

Screening of Feijoa (*Acca Sellowiana* (O. Berg) Burret) Fruit Effect on Proliferation and Apoptosis using Bone Marrow derived Stem Cells Model

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ABSTRACT

Background and Objective: Curative properties of Feijoa sellowiana Bert. (Myrtaceae, *Acca sellowiana*), have been often claimed, while investigations into details of its bioactivities are still lacking. This study investigated the effect of acetonic extract of Feijoa (*Acca sellowiana* (O. Berg) Burret) fruit on proliferation and apoptosis of bone marrow derived stem cells.

Methodology: Human bone marrow stem cells (hBMSCs) were characterized before experiments. MTT Assay was performed to determine the toxicity of Feijoa. The growth kinetics was also investigated until 7 days. Real time PCR assessed the expression of apoptosis genes.

Results: In MTT assay, Feijoa at doses ≤ 5 ng/ml did not show any cytotoxic effect on hBMSCs and increased the cell proliferation until day 4th followed by a decrease until day 7th. Population doubling time (PDT) decreased until day 4th followed by an increase until day 7th. A significant increase in expression of Bax and decrease Bcl-2 expression were recorded on day 7th.

Conclusion: The antioxidant, anti-inflammatory and modulatory activities of Feijoa can explain the increasing effect on cell proliferation till day 4th, but the apoptotic activity of Feijoa noted after four days is reported for the first time denoting to the short term antioxidant, anti-inflammatory and modulatory properties of Feijoa that should be considered for curative activity of this fruit.

Keywords: acca sellowiana, feijoa, bone marrow stem cells, proliferation, apoptosis

INTRODUCTION

Acca sellowiana (O. Berg, Burret) known as Feijoa is a subtropical species of *Myrtaceae* family with medicinal properties that are cultivated in many countries including Iran (1-3). It has several varieties such as apollo, coolidge, gemini, mammoth, moore, and triumph with difference in size, maturation and quality of the fruit. The fruit is oval and has a size of 4-8 cm with a bright waxy green skin and a dense, granular, light, honey-color, juicy and sweet pulp containing translucent seeds (4). The fruit is characterized by high amount of pectin and is used in manufacturing of many confectionery products, such as ice cream, sorbet, etc. (5).

It has essential minerals (e.g., potassium, calcium, magnesium, phosphorus, iodine), vitamins (e.g., vitamin C), terpenes, tannins, steroidal saponins, dietary fibers, flavonoids (e.g., catechin, flavone, quercetin-glycoside, procyanidin B1

and B2), phenolic acids, and polyphenols (6). These components are not only found in the pulp, but also are identified in other parts of the plant such as leaf, peel and flower bud (4). In addition, this fruit is a source of aroma and volatile compounds such as ethyl butanoate, ethyl benzoate and methyl benzoate giving Feijoa a 'unique' flavour profile (4). Whilst information about the health benefits reported from *in vivo* studies is relatively limited, *in vitro* studies have shown a wide-range of biological properties such as antimicrobial, antifungal, and anticancer activities for the Feijoa plant (7-9). It has anti-inflammatory property through TLR2 and NOD2 pathways with significant lower secreted embryonic alkaline phosphatase IC50 concentrations (2,4).

It also has therapeutic properties for solid and hematological cancers inducing apoptosis in cancer cells by caspase activation, p16, p21 and TRAIL overexpression, increasing the histone and non-histone acetylation and inhibition in HDAC pathway (8,10). The positive *in vitro* effect of

Feijoa on cell viability and proliferation, membrane peroxidation, and disaccharidase in Caco-2 and HT-29 cells was reported before (11). Cellular apoptosis following substance use was previously reported showing an increase in Bax gene and death signaling cascade (12,13).

As Feijoa fruit is popular and cultivated easily in several regions for consumption and processing, the present study was undertaken to determine the effect of acetonic extract of Feijoa fruit (*A. sellowiana*) on proliferation and apoptosis using human bone marrow derived stem cells (hBMSCs) as a screening model.

METHODOLOGY

Totally, 100 g of Feijoa fruit was identified and donated by Ramsar Research Center, northern Iran and was used in this study. The study was approved by Yasooj Branch of Islamic Azad University Ethics Committee in Yasuj, Iran (IAUYB-1396 in February 2018). Using a percolator machine as described before (8), the acetonic extract of the fruit was prepared. Briefly, Feijoa fruit was subjected to 0.8% Triton X-100 solution to remove epiphytic hosts that were normally present on the fruit surface. After several washings in distilled water, the fruit was allowed to be dried on a filter paper and was later extracted in a liquefied blender using acetone till being homogenized. The acetonic extract was later centrifuged at 2800 g, while the supernatant was oven-dried at 45°C. Totally, 100 mg of the dry residues were dissolved in 10 ml of sterile physiological TRIS buffer (pH of 7.4) until used.

