Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2016; 8(2); 63-69

ISSN: 0975-5160

Research Article

Increase in Bax/Bcl-2 Ratio Induced by Salvigenin Through Mitochondria in Sw948 and HT-29 Human Colon Cancer Cell Line

Sarvestani N N, Sepehri H*

Department of Animal Biology, School of Biology, College of Science, University of Tehran, Tehran, Iran

Available online:1st May, 2016

ABSTRACT

Constituting an untold diversity of chemical structures makes natural products a valuable source of lead compounds in drug discovery. Multiple pharmacological studies, have led to the observations that polyphenolic antioxidants, such as flavonoids, acquire great therapeutic potential in battle against cancer. This study reports the anticancer effect of salvigenin against HT-29 and SW948 human colon cancer cell line through induction of apoptosis with no significant effect on normal HFFF-2 cell line. This natural polyphenolic compound, salvigenin, is an active ingredient of *Salvia lachnocalyx and Salvia hydrangea*. In this study, it has been shown the ability of salvigenin in induction of Bax/Bcl-2 ratio which lead to initiating apoptosis on human colon cancer cell line. Moreover, we have screened the increase in mitochondrial membrane potential on colon cancer cells treated by salvigenin. Bcl-2 family proteins and mitochondrial dysfunction are probably key regulators of the apoptotic response. Hence, salvigenin trigger one of the important pathways of anticancer feature of natural product. Dual effects of flavonoids, as an antioxidant or pro-oxidant, gives them this capacity to induce cell death from different way. Here it had shown that salvigenin increase ROS production in colon cancer cell line to accelerate cell death.

Keywords: Salvigenin, Apoptosis, Mitochonria, Colon cancer

INTRODUCTION

Salvia species have long history of medicinal and culinary use. Having been the subject of numerous studies to elucidate their biological properties, they have shown to exert multiple effects such as anticarcinogenic, neuroprotective, anti-inflammatory and analgesic Properties[1,2]. Salvigenin, a subtype of Salvia, is a naturally polyphenolic compound extracted from Salvia lachnocalyx and Salvia hydrangea and is present naturally in widely consumed vegetables. This compound has been shown to have cytotoxic effects on human cancer cells like breast adenocarcinoma (MCF-7), colon adenocarcinoma (HT-29) and glioblastoma (SF-268) and a human kidney epithelial cell line[2,3]. One proposed hypothesis involves in anticancer effect of salvigenin is due to its apoptotic stimulation in cancer cells. Two distinct apoptosis way has been determined and categorized as below: intrinsic or mitochondrial and extrinsic or death receptor ligands pathways. While, in the mitochondrial apoptotic pathway, Bcl-2 protein family members regulate initiation of the apoptotic signal, the ratio of Bax/Bcl-2 becomes important factor in apoptotic induction[4,5]. The Bcl-2 family that regulates cell survival is one of the outer mitochondrial membrane proteins. Hence, changes in mitochondrial membrane potential may directly effect on Bcl-2 family protein and regulate intrinsic apoptotic pathway. Bcl-2 family member play its important role by forming channel in mitochondrial outer membrane and lead to release other apoptotic molecules through mitochondria. Thus, it is important to determine whether Bcl-2 family protein involve in flavonoid induce apoptosis by regulating the permeability of the outer mitochondrial membrane[4,5]. On the other hand, the well-known feature of flavonoid is their antioxidant ability due to their aromatic ring with a reactive hydroxyl group. Interestingly, regarding to cell types they can also be as pro-oxidant, increase ROS production and finally reduce cell growth. This property is effective in tumor growth inhibition when cancer cells treated with flavonoid[6].

It was previously documented that eupatorin as a natural flavonoids had an anticancer effect on colon cancer cell line[7]. Subsequently, in this study, we investigated whether salvigenin induce apoptosis through mitochondria by changes in mitochondrial membrane permeability and enhances Bax/Bcl-2 ratio. Moreover, the impact of

