Article

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Ameliorative effect of *Allolobophora caliginosa* extract on hepatotoxicity induced by silicon dioxide nanoparticles

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Abstract

This study aims to evaluate the possible ameliorative effect of earthworm (*Allolobophora caliginosa*) extract (EE) against silicon dioxide nanoparticles (SiNPs)-induced liver injury in male albino rats. The effectiveness of EE was compared with silymarin as a standard hepatoprotective drug. The present work demonstrates the antioxidant activity of EE by 1,1-diphenyl-2-picrylhydrazyl assay. Administration of SiNPs, for 15 consecutive days, caused changes in most of the biochemical parameters, namely, serum aminotransferase enzymes activities (alanine transaminase and aspartate transaminase), alkaline phosphatase activity, total protein, total and direct bilirubin level, malondialdehyde, glutathione reduced, catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase. In addition, administration of SiNPs induced changes in liver tissue architecture. Administration of EE, for subsequent 30 days, to SiNPs exposure demonstrated significant ameliorative effects on nearly all the studied parameters, and such effects were compatible with those of silymarin. In addition, the administration of EE repairs, to some extent, the abnormal architecture of the liver tissue induced by SiNPs.

Keywords

Earthworm extract, liver injury, oxidative stress, silicon dioxide nanoparticles

Introduction

Engineered nanoparticles (NPs) have been mass produced and widely applied with the development of nanotechnology and materials science. NPs are ultrafine particles with lengths in two or three dimensions greater than 1 nm and smaller than 100 nm (ASTM, 2006). Previous studies have demonstrated that NPs induced toxicological effects mainly on lungs, liver, spleen, and kidneys (Chen et al., 2006; Wang et al., 2007). One of the most important engineered nanomaterials is the silicon dioxide nanoparticles (SiNPs), which are used in cosmetics, printer toners, and food (Zhao et al., 2007). Kumar et al. (2010) reported that liver is the target organ for the toxic effects of SiNPs, as SiNPs accumulate in the liver and induce pathological changes.

Commercially manufactured drugs used in the treatment of liver diseases can cause serious side effects. This is one of the reasons why many people worldwide consider complementary or alternative medicine (Soliman, 2011). Earthworms are soil oligochaetes and macroinvertebrates that play an important role in maintaining soil fertility and productivity. Extracting and using biologically active compounds from earthworms have been a traditional practice by many people throughout the world (Ranganathan, 2006).

Several studies have suggested that SiNPs inflict severe liver damage after systemic administration (Fu et al., 2012; Xie et al., 2010). Since NPs are used as food additives or in food processing and packaging, there are concerns that NPs could gain access to the blood stream via gastrointestinal absorption. In addition, there is a little information about the toxic effect of SiNPs after oral administration. Thus, the present

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investigation was designed to describe the toxic potential of SiNPs after oral administration as well as to find out the possible ameliorative effects of earthworm extract (EE) in albino rats.

Materials and methods

Experimental animals

Male albino rats, *Rattus norvegicus* (100–120 g) aged 6–7 weeks were used in this study. Animals were divided and housed in polyacrylic cages (five animals per cage) and bedded with wood shavings in the well-ventilated animal house of the Department of Zoology, Faculty of Science, Cairo University, Giza, Egypt. Animals were fed a standard pelleted ration and provided water *ad libitum*. Ambient temperature was controlled at 22 $\pm$ 3°C with a relative humidity of 50 $\pm$ 15% and a 12-h light/12-h dark photoperiod. Rats were acclimatized to laboratory conditions for 7 days before commencement of the experiment. The protocol was approved by the Faculty of Science, Cairo University, Egypt, and Institutional Animal Care and Use Committee (IACUC). All the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals.

Chemicals

SiNPs were purchased from Sigma-Aldrich (St Louis, Missouri, USA). The given supplier showed that SiNPs were spherical and porous in shape, with a size of 5–15 nm and purity of 99.5%. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich. Silymarin was purchased from SEDICO Pharmaceutical Co. (6 October City, Giza, Egypt). Kits for most of the biochemical analysis determinations were purchased from the Biodiagnostic Company (Dokki, Giza, Egypt). The kit for γ-glutamyltransferase analysis was purchased from Spectrum Company (Obour City, Cairo, Egypt).

Preparation of EE

Earthworms, *Allolobophora caliginosa*, were purchased from Kiro Company in Giza (January 2012). Earthworms were extracted according to the method described by Ishii and Mihara (1992). One kilogram of sexually mature worms were washed with running tap water to remove any undesirable contents attached on the body surface. The living earthworms are placed in 0.65% sodium chloride solution at a temperature of 25°C for a period of 72 h and then were wet ground in chloroform and methanol (1:1 v/v). The resulting suspension was concentrated and dried by lyophilizer apparatus (Edwards, Irvine, California, USA).

Amino acid analysis of EE

The extract was analyzed for the amino acid content using an automatic amino acid analyzer (400, INGOS Ltd, Praha, Czech Republic) following the method described by Block et al. (1958).

Determination of EE antioxidant activity (scavenging activity of DPPH radical)

The DPPH free radical scavenging assay was used for the evaluation of the antioxidant activity of the EE according to the method of Brand et al. (1995). The following concentrations of the EE and ascorbic acid (as positive control) were prepared: 10, 20, 30, 40, 50, and 60 mg/mL in methanol. Briefly, to 2 mL of DPPH, the specified weight of extract was added, and the final volume was adjusted to 4 mL with methanol. The solution was shaken and then incubated at 37°C in the dark for 30 min. Control tubes contained 2 mL of DPPH and 2 mL of methanol only. Methanol was used as a blank. The changes in absorbance (Abs) were measured at $\lambda = 517$ nm using a spectrophotometer (U-2001, model 121-0032, Hitachi, Tokyo, Japan). The inhibition of DPPH radicals was calculated using the following equation:

$$\%\ \text{of radical scavenging activity} = \left[\frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}}\right] \times 100.$$

Physicochemical characterization of SiNPs

The optical absorption of the SiNPs suspension was measured using a double-beam ultraviolet–visible (UV-Vis) near-infrared (NIR) spectrophotometer (Cary 5000, Varian, Palo Alto, California, USA) at wavelength ranging from 200 nm to 800 nm at room temperature. X-ray diffraction (XRD) was used to identify the crystal phase and to estimate the average crystallite size. Structural studies of SiNPs were carried out by transmission electron microscopy (TEM). Samples were resuspended using a 10-mL disposable pipette and then applied to a copper grid coated with an air-dried carbon film. After being negative stained and air-dried at room temperature, the samples were examined using TEM. The average hydrodynamic

size of SiNPs in water was determined by dynamic light scattering (DLS; Zetasizer Nano Series, Malvern, UK). This device can measure particle sizes ranging from 0.6 nm to 6 μm.

