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Achillea Wilhelmsii C. Koch Hydroalcoholic Extract Induces Apoptosis and Alters *LIN28B* and *p53* Gene Expression in Hela Cervical Cancer Cells

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Abstract

Background: Inappropriate activation of the proto-oncogene LIN28B and inactivation of the p53 tumor suppressor, have been shown to have a critical role in tumorigenesis. Previous research has shown therapeutic potential for the use of herbal plants as an alternative strategy for cancer treatment. *Achillae wilhelmsii* C. *Koch* is a plant that has been traditionally used for its medicinal properties. The aim of this study was to investigate the cytotoxic and apoptosis-inducing effect of *Achillea wilhelmsii C. Koch* hydroalcoholic extract (*AWHE*) on HeLa cervical cancer cells and its effect on *LIN28B* and *p53* expression.

Methods: The cytotoxic activity of *AWHE* was evaluated on HeLa cells using a trypan blue exclusion assay. The Annexin V/PI double staining assay was used to evaluate the apoptosis-inducing effect of the extract. The expression of *LIN28B* and *p53* mRNA was measured using the real-time-PCR method.

Results: Treatment with *AWHE* was shown to induce cytotoxicity in both time and concentration-dependent manners ($P<0.05$). The proposition of HeLa cells undergoing apoptosis increased with increasing concentrations of *AWHE* ($P<0.05$). The mRNA levels of *p53* increased following 12, 24, and 48 hours of *AWHE* treatment whereas the mRNA levels of *LIN28B* were significantly decreased after 4 to 12 hours of *AWHE* treatment ($p<0.05$).

Conclusions: Our findings confirmed the pro-apoptotic function of *AWHE* on the cervical cancer HeLa cell line. This indicates that targeting the LIN28B signaling cascade may be a promising therapeutic strategy for cervical cancer. Further research is required to understand the therapeutic effects of *AWHE* in primary human cervical cancer cells and a pre-clinical cervical cancer model.

Keywords: Achillea, Apoptosis, Cervical cancer, LIN28B, p53.

Introduction

Cervical cancer is the third most common malignancy to develop in women worldwide, being one of the leading causes of cancer-related mortalities among women (1). Although the exact etiology of cervical cancer remains to be clearly elucidated, infection with the human papilloma virus (HPV) is present in almost all cases, and therefore likely holds a significant role in the development of cervical cancer (2). Despite the ability to cure early stage cervical cancer through surgery and chemotherapy, recurrence and metastasis remain as a major cause of cervical cancer-related mortality. Prophylactic HPV vaccines against the oncogenic forms of HPV,

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HPV-16 and HPV-18, are an effective means of preventing the development of cervical cancer (3). Current vaccine strategies are based eliciting a neutralizing antibody response against the HPV L1 capsid protein (4).

let-7, as the first known human miRNA, *is* highly conserved across species in sequence and function. Misregulation of *let-7* causes decreased cellular differentiation and triggered the onset of cell-based disorders including cancer (5). Reduced expression of *let-7* microRNA has been linked to increased activity of proto-oncogenes, including the highly conserved RNA binding proteins (RBPs), LIN28A or LIN28B. The exact mechanisms by which these RBPs repress *let-7* biogenesis and tumor suppressor function remains to be elucidated (6). LIN28A and LIN28B have been shown to hold critical roles in development, glucose metabolism, and pluripotency via both *let-7* dependent and independent pathways (7). Correspondingly, the overexpression of *LIN28* has been found to promote tumorigenesis and chemoresistance through the suppression *let-7* (8). These findings suggest a potential therapeutic strategy in targeting the LIN28/let-7 pathway for the treatment of advanced-stage human cancers, including prostate, liver, and cervical cancer (9-11). Lv et al. found that the expression level of Lin28 was closely associated with resistance to paclitaxel chemotherapy treatment. The T47D cancer cell line, which has a high expression of LIN28, was observed to be more resistant to paclitaxel chemotherapy treatment in comparison to MCF7, Bcap 37, and SK-BR-3 cells which have low-level Lin28 expression (12). Song et al. reported that the overexpression of LIN28 suppressed proliferation, migration, and cell cycle progression and induced apoptosis in a gastric cancer cell line (13). Subramanian et al. reported let-7i to be downregulated by multiple cell lines expressing endogenous mutant *p53*. The introduction of let-7i to mutant *p53* cells significantly inhibited migration, invasion, and metastasis of several oncogenes, including E2F5, LIN28B, MYC and NRAS (14).

