Anti-proliferative and apoptosis inducing potential of hydroalcoholic *Achillea wilhelmsii* C. Koch extract on human breast adenocarcinoma cell lines MCF-7 and MDA-Mb-468

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Abstract: *Achillea wilhelmsii* C. Koch contains a variety of components such as flavonoid.The previous studies showed that flavonoid has anti-cancer properties. The aim of the present study was to determine the anti-proliferative and apoptosis-inducing potential of hydroalcoholic *Achillea wilhelmsii* C. Koch extract (HAWE) on MCF-7 and MDA-Mb-468 human breast carcinoma cell lines. the anti-proliferative activity of HAWE was evaluated using MTT, flowcytometry by annexin V/PI double staining, and caspase-3 activity. The results of MTT showed that the ED50 of MCF-7 and MDA-Mb-468 was 25μg/ml of HAWE, 48h after treatment. Flowcytometry by annexin V/PI showed that HAWE induced late apoptosis in MCF-7 and early apoptosis in MDA-Mb-468. In addition, the caspase-3 colorimetric method showed that caspase-3 increased in the MDA-Mb-468 after treatment with HAWE. This study found that the hydroalcoholic extract of *Achillea wilhelmsii* C. Koch induced apoptosis in both the MCF-7 and MDA-Mb-468 human breast carcinoma cell lines.

Keywords: *Achillea wilhelmsii* C. Koch, breast cancer, MTT assay, apoptosis, caspase-3.

INTRODUCTION

In normal conditions, the body has a balance between cell death and cell proliferation. This balance is known as hemostasis and is necessary for normal cell growth.If this balance is disturbed, it leads to cancer(Kim *et al*., 2007). Cancer is a serious concern all over the world and the most common cause of mortality and morbidity after cardiovascular diseases (Saffari *et al*., 2014). Breast cancer is the most common cancer among women; each year nearly 400,000 women suffering from this disease lose their lives, and nearly 234,000 new cases of breast cancer are reported every year(Rastegar *et al*., 2013).The known causes of breast cancer are not so similar to those of other cancers; however, studies indicate various factors that can increase the risk of getting breast cancer, such as genetic predisposition, obesity, pregnancy after the age of 35, exposure to radiation dangers such as UV, a history of cancer in first-degree relatives,a history of breast cancer in one breast, and continuous use of birth control pills overa long period(DeVita *et al*., 2010; Morrow, 1994; Russo *et al*., 1992).

Surgery is the primary treatment for breast cancer and depending on the extent of the cancer, it can take different forms such as complete (simple) mastectomy, radical mastectomy, radical modified mastectomy, extended radical mastectomy and adjuvant treatment such as radiotherapy and chemotherapy(Russo *et al*., 1992; Schwartz and Lillehei, 1974; Townsend Jr *et al*., 2012).

Unfortunately, in most cases, treatment is not effective or leads to unpleasant side effects, hence researchers are trying to use compounds with fewer side effects and to induce apoptosis in cancer cells(Mostafapour Kandelous *et al*., 2016). Natural products can be used as medicines to treat cancer. Since the 1950s, 60% of cancer drugs have been made from natural products or their derivatives (Wang and Sui, 2014).Plants and herbs are low-cost natural products with few side effects that can be used for cancer treatment (Fattahi *et al*., 2013).In this way, not only are cancer cells controlled, but also healthy cells are not damaged (Tabrizi *et al*., 2016).

Achillea wilhelmsii C. Koch is a short, yearling plant 15-40 cm high that contains chemical components such as flavonoid, alkaloid, cineol, and born el (Amjad *et al*., 2012). In traditional medicine, this plant is used for calming stomach pain, and in old times, Iranians used this plant as a drug to treat various health problems such as general fatigue, weakness, neurological diseases, neurasthenia hysteria, and epilepsy (Isfahani *et al*., 2013).

An alcoholic extract of a flowering branch of this plant reduces triglyceride as well as systolic and diastolic blood pressures, while an aqueous extract stimulates humoral and cell-mediated immune response. Aerial parts of

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Achillea wilhelmsii C. Koch have antioxidant properties (Isfahani *et al*., 2013).

