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Effects of Lyophilized Leech Saliva Extract on Cell Migration and Apoptosis in MDA-MB-231 Breast Cancer and HUVEC Cell Lines

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Abstract

Breast cancer, a leading global cause of female mortality, necessitates innovative treatment strategies to alleviate the drawbacks associated with conventional therapies. This study explores the potential of Lyophilized Leech Saliva Extract (LLSE) in addressing this need. The impact of LLSE on the MDA-MB-231 breast cancer cells and HUVECs lines was investigated. This research aims to systematically evaluate LLSE's effects on critical cellular processes, including cell migration, apoptosis, necrosis, and viability. Our findings reveal nuanced responses, with LLSE exerting anti-proliferative effects on cancerous MDA-MB-231 cells and a pro-proliferative influence on healthy HUVECs. The study also scrutinises gene expression dynamics, uncovering intricate patterns involving key regulators: EGF (Epidermal Growth Factor), FGF (Fibroblast Growth Factor), and VEGF (Vascular Endothelial Growth Factor). In summary, this research contributes insights into medicinal leech therapy's potential in cancer treatment. With breast cancer treatment's evolving landscape, exploring alternative, less debilitating options becomes crucial. This study addresses this imperative, providing a foundation for future investigations into the rich bioactive compounds in leech saliva.

Keywords: Cell Death, Cell Viability, Cell Proliferation, Leech Saliva Extract, RT-PCR

1. Introduction

Breast cancer arises from the uncontrolled proliferation of epithelial cells originating in the ducts or lobes of the breast. It stands as a significant global health challenge, claiming the lives of approximately 42,000 women each year (Ben-Dror *et al.*, 2022). It is the cancer that causes the most deaths and is the most prevalent among women worldwide (Abdel-Fattah *et al.*, 2006; WHO, 2021). The issue of resistance to conventional chemotherapeutic drugs employed in the treatment of breast cancer remains a significant challenge for scientists. Furthermore, the severe side effects associated with these drugs pose additional concerns. Addressing these challenges is crucial in improving the efficacy and safety of breast cancer treatment strategies. For this reason, scientists today are searching for alternative, more effective treatment methods with fewer side effects (Nedeljković and Damjanović, 2019). When the history of breast cancer treatment is examined, there is evidence that leech therapy has been used for centuries (Karamanou and Androutsos, 2021).

Leeches are members of the Phylum Annelida, also known as the ringed worms. A few species of leeches, mainly in freshwater, also live in terrestrial areas (Elliott and Kutschera, 2011). Although there are more than 600 known leech species, only a few of them (about 15 species) are found in the medical leech category (Baskova *et al.*, 2008; Sig *et al.*, 2017). Medicinal Leech therapy, also known as hirudotherapy, is one of the ancient practices in the history of medicine (Alam *et al.*, 2016). Hirudotherapy has been found in the oldest inscriptions of Egypt, India, Persia, Europe, China, and Anatolia (Gödekmerdan *et al.*, 2011).

At first, it was believed that leech therapy was healing due to the blood-sucking feature of leeches. With the advances in science, biochemical analyses have shown that the therapeutic effects of medicinal leech therapy are actually due to the wide variety of proteins and peptides contained in the saliva of medicinal leeches and secreted into the host's bloodstream during blood sucking (Hildebrandt and Lemke, 2011). According to their specific properties, these molecules show anti-inflammatory, anti-microbial, analgesic, vasodilator, or

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** **List of Abbreviations** : EGF, Epidermal Growth Factor; FGF, Fibroblast Growth Factor; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; H. verbana, Hirudo verbana; HUVEC, Human Umbilical Vein Endothelial Cell; IC50, Half-maximal inhibitory concentration; LLSE, Lyophilized Leech Saliva Extract; MDA-MB-231, Breast Cancer Cell Line; MTT Assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RTCA, Impedance-based real-time cell analyser; RT-PCR, Real-time Polymerase Chain Reaction; VEGF, Vascular Endothelial Growth Factor.

anaesthetic effects (Sig *et al.*, 2017). The most researched bioactive substances found in medicinal leech saliva are calin and hirudin with anticoagulant properties, bdellins with their anti-inflammatory properties, hyaluronidase with anti-microbial and extracellular matrix degradation activity, acetylcholine and histamine-like substances with their vasodilator effects (Singh, 2010). Previous research has highlighted the potential of leech saliva extract in inhibiting the growth of various tumor types, including gliomas. Studies also show the anti-cancer effects of hirudin, known as an anticoagulant, which is one of the first substances that come to mind regarding leech saliva (Zhao, 2015). Studies have demonstrated the anti-cancer effects of hirudin, an anticoagulant peptide found in leech saliva, which inhibits tumor growth by blocking thrombin and reducing angiogenesis via anti-proliferative properties (Ammar, 2021). Hirudin, along with other bioactive molecules such as eglin and bdellins, contributes to the therapeutic potential of leech saliva by modulating various cellular processes involved in cancer progression (Michalsen *et al.*, 2007; Abdualkader *et al.*, 2013).

Given these promising results of leech therapy in cancer treatment, our study aims to explore the efficacy of lyophilized leech saliva extract (LLSE) on a different cancer type, specifically breast cancer. This experimental study evaluates the effects of different doses of LLSE on cell migration, apoptosis, necrosis, and cell viability of the MDA-MB-231 breast cancer cell line and HUVEC cell line. By examining these parameters, the underlying mechanisms through which LLSE exerts its anti-cancer effects will be understood, and its potential as a novel therapeutic strategy for breast cancer will be explored.

