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Oleuropein prevents ethanol-induced gastric ulcers via elevation of antioxidant enzyme activities in rats

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Abstract Purified oleuropein from olive leaf extract has been shown to have antioxidant effects in our recent studies. Thus, the aim of this study was to assess the antioxidant abilities of oleuropein in comparison with ranitidine in ethanol-induced gastric damages via evaluation of ulcer index inhibition, antioxidant enzyme activities, and lipid peroxidation level. Fifty-six adult male Sprague–Dawley rats were divided into seven equal groups as follows: control group, ethanol group (absolute ethanol 1 ml/rat), oleuropein group (12 mg/kg), and oleuropein (6, 12, and 18 mg/kg) plus ethanol groups, as well as ranitidine (50 mg/kg) plus ethanol group. Pretreatment with oleuropein (12 and 18 mg/kg) significantly increased the ulcer index inhibition (percent), in comparison with oleuropein (6 mg/kg). Glutathione peroxidase (GPx) activity was significantly lower in the ethanol group when compared with the other groups whereas, treatment of rats with oleuropein (12 mg/kg)
significantly increased glutathione content in gastric tissue when compared with the other groups, and lipid peroxidation was significantly reduced in the oleuropein-(12 and 18 mg/kg) and ranitidine-treated animals. Superoxide dismutase (SOD) and catalase (CAT) activities were both much higher in oleuropein-treated rats than the ethanol group, and although there was a moderate increase in SOD and CAT activities in ranitidine-treated rats, the differences were not significant. These findings suggest that oleuropein has beneficial antioxidant properties against ethanol-induced gastric damages in the rat. Therefore, it seems that a combination regimen including both antioxidant and antisecretory drugs may be beneficial in prevention of ethanol-mediated gastric mucosal damages.

Keywords Oleuropein · Antioxidant enzymes · Gastric ulcer · Ethanol

Introduction

During the last decade, a number of studies have focused attention on the crucial role of non-vitamin dietary antioxidants such as polyphenols [1, 3, 30–32]. In this context, phenolic compounds in olives, olive oil, and olive tree leaves have shown antioxidant and anti-inflammatory properties, prevent lipoperoxidation, induce favorable changes of lipid profile, improve endothelial function, and disclose antithrombotic properties [3, 30]. The major constituent of the olive leaves is oleuropein. This phenolic compound is responsible for the bitter taste and pungent aroma of olive derivatives [3, 15] and has been recognized as a powerful hypotensive, hypoglycemic, and antioxidant agent [3, 19, 30, 43, 47]. Oleuropein scavenges superoxide anions and hydroxyl radicals and inhibits the respiratory burst of neutrophils and hypochlorous acid-derived radicals, subsequently inhibiting oxidative stress [3, 9, 45].

The role of oxygen-derived free radicals in the development of pathogenesis in acute experimental gastric lesions induced by ethanol and non-steroidal anti-inflammatory drugs such as indomethacin is well known [7, 11, 13, 20–22]. In this regard, ethanol has been indicated as an oxidative agent in our recent studies [2, 3]. The pathogenesis of ethanol-induced gastric lesions includes the generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radical, which are the major causative factors for mucosal lesions through oxidative damage [8]. Lipid peroxidation, an important parameter for ROS-induced oxidative damage of membrane, is increased in gastric lesions caused by ethanol [8, 16] and plays an important role by impairment of antioxidant enzyme activities [12, 16]. ROS induce oxidative damage in membrane lipids and proteins and deplete glutathione level in gastric ulcers [8]. Oxygen-derived free radicals play an important role in the pathogenesis of digestive damages [27, 38]. Moreover, the involvement of oxygen-derived free radicals is well established in the pathogenesis of mucosal damage caused by indomethacin, ethanol, and other agents besides the inhibition of cyclooxygenase (COX) enzymes [12, 18, 33, 35, 41, 46]. In this regard, ethanol inhibits the synthesis of cytoprotective prostaglandins, produced by COX-1 and COX-2 in the stomach tissue [14, 35]. Indeed, “on one hand, ethanol administration reduces mucus production, gastric mucosal blood flow, bicarbonate secretion, endogenous glutathione and prostaglandin levels, and on the other hand it increases the release of histamine, the influx of calcium ions, the generation of free radicals and the production of leukotrienes” [13]. Therefore, ethanol as an oxidative inducing agent contributes to the development of ulcers.

