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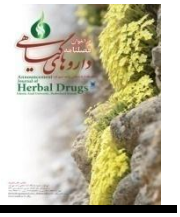

An Investigation of Compositions and Effects of Local Herbal *Glycyrrhiza glabra* and *Mentha pulegium* extracts on *Helicobacterpylori* and Cell Line of stomach Cancer (AGS) by MTT assays

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ABSTRACT

Background & Aim: According to globally development of stomach cancer especially in Ardabil, Iran, as the second major cause of mortality throughout the world, increased drug-resistant bacteria including *Helicobacter pylori* as the most important risk factors for stomach cancer, and side effects of antibiotics and chemical drugs normally used to treat.

Experimental: The current research was conducted to investigate the anticancer and antimicrobial effects of native herbs of liquorice (*Glycyrrhiza glabra*) and pennyroyal (*Mentha pulegium*) extractions for finding a solution with the lowest complications in control or treatment of stomach cancer.The extractions were firstly obtained using Soxhlet and methanol solvent and then their compounds were determined by GC/MS. Antimicrobial activity, MIC and MBC of the extractions were assessed respectively using agar diffusion and broth dilution test and the anticancer effect on stomach cancer (cell line AGS) was assessed by MTT assay. *H. pylori ATCC 26695* was respectively revived and purified on Brucella broth containing 7% citrated horse serum and Columbia agar.

Results: The analysis showed that liquorice extract contains 15 compositions, mainly consists of 26.48% Nonane, 23.38% Ethylcyclohexan, 8.29% 3-Bromodecane, 10.31% trans-2-Heptenal, 8.93% 9-Octadecenamide and 4.68% β-pregna and pennyroyal extract contains 17 compositions, mainly including 3.36% Camphor, 22.79% Pulegone, 4.92% Paramenth-3-n8-l, 8.06% Menthoforan, 7.54% Cis-Isopulegon and 24.58% α-Selinene. The bacteria were resistant or semi-sensitive to common antibiotics, whereas had considerable sensitivity to herbal extracts and liquorice showed almost three times more antibacterial effect. Pennyroyal extract had no cytotoxic effects, but the anticancer effect was observed in liquorice extract with optimal concentration of 25 µg/ml after 48 hours.

Recommended applications/industries: In conclusion, liquorice extract due to the significant health benefits of anticancer and antibacterial activity can be selective and highly effective herbal medicine as an alternative to antibiotics and other chemical drugs.

1. Introduction

Cancer disease is originated from several mutations in transformed cells and other heritable variations in susceptible cells. Disorders in approximately 350 genes have been so far found in human cancers; cancer is epidemiologically responsible for one in eight of deaths all over the world. Gastric or stomach cancer is the fourth most prevalent cancer in the world and adenocarcinoma, or glandular cancer of the stomach is the most common form. Adenocarcinoma can be considered as uncontrolled grow of malignant cells in the stomach. The gastric cancer annually kills about 1 million people worldwide. The prevalence of the disease in men is twice higher than in women. About 2.3 million of cancers were in alimentary tract presenting in pharynx, esophagus, stomach and colorectal region in 2000 (Hashemi *et al*., 2011; Broadhead *et al*., 2010; Zende-Del *et al*., 2013).

The presence of *Helicobacter pylori* and its prone to cause stomach cancer is one of the assumptions embedded in the medical world, while increasing stomach acid, smoking, the use of salted and smoked foods, increased consumption of fried foods are the factors high risk for developing stomach cancer. Endoscopy and radiography of the stomach are diagnostic techniques. There is the possibility of appearing cancer in gastric ulcers. Therefore, if gastric ulcers do not respond to treatment after three months, this will mean an indication for gastric surgery (Lee and Derakhshan, 2013). *H. pylori* as a curved gram-negative and urease-positive bacterium is located in the gastric mucosa, establishes there for years and cause stomach and duodenal ulcers by producing ammonia and toxins. These bacteria are considered as a risk factor for stomach cancer and there is no effective vaccine or treatment against it. The only current therapeutic approach is the use of antibiotics that are accompanied with the possibility of recurrence and re-infection (Brown, 2000; Mobley *et al*., 2001; Kato, 2001).

