Geleneksel ve Tamamlayıcı Tıp Dergisi
Journal of Traditional Medical Complementary Therapies
J Tradit Complem Med. 2023;6(2):142-51

ORIGINAL RESEARCH ORİJİNAL ARAŞTIRMA

DOI: 10.5336/jtracom.2022-92875

The Investigation of Effects of Medicinal Leech Saliva Extract on the Breast Fibroblast Cell Line In Vitro: An Experimental Study

Tıbbi Sülük Tükürük Ekstraktının Meme Fibroblast Hücre Hattı Üzerine Etkilerinin İn Vitro Araştırılması: Deneysel Bir Çalışma

Kübranur ÜNAL[a], Nihan TIRIK[b], Mehmet Emre EROL[b], Leyla İBRAHİMKHANLI[a], Mualla Pınar ELÇİ[b], Hüseyin AYHAN[c]

[a]Department of Medical Biochemistry, Gazi University Faculty of Medicine, Ankara, Türkiye
[b]Department of Medical Biochemistry, Gazi University Institute of Health Sciences, Ankara, Türkiye
[c]Department of Medical Services and Techniques, Ankara Yıldırım Beyazıt University Vocational School of Health Services, Ankara, Türkiye

This study was partially presented as an oral presentation at the 3rd International Science and Innovation Congress, 9-12 June 2022, Online.

ABSTRACT Objective: Medicinal leeches benefit by delivering various bioactive substances from their saliva during sucking. It was aimed to investigate the effects of leech saliva extract (LSE) on breast fibroblast cell line (Hs 578Bst) and HUVEC (CRL-1730) line in vitro. **Material and Methods:** Leech saliva was obtained from medicinal leeches of the Hirudo verbana species. The LSE was obtained from the Hirudo verbana. LSE dose amounts (400 µg/mL, 200 µg/mL, 100 µg/mL and 50 µg/mL) were adjusted for protein concentration. In cell culture; cell viability, apoptosis, cell migration and gene expressions responsible for these effects were evaluated. The effect of LSE on the cell viability was evaluated with MTT assay. The Scratching Test was used to evaluate the effects of LSE on the cell migration. The effect of LSE on mRNA expression levels of fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) was determined with polymerase chain reaction. The effect of LSE on apoptosis was investigated with flow cytometry. **Results:** The doses of LSE (400-50 µg/mL) significantly increased the cell viability compared with the control group for 24 h and 48 h ($p<0.05$). In the scratching test, it was observed that the 50 µg/mL dose induced the cell migration and covered all areas with cells at 24 h. It was observed that 200 µg/mL LSE up-regulated the mRNA expression of FGF, VEGF and EGF compared with the control and other groups ($p<0.001$). All doses of LSE shows no apoptotic or necrotic effects in the cell lines. **Conclusion:** In this study, it was found that the LSE applied at different doses had significant effects on the cell viability, cell migration and gene expression responsible for these effects in the breast fibroblast cell line.

Keywords: Leech saliva extract; cell viability; angiogenesis; apoptosis; proliferation

ÖZET Amaç: Tıbbi sülükler kan emme sırasında tükürüklerinden konakçının kan dolaşımına çeşitli biyoaktif maddeler salarak fayda sağlarlar. Çalışmamızda tıbbi sülük salgı ekstraktının [leech saliva extract (LSE)] meme fibroblast hücre hattı (Hs 578Bst) ve HUVEC (CRL-1730) hattı üzerindeki etkilerinin in vitro olarak araştırılması amaçlanmıştır. **Gereç ve Yöntemler:** Sülük salgısı, Hirudo verbana türü tıbbi sülüklerinden elde edildi. Bu sülük salgısının toplam protein konsantrasyonu belirlendi ve doz miktarları (400 ug/mL, 200 ug/mL, 100 ug/mL ve 50 ug/mL) seri dilüsyon ile ayarlandı. Hücre kültüründe hücre canlılığı, apoptoz, hücre göçü ve bu etkilerden sorumlu gen ekspresyonları değerlendirildi. LSE'nin hücre canlılığı üzerindeki etkisi MTT analiziyle değerlendirildi. LSE'nin hücre göçü üzerindeki etkisini değerlendirmek için Scratch Testi kullanıldı. LSE'nin fibroblast büyüme faktörü [fibroblast growth factor (FGF)], vasküler endotelyal büyüme faktörü [vascular endothelial growth factor (VEGF)] ve epidermal büyüme faktörünün [epidermal growth factor (EGF)] mRNA ekspresyonları üzerine etkisi polimeraz zincir reaksiyonu ile analiz edildi. LSE'nin apoptoz üzerine etkisi flow sitometri ile araştırıldı. **Bulgular:** LSE'nin tüm dozları (400-50 µg/mL) kontrol grubuyla karşılaştırıldığında hem 24 saat hem de 48 saatte hücre canlılığını artırdığı gözlemlendi ($p<0,05$). Scratch testinde, 50 µg/mL dozunun hücre göçünü indüklediği ve 24 saatte tüm alanları hücrelerle kapladığı gözlemlendi. LSE'nin 200 ug/mL dozunun kontrol ve diğer gruplarla karşılaştırıldığında FGF, VEGF ve EGF mRNA ekspresyonunu önemli ölçüde artırdığı gözlemlendi ($p<0,001$). LSE'nin hücre hatlarında nekrotik veya apoptotik etkisi olmadığı tespit edildi. **Sonuç:** Bu çalışmada, farklı dozlarda uygulanan LSE'nin meme fibroblast hücre hattında hücre canlılığı, hücre göçü ve bu etkilerden olası sorumlu gen ekspresyonları üzerinde önemli etkileri olduğu bulundu.

