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Investigation of Compositions and Effects of Local Herbal *Silybum marianum* and *Foeniculum vulgare* extractions on Hospital Acquired Infections (HAI) and Cell Line of liver Cancer (HepG2) by MTT assays

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ABSTRACT

Background & Aim: Increasing of drug resistances and more attention to the hazards of side effects of synthetic drugs has caused extensive researches on medicinal plant resources. The aim of this study was to determination of the chemical composition of native *Silybum marianum* and *Foeniculum vulgare* extractions in Ardabil, Iran; and investigation of their anticancer effect on liver cancer cell line HepG2 and antimicrobial effects on *Staphylococcus aureus* and *Pseudomonas aeruginosa* to propose a solution to control or treatment.

Experimental: The chemical compositions of extractions performed through Soxhlet were analyzed by GC/MS system. The anticancer effect was assayed by MTT method and their antimicrobial activities were tested using disc-diffusion assay as well as MIC and MBC values estimated according to the microdilution method.

Results: The analysis of *S. marianum* extract showed the presence of 29 compounds, mostly (2.63%) Nonane, (4.68%) Camphor, (2.84%) Borneol, (3.65%) Trans-anethole, (8.78%) Viridiflorol, (34.26%) α–Selinene, (2.34%) Longiborn-9-ene, (2.52%) amorphane-B, (5.00%) Erucylamide, (10.15%) o-Terphenyl and (46.8%) α-androst-8-ene; *F. vulgare* extract contained 14 compounds, mainly (5.53%) Fenchone, (5.53%) α- Thujone, (2.84%) Nonane, (79.10%) trans-Anethol and (2.51%) Hentriacontane. The effects of *F. vulgare* extract on Staphylococcus and *S. marianum* extract on Pseudomonas were remarkable. In the combined design, there was no clear effect. MIC and MBC values confirmed the results of the sensitivity test. The cytotoxic effects of extractions on liver cancer cells were the optimum in 25µg/ml after 48 hours.

Recommended applications/industries: According to the results, these compounds are able to replace by chemical preservatives in food and drugs industries.

1. Introduction

Nosocomial infections or hospital-acquired infections (HAI) are a significant cause of public health

problemes, economic burden, morbidity and mortality in hospitalized patients in developed and developing countries (Malhotra *et al*., 2014; Khosravi *et al*., 2014). The most frequent HAI are infections of surgical wounds, urinary tract infections and lower respiratory tract infections. *Staphylococcus aureus and Pseudomonas aeruginosa* are two opportunistic pathogens in hospital that afflict patients with burning damages, respiratory diseases, bacterinemia, septicemia and many of other prevalent infections (Rahimi, Alian, 2013; Rasaei *et al*., 2013). Bacterial resistance to antibiotics is a main problem in the treatment of HAI. Thus, a well-conducted surveillance, prevention programs and alternative drugs may significantly reduce HAI and associated costs (Humphreys, Syth, 2006; Parvin *et al*., 2010). HepG2 is a perpetual cell line consisting of human liver carcinoma cells, derived from the liver tissue of a 15-year-old Caucasian male who had a well-differentiated hepatocellular carcinoma. Hepatocellular carcinoma is, worldwide, the fifth most-common cancer. The morphology of HepG2 cells is epithelial and they have 55 chromosome pairs. HepG2 cells can be grown successfully at a large scale, and secret many plasma proteins, such as transferring, fibrinogen, plasminogen and albumin. Despite some success in controlling infectious diseases, the incidence of non-contagious diseases has dramatically increased; In Iran, cancer after cardiovascular disease and injuries, is the third leading cause of death (Etemadi *et al*., 2008; Alsayyad, Hamadeh, 2007).

Liver cancer is the fifth and seventh most common cancer among men and women, respectively; it has a wide distribution and is a serious threat to human health so that each year more than 500 million people worldwide are infected with the disease and 5-year mortality rate is 95% and after lung and stomach cancers is accounted for most deaths from cancer (Hall, Wild, 2003; Bosch *et al*., 2004). The most important causes of this disease are in most cases including complication of cirrhosis, alcohol intake and chronic hepatitis B and C infections (Nordenstedt *et al*., 2010).

