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Evaluating the Effect of Chloroform and Water Extract of *Satureja khuzistanica* on the Expression of Bcl-2 and Bax in Gastric Cancer Cell Line

Satureja khuzistanica Kloroform ve Su Ekstraktının Mide Kanseri Hücre Hattında Bcl-2 ve Bax Ekspresyonu Üzerine Etkisinin Değerlendirilmesi

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ABSTRACT

Objective: Gastric cancer is a complex disease. Natural antioxidant sources without toxic side effects are suitable for the treatment and management of diseases such as cancer. *Satureja khuzistanica (S. khuzistanica)* is an endemic plant in southern Iran. Despite numerous articles published about this plant, a limited number of studies have been conducted on its anticancer effects. Therefore, this study was conducted to evaluate the cytotoxic and apoptotic effects of this plant on the gastric cancer cell line.

Methods: The antimicrobial activities of chloroform and water extracts were screened against pathogenic bacteria [*Helicobacter pylori (H. pylori)*]. The cytotoxicity effect of the extracts was evaluated using an microculture tetrazolium assay on gastric cancer cell lines (AGS and KATO III) and normal gingival fibroblasts. The expression of Bax and Bcl-2 was evaluated using real-time polymerase chain reaction. Finally, all data were analyzed using REST software.

Results: Both extracts showed good inhibitory activity against *H. pylori*. In addition, extracts showed cytotoxic activity against gastric cancer cell lines. Treatment of *S. khuzistanica* cells increased and decreased the expression of *Bax* and *Bcl-2* apoptotic genes, respectively ($p<0.05$). In addition, in HGF cells, there were no cytotoxic effects or apoptosis induction triggered by the extracts.

Conclusion: The chloroform extract had more cytotoxic and apoptotic effects than the water extract. The extracts could have anticancer and apoptotic effects on the gastric cancer cell line.

Keywords: AGS and KATO-III cell line, apoptotic effect, gastric cancer, *Satureja khuzestanica*

ÖZ

Amaç: Mide kanseri karmaşık bir hastalıktır. Toksik yan etkileri olmayan doğal antioksidan kaynakları kanser gibi hastalıkların tedavisi ve yönetimi için uygundur. *Satureja khuzistanica (S. khuzistanica)*, İran'ın güneyindeki endemik bir bitkidir. Bu bitki hakkında çok sayıda makale yayınlanmış olmasına rağmen antikanser etkileri konusunda sınırlı sayıda çalışma yapılmıştır. Bu nedenle bu çalışma, bu bitkinin mide kanseri hücre hattı üzerindeki sitotoksik ve apoptotik etkilerini değerlendirmek amacıyla yapılmıştır.

Yöntemler: Kloroform ve su ekstraktlarının antimikrobiyal aktiviteleri patojen bakterilere [*Helicobacter pylori (H. pylori)*] karşı tarandı. Ekstraktların sitotoksisite etkisi, mide kanseri hücre dizileri (AGS ve KATO III) ve normal diş eti fibroblastları üzerinde bir mikrokültür tetrazolyum tahlili kullanılarak değerlendirildi. Bax ve Bcl-2'nin ifadesi, gerçek zamanlı polimeraz zincir reaksiyonu kullanılarak değerlendirildi. Son olarak tüm veriler REST yazılımı kullanılarak analiz edildi.

Bulgular: Her iki ekstrakt da *H. pylori*'ye karşı iyi bir inhibitör aktivite gösterdi. Ek olarak ekstraktlar mide kanseri hücre dizilerine karşı sitotoksik aktivite gösterdi. *S. khuzistanica* hücrelerinin tedavisi sırasıyla *Bax* ve *Bcl-2* apoptotik genlerinin ekspresyonunu artırdı ve azalttı ($p<0.05$). Ayrıca HGF hücrelerinde ekstraktların tetiklediği herhangi bir sitotoksik etki veya apoptoz indüksiyonu görülmemiştir.

