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Cytotoxicity Effect of Laserpitium Carduchorum Extract in Mcf-7 Human Breast Cancer Cells Via Cell Cycle Arrest and Induction if Apoptosis

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Abstract--*Laserpitium carduchorum is a perennial herb belonging to the Apiaceae family. In Iran, this plant is found in the Kurdistan Province, Bane Mountains. Several species of genus Laserpitium showed anti-bacterial, anti-fungal, cytotoxic, anti-tumor and anti-inflammatory activities. In this study, we investigated the effect of L. carduchorummethanolic extract on MCF-7 and HEK293 cell line by MTT assay, Induction of apoptosis, cell cycle arrest and expression of some apoptotic genes. After treatment of cells with plant extract, plant was significantly (P < 0.0001) cytotoxic to MCF-7 and cytotoxic to HEK293 at high dose treatment (1000µg/ml). IC50 for each cell evaluated. Proper IC50 for MCF-7 was 55.25µg/ml and 725.9µg/ml for HEK293. Cell cycle arrest and apoptosis were analyzed by using a FACSCanto II (BD Biosciences flow cytometry, cells arrested at S and G2/M phase(P<0.01, P<0.05)**L. Carduchorum** induced a marked increase (P < 0.001) in the percentage of early and late apoptotic. Real-time PCR was performed to analyze the expression of some apoptotic genes (Bax, Bcl-2, Caspase 3 and Caspase 9)Bax, a central cell death regulator, is an indispensable gateway to mitochondrial dysfunction and a major pro-apoptotic member of the B-cell lymphoma 2 was expressed significantly (p <0.05) after cells treatment of cell.*

Keywords--*Laserpitium carduchorum, MCF-7, cell cycle, apoptosis.*

I. INTRODUCTION

Breast cancer is one of the leading cancers globally with the highest rate of cancer deaths in women. Tissue invasiveness and metastatic spread of breast cancer cells are liable for most of the morbidity and mortality allied with the disease(Mansoori et al. 2010). In Iraq, breast cancer is the commonest type of female malignancy, accounting for approximately one-third of the registered female cancers according to the latest Iraqi Cancer Registry (Alwan 2010).

Apoptosis is a type of programmed cell death that is characterized by cell membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation(Nagasaka et al. 2010, Burgess 2013). There are two basic apoptotic signaling pathways: the extrinsic and the intrinsic pathways (Verbrugge et al. 2010).

Bax, a central cell death regulator, is an indispensable gateway to mitochondrial dysfunction and a major pro-apoptotic member of the B-cell lymphoma 2 (Bcl-2) family proteins that control apoptosis in normal and cancer cells. A number of drugs in clinical use are known to indirectly activate Bax. Intriguingly, recent efforts demonstrate that Bax can serve as a promising direct target for small-molecule drug discovery (Liu et al. 2016). Bcl-2, Anti-apoptotic B-cell lymphoma-2 (BCL-2) prevents the induction of apoptosis not only in malignant cells but also in normal cellular lineages. This critical observation has rapidly evolved from merely

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identifying new BCL-2 family members to understanding how their biochemical interactions trigger the cell death process, and, more recently, to pharmacological inhibition of anti-apoptotic BCL-2 function in disease (Opferman and Kothari 2018). Bcl-2 functions by preventing programmed cell death (Vaux et al. 1988).

Plants produce a wide range of chemical compounds that apparently have no direct role in the plants' growth. These compounds are called secondary metabolites. Alkaloids, terpenoids, flavonoids, pigments, and tannins are important constituents of these compounds. Secondary metabolites have biologic effects such as anti-inflammatory, anticancer, contraceptive, and different effects on hematopoietic cells (Mansouri et al. 2015) Finding plants that replace chemotherapy and cumbersome cures of cancer with cytotoxic effects is necessary.Medicinal plants have a special place in the management of cancer. It is estimated that plant- derived compounds in one or the other way constitute more than 50% of anticancer agents (Dashora et al. 2011).

