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Research Article

CYTOTOXIC, APOPTOTIC AND NECROTIC EFFECTS OF *INULA VISCOSA*: AN IN VITRO STUDY ON DIFFERENT CELL LINES

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ABSTRACT. This study aimed to evaluate the cytotoxic, apoptotic and necrotic effects of *Inula viscosa* on mouse fibroblast cell line (L929), human breast cancer cell line (MCF7) and human lung adenocarcinoma cell line (A549). The WST-1 (4-[3-(4-Iodophenyl) -2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, BioVision) test was used to determine cytotoxicity. Apoptotic and necrotic rates were determined by double staining technique, Propidium iodide and Hoechst 33342.The water extract of *Inula viscosa* extract caused cytotoxicity in a dose dependent manner on L929, MCF7, and A549 cell lines with IC_{50} values of 0.217 mg/ml, 0.102 mg/ml, and 0.103 mg/ml, respectively. At all doses, the cell viability of L929 fibroblast cell line was significantly the highest compared to MCF7 and A549 cell lines (p= 0.0001), except for doses of 0.5mg/ml and 0.015625mg/ml. Both apoptotic and necrotic rates changed in a dose dependent manner in all three cell lines and cell death occurred through apoptosis, but mainly necrosis. Except in few concentrations, MCF7 cell line seems to be the most sensitive to *Inula viscosa* extract with the highest apoptotic and necrotic rates (p=0.0001) while L929 fibroblasts seem to the least sensitive with the least apoptotic and necrotic rates (p=0.0001). Further studies should focus on chemical constituents of *Inula viscos*a raised in different region of the territory and additional mechanistic studies to reveal exact mechanism of cell death that may provide additional treatment modalities in cancer cases.

Keywords: *Inula viscosa, MCF7, A549, L929, cytotoxicity, apoptosis, necrosis*.

INTRODUCTION

Traditional herbal medicines are promising agents that have potentials to be used in the treatment of many diseases, including cancer. They exert their effects via activation of the biologically active components found in their structures some of which have strong therapeutic effects. Many plant species contain biologically active compounds that are beneficial for human health, have antioxidant and anti-inflammatory effects [1]. Due to these properties, plants have been regarded as the main sources of new therapeutic agents

for many centuries [2, 3]. In addition to their beneficial effects, herbal remedies used in traditional treatments may also have side effects and interact with some medications [4]. It should be known that green plants may contain toxic and mutagenic ingredients [5].

Inula viscosa is a perpetual herb belonging to the *Asteraceae* family and grows throughout the Mediterranean Basin [6]. Members of Inula family have sesquiterpenoids with a broad biological spectrum of action. As a member of Inula family, *Inula viscosa* has been used for a long time in traditional treatment practices and has anti-inflammatory [7], antipyretic, anthelmintic, antiphlogistic and antiseptic activities [8, 9]. As an herbal remedy, it has been also used in the treatment of diabetes and lung diseases [10, 11]. Numerous studies have been conducted to determine the biologically active contents of *I. viscosa*. The biological mechanisms underlying its anti-inflammatory, antipyretic, anthelmintic, antiphlogistic and antiseptic activities have been attributed to polyphenols ([12] and sesquiterpens [13, 14] in *I. viscosa.* Different studies have shown that *I. viscosa* exerts anti-inflammatory effects by inhibition of COX1, COX2 and iNOS enzymatic activities [13, 15].

Inula viscosa is an aromatic plant that grows widely throughout the Mediterranean Basin [16]. It has been stated that the chemical composition of the *I. viscosa* extract varies by region, thus, such variations in contents may cause deviations in the expected activities [17]. Extract vehicle is another factor effecting the biological activities *I. viscosa*. The water extract has antifungal effects [18, 19] while the extracts in some organic solvents have antibacterial effects [20].

Inula viscosa extract has been previously shown to have various degrees of cytotoxic and apoptosis-inducing effects on some human cancer cell lines [14, 17, 21]. The results of such studies on cancer cell lines are not conclusive on degree of anti- cancer activities. Thus, further studies are needed to characterize the anti-proliferative effects of *I. viscosa*, using different cell lines.

This study aimed to investigate the cytotoxic, apoptotic and necrotic effects of *I. viscosa* on mouse fibroblast cell line (L929), human breast cancer cell line (MCF7) and human lung adenocarcinoma cell line (A549).

MATERIALS AND METHODS

Plant material and extraction protocol

The samples of *I. viscosa* plant were collected from the district of Akhisar in the province of Manisa. The collected plant samples were identified by the Pamukkale University Herbarium a herbarium number has been assigned (registration no: PAMUH2011000004170). The decoction, a widely used method in traditional medicine, was used for extraction. The leaves of the plant were dried in the oven and pulverized. Then, 20 g of powder material was placed in 200 ml of boiling water and boiled in a closed beaker for 5 minutes [3]. After cooling down to room temperature, the boiled suspension was filtered through a filter paper (Whatman No. 1) to remove particles [22]. Fresh extract was prepared prior to each use.

Cell culture

The mouse fibroblast cell line (L929), human breast cancer cell line (MCF7) and human lung adenocarcinoma cell line (A549) were used. The cells were obtained from the cell stocks within the Kırıkkale University Scientific and Technological Research

Application and Research Center. For cultivation of L929 fibroblast, a Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, USA) containing 10% fetal bovine serum (FBS) (Biowest, USA) and 1% Penicillin-Streptomycin (Pen-Strep, Pan Biotech, Germany) was prepared. For MCF7 and A549 cell lines, RPMI-1640 (Sigma-Aldrich, USA) medium was prepared with a mix of 10% FBS and 1% Penicillin-Streptomycin. The cells were seeded in 25 cm² cell culture flasks and kept at 37 ºC conditioned with 5 % CO_2. Trypsin/EDTA (Pan Biotech, Germany) was used for separation of cells from the bottom of the flask during cell passaging.

