

EFFECTS OF HOMEOPATHIC REMEDIES ON MESENCHYMAL STEM CELL PROLIFERATION: A METHODOLOGY STUDY

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ABSTRACT

Purpose: Mesenchymal stem cells are multipotent cells of mesodermal origin, which have the ability to differentiate into other cells in the bone marrow microenvironment under suitable conditions *in vitro*. Homeopathy is a holistic practice method that aims to improve health status with personalized homeopathic medicines. Studies evaluating the effect of homeopathic remedies in the field of stem cells are rarely encountered in the literature. In our study, it has been aimed to observe their proliferative effects.

Material and Methods: Mesenchymal stem cells were exposed to 10%, 20%, 50% and 100% ratios of homeopathic remedies (30°C potency: Aloe, Calendula, Hypericum, Staphysagria) for 24 and 48 hours. The percent viability of cells at 24 and 48 hours was evaluated with the MTT test.

Results: Aloe vera C30 – Staphysagria C30 – Calendula C30 – Hypericum C30 remedies were found to have an effect on mesenchymal stem cell proliferation at all doses, and the results were statistically more significant when maintained in 10% media.

Conclusion: It is thought that the methodology used in this study on working methods with homeopathic remedies will contribute to the literature and many scientific studies to be done in the future.

Keywords: mesenchymal stem cell, homeopathy, methodology, complementary medicine

INTRODUCTION

Presence of colonies morphologically resembling fibroblasts Friedenstein et al. reported in the 1970s and defined them as fibroblast colony forming units (CFU-F) (1). In studies conducted in the following years, these cells were found to be non-hematopoietic multipotent stem cells, and today they are called mesenchymal stem cells (MSCs) due to their ability to differentiate into cells of mesodermal origin (2). MSCs are one of the elements that support bone homeostasis and hematopoiesis in the bone marrow microenvironment, and when suitable conditions are provided *in vitro*, they are the multipotent cells of mesodermal origin that have the

ability to differentiate into bone, muscle, cartilage and fat cells (mesodermal cells) as non-mesenchymal neurons and astrocytes (ectodermal cells) and hepatocytes (endodermal cells) (3). Three defining characteristics for MSCs have been reported by the International Society of Cell Therapy (ISCT): Their ability to adhere to the plastic surface in standard culture media, expressing cell surface markers such as CD105, CD73, CD90 but not having hematopoietic markers such as CD45, CD34, CD14, CD11b, CD79, CD19, HLA-DR and they can differentiate into bone, fat and cartilage cells *in vitro* (4). They have the feature of supporting cells and can be obtained from many tissues (bone marrow, cord blood, amniotic

fluid, placenta, adipose tissue). They are cells that are multiplied in numbers, durable, and have a high potential for use in different areas (5,6). MSCs, which have the ability to repair tissue damage, can be used for treatment purposes. Preclinical and clinical studies show that transplanted MSCs exert their effects either through their differentiation properties or through the release of molecules that restore tissue functions and regulate immune cells (4,6). Perhaps one of the most advantageous features for clinical use is their low immunogenicity and increased immunosuppression. It has also been reported that they may be suitable for allogeneic transplantation due to their ability to evade the immune system (7). MSCs are also used by many disciplines for regenerative purposes: They are used in musculoskeletal diseases (8), gastroenterological diseases (9, 10), cardiovascular diseases, neurological and hereditary diseases (11).

Homeopathy is derived from the Greek words "homeos" and "pathos", meaning similar disease. It is a method based on the principle of similar cures, applied to diseased people who have symptoms similar to those shown when given to healthy people. Here, the application is made with potentiation / dynamization and highly dilute treatment succussion, or treatment products called remedies prepared by trituration method. The roots of homeopathy go back to Hippocrates. Hippocrates said in his works that "if a substance cures a disease, the same substance is also capable of inducing that disease". In homeopathy, plants, minerals and animal origin substances are used as remedy sources, as well as many different substances such as synthetically produced substances. In order to see the usage areas of homeopathic remedies, all substances go through a process called trial/proving beforehand. In this way, the picture of the symptoms elicited by the substance used in healthy human subjects is observed and recorded. When these remedies are used for people with a similar disease picture, the disease picture is eliminated (12).

