Modulation of hepatotoxicity, DNA fragmentation and gene expression of *Solanum nigrum* leaves extract in rats treated with silver nanoparticles

Mosaad A. Abdel-Wahhab[1*], Helmy M.S. Ahmed[2], Sekena H. Abdel-Aziem[3], Aziza A. El-Nekeety[1], Heba A.M. Abd El-Kader[3], Mohamed S. Abdel-Aziz[4], Mohamed A. Hamazawy[5], Hafiza A. Sharaf[6], Hadeer M. Naguib[7], Ezzeldeen S. El-Denshary[2]

[1]Food Toxicology & Contaminants Dept., National Research Center, Dokki, Cairo, Egypt. [2]Toxicology & Pharmacology Dept., Faculty of Pharmacy, Cairo University, Cairo, Egypt. [3]Cell Biology Dept. National Research Center, Dokki, Cairo, Egypt. [4]Microbial Chemistry Dept., National Research Center, Dokki, Cairo, Egypt. [5]Pharmacology & Toxicology Dept., College of Pharmacy, Misr University for Science and Technology, 6th October City, Egypt. [6]Pathology Dept., National Research Center, Dokki, Cairo, Egypt, [7]New Kasr Al-Ainy Hospital, Cairo, Egypt.

ARTICLE INFO

ABSTRACT

The current study aimed to determine the antioxidant compounds in *Solanum nigrum* (*S. nigrum*) leaves extract, to synthesize silver nanoparticles (AgNPs) and to evaluate the protective role of the extract against the hepatotoxicity and genotoxicity of AgNPs compared to CCl₄ in rats. Eight groups of female Sprague-Dawley rats were treated orally for 3 weeks included the control group, CCl₄-trared group (0.1 ml/kg b.w twice a week), AgNPs-treated group (50 mg/kg b.w/day), AgNPs plus CCl₄-treated group, *S. nigrum* leaves extract-treated group (0.5 mg/kg b.w) and the groups treated with AgNPs and/or CCl₄ plus the extract. The results indicated that the extract was rich in the total phenolic, flavoniods and β-carotene. The size of synthesized AgNPs was 30-50 nm. Administration of AgNPs and/or CCl₄ resulted in severe hepatotoxicity and histological changes, increased DNA fragmentation and down regulation of antioxidant gene expression in liver. The extract was safe and succeeded to mitigate the hazards effect of AgNPs and/or CCl₄. It could be concluded that AgNPs have toxic effects and caution should be taken when they use in food or medical application. *S. nigrum* extract succeeded to protect the liver due to its higher content of antioxidant compounds.

INTRODUCTION

Recently, silver nanoparticles (AgNPs) are widely used in different medical products and hygiene application due to their antibacterial (Ayala-Núnez *et al*., 2009), antiviral (Mehrbod *et al*., 2009) and antifungal properties (Kim *et al*., 2008; Abdel-Aziz *et al*., 2014). AgNPs are also used in the disinfection of drinking water (Lv *et al*., 2009), swimming pools antifouling (Yang *et al*., 2009), bedding, toothpaste, washing machines, shampoo, nipples and nursing bottles, deodorants, fabrics, kitchen utensils, toys, filters (MacNee and Donaldson, 2003; Jia *et al*., 2009) and as a promising antibacterial additive to

water-based paints (Holtz *et al*., 2012). AgNPs also modulate cytokine so they promote wound healing (Wong *et al*., 2009). Despite the widespread use of the products containing AgNPs, the subchronic and chronic toxicity data of AgNPs remain rare. Several biological and medical reports suggested that silver ions are released into the blood medical devices and accumulate in the liver and kidney resulting in liver and kidney toxicity which may ultimately lead to death (Park *et al*., 2010). Thus, it is supposed that AgNPs have toxicity however the mechanism of their toxicity is not clear (Tang and Xi, 2008). Several reports suggested that AgNPs showed more toxicity than other metals such as nickel, iron, aluminum and manganese (Braydich-Stolle *et al*., 2005). However, the lack of exposure data on AgNPs in the workplace and their released from the products or into the environment makes it difficult to assess the risks of using these materials.

* *Corresponding Author*
E-mail: *mosaad_abdelwahhab @ yahoo.com*

Several techniques have been used for synthesize of AgNPs including the chemical and physical methods which use huge amount of toxic chemicals with high temperature, so the use of a safe alternative method is of great demand (Quaresma *et al*., 2009). AgNPs can be synthesized by green chemistry using natural organisms which offered a reliable, nontoxic simple, and eco-friendly (Abdel-Aziz *et al*., 2014). Therefore, during the last years researches have used the biological systems for the synthesized of nanoparticle (Tsibakhashvili *et al*., 2010) using microorganisms, enzyme and plant or plant extract as alternatives methods (Nair and Pradeep, 2002). Moreover, the biosynthesis of AgNPs by plant surpasses the other biological methods (Willner *et al*., 2006). The green synthesis of AgNPs using the leaves broth of *Argimone maxicana* produced particles sized of 20 nm effective against many bacterial and fungal pathogens (Khandelwal *et al*., 2010). The aims of the current study were to: (1) determination of total phenolic content, total flavonoids and β-carotene of *S. nigrum* leaves extract, (2) to synthesis and characterized AgNPs using *S. nigrum* leaves extract, (3) to evaluate the hepatotoxicity and cytotoxicity of AgNPs compared to CCl₄ and (4) to evaluate the possible protective role of *S. nigrum* leaves extract against AgNPs-induced hepatotoxicity in rats

MATERIALS AND METHODS

Chemicals and Kits

Carbon tetrachloride (CCl₄) was supplied from Morgan, Cairo, Egypt. Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) kits were purchased from Spectrumdiagnostics Co. (Cairo, Egypt). Alkaline phosphatase (ALP), Gamma Glutamyltransferase (GGT), creatinine, urea, uric acid, catalase (CAT), glutathione peroxidase (GPx), total antioxidant capacity (TAC), tumor necrosis factor alfa (TNF-α), nitric oxide (NO), malondialdehyde (MDA) kits were purchased from Biodiagnostic Co. (Giza, Egypt). All other chemicals used throughout the experiments were of the highest analytical grade available.

Fig. 1: *S. nigrum* plant.

Preparation of *S. nigrum* extract

This process has been performed after collecting the *S. nigrum* (Family: Solanaceae; Fig. 1) leaves during late summer

2015 from canal bank habitat around agriculture area in Dekernis, Dakahlia Governorate, Egypt. The green leaves were thoroughly washed with distilled water to get rid of any strange materials especially dust and dirt. Twenty g of clean leaves were boiled in 50 ml distilled water in Erlenmeyer flask of 500-ml volume for 30 min and the leaf debris were removed by filtration through Whatman filter paper no. 1 and the produced extract was subjected to freeze drying.