Bone marrow (BM) samples were donated by the Bone Marrow Transplantation Center, Shiraz University of Medical Sciences, Shiraz, Iran with the signed consent forms from the patients. BM was diluted with an equal volume of Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) and carefully layered over an equal volume of Ficoll-Paque product (density 1.077 g/mL, Life Sciences, Austria). The interface between the plasma and the Ficoll-Paque layer was centrifuged at 1500 g for 20 minutes to collect mononuclear cells that were later seeded in 75 cm culture flasks containing 15 mL of DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, Germany) and 1% penicillin-streptomycin (Gibco, Germany). It was later transferred in 5% CO₂ incubator at 37°C and saturated humidity. The media change was undertaken every 3 days and subculturing of cells was carried out at 80% confluence. Cells were treated with 0.25% (w/v) trypsin-EDTA (Gibco, USA) until third passage.

The cells were characterized before experiments by morphological assessment under an inverted microscope (Nikon, Japan). Images were provided from cells using a digital camera (Olympus, Japan). Also, cell characterization was performed by *in vitro* differentiation into osteogenic and adipogenic lineages and by flowcytometry too as shown previously (14).

For osteogenic induction, the hBMSCs were transferred into 6-well plates until 80% confluence. The medium was changed with osteogenic medium containing DMEM supplemented with 10% FBS, 100 nM dexamethasone (Sigma, USA), 50 µM ascorbic acid (Merck, Germany), and 10 mM glycerol 3-phosphate (Merck, Germany) for 3 weeks. The media change happened every 2 days and after 21 days, hBMSCs were fixed in 10% formalin for 20 min and following 3x washings with deionized water, they were stained with fresh 1.4% Alizarin Red

solution (Sigma, USA) dissolved in deionized water at pH of 4.1 and were assessed for presence of calcium deposits in red color.

For adipogenic differentiation, the hBMSCs were seeded in 6-well plates containing complete culture medium till 70% confluence. The medium change to adipogenic medium was undertaken with DMEM containing 10% FBS, 100 nM dexamethasone, 100 µM ascorbic acid, and 200 µM of indomethacin (Sigma, USA) for 21 days. The cells were fixed in 10% formalin for 20 min and after three times washing with deionized water, they were stained with fresh 0.5% Oil Red-O (Sigma, USA) dissolved in 2-propanol solution (Merck, Germany) for 2 h. The cells were evaluated for presence of lipid droplets in red color.

Flowcytometry was performed to investigate the presence and expression of mesenchymal markers including CD73 and CD90. Flowcytometry also evaluated absence of hematopoietic markers including CD34, and CD45.

MTT assay was conducted to detect the influence of different doses of Feijoa (5, 50, 500, 5,000, 50,000, 500,000, 5,000,000 ng/mL) on proliferation of hBMSCs. A total of 5,000 cells/200 µl of media were transferred into 96-well plates, while the media was replaced after 24 h with just the culture media (control group), or with Feijoa added to the culture media (experimental group: 5, 50, 500, 5,000, 50,000, 500,000, 5,000,000 ng/mL of Feijoa). After 24 h, 20 µL of a solution containing 5 mg/mL of dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT: Sigma-Aldrich, USA) was added to each well and was incubated at CO₂ incubator at 37°C and saturated humidity for 4 h. The media was gently removed. To dissolve the formazan crystals, 200 µL of DMSO/well (Merck, Germany) was later added.

The cell viability was calculated at optical density of 570 nm on a microplate reader (Floustar Omega, BMG LabTech, Germany) (n=4) using the following formula (15), while Ac and Ab were considered as the absorbance in the control and blank wells: %Survival rate=A sample-(Ab×Ac-Ab)×100.

