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Full Length Research Paper

Anti-inflammatory, analgesic and antioxidant properties of *Bursera morelensis* bark from San Rafael, Coxcatlán, Puebla (México): Implications for cutaneous wound healing

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Bursera morelensis **Ramírez bark is used by people of San Rafael, Coxcatlán, in Puebla State, México, as a traditional remedy to heal skin wounds. In the present study, we evaluated anti-inflammatory, analgesic and antioxidant activities of the methanolic extract (MP) obtained from the bark. Our results demonstrated that MP of *B. morelensis* possesses anti-inflammatory activity by inhibition of paw edema induced by carrageenan in rats. The dose of 50 mg/kg was comparable with indomethacin used as reference standard. The extract (50 mg/kg) also diminished 28.01% of the number of neutrophils migrating into the peritoneal cavity. At the same dose, an analgesic activity of the extract was observed by inhibition of acetic acid induced writhing response in mice. The extract also showed a strong capacity of DPPH radical scavenging with an EC_{50} of 3.05 µg/mL that was comparable with quercetin (EC_{50} = 4.45 µg/mL). The biological activities of the extract are attributed to its mixture of phenolic compounds (50.5%), particularly phenilpropanoids and flavonoids. The anti-inflammatory, analgesic and antioxidant properties shown by the extract favor the wound healing, thus controlling the inflammatory process without inhibiting it. This study supports the traditional use of *B. morelensis* bark for the treatment of acute skin wounds.**

Key words: *Bursera morelensis*, anti-inflammatory, analgesic, antioxidant, wound healing, phenolic compounds.

INTRODUCTION

Wounds are always accompanied by inflammatory processes causing symptoms like reddening and edema of the surrounding tissue (Hayden and Ghosh, 2008) that results in a coordinated influx of neutrophils at the wound site. In addition, various chemical mediators are liberated that is, eicosanoids, prostaglandins, nitric oxide (NO), and cytokines derived from enzymes such as cyclooxygenases (COX) and inducible nitric oxide syntetase (iNOS) are also liberated. Some mediators involved in the inflammatory reaction can extend the repair generating chronic wounds. In addition, the prostaglandins are chemotactic mediators for neutrophils and they are

responsible for fever and pain in the acute inflammation (Pradilla et al., 2004).

In damaged tissues, free radicals are produced by neutrophils and wound related non-phagocytic cells (Griendling et al., 2000). Thus, the wound site is rich in both oxygen and nitrogen reactive species along with their derivatives. The presence of these free-radicals will result in oxidative stress leading to lipid peroxidation, DNA breakage and enzyme inactivation, including the presence of free radical scavenger enzymes (Wiseman and Halliwell, 1996).

The use of anti-inflammatory, analgesic and antioxidant agents are helpful in the therapeutic treatment of wounds and many plants have been shown to possess therapeutic potential with these properties. Species of *Bursera* genus are included among the medicinal plants of popular use and are known for their anti-inflammatory, antitumoral, antibacterial and insecticidal properties. These medicinal effects are attributed to the essential oils, diterpenes, triterpenes, sterols and lignans (Jolad et al., 1977a; Becerra et al., 2001; Canales et al., 2005; Carretero et al., 2008).

B. morelensis Ramírez belonging to the family *Burseraceae*, is endemic from México where it has a wide distribution (Rzedowski et al., 2004). Chemical composition of the species has not been documented and only two lignans with cytotoxic activity has been reported (Jolad et al., 1977b). Traditionally, *B. morelensis* is known as “aceitillo”, “cuajiote”, “palo colorado”, “palo mulato” and “xixote”. Resin and infusions of the bark are used in Mexican folk medicine by people from San Rafael, Coxcatlán Puebla State (México) as a traditional remedy to treat skin wounds. However, until the present investigation, the literature survey revealed that any study had been carried out on the wound healing effect of *B. morelensis* or other biological activities that might be associated with wound healing process such as atiinflammatory, analgesic and antioxidant activities that together can favor the repair of the damage tissues (Khanna et al., 2002). Hence, the aim of this study was to evaluate the anti-inflammatory, analgesic and antioxidant activities of *B. morelensis* bark; such effects are closely related with the wound healing process.

