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Multiple mechanistic action of *Rosmarinus officinalis* L. extract against ethanol effects in an acute model of intestinal damage

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ABSTRACT

The high levels of oxidative stress and inflammation can be present in the etiology of degenerative intestinal pathologies associated with ethanol ingestion. The *Rosmarinus officinalis* L. has exhibited several physiological and medicinal activities. In this investigation, we intended to clarify, for the first time, the antioxidant and anti-inflammatory effects of ethanolic extract of *Rosmarinus officinalis L.* (eeRo) against an acute damage induced by ethanol, specifically in the small intestine of rats. The rats were treated three times, at every 24 h, with eeRo at 500–1000 mg/kg or vehicle, oral gavage. All groups got a single dose of ethanol (2 ml/kg), oral gavage, after 36 h of fasting and 1 h after the last dose of eeRo or vehicle administration. We performed the mensuration of oxidative stress profile in lipid peroxidation in serum and intestine; Na^+/K^+ ATPase, catalase, and superoxide dismutase activities assays only in intestine; and anti-inflammatory evidences of eeRo in myeloperoxidase activity assay only in the intestine. The eeRo was able to protect the animals against the lipid peroxidation in serum and intestine. It prevented the reduction in Na^+/K^+ ATPase and catalase levels induced by ethanol in the intestine. In addition, eeRo increased the superoxide dismutase activity when compared to control and protected the intestine against elevations in myeloperoxidase activity caused by ethanol. Our results suggested that eeRo exerted a significant intestinal protective effect by antioxidant and anti-inflammatory mechanisms. Thus, the eeRo represented a promising agent against intestinal lesions induced by ethanol.

1. Introduction

The breakdown of the normal mucosal defense mechanisms in the intestine [1] by stressors, like ethanol [2], can induce peptic and duodenal ulcers, dyspepsia, bacteremia or translocation of bacterial products [3,4]. Moreover, the peptic ulcer-related gastric cancer ranked fifth among the top 10 causes of cancer [5].

Several factors including environmental and emotional stress, age, diet, genetics and individual behavior among others have been attributed to either predispose or potentiate gastrointestinal mucosal to injury, through enhanced ROS production of mucosal oxidative stress resulting from the disruption of redox control and subsequent alteration in redox signaling [1,2]. These can contribute to the development of degenerative pathologies of the intestine, such as inflammation and cancer [6].

Ethanol is widely consumed and associated with development of gastrointestinal ulcer and cancers [7]. Experimental and clinical studies have demonstrated that ethanol is able to induce the intestinal barrier dysfunction, which has been associated with oxidative stress generation and also the rise of inflammatory process in the intestine [7–9].

In this context, natural products have been proposed as an alternative to avoid several pathological processes, since the plants are a good source of agents with pharmacological effects, either by blocking oxidative stress cascade or acting in cellular targets [10,11]. The *Rosmarinus officinalis* (Lamiaceae) is a perennial herb native to the Mediterranean area and is widely distributed in many parts of the world,

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including Brazil [12]. The *Rosmarinus officinalis L.* extracts are used as condiments for flavoring food, and as a source of antioxidant compounds employed in food conservation [13]. Moreover, this plant's extracts have shown evidences of medicinal properties, as antibacterial [12], antioxidant [14] and antidermatophytic [15], among others. The antioxidant activity of these extracts is related to the presence of phenolic abietane diterpenes, such as carnosic acid and its derivatives, carnosol, rosmadial, rosmanol, rosmanol isomers, and methyl carnosate, and phenolic acids like rosmarinic acid [16–18].

Our goal in this investigation is to clarify, for the first time, the antioxidant effects and anti-inflammatory evidences of eeRo, specifically in the small intestine of rats against an acute damage induced by ethanol and its possible applications in the future as a dietary supplement or medicine to contribute to the health of human and animal intestines.

2. Materials and methods

2.1. Chemicals and instruments

The chlorogenic, acetic and caffeic acids, methanol and hydrogen peroxide were obtained from Merck (Darmstadt, Germany) and ethanol of 99% purity was locally obtained. The Sigma Chemical Co. (St. Louis, MO, USA) provided other reagents like quercetin, kaempferol, carnosic acid and rutin. The high performance liquid chromatography (Shimadzu, Kyoto, Japan) was used for quantitative analysis.

2.2. Animals

The researches were conducted with the use of male wistar rats (270–320 g) from breeding colony of UFSM. The animals were maintained with food and water *ad libitum,* controlled room temperature (22 ± 2 °C), 12 h light/dark cycle and in cages with a maximum of 5 animals. The use of animals was approved by the Committee on Care and Use of Experimental Animal of the UFSM, Brazil (044/2012) and used according to the National Council of Control of Animal Experimentation (CONCEA).

2.3. The extraction of ethanolic extract of R. officinalis L. (eeRo)

The crude *R. officinalis* L. extract (eeRo) was obtained from dried leaves in soxhlet apparatus with ethanol at 100%, 1.5 h, 60–70 °C, according to Barbosa et al. with some modifications [12]. The voucher specimen of *R. officinalis* L. was stored and protected by herbarium of UFSM (SMDB 15.050) and the use of genetic patrimony of this plant was authorized by CNPq (010757/2014-7).