Anahtar Kelimeler: Sülük salgı ekstraktı; hücre canlılığı; anjiyogenez; apopitoz; proliferasyon

Correspondence: Kübranur ÜNAL
Department of Medical Biochemistry, Gazi University Faculty of Medicine, Ankara, Türkiye
E-mail: kubranurunal@gazı.edu.tr

Peer review under responsibility of Journal of Traditional Medical Complementary Therapies.

Received: 10 Aug 2022 ***Received in revised form:*** 23 Jan 2023 ***Accepted:*** 23 Jan 2023 ***Available online:*** 26 Jan 2023

Leeches belong to the Hirudinea class of phylum *Annelida* that is the segmented worms. Among the more than 800 members of the Hirudinea class in the world, there are about 15 species can be used for medicinal purposes. In spite of varies regionally, *Hirudo medicinalis, Hirudo orientalis, Hirudo verbana,* and *Hirudinaria manillensis* are widely known medicinal leeches.[1,2] The treatment method in which these leeches are used is called hirudotherapy also known as the medicinal leech therapy.[3]

Although it is not known clearly when the use of leeches for medicinal purposes started, there are some evidences about their usage in Ancient Egypt.[4] Ibn-i Sina, also known as Avicenna has an important place in the history of medicine, mentioned medical leech therapy in his book "Canon of Medicine" and recommended it, especially for skin diseases.[4,5] As one of the ancient medical practices, medicinal leech therapy lost its popularity in the 20th century. Later on, it has attracted attention again due to the studies conducted in the field of science. It has also been reported that to protect themselves from various diseases, Sultans of Ottoman Empire uses leech therapy in the months May and June every year. Morover, to protect the leech lineage, regulations were made regarding the rules and trade of leech hunting.[6]

In the eighteenth century, along with the Ottomans, the French used leeches in their treatment. During this period, the collection of wild leeches for human therapeutic use was so intense that the central European populations of *H. medicinalis* nearly disappeared.[7] Hirudotherapy is used as a complementary and supportive treatment for many diseases and problems, such as venous congestion, various ulcer forms, migraine and rheumatoid diseases.[8-11]

The main benefits of leech therapy is caused by the various bioactive substances which found in the saliva of medicinal leeches.[12,13] This saliva contains more than 100 bioactive substances. Vasodilator, bacteriostatic, analgesic, anti-inflammatory and anticoagulant properties of those substances can be beneficial against microcirculation disorders, damaged vascular permeability of organs and tissues, hypoxia, blood pressure, decreased immunity and pain.[14] Among these substances, the most studied ones are hirudin and calin shows anticoagulant activity, bdellins have anti-inflammatory properties, hyaluronidase increase permeability as well as has antimicrobial activity, acetylcholine and histamine-like substances promote the dilatation of blood vessels and Factor Xa inhibitors.[4,15,16]

In Türkiye, which is the leader with the largest share in the production and export of medicinal leeches in the world, the Ministry of Health allowed the use of sterile medicinal leeches (*H. medicinalis* and *H. verbana*) in Hirudotherapy since 2014, provided that they are produced from the production site or enterprises approved by the Ministry of Agriculture. Also in Türkiye, hirudotherapy has become an application as a complementary treatment in related centers and can only be performed by a certified medical doctor via using sterile medicinal leeches, thanks to the regulations made by the Ministry of Health.[14,17]

In our study, we investigated the effects of the leech saliva extract (LSE) on the breast fibroblast cell line (Hs 578Bst) and HUVEC (CRL-1730) line *in vitro*. We evaluated cell viability, apoptosis, cell migration and possible gene expression responsible for these effects in the cell culture medium.

MATERIAL AND METHODS

LSE

H. verbana was obtained from a leech farm approved by the Ministry of Agriculture. The leeches were kept in well-ventilated plastic containers filled with chlorine-free and regularly cleared tap water. The leech saliva is obtained using a technique which is used before by Baskova et al., and Abdualkader et al.[18,19] In this method, 0.07 M sodium chloride, and 0.0005 M arginine containing phagostimulatory solution is used. The leeches are fed with this solution. When leeches are entirely satiated, they ceased feeding up with phagostimulatory solution. After that, the LSE is obtained using the milking method that they are squeezed from the posterior end to the anterior end. Thus, medical leech saliva can be obtained without killing the leeches. The saliva was then passed through a 0.22 µm filter (Sartorius minisart, Hannover, Germany), then centrifuged for 10 min at 2,500 rpm at 4 °C. Total protein determination of this su-

pernatant was made according to the Lowry protein assay developed by Lowry et al.[20] Thus, certain doses of LSE (400 µg/mL, 200 µg/mL, 100 µg/mL and 50 µg/mL) which was applied to cells in cell culture were adjusted according to protein concentration of LSE.

THE CELL LINES AND CULTURES

The breast fibroblast (ATCC® Hs 578Bst) cell line and HUVEC (ATCC® CRL-1730™) line were obtained from American Type Cell Collection-ATCC. The medium contained Dulbecco's Modified Eagle Medium, 10% Fetal Bovine Serum and 1% L-glutamine and 1% penicillin-streptomycin. Cells were produced in 25 cm^2 and 75 cm^2 flasks with given medium. The cells were kept under 5% CO_2, 95% air mixture, and humidity at 37 ºC (MCO-18 AIC CVV, Sanyo, Japan) conditions.

THE MTT ASSAY

The cell group without medicinal leech saliva was chosen as the control group. After 24 and 48 h of exposure to the LSE, 20 µl of MTT solution (5%) was added to the wells. The cells in the culture dish were kept in an incubator at 37 °C for 3 hours, and then the medium containing MTT solution was aspirated. At the end of the cell cultivation period, 100 µL of isopropyl alcohol was added to the wells to dissolve the formazan crystals formed by MTT and incubated for 15 min. Each well was read with a microplate reader (BioTek® Synergy HT, USA) at a wavelength of 570 nm.