Local Herbal *Silybum marianum* and *Foeniculum vulgare* extractions in Ardabil, Iran, were studied as herbal medicines. Milk thistle (*S. marianum*), a member of the Compositae family, is an annual or biennial native to the Mediterranean, but now widespread throughout the world. Milk thistle appears to be safe and have multiple health benefits on various liver conditions; liver cirrhosis, alcoholic hepatitis, alcoholic fatty liver, liver poisoning, and viral hepatitis (Kiran Kaur *et al*., 2011).Fennel *(F. vulgare*) is a well known medicinal and aromatic plant spread in Mediterranean area and Central Europe. It is widely cultivated throughout the temperate and tropical regions of the world for its aromatic mature fruits which are used as a culinary spice and flavoring agents in food. Herbal drugs and essential oil of fennel have antispasmodic, diuretic, anti-inflammatory, analgesic and antioxidant, antimicrobial and hepatoprotective effects (Aprotosoaie *et al*., 2010).

Therefore in the present study, the therapeutic effects of Milk Thistle and Fennel using Gas Chromatography Mass Spectrometric (GC-MS) were investigated on hospital acquired infections by *S. aureus and P. aeruginosa* and cell line of liver cancer (HepG2).

2. Materials and Methods

2.1. Preparation of plants extracts

The plants were collected from Ardabil, North-West of Iran and identified according to the flora of Iran. First of all, the seeds were separated and washed by cold water to clean from dust and then dried at room temperature for five days. Next, 100 g of powdered seeds was delivered to Soxhlet apparatus (for 3 hours using n-hexane solvent) to achieve the extractions and then rotary evaporator was used for concentration. Finally, the extractions were stored in sealed glass vials at 4 -5 °C (Ahmad *et al*., 2009).

2.2. Analysis of the extractions

The gas chromatography analysis of the extracts were performed using a HP 5890-series II equipped with Flame ionization detectors (FID), HP-5 (BP-1) (5% phenyl+95% dimethylpolysiloxane) 30 m × 0.25 mm ID, 0.25 µm film thickness fused capillary column and HP Innowax (BP-20; polyethylenglycol) 30m × 0.25 mm ID, 0.25 µm film thickness fused capillary column .The carrier gas was nitrogen (1.2 ml mn^{-1}). The oven temperature program was 1 min isothermal at 50 °C, then 50 - 280°C (BP-1) and 50 – 220 °C (BP-20) at rate of 5°C/min and held isothermal for 1 min.The injection port temperature was 250°C, detector: 280 °C. Volume injected: 1 µl of 1% solution

(diluted in hexane). Percentages of the constituents were calculated by electronic integration of FID peak areas without the use of response factor correction .The analysis of the extractions constituents was run on a Hewlett -Packard GC-MS system (GC: 5890-series II; MSD 5972). The fused-silica HP-5 MS capillary column (30 m x 0.25 mm ID, film thickness of 0.25 µm) was directly coupled to the MS. The carrier gas was helium, with a flow rate of 1.2 ml mn-1. Oven temperature was programmed (50 °C for 1 min, then 50 – 280 °C at 5°C/min) and subsequently, held isothermal for 2 min. Injector port: 250°C ,detector: 280 °C, split ratio 1:50. Volume injected: 1 µl of 1% solution (diluted in hexane): HP 5972 recording at 70 e Volts; scan time 1.5 sec; mass Range 40 - 300 amu. Software adopted to handle mass spectra and chromatograms was a Chem Station. The components of the extractions were identified by comparison of their mass spectra with those of a computer library (Mahmoudi *et al*., 2011, Hajlaoui *et al*., 2010, Hajlaoui *et al*., 2008).

