Antinociceptive Properties of Morusin, a Prenylflavonoid Isolated from *Morus nigra* Root Bark

Márcia Maria de Souza[a], Magali Bittar[a], Valdir Cechinel-Filho[a,*], Rosendo Augusto Yunes[b], Irene Messana[c], Franco Delle Monache[c] and Franco Ferrari[c]

[a] Núcleo de Investigações Químico-Farmacêuticas (NIQFAR)/CCS, Universidade do Vale do Itajaí (UNIVALI), Rua Uruguai, 458, 88302–202, Itajaí-SC, Brazil. Fax: +47 341 7601. E-mail: cechinel@mbox1.univali.rct-sc.br

[b] Departamento de Química, Universidade Federal de Santa Catarina (UFSC), Florianópolis-SC, Brazil

[c] Istituto di Chimica e Chimica Clinica, Universitá Cattolica e Centro Chimica dei Recettori e delle Molecole Biologicamente Attive, C. N. R., Rome, Italy

* Author for correspondence and reprint requests

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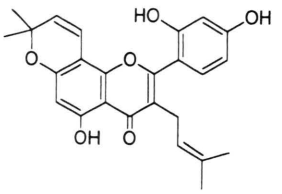
The antinociceptive effects of morusin (**1**), the main prenylflavonoid present in the *Morus nigra* root barks have been investigated in classical models of pain in mice. The results showed that **1** exhibits a promising antinociceptive or analgesic profile by the intraperitoneal route, being more potent than some standard drugs used as reference. The mechanism by which the morusin exerts antinociceptive activity still remains undetermined, but our results strongly suggest that it involves the participation of the opioid system.

Introduction

Morus nigra L. (Moraceae), known as "amoreira-preta" or "sarça-mora" in Brazil, is widely employed in folk medicine of many parts of the world as antiinflammatory, antiinfective, antidiabetic and for the treatment of other pathologies associated with dolorous processes (Graves, 1996; Michalak, 1997).

Acetonic extract of root bark and callus obtained from this plant revealed the presence of various flavonoids and Diels-Alder type adducts (Ferrari *et al.*, 1999). As part of our project to achieve naturally occurring analgesic substances, we selected several plants and evaluated their possible antinociceptive effects in mice. Among others, *M. nigra* caused significative inhibition of abdominal constrictions induced by acetic acid (unpublished results). For this reason, we analysed whether morusin (**1**), the main prenylflavonoid of this plant, exhibits antinociceptive effects in mice. In addition, we compared its potency with some reference drugs, such as aspirin and paracetamol (**2**).



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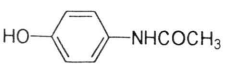

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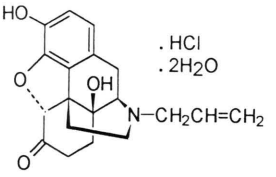

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Material and Methods

Plant material

Roots of *M. nigra* were collected in the Botanical Garden of the University "La Sapienza" of Rome during summer 1994. A voucher specimen

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was deposited at Dipartimento di Biologia Vegetale, Universitá "La Sapienza", Rome, Italy [RO-general Herbarium].

Isolation of morusin

Root barks (400 g) were extracted exhaustively with acetone (20.5 g). Part of the residue (10 g) was chromatographed on silica gel using a $CHCl_3$:MeOH gradient as eluent. The crude compounds obtained were submitted to further purification either on silica gel (n-hexane: ethyl acetate) or LiChroprep. RP8 (MeOH:H_2O, 9:1 v/v), giving morusin (yield=3.5% of 400 g root barks). Spectral data were in accordance with those previously reported (Nomura *et al.*, 1981).

Animals

Swiss mice of both sexes (25–35 g) were housed in automatically controlled temperature conditions (23 ± 2 °C and 12 h light-dark cycles). The animals were given access to water and Nuvital chow *ad libitum* unless otherwise indicated. The animals remained in the appropriate laboratory of University of Itajaí Valey (UNIVALI) until some hours before of the experiments.

