



## CHARACTERIZATION AND ANTI-HEPATIC FIBROSIS ACTIVITY OF AgNPs PRODUCED THROUGH GREEN SYNTHESIS METHOD USING *CITRULLUS COLOCYNTHIS* PLANT EXTRACT

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### Abstract:

Nanoparticles have intriguing properties that could assist the study of regenerative medicine. Silver Nanoparticles (AgNPs) have gained a lot of interest in a variety of industries, particularly pharmaceuticals, agriculture, water purification, filtration of air, the textile industry, and as a catalyst in oxidation reactions. The aim of the current study was to characterize the biosynthesized AgNPs and to evaluate their anti-hepatic activities through in-vivo studies. Biosynthesized AgNPs were characterized using various techniques including UV–VIS spectrophotometry, XRD, FESEM and FTIR analysis. The in-vivo anti-hepatic efficacy of AgNPs were assessed through morphological, biochemical and histological studies. For in-vivo studies, 12 Male Balb/C albino-mice were randomly divided into 3 groups (n = 4) including normal group, disease control group (Liver fibrotic model), and treatment group (liver fibrosis treated with AgNPs). Further, biochemical markers including bilirubin level was assessed for all treatment groups. In CCl<sub>4</sub>-induced fibrotic mice, a considerable increase in glycogen was observed after treatment with AgNPs. Biochemical investigations revealed that biochemical parameters for liver function such as bilirubin, was significantly lowered in AgNPs treated animals. Our findings indicate a preliminary multi-target efficacy for AgNPs against liver fibrotic and thus require further detailed investigation.

**Keywords:** Silver; Nanoparticles; Hepatic; Characterization; Mice; liver

### 1. Introduction

Over time, there has been a rise in interest in applying nanotechnology to enhance present methods of tissue and organ regeneration. Nanoparticles (NPs) are specific colloidal particles that can vary greatly in size, chemical makeup, and componentry. They typically range in size from 10 to 200 nm. Nanoparticles can be used as theragnostic agents or as means of delivery for medications, biological material, or growth factors (GFs) because of their small size and surface chemistry. Numerous

nanoparticle drug delivery systems have undergone rapid clinical development for cancer therapy (Rink *et al.*, 2013). The use of nanoparticles is an intriguing strategy because they frequently exhibit deep tissue entry, permitting more effective delivery of therapeutic substances targeting specific sites. After systemic treatment, they may also offer improved transport characteristics and pharmacokinetics profiles *in vivo* (Waite and Roth, 2012). Additionally, the extraordinary physiochemical characteristics of their tiny size enable the optimization of a wide range of fundamental qualities, including solubility, diffusivity, biodistribution, release characteristics, and immunogenicity. As delivery strategies for tissue repair, a number of different types of nanoparticle compositions have been studied. Direct administration, however, frequently has drawbacks such degradation, non-specificity, and inadequate cell absorption. As a result, greater amounts must be used, which increases the risk of adverse reactions and is therefore not inexpensive. Due to their capacity to transport significant amounts of (insoluble) medicines while preventing their deterioration, nanoparticle-based carriers may improve control over the pharmacokinetics of such drugs. Additionally, nanoparticles can be altered to enable precise cell and tissue targeting in addition to regulated administration of drugs (van and Habibovic, 2017). Regenerative therapy has advanced significantly over the past few decades as a result of the development of nanotechnology, and it now seems to be an exciting option for healing and regenerating damaged tissues and organs. Nanotechnology can affect and even change cellular activity because activity in cells takes place at the nanometer scale, improving the functionality of an organ or tissues in the process (Chaudhury *et al.*, 2014). Among the most popular metallic NPs in the biomedical area, AgNPs are also known as a colloid of nanometer sized particles and are employed primarily for their antibacterial properties. Physical or chemical procedures can be used to create these NPs. In the recent years, natural approaches to synthesize AgNPs have emerged. These techniques decrease silver ions utilizing microbes like bacteria, eukaryotic fungus, or plants. Since harmful chemicals are not used in the process, the method known as bioreduction of silver ions is thought to be more environmentally benign (Fathi-Achachelouei *et al.*, 2019). Research investigated the effects of AgNPs treatment on diabetic and burn wounds in an animal model as a potential wound healing accelerator (Tian *et al.*, 2007).

