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Ethnopharmacological Profiling of *Foeniculum vulgare* Essential Oil and Concentrated Components

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Abstract

The aim of the present study was to evaluate the antioxidant and antifungal activities of *Foeniculum vulgare* Mill. Seeds essential oils extracted by hydro and steam distillation methods under different temperature conditions (110, 115, 120, 125 and 130ºC) and its fractions obtained through low temperature chilling and vacuum distillation methods. The essential oil and fraction that showed maximum antioxidant and antifungal activities were further characterized by GC-MS. Total phenolic and flavonoid contents of each essential oil and its fractions were determined by colorimetric assays. The antioxidant activity of each essential oil and its fractions were investigated by measuring DPPH free radical scavenging and reducing power assays. Disc diffusion assay was used to estimate antifungal activity of each essential oil and its fractions. GC-MS analysis of the most active essential oil extracted by steam distillation method at 125 °C revealed estragole (39.05%) and L-fenchone (25.08%) as the major compounds. The most active fraction HC_{14} revealed estragole (78.89%) and L-limonene (13.04%) as major compounds. The maximum essential oil yield was found to be 0.78% by hydro-distillation at 110 °C. The essential oils and its fractions contained significant amount of total phenolic contents (705.99±0.02 -13.10±0.00 mg/L as GAE) and total flavonoid contents (410.75±0.01 - 3.19±0.17 mg/L as CE). *Foeniculum vulgare* Mill. Seeds essential oils and its fractions also revealed good DPPH radical scavenging activity (99.64±0.04 - 71.00±0.05 %) and reducing power (0.121±0.001 - 0.009±0.000 nm). Overall, pure essential oil extracted at 125 °C by steam distillation method showed the highest antioxidant and antifungal activities.

Keywords: *Foeniculum vulgare* Mill, essential oil, fractions, antioxidant, antifungal

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1. Introduction

Food-borne diseases are one of emergent public health problem world-wide caused by consumption of microbial contaminated food products [1-2]. The existence and growth of microorganisms in foods may lead to spoilage, formation of toxins and quality deterioration of food products. Similarly, lipid peroxidation in fats and food determinate the food products, cause chemical spoilage and rancid flavor that deceases the nutritional and sensory quality of the products [3]. During lipid oxidation reactions, numerous free radicals are formed that responsible for many health problems including diabetes, cardiovascular disease and cancer [4-5]. Antioxidants are our first line of defense against free radical damage, and are critical for maintaining optimum health and well-being [6]. A wide range of synthetic antioxidants such as butylated hydroxyanisole (BHA), propyl gallate (PG) and butylated hydroxitoluene (BHT) are used in many foods to decrease the oxidation process. However, an increasingly negative consumer perception of synthetic antioxidants and food additives has increase researchers interest in finding of food additives and antioxidants from natural sources [7].

Several reports are available on antioxidant and antimicrobial activities of essential oils and their potential uses in preservation of food products [5-8-13]. *Foeniculum vulgare* commonly known as fennel belongs to the family Apiaceae. It is native to Mediterranean area and is also grown commercially in Russia, India, China, and Japan and in central Europe. Fennel is a medicinal and aromatic plant, commonly used to flavor liqueurs, breads, fishes, salads and cheeses. In folk medicine fennel seeds and its essential oil are used to cure various health problems including cardiotonic, lactagogue, gastrointestinal and respiratory system [14]. Moreover, its essential oil is used in cosmetic, perfumes, pharmaceutical, food products [15]. Fennel seeds are a potent source of natural antioxidants, such as phenolic compounds and vitamins C and E, and they show strong antioxidant properties in biological systems and food products [16-17]. The fennel essential oil exhibited a wide and varying chemical composition, depending on part of plant including roots, flowers, stems, fruits, extraction method, phonological state, maturation stages and geological origin [18-20]. Major volatile compounds present in fennel seeds oil are α-pinene, fenchone, α-phellandrene, limonene, *trans*-anethole, estragole and methylchavicol [21].

Numerous studies have shown that different extracts, essential oil and its individual compounds exhibit novel pharmacological activities. Phenolic acids including tannic, gallic, caffeic, cinnamic, chlorogenic, ferulic, and vanillic acids isolated from fennel extract exhibited antitumor activity [22]. To the best of our knowledge, there is little information available about the chemical constituents of *Foeniculum vulgare* seeds essential oil and its antioxidant and antifungal activities, but there is no report available on antioxidant and antifungal activities of its fractions. Therefore, the present study was performed to extract the essential oil from *Foeniculum vulgare* seeds using hydro and steam distillation methods under different temperature conditions, and to fractionate these essential oils through vacuum distillation and low temperature chilling methods. In addition, antioxidant and antifungal activities of essential oil and its fractions performed. Finally, chemical composition of most active essential oil and fraction were evaluated by gas chromatographic mass spectrometric analysis (GC-MS).

2. Material and methods

2.1. Plant material

Fully ripened fennel (*Foeniculum vulgare* Mill.) seeds were collected from cultivated plants from Botanical Garden, University of Agriculture, and Faisalabad, Pakistan (Figure 1). The seeds were identified and authenticated by Dr. Mansoor Hameed, Department of Botany, University of Agriculture, and Faisalabad, Pakistan. The seeds washed with deionized distilled water (DDW) and dried at 30 ̊C in a hot air oven (IM-30 m Irmeco, Germany) to constant weight.

2.2. Chemicals

Gallic acid, catechin, Folin-Ciocalteu, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and homologous series of C_9-C_{24} n-alkanes and various reference chemicals used for identification were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other analytical grade chemicals including anhydrous sodium carbonate, trichloroacetic acid, ferrous chloride, potassium ferricyanide, ammonium thiocyanate, aluminum trichloride and methanol used in this study were purchased from Merck (Darmstadt, Germany), unless stated otherwise. All culture media and standard antibiotic discs were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK).

