Artigo

FIRST SECONDARY METABOLITES FROM *Herissantia crispa* L (Brizicky) AND THE TOXICITY ACTIVITY AGAINST *Artemia salina* LEACH.

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FIRST SECONDARY METABOLITES FROM *Herissantia crispa* L (Brizicky) AND THE TOXICITY ACTIVITY AGAINST *Artemia salina* Leach.The phytochemical investigation of *Herissantia crispa* led to the isolation of seven compounds, identified as: sitosterol 3-*O*-β-D-glucopyranoside, stigmasterol 3-*O*-β-D-glucopyranoside, 3,5,7,4'-tetrahydroxyflavone (kaempferol), 3,5,7,3',4'-pentahydroxyflavone (quercetin), unpublished in the genus *Herissantia*, besides β-sitosterol, kaempferol 3-*O*-β-D-(6''-*E*-*p*-coumaroil) (tiliroside) glucopyranoside and kaempferol 3,7-di-*O*-α-L-ramnopyranoside (lespedin), described for the first time in the species. The structural determination of the compounds was made by means of spectroscopy methods such as Infrared Spectroscopy, ^{1}H and ^{13}C Nuclear Magnetic Resonance, with the aid of two dimensional techniques, and by comparison with literature data. The toxicity activity of the MeOH extract and lespedin on *Artemia salina* Leach. was also carried out.

Keywords: Malvaceae; *Herissantia crispa*; *Artemia salina.*

INTRODUCTION

Malvaceae is a widespread family with about 243 genera and 4225 species,[1] particularly distributed in tropical areas.[2] There are many reports in the literature regarding the use of Malvaceae species in traditional medicine. Some of these uses include snakebites,[3] asthma,[4] diuretic, in treatment of rheumatism and gastrointestinal disorders,[5,6] among others. Chemical studies about the secondary metabolites from Malvaceae have described the occurrence of triterpenes,[7] flavonoids,[8,9] essential oils,[10] sesquiterpenelactones[11] and fatty acids.[12-15]

The genus *Herissantia* is constituted by six species, found in the tropical America, particularly in Antilles, Mexico and South America.[16] However, only *Herissantia crispa* is widespread, being reported from The United States to Argentina.[17] In our phytochemical and pharmacological studies with Malvaceae family, we have described the isolation of a great variety of natural compounds from *Herissantia* and other genera. From *Sida galheirensis* Ulbr., we isolated a 3-methoxyflavonol, a phaephorbide a, a coumarin, a benzoic acid derivative, two sterol glucosides (β-sitosterol and stigmasterol), two flavones (luteolin and apigenin) and two flavonol glucosides (tiliroside and luteolin 7-glucoside). The EtOAc and BuOH extracts from *S. galheirensis* showed a good antioxidant activity using the free radical scaveging assay.[18] The species *Bakeridesia pikelli* MONTEIRO provided a non-glycosyl and two glycosyl steroids, vanillic acid and two flavonol glycosides (kaemfperol-3-*O*-β-D-glucopyranoside and tiliroside). The latter compound showed relaxant activity on guinea-pig ileum.[19] From *Herissantia tiubae* (K. Schum) Brizicky four triterpenes (frideline, lupeol, cycloartenol and cycloeucalenol), a steroid (β-sitosterol) and four phenolic compounds (a benzoic acid derivative, a coumarin and three flavonol glycosides, kaempferol 7-*O*-α-L-rhamnopyranoside, lespedin and tiliroside) were isolated. Cycloeucalenol and cycloartenol also showed a relaxant effect in the guinea-pig ileum.[20] The highly oxygenated flavonoid 4′,5-dihydroxy-3,6,7,8,3′-pentamethoxyflavone played a role in modulating the bacterial resistance to some antibiotics.[21] Preliminary studies carried out with lespedin revealed a relaxing activity on superior mesenteric artery, which could be considered as an important result in the cardiovascular field.[9] Based on the above evidences concerning Malvaceae and the genus *Herissantia*, we focused our study on *Herissantia crispa* L. (Brizicky), an unstudied species which is subject of our continuous investigations in discovering potential natural products with biological activities.