Suspension preparation of SiNPs

Solutions of NPs were prepared by sonication according to the method adopted by Canesi et al. (2010) using Sonics Vibra-Cell sonicator (Newtown, Connecticut, USA). Before each experiment, the solutions were sonicated in an ice bath for 15 min at 100 W, 50% on/off cycle.

Acute oral toxicity study

Acute toxicity studies were performed according to the Organization of Economic Cooperation and Development (OECD) guideline 425 (OECD, 2001). Fifteen healthy male albino rats, fasted overnight, were divided into three groups with five animals in each group. The first group received distilled water and served as controls. The second group was administered SiNPs in suspension at a limit test dosage of 5000 mg/kg body weight. The third group received an aqueous suspension of EE at a limit test dose of 5000 mg/kg body weight. All doses were administered by gastric gavage, and each dose was adjusted to be 2 mL/kg. The animals were observed post dose at 0 min, 30 min, 1 h, 2 h, 4 h, 6 h, and thereafter every day for 14 days.

The chronic oral toxicity study

A repeated dose oral toxicity study was performed according to OECD guideline 407 (OECD, 2008). Fifteen healthy male albino rats were divided into three groups with five animals in each group. First group received distilled water and served as controls. The second and third group received SiNPs suspension at dosage 500 and 1000 mg/kg body weight, respectively. The suspension was administrated daily for 14 days. On the15th day, after overnight fasting, rats were killed, and blood samples were collected into centrifuge tubes without anticoagulant and left for 30 min. Following clot formation, serum was obtained by centrifugation at 3000 rpm/min for 20 min. The serum was stored at −20°C for biochemical analyses.

Experimental design for the pivotal study

Rats were randomly divided into seven groups (nine animals/group) and treated as follows:

- Group I: Served as the control group; rats were administered distilled water (2 mL; vehicle) orally by gastric gavage for 45 days.
- Groups II and III: Rats were orally administered an aqueous suspension of EE at dosages of 500 and 1000 mg/kg body weight/day, respectively for 30 days.
- Group IV: Rats were orally administered SiNPs (1000 mg/kg body weight/day) for 15 days.
- Group V: Rats were orally administered SiNPs (1000 mg/kg body weight/day) for 15 days, followed by EE (500 mg/kg body weight/day) administration daily for an additional period of 30 days.
- Group VI: Rats were orally administered SiNPs (1000 mg/kg body weight/day) for 15 days, followed by an aqueous suspension of EE (1000 mg/kg of body weight/day) administration daily for an additional period of 30 days.
- Group VII: Rats were orally administered SiNPs (1000 mg/kg body weight/day) for 15 days, followed by silymarin (150 mg/kg body weight/day) administration daily for an additional period of 30 days.

At the end of each experiment, rats were killed after being fasted overnight; blood samples were collected in centrifuge tubes without anticoagulant, allowed to clot, and centrifuged at 3000 rpm/min for 20 min. The serum obtained was stored at −20°C until used for biochemical assays. Liver samples were quickly removed, washed with physiological saline, and divided into three parts. One was used for histopathological evaluation, the second for TEM examinations, and the third for biochemical assays.

Biochemical assays

The appropriate kits (Biodiagnostic, Dokki, Giza, Egypt) were used for the determination of serum aminotransferase enzymes activities (alanine transaminase (ALT) and aspartate transaminase (AST)) according to Reitman and Frankel (1957), alkaline phosphatase (ALP) activity (Belfield and Goldberg, 1971), total protein (Henry,1964), albumin level (Doumas et al.,1971), and total and direct bilirubin levels (Walter and Gerade, 1970). A different kit

Table 1. The amino acid contents of EE.

Amino acids	EE (g/100 g)	Amino acids	EE (g/100 g)
Aspartic acid	5.32	Alanine	5.37
Threonine	1.74	Valine	3.4
Serine	2.3	Methionine	0.57
Glutamic acid	7.54	Isoleucine	2.34
Proline	0.07	Leucine	4.31
Glycine	4.96	Tyrosine	1.24
Lysine	2.66	Phenylalanine	0.19
Arginine	1.73	Histidine	1.31

EE: earthworm extract.

(Spectrum Diagnostics, Obour, Cairo, Egypt) was used for the determination of serum γ-glutamyl transferase (GGT) activity (Szasz et al., 1974).

Liver tissue was homogenized (10% w/v) using a Potter Elvehjem homogenizer (Sigma Aldrich) in ice-cold 0.1 M phosphate buffer (pH 7.6) and centrifuged at 3000 rpm/min for 15 min. Using test kits (Biodiagnostic, Egypt), the supernatant was used for the determination of the lipid peroxidation (LPO), which was measured by the formation of malondialdehyde (MDA) (Buege and Aust, 1978), reduced glutathione (GSH) (Beutler et al., 1963), catalase (CAT) (Aebi, 1984), superoxide dismutase (SOD) (Nishikimi et al., 1972), glutathione reductase (GR) (Goldberg and Spooner, 1983), and glutathione peroxidase (GPx) (Paglia and Valentine, 1967).

Histopathological examinations

Liver samples from the left lobe were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological examination using a light microscope.

TEM examination

For electron microscopy, liver tissue samples were collected and cut into small pieces (approximately equal $1 \times 1 \times 1$ mm^3), and immediately fixed in 2.5% glutaraldehyde overnight. The samples were then fixed in 1% (w/v) osmium tetroxide for 2 h, rinsed two times with phosphate buffer, and dehydrated in a graded acetone series ((50–70–90–100–100–100)% (v/v); 10 min/step). The samples were infused in epoxypropane/Epon 812 (50%/50%) solution for 2 h and then infused in Epon 812 again for 2 h under vacuum. The samples were embedded in Epon 812 and reacted at 80°C overnight. The ultrathin sections (75 nm) were stained with lead citrate and uranyl acetate. The sections were examined using TEM (JEM-1400 TEM; JEOL, Japan).

Statistical analysis

Results were expressed as a mean $\pm$ standard error. All data obtained were analyzed by analysis of variance, followed by Student's t test at 95% confidence level. Values of $p < 0.05$ were considered as statistically significant. All computations were performed using Statistical Package for Social Sciences version 15.0 software.

