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Compounds in functional food fenugreek spice exhibit anti-inflammatory and antioxidant activities

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ABSTRACT

The seeds of fenugreek plant (*Trigonella foenum-graecum*) are widely used in the preparation of seasonings, pickles, curry powders and dietary supplements. The fenugreek seeds are also used in traditional medicine to relieve the common cold, arthritic pain and high blood sugar. Therefore, we have investigated the functional food quality of fenugreek seeds by determining the lipid peroxidation (LPO) and cyclooxyganase enzyme (COX) inhibitory activities of their hexane, ethyl acetate, methanolic and water extracts using MTT, LPO, COX-1 and -2 enzyme inhibitory assays. The extracts inhibited LPO by 55–95%, COX-1 by 6–87% and COX-2 by 36–70%, respectively, at 250 μg/ml. Bioassay-guided purification of these extracts yielded triglycerides (**1**–**3**), fatty acids (**4**–**5**), saccharides (**6**–**8**) and flavonoid-C-glycosides (**9**–**11**). The isolates, excluding the saccharides, inhibited LPO and COX-1 and -2 enzymes between the ranges of 8–89%, 4–51% and 15–70%, respectively, at 25 μg/ml. This is the first report of compounds **1**–**8** from fenugreek seeds and the biological activities described herein.

1. Introduction

Fenugreek (*Trigonella foenum-graecum*) is an annual plant in the family of Fabaceae. The seeds of this plant are used by people in Asia, Africa and Mediterranean countries as one of the ingredients in daily diet (Basch, Ulbricht, Kuo, Szapary, & Smith, 2003). Fenugreek seeds also have a long history of medicinal uses in Ayurvedic and Chinese traditional medicines for the amelioration of hypocholesterolemic effects (Sharma, 1986), diabetes (Raju, Gupta, Rao, Yadava, & Baquer, 2001), lack of appetite (Petit et al., 1993), fever (Agababyan, Gevorgyan, Tumadzhyan, Akopyan, & Aristakesyan, 2009), microbial infection (Randhir, Lin, & Shetty, 2004), oxidative effects (Dixit, Ghaskadbi, Moha, & Devasagayam, 2005), and cancer (Shabbee et al., 2009). Today, it is considered as one of the leading functional foods in the market with anecdotal traits ranging from ameliorating diseases to improving health.

The fenugreek seeds contain polyphenolic compounds, which have been correlated to the beneficial health effects of fenugreek (Saleh, Torgils, & Øyvind, 2010). Steroidal saponins in fenugreek seeds increase appetite, while inducing hyperinsulinemia and decreasing plasma total cholesterol levels (Taylor et al., 1997; Yoshikawa et al., 1997). It is also a rich source of polysaccharides and galactomannan (Jiang, Zhu, Zhang, & Sun, 2007). However, reports are not available for its lipophilic constituents and water soluble saccharides other than galactomannan. In addition, potential lipid peroxidation (LPO), cyclooxyganase (COX-1 and -2) enzyme inhibitory activities of its constituents have not been studied. In this study, fenugreek seeds were subjected to bioassay-directed purification to yield bioactive isolates and determined antioxidant and anti-inflammatory activities by assaying the extracts and isolates for their MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide), LPO and COX-1, as well as -2 enzyme inhibitory activities.

2. Materials and methods

2.1. Samples and chemicals

Fenugreek seeds (marketed by Raja Foods Co., India) were purchased from a store in Okemos, Michigan and stored in the laboratory until use. All solvents used for isolation and purification were of ACS reagent grade (Aldrich Chemical Co. Inc., Milwaukee, WI). Yellow [3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide] was used for the MTT antioxidant assay. Butylated hydroxyanisol (BHA), *t*-butyl hydroquinone (TBHQ) and butylated hydroxytoluene (BHT) were purchased from Sigma–Aldrich Chemical Company and used as positive control for LPO assay.

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COX-1 and -2 enzymes were prepared from ram seminal vesicles [Oxford Biomedical Research, Inc. (Oxford, MI)] insect cells cloned with human PGHS-2 enzyme, respectively. Arachidonic acid was purchased from Oxford Biomedical Research Inc. (Oxford, MI). Positive controls [Aspirin, Naproxen and Ibuprofen; Sigma–Aldrich Chemical Co. (St. Louis, MO)] were used in the COX inhibitory assay. Similarly, the nonsteroidal anti-inflammatory drugs Celeberex®, and Vioxx® were a physician's professional samples provided by Dr. Subhash Gupta, Sparrow Pain Centre, Michigan. All enzymes and reagents were stored in the Bioactive Natural Products and Phytoceuticals Laboratory at Michigan State University. NMR spectra were recorded on a 500 (^{1}H NMR) and 125 (^{13}C NMR) MHz Varian VRX instruments. Compounds **1**–**5** and **6**–**11** were dissolved in $CDCl_3$ and DMSO-d_6, respectively. Mass spectrum was recorded on a Waters QT ESI-MS spectrometer in positive mode.

