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Evaluation of Hydroalcoholic Extract of Licorice Root (*Glycyrrhiza glabra* L.) on Wound Healing of Gastrotomized Male Wistar Rats: Histopathological Changes

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ABSTRACT

This study investigated the effects of hydro-alcoholic extract of licorice root on the healing of surgical gastric ulcers in rats. For this experimental study, thirty-six male Wistar albino rats were prepared, and first, a 16 mm incision was made in the greater curvature of the stomach, and then it was sutured in a single layer. The rats were then randomly distributed into three groups (n=12), a control group, and two other groups, which were treated with licorice hydroalcoholic extract at doses of 150 and 300 mg/kg orally via gavage once daily for 21 consecutive days. Wound healing among the groups was compared and a determination was made for the malondialdehyde (MDA) concentration and serum antioxidant capacity. The mean rank of histopathological evaluation on the twentieth day after surgery showed a significant difference between the three groups. The difference in mean rank showed a significant increase between the group of rats treated with the extract at a dose of 300 mg/kg compared to the control group. The amount of MDA in the control group showed a significant increase compared to the groups treated with the extract at doses of 150 and 300 mg/kg. Serum antioxidant capacity in the experimental group treated with extracts showed a significant increase in comparison with the control group. The results of this study showed that lipid peroxidation in a gastrotomy rat treated with licorice root hydroalcoholic extract decreased with a marked increase in antioxidant activity and subsequently accelerated the healing process of the gastric surgery site.

Introduction

Peptic ulcer is a fracture of the gastric and duodenal mucosa.[1] Gastric or peptic ulcer is the most common gastrointestinal disease worldwide.[2] It is estimated that 4 million people worldwide suffer from gastric ulcers each year,[3] and 3,000 to 15,000 die from gastric ulcers.[4] Gastric or duodenal ulcers form for a number of reasons, such as steroid and non-steroidal drugs, smoking, alcohol use, trauma, shock, bacterial infection of *Helicobacter pylori*,[3] gastric cancers following

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infection with *H. pylori*,[5] sepsis, excess salt intake, stress,[3,6] Zollinger-Ellison syndrome (ZES), primary and metastatic malignancies, infectious diseases of cytomegalovirus and cocaine use.[7] Perforation of the stomach and duodenum is the second complication after bleeding that is associated with the clinical symptoms of sepsis and peritonitis.[8] Giant ulcers are defined as gastric ulcers of more than 3 cm or duodenal ulcers of more than 2 cm and require more time to heal due to the nature of chronic diseases.[7] For large wounds, surgical treatment can be one of the few methods of choice performed in perforated wounds.

Today, the use of antibiotics along with surgery is a standard treatment for gastric ulcer perforation.[6] Treatment and prevention of peptic ulcer are associated with decreased gastric acidity or increased protection of the gastric mucosa.[9] Treatment of gastric ulcer with chemical drugs such as omeprazole, metronidazole, ranitidine is expensive and can cause side effects and problems such as autoimmunity and the possibility of recurrence of lesions after the treatment.[10] For this reason, there is an extensive effort to find effective natural and herbal compounds in the treatment of gastric ulcers. The scientific name of licorice plant is *Glycyrrhiza glabra* L. and its English names are Licorice and Liquorice.[11-14] Known compounds in licorice root include flavonoids (liquritin, glabrol), isoflavones, isoliquiritin, liquocoumarin, and stilbenoides. This medical herb has antioxidant, anti-inflammatory, strong immune system, anti-cancer, antimicrobial, anti-viral, anti-parasitic, and anti-cough properties.[15] The yellow color and sweet taste of *G. glabra* L. (Fabaceae) roots are from flavonoids and glycyrrhizin, respectively.[16] Since laboratory rats are one of the most common laboratory models for research on gastrointestinal diseases because of the similarity of their anatomical structure and their organs to the human body, the aim of this study was to evaluate the hydroalcoholic extract of licorice in the process of repair with primary intention in the greater curvature of the stomach, and to determine malondialdehyde (MDA) levels and serum antioxidant capacity in gastrotomized male Wistar rats.

