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Hydroalcoholic Extract of Rosemary (*Rosmarinus officinalis* L.) and its Constituent Carnosol Inhibit Formalin-induced Pain and Inflammation in Mice

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Abstract: The anti-inflammatory and anti-nociceptive properties of *Rosmarinus officinalis* L. (ROL) extract and its major constituent, carnosol in male NMRI mice (W:25-30 g) have been evaluated in the present study. Formalin (2%, 20 μ L) was injected into the plantar portion of the hind paw and resulting pain and inflammation was studied for 60 min. The plant extract, carnosol and other drugs were administered intraperitoneally or subcutaneously 30 min before formalin injection. In a separate experiment, the effects of the extract and carnosol on plasma corticosterone levels and activity of the enzymes cyclooxygenase type 1 and 2 (COX1 and COX2) were investigated. Injection of different doses of ROL and carnosol reduced pain in the phase 2 of the formalin test, which was not inhibited by naloxone and/or memantine. In addition, pretreatment of the animals with ROL and/or carnosol reduces the formalin-induced inflammation. Furthermore, the extract and carnosol did not affect plasma corticosterone levels compared with the control group. Interestingly, both the extract and carnosol inhibited COX1 and COX2 activity. It could be concluded that ROL extract and carnosol suppressed pain and inflammation induced by formalin injection, which may be due to inhibition of COX1 and COX2 enzymes activity.

Key words: Rosemary, pain, formalin test, cyclooxygenase enzyme, carnosol

INTRODUCTION

Rosemary (*Rosmarinus Officinalis* L.) is a Mediterranean herb grown in several parts of the world and its leaves are used as a food additive in Europe, America and Asia (Zargari, 1994). Decoction of aerial parts of the plant produces a folk medicine remedy for the relief of renal colic, spasmodic pain and dysmenorrhea (Al-Sereiti *et al.*, 1999). In addition, ethanolic preparations of rosemary are currently used in Iran as anti-rheumatoid agents and leaves or branch heads are used in the perfume and cologne industry (Zargari, 1994). Phytochemical studies have revealed that rosemary essential oil consists of dismetin, genkwanin, luteolin, hispidulin, apigenin, ursolic acids, carnosic acid and carnosol (Almela *et al.*, 2006; Ibanez *et al.*, 2000, 2003; Martin *et al.*, 2008; Senorans *et al.*, 2000). In addition, rosemary extract contains oleosin and tannins (Okamura *et al.*, 1994; Ramirez *et al.*, 2004; Santoyo *et al.*,

2005). Modern pharmacological studies have indicated that rosemary extract has anti-bacterial (Ramirez *et al.*, 2004), anti-oxidant (Santoyo *et al.*, 2005), anti-diabetic (Soyal *et al.*, 2007), anti-depressive (Abu-Al-Basal, 2010) and anti-cancer activity (Atsumi and Tonosaki, 2007; Huang *et al.*, 1994), protects against UV (Klancnik *et al.*, 2009) and gamma radiation (Lo *et al.*, 2002; Jindal *et al.*, 2006) and ameliorates stress (Machado *et al.*, 2009).

Previous studies have shown that rosemary extract may have analgesic and anti-inflammatory effects (Peng *et al.*, 2007; Chan *et al.*, 1995; Inoue *et al.*, 2005; 2006; Gonzalez-Trujano *et al.*, 2007). In this regard, studies revealed that the ethanolic extract of rosemary inhibited acetic acid-induced pain in mice with an ED 50% of 108.84 mg kg⁻¹ (Takaki *et al.*, 2008). Moreover, the extract inhibited licking and shaking induced by formalin injections. However, the extract did not show any anti-inflammatory activity as evaluated by uric acid

