Article

Flavonoids from the Leaves of *Deguelia utilis* (Leguminosae): Structural Elucidation and Neuroprotective Properties

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Das folhas de *Deguelia utilis* foram isolados cinco flavonoides: 5,3'-di-hidróxi-4'-metóxi-2'',2''-dimetilcromeno-(5'',6'':6,7)-di-hidroflavonol (**1**), 5,3'-di-hidróxi-7,4'-dimetóxi-6,8-dimetilalil-di-hidroflavonol (**2**), 5,3'-di-hidróxi-4'-metóxi-8-prenil-2'',2''-dimetilcromeno-(5'',6'':6,7)-flavanona (**3**), 5,3'-di-hidróxi-7,4'-dimetóxi-6,8-dimetilalil-flavanona (**4**), 3,5,3'-tri-hidróxi-7,4'-dimetóxi-6,8-dimetilalil-flavanol (**5**), juntamente com os estilbenos: 4-metoxilonchocarpeno (**6**) e lonchocarpeno (**7**). Suas estruturas químicas foram elucidadas com base nos seus dados de NMR (ressonância magnética nuclear) e HRESITOF-MS (espectrometria de massas de alta resolução por tempo de vôo, com ionização por eletrospray). Além disso, a fim de investigar o potencial efeito citoprotetor desses flavonoides, foi utilizada uma fração eluída com hexano:AcOEt contendo os sete flavonoides, em um modelo *in vitro* de neurodegeneração, utilizando culturas primárias do hipocampo de ratos neonatal (PND2-P3) expostos à rotenona, um inibidor mitocondrial do complexo I. Houve uma redução significativa da viabilidade celular (19,4 ± 1,6%), quando as culturas foram expostas à rotenona 30 nmol L^{-1} por 72 h. A exposição concomitante das culturas a FR3 (5 μg mL^{-1}) e rotenona 30 nmol L^{-1} resultou em valores de viabilidade celular semelhante ao grupo controle (99,6 ± 4,8%), sugerindo um efeito citoprotetor para essa fração.

Five new flavonoids, 5,3'-dihydroxy-4'-methoxy-2'',2''-dimethylchromene-(5'',6'':6,7)-dihydroflavonol (**1**), 5,3'-dihydroxy-7,4'-dimethoxy-6,8-dimethylallyl-dihydroflavonol (**2**), 5,3'-dihydroxy-4'-methoxy-8-allyl-2'',2''-dimethylchromene-(5'',6'':6,7) flavanone (**3**), 5,3'-dihydroxy-7,4'-dimethoxy-6,8-dimethylallyl-flavanone (**4**), 3,5,3'-trihydroxy-7,4'-dimethoxy-6,8-dimethylallyl-flavanol (**5**), together with the stilbenes 4-methoxylonchocarpene (**6**) and lonchocarpene (**7**) were isolated from the leaves of *Deguelia utilis*. Their chemical structures were established on the basis of NMR (nuclear magnetic resonance) spectral data and HRESITOF-MS (electrospray ionization-high resolution time-of-flight mass spectrometry). Also, in order to investigate potential cytoprotective effects of these flavonoids, we used a fraction eluted with hexane:EtOAc containing all seven flavonoids, in an *in vitro* model of neurodegeneration, using hippocampal primary cultures from neonatal (PND2-P3) rats exposed to rotenone, a mitochondrial complex I inhibitor. There was a significant reduction in cell viability (19.4 ± 1.6%) when the cultures were exposed to 30 nmol L^{-1} rotenone for 72 h. Concomitant exposure of the cultures to the FR3 (5 μg mL^{-1}) and 30 nmol L^{-1} rotenone resulted in values of cell viability similar to control groups (99.6 ± 4.8%), strongly suggesting a cytoprotective effect for this flavonoid-rich fraction.

Keywords: *Deguelia utilis*, flavonoids, leguminosae, timbó

Introduction

Brain energy metabolism is highly dependent on mitochondria oxidative phosphorylation, which is an important source of free radicals. When free radical production exceeds the availability of endogenous and exogenous antioxidants, oxidative stress prevails, leading to cell damage. Not surprisingly, oxidative damage has been implicated in the pathogenesis of a number of neurodegenerative diseases such as Alzheimer´s disease,

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Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, as well as others.[1] Thus, strategies aimed at fighting the excess of free radicals may be beneficial for slowing down or arresting the degeneration process and natural products are an attractive source of antioxidant compounds with promising pharmacological properties. Therefore, natural products continue to play a fundamental role in the discovery of leads for developing new drugs for treating human diseases.[2]

The genus *Deguelia* belonging to the family Leguminosae comprises 20 species, among which *D. rufescens* var. urucu and *D. utilis* are considered the most used by indigenous population of the Amazon region for catching fish. *D. utilis* is a shrub with wide distribution in the Amazon basin, and it is commonly known in the region as "timbó branco". This shrub can reach 3 meters of height and its roots are well known for their piscicide and pesticide properties attributed to its high contents of rotenoids such as rotenone and deguelin.[3-5] Rotenone, which can be extracted from *Deguelia* roots, is a lipophilic mitochondrial complex I inhibitor, and it has been widely used as a neurotoxicant in both *in vivo* and *in vitro* models of Parkinson´s disease.[6]