Materials and Methods

Animal Housing

The present study is an experimental laboratory intervention and was performed in the Animal Home and Pathology Laboratory of Islamic Azad University, Shahrekord Branch, Iran. This study was performed on 36 male Wistar albino rats weighing 250 to 300 g with an age range of 120 to 150 days. The rats were kept at a temperature of 20° C, humidity of 45% and light-dark cycle of 12 hours were provided in polycarbonate cages housing 4 rats. To make sure that the rats adapted to the conditions of the new environment, all experiments were performed one week after the establishment of male Wistar albino rats.[17] All experiment protocols used in this research were approved by the Animal Care Ethics Committee of the Islamic Azad University, Shahrekord Branch.

Preparation of Hydroalcoholic Extract of Licorice

The root of licorice plant with the scientific name of *G. glabra* L., grown in the areas of Kian city of Shahrekord with geographical coordinates of 32.284719° N, 50.894594° E, Chaharmahal and Bakhtiari Province, Iran, picked in December 2019 was transferred to the laboratory of medicinal plants. At the laboratory, this root was approved of by the botanist of the center and was labeled with a herbarium number. The root surfaces were cleaned and dried, and then the milled and hydroalcoholic extract of licorice was stored by soaking with 70% ethanol for 72 hours at room temperature. Every 24 hours, the plant extract was filtered through Whatman No. 1 filter paper and added to the fresh solvent residue, and the resulting extracts were mixed together. The final extract was stored by vacuum distillation and then in an oven at a temperature of less than 40° C. As a result of vacuum distillation and drying in a solvent oven, the extracts were taken out.[17]

Animal Treatment

The rats were randomly divided into three groups of 12, with gastrotomy control group receiving solvent licorice extract, and the other two gastrotomy groups receiving licorice extract at doses of 150 and 300 mg/kg, respectively. Also, a to prepare a dose of 150 and 300 mg/kg body weight, 1 g of licorice extract was dissolved in 10 ml of physiological serum. Then, the gastrotomy treated rats according to their weight were fed orally by gavage for 20 consecutive days.

Method for Determination of Phenol of Licorice Extract

Ten to 20 mg of the prepared ethanolic extract of licorice was dissolved in 60% methanol to achieve a volume of 10 ml. Then, 0.01 ml of this solution was

transferred to the test tube and 0.5 ml from 10% solution of Folin-Ciocalteu reagent was added. After 3 to 8 minutes, 4 ml of 7.5% sodium carbonate solution was added, and the tubes containing the content were kept at laboratory temperature for 30 min, after which the rate of absorption was determined by spectrophotometer at 755 nm wavelengths. Through three replications and based on the standard diagram prepared with concentrations of 12.5, 25, and 50, the total phenols of the extract were measured using a standard curve based on mg/g gallic acid per gram of extract.[18]

Flavonoid Measurement Method of Licorice Extract

An amount of 10 to 20 mg of the prepared licorice extract was dissolved in 60% methanol and was made up to 10 ml. Then 1 ml of this solution was transferred to the test tubes and 1 ml of the prepared 2% aluminum chloride solution was added to it and the absorbance was read after 40 minutes at 415 nm in three replications. Quercetin was used as the standard to draw the calibration curve. The amount of flavonoids was reported based on the equivalent amount of quercetin in each gram of the extract.[19]

Surgical Method

Prior to surgery, all groups were given complete abstinence for 12 hours. Under general injected anesthetic, each rat was premedicated with ketamine (10%, Alfasan, Woerden, Holland) at 70 mg/kg and xylazine (2%, Alfasan, Woerden, Holland) at 5 mg/kg intramuscularly and under complete aseptic conditions. In order to avoid any hypothermia, an electric mattress was used throughout the procedure. The rats were placed lying on their backs, on the operating table, and the midline of the abdomen was normally prepared for surgery, cleaned with povidone iodine 2% and covered with sterile surgical drapes. A 2-cm incision was made in the skin and muscle of the midline of the abdomen and the stomach was removed from the incision, and then a 16 mm incision was made in the bend of the gastric leaf in the glandular section (the corpus region). Then, the edges of the cut were washed with physiological saline solution and immediately sutured with a synthetic absorbable 4-0 polyglycolate, using a single layer Cushing pattern suture technique on all the layers of the stomach wall except the mucosa layer (i.e., serosa, muscularis layers, and submucosa).[20] The white line of the abdominal muscles was simply sutured all over with 4-0 polyglycolate synthetic absorbable suture and the skin of the area was sutured individually with 3–0 silk sutures (Figure 1). All surgical sutures used were made by SUPA factory (Iran). Cefazolin (100 mg/kg) was injected intramuscularly after surgery to prevent possible infection. The rats were returned to the cage after full consciousness and were given water and food from the first day, groups 2 and 3 received 150 and 300 mg/kg of licorice root extract daily by gavage until the day of sampling, respectively. The control group also received the same amount of physiological serum.