2.3. Preparation and culture of microorganisms

The lyophilized standard strains *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 2785 were prepared from ATCC reference center. First, the bacteria were revived in a BHI broth overnight at 30- 35^{o}C; the turbidity samples were isolated and purified on Blood agar and Nutrient agar, respectively (Rahimi, Alian, 2013; Rasaei *et al*., 2013).

2.4. Antibacterial susceptibility testing

Antibacterial activities of extractions were evaluated by the agar-disk diffusion method (ADDM). Overnight bacterial suspensions were first adjusted to 0.5 McFarland turbidity standards (approximate concentration: 1.5×10^{8} cfu/ml). The inoculums of bacterial suspensions were delivered onto Muller Hinton agar plates using a sterile swab, aseptically. The antibiotics Vancomycin (30 µg) for *S. aureus* and Amikacin (30 µg) for *P. aeruginosa* were selected, as positive controls. Two wells were created in each of the plates. One of the wells was poured by antibiotics control (50 µl) and the other was filled by extractions (50, 60 and 70 µl), alone or combined. The plates were incubated at 37 ºC for 18-24 hours. Antibacterial activity was evaluated by Clinical and Laboratory Standards Institute (CLSI) guidelines (Oxoid). Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded (Mahmoudi *et al*., 2011; Kermanshah *et al*., 2011; Baver *et al*., 1996; Jafari *et al*., 2011).

2.5. Determination of inhibitory activity of extracts

The minimum inhibitory concentration (MIC) values were determined for the bacterial strains based on a macro dilution method which were sensitive to the extractions in disk diffusion assay. The inoculums of the bacterial strains were prepared from overnight cultures. The mix of bacteria (one loopful) and dilutions of the extracts were incubated at 37 °C for 24 hours in 1 ml Muller Hinton broth. After this period, the concentration of first tube without turbidity was considered as MIC. In the next stage, the contents (one swab) of the non- growth tubes were cultured in Muller Hinton agar plates. After incubation at 37 °C for 24 hours, the first non-growth plate was considered as minimum bactericidal concentration (MBC) (Mahmoudi *et al*., 2011; Kermanshah *et al*., 2011; Baver *et al*., 1996; Jafari *et al*., 2011).

2.6. Evaluation of anticancer effect of extractions by MTT assay

Cell line of HepG2 was cultured in RPMI- 1640 containing 10% FBC and 5% streptomycin-penicillin and incubated at 37 ° C; then the cells were separated by trypsin 0.25%. 5mg/ml MTT solution (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was prepared in PBS and steriled by 0.2µ filter and stored in a refrigerator. Then the cell suspension (2×10^{4} cells/ ml) were incubated in 96 microplates for 24 hours (CO_2 5% and 90% humidity) and measured by reverse phase microscopy. 20 µl of essential oils (0, 5, 10, 15, 20, 25, 50 and 100 µg/µl concentrations) were added separately. All steps for each dilution were prepared in three separate 96-well plates in parallel and repeated for three consecutive days. Cell suspension without extractions was considered as a negative control. The plates were incubated for 24, 48 and 72 h in a CO_2 incubator. After 24 h – in the first plate– the upper medium was emptied by returning the plate, then fresh medium was added to each well. After the lapse of time, 20 ml MTT solution was added to each well. The plates were covered with aluminum file; after 4 h incubation Cell medium was discarded in each well and 150 ml of DMSO was added in each of the wells to dissolution of produced color. After complete dissolution of the color, intensity of the

absorbance of solution was measured by Elisa plate reader at a wavelength of 570 nm and the percentage of viable cells was calculated by the following formula:

Percentage of viable cells (compared to control cells) = (The optical absorption of cells affected by essential oils in each cell/ The mean of the optical absorption of wells with control cells)×100

All of these steps were performed for the second and third plates after 48 and 72 h. All of the above was repeated three times. Data were analyzed using two-way ANOVA (P <0.05) (Forouzandeh *et al*., 2011; Roudbar Mohammadi *et al*., 2012).