The current study was conducted to evaluate and screen the effect of apoptosis and the anticancer potential of hydroalchoholic *Achillea wilhelmsii* C. Koch extract (HAWE) agent on human breast cancer cell lines MDA-Mb-468 and MCF-7.

METHODS AND MATERIALS

Plant materials

Achillea wilhelmsii C. Koch(Shahraki and Ravandeh, 2013) was collected from the Taftan area (near Khash city) of Iran's Sistan and Baluchistan province. Plant collection was done in spring 2015. Dr. Ali Shahraki, member of the Research Institute of the University of Sistan and Baluchistan confirmed the taxonomic determination of the plant.

Preparation of hydroalcoholic extract of the plant

After the collection, the plant was dried in a dark room, and the aerial parts were separated from the roots. Afterwards, the aerial parts were sliced and gritted into powder, and then a hydroalcoholic solution (70% alcohol) was extracted using a Soxhlet extractor. The solution was filtered and evaporated under a rotary evaporator in order to obtain a hydroalcoholic extract; the solid extract was stored in a freezer at -20°C.

Chemicals and regents

Roswell Park Memorial Institute Medium (RPMI 1640), fetal bovine serum (FBS), trypsin, EDTA, trypan blue, phosphate buffer saline (PBS), streptomycin, and penicillin were purchased from Gibco (Rockville, MD, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA); a caspase-3 colorimetric assay kit was procured from R&D System Co. (Minneapolis, MN, USA); and an annexin V. FITC apoptosis detection kit was purchased from Biovision (San Francisco, CA, USA). All the other materials were of analytical grade and purchased locally.

Cell culture

MCF-7, an estrogen receptor positive (ER+), and MDA-Mb-468, an estrogen receptor negative (ER-),of human mammary adenocarcinoma were purchased from the National Cell Bank of the Pasteur Institute of Iran (NCBI). The cell lines were grown adherently as a mono-layer in 75ml plastic flasks in RPMI 1640 medium supplemented with 10% FBS (heat inactivated 30min, 56°C before use), 100U/ml penicillin,and 100μg/ml streptomycin in incubator under standard cultured condition (95% humidified air, 37°C and 5% CO_2). For enumeration, 30μl of trypan blue (0.2%) stained the same volume (30μl) of cell concentration, and neobar lam was used for counting and viability (more than 95% for adhering cell lines before testing) of the cells.

Cell treatments

For treatment with HAWE, first HAWE as powder was dissolved in DMSO (HPLC grade) and kept in a freezer at -20°C. The cell lines were seeded into sterile 6 or 96 well plates; the cell number was almost equal in all the wells for adhering cell lines to button plates. Incubation was done over night (37°C, 5% CO_2 and 95% air).The medium was aspirated, and different concentrations that were prepared with medium and HAWE were added. The number of cell lines in seeding was different in different tests as indicated therein.

Cell viability assay

MTT assay

Cell viability and anti-proliferation of HAWE were carried out through an MTT reduction assay as described previously(Hashemi *et al*., 2005). The cell lines with a density of 5000 cells per well were seeded in 96 well plates and incubated overnight to reach approximately 80% confluence; the medium used removed cells exposed to the medium containing different concentrations of HAWE (12.5-25-50-100 μg/ml), and then cells were incubated for 24, 48, and 72h.At the end, 20μl of MTT stock solution (5μg/ml in PBS) was added to each well, and then the plate was incubated at 37°C for 4h. The culture medium was discarded, and then 200μl DMSO was added to each well for solubization of Formazan crystals and incubated in a dark place for 2h at room temperature. The absorption was measured by a microplate reader, 570nm; the measurement was repeated in triplicate to confirm the results.

The results were calculated by dividing the percentage of absorbance in the treated cells by the percentage of absorbance in the untreated (control) cells, defined as the viability percentage. ED_{50} was 50%, the concentration range that inhibits the growth of cell lines.

