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Isolation and characterization of polyphenols from *Euphorbia heterophylla* L. (Euphorbiaceae) leaves

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Tostes, João Batista de Freitas[1*]; Silva, Antônio Jorge Ribeiro da[2]; Kuster, Ricardo Machado[3].

[1]Fundação Oswaldo Cruz, Instituto de Tecnologia em Fármacos - Farmanguinhos Rua Sizenando Nabuco, 100, Manguinhos, CEP: 21041-250, Rio de Janeiro, RJ, Brazil.

[2]Universidade Federal do Rio de Janeiro, Instituto de Pesquisa de Produtos Naturais Walter B. Mors, CCS, Bloco H, Ilha do Fundão, CEP: 21941-902, Rio de Janeiro, RJ, Brazil.

[3]Universidade Federal do Espírito Santo, Departamento de Química, Av. Fernando Ferrari, 514, Goiabeiras, Vitória, CEP: 29075-910, Espírito Santo, Brazil.

*Corresponding: joao.tostes@far.fiocruz.br.

Abstract

Many Euphorbiaceae species are recognized as traditional medicinal plants and the oils and latex of many species have also economic importance. Among these, *Euphorbia heterophylla* L. is popularly known in Brazil as “leiteira”, “flor-de-poetas” and wild poinsettia, and has been used traditionally in Africa and India to treat many kinds of health disorders. However, the chemical composition of this species has not been extensively studied so far. In the present investigation, the flavonoids quercitrin (quercetin 3-*O*-*α*-L-rhamnopyranoside), hyperin (quercetin 3-*O*-*β*-D-galactopyranoside), isoquercitrin (quercetin 3-*O*-*β*-D-glucopyranoside) and isoquercitrin 6”-malonate (quercetin 3-*O*-*β*-D-(6”-malonate)-glucopyranoside) were isolated from an ethyl acetate partition of aqueous ketone extract from *E. heterophylla* leaves. The dehydroellagitannins geraniin and phyllantusiin D as well as 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucopyranoside, *trans*-caffeic acid and the coumarin aesculetin (6,7-dihydroxycoumarin) were also isolated. All the compounds were characterized by spectroscopic methods such as ^{1}H and ^{13}C-NMR, COSY, HETCOR, APT and by comparison with literature data. The presence of these substances of relevant bioactivity is reported for the first time in *E. heterophylla* through our study, supporting in this way the medicinal use of this species.

Keywords: Euphorbiaceae. *Euphorbia heterophylla* L.. Polyphenols. Flavonoid glycosides. Dehydroellagitannins. Aesculetin.

Introduction

Euphorbiaceae is among the largest families of Angiosperms (flowering plants), comprising 228 genera and 6547 accepted species names[1]. Many of its species are recognized as traditional medicinal plants, such as *Phyllanthus niruri,* used to treat kidney stones (nephrolithiasis), and *Croton campestris,* used as a laxative, anti-syphilitic and for the treatment of biliary duct infections[2,3]. The oils and latex of many species have also economic importance as *Ricinus communis*, *Aleurites fordii* and *Hevea brasiliensis.* Others like *Manihot*

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glaziovii, *Manihot esculenta*, and *Manihot utilissima* are used in the food industry[4]. Recently, the possibilities of utilization of *Jatropha curcas* seed oil as fuel has been attracted the attention of researchers[5].

Euphorbia is the largest genus in Euphorbiaceae family with approximately 2000 species[1]. As example of medicinal use, it can be mentioned *Euphorbia tirucalli,* whose latex has antitumoral and anti-syphilitic properties[2] and *Euphorbia hirta* that has been used traditionally for respiratory ailments (cough, coryza, bronchitis, and asthma), worm infestations in children, digestive problems, and tumors[6]. *Euphorbia heterophylla* Linnaeus is an annual plant reproducing by seeds, latescent, erect, being able to reach 30-80 cm height. Its leaves are glabrous to slightly pubescent with widely variable form, reaching 4-10 cm length[7]. It is native from the tropical regions of Americas and is widespread in tropical and subtropical regions around the world[7]. In Brazil, it is popularly known as “leiteira”, “flor-de-poetas” and wild poinsettia[8]. In Africa and India, this species has been used traditionally to treat many kinds of health disorders such as arthritis, rheumatism, skin tumors, gonorrhea, erysipelas and many others[9]. In these regions, it is popularly known as milkweed.

Despite its importance, few substances have been isolated from *E. heterophylla* so far: flavonoids such as quercetin, 3-methyl quercetin, kaempferol 3-*O*-arabinoside, kaempferol 3-*O*-glucoside and kaempferol 7-*O*-glucoside along with some triterpenes[10,11]. Therefore, this study aims to contribute with new information on the chemical composition of this species.