According to the absorbance values, cell viability levels were calculated in consonance with the following formula:

Cell viability (%)*=(Mean absorbance value of confirmed treatment well/Average absorbance value of confirmed control well)X100.*

Data were expressed as mean±standard deviation (SD) of not less than three independent experiments.

THE SCRATCHING TEST (THE CELL MIGRATION ASSAY)

The scratching, also known as the cell migration assay, is a technique used to investigate two-dimensional-cell migration in vitro. After creating a scratch, the LSE-applied cells in the certain doses in the Petri dishes (35 mm, high) were kept in the incubator for approximately 24 or 48 h until they completely covered the bottom. The aperture distance was measured in µm. In the scratching assay, cell migration was calculated from the zeroth h to the twenty-fourth h or forty-eighth h.[21]

Measurements were made using the ImageJ v1.0 (Wayne Rasband, NIH USA). Calculation of percent scratching confluence: *% Sctratching confluence=(A-B)*100%/A.*

A: the width of the initial scratch space, B: the width of the scratch space at time 8, 24 and 48h.

The effects of LSE on cell migration were compared with the control group in the breast fibroblast cell and HUVEC lines.

REAL TIME-POLYMERASE CHAIN REACTION

The isolation of mRNA was performed using Hybrid-R (GeneAll, South Korea) and Ribo-Ex Kit (GeneAll, South Korea). Cell samples previously stored in PBS at -80 °C were used for mRNA isolation. cDNA synthesis was performed using EntiLink 1st Strand cDNA Synthesis Kit (ELK Biotechnology, China) after mRNA isolation. The reaction mixture contained 10 µL of real time-polymerase chain reaction (RT-PCR) reagent mix, 2 µL of 10 mM primer mix of target gene [vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) or fibroblast growth factor (FGF)] or house-keeping gene (GAPDH), 1 µL of dNTPs and 15 µL of nuclease-free water. PCR was then performed by ABI 7500 DX system (Applied Biosystems, Foster City, CA). The primer sequences used in the RT-PCR analysis in this study are shown in Table 1.

FLOW CYTOMETRY (ANNEXIN V/PROPIDIUM IODIDE STAINING)

Apoptosis of breast fibroblast and HUVEC cells was estimated with an Annexin V-FITC Apoptosis Detection Kits (BD Pharmingen™, Cat. No: 556570, England) protocol and analysed with flow cytometry (Accuri™ C6 Plus Device, BD Biosciences, United Kingdom).

ETHICAL STATEMENT

Studies on commercially available human cell lines such as the breast fibroblast cell line (ATCC® Hs

TABLE 1: The primer sequences used in RT-PCR.

Gene (Transcript)	Primer sequences (5'-3')
GAPDH forward	GAAGGTGAAGGTCGGAGTCAAC
GAPDH reverse	CAGAGTTAAAAGCAGCCCTGGT
FGF forward	AGTGTGTGCTAACCGTTACCT
FGF reverse	ACTGCCCAGTTCGTTTCAGTG
VEGF forward	AGGGCAGAATCATCACGAAGT
VEGF reverse	AGGGTCTCGATTGGATGGCA
EGF forward	GACAGGCCACCTCGTCG
EGF reverse	TGCGTGAGCTTGTTACTCGT

RT-PCR: Real time-polymerase chain reaction; FGF: Fibroblast growth factor; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor.

578Bst) and HUVEC (ATCC® CRL-1730) require any ethics committee approval, so no ethics approval was needed to conduct the study. The study complied with the principles of the Declaration of Helsinki.

DATA ANALYSIS

Mean±SD were used as the descriptive statistics. The conformity of the data to the normal distribution was checked with the Shapiro-Wilk test. One-way analysis of variance (One-way ANOVA) was used to compare the means of more than two groups, and Student's t-test was used to compare the means of two independent groups. In case of statistical significance as a result of ANOVA, Bonferroni correction was used as a multiple comparison test to find the source of the difference. Data were analysed with the IBM SPSS 21 (IBM SPSS Inc., Chicago, IL) package program. The statistical significance level was taken as 0.05.

RESULTS

LSE INCREASED CELL VIABILITY

In the MTT assay, to understand cell viability in response to treatment, a proliferation of cells is measured. Therefore, LSE is used as an MTT assay treatment agent to understand how it affects the proliferation of the cells. Cells were exposed to LSE in 400-50 mg/mL doses for 24 h and 48 h. Afterwards,

