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ARTICLE

Stability of Lipid Constituents in Radiation Processed Fenugreek Seeds and Turmeric: Role of Phenolic Antioxidants

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Impact of radiation processing on the lipid profile of fenugreek and turmeric was investigated. Oleic and linoleic acid were the dominant fatty acids with an appreciable amount of linolenic acid in both cases. γ-Irradiation did not bring about any significant changes in the fatty acid profile of these spices despite a high content of unsaturation. The ability of aqueous methanolic extract of both spices with high phenolic content to prevent lipid peroxidation suggests a possible role of phenolic constituents in preventing lipid radiolysis. Among the phenolics identified, kaempferol-3-*O*-α-L-rhamnoside, kaempferol 3,7-*O*-α-L-dirhamnoside, quercetin 3,7-*O*-α-L-dirhamnoside, and 3-*O*-α-L-rhamnosyl quercetin are reported here to occur in fenugreek for the first time. The role of phenolic antioxidants in preventing lipid oxidation in the above spices is discussed.

KEYWORDS: Antioxidant activity; flavonoids; γ-irradiation; lipid; fenugreek; turmeric

INTRODUCTION

Fenugreek (*Trigonella foneum*) and turmeric (*Curcuma longa* L.) are two important spices of commerce. In the food industry fenugreek is extensively used for seasoning purposes and as an ingredient of curry powder and sauces. The spice has immense pharmacological importance. Fenugreek seeds possess significant antidiabetic, antiatherosclerotic, anti-inflammatory, antinoiceptive, and antiulcerogenic activities (*1*). Fenugreek is rich in flavonoids such as apigenin, luteolin, orientin, quercetin, vitexin, and isovitexin, which help to strengthen the immune system, improve cellular health, and reduce signs of aging (*2*). Flavonoids are known to possess high antioxidant activities. These compounds are reported to inhibit lipid oxidation and thus play an important role both as additives in stabilizing and extending the shelf life of foods as well as in preventing diseases such as atherosclerosis.

Turmeric, the yellow rhizome of *Curcuma longa* is commonly used as a food coloring agent and also has immense medicinal properties. Turmeric is used in the treatment of cuts, wounds, bruises, sprains, skin disease, and blood purification. Recent studies on turmeric's active ingredient, curcumin, have also found significant potential benefits for people suffering from cystic fibrosis and cancer. Curcumin has enormous potential in the prevention and therapy of cancer. It can suppress tumor initiation, promotion, and metastasis (*3*). Curcumin exhibits antioxidative and free radical scavenging activities. It also enhances activities of other antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase. The compound thus has the ability to protect lipids against oxidative deterioration.

Both the above spices are prone to microbial contamination and insect infestation during storage and transportation resulting in quality deterioration and economic loss. Among the newer nonthermal methods for postharvest hygienization of food, radiation processing using ionizing radiations such as γ-radiation/electron beam occupies a unique position as it is a cold process (*4*). Extensive studies have established the efficacy of this process as a safe method for the preservation of spices without producing any organoleptic changes at the recommended decontamination doses (5−10 kGy) (*5*). With a ban on chemical fumigation the world over because of its adverse effects on human health and the environment, processing of food by γ-radiation/electron beam has gained increased importance. Radiation processing has been known to bring about oxidative changes in lipid constituents (*6*). It is generally agreed that the main reaction involved in oxidative deterioration of food lipids is between oxygen and unsaturated fatty acids. This process of lipid oxidation is a major cause of quality changes in foods, involving aroma, flavor, taste, texture, consistency, and appearance (*7*, *8*). The total lipid content of the Indian variety of turmeric is reported to vary from 5.1%−5.9%. Linoleic (17%), oleic acid (17%), and linolenic (5%) are the major fatty acids reported in this spice (*9*). Fenugreek seed contains about 7.8% lipid constituting mainly of neutral lipids (85%) followed by phospholipids (10%) and glycolipids (5%) (*10*). Unsaturated fatty acids comprising mainly of linoleic (4%), linolenic (22%), and oleic acid (17%) dominate the fatty acid profile. The presence of highly unsaturated fatty acids in fenugreek and turmeric can result in the reduced shelf life of radiation processed spices as a result of oxidative deterioration of lipids and consequent off-flavor development. Despite a high content of unsaturated lipids that are susceptible to oxidative deterioration, the effect of radiation processing on the lipid profile of the above spices has not been explored so far.

The present work, therefore, aims at determining the effect of radiation processing on the major lipid constituents of fenugreek and turmeric at the dose of 10 kGy recommended for the decontamination of spices. The nature of the phenolic constituents in these spices and their potential to scavenge free radicals as

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well as to prevent lipid peroxidation was assessed. The possible role of these compounds in protecting lipid against oxidative deterioration during radiation processing is discussed.

MATERIALS AND METHODS

Materials. Three separate lots of dry fenugreek seeds and turmeric were procured from a local market. The three lots were each divided into two sets and packed in high-density polyethylene bags (0.12 mm thickness). One set was kept as a nonirradiated sample. The other set, was subjected to γ-radiation in air in a cobalt-60 Irradiator (Gamma Cell-220, MDS Nordion Int. Kanata, Ontario, Canada) at a dose rate of 10 Gy/min. Absorbed doses between 2 and 10 kGy were given to the samples. Dosimetry was performed by a Fricke dosimeter (*11*). All the samples were powdered with an electric grinder (Sumeet, Mumbai, India) and sieved (mesh size 40). Both the nonirradiated and radiation processed samples in each lot were analyzed in triplicate for free fatty acid profiles within one week of storage. All standard flavonoids and sugars were procured from Sigma Chemical Co. (United States) Ltd. All solvents (analytical reagent grade) were redistilled before use. TLC was carried out on precoated silica gel 60 F254 (Merck).

