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PROTECTION OF BIOMOLECULES AGAINST *IN VITRO* OXIDATIVE DAMAGE BY THE ANTIOXIDANTS FROM METHANOLIC EXTRACT OF *TRIGONELLA FOENUM- GRAECUM* SEEDS

Anita Dua*[1], S. Vats [2], V. Singh [3] and Ritu Mahajan [4]

Department of Biochemistry [1], University College, Kurukshetra University, Kurukshetra- 136119, Haryana, India
Institute of Pharmaceutical Sciences [2], Kurukshetra University, Kurukshetra- 136119, Haryana, India
Department of Zoology, University College [3], Kurukshetra University, Kurukshetra- 136119, Haryana, India
Department of Biotechnology [4], Kurukshetra University, Kurukshetra- 136119, Haryana, India

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Correspondence to Author:

Dr. Anita Dua

Associate Professor, Department of Biochemistry, University College, Kurukshetra University, Kurukshetra- 136119, Haryana, India

E-mail: anitadua2012@gmail.com

ABSTRACT: Fenugreek (*Trigonella foenum-graecum*) seeds were extracted in 80% methanol to examine the content of various potent antioxidant compounds and their influence on *in-vitro* oxidation of biomolecules i.e. amount. proteins, lipids and DNA. Fenugreek extract had polyphenols (9.47±0.10 mg GAE/g dry seeds) as major antioxidant principle. Gallic acid (170.335µg), caffeic acid (164.550 µg), ellagic acid (184.879 µg) and quercetin (215.814 µg)/g dry weight fenugreek seeds were identified in methanolic extract by HPLC analysis. Other antioxidant compounds ascorbate, tocopherol and riboflavin were present in very low amounts. Antioxidant properties were assayed using DPPH free radical scavenging activity. Fenugreek extract inhibited the metal induced oxidation of proteins and lipids. Presence of the extract could protect DNA against H_2O_2 -induced oxidative stress. IC_{50} value of fenugreek seeds was estimated by these mechanisms. The results of this study indicate that polyphenol rich methanolic extract of fenugreek had efficient free radical scavenging, reducing and metal chelating activity to protect biomolecules like proteins, lipids and DNA against oxidative stress.

INTRODUCTION: Reactive oxygen species (ROS) including free radicals such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH^-) and non-free radical species such as H_2O_2 and singlet ($O_2^{\cdot}$) are generated in body continuously as by product of various essential processes like energy generation, phagocytosis and detoxification reactions [1].

Under normal circumstances, our healthy natural defense system can neutralize these ROS. However, overproduction of ROS from either mitochondrial electron transport chain or excessive stimulation of NAD(P)H or from exposure to environmental pollutants, UV radiations, toxic chemicals, cigarette smoke or stress generating current life style, results in weakened body defense system [2].

ROS can cause nucleic acid mutation, protein oxidation and lipid peroxidation, contributing to the development of various diseases like atherosclerosis, inflammation, neurodegenerative diseases, cataract, cancer and ageing [1-3].

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Antioxidants are the compounds that can scavenge the free radicals or break the chain reaction due to their redox properties. Process of free radical production and their propagation is induced by the metals like iron and copper, therefore, chemical compounds with metal chelating properties also exhibit antioxidant character [3]. Constant supply of antioxidant phytochemicals through dietary supplements can prevent, delay or ameliorate many of the free radicals and can increase the antioxidant capacity [2, 3].

Naturally occurring antioxidants in leafy vegetables, fruits and seeds such as ascorbic acid, vitamin E and phenolic compounds have ability to reduce oxidative damage associated with many diseases including cancer, cardiovascular diseases [3], cataract [4], arthritis and diabetes [5].

Fenugreek (*Trigonella foenum-graecum*) is a widely used herb belonging to the family Leguminosae grown in Asia and Middle Eastern countries. Seeds of the herb are known as insulin stimulating agent and galactagogue. Use of fenugreek seeds can inhibit cholesterol absorption and can lower sugar level, therefore used as a traditional remedy for the treatment of diabetes and hypercholesterolemia in Indian and Chinese medicines [6, 7]. It is reported to have restorative and nutritive properties and to stimulate digestive processes, useful in healing of different ulcers in digestive tract [6].

