Future Natural Products
doi: 10.34172/fnp.2022.04 2022;8(1):15-22 http://futurenatprod.skums.ac.ir

Original Article

Foeniculum vulgare and *Trachyspermum ammi* seed ethanolic extracts: Cytotoxicity assay, *in vitro* toxicity on *Artemia salina* larvae, biocompatibility and antibacterial activity

Samaneh Rahamouz-Haghighi[1*], Malek Hossein Asadi[2], Zahra Gharari[3], Ali Sharafi[4,5]

[1]Department of Plant Production and Genetics, Faculty of Agriculture, University of Zanjan, Zanjan, Iran
[2]Department of Biotechnology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran
[3]Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran
[4]Zanjan Pharmaceutical Biotechnology Research Center, Zanjan University of Medical Sciences, Zanjan, Iran
[5]Department of Pharmaceutical Biotechnology, School of Pharmacy, Zanjan university of Medical Sciences, Zanjan, Iran

Abstract

Background and aims: *Foeniculum vulgare* and *Trachyspermum ammi* belonging to the family Apiaceae are two traditional popular herbs in Traditional Iranian Medicine. Given the wide application of *F. vulgare* and *T. ammi* for treatment of gastrointestinal disorders, the objective of this study was to investigate their biological activities.

Methods: The cytotoxicity of ethanol seed extract of *F. vulgare* and *T. ammi* was evaluated on colorectal cancer (HCT116 and SW480) and human embryonic kidney cell lines (HEK293) by MTT assay. Toxicity and biocompatibility of RBC's also were assessed by *Artemia salina* and hemolysis tests. The antibacterial activities and minimum inhibitory concentrations (MICs) of extracts were measured by disc diffusion and the microtiter broth dilution, respectively.

Results: The proliferation of cancer cells was inhibited by ethanol seed extracts. A moderate degree of cytotoxicity was observed for HCT116 cells growth (IC_{50}=106.46 µg/mL) by *T. ammi* extracts at 72 hours. In addition, the ethanol seed extracts of *F. vulgare* and *T. ammi* exhibited no cytotoxic effects against brine shrimp larvae with LC_{50} of 2071.65 and 1576.92 µg/mL, respectively. The degree of hemolysis for ethanol seed extracts was less than 5% at 400 µg/mL. The maximum antibacterial activity was obtained for ethanol extracts of *F. vulgare* and *T. ammi* against *S. aureus* by disc diffusion (25.8 and 28.3 mm) and MIC (0.55 and 0.39-fold).

Conclusion: The ethanol extracts of *F. vulgare* and *T. ammi* have antiproliferative and antibacterial properties and could be used as adjuvant therapies against gastric and colorectal cancers and pathogenic bacteria.

Keywords: Ajowan, Cytotoxicity assay, Fennel, Hemolysis assay, Minimum inhibitory concentration

***Corresponding Author:**
Samaneh Rahamouz-Haghighi,
Tel:+989151236427,
Email: rahamouz_haghighi.s@yahoo.com

Received: 27 December 2021
Accepted: 17 January 2022
ePublished: 30 June 2022

Introduction

The use of herbal medicines is increasing across the world. In order to advance the appropriate use of herbal medicinal products and demonstrate their capability to serve as sources of new medicines, it is crucial to evaluate medicinal herbs in terms of phytochemical content and biological activities (1).

Foeniculum vulgare Mill (fennel) and *Trachyspermum ammi* L. (ajowan) are two herbs belonging to the family Apiaceae with a broad background of use as medicine and food additive (2). Being a valuable source of bioactive compounds like carvacrol, thymol, p-thymene, terpinene, flavonoids, phenolic glycosides, triterpenes, phytosterols and saponins, *F. vulgare* has attracted growing attention for large-scale cultivation (3). Furthermore, *F. vulgare* has also been reported to contain different phenolic compounds, including rosmarinic acid, caffeoylquinic acid, kaempferol-3-O-glucoside, eriodictyol-7-orutinoside and quercetin-3-O-galactoside, confirming the plant's antioxidant activity (4). *F. vulgare* has also been reported to produce hepatoprotective (5) and immunomodulatory (6) effects as well as pain-killing effect in primary dysmenorrhoea (7). Several studies have shown that *F. vulgare* is effective against various infections such as fungal, bacterial, mycobacterium, viral and protozoal infections (2). The methanol seed extract of *F. vulgare* has been reported to exhibit anti-dementia (8); anti-inflammatory (9); antiplatelet and antithrombotic (10); anticancer and antioxidant properties (11-13). In addition, it has been reported that aqueous *F. vulgare* extract has higher free-radical scavenging activity in comparison to many well-known antioxidants such as vitamin C (14).

Trachyspermum ammi is a well-known plant as a valuable source of biologically active ingredients, which can be applied in pharmaceutical industry (15). Its

essential oil is famous for antibacterial (16), antioxidant (17), antifungal (18), antiviral (19), antitermitic (20), scolicidal (21), anthelmintic (22), antifilarial (23), anti-hyperlipidaemic (24), nematicidal (25), kidney stone inhibitory (26) and mosquito repellent (27) activities. The main components found in *T. ammi* essential oil are thymol and non-thymol fraction, which often includes c-terpinene, p-cymene, alpha-pinene and beta-pinene (28).

The objective of this study was to investigate the biological activities of two medicinal plants *F. vulgare* and *T. ammi* that are used extensively for gastrointestinal disorders in traditional medicine. Given that most studies have been conducted on the essential oils of these medicinal plants, we investigated the *in vitro* biological effects, namely the anticancer, antimicrobial, hemolytic, and toxicity effects of ethanol seed extracts on *A. salina.*

Materials and Methods

Extract preparation

Around 20 g of *F. vulgare* and *T. ammi* seeds were ground and extracted using a Soxhlet extractor containing 500 ml ethanol for 24 hours. Then, obtained extracts were concentrated in a rotatory evaporator under reduced pressure at 45°C for 75 minutes (29).

Cell culture

Colorectal cancer (HCT116 and SW480) and embryonic kidney (HEK293) cells were obtained from the National Cell Bank (Pasteur Institute of Iran, Tehran) and cultured at 37°C in a humidified atmosphere of 5% CO_2 Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum.

