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The Anti-Proliferative Activity of Lyophilised Medicinal Leech (*Hirudo Verbana*) Saliva Extract on Breast Cancer Cell Line (MCF-7) [*]

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Abstract: Cancer is the leading cause of death and morbidity globally and is the second leading cause of death after cardiovascular disease. Breast cancer is the most common cancer in women with a mortality rate of 18 percent. Chemotherapeutics used to treat cancer in modern medicine can have side effects. Medical leech saliva contains substances with anti-proteolytic, platelet aggregation inhibiting, anticoagulant and anti-metastatic activities as natural compounds with fewer side effects. In this study, it was aimed to investigate the anti-proliferative activity of different concentrations of lyophilised medicinal leech secretion extract on MCF-7 (breast cancer) cancer cell lines. For this purpose, cell viability test (XTT) was performed to determine the cytotoxicity after application of various concentrations of medical leech secretion to the cell lines for 24 and 48 hours and the percentage viability of cancer cells was determined at each leech secretion concentration. The IC_{50} value of leech secretion on MCF-7 cell line was calculated as 490.61 µg/ml at 24 hours and 407.04 µg/ml at 48 hours depending on time and dilution rate. It was observed that the anti-proliferation effect of leech secretion applied at different doses on cell viability in MCF-7 cancer cell line increased as the dose and the duration of exposure increased.

Keywords: Cell proliferation, *Hirudo verbana*, medicinal leech, mcf-7 (breast cancer), xtt.

Liyofilize Tıbbi Sülük (*Hirudo Verbana*) Tükürük Ekstraktının Meme Kanseri Hücre Hattı (Mcf-7) Üzerindeki Anti-Proliferatif Aktivitesi

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Öz: Kanser, küresel çapta ölüm ve hastalıkların önde gelen nedenidir ve kardiyovasküler hastalıklardan sonra ikinci önde gelen ölüm nedenidir. Meme kanseri kadınlarda en sık görülen kanser olup ölüm oranı yüzde 18'dir. Modern tıpta kanser tedavisinde kullanıla kemoterapötiklere bağlı olumsuz yan etkiler ortaya çıkabilmektedir. Tıbbi sülük tükürüğü daha az yan etkiye sahip doğal bileşikler olarak anti-proteolitik, trombosit agregasyon inhibitörleri, antikoagülan enzimler ve anti-metastatik ajanlar içermektedir. Bu çalışmada, liyofilize tıbbi sülük salgısı ekstraktının farklı konsantrasyonlarının MCF-7 (meme kanseri) kanser hücre hatları üzerindeki anti-proliferatif etkisinin araştırılması amaçlanmıştır. Bu amaçla, tıbbi sülük salgısının çeşitli konsantrasyonlarının hücre hatlarına 24 ve 48 saat süreyle uygulanmasının ardından sitotoksisiteyi belirlemek için hücre canlılık testi (XTT) yapılmış ve her bir sülük salgısı konsantrasyonunda kanser hücrelerinin yüzde canlılığı belirlenmiştir. Sülük salgısının MCF-7 hücre hattı üzerindeki IC_{50} değeri, zamana ve seyreltme oranına bağlı olarak 24 saatte 490,61 µg/ml ve 48 saatte 407,04 µg/ml olarak hesaplanmıştır. Farklı dozlarda uygulanan sülük salgısının MCF-7 kanser hücre hattında hücre canlılığı üzerindeki anti-proliferatif etkisinin doz ve maruz bırakma süresi uzadıkça arttığı gözlenmiştir.

Anahtar kelimeler: *Hirudo verbana*, hücre proliferasyonu, mcf-7 (meme kanseri), tıbbi sülük, xtt.

