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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2015.09.027

Chemical composition of *Rosmarinus officinalis* essential oil and antioxidant action against gastric damage induced by absolute ethanol in the rat

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ARTICLE INFO

Article history:
Received 13 Apr 2015
Received in revised form 16 Apr 2015
Accepted 25 Sep 2015
Available online 11 Jan 2016

Keywords:
Rosmarinus officinalis L.
Antioxidant activity
Gastric ulcer
Antioxidant enzymes

ABSTRACT

Objective: To evaluate the antioxidant activity of the essential oil obtained from *Rosmarinus officinalis* (*R. officinalis*) in ethanol-induced gastric ulcer model *in vivo*.

Methods: The antioxidant properties of the essential oil obtained from *R. officinalis* were evaluated against gastric injury induced by absolute ethanol. Gastric tissues were prepared to enzymatic assays. The levels of glutathione, lipid peroxides, and the activities of glutathione peroxidase, superoxide dismutase and myeloperoxidase were measured.

Results: Ethanol produced severe hemorrhagic lesions in the stomach with ulcerative lesion of (140.2 ± 37.2) mm^2. In animals pretreated with essential oil of *R. officinalis* (50 mg/kg, *p.o.*), a significant inhibition of mucosal injury of (21.2 ± 7.1) mm^2 (84% inhibition) was observed. The essential oil of *R. officinalis* protected the gastric mucosa probably by modulating the activities of the enzymes (superoxide dismutase and glutathione peroxidase) and increasing or maintaining the levels of glutathione. In addition, lipid peroxides levels were reduced. The essential oil of *R. officinalis* was analyzed by gas chromatography–mass spectrometer and the main constituents were cineole (28.5%), camphor (27.7%) and alpha-pinene (21.3%).

Conclusions: We suggest that the monoterpenes present in the essential oil obtained from *R. officinalis* may be among the active principles responsible for the antioxidant activity shown by essential oil of *R. officinalis*.

1. Introduction

The gastric epithelium is often attacked by physical, chemical or microbiological agents acting in the gastric lumen. Among the numerous injurious and irritant luminal agents, the stomach is a site of massive production and concentration of reactive oxygen species (ROS), which are already well known to take a central role in the pathophysiology of gastric ulcer [1]. Peptic ulcer disease has evolved as a major cause of morbidity and mortality throughout the 20th and 21st centuries [2]. Plant-derived products have shown great potential in treating human diseases, exerting beneficial health effects such as antioxidant properties [3].

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All experimental procedures involving animals in the work were conducted in accordance to recommendations of the Canadian Council on Animal Care and approved by Institutional Animal Care and Use Committee of the Campinas State University (CEEA/IB/UNICAMP, No. 1537-1).

Foundation Project: Supported by Sao Paulo Research Foundation (Grant Number: 10/16965-7) and National Council for Scientific and Technological Development (Grant Number: 303029/2011-0).

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

Rosmarinus officinalis L. (Lamiaceae) (*R. officinalis*) is native to Europe, but it has been cultivated in all Brazilian states. In folk medicine, analgesic, anti-inflammatory and treatment of gastrointestinal disturbances are properties attributed to this species [4,5]. Additionally, various pharmacological studies have demonstrated the analgesic [6], anti-inflammatory [5], and anti-ulcerogenic [7] properties of *R. officinalis*. Although the essential oil obtained from *R. officinalis* has shown antioxidant activity in previous studies [8–10], there is no report of the *in vivo* activity, which motivated the group to evaluate the antioxidant activity of the essential oil obtained from *R. officinalis* in ethanol-induced gastric ulcer model *in vivo* in order to observe whether the traditional use of this medicinal species for gastrointestinal disturbances is justified.

2. Materials and methods

2.1. Animals

Male Unib: WH rats (n = 7, 150–250 g) from Central Animal House of the University of Campinas (UNICAMP; São Paulo, Brazil) 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, (60 ± 1)% humidity and (21 ± 1) °C temperature. Fasting was used prior to the experiment because standard drugs or essential oil treatment were administered orally (by gavage). The experimental protocols were approved by the Institutional Animal Care and Use Committee (CEEA/IB/UNICAMP, no. 1537-1).

2.2. Essential oil

The essential oil of *R. officinalis* was purchased from Laszlo Aromatherapy Ltda. Plants were collected in Caatinga District (João Pinheiro, MG, Brazil), a Cerrado region. Essential oil of *R. officinalis* was isolated from inflorescences, leaves and stems from this species by steam distillation. A flowered "voucher" was identified by Jorge Yoshio Tamashiro of UNICAMP and deposited under the number 150422 at UEC herbarium (Campinas, SP, Brazil).

