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Original Article

# Investigating the Expression of Apoptosis and Metastasis Genes in *Satureja khuzistanica* along with Nisin and Doxorubicin in Colorectal Cancer

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## ABSTRACT

**Background:** Colorectal cancer is the third most common cancer and the second most common cause of cancer-related death worldwide. Common treatments for colorectal cancer include chemotherapy, surgery, immunotherapy, etc. One of the chemotherapy drugs is doxorubicin, but it has many side effects. Recently, the antibacterial and anticancer effects of *Satureja khuzistanica* (*S. khuzistanica*) extract have been proven. Probiotics have been used in recent years to change the intestinal microbiome and treat colon cancer and reduce the side effects of treatments. **Materials and Methods:** In this study, the viability of HT29 colorectal cancer cells was examined using MTT after treatment with the study groups, and the expression of BAX, BCL2, SMAC, P53, MMP9, CASP9, and SUR genes was evaluated by Real time PCR. **Results:** The results showed that the expression of BAX, SMAC, P53, and CASP9 genes increased by 4.21, 3.055, 5.105, and 2.216 ($p<0.0001$) compared to the control after treatment with *S. khuzistanica*, respectively. The expression of BCL2, MMP2, SUR and MMP9 genes decreased by 1.1% after treatment with *S. khuzistanica* compared to the control. The results also showed that the combined treatment of *S. khuzistanica* with Nisin or doxorubicin had a greater effect on changes in gene expression. **Conclusion:** This study could be a therapeutic strategy for using natural substances such as *S. khuzistanica* extract and probiotic Nisin to reduce the dose of chemotherapy.

**Keywords:** *Satureja khuzistanica*, Doxorubicin, Colorectal Cancer, Probiotic, Nisin.

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## INTRODUCTION

Colorectal Cancer (CRC) is the third most common cancer and the second most common cause of cancer death worldwide, with more than two million new cases and one million deaths in 2020.[1] The main treatment for colon cancer is complete removal of the colon, and chemotherapy, radiotherapy, and immunotherapy are used for unresectable cases. However, these treatments are not without side effects and can damage normal cells and cause secondary complications.[2] Doxorubicin is a potent and widely used chemotherapy drug used to treat various types of cancer. It belongs to a class of drugs called anthracyclines and is a mainstay of treatment for many cancers due to its high efficacy.[3] Doxorubicin prevents DNA and RNA replication in cancer cells by binding to DNA and inhibiting the enzyme topoisomerase II. Doxorubicin also damages DNA and cell membranes by producing free radicals and induces programmed cell death (apoptosis) in cancer cells.[4] However, doxorubicin also has significant side effects such as Cardiotoxicity, bone marrow suppression, hair loss, nausea, vomiting, mouth ulcers, and photosensitivity that make its use challenging.[5] To reduce the side effects of chemotherapy drugs, researchers have been interested in using new therapeutic methods, such as Probiotics[6] and plant extracts.[7] Nisin, as a probiotic, is a potent bacteriocin produced by Lactococcus lactis that has antimicrobial and anticancer properties. This compound has no effect on gram-negative bacteria and its anticancer effects on cancer cells have been investigated in research. Nisin can induce selective apoptosis, cell cycle arrest, and inhibit cell proliferation in cancer cells.[8] *Satureja khuzistanica* (*S. khuzistanica*) is a perennial, shrubby, aromatic plant native to Iran, mainly growing in Khuzestan Province. It is a member of the Lamiaceae family and is used in traditional medicine for its medicinal properties.[9] *S. khuzistanica* contains active compounds such as carvacrol, thymol, and rosmarinic acid, which have anti-inflammatory, antibacterial, and antioxidant properties.[10] Research has shown that this plant can be effective in the prevention and treatment of cancer and prevent oxidative stress by inducing apoptosis in cancer cells and neutralizing free radicals.[11] Anticancer drugs exert their

