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Bolax gummifera: Toxicity against *Artemia* sp. of Bornyl and iso-Bornyl Esters

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Abstract

The bioaactivity-guided fractionation of a KB cells cytotoxic methanolic extract of *Bolax gummifera* using the *Artemia* sp.(brine shrimp) toxicity test (ATT), resulted in the isolation of racemic *p*-coumarate (1) and ferulate (2) of borneol as the active components. In order to study structure-activity relationships, optical active *p*-coumarates and ferulates of bornyl and isobornyl alcohols previously synthesised were tested in the ATT. The results show that the (+) isomers are more active that the (-) ones, and that the metoxil group in position 3 does not affect the toxicity against the brine shrimp.

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Introduction

Bolax gummifera (Lam.) Sprengel is a cushion plant that grows in rocky areas of the Argentine and Chilean Patagonia and in the Falkland Islands. It is called "balsam bog" because of the fragrant smell of the white gum which exudes from the aerial parts. The inhabitants of the Falkland Islands, mainly from the countryside, apply the crushed aerial parts of *B. gummifera* for treating external wounds. Some previous results showed that the methanolic extract of *B. gummifera* show to *Pseudomonas aeruginosa* and *Staphylococcus aureus* growth inhibition, and red cells membrane stabilizing activity (Mongelli *et al*, 1997). Since the treatment of skin disorders can be considered when searching for antitumoral compounds, because they reflect diseases states bearing some relevance to cancer symptoms (Cordell *et al*., 1991), the cytotoxicity of this plant was studied. The studies indicated cytotoxicity of the extract against KB cells (IC_{50}=32 μg/ml; SD=9 μg/ml) (Mongelli *et al.*, 2000). Due to the good correlation between cytotoxicity against KB cells and toxicity to the brine shrimp (McLaughlin, 1992), the *Artemia* sp. toxicity test (ATT) was used for isolating the active compounds. Furthermore, in order to begin structure-activity studies, several synthetic isomeric compounds were analysed with ATT.

Experimental

General procedures

Melting points were measured in a Kofler hot stage microscope. Optical rotations were obtained on a Perkin-Elmer 341 MC polarimeter. UV spectra were measured on a UNICAM Helios- spectrometer, in methanolic solution. IR spectra were measured on a Bomem MB-100 FT spectrometer. The EI-MS spectra were acquired using VG TS-250 or Fissons MD-800/GC-800 instruments. The ^1H, ^{13}C, DEPT and 2D-NMR spectra (HMQC, HMBC, COSY) spectra were recorded on a Bruker WP 200SY or Advance 400DRX in $CDCl_3$ solution using TMS as internal reference. TLC were carried out on silica gel Merck 60 plates (Ref. 1.09385) and the spots were detected spraying with a 10% H_2SO_4/EtOH solution followed by heating. Column chromatographies were run on silica gel Merck 60 (0.063-0.2 mm, ref 1.05554) .

Plant material

Bolax gummifera, was collected in the Falkland Islands. A voucher specimen (Mongelli 602) is deposited at the Museo de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

Artemia sp. toxicity test (ATT)

The ATT was performed according standard procedures (Solís *et al.*, 1993). The LC_{50} values were determined in μg/ml, using the Finney Probit (Finney, 1971) analysis computer program. Berberine chloride (Sigma) was used as positive control (LC_{50}=8.4 μg/ml)

Extraction and identification

The air-dried and powdered plant material (200 g) from the aerial parts of *Bolax gummifera* were soaked in *n*-hexane twice for three days at room temp. The extract was concentrated (8.3 g, fraction H), the remaining plant material was then soaked in *n*-hexane/diethyl ether 1:1 three times for three days which gave after solvent evaporation a residue (11g, fraction E). This last fraction was solved in diethyl ether and extracted with a 10% solution of $NaHCO_3$. The aqueous phase was acidulated with 2M HCl and extracted with diethyl ether, the organic layer dried and concentrated to left an acidic residue (200mg, fraction SA). The remaining ethereal solution after bicarbonate extraction was extracted now with 6% NaOH, the aqueous phase was neutralized, extracted with ether, concentrated and worked as usual to give a neutral fraction (9.7 g, faction N) and a low acidic fraction (1.1 g, fraction WA).

