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Gastroprotective activity of oleanolic acid derivatives on experimentally induced gastric lesions in rats and mice

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Abstract

The gastroprotective effect of the triterpene oleanolic acid (OA) was assessed on gastric ulceration in rats. The effect of a single oral dose of OA was evaluated at 50, 100 and 200 mg kg^{-1} in the following models: pylorus ligature (Shay), and aspirin- and ethanol-induced gastric ulcers. A single oral administration of OA at doses of 50, 100 and 200 mg kg^{-1} inhibited the appearance of gastric lesions induced by ethanol, aspirin and pylorus ligature. In the pylorus ligature and aspirin models, the effect of OA at the selected concentrations was comparable with that of ranitidine at 50 mg kg^{-1}. In the ethanol-induced gastric lesion model, OA showed a dose-dependent activity, and at 100 and 200 mg kg^{-1} was as active as omeprazole at 20 mg kg^{-1}. The effect of OA, its acetylated and methoxylated derivatives, oleanonic acid and its methyl ester were assessed on HCl/ethanol-induced ulcers in mice at 200 mg kg^{-1}. OA and its methoxylated (OAM) and acetylated (OAAM, OAA) derivatives proved to be active in this animal model. The semisynthetic derivatives OAM and OAAM had the greatest gastroprotective activity, but their effect was not significantly greater than OA. In an acute toxicity test on mice, intraperitoneal administration of OA showed no toxicity at doses up to 600 mg kg^{-1}.

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Introduction

In recent years, much effort has been directed towards identifying new anti-ulcer drugs from natural resources. Many plants are used in traditional medicine to treat gastrointestinal disorders, including peptic ulcers. Some have originated anti-ulcer drugs, for example carbenoxolone from *Glycyrrhiza glabra*, solon from sophoradin and gefarnate from cabbage (Lewis & Hanson 1991). It has been reported that some triterpenes or their derivatives display gastroprotective activity in different models of induced gastric lesions in animals (Lewis & Hanson 1991; Matsuda et al 1998). Oleanolic acid (OA) is a triterpene widely distributed in the plant kingdom and can be obtained in high yields from the Chilean medicinal plant *Fabiana imbricata* R. et P. (Solanaceae).

We report the gastroprotective activity of OA on three different models of experimentally induced gastric ulcer in rats: pylorus ligature, ethanol and aspirin. Ethanol- and aspirin-induced gastric lesion models were used because they represent some of the most common causes of gastric ulcer in humans. In addition, OA and five derivatives were assessed for gastroprotective effect in the HCl/ethanol ulcer model in mice.

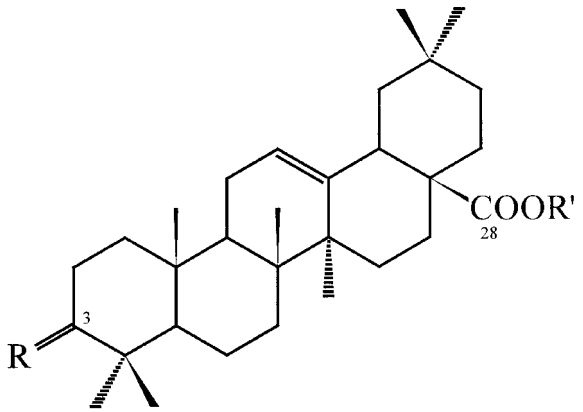
Materials and Methods

Animals

Fasted male Sprague–Dawley rats from the Central Animal House of the Universidad de Talca, 200–250 g, were used to assess the gastroprotective effect of OA in the pylorus ligature (Shay), ethanol- and aspirin-induced gastric ulcer models. Fasted Swiss albino mice, 30±3 g, were used in the HCl/ethanol model. Fasting for 24 h before ulcerogenic assays was used because reference compounds (ranitidine and omeprazole) or OA were administered orally. For acute toxicological effects, Swiss albino mice, 30±3 g, were used. The animals were fed on certified Champion diet with free access to water under standard conditions of temperature, humidity and 12-h light–dark cycle. The protocols were approved by the Universidad de Talca Institutional Animal Care and Use Committee, which follows the recommendations of the Canadian Council on Animal Care (Olfert et al 1993). The number of animals used was determined on the basis of statistical power calculations as reported elsewhere (Antonio & Souza Brito 1998; Souza Brito et al 1998; Hiruma-Lima et al 2001).

