RESEARCH ARTICLE

Identified Substances from the Leaves of *Tephrosia cinerea* (Leguminoseae) Crude Extracts and their Phytotoxic Effects

Antonio Jose Cantanhede Filho[1]*, Lourivaldo Silva Santos[2], Giselle Maria Skelding Pinheiro Guilhon[2], Raissa Priscilla Costa Moraes[2], Reinaldo Araujo Dos Santos[2], Antonio Pedro Da Silva Souza Filho[3], Juliana Feitosa Felizzola[3]

[1]Federal Institute of Education Science and Technology of Maranhao, Campus Sao Luis- Monte Castelo, Sao Luis- MA, Brazil

[2]Federal University of Para, Institute of Exact and Natural Sciences, Belem- PA, Brazil

[3]Embrapa Amazonia Oriental, Agroindústria Laboratory, Belem- PA, Brazil

[*]**Address for Correspondence:** Dr. Antonio Jose Cantanhede Filho, Teacher, Department of Natural Products, Federal Institute of Education, Science and Technology of Maranhao, Sao Luis Campus- Monte Castelo, Getúlio Vargas Avenue 04, CEP 65030-005, Sao Luis- MA, Brazil

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ABSTRACT- This research evaluated the phytotoxic effect of the hexane (H.E), ethyl acetate (EtOAc.E) and methanolic (MeOH.E) crude extracts of the *Tephrosia cinerea* leaves on the seed germination of seeds using two weed species, *Mimosa pudica* (*Malícia*) and *Senna obtusifolia* (*Mata-pasto*), as test plants. The compounds were isolated using classic chromatography techniques and the structural elucidation of the compounds was performed by ^{1}H and ^{13}C NMR (1D and 2D) techniques. The ethyl acetate and methanolic extracts of *Tephrosia cinerea* were the most active, as they inhibited the germination of seeds in 92.0% and 81.0% respectively of *malicia* and *mata-pasto*, the ethyl acetate extract inhibited germination by 81.0% and the methanolic extract by 32.0%. The chemical study led to isolation of cinnamic acid and rotenone from the ethyl acetate extract, and mixture containing triacylglycerol and *β*-sitosterol fatty acids from the hexane extract and the disaccharide trehalose from methanolic extract.

Key-words- Crude extracts, Invasive species, Phytotoxicity, Rotenone

INTRODUCTION

Tephrosia cinerea (Leguminoseae/Papilionoideae) is a Brazilian non-endemic native bush that presents two relevant synonyms, *Tephrosia littoralis* (Jacq). Benth. and *Tephrosia villosior* Benth [1]. Commonly known in Brazil as "anil bravo", this plant is narcotic, vermifuge and poisonous, useful against swollen glands, ulcers and nerve disorders [2].

Four substances have been identified from the leaves of *T. cinerea*: Flemichapparin B, Isolonchocarpin, Anhydrolanceolatin-A and Rutin [2]. Methylapollinin 7-*O*-*β*-glucopyranoside, and cineroside (A) have been isolated from the aerials parts of this species [3].

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Weed or invasive species, which often spoil the crop is one of the most important factor to impose limitations to the world agricultural activity development [4]. The known toxic effects of commercial herbicides on human health range from nausea and vomiting to certain kinds of cancer. As for environmental damages, they can accumulate in the biota or contaminate waters and soil, which may lead to ecological imbalance [5].

Natural products based herbicides and agrochemicals are attractive for many reasons. These products are often considered to not cause environmental damages, causes many of them are easily biodegradable and due to the fact they are, at least partially, water-soluble, favoring their absorption by plants, it's possible to use them in smaller amounts, therefore offering less risks to crop rotation [6-8].

In order to investigate extracts and/or substances that may be used as bioherbicides, *T. cinerea* leaves was subject to chemical investigation and phytotoxic effects evaluation, using germination inhibition assays against two weed species, *M. pudica* (*Malicia*) and *S. obtusifolia* (*Mata-pasto*).

MATERIALS AND METHODS

Plant collection and extraction

T. cinerea leaves were collected on June 2011 in an area belonging to the Atico Seabra Herbarium, located at Maranhao Federal University (UFMA). A voucher specimen (#1256) was deposited in the same Herbarium.

Part of the botanical material (780.2 g) was ground using a knife mill after drying for one week in a low humidity environment. The crude extracts were prepared by maceration with organic solvents for seven days with each solvent in the following sequence: hexane (Hex), ethyl acetate (EtOAc) and methanol (MeOH). The solutions were concentrated in a rotary evaporator, giving finally the hexane (9.3 g), ethyl acetate (5.7 g) and methanolic extracts (5.1 g).