Sonuç: Kloroform ekstraktının su ekstraktına göre daha fazla sitotoksik ve apoptotik etkiye sahip olduğu görülmüştür. Ekstraktların mide kanseri hücre dizisi üzerinde antikanser ve apoptotik etkileri olabilir.

Anahtar Sözcükler: AGS ve KATO-III hücre dizisi, apoptotik etki, mide kanseri, *Satureja khuzestanica*

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INTRODUCTION

Japan ranks first in terms of the incidence of gastric cancer within the world and South Korea, Costa Rica, the former Soviet republics, and Japan rank first to fourth in the world in terms of mortality caused by gastric cancer. Its highest prevalence has been observed in the Far East. In Iran, cancer is the third most common cause of death, and more than 30,000 people die each year due to this disease (1,2). Gastric cancer is a common disease in Azerbaijan and Iran. Ardebil has the highest incidence of gastric cancer in Iran after Gilan province. Genetic factors, environmental factors, and food habits are among the causes of gastric cancer in these provinces. Gastric cancer can be divided into completely separate pathologic subtypes with different epidemiological and prognostic features (3). The spread of gastric cancer is a complex and multistage process. It involves genetic and epigenetic changes in cancer-producing genes, tumor suppressor genes, DNA-modifying genes, cell cycle regulators, and signaling molecules. Gastric cancer may be caused by genomic instability, which can be in the form of microsatellite instability or chromosomal instability (4,5).

The main cause of most cancers is the overexpression of anti-apoptotic proteins, such as Bcl-2, and lack of apoptosis. Increasing the expression of these proteins increases resistance to chemotherapy. Thus, inhibiting the expression or function of these proteins in cancer cells can induce apoptosis. The *Bax* and *Bcl-2* genes, other apoptotic genes, and planned deaths are involved in the incidence and development of gastric cancer (6). Chemotherapy is used to control many cancer diseases. However, side effects (e.g., neurological damage and kidney failure) of chemotherapy and the drugs used are sometimes more hazardous than cancer itself. Thus, to reduce these complications and given its resistance to chemotherapy, modern methods are needed to prevent and increase the chance of treating cancer patients (7). *Satureja khuzistanica (S. khuzistanica)* is a plant with two main effective ingredients, namely thymol and carvacrol, which have anticancer, antioxidant, and antimicrobial effects (8-11). A study on the percentage and ingredients of essential oil of *S. khuzistanica* showed that the mean essential oil content of *S. khuzistanica* was 18.3%. Additionally, 43 compounds were identified in the essential oil of *S. khuzestanica*, which contains 90% of the total carvacrol (11). The objective of this study was to evaluate the antimicrobial, cytotoxic, and anticancer effects of chloroform and water extracts of *S. khuzistanica* on gastric cancer.

MATERIALS AND METHODS

S. khuzistanica was collected from the mountains around Khuzestan from June to August. The plant was identified and sent by the botanists of Khoraman Pharmaceutical Plant Company (Dr. Reza Shahsavari). Approximately 120 g of *S. khuzistanica* powder was mixed with 1 L of chloroform and water. After 2 weeks, extraction was performed by distillation using a rotary evaporator.

Antimicrobial Activity

The antimicrobial activities of chloroform and water extracts were screened against pathogenic bacteria [*Helicobacter pylori (H. pylori)*]. Concentrations of chloroform and water extract of *S. khuzistanica* (25 and 50 mg/mL) were tested against *H. pylori* using the disc diffusion method. About 50 µL of each concentration of the extracts was added to each sterile blank disc (Padtan Teb, Iran). From the fresh culture of *H. pylori*, 1 McFarland solution in brucella broth (Himedia) was prepared, and 100 µL of the solution was taken by sterile swab. Next, it was cultured on a brucella agar medium containing 5% defibrinated sheep blood and 7% inactivated fetal bovine serum. The dried discs of the extracts were placed on the culture at appropriate distances. After placing the discs, the media were kept in a "CO_2" incubator (with 10% "CO_2") at 37 °C for 72 h. Antibacterial activity was determined by measuring the zone of inhibition around the test discs. The growth inhibition diameter was the average of three different measurements. The result was expressed as the inhibition zone diameter and was compared with the standard antibiotics ampicillin (Amp, 10 µg) and chloramphenicol (C, 30 µg).