Laserpitium carduchorum is a perennial herb belonging to the Apiaceae family. In Iran, this plant is found in the Kurdistan Province, Bane Mountains (Ghahreman 1993). Several species of genus *Laserpitium* showed anti-bacterial, anti-fungal, cytotoxic, anti-tumor and anti-inflammatory activities (Rodriguez et al. 1976, Picman 1986, Petrović et al. 2009, Tirillini et al. 2009, Popović et al. 2013). The genus *Laserpitium*is known to be a rich source of biologically active compounds, such as sesquiterpene lactones (Appending et al. 1986, Appendino et al. 1987, Appendino et al. 1993).The aim of the research is to find the cytotoxic effect of *Laserpitium Carduchorum*in cancer andnormal cell line and expression of different apoptotic genes in breast cancer cell line, and measurement of cell proliferation in response to growth factors by MTT kit, and cell cycle under the effect of plant extraction. Also to investigate new treatment from the medicinal plants with less side effect and safe for normal cells as nowadays cancer is rating second fatal disease worldwide.

II. METHODOLOGY

2.1. Plant extraction

The aerial parts of *Laserpitium carduchorum* (200 g) were cut into small pieces and put in the Erlenmeyer to extract the methanolic extract according to the maceration method with shaking. After 72 hours, the methanolic extract was passed through a Whatman filter paper and concentrated in a vacuum at 50°C using a rotary evaporator. The extract was kept in a sterile vial in a dark and cool place for another stage of the test.

2.2. Cell Culture

MCF-7 and HEK 293 Cells were grown in DMEM media supplemented with 10% FBS, 1% penicillin and streptomycin 0.1% amphotericin-B. High glucose media contain L-glutamine and sodium bicarbonate solution. The cells were maintained as monolayers in 25 cm2 plastic tissue culture flasks at 37C° in a humidified atmosphere containing 5% CO2 in air. Exponentially growing cells were used in all the experiments

2.3. MTT *assay*

Cell suspensions were seeded into a 96-well-plate and incubated for 24 h (5- $6x10^3$ per well). Cells were allowed to reach exponential growth by resting at least 24 hours which is equal to one cell cycle duration of selected cells. After removing the medium, 100 µL medium was added to each well. The cells were incubated with 10 µL of 5mg/ml 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT), which was dissolved in 90 µL of medium for 4hr after each treatment. Cells that were life, cleaved the yellow tetrazolium salt to an insoluble precipitate (formazan). The decrease in the percentage of living cells is correlated with the

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amount of formazan precipitate crystals (Fotakis and Timbrell 2006).Then discarded the supernatant, kept the formazan precipitate and added 100 µL DMSO into the wells. The absorbance of the specimen was measured at 540 nm with a BioTek ELx808 microplate reader.Cell viability results were shown as percentages in comparison with the control group To quantify the sensitivity of selected cell types the half-maximal inhibitory concentration (IC50) is the *L.carduchorum* concentration required for a 50% inhibition of cell growth was also measured.

2.4. Cell Cycle Analysis by Propidium Iodide Staining

Cells were seeded at $5x10^5$ cells per well in six-well plates, treated with *L.carduchorum*, and harvested as described above. Cells were washed twice with DPBS and resuspended in 1.2 mL of ice-cold DPBS in polypropylene flow cytometry tubes. Next, 2.8 mL of 100% ice-cold ethanol was added dropwise with gentle vortexing, to achieve a final concentration of 70% ethanol. The fixed cells were stored at -20C° overnight, washed twice by centrifuging at 200x g for 10 min at 4 C° and aspirating the supernatant. Cells were resuspended in freshly prepared propidium iodide (PI) staining solution consisting of 200 µg/mL PI (Sigma-Aldrich), 200 µg/mL DNase-free RNase A (Sigma-Aldrich), and 0.1% (v/v) Triton X-100 (Sigma-Aldrich) in DPBS, incubated at 37 C° for 15 min, and then placed on ice protected from light. Stained cells were analyzed using a FACSCanto II (BD Biosciences) flow cytometer, acquiring at least 50,000 single-cell events per sample. Quantification of the percentage of cells in G0/G1, S, and G2/M phases of the cell cycle was performed using the Watson (Pragmatic) model in FlowJo v10.4.1 (FlowJo, LLC, Ashland, OR, USA).

