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Article

Green Synthesis of *Citrus reticulata* Mediated Silver Nanoparticles

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Abstract: Biosynthesis of nanoparticles for delivery of therapeutic agents has introduced new opportunities in upgrading medical treatment. Plant extracts contains different capping and reducing agents naturally thus provided simpler and less expensive way to synthesize AgNPs. In present work, *Citrus reticulata* mediated stabilised AgNPs was synthesized. Optimum concentration of reactants was achieved by varying the amount of extracts (1-11 ml) and $AgNO_3$ concentration (0.5-3 mM). Surface Plasmon peak of *Citrus reticulata* mediated AgNPs was determined by UV-visible spectrophotometer and functional groups of capping agents were examined by FTIR analysis. Surface Plasmon peaks of *Citrus reticulata* fresh peel, seed, and juice extracts were observed at 420 nm. But in dry peel extract, absorption peak of AgNPs appeared at 410 nm. Colour of different extracts was changed after the reduction of $AgNO_3$ to AgNPs by reducing agents present in the extracts. FTIR analysis showed band peaks at 3316 cm-1 correspond to amide (N-H and O-H) stretching vibrations while alkanes peaks was observed at 1638 cm-1 which showed C=C stretching aromatic ring (flavonoids). Furthermore, *Citrus reticulata* fresh peel mediated AgNPs showed impressive stability up-to 112 days. In conclusion, *Citrus reticulata* fresh peel extract provided an excellent source of reducing agents for synthesizing stabilized AgNPs.

Keywords: Biosynthesis; nanoparticles; plant extracts; *Citrus reticulata*

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1. Introduction

Plants are greatest natural chemists on the earth that start with the transfer of sunlight into chemical energy, a basis of primary products in the biosphere and extend to the astonishingly varied and intricate secondary compounds, which synthesized in their body [1]. Protein based compounds; metallothionins and phytochelators, phytochemicals; polyphenols, tannins and flavonoids are found in naturally occurring compounds, play a key role in different biochemical reaction and for the preparation of nanoparticles [2]. *Citrus reticulata* (Mandarin orange) is one of the famous and most liking fruit of the world which belongs to Rutaceae family. It is reported that *Citrus reticulata* have anti-bacterial [3], anti-fungal [4], anti-diabetic [5], cardio-protective [6], anti-cancer [7], anti-arthritic [8], anti-inflammatory [9], anti-oxidant [10], anti-tubercular, and anti-anxiety medicinal properties [11]. It has diverse and beneficial, capping and reducing phytochemicals like vitamin C, carotenoids and flavonoids and many others [6] which might be involved in the green synthesis of silver

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nanoparticles [12]. It has been reported that different parts of plants and their extracts have their own antioxidant or reducing properties due to secondary metabolites including flavonoids, polyphenol and tannins which play important function in the biosynthesis of Silver nanoparticles (AgNPs) by the reduction of metals [13].

Many physical, electrochemical and chemical methods followed top down and bottom up pathways are being in used for the synthesis of different nanoparticles including AgNPs and nano materials. But the physical and chemical processes are not clean, safe and eco-friendly due to the involvement the of highly technical apparatus, cost [14] and participation of hazards, harmful and explosive chemicals [15,16]. It is therefore, green method was emerged for AgNPs synthesis [13], as it involves low temperature, pressure, and energy as well as it is ecofriendly, radially scalable, safe to handle and cost effective technology with reduced toxicity [17]. Materials having size ranges from 1-100 nm are called nanomaterials which have the greatest importance in the field of nanotechnology. Nanoparticles like gold, silver and copper are prepared by the extract of different parts of plants as ecofriendly product and are being excessively [12] used in the fields of physics, chemistry, biology [18] photography, catalysis, and biological labeling has helped in the development of excessive interest in the field of nanotechnology [19]. Silver nanoparticles are of greatest interest among noble metal nanoparticles due to antibacterial [20], antifungal [21], antitumor [22], antiviral [23] , anti-diabetes [24] and antioxidant activities [18]. AgNPs are used as catalysts, as optical sensors, in textile engineering, in electronics, in optics, most importantly in the medical field as a therapeutic agent [12] and also in food packaging, preservation, soaps, detergents, shampoos and cosmetics [25]. AgNPs have the greatest antimicrobial activities. At very low concentration these are nontoxic to human and have no serious side effects [26].

