ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (9) 507 - 513
doi: 10.30466/vrf.2023.1985987.3726
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Hydroalcoholic extract of *Taraxacum officinale* induces apoptosis and autophagy in 4T1 breast cancer cells

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Article Info

Article history:

Received: 03 January 2023
Accepted: 26 January 2023
Available online: 15 September 2023

Keywords:

Apoptosis
Autophagy
Breast cancer
Dandelion
Taraxacum officinale

Abstract

Triple-negative breast cancer (TNBC) is an aggressive and deadly breast cancer sub-type with limited therapeutic options. Dandelion (*Taraxacum officinale*) exhibiting extensive anti-cancer activity is reported to be effective against TNBC; however, its anti-tumor effect mechanisms have not been fully elucidated. The purpose of this study was to determine the anti-cancer activity of hydroalcoholic extract of dandelion (HADE) on 4T1 cells, and the mechanism of HADE-induced cell death. The effect of HADE on cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase assays. Apoptotic cell death was monitored by flow cytometry. The DNA fragmentation was evaluated by Acridine orange/Ethidium bromide (AO/EB) staining. Nitric oxide (NO) level was detected using Griess assay. The effects of HADE on *Atg-7*, *Beclin-1*, *Bcl2*, *Bax* and *p53* genes were investigated by real-time reverse transcription-polymerase chain reaction. The results showed that HADE inhibited cell growth and proliferation in a dose- and time-dependent manner. The HADE induced 4T1 breast cancer cell death via apoptosis and autophagy. The DNA fragmentation was improved as the concentration of HADE increased. The NO secretion was declined with increasing concentration of HADE. Gene expression analysis confirmed HADE-induced apoptosis and autophagy in cancer cells. The *Bax*, *Bax/Bcl-2* ratio, *p53*, *Beclin-1* and *Atg-7* over-expression as well as *Bcl-2* down-regulation were also evident in treated cancer cells.

Introduction

Breast cancer is the most common malignant tumor among women,[1] ranked first for incidence (2.30 million) and death (685,000) worldwide in 2020, [2] and expecting to increase to 4.40 million in 2070. [3] This cancer is a heterogeneous disease and thus, the patients are classified using different approaches. The current policy categorizes breast cancer based on the detection of three receptors, including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). This appeared as heterogeneity of the results and responses to the treatments within the clinical sub-types.[4] Approximately 10.00 - 20.00% of invasive breast cancers are triple-negative tumors (TNBC) and defined as an absence of ER, PR and lack of HER2 over-expression. This breast cancer sub-type is associated with poor prognosis and the survival rate after metastasis is lower than the other sub-types.[5]

During the past decades, conventional therapeutics including chemotherapy, radiation and hormonal and targeted therapies have been applied. However, these therapeutic options are accompanied by many side effects. Consequently, they become ineffective due to the development of resistance to conventional therapies.[6] Therefore, an investigation for some new efficient and affordable compounds is required. In this regard, screening extracts and bioactive compounds derived from plants has been widely considered a practical approach for discovering novel anti-cancer drugs.[7]

Dandelion (*Taraxacum officinale* L.) is an herbaceous perennial belonging to the Asteraceae family, which can be found in almost every part of the world. Dandelion has been used for a long time as a folk medicine to treat gastrointestinal ailments, cancer, liver problems, eye diseases, osteoarthritis, anemia and eczema.[8] Dandelion contains several useful bioactive phytochemicals such as flavonoids (luteolin, isoquercitrin, and caffeic acid),

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phenolic acids, lactones, sesquiterpenes, triterpenes and alkaloids.[9] Investigations have demonstrated that dandelion exhibits anti-tumor effects in breast,[10] liver,[11] pancreatic,[12] lung,[13] colorectal[14] and ovarian cancers,[15] as well as in leukemia.[16] Studies have indicated that this herb might inhibit the growth, proliferation, cell cycle and invasion of breast cancer cells and may be of great value as novel anti-cancer agents.[10,17,18] However, little is known about the mechanism of action of these effects. The present study was designed to investigate the cytotoxic effects of hydroalcoholic dandelion extract (HADE) on the 4T1 mouse TNBC cell line and the type of cell death induced by cancerous cells.