Cytotoxicity assay

The inhibitory effects of the ethanol seed extracts of *F. vulgare* and *T. ammi* were determined on cancerous and normal cells by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich) (30). Before addition of extracts, the cells were seeded in a 96-well culture plate at a density of 7×10^3 cells/well to reach 80% confluency and extracts were filtered through 0.22 µm membranes. 10 mg of extracts was dissolved in 100 µL of DMSO and then added to 900 µL of medium for the preparation of 12.5, 25, 50, 100, 200 and 400 µg/mL dilutions (31). The extract at final concentrations was added to cells and then incubated for one, two and three days at 37°C in a CO_2 incubator. DMSO was used as negative control. After completion of each incubation turn, 10 µg/mL of MTT solution (20 µL) was added to the cultured cells and incubated for 4h; then the medium was removed by aspiration followed by adding DMSO (200 µL). The absorbance of formazan dye was recorded at 570 and 690 nm using an ELISA plate reader. The inhibition rate of the cell growth was calculated using the following formula:

$$\text{Growth inhibition}(\%) = 1 - \frac{\text{OD extract treated}}{\text{OD negative control}} \times 100$$

The cells in control group showed 100% viability with all studied extracts.

Hemolytic cytotoxicity

Hemolysis assay was performed to determine the biocompatibility of the ethanol seed extracts of *F. vulgare* and *T. ammi* (32). For this purpose, fresh human blood samples were collected in ethylenediaminetetraacetic acid-containing tubes and washed three times with an isotonic saline solution (PBS, pH 7.4, 5 minutes) by centrifugation at 1663 ×g. Then, the red blood cells (RBCs) were re-suspended in the same medium at a final hematocrit of 5%. Afterwards, the ethanol seed extracts at12.5 to 400 µg/mL were added to 0.4 mL of diluted human RBC suspension. All the suspensions were shaken before incubation at 37 °C for 4hours. To remove the non-lysed human RBCs, samples were centrifuged at 5400 ×g for 5 minutes. Next, 100 µL of the obtained supernatant was transferred to a 96-well plate and hemoglobin release was assessed at 545-nm wavelength. Sodium dodecyl sulfate (0.1%) and PBS were used as the positive and negative samples to establish 100% and 0% hemolysis, respectively. The percentage of hemolysis was obtained by the formula below:

$$Hemolysis(\%) = \frac{A\,sample - A\,negative\,control}{A\,positive - A\,negative\,control} \times 100$$

(A)**: Absorbance**

Toxicity assay on A. salina

The cytotoxic activity of the ethanol extracts was assessed by *A. salina* lethality test (33). To this end, brine shrimp cysts were obtained from Urmia University (West Azerbaijan, Iran) and then seeded in a 2000-mL flask containing 1L of 0.6M NaCl and incubated at 28°C for 48 hours. The test was performed on the hatched brine shrimp larvae. To prepare the stock (10 mg/mL), 10 mg of dried ethanol extracts was dissolved in 100 µL of DMSO and then added into 900 µL of the medium. It was then serially diluted to achieve concentrations 0.78125 to 10 mg/mL. For this purpose, 20 µL of the ethanol extracts was loaded to each well of the 96-well plates containing 180 µL of Roswell Park Memorial Institute (RPMI-1640) *medium*. Next, 10 larvae were transferred to each well and incubated at 25°C for 24 hours. After that, survived larvae in each well were counted under a binocular microscope. The artificial sea water containing 10 larvae was used as negative control. The percentages of died larvae were calculated based on the number of survived larvae in the test and control wells. The lethality was determined using Abbott's formula:

$$\text{Lethality}(\%) = \frac{\text{Test} - \text{Control}}{\text{Control}} \times 100$$

Microorganisms

Five pathogenic bacteria including one **g**ram-positive *Staphylococcus aureus* (ATCC 29737) and four **g**ram-negative *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 10031), *Proteus vulgaris* (PTCC 1182) and *Salmonella paratyphi* (ATCC 5702) were

obtained from the Iranian Biological Resource Center. Bacterial strains were grown in Mueller-Hinton broth (QUELAB) at 37°C for 18 hours. Bacterial suspensions were prepared on the basis of 0.5 McFarland (10^8 CFU/mL) standard turbidity and inoculated on the Mueller-Hinton Agar (MHA) medium.

Antibacterial activity

Sterile paper discs (6 mm) were soaked in the ethanol extracts (50 mg/mL), and the discs were left at room temperature until they dried. These discs were used to study the antibacterial activity of extracts. The disk diffusion test was performed to determine the antibacterial activity of ethanol seed extracts of *F. vulgare* and *T. ammi*. The turbidity of test suspensions was compared with that of the McFarland turbidity standard (1.5×10^8 CFU/mL). In order to obtain a uniform microbial growth, inoculums were spread on an MHA plate using a sterile cotton swab. Gentamicin (5 mg/mL) was used as positive control. Next, the plates were incubated at 37 °C for 24 hours. The antibacterial activity and the average diameter of inhibition zones were recorded.

The microtiter broth dilution method was performed for determination of MICs. The MIC values were determined by adding 200 µL of bacterial suspension to each well containing 20 µL of extracts (50 mg/mL) to achieve a final concentration of 5 mg/mL. The 96-well plates were incubated at 37 C for 24 hours. Optical densities (ODs) were recorded from 0 to 24 hours as with the MICs (34).

$$\text{Viability of bacteria}\left(\text{ratio}\right)=\frac{\left(\text{viability time24}\right)}{\left(\text{viability time0}\right)}$$

$$\text{Viability time0}=\frac{\text{OD0 bacterial and extract suspensions}-\text{OD0 extracts}}{\text{OD0 bacterial suspensions}}\times100$$

$$\text{Viability time24}=\frac{\text{OD24 bacterial and extract suspensions}-\text{OD24 extracts}}{\text{OD24 bacterial suspensions}}\times100$$

OD_0: The number of bacteria (10^8 CFU/mL) cultured in 96 well, OD_{24}: The number of bacteria after 24 hours of culture. Values of ≤1 shows inhibition of growth and proliferation of bacteria by extracts.