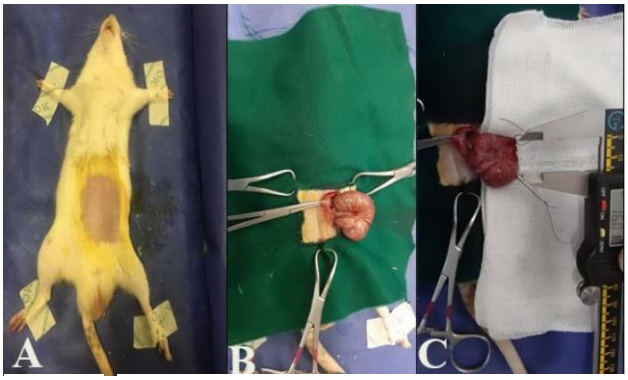

Figure 1. A, Different stages of ligation. B, Removal of the stomach from the abdominal area, C, Incision in the large bend of the stomach and suturing of the incision site.

Evaluation of Gastric Pathology

In order to evaluate the pathology of gastric tissue on days 5, 10 and 20 after surgery, 4 samples from each group were randomly selected and anaesthetized by intramuscular injection of ketamine/xylazine (70 mg/kg ketamine, 5 mg/kg xylazine). Immediate laparotomy was followed by stomach separation that was opened along the lesser curvature and washed out with normal saline to remove gastric contents. Tissue stomach immersed in 10% buffered formalin. All fixed specimens were routinely processed to prepare 5 µm thick paraffin embedded sections for histopathological examination. Moreover, to prepare tissue sections from conventional hematoxylin and eosin (H&E) staining in the previously reported method was used.[17,21] To evaluate the repair, the tissues were studied with a light microscope with a lens 4 × 10 × 40. In the microscopic pathology of gastric ulcer surgery in the rats of different groups, the indicators affecting wound healing with primary intention include inflammation and exudate on the wound surface (0: presence, 1: absence), bleeding at the wound cutting edges (0: presence, 1: absence), re-epithelization of the gastric ulcer (0: lack of repair or very minor, 1: repair of moderate incision of cut mucosa, 2: repair of completely cut mucosa),

penetration of inflammatory cells between glands (0: presence mixed inflammatory cells, 1: lack of mixed inflammatory cells), formation of granulation tissue below the incision site (0: lack, 1: immature granulation tissue including newly established capillaries, fibroblasts and a few collagen fibers (medium), 2: tissue mature granulation consisting of newly established regressive capillaries, slightly fibroblasts, and dense collagen fibers (complete) were used and scored (Table 1).

Table 1. Histopathological grading system of gastric tissue wound healing with primary intention.

Histopathology score	0	1	2
Inflammation on the wound surface	Presence	Absence	-
Bleeding at the site of the cutting edges	Presence	Absence	-
Mucosal re epithelialization	Absence	Moderate	Complete
Infiltration of inflammatory cells between glands	Presence	Absence	-
granulation tissue below the incision site	Absence	Moderate	Complete (mature)

Method of Collecting and Separating Blood Serum

Twelve hours after the last consumption of licorice hydroalcoholic extract (day 20) and removing food from their reach, rats were anesthetized with xylazine and ketamine and after opening their thoracic cavity, blood was drawn from their heart. Blood was poured into tubes with and without anticoagulant, and serum and plasma were separated by a German-made Hitachi centrifuge at 3,000 rpm for 10 minutes. The obtained serum was separated from the blood sample and stored at -70° C to determine the concentration of MDA and to evaluate the antioxidant capacity.

