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RESEARCH

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# *Gentiana lutea* attenuates hepatotoxicity induced by ketoconazole in rats by fortifying the cellular antioxidant defense system

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## Abstract

**Background** Ketoconazole (KET) is a broad-spectrum antifungal drug that has been reported to induce hepatotoxicity in humans and animals.

**Methods** The safe guarding response of Gentiana extract (GEN) against KET-induced hepatotoxicity was investigated in this study using male Wistar rats. GEN ethanol extract was orally administered to rats (1 g/kg b.wt) for 30 days. Beginning on day 26, KET was intraperitoneally administered once daily for 5 days using a dose of 100 mg/kg. The hepatoprotective effects of GEN against liver damage induced by KET were monitored through significant decrements in serum levels of aminotransferase and alpha-fetoprotein as well as recorded hepatic histopathological changes.

**Results** The hepatotoxicity of KET treatment was accompanied with a marked oxidative damage to hepatic proteins, lipids, and DNA, and depletions in natural antioxidants (glutathione and superoxide dismutase). GEN inhibited KET-induced oxidative stress by diminishing lipid peroxidation, protein carbonylation, and oxidative stress in DNA. These free radical mediated effects were greatly decreased with GEN treatment.

**Conclusions** This study suggests that GEN's hepatoprotective effects could be attributed to its antioxidant properties.

**Keywords** Gentian, Ketoconazole, Hepatic injury, Protection

## Background

Ketoconazole (KET) is an antifungal, broad spectrum, drug associated with imidazole (Kinobe et al., 2006). Certain properties of KET have been reported to have anticancer effects. KET, for example, has been used as a part of the remedy for progressive prostate cancer (Patel et al., 2018; Rodriguez & Acosta, 1995). In addition, KET has shown promising anticancer effects among multiple cancer types including glioblastoma, prostate, breast, colon, and bladder cancers (Chen et al., 2019). KET has a suppressive effect on hepatocellular carcinoma (HCC) cell growth through the elicitation of apoptosis (Chen et al., 2019). However, hepatotoxicity induced by KET was reported in humans (Chien et al., 2003; Kim et al., 2003), rabbits (Ma et al., 2003), and rats (Amin & Hamza, 2005; Rodriguez & Buckholz, 2003). KET's major metabolite is N-deacetyl ketoconazole. Such a metabolite can be further processed by the flavin-containing monooxygenase forming a more toxic and reactive metabolite

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(Rodriguez & Acosta, 1995; Rodriguez et al., 1999). Hepatotoxicity in rats subsides in the presence of KET by chelating the reactive metabolite with cellular protein (Rodriguez & Buckholz, 2003). Oxidative stress damage and the reduction of antioxidants in the livers of rats are common factors in KET-induced liver damage (Amin & Hamza, 2005). This result may imply that free radicals are culpable for hepatotoxicity induced by KET.

Natural products have long been the major source of health-promoting remedies (Al-Dabbagh et al., 2018; Amin & Mahmoud-Ghoneim, 2011; Amin et al., 2021; Hamza et al., 2020; Kamal et al., 2018; Mu et al., 2021; Murali et al., 2021) and nanoformulations (Baig et al., 2019; El-Kharrag et al., 2017). Natural product-based antioxidants may play a key role in the inhibition of drug-induced toxic effects. There has been growing evidence that supports that phenolic antioxidants of medicinal plants origin prevents different oxidative stress mediated disorders (Hamza et al., 2021; Soobrattee et al., 2005). Phenolic antioxidants have pharmacological actions which stem primarily from their metal chelating and free radical scavenging properties in conjunction with their effects on gene expression and cell signaling pathways (Soobrattee et al., 2005; Urquiaga & Leighton, 2000). The perennial plant *Gentiana lutea* is used worldwide for the preparation of pharmaceutical and food products (Mudrić et al., 2020). Roots of Gentiana (GEN) [*Gentiana lutea*; Gentianaceae] have been utilized to treat ailments such as jaundice, chronic pharyngitis, fevers, and similar liver damages in Ayurvedic medicine (Dal Cero, 2017; Jiang et al., 2020). Other Gentiana species (*Gentiana olivieri*, *Gentiana manshurica* Kitagawa, *Gentiana macrophylla* Pall, *Gentiana asclepiadea*) have shown their hepatoprotective effects against liver injury induced by carbon tetrachloride (Hongjie et al., 2012; Mihailović et al., 2013; Orhan et al., 2003), ethanol (Lian et al., 2010a), acetaminophen (Wang et al., 2010), and the non-structural protein (NS) 1 of human parvovirus B19 (Sheu et al., 2017) in rats and mice. Various photochemical ingredients such as alkaloid gentianine, xanthone glycosides (Aberham et al., 2007), secoiridoids, swertiamarine, gentiopicroside, and sweroside were all (Xu et al., 2017) isolated from roots of GEN. The known C-glycosylflavone, Isoorientin, was extracted from another species of Gentiana, and it displayed antioxidant (Ko et al., 1998; Nastasijević et al., 2012) and antihepatotoxic (Mihailović et al., 2013; Orhan et al., 2003) properties. Besides this, flavonoids, such as catechin, naringin, myricetin, and morin, as well as phenolic acids, including gallic acid, gentisic acid, chlorogenic acid, vanillic acid, caffeic acid, and syringic acid, were found in the GEN root extract (Mustafa et al., 2016; Sheu et al., 2017). Moreover, we previously reported the defensive capacity of GEN against KET-induced reproductive toxicity in rats (Amin, 2008).

This investigation evaluates the preventive property of GEN against acute hepatotoxicity induced by KET in male Wistar rats and dissect the mechanism underlying these effects. We assess here biomarkers of hepatic toxicity including serum activities of aminotransaminases, alpha-fetoprotein (AFP), as well as examinations of liver histology. The oxidative stress status of the examined liver tissues is also analyzed through assessing the reduced enzyme activities of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) along with the diminished lipid peroxidation (malondialdehyde), protein carbonyl (P.Carbonyl), and DNA fragmentation.

