Studies on Aldose Reductase Inhibitors from Natural Products. II.[1] Active Components of a Paraguayan Crude Drug "Para-parai mí," *Phyllanthus niruri*

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Aldose reductase (AR) inhibitory activity-directed fractionation of the 70% ethanolic extract of Para-parai mí, *Phyllanthus niruri*, has led to the isolation of three active components, ellagic acid (1), brevifolin carboxylic acid (4) and ethyl brevifolin carboxylate (5). Among them, 1 showed the highest inhibitory activity, being about 6 times more potent than quercitrin, which is a known natural inhibitor of AR.

Keywords *Phyllanthus niruri*; Euphorbiaceae; aldose reductase inhibitor; ellagic acid; brevifolin carboxylic acid; ethyl brevifolin carboxylate

In screening tests for biological activities of medicinal plants in Paraguay, Para-parai mí, the whole plant of *Phyllanthus niruri* L. (Euphorbiaceae), which has traditionally been used for dissolution of calculus and as a diuretic in Paraguay, was found to have inhibitory activities against angiotensin converting enzyme (ACE) and rat lens aldose reductase (AR), which plays a significant role in the reduction of aldose to alditol under abnormal conditions such as diabetes. We previously reported the isolation of geraniin as an ACE inhibitor.[3] In this paper, we report the isolation and identification of the active components inhibiting rat lens AR.

The 70% EtOH extract (A) of Para-parai mí which showed high inhibitory activity towards crude rat lens AR ($IC_{50} = 1.0 \times 10^{-6}$ g/ml), was partitioned between *n*-hexane and water to afford the *n*-hexane extract (B), water layer and a precipitate (C) insoluble in both solutions. The water layer was further extracted with $CHCl_3$ and *n*-BuOH successively to afford the $CHCl_3$ extract (D), *n*-BuOH extract (E) and a residue (F) (Fig. 1). The precipitate C, which was most active (Table I), was applied to a column of Sephadex LH-20 to give five compounds **1**—**5**. Compounds **1** and **2** were identified as ellagic acid and gallic acid by comparing them with the respective authentic samples. Compound **3** gave **1**, **2** and D-glucose on hydrolysis with 2 N HCl suggesting it to be corilagin[4] and this identification was cofirmed by direct comparison with an authentic sample.

Compound **4**, mp > 250 °C, gave a positive color reaction to $FeCl_3$ and was also considered to be an ellagic acid analogue from the spectral data. Compound **4** showed a molecular ion peak at *m/z* 292 in the mass spectrum (MS), and its infrared (IR) spectrum suggested the presence of carboxylic acid and α,β-unsaturated lactone moieties (1710, 1680 and 1620 cm^{-1}). Decarboxylation of **4** in a fused glass tube at 140 °C gave brevifolin[4] which was identified by comparison with an authentic sample. As a result, **4** was deduced to be brevifolin carboxylic acid.[5] Compound **4** was methylated with diazomethane to give trimethyl brevifolin carboxylic acid methyl ester, whose melting point (164—166 °C) coincided with the reported value.[5]

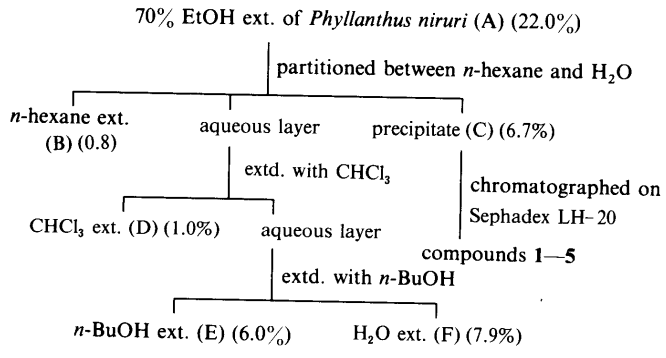

Fig. 1. Fractionation of Biologically Active Constituents of *Phyllanthus niruri*

Values in parenthesis indicate the yield (%) from dried material.

Compound **5**, yellowish powder, showed a molecular ion peak at *m/z* 320 in the MS ($-C_2H_5$ unit more than **4**) and absorption bands at 1720, 1690, 1630 and 1600 cm^{-1} in the IR spectrum, suggesting it to be the ethyl ester of **4**. In addition, the ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum of **5** (given in the Experimental section) was consistent with this structure.

Compounds **1**—**4** have generally been regarded as hydrolysis products of geraniin,[6-9] which was isolated as an ACE inhibitor from this plant[3] and is distributed widely in plants, especially in Euphorbiaceae.[9] This is the first report of the natural occurrence of **5**, but the possibility remains that it might be an artifact derived from geraniin. No other active constituent was found in the extract E.

Inhibitory Effect on Crude Rat Lens AR The isolated compounds **1**—**5** and geraniin were tested for AR-inhibitory activity (Table I). (Geraniin showed no effect and has not been included in Table I.) Among them, **1** exhibited the highest activity ($IC_{50} = 2.0 \times 10^{-7}$ M), being more potent than quercitrin ($IC_{50} = 1.2 \times 10^{-6}$ M), tested as a reference.

TABLE I. Inhibition of Crude Rat Lens AR by Extracts from *Phyllanthus niruri* and Compound **1**—**5**

Extract	IC_{50}(μg)	Compound	IC_{50}(μM)
A	1.0	**1**	0.2
B	>20	**2**	—
C	0.3	**3**	—
D	11.0	**4**	2.4
E	1.2	**5**	5.0
F	3.8	Quercitrin[a]	1.2

a) Quercitrin was assayed previously, and was tested again as a reference in this study.

