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Ameliorative Effects of Alpha Lipoic Acid, Quercetin and Ascorbic Acid against Zinc Oxide Nanoparticles Induced Hepatic Damage: *In vivo*

(Kesan Amelioratif Asid Alfa Lipoik, Kuersetin dan Asid Askorbik terhadap Nanozarah Zink Oksida Aruhan Kerosakan Hepar: *In vivo*)

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ABSTRACT

The current study envisioned to evaluate time related protective effect of quercetin, alpha lipoic acid and ascorbic acid on liver of mice against sub-acute exposure of zinc oxide (ZnO-NP) nanoparticle. Male Swiss albino mice (n=72) were randomly divided into eight groups (n=9, each group). G1 received saline solution 0.9%; G2 received quercetin (100 mg/kg b.w); G3 received alpha lipoic acid (100 mg/kg b.w); G4 received ascorbic acid (100 mg/kg b.w); G5 received ZnO-NPs (50 mg/kg b.w); G6 received ZnO-NPs with quercetin; G7 received ZnO-NPs with Alpha lipoic acid and G8 co-treated with ZnO-NPs and ascorbic acid for 21 consecutive days. Body weight, hepatosomatic index and plasma biochemical parameters (total protein, albumin, globulin, total cholesterol, triglycerides, high density lipoproteins, low density lipoprotein, aspartate aminotransferase, alanine transaminase, *alkaline phosphatase* & bilirubin) were estimated. ZnO showed significant increase in body weight and cause alterations in all biochemical parameters. Co-administration of quercetin (100 mg/kg b.w), alpha lipoic acid and ascorbic acid daily along with ZnO-NPs, significantly ameliorate the dramatic alteration in biochemical parameters and hepatocellular necrosis caused by ZnO nanoparticles. Brine shrimp larvae cytotoxicity assay of ZnO nanoparticles showed 0% mortality. Present study concluded that all three active ingredients showed hepatoprotective effects against nanoparticles induced time dependent toxicity.

Keywords: Alpha lipoic acid and ascorbic acid; quercetin; ZnO-NPs

ABSTRAK

Kajian ini berwawasan untuk menilai kesan perlindungan berkaitan masa kuersetin, asid alfa lipoik dan asid askorbik pada hati tikus terhadap pendedahan sub-akut nanozarah zink oksida (ZnO-NP). Tikus albino Swiss jantan (n=72) dibahagikan secara rawak kepada lapan kumpulan (n=9, setiap kumpulan). G1 menerima larutan garam 0.9%; G2 menerima kuersetin (100 mg/kg b.w); G3 menerima asid alfa lipoik (100 mg/kg b.w); G4 menerima asid askorbik (100 mg/kg b.w); G5 menerima ZnO-NPs (50 mg/kg b.w); G6 menerima ZnO-NPs dengan kuersetin; G7 menerima ZnO-NPs dengan asid alfa lipoik dan G8 dirawat bersama dengan ZnO-NPs dan asid askorbik selama 21 hari berturut-turut. Berat badan, indeks hepatosomatik dan parameter plasma biokimia (jumlah protein, albumin, globulin, jumlah kolesterol, trigliserida, lipoprotein ketumpatan tinggi, lipoprotein ketumpatan rendah, aminotransferase aspartat, transaminase alanin, fosfatase alkali & bilirubin) telah dianggarkan. ZnO menunjukkan peningkatan ketara dalam berat badan dan menyebabkan perubahan dalam semua parameter biokimia. Pemberian bersama kuercetin (100 mg/kg b.w), asid alfa lipoik dan asid askorbik setiap hari bersama-sama dengan ZnO-NPs dengan ketara memperbaiki perubahan dramatik dalam parameter biokimia dan nekrosis hepatosel yang disebabkan oleh nanozarah ZnO. Asai kesitotoksikan larva udang air garam nanozarah ZnO menunjukkan 0% kematian. Kajian ini menyimpulkan bahawa ketiga-tiga bahan aktif menunjukkan kesan hepatopelindung terhadap nanozarah aruhan ketoksikan bersandar-masa.

Kata kunci: Asid alfa lipoik; asid askorbik; kuersetin; ZnO-NPs

Introduction

Nanoparticles show ecotoxicological effects on aquatic and terrestrial biota by travelling over long distances (being suspended in air), resulting in uncontrollable human exposure (Batley, Kirby & McLaughlin 2013). Nanoparticles pose negative effects to different biogeochemical cycles, as they can penetrate soils directly through fertilizers or plant protection products, or indirectly through application to land or wastewater treatment products, such as sludges or biosolids (Gupta & Xie 2018; Tourinho et al. 2012). Due to widespread use of nanoparticles in various sectors (aerospace, automobile, chemical, construction, cosmetics, electronics, energy, engineering, environment, food, medicine, security, sports, telecommunication, textiles and transportation), their toxicity assessment is of major concern (Kim et al. 2017; Ma, Williams & Diamond 2013). Nanoparticles can enter into body through various possible routes such as dermal skin pores, oral, olfactory, injection and respiratory tract and after being entered into body they start accumulating in different organs inducing toxicity and DNA damage by producing reactive oxygen species (ROS) (Sharma et al. 2012a, 2012b). Zinc oxide nanoparticles (ZnO-NPs) are widely used in the production of different products including sunscreens, cosmetics, paints, papers, plastics and building materials (Ben-Slama et al. 2015).

Quercetin (Qur) is a bioflavonoid, polyphenolic compound having free radical scavenging or metal ion chelating property (Kessler, Ubeaud & Jung 2003; Symonowicz & Kolanek 2012). Quercetin, an antioxidant, showed protection against CCl_4 induced hepatic steatosis along with reduced necrotic and apoptotic cell death in rats (Esrefoglu et al. 2015; Jung & Sung 2004). Alpha-lipoic acid (ALA) (dihydrolipoic acid (reduced form), free radical scavenger) may be water soluble or fat-soluble (amphiphilic) can easily cross biological membranes (Bast & Haenen 2002; Skorupa, Michalkiewicz & Jakubczyk 2021). The human body synthesizes alpha lipoic acid naturally in the liver, heart, and testis (Jan et al. 2015). It also protects the cellular membranes by interacting with vitamin C and glutathione, which subsequently recycles vitamin E (Laher 2021) and showing metal chelator properties due to presence of sulphur in it (Zou et al. 2015). ALA is a disulphide compound having eight-carbons which then act as a natural cofactor in pyruvate enzyme, and α-keto dehydrogenase complexes. Antioxidant role of ALA is used in cure of acute liver poisoning and liver cirrhosis and heavy metal poisoning (Pari & Murugavel 2004). Ascorbic acid (Vitamin C) is hydrophilic acting as a reducing agent, antioxidant and free radical scavenger, has been reported to increase glutathione (GSH) levels in the liver and muscle (Shireen et al. 2008). Active extracts of various natural plants have promising effect as an antioxidant in reducing toxic effects of nanoparticles (Abdel-Azim et al. 2015). Unlike alpha lipoic acid which is naturally produced in our body, quercetin a flavonoid and ascorbic acid are used as antioxidant supplements which are not naturally produced in our body and people need to get them from diet. The current study aimed to evaluate the comparative effect of three different antioxidants i.e., quercetin, alpha lipoic acid and ascorbic acid against metal (ZnO nanoparticle, 18 nm, Anatase) induced time related liver toxicity. To the best of our knowledge, there is no report available regarding the beneficial effect of these three antioxidants (quercetin, alpha lipoic acid and ascorbic acid) in one research against conditions where humans or animals were exposed to ZnO NPs along with these antioxidants.