## Methods

### Chemicals

KET was obtained as standard powders (≥ 98% purity, from European Pharmacopoeia, Council of Europe, Strasbourg, France) from the pharmaceutical company Janssen-Cilag (Beerse, Belgium). The root of Gentian (*Gentiana lutea*) was obtained at a traditional herb store (Mountain Rose Herbs, Eugene Oregon). Thiobarbituric acid, SOD enzyme, Folin's reagent, bovine albumin, epinephrine, and H2O2 were received from Sigma Chemical Co. (St. Louis, MO). The other chemicals were acquired from commercial suppliers locally.

### Animals

Adult male albino rats (150–200 g) of the Wistar strain were obtained from the animal house located at the National Research Center (Giza, Egypt). They were cared for using tap water ad libitum as well as a standard pellet diet. They were housed in polycarbonate cages bedded with wood chips under a 12-h light/dark cycle at a room temperature ranging from 22 to 24 °C. Prior to experimental use, the rats were acclimatized to the environment for one-week. All experiments were carried out in accordance with research protocols established by the Animal Care Committee of the NODCAR, Egypt, following the recommendations of the National Institutes of Health's Guide for Care and Use of Laboratory Animals. All efforts have been made to reduce the suffering of animals and the number of animals used. The approval number of the protocol is: NODCAR/II/3/2022.

### Extraction

The liquid-phase microwave-assisted process described by Pan et al. (2001) was used for the extraction of GEN in order to lower the temperature and increase the yield of extraction within a short time frame. These extraction applications are based upon the selective heating of the matrix containing the target extract, while the matrix is

immersed in a solvent such as ethanol and water, which is transparent to the microwaves. This solvent allows for components to be selectively heated within the materials being treated while avoiding excessive energy use. One hundred grams of fully dried GEN roots was mixed with 1000 ml of 70% ethanol. For every 10 g of ground herb, 100 ml of 70% ethanol in a 250-ml was added and mixed in a conical flask. Afterward, the mixtures were irradiated with microwaves for two minutes. Output power strategy of the microwave oven was 300 W. The suspension was irradiated for 25 s with power to get to the desired temperature of about 80 °C, then for another 5 s with the power on to further heat the suspension, and finally for 10 s with the power off to cool. Afterward, gauze is factored in, and the extract is filtered through it before being evaporated under a vacuum using a rotary evaporator at a temperature of 40 °C. Finally, the dried extract was dissolved in 2.5 ml of dimethyl sulfoxide (DMSO) before administration.

#### Determination of total antioxidant capacity of GEN extract

A ferric reducing antioxidant power (FRAP) assay was used to evaluate the total antioxidant capacity (TAC) in the crude extract. The FRAP assay was determined according to the method described by Benzie and Strain (1996). The FRAP assay assesses alterations in absorbance at 593 nm wherein changes result from the conversion of colorless oxidized ferric form into a blue colored ferrous-tripyridyltriazine complex occurs with the help of the electron donating antioxidants.

#### LC–MS of phenolic compounds of GEN extract

Phenolic compounds of GEN extract were detected and quantified using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) with an ExionLC AC system for separation and SCIEX Triple Quad 5500 + MS/MS system equipped with an electrospray ionization (ESI) for detection. A full-scan mode from m/z 500 to 1,200 was carried out. The chromatographic separation was performed using ZORBAX SB-C18 Column (4.6 × 100 mm, 1.8 µm). The mobile phases consisted of two eluents A: 0.1% formic acid in water; B: acetonitrile. The mobile phase was programmed as follows, 2% B from 0 to 1 min, 2–60% B from 1 to 21 min, 60% B from 21 to 25 min, and 2% B from 25.01 to 28 min. The flow rate was set at 0.8 ml/min with an injection volume of 3 µl. Optimization of the HPLC–MS/MS conditions was carried out by varying them in flow injection analysis (FIA) of the analytes. Operating ESI conditions were set as follows: Curtain gas: 25 psi; ion spray voltage: 4500; source temperature: 400 °C; and ion source gas 1 and 2 were 55 psi with a declustering potential: 50; collision energy: 25; collision energy spread: 10. Mass spectra were acquired in both negative and positive ESI modes and elaborated using multiple reaction monitoring (MRM) modes. For the quantitative analysis of major phenolic compounds, similar references (such as catechin, naringin, myricetin, rutin, gallic acid, gentisic acid, chlorogenic acid, vanillic acid, caffeic acid, and syringic acid), at the concentration of 0.1, 0.075, 0.005, 0.0025, and 0.001 mg/ml each, were injected in the same chromatographic conditions. The standard curve was obtained for each standard substance and was used to measure the concentrations of phenolic substances in the extract.

#### Treatment regime

A stock solution for KTZ was prepared by dissolving the powder in 2.5% DMSO. The KET solution was delivered to animals using a volume of 1 ml/100 g b.wt saline solution and was protected from light. Both the control and protected animals received equivalent volumes of saline dependent on their body weight. GEN extract was delivered by gavage orally using a concentration of 1 g/kg of body weight (Amin & Hamza, 2005). The rats were divided into four groups randomly with six rats in each group. The groups received the following care: The KET treatment group received 2.5% DMSO every day for 26 days, and after 3 weeks of the DMSO treatment, rats received an intraperitoneal injection of KET (100 mg/kg b.wt.) every day for 5 days. Male Wistar rats were previously exposed to this hepatotoxic dosage of KET to cause liver toxicity (Amin, 2008). The rats in the GEN + KET group received daily treatment with GEN extract for 26 days, followed by five days of daily treatment with KET at the same dose (100 mg/kg b.wt.) following three weeks of GEN extract. The rats in the GEN group were given the same volume of saline solution (1 ml/100 g) every day for five days after receiving GEN extract daily for 26 days to the animals in the GEN group. The normal control group had a similar regimen of an identical volume of DMSO every day for 26 days before receiving an injection of saline for five days following the completion of the first 21 days of DMSO treatment. 24 h after the last treatment with KET, the vehicle solution administration, the herbal extract, liver, and blood samples were gathered from all the groups.