Statistical analysis

The data were analyzed using SPSS version 21 and the significance of differences between mean values was investigated. Values represented the mean ± standard deviation (SD) of three replications. Duncan's test at significance level (P) of <0.05 was used to investigate the significance of differences among treatments. IC_{50} values were analyzed using ED50plus v1.0.

Results

Cytotoxic activity of ethanolic extracts

To evaluate the growth-inhibitory activity of *F. vulgare* and *T. ammi* ethanol seed extracts, malignant colon cells (HCT116 and SW480) and non-cancerous normal cells (HEK293) were incubated with different concentrations ranging from 12.5 to 400 µg/mL and MTT assay was performed at 24, 48 and 72-hour intervals. Figures 1 and 2 show the effects of ethanol seed extracts of *F. vulgare* and *T. ammi* on *in vitro* proliferation of HCT116, SW480 and HEK293 cells in the MTT assay. In comparison to SW480, untreated and non-cancerous cells, the HCT116 cells treated with different concentrations of the ethanol extracts exhibited a significant decrease in cell viability. Ethanol *T. ammi* extract exhibited a pronounced inhibitory effect on proliferation of HCT-116 cells and decreased their cell viability in a dose-dependent manner in all studied intervals. Highest inhibition on proliferation of HCT-116 cells was observed at 400 µg/mL, resulting in approximately 50%, 66% and 84% inhibition at 24, 48 and 72 hours, respectively (Figure 1A,B,C). Meanwhile, the corresponding values were around 37%, 42% and 57% at 24, 48 and 72 hours, respectively, for ethanol seed extract of *F. vulgare* (Figure 2A,B,C). According to our results, ethanol *T. ammi* seed extract did not exhibit significant anticancer activity against SW-480 cells, however, ethanol *F. vulgare* seed extract was active on SW-480 proliferation at 400 µg/mL when incubated for 72 hours and slightly reduced the viability and proliferation of the cells. The ethanol extracts of both plants showed significant cytotoxic activity against normal cells (HEK-293) growth at 400 µg/mL at 72 hours (Duncan's test, P<0.05).

The IC_{50}s of ethanol seed extracts of *F. vulgare* and *T. ammi* on HCT-116 and SW-480 cell lines at 24, 48 and 72 hours are reported in Table 1. According to the results, ethanol seed extracts produced cytotoxic effects on HCT-116 in a dose and time-dependent manner. The ethanol seed extract of *T. ammi* exhibited marked inhibitory effect on proliferation of HCT-116 cells with IC_{50} of 106.46 µg/mL at 72 hours in comparison to alcoholic *F. vulgare* extract with IC_{50} of 267.39 µg/mL (Table 1).

At all intervals, SW480 cells treated with ethanol seed extract of *F. vulgare* and *T. ammi* exhibited the highest cell viability (Table 1).

Biocompatibility assay

The cytotoxicity of ethanol seed extracts of *F. vulgare* and *T. ammi* at 12.5-400 µg/mL was evaluated on hemolysis of normal human erythrocytes. Neither of ethanol seed extracts exhibited significant cytotoxicity toward human erythrocytes at 400 µg/mL at 4 hours interval. The higher stability of erythrocyte membrane could be related to the incorporation of plant metabolites into erythrocyte membranes, offering them a good resistance and high stability against hemolysis inducted by different extracts.

Toxicity effects of ethanol extracts on A. salina viability

The general toxicity of ethanol seed extracts of *F. vulgare* and *T. ammi* was evaluated against *A. salina*. The percentage of lethality was used as a bioassay indicator for the toxicity of ethanol seed extracts. The lethality (%) of nauplii at concentrations of 1000 µg/mL of *F. vulgare* and *T. ammi* ethanolic extracts was 23.33% to 30.5%, which indicates the moderate toxicity of them against brine shrimp larvae with LC_{50} values of 2071.65 and 1576.92 µg/

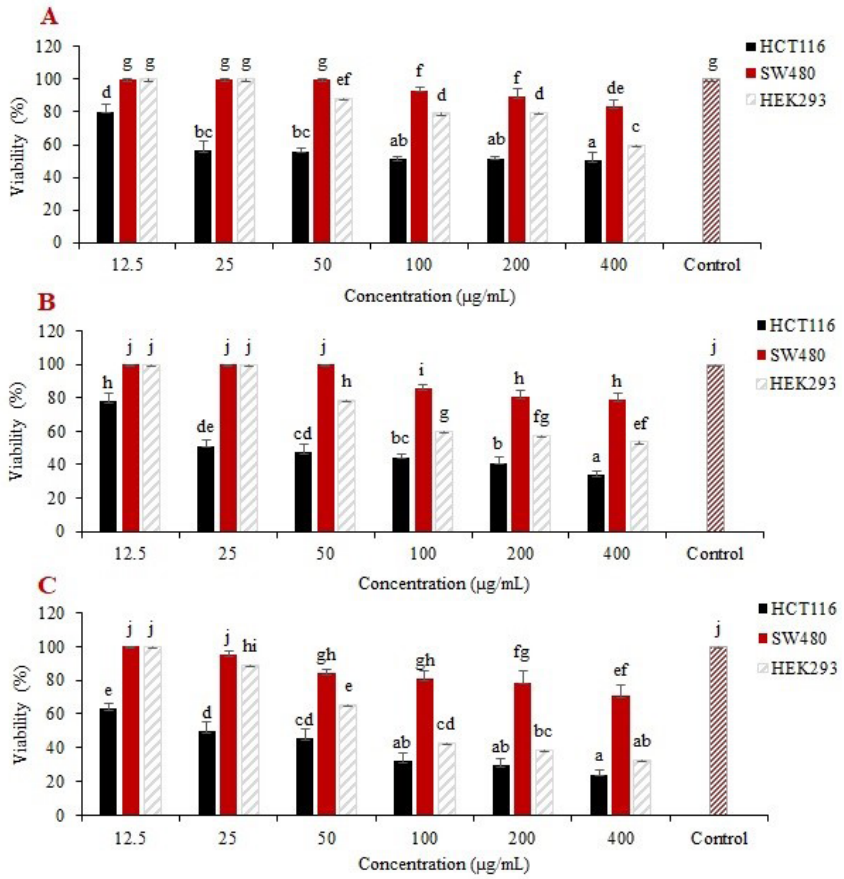

Figure 1. Cytotoxic effect of ethanol extracts of *Trachyspermum ammi* seeds on cancer and normal cells lines; effects of the extract at 24 h (A), 48 h (B) and 72 h (C). Colorectal cancer cells (HCT116 and SW480), embryonic kidney cells (HEK293). Charts with the same letters are not statistically significant (Duncan's test at $P<0.05$).

