Research Article

Green Synthesis of Silver Nanoparticles Using the *Citrus medica* Leaf Extract and Evaluation of their Antioxidant Activity

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Received: 04-02-2021; **Revised:** 22-03-2021; **Accepted:** 28-03-2021; **Published on:** 20-04-2021.

ABSTRACT

The present study Green synthesis of silver nano particles and their biological assessment. The bimolecular compound present in plants to form the silver nanoparticles (AgNPs) from silver nitrate to reduce the silver ions. The biogreen method are advantageous overcome the chemical methods and physical methods. Plant based silver nanoparticles are very useful to the environment. The presence of phytochemical components such as polyphenols, flavonoids, alkaloids, terpenoids, carbohydrate, tannins, saponins, quinones, glycosides, steroids in the aqueous extracts of citrus medica. The Plasmon absorbance of silver nanoparticles at the peak 430nmin the *citrus medica* silver nanoparticles solution *(AgNPs).* FTIR, the presence of Phyto constituents in AgNPs. It was used to find the functional groups. Silver nano particles showed the enhanced antioxidant activities like 56.15±0.41 % at 250µg/ml and highest total phenolic content in 318.67±0.28 mg of Gallic acid/g. *citrus medica* also exhibited the effective antifungal activity, particularly against by *candid albicans.*

Keywords: *Citrus medica*, silver nano particles, *candid albicans,* antioxidant activity FTIR and UV-visible spectroscopy.

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DOI: 10.47583/ijpsrr.2021.v67i02.005

DOI link: http://dx.doi.org/10.47583/ijpsrr.2021.v67i02.005

INTRODUCTION

In numerable methodologies have formulated in the previous years to synthesize silver nanoparticles of specific shape and size depending on their demand. The toxic chemicals and solvents used to synthesis the silver nanoparticles, limits their application in the clinical fields. So, the bio preparation of nanoparticles has a budding of the Biotechnology and Nanotechnology Biotechnology field. It has received the increased attention due to a growing need to develop environmentally better technologies in silver nanoparticles synthesis[1.] The process for making silver nanoparticles used by the plant extracts, and is easily accessible and low cost. The plant extracts act as a reducing and stabilizing agent in the production of nanoparticles. It contains the various concentrations of reducing agents in the different plant extracts.[2] Though, plant-based synthesis of silver nanoparticles are considered as protect, profitable and non-polluting, but they also have some disadvantages in using the plant resources [3.]

Silver is innoxious, bactericidal agent to kill the disease-causing microorganism [4.] Silver nanoparticles are very important materials that have been studied broadly, such as unique electrical, optical and also biological properties. It is applied in various fields like catalysis, biosensing, imaging, drug delivery, nano device fabrication and in medicine[5.]Plant-based nanoparticle synthesis is favored because it is environmentally friendly, worthwhile, and this method for biosynthesis process and human therapeutic use [6.]

Nanotechnology is a blossoming in a science and technology [7.] Now days, green synthesis procedures used for numerous biological systems.[8,9,10] The large fragrant citrus fruit is the citron, with a thick skin, botanically classified as *Citrus medica*. It has the Tanaka and Swingle botanical name systems. It has the four original citrus fruits one of them is citrus, pomelo, papeda and mandarin), These three citrus types like Pomelo, Papeda and Mandarin types are developed through natural hybrid speciation or artificial hybridization. Polygonum and indigofera are combined with a citrus leaf juice is taken after childbirth. Candied peel is widely used in the food industry, especially as an element in fruit cake, plum pudding, sweet rolls and candy [11.]

The solution color was changed and it represents the silver nanoparticles was synthesized then characterized by UV-Visible spectroscopy and Fourier transform infrared (FTIR) spectral measurements used to identify the potential bio molecules.

MATERIALS AND METHODS

Plant sample

The Fresh leaves of citron (*citrus medica)* were collected in the month of October 2016 in Chennai. The leaves were

washed and dry at room temperature and macerated to obtain a fine powder.

Preparation of leaf extract

Powdered Leaves of *citrus medica* were weighed at 20 g and run in a Soxhlet apparatus with 350 ml of distilled water. Prepared extract was allowed to cool and filtered through Whattman paper No. 1 into a clean dry beaker and dried. Finally, this extract was collected and stored in airtight container for future use.

