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 phytochemical and biological studies. Two new natural flavonoids, (3*S*)-7-hydroxy-4'-methoxy-isoflavanone 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 compare the 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.

1. Introduction

Plants rich in antioxidant phenolics have been attracting an increasing attention and nowadays the discovery of new sources of safe and inexpensive antioxidants from natural origin it's mandatory (Fernández et al., 2006), since some synthetic ones showed potential health risks and toxicity (Safer and Nughamish, 1999). *Ononis* L. is a large genus of perennial herbs and shrubs of the Fabaceae family (Ozenda, 1958) known to be very rich in phenolic derivatives (Barrero et al., 1993; Abdel-Kader, 2001; Mhamdi et al., 2015). Some *Ononis* species are favorite food for sheeps, goats, donkeys and other animals; the young shoots are succulent and sweet and in some Saharian countries are eaten as vegetables (Ozenda, 1958; Burnett, 1833). Recently, some herbal teas containing *Ononis* species as components are sold in the market. Several plants belonging to the *Ononis* genus are used as folk medicine remedies for the treatment of jaundice, urinary tract inflammations and kidney stones; besides, some species have been used as wounds, eczema and rheumatic complaints healings, against skin cancer and lesions and topically as antiseptic and antimicrobial agents (Süntar et al., 2011). The ethnopharmacology importance and the richness in flavonoids of *Ononis* genus prompted us to carry out further studies (Mezrag et al., 2013; Bouheroum et al., 2009) on *O. angustissima* Lam., a plant widespread in Sahara regions. An antioxidant-oriented approach was carried out on the aerial part extracts and exudate, leading to the isolation and structural characterization of two new natural flavonoids, (3*S*)-7-hydroxy-4'-methoxy-isoflavanone 3'-β-D-glucopyranoside (**1**), and kaempferol 3-*O*-β-D-glucopyranoside-7-*O*-(2'''-acetyl)-β-D-galactopyranoside (**4**), together with sixteen known phenolic derivatives (**2-3** and **5-18**) (**Fig. 1**). In order to deepen and full characterize the phytochemical profile of *O. angustissima*, LC-HRESIMS and LC-HRESIMS/MS analyses were also performed. Extracts, fractions and pure compounds antioxidant activity was tested by using three different complementary methods and the Relative Antioxidant Capacity Index (RACI) was also calculated. All extracts were also evaluated for their cytotoxic activity.

2. Results and Discussion

In this study all extracts of *O. angustissima* were subjected to the 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-Carotene Bleaching (BCB) and Ferric Reducing Antioxidant Power (FRAP) assays to screen their antioxidant activity. As previously demonstrated (Milella et al., 2014), a single assay cannot determine the antioxidant activity of a phytocomplex; therefore three complementary approaches were used to study the antioxidant potential of *O. angustissima.* Total phenolic content (TPC) was also measured and the results were included in RACI calculation. In this way RACI provided a more comprehensive assessment of the whole extract antioxidant potential. Both *n*-BuOH and crude exudate (Ex-ONA) extracts showed an interesting antioxidant activity and to be a good source of phenolics (**Table S1**); on the basis of these results they were further analysed. The *n*-BuOH extract was subjected to Sephadex LH-20 column chromatography collecting five major fractions (A-E), that were consequently evaluated for their TPC and antioxidant activities (*AA*) (**Table S1**). Fraction E resulted the richest in terms of TPC (199.5 mgGAE/g) followed by fractions C and D (121.5 and 110.9 mgGAE/g, respectively). Fraction E showed the highest DPPH value (143.7 mgTE/g of extract), while the highest BCB value was registered for fraction D demonstrating 57.9%