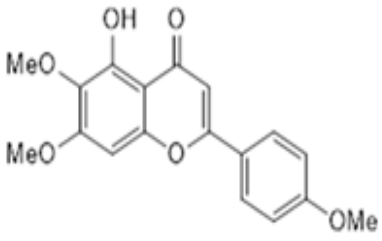

Figure 1: Chemical structure of salvigenin

*Author for Correspondence

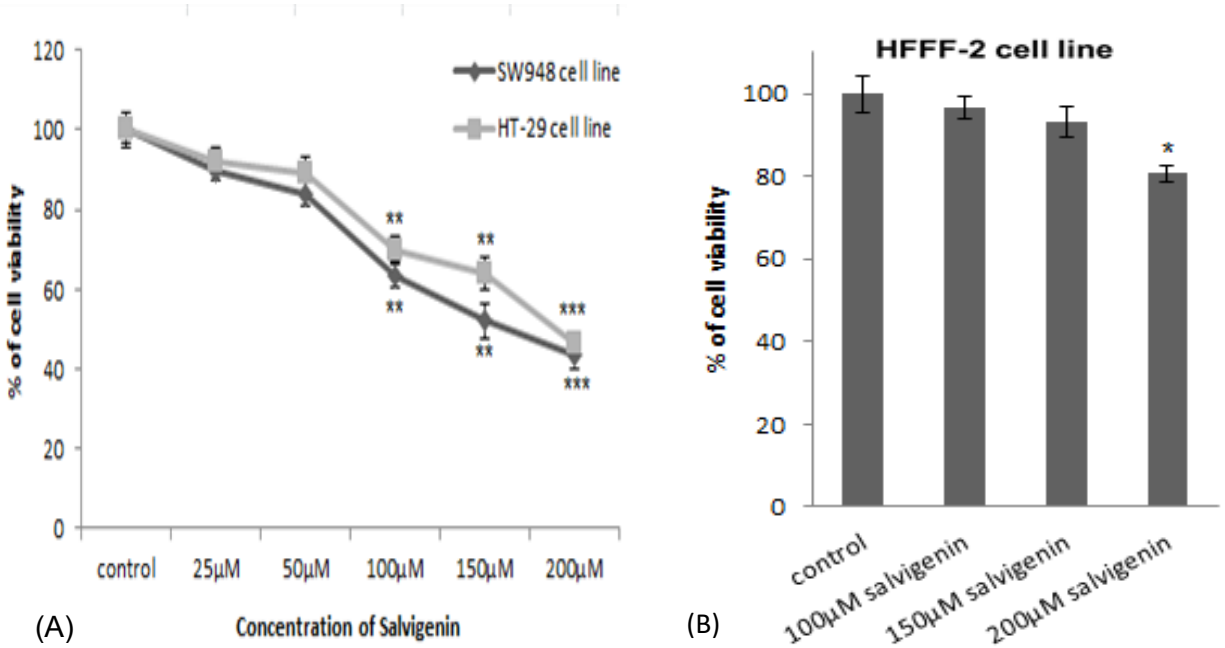

Figure 2: Inhibitory effect of salvigenin on cell viability in HT-29, SW948 and HFFF-2 cell lines. A) Viability of HT-29 and SW948 cells 24 h after exposure to increasing doses of salvigenin (25, 50, 100, 150 and 200 µM). B) Viability of HFFF-2 normal fibroblastic cell line 24 h after exposure to effective doses of salvigenin (100, 150 and 200 µM) as indicated. *Significantly different from control cells. (*P<0.05).

