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REGULAR ARTICLE

Hepatoprotective effect of *Rosmarinus officinalis* and rosmarinic acid on acetaminophen-induced liver damage

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Abstract

The hydroalcoholic extract of the leaves of *Rosmarinus officinalis* (HE), a species of medicinal value, and its constituent rosmarinic acid (RA) were evaluated for hepatoprotective in the acetaminophen-induced liver damage model. Groups of Wistar albino rats (n=6) were pre-administered with HE (100, 250 and 500 mg/kg, p.o.) and RA (10, 25 and 50 mg/kg, p.o.) prior to a single dose of acetaminophen (APAP, 600 mg/kg body weight; p.o). The hepatoprotective activity of HE extract was observed through histopathological analysis and reduction of the level of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). These observations were comparable to the normal group prior to APAP administration. Group that was treated with APAP alone exhibited high levels of transaminases and ALP. The obtained results from the present study suggested that HE may prevent APAP-induced hepatic injuries. RA did not displayed significant hepatoprotective activity against APAP-induced liver damage.

Key words: Rosemary, Hepatoprotective effect, Acetaminophen, Carnosol, Rosmarinic acid

Introduction

Acetaminophen (APAP) is an effective antipyretic-analgesic drug with excellent safety record at therapeutic doses (Larson, 2007). However, the exact mechanisms of APAP-induced toxity are poorly understood. Evidence suggests that glutathione depletion and formation of reactive metabolites somehow triggers the cascade of events leading to hepatotoxicity (Jaeschke and Bajt, 2006). Indeed, APAP overdose depletes glutathione levels and generates free radicals (Jaeschke and Bajt, 2006). Potent antioxidants and several medicinal plants extracts already used to treat liver diseases prevent APAP induced hepatotoxicity (Fahim et al., 1999; Sotelo-Felix et al., 2002a), which have also been used in the treatment of liver diseases (Bhadauria et al., 2008; Saba et al., 2012; Singhal and Gupta., 2012; Liu et al., 2012).

The use of herbal natural products has become increasingly popular worldwide. Many herbs have been developed into herbal supplements, claimed to assist people in adopting a healthy lifestyle. The Mediterranean plant *Rosmarinus officinalis* (L.) (Labiatae), known as Rosemary, grows in many parts of the world and helps treat asthma, eczema, and rheumatism (Fahim et al., 1999). Rosemary extracts possess many biological activities, including antimicrobial (Bernardes et al. 2010, Rasooli et al., 2008), antimutagenic (Furtado et al, 2008), anti-hyperglycemic (Al-Hader et al., 1994), anti-ulcerogenic (Dias et al., 2000), and antioxidant (Ozcan, 2003) actions. The most important antioxidant constituents of this plant are carnosic acid, carnosol, caffeic acid, and its derivative

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rosmarinic acid (RA), which exhibit antioxidant activity (Frankel et al., 1996).

Rosemary extracts exert potent hepatoprotective effects against a variety of hepatotoxic agents, including carbon tetrachloride [CCl_4] (Fahim et al., 1999), *tert*-Butyl hydroperoxide (Joyeux et al., 1990) and cyclophosphamide (Fahim et al., 1999). However, to the best of our knowledge, neither Rosemary extracts nor RA has been evaluated against APAP-induced hepatotoxicity. Therefore, in this study we aimed to assess the hepatoprotective effect of the hydroalcoholic extract of *R. officinalis* leaves (HE) and of its constituent rosmarinic acid (RA) on the APAP-induced hepatotoxicity. The hepatoprotective potential of the HE and RA was compared with silymarin, a known and commercially available hepatoprotective agent.

Materials and methods

Plant material and preparation of the hydroalcoholic extract

Rosmarinus officinalis L. (Lamiaceae) was collected in the urban perimeter of Patrocínio city, state of Minas Gerais, Brazil (18º56'35" S, 46º59'31" W) in May 2007. The plant material was identified by Dr. Milton Groppo. A voucher specimen (collector M. Groppo, number 1871, SPFR 11912) was deposited in the Herbarium of the Departmento de Biologia, Faculdade de Filosofia, Ciências e Letras de ribeirão Preto, Universidade de São Paulo, Brazil (Herbarium SPFR).

The *R. officinalis* leaves were dried in a stove with circulating air (40ºC), and powdered using a blender. The obtained powder (130 g) was exhaustively extracted with Ethanol/H_2O 8:2 (v/v) by maceration at room temperature, followed by filtration. The filtered extract was concentrated under reduce pressure, affording 18.6g of the hydroalcoholic extract of *R. officinalis* leaves (HE).

