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Antiproliferative and Antibacterial Properties of Pethanolic Extract and Essential Oil of *Trachyspermum ammi* and *Foeniculum vulgare* Seeds on Gastric Cancer, *Artemia salina Larvae and Pathogenic bacteria*

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Abstract

Background: *Trachyspermum ammi* and *Foeniculum vulgare* (Apiaceae) have been widely employed in traditional medicine to treat many diseases. Methods: The cytotoxic activity of methanolic extracts and essential oils of *T.ammi* and *F.vulgare* seeds on gastric cancer cell line (AGS) and Human Skeletal Muscle cell line (HSkMC) were assessed by MTT method. *In vitro* toxicity was also evaluated on *Artemia salina*. The antibacterial activity was measured by the Microtiter broth dilution method. Result*:* The proliferation of cancer cells was inhibited by methanolic extracts and essential oils. The results showed a greater degree of cytotoxicity on AGS at the dose of 400µg/mL of methanolic extracts and essential oils of *T.ammi* and *F.vulgare* with IC_{50} values lower than 50 µg/mL at 48 to 72h. so, they can be considered appropriate for further purification and are agree with the US National Cancer Institute. The methanolic extracts and essential oils exhibited cytotoxicity activity against brine shrimp larvae ($LC_{50:}$ 1066.4 µg/mL and 137.5 µg/mL for *T.ammi*) and ($LC_{50:}$ 1267.5 µg/mL and 235.7 µg/mL for *F.vulgare*). The antimicrobial activity of methanolic extracts and essential oils showed maximum inhibitory activity against *S.aureus* with MICs (0.35 and 0.08 fold) and (0.45 and 0.1 fold) for *T.ammi* and *F.vulgare,* respectively. Conclusion: Therefore, our results showed that the methanolic extracts and essential oils of *T.ammi* and *F.vulgare* have antiproliferation and antibacterial properties and could be used as adjuvant therapy against common gastric and pathogenic bacteria.

1. Introduction

Trachyspermum ammi L. and *Foeniculum vulgare* Mill (Apiaceae) commonly known as ajwain and Fennel and they are important and valuable medicinal herbs.

T. ammi (Ajwain) is one of the most popular plants with bioactive medicinal constituents which is exploited at the pharmaceutical level (Ashraf, 2002). Thymol is the main component of essential oil (35–60%), which is a strong antispasmodic, fungicide, and germicide agent. The non-thymol fraction contains, c-terpinene, p-cymene, a-pinene, b-pinene, and other minor components (Zarshenas *et al*., 2014). However, sometimes c-terpinene and p-cymene exceed the thymol content (Omer *et al*., 2014; Moein *et al*., 2015), and in other cases thymol and p-cymene are not among the predominant components (Singh *et al*., 2008). The methanol extracts of *T. ammi* showed significant *in vitro* inhibitory effect on hepatitis C virus (HCV) protease (Hussein *et al*., 2000). The *T.ammi* essential oil exhibited molluscicidal (Singh, 2000); antihyperlipidaemic (Javed *et al*., 2006); anthelmintic (Lateef *et al*., 2006); nematicidal (Park *et al*., 2007); antifilarial (Mathew *et al*., 2008); insecticidal (Chaubey, 2008); kidney stone inhibitory (Kaur *et al*.,2009) and mosquito repellent (Pandey *et al*., 2009). *T. ammi* has been shown to possess antioxidant effects (Chatterjee *et al*., 2013; Gandomi *et al*., 2014).

