The survey of Capparis extracts effects on expressional profile of essential self-renewal genes in MCF7 cell line

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Research Article

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Abstract

Several drugs have been proposed for the treatment of breast cancer, but none has fully treated the disease, so far. this study was to investigate the effect of aqueous-alcoholic extract of unripe *Capparis* fruit as an anticancer agent on expressional pattern of OCT4, NANOG and SOX2 as essential self-renewal pathway genes in MCF7 cell line (human breast carcinoma). MCF7 cells were cultured in RPMI-1640 medium, consisting of different concentrations of aqueous-alcoholic extract of unripe *Capparis* fruit (125, 250, 500, 1000, 1500, 2000, 2500 and 5000 μg/ml) for 48 and 72 hours. MTT assay was used to determine the cell proliferation inhibition (IC50). RT-PCR method was carried out to assess the fold changes of OCT4, NANOG and SOX2 genes. One-way ANOVA was used for the statistical analysis of obtained data. Hydroalcoholic extract of the unripe *Capparis* fruit caused time- and concentration-dependent cell death in MCF7 cells. IC50 was observed at 48h culture period with 4817.51 μg/mL , and 72h with 2724.29 μg/ml fruit extract, respectively. Our results have shown that cell death was induced by increasing *Capparis* extract concentration. According to RT-PCR findings on capparis extract-treated cells, the mean expression of OCT4, NANOG and SOX2 genes decreased after 48 and 72h of incubation with IC50 concentration compared to controls. *Capparis* plant species is able to decrease the expression of self-renewal genes in MCF7 cell line. Therefore, the *Capparis* extract can be considered as a promising candidate for the management of human breast cancer after clinical trials.

1. Introduction

Breast cancer remains at the forefront of global health concerns, despite significant research and scientific advances in treatment and prevention, which has made it one of biomedical research priorities. It is the most common cancer among women worldwide, with a significant increase in its incidence and death in the coming years. The GLOBOCAN 2020 estimates presented indicate that Female breast cancer has now surpassed lung cancer as the leading cause of global cancer incidence in 2020, with an estimated 2.3 million new cases, representing 11.7% of all cancer cases. (Sung et al., 2021)

This highly heterogeneous cancer appears to have complex and aggressive biological nature. However, the complex biology of this cancer is unclear and existing treatment strategies are age-independent (Anastasiadi, Lianos, Ignatiadou, Harissis, & Mitsis, 2017). Treatments have their own complexities for each type of cancer. Pharmaceutical developments often focused on inexpensive anticancer drugs with higher efficacy and lower toxicity. Despite significant advances in breast cancer, there have been reports of drug resistance in many therapeutic trials. Hence, many researchers have turned to some well-known plants that have been shown in experimental studies to have numerous anti-cancer effects, possibly due to the variety of compounds in these herbs, unlike synthetic version. Accordingly, the acquisition of new drugs of plant origin can help to treat a variety of cancers more effectively (Moghadamnia, Kani, Ghasemi-Kasman, Kani, & Kazemi, 2019).

Capparidaceae is a superfamily of plant species embarrassing various phanerogam gymnosperm dialypetalae. *Capparis spinosa* is one of the most widely used species of this family in various fields,
including anti-inflammatory, antioxidant, antimicrobial, anti-cancer and anti-diabetic activities (Vahid, Rakhshandeh, & Ghorbani, 2017). One of the known naturally occurring compounds is flavonoids with potent antioxidant properties and other important activities in cell biology such as preventing the destructive role of free radicals in carcinogenesis by collecting these species. According to previous findings, flavonoids are key players in the prevention of genetic mutations and thus the inhibition of tumorigenesis. The presence of flavonoids in large quantities has been reported in *C. spinosa*, especially quercetin with a molecular mass of 302.23 g/mol and a molecular formula $C_{15}H_{10}O_{7}$. The quercetin has shown various activities, including anti-inflammatory, antibacterial, anticoagulant, anti-atherogenic and anti-hypertensive potentials. In a study, *C. spinosa* was reported to have both volatile and non-volatile ingredients with central functions in the prevention of colorectal cancer (H. Zhang & Ma, 2018).

