South African Journal of Botany 121 (2019) 418–421

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Isolation, identification and characterization of pancreatic lipase inhibitors from *Trigonella foenum-graecum* seeds

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ARTICLE INFO

Article history:
Received 29 July 2018
Received in revised form 14 October 2018
Accepted 29 October 2018
Available online xxxx

Edited by KRR Rengasamy

Keywords:
Lipase inhibitor
Trigonella foenum-graecum
Flavone C-glycoside
Vicenin-1
Isoschaftoside
Schaftoside

ABSTRACT

Activity-guided fractionation of the methanol extract of *Trigonella foenum-graecum* seeds furnished three flavone C-glycosides having pancreatic lipase inhibitory activity. Structures of these compounds were established as vicenin-1 (**1**), isoschaftoside (**2**) and schaftoside (**3**) by detail analysis of ^{1}H, ^{13}C NMR and MS data. These compounds showed percentage inhibition 60.3% (**1**), 33.8% (**2**) and 95.5% (**3**) at the concentration of 250 μg/ml and IC_{50} values of the inhibition were 207 μg/ml (**1**), 330 μg/ml (**2**) and 130 μg/ml (**3**). This is the first report of the isolation of lipase inhibitors from *T. foenum-graecum* seeds. Temperature dependency of ^{1}H NMR of **3** was studied in detail.

1. Introduction

Obesity is a global health problem mainly due to the life-style disorder related to lack of physical activities. It is associated with a large number of chronic diseases and disabilities such as dyslipidemia, fatty liver diseases, osteoarthritis, hypertension, diabetes mellitus and some cancers. Scientists have paid attention for many years to search for lipase inhibitors from natural sources. Although several antiobesity drugs such as phentermine, mazindol, diethylpropion, rimonabant, sibutramine have been introduced during the past few decades, all of them have been withdrawn from the market due to their adverse side effects. Orlistat is the only drug currently available in the market for antiobesity. However it is also claimed to have some side effects including gastrointestinal effects that limit its use (Kang and Park, 2012). Hence it is important to search for new compounds with antiobesity properties without adverse side effects. Inhibition of pancreatic lipase enzyme is one of the approaches in antiobesity drug discovery. Pancreatic lipase is the main lipid digesting enzyme and removes fatty acids from triglycerides, which yield the lipolytic product, monoglyceride and long chain saturated and polyunsaturated fatty acids. Inhibition of pancreatic lipase is an attractive targeted approach for diet induced obesity treatment. Furthermore, pancreatic lipase inhibition is one of the most widely studied mechanisms for determining antiobesity activity of natural products (Birari and Bhutani, 2007). Also pancreatic lipase mediated hydrolysis of triglycerides was shown to be necessary for cholesterol transport from lipid emulsion to the intestinal cells (Young and Hui, 1999). Therefore, inhibition of pancreatic lipase will be beneficial to control obesity and prevent hyperlipidemia and hypercholesterolemia (Young and Hui, 1999). Natural products can be very good candidates for this purpose. The use of natural products in the management and treatment of diseases is more acceptable and offers less risk than the use of synthetic compounds. Several reviews have described the pancreatic lipase inhibitors belonging to diverse classes of compounds, saponins, polyphenols, terpenes, alkaloids, carotenoids, polysaccharides, etc., which are originated from plants and microbes (Birari and Bhutani, 2007; Singh et al., 2015; Lunagariya et al., 2014).

In a continuation of our studies directed towards the search for bioactive compound from natural sources, we investigated *Trigonella foenum-graecum* seeds for its lipase inhibitory activity. *Trigonella foenum-graecum* is a popular spice and medicinal plant. Different groups of bioactive compounds, alkaloids (Zhuo et al., 2010), amino acids (Hilles and Mahmood, 2016), flavonoids (Huang and Liang, 2000; Han et al., 2001), saponins (Taylor et al., 1997), anthocyanins, fiber, lipids, vitamins and traces of inorganic elements have been reported. C-glycosidic flavonoids such as, vitexin, orientin (Adamska

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https://doi.org/10.1016/j.sajb.2018.10.023
0254-6299/

