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Evaluation of antioxidant, oxidative DNA damage protective and antimicrobial activities of *Foeniculum vulgare* plant

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The potential health benefits of *Foeniculum vulgare* (FV), commonly known as fennel, is related to the treatment of cardiovascular disease, digestive disorders, carminative, diuretic, etc. The present study was carried out to evaluate *F. vulgare* plant as new source of natural bioactive molecules having antioxidant potential and DNA damage protection activity caused by reactive oxygen species (ROS). Among ethanol, methanol, hexane, chloroform, butanol, acetone and aqueous extracts, ethanol extract of mature *F. vulgare* plant leaf showed the highest total phenolic content (TPC; 44.11 mg of gallic acid equivalent (GAE)/g) and the lowest inhibitory concentration (IC_{50}) for free radical scavenging activity (FRSA) (1.95 mg/ml), superoxide anion radical scavenging activity (SARSA) (7.02 mg/ml) and hydroxyl radical scavenging activity (23.53 mg/ml). Methanol and hexane extracts had the lowest IC_{50} for lipid peroxidation (LPO) (9.68 mg/ml) and ferric thiocyanate assay (FTC) (0.92 mg/ml), respectively. Ethanol extracts showed the highest reducing power (RP, 1.22 ascorbic acid equivalent (ASE)/ml) proving itself to be the best solvent. In further studies, the antioxidant activity at early and mature stages of plant was compared. Ethanol extract of mature *F. vulgare* leaf had the highest TPC and maximum percentage inhibition for FRSA, SARSA, RP, LPO, FTC, hydroxyl radical scavenging activity and had maximum protection against DNA damage in pBR322 plasmid. Results showed that *F. vulgare* is a rich source of many biomolecules having antioxidant activities and showed potential utility of mature plant for use in herbal drug system or as nutritional supplement.

Key words: Total phenolic content, antioxidant, reducing power, herbal drug.

INTRODUCTION

Reactive oxygen species (ROS), the bye products of cellular metabolism, can damage biomolecules like DNA, RNA, enzymes, lipid, carbohydrate and consequently may adversely affect the immune function (Dhakarey et al., 2005; Shukla et al., 2009; Singh et al., 2009a). Over the past twenty years, interest in the medicinal plants has grown enormously for the use of herbal products and for self medication by the general public (Maffei, 2003). According to World health Organization (WHO), more than 80% of the world population rely on plant based herbal medicine for their primary health care product (Shanmugasunderam, 2005). The overproduction of ROS like hydroxyl radicals, superoxide anion radicals and hydrogen peroxide can contribute to oxidative stress (Braca et al., 2002). Natural antioxidants or phytochemical antioxidants such as polyphenolics, carotenoids, terpenoids, flavonoids and vitamins E and C, are the secondary metabolites of plants and have the potential to neutralize free radical to overcome oxidative stress (Singh et al., 2009b,c). At present, most of the

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available antioxidants are manufactured synthetically. They belong to the class of synthetic antioxidants. The main disadvantage with the synthetic antioxidant is its side effects caused by the intermediates present (Ramamoorthy and Bono, 2007).

Foeniculum vulgare (FV; Family: Umbelliferae) plant is commonly known as fennel and used in Indian houses as home spices and remedies. *F. vulgare* plant is an annual, biennial or perennial aromatic herb, that grows wild in the Mediterranean area and in the Asia minor, but is commonly cultivated in the US and Europe. Fruits, oil and root powder are widely used in Ayurveda as cardiac tonic, brain tonic and to improve sight (www.tradekey.com/brochure/1001-5193395-4/.pdf). In recent years, there has been a global trend towards the use of natural phytochemicals present in fruits, vegetables, oil seed and herbs as antioxidant and functional food (Lee et al., 2002). Fennel seeds, an ancient spice of Indian kitchen, are used as a flavoring agent in many herbal medicines, and to help open obstruction of the liver, spleen and gall bladder. Fennel seeds are commonly chewed after meals, as a mouth freshener to prevent gas and upset stomach. It also helps to stimulate lactation in females and stimulate spermatogenesis in males (Annida et al., 2005). The studies reported in the literature are not sufficient to arrive at a definite conclusion as far as their remedial powers in terms of DNA damage protection as well as hepatoprotective activity are concerned. Also, the bioactive phytochemicals have not yet been fully identified. In view of increasing awareness about side effects of synthetic medicines, there is need to find out some safe alternative drug/nutraceuticals which may be used as nutritional supplement in our day-to-day life in one or the other form.

In the proposed investigation, an attempt has been made to select a solvent which may extract maximum phytochemicals having antioxidant activity. Organic solvents have different polarity and nature to extract the compounds. Earlier, the concentration of phytochemicals (vitamin C, carotenoids, total phenolic, protein, carbohydrate content) in *F. vulgare* plant was reported and its parts is greatly influenced by degree of development (Singh et al., 2010). To ascertain these results, *F. vulgare* plant parts were further explored for its total phenolics, free radical scavenging assay (FRSA), superoxide anion radical, reducing power (RP), lipid peroxidation (LPO), ferric thiocyanate assay (FTC) and OH* radical scavenging activity determinations. The bioactive compounds were also detected along with the DNA damage protective activities of the plant parts.

MATERIALS AND METHODS

Plant samples were collected from vegetable growing areas of Eastern Uttar Pradesh at two developmental stages of the plant. Plant parts of early stage (E) were collected after one and half month from the seedling stage and mature plant (M) parts were collected at fruiting stage. Collected plant samples were washed under running tap water, dried, in sun shade, powdered in a grinder and stored in polythene bags at 4°C. The plant identification was confirmed by National Botanical Research Institute (NBRI), Lucknow, India. The voucher specimens were also deposited at institute.

Chemicals and reagents

Gallic acid, quercetin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, St. Louis, USA. β-carotene, ascorbic acid, Folin Ciocalteau's phenol reagents were the product of E-Merk, Mumbai, India. Nitro blue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride and sodium dodecyl sulphate were purchased from SRL, India. All other reagents and chemicals used were of analytical grade.

Extraction procedure

The different extracts of mature *F. vulgare* leaf were prepared with 20 g of dried plant sample and solvents such as ethanol, methanol, hexane, butanol, chloroform, acetone and water by continuous extraction until decoloration to evaluate and compare the total phenolic content (TPC) and antioxidant activity of different solvent extracts. Ethanol showed better extractable properties hence it was selected to compare the antioxidant activities of *F. vulgare* plant parts. The extracted solvent was evaporated at 40°C in a vacuum rotary evaporator and lyophilized till dryness. The powdered form of plant extracts were stored at -4°C and used for the antioxidant activity determination.

