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Antinociceptive Properties of Extracts and Two Flavonoids Isolated from Leaves of *Danae racemosa*

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The antinociceptive properties of the hydro-methanolic extract (HME) and two flavonoids isolated from *Danae racemosa* have been investigated in several nociceptive rat models. The HME from *D. racemosa* (100-400 $mgkg^{-1}$, i.p.) produced significant dose-related inhibition of acetic acid-induced abdominal constriction. In the same dose range, the HME produced dose-related inhibition in both phases of a formalin-test. Treatment of animals with naloxone (5 $mgkg^{-1}$, i.p.) completely reversed the antinociceptive effect caused by morphine (5 $mgkg^{-1}$, s.c.) and the HME (200 $mgkg^{-1}$, i.p.) when assessed against the first phase of the formalin-test, but this effect was less significant for the HME in the second phase. Furthermore, when assessed via a hot-plate test, the HME (100-400 $mgkg^{-1}$, i.p.) caused a significant increase in response latency. The HME, given daily for to 7 consecutive days, develop tolerance, but did not induce cross-tolerance to morphine. These data demonstrate that the HME elicites pronounced antinociception against several pain models. The actions of the HME involve, at least in part, an interaction with the opioid system, but does not seem to be related with non-specific peripheral or central depressant actions. Finally, the active principle(s) responsible for the antinociceptive action of *D. racemosa* is likely to be partially related to the presence of quercetin and kaempferol.

Key words: *Danae racemosa*, Analgesic, Opioid system, Formalin test, Quercetin, Kaempferol

INTRODUCTION

Danae racemosa (Asparaginaceae) is an erect, much-branched evergreen shrub, with thick unarmed alternate cladophylla and terminal racemese of small whitish flowers, often referred to as *Ruscus*, which is found in Syria, Persia and Transcaucasia (Parsa, 1950; Bailey, 1961; Ghahraman, 1994). *Danae racemosa* is used as an ornamental plant. To our knowledge, the pharmacological properties of *Danae racemosa* remain to be elucidated, but phytochemical studies have demonstrated the presence of rutin in the dried roots of *Danae racemosa* (Nasudari *et al.*, 1972). During our preliminary studies to evaluate the anti-inflammatory properties of the plant (Maleki *et al.*, 2003), it was noticed it would probably possess antinociceptive properties.

In the aim of this study was to investigate the antinociceptive properties of the methanolic extract from leaves of *Danae racemosa* using both chemical and thermal nociceptive rat models. In addition, the different fractions of the methanolic extract were analyzed to isolate the active ingredient responsible for the analgesic activity.

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MATERIALS AND METHODS

Plant material

Leaves of *D. racemosa* were collected in September from a colony growing on the outskirts of Shirgah, in Ghaemshahr city, Mazandaran province, Iran. The plant was classified by Dr. Fathiazad (Department of Pharmacognosy), and a voucher deposited in the Department of Pharmacognosy, Tabriz University of Medical Sciences.

Extraction and isolation

Air-dried and powdered leaves of *D. racemosa* (100 g) were defatted with petroleum ether, with the remaining powder then extracted four times for 24 h, with 1000 mL of 70% methanol-water, while being macerated at room temperature. The hydro-methanolic extract (HME) was

evaporated under vacuum using a rotary evaporator at 50°C. The remaining aqueous extract was successively partitioned with chloroform and ethyl acetate. The ethyl acetate fraction (EAF) was subjected to preparative HPLC, yielding two major flavonoids; quercetin and kaempferol (unpublished data).

Animals

Male wistar rats (180-220 g), with free access to water, but fasted over night (18 h), were used throughout the experiments. Rats were acclimatized to the laboratory for at least 1 h prior to testing, and then used only once throughout the study. The reported experiments were carried out in accordance with the research affairs of Tabriz University of Medical Sciences guidelines for the care of laboratory animals.