Extraction, Isolation, and Lipid Analyses. The method followed was essential according to the procedure reported earlier (*12*). Irradiated and nonirradiated turmeric and fenugreek samples (100 g each) were exhaustively extracted separately with a distilled chloroform/methanol (2:1) mixture (4 × 200 mL) in an omnimixer. Three replicates of chloroform/methanol and 80% aq. methanol extracts were prepared. The respective extracts were concentrated in vacuo. The chloroform/methanol (2:1) extract was saponified (2 N KOH, 80 °C, 1 h). The hydrolysate after removal of nonsaponifiable matter was acidified (2 N HCl), and the free fatty acids liberated were extracted into diethyl ether. The ether layer was washed with distilled water until free of acid and then dried over anhydrous sodium sulfate. The free fatty acids obtained after the removal of solvent was converted to their methylated derivative (diazomethane, room temperature) and then analyzed by GC/MS. GC-MS analysis was carried out on a Shimadzu GC-MS instrument (Shimadzu Corporation, Kyoto, Japan) equipped with a GC-17A gas chromatograph and provided with a DB-5 (J&W Scientific, California, USA) capillary column ((5%-phenyl)-methylpolysiloxane, length, 30 m; id., 0.25 mm; and film thickness, 0.25 μm). The operating conditions were as follows: column temperature programmed from 140−280 °C at the rate of 4 °C/min, held at initial temperature, and at 200 °C for 5 min, and further to 280 °C at the rate of 10 °C/min, held at final temperature for 10 min; injector and interface temperatures, 210 and 230 °C, respectively; carrier gas helium (flow rate, 0.9 mL/min); ionization voltage, 70 eV; electron multiplier voltage, 1 kV. Samples (0.1 μL) were injected in the splitless mode. Peaks were identified by comparing their mass fragmentation pattern with that of standard compounds as well as from the data available in the spectral library (Wiley/NIST Libraries) of the instrument.

The same instrument was used to obtain MS for the pure flavonoids by direct injection of the samples.

Estimation of Fatty Acids. Aliquots of standard linoleic acid ranging in concentration from 1 to 10 μg/mL were injected into the GC, and the plot of peak area vs concentration was then drawn. The plot was found to be linear in the range of 1−8 μg/mL. The concentration of individual components in the sample was obtained from the standard curve and expressed as mg/g of sample.

Extraction and Antioxidant Activity. Irradiated and nonirradiated turmeric and fenugreek samples (100 g each) were successively extracted with hexane (4 × 250 mL), chloroform (4 × 250 mL), 80% aq. methanol (4 × 250 mL), and water (4 × 250 mL).

Estimation of Total Phenols. The total phenols present in 80% aq. MeOH extracts of both the spices were estimated according to the Folin−Ciocalteu method (*13*). To 50 μL of sample, 250 μL of undiluted Folin−Ciocalteu reagent was added. After 1 min, 750 μL of 20% (w/v) aqueous Na_2CO_3 was added, and the volume was made up to 5.0 mL with H_2O. The controls contained all of the reaction reagents except for the extract. After 2 h of incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. Total phenols were determined as gallic acid equivalents (mg gallic acid/g extract), and the values are presented as the means of triplicate analyses.

Antioxidant Activity. One test tube containing 1% solution of only the hexane extract (10 mL) and another test tube containing 1% solution of hexane extract (5 mL) mixed with 1% solution of 80% aq. MeOH extract (5 mL) were irradiated to a dose of 10 kGy. The third test tube contained 10 mL of 1% solution of nonirradiated hexane extract. All of these solutions were diluted to 10 times, and 1 μL of these was injected into the GC. A standard curve was prepared by injecting standard linoleic acid ranging in concentration from 1 to 10 μg/mL, and the protection capacity of the methanol extract toward lipid peroxidation was then measured. A blank methanol was also irradiated and injected to check whether any breakdown products were present during irradiation.

Lipid Peroxidation Assay (14). The total reaction mixture (1.0 mL) contained tris-HCl buffer at pH 7.4 (125 mM) and rat brain homogenate (0.5 mg protein/mL) with or without the test extracts. The reaction was triggered by the addition of ferrous ammonium sulfate (10 μM) and ascorbic acid (200 μM), and incubating the mixture at 370 °C for 30 min. The reaction was terminated by the addition of 2 mL of a TCA−TBA−HCl (2.8% w/v TCA, 0.25 N HCl, and 0.375% w/v TBA) solution and boiling the mixture at 100 °C for 10 min. The extent of lipid peroxidation was measured spectrophotometrically by recording the absorbance at 532 nm after accounting for the appropriate blank.

High Performance Liquid Chromatography (HPLC) Analysis. HPLC analysis was carried out on a Jasco HPLC system from Jasco Corporation (Tokyo, Japan) equipped with a C-18 reverse phase stainless steel column (30 cm × 0.46 cm) and a PDA detector set at a wavelength of 275 nm. Eighty percent aq. methanol extract (20 μL, 0.01% solution) was injected on to the column and then eluted with water, and elution was performed using a solvent system comprising solvents A (1.5% H_3PO_4) and B [acetic acid/CH_3CN/H_3PO_4/H_20 (20:24:1.5:54.5)] mixed using a gradient starting with 80% A, linearly decreasing to 33% A after 30 min, 10% A after 33 min, and 0% A after 39.3 min (column temperature 30 °C). Peaks were identified by comparing their retention times with that of authentic standards injected under identical conditions and also comparing with literature data. Contents of the flavonoids and their glycosides were estimated from a standard curve (correlation coefficient 0.99) prepared using quercetin (linear in the range of 2−15 μg).

Data Analysis. All data are an average of 3 independent analyses, each carried out in triplicate. Thus, a total of 9 replications were performed. Statistical analysis was carried out using the analysis of variance method (Origin 6.1 software), and means were expressed as significantly different or not at 5% level of confidence.

Isolation and Characterization of the Pure Compounds. A separate 80% aq. methanol extract was obtained after successive extractions with hexane (10 L) and chloroform (10 L) from 5 kg of fenugreek. It was evaporated to dryness under reduced pressure, H_2O was added (80 mL), and the suspension was filtered. The filtrate was concentrated and subjected to column chromatography on silica gel. The column was eluted first with $CHCl_3$ followed by an increasing proportion of methanol in chloroform. Two major fractions, $CHCl_3$/MeOH (4:1) and (4:2) obtained were further purified after repeated column chromatography on Sephadex LH-20 with H_2O and H_2O/MeOH (95:5) as eluants. Six major fractions were obtained. Each fraction was purified by preparative TLC using either solvent system $CHCl_3$/MeOH/H_2O/HOAc (80:20:2:1) or solvent system EtOAc/HCOOH/AcOH/H_2O (100:11:11:15). While the former system yielded compounds **1** and **6**, the rest of the compounds were obtained in a pure form using the later system.

