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Zinc citrate incorporation with whey protein nanoparticles alleviate the oxidative stress complication and modulate gene expression in the liver of rats

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ABSTRACT

This study aimed to evaluate the hepatoprotective effect of whey protein nanoparticles (WP-NPs) coated Zinc citrate (Zn) against oxidative stress complications and disturbances in gene expression in rats treated with CCl_4. WP-NPs were used to coat Zn at three levels and amino acids content was determined in WP-NPs and the fabrications. Seven groups of male albino rats included the control group, CCl_4-treated group (0.5 ml/100 g b.w) and the groups treated with CCl_4 plus WP-NPs, Zn and the three Zn-WP-NPs fabrications. Blood and liver samples were collected for different analysis. Particles sizes were 95, 142, 196 and 228 nm and zeta potential values were −95, −114, −85 and −79 for WP-NPs and the three Zn-WP-NPs fabrications, respectively. Twelve amino acids were found in WP-NPs and this number was decreased by increasing Zn content. WP-NPs, Zn and the Zn coated WP-NPs counteracted the disturbances in biochemical, parameters, gene expression and histological changes in CCl_4-treated rats and Zn-WP-NPs was more effective at the low dose. It could be concluded that WP-NPs enhance the effect of Zn and can be used for coating Zn in the preparation of Zn supplementation to enhance its effect and counteract the side effect of excess Zn.

1. Introduction

Zinc (Zn) is well known as an essential trace element that affects several vital processes such as immune function, cell proliferation, the defense against oxidative stress and free radicals generation and activation of antioxidant (Prasad, 2014). The antioxidant activity of Zn is might due be through the maintaining of adequate levels of metallothionein, which is an essential component for Copper/Zn SOD synthesis (Bogani et al., 2013). Zn supplementation reduces lipid peroxidation in protein deficient-fed animals, enhanced reduced glutathione and the activity of SOD (Sidhu et al., 2005). Moreover, the elevated level of LPO in rat tissues was reduced by Zn supplementation (Yu et al., 2014). Additionally, Zn is well known to have a vital role in DNA repair, cell division and differentiation as well as its neuroprotective effects (Soussi et al., 2018).

Moreover, several people think that Zn can cure different ailments such as cancer (Yu et al., 2018), growth failure (Cho et al., 2018), infection (Yousefichaijan et al., 2016), wounds and skin diseases (Pietsch et al., 2009) since there are some evidences that these aliments can result from Zn deficiency. The use of water-soluble Zn compounds such as zinc acetate, zinc gluconate or zinc sulfate in syrups or tablets for the treatment of diarrhea in infants (WHO, 2006). However, zinc acetate and zinc sulfate have a strong metallic, astringent and bitter taste and should be masked. On the other hand, low Zn content of Zn gluconate makes it more expensive. The EU permitted the water insoluble ZnO and the soluble Zn sulfate for the use of supplements or food fortification since they are less expensive (IZiNCG et al., 2004). Wegmüller et al. (2014) reported that Zn citrate is an alternative compound and has promising sensory properties due to its high Zn content, its slightly water soluble its odorless and its relatively low cost. Despite no available data on the human absorption to support the use of Zn citrate, few data suggested that Zn citrate, Zn sulfate and Zn gluconate are absorbed equally and Zn oxide showed slightly less absorption (Siepmann et al., 2005). Moreover, Bertinato et al. (2012)

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showed that Zn citrate was similar bioavailability to other Zn compounds such as Zn sulfate, acetate, gluconate and oxide; however, Zn gluconate and Zn oxide was found to have higher dialyzability than Zn citrate *in vitro* (Guillem et al., 2000).

Previous reports indicated that food fortified with Zn is characterized by some changes in the sensory attributes such as flavor (Salgueiro et al., 2002). Several compounds were used in food fortification but their bioavailability is determined by the effect of gastric pH on their stability and solubility. In this concern, zinc oxide is not expensive and does not possess any adverse effects on sensory perception; however, it is not soluble at gastric pH, so it has a low bioavailability (Gibson and Ferguson, 1998). Recently, nanotechnology has been applied in the food industry (Ko and Gunasekaran, 2006) and nanoparticles display promising tools in the improving of nutraceutical compounds bioavailability, especially those with poor solubility originated from their subcellular size (Shi et al., 2008). The use of encapsulated Zn compounds is effective to increase its bioavailability and prevents the sensory defects. Consequently, development of effective delivery tools to encapsulate and control the release of ZN is of great demand.

Protein-based nanoparticles (NPs) are interesting because their preparation is relatively easy and the distribution of their size can be monitored (MacAdam et al., 2000). Recently, whey protein (WP) has received considerable attention due to its antioxidant bioactivity (Zhang et al., 2012). WP is used widely in the food manufacturers for its nutritional, functional-values and health-promoting (Ramos et al., 2017) including immunomodulation, anti-hypertension and cardioprotection (Martin et al., 2015). The antioxidant properties of WP have been utilized to produce the natural antioxidant used as food additives (Corrochano et al., 2018). WP was utilized as a drug carrier for oral administration and the nano-sized WP is an outstanding choice as a carrier medium of Zn (Ko and Gunasekaran, 2006). Ocak (2010) reported that metal ions have the ability to bind to the functional groups in proteins (i.e. amino and peptide groups, sulphydryl, imidazole, carboxyl). Therefore, the aims of the current study were to synthesis WP nanoparticles (WP-NPs), to utilize WP-NPs as a coating for different levels of zinc citrate (Zn) and to determine the best formula that has antioxidant activity, modulate gene expression and counteract the oxidative stress complications in the liver of rats treated with CCl_4.

2. Materials and methods

2.1. Materials, chemicals and kits

Whey proteins isolate (WPI) 92.6% proteins (w/w) was obtained from Davisco Foods International Inc. (Eden, Prairie, MN, USA). Zinc citrate was obtained from El Nasr. Pharmaceutical Chemicals Co. (Cairo, Egypt). Standards of amino acids, sodium tripolyphosphate (TPP) and RevertAid™ H Minus First Strand cDNA Synthesis Kits were purchased from Sigma Chemical Co. (St. Luis, Mo, USA). ™DNase and the removal reagent kit was purchased from Promega, Co. (Madison, WI, USA). Transaminase (ALT and AST) kits were purchased from Randox, Antrim (UK Co), cholesterol (Cho), triglycerides (TriG), high density lipoprotein (HDL), low density lipoprotein (LDL) and total protein (TP) were purchased from FAR Diagnostics Co. (Via Fermi, Italy), catalase (CAT), nitric oxide (NO), glutathione peroxidase (GPx), superoxide dismutase (SOD) kits were obtained from Eagle diagnostics (Dallas, TX, USA) and malondialdehyde (MDA) kit purchased from Oxis Research™ Co. (USA). TRIZOL reagent was purchased from Invitrogen™ (Carlsbad, CA, USA). All other chemicals used throughout the experiments were of the highest analytical grade available.

2.2. Preparation of whey protein soluble polymers

WPI was dispersed in Milli-Q water to form 8% (w/v) and adjusted with 1 N NaOH to pH 7. The dispersion was kept refrigerated at 4 ^{O}C overnight for complete hydration and was warmed at room temperature and degassed for 20 min under vacuum (560 mm Hg). The dispersion was then heated up in a water bath up to 80 ^{O}C at a heating rate of 8 ^{O}C/min and kept for 15 min at this temperature. The heated suspension was cooled to room temperature in an ice bath and then diluted to 2% (w/v) using Milli-Q water.

2.3. Preparation of WPNPs

WP-NPs were prepared by the pH-cycling method previously described by Giroux et al. (2010). The whey protein polymers dispersion was acidified under stirring to the aggregation pH (6.0, 5.5, or 5.0) with 0.1 N HCl, and $CaCl_2$ was added at a final concentration of 0, 2.5 or 5 mM. Because the addition of $CaCl_2$ decreased the pH, the aggregation pH was adjusted at 0.5 units higher than the target value and corrected if necessary after the addition of $CaCl_2$. The protein concentration after pH adjustment and calcium addition was 1.9% (w/v). The preparation was aged at the aggregation pH for a period of 0, 2.5, 5, 22 or 75 h at 4 ^{O}C to allow the formation of disulfide bonds between protein polymers. After the ageing period, the dispersion was neutralized to pH 7.0 with 0.1 N NaOH and the protein concentration was adjusted to a final value of 1.75% (w/v). Increasing pH restored electrostatic repulsions and disrupted non-covalently linked aggregates.

