

Cytotoxic evaluation of different fractions of *Salvia chorassanica* Bunge on MCF-7 and DU 145 cell lines

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Abstract

Because of antimicrobial, antioxidant, and anticancer potential, *Salvia chorassanica* Bunge (Lamiaceae) has been considered as a popular herb in Iranian traditional medicine. Previous studies have shown remarkable cytotoxic properties of the methanol, *n*-hexane and dichloromethane extract of *S. chorassanica* on human cervical cancer cells. To seek the therapeutic potentials of *S. chorassanica*, this study was undertaken to evaluate the cytotoxic activities of various extracts of this plant on human breast MCF-7 and prostate cancer DU 145 cells. The DU 145 cells were exposed to different concentrations of plant extracts (1-200 µg/ml). Cytotoxic activities were examined using alamarBlue[®] assay and apoptosis was assessed by acridine orange/propidium iodide double staining and evaluation of DNA fragmentation by flow cytometry. Our findings indicated that *n*-hexane and dichloromethane extracts had more cytotoxic activities against DU 145 and MCF-7 cell lines compared with other extracts ($P < 0.05$). The acridine orange/propidium iodide staining showed apoptogenic properties of *n*-hexane and dichloromethane extracts which was consequently confirmed by flow cytometric histogram that exhibited an increase in sub-G1 peak in treated cells as compared with untreated cancer cell lines. Taken together, these observations demonstrated cytotoxic effects of *S. chorassanica* extracts on MCF-7 and DU 145 cell lines which is most likely exerted via apoptosis cell death. Therefore, further investigations on *S. chorassanica* extracts as potential chemotherapeutic agents are warranted.

Keywords: *Salvia chorassanica*; Lamiaceae; Cytotoxicity; Cell line

INTRODUCTION

Cancer is one of the most dangerous multifactorial human disorders with high mortality and morbidity (1). Chemotherapy, radiotherapy, hormone therapy, immune therapy and surgery have been proposed effective modality for cancer treatment and prevention (2). In spite of the progression of cancer treatment approaches, it is still accounting as a prominent dreadful condition (3). Controlling cancer cell proliferation, reducing the risk of tumor progression, improving malignancy eradication have encouraged researchers to seek for novel

treatment options (4). In this regard, various natural product as therapeutic agents used in chemoprevention have been introduced through high throughput screenings (5). Many of the herbal natural compounds and purified secondary metabolites are used as nutraceuticals or dietary supplements (6). Herbal medicine may propose better therapeutic approaches due to higher efficacy, minimal toxicity and lower cost of treatment (7). In addition, the whole natural extract represents the synergistic and antagonistic function of biochemicals compounds in the extract which improves the therapeutic efficacy (8).

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Naturally occurring substances exhibit pivotal role in elimination of malignant cells by modulation of apoptosis signaling (9,10). The programmed mode of cell death is an appreciate defense mechanism that exerted via self-destruction pathway and characterized with morphological and biochemical indicators such as DNA fragmentation, reducing of cell volume and membrane blebbing (11). Therefore, researchers have tried to discover innovative therapeutic agents accomplishing their cytotoxic effects through triggering of apoptosis in malignant cells (12-14). There are numerous reports on cytotoxic activities of some genus in Lamiaceae plants including *Scutellaria* (15), *Lavandula* (16) and *Salvia* (17). The genus *Salvia* (Lamiaceae) are traditional medicinal plants with unique biological properties which have been used due to their antimicrobial, antioxidant, and anti-cancer effects in folk medicine for many years (18).

The prominent constituents of this genus involve sesquiterpenoides, diterpenoides, triterpenes, essential oils and flavonoids (19,20). *Salvia chorassanica* is an Iranian endemic *Salvia* species which grows naturally in Khorasan Razavi province (18). There are a few literatures on biomedical efficacy of *S. chorassanica*.

In our two previous studies we have demonstrated the cytotoxic effect of *S. chorassanica* on leukemic and cervical cancer cells (19). Nevertheless, the comparative cytotoxic effects of different extracts of *S. chorassanica* on MCF-7 and DU 145 have not been reported previously. Thus, this study was planned to explore the cytotoxic effects of *S. chorassanica* on MCF-7 and DU 145 cell lines as two sex related common cancers in individuals.