Due to their special properties, AgNPs have gained a lot of interest in a variety of industries, particularly pharmaceuticals, agriculture, water purification, filtration of air, the textile industry, and as a catalyst in oxidation reactions. Additionally, its main characteristic is strong antibacterial action against a variety of germs without being hazardous to mammalian tissues. (Pirtarighat *et al.*, 2019). Even today, there are many severe and complicated disorders that pose a serious threat to humanity, including diabetes, cancer, Alzheimer's disease, Parkinson's disease, and other heart disease, multiple sclerosis, and several serious inflamed or infectious diseases. The use of nanotechnology allows for the replication or regeneration of tissue that is injured. Tissue engineering, which uses these so-called chemically activated cells, might change the replacement of organs or the usage of synthetic implants (Nikalje, 2015). Over time, more focus has been placed on using nanotechnology to enhance existing methods for tissue and organ regeneration. Nanoparticles most especially have intriguing properties that could assist the study of regenerative medicine. Nanotechnology are solid particles with colloidal structure that can vary greatly in size, surface chemistry, and componentry. They typically range in size from 10 to 200 nm. Nanoparticles can be used as theranostic agents or as delivery systems for medications, genetic material, or growth factors (GFs) because of their size and surface chemistries. In fact, a wide range of nanoparticles, including dendrimers, liposomes, polymer-based nanoparticles, micelles, carbon nanotubes, and a lot more, have been produced for therapeutic purposes. For the treatment of cancer, a number of nanoparticle drug delivery methods have gone through quick development in clinical trials (Rink, *et al.*, 2013). Utilizing physical properties not visible at the microscale is made possible by operations at the level of the nanoscale, including the volume/surface ratio (Nikalje, 2015). The use of nanoshells made of gold to assist in the diagnosis and treatment of cancer and the use of liposomes as vaccine supplements and drug delivery vehicles are two examples of nanomedicine that have previously been tested in mice and are awaiting human trials (Boisseau

and Loubaton 2011). Similar to this, nanomedicine has also been effectively applied in rats for drug rehabilitation. Smaller gadgets used in medicine are less intrusive, more likely to be implanted inside the body, and have significantly faster biological reaction times. Nanotechnology-based drug delivery methods are quicker and more precise than conventional methods (LaVan *et al.*, 2003). The aim of this work was to characterize the biosynthesized AgNPs from *C. colocynthis* Plant extract and to evaluate its anti-hepatic fibrosis activity through in vivo study. The result of the study showed that the AgNPs produced through biosynthesis method from *C. colocynthis* extract efficiently regenerate the CCl<sub>4</sub> induced injured mice liver.

## 2. Materials and Methods

### 2.1 Biosynthesis of AgNPs using *C. colocynthis* Plant Extract

The collection of plant and extract preparation were performed as we have already described in our recent studies. Briefly the *whole plant of C. colocynthis* were collected from district Charsadda. Mud and dust were removed by washing with tap water followed by distilled water and then shed dried for one week at room temperature. To remove any mud and dust, plant was washed with tap water, followed by distilled water and then shad dried completely at room temperature for 1 to 2 weeks. After shad drying, the plant was crushed into fine powder via grinder and were brought to the laboratory in polythene bags. The synthesis of AgNPs from extract of *C. colocynthis* plant was performed as we previously described (Ayaz *et al.*, 2024). Briefly, we prepared aqueous solution of AgNO<sub>3</sub>. The aqueous solution of AgNO<sub>3</sub> (10 mM) were mixed with fresh plant extract of *C. colocynthis* at a ratio of 9:1 by adjusting of pH through the addition of a few droplets of HCl or NaOH.

### 2.2 Characterization of AgNPs

The size, crystal structure, elemental makeup, and a number of other physical features of nanoparticles have all been described using a variety of methodologies. Physical characteristics can frequently be assessed using a variety of methods. Following nanoparticle creation, the size, crystal structure, and chemical makeup of the NPs are also carefully examined. The choosing of the best appropriate method is made more difficult by the various advantages and disadvantages of each methodology; frequently, a combinatorial characterization approach is required. The notion of the approach being used, the data it can provide, or the materials it is intended for are used to categorize various characterization techniques. We outline the primary methods and how they serve the NPs characterization concepts.

