The effects of acetyl shikonin isolated from *Onosma armeniacum* on oxidative stress in ethanol-induced ulcer model of rats

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Aim: We investigated the effects of acetyl shikonin isolated from *Onosma armeniacum* on ethanol-induced gastric injury (ulcer) and on oxidant–antioxidant parameters in the gastric tissue of rats.

Materials and methods: Eighteen albino Wistar male rats were divided into 3 different experimental groups. Group 1 (n = 6) was the control group, group 2 (n = 6) was the ethanol group, and group 3 (n = 6) was the acetyl shikonin + ethanol group. The animals in group 3 were given ethanol (1 mL, 50% v/v) 30 min after acetyl shikonin (40 mg/kg) administration by orogastric tube.

Results: The lowest mean nitric oxide level; the lowest superoxide dismutase, glutathione peroxidase, and glutathione S-transferase activities; and the highest malondialdehyde level and myeloperoxidase activity were obtained in group 2. It was observed that acetyl shikonin increased the activities of antioxidant enzymes and the level of nitric oxide and decreased the level of malondialdehyde and the activity of myeloperoxidase in group 3. In histopathological evaluation, minimal lymphocyte infiltration and edema were seen but no ulcerations were observed in group 3.

Conclusion: The data revealed that the antiulcer effect of acetyl shikonin might be due to an increase in antioxidant enzyme activity and nitric oxide levels, and a decrease in malondialdehyde levels and myeloperoxidase activity.

Key words: Acetyl shikonin, oxidative stress, ethanol-induced ulcer

1. Introduction

Peptic ulceration is a multifactorial disease including some factors such as trauma, stress, sepsis, burns, pulmonary and liver disease, and drugs, but the etiologic factors are unknown in 60%–80% of peptic ulcerations (1). The reactive oxygen species (ROS)-mediated lipid peroxidation is an important occasion of destruction and damage to cell membranes and it is involved in the pathogenesis of acute mucosal injury induced by ethanol (2,3).

The acute administration of ethanol to rats presents gastric mucosal lesions and erosions similar to those occurring in gastric ulcer (4). Intragastric application of absolute ethanol has long been used as a method to induce gastric lesions. Ethanol rapidly penetrates the gastroduodenal mucosa, causing membrane damage, exfoliation of cells, and erosion. The increase in mucosal permeability together with the release of vasoactive products from mast cells, macrophages, and other blood cells may excite vascular injury, necrosis, and ulcer formation (5). Application of absolute alcohol by gastric gavage induces marked damage to the gastric mucosa that is apparent by macroscopic examination.

Many postulates have been hypothesized about the development of gastric mucosal lesions, such as modulation of the nitric oxide system (6), reduction of nonprotein sulfhydryl concentrations (7), and decline of gastric mucosal blood flow (8). Additionally, the formation of lesions may be mediated by oxygen-derived free radicals (9). Oxidative stress and depletion of antioxidants have also been considered crucial in alcohol-induced mucosal damage (10,11).

Some members of the family Boraginaceae that are rich in naphthoquinones are *Alkanna*, *Onosma*, *Arnebia*, *Lithospermum*, and *Echium*. The naphthoquinones found in Boraginaceae species are alkannin/shikonin and/or their derivatives. Shikonin (*R*-configuration) is an

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enantiomer of alkannin (*S*-configuration) (12). Shikonin and its derivatives have biological activity against microorganisms; hence, these compounds may play a role in plant defense (13). In Turkey, *Onosma armeniacum* is a species found especially in the East Anatolian Region (14) and is used for the treatment of wounds, burns, hemorrhoids, gastric ulcer, and tonsillitis (15). Although there is some knowledge about the antioxidant and antimicrobial effects (16) and effects on wound healing (17) and gastric ulcer (18) of *Onosma* species, there are no data in the literature on the gastroprotective effect of acetyl shikonin, which exhibits O_2^- scavenging activity (19).

