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Phytochemical and biological preliminary study of *Himatanthus drasticus* (Mart.) Plumel (Janaguba).

[1]Colares, A.V., [2]Cordeiro, L.N., [1]Costa, J.G.M., [3]Silveira, E.R., [1]Campos, A.R. and [1]*Cardoso, A.H.

[1]*Departamento de Química Biológica, Campus do Pimenta, Universidade Regional do Cariri, Rua Coronel Antonio Luis, 1161, 63105-000, Crato, CE, Brasil.*

[2]*Laboratório de Microbiologia, Faculdade de Medicina de Juazeiro do Norte, Avenida Tenente Raimundo Rocha, s/n, Juazeiro do Norte, CE, Brasil.*

[3]*Departamento de Química Orgânica e Inorgânica, Campus do Pici, Universidade Federal do Ceará, Avenida Mister Hull s/n, 60455-760, Fortaleza, CE, Brasil.*

***Author for Correspondence:** aherzog@urca.br*

ABSTRACT

Himatanthus drasticus (Apocynaceae), popularly known as Janaguba, is a medicinal plant tree, which latex is used by the population of the Cariri region, south of Ceará State, Brazil, as anticancer, among other medicinal uses. The stem bark of *H. drasticus* was subjected to a phytopharmacological investigation in order to identify its major chemical constituents and to evaluate in preliminary in vitro assays for citotoxic, antimicrobial and antinociceptive activities. One compound was isolated and identified as the triterpene lupeol cinnamate. The ethanolic extract was first evaluated biologically as cytotoxic agent against the brine shrimp lethality test, but showed no antimicrobial effect on the tested pathogens *in vitro*. The extract had a significant antinociceptive effect on the writhing test while showed no effect on the hot-plate test. This is the first detailed phytochemical investigation of *H. drasticus* L. growing in Brazil and elsewhere. The isolated compounds are reported from this plant for the first time and their full ^{1}H and ^{13}C-NMR assignments are included.

KEY WORDS: *Himatanthus drasticus*, Janaguba, lupeol, pharmacological study.

INTRODUCTION

Himatanthus drasticus (Mart.) Plumel (Apocynaceae) *Himatanthus drasticus* (Mart.) Plumel (Apocynaceae) is a medium-sized tree growing on firm ground in the South America region, known as janaguba, tiboma, jasmim-manga, raivosa, pau-de-leite, joanaguba e sucuúba (6). Its stem latex wood is popularly used for the treatment of cancer, among a variety of popular medicinal uses (1). Species belonging to the genus *Himatanthus* have been scarcely mentioned in the chemical literature (2) but there is no report about the specie *Himatanthus drasticus*.

Earlier studies of *Himatanthus articulata* recorded the isolation of the acetate and cinnamate of α-amyrin and of β-amyrin. From *H. phageadaenica* were isolated and identified an acetylated mixture of the triterpenes α-amyrin and lupeol, in addition to sitosterol and the iridoid lactones: plumericin, allamandin and isoplumericin, as well as a mixture of plumieride glucoside and sucrose. A glycosylplumieride has been also isolated from *H. lancifolius* (2).

Previous studies about *Himathantus* species showed antileishmanial and trypanocidal (3), gastroprotective (4), spasmolytic (5), antimicrobial (6), antiproliferative (7), cytotoxic (8) antiinflammatory and analgesic (9) and immunoregulatory activities (10).

Despite the widespread consumption of *Himathantus drasticus* in Ceará state, and since at other Brazilian states, there have been no available reports in literature. In the current study, we therefore analyzed the chemical constituents and the effects of Janaguba extract on citotoxic, antimicrobial and antinociceptive tests.

MATERIAL AND METHODS

Plant material and extract preparation

Himatanthus drasticus stem bark was collected at the D line of the Araripe National Forest (FLONA), south of Ceará State, Brazil, and identified by the botanists Manoel Silva Amaro and Edson de Paula Nunes. A voucher specimen (No. 31685) has been deposited in the Herbarium Prisco Correia of the Federal University of Ceará (Fortaleza, Brazil).

Animals

Experiments were performed in male Swiss mice (20 – 25 g) obtained from the Central Animal House of this University. They were housed at 22 ± 2° C under a 12 h light/12 h dark cycle and had free access to standard pellet diet (purina chow) and tap water. For experiments, the animals were deprived of food for 24 h but allowed free access to water. The experimental protocol was approved by the Animal Care and Use Committee of this University in accordance with the guidelines for Care and Use of Laboratory Animals.

Phytochemical screening

Preparation and precipitation of the extract.

The dried stem bark was pulverized into fine powder and was then percolated by absolute ethanol. The *Himatanthus drasticus* ethanolic extract (HDEE) was then evaporated and concentrated *in vaccuo*, provided a precipitate. Methanol and ethyl acetate was added in order to provide a material denominated JANA1 (Figure 1).

JANA1 Nuclear Magnetic Ressonance.