MATERIALS AND METHODS

Plant

B. morelensis Ramirez bark was collected at San Rafael, Coxcatlán, Puebla, México, in August, 2007 and identified by Dr. Oswaldo Téllez Valdés (Laboratory of Natural Products of the Universidad Nacional Autónoma de México (UNAM)). A voucher specimen was deposited at the herbarium IZTA at Facultad de Estudios Superiores Iztacala (IZTA 42123), (UNAM)

Preparation of the extract

The bark of *B. morelensis* (646 g) was shade-dried at room temperature, ground in powder and extracted with 2 L of methanol. The extract was filtered and concentrated *in vacuo* to yield 150.4 g of dry extract. The non polar compounds of methanolic extract were removed by partition with 500 mL of methanol and 500 mL of hexane in a separating funnel. After solvent-solvent extraction, the methanol phase (MP) was removed from the hexane phase (HP). Both extracts were concentrated under low pressure; the yields obtained were hexane 1.53% (w/w) and methanol 10.28% (w/w). The extracts were kept in dark at 4°C until tested.

Phytochemical analysis

Total phenolic compounds

The total phenols of the methanolic phase (MP) were determined with Folin-Ciucalteu reagent (Singleton and Rossi, 1965). Gallic acid was used as reference standard. A standard curve was prepared using solutions of gallic acid in water (0.00625 to 0.2 mg/mL).

An aliquot of 1 mL of stock solution of MP (0.05 mg/mL) was transferred to a tube that contained 7 mL of distilled water and 0.5 mL of the Folin-Ciucalteu reagent. After 5 min of incubation, 1.5 mL of Na_2CO_3 (20%) was added. The mixtures were allowed to stand at room temperature for 120 min. The total phenols were determined by absorption at 760 nm in a spectrophotometer (COLEMAN Junior® II, UV-VIS). The result was expressed as milligrams of gallic acid equivalents per gram dry material.

High performance liquid chromatography

Chemical nature of the compounds in the MP of *B. morelensis* was identified using HPLC (Hewlett Packard 1100 system equipped with a diode array detector-DAD). Analysis was performed with an Allphere ODS-1 C18 column (250 × 46 mm, 5 µm) at 23°C. The flow rate was 0 to 5 min of 1 mL/min; 5 to 7.5 min of 1.5 mL/min. The mobile phase was isocratic [MeOH:AcOH:H_2O (30:5:65)]. The analysis of peaks of chromatogram was determined with ultra-violet (UV) spectra (260 nm) using Chemstation A.09.03 software.

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Na_2CO_3, sodium chloride, gallic acid, acetic acid, Folin-Ciucalteu phenol reagent, indomethacin, heparin, and carrageenan were obtained from Sigma (St Louis MO). Dexamethasone (Merck-Sharp and Dohme) and solvents used (methanol, hexane, water) were analytical and of HPLC grades, purchased from Baker.

Animals

The anti-inflammatory and analgesic activities of MP were evaluated on male Wistar rats (260 to 320 g) and male mice *Mus musculus* CD-1 (25 to 30 g). Animals were housed in a room maintained at 22 ± 1°C with an alternating 12 h light-dark cycle.

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Abbreviations: MP, methanol phase; **HP,** hexane phase; **PPH,** 1,1-diphenyl-2-picryl-hidrazyl; **HPLC,** high-performance liquid chromatography; **DAD,** diode array detector; **MeOH,** methanol; **AcOH,** acetonitrile; **NOM,** norma Oficial Mexicana.

Table 1. Data of reversed phase HPLC-DAD profile (260 nm) of MP of *B. morelensis.*

Phenolic compound	t_R (min)	λ_{max} (nm)
1	2.578	240, 278^{pp}
2	3.014	276^{pp}
3	3.574	238, 278^{pp}
4	4.001	238, 278, 336$^{sh\ flv}$
5	4.534	238, 278, 330$^{sh\ flv}$
6	6.201	238, 304, 318$^{sh\ flv}$
7	8.328	238, 286, 332$^{sh\ flv}$
8	8.734	238, 282, 330$^{sh\ flv}$

t_R: retention time; λ_{max}: maximum absorbance; sh: shoulder peak; compound corresponding to pp = phenylpropanoid; flv = flavonoid.

Food and water sources were available *ad libitum*. All the experimental procedures in the animals were attached to the NOM-062-ZOO-1999, with respect the use and handling in the laboratory animals used in the scientific investigation.

