



# The effect of hydroalcoholic extract of *Cannabis Sativa* on morphology and growth of bone marrow mesenchymal stem cells in rat

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## ABSTRACT

**Background:** One of the most important issues in cellular therapies is the examination of stem cells in different conditions. Given the increasing use of herbal drugs and herbal medicines and the potential effects of herbs on various diseases, the study of stem cells in the mediums of these compounds seems to be necessary.

**Aims:** The current paper was to evaluate the effect of cannabis sativa extract on morphology, growth and maintenance of the characteristics of rat bone marrow stem cells.

**Methods:** In this study, rat bone marrow mesenchymal stem cells were used. The effect of cannabis extract in different concentrations on cell morphology was investigated through microscopic observations and cytogenetic methods. In the context of the study of bioavailability and growth process, we used the mtt assay test and calculated the cell population doubling time and the growth curve plotting.

**Results:** The results show that cannabis extract increases the proliferation of rat bone marrow mesenchymal stem cells compared to control group. Also, the effect of cannabis extract is dependent on its dose. The treated cells with a concentration of 100 ng / ml cannabis extract had the highest growth rate compared to control ( $P < .050$ ).

**Conclusion:** Given the increasing impact of low doses of cannabis extract on the growth of bone marrow mesenchymal stem cells, it seems that the use of effective ingredients in this plant extract is beneficial for cell-therapeutic purposes in autoimmune diseases. On the other hand, higher doses cause cell death and an increase in apoptosis, so, in people consuming these substances, they will decrease the proliferation of stem cells due to increased concentration of substances in the body.

**Keywords:** bone marrow mesenchymal stem cells, cannabis sativa, mitosis, cell morphology

## BACKGROUND

Hashish is cannabis resin. More than 4500 years have passed since the cultivation of this plant and still farmers use different parts of the plant for medicinal purposes. Mesenchymal stem cells play a significant role in restorative and cellular therapies due to the ability to proliferate and differentiate. According to research, the flexibility of these cells has been proven to differentiate into neural, lining, pulmonary, intestine, liver, kidney and spleen cells (6). Despite the importance of mesenchymal stem cells in cell therapy, some of the biological aspects associated with them, especially in vivo, are unknown. Today drug abuse is one of the major medical and social problems of the present time, which has become global. Statistics on addiction show that there are over one billion abusers around the world and this figure is on the rise. Different articles have been referred to various effects of cannabis such as sedation, anxiety, anticonvulsant, analgesic, appetite stimulation, antipyretic, antibacterial and diarrhea treatments, toothache, wound healing, nausea, vomiting and bloating (1). There are several reports of the use of this plant in the field of allergic and inflammatory and neurodegenerative diseases and apoptotic effects, but in all of these studies, the possible toxic effect of this material on

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stem cells is less widely considered. Given the medicinal uses of cannabis and its compounds on the one hand and its harmful effects on the other, as well as the importance of mesenchymal stem cells in the restoration of organs and its application in tissue transplantation and tissue engineering, this research aims at investigating the effect of extract of this plant on morphology and growth process of rat bone marrow mesenchymal stem cells.

## METHOD

The nature of the cannabis female flowers was received from Iran drug control headquarters and confirmed by the expert of the biosystematics center of Shiraz University of Medical Sciences. The cannabis female flowers were powdered and then poured 50 g of powder for 72 hours into 500 ml of 70% ethanol (Merck-Germany), in a percolator machine (Arian Equip-Iran), and after the time has elapsed the valve was opened to gain the extract. It should be noted that, through the detonator, ethanol was added from above to make the solution out of the percolator totally colorless. The solvent was evaporated by rotary device (IKA-Germany) at 45 ° C and 50 rpm. The result was a thick honey-like substance. The material obtained was dried in a desiccator (Pyrex-Germany) connected to a vacuum pump and transferred to a dark color pre-sterilized glass container. In this study, 5 adult two-month male rats with an approximate weight of 200-180 g were purchased from the experimental animals of the Shiraz University of Medical Sciences and transmitted to the Laboratory at Mohammad Rasul Allah Research Tower.

After calm killing of the animals, they were transferred to the tray underneath the laminar hood (JAL TAJHIZ-JTLVC2X-IRAN-), then the bones of the femur and tibia were separated and added to Falcon 15ml (BD-USA) containing PBS (Gibco-USA) in an ice tube.

