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Induction of apoptosis and G_2/M cell cycle arrest by *Scrophularia striata* in a human leukaemia cell line

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Abstract

Objectives: *Scrophularia striata* Boiss (Scrophulariaceae) is a plant that grows in northeastern Iran; it has been used traditionally to treat various inflammatory disorders. This study was designed to investigate cytotoxic effects of *S. striata* extract, on the Jurkat human leukaemia cell line (T-cell leukaemia).

Materials and methods: Phytochemical assay by thin layer chromatography and 2, 2 diphenyl-1-picryl-hydrazyl were used to evaluate main compounds and antioxidant capacity of the plant extract, respectively. Its inhibitory effect on Jurkat cells was evaluated by MTT assay. In addition, cell cycle distribution and apoptotic cell death were evaluated by propidium iodide and annexin V-FITC/ propidium iodide staining.

Results: These showed that the main components present in *S. striata* extract included flavonoids, phenolic compounds and phenyl propanoids. Treatment with extract was significantly cytotoxic to the tumour cell line. In addition, flow cytometry analysis indicated that *S. striata* extract induced cell cycle arrest in G_2/M phase and apoptosis of tumour cells.

Conclusions: Results of the study indicated that *S. striata* extract could inhibit leukaemia cell proliferation by inducing G_2/M phase arrest and apoptosis.

Introduction

Malignancy is one of the leading causes of death in the world. Although traditional cancer therapies, including surgery, chemotherapy and radiotherapy, are the standard methods of patient treatment, they are not fully effective. Using natural agents such as medicinal plants, in tumour therapy arouses extensive interest, aspiring to minimal side effects, better safety and efficiency. Many of the most useful anti-cancer drugs (including vinblastin, vincristine, etoposide and taxol) that have been approved for use in anti-cancer therapy, are plant-derived compounds (1–5); in addition, during our course of study on different medicinal plants, we have found inhibitory effects of several plants on tumour cell proliferation (6,7). *Scrophularia striata* Boiss (Scrophulariaceae) is a plant that grows in northeastern Iran that has been used as a traditional herb for various purposes. Several species of *Scrophularia* have been used in folk medicine since ancient times, as a sedative and for treatment of illnesses such as scrophula, scabies, eczema, psoriasis and tumours. Our previous *in vitro* studies have demonstrated inhibitory effects of *S. striata* extract on nitric oxide production and on pro-inflammatory cytokine (including TNF-α, IL-1β and PGE2) production by macrophages (8,9). In addition, anti-tumour, anti-inflammatory and immunomodulatory activities of some species of *Scrophularia* have been shown by other investigators (10–13). Moreover, several compounds from various *Scrophularia* species with anti-inflammatory and neuroprotective properties (including iridoids and phenyl propanoids) have been isolated. Further, flavonoids, phenolic compounds, quercetin and isorhamnetin *3-O*-rutinoside, with antioxidant activity, have also been identified from *S. striata* (14). In the present study, we investigated cytotoxic effects and induction of apoptosis of *S. striata* in the Jurkat human leukaemia cell line.

Materials and methods

Plant material and extract preparation

Aerial parts of *S. striata* were collected from the Ruin region of northeastern Iran, in May 2010, and air dried at room temperature. A sample was authenticated by Dr

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Faride Attar, from Tehran University, Faculty of Sciences and a voucher specimen (Herbarium No: 36501) was preserved in the herbarium of the Tehran University Faculty of Sciences, Tehran, Iran. Aerial components of the plant were dried, powdered (20 g) and macerated in 80% ethanol solution for 3 days with three changes of solution. Resulting extract was filtered and evaporated under vacuum into the final dried powder of *S. striata.* Extract including phenyl propanoids, phenolic compounds and flavonoids dissolved in dimethylsulphoxide (% 0.1 v/v); this process was performed here.

Phytochemical assay

To be able to identify chemical components of the extract, thin-layer chromatography was performed. A variety of indicators including vanillin sulphuric acid, ferric chloride and natural product polyethylene glycol were used. Indictors were sprayed on prepared thin layers of extract and were observed at 260 and 280 nm wavelengths under UV light.

DPPH assay

DPPH testing was used to evaluate antioxidant capacity of the plant extract. Briefly, one thousand microlitres of selected concentrations (250, 125, 62.5, 31.25, 15.62 and 7.81 μg/ml) of *S. striata* extract in ethanol was added to 4 ml of 0.004% methanolic solution of DPPH. After 60 min incubation at room temperature, absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH percent (I%) was calculated as follows:

$$I\% = \left[\frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}}\right] \times 100$$

A_{blank} is the Absorbance of control reaction (containing all reagents except the test compound).