While preparing homeopathic medicines, they are not processed as a mixture, but as a single main ingredient. Homeopathic medicine is prepared from either pure forms or tinctures of these substances with a unique and special procedure.

Hannemann discovered dynamizing by improving the methods for the preparation of drugs. By shaking the liquid drugs, a new dynamization is provided to the drug. If a liquid medicine is to be prepared, the

tincture of the substance is prepared first. Then the dilution step is started. In the dilution process, water, alcohol or lactose granules are used. With step-by-step dilution, the remedy becomes stronger and stronger. It gets stronger with the gradually decreasing active ingredient. A stronger effect occurs with fewer side effects (13). The fact that the therapeutic power of a substance with higher potency is greater has been confirmed by the continuous clinical observations of all homeopaths. The agitation process adds kinetic energy to the solution. The more shaking and dilution is done, the greater the therapeutic effect of the remedy, even if one molecule of the original substance remains in the solution. The electromagnetic field strength of the original substance can be transferred to the solvent molecules without changing the resonance frequency (14).

Delphinium staphisagria (Stephanskraut) is a plant from the group of buttercup plants (Ranunculaceae) native to Southern Europe. Staphisagria works very strongly on the nervous system, where it has a debilitating effect. As a homeopathic remedy, it is indicated in cases with a painful surgical wound and a clear cut. For the production of the homeopathic medicine Staphysagria, The seed of St. Stephen's plant is crushed with lactose, mixed with alcohol and then fortified (15).

According to the homeopathic principle, homeopathic calendula preparations stimulate the body from the inside to self-heal. Presented globules, as well as main tincture and ointments (complexing agents) for external use. Typical uses of calendula are hairs and lacerations which are common musculoskeletal complaints with skin loss, healing of tears (even with wounds) and a tendency to scarring. Also suitable active ingredient for the purification of teething, muscle fiber tears and operations (16).

Hypericum is used in acute injuries. It can help with wound healing and relieve pain. The homeopathic remedy Hypericum perforatum is used for a wide variety of injuries. Especially nervous system lesions constitute the main area of effect. Therefore, it can be helpful in operations and accidents, especially if nerve tissues such as fingertips, lips or spine are affected (17,18).

Aloe vera is a plant of the lily family. Based on the main tincture prepared with the sap of the plant, different homeopathic dynamizations of the drug are obtained. The area Aloes choose to study is the vascular system. It especially concentrates its effects

on the portal veins. It is used in the most severe periods of acute pathological conditions due to congestion due to the intensity of the obstruction in the blood flow (19).

There are very few articles in the literature investigating the effects of homeopathic remedies on stem cells. The material and method parts of these studies are not clear enough. However, in order for scientific studies to be reproducible by other researchers, the methodology should be written in a clear and understandable way. In this study, it was aimed to clarify the methodology while investigating the effect of homeopathic remedies on MSC proliferation.

MATERIAL AND METHODS

Cell Culture

Human Mesenchymal Stem Cell (Bone marrow origin) (PCS-500-012) (ATCC, US) were seeded in 25 cm² flasks (Corning, Manassas) in 7 ml DMEM (Dulbecco's Modified Eagle Medium) (Biological Industries, Israel) with 10% FBS (Foetal Bovine Serum) (Biological Industries, Israel) and 1% penicillin-streptomycin (Pen-Strep) (Penicilin 10.000 units/ml, Streptomycin 10 mg/ml) (Biological Industries, Israel) and then grown for 24 h in an incubator at 37 °C in an atmosphere supplemented with 5% CO₂. When cells covered 80% of the culture dish surface, trypsinization was performed to generate more cells.

