

Inhibitory and Cytotoxic Activities of *Salvia Officinalis L.* Extract on Human Lymphoma and Leukemia Cells by Induction of Apoptosis

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ABSTRACT

Purpose: *Salvia officinalis L.*, also known as Maryam Goli, is one of the native plants used to Persian medicinal herbs. Hence, the objective of this study was to examine the in vitro cytotoxic activities of a standardized crude methanol extracts prepared from *Salvia officinalis L.*, on a non-Hodgkin's B-cell lymphoma (Raji) and human leukemic monocyte lymphoma (U937), Human acute myelocytic leukemia (KG-1A) and Human Umbilical Vein Endothelial (HUVEC) cell lines. **Methods:** The effect of methanolic extract on the inhibition of cell proliferation and cytotoxic activity was evaluated by Dye exclusion and Micro culture tetrazolium test (MTT) cytotoxicity assay. Cell death ELISA was employed to quantify the nucleosome production result from nuclear DNA fragmentation during apoptosis and determined whether the mechanism involves induction of apoptosis or necrosis. **Results:** The present results demonstrated that methanolic extract at 50 to 800 µg/ml dose and time-dependently suppressed the proliferation of KG-1A, U937 and Raji cells by more than 80% ($p < 0.01$), with ascending order of IC₅₀ values in 24: KG-1A (214.377 µg/ml), U937 (229.312 µg/ml) and Raji (239.692 µg/ml) when compared with a chemotherapeutic anticancer drug, paclitaxel (Toxol), confirming the tumour-selective cytotoxicity. The crude extract however did not exert any significant cytotoxic effect on normal cell line HUVEC (IC₅₀ > 800 µg/ml). Nucleosome productions in KG-1A, Raji and U937 cells were significantly increased respectively upon the treatment of *Salvia officinalis L.* extract. **Conclusion:** The *Salvia officinalis L.* extract was found dose and time-dependently inhibits the proliferation of lymphoma and leukemic cells possibly via an apoptosis-dependent pathway.

Introduction

The conventional modality for cancer therapy includes surgery, radiation and drugs, separately or in combination. An effective anticancer drug should kill cancer cells without affecting abnormal to normal cells. Therefore, the identification of new cytotoxic drug with low side effects on immune system has developed as important area in new studies of immunopharmacology.¹ This ideal circumstance is feasible by inducing apoptosis in cancer cells. Apoptosis (Programmed cell death) an active physiological suicide that occur normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis is characterized by distinct morphological and biochemical changes, including cell shrinkage, membrane blebbing, chromatin condensation, and formation of apoptotic bodies. Maintenance of organelle integrity, condensation and DNA

fragmentation, followed by removal of dying cells by macrophage-mediated phagocytosis.² Oncogenic mutations can disrupt apoptosis and lead to tumor initiation, progression and metastasis. Dysfunction of the apoptotic program can promote tumor initiation, progression and treatment resistance. Defects in apoptosis can result in cancer, autoimmune diseases and spreading of viral infections.³ Traditional medicines have been used for maintaining health, boosting immune system function, prevention, therapy and remission of cancer. Natural product can serve as chemopreventive and chemotherapeutic agent.⁴

Recently, extensive studies have been dedicated to the apoptosis and the role of this process in intervening the lethal properties of anti-neoplastic agents in cancer cells. Anticancer agents induce apoptosis, so that disruption of apoptotic cell death reduces treatment sensitivity. Extensive varieties of natural compounds

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possess significant cytotoxic as well as chemopreventive activity, which act via apoptosis.⁵ Extracts of plants used in traditional medicine also have a similar property. Fifty-eight species of the genus *Salvia* (Lamiaceae) are found in Iran, which 17 are endemic. The genus *Salvia* shows diverse biological activities manifested by the different components that allow for the many medicinal and pharmaceutical applications of the plant materials and/or extracts. In fact, many diterpenes, isolated from plants of several species of the genus *Salvia*, have been demonstrated interesting pharmacological properties, such as antioxidant, anti-microbial,⁶ anti-inflammatory, analgesic,⁷ antipyretic, hemostatic,⁸ hypoglycemic⁹ and antitumor.¹⁰

The objective of this study was to examine the in vitro cytotoxic activities of methanol standardized extract, on a non-Hodgkin's B-cell lymphoma (Raji) and human leukemic monocyte lymphoma (U937), Human acute myelocytic leukemia (KG-1A) and Human Umbilical Vein Endothelial (HUVEC) cell lines, using a MTT cytotoxicity assay. The study also tested whether the mechanism of action involves induction of apoptosis. Cell death ELISA was employed to quantify the nucleosome production resulting from nuclear DNA fragmentation during apoptosis.