Results

Amino acid analysis of EE (*A. caliginosa*)

The amino acid analysis of *A. caliginosa* extracts showed different amino acid constituents. The EE had essential and nonessential amino acids (Table 1). Leucine, as an essential amino acid, represented the highest concentration followed by valine and lysine. On the other hand, the nonessential amino acid glutamic acid showed the highest concentration as compared to both alanine and aspartic acid.

DPPH radical scavenging assay

Figure 1 shows the DPPH radical scavenging activity of EE and ascorbic acid, as a standard, at varying concentrations. It was noticed that the EE showed high radical scavenging activity at a concentration of 10 mg/mL. At concentrations more than 20 mg/mL, the antioxidant activity of EE decreased, compared with ascorbic acid. The radical scavenging activity of EE was inversely proportional to the EE concentration (Figure 1). In contrast, free radical scavenging activity for ascorbic acid was independent of concentration.

Physicochemical characterization of SiNPs

The UV-Vis-NIR spectrophotometer showed an absorption band for SiNPs at 351 nm (Figure 2(a)). The size of SiNPs was calculated from the XRD spectrum shown in Figure 2(b). The 2θ values were observed at 25.5°. The particle size was determined using the Debye–Scherrer formula:

$$D = \frac{0.9\lambda}{\beta \cos\theta}$$

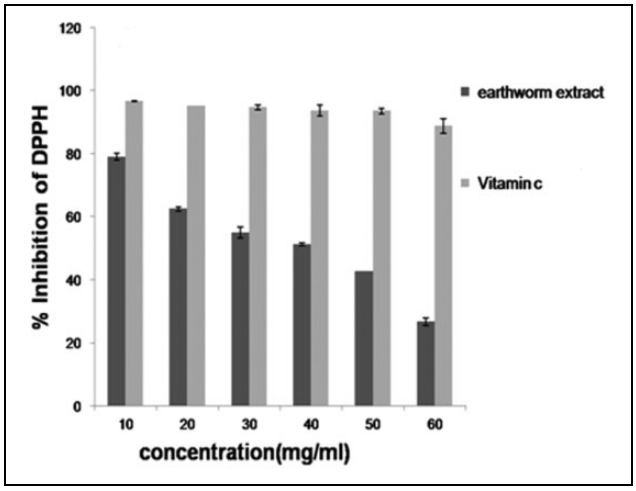

Figure 1. Inhibition of DPPH by EE. DPPH: 1,1-diphenyl-2-picrylhydrazyl; EE: earthworm extract.

where D represents particle size, $\lambda = 0.154$ nm, and β was calculated according to the following equation: $\beta = \left[\left(2\theta_{\text{high}} - 2\theta_{\text{low}}\right) \times 3.14\right]/180$.

The particle size calculated from XRD spectrum employing the above equation was 8.8 nm. Also, XRD analysis revealed that the amorphous nature of SiNPs (Figure 2(b)). Figure 2(c) shows the typical TEM image of SiNPs. This picture revealed that the majority of the particles were polygonal in shape. The TEM average diameter was calculated from measuring over 60 particles in random fields of TEM view. The average TEM diameter of SiNPs was approximately 11 nm. Figure 2(d) represents the frequency of size (in nanometer) distribution of SiNPs. The average hydrodynamic size and zeta potential of SiNPs in water determined by DLS were 1039 nm and −24.5 mV, respectively (Figure 2(e)). Zeta potentials provide quantitative information on the stability of the particles. It is documented that the particles are more likely to remain dispersed if the absolute value of zeta potential is higher than 30 mV. This means that SiNPs are moderately stable.

Acute oral toxicity test

Single oral administration of EE and SiNPs (5000 mg/kg bodyweight) did not show visible signs of toxicity, abnormal behaviors, or mortality, which indicated that the median lethal dose (LD_{50}) of EE and SiNPs was higher than 5000 mg/kg body weight. The effective doses of both EE and SiNPs (used in this study) were selected based on this LD_{50}.

The chronic oral toxicity study

The effect of chronic oral administration of SiNPs on serum ALT and GGT activities

Table 2 shows the activities of serum ALT and GGT in control and SiNPs-treated groups at the two selected dosages (500 and 1000 mg/kg body weight) in male rats. Rats orally administered with SiNPs (1000 mg/kg body weight) for 14 days showed a significant ($p < 0.05$) increase in the ALT activity as compared to the control group (Table 2). The activity of GGT was significantly ($p < 0.05$) increased after oral administration of SiNPs (500 and 1000 mg/kg body weight) for 14 days in rats as compared to the control group (Table 2). It may be concluded that the SiNPs at a dosage of 1000 mg/kg body weight caused more pronounced liver injury after oral administration in comparison with the low dosage (500 mg/kg body weight). Because the higher dosage (1000 mg/kg body weight) affected two important liver biomarkers (ALT and GGT), this dosage was chosen to investigate the toxicity of SiNPs.

Serum enzymes

Table 3 shows that oral administration of EE (500 and 1000 mg/kg body weight) for 30 days did not cause any difference in the activities of serum AST and ALP. However, a significant ($p < 0.05$) increase in the activity of ALT of rats was recorded after administration of EE at a dosage of 1000 mg/kg body weight as compared to the control group. The activity of GGT significantly ($p < 0.05$) decreased after oral administration of EE (500 mg/kg body weight) for 30 days as compared to the control group. On the other hand, a significant ($p < 0.05$) increase was recorded in the activities of ALT, AST, ALP, and GGT of rats after the oral administration of SiNPs as compared to the control group (Table 3). Posttreatment with EE at two selected doses and silymarin was found to ameliorate the undesirable effect of SiNPs on the previous parameters (Table 3).

