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Effects of limonene and essential oil from *Citrus aurantium* on gastric mucosa: Role of prostaglandins and gastric mucus secretion

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ABSTRACT

Essential oil from *Citrus aurantium* and the monoterpene limonene are widely used flavoring agents that are found in some common food items. This specie is also used medicinally throughout the world to treat gastritis and gastric disorders. Therefore, biological assays were performed *in vivo* on essential oil of *C. aurantium* (OEC) and its majority compound limonene (LIM) to evaluate their effect on gastric mucosa. The OEC (250 mg/kg, p.o.) and LIM (245 mg/kg, p.o.) provided effective (99%) gastroprotection against lesions induced by absolute ethanol and NSAID (non-steroidal anti-inflammatory drug) in rats. OEC and LIM do not interfere with gastric H^+ secretion, serum gastrin or glutathione (GSH) level in gastric mucosa. But the gastroprotective action of OEC and LIM occurs due to an increase in the gastric mucus production induced by conserving the basal PGE_2 levels after challenge by agents harmful to the gastric mucosa. Given that LIM and OEC are excellent flavoring agents and also present gastroprotective actions, they can be regarded as a promising target for the development of a new drug for the prevention of gastric damage.

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1. Introduction

Peptic ulcer, a heterogeneous disease of multifactorial etiology, is one of the most common chronic illnesses among working-age adults. In the USA, approximately 4 million individuals have peptic ulcers, while each year 350,000 new cases are diagnosed, about 100,000 patients are hospitalized and at least 3000 people die as a result of this disease [1]. Despite great advances in the understanding of the peptic ulcer illness, its etiology has not been completely elucidated. The basic physiopathological concept is that the peptic ulcer results from disruption in the normal balance between its aggressive factors (secretion and action of acid and pepsin) and defensive aspects (secretion and action of mucus and bicarbonate) [2]. It is also known that several endogenous factors are related to the pathophysiology of gastroprotection including prostaglandin E_2 (PGE_2), somatostatin, nitric oxide (NO) and sulfhydryl (SH) compounds [3]. The etiopathogenesis of gastric ulcer involves genetic factors, physiopathological disturbances and environmental factors such as alcohol, non-steroidal anti-inflammatory drugs (NSAIDs) and *Helicobacter pylori*, among others [4].

The global expansion in the consumption of alcohol and NSAIDs has contributed to the growth of gastric ulcer incidence. The NSAIDs are among the most frequently prescribed pharmacological agents. Although their ability to cause gastrointestinal ulcer was demonstrated many years ago [5], NSAIDs continue to promote serious injuries to the gastric mucosa. The ulcerogenic effect of NSAIDs has been related to the potential of this drug to inhibit the synthesis of PGE_2 [6]. The augmented acid secretion also contributes to this harmful process, as does the fact that NSAID provokes disturbances in the gastric microcirculation, increases neutrophil infiltration, induces TNF-α expression, and disrupts the balance between NO expression and apoptosis [7].

The introduction of H_2 receptor antagonists and proton-pump inhibitors has been associated with an increase in the ulcer cure rate, despite the knowledge that the prolonged use of these medications provokes serious side effects such as hypergastrinemia, defined as a serum gastrin level above the normal range, and reduced pH in gastric lumen [8]. The success of pharmacological treatments to prevent or to cure ulcerative lesions depends not only on blocking acid secretion, but also on augmenting mucosal protective factors including prostaglandins, growth factors, somatostatin, nitric oxide (NO), and sulfhydryl compounds (SHs) [3].

According to Lewis and Hanson [9], among substances found in nature, terpenes constitute the main chemical compound class with antiulcerogenic activity. The specie *Citrus aurantium* L. (Rutaceae

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family), popularly known in Brazil as orange-bitter or orange-sour, is among the species most frequently used for medicinal purposes [10]. The parts of this plant most often utilized by the population for medicinal ends are the fruit peel, flowers and leaves. *C. aurantium* is used for treating gastrointestinal tract disorders and for its diuretic action against tachycardia and rheumatism [11]. Phytochemical analyses of the essential oil in the fresh fruit peel of *Citrus* species revealed the presence of limonene (LIM), a monoterpene as the majority constituent [12]. Limonene is one of the most common terpenes in nature and is the majority constituent of an essential oil series. Its pleasant citric fragrance is commonly used as a flavoring in foods and drinks [12], for which it is classified in the U.S. Code of Federal Regulation as safe. Tests in animals have proven the effectiveness of limonene against some types of cancer including gastric, mammary, pulmonary adenoma and liver [12]. Limonene also has been shown to be effective in relieving gastroesophageal reflux disorder and occasional heartburn but the action mechanism has not been elucidated [13].