*C. spinosa* has been shown reportedly to have outstanding bio-activities such as antioxidant, antimicrobial, anti-inflammatory, anti-diabetic and anti-cancer. Other beneficial effects attributed to this plant include the improvement of cardiovascular disorders, nephropathy and liver failure in diabetic animals, possibly due to the presence of antioxidant agents like phenolic compounds, carotenoids, flavonoids, terpenes and tocopherols (Vahid et al., 2017). Due to the antioxidant and anti-cancer activity of flavonoids and the abundant presence of *C. spinosa*, there is a possibility of reducing the growth and proliferation of cancer cells by hydroalcoholic extraction of this plant (Moghadamnia et al., 2019). Genes involved in regulating the reproduction of stem cells have been identified as molecular markers of cancer, the uncontrolled expression of which is significant in the pathogenesis of cancer, such as SOX2, OCT4 and NANOG genes in the self-renewal pathway. Embryonic stem cell proliferation and survival are highly dependent on the presence of the OCT4 gene along with genes such as NANOG and SOX2. It is a preserved gene in mammals, as a copy factor in the control of the expression of numerous genes.

To the best of our knowledge there has been no study that has investigated the effect of *C. spinosa* on MCF7 cell line. Therefore, in light of these considerations, the current study was undertaken to investigate the effect of *C. spinosa* on MCF7 cell line by detects the gene expression (mRNA) of OCT4, SOX2 and NANOG to eliminate the possible underlying molecular mechanism.

### 2. Materials And Methods

#### 2.1. Plant extract

Fruits of *Capparis spinosa* were collected from the southeastern region of Iran (Kerman, Rafsanjan) in September 2020. These fruits were identified by a local Plant Biotechnologist at Department of Natural Resources, Rafsanjan, Iran. Then, the samples (*Capparis* fruits) were dried in the shade and powdered by electric grinder. The extract of powdered fruits were obtained by 70% ethanol employing Soxhlet extractor. Next, we prepared different concentrations of the extract.

#### 2.2. Cell line provision and cultivation
The MCF7 cell line was purchased from Pasteur Institute of Iran. Then, these cells were kept in Laboratory of molecular medicine University of Rafsanjan Medical Sciences, which were cultivated in RPMI1640 (GIBCO Co. (Grand Island, NY, USA)) medium in the presence of streptomycin (100 mg/ml), penicillin (100 IU/ml) and FBS (10%) (GIBCO,USA), followed by incubation at 37°C with 5% CO₂ and 95% humidity.

2.3. Cell line preparation

The \textit{C. spinosa} extract impact was evaluated on the expression profile of self-renewal genes in MCF7 cell line by preparing cell culture with several passages to obtain the cells in reproductive status. The cells within two groups of test and control were cultured in triplicate for all plant extract concentrations (125, 250, 500, 1000, 1500, 2000, 2500 and 5000 µg/mL). Next, almost 15000 cells per well in 200 µl of cell culture were planted to each well of 96-well plate, followed by incubation in the presence of CO₂ for 24 hours. In the test group, the eight different plant extract concentrations were appended into the cell-containing wells. In the control group, the wells were added with culture medium.

2.4. MTT assay

The cell viability was assayed using MTT test (Bio Idea, Iran). This experiment is on the basis of yellow MTT reduction via active mitochondrial dehydrogenase of intact and living cells to a purple formazan, meaning that the conversion can be directly associated with the number of viable cells (Mosmann, 1983). The cells (15000 cells per well) were planted in the 96-well culture plates and incubated for 24 hours, and subsequently were exposed to different plant extract concentrations (125, 250, 500, 1000, 1500, 2000, 2500 and 5000 µg/mL) for 48 and 72 hours. Then, each well was appended with 10 µl of MTT dye (5 mg/m) and incubated at 37.6°C for four hours. Next, the solution was pulled out, and DMSO solution (80 µl) was poured into all wells for the crystals solubilizing. Finally, the OD was read at a reference wavelength of 570 nm using ELx808 ELISA Reader (BioTek Instruments, USA). The results of the study were statistically analyzed using complete randomized design via SPSS.

2.5. Extraction of total RNA

After 48 and 72 hours of incubation, both test and control cells were harvested via the centrifugation of medium and washed with PBS. RNA purification Kit (Pars Tous, Iran) was utilized to extract the total RNA samples. After dissolving the RNA samples in DEPC-treated water, the measurements were performed using the spectrophotometer (DS- 11 FX; DENOVIX; the USA). The extracted RNA was quantified by 1.5% agarose gel electrophoresis, DNA Green Viewer™ staining and gel documentation system.

