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ANTICARCINOGENIC EFFECT OF ETHANOLIC EXTRACT OF ACHILLEA MILLEFOLIUM ON OSTEOSARCOMA (AN IN VITRO STUDY)

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ABSTRACT

INTRODUCTION: Natural products, with their diverse chemical structures and biological activities, have gained attention for their anticancer potential. While Achillea millefolium (yarrow) has shown promise in cancer research, its effect on osteosarcomaremainsunexplored.

OBJECTIVES: To investigate the anticancer efficacy of the ethanolic extract of Achillea millefolium on osteosarcoma cells (MG-63)versuscisplatin.

MATERIALS AND METHODS: The ethanolic extract of Achillea millefolium (AMEE) was obtained using maceration protocol followed by solvent evaporation. The cell viability of MG-63 cells was assessed using the MTT assay with serial dilutions of AMEE to determine the IC50 concentration and compare it to cisplatin. Apoptosis was analysed using flow cytometry with the Annexin-V assay, and migration was assessed by a wound healing assay. Statistical analysis was conductedwithGraphPadPrism(8.0.2).

RESULTS: The cell viability assay showed that AMEE exerted a dose-dependent cytotoxic effect on MG-63 cells, with an IC50 of 104.5 µg/mL after 48 h. At this concentration, early apoptosis was only 6.28 ± 1.36% compared to 15.42 ± 0.3% for cisplatin. In contrast, late apoptosis was significantly higher for the extract at 12.39 ± 4.5%, while cisplatin reached only 5.23 ± 0.1. The extract also demonstrated significant antimigratory potential, achieving a closure of 46.23 ± 8.0% compared

CONCLUSION: Achillea millefolium extract exhibited significant tumoricidal effects on osteosarcoma cells by reducing cell viability, inducing apoptosis, and inhibiting cell migration, highlighting its potential for cancer therapy.

RUNNING TITTLE: Anticarcinogenic Effect of Ethanolic Extract of Achillea Millefolium on Osteosarcoma (An In Vitro Study)

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INTRODUCTION

Osteosarcoma, or osteogenic sarcoma, is the most prevalent primary bone cancer in children and young adults, with an estimated incidence of about eight cases per million annually [1, 2]. It typically arises in the long bones of the limbs, close to the growth plates in the metaphysis. The femur is the most affected bone, followed by the tibia and humerus. Less frequently, it can also occur in the skull or jaw bones and the pelvis [3].

The treatment of osteosarcoma is challenging, typically involving a combination of preoperative adjuvant chemotherapy, surgical resection, and postoperative chemotherapy [4, 5]. Despite these efforts, traditional chemotherapeutic agents such as cisplatin and doxorubicin often come with a range of side effects and limited precision, negatively affecting patients' quality of life [6]. Furthermore, osteosarcoma is highly resistant to treatment, as it typically requires high initial chemotherapy doses but rapidly develop resistance [7]. The overall 5-year survival rate for osteosarcoma remains around 70%, a figure that has remained unchanged for the past 40 years. For patients with metastatic or relapsed osteosarcoma, the 5-year survival rate is currently only 20-30% [8]. To address this pressing challenge, there is a growing demand for the development of novel, more effective, and complemen-

tary therapeutic strategies to enhance cancer treatment outcomes.

Natural products have emerged as a vital source of potential new drug candidates. Modern research in medicinal plant drug discovery now integrates a range of approaches, including phytochemical analysis, biological assays, and molecular techniques, all of which continue to yield promising leads for combating cancer [9]. In this context, phytochemicals derived from natural products have garnered increasing attention due to their remarkable potency and lower toxicity compared to conventional chemotherapeutic agents [10].

The use of natural compounds as antitumor agents for osteosarcoma treatment has gained significant interest. Several studies have highlighted the potential of bioactive compounds such as phenolics (curcumin, resveratrol, apigenin, baicalin), thio-derivatives (sulforaphane), and triterpenes (raddeanin) for their antitumor effects [11-13]. Additionally, *Achillea millefolium*, known for its rich content of bioactive compounds, has various medicinal applications [14].