The aim of the present work was to synthesize the *Citrus reticulata* mediated AgNPs and to screen which part of *Citrus reticulata* fruit was most active for the synthesis of stabilized AgNPs.

2. Materials and Methods

2.1 Extract preparation

Fresh *Citrus reticulata*s were purchased from local market of Lahore and were washed with distilled water. 5 grams of *Citrus reticulata* peels were weighed and cut into small pieces. Resulting pieces of peel were soaked in a beaker containing 100 ml distilled water and mixed for 2 hrs on magnetic stirrer. After mixing, it was boiled on burner for 10 mins, then filtered and stored at 4 °C [12,38]. Seeds and dry peel extracts were also prepared by using same said method while fresh juice of *Citrus reticulata* was used after centrifugation and filtration.

2.2 Preparation of the silver nitrate solution

Three dilutions (0.5, 1 and 2 mM) of $AgNO_3$ were prepared from 3 mM stock solution of $AgNO_3$ which was prepared by dissolving 0.051 g of $AgNO_3$ in 100 ml distilled water. All these solutions were further used for the synthesis of AgNPs.

2.3 Synthesis of silver nanoparticles

AgNPs were synthesized by mixing 18 ml $AgNO_3$ (0.5 mM) in 5 ml fresh peel extract in volumetric flask and then boiled for 30 mins in water bath. AgNPs were also synthesized by using juice, seeds and dry peel extract separately by same above said procedure. The effects of the reaction conditions such as volume of *Citrus reticulata* peel extract and concentration of $AgNO_3$ solution were evaluated. Different volume of peel extract such as 1 ml, 2 ml, 5 ml and 11 ml were mixed with 18 ml $AgNO_3$

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(0.5 mM) in a volumetric flasks separately to synthesize the AgNPs. Similarly, AgNPs biosynthesis was examined by mixing different concentrations of $AgNO_3$ including 0.5 mM, 1 mM, 2 mM and 3 mM in 5 ml of peel extract used respectively while other conditions remains constant. The stability of AgNPs was also checked at 1, 4, 12, 28, 56, 86 and 112 days after the reaction. UV-visible spectrophotometer was used to analyze the formation of AgNPs [35,39].

2.4 Characterization of silver nanoparticles

2.4.1 Visual analysis

This colour change was noted by visual observation after the mixing of $AgNO_3$ and respective extract [25].

2.4.2 UV-Visible analysis

Spectral analysis of AgNPs prepared by different reaction conditions were observed at the range between 300 to 600 nm by UV–Visible spectrophotometer ($T90^+$ UV/Vis Spectrometer.PG Instrument LTd) [40].

2.4.3 Fourier transform infrared spectrophotometer (FTIR) analysis

For FTIR analysis, the synthesized AgNPs were centrifuged at 12,000 rpm for 30 mins at 0 °C and then Supernatant was discarded and pallet of AgNPs washed with deionized water. Washing step was repeated for three times [41]. The resulting AgNPs pallet was dissolved in methanol and then evaporated [42]. Powder of AgNPs and fresh peel extract were subjected to FTIR (Agilant Microlab, Carry 630 FTIR) for the identification of functional groups in the compounds present in the peel extract and their involvement in the synthesis of AgNPs was determined [43].

3. Results

3.1 Visual analysis

Visual examination was done when the *Citrus reticulata* peel extract was mixed with aqueous solution of 0.5 mM silver nitrate and put on water bath. Colour was changed from pale yellow to brown after 30 mints, which was due to reduction of silver ions and indicated that the AgNPs were formed (Fig 1).

3.2 UV-Visible analysis

Extracts of different parts of *Citrus reticulata* fruit: seed, juice, dry peel and fresh peel were prepared for bio-reduction of $AgNO_3$. The AgNPs formation was monitored with the help of UV-Visible spectroscopy. UV-visible spectra were recorded immediately after the reaction.