Acid hydrolysis of each of the compounds was carried out using 2 N HCl (1 h, 100 °C). The hydrolyzates in each case were extracted with ethyl acetate to obtain the aglycones. The sugars in the neutralized acidic phase were analyzed on cellulose TLC (Merck). Comparison of R_f values with authentic standards as well as GC-MS of their derivatives aided in the identification of both the aglycone and sugar residues. The structures of the aglycones were further identified by comparison of their spectral data (UV, NMR, IR, and MS) with authentic standards and the reported literature data. Antilipid peroxidation capacities of the isolated compounds were studied using the above-mentioned method (*14*).

Spectral Data. Melting points (uncorrected) were determined with a Fisher John apparatus; optical rotations were measured on a JASCO-DIP 370 polarimeter; the IR spectrum was scanned with a JASCO FTIR 4100 spectrophotometer (Jasco Corporation, Tokyo, Japan). The NMR spectra were recorded with a Bruker AC-200 MHz FT NMR spectrometer

(Bruker, Fallanden, Switzerland) using DMSO-d_6, and TMS was used as an internal standard. The usual abbreviations employed areas follows: d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet, J = coupling constant (in Hz), and δ = chemical shift in ppm.

Compound 1 (Kaempferol-3-O-α-L-rhamnoside). Yellow needles; gave a positive HCl−Mg test; m.p., 172−173 °C. $[\alpha]^{24}_D$: −165° ($CHCl_3$, c = 0.65). UV λ_{max} (MeOH) nm: 265, 312 sh, 355. IR ν^{KBr} (cm^{-1}): 3400, 1663, 1607, 1442. EI-MS: m/z 286 $[M\text{-rhamnosyl}]^+$, 285, 258, 229.

Compound 2 (Kaempferol 3-O-β-D-glucosyl (1 → 2)-β-D-galactoside 7-O-β-D-glucoside). Amorphous powder; gave a positive HCl−Mg test; m.p., 220−221 °C. $[\alpha]^{24}_D$: −37° (H_2O, c = 0.09). UV λ_{max}(MeOH) nm: 263, 318 sh, 355. IR ν^{KBr} (cm^{-1}): 1658, 1600, 1488. EI-MS: m/z 448 $[M\text{-}324]^+$, 285.

Compound 3 (Kaempferol 3,7-O-α-L-dirhamnoside). Light yellow crystals; gave a positive HCl−Mg test; m.p., 221−223 °C. $[\alpha]^{24}_D$: −125° (H_2O, c = 0.65). UV λ_{max}(MeOH) nm: 238 sh, 260, 345. IR ν^{KBr} (cm^{-1}) 3450, 1658, 1620, 1395. EI-MS: m/z 433, 286, 153, 121.

Compound 4 (Quercetin 3,7-O-α-L-dirhamnoside). Yellow powder; gave a positive HCl−Mg test; m.p., 211−213 °C. $[\alpha]^{24}_D$: −115° (H_2O, c = 0.55). UV λ_{max}(MeOH) nm: 260, 265, 360. IR ν^{KBr} (cm^{-1}): 3450, 2900, 1660, 1605, 1460, 1350, 1180, 1130, 1100, 1056. EI-MS: m/z 464, 302, 273, 228, 153.

Compound 5 (Quercetin 3-O-β-D-glucosyl (1 → 2)-β-D-galactoside 7-O-β-D-glucoside). Yellow amorphous powder; gave a positive HCl−Mg test; m.p., 198−200 °C. $[\alpha]^{24}_D$: −38° (H_2O, c = 0.09). UV λ_{max}(MeOH) nm: 255, 267 sh, 360. IR ν^{KBr} (cm^{-1}): 3300, 1660, 1603, 1495. EI-MS: m/z 486, 465, 302.

Compound 6 (3-O-α-L-Rhamnosyl Quercetin). Yellow needles; gave a positive HCl−Mg test; m.p., 198−200 °C. $[\alpha]^{24}_D$: −138° (H_2O, c = 0.55). UV λ_{max}(MeOH) nm: 257, 293 sh, 372. IR ν^{KBr} (cm^{-1}): 1655, 1603, 1495. EI-MS: m/z 302, 273, 228, 153.

RESULTS AND DISCUSSION

The average yield of extract obtained from nonirradiated and irradiated fenugreek samples were 7.5 and 8.7%, respectively. A significant increase (at 5% level of confidence) in the extractives when the spice was subjected to radiation processing could possibly be accounted for by the break down of polysaccharides and a consequent increase in extractability. Saponification of the extract and subsequent work up resulted in the isolation of free fatty acids. **Table 1** lists the major fatty acids and their quantitative distribution in nonirradiated and radiation processed fenugreek. The fatty acid profile of fenugreek is dominated by highly unsaturated fatty acids (82.35%). Linoleic acid was the major fatty acid, followed by linolenic and oleic acids. Saturated fatty acids comprise 17.65% of the total lipids. Palmitic acid was the major (10.9%) saturated fatty acid. The percentage of linolenic acid has been shown to vary depending on the variety and agroclimatic conditions. Shahat (*15*) reported a content of 13.8% in Egyptian fenugreek oil, while its content varied from 7%−13% in Indian fenugreek (*16*, *17*). Our results are thus in agreement with the reported literature values (*10*, *18*). Though there is a statistically significant difference in the linoleic acid content between nonirradiated and irradiated fenugreek, looking at the gross changes it amounts to only 2.7%, which may be low enough to bring about significant effects on the sensory quality of the product.

Unlike fenugreek, no significant difference ($p > 0.05$) in the yield of extract could be noted between the nonirradiated (5.81%) and irradiated (5.77%) turmeric samples. The yield of extract is similar to that reported earlier (*9*). The distribution of the fatty acids identified in turmeric is presented in **Table 2**. Total saturated fatty acids comprise 33.5%, while total unsaturated fatty acids account for 66.46% of the total fatty acids identified. Thus, unsaturated fatty acids also dominated the lipid profile of turmeric. Oleic (22%), linoleic (35%), and linolenic (4%) acids were the major fatty acids identified. Besides, myristoleic (3%) and palmitoleic (2.3%) acids were the other unsaturated acids detected in turmeric. Stearic acid was the prominent saturated fatty acid accounting for 21% of the total fatty acids.