Materials and Methods

Preparation of plant extract

Salvia officinalis L. plants were collected from the central part of Iran (Karaj) in April 2011. Mr. Ajani from the Department of Botany, Institute of Medicinal Plants (IMP) of Karaj, Iran identified the plant. A voucher specimen was deposited in the herbarium of the above mentioned (herbarium No.1471). The aerial parts of the plant were separated, shade dried and grinded into powder with mortar and pestle. The prepared powder was kept in tight containers protected completely from light. Extraction of methanolic extract was carried out by macerating 100 g of powdered dry plant in 500 ml of 70% methanol for 48 h at room temperature. Then, the macerated plant material was extracted with 70% methanol solvent by percolator apparatus (2-liter volume) at room temperature. The plant extract was removed from percolator, filtered through Whatman filter paper (NO.4) and dried under reduced pressure at 37 °C with rotator evaporator before being added to ethanol as the solvent. The methanol extract was filtered and concentrated using a rotary evaporator and then evaporated to dryness. Briefly, the concentrated plant extracts were dissolved in dimethyl sulphoxide (DMSO) (SIGMA, USA) to get a stock solution of 10 mg/ml. The sub-stock solution of 0.2 mg/ml was prepared by diluting 20 µL of the stock solution into 980 µL serum-free culture medium, RPMI 1640 (the percentage of DMSO in the experiment should not exceed 0.5). The stock and sub-stock solutions were both stored at 4 °C.

Cell cultures

Burkitt's lymphoma B-cell line (Raji), human leukemic monocyte lymphoma cell line (U937), Human acute myelocytic leukemia cell line (KG-1A) and Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Pasteur Institute of Iran (Bank cell). The cells was grown and maintained in a humidified incubator at 37 °C and in 5% CO₂ atmosphere. RPMI-1640 medium (SIGMA) was supplemented with 0.01 mg/ml heat inactivated Fetal Calf Serum (FCS), 100 units/ml penicillin, and 100 µg/ml streptomycin (ALL FROM INVITROGEN GIBCO) was used for cell cultures. Upon reaching confluency, the cells were passaged. After being harvested from sterile T75 culture flasks (NUNC, DENMARK), the cells were counted using a hemocytometer and cell viability was determined by trypan blue exclusion. Ten thousand cells from log phase cultures were seeded in 100 µl of RPMI medium supplemented with 10% fetal bovine serum per well of 96-well flat-bottom culture plates (NUNC, DENMARK).¹¹ Cells were incubated with the *Salvia officinalis* L. extract for a defined time (12, 24 and 48 hours). Proliferative response and cell death of the *Salvia officinalis* L. extract-treated cells were determined using MTT assay and cell death ELISA Cell Viability Assay, respectively.

MTT Cell viability assay

The assay detects, the reduction of MTT [3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide, Sigma] (a colorimetric technique) by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria and hence for measuring the cytotoxicity cell and viability. 1×10^4 viable cells/well were plated into the 96-well tissue culture plates (NUNC DENMARK), and then incubated at 37 °C overnight. The next day when cells reached >80% confluence, the media were replaced with 200 ml of fresh complete medium containing 50,100,200,300,400,500,600,800 µg/ml concentrations of crude extract, no extract was added to the negative control well. After 12, 24, or 48 h, the supernatants were removed and cell layers were washed with phosphate buffered saline (PBS, INVITROGEN GIBCO) and incubated with MTT (50 ml, 0.5 mg/ml) in RPMI 1640 without FCS for 4 h in a humidified atmosphere at 37 °C according to the manufacturer's protocol. The cell cultures were centrifuged at 1000 g for 5 min and the supernatants were discarded. Subsequently, 200 ml of dimethyl sulfoxide (DMSO, SIGMA) and 25 ml Sorenson buffer were added to dissolve the formazan crystals formed. The optical density (OD) colored solution was quantified at 570 nm wavelengths by an enzyme linked immunoabsorbent assay reader (ELISA READER, BIO-RAD). The absorbance of untreated cells was considered as 100%. Each extract and control was assayed in triplicate in three independent experiments.¹² The concentration of the crude extract that killed 50% of the cells (IC₅₀) was

calculated by EXCEL software and the mean optical density (OD) \pm SD for each group of the replicates calculated. Percent growth inhibition of cells exposed to treatments was calculated as follows: % Inhibition = $100 - (\text{Test OD}/\text{Non-treated OD}) \times 100$.