Materials and Methods

Chemicals. All reagents were obtained from Sigma-Aldrich (Darmstadt, Germany) unless otherwise stated.

Preparation of dandelion extract. Dandelion was collected from Kermanshah, Iran, and processed according to the method previously described.[12] *Taraxacum officinale* identity was confirmed in Natural Resource Center (Karaj, Iran) with herbarium No. 7124. Briefly, the aerial parts of the plant were dried and powdered; 15.00 g of which was dissolved in 150 mL 70.00% ethanol at 25.00 ˚C and kept in the dark for 48 hr. The total homogenate was filtered through grade 42 Whatman filter paper (DD Biolab, Barcelona, Spain), and centrifuged at 8,000 *g* for 5 min. The supernatant was dried at room temperature to allow alcohol evaporation. The powder was dissolved in a serum-free culture medium (Gibco, Grand Island, USA) and filtered through a 0.22 µm filter. The final filtrate was used directly or lyophilized as needed into stock solution.

Cell line and culture. Mouse TNBC cells (4T1; Pasteur Institute, Tehran, Iran) were cultured in RPMI-1640 medium supplemented with 10.00% fetal bovine serum (Gibco, Billings, USA), 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin in a humidified incubator at 37.00 ˚C and 5.00% CO_2.[19]

Viability assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were cultured in 96-well plates at 1.50×10^4 cells per well density for 24 hr. Then, the media containing 0.00, 31.20, 62.50, 125, 250, 500, and 1,000 µg mL^{-1} of the extract were added to each well and incubated for 24, 48 and 72 hr. Then, MTT solution (5.00 mg mL^{-1} in phosphate-buffered saline [PBS]) was added to each well and incubated for 4 hr at 37.00 ˚C. Afterward, reduced formazan crystals were solubilized in dimethyl sulfoxide. After 30 min, the optical density (OD) was measured at 540 nm using an ELISA reader (Sunrise, Tecan, Switzerland). The percentage of cell viability was calculated using the relative formula.[20] The half-maximal inhibitory concentration (IC_{50}) was obtained by non-linear regression and three concentrations of IC_{50}, half of IC_{50} and two-fold IC_{50} were used.

Cytotoxicity test. To evaluate the cytotoxic effect of HADE on cells, lactate dehydrogenase (LDH) activity was measured by a colorimetric LDH kit (Abcam, Cambridge, USA) as previously described.[21] Briefly, cells were incubated in 96-well plates (1.50×10^4 cells per well) overnight at 37.00 ˚C, and on the next day, 300 µL of culture media containing different concentrations of extract were added to each well. The plates were incubated at 37.00 ˚C in 5.00% CO_2 for 24, 48 and 72 hr and 100 µL of the supernatant from each well was added onto the LDH substrate in a new 96-well plate. After 20 min shaking at room temperature, 1.00M HCl was used to stop the reaction and then, absorbance was measured at 450 nm wavelength using the microplate reader (Sunrise).

Apoptosis detection. Evaluation of apoptosis was performed by annexin V-FITC/Propidium Iodide (PI) assay as described elsewhere.[22] The cells were seeded in a 12-well plate for 24 hr and treated with the HADE at a corresponding IC_{50} concentration for 24 hr. Then, cells were harvested and washed three times with cold PBS. After centrifugation for 10 min at 300 *g*, the supernatant was discarded, and the pellets were re-suspended in annexin V-FITC/PI buffer (0.10 M NaOH with pH of 7.40, 1.40 M NaCl and 25.00 mM $CaCl_2$) and left in the dark for 15 min at room temperature. Cells were evaluated by a flow cytometer (Becton Dickinson, San Jose, USA), and the proportions of viable, early apoptotic, late apoptotic and dead cells were evaluated using WinMDI Software (version 2.90; Scripps Institute, San Diego, USA).