2.5. Apoptosis Assay by Annexin V Propidium Iodide Staining

Cells were seeded at $5 x10^5$ cells per well in six-well plates, treated with *L.carduchorum*, and harvested as described above. Cells were washed twice with DPBS and stained with the Annexin-V-FLUOS staining kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. To compensate for the overlapping spectra of annexin V and PI, additional unlabeled and single-labeled samples, which contained dead cells, were prepared. Necrotic cells were prepared by heating a cell suspension in DPBS at 63 C° for 30 min. Cells were analyzed using a FACSCanto II (BD Biosciences), gating out debris and doublets, and acquiring at least 10,000 single-cell events per sample. Quantification of viable (double-negative), early apoptotic (annexin V-positive), late apoptotic (annexin V and PI double-positive) and necrotic cells (PI-positive) was performed using FlowJo v10.4.1 (FlowJo, LLC).

2.6. Quantitative Analysis of Gene Expression

According to the manufacturer's recommendations, the totalamount of RNA was extracted from the MCF7 cells and HEK293cells treated with *L.carduchorum*ineach step (0, 24, 48, 72, and 96 h) by using RNeasy Mini Kit (Qiagen, USA). Concentrations of RNA were determined by UV spectrophotometry (Eppendorf, Germany). The cDNAs were synthesized from 500ng ofDNAase-treated RNA samples with a Quantitect Reverse TranscriptionKit by using oligo (dT) primers. The specific primers used for PCR reactionsare listed in (Table 2-1)and they were synthesized by Pishgam (Tehran,Iran). PCRs were performed by using Master Mix and Cyber Green in an Applied Biosystems Step One™ thermal cycler (Applied Biosystems, USA). The PCR program started with an initial melting cycle to activatethe polymerase for 5 min at 95 °C, followed by 40 cycles of melting 30s at 95 C°), annealing (30 s at 58 C°), and extension (30 s at72C). The quality of PCR reactions was confirmed by melting curve analysis. Efficiency was determined by using a standard curve for each gene

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(logarithmic dilution series of cDNA fromthe tests). For each sample,the reference gene (Gapdh) and target gene were amplified in the same run. The reference genes were approximately equal. The target genes were normalized to a reference gene and expressed relative to a reference gene as calibrator

Table 2.1 list of specific primers used for PCR reactions

Genes	Primer Sequence	Annealing Temperature
CAS 3	Forward: AAGCGAATCAATGGACTCTGG	62 C°
CAS 3	Reverse: CAAGTTTCTGAATGTTTCCCTGAG	
CAS 9	Forward: GCTCTTCCTTTGTTCATCTCC	54 C°
CAS 9	Reverse: CATCTGGCTCGGGGTTACTGC	
BAX	Forward: CGAGAGGTCTTTTTCCGAGTG	60 C°
BAX	Reverse: GTGGGCGTCCCAAAGTAGG	
BCL2	Forward: AGCATCACGGAGGAGGTAGAC	62 C°
BCL2	Reverse: CTGGATGAGGGGGTGTCTTC	
Gapdh	Forward: TGTGACTTCAACAGCAACTCCCAT	62 C°
Gapdh	Reverse: CTCTCTTGCTCTCAGTATCCTTGC	

III. RESULT

3.1. Plant Extraction

Laserpitium carduchorum was collected from Kurdistan province in the west of Iran. A voucher specimen (No.7909) was registered in the herbarium of the Institute of Forests and Rangelands Researches, Sanandaj, Kurdistan Province, Iran. The plant was analyzed by Dr. Dara Dastan from Hamadan University in Iran. After extraction by maceration method yield extract was 7gr in 200gr plant.

3.2. Cytotoxic effect of *L. Carduchorum* on MCF-7, HEK 293 cell lines in vitro

*L.Carduchorum*did not change cell viability percent in normal cells HEK 293 but, changed cell viability percent in breast cancer cells MCF-7 and there was a significant difference between the two cell lines. Increasing concentration of *L. Carduchorum* treatment exhibited a marked decrease in viability percent in breast cancer cells compared with normal cells. The obtained mean viability percent of normal and cancer cells after treatment with different concentrations of *L. Carduchorum* shown in figure (3-1). The result showed a significant decrease in MCF-7 cells by increasing concentration. This decrease is less obvious in the HEK 293 cells compared with the MCF-7 cells.