Preparation of the Cell Culture Medium

In the present study, AGS and KATO III gastric cancer cell lines and gingival fibroblasts were prepared from Pasteur institute and cultured in RPMI enriched with 10% fetal calf serum, 1% Pen strep, and 5% CO_2 at 37 °C.

MTT Test

Microculture tetrazolium (MTT) test is a colorimetric method performed based on the reduction and breakdown of tetrazolium yellow crystals with the chemical formula of 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT). The test was conducted using succinate dehydrogenase enzyme and the formation of insoluble blue crystals. In the present study, the MTT test was performed to evaluate the effect of *S. khuzistanica* on the viability of AGS and KATO III cells and gingival fibroblasts. Accordingly, the cells cultured in a 96-well plate at concentrations of 100, 500, 250, 125, 62.5, and 31.25 µg/mL of the plant extract were treated on normal and cancer cells over 24 h. Then, the medium was replaced with MTT (Sigma Aldrich) solution. After 4 h of incubation in the dark, the MTT solution was replaced with DMSO. The light absorption was then read at 570 nm in an ELISA device. In the next step, the percentage of live cells was calculated using the formula (%) = (ODexp/ODcon) × 100. In this formula, ODexp and ODcon are the light absorption of the exposed and control groups, respectively. All experiments were performed in triplicate.

Generally, to evaluate the gene expression changes, total cell RNA was extracted from the gastric cancer cell line using a solution (Ribospin304-15 Gene All, Korea) according to the manufacturer's instructions. Spectrophotometer and agarose gel electrophoresis were used for quantitative and qualitative analysis of the concentration and purity of the extracted RNA. In this study, cDNA was synthesized from mRNA using the Sina Colon First Starandn cDNA kit according to the manufacturer's instructions. To produce cDNAs with similar concentrations, we measured the volume of each sample so that 1000 ng of RNA was available. Eventually, the cDNA samples were produced and stored at -20 °C until the real-time polymerase chain reaction (RT-PCR).

Primers

Each gene fragment was proliferated by selecting a pair of specific primers for each gene, including forward and reverse prime (Table 1).

RT-PCR was performed using a magnetic induction cycler (MIC) device and RealQ Plus 2x Master Mix Green. For this purpose, 12.5 µL of cyber green mixer master, 3 µL of the cDNA sample, 0.5 µL of forward and reverse primers (10 pmol) in water, and 8.5 µL of nuclease-free water were mixed in 25 µL of the reaction mixture. The *beta-actin (β-actin)* gene was amplified as an internal control, and the fold change in the relative expression of each target mRNA was calculated using the comparative Ct (2-ΔΔCt) method.

Real-time PCR Procedures

In this research, the heat schedule of the RT-PCR device (MIC model) was 95 °C for 15 min in the first stage, 95 °C for 10 s in the second stage, and 49-51-60 °C, respectively, for the *Bax*, *Bcl-2*, and *β-actin* genes C for 15 s, and 72 °C for 20 s in 40 cycles in the last stage. Finally, the data were analyzed by comparing the threshold cycle and the difference in the obtained threshold from the treated cells with the extracts and untreated cells with the target gene ratio (Table 2, 3).

Melting Curve Analysis

Once PCR is completed, the device can plot the melting curve of each sample. Thus, by analyzing the melting curve, it is possible to identify the presence of non-specific bands and the primer dimer (Figure 1).

Statistical Analysis

Statistical analysis of the data was performed using REST software at a significance level of <0.05.