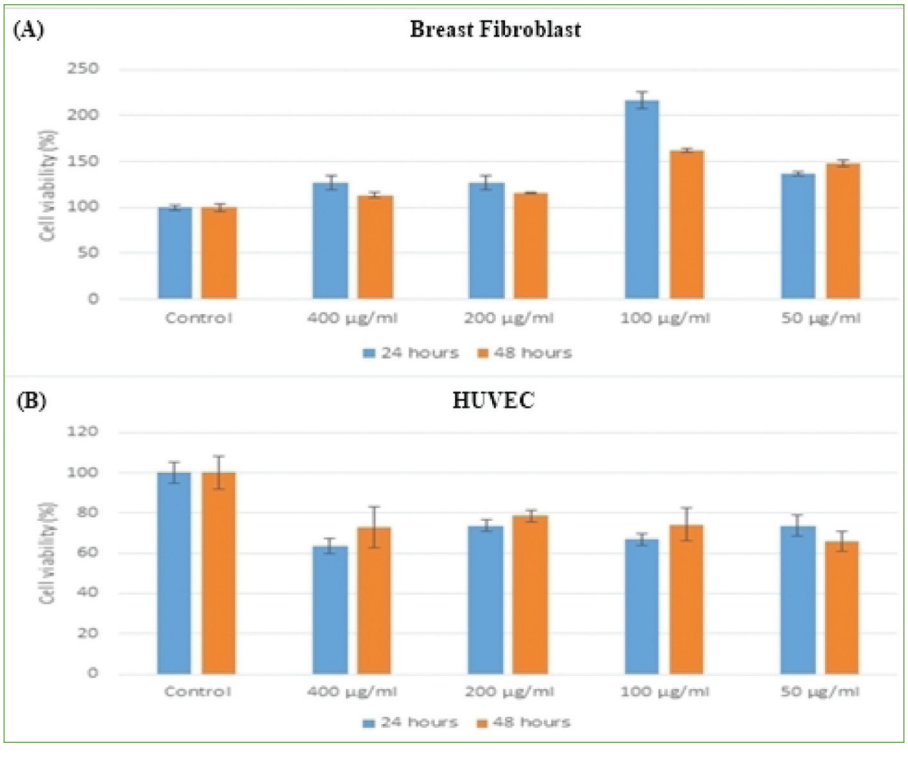

FIGURE 1: MTT assay. The effect of LSE on the breast fibroblast cell line and t HUVEC line viability. **A)** The concentrations of LSE (400-50 µg/mL) increased significantly the cell viability in the breast fibroblast cell line compared to control group for 24 h and 48 h ($p<0.05$); **B)** The concentrations of LSE (400-50 µg/mL) decreased significantly the cell viability in the HUVEC line compared to the control group for 24 h and 48 h ($p<0.05$).
LSE: Leech secretion extract.

the cell viability was compared with the control group, which was accepted as 100%.

In the breast fibroblast cell line, application of different doses of LSE (400-50 µg/mL) increased cell viability significantly ($p<0.05$) compared to the control for 24 h and 48 h (Figure 1A).

However, in the HUVEC line, different dose applications of LSE (400-50 µg/mL) decreased cell viability significantly ($p<0.05$) compared to the control for 24 h and 48 h (Figure 1B). LSE had about 30% anti-proliferation activity on the HUVEC line compared to the control group of percent viability.

LSE INDUCED CELL MIGRATION

To find the best concentration, the doses of LSE in the range of 400-50 µg/mL applied to the cells. Images of the cell culture scratching test model created with LSE was taken under the inverted microscope at zeroth, eighth, twenty-forth, and forty-eighth hours.

In the breast fibroblast cell line, it was observed that 50 µg/mL dose induced the cell migration compared to the control group and covered all areas with cells at 24 h. Although there was some closure in the scratch area at the twenty forth hour in the control group and other doses, complete closure happened in the 50 µg/mL dose (Figure 2A).

In the HUVEC line, it was observed that 50 µg/mL dose covered all areas with cells at forty eighth hour, as in the control group. The other concentrations of LSE induced lesser migration (Figure 2B).

LSE STIMULATED MRNA EXPRESSION OF FGF, VEGF AND EGF

RT-PCR was used to quantify the mRNA expression levels of VEGF, EGF and FGF.

LSE stimulated mRNA expression of FGF, VEGF and EGF in the breast fibroblast cell line. The different doses of LSE (400-50 µg/mL) up-regulated mRNA expression of FGF, VEGF and EGF significantly compared to the control in the breast fibroblast cell line ($p<0.001$). The mean value comparison of each doses of LSE and control groups, it demonstrated

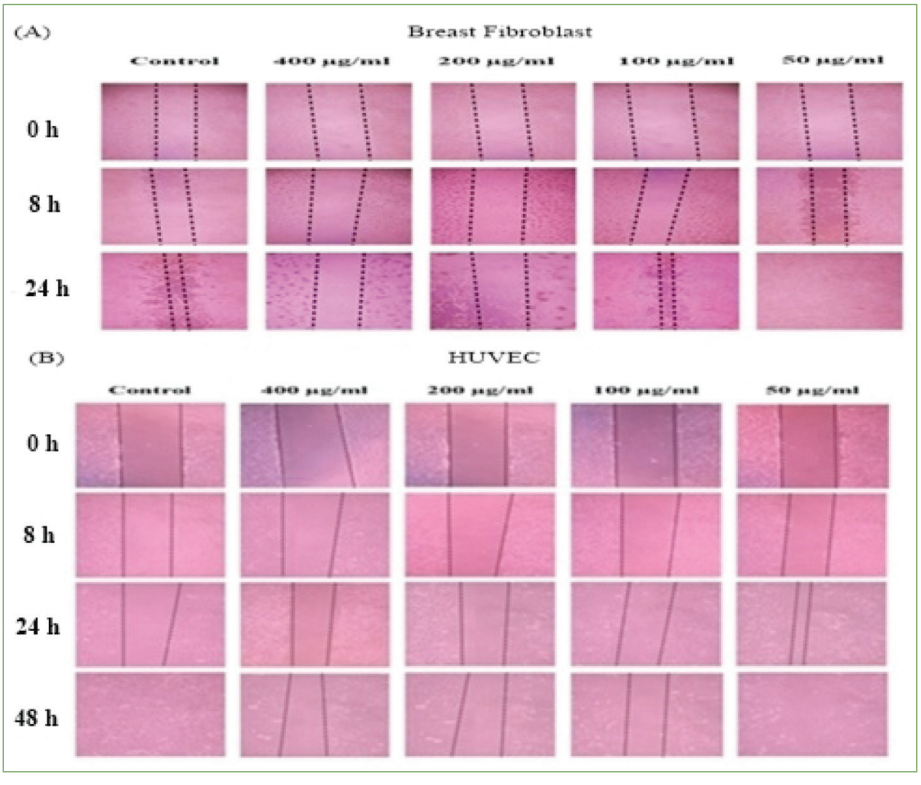

FIGURE 2: Scratching Test. The effect of leech secretion extract on cell migration on the breast fibroblast cell line **A)** and HUVEC line **B).**

