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ARTICLE

Lipid-Lowering and Antioxidant Effects of an Ethyl Acetate Extract of Fenugreek Seeds in High-Cholesterol-Fed Rats

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The purpose of this study was to investigate the relationship between the lipid effects, the phenolic content, and the antioxidative effects of an ethyl acetate extract of fenugreek. Wistar rats fed a standard laboratory diet or cholesterol-rich diets for 16 weeks were used. The plasma lipid levels, total phenolics, and total flavonoïd contents were measured, and the thiobarbituric acid reactive substances (TBARS) and antioxidant activities were examined. Administration of fenugreek ethyl acetate extract significantly lowered the plasma levels of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C), while increasing the plasma level of high-density lipoprotein cholesterol (HDL-C). Furthermore, the content of TBARS and catalase (CAT) and superoxide dismutase (SOD) activities in liver, heart and kidney decreased significantly after oral administration of the extract compared with those of rats fed a cholesterol-rich diet. These lipid effects and in vivo antioxidative effects were correlated with the in vitro phenolic content scavenging ability. In addition, three flavonoids (kaempferol 3-*O*-glycoside, apigenin-7-*O*-rutinoside, and naringenin) were identified by using an LC-MS/MS apparatus. Naringenin was the abundant flavonoid compound in the ethyl acetate extract, and its concentration reaches 7.23 ± 0.09 mg/g of dry extract. These results revealed significant hypocholesterolemic effects and antioxidant activity in an ethyl acetate extract of fenugreek seed, which may be partly due to the presence of flavonoids, especially naringenin.

KEYWORDS: Fenugreek; hypercholesterolemic; ethyl acetate extracts; naringenin; flavonoïds

INTRODUCTION

Many epidemiological studies and clinical investigations have demonstrated a significant decrease in morbidity and mortality from cardiovascular and other diseases among consumers of fruits and vegetables (*1*). The positive influence of these natural products, in part, is attributed to their dietary fiber and antioxidants and, in same cases, to their hypocholesterolemic effects.

Fenugreek (*Trigonella-Foenum-Graecum*) belongs to the leguminous family that grows predominantly in Northern Africa, the Middle East, and Asia. Fenugreek contains 23−26% protein, 6−7% fat, and 58% carbohydrates, of which 25% is dietary fiber (*2*) and saponins (*3*). Nair et al. (*4*) has shown that fenugreek seeds are rich in flavonoïds (> 100 mg/g). Shang et al. (*5*) identified five different flavonoids, namely vitexin, tricin, naringenin, quercetin, and tricin-7-9O-b-Dglucopyranoside to be present in fenugreek seeds. Fenugreek had been long used widely not only as a flavoring agent but also as a folk medicine. Several beneficial actions have been reported, such as appetite stimulation (*6*), anti-inflammatory and antipyretic activity (*7*), antimicrobial activity (*8*), antioxidant effects (*9*), cancer-preventive activity (*10*), and antidiabetic effects (*11*). The hypocholesterolemic properties of fenugreek currently being investigated support the use of fenugreek as a natural antiatherogenic dietary supplement. Sowmya and Rajyalakshmi (*12*) demonstrated that the consumption of 18 g/day of germinated fenugreek seed powder by human subjects resulted in a significant reduction in total cholestertol and LDL levels. Bordia et al. (*13*) have shown that fenugreek significantly decreases the blood lipids (cholesterol and triglycerides) in patients with coronary artery disease. This hypocholesterolemic effect of fenugreek was demonstrated in animal models such as diabetic dogs and rats (*14*, *15*). However, the mechanism of hypocholesterolemic action of fenugreek is still hypothetical.

The current study was designed to investigate the effect of an ethyl acetate extract of fenugreek on cholesterol metabolism. We investigated the hypothesis suggesting a polyphenolic structure for this "hypocholesterolemic agent". Hence, we studied the relationship between the lipid effects, phenolic content, and antioxidative effects of the extract.

MATERIALS AND METHODS

Fenugreek Seed Extract. A sample (100 g) of fenugreek seed powder was extracted with 400 mL of ethyl acetate. The solution was stirred at 120 rpm for 24 h at room temperature. Then, the extract was paper-filtered and transferred into a flask. The ethyl acetate extract was then brought to dryness using a rotating evaporator at 40 °C. The extract was stored at 4 °C to avoid compound degradation.

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Total Phenol Determination. The total phenol content of the extract was determined using the Folin−Ciocalteu technique (*16*). Briefly, a 50 μL aliquot of ethyl acetate extract was assayed with 250 μL of Folin reagent and 500 μL of sodium carbonate (20%, w/v). The mixture was vortexed and diluted with water to a final volume of 5 mL. After incubation for 30 min at room temperature, the absorbance was read at 765 nm in a basin of 1 cm, and total phenol in the extract was expressed as gallic acid equivalents (GAE), using a calibration curve of a freshly prepared gallic acid solution. We performed 10 determinations ($n = 10$). For gallic acid, the curve absorbance versus concentration is described by the equation $y = 0.0012x - 0.0345$ ($R^2 = 0.9997$).

