

Dichloromethane and Methanol Extracts of *Scrophularia oxysepala* Induces Apoptosis in MCF-7 Human Breast Cancer Cells

Samira Valiyari^{1,2}, Behzad Baradaran^{1*}, Abbas Delazar³, Ardalan Pasdaran¹, Fateme Zare^{1,4}

¹ Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

² Student Research Committee, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.

³ Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

⁴ Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.

ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 15 July 2012
Accepted: 30 July 2012
ePublished: 15 Aug 2012

Keywords:
Scrophularia oxysepala
Cytotoxic
MCF-7 cells
Apoptosis
Anticancer
Breast cancer

ABSTRACT

Purpose: Breast cancer is the most common cause of cancer-related death in women worldwide. Therefore, there is an urgent need to identify and develop therapeutic strategies against this deadly disease. This study is the first to investigate the cytotoxic effects and the mechanism of cell death of *Scrophularia oxysepala* extracts in MCF-7 human breast cancer cells. **Methods:** Three extracts of *Scrophularia oxysepala* including the n-hexane, dichloromethane and methanol extracts were examined. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Trypan-blue assays were performed in MCF-7 cells as well as Human umbilical vein endothelial cells (HUVEC) to analyze the cytotoxic activity of the extracts of *Scrophularia oxysepala*. Further, the apoptosis inducing action of the extracts was determined by TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling) test and cell death assay. **Results:** The results showed that the n-hexane extract had no cytotoxic effects but dichloromethane and methanol extracts significantly inhibited cell growth and viability in a dose and time dependent manner without inducing damage to non-cancerous cell line HUVEC. In addition, Cell death assay and DNA fragmentation analysis using TUNEL indicated induction of apoptosis by dichloromethane and methanol extracts of *Scrophularia oxysepala* in MCF-7 cells. **Conclusion:** Our studies suggest that this plant may contain potential bioactive compound(s) for the treatment of breast cancer.

Introduction

Breast cancer, a major clinical problem, is considered as the most common cause of cancer death in women world wide. Breast cancer incidence and death rates have increased in most countries worldwide in the last decades. In the USA alone, breast cancer comprised 28% all new cancer cases and 15% of all cancer death among women.¹⁻³ There are five treatment methods for breast cancer: surgery, chemotherapy, radiation therapy, hormonal therapy and targeted therapies. But some of these methods cause undesired side effects by the non-specific targeting of both normal and cancer cells.^{4,5} Based on this, there are growing searches for the treatment of cancer by using natural products. Among natural sources, medicinal plants have played an important role in the treatment of many forms of cancer. Several plant-derived compound including taxol from *Taxus brevifolia* L., camptothecin from *Camptotheca acuminata*, Decne, vinca alkaloids from *Catharanthus roseus* G. Don and podophyllotoxin from

Podophyllum peltatum L. are used in clinic all over the world.⁶⁻⁸ Previous studies have shown that anticancer properties of medicinal plant extracts can be mediated through the induction of apoptosis.⁹ Apoptosis, programmed cell death is a physiological process that characterized by remarkable morphological features such as cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation. Apoptosis plays a major role as a protective method against carcinogenesis by eliminating damage or abnormal cells.¹⁰⁻¹⁴ Therefore, the identification of medicinal plants that induce apoptosis may help in generating anti-cancer drugs.¹⁵⁻²⁰

Scrophularia is a genus of the family Scrophulariaceae. This genus includes about 3000 species and 220 genera which are widely distributed in central Europe, central Asia and North America, especially in the Mediterranean area.²¹ In traditional medicines, some species of the genus have been used to treat fever,

erythema, eczema, wounds, inflammation of skin, different types of dermatosis (including scabies, tumours and slough), mouth dryness, sore throat, ulcers, goiter, tonsillitis, furunculosis, constipation, prurigo, aching bones, abscesses, fistulas and cancer.²²⁻²⁵