induced-hind limb edema in rats (Takaki *et al.*, 2008). In this study, they showed that rosemary essential oil inhibited carrageenan-induced paw edema tests in rats and acetic acid-induced writhing and hot plate tests in mice, suggesting that rosemary essential oil possesses anti-inflammatory and peripheral anti-nociceptive activity (Takaki *et al.*, 2008; Chen *et al.*, 2011). Investigations of the effects of carnosol as one of the constituents of ROL extract have also shown that carnosol inhibited LPS-stimulated nitric oxide production in Raw 264.7 cells and reduced inflammation (Kuo *et al.*, 2011). In addition, carnosol inhibited pro-inflammatory leukotrienes in intact polymorph nuclear leukocytes (Shingai *et al.*, 2011), inhibited 5-lipoxygenase, antagonized mobilization of intracellular calcium ions and inhibited cyclooxygenase type 2 (COX2) in inflamed skin in male Balb/C mice (Mengoni *et al.*, 2011).

These studies clearly indicate that ROL extract and carnosol interact with some anti-inflammatory factors to reduce inflammation; however, it remains unclear whether ROL extract and carnosol can inhibit cyclooxygenase type 1 (COX1). Considering the important role for COX1 enzyme in pain and inflammation, this study was design for further evaluation of the extract function in this regard. Moreover, studies have shown that some of the plant extracts can induce glucocorticoid release from the adrenal glands, which may be involved in the anti-inflammatory effects of the extract. The possible activity of the extract in this regard also is not clear.

MATERIALS AND METHODS

Study duration: The study conducted from August 2010 until April 2011. All of the studies were performed in the behavioral laboratory section of Neuroscience Research Center, Baqiyatallah (a.s.) University of Medical Sciences, Tehran, Iran. Experimental duration in this study was one hour and animals' response was recorded at same time.

Animals: Male NMRI mice (W: 20-25 g, Pasture Institute, Tehran, Iran) were used in this study. Animals were kept in cages in groups of six at 22±2°C under a 12h/12h light-dark cycle (lights on at 07:00 a.m.). Food and water were provided ad libitum. Each animal was used once and animals were randomly allocated to different experimental groups. Experiments were conducted in accordance with standard ethical guidelines and approved by the local ethics committee (The Baqiyatallah (a.s.) University of Medical Committee on the Use and Care of Animals, 87/534, Nov 21, 2008).

Plant materials: Aerial parts of ROL were collected in July 2009 from the botanical farm at Baqiyatallah Medical University and were identified by the department of Pharmacognosy, Shahid Beheshti University of Medical Sciences and a voucher number (W 342) was deposited at the herbarium. The dried plants were grinded and after maceration, aqua-alcoholic extraction was performed. Briefly, 100 g of ground rosemary was mixed with 500 mL of distilled water and 500 mL of ethanol in a 2000 mL glass balloon for 24 h at 25°C in a mixer on slow mode. The superficial liquid was passed through a paper filter with a 4 micrometer diameter and incubated at 35°C for one week to allow evaporation of water and ethanol. The extract was then dissolved in saline and injected intraperitoneally into animals. By this method, 20 g of extract was obtained from 100 g of ROL.

Drugs: Morphine sulfate (Temad - Iran), dexamethasone, indomethacin, naloxone hydrochloride, carnosol (Sigma-USA) and memantine bromide (TOCRIS-UK) were used in this study. Drugs were dissolved in saline and injected intraperitoneally to the animals in volumes of 10 mL kg⁻¹ except for morphine, which was given subcutaneously. Control groups received saline either subcutaneously or intraperitoneally.

Experimental design

Evaluation of ROL extract and carnosol analgesic activity: Groups of animals (n = 6/group) were treated with saline, morphine, dexamethasone, indomethacin or different doses of carnosol or ROL extract followed 30 min later by intraplantar formalin injection for pain induction.

Evaluation of ROL extract and carnosol anti-inflammatory activity: Groups of animals (n = 6/group) were treated with saline, morphine, dexamethasone, indomethacin or different doses of carnosol or ROL extract followed 30 min later by intraplantar formalin injection for inflammation induction.

Study of opioid or NMDA glutamate receptor inhibition by ROL extract or carnosol-induced analgesia: Groups of animals (n = 6/group) were treated with co-administration of memantine or naloxone followed 30 min later by different doses of carnosol or ROL extract administration. Approximately, 30 min later, pain was induced by intraplantar formalin injection.