Cell death detection

Cell Death Detection ELISA^{PLUS} (ROCHE APPLIED SCIENCE) was used to quantify histone-complexed DNA fragments (nucleosomes) in cytoplasm of the apoptotic cells after induction of apoptosis. Briefly, after incubation with the methanolic extract (at concentrations determined by MTT assay) for 24 h cells were pelleted and lysed. Mouse monoclonal antibodies against single-strand DNA and histones (H1, H2a, H2b, H3 and H4) specifically detected and bound mononucleosomes and oligonucleosomes derived from cells undergoing apoptosis. Biotinylated anti-histone antibodies then fixed the antibody-nucleosome complexes to the streptavidin-coated microtiter plate. The anti-DNA antibodies were conjugated with a peroxidase that reacted with the substrate ABTS [2,2'-azino-di (3- ethylbenzthiazolin-sulfonate)] to form a colored product. The remaining steps were carried out according to the instructions supplied by the manufacturer. The resulting color development, which was proportional to the amount of nucleosomes captured in the antibody sandwich, was measured at 405 nm wavelength using a Benchmark microtiter plate reader (BIO-RAD).¹³ Results were expressed as the apoptotic index (AI), calculated from the ratio of absorbance of treated (apoptotic) sample to that of the untreated (control) sample.

Dye exclusion assay

Cellular cytotoxicity induced by the *Salvia officinalis* L. extract treatment was measured with trypan blue exclusion assay. Briefly, 1×10^4 cells were seeded into 96-well plates and treated with or without (as control) crude extract at specified doses for 12, 24 and 48 h. After the incubation period, the cultures were harvested and washed twice with PBS. The cell pellet was then resuspended with 0.5 ml PBS. Then, 20 μ L of cell was mixed with equal volume of 0.4% trypan blue (SIGMA, USA Merck) and was count by Neubauer haemocytometer (WEBER, ENGLAND) by clear field microscopy (NIKON, JAPAN). Only viable cells were counted. Each extract and control was assayed two times in triplicate.

Statistical Analysis

The data are expressed as mean \pm standard deviation (SD) for at least three independent determinations in triplicate for each experimental point. The data were analyzed using IBM SPSS Statistics 20 software. For all the measurements, Two-way ANOVA followed by Duncan's New Multiple Range Test ($P \leq 0.05$) was used to assess the statistically significance of difference between control and FA treated.

Results

Effects of methanolic extract of *Salvia officinalis* L. on proliferation of leukemia and lymphoma cells

Salvia officinalis L. extract at 50 to 800 μ g/ml exhibited significant dose-dependent inhibitory effects on the proliferation of Raji (Figure 1A), U937 (Figure 1B), and KG-1A cells (Figure 1C), with more than 80% suppression. However, the extract induced no significant suppression on the proliferation of normal HUVEC cells (Figure 1D).