3. Results and discussion

3.1. Chemical composition of extracts

In this study, we intend to determine the chemical composition of extracts of the *S. marianum* and *F. vulgare* collected from Ardabil province, Iran. The extracts were liquid at room temperature; their odors were agreeable. The analysis of *S. marianum* extract showed the presence of 29 compounds and *F. vulgare* extraction contained 14 compounds (Table 1 and 2). The analysis of *S. marianum* extract showed the presence of 29 compounds including Nonane, α -terpinene , α -thujene, β -thujene, Camphor, Borneol, Trans-anethole, Carvacrol, Caryophyllene, α.-Humulene, β - Selinene, Octanone, Spathulenol, Viridiflorol, Crypton, Cuminic aldehyde, α-pinene, β-Ocimene, Lavandulyl acetate, β-Cedranoxide, α - Selinene, Longiborn-9-ene, amorphane-B, Ferruginol, Erucylamide, Phenanthrene, o-Terphenyl and α-androst-8-ene; the highest levels of these compounds were related to Nonane (2.63%), Camphor (4.68%), Borneol (2.84%), Trans-anethole (3.65%), Viridiflorol (8.78%), α – Selinene (34.26%), Longiborn-9-ene (2.34%), amorphane-B (2.52%), Erucylamide (5.00%), o-Terphenyl (10.15%), α-androst-8-ene (8.46%) (Table 1). *F. vulgare* extract contained 14 compounds including α-pinene, Limonene, Fenchone, α- Thujone, β - Thujone, Camphor, Nonane, Fenchyl acetate, Anisyl acetone, α- Humulene, Neophytadiene, Cis-Anethol, trans-Anethol and Hentriacontane; mainly (5.53%) Fenchone, (5.53%) α- Thujone, (2.84%) Nonane, (79.10%) trans-Anethol and (2.51%) Hentriacontane (Table 2).

Some studies were to some extent in line with the present work.

Table 1. The major compounds of *Silybum marianum* extract

	Components	Emergence time	Percentage (%)
1	Nonane	6.940	2.63
2	α -terpinene	8.246	0.25
3	α -thujene	10.847	1.31
4	β -thujene	11.164	0.29
5	Camphor	11.972	4.68
6	Borneol	12.658	2.84
7	Trans-anethole	16.054	3.65
8	Carvacrol	16.675	0.56
9	Caryophyllene	19.573	1.85
10	α.-Humulene	20.433	1.42
11	β - Selinene	20.433	1.08
12	Octanone	21.229	0.20
13	Spathulenol	23.461	0.20
14	Viridiflorol	23.816	8.78
15	Crypton	24.185	1.40
16	Cuminic aldehyde	27.898	0.20
17	α-pinene	28.403	0.34
18	β.-Ocimene	28.403	0.34
19	Lavandulyl acetate	29.030	0.54
20	Calarene epoxide	29.936	0.81
21	β.-Cedranoxide	29.936	0.81
22	α - Selinene	33.383	34.26
23	Longiborn-9-ene	33.940	2.34
24	amorphane-B	37.310	2.52
25	Ferruginol	38.086	1.22
26	Erucylamide	38.623	5.00
27	Phenanthrene	38.849	1.87
28	o-Terphenyl	39.328	10.15
29	α-androst-8-ene	40.001	8.46

Table 2. The major compounds of *Foeniculum vulgare* extract

	Components	Emergence time	Percentage (%)
1	α-pinene	5.976	0.24
2	Limonene	8.615	1.64
3	Fenchone	10.349	5.53
4	α- Thujone	10.853	5.53

5	β - Thujone	11.177	0.80
6	Camphor	11.972	0.44
7	Nonane	12.736	2.84
8	Fenchyl acetate	14.560	0.20
9	Anisyl acetone	18.719	0.40
10	α- Humulene	20.433	0.27
11	Neophytadiene	29.030	0.20
12	*Cis*-Anethol	36.928	0.29
13	*trans*-Anethol	37.543	79.10
14	Hentriacontane	42.297	2.51