2.3. Isolation of the essential oils

Five kilograms of dried *Foeniculum vulgare* seeds were subjected to hydro and steam distillation methods for 4 h under different temperature conditions (110, 115, 120, 125 and 130 ºC), using a pilot scale hydro and steam distiller. The distilled essential oils were dried over anhydrous sodium sulphate, filtered and stored in sealed vials at 4 ̊C, prior to further analyses.

2.4. Fractionation of essential oils

The extracted essential oils were fractionated using different fractionation techniques like low temperature chilling and vacuum distillation methods. Essential oils were fractionated using a laboratory vacuum distillation apparatus as reported in literature [23] with minor modifications. Fifty mL of essential oil was introduced in a round bottom flask connected to fractionating column, condenser and receiving apparatus. The fractionating column was 1.5 m in height and 30 mm in length. The temperature was determined at the top of column, and this temperature was used to define the distillation temperature. The distillation equipment was maintained under vacuum at -760 mmHg. In low temperature chilling, essential oil temperature was allowed to fall 1℃ per min under constant vacuum of -760 mmHg. On lowering temperature freeze part of essential oil was carefully separated from unfreezed part. And this process continued until no more freezing part could be separated.

2.5. Chemical analysis

The chemical analysis of most active essential oil (Steam distilled at 125 °C) and fraction (HC_{14}) were determined by Gas Chromatography–Mass Spectroscopy (GC–MS). GC-MS analysis of the most active essential oil extracted at 125 °C by steam distillation and fraction HC_{14} was performed using Agilent-Technologies 6890N Network gas chromatographic (GC) system, equipped with Agilent-Technologies 7890A inert XL Mass selective detector and Agilent-Technologies 5975C auto injector (Agilent-Technologies). Chromatographic conditions, injector and detector temperatures were set at 220 and 290 °C, respectively. Column temperature was programmed from 80 °C to 220 °C at a rate of 4 °C / min, lower and upper temperatures were held for 3 and 10 min, respectively. Helium was used as carrier gas at a flow rate of 1.5 mL/min. A sample of 1.0 µL was injected, using split mode (split ratio, 1:100). All quantifications were carried out using a built-in data-handling program provided by the manufacturer of the gas chromatograph (Perkin-Elmer). For MS detection, an electron ionization mode with ionization energy of 70 eV was used. Injector and MS transfer line temperatures were set at 220 and 290℃, respectively (Hussain *et al*., 2008).

2.6. Antioxidant activity

2.6.1. Total phenolic contents

To 1.0 mL of each essential oil, its fractions or gallic acid standard solution (20, 40, 60, 80 and 100mg/L), 5mL of Folin-Ciocalteu and 4 mL sodium carbonate (7% w/v) were added and samples were shaken to mix components completely. After keeping all the samples in dark for 30 min, absorbance was measured at 765 nm using a spectrophotometer (model 721D). Reagent solution was expressed as gallic acid equivalent (GAE) in milligram per gram of dry weight basis (Khan *et al*., 2012).

2.6.2. Total flavonoid contents

To 1.0 mL of essential oil, its fractions or catechin standard solution (20, 40, 60, 80 and 100 mg/L) was mixed with 4.0 mL of water in 10 mL volumetric flask followed by addition of 0.3 ml of 5% $NaNO_2$. After 5 min, 0.3 mL of 10% $AlCl_3$ added and after waiting for one more min, 2 mL of 1 M NaOH added and total volume made up to 10 mL using deionized distilled water (DDW). After mixing solution properly, absorbance reading measured at 510 nm using reagent blank. Amount of total flavonoids expressed as catechin equivalent in mg per g dry plant materials [24].

2.6.3. Free radical-scavenging activity

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity was carried out by adding DPPH solution (1 mL, 0.3M) to 2.5 mL solution of essential oil and its fractions or gallic acid standard. Then samples and standards

were incubated at room temperature in the dark for 20 min, finally absorbance was taken at 510 nm. The absorbance of control was prepared by adding 1.0 mL of methanol to 2.5 mL of extract solution without DPPH, while the positive control was prepared by adding 1.0 ml of DPPH solutions to 2.5 ml of gallic acid [25]. The percentage DPPH scavenging activity was calculated using expression.

$$DPPH\ scavenging\ activity\ (\%) = 100 - [(Abs\ sample/Abs\ control) \times 100]$$

2.6.4. Reducing power ability

The essential oil and its fractions (1.0 mL) or gallic acid standard solution (100, 200, 300, 400 and 500 mg/L) was mixed with 2.3 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide ($K_3[Fe(CN)_6]$). The mixture was incubated at 37°C for 20 min. Later on, 10% Trichloroacetic acid (2.5 ml, Sigma) was added to the mixture and centrifuged for 10 min at 1000 rpm, the supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$. After keep1ing solution for 10 min, the absorbance was measured at 700 nm.

