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Hesperidin produces antinociceptive response and synergistic interaction with ketorolac in an arthritic gout-type pain in rats

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ABSTRACT

Hesperidin occurs in greatest concentration in plants from the Rutaceae and Lamiaceae families. In human nutrition it contributes to the integrity of blood vessels and its deficiency in the diet has been linked to abnormal capillary leakiness as well as pain. In this study, the bioflavonoid hesperidin was identified as an active compound in an ethanol extract of the *Rosmarinus officinalis* aerial parts tested in the pain-induced functional impairment model in the rat (PIFIR) as an assay of inflammatory and chronic nociception similar to that observed in clinical gout. Hesperidin produced a dose-dependent and significant response with an $ED_{25} = 1666.72$ mg/kg in comparison to an $ED_{25} = 302.90$ mg/kg for the extract or an $ED_{25} = 0.47$ mg/kg for the reference drug ketorolac in the PIFIR model. Although the antinociceptive response of *R. officinalis* was reverted in presence of the opioid antagonist naloxone (10 mg/kg, s.c.) and the $5HT_{1A}$ antagonist WAY100635 (0.12 mg/kg, s.c.), the hesperidin response was not modified by naloxone (10 mg/kg), WAY100635 (0.12 mg/kg), bicuculline (1 mg/kg, s.c.), flumazenil (10 mg/kg, i.p.) or caffeine (1 mg/kg, s.c.). Nevertheless, it was reduced in presence of capsazepine (10 or 20 mg/kg, s.c.) suggesting the participation of the TRPV1 receptor, which was reinforced when hesperidin significantly reduced the capsaicin-induced nociceptive response. A synergistic interaction was also observed when antinociceptive doses of hesperidin were combined with those of ketorolac producing 15 combinations mainly in additive and supra-additive responses. These results provide evidence for the antinociceptive activity of hesperidin and demonstrate synergistic response when combined with ketorolac, possibly by involvement of the TRPV1 receptor, suggesting their clinical potential in pain therapy.

1. Introduction

Hesperidin is a flavonoid previously called “vitamin P” to indicate that it could decrease capillary permeability and fragility (Morii, 1939). It has been reported that hesperidin prevents vascular leakage through the inhibition of the enzyme hyaluronidase; it also decreases the amount of blood cells and platelet aggregation (Beiler and Martin, 1948; Garg et al., 2001). Besides its effect on vascular permeability, this flavonoid exhibits a wide range of pharmacological activities such as sedative (Fernández et al., 2005; Guzmán-Gutiérrez and Navarrete, 2009), antioxidant (Hirata et al., 2005; Zhang et al., 2002), anticarcinogenic (Hirata et al., 2005; Tanaka et al., 1997), as well as exhibiting anti-inflammatory and analgesic properties (Hirata et al., 2005; Galati et al., 1994; Benavente-García and Castillo, 2008). A preventive effect was also previously reported on the development of adjuvant arthritis, a rat model of rheumatoid arthritis by using Freund's complete adjuvant (Guardia et al., 2001; Li et al., 2008).

Hesperidin is abundantly found in citrus fruits (family Rutaceae) (Benavente-García and Castillo, 2008) and in many plants of the genera Lamiaceae (Garg et al., 2001; Kokkalou and Kapetanidis, 1988). *Rosmarinus officinalis* L. is a plant species of the family Lamiaceae; it is an aromatic evergreen shrub native to the Mediterranean area and now grown in many countries. It is well-known around the world and widely used for flavoring food and beverages, as well as an ingredient in cosmetic products (Al-Sereiti et al., 1999; Altinier et al., 2007); in folk medicine it is mainly used as an antispasmodic, in renal colic, dysmenorrhoea and abdominal colic (Al-Sereiti et al., 1999; Peng et al., 2007). In Mexico, *R. officinalis* aerial parts are prepared as maceration in ethanol and used in topical administration to relief rheumatic pain. A tea made of the boiled leaves is used to improve digestion and to relief stomach-ache (Martínez, 1989). Several phytochemical studies have shown that *R. officinalis* contains essential oils, phenols, diterpenoids, terpenoids and especially flavonoids (Okamura et al., 1994; Zeng et al., 2001). Flavonoids, part of a large

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family of naturally occurring polyphenolic compounds characterized by a common benzo-γ-pyrone structure, are important constituents of the human diet (Miean and Mohamed, 2001). They also have a wide range of biological and pharmacological activities as antioxidant, anti-ageing, antineoplastic, and anti-inflammatory; these compounds also lower of blood cholesterol levels, and improve bone strength (Yang et al., 2006). In a previous study, we observed that the ethanol extract from *R. officinalis* aerial parts produced antinociceptive and anti-inflammatory effects in different experimental models of nociception (González-Trujano et al., 2007). However, the active components and the mechanism of action of the antinociceptive response have not been described so far. The aim of this study was not only to evaluate the antinociceptive effect of hesperidin, as a responsible antinoceptive component of *R. officinalis*, but also to gain some knowledge into its mechanism of action and pharmacological interaction with a non-steroidal anti-inflammatory drug (NSAID) by using the "pain-induced functional impairment in the rat" (PIFIR) model, an assay of inflammatory and chronic nociception similar to that observed in clinical gout (López-Muñoz et al., 1993).

