

***IN VIVO* PROTECTIVE EFFECTS OF GALLIC ACID ISOLATED FROM *PELTIPHYLLUM PELTATUM* AGAINST SODIUM FLUORIDE-INDUCED OXIDATIVE STRESS IN RAT ERYTHROCYTES**

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Gallic acid has been identified as an antioxidant component of the edible and medicinal plant *Peltiphyllum peltatum*. The present study examined its potential protective role against sodium fluoride (NaF)-induced oxidative stress in rat erythrocytes. Oxidative stress was induced by NaF administration through drinking water (1030.675 mg m⁻³ for one week). Gallic acid at 10 mg kg⁻¹ and 20 mg kg⁻¹ and vitamin C for positive controls (10 mg kg⁻¹) were administered daily intraperitoneally for one week prior to NaF administration. Thiobarbituric acid reactive substances, antioxidant enzyme activities (superoxide dismutase and catalase), and the level of reduced glutathione were evaluated in rat erythrocytes. Lipid peroxidation in NaF-exposed rats significantly increased (by 88.8 %) when compared to the control group ($p < 0.05$). Pre-treatment with gallic acid suppressed lipid peroxidation in erythrocytes in a dose-dependent manner. Catalase and superoxide dismutase enzyme activities and glutathione levels were reduced by NaF intoxication by 54.4 %, 63.69 %, and 42 % ($p < 0.001$; vs. untreated control group), respectively. Pre-treatment with gallic acid or vitamin C significantly attenuated the deleterious effects. Gallic acid isolated from *Peltiphyllum peltatum* and vitamin C mitigated the NaF-induced oxidative stress in rat erythrocytes.

KEY WORDS: *antioxidant, catalase, haemolysates, reduced glutathione, superoxide dismutase*

Fluoride and fluorinated compounds occur in water, foods, drugs, air (due to gaseous industrial waste), and are widely used as ingredients of toothpaste and agrochemical products (1). Acute high-level exposure to fluoride is rare and usually results from accidental contamination of drinking water. Moderate-level

chronic exposure (above 1.5 mg L⁻¹, the WHO guideline value for fluoride in water) is more common. It is well-known that chronic long-term exposure to high levels (both the Maximum Contaminant Level and Maximum Contaminant Level Goal for fluoride equal 4 mg L⁻¹) of fluoride leads to dental and skeletal

fluorosis (2). Since numerous scientific reports have documented fluoride-induced toxicity in living systems (3-5), the ever-increasing volume of fluoride-polluted drinking water has been one of the main sources of public concern (3). Recent studies have suggested that fluoride intoxication leads to the down-regulation of antioxidant enzymes (3), an increase in relative oxygen species (ROS), and oxidative stress (4). The prooxidant-antioxidant imbalance caused by fluoride intoxication may lead to multi-organ dysfunctions (5). Excessive ROS production and/or diminished antioxidant defences have been implicated in cancer, diabetes, and cardiovascular diseases (6-8).

Over the last few decades, various laboratories worldwide have been in search of novel antioxidant compounds that could be used as nutritional and/or therapeutic agents. In our laboratory studies, one plant demonstrated increased antioxidant potential: *Peltiphyllum peltatum* (Torr.) Engl. [Synonyms - *Darmera peltata* (Torr.) Voss; *Saxifraga peltata* (Torr. ex Benth.)], which belongs to the Saxifragaceae family (9). This plant is cultivated in Europe for its distinct ornamental features and is commonly known in many localities as Indian Rhubarb or Umbrella Plant. It is known to be edible and is used for medicinal purposes (10, 11). The extracts of its leaves possess potent radical scavenging effects, protect biological macromolecular molecules (e.g., DNA) from oxidative damage, and protect cultured cells from hydrogen peroxide (H₂O₂)-induced cell death (9). Subsequent studies on this plant revealed active antioxidant components such as gallic acid and a number of other related polyphenols (12, 13). The comparative antioxidant and prooxidant effects of gallic acid and the related compounds of *P. peltatum* have been assessed *in vitro* (12). Gallic acid is a ubiquitous natural product with various industrial applications including ink dyes, tanning products, and paper (14). Recent studies have documented that gallic acid and its esters [e.g., (-)-*epi*-gallo catechin-3-gallate] exert antioxidant, anticancer, antiviral, and many other biological effects (15-17). The cytoprotective properties of gallic acid *in vitro* have also been observed previously (18, 19).