Apoptosis assay

The apoptotic effect of HAWE on the cell lines was analyzed by means of a flowcytometry assay, using an annexin V/PI double staining Kit, according to the manufacturer's protocol (Saravani *et al*., 2012). First, $1\text{x } 10^5$ cells per well were seeded in six well plates and incubated over night, and then incubated with or without different concentrations of HAWE for 48h. All cells (treated and untreated cells) were washed two times with cold PBS, and then the cell pellets were stained in a 250μl 1x binding buffer, 2.5μl AnnexinV FITC, and 2.5μl PI. Afterwards, the samples were inculcated in a dark place for 15min at room temperature.

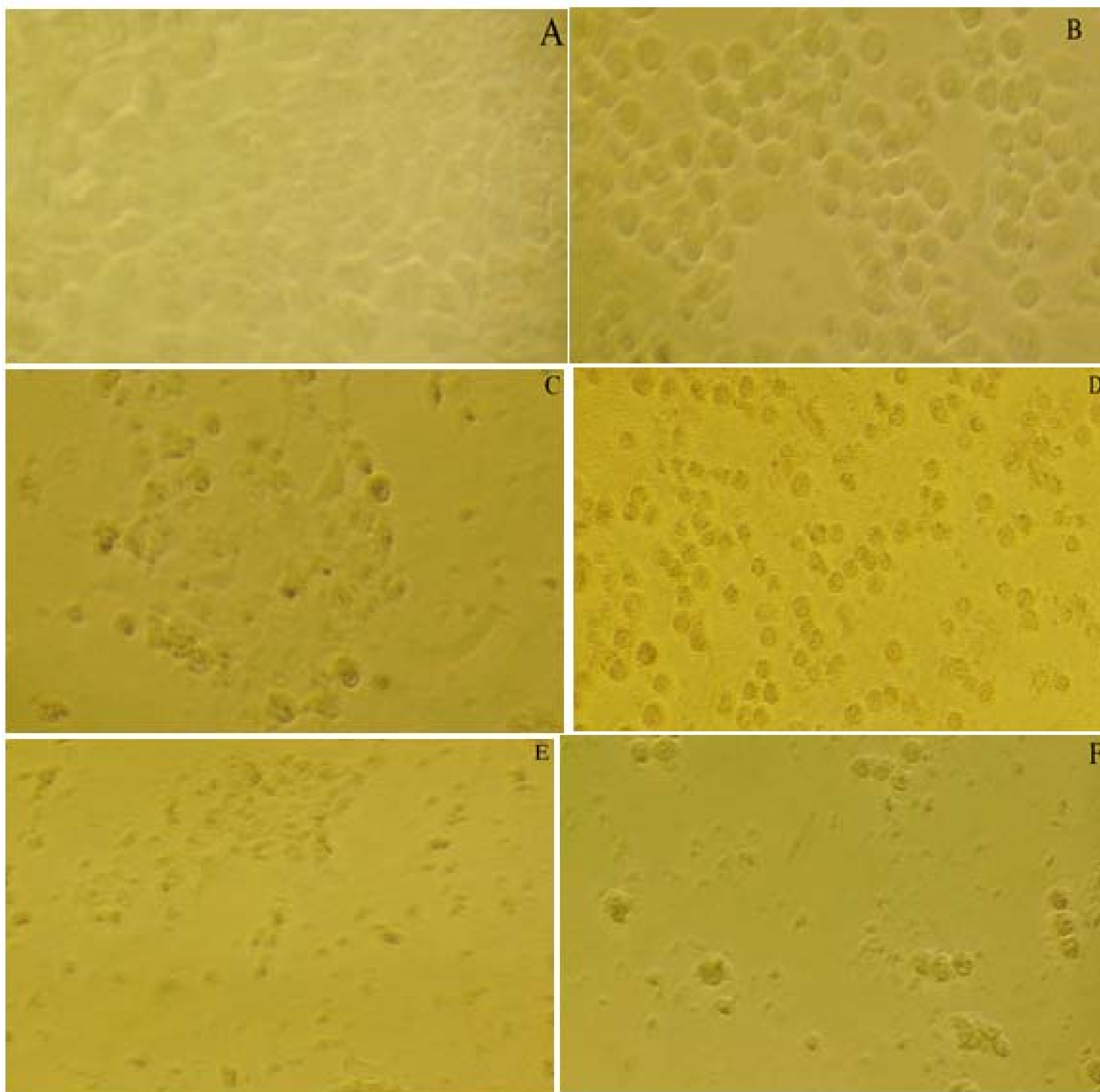

Fig. 1: Effects of HAWE on the morphological alterations of MCF-7 and MDA-Mb-468 cells were monitored for 72h at two different doses of HAWE. Control MCF-7 cells (A) and MDA-Mb-468 (B) dispersed homogeneously with distinct boundaries after over night incubation. MCF-7 cells (50 µg/ml)(C) And MDA-Mb-468 cells (50 µg/ml)(D) MCF-7 (100µg/ml)(E) and the MDA-Mb-468(100µg/ml)(F)

Partec PAS II flowcytometry was used for analysis of the samples. The results consist of four sections: living cells (were not stained with PI or annexin V), early apoptosis (stained with annexin V connected to phosphatidylserine in outer layer of cell membrane), late apoptosis (stained with both annexin V and PI to fragmented DNA), and necrosis cells (stained with PI).

Caspase-3 activity assay

A colorimetric assay kit (R&D system CO, Groding Germany) was used for measuring caspase-3 activity according to the manufacturer's protocol after 24h of seeding $1x10^5$cells per well of each cell line in six well plates. Wells were treated with an ED50 concentration of HAWE at different times (6, 12, and 24h). At the end of these periods, all cell plates were washed two times with cold PBS, then 50µl of Lysis buffer was added to each suspension, and then all tubes were centrifuged for 10 min at 1000g. Supernatants were transferred to new tubes, and a 50µl reaction buffer and 5µl caspase-3 substance were added and then incubated at 37°C for 60 min; the color change was measured in a microplate reader at 405nm. The results were calculated by dividing the percentage of absorbance in the treated cells by the percentage of