Total protein, albumin, total and direct bilirubin

Oral administration of EE (500 and 1000 mg/kg body weight) for 30 days caused no difference in the levels of serum total protein, albumin, total and direct bilirubin of rats as compared to the control group (Table 3). However, a significant ($p < 0.05$)

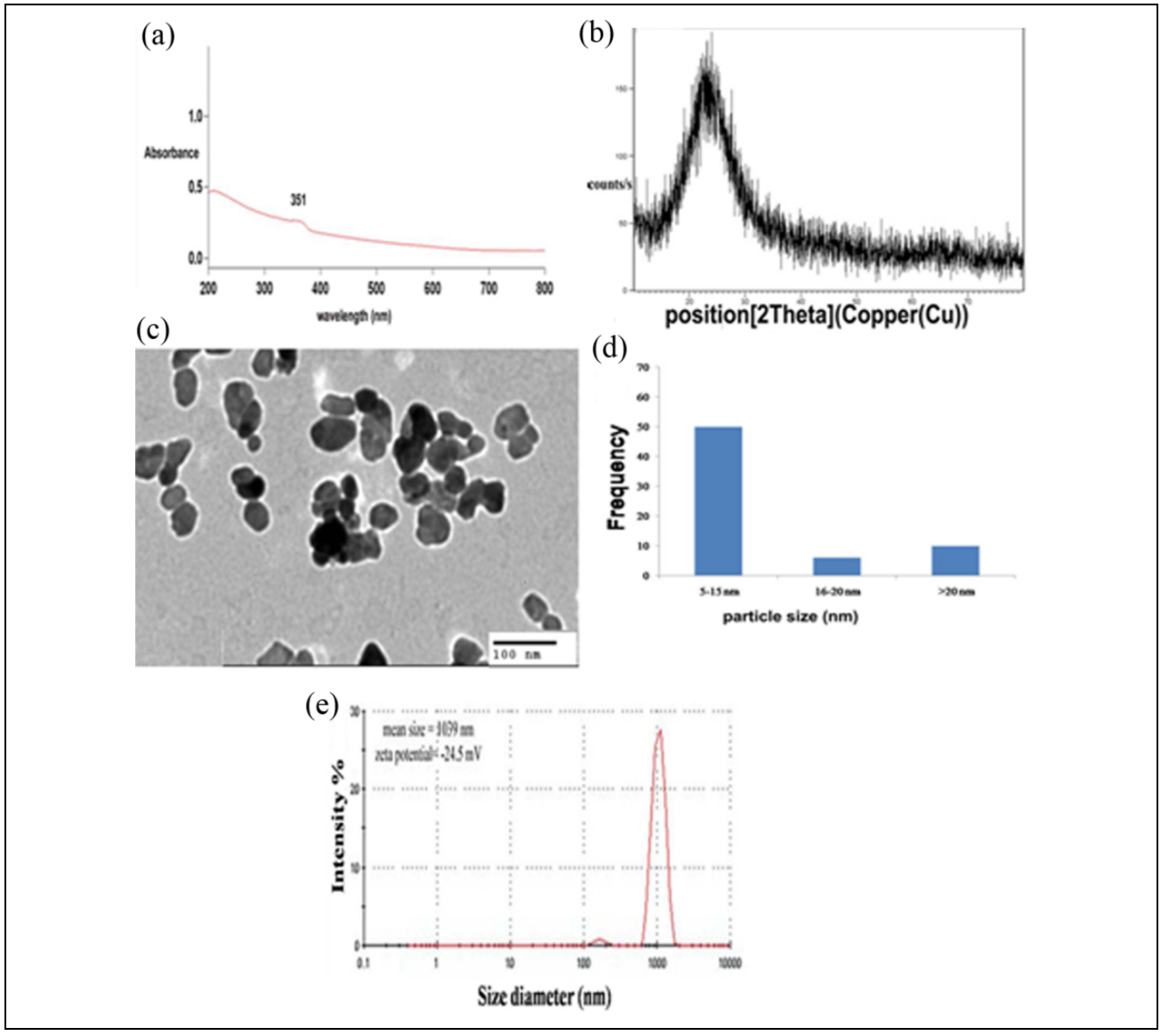

Figure 2. Shows the physicochemical characterization of SiNPs. (a) UV-Vis spectrum of SiNPs; (b) XRD pattern of SiNPs; (c) TEM image; (d) the size distribution histogram generated by using TEM image; and (e) size distribution and zeta potential of SiNPs were determined using DLS. SiNPs: silicon dioxide nanoparticles; UV-Vis: ultraviolet–visible; XRD: X-ray diffraction; DLS: dynamic light scattering.

Table 2. Effect of chronic low and high dose of SiNPs on the serum ALT and GGT activity of rats.[a]

Groups	ALT (U/mL)	GGT (U/L)
Control	11.13 ± 2.13	4.23 ± 0.60
SiNPs (500 mg/kg)	17.50 ± 2.13	6.63 ± 0.64[b]
SiNPs (1000 mg/kg)	29.13 ± 1.92[b]	10.6 ± 0.95[b]

SiNPs: silicon dioxide nanoparticles; ALT: alanine transaminase; GGT: γ-glutamyl transferase.
[a]All data are mean ± SE of five rats.
[b]$p < 0.05$: significant as compared to control group.

increase was noticed in the levels of total and direct bilirubin of rats after the oral administration of SiNPs (1000 mg/kg body weight) for 15 days as compared to the control group (Table 3). The level of total protein of rats decreased significantly ($p < 0.05$) after the oral administration of SiNPs (1000 mg/kg) as compared to the control group. On the other hand, posttreatment with EE at the two selected doses and silymarin was found to ameliorate the undesirable effects induced by SiNPs on most of the previous parameters (Table 3).

Table 3. Serum biochemical parameters of rats under different experimental conditions.[a]

Parameters Groups	ALT (U/mL)	AST (U/mL)	GGT (U/L)	ALP (U/L)	Total protein (g/dL)	Albumin (g/dL)	Total bilirubin (mg/dL)	Direct bilirubin (mg/dL)
Control	24.25 ± 2.58	114.68 ± 1.74	5.73 ± 1.14	245.36 ± 10.9	11.24 ± 1.01	8.54 ± 1.31	1.82 ± 0.23	3.14 ± 0.19
EE (500 mg/kg)	26.25 ± 3.69	120.55 ± 3.96	2.35 ± 0.17[b]	241.6 ± 17.41	10.26 ± 0.85	7.73 ± 1.40	2.14 ± 0.29	2.65 ± 0.34
EE (1000 mg/kg)	30.81 ± 1.36[b]	116.75 ± 9.11	4.11 ± 0.51	233.45 ± 35.8	10.51 ± 1.24	8.12 ± 1.75	2.12 ± 0.16	2.92 ± 0.23
SiNPs (1000 mg/kg)	44.44 ± 2.76[b]	142.33 ± 3.93[b]	9.70 ± 0.59[b]	365.24 ± 19.5[b]	8.52 ± 0.65[b]	10.13 ± 0.57	5.10 ± 0.97[b]	3.98 ± 0.16[b]
SiNPs + EE (500 mg/kg)	26.85 ± 1.96[c]	107.34 ± 4.89[c]	3.68 ± 0.49[c]	174.31 ± 23.32[c]	10.56 ± 0.91	6.13 ± 0.54[c]	3.25 ± 0.18	2.00 ± 0.15[c]
SiNPs + EE (1000 mg/kg)	27.89 ± 2.10[c]	101.35 ± 3.16[c]	3.54 ± 0.55[c]	193.74 ± 17.60[c]	11.21 ± 0.71[c]	6.75 ± 0.47[c]	2.87 ± 0.23[c]	2.16 ± 0.17[c]
SiNPs + silymarin (150 mg/kg)	28.10 ± 2.97[c]	129.32 ± 5.85	3.14 ± 0.43[c]	225.03 ± 22.65[c]	10.59 ± 0.58[c]	6.32 ± 0.51[c]	2.98 ± 0.32	2.87 ± 0.23[c]

ALT: alanine aminotransaminase; AST: aspartate aminotransaminase; GGT: γ-glutamyl transferase; ALP: alkaline phosphatase; EE: earthworm extract; SiNPs: silicon dioxide nanoparticles.