2. Materials and Methods

2.1. Extraction of Leech Saliva

Mediterranean medicinal leech *Hirudo verbana* Carena, 1820 (Clitellata, Hirudinea, Hirudo) is used in this study (Tessler *et al*., 2018). The leeches were obtained from an approved sterile leech farm in Isparta, Turkiye. The exact origin and species of the leeches were identified using a stereo zoom microscope (Euromex NZ.1903-S, Germany) with a camera to diagnose and examine their morphological characteristics. Morphological criteria were used to confirm the species identification of the leeches (Neubert and Nesemann, 1999; Davies and Govedich, 2001). They were maintained by changing their water regularly every 2-3 days. The leech saliva was obtained without sacrificing the leeches (Abdualkader *et al.*, 2011). All leeches were starved for about three months in case they were fed before. A phagostimulatory solution containing 0.07-M NaCl with 0.0005-M arginine was prepared at 37°C to feed the leeches. After the leeches were satiated, they were kept in a plastic test tube immersed in an ice-filled beaker until they were temporarily paralyzed and vomited up all the solution they had absorbed. Then, milking was performed by squeezing the leech from the tail to the head to collect the remainder of the leech saliva. Only the colourless (blood-free) salivary fluids vomited by the fed leeches were pooled. Then, this liquid was passed through a membrane filter (Sartorius minisart, Hannover, Germany) to remove unwanted particles. After centrifuging these substances (10 min, 2500 rpm, 4°C), the supernatant was lyophilized (Christ Lyophilizer - Freeze Dryer - Alpha 2-4 LD, Germany) and used in our experiments. The collected samples were kept homogeneous by creating stock, divided into Eppendorf, stored at -20 °C, and protected from light. Freeze drying or lyophilization is the process of sublimation of ice in a frozen material to minimise the amount of liquid in the materials (Smith, 2012).

2.2. Protein Determination of the LLSE

The total protein concentration of lyophilized leech saliva extract (LLSE) was assessed using the Bradford Protein Assay Kit (ABP Biosciences, USA). This assay allows for rapid and specific measurement, minimizing waiting times and potential proteolysis effects (Kielkopf *et al*., 2020). LLSE doses were adjusted based on protein concentration to the following levels: 800 µg/mL, 400 µg/mL, 200 µg/mL, and 100 µg/mL. The dosage to be used in this study is prepared using serial dilution. To determine the molecular weights of peptides and proteins in LLSE, Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was employed. A 15% resolving gel using the tris-glycine SDS-PAGE method was utilized, with visualization of protein bands achieved through the Coomassie blue dye method. All experiments were conducted under standard conditions at 25 °C.

2.3. Cell Lines and Cell Cultures

Human Breast Cancer Cell (MDA-MB-231™) and Human Umbilical Vein Endothelial Cell (HUVEC – CRL-1730™) lines obtained from ATCC, USA, were utilized in this study. Cells were transported to our laboratory following manufacturer guidelines under cold chain conditions.

2.4. Cell Viability Test (MTT Assay)

Cell viability and proliferation were assessed utilizing the 3 - (4,5 - dimethylthiazol-2-yl) - 2,5 - diphenyltetrazolium bromide (MTT) assay. The dosage to be used in this study was determined by previously published data (Ünal *et al*., 2023). Cells were plated and treated with varying concentrations of LLSE (800 µg/mL, 400 µg/mL, 200 µg/mL, and 100 µg/mL), with untreated cells serving as controls. After incubation for 24 or 48 hours, formazan crystals, indicative of viable cells, were dissolved using isopropyl alcohol. Absorbance was measured at 570 nm (BioTek® Synergy HT microplate reader, USA). Cell viability was calculated as follows:

$$\text{Cell viability (\%)} = (\text{Mean absorbance of treated well} / \text{Average absorbance of control well}) \times 100.$$

Control well values were designated as 100% viable cells. Cell viability and cytotoxicity were expressed as percentages relative to control values. Thiazolyl Blue Tetrazolium Bromide (Sigma, USA) was used in the assay. IC50 and EC50 values were determined through serial dilutions of LLSE.

2.5. Cell Migration Assay

Cell migration experiments were done via an xCELLigence, impedance-based real-time cell analyzer (RTCA) device and cell invasion and migration plate (CIM-plate 16) (ACEA Biosciences Inc., California, USA). The CIM plate consists of a top and a bottom

chamber. The top chamber provides wells to hold the cells and is equipped with gold electrodes below a separating membrane to measure the impedance. The bottom chamber contains the cell media, where the LLSE is added. The migration of cells towards the chemoattractant through the membrane is measured by the changes in impedance values on the gold electrodes below the membrane.

100 µL of the DMEM with 10% FBS and penicillin-streptomycin containing the chemoattractants at 200 µg/mL and 800 µg/mL concentrations were added in each well of the bottom well. Media containing no chemoattractant was used as a Control. Then, the top chamber was placed onto the bottom chamber. Medium without the chemoattractant was added to the wells of the top chamber to moisten the membrane. Then, the CIM plate was placed into the DP station for 30 minutes in the cell culture incubator, and a baseline measurement was taken. Then, the MDA-MB-231 cells and HUVECs were seeded into the wells of the top chamber at $4x10^3$ cells/well concentration (determined via prior optimization measurements), and the cell index measurements were taken at 30-minute intervals. The cell migration index was calculated as follows:

2.6. Real-Time PCR (RT-PCR)

EGF (Epidermal Growth Factor), FGF (Fibroblast Growth Factor), and VEGF (Vascular Endothelial Growth Factor) expression levels were determined by RT-PCR using EnTurbo™ SYBR Green PCR SuperMix kit (ELK Biotechnology, China). Reactions were performed on the ABI 7500 RT-PCR device (Applied Biosystem, USA). According to the $2^{-\Delta\Delta}$Ct method, the expression levels of the housekeeping gene were first determined, and then normalisation was determined according to the control. mRNA isolation was performed via Hybrid-R and RiboEx Kit (Gene-All, South Korea), and cDNA synthesis was performed using the EntiLink 1st Strand cDNA Synthesis Kit (ELK Biotechnology, China). The primer sequences are as follows:

(House-keeping gene) GAPDH, 5'-GAAGGTGAAGGTCGGAGTCAAC-3' and 5'-CAGAGTTAAAAGCAGCCCTGGT-3';

FGF, 5′ -AGTGTGTGCTAACCGTTACCT- 3′ and 5′ -ACTGCCCAGTTCGTTTCAGTG- 3′;

VEGFA, 5′ -AGGGCAGAATCATCACGAAGT- 3′ and 5′ -AGGGTCTCGATTGGATGGCA- 3′;

EGF, 5′ -GACAGGCCACCTCGTCG-3′ and 5′ -TGCGTGAGCTTGTTACTCGT- 3'.

2.7. Flow Cytometry

Apoptosis and necrosis in the MDA-MB-231 and HUVEC cell lines were assessed using Annexin V-FITC Apoptosis Detection Kits (BD Pharmingen™, Cat. No: 556570, England). The analysis was performed via flow cytometry using the Accuri™ C6 Plus device (BD Biosciences, UK).

2.8. Statistical Analysis

Data were analyzed using IBM SPSS 21 (IBM SPSS Inc., Chicago, IL) for statistical analyses and GraphPad Prism 5.0 (GraphPad Software, USA) for IC50 and EC50 determinations. Mean ± standard deviation (mean ± sd) was used as descriptive statistics. One-way ANOVA was used to scrutinize mean disparities across multiple groups, while Student's t-test was utilized to compare means between two distinct groups. Subsequent pairwise comparisons among the groups were conducted using Bonferroni's test at $p < 0.05$ to discern the underlying cause of the observed statistical significance.

IC50 determination and curve fitting were conducted using GraphPad Prism 5.0 software (USA). Each data point represents the average of triplicate samples.

3. Results

3.1. Protein Determination of the LLSE

Analysis of the LLSE using SDS-PAGE revealed the presence of a diverse mixture comprising over 20 proteins/polypeptides, with molecular weights ranging from 13 kDa to 260 kDa (see Figure 1). This indicates a complex protein composition within the LLSE, which may contribute to its bioactive properties.

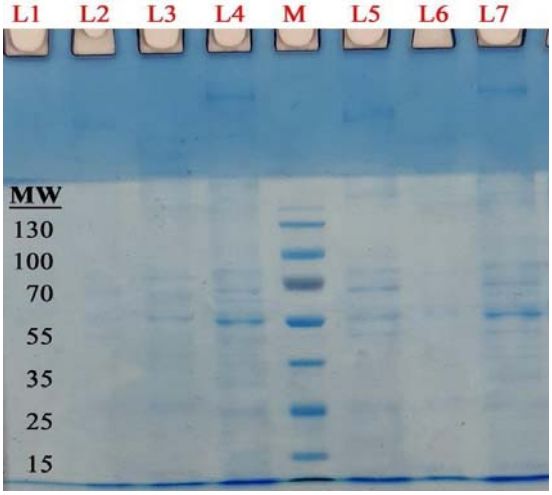


Figure 1. Separation of LLSE peptides and proteins by SDS-PGE method with 15% resolving gel.

M: Molecular Mass Marker (kDa)

Lane 2, 3, 4, 5 ,6 and 7: LLSE

MW: Molecular Weight

3.2. Effect of LLSE on Cell Viability

Following LLSE application for 24 hours or 48 hours in the MDA-MB-231 cell line, IC50 doses were determined to be 470 µg/mL and 467 µg/mL, respectively, indicating a dose-dependent effect of LLSE with consistent efficacy across exposure times (see Figure 2A, and 2B). Conversely, analysis of EC50 results in the HUVEC cell line following LLSE application for 24 hours and 48 hours revealed EC50 doses of 108.4 µg/mL and 150.3 µg/mL, respectively. These findings suggest that a 24-hour LLSE application is preferable, achieving similar outcomes with a lower dose compared to the 48-hour application in terms of proliferation (see Figures 3A and 3B).

The impact of LLSE on proliferation was evaluated in two distinct cell lines: the cancerous MDA-MB-231 cell line and the healthy HUVEC cell line, using the MTT assay. In MDA-MB-231 cells, LLSE doses ranging from 400 to 100 µg/mL showed no significant differences in cell viability compared to the control group at both 24 hours and 48 hours. However, 800 µg/mL resulted in a

considerable decrease in cell viability at both time points ($p < 0.05$) (see Figure 4A and 4B). Conversely, in the HUVEC line, LLSE doses ranging from 800 to 100 µg/mL significantly increased cell viability compared to the control group at both 24 hours and 48 hours ($p < 0.05$) (see Figures 5A and 5B). These results underscore the differential effects of LLSE on cell proliferation between cancerous and healthy cell lines, highlighting its potential as a therapeutic agent with selective cytotoxicity towards cancer cells while promoting viability in healthy cells.