Phytochemical study of *Scrophularia* species identified several bioactive compounds, including iridoids, iridoid glycosides, phenylpropanoid glycosides, phenylethanoid glycosides, resin glycosides, sugar esters, flavonoids, terpenoids and saponins.²⁶⁻³⁵ They have shown a number of biological activities such as anticancer, antioxidative, anticoagulant, antimicrobial, antiprotozoal, antifungal, antidiabetic, anti-inflammatory, choleric, hepatoprotective and neuroprotective activities.³⁶⁻³⁸ Recently, other research groups reported cytotoxic activity of *Scrophularia striata* extract on a human Astrocytoma cell line (1321) without effects on fibroblast cell line.²¹ In present study we aimed for the first time to investigate cytotoxic effects of *Scrophularia oxysepala* extracts and to determine their possible cell death properties on breast carcinoma cancer cells. Furthermore, we evaluated probable side effects of the extracts on Human umbilical vein endothelial cells (HUVEC) as normal cells.

Materials and methods

Preparation of extracts

Scrophularia oxysepala plant was collected from Eastern Azarbaijan province, Iran, in April 2010. A voucher specimen (2821) was deposited at the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences. The leaves and stems were washed, dried and ground to get powder using a blender. Extractions were performed in a Soxhlet apparatus with n-hexane, dichloromethane and methanol, respectively. The different extracts were concentrated by rotary evaporator (Heildolph, Germany) at about 45 °C and then dried in very low pressure. The dried extracts were stored at -20 °C. 20 mg of each extract were dissolved in 100 µL dimethyl sulfoxide (DMSO) and were diluted with RPMI-1640 medium. Then, test solutions were sterilized using 0.22 µm Syringe filters (Nunc, Denmark) and were used as stock solution for further experiments.

Cell culture

MCF-7 cells (Human breast carcinoma cell line) and HUVEC (Human umbilical vein endothelial cells) were purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Cells were grown in RPMI-1640 medium (Sigma, Germany) supplemented with 10% fetal bovine serum (FBS) (Sigma, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, Germany). Cells were incubated in a humidified incubator contain 5% CO₂ at 37 °C. At 80% confluence, cells were rinsed with PBS/0.5% EDTA and harvested from 25cm² flasks using 0.25 % trypsin/EDTA solution (Gibco, U.K). Then, cells were sub-

cultured into 75cm² flasks, 96-well plates or 6-well plates (Nunc, Denmark) according to experiments. The experiments were performed in triplicate.

MTT assay

Cytotoxicity of n-hexane, dichloromethane and methanol extracts from *Scrophularia oxysepala* were assessed in MCF-7 cells as well as HUVEC using the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Roche Diagnostics GmbH, Germany) according to the manufacturer's protocol. This method is based on the ability of viable cells to metabolize yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenases. The cells were seeded in 96-well plates with a density of 10⁴ cells/well/200 µl and incubated for 24 h at 37°C and 5% CO₂. The cells were treated with different concentrations of solvent extracts (10, 20, 50, 100, 150, 200, 300, 400 µg/ml) and 0.2 % (v/v) DMSO (Merck, Germany) as a negative control. After 12, 24 and 48 h treatment 10 µl of MTT labeling reagent was added to each well. The plates were incubated at 37°C and 5% CO₂ for 4 hours. Then, 100µl of the Solubilization solution was added to each well and followed by incubation overnight at 37°C to dissolve formazan crystals. Finally, absorbance was read using an ELISA plate reader (Bio Teck, Germany) at a wavelength of 570 nm. The percentage of cytotoxicity and cell viability were calculated using following equation: % Cytotoxicity = 1- [mean absorbance of treated cells/ mean absorbance of negative control] and % Viability = 100 - % Cytotoxicity.

Trypan blue assay

Cell membrane integrity and direct counting of living and dead cells was evaluated by trypan blue dye exclusion. This dye does not enter living cells, but passes through the membranes of dead cells. MCF-7 cells (10⁴) in 96 well-plates were exposed to same different concentrations of dichloromethane and methanol extracts of *Scrophularia oxysepala* and 0.2 % (v/v) DMSO for 24 hrs. Then, the medium was removed from the wells, and the cells were washed with 200 µL of PBS. Cells were detached by adding 100 µL of 0.5 % trypsin/EDTA. RPMI-1640 medium supplement with 10% FBS (50 µL) and 0.5 % trypan blue (50 µL) (Merck, Germany) were added to each well, and the plates were incubated for 5 min. Then, a 20 µL aliquot was removed and placed on a Neubauer hemacytometer. Finally, the numbers of viable and nonviable cells were counted under a microscope. The number of viable cells was calculated according to the following formula: [The unstained cell count (viable cells) × the dilution of the cell suspension × 10⁴] / the number of hemacytometer squares that were counted. The percent viability was calculated as: [viable cells/ the total cell count] × 100.

