

# Dichloromethane fractions of *Scrophularia oxysepala* extract induce apoptosis in MCF-7 human breast cancer cells

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## ABSTRACT

Breast cancer is a prevalent malignancy among women, especially in developing countries. A large number of anticancer agents with herbal origins have been reported. Hence, herbals may play an essential role in prevention and treatment of cancers. We investigated cytotoxic effects of dichloromethane fractions of *Scrophularia oxysepala* extract on the MCF-7 breast cancer cell line. The cytotoxic activity of *Scrophularia oxysepala* fractions on the MCF-7 cells was assessed using Trypan Blue dye exclusion and MTT (3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) assays. In addition, apoptosis induction was determined using TUNEL (terminal deoxy transferase (TdT)-mediated dUTP nick- end labeling) assay and DNA fragmentation analysis. Quantitative Real-Time PCR was also used for analyzing the changes in Caspase-3, Caspase-9, and Bcl-2 genes' expression. Results revealed an effective inhibition of growth and viability in MCF-7 cells treated with dichloromethane fractions. Cell death assay and DNA fragmentation analysis using the TUNEL test confirmed the induction of apoptosis in the MCF-7 cell line. Further, the fractions have resulted in an increased expression of Caspase-3 and Caspase-9 mRNA, which highlights the possibility of apoptosis in the treatments. The expression study of Caspase-9 mRNA confirmed that, the fractions have triggered apoptosis via intrinsic mitochondrial pathway. In summary, fractions of *Scrophularia oxysepala* extract were found to be promising in growth inhibition and induction of apoptosis in MCF-7 breast cancer cells.

KEY WORDS: *Scrophularia oxysepala*; cytotoxicity; MCF-7 cells; apoptosis; Real Time PCR

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## INTRODUCTION

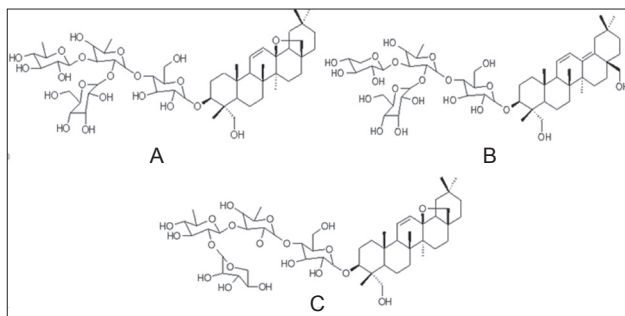
Breast cancer is one of the most prevalent types of cancer among women. Thus, searching for an effective treatment for this disease is a priority [1]. Using herbals for the treatment of malignancies is common in many cultures, because some herbals contain abundant anti-cancer compounds. In addition, useful compounds from these herbals are being used in the production of various modern drugs [2-4].

Scrophulariaceae is a family of flowering plants containing 3000 species and 280 genera that are widely distributed around the world, especially, in Asia and North America [5, 6]. Effects of *S. oxysepala* on cell death mechanisms have been

assessed in previous studies. *S. oxysepala* fractions comprise ingredients such as verbascosaponin and scrokoelzicide (Figure 1) [7-10]. Some herbal compounds are effective in the treatment of cancer by different mechanisms including apoptosis [11, 12].

Apoptosis is a biological process that maintains homeostasis in living beings without causing inflammation [13, 14]. Caspases are cysteine proteases that play a significant role in primary stages of executive phase of apoptosis. During the detection of Caspases, it was specified that Caspase-3 typically is activated by many death signals [15, 16]. Basically, there are two main signaling pathways for cellular apoptosis: a) the mitochondrial or intrinsic pathway that responds to intracellular stimuli and results in cytochrome c release from the mitochondria leading to the activation of Caspase-9; and b) the extrinsic death receptor pathway initiated by ligand binding to extracellular cell death receptors resulting in caspase-8 activation. Both pathways converge at downstream activation of caspase-3 [17].