In many human cancers, apoptosis is downregulated, enabling the uncontrolled proliferation of cancer cells and the development of resistance to chemotherapy. This avoidance of apoptosis makes cancerous cells very difficult to kill. Drugs that restore the normal intrinsic and extrinsic apoptotic pathways have the potential to effectively treat cancers that rely on the downregulation of the apoptotic pathway for continued survival (15). Natural phenolic compounds derived from medicinal herbs and dietary plants include phenolic acids and flavonoids. These compounds contain several bioactive functions that are responsible for their chemopreventive properties and contribute to their ability to induce apoptosis, inhibit cell migration and proliferation (16). *Achillea wilhelmsii* C. Koch is a medicinal plant belonging to the *Asteraceae* genus. This medicinal plant grows throughout different regions of Iran. The hydroalcoholic extract of *Achillea wilhelmsii* C. Koch (*AWHE*) has been shown to have antiproliferative effects in folk medicine. *AWHE* contains several components such as flavonoids, alkaloids and cineol which are responsible for its anti-tumor effects in prostate cancer cells (17, 18).

This study aimed to determine the cytotoxic and apoptosis-inducing effects of *AWHE* on the human HeLa cervical cancer cell line. Additionally, we examined the effect of *AWHE* treatment on the mRNA expression levels of the *LIN28B* oncogene and *p53* tumor suppressor. Using this type of compound may be an effective alternative strategy to conventional cervical cancer therapies.

Materials and methods

The Ethics Committee of Zahedan University of Medical Sciences approved the protocol for the present study (Ethical code: IR.ZAUMS.REC.1396.375).

Plant collection and hydroalcoholic Extraction

The different parts of *Achillea wilhelmsii* C. Koch were collected from various locations of southern Iran throughout March 2018. The plant materials were authenticated taxonomically by the Department of Biology at THE University of Sistan and Baluchistan, Zahedan, Iran (herbarium number: 2345). The collected plant materials were shade dried, and the powdered sample was extracted with 70% ethanol solvent (250 mL) using a Soxhlet apparatus. Following this, the extract was filtered through a Whatman 41 filter

paper, then dried under reduced pressure. The stock solution was prepared using 100 mg of *AWHE* dissolved in dimethyl sulfoxide (DMSO; Sigma), the remaining volume was made up with RPMI1640 media containing 2% inactivated fetal bovine serum (FBS).

Cell culture

The human cervical cancer HeLa cells were obtained from the Cell Repository of the Research Institute of Biotechnology, Ferdowsi University of Mashhad, Iran. HeLa cells were cultured in RPMI 1640 medium (Invitrogen Inc., Carlsbad, CA) containing 10% (v/v) FBS (GIBCO, Grand Island, NY), penicillin (100 IU/mL), and streptomycin (100 ug/mL) and incubated at 5% CO_2, 37 °C. Cells were passaged every 3-4 days. All of the following assays were repeated at least in triplicate.

Analysis of cytotoxicity and cell morphology

Cell survival was quantified using the trypan blue dye exclusion assay (19). Briefly, 2×10^5 cells were seeded in 6-well microplates (SPL Lifesciences) and incubated with different concentrations of *AWHE* ranging from 0 to 200 µg/mL for 24 to 72 hours. Following two washes with phosphate-buffered saline (PBS) (Inoclon, Iran) and trypsinization, 30µL of cell suspension was added to a cryovial and thoroughly mixed with an equal volume of trypan blue dye (Inoclon, Iran) (0.5 % in PBS). Following immediate mixing, a haemocytometer was used for counting the blue-coloured (dead) and uncoloured (live) cells. The percentage growth inhibition was calculated using the following equation, % Inhibition = 100 - (Total cells-dead cells/Total cells) × 100). The half-maximal inhibitory concentration (IC50) for each treatment period was determined via CompuSyn Software (ComboSyn, Paramus, NJ, USA) (20).

Analysis of cell morphology was done following exposure of HeLa cells to varying concentrations of *AWHE* for 48 hours using an inverted phase-contrast microscope (IX71, Olympus Inc.). The images were taken directly by a digital camera (Olympus C-7070, Imaging, Melville, NY, USA).