#### 2.2.1 UV

An UV-visible spectrophotometer was used to test the optical characteristics of the biosynthesized NPs. Shimadzu UV-1650 PC Spectrophotometer was used to record UV-visible spectrum of absorption via a cell made of quartz in order to analyze the production of AgNPs. The samples were put within a cuvette with a 1-cm light path, and the light absorption bands were provided in relation to deionized water. For AgNPs a UV Visible absorption spectrophotometer with a resolution of 1 nm between 200 and 800 nm was utilized. The sample was pipetted into a cuvette with a volume of one milliliter, and it was then examined at the ambient temperature.

#### 2.2.2 FTIR

The produced AgNPs were described in this section using methods based on spectroscopy. FTIR spectral measurements were carried out to identify the potential biomolecules (functional groups) existed in plant extract which is responsible for reducing and capping the bio-reduced nanoparticles. FTIR spectroscopy using FTIR (SHIMADZHU) with a wavenumber range of about 4000–400 cm<sup>-1</sup>. Powdered NPs and potassium bromide (KBr) were mixed and finely ground at a concentration ratio of 1:10 in order to prepare samples for FTIR analysis. The acquired peak was plotted with wavenumber (cm<sup>-1</sup>) in the Y-axis and transmittance percentage (%) in the X-axis. Using a KBr pellet technique, FTIR spectra of powdered materials were captured.

### 2.2.3 XRD

One of the methods that is most frequently used for characterizing NPs is XRD. XRD typically provides information on the crystalline structure and size of NPs. XRD patterns of powdered samples were obtained by using powder X-ray Diffractometer (Bruker, Germany) instrument operating at a voltage of 50 kV and a current of 30 mA. The position and intensity of the peaks will be compared with the reference patterns available from the International Centre for Diffraction Database (ICDD) to determine the composition of the particles. The crystallinity and composition of the NPs were analyzed over a  $2\theta$  range from  $20^\circ$  to  $90^\circ$ .

### 2.2.4 Field emission scanning electron microscopy

FESEM was used to take the image of the sample by scanning with a high-energy beam of electrons. Surface morphology of green synthesized NPs were observed by FESEM (HITACHI, S-416). Nanoparticles samples were placed in a sample chamber and scanning was performed under different magnifications ranging from  $\times 15,000$  to  $50,000$ . To provide good conductivity of the electron beam, the samples were covered with a carbon coating before examining in FESEM. FESEM was performed with an accelerating voltage of 20 kV, counting time of 60 s, and probe current of 45 nA.

### 2.2.5 High-resolution transmission electron microscopy

The size and shape of the nanoparticles were examined by HRTEM (JEOL JEM-3010, Japan). The HRTEM image was recorded by dissolving the synthesized powder sample in double-distilled water in 1:10 ratio and then placed a drop of diluted solution on the surface of copper grid. The surplus solution was then blotted off the grid with filter paper after being permitted to sit for 1 minute. The grids were put in the grid box to dry at room temperature prior to imaging. A speeding up voltage was used for collecting the HRTEM images.

Analyses of particle size were performed using ImageJ 1.47v. Using a Microtrac S3500 particle size analyzer, the hydrodynamic diameters of the particles had been determined. The TEM micrographs were captured using a Philips CM 10 TEM that was operating at 100 kV of high voltage.

## 2.3 In-Vivo studies

For in-vivo studies we divide the mice into three groups (each group includes 4 mice): Group I (Normal group, which received only saline); Group 2 Mice consider as positive control and received  $\text{CCl}_4$  intraperitoneally at a dose of 1  $\mu\text{L/g}$  body weight twice/week for six weeks. Group 3 mice considered as AgNPs treated group (four weeks  $\text{CCl}_4$ -intoxicated mice) and were injected with 0.5 mg/kg dose of AgNPs intraperitoneally, dissolved in DMSO, twice for a week. Mice of all three experimental groups were sacrificed after six weeks to study the regeneration role of AgNPs of hepatic damage caused by  $\text{CCl}_4$ . The anti-hepatic activity of AgNPs was evaluated through morphological and biochemical parameters as we describes previously (Iqbal *et al.*, 2023).

