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ORIGINAL RESEARCH ARTICLE

A Study of Antioxidant Properties and Antioxidant Compounds of Cumin *(Cuminum cyminum)*

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ABSTRACT

Cumin (*Cuminum cyminum*) seeds were extracted in 80% methanol to examine antioxidant properties and content of various potent antioxidant compounds. Antioxidant properties were assayed using DPPH free radical scavenging, inhibition of metal induced oxidation of proteins & lipids and protection of DNA against H_2O_2- induced oxidative stress. IC_{50} value of cumin was estimated by these mechanisms. Cumin extract had polyphenols (7.45±.10 mg GAE/g dry seeds) as major antioxidant principle. Gallic acid (287.948µg), quercetin (336.413µg) and kaempferol (215.814 µg)/g dry weight cumin seeds were identified in methanolic extract by HPLC analysis. Other antioxidant compounds ascorbate, tocopherol and riboflavin were present in very low amount to account for observed antioxidative properties. The results of this study indicate that polyphenol rich methanolic extract of cumin had efficient free radical scavenging and metal chelating activity to protect biomolecules like proteins, lipids and DNA against oxidative stress.

Key words: Antioxidant, polyphenols, cumin, oxidative stress, free radical scavenging.

INTRODUCTION

Free radicals and other reactive oxygen species (ROS) are continuously produced in our body as byproduct of various essential processes like energy generation, phagocytosis and detoxification reactions. Although human body has a defense mechanism to neutralize the ROS, current life style is causing the over production of free radicals, ROS and decreasing the physiological antioxidant capacity. A shift of this balance in favor of excessive ROS is called oxidative stress. ROS work as second messenger in physiological and pathological pathways [1]. ROS can also 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 [2,3,4]. Antioxidants are the compounds that can delay, inhibit or prevent the oxidation of biomolecules like lipids, proteins or nucleic acids. Antioxidants may scavenge the free radicals or break the chain reaction due to their redox properties[5]. 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[6,7]. In general, there are two categories of antioxidants- natural and synthetic. These days, interest has increased in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are restricted due to their carcinogenicity and toxicity [8]. Naturally occurring antioxidants in leafy vegetables, fruits and seeds such as ascorbic acid, Vitamin E and phenolic compounds have abiliy to reduce oxidative damage associated with many diseases including cancer, cardiovascular diseases, cataract, arthiritis, diabetes[3,9].

Herbs and spices have been used for large purposes including medicine, nutrition, fragrance, charms etc. Cumin (*Cuminum cyminum*) is a widely used spice condiment in India, South East Asia and Arabia. Cumin commonly known as jeera or Kashmiri jeera belongs to the family Apiaceae. Cumin is used in vegetarian and non-vegetarian food preparations as an ingredient of garam masala. Cumin is known for its

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carminative, stimulant, diuretic, emmanogogic, antispasmodic and astringent properties. It is also used in treatment of diarrhea, dyspepsia and jaundice[10]. Various herbs and spices have been reported to have antioxidant properties [6-12]. Polyphenols isolated from cumin have been reported to have antioxidant properties [11]. Sultana et al 2010 have found the methanolic extract of cumin better free radical scavenger than standard ascorbic acid and other spices like coriander, ginger, cardamom and cinnamom[10]. Although extracts of various herbs, fruits, vegetables and spices including cumin have exhibited free radical scavenging and metal binding activity [10,12], but no comprehensive study on the antioxidants in cumin and their biomolecules protective function has been reported yet. Since the plant sources are known to contain various biomolecules like ascorbic acid, riboflavin, tocopherols and phenolics having antioxidant properties, which may vary in the mechanism of action or may work in synergetic manner,the present study was undertaken to study the antioxidant activity of methanolic cumin extract and to identify the possible active antioxidant principles in cumin.

MATERIALS AND METHODS

The Cumin (*Cuminum cyminum*) seeds were procured from the local market, identified and authenticated at Department of Botany, Kurukshetra University Kurukshetra. Caeffic acid, ellagic acid, ferulic acid, quercetin, kaempferol, thiobarbituric acid, Bovine serum albumin, calf thymus DNA were purchased from Sigma Chemical Co.MO USA. Diphenyl-picrylhydrazyl (DPPH), acetonitrile, gallic acid, folin-ciocalteau reagent, methanol was purchased from Hi-media, Mumbai, India. All other chemicals and solvents used were of analytical grade.