Detection of apoptosis via flow cytometry

Apoptotic cell death was measured using the Annexin V/PI detection kit (BD Biosciences, San Jose, CA). HeLa cells (300000/well) were seeded in 6-well plates for at least 20 hours then treated with 0 to 200 µg/mL of *AWHE* for 48 hours. Following the incubation period, cells were collected and washed with cold PBS and resuspended in 300 µL of binding buffer. The pelleted cells were then incubated with Annexin V-FITC (5 µL) for 15 minutes and propidium iodide (PI) (5 µL) in the dark at room temperature. After adding 500 µL of the buffered isotonic solution, samples were analyzed using the Partec PAS flow cytometer (Partec, Münster, Germany). The total percentage of apoptotic cells was defined as the sum of both early apoptotic (annexin V^+/PI^-) and late apoptotic (annexin V^+/PI^+) cells. Necrotic cells were annexin V^-/PI^+ (21).

RNA extraction, cDNA synthesis and real-time PCR analysis

Cells were treated with 95.9 µg/mL of *AWHE* (equal to the IC50 value following 48 hours of treatment). Total RNA was isolated from HeLa cells using RNX Plus™ kit (CinnaColon, Tehran, Iran) according to the manufacturer's protocol. After assessing the quality and quantity of extracted RNAs, cDNA was synthesized via a cDNA synthesis kit (TaKaRa, Tokyo, Japan). Briefly, 5 µg of RNA, 0.5 µL random 6-mers, 0.5 µL oligo dT primer, and 1.5 µL RNase free dH2O were placed in a microtube and thoroughly mixed. After incubation and cooling on ice, 2 µL of Buffer and 0.5 µL of reverse transcriptase (RT) enzyme was added. PCR amplification was performed in a total volume of 10µL for 15 minutes at 37 °C, 5 seconds at 85 °C, followed by 10 minutes of holding at 4 °C.

Using a 48-well StepOne™ Real-Time System (Applied Biosystems, Inc., Hercules, CA), the SYBR green real-time PCR method was used to evaluate the mRNA levels of *LIN28B* and *p53* genes after 0 to 24 hours of *AWHE* treatment. The cycling conditions included an initial denaturation step for 10 minutes at 95 °C followed by 35 PCR

cycles (denaturation for 30 seconds at 95 °C and annealing for 30 seconds at annealing temperatures shown in *Table 1*, followed by a final extension for 45 seconds at 72 °C). The specific primers were designed using the GeneRunner software (available at http://www.generunner.com), spanning exon/intron junctions to avoid the amplification of DNA sequences while ensuring that all the transcript variants of both genes and *GAPDH* (as a reference gene) would be amplified (*Table 1*). The relative quantification of gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and expressed as fold changes (22).

Table 1. Primer sequences used for real-time PCR analysis of *LIN28B*, *p53* and *GAPDH*.

Genes	Sequence	Annealing Temperature	Fragment Lengths (bp)	Transcript Variants
***LIN28B* (Forward)**	CTGTCAGAGCATCATGCACATG	58	61	X, and X2
***LIN28B* (Reverse)**	GGGTGGCTGTGCAACATTTT			
***p53* (Forward)**	CCAGCCAAAGAAGAAACCAC	57	194	1, 2, 3, 4, 5, 6, 7, and 8
***p53* (Reverse)**	TATGGCGGGAGGTAGACTGA			
***GAPDH* (Forward)**	AGCCTCAAGATCATCAGCAATGCC	61	105	1, 2, 3, 4, and 7
***GAPDH* (Reverse)**	TGTGGTCATGAGTCCTTCCACGAT			

Statistical analysis

The results were analyzed via SPSS for Windows, Version 16.0 software (IBM SPSS Inc., Chicago, IL, USA) using sample T-test and the non-parametric ANOVA test. The results are expressed as a mean ± standard deviation. A P-value of less than 0.05 were considered statistically significant.