2. Materials and methods

2.1. Animals

Male Swiss albino mice (25–30 g) and Male Wistar rats [Crl(WI)fBR] weighing 180–200 g obtained from Cinvestav-Sur and Instituto Nacional de Psiquiatría "Ramón de la Fuente Muñiz" were used in this study. Animals were housed in a temperature- and light-controlled room under a 12-h light/12-h dark cycle (lights on at 7:00 a.m.) with water and food *ad libitum*. Twelve hours before the experiments, food was suspended, though animals had free access to tap drinking water. All experimental procedures followed the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals (Zimmermann, 1983), and were carried out according to a protocol approved by the local Animal Ethics Committee. The number of experimental animals was kept to a minimum; they were used only once. For each experimental procedure, animal groups consisted of six mice or rats.

2.2. Drugs

The *Rosmarinus officinalis* L. ethanol extract was prepared as previously described González-Trujano et al. (2007). Extract, flumazenil and bicuculline were suspended in vehicle [0.5% tween 80 in saline solution (s.s.)]. Capsaicin was firstly prepared in a stock solution containing 1 mg of capsaicin dissolved in 1 ml of vehicle (0.2% Tween 80 in s.s. and 10% ethanol). Then, 16 μl of this stock solution were diluted with 384 μl of s.s. to yield a concentration of 1.6 μg/40 μl. The extract was administered via oral (p.o.), whereas ketorolac–tromethamine (≥99% purity) and hesperidin (~80% purity) were dissolved in s.s. and administered via intraperitoneal (i.p.). Naloxone, WAY 100635, capsazepine and caffeine were dissolved in s.s and administered via subcutaneous (s.c.). Drugs were freshly prepared on the day of the experiments and administered in a volume of 0.1 ml/100 g body weight rats. Control animals received the same volume of either vehicle or s.s. by the respective route of administration. All pure compounds were purchased from Sigma Chemical Company. To induce nociception, a 20% uric acid suspension in mineral oil was used.

2.3. High pressure liquid chromatography (HPLC) analysis

Samples tested (10 mg) were dissolved in 10 ml of HCl (1 N) in MeOH and filtered to inject 20 μl to the HPLC system.

2.3.1. HPLC-PAD conditions

The HPLC system included a Waters 2795 pump (Waters, Milford MA, USA) equipped with an automatic sample injector module and photodiode detector model 996 (Waters, Milford MA, USA). The separation was performed on a Kromasil C-18 column (150 mm × 4.6 mm, MetaChem Technologies, Inc., Torrance CA, USA). The mobile phase consisted of components A (10 mM phosphoric acid aqueous solution) and B (acetonitrile). The system was run with a linear gradient program: at the beginning 90% A, 10% B; 18 min, 30% A, 70% B; 20 min, 30% A, 70% B; and 22 min, 90% A, 10% B. The flow rate was set at 0.6 ml/min. Chromatograms were detected at 280 nm.

2.4. Antinociceptive activity

2.4.1. Pain-induced functional impairment model in the rat (PIFIR model)

Antinociceptive activity was measured using the PIFIR model, previously described in detail elsewhere (López-Muñoz et al., 1993). The animals were anaesthetized with ethylic ether (J.T.Baker) in an anesthesia chamber (glass dryer Pyrex satured with ether vapors). Nociception was induced by an intra-articular injection of 0.05 ml of 20% uric acid suspended in mineral oil in the knee joint of the right hind limb. The suspension was prepared by grinding 2.0 g of uric acid in a glass mortar (Pyrex) and adding 10 ml of mineral oil. The intra-articular injection was performed through the patellar ligament using a 1 ml glass syringe (Becton, Dickinson LTDA, Brazil) with a 24 gauge needle of 5 mm. Subsequently; an electrode was attached to the plantar surface of each hind paw between the plantar pads. Rats were allowed to recover from anesthesia and placed on a stainless steel cylinder, 30 cm in diameter. The cylinder was rotated at 4 rpm, forcing the rats to walk. The variable to be measured was the time of contact between each hind paw and the cylinder. When the electrode placed on the animal's paw made contact with the cylinder floor, a circuit was closed; the time the circuit remained closed was recorded. The cylinder was rotated for 2-min periods, during which time recordings were made; 28 min rest periods were interspersed between recordings. In all subsequent experiments, analgesic agents were administered 2.5 h after uric acid injection. Thus, this time was considered as time zero for the measurements of the antinociceptive effect. Drugs were given at this time and the time of contact was measured every 30 min for 4 h. All experiments were performed between 7:00 a.m. and 14:00 p.m. After uric acid injection, animals developed a progressive dysfunction of the injured limb. The time of contact of the injured hind limb reached a zero value 2.5 h after uric acid injection. Data are expressed as the percentage of the functionality index (FI%), i.e., the time of contact of injected paw divided by the time of contact of the control left paw multiplied by 100. Recordings were taken every 15 min for the first hour, and every 30 min for the remaining 3 hours. Rats were allowed to rest between recording periods. Recovery of FI% was considered as expression of the antinociceptive effect. Time–response curves were plotted to detect the onset of the antinociceptive effect, as well as dose–response curves to determine the antinociceptive efficacies. For the purpose of this study, inducing nociception in the experimental animals was unavoidable. However, care was taken to avoid unnecessary suffering.

