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Research Paper

Gastroprotective activity of methanol extract and marrubiin obtained from leaves of *Marrubium vulgare* L. (Lamiaceae)

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Abstract

Objectives The purpose of this study was to assess the gastroprotective properties of the methanol extract and the diterpene marrubiin obtained from the leaves of *M. vulgare*.
Methods Assays were performed using different protocols in mice. Studies focusing on mechanisms of gastroprotection were also undertaken.
Key findings In the model of ethanol-induced ulcers, we observed a significant reduction in all the parameters analysed; the curative ratios obtained were 49.31 ± 0.57, 74.31 ± 0.91 and 79.86 ± 0.59 for the groups treated with 50 and 100 mg/kg of extract of *M. vulgare* and omeprazole (30 mg/kg), respectively. For indomethacin-induced ulcers, the percentages of ulcer inhibition were 50.32 ± 5.60, 66.24 ± 4.30, 82.17 ± 04.09 and 67.52 ± 4.38, for the groups treated with 25, 50 and 100 mg/kg *M. vulgare* and positive control (cimetidine), respectively. In both models, the marrubiin (25 mg/kg) produced a significant reduction in all the parameters when compared with the control group ($P < 0.01$). There was also a significant increase in pH and mucus production in the groups treated with *M. vulgare* extract and marubiin. The results also demonstrated that the gastroprotection induced by the extract and marubiin is related to the activity of nitric oxide and endogenous sulfhydryls, which are important gastroprotective factors.
Conclusions The results of this study show that the extract of *M. vulgare* and marrubiin displays antiulcer activity and that this effect can be partly attributed to the isolated diterpene.
Keywords antiulcerogenic activity; diterpene; gastroprotection; marrubiin; *Marrubium vulgare*

Introduction

Peptic ulcers and dyspepsia are conditions that affect thousands of people around the world, and are therefore considered a global health problem.[1–3] Ulcers are usually caused by an imbalance between aggressive and protective factors in the stomach. Some of these factors are acid secretion, *Helicobacter pylori* infection, mucus secretion, blood flow, cell regeneration and prostaglandins.[4] Exogenous factors, such as smoking, anti-inflammatory drugs, alcohol, stress and other factors, are also responsible for the increase in the incidence of gastric ulcers.[5]

The main therapeutic aim in the treatment of ulcers is to control acid secretion using antacids, H2 receptor blockers, anticholinergics or proton pump blockers.[6] However, most of the drugs currently available show limited efficacy and are often associated with severe side effects, such as hypersensitivity, arrhythmia, impotence, gynecomastia and hematopoietic changes. There is therefore a need to develop more effective and less toxic agents.[1]

Marrubium vulgare L. (Lamiaceae), popularly known as white horehound (maromba or marroio in Brazil), is widely used in traditional medicine to treat inflammation, gastrointestinal disorders and respiratory diseases.[7] Several experimental studies have demonstrated its antihypertensive, anti-inflammatory, antioxidant, analgesic, antispasmodic, hypoglycemic and hypolipidemic properties.[8–15] Phytochemically, *M. vulgare* is characterized by the presence of a variety of compounds, such as alkaloids, steroids, lactones, tannins, flavonoids

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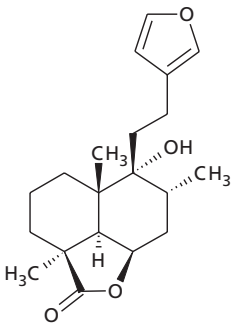

Figure 1 Marrubiin.

and, in particular, terpenes. The most abundant of the terpenes described for this plant is marrubiin, a furan labdane diterpene (Figure 1).[7,12]

In view of the traditional use of this plant for the treatment of gastrointestinal ailments, the fact that there are many terpenes with gastroprotective activity[2,16,17] and that gastroprotective properties of this species have not yet been properly studied, our research group decided to evaluate the antiulcer potential of its methanol extract, as well as marrubiin obtained from *M. vulgare* leaves, in different experimental ulcer models that would contribute to the validation of the antiulcer activity of *M. vulgare* and the diterpene marrubiin.

Materials and Methods

Plant material

The leaves of *M. vulgare* were collected in December 2009 in the town of Bom Retiro (latitude 27°47′50'S and longitude 49°29′21′W), in the state of Santa Catarina, Brazil, and were identified by comparison with a voucher deposited at the FLOR Herbarium (Florianopólis, SC) under number 4725.

