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ORIGINAL RESEARCH

Chemical Composition and Antimicrobial and Cytotoxic Activities of *Foeniculum vulgare* Mill Essential Oils

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Abstract

Background and aim Foeniculum vulgare (*F. vulgare*) Mill, commonly known as fennel, belongs to the Umbelliferae (Apiaceae) family, biennial or perennial herbs disseminated in Mediterranean region and central Europe. This herbal medicine (HM) is considered as a traditional HM, and its parts have been studied.

Methods In this survey, essential oils from seeds collected from three various regions (Kerman, Golestan, and East Azerbaijan Provinces) of Iran were prepared with hydro-distillation and their components were analyzed with gas chromatography (GC) and chromatography time-of-flight mass spectrometry (GC/MS). Antimicrobial and cytotoxic activities of the essential oils were examined with disk-diffusion method on Muller–Hinton agar and Subaru-dextrose Agar, respectively. Additionally, the MTT assay was assessed on breast cancer cell line (MCF-7). The expression of apoptosis-related genes, *Bax* and *Bcl2*, was determined using quantitative real-time PCR (RT-qPCR).

Results The major fractions of essential oils identified by GC and GC/MS included *trans*-anethole (78.47%, 49.64%, 78.68%), fenchone (10.5%, 8.4%, and 10.2%), and limonene (5.9%, 6.70%, and 5.6%), respectively. Fennel oil collected from three various places exerted inhibitory effects on the bacterial growth and higher cytotoxic effects on MCF-7 cancer cell line. In addition, the essential oil increased the expression of *Bax*, but decreased *Bcl2* gene expression significantly ($P < 0.001$).

Conclusion According to our findings, *F. vulgare* essential oil can be considered as a promising agent opening venues for novel antimicrobial and anticancer therapies. Owing to side effects and expensiveness of chemotherapy approaches, HM is a new remarkable insight for future therapies.

Keywords *Foeniculum vulgare* · Antimicrobials · Cell cytotoxicity · Bax · Bcl2

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Introduction

Foeniculum vulgare (*F. vulgare*) Mill or fennel belongs to the Umbelliferae (Apiaceae) family, as biennial or perennial herbs disseminated in Mediterranean and central Europe regions. This herbal medicine (HM) is considered as a traditional HM for its antibacterial, antifungal, anti-inflammatory, antithrombotic, antidiabetic, and hepatoprotective activities [1, 13, 28, 33]. Emergence and spread of multidrug-resistant, extensive drug-resistant, and pandrug-resistant (MDR, XDR, and PDR, respectively) infectious agents are a crisis restraining the eradication of related infections [18, 22].

Although the commercial antibiotics have exerted efficient therapeutic effects for controlling various infectious diseases, several adverse events such as lethal hypersensitivity reactions, unpleasant effects, higher costs, and developed antimicrobial resistance have been reported [11–13]. Additionally, a

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critical global issue in antimicrobial chemotherapy is the extensive antibiotic resistance, leading to the inefficiency of antimicrobial agents [4, 15, 21, 27]. Thus, searching for novel, highly efficient, and safe agents toward developing alternative anti-infections approaches is essential.

In addition, human cancers and carcinoma are a great concern worldwide with high death rate. Hence, investigation and evaluation of novel compounds or alternatives against cancer cell progress seem pivotal.

Several studies have unraveled the efficacy of HM as potential sources of novel anticancer compounds to be appropriate for the development of better therapeutic agents [25, 30]. Nowadays, the antimicrobial traits of HM have been assessed deeply by some authors and are considered as safe compounds [2, 10, 16]. Considering this, the antimicrobial activity of the HM essential oils extracted against various pathogenic microorganisms has been reported to some extent [3]. *F. vulgare* HM widely grows in temperate and tropical areas worldwide. The *F. vulgare*'s aromatic fruits are applied as a culinary spice, and this herb is considered as a traditional and popular HM [26, 29]. Numbers of studies have revealed that *F. vulgare* extract can efficiently control the numerous disorders through antioxidant, anti-infective, anticancer, chemopreventive, cytoprotective, hepatoprotective, hypoglycemic, and oestrogenic traits [14, 31]. Fennel essential oil is rich in anise aldehyde interfering with molecular targets in cells. Several fennel bioactive components including tannic, caffeic, chlorogenic, cinnamic, ferulic, vanillic, and gallic acids have exerted anticancerous, cytotoxic, genotoxic, and apoptotic traits [33]. Because of lipophilic nature of essential oil fraction, they can conveniently penetrate cytoplasmic membranes by free diffusion; furthermore, the antimicrobial and anticancer activities are also attributed to anethole as the fennel seed bioactive compounds [6, 7, 9, 20, 23, 32]. In this study, some funnel anticancer, antimicrobial traits were evaluated.