F.vulgare (Fennel) is interested herb because it contains thymol, thymol methyl-ether, P-thymene, carvacrol, saponins, phenolic glycosides, terpinenes, phytosterols-triterpen and flavonoids (Ebeed *et al*., 2010). There are many phenolic compounds in plant, including kaempferol-3-*O*-glucoside, eriodictyol-7-orutinoside, quercetin-3-*O*-galactoside, caffeoylquinic acid and rosmarinic acid, with antioxidant activities (Parejo *et al*., 2004). Many studies been reported to possess hepatoprotective (Ozbek *et al*., 2003); immunomodulatory (Kaileh *et al*., 2007); and pain reliever in primary dysmenorrhoea (Modaress and

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Asadipour, 2006). *F. vulgare* is effective against infections such as viral, fungal, bacterial, mycobacterium and protozal origin (Badgujar *et al*., 2014). *F.vulgare* seed extract has demonstrated antiinflammatory (Choi and Hwang, 2004); potential antidementia (Joshi and Parle, 2006); antithrombotic and anti-platelet (Tognolini *et al*., 2007); Antioxidant and anticancer (Barros *et al*., 2009; Nickavar and Abolhasani, 2009; Mohamad *et al*., 2011).

The present research is aimed at evaluating biological activities of two medicinal plants *T. ammi* and *F. vulgare* which have been extensively used for gastrointestinal disorders in traditional medicine. Now, *T. ammi* and *F. vulgare* is used by researchers as an adjunct treatment for some types of cancer, although information on the nature of the total extracts of some species is still insufficient. In the present work, we have evaluated the *in vitro* biological effects of methanolic extracts and essential oils, namely toxicity activities to induce cell death in human gastric cancer AGS cell line, *Artemia salina* and pathogenic bacteria. These activities were evaluated by MTT methods, toxicity assay on *A. salina*, and microtiter broth dilution method, respectively.

2. Material and Methods

2.1. Extract Preparation

T.ammi and *F.vulgare* seeds were prepared from the Pakan Bazr company of Esfahan, Iran. 20 g of *T.ammi* and *F.vulgare* seeds were ground and extracted by a Soxhlet extractor containing 500 mL methanol (24 h). Then obtained extracts were concentrated in a rotatory evaporator under reduced pressure at 45 °C for 75 min. After that, extracts put in the shade at room temperature for two weeks to full dryness.

Essential oil Preparation

100 g of *T. ammi* and *F. vulgare* seeds were ground for hydrodistillation in a Clevenger apparatus for 3 h to derive essential oils. The volatile oils were dried over anhydrous sodium sulphate and then kept separately in sealed clean glass vials at 4 °C until use.

2.2. Cell Culture

The Gastric adenocarcinoma cell line (AGS) and Skeletal Muscle cell line (HSkMC) were obtained from the national cell Bank (Pasteur Institute of Iran, Tehran) and was cultured at 37 °C in a humidified atmosphere of 5% CO_2 and Roswell Park Memorial Institute (RPMI-1640) supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (PBS).

2.3. Cytotoxic Assay

The inhibitory effect of the methanolic extracts and essential oils of *T.ammi* and *F.vulgare* seeds on cancer and normal cells was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Before adding extracts, the cells (7×10^3 cells per well) were seeded in a 96-well plate to reach 80% confluency and extracts were filtered by 0.22µm membrane filters. The preparation of extracts according to our previous study (Rahamouz-Haghighi *et al*., 2020b). The methanolic extracts and essential oils with 12.5, 25, 50, 100, 200 and 400 µg/mL concentrations were added to cells and then was incubated for three days at 37 °C in a CO_2 incubator. DMSO was applied as a negative control. One, two and three days after treatment, 20µL MTT solution was added to the cells and incubated for 4 h, then the medium was removed by aspiration followed by adding 200µL DMSO. The absorbance of formazan dye was read using an ELISA plate reader at 570 and 690 nm. The inhibitory rate of the cell growth was calculated using the following formula:

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

2.4. Toxicity Assay on *A. salina*

The general toxicity of the methanolic extracts and essential oils of *T.ammi* and *F.vulgare* seeds on *A.salina* were assessed. *A. salina* eggs were achieved from Urmia University, the West Azerbaijan Province, Iran. The cysts were seeded in a flask containing 35 g of NaCl in 1 L of distilled water. After incubation for 48 h at 28 °C, the larvae hatched. The test was performed on the larvae of brine shrimp (*A.salina* Leach.). The concentrations ranging from 7.8125 µg/mL to 1000 µg/mL were prepared. The preparation of solutions according to our previous study (Rahamooz-Haghighi *et al*., 2020b). After that, 10 nauplii per well were added to the 96-well plates and incubated at 25 °C for 24 h. Afterward, the numbers of surviving nauplii in each well were calculated under a binocular microscope after 24 h. All experimental settings for each concentration were in triplicates. Additionally, the negative control contained only 10 nauplii and artificial sea water. The percentages of nauplii deaths were calculated by considering the number of survivors in the test and control wells. The lethality was determined by Abbott's formula:

$$\text{Lethality (\%)} = \frac{\text{Test} - \text{Control}}{\text{Control}} \times 100 \quad (3)$$

2.5. Microorganisms

The standard pathogenic bacteria culture Gram-positive bacteria *Staphylococcus aureus* (ATCC 29737) and Gram-negative bacteria *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 10031), *Proteus vulgaris* (PTCC 1182) and *Salmonella paratyphi* (ATCC 5702) were obtained from Iranian Biological

Resource Center. The culture collection of bacteria was grown in Mueller-Hinton broth (MHB) at 37 °C for 18h. Bacteria suspensions were prepared on the basis of 0.5 McFarland.

2.6. Antibacterial Activity

The MIC values were determined by 200 µL of standard bacterial suspension and 20 µL of extracts were added to the wells to reach 10 mg/mL concentration. The 96 well plates were incubated at 37 °C for 24 h in the Eliza reader plate. ODs were recorded from 0 to 24 h as the MICs (Rahamooz-Haghighi *et al*., 2021a).

$$\text{Viability of bacteria (Ratio)} = \frac{(\text{viability time24})}{(\text{viability time0})} \quad [4]$$

$$\text{viability time0} = \frac{\text{OD0 bacterial and extract suspensions} - \text{OD0 extracts}}{\text{OD0 bacterial suspensions}} \times 100 \quad [5]$$

$$\text{viability time24} = \frac{\text{OD24 bacterial and extract suspensions} - \text{OD24 extracts}}{\text{OD24 bacterial suspensions}} \times 100 \quad [6]$$

OD_0 is equivalent to the number of bacteria (10^8 CFU/mL) cultured in 96 well, OD_{24} is the number of bacterial after 24 h of culture, and values of one and less than one shows inhibition of growth and proliferation of bacteria by tested extracts.

2.7. Statistical Analysis

The data were analyzed using SPSS v.21 software and the significant difference between means was calculated. Values expressed as mean of three replications ± standard deviation (SD). Duncan's test at P-value<0.05 was used to determine significant differences among treatments. IC_{50} values were analyzed with ED_{50} plus $v_{1.0}$ Software.

3. Result

3.1. Cytotoxic Activity of Methanolic Extracts and Essential Oils

Data presented in Figures 1 and 2 showed the methanolic extracts and essential oils of *T. ammi* and *F. vulgare* were investigated *in vitro* towards AGS, and HSkMC by MTT assay. The cells treated with different concentrations of methanolic extracts and essential oils of *T. ammi* and *F. vulgare* exhibited a significant decrease in viability of AGS cells in comparison to HSkMC cells. There was a significant concentration and time-dependent decrease in the proliferation of AGS cancer cells after treatment with the methanolic extracts and essential oil of *T. ammi* and *F. vulgare* (Fig. 1 and 2).

The methanolic extract of *F.vulgare* had cytotoxic effect on AGS cells in all treatments (Fig. 1a,b,c) but the methanolic extract of *T.ammi* had a significant cytotoxic effect in all treatments after 48 and 72 h of treatment on AGS cells (Fig. 2a,b,c). However, methanolic extracts of two plants at 200 to 400 µg/mL, showed significant cytotoxic activity against HSkMC (Duncan test, p-value<0.05). The essential oils of *T. ammi* and *F. vulgare* showed more cytotoxic effect on AGS cells than the methanolic extracts. It must be mentioned that the cytotoxic effect of essential oils on AGS cells increased along with increasing concentrations of the extract (Fig. 1 and 2d.e,f).