Antioxidant studies

TPC

TPC of powdered plant material was extracted with 50% methanol+1% HCl, filtered and made up to 10 ml each with water. TPC was measured with the method of Ragazzi and Veronese (1973). To 0.1 ml plant extract, 0.5 ml of Folin's reagent (1 N) and 1.0 ml of sodium carbonate was added subsequently. The test mixture was mixed properly and kept at room temperature for 30 min and volume was made up to 12.5 ml with distilled water. The absorbance of this solution was measured at 720 nm. The TPC was reported as mg of gallic acid equivalent (GAE)/g of dry weight (DW).

FRSA

FRSA of the extracts was measured by using DPPH stable radical according to the method of Yen and Duh (1994). Each extract (0.1 ml) was added to freshly prepared DPPH solution (6×10^{-5} M in HPLC grade 2.9 ml methanol) and mixed vigorously. The reduction of the DPPH radical was measured by continuous monitoring of the decrease in absorbance at 515 nm until a stable value was obtained.

Inhibition (%)= [(Blank absorbance-sample absorbance)/blank absorbance] × 100

The inhibitory concentration (IC_{50}) which represents the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, representing a parameter widely used to measure the

antioxidant activity, was calculated from a calibration curve by linear regression. EC_{50} was calculated as IC_{50} (mg/ml)/concentration of DPPH/ml and expressed as mg/mg_{DPPH}. For rational reasons of clarity, the ARP was determined as the reciprocal value of the EC_{50}, representing a comparable term for the effectiveness of antioxidant and radical scavenging capacity:

$ARP = 1/EC_{50} \times 100$

The larger the ARP, the more efficient the antioxidant.

Superoxide anion radical scavenging activity (SARSA)

This assay was based on the capacity of the extract to inhibit the reduction of NBT by the method of Nishikimi et al. (1972). Three milliliters reaction mixture containing different aliquot of plant extracts (50, 100, 150 and 200 µl) with 0.1 M phosphate buffer (pH 7.8), 60 µM PMS, 468 µM nicotinamide adenine dinucleotide reduced (NADH) and 150 µM NBT was incubated for 5 min at ambient temperature. Absorbance was read after 6 min at 560 nm using UV-Vis spectrophotometer. The percentage inhibition of superoxide generation was measured by comparing the absorbance of the control and those of the reaction mixture containing test sample.

RP

RP of the extracts was determined by using slightly modified method of ferric reducing-antioxidant power assay (Apati et al., 2003). Each extract (1.0 ml) was mixed with 2.5 ml of phosphate buffer (0.1 M, pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide and was incubated at 50°C for 20 min. After completion of incubation period, 2.5 ml of 10% (w/v) TCA was added to terminate the reaction. The upper layer (2.5 ml) was diluted with equal volume of deionized water. Finally, 0.5 ml of 0.1% (w/v) $FeCl_3$ was added and after 10 min the absorbance was measured at 700 nm against a blank. RP was expressed as ascorbic acid equivalents (1 ASE = 1 mM ascorbic acid). The ASE value is inversely proportional to RP.

LPO assay

A modified thiobarbituric acid-reactive species (TBARS) assay method of Ohkawa et al. (1979) was applied to measure the LPO formation, using egg homogenate as lipid rich media. Egg homogenate (10% in 0.2 M PBS, 0.5 ml), test extract (0.1 ml) and deionized water (0.85 ml) were mixed in a test tube. Finally, $FeSO_4$ (0.07 M, 0.05 ml) was added to the reaction mixture and incubated at 37°C temperature for 30 min to induce LPO. Thereafter, acetic acid (20%, 1.5 ml), TBA (0.8% prepared in 1.1% sodium dodecyl sulphate, 1.5 ml) and TCA (20%, 0.05 ml) were added, vortexed and then heated in a boiling water bath for 60 min. After cooling, butanol (5 ml) was added to each tube and centrifuged for 10 min at 3000 rpm. The absorbance of the organic upper layer was measured at 532 nm by UV-Vis Spectrophotometer.

FTC assay

The reaction mixture containing 400 µl of different concentration of ethanolic plant extracts, 200 µl of diluted linoleic acid (25 mg/ml in 99% ethanol) and 400 µl of 50 mM phosphate buffer (pH 7.4) was incubated for 15 min at 40°C. A 100 µl aliquot of this was then mixed with a reaction mixture containing 3 ml of 70% ethanol, 100 µl of ammonium thiocyanate (300 mg/ml in DW) and 100 µl of ferrous sulphate. Red color developed was measured at 535 nm (Tsuda et al., 1994).

Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by a mixture of Fe^{3+}-EDTA, H_2O_2 and ascorbic acid and assessed by monitoring the degraded fragments of deoxyribose, through malondialdehyde (MDA) formation (Halliwell et al., 1987). The reaction mixtures contained ascorbic acid (50 µM), $FeCl_3$ (20 µM), EDTA (2 mM), H_2O_2 (1.42 mM), deoxyribose (2.8 mM) with different concentrations of the plant extracts in a final volume of 1 ml, was incubated at 37°C for 1 h and then 1 ml of 2.8% TCA (w/v in water) and 1 ml of 1% TBA (w/v) were added. The mixture was heated in a boiling water bath for 30 min. It was cooled and absorbance was taken at 532 nm.

DNA damage assay

The DNA damage assay was performed using supercoiled pBR322 plasmid DNA according to the method of Lee et al. (2002) with some modifications. Reaction mixture containing 10 µl of plant extract of different concentrations (25 and 50 µg/ml), pBR322 DNA (0.25 µg) and 10 µl Fenton's reagent (30 mM H_2O_2, 500 µM ascorbic acid and 800 µM $FeCl_3$) was incubated for 40 min at 37°C and analyzed on 0.9% agarose gel by staining with ethidium bromide.

Antimicrobial activity assay

Antibacterial activities of the plant extracts were tested using well diffusion method of Bauer et al. (1996). The prepared culture plates were inoculated with selected strains of bacteria using streak plate method. Wells were made on the agar surface with 6 mm cork borer. The extracts were poured into well using sterile syringe. Plates were incubated at 37±2°C for 24 h for bacterial activity. The plates were observed for the zone clearance around the wells. Zone of inhibition was calculated by measuring the diameter of the inhibition zone around well (mm) including the well diameter.

HPLC analysis

The dried extracts were dissolved in HPLC grade methanol (1.0 mg/ml), filtered through sterile 0.22 µm millipore filter and subjected to qualitative and quantitative analysis by using Shimadzu LC-10A (Kyoto, Japan) HPLC instrument. The instrument was equipped with a dual-pump LC-10AT binary system (Shimadzu, Kyoto, Japan) HPLC, a UV detector SPD-10A (Shimadzu, Kyoto, Japan), and a Phenomenex Luna RP, C_{18} column (4.6×250 mm). Data were integrated by Shimadzu Class VP series software. Separation was achieved with an acetonitrile/water containing 1% acetic acid linear gradient program, started with 18% acetonitrile, changing to 32% in 15 min and finally to 50% in 40 min. Results were obtained by comparison of peak areas (λmax = 254 nm) of the samples (mg/100 g dry extract) with that of standards (Prakash et al., 2007a).