The *Achillea* genus, part of the Asteraceae family, includes over 130 perennial herb species found across the Northern Hemisphere, from Europe to Asia, typically in temperate climates with dry or semi-dry conditions [15]. The most well-known and widely distributed species within this genus is *Achillea millefolium* L., commonly referred to as yarrow or milfoil. For more than 3,000 years, *Achillea millefolium* has been utilized in both traditional and alternative medicine for its therapeutic properties, earning its place as one of the most recognized plants in the treatment of various health conditions [16]. It is attributed with a wide range of pharmacological properties, including spasmolytic, anti-inflammatory, analgesic, haemostatic, antidiabetic, cholagogue, antioxidant, antifungal, antiseptic, and liver-protective effects. These benefits are believed to stem from its diverse chemical components, such as sesquiterpenes and phenolic compounds [15, 17-22]. Its essential oil is primarily used to treat conditions like influenza, hemorrhage, dysmenorrhea, diarrhea, and is known for its haemostatic properties [23, 24].

Achillea millefolium has demonstrated notable antitumor properties, including potent antiproliferative effects. It has shown significant antiproliferative activity against various human lung tumor cell lines, with efficacy greater than that of cisplatin [25]. Additionally, it exhibited strong antitumor effects in mice with breast cancer [26]. Compounds extracted from *Achillea millefolium* have been found to exert antitumor effects through modulation of cell cycle progression and apoptotic signalling pathways [27] . Furthermore, *Achillea millefolium* has proven effective against other types of cancer, including breast epithelial adenocarcinoma, leukemia and skin epidermoid carcinoma, suggesting potential for broader anticancer applications [28]. However, there is a lack of research on its anticancer effect on osteosarcoma. This study aims to investigate the anticarcinogenic effect of *Achillea millefolium* on osteosarcoma cell line and to compare its anticarcinogenic effect with Cisplatin on osteosarcoma cell line.

The null hypothesis of this study is that *Achillea millefolium* will exert no anticarcinogenic effect on osteosarcoma cells.

MATERIALS AND METHODS

Cell line and cell culture:

The MG-63 human osteosarcoma cell line (ATCC® CRL-1427™) is authenticated and obtained from the American Type Culture Collection (ATCC). It is originated from a primary culture of bone tissue from a 14-year-old white male patient with osteosarcoma. For this study, these cells were cultured in DMEM and supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin to maintain sterility and optimal growth conditions. The cells were incubated at 37 °C in a 95% humidified atmosphere with 5% CO_2 to ensure proper cell proliferation and viability. All culturing was conducted at the Center of Excellence for Research in Regenerative Medicine and its Applications (CERRMA) at the Alexandria Faculty of Medicine.

Preparation of *Achillea millefolium* Ethanolic Extract:

Achillea millefolium was purchased from Harraz Company in Egypt. The shoots of *Achillea millefolium* were washed and dried in shade, in a relatively dark area, to ameliorate preservation of active compounds. The entire plant was then powdered using a Wiley mill, with smaller particles selected to enhance the efficiency of solvent extraction. An ethanolic extract was prepared following a standard plant extract preparation (maceration) protocol. Specifically, 400 g of the plant material was suspended in 2000 mL of ethanolic solvent (1:5 w/v) for three days with constant agitation. The solution was then filtered, and the solvent evaporated using a rotary evaporator under reduced pressure. The dried extract was stored at −20 °C until use [29].

Grouping

The MG-63 cell line was randomly divided into three groups. Group I, the study group, consisted of the osteosarcoma cell line treated with *Achillea millefolium* ethanolic extract (AMEE). Group II served as the positive control and was treated with the standard chemotherapeutic drug, cisplatin. Group III served as the negative control and received no treatment.

Cell viability analysis:

The cells were seeded in 96 well plates and allowed to adhere overnight at 37°C. Various concentrations

of AMSEE and cisplatin (2, 5, 10, 20, 40, 80 µg/ml) were added to the wells containing MG-63 cells. After 48 hours, the MTT reagent (0.5 mg/ml) was added to the cells, and the plates were incubated for 4 hours at 37°C with 5% CO_2. The formazan crystals formed were solubilized with dimethyl sulfoxide lysis buffer. The absorbance was measured at 570 nm using a spectrophotometer to assess cell viability. GraphPad Prism 9.0 was used to determine the IC50 values of *Achillea millefolium* and cisplatin [28].