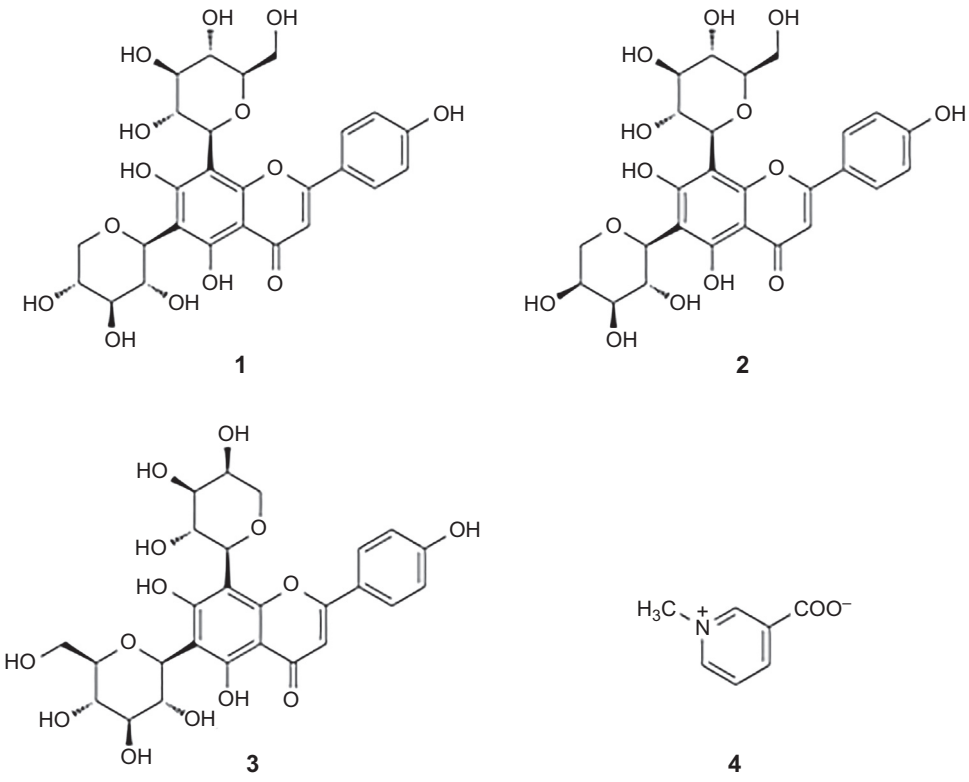

Fig. 1. Structures of compounds **1–4**

and Lutomski, 1971) were isolated from *T. foenum-graecum* seeds. In addition, more than 10 C-glycosidic flavonoids, derived from apigenin, kaempferol and luteolin, have been isolated from *T. foenum-graecum* seeds (Adamska and Lutomski, 1971; Rayyan et al., 2010). C-Glycosidic flavonoids isolated from other plant sources have been investigated for various biological activities like antioxidative (Hollman et al., 2011; Mladenka et al., 2010), anti-inflammatory (Shie et al., 2010), antinociceptive (De Melo et al., 2005), anxiolytic (Sena et al., 2009), antispasmodic (Ragone et al., 2007), antidiabetic (Courts and Williamson, 2009; Islam et al., 2014), antimutagenic (Snijman et al., 2007), antiangiogenic and antiglycation activities (Islam et al., 2014). However, information on the bioactivity of C-glycosidic flavonoids from *T. foenum-graecum* seeds is very limited. In this article we report activity-guided isolation of pancreatic lipase inhibitory substances from the methanol (MeOH) extract of *T. foenum-graecum* seeds. The study led to the isolation of vicenin-1 (**1**), isoschaftoside (**2**) and schaftoside (**3**) along with trigonelline (**4**).