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**FIGURE 1.** Three saponins isolated from *S. oxysepala* DCM fractions A: verbascosaponin B: scrokoelzicide and C: isolated from these fractions

Caspase activation influences specific substrates, causing biochemical and morphological changes in the cells such as cell shrinkage, condensation of chromatin, and cleavage of DNA [18-20]. Thus, the caspase activity can be a biochemical marker for apoptosis. On the other hand, Bcl-2 is an anti-apoptosis protein playing an inhibitory role in apoptosis [21-23]. In the present study, we assessed the cytotoxic effects of dichloromethane (DCM) fractions of *S. oxysepala* extract on MCF-7 human breast cancer cells. In addition, the effect of fraction on the expression levels of Caspase-3 and Bcl-2 genes, as the main indicators of apoptosis, were evaluated. To determine whether the apoptosis was conducted via intrinsic or extrinsic pathway, changes in the expression of Caspase-9 mRNA was analyzed by quantitative real-time PCR.

## MATERIALS AND METHODS

### Preparation of fractions

*S. oxysepala* was collected from a 30 kilometer area in Kaleybar, Garehdagh Mountain during the flowering period. A voucher specimen (2821) was deposited at the Herbarium of the Researches Center for Agriculture and Natural Resources, East Azerbaijan, Iran.

Air-dried and powdered aerial parts of *S. oxysepala* (1800gr) were extracted with n-hexane, dichloromethane and methanol using a Soxhlet apparatus. All extracts were concentrated using a rotary evaporator at 45 °C (Heildolph, Germany) under reduced pressure to obtain a powder or a viscous mass. Five grams of dichloromethane extract were dissolved in the minimum possible amount of methanol and loaded and fractionated in a Sephadex-LH20 column using an isocratic ( $\text{CH}_2\text{Cl}_2$ -MeOH, 1:1) elution. This method yielded fifteen fractions (F). These fractions were intermingled based on pattern similarities resulting from thin layer chromatography using a chloroform system (7:3). From 13 fractions, F3, F4, F6, and F13 fractions were monitored and assessed for their effects and compounds.

### Cell culture

A human breast cancer cell line (MCF-7) and a mouse normal control cell line (L929) were purchased from the Iranian National Cell Bank (Pasteur Institute, Tehran, Iran). Cells were cultured in RPMI-1640 culture medium containing 10% fetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100 µg/ml) (all purchased from Sigma, Germany) and then incubated at 37 °C at 95% humidity and 5 %  $\text{CO}_2$ .

### Cell viability assay

3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) (Sigma, Germany) assay is based on tetrazolium salt breakage by mitochondrial succinate dehydrogenase within living cells. This results in the production of purple formazan crystals becoming dissolved by dimethyl sulfoxide (DMSO). Briefly, cancer cells as well as L929 cell (as a normal control cell) were cultured in 96 well plates and treated with various concentrations (0-300 µg/ml) of dichloromethane fractions for 24 h and 36 h. Untreated cells were also used as the control group. After incubations, MTT solution with a concentration of 5 mg/ml was added to the each well. After incubation in 37 °C, 200 µl of DMSO and Sorenson buffer was added to each well. Thirty minute after incubation at room temperature, absorbance at 570 nm was measured using an ELISA reader (Awareness Technology, USA).

### TUNEL assay

The TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) (Roche, Germany) assay is based on the activity of TdT (Terminal deoxynucleotidyl transferase) (Roche, Germany) enzyme that adds probed nucleotides to the ends of broken DNA strands. Probed nucleotides bind to the free 3'-hydroxyl end of double- or single-stranded DNA. After 24 hours of treatment with fractions, cells were fixed using 4% paraformaldehyde diluted in PBS (pH, 7.4). After twice washing with PBS, cells were incubated in a solution containing 3%  $\text{H}_2\text{O}_2$  in methanol for 10 minutes and then washed again with PBS. Next, cells were incubated in permeabilization solution on ice for 10 minutes. Next, 50 µl of the reaction mixture containing the TdT enzyme and nucleotides was added to the cells and incubated at 37 °C for 1 hour. After washing with PBS three times, slides were incubated with 50 µl of POD (Roche, Germany) streptavidin HRP solution for 30 minutes. After another wash, the cells were incubated with DAB (Sigma, Germany) substrate and stained cells were then analyzed using a light microscope.

