Industrial Crops and Products 44 (2013) 50–55

Contents lists available at SciVerse ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Hepatoprotective effect of gallic acid isolated from *Peltiphyllum peltatum* against sodium fluoride-induced oxidative stress

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ARTICLE INFO

Article history:
Received 20 June 2012
Received in revised form 10 October 2012
Accepted 18 October 2012

Keywords:
Antioxidant enzymes
Lipid peroxidation
Liver function markers
Peltiphyllum peltatum
Oxidative stress
Sodium fluoride intoxication

ABSTRACT

In the present study, the possible protective effects of gallic acid isolated from *Peltiphyllum peltatum* against sodium fluoride (NaF)-induced hepatotoxicity and oxidative stress were evaluated. Rats were intoxicated with 600 ppm NaF through drinking water for one week. Gallic acid (10 and 20 mg/kg) and the positive control, silymarin (10 mg/kg) were administrated for seven days prior to NaF intoxication. 24 h after the treatment period, superoxide dismutase and catalase activities, lipid peroxidation and reduced glutathione levels were measured in the liver. Serum biochemical markers including: alanine transaminase, aspartate aminotransferase, alkaline phosphatase, lipase and α-amylase activities and triglyceride, cholesterol, glucose, total bilirubin, direct bilirubin, total protein and albumin levels were determined. The results demonstrated that pretreatment with gallic acid normalized the sodium fluoride-induced alterations in serum parameters and oxidative stress in hepatic tissue. Fluoride intoxication resulted in an increased level of thiobarbituric acid reactive substances (TBARS) (53.05 ± 2.23 nmol MDA equiv./g tissue) in the liver homogenates in comparison with control group (25.03 ± 1.27 nmol MDA equiv./g tissue). Pretreatment with gallic acid at 20 mg/kg demonstrated significant mitigation in TBARS level (33.95 ± 2.51 nmol MDA equiv./g tissues). Fluoride intoxication did also suppress the superoxide dismutase and catalase activity of hepatic tissue homogenates by 33.87% and 66.87%, respectively. Treatment with gallic acid resulted in a dose-dependent mitigation of the fluoride-mediated suppression of antioxidant enzymes. In conclusion, gallic acid prevented the NaF-induced abnormalities in the serum and hepatic biochemical markers.

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1. Introduction

Fluorinated compounds are largely used all over the world to prevent dental caries and osteoporosis (Azarpazhooh and Main, 2008; Heaney, 1994). In the treatment of osteoporosis, fluorinated compounds such as sodium fluoride (NaF) are also shown to promote bone formation (Fordyce, 2011). The use of fluorinated water and mouth rinses is further seen as the most effective strategies for restriction of dental caries in developing countries (Azarpazhooh and Main, 2008). The large use of these products can lead some toxicological risks as fluoride intoxication is associated with severe damage to different tissues (Nabavi et al., 2012a,b). Depending on the concentration, duration of exposure and cellular type, fluoride intoxication has also shown to induce direct harmful effects on cellular functions (Chien et al., 2006).

Our previous studies demonstrated that fluoride-induced oxidative stress and hepatotoxicity are associated with an imbalance in the oxidant–antioxidant systems of hepatic tissues (Nabavi et al., 2012c). We also showed that fluoride intoxication can cause alterations in the serum markers of normal hepatic functions (Nabavi et al., 2012c). Other studies reported that reactive oxygen species (ROS) have an important role in the initiation and progression of oxidative stress and hepatic injuries induced by fluoride (Shanthakumari et al., 2004). In view of the potential role of antioxidants in reversing hepatotoxicity, several recent studies examined the ameliorative effects of natural antioxidants against experimentally induced oxidative stress (Shanthakumari et al., 2004).

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http://dx.doi.org/10.1016/j.indcrop.2012.10.024

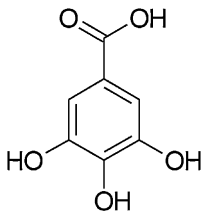

Fig. 1. Chemical structure of gallic acid.