Increasing concentration (1, 10, 50, 100, 500 and 1000 µg/ml) of *L. Carduchorum* showed a noticeable decrease in viability percent in cancer and a slight decrease in normal cell lines compared with the control group. The most significant ($P < 0.0001$) decrease in cell viability percent was observed in the group treated with 100 and 500 µg/ ml that cell viability percent decreased from 100% (in control group) to 24.16% and 22.94% in MCF-7 and 96.18% and 86.59% in HEK 293 cells respectively.

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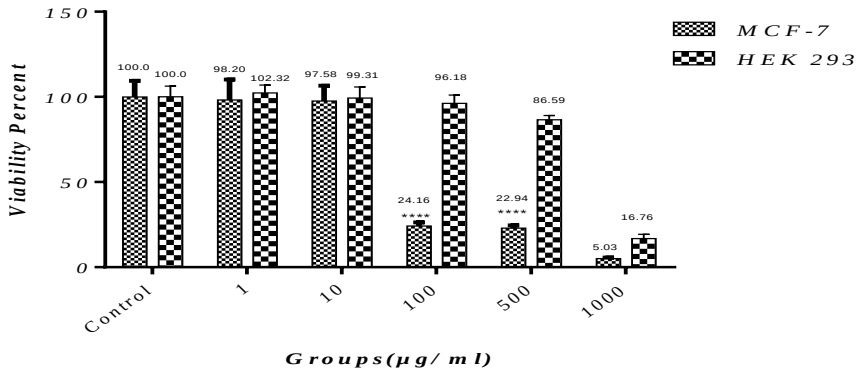

Figure3.1: The mean viability percent of MCF-7 and HEK 293 cells after treatment with different concentrations of *L. Carduchorum.*

3.3. Evaluation of the half-maximal inhibitory concentration (IC50):

The concentration of *L. Carduchorum* extraction required for a 50% inhibition of cell growth (IC_{50}) was obtained by extrapolation from an inhibition curve. The obtained IC_{50} values for HEK 293 cells were (725.9µg/ml) greater than the values obtained for MCF-7 cells (55.25µg/ml). This result showed that breast cancer cells were more sensitive to *L. Carduchorum*, as expected (Fig 3-2).

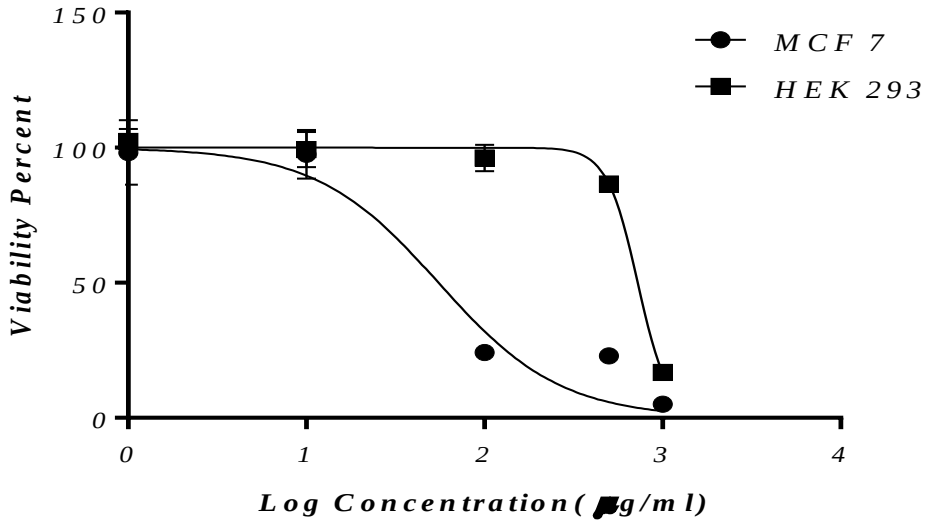

Figure 3.2: The half-maximal inhibitory concentration (IC50) of *L. Carduchorum* in MCF-7 and HEK 293 cells at different concentrations (100,500 and 1000 µg/ml).