Analysis of cell apoptosis by annexin V/PI staining

The cells were seeded in 6-well plates and incubated at 37°C for 24 hours to allow monolayer formation. They were then treated with the extract at a concentration of 104.5 µg/mL and cisplatin at 3.3 µg/mL for 24 hours, followed by two washes with 1 ml PBS. The cells were collected in pellets and stained with annexin V-FITC and propidium iodide (PI) for 5 minutes. Apoptosis was assessed using a flow cytometer, utilizing annexin V and PI as markers [27] .

Scratch wound healing migration assay:

The osteosarcoma cells were seeded to form a confluent monolayer. A 200-µL sterile pipette tip created a scratch line, followed by PBS rinses to remove debris. The cells were treated with AMEE and cisplatin at IC50 concentrations and incubated for 48 hours. The wound width was measured and photomicrographed at 0, 24 and 48 hours using a phase-contrast microscope (Olympus BX41). Cisplatin-treated wells served as positive controls, untreated wells as negative controls. To analyse the images, ImageJ software was used. The relative migration ratio was determined using the following equation[27] :

$$Relative\ migration\ ratio = \frac{distance_{0h} - distance_{time\ interval}}{distance_{0h}} \times 100$$

Statistical analysis

All experiments were performed in triplicate across three independent trials, and the results were expressed as mean ± SD. Data analysis was conducted using GraphPad Prism 9.0 (GraphPad Software, San Diego, California, USA), with a p-value < 0.05 considered statistically significant. Non-linear regression was applied to the MTT assay, while two-way ANOVA with Tukey's post-test for pairwise comparisons was used for the flow cytometry apoptosis assay and migration assay analysis. Error bars represent the standard deviation.

RESULTS

Cytotoxicity results:

Human osteosarcoma cell line, MG-63 was used to study the potential cytotoxic effect of *Achillea millefolium*. The cells were treated with different doses of AMEE and cisplatin (2, 5, 10, 20, 40, 80 µg/ml) for 48 hours. The results showed that AMEE directly inhibited cell growth in a dose-dependent manner. Cell Viability in the MG-63 cells was significantly decreased (50%) for ~ 104.5 µg/ml dose of AMEE. While cisplatin showed an IC50 of 3.3 µg/mL. (Fig.1).

Annexin V/PI staining results

The flow cytometric analysis using Annexin V and PI staining was performed to assess apoptotic and necrotic cell death in cancer cells treated with AMEE and cisplatin at their IC50 concentrations.

Early apoptosis (Annexin V-positive, PI-negative) was minimal for AMEE at 6.28 ± 1.36%, compared to 15.42 ± 0.3% for cisplatin ($p > 0.9999$ vs. control). However, late apoptosis (Annexin V-positive, PI-positive) was significantly higher for AMEE at 12.39 ± 4.55%, versus 5.23 ± 0.20% for cisplatin ($p < 0.0004$ vs. control) (Fig. 2).

Regarding necrosis (PI-positive, Annexin V-negative), AMEE only induced 7.58 ± 0.40%($p < 0.0133$ vs. control), while cisplatin induced significantly more necrosis at 24.12 ± 0.11% (Table 1).

Scratch wound healing assay results:

The extract demonstrated significant antimigratory activity, achieving a wound closure of 5.80 ± 10.23% at 24 hours, compared to cisplatin, which reached 34.10 ± 6.55% ($p < 0.0001$ vs. control). At 48 hours, the extract showed a marginal increase in closure to 8.30 ± 10.2%, whereas cisplatin exhibited a substantial increase in closure, reaching 51.21 ± 13.4% ($p < 0.0001$ vs. control) (Fig.3).

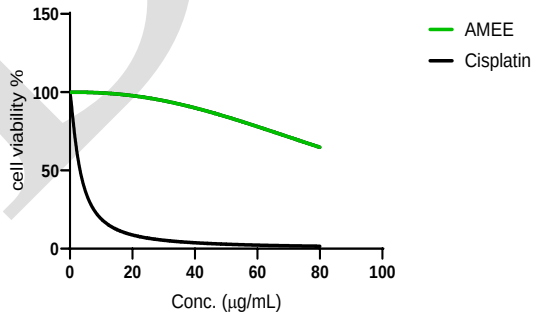
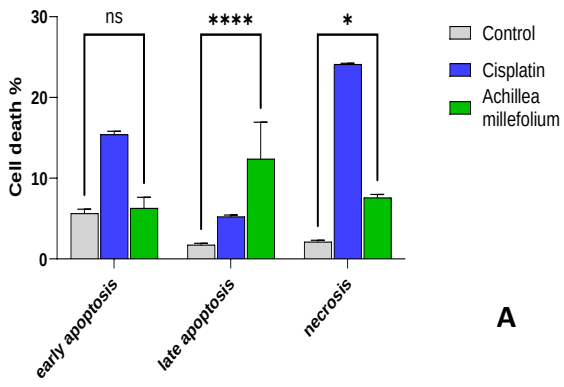


