Growth Inhibition and Apoptosis Induction of *Salvia chloroleuca* on MCF-7 Breast Cancer Cell Line

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Abstract

Fragrant species of the genus *Salvia* have been attributed many medicinal properties, which include anticancer activity. In the present study, cytotoxic properties of total methanol extract of *Salvia chloroleuca* Rech. f. & Aellen and its fractions were investigated on MCF-7, a breast carcinoma cell line. Malignant and non-malignant cells were cultured in RPMI medium and incubated with different concentrations of plant extracts. Cell viability was quantitated by 3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl) -2-(4-sulphophenyl) -2H-tetrazolium (MTS) assay. Apoptotic cells were determined using propidium iodide (PI) staining of DNA fragmentation by flow cytometry (sub-G1 peak). *S. chloroleuca* inhibited the growth of malignant cells in a dose-dependent manner. Among solvent fractions of *S. chloroleuca*, the *n*-hexane and methylene chloride fractions were found to be more toxic compared to other fractions. *S. chloroleuca*-induced a sub-G1 peak in flow cytometry histogram of treated cells compared to control and DNA fragmentation suggested the induction of apoptosis. Administration of N-acetyl cysteine and vitamin C two ROS scavengers also resulted in significant inhibition of cytotoxicity induced by *S. chloroleuca*. These results support a mechanism whereby *S. chloroleuca* induces apoptosis of MCF-7 human breast cells through a ROS-mediated pathway.

Keywords: *Salvia chloroleuca*; Lamiaceae; Cytotoxicity; Apoptosis; ROS.

Introduction

The genus *Salvia* (Lamiaceae) with about 900 different species, divided into five subgenera (*Sclarea*, *Audibertia*, *Jungia*, *Leonia* and *Salvia*), present in diverse areas as the Mediterranean, Central Asia, Pacific Islands, tropical Africa and America (1) of which 17 species are endemic to Iran (2). The known antioxidant, aromatic and antimicrobial properties of different members of the genus *Salvia* made it popular in the cosmetic industry, medicine and as food flavouring and preservation products since ancient times (3, 4).

The genus *Salvia* has widely used in traditional folk medicine. Different parts of the plant especially root (5, 6) are a rich source of different phytochemicals including terpenoids, polyphenols, as well as essential oil (7, 8). *Salvia officinalis*, *S. miltiorrhiza*, *S. plebeia* and *S. menthaefolia* are some species of the genus that have been recognized to possess anticancer and anti-proliferative activity on tumor cells. *Salvia menthaefolia* extract has been shown to take marked anti-proliferative activity against glioblastoma (DBTRG-05MG, T98G,
Plant materials

The roots of *S. chloroleuca* were collected from Hosseinabad valley (2100 m height) in Pivejan on July 2011, a village at 65 km southwest of Mashhad, Razavi Khorasan 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 School of Pharmacy, University of Mashhad Medical Sciences.

The dried root (100 g) was percolated with methanol (MeOH) at room temperature. The whole extract was filtered and the solvent was evaporated under reduced pressure at 40–45°C, to afford crude methanol extract (11.4 g). Methanol extract (10 g) was then resolved in methanol 95% and partitioned successively between *n*-hexane, methylene chloride (CH$_2$Cl$_2$), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH), and finally water based on increasing polarity of the solvent. *n*-Hexane, CH$_2$Cl$_2$, and EtOAc fractions were evaporated under vacuum to yield the residues of 0.27, 2.0, and 2.0 g fraction respectively. Water fraction was freeze dried.

Extracts were stored at 4°C until analysis. A partitioning scheme of *S. chloroleuca* methanol extract is presented in Figure 1 (16).

All of the isolated fractions were dissolved in dimethylsulfoxide (DMSO) and then were subjected to cytotoxic and apoptosis assays.

**Cell culture and treatment**

The human breast cancer cells (MCF-7) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO$_2$. The stock solution of each compound was prepared at 100 mg/mL in dimethylsulfoxide and kept at -20 °C.