Regarding studies about *Deguelia* spp., many phytochemical reports are found in the literature describing the chemistry of the roots.[7-12] To date, however, there are only a few publications about the phytochemical properties of the leaves of these species, and they have reported the presence of several flavonoid compounds with potential antioxidant properties.[13,14]

In this study we sought to perform a phytochemical characterization of the leaves from *D. utilis* and investigated the presence of compounds with potential neuroprotective properties. A flavonoid-rich fraction extracted from *D. utilis* leaves (FR3) was tested for its potential neuroprotective properties in a cell culture model of neurodegeneration induced by rotenone. Our results showed that the use of a very small concentration of the fraction was able to abolish rotenone-induced cell loss, as determined by the MTT colorimetric assay.

Experimental

General

UV spectra were obtained from LC equipped with DAD Prominence SPDM-20A (Shimadzu, Tokio, Japan). NMR spectra including ^{1}H-^{1}H COSY, HETCOR, HMBC experiments, were recorded on a Varian Mercury-300 spectrometer, operating at 300 MHz at ^{1}H and 75 MHz at ^{13}C, using *d*-chloroform as solvent and internal standard. Mass spectral analyses were performed on UltrOTOF-Q

(Bruker, Daltonics Billerica MA, USA) at the cationized ion region. The heated capillary and voltage were maintained at 250 ºC and 3 kV, respectively. A 20 V cone energy for ion extraction and mass spectrometry data were acquired at positive and negative modes for all compounds. HPLC was carried out in a preparative LC-8A Shimadzu system with SPD-10AV Shimadzu UV detector (Tokyo, Japan); using a Phenomenex Gemini C18 column (250 × 10 mm, 5 μm), an isocratic system of water/acetonitrile (3:7) and a flow rate of 4.7 mL min^{-1}. Detection was performed at 270 and 325 nm. All solvents were filtered through a 0.45 μm nylon membrane filter prior to analysis.

Plant material

Approximately 1.3 Kg of green leaves of *D. utilis* (A. C. Sm.) A. M. G. Azevedo (sinonimia: *Lonchocarpus nicou* and *Derris nicou*) were collected in the forest reserve of EMBRAPA -Amazônia Oriental, located in Belém, Pará at 01° S 48° W, 10 m. The plant was identified and a Voucher (Exsiccate IAN 181063) was deposited in the EMBRAPA Herbarium.

Extraction and isolation

An amount of 480 g of dried and powdered *D. utilis* leaves was extracted with 3 L of ethanol at room temperature for 24 h (two times). The solvent was removed under reduced pressure, furnishing 80 g of crude ethanolic residue, from which 50 g were fractionated in a silica gel (40-60 μm, 1:10) column chromatography with gradient elution: hexane-EtOAc 9:1 (FR1 1.85 g), 7:3 (FR2 2.50 g), 5:5 (FR3 11.63 g, AcOEt (FR4 2.75 g) and EtOAc-MeOH 7:3 (FR5 1.22 g) and MeOH (FR6 8.70 g). Fraction FR3 (0.4 g), was sonicated in 4.8 mL of acetonitrile for 1 min. Next 1.2 mL of H$_2$O was added and sonicated again for 1 min. Solution was subjected to solid phase extraction (SPE) in a C18 cartridge (Phenomenex, 1 g of stationary phase *per* 6 mL). After evaporation, the residue (about 260 mg) was purified by semi-preparative HPLC yielding compounds **1** (23.0 mg), **2** (42.4 mg), **3** (13.0 mg), **4** (16.2 mg), **5** (11.4 mg), **6** (21.7 mg) and **7** (10.5 mg), which showed chromatographic peak retention times of 11.3, 20.9, 25.0, 31.8, 37.2, 16.6 and 9.4 min, respectively.

Cell culture and rotenone exposure

Mixed primary hippocampal cultures were prepared from postnatal day 2 (PND2) - PND3 Wistar rats. Pups were sacrificed by decapitation; the brain was rapidly removed and dissected in a petri dish with sterile dissection medium. The hippocampus was isolated free of meninges

and incubated for 5-10 min at 37 °C in a trypsin-EDTA 0.05% solution (Invitrogen) for enzymatic digestion, and then transferred to culture medium DMEM containing 10% fetal bovine serum (FBS), 10% horse serum (HS) and penicillin-streptomycin 10,000 U mL^{-1}. After that, the tissue was mechanically dissociated with a sterile fire-polished Pasteur pipette. The cells contained in the supernatant were then plated at a density of approximately 5×10^5 cells *per* well in plates previously treated with poly-L-lysine (100 µg mL^{-1}) containing DMEM supplemented with 10% FBS, 10% HS and penicillin-streptomycin. The cultures plates were kept in 5% CO_2/95% O_2 at 37 °C. After three days, the cultures were replenished with new medium. Until vehicle or rotenone exposure, the cultures were maintained in DMEM supplemented with 2% FBS, 2% HS, and penicillin-streptomycin.[15,16]