Our result indicates that the methanolic extract of *F.vulgare* showed more cytotoxic on AGS cells than *T.ammi*. The methanolic extract and essential oil of *F.vulgare* exhibited marked inhibition of proliferation on AGS cells with IC_{50} lower than 50 µg/mL at 48 and 72h (Table 1).

Therefore, HSkMC cells treated with methanolic extracts and essential oils of *F.vulgare* and *T.ammi* exhibited the less viability of cells at 72h with IC_{50} values of (235.601 µg/mL and 224.064 µg/mL for methanolic extracts) and (119.68µg/mL and 131.402 for essential oils), respectively (Table 1). So, the methanolic extracts and essential oils exhibited a more cytotoxic effects on AGS cell compared to HSkMC cell and this result is a good option for using *T.ammi* and *F.vulgare* seeds.

3.2. Toxicity Assay of Methanolic Extracts and Essential Oils on *A. salina*

The general toxicity of methanolic extracts and essential oils of *T. ammi* and *F. vulgare* were evaluated against *A. salina*. The percentage of lethality was used as a bioassay indicator for the toxicity of treatments. % lethality of nauplii at different concentrations of methanolic extracts and essential oils of *T.ammi* and *F.vulgare* were depicted in Table 2. The LC_{50} values of methanolic extracts of *T. ammi* and *F. vulgare* seeds exhibited cytotoxicity activity against brine shrimp larvae with LC_{50s}: 1066.4 and and 1267.5µg/mL. At 7.8125 µL/mL methanolic extracts concentration, no toxic effect on *A. salina* was observed. When the methanolic extracts concentration was increased to 15.625 µL/mL, a 2 - 5% death rate of *A. salina* was recorded. The *T. ammi* essential oil showed the highest toxicity with a LC_{50} value of 137.5 µL/mL, followed by the *F.vulgare* essential oil with a LC_{50} of 235.7 µL/mL. At 1000 µL/mL essential oils concentration, the percentage death of *A. salina* was 90 and 85%, respectively.

3.3. Antibacterial Assay

Results for antibacterial activity are shown in Table 3. As evaluated by the Microtiter broth dilution method, methanolic extracts and essential oils of *T. ammi* and *F.vulgare* inhibited Gram-positive bacteria, *S.aureus*

was more susceptible than Gram-negative bacteria. Among Gram-negative species, *S.paratyphi* was more active than other bacteria with a remarkable MIC of (0.65 and 0.33-fold) and (0.45 and 0.15-fold) toward methanolic extracts and essential oils of *T.ammi* and *F. vulgare* (Table 3).

4. Discussion

Apiaceae species are widely found in Iran and they have been commonly used in Iranian traditional medicine to treat various ailments. *T. ammi* and *F. vulgare* showed good anticancer potential on various cancers which are insufficient. However, in the present study, the effect of methanolic extracts and essential oils of *T. ammi* and *F. vulgare* seeds were investigated on the gastric cell line, and also the cytotoxic effects on *A.salina* and antibacterial properties were investigated. The results showed a greater degree of cytotoxic effect of *T. ammi* and *F. vulgare* on AGS.

Table 1 IC_{50} values of ethanolic extraction of *Funiculum vulgare* and *T. ammi* seeds on AGS, HCT116, SW480, HEK293 and HSkMC cell lines as determined in MTT assay

Time (hours)	*F. vulgar* (µg/mL)*e*				*T. ammi* (µg/mL)			
	Methanolic extract		Essential oil		Methanolic extract		Essential oil	
	AGS	HSkMC	AGS	AGS	AGS	HSkMC	AGS	HSkMC
24	200.326	275.774	104.115	222.53	238.262	385.862	148.795	342.638
48	-	249.644	-	162.498	69.8652	257.377	17.2498	179.803
72	-	235.601	-	119.68	-	224.062	-	131.402

Values are mean ± Standard of Deviation, <: lower than 30 µg/mL concentration. Gastric cancer (AGS), Colorectal cancer (HCT116), Embryonic Kidney (HEK293) and Human Skeletal Muscle Cells (HSkMC) at concentration (12.5 - 400µg/mL).