Assessment of necrosis and apoptosis

Apoptosis and necrosis of cells were measured using the Cell Death Detection ELISA^{plus} kit (Roche Diagnostics GmbH, Germany) that quantifies histone associated DNA fragments (mono and oligonucleosomes). MCF-7 cells (10^4) were treated with the same different concentrations of dichloromethane and methanolic extracts of *Scrophularia oxysepala* and 0.2 % (v/v) DMSO at 37°C for 48 hrs. The procedure was performed according to the manufacturer's protocol. Briefly, the culture supernatants and lysate of cells were prepared and incubated in the microtiter plate coated with anti-histone antibody. After color development the results were analyzed spectrophotometrically using an ELISA plate reader at 405 nm.

TUNEL assay for detection of DNA fragmentation

DNA fragmentation was detected by terminal deoxy transferase (TdT)-mediated dUTP nick- end labeling (TUNEL) with the In Situ Cell Death Detection Kit, POD (Roche Diagnostics GmbH, Germany) as described by the manufacturer's protocol. Briefly, (1.5×10^5) MCF-7 cells were subcultured into LabtekTM Chamber Slides and incubated for 24 hrs at 37°C and 5% CO₂. The cells were treated with dichloromethane and methanolic extracts of *Scrophularia oxysepala* at concentrations required for 50% inhibition of growth of MCF-7 cells (LC₅₀) for 24h. Negative control cells were treated with the same final concentration of DMSO present in treated wells [0.2% (v/v)]. After treatment, the cells were fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature and rinsed twice with PBS. Then, the fixed cells were incubated with blocking solution (3% H₂O₂ in methanol) for 10 min and rinsed with PBS. The cells were then incubated in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Subsequently, 50 µl of reaction mixture containing TdT enzyme and nucleotide was added to the cells and incubated for 1 h at 37°C. After washing three times with PBS, the slides were incubated with 50 µl converter-POD streptavidin HRP solution for 30 min, and rinsed three times with PBS. Finally, the cells incubated with DAB and stained cells were analyzed with the light microscope.

Statistical analysis

All the data represented in this study are means±SEM of three identical experiments made in three replicate. Statistical significance was determined by analysis of variance (ANOVA), followed by Duncan test and *p* value ≤0.05 was considered significant. LC50 values were derived from Probit analysis. All analyses were conducted using the SPSS 20.

Results

Cytotoxic effects of *Scrophularia oxysepala* extracts on MCF-7 cells

The cytotoxic effects of *Scrophularia oxysepala* extracts on the growth of MCF-7 cells were determined by MTT and trypan blue assays are shown in Figure 1 and 2. As shown in Figure 1, the treated cells with dichloromethane and methanol extracts in comparison to the untreated control cells exhibited significant decline in viability, whereas the n-hexane extract did not show cytotoxic activity. Moreover, treatment of MCF-7 cells with dichloromethane and methanol extracts showed cell growth inhibition in a time and dose dependent manner. In the higher concentration of these two extracts and the longer time of treatment on cells, the more significant cytotoxicity was achieved. Data analysis of cytotoxicity assay showed that IC₅₀ (dose required for 50% inhibition) of dichloromethane extract of *Scrophularia oxysepala* on MCF-7 cells was 57.5, 22.7 and 19.8 µg/ml for 12, 24 and 48 hrs, respectively. For the methanol extract IC₅₀ was > 400, 180.5 and 100 µg/ml for 12, 24 and 48 hrs, respectively. The IC₅₀ values strongly indicated that the dichloromethane extract has a higher cytotoxic activity on MCF-7 cells than the methanol extract. Direct counting for dead and living cells using the trypan-blue exclusion test showed that 85% dichloromethane extract-treated cells and 67% methanol extract-treated cells with the highest concentration (400 µg/ml) absorbed the dye at 24 h, respectively (Figure 2). Further, we tested dichloromethane and methanol extracts effects on normal non-cancer human umbilical vein endothelial cells. Interestingly, the normal cells were not influenced by these two extracts (Figure 3). Based on the results, dichloromethane and methanol extracts were further used to characterize the cell death mechanism.