Statistical analysis

Statistical analysis was done using prism software. Values from *in vitro* antioxidant activities were reported as mean±standard deviaition (SD) of three determinations. The r^2 value and regression equation were calculated through plotting graph between TPC on x-axis and antioxidant deciding parameters on y axis with the help MS office excel 2007.

Table 1. Total phenolic content of mature FV leaf in different solvent extracts.

Plant sample	Crude amount (g)	TPC (mg/g of GAE of DW)
Ethanol	7.02±0.01	44.11±0.50
Methanol	5.90±0.01	17.81±0.49
Hexane	3.24±0.01	37.11±1.73
Water	5.16±0.01	25.75±1.73
Chloroform	0.38±0.01	ND
Butanol	0.30±0.01	ND
Acetone	0.52±0.01	ND

Values are mean ± SD (n=3); TPC: Total phenolics content; GAE: gallic acid equivalent; DW: dry weight; ND: not detected.

Table 2. Free radical scavenging activity (FRSA) and super oxide anion radical scavenging activity of FV leaf (M) in different solvents.

Solvent extract	$^{1}IC_{50}$	EC_{50}	ARP	$^{2}IC_{50}$
Ethanol	1.95±0.03	84.92±1.32	1.17±0.02	7.02±0.33
Methanol	2.23±0.05	97.24±0.25	1.02±0.05	36.29±0.59
Hexane	41.80±0.34	1817.53±14.28	0.05±0.01	38.98±1.53
Butanol	8.41±0.52	365.94±22.89	0.27±0.01	164.23±3.29
Acetone	36.21±0.44	1574.63±19.16	0.06±0.01	535.34±2.70
Water	ND	ND	ND	ND
Chloroform	90.02±1.65	3809.41±12.12	0.02±0.01	27.55±2.70
Quercetin (Std)	0.04±0.01	1.51±0.05	65.96±1.96	2.33±0.05

Values are mean ± SD (n=3); $^{1}IC_{50}$: FRSA; $^{2}IC_{50}$: Superoxide anion radical scavenging activity; IC_{50}: In mg/ml; EC_{50}: In mg/mg of DPPH; ARP: Antiradical power.

RESULTS

TPC

In different solvents tested, the highest value of TPC was observed in ethanolic extract (44.11 mg/g of GAE) and the lowest value was found in the methanolic extract (17.81 mg/g of GAE), whereas chloroform, butanol and acetone extracts did not show the presence of phenolics (Table 1). The decreasing order of TPC in different solvent was: ethanol>hexane>water>methanol. Hence, ethanol extract was selected for further studies. In *F. vulgare* plant parts, the higher TPC was present in mature *F. vulgare* leaf ethanolic extracts (44.11 mg/g of GAE) followed by early stage of *F. vulgare* leaf (20.94 mg/g of GAE) and seed (8.94 mg/g of GAE) (Figure 1).

FRSA

FRSA value (in terms of IC) among the tested extracts of mature *F. vulgare* leaf ranged between 1.95 and 90.02 mg/ml (Table 2). The ethanolic extract had the lowest value of IC_{50} whereas water extract did not show free radical scavenging activity. The increasing order of FRSA value of different solvent extracts was: ethanol>methanol>butanol>hexane>acetone>chloroform. *F. vulgare* leaf (early and mature) and seed extracts were also subjected for the evaluation of antioxidant activity using DPPH method. Mature leaf extract scavenges the radicals by 45.93, 80.73, 90.44 and 94.44%, respectively when 100, 200, 300 and 400 µg/ml of the plant extract were added to the reaction mixture (Figure 2). However, the concentration of ethanolic mature *F. vulgare* leaf extract required to achieve a 50% reduction in DPPH radicals (IC_{50}) was 1.95 mg/ml followed by early stage leaf (12.26 mg/ml) and seed (23.74 mg/ml). The ethanol extract reported the highest phenolic content and also had the highest scavenging activity. There was a linear correlation between the antioxidant activity and TPC.

SARSA

Among different solvent extracts of mature *F. vulgare* leaf, ethanolic extract showed good potential of superoxide radical scavenging activity (IC_{50}=7.02 mg/ml), whereas aqueous extract did not show any scavenging activity (Table 2). The order of superoxide radical scavenging activity in different solvents was:

ethanol>chloroform>methanol>hexane>butanol>acetone

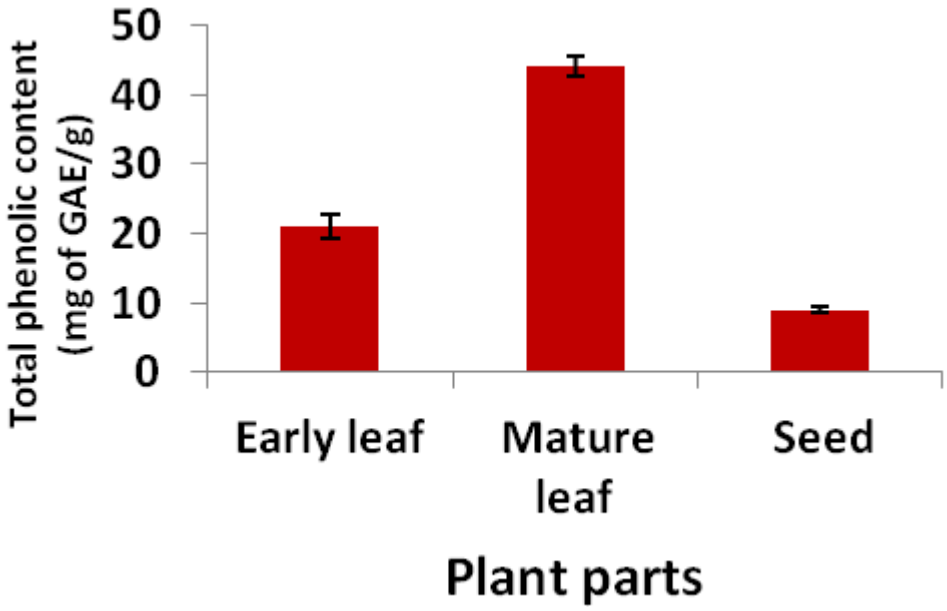

Figure 1. Total phenolic content of ethanolic extracts of *F. vulgare* leaf (early and mature) and seed. Values are mean ± SD; n (number of replications)=3.

