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Investigating the effects of *Salvia chorassanica* Bunge and shoot extracts on gastric cancer cells: evidence of different behavior on various tumor grades

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Running title: Remarkable cytotoxicity of root extract of *Salvia chorassanica* on gastric cancer cells

Abstract

Background: Different *Salvia* species have demonstrated anti-proliferative effects on various cancer cells. Owing to the poor literature on the anti-proliferative effects of *Salvia* species on gastric cancer cells, present study was conducted to determine the anticancer effects of a local Iranian *Salvia*, *Salvia chorassanica*, on two different gastric cell lines.

Methods: Root, stem and leaf extract of *Salvia chorassanica* were prepared through maceration method and were then used to treat the AGS and MKN-45 cell lines in different concentrations. MTT assay was employed to determine the toxicity of all the types of extracts on the two studied cell lines. The expression of *Bax*, *Bcl-2*, *Caspase3*, *MMP2* and *MMP9* genes were determined through reverse transcription Real time PCR (RT-Real time PCR).

Results: Bunge and shoot extracts demonstrated toxicity in both cell lines which were more considerable in AGS cells treated with root extract. In contrary to AGS cells, *Caspase3* gene was up-regulated in all types of treatment while the *MMP2* and *MMP9* genes were down-regulated ($p\text{-value} < 0.001$). Except of the MKN-45 cells treated with leaf extract, *Bax/Bcl-2* expression ratio was decreased in the treatment with all types of *Salvia chorassanica* extracts ($p\text{-value} < 0.001$).

Conclusion: Remarkable low IC50 concentration of root extract in MKN-45 cell line is indicating the significant cytotoxicity of *Salvia chorassanica* against gastric cancer cells. Moreover, gene expression analysis in MKN-45 needs further confirmation on the potential anti-metastatic roles of leaf and root extracts in higher grades of gastric cancer.

Keywords: MKN-45; AGS; cytotoxicity; *Salvia chorassanica*; gene expression

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Introduction

Gastric cancer is the most frequent type of cancer among men and the leading cause of cancer related death among Iranian men and women.¹ In spite of the possibility of management of gastric malignancies in earlier stages, high recurrence rate and poor survival of the patients are still remained as major concerns.² In addition, the efficiency of adjuvant chemotherapy which had improved the overall survival rate of gastric cancer patients is limited to the individuals who have undergone radiotherapy.³ In this regard, complementary medicine as well as herbal medicine has been extensively used to increase the survival of the patients, especially among the Asian population.⁴ Moreover, natural products administration has been shown to be associated with lower post-operation and chemotherapy side effects in gastric patients.⁵ The genus *Salvia* (Lamiaceae) is one of the herbal medicines which has 900 and 17 species in the world and Iran, respectively.⁶ The antimicrobial, anti-inflammatory and antitumor effects of different species of *Salvia* have been reported in various studies.⁷ Anti-proliferative impact of different compounds derived from local Iranian *Salvia* species have been demonstrated on various cancer cells as well. *Salvia officinalis* is one of the most important *salvia* species which its anti-proliferative effects have been reported on different cancer cells.⁸⁻¹⁰ In an investigation on the cytotoxic effects of eleven local *salvia* species on three HL60, K562 and MCF-7 cell lines, the anticancer effects of *S. eremophila*, *S. santolinifolia*, *S. aegyptiaca*, *S. aethiopsis*, *S. hypoleuca* and *S. limbata* was found to be significant.¹¹

Noori *et al.* have shown that Sclareol which has been isolated from *Salvia sclarea* induced a decrease in tumor size of breast cancer mouse model.¹² Moreover, apoptosis mediated cytotoxic effects of *Salvia sclarea* root on four different AGS, HeLa, MCF-7 and PC12 human cell lines would be a further confirmation on the anticancer potential of the Iranian *salvia* species.¹³

Salvia chorassanica is another local Iranian Salvia which has been the focus of anticancer studies. It was found that three compounds derived from the root of Salvia chorassanica can have cytotoxic and apoptotic effects on K562 and HL-60 cells (human leukemia cell lines) in addition to MCF-7 and DU 145 cell lines.^{14,15} Cytotoxic effect of Salvia chorassanica on HeLa cell line was demonstrated to be occurred through the induction of extrinsic apoptosis pathway.¹⁶

Owing to the aforementioned cytotoxic effects of Salvia chorassanica on human cancer cells and limited literature on the study of salvia species on gastrointestinal cell lines, the current study was conducted to determine the extent of cytotoxicity of Salvia chorassanica on two gastric cell lines. In addition, to the best of our knowledge, most of the studies on different Salvia species were performed only on the root extract of the plant. To clarify the medicinal potential of other parts of the plant, the extracts of leaf and stem of Salvia chorassanica were prepared and used to treat the cancer cells as well. Moreover, there is lack of information regarding the effect of Salvia species treatment on gene expression profile and, therefore, the mechanism of possible cytotoxicity was assessed through expression analysis of five key genes involved in apoptosis and metastasis.