Although chemical medicines are employed to heal most of the routine diseases, but these therapies can lead to several disorders and unwanted complications. The reported plants and herbals as natural products with anti-cancer properties have attracted further attentions as alternatives to chemical drugs, since they may have fewer side effects. Numerous investigations have been carried out on traditional medical and herbal medicines in different field of medical science. Use of traditional medicine is one of the ways to achieve new drugs (Hashemi *et al*., 2011; Ghasemi, 2015; Mann, 2002). Extractions of Local *Glycyrrhiza glabra* and *Mentha pulegium* in Ardabil, Iran, were studied as herbal medicines.

Liquorice or licorice (*Glycyrrhiza glabra*) is one of the medicinally important plants belonging to the family Fabaceae, mainly with rhizomes and taproot. It is native to Mediterranean regions of Asia. This plant has a long history of therapeutic purposes in cough, respiratory disorders, hair fall, baldness, piles, gout, weakness in lower back and lower limbs, constipation, allergic rhinitis, chronic hepatitis, Herpes simplex, SARS and HIV, as well as anti-ulcer, anti-microbial, anti-asthmatic and anti-diuretic activities (Vijayalakshmi & Shourie, 2013). Studying the essential oils and extractions of pennyroyal (*Mentha pulegium*), belonging to Lamiaceae family, have indicated major effects on preventing the growth of several spoilage and pathogenic agents because of their own anti-bacterial, anti-spasmodic and anti-inflammatory activities (Gaeini *et al*., 2013; Hassanpour *et al*., 2012; Teixeira *et al*., 2012).

Therefore, in the present study, the compositions of *G. glabra* and *M. pulegium* extractions were detected using Gas Chromatography Mass Spectrometric (GC-MS) to assess the therapeutic effects of the extractions on *H. pylori* and Cell Line of stomach Cancer (AGS) by MTT assays, in vitro.

2. Materials and Methods

2.1. Preparation of plants extracts

The plants were collected from Ardabil, Iran, and their identification was based on the flora of Iran. In the next step, the leaves of *M. pulegium* and the roots of *G. glabra* were separated and washed then by cold water to remove physical pollution like dust. Next, the samples were dried at room temperature at least for seven days. After that, 100 g of powdered parts were

delivered to Soxhlet apparatus (for 3 hours using methanol solvent) to achieve the extracts and then rotary evaporator was used for concentrating. Finally, the extracts were delivered to refrigerator inside sealed glass vials at 4°C to 5°C until testing (Ahmad *et al*., 2009).

2.2. Analysis of the extractions

Extracts compounds were analyzed via gas chromatography. For this purpose, the GC-MS device was set on HP 5890-series II equipped with FID, HP-5 (BP-1, 95% dimethylpolysiloxane + 5% phenyl) 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 nitrogen gas was used as carrier (1.2 ml mn^{-1}). The oven was set on 1 min isothermal at 50°C, and then 50°C - 280°C (BP-1) and 50°C– 220°C (BP-20) at 5°C/min and isothermal condition was continued for 1 min. The temperature of injection port and detector were 250°C and 280°C, respectively. The injection volume was 1 µl of 1% solution diluted in hexane. The electronic integration of FID peak points lacking response factor correction was recruited to calculate constituents' percentages. The constituents of extractions were analyzed using 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 mounted on the MS directly. Helium was recruited as carrier gas and its flow rate was 1.2 ml/min. The oven was adjusted at 50 °C for 1 min, and then 50 – 280 °C at 5°C/min, followed by isothermal condition was two minutes. Other conditions were as follows: 250°C for injector port, 280°C for detector, split ratio of 1:50. The injection volume was 1 µl of 1% solution diluted in hexane; HP 5972 recording at 70 e Volts; 1.5 seconds scan time; mass range of 40 amu to 300 amu. Chem Station Software was utilized to manage mass spectra and chromatograms. The mass spectra of compounds saved on a computer library were used to compare and identify the components of the extractions (Mahmoudi *et al*., 2011; Hajlaoui *et al*., 2010; Hajlaoui *et al*., 2008).