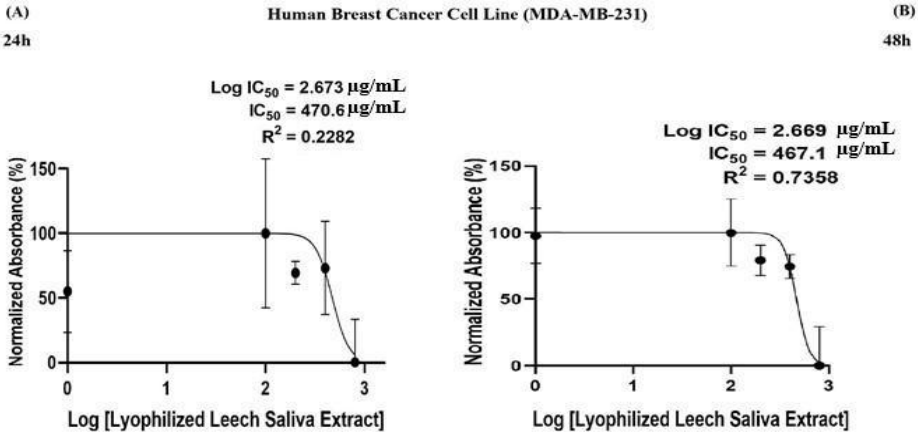


Figure 2: Identification of IC_{50} values in LLSE-exposed **MDA-MB-231** cell line; **(A)** 24h, and **(B)** 48h

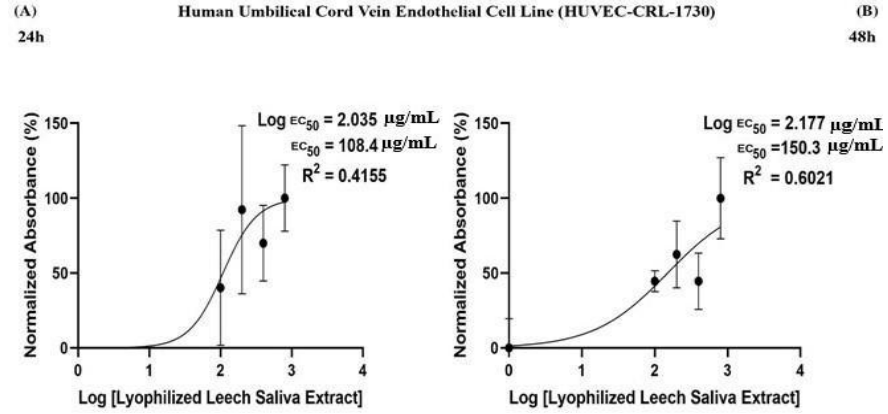


Figure 3: Identification of EC_{50} values in LLSE-exposed **HUVEC** line; **(A)** 24h, and **(B)** 48h

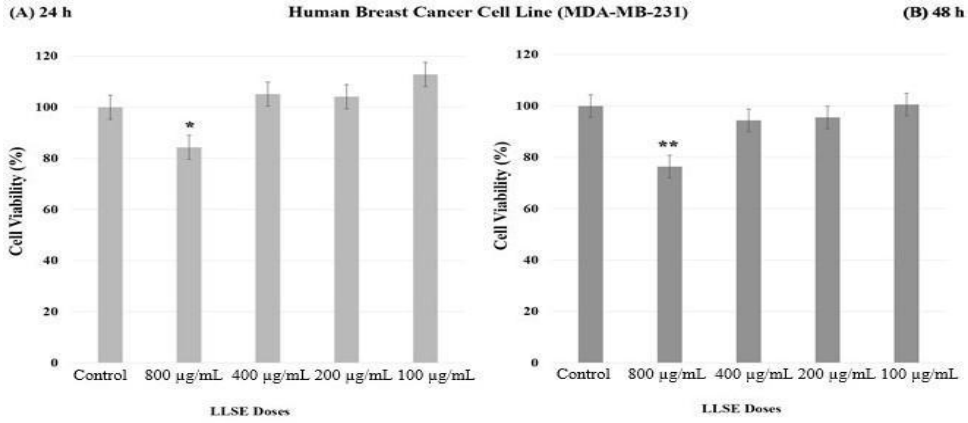


Figure 4: MTT assay for MDA-MB-231 cell line at 24h and 48h

Effects of LSSE on the MDA-MB-231 cell line viability. Data were expressed as mean ± SD of not less than three independent experiments.

All concentrations of LLSE (400–100 µg/mL) did not cause differences, and 800 µg/mL of LLSE decreased the cell viability in **the MDA-MB-231 cell line** compared to the control group at **(A)** 24 h (* $p < 0.05$). **(B)** 48 h)