Materials and Methods

Plant Material

The fully ripened fennel (*F. vulgare* Mill) seeds were collected from the three types of different regions of Iran, including Kerman, Golestan, and East Azerbaijan Provinces. The samples were collected and dried at ambient temperature. The seeds were kept in glass containers for further study.

Extraction and Preparation of the Essential Oil

For extraction of the *F. vulgare* seed essential oil, the dried samples of seeds (mg/100 g, dry matter) were ground into small pieces separately and subjected to hydro-distillation for 5 h using a Clevenger-type apparatus; the obtained oils were dried over anhydrous sodium sulfate.

Gas-Liquid Chromatography-Mass Spectrometry

GC/MS analysis of three sample essential oils was performed by a HP-6890 gas chromatograph (HP-5MS column with 0.25-μm film thickness and 60 m × 0.25 mm i.d.) and with a mass detector (HP-6973) from the company. The analysis was fulfilled on applied silica HP-5MS capillary column (60 m × 0.25 mm i.d.; film thickness 0.25 μm). Additionally, the injector and detector parts were kept at 250 and 300 °C, respectively. Oven temperature program was 60 to 250 °C at the rate of 4°/min and finally held isothermally for 15 min [36].

Identification of Compounds

The compounds were characterized via comparison of retention indices (RI, HP-5) with those reported from previous studies and compared to their mass spectra with the Wiley library or with published mass spectra [34].

Antimicrobial Activity

For assay, the antimicrobial activity of the fennel essential oil, disk-diffusion method on Muller–Hinton agar, and Subaru-dextrose agar were investigated. They were individually tested against a panel of pathogenic agents, including *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus epidermidis* ATCC 12228, and *Candida albicans*. For this approach, the seed essential oils and extracts were dissolved in dimethyl sulfoxide (DMSO 10 mg ml^{-1}) separately and sterilized. Under aseptic conditions, empty or blank sterilized disks (diameter) were impregnated with 100 μl doses of 100, 50, 25, and 12.5 μg ml^{-1} extracts and dosages of 50, 12/5, and 25 μg ml^{-1} essential oil and then put onto the agar surface. Subsequently, the plates were placed at room temperature for 30 min to allow the diffusion of the seed oil or extracts, and then incubated at 37 °C. Following an overnight incubation, the inhibition zone diameter was measured in millimeter and values < 12 mm were considered as a deactivate essential oil against bacteria. Gentamicin and nystatin were applied as control antibiotics for the comparison. Each antibacterial test was conducted in three replicates, and the averages were considered as a mean value. In addition, the essential oil minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was determined using broth microdilution ranging 0.50–512 μg/ml (CLSI version 2016).

Cytotoxicity Assay on Cancer Cell Line

The MTT colorimetric assay was applied to investigate the cytotoxic traits of the fennel essential oil on MCF-7 breast cancer cell line. Briefly, the cells were counted, and next, distributed into 96-well plates at a concentration of 10^4 cells per well, followed by an overnight incubation at 37 °C in a CO_2 incubator. Afterwards, various densities of the fennel essential oil, including 100, 50, 25, 12.5, 6.25, and 3.125 mg/ml were subjected to the cell lines in the 96-well plates for an overnight. Next, the 96-well plate contents were carefully discarded, and the MTT (Sigma Aldrich, Germany) was added to the wells and the reaction mixture was kept in a 5% CO_2 atmosphere at 37 °C for 4 h; by the removal of the MTT dye and the formazan crystals dissolving with isopropanol, the absorbance amounts of the samples were obtained using an ELISA Reader (Oraganon Teknika, Netherlands) at 570 nm. Therefore, the rate of cytotoxicity was measured considering the following formula:

Cell survival rate

$$: (\text{absorbance of control cells}/\text{absorbance of treated cells}) \times 100$$

Additionally, the half-maximal inhibitory concentration (IC50) was measured.