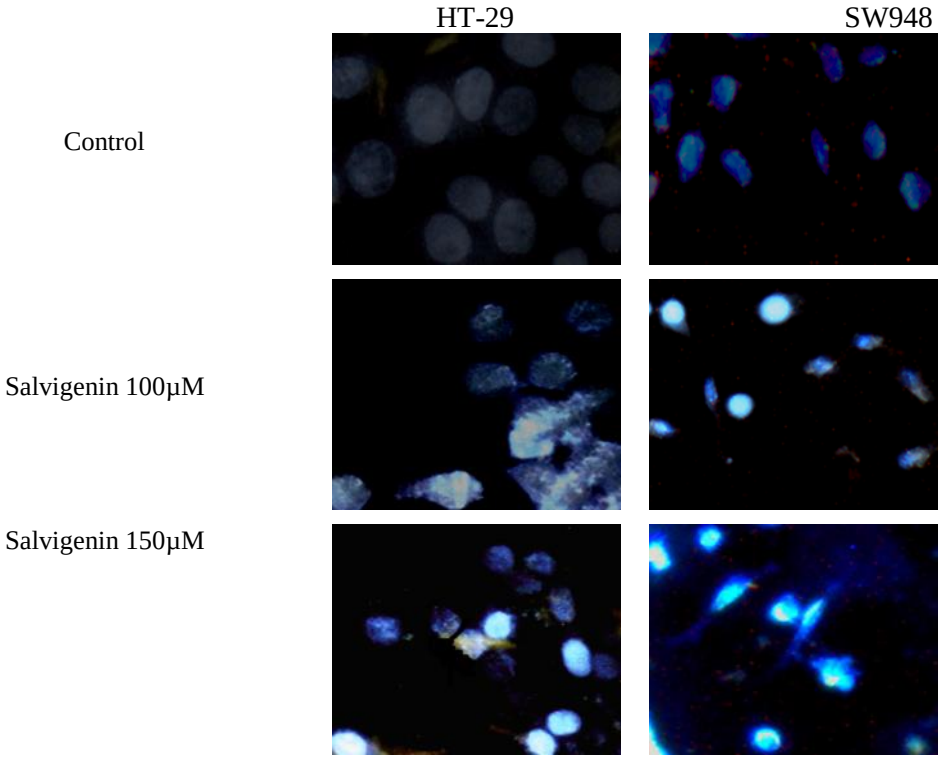

Figure 3: DAPI staining. The cells were exposed 100 and 150 µM eupatorin for 24 hour. The cells were harvested, resuspended in PBS, and incubated with DAPI. The morphological patterns of apoptotic cells are described in the text. All experiments were repeated three times.

salvigenin on ROS production in human colon cancer cell line examined.

MATERIAL AND METHOD

Material

MTT (3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide) and DAPI stain purchase from Sigma Aldrich (MO, USA). Antibody against Bax,

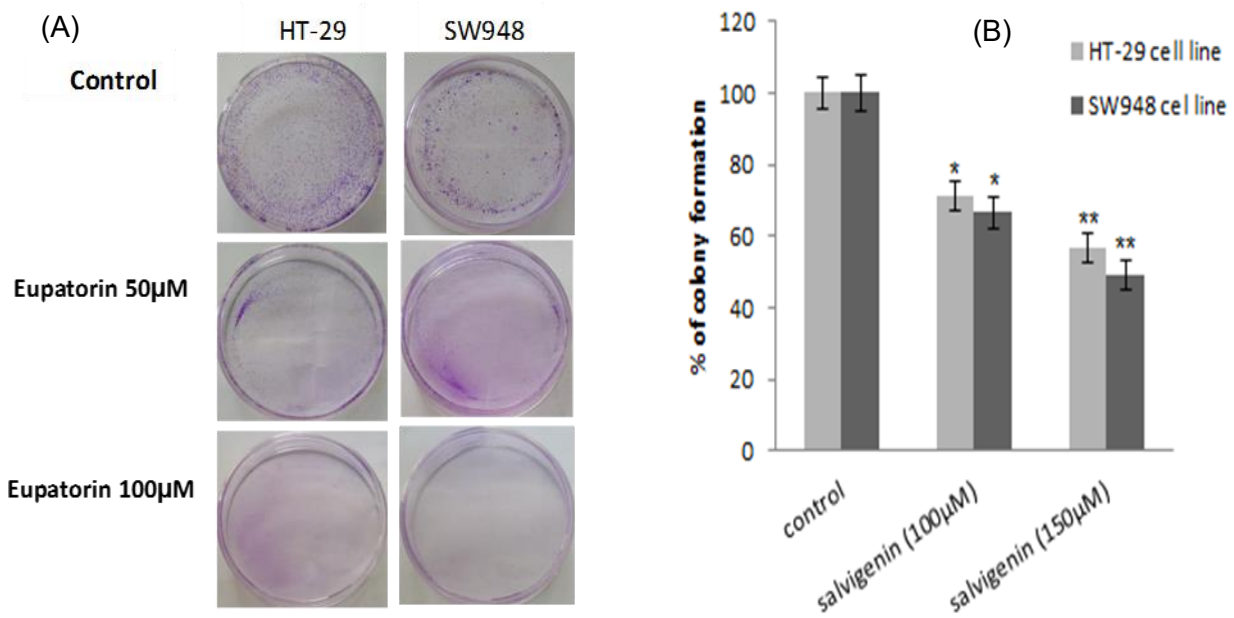

Figure 4: Eupatorin repressed clonal formation of HT-29 and SW948 cells. (A) Morphological analysis and B) Quantities analysis of inhibitory effect of 100 and 150 µM eupatorin for 24 hour. About 1 × 103 HT-29 and SW948 cells were seeded on to the 100 mm plates after the indicated treatment and changed for fresh medium every 3 days. *Significantly different from control cells. ((*P<0.05 and **P<0.01).