Cytotoxicity test

The WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, BioVision) test was used to determine cytotoxicity. Cells were seeded into 96 well plates at $1.0x10^4$ cells/well and left for 24 hours incubation. The test material as prepared above was applied to cells in various concentrations (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) and incubated for 24 hours at 37 °C conditioned with 5% CO_2. Each treatment was prepared in triplicate. Cell culture medium was used as negative control. At the end of 24 hours, the medium in the wells were removed and 100 µl of Phenol Red-free medium (RPMI 1640 Medium, no phenol red, Thermo Fisher Scientific) was placed in all wells. Then 10 µl of WST-1 solution was added. After 2 hours of incubation at 37 ºC, it was read at 440 nm in a microplate reader (AMR-100, Allsheng). Based on the comparison with the controls, the cell viability was calculated using the equation below:

$$Cell\ viability\ \% = \frac{100 \text{ x } OD440e}{\text{OD440b}}$$

OD440e: the value of the optical density of the sample

OD440b: optical density of the control (blind) group

Double staining for apoptosis and necrosis

For the determination of apoptosis and necrosis by double staining, the dye solution was prepared using Propidium iodide (PI, Serva, Finland), Hoechst 33342 (Santa Cruz Biotechnology, USA) and Ribonuclease A (Serva, Finland) [23]. Cells were seeded into 96 well plates at $1.0x\ 10^4$ cells/well and kept for 24 hours at 37 °C conditioned with 5 %. The extract was diluted at various concentrations (1: 1, 1: 2, 1: 4, 1: 8, 1:16, 1: 32,1: 64, and 1: 128), applied to cells and kept for 24 hours at 37 °C conditioned with 5 % CO_2. Treatments were run in triplicate. Cell culture medium was used as negative control. At the end of 24 hours, the media in the wells was removed and 100 µl of the prepared dye solution was added to all wells. After 15 minutes of incubation at 37 °C, the cells were examined under a fluorescent microscope (Leica, DMI 6000B). Apoptotic cells illuminate high intensity brilliant blue fluorescence as a results of Hoechst dye staining, while normal cells illuminate pale low fluorescence [23]. The nuclei of necrotic cells illuminate red fluorescence as a consequence of PI staining. Unlike in normal healthy live cells, PI can pass through the cells with a disintegrated cell membrane and stains the cell nucleus in red [24, 25]. Cells were evaluated in 10 randomly selected areas using the 40X microscope objective in order to determine the number and ratios of apoptotic and necrotic cells.

Statistical analysis

All data were given as mean±standard deviation (X±SD). One-way analysis of variance (ANOVA) was used to compare the groups and then Tukey test was used in post-Hoc analysis. The SPSS software (IBM SPSS Statistics 22.0) was used for statistical analyses. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Wst-1 results

The *I. viscosa* extract caused a dose dependent cytotoxicity on L929, MCF7, and A549 cell lines with IC_{50} values of 0.217 mg/ml, 0.102 mg/ml, and 0.103 mg/ml, respectively (Fig.1.-3.). At the highest dose used (0.5 mg/ml), the cell viability values were 16.48±4.6%, 10.86±3.1%, and 8.95±3.1% in the L929, MCF7, and A549 cell lines, respectively. At a dose of 62.5 µg/ml, the cell viability values were 65.71±3.6%, 39.65±8.9%, and 21.01± 6.3% in L929, MCF7, and A549 cell lines, respectively. At the lowest dose used (3.9 µg/ml), the cell viability values were 120.65±5.2%, 90.67±11.8% and 96.59±1.8% in L929, MCF7, and A549 cell lines, respectively (Table 1.). The IC_{50} values were 0.217 mg/ml, 0.102 mg/ml, and 0.103 mg/ml for L929 fibroblasts, MCF7, and A549 cell lines, respectively.

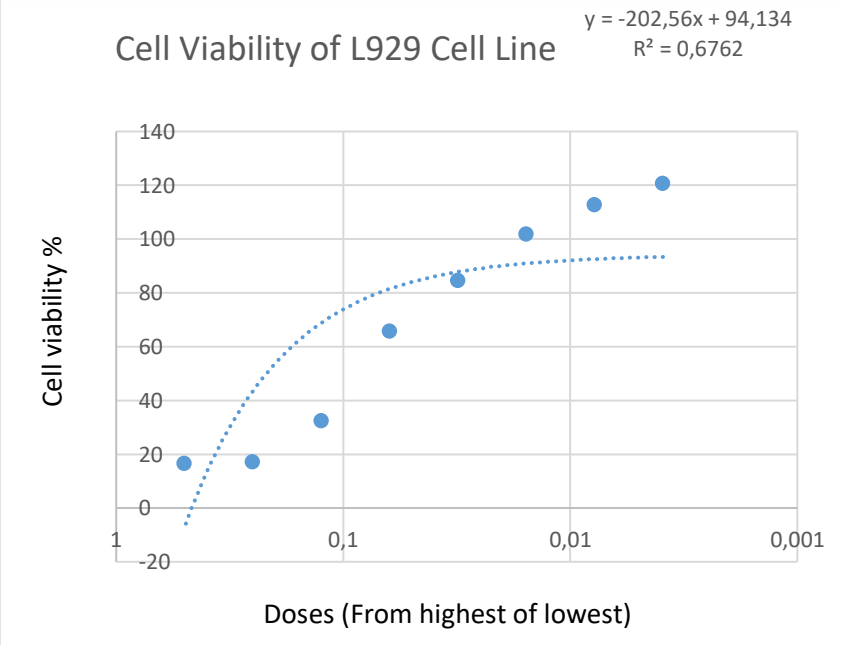

Fig. 1. *Dose dependent change of viability values (%) in L929 cell line exposed to various concentrations of I. viscosa extract. IC_{50}=0.217 mg/ml*

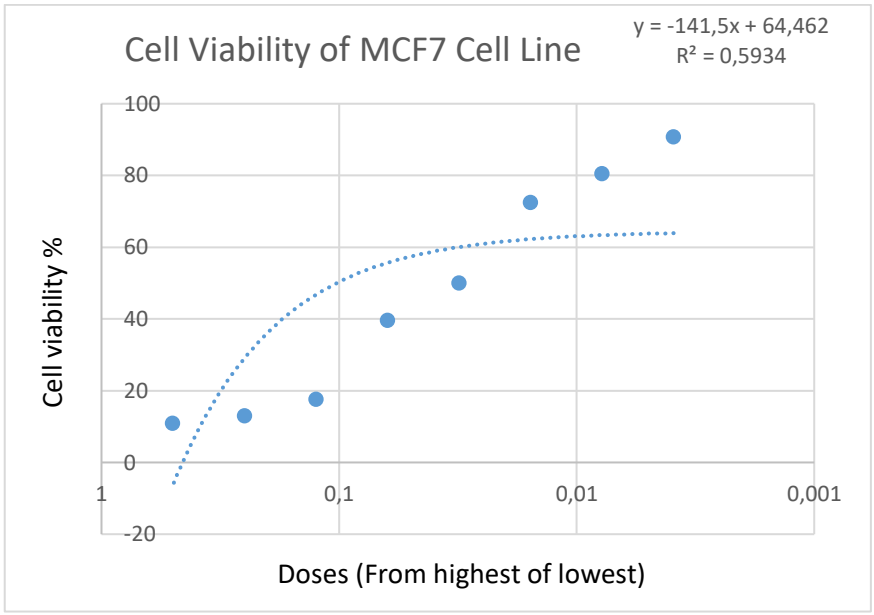

Fig. 2. *Dose dependent change of viability values (%) in MCF7 cell line exposed to various concentrations of I. viscosa extract. IC_{50}=0.102 mg/ml.*

