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ORIGINAL ARTICLE

Antioxidant and α-glucosidase inhibitory activities of *Achillea tenorii*

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Abstract

Context: There is a need for the discovery of novel natural remedies to prevent and treat metabolic disorders such as hyperglycemia, type II non-insulin-dependent diabetes mellitus, and obesity. Several *Achillea* species have been utilized for centuries all around the world and are generally considered effective as hypoglycemic.

Objective: Considering the ethnobotanical uses of *Achillea* genus, we evaluated the *in vitro* inhibitory activity of *Achillea tenorii* Grande (Asteraceae) extract on α-glucosidase, which is a valuable target to prevent and treat metabolic disorders. We also tested its antioxidant activity. Moreover, the phytochemical profile was discussed from a chemotaxonomic point of view.

Materials and methods: *In vitro* α-glucosidase inhibition of crude ethanolic extract obtained from the aerial parts was assayed as well as the *in vitro* antioxidant activity (ABTS, DPPH, and FRAP-FZ tests) was measured. The extract was characterized from a phytochemical point of view by means of spectroscopic analysis.

Results: The extract results endowed with α-glucosidase inhibitory activity (IC_{50} 32 μg/mL) with a particular mechanism of action definable as un-competitive, which differed from the mechanism observed for the best-known α-glucosidase inhibitor (acarbose and miglitol). In addition, a considerable antioxidant potential has been found for *A. tenorii* extract, which resulted mainly constituted by phenolic compounds such as caffeoylquinic acids and flavonoids.

Discussion and conclusions: These results suggest the potential of *A. tenorii* as a possible natural remedy to prevent and treat metabolic disorders of carbohydrates.

Keywords

Phytochemical profile, type II diabetes, un-competitive α-glucosidase inhibition

History

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Introduction

Achillea tenorii Grande (Asteraceae) [syn. *A. virescens* (Fenzl) Heimerl subsp. *virescens* (Grande) Bässler] is considered a member of *A. nobilis* aggregate. There are many endemic species in the genus *Achillea*, among them *A. tenorii*, whose presence is restricted to certain areas; it grows from 900 up to 2200 m (a.s.l.) in stony mountains and high mountain pastures of central and southern Apennines (Conti et al., 2005). As regards its morphological characteristics, this plant is a scapose hemicryptophyte perennial herb with pinnatisect leaves, narrow segments, and small capitula bearing white-yellowish ligulate florets accompanied by pubescent and glandular bracts. Notably, *A. tenorii* differs from other species of the *Achillea* genus for the presence of stolons, short floral tube, and dark bracts (Pignatti, 1982). Several *Achillea* species have been utilized in ethnopharmacology for centuries all around the world. Although the use as traditional remedy depends considerably on national traditions, *Achillea* species are generally considered effective as tonic, anti-inflammatory, spasmolytic, diaphoretic, diuretic, hypoglycemic, anti-bleeding, and wound healing (Nemeth & Bernath, 2008). A large number of phytochemical and pharmacological studies were carried out on different species of *Achillea* genus and recent findings have also confirmed some traditional uses but until now no data are reported for *A. tenorii*.

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Objective

In this work, we elucidated the phytochemical composition of *A. tenorii* polar fraction, discussing the results from a chemotaxonomic point of view. Furthermore, in order to provide information on the potential of *A. tenorii* hydroalcoholic extract as an antioxidant, we performed ABTS, DPPH, and FRAP-FZ tests. Moreover, considering the ethnobotanical use of different *Achillea* species in the treatment of diabetes as well as different studies on the hypoglycemic effect of these plants (Al-Hindawi et al., 1989; Yazdanparast et al., 2007), we have tested the inhibitory activity of *A. tenorii* extract on α-glucosidase, a membrane-bound enzyme of the GH31 family, which plays a key role in carbohydrate absorption. Indeed, this enzyme catalyzes the final step in

the digestive process of carbohydrates, by the hydrolysis of polysaccharides into glucose and related monosaccharides, which can be easily absorbed (El-Kaissi & Sherbeeni, 2011). Therefore, this enzyme may be considered a valuable target in order to reduce the postprandial glucose blood rise and α-glucosidase inhibitors may be useful to prevent and treat metabolic disorders such as type II non-insulin-dependent diabetes mellitus, obesity, and hyperglycemia (Baron, 1998; Taylor & Johnson, 1996).

Materials and methods

General

NMR spectra were recorded on Varian Mercury 300 MHz instrument using CD_3OD or D_2O as deuterated solvents; the chemical shifts were expressed in ppm from tetramethylsilane (TMS).

MS spectra were performed on a Q-TOF MICRO spectrometer (Waters, Manchester, UK) equipped with an ESI source, which was operated in the negative and/or positive ion mode. The flow rate of sample infusion was 10 μL/min with 100 acquisitions per spectrum. Data were analyzed using the MassLynx software developed by Waters (Manchester, UK).