Bcl-2, and β-actin were obtained from Cell Signaling Technology. An Electrochemiluminescence (ECL) reagent was bought from (Amersham Bioscience, USA). Polyvinylidene fluoride (PVDF) was from millipore (Billerica, MA). Rhodamin culture medium, penicillin–streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Gibco, Grand Island, NY, USA).

Cell culture condition

Pasteur Institute (Tehran, Iran) was provided HT-29, SW948 and HFFF-2 cells. These cells grown in RPMI medium (Sigma, Aldrich) and supplemented with 10% horse serum, 5% fetal bovine serum, and 1% antibiotic mixture comprising penicillin–streptomycin, in a humidified atmosphere at 37 °C with 5% CO2. Growth medium was changed three times a week and when they reach 70-80% confluence, subculture again.

Measurement of cell viability

Cell viability was determined by the conventional MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reduction assay. The dark blue formazan crystals formed in intact cells were solubilized in dimethyl sulphoxide and the absorbance was measured at 630 nm. Results were expressed as the percentages of reduced MTT, assuming the absorbance of control cells as 100%.

Treatment condition

Salvigenin were prepared as a stock solution in dimethyl sulfoxide (DMSO). Vehicle final concentration of in the medium was always 0.5%. Salvigenin (25- 200 µgr/ml), were added to HT-29 and SW948 cell cultures medium for 24 hour. According to MTT result, salvigenin at 100 and 150 µg/ml that are significantly reduce cell viability, were added to HT-29 and SW948 human colon cancer cell line for 24 hours for further study.

DAPI staining

Apoptosis was determined morphologically after staining the cells with DAPI followed by fluorescence microscopy inspection. Briefly, HT-29 and SW948 cells were seeded in a 6-well plate and were treated with different concentrations (100 and 150 µg/ml) of salvigenin. After 24 h, the cells were harvested and washed three times with phosphate buffered saline (PBS) and were adjusted to a density of 1×10^6 cells/ml of PBS. DAPI solution (1:1, v/v) was added to the cell suspension in a final concentration of 100 µg/ml. Cellular morphology was evaluated by fluorescence microscope (Zeiss, Germany).

Colony formation

HT-29 and SW948 cell lines were seeded in a 6 well plate at $1x10^3$cells/plate. After pre-incubation time cells were treatment added as describe previously. The cells medium were refresh every three days until 10 days. At least 50 cells mentioned as a colony cluster. Finally, cells were fixed by paraformaldehyde for 20 minute and then staining with crystal violet[8].

Measurement of the mitochondrial membrane potential (MMP)

Rhodamin 123 (Rh123) fluorescent dye used to estimate MMP, as described previously[9]. After treatment, HT-29 and SW948 cells were incubated for 30 min at $37^{\circ C}$ with PBS containing 5 µM Rh123. After washing with PBS, cells were trypsinized at room temperature and resuspended in PBS. The fluorescence intensity was measured by the Varian Cary Eclipse spectrofluorometer with excitation and emission wavelengths of485 and 530 nm, respectively.

Measurement of Intracellular ROS

20, 70-dichlorofluorescein diacetate (DCF-DA) is the fluorescent probe that was used to measure ROS accumulation in cell. After cell treated with salvigenin

Table 1: MMP (rhodamin123 fluorescence) levels in HT-29 and SW948 cells treated with 100 and 150 µM of salvigenin in 528 nm wavelength were measured. The mean of three independent experiments is shown. *significantly different from control. (*P<0.05, **P<0.01)

Treatment	MMP (rhodamin123 fluorescence at 528 nm) HT-29 cell line	MMP (rhodamin123 fluorescence at 528 nm) SW948 cell line
Control	428.54 ±3.73	514.32±4.17
Salvigenin 100µM	589.34±4.46*	718.91±3.23*
Salvigenin 150µM	711.37±3.25**	925.77±2.86**