Fenugreek has also been reported to exhibit pharmacological properties as antitumor, antiviral, antimicrobial, anti-inflammatory, hypotensive and antidiabetic agent [5-8]. Aqueous fenugreek extract is reported to prevent liver damage under alcohol induced hepatotoxicity [6] and apoptosis [9]. Although extracts of various herbs, fruits, vegetables and spices including fenugreek have exhibited free radical scavenging and metal binding activity [8-13] but no comprehensive study on the level of antioxidants in fenugreek seeds and their influence on the damage caused to biomolecules by oxidative stress is reported.

Since the plant sources are known to contain various antioxidants like ascorbic acid, riboflavin, tocopherols and phenolics, which may vary in the mechanism of action or may work in synergetic manner, the present study was undertaken to identify and quantify the possible active antioxidant principles in fenugreek seeds and to study their influence on the oxidative damage induced in biomolecules.

MATERIALS AND METHODS: The fenugreek (*Trigonella foenum-graecum*) seeds were procured from the local market, identified and authenticated at Department of Botany, Kurukshetra University, Kurukshetra. Caffeic acid, ellagic acid, ferulic acid, quercitin, kaempferol, thiobarbituric acid, bovine serum albumin and calf thymus DNA were purchased from Sigma Chemical Co.MO USA. Diphenyl-picrylhydrazyl (DPPH), 5, 5`-Dithiobis-(2-Nitrobenzoic acid) (DTNB), Acetonitrile, gallic acid, folin-ciocalteau reagent and methanol were purchased from Hi-media, Mumbai, India. All other chemicals and solvents used were of analytical grade.

Extraction: Fenugreek seeds were dried at 60^0C in hot air oven till constant weight is attained. Finely powdered fenugreek seeds were extracted with 80% aqueous methanol (1g/10ml) in a shaker at room temperature for 4 hrs. Residue was again extracted with 80% methanol for 2 hrs. Collected extract was filtered through double layered muslin cloth followed by centrifugation at 5000g for 5 min to get clear supernatant. Extract was concentrated in a vacuum evaporator and stored at -20^0C for further use. The extract was diluted appropriately for different experiments.

Estimation of Antioxidants:

1. **Ascorbate:** Ascorbate was estimated by oxidation of dehydroascorbate followed by coupling with 2, 4 dinitrophenylhydrazine under controlled conditions to give red colored osazones [14]. Fenugreek extract was diluted in 5% metaphosphoric acid in presence of 10% stannous chloride. Equal volume of 2% thiourea in 5% HPO_3 was added and incubated at 37^0C for 6 hrs to complete osazone formation. Contents were shifted to ice bath and 5ml of 85% H_2SO_4 was added slowly. After 30 min, the absorbance was read at 540 nm against reagent blank. A calibration curve of ascorbic acid (1-20 µg/ml) was prepared. To determine total ascorbic acid, reduced ascorbate was first oxidized by adding bromine water.

2. **Riboflavin:** To analyze riboflavin content, the extract was diluted to 10 ml with 0.2 M acetate buffer, pH 4.0. One to two drops of caprylic alcohol was added, followed by addition of 3 ml of freshly prepared 4% potassium permanganate solution. Mixture was stirred and within 2 min, 3 ml of H_2O_2-water solution (1:1) was added and pH adjusted to 7.0 with NaOH. The solution was filtered and the fluorescence by the filtrate is measured at 530 nm with excitation at 470 nm using fluorescence spectrophotometer [14]. Standard riboflavin (1 µg/ml) was used for calibration.