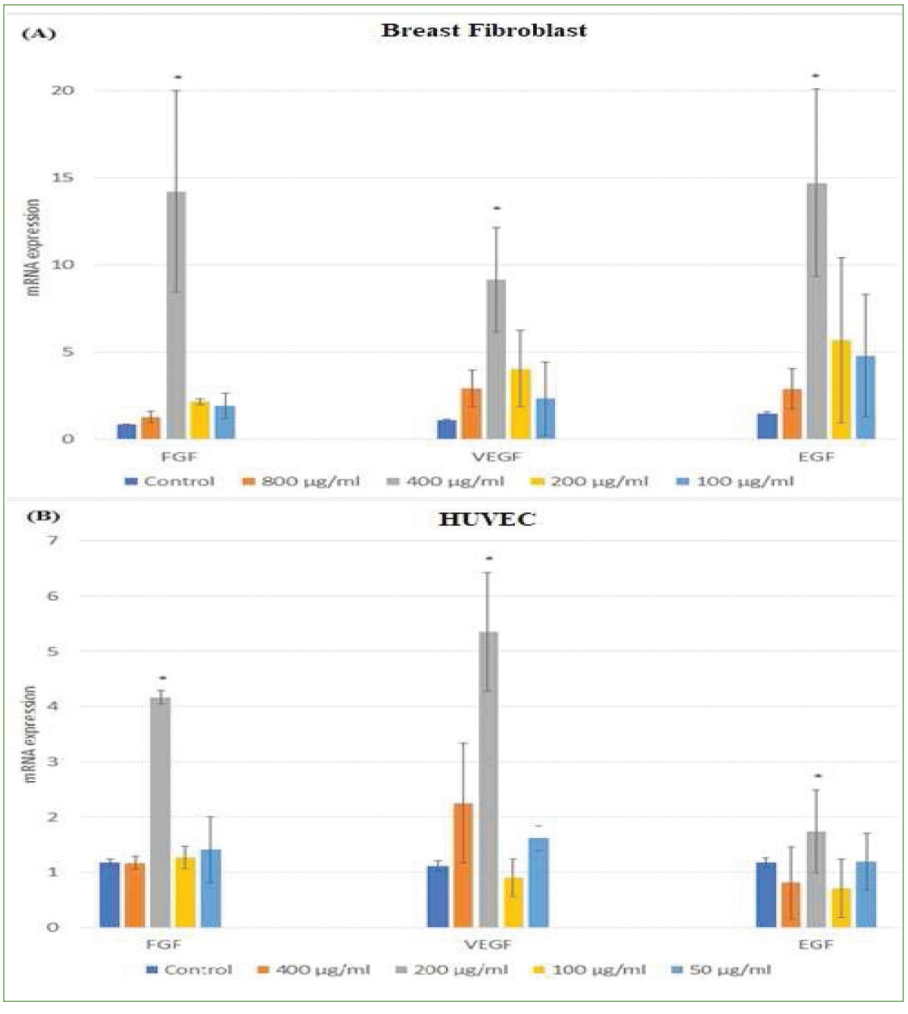

FIGURE 3: RT-PCR analysis. The effect of LSE on mRNA expression of FGF, VEGF and EGF on the breast fibroblast cell line **A)** and HUVEC line **B).**
RT-PCR: Real time-polymerase chain reaction; LSE: Leech secretion extract; FGF: Fibroblast growth factor; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor.

TABLE 2: Comparison of mRNA expression results in the breast fibroblast cell line.

	Groups					
Breast fibroblast	**Control**	**400 µg/mL**	**200 µg/mL**	**100 µg/mL**	**50 µg/mL**	**p value**
FGF $\bar{X}$±SD (n=3)	0.86±0.03	1.28±0.32	14.20±5.79	2.15±0.17	1.92±0.73	p<0.001*
VEGF $\bar{X}$±SD (n=3)	1.11±0.04	2.92±1.05	9.15±2.98	4.04±2.19	2.33±2.09	p<0.001*
EGF $\bar{X}$±SD (n=3)	1.47±0.10	2.88±1.15	14.70±5.38	5.70±4.72	4.78±3.50	p<0.001*

*200 µg/mL of leech secretion extract up-regulated significantly mRNA expressions of FGF, VEGF and EGF compared to the control and other groups;
FGF: Fibroblast growth factor; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor; SD: Standard deviation.

that the significant increase in the expression of FGF, VEGF and EGF is provided by the application of 200 µg/mL (Figure 3A). The comparison of mRNA expression results in the breast fibroblast cell line are shown in Table 2.

In the HUVEC line, 200 µg/mL of LSE up-regulated mRNA expression of FGF, VEGF and EGF statistically significantly compared to control and other doses (p<0.001). No statistically significant difference was found between the other

TABLE 3: Comparison of mRNA expression results in the HUVEC line.

	Groups					
HUVEC	Control	400 µg/mL	200 µg/mL	100 µg/mL	50 µg/mL	p value
FGF X̄±SD (n=3)	1.17±0.06	1.17±0.11	4.17±0.12	1.26±0.20	1.41±0.60	p<0.001*
VEGF X̄±SD (n=3)	1.11±0.09	2.25±1.08	5.35±1.08	0.90±0.34	1.61±0.21	p<0.001*
EGF X̄±SD (n=3)	1.18±0.08	0.80±0.65	1.74±0.75	0.71±0.53	1.18±0.51	p<0.001*

*200 µg/mL of leech secretion extract up-regulated significantly mRNA expressions of FGF, VEGF and EGF compared to the control and other groups;
FGF: Fibroblast growth factor; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor; SD: Standard deviation.