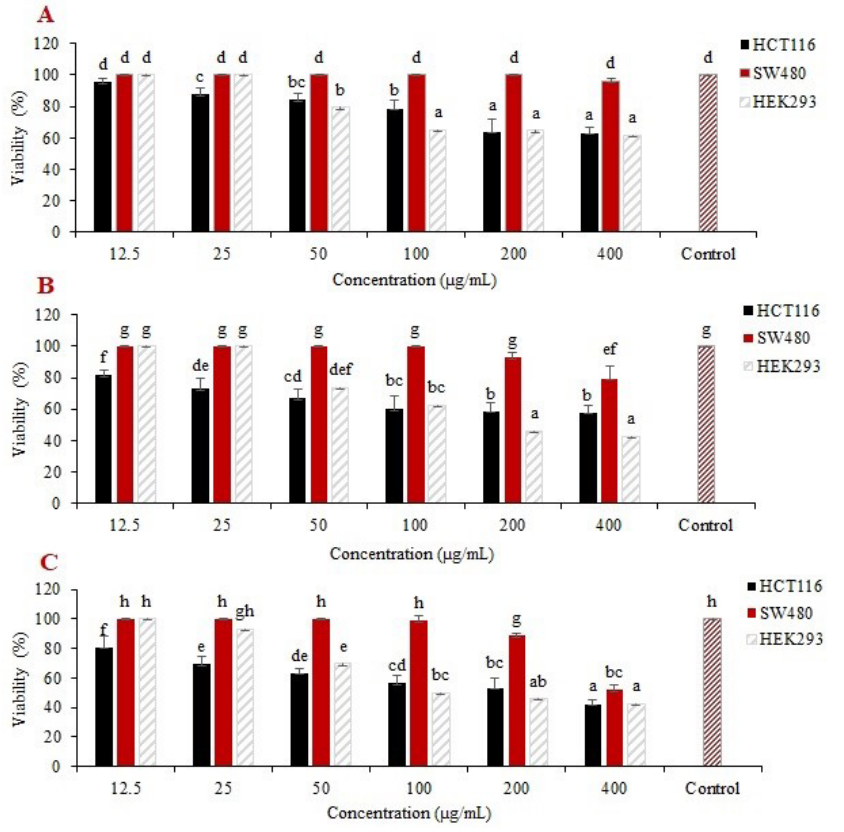

Figure 2. Cytotoxic effect of ethanol extract of *Foeniculum vulgare* seeds on cancerous and normal cells lines; effects of the extract at 24 h (A), 48 h (B) and 72 h (C). Colorectal cancer cells (HCT116 and SW480) and embryonic kidney cells (HEK293). Charts with the same letters are not statistically significant ($P<0.05$).

Table 1. IC_{50} of ethanol extracts of *Foeniculum vulgare* and *Trachyspermum ammi* seeds on HCT116, SW480 and HEK293 cell lines in the MTT assay

Time (h)	*Trachyspermum ammi* (µg/mL)			*Foeniculum vulgare* (µg/mL)		
	HCT116	SW480	HEK293	HCT116	SW480	HEK293
24	294.86	1115.63	484.58	474.34	5676.23	429.27
48	172.03	876.78	344.43	410.20	987.02	275.32
72	106.46	794.22	214.49	267.39	460.16	259.64

Values (µg/mL) are expressed as mean of three replications.
Colorectal cancer (HCT116 and SW480); embryonic kidney (HEK293).

mL, respectively (Figure 3). The LC_{50} values determined for alcoholic extracts were higher than 1000 µg/mL and therefore they were defined as nontoxic.

Antibacterial assay

Results for antibacterial activity of the plant extracts are presented in Table 2. As evaluated by the disc diffusion test, *F. vulgare* and *T. ammi* extracts inhibited the growth of *S. aureus* in comparison to gram-negative bacterial cultures. It was observed that *S. aureus* was more susceptible than gram-negative bacteria (Table 2). Among gram-negative strains, *S. paratyphi* was more susceptible than other bacteria as confirmed with a remarkable MIC of 0.55-fold (Table 3). Among all studied strains, *S. aureus* and *S. paratyphi* were the most susceptible strains to the extracts and the biggest inhibition zones of ethanol seed extracts were obtained for the two strains, so that for *S. aureus* and *S. paratyphi strains*, the inhibition zones were bigger than those created by the antibiotic gentamicin.

Discussion

The findings of the present study clearly demonstrated the anticancer properties of ethanol *F. vulgare* and *T. ammi* seed extracts that are consistent with previous reports on cancer cells, including lymphoblastic cell line (35), MCF7 cell line (6) and human breast cancer cells (MDA-MB231) and the anti-ulcerogenic effect of aqueous *F. vulgare* extract on gastric injuries induced by ethanol in rats (36). The study of Pradhan et al revealed that the normal blood lymphocyte cells treated with methanol extract of *F. vulgare* exerted less percentage of micronucleus compared to the standard drug and also exhibited potent cytotoxicity against B16F10 melanoma cell line (37). The study of Pradhan et al also showed that *F. vulgare* could serve as an anticancer agent against B16F10 melanoma cells and as a cytoprotective agent for normal cells (37). The anticancer activity of methanol *F. vulgare* seed extract has also been shown against breast and liver cancer cells (Hep G2) (13). Numerous studies have reported antitumor activity of *T. ammi* extract against various cancerous cells. Treatment of skin and forestomach with seed extract of *T. ammi* reduced tumor multiplicity of the cells (38). Also, *T. ammi* exhibited a significant cytotoxicity against MCF-7 cell lines at low concentrations (25 µg/mL) (39). In addition, *T. ammi* essential oil and *n*-hexane extract showed cytotoxicity against the liver carcinoma cell line (HepG2). The anti-proliferative activity of the essential oil was higher in comparison to *n*-hexane fraction (40). As well, Khorsandi et al reported that the growth of colon cancer cells was inhibited by reducing cell viability in the presence of *T. ammi* essential oil at 25 µg/mL in the dark (41). For the first time, Vitali et al reported the anticancer effect of *T. ammi* essential oil (IC_{50}:9.61 g/mL) on colorectal carcinoma cells along with its network interaction with the immune system, which can lead to novel exciting applications for healthcare (42). Although the anticancer effects of *T. ammi* oil and its alcoholic extracts have been demonstrated in several *in vitro* studies on colorectal cancers, further clinical studies are needed to investigate this argument. In our previous study, the proliferation of AGS gastric cancer cell line was inhibited by methanolic extracts and essential oils of *T.ammi* and *F.vulgare* with IC_{50} values lower than 50 µg/mL at 48 to 72hours (43).