Results

AWHE inhibits the growth of HeLa cells in a time and concentration-dependent manner

The percentage of viability shown in Fig.1 suggests *AWHE* has anti-proliferative effects on HeLa cells in a concentration- and time-dependent manner. Treatment of HeLa cells with *AWHE* significantly reduced the number of living cells at 24 (P=.004) (Fig.1A), 48 (P=0.0032) (Fig.1B), and 72 (P=0.0034) (Fig.1C) hours of treatment compared to untreated cells. The IC50 values within these exposure periods were calculated by CompuSyn software were determined to be 154.8 µg/mL, 95.9 µg/mL, and 79.04 µg/mL after 24, 48 and 72 hours of treatment, respectively. As shown in *Fig.2*, with increasing concentrations of *AWHE*, morphological changes including cell shrinkage were observed in treated HeLa cells following 48 hours of treatment. These findings verify the results of the trypan blue exclusion assay suggesting that this extract causes an increased rate of cell death in a concentration-dependent manner.

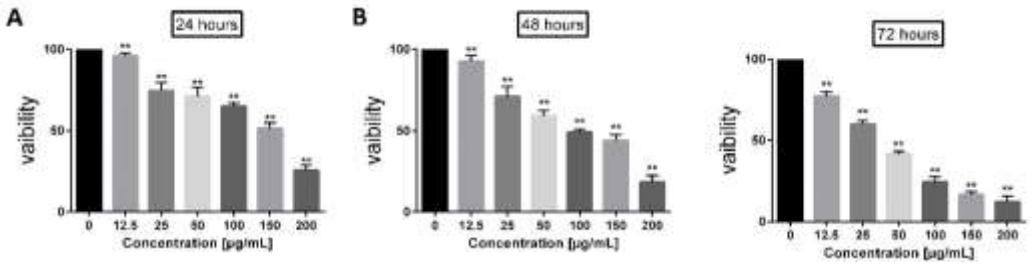

Fig. 1. Cytotoxic effects of *AWHE* following 24 (A), 48 (B), and 72 (C) hours of treatment analyzed by trypan blue exclusion assay. *AWHE* was able to induce cell death in both a time- and concentration-dependent manner (**P < 0.05 compared to the untreated control cells).

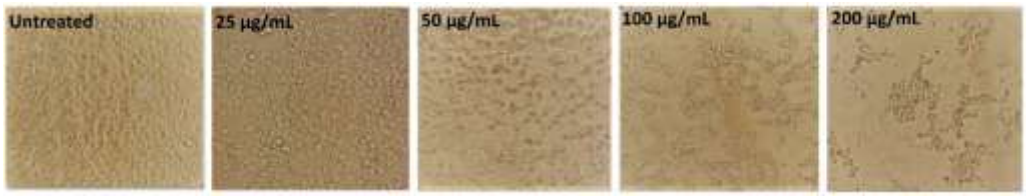

Fig. 2. Morphological changes of HeLa cervical cancer cells treated with varying concentrations of *AWHE* ranging from 0 to 200 µg/mL following 48 hours of incubation. In the *AWHE*-treated HeLa cells, there is a decrease in the number of viable cells, cell shrinkage and rounding observed compared untreated cells.

AWHE Induces Apoptosis of HeLa Cells

The proportion of apoptotic HeLa cells that were untreated (Fig.3A) or treated with either 5050 µg/mL (Fig.3B*)*, 100 µg/mL (Fig.3C*)*, or 200 µg/mL (Fig.3D*)* of *AWHE* were 1.32 (P=0.149 compared with untreated cells), 20.15 (P= 0.0008 compared with untreated cells), 47.46 (P<0.0001 compared with untreated cells), and 80.30% (P<0.0001 compared with untreated cells) respectively. The proportion of necrotic HeLa cells subjected the same concentrations of *AWHE* were 0.1, 0, 0.42, and 0.96% (P>0.5 compared with untreated cells). These findings suggest that necrosis might not be the main pathway responsible for cell-death in the *AWHE*-treated HeLa cells. Different proportions of *AWHE*-treated HeLa cells were viable, undergoing early and late apoptosis, or undergoing necrosis (Fig. 3E). Increased *AWHE* concentrations induced higher rates of late apoptosis in a concentration-dependent manner.

