SUPPLEMENTAL MATERIAL

Antioxidant potential and phytochemical composition of extracts obtained from *Phyllanthus phillyreifolius* by different extraction methods

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Running Head Title: Antioxidants of *Phyllanthus phillyreifolius*

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ABSTRACT: *Phyllanthus phillyreifolius* (Euphorbiaceae), poorly studied plant species, was fractionated using conventional and high pressure extraction techniques such as supercritical fluid and pressurized liquid extractions. Lipophilic substances were extracted with n-hexane and supercritical CO_2 with or without co-solvent ethanol, meanwhile higher polarity fractions were recovered with acetone and 70% ethanol. Antioxidant potential was assessed by various chemical assays, which revealed that 70% ethanol was the most effective solvent for recovery of antioxidants. UPLC-MS phytochemical analysis of hydrophilic extracts confirmed geraniin as the main constituent of *P. phillyreifolius*. Other quantitatively important compounds were phylanthusiin D and elaeocarpusin. Three isomers of tocopherol (α, β and γ) were quantified by HPLC in lipofhilic extracts. Generally, the results from this study revealed high antioxidant potential of *P. phillyreifolius*; consequently the plant may be considering as a promising source of antioxidants for functional foods, nutraceuticals and pharmaceutical formulations.

Keywords: *Phyllanthus phillyreifolius*, antioxidant activity, phytochemicals, geraniin, tocopherols.

1. Experimental

1.1. Plant material

The aerial parts of *Phyllanthus phillyreifolius* were collected in south west of Reunion Island in Nowember 2013 and dried at 37 °C overnight. The voucher specimens (UR-PP2013/1) were deposited in the herbarium of the University of Reunion. Before extraction the plants were ground in a laboratory mill Retsch ZM200 (Retch GmbH, Haan, Germany) using 1 mm particle size sieve.

1.2. Chemicals and reagents

2,2-Diphenyl-1-picryhydrazyl hydrate (DPPH$^{\bullet}$, free radical 95%), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 98%), 2,2´-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox, 97%), Folin–Ciocalteu reagent were purchased from Sigma-Aldrich (Steinheim, Germany); KCl, $Na_2HPO_4 \times 12H_2O$, $K_2S_2O_8$ and NaCl from Merck (Darmstadt, Germany); KH_2PO_4 was from Jansen Chimica (Beerse, Belgium); Na_2CO_3 (98%, anhydrous) from RPL (Grauwmeen, Belgium); $FeCl_3 \cdot 6H_2O$ (>99%), sodium acetate (>99%) from Acros

Organics (Geel, Belgium); 2,4,6-tris(2-pyridyl)- s-triazine (TPTZ) and fluorescein (FL) from Fluka Analytical (Bor-nem, Belgium). Tocopherols, α-T (99.9%), rac-β-T (90 + %), γ-T (99%) and δ-T (95.5%) were purchased from Supelco Analytical (Bellefonte, PA, USA). Reference compounds, gallic acid, rutin and quercitrin hydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA), ellagic acid from Fluka Biochemica (Buchs, Switzerland), geraniin from ALB technology (Mongkok Kowloon, Hong Kong, China). Analytical grade solvents, hexane, acetone, methanol and acetic acid were from StanLab (Lublin, Poland); agricultural origin ethanol (96.6%) from Stumbras (Kaunas, Lithuania). HPLC grade solvents for chromatographic analyses were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ultrapure water was produced using a Simplicity 185 system (Millipore, MA, USA). Carbon dioxide (99.9%) was obtained from Gaschema (Jonava r., Lithuania).

1.3. Preparation of extracts

Ground plant samples were extracted with hexane, acetone, ethanol:water (70:30, v/v) (further 70% ethanol) and supercritical CO_2 using four different extraction methods: Soxhlet, maceration by stirring, supercritical fluid (SFE) and pressurized liquid (PLE) extraction (Figure S1). After extractions, the solvents were removed in a rotary evaporator (Büchi, Flawil, Switzerland) at 42 °C, while residual water was freeze-dried. The amount of extracts was determined gravimetrically (±0.001 g). The extracts are further marked with a lowercase letter depending on extraction method: c – consecutive and p – pressurized. Dry extracts were stored in a freezer prior to further analysis.