Material and Methods

General

Reagents and solvents were PA grade (Vetec or Merck) except those used for HPLC analysis that were UV/HPLC grade (Merck). The ultrapurified water used was Milli-Q (Millipore). The standards were obtained from Sigma-Aldrich or Fluka.

Plant extraction

E. heterophylla was collected on campus at University of the Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro – Brazil, in August 2000. A voucher specimen was deposited at Herbarium of Botanic Garden of Rio de Janeiro. The fresh leaves (540.0 g) were extracted by static maceration with acetone-water (70:30), 7 x 2.0 L for 24 h each one. The extract was grouped, filtered by gravity in paper and the acetone removed by evaporation under reduced pressure at 40 ºC. The residual aqueous phase (4.0 L) was sequentially partitioned 3 times at 1:1 v/v ratio with chloroform, ethyl acetate and *n*-butanol yielding 0.5 g, 6.0 g and 6.9 g, respectively.

Thin Layer Chromatography (TLC) analysis

Extract and fractions were analyzed in silica gel AL 60F_{254} 20 x 20 cm plates (Merck) using NP/PEG reagent for detection of flavonoids[12], acetic acid/sodium nitrite reagent for ellagitannins[13,14], sulfuric orcinol for sugars, $KMnO_4$-$NaCO_3$-bromocresol green-bromothymol blue for malonic acid[15], ferric chloride for phenolic substances[12], UV light 254 nm and 365 nm. TLC solvent systems: (A) ethyl acetate/acetic acid/formic

acid/water (100:11:11:27), (B) ethyl acetate/formic acid/water (100:10:5) and (C) *i*-propanol/ethyl acetate/water (7:2:1).

Medium pressure liquid column chromatography (MPLC)

MPLC was performed in a solvent resistant column (SR) from Pharmacia (26.0 x 2.8 cm) with the stationary phase Toyopearl HW-40C (TosoHaas) and peristaltic pump EP-1 EconoPump (BioRad). The extract (2.5 g) was solubilized in 6.0 mL of ethanol-water (3:2) collecting 24 fractions of 20.0 mL each and then other 26 fractions of the same volume were collected with ethanol-water-acetone (7:2:1), flow-rate of 2.5 mL/min, totalizing 50 fractions, named as UN01 to UN50.

Analytical High-Performance Liquid Chromatography (HPLC)

The Shimadzu chromatograph consisted of LC-10AD pump, diode array detector SPD-M10A, DGU-14A degasser, CTO-10A column oven at 30 °C, Rheodyne injector with a 20 µL loop, SCL-10A interface and controlled by Class-VP software. A Hypersil ODS C18, 250 × 4.0 mm, 5 µm (Thermo Scientific) column was applied using as mobile phase acetonitrile and water adjusted to pH 3.0 with trifluoracetic acid (TFA) (15:85) at flow-rate 1 mL/min. The runs were monitored at 254, 280 and 365 nm.

Semi-preparative HPLC system and conditions

For the semi-preparative purposes, a Shimadzu chromatograph was used composed with LC-10AS pump, diode array detector SPD-M10A or refraction index detector RID-6A, DGU-14A degasser, and a Rheodyne injector with a 500 µL loop. A Supelcosil LC-18, 250 × 10.0 mm, 5 µm (Supelco) column was employed at room temperature using same mobile phase that applied in analytical system at flow-rate 4 mL/min or 5 mL/min. The separations were also monitored at 254, 280 and 365 nm.

Nuclear Magnetic Resonance (NMR)

The ^{1}H and ^{13}C-NMR spectra were obtained in Varian Gemini 200 RT and Bruker Avance DRX-400. Correlations were assigned by two-dimensional techniques of correlated spectroscopy (COSY), carbon-13-proton correlation (HETCOR) and attached proton test (APT).

Flavonoid glycosides hydrolysis

Flavonoid (2.0 mg) were heated in a boiling water for two hours in sealed tube with 2.0 mL TFA. After that, the respective aglycone was adsorbed in XAD-2 mini-column (50 x 5 mm) and the eluate was evaporated adding methanol several times until all the acid has been removed. The aglycone was eluted from the column with methanol.

Reduction of the sugars

The acid-free eluates from XAD-2 were reduced with 3.0 mg of sodium borohydride ($NaBH_4$) (Merck) in 1.0 mL of Milli-Q water cooled in an ice bath. After 2 hours the $NaBH_4$ excess was decomposed with acetic acid 20% v/v (3 drops) and the solution was passed through a column (50 x 5 mm) containing Dowex 50W-X8 resin (acid form) to remove sodium. The eluate was evaporated adding methanol several times until all the

borate has been removed. The residue was solubilized in water, frozen with liquid nitrogen and lyophilized yielding a white solid. Sugar standards were treated the same way.