By considering sterile conditions, the two ends of the bone were discontinued. Then, the bone marrow was washed with a syringe by the DMEM without FBS from the two ends of the bone. Centrifuge (Eppendorf Centrifuge 5702R-Germany) Intra-falcone cell suspension was performed at 1000 rpm at 20 ° C for 5 minutes.

The supernatant cell mass was transferred to a 1 ml of DMEM medium suspended in a 75ml (orang-USA) flask and cultured with 15 ml of DMEM medium containing 15% FBS (Biowest-USA).

The cells were then incubated at 37 ° C and 5% CO<sub>2</sub> (CO<sub>2</sub> Incubator-MEMMERT GERMANY).

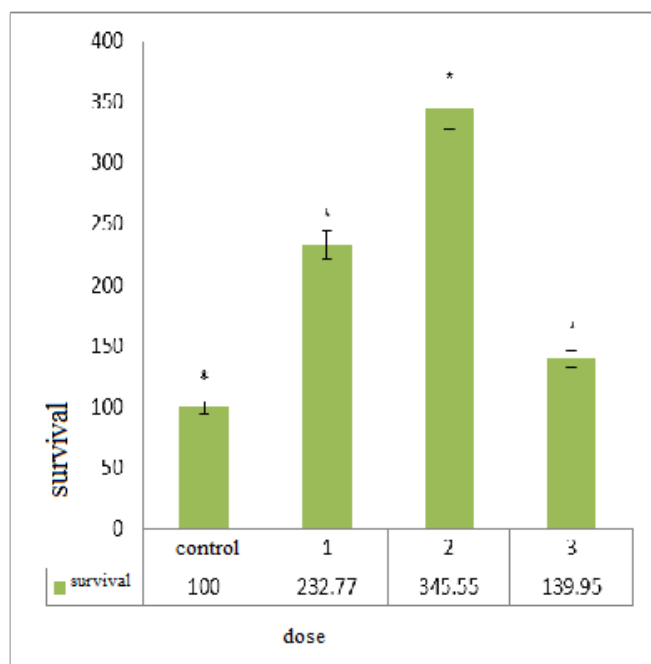
After the cells in the flask floor grew to their maximum, the process of passage was performed up to 4 steps.

To investigate the probable toxicity of cannabis extract in rat bone marrow stem cells, a 96-well (Invitrogen-USA) plate was cultured in a 100 µl cell suspension containing 5000 cells and then, 24 hours later, adding different concentrations of the extract (30-40-50-60-70-80-90-100-110-120-130-140-150-200-300-400-500-600-700-1000-2000 3000-4000-5000-6000 ng / ml) were evaluated for the experimental groups, cell viability and bioavailability of the cells.

The groups were: control, zero control and three experimental groups. The grouping pattern of the experimental groups was based on the number of doses and time, which included group 1: one treatment of cells with one of the concentrations and a test after 24 hours; group 2: one treatment of the cell with one of the concentrations and a test after 72 hours; group 3: three treatments of the cells with one of the concentrations at equal intervals and a test after 72 hours. For each 200 µl of the culture medium, 20 µl of MTT solution (Sigma-Aldrich-Germany) at a concentration of 5 mg / ml PBS was added to each well from a 96-well plate and incubated for four hours. After four hours, the medium on cells was removed and 200 µl DMSO (Sigma-Aldrich-Germany) was added to the wells to lysate the cells and 96 wells were wrapped in foil and centrifuged for 30 minutes. The OD was then recorded at 550 nm with a polarstar omega-device (BMG LABTECH-Germany).

The amount of cells in exposure to cannabis extract was not expressed by toxicity using MTT assay method. Concentrations in the same range were chosen, and the application of the concentration of 10000 ng / ml was carried out to investigate the toxic concentrations. Having added the cannabis extract to the culture medium of experimental groups, the flasks containing stem cells were compared with invert microscope (Nikon ECL IPSE TS100-GAPAN) in terms of cell size and shape.

Population doubling time of cells was calculated in order to monitor the rate of cell growth in laboratory conditions. To calculate the population doubling time of the cells (PDT) the fourth passage cell in a 24-well (Invitrogen-USA) plate with a cell density of 15,000 cells per well for 8 days, were counted each day under the microscope (2 wells per time) by neobar lam (HBG-Germany) after staining with Trypan Blue (Merck-Germany). The average cell was counted at any time and the population doubling time was calculated. Cells of two wells from each plate were counted daily. After the last count, the growth curve of treated and control groups with three different concentrations of cannabis extract was drawn according to the data obtained from the counting of different plates.



