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Bioactive constituents of germinated fenugreek seeds with strong antioxidant potential

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ABSTRACT

Germinated fenugreek seeds rich in bioactive antioxidant substances are used extensively as an important ingredient in daily food preparations and herbal formulations. The present study was carried out to isolate and identify active antioxidant principles from germinated fenugreek seeds. The partitioned extracts, column fractions and sub-fractions of germinated fenugreek seeds were subjected to activity assessment using standard *in vitro* antioxidant assays. Compounds with high antioxidant activity were identified by UV scan, mass analysis, NMR and LC–MS/MS. Bioactivity guided fractionation of the ethyl acetate extract led to isolation of vitexin (1) and isovitexin (2) as the major antioxidant compounds. These compounds were found to exert their effect at various levels of antioxidant action. LC–MS/MS analysis of ethyl acetate extract showed pre-dominance of apigenin, kaempferol and caffeic acid derivatives which may also additionally contribute to the observed antioxidant activity. This is the first report describing the phytochemical composition of germinated fenugreek seeds.

1. Introduction

Fenugreek (*Trigonella foenum-graecum* L.) seeds form a major constituent of Indian spices, are bitter to taste and are known for a long time for their medicinal properties. These seeds are an excellent source of dietary fiber and have been shown to lower blood glucose in various human and animal model studies and exert beneficial effects on serum lipid profile. Several human trials have unequivocally demonstrated the beneficial hypoglycaemic potential of this spice in both type-1 and type-2 diabetes (Srinivasan, 2006). A recent study on the steroidal saponins of fenugreek seeds showed anti-inflammatory and anti-melanogenic activities (Kawabata, Cui, Hasegawa, Takano, & Ohta, 2011). Besides its antidiabetic property the seeds are also shown to have antioxidant activity (Kaviarasan, Naik, Gangabhagirathi, Anuradha, & Priyadarshani, 2007).

Germinated seeds have several beneficial properties over ungerminated seeds. Germination improves *in vitro* protein digestion, as well as fat absorption capacity (Mansour & El-Adawy, 1994) and the extent of germination determines the actual composition. Prolonged germination of 96 h decreased the levels of total unsaturated fatty acids, total lipid, triglycerides, phospholipids and unsaponifiable matter (El-Sebaiy & El-Mahdy, 1983), short-term germination of 68 h increased polyunsaturated fatty acids (PUFA) which are proven to have health benefits (Shakuntala & Naik, 2011). Additionally fenugreek sprouts have shown to be rich in polyphenols, reducing

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sugars and minerals (K, Zn and Fe) indicating the superiority in the use of germinated seed fractions in functional and nutritional foods compared to their ungerminated counterparts (Shakuntala & Naik, 2011). Use of 10% germinated fenugreek seed flour (FSF) into wheat biscuit formula improved their nutritional quality criteria, reduced phytic acid content and enhanced iron bioavailability of FSF-biscuit (Ibrahium & Hegazy, 2009). A recent study demonstrated that impregnation of a fermentable soluble dietary fiber isolated from fenugreek to curcumin increased its bioavailability (Krishnakumar, Ravi, Kumar, Kuttan, & Maliakela, 2012). Germinated fenugreek seeds are also shown to reduce blood sugar levels and cholesterol in diabetic patients (Sowmya & Rajlakshmi, 1999). Previous studies carried out in our laboratory by Dixit, Ghaskadbi, Mohan, and Devasagayam (2005) have shown that germinated fenugreek seeds exhibit high antioxidant activity. An herbal anti-diabetic formulation made up exclusively of germinated fenugreek seeds namely Syndrex® (manufactured by Plethico laboratories, Indore, India) is also reported to possess antioxidant (Dixit, Ghaskadbi, & Devasagayam, 2008) as well as antidiabetic activity in streptozotocin induced diabetic mice (Dixit, Misar, Mujumdar, & Ghaskadbi, 2010). Thus, several studies have demonstrated strong antioxidant activity to germinated fenugreek seeds. To the best of our knowledge a good number of literatures are available on the chemical properties such as mineral content, fatty acid profile, sugar content etc., of germinated fenugreek seeds, but information regarding its phytochemical constituents and their roles as antioxidants is scanty. Present work was thus aimed to identify active antioxidant principles present in germinated fenugreek seeds. For this bioactivity guided fractionation and LC–MS/MS techniques were employed.

