

Polyphenols, flavonoids and antimicrobial properties of methanolic extract of fennel (*Foeniculum vulgare* Miller)

Anita Dua^{1*}, Gaurav Garg² and Ritu Mahajan³

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

²Department of Biotechnology, Maharishi Markandeshwar University, Mullana, India

³Department of Biotechnology, Kurukshetra University, Kurukshetra, India

ABSTRACT

To address the ever increasing resistance of pathogens and need of safer preservatives for food products, herbs are being explored for antimicrobial properties. Methanolic extract of Fennel (*Foeniculum vulgare* Miller) was analyzed for the presence of polyphenolic compounds, flavonoids and antibacterial potential. The extract, rich in flavonoids (9.325 ± 1.25 mg QE/g dry seeds) was subjected to HPLC analysis for identification and quantification of phenolics. Gallic acid (277.131 μ g), caffeic acid (166.062 μ g), ellagic acid (99.476 μ g), quercetin (781.986 μ g) and kaempferol (92.856 μ g)/g dry seeds were identified. Antibacterial activities of methanolic extract of dried fennel seeds were determined against pathogenic bacteria *Bacillus pumilus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* by determining cell damage, growth inhibition zone and minimum inhibitory concentration. Fennel extract caused cell damage and effectively inhibited the growth of *Bacillus pumilus* and *Staphylococcus aureus*. Minimum inhibitory concentration of fennel, determined by agar well diffusion method, was 8.33mg/ml for *B. pumilus* and *S.aureus*. The results suggest that methanolic extract of fennel containing polyphenols including gallic acid, caffeic acid, ellagic acid, quercetin and kaempferol has efficient antimicrobial activity against pathogenic gram positive bacteria under study.

Key words: fennel, polyphenols, antimicrobial properties, MIC

INTRODUCTION

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. Nowadays, the resistance of pathogens against antibiotics is developing much faster than ever. The search for new antimicrobial and antioxidant substances from nature is on great demand. Synthetic chemicals are often used as preservatives in food processing and storage to inhibit food-borne pathogens and to extend shelf life. 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.

In India, some species and herbs have great economic importance, being utilized as foods and in medicine. These possess notable biological activities, in particular the antioxidant and antimicrobial action. Fennel (*Foeniculum vulgare* Miller), a perennial herb belonging to the family Apiaceae, is mainly grown in the temperate regions of the northern hemisphere. It is highly aromatic and flavorful herb with culinary and medicinal uses. Fennel fruit is a dry seed, traditionally used as anti-inflammatory, analgesic, carminative, diuretic and antispasmodic agent[1]. Fennel is used in treatment of glaucoma[2], galactosaemia and hypertension [3]. Several scientific reports have described the inhibitory effect of spices on a variety of microorganisms, although variation for resistance of different microorganisms to a given spice and of the same microorganism to different spices has been observed[4]. Recently there has been considerable interest in the antimicrobial potential of fennel seed extracts and essential oil [5,6,7]. Antimicrobial activity of herbs against different bacteria might be due to the presence of different active phyto-compounds. Among these phenolics, terpenoids and alkaloids are reported to have antimicrobial and antioxidant effects[8,9]. The purpose of the present study was to qualitatively and quantitatively analyze the methanolic extract of fennel for the presence of various polyphenols and flavonoids and to evaluate the antimicrobial effectiveness. The present study was undertaken to investigate the antibacterial properties of fennel in the evaluation of its potential to be an antimicrobial preservative 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. Caffeic acid, ellagic acid, ferulic acid, quercetin and kaempferol were purchased from Sigma Chemical Company, USA. Acetonitrile, gallic acid, folin-ciocalteu reagent, methanol were purchased from Hi-media, Mumbai, India. All other chemicals and solvents used were of analytical grade.

Four enteropathogenic and food-spoiler bacterial strains [two gram negative bacteria i.e. *Escherichia coli* (MTCC 119), *Pseudomonas aeruginosa* (MTCC 741) and two gram positive bacteria i.e. *Staphylococcus aureus* (MTCC 96), *Bacillus pumilus* (MTCC 7411)] were obtained from MTCC, IMTECH, Chandigarh, India.

Extraction

Fennel seeds were dried at 60°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 4 hrs. Residue was extracted with 80% methanol again for 2 hrs. 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 Flavonoids

Flavonoids were estimated by the method described by Zhishen et al. 1999[10] with slight modifications. Distilled water is added to an aliquot of extract to make volume 0.5ml; 30µl NaNO₂ (5%) is added and mixed well. After 5min, 300 µl of AlCl₃ (10%) was added and mixed. After another 5min, 200µl NaOH (1N) was added. Absorbance was read at 510nm against reagent blank. Quercetin was used to prepare a standard curve and flavonoids are expressed as mg quercetin equivalents (QE).