In a previous study (20), we introduced a rat model of fluoride-induced oxidative damage. In order to continue our work on *P. peltatum* and validate the therapeutic potential of gallic acid from natural sources, the present study was designed to study the *in vivo* protective effect of gallic acid against oxidative damages in rat erythrocytes.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin and protein quantification kits were purchased from ZiestChem Company (Tehran, Iran). Glacial acetic acid, 5,5-dithiobis(2-nitrobenzoic acid), heparin, nitro blue tetrazolium chloride, potassium dihydrogen phosphate, reduced glutathione, sodium dihydrogen phosphate, sodium fluoride, trichloroacetic acid, thiobarbituric acid, hydrogen peroxide were purchased from Sigma-Aldrich chemical company (St Louis, MO, USA). All chemical reagents were of analytical grade.

Isolation of gallic acid

As a source for gallic acid, we used *Peltiphyllum peltatum* (Torr.), grown in our medicinal gardens. The isolation and identification of gallic acid from this plant has been described previously (12, 13).

Animals

The experiments were performed on 50 male Wistar rats (8 to 12 week-old, weight 200 g to 250 g). Animal housing was maintained at (24±2) °C, (60±5) % humidity, and a 12 h light/12 h dark cycle. The animals were fed on a standard laboratory pellet diet purchased from the Pasture Institute (Tehran, Iran) and water was given *ad libitum*. The animals were allowed to adapt for two weeks before being used for experiments. All experiments were performed following guidelines from the "Principles of Laboratory Animals Care" (NIH Publication no. 85-23, revised 1996) (21). All efforts were made to minimize animal suffering and reduce the number of animals used.

Study protocols

The rats were randomly divided into five groups of 10 animals each. The control group received isotonic saline solution. One group received sodium fluoride (NaF) at a dose of 1030.675 mg m⁻³ through drinking water for 7 days as described previously (1). The other three experimental groups were treated with gallic acid (10 mg kg⁻¹ and 20 mg kg⁻¹ body mass) or vitamin C (10 mg kg⁻¹ body mass) intraperitoneally for 7 days prior to NaF intoxication. The doses of the tested substances were based on a previous *in vivo* study (1). The rats were anaesthetised with ketamine (60 mg kg⁻¹) and xylazine (5 mg kg⁻¹), sacrificed 24 h after the final dose of NaF, and the blood samples were

collected from cardiac punctures into heparinized tubes.

Preparation of haemolysates

Blood samples were centrifuged at 1000 g for 15 min. After removing the upper layer, the packed erythrocytes were washed with 0.01 mol L⁻¹ of phosphate buffer saline (pH 7.4), and lysed by hypotonic phosphate buffer (0.01 mol L⁻¹). Cell debris was removed through centrifugation at 3000g for 15 min. Blood haemolysates were collected and immediately used for biochemical analysis.

Protein content analysis

Bradford method (22) was used for total protein determination, while the Drabkin & Austin method (23) was used to evaluate haemoglobin content.

Measurement of erythrocytes lipid peroxidation

The level of lipid peroxidation was assessed by measuring the levels of thiobarbituric acid reactive substance (TBARS) formation (24). Briefly, erythrocyte homogenates containing 1 mg protein were mixed with trichloroacetic acid (1 mL, 20 %) and thiobarbituric acid (2 mL, 0.67 %). Reaction mixtures in microcentrifuge tubes were incubated for 1 h at 100 °C. After cooling and removal of precipitate by centrifugation (1000g for 15 min), absorbance was measured at 535 nm using appropriate blank controls. The extinction coefficient of malondialdehyde (MDA) was used for calculating the extent of lipid peroxidation.

Measurement of superoxide dismutase activity

The superoxide dismutase (SOD) activity of erythrocyte haemolysates was assessed by using method of Paoletti et al. (25). In this nitroblue tetrazolium-based assay, one unit of SOD activity is defined as the enzyme level required in inhibiting chromogen generation by 50 %.

