Antinociceptive Effect and GC/MS Analysis of *Rosmarinus officinalis* L. Essential Oil from its Aerial Parts

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Abstract

The rationale of this investigation was to examine the antinociceptive properties of the essential oil obtained from *Rosmarinus officinalis* aerial parts, using a rat model of arthritic pain. The essential oil (100, 300 and 600 mg/kg, *i.p.*) produced a dose-dependent antinociceptive effect, manifested as a significant reduction in the dysfunction in the pain-induced functional impairment model in the rat (PIFIR model), mainly at high doses. Chemical constituents of the essential oil were further analyzed by gas chromatography-mass spectrometry (GC/MS). The major compounds in the essential oil were α-pinene (14.10%), camphene (11.47%), β-pinene (12.02%), myrcene (3.31%), α-phellandrene (7.87%), eucalyptol (8.58%), 2-bornanone (3.42%), camphor (8.75%), isoborneol (3.48%), borneol (4.85%) and borneol acetate (6.49%). The antinociceptive effects of *R. officinalis* essential oil were tested in combination with 0.12 mg/kg WAY100635, *s.c.* (an antagonist of 5-HT_{1A} receptors) or 1 mg/kg naloxone, *i.p.* (an antagonist of endogenous opioids receptors), demonstrating in both cases an inhibition of the antinociceptive response. This study suggests an involvement, at least in part, of the serotonergic system via 5-HT_{1A} receptors and endogenous opioids in the antinociceptive effect of *R. officinalis* essential oil in the PIFIR model.

Key words

antinociception · endogenous opioids · PIFIR assay · 5-HT_{1A} receptors · *Rosmarinus officinalis* L. · Lamiaceae

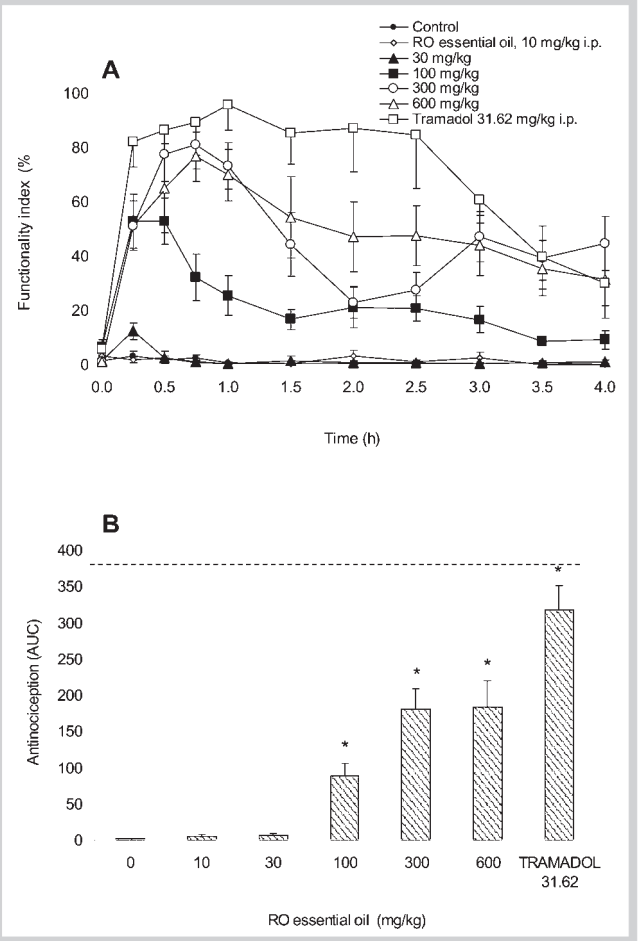

Fig. 1 **A** Time course curves of the antinociceptive effect of RO at 10 (◊), 30 (▲), 100 (■) 300 (○) or 600 (Δ) mg/kg in comparison to the control group (●) and tramadol 31.62 mg/kg (□) in the PIFIR model. **B** Dose-response curve of the antinociceptive effect of RO (10 to 600 mg/kg, *i.p.*) in comparison to the effect of vehicle (0.2% Tween 80 in s.s., *i.p.*) and tramadol (31.6 mg/kg, *i.p.*) in the PIFIR model. Data are expressed as the mean of the area under the curve (AUC) ± SEM of 6 animals. * $p < 0.001$, ANOVA followed by Dunnett's test.

