

Evaluation of Kohlrabi (*Brassica oleracea* Var. Gongylodes) Extract Effect on Mesenchymal Stem Cells Viability and Apoptosis



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ABSTRACT

Background: Cell viability and apoptosis are two crucial factors that may determine cell fate. There are several factors, such as hypoxia, which may be effective in cell processes. Because of its unique features, such as its antioxidant, anti-inflammatory, and anti-apoptosis mechanisms, kohlrabi (*Brassica oleracea* var. gongylodes) extract may be used in the amelioration of cell viability and a decrease in cell apoptosis. In this study, we evaluate the effect of kohlrabi extract on the viability and apoptosis of adipose-derived mesenchymal stem cells (AD-MSCs).

Materials and Methods: In this study, extract from kohlrabi and mesenchymal stem cells from adipose tissue were isolated in a laboratory under sterile conditions. Expression of mesenchymal stem cell (MSC) surface markers, including CD44, CD90, and CD105 was evaluated by flow cytometry method. Besides, CD34 was used as a negative marker. MTT assay was carried out to determine the cell viability. Evaluation of *BCL2* and *BAX* expression levels was performed by real-time PCR.

Results: MSC surface markers were verified by flow cytometry. The obtained results demonstrated a significant difference between the cell viability of the kohlrabi-extract treated and control group over time ($P=0.03$). In addition, the real-time PCR analysis showed that expression levels of *BCL2* significantly increased in hypoxic condition after treatment with leaf extract ($P=0.019$). However, there was no significant expression change in the *BAX* gene.

Conclusion: Our study illustrates that kohlrabi extract may have positive effects on cell survival while having inhibitory effects on apoptosis.

Introduction

Brassicaceae oleracea is one of the top 10 economic plants in the world [1]. Several varieties of *Brassica oleracea*, including broccoli, cauliflower, cabbage, and kohlrabi have an edible taproot and leaves [1].

One of the members of this family is kohlrabi that contains very potent phytochemicals and glucosinolates.

With its special characteristics, such as anti-oxidant, anti-inflammatory, and anti-apoptosis mechanisms, *Brassica oleracea* extract may be used as a suitable means to improve cell viability and to reduce cell apop-

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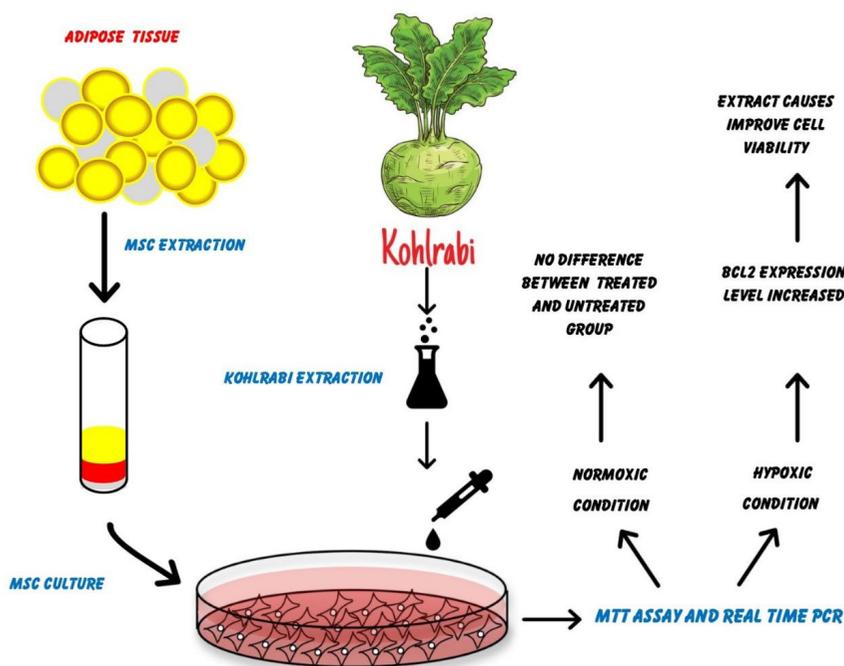


Figure 1. The schematic image summarizes the entire process of this study.



tosis [2]. Besides, *Brassica oleracea* also has several vitamins such as A, C, K, and B, as well as phytochemicals, including glucosinolates and indoles [2].