In an earlier study on the effect of radiation processing on the lipid constituents of nutmeg, an increase in free fatty acid content in the radiation processed spice was reported (*12*). A break down of trimyristicin and consequent release of free myristic acid was demonstrated to account for this increase. The soapy rancid odor perceived in nutmeg when exposed to higher doses restricted the application of radiation processing to doses less than 5 kGy. The breakdown of triacylglycerols to liberate free fatty acids when exposed to ionizing radiation has also been reported in sea food (*19*) and wheat germ (*20*). The role of unsaturated acids, particularly linoleic and linolenic acids, in contributing to the off flavor of radiation processed food stuffs has been reported. Because of the high levels of unsaturation, these fatty acids are susceptible to oxidative damage (*21*). The lipid peroxidation is one of the complex cellular processes and is usually initiated by any of the ROS, which may be generated both because of endogeneous and exogeneous factors. Radiation processing is known to bring about the oxidation of lipid radicals at the olefenic centers resulting in the formation of peroxy radicals that further break down to form carbonyl compounds. These compounds with low aroma thresholds impart off-flavor to foods. Interestingly, no significant difference ($p > 0.05$) was noted in the fatty acid profile between the nonirradiated and irradiated samples (**Tables 1** and **2**) in both spices despite a high content

Table 1. Fatty Acid Composition of Total Lipids of Nonirradiated and γ-Irradiated Fenugreek Seeds[a]

fatty acid methyl ester	nonirradiated (mg/g of fenugreek)	irradiated (mg/g of enugreek)
$C_{14:0}$	0.16 ± 0.03	0.15 ± 0.04
$C_{16:0}$	8.95 ± 0.02	9.01 ± 0.03
$C_{16:1}$	0.15 ± 0.01	0.15 ± 0.02
$C_{18:0}$	3.67 ± 0.04	3.62 ± 0.05
C18:1	12.93 ± 0.05	12.79 ± 0.05
$C_{18:2}$	35.81 ± 0.07	35.73 ± 0.07
$C_{18:3}$	18.1 ± 0.02[b]	17.6 ± 0.03[b]
$C_{20:0}$	1.17 ± 0.04	1.19 ± 0.02
$C_{20:1}$	0.06 ± 0.03	0.07 ± 0.02
$C_{22:0}$	0.36 ± 0.01	0.34 ± 0.01
$C_{24:0}$	0.07 ± 0.01	0.07 ± 0.02

[a] Data are the mean of nine replicates ± standard deviation. Means in rows (irradiation effect) are not significantly different at 5% level of confidence. [b] Means in rows (irradiation effect) are significantly different at 5% level of confidence.

Table 2. Fatty Acid Composition of Total Lipids of Nonirradiated and γ-Irradiated Turmeric[a]

fatty acid methyl ester	nonirradiated (mg/g of turmeric)	irradiated (mg/g of turmeric)
$C_{12:0}$	1.31 ± 0.03	1.43 ± 0.02
$C_{14:0}$	1.03 ± 0.01	1.01 ± 0.01
$C_{14:1}$	1.49 ± 0.02	1.53 ± 0.03
$C_{16:0}$	0.64 ± 0.02	0.65 ± 0.02
$C_{16:1}$	0.63 ± 0.03	0.65 ± 0.01
C18:0	9.52 ± 0.19	9.43 ± 0.02
C18:1	9.86 ± 0.02	9.79 ± 0.02
$C_{18:2}$	15.76 ± 0.01	15.69 ± 0.02
$C_{18:3}$	1.87 ± 0.03	1.92 ± 0.03
$C_{20:0}$	2.44 ± 0.03	2.51 ± 0.01

[a] Data are the mean of nine replicates ± standard deviation. Means in rows (irradiation effect) are not significantly different at 5% level of confidence.

Table 3. Total Phenolic Content in Fenugreek and Turmeric[a]

samples		total phenolics (mg of GAE per gm dry weight)
fenugreek	nonirradiated	81.3 ± 3.15 a
	irradiated	83.7 ± 2.41 a
turmeric	nonirradiated	175 ± 3.92 b
	irradiated	173 ± 5.71 b

[a] Data are the mean of three replicates ± standard deviation. Values followed by similar letters are not significantly different at ($P > 0.05$).

of unsaturated fatty acids. Ramadan et al. (*20*) have also demonstrated that neither thermal nor irradiation treatment had a significant effect on the total lipid recovery or the fatty acid composition despite a high content of linoleic and oleic acids in wheat germ samples. An increase in free fatty acids with radiation dose was, however, reported by these researchers (*20*).

Spices are reported to possess a high concentration of phenolic antioxidants that contribute to their use as preservatives (*22*). Antioxidants such as flavonoids, curcuminoids, and other phenolics generally present in spices act by protecting lipid peroxidaton and quenching free radicals formed during radiation processing. Total phenolic content of turmeric and fenugreek was to be in the range of 173−175 and 81−84 mg of gallic acid equivalent per gm dry weight, respectively. An appreciable content of phenolic compounds in the two spices, thus, suggests a possible role of these compounds in preventing lipid oxidation (**Table 3**).

In order to ascertain the role of phenolics in contributing to the stability of fenugreek and turmeric lipids during radiation processing, extracts obtained using different solvents were tested for their efficiency in quenching free radicals. The 80% aq. methanol extract was found to have the greatest ability to prevent lipid peroxidation as measured by antilipid peroxidation (ALP) capacity in both turmeric and fenugreek. The potential of the different extracts to prevent lipid peroxidation was in the order hexane < chloroform < aqueous < 80% aq. methanol extract (**Figure 1**). Thus, the 80% aq. methanol extract was taken up for detailed study. The antioxidant activities of the 80% aq. methanol extract correlated well with its total phenolic content in both turmeric (175.6 mg/g gallic acid equivalent) and fenugreek (81.3 mg/g gallic acid equivalent). Our results on total phenolic content are comparable to the literature values (*1*, *23*, *24*). A higher ALP activity was noted in turmeric compared to that in fenugreek. High efficiency of these extracts to prevent lipid peroxidation suggests the role of phenolic antioxidants in preventing oxidative deterioration of lipids. No significant difference ($p > 0.05$) in the phenolic content and ALP activities was found between the irradiated and nonirradiated fenugreek and turmeric extracts (**Table 3** and **Figure 1**).

Curcuminoids are reported to be the major phenolics of turmeric. The nature of the curcuminoids in turmeric has been extensively studied and their antioxidant property established. Thus, no attempt was made to identify the individual antioxidant curcuminoids in this spice. Fenugreek is known to contain an appreciable content of phenolics and flavonoids (*1*). The nature of flavonoids in fenugreek stem and seed has been reported. In vitro studies on the antiradical and antioxidant activities of fenugreek seed extracts have also been recently described. However, to the best of our knowledge the role of flavonoids in contributing to the antioxidant activity of fenugreek has not been established so far. It was, therefore, of interest to determine the role of these constituents in contributing to the antioxidant status of the spice.