2.2. Extraction and isolation

The powdered Fenugreek seeds (270 g) were packed in a glass column and eluted sequentially with hexane (2 l), ethyl acetate (EtOAC, 2 l) and methanol (MeOH, 2 l). The eluents were evaporated under vacuum to yield hexane extract (12.5 g), ethyl acetate extract (2.0 g), and methanolic extract (5.5 g), respectively. The ethyl acetate extract showed an identical TLC profile with the hexane extract and hence it was kept aside.

An aliquot of hexane extract (1.5 g) was subjected to a CombiFlash silica gel column, eluted with chloroform–methanol ($CHCl_3$–MeOH, linear gradient, starting at 100:0 in 0 min to 0:100 in 120 min, flow rate: 5 ml/min, 15 ml per fraction) to yield 40 fractions. The fractions were combined to form seven sub-fractions (A, 45 mg; B, 120 mg; C, 1100 mg; D, 200 mg; E, 40 mg; F, 30 mg; and G, 32 mg) based on their silica gel TLC profiles. Purification of an aliquot of fraction A (22 mg) yielded compound **5** (11 mg) by preparative TLC (PTLC) [Hexane–EtOAc (20:1)]. From 60 mg of fraction B, compound **1** (44 mg) was obtained by PTLC [Hexane–EtOAc (10:1)]. An aliquot of combined fractions C and D (80 mg) and repeated purification by PTLC [Hexane–EtOAc (8:1)] yielded compounds **2** (35 mg) and **3** (32 mg). Similarly, fractions E–G (50 mg) were also combined and purified to yield compound **4** (11 mg).

The methanolic extract (3.6 g) was dissolved in $CHCl_3$–MeOH (1:1 v/v; 3 × 100 ml) to yield $CHCl_3$–MeOH soluble (I, 1.2 g) and insoluble portion (II, 2.3 g). Fraction I (1.2 g) was fractionated by using CombiFlash silica gel column, eluted with the solvent system $CHCl_3$–MeOH (4:1) to yield 20 fractions (25 ml/min). The resultant fractions were combined to make four sub-fractions (MA, 78 mg; MB, 290 mg; MC, 500 mg; MD, 160 mg). The major compounds in fraction MB (110 mg) were methyl-O-glycoside (63 mg) and 2,3-dihydroxypropyl-glycoside (26 mg). Fractions MC (100 mg) and MD (150 mg) were, respectively purified by PTLC using solvent systems $CHCl_3$–MeOH–H_2O (4:1:0.1) and (2:1:0.1) to yield compounds **8** (46 mg), **7** (69 mg), and **6** (58 mg). Similarly, purification of fraction II (250 mg) gave sucrose (102 mg) and glucose (66 mg). Fraction II also showed trace amounts of compounds **6–8** and flavonoids by TLC, but were not isolated.

In order to follow the folk methods of its processing before consumption, fenugreek seeds were also extracted under different conditions. Therefore, powdered fenugreek seeds (100 g) were extracted with boiled water (1 l × 3) for 30 min. The combined water solution was centrifuged for 10 min and lyophilised to yield a pale yellow powder (32.2 g).

The dried water extract was then stirred with methanol (1 l) to yield methanol-soluble (I, 8.7 g) and insoluble (II, 23.5 g) fractions. Fraction I showed positive activity for the MTT assay. Hence, an aliquot of fraction I (4 g) was further partitioned with $CHCl_3$–MeOH (1:2, v/v) to yield the fraction soluble (I-A 1.9 g) and insoluble (I-B, 2.0 g). Fraction I-B showed again strong MTT activity, and hence an aliquot of it (1.5 g) was fractionated by CombiFlash C-18 silica gel column chromatography [Gradient system: MeOH–H_2O (5:95) initial to 100% methanol in 60 min, additional 30 min with 100% methanol, flow rate 5 ml/min] to yield 30 fractions (15 ml/fraction). These fractions were combined to make 7 sub-fractions (I-B-1, 30 mg; I-B-2, 530 mg; I-B-3, 10 mg; I-B-4, 22 mg; I-B-5, 125 mg; I-B-6, 260 mg; I-B-7, 190 mg). The MTT assay results showed that fractions I-B-2, I-B-3, I-B-4 and I-B-5 were positive.

Fraction I-B-2 (250 mg) was separated by CombiFlash on C-18 column, eluted with MeOH–H_2O (50:50, 5 ml/min, 15 ml/fraction, 20 fractions) to yield compounds **9** (21 mg) and **10** (17 mg). Although fraction I-B-3 gave strong MTT activity, the quantity was not sufficient to carry out further purification. Fractions I-B-4 and I-B-5 (in total, 145 mg) were combined and fractionated by preparative HPLC (LC-20), eluted with isocratic solvent system (35% MeOH-65% H_2O, 3 ml/min) to yield compound **11** (11.3 mg).