#### Sample preparation

After anesthetization with sodium phenobarbital (150 mg/kg, i.p.), blood was collected from the retro-orbital plexus (Hamza et al., 2020). The rats were also euthanized by cervical dislocation under sodium phenobarbital anesthesia, and livers were removed. Part of the liver was fixed in 10% buffered formalin immediately for histopathological examination. Another part of liver was

homogenized in ice-cold KCl (150 mM) for biochemical determination. The liver tissue weight to homogenization buffer ratio was 1:10. From such a stock, proper dilutions were utilized to determine levels of MDA, GSH, and total proteins. The activities of CAT and SOD were assessed using different buffers. Serum was obtained from centrifuged blood that was collected in centrifuge tubes and centrifuged at 4 °C at 3000 r.p.m. for 20 min.

### Biochemical assays and histopathology

#### *Liver toxicity indices*

Alpha-fetoprotein (AFP) is used as marker for hepatic carcinoma and acute toxic liver damage, and it is produced by the fetal liver (Hudig et al., 1979; Kreczko et al., 2000). An enzyme immunoassay method using a commercial Kit was employed to determine AFP in serum (Calbiotech Inc., 10461 Austin Dr. Spring Valley, I CA, 1978) while following their instruction manual. Serum levels of aspartate aminotransferase (AST), and alanine aminotransferase (ALT), were estimated using Randox (Randox Laboratories Ltd., Antrim, UK) following the available instruction manual.

### Histopathological examination

For histological examinations, pieces of livers were fixed in 10% neutral phosphate-buffered formalin. Using hematoxylin and eosin (H&E), hydrated tissue sections (5 µm in thickness) were stained. Those sections were examined using a Leica DMRB/E light microscope.

### Oxidative stress biomarkers

The method described by Van Dooran et al. (1978) was used to determine the reduced glutathione (GSH) content in the liver homogenate. The basis of the GSH determination method is the reaction of Ellman's reagent 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) with the thiol group of GSH at a pH of 8.0 to give a yellow color to 5-thiol-2-nitrobenzoate anion. Malondialdehyde (MDA) is an individual aldehyde, and in fact the most prevalent one, that results from lipid peroxidation (LP) breakdown in biological systems and it is used as an indirect index of LP (Packer & Cadenas, 2002). As described in Uchiyama and Mihara (1978), the determination of MDA in biological materials is based upon the reaction it has with thiobarbituric acid (TBA) which forms a pink complex with an absorption maximum at 535 nm. By measuring the exponential disappearance of $H_2O_2$ at 240 nm, CAT activity was determined and expressed in units/mg of protein as described by Aebi (1984). The method described by Sun and Zigman (1978) was used to determine the activity of the SOD enzyme in liver homogenate. This method relies upon SOD's ability to inhibit the auto-oxidation of epinephrine to adrenochrome and other derivatives at an alkaline pH, which can easily be monitored in the near-UV region of the absorption spectrum. The method of Reznick & Packer (1994) was used to determine hepatic P.Carbonyl contents. Their method is based on the reaction of 2,4-dinitophenylhydrazine with P.Carbonyl to form protein hydrazones at 370 nm which can be spectrophotometrically detected. Results were expressed as nmol of carbonyl group per milligram of protein with a molar extinction coefficient of 22,000 M/cm.

DNA fragmentation was assessed in deparaffinized sections by terminal deoxynucleotidyl transferase-mediated triphosphate nick-end labeling (TUNEL) by using the Apoptag plus Peroxidase in the Situ Apoptosis Detection Kit (Chemicon International, CA, USA) according to the protocol provided by the manufacturer. This method detects the DNA fragmentation using a process facilitated by terminal deoxynucleotidyl transferase, labeling 3-OH DNA termini with digoxigenin nucleotides. The labeled fragments produced are then allowed to bind to anti-digoxigenin antibody conjugated with peroxidase. To develop color, sufficient peroxidase substrate was applied to specimens. TUNEL slides were observed using the Leica DMRB/E light microscope. DNA fragmentation of hepatocytes was identified by positive TUNEL staining. The TUNEL-positive cells have brown condensed nuclei. The expression of P53 protein was studied using immunohistochemistry in the sections. After deparaffinization and rehydration, the tissue sections were treated for 20 min with 3% hydrogen peroxide to diminish non-specific staining. The sections were put in a microwave oven twice for 5 min while immersed in 10 mM citrate buffer solution (pH 6.0) and then incubated for 20 min with normal goat serum. The sections were incubated overnight at 4 °C with the rabbit primary antibody for P53 protein and then washed with phosphate-buffered saline. The sections were exposed to the avidin biotin peroxidase complex (1/400, Dako) for one hour at room temperature. Using a 0.05% solution of 3,3 diaminobenzidine tetrahydrochloride, 0.03% hydrogen peroxide, and imidazole in Tris HCL buffer (pH7.6), the chromogenic substrate of peroxidase was developed. The sections were counterstained with hematoxylin. For the immunohistochemistry, two sections were made for five rats for each group, and five stages were read for each rat. Now, the TUNEL- and P53-positive cells were calculated by image j program as a percentage of total area and the statistical analysis between groups was calculated. The total protein content of the liver was determined utilizing the Lowry method as modified by Peterson (1977). Absorbance was recorded using a Shimadzu recording spectrophotometer (UV-160) in all measurements.

### Statistical analysis

SPSS (version 20), a statistical program (SPSS Inc., Chicago, IL, USA), was used to carry out the one-way analysis of variance (ANOVA) on our data. Statistical significance between treatment groups was determined using the one-way analysis of variance (ANOVA) followed by a Dunnett's *t* test post hoc analysis for multiple comparisons, with $P<0.05$ being considered as statistically significant. Figures were finished using GraphPad Prism program (version 5) (San Diego, California, USA).

## Results

### Effects on serum markers of liver damage

The activities of serum ALT and AST were significantly increased as compared to the control by about 77% and 55%, respectively, after treatment with KET (Fig. 1). Rats receiving GEN alone did not show any significant change in the serum markers of liver damage. While concomitant treatment with GEN abolished the significant increase in serum ALT activity compared to control group, the pretreatment with GEN attenuated the increase in AST activity by about 47% when compared to the KET-treated group (Fig. 1). In KET-intoxicated rats, serum AFP levels (Fig. 1) were significantly increased by about 564.7% compared to the control group. In both the GEN and GEN + KET groups, levels of AFP did not differ significantly from the control values.