**Figure 1:** Comparison of the mean viability rate in different groups

Groups:

Control: cells without exposure at cannabis extract

1: cells receiving one of dose - test after 24 hours

2: cells receiving one of dose - test after 72 hours

3: cells receiving three doses - test after 72 hours

The current paper has used SPSS version 23 and ANOVA test and post-hoc tests to examine the differences in various aspects of the mtt test and the growth trend.

## RESULTS

One Way Anova Test results in different groups of (controls without exposure to cannabis extract and three experimental groups. 1: one treatment of cells with one of the concentrations and a test after 24 hours; group 2: one treatment of the cell with one of the concentrations and a test after 72 hours; group 3: three treatments of the cells with one of the concentrations at equal intervals and a test after 72 hours) with a significant level of fisher test is in the range of less than 0.001. Therefore, the difference between the mean of the group is confirmed ( $F = 52,283$  and  $P = 0.0001$ ).

Then, Scheffe's post hoc test was used to determine the differences between the groups. Thus, there are a significant difference among groups 1 and 2, 1 and 3, 2 and 3, 2 and control (at a significant level less than 0.001) and groups 1 and control (at a significant level less than 0.05). The mean cell viability rate among different groups is shown in **Figure 1**.

The adhesion property to the plastic container and the morphology of the control and treated with cannabis extract cells were checked on a daily basis using invert microscope. Cannabis extract at concentrations used did not affect the adhesion properties to the plastic container and cell morphology.

The Significance level of fisher test for control groups (without exposure to cannabis extract) and three experimental groups. 1: one treatment of cells with one of the concentrations and a test after 24 hours; group 2: one treatment of the cells with one of the concentrations and a test after 72 hours; group 3: three treatments of the cells with one of the concentrations at equal intervals and a test after 72 hours) is in the range of less than 0.001, so the difference between the groups is confirmed ( $F = 10.286$  and  $P = 0.0001$ ). Tukey's post hoc test was used to determine the difference between groups. Comparison of the mean pair of variables of the growth trend between the different groups indicates that there are significant differences among the control groups and C, B and A, A and C (at a significant level less than 0.001) and B and A (at the significant level less than 0.05), control group and C (at significant level less than 0.0001). The comparison of the mean and their order among the growth groups shows that the group C has the lowest score (10388.78) and the group A has the highest score (36759.33).



**Figure 2:** Microscopic Images of Multiple Cell Groups with a 10x Magnification

A: Controls including cells in a non-cannabis extract, B: 100 ng / ml cannabis extract treated cells, C: 1000 ng / ml cannabis extract treated cells D: 10000 ng / ml cannabis extract treated cells

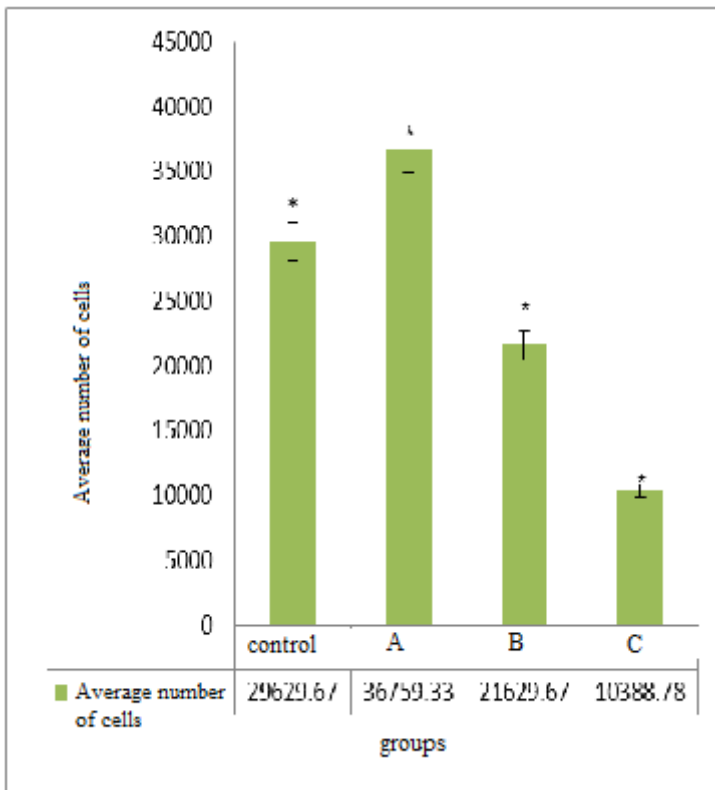
**Table 1:** pdt of mesenchymal bone marrow rat stem cell

