SUPPLEMENTARY MATERIAL

Phytochemical and antioxidant activity studies on *Ononis angustissima* L. aerial parts: isolation of two new flavonoids

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Abstract

Ononis angustissima aerial parts extract and exudate were subjected to a phytochemical and biological analysis. Two new natural flavonoids, (3*S*)-7-hydroxy-4'-methoxy-isoflavane 3'-β-D-glucopyranoside (**1**), and kaempferol 3-*O*-β-D-glucopyranoside-7-*O*-(2'''-acetyl)-β-D-galactopyranoside (**4**), and sixteen known compounds were isolated through a bio-oriented approach. Their structural characterization was achieved by using spectroscopic analyses including 2D NMR. The phytochemical profile of the extracts was also performed and the antioxidant activity of all compounds was tested by three different assays. To get a trend in the results and to facilitate the comparison of antioxidant capacity among the different methods used, the obtained data were transformed to a Relative Antioxidant Capacity Index (RACI).

Keywords: *Ononis angustissima*; Fabaceae; phenolic derivatives; isoflavonoids; antioxidant activity; mass spectrometry.

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EXPERIMENTAL SECTION

General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were recorded on a Perkin-Elmer-Lambda spectrophotometer. CD spectra were measured on a JASCO J-810 spectropolarimeter with a 0.1 cm cell in MeOH at room temperature under the following conditions: speed 50 nm/min, time constant 1 s, bandwidth 2.0 nm. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. The standard pulse sequence and phase cycling were used for DQF-COSY, TOCSY, HSQC, and HMBC experiments. Mass spectrometry analyses were performed on a Q-TOF premier spectrometer (Waters-Milford) coupled with an Alliance HPLC module (Waters-Milford). TLC was performed on precoated Kieselgel 60 F254 plates (Merck); compounds were detected by spraying with $\text{Ce}(\text{SO}_4)_2/\text{H}_2\text{SO}_4$ (Sigma Aldrich) and NTS (Naturstoffe reagent)-PEG (Polyethylene glycol 4000) solutions. Column chromatography was performed over Sephadex LH-20 (Pharmacia) and silica gel (Merck); reversed-phase (RP) HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID10A refractive index detector and a Shimadzu injector, using a C_{18} μ -Bondapak column (30 cm x 7.8 mm, 10 μm , Waters-Milford). GC analyses were performed using a Dani GC 1000 instrument. All spectrophotometric measurements were done in 96-well microplates or cuvettes on a UV/VIS spectrophotometer SPECTROstar Nano (BMG Labtech).

Plant material

Aerial parts of *O. angustissima* were collected in the region of Bechar, on April 2010. The plant was authenticated by Prof. A. Kaabeche, Biology Department, University of Setif, Setif, Algeria. A voucher specimen (OnaAlg110-2010) was deposited at the VARENBIOMOL, Université Constantine, Constantine, Algeria.

Extraction, fractionation and isolation

Air dried aerial parts of *Ononis. angustissima* (980 g) were macerated four times with 70% MeOH solution by replacing the solution every day with fresh solvent. The hydro-alcoholic solutions were concentrated under reduced pressure to dryness and the residue was dissolved in hot water (200 mL) and kept in cold overnight. After filtration, the aqueous solution was successively extracted with CHCl_3 , EtOAc and *n*-BuOH for three times for each solvent, then the CHCl_3 (25.6 g), EtOAc (17.0 g) and *n*-BuOH (9.0 g) extracts were concentrated to dryness. A part of *n*-BuOH extract (2.5 g) was submitted to chromatographic separation on a Sephadex LH-