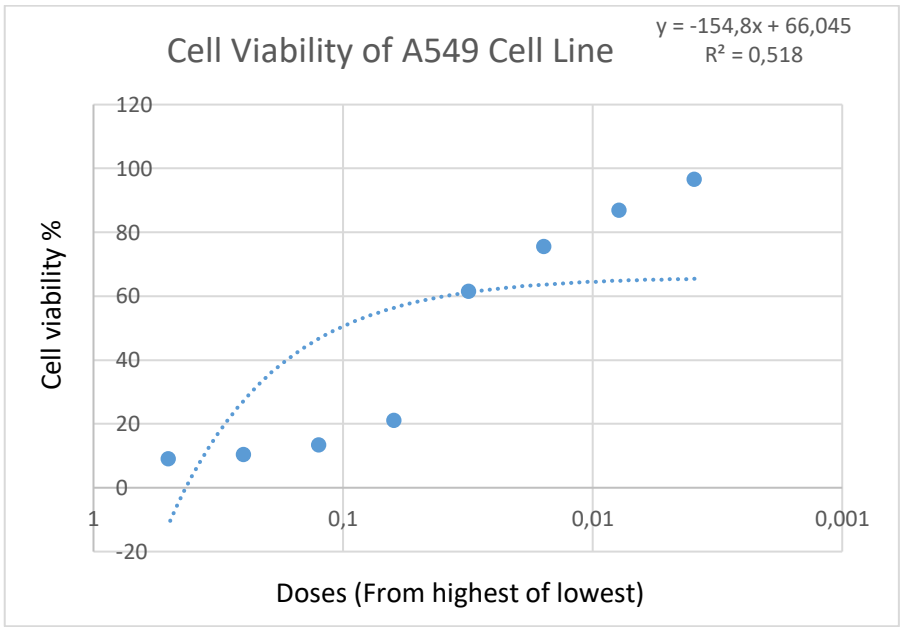

Fig. 3. *Dose dependent change of viability values (%) in A549 cell line exposed to various concentrations of I. viscosa extract. IC_{50}=0.103 mg/ml.*

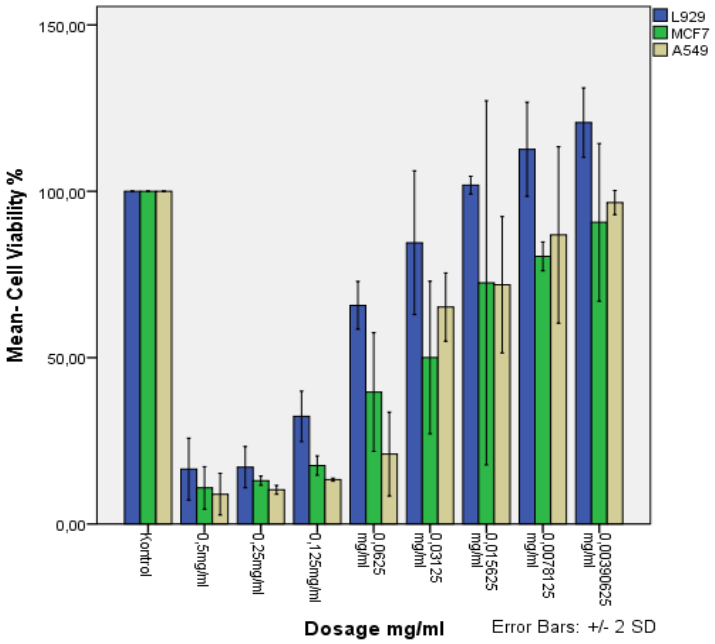

Fig. 4. *Cell viability values in L929, MCF7 and A549 cell lines treated with different concentrations of I. viscosa extract. Data are presented as % mean±standard deviation.*

For L929 cell line, the viability rates were compared not only among the doses but also with control. The difference in comparison with control was statistically significant at doses of 0.0625 mg/ml and above (p=0.0001), but insignificant at the doses of 0.03125 mg/ml and below (p=0.053). For the MCF7 cell line, it was observed that the difference from the control group at doses of 0.03125 mg/ml and above was statistically significant (p=0.0001), and the difference from the control group at doses of 0.015625 mg/ml and below was not statistically significant (p=0.123). For A549 cell, it was observed that the difference from the control group was statistically significant (p=0.0001) at the doses of 0.0625 mg/ml and above, and the difference from the control group was not statistically significant (p=0.099) at doses of 0.03125 mg/ml and below.

When the same doses of plant extract applied to L929, MCF7 and A549 cell lines were compared (Table 1), the difference was statistically significant (p=0.0001) for almost every dose, except for doses of 0.5 mg/ml and 0.015625 mg/ml. At all doses, the cell viability of L929 fibroblast cell line was the highest compared to MCF7 and A549 cell lines. The cell viability ratios of MCF7 and A549 were not statistically significant except at the dose of 0.0625 mg/ml.

Double staining results

Double stained cultures of the cell lines studied were depicted in Fig. 5, 6, 7. Cell nuclei illuminating an intense blue fluorescence under the DAPI filter of the fluorescence microscope (LEICA DMI 6000B) were interpreted as cells undergoing apoptosis while cells with a pale nucleus were interpreted as absence of apoptosis. In cells that undergo necrosis, cell nuclei illuminate red fluorescence under the FITC filter of the fluorescence microscope. The apoptotic and necrotic rates (%) are presented in Table 2. Both apoptotic and necrotic rates changed in a dose dependent manner in all three cell lines. Except in few concentrations, MCF7 cell line seems to be the most sensitive to *I. viscosa* extract

with the highest apoptotic and necrotic rates while L929 fibroblasts seem to the least sensitive with the least apoptotic and necrotic rates (p=0.0001).