Evaluation of ROL extract- and carnosol-mediated suppression of cyclooxygenase enzyme type 1 and 2:

Groups of animals (n = 6/group) were treated with saline, morphine, dexamethasone, indomethacin or different doses of carnosol or ROL extract followed 30 min later by intraplantar formalin injection. Edema fluid was collected 30 min after formalin injection into the plantar portion of the paw. This fluid was used for the enzyme inhibition study.

Evaluation of the ability of ROL extract and carnosol ability to induce corticosterone release from adrenal glands:

Groups of animals (n = 6/group) were treated with saline or different doses of carnosol or ROL extract. Blood sampling from retro-orbital sinus was performed 30 min later. Plasma corticosterone levels were determined by ELISA.

Pain study: Formalin test was performed using the modified method of Hunskaar and Hole (Hunskaar and Hole, 1987). Each animal received 20 μ L of formalin (2%) in the plantar portion of the right hind paw and was placed in a Plexiglas box with the dimensions of 30×30×30 cm (length×width×height). Hind paw position and animal response to formalin injection were evaluated by an observer on a 0 to 3 scale depending on the animal's foot condition. Animals given a score of 0 had no pain and normal movement, those with a score of 1 placed no body weight on the injected foot but put the foot on the ground (claudicating), those with a score of 2 avoiding contacting the bottom of the box with the injected foot and those with a score of 3 bit or licked the injected foot in response to pain. In a pilot study, the pain response over the time was evaluated for formalin in the intact animals. This study showed that the response to formalin contains two distinct phases. One phase initiated with formalin injection and lasts for 5 min. The second phase initiated 15 min after formalin injection and lasts for 50 min. The times 4 and 25 min after formalin injection was chosen as the pick of phase one and two of formalin test. Extract, morphine, carnosol, dexamethasone and indomethacin were injected into the animals 30 min before injection of formalin, while naloxone and memantine were injected into animals 30 min before injection of the extract. Formalin-induced inflammation:

The degree of inflammation induced by formalin was determined as previously described (Fereidoni *et al.*, 2000). In brief, saline was injected into the left hind paw of each animal as a control. The left hind paw of each animal was placed in a container that contained mercury. The

exact weight of the mercury was determined and the mercury weight change was calculated. By calculating the weight change of the mercury due to displacement by the left hind paw (control) and right hind paw (test), foot weight changes were determined after formalin injection and this weight change was converted to volume change by dividing in to 13.6 (density of mercury).

Determination of plasma corticosterone concentration:

Blood samples was taken from retro-orbital sinus (0.5 mL of the blood in 0.5 mL sodium citrate 1%) 30 min after injection of extract, carnosol, or other drugs. Samples were centrifuged at 3000 rpm for 5 min in 4°C and the supernatant serum was collected for detection of corticosterone. Corticosterone concentration was determined by measuring absorbance at 450 nm using an ELISA kit (Rat Corticosterone ELISA kit; EIA-4164; DRG Instruments GmbH, Germany).

COX1 and COX2 enzyme activities: An ELISA kit (Cox Activity Assay Kit, Cayman-USA) was used to measure COX1 and COX2 activities. As mentioned in the previous section, serum from formalin-injected paws was collected using a fine needle (gauge 30) and added to the ELISA kit, which was poured in three wells containing COX1 or COX2 enzymes. After incubation for 30 min at 37°C, the enzyme product was measured by an ELISA reader at 870 nm.

Statistical analysis: Data were expressed as Means±SEM. To analyze the data, one-way Analysis of Variance (ANOVA) followed by Tukey post hoc test was used. $p < 0.05$ was considered statistically significant.