Materials and Methods

In the current study, healthy adult (12 weeks old) male swiss albino mice (n=72) weighing 25-35 g were purchased from University of Veterinary and Animal Science, Lahore, Pakistan. The animals were acclimatized and kept in cages at temperature of 22 ± 1 °C, relative humidity of 60 ± 10%, and a 12 h light/dark cycle. Commercial pellet diet (Purchased from Tolliton market, Lahore) and distilled water were provided for mice *ad libitum* for two weeks. The experimental protocol was performed with the compliance of Research Ethical Review Committee of Zoology Department (Memo number RERC/Zoo/2020/10).

Nanoparticles and antioxidants dosage

Zinc oxide nanoparticles was purchased from U.S Research Nanomaterials, Inc. having size of 18 nm as calculated by scanning electron microscope (Table 1 & Figure 1). Quercetin, alpha lipoic acid were purchased from General Nutrition Corporation Pittsburgh, PA-15222) and ascorbic acid purchased from Mumtaz Pharmacy (License No. 853-A/AIT/9/2015). Animals received sub-acute oral dose 50 mg/kg b.w of ZnO-NPs (Khorsandi et al. 2016), 100 mg/kg b.w of quercetin (1/2 of 200 mg/kg b.w) (23). ZnO (0.25 g) was dissolved in 70 mL ddH_2O to make 0.35% ZnO. Vortexing was performed for a period of 15 min before administering every dose. Quercetin (100 mg/kg b.w) (Abdelbaky et al. 2013), Alpha lipoic acid (50 mg/kg b.w) (Shrivastava, Bhargava & Flora 2014) and ascorbic acid at a dose of 100 mg/kg b.w (Abdel-Daim 2014) was administered to animals.

TABLE 1. ZnO nanoparticles data

Purity	[a]APS	Colour	Crystal phase	Morphology	[b]SSA	True density
99%	18nm	White	single crystal	nearly spherical	20-60 m^2/g	5.606 g/cm^3

[a]Average particle size measured by high resolution SEM with charge compensation system
[b]Specific surface area measured by Brunauer, Emmett and Teler (BET) technique

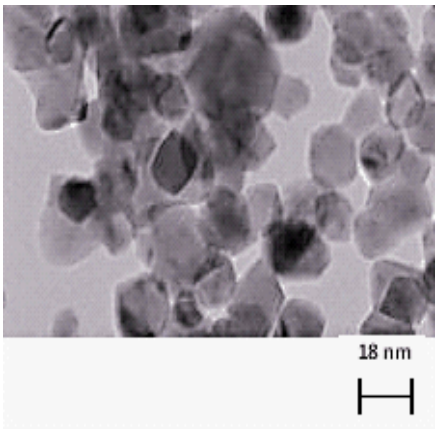


FIGURE 1. Scanning electron microscopy of the ZnO-NPs

EXPERIMENTAL DESIGN

One hundred and twenty (n=72) healthy adult albino mice were randomly divided into eight groups (n=9/ group). G1 (Control) received saline solution 0.9%, G2 (Quercetin) received quercetin, G3(ALA) received alpha lipoic acid, G4(AsA) received ascorbic acid, G5(Zn) received ZnO-NPs, G6 (Zn + Qur) received ZnO-NPs with quercetin, G7(Zn + ALA) received ZnO-NPs with Alpha lipoic acid and G8(Zn + AsA) co-treated with ZnO-NPs and ascorbic acid for 21 consecutive days.

BLOOD SAMPLING

At the end of each week, mice (n=3) were euthanized and their blood samples were collected by cardiac puncture technique using heparin coated syringes. Blood centrifuged at 3000 rpm for 15 min to separate plasma which was stored at -20 °C, until analyzed for biochemical parameters (Plasma proteins, lipid profile, LFTs and RFTs) with respective kits (Crescent Diagnostics, Cat. No. CS. 610, Jeddah Industrial City, Phase III, Kingdom of Saudi Arabia) by chemistry analyzer (URIT-800 chemistry analyser, URIT medical Electronic Co., Ltd. Guangaxi, China). Liver tissues were removed and weighed for calculating organosomatic index (OSI) and histopathological findings (Abdel-Azim et al. 2015).

$$\text{OSI: Organ weight/ Mice weight} \times 100$$

Cytotoxicity assay was performed according to standard protocol (Asaduzzaman et al. 2015).

LIVER HISTOLOGY

Liver tissues were processed for histological examination using the standard protocol of fixation, embedding and staining using hematoxylin and eosin stains (H&E; Drury & Wallington 1980; Srivastava & Yadav 2007). The slides, prepared to a thickness of 5 µm, were studied and photographed under a Trinocular camera-fitted microscope (E- 200, digital microscopic camera- Nikon Japan Ei1-L2).

HISTOLOGY DETAIL

Mice liver tissue were obtained after dissection, cleaned, and put in a saline solution then it was treated with Bouin fixative solution. The tissue was dried out with an alcohol solution of 50, 70, 80, 90, and 96% and pure alcohol for

1 hour. Cleaning was done by putting tissue in a mixture of absolute ethanol (1:1) and xylol for 1 hour. The tissue was put into the infiltration solution, which was kept at 56 °C to 60 °C. Tissue embedding was performed by placing tissue in metal mould with liquid paraffin, heated in an incubator. The mould was then left to freeze. Cutting was done by placing paraffin block in the holder and slicing it into a thin section (5 mm) with a microtome. After cutting, piece of glass was rubbed with Mayer's albumin for attachment then slide was placed in water and stretched on a hot plate. After drying, treated with xylol (30 min). Tissue was treated with Alcohol: xylol (1:1), alcohol 100, 96, 90, 80, 70, and 50% for 3 min each, hematoxylin-eosin for 1-5 min, then washed with water. Tissue drying out was avoided by dripping it with glue, covering it with a glass cover, and letting it dry. Preparations were labeled, and then they were observed under microscope.

STATISTICAL ANALYSIS

One-way ANOVA followed by Dunnett T3 and Tukey's test was performed as the *post hoc* tests. Mixed design ANOVA was also performed to analyze study with time x group and to evaluate difference between different treated groups with respect to three sampling. $p < 0.05$ was considered significant and $p < 0.01$, $p < 0.000$ was considered as highly significant. All the statistical analysis was done using SPSS software (v 19, IBM Corporation, Armonk, NY, USA).

RESULTS

MORTALITY AND BEHAVIOR

After administration of ZnO nanoparticles symptoms of vomiting and severe lethargy were observed during the experimental period by the mice. No mortality was observed throughout the experiment.

