Antinociceptive mechanisms of *Rosmarinus officinalis* extract in mice using writhing test

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Abstract:

Rosemary (*Rosmarinus officinalis*) is a common household plant grows in many parts of the world, including Iran. Rosemary leaves are used in folk medicine as an antispasmodic, analgesic, diuretic and antiepileptic agent. The objective of this study was to investigate the antinociceptive mechanisms of rosemary extract using a writhing test as a model of visceral pain. Possible antinociceptive mechanisms were explored by testing the effects of naloxone (nonselective opioid antagonist), cyproheptadine (nonselective serotonin antagonist), Chicago sky blue 6B (inhibitor of glutamate uptake) and bicuculline (GABAA antagonist) on rosemary extract-induced antinociception. Results showed that rosemary extract (50, 100 and 200 mg/kg, i.p.) induces antinociceptive effects in a dose-dependent manner ($p < .001$). Pre-treatment with naloxone and bicuculline significantly reduced this effect (from 61.04% to 27.56%, and from 60.31% to 42.09%, respectively; $p<0.05$). Naloxone and bicuculline brought forward the onset of first abdominal writhing in comparison with extract ($p < .001$). The analgesic effect and latency induced by the extract significantly increased with Chicago sky blue 6B pre-treatment ($p<0.05$), while cyproheptadine had no effect. These results indicate that the antinociceptive effect of *Rosmarinus officinalis* may be mediated by opioidergic, glutamatergic and GABAergic mechanisms.

Introduction

Pain is a common and distressing feature of many disorders such as tumors, surgical procedures, physical trauma and noxious chemical stimulation (Aliu, 2007). Although pain acts as a warning signal and is primarily protective, excessive pain can lead to side effects such as sweating, apprehension, nausea and palpitation (Raquibul et al., 2010). A wide variety of analgesic drugs are available, but these can have serious adverse effects such as drowsiness, nausea, respiratory depression (as seen with opiates; Laurence, 1997), gastrointestinal bleeding and ulceration (as seen with non-steroidal anti-inflammatory drugs; Mate et al., 2008), and an addictive potential. These adverse effects make the search for new analgesic drugs a necessity. Medicinal plants have been documented to have an advantage in long-term toxicity considerations, and it is expected that bioactive compounds obtained from such plants to have low animal and human toxicity (Fabricant and Farnsworth, 2001).

Rosemary (*Rosmarinus officinalis* L.) is a common household plant grown in many parts of the world (Al-Sereiti et al., 1999). It is native to Europe but has been cultivated across all states in Iran. In folk

medicine, it is used as an antispasmodic for renal colic and dysmenorrhoea (Al-Sereiti et al., 1999). The plant has been reported to have the following pharmacological effects: antibacterial (Del Campo et al., 2000), anti ulcerogenic (Dias et al., 2000), hepatoprotective (Sotelo-Félix et al., 2002), anti depressant (Heinrich et al., 2006), antinociceptive (González-Trujano et al., 2007) and anti-inflammatory (Altinier et al., 2007). However, the antinociceptive mechanism of rosemary is not fully understood. Therefore, the objective of this study was to evaluate these mechanisms in mice using opioidergic, serotonergic, glutamatergic and GABAergic receptor antagonists in a writhing test model.

Materials and Methods

Preparation of crude extract: The aerial parts of *R. officinalis* were collected during the flowering period in Khorasan, north east Iran. Samples of the plant were identified by a botanist from the division of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences. The plant leaves were cleaned, shade dried and coarsely ground. The powdered material was soaked in hydroethanolic (70%) solvent for three days with occasional shaking. It was first filtered through a muslin cloth and then through filter paper, and this process was repeated twice more. The combined filtrate was evaporated in a rotary evaporator under reduced pressure to a thick, semi-solid mass of a dark brown color (Subhan et al., 2007). The percentage yield on the basis of the starting dried material was 15 % for the dried hydroalcoholic extract (W/W).

Animals: 208 male albino NMRI mice weighing 25-30 g purchased from the Pasteur Institute of Tehran were used in all experiments. Animals were housed in a room set at a temperature of 22±2°C, on a cycle of 12h light/12h dark (light on at 7:00 am). Food and water were available *ad libitum*. The animals were allowed to adapt to the laboratory environment for at least 2h before testing and were used only once. All experimental procedures followed the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals (Zimmermann, 1983).

