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Gastroprotective activity of *Loranthus acaciae* flower extract in a rodent model of ethanol-induced ulcer

Manal A. Abbas^{1*}, Yasser I. Kandil^{1,2}, Ahmad M. Disi³, Sahar M. Jaffal³

¹ Faculty of Pharmacy and Medical Sciences, Al-Ahliyya Amman University, 19328, Amman, Jordan

² Biochemistry Department, Faculty of Pharmacy, Al-Azhar University, Cairo 11884, Egypt

³ Department of Biological Sciences, Faculty of Science, The University of Jordan, 11942 Amman, Jordan

* Corresponding author email: m.abbas@ammanu.edu.jo

Manal A. Abbas email: m.abbas@ammanu.edu.jo

Yasser I. Kandil email: kandil.yasser@azhar.edu.eg

Ahmad M. Disi email: ahmadmdisi@yahoo.com

Sahar M. Jaffal email: sjaff333@gmail.com

Abstract

Loranthus acaciae (Loranthaceae) is a perennial green semi-parasitic plant used in ethnopharmacological medicine for healing wounds. The protective effect of *L. acaciae* on gastric ulcer induced by ethanol was investigated in a rat model. Ulcer index and total glutathione level were measured, histological and immunohistochemical studies for the expression of cyclooxygenase-2 were performed. Furthermore, chemical constituents of the flower extract were analyzed. Ulcer index was significantly lowered in *L. acaciae* treated groups. Protection ratios were 75.9%, 98.9% and 70.7% for *L. acaciae* 250 mg/kg, 500 mg/kg, and esomeprazole 40 mg/kg, respectively. Histological examination revealed fewer hemorrhage in mucosa and less edema in submucosa in *L. acaciae* treated groups compared to control. In esomeprazole-treated group, there was mild disruption in the surface epithelium and mild hemorrhage. However, edema and leucocytes infiltration in the submucosa layer were present. Immunohistochemical staining of stomach sections for cyclooxygenase-2 was negative in control group as well as in *L. acaciae* treated groups. Total glutathione level in mucosa layer of the stomach was higher in *L. acaciae* treated groups compared to control. LC-MS analysis revealed the presence of loranthin and rutin as the major constituents. It can be concluded that *L. acaciae* imparted a gastroprotective action against ethanol induced ulcer in rats.

Key points

1. 500 mg/kg *L. acaciae* protected the stomach by 98.9% from ulcerogenic effect of ethanol.

2. *L. acaciae* increased total glutathione level but not COX-2 expression in gastric mucosa.
3. Loranthin and rutin were the major constituents in *L. acaciae* flower extract.

Keywords: *Loranthus acaciae*, *Plicosepalus acacia*, gastroprotective, cyclooxygenase-2, ulcer index.

Draft

Introduction

The perennial green semi-parasitic plant *Loranthus acaciae* (*Plicosepalus acacia*) belongs to the family Loranthaceae (Badr et al. 2013). The nutritional requirement for this mistletoe is not satisfied. Therefore, it penetrates its haustoria into the host plants and siphon off the nutrients (Patel and Panda 2014). *L. acaciae* grows in desert where it parasitizes several species of Acacia. However, it may be found on other plants (Qasem 2009). Therefore, it is commonly called as Acacia strap flower (Ali-Shtayeh et al. 2014).

L. acaciae is economically important because it is used as good camel fodder (Gadour 2004). In folk medicine, it is used for abscesses and tumors (Al-Qura 2008). Also, the fresh whole plant is used as lactagogue and for the treatment of diabetes mellitus (Gadour, 2004; Elegami et al. 2001). A myriad of biological activities has been reported including antimicrobial (Elegami et al. 2001), antihyperglycemic (Aldawsari et al. 2014), antioxidant (Bamane et al. 2012), hepatoprotective (Sasidharan et al. 2010), neuroprotective (Kelsey et al. 2010; Chu et al. 2009) and protective action against cardiovascular diseases (Shargorodsky et al. 2010). In addition, *L. acaciae* was slightly active against *Candida albicans* (Omwenga et al. 2012) and inhibited rabbit jejunum motility (Gadour 2004).