Measurement of catalase activity

The enzymatic activity of erythrocyte lysates was examined following the method of Pari & Latha (26). This assay is based on measuring catalase activity in transforming hydrogen peroxide (H₂O₂) into water. Briefly, erythrocyte homogenates (5 µg protein) were mixed with H₂O₂ (2.1 mL, 7.5 mmol L⁻¹) and enzymatic activity was monitored over 10 min using

UV absorbance at 25 °C. One unit of catalase activity was defined as the amount of enzyme that reduced 1 µmol of H₂O₂ per minute.

Measurement of reduced glutathione (GSH) levels

GSH levels were measured using Ellman's colorimetric method (27). Trichloroacetic acid was added to erythrocyte haemolysates (containing 36 µg protein) to precipitate the proteins. After removal of proteins by centrifugation (12000g, 5 min), the supernatants containing GSH were mixed with 5, 5-dithiobis 2-nitrobenzoic acid solution (Sigma-Aldrich chemical company, St Louis, MO, USA). Absorbances at 417 nm were measured for samples and GSH levels were calculated from standard GSH calibration curves.

Statistical analysis

Results are presented as mean±SD. Statistical analysis was carried out using a statistical package (SPSS 17.0 for Windows®). Differences between group means were estimated using one-way analysis of variance followed by Duncan's multiple range tests. Results were considered statistically significant when $p < 0.05$ or $p < 0.01$.

RESULTS AND DISCUSSION

In order to obtain deeper insight into the therapeutic potential of gallic acid (and its source plant *P. peltatum*), the present study was designed to evaluate the *in vivo* antioxidant effects of the compound by using a rat model of NaF-intoxication.

The results have confirmed our working hypothesis that gallic acid extracted from *P. peltatum* is capable of protecting rat erythrocytes against NaF-induced oxidative damage. Through comparison with known antioxidant positive controls (vitamin C), the positive effect of gallic acid on NaF-mediated lipid peroxidation, as well as on the level of antioxidant defences (superoxide dismutase, catalase and reduced glutathione), was proven. The extent of lipid peroxidation, expressed as TBARS levels in the erythrocytes of the treated groups and untreated controls, is shown in Figure 1. Exposure to NaF significantly increased the TBARS levels when compared to the control group ($p < 0.001$). Pre-treatment with gallic acid (10 mg kg⁻¹ and 20 mg kg⁻¹ body mass) prior to NaF exposure resulted in a dose-

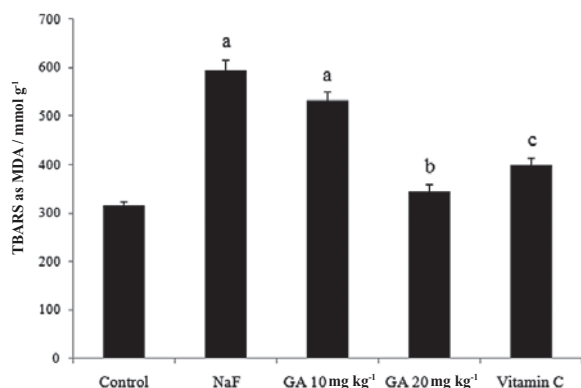


Figure 1 Effects of gallic acid and vitamin C on TBARS levels in erythrocytes of sodium fluoride-intoxicated rats. Data are mean±SD values (n=10); ^a p<0.001 vs. control group, ^b p>0.05 vs. control group, ^c p<0.01 vs. control group.

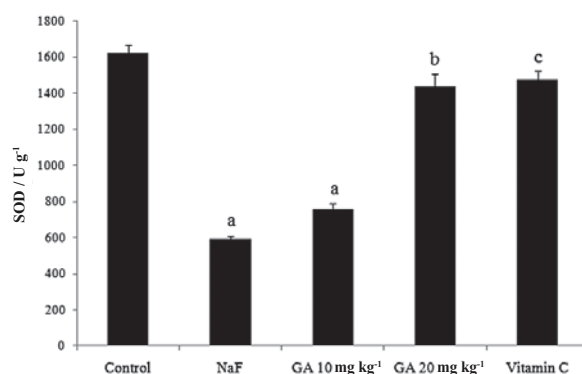


Figure 2 Effects of gallic acid and vitamin C on SOD activity in erythrocytes of sodium fluoride-intoxicated rats. Data are mean±SD values (n=10); ^a p<0.001 vs. control group, ^b p<0.01 vs. control group, ^c p<0.05 vs. control group.