HPLC analysis of the hydroalcoholic extract of *R. officinalis*

HPLC analysis was conducted on a Shimadzu LC-6AD system equipped with a DGU-20A5 degasser, a UV-DAD detector SPD-M20A series with a CBM-20A module, and a Reodyne manual injector. The micromolecules were separated out on a Shimadzu Shim-pack ODS column (250 x 4.60 mm i.d., 5 μm) equipped with a pre-column of the same material. Methanol (MeOH) was HPLC grade (J. T. Baker). Ultrapure water was obtained by passing redistilled water through a Direct-Q UV3 system. Water with 0.1% acetic acid (A) and methanol (B) were used as mobile phases during chromatography. The samples were eluted with A/B (1:1) for 5 min, followed with linear gradient A/B gradient up to 100% B for 25 min, and elution with B for 10 min. The mobile phase flow rate was 1.0 mL min^{-1}. The compounds were identified by comparison of their retention times and UV spectra with those of the corresponding standards acquired from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of rosmarinic acid (RA)

HE (200 mg) was dissolved in MEOH/H_2O (1:1 v/v) and chromatographed over preparative RP-HPLC Shimadzu Shim-pack ODS (particle diameter 5 μm, 250 x 20 mm) column equipped with a pre-column of the same material, using the elution program described in the previous section. RA was isolated after several injections at a flow rate of 10 mL/min.

The chemical structure of RA was established by ^{1}H- and ^{13}C-NMR analysis and by direct comparison of its retention time (R_t) and UV spectral features with those of an authentic chromatographic standard on HPLC (Bernardes et al., 2010). RA purity was estimated to be higher than 95% by both HPLC analysis and ^{13}C NMR spectroscopy.

Experimental animals

Male Wistar albino rats aged 5 weeks and weighing approximately 180 g, were obtained from the animal breeding unit of our laboratory. The rats were individually housed in wire cages and maintained at 22 ± 1ºC at constant humidity in a 12-h light/dark cycle. They were fed ad libitum with a purified diet for one week and were divided into nine groups of six rats. The experiments were carried out in accordance with the current guidelines for laboratory animal care and handling, approved by the local Ethics Committee (Institutional Ethical Committee Number 050/2011).

APAP-induced hepatotoxicity in rats

Acute hepatotoxicity was induced using the method described by Lin et al. (1995), with some modifications. Rats were randomly divided into nine groups (n = 6). In group 1 (normal control group), the rats were orally treated only with water for 7 days. The rats of Group 2 (hepatotoxicity control group) received distilled water for 7 days before APAP administration. Groups 3, 4 and 5 were treated with HE (100, 250 and 500 mg/kg, p.o.) respectively, and groups 6, 7, 8 were treated with RA (10, 25 and 50 mg/kg, p.o.) for 7 days. Group 9 rats (positive control) received the standard drug silymarin (200 mg/kg body weight,

p.o.). On day 8, group 1 rats (normal control) were administered an intraperitoneal injection of 10 mL/kg body weight of 0.9% NaCl solution; while rats belonging to groups 2–8 were intraperitoneally administered a single APAP dose (600 mg/kg body weight in 40% PEG 400 solution). Six hours after administration of isotonic saline or APAP, animals were anaesthetized and sacrificed. Blood samples were collected by cardiac puncture and placed in heparinized tubes for analyses of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). The liver was quickly removed and washed with ice-cold saline, weighed, and stored at -80ºC. The blood and liver samples were assessed for their biochemical and histological activities.

Liver function tests

The liver function tests, including AST, ALT, ALP levels, were conducted on an Auto Analyzer Hitachi, Japan Inc., according to the manufacturer's protocols. Complete blood counts were obtained on a Coulter HmX Hematology Analyzer Beckman Coulter Inc., following the manufacturer's protocols.

Histopathological observation of the liver

The median lobe of the liver was removed from each rat, fixed in 10% formalin solution, dehydrated in ethanol (50%-100%), cleared in xylene, and embedded in paraffin wax. Thick sections (5-6 mm) were cut and stained with hematoxylin and eosin for photomicroscopy (Drury and Wellington, 1980). Liver sections were scored on a scale of 1-4 (0 = Normal liver histology; + = Tiny and short septa of connective tissue without influence on the structure of hepatic lobules; ++ = Nodular transformation of the liver architecture with loss of structure of the hepatic lobules; +++ = Excessive formation and deposition of connective tissue with subdivision of the regenerating lobules and development of scars). Sections were then examined for pathological findings such as centrilobular necrosis, and lymphocytes infiltration (Eidi et al., 2011; Ranawat et al., 2010).