Determination of total phenol and total flavonoid content of *S. nigrum* extract

Total phenol was determined in the *S. nigrum* leaves extract by Folin-Ciocalteau reagent in alkaline medium and was expressed as gallic acid equivalents (Singh *et al*., 2002). However, total flavonoid content was determined according to Kim *et al*. (2003) and was expressed as catechin equivalents.

Determination of β-Carotene

The determination of β-carotene in the plant extract was carried out according to the method described by Wettasinghe and Shahidi (1999). Briefly, 2 ml of β-carotene solution (0.2 mg/ml in chloroform) were pipetted into a round-bottom flask containing 20 μl linoleic acid and 200 μl Tween 20. The solvent was evaporated from the mixture at 40 °C for 10 min. A volume of 100 ml distilled water was added immediately and after the mixture was agitating vigorously, 5 ml aliquots of the resulting emulsion were transferred into test tubes containing different concentration (5-20 mg/ml) of the extracts. The mixture was vortexed and placed in a water bath at 50 °C for 2 h while the absorbance of the tested sample was repeatedly measured every 15 min at 470 nm using a UV-VIS spectrophotometer against a blank solution contained the same concentration of sample without β-carotene. All determinations were performed in triplicates and the total antioxidant activity was calculated based on the following equation:

$$AA = 1 - (A_0 - A_t) / (A^0_0 - A^t_0)$$

where AA is antioxidant activity, A_0 and A^0_0 are the absorbance values measured at initial time of the incubation for samples and control, respectively, while A_t and A^t_0 are the absorbance in the samples and control at t = 120 min.

Biosynthesis of Silver nanoparticles (AgNPs)

AgNPs were biologically synthesized from *S. nigrum* (Family: Solanaceae) leaves extract (Abdel-Aziz *et al*., 2014; Dwivedi and Gopal, 2011). Fifty milliliter of silver nitrate solution (3 mM) were prepared in stopper conical flask and 5 ml of the previously prepared leaf extract were added and left at room temperature for 1h and the produced brownish yellow or reddish brown color indicate the biosynthesis of AgNPs.

Characterization of AgNPs
UV-vis adsorbance spectroscopy analysis

The bioreduction of silver nitrate (AgNO₃) to AgNPs was monitored periodically by UV-vis spectroscopy (Shimazu

2401PC) after the dilution of the samples with deionized water as described by Raut *et al.* (2010) UV-vis spectrograph of AgNPs was recorded by using a quartz cuvette with water as reference and the readings were recorded at a scanning speed of 200 to 800 nm (Leela and Vivekanandan, 2008).

TEM analysis of AgNPs

AgNPs were sampled by TEM analysis using JEOL model 1200 EX electron microscope. The samples were prepared by placing a drop of the suspension of AgNPs solutions on carbon-coated copper grids. After the evaporation of water, the samples on the grids were allowed to dry for 4 minutes and the shape and size of AgNPs were determined from TEM micrographs (Elavazhagan and Arunachalam, 2011).

Experimental animals

Three-month old sexually mature female Sprague-Dawley rats (150-160 gm) were purchased from Animal House Colony, Misr University for Science and Technology, 6 October City, Giza, Egypt. Animals were maintained on the specified diet and housed in filter top polycarbonate cages in a room free from any source of chemical contamination, artificially illuminated (12 h dark/light cycle) and thermally controlled (25 ± 1 $^{\text{O}}$C) at the Animal House Lab, Misr University for Science and Technology. All animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of Misr University for Science and Technology, Giza, Egypt and the National Institute of Health (NIH publication 86-23 revised 1985).

Experimental design

Animals were divided into eight groups (10 rats/group) and were maintained on their respective diet and treated orally for 3 weeks as follows: group1; normal control animals which fed on basal diet and water without any treatment, group 2; animals treated with CCl_4 suspended in corn oil (0.1 ml/kg b.w) twice a week (Feng *et al.,* 2010), group 3; rats treated with aqueous solution of AgNPs (50 mg/kg b.w), group 4; rats were treated with AgNPs plus CCl_4, group 5; rats treated with water extract of *S. nigrum* leaves (0.5 mg/kg b.w), group 6, rats treated with AgNPs plus *S. nigrum* leaves extract, group 7; rats were treated with *S. nigrum* leaves extract plus CCl_4, group 8; rats were treated with AgNPs and CCl_4 plus *S. nigrum* leaves extract.

The animals were observed daily for signs of toxicity during the experimental period. At the end of the treatment period (i.e. day 21), all animals were fasted for 12 hr, then blood samples were collected via the retro-orbital venous plexus under ether anesthesia. The blood samples were used for the determination of ALT, AST, ALP, GGT, createnine, uric acid, urea, NO and TNF-α according to the manufacture instaurations. After the collections of blood samples, animals were sacrificed and samples of liver of each animal were dissected, weighted and homogenized in phosphate buffer (PH 7.4) to give 20% w/v homogenate (Lin *et al.*, 1998). This homogenate was centrifuged at 1700 rpm and 4 $^{\text{O}}$C for 10 min, the supernatant was stored at (-70$^{\text{o}}$C) until analysis and it

used for the determination of MDA. This supernatant was further diluted with phosphate buffer solution to give 2% and 5% dilutions for determination of hepatic GPx (2%), TAC and CAT (0.5%) activities. Samples of liver were collected, excised and fixed in natural formalin and were hydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin. Sections (5 μm thick) were cut and stained with hematoxylin and eosin (HX & E) for the histological examination (Drury & Wallington 1980). Other samples of liver tissues were collected and stored at -70 $^{\text{O}}$C for cytotoxucity studies.