Determination of MDA Concentration and Serum Antioxidant Capacity

Plasma MDA was measured using the calorimetric method of thiobarbituric acid reaction with MDA and color composition with maximum light absorption at 532 nm using a spectrophotometer.[22] Plasma antioxidant capacity was determined using tripyridyl-S-triazine a spectrophotometer at 593 nm.[23]

Data Analysis

To statistically analyze the data, SPSS software version 21 was used. The biochemical results were analyzed by one-way ANOVA and expressed as mean ± standard error of the mean and in case the difference was statistically significant ($p < 0.05$), the data of each two groups were compared by Tukey's test. Wound histopathological changes in the studied groups were evaluated using the nonparametric Kruskal-Wallis test ($p < 0.05$) was considered significant. When p-value was less than 0.05, pairwise group comparisons were performed by the Dunn's test.

Results

Results of Phenols and Flavonoids of Dry Licorice Root Extract

The total amount of licorice root phenol, which was used as a quantification of the calibration curve, was 68.9 mg/g and the total amount of flavonoids was 7.5 mg/g dry weight.

Results of MDA Assessment and Evaluation of Antioxidant Capacity Based on Iron Reduction or Ferric Reducing Antioxidant Power (FRAP) Method

The amount of MDA in the studied groups (experimental control group, experimental group treated with hydroalcoholic extract of licorice root at a dose of 150 mg/kg, and experimental group treated with hydroalcoholic extract of licorice at a dose of 300 mg/kg) proved significant ($p < 0.01$). The amount of MDA in the control group showed a significant increase compared to the group treated with licorice extract at doses of 150 and 300 mg/kg, respectively, ($p < 0.05$) and ($p < 0.0001$). Also, the amount of MDA in the group treated with licorice extract at a dose of 150 mg/kg showed a significant increase compared to the group treated with licorice extract at a dose of 300 mg/kg ($p < 0.05$) (Table 2). There was a significant difference in antioxidant capacity in the studied groups (experimental control group, experimental group treated with hydroalcoholic extract of licorice root at a dose of 150 mg/kg and experimental group treated with hydroalcoholic extract of licorice at a dose of 300 mg/kg) ($p < 0.01$). Serum antioxidant capacity in the experimental group treated with hydroalcoholic extract of licorice at doses of 150 and 300 mg/kg showed a significant increase compared to the control group ($p < 0.05$) and ($p < 0.01$), respectively.

Table 2. Malondialdehyde (MDA) content and serum antioxidant capacity (micromolar) in control and treatment groups.

Expiremental Groups	Group 1	Group 2	Group 3	*p* value
MDA (*μ*mol/L)	46.32 ± 3.52^{a}	34.93 ± 15.72^{b*}	22.57 ± 3.55^{c**}	0.002
FRAP (*μ*mol/L)	331.57 ± 36.88^{b}	446.72 ± 77.79^{a*}	469.57 ± 108.01^{a**}	0.02

Group 1: surgical control treated with normal saline, Group 2: experimental surgery treated with hydroalcoholic extract of licorice at a dose of 150 mg/kg, and Group 3: experimental surgery treated with hydroalcoholic extract of licorice at a dose of 300 mg/kg. Sample in each group = 5, dissimilar letters (a, b, and c) in each row show a statistically significant difference. Data is mean ± standard deviation of the mean. Significant decrease compared to the control group (*p < 0.05 and **p < 0.001).

Table 3. Evaluation of mean rank and (third quarter-middle quarter) of histopathological evaluation in groups of gastrotomy rats under study.