### Effects on hepatic MDA and P. carbonyl

In KET-intoxicated rats, hepatic MDA and P.Carbonyl levels were significantly increased by about 64% and 76%, respectively, compared to the control group (Fig. 2). In both the GEN + KET and GEN groups, levels of MDA and P.Carbonyl did not significantly differ from control values.

### Effects on antioxidant substances

The rats in the KET-treated group showed significant depletion in GSH level and SOD activity in liver by about 18% and 34%, respectively (Fig. 3). Rats pretreated with GEN before KET intoxication showed levels of these oxidative stress markers that did not differ significantly from the control. In respect to CAT activity, there were no significant changes from main values in all the experimental groups.

### Effects on histopathological changes

The hepatocytes in KET-treated rats showed clear cellular degeneration with a loss of distinct liver characteristic configuration (Fig. 4c, d). Focal necrosis was observed in

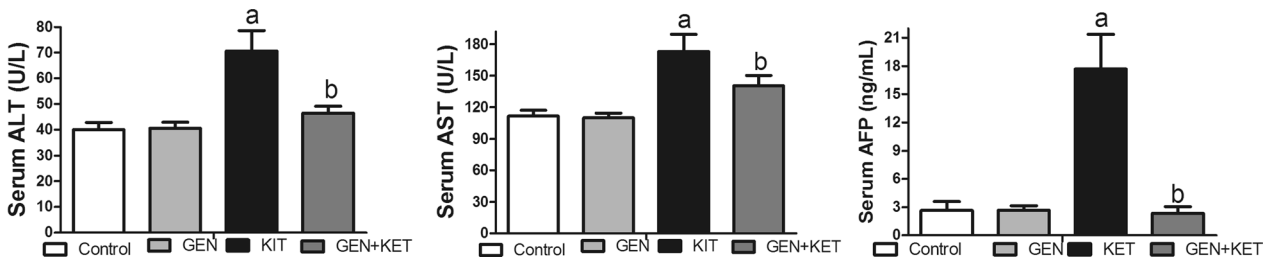


**Fig. 1** Effect of GEN on serum aminotransferases activities (ALT and AST) and AFP concentration in control and KET-treated rats. Columns represent the means ± S.E.M for six animals in each group. Significance was determined by one-way analysis of variance followed by Dunnett's t test: a $P<0.05$ versus control group; b $P<0.05$ versus KET

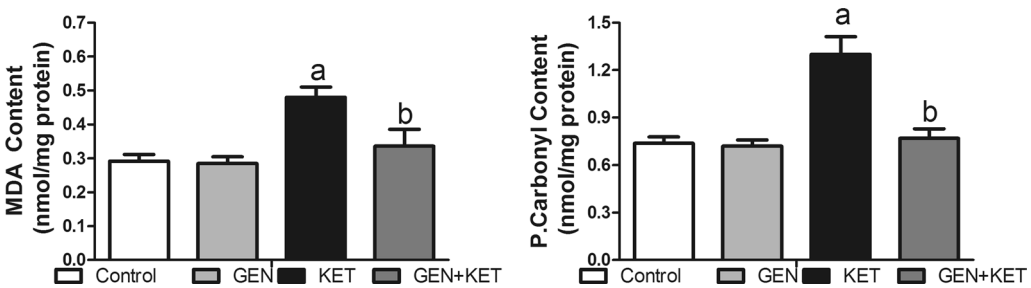


**Fig. 2** Effect of GEN on hepatic MDA and P. Carbonyl contents in control and KET-treated rats. Each column represents the mean ± S.E.M. for six rats in each group. Significance was determined by one-way analysis of variance followed by a Dunnett's t test: a $P<0.05$ versus control group; b $P<0.05$ versus KET

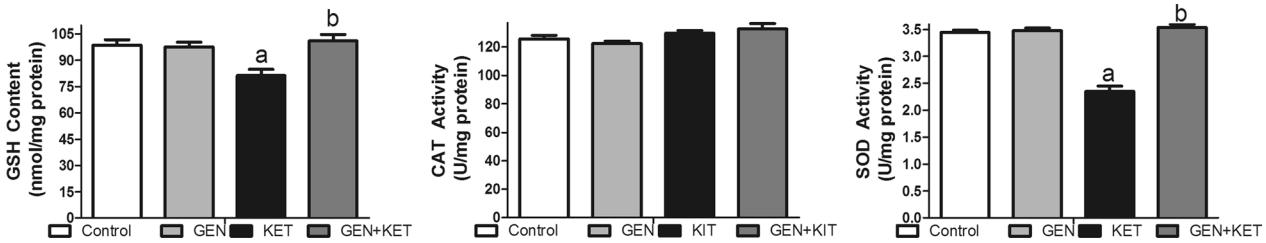


**Fig. 3** Effect of GEN on hepatic GSH content and CAT and SOD activities in control and KET-treated rats. Each column represents the mean ± S.E.M. for six rats in each group. Significance was determined by one-way analysis of variance followed by Dunnett's t test: a $P < 0.05$ versus control group; b $P < 0.05$ versus KET