RESULTS

According to the following equation, the percentage of chloroform and water extracts of the *S. khuzistanica* plants is 7.5%.

$$\frac{\text{the weight of the extract obtained based on gram}}{\textit{the weight of the plant used based on gram}} \times 100 = \text{percentage of the extracts obtained}$$

$$\frac{9.53}{120} \times 100 = 7.5$$

Table 1. The sequence of primers used along with the length of the proliferated fragment

Primer name	Primer sequence	Primer binding temperature	Base pair length
Bax-F	GGTTGTCGCCCTTTTCTA	48.84	108
Bax-R	CGGAGGAAGTCCAATGTC	49.1	108
B-actin-F	GCGAGAAGATGACCCAGAT	50.87	88
B-actin-R	GAGGCGTACAGGGATAGC	50.97	88
Bcl-2-F	GATGTGATGCCTCTGCGAAG	65	93
Bcl-2-R	CATGCTGATGTCTCTGGAATCT	64	93

Table 2. Contents of RT-PCR-master mix per sample for examining a gene as performed according to the time and temperature schedule of Table 3 of RT-PCR

Value based on µL	Substance
12.5	Master mix
8.5	Distilled water
0.5	Forward primer
0.5	Reverse primer
3	cDNA
25	Total volume

RT-PCR: Real-time polymerase chain reaction.

Table 3. RT-PCR test schedule and temperature for genes

Gene	Step	Temperature	Time	Number of cycles
Bax & Bcl-2 &	Initialization	95 °C	15 min	40
β-actine	Denaturation	95 °C	10 sec.	
β-actine	Annealing	51 °C	15 sec.	
Bax	Annealing	49 °C	15 sec.	
Bcl-2	Annealing	64 °C	15 sec.	
Bax & Bcl-2 & β-actine	Extension	72 °C	20 sec	

RT-PCR: Real-time polymerase chain reaction.

Images taken from the growth of AGS gastric cancer cells before and after treatment with *S. khuzistanica* extract are illustrated in Figure 2.

Antimicrobial Activity

The antimicrobial activity of the extracts was measured using the disk diffusion method. The antimicrobial activity of the extracts was found within the 25-50 mg/mL concentration range. The inhibition zones of the bacteria were in the range of 18±0.4 mm. According to the disk diffusion method, all the concentrations used were inhibitory for all the reference bacterial strains.

MTT Results

The MTT assay was performed to find a minimum of 50% killing extract in µg/mL, examine the cytotoxic effect of the extract, and determine the IC_{50} in AGS cells. This test was performed in three replicates. The results of the MTT test at different concentrations of the extract in µg/mL were obtained as a linear chart. In addition, the results showed no significant cytotoxic properties in normal cells. The results for the cancer cells are presented in Figure 3.

RNA Electrophoresis Results

In order to evaluate the quality of the extracted RNAs, three samples were electrophoresed randomly on Agarose gel. For this purpose, 1.5% gel was made and the congealed samples were loaded onto gel and electrophoresed at V100 for 2 min (Figure 4).

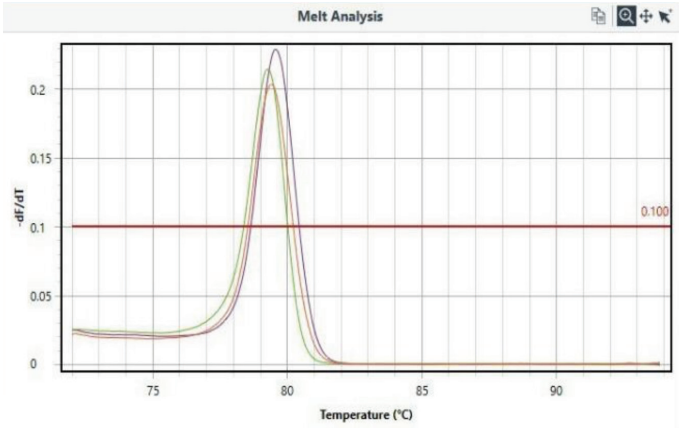

Figure 1. Melting curve for beta-actin gene.