To the best of our knowledge, no unifying concept has developed yet on the mechanism of gastric mucosal damages by various ulcerogens, and in spite of the number of studies published on olive leaf extract and the effects of its constituents, none has focused on the influence of oleuropein on the gastric tissue. We have recently demonstrated oleuropein antioxidant activity against ethanol-induced oxidative stress in the rat testis [3] and liver (unpublished data). Therefore, the protective effects of oleuropein (as a natural antioxidant agent) was investigated on the gastric mucosal damages induced by ethanol in the present study. We also investigated how antioxidant enzyme activities and lipid peroxidation level of gastric tissue varied with fasting and oleuropein therapy in rats.

Materials and methods

Chemicals and kits

Ethanol (99.5 %) and thiobarbituric acid (TBA) were prepared from Merck Chemical Company (KGaA, Darmstadt, Germany). GPx and superoxide dismutase
SOD kits were obtained via Randox® Company (Antrim, UK). Ranitidine tablets were prepared from Chimidar® Pharmaceutical Company (Tehran, Iran), and oleuropein was purified in our laboratory (94 % purity) as described previously [3]. Ranitidine and oleuropein dissolved in distilled water before administration. Other chemicals used were of analytical grade.

Animals

A total of 56 adult male Sprague–Dawley rats (weighing 180–220 g, supplied from Shiraz University of Medical Sciences, Animal House Center, Iran) were housed in temperature-controlled conditions under a 12:12-h light/dark photocycle with food and tap water supplied ad libitum. All rats were treated humanely and in compliance with the recommendations of Animal Care Committee for the Lorestan University of Medical Sciences (Khorram Abad, Iran; approval number: S 16. 10. 89). All of experimental procedures were carried out between 10.00–12.00 AM and all of the treatment applied orally by gavage.

Experimental design

The rats were divided into seven equal groups (eight rats per group) as follows: control group (normal control), ethanol group (negative control), oleuropein group, and oleuropein plus ethanol groups, as well as ranitidine plus ethanol group (positive control). While control and ethanol groups received 1 ml distilled water, the oleuropein group received oleuropein (12 mg/kg body weight, BW), and oleuropein plus ethanol groups received oleuropein at the dosages of 6, 12, and 18 mg/kg BW for ten consecutive days. Thereafter, all the groups were kept fasting for overnight (18 h) but had free access to water. Absolute ethanol (1 ml/rat) was then given to negative control (ethanol group) and oleuropein plus ethanol groups. The positive control group (ranitidine plus ethanol) received one dose of ranitidine (50 mg/kg BW) 120 min before feeding with absolute ethanol. Doses of ranitidine and ethanol were determined according to the previous studies [13, 15, 16] and that of oleuropein based on the average consumption of olive drupes and olive oil in the Mediterranean diet [3–5]. One hour after absolute ethanol administration, all of animals were sacrificed upon light ether anesthesia (Dagenham, UK) by decapitation, and the stomachs were removed, inflated by injecting 2 ml of normal saline, and opened along the greater curvature. Stomachs were gently rinsed with distilled water to remove gastric content and blood clots before scanning. The ulcer index ((UI= [ulcerated area (mm²)/total stomach area (mm²)]×100)) was determined by a digital camera (Panasonic WV-GP240/G, Suzhon, China) and measured according to the method as described previously [15]. Thereafter, ulcer index inhibition (% inhibition=[1−(UI pretreatment/UI non-pretreatment)×100]) was evaluated based on the pretreatment with oleuropein or ranitidine versus ethanol group as a negative control (Fig. 1).

The gastric tissues were removed for biochemical analysis from the antral portion of the stomachs and stored at −70 °C up to 2 months for determination of antioxidant status and lipid peroxidation.