[*] *This study was produced from the master thesis of Özgen TURAL*

INTRODUCTION

Cancer represents the most significant cause of mortality and morbidity on a global scale, ranking as the second leading cause of death after cardiovascular diseases (Bell et al., 2023). In 2020, there were over 2.3 million new cases and 685,000 deaths from breast cancer, representing the most commonly diagnosed cancer worldwide. Breast cancer is the most prevalent form of cancer in women, with a mortality rate of 18%. By 2040, the number of newly diagnosed breast cancer cases is projected to increase by over 40%, reaching nearly 3 million cases annually. Similarly, the number of deaths from breast cancer is projected to increase by over 50% from 685,000 in 2020 to 1 million in 2040 (Arnold et al., 2022). Modern medicine employs a range of therapeutic modalities, including radiotherapy, chemotherapy, anti-cancer drugs, and surgical intervention in the management of cancer. It is acknowledged that certain side effects may manifest during or following the administration of this treatment (Bray et al., 2013). The advancement of technology has facilitated the acceleration of cancer research, leading to the introduction of novel methodologies alongside the cancer treatments that have emerged in recent years. A number of studies have demonstrated that medicinal plant extracts and peptides containing poisons have the potential to be effective anti-cancer agents against a range of cancers when used as a form of therapy (Surh, 2003; Deng et al., 2019).

The use of medicinal leeches has a long history in traditional medicine. Recently, there has been a resurgence of interest in this method due to the discovery of various peptides and protein components with medicinal benefits (Abdualkader et al., 2013; Ayhan & Mollahaliloğlu, 2018). The saliva secreted by medicinal leeches contains bioactive compounds that positively affect physiological and cellular activities. These compounds in the saliva of the leech include anticoagulants, vasodilators, bacteriostatics, anti-inflammatories and analgesics (Ayhan et al., 2021). They have positive benefits such as regulating blood pressure, repairing damaged cells of tissues and organs, and strengthening the immune system (Shakouri & Wollina, 2021). The salivary gland secretions of the leech contain antimetastatic activity as well as a protein called antistasin, which inhibits cancer colonisation (Ammar et al., 2015; Shakouri et al., 2022).

This study aimed to investigate the cytotoxicity of lyophilised medicinal leech *(Hirudo verbana*) secretion on breast cancer cell lines (MCF-7).

MATERIAL AND METHOD

Chemicals and Cell Lines: The MCF-7 cell lines were obtained from the Ankara Yıldırım Beyazıt University Central Research Laboratory Application and Research Center, and the study was conducted in this laboratory. The culture of MCF-7 cell lines was conducted by adding 50 ml of FBS (fetal bovine serum) and 5 ml of penicillin-streptomycin to 500 ml of DMEM (Dulbecco's Modified Eagle Medium) in a laminar safety cabinet. The cells were then seeded in fresh medium in 25 cm^2 flasks and grown in a 37°C incubator with a humid atmosphere containing 5% CO_2 until they reached sufficient density.

Preparation of Medical Leech Secretion: The medical leech specimens of the *Hirudo verbana* species were obtained from a farm that has been approved by the Ministry of Agriculture and Forestry. The leeches were maintained in glass jars filled with chlorine-free tap water, with the water changed every two to three days. The species of leech was identified using a Euromex NZ.1903-S trinocular stereo microscope, in accordance with the methods described by Sawyer (1986), Davies (1991), Neubert & Nesemann (1999) and Saglam (2004). The leeches obtained from the production farm were subjected to a four-month starvation period, during which they were not provided with any sustenance. A solution approximating the composition of human blood fluid, comprising 0.07 M sodium chloride and 0.0005 M arginine, was prepared for the purpose of removing the secretions of starved leeches (Abdualkader, 2011). The leeches were provided with an arginine solution and placed in plastic test tubes, which were then placed in a container filled with ice. The solution was allowed to be vomited up by the leeches. The colourless salivary fluids vomited by the leeches were filtered through a 0.8 µm filter, collected in a pool, and subjected to centrifugation at 3500 rpm and a temperature of +4°C for a period of 10 minutes. The secretions were then frozen at a temperature of -80°C for a period of 24 hours and subsequently placed in a lyophiliser for a period of 48 hours, during which time they were transformed into a solid powder.

Protein Analysis in Medical Leech Secretion Extract: The lyophilised leech secretion to be utilised in the forthcoming application was dissolved in DMEM, and the total protein content of the secretion extract was determined using a Bradford Protein Assay Kit (ABP Biosciences, USA), by the Bradford method (Bradford, 1976). Following the determination of the total protein concentration, the various dose amounts (1200 µg/ml, 600 µg/ml, 300 µg/ml, and 150 µg/ml) to be employed in the study were adjusted by serial dilution.