Park et al. analyzed the secondary metabolite of kohlrabi and reported that carotenoids and phenylpropanoid comprise the main part of kohlrabi's metabolites. β -carotene, lutein, 4-hydroxybenzoic acid, benzoic acid, and caffeic acid are the main ingredients of kohlrabi [2]. Previous studies demonstrated that the secondary metabolites, such as carotenoids and β -carotene, could be affected by cell proliferation and apoptosis [3, 4].

On the other hand, numerous studies have reported the effect of kohlrabi extract on cell proliferation [5-7]. For instance, in an experimental study, it was reported that kohlrabi extract has an anti-proliferative and anti-oxidant effect in a dose-dependent manner on cancer cells [5]. As a consequence, plants such as kohlrabi that contain these secondary metabolites can be used as effective candidates in the processes of cell proliferation and apoptosis.

One of the multipotent stem cells that can differentiate into different cell types like osteoblast, adipocyte, chondrocyte, and myocyte are mesenchymal stem cells (MSCs) [8-10]. Ranera et al. evaluated hypoxic conditions on bone marrow-mesenchymal stem cells (BM-MSCs) and adipose-derived mesenchymal stem cells (AD-MSCs). The difference they observed in the prolif-

eration of BM-MSCs in the hypoxic and normoxic group and AD-MSCs showed variation in cell viability in the hypoxic group [11].

Hypoxia refers to a condition in which oxygen is depleted in cellular niches and this condition can cause several actions such as angiogenesis, innate immunity, metabolism, and stemness induction [12]. Hypoxia-inducible factor 1 α (HIF-1 α) controls the expression of many molecules associated with the cell cycle, including p21, anti-apoptotic factors, such as Bcl-2 [13], and pro-apoptotic proteins, such as p53 [14].

The study has shown that umbilical cord (UC)-derived MSCs adjust metabolism and energy consumption during hypoxia, and they not only reduce cellular injury but also increase UC-derived MSC growth and cell proliferation [15].

Even though stem cells are one of the resistant cells to hypoxia, it can arrest the cell cycle in mammalian cells. Simmons et al. showed that the overall cell number reduced in hypoxic condition [16]. On the other hand, studies showed that cells in normoxic condition are more differentiated than cells in hypoxic condition [17-19].

MSCs are suitable for transplantation due to their self-renewal characteristics and potential for the differentiation process [20, 21]. However, the results of other stud-

ies do not always support this issue. In Geng et al. study, less than 1% of MSCs injected into the left ventricle of CB17 SCID/beige adult mice remained within four days of injection [22]. Protection and surveillance of stem cells are crucial for many applications among cell therapy. Thus, it is necessary to find factors or methods that can affect MSCs' apoptosis.

Apoptosis is a crucial and sensitive action in multicellular organisms. Over- or under-expression of apoptosis genes can have a serious consequence [23, 24]. Therefore, the balance between cell proliferation or surveillance and cell death that determines cell fate is enrolled by apoptosis [25, 26]. *BAX* and *BCL2* genes determine the proceeding of apoptosis as its pivotal that keeps cells in balance.

Bcl-2 proteins are classified into two groups: pro-apoptotic and anti-apoptotic. *BAX* and *BCL2* are part of this classification, respectively. One of the most important mechanisms of apoptosis is the disrupted balance between pro-apoptotic and anti-apoptotic proteins [24]. Apoptosis starts when balance turns to *BAX* and block when the balance is in favor of Bcl-2.

To date, many studies have been done on the factors and methods that impress surveillance of MSCs (to different applications) but no study has been reported so far on the effect of kohlrabi extract on MSCs' viability and apoptosis. Thus, in the present study, we have assessed the effect of kohlrabi extract on MSCs' viability and apoptosis in hypoxic and normoxic conditions (Figure 1).

Materials and methods:

Human tissue

Fresh adipose tissue was obtained from three male patients who underwent liposuction and surgery. Patients provided their informed consent. All test processes performed separately on each sample. The Ethics Committee of Qom Azad University granted permission to collect the tissue (IR.IAU.QOM.REC.1398.022).

Isolation of MSCs

The adipose sample was obtained from subcutaneous adipose tissue and rinsed three times with phosphate-buffered saline (PBS) and then were divided and digested with 2 mg/mL collagenase type I (Sigma, USA) at 37°C for 45 min. Eventually, the suspensions were neutralized and samples were centrifuged at 1800 rpm (310 RCF) and cultured in DMEM medium (Bioidea, Iran)

supplemented with 10% fetal bovine serum (Bioidea, Iran), 100 U/mL penicillin/streptomycin (Bioidea, Iran) at 37°C, 5% CO₂, and 95% humidity. The cell culture medium was replaced every 3 days.