2.3. Identification of essential oil constituents

The essential oil of *R. officinalis* samples were analyzed in a gas chromatographer coupled to an electronic (70 eV) mass spectrometer (GC–MS, Shimadzu, GC-2010) equipped with a capillary column of fused silica (DB-5; 5.30 m × 0.32 mm × 0.25 μm), helium as carrier gas (1.52 mL/min, White Martins, 99.9%), injector at 250 °C, detector at 250 °C and split injection mode. Mass spectrum acquisition was performed at the mass range from 40 to 600 *m/z*. The essential oil (10 μL) was diluted in chloroform to produce 1 mL of chromatographic grade solvent, 1 μL of which was injected as sample at the split ratio of 1:30. The column temperature was heated at 60 °C and programmed at 5 °C/min to 220 °C. The identification of substances was performed by comparing its mass spectra with the GC–MS system database (Nist 62 lib.), the literature and with the Kovats retention indexes [11].

2.4. Drugs and chemicals

The following drugs were used: lansoprazole (Medley, Campinas, Brazil), Tween 80® and acetic acid (Synth, SP, Brazil), absolute ethanol (Merck KGaA, Darmstadt, Germany) and cimetidine (Sigma Chemical Co., St. Louis, USA). The chemicals used in the buffers and other solutions were all of analytical grade. All drugs and reagents were prepared immediately before use.

2.5. Ethanol-induced ulcer

After fasting for 24 h, the experimental groups were submitted to the treatments (*p.o.*) with vehicle, lansoprazole (30 mg/kg), essential oil of *R. officinalis* (6.25, 12.5, 25 and 50 mg/kg) 1 h before induction of gastric injury by absolute ethanol. Animals were killed by CO_2 gas. One hour after ethanol administration, the stomachs were removed, opened along the greater curvature, pressed onto a glass plate, and scanned so that the lesions could be measured by the Avsoft program [12]. The results were expressed as total ulcerated area (mm^2) [13]. Subsequently, the mucosa of each stomach was scrapped off using two glass slices with ice, homogenized in phosphate buffer (0.1 mol/L, pH 7.4), and frozen at −80 °C until biochemical determinations. The protein concentration of the samples was determined following the method described by Bradford [14].

2.6. Myeloperoxidase (MPO) activity

MPO activity in the gastric mucosa was measured by the method proposed by Krawisz *et al.* [15], with minor modifications in Farias-Silva *et al.* [16], to evaluate neutrophil accumulation. Briefly, the samples were centrifuged at 5 200 r/min for 15 min at 4 °C. Aliquots of the supernatant were then mixed with a reaction buffer of 50 mmol/L phosphate buffer, pH 6.8, containing 0.005% H_2O_2 and 1.25 mg/mL o-dianisidine dihydrochloride, measured at 460 nm.

2.7. Estimation of lipid peroxidation (LPO)

The homogenate of the glandular portion of stomach was diluted in 0.15 mol/L KCl (ratio 1:10). Then 0.2 mL of dodecyl sulfate (8.1%), 1.5 mL of acetic acid (20%, adjusted with NaOH solution to pH 3.5), 1.5 mL thiobarbituric acid (0.8% w/v), and 0.3 mL of distilled water were added to 0.5 mL of this homogenate. All samples were left in water bath with thermostat set at 95 °C for 1 h. After this period, the samples were cooled and added to 1 mL of distilled water and 5 mL of the mixture [*n*-butanol + pyridine (15:1, v/v)], shaken in vortex for 1 min, and centrifuged at 3 500 r/min for 10 min. The absorbance of organic layer was determined at 532 nm. 1,1,3,3 Tetraethoxypropane diluted in ethanol was used as standard. The results were expressed as nanomoles of substances that react with thiobarbituric acid per mg of protein (nmol TBARS mg/protein) [17].

2.8. Levels of sulfhydryl contents

Glutathione (GSH) levels of gastric tissue of animals were determined by Ellman's reaction using 5,5′-dithiobis(2-nitrobenzoic acid) as described by Faure and Lafond [18]. The intensity of the yellow color was read at 412 nm.

2.9. Glutathione peroxidase (GSH-Px) activity

GSH-Px activity was quantified by following the decrease in absorbance at 365 nm induced by 0.25 mmol/L H_2O_2 in the presence of reduced GSH (10 mmol/L), nicotinamide adenine dinucleotide phosphate (4 mmol/L), and 1 IU enzymatic activity of glutathione reductase [19].

2.10. Superoxide dismutase (SOD) activity

SOD activity was analyzed by the reduction of nitroblue tetrazolium using a xanthine–xanthine oxidase system, that is, superoxide generation [20].

2.11. Statistical analysis

Results were expressed as the mean ± SEM, and statistical significance was determined by One-way ANOVA followed by Dunnett's *post hoc* test, with the minimum level of significance set at $P < 0.05$.