2. Materials and methods

2.1. General

Extractions were performed using a sonicator (VWR Ultrasound cleaner, model-USC 1700 D). TLC analysis was conducted on silica gel plates (Merck 1.05554, $60F_{254}$, 0.20 mm thickness). TLC spots were located using a UV lamp and by heating after spraying with acidic anisaldehyde. Silica gel (Merck Art. 7734 & 9385) and Sephadex LH-20 were used for column chromatography. ^{1}H NMR and ^{13}C NMR were recorded on a JEOL AL-300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) or a Bruker DRX 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer at the indicated temperature in DMSO-d_6 or CD_3OD solution. ^{1}H Chemical shifts are referenced to the residual proton signal of the solvents (δ 2.49 or 3.30, respectively), while ^{13}C chemical shifts are expressed in reference to the solvent signals (δ 39.5 or 49.0, respectively). ESI-MS was recorded on a Shimadzu LC–MS 2020 spectrometer. UV absorptions were measured on a Thermo Scientific Multiskan GO Microplate spectrophotometer. RP-HPLC was carried out with a Shimadzu LC-6A apparatus attached with STR PREP ODS column (250 mm × 20 mm i.d.) monitored with a UV detector (254 nm). Pancreatic lipase and orlistat (a lipase inhibitor) were purchased from Sigma chemicals (USA).

2.2. Plant material

Seeds of *T. foenum-graecum* was purchased from a local market in Kandy, Sri Lanka. A voucher specimens (WITF/S/11) was deposited at the Department of Biochemistry, Faculty of Medicine, University of Peradeniya, Sri Lanka.

2.3. Extraction and isolation of *1–4*

The dried powdered seeds of *T. foenum-graecum* (1 kg) was successively extracted using an ultra-sonicator with MeOH (2 l × 30 min × 3) at room temperature and solvents were evaporated using a rotary evaporator to give MeOH extract (110 g). 100 g of the dried crude MeOH extract (M) was partitioned with MeOH and *n*-hexane (1: 1) ratio to give *n*-hexane extract (20 g) and defatted MeOH fraction. The MeOH fraction (M1) was evaporated to dryness and partitioned with ethyl acetate (EtOAc) and H_2O (1: 1) ratio. Evaporation of the EtOAc furnished EtOAc extract (9.2 g) and the evaporation of water furnished the water extract (67 g) (Fig. S1). Five extracts, *n*-hexane extract (H), EtOAc extract (E), two MeOH extracts (M and M1) and water extract (W) were subjected to α-amylase and pancreatic lipase inhibition assays. The water extract (10 g) was subjected to chromatography over Sephadex LH-20 (3.5 cm i.d. × 38 cm) with 100% MeOH to give five fractions A_1–A_5. Fraction A_5 was subjected to preparative RP-HPLC using ODS column (250 mm × 20 mm i.d.) with isocratic solvent system MeOH:H_2O (65:35), flow rate 5 ml/min under 254 nm to yield compounds **1** (18.5 mg) and **2** (18.9 mg), and **3** (13.0 mg) in this elution order. The fraction A_2 was chromatographed over silica gel (CH_2Cl_2-

MeOH) to yield compound **4** (67 mg) (Fig. 1). Compounds **1**–**4** were identified as vicenin-1 (**1**), isoschaftoside (**2**), schaftoside (**3**) and trigonelline (**4**) by detailed analysis of NMR and MS data.