Time	Day 4			Day 10			Day 20		
Groups	**Group 1**	**Group 2**	**Group 3**	**Group 1**	**Group 2**	**Group 3**	**Group 1**	**Group 2**	**Group 3**
Histopathological (mean rank)	4.38	5.5	8	3.5	7.33	7.5	3.13^{b}	6^{ab}	8^{a}
Median (Q1-Q3)		2 (2-3)			5 (4-5)			5 (4-6)	
***p* value**		0.222			0.111			0.02	

Group 1: surgical control treated with normal saline, Group 2: experimental surgery treated with hydroalcoholic extract of licorice at a dose of 150 mg/kg, and Group 3: experimental surgery treated with hydroalcoholic extract of licorice at a dose of 300 mg/kg. Non-identical letters (a, b, c) in each day and in each row indicate a statistically significant difference (p < 0.05).

Evaluation of Histopathological Findings

Histopathological evaluation of the wound healing process was carried out in the gastrotomized rats in three groups. Gastric microscopic damage was scored based on the criteria described in the literature.[24] Grading was performed in terms of inflammatory reaction on the mucosal surface, bleeding edge of the wound, exudation of inflammatory cells between the gastric glands, conversion from incomplete wound space to normal tissue and condition of granulation tissue. Based on Table 2, evaluation and ranking were performed on each sample in each group and at any specific time. Based on nonparametric tests, the mean rank in the studied groups on the fourth and tenth days was not significant (p < 0.05). The mean rank of histopathological evaluation on the twentieth day of the study showed a significant difference between the three groups (p < 0.05). In pairwise comparison between groups, this difference was the mean rank between gastrotomy rats treated with hydroalcoholic extract of licorice at a dose of 300 mg/kg compared with the control group (p < 0.05) (Table 3, Figure 2).

Discussion

Gastric or peptic ulcer is the most common gastrointestinal disease.[2] Traditional medicine in different parts of the world has introduced many herbs, including licorice root with anti-inflammatory activity and healing stomach ulcers.[25-27] In the present study, the effects of hydroalcoholic extract of licorice on healing surgical wounds from gastrotomy in male rats were investigated, and it was found that licorice extract accelerates the healing process of gastric incision surgery on the twentieth day after surgery in the gastrotomized group.

There was a statistically significant difference at 300 mg/kg of hydroalcoholic extract of licorice compared to the other groups. The histopathologic results revealed that there was a significant (p < 0.05) decrease in mucosal re-epithelialization, mature granulation tissue below the incision site and elevation mixed inflammatory cells infiltrative between glandular cells of control group compared to treatment group which treated with hydroalcoholic extract of licorice with dose of 300 mg/kg (Table 3). In fact, the purpose of wound healing is to repair defects in damaged tissue.[28] Although, the mechanism of action of licorice extract in the treatment of advanced gastric ulcer has not been the aim of this study, licorice root has been shown to contain triterpenoid saponins (4-20%) especially glycyrrihzin. Most glycyrrhizin is a mixture of the salts of potassium and calcium glycyrrhizic acid,[14] which have been shown to have anti-ulcer and anti-inflammatory properties of saponins.[29] B-Glycyrrhizinic acid is one of the main metabolites of glycyrrhizin, which has strong anti-inflammatory properties in various animal models.[30] Glycyrrhizin also increases the concentration of prostaglandins in the gastrointestinal tract and enhances gastric mucosal secretions and

bicarbonate,[31] and plays an important role in regulating mucosal blood flow.[32] Elevated levels of prostaglandin cause mucus secretion, stimulate cell proliferation, and have a potential healing effect on stomach injuries.[33] Other sweet compounds indicate that Hispaglabridin A, Hispaglabridin B and 4′-O-methylglabridin have anti-inflammatory and antioxidant functions.[34] *G. glabra* extract can be used as a powerful antioxidant that has the activity of eliminating and inhibiting free radicals in order to protect the tissue against tissue damage and against free radicals caused by harmful agents.[35] In other words, the antioxidant properties of flavonoids and tannins improve the wound healing process.[36] In previous studies, the effects of the hydroalcoholic extract of *G. glabra* was evaluated for antiulcerogenic activity in various doses of 50-200 mg/kg p.o.