A surface plasmon peak was observed at 420 nm for fresh peel, seed, and juice mediated AgNPs, while dry peel mediated AgNPs showed a surface Plasmon peak with a maximum absorption at 410 nm. Fresh peel extract was used for further studies because it gave maximum concentration of AgNPs (Fig.2).

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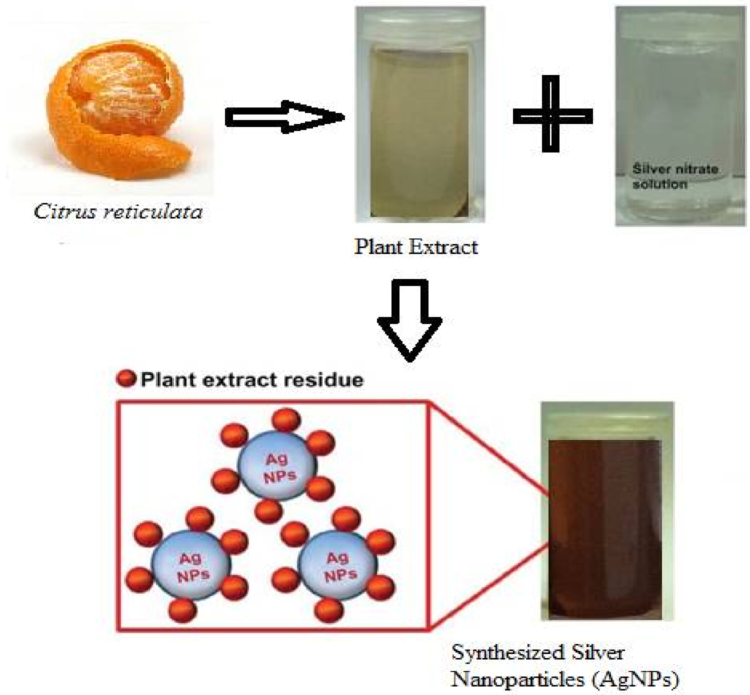

Figure 1: Schematic diagram for the biosynthesis of *Citrus reticulata* mediated silver nanoparticles

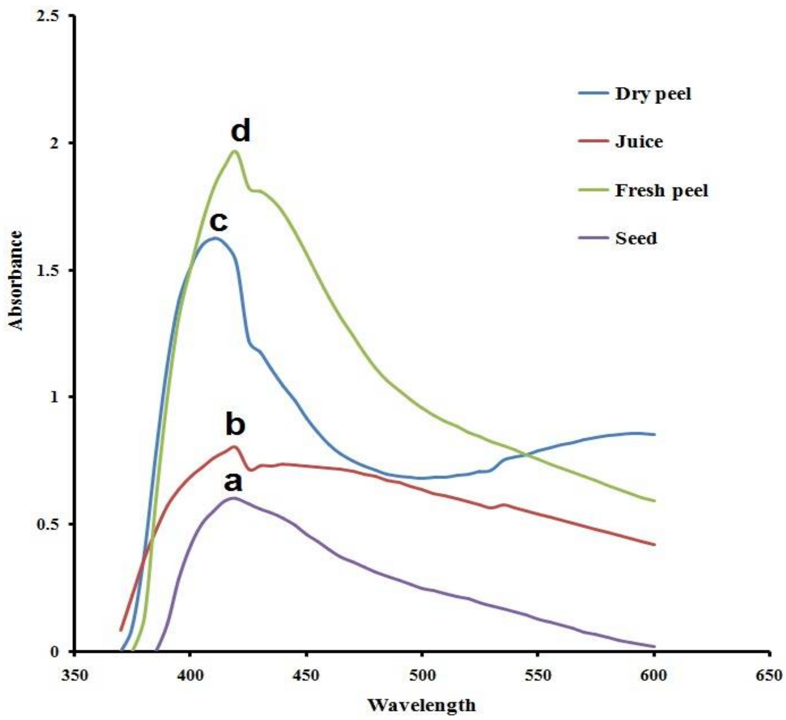

Figure 2: UV-visible absorption spectra of AgNPs synthesized from the extracts of different parts of *Citrus reticulata*. Silver nitrate ($AgNO_3$) was reduced to silver nanoparticles (AgNPs) with extract isolated from different parts of *Citrus reticulata* fruit and UV-visible absorption spectra were recorded immediately after the reaction. Peak (a) shows seed extract mediated green synthesis of AgNPs absorption spectra, (b) represents juice mediated green synthesis AgNPs absorption spectra while (c) exhibits dry peel extract mediated green synthesis of AgNPs absorption spectra and (d) shows fresh peel mediated green synthesis of AgNPs absorption spectra.