The genesis of ethanol-induced gastric lesion with the decrease in gastric mucus amount is of multifactorial origin and is associated with significant production of free radicals leading to increased lipid peroxidation which in turn causes damage to cell and cell

membranes (Khazaei and Salehi 2006). Stomach ulcers are open sores formed due to the damage of the lining of stomach. Since *L. acaciae* is used in ethnopharmacological medicine for healing wound (Gadour 2004). The present work was performed to investigate the antiulcer effect of *L. acaciae* flower extract in ethanol-induced ulcer model and to study its mechanism of action.

Materials and Methods

Plant collection and extraction

L. acaciae flowers were collected in July 2014 from south Jordan. The plant was authenticated by Prof. Dawud Al-Eisawi (a botanist) at the Department of Biological sciences, The University of Jordan. A voucher specimen was deposited at Al-Ahliyya Amman University-Laboratory of Graduate Studies (Lor#7-2014). The flowers were dried at room temperature in a shady place. Fifty grams of dried flowers were grinded using Black and Decker coffee grinder. The coarse powder was macerated in 250 ml absolute methanol for 3 days and then evaporated using rotary evaporator at temperature not exceeding 45 °C. The dried flowers yielded 6 g brownish residue (percentage yield of 12%). The extract was stored in a deep freezer at -20 °C until used.

Anti-ulcer action

Animals were obtained from Al-Ahliyya Amman University animal house. All protocols and procedures for use and care of laboratory animals were approved by the ethical committee at Al-Ahliyya Amman University (ethical approval number AAU-2/4/2018). All rats were fed a standard diet and water. kept under standard animal house conditions

with 12 h light/12 h dark cycle with room temperature at 23 ± 2 °C. Female Wistar rats weighing 220-240 g were used. Ethanol-induced gastric ulcer was produced according to Abbas et al. (2009). Rats were placed in wire-floor cages to prevent coprophagy and were deprived of food 36 h before the experiment. Sucrose (10%) was available *ad libitum* instead of water, removed one hour before rats received their first treatment. Animals were divided randomly into 5 groups (8 rats each). Group I (negative control group) received vehicle (normal saline) by oral intubation (5 ml/kg). Group II (ulcer control group) received normal saline by oral intubation. Group III (standard drug group) orally received 40 mg/kg Esomeprazole (Nexium, AstraZeneca). Group VI and V orally received methanolic flower extract of *L. acaciae* at dose 250 mg/kg and 500 mg/kg, respectively. After 90 minutes, absolute ethanol (5 ml/kg) was administered by oral intubation to all groups except negative control group. Sixty minutes later, animals were sacrificed, stomach was removed, opened along the lesser curvature, washed with cold phosphate buffered saline and photographed. Total lesion area as well as the total area of the glandular portion was measured using Motic software version 2 (Motic China group CO, LTD). Ulcer index was calculated as the following: dividing the total area of the lesions in the stomach by the area of the glandular portion of stomach. Protection ratio of each fraction was calculated using the formula:

$$\text{Protection ratio (\%)} = 100 - \frac{\text{Ulcer index (test)}}{\text{Ulcer index (control)}} \times 100$$

The glandular portion was cut into 2 halves. One half was kept in deep freeze at -80 °C for antioxidant studies and the other half was kept in 10% formalin for further histological and histochemical studies.

Light microscopy

Stomachs were fixed in 10% buffered formalin, correctly oriented and embedded in paraffin. Five µm sections were stained with hematoxylin and eosin and examined using a light microscope (Leica) and photographed using MC 170 HD Leica Camera, Switzerland, and LAS EZ software. The evaluation of histological sections was evaluated by two of the authors in a blinded fashion (A.M. and D.A.). Treatment effects were evaluated for edema, inflammation and for sloughing, necrosis and presence of hemorrhagic lesions in mucosa.

