SUPPLEMENTARY MATERIAL

Phytochemical analysis with the antioxidant and aldose reductase inhibitory capacities of *Tephrosia humilis* aerial parts' extracts

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The aerial parts of *Tephrosia humilis* were tested about their antioxidant potential, their ability to inhibit the aldose/aldehyde reductase enzymes and their phenolic content. The plant material was exhaustively extracted with petroleum ether, dichloromethane and methanol consecutively. The concentrated methanol extract was re-extracted, successively, with diethyl ether, ethyl acetate and n-butanol. All extracts showed significant antioxidant capacity, but the most effective was the ethyl acetate extract. As about the aldose reductase inhibition, all fractions, except the aqueous, were strong inhibitors of the enzyme, with the n-butanolic and the ethyl acetate fractions to inhibit the enzyme above 75%. These findings provide support to the ethnopharmacological usage of the plant as antioxidant and validates its potential to act against the long term diabetic complications.

The phytochemical analysis showed the presence of 1,4-dihydroxy-3,4-(epoxyethano)-5-cyclohexene(**1**), clerodindicin E(**2**), lupeol(**3**), methyl p-coumarate(**4**), methyl 4-hydroxybenzoate(**5**), prunin(**6**), 5,7,2',5'-tetrahydroxyflavanone 7-rutinoside(**7**), protocathechuic acid(**8**), luteolin 7-glucoside(**9**), apigenin(**10**), naringin(**11**), rhoifolin(**12**) and luteolin 7-glucuronate(**13**).

Keywords: Tephrosia; antioxidant activity; DPPH; luminol chemiluminescence; aldose reductase; lipoxxygenase

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1. Experimental

1.1. General experimental procedures - Instrumentation

All solvents and chemicals used were of analytical or HPLC grade and were purchased from Merck (Germany) and Panreac (Spain).

DPPH (1,1-diphenyl-2-picryl-hydrazyl, 90%) and boric acid was from Sigma (Germany), luminol (3-aminophtahydrazine) from Alfa Aesar, EDTA and H₂O₂ from Panreac and CoCl₂·6H₂O from Riedel-de Haen. All reagents and solvents mentioned in the assessment of ALR2 activity in vitro are commercially available and were obtained from Merck, Aldrich, Fluka and Pfizer. DMSO, LLA (Linoleic acid), LOX (Lipoxygenase) and Tris·HCl (pH=9.00) were obtained from Sigma Aldrich Corporation.

Preparative TLC was conducted on 0.1mm cellulose plates (20x20cm) (Merck, Art. 5552), 0.2mm silica gel 60 F254 plates (20x20cm) (Merck Art. 5554) and 0.2mm silica gel 60 plates (20x20cm) (Merck, Art. 5553) and the detection by Naturstoffgenz A and 1% vanillin/H₂SO₄ (Acros Organics).

Column chromatography (CC): Polyamide CC6 Macherey-Nagel (70-160µm) and Silica gel 60, 0.040-0.063mm (Merck, Art.9385), gradient elution with the solvents mixtures indicated in each case; Sephadex LH-20 (Amersham Biosciences) elution with MeOH. Polyamide CC6 Macherey-Nagel (70-160µm) and silica gel 60H (Merck, Art. 7736) were utilized for Vacuum Liquid Chromatography (VLC).

For High Pressure Liquid Chromatography (HPLC), an HPLC SSI Marathon III Pump, an SSI 232C Gradient elution system and a Variable UV-Vis SSI 500 detector with an SSI 232 Clarity Lite software were used, accompanied by an Apollo C-18 Semi-prep column (250mmx5mm, 5µm) and an Apollo C-18 PrepGuard column (33mmx7mm, 5µm). For medium-pressure liquid chromatographic (MPLC) separations, Gilson M312 peristaltic pump and polyamide CC6 Macherey-Nagel (70-160µm) were used.

The UV-Vis spectra on a Hitachi U-2001 spectrophotometer and a Hitachi U-2000 spectrophotometer and the chemiluminescence method was conducted on a Sirius Luminometer (Berthold Detection Systems).

