SUPPLEMENTARY MATERIAL

Phenolic constituents from *Wissadula periplocifolia* (L.) C. Presl. and anti-inflammatory activity of 7,4'-di-*O*-methylisoscutearein

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Abstract: This study reports the first phenolics from *Wissadula* genus (Malvaceae) and the anti-inflammatory activity of 7,4'-di-*O*-methylisoscutearein. By using chromatographic methods five phenolic compounds were isolated from aerial parts of *Wissadula periplocifolia* (L.) C. Presl. The compounds were identified as 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, *trans*-cinnamic acid, tamgermanetin and 7,4'-di-*O*-methylisoscutearein using spectroscopic methods. The flavone 7,4'-di-*O*-methylisoscutearein showed anti-inflammatory activity by inhibiting neutrophils

recruitment in a mice model of pleurisy and by decreasing significantly the production of cytokines IL-1 β and TNF- α .

Keywords: Malvaceae; *Wissadula periplocifolia*; 7,4'-di-O-methylisoscuteallarein; anti-inflammatory activity.

1. Experimental

1.1. Isolation of compounds

The aerial parts of *W. periplocifolia* were collected in Pedra da Boca Park (Araruna City, Paraíba/Brazil) in August 2005 (ICMBio Authorization Number: 46923-2). A voucher specimen (JPB 6498) was authenticated by Profa. Dra. M. F. Agra and deposited at Lauro Pires Xavier Herbarium (JPB/UFPB).

Column chromatography separations (CC) were performed on glass columns packed with Silica gel (ASTM, 230-400 mesh, Merck) in which a 30:1 ratio of silica to dried sample was used. Gel filtration chromatography (GFC) was also carried out using Sephadex LH-20. Thin layer chromatography (TLC) were performed on silica gel PF254 plates and spots were visualized under UV light (254 and 366 nm) and by exposure to iodine or sprayed with vanillin–sulphuric acid reagent.

The plant material was dried in an oven at 40°C for 72 h. After milling, 8.9 kg of powder was macerated with absolute ethanol for 72 h. The obtained ethanolic extract was concentrated with a rotatory evaporator yielding 705 g of crude extract (CEE). 200 g of this extract was solubilized using EtOH:H₂O (9:1) and the obtained solution was sequentially partitioned in separation funnel using hexane, dichloromethane (CH₂Cl₂), ethyl acetate (EtOAc) and *n*-butanol to yield 48 g of hexane (HF), 33 g of CH₂Cl₂ (DF), 28 g of EtOAc (EAF) and 9 g of *n*-butanol (BF) fractions. The various fractions were analysed by ¹H NMR to detect the presence of phenolic constituents and from the obtained results, DF and EAF were chosen for CC.

DF (15g) was subjected to silica column chromatography using glass column (4 cm x 50 cm), eluted with hexane, EtOAc and methanol (MeOH) at flow rate of 15 mL/min, resulting 240 fractions of 30 mL (Table 1S). The fractions were combined through TLC and the pure fractions 16 to 19 and 21 to 24 were identified as compound **1** (17 mg) and compound **2** (20 mg). Combined fractions 25 to 38 (200 mg) were subjected to gel filtration in Sephadex LH-20 (150 g) using a glass column (1.5 cm x 40 cm) and eluted with methanol (flow rate 8 mL/min) in isocratic mode to obtain 25 fractions of 10 mL. Fraction 10 was found pure and coded as compound **3** (12 mg). Fractions 135 to 154 (280 mg) was chromatographed on Sephadex LH-20 column using the same conditions, yielding compound **4** (30 mg).

Table 1S. Solvent systems and fractions eluted from DF silica column chromatography.