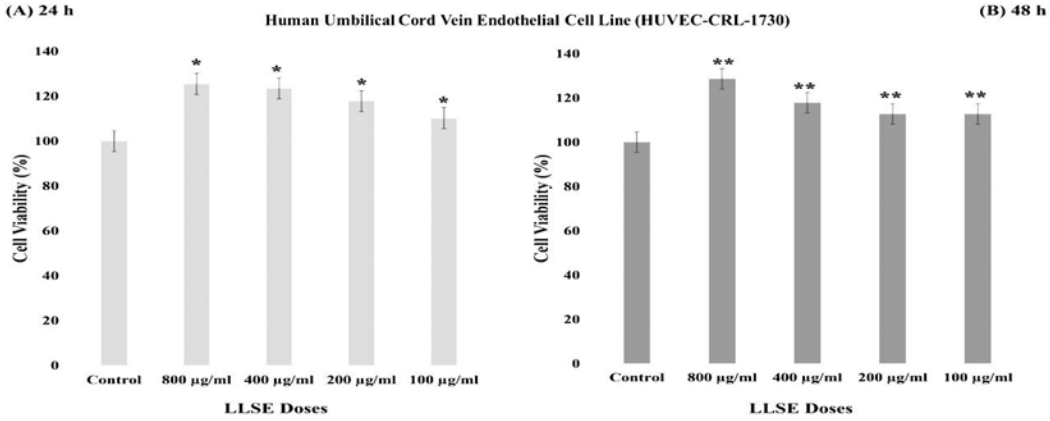


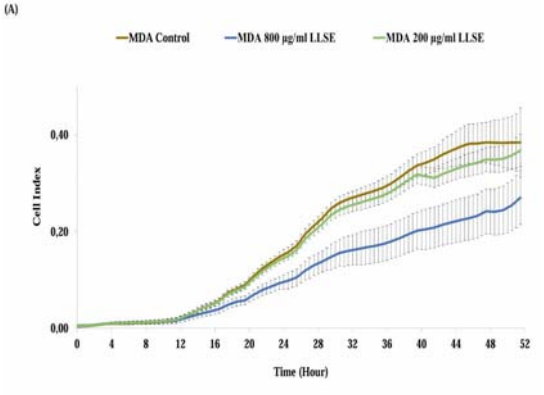
Figure 5. MTT assay for HUVEC line at 24h and 48h

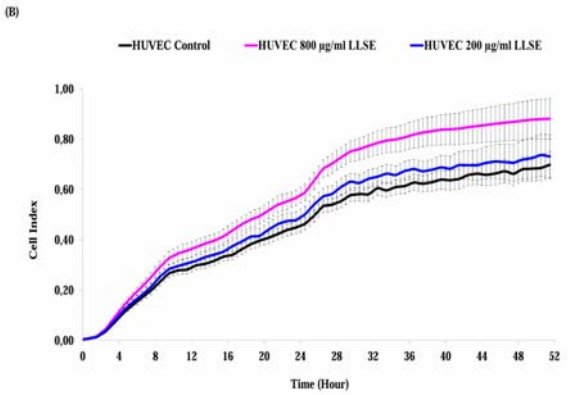
Effects of LSSE on the HUVEC line viability. Data were expressed as mean ± SD of not less than three independent experiments.

All concentrations of LLSE (800–100 µg/mL) significantly increased cell viability in **the HUVEC line** compared to the control group at **(A)** 24 h (* $p < 0.05$). **(B)** 48 h), (** $p < 0.05$).4h and 48 h (** $p < 0.05$).

3.3. Effect of LLSE on Cell Migration

For the MDA-MB-231 cell line, RM analysis showed a statistically significant difference in the cell migration index increase between the dosages ($p < 0.0001$). As shown in Figure 6A, the pairwise comparison indicated that the cell migration index significantly decreased in the 800 µg/mL LLSE compared to the 200 µg/mL LLSE and the control group ($p < 0.0001$).For the HUVEC line, RM analysis showed a statistically significant difference in the cell migration index increase between the dosages ($p < 0.0001$). As shown in Figure 6B, the pairwise comparison indicated that the cell migration index significantly increased in the 200 µg/mL LLSE and the 800 µg/mL LLSE compared to the control group ($p < 0.0001$).





(A) Effects of LLSE on the **MDA-MB-231** cell line.

The graph shows the cell migration index of MDA-MB-231 cells treated with different doses of lyophilized leech saliva extract (LLSE) over a 52-hour period. The groups include the control (no LLSE treatment), 800 µg/mL LLSE, and 200 µg/mL LLSE. The cell migration index was measured every hour for a total of 52 hours. The data points represent the mean ± SD of three independent experiments (n=3).

Error bars indicate standard deviation (SD). A statistically significant difference ($p < 0.0001$) was observed between the groups, with the 800 µg/mL LLSE group showing the most substantial decrease in cell migration index compared to the control and 200 µg/mL LLSE group

(B) Effects of LLSE on **the HUVEC line**.

The graph shows the cell migration index of HUVEC cells treated with different doses of lyophilized leech saliva extract (LLSE) over 52 hours. The groups include the control (no LLSE treatment), 800 µg/mL LLSE, and 200 µg/mL LLSE. The cell migration index was measured every hour for 52 hours. The data points represent the mean ± SD of three independent experiments (n=3). Error bars indicate standard deviation (SD). A statistically significant difference ($p < 0.0001$) was observed between the groups, with the 800 µg/mL LLSE group showing the most substantial increase in cell migration index compared to the control and 200 µg/mL LLSE group.

Figure 6: Migration curves of the MDA-MB-231 cell and HUVEC lines (800 µg/mL LLSE, 200 µg/mL LLSE, and control)

3.4. Effects of LLSE on the mRNA Gene Expressions

In the MDA-MB-231 cell line, FGF mRNA expression significantly decreased in all doses (800, 400, 200, and 100 µg/ mL) compared to the control (* $p < 0.001$). Treatment with 800 µg/mL LLSE notably reduced mRNA expression of EGF, FGF, and VEGF compared to the control group (** $p < 0.001$). Moreover, treatment with 400 µg/mL LLSE significantly increased VEGF and EGF mRNA expression compared to the control and all doses (800, 400, 200, and 100 µg/ mL) (*** $p < 0.001$) (Table 1). Conversely, in the HUVEC line, treatment with 100 µg/mL of LLSE significantly increased mRNA expression of EGF, FGF, and VEGF compared to the control group

and all doses (800, 400, 200, and 100 µg/ mL) (**** $p < 0.001$) (Table 2).

Table 1. mRNA expression levels of EGF, FGF, and VEGFA on the **MDA-MB-231** cell line

Groups	FGF Mean ± SD (n=3)	VEGF Mean ± SD (n=3)	EGF Mean ± SD (n=3)
Control	0,94 ± 0,11	1,15 ± 0,1	1,00 ± 0,10
800 µg/mL	0,35 ± 0,07 *, **	0,85± 0,07 **	0,93 ± 1,17 **
400 µg/mL	0,60 ± 0,06 *	2,57±0,29 ***	2,10 ± 0,39 ***
200 µg/mL	0,37 ± 0, 10 *	1, 58 ± 0,16	1, 56 ± 0,12
100 µg/mL	0,46 ± 0,07 *	1,4 ± 0,24	1,27 ± 0,12
p Value	*$p < 0.001$ **$p < 0.001$	**$p < 0.001$ ***$p < 0.001$	**$p < 0.001$ ***$p < 0.001$

* mRNA expression of FGF was significantly lower in all treated groups compared to the control group, (*p < 0.001).

