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Research Article

Cytotoxic and apoptotic effect of *Lemna minor* L. extract on human osteosarcoma (Saos-2)

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Abstract: Osteosarcoma is a malignant form of bone cancer that responds poorly to chemotherapy and has a significant incidence of recurrence. Recent studies suggest the need for new natural agents to support treatment. Lemna minor is a macrophyte frequently used in traditional treatments and is known to have high antioxidant and antimicrobial properties due to the phenolic compounds it contains. This study investigated the anticancer activity of the *L. minor* on Saos-2 cancer cells and the apoptosis pathways. *L. minor* was extracted in ethanol by microwave technique. The extract showed cytotoxic activity (59-79%) on Saos-2 cells but did not harm healthy human bone cells (hFOB). Expression of bax, bcl-2, caspase-3, and caspase-8 genes was investigated by RT-PCR to examine the apoptosis-inducing pathways. RT-PCR analysis revealed that L. minor induced apoptosis via a mitochondria-dependent pathway by affecting the bax/bcl-2 ratio. The study suggests *L. minor* as a promising natural agent for bone cancer treatment.

1. INTRODUCTION

Osteosarcoma is a malignant bone tumor that mainly affects children, young adults aged 0-24 years, and those over the age of 60 (Beird *et al.*, 2022; Janani *et al.*, 2022). Advances in treatment have led to a rise in survival rates to 60-70% over the last three decades (Du *et al.*, 2015; Mintz *et al.*, 2005;). Despite this improvement, the disease has a high recurrence rate, frequently metastasizing to other body parts, particularly the lungs (Janani *et al.*, 2022; Li *et al.*, 2016). Studies indicate that the survival rates post-metastasis range from 20-30% (Durfee *et al.*, 2016). However, even with numerous recent studies, the metastasis mechanism remains incompletely understood (Li *et al.*, 2016).

The current management of osteosarcoma relies on surgical, chemotherapeutic, and radiotherapeutic interventions (Kazantseva *et al.*, 2022; Li *et al.*, 2016). Nevertheless, it is widely acknowledged that osteosarcoma displays poor responsiveness to chemotherapy and has a notable incidence of recurrence (Bernardini *et al.*, 2018; Mintz *et al.*, 2005). Despite chemotherapy treatments, metastatic rates have remained unaltered over the last two decades (Kazantseva *et al.*, 2022). Conversely, researchers have found that drugs used in osteosarcoma treatment have several side effects, including cardiovascular issues, and suppress the immune system (Abdelrheem *et al.*, 2021). In addition, chemotherapy has been reported to induce apoptosis in healthy cells (Majeed *et al.*, 2022; Wang *et al.*, 2020) While the occurrence of

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osteosarcoma is infrequent, reports suggest the necessity for novel drugs and approaches in addressing this ailment due to various factors, including recurrence following treatment, aggressive metastasis, and inadequate response to chemotherapy (Bernardini *et al.*, 2018; Janani *et al.*, 2022; Kumar *et al.*, 2014). Tarkang *et al.* (2015) recommended that plant-based treatments used in complementary medicine could be integrated with surgical and chemotherapeutic regimens in cancer treatment. Additionally, according to Prakash *et al.* (2007), plants constitute a valuable alternative agent source with minimal or no side effects. Kim and Gilbert (2019) claimed that extracts obtained from plants with high levels of secondary metabolites possessing antioxidant properties could aid in preventing cancer or supporting its treatment. According to Kamran *et al.* (2022), terpenoids, which are secondary metabolites, exhibit synergistic effects specifically against cancer cells that have multidrug resistance when paired with cancer drugs such as doxorubicin, cisplatin, paclitaxel, sorafenib, and 5-fluorouracil. Hence, certain terpenoids hold the potential to enhance traditional chemotherapy's response. Additionally, the World Intellectual Property Organization (WIPO) patent database registers 23 patents related to terpenoids' anticarcinogenic potential in treating various types of cancer. Chai *et al.* (2015) noted that while edible macrophytes have not been studied as thoroughly as terrestrial plants, recent research has shown that they have valuable anticancer properties and represent an untapped natural resource for further study. Macrophytes are photosynthetic organisms visible to the naked eye that live in aquatic environments (Justin *et al.*, 2022; Nassouhi *et al.*, 2018). This category includes macroalgae and bryophytes such as liverwort, moss, fern, and angiosperms (Rejmankova, 2011; Unadkat & Parikh, 2021). Studies with aquatic macrophytes have found that extracts from *Pistia stratiotes* and *Eichhornia crascipes* have cytotoxic effects on bone, lung, breast, and prostate cancers (Abraham *et al.*, 2014; Kumar *et al.*, 2014).