3. Results

3.1. Effects of essential oil obtained from R. officinalis on ethanol-induced acute gastric lesion in rats

Table 1 represents the antiulcer activity observed when the essential oil of *R. officinalis* (6.25, 12.50, 25.00 and 50.00 mg/kg) was administered orally to rats before gastric lesion induced by ethanol. Theses doses were initially used to establish a general profile of the antiulcerogenic activity of the essential oil of *R. officinalis*. These data suggested that essential oil of *R. officinalis* (50.00 mg/kg) produced a gastroprotective effect since they significantly reduced ethanol-induced ulcers (protection of 84%) when compared with respective control. Therefore, with the purpose of investigating the probable gastroprotective mechanisms involved in the action promoted by essential oil of *R. officinalis*, we continued our studies using a single dose 50 mg/kg in subsequent assays.

Table 1
Effects of essential oil obtained from *R. officinalis* on ethanol-induced acute gastric lesion in rats.

Treatment (*p.o.*)	Dose (kg/mg)	Gastric lesions (mm^2)	Inhibition (%)
Sham	–	–	–
Vehicle	–	140.2 ± 37.2	–
Lansoprazole	30.00	40.4 ± 18.8***	71
Essential oil of *R. officinalis*	6.25	63.7 ± 18.6*	54
	12.50	146.7 ± 41.7	–
	25.00	57.5 ± 20.2**	59
	50.00	21.2 ± 7.1***	84

Data are presented as mean ± SEM, $n = 7$. ANOVA followed by Dunnett's test. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

3.2. Effects of essential oil obtained from R. officinalis on MPO activity

The MPO activity was found elevated by four-fold in the vehicle group when compared with sham group (without ulcer induced by ethanol) (Table 2). The data obtained indicated possible antioxidant mechanism promoted by essential oil of *R. officinalis* (50 mg/kg), since a reduction of 58% was observed (Table 2).

Table 2
Effects of essential oil obtained from *R. officinalis* on MPO activity.

Treatment (*p.o.*)	Dose (kg/mg)	IU/mg of protein	Inhibition (%)
Sham	–	15.6 ± 1.7*	–
Vehicle	–	63.2 ± 15.9	–
Lansoprazole	30	28.9 ± 4.6*	54
Essential oil of *R. officinalis*	50	26.5 ± 6.6*	58

Data are presented as mean ± SEM, $n = 7$. ANOVA followed by Dunnett's test. *: $P < 0.05$.

3.3. LPO levels in stomachs from rats with acute gastric lesion induced by ethanol

Table 3 shows that the absolute ethanol significantly increased TBARS. However, essential oil of *R. officinalis* (50 mg/kg) was able to prevent an increase in the amount of TBARS induced by ethanol (Table 3).

3.4. GSH levels, SOD and GSH-Px activities in stomachs from rats with acute gastric lesion induced by ethanol

The administration of ethanol provoked a decrease in GSH levels (55%) and pretreatment with essential oil of *R. officinalis* (50 mg/kg) increased the GSH levels (30%) when compared with the sham group and (138%) when compared with vehicle group. The essential oil of *R. officinalis* group showed a similar GSH-Px activity, similar to those of the sham group (Table 3). The administration of ethanol increased the activity of SOD (78%), while in essential oil of *R. officinalis* group, the activity of this enzyme was maintained at values close to those obtained in the sham group (without ulcer induced by ethanol).

3.5. Chemical composition of the essential oil of R. officinalis

The GC–MS analysis of essential oil of *R. officinalis* indicated three compounds of which the major compounds were three monoterpenes: cineole (28.5%), camphor (27.7%), and alpha-pinene (21.3%) (Table 4).

Table 3
GSH levels, SOD and GSH-Px activities and estimation of LPO levels in stomachs from rats with acute gastric lesion induced by ethanol.

Groups	GSH (nmol/g)	GSH-Px (μmol/min/mg protein)	SOD (IU/mg protein)	LPO (nmol TBARS/mg protein)
Sham	28.7 ± 3.8*	9.0 ± 1.8**	1.9 ± 0.2**	0.10 ± 0.01**
Vehicle	15.7 ± 3.2	15.9 ± 1.9	3.4 ± 0.6	0.52 ± 0.63
Lansoprazole	30.0 ± 1.7*	9.7 ± 1.2*	2.2 ± 0.1*	0.13 ± 0.01**
Essential oil of *R. officinalis*	37.3 ± 1.8***	10.0 ± 0.7*	2.1 ± 0.2**	0.14 ± 0.03**

Data are presented as mean ± SEM, $n = 7$. Rats received 12% Tween 80®, vehicle (10 mL/kg), lansoprazole (30 mg/kg) and essential oil of *R. officinalis* (50 mg/kg). ANOVA followed by Dunnett's test. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Table 4
Chemical composition of the essential oil of *R. officinalis*.

