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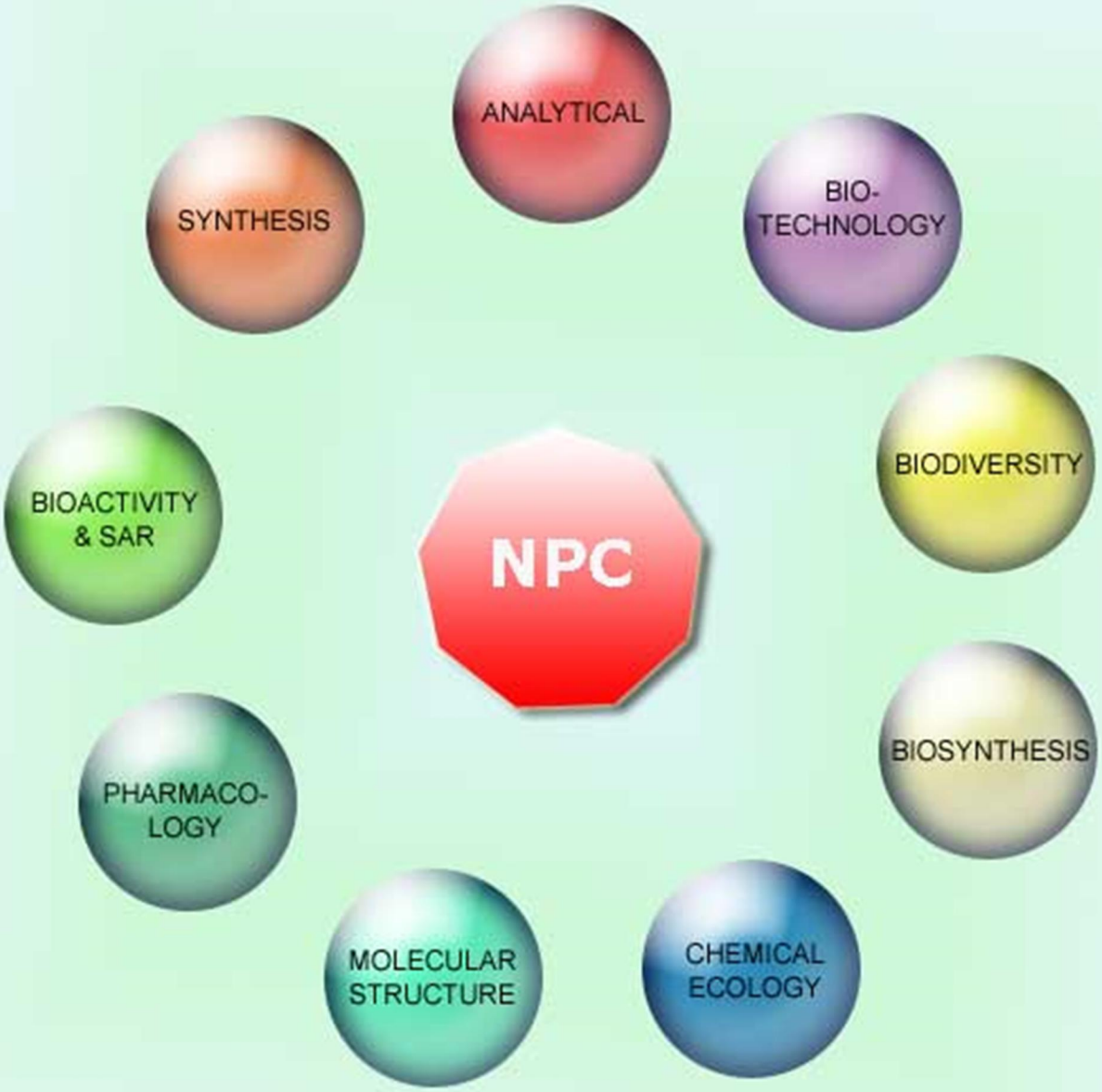

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Isolation, Identification and Usefulness of Antifungal Compounds from *Zuccagnia punctata* for Control of Toxigenic Ear Rot Pathogens