Anti-inflammatory activity

Carrageenan-induced paw edema test

The test was carried out according to the method described by Vazquez et al. (1996) in groups of five rats. Edema was induced injecting 0.1 mL of carrageenan (1%) in the right hind paw, under the plantar aponeurosis. An equal volume of saline solution into the left hind paw was injected. Animals were pretreated with the MP of *B. morelensis* at doses of 25 and 50 mg/kg (intra-peritoneally) one hour before carrageenan injection. Indomethacin (10 mg/kg) and saline solution (0.9%) (10 mL/kg) were used as reference drug and control, respectively. The paw volume was measured before injection of carrageenan or saline solution by the mercury displacement method (Van Arman et al., 1965) and the time course of edema formation was followed over 4 h. The volume increase (Δvolume) of the inflamed paw was estimated by subtracting the volume of the contralateral paw. The anti-inflammatory effect of the treatments was evaluated as degree of edema inhibition.

Neutrophil migration into peritoneal cavity

The neutrophils migration test was performed as described by Vázquez et al. (1996). Four groups of five rats were used. 3 mL of carrageenan (10%) was administered intra-peritoneally to each animal. One hour before the carrageenan injection, the MP of *B. morelensis* were administered subcutaneously in doses of 25 and 50 mg/kg. The reference drug rats group was treated with dexamethasone (1 mg/kg) and the control group received saline solution (10 mL/kg). 4 h later after administration of the carrageenan injection, the rats were sacrificed. Abdominal cavity was washed with 10 mL of phosphate buffered saline solution (5 U/mL of heparin; 5% of bovine serum albumin). 5 mL of this washed solution was withdrawn for cell counts. The total cell counts were done in a Neubauer chamber and differential cell counts were performed by the technique reported by Souza and Ferreira (1985). The leucocytes percentage was determined in the peritoneal fluid. The results are expressed as number of cells/mL of collected fluid.

Analgesic activity

The analgesic activity was evaluated by the acetic acid-induced abdominal writhing response (Collier et al., 1968); four groups of eight mice were used. The mice were injected in the peritoneal cavity with 0.6% acetic acid at a dose of 60 mg/kg. The MP of *B. morelensis* at doses of 25 and 50 mg/kg (*per os*) were administered 30 min prior to treatment with acetic acid. A group of rats were treated with indomethacin (10 mg/kg), used as reference drug, and control group received saline solution (0.9%, 10 mL/kg). The writhes induced by the acetic acid consisted of a contraction of the abdominal muscle together with a stretching of the hind limbs. The analgesic activity was expressed as the writhes scores over 20 min.

Antioxidant activity

A DPPH (1,1-diphenyl-2-picryl-hidrazyl) assay was employed to investigate the antioxidant activity. Samples at various concentrations (2, 4, 6, 8 and 10 µg/mL) of MP were plated out in triplicate in a 96 well plate. 50 µL of concentrations were added into the wells. For the DPPH and methanol controls, 50 µL of methanol were added. A volume of 150 µL of DPPH (100 µM in methanol) was added to the test samples and 150 µL of methanol were added to the control wells. The plate was incubated in the dark, at 37°C for 30 min. The absorbance was read after incubation using a SLT Spectra ELISA reader, at a single wavelength of 515 nm. The percentage of decolourisation was determined (Equation 1) and the efficient concentration value (EC_{50}) was calculated. Quercetin was used as reference standard.

$$\text{Decolourisation (\%)} = [(A_{control} - A_{test}) / A_{control})] \times 100 \quad (1)$$

Where A = absorbance at 515 nm, $A_{control}$ = average absorbance of DPPH - average absorbance of methanol, A_{test} = average absorbance obtained in the wells contained DPPH and test sample.

Statistical analysis

Pharmacological activities (anti-inflammatory and analgesic activities) were presented as mean ± standard error of mean (SEM). Significant differences were assessed by Student's t-test for unpaired samples. Results of the antioxidant activity were obtained from two independent experiments and given as mean ± standard deviation (SD). The differences between values obtained for concentrations of MP and quercetin were done by one-way ANOVA. The EC_{50} values in antioxidant assay were calculated by lineal model. In all the cases, the data distribution was normal and the differences among these were considered significant at $P < 0.05$. Statistical tests were run in Statistica® '99 edition program.