2.2.1. Compound *1*

A colourless wax; ESI-MS: *m/z* 925.2 $[M+Na]^+$ and 903.2 $[M+H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88 (bt., 6H, H-18′ × 2), 0.91 (bt., 3H, H-20″), 4.15 (dd, 2H, *J* = 14, 6 Hz, H-1a, 3a), 4.27 (dd, 2H, *J* = 14, 4, H-1b, 3b), 5.27 (m, 1H, H-2), 5.35 (m, 16H, H-9′, 10′, 12′, 13′, 15′, 16′, 11″, 12″, 14″ and 15″); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.2 (C-18′), 14.4 (C-20″), 22.5, 22.7, 24.8, 24.9, 29.3–29.7 (C-4′–7′ × 2, 4″–9″, 18″ and 19″), 25.6, 25.7 (C-11′, 14′ × 2), 25.9 (C-14″), 27.2 (C-8′ and 17′), 31.5 (C-3″), 31.6 (C-17″), 31.9 (C-3′ × 2), 34.0 (C-2′ × 2), 34.2 (C-2″), 62.2 (C-1 and 3), 68.9 (C-2), 127.9–130.2 (C-9′, 10′, 12′, 13′, 15′, 16′, 11″, 12″, 14″ and 15″), 172.8 (C-1″), 173.2 (C-1′ × 2). According to the spectral data, compound **1** was identified as (11Z,14Z)-11,14-eicosadienoic acid 2,3-bis[((9Z,12Z,15Z)-1-oxo-9,12,15-octadecatrien-1-yl)oxy] propyl ester (Gakwaya et al., 2007).

2.2.2. Compound *2*

A colourless wax; ESI-MS: *m/z* 911.2 [M+Na]+; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.89 (bt., 6H, H-18′ × 2), 0.92 (bt., 3H, H-19″), 4.16 (dd, 2H, *J* = 14, 6 Hz, H-1a, 3a), 4.29 (dd, 2H, *J* = 14, 4, H-1b, 3b), 5.26 (m, 1H, H-2), 5.35 (m, 16H, H-9′, 10′, 12′, 13′, 15′, 16′, 11″, 12″, 14″ and 15″); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.1 (C-18′), 14.2 (C-20″), 22.4, 22.6, 24.8, 24.9, 29.2–29.8 (C-4′ – 7′ × 2, 4″–9″ and 18″), 25.5, 25.7 (C-11′, 14′ × 2), 25.9 (C-14″), 27.3 (C-8′ and 17′), 31.6 (C-3″), 31.6 (C-17″), 31.9 (C-3′ × 2), 34.2 (C-2′ × 2), 34.3 (C-2″), 62.1 (C-1 and 3), 68.9 (C-2), 127.9–130.2 (C-9′, 10′, 12′, 13′, 15′, 16′, 11″, 12″, 14″ and 15″), 172.9 (C-1″), 173.2 (C-1′ × 2). According to the spectral data, compound **2** was identified as (11Z,14Z)-11,14-nondecanoic acid 2,3-bis[((9Z,12Z,15Z)-1-oxo-9,12,15-octadecatrien-1-yl)oxy] propyl ester (Gakwaya et al., 2007).

2.2.3. Compound *3*

A colourless oil; ESI-MS: *m/z* 433.3 [M+Na]+; ^{1}H NMR (500 MHz, in $CDCl_3$): δ 0.91 (bt., 6H, H-5′ × 2), 1.12 (bt., 3H, H-10″), 4.20 (dd, 2H, *J* = 14, 5 Hz, H-1a, 3a), 4.33 (dd, 2H, *J* = 14, 5, H-1b, 3b), 5.28 (m, 1H, H-2), 5.39 (m, 4H, H-3″, 4″, 6″ and 7″); ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.6 (C-10″), 15.3 (C-5′ × 2), 23.6 (C-4′ × 2), 27.8 (C-8″), 31.6 (C-5″), 32.2 (C-9″), 33.9 (C-3′ × 2), 35.2 (C-2′ × 2), 41.2 (C-2″), 62.1 (C-1 and 3), 68.9 (C-2), 127.9–130.2 (3″, 4″, 6″, 7″), 172.9 (C-1″), 173.2 (C-1′ × 2). According to the spectral data, compound **3** was identified as (3Z,6Z)-decanoic acid 2,3-bis-(1-oxo-pentyl-1-yl)oyl propyl ester (Gakwaya et al., 2007).

2.2.4. Compound *4*

A colourless oil; ESI-MS: *m/z* 261.2 [M+Na]+; ^{1}H NMR (500 MHz, in $CDCl_3$): δ 5.21 (4H, m, H-6, 7, 9 and 10), 0.88 (3H, m, 15-Me), 0.19 (2H, t, H-2), 0.21 (4H, m, H-5 and 11), 0.25 (2H,