The growth kinetic of hBMSCs in control and Feijoa groups was determined by trypan blue exclusion test until 7 days, while the cells were cultured in 24-well plates (22,000 cells/well). Totally, 0.4% trypan blue solution (Sigma, USA) was mixed with the cell suspension for cell count. The cells were observed under a phase contrast microscope (Olympus, FSX100) using a Neubauer hemocytometer slide. The PDT was determined using the following formula:

$$PDT = T \times \frac{\ln 2}{\ln \frac{X_e}{X_b}} N = \frac{n_1 + n_2 + n_3 + n_4}{4} \times 2 \times v \times 1000, \text{ while } T \text{ was}$$

the incubation time in h, Xb was the cell number at the beginning of the incubation time and Xe was the cell number at the end of the incubation time, Ln=log_e, v the media volume, n1-n4 the frequency of counts and e=the Euler's number (13).

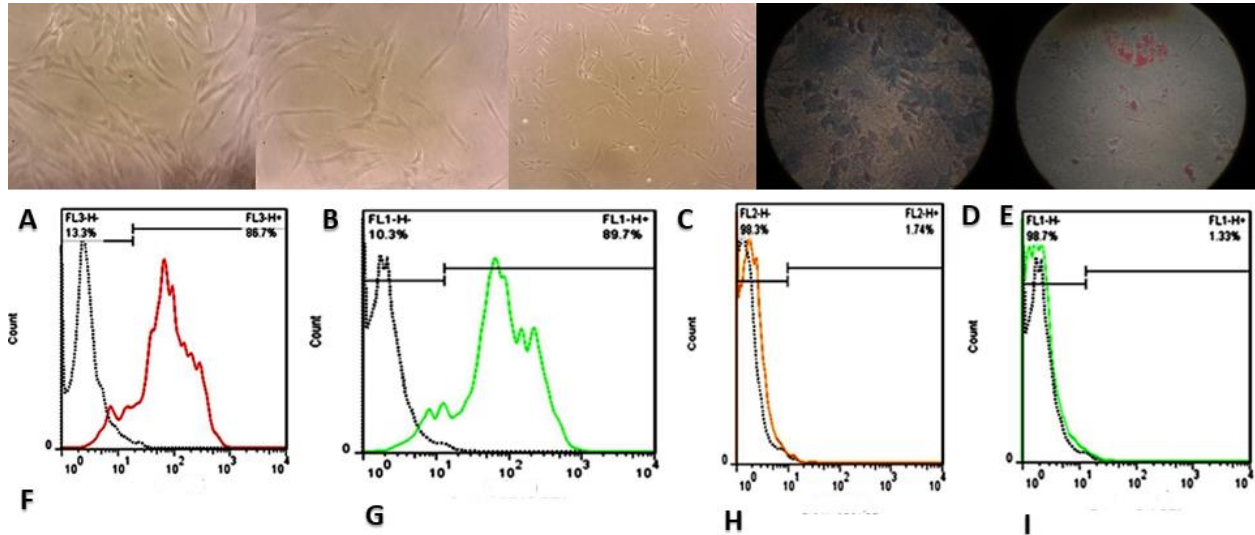
To quantify Bax and Bcl-2 expressions, hBMSCs were harvested for total cellular RNA extraction after subjecting the cells with Feijoa until 7 days using an RNA extraction kit (Cinna Gen Inc., Tehran, Iran). The quality and quantity of recovered RNA were assessed by determining the ratio of optical density (A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀) using Nanodrop™ spectrophotometer (Nanodrop; Thermo Fisher Scientific Inc., USA). The cDNA synthesis was undertaken by 1,000 ng total RNA in a first-strand cDNA synthesis reaction using Revert Aid™ first strand cDNA synthesis kit (Thermo Fisher Scientific Inc., USA).

The Bax and Bcl-2 genes were used as targets and TBP as an endogenous control. The sequences of interest genes were

Table 1. The specific primer sequences of the targeted genes

Genes	Primer sequences	Size (bp)
BAX	Forward: 5'- GCCCTTTTGGTTCAGGGTTTCA -3' Reverse: 5'- CAGCTTCTTGGTGACGCAT -3'	108
Bcl-2	Forward: 5'- ACGAGTGGGATCGGGAGATGTG-3' Reverse: 5'- GCGGTAGCGCGGGGAGAAGTC-3'	245
TBP	Forward: 5'- GGATAAGAGAGCCACGAACCAC-3' Reverse: 5'- TTAGCTGAAAACCAACTTCTG-3'	139

Bp: base pair

**Figure 1.** hBMSCs in Passage 1 (A, x100), Passage 2 (B, x100), and Passage 3 (C, x40). Osteogenic induction (D), and adipogenic differentiation (E). Flow cytometry was positive for mesenchymal markers of CD73 (F) and CD90 (G) and negative for hematopoietic markers of CD34 (H) and CD45 (I)

obtained from the NCBI database and primer sets were designed by primer 3 software as shown in **Table 1**. Real-time PCR was carried out by SYBR Green I as reporter dye and Step One Real Time PCR reactions (Applied Biosystems, USA). In every reaction, 200 nM of each primer was added to target the specific sequence. The PCR condition was set at 94°C for 10 min followed by 40 cycles at 94°C for 15 sec, at 60°C for 60 sec and melting curve analysis ramping of 65-95°C.