Compounds **4** and **5** also showed some effect.

In order to determine the type of inhibition, the kinetics of inhibition of AR by **1** were plotted according to Lineweaver and Burk and **1** was found to be a non-competitive inhibitor at the concentration of IC_{50} (2.0×10^{-7} M), as was seen in the cases of quercitrin and 5,7,4′-trihydroxy-3,6-dimethoxyflavone[1] reported by us.

Results and Discussion

It is currently expected that AR inhibitors will play an important role in the management of diabetic complications such as cataract. Carboxylic acid, flavonoid and hydantoin analogues, *etc.*, have hitherto been examined as AR-inhibitory drugs, but this is the first report of ellagic acid and tannin-related compounds as AR inhibitors. Tannins have many kinds of biological activities, but geraniin, a typical tannin, showed no effect on AR, whereas ellagic acid, considered to be a hydrolysis product of geraniin, showed a strong activity. We are now investigating the AR-inhibitory effect of ellagic acid-related compounds and will report the results in the future.

Experimental

Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Spectral data were obtained as follows: IR spectra with a Hitachi 260-0611 spectrophotometer; ultraviolet (UV) spectra with a Hitachi 270S spectrophotometer; MS with a JEOL JMS-D 200 spectrometer (70 eV); ^{13}C-NMR spectra with a Varian XL-200 (50.3 MHz) spectrometer in dimethyl sulfoxide (DMSO)-d_6. Chemical shifts are given in δ(ppm) values referred to internal tetramethylsilane.

Plant Materials Para-parai mí was purchased from local dealers in Asuncion, Paraguay, and identified as *Phyllanthus niruri* L. Voucher specimens are on deposit at both the Institute of Toyama Medical and Pharmaceutical University and Asuncion University.

Bioassay Crude AR was obtained from the supernatant fraction of the homogenate of rat lens according to the method of Kador and Sharpless,[10] and showed a specific activity of 22 units/mg. One unit was defined as the amount catalyzing the oxidation of 1 μmol of reduced nicotinamide adenine dinucleotide phosphate per minute. Inhibitory effects of the extract and isolated compounds on AR were assayed by the method previously reported.[1,11] Samples were dissolved in DMSO which was found to have no effect on the enzyme activity at below 0.1% concentration.

Extraction and Isolation Dried powder of the material (3.7 kg) was extracted with hot 70% EtOH (1 h × 3) to yield 796 g of the dried extract A. The extract A (786 g) was partitioned between *n*-hexane (3.9 l × 5) and distilled water (3.9 l) to afford an *n*-hexane extract B and an insoluble precipitate C (243 g). The aqueous layer was extracted with $CHCl_3$ and *n*-BuOH successively to yield $CHCl_3$, *n*-BuOH and H_2O extracts, D, E and F. The biologically active C (32 g) was chromatographed on a Sephadex LH-20 column eluting first with 70% MeOH and then with increasing percentages of MeOH to yield compounds **1** (3.6 g), **2** (10 mg), **3** (26 mg), **4** (10 mg) and **5** (25 mg). Ellagic acid (**1**), mp > 300 °C, gallic acid (**2**), mp 240—241 °C (dec.) and corilagin (**3**), mp 193—195 °C (dec.) were identified by direct comparisons with the respective authentic samples (IR, paper partition chromatography).

Brevifolin Carboxylic Acid (4) Yellowish powder, mp > 250 °C (dec. not sharp) (MeOH), yellow–green to $FeCl_3$ reagent. MS *m/z*: 292 (M^+), 246 (M − COOH − H), 218 (246 − CO), 204, 190, 162. *Anal.* Calcd for $C_{13}H_8O_8$: C, 53.44; H, 2.76. Found: C, 53.21; H, 2.90. IR ν_{max}^{KBr} cm^{-1}: 3200, 1710, 1680, 1620, 1590. UV λ_{max}^{MeOH} nm (log ε): 365 (3.79), 350 (3.81), 280 (4.13). A solution of **4** (3 mg) in distilled H_2O (2 ml) was heated in a fused glass tube at 140 °C for 1 h, and the precipitate was recrystallized from MeOH to afford brevifolin (2 mg), which was identified by comparison with an authentic sample (IR). **4** gave trimethyl brevifolin carboxylic acid methyl ester, mp 164—165 °C, with CH_2N_2. (Spectral data could not be obtained due to an insufficient amount of sample).

Ethyl Brevifolin Carboxylate (5) Yellowish powder, mp > 250 °C (dec.) (MeOH), yellow–green to $FeCl_3$ reagent. MS *m/z*: 320 (M^+), 274 (M–OC_2H_5–H), 246, 218 (246 − CO), 204, 190, 162. *Anal.* Calcd for $C_{15}H_{12}O_8$: C, 56.26; H, 3.78. Found: C, 56.02; H, 3.89. IR ν_{max}^{KBr} cm^{-1}: 3150, 1720, 1690, 1630, 1600. UV λ_{max}^{EtOH} nm (log ε): 365 (3.94), 350 (3.96), 276 (4.34). ^{13}C-NMR (50.3 MHz, DMSO-d_6) δ: 192.9, 171.9, 160.0, 149.5, 145.6, 143.5, 140.1, 138.4, 138.3, 114.8, 112.8, 107.9, 60.5, 36.9, 13.8.

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