SERUM BIOCHEMICAL PARAMETERS

Tables 2, 3, 4, and 5 showed the comparative effect of quercetin, alpha lipoic acid and ascorbic acid against ZnO nanoparticle induced toxicity in a time dependent manner. All groups showed highly significant increase in body weight during 3rd week of treatment except quercetin treated (G2) (Table 2). Hepatosomatic index, total protein, albumin, globulin and bilirubin showed non-significant difference between control and treated groups (Table 2). Quercetin, alpha lipoic acid and ascorbic acid treated group (G2, G3, and G4) showed normal plasma value of all biochemical parameters. ZnO nanoparticle treated group (G5) showed significant decline (-36.09%, 45.35%, and -26.32%) in plasma total cholesterol level as compared to control (G1) from 1st to 3rd week. Concomitant administration of ZnO nanoparticles along with quercetin, alpha-lipoic acid and ascorbic acid (G6-G8) significantly decreased plasma total cholesterol value as compared to ZnO nanoparticles (G5) exposure during 3rd week (Table 3). In contrast to ZnO nanoparticles treated group (G5) which showed significant elevation in plasma triglycerides (TG) level (+13.53%) (week 3), Co-administration of ZnO nanoparticle and quercetin (G6) showed non-significant decrease ($p > 0.05$) in plasma triglycerides as compared to ZnO exposed group (G5) whereas, ZnO + Alpha lipoic acid and ZnO + ascorbic acid groups (G7 and G8) showed significant decrease in triglycerides as compared to ZnO exposed group (G5) during 3rd week (Table 3). ZnO nanoparticles treated group showed highly significant decrease in high density lipoprotein (HDL) as compared to control (Table 3). Furthermore, administration of antioxidants (quercetin, alpha-lipoic acid and ascorbic acid) along with Zn-NPs (G6-G8) raised HDL level as compared to exposure of ZnO-NPs alone (G5) in 3rd week of experimental protocol (Table 3). ZnO-NP treated group (G5) declined plasma low density lipoprotein (LDL) (-36.64%) as compared to control group during 3rd week (Table 3). Contrary to Zn + Qur and Zn + AsA; administration of ZnO nanoparticles along with alpha lipoic acid showed highly significant ($p > 0.001$) increase in LDL as compared to ZnO nanoparticles exposed group. ZnO nanoparticles exposure (G5) augmented plasma alanine transaminase (ALT) (+256.85%) and aspartate aminotransferase (AST) (-12.2%) as compared to control (G1) during last week (Table 4). Other groups in which ZnO was given with alpha lipoic acid and ascorbic acid (G7 and G8) decreased plasma ALT level as compared to ZnO treated group (3rd week). ZnO nanoparticle treatment significantly increased (+13.66%, +29.68 % and +73.46%) plasma *alkaline phosphatase* (ALP) level as compared to control in weeks 1, 2 and 3, respectively (Table 4). Concomitant administration of nanoparticles along with three antioxidants i.e., quercetin, alpha-lipoic acid and ascorbic acid (G6, G7, G8), restored the dramatic alterations in plasma ALP concentration during last week of experiment (Table 4). All biochemical parameters showed significant difference ($p < 0.05$) when all group data was analyzed with a time × group design

(Table 6).

TABLE 2. Mean ± SEM of body weight, hepatosomatic index, total protein (g/dl), albumin (g/dl) and globulin (g/dl) after 1st, 2nd and 3rd week of treatment

Groups	Time duration	Body weight	Hepatosomatic index (Liver)	Total protein	Albumin	Globulin
G1 (Control)	1st	25.36±0.22	7.10 ± 0.53	5.51 ± 0.39	2.91 ± 0.14	2.60 ± 0.49
	2nd	27.1± 0.55	6.66 ±0.65	6.06 ± 0.79	3.30 ± 0.46	2.07 ± 0.29
	3rd	29.01±0.56	6.10 ± 0.38	6.44 ± 0.31	3.44 ± 0.11	3.00 ± 0.25
G2 (Qur)	1st	26.83 ± 0.35**	6.60 ± 0.34	5.57 ± 0.44	2.83 ± 0.31	2.74 ± 0.64
	2nd	25.27 ± 0.36	6.60 ± 0.34	5.40 ± 0.25	2.80 ± 0.15	2.60 ± 0.40
	3rd	24.58 ± 0.29**	5.25 ± 0.20	5.53 ± 0.19	2.57 ± 0.42	2.95 ± 0.44
G3 (ALA)	1st	24.92 ± 0.14	6.35 ± 0.30	6.20 ± 0.35	4.41 ± 0.28	2.79 ± 0.18
	2nd	27.14 ± 0.38**	7.48 ± 0.46**	7.83 ± 0.28**	5.89 ± 0.31	3.49 ± 0.12
	3rd	31.76 ± 0.35	8.01 ± 0.43	8.66 ± 0.49	7.21 ± 0.23*	3.61 ± 0.14
G4 (AsA)	1st	28 ± 0.35	6.4 ±0.24	6.31 ± 0.5	3.43 ± 0.24	1.97 ± 0.15
	2nd	28.17 ± 0.33**	6.7 ±1.14	6.61 ± 0.84	3.15 ± 0.17	1.97 ± 0.09
	3rd	30.62 ± 0.41	7.93 ±1.19	7.22 ± 0.61	2.84 ± 0.43	2.12 ± 0.21
G5 (Zn)	1st	22.71±0.38**	5.21 ± 0.23	5.49 ± 0.32	3.49 ± 0.41	2.00 ± 0.10
	2nd	25.16 ± 0.27**	5.03 ±0.53	6.34 ± 0.89	3.48 ± 0.98	1.72 ± 0.39
	3rd	27.41±0.30**	5.79 ± 0.44	5.09 ± 0.32	3.27 ± 0.57	1.82 ± 0.55
G6 (Zn + Qur)	1st	25.07±0.27**	5.84 ± 0.74	7.42 ± 0.27	3.96 ± 0.38	3.46 ± 0.55
	2nd	25.01 ± 0.21**	5.39 ± 0.24	4.84 ± 0.32	2.14 ± 0.43	2.70 ± 0.68
	3rd	26.6±0.12**	5.43 ± 0.35	4.31± 0.16	3.42 ± 0.38	0.89 ± 0.48
G7 (Zn + ALA)	1st	23.60 ± 0.14	5.21 ± 0.08**	4.21 ± 0.22	2.57 ± 0.15	1.64 ± 0.15
	2nd	28.12 ± 0.46**	5.25 **± 0.21****	5.07 ± 0.15	3.32 ± 0.21	1.75 ± 0.21
	3rd	30.21 ± 0.24	5.27 ± 0.24**	6.44 ± 0.27	3.93 ± 0.10	2.51 ± 0.1
G8 (Zn + AsA)	1st	23.78 ± 0.46	5.05 ±0.14*	4.93 ± 0.58	3.22 ± 0.78	1.83 ± 0.12
	2nd	27.91 ± 0.5**	5.48 ±0.72	5.52 ± 0.65	2.09 ± 0.43	1.9 ± 0.12
	3rd	30.35 ± 0.34	6.35 ±0.18	6.79 ± 0.56	1.86 ± 0.02	2.03 ± 0.03

* =versus C (Week 2), b = versus (Week 4); *, b, $p < 0.05$; **, bb, $p < 0.01$

TABLE 3. Mean ± SEM of Total cholesterol (mg/dl), triglycerides (mg/dl), HDL (mg/dl) and LDL (mg/dl) after 1st, 2nd and 3rd week of treatment