Solvent system	Fractions
Hexane	1 to 15
Hexane: EtOAc (9:1)	16 to 31
Hexane: EtOAc (8:2)	32 to 54
Hexane: EtOAc (6:4)	55 to 78
Hexane: EtOAc (1:1)	79 to 96
Hexane: EtOAc (4:6)	97 to 120
Hexane: EtOAc (2:8)	121 to 148
Hexane: EtOAc (1:9)	149 to 160
EtOAc	161 to 188
EtOAc:MEOH (9:1)	189 to 213
EtOAc:MEOH (7:3)	214 to 240

EAF (4 g) was subjected to gel filtration in Sephadex LH-20 (200 g) using a glass column (2.5 cm x 45 cm) and eluted with methanol (flow rate 8 mL/min) in isocratic mode to obtain 45 fractions of 10 mL. The combined fractions 18 to 22 (375 mg) were re-chromatographed using the same method yielding 135 mg of compound **5**.

The pure fractions were analyzed by LC-HRMS, H^1 and C^{13} NMR, besides 2D techniques such as HMQC, HMBC and COSY.

1.2. Identification of isolated compounds

Isolated compounds were identified by 1D and 2D NMR analysis (^1H 400 MHz and ^{13}C 100 MHz - Bruker-Avance III).

In order to confirm the isolated compounds structures the high-resolution mass spectra was obtained by LC-HRMS analysis performed on an Accela 600 HPLC system combined with an Exactive (Orbitrap) mass spectrometer from Thermo Fisher Scientific (Bremen, Germany). Each sample was dissolved in methanol (HPLC grade) to get a concentration of 1 mg/mL. From this solution, 20 μL was injected in the chromatographer. It was used a ACE C_{18} column (150 $\times$ 3 mm, 3 μm) from HiChrom, a flow rate of 300 $\mu\text{L}/\text{min}$, and the mobile phase was composed by 0.1% of formic acid in H_2O (solvent A) and acetonitrile (solvent B) in gradient mode according to the summarized method in the Table 2S.

Table 2S. Gradient method used at LC-HRMS experiment.

Time (min)	A%	B%
0	75	25
15	25	75

The scanning for MS detection (from 100 to 1500 m/z) was performed under ESI polarity switching mode. The obtained data were analysed using the software Xcalibur 2.2 from Thermo Fisher Scientific.

4-hydroxybenzoic acid (*p*-hydroxybenzoic acid) (**1**). White crystalline solid, $\text{C}_7\text{H}_6\text{O}_3$. HR-MS: m/z : 137.0241 $[\text{M} - \text{H}]^-$. ^1H -NMR (DMSO, 400 MHz) δ (ppm): 7.78 (2H, d, $J=8.8$ Hz, H-2 and H-6), 6.83 (2H, d, $J=8.7$ Hz, H-3 and H-5). ^{13}C -NMR (DMSO, 100 MHz) δ (ppm): 121.3 (C-1), 131.5 (C-2 and C-6), 115.1 (C-3 and C-5), 161.6 (C-4), 167.1 (C-7) (Figures 1S to 5S) (Canuto & Silveira, 2010; Scott, 1972).

3-hydroxybenzoic acid (*m*-hydroxybenzoic acid) (**2**). White crystalline solid, $\text{C}_7\text{H}_6\text{O}_3$. HR-MS: m/z : 137.0211 $[\text{M} - \text{H}]^-$. ^1H -NMR (DMSO, 400 MHz) δ (ppm): 9.02 (1H, d, $J=1.4$ Hz, H-2), 8.70 (1H, d, $J=4$ Hz, H-4), 7.49 (1H, dd, $J=8$ and 4 Hz, H-5), 8.20 (1H,

d, J=8 Hz, H-6). ^{13}C -NMR (DMSO, 100 MHz) δ (ppm): 121.6 (C-1), 149.2 (C-2), 130.2 (C-3), 152.5 (C-4), 123.9 (C-5), 135.7 (C-6), 167.0 (C-7) (Figures 6S to 10S) (Canuto & Silveira, 2010; Scott, 1972).

trans-cinnamic acid (**3**). White crystalline solid, $\text{C}_9\text{H}_8\text{O}_2$. HR-MS: m/z : 147.0514 $[\text{M} - \text{H}]^-$. ^1H -NMR (DMSO, 400 MHz) δ (ppm): 7.66 (2H, m, H-2 and H-6), 7.40 (3H, m, H-3, H-4, H-5), 7.55 (1H, d, J=15.1 Hz, H-7), 6.53 (1H, d, J=15.1 Hz, H-8). ^{13}C -NMR (DMSO, 100 MHz) δ (ppm): 135.0 (C-1), 128.6 (C-2; C-6), 129.4 (C-3; C-5), 130.6 (C-4), 143.8 (C-7), 120.7 (C-8), 169.4 (C-9) (Figures 11S to 15S) (Ponce et al., 2009; Scott, 1972).