Immunohistochemical staining for cyclooxygenase-2 (COX-2)

Formalin-fixed, paraffin-embedded sections of the stomach were mounted onto coated slides (Biocare Medical, USA) were placed in oven at 70 °C for 25 min, placed in xylene and hydrated in graded concentrations of alcohol. Antigen retrieval was performed according to Höing et al. (2018) by HIER (heat-induced epitope retrieval) in 0.01 M citrate buffer pH 6.0 in boiling water bath for 15 min. Sections were incubated with primary antibodies monoclonal mouse anti-human COX-2 (R&D System USA; 100 µg mL⁻¹) diluted (1:10 dilution in sterile phosphate buffered saline), pH 7.4, at 4 °C overnight in a humidity chamber. Streptavidin–biotin peroxidase staining was performed

using cell and tissue staining kit HRP-DAB System (R&D System, USA) according to manufacturer directions.

Liquid chromatography-mass spectrometric (LC-MS) analysis

LC-MS separation was performed with the mobile phase containing solvent A and B in gradient, where A was 0.1% (v/v) formic acid in water and B was 0.1% (v/v) formic acid in acetonitrile for the following gradient: 5% B for 5 min and 5-100% B in 15 min and 100% B for 5 min at a flow rate of 0.5 ml/min. Column was Agilent Zorbax Eclipse XDB-C18 (2.1x150 mm x 3.5 μ m), oven temperature 25 °C and the injection volume was 1 μ l and sample concentration was 18 mg/ml in methanol. The eluent was monitored by Shimadzu LC-MS 8030 with electrospray ion mass spectrometer (ESI-MS) under positive ion mode and scanned from 100 to 1000 m/z. ESI was conducted by using a fragmentor voltage of 125 V, skimmer 65 V. High-purity nitrogen (99.99%) was used as drying gas and at a flow rate of 10 L/min, nebulizer at 45 psi and capillary temperature at 350 °C. As a blank, 0.1 % formic acid. Sample was injected to mass detector by using Shimadzu CBM-20A system controller, LC-30AD pump, SIL-30AC autosampler with cooler and CTO-30 column oven.

Determination of total glutathione level in mucosa layer of the stomach

Mucosa layer of the stomach was scraped with a glass slide and stored at -20 °C until used. Homogenization was performed (1:9 wt/vol) in ice-cold cell lysing buffer (Cat. #: 895347. R&D Systems Inc. Minneapolis, USA,) using Teflon homogenizer (Potter-Elvehjem). The lysate was then cold-centrifuged at 12,000 g for 10 min at 4 °C.

Supernatants were collected and assayed for the total protein according to Lowry's method (Lowry, 1951). The deproteinized homogenates were then made into aliquots and used for the assessment of total glutathione.

Gastric total glutathione level was determined according to the method by Eyer and Podhradský (1986). Briefly, the method is based on the reductive cleavage of Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) by SH group of glutathione to yield a yellow color with a maximum absorbance at 405-412 nm. The glutathione concentration was expressed as $\mu\text{mol/mg}$ protein.

Statistical analysis

For all measured parameters, data were expressed as mean $\pm$ standard deviation (SD), one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used for comparison between variables. All data analyses were performed using GraphPad Prism version 6. (GraphPad Software, San Diego, USA) and $p \leq 0.05$ was considered as significant.

Results

Ulcer index was significantly lowered in *L. acaciae* treated groups as well as in Esomeprazole treated group (Figure 1). Protection ratio was 75.9% for *L. acaciae* (250 mg/kg) 98.9% for *L. acaciae* (500 mg/kg) and 70.7% for Esomeprazole.

Gross examination of glandular portion of the stomach in control group (vehicle-treated, ethanol-induced ulcer) revealed severe hemorrhagic lesions (Figure 2A). In *L. acaciae*

(500 mg/kg) and Esomeprazole-treated groups lesions were less pronounced and almost absent in most rats (Figure 2).

Histological examination of rat stomachs with ethanol-induced ulcers treated with vehicle (control group) revealed severe disruption of the surface epithelium, hemorrhage and edema in the submucosa layer with leucocyte infiltration. *L. acaciae* treated groups have fewer hemorrhage and less edema. In Esomeprazole-treated group (standard drug), there was mild disruption in the surface epithelium, mild hemorrhage. However, edema and leucocytes infiltration in the submucosal layer were extensive (Figure 3). Immunohistochemical staining of stomach sections for COX-2 was negative in control group as well as in *L. acaciae* treated groups. Esomeprazole gave positive staining indicating that it induces the expression of COX-2 in stomach (Figure 4).