Groups	Sampling	Total Cholesterol	Triglycerides	HDL	LDL
G1 (Control)	1st	45.94 ± 1.43ee#	141.08 ± 0.86##	6.14 ± 0.27^^##	138.50 ± 0.87e#
	2nd	50.25 ± 5.04	158.83 ± 0.97	7.13 ± 0.15	142.90 ± 0.46
	3rd	50.60 ± 0.63@e##	158.60 ± 8.99	9.00 ± 0.28##	145.00 ± 0.29ee#
G2 (Qur)	1st	39.56 ± 2.43^e (-13.8%)	124.00 ± 2.08@@## (-12.10%)	2.54 ± 0.39bb^^ (-58.63%)	140.00 ±s 1.10e# (+1.0%)
	2nd	40.74 ± 1.84 (18.92)	121.33 ± 1.45* (23.61)	3.28 ± 0.08 (53.99)	141.33 ± 1.64 (1.09)
	3rd	34.85 ± 2.56ee (-31.12%)	116.33 ± 4.33## (-26.65%)	2.37 ± 0.04*bb^^ (-73.66%)	138.47 ± 3.05# (-4.50%)
G3 (ALA)	1st	61.51 ± 2.27^@# (+33.89%)	176.28 ± 3.31* (+24.95%)	8.69 ± 0.26bb^## (+41.53%)	200.2 ± 3.46 (+44.54%)
	2nd	46.96 ± 2.93* (6.54)	159.1 ± 2.56 (0.16)	7.38 ± 0.29 (3.50)	205.4 ± 3.51 (43.73)
	3rd	40.22 ± 2.35^ee (-20.51%)	144.16 ± 2.69## (-9.10%)	6.06 ± 0.14^## (-32.66%)	229.0 ± 3.58# (+57.93%)
G4 (AsA)	1st	46.23 ± 1.58ee (+0.631%)	163.22 ± 1.15** (+15.69%)	3.31 ± 0.36bb^^ (-46.09%)	141.01 ±2.41## (+1.81%)
	2nd	39.9 ± 1.56 (20.59)	153.47 ± 1.63 (3.37)	2.62 ± 0.08 (63.25)	142.67±1.83 (0.16)
	3rd	37.6 ± 1.72ee (-25.69%)	147.01 ± 1.28## (-7.30%)	2.7 ± 0.16*bb^^## (-70%)	145.8±1.41^e## (+0.55%)
G5 (Zn)	1st	29.36 ± 1.68*be (-36.09%)	158.45 ± 1.77* (+12.31%)	4.24 ± 0.53^^ (-30.94%)	119.72 ± 2.91 (-13.55%)
	2nd	27.46 ± 0.38*bb (45.35)	161.18 ± 5.80* (1.47)	2.67 ± 0.88 (62.55)	106.87 ± 2.60*ab (25.21)
	3rd	37.28 ± 0.51**bbe (-26.32%)	180.07 ± 2.34** (+13.53%)	0.90 ± 0.13**bb^^ (-90%)	91.87 ± 3.64*b (-36.64%)
G6 (Zn + Qur)	1st	35.08 ± 1.49 (-23.63%)	171.00 ± 6.08 (+21.20%)	4.21 ± 0.23b (-31.43%)	130.94 ± 0.97 (-5.45%)
	2nd	40.85 ± 1.26b# (18.70)	156.33 ± 6.69 (1.57)	4.73 ± 0.68# (33.66)	128.67 ± 1.85 (9.95)
	3rd	21.94 ± 1.13# (-56.64%)	128.00 ± 2.89 (-19.29%)	4.67 ± 0.41#b (-48.11%)	133.63 ± 1.87 (-7.84%)
G7 (Zn + ALA)	1st	36.41 ± 1.90 (-20.74%)	138.89 ± 2.32## (-1.55%)	5.99 ± 0.15**bb# (-2.44%)	139.2 ± 1.75*# (+0.50%)
	2nd	40.30 ± 2.44 (19.80)	148.55 ± 1.66** (6.47)	7.06 ± 0.17 (0.98)	147.9 ± 2.50 (3.49)
	3rd	28.14 ± 4.02*# (-44.38%)	126.33 ± 1.65## (-20.34%)	9.03 ± 0.26bb## (+0.33%)	160.7 ± 2.24@@## (+10.82%)
G8 (Zn + AsA)	1st	37.08 ± 1.49 (-19.28%)	169.74 ± 1.34# (+20.31%)	4.04 ± 0.53 (-34.20%)	122.53±1.41 (-11.53%)
	2nd	43.52 ± 1.54** (13.39)	160.51 ± 1.94** (1.05)	4.59 ± 0.25* (35.62)	125.63±1.05** (12.08)
	3rd	25.94 ± 1.43# (-48.73%)	152.11 ± 1.22**# (-4.09%)	4.13 ± 0.29## (-54.11%)	126.77±0.72 (-12.57%)

* =versus C (Week 2), b = versus (Week 4); *, b, $p < 0.05$; **, bb, $p < 0.01$
@ =versus Z (Week 2), # = versus Z (Week 4); @, # $p < 0.05$; @@, ## $p < 0.01$

TABLE 4. Mean ± SEM of ALT (IU/L), AST (IU/L), ALP (IU/L) and bilirubin (g/dl) after 1st, 2nd and 3rd week of treatment

Groups	Sampling	ALT	AST	ALP	Bilirubin
G1 (Control)	1st	38.66 ± 10.97##	111.19 ± 5.65e@	133.92 ± 5.08	0.71 ± 0.03
	2nd	31.81± 6.95	125.33 ± 3.93	138.90 ± 5.00	0.66 ± 0.03
	3rd	31.27 ± 5.21^##	118.67 ± 2.96e@	151.12 ± 3.21e##	0.84 ± 0.03
G2 (Qur)	1st	41.31 ± 4.38^## (+6.85%)	112.96 ± 3.01 (+1.59%)	135.92 ± 1.71# (+1.49%)	0.55 ± 0.01 (-22.53%)
	2nd	38.72 ± 3.67 (+21.72)	121.66 ± 2.41 (+2.92)	150.35 ± 2.92 (+8.24)	0.68 ± 0.04 (-3.03)
	3rd	36.51 ± 2.21## (+16.75%)	116.87 ± 6.63@@ (-1.57%)	140.67 ± 3.71e# (-6.91%)	0.84 ± 0.08 (0%)
G3 (ALA)	1st	53.18 ± 2.89## (+37.55%)	96.96 ± 3.01e (-12.79%)	151.68 ± 2.0e## (+13.26%)	0.80 ± 0.06 (+12.67%)
	2nd	42.98 ± 5.22** (+35.11)	127.66 ± 2.41** (-1.85)	147.67 ± 3.7** (+6.31)	0.61 ± 0.02** (-7.57)
	3rd	28.72 ± 3.18@## (-8.15%)	100.87 ± 6.63e@@ (-14.99%)	134.85 ± 5.5# (-10.76%)	0.64 ± 0.01 (-23.80%)
G4 (AsA)	1st	64.04 ± 2.59## (+65.64%)	112.96 ± 3.03 (+1.59%)	130.52±2.81## (-2.53%)	0.65±0.03 (-8.45%)
	2nd	82.48±6.54** (159.28)	134.32±5.6 (7.17)	146.68±1.52 (5.60)	0.68±0.04 (3.03)
	3rd	55.6 ± 6.37## (+77.80%)	121.87 ± 6.63@@ (-2.69%)	147.75±3.73ee (-2.23%)	0.80±0.07 (4.7%)
G5 (Zn)	1st	94.50 ± 5.85b (+144.43%)	179.57 ± 4.26*b^ee (+61.49%)	152.22 ± 4.23 (+13.66%)	0.64 ± 0.01 (-9.85%)
	2nd	119.34 ± 8.51[ab] (275.16)	206.23 ± 2.91[*aabb] (64.54)	180.13 ± 2.17 (29.68)	0.58 ± 0.02 (12.12)
	3rd	142.86 ± 3.78*bb (+356.85%)	269.67 ± 2.60*bb (+12.2%)	262.14 ± 4.27**bb^^@ (+73.46%)	0.55 ± 0.03 (-34.52%)
G6 (Zn + Qur)	1st	46.91 ± 0.91# (+21.33%)	120.27 ± 3.46@## (+8.166%)	128.69 ± 4.35# (-3.90%)	0.50 ± 0.06 (-29.57%)
	2nd	44.89 ± 2.72[##] (41.11)	110.23 ± 5.50[@+++##] (12.04)	123.95 ± 4.19[+##] (10.76)	0.52 ± 0.07 (21.21)
	3rd	43.63 ± 1.13## (+39.52%)	101.33 ± 0.53@## (-14.61%)	117.27 ± 1.60## (-22.3%)	0.61 ± 0.03 (-27.38%)
G7 (Zn + ALA)	1st	45.01 ± 0.91# (+16.42%)	118.27 ± 3.46@ (+6.36%)	130.99 ± 2.4# (-2.18%)	0.49 ± 0.06 (-30.98%)
	2nd	50.22 ± 3.13** (57.87)	107.23 ± 3.18** (14.44)	128.43 ± 3.8** (7.53)	0.63 ± 0.07** (4.54)
	3rd	44.63 ± 2.29## (+42.72%)	108.33 ± 5. 38@@ (-8.71%)	121.27 ± 3.6## (-19.75%)	0.69 ± 0.02 (-17.85%)
G8 (Zn + AsA)	1st	49.96±3.51# (+29.22%)	125.6 ±2.8@ (+12,95%)	110.69±4.35## (-17.34%)	0.58±0.05 (-18.30%)
	2nd	60.24±4.52** (89.37)	107.57±3.18** (14.17)	118.33±2.51** (14.80)	0.58±0.06 (12.12)
	3rd	57.64±11.57## (+84.33%)	99.33±5.38@@ (-16.29%)	101.27±5.6** (-32.98%)	0.60±0.02 (-28.57%)