2.7. Antifungal activity

In order to determine the antifungal activity of essential oil and its fractions, the pathogenic fungi *Aspergillus niger* (AN), *Aspergillus flavus* (AF) and *Ganoderma lucidum* (GL) were obtained from Biological Division of the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. Purity and identity were verified by the Department of Microbiology, University of Agriculture, and Faisalabad, Pakistan. Fungal strains were cultured overnight at 30°C using Potato dextrose agar (PDA, Oxoid). Briefly, 100 µL of suspension of tested microorganisms, containing 10^4 cfu/mL spores of fungal strains spread on PDA medium. The filter discs (6 mm in diameter) were individually soaked with 5 µL of essential oils or fractions and placed on the agar plates which had previously been inoculated with the tested microorganisms. Disc without samples were used as a negative control. Flumequine (30 µg/disc) were used as positive references, to compare sensitivity of strain/isolate in analyzed microbial species. The Petri dishes were kept at 4°C for 2 h. The plates were incubated at 30°C for 48 h for fungal strains. Antifungal activity evaluated by measuring the diameter of the growth inhibition zones in millimeters (including disc diameter of 6 mm) for test organisms and comparing to the controls. The measurements of inhibition zones carried for three sample replications, and values are average of three replicates.

2.7. Statistical analysis

All the experiments were conducted in triplicate and statistical analysis of the data were performed by analysis of variance (ANOVA) using STATISTICA 5.5 (Stat Soft Inc., Tulsa, OK, USA) software. A probability value at $p \leq 0.05$ was considered statistically significant. Data are presented as mean values ± standard deviation calculated from triplicate determinations.

3. Results and discussion

3.1. Percentage yield of essential oil and its fractions

In order to find the highest percentage yield of *Foeniculum vulgare* seeds essential oils in hydro and stream distillation method, experiments were performed at different temperature conditions (110, 115, 120, 125 and 130 °C) and results are shown in Table 1. The maximum percentage yield of essential oil 0.78% was obtained at 110 °C by hydro distillated oil, while minimum essential oil yield 0.44% was obtained at 130 °C by steam distillation method. In both hydro and steam distillation methods, it was observed that as extraction temperature increased percentage yield of essential oil was deceased. This might be due to degradation of essential oil at higher temperature. All the essential oils further separated into different fractions by vacuum distillation and low temperature chilling methods. In vacuum distillation method, fractions were obtained by slow heating essential oil at constant reduced pressure, while in low temperature chilling, fractions obtained by slow cooling of essential oil at constant reduced pressure. The average yield of each fraction in vacuum distillation and chilling method calculated from relationship between the fraction volume and essential oil volume as shown in Table 2 and 3, respectively.

3.2. Chemical composition of most active essential oil and its fraction

The gas chromatographic profiles and chemical compositions of the most active essential oil (steam distilled oil at 125 °C) and its fraction (HC_{14}) are shown in Table 4. Total nine compounds were identified in steam distilled oil at 125 °C that represents (99.8%) of the total amount of essential oil with estragole (39.05%) and L-fenchone (25.08%) as the major compounds followed by *Trans*-anethole (12.22%), L-limonene (12.16%), Dibenzo[b,f]1,5-dioxacyclooctane-4-methoxy-6,12-(ethylideno)- (4.07%), P-acetonylanisole (2.57%), 2-Hydroxy-2-(4-methoxy-phenyl)-N-methyl-acetamide (1.74%), apiol (1.73%) and α-pinene (1.4%). While, five compound compounds were identified in HC_{14} fraction including estragole (78.89%), L-Fenchone (6.38%), L-limonene (13.04%), α-pinene (1.12%) and apiol (0.57%). Previously, Ozcan *et al.* (2006) identified nine compounds from fennel seed oil along with estragole (61.08%) and fenchone (23.46%) as major compounds. Similarly, Mohamad *et al*. (2011) reported estragole (71.099%) as major compound followed by Bicyclo[3,3,1]non-2-en-9-ol,9-methyl (1.944%), P-menth-3-en-1-ol (0.726%), benzaldede (1.944%), 1-methyl-4-(1-methylethyl) (1.6781%); α-pinene (0.8202%), E-citral (0.313%), anethole (0.2718%), L-fenchone (0.1378%), β-pinene (0.1257%) and L-limonene (11.967%). Damjanović *et al.* (2005) identified *trans*-anethole (74.18%), estragole (5.29%), fenchone (11.32%), α-pinene (2.77%) and limonene (2.53%) as major components in *Foeniculum vulgare* seeds essential oil. Such variations in chemical composition of *Foeniculum vulgare* seeds essential oils might be attributed to the different extraction methods, extraction of essential oil at different maturation stages, geological, seasonal and climatically variation [18-20-26].

3.3. Antioxidant activity

3.3.1. Total Phenolic Contents (TPC)

Phenolic compounds are important plant constituents and acting as an antioxidant due to their hydroxyl groups. The amount of total phenolic contents (mg/L) of essential oil, measured as Gallic acid equivalent (GAE) from *Foeniculum vulgare* seeds essential oils extracted through hydro and steam distillation methods under different temperature conditions and its fractions through low temperature chilling and vacuum distillation given in Table 5. The amount of total

phenolic contents from different essential oils and its fractions, extracted from *Foeniculum vulgare* seeds were ranged from (13.10±0.00-705.99±0.02 mg/L as GAE). The maximum total phenolic contents were found in steam distilled essential oil at 125℃ (705.99±0.02 mg/L as GAE), while HV_2 fraction showed minimum total phenolic contents (13.10±0.00 mg/L as GAE). It observed that pure essential oils extracted under different temperature conditions through hydro and steam distillation methods reveals more total phenolic contents than its fractions obtained through low temperature chilling and vacuum distillation methods.

