SHORT REPORT

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Antioxidant Activities of Hydrolysable Tannins and Flavonoid Glycosides Isolated from *Eugenia uniflora* L.

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Abstract: A phytochemical investigation of *Eugenia uniflora*'s leaf extract resulted in the isolation of eleven phenolic compounds: 2,3-di-*O*-galloyl-*β*-D-glucose (**1**), 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**2**), gemin D (**3**), hippomanin A (**4**), oenothein B (**5**), eugeniflorin D_2 (**6**), camptothin A **(7)**, afzelin (**8**), quercitrin (**9**), myricitrin, (**10**) and desmanthin-1 (**11**). These compounds were identified by spectroscopic methods including 1D- and 2D-NMR, UV, IR, and TOF/MS. Compounds **1**, **2**, **3**, **4**, **7**, **8**, **9**, and **11** were isolated from this species for the first time. Ten isolates were evaluated for antioxidant activity by DPPH free radical and Oxygen Radical Absorbance Capacity (ORAC-Fluorecein) assay. Dimeric tannins, oenothein B (**5**), eugeniflorin D_2 (**6**), and camptothin A (**7**) showed a remarkable radical scavenging capacity.

Keywords: Hydrolysable tannins; camptothin A; desmanthin-1; pitanga; DPPH; ORAC assay.

1. Plant Source

Eugenia uniflora L. (Myrtaceae) is a semi-deciduous shrubby tree with edible, cherry-like fruits. Native to Brazil, where it is known as "pitangueira", it is widespread in this as well as in other countries of South America. Its leaves have been used in folk medicine for the treatment of diarrhoea [1], inflammation, rheumatic pains, fever, stomach problems [2], and hypertension [3].

E. uniflora leaves were collected in Anápolis (S 16° 20′ 13′′; W 48° 56′ 19′′; 1034 m), Goiás State, Brazil, in February 2006 and identified by Professor Heleno Dias Ferreira of the Department of General Biology at Universidade Federal de Goiás (UFG). A voucher specimen (UFG-25477) is currently deposited at UFG's Herbarium.

2. Previous Studies

Macrocyclic ellagitannins such as oenothein B, eugeniflorin D_1, and eugeniflorin D_2, along with other phenolic compounds, 1,2,4,6-tetra-*O*-galloyl-*β*-D-glucose, gallocatechin, and myricitrin, have been isolated from *E. uniflora* leaves [4]. Selina-1,3,7(11)-trien-8-one (43%) and Selina-

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1,3,7(11)-trien-8-one epoxide (29%) were identified as the major components of this plant's essential oil [5]. Several biological activities, such as antiinflammatory, antioxidant, antinociceptive, diuretic, hypothermic, hypoglycemic, and antihypertensive were confirmed for this species by *in vitro* and *in vivo* tests [6-10].

3. Present Study

Dried and grounded leaves of *E. uniflora* (1.0 kg) were exhaustively extracted with 50% acetone, using an overhead stirrer apparatus at room temperature. The acetone was removed under reduced pressure and the suspended aqueous extract was filtered to eliminate fats and chlorophylls. Following, a liquid-liquid extraction with ethyl acetate (10 x 150 mL) was carried out. The combined organic phase was evaporated to yield an ethyl acetate extract (15 g). The aqueous layer was freeze-dried to yield a 122 g extract, which was dissolved in methanol (800 mL) to separate soluble (87 g) and insoluble (33 g) methanolic extracts.

The soluble methanolic extract was separated into six 12 g portions. These were subjected to Diaion HP-20 column chromatography (200 g) and eluted with a decreasing polarity gradient of H_2O/MeOH. Five main fractions were combined (*M1-M5*). *M3* (7.1 g), *M4* (6.8 g), and *M5* (12.7 g) were separately applied to Diaion HP-20 CC and eluted with 0-100% MeOH/H_2O to yield ten (*M3.1-M3.10*), six (*M4.1-M4.6*), and four (*M5.1-M5.4*) combined fractions, respectively. Fractions *M3.6* (2.3 g), *M4.3* (1.7 g), and *M5.2* (3.7 g) were separately subjected to Sephadex LH-20 CC (eluting with an increasing polarity gradient of $CHCl_3$/EtOH followed by EtOH/MeOH) to give compounds **1** (26 mg), **3** (540 mg), **5** (270 mg), and **6** (350 mg) from *M3.6*, **3**, **4** (135 mg), and **7** (56 mg) from *M4.3*, and **5** (840 mg) from *M5.2*.