* =versus C (Week 2), b = versus (Week 4); *, b, $p < 0.05$; **, bb, $p < 0.01$
@ =versus Z (Week 2), # = versus Z (Week 4); @, # $p < 0.05$; @@, ## $p < 0.01$

TABLE 5. Comparison of % mortality rate of brine shrimp larvae at different % concentrations of ZnO and TiO_2 by performing cytotoxicity assay

Sr. No	NPs-Conc. (%)	Initial No. of dead larvae 'N'	Dead larvae after 24 hour 'A'	Av. No. of dead larvae in blind samples after 24 h 'B'	Total No. of larvae 'G'	%Mortality rate 'M' = (A-B-N)/(G-N)*100
1.	Zn-0.1	0	0	2	12	0%
2.	Zn-0.2	0	1	2	10	0%
3.	Zn-0.4	0	0	2	16	0%
4.	Zn-0.6	1	2	2	20	0%

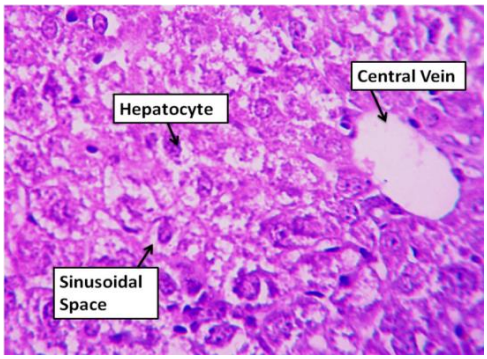
TABLE 6. Outcome of mixed design ANOVA (for analyzing data at three time points) for all biochemical parameters

Biochemical parameters	Between 3 time points (1st , 2nd and 3rd sampling)	Between subjects effects
Total Protein	F(2, 32) =4.841; p<0.005	F(7,16) = 8.868; p=0.000
Albumin	F(14, 32) =7.503; p=0.000	F(7,16) = 14.38; p=0.000
Globulin	F(14, 32) =0.275; p<0.005	F(7,16) = 6.398; p<0.001
Total cholesterol	F(2, 32) = 53.032; p=0.005	F(7,16) = 25.994; p=0.000
Triglycerides	F(2, 32) =28.28; p=0.000	F(7,16) = 39.107; p=0.000
HDL	F(14, 32) = 4.082; p=0.000	F(7,16) = 95.705; p=0.000
LDL	F(14, 32) = 4.101; p=0.05	F(7,16) = 384.148; p=0.000
AST	F(2, 32) =13.884; p<0.001	F(7,16) = 228.712; p=0.000
ALT	F(2, 32) = 3.803; p<0.05	F(7,16) = 101.116; p=0.000
ALP	F(2, 32) =27.939; p<0.000	F(7,16) = 112.217; p=0.000
Bilirubin	F(2, 32) = 12.640; p<0.005	F(7,16) = 10.247; p=0.000

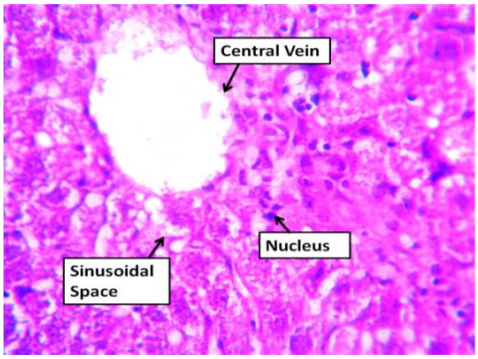
HISTOPATHOLOGICAL EXAMINATION

In the current study control group showed normal hepatocytes structure from 1st to 3rd sampling (Figure 2(a), 2(b), 2(c)). Quercetin treated group showed no changes after 1st sampling. 2nd and 3rd sampling showed infiltration of portal triad, increased sinusoidal spaces and increased size of hepatocytes (Figure 2(d), 2(e), 2(f)). 1st to 3rd sampling of ascorbic acid and alpha lipoic acid treated group showed normal structure of hepatocytes (Figure 2(g), 2(h), 2(i), 2(j), 2(k), 2(l)). In ZnO treated group, the liver structure shows prominent changes along with distortion of central vein, some infiltration in portal vein, irregular hepatocytes and distorted hepatic lobule after 1st and 2nd sampling. 3rd sampling showed a lot of infiltration of red blood cells in central vein, hepatic lobules disarrangement, and more congestion of sinusoidal spaces and swelling of endothelial cells (Figure 2(m), 2(o), 2(p)). Quercetin and ZnO-NP co-treated group showed infiltrated congested central vein, distorted hepatic lobule, congested sinusoidal spaces with necrotic liver structure after 1st and 2nd sampling. After 3rd sampling, the central vein showed normal structure,

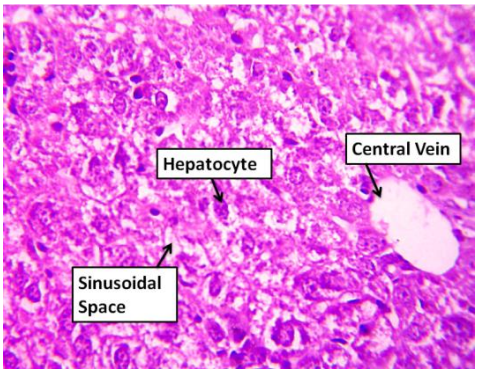
prominent hepatocytes, well-arranged sinusoidal spaces and portal triad (Figure 2(p), 2(q), 2(r)). Ascorbic acid + ZnO-NPs treated group showed mild central vein infiltration, vacuolated hepatocytes, ovoid nucleus, infiltration and normal sinusoidal spaces after 1st sampling and 2nd sampling. 3rd sampling showed shrined nucleus, mild degenerated hepatocytes, vacuolated hepatocytes and sinusoidal spaces (Figure 2(s), 2(t), 2(u)). Concomitant administration of Alpha lipoic acid and ZnO-NPs showed less debris in central vein and rearrangement of hepatocytes around the central vein, nucleus showed recovery and infiltration in portal vein after 1st sampling. 2nd sampling showed occluded central vein with some debris was observed. 3rd sampling showed rearrangement of hepatocytes around the central vein as compared to ZnO treated group (Figure 2(v), 2(w), 2(x)).



