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A new α-glucosidase inhibitor from *Achillea fragrantissima* (Forssk.) Sch. Bip. growing in Egypt

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α-Glucosidase inhibitors (AGIs) represent a class of oral antidiabetic drugs that delay the absorption of ingested carbohydrates, reducing the postprandial glucose and insulin peaks to reach normoglycaemia. In this study, a bioassay-guided fractionation of the ethanolic extract of the aerial parts of *Achillea fragrantissima* (Forssk.) Sch. Bip. growing in Egypt led to the isolation of a new potent AGI; acacetin-6-C-(6″-acetyl-β-D-glucopyranoside)-8-C-α-L-arabinopyranoside (**5**) alongside with four known compounds: chondrillasterol (**1**), quercetin-3,6,7-trimethyl ether (chrysosplenol-D) (**2**), isovitexin-4′-methyl ether (**3**) and isovitexin (**4**). The structure of the new compound (**5**) was elucidated on the basis of its spectral data, including HR-FAB-MS, UV, ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, HSQC and HMBC. The new compound (**5**) exhibited the most significant α-glucosidase inhibitory activity (IC_{50} 1.5 ± 0.09 μg/mL). Under the assay conditions, all the tested compounds were more potent than the positive control acarbose (IC_{50} 224 ± 2.31 μg/mL).

Keywords: *A. fragrantissima*; α-glucosidase inhibitors; chondrillasterol; chrysosplenol-D; isovitexin; acacetin-6-C-(6″-acetyl-β-D-glucopyranoside)-8-C-α-L-arabinopyranoside

1. Introduction

Diabetes mellitus is a debilitating and often life-threatening disease with increasing incidence in rural populations throughout the world. It is a metabolic disorder resulting from a defect in insulin secretion, insulin action or both. As a result, there is a disturbance of carbohydrate, fat and protein metabolism. At least 30 million people throughout the world suffer from diabetes mellitus. In 2030, there will be a 69% increase in numbers of adults with diabetes in developing countries and a 20% increase in developed countries indicating a growing burden of diabetes, particularly in developing countries (Sicree & Zimmet 2010).

The aim of oral therapy in type 2 diabetes is to reach normoglycaemia to prevent later complications (retinopathy, nephropathy, neuropathy and microangiopathy). Reaching near normal preprandial and postprandial plasma glucose levels can significantly diminish the risk of long-term complications (Florence & Yeager 1999). α-Glucosidase inhibitors (AGIs) are among the available glucose-lowering medications. α-Glucosidase is a key enzyme for carbohydrate digestion, located in the brush border of the small intestine. This enzyme has been recognised as a therapeutic target for the modulation of postprandial hyperglycaemia (PPHG), which is the earliest metabolic abnormality to occur in type 2 diabetes mellitus and may lead to the development of cardiovascular complications (Mooradian & Thurman 1999). The AGIs delay,

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but do not prevent, the absorption of ingested carbohydrates, reducing the postprandial glucose and insulin peaks (Stuart et al. 2004). A scientific investigation of traditional herbal remedies for AGIs may provide valuable leads for the development of alternative drugs and therapeutic strategies. Alternatives are clearly needed because of the inability of current therapies to control all of the pathological aspects of diabetes, in addition to the high cost and poor availability of current therapies for many rural populations, particularly in developing countries (Marles & Farnsworth 1995).