Bax and Bcl2 Gene Expression Analysis

The *Bax* and *Bcl2* apoptosis-related gene expression was analyzed using RT-qPCR. At first, the total RNA of fennel essential oil treated and non-treated cells was isolated using an RNA extraction kit, following the manufacturer's guidelines (Qiagen, USA). After OD value checking and DNase treatment, the total cDNA was synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania); the reaction mixture included 5 μl of the 5× reaction buffer, 0.5 μl of a random hexamer primer, 0.5 μl of the oligo dT primer, 1 μg of the total RNA, 2 μl of the deoxynucleotide triphosphate mixture (10 mM), 1 μl of reverse transcriptase enzyme, 1 μl of RNase enzyme inhibitor (20 units/ μl), and double-distilled water (up to a final volume of 20 μl). Additionally, the thermal profile included 25 °C for 5 min (annealing), 42 °C for 60 min, 70 °C for 5 min, and 4 °C for 5 min. The specific primer sequence of the *Bax* and *Bcl2*, target genes, and *β-actin* (internal control) are given in Table 1. Finally, the RT-qPCR reaction was performed using LightCycler (Bioneer, South Korea) at a temperature program as follows: 95 °C for 1 min, 95 °C for 15 s, and 60 °C for 60 s.

Table 1 The primer sequences of target genes

Target genes	Primer sequence (5′ to 3′)
Bax (FW)	TTGCTTCAGGGTTTCATCCAG
Bax (REV)	AGCTTCTTGGTGGACGCATC
Bcl2 (FW)	TGTGGATGACTGAGTACCTGAACC
Bcl2 (REV)	CAGCCAGGAGAAATCAAACAGAG
β-actin (FW)	TCCTCCTGAGCGCAAGTAC
β-actin (REV)	CCTGCTTGCTGATCCACATCT

Statistical Analysis

Statistical analysis was performed using SPSS statistical software version 19.0. Analysis of variance (ANOVA) and *t* tests were applied for comparing two essential seeds' oil. Values were considered as mean ± SEM, and 뭔 < 0.05 was given as a statistically significant level.

Ethical Approval

This study was ethically approved by Islamic Azad University, Roudehen, Iran.

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Results

Essential Oil Yields

According to the results, the yields of essential oils for Kerman, East Azerbaijan, and Golestan Provinces included 1.84%, 1.61%, and 1.77% (*v*/*w*), respectively.

Chemical Composition of Essential Oil

Based on the Table 2, analysis results from Kerman *F. vulgare* seed's compaction essential oil revealed 21 types of various bioactive compounds among which major fractions included *trans*-anethole (78.5%), fenchone (10.5%), limonene (6.9%), and tarragon (2.3%), respectively. In parallel, in Golestan Province, the analysis demonstrated *trans*-anethole (79.6%), fenchone (8.5%), limonene (6.71%), tarragon (2.3%), and α-pinene (1.05%), respectively. Additionally, that from East Azerbaijan Province contained 21 compounds mostly such as *trans*-anethole (78.7%), fenchone (10.2%), limonene (5.6%), and tarragon (2.5%), respectively. In addition, the tested essential oil contained incredible amounts of various other fractions (< 10%) with different chemical compositions (Table 2). The data clearly indicated that the essential oils of fennel are dominated by oxygenated monoterpenes (*trans*-

Table 2 The bioactive fractions of essential oils from fennel grown in Kerman, Golestan, and East Azerbaijan Provinces

No.	Compound name[a]	% Kerman	% Golestan	% East Azerbaijan	RI[b]
1	α-Thujene	0.01	0.01	0.01	926
2	α-Pinene	0.79	1.05	0.83	938
3	Camphene	0.12	0.14	0.12	956
4	β-pinene	0.38	0.35	0.33	977
5	α-Phellandrene	0.12	0.11	0.11	1008
6	Cymene	–	–	0.03	1022
7	Limonene	5.90	6.79	5.65	1024
8	β-Phellandrene	0.24	0.23	0.25	1026
9	*cis*-Ocimene	0.52	0.42	0.46	1039
10	*trans*-Ocimene	0.07	0.06	0.07	1045
11	γ-Terpinene	0.07	0.05	0.06	1055
12	Fenchone	10.54	8.46	10.20	1083
13	α-Terpinolene	0.03	0.03	0.04	1086
14	Camphor	0.22	0.10	0.15	1143
15	Borneol	0.03	–	–	1166
16	Unknown	2.37	2.39	2.53	–
17	*trans*-Anethole	78.47	79.65	78.68	1282
18	Borneol acetate	0.07	0.06	0.42	1184
19	α-Copaene	0.03	0.04	0.03	1378
20	*trans*-Caryophyllene	0.01	0.03	0.01	1419
21	α-Bergamotene	–	0.02	0.01	1435
22	γ-Cadinene	0.01	0.01	0.01	1514