2.4. Coating of zinc citrate by whey protein nanoparticles (Zn-WP-NPs)

Zinc citrate powder was added to the prepared WP-NPs at three concentrations: 7 mg/g WP-NPs (low dose), 14 mg/g WP-NPs (medium dose) and 28 mg/g WP-NPs (high dose). The mixture (250 ml) was then acidified under stirring using 0.1 N HCl to an aggregation pH of 6.0 and 1 ml of 62.5 mg/L $CaCl_2$ solution was added to reach the final Ca concentration of 2.25 mM in the final mixture and left formation disulphide bonds. The dispersion was then brought to pH 7 using 1 N NaOH at room temperature, homogenized, using 2-stage Rannie Homogenizer (Copenhagen, Denmark) at 20 MPa for the first stage and 3.5 MPa for the second stage, respectively. In order to obtain the low average size of nanoparticles, 50 ml of the preparation was kept in solution for particle size, transmission electron microscope and atomic absorption analysis, while the other part of the formula was dried. Two hundred ml from the zinc coated WP-NPs (Zn-WP-NPs) preparation was filtered through 3 μm pore size filter paper before homogenization. The precipitate was then washed using 20 ml Milli Q-water then was collected in a porcelain dish and allowed to dry under vacuum overnight. The large particles in the dried precipitate were then ground using a mortar and pestle.

2.5. Particle size analysis

The z-average diameter and size distribution of WP-NPs alone or Zn-WP-NPs at the three levels were carried out at 25 ± 0.1 °C using Nano ZS/ZEN3600 Zetasizer (Malvern Instruments Ltd., UK) with a He/Ne laser (λ = 633 nm), refractive index 1.35 and scattering angle 90° scattering optics. The z-average diameter (Dz) and polydispersity index (PDI) were recorded by dynamic light scattering (DLS) as described by Giroux et al. (2010). The samples were prepared for TEM according to the method described by Moslehishad and Ezzatpanah (2010). The grid was air dried and examined by TEM using a JEOL JEM-1400 with an accelerating voltage of 100 kV at a magnification of 200,000 x.

2.6. Determination of amino acids

The determination of amino acids in WP-NPs and the three formulations of Zn-WP-NPs at the three levels were carried out by HPLC according to the method described by Heinrikson and Meredith (1984).

Table 1
Primer sequences and expected product sizes for the genes amplified.

cDNA	Forward primer.(5' -3')	Reverse primer (5' -3')	PCR product size bp	Reference
Caspase-3	AAATTCAAGGGACGGGTCAT	ATTGACACAATACACGGGATCTGT	205 bp	Liu et al. (2002)
Bax	AGGATGATTGCTGATGTGGATAC	CACAAAGATGGTCACTGTCTGC	300 bp	Van Der Hoeven et al. (2003)
Bcl2	GCTACGAGTGGGATACTGGAGA	AGTCATCCACAGAGCGATGTT	446 bp	Schoemaker et al. (2002)
GPx	CTCTCCGCGGTGGCACAGT	CCACCACCGGGTCGGACATAC	290	Limaye et al. (2003)
Cu-Zn SOD	GCAGAAGGCAAGCGGTGAAC	TAGCAGGACAGCAGATGAGT	387 bp	Limaye et al. (2003)
CAT	GCGAATGGAGAGGCAGTGTAC	GAGTGACGTTGTCTTCATTAGCACTG	652 bp	Gandhi et al. (2013)
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG-	496	Wiame et al. (2000)

2.7. Experimental animals

Seventy Adult male albino Wistar rats weighing about 120 ± 15 g were purchased from the National Organization of Drug Control and Research (NODCR). Animals were maintained on a standard lab diet (metabolizable energy 12.08 MJ; protein: 160.4; fat: 36.3; fiber: 41 g/kg purchased from Meladco Feed Co., Aubor City, Cairo, Egypt). The animals were 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^{O}C) and humidity (50 ± 5%) at the Animal House Lab., NODCR, Giza, Egypt. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Organization of Drug Control and Research (NODCR) and the National Institutes of Health (NIH publication 86-23 revised 1985).

2.8. Experimental design

After an acclimatization period of 1 week, the animals were divided into seven equal groups (10 rats/group) and treated orally for 4 weeks as follows: group 1, normal control group received buffer as vehicle; group 2, animals treated orally with 3.3% CCl_4 in corn oil (0.5 ml/100 g b.w) twice a week; group 3, animals treated orally with WP-NP (300 mg/kg b.w.) plus CCl_4; group 4, animals treated orally with Zn dissolved in buffer (50 mg/kg b/.w) plus CCl_4 and the groups treated orally with 50 mg/kg b.w of Zn-WP-NPs (LD), Zn-WP-NPs (MD) or Zn-WP-NPs (HD) plus CCl_4. All the three formulas were given to the animals dissolving in the buffer. At the end of the treatment period (i.e. day 28), all animals were fasted for 12 h, then blood samples were collected from the retro-orbital venous plexus under diethyl ether anesthesia. Sera were separated using cooling centrifugation and stored at −20 ^{O}C until analysis. The sera were used for the determination of ALT, AST, total protein, Cho, TG, triglycerides, LDL-Ch, HDL-Ch and NO according to the kits instructions using a spectrophotometer. After the collections of blood samples all animals were sacrificed by cervical dislocation and samples of the liver were collected. Three liver samples were collected from each animal within different treatment groups. The first liver sample was dissected, weighed and homogenized in phosphate buffer (pH 7.4) to give 20% w/v homogenate and was used for the determination of MDA and NO then it was further diluted to give 2% and 0.5% dilution for the determination of hepatic GPX (2%), CAT and SOD (0.5%) activities (Lin et al., 1998). The second liver sample from each animal was used for the determination of the molecular genetic analyses. The third liver sample was used for the histopathological examination. These samples were fixed in 10% neutral formalin and paraffin embedded. Sections (5 μm thickness) were stained with hematoxylin and eosin (Hx & E) for the histological examination (Drury and Wallington, 1980).

2.9. Molecular genetics analyses

2.9.1. Semi-quantitative –PCR

2.9.1.1. Isolation of total RNA. One hundred mg of fresh frozen liver tissue were used for the extraction of RNA by the standard TRIzol® Reagent (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's procedures and recovered in 100 ml of diethylpyrocarbonate (DEPC)-treated water. In order to remove any possible genomic DNA contamination, the total RNA samples were pretreated using DNA free TM DNase and removal reagent kit (Promega, Co) following the manufacturer's procedures. The integrity and quality of the purified RNA were checked through agarose gel electrophoresis (1%) according to the integrity of 18S and 28S of rRNA bands. The RNA quantity was ascertained spectrophotometrically (Jenway 6505, UK) as described by Sambrook and Russell (2001) with an A260/A280 ratio between 1.7 and 1.9. The purified RNA samples were preserved at 80 °C until use.

2.9.1.2. Reverse transcription and semi-quantitative polymerase chain reaction (sq-PCR). Two mg of RNA were reverse transcribed into in a total volume of 20 ml cDNA using the high capacity RNA to PreMix cDNA Kit (iNtRON Biotechnology, Korea). The resulting cDNA was stored at 20 °C for later use or directly used as a semi quantitative PCR template.

2.9.1.3. Gene expression analysis using semi quantitative PCR. Caspase-3, Bcl2, Bax, GPx, SOD and CAT expression were determined in liver using semi quantitative PCR. Oligonucleotide PCR primer pairs were developed for caspase-3, Bcl2 and Bax genes based on the published primer sequences (Table 1). GAPDH amplification was used as the housekeeping gene in semi quantitative PCR analysis. The thermal cycling parameters were: initial denaturation at 94 ^{O}C for 5 min, 30 cycles of amplification at 94 ^{O}C for 60 s for DNA denaturation, annealing at 52–60 ^{O}C for 30 s, extension at 72 ^{O}C for 1 min and a final extension at 72 ^{O}C for 5 min (Abdel-Aziem et al., 2014).

2.9.1.4. Agarose gel electrophoresis. All PCR products were electrophoresed on 2% agarose, stained with ethidium bromide and visualized by UV transilluminator.

2.9.1.5. Semi-quantitative determination of PCR products. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control and was also amplified using its specific primer. The ethidium bromide-stained gel bands were scanned and the intensity of each band was quantified by the computerized Gel-Pro (version 3.1 for window 3). The ratio between the levels of the target gene amplification products and the GAPDH (internal control) was calculated to normalize for initial variation in sample concentration as a control for reaction efficiency (Raben et al., 1996).

2.10. Statistical analysis

All data for biochemical parameters were statistically analyzed by one-way ANOVA using the General Linear Models 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 $P < 0.05$.