Extraction and isolation of marrubiin

The leaves of *M. vulgare* were air dried for 10 days. After this period plant material was powdered, producing particles of 2.86 ± 0.463 mm^2. A quantity of 1.5 kg was macerated with occasional agitation (three times per day) with methanol (4 l), for 7 days at room temperature (25 ± 3°C). After maceration, the solvent was evaporated at 70°C to promote the formation of marrubiin from the premarrubiin of the plant.[18]

Marrubiin (**1**) was isolated from leaves of *Marrubium vulgare* with a yield of approximately 0.1%, according to a previously described method.[18] It was identified on the basis of its spectroscopic data (^{1}H and ^{13}C NMR and IR) as compared to the literature[19,20] and co-TLC with an authentic sample.

Drugs, reagents and solvents

Indomethacin, cimetidine, omeprazole, carbenoxolone, N_G-nitro-L-arginine methyl-ester (L-NAME) and N-ethylmaleimide (NEM) were purchased from Sigma-Aldrich (St Louis, MD, USA). All the other reagents and solvents used were of analytical grade.

Animals

Swiss mice (25–35 g) were provided by the Central Animal House of the Universidade do Vale do Itajaí, Itajaí-SC, Brazil. The animals were housed in groups of six, in standard cages, at room temperature (25 ± 3°C), with 12 h dark/12 h light cycles, and were provided with food and water *ad libitum*. For the experiments, the animals were transferred for the laboratory and allowed an adaptation period of 5 days. Twelve hours prior to the experiments, the food was withdrawn but access to water *ad libitum* was maintained. In all the experiments, the animals were kept in cages with raised, wide-mesh floors to prevent coprophagy. The animals used in the present study were housed and cared for in accordance with Brazilian Federal Government legislation on animal care. The experiments were also authorized by the Ethical Committee for Animal Care of the Universidade do Vale do Itajai, Itajai, Santa Catarina, Brazil.

Evaluation of antiulcer activity

Ethanol/HCl-induced ulcer

The experiment was performed according to the method described by Schmeda-Hirschmann *et al*.[21] After 12 h of fasting, the mice were randomly divided into six groups of six animals each. The first group was given 0.5 ml of vehicle (1% Tween-80 aqueous solution), and the second group was treated with omeprazole (30 mg/kg). The remaining groups received 25, 50 and 100 mg/kg of methanol extract of *M. vulgare* and 25 mg/kg of marrubiin. All the treatments were administered orally by gavage. One hour after treatment, all the mice received 0.5 ml of a 0.3 mol/l HCl/60% ethanol solution (ethanol/HCl) to induce gastric ulcers. One hour later, the animals were sacrificed by cervical dislocation, and the stomachs were removed and opened along the greater curvature. The stomachs were gently rinsed with water to remove the gastric contents and blood clots before scanning. The images obtained were analysed using the parameters described in the section on stomach analysis.

Non-steroidal anti-inflammatory drug-induced ulcers in cholinomimetic-treated mice

The experiment was performed according to the method described by Rainsford,[22] but with some modifications. After 12 h of fasting, the mice were randomly divided into five groups of six animals each. The first group was given 0.5 ml of vehicle (1% Tween-80 aqueous solution), and the second group was treated with cimetidine (100 mg/kg). The remaining four groups received 25, 50 and 100 mg/kg of the extract of *M. vulgare* and 25 mg/kg of the marrubiin isolated compound. All the treatments were administered orally by gavage. One hour after treatment, all the mice received a combination of indomethacin (100 mg/kg, p.o.) and bethanechol (5 mg/kg, i.p.) to induce gastric ulcers. Twelve hours after treatment with indomethacin and bethanechol, the animals were sacrificed by cervical dislocation. The stomachs were removed and opened along the greater curvature. The stomachs were gently rinsed with water to remove the gastric contents and blood clots before scanning. The images obtained were analysed using the parameters described in the section on stomach analysis.