2.4.2. Capsaicin-induced nociception

The procedure used was similar to that described by Déciga-Campos et al. (2010), but by using intraplantar injection of capsaicin in mice. Before testing, the animals were individually placed in a transparent glass cylinder, 20 cm in diameter, which serve as an observation chamber. A mirror was placed behind the chamber to enable unhindered observation. After a 20-min adaptation period, mice were injected 40 μL of capsaicin (1.6 μg/paw) intraplantar in the right hind paw by using a 27-gauge needle. Immediately after capsaicin injection, each animal was returned to the chamber for nociceptive behavior observation. Nociceptive behavior was quantified as the amount of time the animal spent licking the injected paw within a 5-min period measured with a chronometer.

2.5. Experimental design

In the PIFIR model, dose–response curves were constructed in order to know the effective dose twenty-five (ED_{25}) of hesperidin; this dose was compared to that produced by *R. officinalis* extract and the reference drug ketorolac. Once the FI% reached the zero value, rats were administered with vehicle (0.5% tween 80 in s.s.), *R. officinalis* (30, 100, 300, 1000 and 3000 mg/kg), hesperidin (100, 300, 562.3, 1000 and 1778.3 mg/kg), or ketorolac (0.18, 0.32, 0.56, 1, and 1.78 mg/kg). Subsequently, the possible mechanism of action of hesperidin-induced antinociception was investigated by exploring the participation of endogenous opioids, 5-HT_{1A}, $GABA_A$/benzodiazepine, A_1/A_2 and/or TRPV1 (the transient receptor potential vanilloid 1) receptors in different animal groups (at least 6 rats each). In this part of the experiment, one of the following treatments was given when FI% = 0: naloxone (a competitive opioid antagonist, 1 and 10 mg/kg, s.c.); WAY100635 (a selective 5-HT_{1A} receptor antagonist, 0.12 mg/kg, s.c.); flumazenil (a benzodiazepine antagonist, 10 mg/kg, i.p.); bicuculline (a competitive antagonist of $GABA_A$ receptor, 1 mg/kg, s.c.); capsazepine (a TRPV1 competitive antagonist, 10 or 20 mg/kg s.c.), or caffeine (a non selective adenosine antagonist, 1 and 3.2 mg/kg, s.c.). Fifteen minutes later, all groups of animals received hesperidin (1000 mg/kg, i.p.). At the end of the experiment, animals were euthanized. No side effects were observed in any of the studied groups of animals. For synergistic analysis, all doses of hesperidin were combined with lesser doses of ketorolac (0.18, 0.32 and 0.56 mg/kg) giving 15 combinations in total.

2.6. Statistical analysis

Antinociception was estimated as the recovery of the FI% value in the PIFIR model. The cumulative antinociceptive effect during the whole observation period (4 h) was determined as the area under the curve (AUC) of the time course to obtain the dose–response curves and to analyze the whole antinociceptive effect elicited by the *R. officinalis* extract, hesperidin and/or ketorolac, either alone or in combination with an inhibitor or antagonist drug. The AUC was obtained by the trapezoidal rule. The percentage of the antinociceptive effect was calculated with respect to the maximum antinociceptive effect observed in the PIFIR model under the present experimental conditions; its value was 387.5 au (López-Muñoz et al., 1993; González-Trujano et al., 2007; Martínez et al., 2009). The doses producing 25% of the maximum possible effect in the PIFIR model (ED_{25}) of *R. officinalis* extract, hesperidin and ketorolac were calculated by performing a linear regression analysis in the linear portion of the dose–response curves. Statistical differences were determined by ANOVA followed by Dunnett's test. The synergism between hesperidin and ketorolac was calculated using surface of synergistic interaction (SSI) analysis (López-Muñoz, 1994; López-Muñoz and Salazar, 1995). The AUC of the individual effects of each drug were added up to obtain the expected value. If the experimental AUC obtained from the combinations was higher than the theoretical sum of the individual effects, the results were considered to show supra-additive response; if it was similar to the theoretical sum, it was considered to show an additive antinociceptive effect and if it was less than the theoretical sum, it was considered to show sub-additive effect. The AUC values for hesperidin–ketorolac combinations were compared with the expected value by using Student's test. Statistical differences for gastric injury were determined by ANOVA on ranks followed by Dunn's test. Significance was considered for $P<0.05$.

3. Results

3.1. Chemical composition

Thin layer chromatography analysis of an ethanol extract revealed the presence of terpenoids and flavonoids as principal constituents of the ethanol extract from *R. officinalis*; a bio-guided fractionation allowed the isolation of fractions revealing mainly flavonoid components (data not shown). Then, HPLC analysis indicated several peaks in the most active ethyl acetate fraction (7.5 g, 8.33%) with flavonoid content in which a compound 9.37 mg/g was identified as hesperidin (10.836 min) (Fig. 1).