[a]All data are mean ± SEM of nine rats.

[b]$p < 0.05$: significant as compared to control group.

[c]$p < 0.05$: significant as compared to SiNPs group.

Table 4. Liver antioxidant parameters of rats under different experimental conditions.[a]

Parameters Groups	MDA (mg/g.protein)	GSH (nmol/g.protein)	CAT (U/g.protein)	SOD (U/g.protein)	GR (U/g.protein)	GPx (U/g.protein)
Control	27.48 ± 3.26	22.6 ± 2.7	16.1 ± 1.21	377 ± 22.1	26.5 ± 0.25	2.38 ± 0.18
EE (500 mg/kg)	27.58 ± 2.46	33.5 ± 1.62[b]	18.9 ± 0.62	348 ± 28.8	23.6 ± 2.7	2.43 ± 0.40
EE (1000 mg/kg)	23.88 ± 0.85	28.6 ± 2.76	13.1 ± 1.4	410 ± 11.6	21.7 ± 3.3	2.57 ± 0.25
SiNPs (1000 mg/kg)	50.00 ± 4.5[b]	12.5 ± 1.0[b]	5.9 ± 0.5[b]	185 ± 14.2[b]	2.11 ± 0.35[b]	4.39 ± 0.48[b]
SiNPs + EE (500 mg/kg)	25.22 ± 0.94[c]	23.5 ± 2.2[c]	17.2 ± 2.1[c]	355 ± 14.4[c]	17.6 ± 1.42[c]	3.00 ± 0.26[c]
SiNPs + EE (1000 mg/kg)	16.00 ± 0.72[c]	25 ± 4.23[c]	9.8 ± 0.71[c]	540 ± 28.6[c]	20.7 ± 0.89[c]	2.60 ± 0.12[c]
SiNPs + silymarin (150 mg/kg)	18.65 ± 1.35[c]	26.3 ± 1.77[c]	7.6 ± 0.27[c]	357 ± 16.8[c]	4.95 ± 0.70[c]	2.51 ± 0.23[c]

MDA: malondialdehyde; GSH: glutathione reduced; CAT: catalase; SOD: superoxide dismutase; GR: glutathione reductase; GPx: glutathione peroxidase; EE: earthworm extract; SiNPs: silicon dioxide nanoparticles.
[a]All data are mean ± SEM of nine rats.
[b]$p < 0.05$: significant as compared to control group.
[c]$p < 0.05$: significant as compared to SiNPs group.

Liver oxidative stress markers

Oral administration of EE at the two selected doses caused no differences in the liver MDA level of rats as compared to the control group (Table 4). However, SiNPs administration (1000 mg/kg body weight) induced a significant ($p < 0.05$) increase in the level of liver MDA as compared to the control group (Table 4). On the other hand, administration of EE at the two selected doses or silymarin after SiNPs administration caused a significant ($p < 0.05$) decrease in the level of rats MDA as compared to the SiNPs group (Table 4).

Table 4 shows that oral administration of EE increased the level of liver GSH significantly ($p < 0.05$) only at the low dosage (500 mg/kg body weight) as compared to the control group. On the other hand, the level of GSH significantly ($p < 0.05$) decreased after the administration of SiNPs as compared to the control group. Posttreatment, for 30 days, by either EE (500 or 1000 mg/kg body weight) or silymarin (150 mg/kg body weight) significantly ($p < 0.05$) increased the level of GSH as compared to the SiNPs group (Table 4).

No difference was recorded in the activity of liver CAT following the administration of EE (both selected doses) for 30 days as compared to the control group (Table 4). Meanwhile, oral administration of SiNPs caused a significant ($p < 0.05$) decrease in the liver CAT activity as compared to the control group. On the other hand, rats orally treated with either EE (500 or 1000 mg/kg body weight) or silymarin (150 mg/kg body weight) after SiNPs administration showed a significant ($p < 0.05$) increase in their liver CAT activity as compared to the SiNPs group (Table 4).

Oral administration of EE at the selected doses for 30 days did not cause any difference in liver SOD and GR activities of rats as compared to the corresponding control value (Table 4). However, rats that were administered with SiNP suspension for 15 days showed a significant ($p < 0.05$) decrease in their liver SOD and GR activities as compared to the control group. On the other hand, posttreatment with EE and silymarin was found to ameliorate the undesirable effects of SiNPs on the liver SOD and GR activities (Table 4).

Liver GPx activity of rats showed no difference after the administration of EE at the two selected doses as compared to the control group (Table 4). Meanwhile, a significant ($p < 0.05$) increase was recorded in the activity of GPx after SiNPs administration as compared to the control group (Table 4). On the other hand, rats treated either with EE or silymarin after SiNPs administration significantly ($p < 0.05$) reduced their GPx activity as compared to the SiNPs group (Table 4).