20 column (3.5 x 100 cm, flow rate 1.0 mL/min) using MeOH as mobile phase; 55 fractions of 10 mL were collected, analyzed by TLC using CHCl₃:MeOH:H₂O (40:9:1 v/v/v) and *n*-BuOH:AcOH:H₂O (60:15:25 v/v/v) as eluent and grouped to obtain five main fractions (A–E). Fractions B (800.0 mg, 200–300 mL) and D (168.0 mg, 410–480 mL) were separately chromatographed using RP-HPLC with MeOH:H₂O (15:35 v/v) as mobile phase (flow rate 2.0 mL min^{−1}) to yield pure compounds **9** (1.1 mg, *t_R* = 46 min) and **8** (1.6 mg, *t_R* = 50 min) from fraction B, and pure compounds **2** (2.4 mg, *t_R* = 14 min) and **1** (1.6 mg, *t_R* = 27 min) from fraction D, respectively. Fraction C (178.3 mg, 310–400 mL) was chromatographed using RP-HPLC with MeOH:H₂O (35:65 v/v) as mobile phase (flow rate 2.0 mL min^{−1}) to yield pure compounds **7** (1.0 mg, *t_R* = 15 min), **4** (1.0 mg, *t_R* = 27 min), **6** (8.0 mg, *t_R* = 34 min), and **3** (1.5 mg, *t_R* = 44 min). Fraction E (195.8 mg, 490–550 mL) yielded pure compound **5**. At the same time, the fresh aerial parts of *O. angustissima* (500.0 g) were decocted in chloroform for fifteen minutes; the solution was filtered from solids. The filtrate was concentrated to dryness under vacuum to give 20.0 g of extract. A portion of crude exudate extract (Ex-ONA) (17.0 g) was fractionated by silica gel column (3.0 x 120 cm), eluting with a gradient of Me₂CO (0–100%) in petroleum ether (v/v) 9:1, 4:3, 7:3, 1:1, 2:8, 0:10 (6.0 L, 4.0 L, 4.0 L, 3.0 L, 2.0 L, 2.0 L). Fractions of 50 mL were collected and grouped by TLC into 14 fractions (AA–NN). Fraction CC (72.0 mg, 5000–5100 mL) was washed with *n*-hexane and then with petroleum ether leading to compound **15** (12.0 mg) as yellow crystals. Fraction DD (104.0 mg, 5150–5500 mL) gave a large quantity of yellowish crystals: repeated recrystallization with Me₂CO led to the isolation of **11** (35.1 mg). Fraction EE (589.0 mg, 5550–6200 mL) was submitted to a silica gel column (2.5 x 20 cm), eluting with *n*-hexane:CHCl₃ (v/v), 10:0, 3:7, 0:10, (1000 mL, 500 mL, 250 mL) and to Sephadex column LH-20 (3.5 x 100 cm, flow rate 1.0 mL/min), eluting with MeOH, and further purification by preparative silica gel TLC in *n*-hexane:CHCl₃ (v/v), 3:7, yielding compounds **10** (49.0 mg) and **12** (25.0 mg). Fractions FF (196.0 mg, 6250–6700 mL) and GG (87.7 mg, 6750–6900 mL) were chromatographed using RP-HPLC with MeOH:H₂O (7:3 v/v) as mobile phase (flow rate 3.5 mL min^{−1}) to yield pure compounds **11** (1.6 mg, *t_R* = 46 min) and **12** (1.6 mg, *t_R* = 47 min) from fraction FF and compounds **14** (1.0 mg, *t_R* = 35.0 min) and **12** (1.1 mg, *t_R* = 47 min) from fraction GG, respectively. Fraction HH (128.0 mg, 6950–7500 mL) was separated using RP-HPLC with MeOH:H₂O (35:65 v/v) as mobile phase (flow rate 3.0 mL min^{−1}) to yield pure compounds **13** (1.4 mg, *t_R* = 29 min) and **14** (1.6 mg, *t_R* = 31 min). Fraction II (282 mg, 7550–7900 mL) was purified using RP-HPLC with MeOH:H₂O (45:55 v/v) as mobile phase (flow rate 3.5 mL min^{−1}) to yield pure compounds **16** (2.0 mg, *t_R* = 28 min) and **18** (2.6 mg, *t_R* = 35 min). Fraction JJ (8.8 mg, 7950–8100 mL) yielded pure compound **18**. Fraction NN (318.4 mg, 15550–

17100 mL) was subjected to silica gel column (2.5 x 20 cm) eluted with CH₂Cl₂:MeOH (v/v), 10:0, 4:1, 3:2, 0:10 (1L, 800 mL, 500 mL, 500 mL), then purified by preparative silica gel TLC (CH₂Cl₂:MeOH, 4:1 v/v) to obtain compound **17** (23.0 mg). All the compounds met the criteria of $\geq 95\%$ purity, as determined by HPLC and NMR analyses.