Solvents of RPE grade were purchased from Sigma Aldrich (Milan, Italy) or Carlo Erba Reagenti (Milan, Italy); silica gel 60 (70–230 mesh ASTM) were from Fluka (Dresden, Germany); all the other reagents were purchased from Sigma Aldrich (Milan, Italy).

Plant materials

Plant materials were collected from the Majella National Park on June 2011 and the botanical identification was performed by the botanists of the Park (Dr. Mirella Di Cecco and Dr. Giampiero Ciaschetti). A sample of the studied plant is stored in our laboratory under the accession number: AT03062011.

Extraction and isolation of polar compounds

Four consecutive extractions were conducted on *A. tenorii* aerial parts (400 g) using a mixture of ethanol/water (3 L for each extraction, 48 h infusion time). In particular, for the first extraction, ethanol 96% v/v was used, while in the following three extractions, ethanol 80% v/v was used. The obtained extracts were collected separately and, after evaporation of the organic solvent, the extracts were frozen and lyophilized. From the first to the fourth extraction: 10.12, 1.87, 1.52, and 0.89 g of crude extracts were obtained.

The chromatographic screening on TLC revealed the abundance of phenolic compounds, also evidenced by a strong positive reaction with the $FeCl_3$ spray reagent. The four extracts appeared similar from a qualitative point of view while quantitative differences were evident, in particular the third and fourth extracts were constituted mainly of compounds with $R_f. < 0.6$, while the first extract showed the whole molecular pattern and, for this reason, it was subjected to fractionations and tested for biological activity.

Repeated silica gel column chromatography was conducted on 2.05 g of the first extract using different solvent mixtures: butanol saturated with water, mixtures of chloroform/methanol at various percentages, with or without previous saturation with CO_2, and increasing the polarity during the chromatographic run. This separation procedure yielded fractions containing: a mixture of caffeoylquinic acids (chlorogenic acid (**1**) and neochlorogenic acid (**2**) 2:1, (92.9 mg)), caffeic acid (**3**) (13.2 mg), apigenin-7-*O*-β-D-glucopyranoside (**6**) (17.3 mg) and luteolin-7-*O*-β-D-glucopyranoside (**7**) (29.1 mg), from the more polar fractions, and the respective flavone aglycones apigenin (**4**) (8.2 mg), luteolin (**5**) (5.6 mg) from the less polar fractions (Figure 1). All the isolated substances were identified by comparison of experimental spectroscopic data with those reported in the literature and also by direct comparison with standard substances available in our laboratory.

3-*O*-Caffeoylquinic acid (**chlorogenic acid**) (**1**): ^{1}H NMR (300 MHz, CD_3OD) δ: 7.55 (1H, *d, J* = 15.9 Hz, Hβ), 7.04 (1H, *d, J* = 1.9 Hz, H2′), 6.95 (1H, *dd, J* = 8.2, 1.9 Hz, H6′), 6.77 (1H, *d, J* = 8.2 Hz, H5′), 6.26 (1H, *d, J* = 15.9 Hz, Hα), 5.33 (1H, *td, J* = 9.1, 4.5 Hz, H3), 4.22–4.12 (1H, m, H4), 3.72 (1H, *dd, J* = 8.5, 3.1 Hz, H5), 2.29–1.97 (4H, *m*, H2, H6). ^{13}C NMR 75 MHz, D_2O, δ: 179.7 (COOH quin.), 168.9 (COO caff.), 147.5 (C4′), 146.1 (Cβ), 144.2 (C3′), 129.7 (C1′), 122.6

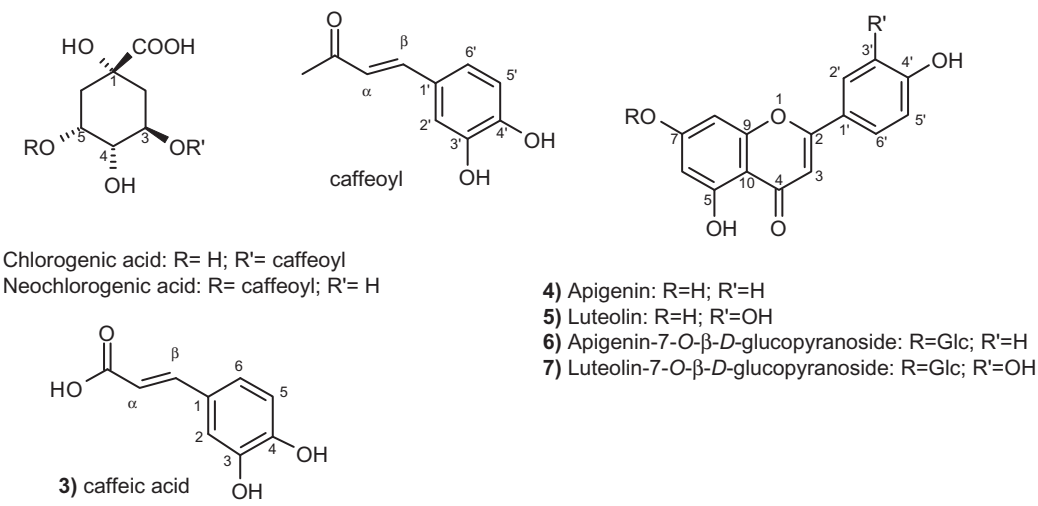

Figure 1. Identified compounds of polar fraction.