Statistical analysis

One-Way ANOVA complemented with the Tukey's test was carried out. All the data are expressed as the mean ± S.E.M.; differences between groups were considered significant when $p < 0.05$.

Results

HPLC analysis of the hydroalcoholic extract of *R. officinalis* leaves (HE, Figure 1) evidenced that rosmarinic acid (RA, **1**, R_t 11.80 min) and carnosol (**2,** R_t 25.17 min) are the main constituents (Figure 2).

Table 1 summarizes the AST, ALT, and ALP levels obtained for all the rat groups. It was observed that the levels of AST, ALT and ALP in the APAP group (Group 2) were higher than in normal control (Group 1) ($p < 0.05$). HE-treated rats (Groups 3, 4 and 5) and RA-treated rats (Groups 6, 7 and 8) had lower AST, ALT and ALP levels compared with APAP-intoxicated rats (Group 2). However, the liver damage caused by APAP administration was higher in the RA groups in comparison with HE groups, as evidenced by the higher levels of liver biochemical parameters in the RA groups. Treatment with HE at the doses of 250 and 500 mg/kg body weight conferred marked protection against APAP-induced liver damage, which effect similar to that of the standard drug silymarin (positive control, group 9). The doses used in this study are in agreement with literature data (Chandan et al., 2008; Olaleye et al., 2010).

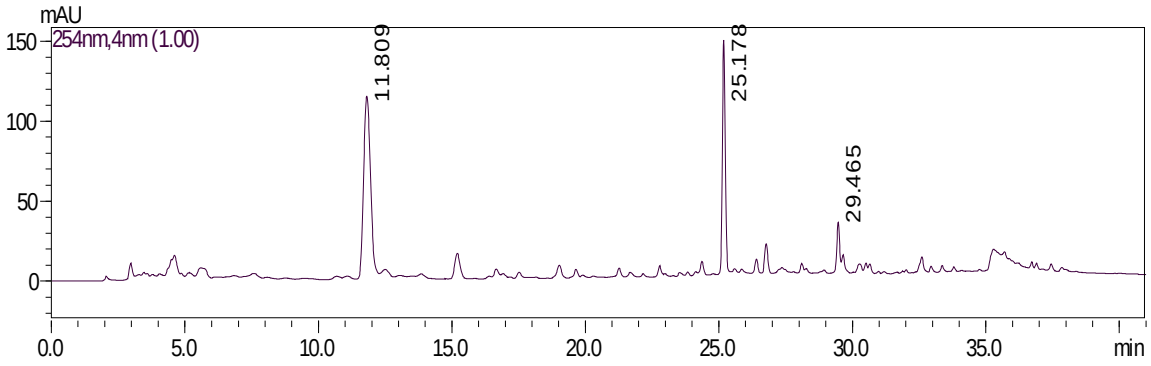

Figure 1. HPLC profile obtained for the ethanol/H_2O (1:1) extract of *R. officinalis* the leaves (HE) showing the major compounds: rosmarinic acid (1, R_t 11.80 min) and carnosol (2, R_t 25.17 min).

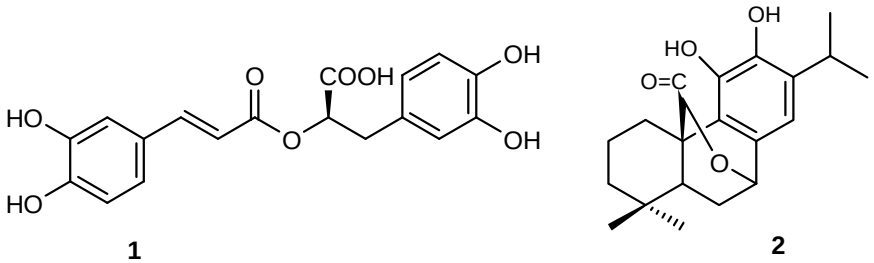

Figure 2. Chemical structures of rosmarinic acid (1) and carnosol (2).

Table 1. Effect of the hydroalcoholic extract of *Rosmarinus officinalis* leaves (HE) and rosmarinic acid (RA) on the serum biochemical parameters of rats during acetaminophen-induced liver damage.