Drugs

The following drugs were used: omeprazole (Losec; Merrell Lepetit Farm. Ltd, Sao Paulo, Brazil), lansoprazole, ranitidine, Tween 80 and aspirin (Sigma Chemical Co., St Louis, MO), ethanol and HCl (Merck, Darmstadt, Germany). Omeprazole and ranitidine were used as standard anti-ulcer drugs. Oleanolic acid (3β-hydroxyolean-12-en-28-oic acid) **1** was isolated from the aerial parts of *Fabiana imbricata* (Solanaceae) as previously reported (Schmeda-Hirschmann & Papastergiou 1994) and recrystallized in hexane–CH_2Cl_2 as colourless crystals, mp 196–198°C; $[\alpha]^{20}$ 35.8 (c = 0.33, $CHCl_3$). The derivative **2** was obtained by methylation of the COOH at C-28, and **3** was obtained by methylation at C-28 and acetylation of the OH at C-3. Oleanolic acid acetate **4** was prepared by acetylation of OA. Methylation was performed with diazomethane. Acetylation was carried out with acetic anhydride/pyridine. Oleanonic acid (3-oxoolean-12-en-28-oic acid) **5** was obtained by pyridinium chlorochromate oxidation of OA. Methylation of **5** with diazomethane yielded the methyl ester **6**. Melting points and $[\alpha]^{20}$ of the derivatives are as follows: **2**, mp 197°C, $[\alpha]^{20}$ 59.2 (c = 0.093, $CHCl_3$); **3**, mp 209°C, $[\alpha]^{20}$ 95 (c = 0.060, $CHCl_3$); **4**, mp 255°C, $[\alpha]^{20}$ 63 (c = 0.103, $CHCl_3$); **5**, mp 169°C, $[\alpha]^{20}$ 98 (c = 0.040, $CHCl_3$); and **6**, mp 180°C, $[\alpha]^{20}$ 90 (c = 0.060, $CHCl_3$). The spectroscopic data of OA and the derivatives were in agreement with those reported by Ahmad & Rahman (1994). The structures of compounds **1**–**6** are presented in Figure 1.



	R	R'	
1	OH, H	H	Oleanolic acid (OA)
2	OH, H	CH_3	OAM
3	OAc, H	CH_3	OAAM
4	OAc, H	H	OAA
5	O	H	OAC
6	O	CH_3	OACM

Figure 1 Structure of oleanolic acid and its semi-synthetic derivatives.

In-vivo toxicity

Acute oral toxicity of OA was assessed in male Swiss albino mice, after fasting for 12 h, using a protocol previously described (Souza Brito et al 1998). Increasing doses of OA were administered intraperitoneally to groups of 10 animals for each dose level (100, 200, 400 and 600 mg kg^{-1}). Animals receiving the vehicle (12% Tween 80, 10 mL kg^{-1}) served as controls. The groups were observed at 0, 30, 60, 120, 180 and 240 min after OA administration and then twice a day for the next 14 days. At the end of this period, the number of survivors was recorded and the acute toxicological effect was inferred on the basis of mortality, expressed as LD50 according to Litchfield & Wilcoxon (1949). The intraperitoneal route was selected because of the large amount of the compound to be administered and the low solubility of OA in the vehicle.

Acute gastric lesions

The gastroprotective activity of OA was assessed on three different experimentally induced gastric ulcer models.