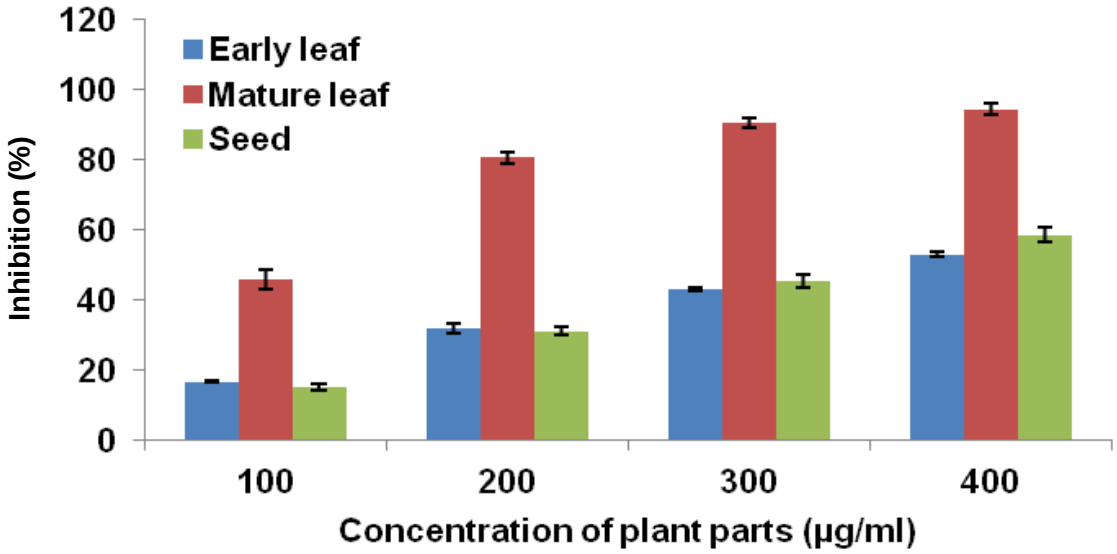

Figure 2. Free radical scavenging activity of different *F. vulgare* plant parts extracts against DPPH radicals. Values are mean ± SD; n=3.

>water. The results presented in Figure 3 showed that mature *F. vulgare* leaf inhibited NBT reduction significantly. Mature *F. vulgare* leaf showed 35.82, 40.33, 64.57 and 68.38% inhibition when 100, 200, 300 and 400 µg/ml plant extract was added to reaction mixture. Whereas, early developmental stage leaf and seed showed 18.50, 38.58, 52.64, 63.82 and 13.53, 26.76, 35.99 and 42.62% inhibition, respectively with same concentration as in mature leaf. Data showed that mature leaf of *F. vulgare* plant had better SARSA than the other parts.

RP

RP is determined to measure reductive ability of antioxidant, which is evaluated by transformation of Fe (III) to Fe (II) in the presence of the plant extracts. Among

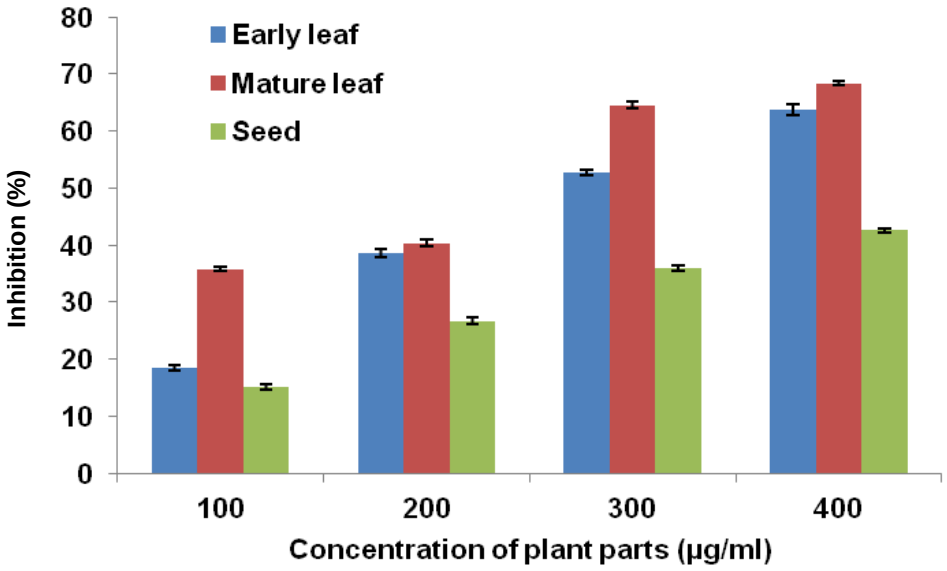

Figure 3. Inhibitory effects of ethanolic extract of *F. vulgare* leaf (early and mature) and seed on superoxide anion radical. Values are mean ± SD; n=3.

Table 3. Reducing power, lipid peroxidation, ferric thiocynate assay and hydroxyl radical scavenging activity of FV leaf (M) in different solvents extracts.

Solvent extract	RP (ASE/ml)	$^{3}IC_{50}$	$^{4}IC_{50}$	$^{5}IC_{50}$
Ethanol	1.22±0.03	37.70±0.46	3.10±0.04	23.53±0.75
Methanol	29.54±2.51	9.68±0.06	1.90±0.06	40.46±0.98
Hexane	4.47±0.16	16.66±2.26	0.92±0.01	63.39±0.80
Butanol	4.65±0.34	25.94±0.65	ND	75.50±1.83
Acetone	14.74±1.13	68.19±10.36	ND	134.96±12.17
Water	6.81±0.51	ND	2.07±0.02	ND
Chloroform	5.30±0.51	87.42±5.99	ND	147.92±7.08
Quercetin (Std)	1.12±0.02	0.007±0.001	0.45±0.01	0.016±0.006

Values are mean ± SD (n=3); RP: Reducing power; $^{3}IC_{50}$: For lipid peroxidation; $^{4}IC_{50}$: For ferric thiocyante assay; $^{5}IC_{50}$: Hydroxyl radical scavenging activity; IC_{50}:In mg/ml.

different solvent extracts of mature *F. vulgare* leaf, ethanolic extract showed maximum RP which is comparable to standard quercetin value (1.12 ASE/ml) (Table 3). The order of RP in different solvent extracts was:

ethanol>hexane>butanol>chloroform>water>acetone>methanol. Among different *F. vulgare* plant parts, mature leaf showed maximum RP (1.22 ASE/ml), whereas early stage leaf and seed has RPs to the tune of 7.13 and 11.13 ASE/ml (Figure 4).

LPO

Among the tested extracts of mature *F. vulgare* leaf, methanol extract had high potential to inhibit the LPO, whereas water extract did not show any inhibition (Table 3). The order of LPO inhibiting capacity of different solvent extract was: methanol>hexane>butanol>ethanol>acetone>chloroform. Ethanol extract of mature *F. vulgare* leaf showed 22.18, 45.94, 58.61 and 76.48% inhibition when 250, 500, 750 and 1000 µg/ml plant sample were added to reaction mixture, whereas ethanolic extract of early stage leaf and seed showed 15.03, 19.43, 35.94, 55.65 and 24.49, 29.68, 44.49, 52.62, respectively with same concentration (Figure 5).