Tissue preparation

The samples were thawed and manually homogenized in cold phosphate buffer (0.1 M, pH 7.4, containing 5 mM EDTA) on liquid nitrogen [2, 3], and debris were removed by centrifugation at 400×g for 5 min (Centrifuge 5415 R; Rotofix 32A, Germany). Supernatants were recovered and used for protein measurement, antioxidant enzymes activities, and glutathione (GSH) and thiobarbituric acid-reactive substances (TBARS) concentrations. Protein content of tissue homogenates was determined using a colorimetric method of Lowry with bovine serum albumin as a standard [29].

![Fig. 1](image-url) Comparison of ulcer index inhibition among the treated groups (ulcer index inhibition was determined based on the pretreatment with oleuropein or ranitidine in comparison with ethanol group as a control). Values represent mean±SEM of ulcer index inhibition (%). Means with different superscripts differ statistically (one-way ANOVA followed by Tukey’s post hoc test; P<0.05). Oleu oleuropein, Eth ethanol, Rani ranitidine
Measurement of GPx activity

The activity of GPx was evaluated with Randox® GPx detection kit according to the manufacturer’s instructions, as described previously [2]. GPx catalyzes the oxidation of GSH by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance was measured spectrophotometrically (S2000 UV model; WPA, Cambridge, UK) against blank at 340 nm. One unit (U) of GPx was defined as $1 \mu$mol of oxidized NADPH per minute per milligram of tissue protein. The GPx activity was expressed as unit per milligram of tissue protein (U/mg protein).

Measurement of SOD activity

The activity of SOD was evaluated with Randox® SOD detection kit according to the manufacturer’s instructions, as described previously [2]. The role of SOD is to accelerate the dismutation of the toxic superoxide ($O_2^-$) produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity is then measured by degree of inhibition of this reaction. One unit of SOD is that which causes 50 % inhibition of the rate of reduction of INT under the conditions of the assay. SOD levels were recorded at 505 nm and through a standard curve and expressed as nanomoles per milligram of tissue protein (nmol/mg protein).

Measurement of CAT activity

Tissue catalase activity was assayed using the method described by Claiborne [10] and was reported by Kheradmand et al. [23]. The reaction mixture (1 ml) consisted of 50 mM potassium phosphate (pH 7.0), 19 mM H$_2$O$_2$, and a 20–50-$\mu$l sample. The reaction was initiated by the addition of H$_2$O$_2$, and absorbance changes were measured at 240 nm (25 °C) for 30 s. The molar extinction coefficient for H$_2$O$_2$ is 43.6 M$^{-1}$ cm$^{-1}$. The CAT activity was expressed as the unit that is defined as micromoles of H$_2$O$_2$ consumed per minute per milligram of tissue protein (µmol H$_2$O$_2$ min$^{-1}$ mg$^{-1}$ tissue protein).

Measurement of total glutathione (GSH content)

Total glutathione was measured by the model as described previously [39] and was reported by Kheradmand et al. [23] and Neamati et al. [34]. In brief, 5 % tissue homogenates were prepared in 20 mM EDTA, pH 4.7, and 100 µl of the homogenate or pure GSH was added to 0.2 M Tris–EDTA (1.0 ml, pH 8.2) buffer (Fluka, Switzerland) and 20 mM EDTA, pH 4.7 (0.9 ml) followed by 20 µl of Ellman’s reagent (10 mM DTNB in methanol). After 30 min of incubation at room temperature, absorbance was read at 412 nm. The blank was prepared with the same method, however, instead of 100 µl of the tissue homogenates, 100 µl of distilled water was applied. Both the blank and sample reaction mixtures were read against water at 412 nm. GSH concentration was calculated on the basis of a millimolar extinction coefficient of 13.6 M$^{-1}$ cm$^{-1}$ and a molecular weight of 307 g. GSH content results were expressed as nanomoles per milligram of tissue protein (nmol/mg protein).