### Cell death ELISA

In order to determine the type of the cell death induced by fractions, an ELISA kit (Roche Diagnostic GmbH, Germany)

was used. First, MCF-7 and L929 cells were cultured in separate flasks. Cells ( $1 \times 10^4$  cells/well) were then treated with DCM fractions of *S. oxysepala* in 75  $\mu\text{g/ml}$  concentrations ( $\text{IC}_{50}$ ). After 24 hours, cells were lysed, and cell lysates were used for apoptosis assessment. Next, 80  $\mu\text{l}$  of immune-reagent solution was added to wells and incubated for 2 hours at room temperature. 100  $\mu\text{l}$  of stop solution was added to each well and absorbances at 405 nm were measured using the ELISA reader.

### DNA fragmentation

DNA fragmentation is one of the most significant biochemical markers for apoptosis. Cells with a density of  $7 \times 10^5$  cells/well were exposed to a fractions extract of *S. oxysepala*. After 24 hours, DNA was extracted using the proteinase K method and sedimentation was done using cold isopropanol. Agarose gel (1.8%) electrophoresis and safe stain<sup>TM</sup> (Cinnagen, Iran) were used to visualize bands under UV light.

### Quantitative real time-PCR

The total RNA was isolated using an RNX PLUS kit (Cinnagen, Iran) according to the manufacturer's instructions. The quality and quantity of isolated RNA were assessed using a NANODROP 2000c spectrophotometer (Thermo Scientific, USA). The RNA was then transcribed to cDNA using a reverse transcriptase kit (Promega, USA). Quantitative RT-PCR (qRT-PCR) was performed using the Corbett Rotor-Gene 6000 system (Corbett Life Science, Australia). PCR was performed in a 20  $\mu\text{l}$  reaction system containing 0.2  $\mu\text{M}$  of each primer, 10  $\mu\text{l}$  of SYBR green reagent (RR820L Takara Bio, Japan), 1  $\mu\text{l}$  of cDNA template and 8.6  $\mu\text{l}$  of nuclease-free water. The primer sequences were as follows: forward: 5'-TCCCTGGAGAAGAGCTACG-3' and reverse: 5'-GTAGTTTCGTGGATGCCACA-3' for  $\beta$ -actin, forward: 5'-ATGGTTTGAGCCTGAGCAGA-3' and reverse: 5'-GGCAGCATCATCCACACATAC-3' for Caspase-3, forward: GCAGGCTCTGGATCTCGGC and reverse:

GCTGCTTGCCTGTTAGTTTCGC for Caspase-9 and forward: 5'-CCTGGGATGACTGAGTACC-3' and reverse: 5'-GGCAGCATCATCCACACATAC-3' for Bcl-2. The PCR cycling was performed by initial denaturation step at 95 °C for 3 min followed by 45 cycles at 95 °C for 10 seconds, 58 °C for 30 seconds and 72 °C for 20 seconds. Relative mRNA expression was measured using the  $2^{-\Delta\Delta\text{CT}}$  method, using  $\beta$ -actin as a reference gene[24].