Fig.1. Dose-dependent curves of cell viability of osteosarcoma cells-treated with AMEE vs cisplatin at different concentrations.



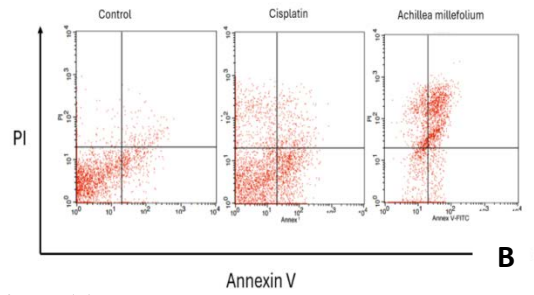


Fig.2. (a) The bar chart showing the percentages of early apoptosis, late apoptosis, and necrosis in MG-63 cells after 48 h of treatment with IC50 doses of AMEE and cisplatin, compared to the control group. Statistical analysis was performed using two-way ANOVA followed by multiple comparisons. Early apoptosis: ns ($p > 0.9999$) for AMEE vs. control; Late apoptosis: **** ($p < 0.0004$) for AMEE vs. control; Necrosis: * ($p < 0.0133$) for AMEE vs. control. Data represent mean ± SD of triplicates. **(b)** Representative scatter plots showing the Annexin/PI apoptosis assay results by flow cytometry at 48 h. The percentages of living cells, early apoptosis, late apoptosis, and necrosis are represented in the lower left, lower right, upper right, and upper left quadrants, respectively.

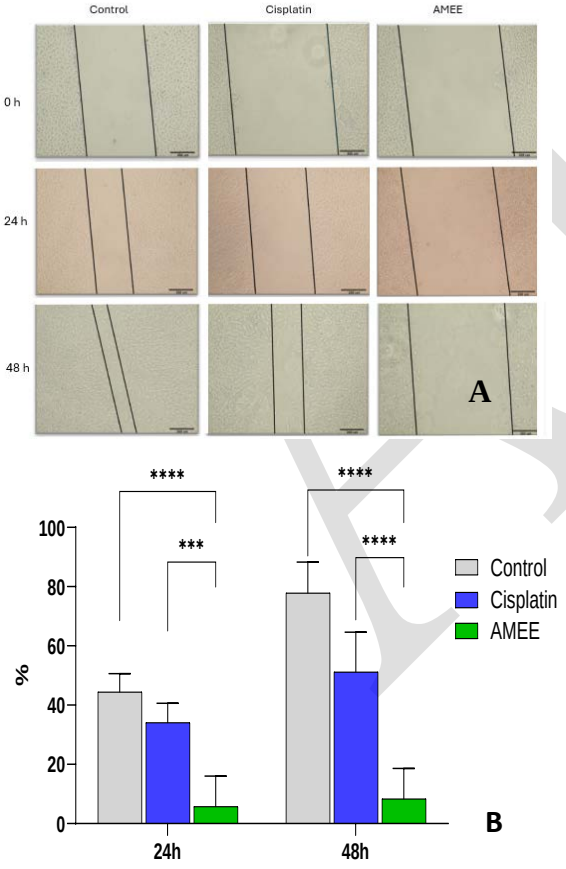


Fig.3: Scratch wound closure rate evaluation a) An inverted microscope photomicrograph displays the progression of the wound healing at 0h, 24h, and 48h for MG-63 cells (×100). b) The bar chart illustrates the percentage of cell migration in the treated groups, relative to the proliferative behaviour of the untreated cells. Statistical analysis was performed using two-way ANOVA followed by multiple comparisons reveals *** = $p<0.001$, and **** = $p<0.0001$. The data is expressed in mean ± SD of triplicates.