Table 2 Percentage death of *Artemia salina* as a function of concentration of methanolic extract and essential oils extracted from *T. ammi* and *F. vulgare* and the LC_{50} values.

Concentration (µg/mL)	*T.ammi*(µg/mL)		*F.vulgare*(µg/mL)	
	Methanolic extract	Essential oil	Methanolic extract	Essential oil
7.8125	0	27	0	20
15.625	2	44	5	35
31.25	6	52	9	42
62.5	13	58	12	55
125	22	64	18	60
250	30	78	25	70
500	35	84	30	75
1000	39.44	90	35	85
LC_{50}	1066.49	137.595	1267.53	235.746

Values are mean (µg/mL) ± Standard of Deviation, LC_{50} determined by ED50plus v1.0 software.

Table 3 MIC values of methanolic extracts and essential oils of *Funiculum vulgare* and *T. ammi* seed against different pathogenic bacteria.

	T.ammi		*F.vulgare*	
	Extract (5mg/mL)	Essential oil (5µL/mL)	Extract (5mg/mL)	Essential oil (5µL/mL)
Gram positive bacteria				
Staphylococcus aureus (ATCC 29737)	0.35±0.05	0.08±0.02	0.45±0.05	0.1±0.02
Gram negative bacteria				
Klebsiella pneumoniae (ATCC 10031)	0.72±0.03	0.4±0.08	1.2±0.3	0.7±0.2
Proteus vulgaris (PTCC 1182)	0.5±0.05	0.35±0.07	0.78±0.05	0.4±0.1
Salmonella paratyphi (ATCC 5702)	0.45±0.11	0.15±0.05	0.65±0.05	0.33±0.02
Escherichia coli (ATCC 10536)	0.85±0.05	0.5±0.05	1.1±0.1	0.57±0.07

The results are expressed as mean ± SD.