3.4. *L. Carduchorum* Induced Cell Cycle Arrest

After observing inhibition of cell growth, we investigated if *L. Carduchorum*induced cell cycle arrestusing flow cytometric analysis of propidium iodide stained DNA. Cells were treated with *L. Carduchorum*at (55.25µg/ml)for MCF-7 cells and (725.9µg/ml) for HEK 293,three samples were analyzed respectively for eachcell and mean of results were analyzed. Results showedasignificant increase in the

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percentageof cells in S and G2/M(P<0.01, P<0.05) and decreased rate of cells in the G0/G1 phase as shown in figure (3-3).

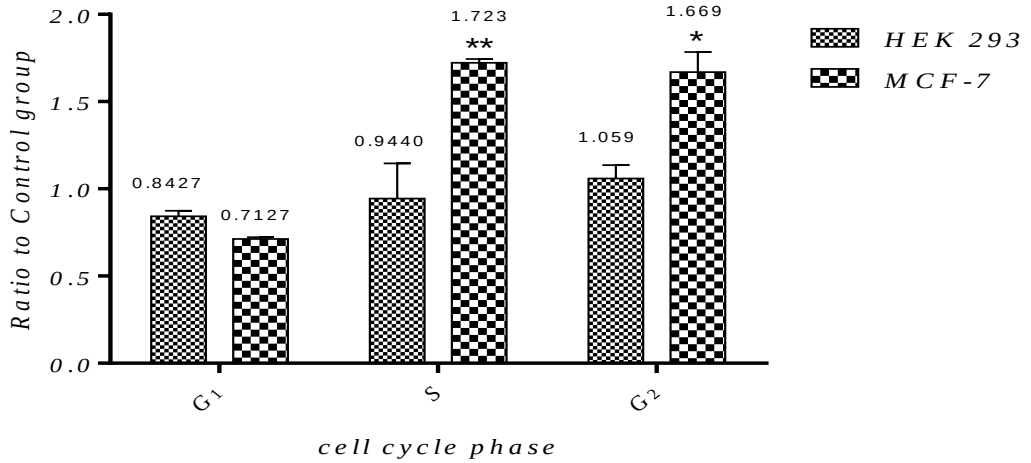

Figure 3.3: cell cycle arrest after treatment of cells MCF-7 and HEK 293 with *L. Carduchorum.*

3.5. *L. Carduchorum*Induced Apoptosis

To determine if ***L. Carduchorum*** inhibited cell growth by inducing apoptosis, cells were stained using the Annexin-V-FLUOS staining kit. Exposure of phosphatidylserine to the outer leaflet of the plasma membrane occurs early in apoptosis and can be detected by flow cytometry using a fluorescently conjugated annexin V, which binds to phosphatidylserine. Viable cells do not stain (annexin V and PI double-negative), early apoptotic cells display increased staining only with annexin V (annexin V positive, PI negative), for late apoptotic cells the cell membrane integrity is lost allowing penetration of PI (annexin V and PI double-positive), and necrotic cells stain with PI only (annexin V negative, PI-positive). ***L. Carduchorum*** induced an increase in annexin V staining in each of the MCF-7 and HEK 293 cell lines, indicative of apoptosis in all studies after cells treated with ***L. Carduchorum*** extract three samples were studied for each cell line, and mean were analyzed in figure (3-4). For MCF-7 cells, (55.25µg/ml)***L. Carduchorum*** induced a marked increase (P < 0.001) in the percentage of early and late apoptotic cells; in contrast, for HEk 293 cells this marked increase occurred with 725.9µg/ml ***L. Carduchorum*** methanolic extract.

