Arabian Journal of Chemistry (2016) 9, S191–S197

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Components and antioxidant, anti-inflammatory, anti-ulcer and antinociceptive activities of the endemic species *Stachys mialhesi* de Noé

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Received 10 January 2011; accepted 3 March 2011
Available online 9 March 2011

KEYWORDS

Stachys mialhesi de Noé;
Flavonoids;
Antioxidant activity;
Anti-inflammatory;
Antinociceptive

Abstract One diterpenoid, horminone **1**, two flavonoid glycosides, apigenin-7-*O*-(6″-*E*-*p*-coumaroyl)-β-D-glucopyranoside **2**, isoscutellarein-7-*O*-(6‴-*O*-acetyl-β-D-allopyranosyl-(1 → 2)-β-D-glucopyranoside) **3**, were isolated from *n*-butanolic extract of the aerial parts of *Stachys mialhesi* de Noé (BESM). Their structures were established on the basis of physical and spectroscopic analysis, and by comparison with the literature data. Antioxidant activity of this extract and the compound **3** was evaluated by the use of the Electron Spin Resonance method in order to visualize the inhibition of the DPPH radical. In this study, we also investigated the anti-inflammatory, anti-ulcer and antinociceptive activities of the BESM in experimental animal models at different doses. Our results showed that the BESM showed a strongest antioxidant activity. It decreased acetic acid induced writhing times; inhibited carrageenan-induced hind paw edema. All of these results suggested that the BESM possesses significant antioxidant, antinociceptive and anti-inflammatory activities.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

1. Introduction

The genus *Stachys* (Lamiaceae) contains about 200–300 species in the world (Rechinger and Hedge, 1982) and is considered to be one of the largest genera of this family. In Algeria, this genus is represented by 14 species including the

http://dx.doi.org/10.1016/j.arabjc.2011.03.005
1878-5352

endemic species *Stachys mialhesi* de Noé (Quezel and Santa, 1962).

To our knowledge, *S. mialhesi* de Noé has not been the subject of any study. However, pharmacological studies confirmed that extracts or components of plants belonging to the genus *Stachys* exert significant antibacterial (Skaltsa et al., 1999), anti-inflammatory (Zinchenko et al., 1981), antitoxic (Zinchenko et al., 1981), anti-nephritic (Hayashi et al., 1994a,b), antihepatitis (Savchenko and Khvorostinka, 1978), anti-anoxia effects (Yamahara et al., 1990), effect on hyaluronidase activity (Takeda et al., 1985) and hypotensive activity (Taked et al., 1997). Some species of this genus are used in folk medicine, especially *S. palustris* L. and *S. sylvatica* L. (Wound wort), as disinfectant, antispasmodic and for treatment of wounds (Gruenwald et al., 2000). In Iran, the aerial parts of *S. inflata* Benth are used to treat infection, asthma, rheumatic and other inflammatory disorders (Maleki et al., 2001). *S. lavandulifolia* Vahl was used as an anxiolytic and sedative (Amin, 1991).

We report here for the first time the isolation and identification of secondary metabolites of the *n*-butanolic extract obtained from the endemic species *S. mialhesi* de Noé aerial parts. The anti-inflammatory, antinociceptive and antioxidant activities of this plant are also investigated.

2. Experimental

2.1. General procedures

^{1}H and ^{13}CNMR spectra were obtained on a Bruker model 300, AMX-400 and AMX-600 spectrometers with standard pulse sequences operating at 300, 400 and 600 MHz in ^{1}H and 125, 150 MHz in ^{13}C NMR. DMSO-d_6 and $CDCl_3$ are used as solvents. EIMS and HRE-IMS were taken on a Micromass model Autospec (70 eV) spectrometer. Column chromatography (CC) was carried out on polyamid, and prep. TLC on silica gel 60 PF254 + 366 plates (20 × 20 cm, 1 mm). Indomethacin (Sigma, USA), Acetylsalicylic acid (El Vasr Co.), Carrageenan (BDH, England), Tween 80 (Sigma, Germany), saline (Sigma, Germany). All the chemicals and reagents used were of the highest grade of purity.