Induction of apoptosis by *Scrophularia oxysepala* extracts on MCF-7 cells

To determine the mechanism of the cytotoxic effects of dichloromethane and methanol extracts of *Scrophularia oxysepala*, apoptosis and necrosis of cells were measured by cell death detection ELISA kit. Our results revealed active MCF-7 cells apoptosis in a dose dependent manner. The highest ratio of apoptotic effect in dichloromethane and methanol extracts was 76% and 66%, respectively (Figure 4). In comparison to the methanol extract, the dichloromethane extract induced the greatest apoptotic activity in MCF-7 cells.

Since DNA fragmentation is one of the hallmarks of apoptotic cell death, in the current study, we confirmed the presence of nucleosomal DNA fragments in cells treated with dichloromethane and methanol extracts of *Scrophularia oxysepala* by TUNEL assay. As shown in Figure 5, after the treatment of MCF-7 cells with 24 hrs IC₅₀ concentration of extracts, the apoptotic cells produced brown stained nuclei, whereas the non-apoptotic cells were not stained with similar observation was found in the negative control cells treated with 0.2% (v/v) DMSO.

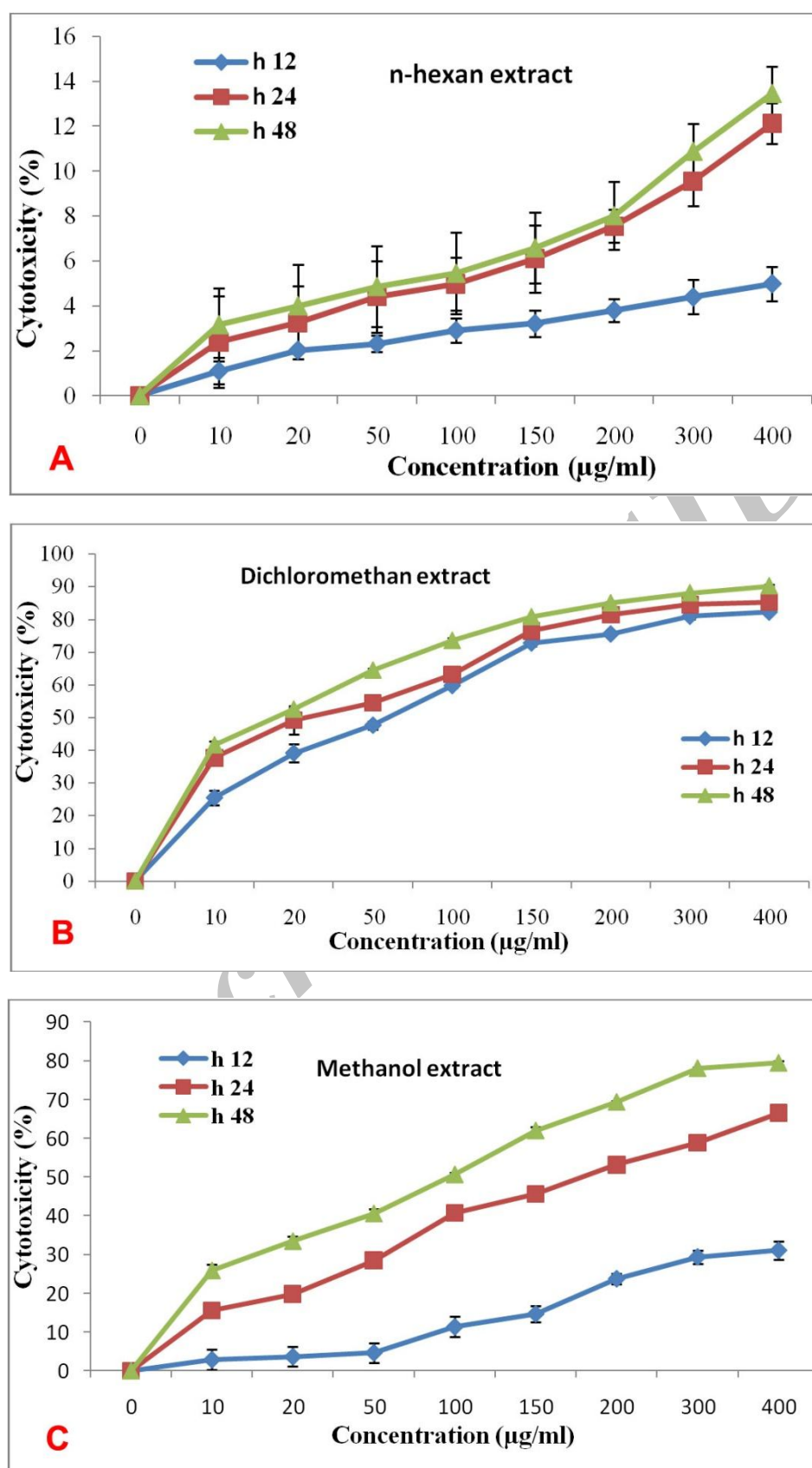


Figure 1. shows the cytotoxic effects of *Scrophularia oxysepala* extracts in MCF-7 cells that were measured using MTT assay. **A.** n-hexan extract., **B.** Dichloromethane extract., **C.** Methanol extract. Data presented are the mean \pm SEM of three independent experiments.

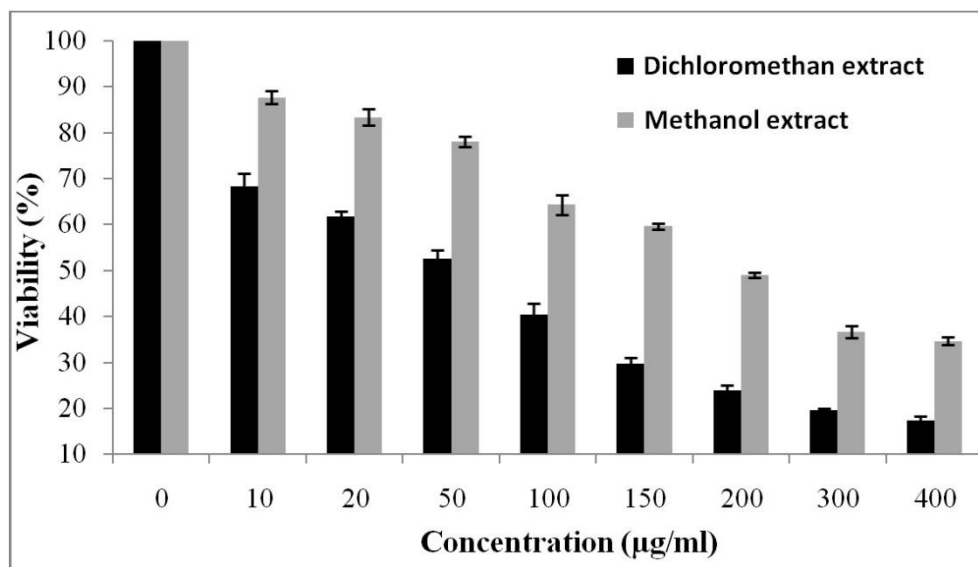


Figure 2. shows inhibition of MCF-7 cells growth that was assessed by the trypan blue exclusion test. Data presented are the mean±SEM of three independent experiments.