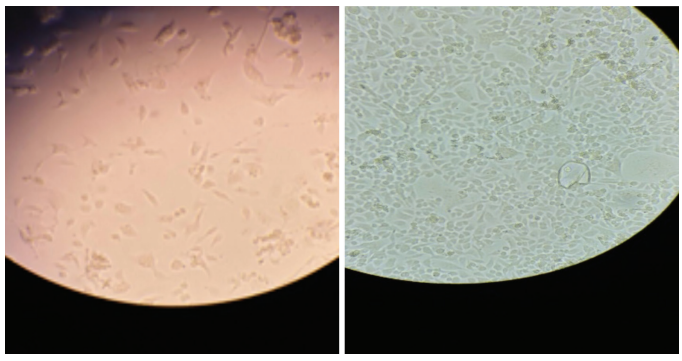

Figure 2. AGS gastric cancer cells before (A) and after (B) treatment with extract.

Results of Real-Time PCR

After obtaining the mean, CTs were analyzed using the following formula: in addition, the results were analyzed using REST software, and the following charts were obtained.

$$\text{Ratio} = \frac{(E\ target)\ \Delta Ct\ target\ (control - sample)}{(E\ ref)\ \Delta\ ref\ (control - sample)}$$

Results of relative expression of the *Bcl-2* gene and *Bax* relative to the *β-actin* gene.

According to the charts of *β-actin* genes as a reference and *Bcl-2* gene and *Bax* as a target, the expression of *Bax* and *Bcl-2* genes after applying *S. khuzistanica* extract increased and decreased, respectively. Therefore, after applying a dose of 125 µg/mL, the expression rate of these genes increased and decreased by approximately 1.9 and 0.227 times, respectively, at a significance level of 0.000.

The RT-PCR results show the relative expression of Bax to Bcl-2 (Figure 5).

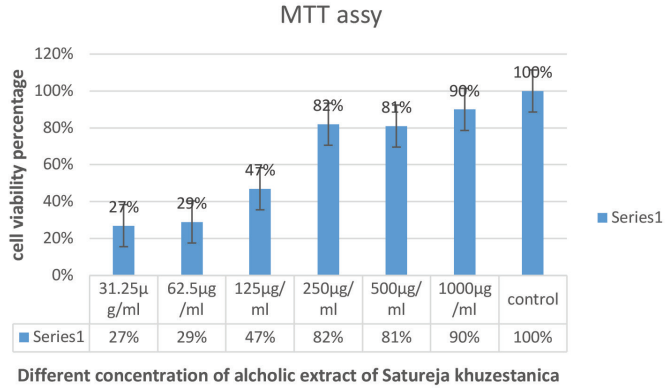

Figure 3. The result of the MTT test to determine the concentration of the extract which killed 50% of the cells.

MTT: Microculture tetrazolium.

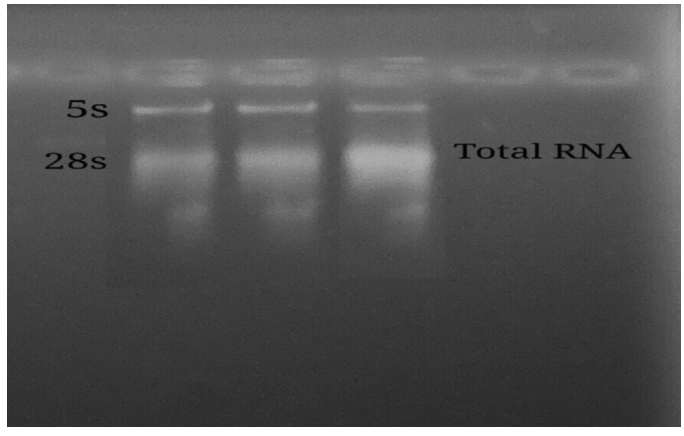

Figure 4. Gel electrophoresis of total RNA extracted from the samples; The quality of extracted RNAs was controlled by putting samples on a 2% agarose gel.