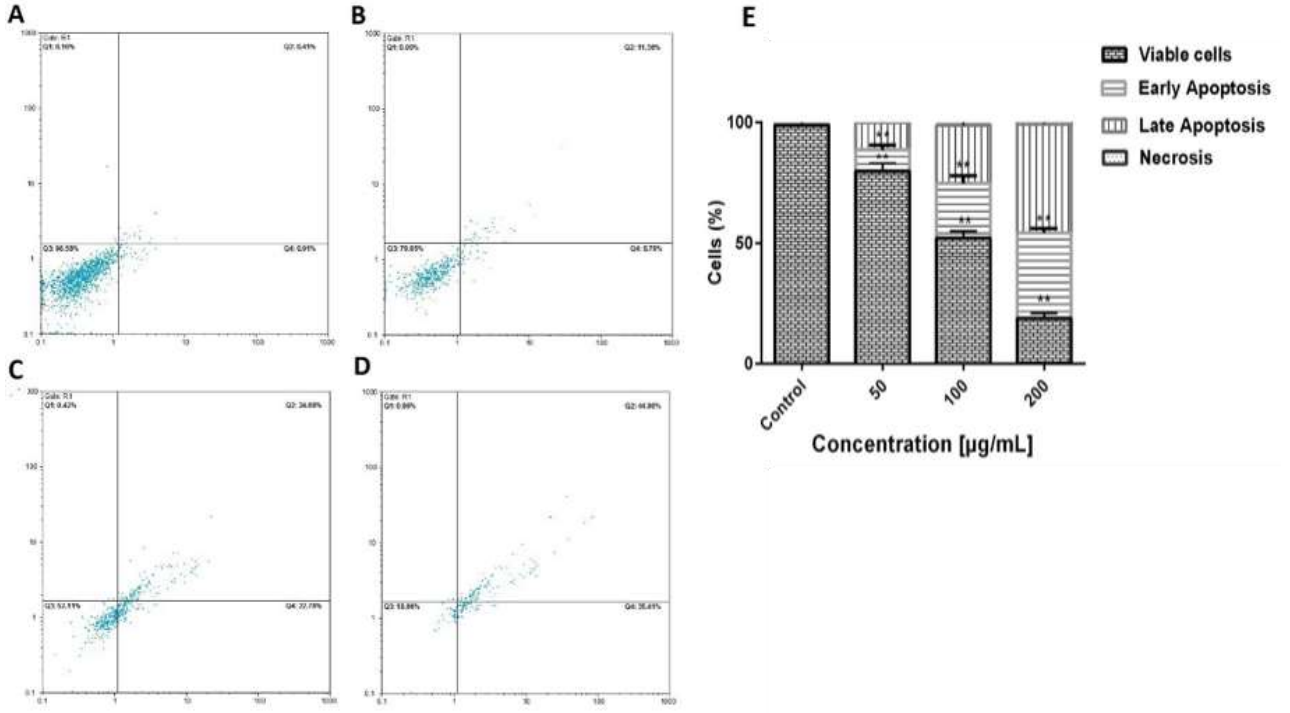

Fig. 3. Flow cytometric determination of apoptosis using Annexin V /PI double staining method following 48 hours of treatment with 0 µg/mL (A), 50 µg/mL (B), 100 µg/mL (C), and 200 µg/mL (D) of *AWHE*. The number of cells undergoing early and late apoptosis was increased in a concentration-dependent manner, indicating that *AWHE* induced marked apoptosis in Hela cervical cancer cells (**P <0.05 compared with untreated cells)

AWHE alters the mRNA levels of both LIN28B and p53 genes

The baseline expression of *LIN28B* and *p53* mRNA was not significantly different (Fig. 4A) (P=0.1). As illustrated in Fig.4B, mRNA levels of *p53* were not affected by *AWHE* treatment following 2, 4, and 6 hours of incubation (P>0.05). However, the *p53* mRNA levels of significantly increased after 12 (5.49±0.16 fold, P<0.0001), 24 (3.25±0.15 fold, P=0.0001), and 48 (2.95±0.21 fold, P=0.0014) hours of incubation compared to untreated cells. The upregulation of *p53* following 12 hours of *AWHE* treatment may indicate p53-induced apoptosis in these cells. The mRNA levels of *LIN28B* showed a different expression pattern compared to *p53*. Following 2 hours of *AWHE* treatment, the mRNA levels of *LIN28B* was not altered (P=0.54), however following 4 (0.32±0.05 fold, P<0.0001), 6 (0.65±0.04 fold, P=0.0003), 12 (0.80±0.12 fold, P=0.0008) and 24 (0.88±0.06 fold, P=0.004) hours of treatment the levels of *LIN28B* were significantly reduced compared to the untreated HeLa cells. Additionally, *LIN28B* mRNA returned to basal levels following 48 hours of treatment, therefore resulting in no significant difference in *LIN28B* mRNA compared to the initial exposure (P=0.08).