of antioxidant activity. On the other hand, the highest FRAP value was obtained from fraction C (29.6 mgTE/g). On the basis of RACI results (**Fig. 2a**), fractions B-E were purified and a total of nine compounds (**1-9**) were characterized (**Fig. 1**) of which two were new natural flavonoids (**1** and **4**). Fraction E showed also the presence of highly polymerized polyphenols. Following the same approach, the TPC content and the *AA* of Ex-ONA fractions (AA-NN) were also studied, demonstrating a higher TPC content than the *n*-BuOH one, according to the respective extract results (**Table S1**). The highest TPC value was observed for fractions II and HH with 639.1 and 527.8 mgGAE/g respectively; these two fractions resulted also the most active in DPPH test (93.3 and 78.2 mgTE/g respectively), together with the NN fraction (190.5 mgTE/g). On the other hand, Ex-ONA fractions CC and DD demonstrated the highest *AA* when measured by BCB test. This phenomenon can be explained by the affinity of the antioxidant complex for the lipids and thus the lipophilic nature of BCB test could be the determining factor (Milella et al., 2014; von Gadow et al., 1997). RACI was calculated (**Fig. 2a**) and fractions LL, KK, MM, and BB, together with the totally inactive fraction AA (data not shown), were not further analysed.

The molecular formula of compound **1** ($C_{22}H_{24}O_{10}$) was determined by HRESIMS spectra (*m/z* 449.1453) and ^{13}C NMR. In the HRESIMS/MS spectrum, fragments at *m/z* 287.05 $[(M-162)+H]^+$ and 272.04 $[(M-162-15)+H]^+$ were observed. The ^1H NMR spectrum of **1** (**Table S2**) showed signals for an oxygenated methine double of doublets at δ 3.85 (1H, dd, *J* = 11.0, 5.0 Hz, H-3), two methylene double of doublets at δ 4.61 (1H, dd, *J* = 13.0, 5.0 Hz, H-2a) and 3.49 (1H, dd, *J* = 12.5, 11.0 Hz, H-2b). The proton signals of ring A were observed at δ 7.75 (1H, d, *J* = 8.0 Hz, H-5), 6.48 (1H, br d, *J* = 8.0 Hz, H-6), and 6.31 (1H, br s, H-8), while in the B-ring, a 1,3,4-trisubstitution was evident from the three signals at δ 7.11 (1H, d, *J* = 8.0 Hz, H-5'), 6.95 (1H, dd, *J* = 8.0, 1.5 Hz, H-6') and 6.97 (1H, d, *J* = 1.5 Hz, H-2') (Cooper et al., 2002). In the ^1H NMR the presence of an anomeric proton at δ 4.84 (1H, d, *J* = 7.6 Hz) and a methoxy group at δ 3.85 (3H, s) was also observed. The 1D-TOCSY spectrum together with information obtained from ^{13}C NMR led to establish the presence a β-glucose unit. Hydrolysis of **1** with 1N HCl, followed by GC analysis through a chiral column of the trimethylsilylated monosaccharide, led to the assignment of the glucose configuration. All the NMR assignments were obtained using 1D-TOCSY, COSY, HSQC, and HMBC NMR correlations. The presence of the methoxy group at C-4' was confirmed by NMR chemical shifts and the HMBC correlation between OMe and C-4', while the correlation of $H\text{-}1_{glc}$ and C-3' located the glucose moiety at C-3'. In order to establish the absolute configuration at C-3, the CD spectrum was recorded (Kuhn et al., 2003; Slade et al., 2005). The negative Cotton effect at 312 nm led to establish the (3*S*) configuration at C-3 of compound **1**. Thus, the structure of **1** was elucidated as (3*S*)-7-hydroxy-4-methoxy-isoflavanone 3'-β-D-glucopyranoside.