Extraction:

Cumin seeds were dried at 60^0C in hot air oven till constant weight is attained. Finely powdered cumin 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 followed by centrifugation at 5000g for 5min 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.

Measurement of antioxidant activity:

DPPH radical scavenging activity:

Different dilutions of the cumin extract were incubated with 1ml of DPPH solution (50x10^{-5}M) in a final volume of 1.1ml. The decrease in absorbance due to the scavenging of DPPH radicals by the spice extract was recorded at 517 nm [11]. 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.

Copper induced egg lecithin peroxidatoin Inhibition:

Lipid peroxidation inhibition was monitored as the amount of malonaldehyde produced by copper induced egg lecithin peroxidation[13]. Different dilutions of cumin extract were added to the reaction mixture containing 2.5mM lecithin and 250 mM $CuCl_2$ in 50 mMTris-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 2ml of TBA reagent containing 0.37 % thiobarbituric acid, 15% trichloroacetic acid, 0.04% butylated hydroxyl toluene and 2% ethanol. Mixture was heated at 100^o C for 15 min and centrifuged at 3000 rpm for 10 min. The absorbance of supernatant at 535 nm is an index of malonaldehyde concentration. Percent inhibition of peroxidation was calculated in presence of different dilutions of spice extract. The graph plotted is used to note the concentration inhibiting 50% of peroxidation.

Copper induced protein oxidative modification:

Oxidative modification in albumin was induced by copper in presence and absence of different dilutions of cumin extract. In brief, the reaction mixture containing albumin (10mg/ml) and 100mM $CuCl_2$ in 50 mMTris–HCl buffer (pH 7.4) in a total volume of 0.3ml was incubated at 37^o C for 2 hr in absence and presence of different concentrations of extract. After incubation, 1.6 ml of 0.125 M phosphate buffer (pH 8.0) containing 12.5mM EDTA plus 10.0 M urea and 0.1ml of 50mM phosphate buffer (pH 7.0) containing 10 mm DTNB were added. The absorbance was recorded at 412nm as an index of cysteine-SH residue [13]. Percent inhibitory ratio was calculated as follows:

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

Protection of DNA against oxidative damage:

Hydroxyl radicals generated by Fenton's reaction were used to induce oxidative damage to DNA[11].The reaction mixture (9 µl) contained

3µg of calf thymus DNA in 20.0 mM phosphate buffer saline (pH 7.4) and different concentrations of cumin extract (0.5, 1.0, 1.5 and 2.0µg) was preincubated for 15 min at ambient temperature. The oxidation was induced by incubating DNA with 1.0 mM FeSO4 and 10.0 mM ascorbic acid for 1 h at 37^{o}C.The reaction was terminated by the addition of loading buffer (xylenecyanol, 0.25%; bromophenol blue, 0.25% and glycerol 30%). The mixture was subjected to gel electrophoresis in 1.5% agarose/ TAE buffer run at 60 V. DNA was visualized and photographed by UV-transilluminator (Geni) system to assess the damage and protection.

Estimation of Antioxidants:

Ascorbate:

Ascorbate was estimated by oxidation of dehydroascorbate followed by coupling with 2,4 dinitrophenylhydrazine under controlled conditions to give red colored osazones[14]. Cumin 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^{o} C 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.

Riboflavin:

To analyze riboflavin content, cumin extract was diluted to 10 ml with 0.2M acetate buffer pH 4.0. One to two drops of caprylic alcohol was added, followed by 3ml of freshly prepared 4% potassium permangnate solution. Mixture was stirred and within 2 min, 3ml 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 470nm using fluorescence spectrophotometer [14]. Standard riboflavin (1 µg/ml) was used for calibration.

Tocopherol:

Tocopherol was extracted from the extract with saturated potassium hydroxide and hexane. Hexane layer was collected and evaporated under nitrogen. The contents were dissolved in ethanol. To one ml of ethanol extract, 0.2ml 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, 0.2ml 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.

Total Polyphenols:

Total phenolic content of the methanolic extract of cumin was estimated by folin-Ciocalteau method [11].Aliquot of the extract was mixed with 2ml of sodium carbonate (2%) and after 2 min, 100µl of folin reagent (IN) was added for colour development, which was read at750 nm after 30 min .Standard curve with gallic acid was used to express results as mg gallic acid equivalent (GAE) / g cumin seeds.