Methods

Preparation of plant extracts

Salvia chorassanica plant was collected from the mountains of Zeshk restricts (Mashhad, Iran) which was confirmed by the Herbal Systematic Lab of Mashhad University of Medical Sciences. Root, stem and leaf of the plant were dried and then blended to be further processed during the extraction. The extraction was performed based on maceration method which included dissolving plants in hydroalcoholic solution (80% ethanol) and shaking at room temperature for 24 hours. Supernatants were removed and transferred to a new container to be

1 dissolved in 80% ethanol solution by further shaking at room temperature for 24 hours.

2 Obtained solutions were filtered and were incubated in cater-closet rotary to remove solvent.

3 Extracts were dried at 30°C for 24 hours and stored at 4°C until further assessments.

4 Cell Culture

5 Two gastric cancer cell lines including MKN-45 and AGS cell lines were purchased from the

6 Iranian Biological Resource Center and were cultured in DMED-F12 (Gibco, USA) and

7 RPMI 1640 (Gibco, USA), respectively. Culture mediums were supplemented with 10% Fetal

8 Bovine Serum (Gibco, USA) and 1% Penicillin Streptomycin (Gibco, USA) and incubated at

9 37°C saturated with 5% CO₂.

10 MTT Assay

11 A total of 10⁴ cells were seeded in each well containing culture medium complemented with

12 10 % FBS, and 1% Penicillin Streptomycin. After 24 hours, both cell lines were treated with

13 different concentrations (v/v) of root, stem and leaf extracts in triplicates. Plates were

14 incubated at 37°C under 5% (v/v) of CO₂ saturation for 24, 48 and 72 hours. Control

15 experiments included two plate series which were separately filled with complete culture

16 medium as negative control and plant extracts diluted by DMSO as vehicle control. MTT

17 assay was performed using cell proliferation kit according to the manufactures instructions

18 (Sigma, St. Louis, MO) to measure the inhibitory effect of different concentration of three

19 types of extracts including root, stem and leaf of salvia Chorassanica on the growth rate of

20 MKN-45 and AGS cells. Cell viability was measured by ELISA reader (Anthons2020,

21 version 1.8.3, UK) and analysis was carried out according to the following equation:

$$22 \% \text{ Cell viability} = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of Control} - \text{Absorbance of blank}} \times 100$$

RNA Extraction and cDNA Synthesis

Both MKN-45 and AGS cell lines were treated with half of the maximal inhibitory concentration (IC50) of each type of extracts in 6 well-plates to be analyzed after 24 hours. Total RNA was extracted from both treated and untreated cell lines using Roche High Pure RNA Isolation Kit (Roche Life Science, UK) according to the manufacturer's instructions. The quality and quantity of isolated RNAs was investigated through Nano-drop and agarose gel electrophoresis (0.8 %). Isolated total RNAs were reversely transcribed to cDNA using the Thermo Fisher Scientific RevertAid Reverse Transcriptase kit (Thermo Fisher Scientific, UK) according to the manufacturer's protocol.

Real time RT-PCR

The specific primer sequences were designed for six genes including *Caspase-3*, *BAX*, *Bcl-2*, *MMP2* and *MMP9* and checked through online NCBI Primer BLAST (Table 1). The Reverse transcription Real time PCR (RT-Real time PCR) reaction was performed on Bio-RAD (CFX Connect Real-Time PCR) and was included 5 ng cDNA, specific primer pairs, 2X SYBR Premix Ex Taq II (Takara, Japan) which was adjusted to final volume of 20 μ l using ddH₂O. Thermal cycling condition included an initial denaturation at 95°C for 5 minutes, followed by 45 cycles of 95°C for 20 s and 60°C for 40s. All the reactions have been done in triplicate and were normalized to the expression of the *ACTB* gene as a reference gene.