LC-MS analysis detected the presence of 12 compounds in the flower extract of *L. acaciae* that constituted 99.8 % of the extract (Table 1). Loranthin was the major constituent and formed 20.9% of the extract.

Total glutathione level in mucosa of the stomach was significantly higher in *L. acaciae* groups compared to ulcer control or Esomeprazole-treated groups (Figure 5).

Esomeprazole has not increased the total glutathione level.

Discussion

Intragastric administration of absolute ethanol has long been used as a reproducible method to induce gastric mucosa lesions in laboratory animals. The effect of acute administration of absolute ethanol to rats on the gastric mucosa is dose-dependent and

appears as early as 30 minutes. The ethanol-induced gastric mucosal lesions and erosions are similar to those occurring in gastric ulcer in humans (Silva and de Sousa 2011). According to Liu and Cho (2000), *Helicobacter pylori* alone is not sufficient to produce ulceration in the gastric tissue. Other exogenous agents, such as non-steroidal anti-inflammatory drugs (NSAID) or ethanol, may also damage the gastric mucosa. In fact, excessive ethanol ingestion results in gastritis characterized by mucosal edema, subepithelial hemorrhages, cellular exfoliation, and inflammatory cell infiltration (Kvietys et al. 1999). Therefore, ethanol is considered a potential risk factor for gastric ulcer disease. It penetrates the gastric mucosa resulting from the solubilization of the protective mucous and exposing the mucosa to the hydrolytic and proteolytic actions of HCl and pepsin, which leads to injury of the lining of stomach. Ethanol can stimulate also acid secretion and disturb vascular endothelium. This will result in increased vascular permeability and edema formation (Kvietys et al. 1999). Also, ethanol triggers the release of superoxide anion leading to an increased oxidative stress (Terano et al. 1989).

In the present study, ethanol-induced ulcer model was used to study the protective effect of *L. acaciae* and compare it to the standard drug Esomeprazole. The protection ratio produced by *L. acaciae* flower extract (500 mg/kg) was 98.9% compared to 70.7% protection produced by 40 mg/kg Esomeprazole. Gross morphological as well as histological findings confirm the effectiveness of *L. acaciae* in protecting the stomach from the necrotizing agent ethanol. Up to our best knowledge, this study represents the first report that *L. acaciae* flower extract protects the stomach from the ethanol-induced ulceration. Similarly, antiulcer effect of other *Loranthus* species was reported like *L.*

micranthus that exerted gastroprotective effects in indomethacin induced ulcer model in rats (Ijioma et al. 2015).

In our study, the proton pump inhibitor Esomeprazole increased the expression of the inducible isoform COX-2 in mucosa as was indicated by increased immunostaining of sections with COX-2 antibody. Similar results were obtained by the proton pump inhibitor lansoprazole that induced an increment of gastric COX-2 expression and PGE-2 production after repeated administrations in rats (Tsuji et al. 2002). In the present study, the vehicle-treated group as well as *L. acaciae* failed to give positive results for COX-2 immunostaining.

LC-MS analysis detected the presence of 12 compounds in the flower extract of *L. acaciae* that constituted 99.8 % of the extract. Loranthin was the major constituent and formed 20.9% of the extract. Catechin, quercetin, rutin, gallic acid, methyl gallate and the polyhydroxylated flavanocoumarin loranthin were isolated from *P. acacia* (Syn. *L. acaciae*) (Badr et al. 2013). Quercetin 3-O glucoside was isolated from fresh flowers of *L. acaciae* (Jayanthi and Merina 2013). Extracts of *L. acaciae* contained reducing sugars, terpenoids and/or steroids, flavonoids and tannins but not alkaloids, saponins, anthraquinones or cardenolides (Waly et al. 2012). The presence of quercetin in flower extract of *L. acaciae* used in this study may explain, at least partially, the antiulcer activity of *L. acaciae*. Previous studies have demonstrated the effectiveness of quercetin in protecting the stomach in ethanol-induced ulcer model (Kahraman et al. 2003; Suzuki et al. 1998).