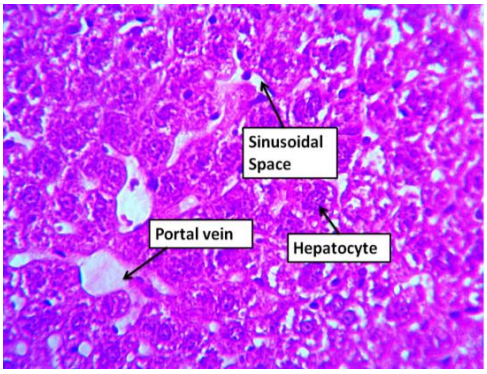
a) Control 1st sampling 40X



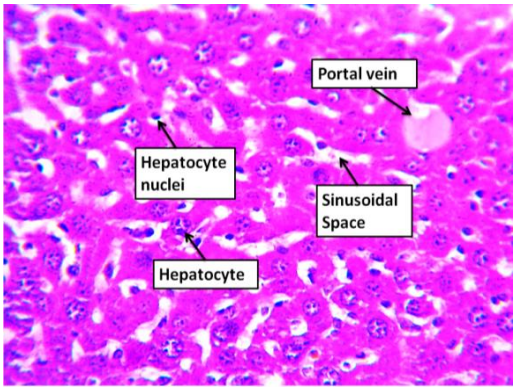
b) Control 2nd sampling



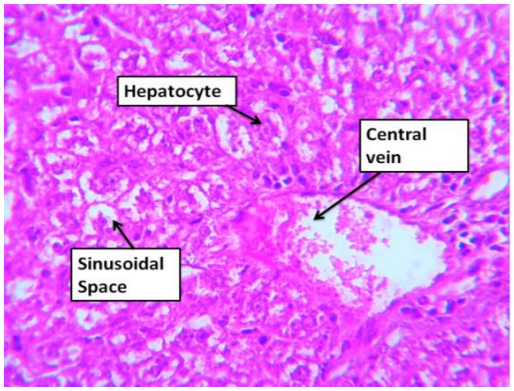
c) Control 3rd sampling



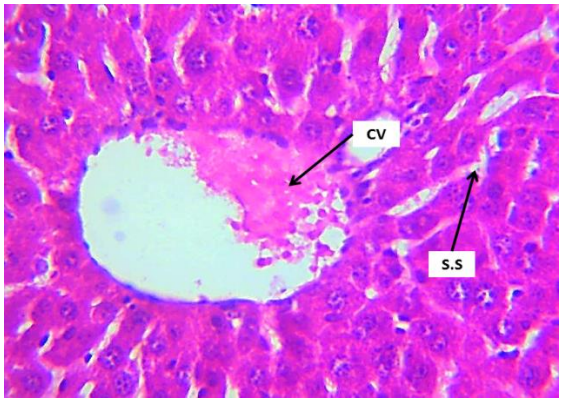
d) Quercetin 1st sampling



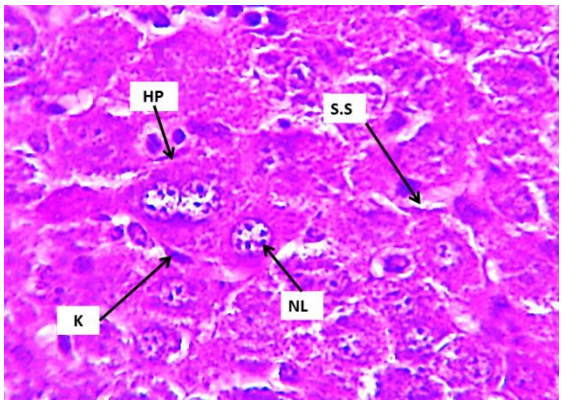
e) Quercetin 2nd sampling



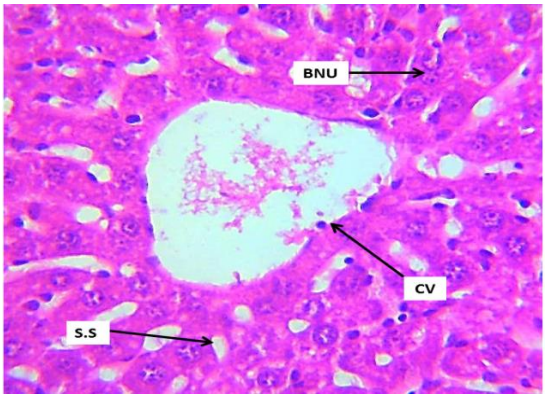
f) Quercetin 3rd sampling



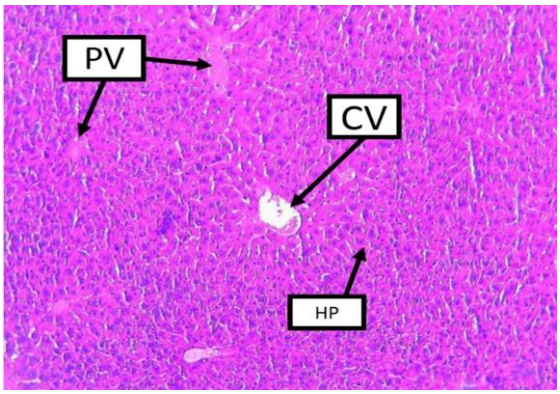
g) Ascorbic acid 1st sampling



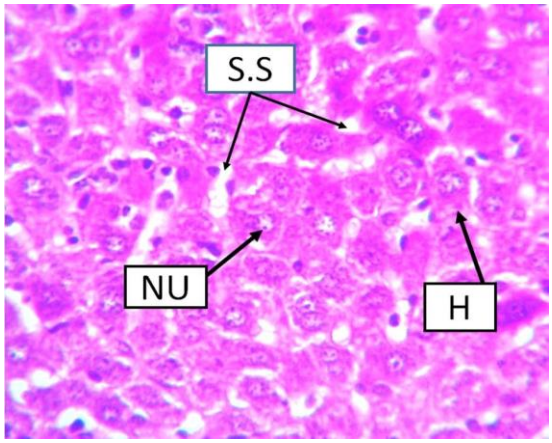
h) Ascorbic acid 2nd sampling



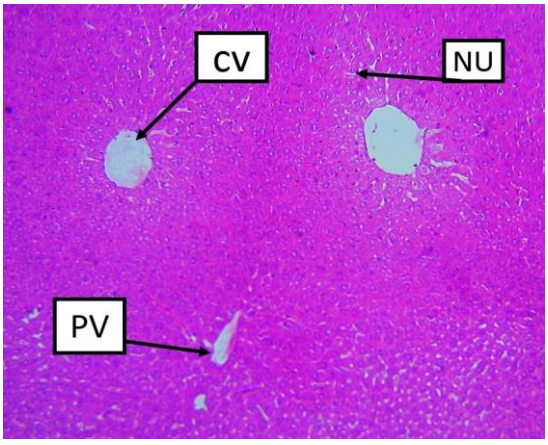
i) Ascorbic acid 3rd sampling



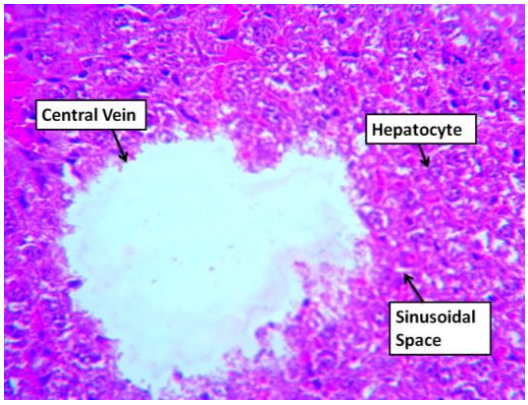
j) Alpha Lipoic acid 1st sampling



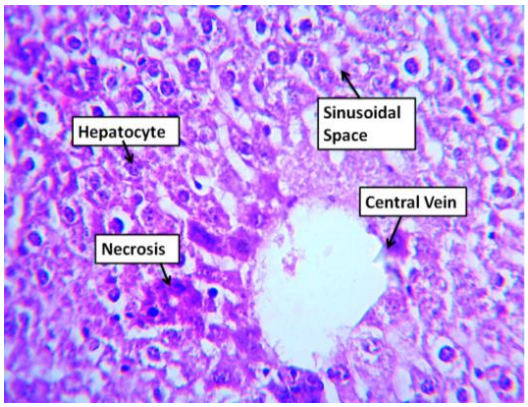
k) Alpha lipoic acid 2nd sampling



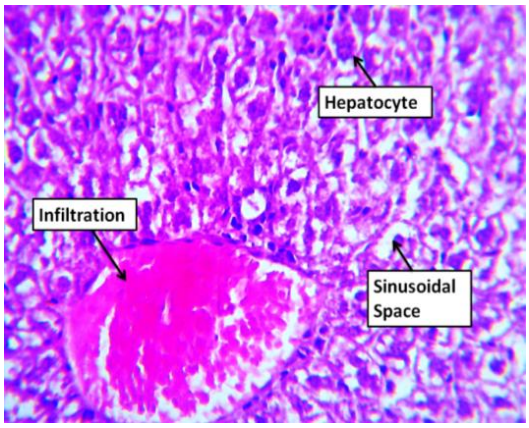
l) Alpha Lipoic acid 3rd sampling



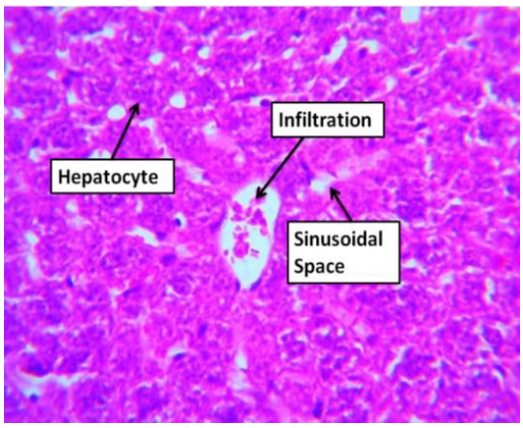
m) ZnO 1st sampling



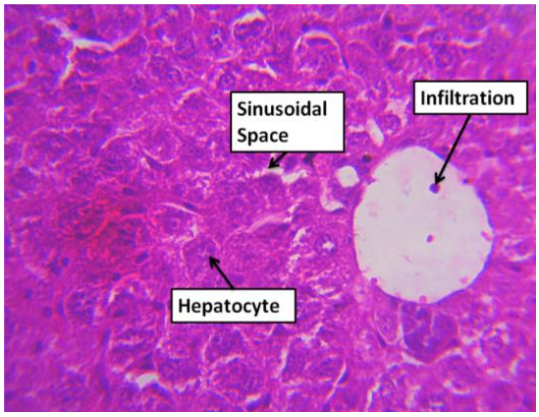
n) ZnO 2nd sampling



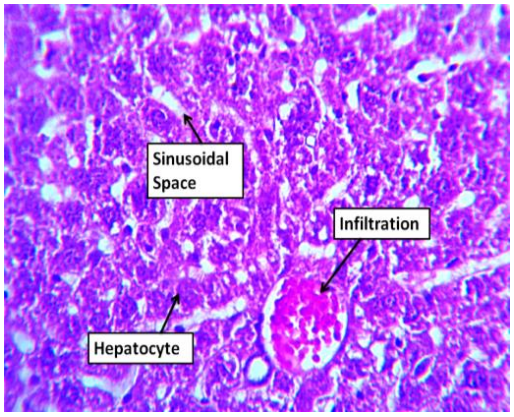
o) ZnO 3rd sampling



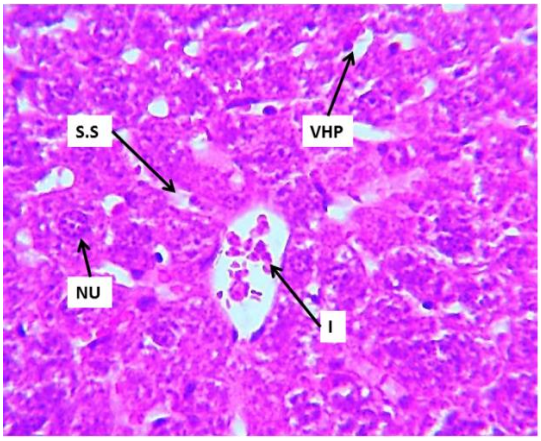
p) ZnO & Quercetin 1st sampling



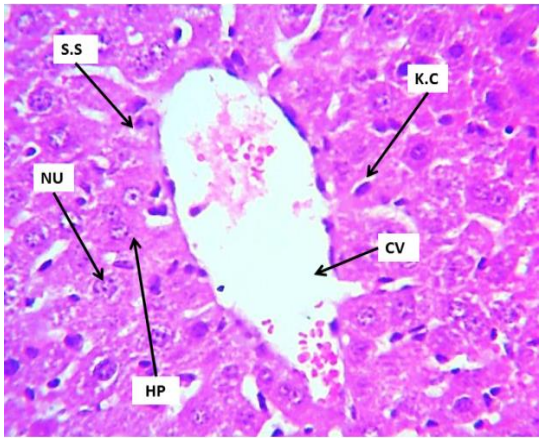
q) ZnO & Quercetin 2nd sampling



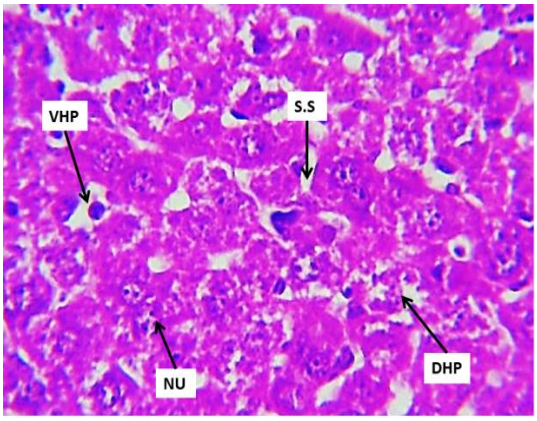
r) ZnO & Quercetin 3rd sampling



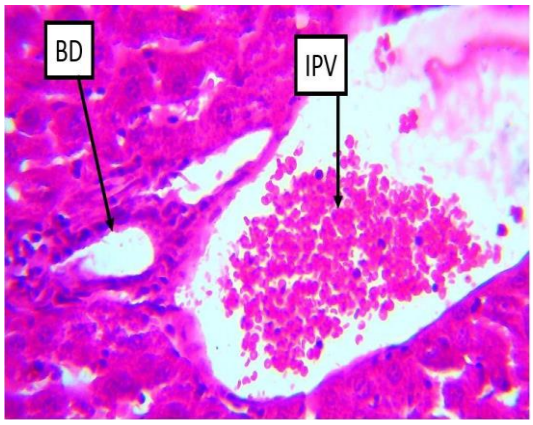
s) Ascorbic acid & ZnO 1st sampling



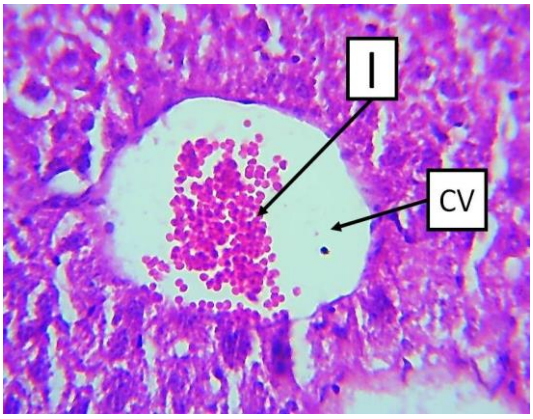
t) Ascorbic acid & ZnO 2nd sampling



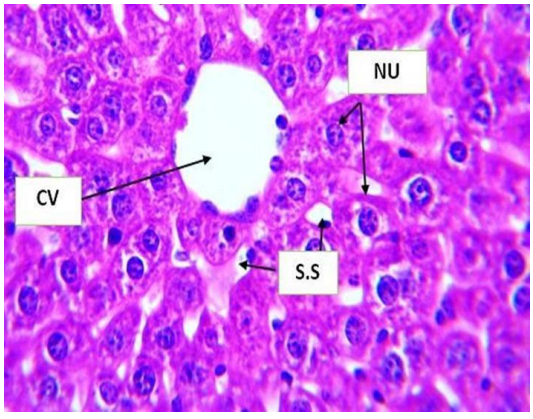
u) Ascorbic acid & ZnO 3rd sampling



v) Alpha lipoic acid & ZnO 1st sampling



w) Alpha lipoic acid & ZnO 2nd sampling



x) Alpha lipoic acid & ZnO 3rd sampling

FIGURE 2. Cross sections of liver showing different treated groups at 40 X (H & E) staining; nucleus (NU), kupffer cells (K.C) and sinusoidal spaces (S.S) vacuolated hepatocytes (VHP), infiltration (I), degenerated hepatocytes (DHP) central vein (CV), normal hepatic lobule (HL), dilated central vein (DCV), necrosis (Nc), bile duct (BD) and hepatic artery (PH)

CYTOTOXICITY ASSAY

Present study used *Artemia salina* (brine shrimp larvae) for cytotoxicity assay placed in sea water, exposed to different concentrations of nanoparticles for the period of 24 h. Cytotoxicity assay showed 0% mortality with ZnO-NP at 0.1- 0.6% concentration in 24 h (Table 5).

DISCUSSION