DNA fragmentation by diphenylamine assay (DPA)

Liver tissues were lysed in 0.5 ml of lysis buffer containing 10 mM tris-HCl (pH 8), 1 mM EDTA, 0.2 % triton X-100, centrifuged at 10,000 rpm for 15 min at 4$^{\text{O}}$C to separate intact chromatin in the pellet from fragmented/damaged DNA in the supernatant. The pellets were resuspended in 0.5 N perchloric acid (P) and 5.5 N perchloric acid was added to supernatants (S) to attain a final concentration of 0.5 N. The samples were incubated at 90 $^{\text{O}}$C for 20 min and centrifuged at 10 000 rpm for 10 min to remove proteins. Subsequently, 160 ml of diphenylamine (DPA) solution [150 mg DPA in 10 ml glacial acetic acid, 150 ml of sulfuric acid and 50 ml acetaldehyde (16 mg/ml)] was added to each sample and incubated at room temperature for 24 h (Gibb *et al.*, 1997). Absorbance was measured at 600 nm using a UV double beam spectrophotometer (Shimdazu 160 A; Shimadzu Co., Japan). The proportion of fragmented DNA was calculated from absorbance reading at 600 nm. The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the equation:

DNA fragmentation = OD of fragmented DNA (supernatant) / [OD of fragmented DNA (supernatant) + OD of intact DNA (pellet)] × 100

DNA gel electrophoresis laddering assay

Apoptotic DNA fragmentation was qualitatively analyzed by detecting the laddering pattern of nuclear DNA according to Lu *et al.* (2002). In brief, liver tissues were homogenized, washed overnight at 37 °C in PBS and lysed in 0.5 ml of DNA extraction buffer (50 mM Tris-HCl, 10 mM EDTA. 0.5% Triton, and 100 μg/ml proteinase K, pH 8.0). The lysate was then incubated with 100 μg/ml DNase-free RNase for 2 h at 37 $^{\text{O}}$C followed by three extractions of an equal volume of phenol/chloroform (1:1 v/v) and a subsequent re-extraction with chloroform by centrifuging at 15,000 rpm for 5 min at 4 $^{\text{O}}$C. The extracted DNA was precipitated in 2 volume of ice-cold 100% ethanol with 1/10 volume of 3 M sodium acetate, pH 5.2 at -20 $^{\text{O}}$C for 1h, followed by centrifuging at 15,000 rpm for 15 min at 4 $^{\text{O}}$C. After washing with 70% ethanol, the DNA pellet was air-dried and dissolved in 10 mM Tris-HCl/1 mM EDTA, pH 8.0. The DNA was then electrophoresed on 1.5% agarose gel and stained with ethidium bromide in Tris/acetate/EDTA (TAE) buffer (pH 8.5, 2 mM EDTA, and 40 mM Tris-acetate). A 100-bp DNA ladder (Invitrogen, USA) was included as a molecular size marker and

DNA fragments were visualized and photographed by exposing the gels to ultraviolet transillumination.

Semi-quantitative -PCR
Isolation of total RNA

RNA was isolated from hepatocytes by the standard TRIzol® Reagent (Invitrogen™, Carlsbad, CA, USA) extraction method. RNA was dissolved in diethylpyrocarbonate (DEPC)-water by passing solution a several times through a pipette tip. To digest DNA residues, RNA was treated with 1 U of RQ1 RNase-free DNase and re-suspended in DEPC water. Purity of RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Integrity was confirmed with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

Reverse transcription and semi-quantitative polymerase chain reaction (sq-PCR)

To perform a semi-quantitative-PCR, 1 µg of isolated total RNA was reverse-transcribed into cDNA with an RT PreMix Kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. In brief, cDNA synthesis was carried out by PCR (Thermo-Cycler 9700, Germany) at 45 ^{O}C for 1h and 95 ^{O}C for 5 min. Then, the PCR mixture consisted of cDNA 2 µl, 10× PCR buffer 2 µl, dNTPs 2 µl, Taq DNA polymerase 0.2 µl, forward/reverse primer 0.2µl and 0.1% diethylpyrocarbonate water for final volume 20 µl were amplified as follows: denaturation at 95 ^{O}C for 5 min and 94 ^{O}C for 30s, annealing at 50 ^{O}C for 30s, extension at 74 ^{O}C for 1 min (35 cycles) and final extension at 72 ^{O}C for 10 min. The primers GPx (GPx-F: 5′-CTCTCCGCGGTGGCACAGT-3′, GPx-R: 5′-CCACCACCGGGTCGGACATAC-3) (GenBank: M21210). The primer Cu-Zn SOD (Cu-Zn SOD- F: 5′-GCAGAAGGCAAGCGGTGAAC-3′, Cu-Zn SOD- R: 5′-TAGCAGGACAGCAGATGAGT-3′) (GenBank: X05634) genes were normalized on the bases of GAPDH GAPDH -F: 5′-CAAGGTCATCCATGACAACTTTG-3′, GAPDH-R: 5′-GTCCACCACCCTGTTGCTGTAG -3′ (Wiame *et al.*, 2000).

Semi-quantitative -PCR

The PCR product was run on a 1.5% agarose gel in Tris-borate-EDTA buffer and visualized over a UV Trans-illuminator. The ethidium bromide-stained gel bands were scanned and the signal intensities were quantified by the computerized Gel-Pro (version 3.1 for window 3). The ratio between the levels of the target gene amplification product and the GAPDH (internal control) was calculated to normalize for initial variation in sample concentration as a control for reaction efficiency. All PCRs were independently replicated three times (Raben *et al.*, 1996)**.**

Statistical analysis

All data were statistically analyzed by analysis of Variance (ANOVA) using the General Linear Model Procedure of the Statistical Analysis System (SAS, 1982). The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio (Waller and Duncan, 1969). All statements of significance were based on probability of $P \le 0.05$.

RESULTS

The results of the current study revealed that the aqueous extract of *S. nigrum* leaves is a promise antioxidant agent due to the higher content of total phenolic and flavoniods (Fig. 2). The data revealed that the extract contained 75.2 ± 2.35 mg/g gallic acid equivalent total phenol and 13.21 ± 1.62 mg/g gallic acid equivalent total flavonoids. Moreover, the results of β-carotene oxidation (Fig. 3) demonstrated also a higher antioxidant activity and the recorded IC_{50} values of β-carotene were 16.23 ± 1.1 µg / ml at the lowest concentration of the aqueous extract (5 mg/L) and increased to 52.16 ± 1.16 µg / ml at the highest concentration of the extract (20 mg/ L).
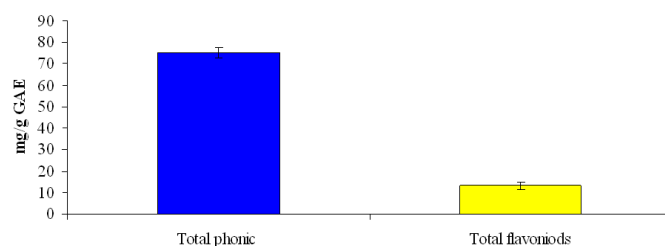