Statistical analysis

Graph Pad Prism V7.04 was implicated to perform all the statistical analysis. The IC50 was calculated using non-linear regression. Normality distribution and variance homogeneity among experiment groups were tested using the Shapiro Wilk test and Brown Forsythe test, respectively. In order to compare quantitative variables among the experiment groups, regular one-way and Two-way ANOVA tests were used based on the dimensions of the data and, the p-value less than 0.05 was assumed as significant.

Results

Effects of three parts of salvia chorassanica extracts on AGS and MKN-45 cell lines

viability

Cell viability percentage was calculated by MTT test and non-linear regression was employed to estimate the IC₅₀ of all the types of extracts. Obtained cell viability of each extract treatment at different points of times and concentrations has been presented for the two studied cell lines (Figure 1-2). Root extract has demonstrated strong cytotoxicity on both cell lines, which was remarkably significant in AGS cells (p-value<0.001). All the three types of extracts were found to be toxic against both cell lines proliferation in a time and dose dependent manner. The IC₅₀ of the treatment with root, stem and leaf extracts following 24 hours on AGS cell were 9.262, 880.4 and 420.1 µg/ml and, on MKN-45, 271.73, 898 and 354.6 µg/ml, respectively.

Effects of three parts of salvia chorassanica extracts on selected genes expression in AGS

cell line

Both AGS and MKN-45 cell lines were treated with IC₅₀ concentration of all the types of extract in 6 well-plate and were analyzed after 24 hours. Expression of *BAX*, *Caspase-3* and *MMP2* genes was significantly down-regulated (p-value<0.001), while *Bcl-2* and *MMP9* genes expression was found to be meaningfully decreased following treatment with root extract (p-value<0.001). The *MMP2* and *Bcl-2* genes were meaningfully up-regulated (p-value<0.001) while the expression of *Caspase-3* was demonstrated to be significantly decreased following treatment with stem extract (p-value=0.01). In the treatment of AGS cells with leaf extract, the expression of *Bcl-2* and *Caspase-3* was found to be significantly increased and decreased, respectively (p-value<0.001) (Figure 3).

Effects of three parts of salvia chorassanica extracts on selected genes expression in MKN-45 cell line

Following the treatment of MKN-45 cells with the IC-50 concentration of root extract, the expression of *BAX* and *Bcl-2* genes was significantly decreased and increased, respectively (p-value<0.001). The same but meaningless pattern was found in the expression of *MMP9* and *Caspase-3* genes, respectively (p-value>0.05). Expression of *BAX*, *BCL-2* and *Caspase-3* genes was significantly increased in response to treatment with leaf extract (p-value<0.001). Expression of *MMP-2* and *MMP-9* genes was shown to be profoundly decreased following treatment with stem and leaf extract (p-value<0.001) (Figure 4).

Discussion

According to the MTT analysis, the root extract had shown the most cytotoxic effect on both studied gastric cancer cells lines and in particular, AGS cells. Although it requires protein expression confirmation, according to the discordant apoptotic genes expression, it seems that the mechanism of root cytotoxicity was independent of apoptosis. Owing to the down-regulation of *MMP2* and *MMP9* genes in MKN-45 as a model of a moderately metastatic cell line, root extract of salvia chorassanica could be a potential herbal medicine in the prevention of the metastatic evolution of gastric cancer cells.¹⁷

Cytotoxic effect of root extract of salvia chorassanica has been previously replicated in Hela cells as well. It was associated with overexpression of *Caspase-3* and *Caspase-8*, which is consistent with our result regarding the *Caspase-3* gene in MKN-45 cells treated with root and leaf extracts.¹⁶ Tayarani-Najaran *et al.* have shown the cytotoxic effect of Taxodione, ferruginol and 6-hydroxysalvinolone derived from salvia chorassanica root on two apoptosis proficient and resistant leukemia cell lines through overexpression of BAX and active Caspase-3 proteins.¹⁴ In the present study, the significantly higher toxicity of salvia