Table 2: ROS levels in HT-29 and SW948 cells treated with 100 and 150 µM of salvigenin in 528 nm wavelength were measured. The mean of three independent experiments is shown. *significantly different from control. (*P<0.05, **P<0.01)

Treatment	ROS (absorbance at 528 nm) HT-29 cell line	ROS (absorbance at 528 nm) SW948 cell line
Control	458.29±2.85	209.25±2.87
Salvigenin 100 µM	589.19±3.14*	367.19±3.64*
Salvigenin 150µM	769.99 ±3.44**	500.1 ±3.71**

(100 and 150 µM), the DCFH-DA solution (10µM) was added to the cell suspension ($1x10^6$/ ml). The mixture was incubated at 37^0C for 1h and then washed twice with PBS. The fluorescence intensity was measured by Varian Cary Eclipse spectrofluorometer with excitation and emission wavelengths of 485 and 530 nm, respectively[10].

Western blotting

After indicated treatment, cells were harvested and the concentration of protein defines by Bradford's method[11]. Specific amount of protein boiled for 5 min. SDS-PAGE separated proteins and then transferred onto a PVDF membrane. Followed by PVDF membrane blocking (5% non-fat dry milk in Tris-Buffered-Saline with Tween (TBST)) for 1 h at room temperature, membrane incubated with primary antibodies overnight at 4 °C. After washed with TBST, the membrane was then incubated with appropriate secondary antibody for 1 h at room temperature. After three times washing with TBST, the Electrochemilu-minescence (ECL) reagent measures the chemiluminescence intensity. The result analysis by measuring integrated density with Image J.

Statistical analyses

Each experiment was performed at least three times, and the results were presented as mean ±S.E.M. One-way analysis of variance (ANOVA) followed by Turkey's test was used to compare the differences between means. A probability value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Cell viability decrease in presence of salvigenin treatment in HT-29 and SW948 human colon cancer cell lines while have no effect on HFFF-2 normal cells

Results indicated that salvigenin reduce cell viability significantly in two types of colon cancer, HT-29 and SW948, at 100 and 150µM. It was shown that HT-29 cells are a little restricted to cell death compare to SW948 and their cell viability decrease about 30% and 32 % while in SW948 the cell viability decrease about 36.6% and 47.7 % at concentration 100 and 150µM of salvigenin, respectively (fig. 2A). Interestingly, these treatments have no effect on normal HFFF-2 cell lines (fig. 2B).

Morphological evaluation of apoptosis

DAPI stain use to label nuclear DNA of cells grown in culture. Salvigenin at 100 and 150µM increase fluorescence intensity in HT-29 and SW948 cell lines as shown in Fig. 3.

Colony formation decrease in presence of salvigenin treatment

Colony numbers significantly decrease in HT-29 and SW948 cell lines treated by 100 and 150µM of salvigenin. At the most effective dose (150 µM/ml) it was reduced to 56.65% in HT-29 and 48.89 % in SW948 cell line, respectively (Fig. 4A and B).

MMP decrease in colon cancer treated by salvigenin

Salvigenin decrease MMP level and increase in Rhd 123 intensity. It has been shown in Table. 1 that rhodamin intensity in presence of the most significant dose of salvigenin (150 µM) in HT-29 and SW948 cell line increase about 1.66 and 1.80 fold respectively.

ROS increase in colon cancer cells treated with salvigenin

To evaluate pro-oxidant activity of salvigenin in cancer cell lines ROS production measured in presence of 100 and 150µM of salvigenin treatment for 24h. Treatment of 150µM salvigenin in HT-29 and SW948 human colon cancer cells lead to increase ROS about 1.68 and 2.39 fold respectively as show in Table. 2.

Bax/Bcl-2 protein ratio increase in colon cancer cells treated by salvigenin

To determine whether important protein in mitochondrial apoptosis pathway like Bax/ Bcl-2 level increase in HT-29 and SW948 cells expose to salvigenin, western blot technique done. It was demonstrated that salvigenin increase Bax/Bcl-2 ratio in presence of 150µM salvigenin about 2.75 fold in HT-29 and about 1.69 fold in SW948 colon cancer cell line as shown in Figure 5.