Polyphenol analysis by HPLC

The methanolic extract was defatted with n-hexane. The defatted extract was treated with 2N HCl to hydrolyze glycosidic bonds. The extract was dried and again dissolved in methanol. It was then subjected to HPLC for qualitative and quantitative analysis of free phenolic contents[11]. The HPLC system (Agilent Technologies Company) was equipped with dual lamp binary system, UV detector, C18 column (i.d. 4.6 mm×150mm, 5µm) and data was integrated by Agilent Chem Station software. Standards and sample extracts were analyzed using the following gradient program (A: 100% acetonitrile B HPLC Grade Water 0min, 5% A: 10min, 15% A: 20 min, 25% A: 30min, 35% A: 40min, 45% A: 50min 55% A). Flow rate was 0.5 ml/min and injection volume was 10µl. 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 –gallic acid, caffeic acid, ellagic acid, ferulic acid, quercetin and kaempferol.

Antibacterial activity

All bacterial cultures were maintained and subcultured regularly on Nutrient agar media (NAM) containing peptone 5 g; beef extract 3 g; sodium chloride 5 g and agar 2% in a final volume of 1l. The size of inoculum was adjusted to approximately 10^8 colony-forming units per ml by suspending the culture in sterile distilled water. Petridishes containing nearly 25 ml of nutrient agar medium were seeded with 100 μ l culture of the respective bacterial strains and kept for 15 min for the absorption of culture.

Estimation of bacterial Cell Damage

The bacterial culture was grown in nutrient broth up to the log phase of the culture. The cultured broth was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was suspended in 20 mM phosphate buffer of pH 7.0. Culture was washed again and turbidity of each suspension was adjusted to 0.5 McFarland units by suspending the cultures in sterile phosphate buffer. To check the amount of cell damage due to fennel extract, 100 μ l of bacterial suspension was incubated with 100 μ l of fennel extract (equivalent to 1.25mg dry seeds per ml extract) diluted in 10% methanol at 37 °C for 30 min in water bath. The incubation mixtures were diluted appropriately to observe absorbance at 260 and 280 nm before and after incubation for 30 min against methanol blank.

Determination of growth inhibition zone

Using a sterile cork borer, nearly 8mm diameter wells were bored in the seeded agar plates and a 100 μ l volume of fennel seed extract (equivalent to 33.33 mg dry seeds) diluted in 10 % methanol was added into the wells. All the plates were incubated at 37°C for 24 h. Antibacterial activity was determined by measuring the zone of growth inhibition around the well using agar well diffusion assay technique [12]. The antimicrobial activity of the fennel extract was compared against the standard drugs, ampicillin and chloramphenicol (concentration 25 μ g/ml; negative control) and 10% methanol (positive control). These tests were performed in triplicate and the mean of growth inhibition zone diameter was taken.

Determination of Minimum Inhibitory Concentration (MIC)

MIC of fennel was determined by the agar well diffusion method. Two fold serial dilutions of fennel extract (33.33mg/100 μ l) ranging from 100-3.125 μ l/ml with concentrations 33.33–1.042mg/ml fennel seeds were made in 10% methanol and 100 μ l of different dilutions of the extract were added into different wells and incubated at 37°C for 24 h to determine the minimum concentration of fennel inhibiting the growth of bacteria under study.

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

Polyphenols can contribute as reducing agents and 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 electron donor and reducing agent. Flavonoids with dense π electron cloud, 4-oxo group with 2-3 double bond and 3,5 hydroxyl groups have higher reducing character. Fennel extract had flavonoids 9.325 ± 1.25 mg QE/g dry seeds. Flavonoids make a major component of the polyphenol pool extracted with ethanol and methanol [13]. Extract of fennel seeds in 80% methanol had fairly high amount of polyphenols 16.506 ± 0.32 mg GAE/g dry weight of seeds [14]. Results in present study indicate that flavonoids in methanolic fennel extract are more than 50% of the total polyphenolic content of fennel. Polyphenolic compounds are usually present as glycosides in plant sources. The fennel seed extract was hydrolyzed with 2N HCl to break glycosidic bonds before analysis by HPLC. Polyphenols extracted from fennel seeds were characterized qualitatively and quantitatively by HPLC (Fig.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, quercetin and kaempferol were identified in the fennel extract (Table1). Quantification of the identified compounds was achieved by comparing the peak area of individual compound with that of standards (2ng/10 μ l). Methanolic extract of fennel had Gallic acid 277.131 μ g, caffeic acid 166.062 μ g, ellagic acid 99.476 μ g, quercetin 781.986 μ g and kaempferol 92.856 μ g/g dry fennel seeds. Roby *et al.* 2012 have also identified gallic acid, caffeic acid, ferulic acid, quercetin, rosmarenic acid and their glycosides in the Egyptian fennel extract. Quercetin and rosmarenic acid are reported to be the major constituents with 15–17 % area among the identified polyphenols [13]. In the present study