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Infusion, tincture and decoction of leaves of *Zuccagnia punctata* Cav. were assayed on growth of *Fusarium verticillioides*, *F. graminearum sensu stricto*, *F. boothii*, *F. meridionale*, *F. subglutinans* and *F. thapsinum*. The tincture showed the lowest IC_{50} on mycelial growth. A diethyl ether fraction of the tincture showed the highest antifungal activity in microdilution assays on *F. verticillioides* and *F. graminearum*. The antifungal constituents were separated by silica gel chromatography and identified as 2′,4′-dihydroxychalcone, 2′,4′-dihydroxy-3′-methoxychalcone and 7-hydroxy-3′,4′-dimethoxyflavone. These chalcones had the lowest MIC and MFC values on *F. verticillioides* and *F. graminearum sensu stricto*. 2′,4′-Dihydroxychalcone was mildly toxic and the remaining identified compounds were non-toxic in the brine shrimp assay. 2′,4′-Dihydroxychalcone in mixtures with commercial food preservatives showed additive effects on *F. graminearum sensu stricto* and synergistic ones on *F. verticillioides*. 2′,4′-Dihydroxy-3′-methoxychalcone showed synergistic effects in mixtures. Our results suggest that addition of chalcones to food preservatives allows reduction in the doses of the preservatives required for control of *Fusarium* species.

Keywords: Chalcones, Food preservatives, *Fusarium*, *Zuccagnia punctata*.

Several species of *Fusarium* cause ear rot diseases in cereals from Argentina [1].They reduce cereal yields and contaminate the grains with mycotoxins noxious to human and animal health. Fumonisins and trichothecenes are among the most common mycotoxins contaminating cereal grains [2]. Exposure to fumonisins produced by *F. verticillioides* has been associated with several diseases in animals including leucoencephalomalacia in equines, pulmonary edema in swine, liver cancer in rats, and immunosuppression in poultry. Epidemiological studies suggest that fumonisins increase the incidence of human esophageal cancer in Africa, Brazil, China, and Italy [3]. Deoxynivalenol (DON), a type B trichothecene produced by *F. graminearum sensu stricto*, is the predominant mycotoxin in small grain cereals. Animal intake of DON is associated with feed refusal, vomiting and suppressed immune functions [4].This mycotoxin is also acutely phytotoxic and acts as a virulence factor on cereal hosts [5].Several public institutions such as the FAO/WHO Expert Committee on Food Additives, the U.S. Food and Drug Administration and the European Commission, have established maximum permissible contents for fumonisins and DON in cereals and derived products for human intake [6a-6b]. These contents can be overpassed in outbreak years of ear rot disease restricting exportation of Argentinian cereal and increasing the mycotoxigenic risk for humans and animals.

There is currently no single robust control measure to manage either the ear rot disease caused by *Fusarium* or the concomitant mycotoxin presence. *Fusarium* proliferates when the grains are not efficiently dried after harvest. To avoid this, grains are often treated with preservatives such as propionic and sorbic acids and their salts [7]. These compounds can control, to some extent, the growth of *Fusarium* species. Nevertheless, they are fungistatic and special care needs to be taken in order to guarantee the efficient coverage of the grains [8]. Factors such as adsorption, hydrolysis, oxidation, and pH level of the substrate, reduce the active concentration of these preservatives. In addition, insufficient application of these compounds may enable fungal growth. Thus, alternative compounds or additives to the current preservatives are necessary to improve the control of the toxigenic *Fusarium* species.