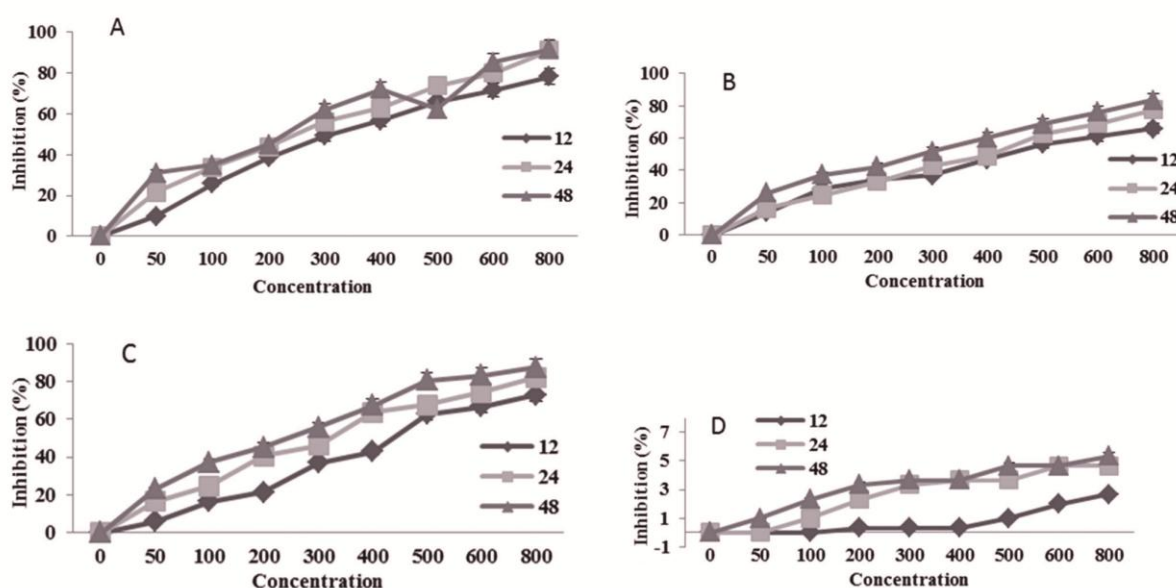


Figure 1. Effect of different concentrations of *Salvia officinalis* L. (A) Raji, (B) U937, (C) KG-1A and (D) HUVEC cell lines in 12, 24 and 48 h. Values represent the mean of three experiments. Standard deviations were less than 0.5.

Table 1 shows the concentrations producing 50% growth inhibition (IC₅₀) of the *Salvia officinalis L.* extract on the three cell lines in three times, which Raji proliferation was most potently suppressed with the lowest IC₅₀ value. Similar inhibitory effects were found in KG-1A, U937 cells after incubation with the *Salvia officinalis L.* extract. Proliferation of Raji cells was most significantly reduced by *Salvia officinalis L.* extract at 100 to 300 µg/ml, result in a lower IC₅₀ value; while KG-1A and U937 cell growth was most significantly reduced from 200 to 400 µg/ml with higher IC₅₀ values. Table 2 illustrates that Raji, KG-1A and U937 cells were similarly susceptible to the cytotoxicity of *Salvia officinalis L.* extract with more than 80% growth suppression; however, HUVEC cells were much less susceptible to the cytotoxic effect of *Salvia officinalis L.* extract. Viability rate was decreased in descending form 50.29%, 47.07% and 44.37% in U937, KG-1A and Raji, respectively (Table 3). In Table 4, Raji, KG-1A and U937 cells was compared to elucidate the cytotoxicity of both *Salvia officinalis L.* extract and Toxol with more than 80% and 90% growth suppression.

Table 1. Concentration producing 50% growth inhibitions (IC₅₀) of *Salvia officinalis L.* extract on the three cell lines in 12h, 24h and 48h.

Cell lines	IC ₅₀ (µg/ml)		
	12 hr	24 hr	48 hr
Raji	239.692	163.187	130.597
U937	304.332	229.312	161.825
KG-1A	345.623	214.377	152.182

Table 2. Percentage of inhibition in IC₅₀ concentration induced by *Salvia officinalis L.* extract on Raji, U937 and KG-1A cell lines respectively.

Time (hr)	Percentage of inhibition (%)		
	Raji	U937	KG-1A
12	44.03	36.18	38.22
24	51.51	46.22	41.70
48	53.81	53.37	49.55

Table 3. Percentage of Viable cells induced by *Salvia officinalis L.* extract in IC₅₀ concentration on the three cell lines. Results are expressed as the mean percentage of dead cells F SD in triplicate experiments with three wells each. Percentage of dead cells was calculated from the ratio of dead cells to total number of cells using trypan blue exclusion test.

Time (hr)	Viable cells (%)		
	Raji	U937	KG-1A
12	53.92	59.81	61.22
24	48.40	51.81	54.88
48	44.37	47.07	50.29

Table 4. Comparison of cytotoxic activity of *Salvia officinalis L.* extract (800 µg/ml) and Toxol (50 µg/ml) on the 3 cell lines.

Cell lines	Growth inhibition %	
	Crude extract	Toxol
Raji	87.00	89.1
U937	75.77	99.4
KG-1A	81.00	89.9

Effects of *Salvia officinalis L.* extract on cell death of leukemia and lymphoma

As determined by MTT assay, *Salvia officinalis L.* extract at 100, 200 and 300 µg/ml in 24 h were chosen for each cell line in cell death detection ELISA. Before determination of *Salvia officinalis L.* extract-induced cell death, cell viability of the four cell lines was evaluated. As shown in Table 3, the proportion of viable Raji cells was increased sharply. However, the percentage of death of U937 and KG-1A cells peaked at 12, 24 and 48 hr, respectively, when incubated with the *Salvia officinalis L.* extract of the same concentration. These results indicate that the apoptotic response of these three cell lines should be evaluated at different time points.