Total Flavonoids. Total flavonoids were measured by the colorimetric assay developed by Zhishen et al. (*17*). A 1 mL aliquot of an appropriately diluted sample or standard solution of catechin (20, 40, 60, 80, and 100 mg L^{-1}) was added to a 10 mL volumetric flask containing 4 mL of doubly distilled (dd) H_2O. At zero time, 0.3 mL of 5% $NaNO_2$ was added to the flask. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. At 6 min, 2 mL of 1 M NaOH was added to the mixture. Immediately, the reaction solution was adjusted to 10 mL by adding 2.4 mL of dd H_2O and thoroughly mixed. The absorbance of the mixture was determined at 510 nm versus a prepared water blank. Total flavonoids of the extract were expressed on a fresh weight basis as mg/100 g of catechin equivalents (CE). Samples were analyzed in five replications.

LC-MS/MS Analysis. The LC-MS/MS experiments were carried out with an Agilent 1100 LC system consisting of degasser, binary pump, auto sampler, and column heater. The column outlet was coupled with an Agilent MSD Ion Trap XCT mass spectrometer equipped with an ESI ion source. Data acquisition and mass spectrometric evaluation were carried out in a personal computer with Data Analysis software (Chemstations). For the chromatographic separation, a Zorbax 300 Å Extend-C-18 Column (2.1 × 150 mm) was used. The column was held at 95% solvent A (0.1% formic acid in water) and 5% solvent B (0.1% formic acid in acetonitrile) for 1 min, followed by an 11 min step gradient from 5% B to 100% B, then it was kept for 4 min with 100% B, and finally, the elution was achieved with a linear gradient from 100% B to 5% B for 2 min.

The flow rate was 200 μL min^{-1}, and the injection volume was 5 μL. The following parameters were used throughout all MS experiments: for electrospray ionization with positive ion polarity the capillary voltage was set to 3.5 kV, the drying temperature to 350 °C, the nebulizer pressure to 40 psi, and the drying gas flow to 10 L min^{-1}. The maximum accumulation time was 50 ms, the scan speed was 26 000 m z^{-1} s^{-1} (ultra scan mode), and the fragmentation time was 30 ms.

The flavonoids and other compounds were identified using a combination of HPLC with diode array detection and liquid chromatography with atmospheric pressure chemical ionization mass spectrometry (ESI-LC-MS/MS) on the basis of their UV spectra and mass spectra and by comparison of the spectra with those of available authentic standards.

DPPH Assay. Different concentrations (5, 10, 20, and 40 μL equivalent to 5, 10, 20, and 40 ppm) of extract and BHT were placed in different test tubes. The volume of the sample/BHT was adjusted to 100 μL by adding MeOH. A methanolic solution (5.0 mL) of 2,2-diphenyl-1-picrylhydrozyl (DPPH; 100 μM) was added to these tubes, and the contents were shaken vigorously. The tubes were held at 27 °C for 20 min (*18*). The control was prepared as above without extracts, and MeOH was used for the baseline correction. The changes in the absorbance of the extract and BHT were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the formula

$$\%\text{radical scavenging activity} = ((\text{control OD}) - (\text{sample OD})/(\text{control OD})) \times 100$$

ABTS Assay. The Trolox equivalent antioxidant capacity (TEAC) assay, which measures the reduction of the radical cation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by antioxidants, was performed as previously described (*19*). Briefly, ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and placing the mixture in the dark at room temperature for 12−24 h before use. For the study, the $ABTS^{\bullet+}$ solution was diluted with ethanol or water (lipophilic and hydrophilic assay, respectively) to an absorbance of 0.70 (±0.02) at 734 nm. For the photometric assay 1 mL of the $ABTS^{\bullet+}$ solution and 100 μL of antioxidant solution were mixed for 45 s and measured immediately after 5 min at 734 nm (the absorbance did not change significantly up to 10 min). Compounds were assayed at five different concentrations determined within the linear range of the dose−response curve. A calibration curve was prepared with different concentrations of Trolox (0−20 μM). Results were expressed as millimolar concentration of Trolox.

Table 1. Composition of the Control Diet (g/kg)

diet ingredient	concn (g/kg)
casein	200
DL-methionine	3
corn oil	155
corn starch	393
sucrose	154
cellulose	50
mineral mix[a]	35
vitamin mix[b]	10

[a] The mineral mixture contained the following (mg/kg of diet): $CaHPO_4$, 17.200; KCl, 4000; MgO, 420; $MgSO_4$, 2000; Fe_2O_3, 120; $FeSO_47H_2O$, 200; trace elements, 400; $MnSO_4H_2O$, 98; $CuSO_4 \cdot 5H_2O$, 20; $ZnSO_4 \cdot 7H_2O$, 0.16; KI, 0.32; sufficient starch to bring to 40 g (per kg of diet). [b] The vitamin mixture contained the following (mg/kg of diet): retinol, 12; cholecalciferol, 0.125; thiamine, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyanocobalamin, 0.1; *p*-aminobenzoic acid, 100; biotin, 0.6; sufficient starch to bring to 20 g (per kg of diet).

