BIOREACTIVE COMPOUNDS AND ANTIOXIDANT PROPERTIES OF METHANOLIC EXTRACT OF FENNEL (*FOENICULUM VULGARE* MILLER)

Anita Dua^{1*}, Mittal Ashwani¹, Gupta Sanjeev², Ritu Mahajan³

¹Department of Biochemistry, University College, Kurukshetra University, Kurukshetra, India

²Department of Zoology, University College, Kurukshetra University, Kurukshetra, India

³Department of Biotechnology, Kurukshetra University, Kurukshetra, India

*Corresponding Author Email: anitadua2012@gmail.com

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ABSTRACT

Methanolic extract of dried Fennel (*Foeniculum vulgare* Miller) seeds was analyzed for the presence of bioreactive compounds: ascorbate, riboflavin, tocopherol and polyphenols with potential antioxidant properties. The extract had low ascorbate ($197.12 \pm 1.82 \mu\text{g}$), riboflavin ($11.97 \pm 0.35 \mu\text{g}$) and tocopherol ($280.33 \pm 5.67 \mu\text{g/g}$ dry seeds) content. However the extract had high polyphenol content ($16.506 \pm 0.32 \text{mg/g}$ dry seeds). Antioxidant activity of the extract was determined by various mechanisms including DPPH free radical scavenging, metal induced protein and lipid oxidation inhibition and protection of DNA against H_2O_2 induced damage. Fennel had excellent free radical scavenging activity with IC_{50} 2.1mg dry seed weight. IC_{50} observed for protection of proteins and lipids against metal ion induced oxidation is 2.1 and 2.5mg dry seed weight respectively. Extract equivalent to 0.5 μg seeds is enough to protect DNA against H_2O_2 induced oxidation. The results suggest that polyphenols are the principal components responsible for high antioxidant potential of methanolic extract of fennel.

Key words: fennel, polyphenols, antioxidant, radical scavenging.

INTRODUCTION

Active oxygen species and free radical-mediated reactions can cause damage to various biomolecules like proteins, lipids and DNA. ROS are highly reactive chemical species playing an important role in the degenerative or pathological processes such as aging^{1,2}, cancer, coronary heart disease³ and Alzheimer's disease⁴. Moreover, there are many epidemiological surveys revealing an association between the people, who have a diet rich in fresh fruits and vegetables and a decrease in the risk of cardiovascular diseases and certain forms of cancer⁵. There were also reports concerning natural compounds like phenolics in fruits, vegetables⁵, herbs and spices⁶ for their antioxidant activities. The bioreactive compounds can inhibit, prevent or delay the oxidation reaction by preventing the initiation and propagation of chain reaction or by scavenging free radicals and terminating chain reaction. Ascorbate, riboflavin, tocopherol and polyphenols are some bioreactive compounds, which can scavenge or terminate ROS⁷. The phenolics are common to many plants, and have evolved as antibacterial and antioxidant agents against environmental stress due to a variety of oxidizing and potentially harmful free radicals. Consumer awareness and concerns over the potential risks of synthetic food additives to human health have renewed the interests in using naturally occurring alternatives. As a consequence, the market of healthy and herbal nutraceuticals constantly addresses its attention to new plant sources offering functional efficacy. Fennel (*Foeniculum vulgare* Miller), a perennial herb belongs to the family Apiaceae. It is highly aromatic and flavorful herb with culinary and medicinal uses. Fennel fruit, a dry seed, is traditionally used as anti-inflammatory, analgesic, carminative, diuretic and antispasmodic agent⁸. Fennel is used in treatment of glaucoma⁸, galactoaquoque and hypertension⁹. Recently there has been considerable interest in the antioxidant potential of fennel seed extracts and essential oil¹⁰⁻¹², but the detail study on the level of bioreactive compounds with antioxidant potential and

protection of biomolecules against oxidation by them has not been reported so far. The purpose of the present study was to determine the concentration of potent bioreactive antioxidants and evaluate the antioxidant effectiveness of the methanol extract of fennel seeds. The present study was undertaken to investigate the antioxidant properties of fennel to evaluate its potential as nutraceutical from natural source.