[a] Identified compounds listed in order of elution from HP-5MS column

[b] Relative Kovats retention indices to C8-C24 n-alkanes on HP-5MS column

anethole, fenchone, and tarragon), monoterpene hydro-carbons (6.5%), and sesquiterpene hydro-carbons (0.35%).

Antimicrobial Activity

The antibacterial effects of the essential oil from the fennel seeds against a panel of microorganisms have been shown in Table 3. Accordingly, all three different essential oils from *F. vulgare* exhibited remarkable antimicrobial effects against several of the microorganisms as compared to the control group ($P < 0.05$), but no antimicrobial effect by the all three essential oils was observed in 6.25 μg/ml. Accordingly, *F. vulgare* fennel essential oil revealed increased antibacterial activity at higher dosages. The fennel essential oils showed remarkable antimicrobial traits against all the tested strains, particularly against *E. coli* and *S. aureus* (Table 3).

The results of the disk diffusion method clarified that *E. coli* and *S. aureus* were the most sensitive microorganisms tested, displaying the inhibition zones of 20 and 18 mm, respectively, with a concentration of 50 μg/ml. The lowest activity was exhibited against *C. albicans* with the lowest inhibition zones of 10 mm at the concentration of 100 μl/disk as compared to gentamicin (4 μg/cup) and nystatin (100 IU/cup) positive controls. Furthermore, the lowest MIC and MBC of essential oil were against *S. aureus* including 64 μg/ml and 128 μg/ml, respectively, followed by 128 μg/ml and 256 μg/ml, respectively, against the both *E. coli* and *S. epidermidis* strains.

Cytotoxicity Assay

The cytotoxicity of *F. vulgare* essential oil was evaluated using the colorimetric MTT assay with various concentrations. The results demonstrated that the *F. vulgare* essential oil conferred inhibitory effect on MCF-7 cells at the concentrations of 50 μg/ml and 100 μg/ml ($P < 0.001$) increasing in a dose-dependent manner. Furthermore, the IC_{50} of *F. vulgare* essential oil in Kerman, Golestan, and East Azerbaijan was calculated to be 14.93, 11.34, and 15.93 mg/ml, respectively (Fig. 1). Among different *F. vulgare* essential oils, that collected from *F. vulgare* in Golestan Province was more cytotoxic against cancerous cells as compared with those from other regions.

Bax and Bcl2 Gene Expression Analysis

Treatment of MCF-7 cell line with IC_{50} of fennel essential oil from Kerman, East Azerbaijan case, and Golestan cases clarified the upregulated expression of *Bax* with values including 3.18 ± 0.52, 2.61 ± 0.92, and 3.62 ± 0.62, respectively.

Table 3 The antimicrobial activities in terms of inhibition zones of fennel essential oil of Kerman, Golestan, and East Azerbaijan Provinces against the selected microorganisms