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therapeutic effects by interfering with cell cycle mechanisms, inhibiting growth, migration, and inducing apoptosis.[12] In its inactive, soluble form, BAX has a globular structure and interacts with other Bcl-2 proteins. During apoptosis, Bax activation involves a conformational transition from a globular to an extended, membrane-inserted conformation.[13] BCL-2 is a protein that helps control cell survival or death by blocking a type of cell death called apoptosis. The tumor suppressor gene TP53 is the most commonly mutated gene in human tumors. It is involved in a variety of pathways such as cell cycle arrest, DNA repair, cell apoptosis, autophagy, and metabolism.[14] Casp9 encodes a member of the cysteine-aspartic acid protease family. The sequential activation of caspases plays a central role in the execution phase of cellular apoptosis.[15] MMPs - Matrix Metalloproteases (MMPs) are a family of enzymes that cleave protein substrates based on a conserved mechanism involving the activation of a water molecule bound to the active site by a zinc ion. MMP-9 and MMP-9 are involved in metastasis.[16] SMAC activates caspases in the cytochrome c pathway and induces apoptosis.[17] In this study, we evaluated the effect of *S. khuzistanica* on the expression of apoptosis and metastasis genes including BAX, BCL2, CASP9, P53, SUR, SMAC, MMP2 and MMP9 genes that play a role in cell migration. We also evaluated the expression changes of genes after combined treatment with doxorubicin and Nisin.

## MATERIALS AND METHODS

### Cell line and cell culture

HT29 cell line was obtained from the Immunology Research Center, University of Tabriz. HT29 cells was cultivated in RPMI1640 culture medium along with 10% FBS and 1% penicillin and streptomycin and incubated in 98% humidity, 5% CO2 and 37°C temperature.

### MTT assay

In the MTT assay, MTT (Methyl Thiazole Tetrazolium) is used, which is converted into purple formazan by living cells, and the amount of formazan production is directly related to the number and metabolic activity of living cells. For this purpose, HT29 cells were cultured in a 96-well plate at a density of 5000 per well and kept in a $CO_2$ incubator for 24 hr. After 24 hr, the old culture medium was removed and fresh culture medium containing different concentrations of *S. khuzistanica* was added to the wells. The cells were incubated for 24 hr. After that the culture medium was removed and MTT solution (0.5 mg/mL) was added to each well. The plate was placed in a $CO_2$ incubator for 24 hr. After incubation, the MTT solution was removed from the wells and 100 µL of DMSO was added to dissolve the formazan crystals. The plate was left at room temperature for 15 min. The absorbance of the samples was measured using an ELISA reader at a wavelength of 570 nm and the percentage of cell viability was calculated using the following formula:

Percentage of viability= (absorbance of the treated sample/the absorbance of the untreated sample) ×100

### RNA extraction

To extract RNA from cell cultures, the RNJia Kit from (Rojet-Iran) was used. 400 µL of buffer A was poured onto the cells and peptized. After 5 min, 300 µL of buffer B was poured and shaken, and incubated for 4 min at room temperature. And centrifuged for 10 min at 12000 g at 15°C. 400 µL of the supernatant was poured into the column and centrifuged for 1 min at 10000 g. 450 µL of cold isopropanol was poured into the column and centrifuged at 10000 g at room temperature. 500 µL of buffer W was poured onto the column and centrifuged at 10000 g. It was allowed to dry for 5 min. Then 70 µL of E buffer was added and kept at room temperature for 1 min and then centrifuged at 130000 rpm and the supernatant containing RNA was collected. The quality and quantity of extracted RNA was assessed using Nanodrop. RNA was stored at -70°C.

### cDNA synthesis

A Rojet kit was used to synthesize the cDNA of the butter. First, 1µg of RNA was poured into a 0.2 mL microtube and 2 µL of RT enzyme, 10 µL of MIXzyme buffer were added to it and the volume was made up to 20 µL with DEPC water. After 20 min of incubation at 25°C, it was incubated for 60 min at 47°C and finally heated for 5 min at 85°C. The synthesized cDNA was stored at -21°C.

### Real Time PCR

To evaluated relative gene expression, the RealQ Plus 2x Master Mix Green (Denmark-Iran) was used. 10 µL of RealQ Plus 2x Master Mix Green, along with 1 µL of forward and reverse primers and 1 µL of cDNA, was poured into a Real Time PCR microtube and made up to 20 µL with deionized water. Amplification was performed according to the temperature protocol in Table 1. The β-Actine gene was considered an internal control. NTC was considered a negative control. For the accuracy of the test, all reactions were repeated twice. Primer sequences, annealing temperatures, and PCR product size was showed in Table 2.