Numerous scientific reports demonstrated that polyphenolic compounds of plant origin display different pharmacological activities that appear to correlate with their potent antioxidant effects (Eslami et al., 2010). Gallic acid (3,4,5-tryhydroxybenzoic acid, Fig. 1) and its derivatives comprise a large group of plant secondary metabolites and endogenous polyphenols (Lattanzio et al., 2009). They are usually found as methylated gallic acid derivatives or galloyl conjugates of catechin and other flavonoid compounds (Nabavi et al., 2012b). Gallnuts, tea, sumac, hazel nut, oak bark and other fruit and vegetable are rich sources of natural gallic acid derivatives (Nabavi et al., 2012b). Our recent studies on the chemistry and pharmacology of the edible plant, *Peltiphyllum peltatum* also revealed that this plant is packed full of gallic acid and various other antioxidants derivatives (Habtemariam, 2008, 2011). To date, numerous biological and pharmacological activity studies have documented that gallic acid (Fig. 1) derivatives possess antiviral, antifungal, anticancer and antioxidant properties (Zhao et al., 2011). Comprehensive evidence on the in vivo antioxidant potential of gallic acid need to be demonstrated. In the present study, we examined the protective role of gallic acid isolated from *P. peltatum* against NaF-induced oxidative stress in rat hepatic tissues. The results of the current study suggest that gallic acid can protect hepatic tissues from oxidative damages induced by NaF through modification of antioxidant enzymes activities, non-enzymatic antioxidant levels and improvement of hepatic function markers.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin and protein measurement kits were purchased from ZiestChem Company (Tehran, Iran). Experimental kits for serum biochemical measurement, glacial acetic acid, 5,5-dithiobis(2-nitrobenzoic acid), nitroblue tetrazolium chloride, heparin, reduced glutathione, potassium dihydrogen phosphate, NaF, silymarin, sodium dihydrogen phosphate, thiobarbituric acid, trichloroacetic acid and hydrogen peroxide were purchased from Sigma–Aldrich Chemical Company (St. Louis, USA). Other chemical reagents and solvents were of analytical grade.

2.2. Plant materials

P. peltatum Engl. grown for several years around a small stream bank in our medicinal garden (Hadlow College, Kent, UK) was used for the experiment. The plant material was collected in July 2009 and voucher specimen (PP2009HAD) was deposited in our Pharmacognosy Laboratory (School of Science) specimen collections for future references.

2.3. Gallic acid isolation

The leaves and rhizomes of *P. peltatum* were extracted separately by soaking the plant materials with absolute alcohol for two weeks (Habtemariam, 2008, 2011). Removal of the solvent under reduced pressure using rotary evaporator yielded the crude extract. Fractionation of the extract with solvents of increasing polarity was carried out as described previously (Habtemariam, 2008, 2011). Solvents of ascending polarity: namely, petroleum ether, chloroform, ethyl acetate, n-butanol and water were used. The ethyl acetate fraction which displayed the most antioxidant activity when assessed by 1,1-diphenyl-2-picryl hydrazyl radical scavenging activity test was taken for the isolation of gallic acid and other antioxidant compounds. RediSep Rf C18 Gold Column (50 g; Presearch, Hampshire, UK) attached to a Teledyne Isco flash chromatography system was used for large scale isolation of gallic acid. Briefly, an isocratic solvent system of 10% methanol in water was maintained for 5 min followed by a linear gradient obtained by raising the composition of methanol to 50% at 15 min. A constant flow rate of 40 ml/min was used throughout the system. The chromatogram was monitored by observing absorbance at dual wavelengths: 214 and 254 nm. Gallic acid was eluted within the first 5 min and repurified by using Sephadex LH-20 column as described previously (Habtemariam, 2008, 2011). Gallic acid isolated from the plant has been checked by HPLC and was of greater than 99% purity.

2.4. Animals

Experiments were performed on male Wistar rats (8–12-week-old, 200–250 g) which were purchased from Pasteur Institute (Amol, Iran). Animals were kept at $24 \pm 2\,^{\circ}C$ with 12/12 h light/dark cycle and $60 \pm 5\%$ humidity. Animals were fed on a standard laboratory pellet diet, water given ad labium and allowed to acclimatize for two weeks before used for experiments.