3.2. Antinociceptive effect of hesperidin in the PIFIR model

At the time when the functionality index reached the zero value, administration of *R. officinalis* extract, hesperidin or ketorolac produced a dose-dependent antinociceptive effect curve (Fig. 2) at doses not affecting the walking ability of the rats during the period of evaluation. *R. officinalis* extract (3000 mg/kg) and ketorolac (1.78 mg/kg) appeared to be the most effective of the assayed compounds; hesperidin (1778.3 mg/kg) showed limited efficacy with a maximum of 25 ± 12%. The antinociceptive effect curves allowed the calculation of an $ED_{25} = 0.47$ mg/kg for ketorolac, i.p.; $ED_{25} = 302.9$ mg/kg for the *R. officinalis* extract p.o. and, finally, an $ED_{25} = 1666.72$ mg/kg for hesperidin i.p. (Fig. 2). All ED_{25} were calculated with respect to the maximum antinociceptive effect observed in the PIFIR model. A lower potency ($ED_{25} = 1666.72$) as well as limited antinociceptive efficacy were observed for hesperidin. The *R. officinalis* extract was considerably less potent than ketorolac though both exhibited similar efficacy. *R. officinalis* extract increased FI% in rats, showing a significant antinociceptive effect starting at 300 mg/kg dosage (Fig. 2) with a maximum antinociceptive peak reached within the first 15 min of evaluation (Fig. 3A). Moreover, *R. officinalis* at a 3000 mg/kg dosage allowed the observation that the maximum effect was reached within the first 15 min, this response remained until the end of the evaluation period (3A). With regard to hesperidin, the antinociceptive effect was significant at 1000 mg/kg (Fig. 2), with a maximum antinociceptive effect detected also within the first 15 min (Fig. 3B) of evaluation.

3.3. Effect of hesperidin on the capsaicin-induced nociception

Hesperidin produced a dose-dependent (10, 30 and 100 mg/kg, i.p.) diminution in the time mice spent licking the injected paw after receiving intraplantar capsaicin (Fig. 4). A significant antinociceptive response ($P<0.05$) was observed in mice treated with 30 mg/kg (19 ± 5 s) and 100 mg/kg (11 ± 6 s) dosages in comparison to the vehicle group (43 ± 10 s) (Fig. 4).

3.4. Analysis of the mechanisms of action of hesperidin

The significant antinociceptive effect of 3000 mg/kg of *R. officinalis* extract was reverted in presence of 10 mg/kg, but not for 1 or 3.16 mg/kg naloxone (Fig. 5A and B). Similarly, pretreatment of WAY100635 reduced the antinociceptive response of the *R. officinalis* extract (Fig. 5A and B). However, when both antagonists were tested in combination with hesperidin (1000 mg/kg), identified as one of the responsible constituents of the antinociceptive activity of *R. officinalis*, no changes were observed (Table 1). Because of this fact, several antagonist or inhibitor drugs were tested in combination with this bioflavonoid in order to investigate its possible antinociceptive mechanism of action. As observed in Table 1, the presence of bicuculline (1 mg/kg), flumazenil (1 mg/kg), or caffeine (1 or 3.2 mg/kg, s.c.) did not modify the antinociceptive effect of hesperidin. On the other hand, pretreatment with capsazepine (10 or 20 mg/kg) reduced 36% the antinociceptive response produced by hesperidin (Table 1). All control groups tested receiving vehicle (s.s. or 0.5% tween 80 in s.s.) or some antagonist or inhibitor drug showed a FI% ≈ 0 throughout the 4-h duration of the experiment (Table 1).

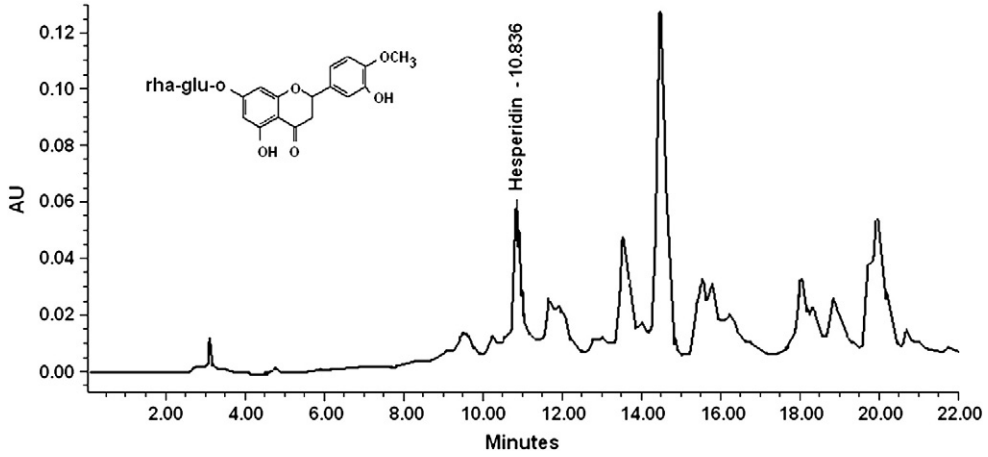

Fig. 1. Representative HPLC profile of the ethanol extract of *R. officinalis* aerial parts. The peak corresponds to hesperidin (TR = 10.836 min) corroborated with a standard.