1.3.1. Conventional extraction procedures

The sample (12 g) was placed into a cellulose thimble and extracted with hexane for 3 h at 69 °C in a Soxhlet extractor (Behr Labor-Technik, Düsseldorf, Germany). Then the residue was re-extracted with acetone. For hydroethanolic (ET) extract 5 g of residue after acetone extraction were extracted 2 times by stirring with 100 mL 70% ethanol at room temperature for 1 h; the extracts were filtered through Whatman No.1 filter paper and combined.

1.3.2. Supercritical carbon dioxide extraction (SFE)

The SFE was carried out in a supercritical fluid extractor Helix (Applied Separation, Allentown, PA) as described elsewhere (Kraujalis and Venskutonis 2013) with slight

modifications. The extractor (320 mm length×14 mm internal diameter) was filled with 15 g of plant sample, its ends were plugged with cotton wool to avoid particle clogging in the system and to eliminate the vessel's dead volume. The conditions for extraction with CO_2 was set as follows: time 120 min (including 10 min of a static extraction time), pressure 47.5 MPa, temperature 45°C, flow rate of CO_2 2–3 SL/min. The volume of CO_2 consumed was measured by a ball float rotameter and a digital mass flow meter in standard liters per minute (SL/min) at standard state (PCO_2 = 100 kPa, TCO_2 = 20 °C, qCO_2 = 0.0018 g/mL). The flow rate of CO_2 in the system was controlled manually by the micro-metering valve (back-pressure regulator) and kept constant during all experiments.

1.3.3. Pressurized liquid extraction (PLE)

The SFE residue was consecutively re-extracted with acetone and then with 70% ethanol using accelerated solvent extraction apparatus ASE 350 (Dionex, Sunnyvale, CA, USA) as described elsewhere (Kraujalis et al. 2013). Ten g of plant sample were placed in the extraction cell containing cellulose filter at the end to avoid solid particles in the collection vial. The cells were preheated 5 min to ensure that the samples reached thermal equilibrium at 10 MPa pressure and 70 °C temperature before 3 extraction cycles, 5 min each (total time 15 min). Afterwards the cell was purged for 60 s with nitrogen to collect the extract in the collection vial.

1.4. In vitro antioxidant activity assessment

Working solution was prepared by dissolving extracts in methanol, except for lipophilic fractions, which were diluted in acetone:methanol (1:9, v/v), at a concentration of 10 mg/mL. Methanol was used for further dilutions needed for every individual assay. Not fully dissolved extracts were treated in the ultrasonic bath ASTRA-SON™, model 9H (Heat Systems Ultrasonics, NY, USA) and filtered. The absorbances were measured with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY) in semi-micro cuvets (Ratiolab GmbH, Dreieich, Germany).

1.4.1. Analysis of total phenolic content (TPC) by Folin-Ciocalteu's assay

TPC was determined spectrophotometrically with Folin-Ciocalteu reagent (FCR) (Singleton et al. 1998). Briefly, 150 µL of extract solution were mixed with 750 µL of diluted with distilled water FCR (1:9, v/v) and 600 µL of 7.5% Na_2CO_3 solution and left in the dark

for 2 hours. Afterwards the absorbance was measured at 760 nm. TPC was calculated from the calibration curve, which was obtained using 150 µL GA solutions (0-80 µg GA/mL ethanol) as a standard and the results were expressed as mg GA equivalents per gram of extract (mg GAE/g DWE) and dry plant material (mg GAE/g DWP).

1.4.2. $DPPH^{\bullet}$ scavenging assay

Radical scavenging capacity (RSC) of extracts against $DPPH^{\bullet}$ was measured by the method of Brand-Williams et al. (1995) with some modifications. $DPPH^{\bullet}$ methanolic solution (~90 µM/L, final absorption 0.80 ± 0.03) was prepared daily before measurements; 1000 µL of $DPPH^{\bullet}$ solutions were mixed with 500 µL of extract solution or MeOH (blank) and left in dark for 2 hours. The decrease in absorbance value was measured at 517 nm. The RSC value was calculated from the calibration curve using 500 µL Trolox solutions (0–50 µmol TE/L MeOH).