## 3.0 RESULTS

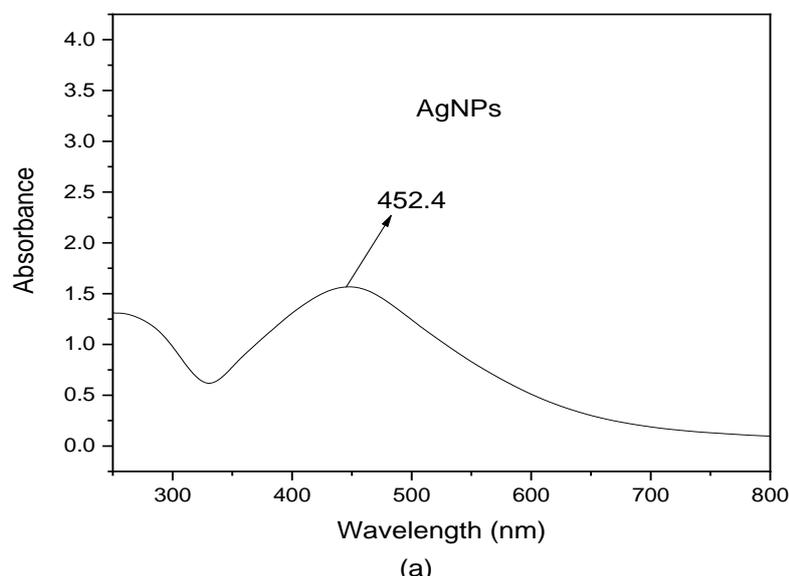
### 3.1 Green synthesized of AgNPs using *C. colocynthis* extract

The plant *C. colocynthis* has numerous biological importance, such as antioxidative, antibacterial, anti-cancerous, anti-inflammatory, anti-microbial and antidiabetic. In general, extracts of this plant showed considerable antioxidant and reducing properties. After boiling in deionized water and filtering through Whatman paper, the aqueous extract of *C. colocynthis* was dark greenish in color. Green synthesis of silver nanoparticle via *C. colocynthis* extract were confirmed as the dark green colour of the solution turned reddish brown as shown. The results indicate that with the passing of incubation time, the color intensity increased, indicating the reduction of Ag ions in  $\text{AgNO}_3$  into  $\text{Ag}^0$  and thus the formation of AgNPs.

### 3.2 Characterization of AgNPs

### 3.2.1 UV–vis spectra analysis

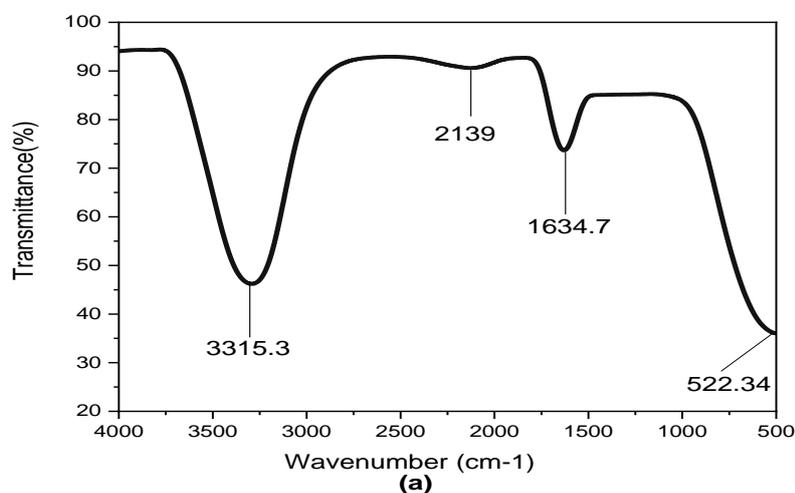
Formation of nanoparticles was determined by UV-visible spectrophotometer. NPs bioreduction was confirmed by subjecting diluted aliquots of the AgNPs to UV-visible spectrophotometry in the range of 200–800 nm. The maximum absorbance at 452.4 was observed in the visible UV spectra, confirming the formation of AgNPs as shown in figure 3.1.