2.2. Plant material

Aerial parts of *S. mialhesi* de Noé were collected on April 2005 at Djebel El-Ouahch Constantine (North Eastern Algerian). The voucher specimen was identified by Professor Gérard De Bélair (University Badji-Mokhtar, Annaba) and was deposited at the Musée botanique de la Ville d'Angers (France) under the reference MBAng2005.10.

2.3. Preparation of the extract

Air-dried and powdered aerial parts (1 kg) of *S. mialhesi* were extracted with 70% MeOH. The residue was dissolved in water and extracted with petroleum ether, dichloromethane, ethyl acetate and *n*-BuOH, successively.

Each solvent free extract was dissolved in Tween 80 (7% aqueous solution) as vehicle and biologically tested in different dose levels. The studied biological activities of the tested extract were compared with the reference standard as well as the controls injected with 0.2 ml vehicle (7% Tween 80).

2.4. Isolation of chemical constituents

The *n*-butanolic extract (10 g) has been chromatographed over polyamid column and eluted with a gradient of toluene/methanol with increasing polarity. Fraction F4, obtained from toluene 92% (50 mg) was subjected to another silica gel column chromatography and eluted with a gradient of dichlormethane/methanol with increasing polarity. Fraction F4.4 (38 mg) was further subjected to preparative silica gel TLC and eluted with dichloromethane/methanol (80:20) leading to compound **1** (7 mg) which was identified as horminone, reported for the first time from *Stachys* genus and compound **2** (25 mg) which was identified as apigenin-7-β-D-(6″-*E*-*p*-coumaroyl)-glucopyranoside, and isoscutellarein-7-*O*-(6‴-*O*-acetyl-β-D-allopyranosyl-(1 → 2)-β-D-glucopyranoside) (**3**). The latter precipitated in all fractions starting from toluene 90% from which 3g of this compound is gathered.

2.5. Animals

Animal studies were conducted in accordance with the internationally accepted principles for laboratory animal use; Animals were obtained from the Animals house colony of the National Research Center, Cairo, Egypt.

Adult albino mice of both sex weighing (20–25 g) and adult male albino rats weighing (120–150 g) were used for determination of the medium lethal dose (LD_{50}), analgesic activity and carrageenan-induced edema, ulcerogenic effect, respectively. The animals were kept under normal laboratory conditions of humidity, temperature (25 ± 1 °C) and light (12 h day:12 h night), and allowed free access to food and water.

2.6. Evaluation of antioxidant activity

2.6.1. Analyses of phenolic compounds

Total phenol concentration was determined according to the Folin–Ciocalteu method, using pyrogallol as a standard. The absorbance was measured at 760 nm on a Uvikon 930 UV/vis spectrophotometer (Kontron instruments) and the results were expressed as pyrogallol equivalents in g per 100 g of the dry material (Kujala et al., 2000).

2.6.2. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging test

The antioxidant activity of BESM and compound (**3**) was assessed on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH, from Sigma) free radical (Fatimi et al., 1993) using ESR (Electron Spin Resonance) spectroscopy (Yamaguchi et al., 2000). Reaction mixtures contained 100 ml test samples and 100 ml DPPH ethanolic solution (5×10^{-4} M). Inhibition ratio was determined by comparison with a water-treated control group. ESR spectra were obtained with a Bruker ESP300E spectrometer using micro-sampling pipettes at room temperature under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.197 mT; scanning field, 349.7 mT; receiver gain, 1.25×10^{5}; sweep time, 11 s; microwave power, 4 mW; microwave frequency, 9.78 GHz. All spectra were recorded at

3 min after homogenization by agitation. The inhibition percentage was calculated by using the double integral of the signal (Eq. 1):

$$\text{Inhibition ratio} = \frac{\text{ref-extract}}{\text{ref-bg}} \quad (1)$$

where ref is the reference signal (DPPH + water), extract is the test signal, bg is the background signal. The data were the means of five measurements.