1.4.3. $ABTS^{\bullet+}$ scavenging assay

The $ABTS^{\bullet+}$ scavenging assay was carried out by the method of Re et al. (1999) with slight modifications. Firstly, phosphate buffered saline (PBS) solution (75 mM/L; pH 7.4) was prepared by dissolving 8.18 g NaCl, 0.27 g, KH_2PO_4, 3.58 , Na_2HPO_4 ×12 H_2O and 0.15 g KCl in 1 L of distilled water. Stock $ABTS^{\bullet+}$ solution was prepared by mixing 50 mL ABTS (2 mM/L PBS) with 200 µL $K_2S_2O_8$ (70 mM/L H_2O) and keeping for 12–16 h at room temperature in the dark. Before each assay, stock $ABTS^{\bullet+}$ solution was diluted with PBS to obtain the working $ABTS^{\bullet+}$ solution with absorbance of 0.80 ± 0.03 at 734 nm. For the analysis, 25 µL of sample or MeOH (blank) were mixed with 1500 µL of working $ABTS^{\bullet+}$ solution and left in dark for 2 hours. The decrease in absorbance value was measured at 734 nm. RSC was calculated from the calibration curve using 25 µL Trolox solutions (0-1800 µmol TE/L MeOH).

1.4.4. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out by the method of (Benzie and Strain 1996) with some modifications. FRAP reagent was prepared by mixing 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$ and acetate buffer (300 mM, pH 3.6) at 1:1:10 (v/v/v). For the measurement, 50 µL of sample or MeOH (blank) were mixed with 150 µL of distilled H_2O and 1500 µL of freshly prepared FRAP reagent. After 2 h incubation in the dark at 37 °C, the

decrease in absorbance was read at 593 nm. A series of Trolox solutions in the concentration ranges of 0 – 800 µM/L MeOH were used for the calibration.

1.4.5. Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was carried out as described by Prior et al. (2003) using fluorescein as a fluorescent probe. Briefly, 25 µL of sample or MeOH (blank) were mixed with 150 µL of fluorescein solution (14 µM/L PBS) in a clear-bottom 96-well black opaque microplate. The mixture was preincubated for 15 min at 37 °C and 25 µL of AAPH solution (240 mM/L PBS) as a peroxyl radical generator immediately added using multichannel pipet. The fluorescence was recorded 120 cycles (every cycle 1 min × 1.1) at 485 excitation and 520 emission wavelengths at 37 °C in a FLUOstar Omega reader (BMG Labtech, Offenburg, Germany). The fluorescence was recorded 120 cycles followed by rapid addition of AAPH. Trolox solutions (0–250 µM/L PBS) were used for calibration. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the curve (AUC) as follows: AUC = $(1+f_1/f_0+f_2/f_0 \ldots f_i/f_0)$, where f_0 is the initial fluorescence reading at time 0 min and f_i is fluorescence reading at time i. The results in all antioxidant capacity evaluation assays (sections 2.4.2-2.4.5) were expressed in µM of Trolox equivalents: µM TE/g DWE or DWP.

1.5. Determination of tocopherols by high performance liquid chromatography (HPLC)

Quantitative determination of tocopherols (α, β, λ and γ) in hexane and SC-CO_2 extracts was performed in an HPLC system as described by Kraujalis and Venskutonis (2013). Perkin Elmer Series HPLC system was equipped with C_{30} reverse-phase column (250 mm×4.6 mm, 5 µm) applying isocratic elution with acetonitrile:methanol:dichlormethane (72/22/6, v/v/v). For analysis extracts and standards were dissolved in mobile phase at final concentration 0.1 mg/mL and 0–10 µg/mL, respectively. Injection volume was 20 µL and flow rate 1 mL/min. Fluorescence detector at 290 nm excitation and 330 nm emission was used for detection. Tocopherols were identified by comparing the retention time of peaks to those of pure standard solutions.