800 µg/mL of LLSE significantly decreased the mRNA expression of FGF, VEGF, and EGF compared to the control (p < 0.001).

400 µg/mL of LLSE significantly upregulated VEGF and EGF mRNA expression compared to the control and all other groups (p < 0.001).

Table 2. mRNA expression levels of EGF, FGF, and VEGF on the **HUVEC** cell line

Groups	FGF Mean ± SD (n=3)	VEGF Mean ± SD (n=3)	EGF Mean ± SD (n=3)
Control	0,95 ± 0,06	1,15 ± 0,06	0,94 ± 0,17
800 µg/mL	1,80 ± 0,41	0,90 ± 0,20	1,18 ± 1,12
400 µg/mL	1,90 ± 0,70	1,93 ± 0,10	1,91 ± 0,29
200 µg/mL	1,19 ± 0,38	1, 96 ± 0,70	1, 39 ± 0,11
100 µg/mL	3,74 ± 0,46 ****	3,58 ± 0,44 ****	2,48 ± 0,36 ****
p Value	****$p < 0.001$	****$p < 0.001$	****$p < 0.001$

100 µg/mL of LLSE significantly upregulated mRNA expression of FGF, VEGF, and EGF compared to the control and all other groups. (*p < 0.001).

3.5. Effects of LLSE on Apoptosis and Necrosis

While drugs or natural products used in cancer treatment are expected to induce cell death in the cancerous cells, this is the opposite for the healthy cells. Therefore, to observe the effects of LLSE on the cancerous and healthy cell lines, cell lines were analysed by flow cytometry after the cells were exposed to LLSE for 24 hours.

It was determined that all doses (800, 400, 200, and 100 µg/ mL) of LLSE on the MDA-MB-231 cell line caused necrosis; the necrosis percentages were determined as 8.5%, 5.8%, 6.9%, and 6.3%, respectively (see Figure 7A). At the same time, 800 µg/mL LLSE was determined as the dose that caused the most necrosis in the MDA-MB-231 cell line. On the other hand, all doses of LLSE (800, 400, 200, and 100 µg/ mL) did not cause any significant apoptosis or necrosis in the HUVEC line (see Figure 7B).

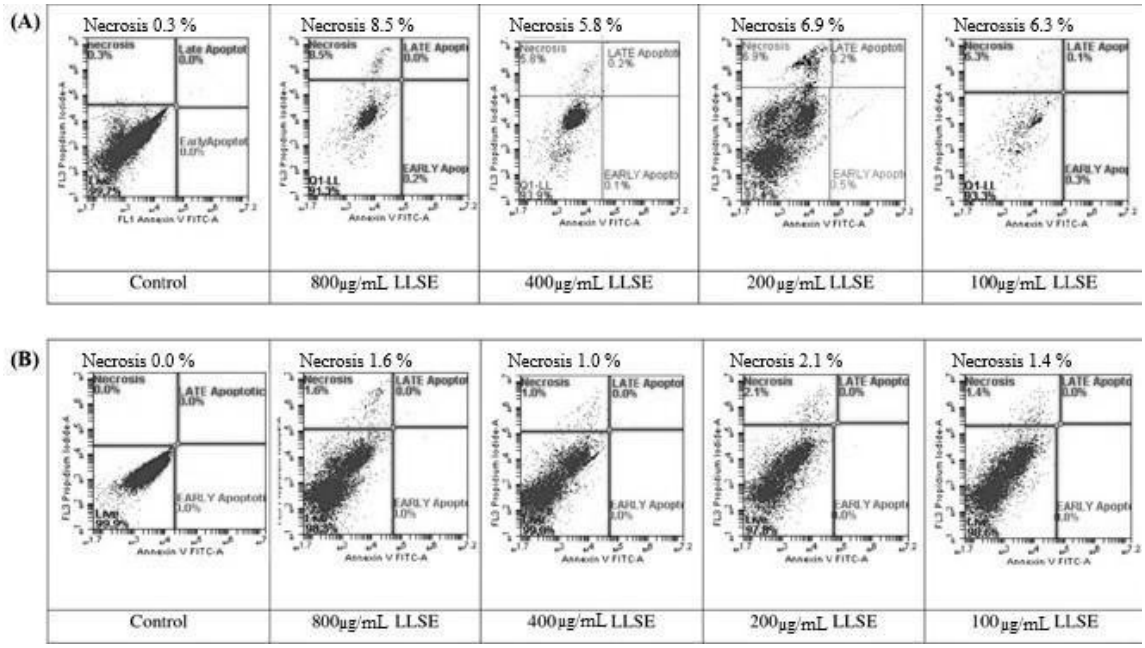


Figure 7: Investigation of apoptotic and necrotic effects of LLSE by flow cytometry analysis, **(A)** the **MDA-MB-231** cell line, **(B)** the **HUVEC** line.

4. Discussion

Imbalances in programmed cell death, a fundamental physiological process, are pivotal in determining cancer cell fate. Cancer initiation arises from DNA alterations, prompting uncontrolled cell division, leading to a disparity between proliferation and apoptosis (Ouyang *et al.,* 2012). Breast cancer, second only to lung cancer in female mortality globally, is linked to lifestyle factors like alcohol consumption and obesity, accounting for approximately 30% of cases (Giaquinto *et al.,* 2022; Öztecik *et al.,* 2023).