Table 3. The mean ± SD of non growth zone of extracts and control antibiotic on *Staphylococcus aureus ATCC1341*

Antibacterial agents	**Concentration** (µl)	**Non growth zone** (mm)
Silybum marianum	50	11.54±1.01(I)
	60	12.17±1.78(I)
	70	12.42±1.5(I)
Foeniculum vulgare	50	28.78±2.1(S)
	60	29.81±1.25(S)
	70	30.13±1.30(S)
Vancomycin	50	20(S)
S = Sensitive; I = Intermediate; *level of significance (P value <0.05)		

Table 4. The mean ±SD of non growth zone of extracts and control antibiotic on *Pseudomonas aeruginosa ATCC2785*

Antibacterial agents	**Concentration** (µl)	**non growth zone** (mm)
Silybum marianum	50	21.04±1.7(S)
	60	22.17±2.1(S)
	70	22.31±1.3(S)
Foeniculum vulgare	50	11.10±2.3(I)
	60	10.88±2.7(I)
	70	11.01±1.4(I)
Amikacin	50	20(S)
S = Sensitive; I = Intermediate; *level of significance (P value <0.05)		

Table 5. The level of MIC and MBC of plant extracts on studied bacteria

Antibacterial agents	***Staphylococcus aureus ATCC1341***		***Pseudomonas aeruginosa ATCC2785***	
	MIC (µg/ml)	**MBC**(µg/ml)	**MIC** (µg/ml)	**MBC**(µg/ml)
Silybum marianum	20	40	10	20
Foeniculum vulgare	5	20	10	40

Table 6. The mean ± SD of non growth zone *, MIC and MBC of combined plant extracts (50 µl) on studied bacteria

Antibacterial agents	***Staphylococcus aureus ATCC1341***	***Pseudomonas aeruginosa ATCC2785***
non growth zone (mm)	28.73±1.20 (S)	19.63±1.25 (S)
MIC (µg/µl)	20	20
MBC (µg/µl)	40	40
S = Sensitive; *level of significance (p value < 0.05)		

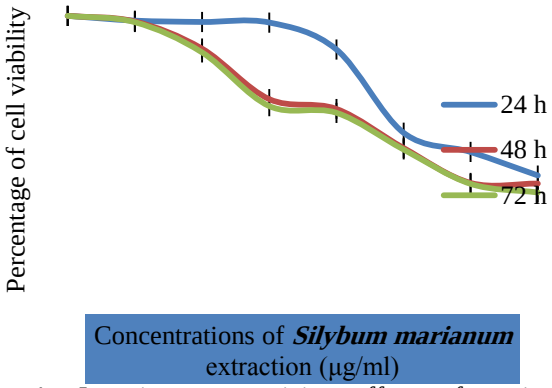

Fig 1. In vitro cytotoxicity effect of various concentrations of *Silybum marianum* extract on liver cancer cell line HepG2 (%).

Raal *et al.* (2012) have determined differences in the essential oil composition of *F. vulgare* obtained from retail pharmacies in Estonia, Norway, Austria and Moldova and from a spice shop in Turkey using capillary GC and GC-MS. Overall joint and main compounds were Fenchone, Estragole, Limonene and Cis-anethole (Raal *et al.*, 2012). In a similar study, difference in the content of *F. vulgare* essential oil has been proven based on a variety of different weather conditions including temperature and rainfall (Clara et

al., 2010). In a study *S. marianum L.* seeds have been fermented using solid-state fermentation with several lactic acid bacteria of Lactobacillus and Pediococcus genera to improve sensory properties (flavor). The composition of volatile compounds of the unfermented and LAB-fermented seeds of this plant has been analyzed using GC-MS. Fermented seeds have shown considerable differences mainly due to the accumulation of higher alcohols, phenolic compounds, flavonoids and 2,2-diphenylpicryl-hydrazyl (DPPH).