Aerial parts of *Rosmarinus officinalis* L. (Lamiaceae) in aroma therapy, a form of alternative medicine that uses volatile liquid plant materials known as essential oils, has been commonly employed in folk medicine since ancient times to minimize painful conditions in humans. Clinical studies have described that aroma therapy with this species decreases pain in arthritic patients [1] and modifies the unpleasantness in pain, as was analyzed in experimentally induced pain in humans [2]. Phytochemical analysis of *R. officinalis* essential oil (RO) has been previously described [3], but with no association to the antinociceptive or anti-inflammatory effect. In a previous study, we observed that an ethanol extract from the aerial parts of *R. officinalis* produced antinociceptive and anti-inflammatory effects in different experimental models of pain [4]. However, to the best of our knowledge, this is the first report on basic pharmacological studies of RO as antinociceptive and anti-inflammatory and its action mechanism. Thus, the purpose of the present study was not only the evaluation of the antinociceptive effect of RO, but also the characterization of its chemical compounds. In addition, the possible involvement of 5-HT_{1A} receptors and endogenous opioids in the antinociceptive effect was evaluated using the pain-induced functional impairment model in the rat (PIFIR model), which induces similar inflammatory and chronic pain to that observed in clinical gout arthritis [5].

At doses from 100 to 600 mg/kg, RO produced a significant ($p < 0.001$) dose-dependent reduction of the dysfunction induced by uric acid in the PIFIR model. The maximal effect obtained at 300 mg/kg was not modified when RO was tested at 600 mg/kg (**Fig. 1**). Temporal course curves showed that almost 80% of the functionality index (FI) was recovered at 300 and 600 mg/kg dosages, with a maximal response observed between 0.5 and 1 h after administration. This maximal antinociceptive effect was reduced by 25 and 45% during the subsequent hour, but recovered 30 min later and was maintained there at 45% functionality until the end of the experiments (1 h more) (**Fig. 1 A**). At a dosage of

Table 1 Constituents identified in the essential oil of *Rosmarinus officinalis* aerial parts by GC/MS analysis.

Peak No.	Identification	Area (%)	RT (s)
1	Tricyclene	< 1	148
2	α-Thujene	< 1	151
3	α-Pinene	14.10	154
4	**Camphene**	**11.47**	**160**
5	**β-Pinene**	**12.02**	**172**
6	**Myrcene**	**3.31**	**178**
7	**α-Phellandrene**	**7.87**	**184**
8	α-Terpinine	1.17	189
9	*o*-Cymene	1.81	194
10	Trifluoroacetyl-α-terpineol	2.01	195
11	**Eucalyptol**	**8.58**	**195**
12	unidentified	< 1	199
13	Carene	1.86	208
14	*cis*-α-Terpineol	1.45	213
15	Terpinolene	< 1	222
16	unidentified	< 1	227
17	Linalool	< 1	235
18	**2-Bornanone**	**3.42**	**245**
19	**Camphor**	**8.75**	**249**
20	**Isoborneol**	**3.45**	**255**
21	**Borneol**	**4.85**	**257**
22	*p*-Menthol	0.08	278
23	**Borneol acetate**	**6.49**	**306**
24	b-caryophyllene	1.38	357