(3S)-7-Hydroxy-4'-methoxy-isoflavane 3' β -D-glucopyranoside (1): yellow amorphous powder; -36.5 (c 0.042, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ): 230 (3.90), 285 (4.01), 330 (3.78); CD [0]25 (c 0.1, MeOH, nm) – 3300 (231 nm), -5150 (312 nm); HRESIMS: m/z 449.1453, calcd for C₂₂H₂₄O₁₀ m/z 449.1447); MS/MS m/z 287.05 [(M-162)+H]⁺, 272.04 [(M-162-15)+H]⁺; 1H and 13C NMR data, see **Table S2**.

Kaempferol 3-O- β -D-glucopyranoside-7-O-(2'''-acetyl)- β -D-galactopyranoside (**4**): yellow amorphous powder; -84 (c 0.033, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ): 287 (3.90), 330 (4.05); HRESIMS: m/z 653.1721, calcd for C₂₉H₃₂O₁₇ m/z 653.1718); MS/MS m/z 491.11, [(M-162)+H]⁺, 447.09 [(M-206)+H]⁺, 285.04 [(M-162-206)+H]⁺; 1H and 13C NMR data, see **Table S2**.

Acid hydrolysis of compounds 1 and 4

Acid hydrolysis of compounds **1** and **4** was carried out as reported before in our previous paper (Dal Piaz et al., 2013) D-glucose and D-galactose were identified as sugar moieties by comparison with retention times of authentic samples (Sigma-Aldrich).

Liquid Chromatography-High-Resolution Electrospray Ionization Mass Spectrometry analyses

Liquid chromatography separation was achieved on a Luna C₁₈ (150 x 2.1 mm, 5 μ m Phenomenex, Palo Alto, USA), using 0.1% formic acid in water (A) and acetonitrile (B) as eluents. Compounds elution was achieved using a linear gradient from 1 % to 50 % of B over 45 minutes. Mass spectra were acquired in positive ion mode, over the 200-800 m/z range. Source temperature was set at 120 °C, capillary voltage 3300 eV, and cone voltage 50 eV. HRESIMS/MS spectra were acquired for the four most abundant ions observed in each MS spectrum (dependent scan mode).

Antioxidant activity assays

DPPH radical-scavenging activity

The ability to scavenge the DPPH free radical was monitored according to the method reported by Milella et al., 2014 with slight modifications. All samples were tested individually at different

concentrations by addition to a methanol solution of DPPH radical (100 μ M). In all experiments Trolox radical scavenging activity was also determined and used as a reference. Activity was expressed as mg of Trolox equivalents per gram of sample (mg TE/g) (Padula et al., 2013).

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out as previously described with slight modifications (Russo et al., 2012). Also in this case Trolox was used as a reference standard and FRAP values were expressed as mg Trolox equivalents per gram of sample (mg TE/g).

β -Carotene bleaching assay

The β -carotene assay was performed as reported by Milella et al., 2014. Since the reaction was temperature sensitive, outer wells of the plate were filled with 250 μ L of water to provide a large thermal mass (Mikami et al., 2009). Butylhydroxytoluene (BHT) was used as positive control and results were expressed as % A.A.

$$\% \text{ AA} = [1 - (A \text{ sample } T_0 - A \text{ sample } T_{180}) / (A \text{ blank } T_0 - A \text{ blank } T_{180})] * 100.$$

Total polyphenolic content (TPC)

TPC was determined according to the Folin-Ciocalteu method (Milella et al., 2014) using Gallic acid as reference standard. Results were expressed as mg Gallic Acid Equivalents (mgGAE)/g of sample.