Discussion

Avoidance of apoptosis is a distinctive feature of cancerous cells, contributing to tumor development, progression, and resistance to therapeutic interventions. In this preliminary study, we investigated the cytotoxic and apoptosis-inducing effects of *AWHE* on the human cervical cancer HeLa cell line. Our results indicate that *AWHE* causes cell death in both time and concentration-dependent manners. These findings corroborate our previous work examining the anti-tumor characteristics of *Achillea wilhelmsii C. Koch* on MCF-7 breast cancer cells (23). Morphological alterations were observed in HeLa cells treated *AWHE*. Increasing concentrations of *AWHE* resulted in more drastic morphological changes. This may be a manifestation of *AWHE*-induced apoptosis. In addition to cell shrinkage, the formation of blebs and the formation of apoptotic bodies are specific characteristics unique to apoptosis (24). The density plots obtained from the Annexin V/PI double staining assay showed that HeLa cells treated with *AWHE* (50-200 µg/mL) were undergoing early and late apoptosis. Among all treated cells, the proportion of late apoptotic cells was higher than the number of early apoptotic cells. The apoptosis-inducing effects mediated by the presence of flavonoids and phenolic acids have been previously shown in a similar study (25), suggesting that the hydroalcoholic extract of *Achillea wilhelmsii* C. Koch maintains the same quantity of these compounds responsible for the inhibitory effects on cell proliferation. Here, we demonstrated that the expression of *p53* was up-regulated following 12, 24 and 48 hours of *AWHE* treatment in HeLa cells compared with untreated cells.

A significant decrease in *LIN28B* mRNA levels was observed after 2 to 24 hours of *AWHE* treatment. Previous work by Ashtiani et al. found that *AWHE* inhibits the proliferation of the PC-3 prostate cancer cell line when exposed to 150µg/ml of *AWHE* following 48 hours of incubation (17). Our findings indicate that HeLa cells are more sensitive to *AWHE* treatment since the same inhibitory effect was observed with lower IC50 values (95.9 µg/mL). Treatment of MDA-Mb-468 cells with *AWHE* has been reported to induce apoptosis in a concentration-dependent manner following 48 hours of treatment, while the caspase3 activity was increased shortly after the beginning of treatment (26). These findings suggest that apoptosis may be the main cell death pathway activated when subjecting breast cancer cells to varying concentrations of *AWHE*. According to these findings, our data indicate that *AWHE* is a potent apoptosis-inducing agent which functions by triggering apoptotic cell death.

So far, very few studies have examined the potential alterations in *LIN28B* and *p53* mRNA expression in female malignancies. The p53 tumor suppressor promotes apoptosis through both transcription-dependent and independent mechanisms. It has been shown that alterations in this process that inhibit p53 function can lead to tumor growth and invasion. Furthermore, the apoptotic activity of this cell cycle checkpoint is controlled in normal cells, but not in tumor-derived cells (27). A novel link between NF-κB and p53 has been proposed by Kasinski and colleagues that includes three miRNAs which are located downstream of p53 activation (28). In this regard, levels of a *let-7* family of miRNAs were significantly downregulated by NF-κB activation. Triggering the NF-κB signalling pathway is required for the regulation of *LIN28B* transcription (29). *LIN28B* has been found to be overexpressed in primary human tumors and human cancer cell lines. This increased expressed is associated with let-7 miRNA suppression (9). Gaining a more comprehensive understanding of the link between *p53* and *LIN28B* genes will provide novel and effective treatment strategies for cervical cancer.

Despite the limitations of our study, we could determine the cytotoxic effects of either hydro- or oil-extract of *Achillea wilhelmsii* C. Koch in HeLa cells. Determining the protein levels of p53 and LIN28B may help corroborate our current findings. Additionally, determining the enzymatic activity of caspase family proteins may help us to better understand which type of cell death pathway is mainly activated in *AWHE*-treated HeLa cells.

Our findings revealed that AWHE has a potent apoptotic-inducing effect on HeLa cervical cancer cells. This hydroalcoholic extract can inhibit *LIN28B* gene expression while up-regulating *p53* expression. Further studies examining more plants of the Asteraceae family and different components of AWHE are needed to identify the role of each active substance of Achillea wilhelmsii C. Koch on the expression of *LIN28B* and *p53*.

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