Peak	Compound	Composition (%)
1	Alpha-pinene	21.3
2	Camphene	8.7
3	Beta-pineno	4.7
4	Beta-myrcene	1.3
5	*p*-Cimeno	1.4
6	Cineole	28.5
7	Gamma-terpineno	0.3
8	Terpinoleno	0.3
9	Camphor	27.7
10	Borneol	2.5
11	Alpha-terpineol	0.7
12	Bornyl acetate	1.3
13	Caryophyllene	1.1

4. Discussion

The gastric ulcer is a complex process that involves ROS generation, extracellular matrix degradation and mitochondrial damage [21]. It results from an imbalance between aggressive gastric luminal acid factors and pepsin and defensive mucosal barrier function [22]. Ethanol is one of the exogenous aggressive factors where ROS are involved in the mucosal damage leading to oxidative stress [23,24]. Considering that antioxidant activity is an important mechanism of action involved in cytoprotection, there has been a considerable interest in the screening of plant extracts and compounds for their potential use as ROS scavengers [16,25]. In this context, the aim of this work was to evaluate the antioxidant properties *in vivo* of the essential oil from *R. officinalis* on rat gastric mucosa submitted to ethanol-induced gastric ulcer. Oral administration of the ethanol solution to the control group clearly produced characteristic hemorrhagic lesions with large linear patches of mucosal necrosis and edema. The oral administration of essential oil of *R. officinalis* at the dose of 50 mg/kg significantly decreased the gastric lesion from (140.2 ± 37.2) mm^2 obtained in the control group, to (21.2 ± 7.1) mm^2 (84%) ($P < 0.001$). Based on the results obtained with essential oil of *R. officinalis*, other assays were developed only with essential oil of *R. officinalis* at the dose of 50 mg/kg, which represents the best results in ethanol model.

The MPO enzyme catalyses the H_2O_2-mediated oxidation of halide ions to hypohalous acids, especially HOCl [26]. Excessive generation of MPO-derived oxidants has been linked to tissue damage and in the initiation and progression of diseases such as gastric ulcer. De-Faria *et al.* have reported that the exposure of gastric mucosa to ethanol caused significant increase in the MPO activity [23]. Thus, the MPO activity was studied in ethanol model as oxidant component of the gastric mucosa. In this parameter, the data obtained indicated possible antioxidant mechanism promoted by essential oil of *R. officinalis* (50 mg/kg), since a reduction of 58% was observed.

The LPO mediated by ROS is an important cause of destruction and damage of cell membranes, and it is involved in the pathogenesis of acute mucosal injury induced by ethanol [23]. The role of LPO in the pathogenesis of gastrointestinal diseases has been confirmed and the ability of absolute ethanol in increasing the amount of TBARS, which is closely related with the gastric damage, is well described in literature [23,27,28]. The essential oil of *R. officinalis* treatment was able to prevent the increase in the amount of TBARS induced by ethanol, showing an antioxidant activity.

The cells of the gastrointestinal tract have an antioxidant defense system capable of preventing the cytotoxicity of ROS through mechanisms that involve the action of enzymes and compounds with potential to scavenge free radicals [16]. SOD and GSH-Px are in the list of enzymes involved in this action. In addition, the mucosa is protected by GSH, the major scavenger of ROS inside cells [29]. The pretreatment with essential oil of *R. officinalis* increased the GSH levels and presented a low GSH-Px activity, indicating a lighter oxidative stress in the stomach of the animals treated with the essential oils. The GSH-Px activity is high in the vehicle group, probably due to the formation of large quantities of H_2O_2.

It has been shown that oxidative stress in the pathogenesis of ethanol-induced acute gastric mucosal injury promotes superoxide anions formation [30]. The superoxide anion is converted by the action of SOD to H_2O_2, which, in turn, is detoxified by GSH-Px. These results indicated that essential oil of *R. officinalis* was able to inhibit the damage induced by ethanol, not allowing the formation of superoxide anions.

The essential oils represent an important part of the folk medicine for their medicinal properties, including the antioxidant activity [31]. The GC–MS analysis of essential oil of *R. officinalis* indicated three compounds: cineole, camphor and alpha-pinene. Although these compounds have been detected in majority, we believe that the antioxidant effect is due to the presence of all the compounds present in essential oil of *R. officinalis*. Recent study shows that the antioxidant properties of essential oils do not always depend on the antioxidant activity of its main component, being very relevant to the concepts of synergism, additivity and antagonism [32].

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors are thankful to São Paulo Research Foundation and National Council for Scientific and Technological Development.

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