Histological studies

Figure 3 shows representative histological sections of the liver. The normal morphology of liver exhibited the well-organized lobular architecture with hepatocytes (H) arranged in chords with regularly aligned sinusoids (S) in the control group (Figure 3(A)). In comparison with the control group, the liver

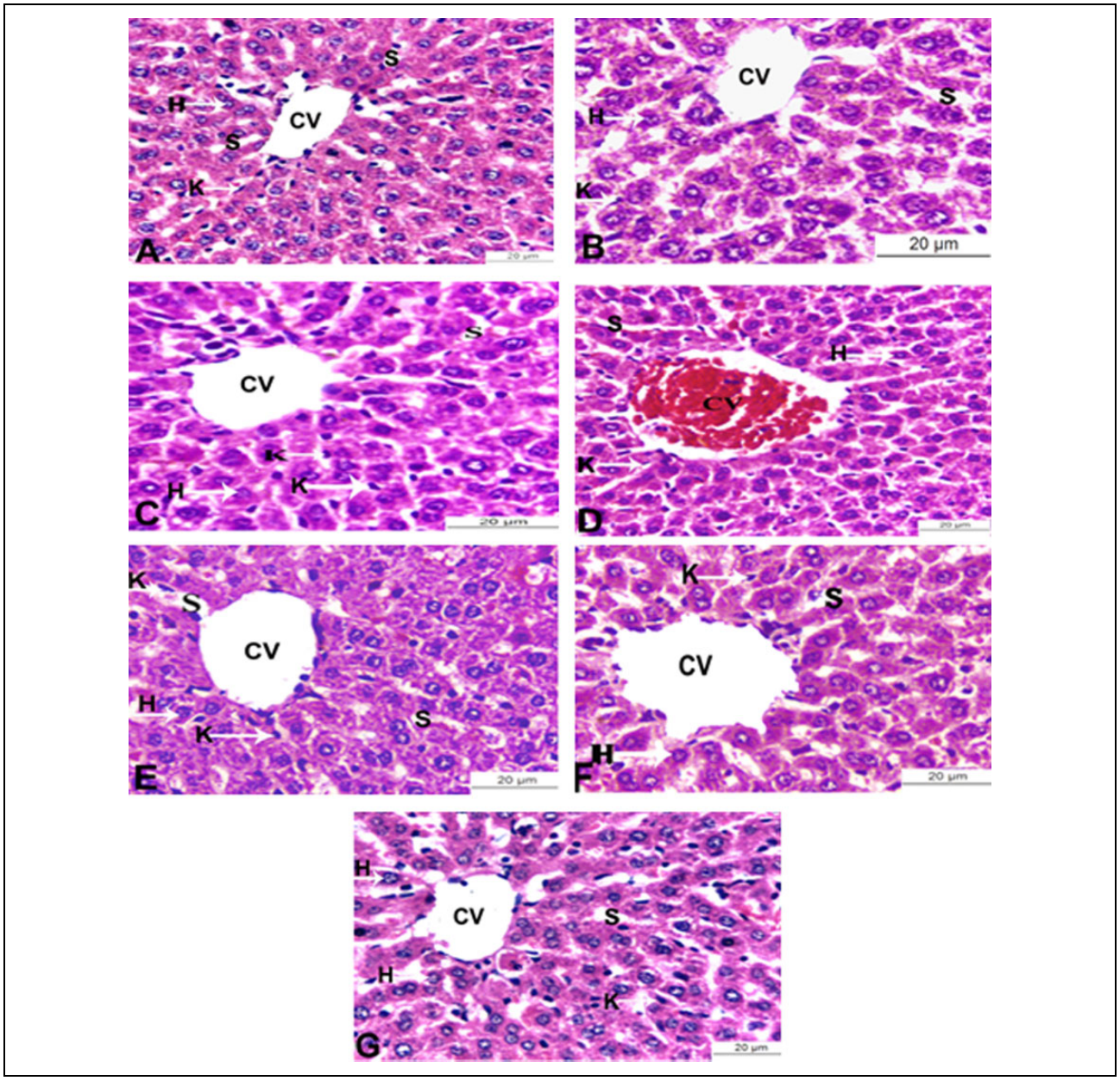

Figure 3. Histological section in liver of rats stained with hematoxylin and eosin at magnification ×40. (A) Photomicrograph showing histological section in control rat. (B) Photomicrograph showing histological section in rat treated with EE (500 mg/kg) only. (C) Photomicrograph of histological section in rat treated with EE (1000 mg/kg) only. (D) Photomicrograph of histological section in rat treated with SiNPs (1000 mg/kg) only. (E) Photomicrograph histological section in rat post treated with EE (500 mg/kg). (F) Photomicrograph histological section in rat post treated with EE (1000 mg/kg). (G) Photomicrograph histological section in rat post treated with silymarin (150 mg/kg). CV: central vein; H: hepatocyte; K: Kupffer cell; S: sinusoid; EE: earthworm extract.

morphology of rats treated with EE at low dose showed disorganization of hepatocytes, sinusoids, and increased Kupffer cells (K; Figure 3(B)). Meanwhile, rats treated with a high dose of EE had more or less a normal liver architecture with few lymphocytes and enlarged Kupffer cell (Figure 3(C)). However, histological alterations were detected in the liver tissue of SiNPs-treated rats. These alterations showed severe liver injury with prominent hemolyzed red blood cells in the central vein and in sinusoidal spaces and degenerated hepatocytes (Figure 3(D)). On the other hand, posttreatment of silymarin showed almost complete normalization of the liver tissue that neither hemolyzed blood cells nor degenerated hepatocytes were