RT = retention time

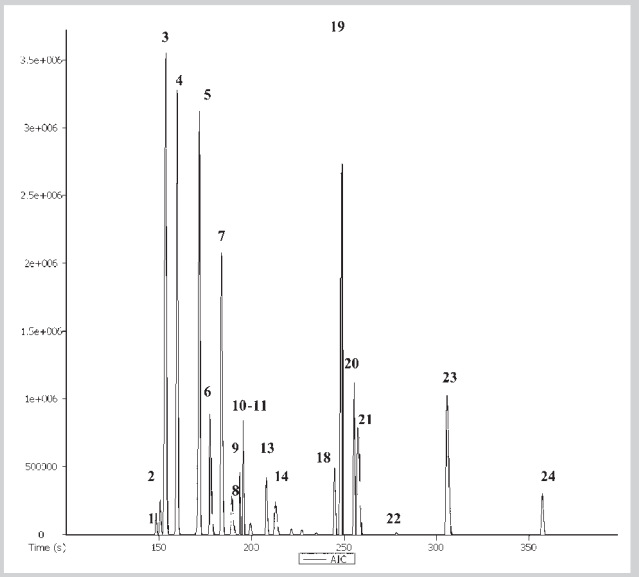

Fig. 2 GC/MS chromatogram showing the peaks of the major components of RO from fresh aerial parts. For data on peak values, see **Table 1**.

100 mg/kg, the maximal antinociceptive effect was almost 55% during the first 30 min, and then the FI% decreased to 20% and remained stable for 1.5 hours more: The FI at the end of the experiment was shown to be 10%. The control rat group [receiving 0.2% Tween 80 in saline solution (s.s.)] as well as 10 mg/kg of RO, exhibited no antinociceptive effects (AUC) along the experiment (**Fig. 1A**). RO antinociception was compared with a 31.6 mg/kg tramadol dosage producing 82% of antinociceptive effects (**Fig. 1B**). Our results support the traditional use of RO and recent studies reporting that this plant alone or in combination with reduced iso-alpha acids and oleanolic acid can relieve arthritic pain [1,6]. However, since high doses (300 and 600 mg/kg) were required to produce a maximal antinociceptive effect that reached 50% of the total antinociceptive response showing two peaks, it will be interesting in future experiments to look for the active compounds involved in this effect.

Table 1 lists the compound name, area (%) and retention time (s) of 24 constituents characterized by GC-MS analysis in the RO obtained from its aerial parts. **Fig. 2** shows the chromatogram where the major peaks were identified as α-pinene (**3**), camphene (**4**), β-pinene (**5**), myrcene (**6**), α-phellandrene (**7**), eucalyptol (**11**), 2-bornanone (**18**), camphor (**19**), isoborneol (**20**), borneol (**21**) and borneol acetate (**23**), as compounds recognized in this essential oil. Compounds **5, 6, 11, 18, 19, 21, 23,** and **24** have been previously identified and isolated from various species other than RO, which have been associated with antinociceptive and anti-inflammatory activities, such as: *Cinnamomum osmophloeum* [7,8], *Cinnamomum camphora* [9], *Hyptis fruticosa* [10], *Nepeta italica* L. [11], *Eucalyptus camadulensis* [12], *Cymbopogon citrates* [13] and *Eremanthus erythropappus* [14].

Fig. 3A shows that rats from control groups receiving s.s. and those injected with WAY 100635 or naloxone alone did not show any recovery in the FI all along the experiment. Administration of each antagonist in the presence of 100 mg/kg of RO prevented the antinociception produced by RO alone (**Figs. 3A** and **B**). As observed in **Fig. 3** reduction of the antinociceptive effect of RO was more pronounced in the presence of naloxone than of WAY 100635 ($p < 0.001$).The specific mechanism of action of RO is unknown. However, since more than one constituent integrates this oil it is possible that more than one mechanism of action could be participating or even that a possible association between various systems may exist. Because an inhibition in the antinociceptive effect of RO was observed with both antagonist drugs (naloxone and WAY 100630) in this study, this suggests that serotonergic and opioid endogenous systems may be involved in its mechanism of action as an antinociceptive as shown in the PIFIR model. *In vivo* and/or *in vitro* studies have described that mechanisms of action suggested for compounds **6, 11, 19, 21, 23**, and **24**, identified and isolated from other plants, produce antinociceptive and anti-inflammatory effects involving the inhibition of some of these mediators – such as cytokines and prostaglandins [7,15,16], transient receptor potential A_1 [17,18], glycine and $GABA_A$ receptors [19], but also adrenergic and opioidergic systems [12,13]. However, until now no study has described the participation of the serotonergic system mediated by 5 HT_{1A} receptors and endogenous opioids in the antinociceptive activity of RO essential oil.