(C6′), 116.2 (C5′), 115.3 (C2′), 114.2 (Cα), 77.1 (C1), 75.9 (C3), 74.3 (C4), 72.9 (C5), 38.9 (C6), 37.0 (C2). ESI-MS: *m/z* $[M+Na]^+ = 377.02$; *m/z* $[M-H]^- = 352.85$.

5-*O*-Caffeoylquinic acid (neochlorogenic acid) (**2**): ^{1}H NMR 300 MHz D_2O δ: 7.31 (1H, *d, J* = 15.9 Hz, Hβ), 6.76 (1H, *d, J* = 1.8 Hz, H2′); 6.64 (1H, *d, J* = 1.8 Hz, H6′); 6.06 (1H, *d, J* = 15.9 Hz, Hα); 5.05 (1H, *s*, H5); 4.08 (1H, *bd*, H3); 3.81 (1H, *bd*, H4); 2.00 (1H, *m*, H6); 1.886 (1H, *m*, H2). ^{13}C NMR 75 MHz, D_2O δ: 180.8 (COOH quin.), 169.8 (COO caff.), 147.9 (C4′), 146.9 (Cβ), 145.09 (C3′), 127.8 (C1′), 123.5 (C6′), 117.05 (C5′), 115.96 (C2′), 115.4 (Cα), 77.59 (C1), 73.73 (C4), 72.49 (C5), 71.9 (C3), 38.9 (C6), 37.9 (C2). ESI-MS: *m/z* $[M+Na]^+ = 377.02$; *m/z* $[M-H]^- = 352.85$.

Caffeic acid (**3**): ^{1}H NMR (300 MHz, CD_3OD) δ: 7.54 (1H, *d, J* = 15.9 Hz, Hβ), 7.04 (1H, *d, J* = 1.9 Hz, H2), 6.93 (1H, *dd, J* = 8.2; 1.9 Hz, H6), 6.78 (1H, *d, J* = 8.2 Hz, H5), 6.22 (1H, *d, J* = 15.9 Hz, Hα). ^{13}C NMR (75 MHz, CD_3OD) δ: 171.08 (COOH), 149.36 (Cβ), 147.06 (C4), 146.69 (C3), 127.72 (C1), 122.87 (C6), 116.44 (C5), 115.42 (C2), 115.05 (Cα). ESI-MS: *m/z* 179.05 $[M-H]^-$.

Apigenin (**4**): ^{1}H NMR (300 MHz, CD_3OD) δ: 7.84 (2H, *d, J* = 8.6 Hz, H2′, H6′), 7.12 (2H, *d, J* = 8.5 Hz, H3′, H5′), 6.82 (1H, *s*, H3), 6.68 (1H, *d, J* = 2.0 Hz, H8), 6.57 (1H, *d J* = 2.0 Hz, H6). ^{13}C NMR (75 MHz, CD_3OD) δ: 182.0 (C4), 165.7 (C7), 163.2 (C5), 162.5 (C2), 162.2 (C4′), 157.6 (C9), 129.1 (C2′, C6′), 122.0 (C1′), 116.8 (C3′, C5′), 105.3 (C10), 103.3 (C3), 99.0 (C6), 94.6 (C8). ESI-MS: *m/z* 269.03 $[M-H]^-$.

Luteolin (**5**): ^{1}H NMR (300 MHz, CD_3OD) δ: 7.41 (1H, *dd, J* = 8; 2 Hz, H6′), 7.37 (1H, *d, J* = 2 Hz, H6′), 6.90 (1H, *d, J* = 8.2 Hz, H5′), 6.68 (1H, *s*, H3), 6.47 (1H, *d, J* = 1.8 Hz, H8), 6.28 (1H, *d, J* = 1.8 Hz, H6). ^{13}C NMR (75 MHz, CD_3OD) δ: 182.4 (C4), 164.7 (C7), 164.4 (C2), 162.5 (C5), 150.4 (C4′), 146.6 (C3′), 123.1 (C6′), 119.5 (C1′), 116.8 (C5′), 104.7 (C10), 99.6 (C6), 96.2 (C8). ESI-MS: *m/z* 287.15 $[M-H]^-$; *m/z* 309.27 $[M+Na]^+$.