2.7. Determination of the medium lethal dose (LD_{50})

For acute toxicity study, groups of five mice of both sexes (20–25 g) were used, one group injected with the corresponding volume 0.2 ml of the vehicle (Tween 80) and served as control and the tested extract were orally administered in doses ranging from 1000 to 10,000 mg/kg body weight. Animals were observed and the mortality rates were recorded within the first 24 h after extract administration. The LD_{50} was determined according to Behrens and Kerber method (Behrens and Kerber, 1935).

2.8. Evaluation of analgesic activity

2.8.1. Acetic acid induced writhing method

Groups of six mice of both sex (20–25 g) were used, one group injected with the corresponding volume 0.1 ml of the vehicle (saline) and served as control and the tested extract and reference drug were orally (p.o.) administered in doses ranging 5–10 g/kg body weight 30 min before of a intraperitoneal (i.p.) freshly prepared acetic acid (2% W/v in saline; pH 2.7; 10 ml/kg body weight) injection.

The animals were then placed immediately to individual in a transparent plastic box. The number of writhes, a response consisting of contraction of an abdominal wall, pelvic rotation followed by hind limb extension, was counted during continuous observation for 20 min beginning from 5 min after the acetic acid injection and the percentage inhibition of writhing was expressed. Acetylsalicylic acid (200 mg/kg, p.o) was used as a reference drug against which the test extracts were compared (Koster et al., 1959).

2.9. Evaluation of anti-inflammatory activity

2.9.1. Carrageenan-induced rat paw edema

The inhibitory activity of the studied extract on carrageenan-induced rat's paw edema was investigated according to the method of Winter et al. (1962).

Adult male albino rats (110–130 g), with free access to water but had been fasted overnight (18 h), received a subplantar injection of 0.05 ml of 1% suspension of carrageenan in saline into the plantar tissue in the right hind paw. An equal volume of saline was injected into the other hind paw and served as control.

Data are expressed as a percentage increase in paw thickness and the differences between treated animals and the control group were expressed at the same time point after carrageenan injection. In this part of the study, rats were allocated randomly to one of six groups each were orally dosed with the test extract, one hour before carrageenan challenge: (a) controls (saline, $n = 6$); (b) *n*-butanolic extract (5000 mg/kg, $n = 6$); (c) indomethacin (5 mg/kg, $n = 6$). Orally, injection of extract or indomethacin was performed 60 min before carrageenan injection.

Four hours after extract administration, the animals were decapitated and the paw was rapidly excised. The average weight of edema was estimated for the treated as well as the control group. The percentage inhibition of weight of edema was also evaluated. Indomethacin was employed as standard against which the test extracts were compared.

2.10. Ulcerogenic effect

Groups of adult male albino rats of six animals each (110–150 g) were fasted overnight, and orally given the tested extract at a dose level of 5 g/kg body weight, for four consecutive days. Four hours following the last dosing, animals were sacrificed, their stomach were removed, opened along the greater curvature, and the numbers of ulcers were assessed by adopting the method of Correl et al. (1979).

2.11. Data and statistical analysis

Data were expressed as mean ± s.e.m, statistical comparisons between different groups were made by analysis of variance (ANOVA) followed by multiple comparison tests (Post hoc Dunnett's). Differences with $p < 0.05$ between experimental groups and control animals were considered statistically significant (Norusis, SPSS INC).