Cytotoxic Evaluation: The 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) test was conducted according to the instructions provided by the manufacturer to assess the cytotoxic impact on the MCF-7 cell line. Following the treatment of the cells with medical leech secretion, the reaction solution was prepared by combining the XTT and activation

solutions with the instructions provided by the kit manufacturer. The cell line was cultured in an incubator at 95% humidity, 5% CO_2, and 37°C. On the day preceding the administration of the treatment, the cell line was uniformly seeded in 96-well plates and subsequently incubated in a CO_2 incubator. A volume of 50 µl of the reaction solution was added to each well and the plates were subsequently incubated in a CO_2 incubator for a period of 2.5 hours. Following a 2.5-hour incubation period, the plates were removed from the incubator and the absorption was measured at 450 nm and 650 nm. Subsequently, the values obtained at 650 nm, corresponding to non-specific absorption, were subtracted from the values obtained at 450 nm. The cells in the positive control group without any secretion were treated with natural rubber extract. The cells in the negative control group, which did not receive any secretion, were considered to be 100% viable. The viability rates of the cells in the other groups were calculated based on the negative control group. The calculation was performed using the formula (experimental group average/control group average) x 100. The XTT test was conducted in triplicate for three incubation rates (1:1, 1:2, 1:4, 1:8, and 1:16 dilution) and two-time points (24 and 48 hours). Additionally, IC50 values were determined by fitting a curve with varying slopes, comparing the response data normalised by the logarithm of the leech saliva concentration.

Statistical Analysis: The IC_{50} data obtained by the XTT test and measured at 24-48 hours were subjected to statistical analysis using the Excel power regression program. The mean, standard deviation, and percent viability were calculated using the IBM SPSS 21 package program (IBM SPSS Inc., Chicago, IL).

RESULTS

Cytotoxic effect of Medical Leech Secretion Extract on cancer cells: Cell viability (%) = Experimental Group at Average Value / Control Group at Average Value x 100.

The cytotoxicity of the control group was accepted as 100% viable during the calculation. According to the power regression of XTT results of different doses of medicinal leech extracts applied to the MCF-7 cell line in the Excel program, IC_{50} value at 24 hours was calculated as 490.61 µg/mL (Figure 1) and 407.04 µg/mL at 48 hours (Figure 2).

The cytotoxic effects of leech secretion on MCF-7 cells are presented in Figure 3. As can be seen from the figure, there is a significant reduction in the viability of breast cancer cells in a dose-dependent manner. According to the 24th hour measurement, the percent viability rates were statistically evaluated and the percentages of viability in dose groups were found as 75 µg/ml 98.62%, 150 µg/ml 75.21%, 300 µg/ml 61.33%, 600 µg/ml 38.66% and 1200 µg/ml 31.64%. At the 48-hour mark, the percent viability rates were as follows: 75 µg/ml, 82.18%; 150 µg/ml, 73.87%; 300 µg/ml, 57.98%; 600 µg/ml, 41.73%; and 1200 µg/ml, 31.79% (Table 1).

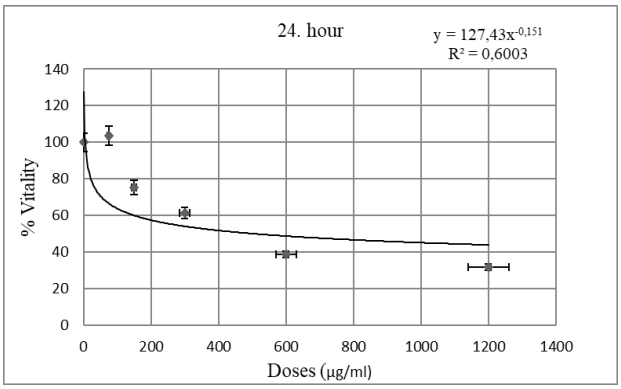

Figure 1. IC_{50} plot relative to lyophilized leech extract at 24 hours.

