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Gastroprotective Activity of Methyleugenol from *Peperomia hispidula* on Ethanol-Induced Gastric Lesions in Rats

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ABSTRACT
Peperomia hispidula is used in traditional Mexican herbal medicine to treat gastric ulcers. However, this use has not been corroborated scientifically. Hence, the gastroprotective activity of *P. hispidula* was herein evaluated, as well as the role of endogenous NO, sulfhydryl groups and prostaglandins in the gastroprotective effect shown by one compound. The activity of *P. hispidula* was evaluated by using an animal model of gastric lesions induced by absolute ethanol in Wistar rats. Methyleugenol was isolated via silica gel column chromatography. The cytoprotective mechanisms of this compound were evaluated in relation to nitric oxide (pretreatment with L-NAME), sulfhydryl groups (pretreatment with NEM) and prostaglandins (pretreatment with indomethacin). The hexane and dichloromethane extracts showed gastroprotective activity, the latter (at 100 mg kg^{-1}) having the greatest effect (91.55±3.12%). Methyleugenol was identified as one of the most active compounds in this extract. The gastroprotective activity of methyleugenol at 100 mg kg^{-1} was not attenuated by pretreatment with L-NAME, NEM or indomethacin. Methyleugenol was identified as one of the compounds of *P. hispidula* that exerts a gastroprotective effect. The results suggest that the gastroprotective mechanism of methyleugenol does not involve nitric oxide, sulfhydryl groups or prostaglandins.

Key words: *Peperomia hispidula*, methyleugenol, gastroprotective effect, medicinal plants, gastric ulcer

INTRODUCTION

Peptic ulcer, a disruption of the mucosal integrity of the esophagus, stomach or duodenum, (Paguigan *et al*., 2014) is a chronic and recurring disorder that is widespread in the world today with increasing morbidity and mortality rates (Junior *et al*., 2014). Although, a peptic ulcer is not deadly, it can lead to more serious complications such as perforations, gastrointestinal bleeding and gastric outlet obstruction (Paguigan *et al*., 2014).

Under normal physiological conditions, the integrity of the aforementioned tissue depends on a balance between aggressive factors (e.g., stress, ethanol exposure and frequent ingestion of non-steroidal anti-inflammatory drugs) and countervailing mucosal defense mechanisms (e.g., mucus and bicarbonate secretion, increased levels of antioxidants and production of prostaglandins) (Spirt, 2004). Nowadays, several classes of drugs are used for the treatment of peptic ulcers, including antibiotics, antacids, H_2 receptor antagonists

and proton pump inhibitors. However, all of these drugs are associated with undesirable side effects, making the development of alternative treatments necessary. In this context, medicinal plants provide an important source of new chemical substances with potential therapeutic effects (Vera-Arzave *et al*., 2012). In traditional medicine of the state of Chiapas, Mexico, *Peperomia hispidula* (Piperaceae), locally known as lenteja, is commonly prepared as an infusion and used to treat gastric ulcers. In spite of the uses of this plant, no report has been issued with a chemical or biological evaluation of *P. hispidula*.

The aim of the present study was to evaluate the gastroprotective activity of *Peperomia hispidula* through a bioassay guided study by using an animal model of gastric lesions induced by absolute ethanol in Wistar rats. Regarding one active compound, the role of endogenous NO, sulfhydryl groups and prostaglandins was explored.

MATERIAL AND METHODS

Animals: All experiments were performed with male Wistar rats weighing 180-220 g, obtained from the animal house of the Universidad Autónoma Metropolitana, Xochimilco campus, Mexico City. The procedures involving animals and their care were conducted in accordance with the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999) and in compliance with international rules on care and use of laboratory animals. Unless otherwise specified, the rats were placed in single cages with wire-net floors and deprived of food 24 h before experimentation. Animals were allowed free access to tap water throughout the experimental procedures. All experiments were carried out with 8 animals per group.

Drugs: The drugs, extracts, fractions and methyleugenol were prepared immediately before use. Carbenoxolone (the reference drug), N^G-nitro-L-arginine methyl ester (L-NAME), N-Ethylmaleimide (NEM) and Indomethacin (IND) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant material: *Peperomia hispidula* (Piperaceae) was collected in the Ejido Zaragoza Nueva Alemania, Municipality of Tapachula, the State of Chiapas, Mexico, during August of 2013. The plant was authenticated by Dra. Dorismilda Martínez-Cabrera. A specimen of the original collection can be found with the voucher number 1429 in the HERITH Herbarium of Instituto Tecnológico de Huejutla of the State of Hidalgo, Mexico.