Zuccagnia punctata Cav. (Fabaceae) is a monotypic species endemic to western Argentina, commonly known as jarilla pispito, puspus and jarilla macho [9]. This plant is used in traditional medicine to treat bacterial and fungal infections [10], and showed antifungal activity on phytopathogenic fungi [11]. The bioactive constituents of *Z. punctata* include polyphenolic compounds such as flavanones, flavones, chalcones and caffeoylesters [12]. In this work, leaf extracts of *Z. punctata* were assayed using *Fusarium* species. Some of the antifungal compounds isolated were identified and assayed as additives to food preservatives. The tincture of aerial parts of *Z. punctata* showed the highest inhibition of mycelial growth of the *Fusarium* species, with IC_{50} values between 120 (80-230) and 330 (280-450) µg/mL (Table 1). The tincture was not able to kill completely the *Fusarium* mycelia, yet at 1.6 mg dry matter/mL, a situation also observed on wood-rot causing basidiomycetes *Ganoderma applanatum*, *Lenzites elegans*, *Pycnoporus sanguineus* and *Schizophyllum commune*, and the phytopathogenic fungi *F. oxysporum*, *Penicillium notatum*, *Trichoderma* spp and *Aspergillus niger* [13]. Microdilution assays indicated that spore germination and early hyphal growth of the *Fusarium* species was more sensitive to the tincture constituents than mycelial growth. The tincture showed MIC and MFC values between 15-150 µg dry matter/mL and 25-150 µg dry matter/mL, respectively (Table 2). In agreement with our results, MIC values of the tincture were reported in the range 100-500 µg dry matter/mL for the soybean pathogens *Phomopsis longicolla*, *Alternaria alternata*, *F. equiseti*, *Colletotrichum truncatum*, *Sclerotium bataticola* and *F. graminearum* [11]. The tincture and its ethereal fraction had a fungicidal effect rather than a fungistatic one with MFC/MIC ratios near to 1. Antifungal activity was absent in the remaining tincture fractions (EAc and Me).

Table 1: The IC_{50} for hyphal growth obtained for infusions, decoctions and tincture of *Zuccagnia punctata* on *Fusarium* species. Upper and lower confidence limits are presented into brackets.

	IC_{50} in mg of dry matter/mL		
Fungal species	Infusion	Decoction	Tincture
F. subglutinans	1.44 (1.31-1.52)	1.25 (1.18-1.31)	0.29 (0.21-0.34)
F. thapsinum	>1.60	1.08 (0.84-1.15)	0.32 (0.19-0.30)
F. boothii	>1.60	>1.60	0.33 (0.28-0.45)
F. meridionale	>1.60	>1.60	0.29 (0.18-0.33)
F. graminearum	>1.60	>1.60	0.27 (0.15-0.35)
F. verticillioides	>1.60	1.10 (0.95-1.20)	0.12 (0.08-0.23)

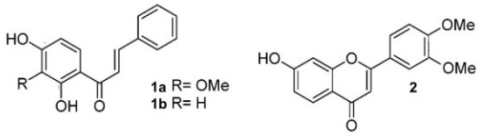

Figure 1: Compounds isolated from the etheral fraction of the tincture of *Zuccagnia punctata*: (**1a**) 2′,4′-dihydroxy-3-methoxychalcone, (**1b**) 2′,4′-dihydroxychalcone, (**2**) 7-hydroxy-3′,4′-dimethoxyflavone.

The ethereal fraction showed brown bands at 366 nm in pools G2 (Rf = 0.45, compound **1a**), G3 (Rf = 0.45, compound **1a**; 0.35, compound **1b**), G4 (Rf = 0.35, compound **1b**) and G5 (Rf = 0.22, compound **2**). These bands inhibited the growth of both *F. verticillioides* and *F. graminearum*. A first HPLC gradient separated compound **1b** (Rt =36.7 min) and compound **1a** (Rt = 38.8 min). Constituents of G5 were separated with a second HPLC gradient, obtaining compound **2** (Rt = 20.2 min). Compound **1a** was identified as 2′,4′-dihydroxy-3′-methoxychalcone, compound **1b** as 2′,4′-dihydroxychalcone, and compound **2** as 7-hydroxy-3′,4′-dimethoxyflavone (Figure 1). 2′,4′-Dihydroxychalcone showed the lowest MIC and MFC values against *F. graminearum* and *F. verticillioides* (Table 3). Compounds **1a** and **1b** were previously reported for leaves of *Z. punctata* [12]. 7- Hydroxy-3′,4′-dimethoxyflavone is reported for the first time. The identified chalcones are natural constituents of food, beverages and propolis, where they are usually found in mg/kg [14]. 2′,4′-Dihydroxychalcone was mildly toxic (LC_{50} 30-100 µg/mL) in the brine shrimp assay, while the remaining identified compounds were non-toxic (LC_{50} >150 µg/mL). In agreement with these results, previous reports indicated that compound **2** is neither genotoxic nor mutagenic, and showed cytoprotective properties on the gastroduodenal tract in rats [15a-15b]. Regarding the commercial food preservatives, sodium sorbate and calcium propionate were non-toxic to brine shrimps (LC_{50} > 2000 µg/mL) while sodium benzoate was mildly toxic (LC_{50} = 50 µg/mL). The antifungal activity of the identified phenolic compounds on the toxigenic *Fusarium* species was several folds lower than that observed for the triazol epoxyconazole and the strobilurin pyraclostrobin, both fungicides often used at cereal flowering for the control of *Fusarium*.