3. Results and discussion

3.1. Characterization of isolated compounds

The *n*-butanolic extract (BESM), obtained after maceration of the aerial parts of *S. mialhesi* with MeOH/H_2O (7:3) followed by filtration, concentration and successive liquid–liquid extractions using petroleum ether, dicholoromethane, ethyl acetate and *n*-butanol, successively, led to the isolation of one diterpenoid, Horminone (**1**), and two flavonoids glycosides, apigenin-7-*O*-(6″-*E*-p-coumaroyl)-β-D-glucopyranoside (**2**) and isoscutellarein-7-*O*-(6‴-*O*-acetyl-β-D-allopyranosyl-(1 → 2)-β-D-glucopyranoside) (**3**) (Fig. 1). These compounds were identified by the use of ^{1}H NMR and ^{13}C NMR, DEPT, and 2D NMR experiments, COSY, HMQC, HMBC in addition with UV spectroscopy.

3.1.1. Horminone (*1*): Yellow crystals

^{1}H NMR (300 MHz, DMSO-d_6) δ: 0. 89 (3H, s, Me-19), 0.97 (3H, s, Me-18), 1.15 (m, H-1α), 1.18 (d, $J = 7.0$ Hz, Me-16), 1.20 (3H, s, Me-20), 1.21 (d, $J = 7.0$ Hz, Me-17), 1.18–1.41 (m, H-3α, β), 1.46 (m, H-2α), 1.48 (m, H-5), 1.52 (dd, $J = 12.6$ Hz, $J = 4.5$ Hz, H-6β), 1.65 (m, H-2β), 1.89 (d, $J = 12.6$ Hz, H-6α), 2.65 (ddd, $J = 4.0$, 4.0, 12.0 Hz, H-1β), 3.01 (s, 7-OH), 3.15 (septet, $J = 7.1$ Hz, H-15), 4.65 (dd, $J = 4.5$ Hz, $J = 1.5$ Hz, H-7), 7.28 (s, OH-12). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 34.8 (C-1), 17.8 (C-2), 40.0 (C-3), 38.1 (C-4), 44.7 (C-5), 24.8 (C-6), 62.2 (C-7), 142.2 (C-8), 146.8 (C-9), 32.0 (C-10), 182.8 (C-11), 150.0 (C-12), 123.2 (C-13), 188.1 (C-14), 22.9 (C-15), 18.7 (C-16), 18.8 (C-17), 32.1 (C-18), 20.7 (C-19), 17.3 (C-20) EISM: m/z 332 $[M]^{+\cdot}$ (Jonathan et al., 1989).

