The Journal of Animal & Plant Sciences, 29(6): 2019, Page: 1587-1595
ISSN: 1018-7081

POST-EXPOSURE HISTOPATHOLOGICAL STUDIES OF ALBINO MICE LIVER TO SILVER NANOPARTICLES PREPARED FROM *(Ocimum tenuiflorum)* GREEN SYNTHESIS METHOD

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ABSTRACT

Industrial and biomedical uses of nanoparticles have significantly increased in recent years. The Preparation of nanomaterials by conventional methods is posing serious hazards to the environment and the living beings in a variety of ways. Due to this setback, the development of some safer nanoparticles is deemed necessary for their potential applications instead of discontinuing their development and use altogether. During the present study, silver nanoparticles (AgNPs) using green synthesis approach were developed and characterization of the newly-synthesized AgNPs was performed by using ultraviolet (UV) visible spectroscopy, Scanning Electron Microscopy (SEM) analysis and Fourier-transform Infrared Spectroscopy (FTIR), revealing UV visible peak at 420 nm and confirming the formation of spherical-shaped AgNPs; SEM imaging also confirmed the spherical-shaped AgNPs with size ranging between 32 and 50nm; FTIR analysis designated the role of alkane and aromatic compounds from the plant extract as capping and reducing agent. Histopathology of albino mice liver was performed after dosing the mice with specific concentrations of the nanoparticles for 7, 14 and 21 days. Micrographs of the liver histology revealed cell distortion, necrosis, apoptosis, and detachment of hepatocytes and other signs of fibrosis in the mice liver exposed to the nanoparticles.

Keywords: Acute toxicity, Albino mice, Green synthesis, Histopathology, Oxidative stress, Silver nanoparticles.

INTRODUCTION

In recent years, nanotechnology has gained significant research interest because of the development of some important biomedical and health applications through the use of nanotechnology; nanomaterials and their potential applications are of serious interest to new researchers (Handy *et al.,* 2008). Development of nanoparticles with special properties is the dire need of time.

Thus far, researchers have prepared a variety of nanomaterials for different applications; most importantly these particles of various shapes range from 1 to 100nm in diameter (Kreyling *et al.,* 2010). Unique features and properties of nanoparticles i.e. magnetic, optical, mechanical, electrical, size, shape, and morphology characterize them as particular particles with novel functionalities. Since ecological parameters and stabilities are greatly influenced by the natural nanoparticles (Pennycook *et al.,* 2011), it is important to investigate these particles and their interactions with the cell and its machinery (Kane and Stroock 2007). Expected adverse effects of these particles on the environment and human health, the risks associated with nanotechnology must be analyzed (Pettitt and Lead 2013).

Various techniques and methods are used for the synthesis of nanoparticles; however, compared to the physical and chemical methods, green synthesis methods are more eco-friendly and cost-effective (Mallikarjuna *et al.,* 2011). Green chemistry deals with novel methods for synthesizing the nanoparticles from plants at neutral pH and room temperature. In this way, it connects the nanotechnology with the plants and plants-mediated synthesis of nanoparticles. Furthermore, these synthesis methods do not require any toxic chemicals, high pressure or temperature; therefore they are proven to be cost-effective as well (Dhuper *et al.,* 2012).

Development of safer nanoparticles is vital for biological systems and living beings. In the present study, AgNPs were synthesized from the plant extract of *Ocimum tenuiflorum* L., synonymously termed as *Ocimum sanctum* L. and commonly known as "Tulsi". It is medicinally important and contains many active ingredients that are effective against numerous ailments (Bindhani and Panigrahi 2015).

MATERIALS AND METHODS

All the chemicals and reagents used during the present study were of analytical grade and purchased from authentic vendors, whereas Tulsi was collected from the Botanical Garden of Government College University, Lahore Punjab, Pakistan.

Preparation of Plant Broth: Ten grams of sun-dried leaves of Tulsi were weighed and washed with double distilled water to remove impurities and dust particles.

Thereafter, these leaves were boiled in 100ml deionized water on a hot plate for 10 minutes. Finally, the extract was filtered and preserved at -20 °C for future use.

Preparation of AgNPs: The broth and 1mM solution of $AgNO_3$(1: 4) ratio were mixed at room temperature and heated up to 65°C while constantly stirring with a magnetic stirrer. Few drops of alkaline solution of NaOH were added for pH maintenance. Color transformation to yellowish-brown confirmed the formation of nanoparticles.