Extraction and preliminary fraction: *Peperomia hispidula* was dried at room temperature (22±2°C) in the shade. After grinding, 4 kg were successively extracted by maceration with hexane, dichloromethane and methanol (three times during 3 days for each solvent). Evaporation of the solvents in vacuum gave 60, 130 and 261 g of syrupy residues, respectively.

The dichloromethane extract showed the most gastroprotective effect (Table 1). A portion (60 g) of this extract was submitted to column chromatography by using a step gradient of hexane/EtOAc (9:1, 1.5 L, F1, 11 g), hexane/EtOAc (7:3, 1.5 L, F2, 19 g), hexane/EtOAc (1:1, 1.5 L, F3, 12 g) and EtOAc (1.5 L, F4, 15 g) as the eluents.

Since, the F2 fraction was the most active (Table 2), 17 g was chromatographed on a silica gel column by using a step gradient of hexane, mixtures of hexane/EtOAc and EtOAc. We obtained 50 fractions of 20 mL each from this procedure. Fractions 15-20 (Hexane/EtOAc, 90:10) yielded an oil

Table 1: Gastroprotective effect of the extracts of *Peperomia hispidula* on ethanol induced gastric lesions in rats

Treatments and dose (mg kg^{-1})	n	UI (mm^2)	Gastroprotection (%)
Control			
-	8	56.25±9.99	-
Hexane extract			
10	8	33.37±6.79	40.66±12.08
100	8	11.33±5.44*	79.85±9.67
Dichloromethane extract			
10	8	20.00±5.57*	64.44±9.90
30	8	17.42±4.27*	69.01±7.59
100	8	4.75±1.75*	91.55±3.12
Methanol extract			
10	8	57.17±1.10	-1.64±3.74
100	8	54.35±6.73	3.37±11.97

*p<0.05 versus control group, UI: Ulcer index

Table 2: Gastroprotective effect of the fractions of the dichloromethane extract on ethanol induced gastric lesions in rats

Treatments	Dose (mg kg^{-1})	n	UI (mm^2)	Gastroprotection (%)
Control	-	8	72.00±12.43	-
F1	100	8	14.57±5.72*	79.76±7.94
F2	100	8	1.00±0.68*	98.61±0.94
F3	100	8	12.66±3.21*	84.92±4.89
F4	100	8	30.00±6.65*	58.33±10.67

*p<0.05 versus control group, UI: Ulcer index

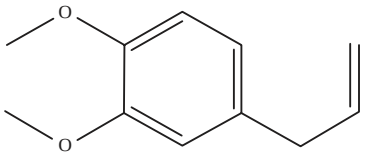

Fig. 1: Methyleugenol

(3.2 g) which was identified as methyleugenol (Fig. 1) by comparing the ^{1}H and ^{13}C NMR spectrum to data reported in the literature (Grosch *et al*., 2013).

Ultra-High Performance Liquid Chromatography- Masses Spectrometry (UHPLC-MS) analysis: Since, all the fractions of the dichloromethane extract (Table 2), were active, a UHPLC-MS assay was conducted in order to determine if methyleugenol was contained in them. An Ultimate 3000 Ultra Performance Liquid Chromatography (UPLC) system (Dionex corp., CA, USA) with Photodiode Array Detection (PAD), was coupled to a Bruker MicrOTOF-QII system by an Electrospray Ionization (ESI) interface (Bruker Daltonics, Billerica, USA) for chromatographic and Mass Spectrometric (MS) analysis. For chromatographic analysis, a Polaris C18 column (3.0 μm, 2.1×100 mm) (Varian) was used. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) using a gradient program of 25-28% (B) in 0-0.5 min, 28-30% (B) in 0.5-1.0 min, 30-85% (B) in 1.0-8.0 min, 85-25% in 8.0-10.0 min and 25% (B) in 10-12 min. The solvent flow rate was 0.3 mL min^{-1}, the column temperature was set to 25°C and the detection wavelength was 240 nm. The conditions of MS analysis in the positive ion mode were as follows: Drying gas (nitrogen), flow rate, 8 L min^{-1}; gas temperature, 180°C; scan range, 50-3000 m/z; end plate offset voltage, -500 V; capillary voltage, 4500 V; nebulizer pressure, 2.5 bar.