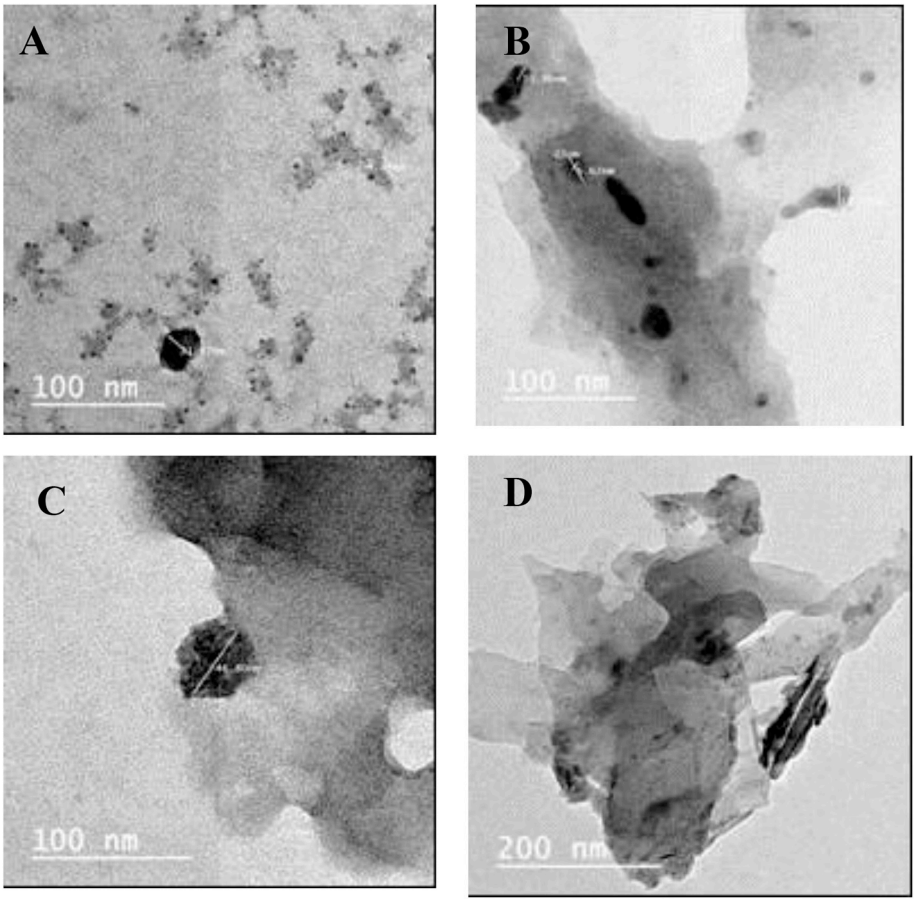

Fig. 1. TEM images for **(A)** WP-NPs, **(B)** Zn-WP-NPs (LD), **(C)** Zn-WP-NPs (MD) and **(D)** Zn-WP-NPs (HD).

3. Results

3.1. Characterization of Zn-WP-NPs

TEM images (Fig. 1 A, B, C and D) showed nearly spherical shape for Zn-WP-NPs. The dark part is corresponding to Zn while the pale part is corresponding to WP-NPs. Moreover, The Zn-WP-NPs were stable during TEM analysis suggesting the strong binding between Zn and WP-NPs. The size distribution of WP-NPs, Zn-WP-NPs (LD), Zn-WP-NPs (MD) and Zn-WP-NPs (HD) by DLS measurements expressed in numbers showed that the composite granules are uniform with an average size of the particles of 95, 142, 196 and 228 nm, respectively (Fig. 2a,b,c and d). WP-NPs alone or Zn loaded WP-NPs at the three doses showed a negative zeta potential value of −95, −114, −85 and −79, and the Zn ratio in the three formulas was 13.8%, 33.9% and 46.92%, respectively.

3.2. Amino acids analysis

The results of amino acids content in WP-NPs, and Zn loaded WP-NPs at the three levels are presented in Table (2). WP-NPs (Fig. 3a) and the Zn-WP-NPs (LD) (Fig. 3b) contained twelve amino acids, although the concentrations of these amino acids were higher in WP-NPs than Zn-WP-NPs (LD). Five amino acids were absent in Zn-WP-NPs (MD) (Fig. 3c); however, seven amino acids were absent in Zn-WP-NPs (HD) (Fig. 3d). Generally, the number of amino acids was decreased by increasing the level of Zn in the mixture.

3.3. Biochemical assays

The biochemical results presented in Table (3) revealed that animals treated with CCl_4 showed a significant increase in ALT and AST accompanied with a significant decrease in total protein (TP) compared to the control or the other treatment groups. Animals treated with WP-NPs alone, Zn alone or Zn coated WP-NPs at the three levels could induce a significant improvement in all the biochemical parameters of liver function towards the control levels although these treatments did not normalize them. The results also indicated that Zn-WP-NPs (LD) was the most effective since it normalized ALT, AST and TP. Moreover, WP-NPs alone or Zn alone could normalize TP and the improvement in all the tested parameters was decreased when the level of Zn was increased (i.e. 14 and 28 mM/g).

The effect of different treatments on lipid profile (Table 4) revealed that treatment with CCl_4 resulted in a significant increase in cholesterol, triglycerides and LDL-Ch accompanied with a significant decrease in HDL-Ch compared to the control or the other treatment groups. Treatment with WP-NPs, Zn and the different levels of Zn loaded WP-NPs resulted in a significant improvement in lipid profile although none of these treatment could normalize these parameters. Additionally, Zn-WP-NPs (LD) showed the best results compared to the other treatments.

The current results also revealed that treatment with CCl_4 induced a significant decrease in the hepatic antioxidant enzyme activity GPX, SOD and CAT compared to the control or the other treatment groups (Table 5). Animals treated with WP-NPs, Zn or Zn coated WP-NPs at the three levels resulted in a significant improvement in the antioxidant enzyme activity although none of these treatments could normalize them. The data also showed that Zn-WP-NPs (LD) was the most effective to improve the antioxidant enzyme activity followed by WP-NPs alone then Zn alone. Additionally, the increased level of Zn e.g. Zn-WP-NPs (MD) and Zn-WP-NPs (HD) reduced the improvement in the tested antioxidant enzyme activity.

The current results also indicated that treatment with CCl_4 induced a significant increase in serum NO and hepatic lipid peroxidation as

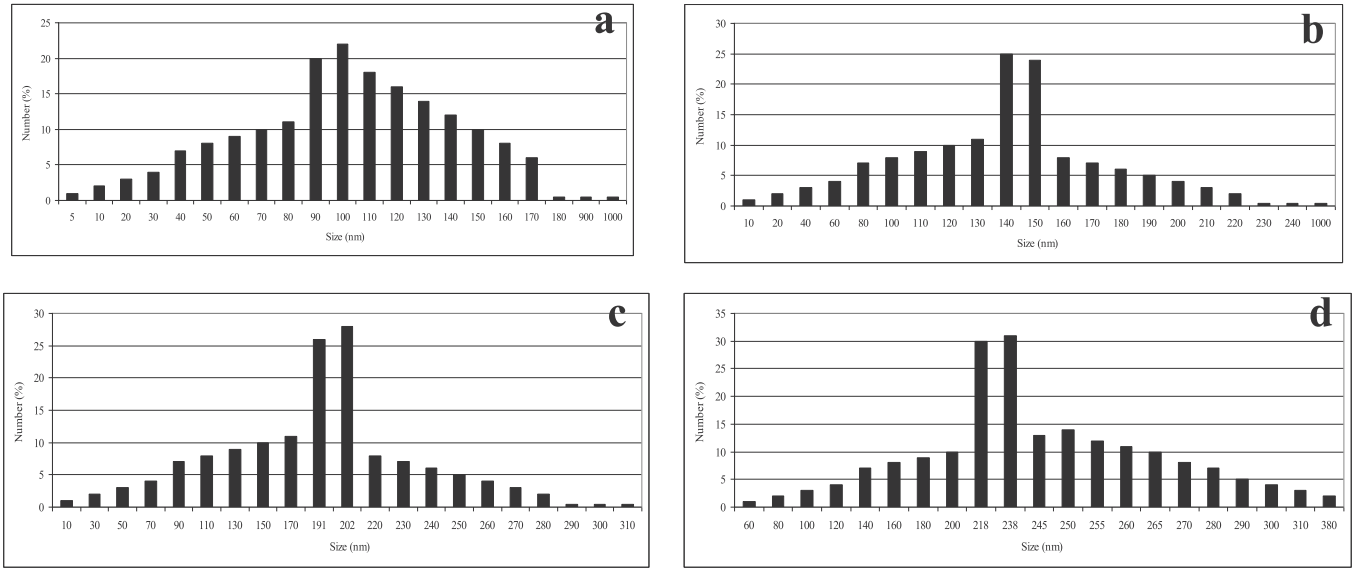

Fig. 2. The size distribution of **(a)** WP-NPs, **(b)** Zn-WP-NPs (LD), **(c)** Zn-WP-NPs (MD) and **(d)** Zn-WP-NPs (HD) by DLS measurements.

Table 2
Amino acids of WP-NPs and Zn loaded WP-NPs at the three levels tested (mg/g).