The antioxidant potential of the above extract was further ascertained on the basis of its ability to prevent lipid peroxidation

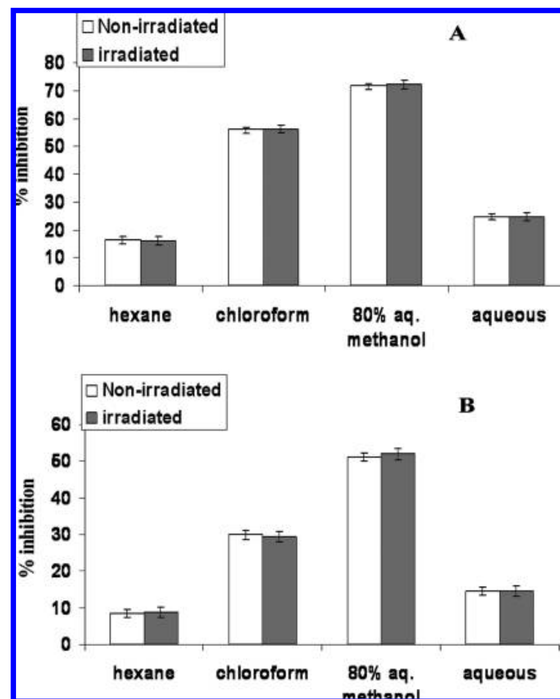

Figure 1. Antilipid peroxidation activities of hexane, chloroform, 80% aqueous methanol, and aqueous extracts of (**A**) turmeric and (**B**) fenugreek seeds at 0.05 mg/mL concentration.

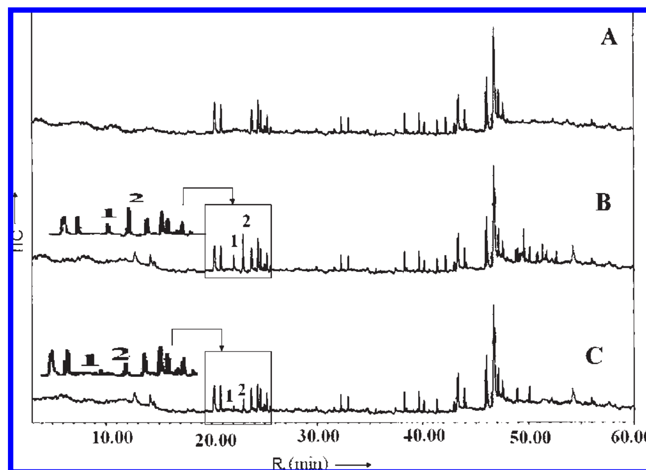

Figure 2. GLC chromatogram of (**A**) hexane extract, (**B**) irradiated hexane extract, and (**C**) hexane extract added with methanol extract and irradiated. (*E*,*Z*)-2,4-Nonadienal (R_t 22.06) and (*E*,*E*)-2,4-decadienal (R_t 22.87) have been designated as 1 and 2, respectively (inset, zoomed chromatogram).

in both hexane extract and standard linoleic acid exposed to γ-radiation at a dose of 10 kGy. **Figure 2** depicts the results of such a study. The appearance of two new peaks at R_t 22.06 and 22.87, identified as (*E*,*Z*)-2,4-nonadienal (R_t) and (*E*,*E*)-2,4-decadienal (R_t), respectively, were observed when irradiated to a dose of 10 kGy (**Figure 2B**). These two compounds are known to be the major oxidized products of unsaturated lipids such as linoleic acid and are routinely used as markers for the detection of fat deterioration. No peaks at R_t values 22.06 and 22.87 were observed (**Figure 2A**) in the case of nonirradiated hexane extract when injected in GC/MS. Interestingly, the addition of 80% aq. methanol extract (5 mL, 1% solution) into the hexane extract during γ-radiation resulted in a 38% and 24% reduction in the

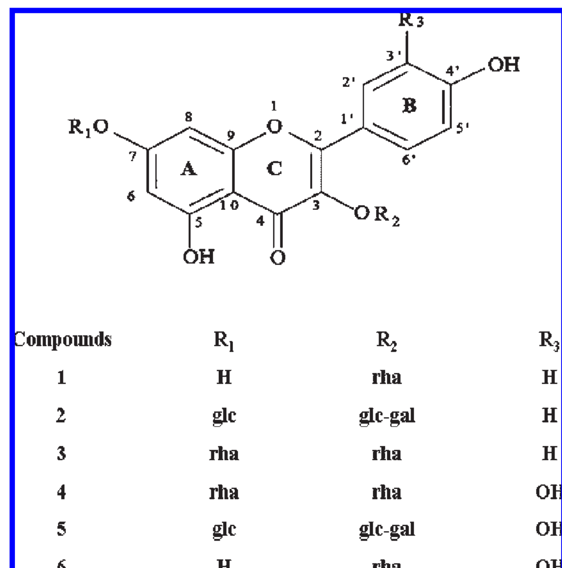

Figure 3. Structures of flavonoid glycosides **1** to **6**.

formation of (*E*,*Z*)-2,4-nonadienal and (*E*,*E*)-2,4-decadienal, respectively (**Figure 2C**). Lacroix et al. (*25*) have also shown that rosemary and thyme, both known to contain natural antioxidants, markedly reduced the radiolytic generation of $C_{10}-C_{19}$ hydrocarbons from linoleic acid by 52.5−80.5% when irradiated at a dose of 10 kGy. A similar reduction in hydrocarbon $C_{14:1}$ (generated during irradiation) was also observed in this present study when irradiated with 80% aq. methanol extract. Since hydrocarbons were not of interest in this study, further detailed data have not been presented here. Since the role of the individual constituents present in the 80% aq. methanol extract in preventing oxidative deterioration of lipids was of interest, the nature of the individual constituents in this extract was further investigated.