2.4. The analysis of eeRo composition by HPLC-DAD assay

The reverse phase chromatographic analyses were carried out under gradient conditions in accordance with Amaral et al. [19]. The mobile phase and samples were filtered (Millipore, 0.45 µm) and degassed (ultrasonic) prior to use. Stock solutions of standards were prepared in mobile phase. The quercetin, kaempferol and rutin standards were used at 0.031–0.250 mg/ml; and the carnosic acid, rosmarinic acid, caffeic acid and chlorogenic acid standards were used at 0.006–0.250 mg/ml. The retention time of reference standards and chromatography peaks of samples were compared to identify the components of samples (DAD spectra at 200 nm–500 nm). The chromatography assay was tested in triplicate and at ambient temperature.

2.5. The treatments and intestinal damages induced by ethanol method

Ethanol was used as an acute agent of intestinal damages according to Robert et al. [20]. The animals received 1 dose of eeRo (500 and 1000 mg/kg, 15 ml/kg, oral gavage administration) or vehicle (tween 80 at 2%, 15 ml/kg; oral gavage administration) every at 24 h, for a total of three times, before ethanol administration. The animals were submitted to fasting for 36 h before they received the last dose of vehicle or eeRo. We administrated a mean of 0.472 g of a single dose of ethanol 70% per rat (2 ml/kg, oral gavage administration) or vehicle 1 h after the animal received the last dose of eeRo or penultimate dose of vehicle. The animals were killed by decapitation 1 h after ethanol administration. The experimental procedure is shown in Fig. 1.

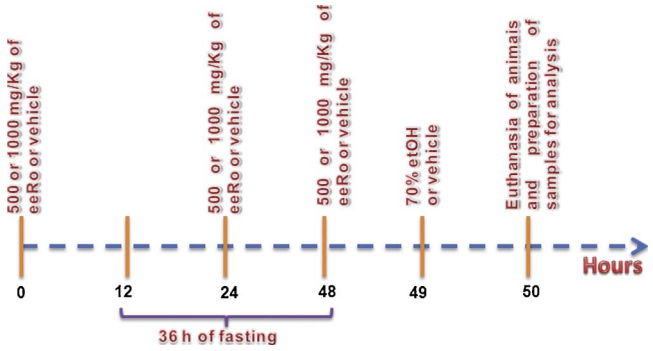

Fig. 1. Diagram showing the design and time-course of experimental procedures (eeRo, ethanolic extract of *R. officinalis* L. at 500 or 1000 mg/kg; vehicle, distilled water (98%) + tween 80 (2%); etOH, ethanol (70%). All drugs were administrated by oral gavage. Data are reported as five to nine animals per group.

2.6. Biochemical tests

The intestine, equal parts of duodenum, jejunum and ileum, were quickly removed, homogenized in 150 mM NaCl (1:4 dilution, w/v) and maintained on ice. The samples were centrifuged to 2000 × *g* at 4 °C for 10 min to get a low speed supernatant (S_1) which was used for determining markers of oxidative damage. Aliquots of S_1 were frozen (−20 °C) for later analysis of enzymatic antioxidant defense system and to protein determination.

2.6.1. Oxidative damage tests

2.6.1.1. Dichlorofluorescein fluorescence method (DCF). The reactive species were measured in S_1 [21]. S_1 aliquots (50 µl), tris–HCl buffer (10 mM, pH 7.4) and 2′,7′-dichlorofluorescein diacetate (1 mM) were incubated in the dark for 1 h to fluorescence measurement (at 488 nm in excitation and at 525 nm in emission, and 1.5 nm in slit widths). The standard curve of oxidized dichlorofluorescein was used to analyze the results expressed in nmol of oxidized DCF/mg protein [22].

2.6.1.2. Lipid peroxidation test. We added 100 µl of aliquots of S_1 to a medium with eeRo, its fractions or gallic acid (1–300 µg), tris–HCl buffer (10 mM, pH 7.4) in an incubation at 37 °C for 1 h. After this incubation, 200 µl of aliquots were removed from the medium and added to thiobarbituric acid at 100 °C for 1 h, inducing a color reaction, which was measured spectrophotometrically at 532 nm. The malondialdehyde curve was used to analyze the results expressed in nmol of MDA/mg protein [23].

2.6.1.3. The oxidized (GSSG) and reduced (GSH) glutathione levels assay. This test is a fluorimetric method used to determine the oxidized (GSSG) and reduced (GSH) glutathione levels in S_1 samples according to Hissin and Hilf [24]. The medium with S_1 aliquots (250 mg protein/ml), phosphate–EDTA buffer at 100 mM, pH 8.0 and HPO_3 at 25% were centrifuged (100,000 × *g* for 30 min, at 4 °C) to obtain GSH and GSSG. The glutathione levels were shown as GSH/GSSG ratio. The measurement of GSH and GSSG was obtained at 350 nm in excitation and at 420 nm in emission.

2.6.1.4. Antioxidant enzymes