3.5. Pharmacological synergistic interaction of hesperidin and ketorolac in the PIFIR model

Fig. 6A shows the antinociceptive effect of 3 different combinations of hesperidin plus ketorolac. A maximal antinociceptive effect was obtained with the hesperidin (562.3 mg/kg) plus ketorolac (0.56 mg/kg) combination giving AUC = 228.31 ± 30.82 au which corresponds to a high supra-additive interaction ($P<0.001$). The other 12 combinations showed additive antinociceptive effects (i.e. the sum of the effects produced by each agent alone). One representative combination of additive response is show in Fig. 6A. Only one combination (1000 mg/kg hesperidin with 0.18 mg/kg ketorolac) exhibited a sub-additive antinociceptive effect (i.e., less than the sum of the effects produced by the each drug alone) (Fig. 6A).

The combination ketorolac plus hesperidin producing potentiation did not cause any gastric injury as the one observed with indomethacin (as positive control) or ketorolac alone in single administration (Fig. 6B).

4. Discussion

In the present study, the antinociceptive activity of the bioflavonoid hesperidin was investigated, as well as its possible mechanism of action and pharmacological interaction with a specific NSAID. Our results give evidence that systemic administration of hesperidin produces antinociceptive effect in the PIFIR model at doses causing no motor dysfunction. We also found that the TRPV1 receptor is partially involved in its mechanism of action, whereas combinations with ketorolac produced synergism mainly as an additive antinociceptive response.

The PIFIR model is a well known test validated to search for new or alternative treatments for arthritic pain. This model uses diluted uric acid injected into the knee joint of the rat right hind limb (i.art.), to render a good representation of clinical gout arthritis (López-Muñoz et al., 1993). In a preliminary study, it was reported that the *R. officinalis* ethanol extract possesses antinociceptive and anti-

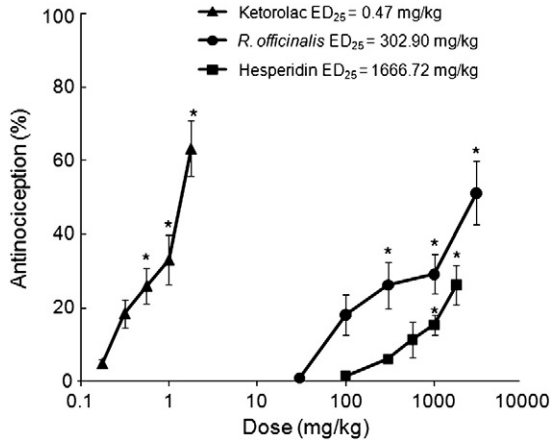

Fig. 2. Dose-response curves of the antinociceptive effect of *R. officinalis* extract (●), hesperidin (■) and ketorolac (▲). Data are expressed as the mean ± S.E.M. of the percentage of the antinociceptive effect with respect to the maximum antinociceptive response in the PIFIR model. *$P<0.001$, ANOVA followed by Dunnett's test.

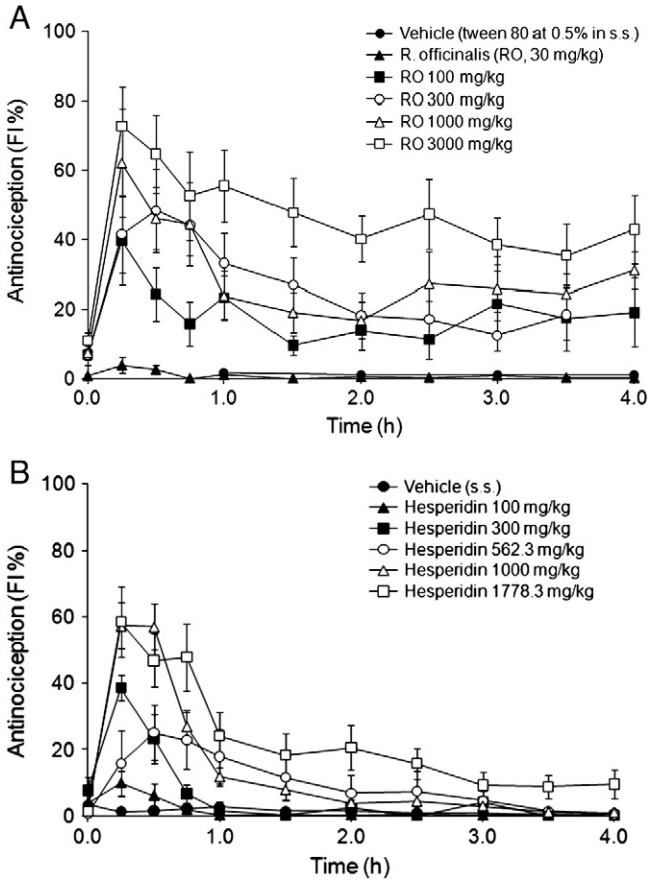

Fig. 3. Time course curves of the antinociceptive effect of (A) *R. officinalis* extract: 30, 100, 300, 1000 and 3000 mg/kg, p.o. and (B) hesperidin: 100, 300, 562.3, 1000 and 1778.3 mg/kg, i.p. in the PIFIR model during a 4 h-period. Each point represents the average of the functionality index in percentage (FI%) ± S.E.M. of at least six animals.