NMR spectra for JANA 1 were recorded on Brucker DPX-300 (300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts (δ in ppm) are given from internal standard $CHCl_3$ (7.26) for ^{1}H NMR, $CDCl_3$ (77.0) for ^{13}C NMR.

JANA1 Infrared.

Infrared spectra was recorded with an FT-IR spectrophotometer (SPECTRUM1000, Perkin Elmer) using KBr pellets.

JANA1 Gas Chromatography.

Chromatography-mass spectrometry analysis was conducted using an Hewlett-Packard 5971A apparatus coupled to an HP – 5890A II gas chromatograph with an fused silica capillary column (30 mm, 0.20 mm, 0.25 mm).

Biological studies

As we had no large quantities of sufficient pure material, the biological studies were conducted with the ethanolic extract (HDEE). Previous studies about *Himathantus* species showed cytotoxic, antimicrobial and analgesic effects, so these activities were chosen to begin the studies.

Brine shrimp bioassay.

Cytotoxicity was evaluated by the brine shrimp lethality bioassay. Brine shrimp (*Artemia salina*) eggs were placed into the marine water and left to incubate for 48 h at 28 ºC in a small tank. HDEE was tested at 1000, 100 and 10 ppm. Then 20 mg of plant extract was dissolved in 2 ml of chloroform (20 mg/2 ml). From this solution 500, 50 or 5 µl was transferred to vials corresponding to 1000, 500 or 100 ppm, respectively. Vials including DMSO were prepared as controls. After incubation, 10 brine shrimp larvae (nauplii) were introduced into vials containing graded concentrations (ranging from 100 to 1000 ppm) of the HDEE. After 24 h, the number of surviving shrimps at each concentration of the extracts was counted and data analyzed with the Finney computer program to determine the LC_{50} at a 95% confidence interval.

Kirby-Bauer Antimicrobial assay.

Pure cultures were maintained in tryptic agar slants (Difco, prepared by adding 1.5 g per 100 mL tryptic soy broth made by following the instructions on the label), transferred using standard aseptic technique to working plates (tryptic agar or Mueller-Hinton agar) and then into sterile 10 mL tryptic soy broth (Difco, prepared according to label instructions) in screw cap culture vials where they were incubated overnight at 37°C prior to being inoculated by swabbing on tryptic agar test plates. The required number of tryptic agar test plates were prepared and allowed to warm to room temperature. Microorganisms from cultures were inoculated into these plates using sterile cotton swabs, taking care to distribute it as evenly as possible over the agar surface. HDEE was prepared for the assay in the following manner. Sterile blanks (6 mm diameter) made of Whatman #3 filter paper, or prepunched (S and S Sterile blanks) were systematically laid out on a clean aluminum foil in such a manner that each extract was provided with three blanks per microorganism. 10 µL of HDEE were placed on the blanks by first applying 5 µL with the pipette, letting dry, then applying another 5 µL, then drying.

Writhing test.

The abdominal constriction test described was used to measure the antinociceptive actions of HDEE. Mice (n=8/group) were pretreated with oral HDEE (200 or 400 mg/Kg, p.o.) 60 minutes prior to intraperitoneal injection of 0.1mL/10g of 0.6% acetic acid to cause a typical stretching response. Writhings or stretchings (abdominal constrictions) were counted for a period of 20 minutes under a double blind observation. The antinociceptive effect of HDEE was measured by calculating the mean reduction in the number of abdominal constrictions, as compared to gum acacia controls.

Hot plate.

The test was carried out to assess the effects of agents on the thermal nociceptive threshold. Mice were placed on 52.5°C hot plate. Mice (n=8/group) were pretreated with oral HDEE (200 or 400 mg/Kg, p.o.).

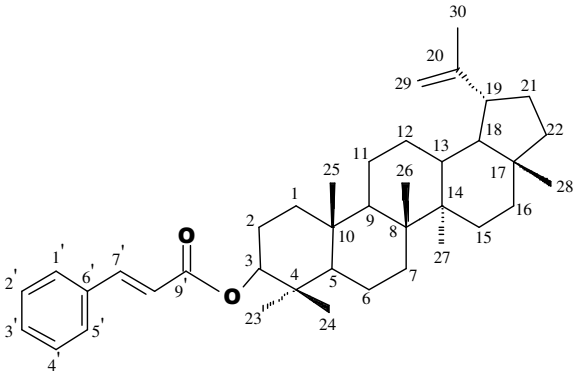

Figure 1. JANA-1.