MATERIALS AND METHODS

Chemicals

MCF-7 (breast cancer cells) and DU 145 (prostate cancer cells) were from National Cell Bank of Iran (NCBI). Roswell park memorial institute medium (RPMI)-1640 medium, fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) and antibiotic (penicillin-streptomycin) were

supplied by Gibco (USA). AlamarBlue[®] (resazurin) was from Sigma (Saint Louis, MO, USA). Acridine orange (AO) was procured from AppliChem (USA) and propidium iodide (PI) was obtained from Sigma (USA).

Plant materials

The roots of *S. chorassanica* were collected from Hosseinabad valley (2100 m height) in Pivejan, a village located 65 km south-west of Mashhad, Khorasan Razavi province, northeast of Iran. The plant was identified by Mr. M.R. Joharchi, from Ferdowsi University of Mashhad Herbarium (FUMH). Voucher specimen (No. 11289) was deposited in herbarium of Faculty of Pharmacy, Mashhad University of Medical Sciences, Iran.

Plant extraction

n-Hexane, dichloromethane (CH₂Cl₂), ethyl acetate (EtOAc) and water (H₂O) extracts were prepared as previously described (21). Briefly, extracts were prepared after successive partitioning among different solvents started with crude dried methanol extract and stored at 4 °C until the use or analysis. A partitioning scheme of *S. chorassanica* methanol extract is presented in Fig. 1.

Sample preparation

To prepare the stock solutions (100 mg/ml), all extracts were dissolved in dimethyl sulfoxide (DMSO). The concentrations of 1-200 µg/ml were then prepared by diluting these solutions with complete media, such that the final concentrations of DMSO did not exceed 0.5%.

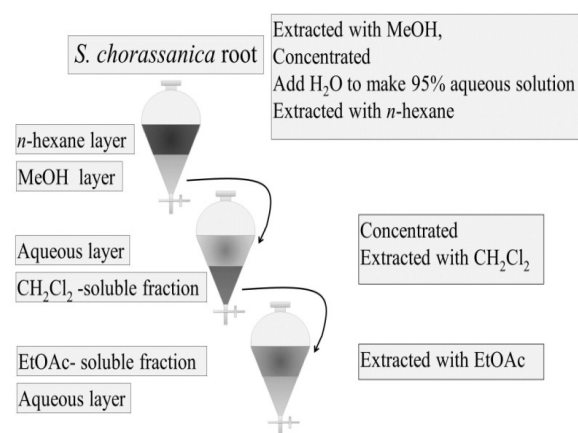


Fig. 1. Extraction scheme of *Salvia chorassanica* using immiscible solvents.

Cells and culture

MCF-7 and DU 145 cells were obtained from the Pasteur Institute of Iran and grown in RPMI-1640 medium containing 10% FBS supplemented with 1% antibiotic (penicillin/streptomycin) at 37 °C in a humidified, 5% CO₂ incubator.

Cytotoxic assay

The inhibitory effects of all prepared extracts of *S. chorassanica* on the proliferation of MCF-7 and DU 145 cancer cells were measured by alamarBlue[®] assay (22). Briefly, the cancer cells were cultured overnight at a concentration of 10⁴ cells/well in 96-well plates. Then incubated with appropriate concentrations (1-200 µg/ml) of methanol, *n*-hexane, CH₂Cl₂, EtOAc and H₂O extracts. The alamarBlue[®] assay was performed after 48 h incubation with extracts. Ten µl of alamarBlue[®] solution was added and incubated for 4 h, and eventually optical density was recorded at 570 and 600 nm using spectrophotometer (Epoch, USA).

Cellular morphology

MCF-7 and DU 145 cells (2 × 10⁵ cells/well) were grown in six-well culture plates in RPMI-1640 supplemented medium and incubated with cytotoxic extracts at a specified concentration for 24 h. After the desired time the cytomorphological changes were visualized under an inverted microscope (Bio Photonic, Brazil) and photographs were taken.

Apoptosis assay

Acridine orange/propidium iodide staining

The human MCF-7 and DU 145 cells (2 × 10⁵ cells/well) incubated with a defined concentrations of cytotoxic extracts for 24 h, then cells were trypsinized and stained with 10 µl of a dye mixture comprising 100 µg/ml PI and 100 µg/ml AO (23). The cells were then mounted on coverslips and observed under a fluorescence microscope.

