

Antioxidants present in lemon peel extract in this study are studied by calculating the scavenging activity. Kelly Oriakhi*et al* studied antioxidant activities in different citrus juice concentration which resonate with the current results (34). Antioxidant activity of 13 Citrus species peels and tissues were studied by Kamran Ghasemi*et al* and the results obtained were similar to current study (24). Antioxidant studies of lemon fruit peels were carried out by Nessma Ahmed El Zawawy and the results are in sync with current work (25). In another study by I. Hinkov*et al*, the antioxidant analysis obtained was similar as the current study. (26)

Table 1: Antimicrobial activity of all extracts against ten microorganisms

Sr No.	Microorg.	Methanol	Methanol Extract	Ethanol	Ethanol Extract	Crude	Aqueous
1.	E. coli	-	14	-	18	7	15
2.	S. aureus	-	22	-	21	17	19
3.	K. pneumoniaee	-	12	-	11	-	-
4.	B. subtilis	-	19	-	19	11	10
5.	P. aeruginosa	-	19	-	18	17	18
6.	S. typhi	-	18	-	17	12	14
7.	S. pyogenes	-	11	-	10	-	-
8.	C. diptheriae	-	25	-	27	26	23
9.	Candida B	-	25	-	23	24	24
10.	Shigella	-	16	-	21	12	15

Table 2: Synergistic antimicrobial activity

Sr No.	Microorg.	Synergistic extracts of	Methanol Extract	Ethanol Extract	Crude	Aqueous
1.	*P. aeruginosa*	Lemon and Amla	12	12	8	11
		Lemon and Kokum	15	14	9	14
		Lemon and Orange	9	9	-	12
2.	*C. diptheriae*	Lemon and Amla	25	22	**25**	20
		Lemon and Kokum	18	19	15	14
		Lemon and Orange	12	12	11	16
3.	*S. typhi*	Lemon and Amla	16	12	9	9
		Lemon and Kokum	13	13	12	13
		Lemon and Orange	11	11	-	13
4.	*Candida B*	Lemon and Amla	25	22	20	17
		Lemon and Kokum	12	18	15	11
		Lemon and Orange	13	13	-	13
5.	*S. aureus*	Lemon and Amla	23	**24**	24	16
		Lemon and Kokum	**29**	23	12	**27**
		Lemon and Orange	13	11	-	-

Figure 1: Test tubes before and after sunlight exposure.

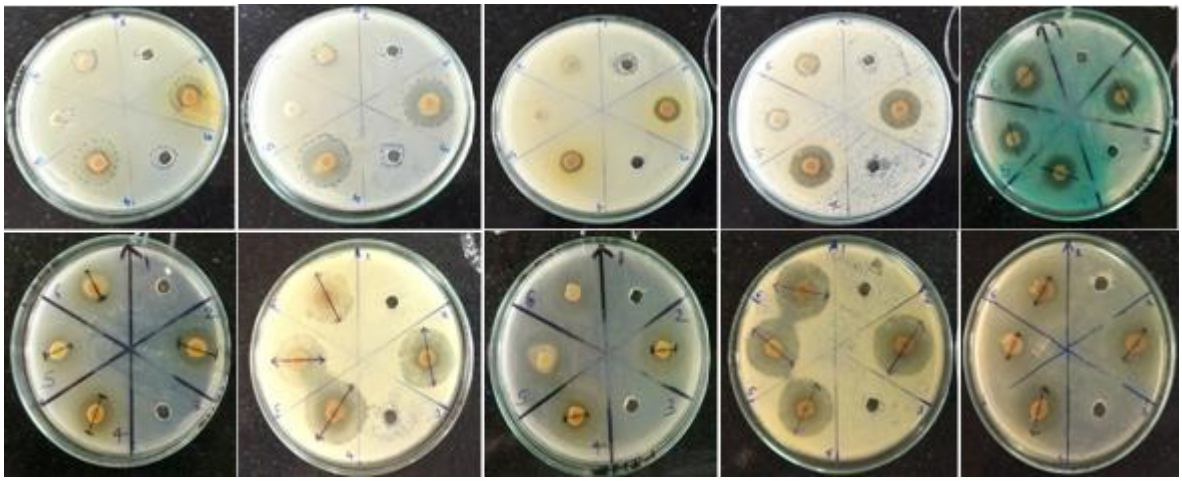

Figure 2: Upper row - Activity against *E. coli, S. aureus, K. pneumoniae, B. subtilis, P. aeruginosa;* Lower row - Activity against *S. typhi, S. pyogenes, C. diptheriae, Shigella* and *Candida B.*