FTC

Among different extracts of *F. vulgare* leaf, hexane extract showed maximum inhibition of linoleic acid oxidation followed by methanol, water and ethanolic extracts, whereas butanol, acetone and chloroform

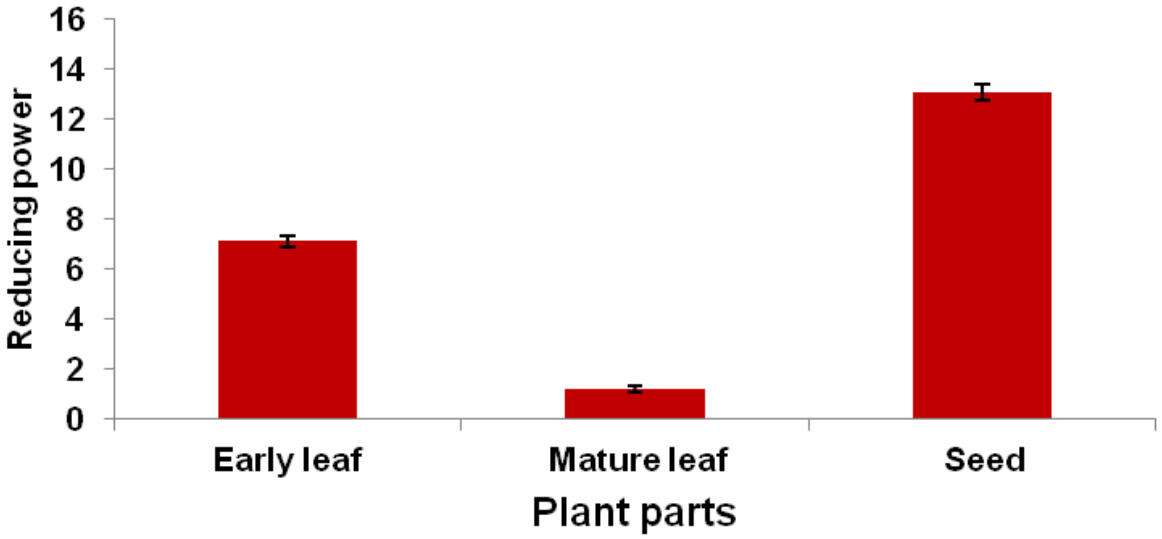

Figure 4. Reducing power (ASE/ml) of ethanolic extract of *F. vulgare* leaf (early and mature) and seed. Values are mean ± SD; n=3.

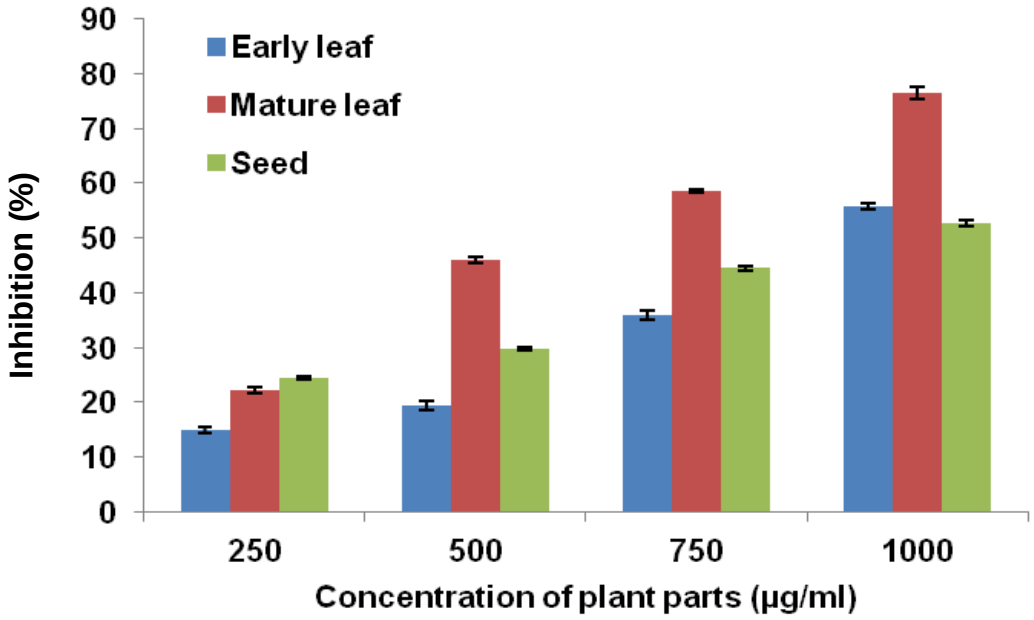

Figure 5. Inhibitory effects of ethanolic extract of *F. vulgare* leaf (early and mature) and seed on lipid peroxidation using egg homogenate as a lipid rich source. Values are mean ± SD; n=3.

extracts did not show any inhibition even at higher concentration (Table 3). In different parts of *F. vulgare* plant, ethanolic extract of mature leaf had maximum inhibition capacity. Mature leaf extract showed 39.10, 56.55, 67.94 and 77.65% inhibition, whereas early stage leaf and seed showed 23.04, 31.31, 43.73, 47.10 and 38.02, 49.72, 53.34, 65.49% inhibition with the administration of 100, 200, 300 and 400 µg/ml plant extracts, respectively (Figure 6).

Hydroxyl radical scavenging activity

In this experiment, protection of DNA by plant extracts against hydroxyl radical induced damage was determined in terms of the damage to its deoxyribose sugar moiety. The effect of *F. vulgare* plant extract on hydroxyl radicals generated by Fe^{3+} ion was measured by determining the degree of deoxyribose degradation, as indicated by thiobarbituric acid-malondialdehyde (TBA-MDA) adduct

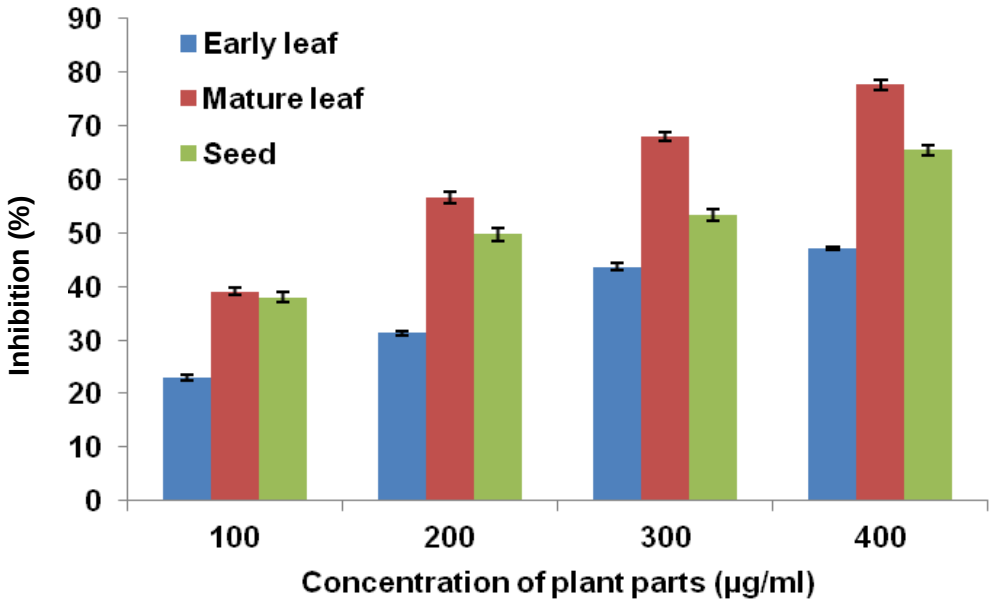

Figure 6. Inhibitory effects of ethanolic extract of *F. vulgare* leaf (early and mature) and seed on ferric ion chelation by ferric thiocyanate assay method. Values are mean ± SD; n=3.