Measurement of lipid peroxidation

The amount of lipid peroxidation was indicated by the content of TBARS in the stomach. Tissue TBARS determined by following the production of thiobarbituric acid-reactive substances as described previously [42] was reported by Alirezaei et al. [2]. In short, 40 µl of homogenate was added to 40 µl of 0.9 % NaCl and 40 µl of deionized H$_2$O, resulting in a total reaction volume of 120 µl. The reaction was incubated at 37°C for 20 min and stopped by the addition of 600 µl of cold 0.8 M hydrochloric acid, containing 12.5 % trichloroacetic acid. Following the addition of 780 µl of 1 % TBA, the reaction was boiled for 20 min and then cooled at 4°C for 1 h. In order to measure the amount of TBARS produced by the homogenate, the cooled reaction was spun at 1,500×g in a microcentrifuge for 20 min, and the absorbance of the supernatant was spectrophotometrically read at 532 nm, using an extinction coefficient of 1.56×10$^5$ M$^{-1}$ cm$^{-1}$. The blanks for all of the TBARS assays contained an additional 40 µl of 0.9 % NaCl instead of homogenate as just described. TBARS results were expressed as nanomoles per milligram of tissue protein (nmol/mg protein).
Statistical analysis

All results are presented as mean±SEM. The statistical differences were applied among all groups by one-way analysis of variance (ANOVA) with Tukey’s post hoc analysis and between the control and oleuropein-treated rats (for gastric erosion index) by an independent-sample t test. A calculated P value of less than 0.05 was considered statistically significant. Statistical analysis was performed using the statistical package Graphpad PRISM version 5 (Graphpad Software Inc., San Diego, CA, USA). Previously, all variables were tested for normal and homogeneous variances by Leven’s statistic test.

Results

We used two models in the present study: ethanol-induced gastric ulcer and fasting-induced gastric erosion to evaluate the effects of ethanol and fasting on the gastric tissue. Based on the present study, fasting could induce gastric erosion, but ulcer was not found in the stomach wall following overnight fasting. In the ethanol-induced ulcer protocol, administration of absolute ethanol to rats produced visible gastric ulcers in all groups, which were localized in the portion of the stomach secreting acid and pepsin (corpus part). It was observed that pretreatment with oleuropein (6, 12, and 18 mg/kg) and ranitidine (50 mg/kg) significantly increased the ulcer index inhibition (percent), in comparison with ethanol group (P<0.05). However, for detection of dose-dependency of oleuropein in the ethanol-induced ulcer protocol, the pretreatments with 12 and 18 mg/kg of oleuropein significantly elevated UI inhibition in comparison with oleuropein 6 mg/kg (P<0.05; Fig. 1). Regarding the fasting-induced erosion protocol in both control and oleuropein groups that were not received absolute ethanol, a significant reduction (P<0.05) in erosion index in oleuropein-treated animals (3.25±1.58) versus control group (23.90±4.80) was observed.

In order to determine the effects of fasting, ethanol, oleuropein, and ranitidine on antioxidant status of gastric tissues, the activities of GPx, SOD, and CAT in all groups were measured (Figs. 2, 3, and 4). GPx activity was significantly lower in the ethanol group compared with the other groups (P<0.05), except for oleuropein (6 mg/kg) plus ethanol group. In this regard, GPx activity is insignificantly higher in the oleuropein (6 mg/kg) plus ethanol group compared with the ethanol-treated rats (P>0.05). SOD activity was significantly higher in the oleuropein plus ethanol groups (12 and 18 mg/kg) compared with the ethanol and oleuropein (6 mg/kg) plus ethanol group (P<0.05). In contrast, ranitidine could not increase SOD activity in the ranitidine plus ethanol group as compared with ethanol-treated rats (P>0.05). Indeed, when ranitidine (as a reference drug) was administered prior to ethanol, it could increase the level of this parameter close to the oleuropein-treated rats. CAT activity significantly elevated in oleuropein (12 mg/kg) plus ethanol groups as compared with ethanol-treated rats (P<0.05), and although the activity of CAT in the oleuropein (6 and 18 mg/kg) plus ethanol groups and ranitidine plus ethanol group were higher compared with the ethanol-treated rats, these enhancements were not statistically significant (P>0.05). Overall, GPx, SOD, and CAT levels in oleuropein

![Fig. 2](image_url)