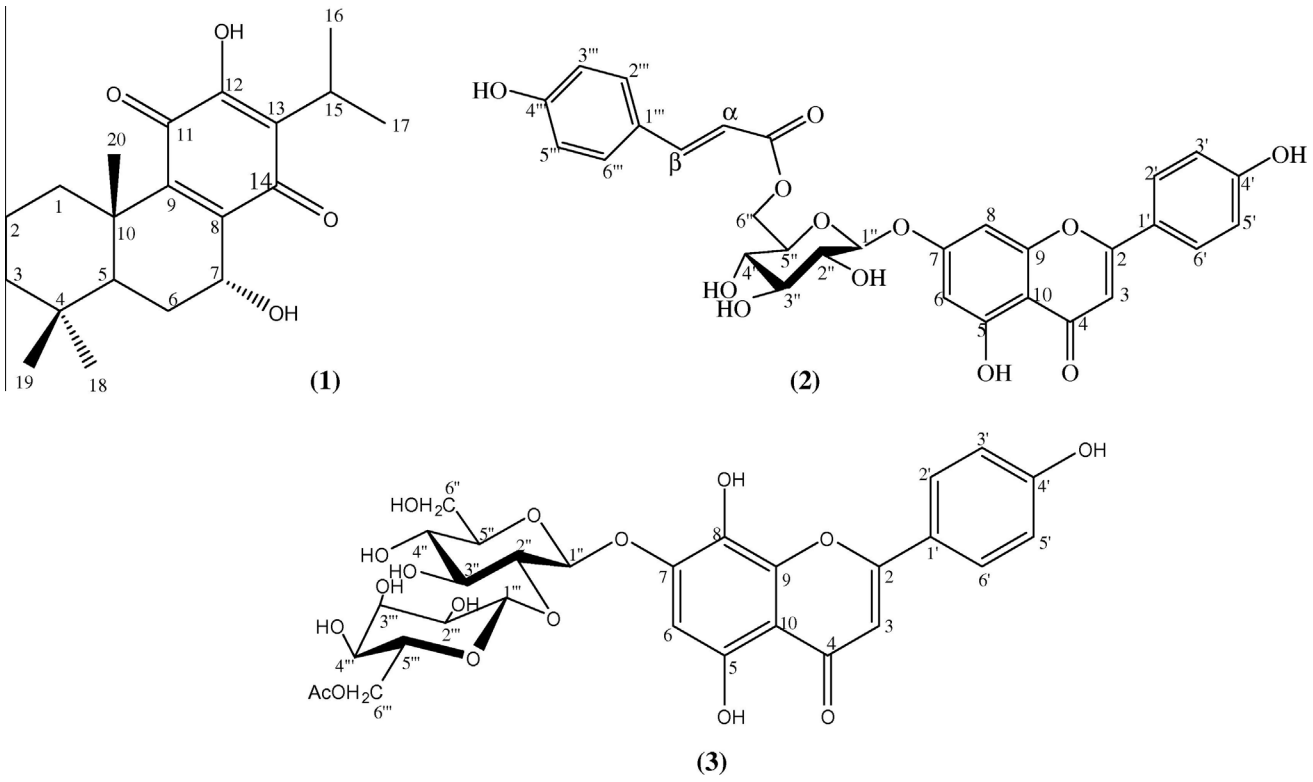

Figure 1 Chemical structures of compounds **1**–**3**.

*3.1.2. Apigenin-7-O-(6″-E-p-coumaroyl)-β-D-glucopyranoside (**2**)*

Yellow powder. UV (λ_{max} in MeOH): gives bands at 317, 268 nm for band I and II, addition of NaOH; 370 and 262, NaOAc; 317 and 268, H_3BO_3; 300, 317, 385 and 268, $AlCl_3$; 322, 380, 421 and 278, 298 while HCl; 323, 377 and 278, 298. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 6.48 (1H, d, J = 2.0 Hz, H-6), 6.82 (1H, d, J = 2.0 Hz, H-8), 6.85 (s, H-3), 6.93 (2H, d, J = 9.0 Hz, H-3′/5′), 7.95 (2H, d, J = 8.4 Hz, H-2′/6′), 12.95 (1H, s, OH-5). 3.85 (1H, td, J = 1.8 H, J = 7.2 Hz, H-5″), 4.16 (1H, dd, J = 12.0, 7.2 Hz, H-6″a), 4.46 (1H, dd, J = 1.8, 11.4 Hz, H-6″b), 5.17 (1H, d, J = 7.8 Hz, H-1″), 6.34 (1H, d, J = 16.2 Hz, H-α), 6.67 (2H, d, J = 9.0 Hz, H-3‴/5‴), 7.38 (2H, d, J = 8.4 Hz, H-2‴/6‴), 7.50 (1H, d, J = 16.2 Hz, H-β), ^{13}C NMR (DMSO-d_6, 150 MHz) δ: 164.6 (C-2), 103.4 (C-3), 182.4 (C-4), 161.8 (C-5), 99.8 (C-6), 163.1 (C-7), 95.1 (C-8), 157.3 (C-9), 105.8 (C-10), 121.4 (C-1′), 128.9 (C-2′/C-6′), 116.4 (C-3′/C-5′), 161.1 (C-4′), 99.9 (C-1″), 73.3 (C-2″), 76.6 (C-3″), 70.3 (C-4″), 74.2 (C-5″), 63.8 (C-6″), 114.1 (C-α), 145.3 (C-β), 125.3 (C-1‴), 130.6 (C-2‴/C-6‴), 116.1 (C-3‴/C-5‴), 160.2 (C-4‴), 166.9 (C═O), HRESIMS: m/z 601.1328 (calcd for $C_{30}H_{26}O_{12}$ Na) (Itokawa et al., 1981). Acid hydrolysis of **2** produced the aglycone moiety and D-glucose.