### Statistical analysis

Relative Expression Software Tool (REST) version 2.0.13 and GraphPad Prism version 8.2.1 were used to examine gene expression, perform statistical analysis, and draw graphs, respectively. $p$ value <0.05 was considered as a significant level. Data were reported as Mean±SEM.

## RESULTS

### Cytotoxicity assay

The viability of HT29 cells of *S. khuzistanica* was measured using the MTT method, and the results showed that a concentration

of 20 µg/mL of *S. khuzistanica* inhibited the growth of 50% of HT29 cells. Figure 1 shows the viability of HT29 cells at different concentrations.

## Realtime PCR

The results of examining the relative expression profile of the BAX gene in the treated groups compared to the control showed that the expression of the BAX gene in all groups treated with *S. khuzistanica*, Nisin+*S. khuzistanica*, and DOX+*S. khuzistanica* increased significantly compared to the untreated group. The highest increase was related to the treatment of DOX+*S. khuzistanica* which increased expression by 11.029 ($p$<0.0001) times compared to the control. The results also showed that there was a significant difference between all treatment groups in a pairwise manner ($p$<0.0001). BAX expression increased 6.192 and 4.211 fold in the Nisin+*S. khuzistanica* and *S. khuzistanica* groups, respectively, compared to the untreated group (Figure 2A).

The results of examining the relative expression profile of the CASP9 gene in the treated groups compared to the control showed that the expression of the CASP9 gene in all groups treated with *S. khuzistanica*, Nisin+*S. khuzistanica*, and DOX+*S. khuzistanica* increased significantly compared to the untreated group. The highest increase was related to the treatment of DOX+*S. khuzistanica* which increased expression by 9.097 ($p$<0.0001) times compared to the control. The results also showed that there was a significant difference between all treatment groups in a pairwise manner ($p$<0.0001). CASP9 expression increased 4.49 and 2.216 fold in the Nisin+*S. khuzistanica* and *S. khuzistanica* groups, respectively, compared to the untreated group (Figure 2B).

The results of examining the relative expression profile of the P53 gene in the treated groups compared to the control showed that the expression of the P53 gene in all groups treated with *S. khuzistanica*, Nisin+*S. khuzistanica*, and DOX+*S. khuzistanica* increased significantly compared to the untreated group. The highest increase was related to the treatment of DOX+*S. khuzistanica* which increased expression by 10.343 ($p$<0.0001) times compared to the control. The results also showed that there was a significant difference between all treatment groups in a pairwise manner ($p$<0.0001). P53 expression increased 7.037 and 5.105 fold in the Nisin+*S. khuzistanica* and *S. khuzistanica* groups, respectively, compared to the untreated group (Figure 2C).

The results of examining the relative expression profile of the SMAC gene in the treated groups compared to the control showed that the expression of the SMAC gene in all groups treated with *S. khuzistanica*, Nisin+*S. khuzistanica*, and DOX+*S. khuzistanica* increased significantly compared to the untreated group. The highest increase was related to the treatment of DOX+*S. khuzistanica* which increased expression by 9.097 ($p$<0.0001) times compared to the control. The results also showed that there was a significant difference between all treatment groups in a pairwise manner ($p$<0.0001). SMAC expression increased 5.272 and 3.055 fold in the Nisin+*S. khuzistanica* and *S. khuzistanica* groups, respectively, compared to the untreated group (Figure 2C).

The results of examining the relative expression profile of the MMP2 gene in the treated groups compared to the control showed that the expression of the MMP2 gene in all groups treated with *S. khuzistanica*, Nisin+*S. khuzistanica*, and DOX+*S. khuzistanica* decreased significantly compared to the untreated group. The highest decrease was related to the treatment of DOX+*S. khuzistanica* which decreased expression by 1.7 ($p$<0.0001) times compared to the control. The results also showed that there was a significant difference between all treatment groups in a pairwise manner except Nisin+*S. khuzistanica* ($p$<0.0001). MMP2 expression decreased 1.4 fold in the *S. khuzistanica* group, compared to the untreated group. There is no significant difference between Nisin+*S. khuzistanica* and untreated group (Figure 3A).