Apoptosis evaluation by detection of sub-G1 peak

The cultured MCF-7 and DU 145 cells were treated with a medium containing certain concentrations of cytotoxic extracts. Then the cells were washed twice and resuspended in PI

solution containing 0.1% sodium citrate plus 0.1% Triton X-100 and incubated at 37 °C for 30 min.

The fluorescence of stained cells was evaluated using a FACScan laser flow cytometer (Becton Dickinson, NJ, USA), and the population of cells was calculated using the WinMDI 2.8 software.

Statistical analysis

The results are expressed as the mean ± SEM and experiments were carried out in triplicate. One-way ANOVA used for statistical analysis and the *P* values ≤0.05 was considered significant.

RESULTS

Cytotoxicity of different extracts of *S. chorassanica*

The data obtained from alamarBlue[®] assay indicated that the CH₂Cl₂ extract had the most cytotoxic effect among other extracts of the *S. chorassanica* against MCF-7 cells (*P*<0.05), while DU 145 cells were more sensitive to the *n*-hexane extract in comparison with others (*P*<0.05) (Fig. 2).

IC₅₀ values for various concentrations of *S. chorassanica* extracts against MCF-7 and DU 145 cell lines are given in Table 1. The IC₅₀ value of CH₂Cl₂ extract against MCF-7 cells after 48 h was determined as 3.8 µg/ml and the IC₅₀ value of *n*-hexane extract against DU 145 cell line after 48 h was found to be 8.1 µg/ml.

AO/PI staining and morphological characterization

Apoptosis is a regulated mechanistic cell death identified by appearance of morphological and cellular features. Morphological alterations of MCF-7 and DU 145 cancer cells after incubation with efficient concentrations of the most cytotoxic extracts were identified using phase contrast inverted and fluorescent microscope.

As shown in Fig. 3 the untreated breast and prostate cancer cells exhibited polygonal shapes, while MCF-7 and DU 145 cells exposed to cytotoxic concentrations of CH₂Cl₂ and *n*-hexane extracts represented cell shrinkage and loss of plasma membrane

Table 1. IC₅₀ values (µg/ml) of various extracts of *Salvia chorassanica* in MCF-7 and DU 145 cell lines.

Cell line	Extracts				
	MeOH	<i>n</i> -hexane	CH ₂ Cl ₂	EtOAc	H ₂ O
MCF-7	13.0	6.1	3.8	>200	>200
DU-145	20.9	8.1	10.5	>200	>200

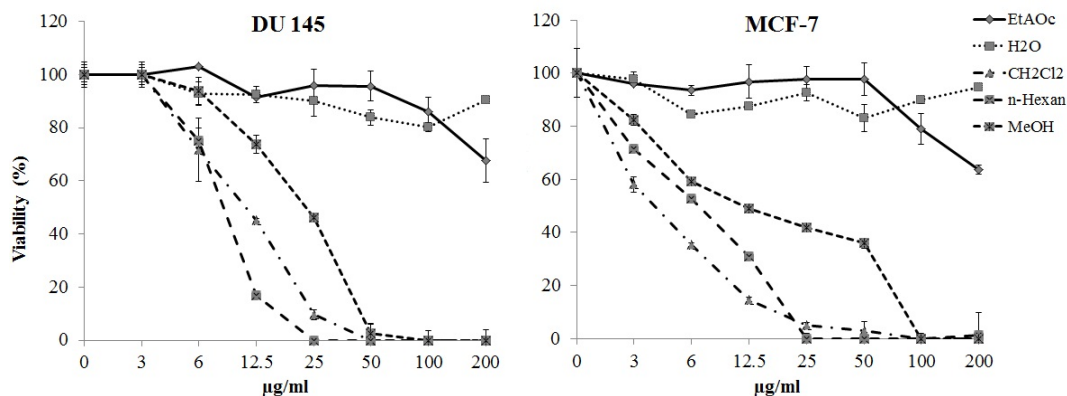


Fig. 2. The cytotoxic effect of various extracts obtained from *Salvia chorassanica*. MCF-7 and DU 145 cancer cells were incubated 48 h in the presence of various concentrations of MeOH, *n*-hexane, CH₂Cl₂, EtOAc, and H₂O extracts of *Salvia chorassanica*. Growth inhibitory effect was determined by alamarBlue[®] assay. Each point represents the mean ± SEM of three replicates.