Verification of human MSC marker expression by flow cytometry method

CD44, CD90, and CD105 markers expression were investigated with the flow cytometry method.

Briefly, the cell's antibodies of the third passage were investigated by flow cytometry. The cells were suspended in 2 × 10⁵/100 μL PBS and incubated at 4°C for 30 min with FITC-labelled antibodies against CD34, CD44, CD90, and CD105 markers. Flow cytometric method was carried out utilizing a FACSCalibur (BD Biosciences) flow cytometer. The obtained data were analyzed by utilizing the WinMDI 2.9.

Kohlrabi extraction procedure

About 100 g well-dried seed and leaf were ground completely, mixed with ethanol 96% as equal as dried material volume and kept at room temperature for 24 h. After passing through filter paper, evaporation was done with rotary to obtain dry and purified samples for downstream applications.

Proliferation assessment

Cell proliferation was evaluated by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as Hayon et al. previously reported [27]. Cells at 80% confluency were collected by trypsinization and cultured for one day in a 96 multi-well plate in the density of 10⁴ cells per well before treatment. To get the optimum incubation time and volume of extraction, the concentration gradient was utilized in 24, 48, and 72 h. In brief, 10 μL of 5 mg/mL MTT solution (PBS+MTT) was added into a 90-μL cultured medium and allowed to incubate at 37°C for 3 h. About 100 μL of DMSO was added into the medium and absorbance was evaluated at 570 nm and 630 nm until 10 min. The process was repeated in three independent times. Moreover, hypoxic condition on cells induced by CoCl₂ based on Majed et al. study [28].

Real time-PCR for apoptosis genes expression

The apoptosis gene was evaluated in vitro after the cells reached the third passage under hypoxic condition. The cells were harvested by trypsinization and total RNA was isolated from cells when cell number reached 10⁴

in each well in various conditions, for three independent repeats using GeneALL kit according to the manufacturer's instruction (Dongnam-ro, Songpa-gu, Seoul, Korea). RNA samples (1 µg) were converted into complementary DNA (cDNA) with a random hexamer primer and reverse transcriptase (GeneALL, Korea) following the manufacturer's protocol (5 min at 25°C, 55 min at 55°C, 5 min at 95°C).

qRT-PCR was carried out by the Applied Biosystems StepOne Plus (ABi) Real-Time System. All reactions were conducted using the SYBR Green qPCR master mix according to the manufacturer's instructions. Reactions were performed in 3 discrete times using 10 µL of the master mix, 1 µM of each primer, 2 µL of cDNA and DNase-free water up to 20 µL volume.

The PCR amplification was performed as follows: 1 cycle of 10 min at 95°C, 40 cycles of denaturation for 15 s at 95°C, annealing for 40 s at 57°C, and elongation for 30 s at 72°C. Amplification was performed during the 65°C-95°C melt. Each gene primer reaction was led to obtaining only one product as a melt curve assessment of qPCR samples.

The qPCR primers of kohlrabi were designed by Oligo 5 and listed in Table 1. GAPDH was used as a reference gene for internal standardization of real-time PCR data.

Statistical analysis of data

Viability and apoptosis gene expression of cells in different conditions were evaluated by t-test in REST software. Results were regarded as significant with a P-value <0.05. The standard deviation of the mean was displayed by the error bars.

Results

MSC characterization

As reported in Figure 2, AD-MSCs identified positive expression for CD90, CD44, and negative expression for CD34 in the third passage.

Confirm multipotent

Also, to verify multipotent human adipose-derived mesenchymal stem cells, the differentiation potential of hAD-MSCs into tri-lineages, including the chondrogenic, osteogenic, and adipogenic lineage was assessed by the real-time PCR procedure. In differentiated hAD-MSCs into adipocyte lineage, the PPAR γ gene expression was significantly higher ($P=0.027$; t-test) than that of undifferentiated hAD-MSCs as control (Figure 2). We also assessed the expression of collagen type II in differentiated hAD-MSCs into chondrocyte ($P=0.032$; t-test) (Figure 2). In the differentiated hAD-MSCs into osteocyte lineage, expression levels of alkaline phosphatase were significantly higher than undifferentiated hAD-MSCs ($P=0.018$; t-test) (Figure 3).