Achillea fragrantissima (Forssk.) Sch. Bip., *Santolina fragrantissima* Forssk. (Compositeae), is also known as lavander cotton or Qaysūm (Arabic). It is globally distributed in Libya (Cyrenaica), Egypt, Palestine, Syria, Arabia and Iraq (Täckholm 1974; Hepper & Friis 1994; Boulos 2002). *A. fragrantissima* is used in Egypt to treat gastrointestinal disturbances, eye infections and smallpox (Ozenda 1991; Sincich 2002). *A. fragrantissima* is reputed for its antidiabetic properties in the folk medicine of the Middle East (Oliver-Bever & Zahnd 1979; Bailey & Danin 1981; Yaniv et al. 1987). Reports on the chemical constituents were mainly concerned with those of the whole herb and included the isolation of monoterpenes, glaucolides and pyran (Abdel Mogib et al. 1989; Ahmed et al. 1995; Marles & Farnsworth 1995); sesquiterpene lactones were also isolated from the aerial parts of the plant (Segal & Dor 1987). Moreover, the essential oil of the aerial parts was studied (Aboutabl et al. 1986a, 1986b; Fleisher & Fleisher 1993; El-Shazly et al. 2004). In an approach to search for AGIs in the extracts of oriental herbs, which are traditionally used as antidiabetic remedies, the goal of our study was to screen the aerial parts of the Egyptian plant *A. fragrantissima* for its α-glucosidase inhibitory activity and to establish a bioassay-guided isolation of new AGIs from this plant.

2. Results and discussion

AGIs such as acarbose (Glucobay)® is known to specifically reduce PPHG (irresponsive to other antidiabetic drugs such as biguanides, sulfonylureas or thiazolidinediones) primarily by interfering with the carbohydrate digesting enzymes and delaying glucose absorption (Godbout & Chiasson 2007). It has also been shown to decrease the risk of progressing to diabetes in subjects with impaired glucose tolerance (Nishioka et al. 1997). In recent years, there is an increasing interest for the isolation of AGIs from medicinal plants especially those which are known for their hypoglycaemic capabilities.

In that view, the *in vitro* α-glucosidase inhibitory activity of the ethanolic (EE) and aqueous (AE) extracts of the aerial parts of *A. fragrantissima* was assessed using *p*-nitrophrnyl-α-D-glucopyranose (*p*-NPG) (Supplementary Table S1). As observed in Supplementary Table S1, EE exerted more significant inhibitory activity (IC_{50} 26.66 ± 1.02 μg/mL). The EE was fractionated by chloroform, *n*-butanol and residual water fractions. Those fractions were, consequently, subjected to *in vitro* assay of α-glucosidase inhibitory activity (Supplementary Table S1), where the chloroform and *n*-butanol fractions exhibited the highest inhibitory activity (IC_{50} 129.7 ± 2.01 and 102.16 ± 1.12 μg/mL, respectively), while the residual water fraction did not exhibit a significant effect at the assayed concentrations. Purification of the chloroform fraction yielded two known compounds, (**1**) and (**2**), and purification of the *n*-butanol fraction yielded a new compound identified as acacetin-6-C-(6″-acetyl-β-D-glucopyranoside)-8-C-α-L-arabinopyranoside (**5**) together with two known compounds (**3**) and (**4**). Structural elucidation of the known compounds was performed by comparing their UV, ^{1}H and ^{13}C NMR spectroscopic data with those in the literature. They were identified as chondrillasterol (**1**) (Goad & Akihisa 1997), quercetin-3,6,7-trimethyl ether (chrysosplenol-D) (**2**) (Ghisalberti et al. 1967; Dendougui et al. 2012), isovitexin-4′-methyl ether (**3**) (Mabry et al. 1970; Markham 1982; Adamska et al. 2003) and isovitexin (**4**) (Mabry et al. 1970; Markham 1982; Choo et al. 2012). The structures of the isolated compounds are shown in Figure 1. Compound **5** was isolated as a yellow powder,