On the basis of the popular indications of this plant for treating gastrointestinal disturbances, the present work aimed to characterize the effects of the essential oil of *C. aurantium* (OEC) and of its majority constituent limonene (LIM) on the gastric mucosa of animals challenged with different ulcerogenic agents that commonly attack the gastric mucosa in humans.

2. Materials and methods

2.1. Essential oil

A plant sample of the specie *C. aurantium* was collected and the exsiccates deposited in the Herbarium "Irina D. Gemtchujnicov"—BOTU, Department of Botany at Unesp, under n° BOTU 23123. After the fruits were collected at Unesp-Botucatu, fresh fruit peels of *C. aurantium* L. were submitted to extraction of essential oil by water vapor with the aid of a Clevenger type device (Marconi, Brazil). The vegetal material (fruits peels) was mixed inside a glass balloon (5 L) with distilled water and put on a heated pad. The essential oil (OEC) obtained was stored in an amber bottle at 5 °C temperature until the accomplishment of the pharmacological experiments and phytochemical analyses.

2.2. Identification of substances

The OEC samples were analyzed in a gas chromatographer coupled to an electronic (70 eV) mass spectrometer (GC-MS, Shimadzu, QP-5000) equipped with a capillary column of fused silica (OV-5, 30 m × 0.25 mm × 0.25 μm), helium as carrier gas (1.0 mL/min, White Martins, 99.9%), injector at 24 °C, detector at 230 °C and *split* injection mode. Mass spectrum acquisition was performed at the mass range from 40 to 500 *m*/*z*. The essential oil (1 μL) was diluted in ethyl acetate to produce 1 mL of chromatographic grade solvent, 1 μL of which was injected as sample at the split ratio of 1:15. The column temperature was heated to 60 °C and programmed at 3 °C/min to 240 °C. The identification was realized through the comparison of its mass spectra with the GC–MS system database (NIST 62 lib.), the literature and with the Kovats retention indices [14].

2.3. Reagents and isolated substances

The following drugs were used: cimetidine, indomethacin, carbenoxolone, N-nitro-L-argenine, N-ethylmaleimide, DTNB (5,5′-ditio-bis 2-nitrobenzoic acid), NADPH, malonyldialdehyde, ruthenium red, capsaicin, Alcian Blue and limonene (Sigma Chemical Co., USA), absolute ethanol (EEL, Brazil), atropine (Ariston, Brazil) as well as mircene and octanal (Acros Organics, USA).

2.4. Animals

Male Swiss albino mice (25–45 g) and male Wistar albino rats (170–250 g) from the UNESP Central Animal House were used. The animals were fed a certified Nuvilab® (Nuvital) diet with free access to tap water under standard conditions of 12 h dark–12 h light and temperature (21 ± 1 °C). All experiments were performed in the morning and followed the recommendations of the Canadian Council on Animal Care [15]. The UNESP Institutional Animal Care and Use Committee approved all of the employed protocols.

2.5. Antiulcerogenic activity

2.5.1. Gastric injuries

Based on their respective specifications, the groups under each experimental model included positive (carbenoxolone or cimetidine) and negative (vehicle-Tween 80 at 8%) controls. Fasting was used prior to all assays because standard drugs were always administered orally (by gavage) or intraduodenally. Moreover, the animals were kept in cages with raised floors of wide mesh to prevent coprophagy. After each experiment the animals were killed; the stomachs were opened along the greater curvature, pressed onto a glass plate, and scanned so that the lesions could be counted with aid of the AVSoft program. The results were expressed as total ulcerated area (mm^2).

2.5.2. Ethanol-induced ulcer

After fasting for 24 h, the experimental groups (male rats, $n = 5$) were submitted to the treatments (p.o.) with vehicle, carbenoxolone (100 mg/kg), OEC (50, 100 or 250 mg/kg) or LIM (245 mg/kg, the dose calculated based on its percentage present in OEC composition) 1 h before induction of gastric injury by absolute ethanol. Animals were killed 1 h after ethanol administration, the stomachs were removed, opened along the greater curvature and the injuries calculated as described previously [16].

2.5.3. NSAID-induced ulcer

The gastric injuries were induced by oral administration of indomethacin 100 mg/kg in male rats ($n = 5$). The treatments (p.o.) with vehicle, carbenoxolone (100 mg/kg), OEC (50, 100 or 250 mg/kg) and LIM (245 mg/kg) were carried out 30 min before administration of the NSAID. Five hours after the NSAID administration the animals were killed and the stomachs removed for lesion quantification [17].