Salvia chorassanica root on AGS cell line is maybe indicating more toxic behavior of this type of Salvia on lower grade gastric cancer cells. In this regards, our finding is in line with more cytotoxicity of Tanshinone IIA (derived from Salvia miltiorrhiza) on MKN-45 cells than on SGC7901 cells as highly metastatic gastric cancer cell line.¹⁸ Sung *et al.* examined the cytotoxicity of Salvia miltiorrhiza root extract dissolved in separate acetone and ethanol solutions on five MCF-7, HCT116, LNCap, A549 and AGS cell lines and demonstrated the highest level of toxicity on AGS and HCT116 (colorectal cancer) cell lines. The IC₅₀ of ethanol (100%) solvent of Salvia miltiorrhiza root was found about 10 µg/ml for AGS and HCT116 cell lines, which was higher than the IC₅₀ concentration of Salvia chorassanica root extract (solved in 80% ethanol) in our AGS cells. In addition, it indicates that the concentration of ethanol used in dissolving the extract may have a pivotal role in cytotoxicity.¹⁹

To the best of our knowledge, there are a few reports on the anti-proliferative effects of the leaf and stem of salvia species on cancer cells. Herein, in contrast to the root, the leaf and stem extracts demonstrated considerable toxicity against gastric cancer cells, which was more significant in MNK-45. Fiore *et al.* investigated the effect of root, stem and leaf of Salvia menthifolia on a glioblastoma cell line (DBTRG-05MG) and have found that leaf extract with IC₅₀ of 112.7 mg/L was the most toxic extract followed by stem and root.²⁰ Leaf extract of Salvia officinalis was cytotoxic to AMN3 and Rhabdomyosarcoma cell lines which was associated with decreased mitotic index. Of note, the lethal concentration of their leaf extract was about 14 fold more than the leaf extract concentration used in the present study.²¹ Comparable lower IC₅₀ concentrations of the leaf and stem extracts of our local Salvia chorassanica maybe indicating their potential in complementary therapeutic plans of gastric cancer patients.

Main metastatic genes, *MMP2* and *MMP9*, were found to be down-regulated in MKN-45 cells following treatment with root, stem and leaf extracts of *Salvia chorassanica*. Anti-metastatic potential of salvia species has been demonstrated in MCF-7 cells treated with *Salvia miltiorrhiza* through inhibition of *MMP9* expression and MAPK/AP-1 signaling pathway.²² To the best of our knowledge, this is the second report on the anti-metastatic characteristics of another *Salvia* species through modulation of *MMP2* and *MMP9* genes expression. However, the anti-metastatic of *Salvia chorassanica* requires further confirmatory assessments including migration assay and protein expression analysis of MMP family members as well as *MMP9* and *MMP2*.

Conclusion

Taken together, anti-proliferative and anti-metastatic effects of the root and leaf extract of *Salvia chorassanica* were primarily found in gastric cancer cells. Although incompatible apoptotic genes expression should be confirmed at the protein level, it may rely on other cell death mechanisms, including necrosis. Autophagy is another mechanism of gastric cancer cells death which should be considered in further investigations.

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Table 1: Primer pair sequences used in RT-Real time PCR reaction

Gene	Sequence (5'-3')	Concentration (μ M)	Product size (bps)
<i>MMP2-F</i>	AACCAGCTGGCCTAGTGATG	500	154
<i>MMP2-R</i>	CTTGGGGCAGCCATAGAAGG	500	
<i>MMP9-F</i>	CCTGGGCAGATTCCAAACCT	300	172
<i>MMP9-R</i>	GTACACGCGAGTGAAGGTGA	300	
<i>Caspase-3-F</i>	CGGCGCTCTGGTTTTTCGTTA	250	120
<i>Caspase-3-R</i>	CAGAGTCCATTGATTCGCTTCC	250	
<i>BAX-F</i>	TCATGGGCTGGACATTGGAC	250	114
<i>BAX-R</i>	GAGACAGGGACATCAGTCGC	250	
<i>BCL-2-F</i>	CTTTGAGTTCGGTGGGGTCA	300	162
<i>BCL-2-R</i>	GGGCCGTACAGTTCCACAAA	300	
<i>ACTB-F</i>	TGGAACGGTGAAGGTGACAG		125
<i>ACTB-R</i>	AACAACGCATCTCATATTTGGAA		