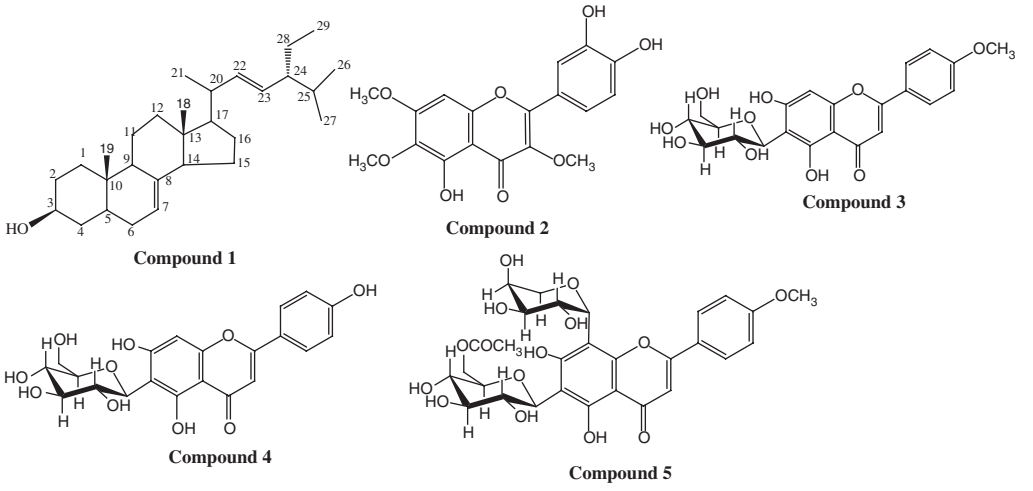

Figure 1. The structure of the isolated compounds.

m.p. 326°C. Its negative HR-FAB-MS revealed a molecular ion peak at *m/z* 619.1481 $[M - H]^-$ (calcd 619.1476), corresponding to a molecular formula $C_{29}H_{31}O_{15}^-$. In positive HR-FAB-MS mode, it revealed a molecular ion peak at *m/z* 643.1479 $[M + Na]^+$ (calcd 643.1475) corresponding to the molecular formula $C_{29}H_{32}O_{15}Na^+$.

Its UV absorptions in MeOH and the shifts observed after the addition of shift reagents were consistent with the presence of a 5,7-dihydroxyflavone structure (Mabry et al. 1970; Markham 1982). The ^{1}H NMR spectrum of this compound revealed resonances characteristic for an $ArOCH_3$ group (δ_H 3.87), two anomeric sugar protons (δ_H 4.6 and 4.81) and an acetyl group (δ_H 2.0), as well as those for aromatic system and other sugar protons. Analysis of the aromatic proton resonances, their corresponding ^{13}C NMR chemical shifts (HSQC) and their long-range ^{1}H–^{13}C connectivities (HMBC) indicated that the aglycone is a derivative of apigenin with the absence of signals for H-6 and H-8. The downfield shift of H-3′,5′ protons (δ_H 7.13) compared to apigenin (c.f. compound **4**, H-3′,5′ at δ_H 6.93) suggested that the 4′-hydroxyl is substituted (Marin et al. 2001). The long-range connectivity detected in HMBC between C-4′ and the methoxy group at δ_H 3.87 confirmed that the aglycone was apigenin 4′-methyl ether (acaetin). In addition, the ^{1}H and ^{13}C NMR spectra of compound **5** exhibited resonances owing to a β-D-glucopyranose moiety and α-L-arabinopyranose moiety whose configuration were deduced from the large coupling constant of H-1″ of the glucopyranose moiety ($J = 9.1$ Hz) and the small coupling constant of arabinopyranose moiety ($J = 2.1$ Hz) (Mabry et al. 1970; Markham 1982). The chemical shift values of the anomeric carbons of the glucose (δ_C 74.3) and arabinose (δ_C 75.0) indicated that the linkage of both sugars was through a C-bond (Mabry et al. 1970; Harborne & Mabry 1982). The information supporting the linkage of the sugar moieties was confirmed from the HMBC spectrum. A prominent long-range correlation between the anomeric protons of the glucose (H-1″, δ_H 4.60) with C-6 (δ_C 108.2) of the aglycone and that between the anomeric proton of arabinose (H-1‴, δ_H 4.81) with C-8 (δ_C 104.8) of the aglycone confirmed the attachment of the glucose at C-6 and the arabinose moiety at C-8 position. Further confirmation for this assignment came from the HMBC cross-peaks observed from H-1″ to C-5 (δ_c 161.1) and C-7 (δ_c 163.5) and that from H-1‴ to C-9 (δ_c 159.2) and C-7 (δ_c 163.5). The ^{13}C NMR resonance of C-6″ of the glucose unit was downfield shifted (c.f. compounds **3** and **4**) suggesting that C-6″ was the site of substitution of the acetyl group (Marin et al. 2001). Further evidence was provided from HMBC cross-peaks (Supplementary Figure S1) observed between $OCOCH_3$ (δ_H 2.0) with both $OCOCH_3$ (δ_c 170.1) and C-6″ (δ_c 63.3). Based on the earlier data, the structure of compound **5** was assigned as