Amino acid	WP-NPs	WP-ZnO-NPs (LD)	WP-ZnO-NPs (MD)	WP-ZnO-NPs (HD)
Aspartic Acid (ASP)	10.28	7.80	7.289	2.858
Glutamic Acid (GLU)	16.02	10.63	10.701	6.010
Serine (SER)	4.63	3.05	3.315	–
Glycine (GLY)	16.24	10.12	12.066	–
Histidine (HIS)	1.14	0.88	–	–
Threonine (THR)	5.86	4.58	–	–
Alanine (ALA)	3.94	2.49	2.807	0.995
Tryptophan (TYR)	1.91	1.21	1.341	0.393
Arginine (ARG)	1.99	1.40	–	–
Methionine (METH)	1.63	1.22	–	–
Valine (VAL)	5.31	3.61	–	–
Proline (PRO)	5.26	3.57	3.770	1.881

indicated by the increased level of MDA compared to the control or the other treatment groups (Table 6). Zn-WP-NPs (LD) was the most effective to reduce these oxidative stress markers resulted from CCl_4 treatment followed by WP-NPs alone then Zn alone. Moreover, the improvement in these markers was decreased with the increased of Zn in the mixture.

3.4. Effect on the mRNA expression apoptotic genes

The changes in mRNA expression of proapoptotic Bax, antiapoptotic Bcl2 and caspase-3 in the liver of rats in different treatment groups compared to the control group and to the housekeeping glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) are represented in Fig (4A, B and C). No significant difference in the mRNA expression of these genes was noticed between the control group and the groups treated with either WP-NPs and/or Zn loaded WP-NPs. Animals treated with CCl_4 alone showed a significant increase in the expression of caspase-3 and Bax mRNA accompanied with a significant decrease in Bcl2 mRNA expression as compared to the control or the other treatment groups. Co-treatment with CCl_4 plus WP-NPs, Zn or Zn-WP-NPs at the three concentrations tested succeeded to improve the mRNA expression of Bax, Caspase-3 and Bcl2 towards the control levels. Moreover, the group treated with CCl_4 plus Zn-WP-NPs (LD) showed the best results and succeeded to restore the expression of these apoptotic genes to the normal levels of the control group.

3.5. Effect on the mRNA expression antioxidant genes

The changes in mRNA of the antioxidant gene expression in the liver of rats in different treatment groups compared to the control group and to the housekeeping glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) are represented in Fig (4 D, E and F). Treatment with CCl_4 induced a significant decrease in GPx, SOD and CAT gene expression compared to the control group or the other treatment groups. Animals treated with WP-NPs or ZnO-NPs were comparable to the control group regarding to GPX and CAT mRNA expression. However, SOD mRNA expression showed a significant decrease in the group treated with Zn alone compared to the control group. The co-treatment with CCl_4 plus Zn coated WP-NPs at the three tested doses resulted in a significant improvement in the mRNA expression of the three antioxidant enzymes, although Zn-WP-NPs (LD) showed the best result since this group was comparable to the control followed by Zn-WP-NPs (MD) then Zn-WP-NPs (HD).

3.6. Histological examination

The histological study of the liver sections of the control group showed normal central and most of the hepatocytes with vesicular nuclei (Fig. 5a). The liver sections of rats treated with CCl_4 showed extensive areas of patchy and confluent hepatocytes necrosis and lobular inflammation at the periphery of the hepatic lobule close to the portal triad, the hepatic cords are distorted due to focal necrosis mononuclear cells infiltration mostly macrophages and lymphocytes around central veins and in portal areas (Fig. 5b). The liver sections of the animals treated with CCl_4 plus WP-NPs showed focal fibrosis and necrosis in the hepatic lobule close to the portal tracts while there are nearly normal hepatic cells around the central vein (Fig. 5c). The liver section of rats treated with CCl_4 plus Zn showed marked improvement in hepatocytes architecture and the portal vein area (Fig. 5d). The liver sections of rats treated with CCl_4 plus Zn-WP-NPs (LD) showed the same picture of patchy lobular fibrotic damage in the form of fatty and necrotic degeneration close to the portal veins (Fig. 6a). However, the liver sections of the rats treated with CCl_4 plus Zn-WP-NPs (MD) showed periportal histological changes in the form of large and small fatty cells, necrosis, fibrosis and apoptotic cells (Fig. 6b). The liver sections of the rats treated with CCl_4 plus Zn-WP-NPs (HD) showed periportal necrosis with inflammatory and ballooning cells (Fig. 6c).

The histochemical staining with Masson Trichrome for collagen

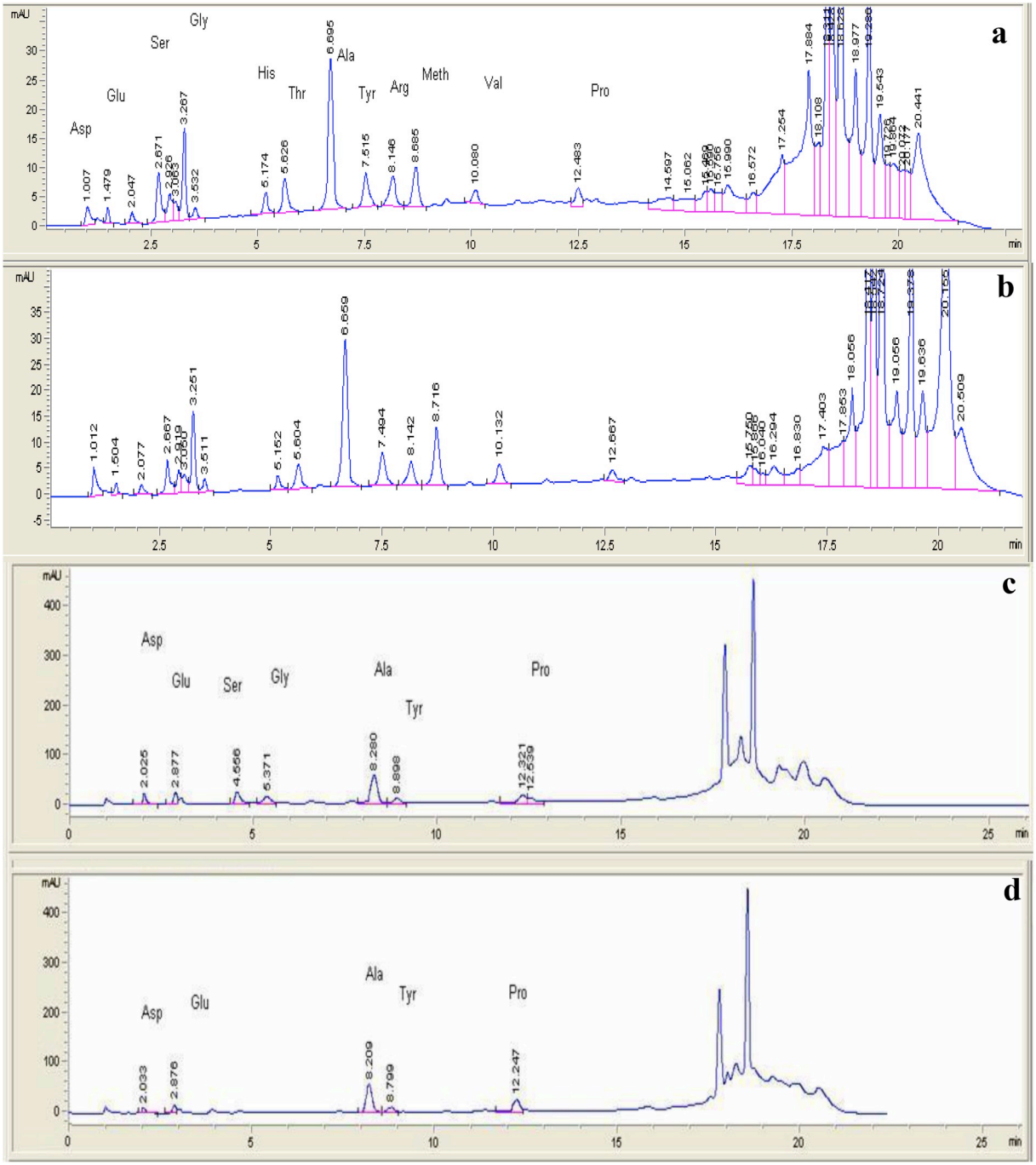

Fig. 3. HPLC chromatogram for amino acids analysis in: (a) WP-NPs, (b) Zn-WP-NPs (LD), (c) Zn-WP-NPs (MD) and (d) Zn-WP-NPs (HD).

fibers examination revealed that the control liver showed fine linear of the collagen fibers around the blood vessels (Fig. 7a). The liver sections of the rats treated with CCl_4 showed fibrosis, prominent increase in connective tissues and collagen between the liver lobules and around the portal tract (Fig. 7b). The liver sections of the rats treated with CCl_4 plus WP-NPs showed that the fibrosis and the collagen fibers are concerned around the portal tract (Fig. 7c). The liver section of rats treated with CCl_4 plus Zn showed marked decreased in collagen fibers or fibrosis around blood vessels (Fig. 7d). The liver sections of the rats treated with CCl_4 plus Zn-WP-NPs (LD) showed a marked increase in collagen fibers or fibrosis around blood vessels (Fig. 7e). The liver of animals treated with CCl_4 plus Zn-WP-NPs (MD) showed a prominent amount of collagen fibers and inflammatory cells (Fig. 7f); however, the liver of animals treated with CCl_4 plus Zn-WP-NPs (HD) showed detected amount of collagen fibers and inflammatory cells around the damaged portal tracts (Fig. 7g).