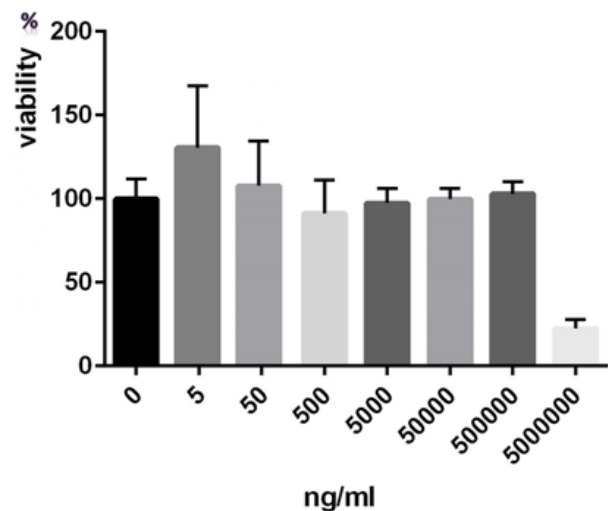
The amplification signals of different samples were normalized to TBP cycle threshold (Ct), and then $2^{-\Delta\Delta Ct}$ method was applied for comparing mRNA levels of underlying groups, which represented as fold-change in data analysis (16). Data were analyzed by independent samples t test, using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). The $p < 0.05$ was considered statistically significant.

RESULTS

Characterization of hBMSCs was undertaken by morphologic investigation revealing that cells at all passages were adherent to the culture plates and were fibroblast like and spindle-shape (**Figure 1A-C**).

The cells in osteogenic media were characterized by Alizarin Red after 21 days denoting to the presence of calcium deposits in red color (**Figure 1D**).

Cell characterization in adipogenic media after 21 days and staining with Oil Red O demonstrated the presence of intracellular lipid droplets in red color (**Figure 1E**).

**Figure 2.** MTT assay of different concentrations of Feijoa after 4 days regarding the cell viability

Final characterization of hBMSCs was undertaken by flowcytometry revealing positive expression and presence of CD73 (**Figure 1F**) and CD90 (**Figure 1G**) and negative expression and absence of CD34 (**Figure 1H**) and CD45 (**Figure 1I**).

MTT assay revealed the least toxicity and apoptosis and the highest viability and proliferation capacity at concentration of ≤ 5 ng/mL of Feijoa until 4 days of treatment of hBMSCs to the Feijoa fruit (**Figure 2**).