MTT assay

The viability of hAD-MSCs treated with kohlrabi extract for 24, 48, and 72 hours were assessed by the MTT assay. Cell viability in group 3 was on average higher than other groups at 72 h. In various times and concentrations, it was shown that MSCs treating by kohlrabi seed and leaf extract in 100 mg/mL has maximum efficiency. As mentioned above, more efficient concentration was selected to further experiment. It should be noted that the maximum viability score belongs to 50 mg/mL at 48 h (Table 3).

Table 1. Real-time PCR primer sequences

Gene	Sequence 5'-3'
<i>BAX</i> Forward	CGGCAACTTCAACTGGGG
<i>BAX</i> Reverse	TCCAGCCCAACAGCCG
<i>BCL</i> Forward	GGTGCCGGTTCAGGTACTCA
<i>BCL</i> Reverse	TTGTGGCCTTCTTGAGTTCG
<i>GAPDH</i> Forward	TGGCTACAGCAACAGGGTG
<i>GAPDH</i> Reverse	CTCTTGCTCTTGCTGGG

Table 2. *BAX* and *BCL2* gene expression

Gene	Extract Source	Control (Non-hypoxic Condition without Extract Treatment)	Normoxic Condition with Extract Treatment	P	Hypoxic Condition without Extract Treatment	P	Hypoxic Condition without Extract Treatment	P
<i>BCL2</i>	Leaf	1	9.6	0.092	3.45	0.074	9.31	0.019
	Seed	1	0.59	0.651	3.75	0.081	1.44	0.649
<i>BAX</i>	Leaf	1	0.35	0.081	0.91	0.93	0.445	0.052
	Seed	1	1.74	0.514	0.94	0.91	0.53	0.421
<i>BCL2/BAX</i>	Leaf	1	27.42	-	3.79	-	21.15	20.92
	Seed	1	0.33	-	3.99	-	2.71	2.72

Just leaf extract has a significant difference in hypoxic condition.



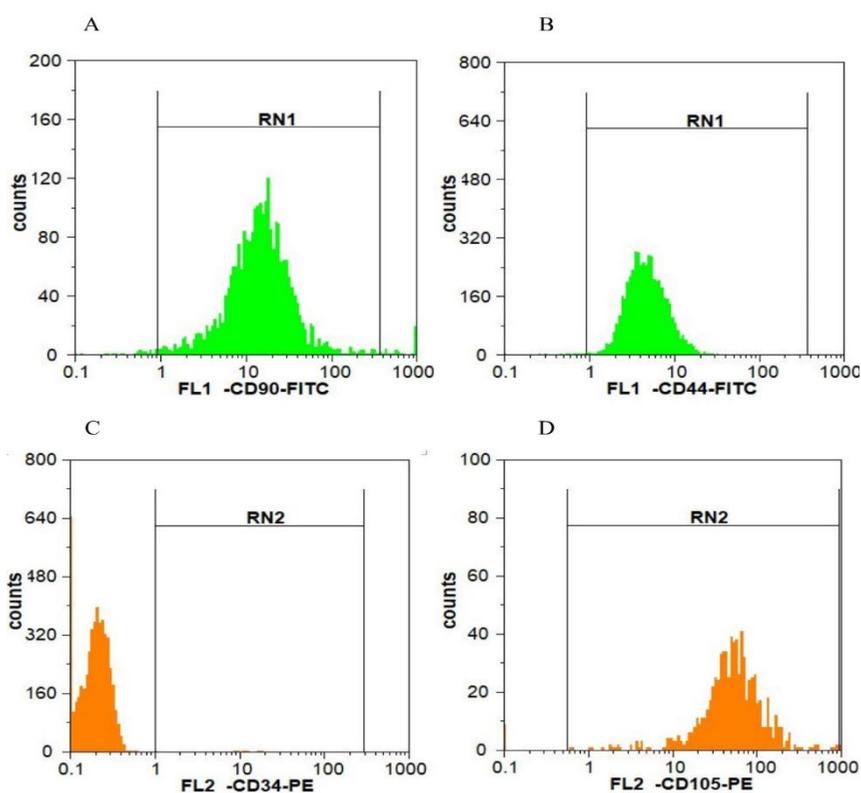
Transcriptional analysis of *BCL2* and *BAX* gene in MSCs

BCL2 and *BAX* as apoptotic genes have a difference in expression after MSCs treating by kohlrabi seed and leaf extract. However, most of these differences were not significant but the *BCL2* gene in MSCs after treating by leaf extract and hypoxic condition showed a significant increase in expression ($P=0.019$). Also, *BCL2/BAX* showed that cells tend to express *BCL2* to apoptosis prevention in comparison with *BAX*. *BCL2* and *BAX* gene

expression in normoxic and hypoxic conditions is shown in Figures 4, 5 and Table 2.