The results of examining the relative expression profile of the MMP9 gene in the treated groups compared to the control showed that the expression of the MMP9 gene in all groups treated with *S. khuzistanica*, Nisin+*S. khuzistanica*, and DOX+*S. khuzistanica* decreased significantly compared to the untreated group. The highest decrease was related to the treatment of DOX+*S. khuzistanica* which decreased expression by 1.2 ($p$<0.0001) times compared to the control. The results also showed that there was a significant difference between all treatment groups in a pairwise manner ($p$<0.0001). MMP9 expression decreased 1.5 and 1.4 fold in the Nisin+*S. khuzistanica* and *S. khuzistanica* groups, respectively, compared to the untreated group (Figure 3B).

The results of examining the relative expression profile of the SUR gene in the treated groups compared to the control showed that the expression of the SUR gene in all groups treated with *S. khuzistanica*, Nisin+*S. khuzistanica*, and DOX+*S. khuzistanica*

**Table 1: Amplification temperature protocol.**

| Reaction | Temperature (°C) | Time | Cycle |
|---|---|---|---|
| Primary denaturation | 95 | 10 min | 1 |
| Denaturation | 95 | 30 sec | 40 |
| Annealing | 52-62 | 30 sec | |
| Extention | 72 | 30 sec | |

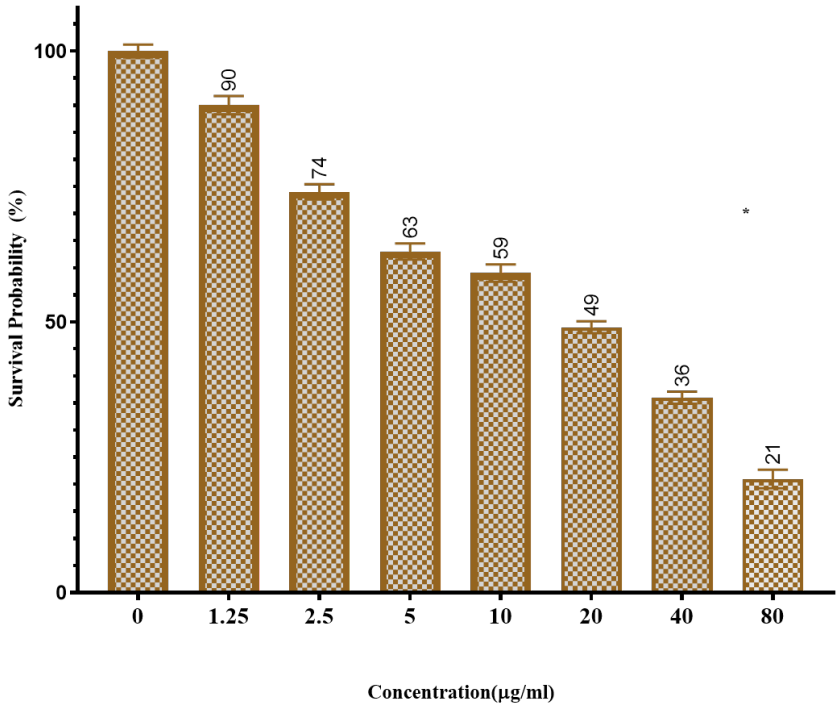


**Figure 1:** Diagram of the effect of different concentrations of *S. khuzistanica* on the viability of HT29 cells.

**Table 2: Primer sequences, annealing temperatures, and PCR product size was showed in the (5′-3′) Direction.**

| Genes | | Primer sequences (5′-3′) | Length (bp) | Annealing temp (°C) | Product size |
|---|---|---|---|---|---|
| Bax | F | GGTTGTGGCCCTTTTCTA | 18 | 55 | 108 |
| | R | CGGAGGAAGTCCAATGTC | 18 | | |
| BCL-2 | F | GATGTGATGCCTCTGCGAAG | 20 | 58 | 92 |
| | R | CATGCTGATGTCTCTGGAATCT | 22 | | |
| MMP2 | F | CACATAGTGATGGTTCCCCTGTT | 23 | 61 | 177 |
| | R | CGGCCACTCAGTAGGTGTCTTT | 22 | | |
| MMP9 | F | ATTTCTGCCAGGACCGCTTCTAC | 23 | 62 | 187 |
| | R | ATCCGGCAAACTGGCTCCTTC | 21 | | |
| SAMC | F | CAGAGGAGGAAGATGAAGTGTG | 22 | 59 | 172 |
| | R | GCGGTTATAGAGGCCTGATCTG | 22 | | |
| CASP9 | F | GCAGGCTCTGGATCTCGGC | 19 | 62 | 152 |
| | R | GCTGCTTGCCTGTTAGTTCGC | 21 | | |
| P53 | F | TGCGTGTGGAGTATTTGGATG | 21 | 59 | 90 |
| | R | TGGTACAGTCAGAGCCAACCTC | 22 | | |
| SURVIVN | F | CCCTTTCTCAAGGACCACCG | 20 | 59 | 196 |