RESULTS

Phytochemical study

High- performance liquid chromatography and total phenolic compounds

In the characterization of compounds of MP of *B. morelensis* the HPLC chromatogram showed eight peaks. The spectral data indicated that they correspond to phenolic compounds, with a λ_{max} among 238 and 336 nm. The spectral UV of peaks 4 to 8 corresponded to the characteristic patterns of flavonoids (Table 1). The result of total phenolic content estimated was 504.54 mg of

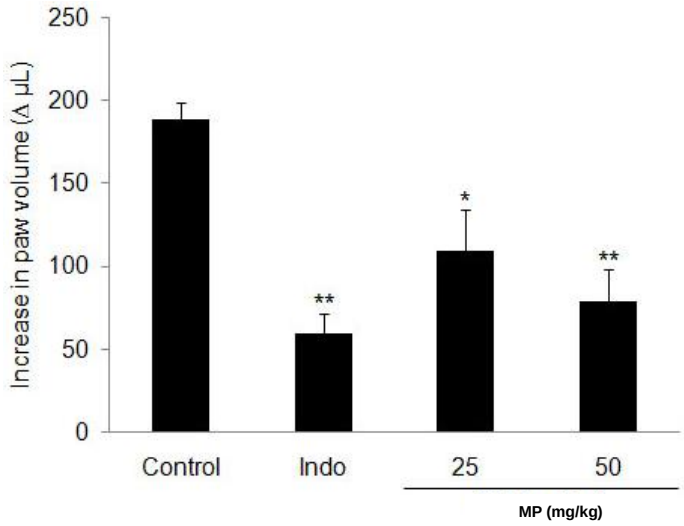

Figure 1. Antiinflammatory effect of MP of *B. morelensis* on the paw edema induced by carrageenan in rats. Carrageenan (100 µg/mL) was administered intraplantarly. MP, saline solution (control group) or indomethacin (indo) (10 mg/kg) (i.p.) was administered 1 h before carrageenan administration. Paw edema was measured at 4 h after the carrageenan injection. Data are expressed as mean ± SEM; n = 5 rats per group. Significantly different compared to control group (*P < 0.05; **P < 0.01) by Student's unpaired t-test.

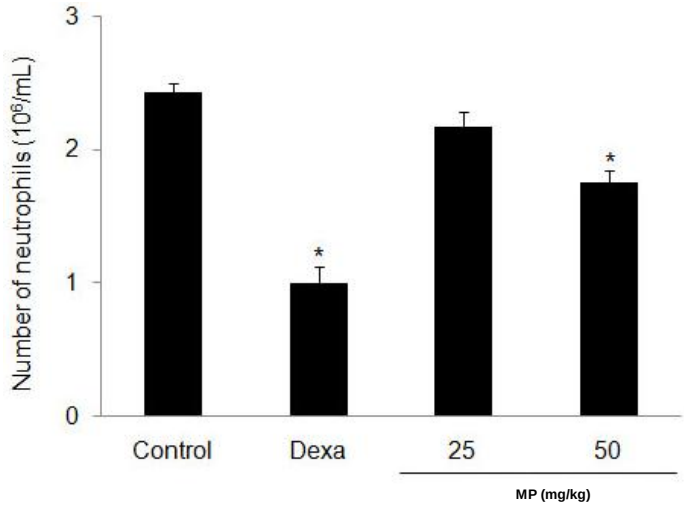

Figure 2. Effect of MP of *B. morelensis* on neutrophils migration into peritoneal cavity in rats. Increasing doses of MP were administered (s.c.) and compared with control (saline solution), dexamethasone (Dexa) (1 mg/kg, s.c.). Values are the mean ± SEM; n = 5 rats per group; N = 20. *Significantly different compared to control group (P < 0.01) by Student's unpaired t-test.

gallic acid equivalents per gram dry sample that corresponded to 50.5% of phenolic compounds.

Anti-inflammatory activity

The carrageenan induced paw edema test showed a maximum volume of edema obtained 4 h, following carrageenan injection in the control group (188.41 ± 9.9 µL of edema). Rats pretreated with dose of MP of *B. morelensis* showed a significant decrease of the edema compared with control in a dose-dependent form (P < 0.05) (Figure 1). The group which was administered the dose of 50 mg/kg showed an increment in the plant volume of 79.32 ± 18.52 µL, corresponding to 57.9% of edema inhibition, while the group tried with 25 mg/kg presented an increment of volume of 109.8 ± 25.24 µL (41.72% edema inhibition). The effect shown by the 50 mg/kg dose was comparable with indomethacin (10 mg/kg) (59.5 ± 12.12 µL edema; 68.42% edema inhibition), used as reference drug, among which there were not significant differences.

In the neutrophils migrating into the peritoneal cavity test, the MP of *B. morelensis* decreased the number of neutrophils in a dose-dependent form. The dose of 50 mg/kg of MP decreased the neutrophils migration by 28.01% and the dose of 25 mg/kg 10.77% (Data not shown) respect to control. The dose of 50 mg/kg and dexamethasone decreased significantly the migration of neutrophils compared to control group (P < 0.01) (Figure 2).