Pharmacological analysis

Abdominal constriction response caused by intraperitoneal injection of diluted acetic acid

The abdominal constriction induced by intraperitoneal injection of acetic acid (0.6%), was carried out according to the procedures described previously (Collier *et al.*, 1968; Souza *et al.*, 1998) with minor modifications. Animals were pretreated intraperitoneally (i.p.) with morusin (**1**) (23.8–142.9 mmol/kg) or standard drugs intraperitoneally 30 min before the acid acetic injection. Control animals received a similar volume of 0.9% NaCl (10 ml/kg , i.p.). In a separate set of experiments, animals were pretreated either with compound 1 (23.8 mmol/kg, i.p.) or with morphine (13.3 mmol/kg, subcutaneously, s.c.) 30 min before the acetic acid injection.We also analysed the effect of naloxone (**3**) (13.8 mmol/kg, i.p.) injected 10 min beforehand, against the antinociceptive effect caused by both morphine and **1.** After the challenge, each mouse was placed in a separate glass funnel and the number of abdominal contractions of the abdominal muscles together with stretching, was cumulatively counted over a period of 20 min. Antinociceptive activity was expressed as the reduction of the number of abdominal contractions between control animals and mice pretreated with morusin and standard drugs.

Formalin-induced pain

The procedure used was essentially similar to that described previously (Hunskaar and Hole, 1987; Hunskaar *et al.*, 1985; Souza *et al.*, 1998). Animals from the same strain were slightly anaesthetized with ether, except when used to analyse the first phase of formalin-induced pain, and 20 ml of 2.5% (0.92% formaldehyde) made up of PBS (phosphate buffered saline containing: NaCl 137 mM; KCl 2.7 mM and phosphate buffer 10 mM) was injected under the plantar surface of the left hindpaw. Animals were acclimatized to the laboratory for at least 24 hrs before the experiments. Two mice (control and treated) were observed simultaneously for 0 to 30 min following formalin injection. The initial nociceptive scores normally peaked after 5 min (first phase, representing the neurogenic pain), and after 15–30 min after formalin injection (second phase, representing the inflammatory pain) (Hunskaar and Hole, 1987). Animals were treated with saline 0.9% (10 ml/kg, i.p.), morusin (**1**) (23.8–142.9 mmol/kg) by the i.p. route or with standard drugs 60 min before formalin injection. After intraplantar irritant application, the animals were immediately placed in a glass cylinder (20 cm diameter). The time spent by animals licking or biting the injected paw was timed with a chronometer and was considered indicative of pain. At the end of the experiments the animals were sacrificed with ether, the paws cut at the tibio-tarsic joint and weighed on an analytical balance to investigate the interference of **1** on formalin-induced inflammatory edema.

Hot-plate test

The hot-plate was used to stimate the latency of responses according to the method described by Eddy and Leimback (1953) with minor modifications. The temperature of the hot-plate was maintained at 56 ± 3 °C. The animals (n=10) were placed on glass funnels in the heated surface and the time between placing the animals and the be-

ginning of licking paws or jumping were recorded as latency of response, in non-treated (saline 10 ml/kg, i.p) or morusin (23.8–142.9 mmol/kg, i.p.) animals.

Statistical analysis

The results are presented as mean ± s.e.m. and the statistical significance between groups was analysed by means of the *t* test or analysis of variance followed by Dunnettós multiple comparison test, when appropriate. *P* values less than 0.05 were considered significant. The ID_{50} values (the dose of compound that reduced formalin- or acetic acid -induced pain by 50% relative to control values) were determined by graphical interpolation from individual experiments, which are accompanied by their respective 95% confidence limits.

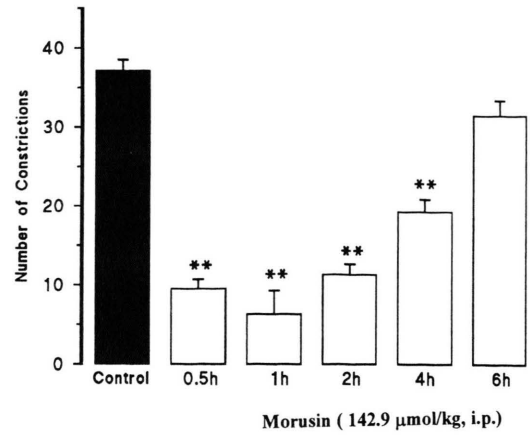

Fig. 2. Time-course of antinociceptive of morusin (**1**) (142.9 μmol/kg, i.p.) against acetic acid-induced pain in mice. Each group represents the mean of 6 to 8 experiments and the vertical bars indicate the s.e.m. **P<0.01 compared with corresponding control value.