The 80% aq. methanol extract after successive washing with petroleum ether and diethyl ether was subjected to column chromatography (CC) on silica gel, followed by CC on Sephadex LH-20. Preparative TLC of the fractions thus obtained yielded purified compounds **1**−**6**. All of these compounds displayed UV absorption data typical of flavonols. The isolated compounds were initially identified as flavonoid glycosides because they gave positive Molisch test results and also Mg-HCl test results. The sugars obtained from the hydrolyzed products were identified from the (i) TLC data compared with the corresponding standards, (ii) GC-MS data of their corresponding acetates and R_t values, and (iii) optical rotatory measurements compared with the authentic standards. The isolated pure compounds were further identified by their spectral data (UV, ^{1}H NMR, ^{13}C NMR, and MS), acid hydrolysis, and by comparison with authentic samples and published literature data. The nature of the flavonoids identified is depicted in **Figure 3**. The hydrolyzed products of compounds **1**, **2**, and **3** showed kaempferol as their aglycone moiety, while that of **4**, **5**, and **6** matched with the standard quercetin. The UV and ^{1}H NMR spectra of these compounds were also in agreement with the above structures.

Compound **1** was obtained as a yellow powder. Complete acid hydrolysis of compound **1** gave kaempferol and rhamnose. The ^{1}H NMR (**Table 4**) spectrum of compound **1** displayed the characteristic signals of the kaempferol nucleus (*26*, *27*). Two doublets at δ 6.41 (J = 2.2 Hz) and 6.63 ppm (J = 2.2 Hz) were assigned to the H-6 and H-8 protons, respectively. A pair of A_2B_2 aromatic system protons at δ 6.90 ppm (J = 8.5 Hz) could be assigned to H- 3′,5′ while that at 7.72 ppm (J = 8.5 Hz) was assigned to H-2′,6′. The ^{13}C NMR signal at δ 115.8 was ascribed

Table 4. ^{1}H NMR Data for Compounds **1**, **3**, **4**, and **6** (DMSO-d_6, 300 MHz)

	compound 1	compound 3	compound 4	compound 6
position	δ_H, mult., J (Hz)	δ_H, mult., J (Hz)	δ_H, mult., J (Hz)	δ_H, mult., J (Hz)
6	6.41, d (2.2)	6.47, d (2.4)	6.49, d (2.4)	6.44, d (2.44)
8	6.63, d (2.2)	6.68, d (2.4)	6.72, d (2.4)	6.70, d (2.44)
2′	7.72, d (8.5)	7.76, d (9.2)	7.41, d (2.0)	7.38, d (2.0)
3′	6.90, d (8.5)	6.96, d (9.2)		
5′	6.90, d (8.5)	6.96, d (9.2)	6.78, d (8.5)	6.79, d (8.4)
6′	7.72, d (8.5)	7.76, d (9.2)	7.40, dd (2.0/8.5)	7.41, dd (2.0/8.5)
		3-*O*-Rhamnose		
1″	5.34, d (1.8)	5.38, d (1.8)	5.31, d (1.4)	5.28, d (1.3)
2″	4.20, dd (1.8/3.8)	4.22, dd (1.8/3.8)	4.15, dd (1.8/3.8)	4.19, dd (1.7/3.8)
3″	3.68, dd (3.0/8.6)	3.72, dd (3.1/8.9)	3.69, dd (3.1/8.9)	3.71, dd (3.5/9.0)
4″	3.29−3.33 m	3.26−3.31 m	3.24−3.29 m	3.23−3.27 m
5″	3.34−3.37 m	3.32−3.36 m	3.32−3.38 m	3.30−3.35 m
6″	0.90 d (5.4)	0.94 d (5.4)	0.96 d (6.0)	0.99 d (6.0)
		7-*O*-Rhamnose		
1‴		5.49, d (1.78)	5.42, d (1.83)	
2‴		4.10, dd (1.8/3.9)	3.99, dd (1.9/3.8)	
3‴		3.78, dd (3.0/9.6)	3.75, dd (3.2/9.7)	
4‴		3.44−3.49 m	3.42−3.46 m	
5‴		3.50−3.55 m	3.49−3.53 m	
6‴		1.15 d (5. 84)	1.11 d (5. 84)	

Table 5. ^{13}C NMR Data for Compounds **1**, **3**, **4**, and **6** (DMSO-d_6, 300 MHz)

	compound 1	compound 3	compound 4	compound 6
position	δ_C	δ_C	δ_C	δ_C
2	156.2	157.7	156.0	156.3
3	134.7	135.6	134.5	133.9
4	178.5	179.1	177.8	178.1
5	164.3	162.8	161.8	163.5
6	100.1	99.3	99.7	100.0
7	165.5	164.1	162.4	162.5
8	95.1	95.4	94.5	95.0
9	157.9	158.6	156.7	157.0
10	105.6	106.8	103.2	102.7
1′	121.2	122.9	120.4	120.7
2′	130.5	132.4	115.7	115.1
3′	115.8	116.7	145.6	146.7
4′	161.6	161.3	148.3	149.8
5′	116.7	116.2	115.9	115.4
6′	130.5	131.4	121.0	120.5
		3-*O*-Rhamnose		
1″	102.9	103.4	102.3	101.9
2″	71.0	71.9	71.3	71.7
3″	72.1	72.5	71.9	72.0
4″	72.8	72.9	72.5	72.2
5″	71.2	71.7	71.4	71.1
6″	17.8	18.1	17.5	17.6
		7-*O*-Rhamnose		
1‴		101.3	102.6	
2‴		71.5	71.1	
3‴		72.3	71.9	
4‴		73.0	72.8	
5‴		71.3	70.8	
6‴		17.7	18.0	

to an oxygen free aromatic carbon (C-3′). The presence of α,β-unsaturated ketone moiety in the flavonol ring was confirmed by ^{13}C NMR (**Table 5**) signals at δ 178.5 (C-4), δ156.2 (C-2), and δ 134.7 (C-3). Comparison with the literature data on the NMR of

flavonol aglycones and sugar moieties suggested an L-rhamnose moiety to be attached to C-3. A doublet at δ 0.90 (5.48 Hz) corresponding to a methyl group and one doublet at δ 5.34 (1.8 Hz), in addition to signals at δ 3−4 suggested the presence of the rhamnosyl moiety. The ^{13}C NMR spectrum also showed signals at δ 71.0, 72.1, 72.8, and 71.2 for hydroxymethine carbons, one signal at 17.8 for one methyl carbon, and at δ 102.9 for the anomeric carbon, confirming the presence of rhamnose as the sugar residue linked to the aglycone. Therefore, compound **1** was identified as kaempferol-3-*O*-α-L-rhamnoside on the basis of acid hydrolysis, spectral data (UV, ^{1}H NMR, ^{13}C NMR, and MS), and comparison with published data (*28*, *29*).