Animals and Diets. Thirty male Wistar rats weighing 120 ± 2 g were purchased from Pasteur Institute (Tunis). The animals were housed identically in an air-conditioned room under a 12 h light:dark cycle at 22−23 °C. The rats were randomly divided into 3 groups of 10 rats each. Group 1 was fed a normal diet (CD) (**Table 1**). Group 2 was fed a high-cholesterol diet (HCD; normal diet supplemented with 1% cholesterol and 0.1% cholic acid). Group 3 was fed a high-cholesterol diet supplement with an ethyl acetate extract of fenugreek (0.125%). The experiment lasted 16 weeks (*20*). The animals were given food and water ad libitum during the experimental period, and the body weight was measured every day. At the end of the experimental period, the rats were killed by decapitation. Blood samples were collected to determine the plasma lipid profile. The livers, hearts, and kidneys were removed and rinsed with physiological saline. All simples were stored at −20 °C until analyzed.

Plasma Lipids. Plasma was separated by centrifugation at 3500 rpm for 15 min at 4 °C. The plasma samples collected were used fresh and subjected immediately to sequential ultracentrifugation in a Beckman L7−-55 ultracentrifuge (Beckman Instruments USA) with a Beckman SW50 rotor. Successive fractions were brought to the appropriate density with solid KBr ($d = 1.019$ kg/L for isolation of VLDL + IDL, $d = 1.063$ kg/L for LDL) and centrifuged respectively at 45 000 rpm for 15 h and at 40 000 rpm for 24 h (*21*). Supernatants were recovered by tube silicing, and after appropriate dilution, triglyceride and cholesterol concentrations were determined by enzymatic methods with commercial kits (ELITECH Diagnostics, Sees, France).

The HDL fractions were determined in the remaining plasma. Plasma concentrations of triglycerides and cholesterol were measured by enzymatic methods with the same commercial kits.

Antioxidant Enzyme Activities. The preparation of the enzyme source fraction in the liver, kidney, and heart tissues was as follows. One gram of liver tissues was homogenized in 2 mL of TBS and centrifuged at 9000 rpm for 15 min at 4 °C. The supernatants were removed and analyzed. The amount of protein in supernatant was measured, according to the method of Lawry et al. (*22*) using bovine serum albumin as standard.

Catalase (CAT) activity was measured using the method of Aebi (*23*). A 20 μL portion of the supernatant was added to a cuvette containing 780 μL of a 100 mM potassium phosphate buffer (pH 7.4); then the reaction was initiated by adding 200 μL of 500 mM H_2O_2 to make a final volume of 1.0 mL at 25 °C. The decomposition rate of H_2O_2 was measured at 240 nm for 15 s, 30 s, and 1 min on a spectrophotometer. A molar extinction coefficient of 0.0041 mM^{-1} cm^{-1} was used to determine the catalase activity. The activity was defined as μmol of H_2O_2 decrease/mg protein/min.

The superoxide dismutase activity (SOD) was measured according to the method of Asada et al. (*24*). A 50 μL portion of the supernatant (diluted 20 times) was mixed with 1 mL of EDTA methionine, tampon phosphate, nitroblue tetrazolium (NBT), and riboflavin and then incubated under light at 25 °C for 25 min. The activity was measured at 580 nm.

One unit was determined as the amount of enzyme that inhibited the oxidation of NBT by 50%. The activity was expressed as units of SOD/mg of protein.

Thiobarbituric Acid Reacting Substances (TBARS) Assay. As a marker of lipid peroxidation production, the TBARS concentration was measured using the method described by Buge and Aust (*25*). A 125 μL portion of the tissue homogenate was mixed with 50 μL of TBS and 125 μL of TCA-BHT, vortexed, and then centrifuged at 1000 rpm for 10 min. A 200 μL portion of the supernatant was mixed with 40 μL of HCl and 160 μL of Tris-TBA, vortexed, and then incubated at 80 °C for 10 min. The resulting coloration intensity was measured at 530 nm.

Statistical Analysis. All data presented are means ± standard deviation (SD). Statistical analyses were calculated using a one-way analysis of variance (ANOVA), followed by Student's *t* test. Differences were considered significant at $P < 0.05$.

RESULTS

Total Phenol and Flavonoid Contents. An ethyl acetate extract of fenugreek seeds was analyzed to determine its total phenol and flavonoid contents by biochemical methods. The total flavonoid content of the ethyl acetate extract of fenugreek was 19.01 ± 0.01 mg catechin equivalents/g of dry weight extracts of fenugreek powder (**Table 2**), and the total phenolic content of the extract of fenugreek was 75.60 ± 0.80 mg gallic acid equivalents/g of dry weight extracts of fenugreek powder (**Table 2**).