*3.1.3. Isoscutellarein-7-O-(6‴-O-acetyl-β-D-allopyranosyl-(1 → 2)-β-D-glucopyranoside) (**3**)*

Yellow powder. UV (λ_{max} in MeOH): gives bands at 308, 326, 277 nm for band I and II, addition of NaOH; 384 and 275, NaOAc; 309, 388 and 276, H_3BO_3; 309, 327 and 276, $AlCl_3$; 322, 345, 421 and 283 while HCl; 322, 343, 420 and 283. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 1.81 (3H, s, OCH_3), 4.87 (1H, d, J = 8.0 Hz, H-1‴), 5.01 (1H, d, J = 7.5 Hz, H-1″), 6.63 (1H, s, H-6), 6.76 (1H, s, H-3), 6.89 (2H, d, J = 8.8 Hz H-3′/H-5′), 7.95 (2H, d, J = 8.8 Hz H-2′/H-6′), 12.29 (1H, s, OH-5). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 164.1 (C-2), 102.6 (C-3), 182.3 (C-4), 152.2 (C-5), 99.4 (C-6), 150.5 (C-7), 127.5 (C-8), 143.7 (C-9), 105.5 (C-10), 121.1 (C-1′), 128.6 (C-2′/C-6′), 115.9 (C-3′/C-5′), 161.3 (C-4′), 100.0 (C-1″), 82.5 (C-2″), 77.1 (C-3″), 69.2 (C-4″), 75.5 (C-5″), 60.5 (C-6″), 102.5 (C-1‴), 70.7 (C-2‴), 77.1 (C-3‴), 66.8 (C-4‴), 71.5 (C-5‴), 63.5 (C-6‴), 20.4 (CH_3 acetyl), 170.3 (C═O), HRESIMS: m/z 675.1547 (calcd for $C_{29}H_{32}O_{17}$ Na) (Lenherr and Mabry, 1987). Acid hydrolysis of **3** produced isoscutellarein, D-glucose and D-allose.

3.2. Antioxidant activity and phenol composition

The antioxidant and free radical scavenging powers of phenolic compounds have been extensively studied for the past 10 years. The roles of OH groups and π electron delocalization in flavonoids have been largely discussed (Rice-Evans et al., 1996).

Many plants which contain flavonoids are known to possess good antioxidant activities by comparison with reference molecules such as vitamin E.

In this study, it was first speculated that *S. mialhesi* de Noé might be a source of natural antioxidants. The performed test confirmed the ability of the BESM and compound (**3**) to scavenge DPPH radical.

The idea that this activity was due to the presence of phenolic compounds led us to investigate the phenolic composition of the BESM.