MATERIALS AND METHODS

Fennel (*Foeniculum vulgare* Miller) seeds were procured from the local market, identified and authenticated at Department of Botany, Kurukshetra University, Kurukshetra, India. Thiobarbituric acid, bovine serum albumin, calf thymus DNA and 5, 5-Dithio-bis (2- nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Company, USA. Diphenyl-picrylhydrazyl (DPPH), 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

Fennel seeds were dried at 50°C in hot air oven till constant weight is attained. Finely powdered seeds were extracted with 80% methanol (1g/10ml) in a shaker at room temperature for 4hrs. Residue was extracted with 80% methanol again for 2hrs. Collected extract was filtered through double layered muslin cloth followed by centrifugation at 5000g for 5min in order to get clear supernatant. Extract was concentrated in a vacuum evaporator and stored at -20°C for further use. The extract was diluted appropriately for different experiments.

Estimation of Bioreactive compounds

Ascorbate was estimated by oxidation of dehydroascorbate followed by coupling with 2, 4 dinitrophenylhydrazine under controlled conditions to give red colored osazones¹³. Fennel 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°C for 6hrs. Contents were shifted to ice bath and 5ml of 85% H_2SO_4 was added slowly. The absorbance was read at 540nm after 30min. A calibration curve of ascorbic acid (1-20 $\mu\text{g}/\text{ml}$) was prepared. To determine total ascorbic acid, reduced ascorbate was first oxidized by adding bromine water. To analyze riboflavin content, fennel extract was diluted to 10ml with 0.2M acetate buffer pH4.0. One to two drops of caprylic alcohol was added, followed by 3ml of freshly prepared 4% potassium permanganate solution. Mixture was stirred and within 2min, 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 was measured at 530 nm with excitation at 470nm. Standard riboflavin (1 $\mu\text{g}/\text{ml}$) was used for calibration purpose. Tocopherol was extracted 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, 0.2ml of 2% bathophenanthroline was added in dark. The contents were mixed thoroughly and 0.2 ml of ferric chloride reagent was added rapidly. After 1min, 0.2ml of 0.01M phosphoric acid (prepared in alcohol) was mixed and read at 534nm. Standard DL tocopherol (1-10 μg) was treated in the same way for preparing the calibration curve. Total phenolic content of the methanolic extract of fennel was estimated by folin-ciocalteau method¹⁴. Aliquot of the extract was mixed with 2ml of sodium carbonate (2%) and after 2min; 100 μl of folin reagent (IN) was added for colour development, which was read at 750nm after 30min. Standard curve with gallic acid was used to express results as mg gallic acid equivalent (GAE) / g fennel seeds.

Measurement of DPPH radical scavenging activity

Different dilutions of the fennel extract were incubated with 1ml of DPPH solution ($50 \times 10^{-5}\text{M}$) in a final volume of 1.1ml as described by Ani *et al*¹⁴. The decrease in absorbance due to the scavenging of DPPH radicals by the extract was recorded at 517nm. The percentage of remaining DPPH after 5min was calculated and the concentration at which 50% of the initial DPPH could be scavenged was noted from the graph.

Measurement of copper induced egg lecithin peroxidation inhibition

Lipid peroxidation inhibition was monitored as the amount of malonaldehyde produced by copper induced egg lecithin peroxidation¹⁵. Different dilutions of the fennel extract were added to the reaction mixture containing 2.5mM lecithin and 250mM CuCl_2 in 50mM Tris- HCl buffer (pH 7.4) in a total volume of 1ml. After incubation at 37°C for 15min, malonaldehyde produced was monitored by adding 2ml of TBA reagent containing 0.37% thiobarbituric acid (TBA), 15% trichloroacetic acid (TCA), 0.04% butylated hydroxyl toluene (BHT) and 2% ethanol. Mixture was heated at 100°C for 15min and centrifuged at 3000rpm for 10min. The absorbance of supernatant at 535nm is an index of malonaldehyde concentration. The graph plotted is used to note the concentration inhibiting 50% of peroxidation.

Measurement of inhibition of copper induced protein oxidative modification

Oxidative modifications in albumin were induced by copper in presence and absence of different dilutions of fennel extract as described by Dua *et al*¹⁵. In brief, the reaction

mixture containing albumin (10mg/ml) and 100mM CuCl_2 in 50mM Tris-HCl buffer (pH 7.4) in a total volume of 0.3ml was incubated at 37°C for 2hr in absence and presence of different concentrations of extract. After incubation, 1.6ml of 0.125M phosphate buffer (pH 8.0) containing 12.5mM EDTA plus 10.0M urea and 0.1ml of 50mM phosphate buffer (pH 7.0) containing 10mM 5,5-Dithio-bis(2-nitrobenzoic acid) (DTNB) were added. The absorbance was recorded at 412nm as an index of cysteine-SH residue. Percent inhibitory ratio was calculated as follows;

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

Measurement of antioxidant activity against oxidative damage to DNA

Hydroxyl radicals generated by Fenton's reaction were used to induce oxidative damage to DNA¹⁴. The reaction mixture (9 μl) contained 3 μg of calf thymus DNA in 20.0mM phosphate buffer saline (pH 7.4) and different concentrations of the extract (0.5, 1.0 μg) was pre incubated for 15min at ambient temperature. The oxidation was induced by incubating DNA with 1.0mM FeSO_4 and 10.0mM ascorbic acid for 1h at 37°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 system and run at 60V. DNA was visualized and photographed by using Chemidoc (Biorad) system to assess the damage and protection.