Furthermore, it observed that overall vacuum distillation fractions exhibited more total phenolic contents as compare to low temperature chilling fractions. In vacuum distillation fractions, HV_{12} fraction exhibited maximum total phenolic contents (539.67±0.10 mg/L as GAE), while HV_2 fraction exhibited minimum total phenolic contents (13.10±0.00 mg/L as GAE). In low temperature chilling fractions, HC_{14} fraction exhibited maximum total phenolic contents (429.30±0.02 mg/L as GAE), while HC_1 fraction exhibited minimum total phenolic contents (18.85±0.01 mg/L as GAE). Moreover, it observed last fraction of all essential oils showed more total phenolic contents than other fractions. No earlier reports are available on total phenolic contents of *Foeniculum vulgare* seeds essential oils and its fractions to compare the results of our present analysis. However, total phenolic contents of different extracts reported in literature. Methanol extract of *Foeniculum vulgare* seeds found to contain total phenolic contents of 29.64 mg/g (Gallic acid equivalent) [21]. In another study, ethyl acetate and diethyl ether extracts of aerial parts of *Foeniculum vulgare* contained 169 g/mg and 70 g/mg of total phenolic contents as caffeic acid equivalent [27].

3.3.2. Total flavonoid contents

Total flavonoids contents (mg/L) of essential oil, measured as catechin equivalent (CE) from *Foeniculum vulgare* seeds essential oils extracted through hydro and steam distillation methods under different temperature conditions and its fractions through low temperature chilling and vacuum distillation are given in Table 5. The maximum amount of total flavonoid contents were found in steam distilled essential oil at 125 ºC (410.75±0.01 mg/L as CE), while HV_{13} fraction showed minimum amount of total flavonoid contents (3.19±0.17 mg/L as CE). All the pure essential oils extracted under different temperature conditions through hydro and steam distillation methods showed higher amount of total flavonoid contents than its fractions obtained through low temperature chilling and vacuum distillation methods. Moreover, it was observed that overall vacuum distillation fractions contained higher total flavonoid contents as compare to low temperature chilling fractions. In vacuum distillation fractions, SV_{13} fraction showed maximum total flavonoid contents (123.47±0.02 mg/L as CE), while HV13 showed minimum total flavonoid contents (3.19±0.17 mg/L as CE). In low temperature chilling fractions, HC_{14} fraction showed maximum total flavonoid contents (128.18±0.04 mg/L as CE), while HC_3 showed minimum total flavonoid contents (6.39±0.11 mg/L as CE). It was also observed that last fraction contained higher amount of total flavonoid contents than other fractions. No earlier reports are available on the total flavonoid contents of *Foeniculum vulgare* seeds essential oil and its fractions for comparison.

3.3.3. Free radical-scavenging activity

Free radical-scavenging activity of *Foeniculum vulgare* seeds essential oils extracted through hydro and steam distillation methods under different temperature conditions and its fractions through low temperature chilling and vacuum distillation are given in Table 5. The examined *Foeniculum vulgare* seeds essential oils and its fractions changed the purple DPPH solution color to yellow that indicates DPPH reduced into DPPH-H. *Foeniculum vulgare* seeds essential oil obtained from steam distillation method at 125 ºC showed highest free radical-scavenging activity (99.64±0.04 %) than all other essential oils and fractions, while SC_6 fraction showed least free radical scavenging activity (71.00±0.05%). All samples showed a significant difference in free radical-scavenging activity and pure essential oils extracted under different temperature conditions reveals more free radical-scavenging activity as compare to its fractions. Furthermore, it was observed that overall vacuum distillation fractions showed higher free radical scavenging as compare to low temperature chilling fractions. In vacuum distillation fractions, SV_3 fraction showed maximum free radical scavenging (96.85±0.03%), while SV_5 exhibited least free radical scavenging (72.15±0.06%).

In low temperature chilling fractions, SC_4 fraction showed maximum free radical scavenging (94.62±0.06%), while SC_6 showed least free radical scavenging (71.00±0.05%). Previously, it was reported that free radical scavenging activity depends on the structural conformations of compounds that contained more phenolic contents may showed higher free radical scavenging [25]. Similar trend was observed in our finding that steam distilled oil at 125 ºC exhibited highest total phenolic contents and reveals maximum free radical scavenging activity (Table 5). Moreover, it was reported that *trans*-anethole is good radical scavenger and antioxidant activity is mostly related to the concentration of *trans*-anethole in essential oil [28]. Our findings are in good agreement with previous study that reported strong free radical scavenging activity of methanol extract of fennel seeds [21]. Another study reported the DPPH radical scavenging activity of water and ethanol extract 47.49% and 36.46% respectively, that was much lower than our finding [16]. Similarly, another study reported 57 % free radical scavenging capacity of the essential oil of aerial parts of *Foeniculum vulgare* through the hydro-distillation method [27].

3.3.4. Reducing power

Measurement of reducing power also imitates the antioxidant activity of plant material [29]. In reducing power assay a ferric ion are reduced to ferrous ions and change the color of reaction mixture. Reducing power depends on the intensity of color. Greater the intensity of the color, greater should be the absorption; consequently, greater should be the reducing power. *Foeniculum vulgare* seeds essential oil obtained from hydro-distillation method at 125 ºC showed highest reducing power, while SC_{17} fraction showed least reducing power. Furthermore, it was observed that overall low temperature chilling fractions showed higher reducing power than vacuum distillation fractions. No earlier reports are available on the reducing power of *Foeniculum vulgare* seeds essential oil and its fractions for comparison. However, few reports are available on reducing power of different extracts of *Foeniculum vulgare* seeds.

Table 1: Percentage yield (g/100g) of essential oils extracted through hydro and steam distillation methods under different temperature conditions from *Foeniculum vulgare* seeds.

Extraction method	Temperature (°C)	Percentage yield (g/100g of dry seeds)
Hydro-distillation	**110**	**0.78±0.039**
	115	0.66±0.045
	120	0.62±0.033
	125	0.62±0.034
	130	0.60±0.040
Steam distillation	**110**	**0.59±0.031**
	115	0.58±0.029
	120	0.56±0.028
	125	0.46±0.023
	130	0.44±0.022

Values are means ± standard deviation of three separate experiments.