The HRESIMS and HRESIMS/MS of compound **4** ($C_{29}H_{32}O_{17}$) displayed a quasimolecular ion peak at *m/z* 653.1721 $[M+H]^+$ and prominent fragments at *m/z* 491.11, $[(M-162)+H]^+$, 447.09 $[(M-206)+H]^+$, and 285.04 $[(M-162-206)+H]^+$, corresponding to the losses of one hexose, one acetylated hexose, and both the sugar units, respectively. The ^{13}C NMR spectrum (**Table S2**) showed 29 signals, of which 12 were assigned to a saccharide portion, 2 to an acetyl group, and 15 to a flavonol moiety. The ^1H and ^{13}C NMR spectra (**Table S2**) of **4** clearly indicated that kaempferol was the aglycone (Agrawal, 1989; Le Gall et al., 2013). Analysis of 1D-TOCSY and DQF-COSY spectra allowed the complete assignments of all proton resonances of the

glucose unit starting from the anomeric proton signal at δ 5.09 and of the galactose unit starting from the H-1 signal at δ 5.54, and the H_2-6 signals at δ 3.70 and 3.56, showing that hydroxyl group at C-2 of galactose was acetylated. These results were consistent with the typical esterification shift of H-2_{gal} signal at δ 5.29 and C-2_{gal} at 73.5 ppm (D'Agostino et al., 1992). The HMBC cross peaks were useful in the determination of the aglycone substitution pattern. Diagnostic long range correlations were observed between H-1_{glc} and C-3, H-1_{gal} and C-7, and H-2_{gal} and COO. The sugar configuration was established as reported for **1**. On the basis of the above results, **4** was characterized as kaempferol 3-*O*-β-D-glucopyranoside-7-*O*-(2'''-acetyl)-β-D-galactopyranoside.

Compound **2** had a molecular ion peak at *m/z* 463.1251 (HRESIMS), corresponding to a molecular formula of $C_{22}H_{22}O_{11}$. The HRESIMS/MS spectrum showed one peak at *m/z* 301.04 $[(M-162)+H]^{+}$, due to the loss of one hexose moiety, and a fragment at *m/z* 245.03 $[(M-162-56)+H]^{+}$, diagnostic of the presence of an isoflavone skeleton (Khun et al. 2003). From 1D and 2D NMR data (**Table S2**), its structure was deduced to be 5',7-dihydroxy-2'-methoxy-isoflavone 4'-*O*-β-D-glucopyranoside, recently described by Ghribi et al. from *O. angustissima* roots (Ghribi et al., 2015). However, chemical shifts of glucose moiety were completely wrongly attributed by these authors; compound **2** correct ^{1}H and ^{13}C NMR values were now assigned in **Table S2** and all its experimental data were listed in Supplementary material.

Compounds **3** and **5-18** were characterized as formononetin 7-*O*-β-D-glucopyranoside or ononin (**3**) (Wang et al., 2013), trifolirhizin (**5**) (Zhao et al., 2009), 9-*O*-methylspinonin (**6**), spinonin (**7**) (Kırmızıgül et al., 1997) citrusin C (**8**) (Hammami et al., 2006), chavicol 1-*O*-β-D-glucopyranoside (**9**) (Higuchi et al., 1977), 2',6'-dihydroxy-4'-methoxydihydrochalcone (**10**) (Orjala et al., 1994), 2',4'-dihydroxychalcone (**11**) (Ghani et al., 2012), dihydroflavokawin B (**12**) (Itokawa et al., 1988), 5,3,4'-trihydroxy-6,7,8-trimethoxyflavone (**13**) (Ruiu et al., 2015), 7-methylapigenin (**14**) (Agrawal et al., 1989), alnetin (**15**) (Asakawa et al., 1971), 5,7-dihydroxy-6,8-dimethoxyflavone (**16**) (Dietz et al., 1981), luteolin 7-*O*-β-D-glucopyranoside (**17**) (Agrawal et al., 1989), and 7-*O*-methylchrysin (**18**) (Heim et al., 2002), by spectrometric and spectroscopic measurements and comparison with the literature data.