2.5. Ethical statement

All experiments were performed according to the instruction of the Principles of Laboratory Animals Care (NIH Publication No. 85-23, revised 1996) and under the norms of Ethical Committee of University of Mazandaran (Iran).

2.6. Study protocols

Rats were divided into five groups consisting of 10 animals per treatment group. Group I served as control group and was fed on standard diet for 14 days. Group II was given NaF at 600 ppm through drinking water for one week. Groups III, IV and V were intoxicated by NaF (600 ppm through drinking water for one week). These three groups were pretreated daily with gallic acid (10 and 20 mg/kg, intraperitoneally) or silymarin (10 mg/kg, intraperitoneally), respectively for a week prior to intoxication. After the last treatment, animals were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg). Hepatic tissues were removed and washed three times with normal saline and kept for tissue homogenate preparation. Hepatic tissues homogenate was prepared in potassium dihydrogen phosphate buffer (100 mM, pH 7.4) containing ethylenediaminetetraacetic acid and then centrifuged ($16{,}000 \times g$, 30 min, $4\,^{\circ}C$). The supernatant of samples were collected and kept at $-70\,^{\circ}C$ for biochemical testing.

2.7. Biochemical analysis

2.7.1. Protein determination

The protein content of test samples was analyzed according to the method of Bradford (1976) using bovine serum albumin as a reference standard.

2.7.2. Determination of lipid peroxidation products

Malonyldialdehyde as an end product of lipid peroxidation was measured through determination of the level of thiobarbituric acid

reactive substances (TBARs) in hepatic tissue homogenates following the method of Chandrasekara and Shahidi (2011). The reaction of thiobarbituric acid with malonyldialdehyde and other peroxidized lipid products, commonly known as "thiobarbituric acid reactive substances", generates a red chromophore that can be measured at 532 nm. All experiments were determined three times and peroxidation value was expressed as nM MDA equiv./g tissues.

2.7.3. Determination of superoxide dismutase activity

Superoxide dismutase activities of hepatic tissue homogenates were examined according to the method of Misra and Fridovich (1972). Sodium carbonate (2 ml, 50 mM), nitroblue tetrazolium (0.8 ml, 25 μm) and freshly prepared hydroxylamine hydrochloride (0.4 ml, 0.1 mM) were added to 0.1 ml of hepatic tissue homogenates (1:10, w/v). Superoxide dismutase activity was measured by measuring absorbance at 560 nm. The activity of superoxide dismutase is expressed as unit/mg protein.

2.7.4. Determination of catalase activity

The Pari and Latha (2004) method has been used for the determination of catalase activity. Each unit of catalase was considered as the amount of enzyme which quenches 1 μmol of hydrogen peroxide in 1 min.

2.7.5. Determination of reduced glutathione activity

The levels of tissue glutathione (GSH) was determined by the method of Ellman (1959) using trichloroacetic acid (5%) to precipitate hepatic tissue proteins and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) for color development. Absorbance of the reaction mixture was then record at 417 nm. All results were expressed as μg/mg protein tissue.

2.7.6. Serum biochemical parameters

Alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triacylglycerol, cholesterol, glucose, lipase, α-amylase, total bilirubin, direct bilirubin, total protein and albumin were determined in serum samples using commercially available kits.

2.8. Statistical analysis

Data are presented as means ± S.D. Statistical differences between group means were analyzed using a one-way analysis of variance followed by Duncan's multiple range tests. Results were considered statistically significant when $p < 0.05$.