Cell culture and cytotoxic assay

HeLa (human cervical carcinoma), Jurkat (human lymphocyte T cells), and human breast adenocarcinoma (MCF7) cell lines were obtained from American Type Cell Culture (ATCC). Cells were maintained in DMEM (HeLa and MCF7) or RPMI 1640 (Jurkat), supplemented with 10% FBS, 100 mg/L streptomycin, and 100 IU/mL penicillin (GIBCO) at 37 °C in a humidified atmosphere of 5% CO₂. Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors (kindly provided by the Blood Center of the Hospital of Avellino, Italy) by using standard Ficoll–Hypaque gradients. Freshly isolated PBMC contained $93 \pm 2.9\%$ live cells. Proliferation of PBMC was induced by phytohemagglutinin (PHA) (10 μ g/mL). Stock solutions (1 mg/mL) of *O. angustissima* extracts were prepared in DMSO and stored at 4 °C. In all experiments, the final concentration of DMSO did not exceed 0.3% (v/v). Cells were seeded in 96-well plates (1×10^4 cells/well). One day after seeding, cells were exposed to increasing

concentrations of extracts (25, 50, 100, 150 µg/mL) or vehicle only and incubated for the established times. The number of viable cells was quantified by MTT assay and CellTiter-Blu Cell Viability assay (Promega) (Mosmann, 1983). Appropriate controls were included in preliminary experiments to exclude any interferences of each compound with the assays. Etoposide was used as positive control.

Statistical analysis

All analyses were performed in triplicate, results are expressed as mean $\pm$ standard deviation (Mean $\pm$ SD) and value of $p < 0.05$ was considered significant. Regression analysis and statistical analysis were carried out using Graphpad Prism 5 software, while Microsoft Excel was used for RACI calculation. Calibration curves of the standards were considered linear if R^2 resulted > 0.99 .

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Figure S1. ^1H NMR spectrum of compound **1** (CD_3OD)

Figure S2. HSQC spectrum of compound **1** (CD₃OD)

Figure S3. HMBC spectrum of compound **1** (CD₃OD)

Figure S4. ^1H NMR spectrum of compound **2** (CD_3OD)

Figure S5. HSQC spectrum of compound **2** (CD₃OD)

Figure S6. HMBC spectrum of compound **2** (CD₃OD)

Figure S7. ^1H NMR spectrum of compound **4** (CD_3OD)

Figure S8. HSQC spectrum of compound **4** (CD₃OD)

Figure S9. HMBC spectrum of compound **4** (CD₃OD)

Figure S10. Total ion current LC-HRESIMS chromatograms obtained for the *n*-BuOH fraction of the aerial parts of *Ononis angustissima*

Table S1. Total phenolic content (TPC) and antioxidant activity (DPPH, BCB, FRAP assays) of *Ononis angustissima* extracts, fractions and isolated compounds