Characterization of AgNPs: SEM (JEOL, JSM-6480LV), FTIR (Shimadzu IR Prestige21),and UV-visible Spectrophotometry (GENESYS 10S UV-Vis) were used for the characterization of the silver nanoparticles synthesized using green synthesis method. The concentrations of these particles were analyzed using lyophilization of the sample as well as using oven-dry techniques. The concentration of the silver ions was analyzed by using Atomic Absorption Spectroscopy.

Model Animals: Male albino mice (8-10 weeks old) were obtained from Government College University, Lahore, and were kept in a metallic cage in an air-conditioned room (22 ±1ºC) with 12:12 hours light to dark cycle. The animals were provided with water and commercially available rat pellets on a regular basis. Before the starting of the experiment, the animals were allowed to acclimatize for 7 days to the laboratory conditions; OECD guidelines were adopted foranimal handling.

Toxicity Studies

Estimation of Toxicity: Estimation of lethal dose 50 (LD_{50}) for green synthesized AgNPs was made by using commercially adopted procedures (Guideline 2001).Doses of the toxicant were administered intravenously through the tail vein of the animal after every 24hour for 4 days. Mortality of the mice was observed and dead mice were removed and counted. Log-probit regression analysis was used for the dosage response (LD_{50} determination).

Sub-lethal dose treatment: In a sub-lethal dose experiment, a total of 40 mice were used. These mice were divided into four groups with 10 mice in each group: G1 (control, having no exposure to AgNPs), G2 (50mg/kg of AgNPs), G3 (75mg/kg of AgNPs), and G4 (100mg/kg of AgNPs). Sampling was performed by sacrificing 3 mice from each group at 7, 14 and 21 days of treatment. Mice were sacrificed to obtain their liver for further histopathological investigations.

RESULTS AND DISCUSSION

Synthesis of Green Synthesized AgNPs: *Ocimum tenuiflorum*-derived AgNPs nanoparticles were found to be stable for more than 5-6 weeks. The finding is consistent with other previously reported studies. For example, *Geranium*-leaf-derived AgNPs were reported to be stable for six weeks (Sastry *et al.,* 2003), while two months stability has been reported for the *O. tenuiflorum*-extract-derived AgNPs (Daniel *et al.,* 2011). Certain biomolecules and other active ingredients present in the plant extracts play important roles as reducing and capping agents and thereby providing stability to the AgNPs. Results obtained during the green synthesis method of AgNPs are in agreement with the previous studies (Banerjee *et al.,* 2014; Sadanand *et al.,* 2016).

Characterization of silver nanoparticles (AgNPs)

UV-Visible spectroscopy:UV-Visible graph showed**a** peak at 420nm which confirmed the formation of spherical-shaped AgNPs (Figure 1). The appearance of peak around 420nm (Figure 1) indicated the formation of spherical-shaped silver nanoparticles. The peak at this range also confirmed the spherical-shaped morphology within size ranging less than 100nm (Dehnavi *et al.,* 2013).The Surface Plasmon Resonance band has special peak ranging from 380 to 440nm for spherical-shaped AgNPs (Stamplecoskie and Scaiano 2010). The yellow color of these AgNPs in suspensions is due to the vibrations, excitations, and surface Plasmon resonance (Abdel-Aziz *et al.,* 2014).Another study reported that the *Ocimum sanctum*-derived AgNPs showed a peak at 406nm on UV-visible graph (Sadanand *et al.,* 2016). Time of the reaction and temperature largely influence the size and shape of the newly formed nanoparticles; hence, this is time and temperature-dependent phenomenon(Song *et al.,* 2012). Results of the present study for the UV-Visible spectroscopy are consistent with the previous findings of Banerjee *et al.,*(2014).

Scanning Electron Microscopy (SEM): Spherical-shaped AgNPs were easily visible with their size ranging from 32 to 50nm on the micrographs (Figure 2a, 2b). SEM analysis of *O. sanctum* mediated AgNPs showed similar results (Vijaya *et al.,* 2014).*Piper longum*-derived AgNPs showed spherical-shaped morphology, with size ranging from 17.6 to 41nm(Jacob *et al.,* 2012). Another study (Niraimathi *et al.,* 2013)by using *Alternanthera sessilis* reported the formation of spherical-shaped AgNPs in the size range of 20 to 30nm. The concentration of $AgNO_3$, leaf broth and temperature of the reaction largely influence the size of the nanoparticles(Song and Kim 2009). The nature of capping and reducing agent present as a part of the plant extract greatly influence the shape of the synthesized nanoparticles(Banerjee *et al.,* 2014).