Qualitative and quantitative analysis of Polyphenolic compounds using HPLC:

The methanolic extract was defatted with n-hexane. The defatted extract was treated with 2N HCl to hydrolyse 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.4.6mm x 150mm,5µm) and data was integrated by Agilent ChemStation software. Standards and sample extract were analysed using the following gradient program (A,100% acetonitrile B,2.5% HPLC grade water) 0min 5%A: 10 min 15%A: 20 min 25%A: 30 min 35%A: 40min 45%A: 50 min 55%A. Flow rate was 0.5ml/min and injection volume was 10µl. Detection was done at 280 nm. Peak area (280nm) 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.

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

Although spices and herbs have been added to foods since ancient times to impart, improve or modify their flavors, their antioxidant properties have added to their applications. Traditional use of spices and herbs in various clinical situations related to oxidative stress indicates their pharmaceutical and medicinal potential. Antioxidants are the chemical moieties which inhibit the production and propagation reactions

of ROS or terminate these reactions when present in small amounts. In the present studies, antioxidant activity of cumin extract was examined by various methods using different mechanisms;DPPH free radical scavenging activity, copper induced lecithin peroxidation, copper induced cysteine oxidation in BSA, peroxide induced damage to DNA. Cumin extract was further analysed for the presence of various possible antioxidant principles.

Antioxidant Activity:

DPPH radical scavenging activity:

Scavenging of DPPH free radicals by the antioxidants in cumin seed extract was determined as decrease in optical density of the reaction mixture. DPPH is a stable free radical which can absorb an electron or hydrogen to become a stable diamagnetic molecule. Cumin extract exhibited a concentration dependent elimination of DPPH free radicals form the reaction mixture (**Fig1**). Methanolic extract equivalent to 5 mg or more of cumin seeds caused 78 % eradication of free radicals in the reaction mixture. IC_{50} of the cumin seeds is 2.3mg for DPPH free radical scavenging activity. These results indicate that antioxidants in cumin seeds are effective electron or hydrogen donors and this activity contributes to the antioxidant capacity of cumin 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 tocopherol content of the chloroform extracts of various spices has been reported [15]. Polyphenols isolated from cumin scavenge free radicals even more effectively than BHA and ascorbate[10,11,12]. 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[16].

Lecithin peroxidation inhibition:

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 susceptible to oxidation. Damage to the membrane lipids can affect permeability and hence various processes related to membrane integrity such as apoptosis, autogenesis and carcinogenesis [4]. 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 cumin extract was determined as thiobarbituric acid reactive substances (**Fig2**). In controls, 19.35±.32nmoles of MDA was produced and the production of MDA was reduced to 6.37±.25 nmoles in presence of the extract equivalent to 4 mg of the cumin seeds. IC_{50}, the amount of cumin required to inhibit the lipid peroxidation, calculated from the curve is2.8 mg. The results indicate that antioxidants from this spice are efficiently preventing the oxidation of lipids induced by metals either by metal chelation or by inhibiting the propagation reactions being hydrogen/electron donor. A decrease in lipid oxidation in presence of cumin [11], parsley [17]and coriander[18] extracts has been reported. This activity has been attributed to the metal chelating property of the polyphenols of the extracts of spices and herbs [12,18]. Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the transition metal catalyzing lipid peroxidation.

Protein oxidation inhibition:

Deleterious impact of oxidative stress in biological systems is related to the damage of proteins, enzymes [19] and various transcriptional factors like NFkB and AP-1[20].BSA was subjected to oxidative modifications by incubation with copper ions in presence and absence of cumin extract(**Fig3**). Although little effect was observed with lower concentrations of seed extract, the process of oxidative modifications was inhibited by 80% in presence of extract equivalent to 10 mg of cumin seeds. Presence of extract equivalent to 4.8 mg spice is enough to inhibit the metal induced protein oxidation upto 50%.The protection of protein and lipid against oxidative damage by cumin extract is an index of metal chelating property of the antioxidants. Methanolic extracts of various herbs and spices are reported to exhibit metal chelating activity comparable to EDTA. Cumin extract inhibited metal induced oxidation to 50% at 200 ppm concentration [12]. Inhibition of metal induced oxidation by the extracts of coriander leaves has been attributed to the presence of polyphenolic compounds in the extracts[18].

Protection of DNA against H_2O_2 induced damage:

Oxidation of nitrogenous bases of nucleic acids may cause mutations [21]. Guanosine is oxidized to

hydroxyl 2 deoxyguanosine and thymine is modified to thymine glycol under oxidative stress caused by carcinogens. 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 (**Fig4**). Presence of extract equivalent to 0.5 µg and 1.0 µg cumin in the incubation mixture could prevent the damage. This protective impact of the extract indicates that antioxidant formulation from cumin seeds can efficiently quench hydroxyl radicals from the reaction mixture.