Pylorus ligature
A total of 30 animals were randomly distributed into five groups and fasted for 48 h with free access to water. A pylorus ligature was performed as described by Shay et al (1945) 1 h after oral administration of OA (50, 100 and 200 mg kg^{-1}), ranitidine (50 mg kg^{-1}) as a positive control, or vehicle (12% Tween 80, 10 mL kg^{-1}). At 4 h after drug administration, animals were killed, the abdomen was opened and another ligature was placed around the oesophagus close to the diaphragm. The stomach was removed, inspected externally and its contents drained into a graduated centrifuge tube and centrifuged at 2000 rev min^{-1} for 10 min. The supernatant volume and pH were recorded. Gastric lesions were evaluated by examining the inner gastric surface with a dissecting binocular microscope. Mucosal lesions were counted and the ulcerative index was determined according to the method of Souza Brito et al (1998). Briefly, the ulcerative index was calculated using the following formula: ulcerative index = (Ax3)+(Bx2)+C, where A corresponds to ulcers >3 mm, B to lesions between 1 and 3 mm, and C to lesions <1 mm.

Aspirin ulcer
A total of 30 animals were randomly distributed into five groups and fasted for 24 h with free access to water before the experiment. At 1 h after oral administration of OA (50, 100 and 200 mg kg^{-1}), ranitidine (50 mg kg^{-1}) or 12% Tween 80 (10 mL kg^{-1}), 200 mg kg^{-1} of aspirin was orally administered to unanaesthetized rats in each group according to Williamson et al (1996). Animals were killed 4 h later. Stomachs were removed, opened and the ulcerative index determined as described above.

Ethanol-induced ulcer
A total of 30 animals were randomly distributed into five groups and fasted for 24 h with free access to water before the experiment. The ethanol-induced lesions assay was carried out according to the method of Morimoto et al (1991). Ethanol (1 mL, 99.5%) was orally administered to animals that had 1 h previously been treated with OA (50, 100 and 200 mg kg^{-1}), omeprazole (20 mg kg^{-1}) or 12% Tween 80 (10 mL kg^{-1}). At 1 h after ethanol administration, animals were killed, the stomachs were removed, opened and the ulcerative index determined as described above.

HCl/EtOH-induced ulcer
This experiment was performed as described by Yesilada et al (1997). A total of 64 mice were randomly distributed into eight groups and fasted for 24 h with free access to water before the experiment. At 50 min after oral administration of OA or its derivatives **2**–**6** (200 mg kg^{-1}), lansoprazole (20 mg kg^{-1}) or 12% Tween 80 (10 mL kg^{-1}), all groups were orally treated with 0.2 mL of a solution containing 0.3 M HCl/60% ethanol (HCl/EtOH) to induce gastric ulcers. Animals were killed by cervical dislocation 1 h after the administration of HCl/EtOH, and the stomachs were excised and inflated by injection of saline (2 mL). The ulcerated stomachs were fixed in 5% formalin for 30 min and opened along the greater curvature. Gastric damage visible to the naked eye was observed in the gastric mucosa as elongated black-red lines, parallel to the long axis of the stomach similar to the HCl/EtOH-induced lesions in rats. The length (mm) of each lesion was measured, and the lesion index was expressed as the sum of the length of all lesions.

Statistical analysis

Results are presented as mean$\pm$s.d. Statistical significance was determined by one-way analysis of variance followed by Duncan's test, with the level of significance set at $P<0.05$. Analysis of variance followed by a post-hoc test is commonly used in this type of gastroprotective study (Antonio & Souza Brito 1998; Souza Brito et al 1998; Hiruma-Lima et al 2001). In our experiments, the Bartlett's test for homogeneity of variance was performed after analysis indicating that groups were homogeneous and that analysis could be carried out (Gad & Weil 1994).

Results

Acute toxicity

At doses up to 600 mg kg^{-1}, intraperitoneal administration of OA did not cause toxicity or mortality in mice. Therefore, the intraperitoneal LD50 for OA should be greater than 600 mg kg^{-1}.