After 7 days *in vitro* (DIV), the cells were pretreated with increasing concentrations of FR3 (3 µg mL^{-1}, 5 µg mL^{-1} and 10 µg mL^{-1}) in order to evaluate its toxicity or FR3 plus 30 nmol L^{-1} rotenone for 72 h. Rotenone was prepared from a stock solution of 1 mmol L^{-1} in 0.1% of dimethyl sulfoxide (DMSO) and then diluted in DMEM to the final concentration. These concentrations of rotenone were chosen, since similar concentrations have been reported in studies of neuron degeneration and/or oxidative stress.[17-19]

Immunocytochemistry

In order to assure that our protocol resulted in a mixture of neurons and glial cells in the primary cultures, cells were plated in wells containing a coverslip of 13 mm in diameter covered with poly-L-lysine. Antibody against the enzyme tyrosine hydroxylase (Millipore, 1:1000) to characterize catecholaminergic (dopaminergic) neurons was used. Microglia cells were stained with OX-42 antibody (Sigma 1:1000) which recognizes the complement receptor type 3 (CR3), and astrocytes were labeled with anti-GFAP antibody (Sigma 1:2000). After 7 DIV, plates were washed with PBS and fixed with 3.7% formaldehyde in PBS for 30 min at room temperature. The cultures were then washed again and incubated overnight at 4 °C with primary antibody. Next, cells were washed and then incubated for 2 h with a donkey biotinylated secondary antibody (Jackson Immuno Research, 1:200). After washing, cultures were incubated for 1 h with Vectastain ABC reagents diluted according to manufacturer's instructions. The visualization of the complex formed was done by incubation of cultures with hydrogen peroxide and DAB (3,3'-diaminobenzidine) in PBS. Slides were coverslipped with mounting medium (Permount), and were analyzed and photographed under a Nikon Labophot 2 microscope (Nikon Microscopy Systems).

Cell viability assay

Cell viability was checked using the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. MTT is a salt that is reduced by the mitochondrial dehydrogenase of viable cells and is detected by this assay. Cultures were exposed to 1 µg mL^{-1} FR3 or FR3 plus 30 nmol L^{-1} rotenone, during 3 days, at 37 °C and 5% CO_2. The cells were then washed and incubated with PBS plus 50 µL of MTT for 3-4 h at 37 °C and 5% CO_2. Subsequently, cells were scraped off, transferred to glass tubes, and the absorbance at 570 nm was measured in a spectrophotometer.

Statistics

Data were expressed as mean ± standard error of mean (SEM). Statistical significance was calculated using the ANOVA associated with Tukey's test. For comparison of control and rotenone values, the Mann-Whitney U test was used. Differences within the groups of $P < 0.05$ were regarded as statistically significant.

Results and Discussion

Phytochemical investigation

The dried and powdered leaves of *D. utilis* were extracted with EtOH. The ethanolic extract was fractionated by silica gel column chromatography, which provided six fractions. Chromatographic separation of the hexane:EtOAc 50% soluble fraction by semi-preparative HPLC led to the isolation of pure compounds **1**-**7** (Figure 1). Compounds **6** and **7** were identified as 4-methoxylonchocarpene and lonchocarpene, respectively, by comparison of their spectral data with those reported in literature.[20,21]

Compoud **1** was obtained as a pale yellow powder. The molecular formula $C_{26}H_{28}O_7$ was determined by HRESITOF-MS at *m/z* 453.1909 [M+H]$^+$ (calc. for $C_{26}H_{29}O_7$, 453.1913). Its ^{1}H NMR spectrum (Table 1) exhibited a typical AX system due to H-2 and H-3 of a dihydroflavonol at δ_H 4.95 (*d*, *J* 11.7 Hz) and 4.47 (*d*, *J* 11.7 Hz), respectively. These assignments were confirmed by the ^{13}C NMR spectrum (Table 2), which showed three C-ring carbon signals at δ_C 82.8 (C-2), 72.4 (C-3) and 196.2 (C-4). The configuration at C-2/C-3 was determined to be *trans* on the basis of the magnitude of J_{H2-H3} 11.7 Hz. Additionally, the ^{1}H NMR spectrum exhibited signals in the aromatic region at δ_H 7.14 (*d*,1H, *J* 1.6 Hz), 7.02 (*dd*, 1H, *J* 8.1, 1.6 Hz) and 6.89 (*d*, 1H, *J* 8.1 Hz), which indicated an AMX spin system of a 1,3,4-trisubstituted phenyl group.
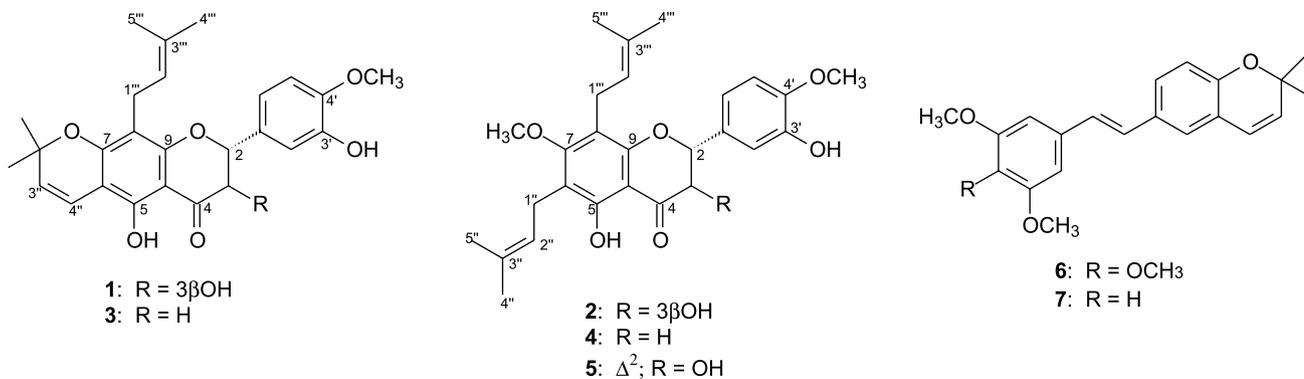
Figure 1. Structures of the compounds isolated from leaves of *Deguelia utilis*: prenyl-urucuol A (**1**), prenyl-isotirumalin (**2**), 3'-methoxylupinifolin (**3**), prenylutiline (**4**), prenylutilinol (**5**), 4-methoxylonchocarpene (**6**) and lonchocarpene (**7**).