3. **Tocopherol:** Tocopherol was extracted from the extracts with saturated potassium hydroxide and hexane. Hexane layer was collected and evaporated under nitrogen. The contents were dissolved in ethanol. To one ml of ethanolic extract, 0.2 ml of 2% bathophenanthroline was added in dark. The contents were mixed thoroughly and 0.2 ml of ferric chloride reagent was added rapidly. After 1 min, 0.2 ml of 0.01M phosphoric acid (prepared in alcohol) was mixed and read at 534 nm. Standard DL-tocopherol (1-10 µg) was treated in same way to prepare a calibration curve.

4. **Total Polyphenols:** Total phenolic content of the methanolic fenugreek extract was estimated by folin-Ciocalteau method [15]. Aliquot of the extract was mixed with 2ml of sodium carbonate (2%) and after 2 min, 100µl of folin reagent (1N) was added for colour development, which was read at 750 nm after 30 min. Standard curve with gallic acid was used to express results as mg gallic acid equivalent (GAE)/g dry fenugreek seeds.

Qualitative and quantitative analysis of polyphenolic compounds using HPLC: The methanolic extract was defatted with n-hexane. The defatted extract was treated with 2 N HCl to hydrolyze glycosidic bonds. The extract dried and again dissolved in methanol, was subjected to HPLC for qualitative and quantitative analysis of free phenolic contents. The HPLC system (Agilent Technologies Company) was equipped with dual lamp binary system, UV detector, Eclipse XDB-C18 column (i.d.4.6 mm x 150 mm, 5 µm) and data was integrated by Agilent Chem Station software. Standards and sample extract were analyzed using the following gradient program (A,100% acetonitrile; B,2.5% HPLC grade water) 0 min, 5%A: 10 min, 15%A: 20 min, 25%A: 30 min, 35%A: 40min, 45%A: 50 min, 55%A. Flow rate was 0.5 ml/min and injection volume was 10 µl. Detection was done at 280 nm. Peak area (280 nm) of the sample is an index of the amount of component and the retention time of individual peaks is used to identify polyphenols by comparing with standard polyphenols; caffeic acid, ellagic acid, ferulic acid, quercetin and kaempferol.

Estimation of Antioxidant activity:

1. **DPPH radical scavenging activity**: Different dilutions of the fenugreek extract were incubated with 1 ml of DPPH solution ($50x10^{-5}$M) in a final volume of 1.1 ml. The decrease in absorbance due to the scavenging of DPPH radicals by the extract was recorded at 517 nm [15]. The percentage of remaining DPPH after 5 min with different dilutions of extract was calculated and the concentration at which 50% of the initial DPPH could be scavenged was noted from the graph.

2. **Lipid peroxidatoin inhibition:** Lipid peroxidation inhibition was monitored as the amount of malonaldehyde (MDA) produced by copper induced egg lecithin peroxidation [14]. Different dilutions of the fenugreek extract were added to the reaction mixture containing 2.5 mM lecithin and 250 mM $CuCl_2$ in 50 mM Tris-HCl buffer (pH 7.4), in a total volume of 1ml. After incubation at 37^o C for 15 min, malonaldehyde produced was monitored as thiobarbituric acid reacting substances by adding 2 ml of TBA reagent containing 0.37 % TBA, 15% TCA, 0.04% BHT and 2% ethanol. Mixture was heated at 100^0C for 15 min and centrifuged at 3000 rpm for 10 min. The absorbance of supernatant at 535 nm is an index of malonaldehyde concentration. The graph plotted is used to note the concentration inhibiting 50% of lipid peroxidation.