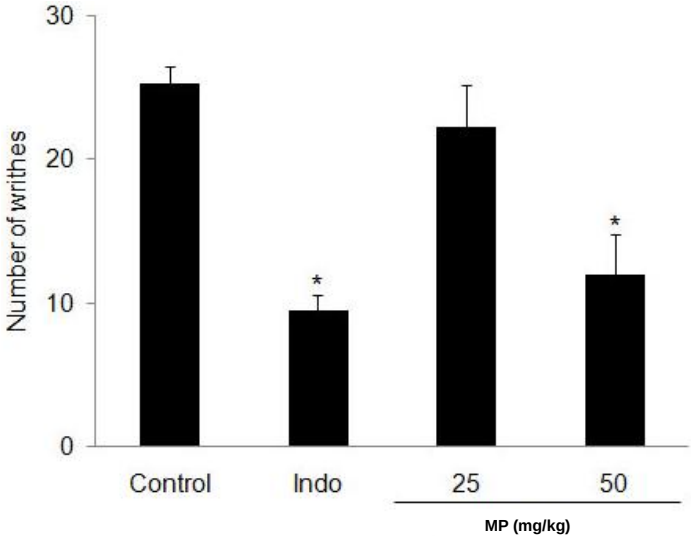

Figure 3. Effect of MP of *B. morelensis* on acetic acid induced writhes in mice. Doses of MP were administered (p.o.) 30 min before the acetic acid injection, saline solution (control group) and indomethacin (Indo; 10 mg/kg) was the reference drug. Data are expressed as mean ± SEM; n = 5 mice per group; N = 20. *Significantly different compared to control group (P < 0.01) by Student's unpaired t-test.

Analgesic effect

The MP reduced the number of writhes induced by acetic acid (Figure 3). The protective effect of MP was shown at

dose of 50 mg/kg, with 12 ± 2.83 writhes corresponding to 52.47% of decrease of writhing response. The protection offered by the oral administration of this extract 30 min before the acetic acid injection was comparable to that of indomethacin (10 mg/kg) (9.5 ± 1.13 writhes; 62.37% of decrease of writhing response) which did not show significant differences, both produced a significant inhibition of acetic acid induced writhing response with respect to control (25.25 ± 1.2 writhes) ($P < 0.01$).

Antioxidant activity

MP of *B. morelensis* displayed antioxidant activity, showing a strong capacity of DPPH radical scavenging. The EC_{50} of MP was of 3.05 µg/mL and not shown significant differences with quercetin (EC_{50} = 4.45 µg/mL).

DISCUSSION

The purpose of this study was to establish the basis for the traditional use of *B. morelensis* on wound healing by means of the evaluation of the anti-inflammatory and analgesic activities on *in vivo* models and antioxidant activity on *in vitro* model. The methanol extract constitute phenolic compounds which are three phenylpropanoids and five flavonoids, and they constitute 50.5% of the compounds in the extract. These results agree with that reported by Dey and Harbone (1989) that phenols are the main compounds in the vegetable barks.

The chemical composition of *B. morelensis* had not been reported previously in the species neither in the *Bursera* genus. It has only been mentioned in the presence of terpenoids in the essential oil and resin of other species of genus, mainly in *B. simaruba* (one of the most studied species). This species constituted mono -, sesqui- and tri-terpenes (Peraza-Sanchez et al., 1995) as well as sterols (Carretero et al., 2008). In the same way, the lignans are present in several species of the genus, as *B. tonkinensis* (Jutiviboonsuk et al., 2005), *B. microphylla* (Cole et al., 1968), *B. permollis* (Wickramaratne et al., 1995), *B. klugii* (Jolad et al., 1977a) and *B. schlechtendalii* (McDoniel and Cole, 1972). Therefore, our results contribute to the knowledge of the chemistry composition of *B. morelensis* and to *Bursera* spp.

The MP showed a significant decrease of the carrageenan induced paw edema in rats in the two assayed dose. Indomethacin showed a maximum inhibition of edema and its effect was similar, statistically to dose of 50 mg/kg of MP of *B. morelensis* which demonstrated that they were equally effective. The initial phase of carrageenan paw edema is mediated by histamine and serotonin, while later phase by prostaglandins, producing edema after migration of leucocytes (Castro et al., 1968; Vinegar et al., 1979). MP inhibited the edematous response produced by injection of carrageenan in the paw of the rats, suggesting that anti-inflammatory activity of extract is possibly mediated by inhibition of the synthesis, release or action of these mediators.