Sugars derivatization

Dried acetonitrile (0.1 mL) and 70 µL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA/TMCS) were added to the final residues from sugars reduction. The sealed tubes were heated to 100 ºC for 2 hours. The products were analyzed by Gas Chromatography (GC) and the identity of the epimeric forms of the free sugars were confirmed against standards in individual injections as well co-injections.

Gas Chromatography (GC)

GC analyses were performed in a Varian Star 3400 CX system with Flame Ionization Detector (FID) using a DB-1 30.0 m × 0.25 mm × 0.25 µm. Injector, detector and column were set at 250ºC, 250ºC and 170ºC, respectively, and the product from sugar derivatization was injected directly (3 µL) at split 1:50.

Results and Discussion

At first, the ethyl acetate extract was analyzed by TLC suggesting the presence of phenolic substances (dark blue spots after sprayed with $FeCl_3$), cinnamic acid derivatives (blue fluorescence under 365 nm after NP/PEG reagent), flavonoids derivatives from luteolin, quercetin or myricetin (orange fluorescence under 365 nm after NP/PEG reagent) and ellagitannins (brown spots after reaction with acetic acid/sodium nitrite). A portion of this extract was fractionated by MPLC according literature[16]. The fractions were analyzed by TLC and grouped according similar profile (UN07-10: 1.05 g, UN11-12: 10.0 mg and UN20-50: 420.0 mg).

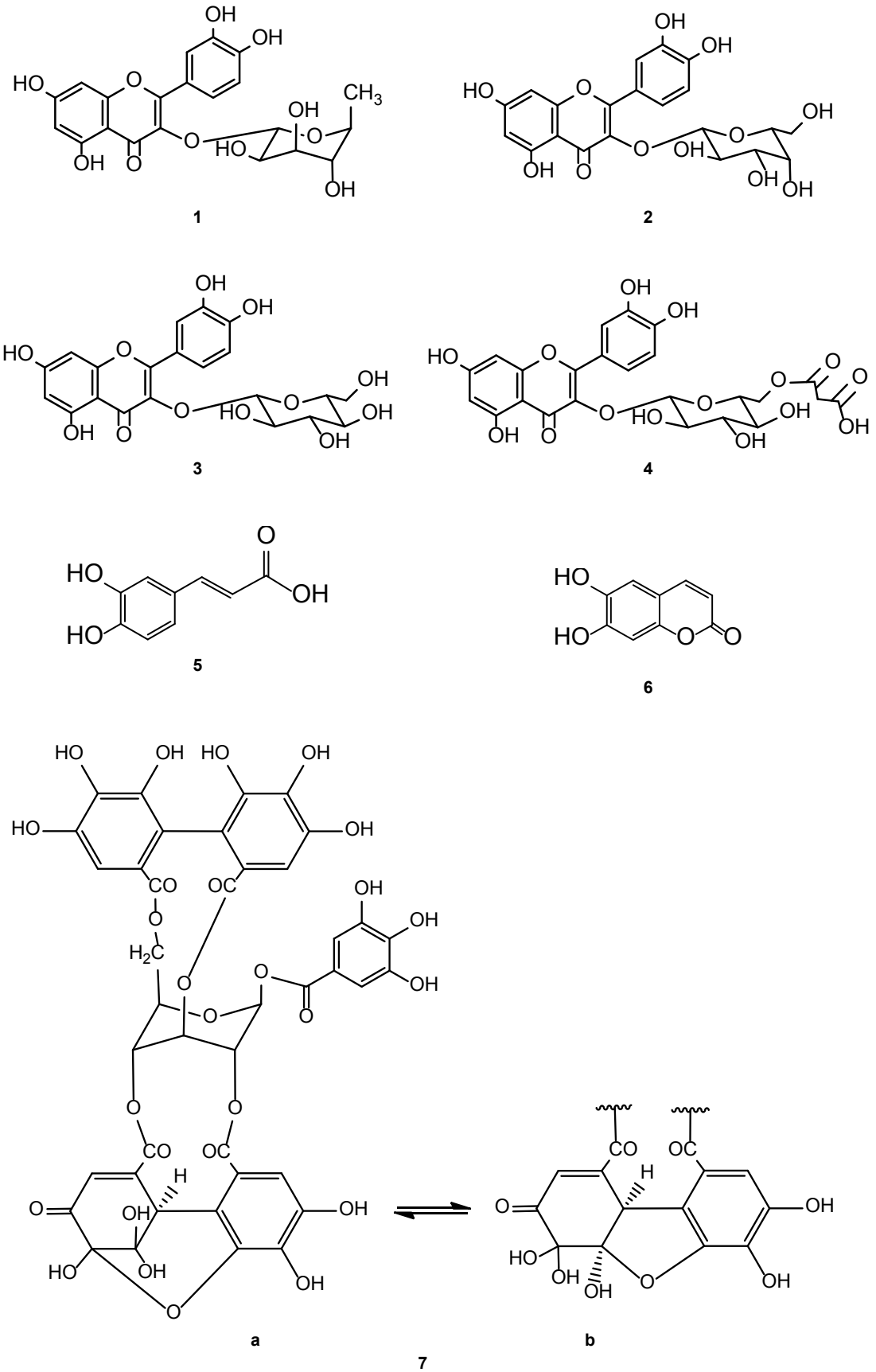
Isolation and characterization of Flavonoids