1.6. Identification and quantification of phenolic compounds by UPLC-MS analysis

Chromatographic separation of analytes was carried out on an Acquity UPLC (Waters, Milford, MA, USA) system equipped with a binary pump, autosampler, photodiode array (PDA) detector, column manager, data station running the Compass acquisition and data

software. Compounds were separated on an Acquity BEH, C18 column (100 mm × 2.1 mm, 1.7 µm) maintained at 40 °C. The eluent system consisted of solvents A (0.1% formic acid in ultra pure water) and B (100% acetonitrile) with a linear gradient programmed as follows: 0.0–14 min, 5% B; 15–17 min, 100% B; 18 min, 5% B. The flow rate was 0.4 mL/min, temperature of sample 12 °C and sample injection volume 1 µL. The effluents from the PDA detector were introduced directly into the quadrupole-time of flight mass spectrometer (Q-TOF) equipped with an electrospray ionization source controlled by HyStar 3.2 SR2 software (Bruker Daltonic, Bremen, Germany). All MS data were recorded in ESI negative ionization mode in a range of 80–1200 m/z, the capillary voltage was maintained at +4000 V. Nitrogen was used as a nebulizer gas at 2.5 bar and drying gas at flow rate of 10 L/min. The peaks were identified by comparing their retention times and parent ions with external standards, references and commercial databases.

Selected phenolics were quantified using an Acquity UPLCTM H-Class equipped with Xevo TQ-S tandem quadrupole mass spectrometer (Waters, Milford, MA) operating in negative electrospray ionization (ESI) mode, capillary voltage was set to 1500 V, cone voltage –20 V, source offset –50 V. Desolvation temperature was 450 °C, desolvation gas flow –1000 L/h, cone gas flow –150 L/h and nebulizer gas flow was set to 7 L/h. Chromatographic separation was performed using the same column and solvents as described above with a linear gradient programmed as: 0.0–7 min, 5% B; 8–9 min, 50,7% B; 10–11 min, 100% B; 12–20 min, 5% B. The flow rate was 0.4 mL/min and sample injection volume was 5 µL. MS detection was achieved in the single-ion-monitoring (SIM) mode. The m/z values and dwell times of components were set as follows: 169.1595m/z and 0.1 s (gallic acid), 301.0957 m/z at 0.025 s (ellagic acid), 447.1595 m/z at 0.025 s (quercitrin), 609.2233 m/z at 0.025 s (rutin), 951.1957 m/z at 0.050 s (geraniin), 991.1000 m/z at 0.025 s (phyllanthusiin D), 1109.1000 m/z at 0.050 s (elaeocarpusin). MassLynx 4.1 software was used for instrument control and data collection. All samples were run in triplicates. The concentrations of phytochemicals were calculated from calibration curves prepared using concentrations of 0.05–50 µg/mL of different standard compounds: gallic acid (y=34937x-16.54; R2=0.9937), ellagic acid (y=6142.6x+16634; R2=0.9954), geraniin (y=6158x+4127; R2=0.9986), rutin (y=45440x+28682; R2=0.9953) and quercitrin (y=65477x+19631; R2=0.9953). Whereas phyllanthusiin D is an artifact condensate of geraniin with acetone, produced during the extraction, and elaeocarpusin is condensation product derived from geraniin and ascorbic acid (Okuda and Ito 2011; Quideau 2009), these compounds, due to a

high structural similarity to geraniin and difficulties to purchase reference standards, were quantified according geraniin calibration curves. The results were expressed both in the dry weight of extracts (DWE) and in the dry weight of the whole plant material (DWP). For the determination of fragmentation patterns of some compounds, direct infusion was made to a Waters TQ-S by deploying collision induced dissociation (CID) using argon as a collision gas at 25 eV and a flow rate of 0.11 mL/min.

1.7 Statistical analysis

All experiments were performed at least in triplicate. The obtained data were analyzed using GraphPad Prism software (Version 5) and expressed as mean ± standard deviation (n = 3). Statistical comparisons were made using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. Differences were considered to be significant when P values were below 0.05 ($P < 0.05$).