Table 1. *Cell viability values in L929, A549 and MCF7 cell lines treated with different concentrations of I. viscosa extract.*

	Cell Viability (%) in Cell Lines			
Doses mg/ml	**L929**	**A549**	**MCF7**	**Control**
0.5	16.48±4.6 $^{a.1}$	8.95±3.1 $^{a.1}$	10.86±3.1 $^{a.1}$	100.00±0^{2}
0.25	17.07±3.1 $^{a.1}$	10.28±0.6 $^{a.2}$	13.01±0.7 $^{a.1.2}$	100.00±0^{3}
0.125	32.34±3.7 $^{b.1}$	13.32±0.2 $^{a.2}$	17.58±1.45 $^{a.2}$	100.00±0^{3}
0.0625	65.71±3.6 $^{c.1}$	21.01±6.3 $^{a.3}$	39.65±8.9 $^{a.b.2}$	100.00±0^{4}
0.0312	84.54±10.7 $^{d.1.3}$	61.55±1.5 $^{b.1.2}$	50.03±11.4 $^{b.c.d.2}$	100.00±0^{3}
0.0156	101.85±1.3 $^{e.1}$	75.55±3.9 $^{b.c.1}$	72.48±27.35 $^{c.e.1}$	100.00±0^{1}
0.0078	112.62 ±7.0 $^{e.f.1}$	86.88±13.25 $^{c.e.2}$	80.43±2.16 $^{d.c.e.2}$	100.00±0$^{1.2}$
0.0039	120.65±5.2 $^{f.1}$	96.59±1.8 $^{d.c.e.2}$	90.67±11.8 $^{e.2}$	100.00±0^{2}
Control	100.00±0 ^{e}	100.00±0 ^{e}	100.00±0 ^{e}	

**Data are given as in mean ± standard deviation*
*** There is no difference between groups with the same number in the same row.*
**** There are no differences among doses with the same letter in the same column.*
***** A p value <0.05 was considered statistically significant.*

Table 2. *Apoptotic and necrotic index values in L929, MCF7 and A549 cell lines treated with different concentrations of I. viscosa extract.*

	L929		**A549**		**MCF7**	
Doses mg/ml	**Apoptotic index (%)**	**Necrotic index (%)**	**Apoptotic index (%)**	**Necrotic index (%)**	**Apoptotic index (%)**	**Necrotic index (%)**
0.5	23.4±2.5 **a.1**	57.1±3.29 $^{a.1}$	25.20±3.44 $^{a.1}$	63.5±2.4 $^{a.2}$	24.52±3.15 **a.1**	68.8±1.48 $^{a.3}$
0.25	20.3±1.53 **a.b.1**	46.03±1.8 $^{b.1}$	17.77±2.84 **b.1**	62.95±2.7 $^{a.2}$	16.99±2.17 **b.1**	65.3±3.9 $^{a.2}$
0.125	22.8±2.08 **a.b.1**	37.68±1.6 $^{c.1}$	12.04±1.78 **c.d.2**	56.77±2.8 $^{b.2}$	9.20±1.20 **c.e.3**	47.42±5.2 $^{b.3}$
0.0625	19.7±1.45 **b.1**	31.86±2.1 $^{d.1}$	14.45±2.43 **c.b.2**	40.7±2.36 $^{c.2}$	5.08±1.76 **d.f.g.3**	24.11±2.72 $^{c.3}$
0.0312	3.81±1.2 **c.1**	7.11±1.76 $^{e.1}$	11.13±2.44 **c.d.e.2**	30.50±0.8 $^{d.2}$	5.70±1.70 **d.e.f.1**	14.24±2.83 $^{d.3}$
0.0156	3.13±2.64 **c.1**	4.78±0.81 **e.f.g.1**	8.04±1.09 **d.e.f.2**	12.44±1.2 $^{e.2}$	8.63±3.78 **d.e.2**	5.83±0.9 $^{e.1}$
0.0078	2.86±1.80 **c.1**	3.31±1.85 **f.g.1**	6.88±3.49 $^{e.f.2}$	10.17±0.5 $^{e.f.2}$	3.60±1.27 **f.g.1.2**	4.0±0.83 $^{e.1}$
0.0039	1.77±0.7 **c.1**	2.8± 0.81 $^{g.1}$	4.01±2.31 $^{f.g.1}$	6.71±1.83 $^{f.2}$	3.26±1.27 **f.g.1**	3.37±2.3 $^{e.1}$
Control	1.28±0.58 **c.1**	1.22±1.21 $^{g.1}$	1.80 ±0.48 $^{g.1}$	1.46 ±1.73 $^{g.1}$	1.64±0.76 **g.1**	1.53±0.85 $^{e.1}$

*Data are given as in mean ± standard deviation
** There are no differences among groups with the same number in the comparison of the same parameter in the same row.
*** There are no differences among doses with the same letter in the same column.
**** A p value <0.05 was considered statistically significant.