Isolation and purification of compounds (1-5)

All three crude extracts were submitted to column chromatography (CC) on silica (70-230 mesh) using hexane/EtOAc and EtOAc/methanol mixtures as eluents in order of increasing polarity. Successive CC procedures were used to purify some fractions. These fractions were analyzed by thin layer chromatography (TLC) in aluminum backed silica gel 60 F254 plates and as revealed in UV light fluorescence chambers (254 and 366 nm) and by a ceric sulphate acid solution, followed by heating.

Fractioning of the ethyl acetate extract (5.7 g) resulted on 58 fractions. Needle-shaped crystals (34.0 mg) precipitated from fractions 10-12, eluted from the column with a Hex- EtOAc 8:2 solutions. These crystals were identified as (E)-cinnamic acid (substance 1). After solvent evaporation, fractions 21–25 (eluted with a Hex-EtOAc 7:3 mixture) were purified through successive washings with a Hex- CH_2Cl_2 1:1solution, at low temperature, giving a syellowish solid, identified as rotenone, substance 2 (24 mg).

The hexane extract (9.3 g) CC fractioning led to 68 fractions. Fractions 4–11, eluted from the column with a Hex - EtOAc 9:1 solution was identified as triacylglycerols, substance 3 (38.8 mg). The major compounds of fractions 12-25 (eluted with Hex - EtOAc 8:2) were fatty esters of *β*-sitosterol (substance 4.12 mg). Fractions 39 and 40, eluted from the column with a Hex-EtOAc 1:1 solution, produced, after precipitation and washing with a Hex-CH_2Cl_2 1:1 mixture, at low temperature, an additional amount of substance 1 (19.0 mg).

The methanolic extract originated 64 fractions after CC fractioning. Fractions 1-4 provided an additional amount of 1 and 2 in a mixture (20.0 mg). A C-18 cartridge filtration a MeOH solution of fractions 18-20 (eluted with a mixture of EtOAc-MeOH result on a mixture of the trehalose disaccharides isomers *α,α*-trehalose, *α,β*-trehalose and *β,β*-trehalose, substance 5 (38 mg).

Nuclear Magnetic Resonance (NMR) Analysis

^1H and ^{13}C (1D e 2D) NMR spectra were obtained from a Varian spectrometer, model MERCURY-300 (300 MHz for ^1H and 75 MHz for ^{13}C), using $CDCl_3$ (1-4) and CD_3OD (5) as solvents. The chemical shifts (δ) were recorded in ppm based on the TMS signal. The experimental data were compared with those found in literature.

Bioassays methodology

Phytotoxic effects assays were performed according to methodologies described in literature [9-11]. *T. cinerea* crude extracts were evaluated (hexane, ethyl acetate, and methanolic extracts). Seeds of the test plants, *M. pudica* and *S. obtusifolia* were collected in the area of Castanhal country in the state of Pará. The seeds went through cleaning process and treated for dormancy break by immersion in sulfuric acid (H_2SO_4) solution for 15 minutes (*M. pudica*) and 20 minutes (*S. obtusifolia*) and then they were dried at room temperature [12].

Seeds germination

A BOD germination chamber was used at the constant temperature of 25ºC and 12 hours photoperiod. Each 9.0 centimeters diameter Petri dish lined with filter paper received 3 ml of each 1% concentrated (m/v) crude extract. After solvent evaporation, the filter paper was moistened with a fungicide aqueous solution (mycostatin-1%), and then were inserted 20 seeds of the recipient plants. The control samples received only the fungicide aqueous solution and considered as germinated seeds, which presented root extension equal to or greater than 2.0 mm. Seeds germination was monitored for a period of 4 days, being the first germination count made 24 hours after assembly of the experiment. Count was realized daily with germinated seeds elimination. The Equation 1 below was used to calculate seeds germination inhibition (I) percentage [13,14].

$$\mathbf{I\ (\%) = [1 - (SG_{sample})] \times 100/SG_{control}} \qquad \mathbf{(1)}$$

Where: SG_{sample} is the number of germinated seeds,
$SG_{control}$ is the number of germinated seeds in the control sample.

For verification of biological effects, experimental lineation was entirely randomized with three repetitions for each one. Bar chart was made in Excel 2013.