2.3. Preparation and cultivation of bacterium

The lyophilized standard strain *H. pylori* ATCC 26695 was prepared from ATCC reference center. First, the bacteria were revived in a Brucella broth medium containing fetal calf serum (FCS) overnight at 30- 35°C; the turbidity samples were isolated and purified on Columbia agar medium (Brown, 2000; Mobley *et al*., 2001;Kato, 2001).

2.4. Antibacterial susceptibility testing

The agar-disk diffusion method (ADDM) was employed for evaluation of extracts antibacterial activities. Firstly, overnight bacterial suspension was prepared according to 0.5 McFarland turbidity standards (approximate concentration 1.5×10^8 cfu/ml). The inoculums of bacterial suspensions were delivered onto Brucella agar medium containing 5% human blood and 7% FCS plates using a sterile swab, aseptically. Four antibiotics including clarithromycin (2 µg), tetracycline (30 µg), metronidazole (5 µg) and amoxicillin (10 µg) were selected as positive controls. To prepare the discs of extractions, 20µl of diluted extract was absorbed into blank discs that were completely dried inside the oven at 40ºC for 24 hours. A disk impregnated with the solvent was considered as negative control. The discs were put on the plate containing Mueller Hinton agar medium by sterile forceps at regular intervals and then incubation was done at 37°C for 3-5 days in microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2). The assessment of antibacterial activity was based on Clinical and Laboratory Standards Institute (CLSI) guidelines (Oxoid). The non-growth zone diameter of strains resistant to clarithromycin, metronidazole, amoxicillin and tetracycline was respectively less than any size, 16 mm, 11mm and 20 mm. The mean diameter of the inhibition zone was calculated on the basis of recorded experiments in triplicate (Tomatari *et al*., 2010; Kermanshah *et al*., 2011; Mahmoudi *et al*., 2011).

2.5. Determination of inhibitory activity of extracts

The macrodilution method was considered to determine the minimum inhibitory concentration (MIC) values of the bacterial strains recognized as sensitive to the extracts in disk diffusion assay. The overnight bacterial cultures were inoculums of the strains. The

resulting mixture containing one loopful of bacteria and dilutions of the extracts in 1 ml Mueller Hinton broth were incubated at 37°C for 24 hours. In the next step, MIC value was considered as the concentration of first tube without turbidity. One swab of the non-growth tubes was cultured on Mueller Hinton agar plates, which were incubated at 37°C for 24 hours. The first non-growth plate indicated minimum bactericidal concentration (MBC) (Kermanshah *et al*., 2011; Mahmoudi *et al*., 2011; Baver *et al*., 1996; Jafari *et al*., 2011).

2.6. Evaluation of anticancer effect of extracts by MTT assay

The cell line of AGS 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%. Five mg/ml MTT solution (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was prepared in PBS and sterilized by 0.2µ filter and stored in a refrigerator. Next, the cell suspension (2×10^4 cells/ml) was incubated in 96 microplates for 24 hours (CO_2 5% and 90% humidity) and measured by reverse phase microscopy; 20 µl of extractions (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. The 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 inverting the plate, then fresh medium was added to each well. After the lapse of time, 20 ml of MTT solution was poured to each well. The plates were covered with aluminum file; after 4 h incubation, the 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 equation:

Percentage of viable cells= (The optical absorption of cells affected by extractions in each well/ The mean of 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 procedures were carried out in three replications. Data were analyzed using two-way ANOVA ($P < 0.05$) (Forouzandeh *et al*., 2014; Roudbar Mohammadi *et al*., 2012).

3. Results and discussion

Gastric 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 nonsteroidal anti-inflammatory drugs are not useful in some circumstances because of their side effects (Moghimi-Dehkordi *et al*., 2008; Pourhoseingholi *et al*., 2007). Therefore, the present study aimed to detect some new anti-cancer, cell line AGS, compounds especially medicinal plants extractions including *G. glabra* and *M. pulegium* in Ardabil, Iran, to find a solution with the lowest side effects in the control or treatment.