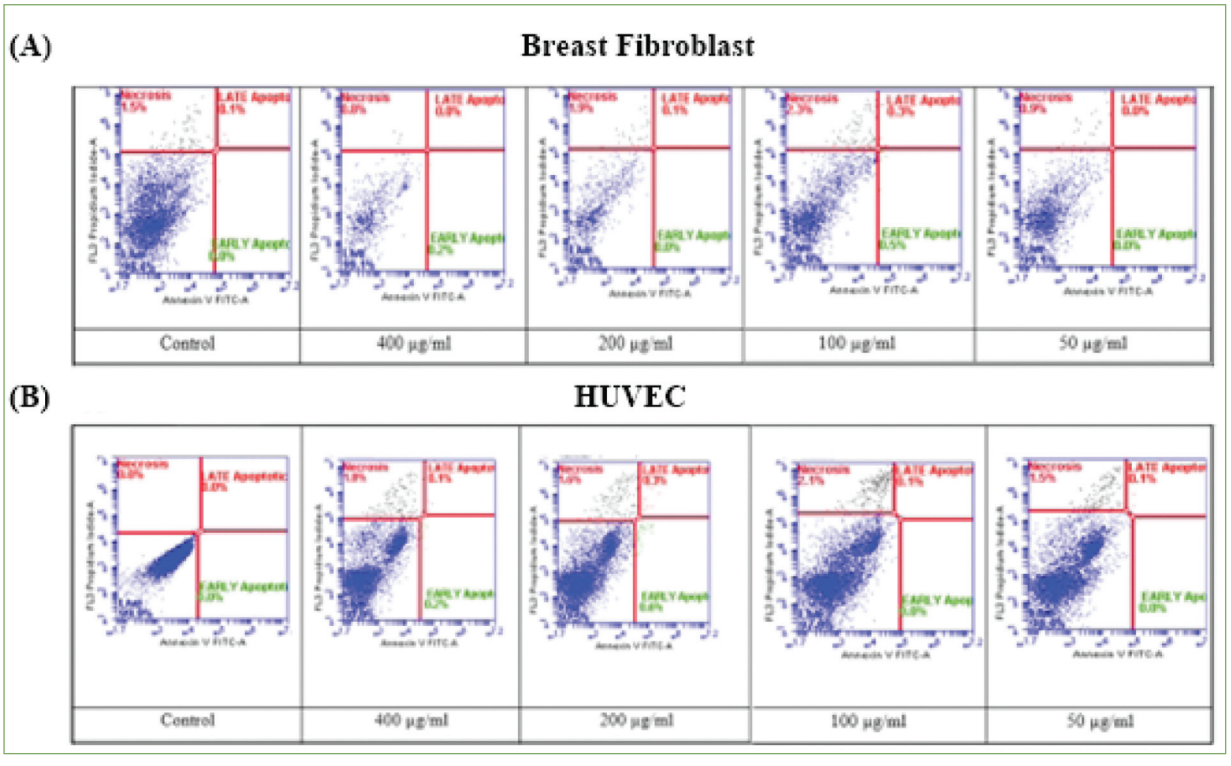

FIGURE 4: The flow cytometry analysis. The effect of leech secretion extract on apoptosis and necrosis on the breast fibroblast cell line **(A)** and HUVEC line **(B)**.

groups (Figure 3B). The comparison of mRNA expression results in the HUVEC line are shown in Table 3.

LSE SHOWS NO APOPTOTIC OR NECROTIC EFFECTS

In the flow cytometry analysis, the cell viability rates were close to 100% in the breast fibroblast cell and HUVEC lines as in the control groups. It was determined that the doses of LSE (i.e., 400 µg/mL, 200 µg/mL, 100 µg/mL and 50 µg/mL) had caused neither necrotic nor apoptotic effects in the cell lines (Figure 4).

DISCUSSION

The salivary glands of medicinal leeches contain more than 100 bioactive substances. Medical leech therapy is frequently used as a traditional and complementary treatment method in Türkiye and worldwide. Nowadays, there are several ongoing research studies that investigate curative effects of bioactive substances of medicinal leeches.[14]

In a recently published article, they observed the effects of encapsulated medicinal LSE on the inhibition of angiogenesis of breast cancer cells by targeting VEGF-A in vitro. They obtained the leech saliva

from *H. medicinalis* and used it on a breast cancer cell line (MCF-7) as the cancerous cell line and the HUVEC line as the control. In that study, encapsulated LSE which was loaded into liposomes was applied to cell lines, and its effect was examined by MTT assay. While liposomal LSE 97% anti-proliferative activity on the MCF-7, it was 10% in the HUVEC. Also, in the same study, they examined the effect of liposomal LSE on the migration of the MCF-7 cells and HUVEC with a scratching test. Results of the scratching test showed that liposomal LSE has the ability to suppress cell migration in the MCF-7 cell line. The space was completely closed at 48 h by HUVEC cells, while there was no closure at any hour in MCF-7 cells. Along with this information, it was reported that VEGF-A gene expression in the MCF-7 cell line was decreased statistically after liposomal LSE application compared with the control.[22]

Our findings revealed that LSE increase cell proliferation in the breast fibroblast cell line, and decrease cell proliferation in the HUVEC line about 30% compared with the control groups. Also, in our study, the results of scratching test show that LSE has the ability to induce cell migration in both the breast fibroblast cell line and HUVEC line. In addition, we found that mRNA expression of FGF, VEGF and EGF up-regulated significantly after 200 µg/mL LSE application in both the breast fibroblast cell line and HUVEC line. The reason for the differences in the effects of LSE in vitro may be depend on the selected type of leech and cell line. While Shakouri et al., used *H. medicinalis* as a medicinal leech in their study, we used *H. verbana*.[22] Also, while the type of cell line, which used in their study was a breast cancer cell line, we used the healthy breast fibroblast cell line.