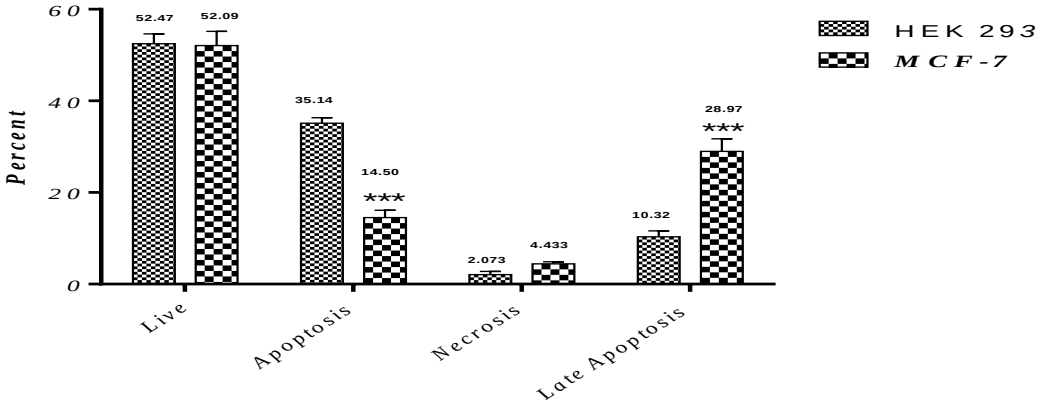

Figure 3.4: Induction of apoptosis after treatment with L.carduchorum for MCF-7 and HEK 293 cell lines

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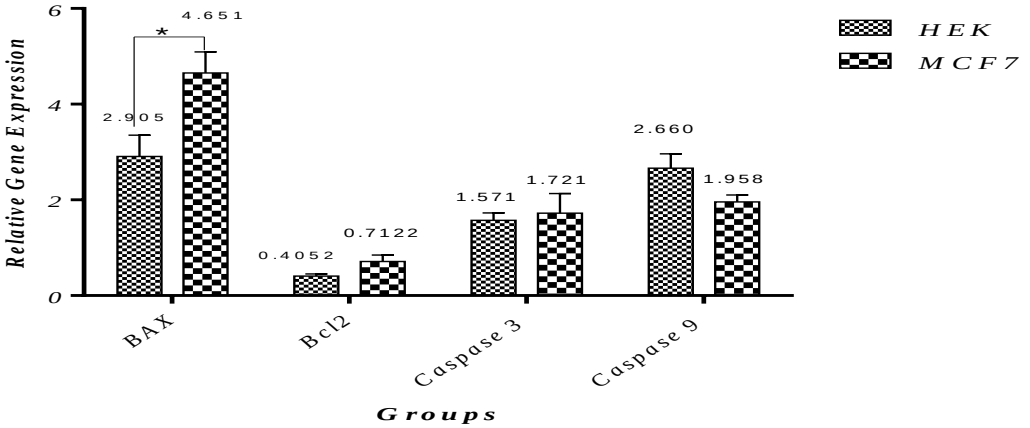
3.6. Gene Expression in the Differentiation Procedure

Real-time PCR was performed to analyze the expression of some apoptotic genes (Bax, Bcl-2, Caspase 3 and Caspase 9) in MCF-7 and HEK 293cells after they treated with methanolic extraction of *L. Carduchorum*. After studying three reactions, the mean of gene expressions was analyzed andthe expressions of Bax were significantly increased ($p < 0.05$) in HEK 293 and MCF-7experimental groups. While there was a slight increase in Caspase 3 and 9 and a decrease in anti-apoptotic gene Bcl-2. The results demonstrated that *L. Carduchorum*



extraction had a positive effect in the expression of the apoptotic geneBax, results shown in(Fig 3-5).

Figure 3.5: Expression of apoptotic genes after treatment with *L. carduchorum.*

IV. DISCUSSION

Natural products due to fewer side effects and higher pharmacological potential are considered important targets for development of drugs, *Laserpitium carduchorum*is frequently used as a spice and in Bane folk medicine the aerial parts ofthis are used to treat urinary infections. The antioxidant activities of different extracts of *L.carduchorum*at different developmental growth stages were examined(Dastan et al. 2016). Several methods have been set up for the measurement of antioxidant activity of plant extracts including DPPH, FRAP and TEAC assays. Free radical scavenging capacities of different extracts of *L. carduchorum*at different phenological stages measured by DPPH assay. According to the results, the highest scavenging activity was found for ethyl acetate fraction of methanolic extracts (E-M) (IC50= 31.1, 38.8 and 55.7 µg/ml for flowering, seed ripening and vegetative, respectively), followed by methanolic (M) and water fraction of methanolic extract (W-M) of plant at different phenological stages(Dastan et al. 2016).