Vicenin-1(**1**): ^{1}H-NMR(300 MHz, DMSO-d_6, 90 °C) δ: 13.66 (0.60H, HO-5), 7.94 (2H, d, J = 8.7 Hz, H-2′, H-6′), 6.94 (2H, d, J = 8.7 Hz, H-3′, H-5′), 6.68 (1H, s, H-3), 4.93 (d, J = 9.6 Hz, Glc-H-1), 4.66 (d, J = 9.9 Hz, Xyl-H-1), 4.64–3.17 (other sugar protons); ^{13}C-NMR (75 MHz, DMSO-d_6, 90 °C) δ: 181.9 (C-4), 163.7 (C-2), 161.0 (C-4′), 160.8 (C-5), 159.3 (C-7), 154.1 (C-8a), 128.3 (C-2′/6′), 121.3 (C-1′), 115.6 (C-3′/5′), 108.4 (C-6), 103.4 (C-8), 102.4 (C-4a), 102.2 (C-3), 81.2 (Clc-C-1), 78.7 (Clc-C-8), 78.2 (Xyl-C-3), 74.2 (Xyl-C-1), 71.6 (Clc-C-1), 70.7 (Clc-C-2), 70.0 (Xyl-C-2), 70.0 (Xyl-C-4), 69.7 (Glc-C-4), 69.5 (Xyl-C-4). Two sets of signals were observed in the ^{1}H (300 MHz) and ^{13}C (75 MHz) spectra recorded in CD_3OD at 25 °C, e.g., δ_H: 5.12 and 4.88 (0.5H each, d, J = 10.0 Hz, Xyl-H-1), 5.00 and 4.81 (0.5H each, d, J = 10.0 Hz, Glc-H-1), δ_C: 77.2 and 77.1 (Xyl-C-1), 75.9 and 75.0 (Glc-C-1); ESI-MS (positive mode) *m/z*: 565 $[M + H]^+$.

Isoschaftoside (**2**): ^{1}H-NMR (500 MHz, DMSO-d_6, 90 °C) δ: 13.59 (0.79H, brs, HO-5), 7.95 (2H, d, J = 8.3 Hz, H-2′, H-6′), 6.93 (2H, d, J = 8.3 Hz, H-3′, H-5′), 6.70 (1H, s, H-3), 4.84 (1H, d, J = 10.2 Hz, Ara-1), 4.72 (1H, d, J = 9.3 Hz, Glc-1), 4.70–3.30 (other sugar protons); ^{13}C-NMR (125 MHz, DMSO-d_6, 90 °C) δ: 181.9 (C-4), 163.8 (C-2), 170.0 (C-5), 160.8 (C-4′), 158.0 (C-7), 157.7 (C-8a), 128.3 (C-2′/6′), 121.3 (C-1′), 115.6 (C-3′/5′), 107.9 (C-6), 105.1 (C-8), 103.5 (C-4a), 102.6 (C-3), 81.4 (Glc-C-5), 78.6 (Glc-C-3), 74.1 (Ara-C-1), 73.8 (Ara-C-3), 73.6 (Glc-C-1), 70.9 (Glc-C-2), 70.4 (Glc-C-4), 69.9 (Ara-C-5), 69.1 (Ara-C-2), 68.3 (Ara-C-4), 61.1 (Glc-C-6); ESI-MS (positive mode) *m/z*: 565 $[M + H]^+$.

Schaftoside (**3**): ^{1}H-NMR (300 MHz, DMSO-d_6, 80 °C) δ: 13.72 (0.55H, brs, HO-5), 8.05 (2H, d, J = 8.6 Hz, H-2′, H-6′), 6.92 (2H, d, J = 8.6 Hz, H-3′, H-5′), 6.71 (1H, s, H-3), 4.80 (1H, d, J = 9.6 Hz, Ara-H-1), 4.75 (1H, d, J = 9.9 Hz, Glc-H-1), 4.64–3.52 (m, other sugar protons); ^{13}C-NMR (75 MHz, DMSO-d_6, 80 °C) δ: 182.0 (C-4), 163.8 (C-2), 161.2 (C-4′), 160.8 (C-5), 159.3 (C-7), 154.1 (C-8a), 128.7 (C-2′/6′), 121.1 (C-1′), 115.7 (C-3′/5′), 108.2 (C-6), 104.0 (C-8), 103.3 (C-4a), 102.2 (C-3), 81.0 (Glc-C-5), 78.5 (Glc-C-3), 74.8 (Ara-C-3), 74.3 (Ara-C-1), 73.3 (Glc-C-1), 70.8 (Glc-C-2), 70.5 (Glc-C-5), 70.0 (Glc-C-4), 68.9 (Ara-C-4), 68.5 (Ara-C-2), 60.8 (Glc-C-6); ESI-MS (positive mode) *m/z*: 565 $[M + H]^+$.