In a study, it was shown that the hypertrophy and fibrosis caused by Angiotensin II (ANG II) on myocardial cells were significantly reduced with the application of leech extract. It was found that leech extract increases the myocardial cell viability and decreases the cell size growth due to ANG II effect. As a result of this study, it was hypothesized that leech extract may be effective for treating of cardiac hypertrophy and fibrosis.[23]

In another study, the researchers evaluated the effect of applying different forms of leech extracts from *Whitmania pigra* to the human endothelial cell line (EA. Hy926). In the MTT assay, it was shown that leech saliva at 48 h cause proliferation on the human endothelial cell line.[24] Different than our results, they observed proliferation of cells. This difference may be due to the use of distinct cell lines and types of leeches.

In a study where the effect of *H. manillensis* medicinal leech saliva on the small cell lung cancer cell line (SW1721) is investigated, leech saliva was used in combination with different anticancer drugs used routinely. As a result, it was observed that the combination of leech saliva with anticancer drugs decreased significantly the cell viability and cell migration in the SW1721 cell line compared with the application of anticancer drugs alone.[25]

In the study by Pang et al., the effect of hirudin obtained from leech saliva on diabetic mouse kidney glomerular endothelial cell with renal angiopathy was investigated.[26] Hirudin applied to the glomerular endothelial cell line of diabetic mice with angiopathy was found to inhibit cell migration in an in vitro wound model. Also in this study, they examined that VEGF protein expression levels, which are important for angiogenesis. Hirudin administered to the glomerular endothelial cell line of diabetic mice with angiopathy has been shown to inhibit VEGF protein expression.

Similar to this study, the HUVEC cell line as an endothelial cell type was used in our study. However, mentioned study differs from our study in a way that they use cells with damaged microangiopathy rather than healthy endothelial cells. The inhibitory effect of hirudin used in this study in an in vitro wound model shows that hirudin has therapeutic efficacy in renal angiopathy. Also, in our study, while medicinal leech saliva increased gene expressions in healthy-cell lines, we believe that the use of unhealthy-cell lines in this study is responsible for the decreased gene expressions.

When we look at the literature in general, natural or synthetic organic compounds obtained from medicinal leeches from them and their saliva are used in

various scientific studies. In this context, both in vitro and in vivo studies have been conducted.[27,28] When the studies in which the effect of leech saliva on cells are evaluated with the various methods, it can be seen that there are quite different findings in the literature. We predict that the findings may vary depending on the type of selected leech, the protein concentration of the saliva, and the chosen cell line, and so on.

CONCLUSION

In our study, we investigated the effects of medicinal LSE applied to healthy-cell lines in vitro. Within the scope of the study, cell viability, cell migration, apoptosis and possible gene expressions responsible for these effects were examined in cell lines. The results obtained from the LSE may be interpreted as a new potential agent for wound healing and angiogenesis because of its potential to stimulate the release of certain proteins and growth factors which may also participate in cell proliferation and migration depending on applied doses.

In the data obtained from our study, differences are observed according to the cell line we used. There are also some differences between our study and those in the literature. The reason for this difference may because of the type of leech, the protein concentration of the saliva, and the type cell line.

In conclusion, the finding obtained from our study can be illuminating for further in vitro studies. In addition, it provides important contributions to the existing literature about the mechanism of action of medicinal leech saliva. Our study also predicts that medicinal LSE can be used as a promising adjunct agent for medical treatments. However, more studies with more precise methodologies are needed to confirm leech therapy's potential therapeutic effect.

Acknowledgment

We want to thanks to the Gazi University Medical School and Gülhane Health Sciences Institute R&D Laboratories.

Source of Finance

This study was financial supported way Gazi University Scientific Research Projects Coordination Unit (Project no: TYL-2021-7342).

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Kübranur Ünal, Nihan Tırık;* ***Design:*** *Kübranur Ünal, Nihan Tırık;* ***Control/Supervision:*** *Kübranur Ünal, Nihan Tırık;* ***Data Collection and/or Processing:*** *Kübranur Ünal, Nihan Tırık, Hüseyin Ayhan, Leyla İbrahimkhanlı, Mualla Pınar Elçi;* ***Analysis and/or Interpretation:*** *Kübranur Ünal, Nihan Tırık;* ***Literature Review:*** *Kübranur Ünal, Nihan Tırık, Mehmet Emre Erol;* ***Writing the Article:*** *Kübranur Ünal, Nihan Tırık, Mehmet Emre Erol;* ***Critical Review:*** *Kübranur Ünal, Nihan Tırık, Mehmet Emre Erol;* ***References and Fundings:*** *Kübranur Ünal, Nihan Tırık;* ***Materials:*** *Kübranur Ünal, Nihan Tırık, Hüseyin Ayhan, Leyla İbrahimkhanlı, Mualla Pınar Elçi.*

REFERENCES

1. Ayhan H, Mollahaliloğlu S. Tıbbi sülük tedavisi: hirudoterapi [Medicinal leech therapy: hirudotherapy]. Ankara Medical Journal. 2018;18(1):141-8. [Crossref]
2. Baskova IP, Kostrjukova ES, Vlasova MA, Kharitonova OV, Levitskiy SA, Zavalova LL, et al. Proteins and peptides of the salivary gland secretion of medicinal leeches Hirudo verbana, H. medicinalis, and H. orientalis. Biochemistry (Mosc). 2008;73(3):315-20. [Crossref] [PubMed]
3. Alam SS, Ahmad W, Yunus SS. Irsal-e-Alaq (leech therapy/hirudo therapy) in surgical diseases: a review. IJMR. 2016;1(4):23-7. [Link]
4. Abdullah S, Dar LM, Rashid A, Tewari A. Hirudotherapy/leech therapy: applications and indications in surgery. Arch Clin Exp Surg. 2012;1(3):172-80. [Link]
5. Itrat M, Zarnigar HN, Hague N. Historical aspects of leech therapy: a critical review. Int J Health Sci Res. 2013;3(7):78-83. [Link]
6. Parlakpınar H, Polat S. Hirudoterapi (tıbbi sülük uygulaması) tarihçesi. Parlakpınar H, editör. Hirudoterapi (Tıbbi Sülük Uygulaması). 1. Baskı. Ankara: Türkiye Klinikleri; 2021. p.1-6.
7. Hildebrandt JP, Lemke S. Small bite, large impact-saliva and salivary molecules in the medicinal leech, Hirudo medicinalis. Naturwissenschaften. 2011;98(12):995-1008. [Crossref] [PubMed]
8. Shankar KM, Rao SD, Umar SN, Gopalakrishnaiah V. A clinical trial for evaluation of leech application in the management of Vicarcikā (Eczema). Anc Sci Life. 2014;33(4):236-41. [Crossref] [PubMed] [PMC]