Formalin-induced licking

Formalin-induced hindpaw licking was induced in male wistar rats (180-220 g) by an intraplantar injection of 50 µL 5% formalin (Merck, Germany). The time spent licking the injected paw was measured with a chronometer, and considered indicative of pain (Hunskaar and Hole, 1987; Dubuisson and Dennis, 1977). Animals were treated intraperitoneally with the HME (100-400 $mgkg^{-1}$), EAF from *D. racemosa* (200-400 $mgkg^{-1}$), quercetin (50-200 $mgkg^{-1}$), kaempferol (60-240 $mgkg^{-1}$) or the vehicle 80 min prior to the formalin injection. After the intraplantar injection of formalin, the animals were immediately placed into a 20 cm diameter glass cylinder, and the time spent licking the injected paw determined.

To investigate the involvement of the opioid system in the antinociceptive effect of the HME from *D. racemosa*, animals were pre-treated with naloxone (5 $mgkg^{-1}$, i.p.) 15 min prior to the administration of HME (200 $mgkg^{-1}$, i.p.), morphine (5 $mgkg^{-1}$, s.c.) or the vehicle (Ferreira *et al.*, 2000). The nociceptive responses caused by the formalin injection were recorded 80 min after drug administration (Maleki *et al.*, 2003). The other groups of animals received only the HME, morphine, naloxone or the vehicle 80 min prior to the formalin injection.

Acetic acid-induced abdominal constriction

The abdominal constriction induced by an i.p. injection of acetic acid (1%) was performed according to the procedures described previously (Ferreira *et al.*, 2000). Animals were pre-treated with different doses of the HME from *D. racemosa* (100-400 $mgkg^{-1}$, i.p.) or the vehicle 80 min prior to the acetic acid injection. After challenge, the rats were placed in separate boxes, and the number of abdominal constrictions cumulatively counted over a period of 30 min following the acetic acid injection.

To study the involvement of the opioid system in the antinociceptive effect of the HME from *D. racemosa* in the writhing test, animals were pre-treated with naloxone (5 $mgkg^{-1}$, i.p.) 15 min prior to the administration of HME (200 $mgkg^{-1}$, i.p.), morphine (5 $mgkg^{-1}$, s.c.) or the vehicle. The constrictive responses caused by the acetic acid injection were recorded 80 min following the drug administration. The other groups of animals received only the HME, morphine, naloxone or the vehicle 80 min prior to the acetic acid injection.

Hot-plate test

The hot-plate test was used to measure the response latencies according to the previously described method (Filho *et al.*, 1997). In these experiments, the hot-plate (Sabz Negar, MGH 787 Iran) was maintained at 56 ± 1°C. Animals were placed into a 24-cm diameter glass cylinder on the heated surface, with the time(s) between placement and shaking or licking of the paws or jumping recorded as the response latency. All animals were selected 24 h prior to the test on the basis of their reactivity in the model by eliminating those rats that remained on the apparatus (maintained at 56 ± 1°C) for up to 8 sec. The reaction time was recorded for the control rats and for the animals pretreated with the HME from *D. racemosa* or the vehicle 80 min prior to the test. A latency period of 30 sec was defined as complete analgesia.

Rota-rod test

To evaluate the possible non-specific muscle relaxant or sedative effects of the HME from *D. racemosa*, the rats were tested on a rota-rod, as described previously (Hamm *et al.*, 1994). The animals were selected 24 hour prior to the test by eliminating those rats not remaining on the bar for two consecutive periods of 120 sec. Animals were treated with the HME from *D. racemosa* (200 $mgkg^{-1}$, i.p.) or with a vehicle injection 80 min prior to the test. Results are expressed as the times the animals remained on the rota-rod, with a cut-off time of 120 sec.

Statistical analysis

All results are expressed as the mean±sem. The statistical significance of differences between groups was obtained by means of a one-way analysis of variance (ANOVA), followed by a Student Newman-Keuls post-test. P-values less than 0.05 were considered significant.

RESULTS

Antinociceptive effects of the hydro-methanolic extract from *D. racemosa*

Formalin-induced pain

An intraplantar injection of 5% formalin evoked a characteristic biphasic licking response in the rats. The dura-

tion of licking (mean±s.e.m.) for the first and second phases (0-5 and 15-30 min) were 72.44±5.6 and 179.67±30.4 sec, respectively. As shown in Fig. 1, pre-treatment (80 min) with different doses (100-400 mgkg^{-1}, i.p.) of the HME from the leaves of *D. racemosa* caused significant dose-related inhibition against both phases of formalin-induced pain in rats. The results showed that the HME exhibited potent and graded antinociception when administered via an i.p. route in the early phase of the formalin test, used as a model of neurogenic pain (Fig. 1).