Cytotoxicity Determination (Cell Viability)

In our study, MTT (3-4,5-dimethyl-thiazolyl-2,5-diphenyltetrazolium bromide) method based on metabolic activity measurement was used to evaluate cell proliferation, viability and cytotoxicity. MTT (Serva, Germany) solution was dissolved at 5 mg/ml in empty media without FBS and Pen-Strep according to the media of the cell group to be used (in DMEM). Then, it was prepared by using a 0.22 µl filter in a 15 ml conical sterile tube. After pipetting 20 µl of MTT solution into each well, it was kept in the incubator for 4 hours. The supernatant, which turned orange after 4 hours, was carefully aspirated without damaging the cells at the bottom. 100 µl of isopropyl alcohol was added to each well, and the plates were wrapped with foil and incubated for 3-4 hours at room temperature and in the dark. At the end of the procedure, the plate was gently shaken and read in an ELISA plate reader at 570 nm wavelength. The viability of cells was calculated with the formula:

$$\% \text{ VITALITY RATE} = \frac{\text{Absorbance of the test well}}{\text{Absorbance of the control well}} \times 100$$

Study 1

The product suitable for use was prepared by adding 3 globules at 30°C to 330 ml of distilled water and shaking by hitting hard the bottom of the bottle 10 times after 30 minutes. Preparation of the remedies for use was done in the same way in all studies.

Trypsinized MSCs were seeded into 96 well plates with 100 µl of cell solution, with 20,000 cells in each well, using a multi-channel pipette. Since the effect of FBS on the function of the remedy was not known, it was decided to reduce the 10% FBS in the culture medium to 2% and to remove the antibiotics, as in the study of Marzotto M. et al. the next day. The media in all the wells of the plates was carefully removed without damaging the cells at the bottom. 200 µl of DMEM (2% FBS, 1% L-glutamine) was added to each well. Four different remedies of 10% (25 µl) and 20% (50 µl) were placed in the groups, each at 30C potency (Aloe, Calendula, Hypericum, Staphysagria). Percent viability of cells was evaluated by MTT at 24th and 48th hours. The wells with only MSC and DMEM, to which we did not add any remedy, formed the control group. In addition, 2 separate water control groups were formed by adding 25 µl and 50 µl water to MSC, DMEM. The groups were planned as follows, and their proliferation was also checked at 24th and 48th hours:

Group 1: Control (200 µl DMEM)

Group 2: Water Control 10% (200 µl DMEM + 25 µl water)

Group 3: Staphysagria 10% (200 µl DMEM + 25 µl Staphysagria)

Group 4: Hypericum 10% (200 µl DMEM + 25 µl Hypericum)

Group 5: Calendula 10% (200 µl DMEM + 25 µl Calendula)

Group 6: Aloe 10% (200 µl DMEM + 25 µl Aloe)

Group 7: Water Control 20% (200 µl DMEM + 50 µl water)

Group 8: Staphysagria 20% (200 µl DMEM + 50 µl Staphysagria)

Group 9: Hypericum 20% (200 µl DMEM + 50 µl Hypericum)

Group 10: Calendula 20% (200 µl DMEM + 50 µl Calendula)

Group 11: Aloe 20% (200 µl medium DMEM + 50 µl Aloe)

Study 2

In this study, unlike Study 1, the remedies were dissolved in 330 ml of media (containing 2% FBS) instead of 330 ml of distilled water. Cells were also seeded in DMEM medium containing 2% FBS, 1% L-glutamine. Other processing steps were set up as in Study 1 and repeated. Additionally, in Study 2, a

group with 200 µl of direct remedy was created. Thus, 10%, 20% and 100% (25 µl, 50 µl, 200 µl) groups were prepared, and a total of 15 groups were formed to evaluate the percentage of cell viability with MTT at 24th and 48th hours.

Study 3

In this study setup, the remedies were prepared by dissolving them in 330cc distilled water as in Study 1 and in 330cc media (containing 5% FBS instead of 2% FBS) as in Study 2. Unlike the first two studies, cells were seeded in DMEM containing 10% FBS, 1% L-glutamine. Remedies were placed in the ratios of 10%, 20%, and 100% with a total volume of 200 µl in each well, and a total of 26 groups were prepared as water and medium groups to evaluate the percentage of cell viability with MTT at 24th and 48th hours.