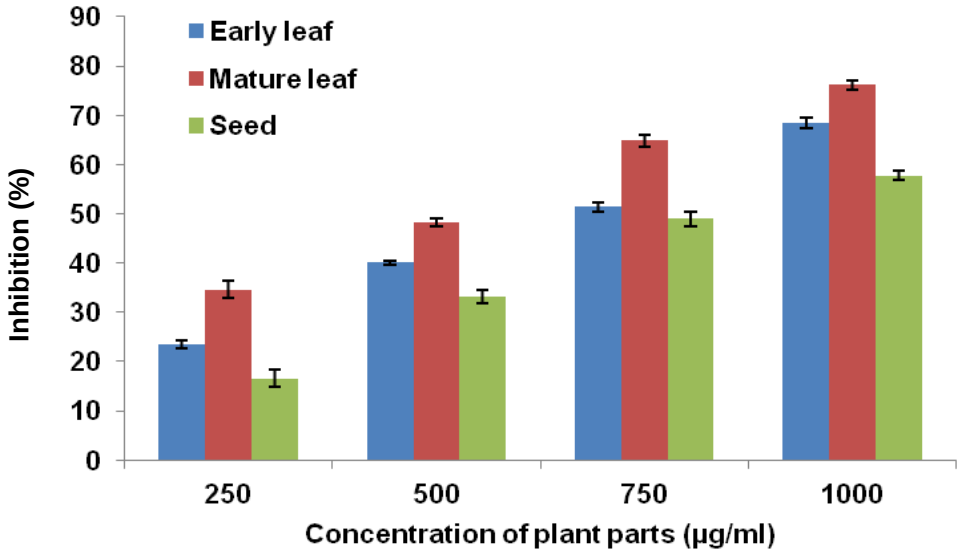

Figure 7. Inhibitory effects of ethanolic extract of *F. vulgare* leaf (early and mature) and seed on hydroxyl radical-mediated deoxyribose degradation. Values are mean ± SD; n=3**.**

formation. Out of different solvent extracts of *F. vulgare* leaf, ethanolic extract showed maximum scavenging of hydroxyl radical, whereas water extract did not show scavenging activity (Table 3). The decreasing order of hydroxyl radical scavenging activity of different extracts is as follows: ethanol>methanol>hexane>butanol>acetone> chloroform. The mature ethanolic leaf extract showed 34.64, 48.32, 64.87 and 76.20% inhibition when 250, 500, 750 and 1000 µg/ml concentrations were applied, while ethanolic extract of early leaf and seed showed 23.45, 40.10, 51.43, 68.38 and 16.61, 33.16, 48.94, 57.78% inhibition, respectively with same concentrations (Figure 7).

DNA damage

Hydroxyl radical scavenging activity of *F. vulgare* plants were further explored by the protection of plasmid pBR322 DNA against Fenton reagent induced damage.

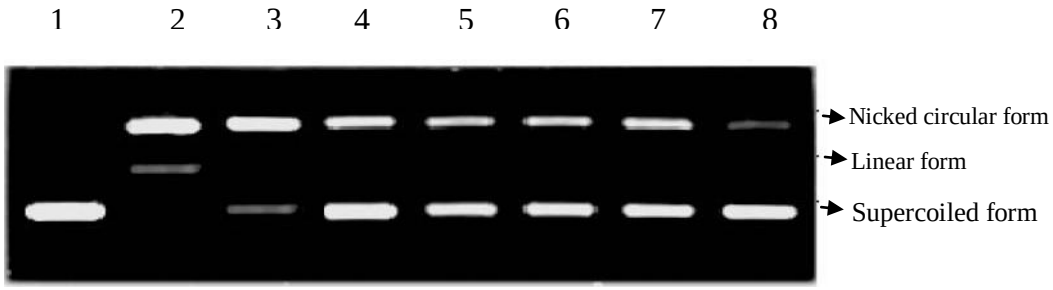

Figure 8. Concentration dependent protection offered by *F. vulgare* leaf (early and mature) extract on native pBR322 DNA nicking caused by hydroxyl radicals. Lane 1: DNA; Lane 2: DNA + Fenton reagents; Lane 3: DNA+ Fenton reagent + 25 µg/ml *F. vulgare* Leaf (E) extract; Lane 4: DNA + Fenton reagent + 25 µg/ml *F. vulgare* leaf (M) extract; Lane 5: DNA + Fenton reagent + 25 µg/ml *F. vulgare* seed; Lane 6: DNA + Fenton reagent + 50 µg/ml *F. vulgare* leaf (E) extract; Lane 7: DNA + Fenton reagent + 50 µg/ml *F. vulgare* leaf (M) extract; Lane 8: DNA + Fenton reagent + 50 µg/ml *F. vulgare* seed extract.

The results obtained through gel electrophoresis (Figure 8) showed that the extract of mature *F. vulgare* leaves have significant DNA damage protective activity in comparison to early stage *F. vulgare* leaves and seed under *in vitro* condition. Addition of Fenton reagent to a mixture containing DNA and different concentration (25 and 50 µg/ml) of ethanolic *F. vulgare* leaf (early and mature) and seed extracts showed a significant reduction in formation of nicked circular form DNA and increased supercoiled or native forms of plasmid DNA with increase in plant extracts concentration. Mature leaf extract showed more protection in comparison to early stage leaf and seed at same concentration.

Antimicrobial activity

F. vulgare leaf (early and mature) and seed extracts were tested for antimicrobial activity by disc diffusion method against the bacterium *Bacillus cereus*. The diameters of growth inhibition zone ranged from 0.60 to 2.80 mm with the highest inhibition zone observed at 500 µg/ml concentration in mature *F. vulgare* leaf, whereas the lowest inhibition zone was found in *F. vulgare* seed at 400 µg/ml concentration against *B. cereus* (Table 4).