Lemna minor is a freshwater floating perennial aquatic plant of the family Lemnaceae (Klaus *et al.*, 2013; Petrova Tacheva *et al.*, 2020). *L. minor* is frequently used in ecotoxicological studies due to its rapid growth, adaptability to laboratory conditions, ease of harvesting, and high biomass production (Alkimin *et al.*, 2019; Ceschin *et al.*, 2020; Khan *et al.*, 2020; Nassouhi *et al.*, 2018; Panfili *et al.*, 2019). Although current studies focus mainly on the removal of heavy metals and toxic chemicals, *L. minor* has a wide range of applications, including feed and food production, biofuel and biogas production, and biotechnological research (Bog *et al.*, 2019; Yahaya *et al.*, 2022). In addition to these studies, *L. minor* is a plant that has been widely used in traditional medicine and homeopathy for many years (Petrova-Tacheva *et al.*, 2019). *L. minor* is known to be used externally as an antipyretic, diuretic, and anti-inflammatory for upper respiratory tract and chronic rheumatic diseases, as well as for eczema, acne, wound healing, and insect bites (Al-Snafi, 2019). Many commercial products derived from L. minor extract are used to treat allergic asthma, rhinitis and nasal congestion. Lemna species contain phenolic compounds such as gallic acid, tannins, flavonoids, anthocyanins, and quercetin, and compounds known as steroids such as thiols and terpenes, suggesting that they may have antimicrobial, antioxidant, and even anticarcinogenic properties (Gonzalez-Renteria *et al.* 2020). Many studies have shown that *L. minor* is an effective antimicrobial (Dafalla, 2015; Gonzalez-Renteria *et al.*, 2020; Gülçin *et al.*, 2010; Tan *et al.*, 2018) and antioxidant (Gülçin *et al.*, 2010; Iskandar *et al.*, 2019; Kim *et al.*, 2012, Saritha & Saraswathi, 2014, Tran *et al.*, 2021). Despite this information cancer studies on *L. minor* are very limited. Within the scope of this study, the anticarcinogenic activity of *L. minor* extract, extracted by microwave method and dried with a lyophilizer, on Saos-2 was investigated. To understand the mechanism of the observed activity, the expressions of bax, bcl-2, caspase-3, and caspase-8 genes were determined by real-time PCR

2. MATERIAL and METHODS

The study was carried out at the Life Sciences Application and Research Center Laboratories of Gazi University (Microbiology, Molecular Biology, and Chemistry Research Laboratories).

The cytotoxicity study was conducted to analyze the efficacy of *L. minor* extracts on cancer cells. Subsequently, RT-PCR was employed to determine the pathway and gene expression induced by *L. minor* extracts with anticarcinogenic activity in the apoptosis study.

2.1. Development and Identification of *L. minor*

The *L. minor* used in the study was obtained from an aquarium store and cultivated at the Hydrobiology Laboratory in the Biology Department of Ankara University. The macrophyte was identified by comparing its morphological features to those described in Güner and Ekim (2014). *L. minor* was cultured in 40% Hoagland medium under a 12:12 light: dark photoperiod at 19.3±2.1 °C, as reported by Ergönül *et al.* (2019).

2.2. Plant Extraction

The plants were harvested and washed with distilled water to eliminate any environmental contaminants. After washing, the plants were air-dried on blotters under normal conditions. Dried *L. minor* was then powdered using a porcelain mortar. An optimization study determined the ideal conditions for extracting phenol from the plants. The study revealed that the highest phenol concentration was achieved at 850 watts, 60% ethanol, and 90 seconds. These conditions were used consistently throughout the study (Dogan *et al.*, 2022). To extract the phenol, one gram of the dried plant sample was mixed with 20 milliliters of 60% ethanol (99.5% Merck, Germany) and subjected to extraction using a microwave device at 850 watts for 30 seconds, done in three runs. The resulting extracts were separated by centrifugation at 2500 revolutions per minute for 20 minutes and filtered through Whatman No. 1 filter paper placed in the upper phase (Yağcıoğlu, 2015). The extracts were then freeze-dried in a lyophilizer (Christ, Alpha 1-2) and stored at +4˚C until analysis (Dogan *et al.*, 2022; Karami *et al.*, 2015).