The aims of this study were to determine whether a treatment with acetyl shikonin (40 mg/kg) might reduce gastric ulceration induced by ethanol and, if so, to determine the potential changes in the activities of certain antioxidant enzymes such as superoxide dismutase (SOD), glutathione S-transferase (GST), and glutathione peroxidase (GPx). Malondialdehyde (MDA) levels, nitric oxide (NO) levels, and myeloperoxidase (MPO) activities were also measured. Thus, the present study was intended to evaluate the possible mechanisms of any acetyl shikonin protective effects on ethanol-induced gastric ulcers in rats.

2. Materials and methods

2.1. Chemicals
All reagents used for laboratory experimentation were purchased from commercial sources (Sigma and Merck Chemicals).

2.2. Plant material
The roots of *Onosma armeniacum* were collected from Erzurum, Turkey, in July 2005. It was authenticated by Dr Ufuk Özgen of the Pharmacognosy Department at Atatürk University. A voucher specimen was deposited in the herbarium of the Faculty of Pharmacy, Ankara University, Ankara, Turkey (AEF 23796).

2.3. Isolation methods
The roots (400 g) of *Onosma armeniacum* were powdered and extracted using a 3×1000 mL *n*-hexane–dichloromethane mixture (1:1) under reflux for 3 h for each extraction. The combined extracts were evaporated under reduced pressure to give a concentrated extract. The extract (8.2 g) was subjected to silica gel column chromatography in which the elution was performed using an *n*-hexane–ethyl acetate mixture with gradient elution. Similar fractions as determined by thin layer chromatography were combined. Elution with *n*-hexane–ethyl acetate (8:2) gave acetyl shikonin. Preparative thin layer chromatography was used to obtain pure compounds. The purity of the obtained acetyl shikonin was controlled by high-performance liquid chromatography (HPLC). The HPLC analysis was performed on a Thermoquest

HPLC system equipped with a DAD detector (Thermo UV6000). The HPLC conditions were column: RP-C18 column (250×4.6 mm, 5 µm particle size, ACE®); mobile phase: acetonitrile-methanol-water with 2% acetic acid (60:20:20); elution: isocratic; temperature: ambient; flow rate: 1.0 mL/min; injection volume: 20 µL; wavelength: 525 nm.

2.4. Animals
This study used 18 (12 in the ulcer model) 12-week-old male albino Wistar rats (200–250 g) received from the Medical Experimental Research Center at Atatürk University. The animals were fed under normal conditions (22 °C) in separate groups before the experiments. Animal experiments were performed in accordance with the ethics rules for laboratory animals and were approved by Atatürk University's local animal care committee (approval number 2007.2.1/3 from 21.06.2007).

The animals were randomly assigned to different experimental groups. Group 1 (n = 6) was the control group, group 2 (n = 6) was the ethanol group, and group 3 (n = 6) was the acetyl shikonin + ethanol group. Acetyl shikonin (40 mg/kg) was administered by orogastric tube to group 3. For this experiment, acetyl shikonin was dissolved in Tween 80 (1%). The animals in both groups 2 and 3 were given ethanol once (1 mL, 50% v/v) with the difference that for group 3, the ethanol was given 30 min after acetyl shikonin was administered by orogastric tube.

The control group received an equal volume of Tween 80 (1%) orally as a vehicle. Thirty minutes after ethanol administration, the stomachs of all rats were excised under general anesthesia with 25 mg/kg thiopental. The gastric tissues were put into containers and put in ice after appropriate labeling.

2.5. Biochemical analysis
For all assays except those for MDA level and MPO activity, a portion of each tissue was homogenized in a phosphate buffer of pH 7.5 with a homogenizer. Tissue homogenates were centrifuged for 15 min at 10,000 rpm and 4 °C, and then the supernatants were removed for analysis and stored at –80 °C.

2.5.1. GST activity
The supernatant's GST activity was measured using 1-chloro-2,4-dinitrobenzene and glutathione at 340 nm (20). Results were expressed as U/g protein.