Apigenin-7-*O*-β-D-glucopyranoside (**cosmetin**) (**6**): ^{1}H NMR (300 MHz, CD_3OD) δ: 7.85 (2H, *d, J* = 8.5 Hz, H3′, H5′), 6.92 (2H, *d, J* = 8.6 Hz, H2′, H6′), 6.79 (1H, *s*, H8), 6.62 (1H, *s*, H3), 6.48 (1H, *s*, H6), 5.07 (1H, *d, J* = 7.0 Hz, H1″), 4.12–3.50 (overlapped sugar signals). ^{13}C NMR (75 MHz, CD_3OD) δ 184.0 (C4), 166.7 (C7), 164.7 (C5), 163.8 (C2), 162.8 (C4′), 158.9 (C9), 129.6 (C2′, C6′), 123.0 (C1′), 117.0 (C3′, C5′), 107.0 (C10), 105.3 (C3), 104.1 (C6), 101.6 (C1″), 78.5 (C5″), 77.3 (C3″), 74.7 (C2″), 71.3 (C4″), 62.5 (C6″). ESI-MS: *m/z* 431.26 $[M-H]^-$.

Luteolin-7-*O*-β-D-glucopyranoside (**cynaroside**) (**7**): ^{1}H NMR (300 MHz, CD_3OD) δ: 7.42 (2H, *bd, J* = 8.2, H6′, H2′), 6.89 (1H, *d, J* = 8.5, H5′), 6.79 (1H, *bs*, H8), 6.68 (1H, *bs*, H3), 6.47 (1H, *s*, H6), 5.09 (1H, *d, J* = 7.2, H1″). ^{13}C NMR (75 MHz, CD_3OD) δ 184.2 (C4), 166.7 (C7), 164.8 (C2), 162.8 (C5), 151.0 (C4′), 147.1 (C3′), 124.1 (C6′), 120.5 (C1′), 117.1 (C5′), 104.2 (C10), 101.2 (C1″), 99.5 (C6), 96.1 (C8), 77.9 (C5″), 75.4 (C3″), 74.7 (C2″), 72.9 (C4″), 62.51 (C6″). ESI-MS: *m/z* 471.14 $[M+Na]^+$; *m/z* 447.23 $[M-H]^-$.

Biological activities

DPPH and ABTS

DPPH assay was performed according to Brand-Williams et al. (1995), with some modifications. Different stock solutions of plant extract were prepared in ethanol/water (20% v/v) to have different final concentrations (5, 10, 30, 50, and 100 μg/mL in the assay) in order to calculate the IC_{50} value. DPPH methanol solution was added to different concentrations of extract and allowed to react at room temperature. Assay was performed in a final volume of 2.0 mL. After 20 min, the absorbance (Abs) values were measured at 517 nm and converted into the percentage antioxidant activity using the following formula:

$$\text{Scavenging capacity } \% \left[1 - (\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}) \times 100\right]$$

DPPH solution plus methanol only was used as a negative control, Trolox (Tr) at different concentrations (from 5 to 50 μM) was used as a positive control and Tr was used for the calculation of total antioxidant capacity (TAC), expressed in mmol Tr eq/g of extract.

ABTS assay was performed according to Arnao et al. (2001), with some modifications. $ABTS^{\bullet+}$ radical was generated by mixing a 2.0 mM ABTS solution with 7.0 mM $K_2S_2O_8$ and incubating in the dark for 24 h at room temperature. Before usage, the $ABTS^{\bullet+}$ solution was diluted (1–25 mL methanol) to get an Abs of 0.7 at 734 nm. 0.9 mL of the diluted $ABTS^{\bullet+}$ solution to 10 μL of Tr (positive control) or extract stock solutions was added to have a final concentrations of 5, 10, 15, 20, 50, and 100 μg/mL. The Abs at 734 nm was recorded after 1.0 min. The IC_{50} values both in ABTS and in DPPH were calculated by linear regression of plots, where *x*-axis represents sample or Tr concentrations, and *y*-axis represents the average percentage of scavenging capacity from three independent experiments with duplicate samples.

FRAP-ferrozine assay

The colorimetric method was used to determine the capacity of the extract to reduce the ferric ions (Fe^{3+}) in ferrous ions (Fe^{2+}), which was obtained following the method proposed by Beker et al. (2010) with some modifications.

A calibration curve was created for the complex Fe^{2+}/ferrozine (FZ) using increasing concentrations of $FeCl_2$ (from 0 to 100 μM) and 0.5 mM FZ (in distilled water) in a final volume of 1 mL. 200 μL of a previously prepared mixture containing $FeCl_3$ (1 mM) and FZ (5 mM) were added to 100 μL of Tr (concentrations range from 6.0 to 300 μM) or extract (concentrations range from 10 to 50 μg/mL). The Abs was read in the microplate reader at 570 nm after 5 min incubation at room temperature. The Abs of $FeCl_3$/FZ mixture was subtracted from that obtained with Tr or samples. In order to build a dose–response curve for Tr, the Fe^{2+} amount produced by different concentrations of this antioxidant was calculated from the calibration curve.

Based on the dose–response curve, FRAP unit, which represent the sample amount capable of producing 100 μM Fe^{2+}, was calculated. TAC (mmol Tr/g of extract) value was calculated comparing the result obtained from the extract to that of Tr. The assay was performed in duplicate and repeated three times.