acacetin-6-C-(6″-acetyl-β-D-glucopyranoside)-8-C-α-L-arabinopyranoside which is a new natural product. The structure of compound **5** is shown in Figure 1.

All the isolated compounds were tested for *in vitro* α-glucosidase inhibitory activity (Supplementary Table S1), and they exhibited significant activities represented by small IC_{50} values. The new compound **5** (acacetin-6-C-(6″-acetyl-β-D-glucopyranoside)-8-C-α-L-arabinpyranooside) exhibited the most significant inhibitory activity against α-glucosidase in a concentration-dependent manner with IC_{50} of 1.5 ± 0.09 μg/mL followed by compound **2** (quercetin-3,6,7-trimethyl ether) which had an IC_{50} of 14.5 ± 0.89 μg/mL. The potent inhibitory activity of compound **5** may be attributed to the presence of two sugars, as being a di-glycoside may exert a stronger competitive action in inhibiting the target enzyme. However, compounds **3** and **4** were relatively less active with IC_{50} values of 83.57 ± 0.59 and 34.37 ± 1.09 μg/mL, respectively. Compound **1** (chondrillasterol) expressed the lowest activity (IC_{50} 138 ± 2.01 μg/mL) relative to the tested compounds, and this could be due to its steroidal nature which differs from that of the other four phenolic compounds. Under our specified assay conditions, all the tested compounds were more potent than the positive control acarbose (224 ± 2.31 μg/mL). The results are in accordance with published data for the α-glucosidase activity of compound **4** (Choo et al. 2012), but this is the first report examining the activity of compounds **2**, **3** and **5**. This report observes (for the first time) the α-glucosidase inhibitory activity in *A. fragrantissima* and assigns the five isolated compounds as active α-glucosidase-inhibiting components. Recent researches demonstrated that AGIs not only exhibit high promise as therapeutic agents for the treatment of metabolic disorders, such as type 2 diabetes and obesity through delaying the digestion of ingested carbohydrates and consequently suppressing the increase in PPHG, but also exert antiviral and antimetastatic activities (Goss et al. 1995). This makes our isolates compounds with multiple therapeutic activity.

3. Experimental

3.1. *Chemicals and equipments*

Silica gel H (Merck, Darmstadt, Germany) for vacuum liquid chromatography (VLC), silica gel 60 (70–230 mesh ASTM, Fluka, Steinheim, Germany) and Sephadex LH 20 (Pharmacia, Stockholm, Sweden) were used for column chromatography. Thin-layer chromatography (TLC) was performed on silica gel GF_{254} precoated plates (Fluka) using the following solvent systems: S_1, hexane–ethyl acetate (90:10); S_2, hexane–ethyl acetate (80:20) and S_3, ethyl acetate–methanol–water–formic acid (100:16.5:13.5:2.5). The chromatograms were visualised under UV (at 254 and 366 nm) before and after exposure to ammonia vapour and spraying with $AlCl_3$, as well as after spraying with anisaldehyde/sulphuric acid reagent.