In conclusion, to the best of our knowledge, this is the first study demonstrating the antinociceptive effects of RO essential oil in which the involvement of 5 HT_{1A} receptors and endogenous opioids have been suggested to account for the experimentally induced nociception. This suggests an advantageous therapeutic activity for *R. officinalis* as medicinal plant acting by different mechanisms of action to produce analgesic and anti-inflammatory effects, where compounds **5, 6, 11, 19, 21, 23,** and **24** may be, in part, responsible for this activity. Since it is a pilot study and a maximal response was observed at doses that might not correlate with the traditional use, further detailed experiments

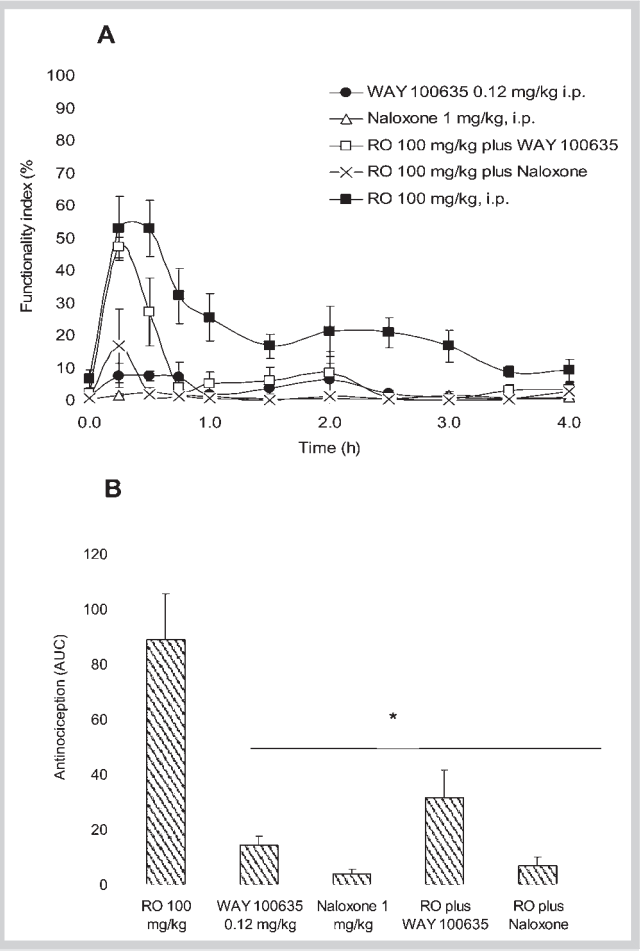

Fig. 3 **A** Time course curves of the FI% produced by RO at 100 mg/kg (■) alone, or in the presence of either 0.12 mg/kg WAY 100635 (□) or 1 mg/kg naloxone *i.p.* (X) and, in comparison to WAY 100635 (●) or naloxone (Δ) alone. **B** Dose-response curve of RO antinociceptive effect represented as AUC. Each point represents the mean ± SEM of 6 animals. * $p < 0.001$, ANOVA followed by Dunnett's test.

are ongoing to investigate which of these compounds are mainly involved in this kind of pain and by using some of these mechanisms of action.

Materials and Methods

Male Wistar rats weighing 180–200 g [Crl(WI)BR] (Cinvestav-Sede Sur and Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz) housed under controlled conditions were used in this study. All experimental procedures followed the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals [20], and were carried out according to a protocol approved by the local Animal Ethics Committee and in compliance with national (NOM-062-ZOO-1999) and international rules on care and use of laboratory animals.

