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Biebersteinia orphanidis Boiss. Shows antioxidant and anti-inflammatory activity

N.Miceli[a], M.F. Taviano[a], O. Tzakou[b], A. Yannitsaros[c], D. Vassiliades[d], D. Giuffrida[e], E.M. Galati[a,*]

[a] *Pharmaco-Biological Department, School of Pharmacy, University of Messina, Vill. SS. Annunziata, 98168 Messina, Italy*
[b] *Department of Pharmacognosy and Chemistry of Natural Products, School of Pharmacy, University of Athens, Panepistimiopolis 15771 Athens, Greece*
[c] *Institute of Systematic Botany, Department of Biology, University of Athens, Panepistimiopolis, 157 84 Athens, Greece*
[d] *24 Issiodou Street, Gr-106 74 Athens, Greece*
[e] *Department of Organic and Biological Chemistry, University of Messina, Vill. S. Agata, 98166 Messina, Italy*

Corresponding author Tel.: +090-3533112; Fax: +090- 3533142
Correspondence: emgalati@unime.it (E.M. Galati).

Abstract:
Leaves of *Biebersteinia orphanidis* Boiss., the only representative of the monotypic family *Biebersteiniaceae* found in Europe, were subjected to biological and phytochemical studies. The leaf methanolic extract showed a moderate toxicity on brine shrimp (LC_{50} =1666.6 µg/ml). The radical scavenging activity of *B. orphanidis* methanolic extract was assayed by the DPPH method (IC_{50}=22.75 µg/ml). Moreover, the anti-inflammatory activity of the extract (50 mg/kg i.p.) in the carrageenan-induced paw oedema in rat was studied. In this experimental model an inhibition of paw oedema, significant from first to fifth hour, was observed; the maximum effect was at the first hour (70.3%). The anti-inflammatory activity of the extract can be due to its inhibitory effects both on release of mediators, as serotonin, histamine and kinins, and on cyclooxygenase activity. By HPLC analysis the cinnamic acids, caffeic, ferulic and *p*-coumaric were identified. The total phenolic content of the extract, evaluated by the Folin-Ciocalteau method, was 42 mg/g. The anti-inflammatory effect of *B. orphanidis* methanolic extract could be due to the synergistic action both of flavonoids and cinnamic acids, and it could be related to the free radical scavenging activity of these polyphenol compounds towards oxygen reactive species.

Keywords: *Biebersteinia orphanidis Boiss.*, Polyphenols, Cinnamic acids, Anti-inflammatory activity, DPPH test.

1. INTRODUCTION

Biebersteinia Stephan is a small, predominantly Asiatic genus of five rare, herbaceous species of plants, distributed in the mountainous semi-arid areas of central and western Asia and the eastern Mediterranean as far as Greece (1). *Biebersteinia orphanidis* Boiss. is the only representative of the genus found in Europe, first discovered in 1851 by Th. Orphanidis in the northern Peloponnesus, afterwards was not seen again in Greece until rediscovered 143 years later (2). This genus was traditionally included in the *Geraniaceae* family, but questions have frequently been raised as to its systematic placement (3, 4, 5). A recent phylogenetic analysis of *rbc*L and *atp*B gene sequences of *B. orphanidis* indicated that the genus was affiliated to the Sapindales (6). The results of an investigation of the $C_{16:3}/C_{18:3}$ fatty acid balance in *B. orphanidis* was in agreement with the previous findings of the DNA analysis (7). Phytochemically the genus has not been analyzed in detail. Leaf surface extracts of *B. orphanidis* have yielded a complex mixture of five flavones with the unusual 5,7-dihydroxy-6,8-dimethoxy A ring substitution pattern, along with gardenin B, luteolin, apigenin, acacetin and the coumarin umbelliferone (8). The internal leaf flavonoids include the 7-glucosides of apigenin, luteolin and tricetin, together with the 7-rutinosides of apigenin and luteolin (8).