Group 1: Control (200 µl DMEM)

Group 2: Staphysagria 30 10% (180 µl DMEM + 20 µl Staphysagria)

Group 3: Staphysagria 30 20% (160 µl DMEM + 40 µl Staphysagria)

Group 4: Staphysagria 30 100% (200 µl Staphysagria)

Group 5: Hypericum 10% (180 µl DMEM + 20 µl Hypericum)

Group 6: Hypericum 20% (160 µl DMEM + 40 µl Hypericum)

Group 7: Hypericum 100% (200 µl Hypericum)

Group 8: Calendula 10% (180 µl DMEM + 20 µl Calendula)

Group 9: Calendula 20% (160 µl DMEM + 40 µl Calendula)

Group 10: Calendula 100% (200 µl Calendula)

Group 11: Aloe 10% (180 µl DMEM + 20 µl Aloe)

Group 12: Aloe 20% (160 µl DMEM + 40 µl Aloe)

Group 13: Aloe 100% (200 µl Aloe)

Study 4

In this working setup, the remedies were thawed with 330 cc of media without FBS. Cells were cultivated in DMEM containing 2% FBS and 1% L-glutamine. The water group was not used. A total of 17 groups were prepared at 10%, 20%, 50% and 100%, and at 24th-48th hours (Medium Control 10,20,50 removed).

Study 5

While the remedies were prepared with blank media as in Study 4, in this study, cells were seeded on plates by adding media containing 10% FBS. A total of 13 groups were prepared as 10%, 20% and 50% (25µl, 50µl, 100µl) and at 24th-48th hours.

Statistical Analysis

SPSS software version 24.0 was used in this study, and $p < 0.05$ was taken as statistically significant. The nonparametric Wilcoxon test was used when comparing the 12th and 24th hours as two dependent groups. The nonparametric Mann-Whitney-U test was used to compare the groups in water and media,

which are two independent groups. The nonparametric Kruskal-Wallis test was used when comparing the multiple independent groups of 10%, 20%, 50% and 100%. Low-to-high mean-rank values in nonparametric tests indicate which group has a large or small value.

RESULTS

Study 2 was designed because there was no significant difference between the water group and the studied groups in Study 1 and there was a cytotoxic effect within the group. In Study 2, remedies were prepared with medium, and no statistically significant difference was found between and within the groups.

In Study 3, a statistically significant increase in cell viability was observed in the media group from the 24th hour to the 48th hour in the within-group evaluation ($p < 0.05$) (except the Aloe 30-100% group) (Figure 1). In the water group, a statistically significant increase was observed at 10% and 20% doses, and a significant cytotoxic effect was observed at 100% doses (Figure 2).

In Study 3, when the effect of MSCs of remedies prepared in medium and water (Staphysagria, Hypericum, Calendula, Aloe) on cell viability rates was evaluated by MTT test between groups; statistically insignificant proliferation was observed at the 48th hour at the dose of Stap30-10% in the medium group, while antiproliferative (Stap30-20%, aloe30-20% etc.) and cytotoxic effects (at 100% doses) were observed in the other groups (Figure 3). In the water group, a statistically insignificant proliferation was observed at the dose of Cal30-10% at the 48th hour, and antiproliferative (Stap30 10%-20%, aloe30 10%-20% etc.) and cytotoxic effects (100% doses) were observed in the other groups (Figure 4).

In Study 4, different from Study 3, different concentrations of the remedies prepared with non-FBS medium were applied to the cells, and again, unlike in Study 3, a 50% dose was added. Statistically significant proliferation was observed in the within-group evaluation from the 24th hour to the 48th hour. A statistically significant proliferation was observed between the groups only at 10% and 20% doses, and no proliferative effect was observed in the other groups. Considering these results, Study 5 was planned.