Statistical analysis

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

RESULTS AND DISCUSSION

Antioxidants are the chemical moieties which inhibit the production and propagation reactions of ROS or terminate these reactions when present in small amounts. Ascorbate, riboflavin, tocopherol and polyphenols have redox potential high enough to scavenge or terminate ROS⁷ and in turn get reduced by enzymatic system of cells. 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. The fennel seed extract was analyzed for the presence of these biomolecules, known to have antioxidant activity. The fennel seed extract had total ascorbate 197.12 μg , tocopherol 280.33 μg and riboflavin only 11.97 $\mu\text{g}/\text{g}$ dry seeds. Reduced ascorbate is 35% of the total ascorbate (Table 1). Extract of fennel seeds in 80% methanol had comparatively higher amount of polyphenols 16.506 $\pm$ 0.32mg GAE/g dry weight of seeds. Polar solvents are better medium for polyphenol extraction. Comparatively lower amount of polyphenols (2mg GAE/g dry seeds) are extracted with water than 9mg GAE/g dry seeds with ethanol¹⁰. Anwar *et al*¹¹ have reported that methanol and ethanol extracts of fennel seeds had polyphenols 6.27 and 9.67mg GAE/g dry seeds. Higher amount of polyphenols in methanol extracts is reported as compared to other solvents like ethanol, diethyl ether and hexane¹². Higher amount of polyphenols could be extracted from fennel seeds using 80% methanol in the present study. Amount of polyphenols extracted depends upon the polarity of medium as well as time and mode of extraction.

Table 1: Bio-reactive Compounds of Fennel

Compound	Amount ($\mu\text{g/g dry wt.}$)
Oxidized Ascorbate	133.76 ± 3.15
Reduced Ascorbate	63.36 ± 4.36
Total Ascorbate	197.12 ± 1.82
Riboflavin	11.97 ± 0.35
Tocopherol	280.33 ± 5.67
Total Polyphenols	$16.506 \pm 0.32^*$

*(mg/g dry wt.)

Figure 1: DPPH Free radical scavenging activity in presence of extract equivalent to different amounts of fennel seeds

Figure 2: Inhibition of metal induced lipid oxidation in presence of extract equivalent to different amounts of fennel seeds

Figure 3: Inhibition of metal induced modifications in BSA in presence of extract equivalent to different amounts of fennel seeds

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

Polyphenolic compounds are usually present as glycosides in plant sources. 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 has most potent antioxidant activity⁷. Presence of high amount of polyphenols indicates high efficacy of fennel seeds as an antioxidant.

Antioxidant activity

Scavenging free radicals such as hydroxyl or superoxide radicals and terminating chain reaction, chelating metal ions and inhibiting ROS production, donating electrons or hydrogen to terminate chain reactions are some of the ways by which antioxidants reduce oxidation. Various antioxidant bioreactive compounds vary in their mechanism of action. The antioxidant activity of a natural source is generally related to either of these activities or as a synergist. Tocopherols and polyphenols act as primary antioxidants while ascorbic acid may regenerate oxidized primary antioxidants reductively. Synergism between various antioxidants has been reported¹⁶. Fennel seed extract was examined for antioxidant activity by the methods based on different principles i.e. DPPH free radical scavenging, copper induced lecithin peroxidation, copper induced cysteine oxidation in BSA and peroxide induced damage to DNA.

DPPH radical scavenging activity

Scavenging of DPPH by the antioxidants in fennel 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. Fennel extract exhibited a concentration dependent elimination of DPPH free radicals (Figure 1). Methanolic extract corresponding to 5mg of fennel seeds caused scavenging of 78% of the free radicals in the reaction mixture. IC_{50} of the herb is 2.1mg for DPPH free radical scavenging activity. Decrease in DPPH radicals to 36.45% and 47.49% with 250µg of aqueous and ethanol extract of fennel seeds respectively has been reported¹⁰ although BHA and BHT could scavenge 95% free radicals at this concentration. These results indicate that antioxidants in fennel seeds are effective electron or hydrogen donors and this activity contributes to the antioxidant capacity of fennel seeds. A positive correlation between the radical scavenging activity and polyphenol content of the chloroform extracts of various spices has been reported¹⁷. Polyphenols from other herbs and spices are also reported to have DPPH free radical scavenging activity¹⁸. Although more polyphenols can be extracted with ethanol than methanol, but DPPH free radical scavenging activity of both extracts are comparable¹¹.

