Isolation and Structure Determination of Bioactive Isoflavones from Callus Culture of *Dipteryx odorata*

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Crude extracts of callus and roots of *Dipteryx odorata* were analyzed by HPLC to detect and quantify isoflavone contents. Based on spectroscopic and X-ray crystallography data the structures of two isoflavones were elucidated as 7-hydroxy-4′,6-dimethoxyisoflavone and 3′,7-dihydroxy-4′,6-dimethoxyisoflavone. The production of dry biomass of 7-hydroxy-4′,6-dimethoxyisoflavone in cultured callus was 4.12 mg/g, approximately eleven fold higher than the amount accumulated in roots of *D. odorata* wild-growing plants. The 7-hydroxy-4′,6-dimethoxy-isoflavone was effective against glyceraldehyde-3-phosphate dehydrogenase from *Trypanosoma cruzi*. This is the first report on those bioactive isoflavones accumulated in callus of *D. odorata*.

Key words *Dipteryx odorata*; isoflavone; callus culture; GAPDH inhibitor

Great commercial value has been credited to the seeds of *Dipteryx odorata* WILLD. (AUBL.) (Leguminoseae) native from Amazon and also known as tonka beans or cumaru, because they are considered rich source of coumarins, used in cosmetics and perfumery.[1)] The presence of umbelliferone in seeds of tonka beans was described by Sullivan who also reported other natural compounds like isoflavonoids, triterpenoids, fatty acids and cassane diterpenoids on seeds of this species.[2)] Additionally some micromolecules specific of the heartwood of *D. odorata* as isoflavones retusin, retusin 8-methyl ether, 3′-hydroxyretusin 8-methyl ether, odoratin, dipteryxin, dipteryxine and odoratine; the triterpenes lupeol and betulin; and methyl esters of fatty acids have been isolated.[3,4)]

Isoflavonoids have gained considerable importance due to the diverse broad of biological activities correlated to them, including antioxidative, oestrogenic, inseticidal, piscicidal, antifungal, antimicrobial and contraceptive properties.[5,6)]

The pharmacological properties of isoflavonoids and the correlation between the consumption of isoflavonoids and reduction on the level of incidence of cardiovascular disease and cancer have increase the interest in the search for biologically active compounds from plant species of both, leguminous and non-leguminous sources.[7)]

In this work we report the establishment of *D. odorata* callus cultures focusing on a comparative study related to the production of tripanossomatidal GAPDH inhibitory isoflavones accumulated in roots of the intact plant and in callus cultures of this species.

Experimental

Plant Material *Dipteryx odorata* plants were collected in the campus of Universidade do Amazonas in Manaus, Brazil. Voucher specimen is deposited under the number HUAM 7276 in the Herbarium of the Universidade do Amazonas.

Seed Disinfestations Seeds of *D. odorata* were soaked in sterile distilled water and maintained under agitation overnight. Seeds were then immersed in 2% Benomyl solution for 4 h under agitation, washed in 70% hydroalcoholic solution, dipped in 1% CaClO for 40 min, washed for three times with sterile distilled water and inoculated into glass flasks (8.5 cm×2.5 cm i.d.) containing 5 ml MS medium[8)] added with 30 g/l sucrose and 2.0 g/l Phytagel, pH was adjusted to 6.0 before autoclaving. Flasks were sealed with polypropylene closures (Bellco), autoclaved at 121 °C and 105 kPa for 20 min and maintained at 25±2 °C, 55—60% relative humidity under 16 h photoperiod with 40 μmol m^{-2} s^{-1}, 85 W cool-white GE fluorescent lamps.

Callus Induction Leaves, hypocotyl, and roots of *D. odorata* seedlings produced *in vitro* were used as explants for callus induction. Explants were inoculated onto semisolid MS medium supplemented with 2.0 mg/l 2,4-D, 0.5 mg/l 6-BAP and 340 mg/l KH_2PO_4 and 2.0 g/l Phytagel, pH ajusted to 6.0. Callus were harvested and subcultured in 30-d intervals.