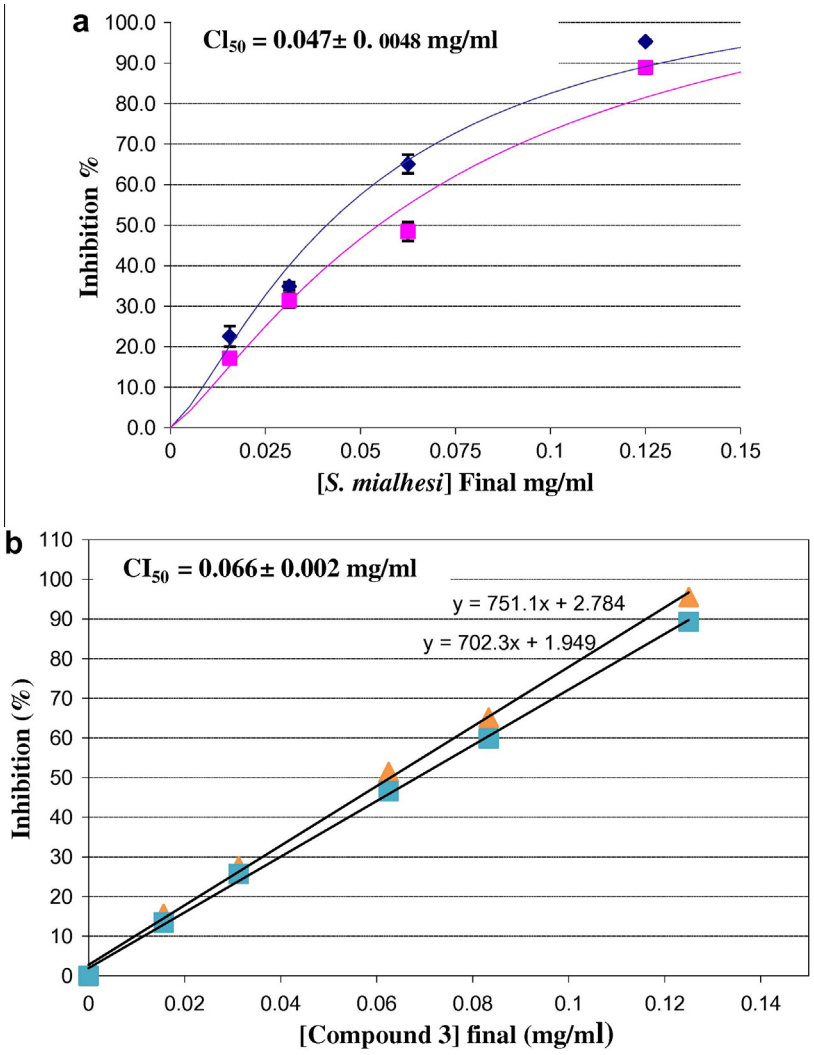

Figure 2 (a) IC_{50} of BESM. (b) IC_{50} of compound (**3**).

Fig. 2a,b.shows the concentration of DPPH which gave 50% inhibition (IC_{50}) of BESM and compound (**3**). By comparison, the value of IC_{50} of vitamin E was, under the same experimental conditions, 0.025 mg/ml. Quercetin IC_{50} values were used as a reference (Rice-Evans et al., 1996). In the DPPH test, quercetin IC_{50} was 0.012 mg/ml. The extract IC_{50} (0.047 mg/ml) was the result of a mixture of molecules, with or without antioxidant activity. The IC_{50} value 0.066 mg/ml was the result of a pure molecule (**3**) (Table 1).

3.3. Acute toxicity

The result of the acute toxicity for BESM showed that the tested extract assayed up to 10 g/kg did not prove to be toxic since it did not induce mortality or toxic manifestations in mice up to 24 h after administration.

3.4. Antinociceptive effect

The results (Table 2) demonstrate that the BESM, when administered orally at different doses (10,000, 5000 mg/kg), caused an inhibition of the writhing response induced by acetic acid (i.p.) ($p < 0.05$). The significant protective effect was dose-dependent. The percentage inhibitions of writhing produced by the extract were 77.11, 58.22, respectively, for each dose. The standard drug acetylsalicylic acid (200 mg/kg), exhibited about 72.99 inhibitions.

Thus, the results obtained from the antinociceptive effect of the BESM revealed the existence of a peripheral analgesic property (Lee et al., 1994).

The acetic acid induced abdominal constriction method elucidated peripheral activity. The BESM was shown to possess antinociceptive activity at the tested doses. This was evident in all the nociceptive models, which indicates that it possesses

Table 1 Phenolic composition and IC_{50} DPPH of the BESM and compound (**3**).