| | R | GTTCCTCTATGGGGTCGTCA | 20 | | |
| β-Actine | F | GCGAGAAGATGACCCAGAT | 19 | 56 | 88 |
| | R | GAGGCGTACAGGGATAGC | 18 | | |

decreased significantly compared to the untreated group. The highest decrease was related to the treatment of DOX+*S. khuzistanica* which decreased expression by 76.9 ($p$<0.0001) times compared to the control. The results also showed that there was a significant difference between all treatment groups in a pairwise manner ($p$<0.0001). SUR expression decreased 4.5 and 1.5 fold in the Nisin+*S. khuzistanica* and *S. khuzistanica* groups, respectively, compared to the untreated group (Figure 3C).

The results of examining the relative expression profile of the BCL2 gene in the treated groups compared to the control showed that the expression of the BCL2 gene in all groups treated with *S. khuzistanica*, Nisin+*S. khuzistanica*, and DOX+*S. khuzistanica* decreased significantly compared to the untreated group. The highest decrease was related to the treatment of DOX+*S. khuzistanica* which decreased expression by 2.2 ($p$<0.0001) times compared to the control. The results also showed that there was a significant difference between all treatment groups in a pairwise manner ($p$<0.0001). BCL2 expression decreased 4.5 and 1.5 fold in the Nisin+*S. khuzistanica* and *S. khuzistanica* groups, respectively, compared to the untreated group (Figure 3D).

## DISCUSSION

Colorectal Cancer (CRC) is the second leading cause of cancer death in the world. Common treatments for colorectal cancer include surgery, radiotherapy, and chemotherapy.[18] Drugs exert their therapeutic effects by interfering with cell cycle mechanisms, inhibiting growth, migration, and inducing apoptosis. Some plant extracts such as *S. khuzestanica*[7] and probiotics such as Nisin[6] also have a similar effect on cancer cells. BAX, BCL2, CASP9, P53, SUR, and SMAC genes are involved in the control of apoptosis. MMP2 and MMP9 genes are involved in cell metastasis.

Esmaeili-Mahani showed that *S. khuzistanica* inhibits the growth of MCF-7 cancer cells. Western blot results showed that the expression of caspase 3 protein increased after treatment with *S. khuzistanica*. Cyclin D protein expression was also measured. They showed that the expression of cyclin D protein decreased after treatment with *S. khuzistanica*.[19] The results of our study