Davis (9) refers that the fruits of *B. orphanidis* are used for making a quite palatable "coffee". In Greece the plant is well known to certain shepherds because of the healing properties they attribute to it. They use especially the rhizome to treat sick sheep and probably other livestock, but they use it as a medicine for themselves (10). All the other species of the genus are used medicinally by local people. The Tibetan species *B. heterostemon* has been found to have hypotensive and immunity-stimulating effects, among other properties (11). *B. odora* Stephan is used by locals in the Himalayas for 60 to 70 different cures (10). The root of *B. multifida*, an Iranian native plant, has been used topically for the treatment of musculoskeletal disorders as a folk medicine. The anti-inflammatory and analgesic effects of the root extract were studied using carrageenan-induced oedema and formalin tests (12).

In order to give a scientific base to the empiric use of some species of *Biebersteinia* genus, and to characterize the active principles responsible for biological activities, we studied the antioxidant and anti-inflammatory activities of *B. orphanidis* leaf methanolic extract, and we carried out further phytochemical analysis.

2. EXPERIMENTAL

2.1. Plant material

Biebersteinia orphanidis Boiss. is a perennial herb, glandular pubescent throughout, with stem erect (35-50 cm). The leaves, up to 35 cm, are alternate, petiolate, and pinnate. The flowers are actinomorphic, grouped in terminal spikes. The fruit is composed of five mericarps (6 x 4 mm) rugose, dark brown (13) (Fig. 1).
Mature leaves were collected from living specimens of B. orphanidis on Mt Killini (N. Peloponnisos, Greece) at an altitude of 1500 m, in May 1998 (weather in May, at this locality, is cool, sunny, with occasional rains or showers; humidity is averagebriefly) by D. Vassiliades; a voucher specimen of the collection (V. 133) has been deposited in the Herbarium of the University of Athens (ATHU).
For our experiments we used leaves of *B. orphanidis* picked during the flowering stage.

2.2. Extraction

Air-dried leaves (50 g) were powdered and extracted with 80% methanol for 2 days in dark to prevent active principles alteration. The extract was filtered and 100 ml of petroleum ether was added to obtain two phases: the light one, green, containing the chlorophyll, and the bottom one, yellow-brown, containing the polyphenol compounds. The extraction was carried out up to complete chlorophyll elimination, until the petroleum ether was colourless. The phase containing the polyphenol compounds was isolated and concentrated to dryness *in vacuum*. The yield was 16.57% w/w.

2.3. Cinnamic acids identification

An aliquot of methanolic extract was submitted to thin-layer chromatography (TLC) using a mixture of chloroform-methanol-acetic acid (90:10:1) as developing solvent (14). The chromatograms were developed at room temperature (20 °C).
Compounds were identified by comparison of their retention times (R_F) with those corresponding to pure standards. Caffeic acid, ferulic acid, and *p*-coumaric acid were identified. The plates were sprayed with two different reagents: ferric chloride reagent and vanillin-sulfuric acid reagent. Ferric chloride reagent was prepared dissolving 1 g anhydrous ferric chloride in 100 ml methanol (15). Ferulic acid and *p*-coumaric acid gave orange spots; caffeic acid gave a bluish-green spot. Vanillin-sulfuric acid reagent was prepared by dissolving 0.5 g vanillin in 100 ml sulphuric acid-ethanol (40:10) (16). The plates were observed after heating at 120 °C for 20 min. Ferulic acid and *p*-coumaric acid gave purple spots.
Cinnamic acids were also identified by high performance liquid chromatography (HPLC) using a Shimadzu (Italy) HPLC apparatus, equipped with two LC-10AD-Vp pumps, an SCL-10A-Vp system controller, a GT-154 on line degassing device, a Rheodyne injector with 5 µl loop, a BER 1000 P oven, an SPD-M10A Vp diode array detector, and a Digital venturis FX-2 computer. A Discovery C18 (5 µm, particle size) column (15 cm x 2.1mm) fitted with a guard column (5 µm particle size; 2 cm x 4.6 mm) was used. The mobile phase consisted of two eluents: water (W) and acetonitrile (A), adjusted to pH 3.0 with formic acid. To achieve separation, flow rate was set at 0.8 ml/min and the following gradient was used: initial 90% W and 10% A; 20 min linear change to 70% W and 30% A; 20 min linear change to 5% W and 95% A; maintained for 5 min; 5 min linear change to 90% W and 10% A; maintained for 10 min for re-equilibration at initial eluent composition before a new injection. The column temperature was set at 30 °C. Detection was at 323 nm and spectrograms were recorded between 200 and 410 nm at the apex of each peak.
Pure standards of caffeic acid, ferulic acid, and *p*-coumaric acid were purchased from Extrasynthese (Genay, France). Compounds in the samples were identified by comparison of retention times and UV-spectra for each peak with the corresponding standards.