α-Glucosidase inhibitory assay

α-Glucosidase from *Saccharomyces cerevisiae* (E.C. 3.2.1.20) was purchased from Sigma Aldrich Co (St. Louis, MO) and

α-glucosidase inhibition assay was performed according to Li et al. (2005) with slight modifications. 3 mU of enzyme (one unit will liberate 1.0 μmol of D-glucose from *p*-nitrophenyl-α-D-glucopyranoside per min at pH 6.8 at 37 °C) prepared in 0.1 M phosphate buffer pH 6.8 were incubated for 10 min with test samples at different concentrations: from 5 to 100 μg/mL or pure standard compounds (luteolin, chlorogenic acid) from 0.01 to 1.5 mM, to a final volume of 100 μL. The synthetic substrate *p*-nitrophenyl-α-D-glucopyranoside (p-NPG), prepared in buffer, was added to the preincubated mixture at a final concentration of 2 mM, to start the reaction with a final volume of 200 μL. After 5 min (t_5), 50 μL of NaOH 0.1 M was added and the absorbance at 405 nm was recorded in the microplate reader under a constant temperature of 30 °C. The initial absorbance of the samples at t_0 was established through their blanks (containing all the reagents except for the enzyme). The specific activity of the enzyme referred to *p*-NPG was 14 U/mg as reported by the purchaser.

The IC_{50} value (concentration necessary for 50% inhibition) was calculated by constructing a logarithmic curve showing sample concentrations on *x*-axes and the percentage inhibition on *y*-axes. The percentage of inhibition of enzyme activity was calculated by the following formula:

$$\%\ \text{Inhibition} = \left[\frac{1 - \left(\Delta\text{Abs}/\min_{\text{sample}}\right)}{\left(\Delta\text{Abs}/\min_{\text{nega contr}}\right)}\right] \times 100$$

A negative control was obtained by adding water instead of samples and ΔAbs values were calculated as Abs t_{10} − Abs t_0 and referred to 1 min.

The Lineweaver–Burk (L–B) plot was constructed in order to calculate the kinetic parameters (K_m expressed in mM and v_{max} in nkat) of the enzymatic reaction with and without samples at the IC_{50} concentrations. Different *p*-NPG concentrations were used in the range 2–0.25 mM; the rate of the enzymatic reaction expressed in μkat was calculated from ΔAbs min, considering p-nitrophenolate ε at 405 nm = 18.5 $mM^{-1}\,cm^{-1}$ and light path length = 0.8 cm.

Statistical analysis

Values are expressed as the mean ± SD of three independent experiments with samples in triplicate. Statistical analysis was performed using Graph Pad Prism 4 software (GraphPad Inc., La Jolla, CA) by one-way analysis of variance (ANOVA), considering significant differences at *p*-values <0.05.

Results and discussion

The polar fraction of *A. tenorii* consisted predominantly by phenolic compounds. In particular, a high amount (about 1% w/w) of two caffeoyl-quinic acid derivatives, namely chlorogenic acid (**1**) (Bai et al., 2011), neochlorogenic acid (**2**) (dos Santos et al., 2004) and free caffeic acid (**3**) (Bai et al., 2011) were obtained. Additionally, flavonoid aglycones, apigenin (**4**) and luteolin (**5**), together with their respective 7-*O*-β-D-glucosides, cosmetin (**6**) and cynaroside (**7**) (Ivancheva & Kuzmanov, 1986; Oyama & Kondo, 2004; Park, 2007; Van Loo et al., 1986; Wada, 1985) were identified. Apigenin and its glucoside as well as luteolin were previously detected in other species of this genus, in particular *A. millefolium* (Huo et al., 2013) and *A. nobilis* (Marchart et al., 2003). Conversely, unlike in other *Achillea* species, no glucuronide derivatives of flavones were identified in *A. tenorii* following the reported fractionation methodology. These metabolites seem to be highly conserved in this genus and they were identified both in *A. millefolium* (Benedek et al., 2007) and in *A. nobilis* (Solomko et al., 1978), which belong to the same group as *A. tenorii*, as well as in *A. collina* (Kasaj et al., 2001) and *A. cartilaginea* (Zapesochnaya & Ban'kovskii, 1976), which belong to a different group. The apparent absence of glucuronide derivatives, or their presence in trace amount, may be dependent on the phenological phase of the plant's development but may also be a metabolic characteristic of the species *A. tenorii*. This aspect should be confirmed with further studies.

Table 1. Antioxidant activity of *A. tenorii* hydroalcoholic extract was measured with ABTS, DPPH, and FRAP-FZ tests and expressed in IC_{50} values (μg/mL) or FRAP unit, which represent the sample amount which produces 100 μM Fe^{2+} and total antioxidant capacity (TAC) expressed in mmol Tr eq/g of extract.