**Figure 3.1.** UV–vis spectrum of AgNPs synthesized from  $\text{AgNO}_3$  via aqueous extract of *C. colocynthis*.

### 3.2.2 FTIR spectroscopy analysis

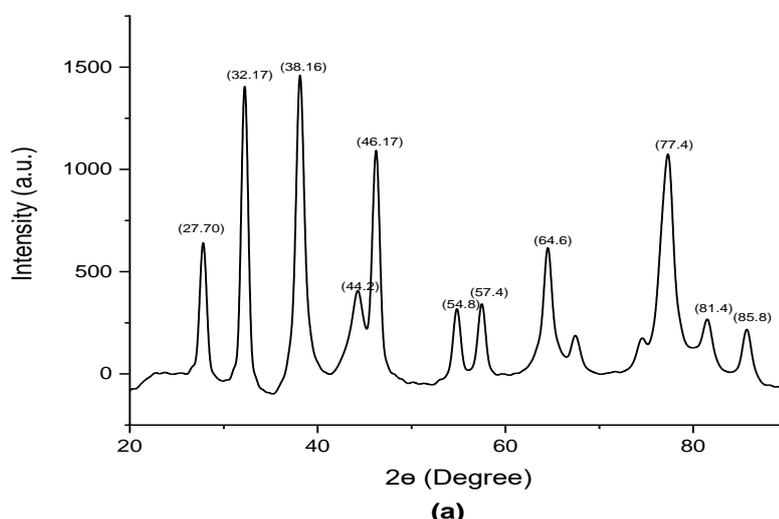
FTIR analysis was carried out to further identify the prominent bioactive molecules responsible for the stability and capping of the AgNPs. The different functional groups of plant extract with synthesized NPs was measured by FTIR in the range of 4000–500  $\text{cm}^{-1}$ . In the IR spectrum of AgNPs, the peaks in the range 3315.30  $\text{cm}^{-1}$  correspond to hydroxyl groups (OH). Absorption peak at 2139  $\text{cm}^{-1}$  represents the C–H stretch of alkynes groups. The characteristic absorption peak at 1634.7  $\text{cm}^{-1}$  in the spectrum is due to the stretching vibrations of carboxylates. A weaker band at 522.34  $\text{cm}^{-1}$  is the result of C–N–C bending in amines. Thus, the main functional groups involved in bioreducing of  $\text{AgNO}_3$  into AgNPs are these functional groups of *C. colocynthis*.



**Figure 3.2.** FTIR spectra of (a) AgNPs showing different peaks which show different functional group of *C. colocynthis* on NPs surface.

### 3.2.3. XRD analysis

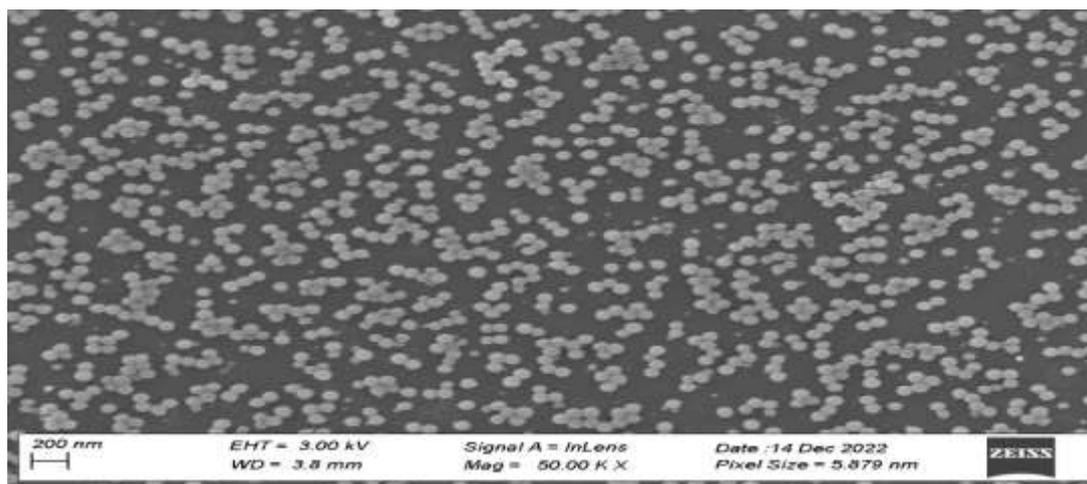
The purity and crystalline structure of NPs were confirmed by XRD pattern with strong diffraction peaks analyzed at different  $2\theta$ . The XRD pattern of the biologically synthesized AgNPs shows several peaks (Fig. 3.3), where the four main peaks located at  $38.10^\circ$ ,  $44.20^\circ$ ,  $64.41^\circ$  and  $77.39^\circ$ , corresponding to the (111), (200), (220) and (311) planes, respectively, to the facets of face-centered cubic (FCC) crystal structure of AgNPs (JCPDS, No. 04-0783). The presence of these sharp diffraction peaks indicate the better crystallinity of the biologically synthesized AgNPs. There are more peaks in the diffractogram at different degrees. These peaks reveal the crystallization of some plant metabolite moieties on the surface of the AgNPs, which is in agreement with Shanmuganathan *et al.*, results (Shanmuganathan *et al.*, 2018). Apart from these peaks, some other peaks have been identified to be due to AgCl or AgNO<sub>3</sub>, which might have not been reduced and hence remained in the sample in minute quantity (Rajendran, *et al.*, 2015). The average distribution of AgNPs formed in the bioreduction process was found to be around 30nm.