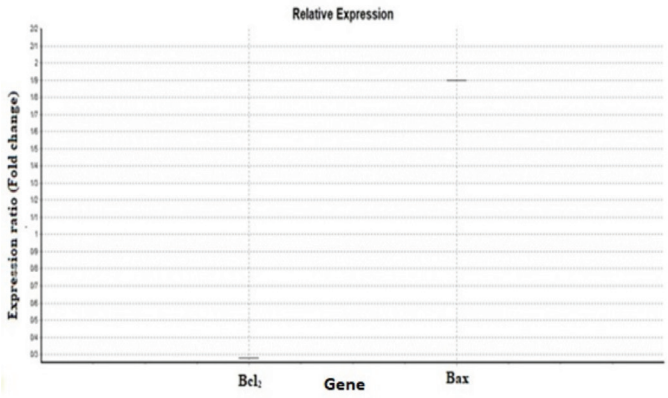

Figure 5. RT-PCR results of relative expression of Bax to BCL-2.
RT-PCR: Real-time polymerase chain reaction.

DISCUSSION

Traditional medicine is effective in many diseases, and its use for treating diseases has been proven. Anxiolytic sources without toxic side effects are a good approach for the treatment and control of diseases such as cancer. Several studies have been conducted on the benefits of *S. khuzistanica* in medicine. For example, Rezvanfar et al. (12) investigated the benefits and protective effects of *S. khuzistanica* on cyclophosphamide-induced hemorrhagic cystitis in a rat model. The results showed that the *S. khuzistanica* extract protects against cyclophosphamide-induced hemorrhagic cystitis in a rat model by reducing radical-induced toxic stress (12).

In another study, Malmir et al. (13) studied new flavonoid monoterpenes in *S. khuzistanica*. The results showed that *S. khuzistanica* had a beneficial antidiabetic and antioxidant effect. In addition, Saidi (14) studied *S. khuzistanica* and its chemical and antioxidant properties and showed that this plant is a rich source of antioxidant compounds. They also examined the effects of *S. khuzistanica* on inhibiting the expression of iNOS in the macrophage cell line J774A.1. The results showed that *S. khuzistanica* had anti-inflammatory effects through its effect on iNOS gene expression (15).

Esmaeili-Mahani et al. (16,17) investigated the effect of *S. khuzistanica* on the tolerance of pain induced by morphine by inhibiting the activation of glial cells. According to their results, *S. khuzistanica* can have beneficial effects in inhibiting drug tolerance and reducing glial cell activation. Sharafati-Chaleshtori et al. (18) showed that carvacrol of *S. khuzistanica* reduces the viability rate of cancer cell degeneration of the genome, and genome degradation at concentrations close to IC_{50} is more significant. Saffari studied the effect of *S. khuzistanica* on MCF-7 breast cancer cells. These authors showed that the rate of cell apoptosis increased with increasing concentration and time. Furthermore, the alcoholic extract of MCF-7 breast cancer cells showed anticancer activity (19).

Ahmadi et al. (20) evaluated the protective effect of *S. khuzistanica* essential oil on ovarian failure caused by busulfan. The results showed that busulfan significantly reduced the number and quality of oocytes, fertilization rate, embryo growth before implantation, and embryo quality. *S. khuzistanica* essential oil significantly reduced the adverse effects of busulfan. According to their results, *S. khuzestanica* essential oil can protect the fertility of the female sex against damages caused by busulfan (20). Sazgar et al. (2017), combining the hydroalcoholic extract of celeriac and Satureja, showed that it had a toxic effect on HeLa cancer cells, whereas this extract did not have a toxic effect on normal fibroblasts. Shirali and Alizadeh (21) evaluated the antimicrobial properties of the essential oil of *S. khuzistanica*. The results showed that phenolic compounds such as carocrole, thymol, and gamma-terpinene in essential oil compounds have antimicrobial properties in this plant. Moreover, an increase in phenolic compounds of the essential oil is directly associated with an increase in antimicrobial activity, which prevents lipid oxidation and heart coronary and cancer diseases (21).