Zone of inhibition (mm)								
Provinces	Concentration microorganism	50 μg/ml^{-1}	25 μg/ml^{-1}	12.5 μg/ml^{-1}	6.25 μg/ml^{-1}	Positive control GA	Positive control Ni	Negative control DMSO
Kerman	*S.* aureus	18 ± 1	15 ± 0.5	7 ± 0.3	–	28 ± 2	–	–
	S. epidermidis	14 ± 1.5	13 ± 1.75	5 ± 0.8	–	27 ± 3.8	–	–
	E. coli	20 ± 0.5	17 ± 0.75	10 ± 1	–	25 ± 2.2	–	–
	P. aeraginosa	13 ± 1.25	11 ± 1.2	6 ± 1.2	–	28 ± 1.9	–	–
	C. albicans	10 ± 1	6 ± 0.5	3 ± 0.1	–	–	28 ± 2.5	–
Golestan	*S.* aureus	19 ± 1.3	16 ± 3.2	12 ± 1.1	–	28 ± 2.8	–	–
	S. epidermidis	17 ± 1	11 ± 1.4	9 ± 0.5	–	28 ± 3.2	–	–
	E. coli	19 ± 2.3	16 ± 1.8	11 ± 0.8	–	26 ± 3.5	–	–
	P. aeraginosa	13 ± 1.7	10 ± 1.2	8 ± 0.5	–	27 ± 1.9	–	–
	C. albicans	9 ± 0.3	5 ± 1.2	2 ± 0.3	–	–	28 ± 3	–
East Azerbaijan	*S.* aureus	19 ± 1.9	15 ± 1.1	9 ± 0.8	–	27 ± 2.2	–	–
	S. epidermidis	19 ± 1.2	10 ± 0.8	8 ± 0.3	–	26 ± 3.6	–	–
	E. coli	19 ± 1.8	16 ± 1.2	11 ± 0.8	–	27 ± 1.9	–	–
	P. aeraginosa	19 ± 1	9 ± 0.6	7 ± 0.5	–	28 ± 2.2	–	–
	C. albicans	11 ± 0.5	8 ± 0.4	4 ± 0.5	–	–	28 ± 3.3	–

GA gentamycin, *Ni* nistatin, *S. aureus Staphylococcus aureus*, *S. epidermidis Staphylococcus epidermidis*, *E. coli Escherichia coli*, *P. aeraginosa Pseudomonas aeruginosa*, *C. albicans Candida albicans*

Moreover, *Bcl2* gene expression was downregulated in all cases (0.31 ± 0.18, 0.43 ± 0.72, and 0.26 ± 0.49, respectively) (Fig. 2). Overall, the results of gene expression in treated cancer cells indicated the apoptosis-inducing effect of the *F. vulgare* essential oil.

Discussion

It has been well known that the extensive antibiotic resistance by microorganisms has been dramatically increased in recent decades worldwide needing unmet substitutes or alternatives. Additionally, cancer chemotherapy has conferred similar problems. For a long time, HM has displayed indispensable role in the infection eradication and cancer therapy approaches as they are potential sources of novel anticancer and antibiotic bioactive compounds. Major bioactive compounds of fennel according to previous studies mostly include *trans*-anethole (between 81.63% and 87.85%), estragole, fenchone, and limonene depending on the chemotype [33]. In the current study, the bioactive compounds of *F. vulgare* from various areas was nearly similar exhibiting no considerable effects by the various ecological factors. Similar to our data, in a study with the assessment of the extracted essential oils from three different

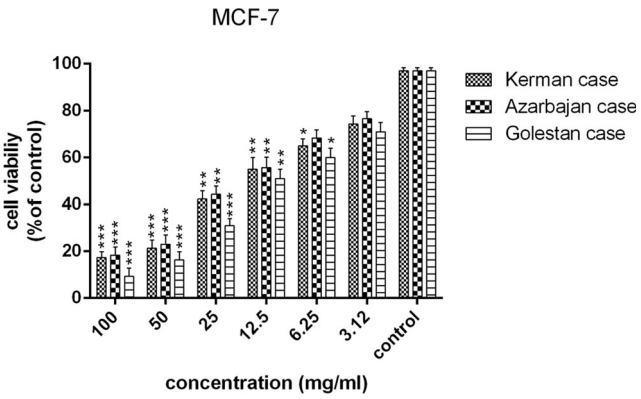

Fig. 1 Survival rate of MCF-7 cancer cells against different concentrations of *F. vulgare* essential oil over a 24 h. Results have been reported as survival rate compared with control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; $n = 3$)