The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonics). During the development of the UHPLC method, external instrument calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Billerica, MA, USA) directly connected to the interface, with a sodium formate cluster solution. The calibration solution was injected at the beginning of each run and all the spectra were calibrated prior to the extract analysis.

Gastric lesions induced by absolute ethanol: The extracts, fractions, methyleugenol (suspended in 0.5% Tween 80) or reference drug (dissolved in water) was administered orally 30 min before gastric lesion induction by the oral application of absolute ethanol (1 mL). Two hours later, the animals were sacrificed in a CO_2 chamber. The stomachs were then removed, inflated with formalin and placed in 2% formalin for 5 min before being opened along the greater curvature to quantify gastric lesions. The lesions were measured under a dissection microscope with an ocular micrometer. The ulcer index was calculated as the sum of all lesions (area in mm^2) in the stomach of each animal and then the gastroprotection (%) was calculated according to Rojas-Martínez *et al*. (2013).

Ethanol-induced gastric mucosal lesions in L-NAME pretreated rats: To assess the participation of endogenous NO in the gastroprotective effect of methyleugenol, the rats fasted for 24 and divided into four groups according to pretreatment: one group received the vehicle (saline solution, i. p.) and three groups received L-NAME (70 mg kg^{-1}, i. p.) an inhibitor of Nitric Oxide Synthase (NOS). Thirty minutes after administration, two groups (including the control) received the vehicle (0.5% Tween 80, p. o.) and the other methyleugenol (100 mg kg^{-1}, p. o.) or carbenoxolone (100 mg kg^{-1}, p. o.). After 30 min all groups were orally treated with 1 mL of absolute ethanol and 2 h later the animals were sacrificed, the stomachs excised and the ulcer index was determined.

Ethanol-induced gastric mucosal lesions in NEM pretreated rats: The NEM, a blocker of sulfhydryl groups, was used to assess the participation of endogenous sulfhydryls in the protective effects of methyleugenol. This inhibitor was dissolved in saline solution, subcutaneously injected (10 mg kg^{-1}) into the animals of 3 groups, 30 min before the oral administration of either the vehicle, methyleugenol (100 mg kg^{-1}, p. o.) or carbenoxolone (100 mg kg^{-1}, p. o.). A control group received no treatment. Thirty minutes after these treatments absolute ethanol was given to each rat in these four groups and 2 h later animals were sacrificed, the stomachs excised and the ulcer index was measured.

Ethanol-induced gastric mucosal lesions in indomethacin pretreated rats: To assess the participation of endogenous prostaglandins in the gastroprotective effect of methyleugenol, rats were fasted for 24 h and divided into four groups. One group received a subcutaneous injection of $NaHCO_3$ (5 mM) in saline solution and other three groups received an injection of indomethacin (10 mg kg^{-1} dissolved in $NaHCO_3$ at 5 mM) by the same route. After 75 min two groups (including the control) received the vehicle (0.5% Tween 80) and the other received methyleugenol (100 mg kg^{-1}, p. o.) or carbenoxolone (100 mg kg^{-1}, p. o.). After 30 min, all groups were treated with 1 mL of absolute ethanol and 2 h later the rats were sacrificed, the stomachs removed and the ulcer index was measured.

Statistical analysis: Data is presented as the Mean±SEM of 8 rats per group. Statistical significance between treatments was evaluated by the Kruskal-Wallis test, followed by Dunn's multiple comparison test, with $p \leq 0.05$ considered as significant.