The current study showed increase body weight as a result of ZnO exposure which might be related to increase cholesterol synthesis (Hong et al. 2013; Mousavi et al. 2016). Quercetin treatment decreased body weight which might be due to ability of quercetin to reduce cholesterol synthesis because quercetin decreases *de novo* fatty acid and triacylglycerol (TAG) synthesis (Czerny et al. 2000; Gnoni, Paglialonga & Siculella 2009; Peredo-Escárcega et al. 2015). Increased plasma cholesterol level in ZnO intoxicated groups might be due to mobilization of free fatty acids from the adipose tissue to blood stream and increase level of acetyl CoA, resulting in increased synthesis of cholesterol (Rubins et al. 1999). Quercetin administration along with either ZnO decreased total cholesterol level which might be due to the ability of quercetin to reduce body fat (Gnoni, Paglialonga & Siculella 2009).

In the present study, ZnO NP treated group showed decrease in HDL concentration which is related to cirrhotic liver disease which was observed in previous study (Mandal et al. 2013). Contrary to previous studies in which liver toxicity is related to increase LDL level, present study showed that ZnO-NP nanoparticles decreased plasma LDL level which is again showing liver damage as evident from raised liver enzymes. As another study highlighted this rare phenomenon of low LDL and high HDL associated with liver damage in US population (10 million) (Jiang et al. 2014). Another study reported that ZnO-NPs induced atherosclerotic alterations both *in vivo* and *in vitro* which might be related to decrease LDL concentration as ZnO nanoparticles induces oxidative stress in tissue and LDL taken up by macrophages and further deposited in atheromatous plaques that mature into atherosclerotic lesions, leading to decrease LDL concentration (Kunjathoor et al. 2022; Yan et al. 2017). Antioxidants (quercetin, ALA, AsA) administration along with ZnO attenuates the alterations in the lipid profile which might be related to their metal chelating and free radical scavenger activity (Camiolo et al. 2019; Selvakumar et al. 2013; Shireen et al. 2008). ZnO-NP increased triglyceride level, which might be related to impaired clearance of chylomicrons (Vaziri 2003). Liver is a major organ subjected to damaging effects of xenobiotics and elevated plasma liver enzymes indicating hepatocyte necrosis further leading to hepatotoxicity. The obtained results of present study showed the increase in AST, ALT and ALP concentration after the ZnO exposure which might be due to hepatocellular damage during oxidative stress imposed by nanoparticles (Wang et al. 2008, 2007). Quercetin, alpha lipoic acid and ascorbic acid administration along with nanoparticles in experimental groups mitigates the toxic alterations of liver enzymes which might be due to their free radical scavenging activity (Miltonprabu et al. 2017; Pari & Murugavel 2004; Shireen et al. 2008).