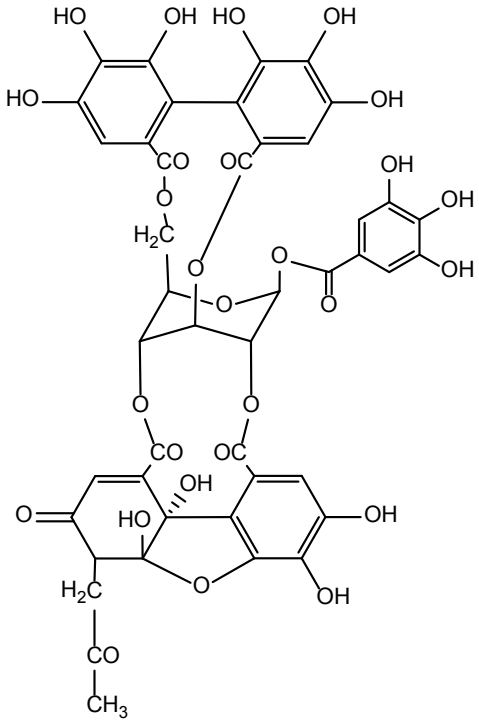
All the substances isolated in this study are structurally represented (**FIGURE 1**).

UN11-12 from the MPLC contained a pure compound according to the TLC and HPLC analysis, which chemical structure was confirmed by ^{1}H and ^{13}C-NMR results plus comparison with literature data[17], and was proved to be the substance quercitrin (quercetin 3-*O*-α-L-rhamnopyranoside) (**1**).

TLC analysis of UN07-10 suggested a mixture of flavonoids (orange fluorescence in a 365 nm UV lamp after NP/PEG reagent suggesting luteolin, quercetin or myricetin derivatives) and cinnamic derivatives (blue fluorescence spots in 365 nm UV lamp after NP/PEG reagent). By HPLC analysis, it was possible to verify that the UV spectra of some components were very similar to rutin standard. Therefore, a portion of this fraction (570.0 mg) was submitted to semi-preparative HPLC yielding five fractions, FR1 to FR5, that were dried in rotavapor: FR1 20.0 mg (pale yellow solid), FR2 30.0 mg (yellow solid), FR3 30.0 mg (yellow solid), FR4 10.0 mg (yellow solid), and FR5 20.0 mg (yellow solid).

FIGURE 1: Substances isolated from *Euphorbia heterophylla* in the present study.





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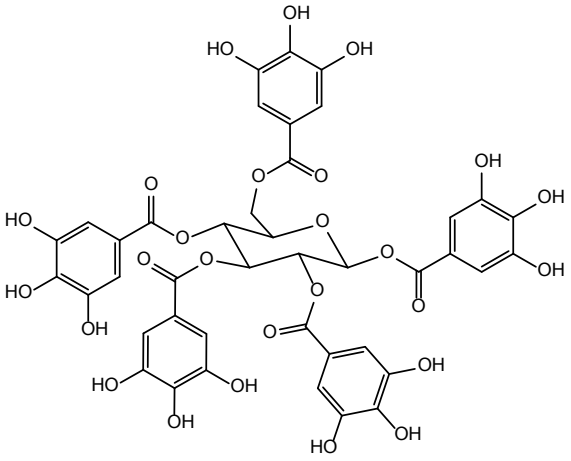

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Legend: Quercitrin (quercetin 3-*O*-α-L-rhamnopyranoside) **1**, Hyperin (quercetin 3-*O*-β-D-galactopyranoside) **2**, Isoquercitrin (quercetin 3-*O*-β-D-glucopyranoside) **3**, Isoquercitrin 6”-malonate (quercetin 3-*O*-β-D-(6”-malonate)-glucopyranoside) **4**, *trans*-Caffeic acid **5**, Aesculetin **6**, Geraniin **7**, Phyllantusiin D **8**, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranoside **9**.