3. Results

The levels of TBARs in hepatic tissue homogenates were summarized in Fig. 2. In comparison to untreated control groups, NaF intoxication increased the levels of TBARs of hepatic tissues by over 2-fold. Pretreatment with gallic acid reduced the hepatic TBARS levels in a dose-dependent manner (Fig. 2). Figs. 3 and 4 show the effect of NaF treatment on superoxide dismutase and catalase activities of hepatic tissue homogenates obtained from animals treated in the presence or absence of gallic acid. The results clearly revealed that NaF intoxication resulted in impairment of both enzyme activities when compared to the control group. Pretreatment of rats with gallic acid (10 and 20 mg/kg) restored in a dose-dependent manner the activity of the enzymes in hepatic tissues. The levels of reduced glutathione in rat hepatic tissues of all experimental groups were summarized in Fig. 5. It is evident that NaF exposure resulted in the reduction of reduced glutathione levels in hepatic tissues by about half. Gallic acid pretreatment resulted in an increase in the levels of reduced glutathione level. For comparison purposes, the activity of the well known hepatoprotective agent, silymarin, was tested at the dose of 10 mg/kg. As shown in Figs. 2–5, gallic acid at the dose of 20 mg/kg was a better hepatoprotective agent when compared with silymarin results in the measurement of TBARS, catalase and superoxide dismutase assays while similar response was obtained in reduced glutathione measurement. In order to gain further insight into the hepatoprotective activity of gallic acid, the serum levels of hepatic function markers in all treatment groups were measured (Table 1). According to these results, one week intoxication with NaF (600 ppm through drinking water) induced significant changes in serum hepatic function markers. The overall trend was that gallic acid and silymarin mitigate these deleterious changes in a dose-dependent manner (Table 1). Gallic acid at 20 mg/kg significantly restores the serum activities of ALT, AST and ALP and the levels of triacylglycerol, cholesterol, glucose, lipase, α-amylase, total bilirubin, direct bilirubin, total protein and albumin ($p > 0.05$ vs. control).

4. Discussion

ROS such as hydrogen peroxide, hydroxyl radical and superoxide anion play crucial role in initiation and progression of chemical-induced intoxications and/or oxidative stress (Babior, 1992). Under oxidative stress disease conditions such as cardiovascular, diabetes and neurodegenerative disorders, ROS and reactive nitrogenous species (RNS) directly participate in the pathogenesis of these diseases (Mates et al., 1999). Our body is well equipped with various antioxidant enzyme systems such as superoxide dismutase, catalase and non-enzymatic antioxidants such as reduced glutathione (Gul et al., 2000). The generation of high levels of ROS and RNS or any disturbance in the oxidant–antioxidant status can result in oxidative damage to molecules (DNA, proteins and lipids), tissues or organs (Chandrasekara and Shahidi, 2011).

Gallic acid (Fig. 1) is a plant polyphenolic compound that can exist in free or large polymeric forms. Gallic acid is largely found in different foods such as tea leaves, grapes and gallnuts (Nabavi et al., 2012b). Different biological activities including antimutagenic, anti-viral and antioxidant activities were reported for gallic acid (Zhao et al., 2011). The hepatoprotective activity of gallic acid against paracetamol (Rasool et al., 2010) and carbon tetrachloride-induced toxicity (Tung et al., 2009) has also been reported previously. It is also well known that gallic acid, as a natural originated polyphenolic antioxidant agent, possesses scavenging effect against ROS and RNS (Stupans et al., 2002). In the present study, the hepatoprotective activity of gallic acid extracted from *P. peltatum* was studied in the model of NaF-induced hepatotoxicity. We have previously shown that pretreatment of animals with antioxidants could mitigate the NaF-induced hepatotoxicity, probably through scavenging of ROS and/or suppressing oxidative stress (Nabavi et al., 2012c). In the present study, the hepatoprotective effect of gallic acid against NaF-induced hepatotoxicity is demonstrated for the first time. It has been suggested by various authors that the phenolic hydroxyl groups in gallic acid structure are responsible for its ability to suppress oxidative stress (Lu et al., 2006). Phenolic compounds can easily lose a proton through homolytic cleavage of the O—H bond and form stabilized phenoxy radical (Andersson et al., 1996). Phenolic compounds such as gallic acid have two or more hydroxyl groups moieties in their chemical structural which can further lead to even a more stabilized phenoxyl radical (Andersson et al., 1996). Hydroxyl radicals are therefore essential for proton donating and the antioxidant ability of gallic acid (Andersson et al., 1996). In the current study, intraperitoneal administration of gallic acid prior to NaF intoxication prevented hepatotoxicity and oxidative stress. Lipid peroxidation is the primary common marker of oxidative stress and is generally expressed in tissues as TBARS. TBARs such as