RESULTS AND DISCUSSION

Phytotoxic effects of *T. cinerea* crude extracts on Seeds germination

The seeds germination inhibition of each extract (ethyl acetate, hexane and methanolic) on invasive species *M. pudica*, and *S. obtusifolia* are shown in Fig 1. The hexane extract inhibited 48.0% germination of the *M. pudica* while the ethyl acetate and the methanolic extract inhibited 92.0% and 81.0% respectively. The hexane extract inhibited 3.3% germination of the *S. obtusifolia*, and the ethyl acetate and the methanolic extract, 81.0% and 32.2% respectively.

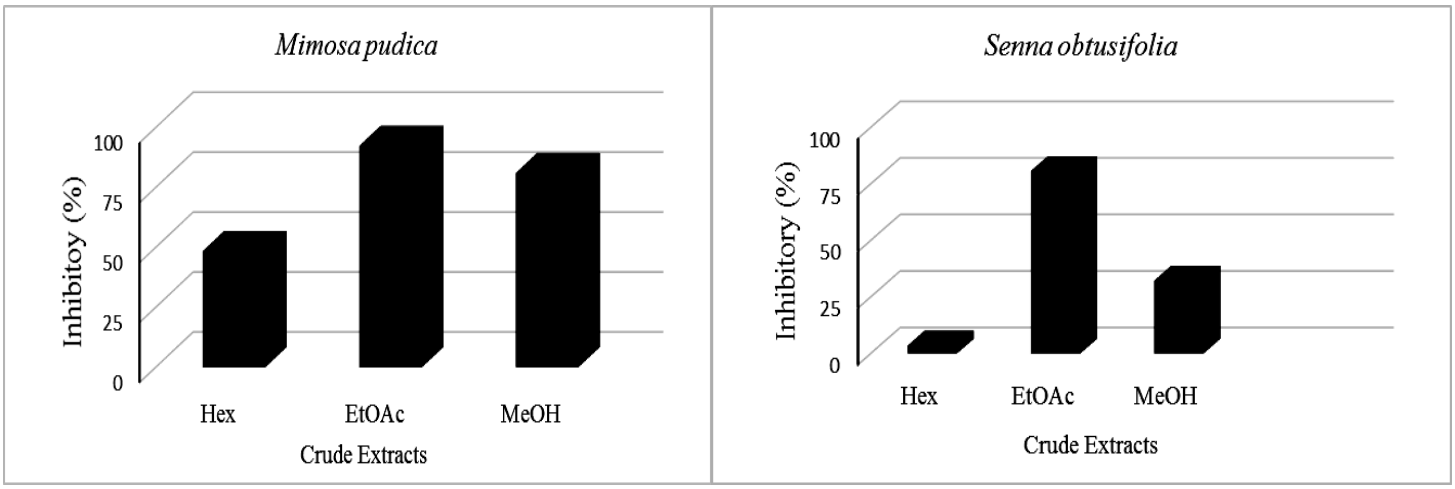

Fig. 1: Seed germination inhibitory effects of the crude extracts of *T. cinerea* leaves, 1% (m/v) in relation to the control test, using distilled water

The inhibition of seed germination the weed species *M. pudica* and *S. obtusifolia* caused by *T. cinerea* extracts (hexane, ethyl acetate and methanolic extracts) is shown in Fig 1. The hexane extract inhibited 48.0% of the seed germination of *M. pudica*, while the ethyl acetate and the methanolic extracts inhibited 92.0% and 81.0%, respectively. Inhibition of 3.3% on the germination of *S. obtusifolia* seeds was observed when the hexane extract was present, and of 81.0% and 32.2% when the ethyl acetate and the methanolic extracts were tested respectively

Substance identification by NMR

Substances obtained from crude extracts of *T. cinerea* leaves were identified as (*E*)-cinnamic acid (1) and rotenone (2). A mixture of triacylglycerol (3), *β*-sitosterol fatty esters (4) and trehalose disaccharides (5). Structures for the substances 1 to 5 are shown in Fig 2.