Groups	Treatment mg/kg	ALT (IU/l)	AST (IU/l)	ALP (IU/l)
1	Control	68.16 ± 1.07	125.33 ± 2.57	134.16 ± 6.12
2	APAP	7335.01 ± 4.17*	8295.16 ± 3.16*	471.66 ± 8.13*
3	HE 100	121.66 ± 2.12**	428.33 ± 13.13**	190.66 ± 4.87**
4	HE 250	95.33 ± 4.17**	418.33 ± 4.75**	118.33 ± 13.38**
5	HE 500	72.5 ± 3.68**	191.83 ± 10.08**	114.01 ± 5.66**
6	RA 10	2098.33 ± 2.13	3075.13 ± 4.33	397.17 ± 6.98
7	RA 25	1517.37 ± 4.13	5065.33 ± 9.87	462.23 ± 7.06
8	RA 50	2057.19 ± 1.23	4062.66 ± 5.66	380.61 ± 2.03
9	Silymarin	147.31 ± 4.12**	328.13 ± 14.9**	276.82 ± 9.78**

* $p < 0.05$ significantly different from control group.
** $p < 0.05$ significantly different from APAP group.

Table 2. Effect of the hydroalcoholic extract of *Rosmarinus officinalis* leaves (HE) and rosmarinic acid (RA) on the histopathology of the liver of rats administered with a single dose of acetaminophen.

Groups	Microscopic observation			
	Necrosis	Hemorrhage	Disorganized sinusoid	Lymphocyte infiltration
1	0	0	0	0
2	+++	+++	+++	++
3	+	+	+	+
4	++	+	++	+
5	++	+	++	+
6	+++	++	++	++
7	+++	++	++	++
8	+++	++	++	++
9	++	++	++	++

0 Normal liver histology; + Tiny and short septa of connective tissue without influence on the structure of hepatic lobules; ++ Nodular transformation of the liver architecture with loss of structure of hepatic lobules; +++ Excessive formation and deposition of connective tissue with subdivision of the regenerating lobules and with development of scars.

Biochemical effects of HE and the pure compound RA were supported by the histopathological findings (Figure 3). The histopathological analyses through microscopic observations of the livers are summarized in Table 2. The animals treated with APAP (Group 2) compared with normal group (Group 1) revealed extensive livers injuries characterized by severe hepatocellular degeneration and necrosis. Rats pretreated with 500 mg/kg body weight HE (group 5) and RA (groups 7, 8 and 9) presented severe liver degeneration, characterized by coalescing centrilobular necrosis, congestion, mononuclear cell infiltration in the centrilobular region, and disrupted hepatocyte cords. Rats pretreated with 100 mg/kg body weight HE (group 3) exhibited only diffuse fatty degeneration throughout the hepatic lobule; rats pretreated with 250 mg/kg body weight HE (group 4) showed diffuse fatty degeneration, which was more severe in the centrilobular region. APAP-evoked congestions and inflammatory cell infiltration decreased considerably decreased following HE pretreatment.