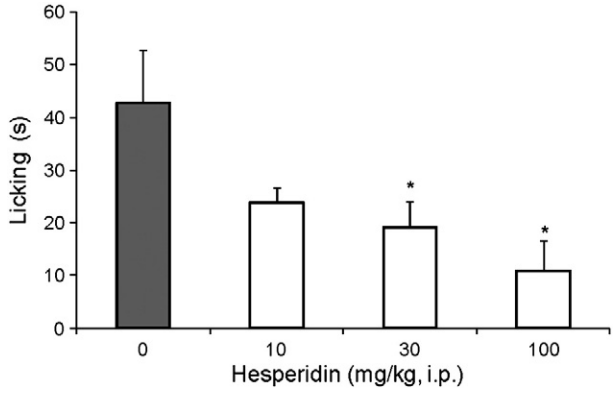

Fig. 4. Antinociceptive effect of hesperidin (10, 30 or 100 mg/kg, i.p.) on the time spent by each mice licking its injected paw with 40 µl of capsaicin (1.6 µg/paw). Each bar represents the average ± S.E.M. of at least 6 animals. *$P = 0.015$, ANOVA followed by Dunnett's test.

inflammatory activity in nociceptive models such as acetic acid-induced writhing and formalin test in mice, and the PIFIR model in rats (González-Trujano et al., 2007). In the PIFIR model, the *R. officinalis* ethanol extract significantly reduced in a dose-dependent fashion the loss of functionality caused by the administration of uric acid into the knee joint. Our results and those reported by Altinier et al. (2007) are in agreement with the use given to *R. officinalis* in traditional medicine and with recent clinical studies reporting that a combination of *R. officinalis* extract with reduced iso-alpha acids and oleanolic acid decrease pain in patients with osteoarthritis and rheumatoid arthritis (Lukaczer et al., 2005). After bio-guided fractionation of the active ethanol extract, it was possible to isolate and identify the flavanone glycoside called hesperidin as partially responsible for the antinociceptive activity of *R. officinalis*. In our present study, hesperidin showed a significant and dose-dependent antinociceptive response in the PIFIR model. Hesperidin was 5.5 times less potent (ED_{25}) than its original extract suggesting that others compounds must be involved in the effect of *R. officinalis*, though the analgesic property of hesperidin was confirmed.

Table 1
Analysis of the possible antinociceptive mechanism of action of hesperidin in the PIFIR assay.

Treatment	Dose (mg/kg)	AUC	Antinociception (%)
Vehicle	–	8.43 ± 2.36	2.18 ± 0.61
Naloxone	1	8.15 ± 4.26	2.10 ± 1.10
	10	4.41 ± 2.57	1.14 ± 0.66
WAY100635	0.12	15.16 ± 3.65	3.91 ± 0.94
Bicuculline	1	0.84 ± 0.57	0.22 ± 0.15
Flumazenil	10	5.69 ± 2.86	1.47 ± 0.74
Caffeine	1	2.55 ± 0.85	0.66 ± 0.22
	3.2	1.76 ± 0.45	0.45 ± 0.12
Capsazepine	10	0.16 ± 0.16	0.04 ± 0.04
	20	0.32 ± 0.16	0.08 ± 0.04
Hesperidin plus	1000	59.41 ± 11.32	15.33 ± 2.92[a]
Naloxone	1	43.98 ± 15.27	11.35 ± 3.94[a]
	10	106.56 ± 7.05	27.50 ± 1.82[a]
WAY100635	0.12	57.18 ± 4.83	14.76 ± 1.25[a]
Bicuculline	1	75.40 ± 11.28	19.46 ± 2.91[a]
Flumazenil	10	98.21 ± 11.99	25.34 ± 3.09[a]
Caffeine	1	87.26 ± 28.78	22.52 ± 7.43[a]
	3.2	61.50 ± 26.73	15.87 ± 6.90[a]
Capsazepine	10	30.90 ± 10.27	7.97 ± 2.65
	20	28.32 ± 13.73	7.31 ± 3.54

Data are represented as the mean of the percentage of antinociception ± S.E.M of six repetitions based in the maximum antinociceptive effect calculated as the area under the curve (AUC) obtained in the PIFIR model. [a]$P<0.001$ ANOVA followed by Dunnett's test.