In the current study, hepatocellular necrosis resulting from ZnO nanoparticles might be due to ability of ZnO-NPs to induce oxidative stress and increase rate of lipid peroxidation (Sharma et al. 2012b). Histopathological observation of liver tissue is co-related with increased liver enzymes caused due to hepatocellular necrosis induced by ZnO-NPs in current study. Another study reported that ZnO NPs induced a number of morphological alterations including enlargement, elongation, angulations, swelling, cristolysis, lacking cristae, and burst membranes in mice hepatocyte mitochondria (Almansour et al. 2015). Present study showed ameliorative effects of quercetin against ZnO induced damage. Previous studies showed that quercetin interact with hydroxyl, superoxide, alkoxyl and peroxyl radicals subsequently scavenging all free-radicals (Choi et al. 2003). Hepatoprotective effect of ascorbic acid related to property of ASA acting as an electron donor or reducing agent, effectively scavenges singlet oxygen, superoxide, hydroxyl, water soluble peroxyl radical and hypochlorous acid further supporting the findings of present study (Sminorff & Wheeler 2000). In the current study, ALA showed protection against ZnO induced hepatotoxicity which might be related to its antioxidant property as previous studies reported protective effect of ALA against alcohol-induced damage, mushroom poisoning, metal toxicity, CCl_4 poisoning and n-6 unsaturated fatty acids induced apoptosis in the liver cells, fibrosis development and inflammation in young rats' liver (Kaya-Dagistanli et al. 2013).

In the current study, cytotoxicity assay of ZnO nanoparticles showed 0% mortality. According to previous studies, toxicity of ZnO is dose and time dependent (Ates et al. 2013a, 2013b). Extended duration and dose would show more evident effects of nanoparticles and dietary antioxidants. More pronounced antioxidant activity of these dietary compounds can be shown by measuring glutathione activity i.e., Glutathione S transferase (GST),

and Superoxide dismutase (SOD). Further details about ZnO nanoparticles toxicity and protective effect of antioxidants could be obtained in chronic study, dose dependent study and more mechanistic approach.

Conclusions

Present study demonstrated that dietary antioxidants like quercetin (Polyphenolic flavonoids), alpha lipoic acid (Lipid base antioxidant) and ascorbic acid (Vitamin C) have unique properties and they showed hepatoprotective effects against nanoparticles i.e., ZnO-NPs. All antioxidants showed remarkable potential, counter-acting against dramatic alterations in body weight, lipid profile (with high HDL and low LDL), liver enzymes and hepatocellular necrosis as done by ZnO-NPs in a time dependent manner.

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