Phytochemical analysis

An aqueous extract of the *citrus medica* was identified by the presence of photochemical viz. polyphenols, alkaloids, terpenoids, flavonoids, carbohydrates, steroids and steroids by following standard procedures[12]

Silver nanoparticles

100ml of 5% leaf extract was added to the silver nitrate solution (1mM) and incubated at room temperature. Incubation in a dark room for three days and dark brown solutions was formed to indicate the formation of silver nanoparticles. Control also maintained without the plant extract.

Ultra Violet -Visible Spectra

To reduce the silver ion in aqueous extract solution. It was observed by UV- Vis spectroscopy and absorption spectra of the extract contain a metal ion concentration was measured by Perkin- Elmer Lamda- 45 spectrophotometer in 300-1000 nm range.

FTIR-Fourier Transform Infrared Spectroscopy

The Sample was measured by a spectral range of4000-400cm-^{1}with resolution of 4 cm^{-1}. Powder diffraction measurements were followed by FTIR. To study the functional groups of the formation of silver nanoparticles by FTIR spectra.[13]

Antioxidant Capacity

Antioxidant property and total phenolic content in the aqueous extract and *Citrus medica* Silver nanoparticles solution were determined by using the DPPH assay.

DPPH

The free radical activity of the extract against 2, 2-dipheny-1-picryl hydrazyl(DPPH). 0.1mM DPPH in ethanol was prepared and to this prepared solution of the extract in different concentrations from 50-250µg /µl, 1ml of DPPH and 0.95 ml of TrisHCl were added. This solution was incubated at room temperature and was measured at 517 nm. The free radical was measured from the aqueous extract and *citrus medica* silver nanoparticles solution.

Total phenolic content

Folin - Ciocalteu method was used to determine the total phenolic content.0.1 ml of each sample extract was diluted to 3 ml with distilled water and to add Folin-Ciocalteu reagent 0.5 ml. 3min after, 20% of 2ml sodium carbonate was added to this mixture. The color was formed and its absorbance was measured at 650nm. Different concentrations of Gallic acid solution were used for the standard calibration curve. The result was expressed as Gallic acid equivalent/100mg of plant sample[14].

Oral candidiasis

Yeast *Candida* to cause the infection is an oral candidiasis. The organism has the ability to transform into a pathogenic hyphael form. It is an infection caused by a species of the yeast *Candida*.

Isolation of C*andida sp*., from throat swab

Candida albicans grow well on sabouraud dextrose agar. 24 hours after incubation at 25-37°c, Cream colored colonies were appeared and colonies have a distinguishable yeast smell (Figure no.1)

Pure culture isolation

C*andida albicans* was streaked into the SDA plate as a pure culture.

Antifungal activity

Broth microdilution

NCCLS protocol was used for the antifungal susceptibility testing on *candida albicans.* Seven-day old *candida albicans* cultures which were grown on potato dextrose agar at 28°C these suspensions were used in future. 10 ml of distilled water was added to the fungal colonies, and the suspensions were made by scraping the surface with the tip of a sterile loop. This loop containing hyphal fragments and fungal conidia was removed and transferred to sterile tube. The last suspension with 0.5 Mac farland concentration of conidia and hyphal fragment was then prepared. The above suspension of 100µ1 was inoculated to a microtiter plate containing culture medium with plant extracts and nanoparticles solution. The plates were incubated for two days at 28°C. It has been investigated every half an hour. This assay was carried out in twice. The MIC was read by the lowest concentrations that stop any visible growth.

RESULTS AND DISCUSSION

Phytochemical analysis

Phytochemical screening of *citrus medica* contain the presence of constituents like polyphenols, flavonoids, alkaloids, terpenoids, carbohydrate, tannins, saponins, quinones, glycosides, steroids and absence of constituents like glycosides and cardiac glycosides show table 1.

Table 1: Phytochemical screening of C*itrus medica* (Aqueous extract)

Phytoconstituents	Presence/Absence
Carbohydrate	+
Tannin	+
Saponin	+
Flavonoids	+
Alkaloids	+
Quinones	+
Glycosides	-
Cardiac glycosides	-
Terpenoids	+
Phenols	+
Coumarins	+
Steroids	Steroid
Phlobatannin	+
Anthraquinones	+

Silver nanoparticles

To reduce the silver ions into silver nanoparticles in the plant extract or natural resources can be observed by a change in color.

Before incubation

After incubation

Figure 1: Synthesis of silver nanoparticles.