4. Discussion

The results of the current study revealed that the synthesized WP-NPs have a nearly spherical shape. The incorporation of Zn did not affect the particles shape, although it affects the average particles size and zeta potential. The increased in particles size by the incorporation by WP was reported previously by Gülseren et al. (2012) who reported that WP-NPs can be synthesized and the amount of zinc incorporated in the WP-NPs was within the range of daily zinc requirements for healthy adults.

The particles size was slightly higher than those reported by

Table 3
Effect of WP-NPs, Zn ad Zn loaded WP-NPs on liver function parameters in rats treated with CCl_4.

Parameter Groups	ALT (U/L)	AST (U/L)	T. Protein (g/dl)
Control	30.0 ± 0.58^{a}	31.90 ± 1.43^{a}	5.57 ± 0.05^{a}
CCL_4	65.0 ± 4.93^{b**}	81.33 ± 3.93^{b**}	3.34 ± 0.08^{b**}
WP-NPs + CCL_4	39.67 ± 4.98^{c*}	48.33 ± 0.67^{c*}	5.25 ± 0.08^{a*}
Zn + CCL_4	46.67 ± 2.91^{d*}	35.67 ± 1.20^{d*}	5.90 ± 0.21^{a*}
Zn-WP-NPs (LD) + CCl_4	31.0 ± 2.03^{a*}	32.67 ± 0.88^{a*}	5.09 ± 0.12^{a*}
Zn-WP- NPs (MD) + CCl_4	34.0 ± 2.65^{e*}	45.67 ± 1.45^{e*}	5.72 ± 0.15^{a*}
Zn-WP- NPs (HD) + CCl_4	37.67 ± 2.03^{c*}	46.67 ± 4.33^{e*}	4.02 ± 0.33^{a*}

Data are presented as mean ± SE.
Within each column, means superscript with different letters (a, b, c, …) are significantly different ($P < 0.05$).
*Significant at $P < 0.05$ compared to CCl_4 alone group.
**Significant at $P < 0.01$ compared to the control group.

Gülseren et al. (2012) which may be due to the difference in the preparation method. In another study, Gülseren et al. (2012a) showed that WP-NPs nanoparticles can be obtained using desolvation and the particles obtained were stable at pH 3 (acidic pH). It was also suggested that metal ions have the ability to bind to carboxyl, imidazole, amino acids, peptides and sulphydryl as the functional groups in the proteins (Ocak, 2010). The size distribution of the prepared WP-NPs and Zn loaded WPs at the three levels by DLS measurements revealed that the particles size was uniform for each granule with an average size of 95, 142, 196 and 228 nm, respectively. These results were similar to those reported by Shi et al. (2008) and Gülseren et al. (2012). It is worthy to mention that the percentage of zn was differ (i.e 13.8%, 33.9% and 46.92%) in the three formulas due to the difference in zn level in the formulas (7, 14 and 28 mg/g WP-NPs) which are in the range suggested by WHO and UNICEF (2001).

The determination of amino acids in WP-NPs showed the occurrence of twelve amino acids and the most abundant amino acids were glycine, glutamic acid and aspartic acid; however, the least abundant amino acids were histidine, methionine, tryptophan then arginine. Despite no available data on amino acids composition in WP-NPs, previous reports showed that WP isolate contains high concentrations of aspratate, threonine and alanine and low concentration in proline, glutamate and phenylalanine (Kalman, 2014).

The current results also showed that the low level of Zn-NPs coated WP-NPs have the same amino acids but in a lesser concentration compared to WP-NPs alone. Moreover, the increased level of Zn resulted in the decrease in amino acids content in the loaded NPs. Interestingly, histidine, threonine, arginine, methionine and valine were completely absent in Zn-WP-NPs (MD). Beside these five amino acids, serine and glycine were also completely absent in Zn-WP-NPs (HD). The changes in adsorbability of WP-NPs to the surface of Zn are affected by the amount of WP-NPs since we used only one level of WP-NPs for the preparation of the three concentrations to coating Zn. According to Saptarshi et al. (2013), the kinetics of the protein adsorption on the surface of NPs can be affected by many factors, including the amount of available proteins which interact with NPs surface. Moreover, it was reported that the interaction of Zn with protein (i.e. BSA) did not show any structural perturbation to the general structure, although minor partially configured changes were suggested (Bardhan et al., 2009), it may induce denaturation for the protein (Chatterjee et al., 2010).

The *in vivo* study was conducted to compare the protective role of WP-NPs, Zn and Zn loaded WP-NPs at different concentrations against CCl_4-induced hepatotoxicity, disturbances in gene expression and oxidative stress complication in rats. The selective doses of CCl_4, WP-NPs and Zn were literature based (Abdel-Wahhab et al., 2012; Gad et al., 2011; Tizhe et al., 2018, respectively). Several reports have shown that CCl_4-induced hepatotoxicity can be prevented by the supplementation of antioxidants due to their efficiency in the treatment of liver disorders (El-Denshary et al., 2012; Vuda et al., 2012). In the current study, animals treated with CCl_4 showed a significant increase in ALT, AST, cholesterol, triglycerides, LDL-Ch, nitric oxide and MDA with a concomitant decrease in total protein, HDL-Ch, and the antioxidant enzyme activity GPx, SOD and CAT. CCl_4 also induced histopathological and histochemical changes in the liver included extensive areas of patchy and confluent hepatocytes necrosis, lobular inflammation, focal necrosis, mononuclear cell infiltration around central veins and in portal areas beside the prominent increase in connective tissues and collagen between the liver lobules and around the portal tract.

The mechanism of CCl_4-induced hepatotoxicity is well documented previously and includes two phases: in the first phase, CCL_4 is converted to its free radical form (CCl^{-3}) by Cyt P-450 (Noguchi et al., 1982). However, in the second phase, CCl^{-3} reacts very quickly with oxygen (O_2) to form CCl_3OO^- which is highly reactive radical compared to CCl^{-3} (Packer et al., 1978). The generation of these free radicals attacks the microsomal lipids resulting in the lipid peroxidation and bind covalently to the microsomal protein and lipids (Gad et al., 2011; El-Denshary et al., 2012). Consequently, these phases resulted in the generation of ROS (reactive oxygen species) including the hydroxyl radical, superoxide anion O_2 and H_2O_2 (Dutta et al., 2018). The elevation levels of ALT and AST activity reported herein in the group treated with CCl_4 indicated the acute hepatic necrosis (Kaplan, 1987) and the decreased level of total protein suggested liver necrosis and/or kidney dysfunction (Abdel-Wahhab et al., 2007).

It is well documented that lipids are playing a critical role in the incidence of hepatic disease. In the current study, treatment with CCl_4 resulted in the destruction of lipid metabolism The increase in the cholesterol levels suggested the increased esterification of fatty acids, which decreases the excretion of the cellular lipids and the inhibition of FA b-oxidation (Fernandez and West, 2005). It was reported that CCl_4 induced a stimulation of acetate transfer into hepatocytes, increase the synthesis of cholesterol, FA and triglycerides from acetate as well as

Table 4
Effect of WP-NPs, Zn-NPs and Zn loaded WP-NPs on serum lipid profile in rats treated with CCl_4.

Parameter Groups	Cholesterol (mg/dl)	TriG (mg/dl)	HDL-Ch (mg/dl)	LDL-Ch (mg/dl)
Control	72.64 ± 0.82^{a}	52.56 ± 2.18^{a}	39.48 ± 0.20^{a}	22.65 ± 0.72^{a}
CCL_4	117.79 ± 2.56^{b**}	179.04 ± 8.40^{b**}	18.33 ± 1.06^{b**}	63.65 ± 6.59^{b**}
WP-NPs + CCL_4	93.76 ± 0.53^{c*}	98.12 ± 2.97^{c*}	33.03 ± 1.31^{c*}	49.22 ± 1.97^{c*}
Zn + CCL_4	92.42 ± 3.19^{c*}	96.52 ± 3.59^{d*}	34.92 ± 1.28^{c*}	38.20 ± 0.53^{d*}
Zn-WP-NPs (LD) + CCl_4	86.66 ± 1.01^{d*}	94.91 ± 1.15^{e*}	28.87 ± 1.28^{d*}	38.81 ± 0.58^{d*}
Zn-WP- NPs (MD) + CCl_4	91.17 ± 0.80^{c*}	129.11 ± 0.74^{f*}	22.52 ± 2.10^{e*}	42.83 ± 0.97^{e*}
Zn-WP- NPs (HD) + CCl_4	92.33 ± 0.35^{c*}	134.53 ± 3.88^{e*}	24.49 ± 0.86^{e*}	40.48 ± 1.02^{e*}

Data are presented as mean ± SE.
Within each column, means superscript with different letters (b, c, d, …) are significantly different ($P < 0.05$) compared to the control group (a).
*Significant at $P < 0.05$ compared to CCl_4 alone group.
**Significant at $P < 0.01$ compared to the control group.