RESULTS

Bioassay-guided fractionation and isolation of methyleugenol: The effect of different extracts of *P. hispidula*

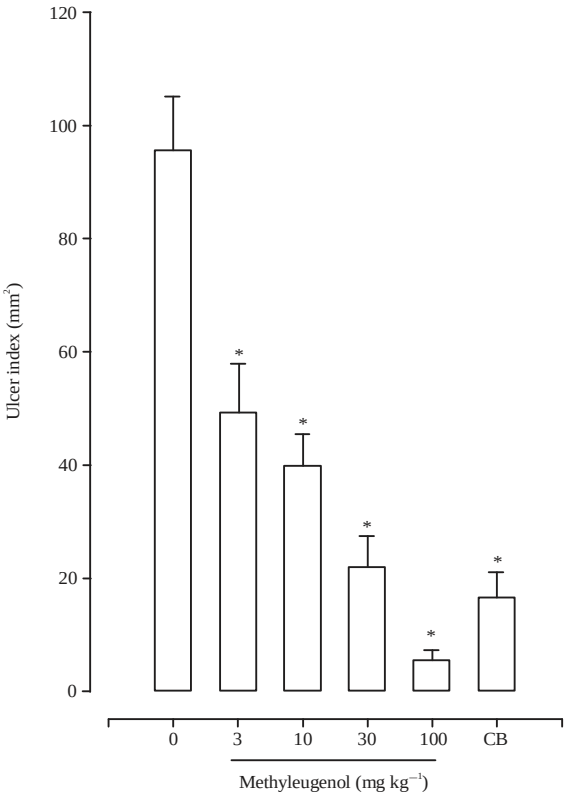

Fig. 2: Effect of different doses of methyleugenol (3-100 mg kg^{-1}) or carbenoxolone (CB: 100 mg kg^{-1}) on gastric lesions induced in rats by absolute ethanol. Bars represent the Mean±SEM (n = 8), *p<0.05 vs., respective control, Dunn's multiple comparison test after the Kruskal-Wallis test

on gastric lesions induced by ethanol is shown in Table 1. The ulcer index of the control group (56.25±9.99 mm^2) and that of the methanol extract were both similar, indicating inactivity. On the other hand, the hexane and dichloromethane extracts reduced the lesion area compared to the control group. The dichloromethane extract showed the greatest activity, providing the maximum gastroprotective effect at 100 mg kg^{-1} (91.55±3.12%).

All the fractions obtained from the dichloromethane extract reduced gastric lesions compared to the control group (Table 2). The F2 was the most active fraction, with 98.61±0.94% of gastroprotection at a dose of 100 mg kg^{-1}. Methyleugenol (Fig. 1), isolated from the F2 fraction, is one of the compounds responsible for the gastroprotective activity of *P. hispidula*. The effect of this compound was found to be dose dependent, reaching its maximum effect (93.62±1.7%) at a dose of 100 mg kg^{-1} (Fig. 2). Carbenoxolone was used as reference drug and at the same dose showed a gastroprotective effect of 78.53±5.36%.

UHPLC-MS analysis: ESI-MS analysis of the four fractions showed a molecular weight of m/z 201.0894, corresponding to the sodium adduct of methyeugenol ($C_{11}H_{14}O_2Na$, Exact Mass: 201.0891). The UHPLC-MS quantitative analysis showed that methyeugenol constitutes 7.8, 30.2, 8.5 and 8.7% of F1, F2, F3 and F4, respectively.

Ethanol-induced gastric mucosal lesion in L-NAME pretreated rats: After pretreatment with L-NAME, methyleugenol (100 mg kg^{-1}) showed the same gastroprotection (14.5±5.21 mm^2) afforded by treatment with methyleugenol alone (Fig. 3a). Hence, it seems that nitric oxide is not related to the gastroprotective effects promoted by methyleugenol. On the other hand, the result obtained with carbenoxolone was in accordance with the literature (Fig. 3a).

Ethanol-induced gastric mucosal lesion in NEM-pretreated rats: The pretreatment of methyleugenol (100 mg kg^{-1}) with NEM resulted in an ulcer index (12.92±8.14 mm^2), which was not statistically different from that obtained with methyleugenol treatment alone (Fig. 3b). The results demonstrate that sulfhydryl groups are not involved in the gastroprotective effect promoted by the methyleugenol. Contrarily, endogenous sulfhydryl compounds do in fact play a role in the mechanism of action of carbenoxolone (Fig. 3b).