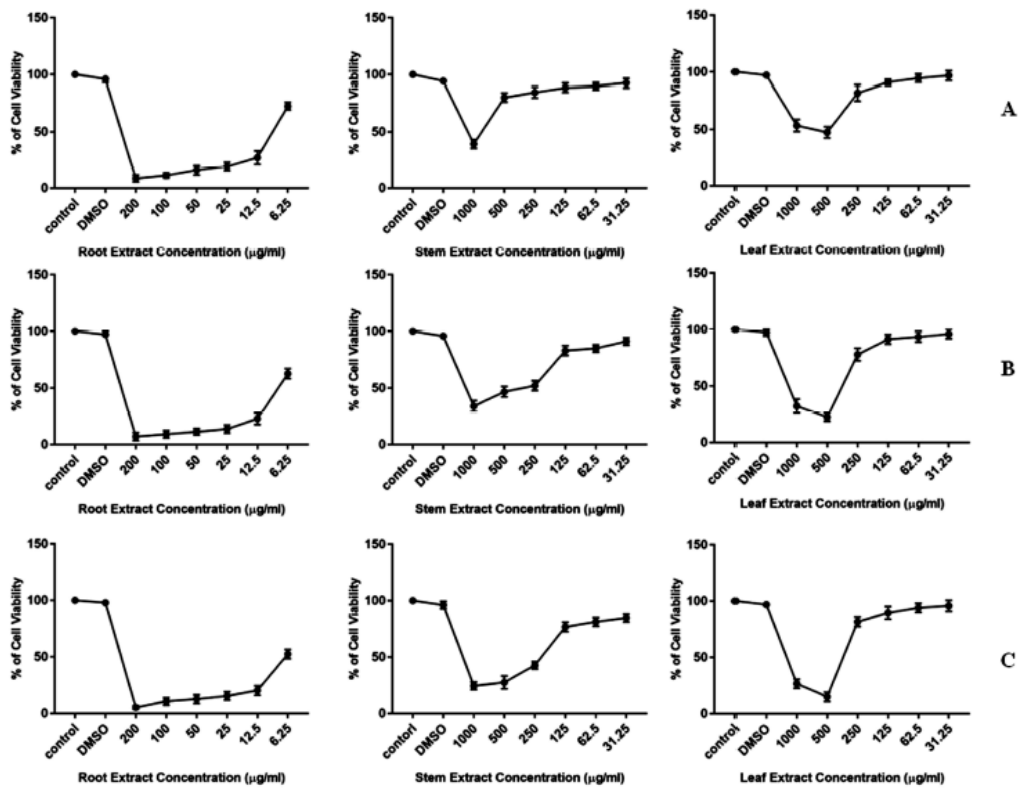


Figure 1: Cell viability percent of AGS cells treated with different concentrations of the A: root after 24, 48 and 72 hours; B: stem after 24, 48 and 72 hours and, C: leaf after 24, 48 and 72 hours.