Nociception induction in mice: Nociception in mice was induced by an intraperitoneal (i.p.) injection of 0.6% acetic acid in a volume of 10ml/kg as described by Koster et al. (1959). The induced nociceptive behavior (writhes) was characterized by a contraction of the abdominal muscles, accompanied by an elongation of the body and extension of the hind limbs.

Experimental procedures: Animals were pre-treated 30 min before acetic acid administration with one of the following: 5% DMSO (dimethyl sulfoxide) as vehicle, indometacine (5 mg/kg) or different doses of the extract (50, 100 and 200 mg/kg) (Table 1). Eight mice were used in each group. To investigate the antinociceptive mechanisms of the extract, mice were pre-treated with naloxone (2 mg/kg), an opioidergic receptor antagonist; cyproheptadine (4 mg/kg), a serotonergic receptor antagonist; Chicago sky blue 6B (100 µg/kg), an inhibitor of glutamate uptake; and bicuculline (2mg/kg), a GABAA receptor antagonist. After 15min, either the vehicle or the highest dose of plant extract (200mg/kg) was given to eight mice in each group, followed by administration of 0.6% acetic acid (Tables 2-5). Immediately after acetic acid injection, the total number of writhes and onset of first abdominal writhing (latency) were determined over 30 mins. Indometacine was administered as a reference drug for comparison. The dose rate was chosen on the basis of preliminary and previous studies (Van Riezen et al., 1972; Bero et al., 1987; Sugiyo et al., 2009; Zendehdel and Babapour, 2010; Zendehdel et al., 2011). All drugs were dissolved in 5% DMSO and the control group received the 0.5 ml vehicle alone.

Determination of antinociceptive activity: Antinociceptive effect was expressed as inhibition percent of abdominal constrictions using the ratio:

$$\frac{\text{(Controlmean-treatedmean)}}{\text{Controlmean}} = \%\ \text{writhing pain score}$$

Table 1: Effect of hydroethanolic extract of *Rosmarinus officinalis* in acetic acid-induced writhing in mice. Control: Vehicle (5% DMSO). Different superscript (a, b, c, d and e) indicate significant differences between groups (P < 0.001); n = 8 for each group.

Group	Dose (mg/kg)	Latency time (sec)	Writhing count (mean ± SEM)	Inhibition (%)	p-value
Control	10	188±13[a]	74.53±8.29[a]	---	---
R. officinalis	50	290±18[b]	52.67±6.59[b]	29.30	0.001
R. officinalis	100	456±30[c]	39.67±4.49[c]	46.75	0.001
R. officinalis	200	610±48[d]	28.83±4.86[d]	61.30	0.001
Indomtacine	5	794±84[e]	13.33±2.15[e]	83.1	0.001

Table 2: The effect of naloxone on *R. officinalis*-induced antinociception in acetic acid-induced writhing in mice. Control: Vehicle (5% DMSO). Different superscript (a, b, and c) indicate significant differences between groups (p<0.001); n = 8 for each group.

Group	Dose (mg/kg)	Latency time (sec)	Writhing count (mean ± SEM)	Inhibition (%)	p-value
Control	10	179±12[a]	76.17±9.29[a]	---	
R. officinalis	200	643±54[b]	29.67±4.43[b]	61.04	< 0.001
Naloxone	2	175±18[a]	84.50±9.30[a]	---	
Naloxone+*R.officinalis*	2+200	371±24[c]	55.17±6.39[c]	27.56	< 0.001
					<0.05 vs. *R. officinalis*

Table 3: The effect of cyproheptadine on *R. officinalis*-induced antinociception in acetic acid-induced writhing in mice. Control: Vehicle (5% DMSO). Different superscript (a, b, and c) indicate significant differences between groups (p<0.001); n = 8 for each group.