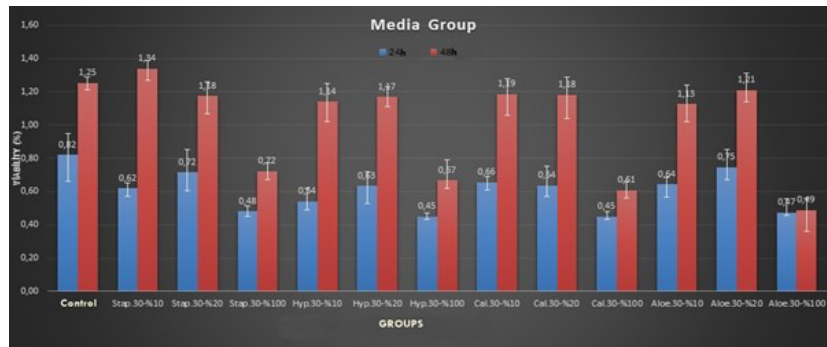


Figure 1. Intragroup cell viability results of the remedies prepared in medium groups of Study 3.

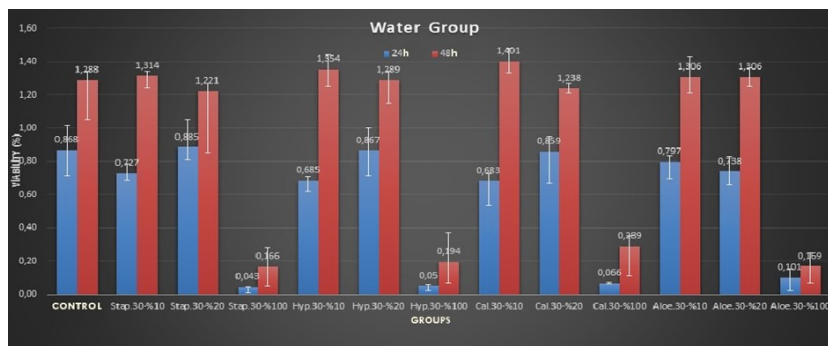


Figure 2. Intragroup cell viability results of the remedies prepared in medium groups of Study 3.

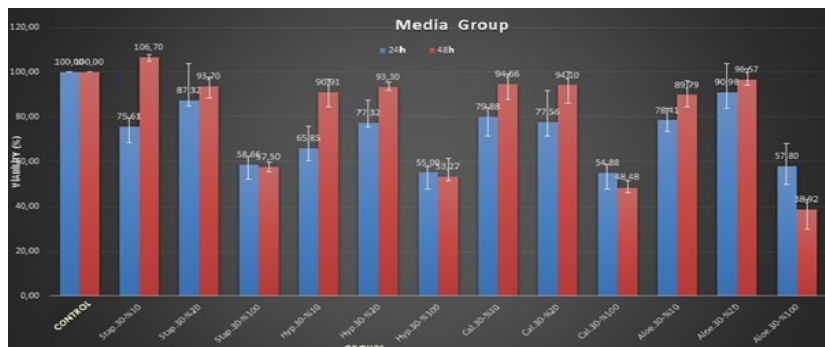


Figure 3. Intergroup cell viability results of the remedies prepared in medium groups of Study 3

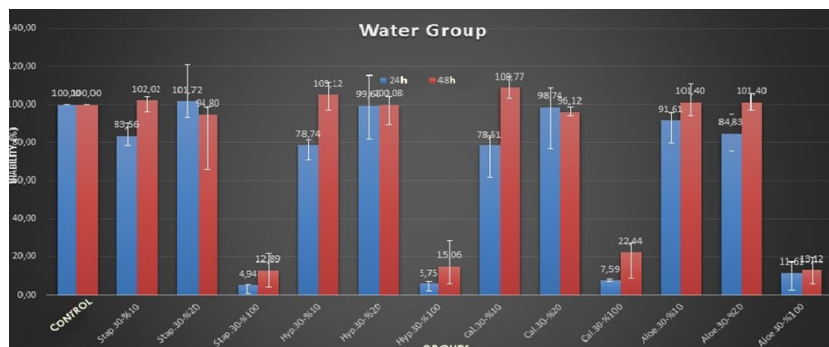


Figure 4. Intergroup cell viability results of remedies prepared in water groups of Study 3