Human umbilical cord blood samples (50 mL) were collected from a fresh umbilical cord attached to the placenta by gravity flow in sterile 50 mL syringe containing citrate buffer.
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as an anticoagulant. The sample diluted with an equal volume of Phosphate Buffered Saline (PBS), then layered over Ficoll-Hypaque density gradient separation solution (1.077 g/mL), and centrifuged at 800 g for 20 min at room temperature. The mononuclear cell layer was removed, washed twice in PBS and resuspended in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, and 100 U/mL penicillin and 100 mg/mL streptomycin. This study protocol was approved by the ethical committee of Mashhad University of Medical Sciences.

For MTS assay, cells were seeded at 10^4 cells per well onto 96-well culture plates. For assay of apoptosis, cells were seeded at 10^4 cell per well onto a 24-well plate. For each concentration and time course study there was a control sample that remained untreated and/or received the equal volume of DMSO.

Cell viability

The MTS assay (17), is based on the reduction, by mitochondrial dehydrogenase in metabolically active cells, of the novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl) -2-(4-sulphophenyl) -2H-tetrazolium inner salt (MTS), to the colored, water-soluble formazan that absorbs at 490 nm. About 10^4 MCF-7 cells were seeded in each well of a 96-microwell plate and treated with various concentrations of each fraction of S. chloroleuca. After incubation for 48 h, CellTiter 96® aqueous one solution reagent (Promega, Madison, WI, USA), which is composed of the novel tetrazolium compound MTS and an electron coupling reagent phenazine methosulfate (PES, a redox intermediary), was added to each well according to the manufacturer’s instructions. After 3 h in culture, the cell viability was determined by measuring the absorbance at 490 nm using an ELISA microplate reader (Awareness, Palm City, FL, USA). The cytotoxicity of methanol extract of S. chloroleuca and its fractions was expressed as IC_{50} which was calculated using Graph Pad Software (Graph Pad prism 5 software) and presented as mean±SEM of three independent experiments with three replicates for each concentration fraction of S. chloroleuca fractions.

Cell morphology

The MCF-7 cells were plated in 96-well plates at a density of 10^4 cells/well and grown for 48 h in order to attach to the surface of the plates completely. The cytotoxicity of methanol extract of S. chloroleuca and its fractions were added in different concentrations (0, 7.8, 15.6, 31.2, 62.5, 125 and 250 µg/mL) to the cells and then the cells were grown at 37°C in a humidified atmosphere with 5% CO_2 for 48 h. For cell morphology experiments, the culture plates were examined and photographed by the inverted light microscope.

PI staining

Apoptotic cells were detected using PI staining of treated cells followed by flow cytometry to detect the so-called sub-G1 peak (18, 19).

It has been reported that DNA fragmentation creates small fragments of DNA that can be eluted following incubation in a hypotonic phosphate-citrate buffer. When stained with a quantitative DNA-binding dye such as PI, cells that have lost DNA will take up less stain and will appear to the left of the G1 peak. Briefly, 10^4 MCF-7 cells were seeded in each well of a 24-well plate and treated with CHCl_2 fraction of S. chloroleuca in different concentrations (0, 25, 50 and 100 µg/mL) for 48 h. Floating and adherent cells were then harvested and incubated at 4°C overnight in the dark with 750 μL of a hypotonic buffer (50 μg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100) before flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson). 10^4 events were acquired with FACS.

DNA fragmentation

Isolation of apoptotic DNA fragments was performed, based on the modified method previously described (20). In brief, the MCF-7 cells were incubated with CHCl_2 fraction of S. chloroleuca in different concentrations (0, 12.5, 50, 100 and 200 µg/mL) for 48 h.

The formation of high molecular weight and oligonucleosomal DNA fragments was examined by agarose gel electrophoresis. Cells (10^6 cells) were seeded onto 6 well plates and treated for 48 h. The cells were collected by
centrifugation at 800 g for 7 min. The DNA from treated and untreated cells was extracted as explained below: cells were incubated with 50 µL of lysis buffer (20 mM Tris, 20 mM EDTA, 200 mM NaCl and 1% SDS) and 2 µL RNase A (500 µg/mL) for 1 h at 37ºC. The cells were further incubated at 50ºC for 1 h after adding 2.5 µL of 10 mg/mL Proteinase K which had been preheated in 37ºC for 30 min. The lysate was mixed with 10 mL of loading solution (30% Ficoll, and 1% bromophenol blue in TBE), The DNA samples were separated in 2% agarose gel electrophoresis at 120 V, 30 min and visualized with ethidium bromide.