	Control	100	1000	10000	
-1					
0					
1	27.4394	18.60782	23.80464	20.08534	
2	57.81382	40.40815	40.89519		pdt
3	55.82345	60.61222	-31.0021		
4	67.82832	60.5564	631.432		
5	119.9745	74.45199	789.29		
6	114.5943	84.05903	225.8592		
7	150.5762	99.50163	130.2547		

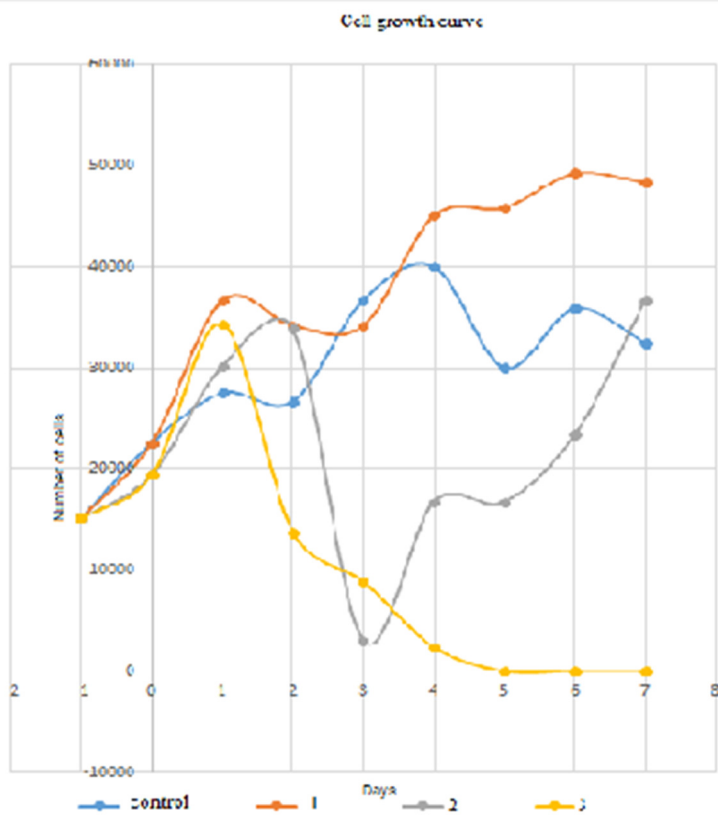
The calculated population doubling time of the control group was 27.5 hours since the first day, and the maximum number of cells was seen on day four. The cell population in of experimental group 1, which received 100 ng / ml of cannabis extract, doubled after 18.6 hours. For group 2 that received 1000 ng / ml cannabis extract, this time were 23.8 hours, and the cell population of the experimental group 3 receiving 10,000 ng / ml of cannabis extract doubled after 20 hours.

In all three experimental groups, the doubling time after the first day was shorter than control. According to the pdt table, these times for the second day are less in groups 1 and 2 compared to control, which indicate the rapid growth of cells in concentrations of 100 and 1000 ng / ml, but this is not true of group 3 because the number of cells is decreasing. On the last day of the study, the growth trend also saw a shorter doubling time for groups 1 and 2 than the control group.

Having plotted the growth curve, it was determined that exposure to cannabis in doses of 100, 1000, 10,000 ng / ml, first increased the number of cells, and with time, the number of cells decreased. The growth curve of the cell has been compared in normal and in applied doses in **Figure 3**.



**Figure 3:** The structure of the mean values of the number of cells among different groups  
 A: Controls including cells in a non-cannabis extract, B: 100 ng / ml cannabis extract treated cells, C: 1000 ng / ml cannabis extract treated cells D: 10000 ng / ml cannabis extract treated cells



**Figure 4:** Cell growth curve  
 A: Controls including cells in a non-cannabis extract- 1: 100 ng / ml cannabis extract treated cells- 2: 1000 ng / ml cannabis extract treated cells- 3: 10000 ng / ml cannabis extract treated cells

## DISCUSSION

The observation of the morphology of the cells in the control and treated groups by cannabis extract was confirmed by their mesenchymal form based on the fusiform and adhesion to the flask floor. The presence of cannabis extract in the applied concentrations failed to affect the adhesion property to Flask floor or cellular appearance, and these results are similar to those of previous studies on the proven mesenchymal stem cell properties (10, 14, 16).