DNA fragmentation evaluation. The amount of DNA fragmentation induced by HADE was quantified by the diphenylamine colorimetric method as described previously.[23] The absorbance of samples was measured at 600 nm by a spectrophotometer (Bio-Tek Instruments, Highland Park, USA). The DNA fragmentation was presented as percentages and calculated by the following formula:

$$DNA\ fragmentation\ (\%) = (OD_{660}\ [A]\ /OD_{660}\ [B] - OD_{660}\ [A]) \times 100$$

Autophagy assessment. Autophagy induction was measured by Acridine orange/Ethidium bromide (AO/EB).[24] The cells were cultured in a 24-well plate and treated with different concentrations of the HADE after which they were stained for 20 min in the dark. After washing with PBS, the morphological changes were assessed using a fluorescence microscope (BX60F5; Olympus, Tokyo, Japan). Experiments were repeated twice. The results were presented as percentages.

Nitric oxide (NO) assay. The Griess assay was applied to measure NO levels in the cells. Briefly, 300 µL of the culture media were deproteinized by zinc sulfate and centrifuged at 10,000 *g* for 10 min at 4.00 ˚C. An amount of 100 µL of supernatant was mixed with 100 µL vanadium (III) chloride (8.00 mg mL^{-1}) in a microtiter plate (Nunclon, Roskilde, Denmark) and then, 50.00 µL N-(1-Naphthyl)

ethylenediamine dihydrochloride (0.10%), 50.00 µL sulfanilamide (2.00%) and Griess reagents were added to the plate. After 30 min incubation at 37.00 ˚C, the absorbance was measured at 540 nm by the microplate reader (Sunrise). The concentration of NO in medium samples was determined from a standard linear curve established by 0.00 - 150 µmol L^{-1} sodium nitrite.[25]

Gene expression analysis. Total RNA was extracted from the cells using Trizol reagent (Invitrogen, Waltham, USA) according to the manufacturer's protocol. A commercial kit (PrimeScript First Strand cDNA Synthesis Kit; TaKaRa Bio Inc., Nojihigashi Kusats, Japan) was utilized for cDNA synthesis. The target genes were quantified by SYBR Premix Ex Taq II (TaKaRa Bio Inc.) in a real-time reverse transcription-polymerase chain reaction system (Applied Biosystems, Foster City, USA). The results were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a reference gene and relative to the control group. The related primers were designed using Oligo7 software (DBA Oligo, Inc., Colorado Springs, USA) and synthesized by CinnaGen Company (Tehran, Iran). The sequences of the primers are as follows: *Atg-7*: F: 5´-TTGACATGAGTGCTCCCACC-3´ and R: 5´-AGACAGAGGGCAGGATAGCA-3´; *Bcl-2*: F: 5´-TGTGGATGACTGAGTACCTGA-3´and R: 5´-CAGCCAGGAG AAATCAAACAG-3´; *Bax*: F: 5´-CCGGCGAATTGGAGATGAA CT-3 and R: 5´-CCAGCCCATGATGGTTCTGAT-3': *p53*: F: 5´-AGAGACCGCCGTACAGAAGA-3´and R: 5´-GCATGGGCA TCCTTTAACTC-3´; *Beclin-1*: F: 5'-ATGGAGGGGTCTAAGGC GTC-3' and R: 5'-TGGGCTGTGGTAAGTAATGGA-3'; *Gapd*h: F: 5´-AGGTCGGTGTGAACGGATTT G-3´ and R: 5´-TGTAGAC CATGTAGTTGAGGTCA-3´.

Statistical analysis. The SPSS software (version 19.0; IBM Corp., Armonk, USA) was used for all statistical analyses. The Shapiro-Wilk test was used to test the normal distribution of the data. The homogeneity of variances between the groups was evaluated by Levene's test. The comparisons were performed by the one-way analysis of variance and Student's *t*-test. Data were expressed as mean ± standard deviation. Differences were considered to be statistically significant when $p < 0.05$.

Results

As shown in Figure 1A, the results of the LDH test revealed that HADE was toxic against breast cancer cells compared to the control group. This cytotoxicity was also in a concentration- and time-dependent manner (Fig. 1A). The viability of the cells exposed to HADE was significantly inhibited in a dose- and time-dependent manner (Fig. 1B). The IC_{50} values obtained by MTT test were 330.21, 262.38 and 145.90 µg mL^{-1} for 24, 48 and 72 hr, respectively.