FTIR of the AgNPs: Pure extract of*Ocimum tenuiflorum* leaves, when tested for FTIR analysis, prominently showed absorption bands at the following wave number: 3319, 2929, 2883, 2393, 2347, 1606, 1400, 1313, 1060, 825, and 784 cm^{-1}.

FTIR spectrographs of AgNPs, when compared with the spectrum of extract showed that magnitudes of some peaks shifted slightly within the range of 1-10cm^{-1}. While bands at 825 and 784 cm^{-1} disappeared in the spectrum of AgNPs (Figure 3); these bands are concerned with the aromatic stretching and alkane compounds in the extract. Further, it proved that functional groups of these peaks were consumed as capping and reducing agent for the formation of AgNPs.

Previouslyreported studies of FTIR analysis of the *Ocimum tenuiflorum* plant extract generally agree with the results of the present study with minor variations in the peaks(Daniel *et al.,* 2011), plant extract of *O. tenuiflorum* has many active biological molecules, such as carboxylic acid, alcohol, and amine. As AgNPs are formed, slight changes in theposition of peaks were recorded on the spectrum; a few peaks were reduced while a few others diminished. The peak at 1230cm^{-1}on the spectrum diminished totally(Daniel *et al.,* 2011); 1230cm^{-1} peak indicated the C-O-C and C-OH vibrations present in the molecules(Gole *et al.,* 2001).Stretching of O-H and H-bonded compounds, such as alcohols and phenols was indicated at 3419 cm^{-1}spectrum of the FTIR. A band at 2929cm^{-1} indicated the presence of carboxylic acids. A peak at 1606cm^{-1} indicated the presence of amine due to the bending of N-H bonds. Other peaks on the spectrum corresponded with the presence of other molecules, such as ether, ester, aromatic compounds, aldehydes, and ketones in the plant extract of the *Ocimum* species (Mallikarjuna *et al.,* 2011).These results are in agreement with findings of the present study in terms of the consumption of Alkane and aromatic compounds as capping and reducing agent.

Many other studies have reported various types of biomolecules and functional groups in the plant extracts which might act as reducing and capping agents as well; for example,protein in *Capsicum annuum* (Li *et al.,* 2007), flavonoids, polyol and terpenoids in *Cinnamomumcamphora* leaf extract (Huang *et al.,* 2007), phyllanthin in *Phyllanthus amarus*(Kasthuri *et al.,* 2009),caffeine and theophylline in tea plant (Krishnaraj *et al.,* 2010) have played the role as capping and reducing agents for the formation of AgNPs, and other types of the nanomaterials.

Atomic Absorption Spectroscopy: Total silver content of the AgNPs was estimated after complete digestion was found to be 10.92 ± 0. 84 µg/ml. The concentration of AgNPs was estimated using the oven drying method and was found to be 3.8 ± 0.2 mg/ml.Time-dependent studies of the reaction of the AgNPs formation with the help of AAS showed that the free silver ion went on decreasing with the time which confirmed the utilization of the silver ions in the formation of AgNPs(Awwad *et al.,* 2012).

Evaluation of LD_{50} (Lethal Dose) Value: The LD_{50} value was estimated to be 812 mg/kg for green synthesized AgNPs.Higher values of the LD_{50} make these particles less toxic to the environment; hence, proving them to be safer to the living being. On the other hand, the LD_{50} value of the size-dependent chemically synthesized AgNPs amounted to 169mg/kg and 213.8mg/kg for 20nm, 354 and 391.5 mg/kg for 50nm AgNPs in the albino mice. The present study further revealed that the smaller-sized particles exhibit more toxic effects compared to the particles of larger sizes (Elkhawass *et al.,* 2015). Since other studies (Elkhawass *et al.,* 2015)used mainly chemically synthesized AgNPs and observed lower values of LD_{50} for the albino mice compared to the estimated values of the LD_{50} for green synthesized AgNPs in the present study, it proves that the green synthesized AgNPs are relatively safe to the environment. Size and dose-dependent activity of the AgNPs might be another possible explanation for this phenomenon; particles of smaller sizes are more penetrating inside the cell compared to the larger sized particles, thereby proving to be more fatal to the cell(Kim *et al.,* 2010).