Table 1: Annexin V-FITC Apoptosis Assay Results Demonstrating Percentages of Cell Death Types

	Negative control	Positive control (cisplatin)	*Achillea millefolium*
Early apoptosis	5.63%	15.42%	6.28%
Late apoptosis	1.73%	5.24%	12.39%
Necrosis	2.11%	24.12%	7.58%

DISCUSSION

Osteosarcoma is a rare and aggressive bone cancer primarily affecting children and young adults developing typically in the long bones [1, 3]. The standard treatment for osteosarcoma involves a combination of doxorubicin, cisplatin, and high dose methotrexate, administered before and after surgery [30]. While this regimen improves survival in patients with non-metastatic disease, the overall 5-year survival rate remains under 40% This is primarily due to the high rate of lung metastasis, which occurs in about 80% of osteosarcoma cases , resulting in poor outcomes [31]. Moreover, the chemotherapeutic drugs often cause severe side effects [32]. These challenges underscore the urgent need for more effective and less toxic therapies.

Despite treatment advances involving surgery and chemotherapy, the prognosis for patients with osteosarcoma remains poor. The challenges in terms of treatment and prognosis are mainly due to chemotherapy resistance of the cancer cells, severe side effects, and high rates of metastasis, make it difficult to treat effectively [7]. Consequently, there is a critical need for the development new treatment options. Natural products, particularly plant-derived compounds, present promising alternatives for selectively targeting osteosarcoma cells, enhancing treatment outcomes and minimizing toxicity to normal tissues This study investigated the anticarcinogenic potential of *Achillea millefolium* in osteosarcoma MG-63 cells. The ethanolic extract demonstrated significant in vitro tumoricidal activity, reducing cell viability, inducing apoptosis, and inhibiting the migration of cancer cells.

In the current study, the dose-dependent cytotoxic nature *Achillea millefolium* (AMEE) that increases with higher concentrations is a common characteristic for many natural plant-derived compounds. This result is significant, as it highlights the potential of *Achillea millefolium* as an anticancer agent for osteosarcoma. These findings are consistent with previous research, conducted by Hashemi et al., which investigated the cytotoxic

effects of *Achillea millefolium* hydroalcoholic extract on AGS gastric cancer cells. In their study, a concentration of 64 µg/mL was found to inhibit AGS cell growth, reducing cell survival to 47% after 48 hours of treatment. This suggests that *Achillea millefolium* extract can exert a cytotoxic effect across different cancer cell lines, including both gastric cancer and osteosarcoma, supporting the general anti-cancer potential of the extract.

Varying sensitivities of different cancer cell types to *Achillea millefolium* extract have been observed. A key comparison between our study and that of Hashemi et al. lies in the concentration required to achieve similar effects. In the case of MG-63 osteosarcoma cells, the IC50 was 104.5 µg/mL, which is higher than the 64 µg/mL concentration used in Hashemi et al.'s study on AGS gastric cancer cells. This suggests that osteosarcoma cells are more resistant to the anticancer potential of the extract. The cancer cells often exhibit distinct molecular characteristics, such as variations in cell signaling pathways, drug uptake, and resistance mechanisms, which may explain the higher concentration needed to inhibit osteosarcoma cell growth. Thus, while the extract demonstrates cytotoxic effects on both cancer types, these findings indicate that osteosarcoma cells might require higher doses or exhibit increased resistance for effective inhibition [33].

Similarly, Haidara et al. demonstrated that casticin, a flavonoid compound isolated from *Achillea millefolium*, exerts a cytotoxic effect on both AdrC (adriamycin-resistant) and MCF-7 breast cancer cell lines [27]. Notably, casticin exhibited a similar IC50 of 2 µM for both cell lines, suggesting its potent and comparable efficacy across these different cancer types. This aligns with the broader observation that natural products like *Achillea millefolium* and its bioactive components, such as casticin, may have wide-ranging anticancer effects, though the precise dosage required can vary depending on the cancer cell type and its inherent resistance mechanisms [27].