Several phenolic compounds with either low or no biological activity have synergistic antifungal effects in mixture with biocides or biostatic compounds [16]. To check this possibility, the identified chalcones were assayed in mixtures with the food preservatives sodium benzoate, calcium propionate and sodium sorbate. When assayed alone, the inhibitory activity of the phenolic compounds was about 2 folds lower than that of the food preservatives on *F. graminearum* and *F. verticillioides* (Table 4).The FICI values of the mixtures containing 2′,4′-dihydroxychalcone indicated an additive interaction on *F. graminearum sensu stricto* and a synergistic one on *F. verticillioides*. The mixtures containing 2′,4′-dihydroxy-3′-methoxychalcone showed synergistic interactions on both *Fusarium* species. The dominance of synergistic interactions suggests that the chalcones exert their antifungal action by a mechanism different from that of the food preservatives. Sorbates, propionates and benzoates enter the fungal cells in undissociated form and accumulate in the cytosol, generating a disbalance in cellular homeostasis and affecting the activity of several endogenous enzymes [17]. The action mechanism of chalcones is unknown [18], but it was shown that they do not disrupt the fungal membranes up to 4xMFC and do not act by inhibiting the fungal cell wall. Although the fungitoxicity of the chalcones was lower than that of the synthetic fungicides and food preservatives, these phenolic compounds appear to be promising as additives of food preservatives. Lower doses of these xenobiotics were required for control of the *Fusarium* species when they were assayed in mixtures with the chalcones. Further *in vivo* assays are required in order to check the antifungal potential of the mixtures.

Table 2: Minimum inhibitory concentration of fungal growth (MIC) and minimum fungicidal concentration (MFC) obtained on *Fusarium* species for the tincture of *Zuccagnia punctata* and its ethereal fraction.

Fungal Species	Pyraclostrobin µg/mL		Epoxiconazole µg/mL		Tincture µg/mL		Ethereal fraction µg/mL	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
F. verticillioides	25	50	2.5	5	100	100	100	100
F. subglutinans	25	50	25	25	100	100	100	100
F. bothii	50	50	25	25	150	150	250	250
F. meridionale	20	80	4	8	80	80	100	250
F. graminearum sensu stricto	20	40	4	8	15	25	25	50

Table 3: Minimum inhibitory concentration of fungal growth (MIC) and minimum fungicidal concentration (MFC) of fungicides and phenolic compounds identified in the ethereal fraction of the tincture of *Zuccagnia punctata*.

	Fusarium verticillioides		*Fusarium graminearum sensu stricto*	
	MIC µg/mL	MFC µg/mL	MIC µg/mL	MFC µg/mL
Pyraclostrobin	25	50	20	40
Epoxyconazole	2.5	5	8	25
2′,4′-Dihydroxychalcone	50	50	25	25
2′,4′-Dihydroxy-3′-methoxychalcone	100	100	50	50
7-Hydroxy-3′,4′-dimethoxyflavone	400	550	250	425

Table 4: Joint action of chalcones and food preservatives on growth of *Fusarium verticillioides* and *F. graminearum.*

Compounds assayed	FICI[1]	
	Fusarium graminearum sensu stricto	*Fusarium verticillioides*
2′,4′-dihydroxychalcone		
+ sodium benzoate	0.75	0.31
+ calcium propionate	0.75	0.50
+sodium sorbate	0.75	0.38
2′,4′-dihydroxy-3′-methoxy chalcone		
+ sodium benzoate	0.15	0.05
+ calcium propionate	0.08	0.08
+ sodium sorbate	0.06	0.04

[1]Interpretation of FICI: ≤ 0.5, synergy; 0.5-4.0, no interaction; > 4.0, antagonism

Experimental

Microorganisms: Strains of ear rot pathogens from north and central Argentina (*F. verticillioides*, *F. subglutinans*, *F. bothii*, *F. meridionale*, *F. graminearum sensu stricto* and *F. thapsinum*) were provided by the Laboratory of Biology of Bioactive Agents and Phytopathogens (LABIFITO – National University of Tucuman).