2.4. Determination of total phenolic content

The total phenolic content of *B. orphanidis* methanolic extract was evaluated by the Folin-Ciocalteau method (17). 100 µl of the sample, opportunely diluted, were added to 200 µl of Folin-Ciocalteau solution. After 3 min, 2 ml of water and 1 ml of sodium carbonate (15%) were added; then, the test tubes were shaken and allowed to incubate in darkness for 2 hours. The assay was carried out in triplicate. A blue coloration was developed, and the absorbance was read at 765 nm. Results were expressed as mg of gallic acid equivalent/g methanolic extract.

2.5. Radical scavenging activity on DPPH radical

Free radical scavenging activity of *B. orphanidis* methanolic extract was determined using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method (18). An aliquot (0.5 ml) of methanolic solution containing different amounts of *B. orphanidis* extract was added to 3 ml of freshly prepared methanolic DPPH (Sigma-Aldrich S.r.l., Italy) solution (0.1 mM); the maximum concentration of the extract employed was 100 µg/ml. The optical density change at 517 nm was measured 10 min later by a spectrophotometer (Shimadzu UV-1601, Italy). The antioxidant assay was carried out in triplicate and the readings were averaged.
The scavenging activity was measured as the decrease in absorbance of the samples versus DPPH standard solution. Results were expressed as "percentage inhibition" of the DPPH and "mean inhibiting concentration" (IC_{50}) of the DPPH. IC_{50} parameter is defined as the concentration, in µg/ml, of substrate required to inhibit DPPH radical formation by 50% and it was calculated by using the Litchfield and Wilcoxon test (19).

2.6. Biological assay

2.6.1. LC_{50} determination

LC_{50} determination was carried out according to the method of Meyer et al. (20). *Artemia salina* L. (Artemiidae), the brine shrimp larva, is an invertebrate used in the alternative test to determine toxicity of chemical and natural products. For the determination, 50 mg of extract were dissolved in propylene glycol (1 ml), then diluted in 4 ml of brine (33 g sea salt per litre distilled water). Different amounts of the solution (5, 50, and 500 µl) were transferred to vials to correspond to 10, 100 and 1000 µg/ml final concentrations of methanolic extract. Ten brine shrimp larvae, taken 48 h after initiation of hatching in brine, were added to each vial, and the final volume of each vial was adjusted to 5

ml using the artificial seawater; the control group was exposed to 5:1 brine:propylene glycol. The assay was carried out in triplicate. After 24 h of incubation at 25-28 °C, the vials were observed using a magnifying glass and the number of survivors in each vial were counted and noted.
LC_{50} was determined from 24 hours counts using the probit analysis method described by Finney (21).

2.6.2. Anti-inflammatory activity

2.6.2.1. Animals

Adult male Wistar rats (180-200 g) were used in the experiment. They were kept in standardized conditions (temperature 22 ± 2 °C; humidity 60 ± 4%; natural lighting), fed with a standard diet and water was provided *ad libitum*. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192), as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).
The rats, divided into three groups of ten animals each, were kept fasting 18 hours before the experiment.

2.6.2.2. Carrageenan-induced paw oedema in rats

B. orphanidis anti-inflammatory activity was evaluated according to the method described by Winter et al. (22). Animals were divided into three groups of ten rats each:

- Group I received by intraperitoneal route (i.p.) *B. orphanidis* methanolic extract, at the dose of 50 mg/kg, dissolved in propylene glycol in a volume of 0.25 ml/100 g body weight
- Group II was treated by gavage with indomethacin, as reference drug, at the dose of 5 mg/kg, suspended in 0.5% carboxymethyl cellulose in a volume of 1 ml/100 g body weight
- Control group (III) received only the vehicle (propylene glycol).