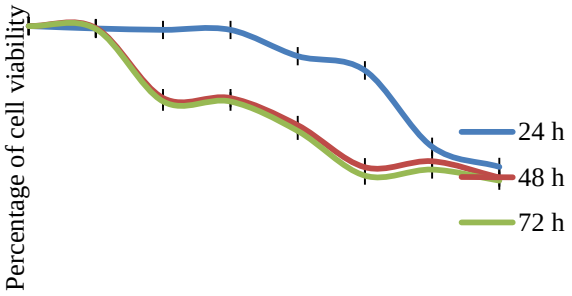

Fig 2. In vitro cytotoxicity effect of various concentrations of *Foeniculum vulgare* extract on liver cancer cell line HepG2 (%).

3.2. Antibacterial activity, MIC and MBC

The results of antibacterial activity of native medicine plants extractions on the bacteria by the disk diffusion agar susceptibility test method has been shown in Table 3 and 4 (P_{value} <0.05) and the results of broth macrodilution test method showing the levels of MIC and MBC has presented at Table 5, as well as Table 6 indicates antibacterial test results for combined design of plants (P value <0.05). This study has been revealed that fermented *S. marianum* seeds are a suitable antimicrobial additive for natural flavouring of baked goods (Juodeikiene *et al*., 2013).

Liver cancer is one of the major causes of cancer related death in the world, even though its incidence has decreased over the past decade. Chemical and synthetic drugs such as non-steroidal anti-inflammatory drugs are not useful in some circumstances because of their side effects (Etemadi *et al*., 2008; Alsayyad, Hamadeh, 2007; Nordenstedt *et al*., 2010). Therefore, the present study was necessary to detect some new anti-cancer, cell line HepG2, compounds especially medicinal plants including Milk Thistle and Fennel in Ardabil, Iran, to find a solution with the lowest side effects in the control or treatment.

The averages live of liver cancer cell line HepG2 (%) rather than control cells in the presence of various concentrations (µg/ml) of indigenous medicinal plants extractions have been shown in Figures 1 and 2. In a similar study conducted in 2013 in Palestine, it has been showed that pilot array of concentrations of *S. marianum* and *O. narbonense* had no cytotoxic effect on the hepatic cell line HepG2 up to 500 µg/ml, but effectiveness was significant and optimum increasing concentration to 500 µg of plant extract/ml of HepG2 cell culture medium as measured by the viability MTT assay (Ahmad Ghareeb, 2013).

Another research has been performed by Villarini *et al.* (2014) in Italy to evaluate the in vitro cytotoxic, genotoxic, and apoptotic activities of estragole 1 in the essential oil of *Foeniculum vulgare* (fennel) on HepG2 human hepatoma cell line. Toward this end, an MTT cytotoxicity assay, a trypan blue dye exclusion test, a double-staining (acridine orange and DAPI) fluorescence viability assay, a single-cell microgel-electrophoresis (comet) assay, a mitochondrial membrane potential (Δψm) assay, and a DNA fragmentation analysis have been conducted. In terms of potential genotoxic effects, the comet assay indicated that estragole 1 was not able to induce DNA damage nor apoptosis under the experimental conditions used (Villarini *et al.*, 2014). Also the results of the study and analysis of data related to MTT assay test (Level of confidence 95%) showed that extracts had significant cytotoxic effects on the liver cancer cell line HepG2; while the onset and intensity of the effects were different. The influence of two plant extracts did not show significant and remarkable difference up to concentration of 20 µg /ml, but beyond this range the effects intensified significantly, and were strangely very close and almost similar to each. Effectiveness was significant and optimum after 48 hours by increasing concentration to 25µg/ml; but intensity of the influence of subsequent concentrations was steady and had no significant effects. According to the statistical analysis, for two medicinal plant extracts-separately and compared with each other- time and extract concentration had significant effects on the mean survival (%) of liver cancer cells compared to

control cells; however, these two parameters had no synergistic effects.