### Statistical analysis

All experiments were assayed in triplicate ( $n = 3$ ). Data are expressed as means  $\pm$  S.D. Analysis of variance (ANOVA) followed by a two-tailed unpaired t-test was used to determine the significant differences between groups. The differences were considered to be statistically significant at  $p < 0.05$ . All statistical analyzes were performed using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

## RESULTS

### Effects of the DCM fractions of *S. oxysepala* extract on cell viability

Cytotoxic effects of DCM fractions of *S. oxysepala* extract on cell viability were assessed using an MTT assay. As shown in Figures 2 and 3, all four DCM fractions (F3, F4, F6, and F13) decreased the viability of the both MCF-7 and L929 cells in a dose-dependent manner (relative to the blank control) after 24 and 36 hours. The F4 and F13 fractions were more cytotoxic compared to the F3 and F6 fractions ( $p < 0.05$ ). Therefore, we used F4 and F13 fractions in the next experiments. Table 1 shows the  $\text{IC}_{50}$  (50% inhibitory concentrations) values of the DCM fractions on the cells. The F4 and F13 fractions had a lower  $\text{IC}_{50}$  values in comparison to the F3 and F6 fractions. In addition, the cytotoxic effects of DCM fractions of *S. oxysepala* extract on the MCF-7 cells were significantly higher than on the L929 cells ( $p < 0.05$ ).

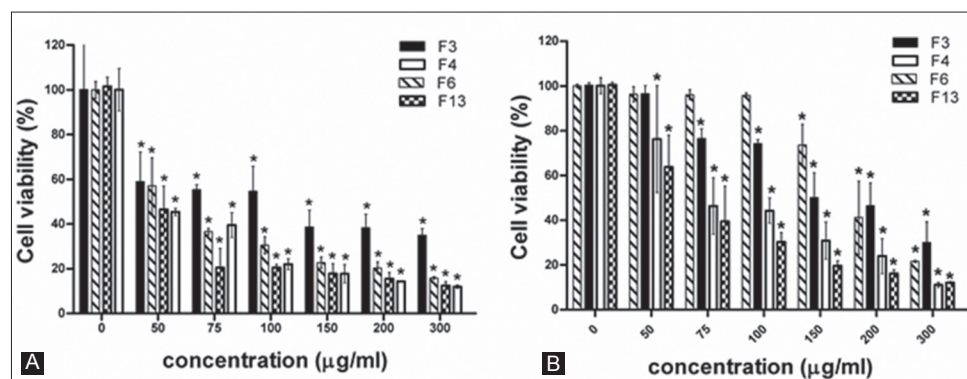


FIGURE 2. Effects of *S. oxysepala* dichloromethane fractions on viability of MCF-7 cells, (A) (24) hr and (B) 36 hr. Values are presented as means ( $n = 3$ )  $\pm$  S.E. \*significant statistical difference ( $p < 0.05$ ) relative to the blank control.

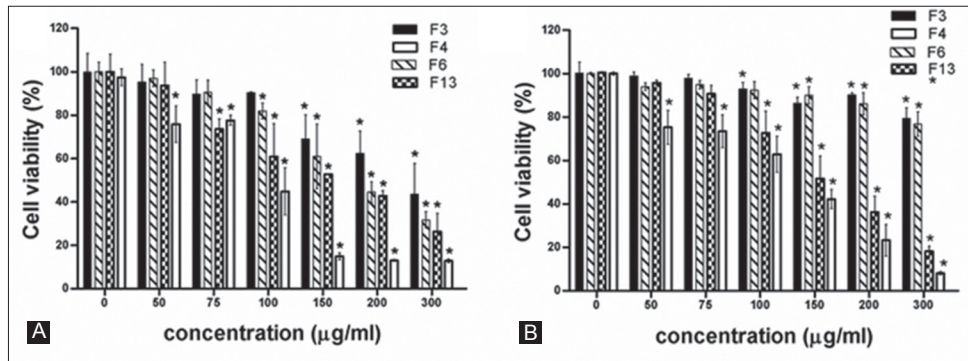


FIGURE 3. Effects of dichloromethane fractions of *S. oxysipala* on L929 cell line viability. Cells for (A) 24 hr and (B) 36 hr. Data are presented as means (n = 3) ± S.E. \*sp< 0.05 normalized the control.

TABLE 1. (IC<sub>50</sub>) concentrations of DCM fractions of *S. oxysipala* for L929 and MCF-7 cell lines after 24 and 36 hr incubations.