A_{sample} is the Absorbance of test compound. Extract concentration providing 50% inhibition (IC_{50}%) was calculated from the graph inhibition percentage against extract concentration. IC_{50}% values were compared to IC_{50}% value of a 'standard' antioxidant, in this case ascorbic acid (AA), obtained by the same procedure.

Measurement of total phenolic compounds

Total phenolic content of the dry herb was determined by using Folin-Ciocalteau assay.

One aliquot (1 ml) of extract or standard solution of gallic acid (20, 40, 60, 80 and 100 mg/l) was added to 25 ml in a volumetric flask, containing 9 ml of distilled deionized water (dd H_2O) and a reagent blank using dd H_2O was prepared. One millilitre Folin-Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 ml 7% Na_2CO_3 solution was added to the mixture, the solution was diluted to 25 ml with dd H_2O and mixed. After incubation for 90 min at room temperature, absorbance against the prepared reagent blank was determined at 750 nm. Data of total phenolic contents are expressed as milligrams gallic acid equivalents (GAE) per gram dry weight (mg_{GAE}/g_{DW}). All samples were analysed in duplicate.

Cell culture

Jurkat tumour cell line (T cell leukaemia) prepared from the National Cell Bank of Iran and maintained by culturing in RPMI 1640 medium (Sigma, St Louis, MO, USA), was supplemented with 10% heat-inactivated foetal calf serum (Gibco-BRL Grand Island, NY, USA), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Cell viability was determined using the trypan blue dye exclusion test.

Cell viability assay

Effects of *S. striata* extract on the Jurkat human tumour cell line were determined using MTT (3-(4, 5-dimethylthiazoyl)-2, 5- diphenyltetrazolium bromide) assay. Briefly, cells were added to flat-bottomed micro-culture plates in the presence or absence of selected concentrations (50, 100, 200 and 400 μg/ml) of the extracts (in triplicate) and incubated at 37 °C, in 5% humidified CO_2 for 48 h. Then, 10 μl of MTT (5 mg/ml, Sigma) was added to each well and incubation continued for further 4 h at 37 °C. In each well, 100 μl/well solubilization solution, containing isopropanol and 10% SDS in 0.01 M HCl, was added. After complete solubilization of formazan crystals, plates were read at 570 nm on an ELISA reader; reference wavelength was 690 nm. Mean optical density (OD) ± SD for each group of replicates was calculated. Percent inhibition of cells exposed to various treatments was obtained as follows:

$$\%\text{Cell viability} = 100 - \left[\left(\frac{\text{Test OD}}{\text{Non-treated OD}}\right) \times 100\right].$$

Annexin V-FITC/PI apoptosis assay

To determine apoptosis, an Annexin V–FITC apoptosis Detection Kit (Invitrogen, Carlsbad, CA, USA) was used, according to the manufacturer's protocol. Briefly, 1×10^5 cells/ml were treated with the selected concentrations (50, 100, 200 and 400 μg/ml) of *S. striata*

extract, for 24 h at 37 °C. Cells were then harvested and re-suspended in binding buffer. They were then stained with 10 μl AnnexinV–FITC and 5 μl propidium iodide (PI) for 15 min at room temperature in the dark. Apoptotic index was immediately determined by flow cytometry.

Cell cycle analysis

Cells (1×10^5 cells/ml) were treated with the selected concentrations (50, 100, 200 and 400 μg/ml) of the extract for 24 h; they were then centrifuged and fixed in 70% ethanol. After washing, cells were resuspended in 1 ml PBS containing 10 mg/ml RNase and 1 mg/ml PI (Sigma); they were then incubated for 1 h at 37 °C in the dark. Thereafter, cells were analysed on a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Taxol treatment (1.75 μg/ml) was used as positive control.

DNA fragmentation analysis

In this study, isolation of fragmented DNA from Jurkat cells cultured in 24-well plates was carried out according to the procedure of Amirghofran *et al.*, with modifications (7). Briefly, 2×10^6 cells/ml were treated with plants extract then collected by centrifugation (2000 g, 10 min). Pellets were resuspended in 0.5 ml DNA lysis buffer (2% SDS, 10 mM EDTA, 10 mM Tris-Hcl, pH 8.5) and lysate was immediately incubated with 0.1 mg/ml proteinase k (Sigma) before being incubated for 3 h at 37 °C. After addition of isopropanol, DNA was precipitated with 70% ethanol. The suspension was then centrifuged, and DNA treated with 100 μl 10 mM Tris-HCl (pH 7.5) and 0.5 mg/ml RNase A (Boehringer Mannheim, Mannheim, Germany), at 37 °C for 24 h. Samples were then loaded into 2% agarose gels containing ethidium bromide, and were electrophoresed. DNA bands was visualized under ultraviolet illumination and photographed.