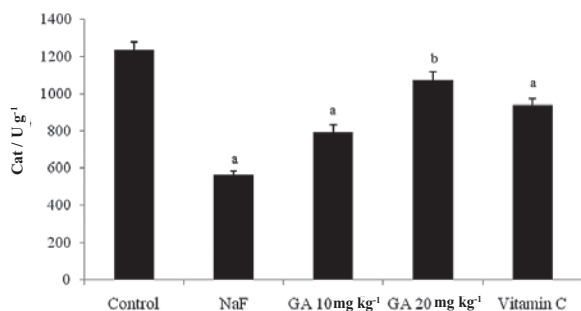


Figure 3 Effects of gallic acid and vitamin C on catalase activity in erythrocytes of sodium fluoride-intoxicated rats. Data are mean±SD values (n=10); ^a p<0.001 vs. control group, ^b p<0.01 vs. control group, ^c p<0.05 vs. control group.

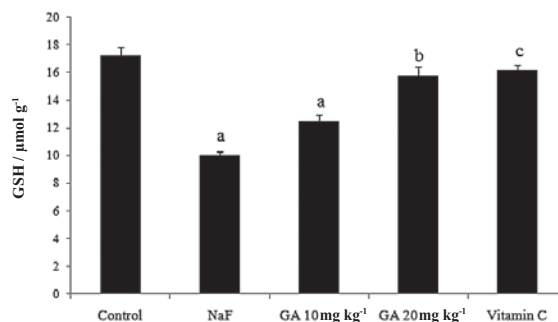


Figure 4 Effects of gallic acid and vitamin C on GSH levels in erythrocytes of sodium fluoride-intoxicated rats. Data are mean±SD values (n=10); ^a p<0.001 vs. control group, ^b p<0.01 vs. control group, ^c p<0.05 vs. control group.

dependent reduction of TBARS levels in rat erythrocytes. At 20 mg kg⁻¹ body mass, gallic acid completely reversed the NaF-induced increase in TBARS levels ($p<0.05$ vs. control group). The positive control, vitamin C, tested at 10 mg kg⁻¹ also ameliorated the NaF-induced rise in TBARS levels (Figure 1).

As shown in Figure 2 and 3, the activity profiles of rat erythrocyte superoxide dismutase and catalase were very similar. The two enzyme activities in erythrocytes significantly ($p<0.05$) decreased in NaF-exposed rats when compared to untreated control rats. Rats pre-treated with gallic acid appeared to have erythrocytes with higher levels of enzyme activity than the NaF-treated groups (Figure 2 and 3). At 20 mg kg⁻¹, the protective role of gallic acid on SOD activity was comparable to ascorbic acid, whereas the

effect of gallic acid on catalase activity was more pronounced than for vitamin C (Figure 2 and 3).

GSH levels in rat erythrocytes from the NaF-treated group were significantly lower than in the untreated control groups (Figure 4). Administration of gallic acid before NaF intoxication mitigated the reduced GSH levels in a dose dependent manner. As with the remaining antioxidant parameters studied, the positive control vitamin C was also able to reverse the *in vivo* prooxidative effect of NaF (Figure 4).

Previous reports on the protective effects of gallic acid are ambiguous. Hsieh et al. (28) found that, under *in vitro* conditions, gallic acid protected human erythrocytes from oxidative damage. In contradiction to this finding, Ximenes et al. (29) suggested that gallic acid is prooxidative in cellular systems and could

exacerbate the drug-induced prooxidative damage in human erythrocytes. The prooxidative effect of gallic acid at the molecular level (30) and oxidative-based induction of cell death in cancer cells (31, 32) have also been documented. Furthermore, the *in vitro* induction of cancer cells through the generation of ROS has also been linked to the depletion of antioxidant defences (32). Previous studies have also suggested that fluoride intoxication leads to excessive ROS generation, and inhibition of endogenous antioxidant enzymatic activities (33), increased lipid peroxidation, and cell injury (34).