The MP decreased the number of neutrophils migrating into the peritoneal cavity, confirming the anti-inflammatory activity of the extract. It has been suggest that mechanism of action of anti-inflammatory agents that inhibit the neutrophils migration is related to inhibitory action of the arachidonic acid pathway (Higgs et al., 1979), that is the substrate for prostaglandins production (Blackwell et al., 1980) which are chemical mediators that play a role in the neutrophils chemotaxis in the acute inflammation (Kumara et al., 2007) by induction of capillary permeability (Amico-Roxas et al., 1984). The MP displayed a significant anti-inflammatory activity through inhibition of neutrophils migration, suggesting that probably the extract possesses this mechanism of action, inhibiting the synthesis of chemical mediators derived by this pathway, such as prostaglandins by inhibition of vascular permeability.

The results obtained indicate that MP can be used as an anti-inflammatory agent because the necessary dose to diminish edema is relatively low in comparison to previous studies that evaluated the anti-inflammatory effect of other species such as *B. simaruba.* In these studies, the anti-inflammatory effect was obtained with 78 mg/kg of hexanic extract (Carretero et al., 2008) and 80 mg/kg of methanolic extract (Abad et al., 1996). The anti-inflammatory activity of *B. simaruba* was attributed to the methyl-β-peltatin A (Noguera et al., 2004) and sterols (Carretero et al., 2008).

In the analgesic potential of MP, the extract inhibited acetic acid induced writhing response in mice one hour after oral administration at the dose of 50 mg/kg. The MP can be considered a potent analgesic for its similar effect to indomethacin which possesses analgesic and anti-inflammatory properties. In our results of anti-inflammatory and analgesic activities of MP, a general correlation dose-dependent was observed. This suggests the presence of compounds with a similar mechanism for both activities.

During the inflammatory process, the reactive oxygen species are produced, aggravating the disorders in the tissues (Lopes et al., 2005). The DPPH assay showed that the MP was capable to scavenge free-radicals for what a good antioxidant can be considered because its effect was similar to quercetin. The ability of the extract to act as reducer and DPPH free-radicals scavenger suggests that it is an electron donor reacting with free-radicals to convert them to more stable products, finishing the chain reaction.

According to the above mentioned, the biological effects of MP observed in this study may be attributed to its flavonoid rich composition that possess diverse pharmacological activities, including analgesic (Cárdenas

et al., 1993; Borgi et al., 2008), antioxidant and anti-inflammatory (De Bruyne et al., 1999; Peterson and Dwyer, 2000; Abdel-Hameed, 2009).

The anti-inflammatory and antioxidant properties of the phenolic compounds have been related with the number of hydroxy groups in the molecules (Soobrattee et al., 2005). Anti-inflammatory activity of the flavonoids is due to the inhibition of enzymes like phospholipase A_2, cycloxygenase and 5-lipoxygenase during the metabolism of the arachidonic acid (Torel et al., 1986; Middleton et al., 2000; Tenorio et al., 2006). On the other hand, the analgesic activity of flavonoids has been documented mainly in glycosyl flavonoids (Cárdenas et al., 1993; Borgi et al., 2008) which have both central and/or peripheral analgesic actions. The flavonoids with central analgesic property are opioid-like analgesics (that is, quercetin), they inhibit non-inflammatory pain. Peripheral analgesics (that is, penta-O-ethylquercetin) inhibit inflammatory pain acting on inflammatory mediators especially the prostaglandins synthesis (Picq et al., 1991).

Finally, the antioxidant capacity of flavonoids is due to their potential of chelation of transition metal ions (particularly iron and copper) and to donate hydrogen atoms and to transfer electrons to free radicals mainly the hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^{\bullet-}$), and lipid peroxyl radical ($LOO^{\bullet}$) that stabilize and delocalize their unpaired electron (Rise-Evans et al., 1997).

Conclusion

B. morelensis is used by folk medicine for wound healing. In addition, our results suggest that the combination of the biological properties shown by the extract (anti-inflammatory, analgesic and antioxidant) could be related to this medicinal effect. Control of the inflammatory phase could take place by inhibition of chemical mediators like prostaglandins. Likewise, the decrease of free-radicals diminishes the oxidative damage in the tissues and increases the proliferation of fibroblasts which are responsible for collagen synthesis in the wounds. The exact mechanisms of action and the active principles for such activities remain to be confirmed. Finally, this study supports the traditional use of *B. morelensis* bark for the treatment of acute wounds.

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