3. **Protein oxidative modification:** Oxidative modification in albumin was induced by copper in presence and absence of different dilutions of fenugreek extract. In brief, the reaction mixture containing albumin (10 mg/ml) and 100 mM $CuCl_2$ in 50 mM Tris–HCl buffer (pH

7.4) in a total volume of 0.3 ml, was incubated at 37°C for 2 hrs in the absence and presence of different concentrations of the extract. After incubation, 1.6 ml of 0.125 M phosphate buffer (pH 8.0) containing 12.5 mM EDTA plus 10.0 M urea and 0.1 ml of 50 mM phosphate buffer (pH 7.0) containing 10 mM DTNB were added. The absorbance was recorded at 412 nm as an index of cysteine-SH residue [14]. Percent inhibitory ratio was calculated as follows:

% inhibition = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$

4. **Protection of DNA against oxidative damage:** Hydroxyl radicals generated by Fenton's reaction were used to induce oxidative damage to DNA [15.] The reaction mixture (9 µl) containing 3 µg of calf thymus DNA in 20.0 mM phosphate buffer saline (pH 7.4) and different concentrations of fenugreek extract (0.5, 1.0 and 1.5 µg) was preincubated for 15 min at ambient temperature. The oxidation was induced by incubating DNA with 1.0 mM $FeSO_4$ and 10.0 mM ascorbic acid for 1 hour at 37°C. The reaction was terminated by the addition of loading buffer (Xylene cyanol, 0.25%; bromophenol blue, 0.25% and glycerol, 30%). The mixture was subjected to gel electrophoresis in 1.5% agarose/TAE buffer run at 60V. DNA was visualized by UV-transilluminator (Genei) system and photographed by Chemidoc (Biorad) to assess the damage by H_2O_2 and protection by fenugreek extract.

Statistical analysis: The statistical analyses were performed with the statistical software SPSS/Windows (SPSS 10.0.LNK). The results were expressed as the means ± SEM to show variations in a group. Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION: Herbs, spices and condiments are an integral part of human diet to impart flavor, taste and color to the food. Several healthy benefits of consumption of a number of such spices and herbs including a digestion stimulating action, a hypolipidemic effect, antidiabetic influence, presence of antimutagenic, anti-inflammatory, anticarcinogenic and antioxidant potential have been reported [3,7].

Traditional use of spices and herbs in various clinical situations related to oxidative stress indicates their pharmaceutical and medicinal potential. Various secondary metabolites and phytochemicals can inhibit the production and propagation reactions of ROS or terminate these reactions when present in small amounts. In the present studies, fenugreek seed extract was analyzed for possible antioxidant principles. The capability of methanolic extract to protect various biomolecules against oxidative damage was examined to find out the probable mechanism of interaction of the antioxidants from fenugreek seeds with cellular components.

Antioxidants: Ascorbate, riboflavin, tocopherol and polyphenols have redox potential high enough to scavenge or terminate ROS [2]. To understand the mechanism of action of antioxidants in fenugreek seeds, the seed extract was analyzed for the presence of these metabolites. The fenugreek seeds had low methanol extractable ascorbate, riboflavin and tocopherol (**Table 1**).

Total ascorbate and tocopherol are 99.97±4.62 µg and 22.0±1.54 µg/ g dry seeds, whereas riboflavin is only 0.115±0.04 µg/g dry seeds. Methanolic extract of fenugreek had considerable amount of polyphenols 9.470±0.10 mg GAE/g dry seeds. Much less yield of 5-6 mg polyphenols/g dry seeds is reported with polar solvents like ethanol [10], whereas more phenolics could be extracted from fenugreek seeds with distilled water than polar solvents [13].

TABLE 1: CONCENTRATION OF VARIOUS ANTIOXIDANT COMPOUNDS IN FENUGREEK EXTRACT

Antioxidant compound	Amount (µg/g dry wt. seeds)
Total Ascorbate	99.97±4.62
Reduced Ascorbate	36.61±2.63
Oxidized Ascorbate	63.63 ±2.23
Riboflavin	0.115 ±0.04
Tocopherol	22.0 ±1.54
Total Polyphenols	9.470 ±0.10*
Gallic acid	170.335
Caffeic acid	164.550
Ellagic acid	184.879
Quercetin	215.814

*mgGAE/g

Polyphenolic flavonoids are reported to be the active antioxidant constituent of the aqueous and methanolic extracts of herbs including fenugreek [9-13].