Wounds induced in rats by indomethacin,[14] and glacial acetic acid,[37] but in our study the effect of hydroalcoholic extract of licorice on the repair process of gastrotomy surgery with primary intention was investigated. Maintaining gastric blood flow is very important to protect the gastric mucosa against various harmful factors. Researchers have shown that reduced blood flow in the mucosa and submucosa layers cause ischemia of the gastric mucosa and impairs tissue resistance and ulcers.[38]

The genus Glycyrrhiza is known to inhibit 11 beta-hydroxysteroid dehydrogenase (11β-HSD2) and inhibits corticoid metabolism and increases mineralocorticoid or pseudohyperaldosteronism in the blood.[39] Licorice root has been reported to have anti-inflammatory activity similar to the steroid hormone (hydrocortisone) by inhibiting the activity of the enzyme phospholipase A2. Glycyrrhizic acid also inhibits cyclooxygenase activity and prostaglandin E2 *(*PGE2*)* formation and indirectly prevents the formation of vascular thrombosis.[40] In fact, the anti-inflammatory effects of glycyrrhizin are similar to those of glucocorticoids and the mineral corticosteroids, which exert their protective effect by maintaining local defense agents, inhibiting pathogens, and maintaining the integrity of the gastric mucosa.[41] Also, the antibacterial activity of licorice root on gram-negative and gram-positive bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Bacillus subtilis* is due to the presence of secondary metabolites such as saponins, alkaloids and flavonoids.[42-44] Licorice extract is effective in accelerating wound healing by preventing the colonization of bacteria on the surface of gastric