HPLC analysis

F. vulgare leaf (early and mature) and seed extracts were also examined for their phenolics composition using HPLC. Data from HPLC analysis revealed that the concentration of phytochemicals increases during the development of plant. In *F. vulgare* plant, the content of chlorogenic acid, caffic acid, rutin, quercetin, kaempferol and myricetin in early stage *F. vulgare* leaf were 1.92, 0.39, 1.47, 1.09, 0.27, and 0.68 mg/100 g, respectively whereas in mature *F. vulgare* leaf, these were 1.98, 0.90, 1.68, 1.00, 0.19 and 1.48 mg/100 g, respectively (Table 5). HPLC analysis of early and mature leaf showed that the concentration of detected phytochemicals such as chlorogenic acid, caffic acid, rutin, and myricetin increased by 3.03, 56.66, 12.50 and 54.05%, respectively with maturity of the plant leaf whereas quercetin and kaemferol decreased by 7.40 and 29.62%.

DISCUSSION

Extraction of phenolics with different solvents showed that ethanol is most suitable in comparison to other solvents tested. This is in agreement with a study by Taso and Deng (2004) which also showed that phenolic acids are generally better extracted using alcohol solvents. Hence, ethanol extract was selected for further studies. It has been observed that TPC are mainly responsible for the antioxidant activity of plants. Heim et al. (2002) reported that the beneficial effects of fruits, vegetables and various medicinal plants are due to the presence of phenolic compounds. Reports also suggest that the phenolic compounds could be major contributor of antioxidant activity, and therefore, plants containing high concentration of phenolic compounds could be good source of natural antioxidants.

Our studies show that the mature *F. vulgare* leaf extracts significantly reduced the DPPH radicals in dose dependent manner in comparison to the early stage leaf and seed. In its radical form, DPPH* absorbs at 517 nm, but upon reduction by an antioxidant, its absorbance decreases (Gulcin et al., 2007). Antioxidants react with DPPH radical and convert it into α,α-diphenyl-p-picryl hydrazine (Singh et al., 2002). DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. It is reported that the decrease in the absorption of DPPH radical caused by phenolic compounds is due to the reaction between antioxidant molecules and radicals resulting in the scavenging of the radical by hydrogen donation and is

Table 4. Antimicrobial activity of FV plant and seed extracts.

Plant sample	Inhibition zones (mm)				
	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml	500 µg/ml
leaf (E)	ND	ND	0.80±0.30	0.95±0.50	1.60±0.40
leaf (M)	ND	ND	1.20±0.36	1.40±0.51	2.80±0.46
Seed	ND	ND	ND	0.60±0.18	0.90±0.20

Values are mean ± SD (n=3); FV: *Foeniculum vulgare*; E: early stage; M: mature stage; ND: not detected.

Table 5. Quantification of phytochemicals of FV (E&M) and seed using HPLC analysis.

Sample name	Chlorogenic acid (mg/100 g)	Caffeic acid (mg/100 g)	Rutin (mg/100 g)	Quercetin (mg/100 g)	Kaempferol (mg/100 g)	Myricetin (mg/100 g)
Leaf (E)	1.9169	0.38742	1.46519	1.08901	0.27146	0.68426
Leaf (M)	1.9782	0.89977	1.68158	1.00153	0.19379	1.48888
Seed	0.93738	0.31462	1.25694	0.54334	0.1755	0.84815

responsible for change in colour from purple to yellow. A study by Albano et al. (2012), showed that essential oil of *F. vulgare* has almost similar IC_{50}=2.34 mg/ml as reported by them in the case of mature *F. vulgare* leaf. Kim et al. (2011) reported that hot water fennel extract showed 10.48% DPPH inhibition at 1000 µg/ml concentration which is very low. This study also showed no DPPH inhibition by water extract of *F. vulgare* even at higher concentration.

The SARSA of *F. vulgare* plant was monitored by PMS-NADH-NBT reduction system, a non-enzymatic method. In this method, $O_2^{\bullet-}$derived from dissolved oxygen by PMS-NADH coupling reaction reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. The decrease in color intensity showed that antioxidant present in the plant extracts scavenge the superoxide radical. Superoxide anion is a weak oxidant which gives rise to generation of powerful and dangerous hydroxyl radical and singlet oxygen. Both contribute to oxidative stress. The color intensity decreases with the addition of plant extract to reaction mixture. The plant extract reduce the superoxide anion and inhibit the formation of blue formazan complex (Cos et al., 1998; Parejo et al., 2004). Kim et al. (2011) also reported mild inhibition of superoxide in water extract of *F. vulgare* plant. Almost similar result was observed in the case of SARSA with no inhibition of superoxide.

Sathisha et al. (2011) hypothesized that antioxidant activity and RP are related to each other. The RP of a compound may serve as a significant indicator of its potential antioxidant activity (Prakash et al., 2007b). The RP of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued proton abstraction and radical scavenging activities (Diplock, 1997). The reducing compound present in the plant extract reduce the ferricyanide to ferrocyanide which develops perl's Prussian blue color.

The developed color intensity by ferrocyanide is measured at 700 nm. With regards to RP, higher reducing capacity might be attributed to higher amount of TPC and flavonoids (Lee et al., 2007). It was observed in this study that mature *F. vulgare* leaf showed maximum RP followed by early stage leaf and seed showing increase in phenolic contents during development from early to mature leaf. Seed has shown the minimum value. This is well correlated with the studies mentioned earlier.

LPO is a consecutive process of oxidative degradation of polyunsaturated fatty acids present in the biological membranes and production of a variety of secondary products including several aldehydes such as MDA. LPO increased during the lung damage by oxygen toxicity and liver damage by various drugs such as, herbicides and pesticides particularly, those which deplete intracellular GSH levels. MDA is the major reactive aldehyde resulting from the peroxidation of polyunsaturated fatty acid present in biological membrane (Vaca et al., 1988).

MDA reacts with TBA and produce pink colored products. It has also been proposed that the effect of dietary fat on cancer occurs through the activation of procarcinogens to ultimate carcinogens by fat oxidation products such as lipid hydro-peroxide. Bartsch (1996) reported that MDA forms adducts with DNA adenine and cytosine which contribute to carcinogenicity and mutagenicity. So, the plant parts having better protection against free radical induced LPO may be used as anti-LPO as well as anticarcinogenic/antimutagenic substances. The high LPO scavenging effects in mature *F. vulgare* leaf observed in our experiment may be due to the high contents of phenolic compounds or other radical scavengers present in the extracts which can terminate

the peroxidation chain reaction easily and quench ROS or nitrogen species, thereby inhibiting the oxidation of lipid and other biological molecules. LPO inhibitory activity mainly depends on the solubility of the compounds present in the respective extracts (Son and Lewis, 2002). It has been reported that the LPO is one of the cause of occurrence of cardiovascular disease and cancer. Therefore, the inhibition of peroxidation by the plant extracts indicates its antioxidant potential.

