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 (IC50 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.

Introduction

Achillea tenorii Grande (Asteraceae) [syn. A. virescens (Fenzl) Heimerl subsp. virecens (Grande) Büssler] 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.

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 CD3OD or D2O 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); all the other reagents 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 FeCl3 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 Rf < 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 CO2, 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)), caffeeic 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): 1H NMR (300 MHz, CD3OD) 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, H4), 1H NMR (300 MHz, D2O) δ: 179.7 (COOH quin.), 168.9 (COOα), 147.5 (COOβ), 144.2 (Cβ), 129.7 (C1'), 122.6 (C1).
(C6), 116.2 (C5), 115.3 (C2'), 114.2 (Cx), 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):** ¹H NMR 300 MHz, D₂O 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, Hx); 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). 

**Caffeic acid (3):** ¹H NMR (300 MHz, CD₃OD) 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, Hx). ¹³C NMR (75 MHz, CD₃OD) 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 (Cz). 

**Apigenin (4):** ¹H NMR (300 MHz, CD₃OD) 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, dJ = 2.0 Hz, H6). ¹³C NMR (75 MHz, CD₃OD) δ: 182.00 (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'), 105.3 (C10), 103.3 (C3), 99.0 (C6), 94.6 (C8). 

**Luteolin (5):** ¹H NMR (300 MHz, CD₃OD) 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). ¹³C NMR (75 MHz, CD₃OD) 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). 

**Apigenin-7-O-β-D-glucopyranoside (cosmetin) (6):** ¹H NMR (300 MHz, CD₃OD) 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
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 (t₀), 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 t₀ 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 IC₅₀ value (concentration necessary for 50% inhibition) was calculated by constructing a logistic 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:

\[
\% \text{ Inhibition} = \left(1 - \frac{(\Delta \text{Abs} / \text{min sample})}{(\Delta \text{Abs} / \text{min} \text{ neg. contr})}\right) \times 100
\]

A negative control was obtained by adding water instead of samples and ΔAbs values were calculated as Abs t₁₀ − Abs t₀ and referred to 1 min.

The Lineweaver–Burk (L–B) plot was constructed in order to calculate the kinetic parameters (Kₘ expressed in mM and vₘₐₓ in nkat) of the enzymatic reaction with and without samples at the IC₅₀ 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 cyanaroside (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 (Zapesochnya & 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.

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 (IC₅₀ 22.49 ± 2.01 μg/mL) and DPPH (IC₅₀ 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 IC₅₀ value ranges from 55.0 to 33.0 μg/mL) (Ardestanti & 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 Fe²⁺–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.
Moreover, according to our results (Figure 2) extracts of *A. tenorii* are endowed with an interesting α-glucosidase inhibitory activity (the IC₅₀ value is 32.07 ± 2.2 mg/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 IC₅₀ value of 47 ± 1.5 μM (13.23 mg/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 (IC₅₀ 1.3 ± 0.2 mM; 461 μg/mL).

The variation of α-glucosidase kinetic parameters (*Kₘ* and *vₘₐₓ*) 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 enzyme is treated with this compound, *vₘₐₓ* is altered while no change in the *Kₘ* 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.

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