

Apoptosis Cell Death Effect of *Scrophularia Variegata* on Breast Cancer Cells via Mitochondrial Intrinsic Pathway

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Article info

Article History:

Received: 25 January 2015

Revised: 30 May 2015

Accepted: 13 June 2015

ePublished: 19 September 2015

Keywords:

- *Scrophularia variegata*
- Apoptosis
- Breast cancer cell line
- Caspase

Abstract

Purpose: *Scrophularia variegata* M. Beib. (Scrophulariaceae) is an Iranian medicinal plant which is used for various inflammatory disorders in traditional medicine. In this study we evaluated the anti-cancer and cytotoxic effects of the *Scrophularia variegata* (*S. variegata*) ethanolic extract on the human breast cancer cell line.

Methods: The cytotoxicity effect of the extract on MCF-7 cells was evaluated by MTT assay. In addition, Caspase activity, DNA ladder and Cell death were evaluated by ELISA, gel electrophoresis and Annexin V-FITC/PI staining, respectively.

Results: The *S. variegata* extract showed significant effect cytotoxicity on MCF-7 human breast cancer cell line. Treatment with the extract induced apoptosis on the breast cancer cells by cell cycle arrest in G2/M phase. The results indicated that cytotoxicity activity was associated with an increase of apoptosis as demonstrated by DNA fragmentation as well as an increase of the amount of caspase 3 and caspase 9. In addition, the phytochemical assay showed that the extract had antioxidant capacity and also flavonoids, phenolic compounds and phenyl propanoids were presented in the extract.

Conclusion: Our findings indicated that *S. variegata* extract induced apoptosis via mitochondrial intrinsic pathway on breast cancer by cell cycle arrest in G2/M phase and an increase of caspase 3 and caspase 9. However future studies are needed.

Introduction

Breast cancer is one of the leading causes of death among women in the world. At the present, using of natural compounds such as medicinal plants in cancer therapy has aroused general because of its minimal side effect, safety and efficiency.^{1,2} *Scrophularia variegata* is an Iranian medicinal plant and which is used for various inflammatory disorders in traditional medicine. Our previous findings demonstrated that *Scrophularia* species had the anti-cancer activity by induction of apoptosis and inhibition of matrix metalloproteinases, anti-asthmatic, neuroprotective, inhibitory effect on the nitric oxide and pro-inflammatory cytokines production.³⁻¹⁰ In the present study we investigated the cytotoxic effect and induction of apoptosis of *S. variegata* in the MCF-7 human breast cancer cell line.

Materials and Methods

Plant material and preparation

The plant was collected from Taleqan region (Alborz province) in May 2010, in Iran. A voucher specimen was deposited in the herbarium of the Institute of Medicinal Plants (IMP). Aerial parts of the plant were dried, powdered (100 g) and macerated with a 90% ethanol

solution for 3 days with three changes of the solution. The resulting extract was filtered and evaporated under vacuum into a dried powder.

Phytochemical, anti-oxidant and total flavonoid assay

We analyzed the chemical components of extract by thin layer chromatography (TLC). In addition, the antioxidant capacity of the plant extract by the DPPH (2, 2-diphenylpicrylhydrazyl) test and also total flavonoid content were estimated by aluminum chloride colorimetric assay as described previously.³

Cell culture and cell viability assay with MTT test

The MCF-7 human breast cancer cell line and normal human fibroblast cells (L929) were prepared from the National Cell Bank of Iran (NCBI) and maintained by culturing in RPMI 1640 medium (Sigma, St Louis, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco, USA). The cell viability was assayed by MTT (3-(4, 5-dimethylthiazoyl)-2, 5-diphenyltetrazolium bromide) as previously described.³

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Cell apoptosis and cell cycle assay

Detection of apoptosis was evaluated with an Annexin V-FITC apoptosis Kit (Invitrogen, USA) according to the manufacturer's protocol. Moreover, the cell cycle distribution was measured by PI staining as previously described.³

DNA fragmentation analysis and measurement of caspase activity

To confirm breast cancer apoptosis, we evaluated the fragmented DNA from MCF-7 cells by gel electrophoresis as previously described.³ In addition, caspase-3 and caspase-9 activity were assessed according to the manufacturer's instruction of the caspase colorimetric assay kit (R&D systems).

Statistical analysis

Data represented as mean±standard deviation. Statistical analyses were carried out by one-way analysis of variance (ANOVA) and a post-hoc Bonferroni's test to express the difference among the groups. All analyses performed using SPSS software16. Data considered statistically significant at $P<0.05$.

Results and Discussion

Antioxidant activity and total phenolic compounds

The results showed that the extract had the strong antioxidant and free radical scavenging capacities. These results are shown in Tables 1 and 2.