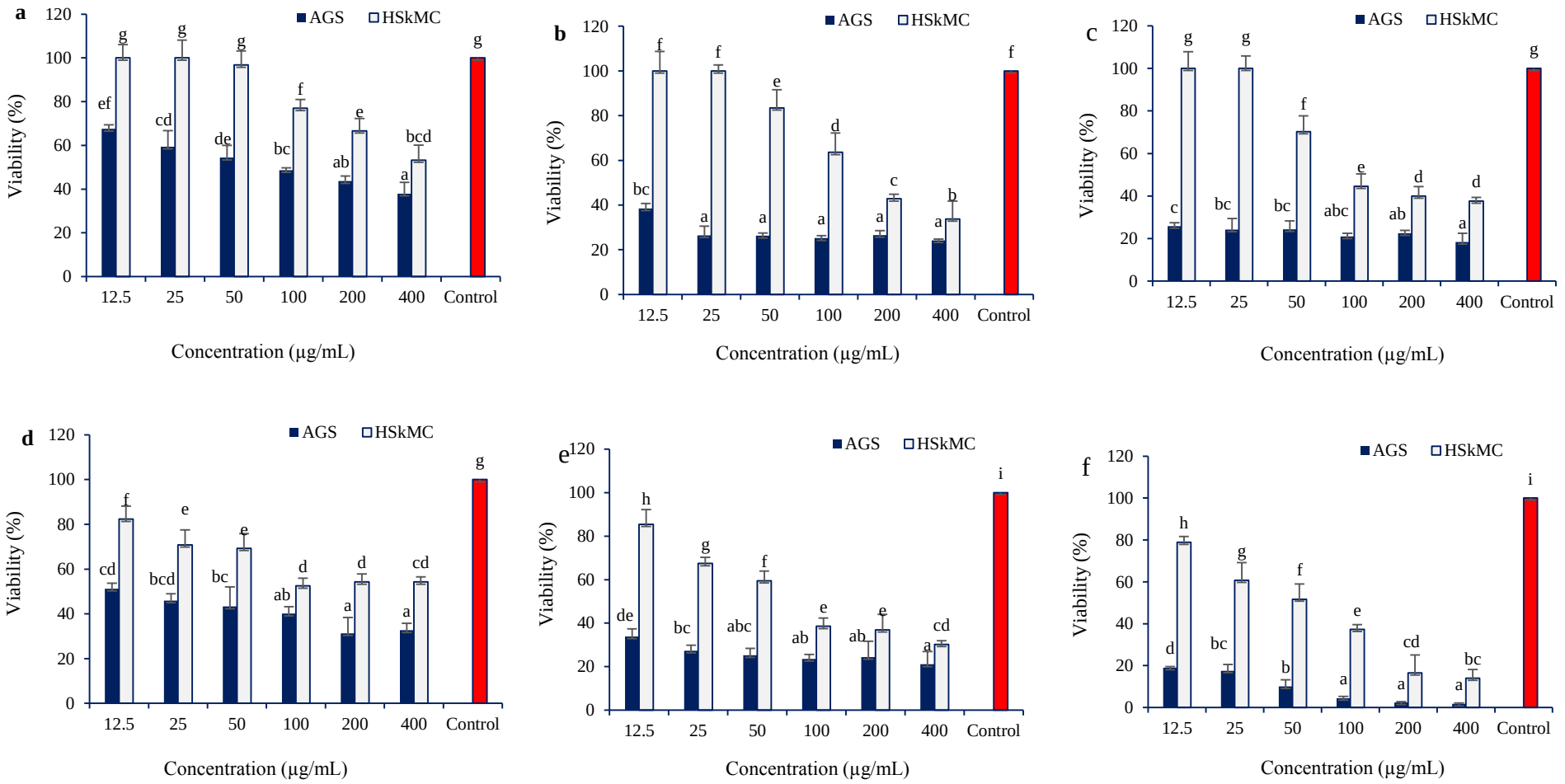

Fig 1 Cytotoxic effects of methanolic extract and essential oil of *F. vulgare* seeds on cancer and normal cells lines. (a) Effect of methanolic extract in 24 h (b) Effect of methanolic extract in 48 h (C) Effect of methanolic extract in 72 h, (d) Effect of essential oil in 24 h (b) Effect of essential oil in 48 h (C) Effect of essential oil in 72 h. Gastric cancer (AGS), and Human Skeletal Muscle Cells (HSkMC) at concentration (12.5 - 400µg/mL). Duncan test was used for mean comparison ($p<0.05$). Charts with the same letters are not statistically significant.