Stomach analysis

The images obtained were analysed using specific EARP software to measure each lesion point. The ulcers were classified

as foolows: level I, ulcer area <1 mm^2; level II, ulcer area 1–3 mm^2; level III, ulcer area >3 mm^2. The total area of lesion and the percentage of lesion area in relation to total stomach area were determined. The ulcerative lesion index (ULI) was calculated as follows:

$$1\times(\text{number of ulcers level I})+2\times(\text{number of ulcers level II})+3\times(\text{number of ulcers level III})$$

The cure ratio was determined as follows: $100 - (ULI_{treated} \times 100/ULI_{control})$.[23]

Determination of gastric secretion

The assay was performed using the method of Shay *et al*.[24] with some modifications. The animals were divided into groups ($n = 6$) according to the treatment used, as previously described. After 24 h of fasting, the animals were anesthetized with thiopental sodium (10 mg/kg, i.p.), the abdomen was incised and the pylorus was ligated. Immediately after pylorus ligature, extract of *M. vulgare* and marrubiin were administered at doses of 25, 50, 100 and 25 mg/kg, respectively. Cimetidine (100 mg/kg) was used as positive control, and 1 ml of vehicle (1% Tween-80 aqueous solution) was administered as negative control. All the samples were administered intraduodenally. Four hours later, the animals were sacrificed by cervical dislocation, the abdomen was opened and another ligature was placed at the esophageal end. The stomachs were removed and the gastric contents collected and centrifuged at 3000 rpm (8000*g*, 25°C, 10 min). The volume of gastric-juice acid (ml) and the pH values were determined. The total acid secretion in the gastric juice in the supernatant volume was determined by titration as pH 7.0, using a 0.01 mol^{-1} NaOH solution and phenolphthalein as indicator.

Determination of mucus in gastric content

This assay was performed according to the methodology described previously by Sun *et al*.,[25] with some modifications. The mice were divided into groups ($n = 6$). After 24 h of fasting, the abdomen was incised under anesthesia and the pylorus was ligated. Immediately after pylorus ligature, 25, 50 and 100 mg/kg of methanol extract of *M. vulgare* and 25 mg/kg of marrubiin were administered intraduodenally. Carbenoxolone (250 mg/kg) was used as positive control and 0.5 ml of vehicle (1% Tween-80 aqueous solution) was administered as negative control. The animals were killed 4 h after the drug treatments. The stomach contents were immersed in 10 ml of 0.02% Alcian blue 0.16 M sucrose/0.05 M sodium acetate solution, pH 5.8, and incubated for 24 h at 20°C. The Alcian blue binding extract was centrifuged at 3000 rpm for 10 min. The absorbency of the supernatant was measured by spectrophotometry at 615 nm. The free mucus in the gastric content was calculated from the amount of Alcian blue binding (mg/wt tissue (g)).

Role of nitric oxide and sulfhydryl compounds in gastric protection

These experiments were based on the method of Matsuda *et al*.,[26] with some modifications. Male Swiss mice, having fasted for 24 h, were divided into eight groups, four of which received saline (i.p.) while the other four received 70 mg/kg of nitric oxide (NO) synthase inhibitor (L-NAME) or 10 mg/kg of sulfhydryl depleter (NEM). Thirty minutes after the pretreatment, the oral treatments were applied by gavage (saline, carbenoxolone 100 mg/kg, extract of *M. vulgare* 100 mg/kg and marrubiin 25 mg/kg). Sixty minutes later, 0.5 ml of a 0.3 mol/l HCl/60% ethanol solution was given orally by gavage to each rodent, and the animals were sacrificed after 1 h. The stomachs were removed to determine the extent of gastric lesion. The images obtained were analysed using the parameters described in the section on stomach analysis.

Acute toxicity study

The acute toxicity study was performed as described in 'Guidelines for Testing of Chemicals – Acute Oral Toxicity – Fixed Dose Procedure'.[27] Five female rats were treated orally with a single dose of 2000 mg/kg extract of *M. vulgare* extract or marrubiin dissolved in 1% Tween-80 aqueous solution. After administration, the animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention during the first 4 h) and daily thereafter for a period of 14 days. Once a day the animals were observed, looking principally for changes in skin, fur, eyes and mucous membrane (nasal), and also autonomic changes (salivation, lacrimation, perspiration, piloerection, urinary volume and defecation) and alterations to the central nervous system (ptosis, drowsiness, gait, tremors and convulsion). Food and water were provided throughout the experiment.

Statistical analysis

The data are reported as mean ± standard error of the mean (SEM) and were compared using one-way analysis of variance (ANOVA) followed by Tukey's test, with P values < 0.05 considered significant.