DISCUSSION

Flavonoids are polyphenolic compounds that exist in many dietary plants. In recent years, there have been many studies which, documented biological properties of

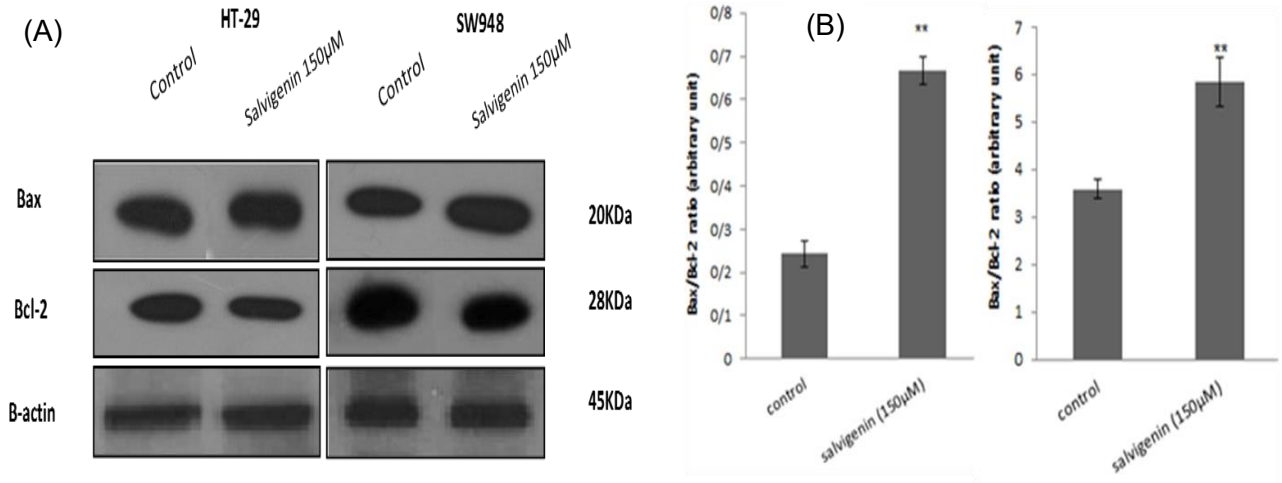

Figure 5: Bax and BCL-2 levels in HT-29 and SW948 cells treated with salvigenin. A) HT-29 and SW948 cells were treated with salvigenin (150m μM) for 24 h. Twenty μg proteins were separated on SDS-PAGE, western blotted, probed with anti-Bax and anti-Bcl-2 antibodies and reprobed with anti-β-actin antibody (One representative western blot was shown; n=3). The densities of Bax/Bcl-2 bands were measured and the ratio were calculated. The median of three independent experiments is shown. *Significantly different from control cells. (**P<0.01).

flavonoids in cancer prevention[12]. In particular, direct exposure of these compounds to intestinal epithelia makes them more dominantly the subject of research on the GI tract[13]. In a recent in vivo study, salvigenin has shown to be a tumor suppressor and possess immunomodulatory effect[1]. Taking this further, differently this study was designed to evaluate in vitro apoptosis activity through changes in mitochondria in human colon cancer cell lines. The cytotoxic activity of salvigenin determined in this study on HT-29 and SW948 human colon cancer certify its cytotoxic activity, which was determined on other cancer cell lines such as breast adenocarcinoma (MCF-7), glioblastoma (SF-268) and a human kidney epithelial cell line. In this study, normal HFFF-2 cells were also found to significantly less sensitive to salvigenin than HT-29 and SW948 human colon cancer cell line.