Evaluation of Liver Histology: Histology of the liver tissues after exposure to AgNPs showed more toxicity at the higher concentrations (dose), i.e., 100 mg/kg as compared to the control and lower concentrations dose groups. These hepatotoxic effects mainly include necrosis, hepatocytes degeneration, and apoptosis.The liver histology for the control mice was very normal. Liver contained prominent hepatocytes of hexagonal shapes having central nuclei around the very clear and prominent central veins, which were used as a point of reference during taking micrograph of the prepared slides. Central portal veins were also seen prominently which were connected to the central veins. Normal liver histology is presented in figures 4A, 5A, and 6A.

In case of the AgNPs-exposed mice, the liver hepatocytes were gradually distorted, some cell observed the increasing degree of necrosis and apoptosis, which resulted in cell detachment and enlarged nuclei at higher dosing rates of the AgNPs. Increased oxidative stress to the liver resulted in the damaging of the central vein and widened it to some extent. Increase in size of the hepatocytes was also observed. The collagen masses were also seen in the central vein on high concentrations of the AgNPs dosing, which were supposed to be the result of fibrosis as shown in figures 4B-D, 5B-D, and 6B-D. The effect was more prominent in the mice liver which was exposed to higher concentrations of AgNPs for longer periods.

Numerous studies have confirmed that the target organ for the toxicity of the AgNPs is mainly the liver of the animal (Sung *et al.,* 2008). AgNPs are highly toxic to the liver cells of rat (Hussain *et al.,* 2005), particularly to the primary hepatocytes (Gaiser *et al.,* 2012). Liver cells are composed of a high amount of thiol rich proteins such as glutathione, which tend to accumulate the silver nanoparticles in the hepatocytes (Knetsch and Koole 2011).

AgNPs caused toxicity to the liver which consequently induced oxidative stress in the body (Piao *et al.,* 2011). Previous studies on albino mice (Arora *et al.,* 2009) reported the hepatotoxicity caused by the AgNPs, where oxidative stress induced further biochemical reactions in the liver cells, i.e. Apoptosis, isolations, reduced hepatocytes viability, and sometimes tissue injuries.

Different level of liver toxicity was developed by the different type, concentration, and size of AgNPs. Chemically synthesized AgNPs induced severe toxicity as compared to the biologically synthesized NPs. AgNPs in high concentrations were more toxic to the liver cells. A histological examination showed cell rounded off with detaching activity in sub-lethal AgNPs dose.

AgNPs, directly and indirectly affect and reduce the mitochondrial activity of the liver cells, which leads to the shortage of available energy for cellular functioning. Macrophages tried to remove the AgNPs by the phagocytosis process, which eventually led to the generation of higher oxygen radicals within the hepatocytes (Sardari *et al.,* 2012). Results of the current study are totally in compliance with the previous studies by (Kim *et al.,* 2010). We have suggested that the repeated dose of the AgNPs intravenously induce mild to moderate toxicity in case of green synthesized AgNPs.

Table 1. Peaks obtained on FTIR spectrum with Associated Functional groups and biomolecules.

No	Peak	Functional Group	Compound Present
1	3319	OH	Alcohols and Phenols
2	2929	O-H	Carboxylic Acids
3	2883	-CH3	Alkane
4	1606	C=O, C=C	Carbonyl group, Ketones, aldehydes, alkenes
5	1400	C-C	Aromatic compounds, alkanes
6	1313	C – O-C, C-OH	Carboxylic Acids, Alcohols, ethers, esters
7	1060	C-O	Carbonyl, Alcohols
8	825	C-H bond	Alkane, Aromatic compounds
9	784	C-H bond	Alkane, Aromatic compounds