However, Roby et al have reported that methanolic extracts are more efficient in free radical scavenging than ethanol, diethyl ether or hexane extracts¹².

Copper induced egg lecithin peroxidation inhibition

Metal ions such as iron and copper can induce oxidation of lipids leading to the production of peroxy radicals, which in turn propagate chain reaction and generate ROS, to accelerate lipid oxidation. 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 carcinogenesis¹⁹. Malonaldehyde produced by copper induced oxidation of egg lecithin in presence and absence of different dilutions of fennel extract was determined as thiobarbituric acid reactive substances (Figure 2). In controls, 19.35 ± 0.32 nmol of MDA was produced and the production of MDA was reduced to 3.907 ± 0.21 nmol in presence of the extract equivalent to 6mg of the fennel seeds. IC_{50} calculated from the curve is 2.1mg. Linoleic acid oxidation was inhibited to 70% when 5mg of seed extract in 80% ethanol is added to the reaction mixture, where as similar amount of BHT has caused 92% inhibition of the process¹¹. Oktay *et al*¹⁰ have reported 91-99% inhibition of lipid peroxidation by aqueous and ethanol extract of fennel, which is comparable to BHA and BHT. 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 oxidation catalyzing transition metal in lipid peroxidation. This activity has been attributed to the metal chelating property of the polyphenols of the extracts of spices and herbs¹⁶⁻¹⁸.

Inhibition of copper induced protein oxidative modification

Deleterious impact of oxidative stress in biological systems is related to the damage of proteins, enzymes²⁰ and various transcriptional factors like NFkB and AP-1²¹. Oxidation of the sulphhydryl groups of cysteine to cystine may cause changes in the structure and functions of the proteins. These proteins may be enzymes, hormones or components of immune system. BSA was subjected to oxidative modifications by incubation with copper ions in presence and absence of fennel extract (Figure 3). A concentration dependent increase in inhibition of protein oxidation was

observed with fennel extract. The process of oxidative modifications was completely inhibited in presence of extract equivalent to 6mg of fennel seeds. Presence of extract equivalent to 2.5mg herb seeds is enough to inhibit the metal induced protein oxidation up to 50%, which is comparable to IC₅₀ for copper induced lecithin oxidation and DPPH scavenging property of the extract. Due to the lack of data on the percent inhibition of protein oxidation by fennel seed extract, we could not compare the results of our study with literature. However, the protection of protein and lipid against oxidative damage by fennel extract is an index of metal chelating and reducing property of fennel. Methanolic extracts of various herbs and spices are reported to exhibit metal chelating activity comparable to EDTA¹⁸.

Antioxidant activity against H₂O₂ induced damage to DNA

Hydroxyl radicals can oxidize and damage DNA and RNA in cells causing mutations²². Guanosine is oxidized to hydroxyl-2-deoxyguanosine and thymine is modified to thymine glycol under oxidative stress caused by carcinogens¹. Oxidative stress generated by Fenton's reaction can cause breaks in calf thymus DNA. Incubation of DNA with FeSO₄ and ascorbate has caused damage to DNA and damaged DNA moves to a greater extent in the gel (Figure 4). Presence of extract equivalent to 0.5µg and 1.0µg fennel in the incubation mixture could prevent the damage. This protective impact of the extract indicates that antioxidant formulation from fennel seeds can efficiently quench and remove hydroxyl radicals from the reaction mixture.

Concerns over the potential risks of synthetic food additives to human health and consumer awareness have directed the interests in using naturally occurring antioxidants. The market of health and herbal nutraceuticals is addressing its attention to rich plant sources offering functional efficacy. The present study revealed that fennel seed is a rich source of natural antioxidant principle. Polyphenol rich methanol extract of fennel seeds exhibited good free radical scavenging and 100% protection of lecithin, protein and DNA against metal ion induced oxidation and peroxidation. Fennel seeds can play an important role as antioxidant agent and it has good potential to be applied in the food and pharmaceutical industry. Possible use of fennel seeds as an antioxidant nutraceutical needs to be explored further.

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