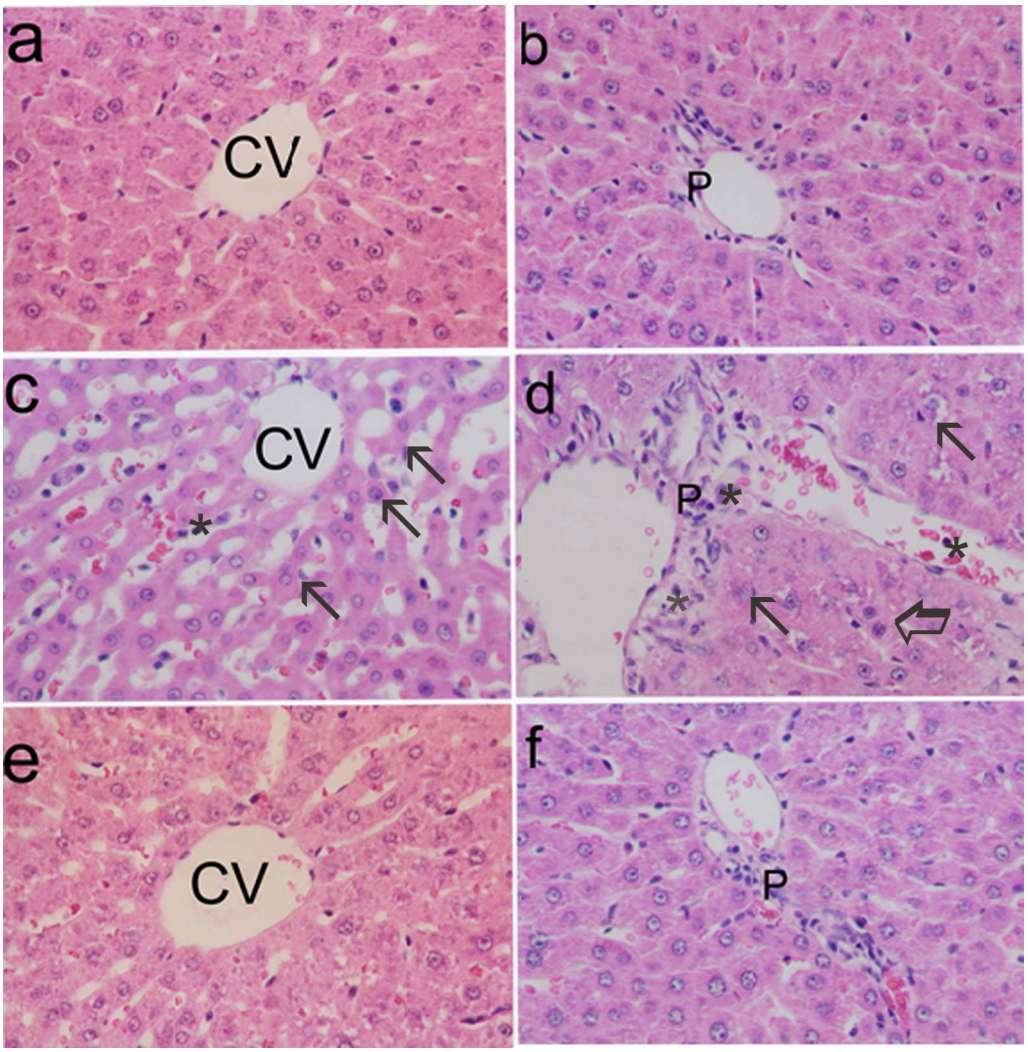


**Fig. 4** Photomicrographs of liver sections in central area (**a**) and portal area (**b**) from control rats showing the normal arrangement of hepatocytes. Liver section of KET-treated rats (**c**) showing extensive degeneration of hepatocytes (thin arrows), focal necrosis (thick arrows), and inflammatory cell infiltration * in central vein (cv) and portal area p (**d**). Group of rats pretreated with GEN before KET (**e**, **f**) exhibiting relatively normal appearance of hepatocytes with moderate degeneration and focal vacuolated cells (H&E, X 400)

all areas of the lobule. Degenerated cells and karyolysis or pyknosis of nuclei were the main form of marked changes in the liver. Histopathological changes in the liver also included congestion in central veins as well as sinusoids and an enlargement of the portal area. Some infiltrations of mixed inflammatory cells around the necrotic hepatocytes were observed, particularly in portal areas. Treatment with GEN prevented both necrotic changes as well as histopathological changes induced by KET treatment. Apart from a few scattered degenerated cells, Fig. 4e, f shows normal appearance. GEN alone did not induce any histopathological changes (data not shown).

#### TUNEL and P53 immunohistochemically assays in KET-treated rats

The TUNEL and P53 were immunohistochemically assessed as markers of DNA strand breaks. In Fig. 5, immunohistochemical study showed that the KET group revealed evident TUNEL and P53 staining in hepatocytes, especially in centrilobular (CV) and periportal areas (P). GEN attenuated the numbers of TUNEL- and P53-positive cells.

#### Total antioxidant capacity of GEN extract

The FRAP assay is a test that was recently developed for measuring TAC. In the present study, GEN has TAC equal to 4.27 m mol/g of crude extract (data not shown).

#### Phenolic compounds of GEN extract

Figure 6 presents the chromatogram of the GEN extract obtained by HPLC–DAD analysis at 280 nm. HPLC–MS/MS was used for confirmation of peak identification, by comparing the retention time and precursor–product ions transitions and for quantitation, by comparing the peak areas of the product ions of the compounds identified with those of the standards in MRM modes.

Based on HPLC–MS/MS analysis, the most abundant phenolic compounds were naringenin, gallic acid, 3,4-dihydroxybenzoic acid, syringic acid, ferulic acid, and quercetin which were determined with concentrations compounds 266.45, 109.87, 60.07, 58.72, 45.70, and 45.70 mg/g, respectively. Catechin, coumaric acid, and caffeic acid were also detected at concentrations of 26.52, 20.06, and 6.21 mg/g, respectively (Table 1).

## Discussion

KIT treatment clearly caused liver impairments as evidenced by histological and biochemical changes, including an increase in liver function enzymes, a decrease in the antioxidant profile, and hepatic oxidative damage (Amin & Hamza, 2005; Rodriguez & Buckholz, 2003). Therefore, this study was conducted to investigate how GEN protected the liver from the hepatotoxicity that was caused by KET. The present study presented a convincing evidence that GEN has a protective effect against KET-induced acute liver damage. Our results showed that cotreatment with GEN effectively reduced tissue oxidative stress and successfully shielded the hepatic tissues from the harmful effects of KET. Here, the hepatic damage induced by KET was demonstrated by an elevation of serum AST and ALT activities and confirmed through histological alteration in the liver. The most common liver transaminases are ALT and AST. ALT is present only in the cytoplasm of hepatocytes, while AST is present in both the cytoplasm and mitochondria. In liver tissue, there are several enzymes which have been considered as effective biochemical markers to evaluate liver injury. Disintegration of the hepatic cell membrane of the liver is evidenced by elevations of AST and ALT levels in circulation (Plaa & Zimmerson, 1997). The severe increase in serum AFP was observed in KET-treated rats which is indicative of acute liver damage (Hudig et al., 1979; Kreczko et al., 2000). These results correlated with other reports where, after an acute dose, KET has been shown to induce an elevation in serum aminotransferase activities and hepatic histopathological changes in male rats (Amin & Hamza, 2005; Rodriguez & Buckholz, 2003). Concomitant treatment of KET-intoxicated rats with GEN significantly attenuated the severity of histopathological injury and the increase in serum aminotransferase and AFP levels. These results demonstrated that GEN has a remarkable hepatoprotective effect on KET-induced liver damage. Comparable hepatoprotective effects of different species of Gentiana were previously reported in different models of hepatotoxicity (Lian et al., 2010b; Mihailović et al., 2013).