Flavonoids and 4-Hydroxyisoleucine Identification. HPLC analysis of the ethyl acetate extract showed the presence of peaks with flavonoid-type UV spectra (two bands, λ_{max} of band 1 between 320 and 350 nm and λ_{max} of band 2 between 250 and 270 nm) and interfering peaks of other phenolics. As the flavonoids were present at low concentrations, it was not possible to isolate them in sufficient amounts for identification by NMR spectroscopy. **Table 3** gives each of the identified compounds in elution order. The structure assignment of flavonoids for which no standards were available was based on a systematic search for molecular ions using extracted ion mass chromatograms and comparing those with data in the literature (*26*). For example, the ESI mass spectrum in positive mode of compound **2** exhibited a base peak $[M + H]^+$ at *m*/*z* 449 (**Table 3**) and an aglycone ion at *m*/*z* 287. The loss of 162 amu from the intermediate ion is due to the loss of glucose. The λ_{max} values of the UV spectrum at 345 and 265 nm suggest that flavonoid **2** is a kaempferol 3-*O*-hexoside, and the results of the MS and UV spectra combined suggest that compound **2** could be kaempferol 3-*O*-glucoside. The identification was also confirmed by cochromatography with authentic standards as kaempferol 3-*O*-glucoside. Flavonoid **3** showed an $[M + H]^+$ ion at *m*/*z* 579 with significant fragments at *m*/*z* 433 and 271. The loss of 146 amu from the pseudomolecular ion represents the sugar rhamnose, and the loss of 162 amu from the intermediate ion is due to the loss of glucose. The combined UV spectra (λ_{max} 338, 266 nm) and MS results of compound **3** suggested that it could be an apigenin 7-*O*-glycoside. This identification was confirmed after acid hydrolysis (*27*) of ethyl acetate extract, followed by HPLC analysis of the reaction products, indicated that apigenin was present in its aglycone form. In the same way, compound **4** was identified as naringenin by comparing its HPLC retention time, UV spectra, and mass spectra with the data obtained from standard in-house libraries.

Another compound (amino acid), 4-hydroxyisoleucine (**1**), was detected. Its identification was confirmed by using an LC-MS apparatus in the positive mode. The spectrum exhibited a molecular ion at *m*/*z* 148 $[M + H]^+$ with fragments at *m*/*z* 138, 130, and 118. The obtained mass fragments agreed with those described previously (*28*).

Quantification of Flavonoids. The concentrations of individual flavonoids in fenugreek ethyl acetate extract were determined by the HPLC method (**Table 4**). The flavonoid levels ranged from 3.2 to 7.32 mg/g of dry extract. The predominant flavonoid in the analyzed extract was naringenin (7.32 mg/g of dry extract). This compound presented 38% of the total flavonoids in an ethyl acetate extract of fenugreek. The other flavonoids kaempferol 3-*O*-glycoside and apigenin 7-*O*-rutinoside presented 26.88% and 16.83% of total flavonoids in this extract, respectively.

DPPH and ABTS Radical Scavenging Assay. The antioxidant activity of fenugreek extract was tested by measuring its capacity to scavenge DPPH and ABTS radicals. The ethyl acetate extract of fenugreek exhibited high antioxidant activity with 13.36 mmol/L Trolox equivalent antioxidant capacity. In the case of the DPPH assay, a high scavenging potential (89.91%) was obtained with the ethyl acetate extract of fenugreek (**Table 5**). As positive controls, the inhibition percent of radicals and TEAC against DPPH and ABTS+ of BHT were 96% and 20.13 mmol/L, respectively, which indicated that the antioxidant activity of the ethyl acetate extract of fenugreek was lower than that of BHT.

Body and Organ Weights. The body weight increased in all groups throughout the treatment without any significant differences between them (**Figure 1**). There were no differences in the heart and kidney to body weight ratios (**Table 6**). However, the liver to body weight ratio increased in rats fed a cholesterol-rich diet (HCD) compared with the rats fed a control diet (CD) (**Table 5**).

Serum Lipids. Figure 2 shows the serum lipid levels at the end of the experiment. After 16 weeks of treatment, the TC, TG, (VLDL+IDL)-C, (VLDL+IDL)-TG, and LDL-C concentrations of rats fed a cholesterol-rich diet (HCD) showed a significant increase compared with those in the rats fed a normal diet (CD). A significant decrease of HDL-C concentration of rats in the HCD group was observed ($p < 0.05$). The administration of ethyl acetate extract of fenugreek reduced concentrations of TC, TG, (VLDL+IDL)-C, (VLDL+IDL)-TG, and LDL-C than those rats receiving an HCD by 14%, 23.3%, 43.9%, 31.3%, and

Table 2. Total Phenol and total Flavonoid Contents of an Ethyl Acetate Extract of Fenugreek Seed[a]

extract	phenolic content (mg of GAE/g of dry weight extracts)	flavonoid content (mg of CE/g of dry weight extracts)
ethyl acetate	75.60 ± 0.80	19.01 ± 0.01

[a] Values are means ± SD of three determinations. Abbreviations: GAE, gallic acid equivalents; CE, catechin equivalents.