The NMR spectra were run on a Varian 600MHz (599.833MHz for ¹H-NMR and 150MHz for ¹³C-NMR), a Bruker DPX 300 (300.13MHz for ¹H-NMR and 75.5MHz for ¹³C-NMR) and a Bruker AMX 500 (500.1MHz for ¹H-NMR and 125.5MHz for ¹³C-NMR) with the solvents CDCl₃, CD₃OD- d₄ and DMSO-d₆ indicated in each case.

The mass spectra were recorded on a HR-MS LTQ Orbitrap velos spectrometer (ThermoScientific), a Shimadzu GC/MS QP 2010 mass spectrometer (Shimadzu GC 2010-gas chromatograph), an MS/MS TQ Detector-Ultra Performance LC spectrometer (Acquity Waters) and a LC-MS (Shimadzu 2020) spectrometer.

1.2. Plant material

Aerial parts of *Tephrosia humilis* were collected in July 2006 from the region of the Central African Republic. The plant material was air-dried in the shade, packed in tightly closed containers and stored for phytochemical and biological studies. Species identification was carried out by Dr. Nicodeme D. N'Dinda from the Agriculture and Livestock Institute of Bangui, Central African Republic. Plant material was collected following international bioethics guidelines. A

voucher specimen was deposited in the herbarium of the Laboratory of Pharmacognosy, School of Pharmacy, Aristotle University of Thessaloniki, under the code No 1214-YAKORO.

1.3. Extraction and isolation of compounds

The plant material (300g) was put into a Soxhlet apparatus 1l and was exhaustively extracted with petroleum ether, dichloromethane and methanol consecutively. The obtained extracts were evaporated under vacuum to dryness. The dry remaining of the methanolic extract (44.68g – 14,89%) was dissolved in hot water, filtrated and partitioned with the following solvents of increasing polarity: diethyl ether, ethyl acetate and *n*-butanol. The organic layers were evaporated under vacuum to dryness. Finally the remaining aqueous extract was collected. The dry weights of the extracts were 3.06g (1,02%), 1.34g (0,45%), 6.48g (2,16%) and 15.63g (5,21%) respectively. The ethyl acetate extract additively gave during concentration a residue, which was also evaluated.

4.51g of the dichloromethane extract (5.21g – 1,74%) was subjected to VLC (7.0 x 10cm) on silica gel (Merck, Art. 7736) using $C_6H_{14}-CH_2Cl_2-MeOH-H_2O$ mixtures of increasing polarity as eluents to yield 15 fractions (MTH-A – MTH-P). Fraction MTH-K (363.5mg; eluted with CH_2Cl_2-MeOH 92:8) was applied to CC (21 x 2.0cm) on silica gel (Merck, Art. 9385) and yielded compound **1** (135.9mg; eluted with CH_2Cl_2-MeOH 94:6). Fraction MTH-N (211.3mg; eluted with CH_2Cl_2-MeOH 80:20) was submitted to CC (21 x 2.0cm) over silica gel (Merck, Art. 9385) and yielded compound **2** (7.7mg; eluted with CH_2Cl_2-MeOH 94:6).

1.14g of the diethyl ether extract was subjected to CC (21 x 1.8cm) on silica gel (0.040-0.063mm, Merck Art.9385) using $C_6H_{14}-CH_2Cl_2-MeOH-H_2O$ mixtures of increasing polarity as eluents and afforded 41 fractions (TDS₁ – TDS₄₁). Fraction TDS₈ (8.8mg; eluted with $C_6H_{14}-CH_2Cl_2$ 75:25) was applied to CC (40 x 1.3cm) over Sephadex LH-20 (MeOH) and yielded compound **3** (1.6 mg). Fraction TDS₁₅ (34.3mg; eluted with $C_6H_{14}-CH_2Cl_2$ 25:75) was applied to CC (70 x 1.4cm) over Sephadex LH-20 (MeOH) and yielded compound **4** (2.9mg) and compound **5** (2.2mg).