4. Conclusion

Each year in the mountainous areas in Ardabil, the large amount of variety of herbs grew self- propelled, particularly plants that studied in this work, and later destroyed without any use. With regard to the effective ingredients of these plants on growth inhibition of bacteria according to results of our study and other researches, they could be simply exploited as affordable and available source of bio-pharmaceuticals. In this work, *S. marianum* and *F. vulgare* were introduced as plants with antioxidant and anti bacterial properties. The cytotoxic effects were optimum in 25µg/ml after 48 hours. The effects of extracts were different on Staphylococcus (gram-positive) than Pseudomonas (gram-negative) bacteria maybe because of differences in the cell wall type of bacteria and/or plant compositions. According to the results, these compounds are able to replace by chemical preservatives in food and drugs industries as well as they also can be investigated on other pathogens and cancers to find useful solutions to overcome them.

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6. References

Ahmad A, Alkarkhi A. F. M, Hena S, Khim L. H. Extraction, Separation and Identification of Chemical Ingredients of *Elephantopus Scaber L.* 2009.Using Factorial Design of Experiment. *International Journal of Chemistry*, 1(1): 36- 49.

Ahmad Ghareeb B. A. 2013.Assessment of Cellular Responses to Plant Extracts in Jenin Area Ranges (*Ornithoganum narbonense* and *Silybum marianum*), Problems and Solutions. *Advances in Life Science and its Applications (ALSA)*, 1(4): 84-90.

Alsayyad J, Hamadeh R. 2007. Cancer incidence among the Bahraini population: A five-year (1998-2002 experience. *Ann Saudi Med*, 27(4): 251-258.

Aprotosoaie AC, Şpac A, Hăncianu M, Miron A, Tănăsescu Vf, Dorneanu V, Stănescu U. 2010. The chemical profile of essential oils obtained from fennel fruits (*Foeniculum vulgare mill.*). *FARMACIA*, 58(1): 46-53.

Baver AW, Kirby WM, shreeis JC, turck M. 1996.Antibiotic Susceptibity testing by astandardized single disc method. *Am. J. Chin. Pathol*, 45: 493-496.

Bosch FX, Ribes J, Diaz M and Cleries R. 2004. Primary liver cancer: Worldwide incidence and trends. *Gastroenterology*, 127(5): 5-16.

Clara AA, Şpac1 A, Hăncianu1m, Miron1 A, Floria TV, Dorneanu V, Stănescu U. 2010. The chemical profile of essential oils obtained from fennel fruits (*foeniculum vulgare mill*.). *FARMACIA*, 58(1): 46-53.

Etemadi A, Sadjadi A, Semnani SH, et al. 2008. Cancer Registry in Iran: A brief overview. *Arch Iran Med* ,(5)11: 577-580

Forouzandeh F, Salimi S, Naghsh N, Zamani N, Jahani S. 2014.Evaluation of anti-cancer effect of *Peganum harmala* L hydroalcholic extract on human cervical carcinoma epithelial cell line (HeLa). *J Shahrekord Univ Med Sci*, 16(4): 1-8.

Hall AJ, Wild CP. 2003. Liver cancer in low and middle income countries. *BMJ*, 326 (7397): 994-995.

Hajlaoui H, Ben A. F, Snoussi M, et al. 2010. Effect of *Mentha longifolia L. ssp longifolia* essential oil on the morphology of four pathogenic bacteria visualized by atomic force microscopy. *African Journal of Microbiology Research J*, 4 (11): 1122-1127.

Hajlaoui H., Snoussi M., Ben Jannet H., et al. 2008. Comparison of chemical composition and antimicrobial activities of *Mentha longifolia L. ssp. longifolia* essential oil from two Tunisian localities (Gabes and Sidi Bouzid). *Annals of Microbiology J*. 58 (3): 513- 520.

Humphreys H, Syth ET. 2006. Prevalence surveys of healthcare-associated infections: what do they tell us, if anything? *Clinical Microbiology and Infection*, 12(1): p. 2-4.