The plant residue was left in acetone three times for three days (11g, fraction K) and lastly the residue was extracted with methanol, which on solvent evaporation afforded a syrup (4.3g, fraction M). The activity was concentrated in the AD and AF fractions which were purified by chromatography on silica gel using n-hexane/ethyl acetate mixtures as the eluent. The main components of these factions were isolated and identified as the esters (±) **1** and (±) **2**.

Bornyl p-coumarate (±)**1**: *Rf*: 0.3 (95:5 Benzene/ethyl acetate). mp: 143 °C (*n*-hexane/CH_2Cl_2). U.V. (MeOH) $\lambda_{máx}$ = 211, 228, 315 nm. IR. (KBr) ν cm^{-1}): 3268, 2957, 2878, 1674, 1628, 1605, 1586, 1514, 1454, 1379, 1339, 1277, 1194, 1173, 1024, 970, 835. ^1H NMR (ppm): 7.65 (d, J=15.9 Hz, 1H, H_3); 7.45 (d, J=8.6 Hz, 2H, H-2'/H-6'); 6.89 (d, J=8.6 Hz, 2H, H-3'/H-5'); 6.35 (d, J=15.9 Hz, 1H, H-2); 5.00 (ddd, J=9.9, 3.4, 2.0 Hz, 1H, H-2"); 2.45-2.35 (m, J=13.8, 9.9, 4.5, 3.4 Hz, 1H, H-3"b); 2.10-2.00 (m, 1H, H-6"b); 1.80-1.73 (m, 1H, H5"b); 1.70 (t, J=4.5 Hz, 1H, H-4"); 1.40-1.30 (m, 1H, H6"a); 1.30-1.20 (m, 1H, H5"a5"a), 1.00-1.10 (dd, J=13.8, 3.4 Hz, 1H, H-3"a); 0.93 (s, 3H, H-8"); 0.89 (s, 3H, H-9"); 0.87 (s, 3H, H-10"). ^{13}C NMR: 168.53 (C-1); 158.35 (C-4'), 144.64 (C-3); 129.99 (C-2'/C-6); 126.95 (C-1'), 115.99 (C-3'/C-5'); 115.72 (C-2), 80.37 (C-2"); 49.00 (C-7"); 47.86 (C-1"); 45.04 (C-4"); 36.815 (C-3"); 27.28 (C-6"); 28.07 (C-5) 18.82 (C-8"); 19.69 (C-9"); 13.50 (C-10"). EI-MS: *m/z* (rel.int.) = 300 (M^+, $C_{19}H_{24}O_3$)(3); 164(4); 147(100); 119(20); 91(43); 65(32); 55(47).

Bornyl ferulate (±) **2**: R_f: 0.4 (95:5 Benzene/ethyl acetate). Oily. IR ν(cm^{-1}): 3405, 2955, 2878, 1699, 1634,

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1603, 1516, 1454, 1429, 1385, 1267, 1177, 1123, 1036, 982, 847, 818, 758, 665. 1H NMR δ(ppm): 7.55 (d, J= 15.9 Hz, 1H, H3); 7.10 (dd, J=8.1,1.8 Hz, 1H, H6'); 7.08 (d, J=1.8 Hz, 1H, H2'); 6.90 (d, J=8.1 HZ, 1H, H5'); 6.35 (d, J=15.9 Hz, 1H, H2); 5.00 (ddd, 1H, H2") 2.40-2.30 (m, 1H, H3"b); 2.20-2.00 (m, 1H), H6"b; 1.90-1.75 (m, 1H, H5"b); 1.70 (t, 1H, H4"); 1.45-1.25 (m, 2H); 1.10-1.00 (dd, 1H, H3"a); 0.94 (s, 3H, H8"); 0.89 (s, 3H, H9"); 0.88 (s, 3H, H10"). ^{13}C NMR δ (ppm): 168.76 (C1), 147.97 (C4'); 146.88 (C3'); 144.24 (C3); 127.30 (C1'); 123.02 (C6'); 116.41 (C); 114.79 (C2); 109.57; 79.87 (C2''); 56.06 (C10'); 49.03 (C7''); 47.91 (C1''); 45.13 (C4''); 36.94 (C3''); 28.15 (C5"); 27.37 (C6"); 18.91 (C8"); 19.76 (C9"); 13.53 (C10"). EI-MS: *m/z* (rel.int.) = 330 (M+ $C_{20}H_{26}O_4$); 194(8); 177(18); 119(12); 84(100); 69(12).