Except for the FR1, the fractions presented separated compounds with high purity grade by TLC and HPLC and were submitted to ^{1}H and ^{13}C-NMR analysis. The proposed structures were confirmed by comparison with literature: hyperin (quercetin 3-*O*-β-D-galactopyranoside) (FR2, compound **2**)[17], isoquercitrin (quercetin 3-*O*-β-D-glucopyranoside) (FR3, compound **3**)[17], isoquercitrin 6”-malonate (quercetin 3-*O*-β-D-(6”-malonate)-glucopyranoside) (FR4, compound **4**) [18, 19] and compound **1** (FR5)[16].

The flavonoid glycosides **1**-**4** were submitted to hydrolysis and the products of the reactions were analyzed by TLC. The resulting compounds were co-chromatographed with quercetin presenting same R_f and color (solvent system C, R_f 0.80, orange fluorescence in UV 365 nm light after NP/PEG reagent). The malonic acid was identified by co-chromatography with standard (system C, $KMnO_4$-$NaCO_3$-bromocresol green-bromothymol blue, white spot in blue background). The sugars *β*-D-glucose (Glc), *β*-D-galactose (Gal) and *α*-L-rhamnose (Rha) were identified by co-chromatography (system C, sulfuric orcinol, R_f 0.38, 0.30 and 0.64, respectively).

The identity of the epimeric forms of the free sugars Glc and Gal, after reduction and silylation were confirmed by GC against standards in individual injections as well co-injections (t_R Glc 17.93 min and t_R Gal 18.19 min).

Flavonoids are known to be very bioactive substances making them good candidates for therapeutic agents[20]. These substances have recently been targeted by *in vitro* studies aimed at combating the fungus *Candida albicans*, which causes lethal opportunistic infections in people with immune deficiencies such as

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that caused by the HIV virus[21]. Kaempferol, quercetin and some of its glycosylated derivatives have shown considerable effects against this species of fungus[21] and can then explain the use of *E. heterophylla* as antifungal agent.

Isolation and characterization of *trans*-caffeic acid and aesculetin

FR1, that presented two spots of blue fluorescence in UV 365 nm light after NP/PEG reagent, was subjected to a new chromatographic separation by semi-preparative HPLC and two fractions were collected, evaporated, and analyzed by ^{1}H-NMR spectroscopy. NMR spectra were compared with literature data[22,23] allowing the identification of *trans*-caffeic acid (compound **5**, 1.4 mg) and the coumarin aesculetin (6,7-dihydroxycoumarin) (compound **6**, 4.0 mg). Possessing strong antioxidant activity, literature sources show that the compound **5** increase collagen production and prevention of premature aging[24]. This phenolic compound also demonstrated antimicrobial activity and may be a promise in the treatment of skin diseases[24]. Many pharmacological activities such as anti-inflammatory, anticoagulant, antioxidant, liver-protective, antidiabetic, antibacterial and antitumor is attributed to the coumarin **6**[25].

Isolation and characterization of hydrolysable tannins

TLC of UN20-50 (system A, acetic acid/$NaNO_2$ reagent, brown colors) indicated the presence of ellagitannins. This fraction was subjected to semi-preparative HPLC, yielding three other fractions, which were collected and dried in rotavapor. Each fraction presented high purity by TLC and HPLC analyses and were submitted to ^{1}H and ^{13}C-NMR analyses. The data were compared with literature[13,26,27] confirming the proposed structures: the dehydroellagitannins geraniin (FRB 1, compound **7**, beige solid, 65.2 mg) and phyllantusiin D (FRB 2, compound **8**, beige solid, 8.6 mg) as well as 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranoside (FRB 3, compound **9**, white solid, 19.3 mg).

The dehydroellagitannin **7** was previously isolated from *Phyllantus amarus* and demonstrated protection of mouse liver slices against ethanol-induced cytotoxicity[28]. A review on this substance demonstrated that it is a suitable candidate for further study into its potential pharmaceutical, nutritional, cosmetic and industrial application[29]. This tannin exists in two isomeric forms, as shown above (**FIGURE 1**). Compound **8** is an uncommon hydrolysable tannin previously isolated from *Phyllantus amarus*[30] and has been considered an artefact by some researchers, generated during acetone extraction[27]. Literature data suggested that this tannin has strong antioxidant activity[31]. In addition, several *in vitro* studies and some *in vivo* studies showed that compound **9** exhibits multiple biological activities making it of great potential in the therapy and prevention of several serious diseases such as cancer and diabetes[32-34].

Spectroscopic data obtained for the isolated compounds by ^{1}H and ^{13}C-NMR are shown below.