However, the anticancer activities of *L. Carduchorum* against MCF-7 human breast cancer cells as well as its effects on cell apoptosis, cell cycle arrest, and activation of apoptotic signaling pathways have not been reported so far to the best of our knowledge. Cell cycle arrest and apoptosis induction are regarded as effective strategies to stop the uncontrolled cell growth by eliminating cancerous cells. Apoptosis especially acts as a protective mechanism that abolishes harmful or damaged cells prior to the appearance of malignancy (Buolamwini 2000, Murray 2004, Michaelis et al. 2011).Initially, the MTT assaywas used to evaluate theeffects

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of *L. Carduchorum* on cell viability, After cells were treated with *L. Carduchorum* methanolic extract the results demonstrated that the plant was significantly cytotoxic to MCF-7 breast cancer cell line (100µg/ml) and cytotoxic to HEK293 normal cell line in high dose treatment (1000µg/ml). The present study reported anticancer potentials *in vitro*,methanolic extract of aerial parts of *L. Carduchorum*was tested against human breast cancer (MCF- 7) cell line and normal human embryonic kidney cell line (HEK 293) by MTT assay, cell cycle arrest, induction of apoptosis and expression of some apoptotic gene analysis. The different concentrations of aliquots with optimum IC50 valuesrecorded potential anticancer. The induction of cytotoxicity (alteration in morphologic characteristics) was recorded in a concentration-dependent manner; the increased concentration of extracts induced a higher rate of cellular damages (MTT, expression of Bax gene, cell cycle arrest, and apoptosis).Furthermore, using flow cytometry, the effects of this drug on apoptosis induction were studied and indicated that *L. Carduchorum*induced both early and late apoptosis in these cells. After treatment with a (55.25µg/ml) dose of *L. Carduchorum*, the percentage of apoptotic cells increased to 35.14% and28.97%. Cell cycle arrest is a common cause of growth inhibition. To determine whether the effect of extracts involvesalterations in cell cycle progression, cell cycle distribution analysis flow cytometry using propidium iodide as a probe was used to study the effects of this compound on cell cycle progression. The binding of water-soluble, DNA intercalating propidium iodide correlates with the amount of DNA within a given cell, and the relative content of DNA indicates the distribution of a population of cells throughout the cell cycle (Heidari et al. 2014). *L. Carduchorum* induced sub-G2 cell cycle arrest and led to a significant increase of sub-G2cells in a dose-dependent manner. Further, it was shown that *L. Carduchorum* could inhibit MCF-7 cancer cell invasion in a concentration-dependent manner. These findings are promising since it is well established that breast cancer is one of the most invasive cancers and *L. Carduchorum* could inhibit this behavior. Finally, the effects of *L. Carduchorum* on the expression levels of various proteins including Bax, Bcl-2, Caspase 3 and Caspase 9 were studied using RT-PCR assay.*Bax*is a proapoptotic *Bcl-2* family member that plays a key role in the induction of mitochondrial-dependent apoptosis. *Bcl-2* is an antiapoptotic member that can neutralize *Bax*function in the initiation of cell death. Proteins that stimulate or inhibit apoptosis determine the fate of the cell for death or survive.High *Bax*expression stimulates sensitivity to apoptotic agents and decreases tumor enlargement (Bargou et al. 1996), Effect of *L. Carduchorum* on the MCF-7 and HEK293 is evaluated by investigation of expression levels of some apoptotic genes by RT-PCR. As a result of this analysis, it was determined that *Bax*gene expressions significantly increased in *L. Carduchorum* -treated MCF-7. But, the *Bcl-2* gene expression was decreased (Figure 3-5). These results were interpreted as *L. Carduchorum*inhibits cell differentiation by increasing the expression of these genes (Bax, Caspase-3, and Caspase-9).

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