Hemolysis is an indicator of RBC lysis by cytotoxic agents. Some plant-derived phytochemicals can exert hemolytic activity (44). Therefore, the toxicological effects of plant-derived constituents on animal or human erythrocytes have been studied *in vitro* using hemolysis tests (45-46).

Ruebhart et al reported that an ethanol extract with LC_{50} of over 1000 µg/mL was determined as non-toxic (47). Because the LC_{50} values determined for alcoholic seed extracts on *A. salina* were higher than 1000 µg/mL, they were defined as nontoxic. However, in another study, essential oils of *T.ammi* and *F.vulgare* showed cytotoxic activities against *A. salina* (LC_{50}:137.5 µg/mL and 235.7 µg/mL, respectively) (43).

The obtained results are in agreement with previously reported observations, including anti-mycobacterial and anti-candidal activities of *F. vulgare* essential oil and seed extract (48,49). In addition, moderate antibacterial activity of *aqueous and organic F. vulgare extracts* was observed in several *in vitro* studies (26,50). Furthermore, the hydro-ethanol extract of *F. vulgare* demonstrated antibacterial activity against *Campylobacter jejuni* and *Helicobacter pylori* (51,52).

The antimicrobial activity of various extracts from *T. ammi* has been observed in similar researches against *E. coli*, *P. aeruginosa*, *S. typhi* and *S. aureus* (53,54). The ancient uses of *T. ammi* seeds in the treatment of gastrointestinal disorders have also been confirmed in the study of Kaur and Arora. Their study revealed that organic and aqueous seed extracts of *T. ammi* showed antimicrobial activity (26). Various studies have confirmed the antibacterial activity of *T. ammi* seed essential oil (16, 43, 55-57). According to the results of Abdel-Hameed et al, *T. ammi* essential oil and *n*-hexane *T. ammi* extract exhibited antibacterial activity against five microorganisms (40). Vitali et al reported that *T. ammi* oil could exhibit higher inhibition zones on *S. aureus* and *Candida albicans* growth than reference antibiotics (42). Generally, in the current study, *S. aureus* was more sensitive than gram-negative bacteria strains,

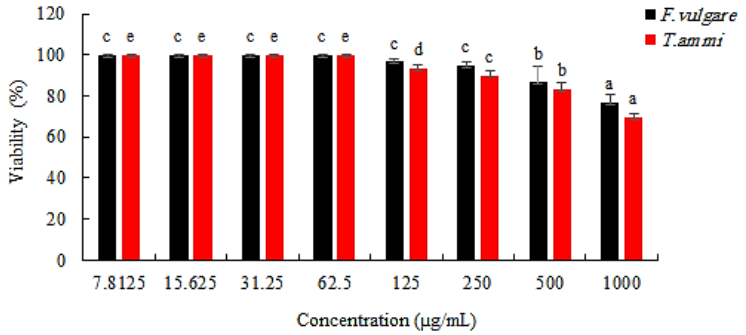

Figure 3. Toxicity effects of ethanol extracts of *F. vulgare* and *T. ammi* on *A. salina* viability. Charts with the same letters are not statistically significant (*P* value < 0.05).

Table 2. Antimicrobial activities of ethanol extracts of *Foeniculum vulgare* and *Trachyspermum ammi* seeds on pathogenic bacterial growth

	Extract (50 mg/mL)		Standards (10 µg/mL)
	T. ammi	*F. vulgare*	Gentamicin
Gram positive bacteria			
Staphylococcus aureus (ATCC 29737)	28.3	25.8±1.5	18±2.2
Gram negative bacteria			
Klebsiella pneumoniae (ATCC 10031)	16	12.5±2.5	17±2.5
Proteus vulgaris (PTCC 1182)	20.5	15±0.5	26±2.2
Salmonella paratyphi (ATCC 5702)	18.5	16.5±1.2	14±0.0
Escherichia coli (ATCC 10536)	15.6	14.4±0.5	16±1.7

Data are expressed as mean ± standard deviation.

Table 3. Minimum inhibitory concentrations of ethanol extracts of *Foeniculum vulgare* and *Trachyspermum ammi* seed against different pathogenic bacteria

	Extract (5 mg/mL)	
	T. ammi	*F. vulgare*
Gram positive bacteria		
Staphylococcus aureus (ATCC 29737)	0.39±0.05	0.55±0.03
Gram negative bacteria		
Klebsiella pneumoniae (ATCC 10031)	0.85±0.02	1.34±0.06
Proteus vulgaris (PTCC 1182)	0.56±0.02	0.84±0.11
Salmonella paratyphi (ATCC 5702)	0.55±0.05	0.75±0.06
Escherichia coli (ATCC 10536)	0.92±0.08	1.22±0.12

Data are expressed as mean ± standard deviation. Results report 24-hour growth to initial growth ratio (fold increase and decrease).

which could be related to its outer peptidoglycan layer with a less effective permeability barrier (58).