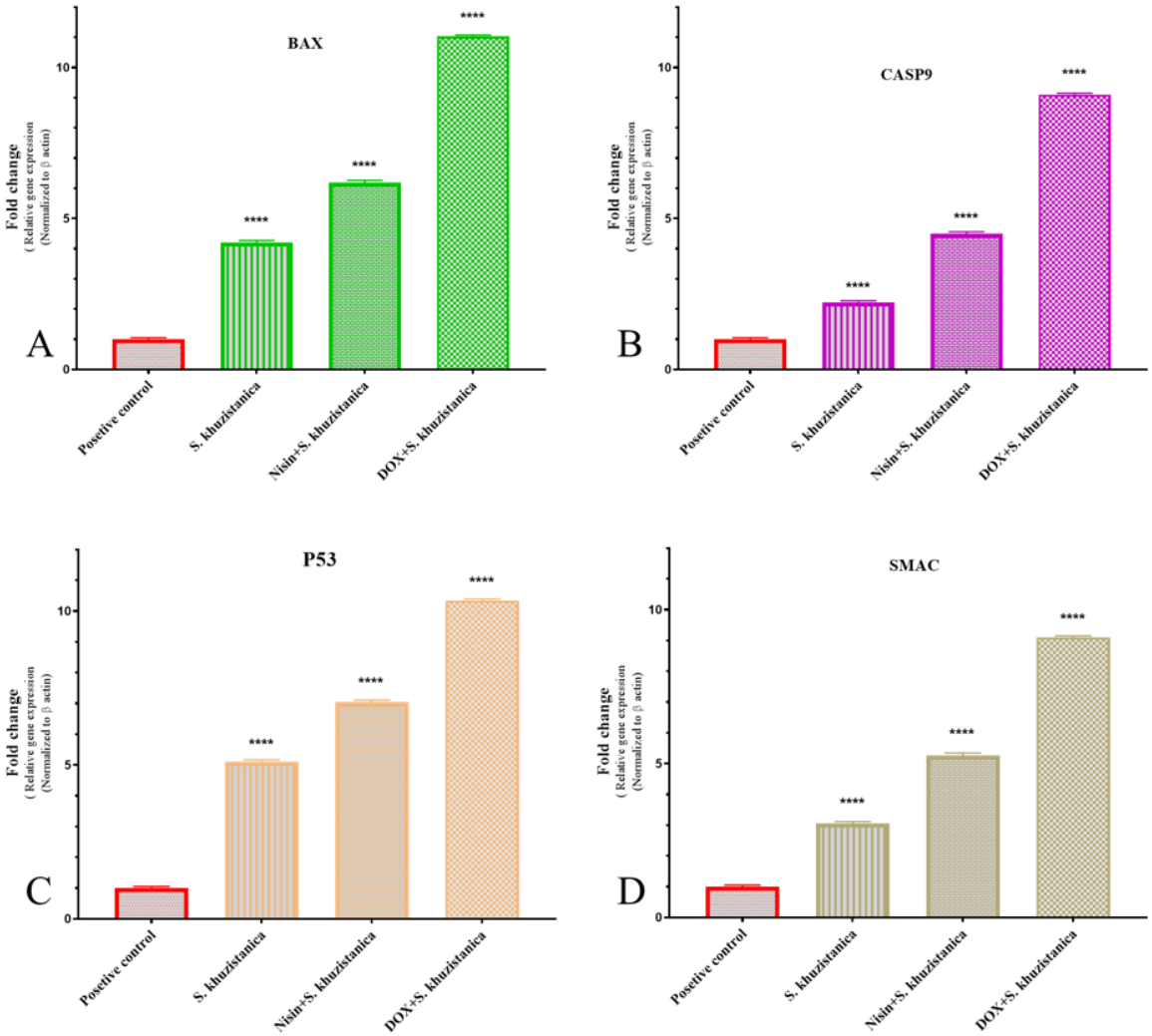


**Figure 2:** Comparative chart of relative expression of BAX, CASP9, P53 and SMAC gene (*, **, *** and **** indicate significance at the $p$<0.05, $p$<0.01, $p$<0.001 and $p$<0.0001 levels respectively).