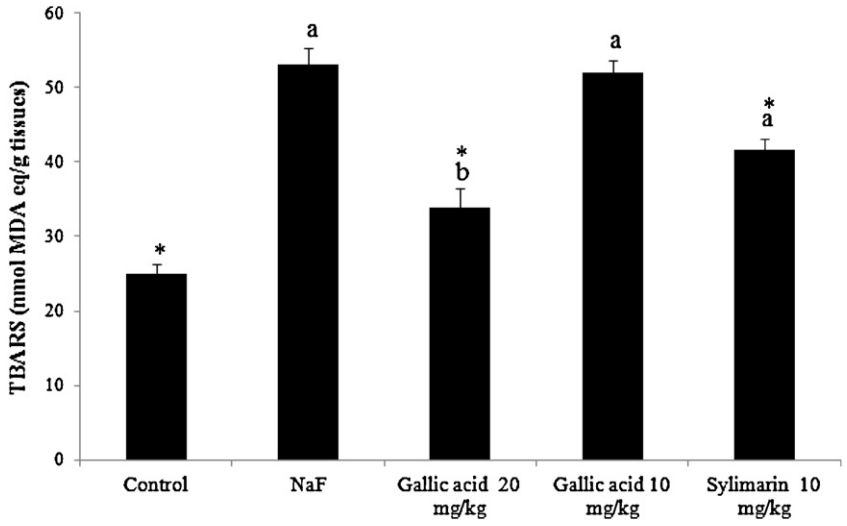

Fig. 2. Thiobarbituric acid reactive substances (TBARS) levels in rat liver homogenates. Data are mean ± S.D. values ($n = 10$). [a] $p < 0.001$ versus control group. [b] $p < 0.01$ versus control group. * $p < 0.001$ versus NaF group.

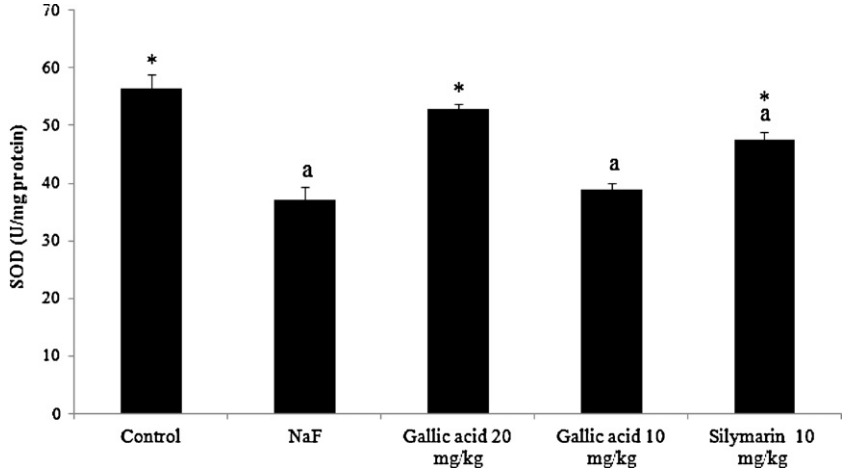

Fig. 3. Superoxide dismutase activity in rat liver homogenates. Data are mean ± S.D. values ($n = 10$). [a] $p < 0.001$ versus control group. * $p < 0.001$ versus NaF group.

MDA are end products of polyunsaturated fatty acids oxidation and are used as index of lipid peroxidation (Pamplona, 2011). The present study revealed that one week of intoxication of rats by NaF significantly increase TBARS levels in hepatic tissue when compared with control group. This increased level of lipid peroxidation is known to be mediated by iron ions that generate hydroxyl radical via the Fenton-type reactions (Kokilavani et al., 2005). As an endogenous antioxidant defense system, glutathione