The signals observed at δ_H 6.63 and 5.52 (*d*, 1H each, *J* 9.9 Hz) and 1.44 (6H, *s*) revealed a 2,2-dimethylchromene ring attached to an aromatic ring, and the singlet at δ_H 11.42 indicated the presence of a hydroxyl group bonded to C-5 and one OMe group at δ_H 3.91 (δ_C 55.9) bonded to the aromatic ring.

All the couplings were confirmed through analysis of the ^1H-^1H COSY spectrum. In addition to the signals related to C-ring carbons, the ^{13}C NMR spectrum (Table 2) of **1** exhibited 25 other signals attributed to twenty six carbons with the aid of the HETCOR and HMBC experiments. The 2,2-dimethylchromene ring linked to ring A at C-6 and C-7 and C-prenyl group attached at C-8 were deduced from the $^{2,3}J$ correlations of the signal at δ_H 5.52 (*d*, *J* 9.9 Hz H-3'') and δ_H 11.42 (OH-5) with δ_C 103.1 (C-6), as well as δ_H 6.63 (*d*, *J* 9.9 Hz, H-4'') and δ_H 3.17 (*t*, *J* 7.2 Hz, H-1''') with the oxidized carbon at δ_C 160.6 (C-7). The location of the OMe and OH groups at C-4' and C-3' of the aromatic B-ring, respectively, was supported by the combination of the substitution pattern on the aromatic ring (1,3,4-trisubstituted) observed in the ^1H NMR spectrum with the $^{2,3}J_{C,H}$ correlations from the signals at 7.02 (H-6'), 6.89 (H-5'), 7.14 (H-2') and 3.91(OMe-4') to oxidized aromatic carbon at δ_C 147.1 (C-4') and from both H-5' and H-2' to another oxidized aromatic carbon at δ_C 145.6 (C-3'). To confirm these positions a NOE experiment was carried out. Irradiation at δ_H 6.89 (H-5') showed nuclear over house effect with the signals at δ_H 7.02 (H-6') and δ_H 3.91 (OMe). All correlations are observed in Figure 2. Therefore, the structure of **1** was determined as 5,3'-dihydroxy-4'-methoxy-2'',2''-dimethylchromene-(5'',6'':6,7)-dihydroflavonol, which we named prenyl-urucuol A.

Compoud **2** was obtained as a yellow amorphous powder and its molecular formula was determined to be $C_{27}H_{32}O_7$ based on its HRESITOF-MS at *m/z* 467.2117 [M-H]$^+$ (calc. for $C_{27}H_{31}O_7$, 467.2069). The ion peaks at
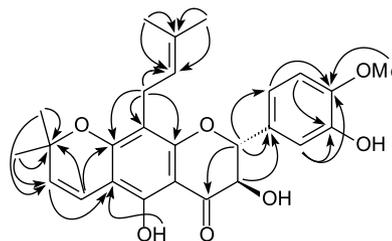
Figure 2. Key HMBC correlations of compound **1**.