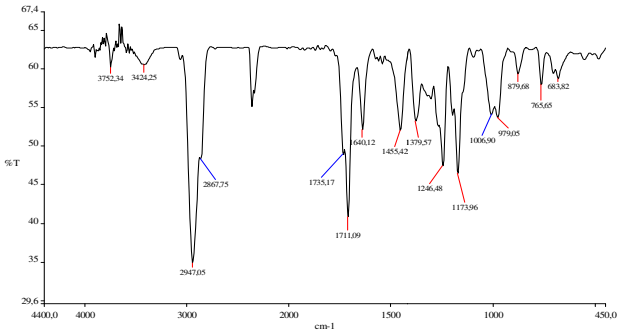

Figure 2. JANA-1 infrared spectra

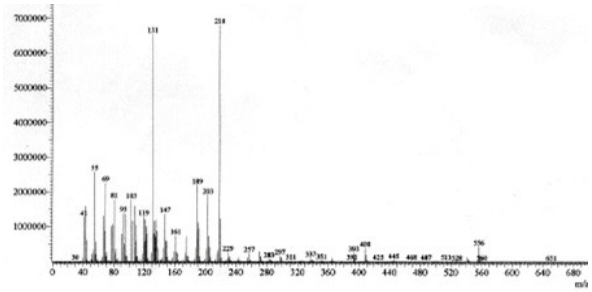

Figure 3. JANA-1 mass spectra.

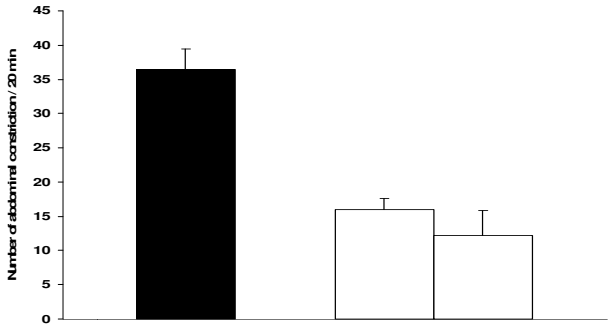

Figure 4. Antinociceptive effect of HDEE on the abdominal constriction test induced bu acetic acid in mice. Values are expressed as mean ± S.D. The number of animal used for each group was 8. * p < 0.05 and ** p < 0.01, ANOVA, Tukey´s test.

The response latency to either a hind-paw lick or jump was recorded (0, 30´, 60´ and 90´) after the treatment. In the absence of a response, the animals were removed from the hot plate at 60 s to avoid tissue injury, and a 60 s latency was assigned as the response.

Statistical analysis

All values are expressed as the mean ± SEM. ANOVA and Student Newman Keul´s tests were used to verify the statistical significance of the differences between groups. Differences were considered to be significant when $p < 0.05$.

RESULTS

Phytochemical screening

JANA1 NMR, IR and GC spectra

From ^{1}H and ^{13}C NMR data spectra of JANA1 we can propose the structure of this isolated substance as a triterpene lupeol cinnamate, showed in Figure 1. Similar finding was previously reported from *Himatanthus phagedaenica* (11). Infrared data (Figure 2) were fundamental for structural determination of JANA1. We can observe an intense absorption at 1711 cm^{-1}, indicating the presence of the carbonilic ester

group and characteristic benzene ring absorption at 1455 – 1356 cm^{-1}.

The mass spectra (Figure 3) showed the base peak at m⁄z 218, and the molecular ion peak at m⁄z 556, corresponding exactly to the molar mass for the proposed molecular formula of lupeol cinnamate.

Biological studies

Brine shrimp bioassay.

HDEE has been tested for their cytotoxic activity by the brine shrimp method and showed a marked significant activity (LC_{50} = 257 ppm).

Kirby-Bauer Antimicrobial assay.

HDEE had no antimicrobial effect on the tested pathogens *in vitro* (*Enterobacter, Streptococcus* and *E.* coli).

Antinociceptive tests.

The extract at both doses tested significantly reduced the number of mouse abdominal constrictions induced by 0.6 % acetic acid solution in a dose dependent manner (Table 1). In hot plate test HDEE showed no significant antinociceptive activity (data not shown).

DISCUSSION

Phytochemical analysis of stem bark ethanolic extract of "janaguba" allowed the identification and isolation of the triterpene lupeol cinammate. Their stem latex wood is popularly used for the treatment of cancer (1). The results of this study showed that the stem bark extract of *Himathantus drasticus* has cytotoxic effect. Taking into account the good correlation between the toxicity on *Artemia salina* with that on tumor cell lines (e.g, KB, P-388, L5178Y and L1210) (12) and that lupeol is an attractive antitumor-promoting agent (13), this result can be a confirmation of the popular use of *Himatanthus drasticus*.

As the *Himathantus* genus is used in popular medicine as an antiinflammatory and analgesic remedy (14), the antinociceptive effect of HDEE was evaluated using peripheral and central tests. HDEE had a significant antinociceptive effect on the writhing test while showed no effect on the hot-plate test. Thus, the extract may not act via central mechanisms. It has also been shown that some plants such as *Hunteria zeylanica* (15) and *Ocotea suaveolens* (16) decrease stretching induced by acid acetic acid but have no effect on heat-induced pain. This mechanism underlying the antinociceptive effect of janaguba can be relationated to the lupeol presence (17, 18).

In addition such results support the traditional use of *Himatanthus drasticus* and suggest that traditional folk medicine, ethnobothanics, could be used as a guide in our continuing bioprospection search for new natural products with potential medicinal properties.

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