Ethanol-induced gastric mucosal lesion in indomethacin-pretreated rats: Pretreatment with indomethacin followed by methyleugenol (100 mg kg^{-1}) administration gave the same gastroprotection (26.21±8.78 mm^2) as that afforded by treatment with methyleugenol alone. This result shows

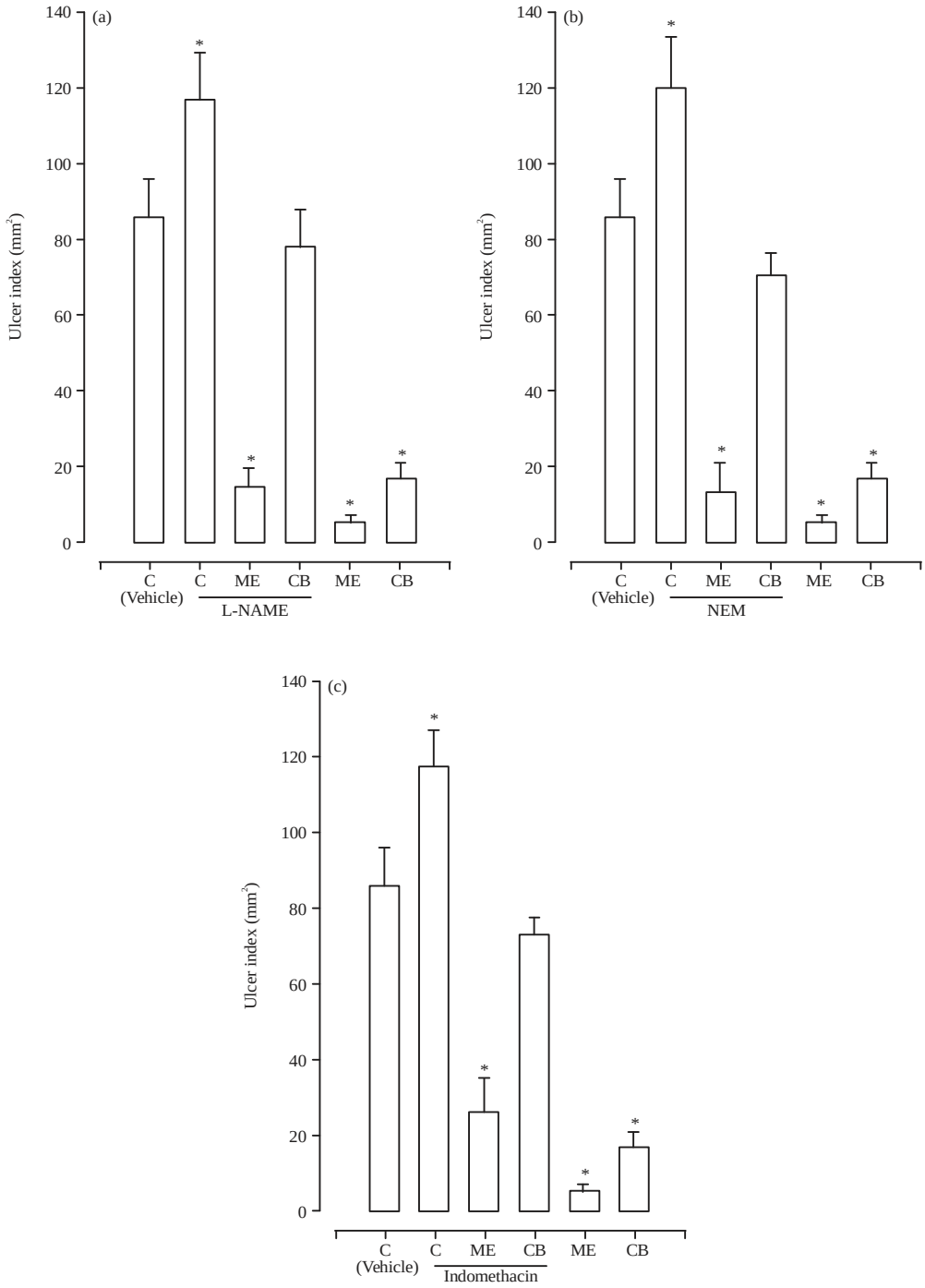

Fig. 3(a-c): Effect of methyleugenol (ME) or carbenoxolone (CB) at 100 mg kg^{-1} on gastric lesions induced by ethanol in rats pretreated with (a) L-NAME (70 mg kg^{-1}), (b) NEM (10 mg kg^{-1}) and (c) Indomethacin (10 mg kg^{-1}), Bars represent the Mean±SEM (n = 8), *p<0.05 vs., respective control, Dunn's multiple comparison test after the Kruskal-Wallis test

that endogenous prostaglandins are not implicated in the action mechanism of methyleugenol. On the other hand, prostaglandins do indeed play a role in the action mechanism of carbenoxolone (Fig. 3c).