m/z 467 are 14 mass units more than the corresponding peaks in **1**, suggesting one additional methyl group. The presence of two γ,γ-dimethylallyl groups was evidenced by ^1H NMR signals at δ_H 3.31 (*d*, *J* 6.6 Hz, H-1'') and 3.22 (*t*, *J* 5.7 Hz, H-1'''); 5.21 (*t*, *J* 6.6 Hz, H-2'') and 5.12 (*t*, *J* 6.9 Hz, H-2'') and four singlets at δ_H 1.70 (H$_3$-4''), 1.79 (H$_3$-5''), 1.66 (H$_3$-4''') and 1.58 (H$_3$-5'''). An OH-5 signal at δ_H 11.27 was also observed. The aromatic and C-ring proton signals of **2** (Table 1), as well as B and C-ring carbon signals (Table 2) were very similar to those of **1**. In the ^{13}C NMR spectrum two signals at δ_C 55.9 and δ_C 61.6 were observed, suggesting a diorthossubstituted OMe group. Thus, the difference between **1** and **2** is in ring A. The γ,γ-dimethylallyl moiety and one OMe group were confirmed as being attached at C-6 and C-7 (A-ring), respectively, based on the long-range correlations from the signal at δ_H 3.31 (H-1'') and δ_H 11.27 (OH-5) with δ_C 116.0 (C-6), and δ_H 3.22 (H-1''') and OMe-7 to C-7 (δ_C 165.9). From these results, the structure of **2** was identified as 5,3'-dihydroxy-7,4'-dimethoxy-6,8-dimethylallyl-dihydroflavonol, named prenyl-isotirumalin.

Compoud **3** was obtained as a yellow amorphous powder and its molecular formula was determined to be $C_{26}H_{28}O_6$ based on its HRESITOF-MS at *m/z* 437.1998 [M+H]$^+$ (calc. for $C_{27}H_{31}O_7$, 437.1964). The ^1H and ^{13}C NMR data (Tables 1 and 2) of compound **3** were very similar to those reported for **1**, indicating the presence of 1,3,4-trisubstituted and pentasubstituted aromatic rings, as

well as a 2,2-dimethylchromene ring. The main differences between the spectroscopic data of compound **3** are signals for three double duplets at δ_H 5.31 (*dd, J* 3.0 and 12.9 Hz, H-2), δ_H 2.81 (*dd, J* 3.0 and 17.1 Hz, H-3β) and δ_H 3.05 (*dd, J* 17.1 and 12.9 Hz, H-3α), which showed correlations with the signals at δ_C 78.5 (C-2) and 43.3 (C-3), respectively, in the HETCOR spectrum, suggesting it to be a flavanone. The substitution pattern of B-ring showed the same AMX system as that of compounds **1** and **2**. The presence of OH at C-3' and OMe at C-4' was supported by means ^3J correlation at δ_H 5.71 signal, attributed to OH phenolic, with δ_C 112.5 (C-2') and δ_C 145.7 (C-3') as well as by irradiation at δ_H 3.92 that showed NOE with the signal at δ_H 6.87 (*d, J* 8.4 Hz, H-5'). The presence of OH-5 was confirmed by the signal at δ_H 12.25. Thus, compound **3** is 5,3'-dihydroxy-4'-methoxy-8-allyl-2'',2''- dimethylchromene -(6,7:5'',6'') flavanone, named 3'-methoxylupinifolin.

Compoud **4** was obtained as a yellow amorphous powder and its molecular formula was determined to be $C_{27}H_{32}O_6$ based on its HRESITOF-MS at *m/z* 453.2270 [M+H]$^+$ (calc. for $C_{27}H_{33}O_6$, 453.2277). The ^1H and ^{13}C NMR data (Tables 1 and 2) of compound **4** were very

similar to those reported for **2** and **3**. The ^1H NMR spectrum showed signals for three double duplets at δ_H 5.30 (*dd, J* 3.0 and 12.9 Hz, H-2), δ_H 2.82 (*dd, J* 3.0 and 17.1 Hz, H-3β) and δ_H 3.06 (*dd, J* 17.1 and 12.9 Hz, H-3α), which showed correlations with the signals at δ_C 78 .4 (C-2) and 43.5 (C-3), respectively, in the HETCOR spectrum, thus compound **4** is a flavanone. The substitution pattern of B-ring showed the same AMX system as that of **1**, **2** and **3** for B aromatic ring. The presence of OH-5 was confirmed by the signal at δ_H 12.05. The presence of two γ,γ-dimethylallyl groups was evidenced by signals at δ_H 3.29 (*d, J* 6.6 Hz, H-1'') and 3.25 (*d, J* 6.7 Hz, H-1'''); 5.21 (*tt, J* 0.9 and 7.8 Hz, H-2'') and 5.15 (*tt, J* 1.5 and 7.8 Hz, H-2''') and four singlets at δ_H 1.68 (H$_3$-4''), 1.77 (H$_3$-5''), 1.66 (H$_3$-4''') and 1.63 (H$_3$-5'''). The signal observed at δ_H 3.75 (δ_C 61.6) suggest the presence of a diorthosubstituited OMe group as observed for **2**. Thus the compound **4** is 5,3'-dihydroxy-7,4'-dimethoxy-6,8-dimethylallyl-flavanone named prenylutiline.