<i>Extract</i>	<i>DPPH</i> <i>mgTE/g*</i>	<i>BCB</i> <i>%AA**</i>	<i>FRAP</i> <i>mgTE/g*</i>	<i>TPC</i> <i>mgGAE/g***</i>
Ex-ONA	17.5±1.4	34.9±2.7	84.6±4.1	236.0±16.8
<i>n</i> -BuOH	22.2±1.9	32.2±2.6	62.7±5.6	81.1±6.1
<i>Fractions</i>				
A	11.2±0.4	0.0±0.0	4.3±0.7	15.4±0.9
B	32.0±2.1	8.3±0.6	19.6±1.5	25.8±1.7
C	36.7±3.0	10.1±0.9	29.6±2.5	121.5±12.1
D	58.8±4.1	57.9±2.5	21.2±2.0	110.9±9.6
E	143.7±9.2	25.6±2.2	8.3±0.9	199.5±11.7
BB	5.1±0.5	4.9±0.3	39.1±2.6	61.0±4.7
CC	7.2±0.6	43.9±3.3	10.7±1.0	158.4±12.4
DD	25.9±1.9	46.4±4.2	5.5±0.4	169.3±9.1
EE	83.3±6.2	3.2±0.1	51.6±3.2	83.8±6.5
FF	16.4±1.8	1.9±0.1	44.1±4.1	268.9±11.9
GG	37.7±2.8	4.1±0.5	100.6±6.4	360.3±14.2
HH	78.2±6.5	6.0±0.7	194.7±9.9	527.8±16.3
II	93.3±9.1	8.1±0.6	56.4±4.2	639.1±19.4
KK	15.1±1.0	1.2±0.1	18.2±1.2	83.2±5.7
LL	11.5±0.9	2.2±0.3	14.8±1.0	72.4±4.9
MM	14.4±1.1	3.2±0.4	15.4±1.2	91.6±6.7
NN	190.5±11.3	32.7±2.6	13.5±0.9	194.2±11.4
<i>Pure compounds</i>				
1	64.7±2.6	76.9±2.8	45.2±3.2	
2	78.5±4.2	36.2±2.5	88.0±6.2	
3	44.9±3.7	65.1±6.1	20.4±1.9	
4	41.7±5.4	33.4±2.6	9.7±1.1	
5	7.3±0.9	15.3±0.9	30.3±2.0	
6	15.5±1.2	46.7±2.8	1.1±0.2	
7	25.3±1.9	44.5±2.6	5.9±0.6	
8	18.5±1.4	4.4±0.3	15.8±1.3	
9	22.5±1.6	3.5±0.2	12.5±1.1	
10	84.3±6.2	7.5±0.7	63.2±3.2	
11	29.9±1.7	49.0±2.5	7.5±0.7	
12	1.4±0.2	36.8±2.6	6.0±0.8	
13	328.0±14.5	36.0±1.9	37.8±2.9	
14	60.5±4.5	78.4±5.2	8.0±1.0	
15	17.2±1.3	46.9±3.9	12.7±1.4	
16	18.6±1.9	6.0±1.0	83.1±1.8	
17	490.7±19.4	22.9±2.1	38.7±3.1	
18	3.4±0.5	43.7±3.6	11.0±1.0	
BHT****		87.1±4.5		

Values are the mean of three determinations ($P < 0.05$). Ex-ONA = crude exudate extract. *n*-BuOH = *n*-butanol extract. *Milligrams of Trolox Equivalents per g of Extract/Fraction/Pure compound; **Antioxidant activity at [0.1 mg/mL]; ***Milligrams of Gallic Acid Equivalents per g of Extract/Fraction/Pure compound; ****BHT = butylhydroxytoluene.

Table S2. ^1H and ^{13}C -NMR data of compounds **1**, **2**, and **4** (CD_3OD , 600 MHz, J in Hz)^a