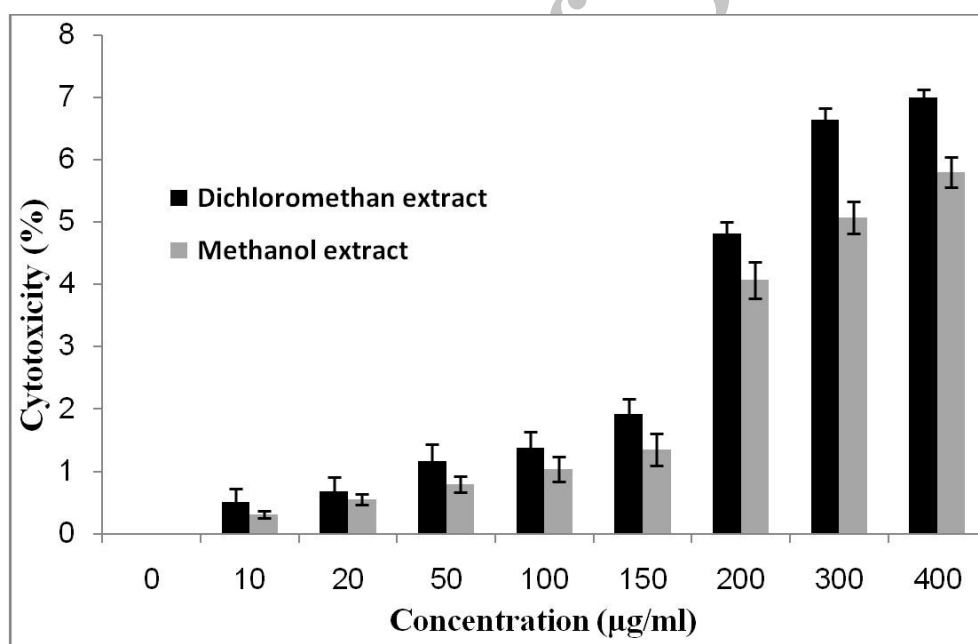


Figure 3. shows effects of *Scrophularia oxysepala* extracts in non-cancer human umbilical vein endothelial cells (HUVEC). Data presented are the mean±SEM of three independent experiments.

Discussion

A successful anticancer drug should kill cancer cells without causing excessive side effects to normal cells. This ideal situation is achievable by apoptosis induction in cancer cells.⁹ Increasing evidence demonstrates that plants are an important source of bioactive compounds that can induce apoptosis in human cancer cells.^{7,8} In previous studies using extract from *Limnophila indica* (Scrophulariaceae) cytotoxic activity has been observed.³⁹

In the present study, as a first step to provide scientific evidence for anti-cancer property of *Scrophularia*

oxysepala, the cytotoxic and apoptotic activity of n-hexane, dichloromethane and methanol extracts from *Scrophularia oxysepala* on MCF-7 human breast cancer cells were evaluated. The results showed that dichloromethane and methanol extracts inhibited the growth of MCF-7 cells through the induction of apoptosis. The activities of this plant may be due to the presence of highly iridoid glycosides that occurred in genus *Scrophularia*. The IC₅₀ values strongly indicated that the dichloromethane extract had a more potent cytotoxic effect on MCF-7 cells than the methanol extract. Thus, the dichloromethane extract may contain

more cytotoxic compound(s) than the methanol extract. Moreover, dichloromethane and methanol extracts did not inhibit normal human umbilical vein endothelial cells. The different sensitivity to dichloromethane and methanol extracts between human breast carcinoma cells and normal cells suggested dichloromethane and methanol extracts as a chemotherapeutic drug. There is evidence that naturally occurring compounds and many chemotherapeutic agents can trigger the apoptosis of cancer cells.

It clear that in apoptosis, the earliest recognized morphological changes are chromatin condensation and nuclear fragmentation.⁴⁰ Progression of the condensation is accompanied by convolution of the nuclear followed by breaking up of the nucleus into

discrete fragments.¹³ Based on this, to confirm the induction of apoptotic process by dichloromethane and methanol extracts cell death assay and DNA fragmentation analysis were performed. Cell death assay as a photometric enzyme-immunoassay for the quantitative determination of histone-associated DNA fragments, revealed that dichloromethane and methanol extracts of *Scrophularia oxysepala* induced the apoptosis in a dose dependent manner. Furthermore, DNA fragmentation that happened in the late stage of apoptosis was confirmed by TUNEL assay. Therefore, the results show the potential of the dichloromethane and methanol extracts of *Scrophularia oxysepala* as an anticancer research agent to inhibit cell growth and trigger apoptosis.