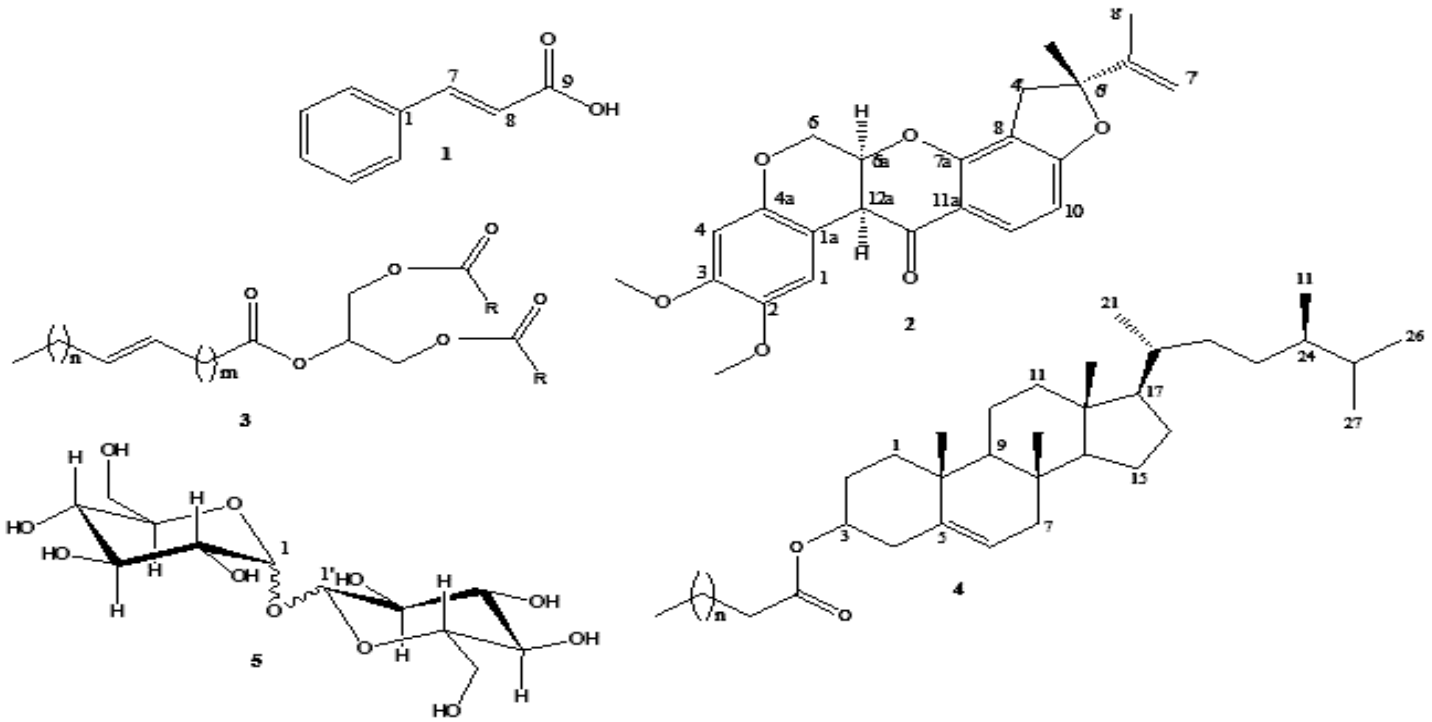

Fig. 2: Isolated and identified substances in crude extracts from *T. cinerea* leaves

Confirmation of the structures of the isolated substances (1 and 2), as well as the substances identified in mixtures (3-5), was given by ^{1}H and ^{13}C NMR spectra analysis and comparison with literature data [15-19]. Identification of substance 2 was also was given by DEPT-135 and correlation maps COSY, HMBC e HSQC analysis and comparison to literature data [16].

Substance 1: ^{1}H NMR (300 MHz, $CDCl_3$), δ (mult., J in Hz, H): 6.48 (d, 16.0, H-8), 7.40 (m, H-2/H-6), 7.56 (m, H-3/H-4/H-5), 7.81 (d, 16.0, H-7). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 117.3 (C-8), 128.3 (C-3/C-5), 128.8 (C-2/C-6), 130.6 (C-4), 135.9 (C-1), 147.1 (C-7), 172.5 (C-9). These data are in agreement with data found in literature for cinnamic acid [15].