Table 5
Effect of WP-NPs, Zn and Zn loaded WP-NPs on hepatic antioxidant enzyme activity in rats treated with CCl_4.

Parameter Groups	GPX (U/g)	SOD (U/g)	CAT (mu/g)
Control	518.76 ± 32.77^{a}	3675.50 ± 60.12^{a}	668.33 ± 48.24^{a}
CCL_4	246.41 ± 4.87^{b**}	2525.58 ± 106.26^{b**}	286.67 ± 41.33^{b*}
WP-NPs + CCL_4	337.19 ± 8.13^{c*}	3325.20 ± 174.91^{c*}	484.0 ± 33.18^{c**}
Zn + CCL_4	324.23 ± 4.87^{c*}	3243.93 ± 175.05^{d*}	525.33 ± 20.51^{d*}
Zn-WP-NPs (LD) + CCl_4	463.64 ± 9.45^{d*}	3434.41 ± 117.53^{e*}	575.0 ± 19.55^{e*}
Zn-WP- NPs (MD) + CCl_4	415.01 ± 10.37^{e*}	3291.31 ± 125.83^{f*}	563.0 ± 7.94^{f*}
Zn-WP- NPs (HD) + CCl_4	308.11 ± 29.35^{c*}	3100.25 ± 50.36^{g*}	550.0 ± 19.70^{g*}

Data are presented as mean ± SE.
Within each column, means superscript with different letters (b, c, d, …) are significantly different ($P < 0.05$) compared to the control group (a).
*Significant at $P < 0.05$ compared to CCl_4 alone group.
**Significant at $P < 0.01$ compared to the control group.

Table 6
Effect of WP-NPs, Zn and Zn-WP-NPs on serum NO and hepatic lipid peroxidation (MDA) in rats treated with CCl_4.

Parameter Groups	NO (μmol/L)	MDA (nmol/g)
Control	44.83 ± 2.48^{a}	411.75 ± 22.21^{a}
CCL_4	68.02 ± 1.66^{b**}	546.58 ± 5.35^{b**}
WP-NPs + CCL_4	50.21 ± 2.09^{c*}	418.87 ± 13.86^{a*}
Zn + CCL_4	55.25 ± 3.18^{c*}	419.86 ± 10.41^{a*}
Zn-WP-NPs (LD) + CCl_4	54.07 ± 1.43^{d*}	404.40 ± 12.38^{d*}
Zn-WP- NPs (MD) + CCl_4	59.13 ± 1.74^{e*}	422.67 ± 11.41^{e*}
Zn-WP- NPs (HD) + CCl_4	66.91 ± 1.69^{f**}	424.57 ± 12.31^{e*}

Data are presented as mean ± SE.
Within each column, means superscript with different letters (b, c, d, …) are significantly different ($P < 0.05$) compared to the control group (a).
*Significant at $P < 0.05$ compared to CCl_4 alone group.
**Significant at $P < 0.01$ compared to the control group.

stimulates the esterification of lipids (Weber et al., 2003). Furthermore, CCL_4 was also reported to inhibit apo-lipoprotein synthesis consequently, reduces the lipoprotein synthesis (Kamalakkannan et al., 2005).

Animals treated with CCl_4 also showed an increase in MDA and NO suggesting that these animals undergo oxidative stress. MDA is the end product of lipid peroxidation and it is generally used as a marker of oxidative stress and free radical mediated toxicity (Dutta et al., 2018). These free radicals attack the highly unsaturated fatty acids in cell membrane resulting in the peroxidation of lipid and are considered the key process in several pathological events of oxidative stress (Pang et al., 2017). The elevated level of NO reported herein revealed that CCl_4 affects the function of macrophage (El-Denshary et al., 2012) and showed the harmful effects of CCl_4 on liver similar to those reported previously in the literature (El-Denshary et al., 2012; Pérez-Cabeza de Vaca et al., 2018).

The living body has its effective mechanism to defense against, prevent and neutralize free radicals through the regulation of endogenous antioxidants such as catalase, superoxide dismutase and glutathione peroxidase which form a common protective system against ROS. In the current study, the alteration in the antioxidant status in hepatic tissue is a manifestation of the oxidative stress of CCl_4 and its metabolites. SOD and GPX, the enzymes responsible for free radical scavenging in the cells, were declined significantly in the rats administrated CCl_4. SOD has an essential role in the elimination of ROS resulted from peroxidative process in hepatic tissues (Abdel-Wahhab et al., 2006). This enzyme converts superoxide to H_2O_2 which in turn convert by CAT to H_2O (Kiokias et al., 2018). Thus, the elevation of MDA and NO levels and the reduced activity of SOD, CAT and GPX suggested the formation of free radical which induce the initiation of chain reactions and the formation of direct and indirect bond with the cellular molecules (proteins, nucleic acids, carbohydrates and lipids) resulting in the impairment of crucial cellular processes (Sarhan et al., 2012).

The reduction in antioxidant enzyme activity was confirmed by the significant decrease in their mRNA expression which may be due to the oxidation of the transcription factors and/or the decrease in mRNA half lives. The current results are in agreement with the previous results published in the literature (Diab et al., 2018; Rahmouni et al., 2018). Moreover, CCl_4 increased the hepatic mRNA expression of Bax and caspase-3 and decreased the mRNA of Bcl2. It was reported that Bcl2 protein is layered the surface of mitochondria and prevents the leakage of cytochrome c in the plasma; however, Bax induces the leaking out of cytochrome c through the punching of the holes of mitochondrial membrane (Kroemer et al., 2007). Additionally, the caspase-3 dependent apoptotic pathway is activated when the balance between Bax and Bcl2 is broken (Peng et al., 2016). It is well known that ROS cause a disturbance in the MMP (mitochondrial membrane potential) due to the physiological response to apoptosis (Ramachandran et al., 2000) and Bax promotes the formation of mitochondrial apoptosis-induced channel because of the release of mitochondrial cytochrome c (Dejean et al., 2006). Hence, the release of cytochrome c compounds into the cytoplasm activates caspase-9 then Caspase-3 and promotes the apoptosis process (Antonsson, 2004).

The histological and histochemical results reported herein in the liver of animals treated with CCl_4 alone were similar to those reported in the previous reports which showed that CCl_4-induced fibrosis, prominent increase in connective tissues and collagen between the liver lobules and around the portal tract. Similar observations were reported previously (Pegoraro et al., 2018; Rathee et al., 2018).

The results of the current study showed that animals treated with CCl_4 plus WP-NPs, Zn or Zn-WP-NPs at the three tested levels showed a significant improvement in all the tested parameter, histological and histochemical picture of the liver tissue. Meanwhile, these treatments could normalize most of the tested parameters. Zn is the second very important trace element in the body following iron (Vallee and Falchuk, 1993) and has crucial roles in the human body since it is a structural protein component and a cofactor in several enzymes which are very important for the biochemical reactions (Ackland and Michalczyk, 2006). It was reported also that Zn has a protective role against oxidative damage to the cells, organelles and molecules *in vitro* (Bray and Bettger, 1990) and showed antioxidant activity (Klotz et al., 2003). However, the excess Zn may lead to symptoms of toxicity such as the decrease of hepatic GSH and increase in AST and ALT (Ahangar et al., 2017).

Moreover, WPs were reported to have health benefits such as anticancer, antioxidant and antihypertensive properties beside their role in the protection against the infection by bacteria and virus (Yalcın, 2006). WPs also have immuno-enhancing activity due to the enhancement of glutathione (GSH) synthesis, the most important tool in the protection against oxidative damage (Hassan et al., 2012). It is also rich in sulfhydryl compounds and protect against ethanol-induce damage to the gastric mucosa via stimulation of the synthesis of GSH (Rosaneli et al., 2002).