D) Synthesis of Silver nanoparticles

For synthesis of silver nanoparticles, the tubes were exposed to direct sunlight containing the solution of silver nitrate solution along with methanol, ethanol, crude and aqueous extracts of lemon peel respectively. The obserbedcolour change observed denoted synthesis of silver nanoparticles and is in accordance with previous work performed by S. Najimu Nisha *et al* and Chandrasekar S. *et al*. as in the references (27), (29)

Table 3: Tests for phytochemical analysis

S.No.	*Test*	*Methanol*	*Ethanol*	*Crude*	*Aqueous*
1.	**SAPONINS**	+	+	+	+
2.	**FLAVONOID**				
	A) NaOH Test	-	-	-	+
	B) FeCl3 Test	+	+	+	+
3.	**TANNINS**				
	A) FeCl3 Test	+	+	+	+
	B) Br Water Test	+	+	+	+
4.	**PHENOL**				
	A) FC reagent Test	-	-	-	-
	B) FeCl3 Test	-	-	-	-
5.	**Steroid**	-	-	-	-
6.	**Protein**	+	+	+	+
7.	**Reducing Sugar**	+	+	+	+
8.	**Terpenoid**	+	+	+	+
9.	**Fixed Oil**	+	+	+++	++

Table 4: Readings of DPPH absorbance

Sample	*Absorbance*	*Colour Observed*
Lemon	0.044	Yellow
Control	2.834	Purple
Methanol (Blank)	0.000	Colourless

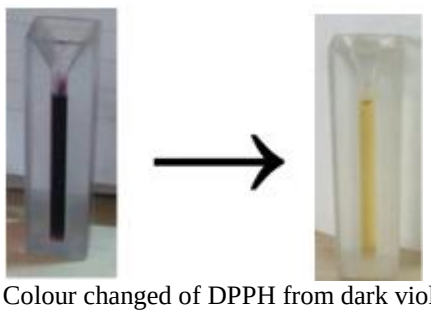

Figure 3: Colour changed of DPPH from dark violet-brown to light yellow. Scavenging activity of the lemon peel

aqueous extract was obtained to be 98.4 %Test tubes before and after sunlight exposure.

E) Antimicrobial activity of synthesized Silver nanoparticles

The aqueous extract was used to synthesize silver nanoparticles. The green synthesis of silver nanoparticles from *Citrus limon*fruit peel extracts showed the maximum antibacterial activity against *P. aeruginosa* 19 mm), *C. diptheriae* (15 mm), *E. coli* (21 mm) and *Candida B* (14 mm)after 15, 30, 45 and 60 minutes of exposure to direct sunlight respectively.Chandrasekar S. *et al*. reported the similar results for *E. coli* and *S. aureus* (29). The zones of inhibition for *E. coli* and *Bacillus subtilis* obtained by Praphulla Rao *et al* support the present results (30).Aysha O.S. *et al* reported results for antimicrobial activity of silver nanoparticles against *K. Pnemoniae and P. aeruginosa* which were similar to the results obtained below (31).

Table 5: Antimicrobial activity of nanoparticles

SR NO.	MICROORG.	15 Minutes	30 Minutes	45 Minutes	60 Minutes
1.	*E. coli*	19	11	21	14
2.	*S. aureus*	11	-	12	-
3.	*K. pneumoniaee*	15	11	18	9
4.	*B. subtilis*	11	-	10	10
5.	*P. aeruginosa*	19	-	18	-
6.	*S. typhi*	12	10	17	11
7.	*S. pyogenes*	16	13	16	14
8.	*C. diptheriae*	15	16	14	12
9.	*Candida B*	15	15	16	14
10.	*Shigella*	12	10	15	9