Group	Dose (mg/kg)	Latency time (sec)	Writhing count (mean ± SEM)	Inhibition (%)	P value
Control	10	178±16[a]	76.37±8.51[a]	---	
R. officinalis	200	640±45[b]	30.67±6.49[b]	59.84	< 0.001
Cyproheptadine	4	174±19[a]	80.17±9.66[a]	---	
Cyproheptadine +*R.officinalis*	4+ 200	587±68[b]	32.00±5.75[b]	58.09	< 0.001

Acute toxicity: Mice were divided into control and test groups (n=8). The first group served as normal control. *R. officinalis* extract was administered IP to different groups at the increasing doses of 400, 800, 1600, 3200 and 6400 mg/kg. After the injection of extracts, mice were allowed to have food and water *ad libitum* and all animals were monitored for possible mortality cases and behavioral changes for 72 hours.

Statistical analysis: Data were presented as mean values ± SEM. Statistical analysis was carried out by one-way analysis of variance (ANOVA) with Tukey's post-hoc test. P-values less than 0.05 were considered to indicate statistical significance.

Results

Antinociceptive effect of *R. officinalis:* A hydroethanolic extract of *R. officinalis* (50, 100 and 200mg/kg) induced antinociceptive effects in a dose-dependent manner (p<0.001, Table 1). Inhibition percentages of abdominal constrictions for the different doses and indomethacin were 29.3%, 46.8%, 61.3% and 83.1%, respectively. Administration of the extract significantly decreased the time to onset of first abdominal writhing, compared to the control group (p< 0.001, Table 1).

Effect of naloxone, cyproheptadine, Chicago sky blue 6B and bicuculline on the antinociceptive action of *R. officinalis*

Pre-treatment with naloxone and bicuculline significantly decreased the analgesic effect of rosemary extract at 200 mg/kg; from 61.04% to 27.56% and from 60.31% to 42.09%, respectively (p< 0.05, Tables 2 and 5). The time to onset of first abdominal writhing significantly decreased in the presence of naloxone and bicuculline (p<0.001, Tables 2 and 5). Furthermore, the analgesic effect and

Table 4: The effect of Chicago sky blue 6B on *R. officinalis*-induced antinociception in acetic acid-induced writhing in mice.

Group	Dose (mg/kg)	Latency time (sec)	Writhing count (mean ± SEM)	Inhibition (%)	P value
Control	10	179 ± 14^{a}	75.67 ± 9.15^{a}	---	
R. officinalis	20	638 ± 67^{b}	30.47 ± 4.49^{b}	59.73	< 0.001
Chicago sky blue 6B	10	207 ± 31^{a}	77.83 ± 11.35^{a}	---	
Chicago sky blue 6B+ *R. officinalis*	10+200	853 ± 95^{c}	21.53 ± 2.33^{c}	71.54	< 0.001
					<0.05 vs. *R. officinalis*

Table 5: The effect of bicuculline on *R. officinalis*-induced antinociception in acetic acid-induced writhing in mice. Control: Vehicle (5% DMSO). Different superscript (a, b, and c) indicate significant differences between groups ($p< 0.001$); n = 8 for each group.

Group	Dose (mg/kg)	Latency time (sec)	Writhing count (mean ± SEM)	Inhibition (%)	P value
Control	10	184 ± 19^{a}	79.33 ± 8.29^{a}	---	
R. officinalis	20	635 ± 59^{b}	31.45 ± 3.49^{b}	60.31	< 0.001
Bicuculline	10	178 ± 23^{a}	76.50 ± 8.58^{a}	---	
Bicuculline+*R. officinalis*	10+200	302 ± 36^{c}	44.50 ± 4.54^{c}	42.09	< 0.001
					<0.05 vs. *R.officinalis*

latency induced by the extract significantly increased with Chicago sky blue 6B pre-treatment ($p<0.05$, Table 4), while cyproheptadine had no effect on pain response induced by rosemary extract (Table 3).

Toxicity effects: Administration of *R. officinalis* extract, at the doses of 400, 800, 1600, 3200 and 6400 mg/kg, had no effect on behavioral responses and no mortality was observed during the observation period of 72 h after administration.

Discussion

In the present study, we did not observe any mortality in mice given doses of up to 6.4 g/kg of *R. officinalis* extract. Therefore, we suggest that the extract has no lethal toxicity in mice.