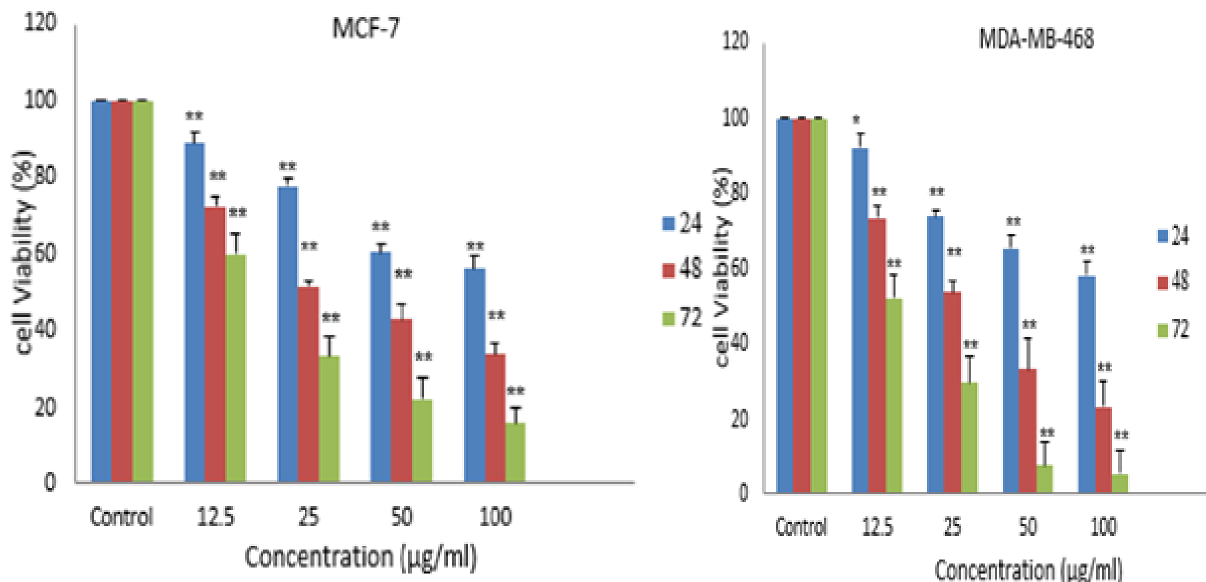

Fig. 2: Effect of HAWE in inhibition of cell growth of the breast cancer MCF-7 and MDA-Mb-468 cell lines. Cells were treated with different concentrations of HAWE for 24, 48 and 72 h, and proliferation was measured withan MTT assay. HAWE reduced cell proliferation in MCF-7 (25µg/ml) and MDA-Mb-468 (25µg/ml) breast cells in a time- and dose-dependent manner. Each value is presented as a mean ± SD of three experiments (each triplicate). *P <0.05; **P<0.01 compared to untreated control groups.

absorbance in the untreated (control) cells in a time-dependent manner.

STATISTICAL ANALYSIS

The SPSS 16 software package (SPSS INC, Chicago, IL, USA) was used for statistical analysis. All the data was represented as mean±SD, and the nonparametric ANOVA exam was used for statistical analysis between the groups. In the present study, P<0.05 was considered statistically significant.

RESULTS

Analysis of cell morphology

The morphology of the MCF-7 cell line changed in a dose- and time-dependent manner; in low concentrations, the cells were deformed, and with increasing dose and time, granulated cellular contents, dropsy, and shrinkage were increased, even, after 48h (at a 100 µg/ml concentration) and 72h (at 50µg/ml and 100µg/ml concentrations), the rupture of membranes and the release content of cytosol were clearly observed. The MDA-Mb-468 cell line had approximately the same condition (Figure 1).

Cell viability (MTT assay)

The effects of HAWE on the MCF-7 and MDA-Mb-468 cell lines were examined. The cells were exposed to different concentrations (0-12.5-25-50-100µg/ml) of HAWE for 24,48, and 72h. After these periods, the cell lines' viability was measured by an MTT assay. Concentration 25µg/ml of HAWE had significant inhibitory effects on both cell lines: 51,67 ±1.527 for MCF-7 and 53,67±1.577 for MDA-Mb-468 after treatment for 48h.This dose (25µg/ml) and time (48h) can inhibit growth by 50% in both cell lines. Figure 2 shows the results of MTT in both cell lines, showing a significant difference in the dose-time manner (P<0.001).

Induction of apoptosis by HAWE

The cell lines were stained with annexin V/PI and analyzed using flowcytometry to explore whether HAWE shows cytotoxicity through induction of apoptosis. To do so, the cell lines were treated in appropriate doses (0-12,5-25-50-100 µg/ml) for 48h. The cell population shifted from viable (annexin V-/PI-) to early apoptosis (annexin V+/PI-) and late apoptosis (annexin V+/PI+) in high doses and in the MDA-Mb-468 cell line more towards early apoptosis (annexin V+/PI-) through an increase in the dose,based on the results of annexinV/PI double staining also in the MCF-7 cell line through induction of late apoptosis (annexin V+/PI+).These shifts were significantly different in both cell lines (P<0.001) (Figures3&4).

Caspase-3 activity

Caspase enzymes play an important role in apoptotic responses; therefore,the effect of HAWE in inducing apoptosis in the cell lines was investigated by measuring the activity of caspase-3. The treatment of the MDA-Mb-468 cell line of breast cancer in a 25µg/ml concentration

of HAWE was observed. Also, a significant increase in caspase-3 activity of MDA-Mb-468 was observed after treatment with HAWE in ED50 (25µg/ml) at various times (6, 12, 24h), and the amount of caspase-3 differed significantly between treated and untreated cell lines (P<0.001); however, there was no such increase in the MCF-7 cell line (Figure 5).