In order to elucidate the mechanisms of apoptosis of colon cancer cells induce by salvigenin, the effect of this natural flavonoids on Bcl-2 protein family and mitochondrial membrane potential was then investigated. Bcl-2 being the underpinning of cell death, is the rational for the focus of this study. Programmed cell death can be divided into several categories such as apoptosis and autophagic death[14]. The Bcl-2 family of proteins not only regulates apoptosis, but also controls non-apoptotic programmed cell death that depends on the autophagy genes. At a mitochondrial levels, the BCL-2 family members which play a central role in determining whether a cell die or will live, reside upstream of irreversible cellular damage[15, 16]. The result by this study showed that treatment by salvigenin change the level of anti-apoptotic Bcl-2 and proapoptotic Bax protein. The Bax/Bcl-2 ratio increase in presence of salvigenin treatment that means dimerization of Bax monomers happen in mitochondria and the expression of Bax inhibitor, which is Bcl-2, decreased. Hence, apoptosis initiated at first steps. Bcl-2 protein is responsible for blocking early apoptotic events such as morphological changes in nuclear DNA in cells[17]. As shown by DAPI staining in results, treatment by salvigenin in HT-29 and SW948 cells increase morphological nucleic feature of apoptosis that could be blocked by Bcl-2 expression in live cells. Bcl-2, a protein of Bcl-2 family associated with involvement in apoptosis, is shown to exert an effect on mitochondrial membrane potential. It has been established that pro-apoptotic Bcl-2 family member such as Bax, induce apoptotic mitochondrial membrane potential loss in isolated mitochondria and Bcl-2 blockage leading to a decline in mitochondrial membrane potential[18,19]. Additionally, it is the subject of discussion that loss in mitochondrial membrane potential can either be an early event in apoptosis or be a consequence event in cell death[20]. Suggested by previous investigations, depending on the cell type and apoptotic stimuli, mitochondrial dysfunction might be one of the starter events in apoptosis[21]. In this study, it was demonstrated that following salvigenin treatment on two different colon cancer cell lines, Bax/Bcl-2 ratio increase in favor to apoptosis. Subsequently, this will lead to a decrease in MMP and release of Rd123, which results an increase in fluorescence absorbance in apoptotic cells. The onset of the MMP loss is followed by a collapse of MMP, which is followed by Bax association with mitochondria[22,23]. Hence its suggested that in HT-29 and SW948 human colon cancer cell lines the loss in mitochondrial membrane potential is a trait of apoptosis. Mitochondria is one of the major sites of ROS generation, as well as the location where the localization of Bcl-2. Many of the effects of ROS including DNA strand breaks and membrane blebbing are part of apoptosis hallmark, which can be reversed by Bcl-2[24,25]. Here it has been demonstrated that not only the anti-apoptotic capacity of Bcl-2 is attenuated in presence of salvigenin treatment, but also, ROS production is not inhibited by Bcl-2 in colon cancer cell line. From other prospective, flavonoids can be either antioxidant or pro-oxidant[26,27]which are the features driven from their structures[28]. It was previously determined that

some flavonoids and polyphenols are chemically antioxidants. It means they can scavenge free radical species[29]. In contrast, there is evidence that some of the apoptotic effects of these compounds on cancer cells may be related to ROS production or pro-oxidant activity[24]. Another study has suggested that reactive oxygen species have an impact on the expression of Bcl-2 family proteins through regulating their phosphorylation and ubiquitination[30,31]. In this study we ducumented that colon cancer cell line which are treated by salvigenin, represent a higher amount of ROS in method using DCF-DA as a fluorescent probe. This could be an indication of salvigenin role in apoptosis progression could be due to ROS accumulation. The present study is a novel to show the efficacy of treatment of natural product, salvigenin, which increase the Bax/Bcl-2 ratio through mitochondrial apoptosis pathway in HT-29 and SW948 human colon cancer cell lines.

Conflict of interest statement

The authors have no conflict of interest.

ACKNOWLEDGEMENT

This study was support by a research grant from the Colleges of Science of University of Tehran. Kind thanks to Dr. Mahdi Moridi Farimani because of giving salvigenin to us.