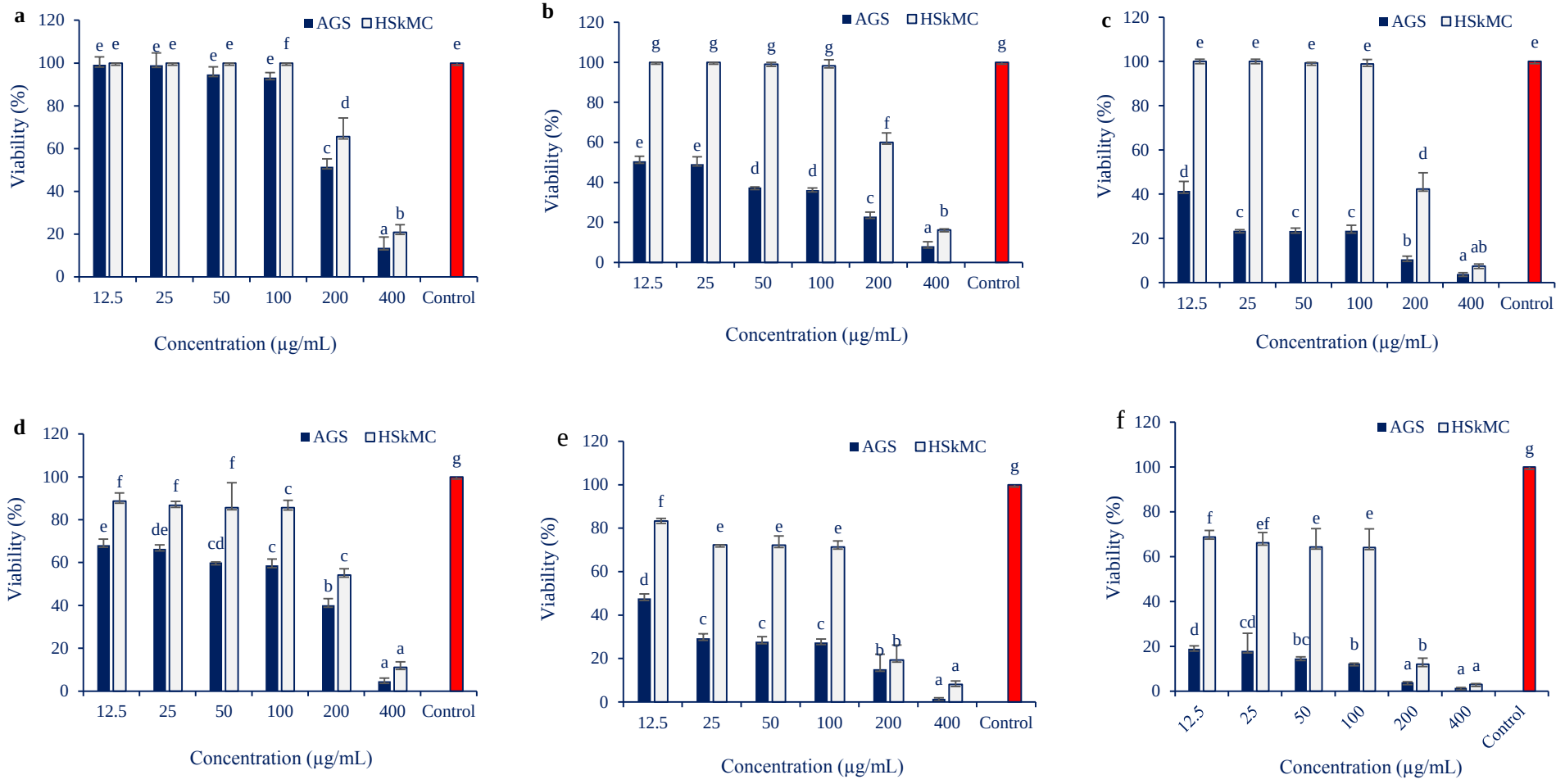

Fig 2 Cytotoxic effects of methanolic extract and essential oil of *T. ammi* seeds on cancer and normal cells lines. (a) Effect of methanolic extract in 24 h (b) Effect of methanolic extract in 48 h (C) Effect of methanolic extract in 72 h, (d) Effect of essential oil in 24 h (b) Effect of essential oil in 48 h (C) Effect of essential oil in 72 h. Gastric cancer (AGS), and Human Skeletal Muscle Cells (HSkMC) at concentration (12.5 - 400µg/mL). Duncan test was used for mean comparison ($p<0.05$). Charts with the same letters are not statistically significant.

The methanolic extract and essential oil of *T.ammi* seeds showed IC_{50} lower than 50 µg/mL at 72h. Although, *F.vulgare* and *T.ammi* can be considered appropriate for further purification and are agree with the US National Cancer Institute (Rajkumar *et al*., 2009).