The results shown in Fig. 2 indicated that the antinociceptive effect of morphine (5 mgkg^{-1}, s.c) was fully reversed by prior treatment of the animals with naloxone (5 mgkg^{-1}, i.p.) against both phases of formalin-induced pain ($P<$ 0.001); whereas, pre-treatment of the animals with naloxone (5 mgkg^{-1}, i.p.) completely reversed the antinociceptive effect of the HME from *D. racemosa* (200 mgkg^{-1}, i.p.) only during early phase (Fig. 2) ($P<0.001$); during the second phase (15-30 min) the reverse was significant, but less marked ($P<0.05$).

The results presented in Fig. 3 show that pretreatment of the animals with morphine (5 mgkg^{-1}, s.c. once a day for 7 consecutive days) produced significant and complete tolerance to the antinociceptive effect caused by either morphine or the HME from *D. racemosa* (100 mgkg^{-1}, i.p.), compared with the animals pretreated with saline (10 mlkg^{-1}, i.p. once a day for 7 consecutive days). However, treatment of the animals with the HME (100 mgkg^{-1}, i.p. once a day for 7 consecutive days) significantly changed the antinociceptive action, but did not change the antinociception caused by morphine when assessed against both phases of the formalin test (Fig. 3).

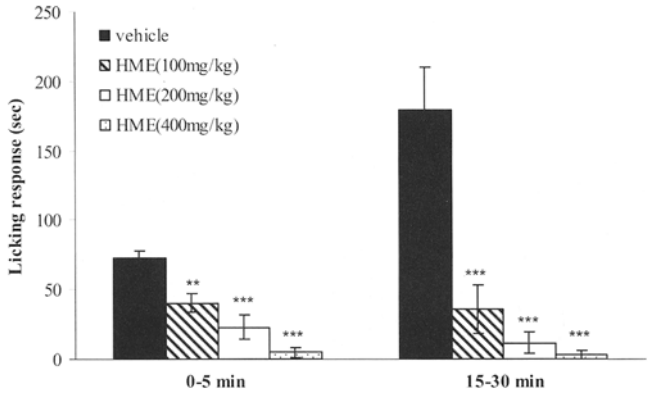

Fig. 1. Effects of different doses of the hydro-methanolic extract (HME, 100-400 mgkg^{-1}) from *D. racemosa,* administered intraperitoneally, against the first and second phases (0-5 and 15-30 min) of formalin-induced nociception in rat, respectively. Each point represents the mean±sem of six animals. The asterisks denote the significance levels compared with the vehicle groups (ANOVA), $^{**}P<0.01$, $^{***}P<0.001$ ($F=18.920$; df=47).

Abdominal constriction response caused by acetic acid

The results presented in Fig. 4A show that the HME from *D. racemosa* (100-400 mgkg^{-1}, i.p.), administered 80 min prior to the test, caused a significant dose-related inhibition of the acetic acid-induced abdominal constriction response. Treatment of the animals with morphine (5 mgkg^{-1}, s.c., 80 min beforehand) also produced marked inhibition of the acetic acid-induced writhing response (Fig. 4B). Pretreatment of the animals with naloxone (5 mgkg^{-1}, i.p.) fully reversed the antinociceptive effects of morphine, but did not change significantly the antinociception caused by the HME from *D. racemosa* in the writhing test (Fig. 4B).