α-Glucosidase enzyme from brewer's yeast (EC 3.2.1.20), the substrate, *p*-NPG, and phosphate buffer (pH 6.8) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The positive control, acarbose, was purchased from Bayer Pharmaceuticals Pty, Ltd (Montville, NJ, USA).

Melting points (uncorrected) were determined on an Electrothermal 9100 (Markham, Ontario, Canada). UV spectra were measured using a Shimadzu UV 240 (P/N 204-58000) spectrophotometer (Kyoto, Japan). HR-FAB-MS was measured in the JEOL JMX-AX 505, HAD mass spectrophotometer (Tokyo, Japan) at an ionisation voltage of 70 eV. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) were measured on a Varian Mercury-VX-300 instrument (Palo Alto, CA, USA). The NMR spectra were recorded in $CDCl_3$ and DMSO-d_6 and chemical shifts were given in δ (ppm) relative to TMS as internal standard.

3.2. *Plant material*

The aerial parts of *A. fragrantissima* (Forssk.) Sch. Bip. (Family: Asteraceae) were obtained from the north coast of Alexandria, Egypt, during spring 2008. The plant was kindly identified at

the Department of Botany, Faculty of Science, Cairo University, Giza, Egypt. A voucher specimen (AF-2008-51) has been deposited at the museum of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

3.3. *Extraction and fractionation*

The air-dried powdered aerial parts of *A. fragrantissima* (650 g) were extracted by cold percolation with 95% ethanol (3 × 2 L) till exhaustion. The EE was concentrated under reduced pressure to give 110 g of a brown residue. Twenty grams of the EE was stored for the biological study, while 40 g was suspended in distilled water and subjected to liquid–liquid fractionation using chloroform- and *n*-butanol-saturated with water. Fractions were separately concentrated under reduced pressure to yield 10 and 11 g of the chloroform and *n*-butanol fraction, respectively, in addition to 19 g residual water fraction. The AE of *A. fragrantissima* was prepared by the extraction of 100 g powdered aerial parts with boiling distilled water (2 × 1 L), the water was evaporated under reduced pressure to yield 10 g residue which was stored for biological activity study.

3.4. *Assay for α-glucosidase inhibitory activity*

The assay was performed to measure the α-glucosidase inhibitory activity of EE: its three fractions and the five isolated compounds. The enzyme inhibition studies were carried out spectrophotometrically in a 96-well microplate reader using a procedure reported by Li et al. (2005). A total 60 μL reaction mixture containing 20 μL of 100 mM phosphate buffer (pH 6.8), 20 μL of 2.5 mM *p*-NPG (Biochem, Ontario, Canada) in the buffer and 20 μL of the inhibitor in DMSO for the EE, its three fractions and the compounds **2**–**5**, in chloroform for compound **1** were added to each well, followed by 20 μL of 10 mM phosphate buffer (pH 6.8) containing 0.2 U/mL α-glucosidase enzyme to the mixture of treatment-terminated wells. The plate was incubated at 37°C for 15 min, and then 80 μL of 0.2 mol/L sodium carbonate solution was added to stop the reaction. Following that, the absorbance was recorded at 405 nm using a SpectraMax 340 (Molecular Devices, Orleans Drive Sunnyvale, CA, USA) spectrometer. Controls contained the same reaction mixture except that the same volume of phosphate buffer was added instead of the inhibitor solution. Acarbose (Bayer, Barmen, Germany) was dissolved in water and used as a positive control.

$$\text{Inhibition } \% = [(A_B - A_A)/A_B] \times 100\%,$$

Where A_B is the absorbance of the control sample and A_A is the absorbance of test sample. The 50% inhibitory concentration (IC_{50} μg/mL) of the active samples against yeast glucosidase was calculated. The results are presented in Supplementary Table S1.