	IC <sub>50</sub> (µg/mL)			
	F3	F4	F6	F13
Time 24 h				
L929	204.9	95.09	145.9	119.7
MCF-7	74.10	41.26	47.24	43.40
Time 36 h				
L929	325.5	238.5	70085	143.3
MCF-7	124.4	76.17	170.3	56.06

### Apoptotic effects of the DCM fractions of *S. oxysipala*

Apoptosis was monitored using TUNEL, DNA fragmentation, and ELISA cell death assays. In addition, expression levels of Caspase-3 and Bcl-2 apoptotic genes were measured using qRT-PCR.

DNA damage is a sign of apoptosis. First, apoptosis was evaluated using a TUNEL test. As shown in Figure 4, cell nuclei became brown after 24 hours of treatment with IC<sub>50</sub> of the F13 fraction, while this did not occur in control cells. Moreover, treatment with all DCM fractions resulted in marked DNA fragmentation in the MCF-7 cells, although not in L929 cells, which revealed the specific apoptotic effect of the extract on tumor cells (Figure 5).

Next, cell death ELISA assay was used to determine the type of cell death. Twenty-four hours after treatment, the cells were lysed and then cell lysates were assessed for the presence of DNA-nucleosome fragments. Results showed that a 24-hour exposure to F4 and F13 fractions led to 3.5- and 2.6-fold increases, respectively, in apoptosis in comparison with blank controls (p<0.05). However, DCM fractions had a less apoptotic effect on the L929 normal cells compared to the MCF-7 tumor cells (Figure 6).

To further confirm the apoptotic effects of DCM fractions of *S. oxysipala* on the cells, the expression levels of Caspase-3 and Bcl-2 genes were examined. After a 24-hour treatment with IC<sub>50</sub> concentrations of DCM fractions, relative mRNA expression was quantified using qRT-PCR.

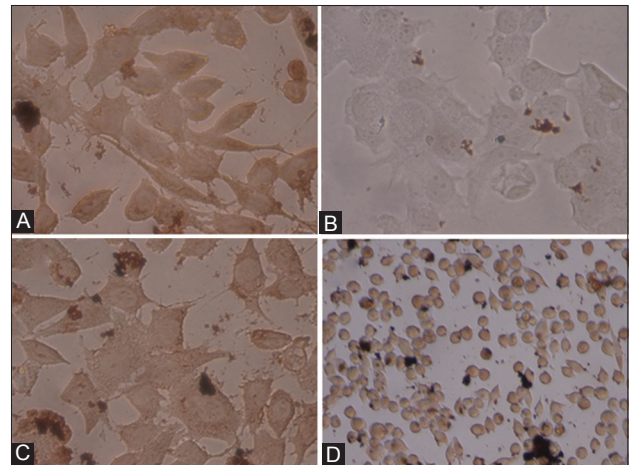


FIGURE 4. Apoptotic effects of DCM (fraction F4) of *S. oxysipala* on MCF-7 cells observed by TUNEL assay. (A) L929 control, (B) MCF-7 control, (C) treated L929 and (D) treated MCF-7. Arrows indicate representative apoptotic cells. A-D: 200x magnification.