**Figure 3.3.** Characteristic peaks confirmed the crystalline structure of (a) AgNPs

### 3.2.4. Field emission scanning electron microscopy

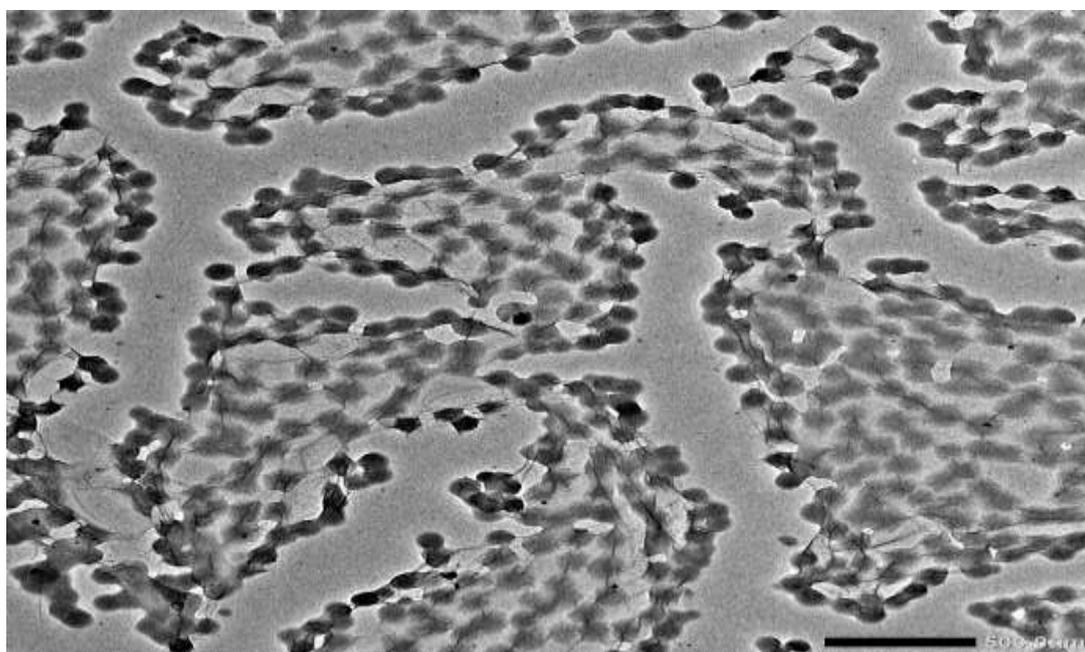
FESEM of crystalline NPs was done to analyze the surface and shape. The FESEM micrograph for Ag-capped with plant extract is given in figure 3.4. On the carbon-coated mounting grid a dried powder of AgNPs was used. Three different scanning scales were used to observe the topography and shape of the crystalline AgNPs, i.e., 100,000 $\times$ , 150,000 $\times$ , and 50,000 $\times$ . The SEM results show that the AgNPs have a uniform morphology and spherical in shape.



**Figure 3.4.** SEM micrographs of AgNPs at 50,000× magnification with prominent spherical NPs in the clusters.

### 3.2.5. High-resolution transmission electron microscopy

HRTEM was used to observe the AgNPs size and shape. The results shown by HRTEM a different frequency size distribution of AgNPs. Information on the mean size and SD was calculated by measuring the synthesized AgNPs in random fields of view (Figure 3.5). TEM showed an average particle core size of  $30.24 \pm 2.25$  nm for AgNPs, although size data provided by the manufacturer were range from 10 to 50 nm. All the nanoparticles were “homogeneous” surrounded by a faint and thin layer of organic capping material which might be plant extract. The results matched previously reported by El-Refai *et al*, (2018).