Results of the present study are in good agreement with previous reports and confirm that NaF administration increased levels of lipid peroxidation and reduced SOD and catalase activities. Furthermore, we found that glutathione levels in erythrocytes diminished after NaF exposure, suggesting an induction of oxidative stress. These findings, together with the results obtained for the positive control, vitamin C, were in agreement with our previous study (20) where *in vivo* amelioration of NaF-mediated oxidative stress was recorded in curcumin pre-treated rats. Our finding that gallic acid effectively protected rat erythrocytes suggests that the antioxidant effect of the compound at the tested dosage *in vivo* was more prevalent than its prooxidative effects. The ameliorative effect of gallic acid on lipid peroxidation *in vivo* was also in agreement with other studies (35, 36, 37, 38). Although Li et al. (36) revealed the potential of gallic acid in increasing the GSH/oxidized glutathione (GSSG) ratio in senescence-accelerated mice, our result appears to be the first evidence on the beneficial effect of gallic acid on *in vivo* NaF-mediated oxidative stress in rat erythrocytes.

CONCLUSION

The use of fluorine in common everyday products such as toothpaste, drugs, and agrochemicals implies that unwanted side effects are practically inevitable. Our finding that gallic acid mitigates such adverse effects on rat erythrocytes underscored its protective or therapeutic potential. However, more studies are needed to investigate the molecular mechanism by which the *in vivo* protective effect of gallic acid against NaF intoxication is exerted (e.g., the up-regulation of microRNAs). Also, future studies should focus on the possible effect of other *P. peltatum* active compounds and their pharmacokinetics.

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Sažetak

ZAŠTITNI UČINCI GALNE KISELINE IZDVOJENE IZ *PELTIPHYLLUM PELTATUM* NA RAZINU OKSIDATIVNOG STRESA IZAZVANOG NATRIJEVIM FLUORIDOM U ERITROCITIMA ŠTAKORA

Galna kiselina u ranijim je istraživanjima potvrđena kao važna antioksidativna sastavnica jestive i ljekovite biljke *Peltiphyllum peltatum*. U ovome istraživanju ispitan je njen potencijalni zaštitni učinak protiv oksidativnog stresa izazvanog natrijevim fluoridom (NaF) u eritrocitima štakora. Oksidativni stres izazvan je davanjem NaF eksperimentalnim životinjama putem vode za piće ($1030,675 \text{ mg m}^{-3}$) tijekom jednog tjedna. Galna kiselina životinjama je davana intraperitonealno u dozama 10 mg kg^{-1} i 20 mg kg^{-1} na dan. Životinje iz pozitivno kontrolne skupine svaki su dan, osim NaF, intraperitonealno primale vitamin C u dozi 10 mg kg^{-1} . U eritrocitima štakora primjenom TBARS-testa izmjerene su razine lipidne peroksidacije, aktivnosti antioksidativnih enzima (superoksid dismutaze i katalaze) te razina reduciranoga glutaciona. Lipidna peroksidacija u štakora izloženih NaF osjetno se povećala (88,8 %) u usporedbi s kontrolnom skupinom ($p < 0,05$). Prethodni tretman galnom kiselinom smanjio je razinu lipidne peroksidacije u eritrocitima, ovisno o dozi. Toksičnost NaF smanjila je aktivnost enzima katalaze i superoksid dismutaze i razine glutaciona za 54,4 %, 63,69 %, odnosno 42 % ($p < 0,001$; u usporedbi s kontrolnom skupinom). Dobiveni rezultati upućuju na pozitivne učinke predobrade štakora galnom kiselinom izoliranom iz *Peltiphyllum peltatum* i vitaminom C na razinu oksidativnog stresa izazvanog natrijevim fluoridom u eritrocitima štakora.

KLJUČNE RIJEČI: *antioksidansi, hemolizati, katalaza, reducirani glutation, superoksid dismutaza*

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