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The reaction of AgNPs formation was monitored for the varying concentrations of fresh peel extract (1, 2, 5 and 11 ml), keeping $AgNO_3$ concentration constant. It was observed that colorless solution of $AgNO_3$ was converted into light brown for 1 ml, brown for 2 ml, radish brown for 5 ml and very light brown for 11 ml . It was also found that concentration of synthesized AgNPs was decreased with increasing volume of fresh peel extract (Fig.3). Furthermore, AgNPs synthesis was examined with varying concentrations of $AgNO_3$ (0.5, 1, 2 and 3 mM) at constant volume of fresh peel extract (5ml). An increasing trend for AgNPs synthesis was observed with increased concentration of $AgNO_3$ firstly then it was decreased for 3 mM $AgNO_3$ (Fig.4).

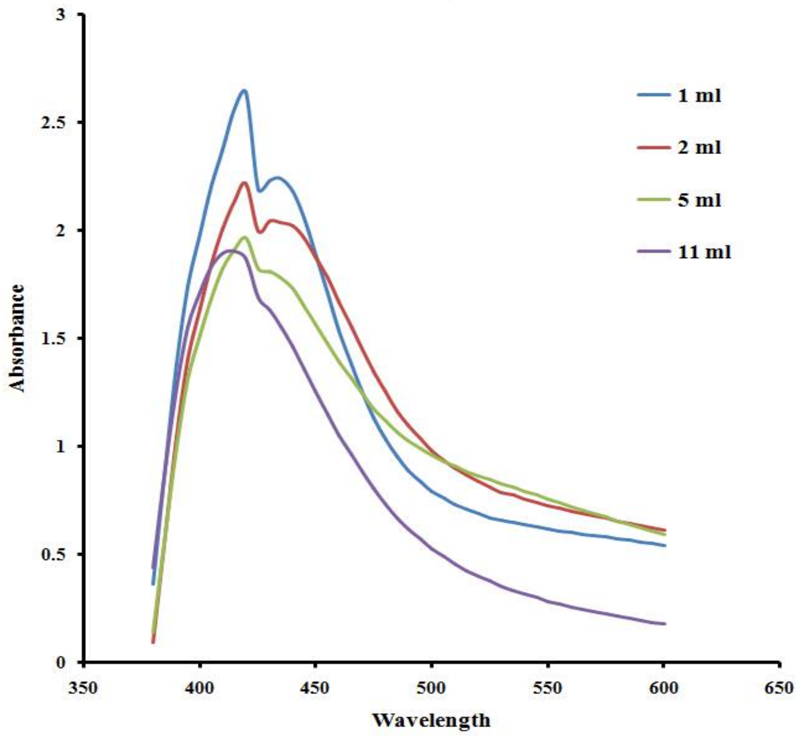

Figure 3: UV-visible absorption spectra for Fresh peel extract dependent study for AgNPs synthesis. Aqueous extract of *Citrus reticulata* fresh peel was made by continuous stirring along with boiling and then used as reducing agent for the bio-reduction of $AgNO_3$ to silver. Concentration of $AgNO_3$ was kept constant and varying volume of "Fresh peel" extract was used for the reduction of silver and UV-visible spectra were recorded immediately after the reaction.

After successful green synthesis of AgNPs, stability of synthesized AgNPs from fresh peel extract as reducing and capping agent was monitored by recording UV-Visible spectra at different time including 1, 4, 12, 28, 56, 86 and 112 days. It was found that, for 0.5 mM silver nitrate solution, concentration of stabilized AgNPs increased until 28 days after the reaction gradually, and then it decreasing as absorption intensity decreased with time. Same trend was observed when we took 1 mM $AgNO_3$ solution instead of 0.5 mM (Fig.5 A-B).