2.6. Cell harvesting to construct cDNA

The cDNA was constructed by a Reverse Transcriptase Kit (Pars Tous, Iran), which was kept at -20°C or instantly used for quantitative RT-PCR. The RNA required for being converted to cDNA via reverse transcriptase was utilized as the target in the RT-PCR. Before synthesizing cDNA, the RNA treated by DNase I to digest contaminating genomic DNA. For this purpose, a mixture of total RNA, 10 µl of DEPC, random hexamer primers and oligo dT was incubated at 70°C for 10 min. Then, the RT-mix (10 µl) was
appended into a microtube, followed by incubation at 42°C for 60 min to construct the cDNA. Finally, the mixture was exposed to 95°C for 5 minutes for the inactivation of RT enzymes.

**2.7. Primer design**

Primer (Pishgam Biotech Co., IRAN) design version 3 software was employed to design the sequence of both reverse and forward primers for SOX2, OCT4, NANOG and Beta-actin genes, followed by a blast on NCBI to ensure their integrity (Table 1).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'→3')</th>
<th>Reverse (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT4</td>
<td>CCTGAAGCAGAAGAGGATCACCC</td>
<td>AAAGCGGCAGATGGTCGTTTGG</td>
</tr>
<tr>
<td>SOX2</td>
<td>GCTACAGCATGATGCAGGACCA</td>
<td>TCTGCGAGCTGGTCATGGAGTT</td>
</tr>
<tr>
<td>NANOG</td>
<td>CTCCAACATCCTGAACCTCAGC</td>
<td>CGTCACACCATTGCTATTCTTGG</td>
</tr>
<tr>
<td>β-actin</td>
<td>CACACCTTCTACAATGAGC</td>
<td>ATAGCACAGCCTGGATAG</td>
</tr>
</tbody>
</table>

**2.8. The gene amplification**

First, the primers were prepared and then the cDNA mRNA was amplified using the RT-PCR target genes. Each well of a PCR plate were added with the reverse and forward primers (2 µl each), Cdna (Pars Tous, Iran) (2 µl), SyberGreen MasterMix (Pars Tous, Iran) (10.4 µl) and DNase-free water (5.6 µl) in a final volume of 20 µl, followed by covering the plate surface via a specific adhesive to avoid evaporation. The plates were positioned in Applied Biosystems real-time PCR device to run the amplification according to the manufacturer's instructions (the cycles for 15 s at 95°C, for 30 s at 58–62°C, and for 30 s at 72°C with triplicate replications). We analyzed the data and curves obtained from the apparatus (ct numbers). The internal control was beta-actin gene and the formula of $2^{-\Delta\Delta ct}$ was used to analyze the data (Karimabad et al., 2017).

**2.9. RT-PCR processes**

The RT-PCR method was utilized to determine the expression profiles of OCT4, NANOG and SOX2, self-renewal genes. The MCF-7 cells were exposed to extract IC$_{50}$ concentration for 48 and 72 hours.

The RT-PCR method was conducted to measure the mRNA level in the treated and untreated cancer cells using a reaction mixt (50 µl) consisting of ddH$_2$O (5 µl), reverse primer (5 µl), forward primer (5 µl), Taq Man (25 µl), Universal Master Mix, labeled (FAM/MGB and or JOE/TAMRA) probe (5 µl), reverse transcriptase (1 µl), random hexamer (2 µl) and purified RNA (2 µl).

**2.10. Statistical analysis**

To statistically analysis the data SPSS software (version 21) was applied. All experiments were performed three times in each individual sample and all of obtained results were presented as the mean...
value of those three. Data were also analyzed by ANOVA and repeated measures of a p value less than 0.05 was considered significant.

### 3. Results

Hydroalcoholic extract of the unripe *Capparis* fruit caused time- and concentration-dependent cell death in MCF7 cells. IC50 of the drug was observed at 48h culture period with 4817.51 µg/mL fruit extract, and 72h with 2724.29 µg/ml fruit extract, respectively. Our results have shown that cell death was induced by increasing *Capparis* extract concentration (Table 2).

<table>
<thead>
<tr>
<th>The hydro-alcoholic capparis extract IC50 (µg/mL)</th>
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<tbody>
<tr>
<td>Time</td>
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<tr>
<td>Concentrations</td>
</tr>
</tbody>
</table>

The expression of OCT4 gene was down-regulated in all eight plant extract concentrations after 48 and 72 hours of treatment, but the highest decrease in the expression of OCT4 gene was seen after 72 hours of cell treatment and the plant extract concentration of 100 µg/ml (Fig. 1).