Results

In the ethanol/HCl-induced ulcer model, treatment with the methanol extract of *M. vulgare* (50 and 100 mg/kg) and omeprazole (30 mg/kg) significantly reduced the lesion index, the total injured area and the percentage injured area, compared with the control group ($P < 0.05$ and $P < 0.01$) The curative ratios obtained were 49.31 ± 0.57, 74.31 ± 0.91 and 79.86 ± 0.59 for the groups treated with 50 and 100 mg/kg of methanol extract of *M. vulgare* and omeprazole (positive control, 30 mg/kg), respectively. The dose of 25 mg/kg of the methanol extract of *M. vulgare* showed no significant results. In contrast, the main compound isolated from the plant, the diterpene marrubiin (25 mg/kg), significantly reduced all parameters when compared with the control group ($P < 0.01$) (Table 1).

In the indomethacin/bethanecol-induced ulcer model, it was observed that the extract of *M. vulgare* (25, 50 and 100 mg/kg) and the positive control cimetidine (100 mg/kg) significantly reduced all parameters evaluated when compared with the control group ($P < 0.01$). The percentages of ulcer inhibition were 50.32 ± 5.60, 66.24 ± 4.30, 83.17 ± 04.09 and 67.52 ± 4:38 for the groups treated with 25, 50 and 100 mg/kg *M. vulgare* and positive control (cimetidine), respectively (Table 2).

Table 1 Effects of methanol extract of *M. vulgare*, marrubiin and omeprazole on ethanol-induced ulcers in mice

Treatment (p.o)	Dose (mg/kg)	Total area of lesion (mm^2)	Percentage of lesion area	Ulcer lesion index	Curative ratio (%)
Control	–	33.97 ± 7.97	11.08 ± 2.94	28.80 ± 2.10	–
Omeprazole	30	4.13 ± 1.67**	1.56 ± 0.56**	5.80 ± 2.57**	79.86 ± 0.59
Extract	25	21.35 ± 5.25	5.78 ± 1.20	18.20 ± 2.57	36.81 ± 1.14
	50	11.90 ± 3.40	4.14 ± 1.15	14.60 ± 3.07**	49.31 ± 0.57
Marrubiin	100	3.20 ± 0.76**	1.41 ± 0.16**	7.40 ± 2.01**	74.31 ± 0.91
	25	3.32 ± 1.16**	1.42 ± 0.58**	11.00 ± 2.38**	44.44 ± 11.09

Results presented as mean ± SEM for six mice. Statistical comparison was performed using ANOVA followed by the Tukey test. $^{**}P < 0.01$ compared with the control group.

Table 2 Effects of methanol extract of *M. vulgare* and cimetide on nonsteroidal anti-inflammatory drug/bethanechol-induced ulcer in mice

Treatment (p.o)	Dose (mg/kg)	Total area of lesion (mm^2)	Percentage of lesion area	Ulcer lesion index	Curative ratio (%)
Control	–	11.30 ± 1.49	3.53 ± 0.47	31.40 ± 5.60	–
Cimetidine	30	3.99 ± 0.59**	1.44 ± 0.11**	10.20 ± 0.80**	67.52 ± 4.38
Extract	25	3.67 ± 1.14**	1.72 ± 0.26**	15.60 ± 1.03**	50.32 ± 5.60
	50	2.94 ± 0.57*,**	1.14 ± 0.28*,**	10.60 ± 0.92**	66.24 ± 4.30
	100	2.68 ± 0.91*,**	0.73 ± 0.26*,**	5.60 ± 1.63*,**	82.17 ± 4.09*
Marrubiin	25	2.34 ± 0.65*,**	0.61 ± 0.21*,**	4.10 ± 1.16*,**	86.94 ± 2.75*

Results presented as mean ± SEM for six mice. Statistical comparison was performed using ANOVA followed by the Tukey test. $^{*}P < 0.05$, compared with the cimetidine group; $^{**}P < 0.01$ compared with the control group.

Table 3 Effects of methanol extract of *M. vulgare*, marrubiin and cimetidine, administered intraduodenally, on the biochemical parameters of gastric juice obtained from pylorus-ligature in mice

Treatment (p.o)	Dose (mg/kg)	Volume (ml)	pH	[H+] mEq/l/4 h
Control	–	0.74 ± 0.05	3.62 ± 0.13	155.21 ± 16.02
Cimetidine	100	0.38 ± 0.02*	5.49 ± 0.17**	40.55 ± 1.23**
Extract	25	0.39 ± 0.06*	3.91 ± 0.18	123.34 ± 13.40
	50	0.36 ± 0.09*	4.80 ± 0.41**	83.05 ± 15.13**
Marrubiin	100	0.33 ± 0.08**	5.01 ± 0.24**	48.36 ± 4.36**
	25	0.37 ± 0.10**	4.36 ± 0.32**	94.99 ± 15.98**

Results presented as mean ± SEM for six mice. Statistical comparison was performed using ANOVA followed by the Tukey test. $^{*}P < 0.05$ compared with the control group; $^{**}P < 0.01$ compared with the control group.