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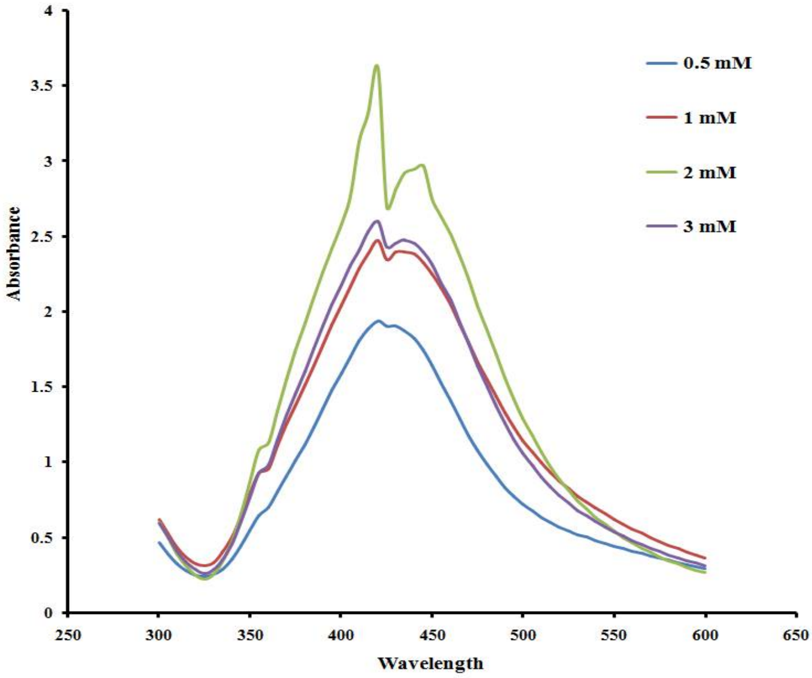

Figure 4: Silver nitrate ($AgNO_3$) concentration dependent UV-visible absorption spectra for fresh *Citrus reticulata* peel mediated AgNPs. Different dilutions of $AgNO_3$ solution (0.5 mM, 1 mM, 2 mM and 3 mM) were prepared and mixed with fresh peel extract (5 ml) to synthesized AgNPs and then immediately their UV-visible spectra were recorded.