In order to evaluate the oxidative stress induced by KET, MDA, P.Carbonyl, DNA fragmentation were analyzed as markers of lipid, protein, and DNA oxidation, respectively. The KET-induced hepatotoxicity in this study was accompanied with elevation in MDA, P.Carbonyl, and DNA fragmentations (TUNEL assay). Therefore, the most likely mechanism for the liver toxicity of KET may be through reactive oxygen species (ROS) generation and the oxidative damage to lipid membranes, proteins, and DNA. Both antioxidant enzymes and non-enzymatic antioxidants are parts of a well-developed antioxidant system of defense that all organisms possess to maintain the cellular redox state. SOD and CAT are the major antioxidant enzymes which help to protect the body from superoxide radicals and hydrogen peroxide (Valko et al., 2006). GSH is an abundant thiols antioxidant present in the liver and represents its major detoxifying agent, thus enabling the body to get rid of toxic compounds and heavy metals (Valko et al., 2006). KET induced depletion in GSH and SOD levels in liver, both

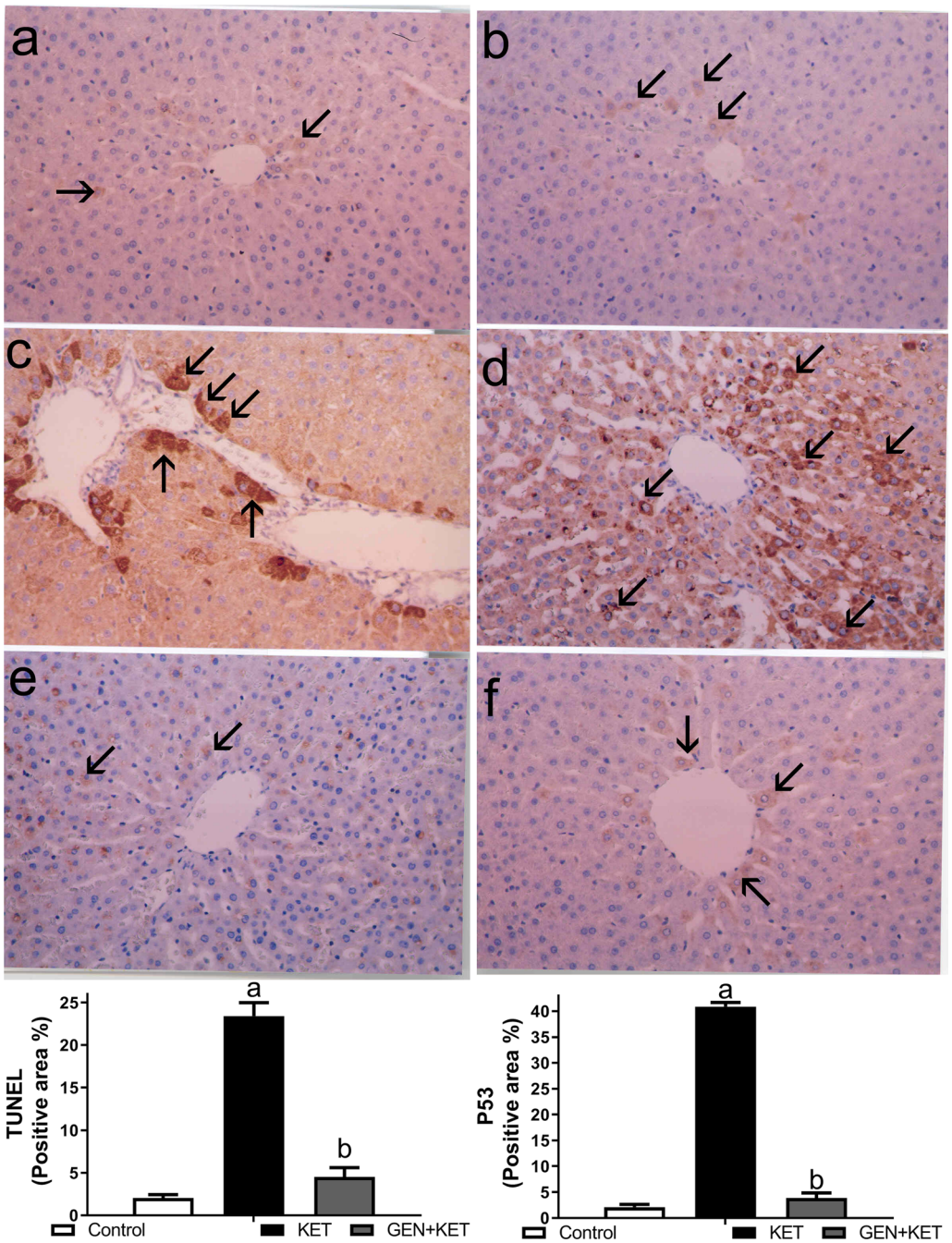


**Fig. 5** Photomicrographs of liver sections showing the effect of GEN on KET-induced DNA strand breaks (TUNEL assay) and increased in P53 protein expression. In liver section of control rats some cells are positive for TUNEL staining (**a**) and P53 immunoreactivity (**b**), the hepatocyte nuclei and cytosol stained brown (arrows). Liver sections of KET-treated rats showing extensive TUNEL-positive cells (**c**), and P53-positive cells (**d**), (arrows). Group of rats treated with GEN + KET had nearly normal appearance of hepatocytes with a marked reduction in TUNEL-positive cells (**e**) and P53-positive cells (**f**). Sections were counterstained with hematoxylin (X 200). Columns represent number of TUNEL- and P53-positive cells as a percentage of total area (5 scope field count for five animals in each group), and values are expressed as mean ± SEM. Significance was determined by one-way analysis of variance followed by Dennett's *t* test:. a and b indicate significant differences relative to control and KET groups, respectively