2.5.4. Evaluation of the gastric juice parameters

Male rats were randomly divided into 6 groups of 7 animals each that fasted for 24 h with free access to water. Thirty minutes after oral treatment or immediately after intraduodenal administration of a single dose of OEC (250 mg/kg), LIM (245 mg/kg), and cimetidine (100 mg/kg) as positive control or vehicle, pylorus ligature was performed [18]. Four hours later the animals were sacrificed, the abdomen opened and another ligature placed around the esophagus close to the diaphragm. The stomach was removed, inspected internally, and its contents drained into a graduated centrifuge tube and centrifuged at 2000 × *g* for 15 min. The total acid content of gastric secretion was determined by titration to pH 7.0 with 0.01 N NaOH using a digital burette (E.M., Hirschmann Technicolor, Germany). The total concentration of acid was expressed as mequiv./mL/4 h.

2.6. Evaluation of mucosal protective factors

2.6.1. Determination of mucus adhering to the gastric wall

After 24 h of fasting the rats, under anesthesia, were submitted to longitudinal incision slightly below the xiphoid apophysis

for the pylorus ligature. The administration (p.o.) of the vehicle, carbenoxolone (200 mg/kg), OEC (250 mg/kg) and LIM (245 mg/kg) was performed 1 h before the ligature. After 4 h, the animals were killed, the glandular portion of the stomach was separate, weighed and immersed in Alcian Blue solution for the mucus quantification procedure. The absorbencies were measured in a spectrometer at 598 nm and the results expressed as μg of Alcian Blue/g of tissue [19].

2.6.2. Quantification of serum gastrin

The oral administration of the vehicle, cimetidine (100 mg/kg), OEC (250 mg/kg), and LIM (245 mg/kg) was carried out 1 h before the pylorus ligature in male rats ($n = 6$) [18]. Four hours after the ligature the animals were killed and the blood was collected for the serum quantification of gastrin through the immune-enzymatic method, utilizing a kit manufactured by the company Assay Designs (USA).

2.6.3. Determination prostaglandin (PGE_2) levels

The methodology was according to Curtis et al. [20]. The animals (male rats, $n = 5$) had been divided randomly into the groups sham, vehicle, vehicle + NSAID, OEC, OEC + NSAID, LIM and LIM + NSAID. First NSAID was administered (indomethacin 30 mg/kg, s.c.), and 30 min afterwards the animals were treated (p.o.) with vehicle, cimetidine (100 mg/kg), OEC (250 mg/kg) or LIM (245 mg/kg). Thirty minutes after the oral treatment, the rats were killed and the stomachs removed. The prostaglandin E_2 level was quantified with an immune-enzymatic kit dosage kit from R&D Systems (USA).

2.6.4. Quantification of total glutathione (GSH)

Male rats ($n = 5$) were treated (p.o.) with vehicle, carbenoxolone (100 mg/kg), OEC (250 mg/kg) or LIM (245 mg/kg) 1 h before inducing gastric lesions by absolute ethanol [16]. One hour after the administration of the harmful agent, the stomachs were removed, opened and washed with saline solution so that stomach samples could be collected, weighed and homogenized. This method was based on the total oxidation of glutathione utilizing reagent DTNB (5,5′-dithiobis 2-nitrobenzoic acid), followed by reduction of the oxidized form with the enzyme glutathione reductase and NADPH [21]. The concentration of reduced glutathione was determined by the reduction speed of DTNB that generates detectable staining in a spectrophotometer at 412 nm.

2.6.5. Evaluation of intestinal motility

The effect of OEC on intestinal motility in mice ($n = 10$) was tested using the charcoal method of Stickney and Northup [22], with modifications. These animals had fasted for 6 h but were allowed free access to water. Five groups of mice were pretreated orally with vehicle, atropine (5 mg/kg) or OEC (50, 100 or 250 mg/kg). After 30 min, each animal received activated charcoal 10% (10 mL/kg, p.o.). All animals were killed 30 min later and the small intestine rapidly dissected out to enable immediate measurement of the distance traversed by the charcoal meal from the pylorus to the ileocecal junction, expressed as a percentage of the total distance and the values were transformed to arcsine for statistical analyses.

2.6.6. Evaluation of the antioxidant activity in vitro

The antioxidant activity of OEC and LIM was evaluated according to the method described by Stocks et al. [23], which is based on the colorimetric determination of the formation of malonyldialdehyde (MDA) induced by lipidic peroxidation utilizing ferrous sulfate and acid ascorbic acid in lipidic membranes of rat brain. The lipidic peroxidation was determined by the reaction of the MDA with thiobarbituric acid.