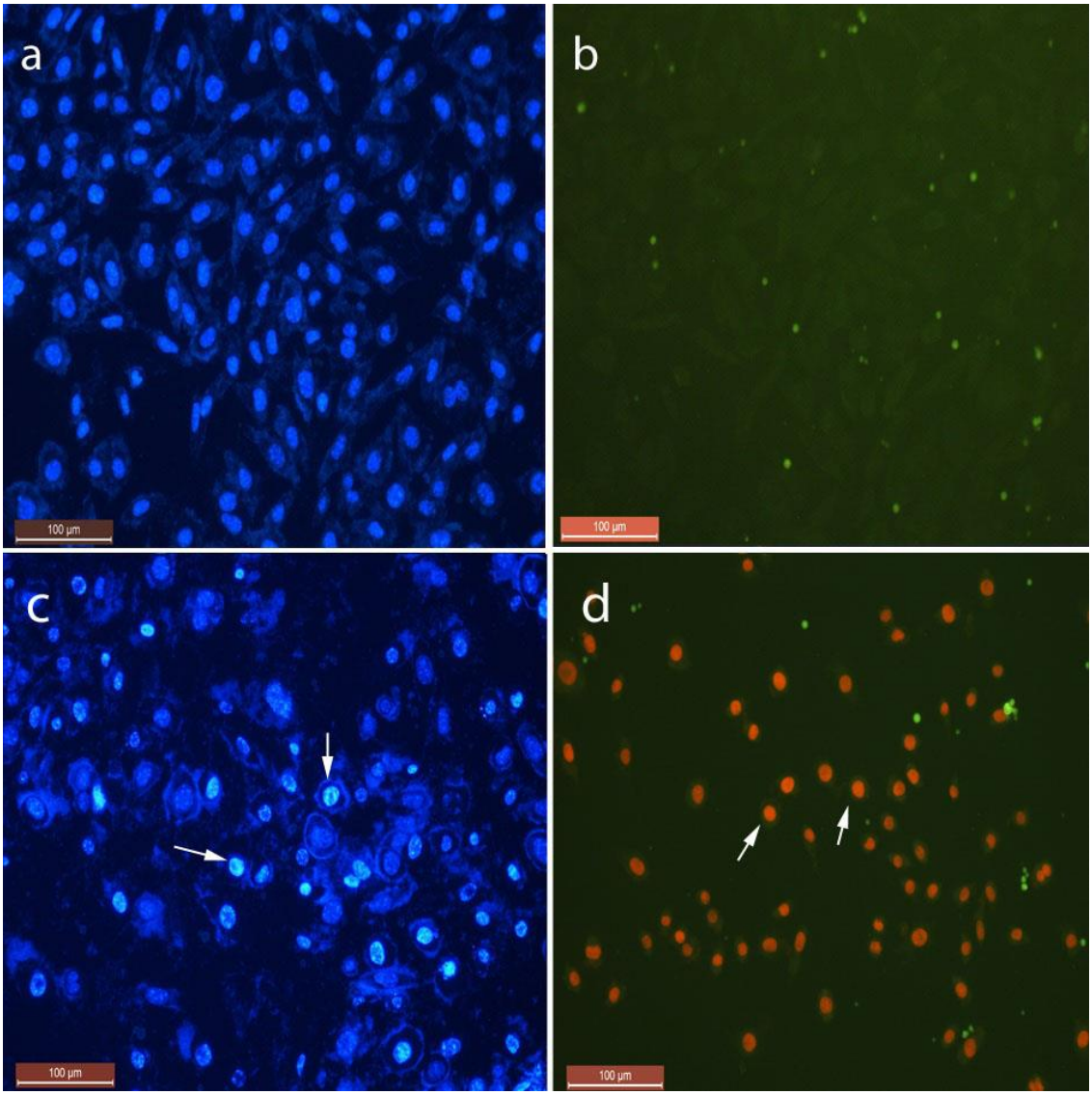

Fig. 5. *Double staining images of L929 cell line treated with 0.5 mg/ml of I. viscosa extract. A) In control group under the DAPI filter the fluorescence microscope, cultured cells appeared in pale blue, indicating absence of apoptosis, B) control group FITC filter, cells illuminated green fluorescence C) In I. viscosa extract applied cultures, apoptotic cells (arrows) were depicted as their nuclei illuminated brilliant blue fluorescence under the DAPI filter, and D) Necrotic cells (arrows) were indicated by their red fluorescence under the FITC filter of the fluorescence microscope in their nucleus seen. Propidium Iodide and Hoescht 33342 were used for double staining.*

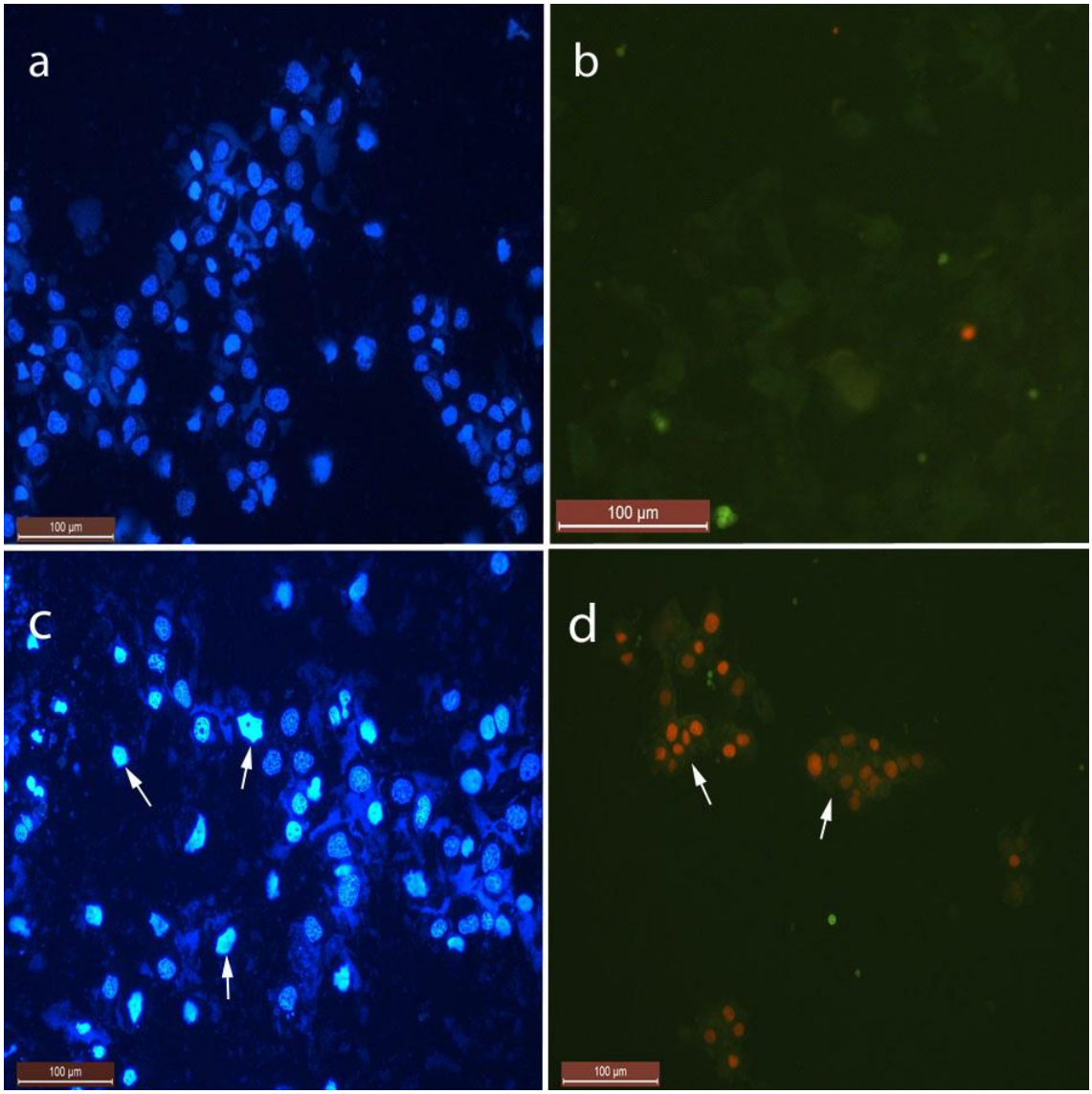

Fig. 6. *Double staining images of MCF7 cell line treated with 0.5 mg/ml of I. viscosa extract. A) In control group under the DAPI filter the fluorescence microscope, cultured cells appeared in pale blue, indicating absence of apoptosis, B) control group FITC filter, cells illuminated green fluorescence C) In I. viscosa extract applied cultures, apoptotic cells (arrows) were depicted as their nuclei illuminated brilliant blue fluorescence under the DAPI filter, and D) Necrotic cells (arrows) were indicated by their red fluorescence under the FITC filter of the fluorescence microscope in their nucleus seen. Propidium Iodide and Hoescht 33342 were used for double staining.*