Upon acid hydrolysis, compound **2** afforded kaempferol, glucose, and galactose. The appearance of three anomeric proton signals in the ^{1}H NMR spectrum confirmed that compound **2** is a kaempferol triglycoside. Three sugar moieties were identified as two glucoses and one galactose from the ^{13}C NMR spectral data. From the ^{13}C NMR spectrum, the presence of a (1 → 2) linked glucosyl-galactosyl unit was suggested because of a downfield shift of C-2′′ (from 71.4 to 80.0 ppm) and an upfield shift of C-1′′ of galactosyl moiety (from 102.5 to 98.5 ppm) in this structure (*28*, *30*, *31*). The anomeric carbon at δ 98.5 ppm and proton shifts (5.67 ppm, 1H, d, J = 7.7 Hz) of the galactosyl moiety indicated the direct connection of the galactosyl moiety at C-3 of the kaempferol moiety. Comparison of the carbon shifts of the aglycone with those of published data for kaempferol 3-glycosides (*30*, *32*) revealed an upfield shift of C-7 and downfield shifts at C-6, C-8 and C-10 implying glycosylation of C-7 of the aglycones (*33*). The H-6 and H-8 signals in the ^{1}H NMR spectrum supported further evidence for this assignment. An anomeric proton signal in the ^{1}H NMR spectrum was assigned to the glucosyl moiety attached to C-7 position of the aglycone. All data obtained from ^{1}H NMR and agreed well with this assignment. Therefore, compound **2** was established as kaempferol 3-*O*-β-D-glucosyl (1 → 2)-β-D-galactoside 7-*O*-β-D-glucoside, a kaempferol glycoside.

Compound **3** was obtained as light yellow crystals. In the ^{1}H NMR (**Table 4**) spectrum of compound **3**, H-6 and H-8 protons appeared separately as doublets at δ 6.47 (J = 2.4 Hz) and 6.68 (J = 2.4 Hz) ppm. The B ring had four aromatic protons that split into two doublets at δ 7.76 (J = 9.2 Hz) and 6.96 (J = 9.2 Hz). They were assigned to H-2′/H-6′ and H-3′/H-5′. The ^{13}C NMR (**Table 5**) signal at δ 116.7 was assigned to an oxygen free aromatic carbon (C-3′). The signals at δ 157.7 and δ 135.6 were assigned to C-2 and C-3, respectively, of the C ring. The methyl groups of sugar moieties showed doublets at δ 0.94 (3H) and 1.15 (3H). Two anomeric protons, H-1′′ and H-1′′′ were observed at δ 5.38 (1.8 Hz) and 5.49 (1.78 Hz), respectively, as narrow doublets for the α-configuration of the glycosidic linkage. Data obtained from its mass spectra also showed the M^{+} as m/z 577 corresponding to the molecular formula $C_{27}H_{29}O_{14}$. Therefore, flavonoid **3** was identified as kaempferol 3,7-*O*-α-L-dirhamnoside (*34*).

Compound **4** was obtained as a yellow powder. Acid hydrolysis of compound **4** yielded quercetin and rhamnose. In the ^{1}H NMR (**Table 4**) spectrum of compound **4**, meta-coupled aromatic protons appeared as two doublets at δ 6.49 (2.4 Hz) and 6.72 (2.4 Hz), which were clearly attributed to H-6 and H-8 protons, respectively. Doublet signals at δ 7.41 (2.0 Hz) and at δ 6.78 (8.5 Hz) were assigned to H-2′ and H-5′, respectively, whereas H-6′ appeared as a doubled doublet at δ 7.40 (2.0 and 8.5 Hz). Both the ^{1}H NMR (**Table 4**) and ^{13}C NMR (**Table 5**) spectra were comparable with the NMR spectra of the authentic sample as well as the published data (*26*, *27*, *30*, *35*). The secondary methyl groups of sugar moieties showed doublets at δ 0.96 (3H) and 1.11 (3H). H-1′′ and H-1′′′ rhamnosyl protons were observed at δ 5.31 (J = 1.4 Hz) and 5.42 (J = 1.83 Hz), respectively. These data are in agreement with the reported literature values (*36*). Thus, flavonoid **4** was identified as quercetin 3,7-*O*-α-L-dirhamnoside.

Compound **5** was obtained as a yellow amorphous powder. It released quercetin, glucose, and galactose upon complete acid hydrolysis. The ^{1}H and ^{13}C NMR spectral data confirmed the

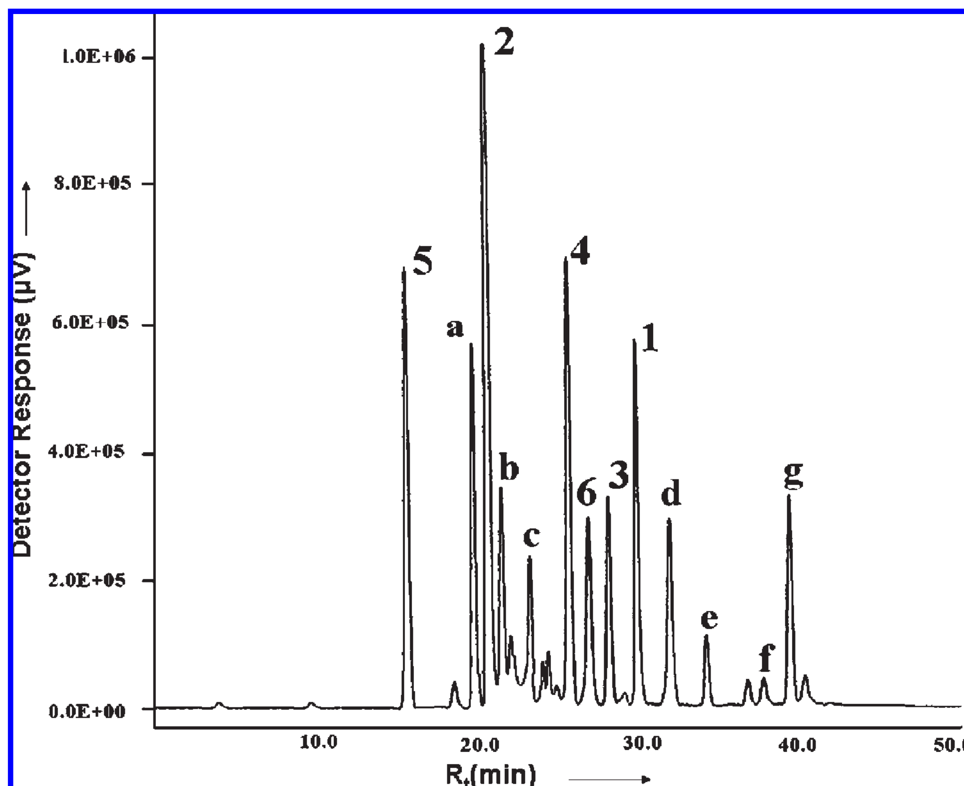

Figure 4. HPLC profile of the 80% aq. methanol extract obtained from fenugreek seeds (refer to **Table 6** for peak numbers and letters).