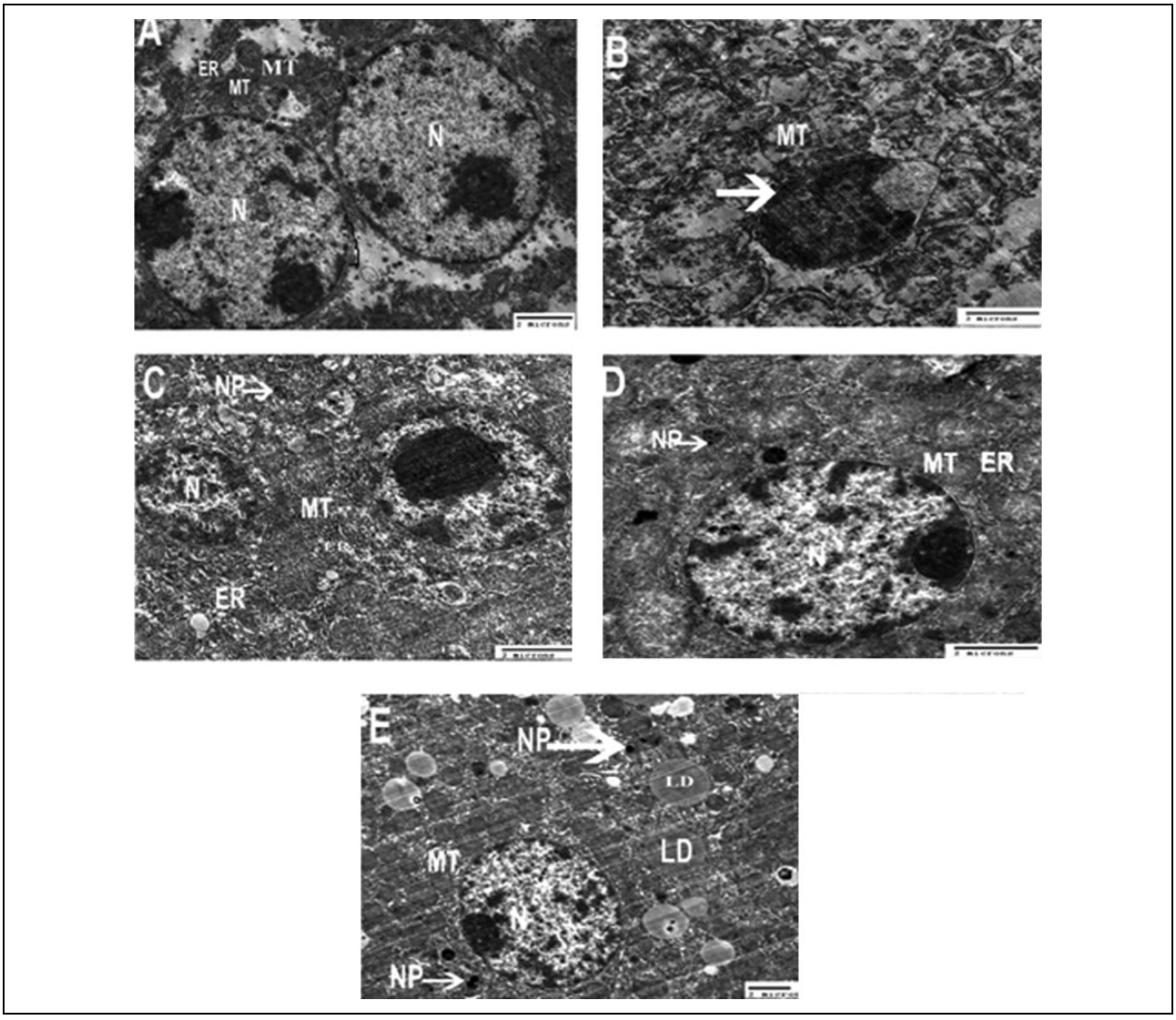

Figure 4. TEM images of rat liver. (A) Image of control rat (magnification ×6000). (B) Rat administrated with SiNP (1000 mg/kg); SiNPs were dispersed throughout the cytoplasm (magnification, ×8000). (C) SiNP-treated rat with EE (500 mg/kg; magnification ×8000). (D) SiNPs-treated rat with EE (1000 mg/kg; magnification ×8000). (E) SiNP-treated rat with silymarin (150 mg/kg; magnification, ×4000). White arrow denotes the presence of pyknotic nucleus with condensed electron dense chromatin with nuclear halo. N: nucleus, MT: mitochondria, ER: endoplasmic reticulum, NP: nanoparticles, LD: lipid droplets; SiNPs: silicon dioxide nanoparticles; TEM: transmission electron microscopy; EE: earthworm extract.

observed as compared to the SiNPs-treated rats (Figure 3(G)). Meanwhile, posttreatment of EE at two doses exhibited partial recovery against the SiNPs-induced toxicity (Figure 3(E) and (F)).

TEM studies

Ultrastructure of hepatocyte in albino rats is shown in Figure 4. Regular morphology of the liver cell with many cytoplasmic organelles such as mitochondria (MT) and endoplasmic reticulum (ER) were observed (Figure 4(A)). The SiNPs-treated group showed severe liver injury with mitochondrial destruction, pyknotic nucleus with condensed electron dense chromatin with nuclear halo (Figure 4(B)). These results suggested that SiNPs could damage the structure of rat hepatocyte. The internalized NPs could lead to the damage of cell membrane. On the other hand, posttreatment with EE at the two selected doses and silymarin showed almost complete normalization of the liver tissue with well-developed cell organelles such as MT and ER, and the SiNPs dispersed in cytoplasm

with cluster or individual form with or without membrane binding (Figure 4(C) to (E)). These results confirmed that the EE at the two selected doses have a potential ameliorative effect against that SiNPs-induced hepatotoxicity.

Discussion

The NPs have the ability to enter, translocate within, and damage living organisms (Shah and Belozerova, 2009). Liver is the major organ for biotransformation of toxins, and it may be the first organ to be exposed to NPs that are able to enter the circulation (Nalabotu et al., 2011).

It has been suggested that the physicochemical properties of NPs should be appropriately characterized before their nanotoxicity research (Balbus et al., 2007; Li et al., 2011; Murdock et al., 2008; Yu et al., 2009). We utilized XRD, TEM, and DLS techniques to characterize the present SiNPs. The amorphous nature of SiNPs was confirmed by XRD. TEM showed that the majority of the NPs had a polygonal shape, smooth surface, and an average diameter of 11 nm. The average hydrodynamic size of SiNPs in water as determined by DLS was 1039 nm. We observed that the hydrodynamic size of SiNPs measured by DLS was approximately 10 times higher than those calculated from TEM. The higher size of NPs in aqueous suspension as compared to TEM size might be due to the tendency of particles to agglomerate in aqueous state. This finding is supported by other investigators (Ahamed et al., 2010; Bai et al., 2009). The tendency of particles to form aggregates depends on the surface charge. The surface charge of SiNPs determined as zeta potential was −24.5 mV.

This study has revealed a significant increase in the activities of serum AST and ALT after SiNPs administration. This finding indicates hepatocytes damage that alter their transport function and membrane permeability as well as leakage of enzymes from the cells. In consonance with the present result, some investigators registered an increase in the activity of serum AST and ALT levels subsequent to SiNPs administration (Fu et al., 2012; Liu et al., 2011).

GGT, a microsomal enzyme that is present in the membrane of the ER of the hepatocyte, has a diagnostic value in hepatobiliary diseases since it is extensively released from damaged hepatic cells to the blood stream (Khalaf et al., 2009). The high activity of serum GGT after SiNPs administration, recorded in this study, may be an indication of unhealthy state of the hepatocytes' membrane integrity and their functionality of such cells in the experimental animals.

An increased activity of ALP that occurs due to *de novo* synthesis by liver cells is a reliable marker of hepatobiliary dysfunction due to damage (Muriel and Escobar, 2003). In this study, rats showed elevated serum ALP levels due to SiNP intoxication. A partial similarity (similarity only regarding the result not the route of administration) with the present finding, Ivanov et al. (2012) disclosed an elevation in the level of serum ALP of rat after intravenous administration of SiNPs, and they added that this indicates the induction of hepatotoxicities.