One hour after these administrations, each rat received a sub plantar injection of 0.05 ml of 1% λ Carrageenan type IV suspension (Sigma-Aldrich S.r.l., Italy) in its right hind limb. Paw volume was measured by a water plethysmometer (Ugo Basile 7150, Italy), before the treatment, and 1, 2, 3, 4 and 5 hours after carrageenan injection. The increase in volume was taken as the volume of oedema and was determined for each rat. The percentage of oedema inhibition in treated animals versus control was calculated (23).
Data were expressed as mean ± S.E. of 10 determinations; the results were statistically analyzed by Student's "t" test; $P < 0.01$ versus control was taken as significant.

3. RESULTS

3.1. Cinnamic acids identification
By HPLC analysis caffeic acid (R_F 11.499), ferulic acid (R_F 18.027), and *p*-coumaric acid (R_F 22.539) were identified (Fig.2).

3.2. Determination of total phenolic content
The total phenolic content of *B. orphanidis* methanolic extract, evaluated by the Folin-Ciocalteau method (17), was 42 mg/g extract.

3.3. Radical scavenging activity on DPPH radical
B. orphanidis methanolic extract showed, at the concentrations assayed, radical scavenging effects on DPPH radical. Actually, it inhibited DPPH formation by 50% at a concentration of 22.75 µg/ml (IC_{50}) (Table 1).
Table 1 : Radical scavenging effects of *B. orphanidis* methanolic extract on DPPH radical.

Dose (µg/ml)	DPPH percentage inhibition*
100	93.18 ± 3.18
50	86.24 ± 3.24
20	43.95 ± 3.95
10	33.80 ± 3.80

*Mean of three determination ± S.D..

3.4. Biological assay

3.4.1. LC_{50} determination
In the brine shrimp assay (20), *B. orphanidis* methanolic extract showed a moderate toxicity; in fact LC_{50} was 1666.6 µg/ml.

3.4.2. Carrageenan-induced paw oedema in rats

In the experimental model of Winter et al. (1962) (22), methanolic extract, at the dose assayed, showed a marked anti-inflammatory activity. The inhibition was significant from first to fifth hour; the maximum effect (70.3 %) observed was at the first hour (Table 2).

4. DISCUSSION

From the results it is evident that *Biebersteinia orphanidis* methanolic extract displays a low toxicity in the *Artemia salina* test. This method provides a simple and inexpensive toxicity test, because the toxicity of a drug against *Artemia salina* is correlated with its cytotoxic activity. (24). Clearly, methanolic extract active principles are not cytotoxic, since LC_{50} is > 1000 µg/ml.
The results of this study indicate that *B. orphanidis* methanolic extract exhibits radical scavenging activity in DPPH test. Phytochemical analysis revealed that polyphenols are the typical active principles of this plant. Actually the plant has high content in polyphenols and contains, in addition to flavonoids (8), cinnamic acid derivatives. All these compounds possess high antioxidant activity (25).

Moreover the extract showed anti-inflammatory activity in the carrageenan-induced paw oedema test; this experimental model is commonly used for evaluation of anti-inflammatory activity of drugs. The paw oedema, induced by sub-plantar injection of carrageenan, is a biphasic event: the first phase, observed around 1 hour, involves the release of serotonin, histamine and kinins; the second phase is mediated by prostaglandins (26). The oedema formation is probably due to the stimulation of the synthesis and/or release of these mediators, especially the cyclooxygenase (COX) products. In this experimental model, *B. orphanidis* methanolic extract exhibited significant anti-inflammatory activity from first to fifth hour after

carrageenan injection. Indomethacin, a COX inhibitor, at a dose of 5 mg/kg, provoked a marked reduction of oedema from third to fifth hour. Therefore, the anti-inflammatory activity of the extract is probably due to its inhibitory effects both on release of mediators and on COX activity.