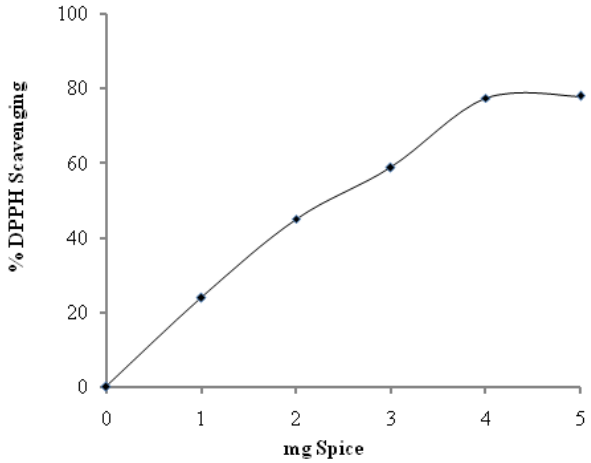
Antioxidants:

Ascorbate, riboflavin, tocopherol and polyphenols have redox potential high enough to scavenge or terminate ROS [5,6]. 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. To understand the mechanism of action of antioxidants in cumin seeds, the seed extract was analyzed for the presence of these biomolecules known to have antioxidant activity.

The Cumin seeds had low methanol extractable ascorbate, riboflavin, tocopherol (**Table 1**). Total ascorbate and tocopherol are 188.67±2.32µg and 54.0±2.51 µg/ g dry seeds, where as riboflavin is only 1.25 µg/ g dry seeds. Methanolic extract had considerable amount of polyphenols 7.446+.10 mg GAE/g dry cumin seeds. Polyphenols have good antioxidant potential both as free radical scavenger and inhibitor of metal induced oxidation [7]. Polyphenolic flavanoids are reported to be the active antioxidant constituent of the aqueous [17]and methanolic extracts of herbs including Cumin [11,12,22].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 cumin were characterized qualitatively and quantitatively by HPLC (**Fig 5 and Table 1**). The identification of polypheols was done by comparing retention time of the peaks with that of standard compounds. Gallic acid, quercetin and kaempherol could be identified in cumin seed extract by HPLC. Quantification of the identified compounds was achieved by comparing the peak area of individual compound with that of standards (2ng/10µl).Methanol extract of cumin had 287.948 µg gallic acid, 336.413µg quercetin and 215.814µg kaempferol/g cumin 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 the flavanols have most potent antioxidant activity [6,7].

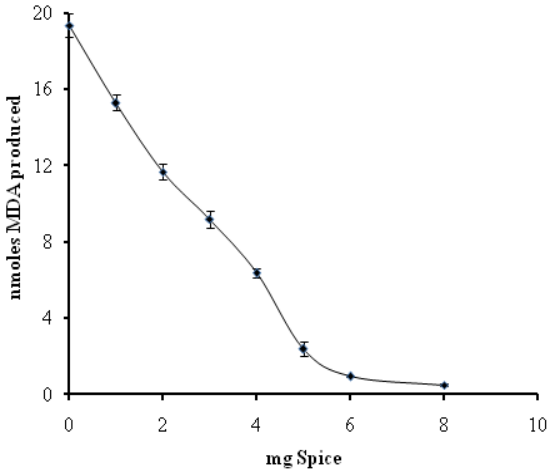
The results of the present study indicate that polyphenols including gallic acid, quercetin and kaempferol are responsible for the high free radical scavenging and metal chelating activity of cumin. Cumin seeds with high efficacy as antioxidant have great potential to be used as pharmaceutical and neutraceutical agent. Use of natural antioxidants occurring in cumin, as a whole or in extracted form as a component of composite food formulations in order to stabilize them or enrich in antioxidant properties may be explored.

Fig 1: DPPH free radical scavenging in presence of cumin extract



DPPH Free radical scavenging activity in presence of extract equivalent to different amounts of cumin seeds is expressed as % decrease in OD at 512 nm .OD of the reaction mixture in absence of extract is used as control i.e. 100%.