Results

The results shown in Fig. 1 indicate a significant (P < 0.05) and dose-dependent antinociceptive effect of morusin (**1**) given by i.p. 30 min beforehand, inhibiting acetic acid-induced writhing responses in mice. As can be observed, it presented a ID_{50} value (and 95% confidence limits) of 72.1 (50.5–113.6) mmol/kg. At 142.9 mmol/kg (60 mg/kg), i.p. **1** caused 78.3 ± 2% of inhibition. It was more potent than two well-known analgesic and antiinflammatory drugs, aspirin and paracetamol, which presented ID_{50} of 133 and 125 mmol/kg, ip, respectively, in the same experimental model (Gaertner *et al.*, 1999). The antinociceptive effect was observed over a long time period, extending its action until 4 hours after the algesic stimuli with acetic acid (Fig. 2). When the animals were treated

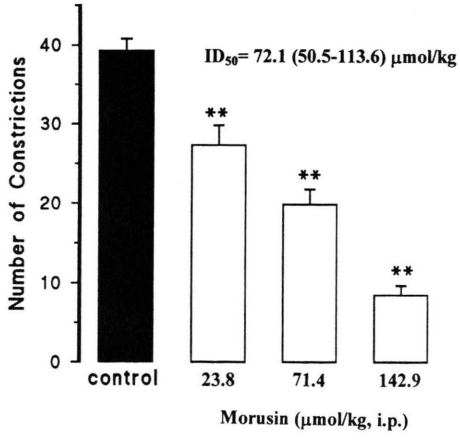

Fig. 1. Antinociceptive effect for morusin (**1**) given intraperitoneally, against acetic acid-induced abdominal constrictions in mice. Each group represents the mean of 6 to 8 experiments and the vertical bars indicate the s.e.m. **P<0.01 compared with corresponding control value.

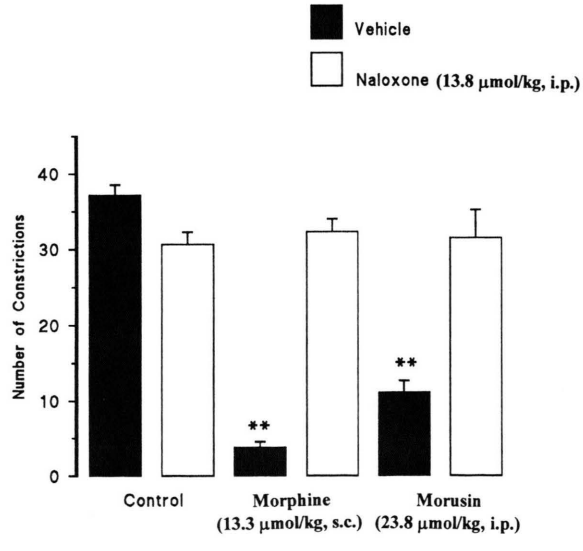

Fig. 3. Effect of naloxone on the antinociceptive profile caused by morphine and morusin (**1**) against acetic acid-induced pain in mice. Each group represent the mean of 6 to 8 experiments and the vertical bars indicate the s.e.m. **P<0.01 compared with corresponding control value.

with naloxone (13.8 mmol/kg, i.p.), a non-selective morphine receptor antagonist, 10 min before the noxious stimuli, morusin (142.9 mmol/kg, i.p.) reversed the antinociceptive effect when compared to morphine (Fig. 3).

In the formalin test, **1** significantly inhibited ($P<0,05$) dose-dependently both the first and second phases by the systemic route (Fig. 4). The calculated ID_{50} 's values for 1^{th} and 2^{th} phases were 67.1 (40.7–111.9) and 63.8 (38.7–106.7) mmol/kg for the systemic route, with maximum inhibitions (MI) of 66.8 ± 2.4 and 81.0 ± 4%, respectively. The standard drugs dose-dependently prevented only the inflammatory effects (second phase), with ID_{50} of about 120 mmol/kg (Gaertner *et al.*, 1999). Morusin was ineffective in antagonizing the formalin-induced hindpaw oedema (results not shown).

In the hot-plate test, **1** ((23.8–142.9 mmol/kg, i.p.) was capable of increasing dose-dependently the latency period of pain induced by heating of the plate (Fig. 5).

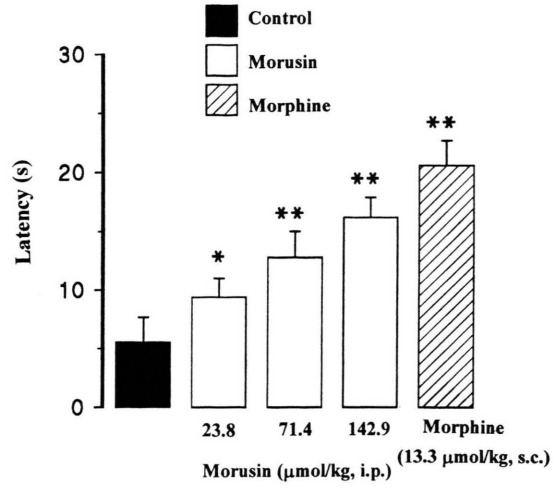

Fig. 5. Antinociceptive effect of morusin (**1**) and morphine in the hot-plate test in mice. Each group represents the mean of 6 to 8 experiments and the vertical bars indicate the s.e.m. *$P<0.05$ and **$P<0.01$ compared with corresponding control value.