To further emphasize the extract's selective cytotoxicity, Ghavami et al. assessed the cytotoxic effects of *Achillea millefolium* across various tumor cell lines, including breast cancer, gastric and colorectal adenocarcinoma, lung carcinoma, melanoma, and liver hepatoma. The highest IC50 (66.00 µg/mL) was observed against liver hepatoma cell line PLC/PRF/5, while the lowest (22.051 µg/mL) for AGS (gastric adenocarcinoma) [28]. Building on these findings, the anticancer potential of *Achillea millefolium* was further explored through its ability to induce apoptosis, a key mechanism in cancer cell death. Flow cytometric analysis using Annexin V/PI staining revealed that at the IC50 concentrations early apoptosis was high for cisplatin (15.42 %) but not for AMEE (6.28 %). This suggests that cisplatin's effect on apoptosis is milder, as it primarily induces high levels of early apoptosis. Early apoptosis is generally reversible, meaning that the cells might not necessarily commit to full cell death at this stage. In contrast, AMEE's effect, with a significantly higher level of late apoptosis (12.39% versus 5.23% for cisplatin), suggests a more irreversible form of cell death, which may lead to more effective elimination of cancer cells. This difference highlights the potential for AMEE to promote a more definitive form of apoptosis, compared to the transient, reversible early apoptosis induced by cisplatin. This goes with the results of the present study, Abou baker evaluated the apoptotic effect of *Achillea millefolium* ethyl acetate fraction on cervical cancer HeLa cell line and demonstrated that the fraction caused an increase of 24.38% of cells undergoing apoptosis when compared to the control [34]. Moreover, Pereira et al. showed that at a concentration of 100 µg/mL, the *Achillea millefolium* hydroethanolic extract induced 16.63% apoptosis in the non-small cell lung cancer cell line NCI-H460 and 38.96% apoptosis in human colorectal adenocarcinoma HCT-15 cells, highlighting its strongest effect on the latter [35]. In contrast, our study found that AMEE induced a total of 18.67% apoptosis at a concentration of 104.5 µg/mL in osteosarcoma cells. This suggests that while AMEE exerted a notable apoptotic effect on the osteosarcoma cell line, other cancer cell lines, such as breast cancer or colorectal carcinoma, appear to be more sensitive to the anticancer potential of the extract. Additionally, a recent study by Qurtam and Nasr demonstrated that *Achillea millefolium* dichloromethane extract exerted a pro-apoptotic effect on MDA-MB-231 human breast cancer cells. This effect was mediated through the upregulation of pro-apoptotic genes, including tumour protein p53, BCL2 associated X, caspase 3 and caspase 9 [36].

In cancer metastasis, the migration and invasion of tumor cells are critical steps in the spread of cancer to distant organs. Therefore, inhibiting these processes can be an effective strategy to limit cancer progression. In this study, AMEE strongly hindered the migration of osteosarcoma cells at 24 and 48 hours (5.80 ± 10.23% and 8.30 ± 10.2% respectively). In contrast, cisplatin, a standard chemotherapy agent exhibited a more substantial increase in closure (34.10 ± 6.55% and 51.21 ± 13.4% respectively) %. These results suggest that AMEE demonstrates inhibitory effects on osteosarcoma cell migration with its efficacy being substantially higher than that of cisplatin. This indicates its potential of the extract to limit cancer metastasis, warranting further investigation into its mechanisms and possible synergistic effects when combined with conventional treatments. Our findings regarding the anti-migratory potential of AMEE align with those of Qurtam and Nasr, who demonstrated that *Achillea millefolium* dichloromethane

extract also exerted a strong anti-migratory effect on MDA-MB-231 human breast cancer cells [36]. Similarly, *Achillea alimeana* has been shown to exhibit both anti-migratory and anti-invasive properties by inhibiting the migration and invasion of two different human lung cancer cell lines, A549 and H1975. These consistent results further support the anti-metastatic potential of *Achillea* species in various cancer types [37].

CONCLUSION

The combined effects of cytotoxicity, apoptosis induction, and anti-migratory activity on osteosarcoma MG-63 cell line suggest that *Achillea millefolium* extract could exert a multifaceted anticancer mechanism. This contributes to the growing body of evidence supporting the anticancer properties of natural products, particularly those derived from medicinal plants. Further studies, including proteomic and genomic analyses, could provide insight into the specific molecular targets of *Achillea millefolium* compounds responsible for these effects. Furthermore, identification of the active components of the extract would be essential to determine which compounds are primarily responsible for the observed bioactivities. Therefore, optimizing its therapeutic potential and its development it into a more targeted anti-cancer agent.

CONFLICT OF INTEREST

The authors declared that they have no conflicts of interest.

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