The apoptotic state of 4T1 cells treated with 165 µg mL^{-1} HADE increased to 15.60% compared to 4.00% in the control group. While, the apoptosis reached 64.90% and 73.30% respectively in the 330 and 660 µg mL^{-1} HADE groups. The viability of cells decreased from 94.10% in control group to 22.90% in 660 µg mL^{-1} treated group.

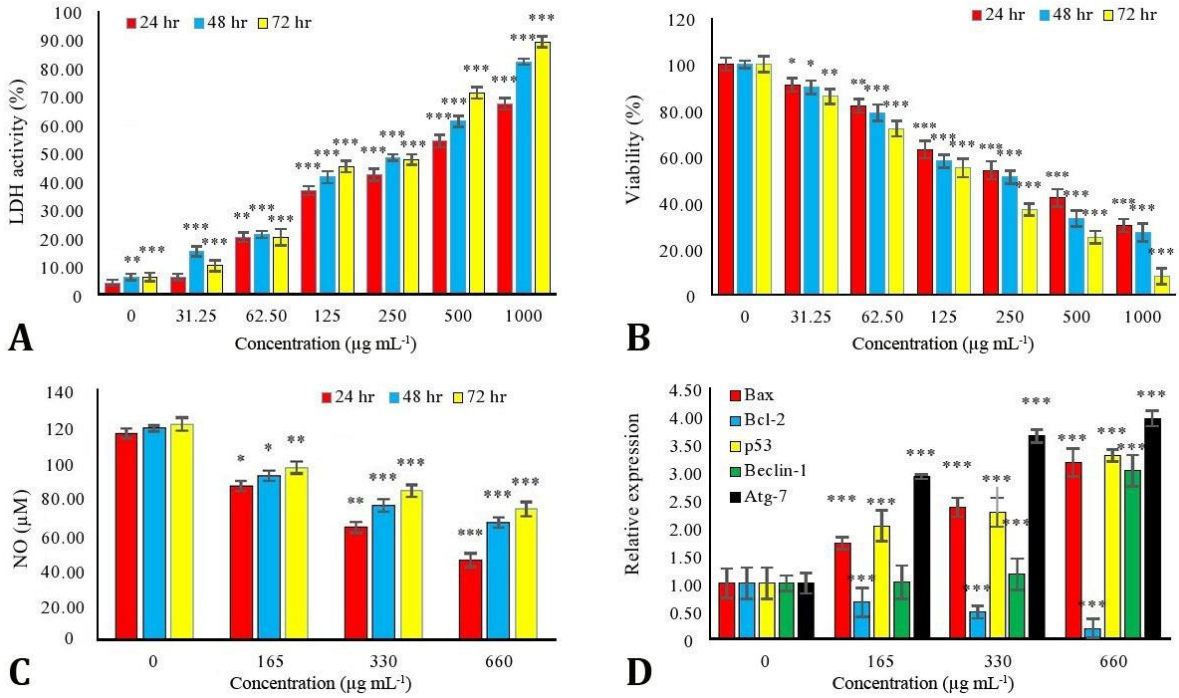

Fig. 1. Effects of different concentrations of hydroalcoholic extract of dandelion on 4T1 breast cancer cells. **A)** Cytotoxic effects evaluation through lactate dehydrogenase (LDH) activity assay; **B)** Effects on the cell viability (proliferation); **C)** Effects on nitric oxide (NO) production; and **D)** Effects on the expression of the genes involved in apoptosis and autophagy. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

According to the analysis of cytoplasmic membrane integrity, the tested concentrations of HADE were significantly cytotoxic to 4T1 cells compared to the control group (Figs. 2 and 3A). Also, DNA fragmentation was significantly increased (Fig. 3B).

The NO production in 4T1 cells was significantly ($p < 0.05$) decreased in all three concentrations of HADE after 24 hr incubation in a dose-dependent manner (Fig. 1C).