ABTS		DPPH		FRAP-FZ	
IC_{50}	TAC	IC_{50}	TAC	FRAP unit	TAC
22.49 ± 2.01	1.50	31.41 ± 3.13	0.71	21.56 ± 1.51	0.53

Regarding the biological activity, *A. tenorii* hydroalcoholic extracts were endowed with considerable antioxidant properties, showing positive results in all tests performed (DPPH, ABTS, and FRAP-FZ) (Table 1). This activity is probably attributable to the high content of secondary metabolites derived from the shikimate pathway, in particular, flavonoids and phenolic acids. Indeed, these kinds of natural compounds are endowed with strong antiradical activity due to their characteristic conjugated ring structure and their hydrogen donor ability, conferred by the presence of hydroxyl groups. As highlighted by the results obtained using the ABTS (IC_{50} 22.49 ± 2.01 μg/mL) and DPPH (IC_{50} 31.41 ± 3.13 μg/mL) tests, the extract has an interesting electron donor and hydrogen donor potential. These results, along with the aforementioned phenolic composition, are comparable with those reported for other *Achillea* species (the IC_{50} value ranges from 55.0 to 33.0 μg/mL) (Ardestani & Yazdanparast, 2007; Barış et al., 2011; Candana et al., 2003). In order to determine the reducing power, the FRAP-Ferrozine (FZ) test was also performed. This assay represents an improved form of the original FRAP test (Benzie & Strain, 1996). In particular, the Fe^{2+}–FZ complex is stable and spectrophotometrically detectable over a wide range of pH, this makes the assay performable at physiological pH instead of the acidic one required by the original method. The TAC value, obtained for *A. tenorii* using the FRAP-FZ test (0.53 mmol Tr eq/g), is comparable with that reported for *Camellia sinensis* (0.63 mmol Tr eq/g) and *Salvia officinalis* (0.31 mmol Tr eq/g) extracts (Beker et al., 2010), which are well known for their antioxidant activity.

Statistical analysis was performed on TAC values obtained from ABTS, DPPH, and FRAP-FZ and the extract was particularly active in the ABTS test ($p<0.01$) while no difference was evident between results obtained using DPPH and FRAP-FZ.

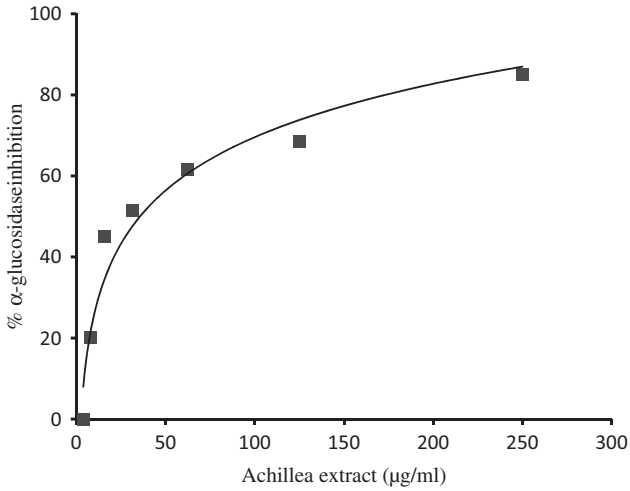

Figure 2. IC_{50} plot of α-glucosidase inhibition calculated using 2 mM p-NG and expressed in μg/mL.

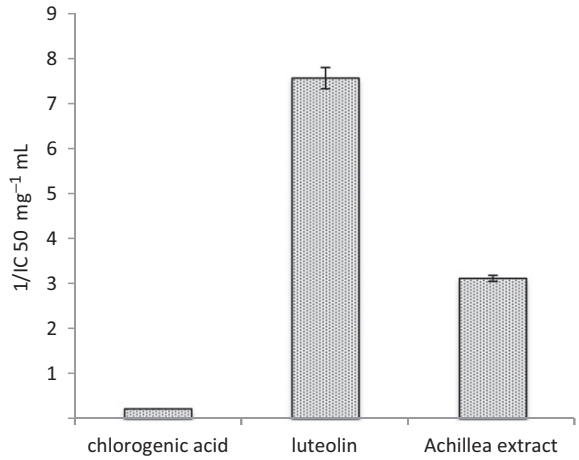

Figure 3. α-Glucosidase inhibition of chlorogenic acid, luteolin, and *Achillea tenorii* extract expressed in $1/IC_{50}$ ($mg^{-1} \times mL$).

Moreover, according to our results (Figure 2) extracts of *A. tenorii* are endowed with an interesting α-glucosidase inhibitory activity (the IC_{50} value is 32.07 ± 2.2 μg/mL). Hypothesizing that the activity exhibited by the extract was probably due to flavonoids, in particular luteolin, which is a well-known α-glucosidase inhibitor (Ha et al., 2012) the purified compound was tested, giving an IC_{50} value of 47 ± 1.5 μM (13.23 μg/mL) (Figure 3). Moreover, considering the high content of chlorogenic acid recognized in the extract, pure standard of this compound was also tested and an activity considerably lower than luteolin was found (IC_{50} 1.3 ± 0.2 mM; 461 μg/ml).