Table 6. Retention Time and Composition of Major Flavonoids and Their Glycosides Identified in the Fenugreek Seeds Obtained from the HPLC Profile

compounds	R_t (min)	amount (mg/kg)
quercetin 3-*O*-β-D-glucosyl (1 → 2)-β-D-galactoside 7-*O*-β-D-glucoside (**5**)	15.74	10.1
vitexin (**a**)	19.78	8.64
kaempferol 3-*O*-β-D-glucosyl (1 → 2)-β-D-galactoside 7-*O*-β-D-glucoside (**2**)	20.75	12.66
kaempferol 3-*O*-glucoside-7-*O*-rhamnoside (**b**)	21.50	5.38
isovitexin (**c**)	23.30	4.28
quercetin 3,7-*O*-α-L-dirhamnoside (**4**)	25.68	10.02
3-*O*-α-L-rhamnosyl quercetin (**6**)	27.01	4.96
kaempferol 3,7-*O*-α-L-dirhamnoside (**3**)	28.08	5.84
kaempferol-3-*O*-α-L-rhamnoside (**1**)	29.78	8.74
quercetin (**d**)	32.44	4.91
luteolin (**e**)	34.15	2.44
kaempferol (**f**)	37.79	1.62
apigenin (**g**)	39.01	28.9

presence of a quercetin molecule in the structure. The ^{1}H and ^{13}C NMR spectral data of the sugar moieties of **5** resembled those of compound **2**. Carbon shifts of the aglycone showed typical 3,7-disubstitution of quercetin as in compound **3** (*30*, *35*). Thus, the structure of compound **5** was established as quercetin 3-*O*-β-D-glucosyl (1 → 2)-β-D-galactoside 7-*O*-β-D-glucoside, a glycoside of quercetin.(*33*)

Compound **6** was obtained as a yellow amorphous powder. Upon acid hydrolysis, compound **6** yielded quercetin and rhamnose. The ^{1}H NMR spectrum (**Table 4**) of compound **6** showed two doublets at δ 6.79 (8.4 Hz) and δ 7.38 (2.0 Hz), and one doubled doublet at δ 7.41 (2.0 and 8.5 Hz), which were assigned to H-5′, H-2′, and H-6′, respectively, suggesting the presence of a 3,4-dioxygenated B ring of the flavonoid. The ^{13}C NMR spectrum (**Table 5**) showed signals at δ 146.7 and 149.8 assigned to quarternary aromatic carbons C-3′ and C-4′, respectively, and signals at δ 115.1, 115.4, and 120.5, which were assigned to the methine aromatic carbons C-2′, C-5′, and C-6′, respectively. This confirmed the 3,4-dioxygenated pattern of B ring. The remaining signals in the ^{1}H and ^{13}C NMR spectra had chemical shifts similar to those of compound **1**. From the comparison of the spectral data with reported literature values, compound **6** was identified as 3-*O*-α-L-rhamnosyl quercetin (*37*).

Figure 4 illustrates the HPLC profile of 80% aq. methanol extract, while the nature of the constituents identified is presented in **Table 6**. Flavonoids and their glycosides were found to be the major constituents in the 80% aq. methanol extract. Thus, the role of flavonoids in contributing to the ALP activities was established. Among the constituents identified in this study, compounds **a**, **b**, **c**, **d**, **e**, **f**, and **g** have already been reported in fenugreek. Compounds **1**−**6** are reported here for the first time to occur in fenugreek seeds. Compounds **4**−**6** demonstrated higher antioxidant activity compared to that of compounds **1**−**3** (**Table 7**). Flavonoids with *O*-dihydroxyl or vicinal-trihydroxyl groups, including quercetin, myricetin (flavonol), luteolin (flavone), and (−)-epigallocatechin gallate (EGCG; flavanol), were demonstrated to have the ability to prevent the formation of malondialdehyde (*38*). The antioxidant activity of flavonoids depends on the structure and substitution pattern of the hydroxyl groups. The essential requirement for effective radical scavenging is a 3,4-orthodihydroxy configuration in the B ring and a 4-carbonyl group in the C ring (*39*) . The presence of 3,4-orthodihydroxy substitution in the B ring of quercetin explains the higher antioxidant activities of compounds **4**−**6**. The anti-oxidative property of curcumin in preventing lipid oxidation has been demonstrated earlier (*40*, *41*). The mechanism involved a

Table 7. Anti-Lipid Peroxidation Capacities of the Flavonoids and their Glycosides Isolated from Fenugreek Seeds

compounds	peroxidation inhibition (%)
kaempferol-3-*O*-α-L--rhamnoside (**1**)	15.3
kaempferol 3-*O*-β-D-glucosyl (1 → 2)-β-D-galactoside 7-*O*-β-D-glucoside (**2**)	16.1
kaempferol 3,7-*O*-α-L-dirhamnoside (**3**)	15.7
quercetin 3,7-*O*-α-L-dirhamnoside (**4**)	35.2
quercetin 3-*O*-β-D-glucosyl (1 → 2)-β-D-galactoside 7-*O*-β-D-glucoside (**5**)	33.4
3-*O*-α-L-rhamnosyl quercetin (**6**)	39.9

chain-breaking reaction at the 3′-position of the curcumin with the lipid, followed by a subsequent intramolecular Diels−Alder reaction. The phenolic and methoxy groups on the benzene rings and the 1,3-diketone system are the two important structural features that contribute to its antioxidant properties. Several other studies have also demonstrated the role of the above compounds in preventing oxidative deterioration of lipids. Thus, the presence of flavonoids and curcuminoids in fenugreek and turmeric, respectively, could account for the stability of the lipid fraction of the above spices during radiation processing.

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