750mg of the ethyl acetate extract was chromatographed on a polyamide (70-160µm) CC-6 column (39 x 2.2 cm) with a water–methanol gradient to yield 16 fractions (TAP₁-TAP₁₆). Fraction TAP₃ (48.5mg; eluted with 100% H₂O) was applied to CC (55 x 1.3cm) over Sephadex LH-20 (MeOH) and yielded 23 subfractions. Subfractions 17-21 (11.0mg) were dried, dissolved in a minimum volume of water and were subjected to preparative TLC on cellulose plates with CAW (CH_2Cl_2 :Acetic acid:Water 50:45:5) as elution solvents and afforded compounds **6**(4.4mg) and **7**(1.9mg). Fraction TAP₅ [28.5mg; eluted with H₂O-MeOH (from 90:10 to 80:20)] was applied to CC (55 x 1.3cm) over Sephadex LH-20 (MeOH) and yielded 5 subfractions (TAP_{5A}-TAP_{5E}). Subfraction TAP_{5C} (6.7mg) was dried, dissolved in a minimum volume of water and was subjected to preparative TLC on cellulose plates with CAW (CH_2Cl_2 :Acetic acid:Water 50:45:5) as elution solvents and afforded six separate bands. The band with the deep purple luminescence was separately extracted with methanol and rechromatographed with preparative TLC on cellulose plates with AcOH 15%. The band corresponding to the compound **8** was seen as a distinct purple band and was separately extracted with methanol and concentrated (1.1mg). Fraction TAP₈ [37.3mg; eluted with H₂O-MeOH (from 70:30 to 60:40)] was applied to CC (65.0 x 1.4cm) over Sephadex LH-20 (MeOH) and yielded compound **9** (3.1mg). Fraction TAP₁₂ (19.2mg; eluted with H₂O-MeOH 20:80) was subjected to preparative TLC on cellulose plates with CAW

(CH₂Cl₂:Acetic acid:Water 50:45:5) as elution solvents and afforded seven separate bands. The band with the yellow luminescence was separately extracted with methanol and was rechromatographed and applied to CC (65.0 x 1.4cm) over Sephadex LH-20 (MeOH) and yielded compound **10** (1.1mg).

3.37mg of the butanol extract was chromatographed on a polyamide (70-160µm) CC-6 column (39 x 2.2 cm) with a water–methanol gradient to yield 11 fractions (TBP₁-TBP₁₁). Fraction TBP₃ (104.9mg; eluted with 100%H₂O) was subjected to preparative TLC on cellulose plates with EAW (Ethyl acetate:Acetic acid:Water 4:1:2) as elution solvents and yielded compound **11**(5.0mg). Compound **12** was isolated from the TBP₅ (201.4mg; eluted with H₂O-MeOH (from 100%H₂O to 80:20) fraction with HPLC (eluted at the 34min), [Rp C-18 (250x7mm, 5µm)], and with the following gradient program: (A) AcOH (2%) and (B) MeOH, 53% A at 0 min, 52% A for 40min at a flow rate of 1.7 ml/min and the detection was monitored at 268nm. Fraction TBP₁₁ [163.0mg; eluted with H₂O-MeOH (from 40:60 to 100%MeOH)] was subjected to preparative TLC on cellulose plates with CAW (CH₂Cl₂:Acetic acid:Water 50:45:5) as elution solvents and yielded compound **13**(2.1mg).

1.4. Pharmacological study

1.4.1. Evaluation of the antioxidant activity using the DPPH[•] method

The activity of 1,1-diphenyl,2-picrylhydrazyl (DPPH[•]) radical scavenging activity was investigated according to the method described by Parejo et al. 2000. Different concentrations of all extracts were prepared . An aliquot of 25 µl of diluted sample was added to 975µl DPPH[•] solution (2 x 10⁻⁵M) and the mixture vortexed. The decrease in the absorbance was determined at 515nm when the reaction reached a plateau, using a U-2000 HITACHI spectrophotometer, in a 10 mm quartz cuvette. For the samples well diluted in methanol, methanol was used to zero the spectrophotometer. The absorbance of the DPPH[•] radical without any sample was measured. The DPPH[•] concentration in the reaction medium was calculated from the calibration curve.

For each sample concentration tested, the percentage of DPPH[•] remaining in the steady state, was calculated in the following way:

Percentage of remaining DPPH[•]=[DPPH[•]]_{at t=T}/[DPPH[•]]_{at t=0}, where T is the time necessary to reach the steady state.