Fig. 2: Total plenolic and total flavoniods concentrations in the aqueous extract of *S. nigrum* leaves
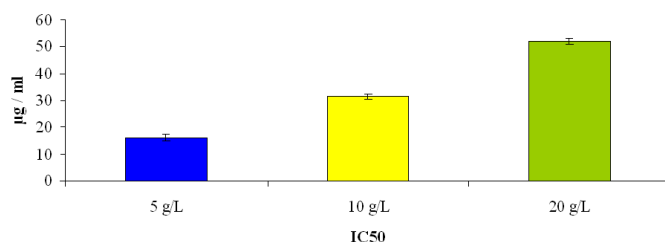

Fig. 3: IC_{50} of β-carotene content in the aqueous extract of *S. nigrum* leaves

Synthesis and characterization of AgNPs

The results of AgNPs synthesize showed that addition of 200 µg of the *S. nigrum* leaves extract to 50 ml of 5 mM aqueous silver nitrate ($AgNO_3$) and the incubation overnight at 40 ^{O}C in dark resulted in the formation of the brown solution which indicated the biosynthesis of AgNPs (Fig. 4a). Spectrophotometric study of the produced brown colored solution through the rage spectra 190-800 nm using Shimadzu UV/VIS 2401PC showed a maximum absorption at 450-500 nm (Fig. 4b). The TEM analysis revealed that the size of AgNPs ranged between 30 and 50 nm (Fig. 4c). The results of *in vivo* study indicated that the treatment with AgNPs and/or CCl_4 resulted in a significant increase in serum AST, ALT, ALP, GGT, creatinine, uric acid, urea, TNF-α and NO (Table 1). Animals treated with *S. nigrum* leaves extract showed a significant increase in GGT and a significant decrease in urea, TNF-α and NO however, the other biochemical parameters were comparable to the control group.
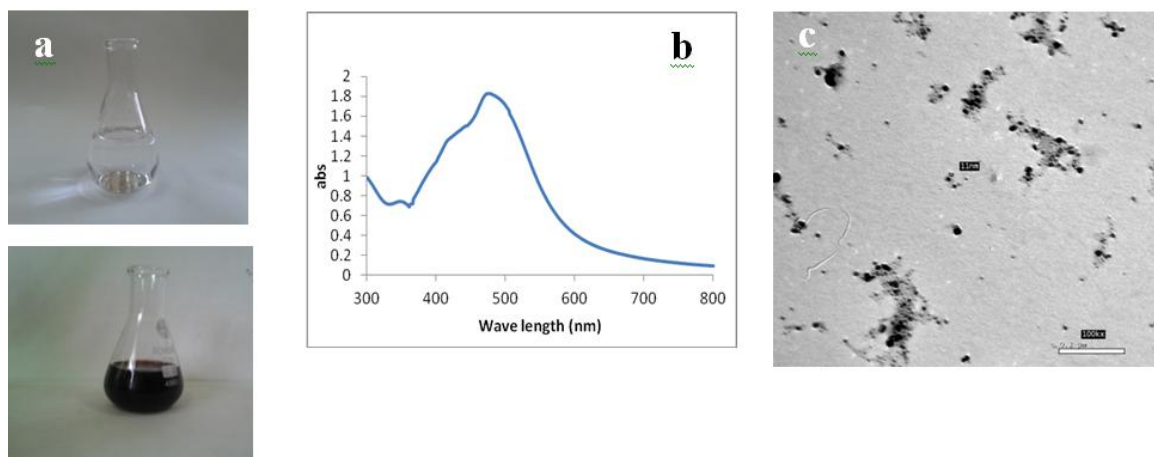
Fig. 4: Changes of the silver nitrate colour to reddish-brown after the addition of *S. nigrum* leaves extract when added to silver nitrate (3 mM) **(a)** and the produced solution showed Uv/Vis absorbance at 460 nm **(b)**. Transimittance electron microscope (TEM) image of the prepared AgNPs revealed that the produced particles were in the nano form and their sizes ranged from 2 to 16 nm **(c)**.

Table 1: Effects of *S. nigrum* leaves extract on serum biochemical parameters in rats treated with AgNPs and/or CCl₄.

Groups parameters	Control	AgNPs	CCl$_4$	AgNPs + CCl$_4$	SNLE	AgNPs + SNLE	CCl$_4$ + SNLE	AgNPs + CCl$_4$ + SNLE
AST (U/L)	88.20 ± 1.52^a	109.80 ± 2.39^c	157.40 ± 9.39^b	193.86 ± 1.28^d	90.14 ± 2.95^a	87.43 ± 0.84^a	93.40 ± 2.27^a	89.32 ± 2.09^a
ALT (U/L)	20.20 ± 1.49^a	26.43 ± 2.23^c	42.80 ± 3.34^b	67.60 ± 2.77^d	21.143 ± 2.51^a	17.29 ± 1.38^a	27.60 ± 1.99^c	28.0 ± 1.64^c
ALP (U/L)	119.61± 8.84^a	145.17 ± 9.77^c	478.83 ± 38.47^b	482.03 ± 11.33^b	120.84 ± 1.83^a	207.92 ± 5.29^d	157.71 ± 19.41^e	184.28 ± 12.75^f
GGT (mg/dl)	9.42 ± 1.30^a	23.14 ± 1.53^c	44.4 ± 1.86^b	52.36 ± 1.62^e	11.00 ± 1.13^d	13.17 ± 1.03^d	15.20 ± 1.20^f	18.20 ± 1.32^g
Creatinine (mg/dl)	1.15 ± 0.12^a	3.12 ± 0.49^c	5.95 ± 0.64^b	9.18 ± 0.07^e	1.14 ± 0.07^a	2.18 ±0.04^d	3.19 ± 0.08^c	4.18 ± 0.06^f
Uric acid (mg/dl)	2.34 ± 0.45^a	3.05 ± 0.36^c	5.78 ± 0.29^b	8.16 ± 0.38^d	2.32 ± 0.28^a	2.46 ± 0.21^a	3.09 ± 0.39^c	4.86 ± 0.34^e
urea (mg /dl)	29.99 ± 5.13^a	34.74 ± 1.01^c	46.84 ± 3.50^b	58.37 ± 2.70^e	25.49 ± 1.31^d	29.79 ± 2.68^a	32.79 ± 8.75^c	35.33 ± 1.26^c
TNF-α (mg/dl)	74.60 ± 0.93^a	91.57 ± 0.20^c	112.94 ± 0.84^f	135.20 ± 1.74^f	69.71 ± 3.57^d	84.57 ± 3.29^e	97.00 ± 1.09^c	100.00 ± 2.22^c
NO (µmol/L	710 ± 34.06^a	750 ± 29.44^c	919.16 ± 44.91^b	994 ± 22.49^e	665 ± 44.03^d	718.57 ±77.44^a	725 ± 20.12^a	788 ± 26.91^f

Within each raw, means superscript with different letters (a,b,c…) are significantly different (P≤0.05).
SNLE: *S. nigrum* leaves extract.