UV-visible spectrophotometer (Characterization of silver nanoparticles)

Addition of plant extract to the silver nitrate solution, this solution turned to a brown color. UV-Vis spectroscopy for the structural characterization of silver nanoparticles. The UV–visible absorption spectra shown the reduction reactions are shown in Figure no. 2 for c*itrus medica*. The UV-Vis spectrum of *citrus medica* shows the maximum absorbance (lmax) of 0.369 at 430 nm which gradually reached 0.153 at 331nm, due to the formation of SNPs and after 600nm it was fully saturated and figure no. 2. It desired the reduction of silver nanoparticle in the *citrus medica extract*. Absorbance peak at 364nm for the formation of silver nanoparticles solution, the particles are polydispersed at the widening peak[15].

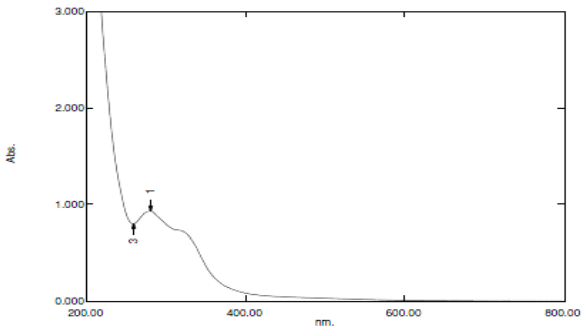

Figure 2: UV/visible spectra of *citrus medica*

FTIR

The functional groups were identified by the FT-IR in the present in the extract. FT-IR spectra after the treatment of AgNO3 are shown in Figure no. 3 for citrus *medica*.

The main peak suppression was seen in the finger print region (500– 1500 cm^{-1}) in the extract. It indicates the phenolic group's participation in the ion exchange reactions like 2924-2090, 1377–1645, 1315–1037 and 900–670 cm^{-1}. The peaks of 1645, 1460 and 1377 cm^{-1} are transferred to latest positions 1647, 1454 and 1092 cm^{-1}. In reduction reaction, these peaks were present that is some new little peak at 1645 (for aromatic C55C), 1460(for phenolic OH in-plane bend), and 1377 (for phenolic C–O stretch) absorption of free hydroxyl groups which are not participating in the reduction reaction while the peak band at 3200-3600.

Nanoparticles were formed in the presence plant extract, these were separated, washed with water, dried and analyzed by FTIR. The figure shows significant peaks at 1645 (C55C), 1377 (OH group), along with small peaks declined in 753, 656 and 602 of 800– 600 cm^{-1} region (C–H out of plane bend) which are of characteristic of aromatic phenols.

DPPH ASSAY

CmNPs on DPPH radical was found to be maximum of 56.15±0.41% at 250µg/ml Silver nanoparticles of citrus medica show remarkable scavenging activity when compared (table 2) with aqueous extract of *citrus medica*.

Total phenol content assay

Spectrophotometric quantitative estimation using Folin-Ciocalteu's method dose levels 100 to 500µg/ ml. CmAgNPs showed the 17.2± 0.08 to 318.67±0.28µg/ml Gallic acid equivalent in different concentration. The highest phenolic content in-318.67± 0.28 µg/ml Gallic acid compared (table 3) to the crude extract.

Isolation of *Candida sp*., from throat swab

Candida albicans grow on the sabouraud dextrose agar. (Figure 4)