2.3. Cell Lines

The research utilized a human bone cancer cell line (Saos-2 ATCC HTB-85) and normal human osteoblast (hFOB ATCC CRL-3602) as control. These cell lines were purchased from the American Type Culture Collection (ATCC). The cells stored in liquid nitrogen were thawed in a 37°C water bath containing 10% ice and transferred to the cell medium. To determine the number of viable cells, 1 mL cell suspension was prepared and centrifuged. The obtained pellet was stained with trypan blue to distinguish between live and dead cells, with dead cells appearing blue. The trypan blue-cell mixture was placed on a Thoma slide and live cells were counted under an inverted microscope, according to Doğan (2011).

Cells were grown in RPMI 1640 medium (Capricorn, Germany) containing 1% penicillin/streptomycin antibiotic (Capricorn, Germany) and 10% fetal bovine serum (FBS, Capricorn, Germany) at 37°C in a 5% carbon dioxide atmosphere. The medium was changed every 2 days. Cells showing 80-90% spreading in the flasks were removed with trypsin/EDTA and counted (Doğan, 2023; Morita *et al.*, 2002, Uçar *et al.* 2016).

2.4. Investigation of Cytotoxicity

In 96-well plates, $2x10^5$ cells were transferred to each well and allowed to grow. *L. minor* extract was dissolved in a cell medium. Concentrations of 5000-10 µg/mL were prepared by serial dilution (Agan *et al.*, 2020). The prepared samples were applied to developing healthy and cancer cells and cultured for 12 and 24 hours. At the end of the incubation, the cytotoxicity level was measured by colorimetric evaluation using the MTT kit (Biotium, USA). 20 µL MTT was added to all wells and incubated at 37°C for 3 hours. After 3 hours, the samples in the wells were removed, and the dye was dissolved with DMSO and measured at a wavelength of 570 nm (Epoch, BioTek). Cells without extract were used as a control. The control was considered 100% viable and % inhibition and IC_{50} values were calculated accordingly (Kaya *et al.*, 2024; Luca *et al.*, 2022).

2.5. Identification of Apoptotic Cells

IC_{50} values for the *L. minor* extract were obtained through cytotoxicity analysis. The mRNA expression levels of genes involved in the regulation of apoptosis, bax, bcl2, caspase3, and caspase8, were measured by quantitative real-time PCR (qPCR) (Janani *et al.*, 2022; Wang *et al.*, 2020). RNA was extracted from cells treated with plant extract and after cDNA synthesis, the PCR products were labeled with SYBR green dye (Kuang *et al.*, 2018).

2.5.1. *Total RNA isolation*

Total RNA isolation was performed using the Hybrid-R kit protocol (GeneAll, South Korea). The extracted RNA was quantified and evaluated for purity using Qubit4 (Invitrogen). Samples with an absorbance range of A_{260}/A_{280} ratio between 1.8-2.0 were chosen to initiate complementary DNA (cDNA) synthesis (Kuang *et al.*, 2018).

2.5.2. *cDNA synthesis*

The cDNA synthesis of isolated and quantified mRNA samples abided by the protocol of the cDNA Synthesis Kit (ABT, Wizbio WizScript™ W2211). To begin with, 10 µL of the sample was added to the mixture outlined in Table 1 before undergoing cDNA synthesis in the Applied Biosystems Veriti heat cycler model, following the parameters depicted in Table 1. Eventually, the cDNA samples were frozen at -20°C until carrying out the qPCR study.

Table 1. cDNA synthesis mixture and conditions.