The antioxidant activity of all pure compounds using DPPH, BCB, and FRAP tests was assayed (**Table S1**). Among isolated compounds from the *n*-BuOH fractions (**1-9**), compound **2** showed the highest DPPH and FRAP values, while the other isoflavones **1** and **3** were less active (**Table S1**). This difference could be ascribed to the loss of free –OH on aromatic rings in **1** and **3**. Our data also confirmed that the presence of 2,3 double bond increases the activity of compound **2** when it is compared with that of **1**. These results are in agreement with those recently shown on similar molecules (Ghribi et al., 2015). Moreover, compound **5** showing no free –OH groups and 2,3 double bond, displayed the lowest activity among isoflavone derivatives (**Table S1**). It is possible to assess that **17** and **13** are the most active flavones (**Fig. 2b**), both exerting the catechol group on ring B, while compounds **8** and **9** showed the lowest values, both without any free phenolic hydroxyl group. RACI (**Fig. 2b**), combining the antioxidant results obtained from different assays, showed that flavones and isoflavones are the most active compounds, and confirmed the importance of the adjacency of two hydroxyl groups in the *ortho*-diphenolic arrangement (Heim et al., 2002; Lee et al.,

2008; Rice-Evans et al., 1996). The interesting antioxidant potential obtained for *O. angustissima* extracts and fractions could be only partially attributed to the activity of pure isolated compounds: the presence of minor constituents could better clarify this activity. Therefore, the *n*-BuOH extract was subjected to LC-HRESIMS and LC-HRESIMS/MS analyses, in order to provide wider information concerning the polyphenols contents of *O. angustissima* aerial parts (**Fig. S10**). Thirty-two main species were revealed, including compounds **1**-**18**. On the basis of accurate molecular weight determination, and taking into account the main fragments observed in the MS/MS spectra, the structure of further fourteen derivatives was proposed (**Table S3**). Particularly, nine other flavones (compounds **a**-**e**, **g**, **h**, **j**, **l**) and four isoflavones (**f**, **k**, **m**, **n**) were detected: the latter were distinguished by the loss of 56 amu fragment typically observed only in the MS/MS spectra of isoflavones (Cooper et al., 2002). The hydroxylation pattern on A and B rings could be recognized taking into account the different ions $^{1,3}A^+$ related to C-ring fragmentation (Wu et al., 2004). The following ions suggested the hydroxylation pattern of ring A for compounds **a**-**h** and **j**-**n**: *m/z* 137 $(C_7H_5O_3)^+$ indicated one hydroxy, 153 $(C_7H_5O_4)^+$ two hydroxy, 167 $(C_8H_7O_4)^+$ one hydroxy and one methoxy, 181 $(C_9H_9O_4)^+$ two methoxy, 183 $(C_8H_7O_5)^+$ two hydroxy and one methoxy, 197 $(C_9H_9O_5)^+$ two methoxy and one hydroxy, 213 $(C_9H_9O_6)^+$ two methoxy and two hydroxy, 227 $(C_{10}H_{11}O_6)^+$ three methoxy and one hydroxy groups, respectively. Moreover, one putative aurone (compound **i**) was also proposed. These results are in agreement with the detected antioxidant activity.

Finally, the cytotoxic activity of crude *O. angustissima* extracts was investigated against healthy PBMC and three human cancer cell lines HeLa, Jurkat, and MCF7. All the extracts were inactive at the tested concentrations. The absence of cytotoxicity on healthy PBMC could suggest the use of *O. angustissima* in herbal drugs preparation.

3. Conclusion

Spectrometric and spectroscopic analyses including 2D NMR allowed to isolate and identify two new natural secondary metabolites and sixteen known compounds from *O. angustissima* aerial parts belonging to the flavonoid, isoflavonoid, phenylpropanoid, spinonin and chalcone classes. Flavonoids and isoflavonoids reported the highest relative antioxidant capacity index and some structure–activity relationships based on the presence of free OH groups, different substituents on aromatic rings and the presence of 2,3 double bond in C ring, were established.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary material

Experimental section, NMR spectra of compounds **1** and **4,** NMR data of compounds **1**, **2** and **4**, total ion current LC-HRESIMS chromatograms of *n*-BuOH fraction of the aerial parts, data of total phenolic content and antioxidant activity of *Ononis angustissima* extracts, fractions and isolated compounds, and identification of the major compounds observed in aerial parts *n*-BuOH fraction by HR-LCMS analysis are available as Supplementary material.

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