Table 2: Fractionation of *Foeniculum vulgare* seeds essential oil by vacuum distillation.

Extraction temperature (°C)	**Fractions**	**Fractionation temperature (°C)**	**Yield % (v/v)**
110	HV_1	158	33.3±1.66
	HV_2	194.5	20±1.1
	HV_3	226	33.3±1.31
	HV_4	262	13.33±0.39
	SV_1	180	33.3±1.29
	SV_2	202	26.66±1.30
	SV_3	232	16.66±0.58
	SV_4	247	16.66±0.49
115	HV_5	175	33.3±1.20
	HV_6	197	13.33±0.45
	HV_7	213	26.66±1.25
	HV_8	234	26.66±1.22
	SV_5	162	26.66±1.20
	SV_6	200	20±1.1
	SV_7	240	33.3±1.65
	SV_8	265	20±1.2
120	HV_9	167	33.3±1.25
	HV_{10}	193	20±1.5
	HV_{11}	221	46.66±2.33
	SV_9	185	33.3±1.23
	SV_{10}	215	23.33±1.16
	SV_{11}	230	43.33±2.11
125	HV_{12}	183.7	36.66±1.83
	HV_{13}	204.5	20±1.2
	HV_{14}	224	43.33±1.29
	SV_{12}	202	50±1.35
	SV_{13}	227	23.33±1.21
	SV_{14}	249	26.66±1.19
130	HV_{15}	159	43.33±1.30
	HV_{16}	201	33.3±1.21
	HV_{17}	236	23.33±1.26
	SV_{15}	205	40±1.5
	SV_{16}	222	20±1.05
	SV_{17}	245	40±1.5

Values are means ± standard deviation of three separate experiments.
HV Vacuum distillation fractions of hydro distilled essential oil; SV Vacuum distillation fractions of steam distilled essential oil

Table 3: Fractionation of *Foeniculum vulgare* seeds essential oil by low temperature chilling under vacuum.

Extraction temperature (°C)	Fractions	Fractionation temperature (°C)	Yield % (v/v)
110	HC_1	-26.7	33.3±1.32
	HC_2	-25.2	33.3±1.32
	HC_3	-29.6	16.66±0.83
	HC_4	-31	16.66±0.93
	SC_1	-30	26.66±1.34
	SC_2	-32.2	33.3±1.38
	SC_3	-32.7	20±0.8
	SC_4	-33.5	20±0.10
115	HC_5	-27.1	33.3±1.45
	HC_6	-30.7	16.66±0.84
	HC_7	-32	33.3±0.99
	HC_8	-39.3	16.66±0.83
	SC_5	-23.9	46.66±1.39
	SC_6	-29	30±1.23
	SC_7	-32.8	13.33±0.67
	SC_8	-37.4	10±0.3
120	HC_9	-24	33.3±1.45
	HC_{10}	-28.1	26.66±1.34
	HC_{11}	-29.9	16.66±0.91
	SC_9	-29.2	43.33±1.30
	SC_{10}	-32.9	33.3±0.10
	SC_{11}	-35.3	23.33±0.69
125	HC_{12}	-25.3	46.66±2.34
	HC_{13}	-27.9	33.3±1.67
	HC_{14}	-28.5	20±0.12
	SC_{12}	-30.3	46.66±2.45
	SC_{13}	-33	36.66±1.84
	SC_{14}	-36.4	16.66±0.85
130	HC_{15}	-28.2	33.3±1.51
	HC_{16}	-29	20±0.12
	HC_{17}	-30.9	46.66±2.34
	SC_{15}	-30.7	46.66±2.45
	SC_{16}	-31.4	43.13±2.66
	SC_{17}	-33.2	10.2±0.51

Values are means ± standard deviation of three separate experiments.
HC Low temperature chilling fractions of hydro distilled essential oil; SC Low temperature chilling fractions of steam distilled essential oil.

Table 4: GC-MS of most active *Foeniculum vulgare* seeds essential oil (steam distilled oil at 125 ºC) and fraction (HC_{14}).

Sr. no.	Component	R.I.	% of total	
			Steam distilled oil at 125 ºC	Fraction (HC_{14}).
1	α-pinene	936	1.4±0.0	1.12±0.0
2	L-limonene	1030	12.16±0.3	13.04±0.4
3	L-Fenchone	8.352	25.08±0.7	6.38±0.2
4	Estragole	10.909	39.05±0.9	78.89±1.7
5	*Trans*-anethole	12.412	12.22±0.3	---
6	P-acetonylanisole	13.950	2.57±0.0	---
7	2-Hydroxy-2-(4-methoxy-phenyl)-N-methyl-acetamide	16.787	1.74±0.0	---
8	Apiole	1686	1.73±0.0	0.57±0.0
9	Dibenzo[b,f]1,5-dioxacyclooctane-4-methoxy-6,12-(ethylideno)-	25.34	4.07±0.1	---

Table 5: Antioxidant activities of *Foeniculum vulgare* seeds essential oils extracted through hydro and steam distillation methods under different temperature conditions and its fractions through low temperature chilling and vacuum distillation.