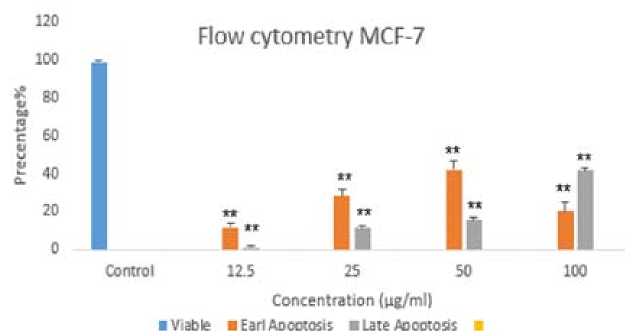

Fig. 3: Flowcytometric evaluation of apoptosis in MCF-7 cells using annexin-V/PI double staining. After 48h treatment, HAWE resulted in a significant increase in late apoptotic cells and a moderate increase in early apoptotic cells, in a dose-dependent manner. Results, mean ± SD three independent experiments. *P < 0.05; **P < 0.01 compared to untreated control groups.

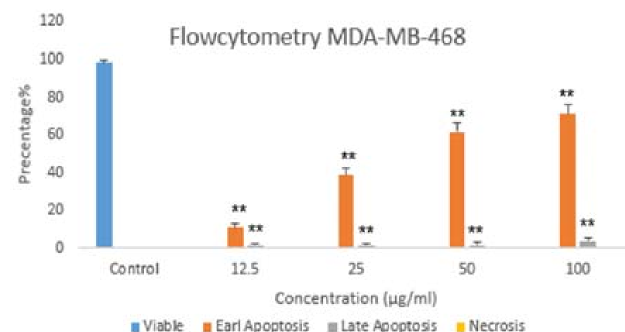

Fig. 4: Flowcytometric evaluation of apoptosis in MDA-Mb-468 cells using annexin-V/PI double staining. After 48h treatment, HAWE resulted in a significant increase of early apoptotic cells and a moderate increase in late apoptotic cells. If there was no increase in late cells, in a dose-dependent manner. Results, mean ± SD three independent experiments. *P<0.05; **P<0.01 compared to untreated control groups.

DISCUSSION

Breast cancer has the highest prevalence among women compared to other cancers, and surgery is the main therapy for this disease, with chemotherapy, radiotherapy, hormone therapy, and gene therapy used as minor therapies (Zhu *et al*., 2015). However, these therapies have several problems. For example, in surgery, in addition to tumor cells, healthy cells are also removed; the radiation used in minor therapy harms normal cells. Moreover, these treatments are expensive (Yu *et al*., 2013). Thus, many researchers have focused on low-priced new drugs with a natural origin that induce apoptosis in tumor cells, but not in healthy cells,and are especially prepared using local and traditional medicines (Cao *et al*., 2011).