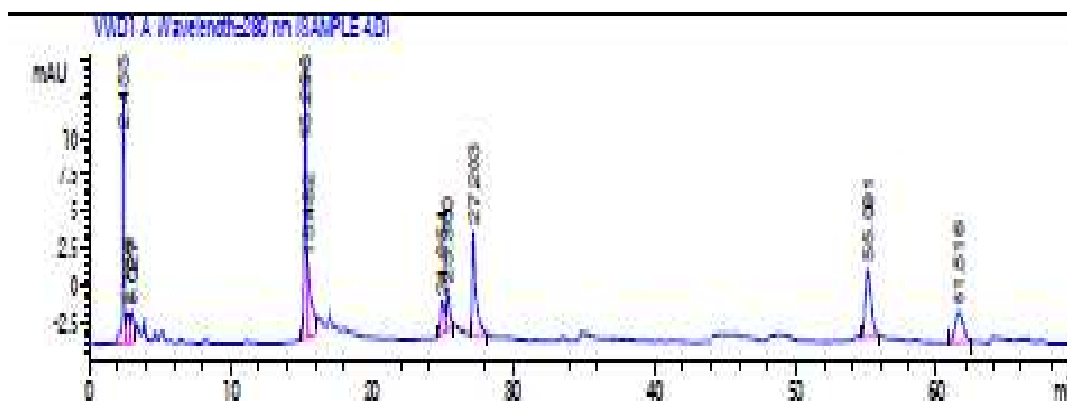
gallic acid, caffeic acid and quercetin are identified as the major constituents with 13-20% area of the identified polyphenols. The finding reported herein confirms both the presence of several flavonoids and appreciable amounts of gallic acid, caffeic acid and quercetin. Reducing property of polyphenols increases with the number of hydroxyl groups and the density of π electron cloud.

Determination of antibacterial activity

Antibiotics are generally an efficient means of treating bacteria infections. Treatment with antibiotics is not only expensive but the risk of bacterial resistance to antimicrobial agents and side effects such as acidity, burning sensation and damage to natural fauna of intestine are also involved [17]. The antimicrobial potential of the methanolic extract of fennel seeds Gallic acid, among the simple phenolics and quercetin among the flavanols are the most potent reducing agents [15,16]. Presence of high amount of gallic acid, quercetin and kaempferol along with other polyphenols in the extract indicates high efficacy of fennel as reducing agent and possible role as antimicrobial agent against pathogenic bacteria *E.coli*, *P.aeruginosa* causing gastroenteritis, urinary tract infections, *B.pumilus* causing stomach cramps, food poisoning and *S.aureus* causing pneumonia, food poisoning and toxic shock syndrome (TSS) was examined.

Cell damage to the bacteria was examined as increase in absorbance of the exogenous solution at 260 and 280 nm after incubation of bacterial cells with fennel extract. A considerable increase in absorbance at 260 nm is observed with *B.pumilus* followed by *S. aureus*, however, negligible change was there with *E.coli* (Table 2). The results indicate that fennel extract caused damage to the bacterial cell membranes of gram positive bacteria leading to the leakage of the biomolecules from cells, but *E.coli* and *P.aeruginosa* were less affected. Extracts of herbs rich in antioxidants like ascorbic acid, tocopherols, flavonoids and polyphenols are reported to cause cell damage and leakage of biomolecules from the damaged membranes of microbes [18,19,20-24]. Growth of *S.aureus* and *B.pumilus* is inhibited in the presence of fennel extract. The growth inhibition zone in presence of extract equivalent to 33.33 mg fennel seeds was 11.27 mm for *S. aureus* and 12.67 mm for *B.pumilus*, although ampicillin and chloramphenicol (25 μ g /100 μ l) were more efficient and growth inhibition zone diameter of 18-44 mm was observed (Table 3). Fennel extracts are reported to be effective against all types of bacteria but the effect is more pronounced against gram positive bacteria [7, 13]. Least activity is reported against *E.coli* and maximum growth inhibition zone diameter of 29 mm is reported against *B.subtilis* with fennel extract containing 30 mg/ml dry wt [13].