Table 3. Flavonoids Detected in Fenugreek Extract with Their Retention Times, UV spectra and Mass Spectral Data

no.	flavonoid	retention time (mn)	UV λ_{max} (nm)	$[M + H]^+$ (*m*/*z*)[a]	$[I + H]^+$ (*m*/*z*)[b]	$[A + H]^+ m/z$[c]
1	4-hydroxyisoleucine	1.6	260	148		
2	kaempferol 3-*O*-glycoside	8.7	265, 345	449		287
3	apigenin 7-*O*-rutinoside	12.3	338, 266	579	433	271
4	naringenin	13	288, 330	273		

[a] APCI-MS (positive mode) data for the protonated molecular ion. [b] APCI-MS (positive mode) data for protonated intermediate molecular ions. [c] APCI-MS (positive mode) data for the protonated aglycone ion.

Table 4. Flavonoid Contents in Ethyl Acetate Extract of Fenugreek (mg/g of Dry Extract)[a]

flavonoid	concn (mg/g of dry extract)
kaempferol 3-*O*-glycoside	3.2 ± 0.12
apigenin 7-*O*-rutinoside	5.11 ± 0.15
naringenin	7.23 ± 0.09

[a] Values are means ± SD of three determinations.

Table 5. Antiradical and Antioxidant Activities of an Ethyl Acetate Extract of Fenugreek Seed[a]

fenugreek extract	TEAC (mmol/L)	% inhibition per DPPH
ethyl acetate	13.36 ± 0.32	89.91 ± 3.09
BHT	20.13 ± 0.61	96 ± 2.11

[a] Values are means ± SD of three determinations.

Table 6. Effect of Fenugreek Extract on the Liver, Kidney, and Heart to Body Weight Ratios (g/100 g of Body Weight)[a]

organ	CD	Ch	Eacé
kidney	0.37 ± 0.01[a]	0.36 ± 0.03[a]	0.37 ± 0.02[a]
heart	0.39 ± 0.01[a]	0.35 ± 0.03[a]	0.35 ± 0.01[a]
liver	4.62 ± 0.1[a]	4.82 ± 0.04 [b]	4.61 ± 0.05[a]

[a] Abbreviations: CD, standard diet; Ch, high-cholesterol diet (HCD); Eacé, HCD + ethyl acetate extract of fenugreek. Values are means ± SD for ten rats per group. Bars with different letters differ, $P < 0.05$.

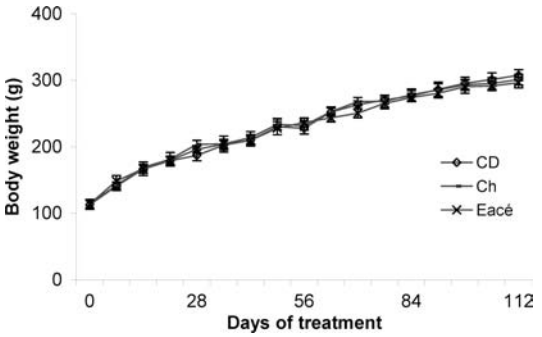

Figure 1. Growth curves of rats fed a standard diet or a cholesterol-rich diet during the 16-week feeding period. Abbreviations: CD, standard diet; Ch, high-cholesterol diet (HCD); Eacé, HCD + ethyl acetate extract of fenugreek. Each bar represents means ± SD from 10 rats.

36.6%, respectively. The concentration of HDL-C of rats treated with ethyl acetate extracts of fenugreek increased significantly compared with those of rats in the HCD group ($p < 0.05$).

Antioxidant Enzyme Activities. The CAT and SOD activities significantly increased in the kidney, heart, and liver (18.5% and 22.3%, 20.4% and 29.1%, and 24.8% and 67.8%, respectively) of rats fed a cholesterol-rich diet compared to the control diet group. The increase was significantly lowered ($p < 0.05$) in the HCD rats supplemented with an ethyl acetate extract of fenugreek (**Figures 3** and **4**)

TBARS levels. The TBARS levels were significantly increased ($p < 0.05$) in the liver, heart, and kidney of the animals fed a high-cholesterol diet compared to the control diet group. In the HCD groups of rats, this increase was significantly reduced in the presence of an ethyl acetate extract of fenugreek (**Figure 5**).

DISCUSSION

The present study investigated the effect of an ethyl acetate extract of fenugreek on the lipoprotein profile in rats fed a hypercholesterolemic diet. The results showed that the extract