Table 1. Antioxidant capacity and total phenolic compounds of *S. variagata* extract

Total flavonoids, (mg RE/1g de)	DPPH radical scavenging activity, IC50% (mg/l)	Ascorbic acid equivalent of the extract antioxidant capacity (mg/g)
51.93±4.43	299.22±0.03	31.5

Table 2. Phytochemical results of *Scrophularia variagata* extract

Compounds	Reagents	Standards	Results
Phenylpropanoids and Terpenoids	Vanillin sulfuric acid	Cinamic acid	+
Phenolic compounds	Ferric chloride	Nepitrin	+
Flavonoides	Natural product reagent	Quercetin	+

Cytotoxicity effect of *S. variagata* extract on MCF-7 tumor cell

In this study normal human fibroblast cells (L929) were used as normal cells compared with MCF-7 human breast cancer cell. In a preliminary experiment on L929, the results indicated that extract up to 200 µg/ml did not any significant toxicity for 48 h (Data not shown). On the other hand, a successful antitumor drug should kill cancer cells without causing excessive side effects to normal cells that this ideal situation is achievable by apoptosis induction in cancer cells. As shown Figure 1, the extract significantly ($p<0.05$) inhibited MCF-7 cell

growth in dose and time dependent manner. So, the results indicate that the extract can induce a cytotoxicity effect in MCF-7 human breast cancer cell line.

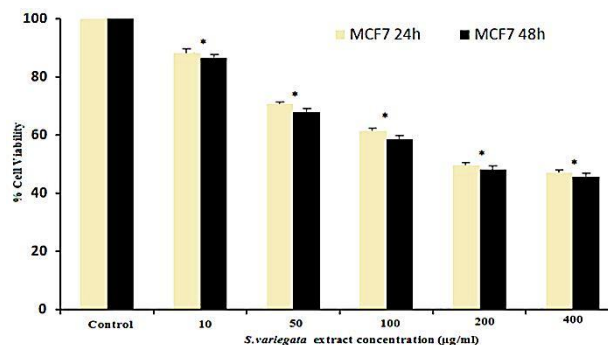


Figure 1. Cytotoxicity effect of *S. variagata* on MCF-7 tumor cell line.

The results showed that the extract significantly ($*P<0.05$) inhibited MCF-7 cell growth in dose and time dependent manner compared with non-treated (control group) after 48 h. The results shown are representative of three independent experiments.

Effect of *S. variagata* extract in MCF-7 apoptosis and cell cycle arrest

Apoptosis is a normal physiologic process that plays an important role in homeostasis and growth of the normal and cancer cells also dysregulation of apoptosis is usually considered as a major cancer property.^{11,12}

In the present study, to determine whether the toxicity effect of *S. variagata* extract on MCF-7 cells was associated to apoptotic cell death, the apoptosis induction were measured by annexin V-FITC/PI staining. As shown in Figure 2, our findings indicated that the amount of apoptotic cells increased with increasing concentration of *S. variagata* extract. These results indicated that the cytotoxic effects of *S. variagata* extract could be mediated by the induction of apoptosis via mitochondrial intrinsic pathway in MCF-7 cells. Moreover, some studies showed that other genus of *scrophularia* such as *S. floribunda* and *S. striata* extract induced apoptosis in tumor cells through induction of cell cycle arrest.^{3,13} Our findings demonstrated that *S. variagata* extract can induce a G2/M phase cell cycle arrest in MCF-7 cells in a dose-dependent manner.

Effects of *S. variagata* extract on caspases induction and DNA fragmentation

To confirm the effects of extract on the induction of apoptosis in the breast cancer cell line, the extract was examined for the appearance of DNA ladder and induction of caspases in treated cells. Our results demonstrated that increasing of the caspase-3 and caspase-9 activity and internucleosomal DNA fragmentation was dose dependently apparent in the cells indicating that the extract can cause apoptosis in the MCF-7 cells (Figure 3 A, B). Therefore, the findings in this study showed that the *Scrophularia variagata* could be as an anticancer agent against breast cancer by cell growth inhibition and apoptosis induction.