RESULTS

Effect of different doses of ROL extract and carnosol on formalin-induced pain:

Different groups of animals received saline (10 mL kg^{-1} , i.p.), dexamethasone (10 mg kg^{-1} , i.p.), indomethacin (10 mg kg^{-1} , i.p.), morphine (10 mg kg^{-1} , s.c.), ROL extract (10, 20, 30, 40 and 50 mg kg^{-1} , i.p.), or carnosol (0.5, 1 and 2 mg kg^{-1} , i.p.) 30 min before formalin injection. Animal responses were evaluated 30 min later. Results indicated that neither the extract nor carnosol could suppress the acute phase of formalin-induced pain [F(11, 61) = 7.3, $p < 0.01$] (Fig. 1a). However, ROL extract and carnosol suppressed pain in the second phase of formalin test [F(11, 61) = 10.28, $p < 0.0001$] (Fig. 1b).

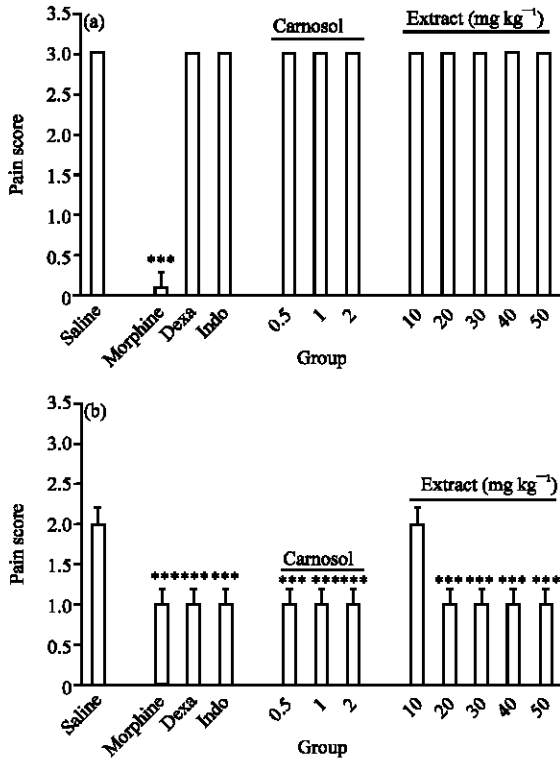


Fig. 1(a-b): Effect of ROL extract and carnosol on (a) phase 1 and phase 2 of the formalin test in mice. Extract and carnosol did not inhibit phase 1 but inhibited phase 2 of the formalin test. Data are Means±SEM for 6 mice, ***p<0.0001 different from experimental groups

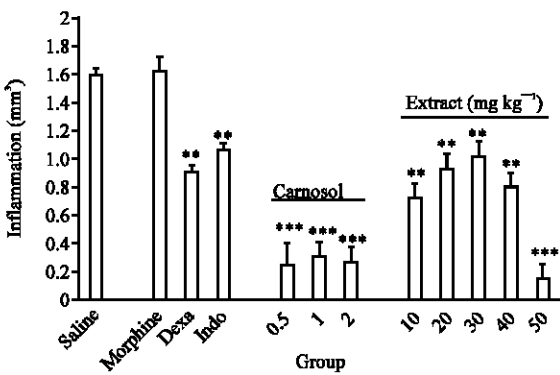


Fig. 2: Effect of ROL extract and carnosol on inflammation induced by formalin in mice. Data are Means±SEM for 6 mice, **p<0.01, ***p<0.001 different from saline-treated control group

Effect of ROL extract and carnosol on formalin-induced inflammation: As the Fig. 2 shows, the ROL extract (10, 20, 30, 40 and 50 mg kg⁻¹, i.p.) and carnosol (0.5, 1 and

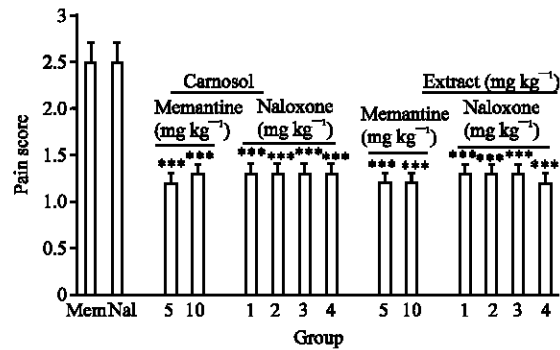


Fig. 3: Effect of opioid and NMDA receptor inhibition on the ROL extract and carnosol effect on phase 2 of formalin pain. Neither naloxone nor memantine blocked the effect of the extract or carnosol. Data are Means±SEM for 6 mice ***p<0.01 different from saline-treated control group

2 mg kg⁻¹, i.p.) could strongly suppress the inflammation induced by formalin injection [F(11, 61) = 11.02, p<0.0001] (Fig. 2). This effect is comparable to the effects of dexamethasone (10 mg kg⁻¹, i.p.) and indomethacin (10 mg kg⁻¹, i.p.) (Fig. 2).