Bibliographic data report the anti-inflammatory activity of polyphenols (27). Flavonoids can inhibit several stages of the inflammatory process, such as the increase in capillary permeability, granulation tissue formation, leukotriene production, and they can influence the stimulating activity of mast cells, lymphocytes and platelets (28). The phenolic acids could act synergistically to polyphenols (29). The anti-inflammatory activity of the related coumaric, caffeic, and ferulic acids has been evaluated on different mechanisms involved in the inflammatory process (30). Fernàndez et al. (31) report a moderate anti-inflammatory effect on carrageenan-induced oedema of phenolic acids. The cinnamic acid derivatives seem to inhibit the enzymatic activity of 5-lipooxygenase and COX (32).

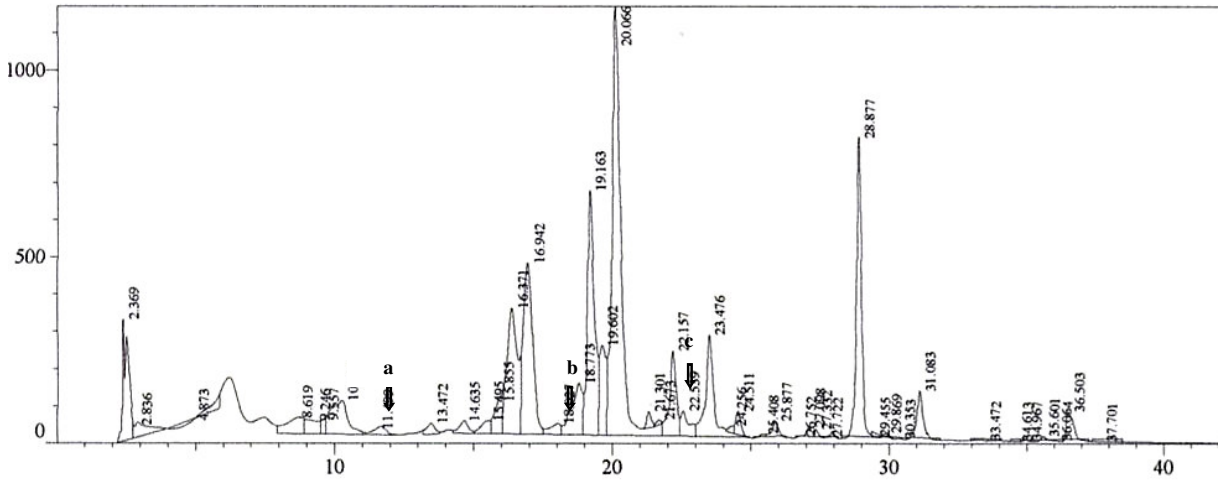
Besides an inflammatory stimulus increases highly reactive oxygen products by neutrophils and the polyphenols inhibit the enzymes responsible for free radicals production (33) and show a direct interaction with superoxide ion (34). Therefore the anti-inflammatory effect of *B. orphanidis* methanolic extract could depend upon the scavenging properties of the polyphenols towards reactive oxygen species (35).

Table 2: Effect of *B. orphanidis* leaf methanolic extract on the carrageenan-induced paw oedema in rat

Treatment	***Dose*** (mg/kg)	Percentages of oedema inhibition (mean ± S.E.)				
		1 h	2 h	3 h	4 h	5 h
MeOH extract (i.p.)	50	70.5 ± 0.1*	69.2 ± 1.2*	62.0 ± 0.5*	51.3 ± 0.1*	36.2 ± 0.9*
Indomethacin (os)	5	16.0 ± 0.8	11.3 ± 0.5	53.9 ± 0.5*	50.1 ± 1.0*	34.0 ± 0.4*

Values are expressed as mean ± S.E.M (n = 10) * P < 0.01 compared with the control group; Student's "t"- test.

Fig. 2 HPLC profile at 323 nm of *B. orphanidis* leaf methanolic extract. (a) Ferulic acid (R_F 18.027); (b) caffeic acid (R_F 11.499); and (c) *p*-coumaric acid (R_F 22.539).



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  Grand Hall Olympia London (UK)
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- **June, 29 – 30, 2005**
  The Royal Norfolk Show
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- **August 23-26, 2005**
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- **August 21-25, 2005**
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- **September 11-15, 2005**
  Medicine and Health in the Tropics.
  Marseille, France
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- **November 30 - December 4, 2005**
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