Mixture	Volume (µL)
20X dNTP mix	1
WizScript™ RTase	1
RNase free water	3.5
10X reaction buffer	2
Random hexamer	2
RNase Inhibitor	0.5
RNA Sample	10
Total	20

Temperature (°C)	Time (Minute)	Cycle
25	10	1
37	20	6
85	5	1
4	∞	∞

2.5.3. *Determination of gene expression by Real Time PCR*

Changes in the expression of bax, bcl-2, caspase-3, and caspase-8 genes in Saos-2 cells treated with *L. minor* extract compared to untreated cells were evaluated using RT-PCR (Applied Biosystems, 7500 Fast Real-Time PCR). The PCR reaction mixture contained 12.5 µL of 2x SYBR Green qPCR master mix, 1 µL of cDNA, 2 µL of primer mix (10 µM stock solutions), and 9.5 µL of PCR grade water. RT-PCR was programmed by following conditions for one cycle: 95 °C for 10 min and 40 cycles: 95 °C for 10 s and 60 °C for 30 s. Analysis of the expression levels of the genes was carried out using the $2^{-\Delta\Delta Ct}$ method. The ACTB gene was determined as a housekeeping gene. Primers are listed in Table 2.

Table 2. Primers used to determine gene expression.

Primer	Primer Sequence
BAX-f	GATGGACGGGTCCGGGG
BAX-r	CGATCCTGGATGAAACCCTGA
BCL2-f	GGATAACGGAGGCTGGGATG
BCL2-r	TGACTTCACTTGTGGCCCAG
CASP3-f	ATTTGGAACCAAAGATCATACATGG
CASP3-r	TTCCCTGAGGTTTGCTGCAT
Caspase 8-f	GCGGAGGGTCGATCATCTAT
Caspase 8-r	CACAACTCCTCCCCTTTGCTG
ACTB-F	CATCCTCACCCTGAAGTACC
ACTB-R	TGAAGGTCTCAAACATGATCTG

3. RESULTS

3.1. Investigation of Cytotoxicity

Saos-2 cells were cultured and transferred to 96-well plates at 80% confluence. Time and concentration experiments were conducted using *L. minor* extract concentrations ranging from 0-5000 µg/mL, and cytotoxicity was observed at 12 and 24 hours. At 12 hours, no cytotoxic effects were observed at initial concentrations of 10, 20, 40, and 78 µg/mL, but effectiveness was observed at concentrations of 156 µg/mL and above. Cytotoxicity was detected at all concentrations within 24 hours of applying the extract. The highest mortality rate was 79% after 12 hours of application, increasing to 95% after 24 hours. The death rate of cancer cells appears to vary depending on the concentration and duration of treatment, with greater concentrations resulting in increased cancer cell death (Figure 1).

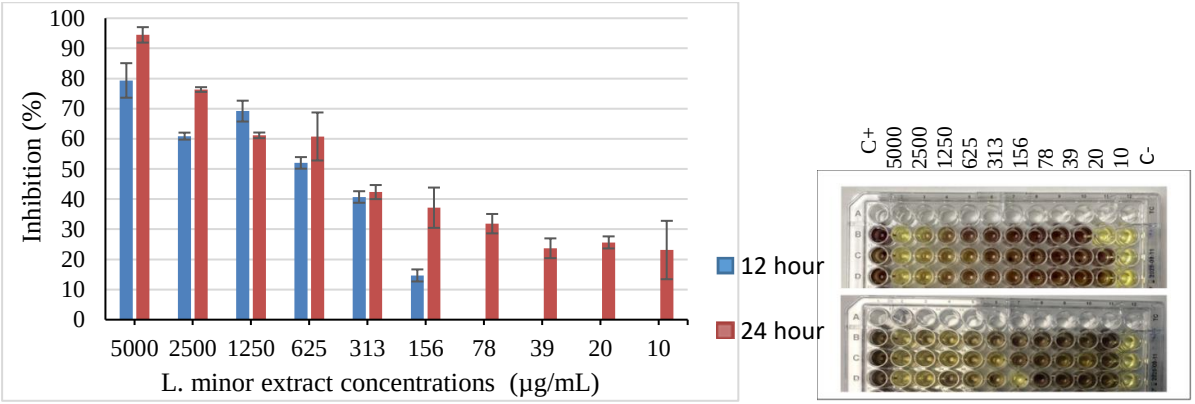

Figure 1. Inhibitory effect of *L. minor* accumulations on bone cancer cell line (Saos-2) at 12 and 24 hours. C+(Positive control): extract-free cells, C-(Negative control): medium without cells and extracts.