Results and Discussion

In order to identify the active components present in the cytotoxic methanolic extract of *Bolax gummifera* the ATT was employed to bioguided the phytochemical isolation. The LC_{50} obtained for the methanolic crude extract was 142 μg/ml (95 % confidence interval=238-87μg/ml). The sequential extraction of the plant material with solvents of increasing polarity was monitored by the ATT to check the toxicity. The 1:1 hexane/diethyl ether extract showed the highest activity and was further fractionated with $NaHCO_3$ (SA fraction) and NaOH (WA fraction). The activity was localized in these basic extracts, which were chromatographed on silica gel. The main components **1** and **2** showed to be more active than the crude fractions and were identified by spectroscopic methods as (±) bornyl *p*-coumarate (**1**) and (±) bornyl ferulate (**2**), respectively (Figure 1), by comparison with published spectral data (Suire *et al*., 1982 y Zschocke *et al*., 1997).

The biological behavior of optically active substances can be different and ferulates of bornyl and isobornyl alcohols previously synthesised were tested in the ATT. from the racemic mixture (Eliel *et al.*, 1994). For this reason, the optical active *p*-coumarates The chemical structure of the isobornyl esters can be observed in figure 2.

The synthetic esters evaluated were: (±) bornyl *p*-coumarate (±**1**), (-) bornyl *p*-coumarate (-**1**), (+) bornyl *p*-coumarate (+**1**), (±) bornyl ferulate (±**2**), (-) bornyl ferulate (-**2**), (+) bornyl ferulate (+**2**), (±) isobornyl *p*-coumarate (±**3**), (-) isobornyl *p*-coumarate (-**3**), (+) isobornyl *p*-coumarate (+**3**), and (±) isobornyl ferulate (±**4**), (-) isobornyl ferulate (-**4**), (+) isobornyl ferulate (+**4**). The results are showed in Table 2.

No activity differences can be observed between the compounds (**1**) and (**2**), or the compounds (**3**) and (**4**), suggesting that the metoxyl group do not change the toxicity. The results obtained by mixing the (+) and (-) isomers were similar to those observed for the natural racemic mixtures. On the other hand, the results show that the (+) isomers are more active that the (-) ones.

Studies are in progress to determine if these structure-activity relationships are the same for the cytotoxicity against the KB cells.

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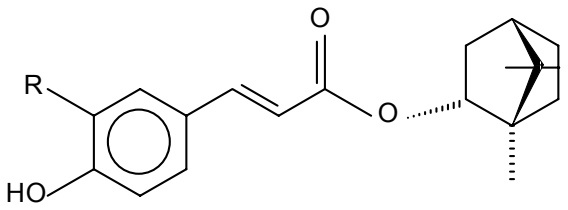
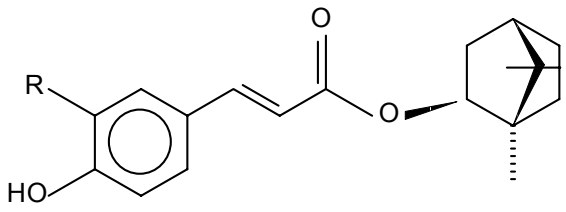
Table 1. Results of the ATT

COMPOUND	LC_{50} (95 % confidence interval) (μg/ml)
(±) **1**	14.10 (15.50-0.20)
(+) **1**	1.60 (5.30-0.10)
(–) **1**	6.40 (11.40-1.50)
(±) **2**	17.20 (33.20-8.30)
(+) **2**	5.60 (9.90-1.40)
(–) **2**	25.00 (63.00-0.80)
(±) **3**	13.00 (19.20-2.10)
(+) **3**	3.40 (12.00-0.90)
(–) **3**	12.10 (17.20-0.80)
(±) **4**	16.00 (19.00-2.70)
(+) **4**	2.20 (4.2-0.9)
(–) **4**	13.00 (20.00-8.00)



1: R= H **2**: R= OMe

Figure 1. Chemical structure of bornyl *p*-coumarate (**1**) and bornyl ferulate (**2**)



3: R= H **4**: R= OMe

Figure 2. Chemical structure of isobornyl *p*-coumarate (**3**) and isobornyl ferulate (**4**)