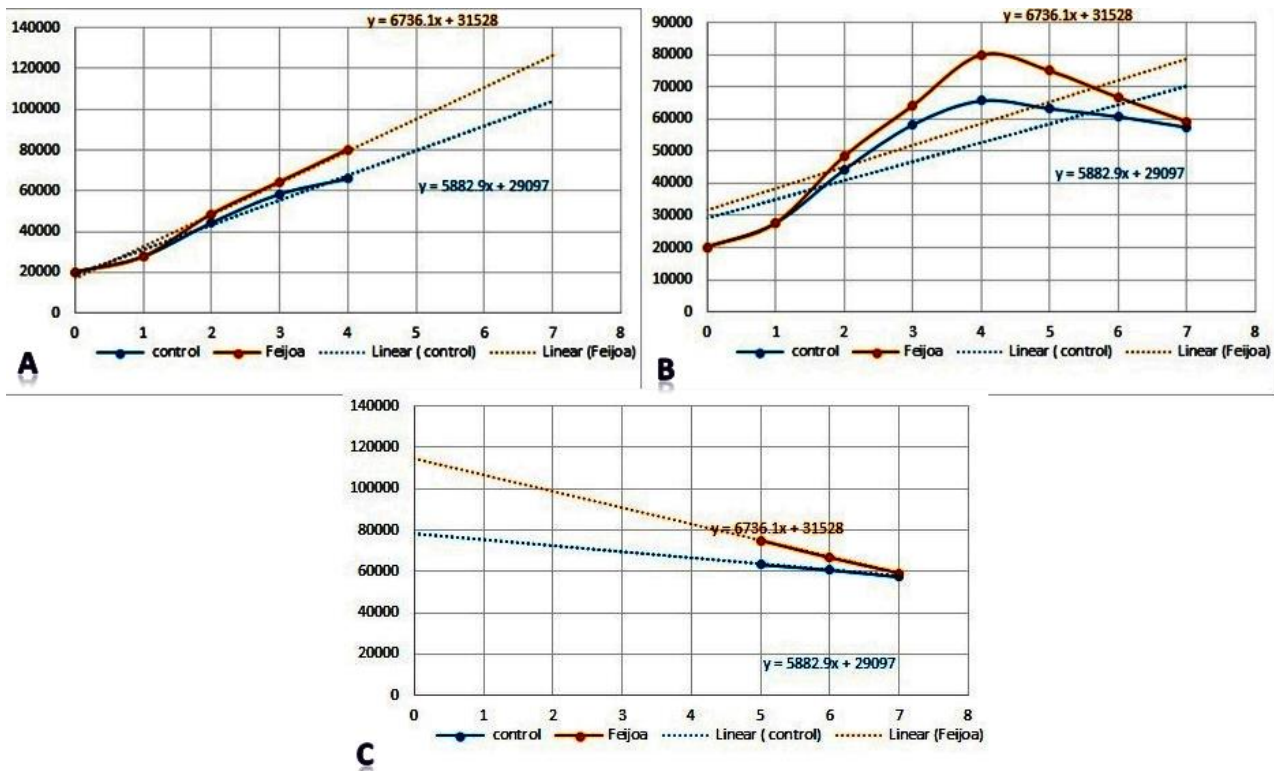


Figure 3. Growth kinetics of cells treated with 5 ng/ml of Feijoa in comparison to the control group plotted for seven days

Table 2. PDT of control and Feijoa group

Variable	Population doubling time (n)	
	Control	Feijoa (5 ng/ml)
Day		
0	0	0
1	21	19
2	31	29
3	42	36
4	58	50
5	75	76
6	94	96
7	125	128

When plotting the growth curve of hBMSCs exposed to 5 ng/mL of Feijoa fruit, an increasing trend in cell proliferation was noted till day 4th, followed by a decrease in cell proliferation after seven days, when compared to the control

group, and the difference between groups was not statistically significant (**Figure 3**). When Feijoa at the dose of 5 ng/mL was added to the culture media, a decrease in PDT of hBMSCs happened until day 4th, followed by an increase in PDT after seven days in comparison to the control group, and the difference was not statistically significant (**Table 2**).

Real time PCR revealed an increase in expression of Bax gene, when hBMSCs were treated with 5 ng/mL of Feijoa after seven days in comparison to the control group, and the difference was statistically significant ($p \leq 0.01$, **Figure 4A**). The expression of Bcl-2 gene in Feijoa-treated cells (5 ng/mL) decreased after seven days, when compared to the control group and the difference was statistically significant ($p \leq 0.01$, **Figure 4B**). The ratio of Bax to Bcl-2 (Bax/Bcl-2) for treated cells with Feijoa demonstrated a rise after seven days, when compared to the control group (**Figure 4C**).