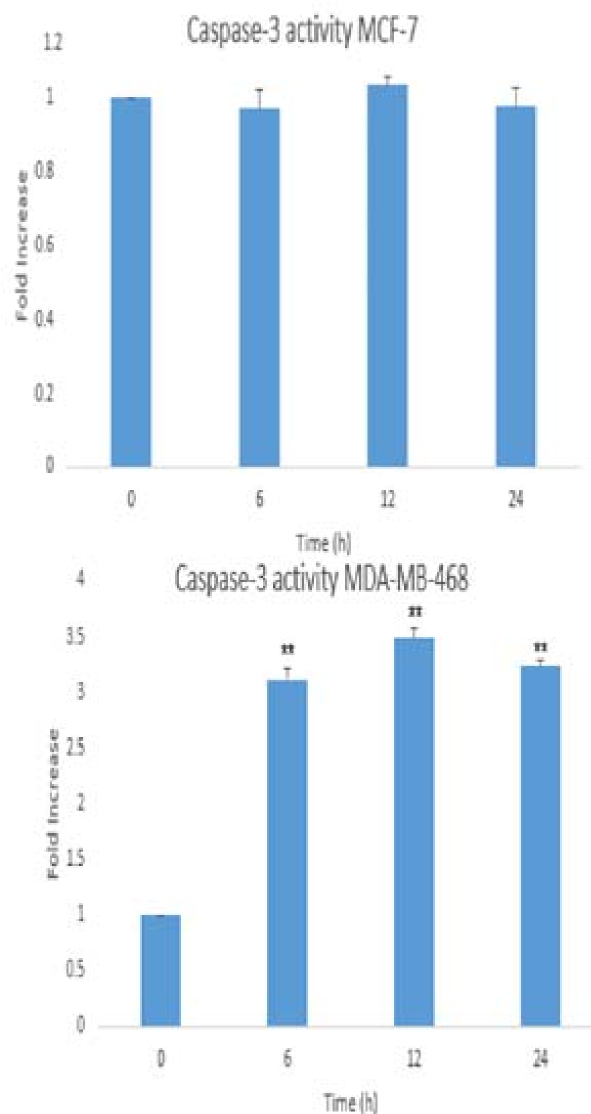

Fig. 5: The effect of HAWE on caspase-3 activity in MCF-7and MDA-Mb-468cells. Cells were incubated to a concentration of HAWE (25µg/ml) in a time-dependent manner (6, 12, 24h). *P<0.05; **P<0.01 compared to untreated control groups.

A. wilhelmsii is commonly known as “Bumadaran” in Persian herbal medicine. Previous studies have shown that it has antioxidant properties and contains flavonoids and alkaloids, compounds that are known as anticancer components. Interestingly, the hydroalcoholic extract of *Achillea wilhelmsii* C. Koch has antioxidant properties (Csupor-Löffler *et al*., 2009; Isfahani *et al*., 2013; Özgen

et al., 2004). In the current study, the anticancer and apoptotic activities of HAWE were investigated.The results showed that HAWE exhibited cytotoxicity towards the MCF-7 and MDA-Mb-468 human breast cancer cell lines with an ED50 of 25µg/ml for both; this was determined by means of an MTT assay.To determine the type of cell death (apoptosis or necrosis),annexinV/PI double staining was used. The findings showed that HAWE induced apoptosis in MCF-7 and MDA-Mb-468, while the apoptotic cells of MCF-7 and MDA-Mb-468 were shifting to late apoptosis and early apoptosis, respectively.

Apoptosis is a programmed cell death that regulates normal physiological processes and plays an essential role in the progress and maintenance of tissue homeostasis(Offen *et al*., 2000). Apoptosis has two main pathways, an extrinsic apoptosis pathway (death receptor-dependent pathway) and an intrinsic apoptosis pathway (mitochondria-dependent apoptosis)(Francis *et al*., 2000). Briefly, in the extrinsic apoptosis, the interaction between the death ligand and the death receptor leading to caspase-8 activation as a starter caspase, activated caspase-8 then activates caspase-3(Lugnier, 2006), while in the intrinsic apoptosis pathway, cytochrome C is released from mitochondria leading to caspase-9 activation as a starter caspase, activated caspase-9 then activates caspase-3(Corbin and Francis, 1999). Thus, in this study, caspase-3 activity was measured:it was found that caspase-3 increased in the MDA-Mb-468 cell line in a time-dependent manner (25µg/ml of HAWE). Caspase activity did not change in a time-dependent manner (25µg/ml of HAWE) because the MCF-7 cell line did not express caspase-3 due to deletion (functional 47bp) inside the exon 3 of capsea-3 gene. Therefore, the induction of apoptosis in the MCF-7 cell line was treated with HAWE, probably due to a caspase-independent or a non-caspase-3-dependent mechanism (Yu *et al*., 2013).

There is little data regarding the effect of *Achillea wilhelmsii* C. Koch on cell lines. In an investigation by Dalali et al.,a methanol extract (leaf aerial part) of *A.wilhemsii* had a cytotoxic effect on HT-29 cell line human colon adenocarcinoma in a 50µg/ml concentration (Isfahani *et al*., 2013). Other species of *Achillea* such as *Achillea millefolium* also inhibited the growth of the DU-145 cell line human prostate carcinoma (Shahani *et al*., 2015), MCF-7 (IC50=0.1250µM), and HeLa (IC50=0.0819µM) (Csupor-Löffler *et al*., 2009). These different effects of concentration are due to different cell lines and, of course, different extracts.

CONCLUSION

The current findings showed that hydroalcoholic extract of *Achillea wilhelmsii* C. Koch can induce apoptosis in both the MCF-7 and MDA-Mb-468 cell lines of human breast cancer.These findings provide evidence that *Achillea wilhelmsii* C. Kochhas potential as an anti-breast cancer agent.

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