Statistical analysis

Data are presented as mean ± SD. Statistical analyses were performed by one-way analysis of variance (ANOVA) and a *post–hoc* Bonferroni test to express the difference among the groups. All analyses were performed using SPSS 16. Data were considered statistically significant at $P < 0.05$.

Results

Chemical components of the extract

Phytochemical assay by thin layer chromatography revealed *S. striata* extract's main components including phenyl propanoids, phenolic compounds and flavonoids (Table 1).

Measurement of total phenolic compounds and antioxidant activity

Phenolic compounds are well recognized as antioxidants which can act as free radical terminators (15) and have been known to show medicinal activity as well as to exhibit physiological functions (16). In this study, we used Folin-Ciocalteau assay for standardizing aerial parts of *S. striata* and quantity of total phenolic compounds; flavonoids and phenolic compounds of dry herb were measured respectively, as shown in Table 2, with gallic acid as standard. In addition, free radical scavenging capacities of the extract were measured by DPPH assay and results are also provided in Table 2, with ratios (IC_{50}%) AA/(IC_{50}%) extract shown. These represent AA equivalent of the extract antioxidant capacity, that is, amount of AA in milligram equivalent to one gram of extract.

Effect of S. striata *extract on tumour cell viability*

In this study, normal human peripheral blood mononuclear cells (PBMCs) were used as normal cells compared to the test Jurkat human leukaemia cells. In a preliminary experiment on PBMCs, results indicated that extract up to 400 μg/ml had no significant toxicity over 48 h. Thus, the Jurkat cells were incubated with selected concentrations (0–400 μg/ml) of extract for 48 h. As shown Fig. 1, extract significantly ($P < 0.05$) inhibited Jurkat cell proliferation in a dose- and time-dependent

Table 1. Phytochemical results of *Scrophularia striata* extract

Compounds	Reagents	Standards	Results
Phenylpropanoids and terpenoids	Vanillin sulphuric acid	Cinamic acid	+
Phenolic compounds	Ferric chloride	Nepitrin	+
Flavonoids	Natural product reagent	Quercetin	+

Table 2. Measurement of phenolic compounds and antioxidant capacity of *Scrophularia striata* extract

Total phenolic compounds in dry herb (mg_{GAE}/g_{dw})	DPPH radical scavenging activity, IC_{50}% (mg/l)	Ascorbic acid equivalent of the extract antioxidant capacity (mg/g)
10.96	316.69	29.8

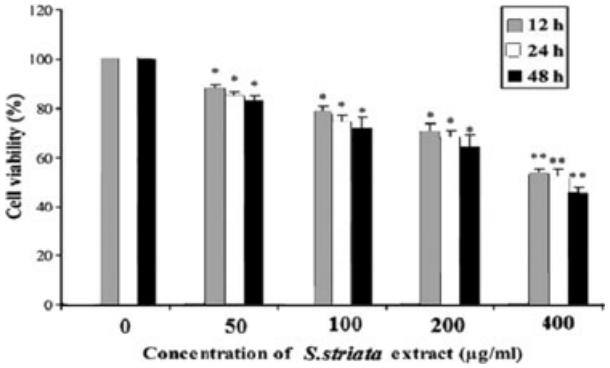

Figure 1. Effect of *Scrophularia striata* extract on viability of Jurkat human leukaemia tumour cells. Tumour cells were incubated with selected concentrations (0–400 µg/ml) of extract for 48 h. Results indicated that the extract significantly (*$P < 0.05$, **$P < 0.001$) inhibited Jurkat cell population growth in a dose- and time-dependent manner compared to non-treated (control group) cells. Results shown are representative of three independent experiments.

manner. For example, treatment with 400 µg/ml of extract for 48 h resulted in 58% Jurkat cells proliferation inhibition. Results indicate that this extract can induce a cytotoxic effect on Jurkat human leukaemia cells.