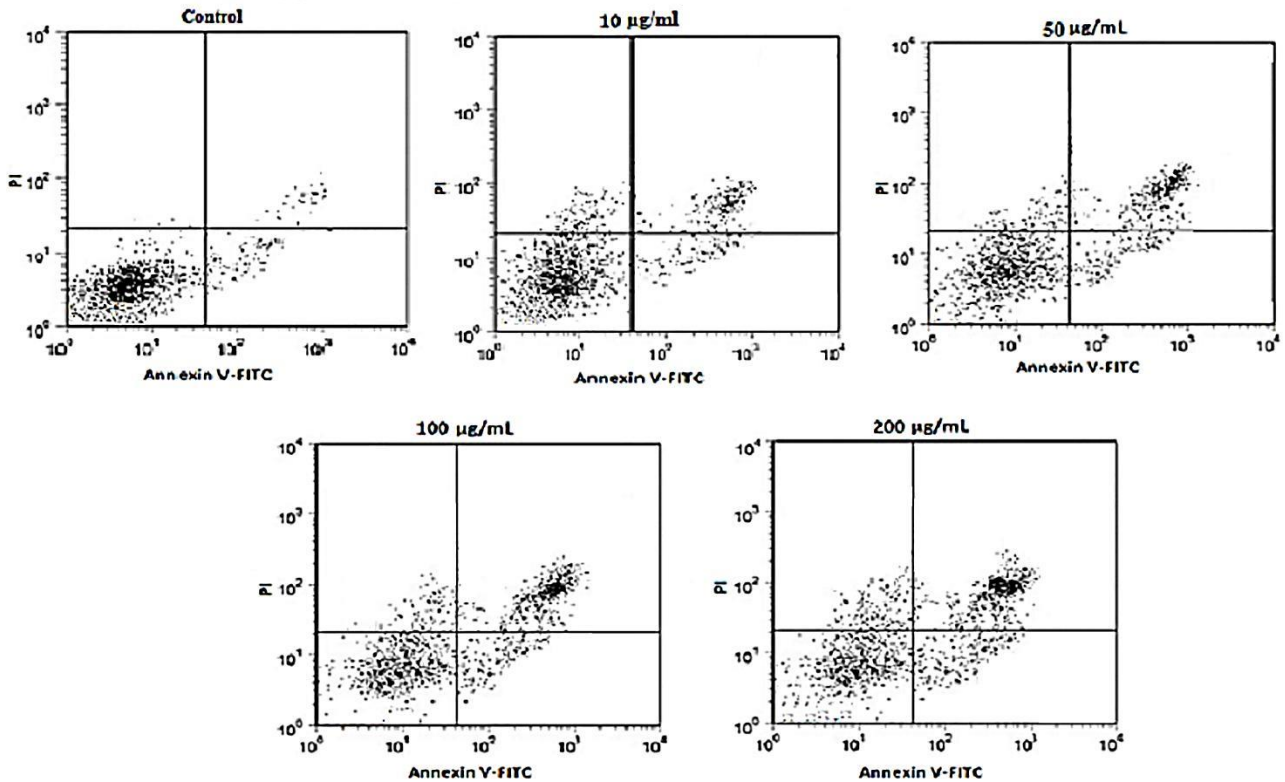


Figure 2. Effect of *S. variegata* extract on the inducing of apoptosis and cell cycle arrest. Apoptosis inducing effect of *S. variegata* extract on MCF-7 human breast cancer cell line evaluated by Annexin V-FITC (AV)/PI method. The results shown are representative of three independent experiments.

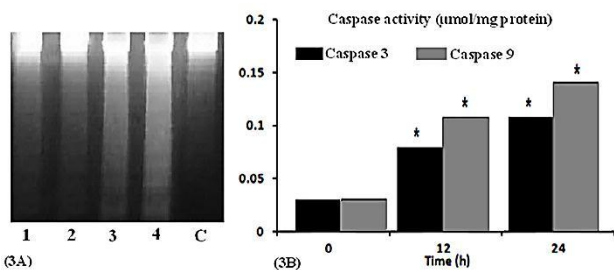


Figure 3. DNA fragmentation in MCF-7 tumor cell and caspases activation.

(A) DNA fragmentation in MCF-7 breast cancer cells after treatment with 0-200 µg/ml of the *S. variegata* extract 1; 10µg/ml, 2; 50µg/ml, 3; 100µg/ml, 4; 200µg/ml, C; negative control. DNA laddering typical for apoptotic cells is visible for cells treated with the *S. variegata* extract.

(B) The activity of caspase-3 and caspase-9 significantly (* $P < 0.05$) is increased in time-dependent manner in MCF-7 cells after the treatment with extract.

Conclusion

Our in vitro study demonstrated that *S. variegata* has cytotoxic activity on MCF-7 breast cancer cell line. The ability of it medicinal plant to induce apoptosis through G2/M phase cell cycle arrest and an increase in caspases activity on human breast cancer cell line candidate it for further studies as a potential natural anti-cancer agent.

Acknowledgments

This study was supported by Iran National Science Foundation. We also thank of deputy for Research,

Qazvin University of Medical Sciences, Qazvin, Iran and, Institute of Medicinal Plants (IMP), ACECR, Iran.

Ethical Issues

Not applicable.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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