DISCUSSION

Gastric ulcers are a global health problem resulting in high morbidity and mortality rates. Considering the growing

concern over the adverse effects of classic treatments for gastric ulcers, new effective drugs are being sought. Natural products are an important source of new therapeutic options (Al-Mofleh *et al*., 2008).

Accordingly, the gastroprotective activity of *P. hispidula* was herein evaluated on ethanol-induced gastric ulcer lesions. Ethanol induces both wide ulcers and petechial lesions within a relatively short time, which makes this technique suitable for screening anti-ulcer drugs. After rapidly penetrating the gastric mucosa, ethanol-induced injury is characterized by membrane damage, erosive hemorrhagic lesions with diffuse coagulative cell necrosis, cell exfoliation, multiple superficial erosions, marked vascular congestion and ulcer formation (Seiki *et al*., 1990).

Bioassay guided fractionation showed that the hexane and dichloromethane extracts were active (Table 1) and the methanol extract inactive. Although, there was important activity in the hexane extract, the greatest activity was shown by the dichloromethane extract. All fractions obtained from the latter extract demonstrated activity (Table 2).

From the most active fraction (F2), methyleugenol was isolated and identified as having gastroprotective activity (Fig. 1). However, at dose of 10 mg kg^{-1} the dichloromethane extract was more active than methyleugenol, indicating that this extract has more than one active compound. In the same sense, F2 contained other active compounds, because at a dose of 100 mg kg^{-1} it showed a gastroprotective value that was not significantly different from methyleugenol at the same dose.

Methyleugenol, an alkenylbenzene, is widely distributed in a variety of plant species as a constituent of essential oils, including basil, nutmeg, mace, anise, laurel leaves and fruits (Abdo *et al*., 2001). It is used as a flavoring agent in foods such as jellies, baked goods and ice cream and as a fragrance in soaps, lotions and perfumes. This compound is a central nervous system depressant with anesthetic, hypothermic, anti-anaphylaxis, antinociceptive, anti-inflammatory, myorelaxant and anticonvulsant properties (Abdo *et al*., 2001; Choi *et al*., 2010). This is the first report, to our knowledge, about the gastroprotective activity of methyleugenol on ethanol-induced gastric lesions.

The effect of this compound was found to be dose dependent, with protective activity beginning at a dose of 3 mg kg^{-1} and reaching the maximum level (93.62±1.7%) at 100 mg kg^{-1}. Interestingly, a structurally related compound, eugenol, has been reported to have gastroprotective activity in the same model, although higher doses were required to reduce the lesion area. Whereas, 100 and 250 mg kg^{-1} had a significant effect, 50 mg kg^{-1} of eugenol did not display any relevant activity. The fact that methyleugenol presents gastroprotective activity at a dose as low as 3 mg kg^{-1} shows that one different functional group in the structure greatly improved pharmacological activity (Santin *et al*., 2011).

Some studies indicate that methyleugenol induces carcinogenic activity, the liver being the principal target organ. However the doses herein evaluated (3-100 mg kg^{-1}) were much lower than the lethal doses LD_{50} (850-1560 mg kg^{-1}) reported for methyleugenol in rats (Abdo *et al*., 2001; Beroza *et al*., 1975). Hence, the animals in the carcinogenic studies received methyleugenol in doses far above the levels consumed by humans, making the relevance of these results questionable. Several authors point out that there should be no cause for concern in regard to the current levels of exposure for humans (Robison and Barr, 2006; Waddell *et al*., 2004; Ding *et al*., 2011). Likewise, carcinogenicity is apparently not a problem at the doses herein employed in animals.

The UHPLC-MS analysis of the four fractions obtained from the dichloromethane extract indicates that methyleugenol is present in all of them. This compound has the highest percentage in the most active fraction (F2), which can explain its greater activity. On the other hand, F3 showed a similar gastroprotective effect as that obtained with F2, but with a lower percentage of methyleugenol (8.5%). Hence, it appears that in this fraction there are other active compounds that contribute to the gastroprotective effect. The F3 fraction was not further evaluated in this study.