Fig.1 HPLC analysis for identification and quantification of phenolic compounds



Minimum inhibitory concentration of the extract observed in this study is equivalent to 8.33 mg/ml fennel seeds for *S.aureus* and *B.pumilus* as compared to 0.2-0.8 μ g/ml for standard antibacterial compounds tested (Table 4). Fennel extract is required in higher concentration to inhibit the growth of *E.coli* or *P.aeruginosa*. These results are in agreement to the other reports in literature [6,7,13,25]. Roby et al 2013 have reported 12.5-15 mg/ml minimum concentration required to inhibit the growth of various bacteria [13]. However Anwar et al 2009 have reported higher MIC value of 62.5 mg/ml for *B.subtilis* and 259 mg/ml for *E.coli* [7]. To add, methanolic extracts are inhibiting bacterial growth more efficiently than extracts in other solvents like ethanol, diethyl ether and hexane [13]. Growth of bacteria is sensitive to the redox potential of the media. Moderately reducing environment of the growth medium can contribute in part to the growth inhibition of various bacteria [18]. Methanolic extract of fennel seeds has high

amount of polyphenols and flavonoids indicating its reducing character, which may in part explain the inhibition of bacterial growth. Metal ion chelating property of the phytochemicals in the extract may also be contributing by leading to the deficiency of essential metal ions in the growth medium or binding to the thiol groups at the active sites of various microbial enzymes (such as trypsin and other proteases) rendering them inactive and inhibiting the growth of microbes.

Table1 Polyphenols of Fennel (*Foeniculum vulgare*)

Compound	Retention Time(min)	Amount ($\mu\text{g/g dry wt.}$)
Flavonoids	-	9.325 \pm 1.25*mg
Gallic acid	2.445	277.131
Caffeic acid	15.296	166.062
Ellagic acid	25.300	99.476
Quercetin	55.091	781.986
Kaempferol	61.516	92.856

*(mg/g dry wt.)

Table 2 Cell damage in presence of fennel extract (1.25mg seed wt.)

Organism	Δ O.D./260nm	Δ O.D./280nm
<i>E.coli</i>	0.055	0.010
<i>P.aeruginosa</i>	0.142	0.083
<i>S.aureus</i>	0.208	0.234
<i>B.pumilis</i>	0.670	0.280

Table 3 In vitro antibacterial activity of fennel extract and standard antibiotics

Compound	Concentration of the compound	Diameter of growth inhibition zone(mm)			
		<i>E.coli</i>	<i>P.aeruginosa</i>	<i>S.aureus</i>	<i>B.pumilis</i>
Fennel extract	33.33(mg seeds/ml)	-	-	11.27 \pm 0.35	12.67 \pm 0.25
Chloramphenicol	25 ($\mu\text{g/ml}$)	17.53 \pm 0.25	24.66 \pm 0.15	23.63 \pm 0.16	26.33 \pm 0.31
Ampicillin	25 ($\mu\text{g/ml}$)	35.66 \pm 0.21	44.10 \pm 0.26	41.40 \pm 0.20	38.56 \pm 0.31
Methanol	10%	0	0	0	0

Table 4 Minimum inhibitory concentration (MIC) of fennel extract and standard antibiotics

Compound	<i>E.coli</i>	<i>P.aeruginosa</i>	<i>S.aureus</i>	<i>B.pumilis</i>
Fennel	\geq 33.33	\geq 33.33	8.33	8.33
Chloramphenicol	0.2	0.4	0.4	0.4
Ampicillin	0.5	0.5	0.8	0.5

The values for fennel are in mg dry wt of seeds /ml and for standard antibiotics are in $\mu\text{g/ml}$

CONCLUSION

To inhibit food born pathogens and to extend shelf life, synthetic chemicals with antimicrobial properties are often used as preservative in food processing and storage. Concerns over the potential risks of synthetic food additives to human health and consumer awareness have directed the interests in using naturally occurring alternatives. 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 with antibacterial activity against gram positive bacteria. Polyphenolic compounds of the methanol extract of fennel seeds contain gallic acid, caffeic acid, ellagic acid and flavanolquercetin and kaempferol. Fennel extract could inhibit the growth of gram positive bacteria with a MIC value of 8.33 mg/ml. Fennel seeds can play dual role as antioxidant and antibacterial agent, and has good potential to be applied in the food and pharmaceutical industry. Possible use of fennel seeds as an antioxidant, antimicrobial nutraceutical and as food preservative needs to be explored further.

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