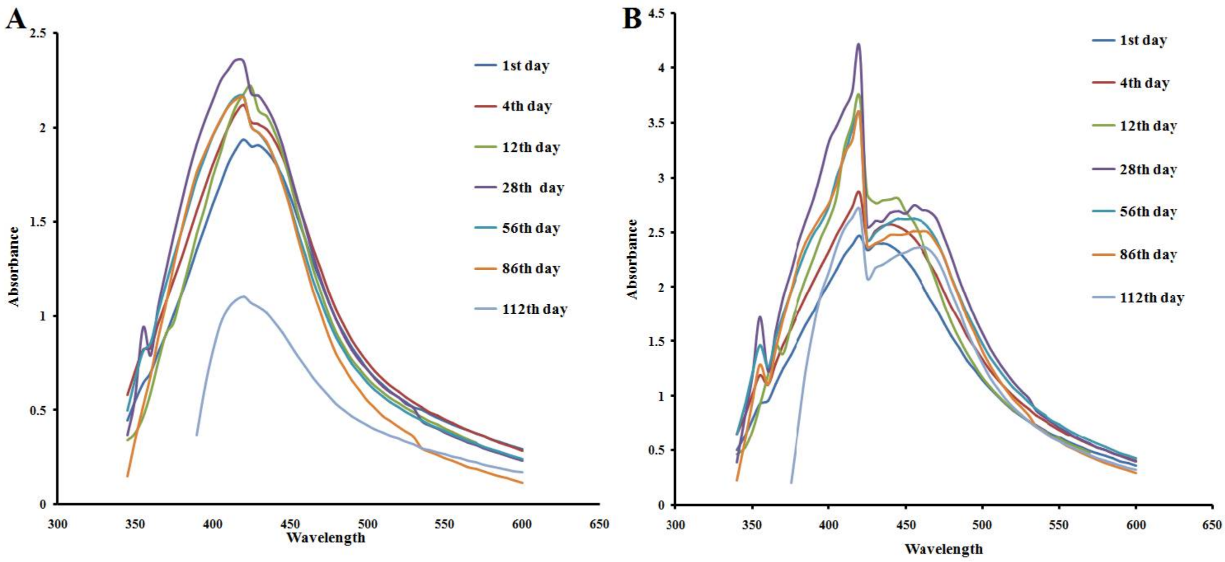

Figure 5: UV-visible absorption spectra for the stability of synthesized AgNPs. (A) AgNPs synthesized by *Citrus reticulata* fresh peel extract (5 ml) and 0.5 mM $AgNO_3$ (B) AgNPs synthesized by *Citrus reticulata* fresh peel extract (5 ml) and 1 mM $AgNO_3$ and UV-visible spectra were recorded immediately and after different days to determine stability of AgNPs.

FTIR Analysis

FTIR spectroscopy is useful in probing the chemical composition of the surface of the AgNPs and the local molecular environment of the capping agents on the nanoparticles. FTIR analysis was used for the characterization of the analysis of both, plant extracts and the resulting AgNPs. Transmittance spectra were observed in the region of 1000 to 4000 cm^{-1}. Bands peaks were observed at 3316, 1638, 1522, 1205, 1158, 1099, 1052, and 1029 cm^{-1}. These peaks are known to be associated with the stretching vibrations for N-H and O-H,–C=C–, C–N stretch, C–O and C–O (polyols) respectively. The bands below 3000 cm^{-1} is due to C-H stretching and C-N stretching (Fig.6 A-B).