Conclusion

Foeniculum vulgare and *T. ammi* are widely cultivated in Iran. They have long been commonly used in traditional Iranian medicine for the treatment of various human ailments. Taken together, *F. vulgare* and *T. ammi* alcoholic extracts showed favorable anticancer potential against HCT-116 cancerous cells. In addition, the toxicity assay of ethanol extracts against brine shrimp larvae confirmed their non-toxicity. These extracts also exhibited promising antibacterial effect against pathogenic bacterial strains. *F. vulgare* and *T. ammi* are used in traditional medicine to alleviate gastrointestinal disorders and microbial infections, making exploration of their potential for the development of novel effective chemotherapeutic agents advisable. However, further research and complementary tests are needed to evaluate the potential of these plants for the treatment of different types of cancers and etiologic agents including viruses, bacteria, and fungi.

Acknowledgements

The authors would like to thank the officials of the School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran.

Authors' Contribution

S.R.H: Project administration, Investigation, Formal analysis, Writing – original draft and editing. M.H.A: Funding, Supervision, and Conceptualization. A.S.H: Funding and Supervision. Z.G.H: editing the English version manuscript.

Conflict of Interest Disclosures

The authors declare that there is no conflict of interests.

Ethical Approval

The ethics approval was obtained from Zanjan University of Medical Sciences (with the ethical code of IR.ZUMU.REC.1400.447).

References

1. Parekh J, Chanda S. In vitro antibacterial activity of the crude methanol extract of *Woodfordia fruticosa* Kurz. flower (Lythraceae). Braz J Microbiol. 2007;38(2):204-7. doi: 10.1590/s1517-83822007000200004.
2. Badgujar SB, Patel VV, Bandivdekar AH. Foeniculum vulgare Mill: a review of its botany, phytochemistry, pharmacology, contemporary application, and toxicology. Biomed Res Int. 2014;2014:842674. doi: 10.1155/2014/842674.
3. Ebeed NM, Abdou HS, Booles HF, Salah SH, Ahmed ES, Fahmy KH. Antimutagenic and chemoprevention potentialities of sweet fennel (*Foeniculum vulgare* Mill.) hot water crude extract. J Am Sci. 2010;6(9):831-42.
4. Parejo I, Jauregui O, Sánchez-Rabaneda F, Viladomat F, Bastida J, Codina C. Separation and characterization of phenolic compounds in fennel (*Foeniculum vulgare*) using liquid chromatography-negative electrospray ionization tandem mass spectrometry. J Agric Food Chem. 2004;52(12):3679-87. doi: 10.1021/jf030813h.
5. Ozbek H, Uğraş S, Dülger H, Bayram I, Tuncer I, Oztürk G, et al. Hepatoprotective effect of *Foeniculum vulgare* essential oil. Fitoterapia. 2003;74(3):317-9. doi: 10.1016/s0367-326x(03)00028-5.
6. Kaileh M, Vanden Berghe W, Boone E, Essawi T, Haegeman G. Screening of indigenous Palestinian medicinal plants for potential anti-inflammatory and cytotoxic activity.