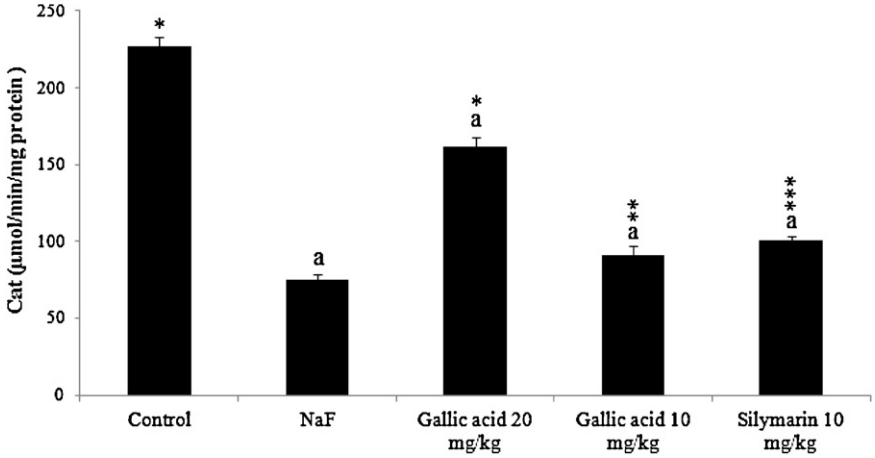

Fig. 4. Catalase activity in rat liver homogenates. Data are mean ± S.D. values ($n = 10$). [a] $p < 0.001$ control group. * $p < 0.001$ versus NaF group. ** $p < 0.05$ versus NaF group. *** $p < 0.01$ versus NaF group.

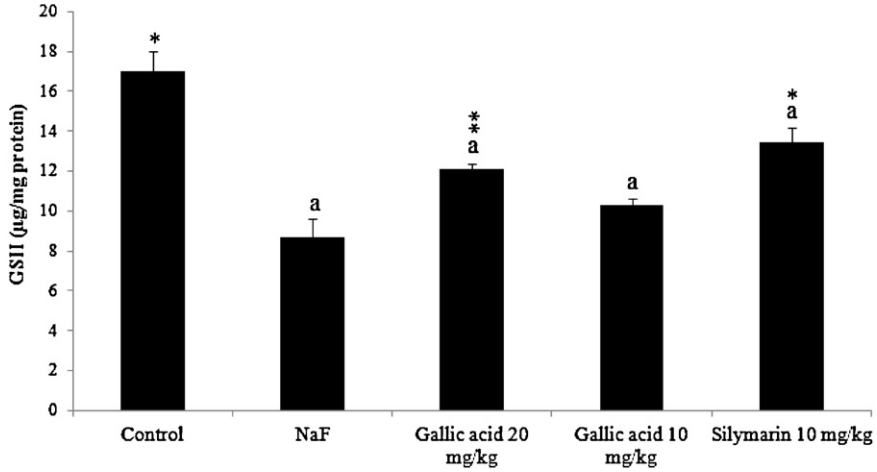

Fig. 5. Levels of reduced glutathione in rat liver homogenates. Data are mean ± S.D. values ($n = 10$). [a] $p < 0.001$ control group. * $p < 0.001$ versus NaF group. ** $p < 0.01$ versus NaF group.

Table 1
Effect of gallic acid on serum biochemical parameters in sodium fluoride induced hepatotoxicity in rat.