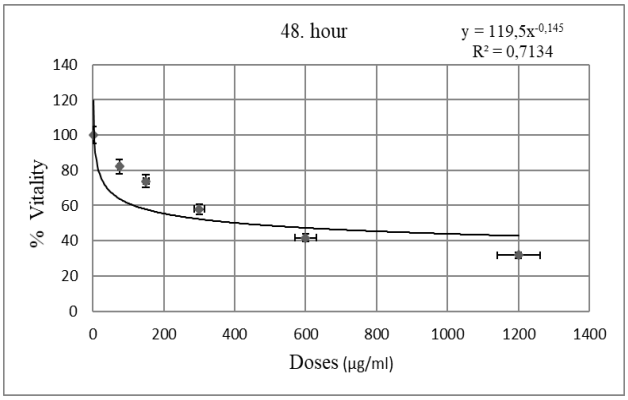

Figure 2. IC_{50} plot relative to lyophilized leech extract at 48 hours.

Table 1. Cell viability (%) was measured at 24 and 48 hours according to the absorbances obtained as a result of the XTT test.

	Negative Control	1200 µg/ml	600 µg/ml	300 µg/ml	150 µg/ml	75 µg/ml	Positive Control
24 hour	%100	%31,64	%38,66	%61,33	%75,21	%98,62	0,62
48 hour	%100	%31,79	%41,73	%57,98	%73,87	%82,18	0.78

It was observed that an increase in the concentration of leech secretion extract at 24 and 48 hours resulted in a linear decrease in viability rates (Figure 3). These values demonstrate the significant cytotoxic effect of leech secretion extract on MCF-7 cells.

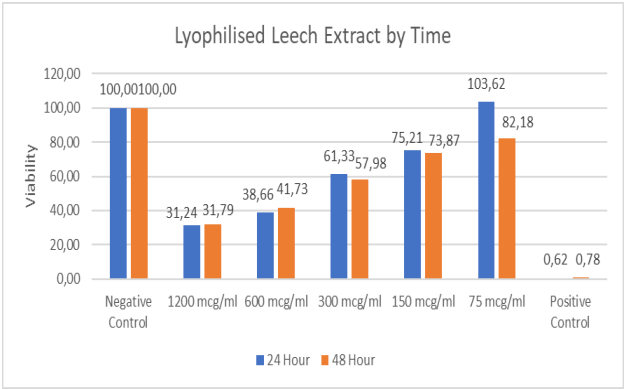

Figure 3. Effects of different concentrations of leech secretion extract on MCF-7 cell viability.

DISCUSSION

The etiology of cancer is attributed to a deficiency in DNA repair or mutations during DNA replication. The principal risk factors are environmental factors, lifestyle habits, and the genetic background of individuals (Shaheen et al., 2011; Nenclares & Harrington, 2020). Breast cancer is the most prevalent form of cancer among women globally, accounting for the majority of cancer-related mortalities (Akram et al., 2017). The National Cancer Institute (NCI) categorizes cancer treatments into five main groups: chemotherapy, radiotherapy, surgery, immunotherapy, and stem cell transplantation (Hanahan & Weinberg, 2011). Nevertheless, despite the extensive scientific studies conducted in this field, patients are typically exposed to adverse effects associated with these treatment modalities. It is, therefore, necessary to identify agents with effective pharmacological activities, low cost, and minimal disease resistance, as well as natural compounds with fewer side effects, for use in cancer treatment (Muhammad et al., 2022; Aysin et al., 2024).

Medical leech therapy is a well-established traditional and complementary treatment method in Turkey and across the globe. Historically, leech saliva has been employed in traditional Chinese medicine as a treatment for various cancers, including esophageal, stomach, bowel, uterine, and breast cancer (Guo, 2006). Currently, a multitude of scientific studies are being conducted with leeches themselves or their secretions, and drugs are being derived from them for use in the treatment of certain diseases (Gödekmerdan et al., 2011). In Russia, the use of Piyavit, an antithrombotic derived from leeches, was authorised in 1993 (Minkin, 1990; Baskova et al., 1995). Furthermore, pharmaceuticals such as Hirudin (Transgeen-France), Eglin (CIBA/Geigy-Switzerland), Gelin (EuroBioPharm-Netherlands), Ghilanten (Merrel Dow Research Inst.- USA), and Orgelase (Biopharm-UK) are also manufactured by companies (Salzet, 2001). The secretions of the leech salivary glands have been demonstrated to possess antimetastatic activity. The saliva of the leech contains a protein, antistasin, which has been demonstrated to inhibit cancer colonisation. The secretions have been found to contain anti-proteolytic, platelet aggregation inhibitors, and anticoagulant enzymes (Baskova et al., 2008; Alaama et al., 2024). Moreover, anti-tumour activity has been identified in other components, including hyaluronidase (Ammar et al., 2015). In the present study, we, therefore, sought to investigate the effect of secretion from the medicinal leech *Hirudo verbana* on cell viability when applied to the MCF-7 cancer cell line. To date, research has been conducted into the antiproliferative or anticancer activities of leech secretion in a range of cancer types. To illustrate, a study assessing the efficacy of *H. medicinalis* medicinal leech secretion employed the breast cancer cell line (MCF-7) as a cancer cell line and the HUVEC cell line as a control group. The direct application of leech secretion was observed to exhibit 50% antitumor activity on MCF-7 cancer cells. However, the antitumor activity rate was found to increase to 97% when the leech secretion was administered in a liposomal form. Furthermore, the application demonstrated a 10% antiproliferative effect on healthy cell lines (HUVEC) (Shakouri, 2022). In this study, we diverged from Shakouri's approach by investigating the secretion of the leech of the *H. verbana* species, which is widely distributed in natural habitats in our country and worldwide. This is the first study to examine this phenomenon.