While cytotoxic drugs remain crucial in cancer therapy, their severe side effects drive interest in traditional and complementary medicine approaches (Muhamad *et al.,* 2012). Leech saliva extract emerges as a potential candidate for cancer treatment due to its rich composition of over a hundred bioactive compounds influencing cell proliferation and migration in a dose-dependent manner (Shakouri *et al.,* 2022).

Our investigation revealed distinct effects of LLSE at varying doses on cancerous versus healthy cell lines. Specifically, in the MDA-MB-231 cell line, LLSE exhibited dose-dependent reductions in cell proliferation and migration, with the 800 µg/mL dose proving the most efficacious—additionally, LLSE induced apoptosis and necrosis in these cancer cells. Conversely, in the HUVEC line, LLSE promoted cell proliferation and migration *in vitro*, with no significant apoptosis or necrosis observed compared to the control group.

The dose-dependent response of MDA-MB-231 cells to LLSE, leading to decreased cell viability, is particularly noteworthy. Variations in LLSE response were identified based on dosage, duration (24 h and 48 h), and cell line selection using the MTT assay. An IC50 value of 470 µg/mL was calculated, with no discernible difference between 24-hour and 48-hour LLSE administrations in the MDA-MB-231 cell line. Notably, the 800 µg/mL dose of LLSE consistently reduced cell viability in both 24-hour and 48-hour assays. Given that this dose exceeded the IC50 value, it likely accounts for the significant decrease in cell viability. The differential findings between the 800 µg/mL dose and the IC50 value (470 µg/mL) highlight the dose-dependent nature of LLSE's effects. Specifically, while the IC50 value indicates the concentration at which LLSE reduces cell viability by 50%, the 800 µg/mL dose surpasses this threshold and results in a more pronounced reduction in cell viability, which suggests that higher concentrations of LLSE may enhance its cytotoxic effects on MDA-MB-231 cells, potentially leading to more effective inhibition of cancer cell proliferation and survival. Additionally, the consistency of the 800 µg/mL dose's impact across both 24-hour and 48-hour time points underscores its robust efficacy, making it a critical focal point for understanding LLSE's therapeutic potential. Furthermore, a separate study on the breast fibroblast cell line (Hs 578Bst) demonstrated that non-lyophilized leech saliva augmented cell proliferation, migration, and mRNA expression associated with these effects (Ünal *et al.*, 2023). This finding underscores the differential impact of LLSE on cancerous versus healthy cell lines, suggesting its potential as a targeted therapeutic agent.

Cell migration assays revealed an inhibitory effect of LLSE on the migration index of MDA-MB-231 cells, reinforcing its potential role in impeding cancer cell spread. In the literature, migration assay may be used to measure the invasion ability of cancer cell lines (Tan *et al.,* 2023). LLSE demonstrated a stimulating effect on the migration index of HUVEC cells, suggesting potential angiogenic or wound-healing properties in healthy cells. These findings are consistent with prior research indicating the contextual effects of leech saliva, notably highlighting the efficacy of the 800 µg/mL dose above the IC50 in enhancing cell migration.

In a study evaluating LLSE's impact on cell migration, breast cancer cells (MCF-7) and HUVEC lines were investigated. A scratching assay revealed complete coverage of the scratch area by HUVEC cells treated with LLSE after 48 hours, contrasting with the lack of closure observed in MCF-7 cells. This result suggests that LLSE may inhibit the migration of breast cancer cells (Shakouri *et al.,* 2022). Similarly, hirudin, derived from medicinal leech saliva, inhibited cell migration in a glomerular endothelial cell line, particularly in diabetic mice with microangiopathy (Pang *et al.,* 2020).

In vitro studies suggest that LLSE decreases cell migration in cancerous and pathological cell lines; it enhances migration in healthy cell lines (Ünal *et al.,* 2023). Moreover, LLSE's effects on wound healing have been investigated in both *in vitro* and *in vivo* models, with several studies demonstrating positive outcomes (Zakian *et al.,* 2022; Koeppen *et al.,* 2020).

As one of the critical regulators of angiogenesis, VEGF (vascular endothelial growth factor) has crucial roles in endothelial cell proliferation and migration and promotes neovascularization through its binding to the VEGF receptor (VEGFR) (Ferrara, 2004). EGF (epidermal growth factor) is important in embryonic development, development, and wound healing. It binds to the epidermal growth factor receptor (EGFR) and activates signal cascades with many effects, such as cell proliferation, reduced apoptosis, and angiogenesis (Jorissen *et al.*, 2003). Finally, FGF (fibroblast growth factor) is one of the growth factors involved in soft-tissue growth and regeneration, primarily through its interaction with fibroblast growth factor receptors (FGFRs) (Turner and Grose, 2010).

In the MDA-MB-231 cell line, treatment with 800 µg/mL LLSE reduced mRNA expression for EGF, FGF, and VEGF compared to the control group. Additionally, treatment with 400 µg/mL LLSE significantly increased VEGF and EGF mRNA expression compared to the control and all other treatment doses. In contrast, in the HUVEC line, treatment with 100 µg/mL LLSE led to a significant up-regulation of mRNA expression for EGF, FGF, and VEGF compared to all other doses. In a study conducted by Shakouri *et al.* on the effect of *H. medicinalis* saliva on tumour angiogenesis, it was shown that leech saliva application reduced VEGF-A gene expression in the MCF-7 cancer cell line. (Shakouri *et al*., 2022).