**Fig. 2** Comparison of glutathione peroxidase (GPx) activity among the control and treated groups. Values represent mean±SEM of enzyme activity (unit/mg protein of gastric tissue). **Means with different superscripts** differ statistically (one-way ANOVA followed by Tukey’s post hoc test; P<0.05). **Oleu** oleuropein, **Eth** ethanol, **Rani** ranitidine
The administration of absolute ethanol to fasted rats resulted in severe gastric hemorrhage, which was visible from the outside of the stomach as thick reddish-black lines. Whereas pretreatment with oleuropein significantly reduced gastric ulcers induced by ethanol, and the best result was obtained in oleuropein (12 mg/kg) plus ethanol group. In this regard, the gastroprotective effect of oleuropein (6 mg/kg) was similar to that achieved by pretreatment with ranitidine (50 mg/kg) as a reference anti-ulcer drug. Hence, our results showed that oleuropein (12 and 18 mg/kg) or ranitidine could suppress TBARS concentration \((P<0.05)\). In this regard, the gastric concentration of TBARS in the oleuropein group was significantly lower compared with the other groups \((P<0.05; \text{Fig. 6})\).

Discussion

In the present study, the administration of absolute ethanol to fasted rats resulted in severe gastric hemorrhage, which was visible from the outside of the stomach as thick reddish-black lines. Whereas pretreatment with oleuropein significantly reduced gastric ulcers induced by ethanol, and the best result was obtained in oleuropein (12 mg/kg) plus ethanol group. In this regard, the gastroprotective effect of oleuropein (6 mg/kg) was similar to that achieved by pretreatment with ranitidine (50 mg/kg) as a reference anti-ulcer drug. Hence, our results showed that oleuropein (12 and 18 mg/kg) or ranitidine could suppress TBARS concentration \((P<0.05)\). In this regard, the gastric concentration of TBARS in the oleuropein group was significantly lower compared with the other groups \((P<0.05; \text{Fig. 6})\).
results showed that ethanol-induced gastric ulcer may be successfully treated with the olive phenolic micronutrient, oleuropein, possibly through its antioxidant effects and the suppression of oxidative stress. In this setting, oleuropein prevents gastric oxidative damages, improves total antioxidant capacity and cell membrane integrity (as shown by antioxidant status), and reduces lipid peroxidation (as shown by TBARS concentration) of stomach wall. Our data also demonstrated antioxidant effects of oleuropein against fasting-induced gastric erosions. Overall, these results support and extend previous reports suggesting that ethanol intoxication generally impairs the gastric antioxidant defense system and induces lipid peroxidation in experimental animals [8, 15, 35].

It is well-known that gastric ulcer results from an imbalance between the pro-oxidant and the antioxidant systems in favor of the former in the stomach [7]. Gastric cell and tissue injury associated with acute and chronic inflammation is due to the toxicity of ROS generated in stomach [27, 28]. Gastric mucosal integrity is maintained by a dynamic process of cell death and cell proliferation. Among various factors involved in gastric mucosal lesions, oxidative damage and apoptotic cell death [8, 11] play significant roles in the loss of gastric mucosal integrity caused by various ulcerogens. Indeed, ulcers develop when oxidative damage and apoptosis predominate over the healing process by cell proliferation; in contrast, various growth factors, nitric oxide, endothelin, angiogenesis, mitogen-activated protein kinases, and some of the oncogene such as c-myc, c-Ha-ras, and c-fos participate in ulcer improvement [8]. In the present study, the gastroprotective effect of oleuropein was better than that achieved by the pretreatment with 50 mg/kg of ranitidine, applied exactly 2 h before the absolute ethanol administration. It is well-known that ranitidine is an antisecretory drug and frequently used as a reference drug in experimental studies of gastric erosion and in clinical practice, as well [15]. Hence, it seems that a combination regimen including both antioxidant and antisecretory drugs may be beneficial in either preventing direct mucosal cell damage or supplementing antioxidant capabilities, because ranitidine
used as antisecretory medications for prevention of ethanol-mediated gastric mucosal damages, where acid and pepsin contribute to the development of ulcers [35].