Any change in the concentration of serum protein and albumin indicates a change in the normal liver function (Ahmad et al., 1992). The significant reduction, recorded in this study, in the total protein concentration of the SiNPs-treated rats may be explained as a reduction in the protein synthetic function of the liver, which could be as a result of possible damage to the hepatocytes induced by SiNPs.

Bilirubin, a major breakdown product of hemoglobin, rises when there is liver injury or damage (Sanjiv, 2002). Elevation of total bilirubin in this study results from decreased uptake and conjugation of bilirubin by the liver due to liver cell dysfunction; meanwhile, the increased levels of direct or conjugated bilirubin are due to that decreased secretion from the liver or obstruction of the bile ducts (Sanjiv, 2002).

In this study, posttreatment with EE at the two selected doses as well as silymarin for 30 days after SiNPs administration caused a significant decrease in the serum activities of AST, ALT, GGT, ALP, total and direct bilirubin, and albumin to nearly normal levels. These findings indicate that EE could be stabilizing the plasma membrane as well as repair the hepatic tissue damage caused by SiNPs. This observation correlates with the finding of Balamurugan et al. (2008) who reported a hepatoprotective effect of EE from *Lampito mauritii* against the liver injury induced by paracetamol.

Antioxidant defense system plays an important role in the elimination of oxygen radical *in vivo*, which includes enzymatic scavengers (SOD, CAT, and GPx) and some small antioxidant molecules such as GSH (Olsvik et al., 2005). Oxidative stress has been reported as toxic mechanism of SiNPs-induced apoptosis and inflammation in various cell types (Park and Park, 2009). LPO has been used extensively as a

biomarker of oxidative stress *in vivo* (Sayeed et al., 2003). LPO is estimated by measuring the content of MDA. The enhancement of lipid peroxides in the rat liver caused by SiNPs, through MDA elevation, implicated an oxidative attack that was activated by a reduction of the antioxidative defense mechanism (Kong et al., 2007). GSH, one of the major tripeptide nonenzymatic biological antioxidants present in the liver,, is concerned with the removal of free radicals and maintenance of membrane protein and thiols and a substrate for GPx (Jollow, 1980). Lii et al. (1998) reported that a decrease in GSH level in the hepatotoxicated animals might have been due to an increased scavenging of reactive substances that were produced as a result of the necrotic and/or static state of the hepatocytes. This study confirmed the results of Akhtar et al. (2010) and Ye et al. (2010) who suggested that SiNPs induced lipid peroxidation that contributed to the depletion of tissue level of GSH.

CAT is one of the important enzymes in the supportive team of defense against reactive oxygen species (ROS). The inhibition of liver CAT activity following SiNPs intoxication, in this study, may be attributed to the enhancement of the peroxidation end product MDA. Durairaj et al. (2008) reported that the reduction in the activity of CAT may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide (H_2O_2). In this study, activity of liver SOD decreased after the oral administration of SiNPs, which may suggest that SiNPs could trigger the generation of ROS and resulted in injuries of the tissues (Park and Park, 2009). Liu et al. (2010) reported that a large amount of SOD was consumed to diminish ROS level. Thus, this finding reminds us that the damage in the liver may be related to the decreased activity of SOD caused by SiNPs and this is in consonance with Yang et al. (2009). GR is important in recycling oxidized GSH back to GSH (Akhtar et al., 2010). In this study, the administration of SiNPs caused significant decrease in GR activity, which is consistent with a marked decrease in GSH levels. In accordance with this result, Akhtar et al. (2010) reported that GR-specific activity in human lung epithelial cells (A549 cells) decreased significantly after treatment with SiNPs at a concentration of 400 μg/mL. GPx plays an important role in catalyzing H_2O_2 and other lipid hydroperoxides reduction into nontoxic products at the expense of GSH. In this study, a significant increase in the level of liver GPx after SiNPs administration indicates the removal of the H_2O_2 in the metabolism and avoids greater oxidative damage in the body (Hao and Chen, 2012). In accordance with the present results, Munteanu et al. (2010) reported that GPx-specific activity in MRC-5 cells treated with 6.3×10^5 SiNPs per cell increased significantly after 48 and 72 h of exposure.

An analysis of EE (*A. caliginosa*) shows high levels of the precursor amino acids of GSH which are glycine, glutamine, and cysteine. It was reported that the administration of such amino acids like methionine (Lieber et al., 1990) and cysteine (Anuradha and Vijayalakshmi, 1995) replete the levels of antioxidants and minimizes the oxidative stress. This study has revealed that the EE administration alone for 30 days at the selected doses causes a significant increase in the liver GSH level.

In this study, posttreatment with EE at the two selected doses and silymarin restores the alterations in the levels of liver MDA, GSH, SOD, GPx, GR, or CAT caused by SiNPs administration toward the normal levels. These findings indicated that the EE administration reduced LPO in liver and removed free radicals as EE administration only enhanced the level of liver GSH. Balamurugan et al. (2008) indicated that EE reduces oxidative stress by suppressing the formation of ROS and protecting the antioxidant machinery. Again, another postulated cellular mechanism to prevent the oxidative stress damage is the modulation of the genes of antioxidant enzymes, such as SOD and CAT (Crawford, 1999; Shull et al., 1991). In consonance with this study, several investigators reported an increase in the activities of CAT, SOD, and GSH subsequent to EE administration (Omar et al., 2012; Prakash et al., 2008).

The TEM pictures showed that SiNPs were dispersed in the cytoplasm and accumulated in some organelles. Morphological changes of cell injury were also observed from the TEM image such as the destruction of mitochondrial structure. The internalized NPs could lead to the damage of the membranes of cells and MT of the cells directly by penetrating membrane and inducing other structure injury (Li et al., 2003). The injury of MT induced by NPs may be due to ROS production. MT play important roles in some biological processes, such as supplying cellular energy, cellular differentiation, and cell apoptosis. Thus, the mitochondrion is one of the most sensitive organelles to many exogenous compounds. Unfried et al. (2007) reported that there is a close relationship between MT and ROS production. MT were considered as major cell compartments relevant for possible

NP toxicity. The NPs could localize in the MT and induce mitochondrial structure damage through oxidative stress (Li et al., 2003; Pan et al., 2009; Xia et al., 2006).

Based on the results of this study, it was concluded that SiNPs have the potential to cause toxicity through its adverse influences on liver function and structure as well as on some serum biochemical parameters. The curative ability of EE to attenuate these influences is believed to be related to its intrinsic antioxidant properties, and these properties were comparable to the control drug silymarin. However, further studies are necessary to isolate and identify the principal components of EE and elucidate the mechanisms of their action.

Conflict of interest

The authors declared no conflicts of interest.

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