The fact that a substance exhibits different effects at different doses in cell culture studies indicates that the substance in question has a complex interaction with biological mechanisms in the cells. This phenomenon indicates that cells exposed to a particular substance show various responses depending on the substance (Lau *et al.,* 2020). The effects observed on different cell lines may be

due to the diversity of factors such as cell receptors, signalling pathways, gene expression, and metabolism, depending on the interaction of the substance with the cells. This diversity may be based on biological differences between cell types (Bondos *et al*., 2022).

In our study, all doses of LLSE caused apoptosis and necrosis in the MDA-MB-231 cell line, with the 800 µg/mL dose causing the most necrosis, while in the HUVEC line cell viability was found to be close to 100% in the flow cytometry analysis performed after applying LLSE, and it was determined that the cells neither underwent apoptosis nor necrosis significantly.

When the literature is examined, there are no studies on the effect of direct leech saliva application on apoptosis or necrosis, as in our study. Instead, studies investigate hirudin's effect on apoptosis or necrosis from the medicinal leeches' saliva (Zhu *et al.,* 2019). These results show that hirudin attenuates apoptosis induced by thrombin in the HMVECs (Human Microvascular Endothelial Cells). A healthy endothelial cell line was used in our study as well. In a study, the effects of hirudin found in the salivary secretion of *H. medicinalis* were observed in glioblastoma tumour cell lines (LN-229 and U251). In this context, it was shown that hirudin treatment drove glioma cells to apoptosis and inhibited glioma cell survival (Zhao, 2015). In another study, the effect of hirudin on bladder cancer cell lines (EJ and J82) and their exosomes was investigated.

Flow cytometry analysis performed after hirudin administration showed that apoptosis significantly increased in both bladder cancer cell lines. It is shown that hirudin significantly induces bladder cancer cell apoptosis (Shen *et al.,* 2022). When the effects of leech saliva on cell death were evaluated by flow cytometry, quite different results can be found in the literature. While hirudin obtained from leech saliva increases the apoptosis in cancer cells, it protects healthy cells from apoptosis. Similarly, it was determined that the cells underwent apoptosis and necrosis after LLSE application in the MDA-MB-231 cell line, while the opposite effect was observed in the HUVEC cell line. Cancer cells often exhibit distinct receptor expression, cellular signaling pathways, and tumor microenvironment profiles compared to healthy cells. The differential responses to varying drug doses can be attributed to several interconnected factors, including pharmacokinetics, receptor saturation, and cellular adaptation mechanisms (Hanahan and Weinberg, 2011; Maman and Witz, 2017). As a result, that can lead to differential responses to bioactive compounds found in leech saliva.

The apoptosis and necrosis assessments further contribute to the nuanced understanding of LLSE's effects. MDA-MB-231 cells exhibited apoptosis and necrosis, particularly at higher doses, indicating a potential cytotoxic impact. Conversely, HUVEC cells showed no significant apoptosis or necrosis, supporting that LLSE might spare healthy cells from such detrimental effects.

Medicinal leech saliva, a complex mixture of bioactive substances, prominently features hirudin, a potent and natural thrombin inhibitor with unique anticoagulant properties. Unlike heparin, hirudin operates independently of cofactors, simplifying its application in preventing blood clot formation (Ünal *et al.,* 2023). The saliva also contains destabilase and vasodilator substances like acetylcholine, alongside other notable components including bdellins, satin, and LCI (leech carboxypeptidase inhibitor) (Liu *et al.,* 2019). These substances stand out for their anti-inflammatory effects, adding another dimension to the therapeutic potential of medicinal leech saliva in areas ranging from anticoagulation to wound healing and vascular health.

Among the limiting factors of the study are budget constraints and the insufficient quantity of available LLSE, which have hindered the conduct of comprehensive tests on the biological activity of LLSE. Nevertheless, meaningful data were obtained when dose groups with limited LLSE were compared with the control. However, the findings from the comparisons provided valuable insights into the effects of LLSE. Future studies exploring the molecular mechanisms of LLSE's effects on different doses and cell lines could reveal important information. This detailed investigation may help us understand how LLSE influences specific genes, signalling pathways, and cell metabolic processes. Molecular-level analyses like these can contribute to a more comprehensive understanding of how LLSE acts at the cellular level and may aid in evaluating potential therapeutic applications.

5. Conclusion

In conclusion, this study provides valuable insights into the differential effects of LLSE on breast cancer cells (MDA-MB-231) and healthy endothelial cells (HUVEC). The observed anti-proliferative, anti-migratory, and potential pro-apoptotic effects in the breast cancer cell line suggest that LLSE possesses promising properties for cancer therapy. Conversely, the enhanced cell viability and migration, along with the absence of apoptosis or necrosis in healthy cells, suggest a context-dependent and selective impact.

The nuanced findings underscore the need for further investigations to decipher leech saliva's intricate mechanisms and potential therapeutic applications in cancer treatment and wound healing. The dual nature of LLSE's impact, inhibiting cancer cells while promoting health in endothelial cells, opens avenues for targeted therapeutic development.

Our results have shown that medicinal leech saliva extract is an intelligent biological agent that works in different ways according to the characteristics of the cell line. Different and variable effects of leech saliva on the cell viability, migration, and death of various cell lines may depend on the selected medicinal leech species, the protein dose or content of the leech saliva, and the applied cell line. We hope this data, reported for the first time, will pave the way for further *in vitro* and *in vivo* studies. Further translational research is warranted to validate these *in vitro* findings and explore the clinical relevance of leech saliva in cancer therapy.

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Conflict of Interests

There are no conflicts of interests between the authors and family members of the scientific and medical committee members or members of the potential conflicts of interest, counselling, expertise, working conditions, share, holding, and similar situations in any firm.

Ethics approval

Ethics committee approval is not required as commercially available cell lines were used in this study.

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