N-trans-3-hydroxy-4-methoxy cinnamoyltyramine (tamgermanetin) (**4**). Amorphous solid, $\text{C}_{18}\text{H}_{19}\text{O}_4\text{N}$; HR-MS: m/z : 314.1306 $[\text{M} - \text{H}]^+$. ^1H -NMR (DMSO, 400 MHz) δ (ppm): 7.11 (1H, d, J=1.1 Hz, H-2), 6.78 (1H, d, J=8.10 Hz, H-5), 6.98 (1H, d, J=8.10 Hz, H-6), 7.30 (1H, d, J=15.7 Hz, H-7), 6.43 (1H, d, J=15.7 Hz, H-8), 8.02 (1H, t, N-H), 3.34 (2H, m, H-8'), 2.94 (2H, t, H-7'), 7.01 (2H, d, J=8 Hz, H-2' and H-6'), 6.68 (2H, J=8 Hz, H-3' and H-5'). ^{13}C -NMR (DMSO, 100 MHz) δ (ppm): 127.1 (C-1), 111.3 (C-2), 148.3 (C-3), 148.7 (C-4), 116.1 (C-5), 122.2 (C-6), 139.2 (C-7), 119.6 (C-8), 166.1 (C-9), 127.1 (C-1'), 130.1 (C-2' and C-6'), 115.6 (C-3' and C-5') (Figures 16S to 20S) (Nawwar et al., 2013).

7,4'-di-*O*-methylisoscuteallarein (5,8-dihydroxy-7,4'-dimethoxyflavone) (**5**). Yellow powder, $\text{C}_{17}\text{H}_{14}\text{O}_6$; HR-MS: m/z : 313.0714 $[\text{M} - \text{H}]^-$. ^1H -NMR (DMSO, 400 MHz) δ (ppm): 6.87 (1H, s, H-3), 12.44 (1H, s, 5-OH), 6.56 (1H, s, H-6), 8.12 (2H, d, J = 8 Hz, H-2', H-6'), 7.13 (2H, d, J = 8 Hz, H-3', H-5'), 3.90 (3H, s, -OCH₃-C7), 3.86 (3H, s, -OCH₃-C4'). ^{13}C -NMR (DMSO, 100 MHz) δ (ppm): 164.0 (C-2), 103.6 (C-3), 182.9 (C-4), 153.6 (C-5), 96.2 (C-6), 154.8 (C-7), 126.8 (C-8), 145.0 (C-9), 104.4 (C-10), 123.5 (C-1'), 129.0 (C-2', C-6'), 115.1 (C-3', C-5'), 162.90 (C-4'), 56.9 (-OCH₃-7), 55.1 (-OCH₃-4') (Figures 21S to 25S) (Gomes et al., 2011).

Figure 1S. ¹H NMR (400 MHz, DMSO) spectrum of compound **1**.

Figure 2S. ¹³C DEPTQ-135 NMR (100 MHz, DMSO) spectrum of compound **1**.

Figure 3S. HMQC spectrum (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz, DMSO) of compound **1**.

Figure 4S. HMBC spectrum (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz, DMSO) of compound **1**.

Figure 5S. HRMS spectrum of compound **1**.

Figure 6S. ¹H NMR (400 MHz, DMSO) spectrum of compound **2**.

Figure 7S. ^{13}C DEPTQ-135 NMR (100 MHz, DMSO) spectrum of compound **2**.

Figure 8S. HMQC spectrum (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz, DMSO) of compound **2**.

Figure 9S. HMBC spectrum (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz, DMSO) of compound **2**.

Figure 10S. HRMS spectrum of compound **2**.

Figure 11S. ^1H NMR (400 MHz, DMSO) spectrum of compound **3**.