Hot-plate assay

Treatment of animals with the HME from *D. racemosa* (100, 200 and 400 mgkg^{-1}, i.p., 80 min prior) or morphine (5 mgkg^{-1}, s.c., 30 min prior) caused a significant ($P<0.05$)

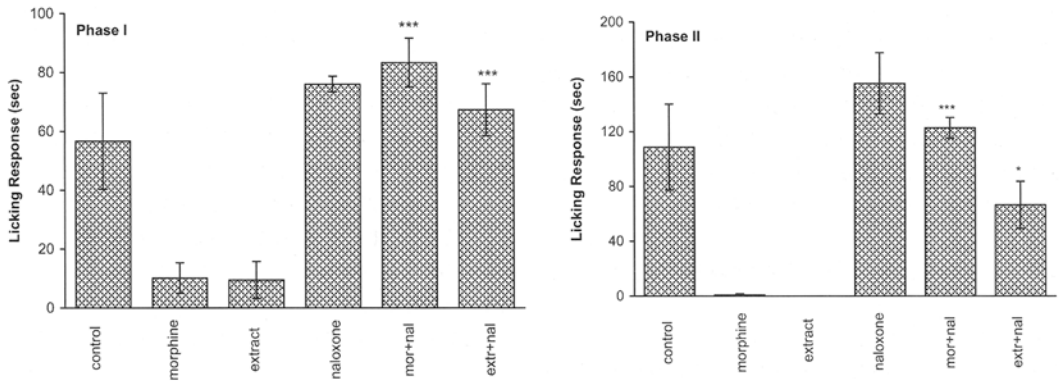

Fig. 2 Effects of pretreatment of the animals with naloxone on the antinociceptive action caused by morphine and the HME from *D. racemosa* (200 mgkg^{-1}, i.p.) against the first and second phases (0-5 and 15-30 min) of formalin-induced nociception in rat, respectively. Each column represents the mean±s.e.m. of six to eight animals. The asterisks denote the significance levels, $^{*}P<0.05$, $^{***}P<0.001$ compared with the agonist plus antagonist versus the agonist plus vehicle ($F_{phase\ I}=13.171$, $F_{phase\ II}=13.758$; df=35).

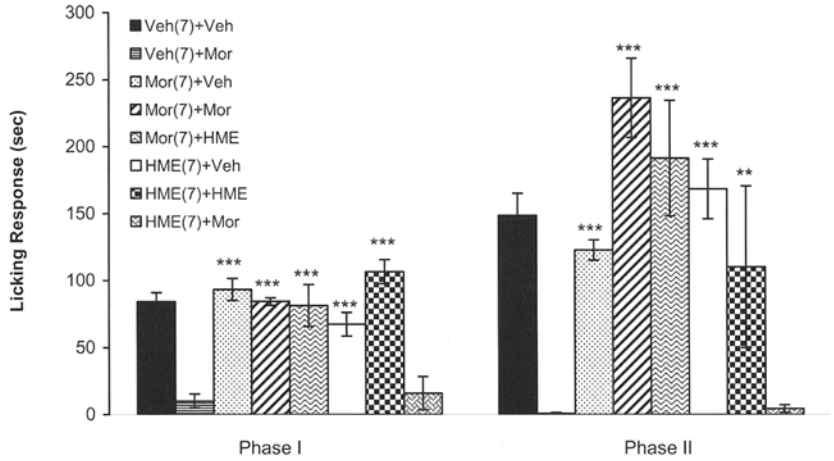

Fig. 3 Effects of cross-tolerance of the animals pretreated with morphine (Mor) (5 mgkg^{-1}, s.c.), hydro-methanolic extract (HME) (100 mgkg^{-1}, i.p.) or saline (Veh) (10 mlkg^{-1}, i.p.) on the antinociceptive action caused by morphine (5 mgkg^{-1}, s.c.) and HME from *D. racemosa* (100 mgkg^{-1}, i.p.) against the phase I & II (0-5 and 15-30 min, respectively, of formalin-induced nociception in rats. Each column represents the mean±sem of six rats. $^{**}P<0.01$, $^{***}P<0.001$ compared with the vehicle (7 days) + morphine ($F_{phase\ I}$=12.172, $F_{phase\ II}$= 5.396; df=41).