Plants have been used for many years as medicines by population, providing good sources of pharmacologically actives substances and improving the therapeutic arsenal. However, many plants are known to be toxic. For this reason, researches are carried out in order to determine their pharmacological action and toxicity.[22]

There is a currently tendency to call for substituting the use of laboratory animals in toxicological tests, due to the high cost and the animal suffering caused by these tests. Alternative methods include procedures that could replace experiments performed with animals, reduce the number of animals used in each test, or refine the existing methodology in order to lessen pain and stress.[22]

Artemia salina L., the brine shrimp, is an invertebrate component of the fauna of saline aquatic and marine ecosystems. It can be used in a laboratory bioassay in order to determine toxicity through the estimation of the medium lethal concentration (LC_{50}). This method has been used in research in order to evaluate toxicity and biological actions as a screening method mainly for products of plant origin. One basic premise here is that toxicology is simply pharmacology at higher dose; thus, if we find toxic compounds, a lower, non-toxic dose might elicit a useful, pharmacological perturbation on a physiologic system.[23]

In the present study we describe the isolation of the first compoun-

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ds from *H. crispa* and also the evaluation of the cytotoxic activity of its MeOH extract and lespedin on *Artemia salina* Leach.

EXPERIMENTAL

General procedures

NMR spectra were measured in $CDCl_3$, CD_3OD and pyridine-d_5 and recorded on a Mercury Varian instrument operating at 200 and 50 MHz for ^1H and ^{13}C, respectively. The solvent signal was used as internal standard. IR spectra were measured on a Perkin-Elmer, FT-IR-1750 spectrometer in KBr pellets. Chromatography columns were carried out on silica gel (Merck) and Sephadex LH-20 (Pharmacia, Upsala-Sweden). TLC were performed on silica gel PF_{254} plates and the spots were visualized under UV light (244 and 366 nm) and by spraying with 5% $FeCl_3$ reagent.

Plant material

The whole plant of *H. crispa* was collected in Pedra da Boca, in the city of Araruna, state of Paraíba, on September 2003. The botanical identification was made by Dr. M. de F. Agra and a voucher specimen was deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), Universidade Federal da Paraíba (Voucher No. 6237).

Extraction and isolation

Dried and powdered whole plant (7 kg) of *H. crispa* was exhaustively extracted with methanol at room temperature. The solvent from the combined extract was evaporated to dryness under reduced pressure to yield 237 g of a dark solid. This residue was suspended in methanol:H_2O (7:3) and successively partitioned with hexane, $CHCl_3$, EtOAc and butanol. The hexane extract (11 g) was submitted to column chromatography using silica gel as stationary phase and eluted with hexane, EtOAc and MeOH with increasing polarity. In this process, fractions of 50 mL were collected. Fractions 70-75 were analysed by TLC, combined and recrystallized with $CHCl_3$, yielding β-sitosterol (**1**) (720 mg). The chloroform extract was also chromatographed on silica gel 60 column using hexane, $CHCl_3$ and MeOH (fractions of 70 mL). After TLC analysis, fractions 120-125 were gathered and then recrystallized with $CHCl_3$:MeOH (1:1), yielding a mixture of sitosterol 3-*O*-β-D-glucopyranoside (**2**) and stigmasterol 3-*O*-β-D-glucopyranoside (**3**) (300 mg). The ethyl acetate extract (4.58 g) was subjected to a Sephadex LH-20 gel column eluted with MeOH (fractions of 15 mL). Fractions 11-18, 19-20 and 21-22 were combined after observation on TLC (SiO_2, $CHCl_3$:MeOH, 5% $FeCl_3$) and led to the isolation of kaempferol 3-*O*-β-D-(6''-*E*-*p*-coumaroil) glucopyranoside (tiliroside) (**4**, 350 mg), 3,5,7,4'-tetrahydroxyflavone (**5**) (80 mg) and 3,5,7,3',4'-pentahydroxyflavone (**6**) (20 mg), respectively. The butanol extract was applied to a Sephadex LH-20 column eluted with MeOH, affording 18 fractions of 15 mL. Pooled fractions 11-16 gave pure kaempferol 3,7-di-*O*-α-L-ramnopyranoside (**7**) (1.93 g) (Figure 1).