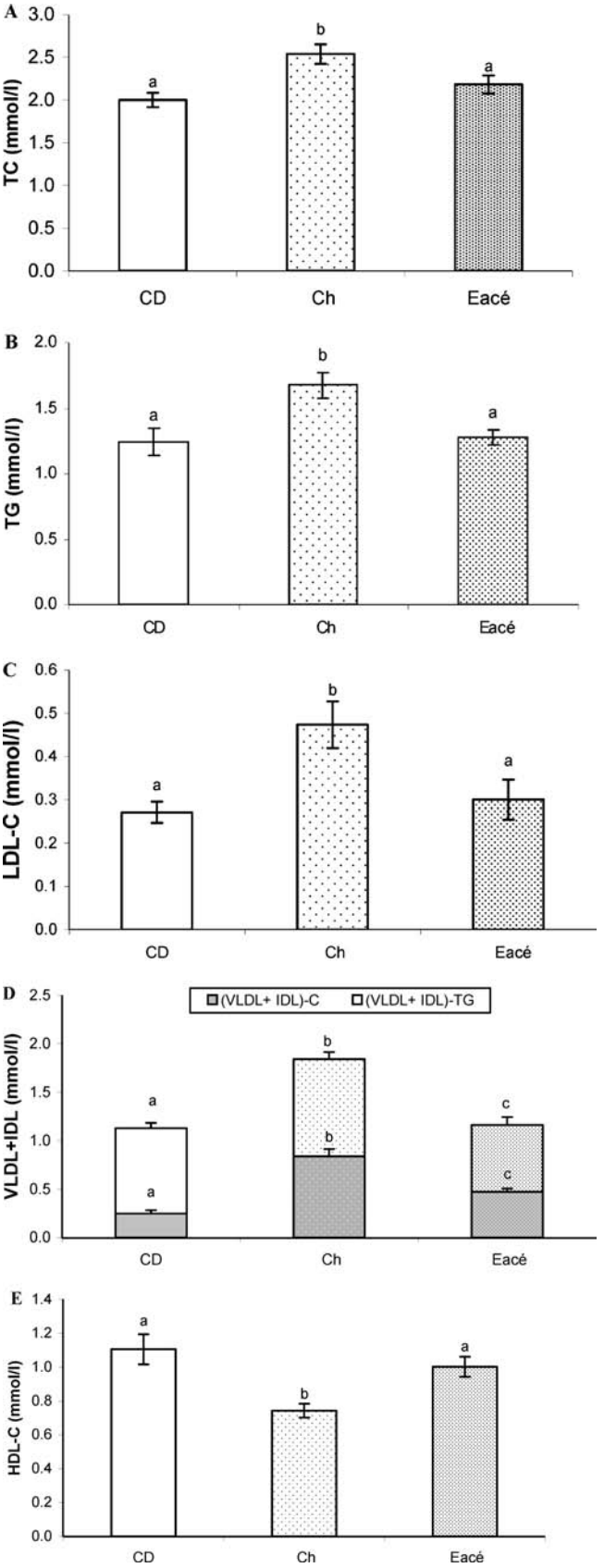

Figure 2. Effects of fenugreek extracts on rat TC (**A**), TG (**B**), (VLDL+ IDL)-C and (VLDL+ IDL)-TG (**C**), LDL-C (**D**), and HDL-C (**E**). Abbreviations: CD, standard diet; Ch, high-cholesterol diet (HCD); Eacé, HCD + ethyl acetate extract of fenugreek. Each bar represents means ± SD from 10 rats. Bars with different letters differ, $P < 0.05$.

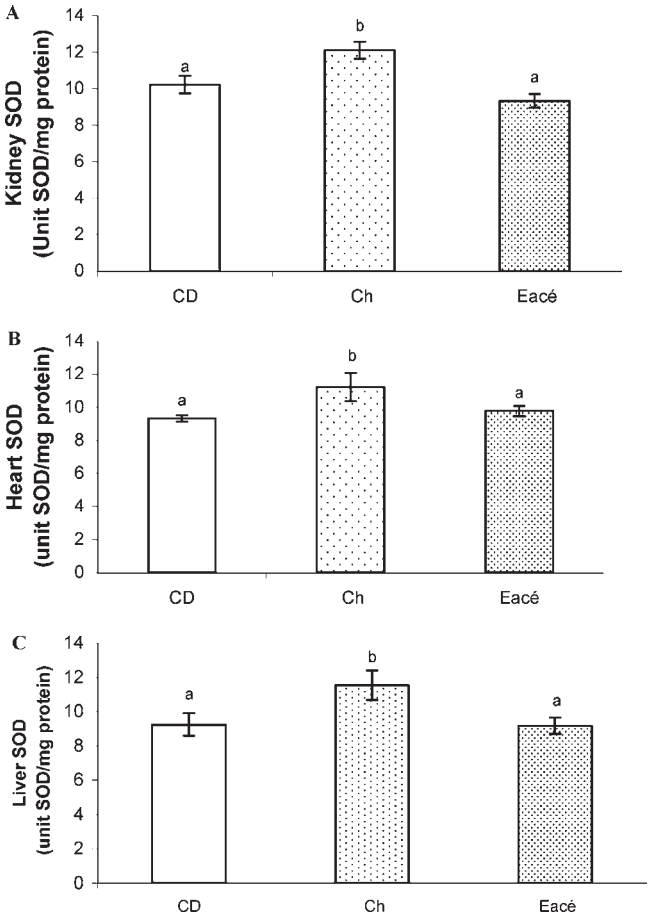

Figure 3. Effects of a fenugreek extract on rat kidney (**A**), heart (**B**), and liver (**C**) SOD activity. Abbreviations: CD, standard diet; Ch, high-cholesterol diet (HCD); Eacé, HCD + ethyl acetate extract of fenugreek. Each bar represents means ± SD from 10 rats. Bars with different letters differ, $P < 0.05$.