Quercitrin (quercetin 3-*O*-α-L-rhamnopyranoside) (**1**): ^{1}H-NMR (DMSO-d_6, 200 MHz, TMS, δ): 7.28 (*dd*, *J* = 1.99/5.55 Hz, H-6'), 7.22 (*d*, *J* = 1.99 Hz, H-2'), 6.86 (*d*, *J* = 8.24 Hz, H-5'), 6.38 (*d*, *J* = 1.61 Hz, H-8), 6.20 (*d*, *J* = 1.61 Hz, H-6), 5.26 (*d*, *J* = 1.33 Hz, H-1'' Rha), 4.80-3.00 (*m*, H-2'' to H-5'' Rha). ^{13}C-NMR (DMSO-d_6, 50 MHz, TMS, δ): 177.67 (C-4), 164.09 (C-7), 161.22 (C-5), 157.50 (C-2), 156.37 (C-9), 148.35 (C-4'), 144.11 (C-3'), 134.19 (C-3), 121.02 (C-1'), 120.71 (C-6'), 115.39 (C-5'), 115.63 (C-2'), 101.79 (C-1'' Rha), 104.03 (C-10), 98.61 (C-6), 93.54 (C-8), 71.70 (C-4'' Rha), 70.80 (C-3'' Rha), 70.46 (C-2'' Rha), 69.98 (C-5'' Rha), 17.14 (C-6'' Rha).

Hyperin (quercetin 3-*O*-β-D-galactopyranoside) (**2**). ^{1}H-NMR (DMSO-d_6, 200 MHz, TMS, δ): 7.66 (*dd*, *J* = 2.04/8.44 Hz, H-6'), 7.54 (*d*, *J* = 2.14 Hz, H-2'), 6.82 (*d*, *J* = 8.54 Hz, H-5'), 6.42 (*s*, H-6), 6.22 (*s*, H-6), 5.38 (*d*, *J* = 6.66 Hz, H-1 Gal), 5.10-3.40 (*m*, H-2 to H-6 Gal). ^{13}C-NMR (DMSO-d_6, 50 MHz, TMS, δ): 177.48 (C-4), 164.14 (C-7), 161.22 (C-5), 156.30 (C-2), 156.22 (C-9), 148.46 (C-4'), 144.82 (C-3'), 133.38 (C-3), 121.09 (C-1'), 121.99 (C-6'), 115.94 (C-5'), 115.19 (C-2'), 101.79 (C-1 Gal), 103.91 (C-10), 98.68 (C-6), 93.51 (C-8), 75.84 (C-5 Gal), 73.18 (C-3 Gal), 71.21 (C-2), 67.92 (C-4 Gal), 60.14 (C-6 Gal).

Isoquercitrin (quercetin 3-*O*-β-D-glucopyranoside) (**3**): ^{1}H-NMR (DMSO-d_6, 200 MHz, TMS, δ): 7.62 (H-2' and H-6'), 6.88 (*d*, *J* = 8.92 Hz, H-5'), 6.46 (*d*, *J* = 1.74 Hz, H-8), 6.24 (*d*, *J* = 1.74 Hz, H-6), 5.50 (*d*, *J* = 7.37 Hz, H-1'' Glu), 5.20-3.10 (*m*, H-2'' to H-6'' Glu). ^{13}C-NMR (DMSO-d_6, 50 MHz, TMS, δ): 177.39 (C-4), 164.08 (C-7), 161.19 (C-5), 156.28 (C-2), 156.28 (C-9), 148.41 (C-4'), 144.76 (C-3'), 133.31 (C-3), 121.55 (C-1'), 121.14 (C-6'), 116.18 (C-5'), 115.17 (C-2'), 100.88 (C-1'' Glu), 103.93 (C-10), 98.61 (C-6), 93.45 (C-8), 77.50 (C-3'' Glu), 76.49 (C-5'' Glu), 74.07 (C-2'' Glu), 69.92 (C-4'' Glu), 60.95 (C-6'' Glu).

Isoquercitrin 6"-malonate (quercetin 3-*O*-β-D-(6"-malonate)-glucopyranoside) (**4**): ^{1}H-NMR (DMSO-d_6, 200 MHz, TMS, δ): 7.54 (*d*, *J* = 2.22 Hz, H-6'), 7.48 (*d*, *J* = 2.26 Hz, H-2'), 6.86 (*d*, *J* = 8.25 Hz, H-5'), 6.42 (*d*, *J* = 1.86 Hz, H-8), 6.22 (*d*, *J* = 1.86 Hz, H-6), 5.38 (*d*, *J* = 6.79 Hz, H-1" Glu), 4.40-4.00 (large, H-2" to H-6" Glu). ^{13}C-NMR (DMSO-d_6, 50 MHz, TMS, δ): 177.29 (C-4), 167.93 (C-3"' malonyl), 166.95 (C-1"' malonyl), 164.18 (C-7), 161.16 (C-5), 156.31 (C-2), 156.69 (C-9), 148.55 (C-4'), 148.88 (C-3'), 133.11 (C-3), 121.34 (C-6'), 120.98 (C-1'), 121.34 (C-6'), 116.21 (C-5'), 115.41 (C-2'), 101.09 (C-1'' Glu), 103.83 (C-10), 98.67 (C-6), 93.54 (C-8), 76.15 (C-3'' Glu), 73.90 (C-2'' and C-5'' Glu), 69.47 (C-4'' Glu), 63.34 (C-6'' Glu), 41.77 (C-2"' malonyl).