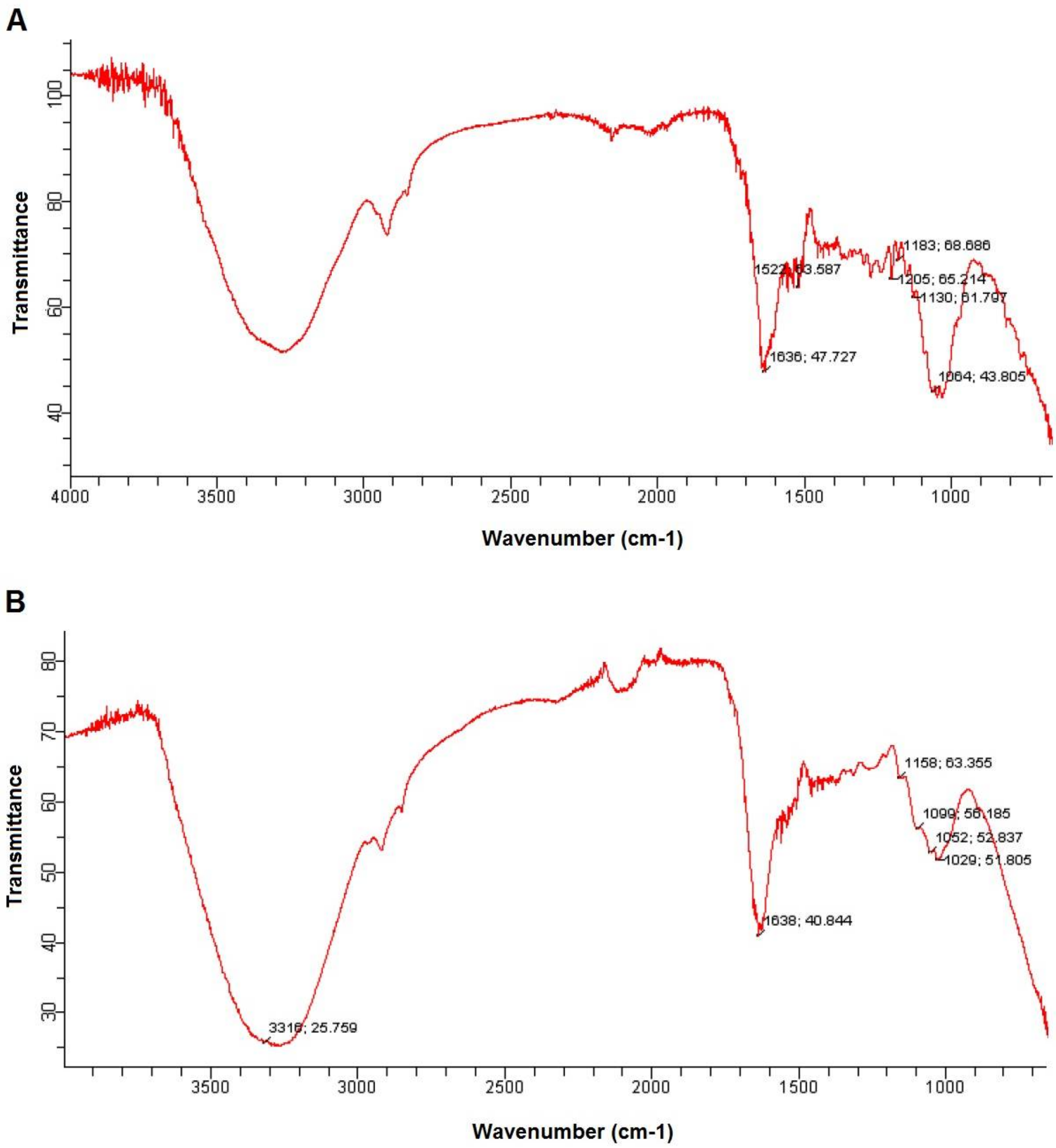

Figure 6: FTIR transmittance spectra for functional group of capping and reducing agents. (A) Shows FTIR transmittance spectra for *Citrus reticulata* fresh peel extract and (B) Shows FTIR transmittance spectra of *Citrus reticulata* fresh peel extract mediated AgNPs.

4. Discussion

Nanotechnology is one of the growing area of research in the life sciences especially biotechnology to improve the human health. For the delivery of therapeutic agents and other activities against different diseases, synthesis of stable and versatile silver nanoparticles (AgNPs) depends on the size and shape. In the present study different parts of *Citrus reticulata* (*Citrus reticulata*) fruit including fresh peel, dry peel, seeds and juice were used for the biosynthesis of silver nanoparticles (AgNPs). Change in color to brown was observed after mixing it with Silver nitrate ($AgNO_3$) due to the reduction of silver ions, which indicated the formation of AgNPs as previously observed in several investigations [12,27]. This change in colour is due to phenomena known as surface plasmon resonance (SPR). This phenomenon is because of the oscillatory movement of electrons present in conduction band, in response of electromagnetic radiation [28]. Under Ultraviolet (UV) inspection, nanoparticles give a characteristics absorption band due to excitation of surface plasmons [29]. Presently, the confirmation of the AgNPs formation was also made via UV-visible spectrophotometer. Absorption peak from UV-vis spectra are also used as prediction tool for stability and size of particles [30]. According to Mie's theory, the peak shifted to be observed in an absorption spectrum when the mean diameter of the particles changes [31].

$AgNO_3$ was reduced with four extracts (seed, juice, dry peel, wet peel) and UV-vis absorption spectra were recorded immediately after the reaction. We found that the maximum absorption for seed and fresh peel and juice mediated AgNPs was obtained at 420 nm, while in case of dry peel extract it was recorded at 410 nm by UV-vis spectrophotometer. The resulting *Citrus reticulata* mediated AgNPs might be spherical in shape. Previously it was found that gelatin mediated spherical AgNPs showed a characteristic SPR peak at around 400 nm [28]. Peel mediated AgNPs found to be in high concentration when compared with AgNPs synthesized from seed extract and juice. The two main compositional differences between peel and juice components are that the peel contains a higher concentration of ascorbic acid than the juice, and that the peel also contains higher concentrations of active components including d-limonene, hesperidin, naringin, and auraptene than do the juice [4].

The maximum concentration of AgNPs was obtained by fresh peel extract as compared to dry peel. It may because of the reason that fresh peel has more essential oil components as compare to dry peel which has less essential oil contents due to evaporation of these antioxidant volatiles during the process of drying [32]. It was also found that the maximum absorbance value (which represents the concentration of nanoparticles) was changed for different samples under study. According to Beer-Lambert law, absorbance relies upon concentration of suspension, thus higher concentration of NPs leads to higher value of absorbance [33]. Previously it was concluded that the absorption wavelength might be represented the estimated nanoparticles size. We supposed the estimated size of *Citrus reticulata* mediated AgNPs size might be in the range of 30-50 nm as presently synthesized AgNPs absorption spectra was recorded at 410 and 420 nm [30].