Polyphenols have good antioxidant potential both as free radical scavenger and inhibitor of metal induced oxidation [2, 3]. Polyphenols can contribute as metal ion chelators due to the presence of various hydroxyl radicals. The π electron cloud of one or more benzene rings makes them suitable as antioxidants. Polyphenolic compounds are usually present as glycosides in plant sources. The extract was hydrolyzed with 2N HCl to break glycosidic bonds before analysis by HPLC. Polyphenols extracted from fenugreek seeds were characterized qualitatively and quantitatively by HPLC (**Table 1**).

The identification of polyphenols was done by comparing retention time of the peaks with that of standard compounds. Gallic acid, caffeic acid, ellagic acid and quercetin could be identified in fenugreek seed extract by HPLC. Quantification of the identified compounds was achieved by comparing the peak area of individual compound with that of standards (2 ng/10 µl). Methanol extract of fenugreek seeds had 170.335 µg gallic acid, 164.550 µg caffeic acid, 184.879 µg ellagic acid and 215.814 µg quercetin/g seeds.

Antioxidant activity of the polyphenols increases with the number of hydroxyl groups and the density of π electron cloud. Gallic acid among the simple phenolics and quercetin among flavonoids have most potent antioxidant activity [14].

DPPH radical scavenging activity: DPPH is a stable free radical which can absorb an electron or hydrogen to become a stable diamagnetic molecule. Scavenging of DPPH free radicals by the plant extracts is assayed to estimate the reducing power or hydrogen/electron donating character of the antioxidants. The extract exhibited a concentration dependent elimination of DPPH free radicals in the reaction mixture (**Fig. 1**).

Methanolic extract equivalent to 5 mg or more of fenugreek seeds caused 78 % eradication of free radicals in the reaction mixture. IC_{50} of the fenugreek seeds is 3.6 mg for DPPH free radical scavenging activity. These results indicate that antioxidants in fenugreek seeds are effective electron or hydrogen donors and this activity contributes to the antioxidant capacity of fenugreek seeds. Tocopherols and polyphenols are reported to contribute reducing properties and antioxidant characteristics in different herbs and spices.

A positive correlation between the DPPH radical scavenging activity and polyphenol content of the chloroform extracts of various spices has been reported [12].

Methanol and aqueous extracts of various plant sources are reported to have better free radical scavenging activity than dichloromethane or ethyl acetate extracts indicating the polar nature of the antioxidant biomolecules [13.] DPPH scavenging activity of fenugreek seed extracts in ethanol and methanol are correlated to the total polyphenolic content of the extracts [10]. High polyphenol content, with quercetin in the fenugreek extract seems to be the major free radical scavenger in the present study.

Lecithin peroxidation inhibition: Polyunsaturated lipids are susceptible to oxidative attack by free radicals and reactive oxygen species, causing the onset and propagation of oxidative chain reactions. Lipid oxidation brings about chemical changes leading to the spoilage of fats and fatty acids of foods. Membrane lipids of cells have polyunsaturated fatty acids so are more prone to oxidation. Damage to the membrane lipids can affect permeability and hence various processes related to membrane integrity such as apoptosis, autogenesis and carcinogenesis [2, 3].

Metal ions such as iron and copper can induce oxidation of lipids leading to the production of peroxy radicals. Peroxy radicals propagate chain reaction and generate ROS, which can further accelerate lipid oxidation. Malonaldehyde produced by copper induced oxidation of egg lecithin in presence and absence of different dilutions of fenugreek extract was determined as thiobarbituric acid reactive substances (**Fig. 2**).

In controls, 19.35±0.32 nmoles of MDA was produced and the production of MDA was reduced to 5.223±0.45 nmoles in presence of the extract equivalent to 5 mg of the fenugreek seeds. IC_{50}, the amount of fenugreek seeds required to inhibit the lipid peroxidation to 50%, calculated from the curve is 2.0 mg.

The results indicate that antioxidants from this herb are efficiently preventing the oxidation of lipids induced by metals either by metal chelation or by inhibiting the propagation reactions being hydrogen/electron donor.

Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the transition metal catalyzing lipid peroxidation.

Lower IC_{50} for lipid oxidation inhibition than DPPH scavenging indicates that metal chelation in addition to reducing properties of polyphenols is contributing to the antioxidant activity in protecting lipids against oxidation. A decrease in lipid oxidation in liver cells in the presence of fenugreek seeds has been reported [5, 9]. Inhibition of lipid peroxidation by the polyphenols from other plant sources [12, 14, 15] has also been reported.

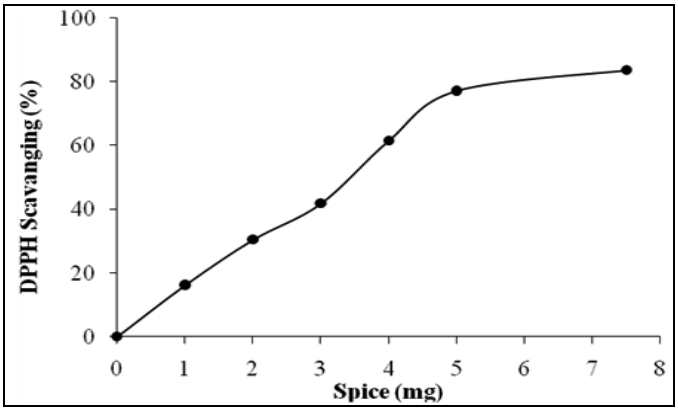

FIG. 1: DPPH FREE RADICAL SCAVENGING IN PRESENCE OF FENUGREEK EXTRACT

Protein oxidation inhibition: Oxidative modifications were induced in BSA by incubation with copper ions in presence and absence of fenugreek extract (**Fig. 3**). The process of oxidative modifications was inhibited by 52.6% in presence of extract equivalent to 10 mg of fenugreek seeds. Presence of extract equivalent to 9.2 mg dry seeds is required to inhibit the metal induced protein oxidation upto 50%.

Sulphydryl groups of the proteins are susceptible to oxidation in presence of ROS generated by metal ions. The side chains of the cysteine residues are oxidized to cystine. The sulphydryl groups play an important role in the structure and function of proteins. Deleterious impact of oxidative stress in biological systems is related to the damage of proteins, enzymes and various transcriptional factors like NFkB and AP-1 [1, 5, 16]. The protection of protein and lipid against oxidative damage by fenugreek extract is an index of metal chelating and reducing property of the antioxidants. Inhibition of metal induced oxidation by the extracts of various herbs has been attributed to the presence of polyphenolic compounds in the extracts. IC_{50} for protection of protein oxidation is higher than that for lipid oxidation, indicating that electron transfer to lipids is more efficient than to proteins.