Compoud **5** was obtained as a yellow amorphous powder and its molecular formula was determined to be $C_{27}H_{30}O_7$ based on its HRESITOF-MS at *m/z* 467.2064 [M+H]+ (calc. for $C_{27}H_{31}O_7$, 467.2069). The presence of

Table 1. ^1H NMR chemical shifts (δ_H in ppm) and coupling constant (*J* in Hz) of compounds **1-5** in CDCl$_3$

H	1	2	3	4	5
2	4.95 (d, 11.7)	4.96 (d, 12.0)	5.31 (dd, 12.9 and 3.0)	5.30 (dd, 12.9 and 3.0)	-
3	4.47 (d, 11.7)	4.50 (d, 12.0)	β 2.81 (dd, 17.1 and 3.0) α 3.05 (dd, 17.1 and 12.9)	β 2.82 (dd, 17.1 and 3.0) α 3.06 (dd, 17.1 and 12.9)	-
2'	7.14 (d, 1.6)	7.14 (d, 2.1)	7.04 (d, 1.8)	7.03 (d, 1.8)	7.77 (d, 1.8)
5'	6.89 (d, 8.1)	6.90 (d, 8.4)	6.87 (d, 8.4)	6.87 (d, 8.2)	6.97 (d, 8.4)
6'	7.02 (dd, 8.1 and 1.6)	7.00 (dd, 8.4 and 2.1)	6.92 (dd, 8.4 and 1.8)	6.91 (dd, 8.2 and 1.8)	7.82 (dd, 8.4 and 1.8)
1''	-	3.31 (d, 6.6)	-	3.29 (d, 6.6)	3.41 (d, 6.0)
2''	-	5.21 (t, 6.6)	-	5.21 (tt, 7.8 and 0.9)	5.24 (br d, 7.8)
3''	5.52 (d, 9.9)	-	5.49 (d, 9.9)	-	-
4''	6.63 (d, 9.9)	1.70 (s)	6.63 (d, 9.9)	1.68 (s)	1.81 (s)
5''	1.44 (s)	1.79 (s)	1.44 (s)	1.77 (s)	1.70 (s)
6''	1.44 (s)	-	1.43 (s)	-	-
1'''	3.17 (t, 7.2)	3.22 (t, 5.7)	3.21 (d, 7.2)	3.25 (d, 6.7)	3.58 (d, 6.0)
2'''	5.12 (bt)	5.12 (t, 6.9)	5.15 (t, 6.7)	5.15 (tt, 7.8 and 1.5)	5.24 (br d, 7.8)
3'''	-	-	-	-	-
4'''	1.65 (s)	1.66 (s)	1.66 (s)	1.66 (s)	1.86 (s)
5'''	1.62 (s)	1.58 (s)	1.66 (s)	1.63 (s)	1.72 (s)
OMe-4'	3.91 (s)	3.92 (s)	3.92 (s)	3.92 (s)	3.98 (s)
OMe-7	-	3.77 (s)	-	3.75 (s)	3.80 (s)
OH-5	11.42 (s)	11.27 (s)	12.25 (s)	12.05 (s)	11.86 (s)
OH	5.80 (br s)	5.75 (br s)	5.71 (br s)	-	-

^1H NMR data were recorded at 300 MHz. Multiplicity and coupling constant (*J*, Hz) are in parenthesis.

two γ,γ-dimethylallyl groups was evidenced by ^{1}H NMR signals (Table 1) at δ_H 3.41 (*d*, *J* 6.0 Hz, H-1") and 3.58 (*d*, *J* 6.0 Hz, H-1'''); 5.24 (*br d*, *J* 7.8 Hz, H-2"), as well as four singlets at δ_H 1.81 (H$_3$-4"), 1.70 (H$_3$-5"), 1.86 (H$_3$-4''') and 1.72 (H$_3$-5'''). In the ^{1}H NMR spectrum a characteristic proton signal of OH-5 at δ_H 11.86 was observed as well as two singlets at δ_H 3.98 (56.0) and 3.80 (62.0) suggesting a diorthossubstituted OMe group as observed for compounds **2** and **4**. The same AMX patterned substation of the B-ring of compounds **1-4** was observed. Based on the ion peak at *m/z* 467 and the presence of the 27 signals in the ^{13}C NMR spectrum, compound **5** is a flavonol. An OMe was establish at C-4 by means of correlations between δ_H 6.97 (*d*, *J* 8.4 Hz, H-5'), 7.82 (*dd*, *J* 1.8 and 8.4 Hz, H-6') and 3.98 (OMe) with δ_C 148.2. Thus compound **5** is 3,5,3'-trihydroxy-7,4'-dimethoxy-6,8-dimethylallyl-flavanol named prenylutilinol.