The obtained extractions were liquid at room temperature and their odors were agreeable. The analysis of *M. pulegium* extraction showed the presence of 17 compounds, including Nonane, α-Decene, α-Thujone, Camphor, Borneol, Spathulenol, 1,8-Cineole, Paramentha 3,8-dien, Pulegone, Paramenth-3- n 8-l, Menthoforan, Cis- Isopulegon, 2-β-Pinene, neo iso dihydrokarool, α-Selinene and 2-cyclohexane-1-van. Among these, the highest levels of these compounds were 3.36% Camphor, 22.79% Pulegone, 4.92% Paramenth-3-n8-l, 8.06% Menthoforan, 7.54% Cis-Isopulegon and 24.58% α – Selinene (Table 1). *G. glabra* extraction contained 15 compounds, including Nonane, Ethylcyclohexan, α-Decene, 3-Bromodecane, 1-Hexadecanol, trans-2-Heptenal, 1H-Indene, Trans-anethole, cerylcerotinate, Lignocerol, 9-Octadecenamide, Adogen 4, β-pregna, Isochiapin b and Hahnfett; mainly 26.48% Nonane, 23.38% Ethylcyclohexan, 8.29% 3-Bromodecane, 10.31% trans-2-Heptenal, 8.93% 9-Octadecenamide

and 4.68% β-pregna (Table 2). In general, the researches in line with present study are low; however, it is referred to some of them.

Table 1. Major compounds of Mentha pulegium extract

	Components	Retention time	Percentage (%)
1	Nonane	5.830	1.89
2	α - Decene	8.033	1.87
3	α- Thujone	10.853	1.79
4	Camphor	11.972	3.36
5	Borneol	12.658	2.04
6	Spathulenol	14.124	0.80
7	1,8 Cineole	18.329	11.46
8	Paramentha 3,8-dien	21.561	1.66
9	Pulegone	22.059	22.79
10	Paramenth-3- n 8-l	23.461	4.92
11	Menthoforan	26.539	8.06
12	Cis- Isopulegon	27.121	7.54
13	2-β-Pinene	27.159	2.25
14	α-Pinene	28.403	1.28
15	neo iso dihydrokarool	30.738	1.09
16	α - Selinene	33.383	24.58
17	2- cyclohexane-1-van	38.369	2.63

Table 2. Major compounds of *Glycyrrhiza glabra* extract

	Components	Retention time	Percentage (%)
1	Nonane	5.013	28.48
2	Ethylcyclohexan	7.392	23.38
3	α - Decene	8.033	1.06
4	3-Bromodecane	12.736	8.29
5	1-Hexadecanol	13.182	1.17
6	trans-2-Heptenal	13.324	10.31
7	1H-Indene	14.288	2.76
8	Trans-anethole	16.080	3.29
9	cerylcerotinate	21.416	1.49
10	Lignocerol	37.316	0.85
11	9-Octadecenamide	38.623	8.93
12	Adogen 42	39.005	1.59
13	β - pregna	40.965	4.68
14	Isochiapin b	41.903	0.85
15	Hahnfett	44.341	2.87

The aqueous extract of *G. glabra* root and polysaccharides showed strong anti-adhesive activity under a fluorescent microscopy of human gastric mucosa aliquots with fluorescent-labeled *H. pylori* (Wittschier *et al*., 2009).

Table 3. Mean (±SD) non-growth zone, MIC and MBC of medicinal plants extracts and control antibiotics on *Helicobacter pylori* ATCC 26695

Antibacterial agents	Concentration (µg/ml)	Non growth zone (mm)	MIC (µg/ml)	MBC (µg/ml)
M. Pulegium extract	20	17.1±1.9	20	40
G. glabra extract	20	53.9±1.6	10	20
Combined extract	20	48.1±3.2	10	40
Clarithromycin	2	1.3±0.9 (I)		
Metronidazole	5	8.2±2.1 (R)		
Amoxicillin	10	10.6±0.3 (I)		
Tetracycline	30	28.9±1.4 (S)		

S = Sensitive; R = Resistant; I = Intermediate

Lakshmi and Geetha (2011) in a review article stated that Glycyrrhizinic acid (GA) as a key compound of licorice shows antiulcer effect by increasing the local concentrations of prostaglandins that promote mucous secretion and cell proliferation in the stomach. The examination of GA against 29 strains of *H. pylori* by MIC using agar dilution method in Brain Heart Infusion broth had rapid bactericidal activity. The effect of this herb against clarithromycin-resistant strains can be promising horizon as an alternative therapeutic agent to control *H. pylori* (Lakshmi & Geetha, 2011).