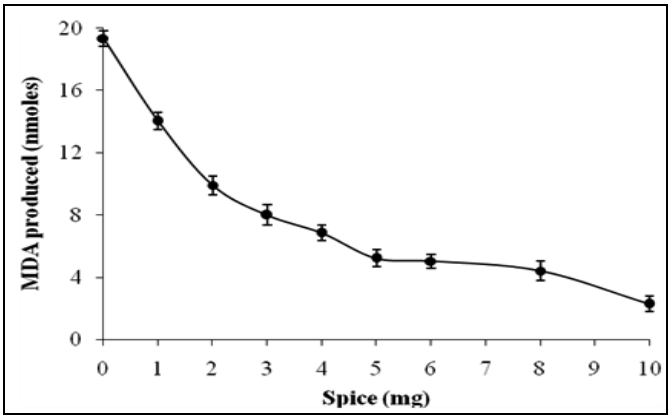

FIG. 2: INHIBITION OF LIPID PEROXIDATION IN PRESENCE OF FENUGREEK EXTRACT

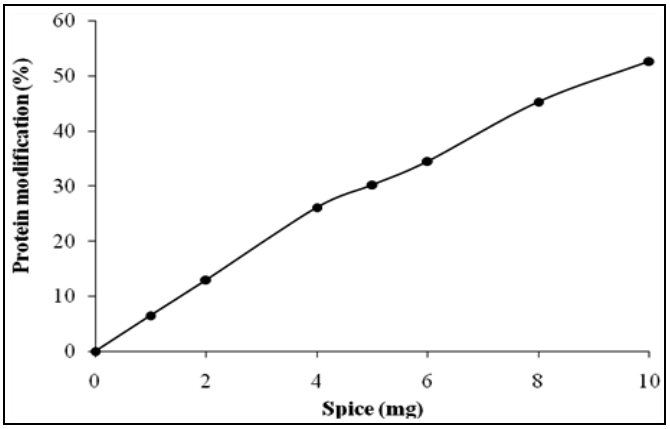

FIG. 3: INHIBITION OF PROTEIN OXIDATION IN PRESENCE OF FENUGREEK EXTRACT

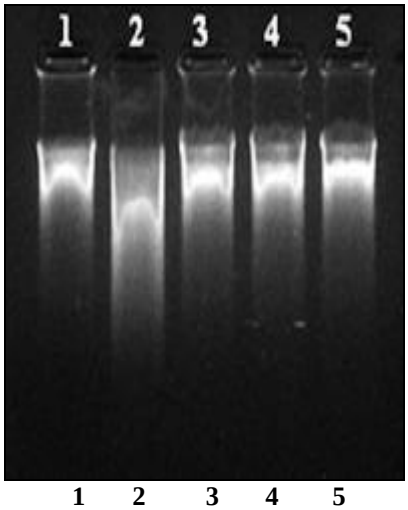

FIG. 4: PROTECTION OF DNA AGAINST OXIDATIVE STRESS BY FENUGREEK EXTRACT; Lane 1- control DNA (1.5 µg); Lane 2- DNA + Fenton's reagent with ascorbic acid; Lane 3- fenugreek (0.5 µg) + DNA + Fenton's reagent with ascorbic acid; Lane 4-fenugreek (1.0 µg) + DNA + Fenton's reagent with ascorbic acid; Lane 5- fenugreek (1.5 µg) + DNA + Fenton's reagent with ascorbic acid.

Protection of DNA against H_2O_2 induced damage: Oxidative stress generated by Fenton`s reaction *in vitro* can cause breaks in calf thymus DNA. Incubation of DNA with $FeSO_4$ and ascorbate has caused damage to DNA and damaged DNA moves to a greater extent in the gel (**Fig. 4**). Presence of extract equivalent to 0.5 µg, 1.0 µg and 1.5 µg fenugreek seeds in the incubation mixture could prevent the damage. This protective impact of the extract indicates that antioxidant formulation from fenugreek seeds can efficiently quench hydroxyl radicals from the reaction mixture. Oxidation of nitrogenous bases of nucleic acids may cause mutations [1-3].

Guanosine is oxidized to hydroxyl-2-deoxyguanosine and thymine is modified to thymine glycol under oxidative stress caused by carcinogens. Protection of DNA and other nucleic acids against oxidative damage by the antioxidants present in the fenugreek seeds can prevent, delay or reduce oxidative stress. Acharya *et al.*, 2011 have also reported an inhibition of DNA damage by fenugreek seed extracts [9].

CONCLUSION: The results of the present study indicate that methanolic extract of fenugreek seeds has polyphenols including gallic acid, caffeic acid, ellagic acid and quercetin, responsible for the high free radical scavenging and metal chelating activity of fenugreek seeds. Antioxidants of the methanolic fenugreek seed extract protect important biomolecules like lipids, proteins and DNA against oxidative stress. Polyphenols from fenugreek seeds are more efficient in protecting lipids than proteins. Natural antioxidants present in fenugreek, as a whole or in extracted form as a component of composite food formulations may be explored in order to stabilize or enrich food with antioxidant properties. Fenugreek seeds, with high efficacy as antioxidant, have great potential to be used as pharmaceutical and nutraceutical agent.

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