Aerial parts of RO were collected in April 2007 in the State of Morelos, Mexico. MSc Abigail Aguilar identified the specimen and a voucher specimen (IMSSM-15005) was deposited in the herbarium of the IMSS in Mexico City for future reference. Fresh collected aerial parts of RO were cut into small bits (2 900 g) and kept in a 9 L container; extraction was carried out by hydrodistillation for 1 h. The final product yielded 0.2% of transparent liquid oil. Samples were injected to an Agilent 6890 N gas chromatograph with an automatic liquid sampler Agilent 7683B coupled to a LECO Pegasus 4D mass spectrometer. A DB-5MS fused silica capillary column (10 m × 0.18 µm, film thickness 0.18 µm), at 250 °C, and under bubbling 99.99% helium carrier gas with a 1 mL/min flow rate was used. The column oven was temperature-programmed; initial temperature 40 °C (0.5 min) until 300 °C at 20 °C/min. The injector and detector temperatures were both 200 °C; electron energy 70 eV. Masses were scanned from 45–800 amu. The constituents of RO essential oil were characterized by matching their 70 eV mass spectra with compound libraries (NIST/EPA/NIH Mass Spectral Library, Version 2.0).

In order to block the 5-HT_{1A} agonist effect, WAY 100635 (*N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-2-pyridinylcyclohexanecarboxamide maleate; Sigma) was applied. On the other hand, as an antagonist of endogenous opioids, naloxone was used (Sigma). Both were dissolved in s.s. and administered 15 min before antinociceptive treatment. To induce nociception, 20% uric acid (Sigma) suspended in mineral oil was employed. Tramadol (Grünenthal de Mexico) was used as reference drug and diluted in s.s. All drugs were of the highest-purity grade. Doses of each agent refer to the free base and they were freshly prepared on the day of the experiments.

Antinociceptive activity was assessed using the PIFIR model as previously described [5]. After the uric acid injection, animals developed a progressive dysfunction of the injured limb. The variable measured in this model is the time of contact between each of the hind paws and a rotating cylinder. The time of contact of the injured hind limb reached a zero value at 2.5 h after uric acid injection. At this moment, the antinociceptive treatment was administered. This time was considered as time zero for the measurement of the antinociceptive effect, thus allowing the determination of the time course of this effect. Antinociception was estimated as the recovery of the time of contact. Data are expressed as the functionality index percent (FI%), i.e., the time of contact of the injected paw divided by the time of contact of the control left paw multiplied by 100. Once the FI% was zero, different groups of rats received one of the following treatments in a volume of 0.1 mL/100 g of body weight: 1) Control: *i.p.* injection of either s.s. or 0.2% Tween 80 in s.s.; 2) RO essential oil (10, 30, 100, 300 or 600 mg/kg *i.p.*); 3) WAY 100635 [0.12 mg/kg, subcutaneous (*s.c.*)] plus RO essential oil (100 mg/kg, *i.p.*) or 4) naloxone (1 mg/kg, *i.p.*) plus RO essential oil (100 mg/kg, *i.p.*) and, 5) WAY 100635 or 6) naloxone alone. Recordings were taken every 15 min for the first 1 h and thereafter every 30 min until 4 h had elapsed. Recovery of FI% was considered as the expression of the antinociceptive effect. The time-response curve of RO essential oil was plotted to detect the onset and maximum value of the antinociceptive effect, whereas dose-response curves were used to determine the significant antinociceptive doses. For the purpose of this study, inducing nociception in the experimental animals was unavoidable. However, care was taken to avoid unnecessary suffering.

Data are expressed as the mean ± standard error of the mean (SEM) of six repetitions. The area under the curve (AUC) values were calculated from the respective temporal course curves obtained in the PIFIR model, which was considered as an expression of the overall antinociceptive activity during the 4-h observation period (maximal value reached 378.5 ua); AUC was calculated using the trapezoidal rule. All data were compared by analysis of

variance (ANOVA) followed by Dunnett's test using SIGMA STAT® software, version 2.3. $p < 0.05$ was considered statistically significant.

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