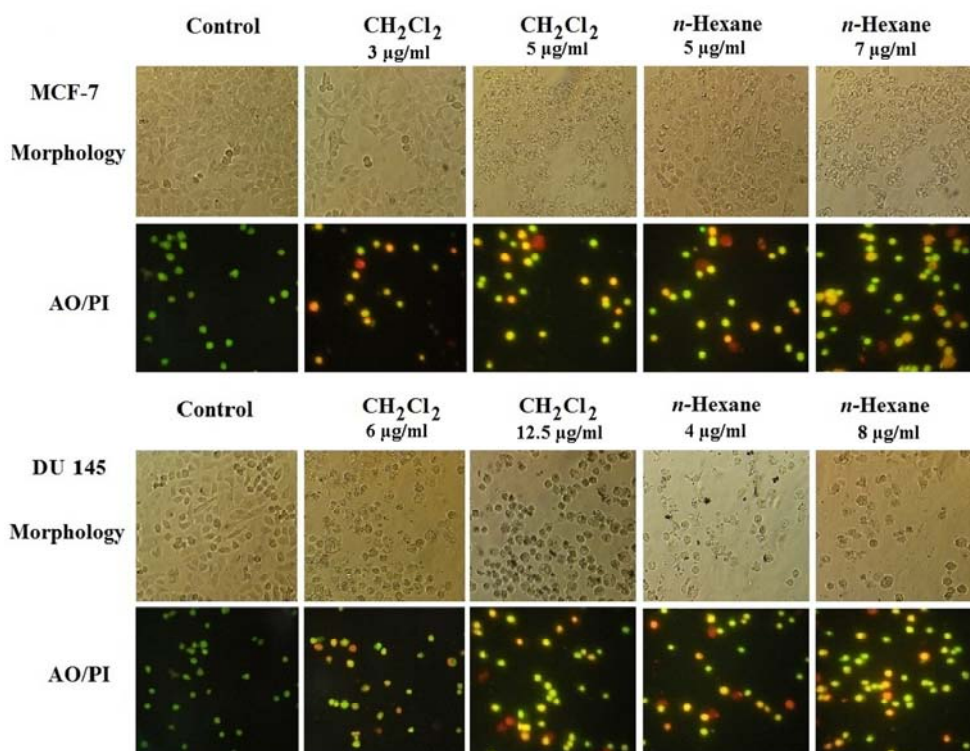


Fig. 3. Apoptotic morphological features under exposure with various concentrations of CH₂Cl₂ and *n*-hexane extracts of *Salvia chorassanica* on MCF-7 and DU 145 cancer cells. Morphological visualization performed under inverted microscope and acridine orange/propidium iodide double staining was examined under fluorescent microscope.

integrity as the most dominant characteristics of apoptosis induction.

To evaluate whether cytotoxic extracts can trigger apoptosis, the MCF-7 and DU 145 cells were double stained with AO/PI.

As illustrated in Fig. 3, AO/PI fluorescence double staining showed that MCF-7 and DU 145 cells treated with IC₅₀ concentrations of *S. chorassanica* extracts were stained green to yellow and yellow to orange color which represents the involvement of apoptosis in cytotoxic activity of *S. chorassanica*.

Sub-G1 peak and DNA fragmentation

The pro-apoptotic effect of *S. chorassanica* was evaluated by flow cytometric analysis of cells in hypotonic phosphate citrate buffer containing PI. As shown in Fig. 4 treatment of MCF-7 cells with CH₂Cl₂ and *n*-hexane extracts of *S. chorassanica* significantly increased the sub-G1 peak, which was indicative of DNA fragmentation. In Fig. 4 flow cytometric histograms of prostate cancer cells treated with *n*-hexane and CH₂Cl₂ for 48 h showed a concentration-dependent sub-G1 peak increment.