Bioassay with *Artemia salina* – Determination of lethal concentration 50% (LD_{50})

Brine Shrimp eggs (*Artemia salina*) were obtained from Labetox-LTF-UFPB. They were hatched in natural seawater and incubated for 24 h, at 28 °C, with continuous side illumination (40 W lamp). The nauplii did not receive food, because they are fed in their yolk-sac in 48 h [24] and the larvae are viable for 36-48 h.[25] After hatching, 10-15 nauplii were collected and put in tubes containing different concentrations of the MeOH extract (MeOHE) or kaempferol 3,7-di-*O*-α-L-raminopyranoside (lespedin) (five dilutions, 10-1000 μg/mL). Three replications were used for each concentration and the experiment was repeated twice. A parallel series of tests with the blank control was always conducted. The method used was that of McLaughlin.[26] The lethal concentration for 50% mortality after 24 h of exposure (LC_{50}) was determined using the probit method. As the measure of toxicity of the extract, LC_{50} value lower than 1000 μg/mL is considered bioactive.[27]

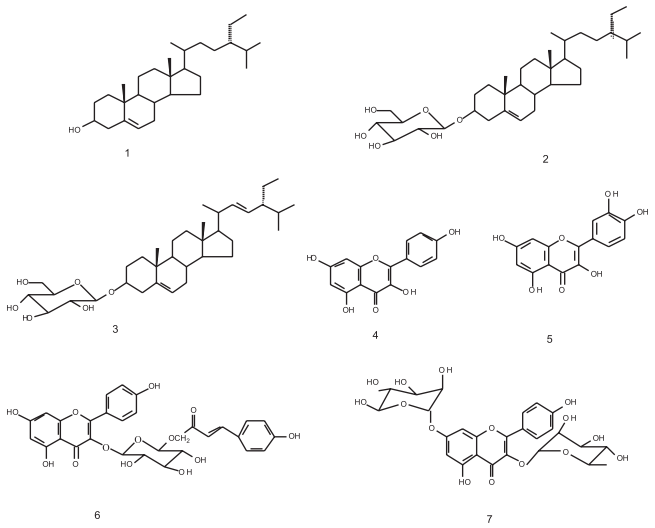

Figure 1. *Chemical constituents isolated from H. crispa*

3,7-di-*O*-α-L-ramnopyranoside (lespedin) (**7**): IR (KBr) $\bar{\nu}_{max}$ 3374 cm^{-1} 2981 e 2937 cm^{-1}, 1659 cm^{-1} 1602 and 1449; ^1H NMR [(200 MHz, CD_3OD, δ (ppm), *J* (Hz)]: 7.72 (*d*, *J* = 8.8 Hz, H-2′,6′), 6.90 (*d*, *J* = 8.8 Hz, H-3′,5′), 6.63 (*d*, *J* = 2.1 Hz, H-8), 6.38 (*d*, *J* = 2.1 Hz, H-6), 5.55 (*d*, *J* = 1.6 Hz, H-1″), 5.36 (*d*, *J* = 1.6 Hz, H-1‴), 4.26-3.30 (*m*, H-2″-5″, H-2‴-5‴), 1.26 (*d*, *J* = 5.8 Hz, H-6″), 0.93 (*d*, *J* = 5.6 Hz, H-6‴); [(50 MHz, $CDCl_3$, δ (ppm)]: 179.63 (C-4), 163.41 (C-7), 162.85 (C-5), 161.69 (C-4′), 159.69 (C-9), 157.90 (C-2), 136.39 (C-3), 131.96 (C-2′,6′), 122.28 (C-1′), 116.56 (C-3′-5′), 107.45 (C-10), 103.42 (C-1‴), 100.47 (C-1″), 99.73 (C-6), 95.57 (C-8), 73.54 (C-4″), 73.13 (C-4‴), 72.03 (C-3″, 3‴,5‴), 71.87 (C-2‴), 71.62 (C-2″), 71.22 (C-5″), 18.09 (C-6″), 17.66 (C-6‴).