**Determination of ROS**

An increased reactive oxygen species generation can induce apoptosis (21). MCF-7 cells were cultured on 96-well microplate to 1×10⁴ cells/well. Cells were treated with 250 mM CH₂Cl₂ fraction of *S. chloroleuca* for 24 h, washed, then incubated with 20 mM ROS-specific dye, CM-H₂DCFDA that is specific for hydrogen peroxide (H₂O₂) in HBSS for 30 min at 37°C. NAC (3 and 6 µM) or Vit C (3 and 6 µM) was added 1 h before and during CH₂Cl₂ fraction of *S. chloroleuca* treatment. The fluorescence intensity was analyzed with an excitation wavelength at 485 nm and emission at 530 nm (22, 23).

**Statistical analysis**

One way analysis of variance (ANOVA) and Bonferroni’s posthoc were used for data analysis. All results were expressed as mean ± SEM and p values below 0.05 were considered statistically significant.

**Result**

**Inhibition of cell viability**

Inhibition of cell viability caused by total methanol extract of *S. chloroleuca* and its fractions was examined using MTS assay.

In order to compare the cytotoxicity of total methanol extract of *S. chloroleuca* and its fractions the MCF-7 cells were incubated with different concentrations for 48 h. The results showed methanol extract of *S. chloroleuca* and n-hexane and CH₂Cl₂ fractions decreased cell viability of cells in a concentration-dependent manner (Figure 2). This toxicity was coupled with morphological changes including decrease in cell volume and rounding of the cells. The substantial morphological changes observed in treated MCF-7 cells were examined and photographed by the inverted light microscope. Damaged cells became round and shrunken, while the untreated cells remained normal in size and shape (Figure 2). The doses inducing 50% cell growth inhibition (IC₅₀) against MCF-7 cells for total methanol extract of *S. chloroleuca* and n-hexane, CH₂Cl₂, EtOAc, n-BuOH, and H₂O fractions are presented in Table 1. In comparison, the cytotoxic effect of total methanol extract of *S. chloroleuca* and n-hexane, CH₂Cl₂, EtOAc, n-BuOH, and H₂O fractions on normal lymphocyte isolated from peripheral blood was minimal (Figure 2).

We used 700 nM of Paclitaxel as a positive control.

**Apoptosis induction by CH₂Cl₂ fraction in MCF-7 cells**

Apoptosis following treatment with CH₂Cl₂ solvent fraction of *S. chloroleuca* was measured,

<table>
<thead>
<tr>
<th>Solvent</th>
<th>IC₅₀ (µg/mL)</th>
<th>IC₅₀ range</th>
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<tr>
<td>MeOH</td>
<td>60.25</td>
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<td>n-hexane</td>
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<tr>
<td>CH₂Cl₂</td>
<td>25.49</td>
<td>25.49 to 30.99</td>
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<tr>
<td>EtOAc</td>
<td>63.14</td>
<td>63.14 to 85.71</td>
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<tr>
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<td>250&lt;</td>
<td>250&lt; to ...</td>
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<tr>
<td>H₂O</td>
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<td>250&lt; ...</td>
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with PI staining and flow cytometry aiming to detect the sub-G1 peak resulting from DNA fragmentation. The MCF-7 cells treated with 12.5, 25, 50 µg/mL CHCl₂ solvent fraction of S. chloroleuca for 48 h induced a sub-G1 peak in flow cytometry histogram compared to untreated control cells (Figure 3A).

Another characteristic event of cell apoptosis is the fragmentation of genomic DNA into integer multiples of 180-200 bp units producing a characteristic ladder on agarose gel electrophoresis. This event was observed in the MCF-7 cells within 48 h after treatment with CHCl₂ solvent fraction of S. chloroleuca at 12.5, 50, 100 and 200 µg/mL (Figure 3B).