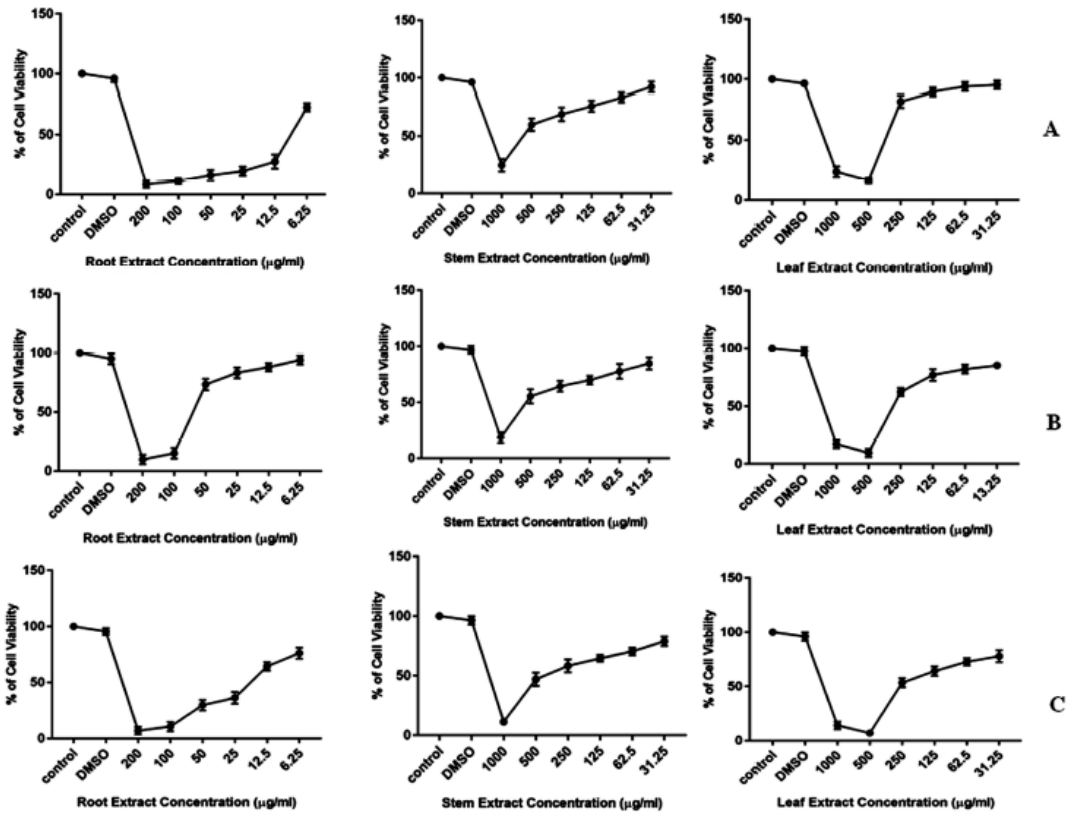


Figure 2: Cell viability percent of MKN-45 cells treated with different concentrations of the A: root after 24, 48 and 72 hours; B: stem after 24, 48 and 72 hours and, C: leaf after 24, 48 and 72 hours.

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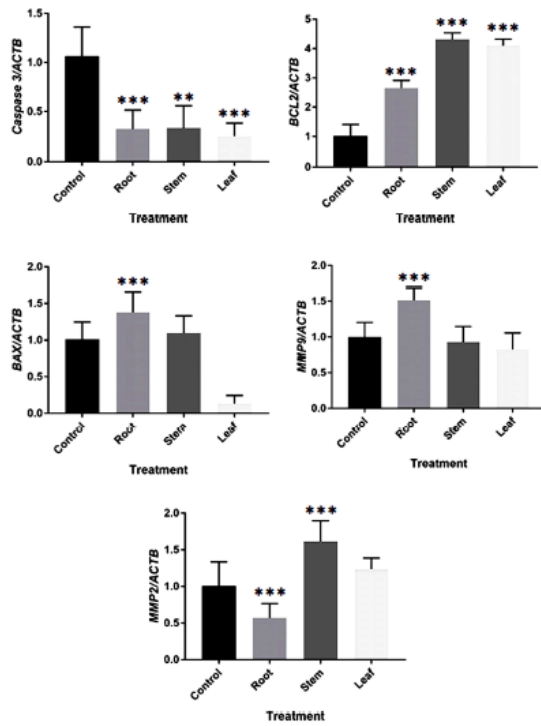


Figure 3: Gene expression analysis 24 hours after treatment with IC50 concentrations of root, stem and leaf extracts of *Salvia chorassanica* in AGS cell line. p-value < 0.001: *** and p-value = 0.01: **.

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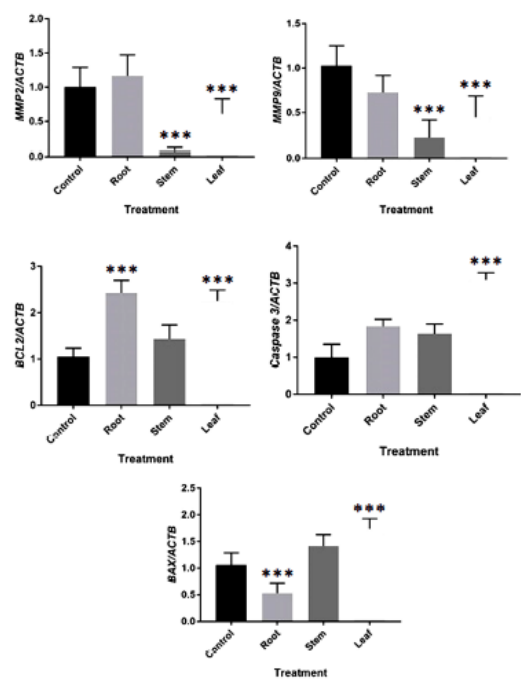


Figure 4: Gene expression analysis 24 hours after treatment with IC50 concentrations of root, stem and leaf extracts of *Salvia chorassanica* in MKN-45 cell line. p-value < 0.001: ***.

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