Figure S1. Yield of *P. phillyreifolius* lipophilic (a) and hydrophilic (b) extracts obtained by various extraction methods and solvents.

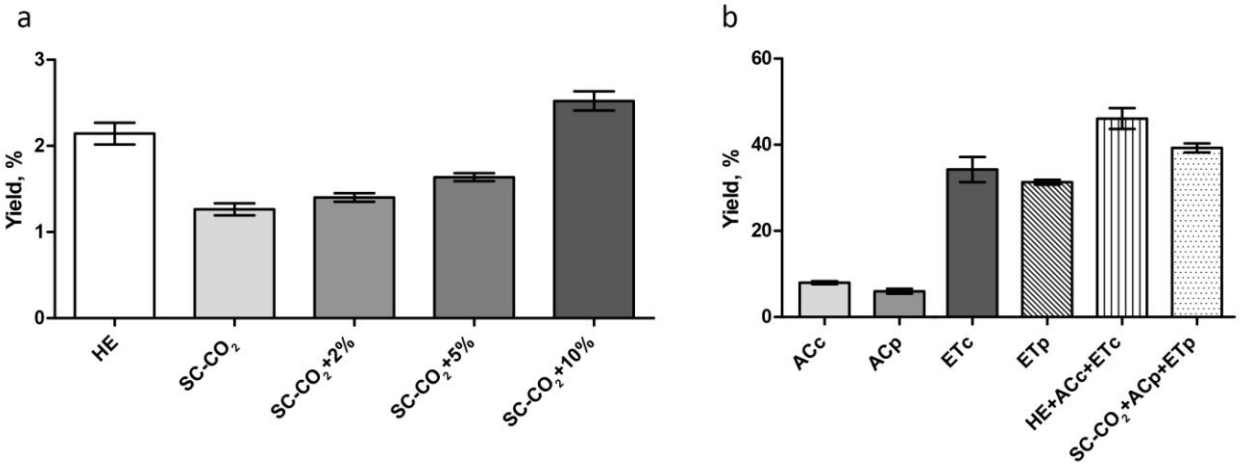

Table S1. Concentration of tocopherols (T) and oxygen radical scavenging capacity ($ORAC_{FL}$) of lipohilic *P. phillyreipholius* extracts (in mg/g and μM TE/g DWE); recovery of T and antioxidants, as expressed in $ORAC_{FL}$ by hexane and SFE (in mg/g and μM TE/g DWP).

Sample	α-T		β-T		γ-T		Total tocopherols		$ORAC_{FL}$	
	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
HE	17.9±0.55^{b}	0.38^{d}	2.19±0.08^{b}	0.05^{c}	0.48±0.00^{b}	0.01^{c}	20.6±0.63^{b}	0.44^{d}	159±5.81^{a}	3.40^{a}
SC-CO_2	21.1±0.93^{c}	0.27^{a}	2.38±0.11^{c}	0.03^{a}	0.59±0.00^{d}	0.007^{a}	24.1±1.04^{c}	0.30^{a}	290±13.1^{b}	3.66^{a}
SC-CO_2+2%	24.9±0.41^{d}	0.35^{c}	2.96±0.06^{d}	0.04^{b}	0.60±0.01^{d}	0.008^{a}	28.4±0.48^{d}	0.40^{c}	322±8.71^{c}	4.51^{b}
SC-CO_2+5%	16.8±0.52^{b}	0.28^{a,b}	2.47±0.11^{c}	0.04^{b}	0.53±0.02^{c}	0.009^{b}	19.8±0.65^{b}	0.32^{a,b}	423±13.6^{d}	6.93^{c}
SC-CO_2+10%	11.3±0.33^{a}	0.29^{b}	1.70±0.06^{a}	0.06^{d}	0.39±0.01^{a}	0.01^{c}	13.4±0.40^{a}	0.34^{b}	514±20.8^{e}	13.0^{d}

Values represented as mean ± standard deviation (n=3); columns with different letters differ significantly for Tukey's test at $p < 0.05$; DWE – dry weight extract; DWP – dry weight initial plant

Table S2. Total phenolic content (TPC) (in mg GAE/g DWE) and antioxidant capacity (in μM TE/g DWE) of *P. phillyreipholius* extracts and recovery of antioxidants by various extraction processes (in mg GAE/g or μM TE/g DWP).