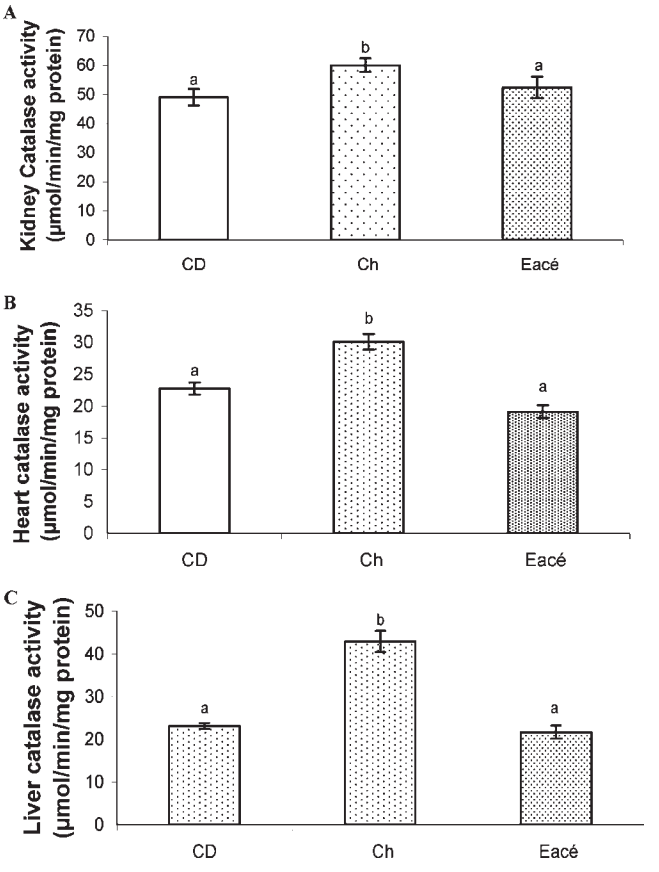

Figure 4. Effects of a fenugreek extract on rat kidney (**A**), heart (**B**), and liver (**C**) catalase activity. Abbreviations: CD, standard diet; Ch, high-cholesterol diet (HCD), Eacé, HCD + ethyl acetate extract of fenugreek. Each bar represents means ± SD from 10 rats. Bars with different letters differ, $P < 0.05$.

of fenugreek reduced hypercholesterolemia. This effect was correlated with the in vitro antioxidant capacity of the extract. The ethyl acetate extract has a very potent antioxidant effect in cholesterol-fed rats.

The rats in the HCD group had higher concentrations of TC, TG, (VLDL+IDL)-C, (VLDL+IDL)-TG, and LDL-C in serum than in those of the control group, indicating that the hypercholesterolemic model was successfully established.

Our data demonstrated that the administration of an ethyl acetate extract of fenugreek in rats fed a cholesterol-rich diet reduced the levels of TC, TG, (VLDL+IDL)-C, (VLDL+IDL)-TG, and LDL-C. The reduction of TC induced by an ethyl acetate extract of fenugreek might be attributed to their increase of fecal bile acid and cholesterol excretion and/or to the decrease of cholesterol biosynthesis and esterification. We did not evaluate these hypotheses in our work, but we can find some evidence in the literature.

Choi et al. (*29*) demonstrated that the administration of hesperetin 7-*O*-lauryl ether, a compound derived from hesperetin (one of the most abandant flavonoids from citrus fruits such as lemons, oranges, and grapefruit), in hypercholesterolemic rats elevated fecal bile acid excretion. This increase lowered the reabsorption of bile acid by the enterohepatic circulation, thereby lowering the bile acid pool and activating the bile acid synthesizing enzyme in the liver.

The inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, was reached with some phenolic compounds. Hence, Kim et al. (*30*) indicated that supplementation with hesperetin or its metabolites sighificantly inhibited hepatic cholesterol biosynthesis and esterification. Other authors reported that the administration of naringenin inhibited the HMG-CoA reductase in humans (*31*), in high-cholesterol-fed rats (*32*), and in high-cholesterol-fed rabbits (*33*).

Cholesterol acyl transferase (ACAT) is a key enzyme involved in the esterification and absorption of cholesterol, secretion of hepatic LDL cholesterol, and cholesterol accumulation in the arterial wall (*34*). Therefore, ACAT inhibitors are also exepted to be cholesterol-lowering and antiatherosclerotic agents (*30*). Wilcox et al. (*35*) and Borradiale et al. (*36*) provided evidence that naringenin and hespertin not only decrease cholesterol but also inhibit ACAT activity.

Hence, different results and studies are in agreement with the hypothesis of a polyphenolic nature and antioxidant properties of the lipid-lowering compound of interest. From that point of view, we studied the relationship between the lipid effects, the phenolic content, and the antioxidative effects of the extract.