In the present study, *L. acaciae* increased total glutathione level in gastric mucosa. The higher dose (500 mg/kg) produced significantly higher protection ratio compared to the lower one (250 mg/kg). On the other hand, the lower dose elevated the total GSH more than the higher dose. However, this elevation was not significant, and this may indicate the presence of other mechanisms for the gastroprotective action. It is well established that ethanol is metabolized in the body with the liberation of superoxide anion and hydroperoxy free radicals. The liberated free radicals are implicated in acute and chronic ulceration in the stomach caused by ethanol (Wasman et al. 2010). It has been suggested that maintenance of gastric endogenous sulfhydryl compounds is an important mechanism to resist challenge with ethanol. In fact, ethanol induced damage to the gastric mucosa was associated with a significant decrease in glutathione gastric tissue values in experimental animals (Szabo et al. 1981). The methanolic extract of *L. acaciae* was rich in phenolic compounds and have high antioxidant activity *in vitro* (Bamane et al. 2012). Loranthin was detected in *L. acaciae* extract in our study. Previous studies have shown significant free radical scavenging activity of this compound *in vitro* (Badr et al. 2013).

Conclusions

In conclusion, *L. acaciae* imparted a gastroprotective action against ethanol induced ulcer in rats. One mechanism by which it exerted this effect is by decreasing oxidative stress through the elevation of total glutathione content in the mucosa of stomach. Other mechanisms may exist and need further elucidation as well as alterations in the biochemical parameters such as PGE-2. Future studies are needed to investigate the anti-

ulcer effect of the major active constituent “loranthin” present in *L. acaciae*. Also, the study of the anti-ulcer effect of this plant in other ulcer models such as NSAID-induced ulcer model and its effect against *H. pylori* is strongly recommended.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1: Chemical composition of *L. acaciae* flower methanolic extract as detected by LC-MS

Item #	Compound	%
1.	Campesterol	5.7
2.	Catechin	9.3
3.	(+)-Catechin 8-C-rhamnoside	2.7
4.	Epicatechin 3-O-gallate	8.8
5.	Linamarin	7.9
6.	Peltatoside	9.7
7.	Loranthin	20.9
8.	Lupeol	3.6
9.	Lupinine	8.4
10.	Quercetin	6.2
11.	Quercetin 3-O- β -D-glucopyranoside	6.4
12.	Rutin	10.2

Figure 1. Ulcer indices in ethanol-induced ulcer groups. Untreated ulcer control, *L. acaciae* and esomeprazole treated groups

Figure 2. Stomachs of ethanol-induced ulcers groups received vehicle (control) (A), 250 mg/kg *L. acaciae* flower extract (B), 500 mg/kg *L. acaciae* flower extract (C), and 40 mg/kg Esomeprazole (Standard drug) (D).

Figure 3. (A,C) Histological sections of rat stomachs with ethanol-induced ulcers treated with vehicle (control): Severe disruption of the surface epithelium, hemorrhage (white arrow in A) and edema in the submucosa layer with leucocyte infiltration (black arrow in C). (B, D) *L. acaciae* flower extract (500 mg/kg) has fewer hemorrhages (white arrow in B) and less edema (black arrow in D). Low dose of *L. acaciae* flower extract (250 mg/kg) has also few hemorrhages (white arrow in E). In Esomeprazole-treated group (standard drug), there was mild disruption in the surface epithelium and mild hemorrhage. However, edema and leucocytes infiltration in the submucosal layer were extensive (black arrow) (F) (H&E stain).

Figure 4. Immunohistochemical staining of stomach sections for COX-2. (A) Ethanol-induced ulcer in stomach of rats treated with vehicle (control), (B) *L. acaciae* flower extract 500 mg/kg and (C) Esomeprazole (Standard drug). Note the positive staining (arrows point to darkly staining dots) in C only.

Figure 5. Total glutathione level in stomachs of studied groups.

535x412mm (300 x 300 DPI)