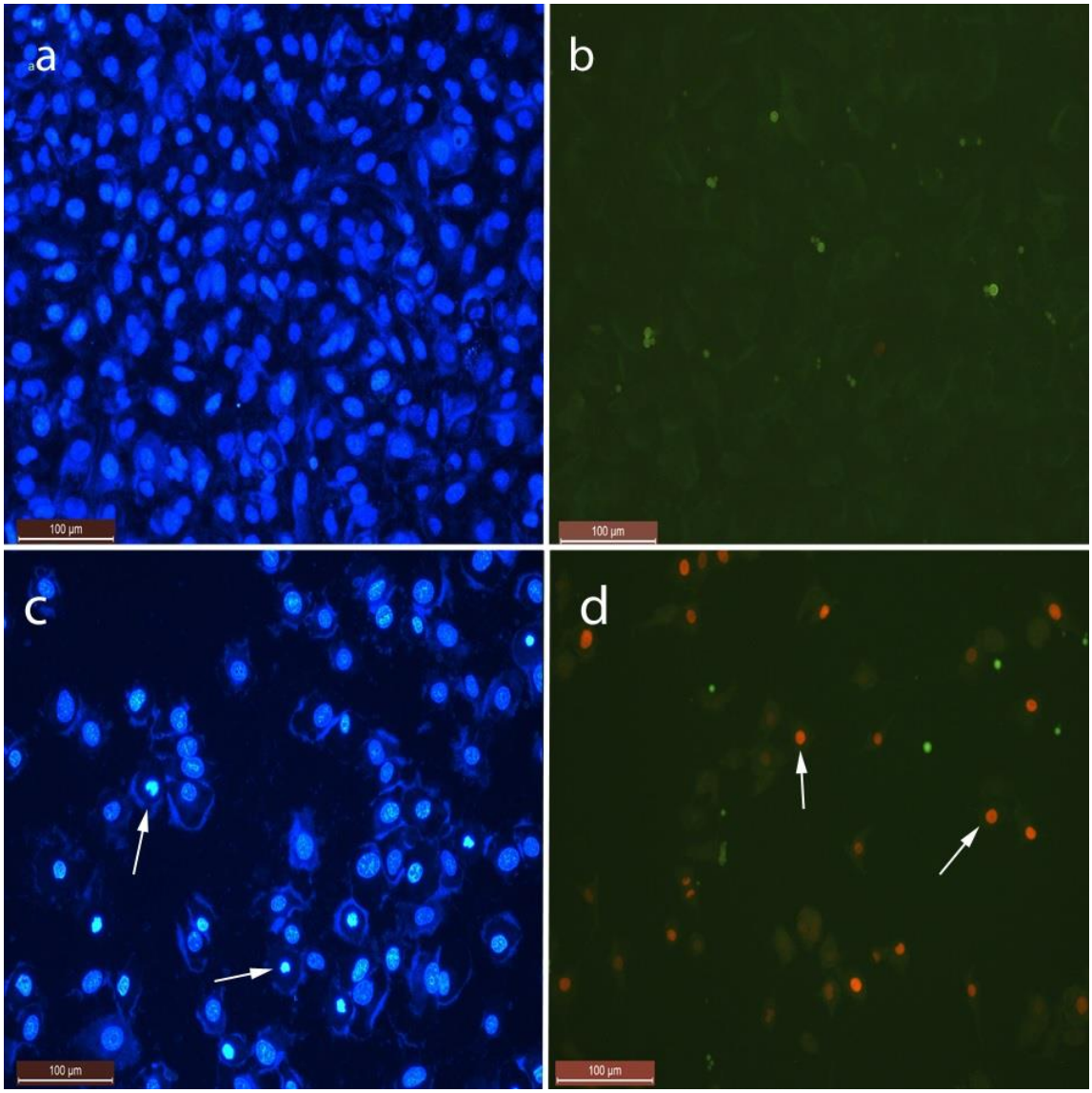

Fig. 7. *Double staining images of A549 cell line treated with 0.5 mg/ml of I. viscosa extract. A) In control group under the DAPI filter the fluorescence microscope, cultured cells appeared in pale blue, indicating absence of apoptosis, B) control group FITC filter, cells illuminated green fluorescence C) In I. viscosa extract applied cultures, apoptotic cells (arrows) were depicted as their nuclei illuminated brilliant blue fluorescence under the DAPI filter, and D) Necrotic cells (arrows) were indicated by their red fluorescence under the FITC filter of the fluorescence microscope in their nucleus seen. Propidium Iodide and Hoescht 33342 were used for double staining.*

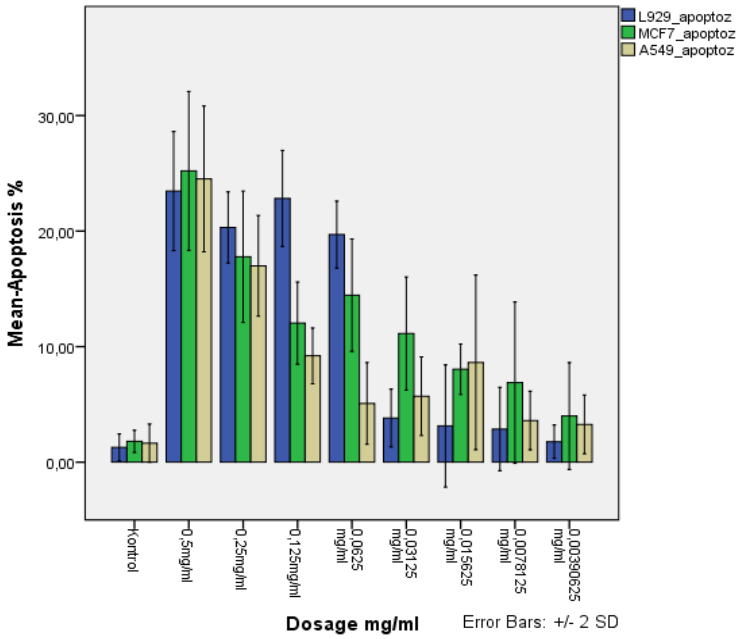

Fig. 8. *Apoptosis values in L929, MCF7 and A549 cell lines treated with different concentrations of I. viscosa extract. Data are given as % Mean±Standard Deviation.*