Trigonelline (**4**): ^{1}H-NMR (500 MHz, CD_3OD, 25 °C) δ 9.20 (1H, s, H-2), 8.89 (1H, d, J = 7.9 Hz, H-6), 8.88 (1H, d, J = 6.0 Hz, H-4), 8.02 (1H, dd, J = 7.9, 6.0 Hz, H-5), 4.44 (3H, s, N-CH_3); ^{13}C-NMR (125 MHz, CD_3OD, 25 °C) δ 166.8 (COO^-), 147.5 (C-2), 146.8 (C-6), 145.9 (C-4), 139.9 (C-3), 128.5 (C-5), 53.8 (N-CH_3); ESI-MS (positive mode) *m/z*: 138 $[M + H]^+$.

Experimental procedures for lipase inhibition assay and α-amylase inhibition assay are given as supplementary materials.

3. Results and discussion

The dried seeds of the *T. foenum-graecum* were extracted with MeOH. The dried MeOH extract (M) was partitioned with n-hexane and MeOH to give n-hexane (H) and MeOH (M1) fractions. Dried MeOH fraction (M1) was partitioned with EtOAc and water to give dried EtOAc fraction (E) and dried water fraction (W). Schematic diagram for the preparation of extracts is given with the supplementary material as Fig. S1.

The extract/fractions (M, H, M1, E and W) were subjected to lipase (Choi et al., 2003) and α-amylase inhibition assays (Bernfeld, 1955). Results are given in the Table 1. All the extracts/fractions exhibited higher percentage lipase inhibitory activity than amylase inhibitory activity. The highest lipase inhibitory activity (70.11%) was exhibited by the water fraction (W). The MeOH and EtOAc fractions (M1 & E) showed an increased lipase inhibitory activity compared to that of the MeOH extract (M), but the hexane fraction showed a decrease in the lipase inhibitory activity (Table 1). Out of the four fractions, only the EtOAc (E) and methanol (M1) fractions showed weak α-amylase inhibitory activity, whereas the water (W) and hexane (H) fractions displayed α-amylase activation. The water extract (W) was then subjected to chromatography over Sephadex LH-20 to give five fractions A_1–A_5. The results of pancreatic lipase inhibition assay for the fractions A_1–A_5 are shown in the Table 2.

The sub-fraction A_5 showed the highest lipase inhibitory activity among the five fractions while the A_2 and A_3 fractions showed moderate lipase inhibitory activity. However the fraction A_4 exhibited a mild activation of lipase enzyme. The most active fraction A_5 was subjected to preparative RP-HPLC using an ODS column with isocratic solvent system MeOH:H_2O (35:65) to furnished compounds **1**, **2** and **3**. The TLC analysis indicated that the fraction A_2 contained a highly UV (254 nm) active compound **4**, which was isolated by chromatographic separation of the A_2 over silica gel. Compounds **1**–**4** (Fig. 1) were identified as vicenin-1 (apigenin 6-*C*-β-D-xylopyranosyl-8-*C*-β-D-glucopyranoside) (**1**) (Yasukawa et al., 1986; Xie et al., 2003), isoschaftoside (apigenin 6-*C*-α-L-arabinopyranosyl-8-*C*-β-D-glucopyranoside) (**2**) (Xie et al., 2003), schaftoside (apigenin 8-*C*-α-L-arabinopyranosyl-6-*C*-β-D-glucopyranoside) (**3**) (Yasukawa et al., 1986; Xie et al., 2003), and trigonelline (**4**) (Marchesini et al., 2009) by detail analysis of NMR and MS data as well as by comparison with the reported data. The isomeric glycosides **2** and **3** showed similar ^{13}C NMR data, but they were able to be differentiated from each other based on the shift difference $\Delta(\delta_{H\text{-}1 \text{ of } Ara} - \delta_{H\text{-}1 \text{ of } Glu})$ in ^{1}H NMR recorded in DMSO-d_6 at an elevated temperature: ca. 0.05 ppm for **3** and more than two-fold difference for **2** compared to that of **3**. The three apigenin C-diglycosides (**1**–**3**) has been previously characterized as major constituents of *T. foenum-graecum* seeds of Polish origin (Król-Kogus et al., 2014). Inconsistent with these findings isolation of apigenin 6-*C*-β-xylopyranosyl-8-*C*-β-galactopyranoside and luteolin 8-*C*-β-glucopyranoside were reported as the major constituents of the same plant seeds cultivated in Israel (Rayyan et al., 2010).