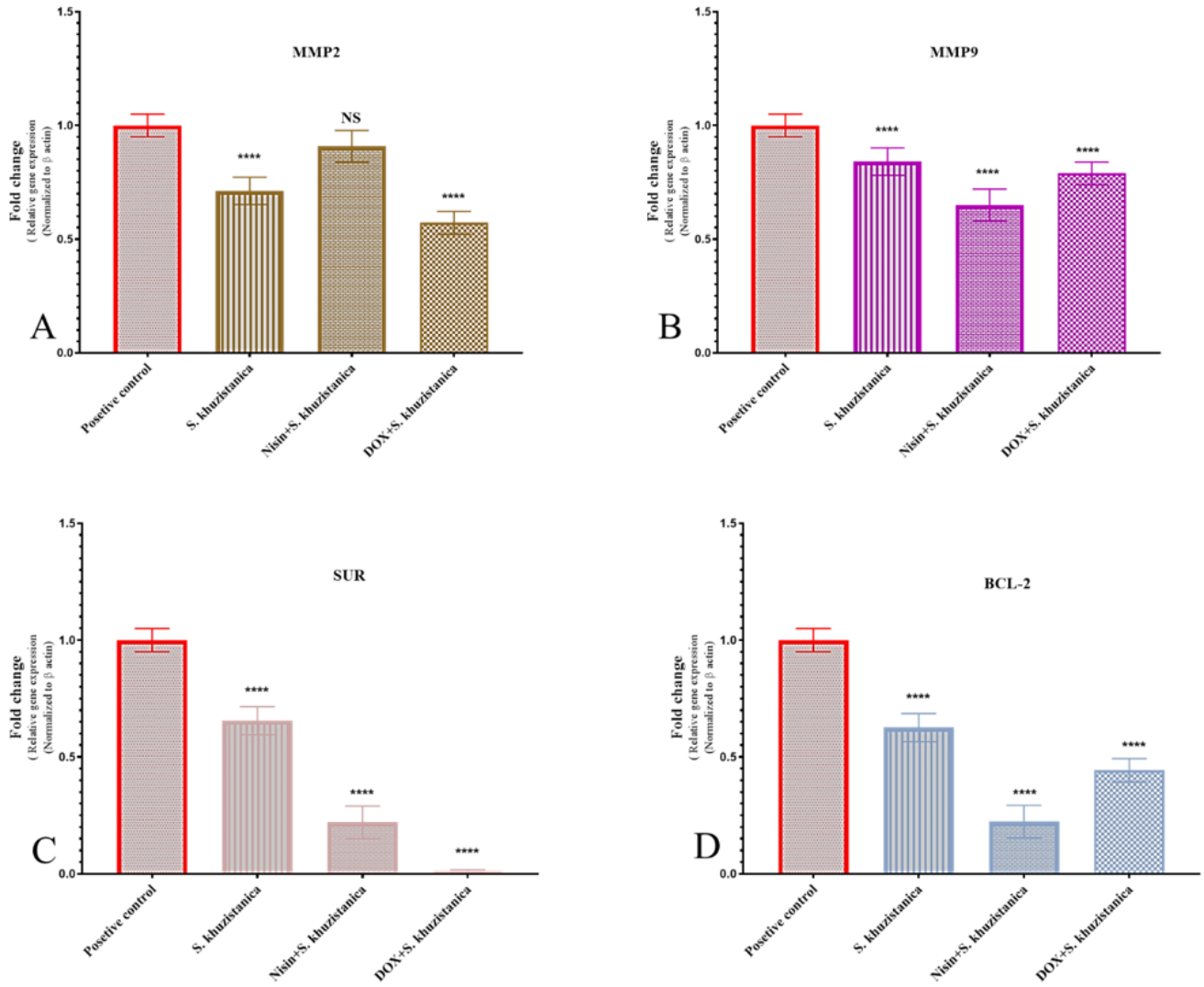


**Figure 3:** Comparative chart of relative expression of MMP2, MMP9, SUR and BCL-2 genes. (*, **, *** and **** indicate significance at the $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$ levels respectively).

showed that the expression of the CASP9 gene, which plays a role in the intrinsic pathway of apoptosis and causes the activation of CASP3, increased after treatment with *S. khuzestanica*.

Khojasteh *et al.*, investigated the effect of S. on the viability of MCF-7 breast cancer cells and HepG-2 liver cancer cells and showed that the viability of breast and liver cancer cells was reduced after treatment with *S. khuzestanica*. They also measured the activity of caspases and showed that the activity of caspase 8 was increased.[20] Aghaei *et al.*, investigated Apatinib on the viability of MDA-MB-231 breast cancer cells and evaluated the expression of apoptosis genes and showed that the viability of breast cancer cells was reduced after treatment with S Apatinib. And the expression of apoptosis genes were changed.[21] Yousefzadi *et al.*, conducted a study to investigate the effect of *S. khuzestanica* on the viability of cancer cell lines, SW480, MCF7, JET, and showed that *S. khuzestanica* reduced the viability of all three cell lines and a lower dose was required to inhibit cell growth for SW480 cancer cells.[22] In this study, we also examined the viability of colorectal cancer cells and showed that *S. khuzestanica* reduced cell viability and the expression of BCL-2 decreased and BAX increased.