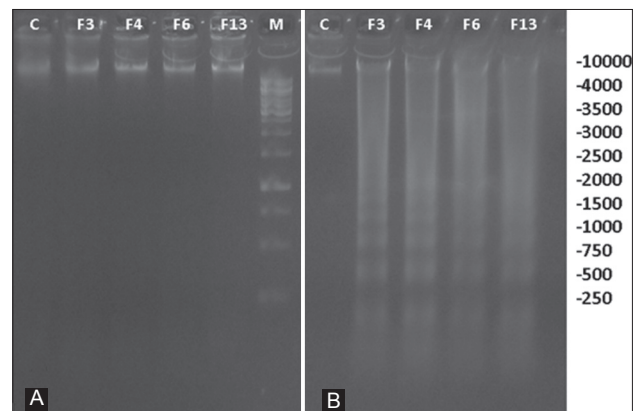


FIGURE 5. DNA fragmentation assay in cells exposed to IC<sub>50</sub> concentration (75 µg/mL) of *S. oxysipala* for 24 hr. (A) L929 Normal cells (left to right: Control, F3, F4, F6, F13 fractions) AND (B) MCF-7 breast cancer cell line (left to right: Control, F3, F4, F6, F13 fractions), M= size marker. DNA fragments were separated on 1.8% agarose gel electrophoresis.

Data showed that treatment of MCF-7 cells with F4 and F13 fractions caused 11.26 and 2.83fold increases in the expression level of Caspase-3 mRNA, respectively, while the expression level of Bcl-2 mRNA was increased, respectively, by 4.16- and 1.62-fold (compared to blank controls). However, in

24 hour exposures of the L929 cells with F4 and F13 fractions, the increase in expression levels of Caspase-3 mRNA were 1.12 and 1.33 fold, respectively, and the expression levels of Bcl-2 mRNA were 1.15 and 2.05, respectively, relative to blank controls (Figure 7).

## DISCUSSION

Breast cancer is one of the most frequent types of cancer among women all over the world [25]. Because of its high mortality and morbidity, there is a great deal of interest in finding an appropriate medication for the treatment of breast cancer. Among these treatments, medicinal plants are considered to be important sources of therapeutic substances and a variety of plants have been shown to contain bioactive and anti-cancer compounds [26-29]. For example, different species of the *Scrophularia* genus have bacteriostatic and anti-inflammatory properties [30, 31]. According to the literature, some *Scrophularia* species contain high quantities of saponins[32-35], olean, epoxy olean, dammaraneand terpenoids[27, 28]. Many chemicals such as phenol glycosides and some phenilicacids extracted from *Scrophularia* species

have significant cytotoxicity and cytostaticity. In a recent study, the inhibitory impacts of *S. striata* extract on leukemic cells by arresting G2/M phase and apoptosis has been reported[36, 37].

In our previous study, we assessed the anti-proliferative and cytotoxic effects of three *S. oxysepala* extracts (n-hexane, DCM, and methanol) on the MCF-7 breast cancer cell line. While the n-hexane extract did not exhibit any cytotoxic impacts, the DCM and methanol extracts significantly inhibited cancerous cells in a dose and time-dependent manner. At the same time, these had no significant adverse effects on human normal cells (HUVEC)[12, 38]. In the present study, we have further investigated the active components of the *S. oxysepala* DCM extract. For this purpose, we prepared four fractions of this extract: F3, F4, F6, and F13. We found that F4 and F13 fractions indicated more cytotoxic effects with no significant inhibitory effects on L929 cells, and nontoxic effects of these fractions on murine normal cells (L929) could be promising for potential use in future *in vivo* studies.

Besides the morphologic characteristics of the cells treated with these fractions, DNA fragmentation confirmed the occurrence of apoptosis. In addition, results of cell death ELISA showed that the fractions above have induced apoptosis in MCF-7 cells. Apoptosis, as the most acceptable form of cell death, depends on a Caspase proteolytic system. In other words, Caspase family members are required for the initiation and execution of apoptosis[39]. Caspase-3 is a major downstream effector in apoptosis and plays a crucial role in DNA fragmentation and chromatin condensation. Meanwhile, anti-apoptotic proteins such as Bcl-2 play important inhibitory roles in apoptotic processes and are shown to increase cell viability [40-44].

Results of quantitative RT-PCR indicated that *S. oxysepala* DCM fractions resulted in increased expression of Caspase-3 mRNA. Moreover, L929 normal cells showed no significant changes in Caspase gene expression patterns. In malignant cells, a lower rate of increase in Bcl-2 gene expression was evident 24 hours after exposure to the fractions. Giessrigl *et al.* also reported antineoplastic effects of *Scrophularia* components.