Table 2: Effects of *S. nigrum* leaves extract on MDA and antioxidant parameters in liver of rats treated with AgNPs and/or CCl₄.

Parameters Groups	MDA (nmol/g tissue)	TAC (nmol/g tissue)	CAT (nmol/g tissue)	GSH (mg/g tissue)
Control	69.45 ± 5.25^a	528.50 ± 13.35^a	763.33 ± 20.67^a	6.92 ± 0.38^a
AgNPs	85.72 ±8.16^c	470.43 ± 1.81^c	608.00 ± 6.76^c	4.44 ± 0.38^c
CCl$_4$	101.23 ± 8.83^b	421.99 ± 12.17^b	503.00 ± 27.81^b	3.64 ± 0.24^b
CCl$_4$ + AgNPs	113.97 ±8.81^e	361.60 ± 2.50^f	403.20 ± 5.31^e	2.25 ± 1.09^f
SNLE	61.39 ±8.56^d	593.50 ± 3.27^d	891.50 ± 9.061^d	7.92 ± 0.66^d
AgNPs + SNLE	70.90 ±6.61^a	502.43 ± 3.06^e	760.57 ± 17.11^a	5.02 ± 0.97^e
CCl$_4$ + SNLE	73.16 ±8.35^a	492.33 ± 10.96^g	663.83 ± 16.79^f	4.36 ± 1.46^c
CCl$_4$ + AgNPs + SNLE	89.52 ±6.40^f	435.20 ± 24.54^b	652.00 ± 31.47^f	3.61 ± 0.46^b

Within each column, means superscript with different letters (a, b, c…) are significantly different (P≤0.05).
SNLE: *S. nigrum* leaves extract.

Moreover, the extract succeeded to induce a significant improvement in the groups treated with AgNPs and/or CCl₄ towards the normal level of the control group (Table 1).

Animals treated with AgNPs and/or CCl₄ showed a significant increase in hepatic MDA accompanied with a significant decrease in GPx, TAC and CAT (Table 2). However treatment with the extract alone resulted in a significant increase in GPx, TAC and CAT accompanied with a significant decrease in MDA level.

The combined treatment with the extract plus AgNPs and/or CCl₄ resulted in a significant improvement in the antioxidant enzymes activity and MDA level towards the control level although it did not normalize these parameters except MDA in the group treated with the extract plus AgNPs (Table 2).

DNA fragmentation by diphenylamine (DPA)

The results presented in Table (3) indicate that treatment with CCl_4 and/or AgNPs induced DNA damage in liver cells as evaluated by measuring the level of fragmented DNA colorimetric using diphenylamine (DPA). The recorded percentage of DNA fragmentation in liver cells of rats treated with CCl_4 and AgNPs were high (33.2 ± 2.9 and 25.9 ± 1.8, respectively) compared to the untreated control group (8.0 ± 0.54). Meanwhile, treatment with *S. nigrum* extract decreased the percentage of DNA fragmentation as compared to the control (7.1 ± 0.69). Co-treatment with *S. nigrum* extract plus AgNPs and/or CCl_4 succeeded to induce a significant improvement in the percentage of DNA fragmentation towards the control value (15.7 ± 0.42, 16.2 ± 0.77 and 21.9 ± 0.86) although the percentage of DNA fragmentation was still higher than the control (Table 3). Moreover, DNA fragmentation in different treatment groups was also detected by gel electrophoresis as a DNA ladder representing a series of fragments (Fig. 5).

Table 3: Effect of *S. nigrum* extract on the percentage of DNA fragmentation in liver of rats treated with AgNPs and/or CCl_4.

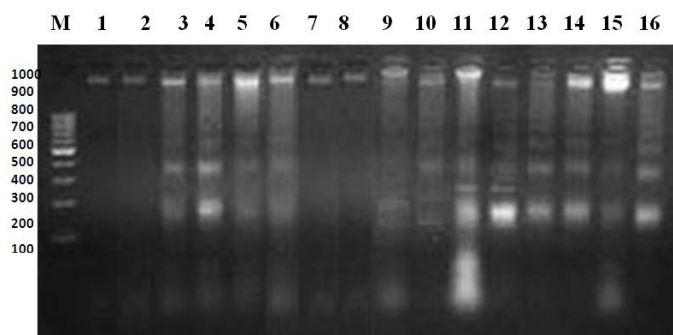| Treatment	DNA fragmentation % (M ± SE)	% of Change compared to control
Control	8.0 ± 0.54^a	--
AgNPs	25.9 ± 1.8^c	+ 17.9
CCl_4	33.2 ± 2.9^d	+ 25.2
AgNPs + CCl_4	36.6 ± 1.8^d	+ 28.6
SNLE	7.1 ± 0.69^a	- 0.9
AgNPs + SNLE	15.7 ± 0.42^b	+ 7.7
CCl_4 + SNLE	16.2 ± 0.77^b	+ 8.2
AgNPs + CCl_4 + SNLE	21.9 ± 0.86^c	+ 13.9

Fig. 5: Effects of SNLE on DNA fragmentation in hepatic tissue of rats treated with AgNPs and/or CCl_4. Agarose gel electrophoretic pattern of DNA isolated from liver tissue of different groups. Lane M: 100-bp ladder marker, Lanes 1,2: Control, Lanes 3,4: CCl_4 Lanes 5,6: AgNPs, Lanes 7,8: SNLE, Lanes 9,10: AgNPs plus SNLE, Lanes 11,12: AgNPs plus CCl_4, lanes 13,14: SNLE plus CCl_4 and Lanes 15,16: AgNPs plus CCl_4 and SNLE.