	Phenolic composition (g/100 g equiv. pyrogallol)	IC_{50} DPPH (mg/ml)
BESM	17.9 ± 0.1	0.047 ± 0.0048
Compound (**3**)		0.066 ± 0.002

Table 2 Effect of the BESM on acetic acid induced abdominal constriction in mice.

Animal groups	Dose (mg/kg)	Number of writhing X′ ± s.e.m[b]	% Protection
Control	–	24.33[a] ± 1.86	–
Acetylsalicylic acid	200	6.57[*] ± 0.87	72.99
n-Butanol extract	5000	10.17[*,a] ± 0.87	58.22
n-Butanol extract	10,000	5.57[*] ± 1.23	77.11

[*] Significantly different from control value at $p < 0.05$.
[a] Significantly different from acetylsalicylic acid (200 mg/kg) value at $p < 0.05$.
[b] Each value represents the mean (number of writhing) ± s.e. of the number of animals in each group ($n = 6$).

Table 3 Anti-inflammatory activity of the BESM in Carrageenan induced rat's paw edema.

Animal groups	Dose (mg/kg)	% increase in weight of paw edema (g) X′ ± s.e.m[b]	% Protection
Control	–	71.94[a] ± 3.28	–
Indomethacin	5	11.97[*] ± 3.23	83.36
BESM	5000	34.51[*,a] ± 4.4	52.03

The results demonstrate that the BESM can play a significant role in the inhibition of pain and inflammatory processes.
[*] Significantly different from control value at $p < 0.05$.
[a] Significantly different from indomethacin (5 mg/kg) value at $p < 0.05$.
[b] Each value represents the mean (increase in rat's paw edema) ± s.e. of the number of animals in each group ($n = 6$).

both central and peripherally mediated activities (Vongtau et al., 2004).

3.5. Effects of the BESM on carrageenan-induced paw edema

Oral injections of BESM (5000 mg/kg) decreased the carrageenan-induced inflammation significantly ($p < 0.05$).

The results presented in Table 3 indicated that BESM exhibited a significant anti-inflammatory activity, where it significantly decreased the weight of edema: 52% induced by carrageenan in the rat's paw (Table 3) using indomethacin (5 mg/kg) as a reference drug.

Carrageenan-induced inflammation is a suitable method for evaluation of the anti-inflammatory effects of the agents (Winter et al., 1962). The inflammation consists of two phases. It appears that the early phase is related to the production of histamine, 5-hydroxytryptamin, bradykinins and cyclooxygenase products, while the delayed phase has been linked to neutrophil infiltration, as well as to the continuing of the production of arachidonic metabolites (Salvemini et al., 1996; Boughton-Smith et al., 1999). BESM was capable of attenuating both early and delayed phases of carrageenan-induced inflammation. The extract in the higher dose failed to inhibit the paw edema associated with the second phase.

Compared to a standard non-steroidal anti-inflammatory drug (NSAID), indometacin, the BESM decreased licking response similar to high doses of indomethacin.

Flavonoids were considered to be the active components responsible for the biological actions of the genus *Stachys* (El-Ansari et al., 1991).

Anti-ulcer activity. The BESM was found to be devoid of ulcerogenicity at the tested dose level (5000 mg/kg body weight) compared to indomethacin (5 mg/kg body weight, ulcer index 21.6) used as reference drug (Makhlouf et al., 2002).

In the *n*-butanol-induced stress gastric ulcer test in rats, it was shown that the tested extract produced significant dose-dependent gastroprotective activity.

In conclusion, this study has shown that the BESM exhibited significant antioxidant, antinociceptive and anti-inflammatory effects in laboratory animals. We can also conclude that *S. mialhesi* de Noé is a natural source of isoscutellarein 7-*O*-(2″-*O*-6‴-*O*-acetyl-β-D-allopyranosyl-β-D-glucopyranoside

Acknowledgments

The authors are grateful to the ANDRS and DG-RSDT/MESRS for the financial support.

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