The HADE-treated cells significantly ($p < 0.05$) increased the expression of *Bax*, *p53*, *Beclin-1* and *Atg-7* in a concentration-dependent manner. The expression of *Bcl-2* was significantly ($p < 0.05$) reduced in all HADE-treated groups (Fig. 1D).

The results of AO/ EB staining showed that HADE induced autophagy in 4T1 cells (Fig. 4). Cells in the control group without any treatment were viable and emitted green fluorescence, indicating that they absorbed the EB dye. The cells in control group showed autophagy as 1.58 ± 0.34, 1.66 ± 0.40 and 1.69 ± 0.31 percent at 24, 48 and 72 hr incubation, respectively. The cells treated with half of the IC_{50} concentration at 24 hr exhibited small cytoplasmic staining because of slight autophagy (65.19 ± 3.20%). Treatment with IC_{50} concentration (82.34 ± 1.74%) and two-fold IC_{50} (92.81 ± 1.06%) showed abundant cytoplasmic acidic vesicular organelles formation as a characteristic of autophagy. The autophagic cells were increased with the increasing time of incubation, reaching the highest level (114.97 ± 6.31%) in two-fold IC_{50} concentration at 72 hr incubation.

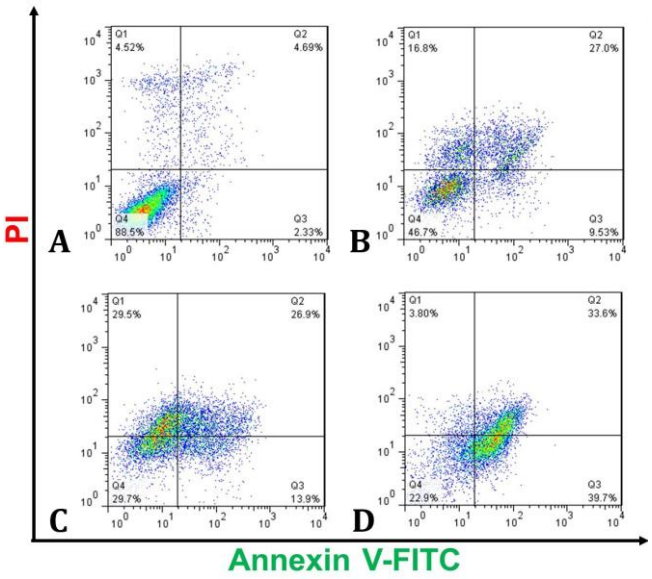

Fig. 2. Flow cytometry diagrams of annexin V-FITC/ Propidium Iodide (PI) double-staining in 4T1 cells. **A)** Untreated cells; **B)** Treated with 165 µg mL^{-1} of dandelion extract; **C)** Treated with 330 µg mL^{-1} of dandelion extract; and **D)** Treated with 660 µg mL^{-1} of dandelion extract; Q4: Viable and non-apoptotic cells (annexin V-/PI-); Q3: Early apoptotic cells (annexin V+/PI-); Q2: Late apoptotic cells (annexin V+/PI+); Q1: Necrotic cells (annexin V-/PI+).