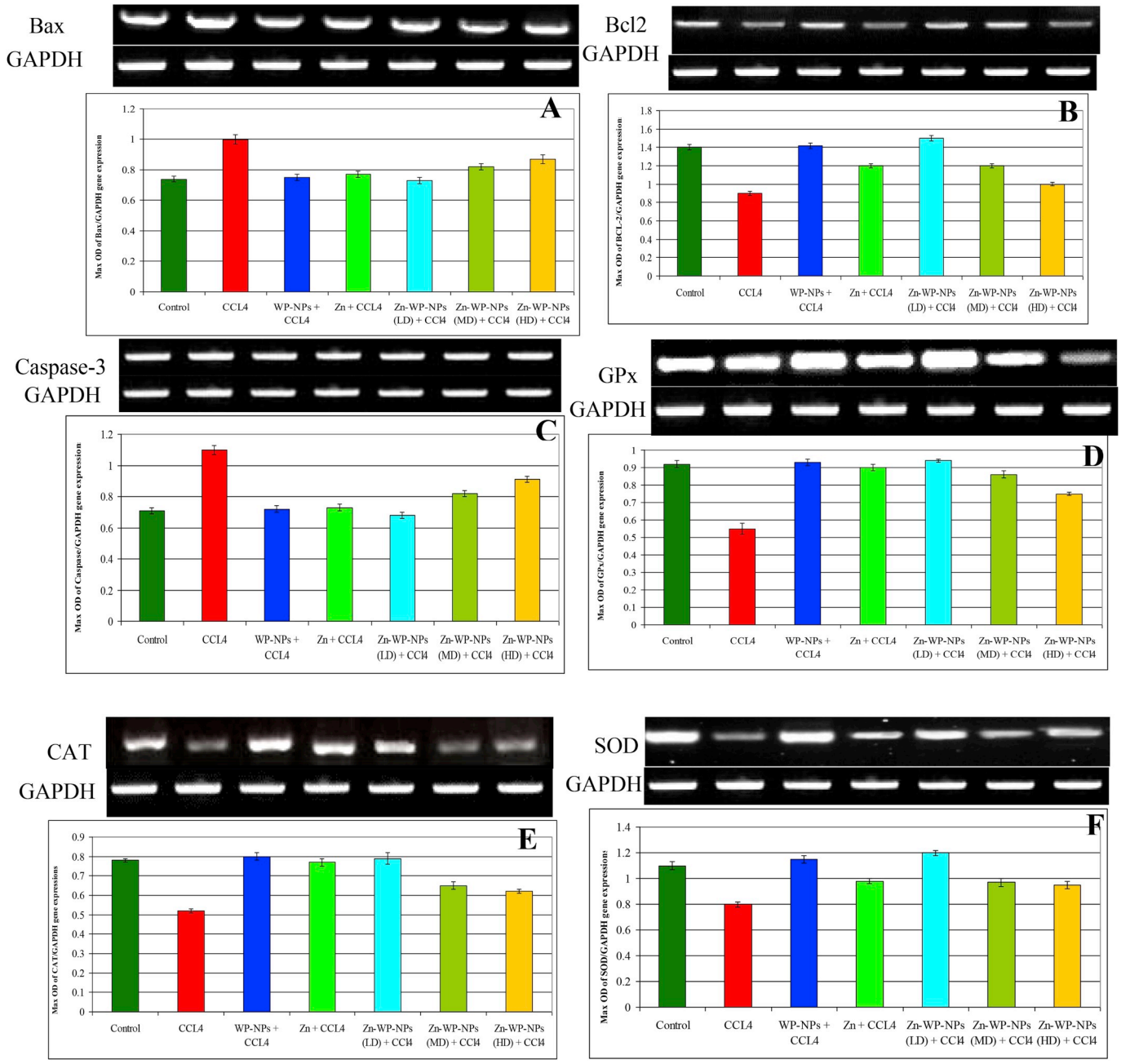

Fig. 4. Effect of Zn coated WP-NPs on caspase-3 (A), Bax (B), Bcl2 (C), GPX (D), CAT (E) and SOD (F) gene expression level in the liver of rats treated with CCl_4. The results illustrated are normalized to the level of GAPDH level and the data are the mean of OD for each gene divided by that for GAPDH.

The protective role of Zn was reported as it acts as complex antioxidant and participates in chelator and enzymatic activities, antioxidative inhibits lipid peroxidation, stabilizes cell membranes, free radical scavenging and repair the sites of molecular damage (Stehbens, 2003). Moreover, Zn enhances defense and immune function through increase the leucocytes function, the production of thymulin and cell-mediated immunity (Sidhu et al., 2004a). The current results were in agreement with the published results which reported the hepatoprotective effects of Zn against different liver toxicants such as CCl_4, ethanol, nickel and cadmium (Sidhu et al., 2004b). The mechanism by which Zn induces its protection was suggested as Zn maintains the homeostasis via the regulation of protein synthesis, the generation of free radical, lipid peroxidation and intracellular Ca (Dhawan and Goel, 1996).

In the current study, animals treated with WP-NPs alone were comparable to the control group in most of the tested parameters. Moreover, WP-NPs alone or in combination with Zn at the three levels could improve all these parameters in the animals treated with CCl_4. Previously; we reported that WPC protects the liver against CCl_4 toxicity through its antioxidant property (Gad et al., 2011) which mainly is owing to its ability to increase the levels of tissues GSH (Bayrama et al., 2008). This increase in GSH level is mainly due to the high content of amino acids cysteine, α-lactalbumin, β-lactoglobulin and the bovine serum albumin in WPs (Morr and Ha, 1993). Cysteine is well known to be responsible for GSH regulation and the anticarcinogenic tripeptide showed a protective effect against cell damage (Bounous et al., 1989).

Additionally, Ashoush et al. (2013) suggested that the protective role of Zn is a result of the increase in total GSH levels. It is of interest to mention that the group treated with WP-NPs–Zn (LD) showed the best results which suggest the synergistic effect of both agents. However, the

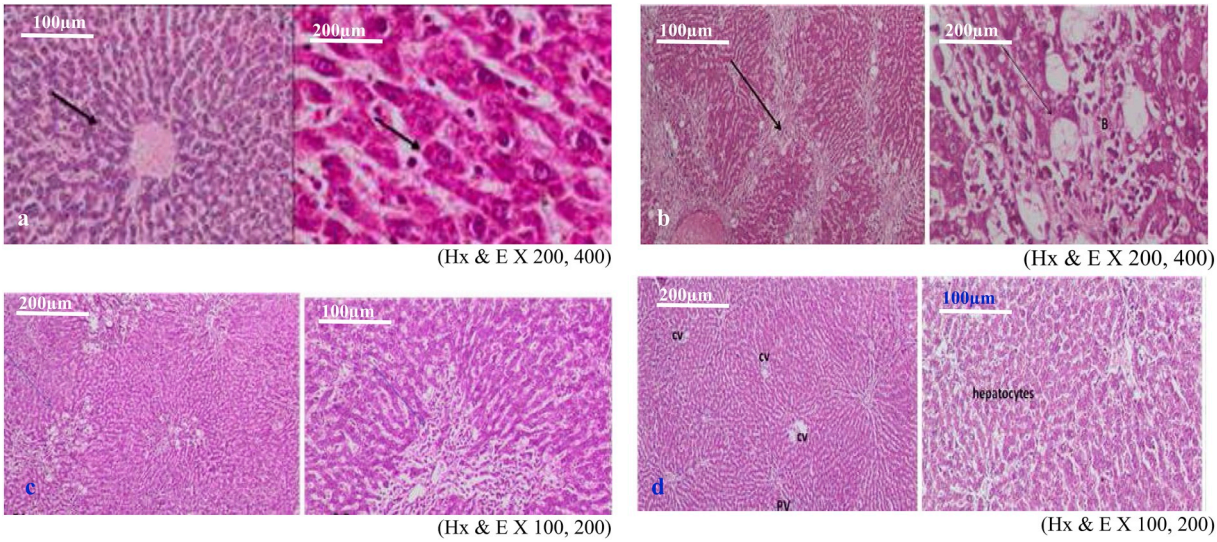

Fig. 5. Photomicrograph of liver sections of **(a)** Low and high power of sections from control liver of a rat showing the central vein and most of hepatocytes are normal and have vesicular nuclei, **(b)** rats treated with CCl_4 showing extensive areas of patchy and confluent hepatocytes necrosis and lobular inflammation, at the periphery of the hepatic lobule close to the portal triad, the hepatic cords are distorted due to focal necrosis, mononuclear cells infiltration mostly macrophages and lymphocytes around central veins and in portal areas (low power), and cellular ballooning damage with large and small fatty droplets and nuclear pleomorphism, the apoptic cells, the massive necrosis and fibrosis are seen, **(c)** sections from liver rats treated with CCl_4 plus WP-NPs showing focal fibrosis and necrosis in the hepatic lobule close to the portal tracts while there are nearly normal hepatic cells around the central vein (high power), **(d)** rat liver treated with Zn plus CCl_4 showing marked improvement in hepatocytes archetictuer and in the portal vein area.

groups treated with WP-NPs-Zn at the medium or the high level showed less improvement depending on the level of Zn in the mixture since this improvement was decreased by increasing the Zn level. This could be explained by the amount of amino acids in the mixture since we observed the absence of five amino acids in Zn-WP-NPs (MD) including HIS, THR, ARG, METH and VAL and the absence of seven amino acids in Zn-WP-NPs (HD) including SER, GLY, HIS, THR, ARG, METH and VAL. The absence of these amino acids reflects the antioxidant potential of these mixtures which affect the intestinal mechanism and neuropeptide gene expression (Corrochano et al., 2018; Park et al., 2018).