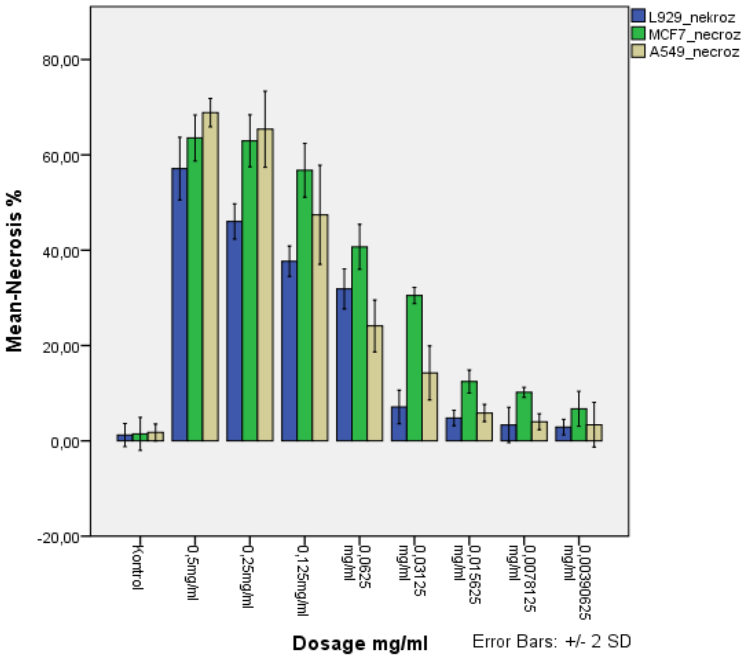

Fig. 9. *Necrosis values in L929, MCF7 and A549 cell lines treated with different concentrations of I. viscosa extract. Data are given as % Mean±Standard Deviation.*

Plant extracts with medicinal properties as a part of traditional medicine provide significant treatment modalities in different disease processes. Possessing various bioactive compounds sesquiterpenes, sesquiterpenes acids, azulenes, lactones, flavonoids, and essential oils [3, 17]. *Inula viscosa* plant is widely used in traditional

medicine and has been proposed for anticancerous activities. In this study, various concentrations of the *I. viscosa* extract obtained by the decoction method, were evaluated for cytotoxic, apoptotic and necrotic activities on normal mouse fibroblast cell line (L929), human breast cancer cell line (MCF7) and human lung adenocarcinoma (A549). In support to some previously reported studies conducted on different cancer cell lines, we found *I. viscosa* plant extracts exerted various degrees of cytotoxic effect on all cell lines tested in a dose dependent manner.

Cancer cell lines seem to be more sensitive to *I. viscosa* extract compared to normal cells [26]. Brahmi-Chendouh et al. [27] concluded that *I. viscosa* extract is able to inhibit cell viability by impairing the mitochondrial redox activity of neuroblastoma, hepatoblastoma and colon carcinoma cells while it does not affect HaCaT cells, immortalized human keratinocytes. Another study by Bar-Shalom et al. [26] found that the water extract of *I. viscosa* extract decreased cell viability of HCT116 and Colo320 cancer cell lines in a time and dose dependent manner while normal primary liver fibroblasts were not affected by the treatment following application of 100-300 µg/ml *I. viscosa* extract. In our study; although twice as resistant as cancer cell lines used, L929 normal mouse fibroblasts are affected by *I. viscosa* extract with an IC_{50} value of 206.7 µg/ml. Such as difference may be due to the different primary cell lines used as the extraction solvents used in both studies are same, distilled water. Additional studies could be conducted to reveal if there is a difference in chemical constituents of these plants as they are harvested in different regions. The present study found that cancer cell lines used, MCF7, and A549 cell lines, are at least twice as sensitive to *I. viscosa* extract as L929 cells, a cell line of normal fibroblasts. The present study cannot conclude that I. viscosa extract does not affect normal cells; however, it can proclaim that it exerts more toxic in MCF7, and A549 cancer cell lines compared to L929 normal fibroblasts. The lower cell viability in cancerous cell line may lie behind the fact that the cell membrane permeability of cancerous cells is higher than that of normal cells [28]. In addition, the cell cycle of cancerous cells may be different from normal cells because cancer cells divide more rapidly [29].

The chemical constituents and thus cytotoxicity of the *I. viscosa* extract may also differ depending upon extract solution used. For example, Benbacer et al. [21] used methanol and hexane extracts of *I. viscosa* to demonstrate its cytotoxicity and antiproliferative effects on SiHa and Hela cell lines of human cervix cancer cells. The IC_{50} values calculated for hexane and methanol extracts *I. viscosa* on the SiHa cell line were 9.56±1.68 µg/ml and 52.83±3.28 µg/ml. In the Messaoudi et al. [17] study, ethyl acetate and ethanol were used as extraction solutions. In the mentioned study, IC_{50} values obtained from MCF7 cell line were calculated as 285.42±1.51 and above 500 µg/ml in ethyl acetate and ethanol extracts of the plants harvested in Imouzzer region, respectively. In the present study, IC_{50} value was calculated as 101.65 µg/ml for MCF7 cell line. Compared to our study, Messaoudi et al. [17], the higher IC_{50} values calculated for the MCF7 cell line may be due to the difference in the extraction protocol as the plant extract varies in the content, amount or density of biological active substances released from the plant during extractions with organic solvents and water. In Ozkan et al. [30] study, IC_{50} of water extract of *I. viscosa* in MCF7 was above 200 µg/ml, which twice as high as that found in the present study. Messaoudi et al. [17] reported that the biological active components of the extracts of *I. viscosa* harvested from different regions are also different. In this context, Messaoudi et al. [17] harvested plants from three different regions of Morocco, and the plant used in extraction in our study was collected from