General Analytical Procedures TLC was carried out on silica gel G pre-coated plates (Sigma-Aldrich) using $CHCl_3$–MeOH (9 : 1) as solvent system. Spots were visualized employing iodine vapor as detection agent. Silica gel (Merck, 70—230 mesh) was used in column chromatography. Quantitative analysis were carried out on a Shimadzu Liquid chromatograph (LC-10AD) with a reverse phase C_{18} Supelcosil column (25 cm×4.6 mm id, 5 μm) under linear gradient elution of MeOH : H_2O 50%→85% (20 min), MeOH 100% (5 min), flow rate (0.9 ml/min), coupled to a photodiode array detector (SPD-M10A) set at 254 nm.

Extraction and Isolation Fresh callus (1.22 kg) were dried and extracted with $CHCl_3$ at room temperature. The chloroformic extract was then concentrated to 2.05 g residue, which was fractionated with $CHCl_3$: MeOH mixtures (98 : 2→1 : 1), AcOEt and MeOH respectively on silica gel column. Fractions containing isoflavones were further rechromatographed on silica gel affording isoflavone **1** (191 mg) and isoflavone **2** (57 mg), which were used as standards in the quantitative analysis.

Quantitative Analysis *D. odorata* callus (4.3 g) and roots (2.0 g) were dried and extracted separately with chloroform at room temperature for 20 min in ultrasonic bath. Crude extracts were dissolved in MeOH–H_2O (2 : 8) and then partitioned successively with hexane and $CHCl_3$. This procedure was repeated for three times. The chloroform phase of each extract was evaporated till dryness, dissolved in methanol, filtered through a 0.45 μm nylon membrane filter and analyzed through HPLC.

The quantitative analysis of isoflavones **1** and **2** were performed and a four point calibration curve (mean values of triplicates), was outlined with external standard within the range 0.004—0.088 μg/μl for **1** and 0.004—0.400 μg/μl for **2**. The curve presented linear detector response and correlation coefficients 0.99733 and 0.99744 respectively. The calibration plot was obtained by measuring respective peak areas.

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NMR Spectrometry All ^{1}H- and ^{13}C-NMR spectra were measured using a Bruker DPY300 spectrometer set at 300 MHz and 75 MHz, respectively, in DMSO-d_6 with TMS (tetramethylsilane) as internal standard.

X-Ray Crystal Data for **1**: $C_{17}H_{14}O_5$, MW=298.28, monoclinic, $P2_1/c$, a=6.751(1), b=28.470(1), c=7.393(1) Å, β=101.34(1)°, V=1393.2(3) Å^3, Z=4, D_{calc}=1.422 g/cm^3, Mo$K\alpha$ (λ=0.71073 Å), μ=0.105 mm^{-1}, F(000)=624. A total of 16844 reflections were collected at T=23±2 °C, using an Enraf Nonius Kappa CCD diffractometer with graphite monochromated Mo$K\alpha$ radiation. A prismatic crystal of dimensions 0.12×0.08×0.04 mm was used to collect data up to θ=50°. The structure was solved by direct methods and Fourier techniques and refined by full matrix least squares on F^2 to $R1$=0.055, $WR2$=0.1413, S=1.061, using 2439 independent reflections (R_{int}=0.076) with $I>2\sigma(I)$ and 213 parameters. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were positioned on stereochemical grounds and refined with the riding model. A final difference electron density map showed largest peak and hole of 0.222 and −0.186 e/Å^3 respectively.

Crystal Data for **2**: $C_{17}H_{14}O_6$, MW=314.29 monoclinic, $P2_1/c$, a=6.813(1), b=28.529(1), c=7.237(1) Å, β=101.23(2)°, V=1379.7(3) Å^3, Z=4, D_{calc}=1.454 g/cm^3, Mo$K\alpha$ (λ=0.71073 Å), μ=0.109 mm^{-1}, F(000)=630. A total of 7321 reflections were collected at T=23±2 °C, using an Enraf Nonius KappaCCD diffractometer with graphite monochromated Mo$K\alpha$ radiation. A prismatic crystal of dimensions 0.09×0.06×0.04 mm was used to collect data up to θ=50°. The structure was solved by direct methods and Fourier techniques. The keto oxygen atom was disordered. Because of their large correlation, the occupancy and thermal factors of this atom were not refined simultaneously. The former was refined for various fixed values of the latter until a minimum of $WR2$ was attained. The occupancy factor converged to a value of 0.30 and this result, together with the facts that unit cells are nearly the same and space groups are identical, lead to the conclusion that the structure is a disordered arrangement of 70% of compound **1** and 30% of compound **2**. The structure was refined by full matrix least squares on F^2 to $R1$=0.071, $WR2$=0.1744, S=0.982, using 2408 independent reflections (R_{int}=0.121) with $I>2\sigma(I)$ and 221 parameters. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were positioned on stereo-chemical grounds and refined with the riding model. A final difference electron density map showed largest peak and hole of 0.314 and −0.296 e/Å^3 respectively.