The use of an abdominal constriction (writhing) model for detection of antinociceptive activity has been reported to be more sensitive to other models such as tail flick and formalin tests (Collier et al., 1968; Bentley et al., 1981). According to our findings, hydroethanolic extract of *R. officinalis* led to a significant reduction in pain response in a dose-dependent manner, compared to the control group. This plant possesses phenolic diterpenes, such as carnosic acid, carnosol, rosmanol, epi- and iso-rosmanol and the phenolic constituent rosmarinic acid (Yesil-Celiktas et al., 2010). Previous studies have found rosmarinic acid in lemon balm (Melissa officinalis L.) to have pharmacological effects, including anti-inflammatory and analgesic activity (Mills and Bone, 2000; Guginski et al., 2009). Thus, the antinociceptive effects of *R. officinalis* extract may be mediated by its phenolic component, especially rosmarinic acid. To investigate the antinociceptive mechanisms of the extract, we examined the possible involvement of opioidergic, serotonergic, glutamatergic and GABAergic receptor antagonists on *R. officinalis*-induced antinociception. Our findings showed that pre-treatment with naloxone significantly decreased the antinociceptive effect of the extract. There is evidence that opioid agents exert their analgesic effects via supraspinal (m1, k3, d1, s2) and spinal (m2, k1, d2) receptors (Reisine and Pasternack, 1996). The antinociceptive activity of opioid agonists, opioid partial agonists and non-steroidal anti-inflammatory agents can be determined by the writhing test (Vogel and Vogel, 1997). However, primary sensory nerve endings as well as cell bodies within the dorsal and trigeminal ganglia possess ?- ?- and k-opioid receptors (Rau et al., 2005) and potent analgesia is achieved when opioids are administered peripherally (LeBon et al., 2009). Thus, the antinociceptive activity of the extract maybe acts on

both the central and peripheral nervous system, although we need more research in this area.

Since the activity of *R. officinalis* extract was significantly, but not completely, reversed by naloxone, it is possible that other mechanisms such as glutamatergic and GABAergic systems are involved. Our data showed that pre-treatment with Chicago sky blue 6B significantly increased the antinociceptive effects of the extract. Several lines of evidence have shown that excitatory amino acids, such as glutamate and aspartate, are important for the transmission of nociceptive inputs; therefore significant changes may occur within the spinal cord in response to peripheral nociception or inflammation (Castillo et al., 1995). These include changes in excitatory amino acid synthesis and release. For example, increased glutamate concentration in the spinal cord was found to correlate with noxious stimulation (Skilling et al., 1988). Feng et al. (2003) reported that intraperitoneal injection of acetic acid stimulated sensory neurons to release glutamate and aspartate, resulting in an increased concentration of these amino acids in the CSF. Therefore, activation of the sensory nervous system and release of excitatory amino acids following acetic acid injection may be considered as a part of the host defense response.

The results obtained from this research showed that the antinociceptive effect of rosemary extract was decreased with bicuculline pre-treatment. Pain involves a set of ascending pathways that convey nociceptive information from peripheral nociceptors to higher levels of the central nervous system, as well as descending pathways that modulate the information. When there is no ascending nociceptive input, the descending inhibitory pathways are tonically inhibited by inhibitory Gamma-aminobutyric acid-mediated (GABAergic) neurons within the brain stem. Nociceptive input activates the descending inhibitory pathways by removing the tonic inhibition through activation of inhibitory neurons, such as opioidergic neurons (Ohashi et al., 2003). It has been reported that *R. officinalis* increases the GABA content in the brain (Abdul-Ghani et al., 1987) and GABA is known to be correlated with the action of morphine. The antinociceptive effect of morphine has found to be enhanced by amino-oxyacetic acid, but decreased by bicuculline (Yoneda et al., 1976). GABA content is known to increase significantly with the antinociceptive action of morphine in the dorsal part of the spinal cord, the ventrolateral part of the ventral thalamic nucleus and the lateral spinothalamic tract (Yoneda et al. 1977). Thus, it is likely that GABA plays a part in nociceptive inhibition in these areas and the GABAergic system may be another possible route involved in the action of this medicinal plant.

In conclusion, the present study proposes that antinociceptive effect of *Rosmarinus officinalis* may be mediated by opioidergic, glutamatergic and GABAergic mechanisms.

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