The percentage of methyleugenol present in the F4 fraction was the same as that contained in the F3 fraction. However, F4 had less activity (58% gastroprotection), perhaps because compounds are present in this fraction that inhibit the effect of methyleugenol. This was not the case with fraction F1, which showed important activity (79% of gastroprotection) even though the percentage of methyleugenol (7.8%) is the least of all fractions. Hence, there are probably less compounds that inhibit the effect of methyleugenol in this fraction.

The contribution of several systems in gastric mucosal defense is well documented, including prostaglandins (PGs), sulfhydryl (SH) compounds and Nitric Oxide (NO). The latter molecule is synthesized by Nitric Oxide Synthase (NOS) from L-arginine. The constitutive forms of NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS), play a physiological role in the homeostasis of the gastrointestinal tract. The inhibition of these enzymes can result in disturbance of gastrointestinal motility, blood flow and acid secretion (Beserra *et al*., 2011). Hence, we evaluated the possible involvement of NO in the gastroprotective mechanism of methyleugenol. Since, methyleugenol maintained its gastroprotective activity in the animals pretreated with L-NAME (Fig. 3a), we conclude that NO does not play a part in the mechanism of this compound.

It has been demonstrated that ethanol-induced ulcers are associated with reduced levels of SH compounds in the gastric mucosa, especially intracellular glutathione. The recovery of SH levels seems to be important in the gastroprotection exerted by some drugs (Nagy *et al*., 2007). Moreover, the 1,1-diphenyl-2-picrylhydrayl assay has demonstrated that methyleugenol has a direct, though weak, free radical scavenging activity (Choi *et al*., 2010). In light of this, the present study evaluated the role of SH compounds in the gastric protection promoted by methyleugenol. However,

pretreatment with NEM did not attenuate the gastroprotective effect of methyleugenol, suggesting that sulfhydryl groups are not implicated in its activity (Fig. 3b).

The aforementioned results are in accordance with the information reported for eugenol, that gastroprotection is not closely related to NO or SH activity (Santin *et al*., 2011). Apparently, the change from a hydroxyl (eugenol) to methoxyl (methyleugenol) group does not cause these factors to be implicated in the gastroprotective mechanism of the latter compound.

On the other hand, eugenol enhances mucus production (Santin *et al*., 2011). It is known that the prostaglandin PGE_2 plays an important role in protecting the mucosa by stimulating the secretion of mucus and bicarbonate, thus maintaining the local blood flow and increasing the resistance of epithelial cells to potential damage (Hawkey and Rampton, 1985). To test the possibility that prostaglandins are implicated in the case of methyleugenol, we inhibited PGs with indomethacin, finding that methyleugenol continued to exert the same gastroprotective effect (Fig. 3c). Thus, it appears that PGs are not implicated in the gastroprotective mechanism of methyleugenol.

A previous study reported that methyleugenol does not affect the activity of COX-1 or COX-2 and has an antinociceptive effect on formalin-induced hyperalgesia in mice (Yano *et al*., 2006). Considering that the search for novel plant-derived or synthetic molecules devoid of ulcerogenic propensity is an active area of investigation (Ali *et al*., 2014), the study of methyleugenol could represent a therapeutic option, especially taking into account that its activity encompasses an antinociceptive (Yano *et al*., 2006) and gastroprotective effect (the latter reported presently).

According to the results of the current study, the three mechanisms known to be involved in enhancing the gastric mucosal defense are not implicated in the gastroprotective activity of methyleugenol. However, it has been reported that this compound inhibits the release of histamine by mast cells (Shin *et al*., 1997) and inhibits the contraction induced by acetylcholine and histamine in isolated guinea pig ileum (Lima *et al*., 2000). Considering that acetylcholine and histamine are involved in some of the pathways regulating acid secretion (Sachs *et al*., 2014), future studies should examine whether methyleugenol provides protection of the gastric mucosa by acid secretion inhibition due the antagonism of acetylcholine and histamine receptors.

CONCLUSION

We herein reported the first scientific evidence for the ethnobotanical use of *Peperomia hispidula*. Methyleugenol was identified as one of the gastroprotective compounds in this plant and its mechanism of gastroprotective action was found not to be related to endogenous NO, sulfhydryl groups or prostaglandins. Further studies are necessary to isolate other active compounds from this plant and to explore whether the mechanism of action of methyleugenol involves an antisecretory effect.

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