2.5.2. GPx activity
The supernatant's GPx activity was measured using decrease in absorbance caused by oxidation of NADPH to NADP at 340 nm (21). Results were expressed as U/g protein.

2.5.3. SOD activity
Cu–Zn SOD activity was measured by the reduction in nitro blue tetrazolium at 560 nm by the xanthine–

xanthine oxidase system, which is a superoxide generator. Enzymatic activity leading to 50% inhibition was accepted as 1 unit (22). Results were expressed as U/g protein.

2.5.4. MPO activity
Tissue samples were homogenized in a 50 mM K_2HPO_4 buffer at pH 6.0. The pellets were then suspended in a 50 mM K_2HPO_4 buffer containing 0.5% hexadecyltrimethylammonium bromide. MPO activity in gastric tissues was measured using a procedure described by Wei et al. (23) that used 4-aminoantipyrine/phenol as a substrate. MPO activity was expressed as U/g protein.

2.5.5. MDA levels
Gastric tissue samples were homogenized so that each gram of tissue contained 9 mL of a 1.15% KCl solution. MDA was determined by spectrophotometry of the pink-colored product of the thiobarbituric acid-reactive substances complex at 532 nm (24). Results were expressed as µmol/g protein.

2.5.6. NO levels
Gastric tissue NO levels were measured as total nitrite + nitrate levels with the use of the Griess reagent (25). The Griess reagent consists of sulfanilamide and N-(1-naphthyl)-ethylenediamine. NO levels were expressed as nmol/g protein.

The supernatant's protein concentration was measured using the method described by Bradford (26). Results were expressed as g/L.

2.6. Pathological examination
Ulcer areas on the stomach surfaces were examined macroscopically and measured on square millimeter paper. Ulcer areas were represented as mm². Gastric tissue samples were obtained from the ulcerated areas and sent to the pathology laboratory in 10% formaldehyde. After 10%

formalin fixation, the tissue samples were dehydrated and embedded with paraffin according to standard histological protocols. After the follow up, 5-µm-wide sections were obtained and stained with hematoxylin and eosin. They were evaluated under a light microscope.

2.7. Statistical analysis
The results were given as medians (minimum–maximum). Principally, the Kruskal–Wallis test was used to assess the differences between the groups. The difference between the groups was specified using the Mann–Whitney U test. Correlation analyses were performed using Spearman's rank test. A Bonferroni correction was done and the significance level was redetermined so that $P < 0.017$ was considered significant.

3. Results
The administration of 50% ethanol in group 2 caused hemorrhagic necrotic areas that were observed macroscopically (ulcer area: 23.5 ± 7.8 mm²). No ulcer areas were observed in the acetyl shikonin application group. In the pathological examination, while the gastric tissue samples of the control group were consistent with normal gastric tissue, the group 2 samples exhibited exfoliation on the stomach surface, ulceration, bleeding, lymphocyte infiltration, and edema. In group 3, minimal lymphocyte infiltration and edema were found, but no ulceration was observed (data not shown).

The effects of ethanol intake alone and following administration of acetyl shikonin on the contents of MDA and NO, as well as the enzyme activities of SOD, GST, GPx, and MPO in rat gastric tissue, are shown in the Table. Ethanol administration (50%, 1 mL) markedly stimulated lipid peroxidation in the gastric tissues. The MDA concentrations were 274.69 (139.37–427.11) and

Table. The effects of ethanol intake alone and after administration of acetyl shikonin on the levels of MDA and NO and the activities of GST, GPx, SOD, and MPO in the gastric tissue of rats.