Discussion

This paper extends previous findings where we have shown that *M. nigra* possesses active constituents which exhibit marked antinociceptive activity (unpublished results). The results reported in the present investigation demonstrate for the first time that morusin (**1**), the main prenylflavonoid present in *M.nigra* root bark (Ferrari *et al.*, 1999), exerts antinociceptive effects on different animal models *in vivo*.

We have observed that the antinociceptive effects of morphine and that of **1** were partially but significantly reversed by naloxone suggesting that **1** is effective in abolishing acetic acid-induced pain

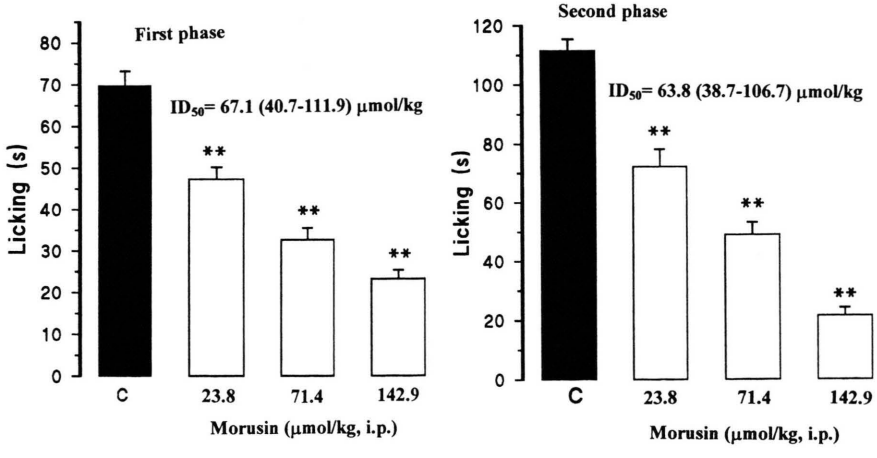

Fig. 4. Effect systemic of morusin (**1**) against the first (0–5 min) or against the second phase (15–30 min) in the formalin-induced pain. Each group represents the mean of 6 to 8 experiments and the vertical bars indicate the s.e.m. **$P<0.01$ compared with corresponding control value. Licking (s) indicate the time spent by animals licking or biting the injected paw.

in an opioid way, which was confirmed by the antinociceptive effects in the hot-plate test, a technique that has a selectivity for opioid-derived analgesics (Abbott and Melzack, 1982; Abbott and Franklin, 1986). It is interesting to note that morusin, on formalin-induced pain, a test which defines two distinct periods of response, i.e., "early response" and "late response" inhibited both phases of pain, as further evidence that the mechanism of action involve opioid receptors (Hunskaar *et al.*, 1985). It is important to mention that morusin failed to affect the edematogenic response associated with the second phase of the formalin test (results not shown), suggesting that its antinociceptive activity is unrelated to an inflammatory effect (Vaz *et al.*, 1996).

Although this is the first report about the antinociceptive action of **1**, some authors have demonstrated its different kind of activities. It has been effective against gram-positive bacteria and some species of fungi and has shown anti-tumor promoting activity in two-stage mouse skin carcinogenesis experiments (Alves *et al.*, 1988). More recently, it was reported that **1** showed significant effects on arachidonic acid-, collagen-, and -platelet aggregation factor (PAF) assays (Ko *et al.*, 1997).

In summary, our results demonstrate for the first time that morusin (**1**) exerts a marked antinociceptive effect by the systemic route, in distinct models of nociception in mice, consisting of the main active component of *M. nigra*. It was about 2-fold more potent than some drugs clinically employed against dolorous processes.

The mechanism by which the morusin exerts antinociceptive activity still remains undetermined, but our results strongly suggest that it involves the participation of the opioid system. Considering its promising pharmacological action and good yield from the plant, **1** might be further used as a model to obtain new more potent derivatives and/or selective analgesic drugs.

Acknowledgements

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