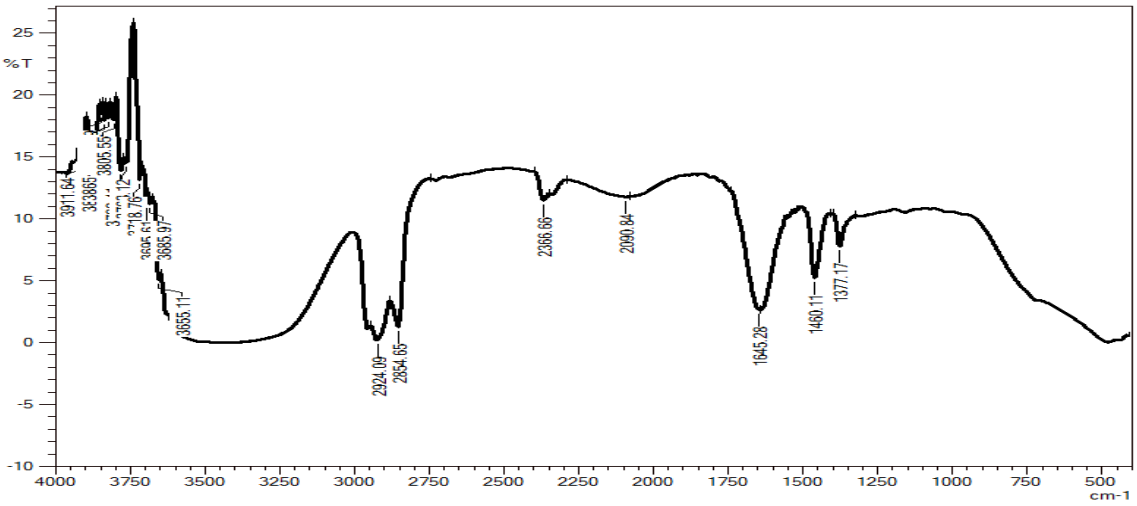

Figure 3: FTIR spectra of synthesized silver nano particles (*citrus medica* AgNPs) was measured in % transmittance in the wave number frequency range of 4000-500 cm^{-1}

Table 2: Antioxidant activity was determined in *citrus medica* silver nanoparticles (CmAgNPs) and aqueous extract of *citrus medica* (Cm*).*

Samples	**% DPPH radical scavenging activity**				
Concentrations	**50 µg/ml**	**100 µg /ml**	**150 µg /ml**	**200 µg /ml**	**250 µg /ml**
CmAgNP	8.64± 0.06	13.79± 0.09	25.08±0.2	39.37± 0.25	56.15±0.41
Aqueous extract of *citrus medica*	6.88± 0.04	12.24± 0.08	22.59± 0.15	29.27± 0.21	35.20±0.24

Values are presented as mean ± standard error from triplicate investigation.

Table 3: Total phenolic content was determined in *citrus medica* silver nanoparticles (CmAgNPs) and Aqueous extract of *citrus medica.* Values are presented as mean ± standard error from three individual experiments.

Samples	**Total phenolic content**				
Concentrations	**100µg/ml**	**200µg/ml**	**300µg/ml**	**400µg/ml**	**500µg/ml**
Assay	17.24 ± 0.08	81.52 ± 0.09	151.52 ± 0.10	200.10 ± 0.20	318.67± 0.28
Aqueous extract of *citrus medica*	14.67 ± 0.07	28.34 ± 0.82	44.87 ± 0.93	69.76 ± 0.97	82.3 4 ± 0.08

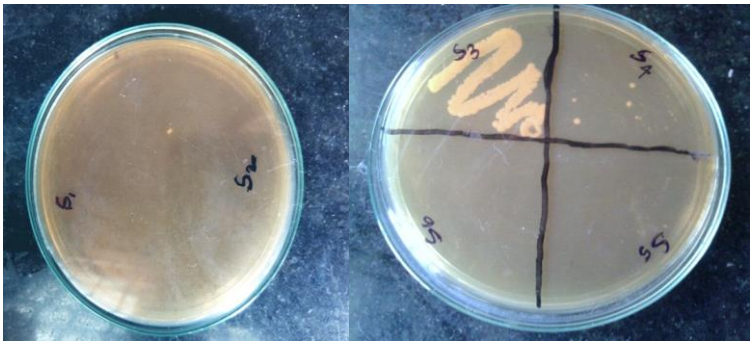

Figure 4: Isolation of *Candida sp*., from throat swab

Antifungal activity:

Ag-based compounds and Ag ions have the powerful antifungal effects. These inorganic Nano Particles contain a unique advantage over the traditional plant extracts. The main problem was caused by the plant extract is multi drug resistance. Therefore, a way to overcome these problems. It is used for an antimicrobial agent in various fields because of their growth was-inhibited in various microorganisms.

Antifungal activity reveals the growth of *candid albicans* was inhibited at concentration of 100µg/ml. The O.D values were decreased in the silver nanoparticles solution because more turbidity in the solution. But *citrus medica* extract contains less turbid so O.D values were increased. AgNP had shown significant inhibitory effects on *candid albicans* (Fig .4).