The results of the current studies clearly exhibited the anticancer activities of *T. ammi* and *F. vulgare* that are consistent with previous studies on different cancer cells, including, *T. ammi* seed extract reduced to forestomach and skin tumor multiplicity (Singh and Kale, 2010). *T. ammi* showed high cytotoxicity against MCF-7 cell (Ramya *et al*., 2017). The n-hexane extract and essential oil of *T. ammi* showed anticancer properties on the liver carcinoma cell (HepG2) that essential oil showed higher activity than the n-hexane extract (Abdel-Hameed *et al*, 2014). The researchers represented that *T. ammi* essential oil reduced the viability of colon cancer cells (Khorsandi *et al*., 2021). On the other hand, (Vitali *et al*., 2016) reported the cytotoxic activity of *T.ammi* essential oil on colon carcinoma cells together with its interaction with immune system cells (Vitali *et al*., 2016).

The cytotoxicity of *F.vulgare* extracts was proved on five different lymphoblastic cell lines (Zidorn *et al*., 2005), *F. vulgare* aqueous extract showed the antiulcerogenic properties on ethanol caused gastric lesions in rats (Birdane *et al*., 2007). *F. vulgare* extracts exhibited a cytotoxic effect on human breast cancer (MDA-MB231), murine fibrosarcoma (L929sA) and MCF7 cell lines (Kaileh *et al*., 2007). *F. vulgare* methanolic extract exhibited less percentage of micronucleus than standard drug on normal human blood Lymphocyte culture and also showed potent antitumor activity on B16F10 melanoma cell (Pradhan *et al*., 2008). The results of (Pradhan *et al*., 2008) also expressed that *F.vulgare* could be a cytoprotective to normal cells and antitumor agent. Anticancer activity of *F. vulgare* seed methanolic extract was also assessed on liver cancer Hepg-2 cells and breast (Mohamad *et al*., 2011). In similar studies, the growth of AGS cells were also inhibited by alcoholic extracts and essential oil of *Pimpinella anisum* (Apiaceae) and *Acorus calamus* (Araceae) (Rahamooz Haghighi *et al*., 2016;2017).

Therefore, if LC_{50} detected for *T. ammi* and *F. vulgare* be more than 1000 µg/mL, it is defined as non-toxic (Ruebhart *et al*., 2009).The LC_{50} determined for methanolic extracts were not low than 1000 µg/mL and they were not defined as toxic but The LC_{50} determined for essential oils were toxic. Therefore, through various *in vitro* studies, the toxicological effect of plant-derived constituents has been studied towards *A. salina* using (Rahamouz-Haghighi *et al*., 2021b).

Some studies report the antimicrobial activity of *T. ammi* extracts. The various extracts of *T. ammi* showed antibacterial properties on *S. aureus*, *E. coli*, *S. typhi*, and *P. aeruginosa* (Ahmad *et al*., 1998; Patel et al., 2008). The organic and aqueous extracts of *T.ammi* seeds showed antibacterial activity (Kaur and Arora 2009). The essential oil of *T. ammi* seeds showed antibacterial activity (Moein *et al*., 2015). (Abdel-Hameed, 2014), reported the *T. ammi* essential oil and n-hexane extract exhibited antimicrobial activity against five microorganisms (Abdel-Hameed *et al*, 2014). The essential oil of *T. ammi* exhibited inhibition zones higher on *Candida albicans* and *S. aureus* than reference antibiotics (Vitali *et al*., 2016).

On one hand, organic and aqueous extracts of *F.vulgare* exhibited moderate antibacterial activity (Kaur and Arora, 2008; 2009). Aqueous-ethanol extract of *F. vulgare* exhibited antibacterial activity on *Helobacterium pylori* and *Campylobacter jejuni* which are the causes of gastrointestinal disorders (Mahady *et al*., 2005; Cwikla *et al*., 2009).

5. Conclusion

The present study showed that the methanolic extracts and essential oils of *T. ammi* and *F. vulgare* have antiproliferation and antibacterial properties and could be used as adjuvant therapy against common gastric and pathogenic bacteria. We evaluated the effects of medicinal plants, including this plant on the growth and proliferation of other cancer cells as colorectal cancer cell lines with complementary tests. *T. ammi* and *F. vulgare* are used in traditional medicine caused their exploration for the development of novel effective chemotherapeutic agents.

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