Manisa province. Another reason for the difference in IC_{50} values of the MCF7 cell lines in the two studies may be that the plants were harvested from different regions. The chemical composition of the *I. viscosa* extract differs according to the region where the plant is grown [17]. Thus, the cytotoxicity induced by *I. viscosa* extracts varies by regions where the plant raised. Having harvested *I. viscosa* form different regions of Morocco and applied to MCF7 and MDA-MB231 cells, Messaoudi et al. [17] reported that the biological active components of the extracts of *I. viscosa* harvested from different regions were different that influences the plant cytototoxicity on cells. For instance, the IC_{50} value of ethanol extract of *I. viscosa* harvested in Sefrou and exposed to MDA-MB-231 cells was 195.42±1.81 µg/ml while it was 392.12±2.56 µg/ml for the ethanol extract of the plant harvested in Taounate. The IC_{50} value of ethanol and ethyl acetate extracts of *I. viscosa* harvested in in Sefrou region were 195.42±1.81 µg/ml and 112.20±1.28 µg/ml, respectively. In the present study, we harvested *I. viscosa* in the province of Manisa and extracted in water. The IC_{50} values were 0.217 mg/ml, 0.102 mg/ml, and 0.103 mg/ml for L929 fibroblasts, MCF7, and A549 cell lines, respectively. Future studies should focus on determination of chemical constituents *I. viscosa* harvested in the province of Manisa and further cytotoxicity studies upon extraction in different solutions.

The cytotoxic activity of *I. viscosa* was mainly attributed to constituents such as nepetin, 3-O-methylquercetin, 3,3'-di-Omethylquercetin, and hispidulin and their ability to induce apoptosis [27]. Having incubated MCF7 cells with 3-O-methylquercetin or 3,3′-di-O-methylquercetin, two constituents of *I. viscosa* plant, Talib et al. [31] proclaimed that *I. viscosa* plant induces cell death through apoptosis in cancer cells. Bar-Shalom at al. [26] found an apoptotoic rate of 16-23% in 24-hour application of *I. viscosa* extract to human colorectal cancer cell lines (HCT116, Colo320). Benbacer at al. [21] found an apoptosis rate of 9-18% in human cervix cancer cell lines (SiHa and HeLa) in 24 hours of application of *I. viscosa* extract. These apoptosis rates are comparable to those found in the present study (16.99-25.20%). The results of the present found that *I. viscosa* plant extract induces cell death not only through apoptosis but also necrosis in all cell lines tested. Importantly, our double staining assay found that the majority of cell deaths induced by *I. viscosa* plant extract seems to be occur through necrosis in a dose dependent manner. In this perspective, our results differ from those of the study by Bar-Shalom at al. [26] which reported a necrotic rate of 1.5-4.4% while the apoptotic rate was 12-21.7% at a dose of 300 µg/ml for the plant extract. As reviewed by Fiers et al. [32] cell death occurs through necrosis due to mitochondrial damages in L929 fibroblasts. Brahmi-Chendouh et al. [27] identified various bioactive chemical compounds in *I. viscosa* and proclaimed that the cytotoxic of compounds in *I. viscosa* mainly impair the mitochondrial redox activity in neuroblastoma, hepatoblastoma and colon carcinoma cell lines. As cited by Marshall and Baines [33] generation of mitochondrial reactive oxygen radicals (ROS) induced by some mediators, for example TNFα, may lead cells to cell death in which some of the mediators leading to cell death are common for both apoptosis and necrosis. The same authors strongly support a new process of cell death, necroptosis, as others [34]. Having these in mind and our double staining results, we proclaim that cell death occurs not only through apoptosis but also necrosis as a result of exposition to *I. viscosa* extract. Compared to MCF7 and A549, L929 normal mouse fibroblasts seems to be more resistant to *I. viscosa* induced apoptosis and necrosis. Additional studies are needed to reveal the exact mechanism of cell death induced by *I. viscosa* extracts in cancer normal cells.

Inula viscosa extract also caused cytotoxicity not only mammalian cells but also plant cells. Presence of a cellulose wall in plant cells, unlike animal or human cells, may be

more resilient to cytotoxicity. Çelik et al. [3] demonstrated cytotoxic and genotoxic effects of *I. viscosa* extract by applying *I. viscosa* extract doses as high as 5 mg/ml and 10 mg/ml to *Allium cepa* meristem cells. Thus, *I. viscosa* extract has potentially cytotoxic not only cancer cells but also normal mammalian and plant cells despite higher doses needed. In this regard, the anticancerous properties of *I. viscosa* should be revisited by further mechanistic studies.

There are various methods of detecting apoptosis e.g. TUNEL, Annexin V assay, ISNT Labeling, mitochondrial detection, H2A.X Assays, etc [35, 36]. Assays such as release of lactate dehydrogenase activity assay has been used to detect cell necrosis [37]. Every technique has same advantages and disadvantages by means of price, sensitivity, optimization, and workload. Thus, despite presence of various methods of apoptosis, there are still challenges to overcome and efforts to find new and more reliable targets [38, 39]. In the present study, a double staining method (Propidium iodide and Hoechst33342) was employed for detection apoptosis and necrosis. Apoptotic cells also show morphological changes such as chromatin condensation and nuclear fragmentation [40]. Apoptotic cells illuminate high intensity brilliant blue fluorescence as a results of Hoechst dye staining, while normal cells illuminate pale low fluorescence [23]. The nuclei of necrotic cells illuminate red fluorescence as a consequence of PI staining. Unlike in normal healthy live cells, PI can pass through the cells with a disintegrated cell membrane and stains the cell nucleus in red [24, 25]. The double staining method used in the present study detect apoptosis and necrosis has been one of the most commonly preferred techniques. Using the double staining method techniques, we demonstrated that *I. viscosa* induced cell death occurs not only via apoptosis but also necrosis.

CONCLUSION

As a result, the water extract of *I. viscosa* exerts cytotoxic effects on L929 fibroblasts, A549, and MCF7 cell lines in a dose dependent manner with the IC_{50} values of 0.217 mg/ml, 0.103 mg/ml, and 0.102 mg/ml, respectively. Cell death occurs through apoptosis but mainly necrosis. L929 normal fibroblast cells are more resistant to *I. viscosa* induced cytotoxicity compared to A549, and MCF7 cancer cell lines. Further should focus on chemical constituents of *I. viscosa* raised in different region of the territory and additional mechanistic studies to reveal exact mechanism of cell death that may provide additional treatment modalities in cancer cases.

Conflict of Interest. The author declared that there is no conflict of interest.

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