The crystal structures were deposited at the Cambridge Crystallographic Data Centre and allocated the deposition numbers CCDC 224005 and 224006.

Preparation and Purification of Recombinant *T. cruzi* GAPDH TcGAPDH was overexpressed and purified as reported by Souza *et al.*[9] It is maintained in the Crystallography Laboratory of the Universidade de São Paulo, São Carlos, SP, Brazil, where the enzymatic inhibition studies were carried out.

***T. cruzi* GAPDH-Activity** TcGAPDH activity was determined according to a modification of a previously reported procedure.[10] Reduced NADH was measured spectrophotometrically at 340 nm at 30 s interval. The reaction medium was 50 mmol·l^{-1} Tris–HCl pH 8.6 buffer, 1 mmol·l^{-1} EDTA, 1 mmol·l^{-1} β-mercapto-ethanol, 30 mmol·l^{-1} Na_2HAsO_4, 2.5 mmol·l^{-1} NAD^+, 0.3 mmol·l^{-1} glyceraldehyde-3-phosphate and 4—9 μg protein, in a total volume of 1000 μl. The reaction was initiated by the addition of enzyme.

The specific activity (unit=U) of the enzyme was calculated as below:

$$(\mathrm{U\cdot mg^{-1}}) = \{(\Delta \text{ absorbance}/\Delta t) \times \text{volume of cell}\}/6.22 \times \text{volume of enzyme} \times [\text{enzyme}]$$

Where Δt=0.5 min; volume of cell=1.00 ml; $^{\varepsilon}$NADH=6.22 (mmol·l^{-1})$^{-1}$ cm^{-1}; volume of enzyme=0.005 ml; [enzyme] concentration of enzyme in mg·ml^{-1}.

***T. cruzi* GAPDH-Inhibitory Activity** The inhibitory activity was recorded using the reaction medium as above, in a total volume of 1000 μl. Absorbance was read at 340 nm at 30 s interval. Isoflavonoids were tested at 25 and 50 μg·ml^{-1} in 10% DMSO using 5 μl of GAPDH at 0.90 mg·ml^{-1}.

In each case, a blank experiment was performed with 10% DMSO in the reaction medium and was used as the positive control. The specific activity of TcGAPDH was not significantly affected by the presence of 10% DMSO.

Data were means of 3 repetitions and values as percent of control were used as follows:

$$\%\text{ inhibitory activity} = \{(\mathrm{U\cdot mg^{-1}control} - \mathrm{U\cdot mg^{-1}compound})/\mathrm{U\cdot mg^{-1}control}\} \times 100$$

Results and Discussion

Callus induction at the cut edge of all types of explants tested occurred and developed within 14—15 d of inoculation. After 3 subcultures callus initiated from leaves or hypocotyl became dark showing necrosis. Only callus induced from roots maintained vigorous growth after several subcultures and therefore were selected for the phytochemical and biological studies.

UV spectra of compounds **1** and **2** detected in the chloroform extract of *D. odorata* callus, showed two maximal absorption at 258 nm and 323 nm, and 257 and 319 respectively, typical of isoflavones.[11] Spectral data (^{13}C-NMR, Table 1) resembled those published for 4′,6-dimethoxy-7-hydroxy-isoflavone (**1**) known as afrormosin[12] and castanin[13] respectively and 4′,6-dimethoxy-5′,7-dihydroxy-isoflavone (**2**) known as odoratin, which have been previously detected in *D. odorata* and *Glycine max* respectively.[4,5,11,12] Final confirmation of the structure of compounds **1** and **2** was achieved through X-ray diffraction analysis (Figs. 1, 2).