2.6.7. Determination of the role of nitric oxide (NO) and sulfhydryl compounds (SH) in gastric protection

Male rats ($n = 5$) were divided into 6 groups and pretreated (i.p.) with saline, L-NAME (N-nitro-L-arginine methyl 70 ester mg/kg) an inhibitor of the NO synthesis or NEM (N-ethylmaleimide, 10 mg/kg) a blocker of SH compounds [24]. Thirty minutes after the pretreatment the animals were administered (p.o.) vehicle, carbenoxolone (100 mg/kg) or OEC (250 mg/kg). After 60 min all the groups received 1 mL absolute ethanol to induce gastric ulcers. One hour after receiving ethanol the rats were killed for determination of gastric lesions.

2.6.8. Evaluation of the participation of sensorial nerves

This method was carried out according to Pongpiriyadacha et al. [25], with modifications. To evaluate the possible involvement of the vanilloid receptors (VR-1) in the protective effect of OEC, rats were pretreated with ruthenium red (RR) (6 mg/kg, s.c.), a VR-1 receptor antagonist of neurons sensitive to capsaicin. Male rats ($n = 5$) were divided into 6 groups, namely, 3 groups pretreated with RR and the other 3 with saline. Thirty minutes after the pretreatment the animals were treated orally with vehicle, capsaicin (4 mg/kg) or OEC (250 mg/kg). After 60 min all animals received 1 mL of absolute ethanol (p.o.), to induce gastric ulcers. One hour after the administration the rats were killed to enable characterization of the gastric injury area.

2.7. Statistical analysis

Results were expressed as mean ± S.E.M. and statistical significance was determined by one-way analysis of variance followed by Dunnett's or Tukey's test with $P < 0.05$ defined as significant.

3. Results

Chromatographic analysis of four OEC samples indicated that the majority compound is a monoterpene called limonene (97.83%), while mircene comprises 1.43% and octanal 0.45% of the total OEC composition (Table 1).

In both the NSAID- and absolute ethanol-induced gastric ulcer models (Table 2), the OEC provided significant gastric protection at doses of 250 and 100 mg/kg, with the former dose being more effective in the two models. In both models LIM, the majority constituent of OEC, also presented effective (99%) gastroprotection when challenged by these two agents harmful to gastric mucosa. The LIM dose used in the experiments was calculated by applying its percentage in the OEC composition (97%) to the more effective OEC dose (250 mg/kg).

The gastric juice parameters of the rats submitted to the treatment with the essential oil and limonene administered by different routes (Table 3) demonstrated that the oral treatment with OEC and LIM diminished the H^+ concentration in the gastric juice without modifying its volume. Although the systemic evaluation of the intraduodenal OEC and LIM administration showed no modification of the H^+ concentration, gastric juice volume was diminished under this administration route.

Table 1
Chemical composition of essential oil from fruit peels of *Citrus aurantium* (OEC) measured by gas chromatograph coupled to a mass spectrometer.

	Sample 1	Sample 2	Sample 3	Sample 4	Total
Myrcene (%)	1.38	1.20	1.45	1.42	1.43
Octanal (%)	0.37	0.54	0.42	0.34	0.45
Limonene (%)	98.25	97.94	97.48	98.00	97.83

Table 2
Effect of essential oil (OEC) and limonene (LIM) from *Citrus aurantium* under models of gastric ulcer induced by absolute ethanol and NSAID in rats.

Experimental models	Treatments (v.o.)	Dose (mg/kg)	U.A. (mm^2)	Protection (%)
NSAID	Vehicle	–	74.90 ± 15.20	–
	Cimetidine	100	1.70 ± 1.07**	97.7
	OEC	50	40.52 ± 14.27	45.9
		100	30.35 ± 9.35*	59.5
		250	0.40 ± 0.40**	99.5
	LIM	245	0.70 ± 0.40**	99.0
Ethanol	Vehicle	–	187.60 ± 29.21	–
	Carbenoxolone	100	10.40 ± 5.97**	94.4
	OEC	50	91.40 ± 20.62*	51.2
		100	14.17 ± 8.18**	92.4
		250	1.00 ± 1.00**	99.4
	LIM	245	1.50 ± 1.50**	99.2

Ulcer areas are presented as mean ± S.E.M.
* Dunnet's test, significantly different from negative control group treated with vehicle, $P < 0.05$.
** Dunnet's test, significantly different from negative control group treated with vehicle, $P < 0.01$.

Table 3
Effects of essential oil (OEC) and limonene (LIM) obtained from *Citrus aurantium* on gastric juice parameters in rats submitted to pylorus ligature.