Effect of S. striata *extract on apoptosis of Jurkat cells*

To determine whether the cytotoxic effect of *S. striata* extract on Jurkat cells was related to apoptotic cell death, we evaluated phosphatidylserine exposure as an early marker of apoptosis, using annexin V-FITC/PI double staining. In control (untreated) groups, 92.2% cells were viable, 7.5% cells were in the early and late stages of apoptosis (lower right + upper right). While, after 24-h treatment with *S. striata* extract, the apoptotic cell population increased from 19.2 to 53.3% when extract concentration increased from 50 to 400 µg/ml. As shown in Fig. 2, percentages of Annexin V-positive cells increased gradually in a dose-dependent manner after *S. striata* extract treatment, suggesting that the extract induced the apoptotic response.

Effect of S. striata *extract on Jurkat cell cycle distribution*

To determine whether anti-tumour and proliferation-inhibitory effects of *S. striata* extract on Jurkat cells was related to induction of cell cycle arrest, distribution of cells in different phases of the cell cycle was evaluated by measuring DNA content. As shown in Fig. 3, after treatment with different doses of *S. striata* extract for 24 h, proportions of cells in G_2/M increased from 4.3 to 28.2% compared to control groups. In addition, proportion of cells in G_0/G_1 fell from 36.8 to 19.1% compared to control groups while percentages of S phase cells almost retained the same levels. Results indicated that *S. striata* extract induced G_2/M cell cycle arrest in these Jurkat cells in a dose-dependent manner.

Effect of S. striata *extract on Jurkat cell DNA fragmentation*

To confirm effects of the extract on induction of apoptosis in the cell line, DNA products were examined for appearance of characteristic DNA laddering in treated cells. Ladder formation was detected after exposing the cells with 100–200 µg/mL of extract. As shown in Fig. 4, increased inter-nucleosomal DNA fragmentation was dose dependently apparent in the cells, indicating that the extract caused the cells to undergo apoptosis.

Discussion

In this study, we evaluated cytotoxic activity of *S. striata* extract on Jurkat human leukaemia cells. We found that *S. striata* extract inhibited Jurkat cell population growth in a dose- and time-dependent manner. Moreover, results indicated that extract up to 400 µg/ml had no significant toxicity to normal PBMCs. It has been shown previously that some anti-cancer effects of medicinal plants are based on induction of apoptosis (6,7,17–19). Apoptosis is a normal physiological process that plays an important role in homeostasis and expan-

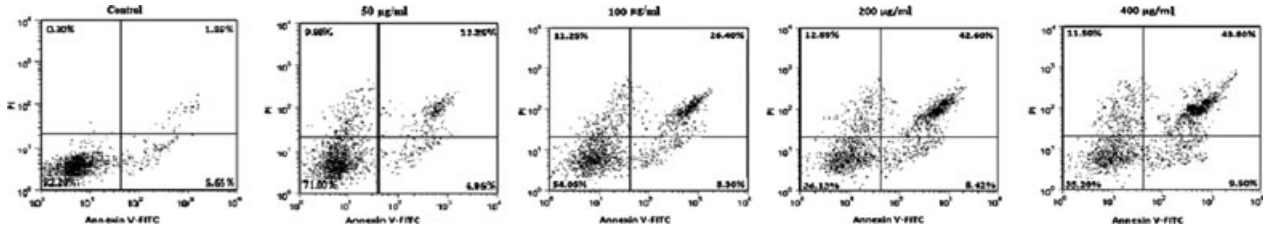

Figure 2. Effect of *Scrophularia striata* extract on induction of apoptosis in Jurkat cells. Apoptosis-inducing effect of *S. striata* extract on Jurkat human leukaemia cells evaluated by the annexin V-FITC (AV)/PI method. Dot-plot graphs show viable cells (AV^-/PI^-), early phase apoptotic cells (AV^+/PI^-), late phase apoptotic cells (AV^+/PI^+) and necrotic cells (AV^-/PI^+).

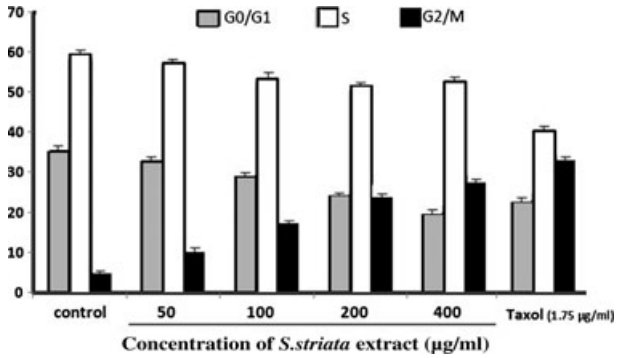

Figure 3. Effect of *Scrophularia striata* extract on cell cycle of Jurkat cells. Jurkat cell line treated with selected concentrations of *S. striata* extract for 24 h. Distribution of cells in different phases of the cell cycle was evaluated by measuring DNA content. Results showed that *S. striata* extract induced G_2/M cell cycle arrest in Jurkat cells in a dose-dependent manner. Taxol treatment was used as positive control. Results shown are representative of three independent experiments.