FTC is used to measure the production of peroxides at the initial stage of oxidation while lipid LPO test is used to measure the secondary products of oxidation such as aldehydes and ketones (Farag et al., 1989). The complex formed with Fe^{3+} and thiocyanate produce a deep red colour, which is detectable at 500 nm. The advantage of using ammonium thiocyanate over other coloring reagents is that binding of iron by thiocyanate ion is specific to Fe^{3+} only. Transitional metals, especially iron has the ability to generate free radicals from peroxides by Fenton reagent. Fe^{2+} has the capability to induce production of oxyradicals and LPO and hence samples showing Fe^{2+} reduction in the reaction mixture are considered to protect cellular damage induced by oxidative stress.

On the other hand, phenolic compounds can also inhibit their formation by chelating with metal ions. Our result with ammonium thiocyanate experiments showed that the mature *F. vulgare* leaf extract is an active scavenger of Fe^{3+} ion which is in agreement with the work done on known Fe^{3+} scavengers (Singh et al., 2009a). Chelating agent present in plant extracts may inactivate metal ions and potentially inhibit the metal dependent generation of free radicals.

DNA is also a major target of free radical to cause DNA damage. Under normal physiological conditions, the endogenous production of free radicals may lead to a minimal damage in DNA which is needed to induce the defense systems and DNA-repair mechanisms. However, if this production of free radicals increase, oxygen radicals may attack the DNA at either sugar (deoxyribose) or the phosphodiaster chain of supercoiled plasmid DNA, giving rise to a large number of products. Attack at a sugar moiety or phosphodiaster chain ultimately leads to strand break. This study suggests that mature leaves of *F. vulgare* plant contain compounds that are capable of acting as antioxidants by reducing free radical-induced DNA damage. Hydroxyl radical is one of the most ROS and can reacts with lipid, carbohydrates, amino acids and nucleic acid in the body and the prevention of such harmful reaction is highly advantageous (Sasaki et al., 1996; Braner and Davidson, 1997).

Hydroxyl radicals can be formed by the Fenton reaction in the presence of transition metals such as Fe^{3+} and H_2O_2, which is known to be the most reactive of all the reduced forms of dioxygen, capable of damaging almost every molecule found in living cells (Rollet-Labelle et al., 1998). In the present experiment, plasmid pBR322 DNA was treated with Fenton reagent. It interacts with the supercoiled DNA which is broken into open circular and nicked circular forms. Hydroxyl radical generated by Fenton's reagent, attacks on nitrogenous bases of DNA and sugar moiety of supercoiled pBR322 plasmid DNA, resulting in nicked circular form due to breakage of sugar phosphate backbone of nucleic acid. The damage was effectively minimized by treatment with mature *F. vulgare* leaf extract as has been shown in our results. The DNA damage may cause a number of genetic disorders (Moskovitz et al., 2002). Damage in one of the phosphodiaster chain of the supercoiled DNA produces a relaxed open circular form. Further cleavage near to the first breakage results in linear double stranded DNA molecules. The formation of circular form of DNA is indicative of single strand break and the formation of linear form of DNA is indicative of double stranded breakage. The mechanism of action of hydroxyl radical induced by the Fenton reagent on plasmid DNA is given as:

Ascorbic acid (reduced) + Fe^{3+} → Ascorbic Acid (oxidized) + Fe^{2+}

$Fe^{2+} + H_2O_2 + Fe^{3+} \rightarrow OH^- + OH^{\cdot}$

$OH\cdot$ + DNA → DNA damage product + OH^-

Gulfraz et al. (2008) also reported that essential oil and seed extracts of *F. vulgare* exhibit different degrees of antimicrobial activities depending on the doses applied which is similar to the result of this study. Kaur and Aurora (2010) reported that the essential oils extracted from the seeds of *F. vulgare* have also been shown to possess antibacterial activity against human pathogenic bacteria. Mature leaf of *F. vulgare* has potential antibacterial activity, which can be further exploited to develop new drugs to treat infections produced by important multiresistant pathogenic bacteria.

HPLC is a sensitive and reliable method for analyzing compounds present in plant. In this study, HPLC analysis of *F. vulgare* leaf clearly indicates that the phytochemical concentration vary with the development of plant in which mature plant parts has higher concentration in comparison to immature plant parts. In a study, Khalil et al. (2007) reported that HPLC analysis of fennel plant has 5.98 mg/100 mg chlorogenic acid content which is higher as compared to our result which could not be explained. The results obtained through HPLC analysis also suggested that the mature *F. vulgare* plant parts are more important in comparison to early stage plant in as much nutraceutical quantities are concerned.

Correlation between TPC in the plant extract in relation to their antioxidant activity

Total phenolics are mainly attributed to the antioxidant activity. The correlation between TPC and free radical scavenging activity of *F. vulgare* plant extracts had a correlation coefficient of R^2=0.930 (y=0.032x-0.317). This

suggests that in *F. vulgare* plant, 93% antioxidant activity is contributed by phenolics compound. The remaining antioxidant activity is due to non phenolics compounds. Activity may also come from the presence of other secondary metabolites such as volatiles oils, flavonoids, metalloprotein, vitamins, etc. A high correlation also exist between the total phenolics and RP of *F. vulgare* plant (R^2=0.966).

This study demonstrated that the concentration of phytochemicals vary during the development of *F. vulgare* plant and found that mature leaf part had more antioxidant activity and phytochemicals in comparison to early stage *F. vulgare* leaf. The presences of polyphenolics in the plant parts are mainly responsible for their overall antioxidant activity. Mature *F. vulgare* leaf exhibited strong and effective *in vitro* antioxidant activity by chelation to metal ions as well as scavenging free radicals. So, it can be stated that mature *F. vulgare* leaf may be good and easily accessible source of nutraceutical compounds in comparison to early stage *F. vulgare* leaf and seed.

It is also clear that ethanol is suitable for better extraction of phytochemical over the other tested solvents as it can extract phenolic content in a better way. Mature *F. vulgare* leaf deserves more intensive study including its *in vitro* antioxidant activity, bioavailability and possible protection against cardiovascular diseases to understand their real potential as nutraceutical.

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ABBREVIATIONS

ASE, Ascorbic acid equivalent; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **DW,** dry weight; **FRSA,** free radical scavenging activity; **FV,** *Foeniculum vulgare*; **FTC,** ferric thiocyanate assay; **GAE,** gallic acid equivalent; **IC_{50},** inhibitory concentration; **LPO,** lipid peroxidation; **MDA,** malondialdehyde; **NBT,** nitro blue tetrazolium; **PMS,** phenazine methosulphate; **RP,** reducing power; **ROS,** reactive oxygen species; **SARSA,** superoxide anion radical scavenging activity; **TBA,** thiobarbituric acid; **TBARS,** thiobarbituric acid-reactive species; **TCA,** trichloroacetic acid; **TPC,** total phenolic content.

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