J Ethnopharmacol. 2007;113(3):510-6. doi: 10.1016/j.jep.2007.07.008.
7. Modaress Nejad V, Asadipour M. Comparison of the effectiveness of fennel and mefenamic acid on pain intensity in dysmenorrhoea. East Mediterr Health J. 2006;12(3-4):423-7.
8. Joshi H, Parle M. Cholinergic basis of memory-strengthening effect of *Foeniculum vulgare* Linn. J Med Food. 2006;9(3):413-7. doi: 10.1089/jmf.2006.9.413.
9. Choi EM, Hwang JK. Antiinflammatory, analgesic and antioxidant activities of the fruit of *Foeniculum vulgare*. Fitoterapia. 2004;75(6):557-65. doi: 10.1016/j.fitote.2004.05.005.
10. Tognolini M, Ballabeni V, Bertoni S, Bruni R, Impicciatore M, Barocelli E. Protective effect of *Foeniculum vulgare* essential oil and anethole in an experimental model of thrombosis. Pharmacol Res. 2007;56(3):254-60. doi: 10.1016/j.phrs.2007.07.002.
11. Barros L, Heleno SA, Carvalho AM, Ferreira IC. Systematic evaluation of the antioxidant potential of different parts of *Foeniculum vulgare* Mill. from Portugal. Food Chem Toxicol. 2009;47(10):2458-64. doi: 10.1016/j.fct.2009.07.003.
12. Nickavar B, Abolhasani FA. Screening of antioxidant properties of seven Umbelliferae fruits from Iran. Pak J Pharm Sci. 2009;22(1):30-5.
13. Mohamad RH, El-Bastawesy AM, Abdel-Monem MG, Noor AM, Al-Mehdar HA, Sharawy SM, et al. Antioxidant and anticarcinogenic effects of methanolic extract and volatile oil of fennel seeds (*Foeniculum vulgare*). J Med Food. 2011;14(9):986-1001. doi: 10.1089/jmf.2008.0255.
14. Satyanarayana S, Sushruta K, Sarma GS, Srinivas N, Subba Raju GV. Antioxidant activity of the aqueous extracts of spicy food additives--evaluation and comparison with ascorbic acid in in-vitro systems. J Herb Pharmacother. 2004;4(2):1-10.
15. Ashraf M. Salt tolerance of cotton: some new advances. Crit Rev Plant Sci. 2002;21(1):1-30. doi: 10.1080/0735-260291044160.
16. Moein MR, Zomorodian K, Pakshir K, Yavari F, Motamedi M, Zarshenas MM. Trachyspermum ammi (L.) sprague: chemical composition of essential oil and antimicrobial activities of respective fractions. J Evid Based Complementary Altern Med. 2015;20(1):50-6. doi: 10.1177/2156587214553302.
17. Gandomi H, Abbaszadeh S, JebelliJavan A, Sharifzadeh A. Chemical constituents, antimicrobial and antioxidative effects of *Trachyspermum ammi* essential oil. J Food Process Preserv. 2014;38(4):1690-5. doi: 10.1111/jfpp.12131.
18. Kedia A, Prakash B, Mishra PK, Dwivedy AK, Dubey NK. *Trachyspermum ammi* L. essential oil as plant based preservative in food system. Ind Crops Prod. 2015;69:104-9. doi: 10.1016/j.indcrop.2015.02.013.
19. Hussein G, Miyashiro H, Nakamura N, Hattori M, Kakiuchi N, Shimotohno K. Inhibitory effects of sudanese medicinal plant extracts on hepatitis C virus (HCV) protease. Phytother Res. 2000;14(7):510-6. doi: 10.1002/1099-1573(200011)14:7<510::aid-ptr646>3.0.co;2-b.
20. Seo SM, Kim J, Lee SG, Shin CH, Shin SC, Park IK. Fumigant antitermitic activity of plant essential oils and components from ajowan (*Trachyspermum ammi*), allspice (*Pimenta dioica*), caraway (*Carum carvi*), dill (*Anethum graveolens*), geranium (*Pelargonium graveolens*), and litsea (*Litsea cubeba*) oils against Japanese termite (*Reticulitermes speratus* Kolbe). J Agric Food Chem. 2009;57(15):6596-602. doi: 10.1021/jf9015416.
21. Moazeni M, Saharkhiz MJ, Hosseini AA. In vitro lethal effect of ajowan (*Trachyspermum ammi* L.) essential oil on hydatid cyst protoscoleces. Vet Parasitol. 2012;187(1-2):203-8. doi: 10.1016/j.vetpar.2011.12.025.
22. Lateef M, Iqbal Z, Akhtar MS, Jabbar A, Khan MN, Gilani AH. Preliminary screening of *Trachyspermum ammi* (L.) seed for anthelmintic activity in sheep. Trop Anim Health Prod. 2006;38(6):491-6. doi: 10.1007/s11250-006-4315-6.
23. Mathew N, Misra-Bhattacharya S, Perumal V, Muthuswamy K. Antifilarial lead molecules isolated from *Trachyspermum ammi*. Molecules. 2008;13(9):2156-68. doi: 10.3390/molecules13092156.
24. Javed I, Iqbal Z, Rahman ZU, Khan FH, Muhammad F, Aslam B, et al. Comparative antihyperlipidaemic efficacy of *Trachyspermum ammi* extracts in albino rabbits. Pak Vet J. 2006;26(1):23-9.
25. Park IK, Kim J, Lee SG, Shin SC. Nematicidal activity of plant essential oils and components from ajowan (*Trachyspermum ammi*), allspice (*Pimenta dioica*) and litsea (*Litsea cubeba*) essential oils against pine wood nematode (*Bursaphelenchus xylophilus*). J Nematol. 2007;39(3):275-9.
26. Kaur GJ, Arora DS. Antibacterial and phytochemical screening of Anethum graveolens, *Foeniculum vulgare* and *Trachyspermum ammi*. BMC Complement Altern Med. 2009;9:30. doi: 10.1186/1472-6882-9-30.
27. Pandey SK, Upadhyay S, Tripathi AK. Insecticidal and repellent activities of thymol from the essential oil of *Trachyspermum ammi* (Linn) Sprague seeds against *Anopheles stephensi*. Parasitol Res. 2009;105(2):507-12. doi: 10.1007/s00436-009-1429-6.
28. Zarshenas MM, Moein M, Mohammadi Samani S, Petramfar P. An overview on ajwain (*Trachyspermum ammi*) pharmacological effects; modern and traditional. J Nat Remedies. 2014;14(1):98-105.
29. Rahamooz-Haghighi S, Asadi MH. Anti-proliferative effect of the extracts and essential oil of *Pimpinella anisum* on gastric cancer cells. J HerbMed Pharmacol. 2016;5(4):157-61.
30. Plumb JA. Cell sensitivity assays: the MTT assay. In: Langdon SP, ed. Cancer Cell Culture: Methods and Protocols. Vol 88. Totowa, NJ: Humana Press; 2004. p. 165-9. doi: 10.1385/1-59259-406-9:165.
31. Rahamooz Haghighi S, Asadi MH, Akrami H, Baghizadeh A. Anti-carcinogenic and anti-angiogenic properties of the extracts of *Acorus calamus* on gastric cancer cells. Avicenna J Phytomed. 2017;7(2):145-56.
32. Aghajanzadeh M, Zamani M, Rashidzadeh H, Rostamizadeh K, Sharafi A, Danafar H. Amphiphilic Y shaped miktoarm star copolymer for anticancer hydrophobic and hydrophilic drugs codelivery: synthesis, characterization, in vitro, and in vivo biocompatibility study. J Biomed Mater Res A. 2018;106(11):2817-26. doi: 10.1002/jbm.a.36468.
33. Rajabi S, Ramazani A, Hamidi M, Naji T. *Artemia salina* as a model organism in toxicity assessment of nanoparticles. Daru. 2015;23(1):20. doi: 10.1186/s40199-015-0105-x.
34. Rahamooz-Haghighi S, Bagheri K, Sharafi A, Danafar H. Establishment and elicitation of transgenic root culture of *Plantago lanceolata* and evaluation of its anti-bacterial and cytotoxicity activity. Prep Biochem Biotechnol. 2021;51(3):207-24. doi: 10.1080/10826068.2020.1805757.
35. Zidorn C, Jöhrer K, Ganzera M, Schubert B, Sigmund EM, Mader J, et al. Polyacetylenes from the Apiaceae vegetables carrot, celery, fennel, parsley, and parsnip and their cytotoxic activities. J Agric Food Chem. 2005;53(7):2518-23. doi: 10.1021/jf048041s.
36. Birdane FM, Cemek M, Birdane YO, Gülçin I, Büyükokuroğlu ME. Beneficial effects of *Foeniculum vulgare* on ethanol-induced acute gastric mucosal injury in rats. World J Gastroenterol. 2007;13(4):607-11. doi: 10.3748/wjg.v13.i4.607.
37. Pradhan M, Sribhuwaneswari S, Karthikeyan D, Minz S, Sure P, Chandu AN, et al. In-vitro cytoprotection activity of *Foeniculum vulgare* and *Helicteres isora* in cultured human blood lymphocytes and antitumour activity against B16F10