As shown in Figure 2, the *Salvia officinalis L.* extract at 300 µg/ml could strongly induce apoptosis of Raji cells after 24 h in a dose-dependent manner. Effects of *Salvia officinalis L.* extract on proliferation of (A) Raji, (B) KG-1A, (C) U937, and (D) HUVEC. Cells were incubated with increasing concentrations (50–800 µg/ml) of *Salvia officinalis L.* extract in culture medium for 24 h (Raji, U937, KG-1A and HUVEC) and the proliferative response was then assessed with MTT assay. The possible mechanism was via induction of apoptosis, as evidenced by the significant increase in nucleosome production at 200 and 300 µg/ml of *Salvia officinalis L.* extract after incubation for 24h but the ratio of apoptosis was constant approximately 50±3%.

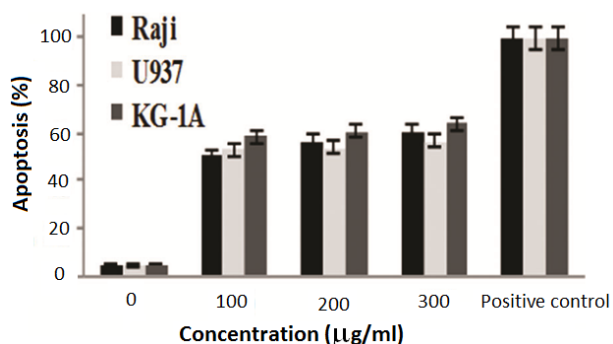


Figure 2. Effects of *Salvia officinalis L.* extract on cell death of Raji, U937, and KG-1A. Cells were incubated with the *Salvia officinalis L.* extract in culture medium at concentrations derived from IC₅₀ (100, 200 and 300 µg/ml) for 24 h.

Discussion

The current study has demonstrated that methanolic extract of a commonly used in Persian medicinal herb, *Salvia officinalis L.* in its natural form, could significantly suppress the proliferation of Burkitt's lymphoma B-cell line (Raji), human leukemic monocyte lymphoma cell line (U937) and Human acute myelocytic leukemia cell line (KG-1A) in vitro by means of the MTT assay. Such antiproliferative activity of *Salvia officinalis L.* extract was characterized by the time and dose-dependent and tumor-selective manner, as reflected by the comparatively low IC50 values and the absence of significant effects on normal HUVEC cells respectively. On the contrary, Toxol at optimal in vitro concentration non-selectively induced at least 90% growth suppression on all the studied cell lines (Table 4). These results suggested that when compared with the commonly used chemotherapeutic antitumor drug (e.g. Toxol), the *Salvia officinalis L.* extract, albeit at higher concentration, could induce comparable antitumor activity with much less cytotoxic effects on normal cells.

In Raji, U937 and KG-1A cell lines viability were decreased (from 79% to 13%, from 81% to 23% and from 85% to 19%) with increasing treatment concentration and (from 53% to 44%, from 61% to 50% and from 63% to 47%) treatment time respectively. In order to determine the antiproliferative activity of *Salvia officinalis L.* extract is shown by induction of apoptosis, cell death detection ELISA was used to quantify the nucleosome production during nuclear DNA denaturation of apoptotic cells. Before evaluating the *Salvia officinalis L.* extract-induced apoptosis, the percentages of death of the 3 cell lines at 24h were determined since the incubation time necessary to induce apoptosis can show broad variation. For Raji cells, the sharp increase in percentage of dead cells paralleled the significant nucleosome production up regulated by the *Salvia officinalis L.* extract from 12 to 48 h, suggesting the dose- and time-dependent induction of apoptosis. Similar results were seen in KG-1A and U937 cells. These results suggested that the extract exerts its antitumor effect on these leukemia and lymphoma cells possibly via an apoptosis-dependent pathway. Therefore, further studies are required to evaluate whether cell cycle arrest or apoptosis induction contribute more to the *Salvia officinalis L.* extract-induced cytotoxic effect on panel cells. In order to elucidate the cytotoxic activities of *Salvia officinalis L.* extract on the growth of leukemia and lymphoma cells of other sub-types, acute T-cell leukemia cell line (Jurkat) and Hodgkin's lymphoma may become the target cells used in our future studies. In addition, mechanistic studies on cell cycle arrest and early apoptotic events may be conducted to delineate other possible antitumor mechanisms of the *Salvia officinalis L.* extract. Besides, future in vivo antitumor studies will

be performed in order to confirm these in vitro results. In conclusion, this study provides the evidence that in vitro cytotoxic activity of a methanolic standardized extract from wild *Salvia officinalis L.* was found dose-dependently inhibit the proliferation of lymphoma and leukemic cells possibly via an apoptosis-dependent pathway.

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Conflict of interest

The authors report no conflicts of interest.

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