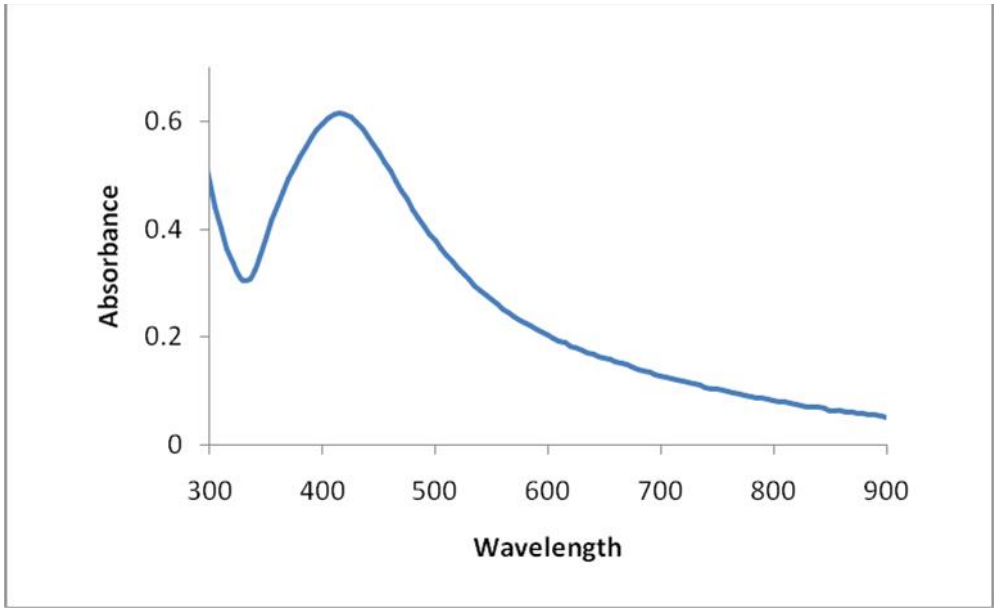
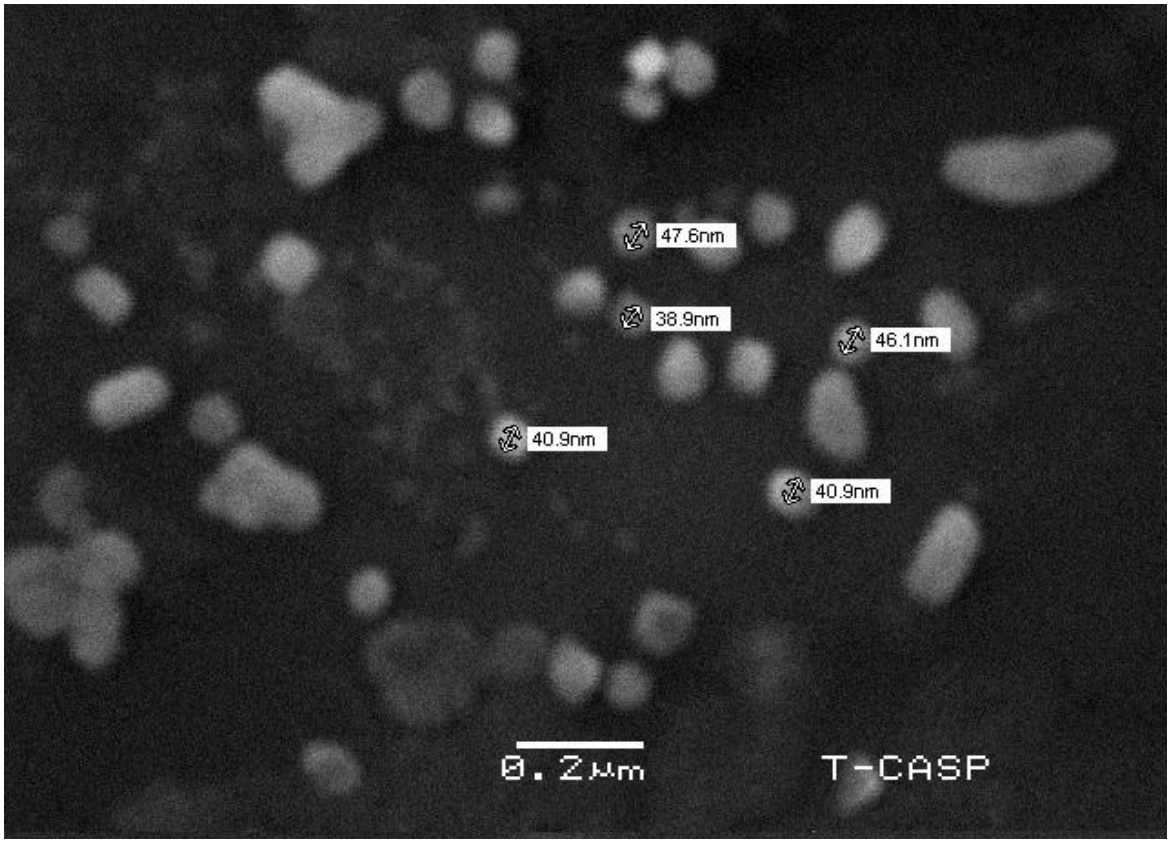