Effect of opioid and glutamate receptor inhibition on rosemary extract- or carnosol-induced analgesia: The effect of naloxone (an opioid receptors antagonist) and memantine (a NMDA glutamate receptor antagonist) on the extract-and carnosol-induced analgesia is shown in Fig. 3. As is clear, pretreatment of the animals with naloxone (1, 2, 3 and 4 mg kg⁻¹, i.p.) and/or memantine (5 and 10 mg kg⁻¹, i.p.) did not inhibit the effect of extract-or carnosol-on pain inhibition [F(16, 84) = 9.81, p<0.001] (Fig. 3).

Effect of ROL extract and carnosol on suppression of COX1 and COX2: The results from in vitro study showed that ROL extract and carnosol were able to inhibit the activity of the enzyme cyclooxygenase type 1 (30%) [F(11, 61) = 11.14, p<0.0001] (Fig. 4a) and type 2 (55%) [F(11, 61) = 15.33, p<0.0001] (Fig. 4b).

Effect of intraperitoneal administration of ROL extract and carnosol on plasma corticosterone levels: The effect of ROL extract and carnosol on blood corticosterone levels is shown in Fig. 5. As is clear in the figure, the extract did not increase plasma corticosterone levels in the experimental groups compared with the control group [F(8, 48) = 0.133, p>0.05] (Fig. 5).

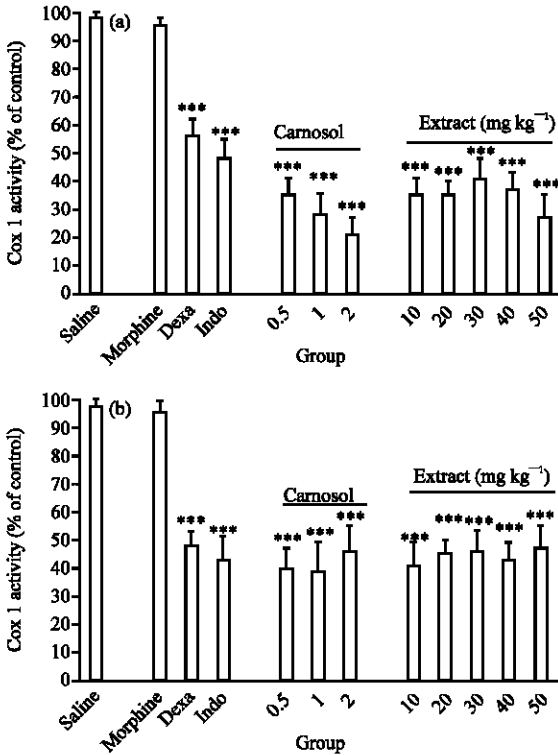


Fig. 4(a-b): Inhibition of COX1 (a) and COX2, (b) by the ROL extract and carnosol in vitro. Data are Means±SEM, for 3×3 well, ***p<0.001 different from control group

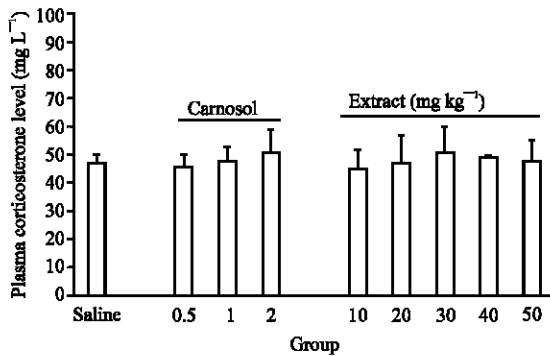


Fig. 5: Effect of ROL extract and carnosol on corticosterone plasma levels in mice. Data are Means±SEM for 6 mice

DISCUSSION

The results of this study indicate that the aqua-alcoholic ROL extract and its constituent carnosol suppresses pain and inflammation induced by formalin injection, possibly by inhibiting COX1 and COX2 activity.