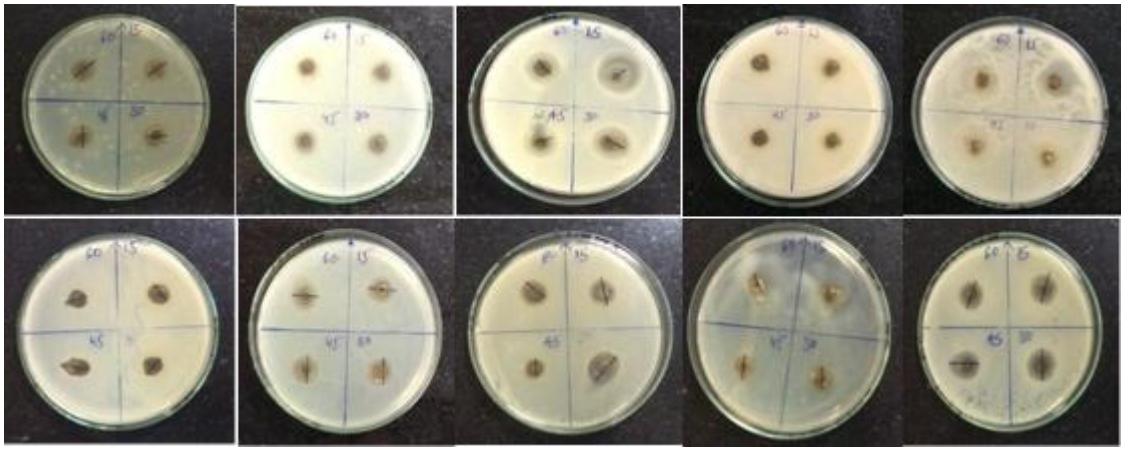

Figure 4: Upper row - Activity against*E. coli, S. aureus, K. pneumoniae, B. subtilis, P. aeruginosa;* Lower row - Activity against *S. typhi, S. pyogenes, C. diptheriae, Shigella* and *Candida B.*

F) Characterization of Silver nanoparticles

The peaks obtained of the silver nanoparticles in the U.V. Spectrophotometer were similar to those obtained by Praphulla Rao *et al* (30). Characterization of nanoparticles was done initially by U.V. Spectrophotometer. The previously studies have obtained the C–O group, C–H bond and N-H bond and the current work is in accordance with the literature cited (28). Also, some work has shown the presence of C-O, C=C and C-H groups (35). Thus, the current work is in harmony with the literature cited. The size of the nanoparticles ranged from 20 nm to 50 nm. The range of nanoparticles obtained in current study falls in the same range reported previously by researchers synthesizing silver nanoparticles from lemon peel extracts which substantiates the current work (31)(35).

Table 6: Peaks obtained by U.V. visible spectrophotometer. The pellet was now obtained of aqueous extract was used for further characterization of FTIR and TEM

SR. NO	*Extract*	*Wavelength (nm)*
1.	Methanol	452
2.	Ethanol	452
3.	Crude	452
4.	Aqueous	452