Figure1. UV–Visible spectrum of the green synthesized AgNPs



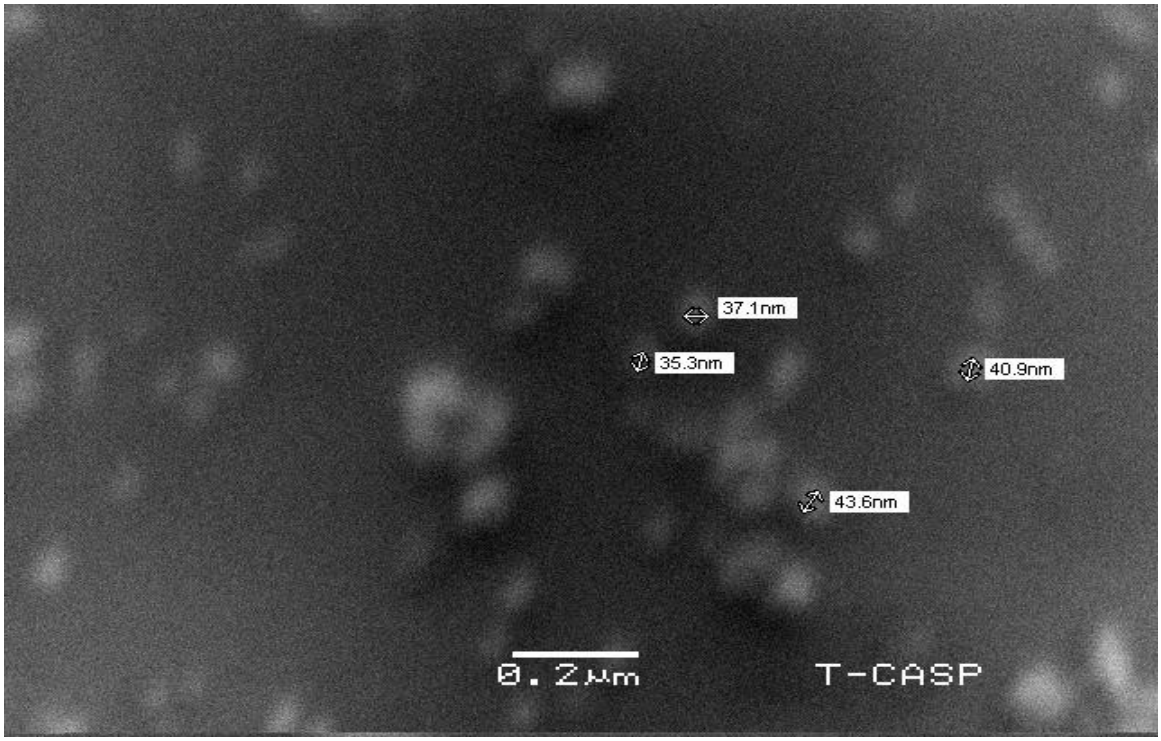


Figure2. Scanning Electron Microscopy (SEM) images of the AgNPs

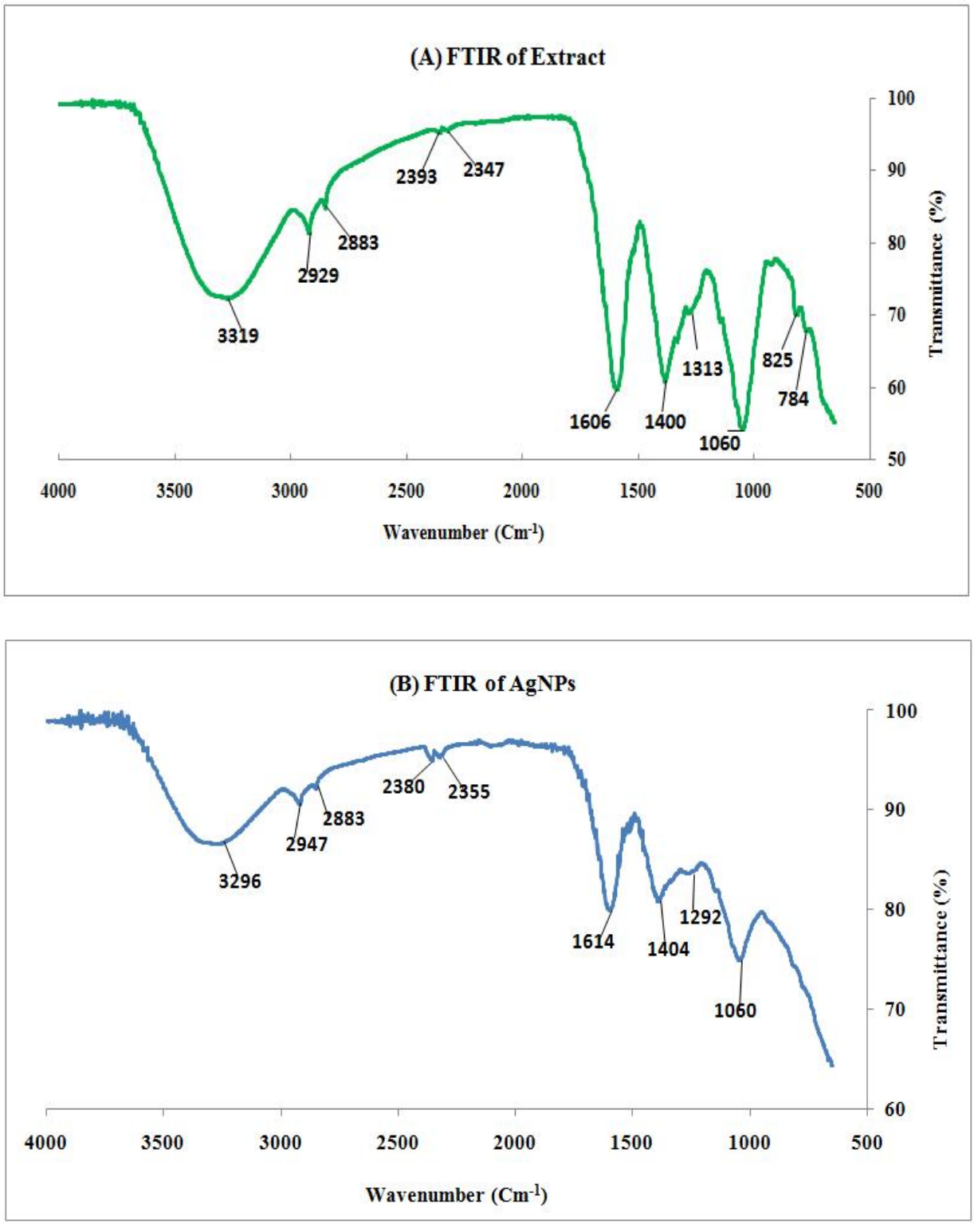


Figure3FTIR Spectrum of the (A) extract of