REFERENCES

1. Noori S, Hassan ZM, Yaghmaei B, Dolatkhah M. Antitumor and immunomodulatory effects of salvigenin on tumor bearing mice. Cellular immunology 2013; 286(1):1621.
2. Mansourabadi AH, Sadeghi HM, Razavi N, Rezvani E. Anti-inflammatory and Analgesic Properties of Salvigenin, Salvia officinalis Flavonoid Extracted. Advanced Herbal Medicine 2015; 1(3):31-41.
3. Kamatou GP, Van Zyl RL, Davids H, Van Heerden FR, Lourens AC, Viljoen AM. Antimalarial and anticancer activities of selected South African Salvia species and isolated compounds from S. radula. South African Journal of Botany 2008;74(2):238-43.
4. Brunelle JK, Letai A. Control of mitochondrial apoptosis by the Bcl-2 family. Journal of cell science 2009; 122(4):437-41.
5. Joza N, Susin SA, Daugas E, Stanford WL, Cho SK, Li CY, Sasaki T, Elia AJ, Cheng HY, Ravagnan L, Ferri KF. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 2001;410(6828):549-54.
6. Rice-evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free radical research. 1995; 22(4):375-83.
7. Sarvestani N N, Sepehri H, Farimani M M. Anticancer Effect of Eupatorin via Bax/Bcl-2 and Mitochondrial Membrane Potential Changes through ROS Mediated Pathway in Human Colon Cancer. International journal of phamacognosy and phytochemical research. 2015; 7(6); 1039-1046.
8. Franken N, Rodermond H, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nat Protoc. 2006;1(5):2315-2319.
9. Poppe M.C, Reimertz H, Dussmann AJ, et al., 2001. Dissipation of potassiumand proton gradient inhibits mitochondrial hyperpolarization and cytochrome C release during neural apoptosis. Journal of Neuroscience 21; 4551–4563.
10. Degli Esposti M. Measuring mitochondrial reactive oxygen species. Methods. 2002 Apr 2;26(4):335-40.
11. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72 :248-254.
12. Middleton E, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacological reviews 2000 ;52(4):673-751.
13. Pierini R, Gee JM, Belshaw NJ, Johnson IT. Flavonoids and intestinal cancers. British Journal of Nutrition 2008 ;99(E-S1):ES53-9.
14. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nature cell biology 2004; 6(12):1221-8.
15. Elmore S. Apoptosis: a review of programmed cell death. Toxicologic pathology. 2007 Jun 1;35(4):495-516.
16. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004 Jan 23;116(2):205-19.
17. Liu XH, Yao S, Kirschenbaum A, Levine AC. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. Cancer research. 1998 Oct 1;58(19):4245-9.
18. Kroemer G. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nature medicine. 1997 Jun 1;3(6):614-20.
19. Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, Newmeyer DD. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Molecular cell. 2005 Feb 18;17(4):525-35.
20. Ly JD, Grubb DR, Lawen A. The mitochondrial membrane potential ($\Delta\psi m$) in apoptosis; an update. Apoptosis. 2003 Mar 1;8(2):115-28.
21. Green DR, Reed JC. Mitochondria and apoptosis. Science. 1998 Aug 28;281(5381):1309.
22. Zamzami N, Kroemer G. Apoptosis: mitochondrial membrane permeabilization–the (w) hole story?. Current Biology. 2003 Jan 21;13(2):R71-3.
23. Smaili SS, Hsu YT, Sanders KM, Russell JA, Youle RJ. Bax translocation to mitochondria subsequent to a rapid loss of mitochondrial membrane potential. Cell death and differentiation. 2001 Sep 1;8(9):909-20.
24. Li D, Ueta E, Kimura T, Yamamoto T, Osaki T. Reactive oxygen species (ROS) control the expression of Bcl-2 family proteins by regulating their

phosphorylation and ubiquitination. Cancer science. 2004 Aug 1;95(8):644-50.
25. Kuwana T, Newmeyer DD. Bcl-2-family proteins and the role of mitochondria in apoptosis. Current opinion in cell biology. 2003 Dec 31;15(6):691-9.
26. Procházková D, Boušová I, Wilhelmová N. Antioxidant and prooxidant properties of flavonoids. Fitoterapia. 2011 Jun 30;82(4):513-23.
27. Fukumoto LR, Mazza G. Assessing antioxidant and prooxidant activities of phenolic compounds. Journal of agricultural and food chemistry. 2000 Aug 21;48(8):3597-604.
28. Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. Free Radical Biology and Medicine. 1997 Dec 31;22(5):749-60.
29. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. The Journal of nutritional biochemistry. 2002 Oct 31;13(10):572-84.
30. Hildeman DA, Mitchell T, Aronow B, Wojciechowski S, Kappler J, Marrack P. Control of Bcl-2 expression by reactive oxygen species. Proceedings of the National Academy of Sciences. 2003 Dec 9;100(25):15035-40.
31. Jacobson MD. Reactive oxygen species and programmed cell death. Trends in biochemical sciences. 1996 Mar 31;21(3):83-6