Acute gastric lesions

The effects of OA on the three models of induced gastric lesions are shown in Table 1. A single oral administration of OA at doses of 50, 100 and 200 mg kg^{-1} inhibited the appearance of gastric lesions induced by ethanol, aspirin

Table 1 Effects of oleanolic acid (OA) at 50, 100 and 200 mg kg^{-1} on pylorus ligature, and aspirin- and ethanol-induced gastric ulcers in rats.

Experiment	Treatment (p.o.)	Dose (mg kg^{-1})	Ulcerative index (mean±s.d.)
Pylorus ligature	Control	–	93.7±6.9
	Raniditine	50	50.2±4.4**
	OA	50	62.0±12.7**
	OA	100	45.2±5.4**
	OA	200	49.3±14.0**
Aspirin-induced gastric ulcer	Control	–	69.8±12.8
	Raniditine	50	35.8±9.4**
	OA	50	40.7±9.7**
	OA	100	43.2±8.1**
	OA	200	41.0±9.8**
Ethanol-induced gastric ulcer	Control	–	73.3±12.7
	Omeprazole	20	40.6±11.1**
	OA	50	60.6±7.4*
	OA	100	50.8±3.3**
	OA	200	46.8±7.7**

*$P < 0.05$, **$P < 0.01$, significantly different compared with corresponding control (analysis of variance followed by Duncan's test).

Table 2 Gastroprotective effect of oleanolic acid (OA, **1**), its semisynthetic derivatives **2**–**6** and lansoprazole in gastric ulcer induced by HCl/EtOH in mice.

Treatments (p.o.)	Dose (mg kg^{-1})	Lesion index (mm)
Control	–	39.3±2.9
Lanzoprazole	20	9.8±2.1**
OA (**1**)	200	18.4±1.1**
OAM (**2**)	200	16.9±3.3**
OAAM (**3**)	200	15.7±5.6**
OAA (**4**)	200	24.6±2.1*
OAC (**5**)	200	36.2±7.1
OACM (**6**)	200	25.4±3.7*

Results are expressed as means±s.d. *$P < 0.05$, **$P < 0.005$, significantly different compared with corresponding control (analysis of variance followed by Duncan's test).

and pylorus ligature. In the pylorus ligature and aspirin models, the effect of OA at the selected concentrations was comparable with that of ranitidine at 50 mg kg^{-1} (Table 1). In the ethanol-induced gastric lesion model (Table 1), OA showed a dose-dependent activity, and at 100 and 200 mg kg^{-1} was as active as omeprazole at 20 mg kg^{-1}. In pylorus-ligated rats, no significant modifications in gastric volume and gastric pH were observed (data not shown).

The gastroprotective effects of OA and its synthetic derivatives **2**–**6** on the HCl/EtOH-induced ulcers are presented in Table 2. At 200 mg kg^{-1}, OA and derivatives **2**–**4** proved to be active in this animal model. The effects of derivatives **1**–**3** were similar to a single dose of lansoprazole at 20 mg kg^{-1}.

Discussion

Intraperitoneal administration of OA in mice did not cause toxicity or mortality at doses up to 600 mg kg^{-1}. Singh et al (1992) reported similar findings in mice and rats with an oral LD50 greater than 2 g kg^{-1}. OA displayed gastroprotective effects in three different experimentally induced gastric ulcer models in rats. The selected models represented different mechanisms of gastric ulcerogenesis (Desai & Parmar 1993). In a study of the protective effects of OA oligoglycosides on EtOH- and indometacin-induced gastric lesions in rats, Matsuda et al (1998) did not find an effect of OA on EtOH-induced gastric mucosal lesions in doses up to 50 mg kg^{-1}. Our results indicated a mild, but significant, effect of OA at 50 mg kg^{-1} ($P < 0.05$).