the *O. tenuiflorum* (B)AgNPs of the *Ocimum tenuiflorum.*

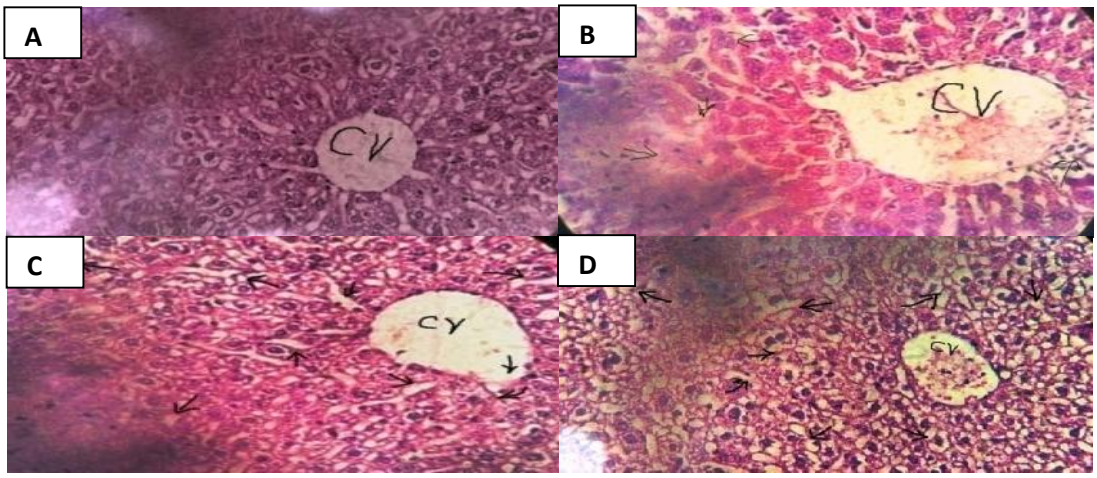


Figure 4. Liver histology at 7days post-treatment of green synthesized AgNPs at concentrations(A) Control (B) 50mg/kg dose(C) 75mg/kg dose(D) 100mg/kg dose