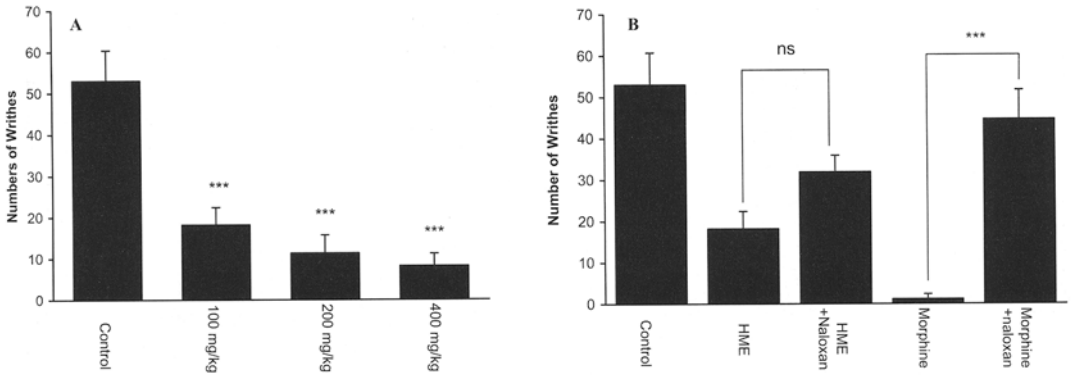

Fig. 4 (A) Effects of the hydro-methanolic extract (HME) (100-200 mgkg^{-1}, i.p.) against acetic acid-induced writhing in rats, $^{***}P<0.001$ compared to the control group (F=17.013; df=23); (B) HME (100 mgkg^{-1}, i.p.), morphine (5 mg.kg^{-1}, s.c.) and naloxone (5 mg.kg^{-1}, i.p.) against acetic acid-induced writhing in rats, ns: not significant, $^{***}P<0.001$ compared to the agonist plus antagonist versus the agonist plus vehicle (F=14.847; df=29). Each bar represents the mean±sem of six to eight animals.

increase in the latency response in the hot-plate test (from 15.20±1.7 sec in control group to 22.33±3.5, 26.00±1.6 and 27.50±1.4 sec in the HME-treated groups, respectively, and to 27.50±1.4 in the morphine-treated group). However, the same dose of the HME given via an i.p. route did not significantly affect the motor performance of the animals (control response in the rota-rod test was 120 sec versus 120 sec in the presence of the HME, N=6).

Antinociceptive effects of EAF, quercetin and kaempferol

Formalin-induced pain

As shown in Fig. 5A, when the ethyl acetate fraction (EAF) of the leaves of *D. racemosa* was administered (200-400 mgkg^{-1}, i.p., 80 min prior) to rats, it produced dose-related inhibition of the formalin-induced licking response, which was significant at doses of 300 ($P<0.05$) and 400 mgkg^{-1} ($P<0.001$) during the first phase. In the second phase, the antinociceptive effect of the EAF was also dose-dependent and highly significant ($P<0.001$) at all doses (Fig. 5A).

The two major flavonoids, quercetin and kaempferol, were isolated from the EAF at 25 and 30.2%, respectively. Quercetin, which was administered (50-200 mgkg^{-1}, i.p) 80 min prior to the test, affected the nociception associated with both phases of formalin-induced pain (Fig. 5B), and was significant at all doses. The antinociceptive effect of kaempferol (60-240 mgkg^{-1}, i.p) was only significant ($P<0.05$) at the higher dose during both phases (Fig. 5C).

DISCUSSION

Preliminary studies by our group have recently demonstrated that the hydro-methanolic extract (HME) obtained