melanoma cell line. Res J Pharm Technol. 2008;1(4):450-2.
38. Singh B, Kale RK. Chemomodulatory effect of *Trachyspermum ammi* on murine skin and forestomach papillomagenesis. Nutr Cancer. 2010;62(1):74-84. doi: 10.1080/01635580903191478.
39. Ramya N, Priyadharshini XX, Prakash R, Dhivya R. Anti-cancer activity of *Trachyspermum ammi* against MCF7 cell lines mediates by p53 and Bcl-2 mRNA levels. J Phytopharmacol. 2017;6(2):78-83.
40. Abdel-Hameed ES, Bazaid SA, Al Zahrani O, El-Halmouch Y, El-Sayed MM, El-Wakil E. Chemical composition of volatile components, antimicrobial and anticancer activity of n-hexane extract and essential oil from *Trachyspermum ammi* L. seeds. Orient J Chem. 2014;30(4):1653-62.
41. Khorsandi K, Kianmehr Z, Ghelichkhani E. Combination effect of red light irradiation and *Traychspermum ammi* essential oil on colorectal cancer cells (SW480). Lasers Med Sci. 2022;37(2):1031-40. doi: 10.1007/s10103-021-03350-w.
42. Vitali LA, Beghelli D, Biapa Nya PC, Bistoni O, Cappellacci L, Damiano S, et al. Diverse biological effects of the essential oil from Iranian *Trachyspermum ammi*. Arab J Chem. 2016;9(6):775-86. doi: 10.1016/j.arabjc.2015.06.002.
43. Rahamouz-Haghighi S, Asadi MH, Baghizadeh A. Antiproliferative and antibacterial properties of methanolic extract and essential oil of *Trachyspermum ammi* and *Foeniculum vulgare* seeds on gastric cancer ,*Artemia salina* larvae and pathogenic bacteria. EPP. 2021;2(2): 42-50.
44. Lakshmi KS, Sangeetha D, Sivamani S, Tamilarasan M, Rajesh TP, Anandraj B. In vitro antibacterial, antioxidant, haemolytic, thrombolytic activities and phytochemical analysis of *Simarouba glauca* leaves extracts. Int J Pharm Sci Res. 2014;5(2):432-7.
45. Gandhi VM, Cherian KM. Red cell haemolysis test as an in vitro approach for the assessment of toxicity of karanja oil. Toxicol In Vitro. 2000;14(6):513-6. doi: 10.1016/s0887-2333(00)00046-1.
46. Rahamooz-Haghighi S, Bagheri K, Danafar H, Sharafi A. Anti-proliferative properties, biocompatibility, and chemical composition of different extracts of *Plantago major* medicinal plant. Iran Biomed J. 2021;25(2):106-16. doi: 10.29252/ibj.25.2.106.
47. Ruebhart DR, Wickramasinghe W, Cock IE. Protective efficacy of the antioxidants vitamin E and Trolox against *Microcystis aeruginosa* and microcystin-LR in *Artemia franciscana* nauplii. J Toxicol Environ Health A. 2009;72(24):1567-75. doi: 10.1080/15287390903232459.
48. Abed KF. Antimicrobial activity of essential oils of some medicinal plants from Saudi Arabia. Saudi J Biol Sci. 2007;14(1):53-60.
49. Del Rayo Camacho-Corona M, Ramírez-Cabrera MA, González-Santiago O, Garza-González E, de Paz Palacios I, Luna-Herrera J. Activity against drug resistant-tuberculosis strains of plants used in Mexican traditional medicine to treat tuberculosis and other respiratory diseases. Phytother Res. 2008;22(1):82-5. doi: 10.1002/ptr.2269.
50. Kaur GJ, Arora DS. In vitro antibacterial activity of three plants belonging to the family Umbelliferae. Int J Antimicrob Agents. 2008;31(4):393-5. doi: 10.1016/j.ijantimicag.2007.11.007.
51. Mahady GB, Pendland SL, Stoia A, Hamill FA, Fabricant D, Dietz BM, et al. In vitro susceptibility of *Helicobacter pylori* to botanical extracts used traditionally for the treatment of gastrointestinal disorders. Phytother Res. 2005;19(11):988-91. doi: 10.1002/ptr.1776.
52. Cwikla C, Schmidt K, Matthias A, Bone KM, Lehmann R, Tiralongo E. Investigations into the antibacterial activities of phytotherapeutics against *Helicobacter pylori* and *Campylobacter jejuni*. Phytother Res. 2010;24(5):649-56. doi: 10.1002/ptr.2933.
53. Ahmad I, Mehmood Z, Mohammad F. Screening of some Indian medicinal plants for their antimicrobial properties. J Ethnopharmacol. 1998;62(2):183-93. doi: 10.1016/s0378-8741(98)00055-5.
54. Patel JD, Patel DK, Shrivastava A, Kumar V. Screening of plant extracts used in traditional antidiarrhoeal medicines against pathogenic *Escherichia coli*. Sci World. 2008;6(6):63-7. doi: 10.3126/sw.v6i6.2636.
55. Mayaud L, Carricajo A, Zhiri A, Aubert G. Comparison of bacteriostatic and bactericidal activity of 13 essential oils against strains with varying sensitivity to antibiotics. Lett Appl Microbiol. 2008;47(3):167-73. doi: 10.1111/j.1472-765X.2008.02406.x.
56. Kumar A, Mishra RK, Srivastava S, Tiwari AK, Pandey A, Shukla AC, et al. Role of phylogenetic analysis for anti-bacterial activity of essential oil of *Trachyspermum ammi* L. against water borne pathogens. Adv Environ Biol. 2011;5(6):1271-8.
57. Paul S, Dubey RC, Maheswari DK, Kang SC. *Trachyspermum ammi* (L.) fruit essential oil influencing on membrane permeability and surface characteristics in inhibiting food-borne pathogens. Food Control. 2011;22(5):725-31. doi: 10.1016/j.foodcont.2010.11.003.
58. Nostro A, Germanò MP, D'Angelo V, Marino A, Cannatelli MA. Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Lett Appl Microbiol. 2000;30(5):379-84. doi: 10.1046/j.1472-765x.2000.00731.x.

Cite this article as: Rahamouz-Haghighi S, Asadi MH, Gharari Z, Sharafi A. *Foeniculum vulgare* and *Trachyspermum ammi* seed ethanolic extracts: Cytotoxicity assay, *in vitro* toxicity on *Artemia salina* larvae, biocompatibility and antibacterial activity. Future Nat Prod. 2022;8(1):15–22. doi: 10.34172/fnp.2022.04.