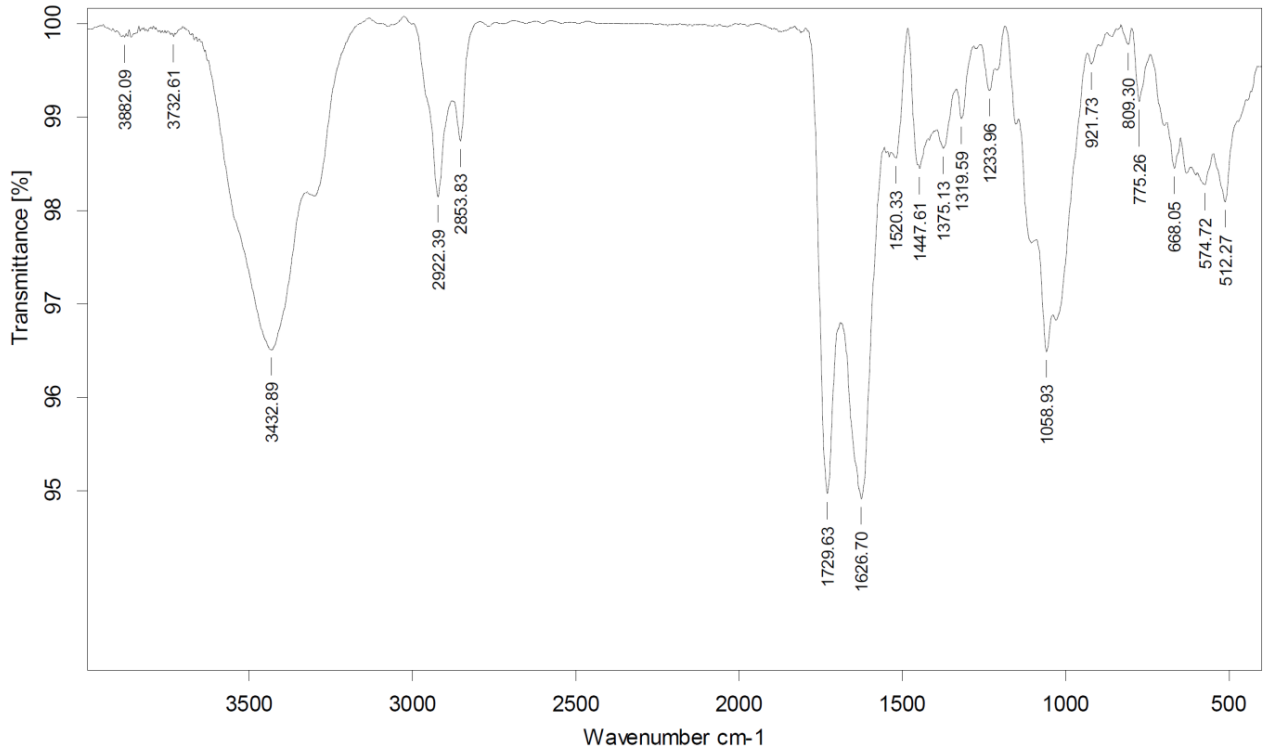

Figure 5: FTIR analysis showing peaks of functional compounds present in sample

Table 7: Description of the peaks obtained in FTIR analysis

Sr No.	Wavenumber (nm)	Functional Group	Description
1.	1626.70	C=C	Medium Bond, Alkene, Aromatic Ring
2.	1729.63	C=O	Strong Bond
3.	1058.93	C-O	Strong Bond, Primary Alcohol
4.	3432.89	N-H	Medium Bond, Primary Amine
5.	2922.39	C-H	Medium Bond, Alkane
6.	512.27	C-I	Strong Bond
7.	574.72	C-BR	Strong Bond

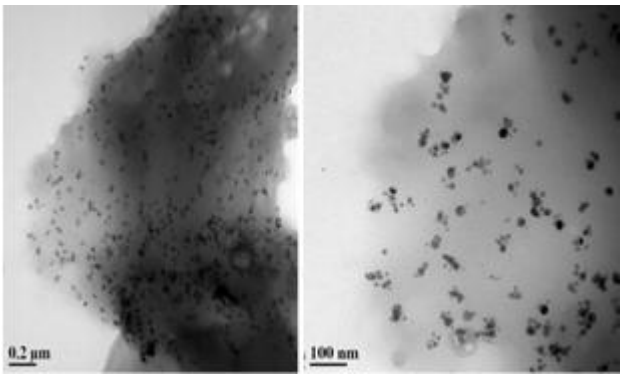

Figure 6: Results of nanoparticles observed at 0.2 µm and 100 nm showing round nanoparticles.

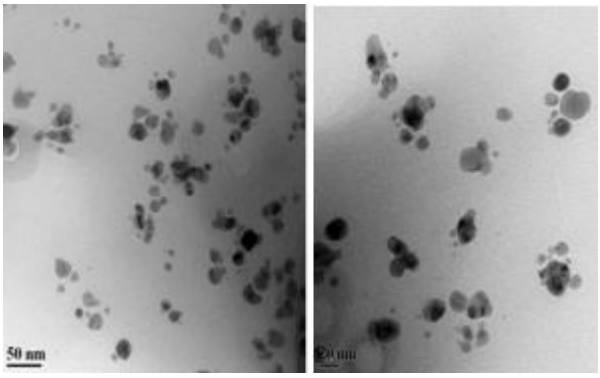

Figure 7: Results of nanoparticles observed at 50 nm and 20 nm showing round nanoparticles.

4. Conclusion

Lemon has many pharmacological activities and hence was utilized for green synthesis of AgNPs. The silver nanoparticles were produced by green synthesis from lemon peel extracts by exposing them to direct sunlight for different lengths of time. The UV spectral peaks for silver nanoparticles ranged from 445 nm to 550 nm. Ten microorganisms were used to test the antibacterial activity by agar well diffusion method and it was found that silver nanoparticles were effective for reducing the growth of bacteria.The synthesized AgNPs were characterized and the size of the nanoparticles ranged between 20 nm and 50 nm. FTIR analysis was performed and presence of C=C, C=O, C-O, N-H, C-H, C-I and C-Br groups was detected. From the present study we conclude that green synthesis of silver nanoparticles from *Citrus limon*fruit peel extracts possess very good antibacterial activity against selected microorganisms. Moreover, they also showed synergistic effect on the antimicrobial activity against gram-positive and gram-negative microorganisms. Green synthesis of silver nanoparticles can potentially eliminate the problem of using chemical agents that may have adverse effects, thus making the nanoparticles more compatible with the eco-friendly approach. Hence, the obtained results are promising and prove to be an important step in this direction, making it a cost-effective and ecofriendly alternative to the conventional approaches.

5. Conflicts of interest

We declare no conflict of interest involved in this study.

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