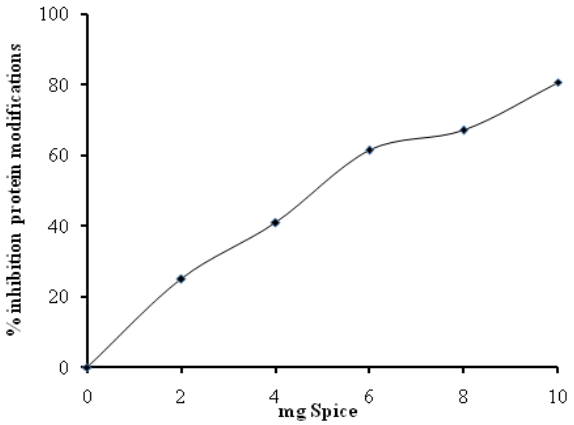
Fig 2: Inhibition of lipid peroxidation in presence of cumin extract



Inhibition of metal induced lipid oxidation in presence of extract equivalent to different amounts of cumin seeds is expressed as

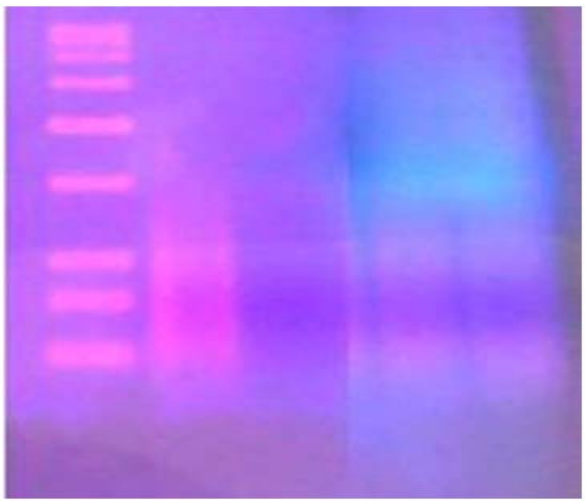
decrease in malonaldehyde production as compared to control (in absence of extract).

Fig. 3 Inhibition of protein oxidation in presence of cumin extract



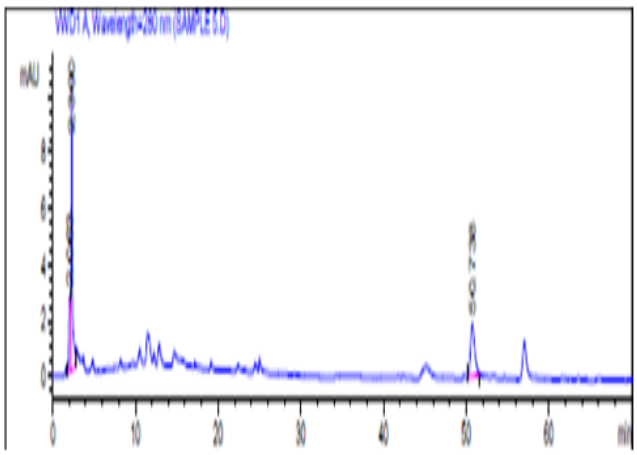
Inhibition of metal induced modifications in BSA in presence of extract equivalent to different amounts of cumin seeds is expressed as % decrease in OD at 412nm as compared to control (in absence of extract).

Fig 4: Protection of DNA against oxidative stress by cumin extract



Protection of DNA from H_2O_2 induced damage in presence of extract equivalent to different amounts of cumin seeds; Lane 1-DNAmarker; 2-control DNA(1.5µg); 3-DNA+Fenton's reagent with ascorbic acid; 4-cumin(0.5µg)+DNA+Fenton's reagent with ascorbic acid; 5-cumin(1.0µg)+DNA+Fenton's reagent with ascorbic acid.

Fig 5: HPLC analysis of polyphenolic compounds of cumin extract



HPLC analysis of the cumin extract; peak at retention time 2.300, 50.738 and 57.474 min are identified as gallic acid, quercetin and kaempferol respectively.

Table 1: Amount of Various Antioxidant Compounds in Cumin Extract

Antioxidant compound	Amount (µg/g dry wt. seeds)
Total Ascorbate	188.67±2.32
Reduced Ascorbate	68.99±5.63
OxidisedAscorbate	119.68 ±5.51
Riboflavin	1.25 ±0.04
Tocopherol	54.0 ±2.51
Total Polyphenols	7.446 ±0.10*
Gallic acid	287.948
Quercetin	336.413
Kaempferol	215.814

**mg/g; Values = mean ± SD*

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