The SOX2 gene expression alteration take place after 48 and 72 hours of cell treatment and the plant extract concentration of 40 µg/ml. The expression pattern of SOX2 gene was down-regulated but the highest decrease in the expression of SOX2 gene was seen after 72 hours of cell treatment and the plant extract concentration of 100 µg/ml (Fig. 2).

The expression of NANOG gene was decreased after treatment of MCF7 cell line with the plant extract concentrations, and the greatest down-regulation was seen after 72 hours (Fig. 3).

To conclude, the results of this study showed a significant difference in cell viability following the use of different concentrations of plant extracts after 48 and 72 hours of incubation. The RT-PCR findings revealed that the *C. spinosa* extract caused a decrease in the expression levels of SOX2, OCT4 and NANOG genes when comparing with the control cells.

The IC50 concentration of *C. spinosa* extract capable of inhibiting the MCF-7 cell proliferation was estimated to be 70 mg/ml when comparing with control group.

The Cell division is made possible by genes implicated in the processes of self-renewal pathway with differential activities like SOX2, OCT4 and NANOG. Hence, the present study was designed to determine the expression pattern of these genes in the MCF7 cell line. The genes involved in regulating the self-renewal pathway of stem cells have been identified as molecular markers of cancer, the uncontrolled expression of which is significant in the tumorigenesis (M. Zhang & Rosen, 2006).
4. Discussion

The cancer death is still high despite the application of various treatment strategies such as radiotherapy, chemotherapy and surgery. On the other hand, these therapeutic procedures are associated with disadvantages such as the destructive effects of radiotherapy and chemotherapy on normal cells (Gul, Mansoor, Gul, & Arshad, 2020). Accordingly, naturally occurring products have aroused widespread attentions due to their anti-cancer potentials. Our results have shown Hydroalcoholic extract of the unripe Capparis fruit caused time- and concentration-dependent cell death in MCF7 cells. Also we have shown that cell death was induced by increasing Capparis extract concentration. Our data showed that the mRNA expression of OCT-4, NANOG and SOX2 expression was decreased in MCF7 cells, once treated with extract of Capparis fruit compared to control cells. Furthermore, the sphere-forming ability of cancer cells was impaired upon with extract treatment, proposing that with extract might have been interfered with the tumor-initiating ability of these cells. Although previous reports have proposed an evident link between multipotency- and pluripotency-associated marker expression and the sphere-forming ability of cancer cells (Chiou et al., 2010; Gharibi, Ghuman, & Hughes, 2012; Jogezai, Mansoor, Gul, & Arshad, 2020).

The effects and chemical composition of C. spinosa extract have been repeatedly studied by various researchers around the world, especially with a focus on different cancer cells. For example, Al-Assadi et al. reported a significant reduction in the growth and proliferation of HeLa and Hep-2 tumor cell lines in exposure to C. spinosa extract (AL-Asady, Khalil, & Barwari, 2012). Yasaman Moghadamnia et al. examined the antioxidant levels and antitumor activity of C. spinosa hydroalcoholic extract, which were evaluated by FRAP and MTT methods, sequentially. One of the main constituents of C. spinosa extract was reported to be quercetin based on the HPLC findings. Moreover, the MTT assay findings confirmed a decrease in the proliferation rate of cancer cell lines in exposure to C. spinosa extract, as well as the FRAP results revealed a strong antioxidant activity for this extract. An anti-inflammatory activity has been reported for the quercetin present in C. spinosa extract. This compound has a significant anti-tumor performance, which makes it a promising natural candidate for cancer therapy (Moghadamnia et al., 2019).

In line with the present study, Chiou et al. reported overexpression of the SOX2 gene in cancer stem cells extracted from oral squamous cell carcinoma. There are reports of overexpression of this gene in different sexual cell tumors and sarcoma bone marrow tumors (Chiou et al., 2010). The NANOG, SOX2 and OCT4 genes have been reported to cooperate with each other to generate central networks in the regulation of stem cell self-regeneration, and each gene in this ternary group is able to regulate two other genes. The three genes act synergistically as transcriptional activators of other genes implicated in self-regeneration and as inhibitors of the function of genes implicated in differentiation (Gharibi et al., 2012). Two key theories describe the development of cancer. In the first theory, all tissue cells are considered susceptible to cancer. In the second theory, cells exist in tissues called adult stem cells. These cells will be cancer stem cell and become cancerous for unclear causes (alterations in the expression of determined genes) (Sell, 2004). "Stem Cell Origin of Cancer" is a novel theory based on which numerous tumors are derived from stem cells in tissues. According to this theory, one of the key factors implicated in
carcinogenicity is the factors capable of inhibiting the process of differentiation or uncontrolled proliferation of tissue stem cells (Jiang, Peng, Zhang, Cho, & Jin, 2012). Another piece of evidence for this theory is the similarity between stem cells and the small population of cancer cells. These small cell populations can resist chemotherapy-inducing factors and apoptosis, as one of the cancer-remitting agents following degradation of primary tumors (Gostjeva & Thilly, 2005). NANOG and OCT4 are prominent genes with the ability to self-regenerate that activate stem cells and impede genes initiating differentiation, and thus preserve the self-regenerative potential of stem cells (Avery, Inniss, & Moore, 2006). There are three variants of A, B and B1 for OCT4 gene. Variant B1 of the OCT4 gene has recently been discovered, with a high expression level in the cancer cells, depending on the degree of malignancy that can determine the diagnosis of cancer and its malignancy (Niwa, Miyazaki, & Smith, 2000).