The variation of α-glucosidase kinetic parameters (K_m and v_{max}), induced by *A. tenorii* extracts, was also investigated. Fitting the inhibition data using a Lineweaver–Burk (L–B) plot indicated an un-competitive mechanism of action. As shown in Figure 4, treating the enzyme with the extract altered both kinetic parameters (Table 2). This uncommon kind of inhibition occurs when the inhibitor binds only the enzyme–substrate complex preventing products formation.

Luteolin has been previously reported as an un-competitive α-glucosidase inhibitor (Ha et al., 2012), in fact when the

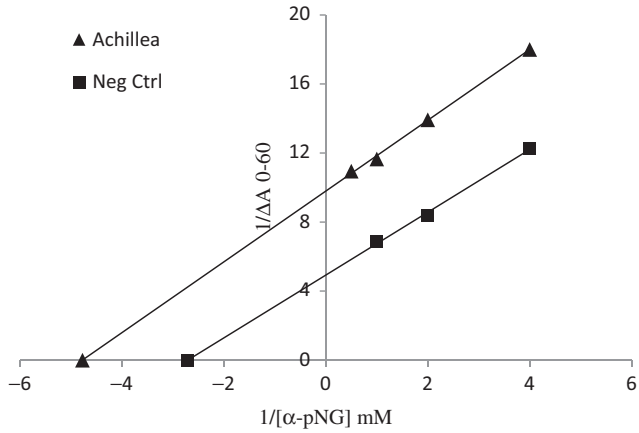

Figure 4. Lineweaver–Burk plot of α-glucosidase and p-NPG, without (■) and with (▲) *Achilea tenorii* extract at the IC_{50} concentration.

Table 2. Kinetic parameters of f α-glucosidase and p-NPG without (negative control) and with *Achilea tenorii* extract at the IC_{50} concentration (32 μg/mL). K_m was expressed in mM and v_{max} in nKat (nmol/s).

	Negative control	*A. tenorii* treated
K_m (mM)	0.37	0.021
v_{max} (nKat)	0.56	0.028

enzyme is treated with this compound, v_{max} is altered while no change in the K_m value is recorded. This suggests that compounds, other than luteolin, present in the extract, may contribute to the total inhibitory activity of *A. tenorii* conferring the un-competitive mechanism of inhibition. Conversely, voglibose and miglitol, the best known α-glucosidase inhibitors, act through a competitive mechanism of action, binding in the active site of the enzyme; this mechanism is characteristic for most of the α-glucosidase inhibitors which are definable as sugar mimetics (Kato et al., 2012). It is noteworthy that un-competitive inhibitors are considered more promising than competitive inhibitors as therapeutic agents, considering that their activity is not lost as the concentration of substrate builds up (Westley & Westley, 1996).

Conclusions

The overall phenolic composition of *A. tenorii* is comparable to that reported for the genus *Achillea* and a high amount of polyphenols (caffeoylquinic acids and flavonoids) was isolated from the extract. However, from a chemotaxonomic point of view the absence of glucuronide derivatives of flavonoids, mainly apigenine derivatives, which represent metabolites highly conserved in this genus is noteworthy.

Regarding the biological activity, *A. tenorii* can be considered as an accessible source of natural antioxidants. Moreover the combination of the antioxidant and α-glucosidase inhibitory activities makes this plant particularly promising in the management of diabetes mellitus, being able to affect different biological targets involved in this disease. Moreover,

the importance of novel α-glucosidase inhibitors are of growing importance considering that acarbose and miglitol, which are the only drugs used in therapy, are characterized by several side effects such as gastrointestinal complaints (Kato et al., 2012). In addition, α-glucosidase inhibitors are of particular interest due to their potential as antiviral and anti-metastatic agents (Elbein, 1991).

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Declaration of interest

The authors report that there are no declarations of interest.

References

Al-Hindawi M, Al-Deen IH, Nabi MH, Ismail MA. (1989). Anti-inflammatory activity of some Iraqi plants using intact rats. *J Ethnopharmacol* 26:163–8.

Ardestani A, Yazdanparast R. (2007). Antioxidant and free radical scavenging potential of *Achillea santolina* extracts. *Food Chem* 104: 21–9.

Arnao MB, Cano A, Alcolea JF, Acosta M. (2001). Estimation of free radical-quenching activity of leaf pigment extracts. *Phytochem Anal* 12:138–43.

Bai G, Cao X, Zhang H, et al. (2011). Direct screening of G-quadruplex ligands from *Kalopanax septemlobus* (Thunb.) Koidz extract by high performance liquid chromatography. *J Chromatogr A* 1218:6433–8.

Barış D, Kızıl M, Aytekin Ç, et al. (2011). *In vitro* antimicrobial and antioxidant activity of ethanol extract of three *Hypericum* and three *Achillea* species from Turkey. *Int J Food Prop* 14:339–55.

Baron AD. (1998). Postprandial hyperglycaemia and alpha-glucosidase inhibitors. *Diabetes Res Clin Pr* 40:S51–5.