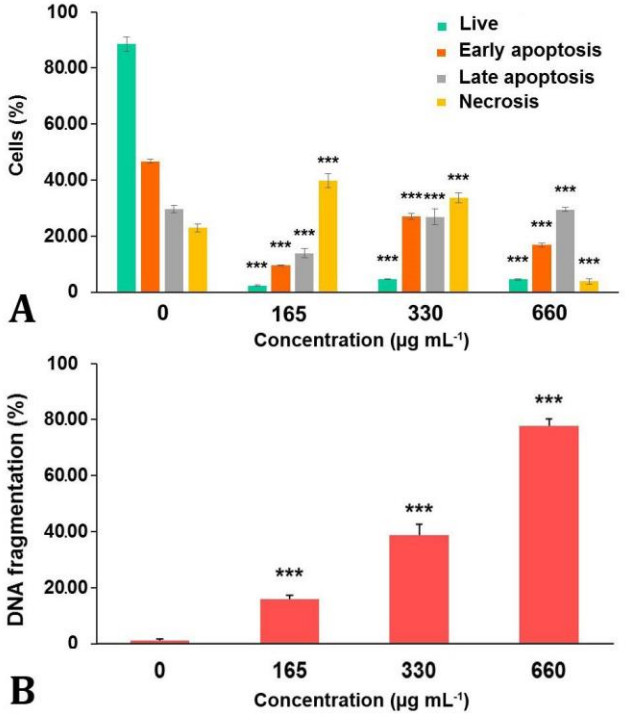

Fig. 3. Evaluation of the effects of increasing dandelion extract concentrations on **A)** apoptotic status (necrosis, late apoptosis, early apoptosis and live); and **B)** DNA fragmentation using flow cytometry in T41 breast cancer cells. *** $p < 0.001$.

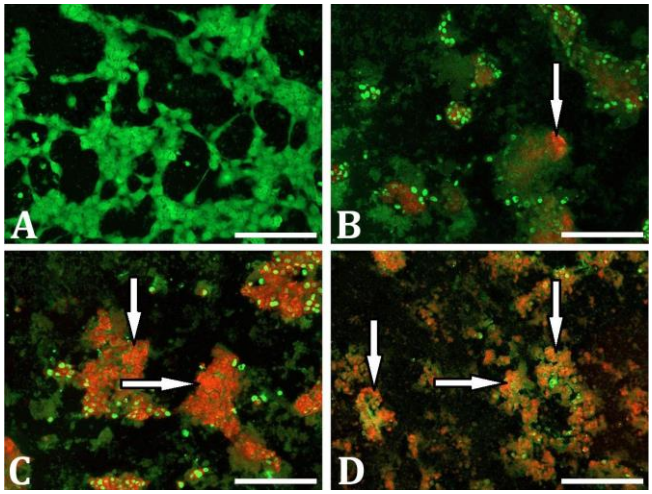

Fig. 4. Effects of different concentrations of hydroalcoholic extract of dandelion on autophagy in 4T1 breast cancer cells. Acidic vesicular organelles are accumulated in autophagic 4T1 breast cancer cells (white arrows). **A)** Untreated cells; **B)** Treated with 165 µg mL^{-1} of dandelion extract; **C**) Treated with 330 µg mL^{-1} of dandelion extract; and **D)** Treated with 660 µg mL^{-1} of dandelion extract, (AO/ EB staining; bars = 200 µm).

Discussion

The present study showed the anti-cancer activity of HADE through autophagy and apoptosis in the 4T1 cell line *in vitro*. Apoptosis is an essential series of events

leading to programmed cell death, and the potential mechanisms for the apoptotic process involve the balance between apoptosis induction and its inhibition. Regulation of apoptosis can directly influence the response of cancer to chemotherapeutic drugs[26] and has been proposed as a promising target for cancer chemotherapy.[27] Autophagy is an intra-cellular degradative process playing dual roles in tumor suppression and promotion in several cancers. Cancer cells under oxidative stress being treated with chemotherapeutic agents regulate autophagic pathways.[28] Some anti-cancer drugs can regulate autophagy; therefore, autophagy-regulated chemotherapy could have a role in the survival or death of a cancer cell.[29] Reduced and abnormal autophagy inhibits the degradation of damaged components or proteins in oxidative-stressed cells, leading to the development of a cancer.[30]

One of the most common hallmarks of cancer comprises the ability of cells to evade apoptosis. In the absence of effective apoptosis, autophagy can be an alternative route of cell death. However, many tumors display deficiencies in autophagy that may result in genomic instability and further anti-cancer drug resistance.[31]

The expression of *Beclin-1*, *P53*, *Ba*x and *Atg-7* significantly increased by HADE treatment in a dose-dependent manner in the present study. In contrast, the expression level of *Bcl-2* was decreased by HADE treatment. It is well known that *Beclin-1* controls the crosstalk between autophagy, apoptosis and inflammasome activation.[28] The *Atg-7* is an autophagy-promoting gene playing a critical role in regulating autophagy.[32] In cancer cell lines and animal models, the loss of *Beclin-1* resulted in a reduction of autophagy and an increased cancer cell proliferation, highlighting its tumor suppressor role.[33]