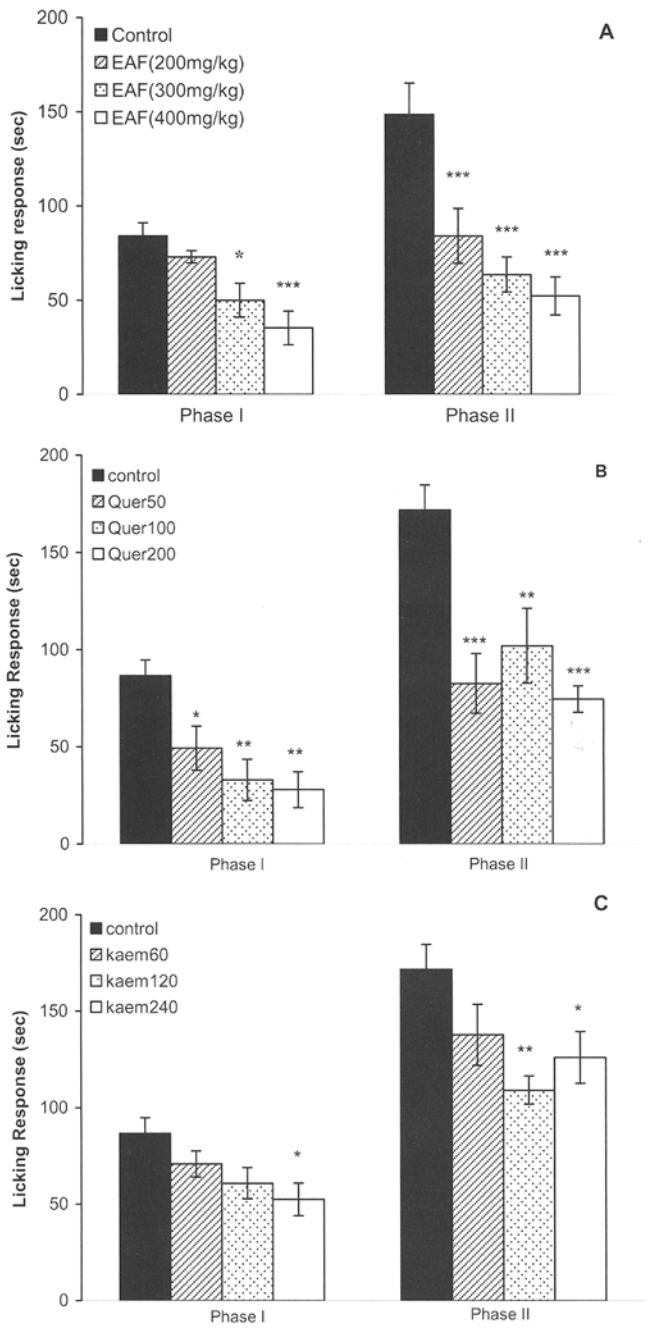

Fig. 5 (A) Effects of the ethyl acetate extract (EAE) (200-400 mgkg^{-1}, i.p.), (B) Quercetin (50-200 mgkg^{-1}, i.p.), (C) Kaempferol (60-240 mg.kg^{-1}, i.p.) against phases I & II (0-5 and 15-30 min, respectively) of formalin-induced nociception in rat. Each bar represents the mean±sem of six to eight animals. (ANOVA) *P<0.05, **P<0.01 compared to the control group (F_A=8.876, F_B=11.032, F_C=7.257; df=23).

from *D. racemosa* has a significant antinociceptive effect against the formalin-induced licking response in rat (Maleki *et al.*, 2003). In the present study, these initial observations were confirmed and extended by demonstrating that the HME of this plant, administered intraperitoneally, produced dose-related and marked antinociception in several models of chemical nociception, namely the formalin-induced licking response and acetic acid-induced abdominal constriction. Furthermore, HME was found to produce significant antinociception in the hot-plate assay.

In the hot-plate test, the intraperitoneal treatment of the HME showed an antinociceptive effect on the acute noxious thermal stimulation. The paw-licking hot-plate response is complex supraspinally organized behavior (Koster *et al.*, 1959). The effectiveness of the HME in the hot-plate test was proposed to indicate that the analgesic agent(s) acts primarily at the spinal medulla and/or higher central nervous system levels or by an indirect mechanism (Yaksh and Rudy, 1977; Hunskaar *et al.*, 1985; Tjølsen *et al.*, 1992). This result; therefore, might suggest that the HME includes component(s) of action with a central mechanism.