The results of antibacterial activity of native medicinal plants extractions on the bacteria by the disk diffusion agar susceptibility test method and the results of MIC and MBC rates have been shown in Table 3 ($P_{value}<0.05$).

Table 4. Mean survival rate of stomach cancer cell line of AGS (percentage) compared to control cells in presence of various concentrations of *Glycyrrhiza glabra* extract.

Concentrations (µg/ml)	Time (hour)		
	24	48	72
0 (control)	100	100	100
5	98.11±1.1	92.25±0.5	94.36±1.8
10	98.31±1.2	90.17±0.7	82.62±1.7
15	95.62±0.3	75.36±1.2	70.75±0.6
20	75.28±0.8	35.82±2.1	34.84±0.3
25	65.72±2.1	10.02±1.6	9.36±0.2
50	25.12±1.4	10.00±1.6	9.2±0.9
100	10.1±0.5	9.85±1.3	8.2±0.4

Table 5. Mean survival rate of stomach cancer cell line of AGS (percentage) compared to control cells in presence of various concentrations of *Mentha pulegium* extract.

Concentrations (µg/ml)	Time (hour)		
	24	48	72
0 (control)	100	100	100
5	99.26±0.3	99.47±0.4	99.39±1.3
10	99.25±0.3	96.86±0.5	99.21±1.8
15	98.75±0.3	96.36±0.5	99.22±0.6
20	99.2±1.1	95.36±0.1	97.01±0.6
25	98.75±0.9	96.89±0.8	98.26±0.7
50	98.75±0.8	97.36±0.9	96.22±0.4
100	97.13±0.1	97.45±1.1	96.57±0.3

A research has been conducted to compare the antimicrobial activities of ethanolic and aqueous extracts from licorice leave and root against *Bacillus subtilis, Enterococcus faecalis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli*, and *Candida albicans* using paper disc agar diffusion, MIC and MBC. The root and leave extracts had dose-dependent activity against *Candida albicans* and gram-positive bacteria. The most active extract against gram-positive bacteria was the ethanolic extract of the leaves (Irani *et al*., 2010).

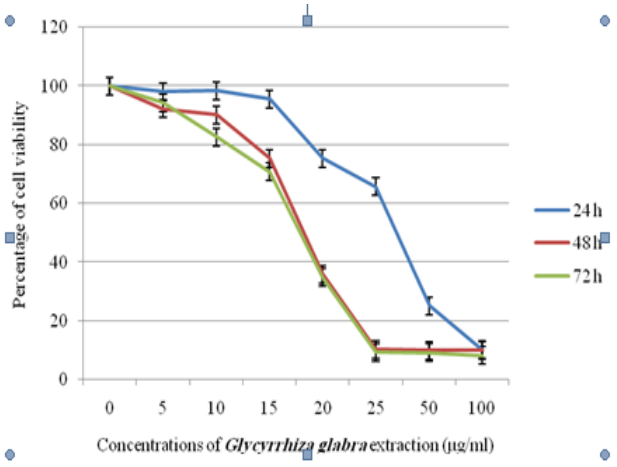

Fig 1. In vitro cytotoxicity effect of various concentrations of *Glycyrrhiza glabra* extract on stomach cancer cell line of AGS (percentage) at different times