The X-ray crystal structure of compound **1** has already been reported as crystallized in the orthorhombic system, space group $Pca2_1$.[12] In our study, both compounds **1** and **2** crystallize in the monoclinic system, space group $P2_1/c$ and they are crystallographicaly isomorphic. Compound **1** is basically identical to the form previously reported except for the dihedral angle between the substituent phenyl and the fused ring system of 47.67(6)° (53.9° in the orthorhombic

Table 1. ^{13}C-NMR Spectral Data for Compounds **1** and **2**, 75 MHz in DMSO-d_6 (Chemical Shift δ ppm)

Carbon	**1**	**2**
2	153.1	153.1
3	124.7	125.1
4	174.4	174.4
5	104.9	104.9
6	147.1	147.1
7	152.9	152.9
8	103.0	103.0
9	151.9	151.8
10	116.4	116.6
1	122.8	123.0
2′	130.2	116.4
3′	113.8	146.2
4′	159.1	147.6
5′	113.8	112.1
6′	130.2	119.9
OMe	56.0	55.8
OMe	55.3	55.0

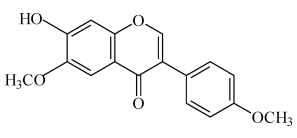

Fig. 1. 7-Hydroxy-4′,6-dimethoxyisoflavone; Afrormosin

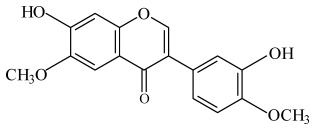

Fig. 2. 3′,7-Dihidroxy-4′,6′-dimethoxyisoflavone; Odoratin

form). Compound **2** was obtained as a disordered structure formed from 70% of compound **1** and 30% of compound **2**, as determined by the site occupation least squares refinement of the keto oxygen atom. The dihedral angle, in this case, was 45.8(1)°. In both compounds the phenyl substituent and the fused ring system were planar (root mean square deviation of fitted atoms 0.003 and 0.014 for compound **1** and 0.007 and 0.011 for compound **2**, respectively).

HPLC quantitative analysis of the chloroform extracts from callus and roots of *D. odorata* revealed that the main constituents in both extracts were isoflavonoids **1** (t_R= 14.43 min) and **2** (t_R=13.70 min). HPLC analysis showed that the production of the 7-hydroxy-4′,6-dimethoxyisoflavone (**1**) and 3′,7-dihydroxy-4′,6-dimethoxyisoflavone (**2**) were 4.12±0.16 mg/g d.w. and 1.54±0.33 mg/g d.w. respectively in callus cultures and 0.37±0.03 mg/g d.w. and 0.48±0.05 mg/g d.w. in roots cultured *ex vitro*. Obtained results demonstrated that isoflavone **1** accumulation in callus cultures is 11 times higher than in roots.

The chemical investigation carried out in this work, lead to the detection of 2 isoflavones in callus culture of *D. odorata* and as far as the authors know, this is the first time that the occurrence of isoflavones in callus of that species is reported.

A considerable difference in isoflavone production was observed when comparing the accumulation in intact plants organ and callus cultures.

Efforts have been made to elucidate genes related to plant secondary metabolism, in order to investigate the gene expression pattern of key enzymes which regulate the main biosynthetic routes of isoflavonoids and other compounds. This information might be useful, in the search for enhanced metabolite production through genetic and biochemical manipulation.[14)]

Compound **1** was evaluated for the ability to inhibit the enzymatic activity of the protein glycosomal GAPDH from *T. cruzi*. The activity of isoflavone **1** was comparable to those the polymethoxylated flavones which act as strong inhibitors. The 50% inhibitory concentration value (IC_{50}) was 84 μM, while in the 3′,4′,5′,5,7-pentamethoxyflavone was 81 μM.[15)] The positive control used was the coumarin chalepin (IC_{50} 64 μM).[15)] Thus, clearly the here reported isoflavonoids deserve more attention as micromolar inhibitors of GAPDH, mainly due to the fact that the conditions for the *D. odorata* callus cultures may be manipulated and once optimized will afford a highly attractive source to various bioactive analogues of the isoflavonoid series.

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