**CHCl₂ solvent fraction of S. chloroleuca increased the production of ROS**

The intracellular level of ROS was measured, to determine whether CHCl₂ solvent fraction of S. chloroleuca-induced apoptosis is mediated by oxidative stress. As shown in Figure 4A, treatment of cells with CHCl₂ solvent fraction of S. chloroleuca increased the production of ROS significantly. For each time point, the 520 nm fluorescence level was measured in control and CHCl₂ solvent fraction of S. chloroleuca treated cells. The ratio obviously indicates a ROS production in CHCl₂ solvent fraction of S. chloroleuca-treated MCF-7 cells (Figure 4). In addition, the co-treatment of cells with CHCl₂ solvent fraction of S. chloroleuca and either NAC (3 and 6 mM) or Vit C (3 and 6 mM) antioxidant inhibited the CHCl₂ solvent fraction of S. chloroleuca -induced ROS production significantly (Figure 4B) indicating that the fluorescence augmentation was related to ROS production.

**Discussion**

Cytotoxic properties of total methanol extract of S. chloroleuca and its fractions were investigated on MCF-7, a breast carcinoma cell line as the most common neoplasm in women around the world (24). Extracts were tested for cytotoxic activity at a range of 0-250 µg/mL after 48 h of treatment.

There are few reports on biological activity of the S. chloroleuca. Yousefzadi et al., reported
### Apoptosis Induction of Salvia chloroleuca

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<table>
<thead>
<tr>
<th>MeOH</th>
<th>0.0 µg/mL</th>
<th>7.8 µg/mL</th>
<th>15.6 µg/mL</th>
<th>31.2 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5 µg/mL</td>
<td>125 µg/mL</td>
<td>250 µg/mL</td>
<td>700 nM</td>
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<tr>
<td>n-hexane</td>
<td>0.0 µg/mL</td>
<td>7.8 µg/mL</td>
<td>15.6 µg/mL</td>
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<td>62.5 µg/mL</td>
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<td>250 µg/mL</td>
<td>700 nM</td>
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<tr>
<td>CH$_2$Cl$_2$ fraction</td>
<td>0.0 µg/mL</td>
<td>7.8 µg/mL</td>
<td>15.6 µg/mL</td>
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Paclitaxel
the in-vitro antimicrobial activity of the essential oil of *S. chloroleuca* against seven Gram-positive and Gram-negative bacteria and three fungi (25).

The anti-proliferative and cytotoxic effect of many *Salvia* species on several cancer cell lines have been reported previously (9, 10, 12, 26). In a previous study Badisa *et al.*, were evaluated eight crude extracts of five *Salvia* species for cytotoxic activities against brine shrimps and four human cancer cell lines (HCA, HepG2, MCF-7, HPC) (27).

In another study in-vitro anti-proliferative screening investigation of crude methanol extracts of six *Salvia* species: *S. dominica* L. leaves, *S. lanigera* Desf. aerial parts, *S. menthaefolia* Ten. roots, *S. palaestina* Benth. aerial parts, S. *sclarea* L. roots and *S. spinosa* L. aerial parts, revealed growth inhibitory activity with IC$_{50}$ values ranged from 90 to 400 μg/mL (9).

It was also reported that CH$_2$Cl$_2$ extract from *S. plebeia* R. Br. may inhibit the cancer cell proliferation by inducing cell apoptosis (12).

However there was not any similar investigation on *S. chloroleuca*.

In our study methanol extract obtained from *S. chloroleuca* has a cytotoxic and apoptotic activity on MCF-7 cells. In order to gain insight into the nature of the active principles responsible for the cytotoxic activity, the methanol extract was fractionated using solvents of increasing polarity. The results observed with the entire *S. chloroleuca* extract and n-hexane, CH$_2$Cl$_2$, EtOAc, n-BuOH, and H$_2$O solvent fractions of *S. chloroleuca* were calculated 60.25, 28.06, 25.49, 63.14, >250, and >250 respectively.