RESULTS AND DISCUSSION

The structural assignments of compounds **1-7** were made based on the spectral analysis and are in good agreement with those reported in the literature. Thus, their structures were identified as β-sitosterol (**1**),[26] sitosterol 3-*O*-β-D-glucopyranoside (**2**),[28] stigmasterol 3-*O*-β-D-glucopyranoside (**3**),[28] kaempferol 3-*O*-β-D-(6''-*E*-*p*-coumaroil) glucopyranoside (**4**) (tiliroside),[29] 3,5,7,4'-tetrahydroxyflavone (kaempferol) (**5**)[30] and 3,5,7,3',4'-pentahydroxyflavone (quercetin) (**6**).[31] Compounds **2**, **3**, **5** and **6** are being reported here for the first time in the genus *Herissantia*. Compound **4** (tiliroside) was previously isolated from Malvaceae species, for example *Herissantia tiubae*,[8] *Sida galheirensis*[18] and *Bakeridesia pickelli*.[19] The isolation of compound **7** was also reported from the leaves of *H. tiubae*.[9]

In this work, we examined the biological activities of the MeOHE and lespedin on the bioassay of *A. salina.* This bioassay was made twice and the lethal concentration (LC_{50}) was the result of the mean of these two tests. MeOHE and lespedin showed LC_{50}=629.24 μg/mL (Figure 2) and LC_{50} = 611.25 μg/mL (Figure 3); they were considered bioactive due to the fact that their capacity to kill 50% of larvae is lower than 1000 μg/mL. The LC_{50} of lespedin was similar to those

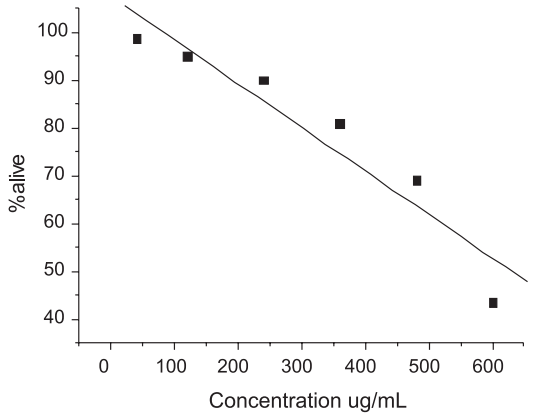

Figure 2. *Effect of MeOHE of H. crispa on bioassay of A. salina*

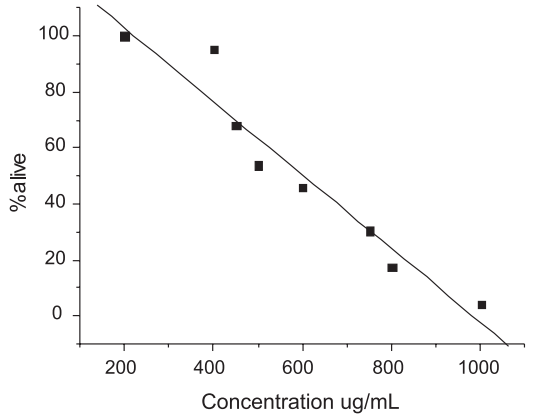

Figure 3. *Effect of lespedin of H. crispa on bioassay of A. salina*

of the MeOHE; then, it is possible that this compound contribute in a broad range to the overall bioactivity of the extract. Further studies on the MeOHE of *H. crispa* will be carried out to better define the spectrum of its biological activity and correlate it to the chemical composition of its extracts.

The result reported here not only provided an insight into the toxic nature of the extract, but also an opportunity for selection of a bioactive extract for initial fractionation and further studies in toxicological and pharmacological assays. Thus, more specific studies will be done to investigate the toxicity of the MeOHE and lespedin of *H. crispa* in animal models.

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