Route	Treatments	Dose (mg/kg)	*N*	Gastric juice volume (mL)	$[H^+]$ mequiv./mL/4 h
Intraduodenal	Vehicle	–	5	5.73 ± 0.31	8.34 ± 0.41
	Cimetidine	100	5	2.55 ± 0.42**	2.26 ± 0.56**
	OEC	250	5	2.14 ± 0.35**	7.09 ± 0.65
	LIM	245	5	2.71 ± 0.31**	7.67 ± 0.67
Oral	Vehicle	–	5	6.13 ± 0.57	13.11 ± 0.36
	Cimetidine	100	5	7.8 ± 0.45	10.30 ± 0.71*
	OEC	250	5	8.15 ± 0.63	9.22 ± 1.16*
	LIM	245	5	9.16 ± 1.11	9.66 ± 0.61*

Data are presented as mean ± S.E.M.
* Dunnet's test, significantly different from negative control group treated with vehicle, $P < 0.05$.
** Dunnet's test, significantly different from negative control group treated with vehicle, $P < 0.01$.

The serum gastrin levels in animals treated with OEC and LIM, presented in Fig. 1, indicate that neither treatment exerted an antisecretory effect on the gastric mucosa. But those that received cimetidine displayed a significant rise in serum gastrin in response to antisecretory acid activity.

Fig. 2 shows that the animals treated with OEC and LIM augmented the amount of mucus adhering to the gastric mucosa, thus confirming the gastroprotective activity of OEC and its majority compound (LIM).

Through the total quantification of mucosal GSH (Fig. 3), it was possible to observe that neither OEC nor LIM increased the basal levels of gastric GSH in rats submitted to the different treatments. Neither treatment hindered the ethanol-induced degradation of

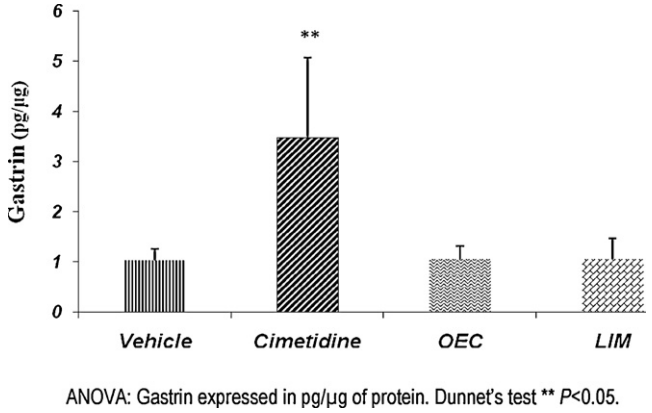

Fig. 1. Quantification of gastrin serum levels in rats treated with essential oil (OEC) and limonene (LIM) from *Citrus aurantium*. ANOVA: gastrin expressed in pg/µg of protein. Dunnet's test $^{**}P < 0.05$.

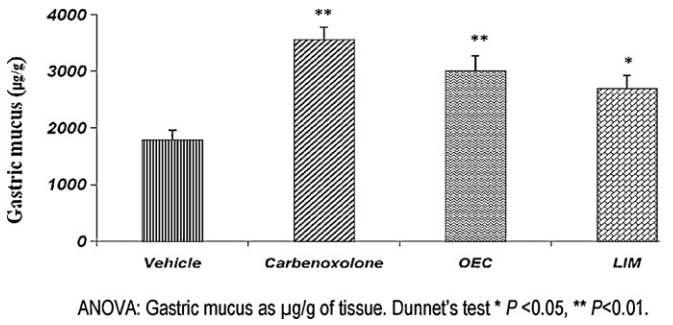

Fig. 2. Quantification of adherent mucus in gastric mucosa of rats treated with essential oil (OEC) and limonene (LIM) from *Citrus aurantium*. ANOVA: gastric mucus as µg/g of tissue. Dunnet's test $^{*}P < 0.05$, $^{**}P < 0.01$.

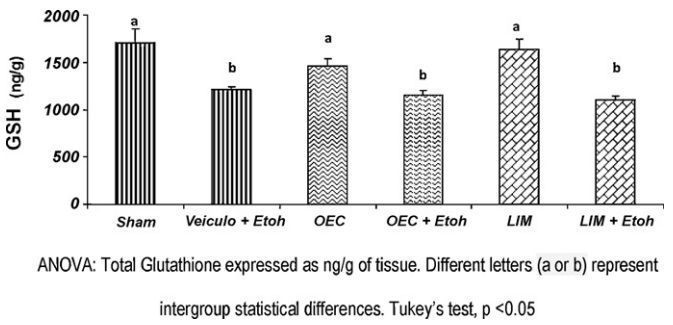

Fig. 3. Glutathione quantification of gastric mucosa in rats treated with essential oil (OEC) and limonene (LIM) from *Citrus aurantium*. ANOVA: total glutathione expressed as ng/g of tissue. Different letters (a or b) represent intergroup statistical differences. Tukey's test, $P < 0.05$.