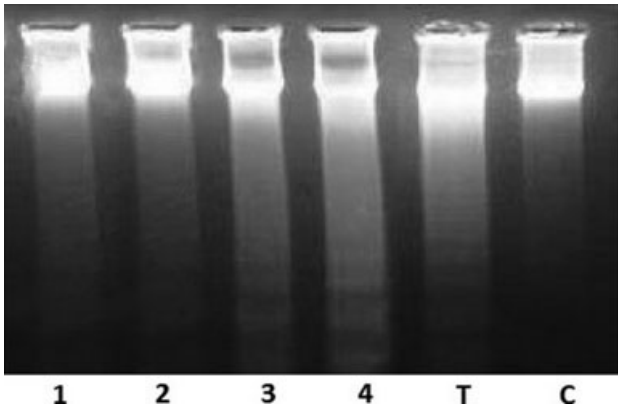

Figure 4. Effect of Scrophularia striata on DNA fragmentation in Jurkat cell line. Cell line after treatment with 1 = 50µg/ml, 2 = 100µg/ml, 3 = 200µg/ml, 4 = 400µg/ml, T = Taxol µg/ml, C = negative control. DNA laddering typical for apoptotic cells are visible for the cells treated with the extract.

sion of normal and cancer cells (20,21); also dysregulation of apoptosis is usually considered to be a major cancer cell property (22). Also, many studies have shown that apoptosis is an important mechanism by which various anti-cancer agents exert antitumour effects (23,24). In previous studies, cytotoxic activity of some species of *Scrophularia* have been reported on cancer cell lines (12,25). Here, to determine whether cytotoxicity of *S. striata* extract on Jurkat cells was related to apoptotic cell death, percentages of apoptotic cells were measured by annexin V-FITC/PI staining. Our results indicated that proportions of apoptotic cells increased with increasing concentration of *S. striata* extract. These data indicated that cytotoxic effects of *S. striata* extract seemed to be mediated by induction of apoptosis in the Jurkat cells. In addition, one previous study has shown that a further genus of Scrophularia, *S. floribunda* extract, induced apoptosis in tumour cells through induction of cell cycle arrest (25). Thus, in this study, to determine whether induction of apoptosis and antitumour effects of *S. striata* extract on Jurkat cells was associated with induction of cell cycle arrest, distribution of cells in different phases of the cell cycle was evaluated by measuring intracellular DNA content. Our finding indicate that *S. striata* extract induced G_2/M phase cell cycle arrest in Jurkat cells in a dose-dependent manner. On the other hand, the antioxidant, anti-inflammatory and immunomodulatory activities of some species of Scrophularia have also been shown by several investigators (10,12,26); *S. striata* has been used to treat various inflammatory disorders (8,26,27). Our previous studies indicated the inhibitory effect of *S. striata* extract on pro-inflammatory mediator production by macrophages including nitric oxide, TNF-α, IL-1β and PGE2 production and also suppressive effects on matrix metalloproteinases in the Wehi-164 tumour cell line *in vitro* (8,9,13). In addition, antioxidant properties of compounds such as iridoide glycosides and phenylpropanoid esters isolated from *S. buergeriana* have been reported (28–31). However, in our previous and present studies, phenolic compounds, phenyl propanoids and two flavonoids, quercetin and isorhamnetin *3-O*-rutinoside, were identified from extracts of this plant (14). Several studies showed that quercetin is a dietary antioxidant and also it has anti-inflammatory and anti-tumour effects (32–36). Moreover, other studies have shown that isorhamnetin *3-O*-rutinoside induces apoptosis in human chronic myelogenous erythroleukaemia cells (K562) (37,38). This plant extract in the present and previous studies is indicated as having antioxidant, anti-inflammatory and antitumour properties. Whether these compounds are the main responsible agents for anti-tumour effects of *S. striata* and are involved in this activity needs further investigation. In conclusion, our findings here show that *S. striata* has cytotoxic activity on Jurkat human leukaemia tumour cells. The ability of this plant to induce apoptosis through G_2/M phase cell cycle arrest on the leukaemic cell line makes it a candidate for further studies to discover the active components involved and mechanisms by which they induce apoptosis.

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Conflict of interest

None.

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