Saxami *et al.*, conducted a study to investigate the beneficial effects of two potential probiotic *Lactobacillus strains, Lactobacillus pentosus* B281 and *L. plantarum* B282. The results showed that these probiotics significantly reduced cell proliferation in Caco-2 cells. They also caused an arrest in the G1 phase of the cell cycle and a decrease in the expression of specific cyclin genes.[23] The results also showed that the probiotic Nisin reduced the viability of HT29 cells. Kourpeti *et al.*, conducted the effects of *Lactobacillus casei* on colon cancer and the results showed that live *Lactobacillus casei* and its bacterial components had a significant antiproliferative effect on mouse (CT26) and Human (HT29) colon cancer cell lines. (78% for HT29 and 52% for CT26 cells) were observed. Furthermore, live *L. casei* induced apoptotic cell death in both cell lines as demonstrated by Annexin V and propidium iodide staining.[24] Our results showed that Nisin combined with *S. khuzestanica* reduced the growth of HT29

cancer cells and altered the expression of apoptotic genes and cell migration.

Jadid *et al*., studied the effect of melatonin and doxorubicin on Caco2 colorectal cancer cells. They evaluated the apoptosis rate, migration rate, and expression of BAX, BCL2, MMP2, MMP9, and SMAC genes and showed that in the combined treatment, the apoptosis rate of cancer cells was higher and the migration rate of cancer cells was lower than in the single treatment with each of melatonin and doxorubicin. Also, the expression of BAX and SMAC was increased in all treatments compared to the control, and the expression of BCL2, MMP2, and MMP9 was decreased compared to the control.[25] Safavi *et al*., evaluated the combined therapeutic effect of *Scrophularia* extract with DOX on the expression of BAX, BCL2, SAMC, SURVIVIN, CASP9, P53, MMP9 genes in AGS and MKN28 cancer cells. The results showed that the expression of BAX, SAMC, CASP9, and P53 genes increased more in the combined treatment of *Scrophularia* with DOX than in the treatment of DOX alone, and the expression of SURVIVIN and MMP9 genes decreased more in the combined treatment of *Scrophularia* with DOX than in the treatment of DOX alone.[26]

Our results showed that when *S. khuzestanica* is combined with Nisin or doxorubicin. It changes the expression of apoptosis and metastasis genes more than *S. khuzestanica* alone.

## CONCLUSION

According to the results of this study, it can be concluded that *S. khuzestanica* and Nisin can be considered as a complement to chemotherapy drugs in the treatment of colorectal cancer.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**CRC:** Colorectal cancer; ***S. khuzistanica*:** *Satureja khuzistanica*; **MMPs:** Matrix metalloproteases; **REST:** Relative Expression Software Tool.

## ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

This project was approved by the university's ethics committee with ID: IR.IAU.TABRIZ.REC.1403.334.

## AUTHOR CONTRIBUTIONS

E.K and Z.H.B were involved in planning and supervised the work and proof outline H.S and drafted the manuscript and designed the figures. All authors discussed the results and commented on the manuscript.

## SUMMARY

Various methods are being investigated for the treatment of colorectal cancer. The use of plant extracts is one of these methods. In this study, it was found that *S. khuzistanica*, which is native to Iran, has apoptotic and anti-metastatic properties on colorectal cancer cells. In this study, the expression of genes related to apoptosis and metastasis, such as BAX, BCL2, SMAC, P53, MMP9, CASP9, and SUR, was investigated, and the results showed that *S.* khuzistanica changes the expression of these genes in favor of apoptosis and inhibition of metastasis. Our results also showed that the use of the Nisin probiotic is also effective in inducing apoptosis and inhibiting metastasis, and this effect is greater when used as a combination therapy. The results show that *S. khuzistanica* extract can be introduced as a complementary treatment.

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