Table 4
Effect of *Citrus aurantium* essential oil (OEC) on gastric motility under the activated charcoal model.

Treatments (v.o.)	Dose (mg/kg)	% of intestine traveled by charcoal
Vehicle	–	72.3 ± 12.5
Atropine	30	25.8 ± 5.3**
OEC	250	61.3 ± 16.2
	100	69.9 ± 19.8
	50	53.0 ± 11.8

Data are presented as mean ± S.E.M.
** Dunnet's test, significantly different from negative control group treated with vehicle, $P < 0.01$.

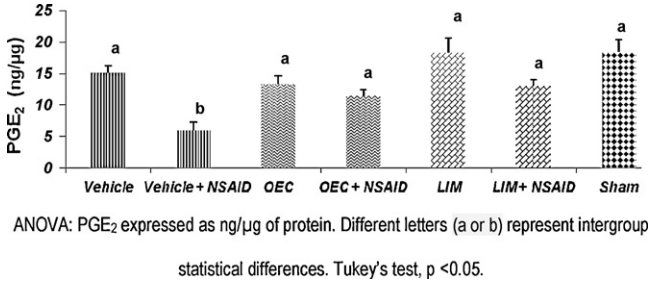

Fig. 4. Quantification of PGE_2 levels in gastric mucosa of rats treated with essential oil (OEC) and limonene (LIM) from *Citrus aurantium*. ANOVA: PGE_2 expressed as ng/μg of protein. Different letters (a or b) represent intergroup statistical differences. Tukey's test, $P < 0.05$.

GSH, since its levels in animals treated with OEC and LIM that had ingested ethanol were the same as in those that had received both the vehicle and ethanol.

Table 4 shows that animals treated with OEC presented the same gastrointestinal transit as the mice treated with vehicle. But the group that received atropine displayed diminished gastric motility as expected.

Table 5 shows that even with the blockade of VR-1 receptors observed by ruthenium red, the OEC maintained its protective action and hence did not stimulate the sensitive neurons of the gastric mucosa. The same was observed when the rats were pretreated with L-NAME, an NO-synthase inhibitor. The OEC continued exerting its gastroprotective effect without the action of NO-synthase, thereby showing that its activity does not depend on NO. But in the animals pretreated with NEM, a sulfhydryl (SH) inhibitor, the OEC stopped acting on gastric mucosa. These results demonstrated that the activity of the OEC is directly related to the presence of SH compounds in the gastric mucosal barrier.

Fig. 4 shows that, when administered jointly with DAINE (indomethacin 30 mg/kg, s.c.), a cyclooxygenase inhibitor, both OEC and LIM maintained high PGE_2 levels, similar to the sham and vehicle-only groups, without modifying their basal PGE_2 levels. The same did not occur under treatments with vehicle and DAINE, since the DAINE administration significantly diminished the PGE_2 levels in these groups.

4. Discussion

The functional integrity of gastric mucosa depends on a balance between aggressive factors and protective mechanisms. Thus, the success of gastric pharmacological treatment relies not only on the blockade of acid secretion, but also on augmentation of the protective factors of the gastric mucosa [26]. The mucosal protective agents consist of three functional factors: mucus secretion, microcirculation and motility [27]; two humoral factors: prostaglandins and nitric oxide [28]; as well as neuronal sensitivity to capsaicin [29]. This ability of certain endogenous factors to protect the gastric mucosa against damage to the gastric epithelium through mechanisms not related to acid secretion inhibition was first denominated "cytoprotection" and then characterized as "gastroprotection" [30,31].

The aggressive properties of non-steroidal anti-inflammatory drugs (NSAIDs) in the gastrointestinal tract continue to be greatest impediment of their use in the treatment of inflammatory illnesses such as rheumatoid arthritis [32]. The inhibition of prostaglandin synthesis is known to be the main ulcerogenic mechanism of the NSAIDs, besides provoking damage to the vascular endothelium, reduction of the blood flow, formation of obstructive micro-thrombi and activation of neutrophils [33]. In the experimental induction of

Table 5
Effect of oral *Citrus aurantium* essential oil (OEC) treatment, under the ethanol-induced gastric lesion model, on rats pretreated with ruthenium red, L-NAME and NEM.