Parameters	Group 1 (n = 6) (control)	Group 2 (n = 6) (ethanol)	Group 3 (n = 6) (acetyl shikonin + ethanol)
MDA (µmol/g protein)	59.73 (38.81–79.56)	274.69 (139.37–427.11)*	106.83 (54.55–201.85)**
NO (nmol/g protein)	2349.5 (21811.1–2705.1)	1057.5 (694.1–1209.1)*	1401.7 (1101.6–2627.1)**
GST (U/g protein)	38.69 (25.13–64.26)	12.69 (3.44–14.39)*	26.06 (13.97–35.65)**
GPx (U/g protein)	302.75 (213.44–397.78)	143.83 (78.50–162.96)*	206.43 (184.32–284.13)**
SOD (U/g protein)	135.51 (96.08–150.62)	50.47 (27.35–62.19)*	81.00 (58.02–113.82)**
MPO (U/g protein)	18.23 (7.39–32.78)	39.56 (31.40–56.77)*	26.41 (12.78–39.83)**

Data are presented as medians (minimum–maximum). Ethanol (50% v/v) was given by orogastric tube (1 mL per rat). Acetyl shikonin (40 mg/kg) was given orally 30 min before ethanol administration.
* = significantly different from the control group at $P < 0.01$, ** = significantly different from the ethanol group at $P < 0.01$.

106.83 (54.55–201.85) µmol/g protein in the rats that were treated with ethanol alone and ethanol combined with acetyl shikonin, respectively (Table). Moreover, pretreatment with acetyl shikonin significantly prevented lipid peroxidation induced by ethanol. In group 2, ethanol administration significantly reduced the SOD, GST, and GPx enzyme activities and NO concentration in the gastric tissue as compared with the intact group rats. There was a significant negative correlation between the serum antioxidant enzyme (GST, GPx, and SOD) levels and the serum NO and MDA levels (P < 0.01). We also found a negative correlation between MPO levels and GST, GPx, and SOD activities and NO levels (P < 0.01). There was a positive correlation between MPO and MDA levels (P = 0.013).

4. Discussion
Ethanol in 50% concentration as used in this study is a model of mucosal barrier injury. In our investigation, we confirmed that exposing gastric mucosa to oxidative stress, induced by ethanol administration (10), leads to the generation of lipid peroxides, as expressed by an increase in the gastric tissue level of MDA accompanied by an impairment of antioxidative defense mechanisms, such as a reduction in SOD, GST, and GPx activities. Pretreatment of rats with a single dose of acetyl shikonin isolated from *Onosma armeniacum* could partly reduce the gastric lesions induced by acute intake of ethanol. The acetyl shikonin significantly decreased the gastric tissue MDA content and MPO activities, while it increased the gastric tissue levels of NO compared to animals receiving ethanol alone. The gastric tissue activities of SOD, GST, and GPx were markedly elevated following the administration of acetyl shikonin.

It has been suggested that alkannin, shikonin, and their derivates have some biological effects such as wound healing, antitumor, antimicrobial, antithrombotic, and antiinflammatory functions (27). Shikonin and alkannin exhibit the same level of O_2^- scavenging activity, which is more potent than the activity of L-ascorbic acid. The O_2^- scavenging activity of shikonin is considered to play an important role in wound healing (19). Hayashi et al. reported that acetyl shikonin extracts were effective for the treatment of cutaneous injuries. When applied topically, the extracts showed increased proliferation of granuloma tissue as well as antiinflammatory, mildly antipyretic, and analgesic effects (28).

Some studies have reported that oxygen-generated free radicals and lipid peroxidation may play important roles in the pathogenesis of ethanol-induced gastric lesions (2,3). Ethanol induces a rapid and strong vasoconstriction accompanied by rapid and vigorous arteriolar dilatation. The oxyradicals generated during the ischemia–

reperfusion provoke severe changes at the cellular level leading to cell death (29). Ethanol weakens the body's defense mechanism against free radical attacks (30). Chronic alcohol intake has harmful effects on the organism (31). Alcohol intake has been shown to be associated with marked oxidative damage to gastric tissue. Several studies have shown the cytoprotective role of some antioxidants in the prevention and healing of gastric lesions (32,33). Ito et al. (34) reported that probucol, a lipid-lowering agent with antioxidant properties, may partly protect gastric mucosa from acute gastric mucosal injury and promote the healing of chronic gastric ulcers by its antioxidant activity. However, quercetin, alpha-tocopherol, nifedipine, and tetracycline were found to possess components that are gastric cytoprotective and promote gastric ulcer healing. The antiulcer effects of these compounds may partly result from their free radical-scavenging properties (32). In addition, Bilici et al. (33) demonstrated that melatonin prevented ethanol-induced gastric mucosal damage, presumably due to its antioxidant property. Therefore, it was reported that some antioxidant agents like rutin, a natural flavone, may have gastroprotective effects in gastric ulceration via decreased ROS production and/or increased antioxidant enzyme activity (11).