The chemical analysis of *M. pulegium* essential oil using GC/MS indicated 22 components (95.30%), which the major substances were Pulegon (31.54%), 1,8 Cineol (15.89%), Menthoforan (11.8%) and Cis-Isopulegon (9.74%). The obtained MIC value for the tested essential oil under different temperature and pH values was in the range of 75-1200 µg/ml. Potent antibacterial activity of this essential oil was confirmed y the results of MIC and membrane cell damage observed in the electron microscopy assessments. Eventually, they claimed that this essential oil along with other agents could be effective on preventing foods against pathogenic and toxigenic microorganisms (Mahmoudi *et al*., 2011). The essential oils have important compounds with potent antimicrobial properties. The results show this antimicrobial effect due to bacterial cell wall damages at MIC concentrations observed by Atomic force microscopy

(AFM). It should be noted that this essential oil could be recruited in food as natural preservatives against the leading agents of food spoilage (Hajlaoui *et al*., 2010). Lorenzo *et al*. (2002), in Uruguay, studied the essential oils composition of leaves of *M. pulegium L.* and *M. rotundifolia (L.) Huds* extracted using hydrodistillation and analyzed by GC-FID and GC-MS. The major group of constituents was oxygen-containing monoterpenes in both essential oils. The main substrates in *M. pulegium* were pulegone, isomenthone and menthone, but in *M. rotundifolia* were piperitenone oxide and (Z)-sabinene hydrate. Multidimensional gas chromatography for *M. pulegium* essential oils determined enantiomerically pure (-)-menthone, (+)-isomenthone, (+)-isomenthol, (-)-menthol and (+)-pulegone (Lorenzo *et al*., 2002).

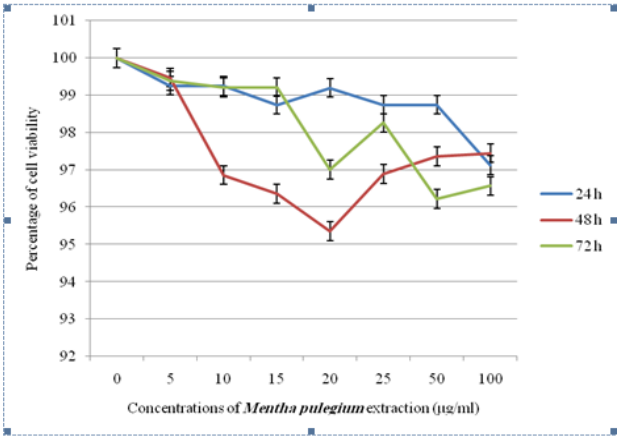

Fig 2. In vitro cytotoxicity effect of various concentrations of *Mentha pulegium* extract on stomach cancer cell line of AGS (percentage) at different times.

The averages live of stomach cancer cell line AGS (percentage) rather than control cells in the presence of various concentrations (µg/ml) of indigenous medicinal plants extractions have been shown in Tables 4 and 5 as well as Figures 1 and 2.

The results of present study and analysis of data related to MTT assay test (Level of confidence 95%) showed that the extraction of *G. glabra* had significant cytotoxic effects on the gastric cancer cell line AGS; while the onset and intensity of the effects were different and *M. pulegium* had no significantly effect on the gastric cancer cell line AGS. The influence of *G. glabra* did not show significant and remarkable difference up to concentration 20µg/ml; but after this range, the effects were started and intensified significantly. Effectiveness was significant and optimum after 48 hours increasing concentration to 25µg/ml; intensity of the influence of subsequent concentrations was steady and had no significant effects. According to the statistical analysis, time and concentration parameters had significant effects on the mean survival rate of gastric cancer cells compared to control cells.

4. Conclusion

Numerous species of useful medicinal plants grown self-propelled, including the herbs in the current study, are destroying without any application in the mountainous areas of Ardabil. According to the antibacterial effects of herbs obtained from our experiments, we can conclude that these valuable plants could be employed as affordable and available sources of bio-pharmaceuticals. Stomach cancer is really important in Ardabil Province, in northwestern Iran. In this work, *G. glabra* extraction showed antioxidant properties; the cytotoxic effects were optimum in 25µg/ml after 48 hours. The antibacterial effects of extractions were observed on *H. pylori*. According to the results, these compounds are able to replace by chemical preservatives in food and drug industry. Moreover, they also can be investigated on other pathogens and cancers to find useful solutions to overcome them.

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