The aim of this novel study was to investigate for the first time the impact of various concentrations of *capparis* fruit extract (125, 250, 500, 1000, 2000, 2500 and 5000 µg/mL) on the expression profile of SOX2, OCT4 and NANOG genes in the MCF7 cell line after 48 and 72 hours. Our observations showed a decrease in the expression of these genes in the presence of this extract both concentration-dependently and time-dependently. The expression of NANOG, OCT4 and SOX2 showed the highest downregulation at the *capparis* fruit extract concentration of 100 µg/ml and the incubation time of 72 hours. The investigations on have revealed a source of bioactive ingredients with anti-tumor, cytotoxic, anti-mutagenic, inhibitory and immunostimulatory features. One of the main chemical composition of *C. spinosa* is quercetin, with anti-tumor potential (Moghadamnia et al., 2019).

### 5. Conclusion

According to the findings of this study, the hydroalcoholic extract of unripe *Capparis* fruit was able to reduce the expression of the main genes controlling the self-renewal pathway (NANOG, OCT4 and SOX2) in the MCF7 cell line, thus resulting in inhibition of cancer cell proliferation. We believe our findings are valuable enough to be exploited in the production of herbal medicines to study the molecular mechanisms of different cancer pathways. It is suggested that further studies could be performed on the effects of such plant compounds on the molecular and cellular pathways involved in the regulation of cell proliferation.

### Abbreviations

Michigan Cancer Foundation-7 (MCF-7); Real-time-PCR (RT-PCR); Embryonic cancer stem cells (ECSCs); optical density (OD); Dimethyl sulfoxide (DMSO); Statistical package for the social sciences (SPSS); octamer-binding transcription factor 4 (OCT4); SRY-Box Transcription Factor 2 (SOX2); Homeobox protein NANOG (NANOG); Analysis of Variance (ANOVA); Roswell Park Memorial Institute (RPMI); (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay (MTT); *Capparis spinosa* (*C. spinosa*); fetal bovine serum (FBS); The enzyme-linked immunosorbent assay (ELISA); phosphate-buffered saline (PBS); Diethyl pyrocarbonate (DEPC); Complementary DNA (cDNA); The
National Center for Biotechnology Information (NCBI); High Performance Liquid Chromatography (HPLC); Fluorescence Recovery After Photobleaching (FRAP);

**Declarations**

**Acknowledgement**

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**Informed consent**

No informed consent

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**References**


Figures
Figure 1

Expression of OCT4 mRNA, against β-actin as an internal control, in treated MCF7 cells by alcoholic extract of Capparis compared to control cells.

The mRNA expression of OCT4 was detected after treating the MCF7 cell lines at IC50 concentrations for 48 and 72 h by real time PCR. Values are the average of triple determinations with the SD. indicated by error bars. *P< 0.05 compared to control
Expression of SOX2 mRNA, against β-actin as an internal control, in treated MCF7 cells by alcoholic extract of Capparis compared to control cells.

The mRNA expression of SOX2 was detected after treating the MCF7 cell lines at IC50 concentrations for 48 and 72 h by real time PCR. Values are the average of triple determinations with the SD. indicated by error bars. *P< 0.05 compared to control
Figure 3

Expression of NANOG mRNA, against β-actin as an internal control, in treated MCF7 cells by alcoholic extract of Capparis compared to control cells.

The mRNA expression of NANOG was detected after treating the MCF7 cell lines at IC50 concentrations for 48 and 72 h by real time PCR. Values are the average of triple determinations with the SD. indicated by error bars. *P< 0.05 compared to control