Discussion

Hypoxia is known as one of the pivotal environmental factors that can affect cells in the areas of cell viability, proliferation capacity, migration pattern, differentiation, and metabolism. A low oxygen concentration in hypoxic condition can lead to the production of reactive oxygen

**Figure 2.** Characterization of human MSC

A, B, and D are related to CD90, CD44, and CD 105, respectively that were positive; C is related to CD34 that was negative; CD90, CD44 and CD105 are MSC markers



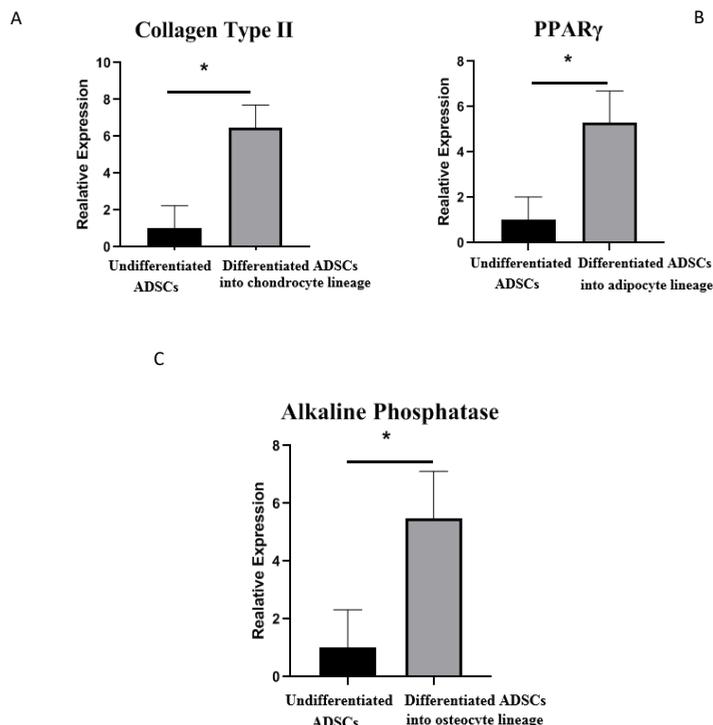


Figure 3. mRNA expression of chondrogenic (A), adipogenic (B), and osteogenic (C)

Markers assessed after two weeks by real-time PCR analysis in ADSCs to confirm their capacity for chondrogenic, adipogenic, and osteogenic differentiation.

species, which are responsible for the improvement of cell damage [29, 30].

Ejtehadifar et al. reported that hypoxia preconditioning can affect the cell survival and genetic instability of MSCs and offer new expectations to ameliorate poor engraftment after transplantation [31].

Bassan et al. evaluated the effect of Brassica juncea extract on cancer cell viability and concluded that there was a direct relation between extraction dose and cancer cell inhibition. So cell viability was high in low dose concentration compared with the high dose concentration [32]. It should be noted that in our study, MSCs were evaluated.

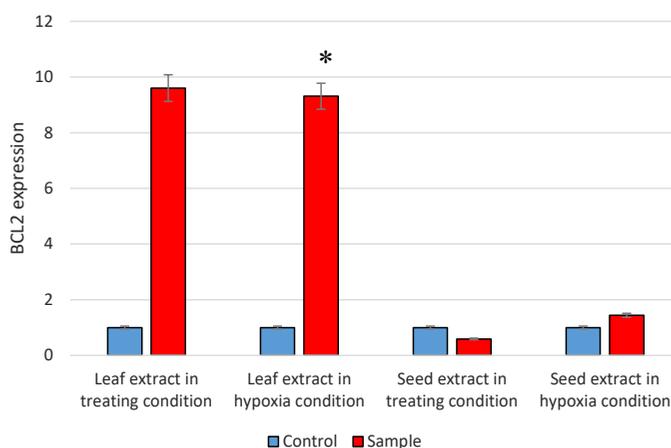


Figure 4. BCL2 gene expression that treated by kohlrabi leaf and seed extract, leaf extract showing a significant difference in hypoxic condition