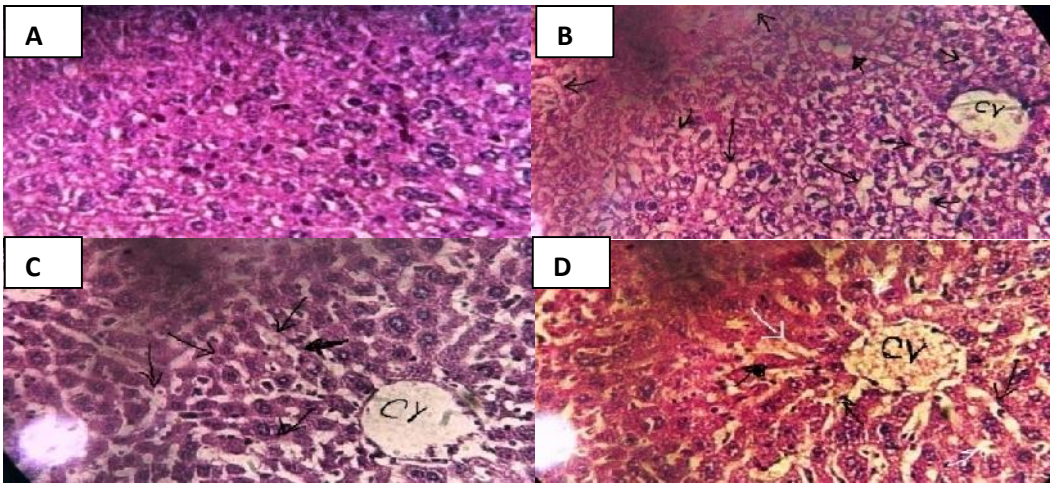


Figure 5. Liver histology at 14 days post-treatment of green synthesized AgNPs at concentrations(A) Control (B) 50mg/kg dose (C) 75mg/kg dose(D) 100mg/kg dose

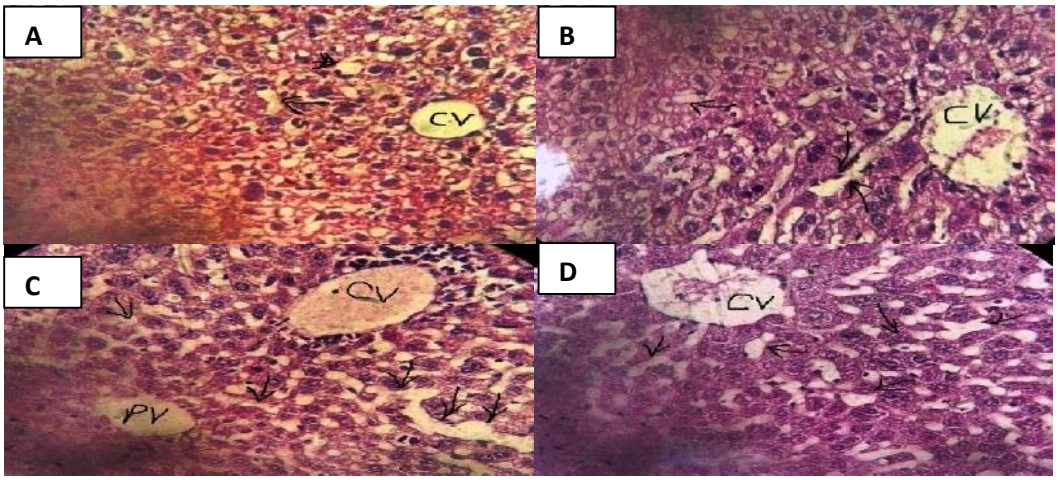


Figure6. Liver histology at 21 days post-treatment of green synthesized AgNPs at concentrations: (A) Control (B) 50mg/kg dose (C) 75mg/kg dose(D) 100mg/kg dose

Conclusions: Green synthesis approach yields safer and cost-effective nanoparticles. However, green synthesized nanoparticles are toxic at relatively higher concentrations than the nanoparticles derived through chemical synthesis. Nonetheless, the former nanoparticles are comparatively less toxic to the biological system(mammalian model).

Recommendations: Chemically synthesized nanoparticles may be replaced with green synthesized nanoparticles for various applications. More and more plant species for nanoparticles synthesis should be explored.

Acknowledgments: The authors are thankful to the Centre of Advanced Studies in Physics (CASP), Government College University, Lahore, for the provision of analytical facilities.

Conflict of Interest: The authors declare that they have no conflict of interest.

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