Another mechanism in the pathogenesis of mucosal lesions provoked by ethanol may be circulating neutrophils (2). The leukocytes might create gastric ulcerations through various mechanisms, such as the production of reactive oxygen metabolites or the release of proteases and lipid mediators (35). In our ethanol-induced gastric ulcer model, acetyl shikonin induced a decline in the MPO activity as an index of neutrophil infiltration via a possible inhibitor effect on leukocyte functions.

Various plants are used in clinical and folk medicine in many countries. The extracts of several plants have been used as therapeutic agents. Herbs and spices are used as sources of natural antioxidants that can protect from oxidative stress. Thus, spices and herbs may play an important role in the prevention of diseases resulting from lipid peroxidation (36).

It has been reported that tea catechin may primarily protect gastric mucosa from acute gastric mucosal injury and promote the healing of chronic gastric ulcers through its antioxidant activity and gastric mucus-increasing actions (37). Zamora et al. showed that the gastric ulcer index was significantly reduced in rats pretreated with ozonized sunflower oil (OSO) as compared with ethanol-treated controls. Although no significant reduction of thiobarbituric acid-reactive substances content was found in rats pretreated with OSO, SOD and GPx activities were significantly increased in their gastric mucosa as compared to those in rats treated with ethanol alone. They demonstrated that OSO pretreatment exerts protective

effects in ethanol-induced gastric ulcers in rats (38). In another study, it was shown that the gastroprotective effect of rutin in the experimental lesions induced by 50% ethanol could be related to its antioxidant properties, which reduce the levels of lipid peroxides and increase the activity of the antioxidant enzyme GPx (11).

It is well known that NO is involved in the modulation of gastric mucosal integrity and is important in the regulation of acid and alkaline secretion, mucus secretion, and gastric mucosal blood flow (39). NO has been recognized as a factor in the prevention of oxidative stress, for example by the inhibition of leukocyte adherence (40). This was based on the decrease in neutrophil infiltration of gastric tissue, resulting in a reduction in oxidative tissue damage. We observed that NO levels were decreased in the damaged gastric tissue, and so our finding is harmonious with the literature (18). GPx is an important enzyme that plays a role in the elimination of H_2O_2 and lipid hydroperoxides in the gastric mucosal cell. The antioxidant activity of GPx is coupled with the oxidation of reduced GST. Inhibition of this enzyme may result in the accumulation of H_2O_2. Acetyl shikonin might protect rat gastric tissue through its ability to increase the synthesis of free-radical scavenging enzymes in the mucosa.

The results of our experiments indicate that the gastric mucosal lesions caused by 50% ethanol lead to a decrease in the activity of antioxidant enzymes. Intensification of ROS production results in lipid peroxidation, expressed by a tissue increment of MDA levels. These phenomena are accompanied by an impairment of antioxidative properties of cells supported by our finding of a decrease in the SOD, GST, and GPx activity in the gastric mucosa.

In conclusion, the data of our study suggest that acetyl shikonin can inhibit ethanol-induced gastric lesions in rats and that acetyl shikonin has an obvious gastroprotective effect and antioxidant properties. Although the exact mechanism underlying these actions is unclear, the possible mechanisms of the antiulcer effect of acetyl shikonin may be due to inhibition of lipid peroxidation and neutrophil activation, preventing the loss of gastric tissue antioxidant enzymes such as SOD, GST, and GPx in ethanol-induced ulcer models of rats.

Acknowledgments

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