9. Bakhshi M, Jalalian B, Valian M, Shariati S, Saeidi T, Ranjbar H. Can leech therapy be used as an alternative treatment for controlling migraine headache? A pilot study. Acta Facultatis Medicae Naissensis. 2015;32(3):189-97. [Crossref]
10. Rai PK, Singh AK, Singh OP, Rai NP, Dwivedi AK. Efficacy of leech therapy in the management of osteoarthritis (Sandhivata). Ayu. 2011;32(2):213-7. [Crossref] [PubMed] [PMC]
11. Weinfeld AB, Yuksel E, Boutros S, Gura DH, Akyurek M, Friedman JD. Clinical and scientific considerations in leech therapy for the management of acute venous congestion: an updated review. Ann Plast Surg. 2000;45(2):207-12. [Crossref] [PubMed]
12. Ünal K, Erol M, Ayhan H. Literature Review on the Effectiveness of Medicinal Leech Therapy in the Wound Healing. Ankara Medical Journal. 2023; 23(1):151-64. [Crossref]
13. Öner Küçük M, Yaman O. Tıbbi sülük terapisi (hirudoterapi) [Medical leech therapy (hirudotherapy)]. Journal of Biotechnology and Strategic Health Research. 2019;3(Özel sayı):29-46. [Crossref]
14. Gödekmerdan A, Arusan S, Bayar B, Sağlam N. Tıbbi sülükler ve hirudoterapi [Medicinal leeches and hirudotherapy]. Turkiye Parazitol Derg. 2011;35(4):234-9. [Crossref] [PubMed]
15. Glyova O. Modern Hirudotherapy-A Review. Biotherapeutics, Education and Research Foundation: The BeTER LeTTER. 2005;2(1):3. [Link]
16. Ünal K, Erol ME. Tıbbi sülük salgisinin biyokimyasi. Karaman O, editör. Sağlık & Bilim 2022: Medikal Araştırmalar IV. 1. Baskı. İstanbul: Efeakademi Yayınları; 2022. p.215-28.
17. Resmî Gazete (27.10.2014, Sayı: 29158) sayılı Geleneksel ve Tamamlayıcı Tıp Uygulamaları Yönetmeliği; 2014. (Erişim tarihi: 08/07/2023). Erişim linki: [Link]
18. Baskova IP, Zavalova LL, Basanova AV, Sass AV. Separation of monomerizing and lysozyme activities of destabilase from medicinal leech salivary gland secretion. Biochemistry (Mosc). 2001;66(12):1368-73. [Crossref] [PubMed]
19. Abdualkader AM, Merzouk A, Ghawi AM, Alaama M. Some biological activities of Malaysian leech saliva extract. IIUM Engineering Journal. 2011;12(4):1-9. [Crossref]
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265-75. [Crossref] [PubMed]
21. Grada A, Otero-Vinas M, Prieto-Castrillo F, Obagi Z, Falanga V. Research techniques made simple: analysis of collective cell migration using the wound healing assay. J Invest Dermatol. 2017;137(2):e11-e16. [Crossref] [PubMed]
22. Shakouri A, Kahroba H, Hamishekar H, Abdolalizadeh J. Nanoencapsulation of Hirudo medicinalis proteins in liposomes as a nanocarrier for inhibiting angiogenesis through targeting VEGFA in the Breast cancer cell line (MCF-7). Bioimpacts. 2022;12(2):115-26. [Crossref] [PubMed] [PMC]
23. Wang CH, Pandey S, Sivalingam K, Shibu MA, Kuo WW, Yu-LanYeh, et al. Leech extract: a candidate cardioprotective against hypertension-induced cardiac hypertrophy and fibrosis. J Ethnopharmacol. 2021;264:113346. [Crossref] [PubMed]
24. Xu L, Li X, Zhang E, Liang H, Li W, Wang S, et al. The effect of leech extracts on endothelial cell coagulation-related factors and endothelial dysfuction-related molecules. Clin Exp Hypertens. 2019;41(3):220-30. [Crossref] [PubMed]
25. Merzouk A, Ghawi A, Abdualkader A, Abdullahi A, Alaama M. Anticancer effects of medical Malaysian leech saliva extract (LSE). Pharm Anal Acta S. 2012;15:1-5. [Crossref]
26. Pang X, Zhang Y, Peng Z, Shi X, Han J, Xing Y. Hirudin reduces nephropathy microangiopathy in STZ-induced diabetes rats by inhibiting endothelial cell migration and angiogenesis. Life Sci. 2020;255:117779. [Crossref] [PubMed]
27. Ünal K, Erol ME. Tıbbi sülük tedavisinin bilimsel değeri [The scientific value of the medicinal leech therapy]. Anadolu Tıbbı Dergisi. 2023;1(3):33-42. [Link]
28. Yingxin G, Guoqian Y, Jiaquan L, Han X. Effects of natural and recombinant hirudin on VEGF expression and random skin flap survival in a venous congested rat model. Int Surg. 2013;98(1):82-7. [Crossref] [PubMed] [PMC]