The *p53* as a tumor suppressor is connected with both apoptosis and autophagy cell death types. In apoptosis, *p53* enhances the expression of *Bax* and decreases the expression of *Bcl-2*. The ratio of pro-apoptotic *Bax* to anti-apoptotic *Bcl-2* controls the mitochondrial pathway of apoptosis.[34] One crucial link between autophagy and apoptosis is *Bcl-2* which inhibits the function of *Beclin-1* in autophagy. Dysfunction of autophagy may result in a shift to apoptosis, and in some cases necrosis. Increased concentration of HADE in this study significantly elevated the apoptosis and DNA fragmentation rates in 4T1 cells. Internucleosomal DNA fragmentation is one of the morphological signs of apoptosis.[35] In the present study, HADE decreased NO production in breast cancer cells. Nitric oxide is a key player in the progress and repression of tumorigenesis, depending on the source and concentration, and induces DNA damage, affects the DNA damage repair response and controls cell cycle arrest. In the tumor micro-environment, moderate NO secreted from tumor and endothelial cells could induce angiogenesis and epithelial-to-mesenchymal transition, expressing an aggressive phenotype.[36]

Different parts of dandelion are rich sources of various phytochemicals including phenolic acids and terpenes.[37] Some known bioactive phytochemicals in dandelion root extract (DRE) activated both the apoptotic and autophagic cell death pathways in HT-29 colorectal cancer cells, emphasizing the caspase-8 and caspase-3 pathways activation.[12,14] It has also been reported that DRE could induce a pro-death type of autophagy which corresponded with the destabilization of the mitochondrial membrane potential in human pancreatic cell lines.[14] The DRE has selectively inhibited cell growth, proliferation and invasion, and also induced cell apoptosis in esophageal squamous cell carcinoma (ESCC) cells. Moreover, the oral administration of DRE arrested the growth of tumors, caused a dose-dependent decline in *p-Akt*, *Ras*, *Raf*, *PI3K*, *Bcl-2* and *pERK1/2* and increased *Bax* at the protein level in human ESCC xenograft models.[38] Aqueous DRE effectively induces apoptosis in human acute T-cell leukemia cell line and dominant-negative Fas-associated death domain protein Jurkat cells in a dose- and time-dependent manner. Early activation of caspase-8 and the subsequent activation of caspase-3 indicated that DRE might induce exogenous or receptor-mediated apoptosis.[39]

In a recent study, dandelion extract arrested the cell cycles in 4T1 cells.[10] Moreover, ethanolic and methanolic extracts of *T. officinale* suppressed the proliferation of human breast cancer stem cell lines in two-dimensional and three-dimensional cell culture platforms. This inhibitory effect was via death receptor signaling pathways and reactive oxygen species production.[40] Dandelion also could influence the micro-environment of breast cancer cells as it has promoted the polarization of macrophages from M2 to M1 phenotype. The dandelion extract suppressed *IL-10*, *STAT3* and *PD-L*1 expressions and inhibited proliferation, migration and invasion of cancer cells.[17]

A new β-branched glucomannan from dandelion leaves mounted onto a nano-carrier promoted apoptosis and induced arrest in the S phase and G2/M, up-regulated *Beclin-1* and *Bax* and inhibited *Bcl-2* expression. It also exerted a marked cytotoxic effect against non-small lung carcinoma cells.[13]

In the present study, hydroalcoholic extract of dandelion induced both apoptosis and autophagy in 4T1 breast cancer cells, suggesting that dandelion can be used to inhibit the growth and proliferation of cancer cells. However, further *in vitro* and *in vivo* experiments are required to define the therapeutic composition and doses of dandelion products.

Acknowledgments

The authors would like to thank the director and staff of the Medical Biology Research Center, Health Technology Institute, Kermanshah University of Medical

Sciences, Kermanshah, Iran, for providing laboratory facilities and for their precious support. This study was supported by the University of Tabriz, Tabriz, Iran.

Conflict of interest

The authors report that there are no competing interests to declare.

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