Regarding the parameters of gastric secretion it was observed that the extract of *M. vulgare* at doses of 50 and 100 mg/kg, marrubiin at a dose of 25 mg/kg and the positive control (cimetidine, 100 mg/kg) caused increased gastric pH and decreased the concentration of H^+ ions compared with the control group ($P < 0.01$) (Table 3). Assessment of the free gastric mucus in the tissue showed that the extract of *M. vulgare* at doses of 25, 50 and 100 mg/kg and marubiin at 25 mg/kg significantly enhanced mucus production when compared with the control ($P < 0.01$) (Table 4).

Pre-treatment with L-NAME revealed that the gastroprotective effects of the extract and marrubiin are strongly related to NO synthesis. The percentage of ulcerative lesions was increased from 1.17 ± 0.27% for the group treated with extract to 13.23 ± 1.34% for extract and L-NAME, while the increase was from 1.42 ± 0.58 for the group treated with marrubiin to 26.33 ± 3.62% for marrubiin and L-NAME.

Table 4 Effects of methanol extract of *M. vulgare*, marrubiin and carbenoxolone on Alcian blue binding to free gastric mucus from pylorus ligature in mice

Treatment (p.o)	Dose (mg/kg)	Alcian blue bound (mg/wt tissue (g))
Control	–	5.16 ± 0.26
Carbenoxolone	250	7.09 ± 0.42**
Indomethacin	100	3.07 ± 0.24
Extract	25	6.10 ± 0.41**
	50	6.78 ± 0.70**
Marrubiin	100	7.04 ± 0.25**
	25	6.52 ± 0.34**

Results presented as mean ± SEM for six mice. Statistical comparison was performed using ANOVA followed by the Tukey test. $^{*}P < 0.05$ compared with the control group; $^{**}P < 0.01$ compared with the control group.

Pre-treatment with NEM showed that there was a clear dependence on SHs for marrubiin but only a slight dependence for the extract. The percentage of ulcerative lesions was increased from 1.57 ± 0.19% for animals treated with extract to 10.11 ± 0.81% for extract and NEM, while the increase was from 1.42 ± 0.58% for animals treated with marrubiin to 38.57 ± 5.22% for marrubiin and L-NEM (Figures 2 and 3).

In the acute toxicity study, no signs of toxicity were observed after the administration of *A. satureoides* extract at a dose of 2000 mg/kg. There were also no changes in food and water consumption during the period of observation.