OA and its derivatives **2**–**6** were evaluated at 200 mg kg^{-1} in the HCl/EtOH model in mice. The dose was selected because previous experiments at 50, 100 and 200 mg kg^{-1} showed a significant gastroprotective effect of OA at 200 mg kg^{-1} (data not shown). The semisynthetic derivatives **2** and **3** presented the greatest gastroprotective activity, but their effects were not significantly greater than OA. Methylation of the COOH function at C-28 with a free or sterified (acetylated) hydroxy group at C-3 did not affect the gastroprotective activity. Oxidation of the hydroxy group to ketone drastically reduced the activity of the triterpene as shown with compound **5**.

The absence of significant effects on gastric volume and pH in pylorus-ligated rats suggests that endogenous prostaglandins (PGs) did not mediate the gastroprotective action of OA. However Lewis & Hanson (1991) have pointed out that triterpenoids such as carbenoxolone and OA maintains the PG content of gastric mucosa at high levels owing to an inhibitory action on PG catabolizing enzymes. Similar explanations for the effect of other anti-ulcer agents of plant origin have been proposed (Baker 1994). The mechanism of the gastroprotective effect of OA is not well understood. The inhibitory effect of OA on enzymes other than PG catabolizing enzymes are presented here. Recent reports on the bioactivity of OA pointed to its inhibitory effect on HIV-1

protease with an IC50 of 10 μg mL^{-1} (Ma et al 2000), inhibition of DNA polymerase β (Deng et al 1999), cytotoxicity against cultured human tumour cell lines (Kim et al 2000), chitin synthase II inhibitory activity with an IC50 of 5.6 μg mL^{-1} (Jeong et al 1999), inhibition of 12-*O*-tetradecanoylphorbol-13-acetate stimulated ^{32}Pi-incorporation into phospholipids of cultured cells (Kinoshita et al 1999), and inhibition of insoluble glucan synthesis from *Streptococcus mutans* (Kozai et al 1999).

Several biological activities have been associated with OA and its close relative ursolic acid. OA is widely distributed in plants and occurs in several medicinal and food plants in variable concentrations. The pharmacology of OA and ursolic acid has been revised by Liu (1995). Both compounds are effective against chemically induced liver injury and have anti-inflammatory and antihyperlipidaemic properties in laboratory animals and antitumour-promotion effects.

OA and its glycosides display hepatoprotective activity. Kinjo et al (1999) studied the protective effects of OA saponins and its derivatives on in-vitro immunological liver injury of primary cultured rat hepatocytes. OA showed both hepatoprotective action and weak hepatotoxicity, while the effect of the saponins represented a balance between hepatoprotective action and hepatotoxicity. The protective effects of OA against carbon tetrachloride (CCl_4)-induced hepatotoxicity may, at least in part, be owing to its ability to block bioactivation of CCl_4 mainly by inhibiting the expression and activity of cytochrome P450 2E1 (Jeong 1999).

Other studies on OA and its derivatives include the anti-HIV activity of OA, pomolic acid and structurally related triterpenoids (Kashiwada et al 1998). OA inhibited HIV-1 replication in acutely infected H9 cells with an EC50 of 1.7 μg mL^{-1}, and inhibited H9 cell growth with an IC50 of 21.8 μg mL^{-1}, being less toxic than ursolic acid. OA derivatives proved to be even more active than the natural product.

In conclusion, this study demonstrates that OA displays a significant gastroprotective effect against gastric ulcers induced by pyloros ligature and ethanol in rats. The gastroprotective effect was dose-dependent in the ethanol model and was not different at doses of 100 and 200 mg kg^{-1}. Some structure–activity relationships can be deduced from the experiment with OA and its derivatives. Oxidation of the OH at C-3 reduced the activity of the triterpene, whereas methylation of the COOH at C-28 with or without acetylation at C-3 did not affect the gastroprotective effect of the compounds. More studies are necessary to determine the mechanism of action of OA and its derivatives.

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