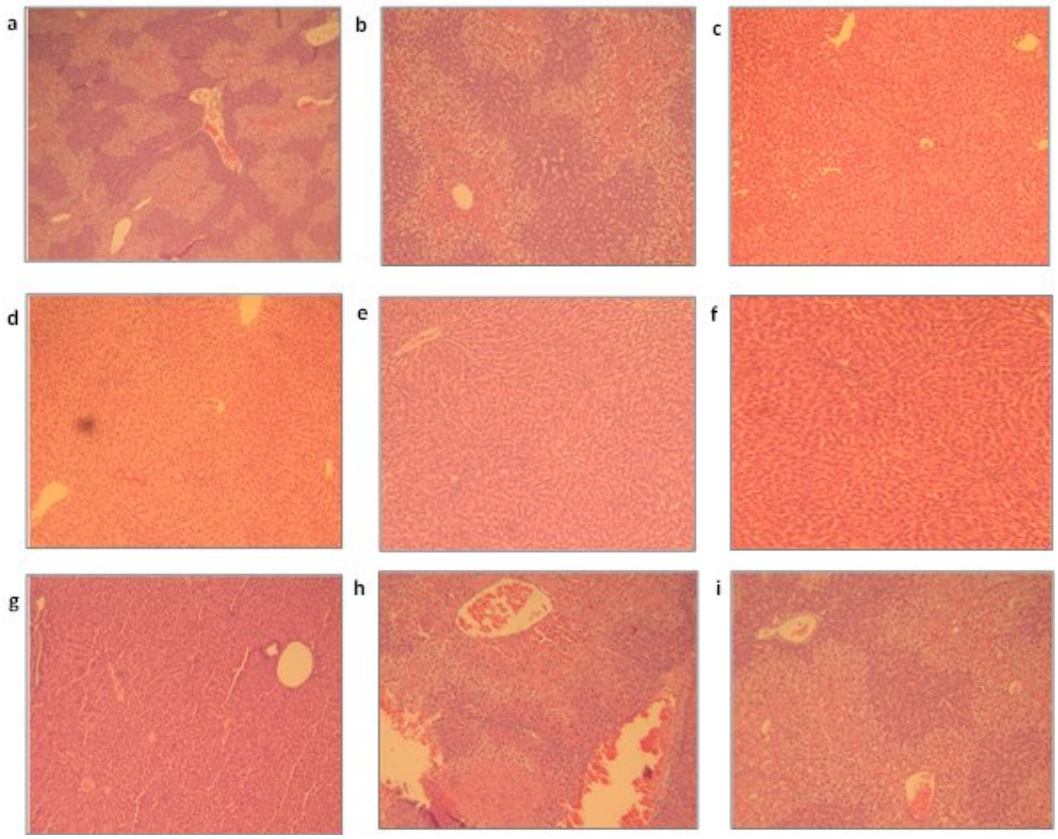

Figure 3. Hepatoprotective effect of HE and RA against APAP-induced hepatotoxicity in rats (H&E staining, magnification= 100x) (a) Pretreatment with distilled water + APAP. The liver section reveals submassive inflammation with cells undergoing necrosis around the perivenular area; (b) pretreatment with silymarin (200 mg / kg) + APAP; (c) pretreatment with distilled water + saline; (d) pretreatment with HE 100 mg/kg + APAP; (e) pretreatment with HE 250 mg/kg + APAP; (f) pretreatment with HE 500 mg/kg + APAP; (g) pretreatment with RA 50 mg/kg +APAP; (h) pretreatment with RA 25 mg/kg + APAP; (i) pretreatment with RA 10 mg/kg + APAP.

Discussion

Liver disease is one of the major causes of morbidity and mortality and affects people of all ages throughout the world. The drugs that are currently available to treat this condition pose serious drawbacks, which justifies the search for new hepatoprotective agents (Sintayehu et al., 2012). In this context, the use of plant extracts and isolated compounds with antihepatotoxic properties can help prevent and treat liver disease.

In this study, we found that HE prevented APAP-induced elevation of ALT, AST, and ALP levels in a dose-dependent manner: pretreatment with 100 mg/kg and 250 mg/kg body weight HE significantly reduced the lesions; pretreatment with a higher HE dose (500 mg/kg body weight) did not prevent liver damage. The histopathological findings obtained for the liver of rats pretreated with 500 mg/kg body weight HE and any RA dose were the same as those recorded for negative control rats (which received APAP only). Taken together, our results show that administration of low doses of HE is sufficient in exerting protective against APAP induced hepatotoxicity.

Foods rich in antioxidants have also been proposed as a tool to prevent liver damage (Morisco et al., 2008). It is generally assumed that these antioxidant compounds may scavenge free radicals and regulate the activity and/or expression of certain enzymatic systems implicated in relevant physiological processes like the metabolism of xenobiotics in the liver (Singletary and Rokusek, 1997; Subbaramaiah et al., 2002). Synergistic interactions amongst the antioxidants present in the

HE of *R. officinalis* leaves might reduce serum ALT, ALP, and AST levels. The presence of the carnosol in the HE of *R. officinalis* may contribute to hepatoprotetive activity displayed by this extract (Sotero-Féliz et al., 2002b) Thus, antioxidant constituents present in extracts of Rosemary might have been responsible for their ability to reduce the acetaminophen induced lipid peroxidation (Lamaison et al., 1990; Haraguchi et al., 1995; Munne-Bosch et al., 1999).

Conclusion

The results of the present study suggest that the hydroalcoholic extract of the *Rosmarinus oficcinalis* leaves reduce the acetaminophen-induced hepathoxicity in rats, and this effect was confirmed by the biochemical and histomorphological findings. These results also provide scientific evidence for the use of the plant in the treatment of liver diseases.

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