Similarly, an in vitro investigation of the effects of the extract of *H. verbana* medicinal leech secretion on the mammary fibroblast cell line was conducted, the results of which were revealed. The findings of this study indicate that the extract of leech secretion when administered at varying doses, exerts notable influences on cell viability, cell migration, and gene expressions that may be implicated in these effects within the breast fibroblast cell line (Ünal et al., 2023). Furthermore, investigations were conducted to ascertain the effects of the same *H. verbana* medicinal leech secretion on human umbilical cord vein endothelial cell (HUVEC-CRL-1730), breast fibroblast (HTB-125), and mesenchymal stem cell (PCS-500-012) lines. These studies focused on elucidating the underlying mechanisms of action, particularly about cell viability, wound healing, apoptosis, and gene expressions. The leech secretion was administered at defined doses to the cells produced by cell culture using the MTT assay to assess cell viability and proliferation. The impact of leech secretion on wound healing was assessed using an in vitro wound model. Gene expression of VEGF, EGF, and FGF was determined in cells treated with varying doses of leech secretion through RT-PCR. The potential impact of leech secretion on apoptosis was examined through flow cytometry. Regarding cell proliferation, the cell viability of the breast fibroblast cell line treated with varying doses of leech secretion was observed to be markedly elevated in comparison to the control group ($p < 0.001$). In the flow cytometry analysis, the cell viability rates approached 100% in the HUVEC and mammary fibroblast cell lines, whereas necrosis was observed in the mesenchymal stem cell line (Tırık, 2022). It can therefore be hypothesised that leech secretion has anti-cancer potential against cancer cells due to the bioactive compounds it contains.

The results of our study corroborate previous findings, as evidenced by the confirmation of lower IC_{50} values for cancer cells. It is postulated that this cytotoxic and apoptotic effect may be due to bioactive compounds such as hirudin, hyaluronidase, and antistasin present in

leech secretion. In light of these findings, it can be concluded that leech secretion may contribute to cancer treatment with its cytotoxic activity as a natural agent. However, further studies in detail with various cell lines and experimental setup are needed to explore the mechanism of action.

CONCLUSION

The findings of our study have significant implications for the development of drugs that can effectively address the various facets of cancer treatment. Despite the growing interest in leech and its bioactive compounds, there is a need for further scientific evidence to ascertain their potential anticancer properties. Future research should prioritise in vitro and in vivo studies to elucidate the pharmacological mechanism of leech and its bioactive compounds.

In this context, significant data were obtained on the leech secretion extract, which we hypothesise may represent a novel potential agent. A comparison of the antiproliferation effect of leech secretion on cells in our study with that of previous studies in the literature suggests that the findings may vary depending on the type of leech selected, the protein concentration of the secretion obtained from the leech, and the cell line selected by the researchers. The results presented in this study represent a novel contribution to the existing literature on the mechanism of action of medicinal leech secretion and provide a foundation for further research. Nevertheless, further investigation and support in vivo are required to elucidate the pathways and molecular mechanisms of action of medicinal leech secretion. The present study demonstrates that medicinal leech secretion extract may serve as a promising biotherapeutic agent, offering the potential to enhance medical treatments through its use as an adjuvant.

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