Figure 12S. ^{13}C DEPTQ-135 NMR (100 MHz, DMSO) spectrum of compound **3**.

Figure 13S. HMQC spectrum (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz, DMSO) of compound **3**.

Figure 14S. HMBC spectrum (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz, DMSO) of compound **3**.

Figure 15S. HRMS spectrum of compound **3**.

Figure 16S. ^1H NMR (400 MHz, DMSO) spectrum of compound **4**.

Figure 17S. ^{13}C DEPTQ-135 NMR (100 MHz, DMSO) spectrum of compound **4**.

Figure 18S. HMQC spectrum (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz, DMSO) of compound **4**.

Figure 19S. HMBC spectrum (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz, DMSO) of compound **4**.

Figure 20S. HRMS spectrum of compound **4**.

Figure 21S. ¹H NMR (400 MHz, DMSO) spectrum of compound **5**.

Figure 22S. ¹³C DEPTQ-135 NMR (100 MHz, DMSO) spectrum of compound **5**.

Figure 23S. HMQC spectrum (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz, DMSO) of compound **5**.

Figure 24S. HMBC spectrum (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz, DMSO) of compound **5**.

Figure 25S. HRMS spectrum of compound **5**.

1.3. Anti-inflammatory activity of 7,4'-di-O-methylisoscuteallarein (**5**)

1.3.1. Animals

Male C57Bl/6 mice weighing 20-30 g were obtained from the Oswaldo Cruz Foundation breeding unit. The animals were maintained with food and water *ad libitum* in a room with the temperature ranging from 22 to 24 °C and a 12 h light/dark cycle. This study was carried out in accordance with the recommendations of the Brazilian National Council for the Control of Animal Experimentation (CONCEA). The protocols were approved by the Animal Welfare Committee of the Oswaldo Cruz Foundation (CEUA/FIOCRUZ protocol L-002/08).

1.3.2. Treatments

For *in vivo* experiments, animals were orally (po) pre-treated with compound **5** (2.5 mg/kg) or dexamethasone (2.5 mg/kg, po). DMSO (0.1% v/v) was given as negative control. All treatments were performed 1h before PBS challenge. For *in vitro* experiments, cells were treated with compound (1 or 10 μM), 1h before the stimulus. Non-treated cells received supplemented RPMI medium with DMSO (0.1% v/v).

1.3.3. LPS-induced pleurisy

Male C57Bl/6 mice orally pre-treated with compound **5** or dexamethasone (2.5 mg/kg) received an intrapleural injection of LPS (250 ng / cavity) dissolved in 100 µL of PBS. Four hour after the LPS injection, the animals were euthanized by exposure to an atmosphere of CO₂, and the pleura were surgically exposed. The pleural lavage was collected from mice by washing the pleural cavity with 1 mL of PBS and with heparin (20 U/mL).

1.3.4. Leukocyte counts

Total leukocyte counts were performed using a Neubauer chamber under a light microscope after diluting the pleural lavage samples in Turk fluid (2% acetic acid). Differential counts were performed by microscopy with an objective lens at 100x magnification.

1.3.5. Peritoneal macrophage culture and cytokine productions analysis

Peritoneal macrophages from C57Bl/6 mice were obtained 4 days after the injection of 4% thioglycolate by washing the peritoneal cavity with RPMI 1640 medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. Cells had the concentration adjusted to 2×10^6 /mL and were plated in 24-well culture plates (500 µL) at 37°C in 4% CO₂ atmosphere overnight. Following incubation, cells were pre-treated with compound **5** (1 or 10µM) and 1h later, stimulated with LPS (500 ng/mL) (Leite et al., 2014). The concentrations of cytokines in the supernatants of the macrophage culture were determined by ELISA 24 hours after stimulation with LPS, using DuoSet kits according to the manufacturer's instructions (R&D Systems). Of note, Compound **5** at 1 or 10 µM did not affect cell viability.

1.3.6. Statistical analyses

Data were analyzed by one-way ANOVA followed by Tukey's test using GraphPad Prism software (GraphPad, San Diego, CA). The values were expressed as the means $\pm$ S.E.M. Differences with $p < 0.05$ were considered significant.

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