Table 2. ^{13}C NMR chemical shifts (δ_C in ppm) of compounds **1-5** in CDCl$_3$

C	δ_C				
	1	**2**	**3**	**4**	**5**
2	82.8	82.8	78.5	78.4	145.6
3	72.4	72.8	43.3	43.5	136.0
4	196.2	197.5	196.4	197.5	175.8
5	155.8	158.9	156.3	159.5	156.1
6	103.1	116.0	102.8	115.5	117.3
7	160.6	165.9	160.0	165.0	162.5
8	109.2	114.6	108.6	114.0	113.6
9	159.2	158.2	159.3	158.2	152.4
10	100.2	103.0	102.6	105.3	106.1
1'	129.6	129.6	132.0	132.0	124.3
2'	113.5	113.4	112.5	112.5	113.7
3'	145.6	145.6	145.7	1457	145.7
4'	147.1	147.0	147.0	146.7	148.2
5'	110.3	110.3	110.5	110.5	110.4
6'	119.5	119.5	117.8	117.8	121.0
1"	-	22.5	-	22.2	22.5
2"	78.4	122.5	78.0	122.8	122.4
3"	126.2	131.9	126.0	131.6	132.0
4"	115.4	25.7	115.6	25.7	17.9
5"	28.3	17.8	28.3	17.9	25.6
6"	28.3	-	28.3		-
1'''	21.2	22.2	21.4	22.7	22.8
2'''	122.1	122.5	122.5	123.0	122.8
3'''	131.3	131.6	131.0	131.4	132.4
4'''	25.7	25.7	25.8	25.7	18.1
5'''	17.7	17.7	17.8	17.8	25.7
OMe-7	-	61.6	-	61.6	62.0
OMe-4'	55.9	55.9	56.0	56.0	56.0

Neuroprotective properties

Immunocytochemical analysis indicated that our culture procedures resulted in a mixed pattern of neurons and glia (Figure 3) with the following composition: 10.38% (± 3.75) of the neurons were catecholaminergic (positive for anti-tirosine hidroxylase antibody), 14.57% (± 2.74) were astrocytes (positive for anti-GFAP antibody) and 9.58% (± 1.97) were microglia (positive for OX-42 antibody), out of the total cell number revealed by counterstaining with Nissl.
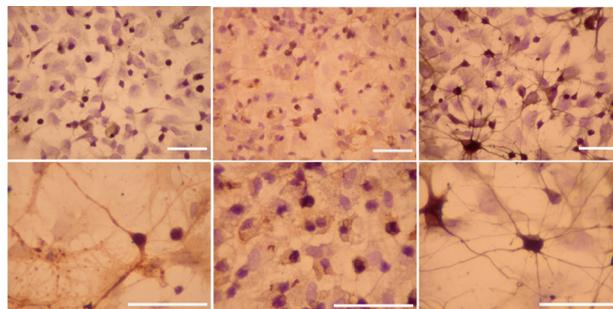
Figure 3. Immunocytochemical caracterization of hippocampal primary cultures as a mixture of neurons and glial cells. Catecholaminergic neurons (TH-positive cells) are shown in the left column, microglia (OX-42 positive-cells) in the middle and astrocytes (GFAP-positive cells) in the right column. Bar = 50 μm.

In the cultures, exposure to 30 nmol L^{-1} rotenone for 72 h resulted in significant reduction (19.4%) in cell viability when compared to control cultures (Figure 4). Exposure to DMSO in the same concentration used to dilute rotenone had no statistically significant effect on cell viability. Likewise, the addition of increasing concentrations (3, 5 and 10 μg mL^{-1}) of the FR3 fraction tested in this study had no significant effect on the viability of the hippocampal cells. On the other hand, concomitant exposure of the cultures to 30 nmol L^{-1} rotenone and FR3 fraction of *Deguelia* leaves
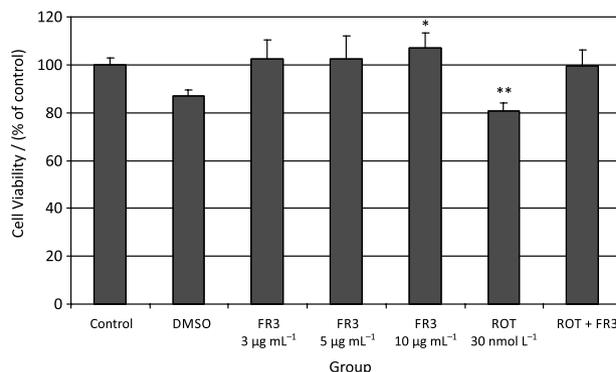
Figure 4. Effect of FR3 on the cell viability of primary hippocampal cells in rotenone-treated cultures. 100% corresponds to the total number of cells after 7 DIV in untreated controls. Values represent the means ± SEM of three independent experiments with two wells in each treatment. *p < 0.05 compared to control group. **p < 0.05 compared to rotenone group.

(5 μg mL⁻¹) for 72 h resulted in increased cell viability (to 99.6%), when compared with cell cultures treated with rotenone alone (negative control). These results suggest that FR3 provided significant protection to hippocampal cells against rotenone-induced toxicity.

Importantly, the FR3 fraction is a flavonoid mixture that contains substances **1** to **7**. Several studies attribute significant neuroprotective effects to flavonoid compounds, and this has been frequently associated with the antioxidant properties of these substances,[22-24] which could explain the observed cytoprotection effects of FR3 in our animal model of neurodegeneration. Thus, we propose that such an effect is related to the high contents of flavonoids present in the leaves. Noteworthy is the fact that the same species provides high concentration of rotenone, a known neurotoxic compound,[25] together with neuroprotective substances present in its leaves.