The antioxidant capacity of each sample was expressed as the amount of sample necessary to decrease the initial DPPH[•] concentration by 50% (EC₅₀). The antiradical efficiency (AE) is calculated as follows: AE=1/EC₅₀.

1.4.2. Estimation of the antioxidant activity using the Co(II)/EDTA – induced luminol chemiluminescence method

The antioxidant activity was also determined using the Co(II)/EDTA – induced luminol chemiluminescence method, according to Gülşen et al. (2007), with some modification. The chemiluminescence measurements were carried out on Serius Single Tube Fluorimeter (Berthold Detection Systems). At least three different dilutions of the extracts were prepared. 1ml of borate buffer (0.05M, adjusted to pH 9 with 1M NaOH), containing 1mg/ml EDTA and 0.2mg/ml of CoCl₂·6H₂O was added to 100µl of luminol solution (5.6 x 10⁻⁴M) in borate buffer (0.05M, adjusted to pH 9 with 1M NaOH) in a test tube and the mixture vortexed for 15s. The above aliquot was then deposited on the bottom of a

10x10 glass cuvette using precision pipettes. An aliquot of 25µl of sample and 25µl of H₂O₂ solution (5.4 x 10⁻¹M) were then added into the cuvette, the solution was thoroughly mixed, and the CL measurements were recorded. The instantaneous reduction in the CL intensity, elicited by the addition of the sample, was recorded as I, and the CL intensity in the absence of the sample was recorded as I₀. The ratio I₀/I was calculated. This ratio vs. µg extract/ml was plotted for three prepared dilutions of each extract and a linear regression was established in order to calculate IC₅₀. IC₅₀ is the amount of sample needed to decrease, by 50%, the CL intensity, according to the equation:

$$[I_0/I = a \text{ (mg extract/ml)} + b]$$

The antiradical efficiency (AE = 1/IC₅₀) was also calculated. Results were also expressed as standard equivalents using quercetin and trolox, on the basis of the IC₅₀ value.

1.4.3. Soybean lipoxygenase inhibition

The bioassay was performed according to Charami et al. (2008). All extracts were initially dissolved in DMSO (approximately 50 mg in 2 mL DMSO). The incubation mixture consisted of 100µl of the test sample, 100µl of sodium linoleate (0.1 mM) and 0.2mL of the enzyme solution (1/3 x 10⁻⁴, w/v in saline). After incubation at room temperature for 3 min, the conversion of sodium linoleate to 13-hydroperoxylinoleic acid was recorded at 234 nm when the absorbance reached a plateau.

1.4.4. ALR2 & ALR1 inhibitory activity and selectivity

Male Wistar rats, 8–9 weeks old, weighing 200–250 g, were used. The animals came from the Breeding Facility of the Institute of Experimental Pharmacology and Toxicology, Dobra Voda (Slovak Republic). The study was approved by the Ethics Committee of the Institute and performed in accordance with the Principles of Laboratory Animal Care (NIH publication 83–25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, Part 139, July 9th 2003). The animals in light ether anesthesia were killed by exsanguinations of the carotid artery and the eye globes were excised. The lenses were quickly dissected and rinsed with saline. The homogenate was centrifuged at 10,000g at 0–4 °C for 15 min. The supernatant was precipitated with saturated ammonium sulfate at 40% salt saturation and this solution was centrifuged at 10,000g at 0–4 °C for 15 min. The latter supernatant was either used directly or stored for maximum 24 h at -80 °C. As for ALR1, the kidneys were removed from the rats of both sexes following euthanasia and were homogenized in a knife homogenizer followed by processing in a glass homogenizer with a teflon pestle in 3 vol of 10 mM sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose, 2.0 mM EDTA dipotassium salt, and 2.5 mM β-mercaptoethanol. The homogenate was centrifuged at 10,000g at 0–4 °C for 30 min and the supernatant was subjected to ammonium sulfate fractional precipitation at 40%, 50%, and 75% salt saturation. The pellet obtained from the last step, possessing ALR1 activity, was redissolved in 10 mM sodium phosphate buffer, pH 7.2, containing 2.0 mM EDTA dipotassium salt and 2.0 mM β-mercaptoethanol to achieve total protein concentration of approx. 20 mg/mL. DEAE DE 52 resin was added to the solution (33 mg/mL) and after gentle mixing for 15 min removed by centrifugation. The supernatant containing ALR1 was then stored in smaller aliquots at -80 °C.