Plant material and preparation of extracts: Aerial parts of *Zuccagnia punctata* Cav collected in February-March 2010 in Tafí del Valle (Tucuman, Argentina) were identified by comparison with voucher specimens deposited at the Herbarium of Fundación Miguel Lillo (Argentina). They were air dried and powdered in a Wiley mill. A portion of the powder (10 g) was extracted by boiling in 100 mL of distilled water (decoction), by adding to 100 mL of boiling water (infusion), and by shaking with 100 mL 96% ethanol

for 7 days at 37°C. The extracts were filtered, and freeze dried. Tinctures were vacuum dried at 30°C. The dry residues, dissolved in methanol, were stored in the dark at 4°C.

Hyphal radial growth assay: Dilutions (1–1.6 mg dry matter/mL) of each methanolic extract were prepared in 96% ethanol [14]. An aliquot of 500 µL of each dilution was incorporated into 4.5 mL of molten malt extract-peptone (MEP) medium (15g/L malt extract,5g/L peptone, 20g/L sucrose), vigorously vortexed, and dispensed into 10 cm diameter Petri dishes. Each Petri dish was inoculated in the center with a plug (3 mm diameter) taken from a fresh culture of *Fusarium*. Growth controls contained MEP medium with 10% 96% ethanol. The Petri dishes were incubated for 5 days at 25°C. Then, 2 diameters of each fungal colony were measured at right angles to each other and averaged. Percentage of growth inhibition was [(average diameter of fungal colony in the growth control – average diameter of fungal colony exposed to an extract concentration) x 100]/average diameter of fungal colony in the growth control. The concentration required to inhibit 50% fungal growth (IC_{50}) was calculated by Probit analysis [19] using XLSTAT Version 7.5.2 (Addinsoft, USA).

Separation of antifungal constituents: Dry tincture (300 g) was successively extracted with diethyl ether, ethyl acetate and methanol. The organic fractions were vacuum dried at 30°C, dissolved in methanol and identified as EEt, EAc and Me, respectively. They were tested by the microdilution method. Dry EEt (800 mg dry matter) was loaded onto a column (20 x 3 cm) packed with 36 g of silica gel (0.04-0.063 mm), which was eluted with 80 mL of *n*-hexane, and 80 mL of *n*-hexane/ethyl acetate (6:1.7 v/v). Fractions of 1 mL each were collected and analyzed by TLC on silica gel with *n*-hexane/ethyl acetate (6:1,7 v/v) as mobile phase. The chromatograms were visualized under UV light (365 nm) and the fractions were grouped into 5 pools (G1-5),evaporated to dryness and dissolved in methanol. The pools were bioautographed on *F. graminearum* and *F. verticillioides*. A volume of 20 µL of pools G2-4 was injected into a Gilson HPLC equipped with a C18 column (5 µm, 250 x 4,6 mm). Elution was followed at 254 nm and the flow rate was 0.7 mL/min. Solvents A (0.1% formic acid in water) and B (20 % solvent A + 80 % acetonitrile) were used in the following program: 40% to 60% B in 15 min, hold 60% B for 15 min, 60% to 80% B in 10 min, and hold 80% B for 5 min. Components of pool G5 were separated with the gradient: 30% to 60% B in 5 min, 60% to 80% B in 10 min, hold 80% B for 5 min, 80% to 100% B in 10 min. The peaks containing the antifungal constituents were recovered by using a semi-preparative C18 column (5 µm, 250 x 10 mm ID), with a loop of 500 µL, a flow rate of 3 mL/min, and absorbance at 254 nm. The collected peaks were evaporated to dryness and dissolved in methanol. Then, the absorbance spectra (200-600 nm) were recorded in a Beckman DU650 spectrophotometer and compared with bibliographic data [20]. The IR spectra, in KBr pellets, were recorded on a Fourier-transform infrared spectrophotometer (Shimadzhu FTIR-8201 PC). The spectral data were compared with those obtained from 2′,4′-dihydroxychalcone (IndoFine, USA), 2′,4′-dihydroxy-3′-dimethoxychalcone, and 7-hydroxy-3′,4′-dimethoxyflavone, kindly provided by Dr Rossi-Bergmann (Federal University of Río de Janeiro) and Dr Akareshov (Eurasian National University, Kazakhstan), respectively. The dry residues recovered by semi-preparative HPLC were dissolved in ethyl acetate (1 mg of dry matter/mL) and injected into a GC Shimadzu equipped with a SPB-1 (100 % dimethylpolixylosane) capillary column (28.5 m x 0.25 µm ID, 0.25 µm film thickness). Helium was the mobile phase; flow 1 mL/min; split 1/10; injector temperature 250ºC. Temperature program was 50°C (3 min), 50-280°C (2 min), and 280-310°C (6 min). The GC was coupled to a single quadrupole mass detector (EI 70 eV, ion source at 230°C and interface at 300°C. The constituents were identified by matching their mass spectra with those stored in the Wiley/NIST database and also with published data [21a-21b].