Substance 2: ^{1}H NMR (300 MHz, $CDCl_3$) δ (mult., H): 1.74 (ls, H-8'), 2.95 (dd, 15.8 and 8.1, α H-4'), 3.31 (dd, 15.8 and 8.1), 3.74/3.78 (s, 2 x OCH_3), 4.15 (d, 12.0), 4.56 (d, 12.0), 4.90 (d, 3.6, H-6a), 4.91 (ls, H-7'α), 5.05 (m,), 6.46 (s, H-1), 6.75 (ls, H-4). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 17.0 (C-8'), 31.1 (C-4'), 44.4 (C-12a), 55.7/56.2 (2 x OCH_3), 66.1 (C-6), 72.1 (C-6a), 87.7 (C-5'), 100.8 (C-1), 104.7 (C-1a), 104.8 (C-11), 110.2 (C-4), 112.4 (C-7') 112.8 (C-8), 113.2 (C-11a), 129.8 (C-10), 142.9 (C-6'), 143.7 (C-2), 147.2 (C-4a), 149.3 (C-3), 157.8 (C-7a), 167.3 (C-9), 188.9 (C-12). These data are in agreement with data found in literature for rotenone [16].

Substance 3: ^{1}H NMR (300 MHz, $CDCl_3$), δ (H): Several signals between 0.84-0.88 ppm (methyl hydrogens), 2.30-2.32 ppm (carbonyl α-hydrogens), 2.34-2.08 ppm (methylene allylic hydrogens), 2.74-2.79 ppm (bis-allylic hydrogens), 1.59 ppm (carbonyl β-hydrogens), two around 4.3-4.0 ppm (oxidized methylene hydrogens from the glycerol moiety), intense signals between 1.24-1.30 ppm (other equivalent methylene hydrogens), 5.00-5.34 ppm (olefinic hydrogens derived from unsaturated fatty acids and oxidized methine hydrogen derived from glycerol). All ^{1}H and ^{13}C NMR signals were assigned by comparison with literature data for triacylglicerols [17].

Substances 4: ^{1}H NMR (300 MHz, $CDCl_3$), δ (H): Several signals between 0.67-0.86 ppm (methyl hydrogens), (methylene hydrogens of the fatty acid moiety), a multiplet at 4.55 ppm-(H-3 the high chemical shift is an evidence that carbon C-3 is esterified), a large doublet at 5.34 ppm with *J* = 5.7 Hz (olefinic hydrogen H-6). ^{13}C NMR (75 MHz, $CDCl_3$): 73.6 ppm (C-3), 140.7 ppm (C-5), 121.7 (C-6) ppm, among others. ^{1}H and ^{13}C NMR data for *β*-sitosterol fatty esters were compared to literature's [18].

Substance (5): ^{1}H NMR (300 MHz, CD_3OD), δ (H): Several signals between 3.00-3.93 ppm (oxidized methylene and methine hydrogens of a sugar unit), doublets at 4.51 ppm, J= 8.1 Hz and 5.14 ppm, J= 3.6 Hz (anomeric hydrogens H-1 e H-1'). ^{13}C NMR (75 MHz, CD_3OD): signals between 61.7-69.1 ppm (oxidized methylene carbons), signals between 70.9-82.8 ppm (oxidized methine carbons) and signals between 93.7-102.9 ppm (anomeric carbons) that are characteristic of mixtures of the isomers of the disaccharide trehalose (*α,α*-trehalose; *α,β*-trehalose; *β,β*-trehalose). The major component was *α,β*-trehalose. The NMR data is in accordance to literature data [19].

The presence of (*E*)-cinnamic acid (1) and rotenone (2) in both EtOAc and MeOH extracts, justifies the seeds germination inhibition effects on the test plants. Substance (1) was also tested in seeds germination inhibition and radicle and hypocotyl growth experiments for the same test plants and the inhibition was 99% at 0.1% concentration [20]. Rotenone (2), which is commonly found in legumes belonging to *Derris* and *Lonchocarpus* genera, as well as *Tephrosia* genus popularly known as Timbos, also has a potent phytotoxic effect against the same test plants [21].

CONCLUSIONS

Chemical study of the crude extracts from *T. cinerea* leaves showed that this species is an important source of phenolic acids and rothenoids, classes of substances usually associated with *T.* genus. Evaluation of seed germination inhibition using *M. pudica* and *S. obtusifolia* as test plants shows important inhibition effects were caused by the ethyl acetate and methanol extracts, the hexane extract was less effective on the inhibition. *M. pudica* was the most sensitive species to all tested extracts. The seed germination inhibition of the ethyl acetate and methanol extracts can be related to the presence of (*E*)-cinnamic acid (1) and rotenone (2) in these extracts. Therefore, this study allows one to conclude that the crude extracts of *T. cinerea* leaves have allelopathic potential and can be used in composition of bioherbicides for the tested invasive species seeds germination control. New studies must be carried out as well to evaluate possible phytotoxic effects of these same plants on seedlings (radicle and hypocotyl) development.

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