Effect of different treatments on mRNA gene expression

The mRNA expression of GPx (Fig. 6) and SOD (Fig. 7) were measured by semi-quentitative PCR. The results showed that the expression of the two antioxidant enzymes were decreased significantly in animals treated with AgNPs and/or CCl_4 compared to the control group. On the other hand, the expression of either GPx or SOD enzymes were increased significantly in the animals treated with the extract alone. Animals treated with AgNPs and/or CCl_4 plus *S. nigrum* showed a significant improvement in GPx and

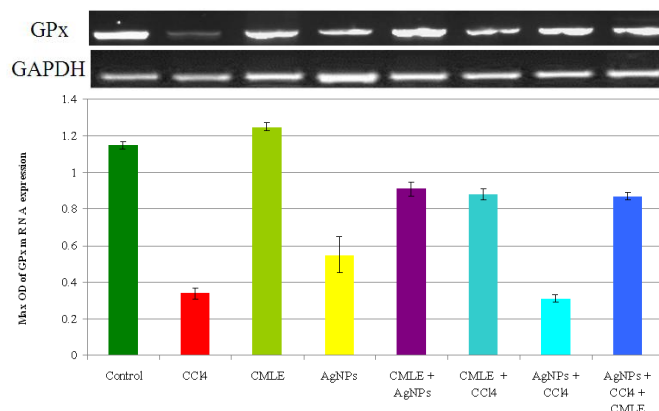SOD gene expression however they were still lower than the control value.

Fig. 6: Effect of *S. nigrum* leaves extract (SNLE) on GPx gene expression level in liver of rats treated with AgNPs and/or CCl_4. The results illustrated are normalized to the level of GAPDH level and the data are the mean of intensity for each gene divided by that for GAPDH.
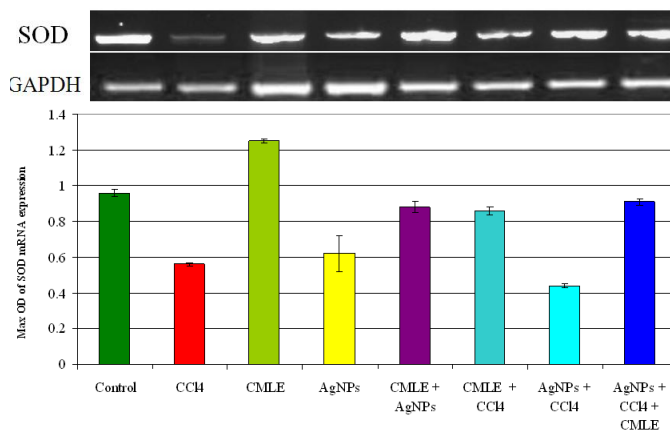

Fig. 7: Effect of *S. nigrum* leaves extract (SNLE) on SOD gene expression level in liver of rats treated with AgNPs and/or CCl_4. The results illustrated are normalized to the level of GAPDH level and the data are the mean of intensity for each gene divided by that for GAPDH.

The histological examination of the liver tissue of the control rats showed normal hepatic lobules formed of radially arranged cords of liver cells extended from the central vein, separated by blood sinusoid (Fig. 8a). The microscopic examination of the liver sections of rats treated with CCl_4 showed sever vacuolar degeneration of cytoplasm of hepatocytes, while nuclei were polymorphism as well as pyknotic (arrows), karyorrhecttic and apoptotic manifested by eosinophilc cytoplasm and condensed nuclei (Fig. 8b). The liver of rat treated with AgNPs showed vacuolar degeneration of cytoplasm of hepatocytes foci of necrosis, nuclei of different forms of degeneration and pyknosis, kryolysis especially eosnphilic cytoplasm (Fig. 8c). The liver section of rats treated with the extract alone showed some degeneration of hepatocytes cytoplasm, multiple foci of necrosis and pyknosis nuclei as well as forms of nuclear degeneration around nodule of inflammatory cell infiltration (Fig. 8d).
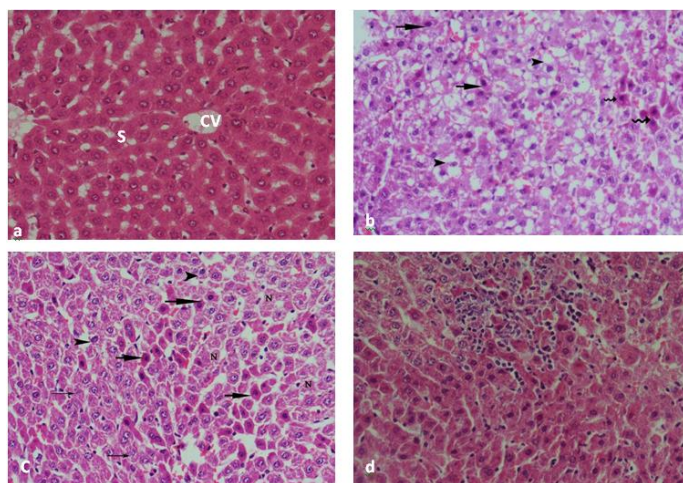
Fig. 8: Photomicrograph of liver section of **(a)** normal control rats showing normal hepatic lobules formed of radially arranged cords of liver cells extended from the central vein (cv), separated by blood sinusoid, **(b)** rats treated with CCl$_4$ showing sever vacuolar degeneration of cytoplasm of hepatocytes (arrowheads), while nuclei were polymorphism as well as pyknotic (arrows), karyorrhecttic and apoptotic manifested by eosinophilc cytoplasm and condensed nuclei (weave arrows), **(c)** rat treated with AgNPs showing vacuolar degeneration of cytoplasm of hepatocytes foci of necrosis (N), nuclei of different forms of degeneration, pyknosis (arrowe head), kryolysis (thin arrows), especially eosnophilic cytoplasm and **(d)** rats treated with the extract alone showing some degeneration of hepatocytes cytoplasm, multiple foci of necrosis, pyknosis nuclei as well as forms of nuclear degeneration around nodule of inflammatory cell infiltration.
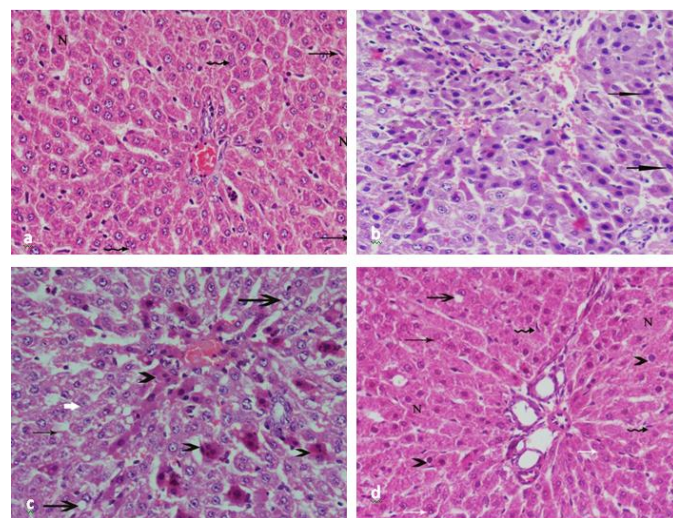