Microdilution assays: The antifungal activity of the tincture, tincture fractions, its constituents, and the xenobiotics was evaluated by the microdilution method [22]. Petri dishes containing 7 day-fungal colonies grown in Spezieller Nährstoffarmer Agar were washed with 2 mL of 0.9% sterile saline solution. The working suspension was prepared by diluting to 2×10^4 CFU/mL in RPMI 1640 medium. The assays were performed in 96-well-microplates. In each well, 100 µL of working suspension was added to 100 µL of each dilution of the tincture, tincture fraction, or its identified constituents prepared in the same medium with final concentrations between 500 and 5 µg of dry matter/mL of medium, with 1% (v/v) of methanol. Growth controls contained 100 µL of RPMI 1640 medium with 1% methanol instead of a dilution. The mentioned fungicides and food preservatives were assayed at final concentrations ranging from 100 to 0.5 µg/mL of medium in 1% methanol. Each concentration of the extract fractions, as well as the positive and negative controls, were replicated 4 times, and the experiments were repeated twice. The microplates were incubated for 72 h at 25°C. Then, the minimum concentration required to inhibit 100% fungal growth (MIC) was visually determined. Aliquots of 10 µL from wells corresponding to the MIC and higher concentrations were placed in Petri dishes containing MEP medium and incubated for 72 h at 25ºC. The lowest concentration of antifungal compound yielding no growth in the MEP medium was considered the minimum fungicidal concentration (MFC).

Bioautographic method: The ethereal fractions and the pools G1-5 were spotted onto TLC plates of silica gel. Then, they were separated in hexane: ethyl acetate (6:1.7, v/v). The chromatograms were left to dry in a laminar flow cabinet. Then, 5 mL of MEP medium, with 0.8% agar containing 10^5 microconidia of *F. verticillioides* or macroconidia of *F. graminearum* were applied to each TLC plate, and incubated for 3 days at 25°C. Absence of growth indicated the presence of antifungal compounds. Each bioautography was performed in triplicate.

Joint effect of chalcones and food preservatives: Compounds **1** and **2** were assayed in mixtures with sodium sorbate, calcium propionate, and sodium benzoate by the chessboard technique and the inhibitory fractionated concentration (FICI) was calculated as: FICI = (Concentration of A in MICA+B/Concentration of A in MICA) + (Concentration of B in MICA+B/Concentration of B). Interpretation of FICI: ≤ 0.5, synergy; 0.5-4.0, no interaction; > 4.0, antagonism [23].

Toxicity assay: Brine shrimp eggs were hatched by incubation in 3.8% NaCl at 25ºC under a lamp (2000 Lux). Ten larvae were placed per well in 24 well-microplates containing sea water and increasing concentrations of the ethereal fraction, its identified constituents or a xenobiotic (15.6 to 1000 µg of dry matter/mL) in a final volume of 1 mL [24]. Controls contained only 1 mL of sea water. Microplates were exposed to the same light at 25°C. After 24 h, survivors were counted and the concentrations required to kill 50% of the brine shrimps (LC_{50}) were estimated by Probit analysis [19]. Interpretation of LC_{50}: <1.0 µg/mL, highly toxic; 1.0-10.0 µg/mL, toxic; 10.0-30.0 µg/mL, moderately toxic; 30-100 µg/mL, mildly toxic; > 100 µg/mL, non-toxic.

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