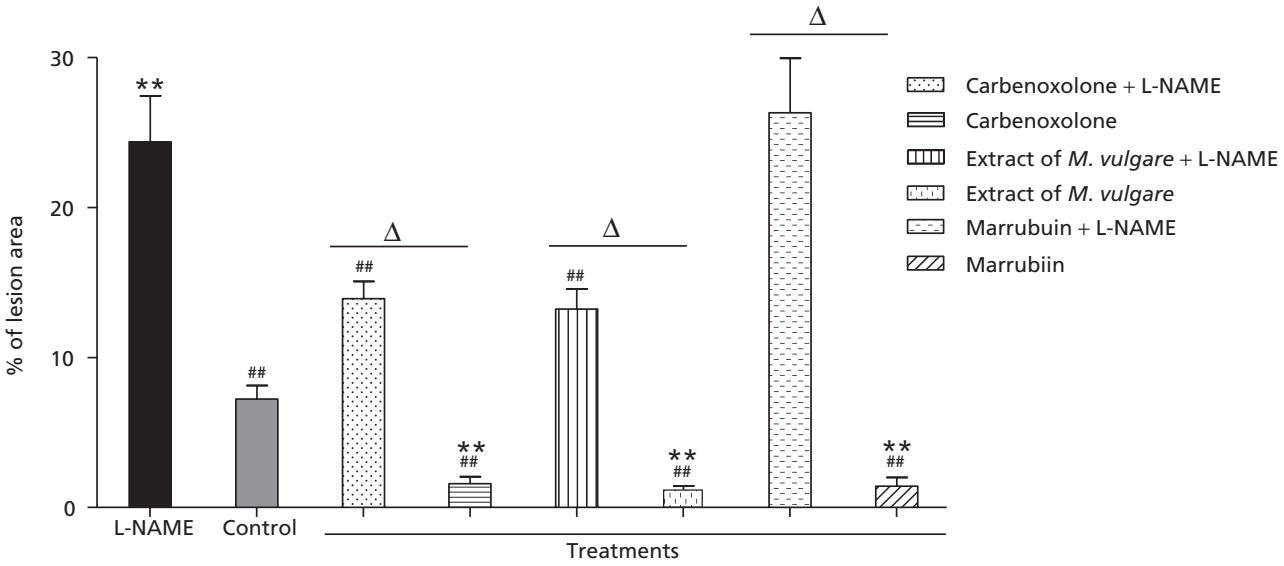

Figure 2 Effects of methanol extract of *M. vulgare* (100 mg/kg), marrubiin (25 mg/kg) and carbenoxolone (200 mg/kg) in lesions induced by ethanol in mice pre-treated with L-NAME (70 mg/kg, i.p.) or with saline (control group). Results are mean ± SEM of six mice. Statistical comparison was performed using ANOVA followed by Tukey test. $^{**}P < 0.01$ when compared with the control group, $^{\#\#}P < 0.01$ when compared with the L-NAME group. Δ indicates intragroup difference considering pre-treatment with L-NAME and pre-treatment with saline ($P < 0.01$).

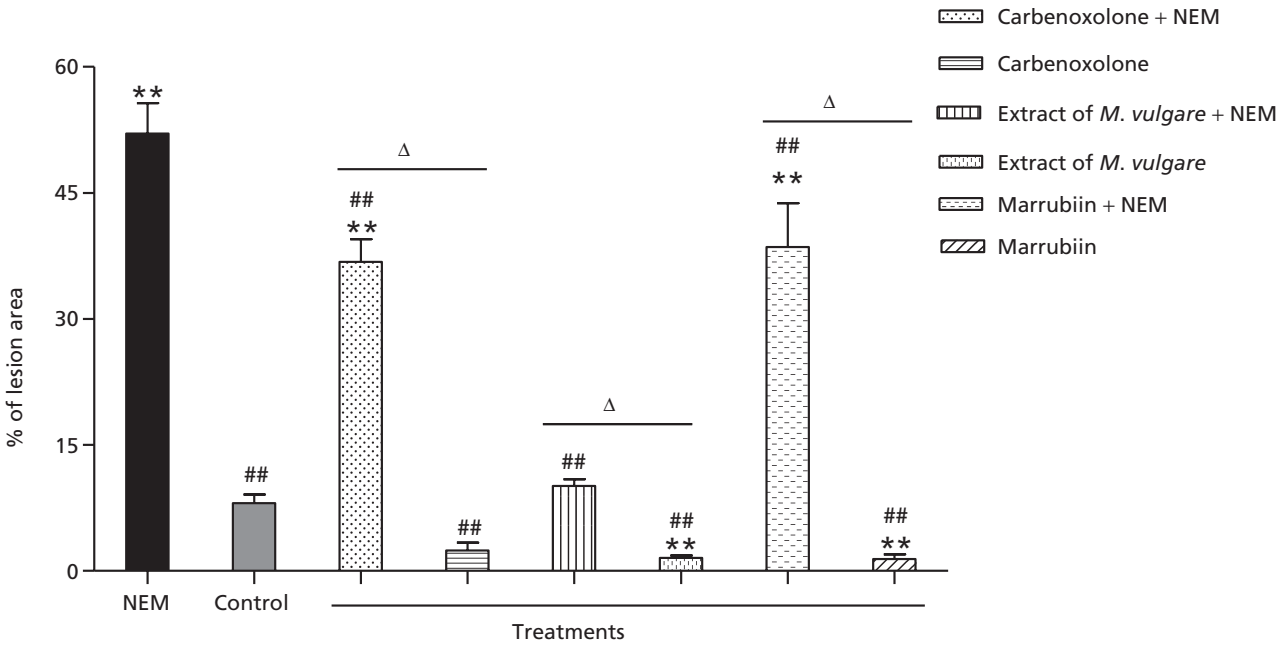

Figure 3 Effects of methanol extract of *M. vulgare* (100 mg/kg), marrubiin (25 mg/kg) and carbenoxolone (200 mg/kg) in lesions induced by ethanol in mice pre-treated with NEM (10 mg/kg, i.p.) or with saline (control group). Results are mean ± SEM of six mice. Statistical comparison was performed using ANOVA followed by Tukey test. $^{**}P < 0.01$ when compared with the control group, $^{\#\#}P < 0.01$ when compared with the NEM group. Δ indicates intragroup difference considering pre-treatment with NEM and pre-treatment with saline ($P < 0.01$).