Fig. 9: Photomicrograph of liver section of **(a)** rats treated with AgNPs plus the *S. nigrum* extract showing congested central vein, multiple areas of necrosis (N), foci of inflammatory cells infiltration invade sinusoid, increase in hypertrophied kupffer cells (thin arrows), increase in binucleation (weavy arrows) could be observed, **(b)** rats treated with AgNPs plus CCl$_4$ showing swelling of the hepatocytes, degeneration, congestion and dilation of blood vessels, mononuclear inflammatory cells observed in sinusoids, marked of nuclear degeneration in the form of necrosis (N), kryolysis, condensed chromatin (arrows), and prominent diffuse of basophilic, eosinophilic cytoplasm in hepatocytes around portal area, **(c)** rat treated with CCl$_4$ plus *S. nigrum* extract showing hepatocytes suffered from fatty degeneration (arrow) beside vacuolar degeneration (thin arrow) scattered in some hepatocytes, of liver lobules, congested central vein with cellular infiltrates, nuclear damage (white arrow) as well as apoptotic cells manifested by eosinophilc cytoplasm with dark nuclei (arrowheads) still present and **(d)** proliferated bile ducts, fatty degeneration, nuclear damage (pyknotic, keryolysis and focal necrosis), few hepatocytes with binucleation also noticed.

The examination of liver sections of rats treated with AgNPs plus the extract showed congested central vein, multiple areas of necrosis, foci of inflammatory cells infiltration invade sinusoid, increase in hypertrophied kupffer cells and increase in binucleation could be observed (Fig. 9a).

However, the liver sections of rats treated with AgNPs plus CCl$_4$ showed swelling of the hepatocytes, degeneration, congestion and dilation of blood vessels, mononuclear inflammatory cells observed in sinusoids, marked of nuclear degeneration in the form of necrosis, kryolysis, condensed chromatin, prominent diffuse of basophilic and eosinophilic cytoplasm in hepatocytes around portal area (Fig. 9b). the liver sections of rat treated with CCl$_4$ plus plant extract showed that the hepatocytes suffered from fatty degeneration beside vacuolar degeneration scattered in some hepatocytes of liver lobules, congested central vein with cellular infiltrates and nuclear damage as well as apoptotic cells manifested by eosinophilc cytoplasm with dark nuclei was still present (Fig. 9c). The liver section of animals treated with AgNPs plus CCl$_4$ and the plant extract showed proliferated bile ducts, fatty degeneration, nuclear damage (pyknotic, keryolysis and focal necrosis) and few hepatocytes with binucleation also noticed (Fig. 9d).

DISSCUSTION

The wide use of AgNPs may increase the potential accumulation of AgNPs in the food chain (Oberdörster *et al.*, 2005) resulting in organ toxicity to alive organism. In general, the toxicity of different sizes of AgNPs was evaluated *in vitro* but to less extent *in vivo* studies. Consequently, the potential effects of AgNPs in humans remain poorly understood. In the current study, AgNPs were synthesized using the water extract of *S. nigrum* leaves suggesting that the extract was able to reduce silver ions to AgNPs when incubated for 24 h in dark. The change in color from yellow to reddish brown indicated the formation of AgNPs. The change in color of AgNO$_3$ has been observed by several investigators and suggesting that the change in color appeared due to the surface plasmon resonance of deposited AgNPs (Khandelwal *et al.*, 2010; Saxena *et al.*, 2010; Abdel-Aziz *et al.*, 2014). The TEM analysis showed that the sizes of the synthesized AgNps ranged from 30-50 nm and confirmed the formation of AgNPs. In the current study, animals treated with AgNPs showed sever toxicity to liver and kidney as indicated by the elevation of serum enzymes activity, kidney function tests, the proinflammatory mediator TNF-α and oxidative stress markers (NO and MDA) accompanied with the significant decrease in antioxidant enzymes activity. Although the toxicity resulted from AgNPs was lower than that resulted from CCl$_4$, but the general conclusion revealed that these AgNPs can induce liver damage after the exposure even for short time period. The results of the current study are consistent with previous reports with respect to the liver as the target organ after oral administration of AgNPs which was found to increase ALT, AST, ALP and GGT (Kim *et al.,* 2007; Sung *et al.,* 2008). The significant decrease in GSH,

CAT and TAC level accompanied with a significant increase in MDA, NO and TNF-α in serum of rats treated with AgNPs compared with control group reported herein suggesting that AgNPs induced to oxidative stress and supported the previous results of other investigators (Arora *et al.,* 2009). One of the most important mechanisms of AgNPs-induced toxicity is the generation of reactive oxygen species (ROS) resulted in oxidative stress. Oxidative stress is subsequent production of inflammatory mediators, DNA damage and apoptosis (Ryter *et al.*, 2007; El Mahdy *et al.,* 2014). In this concern, Kim *et al.* (2010) suggested that AgNPs increase the levels of ROS, lipid peroxidation and decrease GPx as a mechanism of liver damage. Moreover, AgNPs caused membrane damage, leakage of lactate dehydrogenase, reduction in glutathione peroxide and superoxidase dismutase activity and cell viability in human hepatoma cell line (HL-7702) (Song *et al.*, 2012).

Regarding to CCl$_4$ as a model for studying free radical-induced liver injury, several studies have reported the involvement of oxidative stress in CCl$_4$-induced liver damage (Nogueira *et al.*, 2009; Ma *et al.*, 2012). The liver damage caused by CCl$_4$ is characterized by inflammation in early stage. In the damaged hepatocytes, CCl$_4$ is metabolized by cytochrome P450 into trichloromethyl radical, a highly reactive species that triggers lipid peroxidation and lead to hepatotoxicity (Deng *et al.*, 2012). The toxic effects of CCl$_4$ reported herein were similar to those reported in the literature. Animals treated with CCl$_4$ alone or in combination with AgNPs showed an elevation in the proinflammatory mediator TNF-α suggesting chronic hepatotoxicity (Bruccoleri *et al.*, 1997). TNF-α is a central regulator for many bioactive molecules including those responsible for chronic inflammation, induction of acute phase proteins, cell proliferation and cytotoxicity and its elevated expression is associated with potentiating hepatotoxicity.