Jafari A, Ghane M, Arastoo SH. 2011.Synergistic antibacterial effects of nano zinc oxide combined with silver nanocrystales. *African Journal of Microbiology Research*, 5(30): 5465-5473.

Juodeikiene G, Cizeikiene D, Ceskeviciute V, Vidmantiene D, Basinskiene1 L, Akuneca I, et al. 2013. Solid-State Fermentation of Silybum marianum L. Seeds Used as Additive to Increase the Nutritional Value of Wheat Bread. Food Technol. *Biotechnol*, 51 (4): 528–538.

Kermanshah H, Hashemi K.S, Arami S, Mirsalehian A, et al. 2011. Antibacterial effect of hydro alcoholic extract of *Saliva Officinalis* and *Mentha longifolia*on three bacteria cause tooth decay in vitro. *J Shaheed Beheshti University of Dental*, 4: 232-237.

Khosravi AD, Parhizgari N, Abbasi Montazeri E, Mozaffari A, Abbasi F. 2013.The Prevalence of Bacteria Isolated From Endotracheal Tubes of Patients in Golestan Hospital, Ahvaz, Iran, and Determination of Their Antibiotic Susceptibility Patterns. Jundishapur J Microbiol, 6(1):67-71.

Kiran Kaur A, Wahi AK, kumar B, Bhandari A, Prasad N. 2011. Milk Thistle (*Silybum marianum*): A review. *International Journal of Pharma Research and Development (ijprd)*, 3(2): 1-10.

Mahmoudi R., Ehsani A., Tajik H., et al. 2011. Antimicrobial effects of *Mentha Longifolia L.* Essential oil and *Lactobacillus casei* against *staphylococcus aureus* in Iranian White Cheese. *Archive of SID3 J*, 1: 147-161.

Malhotra S, Sharma S, Hans C. 2014. Prevalence of Hospital Acquired Infections in a tertiary care hospital in India. *International Journal of Medicine and Medical Sciences*, 1(7): 91-94.

Nordenstedt H, White DL, El-Serag HB. 2010. The changing pattern of epidemiology in hepatocellular carcinoma. *Dig Liver Dis*, 42(3): 206-214.

Parvin N, Validi M, Banitalebi M, Mobini GhR, Ashrafi K, Farrokhi E, Rafieian M, Akbari N, Safdari F, Rafei-Vardanjani L. 2010.Effect of medicinal smokes on some nosocomial infection factors. *Shahrekord University of Medical Sciences*, 12(2):76-84.

Raal A, Orav A, Arak E. 2012. Essential oil composition of *Foeniculum vulgare Mill*. Fruits from pharmacies in different countries. *Nat Prod Res*, 26(13):1173-1178.

Rahimi E, Alian F. 2013. Presence of enterotoxigenic *Staphylococcus aureus* in cow, camel, sheep, goat, and buffalo bulk tank milk. *Veterinarski Arhiv*, 83 (1): 23-30.

Rasaei S, Sadeghifard N, Zolfaghari MR, Ghafoorian S, Shakib P, Valizadeh A. 2013. Study of PAPI-1 Frequency in Clinical Strains of *Pseudomonas Aeruginosa*. *Scientific Journal of Ilam University of Medical Sciences*, 21(5):67-72

Roudbar Mohammadi Sh, Roudbari M, Vahidi M, Mohammad Hassan Z, Darabi N. 2012. Evaluation of Anti-tumor activity of *Candida albicans* Cell wall fraction on human Fibrosarcoma cell in vitro. *J Army Univ Med Sci*, 10 (2): 103-110.

Villarini M, Pagiotti R, Dominici L, Fatigoni C, Vannini S, Levorato S et al. 2014. In vestigation of the Cytotoxic, Genotoxic, and Apoptosis-Inducing Effects of Estragole Isolated from Fennel (*Foeniculum vulgare*). *J. Nat. Prod.*, 77 (4): 773–778.