3.5. *Purification of the bioactive fractions*

Five grams of the chloroform fraction was chromatographed over a VLC column (3 cm D × 10 cm L, silica gel H, 50 g). Gradient elution was carried out using *n*-hexane–methylene chloride, methylene chloride–ethyl acetate and ethyl acetate–methanol mixtures. Fractions of 100 mL each were collected and monitored by TLC to yield two main fractions (A and B). Fraction A (40–45% methylene chloride–*n*-hexane) was rechromatographed over a silica gel 60 column, using 4% ethyl acetate–*n*-hexane as eluent to give compound **1** (120 mg, white needle-shaped crystals $R_f = 0.38$ in S_2, m.p.172–174°C). Fraction B (65–90% ethyl acetate–methylene chloride) was purified over several Sephadex LH-20 columns using chloroform–methanol (1:1) mixture as eluent to yield compound **2** (110 mg, yellow amorphous powder, $R_f = 0.58$ in S_2).

Six grams of the *n*-butanol fraction was chromatographed over a VLC column (3 cm D × 10 cm L, Silica gel H, 50 g). Gradient elution was carried out using methylene chloride–ethyl acetate and ethyl acetate–methanol mixtures. Fractions of 100 mL each were collected and

monitored by TLC to yield two main fractions (C and D). Fraction C (30–35% methanol–ethyl acetate) was purified over several Sephadex LH-20 columns using methanol and methanol–water (1:1) mixtures as eluent to yield compound **3** (165 mg, yellow amorphous powder, $R_f = 0.18$ in S_4) and compound **4** (113 mg, yellow amorphous powder, $R_f = 0.30$ in S_4). Fraction D (45% methanol–ethyl acetate) was purified as for fraction C to yield compound **5** (75 mg, yellow amorphous powder, $R_f = 0.39$ in S_4).

3.5.1. *Compound **5***

UV/vis λ_{max} nm: (MeOH) 272, 333 (NaOMe) 277, 326, 399 ($AlCl_3$) 260, 279, 333 ($AlCl_3$/HCl) 260 sh, 279, 345 (NaOAc) 277, 301, 334 (NaOAc/H_3BO_3) 272, 334. ^{1}H NMR: δ ppm (300 MHz, DMSO) 2.00 (3H, s, OCOC*H*$_3$), 3.87 (3H, s, OCH_3-4′), 4.60 (1H, 1H, d, $J = 9.1$ Hz, H-1″), 4.81 (1H, d, $J = 2.1$ Hz, H-1‴), 6.60 (1H, s, H-3), 7.13 (2H, d, $J = 7.8$ Hz, H-3′,5′), 7.85 (2H, d, $J = 7.8$ Hz, H-2′, 6′). ^{13}C NMR: δ ppm (75 MHz, DMSO) 20.5 (OCO*C*H_3), 56.5 (C4′–O*C*H_3), 63.3 (C-6″), 67.4 (C-5‴), 68.9 (C-4‴), 70.2 (C-2″), 70.5(C-4″), 71.0 (C-3‴), 74.3 (C-1″), 74.3 (C-2‴), 75.0 (C-1‴), 79.1 (C-3″), 80.8 (C-5″), 102.1 (C-3), 102.1 (C-10), 104.8 (C-8), 108.2 (C-6), 115.9 (C-3′,5′), 121.7 (C-1′), 128.6 (C-2′, 6′), 155.3 (C-2), 159.2 (C-9), 161.0 (C-4′), 163.5 (C-7), 170.1 (O*C*OCH_3), 181.5 (C-4).

3.6. *Statistical analysis*

The results were expressed as the mean ± standard error. The means were compared using ANOVA test (MSTATC software, East Lansing, MI, USA) and the Duncan's multiple-range test. Values were determined to be significant when p was < 0.05.

4. Conclusion

This work suggests the use of *A. fragrantissima* as a source for new AGIs that may serve as models for antihyperglycaemic drug development.

Supplementary material

Supplementary material relating to this article is available online, alongside Table S1 and Figure S1.

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