Sample	TPC		DPPH		ABTS		FRAP		ORAC	
	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
ACc	429±1.10[a]	33.5[b]	3559±21.8[a]	278[b]	8190±319[b]	640[b]	5465±103[a]	427[b]	3669±142[d]	287[b]
ACp	420±14.8[a]	24.6[a]	4094±130[b]	240[a]	7963±241[b]	466[a]	6262±302[b]	366[a]	2834±96.6[a]	166[a]
ETc	429±1.19[a]	133[c]	3278±55.1[a]	1012[c]	5804±170[a]	1791[c]	5649±210[a]	1743[d]	3259±127[c]	1006[d]
ETp	510±24.0[b]	147[d]	4288±137[c]	1235[d]	7311±269[b]	2106[d]	5349±217[a]	1541[c]	2918±94.1[b]	841[c]
Σ(ACc and ETc)	-	166[e]	-	1290[e]	-	2430[e]	-	2170[f]	-	1292[f]
Σ (ACp and ETp)	-	171[f]	-	1475[f]	-	2572[f]	-	1907[e]	-	1006[e]

Values represented as mean ± standard deviation (n=3); columns with different letters differ significantly for Tukey's test at $p < 0.05$; DWE – dry weight extract; DWP – dry weight initial plant.

Table S3. Chemical profile of *P. phillyreipholius* hydrophilic extracts by UPLC-MS.

Compound	Molecular formula	RT (UPLC)	$[M-H]^-$ $[M-2H]^{2-}$	MS Fragments	ACc		ACp		ETc		ETp	
					mg/g DWE	mg/g DWP	mg/g DWE	mg/g DWP	mg/g DWE	mg/g DWP	mg/g DWE	mg/g DWP
Gallic acid[a]	$C_7H_6O_5$	1.00	169.0141	-	0.67±0.01	0.05	0.59±0.03	0.04	0.59±0.01	0.18	0.61±0.03	0.17
Ellagic acid[a]	$C_{14}H_6O_8$	3.05	300.9984	-	56.6±0.80	4.42	38.54±0.05	2.25	48.70±0.53	15.03	31.45±1.38	9.06
Geraniin[a]	$C_{41}H_{28}O_{27}$	2.20	951.0740 475.033	-	262±7.66	20.5	226±14.6	13.2	236±12.3	72.7	248±6.66	71.5
*Elaeocarpusin[b]	$C_{47}H_{34}O_{32}$	2.55	1109.0943 554.0445	1048.48, 972.56, 300.91 $[EA-H]^-$	24.9±0.93	1.95	30.3±0.97	1.77	15.5±0.57	4.08	14.2±0.77	4.77
*Phyllanthusiin D[b]	$C_{44}H_{32}O_{27}$	3.15	991.1062 495.0490	990.61, 300.91 $[EA-H]^-$	178±6.19	13.9	35.6±2.01	2.08	nd	nd	nd	nd
Rutin[a]	$C_{27}H_{30}O_{16}$	3.1	609.1454	-	1.43±0.00	0.11	0.19±0.00	0.01	4.19±0.04	1.29	4.38±0.03	1.26
Quercitrin[a]	$C_{21}H_{20}O_{11}$	3.55	447.0924	-	0.57±0.03	0.04	0.54±0.01	0.03	1.22±0.05	0.38	1.12±0.01	0.32
		Σ			524±15.6	41.0	331±17.7	19.4	306±13.5	93.6	300±8.88	87.1

[a]Confirmed by a standard; [b]Confirmed by a reference; *expressed as geraniin equivalent; nd: not detected. Values represented as mean ± standard deviation (n=3), DWE – dry weight extract; DWP – dry weight initial plant.

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