Several studies of ROL extract and carnosol have reported its pharmacological effect on inflammation and pain. Specifically, it has been shown that rosemary extract can reduce formalin-induced pain and inflammation in rodents (Gonzalez-Trujano *et al.*, 2007) and humans (Inoue *et al.*, 2005, 2006). Previous studies have revealed that formalin-induced pain and inflammation results in part from induction of COX1 and COX2 enzymatic activity (Ferreira, 1980; Ferreira and Lorenzetti, 1981; Ferreira *et al.*, 1978). Going one step further, one can conclude from our data that the extract may inhibit formalin-induced pain and inflammation via such a mechanism. Moreover, previous studies have shown the anti-depressive effects of the extract in mice, which resulted from modulation of the dopaminergic pathway in the brain (Machado *et al.*, 2009). Because the dopamine pathway is involved in pain suppression (Zarrindast *et al.*, 2002), it had been speculated that the extract might have a similar effect on formalin-induced pain. In the current study, the extract suppressed pain in the chronic phase at different doses but chronic pain could not be suppressed when the animals were pre-treated with naloxone (an opioid receptor antagonist) (Abbott *et al.*, 1982; North, 1978) and memantine (an N-Methyl-D-Aspartate glutamatergic receptor antagonist) (Kavirajan, 2009). These findings clearly rule out the possible involvement of these two major pain suppression systems in the extract's mechanism of action. Moreover, ROL extract suppressed inflammation in the formalin test. In agreement with our findings, previous studies have also shown that the extract inhibited inflammation induced by formalin in rodents (Inoue *et al.*, 2005, 2006; Gonzalez-Trujano *et al.*, 2007). Collectively, our results and previous findings suggest two possible mechanisms of action for the extract.

First, ROL extract might affect production of prostaglandins, which are important factors for induction of inflammation (Coutaux *et al.*, 2005; Gronert, 2008; Simmons *et al.*, 2004). There are at least two types of the cyclooxygenase enzyme, namely COX1 and COX2 (Simmons *et al.*, 2004), upon which the extract might act and we examined the effect of the extract on these enzymes in vitro in the third part of this study. The in vitro tests showed that the extract suppressed COX1 and COX2 activity to a similar degree as indomethacin. Therefore, we concluded that ROL extract, by inhibition of enzymes involved in the inflammatory response, decreases inflammatory mediators and suppresses inflammation caused by formalin. The functional mechanism(s) of COX1 and COX2 inhibition by ROL extract remain unknown and require further research. Second, the extract may induce release of the hormone

corticosterone from adrenal glands. Some studies have demonstrated the anti-depressive effects of ROL extract in laboratory mice, which results from dopamine release in the brain (Machado *et al.*, 2009). These neurotransmitters are thought to be involved in release of Corticotropin Releasing Factor (CRF) from the hypothalamus and adrenocorticotropin (ACTH) from the anterior pituitary gland (Dedovic *et al.*, 2009), which can control corticosterone release in adrenal glands. In this study, blood analysis of mice that received different doses of the extract demonstrated that these mice had similar levels of corticosterone as the controls and the extract did not induce corticosterone release. Thus, we conclude that the extract inhibits formalin-induced pain and inflammation by mechanisms other than corticosterone release.

CONCLUSION

The major finding of the current study is that ROL extract controls pain and inflammation through inhibition of COX1 and COX2 enzymatic activity and other potential mechanisms, such as endogenous opioid and glutamate system activity, can be excluded. Moreover, the extract did not induce corticosterone release from the adrenal glands to achieve its inhibitory effect on formalin-induced pain and inflammation.

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