The ethyl acetate extract of fenugreek was examined for its antiradical and in vitro and in vivo antioxidant activities. The ethyl acetate extract of fenugreek showed higher antiradical and in vitro antioxidant activities. Likewise, the administration of an ethyl acetate extract of fenugreek to the rats fed a cholesterol-rich diet significantly decreased the content of TBARS and the activities of SOD and CAT in the liver, heart, and kidney. This increase in SOD and CAT activities is seen as an adaptation process to face the free radical production. The neutralization of the oxidative burst with ethyl acetate extract probably eliminated

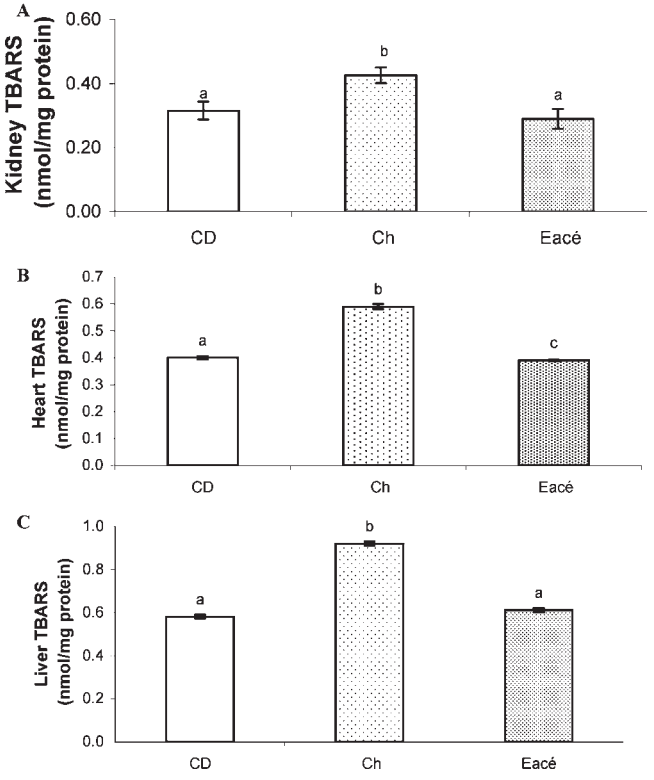

Figure 5. Effects of fenugreek extracts on rat kidney (**A**), heart (**B**), and liver (**C**) MDA levels. Abbreviations: CD, standard diet; Ch, high-cholesterol diet (HCD); Eacé, HCD + ethyl acetate extract of fenugreek. Each bar represents means ± SD from 10 rats. Bars with different letters differ, $P < 0.05$.

the need of SOD and CAT participation to scavenge the free radicals.

It was logical, then, to wonder if these antiradical and in vitro antioxidant activities could be correlated with the total phenolic and flavonoid content of the extract. However, three flavonoids were identified by LC/MS analysis: kaempferol 3-*O*-glucoside, apigenin 7-*O*-glycosides, and naringenin. This result suggested that the protective effect exhibited by the ethyl acetate extract is probably due to its flavonoid content, particularly naringenin, which is the most abundant flavonoid in the ethyl acetate extract (38% of the total flavonoid). The flavonoids identified in fenugreek seeds are reported to have potent antioxidant properties (*37*). The scavenging activities of these flavonoid substances are attributed to the active hydrogen-donating ability of the hydroxyl substitutions (*38*). Kaviarasan et al. (*39*) demonstrated that the presence of phenolic substances, especially naringenin and quercetin, in fenugreek seeds could be responsible for $^{\bullet}$OH radical scavenging activity. Other studies have reported that naringin, a glycone of naringenin, can slightly inhibit lipid peroxidation in rats (*40*) and has an inhibitory effect against cytochrome P450 (CYP3A) activity in human liver microsomes (*41*).

In conclusion, we found in this model of experimental hypercholesterolemia that supplementation with an ethyl acetate extract of fenugreek exhibited an antihyperlipidemic action, reduced the lipid peroxidation process, and regulated the antioxidant system. These effects are mainly supported by the flavonoid contents, especially naringenin, which is the most abundant flavonoid in this extract. Further investigations are needed to establish definitive evidence for the implication of naringenin in the effects of the ethyl acetate extract of fenugreek on the cholesterol metabolism of rats.

ABBREVIATIONS USED

Ch, cholesterol; VLDL, very low density lipoprotein; LDL, low-density lipoprotein; IDL, intermediate density lipoprotein; HDL, high-density lipoprotein; VLDL-C, VLDL-cholesterol; VLDL+IDL-C, VLDL+IDL-cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; VLDL+IDL-TG, VLDL+IDL-triglycerides; TC, total cholesterol; TG, triglycerides; HCD, high-cholesterol diet; SOD, superoxide dismutase; CAT, catalase; TBARS, thiobarbituric acid reacting substances; PBS, phosphate buffer saline; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); LC-MS, liquid chromatography−mass spectrum; MeOH, methanol; DPPH, 2,2-diphenyl-1-picrylhydrozyl; TEAC, Trolox equivalent antioxidant capacity; GAE, gallic acid equivalents; CE, catechin equivalents; HMG-CoA, 3-hydroxy-3-methylglutaryl-Coenzyme A; ACAT, cholesterol acyl-transferase.

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