It has been documented that ^{1}H and ^{13}C NMR spectra of flavone C-glycosides occasionally suffer from line broadening or doubling and in some cases elevation of temperature was necessary to achieve a better NMR spectra (Markham and Geiger, 1994). This aptitude was observed more or less in the ^{1}H and ^{13}C spectra of compounds **1**–**3**, which disturbed their structure elucidation by the spectra obtained at

Table 1
Lipase and α-amylase inhibitory activities for extracts of *T. foenum-graecum* seeds.

Crude extract	Percentage lipase inhibitory activity*	Percentage amylase inhibitory activity*
Crude MeOH extract (M)	25.42	8.69
Hexane fraction (H)	14.29	−9.15
MeOH fraction (M1)	47.22	4.57
EtOAc fraction (E)	36.45	9.81
H_2O fraction (W)	70.11	−7.84
Orlistat	98.80	–
Acarbose	–	87.67

* Concentration at 1 mg/ml.

Table 2
Lipase inhibitory activity of fractions A_1 – A_5 and compounds **1**–**4.**

Fraction/compound	Percentage lipase inhibitory activity[a]
A_1	9.18
A_2	21.3
A_3	22.6
A_4	−2.3
A_5	88.1
Compound **1**	60.3
Compound **2**	33.8
Compound **3**	95.5
Compound **4**	5.90

[a] Concentration at 1 mg/ml (Fr. A_1 – A_5) & compounds **1**–**4** at the concentration of 250 μg/ml.

room temperature. It will be informative to show temperature dependency of the NMR spectrum broadening for compound **3**, since it showed the most heavy ^{1}H line broadening and negligible detection of most of ^{13}C signals among the three compounds. Fig. S2 in the supplementary materials illustrates the ^{1}H NMR spectra of compound **3** recorded at various temperatures. As can be seen in Fig. S2 (A), interpretation of the spectrum at 25 and 40 °C were technically impossible because of the heavy line broadening of sugar and H-2′/6′ proton signals. These signals became sharper upon elevating the temperature and they reached expected patterns about 80 °C. The ^{13}C NMR spectra of compounds **2** and **3** were also recorded at the elevated temperature and the data were carefully compared with the reported values (Yasukawa et al., 1986; Xie et al., 2003), which supported the aforementioned identification of **2** and **3**.

Compounds **1**–**3** belong to the class of flavonoid C-glycosides while compound **4** is a non-indole-benzopyrrole alkaloid. Among the three glycosides schaftoside (**3**) showed the most potent inhibitory effect on pancreatic lipase, with IC_{50} 130 μg/ml. Vicenin-1 (**1**) and Isoschaftoside (**2**) and showed lipase inhibitory activity with IC_{50} 207 μg/ml and 330 μg/ml, respectively. Trigonelline (**4**) showed negligible lipase inhibitory activity. This is the first report of lipase inhibitory activity of vicenin-1, although isoschaftoside and schaftoside were previously reported to show lipase inhibitory activity (Tao et al., 2015).

4. Conclusion

In conclusion this study led to the isolation of vicenin-1 (**1**), isoschaftoside (**2**) and schaftoside (**3**) along with trigonelline (**4**) from the methanol extract of *T. foenum-graecum*. These compounds showed percentage lipase enzyme inhibition 60.3% (**1**), 33.8% (**2**) and 95.5% (**3**) at the concentration of 250 μg/ml and IC_{50} values of the inhibition were 207 μg/ml (**1**), 330 μg/ml (**2**) and 130 μg/ml (**3**). This is the first report of the isolation of lipase inhibitors from *T. foenum-graecum* seeds and these results proved the potential of *T. foenum-graecum* as a natural lipase inhibitor, which helps to control the obesity which is a risk factor of cardiovascular diseases.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sajb.2018.10.023.

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