trans-Caffeic acid (**5**): ^{1}H-NMR (acetone-d_6, 200 MHz, TMS, δ): 7.41 (*d*, *J* = 15.90 Hz, H-7), 7.04 (*d*, *J* = 2.10 Hz, H-2), 6.90 (*dd*, *J* = 1.90/8.30 Hz, H-6), 6.74 (*d*, *J* = 8.20 Hz, H-5), 6.14 (*d*, *J* = 15.80 Hz, H-8).

Aesculetin (6,7-dihydroxycoumarin) (**6**): ^{1}H-NMR (acetone-d_6, 200 MHz, TMS, δ): 7.77 (*d*, *J* = 9.70 Hz, H-4), 7.05 (*s*, H-5), 6.80 (*s*, H-8), 6.15 (*d*, *J* = 9.70 Hz, H-3).

Geraniin (**7**): ^{1}H-NMR (acetone-d_6 + D_2O, 400 MHz, TMS, δ): a form, 7.26 (*s*, H-3 D ring), 7.19 (*s*, H-2 and H-6 galloyl A ring), 7.13 (*s*, H-3/3' B/C ring), 6.65 (*s*, H-3/3' B/C ring), 6.54 (*br s*, H-1 Glu), 6.51 (*s*, H-3' E ring), 5.51 (*br s*, H-2 Glu), 5.50 (*br s*, H-4 Glu), 5.40 (*br s*, H-3 Glu), 5.15 (*s*, H-1' E ring), 4.20 - 4.40 (*m*, H-6 Glu); b form, 7.34 (*s*, H-3 D ring), 7.17 (*s*, H-2 and H-6 galloyl A ring), 7.07 (*s*, H-3/3' B/C ring), 6.64 (*s*, H-3/3' B/C ring), 6.54 (*br s*, H-1 Glu), 6.51 (*s*, H-3' E ring), 6.24 (*d*, *J* = 1.34 Hz, H-3' E ring), 5.51 (*br s*, H-2 Glu), 5.50 (*br s*, H-3 Glu), 5.40 (*br s*, H-4 Glu), 4.92 (*d*, *J* = 1.34 Hz, H-1' E ring), 4.76 (*m*, H-5 Glu) 4.20 - 4.40 (*m*, H-6 Glu). ^{13}C-NMR (acetone-d_6 + D_2O, 100 MHz, TMS, δ): a form. 191.81 (C-4' E ring), 168.43 (C-7 C ring), 166.18 (C-7 B ring), 165.57 (C-7' E ring), 165.39 (C-7 D ring), 164.72 (C-7 A ring), 154.38 (C-2' E ring), 145.95 (C-3 and C-5 A ring), 145.76 (C-4 D ring), 145.40 (C-4 C ring), 145.25 (C-6 B/C ring), 144.67 (C-4 B ring), 143.33 (C-6 D ring), 139.87 (C-4 A ring), 139.04 (C-5 D ring), 137.86 (C-5 B ring), 136.46 (C-5 C ring), 128.61 (C-3' E ring), 125.50 (C-2 C ring), 124.52 (C-2 B ring), 120.06 (C-1 A ring), 119.26 (C-2 D ring), 117.13 (C-1 B ring), 115.72 (C-1 D ring), 115.29 (C-1 C ring), 113.38 (C-3 D ring), 110.79 (C-2 and C-6 A ring), 110.59 (C-3 B ring), 107.79 (C-3 C ring), 96.21 (C-5' E ring), 92.37 (C-6' E ring), 90.79 (C-1 Glu), 72.55 (C-5 Glu), 69.93 (C-2 Glu), 65.86 (C-4 Glu), 63.79 (C-6 Glu), 63.25 (C-3 Glu), 46.09 (C-1' E ring); b form, 194.56 (C-4' E ring), 168.43 (C-7 C ring), 166.18 (C-7 B ring), 165.71 (C-7' E ring), 164.98 (C-7 D ring), 164.87 (C-7 A ring), 149.12 (C-2' E ring), 147.05 (C-6 D ring), 145.95 (C-3 and C-5 A ring), 145.67 (C-