Extraction Temperature (°C)	Mode of Essential Oil Extraction & Fractions	Total Phenolic Contents (mg/L of seeds essential oil and its fractions, measured as Gallic acid equivalent)	Total flavonoid contents (mg/L of seeds essential oil and its fractions, measured as Catechin equivalent)	% Free Radical Scavenging Activity
110	Hydro-distilled essential oil at 110 °C	247.38±0.06	62.01±0.02	95.38±0.03
	HC_1	18.85±0.01	11.42±0.1	83.27±0.01
	HC_2	31.70±0.001	19.29±0.01	91.64±0.09
	HC_3	52.27±0.23	6.39±0.11	76.62±0.11
	HC_4	65.12±0.02	12.27±0.12	87.02±0.01
	HV_1	113.81±0.01	11.24±0.02	94.81±0.02
	HV_2	13.10±0.00	12.94±0.01	88.72±0.1
	HV_3	139.33±0.01	23.96±0.11	87.02±0.03
	HV_4	126.39±0.1	23.03±0.23	93.94±0.12
	Steam distilled essential oil at 110 °C	219.36±0.01	113.25±0.11	98.24±0.1
	SC_1	95.97±0.001	42.58±0.12	84.25±0.02
	SC_2	77.97±0.01	21.82±0.02	93.19±0.03
	SC_3	65.14±0.01	41.40±0.01	91.17±0.1
	SC_4	209.76±0.02	18.07±0.1	94.62±0.06
	SV_1	141.98±0.02	51.34±0.23	87.03±0.17
	SV_2	146.52±0.01	71.85±0.11	75.26±0.11
	SV_3	153.38±0.02	82.92±0.1	**96.85±0.03**
	SV_4	134.10±0.02	15.57±0.001	85.47±0.05
115	Hydro-distilled essential oil at 115 °C	344.89±0.01	139.91±0.12	95.87±0.14
	HC_5	41.98±0.13	73.99±0.10	86.90±0.11
	HC_6	43.27±0.02	65.85±0.03	75.26±0.09
	HC_7	59.12±0.01	80.76±0.01	76.94±0.08
	HC_8	74.54±0.1	22.24±0.09	92.49±0.03
	HV_5	136.50±0.01	19.13±0.03	93.64±0.15
	HV_6	144.81±0.01	29.59±0.11	94.51±0.06
	HV_7	147.38±0.01	31.98±0.10	87.39±0.11
	HV_8	126.39±0.02	23.30±0.01	88.77±0.1
	Steam distilled essential oil at 115 °C	394.25±0.02	114.81±0.02	96.90±0.05
	SC_5	54.84±0.01	51.33±0.01	81.19±0.03
	SC_6	55.69±0.03	61.85±0.15	71.00±0.05
	SC_7	85.68±0.01	72.92±0.01	87.51±0.04
	SC_8	319.61±0.01	55.57±0.001	94.21±0.1

	SV_5	135.38±0.01	73.97±0.07	72.15±0.06
	SV_6	147.38±0.01	56.18±0.16	93.21±0.03
	SV_7	157.75±0.02	37.47±0.05	91.98±0.11
	SV_8	161.52±0.01	13.86±0.01	76.19±0.04
120	Hydro-distilled essential oil at 120 °C	226.13±0.01	88.05±0.02	97.58±0.09
	HC_9	70.26±0.02	14.57±0.01	84.15±0.03
	HC_{10}	164.69±0.21	65.51±0.10	77.32±0.12
	HC_{11}	52.68±0.23	11.30±0.02	91.00±0.10
	HV_9	146.52±0.01	61.82±0.03	95.98±0.01
	HV_{10}	156.38±0.001	72.43±0.01	83.33±0.23
	HV_{11}	137.10±0.01	18.73±0.10	92.20±0.03
	Steam distilled essential oil at 120 °C	167.99±0.02	198.05±0.04	94.95±0.11
	SC_9	84.40±0.13	101.94±0.02	82.84±0.04
	SC_{10}	78.45±0.01	81.49±0.01	74.82±0.09
	SC_{11}	195.37±0.12	33.99±0.10	73.72±0.09
	SV_9	343.87±0.01	23.15±0.05	91.52±0.01
	SV_{10}	133.64±0.01	39.01±0.01	90.53±0.17
	SV_{11}	168.37±0.13	25.44±0.10	84.85±0.04
125	Hydro-distilled essential oil at 125 °C	687.23±0.01	358.14±0.02	98.76±0.03
	HC_{12}	112.16±0.01	8.07±0.03	91.25±0.05
	HC_{13}	74.20±0.02	118.28±0.10	83.22±0.01
	HC_{14}	132.04±0.01	**128.18±0.04**	91.56±0.10
	HV_{12}	**539.67±0.1**	112.4±0.001	87.63±0.09
	HV_{13}	167.95±0.02	3.19±0.17	76.91±0.03
	HV_{14}	172.23±0.11	19.20±0.01	81.19±0.11
	Steam distilled essential oil at 125 °C	**705.99±0.02**	**410.75±0.01**	**99.64±0.04**
	SC_{12}	165.80±0.01	103.76±0.02	75.02±0.15
	SC_{13}	177.29±0.01	121.93±0.01	84.98±0.07
	SC_{14}	429.30±0.02	99.44±0.03	94.52±0.04
	SV_{12}	176.00±0.01	110.03±0.01	83.16±0.01
	SV_{13}	185.08±0.1	123.47±0.02	75.72±0.03
	SV_{14}	179.14±0.01	33.21±0.04	93.25±0.01
130	Hydro-distilled essential oil at 130 °C	373.09±0.01	122.64±0.11	92.70±0.02
	HC_{15}	87.40±0.01	19.20±0.17	82.97±0.04
	HC_{16}	158.11±0.02	29.56±0.1	85.23±0.11
	HC_{17}	240.99±0.01	12.64±0.09	71.43±0.02
	HV_{15}	170.09±0.13	81.57±0.01	83.33±0.01
	HV_{16}	132.38±0.02	18.74±0.1	75.65±0.04
	HV_{17}	145.15±0.12	75.64±0.01	90.63±0.11
	Steam distilled essential oil at 130 °C	263.40±0.01	132.85±0.05	94.09±0.09
	SC_{15}	86.54±0.13	82.35±0.02	91.40±0.04
	SC_{16}	158.78±0.11	29.56±0.04	86.28±0.01
	SC_{17}	241.64±0.23	12.25±0.12	90.56±0.09
	SV_{15}	170.94±0.1	43.35±0.11	93.20±0.05
	SV_{16}	178.23±0.12	31.95±0.04	77.17±0.10
	SV_{17}	212.07±0.32	79.35±0.01	87.70±0.07