Discussion

Current options for the treatment of gastric ulcers include antacids and antisecretory drugs, mainly antagonists of histamine-2 receptors and proton-pump inhibitors, which block acid gastric secretion.[28] These treatments are effective, but can cause many adverse effects, such as hypersensitivity, arrhythmia, impotence, gynecomastia and hematopoietic disorders.[3,29] Research and development of new antiulcer therapies is therefore focused on the search for agents that combine efficacy and lower toxicity, and in this context medicinal plants are a promising source of new candidate compounds.[4]

M. vulgare is a plant used for treating various diseases, including gastric ulcers.[7] However, to best of our knowledge there are no studies in the scientific literature to support this activity. For this reason, we have evaluated the possible antiulcer activity of methanol extract obtained from leaves of *M. vulgare* and its main compound, the diterpene marrubiin.

First, the gastroprotective activity was assessed using the models of ulcers induced by ethanol/HCl and indomethacin/bethanecol, which are among the most widely used tests for screening compounds with antiulcer activity. Ethanol is a potent ulcerogenic agent that acts by dissolving the gastric mucus, resulting in increased flow of Na^+ and H^+ in the lumen, stimulating the secretion of histamine, pepsin and H^+.[3,30,31] Moreover, ethanol causes the tissue to enter a state of blood stasis, leading to severe necrotizing lesions.[32]

In this model, we found that treatment with methanol extract of *M. vulgare* (50 and 100 mg/kg) and omeprazole (30 mg/kg) significantly reduced the lesion index, the total injured area and the percentage injured area, compared with the control group. On the other hand, the main compound isolated from the plant, the diterpene marrubiin (25 mg/kg), significantly reduced all parameters when compared with the control group. These results indicate that the extract has cytoprotective activity that is correlated, at least in part, with the presence of marrubiin.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin act by causing ulcerogenic effects because prostaglandin synthesis is inhibited through inhibition of cyclooxygenase.[33] Prostaglandins play an important role in the stomach, stimulating the secretion of protective factors such as bicarbonate ions, mucus and producing increased mucosal blood flow.[4] Thus, the suppression of prostaglandin synthesis by NSAIDs in the gastric tissue is a damaging process. The screening of extract of *M. vulgare* for antiulcer activity was therefore also performed using the model of ulcers induced by NSAIDs. It was observed that the extract of *M. vulgare* (25, 50 and 100 mg/kg) and the positive control cimetidine (100 mg/kg) significantly reduced all parameters evaluated when compared with the control group. These results suggest the possible involvement of prostaglandin and mucus production in the antiulcer effect of the extract.

Based on the data obtained in acute ulcer tests, we studied whether the extract of *M. vulgare* and marrubiin could somehow be interfering in gastric secretion, since the drugs that are mainly used in ulcer therapy currently act through this mechanism. To this end we performed the pylorus ligature test, which provides important parameters for evaluating the involvement of compounds on gastric secretion because the stretching of the mucosa caused by pyloric obstruction leads to increased release of acetylcholine, which acts directly on the parietal cells, inducing the secretion of hydrochloric acid.[24,29,34] In this model we observed that the extract of *M. vulgare* at doses of 50 and 100 mg/kg, marrubiin at a dose of 25 mg/kg and the positive control (cimetidine, 100 mg/kg) caused increased gastric pH and decreased the concentration of H^+ ions, compared with the control group. These results suggest that the extract and marrubiin interfere with gastric acid secretion.

The mucus coating is important because it protects the mucosa, as well as facilitating the repair of damage caused to the gastric epithelium.[35] It was therefore important to assess whether the extract and marrubiin affect the production of gastric mucus. The extract of *M. vulgare* at doses of 25, 50 and 100 mg/kg, as well as marubiin at 25 mg/kg, significantly enhanced mucus production when compared with the control.