Macrophytes with cytotoxic effects on cancer cells should lack cytotoxicity on healthy cells. For control purposes, this study utilized the hFOB cell line, a healthy human epithelial cell. The results demonstrated that low extract dosages generally did not harm healthy cells, but rather enhanced cell growth. Furthermore, at a concentration of 313 µg/mL, *L. minor* extract exhibited a 42% cancer cell mortality rate, yet increased the growth of healthy cells by 25%. At this concentration, *L. minor* did not harm the healthy cells or increase their development. However, when the concentration exceeded 625 µg/mL, the extracts were observed to cause cell inhibition. The results indicate that the extracts are non-toxic, with IC_{50} values below the inhibitory concentration of 625 µg/mL (Figure 2).

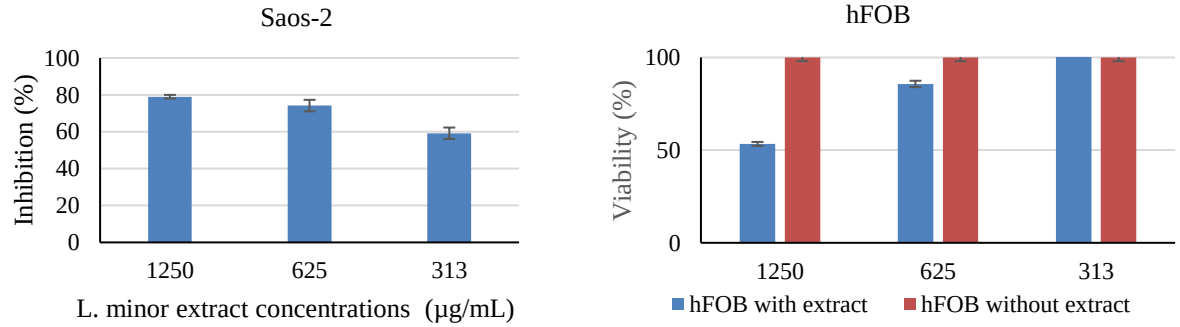

Figure 2. Effect of *L. minor* concentrations in the IC_{50} range on cells A. Saos2: cancer cell, B. hFOB: healthy cell at 24 h.

3.2. Identification of Apoptotic Cells

In this study**,** compared with control cells, the expression of bax, caspase-3, and caspase- 8 *L. minor* treated groups was significantly increased, whereas the level of bcl-2 was decreased (Figure 3).

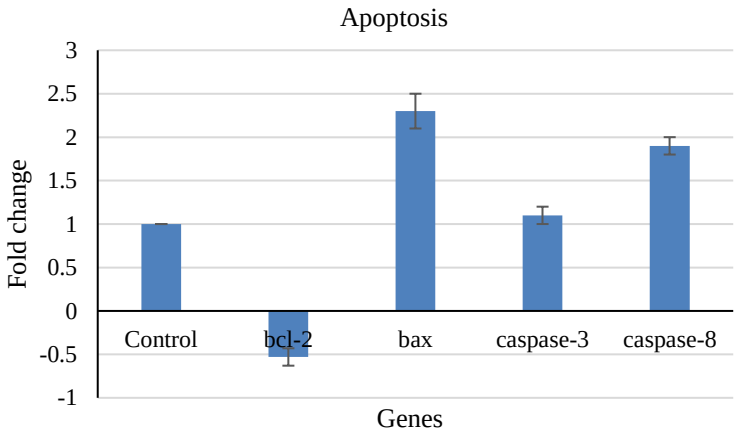

Figure 3. Bcl-2, bax, caspase-3, and caspase-8 expression.