Values are means ± standard deviation of three separate experiments.
HV Vacuum distillation fractions of hydro distilled essential oil
SV Vacuum distillation fractions of steam distilled essential oil
HC Low temperature chilling fractions of hydro distilled essential oil
SC Low temperature chilling fractions of steam distilled essential oil

Table 6: Antifungal activity of *Foeniculum vulgare* seeds essential oils and its fractions against *Aspergillus niger*, *Aspergillus flavus* and *Ganoderma lucidum* by using disk diffusion assay.

Extraction Temperature (°C)	Mode of Oil Extraction & fractionation	*Aspergillus niger*	*Aspergillus flavus*	*Ganoderma lucidum*
		Zone of inhibition (mm)		
110	Hydro-distilled essential oil at 110 °C	18.14±0.81	13.88±0.64	18.62±0.91
	HC_1	11.64±0.58	5.93±0.25	16.21±0.85
	HC_2	13.1±0.65	7.74±0.39	15.94±0.77
	HC_3	9.86±0.50	5.21±0.25	9.17±0.46
	HC_4	9.11±0.46	8.45±0.43	16.08±0.69
	HV_1	8.67±0.51	9.04±0.48	17.69±0.78
	HV_2	15.43±0.75	6.11±0.31	13.77±0.64
	HV_3	8.98±0.49	10.02±0.55	12.67±0.61
	HV_4	7.69±0.38	2.56±0.13	8.09±0.39
	Steam distilled essential oil at 110 °C	17.46±0.72	18.37±0.98	19.87±0.89
	SC_1	13.7±0.68	5.78±0.29	8.78±0.43
	SC_2	9.77±0.49	9.89±0.50	9.37±0.45
	SC_3	12.75±0.64	11.7±0.55	7.74±0.34
	SC_4	8.68±0.44	15±0.77	2.99±0.13
	SV_1	7.5±0.41	6.66±0.35	15.45±0.38
	SV_2	6.89±0.35	8.64±0.42	8.56±0.33
	SV_3	10.6±0.54	3.13±0.15	12.7±0.52
	SV_4	5.78±0.29	6.99±0.39	4.53±0.32
115	Hydro-distilled essential oil at 115 °C	15.13±0.61	12.15±0.60	17.34±0.74
	HC_5	6.79±0.34	9.11±0.45	12.47±0.53
	HC_6	14.33±0.72	5.04±0.25	9.08±0.19
	HC_7	9.55±0.48	8.34±0.40	7.99±0.41
	HC_8	15.1±0.76	6.87±0.39	6.89±0.29
	HV_5	13.44±0.63	7.14±0.35	12.78±0.59
	HV_6	6.64±0.34	4.76±0.23	7.43±0.32
	HV_7	5.25±0.25	2.49±0.15	8.91±0.54
	HV_8	12.55±0.62	5.68±0.23	9.72±0.38
	Steam distilled essential oil at 115 °C	17.63±0.50	14.75±0.75	21.45±1.02
	SC_5	**16.13±0.83**	7.90±0.40	10.56±0.38
	SC_6	9.77±0.50	9.41±0.47	9.84±0.32
	SC_7	13.21±0.71	8.54±0.42	5.78±0.19
	SC_8	8.73±0.45	11.56±0.60	11.7±0.45
	SV_5	11.03±0.57	9.88±0.44	7.65±0.28
	SV_6	14.98±0.79	7.36±0.69	3.23±0.12
	SV_7	9.13±0.45	6.22±0.31	9.21±0.41
	SV_8	8.95±0.47	13.74±0.67	7.39±0.27
120	Hydro-distilled essential oil at 120 °C	16.56±0.90	10.10±0.55	15.97±0.69
	HC_9	3.64±0.12	13.34±0.76	11.98±0.47
	HC_{10}	11.3±0.61	7.09±0.35	13.55±0.56
	HC_{11}	9.88±0.34	9.16±0.58	8.75±0.35
	HV_9	5.7±0.25	5.75±0.28	13.6±0.52
	HV_{10}	9.13±0.46	8.45±0.45	7.47±0.35
	HV_{11}	4.89±0.24	3.53±0.15	6.43±0.23
	Steam distilled essential oil at 120 °C	19.34±0.95	13.88±0.56	17.89±0.77
	SC_9	15.6±0.71	11.33±0.52	15.41±0.67
	SC_{10}	4.14±0.22	6.54±0.33	12.14±0.51
	SC_{11}	7.13±0.36	3.99±0.15	7.52±0.33