Part of the ethyl acetate extract (7.0 g) was subjected to Sephadex LH-20 CC (200 g) and eluted with a stepwise gradient of $CHCl_3$/EtOH (7:3 to 0:10) followed by EtOH/MeOH (9:1 to 1:9) to afford sixteen combined fractions (*EA.1-EA.16*). Compounds **2** (92 mg) and **10** (63 mg) were obtained from *EA.11*. Fractions *EA.3* (2.8 g) and *EA.5* (3.0 g) were separately subjected to Sephadex LH-20 CC eluting with the same gradient of $CHCl_3$/EtOH followed by EtOH/MeOH to afford compounds **8** (201 mg) and **9** (114 mg) from *EA.3*, and **11** (97 mg) from *EA.5*.

The structure elucidation of all isolated compounds (Figure 1) was determined by spectroscopic methods (ESI-TOF MS, 1D, and 2D NMR) and by comparison with data from the literature. They were identified as 2,3-di-*O*-galloyl-*β*-D-glucose (**1**) [11], 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**2**) [12], gemin D (**3**) [13], hippomanin A (**4**) [13], oenothein B (**5**) [14], eugeniflorin D_2 (**6**) [4], camptothin A (**7**) [15,16], afzelin (**8**) [17], quercitrin (**9**) [17], myricitrin (**10**) [17] and desmanthin-1 (**11**) [18]. The spectral data of the isolated compounds are described in the supporting information section.

DPPH radical scavenging activity: The antioxidant capacity of ten pure compounds was determined by a modification of the DPPH method proposed by Brand-Williams *et al.* [19]. A solution of each compound in methanol (0.3 mL) was added to a methanol solution of DPPH radical (0.1 mM, 2.7 mL, Sigma). After 30 minutes of incubation at room temperature in the dark, absorbance was measured at 515 nm. The control was prepared with 0.3 mL methanol and 2.7 mL DPPH methanolic solution (0.1 mM). The blank solution consisted of 0.3 mL of each sample in methanol plus 2.7 mL of methanol. Results obtained by DPPH assay were generated by three independent experiments for each compound and carried out in duplicate. The percentage of scavenging activity (% SA) was evaluated from the decrease value of 515 nm absorption, which was calculated by the following equation.

$$\% \text{ SA} = [A_{control} - (A_{sample} - A_{blank}) / A_{control}] \times 100$$

Oxygen Radical Absorbance Capacity (ORAC-Fluorecein) assay: it was performed as described by Dávalos et al. [20] for the same ten pure compounds. All reagents were obtained from Sigma-Aldrich and were diluted with a 75 mM phosphate buffer (pH 7.4), the final volume was 2 mL. Compounds

(200 µL) and fluorescein (1200 µL, 70 nM) were mixed in a cell and incubated for 15 min at 37 ºC. AAPH solution (600 µL, 24 mM) was added and the fluorescence was recorded every 2 min for 80 min at respective excitation and emission wavelengths of 485 and 520 nm. The same procedure was repeated with a blank (fluorescein + AAPH) for each compound.

Trolox (Sigma-Aldrich) was used as standard in both antioxidant assays for the calibration curves: DPPH assay (10 – 90 µM; $y = 5.8431 + 0.4465x$; $r^2 = 0.9946$) and ORAC assay (0.2 – 4.2 µM; $y = 0.4881 + 1.4511x$, $r^2 = 0.9970$). Results were expressed as µmol of Trolox equivalent/µmol of pure compound. Elagic acid (Sigma) was the positive control in both assays. Results were tested by one-way analysis of variance ($p < 0.05$) followed by Tukey's test.