**Figure 3.5.** HRTEM images of representative. AgNPs HRTEM images show the size of AgNPs . The scale bars are 500 nm.

## 3.3. In-Vivo Studies Result

### 3.3.1. Effect on liver weight

The result showed that the liver weight of  $\text{CCl}_4$  treated mice (3.36 gm) was significantly increased as compared to normal liver. In group 3 mice the liver weight was significantly reduced (2.85 gm, closely to normal), by the treatment with AgNPs (Table 3.1). Thus therapeutic power of AGNPs treatment

was significantly high on CCl<sub>4</sub> injured mice.

**Table 3.1** Effect of AgNPs treatment on liver weight

Groups of mice	Group I (Normal)	Group II (Positive Control)	Group III (AgNPs Treated)
Weight in gm	2.95	3.36	2.85

### 3.3.2. Serum Level of Bilirubin

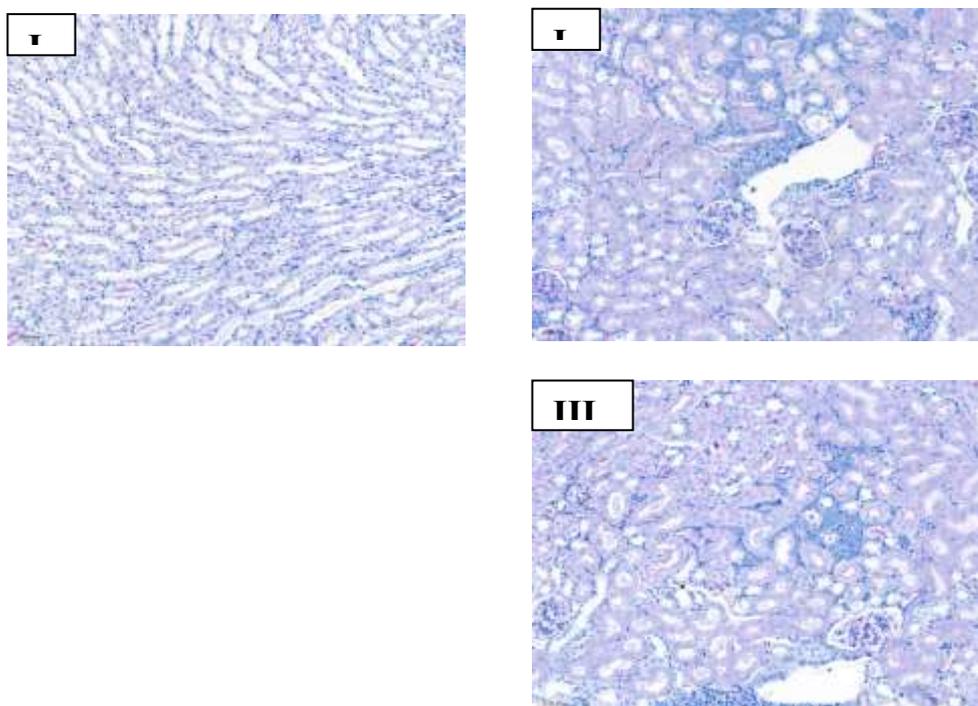
The serum bilirubin levels were analyzed in all groups of mice to estimate the effect of AgNPs on liver fibrotic model. The biochemical result of total bilirubin is very high in group II mice as compared to Group I, mice. However, serum level of bilirubin of group III (0.41 mg/dl) show decreased as compared to group II (1.178 mg/dl) as shown in Table 3.2. Thus value of serum bilirubin level in group III (AgNPs treated mice) is more closed to group I i.e. normal mice (0.24 mg/dl).

**Table 3.2** Effect of AgNPs treatment on liver weight

Groups of mice	Group I (Normal)	Group II (Positive Control)	Group III (AgNPs Treated)
Bilirubin (mg/dL)	0.24	0.98	0.41

### 3.3.3 PAS staining analysis

PAS staining was performed to evaluate the glycogen storage level in all groups of mice. PAS result of normal mice liver showed strong positive reaction of glycogen granules in the cytoplasm of hepatocytic cells (Figure 3.6). Conversely, weak positive reaction was noted in CCl<sub>4</sub> treated group. PAS staining results of AgNP treated liver section demonstrate high quantity of glycogen storage as close to normal group.