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4 D ring), 145.25 (C-4 C ring), 145.25 (C-6 B/C ring), 144.59 (C-4 B ring), 139.87 (C-4 A ring), 137.49 (C-5 D ring), 137.86 (C-5 B ring), 136.46 (C-5 C ring), 125.03 (C-3' E ring), 125.35 (C-2 C ring), 124.35 (C-2 B ring), 120.06 (C-1 A ring), 119.92 (C-1 D ring), 117.13 (C-1 B ring), 116.92 (C-2 D ring), 115.18 (C-1 C ring), 113.38 (C-3 D ring), 110.79 (C-2 and C-6 A ring), 110.32 (C-3 B ring), 108.00 (C-3 C ring), 92.37 (C-5' E ring), 92.37 (C-6' E ring), 91.77 (C-1 Glu), 73.17 (C-5 Glu), 70.45 (C-2 Glu), 66.79 (C-4 Glu), 63.79 (C-6 Glu), 62.29 (C-3 Glu), 51.89 (C-1' E ring).

Phyllantusiin D (**8**): ^{1}H-NMR (acetone-d_6 + D_2O, 400 MHz, TMS, δ): 7.15 (*s*, H-3 D ring), 7.10 (*s*, H-2 and H-6 galloyl A ring), 6.95 (*s*, H-3/3' B/C ring), 6.60 (*s*, H-3/3' B/C ring), 6.50 (*br s*, H-1 Glu), 6.21 (*s*, H-3' E ring), 5.50 (*br s*, H-2 Glu), 5.43 (*br s*, H-3 Glu), 5.37 (*br s*, H-4 Glu), 4.85 (br *s*, H-1' E ring), 4.80 (*t*, *J* = 9.00 Hz, H-5 Glu), 4.71 (*t*, *J* = 9.00 Hz, H-6 Glu), 4.34 (*dd*, *J* = 7.90/10.60 Hz, H-6 Glu), 3.40 (*d*, *J* = 15.90 Hz, H-7' acetonyl), 2.93 (*d*, *J* = 15.90 Hz, H-7' acetonyl), 2.14 (*s*, H-9' acetonyl). ^{13}C-NMR (acetone-d_6 + D_2O, 100 MHz, TMS, δ): 165.42 (C-7 A ring), 165.40 (C-7 D ring), 145.75 (C-3 and C-5 A ring), 144.73 (C-4 B ring), 126.74 (C-3' E ring), 119.68 (C-1 A ring), 110.63 (C-2 and C-6 A ring), 110.28 (C-3 B ring), 107.78 (C-3 C ring), 91.83 (C-1 Glu), 81.00 (C-5' E ring), 72.88 (C-5 Glu), 70.07 (C-2 Glu), 66.48 (C-4 Glu), 63.67 (C-6 Glu), 62.14 (C-3 Glu), 51.68 (C-1' E ring), 50.00 (C-7' acetonyl), 31.88 (C-9' acetonyl).

1,2,3,4,6-Penta-*O*-galloyl-β-D-glucopyranoside (**9**): ^{1}H-NMR (acetone-d_6 + D_2O, 400 MHz, TMS, δ): 7.17 (*s*, H-2 and H-6 galloyl), 7.10 (*s*, H-2 and H-6 galloyl), 7.05 (*s*, H-2 and H-6 galloyl), 6.97 (*s*, H-2 and H-6 galloyl), 6.33 (*d*, *J* = 8.40 Hz, H-1 Glu), 6.00 (*t*, *J* = 9.80 Hz, H-3 Glu), 5.63 (*m*, H-2, H-4 and H-5 Glu), 4.55 (*d*, *J* = 12.5 Hz, H-6 Glu), 4.38 (*dd*, *J* = 4.50/12.50 Hz, H-6 Glu). ^{13}C-NMR (acetone-d_6 + D_2O, 100 MHz, TMS, δ): 166.37 (C=O galloyl), 165.92 (C=O galloyl), 165.71 (C=O galloyl), 165.65 (C=O galloyl), 164.96 (C=O galloyl), 145.97 (C-3 and C-5 galloyl), 139.76 (C-4 galloyl), 139.10 (C-4 galloyl), 138.97 (C-4 galloyl), 121.33 (C-1 galloyl), 120.53 (C-1 galloyl), 120.44 (C-1 galloyl), 119.83 (C-1 galloyl), 110.05 (C-2 and C-6 galloyl), 93.29 (C-1 Glu), 73.90 (C-5 Glu), 73.25 (C-3 Glu), 71.10 (C-2 Glu), 69.25 (C-4 Glu), 62.76 (C-6 Glu).

Conclusion

The presence of the substances isolated in this study represents additional information on the chemistry of the species, since there is no report in the literature of their isolation from *E. heterophylla*. Furthermore, important biological activities related to those compounds could contribute to justify the medicinal use of *E. heterophylla* by some populations.

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