Pretreated rats	Treatment	Dose (mg/kg, p.o.)	Ulcer area (mm^2)	Inhibition (%)
Saline	Vehicle	–	218.65 ± 41.64	–
	Capsaicin	4	31.42 ± 15.05**	85.6
	OEC	250	0.0 ± 0.0**	100
Rhutenium red (s.c.)	Vehicle	–	581.08 ± 41.52	–
	Capsaicin	4	234.86 ± 91.85**	59.6
	OEC	250	18.23 ± 6.64***	96.8
Saline	Vehicle	–	218.0 ± 28.50	–
	Carbenoxolone	100	45.0 ± 9.11*	79.3
	OEC	250	11.0 ± 0.83**	94.9
L-NAME (i.p.)	Vehicle	–	296.0 ± 25.61	–
	Carbenoxolone	100	79.0 ± 7.20**	73.3
	OEC	250	24.0 ± 3.0***	91.9
Saline	Vehicle	–	250.0 ± 61.80	–
	Carbenoxolone	100	33.97 ± 6.0**	86.4
	OEC	250	7.91 ± 3.71***	96.8
NEM (i.p.)	Vehicle	–	390.60 ± 42.91	–
	Carbenoxolone	100	161.53 ± 27.0*	58.6
	OEC	250	290.59 ± 67.90	25.6

Data are presented as mean ± S.E.M.
* Dunnet's test, significantly different from negative control group treated with vehicle, $P < 0.05$.
** Dunnet's test, significantly different from negative control group treated with vehicle, $P < 0.01$.
*** Dunnet's test, significantly different from negative control group treated with vehicle, $P < 0.001$.

gastric ulcers by a NSAID, both the essential oil of *C. aurantium* and limonene presented gastroprotection, the former at doses of 100 and 250 mg/kg and the latter at 245 mg/kg (Table 2). Given that the ulcerogenic properties of NSAIDs are due to the fact that they diminish the protective factors of the mucosa such as PG and mucus [34], it can be affirmed that the antiulcerogenic activity of both OEC and LIM observed in this model must augment these mucosal protective factors.

The ulcerogenic activity of ethanol is driven by its capacity to dissolve the constituent gastric mucus while concomitantly diminishing the transmucosal action potential, thus increasing the flow of Na^+ and H^+ in the lumen and stimulating the secretion of histamine, pepsin and H^+ ions. Furthermore, ethanol also diminishes the levels of protein, DNA and RNA, leading to sanguineous stasis and consequently a lesion on the tissue [35]. Considering that the OEC (250 mg/kg) and LIM (245 mg/kg) exerted 99% protection on the gastric mucosa, it is undeniable that these two substances exert substantial protective action on the gastric mucosa. These results also indicate a possible cytoprotective activity, since ethanol acts directly on gastric mucosal cells.

Given that some pharmacologic agents that inhibit the H^+/K^+-ATPase receptor, including histaminergic and cholinergic antagonists, act antiulcerogenically to reduce acid secretion in the stomach [36], the present work evaluated the antisecretory action of the OEC and LIM for local (p.o.) or systemic action (i.p.) through the pylorus ligature model. The animals that had received OEC and LIM orally showed diminished H^+ concentrations in the gastric juice, whereas those ingesting these treatments intraduodenally showed unmodified H^+ concentration (Table 3). In this model the serum gastrin levels were also unchanged by either compound (Fig. 1). These results indicate therefore that the OEC and LIM do not exert antiulcerogenic action in an antisecretory manner. The reduction of the H^+ concentration in the groups receiving OEC and LIM orally can be explained by the increase in the amount of gastric mucus that the treatments can provoke, given that this mucus neutralizes the H^+ secreted on the gastric lumen. This result is highly significant in the ongoing search for an antiulcerogenic therapy since the prolonged use of proton-pump inhibitors and H_2 blockers can provoke serious side effects including hypergastrinemia by means of reduced pH in the gastric lumen [8].