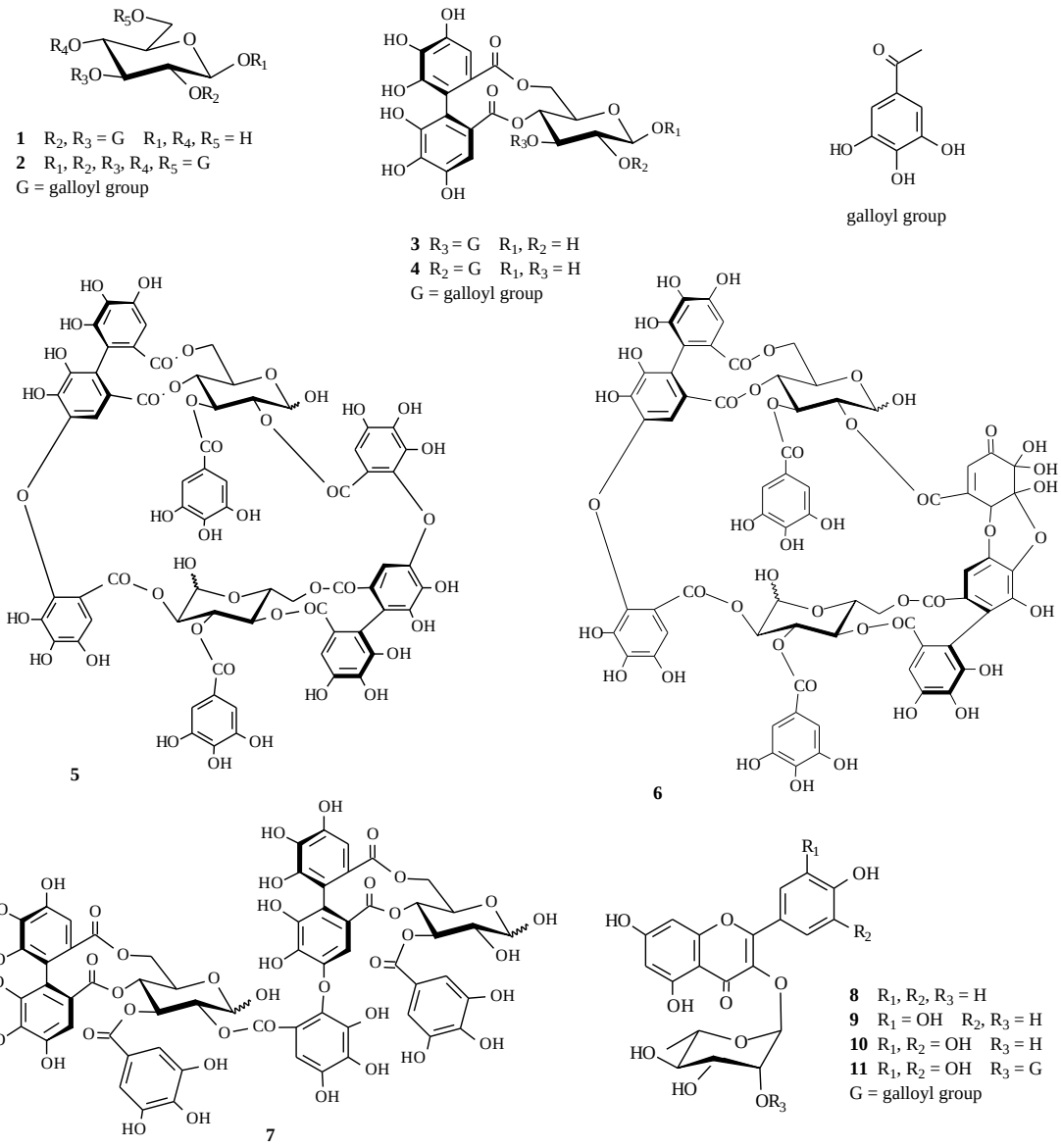

Figure 1. Structures of compounds **1**-**11**

As shown in Table 1, all phenolic compounds tested in this study exhibited inhibitory activity against DPPH and peroxyl radicals. Flavonol-3-*O*-glycosides afzelin and quercitrin showed the lowest scavenging activity and were very similar to elagic acid (positive control), whereas myricitrin, a flavonol with a 3',4',5'-trihydroxyl group, almost doubled the activity in both assays. The presence of three orto-hydroxyls in the B ring, in addition to galloyl groups attached to the structure, may