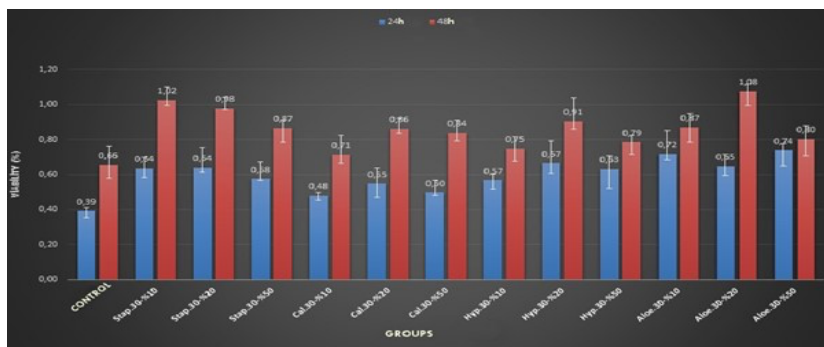


Figure 5. Intragroup cell viability results of the remedies prepared in medium groups of Study 5

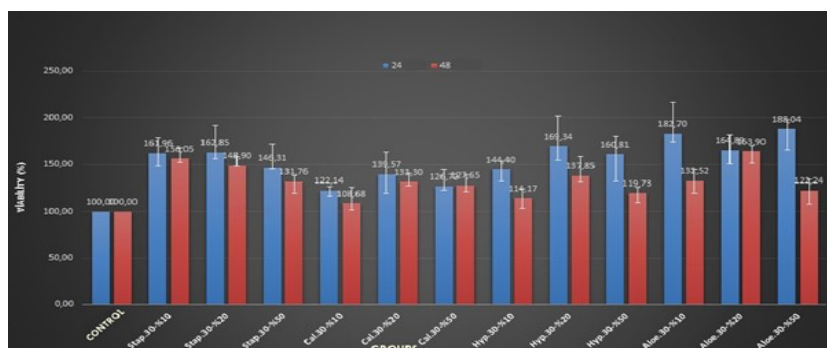


Figure 6. intergroup cell viability results of the remedies prepared in medium groups of Study 5

In Study 5, there was an increase in cell viability in all remedies and at all doses at the 24th and 48th hours in the within-group evaluation, and these increases were found to be statistically significant (except the Aloe 30-10% group) (Figure 5).

In the evaluation between the groups, a statistically significant proliferation was observed in cell viability compared to the control group as a result of MTT analysis at the 24th and 48th hours in all study groups (p<0.05) (Figure 6) (Table 1).

A statistically significant difference was found in cell viability from the 24th hour to the 48th hour in the in-group evaluation in the aquatic environment. However, in the evaluation between the groups, a cytotoxic and antiproliferative effect was observed in some groups in the aquatic environment, but there was no statistically significant difference in the groups with increased cell viability compared to the control.

In the 4th and especially in the 5th study, the remedies were prepared with non-FBS media, and the results were found to be more statistically significant.

Between doses of 10-20-50-100% (25µl, 50µl, 100µl, 200µl); proliferation was observed at doses of Staphysagria C30 10%, Staphysagria C30 20%,

Calendula C30 20%, Hypericum C30 20%, Aloe C30 50% at 24 hours and 20% at 48 hours.

Results were statistically more significant when MSC were maintained in 10% media, which is necessary for their survival.

In Study 5, Aloe vera C30 – Staphysagria C30 – Calendula C30 – Hypericum C30 was found to have an effect on proliferation at all doses.

DISCUSSION

In our study, we observed proliferative effects of homeopathic remedies on mesenchymal stem cells. It was found that Aloe vera C30 – Staphysagria C30 – Calendula C30 – Hypericum C30 remedies has a significant effect on mesenchymal stem cell proliferation at all doses, and the results were statistically more significant when maintained in 10% media.

Mesenchymal stem cells are a type of adult stem cells that have been widely recognized in recent years. The addition of certain chemical and morphogenesis factors to the culture medium of MSC cells induces their proliferation and differentiation into a specific cell type (20).

Table 1. Cell viability and p values between the groups at 24th and 48th hours of the medium groups of Study 5.