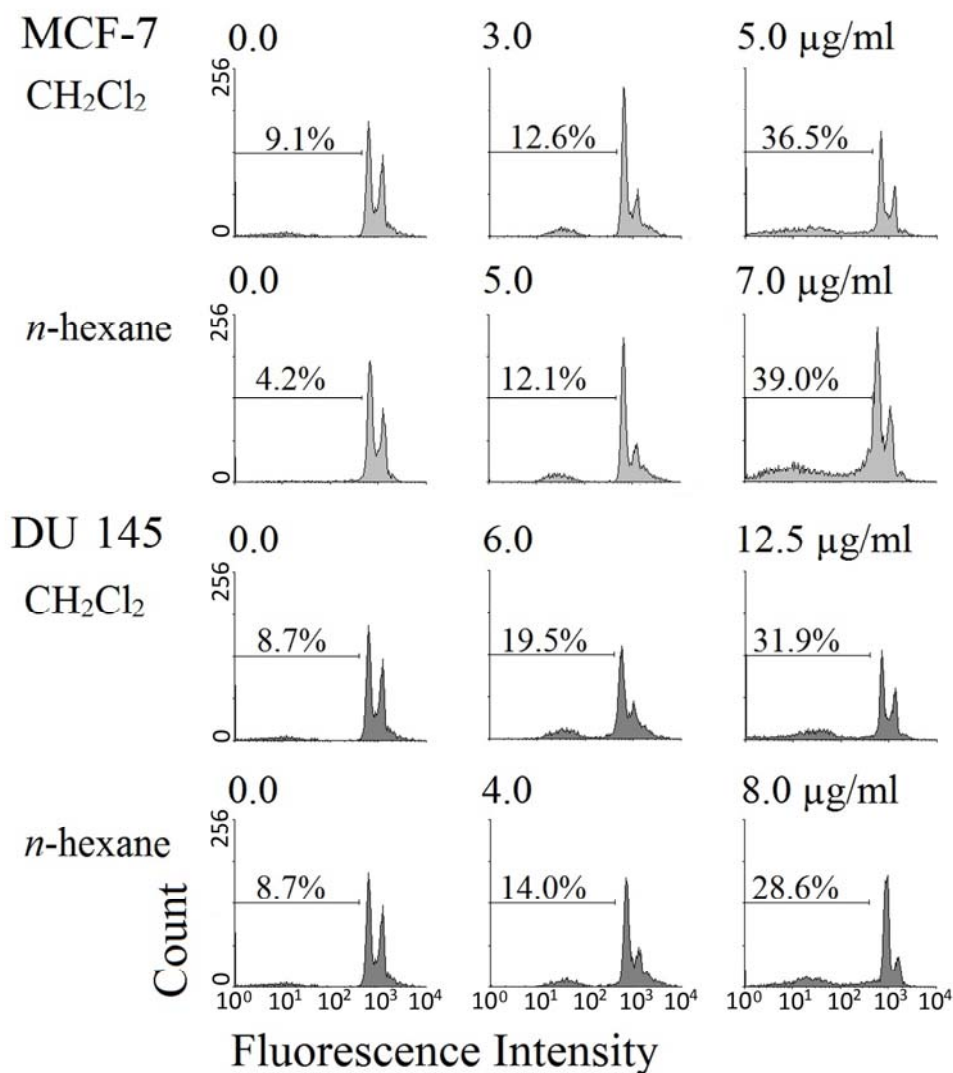


Fig. 4. Detection of apoptosis using flow cytometric analysis of cells stained with propidium iodide. MCF-7 and DU 145 cells remained untreated or treated with CH₂Cl₂ and *n*-hexane extracts of *Salvia chorassanica*. As shown increasing sub-G1 peak in treated cells in comparison with control untreated cells demonstrated the involvement of apoptosis.

DISCUSSION

Breast cancer is the most serious type of malignancies in women which has the metastasis potential to other parts of the body and prostate cancer is the common leading cause of cancer-related deaths in men all over the world (24,25). Despite recent progression in cancer medicinal modalities, attempting to find alternative strategies to eradicate invasive cancer cells is going on (6). However, folk medicine utilized natural extract with therapeutic properties including anti-inflammatory, antioxidant and cytotoxic characteristic which have been isolated from terrestrial and marine resources. Many of the investigated substances play an important role in cancer cell elimination and have entered to clinical trials that means nature represents a promising alternative to enhance efficacy of cancer chemotherapy strategies (2).