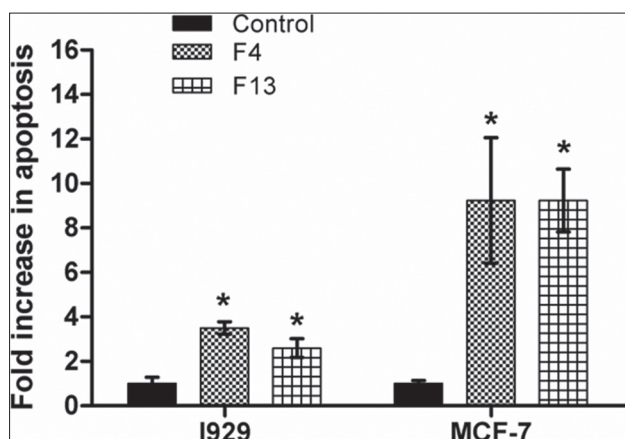


FIGURE 6. Effects of dichloromethane fractions of *S. oxysepala* (IC<sub>50</sub> = 75 µg/mL) on apoptosis of MCF-7 and L929 cells after 24 hr detected by cell death ELISA assay. Data are represented as the fold increase in apoptosis and expressed as means ± S.E. \*signified (p < 0.05) relative to the control.

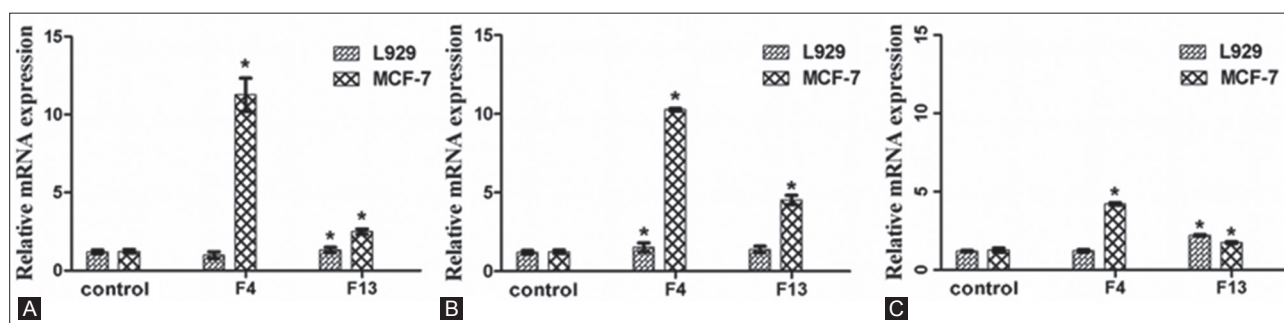


FIGURE 7. Effects of *S. oxysepala* DCM fractions on (A) Caspase-3, (B) Caspase-9 and (C) Bcl-2 mRNA expression in MCF-7 and L929 cells at 24 hr. Relative expression was acquired by qRT-PCR using  $2^{-\Delta\Delta Ct}$  method. The results are presented as mean ± SD (n = 3); \*p < 0.05 versus control.

They concluded that growth inhibition and cell death was due to the activation of Caspase-3 and down-regulation of cell cycle regulators, proto-oncogenes and NF- $\kappa$ B gene expression [45].

Results of an MTT assay indicated that an increased dose of fractions led to more intense cytotoxic effects. Nevertheless, major cytotoxic effects were detected in 24 hours rather than 36 hours, and extended time of incubation resulted in an increase in IC<sub>50</sub> value. Consequently, it can be concluded that there is a half-life for the mentioned fractions, and repeated doses, at 24-hour intervals, are needed to re-establish the effects.

## CONCLUSION

The present study demonstrates that, dichloromethane fractions of *Scrophularia oxysepala* induce apoptosis in MCF-7 breast cancer cells in a dose-dependent manner via mitochondrial intrinsic pathway.

## DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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