The changes in mRNA expression of Caspase-3, Bax, Bcl2 and the antioxidant enzymes GPX, Cu-Zn-SOD and CAT reported in the CCl_4-treated rats confirmed the occurrence of oxidative stress status in these animals. It was reported that oxidative stress is considered to induce apoptosis and several agents that have apoptosis effects are considered oxidants or stimulators of the oxidative metabolism in cells (Ramachandran et al., 2000). Hence, WP-NPs, Zn and their mixture could counteract the oxidative stress resulted from the exposure to CCl_4 and confirmed that the protective role of these agents work at the cellular level via the enhancement of DNA repair system and/or DNA synthesis (Zhou et al., 2008, 2017).

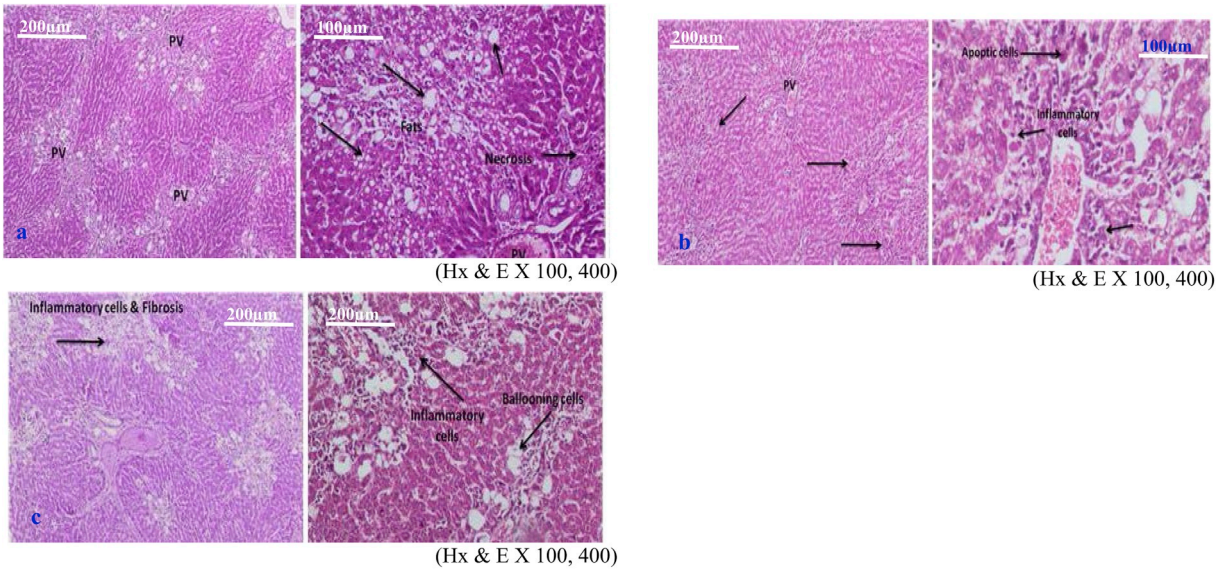

Fig. 6. Photomicrograph of liver sections of **(a)** rat treated with CCl_4 plus Zn-WP-NPs (LD) showing the same picture of patchy lobular fibrotic damage in the form of fatty and necrotic degeneration close to portal veins, **(b)** rats treated with CCl_4 plus Zn-WP-NPs (MD) showing periportal histological changes in the form of large and small fatty cells, necrosis, fibrosis and apoptotic cells and **(c)** rats treated with CCl_4 plus Zn-WP-NPs (HD) showing periportal necrosis with inflammatory and ballooning cells.

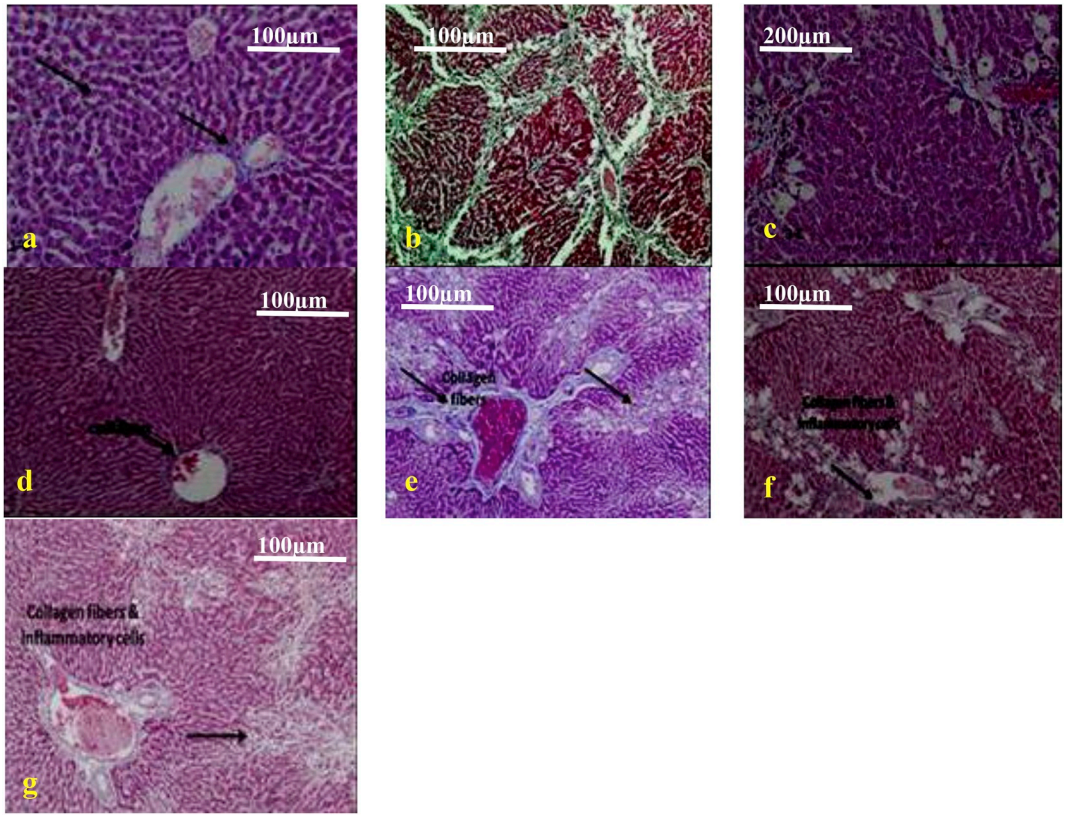

Fig. 7. Photomicrograph of liver sections stained with Masson Trichrome of **(a)** control rat showing fine linear of the collagen fibers are present around the blood vessels, **(b)** rats treated with CCl_4 showing massive necrosis and fibrosis and prominent increase in connective tissues and collagen between the liver lobules and around the portal tract, **(c)** rats treated with WP-NPs showing the fibrosis and collagen fibers concerned around the portal tract, **(d)** rats treated with CCl_4 plus Zn showing marked decreased in collagen fibers or fibrosis around blood vessels, **(e)** rats treated with CCl_4 plus Zn-WP-NPs (LD) showing marked increase in collagen fibers or fibrosis around blood vessels, **(f)** rats treated with CCl_4 plus Zn-WP-NPs (MD) showing prominent amount of collagen fibers and inflammatory cells and **(g)** rats treated with CCl_4 plus Zn-WP-NPs (HD) showing prominent amount of collagen fibers and inflammatory cells
(Masson Trichrome a,b,v,e,f, and g X 200 & c X 100).

5. Conclusion

The results of the current study demonstrate that WP could be synthesized in a nano form with an average particles size of 95 nm. The average size of Zn coated WP-NPs ranged from 142 to 228 nm depending on the amount of Zn used. Twelve amino acids were detected in WP-NPs and Zn-WP-NPs low dose, but some of these amino acids were absent when ZN was loaded WP-NPs and the number of absent amino acids was increased by the increase in Zn content in the loaded mixture. WP-NPs, Zn and their mixtures showed a protective effect against oxidative stress complication resulted from CCl_4. The improvement of both agents or their combinations in the biochemical parameters, gene expression, histological and histochemical picture of the liver tissue revealed that Zn loaded WP-NPs at a dose of 7.5 mM/g was more effective than Zn alone, WP-NPs alone or the medium or high level of Zn in the mixture. Thus WP-NPs can be applied for coating Zn used in supplement preparations to enhance the effect of Zn and counteract the side effect of excess Zn.

Conflicts of interest

There is no conflict of interest in this study.

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Transparency document

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