Beker KI, Güçlü K, Demirata B, Apak R. (2010). A novel antioxidant assay of reducing capacity measured using ferrozine as the colour forming complexation reagent. *Anal Methods* 2:1770–8.

Benedek B, Gjoncaj N, Saukel J, Kopp B. (2007). Distribution of phenolic compounds in Middleeuropean taxa of the *Achillea millefolium* L. aggregate. *Chem Biodivers* 4:849–57.

Benzie IFF, Strain JJ. (1996). The ferric reducing ability of plasma (FRAP) as a measure of ''antioxidant power'': The FRAP assay. *Anal Biochem* 239:70–6.

Brand-Williams W, Cuvelier ME, Berset C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensm Wiss Technol* 28: 25–30.

Candana F, Unlub M, Tepec B, et al. (2003). Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* Afan. (Asteraceae). *J Ethnopharmacol* 87:215–20.

Conti F, Abbate G, Alessandrini A, Blasi C. (2005). *An Annotated Checklist of the Italian Vascular Flora*. Rome: Palombi Press.

dos Santos AR, Pereira de Barros M, Santin SM, et al. (2004). Polar constituents of the leaves of *Machaonia brasiliensis* (Rubiaceae). *Quim Nova* 27:525–7.

Elbein AD. (1991). Glycosidase inhibitors as antiviral and/or antitumor agents. *Semin Cell Biol* 2:309–17.

El-Kaissi S, Sherbeeni S. (2011). Pharmacological management of type 2 diabetes mellitus: An update. *J Curr Diabetes Rev* 7:392–405.

Ha TJ, Lee JH, Lee MH, et al. (2012). Isolation and identification of phenolic compounds from the seeds of *Perilla frutescens* (L.) and their inhibitory activities against α-glucosidase and aldose reductase. *Food Chem* 135:1397–403.

Huo CH, Li Y, Zhang ML, et al. (2013). Cytotoxic flavonoids from the flowers of *Achillea millefolium*. *Chem Nat Compd* 48:958–62.

Ivancheva S, Kuzmanov B. (1986). Flavonoids in genus *Achillea, A. nobilis*-group. *Bull Liaison – Groupe Polyphenols* 13:577–9.

Kasaj D, Krenn L, Reznicek G, et al. (2001). Flavonoids in *Achillea collina*. *Sci Pharm* 69:75–83.

Kato E, Oikawa K, Takahashi K, Kawabata J. (2012). Synthesis and the intestinal glucosidase inhibitory activity of 2-aminoresorcinol derivatives toward an investigation of its binding site. *Biosci Biotech Biochem* 76:1044–6.

Li T, Zhang XD, Song YW, Liu JW. (2005). A microplate-based screening method for α-glucosidase inhibitors. *Chin J Clin Pharmacol Ther* 10:1128–34.

Marchart E, Hattenberger A, Krenn L, Kopp B. (2003). Analysis of flavonoids in *Achillea nobilis* L. by capillary electrophoresis. *Sci Pharm* 71:133–45.

Nemeth E, Bernath J. (2008). Biological activities of yarrow species (*Achillea* spp.). *Curr Pharm Des* 14:3151–67.

Oyama K, Kondo T. (2004). Total synthesis of apigenin 7,4′-di-O-β-glucopyranoside, a component of blue flower pigment of *Salvia patens*, and seven chiral analogs. *Tetrahedron* 60:2025–34.

Park Y. (2007). Spectral assignments and reference data: ^{1}H and ^{13}C-NMR data of hydroxyflavone derivatives. *Magn Reson Chem* 45: 674–9.

Pignatti S. (1982). *Flora d'Italia, vol. 3*. Bologna, Italy: Edagricole, 84.

Solomko OV, Dzhumyrko SF, Kompantsev VA. (1978). Flavonoids of *Achillea nobilis*. *Khim Prir Soedin* 2:266–7.

Taylor R, Johnson AB. (1996). Does suppression of postprandial blood glucose excursions by the alpha-glucosidase inhibitor miglitol improve insulin sensitivity in diet-treated type II diabetic patients? *Diabetes Care* 19:559–63.

Van Loo P, De Bruyn A, Budesinsky M. (1986). Reinvestigation of the structural assignment of signals in the proton and carbon-13 NMR spectra of the flavone apigenin. *Magn Reson Chem* 24:879–82.

Wada H. (1985). Chemical and chemotaxonomic study of pteridophytes. LIX. Studies of the chemical constituents of *Alsophila spinulosa* Tryon. *Chem Pharm Bull* 33:4182–7.

Westley AM, Westley J. (1996). Enzyme inhibition in open systems. Superiority of uncompetitive agents. *J Biol Chem* 271: 5347–52.

Yazdanparast R, Ardestani A, Jamshidi S. (2007). Experimental diabetes treated with *Achillea santolina*: Effect on pancreatic oxidative parameters. *J Ethnopharmacol* 112:13–18.

Zapesochnaya GG, Ban'kovskii AI. (1976). Flavonoid from *Achillea cartilaginea*. *Khim Prir Soedin* (6):814–15.