**Figure 3.6.** PAS staining results in liver sections showing; (I): high glycogen storage in normal group, (II): decreased glycogen storage in CCl<sub>4</sub> treated group, (III) Significant positive PAS reaction of glycogen granules in the cytoplasm of hepatocytes of AgNPs treated group.

## 4. Discussion

Hepatic fibrosis occurs in response to long-lasting liver damage where excessive buildup of fibrillar extracellular matrix occurs. This ECM is gradually replaced by an interstitial collagens, proteoglycans and fibronectin that leads to parenchymal cell damage and abnormal functioning of hepatocytes. As

the result its effects the normal function and architecture of liver. Chronic injury of the liver may be caused due to viral hepatitis, autoimmune, cholestatic, toxic compound, metabolic disorders such as nonalcoholic steatohepatitis (Sayaf *et al.*, 2018). One alternative way for treating liver fibrosis is nanotechnology. The science and technology of employing molecular instruments and molecular understanding of the body of humans to relieve pain, diagnose, cure, and avoid diseases and severe harm are collectively referred to as nanomedicine (El-Refai *et al.*, 2018).

According to another definition, the use of nanotechnology is the research and development that goes into the design, manufacturing, character development, and use of substances and devices whose tiny functional organization, in at least one dimension, is on the nanometer scale (between one and one hundred nanometers), or one billionth of a meter (Saini *et al.*, 2010). Because of features like reduced toxicity, enhanced breakdown, and bioavailability, AgNPs have been used for a variety of medicinal reasons. Due to their tiny dimensions, they have been observed interacting with biological systems without difficulty. AgNPs can be made in a variety of ways, but green synthesis is thought to be the most important one right now because it has so many benefits over chemical synthesis. AgNPs have been biosynthesized using a variety of biological sources, and these nanoparticles are utilized in a number of different industries. However, the most popular technique for biosynthesizing nanomaterials has recently been the production of AgNPs utilizing plants. AgNPs made from *Andrographis paniculata* are reported to have liver-protective qualities (Zhang *et al.*, 2019). In the current studies CCl<sub>4</sub> treated mice was used to study the effect of AgNPs for regeneration of liver fibrosis. The mice were treated with CCl<sub>4</sub> (1µl/g body weight) twice weekly for 4 weeks to prepared liver fibrosis mouse model. It has been concluded that after CCl<sub>4</sub> administration mice liver profile changes by causing alteration in liver weight from normal to disease state. In this study the liver morphology regarding weight showed that, mice treated with CCl<sub>4</sub> show a more increase in liver weight, compared to normal liver. Group III mice have more similarity to group I due to strong therapeutic effect of AgNPs (Table 3.1). our studies supported the previous literature as described (Huma el al., 2022; Iqbal *et al.*, 2023).

Liver is responsible for secreting many important enzymes for several biological functions such as detoxification of harmful substances as well as biological degradation. In the current study, the consequence of AgNPs was noted at serum bilirubin level in liver of mice disease model having injury induced by CCl<sub>4</sub>. After AgNps treatment (group III) reinstated the increase of liver enzymes as related to group II but a noteworthy decrease in liver enzyme was observed is group II to normal level (Table 3.2). Repossession of normal function of liver after AgNps treatment was also inspected histopathologically. The histopathological examination of mice liver treated with AgNps was evaluated by storage of glycogen storage through PAS staining. The liver of the group II mice were morphological and had less glycogen storage as compared to normal. After AgNPs treatment the glycogen level restored to normal as shown in Figure no 3.6. Our results support. Supported the previous studies as described (Khan el al., 2023). Therefore, from all these reports it was finally determined that the AgNPs showed a decrease effect on the reduction of hepatic fibrosis in CCl<sub>4</sub> injured mice.

## 5. Conclusion:

The current study demonstrated that AgNPs have robust regenerative capability on the reduction of liver fibrosis upon CCl<sub>4</sub> injured mice. AgNPs causes reduction of CCl<sub>4</sub> induce liver fibrosis by decreasing hepatic fibrogenesis and enhancing liver regenerative ability. Thus, from histopathological, biochemical and morphological results showed that AgNPs have high therapeutic effect on reduction of liver fibrosis.

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**Conflict of Interest:** All the authors declare that they have no conflict of interest.

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