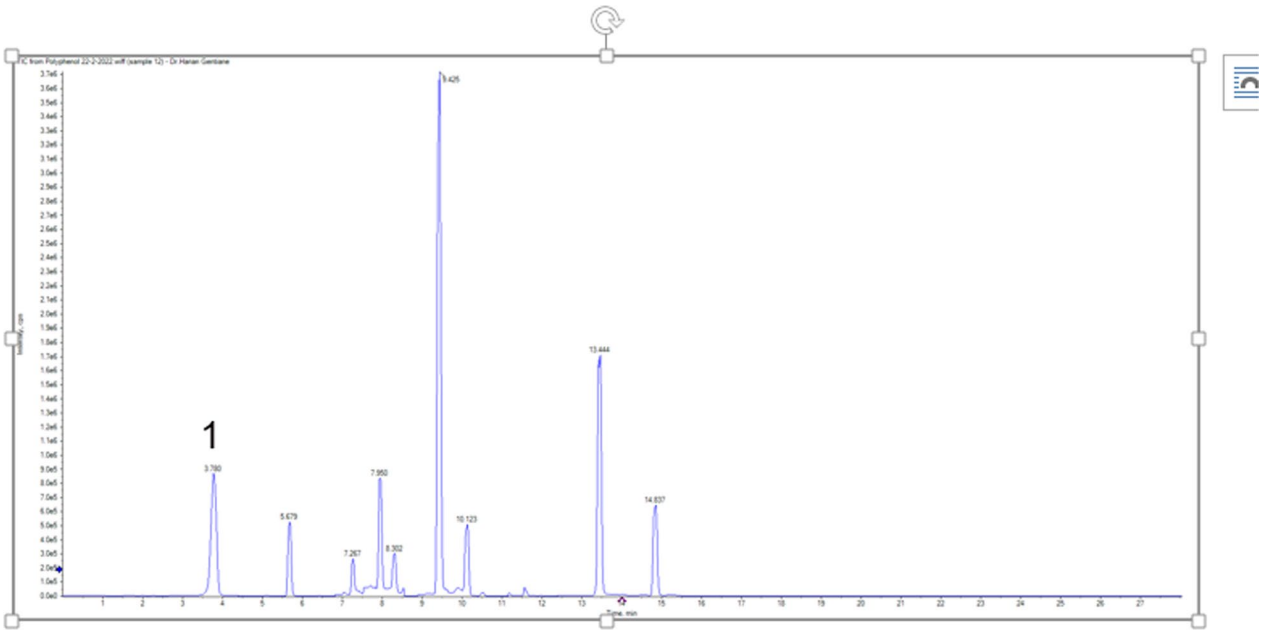


**Fig. 6** HPLC–DAD chromatogram of the GEN extracts

**Table 1** Quantitative results for flavonoids and phenolic acids of gentian by HPLC–MS/MS

| Peak number | Compound | Observed *m/z* | Retention time | Conc. (mg/g) |
|---|---|---|---|---|
| 1 | Gallic acid | 168.9/124.9 | 3.79 | 109.87 |
| 2 | 3,4-Dihydroxybenzoic acid | 152.9/109 | 5.68 | 60.07 |
| 3 | Catechin | 288.8/244.9 | 7.27 | 26.52 |
| 4 | Caffeic acid | 178/135 | 7.95 | 6.21 |
| 5 | Syringic acid | 196.8/181.9 | 8.30 | 58.72 |
| 6 | Coumaric acid | 162.9/119 | 9.46 | 20.06 |
| 7 | Ferulic acid | 192.8/133.9 | 10.12 | 45.70 |
| 8 | Quercetin | 301/151 | 13.44 | 37.14 |
| 9 | Naringenin | 271/119 | 14.84 | 266.45 |

of which catalyze the removal of free radicals (Packer & Cadenas, 2002). This also indicates that the overproduction in ROS and oxidative stress could be attributed to depletion in antioxidants herewith observed. This oxidative stress damage is consistent with our previous study in which KET at the same acute dose induced DNA fragmentations, an increase in lipid peroxidation, and depletions of SOD and GSH in the liver of rats (Amin & Hamza, 2005). GEN crude extract helped preventing the increase in hepatic MDA, P.Carbonyl, and DNA fragmentation. GEN treatment also prevented the depletion in hepatic GSH and SOD levels induced by KET. This result demonstrated that GEN antioxidant properties are primarily involved in alleviating KET-induced liver damage. GEN treatment by itself was shown to have no effect on levels of hepatic antioxidants which indicates that GEN is not functioning through the induction of these antioxidants. There is a significant increase in the number of TUNEL-positive cells after treatment with KET, particularly in the damaged areas of liver. The TUNEL assay detects single-strand DNA breaks as well as double-strand DNA breaks, and this DNA fragmentation was confirmed previously in the liver of KET-treated rats (Amin & Hamza, 2005). The TUNEL-based evidence of DNA fragmentation presented here was further supplemented with an increased accumulation of P53 protein. The accumulation of P53 protein could have been a brief response to DNA fragmentation, which supports the