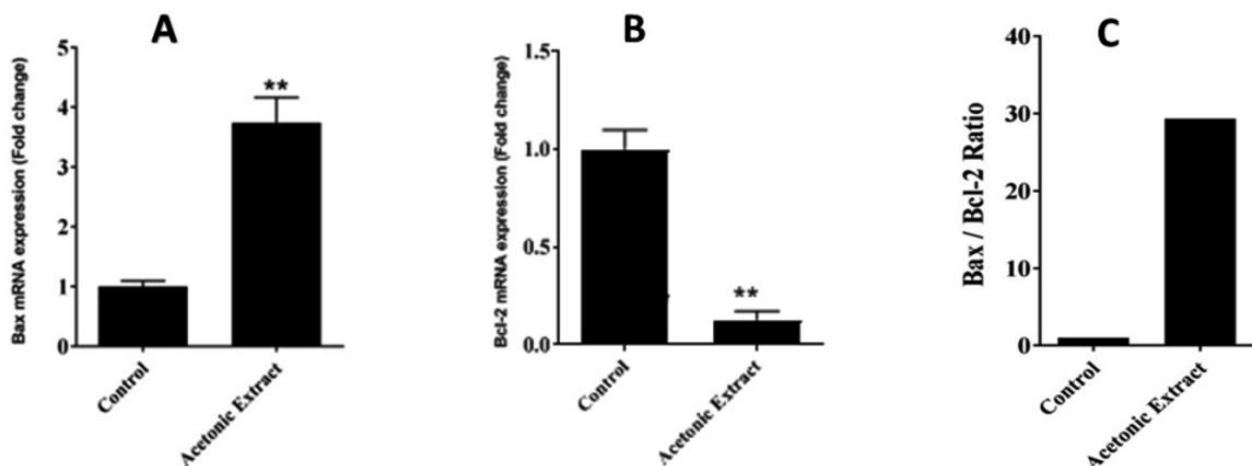


Figure 4. Expression of Bax (A) and Bcl-2 (B) genes and their ratio (C), when hBMSCs were treated with 5 ng/mL of Feijoa in comparison to the control group (** $p < 0.01$)

DISCUSSION

In our research identical to other studies, hBMSCs had mesenchymal properties confirmed morphologically, by osteogenic and adipogenic induction and by flowcytometry (14,17). Feijoa fruit used in this study is commonly consumed as fresh fruit and is also commercially available as processed product in several foods, in ice cream, yoghurt and jam too (10). There is a general agreement that Feijoa fruit has health-promoting effects due to presence of bioactive constituents in the fruit (18).

Our findings demonstrated an increasing trend in cell proliferation until day 4th. The increasing trend in proliferation of hBMSCs during the first 4 days of our study can be related to antioxidant, anti-inflammatory and modulating activities of the Feijoa (4,11,19,20) due to presence of several essential minerals, vitamins, phenolic acids, and polyphenols in the fruit (6). These properties made the fruit to have nutritional value to protect human red blood cells from mercury-induced cellular toxicity (21).

To explain this decreasing effect on proliferation of hBMSCs after seven days can be due to anticancer property of Feijoa (22) and an improvement in the lactase and sucrase-isomaltase activities and the inhibitory effects of Feijoa on proliferation of cancer cells (8,23). Bontempo et al. showed that the flavone content in the fruit demonstrates inhibitory activities on deacetylase enzymes thus hyper-acetylates histones and non-histone targets in leukemia cell lines and patients blasts confirming the anti-cancer potential of Feijoa. They believed that normal myeloid CD34⁺ progenitors in culture are not affected by Feijoa, thus excluding adverse side effects for normal progenitor cells (8). They illustrated that Feijoa acetic extract was not toxic and displayed cancer-cell selectivity. We showed that Feijoa increased the proliferation of hBMSCs until day 4th and there was a decrease in cell proliferation after seven days.

Our findings revealed that Feijoa at the dose of ≤ 5 ng/mL significantly increased the proliferation of hBMSCs. The positive *in vitro* effect of Feijoa on cell viability and proliferation, membrane peroxidation, and disaccharidase in Caco-2 and HT-29 cells was reported before (11). Cellular apoptosis following substance use was previously reported showing an increase in Bax gene and death signaling cascade too (12,13).

Our findings revealed an increase in expression of Bax and a decrease in Bcl-2 genes when hBMSCs were treated with 5 ng/mL of Feijoa until day 7th in comparison to the control group illustrating that Bax and Bcl-2 play important roles in apoptosis and cell proliferation (8,24-27). Bcl-2 was shown to have two classes of pro-apoptotic (Bax, Bad, Bid, Bik) and anti-apoptotic proteins (Bcl-2, Bcl-XL, Bcl-W). The Bax protein can trigger apoptosis by an increase in the opening of the mitochondrial voltage-dependent anion channels. It can induce a loss in the membrane potential too (24). The anti-apoptotic proteins can delay the mitochondrial release of cytochrome-c and the pro-apoptotic proteins can also activate such releases (28). The ratio of Bax to Bcl-2 denotes to the susceptibility of a cell to apoptosis (29). The p16, p21 and TRAIL were demonstrated as some of the molecular effectors of the pro-apoptotic Feijoa activity too (8). So, our results are in line with the literature (8,24-29).

CONCLUSION

The increase in proliferation and decrease in PDT of hBMSCs until day 4th while cells were subjected to Feijoa fruit were demonstrated to have an important role in regenerative medicine, particularly when targeting Feijoa for cell transplantation purposes during a shorter time interval.

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