drastically enhance flavonoids' antioxidant activity, as is the case of desmanthin-1. The increase in the number of galloyl groups positively affects the scavenging capacity, as is the case of the two galloylglucoses (Table 1). The same trend had already been reported, using the DPPH assay, for other gallotannins as well as for quinic acid and shikimic acid esterified with gallic acid [21]. However, previous studies employing the ORAC antioxidant assay presented conflicting results for epigallocatechin and epigallocatechin gallate, two monomers of condensed tannins, and the flavonoids kaempferol, quercetin and myricetin [22, 23], showing no direct relation between the antioxidant capacity and the number of hydroxyl or galloyl groups [22].

Ellagitannins have at least one hexahydroxydiphenoyl (HHDP) group, which constrains the molecule and may pose spatial hindrance to the approach of DPPH and peroxyl radicals. This could be the reason for the lower antioxidant capacities of gemin D compared to 2,3-di-*O*-galloyl-*β*-D-glucose. The molecular structures of galloylglucoses are more flexible and in general have stronger reducing effects than ellagitannins [24].

Dimeric ellagitannins, eugeniflorin D_2, camptothin A, and oenothein B exhibited higher scavenging capacities than monomers, which can be explained by the considerable number of phenolic hydroxyl groups present in their molecules [21]. Polyphenol radicals were produced through the reaction with DPPH or peroxyl radicals. These highly reactive species undergo C-C and C-O coupling reactions to form dimers, which results in the interruption of the radicals' chain reaction [24].

In summary, in this study the radical scavenging capacities increased together with an increase in the number of phenolic hydroxyl groups; these were observed for the three classes of compounds: flavonoids, galloylglucoses, and ellagitannins. In addition, oenothein B, the major compound of this specie, showed the highest antioxidant activity, this fact contributes to explain the highest radical scavenging capacities of the polar extracts of *E.uniflora* [7] and supports the potential use of this plant in preventing the development of many human diseases [6, 8-10].

Table 1. Antioxidant activity of isolated compounds[1]

Compound	DPPH assay TE[2]	ORAC assay TE[2]	Number of Phenolic OH	MW g/mol
Elagic acid[3]	0.47 ± 0.01^{e}	4.77 ± 0.43^{g}	4	302.20
Flavonoids				
Quercitrin (**9**)	0.42 ± 0.03^{e}	2.96 ± 0.20^{h}	4	448.38
Afzelin (**8**)	0.45 ± 0.04^{e}	3.79 ± 0.33^{gh}	3	432.38
Myricitrin (**10**)	0.86 ± 0.07^{d}	8.09 ± 0.37^{ef}	5	464.38
Desmanthin-1 (**11**)	1.26 ± 0.10^{c}	14.06 ± 0.43^{d}	8	616.48
Galloylglucoses				
2,3-di-*O*-galloyl-*β*-D-glucose (**1**)	0.93 ± 0.10^{d}	9.33 ± 0.48^{e}	6	484.08
1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**2**)	1.42 ± 0.22^{c}	21.06 ± 0.25^{c}	15	940.68
Ellagitannins				
Gemin D (**3**)	0.79 ± 0.12^{d}	7.42 ± 0.39^{f}	9	634,45
Eugeniflorin D_2 (**6**)	1.93 ± 0.12^{b}	22.24 ± 0.44^{c}	18	1584.15
Camptothin A (**7**)	2.85 ± 0.09^{a}	24.02 ± 0.24^{b}	20	1418.16
Oenothein B (**5**)	3.07 ± 0.02^{a}	26.08 ± 0.53^{a}	22	1568.15

[1]Results are presented as the mean (n = 3) ± SD. [2]TE (Trolox equivalent) expressed as μmol of Trolox equivalent/μmol of pure compound. [3]Standard reference. Averages followed by the same letter in the column did not share significant differences at 5% probability by Tukey's test.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP.

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