Position	1 δ_{H}	δ_{C}	2 δ_{H}	δ_{C}	4 δ_{H}	
2a	4.61dd (13.0, 5.0)	72.9	8.11 s	155.2		161.0
2b	3.49 dd (12.5, 11.0)					
3	3.85 dd (11.0, 5.0)	51.6		121.6		134.9
4		192.5		177.5		178.2
5	7.75 d (8.0)	129.6	8.02 d (8.5)	127.4		160.0
6	6.48 br d (8.0)	112.2	6.90 dd (8.5, 1.5)	116.9	6.51 d (1.5)	100.1
7		167.8		166.5		165.0
8	6.31 br s	102.9	6.80 d (1.5)	102.6	6.80 d (1.5)	94.6
9		164.7		158.9		157.4
10		113.1		114.6		104.0
1'		121.9		122.1		121.0
2'	6.97 d (1.5)	113.2		148.0	8.08 d (8.0)	131.5
3'		147.0	7.05 s	102.6	6.90 d (8.0)	116.1
4'		149.0		149.3		158.0
5'	7.11 d (8.0)	117.5		141.7	6.90 d (8.0)	116.1
6'	6.95 dd (8.0, 1.5)	123.0	6.78 s	117.3	8.08 d (8.0)	131.5
1''	4.84 d (7.6)	102.1	4.84 d (7.5)	103.1	5.09 d (7.5)	100.6
2''	3.49 br t (8.5)	73.4	3.31 br t (9.0)	74.3	3.50 br t (8.5)	73.8
3''	3.34 t (9.0)	77.2	3.42 t (9.0)	77.2	3.49 t (9.0)	77.0
4''	3.43 t (9.0)	70.3	3.28 t (9.0)	71.2	3.42 t (9.0)	70.2
5''	3.46 m	76.8	3.42 m	77.2	3.79 m	77.0
6''a	3.72 dd (12.0, 5.0)	61.6	3.62 dd (12, 5.0)	62.1	3.94 dd (12.0, 5.0)	62.5
6''b	3.85 dd (12.0, 2.5)		3.87 dd (12, 3.5)		3.72 dd (12.0, 2.5)	
OCH_3	3.85 s	56.0	3.91 s	56.0		
1'''					5.54 d (7.0)	100.0
2'''					5.29 br t (9.0)	73.5
3'''					3.49 dd (9.0, 3.5)	77.0
4'''					3.73 m	72.1
5'''					3.88 m	69.4
6'''a					3.70 dd (12.0, 5.0)	62.5
6'''b					3.56 dd (12.0, 2.5)	
COCH_3						171.4
COCH_3					2.15 s	22.0

^a Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

Table S3. Identification of the major compounds observed in the HR-LCMS analysis of *n*-BuOH extract of *O. angustissima* in positive ion mode. Peaks numbering is consistent within the LC-MS profile shown in **Figure S10**

Peak	[M+H] ⁺	Main fragments (<i>m/z</i>)	Tentative assignment/compound ¹	Theoretical MW
A	611.1625	449.12, 287.09, 153.04		610.1534
B	595.1677	433.11, 271.08, 153.02		594.1585
4	653.1721	491.11, 447.09, 285.04, 153.01	4	652.1640
9	297.1343	135.00	9	296.1260
5	447.1302	285.05, 165.01	5	446.1213
17	449.1092	287.06, 153.02	17	448.1006
C	299.0924	284.07, 197.04, 179.03		298.0841
14	285.0770	270.06, 167.02	14	284.0685
D	285.0772	270.05, 183.04, 168.03		284.0685
E	285.0775	270.04, 167.03, 152.03		284.0685
8	327.1451	165.01	8	326.1366
6	475.1612	313.06, 193.01	6	474.1526
7	445.1505	283.02, 163.01	7	444.1420
11	241.0873		11	240.0786
3	431.1353	269.04, 254.02, 213.01	3	430.1264
12	287.1289	272.09, 183.07	12	286.1205
18	269.0820	254.05, 167.02, 152.01	18	268.0736

F	465.1041	405.09, 349.05, 269.03		464.0955
16	285.0781	270.05, 183.01, 168.01	16	284.0685
2	463.1251	301.04, 286.03, 245.03, 137.01	2	462.1162
g	287.0563	169.03, 141.02		286.0477
h	269.0821	254.06; 167.05, 152.03		268.0736
i	269.0820	254.05, 159.01 111.01		268.0736
j	283.0982	268.07, 181.04		282.0892
15	329.1031	314.07, 227.03, 212.02	15	328.0947
10	273.1132	258.09, 169.03	10	272.1049
1	449.1453	287.05, 272.04, 137.01	1	448.1369
13	361.0930	346.07, 227.04, 212.03	13	360.0845
k	299.0926	284.08, 243.07, 167.05		298.0841
l	255.0663	153.02		254.0579
m	329.1046	314.10, 273.08, 197.03		328.0947
n	313.1095	298.10, 257.08, 181.6		312.0998

hex = hexose