Conclusions

The present phytochemical study of the leaves of Deguelia utilis afforded five new flavonoids, named as prenyl-urucuol A (**1**), prenyl-isotirumalin (**2**), 3'-methoxylupinifolin (**3**), prenylutiline (**4**) and prenylutilinol (**5**). A fraction containing all these flavonoids (FR3) showed significant cell viability when compared with cell cultures treated with rotenone, a mitochondrial complex I inhibitor, strongly suggesting a cytoprotective effect for this fraction.

Supplementary Information

¹H NMR, ¹³C NMR spectra, HMBC correlations for compounds **1**-**5** are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

References

1. Migliore, L. L.; Coppedè, F.; *Mutat. Res.* **2009**, *674*, 73.

2. Newman, D. J.; Cragg, G. M.; Snader, K. M.; *J. Nat. Prod.* **2003**, *66*, 1022.

3. Harbone, J. B.; Mabry, T. J.; Mabry, H.; *The Flavonoids*, 2ⁿᵈ ed.: Chapman & Hall: London, 1975, p. 376.

4. Lin, Y. L.; Chen, Y. L.; Kuo, Y. H.; *J. Nat. Prod.* **1993**, *56*, 1187.

5. Filho, R. B.; Gottlieb, O. R.; Mourão, A. P.; *Phytochemistry* **1975**, *14*, 261.

6. Horowitz, M. P.; Greenamyre, J. T.; *Clin. Pharmacol. Ther.* **2010**, *88*, 467.

7. Kaquadji, M.; Agban, A.; Mariotte, A. M.; *J. Nat. Prod.* **1986**, *49*, 281.

8. Garcia, M.; Kano, M. H. C.; Vieira, D. M.; do Nascimento, M. C.; Mors, W. B.; *Phytochemistry* **1986**, *25*, 2425.

9. Fang, N.; Casida, J. E.; *J. Agric. Food Chem.* **1999**, *47*, 2130.

10. Magalhães, A. F.; Tozzi, A. M. G. A.; Magalhães, E. G.; Moraes, V. R. S.; *Phytochemistry* **2001**, *57*, 77.

11. Lawson, M. A.; Kaquadji, M.; Allais, D. P.; *Tetrahedron Lett.* **2006**, *47*, 451.

12. Lawson, M. A.; Kaquadji, M.; Chulia, A. J.; *Tetrahedron Lett.* **2008**, *49*, 2407.

13. Lôbo, L. T.; da Silva, G. A.; Ferreira, M.; da Silva, M. N.; Santos, A. S.; Arruda, A. C.; Guilhon, G. M. S. P.; Santos, L. S.; Borges, R. S.; Arruda, M. S. P.; *J. Braz. Chem. Soc.* **2009**, *20*, 1082.

14. Lôbo, L. T.; da Silva, G. A.; Freitas, M. C. C.; Souza Filho, A. P. S.; da Silva, M. N.; Arruda, A. C.; Guilhon, G. M. S. P.; Santos, L. S.; Santos, A. S.; Arruda, M. S. P.; *J. Braz. Chem. Soc.* **2010**, *21*, 1838.

15. Costa, E. T.; Oliveira, D. S.; Meyer, D. A.; Ferreira, V. M. M.; Soto, E. E.; Frausto, S.; Savage, D. D.; Browning, M. D.; Valenzuela, C. F.; *J. Biol. Chem.* **2000**, *275*, 38268.

16. Gao, H. M.; Hong, J. S.; Zhang, W.; Liu, B.; *J. Neurosci.* **2002**, *22*, 782.

17. Grammatopoulos, T. N.; Ahmadi, F.; Jones, S. M.; Fariss, M. W.; Weyhenmeyer, J. A.; Zawada, W. M.; *Brain Res.* **2005**, *1045*, 64.

18. Testa, C.M.; Sherera, T. B.; Greenamyre, J. T.; *Mol. Brain. Res.* **2005**, *134*, 109.

19. Tiwari, M.; Lopez-Cruzan, M.; Morgan, W. W.; Herman, B.; *J. Biol. Chem.* **2011**, *286*, 8493.

20. Fang, N.; Casida, J. E.; *J. Nat. Prod.* **1999**, *62*, 205.

21. Kaouadji, M.; Agban, A.; Mariotte, A. M.; Tissut, M.; *J. Nat. Prod.* **1986**, *49*, 281.

22. Seaton, T. A.; Cooper, J. M.; Schapira, A. H.; *Brain Res.* **1997**, *777*, 110.

23. Moldzio, R.; Radad. K.; Krewenka, C.; Kranner, B.; Duvigneau, J. C.; Wang, Y.; Rausch, W. D.; *J. Neural Transm.* **2010**, *117*, 5.

24. Radad, K.; Moldzio, R.; Taha, M.; Rausch, W. D.; *Phytother. Res.* **2009**, *23*, 696.

25. Da Costa, J. P. C.; Alves, S. M.; Belo, M.; *Acta Amaz.* **1999**, *29*, 563.