The results of present study showed that *S. chorassanica* induce cytotoxicity against MCF-7 and DU 145 cell lines. *n*-Hexane and CH₂Cl₂ extracts of *S. chorassanica* are among the active fractions with high cytotoxic effects against MCF-7 and DU 145 cancer cells while the methanol extract had moderate cytotoxicity and H₂O, EtOAc extracts showed minimal effects. Different potency of the extracts can be associated with distinct constituents in each extracts. It should be noted that this finding is in accordance with our previous study. Previously we have indicated that CH₂Cl₂ and *n*-hexane extracts of *S. chorassanica* have cytotoxic effect against HeLa cervical cancer cells with IC₅₀ value of 2.38 and 5.45 µg/ml for CH₂Cl₂ and *n*-hexane extracts, respectively via induction of apoptosis (19). In addition, in a recent study we evaluated the cytotoxic and apoptosis inducing effect of *S. chorassanica* extracts and their active constituents such as taxodione, ferruginol and 6-hydroxyalvinolone against K-562 and HL-60 cancer cells and showed that CH₂Cl₂ is the most cytotoxic extract and ferruginol is the most potent compounds among tested constituents (18). Apoptotic resistance is a key complication that interfere with cancer therapeutic methods and therefore, recruitment of apoptosis signaling possess beneficial

effects and improve the cytotoxic potential of phytochemicals (26).

The pro apoptotic activity of *S. chorassanica* extracts was evaluated using fluorescence microscopy. The morphological alterations such as cell shrinkage, membrane blebbing, increased cytoplasmic density, nuclear disintegration, activation of caspase cascade and formation of apoptotic bodies were considered as obvious apoptosis hallmarks (18).

The fluorescence micrographs showed that untreated cancer cells exhibited bright green fluorescence as the live cells which indicates intact membrane excluded PI, but permits uptake of AO. On the other hand, MCF-7 and DU-145 treated cells revealed more yellow-orange fluorescence which demonstrated apoptotic cells could not exclude the dyes and stained with yellow-orange fluorescence.

Apoptosis inducing activity of *S. chorassanica* extracts was further assessed by flow cytometry using PI to evaluate cell membrane integrity and DNA fragmentation.

It should be notified that nuclear fragmentation produces small fragments of DNA that can be stained with a quantitative DNA-binding dye such as PI in a hypotonic phosphate-citrate buffer. It is proved that apoptosis cell death characterized by accumulation of a subpopulation of cells in sub-G1 area which is indicating endonuclease activation and subsequent leakage of DNA from the cells. Flow cytometric histogram of the *S. chorassanica* extract treated cells showed a sub-G1 peak in MCF-7 and DU-145 cells which exhibit the involvement of an apoptotic process in the cells. Collectively, apoptosis recruitment has pivotal role in cytotoxic mechanism of *S. chorassanica* extracts.

There are different reports on biomedical properties of *Salvia* genus (18). Xavier and colleagues examined the cytotoxic effect of *S. fruticosa* and *S. officinalis* on two human colorectal cancer cells and showed that these extracts can significantly inhibit colorectal cancer cell growth and induce apoptosis in both colorectal cell line (27). Tundis and coworkers have proven the growth inhibitory effect of extracts and isolated metabolites of

S. leriifolia Benth. on amelanotic melanoma, colorectal adenocarcinoma, lung large cell carcinoma, malignant melanoma and hepatocellular carcinoma cells and indicated that the *n*-hexane and CH₂Cl₂ extracts showed the strongest cytotoxic activity against melanoma cells and the EtOAc extract was the most active extract against lung large cell carcinoma cell line. Nevertheless, buchariol, a sesquiterpene obtained from CH₂Cl₂ extracts and naringenin, a flavonoid isolated from the EtOAc extract showed remarkable cytotoxicity against melanoma and lung carcinoma (28). Recently, Zare Shahneh and colleagues reported that *S. officinalis* L. methanol extract could be cytotoxic against lymphoma and leukemic cells through apoptosis induction (29). However, there are rare information related with therapeutic efficacy of Iranian *Salvia* species and this study proved the cytotoxic and apoptogenic effects of various extracts of *S. chorassanica* on two frequent cancer cell lines including human breast carcinoma and prostate cancer cells.

CONCLUSION

Taken together, the present study proved the cytotoxic properties of CH₂Cl₂ and *n*-hexane extracts of *S. chorassanica* that proposed this popular plant for anticancer evaluations. There is need for further investigation and to explore the accurate mechanisms involved in apoptosis cell death and to provide more convincing evidence related to anticancer potential of *S. chorassanica* extract and its active compounds.

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