	Viability (%)			
	Median (Min-Maks)			
	24 hours	(p)	48 hours	(p)
Med-Contol	39 (25 - 40)		66 (58 - 76)	
Med-Stap.30-%10	64 (58 - 70)	0,036	1,02 (100 - 110)	0,026
Med-Stap.30-%20	64 (61 - 80)		0,98 (97 - 104)	
Med-Stap.30-%50	58 (57 - 67)		0,87 (69 - 100)	
Med-Cal.30-%10	48 (46 - 50)	0,022	0,71 (67 - 98)	0,121
Med-Cal.30-%20	55 (47 - 64)		0,86 (84 - 92)	
Med-Cal.30-%50	50 (48 - 57)		0,84 (69 - 101)	
Med-Hyp.30-%10	57 (52 - 60)	0,01	0,75 (68 - 92)	0,007
Med-Hyp.30-%20	67 (61 - 79)		91 (86 - 104)	
Med-Hyp.30-%50	63 (52 - 71)		79 (62 - 82)	
Med-Aloe.30-%10	72 (68 - 95)	0,39	87 (79 - 137)	0,1
Med-Aloe.30-%20	65 (59 - 72)		108 (90 - 136)	
Med-Aloe.30-%50	74 (65 - 78)		80 (71 - 88)	

With people returning to nature and traditional medicine, the importance of traditional medicine is increasing day by day. According to the World Health Organization (WHO), not only the direct use of plant components as therapeutic agents is important, but also the use of these plants as base materials for the synthesis of drugs or as models for pharmacologically active compounds (21).

It is important to perform *in vitro/in vivo* studies to bridge the gaps between conventional uses and *in vitro* studies, pharmacological studies, toxicity profiling and clinical trials.

The aim of this study is to evaluate the proliferative effect of the homeopathic medicine Aloe vera C – Staphysagria C – Calencula C – Hypericum C on mesenchymal stem cells.

In the branch of homeopathy, it is essential that the substances are selected according to their energy-giving properties and that they can treat diseases by strengthening the body's defense mechanism (22).

The aim is to preserve the healing properties of homeopathic medicines and to eliminate their harmful effects. Scientific studies have shown that tannic acid and polysaccharide in Aloe vera content provide wound healing and tissue repair activity. Containing high mannose and gibberellin growth hormone, glucomannan stimulates its development and proliferation by interacting with fibroblast growth factor receptors. In addition to improving collagen synthesis, it has a positive effect on the wound healing process by increasing the quality of collagen and increasing the degree of cross-linking (23-26).

Staphisagria contains diterpenoid alkaloids, flavonoids, sterols and aliphatic acids. There is a

study showing that staphisagria stimulates endothelial cell proliferation and angiogenesis, followed by specific signaling mechanisms, that VEGF is converted into dermal components of the skin and promotes hair growth (27).

Calendula officinalis contains pharmacologically active coumarins, farradiol, oleanolic acid, carotenoids, flavonoids components. The results of the research showed that calendula officinalis extract reduced the severity of skin reactions and oral mucositis due to radiotherapy (28,29).

Hypericum contains naphthodiantron compounds, phloroglucinols, flavonoids, biflavones, phenolic acids, proanthocyanidins. It has been determined that the wound healing effect occurs when the plant stimulates fibroblasts and the production of collagen increases accordingly (30-36).

In support of this information, in our study, Aloe vera C30 – Staphysagria C30 – Calencula C30 – Hypericum C30 was found to have an effect on mesenchymal stem cell proliferation at all doses. In addition, the results were statistically more significant when maintained in 10% media, which is necessary for the survival of these cells.

CONCLUSION

In summary, our study shows that the remedies to be used in cell culture studies should be prepared in media without FBS instead of water, and that the cells to be studied (depending on the cell type, DMEM containing 10% FBS, 1% L-glutamine was used for MSCs in our study) should be cultured in the environment necessary for their survival. In addition, it is thought that using this method will contribute to

the literature and many future scientific studies by using different remedies in different studies (wound healing, therapeutic treatment for cancer, etc.).

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Conflict of interests: None.

Ethical approval: Ethical approval is not required as it is an in vitro study.

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