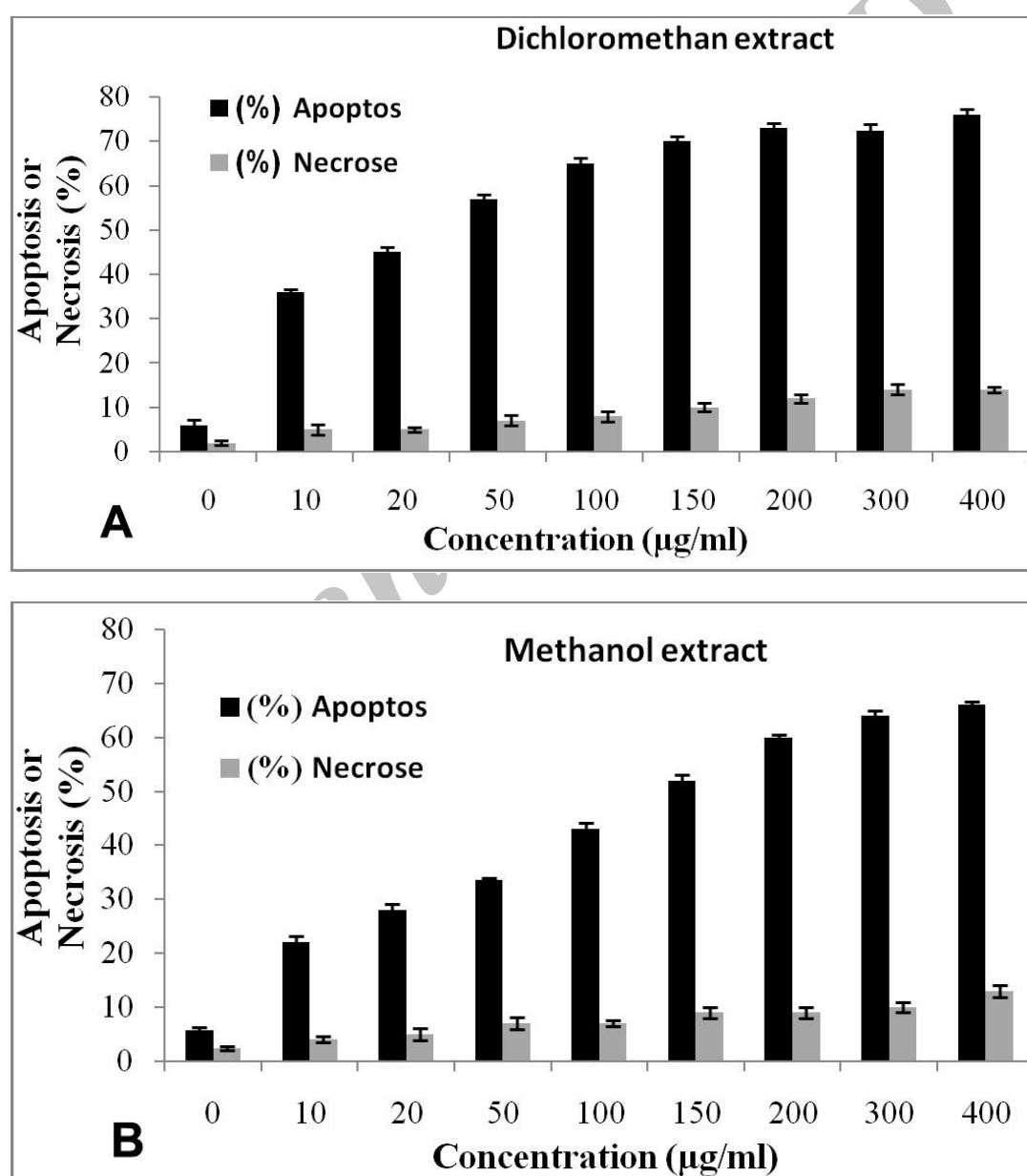


Figure 4. shows the percentages of apoptosis and necrosis by Cell Death assay in MCF-7 cells. **A.** Dichloromethane extract., **B.** Methanol extract. Data presented are the mean \pm SEM of three independent experiments.