ALR2 and ALR1 activities, according to Chatzopoulou et al. (2011), were assayed spectrophotometrically by determining NADPH consumption at 340nm. In order to determine ALR2 inhibitory activity, D,L-glyceraldehyde was used as a substrate and the measurements took place at 30°C, whereas ALR1 inhibitory activity was determined with

D-glycuronate as a substrate and the measurements took place at 37°C. The experiments were performed in triplicate. The average reaction rate was ~0.002 Absorbance Units of NADPH per minute. The % inhibition is calculated from the ratio $[(V_{\text{control}} - V_{(+\text{inhibitor})})/V_{\text{control}}] \times 100$, where V_{control} is the reaction rate of the control and $V_{(+\text{inhibitor})}$ is the reaction rate of the sample.

Figure S1. *Tephrosia humilis*

Figure S2. Aerial parts of *Tephrosia humilis*

Figure S3. Structures of compounds **1-13**

Figure S4 (Detail of the NMR spectrum of naringin)

Table S1 - Antioxidant capacities of the extracts of *Tephrosia humilis* expressed as EC₅₀ and IC₅₀ and AE

	DPPH [•] radical scavenging method		Chemiluminescence method	
Sample (extract)	EC ₅₀ ±SD ^A	AE ^B	IC ₅₀ ±SD ^C	AE ^D
Initial Methanolic	1.27301 ±0.04	0.78554	0.4510 ±0.006	2.217
Diethyl Ether	1.324513 ±0.04	0.75499	0.2568 ±0.03	3.963
Ethyl Acetate	0.433928 ±0.03	2.30453	0.073137 ±0.00002	13.673
Ethyl acetate residue	0.65396 ±0.03	1.52915	0.9480 ±0.13	1.054
Butanolic	0.54502 ±0.02	1.8348	3.6630 ±0.87	0.290
Aquatic	4.48602 ±0.1	0.22291	3.6376 ±0.02	0.275

^A Efficient concentration (mg antioxidant/mg DPPH[•]): Amount of antioxidant needed to decrease the initial DPPH[•] concentration by 50%.

^B Antiradical efficiency: 1/EC₅₀.

^C Efficient concentration (mg antioxidant/ml): Amount of antioxidant needed to decrease the initial chemiluminescence intensity by 50%.

^D Antiradical efficiency: 1/IC₅₀.

AE_{Quercetin}(DPPH):14.71 / AE_{Quercetin}(Chemiluminescence):3.125

AE_{Trolox}(DPPH): 5.59 / AE_{Trolox}(Chemiluminescence):0.4

Table S2 - Percentage soybean LOX inhibitory activity of extracts

Extract (25 mg/mL)	% inhibition* of soybean LOX in 3 min
Diethyl Ether	68.2
Ethyl Acetate	75.3
Ethyl acetate residue	82.5
Butanolic	62.7
Aquatic	41.1

*Number of experiment: n=3

Coumarin: 36% inhibition of soybean LOX in 3 min

Nordihydroguaiaretic acid (NDGA): 83% inhibition of soybean LOX in 3 min

Table S3 - Inhibitory effect of *Tephrosia humilis* extracts on aldose and aldehyde reductase enzyme
(in final concentration that of 50µg dry extract/ml solvent)

Extracts	ALR2 inh.±SD ^A	ALR1 inh.±SD ^A	Selectivity index ^B (SI)
Dichloromethane	36.87 ±0.00	12.15 ±1.41	3.03
Diethyl Ether	78.44 ±3.09	61.06 ±5.00	1.28
Ethyl Acetate	78.86 ±2.23	56.05 ±1.25	1.41
Ethyl acetate residue	82.33 ±1.78	76.20 ±6.79	1.08
Butanolic	73.44±1.77	56.82±4.46	1.29
Aquatic	52.35±1.77	41.95±4.14	1.25

^A Number of experiment: n=3

^B Defined as ALR2 inh./ALR1 inh.

Sorbinil: 45% ALR2 inhibition at the same concentration

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