From the literature search of the related genera and families, it is possible to predict the cytotoxic compounds that might be present in
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S. chloroleuca extract. Extraction with solvents of increasing polarity helps to predict specific classes of compounds (28).

The IC\textsubscript{50} values of the fractions and methanol extract are compared in Table 1. An active constituent(s) of intermediate polarity is thus likely to be responsible for the observed cytotoxicity and future bioassay guided fractionation needs to focus on the CH\textsubscript{2}Cl\textsubscript{2} fraction. These results suggest that CH\textsubscript{2}Cl\textsubscript{2} fraction obtained from S. chloroleuca could be used as a potential apoptosis inducing agent, and that the CH\textsubscript{2}Cl\textsubscript{2} fraction obtained from S. chloroleuca consists of a key component for cytotoxic activity.

Apoptosis induction protects organisms against tumor development (29, 30). Ladder-like pattern of DNA fragmentation into the multiples of 180 bp has been considered as a biochemical hallmark of apoptosis (31).

Anti-cancer effect through ROS induction has been reported for many naturally occurring compounds including d-Limonene, a bioactive food component from citrus (32, 33).

To investigate the mechanism of the CH\textsubscript{2}Cl\textsubscript{2} solvent fraction of S. chloroleuca-induced cytotoxicity, we examined the effect of CH\textsubscript{2}Cl\textsubscript{2} solvent fraction of S. chloroleuca on ROS levels in MCF-7 cells. The results indicate that cellular ROS were strongly induced by CH\textsubscript{2}Cl\textsubscript{2} solvent fraction of S. chloroleuca, suggesting that CH\textsubscript{2}Cl\textsubscript{2} solvent fraction of S. chloroleuca function as pro-oxidants in cancer cells.

ROS accumulation contributes to the cytotoxicity induced by CH\textsubscript{2}Cl\textsubscript{2} solvent fraction of S. chloroleuca. We next investigated whether the ROS accumulation is required for the potentiated cytotoxicity induced by CH\textsubscript{2}Cl\textsubscript{2} solvent fraction of S. chloroleuca-treatment. As shown in Figure 4A, both the ROS scavengers Vit C and NAC suppressed the CH\textsubscript{2}Cl\textsubscript{2} solvent fraction of S. chloroleuca-induced cytotoxicity.

These results suggest that induction of ROS is crucial for CH\textsubscript{2}Cl\textsubscript{2} solvent fraction of S. chloroleuca-induced cytotoxicity in MCF-7 cells.

Our finding in this study also showed that CH\textsubscript{2}Cl\textsubscript{2} solvent fraction of S. chloroleuca...
induced significant cellular ROS accumulation in cancer cells, which substantially contribute to cytotoxicity in CH$_2$Cl$_2$ solvent fraction of S. chloroleuca treated cell.

Regarding the sequential extraction with solvents of ascending polarity, the fractionation of S. chloroleuca with n-hexane and CH$_2$Cl$_2$ generated fractions that were cytotoxic for tumor cells. This can be explained by the low polarity of the solvents used in these fractions, which extract low-polar compounds that are either successfully absorbed through the cell or have cytotoxic activity. Therefore, it is presumed that most of the cytotoxic compounds or the compounds with the highest potency were concentrated in the n-hexane and CH$_2$Cl$_2$ fractions.

In conclusion, this study determined an anti-cancer effect of CH$_2$Cl$_2$ fraction obtained from S. chloroleuca mediated by the induction of apoptosis, which is associated with DNA fragmentation and induction of ROS, in MCF-7 cells. As apoptosis has become a new therapeutic target in cancer research, these results confirm the potential cytotoxic activity of n-hexane and CH$_2$Cl$_2$ fractions obtained from S. chloroleuca in human breast cancer cells and n-hexane and CH$_2$Cl$_2$ fractionation are more efficient than methanol extraction. n-Hexane and CH$_2$Cl$_2$ fractions can extract a compounds that has valuable cytotoxic activity. However, further investigation on the active principals of CH$_2$Cl$_2$ fraction obtained from S. chloroleuca is necessary to be elaborated.

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**References**


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