Biochemical parameters	Normal untreated control	NaF treated control	NaF and gallic acid (10 mg/kg i.p.)	NaF and gallic acid (20 mg/kg i.p.)	NaF and silymarin (10 mg/kg i.p.)
ALP (U/L)	189.0 ± 10.1	665.0 ± 12.6****	463.5 ± 6.3***	266.3 ± 11.3*	345.0 ± 18*
AST (U/L)	232.8 ± 8.9	488.4 ± 10.5****	378.8 ± 9.2***	270.5 ± 9.2*	303.0 ± 7.8*
ALT (U/L)	9.2 ± 1.0	38.5 ± 1.1***	36.1 ± 1.6***	15.1 ± 1.0*	31.5 ± 1.4***
Triacylglycerol (mg/dL)	142.8 ± 13.7	275.3 ± 16.5****	243.2 ± 10.5****	168.8 ± 11.4*	223.2 ± 11.9***
Total bilirubin (mg/dL)	2.5 ± 0.3	8.5 ± 0.8****	7.1 ± 0.5****	3.7 ± 0.2*	5.8 ± 0.55***
Direct bilirubin (mg/dL)	1.1 ± 0.1	4.2 ± 0.2****	1.9 ± 0.2**	1.4 ± 0.3*	2.0 ± 0.2**
Total protein (g/dL)	12.0 ± 0.2	7.1 ± 0.3****	10.4 ± 0.4**	12.7 ± 0.2*	10.1 ± 0.3**
Albumin (g/dL)	6.6 ± 0.2	2.2 ± 0.3****	6.1 ± 0.2*	7.1 ± 0.3*	4.3 ± 0.2*
Glucose (mg/dL)	46.5 ± 1.9	90.1 ± 2.5****	58.3 ± 2.0*	39.0 ± 1.5*	65.6 ± 2.0*
Lipase (U/L)	131.5 ± 0.9	106.8 ± 5.7**	126.7 ± 5.3*	147.7 ± 4.8*	123.6 ± 6.8*
α-Amylase (U/L)	3132.8 ± 53.4	1914.8 ± 17.6****	2658.8 ± 86.2**	3132.6 ± 42.2*	2301.0 ± 65.0****
Cholesterol (mg/dL)	64.0 ± 3.7	159.1 ± 7.4****	129.3 ± 1.2****	86.0 ± 7.0*	103.5 ± 5.5***

Values are mean ± SE ($n = 10$). Data for normal animals are considered as base-line data; there was no significant base-line difference between the groups.
* $p > 0.05$ versus normal.
** $p < 0.05$ versus normal.
*** $p < 0.01$ versus normal.
**** $p < 0.001$ versus normal.

plays a crucial role through its catalytic role in disulfide exchange reactions (Nabavi et al., 2012c). Under oxidative stress conditions, glutathione is oxidized to make disulphide link to form oxidized glutathione. After fluoride intoxication, significant depletion of the reduced glutathione level was evidenced in rat liver, corroborating the state of oxidative stress (Ghosh et al., 2011). Pretreatment with gallic acid prevented the NaF-induced depletion of glutathione. In addition to the thiol based antioxidants, the cellular antioxidant defense system also comprised with enzymatic antioxidants such as superoxide dismutase and catalase. NaF intoxication caused ROS/RNS accumulation in hepatic tissue that could lead to a decrease in superoxide dismutase activity. Fluoride intoxication also decreased catalase activity probably through reducing the level of nicotinamide adenine dinucleotide phosphate required for catalase activation process (Kirkman and Gaetani, 1984). Furthermore, fluoride-induced hepatic damage is likely to disturb tissue transport function and membrane permeability that results in enzymes leakage (e.g., ALT, AST and ALP) from hepatic cells. This process is usually associated with the alteration of many other serum parameters such as bilirubin, albumin, glucose and cholesterol levels (Tung et al., 2009). Our observation of significant release of alkaline phosphatase, AST, alanine transaminase as well as change in other serum parameter was indicative of severe NaF-induced damage to hepatic tissue membranes (Tung et al., 2009). These classical hepatotoxicity markers together with the depletion of enzymatic antioxidant activity after one week fluoride intoxication (600 ppm) through drinking water were in agreement with the induction of oxidative stress-induced hepatotoxicity. Intraperitoneal administration of gallic acid (10 and 20 mg/kg) prior to fluoride intoxication normalized the serum parameters and NaF-induced alteration of enzymatic antioxidant defenses, level of reduced glutathione as well as lipid peroxidation in hepatic tissue.

5. Conclusion

We conclude that intraperitoneal administration of gallic acid isolated from *P. peltatum* before NaF exposure mitigates the hepatotoxicity and the instauration of oxidative stress in rat hepatic tissues. The results of the present study revealed that *P. peltatum* and other herbal sources of gallic acid can be used for the protection of hepatic tissues against fluoride-induced oxidative stress. Future studies should be directed on the mechanism of hepatoprotective action of gallic acid.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

This study was partly supported by the research grant of University of Greenwich (UK) and National Elite's Foundation of Iran (Iran) for this study. We also dedicate this paper Mrs. Seyed Maryam Nabavi and to memory of Mr. Seyed Ali Asghar Nabavi.

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