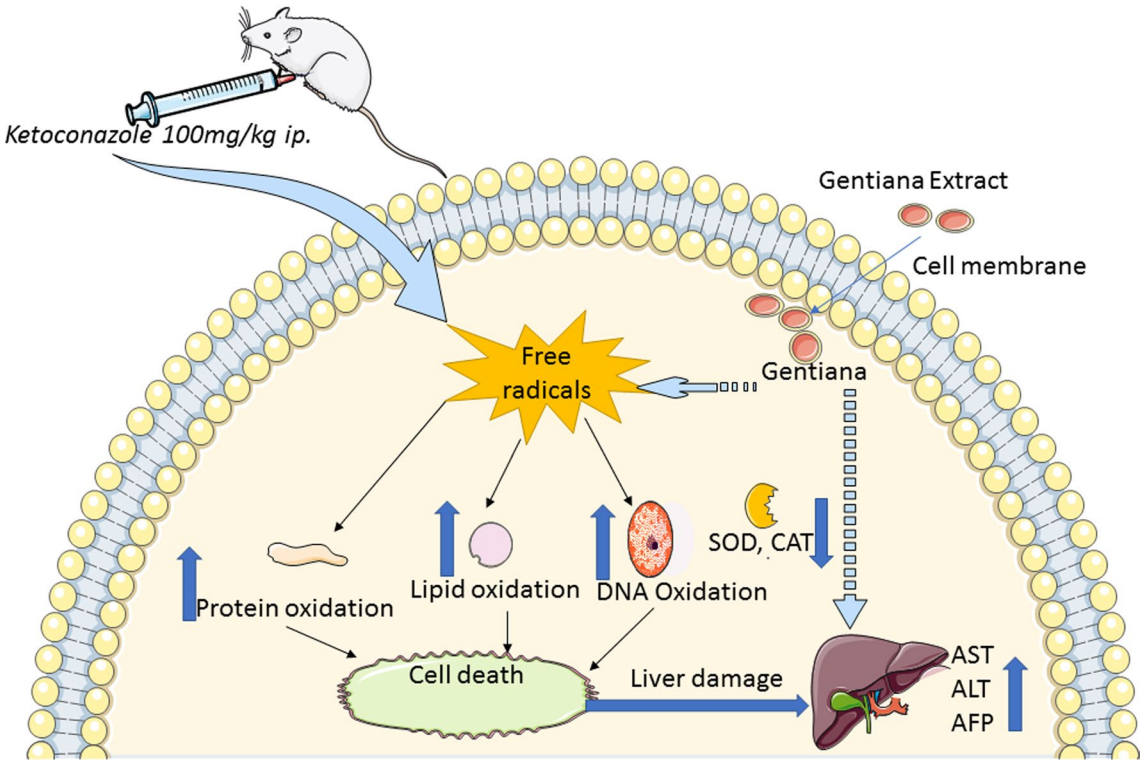


**Fig. 7** The overview of cellular oxidative stress and toxicity in liver tissue of rats after ketoconazole treatment. The speculative pathway indicates the hepatotoxicity effect of ketoconazole attributed to oxidative damage to hepatic proteins, lipids, and DNA, and depletions in natural antioxidants (SOD, CAT), while Gentiana prevented the hepatotoxicity of ketoconazole via its antioxidant effects (fragmented arrows)

cellular repair of DNA damage or triggering apoptosis. P53 proteins typically cause cell-cycle arrest or promote apoptosis in response to cell stress or DNA damage. That helps preventing the replication of damaged DNA and consequently defends the integrity of the genome (Szoke et al., 2005). GEN crude extract was able to prevent the increase in hepatic DNA fragmentation. These results are consistent with previous results with another species of Gentiana that protected human cells against DNA oxidation induced by free radicals (Hudecová et al., 2012).

Gentiana hepatoprotection has been demonstrated by different models of hepatotoxicity (Hongjie et al., 2012; Lian et al., 2010a; Wang et al., 2010). *Gentiana asclepiadea* L. extracts induced hepatoprotective protection against CCl4-induced hepatotoxicity, and such protection was accompanied by impairment of hepatic oxygen stress (Mihailović et al., 2013). The active substances extracted from Gentiana induced hepatoprotective effects against d-GalN/LPSr-induced toxicity in rats and were associated with anti-apoptotic activities (Lian et al., 2010b). As for *Gentiana macrophylla* pall, the effect of hepatic protection in mice against NS 1 of human parvovirus B19 was accompanied by a decrease in inflammation (Sheu et al., 2017).

GEN might reduce free radical formation as well as decompose and quench free radicals. This crude extract's antioxidant activity was confirmed in this study by FRAP assay and in previous work with 2,2-diphenyl-1-picrylhydrazyl (Nastasijević et al., 2012) which indicates the ability of the extract to inhibit free radicals. Previous research on the GEN plant proved the presence of many types of flavonoids such as catechin, naringin, myricetin, and morin, and phenolic acids, including gallic acid, gentisic acid, chlorogenic acid, vanillic acid, caffeic acid, and syringic acid (Mustafa et al., 2016; Sheu et al., 2017). This is consistent with the flavonoids estimated in the present investigation and including naringenin, gallic acid, 3,4-dihydroxybenzoic acid, syringic acid, ferulic acid, quercetin catechin, coumaric acid, and caffeic acid. Those GEN-isolated flavonoids may work together to increase GEN antioxidant's capacity and thereby confer more potent protection against oxidative stress and hepatotoxicity (Fig. 7).

## Conclusions

GEN crude extract has the potential to prevent hepatotoxicity and oxidative damage caused by an acute dose of KET in liver tissues of male albino rats. Presented data

provide sufficient evidence of the benefits of ethanolic crude extract of GEN administration to protect humans from hepatic toxicity induced by KET. That notion may pave the way for developing a combined therapeutic strategy to alleviate KET-associated side effects.

**Abbreviations**

| | |
|---|---|
| AST | Aspartate aminotransferase |
| ALT | Alanine aminotransferase |
| AFP | Alpha-fetoprotein |
| GEN | Gentiana extract |
| KET | Ketoconazole |
| DMSO | Dimethyl sulfoxide |
| FRAP | Ferric reducing antioxidant power |
| GSH | Glutathione |
| CAT | Catalase |
| MDA | Malondialdehyde |
| P.Carbonyl | Protein carbonyl |
| SOD | Superoxide dismutase |
| ROS | Reactive oxygen species |
| H&E | Hematoxylin and eosin |
| TUNEL | Terminal deoxynucleotidyl transferase-mediated triphosphate nick-end labeling |
| TAC | Total antioxidant capacity |

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**Author contributions**
AAH, MG, AAb, YA, and AA designed the study. AAH, MG, AAb, YA, and AA performed the experiments and did the statistical analysis. AAH, MG, AAb, YA, and AA assisted with methodology and contributed resources. AAH, MG, AAb, YA, and AA wrote the first draft of the manuscript, and all authors contributed to the editing of the revised manuscript and approved the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**
All data of this article are available.

## Declarations

**Ethics approval and consent to participate**
All experiments were carried out in accordance with research protocols established by the Animal Care Committee of the National Organization for Drug Control and Research (NODCAR), Egypt, following the recommendations of the National Institutes of Health's Guide for Care and Use of Laboratory Animals. All efforts have been made to reduce the suffering of animals and the number of animals used. Approval number of the protocol is: NODCAR/II/3/2022.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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