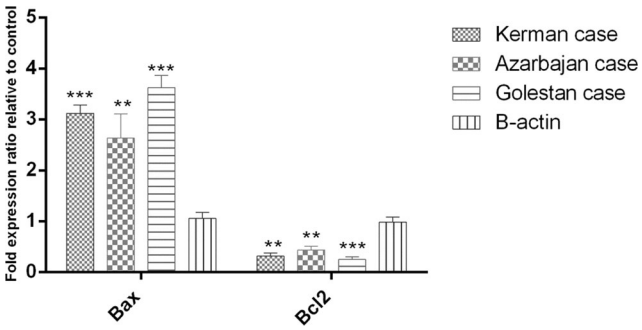

Fig. 2 The expression level of Bax and Bcl2 genes comparing to the control gene. The expression ratios of Bax and Bcl2 genes comparing to the reference gene (β-actin) in the MCF-7 cells treated with the *F. vulgare* essential oil in 24 h were changed (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; $n = 3$)

regions of grown cultivars of Egyptian fennel confirmed the presence of 18 major monoterpenoids including *trans*-anethole, estragole, fenchone, and limonene with different percentage. Furthermore, *trans*-anethole (70.1%), fenchone (8.6%), methyl chavicol (4.7%), and limonene (3.1%) have been mentioned as the major compounds identified in the essential oil from *F. vulgare* in India [36]. Moreover, in another study, the major components of the essential oil of Turkey bitter fennel (*F. vulgare* spp. piperitum) grown were estragole (61.08%), fenchone (23.46%), and limonene (8.68%) [35]. Aldehyde-containing essential oils can react with cellular nucleophiles leading to cytotoxic activities. It is noteworthy that fennel essential oil exhibited hemolytic activity at a concentration of 1100 mg/l on cancer cells as well as cell deformation and affecting the integrity of cell membranes [33]. Anethole, the major fraction of fennel, has exhibited cytotoxicity on RC-37 cell line at 100 mg/l, ATP depletion, and DNA degradation [33]; therefore, similar to previous studies, this fraction is possibly the main anticancerous agent.

We observed that the *F. vulgare* essential oil exerted cytotoxic effects against MC-F7 cancer cells at the concentrations of 50 μg/ml and 100 μg/ml ($P < 0.001$) in a dose-dependent manner.

The results of gene expression in treated cells unraveled that the *F. vulgare* essential oil can induce the apoptosis indicating anticancerous potential of the essential oils extracted from *F. vulgare* Mill seeds. Similarly, some previous studies have suggested the anticancer and antidiabetic traits of some compounds of this HM [9, 19, 24]. Noticeably, estragole (methyl chavicol), another major fraction of fennel, has exhibited anticancer traits in rodents.

In this study, the antibacterial activity of the essential oil was assessed and the results were in agreement with findings of Al-zoreky and Nakahara indicating that Gram-negative bacteria strains, especially *E. coli*, have lower sensitivity to fennel seed extracts. It has been reported that the fennel essential oils exhibited an inhibitory effect against all studied fungi and bacteria, but fennel seed extracts exerted no desirable antimicrobial activity. Furthermore, the highest activity of essential oil was against *S. aureus* with MIC and MBC of 64 μg/ml and 128 μg/ml, respectively, followed by 128 μg/ml and 256 μg/ml, respectively, against the both *E. coli* and *S. epidermidis* strains. The antimicrobial activities of the essential oils, ethanol, a phenylpropanoid derivative, and scopoletin have been clarified against foodborne pathogens and human pathogenic bacteria, such as *Campylobacter*, *Helicobacter* genera, and drug-resistant *Acinetobacter baumannii* [1, 13]. The antifungal activity of fennel extracts has been clarified by Khosravi et al. [5, 8, 17]. Owing to the spread of new strains and re-emerging infectious agents and evolution of non-susceptibility against synthetic antibiotics, the in-depth assessment of *F. vulgare* oil and seed extracts and its formulations can be promising giving insights as novel and effective alternatives for the eradication of various infections.

In conclusion, our findings confirmed the antimicrobial (mostly against *S. aureus*) and anticancer activities of the essential oils extracted from *F. vulgare* Mill seeds. Our data also outlined the apoptotic-inducing traits of *F. vulgare* through Bax- and Bcl2-mediated pathways, though more in-depth studies are essential toward elucidation of cellular mechanisms related to fennel anticancerous traits. The bioavailability, pharmacokinetics, and physiological pathways affected by fennel fractions should be also characterized profoundly.

Author Contributions MT conceived the ideas and collected data; AGH and SKM analyzed the data and led the writing.

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Compliance with Ethical Standards

This study was ethically approved by Islamic Azad University, Roudehen, Iran.

Conflict of Interest The authors declare that they have no conflict of interest.

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