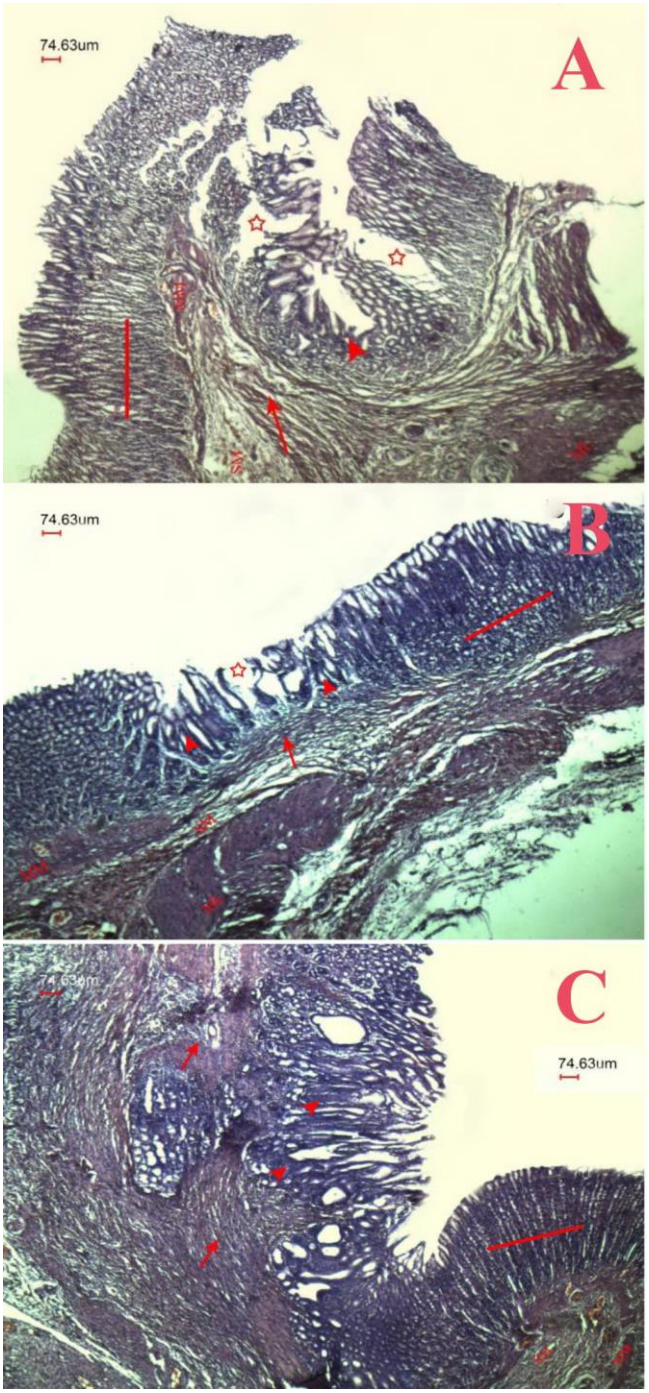

Figure 2. **A,** Microscopic view of the surgical repair site in the control group after 20 days. The wound gap (red stars), which is not covered by the mucosal covering tissue, the space under the mucosa is filled with immature granulation tissue (red arrows). The incision of the wound (arrow), which is not completely covered by the mucosal covering tissue. **B, C,** Groups treated with hydroalcoholic extract of licorice at a dose of 150 and 300 mg/kg after 20 days. The gap wound (red star), and in the underlying layer is covered by a mucous membrane weave (red arrowhead). The wound space under the mucosa is filled with mature granulation tissue (red arrows). Glandular gastric walls retained their normal histological (red line) (H&E, ×40). Abbreviations: MM: muscularis mucosae, SM: submucosa, ML: muscularis layers.

mucosal ulcers and increasing the formation of newly established capillaries in the granulation tissue under the mucosa.

MDA is one of the main products of lipid peroxidation which has strong biological toxicity and can delay the wound healing process.[37] Previously, studies had shown that MDA can directly indicate oxidative stress.[45] Increasing and decreasing the amount of MDA indicates the power of fat peroxidation and indirectly reflects the amount of cell damage.[46] In the control group, the amount of MDA compared to the groups treated with licorice extract showed a significant increase, which indicates the severity of mucosal damage and delay in wound healing (Figure 2a).

Measuring serum antioxidant capacity can be the best criterion for assessing the body's antioxidant status under oxidative stress.[47] In the present study, the rate of lipid peroxidation in the gastrotomized control group was significantly higher than the two groups treated with licorice hydroalcoholic extract, so it can be concluded that in gastrotomized rats, disorders of the oxidative-antioxidant system increased production lipid peroxidation. In this study, there was no significant difference in the amount of serum antioxidant capacity between the two groups treated with licorice hydroalcoholic extract, but the amount of serum antioxidant capacity in the control group showed a significant decrease compared to the experimental groups treated with licorice hydroalcoholic extract. The results of this study showed that after gastrotomy, serum lipid peroxidation increased and the amount of antioxidants decreased, indicating an imbalance between free radical production and antioxidant capacity in the gastrotomy control group.

In this study, we evaluated the effect of hydroalcoholic extract of licorice at doses of 150 and 300 mg/kg body weight orally on the wound healing process with the primary intention of gastric ulcer in rats, which increased significantly on day 20 in the dose-treated group. In group treated with 300 mg/kg hydroalcoholic extract of licorice showed the wound repair in comparison with the control group. Microscopic results of the operated gastric tissue showed that the hydroalcoholic extract at a dose of 300 mg/kg increased the mean rank of wound healing compared to the control group (Table 3).

The results of this study showed that licorice root is useful as an alternative due to its plant phenols, especially flavonoids, which have a wide range of biological activities, including antioxidant and anti-inflammatory activity.[48] Many studies with emphasis on different plant compounds derived from licorice root with biological activities including antioxidant and anti-inflammatory activity with different experimental models have shown that flavonoids act as antioxidants in different ways.[49,50] Frattaruolo studies showed that among the three compounds isolated from licorice, lycoflavonone has the best antioxidant activity, and inhibited the expression of alpha necrosis factor, interleukin-1 beta and interleukin-6 induced by the lipopolysaccharide wall of bacteria lycoflavonone by decreased expression of Inducible nitric oxide synthase (iNOS) levels and cyclooxygenase (COX) interferes with nitric oxide and PGE2 mediated inflammatory cascade. The results of our study could be helpful in finding newer treatments using herbal antioxidant stores to improve the antioxidant system in patients undergoing gastrotomy. The results of this study showed that the rate of lipid peroxidation in the gastrotomy rat model treated with hydroalcoholic extract of licorice root decreased with a marked increase in antioxidant activity, which accelerated the re-epithelization of the gastric ulcer. However, further studies are needed to molecularly evaluate the function of flavonoids.

Conflict of Interest

The authors declare no conflict of interest.

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