Loizzo et al. (22) investigated the anticancer effects of the essential oil of Satureja. They studied the cellular cytotoxicity of this essential oil on various cancer cells, including breast cancer. They reported that Satureja essential oils could inhibit breast cancer cells, and this ability increases with increasing concentration (22). This study showed that the treatment of cells with *S. khuzistanica* leads to an increase and reduction in the expression of apoptotic *Bax* and *Bcl-2* genes. In addition, they reported that the extract of this plant had significantly increased anti-apoptotic properties against the AGS cell line. Therefore, the expression of *Bax* and *Bcl-2* genes changes in gastric cancer cells. A significant reduction in *Bax* gene expression as a pro-apoptotic regulator of apoptosis and an increase in *Bcl-2* gene expression as an anti-apoptotic regulator demonstrate the high capacity of *S. khuzistanica* to reduce the incidence of cell apoptosis. *S. khuzestanica* significantly increases apoptosis by reducing the expression of Bcl-2 and increasing the expression of Bax (23). In addition, this study provided significant information on the correlation between busulfan and apoptosis due to oxidative stress in sperm and testicular tissue in adult rats. An imbalance between the production of ROS and the reduction of antioxidant mechanisms resulted in the toxicity induced by oxidative stress in adult male rats after treatment with busulfan. However, a 1-week treatment with *S. khuzistanica* in rats treated with busulfan-*S. khuzistanica* reduced the apoptosis induced by oxidative stress, cytotoxicity, and genotoxicity in sperm [Nasimi et al. (23), 2018]. Natural substances in *S. khuzistanica* extract can increase and decrease the relative expression of *Bax* and *Bcl-2* genes, respectively. This study showed that the expression of *Bax* and *Bcl-2* genes after application of *S. khuzistanica* extract (125 µg/mL) increased and decreased, respectively. These results suggest that the expression of *Bax* and *Bcl-2* genes can be changed by the application of *S. khuzistanica* extract. Data from a study show that *S. khuzistanica* has anticancer effects on MCF-7 cancer cells and shows synergistic effects in combination with vincristine (17).

In a previous study, the methanol extract of *S. khuzistanica* induced cytotoxic effects in a dose-dependent manner. In this regard, almost half of the cells were destroyed during 24 h with 125 µg/mL, and MCF-7 was detected as the most sensitive cell line (24,25).

CONCLUSION

In our study, the results of antimicrobial activity showed that among the extracts, chloroform extract had a more powerful antimicrobial effect than water extracts against *H. pylori* bacteria. The antimicrobial effect of thymol and carvacrol is due to the damage to membrane integrity induced by a change in pH hemostasis.

In the cytotoxic assay, chloroform and water extracts exhibited cytotoxic effects on the gastric cancer cell lines (AGS and KATO III). Chloroform extract had a more powerful cytotoxic effect than water extract on gastric cancer cell lines.

Dysregulation of the mitochondrial apoptotic pathway is one of the most important events during carcinogenesis. The Bcl-2 protein family, including anti-apoptotic (Bcl-2 and Bcl-xl) and pro-apoptotic (Bax and Bak) members, plays an essential role in regulating this pathway. According to our results, the Bax/Bcl-2 ratio was significantly ($p<0.05$) correlated with the gastric cancer cell line.

This is the first report demonstrating the potential pro-apoptotic effects of *S. khuzistanica* extracts on gastric cancer cell lines (AGS and KATO III). However, further investigations of the extracts in animal models with gastric cancer are needed for additional understanding of their *in vivo* activity.

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Ethics

Ethics Committee Approval: This study was approved by the Bonab Islamic Azad University (approval number: 1394.625).

Informed Consent: It wasn't obtained.

Author Contributions

Concept: B.B., Design: L.A., H.S., Data Collection or Processing: B.B., H.S., Analysis or Interpretation: A.T., Literature Search: F.A.R., G.S., S.K., Writing: B.B., H.S.

Conflict of Interest: No conflict of interest is declared by the authors.

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