4. DISCUSSION and CONCLUSION

Cancer is the second leading cause of death worldwide (Abdelrheem *et al.*, 2021; Janani *et al.*, 2022). Osteosarcoma, a type of malignant bone cancer that mainly affects children and young adults aged 0-24 years, accounts for 5% of all childhood cancers (Majeed *et al.*, 2022). Although osteosarcoma is rare, there is an urgent need for alternative medicines and new approaches due to factors such as treatment relapse, aggressive metastasis, and poor response to chemotherapy (Bernardini *et al.*, 2018; Janani *et al.*, 2022; Kumar *et al.*, 2014,). Chai et al (2015) have highlighted the potential of macrophytes in cancer research, although there has been a limited amount of research carried out in this area. Abraham *et al.* (2014) reported that the hexane and methanol extracts from *P. stratiotes* leaves caused a 60% inhibition on human bone cancer MG 63. Bernardini *et al.* (2018) investigated the effect of *Padina pavonica* algae extracts on human bone cancer Saos-2 and MNNG. As a result of the study, they determined that the extract had a cytotoxic effect on both cell lines. In the proliferation study, they identified a significant decrease in Saos-2 proliferation - by 70% - when an IC_{50} dose of the extract applying. In a different study, it was determined that there was almost 90% inhibition of the human bone cancer U2OS cell line incubated with *Nymphoides peltata* (Little Water Lily) extract obtained with 10% methanol for 48 hours (Du *et al.* 2015). The results of our study indicate that *L. minor* exhibited high cytotoxic activity against the Saos-2 cell line. Cytotoxicity was found to be 79% in 12 hours of incubation and 95% in 24 hours of incubation. In comparison to other studies, it is evident that the rate of killing bone cancer cells is significantly high. This activity is believed to be related to the compounds such as secondary metabolites contained in macrophytes. A body of evidence indicates that antioxidants and anticarcinogenic effects are linked, with phenolic compounds playing a pivotal role in cancer prevention (Janani *et al.*, 2022). The phytochemical content of *L. minor* used in this study was analyzed by GC-MS in previous studies. It was found that *L. minor* extract has a vibrant content, especially in phytol, thymol, hexanal, 2,4-di-tert-butylphenol, 5-tetradecane, cetene (Dogan *et al.*, 2022). These compounds are thought to contribute to the high anticancer activity of our plant.

Despite numerous studies conducted in recent years, the mechanism underlying the development and metastasis of osteosarcoma is still not fully understood. Nevertheless, the consensus of these studies is that apoptosis in osteosarcoma is related to bcl-2/bax balance or caspase activation (Imran *et al.*, 2021; Wang *et al.*, 2020). Wang *et al.* (2020) also suggest that apoptosis in osteosarcoma occurs through a mitochondria-dependent pathway with increased pro-apoptotic bax expression and decrease bcl-2 expression. Recent studies have shown that metabolites obtained from plants induce human cancer cells to apoptosis. Janani *et al.* (2022) reported that certain plant extracts, such as Merremia emarginata caused apoptosis in Saos-2 by increasing bax expression and decreasing bcl-2 expression. Kamran *et al.* (2022) stated that terpenoids such as phytol, thymol, carvacrol, and thymoquin derived from plants induced the

death of human cancer cells such as Saos-2, MG 63 by using caspase-9, caspase-3 pathways and regulating bax/bcl-2 balance. Similarly, Zhu (2022) showed that pseudolaric acid B (PAB) a natural diterpenoid, down-regulated the expression of Bcl-2 and pro-caspase-3 and increased the expression of bax and caspase 3, leading to human bone cancer (MG 63) to apoptosis via the mitochondrial pathway. In this experiment, the human osteosarcoma cell line Saos-2 was first treated with *L. minor* extract. The expression of bax and caspase-8 was significantly increased in the *L. minor* extract groups, whereas the level of bcl-2 was decreased when compared with the control cell. In our study, after the treatment of L. *minor* extract, the cell viability was decreased. Our study results revealed that *L. minor* extract directs cancer cells to apoptosis through a mitochondria-dependent pathway maintaining the bax/bcl-2 balance. A significant increase in caspase 8, the initiator gene of apoptosis, was observed in cells treated with *L. minor*. However, no significant increase was observed in caspase-3, which is considered a lethal caspase. Caspase-8 is responsible for the initiation of apoptosis via the extrinsic pathway. Activated caspase-8 activates lethal caspase-3/caspase-7, leading the cancer cell to apoptosis (Boice, A., and Bouchier-Hayes, 2020). It is thought that *L. minor* initiates apoptosis with caspase 8, but may subsequently activate caspase-7 instead of the studied caspase-3. The results we obtained showed that our study is compatible with previous similar studies.

The high rate of recurrence and metastasis of bone cancer, coupled with the fact that chemotherapy also damages healthy cells, underscores the necessity of integrating traditional treatments with chemotherapy and drugs in bone cancer treatments. The study demonstrated that the *L. minor* macrophyte had a lethal effect on the human bone cancer Saos-2 cell line, but did not harm healthy human cells. These findings indicate that *L. minor* has potential as a treatment for osteosarcoma.

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Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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