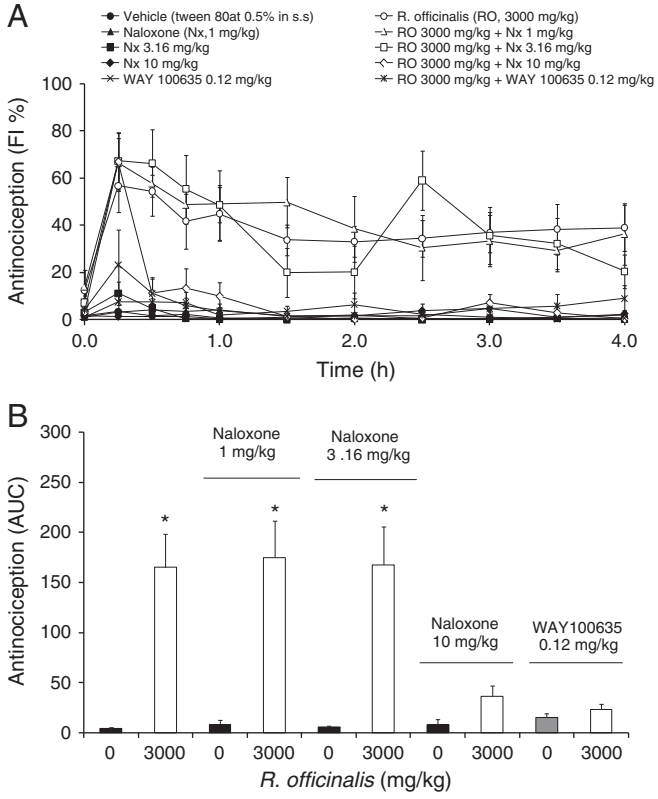

Fig. 5. (A) Time course curves of the antinociceptive effect of *R. officinalis* at 3000 (o) mg/kg alone and in the presence of naloxone (Nx) at 1.0 (Δ), 3.16 (□), and 10 (◊) mg/kg or WAY 100635 at 0.12 (Ж) mg/kg expressed as the percentage of the functionality index (FI%) in the PIFIR assay during a 4-h-period. These effects were compared with the effect observed by administering vehicle (●) and Nx 1.0 (▲), 3.16 (■), and 10 (♦) mg/kg or WAY 100635 at 0.12 (X) mg/kg. (B) Area under the curve (AUC) obtained from the FI% graph showing the administration of *R. officinalis* (3000 mg/kg) alone and in presence of Nx 1, 3.16, and 10 mg/kg or WAY 100635 0.12 mg/kg. Each point represents the average of the AUC ± S.E.M. of at least 6 animals. *$P<0.001$, ANOVA followed by Dunnett's test.

The precise mechanism of action in the antinociceptive response of either *R. officinalis* crude extract or hesperidin is not yet understood. It has been reported that inhibition of the acetic acid-induced abdominal contractions in mice elicited by *R. officinalis* was totally reverted in the presence of naloxone (Hosseinzadeh and Nourbakhsh, 2003). In our present study, both naloxone (an opioid antagonist) and WAY 100635 (a high potent and selective 5-HT_{1A} antagonist) (Foster et al., 1995) prevented the effect of *R. officinalis* in the PIFIR model. These results reinforce the hypothesis that not only the opioid system, but also the serotonergic system through 5-HT_{1A} receptors are involved in the antinociceptive mechanisms of action of this plant. Although for generations it has been suggested that opioid drugs act solely on the central nervous system, producing analgesia by interaction with cerebral and spinal opioid receptors, several studies in animals (Stein et al., 1989; Kolesnikov et al., 1996) and humans (Tegeder et al., 2003) have demonstrated the contribution of a peripheral opioid mechanism in antinociception, particularly prominent under painful inflammatory conditions (Stein et al., 1989; Kolesnikov et al., 1996). As a result there is the possibility that the antinociceptive effect of *R. officinalis* could be mediated centrally and/or peripherally by the opioid system. In addition, it has been reported the involvement of the 5-HT_{1A} receptor as a novel analgesic and high-efficacy receptor (Bardin et al., 2003; Colpaert et al., 2002) participating in a mechanism of central analgesia. It is known that activation of post-synaptic 5-HT_{1A} receptors in the periaqueductal grey substance produces a facilitatory influence of opioids upon K^+-currents in isolated neurons (Jeong et al., 2001). In agreement with Kishimoto et al. (2001), the possibility that this action is related to the engagement of descending inhibition is favored by the finding that the