In the current study, liver damage induced by CCl$_4$ and/or AgNPs was confirmed by DNA fragmentation percentage and DNA ladder assay banding pattern and revealed cellular correlates of damage after CCl$_4$ and/or AgNPs administration. On the other hand, CCl$_4$ is well known to degrade DNA in liver tissue of rats by generating free radicals and increase DNA fragmentation (Alkreathy *et al.*, 2014). Regarding the gene expression level, the current findings indicated that, CCl$_4$ induced down-regulation of the examined antioxidant enzymes (SOD and GPx) gene expression and liver fibrosis in mice (Chen *et al.,* 2013). The genotoxic effects of AgNPs were represented by the increase in DNA fragmentation and the disturbance of gene expression which may be attributed to oxidative stress created by AgNPs exposure. The genotoxicity of AgNPs was evaluated by various *in vitro* and *in vivo* studies and confirmed the findings obtained in the current study and suggested that AgNPs cause chromosomal aberrations and DNA damage and capable to induce proliferation arrest in rats and human cell lines (Ji *et al.*, 2007; Asharani *et al.*, 2009) although the exact mechanism through which AgNPs induce genotoxicity still remains unclear. Asharani *et al.* (2009) found that AgNPs can damage DNA indirectly by increasing ROS

production or by decreasing ATP production inducing mitochondrial damage which impairs energy-dependent DNA repair mechanisms. Moreover, Cha *et al.* (2008) and Yang *et al.* (2009) suggested that direct DNA damage occurred by Ag+ released from AgNPs themselves. Furthermore, it was reported that AgNPs increase DNA-strand breaks in human testicular embryonic carcinoma cells by increase ROS production (Asare *et al.*, 2012). The mechanism of AgNPs induced hepatotoxicity was discussed by Sardari *et al.* (2012) who suggested that the removing of nanoparticles from the liver by macrophages due to phagocytosis process and the repetition of this process produces higher oxygen radicals. On the other hand, Loghman *et al.* (2012) concluded that the cytotoxicity of AgNPs is mainly due to the mitochondrial activity which is decreased with the increase in the concentration of AgNPs inducing drastic reduction of mitochondrial function, increased membrane leakage, necrosis and induction of apoptosis.

The histopathological examination of liver revealed that various alterations denoting the hepatotoxic effect of AgNPs and/or CCl$_4$ including vacuolar degeneration of the cytoplasm in the hepatocytes, foci of necrosis, nuclei of different forms of degeneration, pyknosis, karyolysis especially eosnophilic cytoplasm and dark nuclei. Similar necrosis, hepatocellular degeneration, and individual apoptosis were reported and confirmed that the liver is the target organ for the effect of AgNPs (Sung *et al.,* 2008; Kim *et al.*, 2010; El Mahdy *et al.*, 2014).

Treatment with *S. nigrum* significantly lowered the elevation of hepatic enzyme markers, kidney indices induced by CCl$_4$ and/or AgNPs as well as decreased MDA and DNA fragmentation in the liver tissue. The extract also increased the antioxidant capacity and decreased the inflammatory cytokines consequently, repressed the production of free radicals and the subsequent liver damage (Raju et al., 2003). Similar to these observations, Lin et al. (2008) reported that *S. nigrum* extract protects the liver against CCl$_4$-induced oxidative damage in the liver through its the high content of polyphenols, alkaloids and saponins which contributes free radical scavenging and antioxidation activities. The hepatoprotective effect of the *S. nigrum* leaves extract reported in the current study suggested that these compounds play an important role in plasma membrane stabilization and the repairing of liver damage caused by CCl$_4$ and/or AgNPs. Furthermore, the phenolic compounds contribute directly to antioxidative action (Awika et al., 2003) probably due to their redox properties which allow them to act as hydrogen donors, reducing agents and singlet oxygen quenchers (Chang et al., 2001). The extract also showed a significant decrease in cytokine level and the inflammation process indicated that it has anti-inflammatory properties via decreasing the ROS generation which involved in inflammation process (Ravi et al., 2009). Moreover, Hsu et al. (2009) reported that *S. nigrum* increased expression of glutathione S-transferase-alpha and -mu, the level of transcription factor Nrf2, glutathione peroxidase, superoxide dismutase-1, and catalase. These results are in agreement with previous studies that have reported hepatoprotective and

antioxidant effects of extracts containing flavonoids and other phenolic compounds (Kukrić *et al.*, 2012; Abdel-Aziz *et al.*, 2014). Taken together, strengthening the antioxidant defense system and the free radical scavenging activity can result in positive curative effects on liver injuries (Nagalekshmi *et al.*, 2011). Moreover, Padmashree *et al.* (2014) reported that *S. nigrum* leaves extract is rich in mineral content (Cu, Fe, Na, K, Zn, Cu, Mg and P) and nutrition composition (ascorbic acid, total carotenoids, total chlorophyll, total phenols, total flavonoids and total tocopherol) which have great potential as natural antioxidants. Similar results were reported by Das *et al.* (2015) who suggested higher total phenol and total falvonoid content in *S. nigrum* leaves extract. Moreover, Aboul-Enein *et al.* (2014) isolated 8 active compounds identified as Naphtho [2,1-b]furan-2(1H)-one, 2,3 Dihydroxypropyl elaidate, 5-Bromosalicylaldehyde, 12-sulfanyldodecanoic acid Usnic acid monoacetate, decahydro-3a,6,6,9a-tetramethy, 8-Azabicyclo [3.2.1] octane-2-carboxylic acid, 3-hydroxy-8-methyl,(2-endo,3-exo), Niclofen, and Trilinolein. These compounds also showed a potent antioxidant and anticancer activity.

CONCLUSION

AgNPs can be synthesized simply using *S. nigrum* leaves extract in a size ranged from 30- 50 nm. *S. nigrum* leaves extract was found to be rich in total phenolic compounds, total flavonides and β-carotene. AgNPs affect negatively the liver function enzymes, oxidative stress markers, antioxidant parameters, tumor markers, DNA fragmentation and antioxidant gene expression indicating the hepatotoxic and genotoxic effect. These hepatotoxic and genotoxic effects resulted when rats treated with 50 mg/kg b.w for three weeks and particles size ranged from 30-50 nm. However, this toxicity was less than that caused by CCl_4 at the tested dose of 0.1 ml/kg b.w. *S. nigrum* leaves extract succeeded to counteract these toxic effects due to its higher content of phenolic and flavonoid compounds which exhibited antioxidant and free radical scavenging activities. *S. nigrum* leaves may be promise candidate to be used as a hepatoprotectant against AgNPs-induced hepatotoxicity.

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