Gastric mucus is the first line of defense against acid and adheres together with bicarbonate secreted by the epithelium to serve as a barrier against self-digestion [37]. The results obtained in the present work show a significant increase in the amount of adherent mucus in the animals treated with OEC and LIM (Fig. 2) thus justifying the previously observed gastroprotective action. Mucus is an important protective factor for the gastric mucosa and, when present in a viscous, elastic, adherent form in a transparent gel composed of 95% water and 5% glycoprotein, it can act as antioxidant reducing damage in the mucosa provoked by free radicals [38]. It was demonstrated that ulcer induction by ethanol is associated with reduced levels of SH compounds, especially intracellular glutathione (GSH) [35]. In light of this, the present study evaluated the roles of these compounds in the gastric protection promoted by OEC and LIM. Fig. 3 shows that the OEC and LIM were unable to conserve the normal GSH levels when challenged by ethanol, demonstrating that the protective action displayed by these substances is unrelated to the elevated antioxidant capacity of the cell, since they did not present activity, as confirmed by the lipoperoxidation (LPO) test performed *in vitro* (data not shown). Furthermore, immunohistochemical staining for SOD in stomach samples of the rats submitted to ethanol-induced ulcer revealed no alteration in the expression of this enzyme (data not shown), since SOD plays a essential role in mucosal antioxidant activity [39]. These findings indicate that the gastroprotective activities of OEC and LIM are not related to increased mucosal antioxidant capacity but rather to the augmented gastric mucus production. This hypothesis was confirmed in the present experiment in which the animals were pretreated with a sulfhydryl (SH) inhibitor. SH limits the production of free radicals, thus protecting the cell [40]. SH protects the mucus to unite its subunits by disulfide bridges. If these bridges are reduced, the mucus becomes more soluble and is more prone to harmful agents [32]. On that basis, the animals were pretreated with NEM, an SH inhibitor, to evaluate the interference of this protection mechanism in the OEC action. We can conclude that the SH inhibition decomposed the mucus structure and that without mucus the OEC lost its gastroprotective effect (Table 5).

The neurons sensitive to capsaicin play an important role in the defense mechanisms of gastric mucosa. The VR-1 receptors in the gastrointestinal mucosa are involved in regulating gastric motility and acid secretion, in increasing blood flow through the action of the calcitonin gene-related peptide (CGRP), and in stimulating bicarbonate secretion, mucus secretion and maintaining mucosal integrity in the presence of harmful agents [41]. In this experimental model it was possible to observe that the OEC does not exert its gastroprotective action by activating capsaicin-sensitive neurons. Therefore, the blockade of VR-1 receptor of sensitive neurons by rhutenium red (RR) pretreatment revealed that OEC had exerted continuous gastroprotection (Table 5). Besides capsaicin, another mediator involved in the gastroprotection is nitric oxide (NO), which is synthesized by NO-synthase (NOs) and plays an important role in modulating the defense of gastric mucosa by regulating mucus secretion [42], enhancing blood flow [43] and inhibiting neutrophil aggregation [44]. The evaluation of NO participation in the gastroprotection promoted by OEC demonstrated that despite the inhibition of NO by the action of the L-NAME-blocking NOs, OEC continued exerting its effect (Table 5), and no alteration was observed in the expression of this enzyme in the OEC-treated animals by immunohistochemical staining, thus confirming that OEC's protective mechanism is not related to NO synthesis.

Amongst the existing humoral factors in the mucosa, the prostaglandin PGE_2 plays an important role in protecting the mucosa by stimulating the secretion of mucus and bicarbonate, maintaining mucosal blood flow and increasing the resistance of epithelial cells against potential damage by cytotoxins [45]. Fig. 4 demonstrates that even with the administration of a non-selective COX inhibitor (indomethacin), which consequently caused a decrease in PGE_2 levels, both OEC and LIM were able to maintain PGE_2 at levels similar to those found in normal rats, without modifying its basal levels in the gastric mucosa. Considering that gastric mucus synthesis is controlled by PGE_2 the modulating action of OEC and LIM on PGE_2 synthesis explains the fact that these treatments augmented gastric mucus secretion by increasing the gastric mucosal protection, confirming the gastroprotective actions promoted by the OEC and LIM. Probably this action mechanism explains the relief from gastroesophageal reflux disorder already described for limonene but not completely elucidated [13]. Besides being popular flavoring agents found in common food items, the essential oil from *C. aurantium* and its majority constituent limonene present substantial antiulcerogenic and gastroprotective actions that can be regarded as a promising target for the development of a new drug for the prevention of gastric ulcer.

5. Conclusion

Through the results of this study we can conclude that the antiulcerogenic and gastroprotective actions promoted by the essential oil of *C. aurantium* (OEC) are due to the presence of limonene (LIM), which accounts for about 97% of its composition, and that these effects are directly related to an increase in the gastric production of mucus rooted in the modulating action that these compounds exert on PGE_2 levels. These results indicate that OEC and LIM constitute

an interesting adjuvant to NSAID in the treatment of chronic inflammatory illnesses, with the prospect of annulling the aggressive gastric effect of these drugs on gastric mucosa without promoting alterations in physiological functions of the stomach.

Conflict of interest

There is no conflict of interest.

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