To investigate the influence of endogenous NO on the gastroprotective effects of the extract *M. vulgare* and marubiin, mice were pretreated with an inhibitor of nitric oxide synthase (NOS), L-NAME, an analogue of L-arginine, which is hydrolysed to produce L-nitro arginine, inhibiting NOS activity. NO is an important endogenous transmitter released by the endothelial cells when the mucosa is exposed to damaging agents. Its main function is to protect the gastric mucosa of the vasopressor agents and inhibit gastric acid secretion of the parietal cells.[36] Our data demonstrate that the gastroprotection induced by the extract of *M. vulgare* and marubiin is strongly related to nitric oxide activity.

SHs are important to protect the gastric mucosa, especially when reactive oxygen species are involved in lesions of the mucosa.[37] To investigate the involvement of endogenous sulfhydryls in the gastroprotective effects of the extract of *M. vulgare* and marrubiin, NEM, a thiol blocker of SHs, was used. The data suggest that the gastroprotective mechanism of the extract of *M. vulgare* is slightly related to SHs. On the other hand, gastroprotection mediated by marrubiin seems to be much more closely related to SHs.

Marrubiin is one of the main active compounds of *M. vulgare*, and is interesting from a medical point of view because of its antinociceptive, anti-inflammatory and vasorelaxant activities.[14,15] Moreover, some marrubiin derivatives also show analgesic potential when evaluated against some classical models of pain in mice.[12] However, despite its interesting biological activities, no study has been carried out to evaluate the gastroprotective potential of this compound. Antiulcer and gastroprotective activities of diterpenes have previously been described in several studies, some examples of which we will now describe. From *Aparisthmium cordatum* bark extract a furan diterpenoid with a clerodane skeleton, called aparisthman, was isolated and tested in dose of 100 mg/kg, v.o.. The results revealed a significant decrease in gastric injury induced by indomethacin/bethanechol, ethanol, pylorus ligature and hypothermic restraint-stress in animals. These effects seem to be related to an increase in the defensive mechanisms of the stomach, such as prostaglandin synthesis and mucus production. Similar effects were also observed for marrubiin in this study.[38] Schmeda-Hirschmann *et al*.[39] isolated three diterpenes (imbricatolic acid, 15-hydroxyimbricatolal and 15-acetoxyimbricatolic acid) from the resin of

Araucaria araucana, and assessed these for gastroprotective effects at 50, 100 and 200 mg/kg. A dose-related gastroprotective effect was observed for the three terpenes with significant activity at doses up to 100 mg/kg. Rodrigues *et al*.[40] evaluated the quinoid diterpenes barbatusin and 3β-hydroxy-3-deoxibarbatusin, isolated from *Plectranthus grandis*, in ethanol-induced gastric injury in mice. Doses of 5 and 10 mg/kg significantly reduced gastric lesions. The authors reported that the gastroprotection observed was related to prevention of the depletion of gastric mucus and gastric mucosal non-proteic-sulfhydryl groups as well as an increase in thiobarbituric acid-reactive species.

In addition, data obtained in a test for acute oral toxicity by a procedure of fixed dose (OECD 420, 2001) suggest that the toxic dose of *M. vulgare* extract is higher than 2000 mg/kg. It would therefore be classified in category 5 for toxicity under the criteria of the GHS (Globally Harmonized Classification System for Chemical Substances and Mixtures).

It is important to point out that the combination of the anti-inflammatory, antinociceptive and gastroprotective effects in the same plant extract or compound is interesting, and should be taken into account because of the serious limitations of a large number of anti-inflammatory and analgesic agents that produce gastric irritation, bleeding and mucosal cellular damage.

Conclusions

In conclusion, the results of this study show that the extract of *M. vulgare* and marrubiin displays antiulcer activity, and this effect can be partly attributed to the isolated diterpene. This compound could be used in further studies as a model to obtain more effective derivatives, looking for new and efficacious medicinal agents to treat gastric ulcers. Moreover, the data suggest that the gastroprotection presented by *M. vulgare* and marrubiin is related to its ability to stimulate the synthesis of mucus (an important gastroprotective factor) and to decrease acid gastric secretion (aggressive factor). It is dependent on nitric oxide activity and sulfhydryl groups, which are important gastroprotective factors that have vasodilator effects, inhibit gastric acid secretion and are antioxidants in gastric mucosa. However, further pharmacological and toxicological investigations are required to enable their use in the treatment of gastric ulcers.

Declarations

Conflict of interest

The author(s) declare(s) that they have no conflicts of interest to disclose.

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