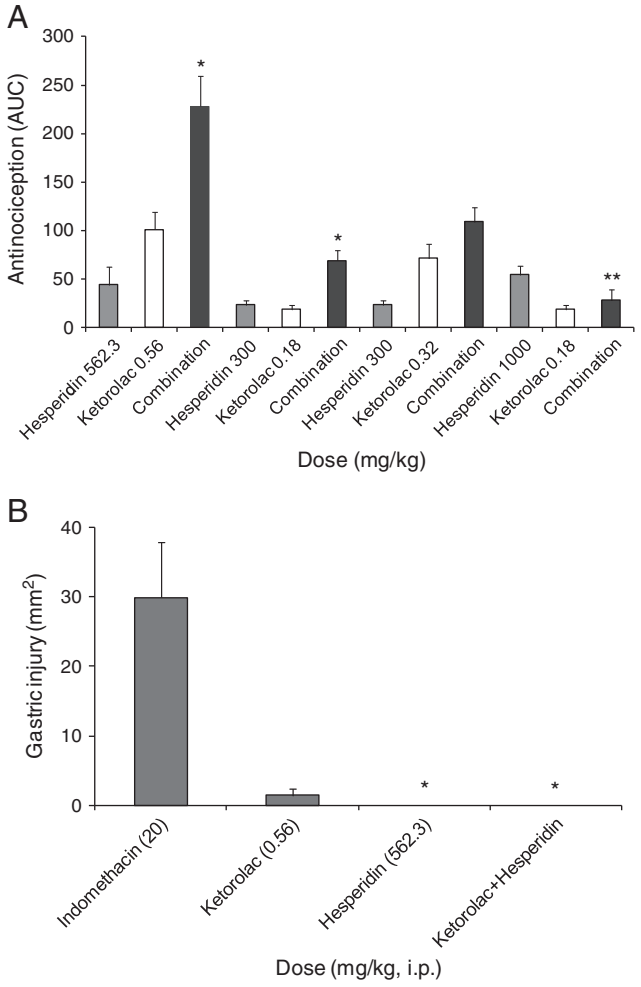

Fig. 6. (A) The antinociceptive effect produced by different combinations of ketorolac and hesperidin that showing synergism of potentiation (high supra-additive interaction) or additive response, as well as antagonism in the last case. Each interaction is represented by the mean of six animals for each combination. *P<0.05; **P = 0.006. (B) Gastric injury produced by indomethacin at 20 mg/kg, i.p. (as positive control) compared to the gastric injury of hesperidin (562.3 mg/kg, i.p.) or ketorolac (0.56 mg/kg, i.p.) alone and in the combination corresponding to a high supra-additive interaction. *P<0.05 ANOVA on ranks followed by Dunn's test.

synergistic action of μ-opioids and 5-HT_{1A} agonists may produce analgesic effectiveness.

The participation of opioid receptors in the antinociceptive effect of hesperidin (0.7 and 1 mg/kg, i.p.) was early reported by Loscalzo et al. (2008) when naltrexone (5 mg/kg, i.p.) was shown to inhibit abdominal constrictions in mice. However, in our present study no changes were observed in presence of 1 or 10 mg/kg of naloxone in the antinociceptive response using the PIFIR model. Other receptor antagonists like WAY100635, bicuculline, flumazenil or caffeine did not modify the antinociceptive activity of hesperidin. Nevertheless, capsazepine, a TRPV1 selective antagonist, partially prevented the hesperidin response in the PIFIR model. The vanilloid receptor TRPV1 is a homotetrameric, non-selective cation channel which is highly selective for calcium. It is found in the central and peripheral nervous systems abundantly expressed in nociceptors (c-fibers). This receptor is involved in the transmission and modulation of pain, as well as in the integration of various painful stimuli (Cui et al., 2006). The precise mechanisms of receptor activation by various agents have not been fully established although such stimuli appear to alter protein conformation and stability through specific amino acid residues on the receptor, which results in ion influx and disruptions of structural gating (Veronesi and Oortgiesen, 2006). Hence, both TRPV1 agonist and antagonist drugs are being evaluated as potential antinociceptive and analgesic drugs as observed in preclinical and clinical trials (Wong and Gawa, 2009). It has been described that capsaicin, the main capsaicinoid in chili peppers, produces pain by selectively activating polymodal nociceptive neurons (Caterina et al., 2000). The capsaicin-induced activation is mediated by TRPV1 receptors which can be selectively and competitively antagonized by capsazepine (Dray, 1992). In agreement with Loscalzo et al. (2008), it is possible that hesperidin participates in various brain receptors implicated in the control of numerous behavioral and physiological functions, such as sedation (Guzmán-Gutiérrez and Navarrete, 2009) and antinociception (present study). Since hesperidin was capable to reduce capsaicin-induced nociception and partial prevention of the antinociceptive effect of hesperidin was observed with capsazepine in the PIFIR model, our results provide the first evidence that the antinociceptive response of the bioflavonoid hesperidin involves the participation of TRPV1 receptors, which act as modulators of this response in a model of arthritis gout-type nociception in rats. Nevertheless, further investigation is necessary to explain the specific involvement of TRPV1 and other possible mediators in the antinociceptive mechanisms of action of hesperidin.

In this study, interaction of ketorolac, a NSAID possessing significant analgesic efficacy in the treatment of moderate to severe pain (Forrest et al., 2002), and hesperidin produced a positive synergism in the antinociceptive response in the PIFIR model. Potentiation was even obtained when 0.56 mg/kg ketorolac + 562.3 mg/kg hesperidin were combined. It has been reported that 1 mg/kg of ketorolac produces 26% of gastric injury as adverse effect in rats (López-Muñoz et al., 2004). In this study, it was also observed a major gastric lesion of 2 mm^2 in one of six rats when 0.56 mg/kg of ketorolac alone were administered. However, in the combination causing potentiation no gastric injury was observed. It has been suggested that the combination of analgesic drugs may have synergistic effects, and a specific combination may produce better antinociceptive response with reduced adverse effects (López-Muñoz et al., 2004). The positive synergism in the antinociceptive response observed with the combination of this bioflavonoid and a NSAID without adverse effects of this clinical drug suggests their potential usefulness in the therapeutic of pain.

In conclusion, the results in the present study demonstrate the antinociceptive properties of hesperidin and in turn the additive and supra-additive response when combined with the NSAID ketorolac. The pharmacological response of hesperidin was partially modulated by the participation of the TRPV1 receptor, but not by endogenous opioids, 5-HT_{1A}, $GABA_A$/benzodiazepine or adenosine receptors, at least with regard to the kind of nociception explored in the PIFIR model.

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The authors declare no conflicts of interest.

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