The results of the statistical analysis of the mtt test numbers indicated that the mean cell viability rate was higher in the different groups of cells exposed to cannabis extract than control, so the compounds contained in the extract of this plant were at least with the values and times recorded in this study, had an increasing effect on the viability of cells

Cells that received a single dose of extract and tested after 72 hours had the highest mean of viability rates, Cells that were once exposed at one time and tested 72 hours hold in second and cells that were three times exposed to the cannabis extract at three times and tested after 72 hours hold the third place. Noteworthy, the greater percentage of viability in the cells of the three groups was mentioned compared to the control group.

The concentrations used to test the mtt were not toxic. Three concentrations were used to continue the tests, two of which were in the range of effective concentrations of mtt with one was more than that.

The cells were counted during their treatment, during which time, the process of stem cell proliferation was impaired and the cells could be investigated, such as mitosis, apoptosis and niche of cells in controlled environments.

Growth curve analysis shows that all cell groups are growing in the first day, and this increase is related to the treated group with the lowest dose of cannabis extract, the group treated with the highest dose of cannabis extract, the control group and finally the treated group with a moderate dose of cannabis extract, which this latter case is still in the ascending course. This effect is likely to be due to the antioxidant effect, anti-inflammatory properties, or the beneficial effects of cannabis extract along with growth factors, and this effect is also confirmed by the MTT result. The function of cannabinoid receptors, CB (1) and CB (2), can also be considered as components of a new regulatory pathway for the involvement of a cannabinoid system in the stem cell viability (2, 4, 8, 9, 15, 16, 19).

During the second day, the growth curve of all groups is decreasing and this course is irreversible for the treated group with the highest dose of cannabis extract, which indicates the high dose toxicity of the extract and the increase in apoptosis compared to mitosis. This is true for the three-dose group for three days in MTT. On the third day, the treated group with the highest dose of cannabis extract continued its downward trend, while the group treated with the moderate dose of cannabis extract was the lowest and control groups and treated group with the lowest dose of cannabis extract are ascending. The fact that delta-9-tetrahydrocannabinol causes a decrease in cellular metabolism and induction of apoptosis has been investigated several times; and it has been shown that this substance can disrupt the pathway of apoptosis and create imbalances in the death of apoptotic and necrotic cells (5, 7, 11, 12). Therefore, it is likely to be a reason of the increase in apoptosis in the groups receiving the moderate and high doses of cannabis extract.

During the fourth day the control group was at their highest level, the treated group with the highest dose of cannabis extract was the descending trend and the other two groups were ascending.

The fifth day the death of all the treated cells with maximum dose of the extract happened. And other groups with a descending trend showed a slight ascending trend in the following day. The results show the toxicity of the extract in high-dose environments and induction of apoptosis, and the antioxidant effect of the low dose extract results in a higher level of the curve than the control.

The duration of use of cannabis is also considered to be an important factor in inducing apoptosis, since, as demonstrated in previous studies, cannabis compounds in the long run can reduce the responsiveness of cannabinoids by affecting the number of cannabinoid receptors and reduce the anti-inflammatory and anti-oxidant responses, consequently, mitotic effects, and increases in apoptosis.

In previous studies, the carcinogenesis of cannabis compounds has also been mentioned, but there is no consensus on this (13, 17, 18).

There is a consensus in all studies conducted on the effect of cannabis on the induction of apoptosis, but all have overlooked the role of the dose (2, 3, 5, 7, 11, 21, 22). But some people have said that the delta-9-tetrahydrocannabinol in the extract acts as an antioxidant for cell viability, while at the same time high concentrations of this substance cause toxicity and increase apoptosis (9).

## CONCLUSION

Rat bone marrow stem cells retain their mesenchymal properties after exposure to cannabis in non-lethal concentrations.

The exposure to different doses of cannabis extract with stem cells extracted from bone marrow has shown that the growth process of the cells is influenced by the dose of cannabis extract and low doses cause more growth and high doses have a lethal effect on it.

The daily bone marrow stem cells exposure to cannabis extract has shown to increase apoptosis over time.

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## CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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## ETHICS COMMITTEE APPROVAL

This research has been approved by the Committee on Ethics of Islamic Azad University of Shiraz coded: IR.MIAU.REC.1396.807.

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