	SV_9	8.67±0.44	8.71±0.43	9.44±0.37
	SV_{10}	9.11±0.56	5.67±0.31	6.5±0.24
	SV_{11}	11.0±0.65	2.44±0.10	13.7±0.58
125	Hydro-distilled essential oil at 125 °C	22.54±1.10	21.89±1.05	23.74±1.13
	HC_{12}	3.16±0.15	12.2±0.61	14.11±0.66
	HC_{13}	8.76±0.46	14.74±0.75	15.99±0.79
	HC_{14}	9.43±0.51	**17±0.85**	11.65±0.58
	HV_{12}	3.44±0.12	11.9±0.59	7.88±0.42
	HV_{13}	8.3±0.41	5.13±0.25	9.13±0.45
	HV_{14}	9.60±0.46	6.66±0.31	10.19±0.50
	Steam distilled essential oil at 125 °C	**24.33±1.40**	**28.26±1.53**	**25.56±1.47**
	SC_{12}	15.1±0.90	15.4±0.78	**18.67±0.93**
	SC_{13}	13.7±0.71	10.7±0.54	17.32±0.87
	SC_{14}	9.76±0.51	9.15±0.47	15.75±0.77
	SV_{12}	10.03±0.50	6.9±0.34	10.54±0.52
	SV_{13}	7.87±0.39	13.14±0.67	12.77±0.64
	SV_{14}	9.81±0.47	16.22±0.83	9.98±0.43
130	Hydro-distilled essential oil at 130 °C	15.17±0.81	10.2±0.75	13.89±0.65
	HC_{15}	11.8±0.59	8.45±0.43	11.77±0.54
	HC_{16}	9.74±0.42	6.34±0.33	9.04±0.39
	HC_{17}	5.4±0.27	7.88±0.40	10.19±0.62
	HV_{15}	8.64±0.42	9.27±0.46	3.22±0.14
	HV_{16}	9.72±0.46	6.42±0.31	7.43±0.31
	HV_{17}	6.17±0.31	3.94±0.19	8.51±0.34
	Steam distilled essential oil at 130 °C	11.76±0.61	15.57±0.78	14.78±0.63
	SC_{15}	9.88±0.52	12.24±0.62	7.99±0.59
	SC_{16}	6.17±0.35	8.53±0.43	2.77±0.14
	SC_{17}	11.3±0.55	11.06±0.54	11.09±0.48
	SV_{15}	12.9±0.65	7.89±0.40	13.4±0.57
	SV_{16}	8.65±0.44	3.09±0.14	6.23±0.22
	SV_{17}	4.54±0.21	9.37±0.46	9.12±0.45
Positive control	-----	23.66±1.25	13.94±0.69	14.04±0.79

Values are means ± standard deviation of three separate experiments.
Diameter of inhibition zone (mm) excluding disc diameter of 6 mm.
Positive control for fungi was Flumequine (30µg/disk)
HV Vacuum distillation fractions of hydro distilled essential oil
SV Vacuum distillation fractions of steam distilled essential oil
HC Low temperature chilling fractions of hydro distilled essential oil
SC Low temperature chilling fractions of steam distilled essential oil

Our results are comparable with the previous investigation which reported reducing power of fennel seed methanol and ethanol extract in range of 0.20–1.85 nm [26].

3.4. Antifungal activity

Inhibition zones of *Foeniculum vulgare* seeds essential oils extracted under different temperature conditions, its fractions and standard drugs (Flumequine) against three fungal strains (*Aspergillus niger*, *Aspergillus flavus* and *Ganoderma lucidum*) are given in Table 6. The values of inhibition zones of essential oils and its fractions against the three fungal strains were in range of (28.26-2.49 mm) and (23.66-13.94 mm) for positive control. As evidenced by the inhibition zones, all essential oils extracted under different temperature conditions showed larger inhibition zones than standard drug, while all fractions showed smaller inhibition zones than pure essential oils. Difference in inhibition zones in pure essential oils and fractions clearly indicates presence of synergetic effect of compounds present in essential oil. *Foeniculum vulgare* seeds essential oil extracted by steam distillation method at 125°C reveals highest antifungal activity with largest inhibition zones (24.33 mm, 28.26 mm and 25.56 mm) against *Aspergillus niger*, *Aspergillus flavus* and *Ganoderma lucidum*, while standard drug Flumequine (30 µg/disc) exhibited (23.66, 13.94 and 14.04 mm inhibition zones), respectively.

Overall, pure essential oils extracted by steam distillation method showed larger inhibition zones (28.26-11.76 mm) than essential oils extracted by hydrodistillation method (23.74-10.1 mm), while in different fractionation methods, low temperature chilling fractions showed larger inhibition zones (18.67-2.77 mm) than vacuum distillation

fractions (17.69-2.44 mm). GC-MS analysis of most active essential oil and fraction showed estragole and fenchone as major compounds that might be responsible for their antifungal activity. Previously, it was reported that *trans*-anethole, estragole and fenchone are major compound of *Foeniculum vulgare* seeds essential oil and responsible for antimicrobial activities [28-30-31]. Our findings are in good agreement of with previous studies that reported antifungal activity of steam and hydro-distilled essential oil of *Foeniculum vulgare* seeds against *Aspergillus niger* with inhibition zone of (22.7 mm) and (28 mm) by disc diffusion assay, respectively [26-32]. Another study, reported antifungal activity of *Foeniculum vulgare* fruit essential oil against *Aspergillus niger* and *Candida albicans* with minimum inhibition zones (16.0 mm and 19.0 mm, respectively) much lower than our finding [28]. Furthermore, no earlier reports are available on antifungal activity of *Foeniculum vulgare* seeds fractions for comparison.

4. Conclusions

This study, first time reports the bioactivities of essential oil fractions. Following major conclusions can be withdrawn from following study.

- In both hydro and steam distillation methods, it was observed that as extraction temperature increased percentage yield of essential oil was deceased.
- Total nine compounds were identified in steam distilled oil at 125 ºC that represents (99.8%) of the total amount of essential oil with estragole (39.05%) and L-fenchone (25.08%).
- The maximum antioxidant activity was found in steam distilled essential oil at 125℃. The values of inhibition zones of essential oils and its fractions against the three fungal strains were in range of (28.26-2.49 mm) and (23.66-13.94 mm) for the positive control.

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