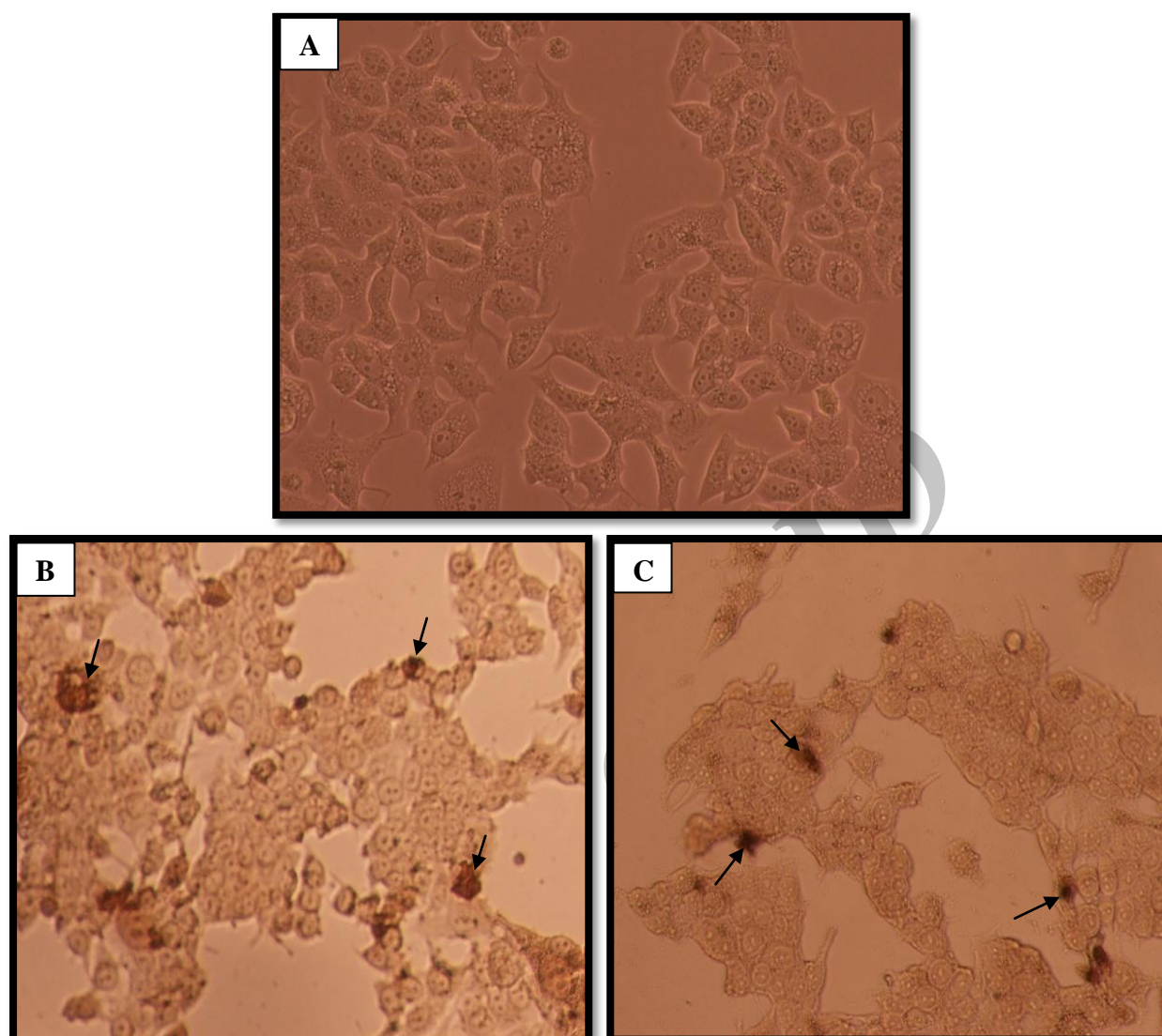


Figure 5. shows effect of *Scrophularia oxysepala* extracts on MCF-7 cells showing characteristic apoptotic morphology in TUNEL assay. **A.** Control., **B.** Dichloromethane extract., **C.** Methanol extract. Arrows indicate representative apoptotic cells. A-C: 200x magnification.

Conclusion

Our studies demonstrate the in vitro cytotoxic and apoptotic activity of dichloromethane and methanol extracts of *Scrophularia oxysepala* in Human breast carcinoma cells, thus possibly suggesting a new potential chemotherapeutic agent for the treatment of breast cancer and are not deleterious towards non-cancerous cells.

Acknowledgements

This study was supported by grants from the Immunology Research Center of Tabriz University of Medical Sciences.

Conflict of interest

The authors report no conflicts of interest.

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