Caroling *et al.*, 2013 reported that the noble metal silver displays characteristic absorbance at around 410-430 nm. They suggested that Polyol components, flavonoids and terpenoids are mainly responsible for the reduction of silver ions. Thus the study suggested that aqueous active components like flavonoids, xanthones, and tannins of *G. mangostana* extract might reduce the silver ions [18]. As, it was examined that maximum concentration of AgNPs when fresh peel extract was mixed with $AgNO_3$. It is therefore, for optimization of reaction parameters, the fresh peel extract was further used in the biosynthesis of AgNPs. We observed that colorless solution of $AgNO_3$ was converted into light brown for 1 ml, brown for 2 ml, radish brown for 5 ml and very light brown for 11 ml. Absorption peak was recorded at 410-420 nm for all volume of fresh peel extracts (1 ml, 2 ml, 5 ml and 11 ml). The range of between 410-420 nm of absorption spectra indicated that synthesized AgNPs might have estimated particle size range 30-50 nm [30]. Some literature proved that when the amount of extract is increased the concentration of AgNPs also increased [34]. But in present study we found inverse trend, this might be because of low concentration of $AgNO_3$ (0.5 mM). In AgNPs dependent study, we examined that absorption of AgNPs increased with increasing concentration of

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$AgNO_3$. Depending on their size and shape, nanoparticles are expected to display one or more surface plasmon (SP) bands: Small spherical nanoparticles exhibit a single SP band at small wavelengths, whereas large anisotropic particles reveal two or three SP bands at longer wavelengths [35]. Therefore, the peaks at 440 nm in fig.3 are likely due to larger spherical size of AgNPs. We monitored that fresh peel mediated AgNPs by using both concentration of AgNO3 solution 0.5 mM and 1 mM separately showed impressive stability of AgNPs at different time including 1, 4, 12, 28, 56, 86 and 112 Days. Furthermore, it was noted that from 1 to 28 days of the preparation, intensity of the absorption peak increased and after this peak value started to decrease.

The FTIR analysis in present study is showing that the broad band around 3316 cm^{-1} is related to amide (N-H and OH) stretching vibrations and alkanes. The bands below 3000cm^{-1} is due to C-H stretching and C-N stretching. The intense absorption observed at 1638cm^{-1} might be a characteristic of the C=C stretching aromatic ring and this result agree with the result of the Thin layer chromatography (TLC) test of previous study, which referred to the active ingredient in the *Citrus reticulata* peel that causes the reduction of Ag^+ ions and it was found in that the effective group is flavonoids which led to the bioreduction of aqueous silver ions (Ag^+) [12]. Polyols and phenols reduce silver ions and oxidize unsaturated carbonyl groups, which leads to a broad peak at approximately 1645 cm^{-1} [36]. This peak at 1638 may also correspond to Ag–O stretching vibration [37].The bands between 1200cm^{-1} to 1000cm^{-1} are attributed to C-O (esters, ethers and polyol) and C-N stretching vibrations (for aliphatic amines), $-CH_2$, $-CH_3$ vibrations, and –C-O-H (for alcohols) and –C-O-R (for ethers or esters) vibrations as shown by literature [38]. The similarity in plant extract spectra and their respective AgNPs might be attributed to the coating of the phytochemicals present in extract on the surface of AgNPs [27].

5. Conclusion

It is concluded that the *Citrus reticulata* fruit extract might be very useful as bio-reductant for the synthesis of AgNPs with impressive stability even after 112 days of the reaction. This indicated that fresh peel extract may have good reducing and capping agents like amide, alkanes, flavonoids, polyols, phenols, esters and ethers (examined in FTIR analysis) which might be involved in the synthesis and stability of AgNPs. To unexposed the hidden properties of fresh peel mediated AgNPs further research may be done to examine the antimicrobial activities.

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

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