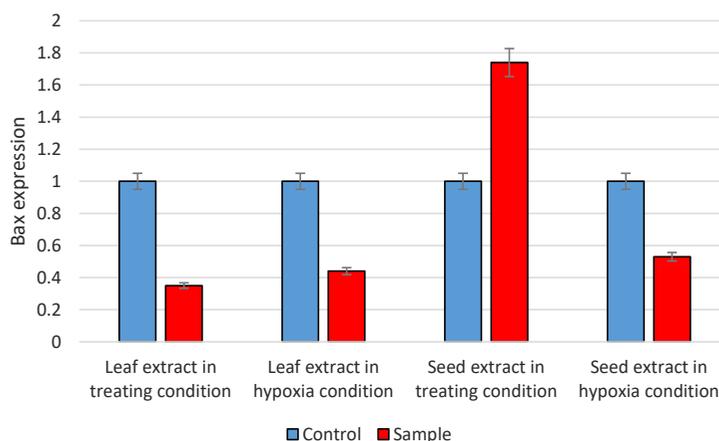


Figure 5. BAX gene expression treated by kohlrabi leaf and seed extract



Table 3. Comparison of MSCs viability in treating by leaf and seed extract, generally, the more efficient dose to viability was 100 mg/mL

Time	Extract Source	Control (Non-hypoxic Condition without Extract Treatment)	Normoxic Condition with Extract Treatment	Hypoxic Condition without Extract Treatment	Hypoxic Condition without Extract Treatment
24 h	Leaf	100	78	65	70
	Seed	100	62	60	60
48 h	Leaf	100	102	55	75
	Seed	100	95	45	70
72 h	Leaf	100	110	45	80
	Seed	100	105	40	75



Results of Yang et al. study showed that different concentration of cabbage from 10 µg/mL to 500 µg/mL did not significantly different, but in 500 µg/mL to 2000 µg/mL concentration, the cell viability reduced [33]. In our study, cell viability generally improved with increasing dose concentration. Leaf extract increased the cell viability according to dose and time gradient. However, the optimal dose was 100 mg/mL. In Basciano et al. study, cell viability increased from the second passage onwards [34]. The results of our cell viability agree with those reported by Basciano et al. [34].

In the current study, the isolated AD-MSCs displayed a high expression level of markers related to MSCs, including CD44, CD90, and CD105. Our flow cytometry results are in consistent with that reported by Sheykhasan et al. (2015) that freshly isolated AD-MSCs high-level express several surface MSC markers, including CD44, CD90, and CD105 [35].

Evaluation of the antiapoptotic effect of kohlrabi extract was comparable with Yang et al. study. In this study,

BCL2 expression was significantly increased in cells treated by cabbage extract [33].

The relative mRNA levels of the *BCL2* and *BAX* have not shown a significant difference between leaf and seed extract in normal and hypoxic conditions except in *BCL2* expression of leaf extract in hypoxic condition (Table 2). *BCL2* expression after treating by leaf extract in the hypoxic group significantly increased, so that leaf extract can inhibit the apoptosis, and shifts cells to survive. Moreover, we also determined the ratio of *BCL2/BAX* that turns to *BCL2* and apoptosis prevention in this study.

Kiani et al. showed that *Brassica oleracea* extract could increase the proportion of *BAX/BCL2* and induce apoptosis in cancer cells [36]. It seems that different varieties of Brassica have various effects on cells, which can this effects vary between MSCs and cancer.

Regarding the lack of any similar study, we discussed our results with others that studied other varieties of the Brassica family. Despite the paucity of studies on kohlrabi

rabi extraction and MSCs viability, the present study showed that kohlrabi extraction can improve cell viability and this claim was confirmed by evaluation of *BCL2* and *BAX* expression.

Conclusion

So far, no study has been performed on the effect of kohlrabi extract in MSCs and this study sheds light on the effect of kohlrabi extract on MSCs viability. Our study illustrates that kohlrabi extract may have positive effects on cell survival while having inhibitory effects on apoptosis.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article. The participants were informed about the purpose of the research and its implementation stages; they were also assured about the confidentiality of their information; moreover, they were free to leave the study whenever they wished, and if desired, the research results would be available to them.

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Authors' contribution's

All authors were equally contributed in preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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