The results of the present study also demonstrated that HME-treated rats showed decreased nociceptive behaviors induced by intraplantar formalin during both the 1st and the 2nd phases in a dose-dependent manner. The acute 1st phase of the nociceptive response in the rat formalin test lasts for about 5 min after the formalin injection, which is followed by the tonic 2nd phase, which is persistent from 20 to 40 min after the formalin injection (Yaksh and Rudy, 1977; Hunskaar *et al.*, 1985; Tjølsen *et al.*, 1992; Chung *et al.*, 2001). It is widely accepted that the 1st and 2nd phases result from the direct effects on nociceptors activating the primary afferent fiber and the tonic inflammatory nociceptive responses, respectively (Tjølsen *et al.*, 1992; Ahmadiani *et al.*, 2000; Maleki *et al.*, 2001). Notably, the effect of the HME was more prominent during the 2nd tonic inflammatory phase as well as the 1st phase. It is well known that nonsteroidal anti-inflammatory drugs, such as aspirin, indomethacin and diclofenac, are largely ineffective or caused very weak inhibition against the early phase of the formalin test (Malmberg and Yaksh, 1992; Vaz *et al.*, 1996); but can attenuate, in a dose-related manner, the second phase of the formalin-induced licking response ((Malmberg and Yaksh, 1992). It has also been reported that morphine and some tachykinin receptor antagonists, non-selective excitatory amino acid antagonists, and both B_1 and B_2 bradykinin receptor antagonists are able to inhibit both phases (McCarson and Krause, 1995). Therefore, our results relating to the antinociceptive action of HME suggest the HME may be more effective on tonic inflammatory pain and probably acts on the central nervous system when systemically administered.

The abdominal constriction elicited by acetic acid has also been used to assess the potential analgesic activity of drugs. It is postulated that acetic acid acts indirectly by releasing endogenous mediators, which stimulate the nociceptive neurons, is sensitive to nonsteroidal anti-inflammatory drugs, narcotics and other centrally acting drugs (Gorski *et al.*, 1993; Gallantine and Meert, 2004). This

writhing response has been considered a visceral inflammatory pain model (Vyklicky, 1979). The HME treatment diminished the number of writhing responses in a dose-dependent manner in acetic acid-induced visceral nociception. Therefore, it can be concluded that HME may be effective on tonic inflammatory pain.

The mechanism(s) by which the HME from *D. racemosa* produces antinociception in the models of nociception are still not completely understood. Our results show; however, that the HME antinociception seems likely to be modulated by the opioid system, because the non-selective opioid antagonist naloxone, at a dose that completely reversed morphine-induced antinociception (McGaraughty *et al.*, 2005; Heidari *et al.*, 2006), consistently reversed the antinociception as a result of the HME from *D. racemosa* in assessments conducted in both phases of the formalin test. The results of the present study also provide experimental support that the treatment of animals with the HME from *D. racemosa*, given once a day for 7 consecutive days, resulted in tolerance, but did not induce cross-tolerance to morphine; whereas, the treatment of animals with morphine, given once a day for 7 consecutive days, resulted in self tolerance and to the HME from *D. racemosa*. In addition, HME had a marked antinociceptive effect when tested against radiant heat in the hot-plate assay, widely reported as being quite sensitive to morphine and opioid agonists. Also, the antinociception caused by the HME is unlikely to be secondary to its depressant and/or non-specific central effects, as revealed by the lack of any detectable effect when tested in the rota-rod test.

The animals treated with the HME showed no symptoms of diarrhea, piloerection, convulsion and sleepiness. The results of the present study also indicate that the HME from *D. racemosa* leaves, administered intraperitoneally up to 7 consecutive days, did not induced irritation on the gastric mucosa in rats. However, these findings are preliminary; therefore, further studies will be required to clarify this point.

The chemical studies carried out on this HME allowed the two flavonoids in the EAF from *D. racemosa* to be isolated and identified, which seem to be responsible for the antinociceptive properties reported for the EAF. From the IR, UV, NMR and mass spectra data, it was possible to identify and elucidate compounds, such as quercetin and kaempferol. At ID_{50} level, quercetin was equal to the EAF, but not kaempferol, when assessed against formalin-induced neurogenic pain.

CONCLUSION

In summary, this study has confirmed those conducted previously, where the HME obtained from *D. racemosa* elicited systemic dose-related antinociception in chemical models of nociception in rats. The precise mechanism of action is presently unknown, but seems to be unrelated with non-specific effects, such as muscle relaxation or sedation actions; however, at least in part, appears to involve participation of the opioid system. Finally, the isolated flavonoids; identified as quercetin and kaempferol, contribute to the explanation of the antinociceptive properties reported for the EAF. Further pharmacological and chemical studies are being conducted to characterize the precise mechanism(s) responsible for the antinociceptive action, and also to identify other active compounds present in *D. racemosa*.

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