Table 4: Antifungal activity of *Citrus medica*

Microbial dilution			
Time in hrs	**Blank**	***Citrus medica* extract**	***Citrus medica* Silver Nano particles**
0 min	0.367	0.341	0.317
0.5	0.253	0.370	0.347
0.75	0.352	0.355	0.352
1 hr	0.364	0.358	0.357
2 hr	0.418	0.41	0.393
3 hr	0.453	0.454	0.386
4 hr	0.486	0.502	0.44
24 hr	1.169	1.237	1.104

DISCUSSION

To add the plant extract in the $AgNO_3$ solution, the reduction of pure Ag silver ions which can be measured in the UV –visible spectra of the solution.[17,18] As mentioned in *Gleichenia Pectinata* AgNPs, the SPR peak present in the 410-460 nm regions is allocated to the silver nanoparticles.[19] UV-visible spectrophotometry used for the faster identification and characterization. It gives the powerful absorbance band is called SPR, 400-500 nm range, because the attraction between light and mobile surface electrons of silver nanoparticles [20-23] Elias *et al.,* 2015 reported that the silver nanoparticles from water extract of *Citrus reticulata* peel reveal the localized surface Plasmon bands at the same regions. The FTIR spectrum assigned the different functional groups present at different positions and functional bimolecularis carboxylic, alcohols and phenols. The silver ions can be reduced from that addition of the acid, in that 706 acids can be involved in the reduction process, because the functional group is responsible for the stabilization of silver nanoparticles. Metallic salts are interacting with biological components because the functional groups are responsible for the reduction tonanoparticles[24.]

Terpenoid, alcohol, and carbonyl group serving as strong binding sites for AgNPs in the FTIR result[25,26]. The reduction and capping of silver nanoparticles are formed due to these phytochemical compounds as revealed by FTIR and photochemical studies are alkaloids, flavonoids, tannins, terpenes and quinines.[27] Injury to the cell membranes due to the accumulation of hydrogen peroxide it leads to the free oxygen radical. The antioxidant capacity of silver nanoparticles, the functional groups attached to them. It found in the medicinal plants. The Anti-oxidant activity of silver nanoparticles from various medicinal plants has been studied, e.g. *Abutilon indicum.*[28] *Capsicum frutescens, Allium sativum*, *Cassia occidentalis* [29] and *Zingiber officinale*[30.]*Staphylococcus aureus*, *Proteus, vulgaris, K. pneumoniae, E. coli, B. subtilis and P. aeruginosa* these organisms were killed by an ethanolic extract of peels of C. *medica L*[31.32] Extract of *C.medica* L. has an antimicrobial agent to kill the Gram-positive, Gram-negative bacteria and two fungi A. *niger* and A. *flavus*.

The synthesized silver nanoparticles from *Citrus sinensis* showed the antibacterial activity maximum zone inhibition *E.coli* (17 mm) at 2mM $AgNO_3$ and *S.aureus* (16.5 mm) at 2mM $AgNO_3$[33, 34.] Likewise, our present results suggested that the plant mediated green, silver nanoparticles have been a good antibacterial activity which can be used as an effective material for biological applications. The subsequent intake of AgNPs that could interfere with enzymes involved in the respiratory chain of the cell for the Gram-negative bacteria have their outer membrane selectivity has modified [35.]

MBC and MIC were used for the germicidal properties of silver nanoparticles. MIC is defined as the minimum concentration to prevent the growth of a microorganism is an antimicrobial an agent.[36] The medicinal properties of silver have been known for millennium. Silver nanoparticles are synthesized from different plant extracts, have been used for the analyzing their antimicrobial activities against the different microbes. The activity of the *Gleichenia Pectinata* AgNPs act against the *P. aeruginosa and E. coli and C. albicans*[37]

CONCLUSION

Silver nanoparticles are very important for the free of toxic chemicals and provide an effective synthesis of expected products in an economic manner. In this present work, we developed an ecofriendly and suitable green chemistry method for the synthesis of silver nanoparticles from the leaves of *Citrus medica* as reducing agent and this aqueous extract was used for the production of AgNPs at room temperature by green approach. Production of AgNPs after incubation was identified by the color change.600nm that occurs due to Surface Plasmon Resonance(SPR) during there action with the organic compounds present in the aqueous extract of *Citrus medica*. The formation of AgNPs was confirmed by UV-Vis spectrum and Surface Plasmon broad peak observed nearby 430 nm. The FTIR spectrum indicated the different functional bimolecular compound present at different position such as phenols, alkaloids,

alcohols and carboxylic acid which is involved in the reduction of silver ions. In addition, *Citrus medica* of aqueous extract mediated green synthesized silver nanoparticles showed excellent antifungal properties.

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Source of Support: None declared.

Conflict of Interest: None declared.