2. Material and methods

2.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonate) ABTS, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma Chemical Co., St. Louis, MO, USA. HPLC grade acetonitrile, methanol and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Other chemicals were from one of the following companies: SRL (New Delhi, India), BDH (Mumbai, India), Hi-media (Mumbai, India) or Merck (Darmstadt, Germany).

2.2. Biologicals

Male or female Wistar rats weighing 250 ± 20 g were purchased from animal house facility of Institute of Vetenary and Biological Sciences, Pune. They were housed in polypropylene cages maintained at 25 ± 2 °C with 12:12 h light and dark cycle. They were provided feed and water *ad libitum*. Prior approval was obtained from the Pune University Institutional Animal Ethical Committee for the protocols involving animals (Registration No. 538/02/c/CPCSEA). The animals were used for isolation of liver mitochondria.

2.3. Preparation of extract

Fenugreek seeds (3000 g) were purchased from the local market, soaked in water for 8 h and were germinated for 24 h. These were air-dried in shade and powdered (40 mesh size). The powder (300 g) was sequentially extracted with 1 L each of diethyl ether (DE) (0.74 g), ethyl acetate (EA) (7.4 g) and *n*-butanol (*n*-but) (250.3 g). Each step of extraction was repeated three times. The extracts were concentrated under vacuum to dryness using a rotary evaporator (Büchi, Essen, Germany), dissolved in methanol and assayed for their antioxidant potential using standard biochemical assays.

2.4. Isolation of compounds

Ethyl acetate (EA) extract (5.5 g) was fractionated on a silica gel column (35 cm × 2.5 cm i.d., 200–400 mesh) using stepwise gradient elution with the solvents chloroform/methanol (99:1–50:50, v/v). Fifteen milliliters of fractions were collected and analyzed on Silica gel F_{254} thin layer chromatography plates using chloroform/ethyl acetate/methanol/water (1:8:1.5:0.8, v/v/v/v) as developing reagent. The compounds were visualized under UV light at the wavelength of 254 and 336 nm. Elution with chloroform/methanol (97:3, v/v) yielded fraction 1 (1.8 g). Subsequent fractionation with chloroform/methanol (90:10) yielded fractions 2 (0.95 g) and 3 (1.1 g). The remaining 5 fractions were obtained using various proportions of chloroform and methanol as 85:15 (fraction 4, 0.14 g), 80:20 (fraction 5, 0.26 g), 75:25 (fraction 6, 0.20 g), 70:30 (fraction 7, 0.16 g) and, finally 50:50 (fraction 8, 0.17 g). Preliminary evaluation of antioxidant activity of the fractions was carried out using standard biochemical assays. Fractions 1, 2 and 3 showed higher antioxidant activity than the other fractions. These fractions were subjected to preparative thin layer chromatography (PTLC) using glass plates coated with silica gel G and a fluorescent indicator. Fractions were applied to the plates, and separated using chloroform/ethyl acetate/methanol/water (1:8:1.5:0.8, v/v/v/v). Bands were visualized under UV light at the wavelength of 254 and 336 nm. Separated fractions were scrapped, dissolved in methanol and filtered. Three bands (B1-B3) were obtained from fractions 1, 2 and 3, respectively and were assessed for their antioxidant activity. Bands 2 and 3 showed high antioxidant activity and therefore were further identified. Band 2 yielded compound 1 whereas band 3 yielded compound 2. Purity of the isolated bands was checked using HPLC. The compounds were further identified based on UV spectra, mass spectra, melting point and NMR data.

2.4.1. Compound 1 (Vitexin)

Yellow amorphous powder, UV λ_{max} (MeOH) nm, 267, 338; mp: 264 °C; gave positive Mg–HCl test; gave positive Molisch test; was resistant to acid hydrolysis; APCI–MS: *m/z* 433 $[M+H]^+$, 415, 271; NMR: ^{1}H-NMR (200 MHz, DMSO-d6): δ 3.53 (1H, m, H-5″), 3.56 (1H, m, H-3″), 3.57 (1H, m, H-4″), 3.77 (1H, dd, *J* = 12.2, 5.6 Hz, H-6a″), 3.75 (1H, dd, *J* = 12.2, 2.1 Hz, H-6b″), 4.12 (1H, t, *J* = 9.4 Hz, H-2″), 4.88 (1H, d, *J* = 9.8 Hz, H-1″), 6.58 (1H, s, H-3), 6.77 (1H, s, H-6), 6.92 (2H, d, *J* = 8.4 Hz, H-3′,5′), 7.88 (2H, d, *J* = 7.4 Hz, H-2′, 6′); ^{13}C-NMR (200 MHz, DMSO d6):