Oleuropein is able to chelate metal ions, such as Cu$^{2+}$ and Fe$^{2+}$, which catalyze free radical generation reactions [3, 6]. Oleuropein and its metabolite hydroxytyrosol both possess the structural requirement (a catechol group) needed for optimum antioxidant and/or scavenging activity [1, 3]. Oleuropein is rapidly absorbed from the intestine with $t_{\text{max}}$ of 2 h reaching a peak of 200 ng/ml of plasma after administration of 20 mg/kg oleuropein in rats [17]. In addition, oleuropein has been detected in plasma only in its glycoside form suggests that it is absorbed intact from the intestine [1]. Hence, the high availability of oleuropein in its active form in vivo may explain the positive impact on the enzymatic and non-enzymatic (GSH content) antioxidants in our study. Although pretreatment with all three doses of oleuropein significantly reduced gastric ulcers induced by ethanol in comparison with non-treated rats, the best inhibition of ulcer index was performed in pretreated rats with 12 mg/kg of oleuropein. The higher dose (18 mg/kg) did not show the greatest effect. Thus, it seems that there is no dose-dependency of oleuropein. On the basis of our results, we conclude that doses of 6 and 12 mg/kg could cause antioxidant effects, and a higher dose causes pro-oxidative effects and consequently worse results, since it has happened in experimental studies of natural antioxidants previously [16, 26, 36].

All cells are able to defend themselves from damaging effects of oxygen free radicals by way of their own antioxidant mechanisms, including enzymatic and non-enzymatic antioxidant systems [34]. GPx and CAT are two key antioxidant enzymes that can decompose hydrogen peroxide to water [2, 3, 24, 44]. SOD, another antioxidant enzyme in cells, rapidly converts superoxide anion (O$^{2-}$) to less dangerous hydrogen peroxide (H$_2$O$_2$), and then GPx and CAT can decompose H$_2$O$_2$ to water [2, 3, 23, 25]. Although H$_2$O$_2$ is not a particularly reactive product, it may be reduced to the highly reactive metabolites hydroxyl radicals and/or single oxygen [37]. In addition, reduced glutathione (GSH) is a tripeptide that is present in all mammalian cells and has a major cytoprotective function as a reductant and co-factor for certain antioxidant enzymes such as GPx, and this function of GSH is of particular importance in defense against oxidative stress [34]. In this sense, GSH and other antioxidants play a critical role in limiting the propagation of free radical reactions, which would otherwise result in extensive lipid peroxidation [40]. In the present study, oleuropein caused significant increases in the levels of antioxidant enzymes and GSH content for both oleuropein (12 and 18 mg) plus ethanol-treated groups in comparison with ethanol group. On the other hand, gastric GSH content was not depleted in the oleuropein- and ranitidine-treated rats, suggesting that oleuropein and ranitidine possessed antioxidant effects and preserved the cellular antioxidant stores.

Herein, we could exclude the fasting-induced erosions from ethanol-induced ulcers via two protocols. In this regard, we used oleuropein 12 mg/kg, the median dose of applied oleuropein in pretreated groups, for determination of lesion index and antioxidant enzyme activities in comparison with controls. Our data showed an increase in gastric GSH content and consequent a decrease in TBARS concentration in oleuropein-treated rats. The antioxidant effect of oleuropein results from its ability to scavenge ROS, produced by fasting or ethanol, which initiate lipid peroxidation [15]. It is widely accepted that lipid peroxidation is a mechanism of cellular injury [2, 3, 15, 16]. Thus, we measured the TBARS concentrations as an indicator of lipid peroxidation in gastric wall. Our results showed that there was a significant increase in the lipid peroxidation level in ethanol-treated rats. In contrast, significant decreases in the TBARS concentration were observed by the administration of oleuropein 12 and 18 mg/kg and ranitidine 50 mg/kg. However, being pretreated with oleuropein 6 mg/kg could not prevent lipid peroxidation adequately.

As previously mentioned, gastric ulcer is an oxidative state where acid and pepsin contribute to the development of the condition, and therefore antioxidant enzyme activities and GSH content as well as TBARS concentration explain the gastric oxidative–antioxidant imbalance. However, no unifying concept has developed yet on the mechanism of gastric mucosal damages by various ulcerogens. Hence, it will be interesting to examine whether a combination regimen, including both antioxidant and antisecretory drugs, may be beneficial in preventing mucosal cell damages in clinical trials.

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