Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2015; 7(6); 1039-1046

ISSN: 0975-4873

Research Article

Anticancer Effect of Eupatorin via Bax/Bcl-2 and Mitochondrial Membrane Potential Changes through ROS Mediated Pathway in Human Colon Cancer

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Available Online: 4th October, 2015

ABSTRACT

Oncology has been the most active therapeutic area within the past few decades. Given that natural product and natural product inspired compounds account for over half of anticancer agents, new templates for future drug design could be procured from natural sources. Eupatorin, a flavonoid with wide range of phytomedical activity, is an active ingredient of Salvia mirzayanii, which is isolated from a native plant of Iran. In this study, we investigated the apoptosis activity of this compound on HT-29 and SW948 human colon cancer cell lines. The compound showed ability to decrease the cell viability with micromolar range of IC50 without significant decrease in viability of HFFF-2 normal cell line. Moreover, it was observed that eupatorin induce apoptosis via mitochondrial pathway by decreasing mitochondrial membrane potential and increase in the ratio of Bax/Bcl-2 expression. Additionally, ROS level was measured in presence of eupatorin to determine how natural flavonoid act as a pro-oxidant and cause excessive amount of ROS involved in apoptosis .

Keyword: Eupatorin, Apoptosis, Colon Cancer, Mtochondria, Bax protein, Bcl-2 protein

INTRODUCTION

Colon cancer is the second most lethal and the third most spread type of cancer worldwide[1]. By estimation, 1 million incident cases occur every year, predominantly in western nations due to this particular type of cancer, which lends itself to prevention, screening and early detection. In recent years, a large number of studies have ascribed a protective effect to flavonoids and foods containing these compounds against colon cancer. In particular, flavonoids are plant secondary metabolites that are ubiquitous in fruits, vegetables, nuts, seeds, and plants[2,3].

Salvia mirzayanii, which has been grow in middle and south part of Iran, is from Labiatae family. It was reported that in traditional medicine it was used for diabetes, wound healing, headache and hyper-cholesterolemia. In addition, various biological activities were determined for this plant like, antioxidant and antibacterial[4-7]. In this study, we showed eupatorin is one of the effective constituents of *Salvia mirzayanii*. Researches attributed large variety of pharmacological feature to eupatorin as a flavone[8]. Eupatorin has been shown to have anti-inflammatory effects in a mouse edema model and also antiproliferative activity in human gastric adenocarcinoma (MK-1), human uterus carcinoma (HeLa), and murine melanoma (B16F10) cell lines[9,10].

Disabling of apoptosis, a natural physiologic process of programmed cell death, has been widely recognized as a hallmark of most types of cancer. When the colorectal epithelium alters to carcinoma, in particular, it correlated with a progressive inhibition of apoptosis[11]. Tumor growth, induction of neoplastic progression, and resistance to cytotoxic anticancer agents occurred following apoptosis deletion. For that reason, natural product compounds with the ability to induce apoptosis along with fewer side effects on normal cells can be consider as cancer chemopreventive and/or chemotherapeutic agents[12]. Two keys molecular signaling pathways lead to apoptotic cell death. One is the intrinsic or the mitochondria-mediated pathway activated from inside the cell by apoptotic stimuli. The other is extrinsic or death receptor-mediated pathways activated from outside the cell by the binding of death ligands to cell surface receptors. While mitochondria play key role in intrinsic apoptosis pathway, it can be involved in extrinsic pathway indirectly. Intrinsic pathway of apoptosis through mitochondria is tightly controlled by a variety of regulators, including the Bcl-2 family proteins[13]. Moreover, disruption in mitochondrial potential is one of the mechanisms during apoptosis that involved in

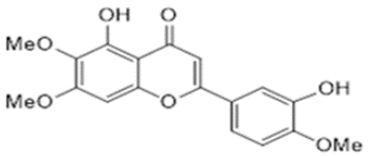

Figure 1: Chemical structure of eupatorin

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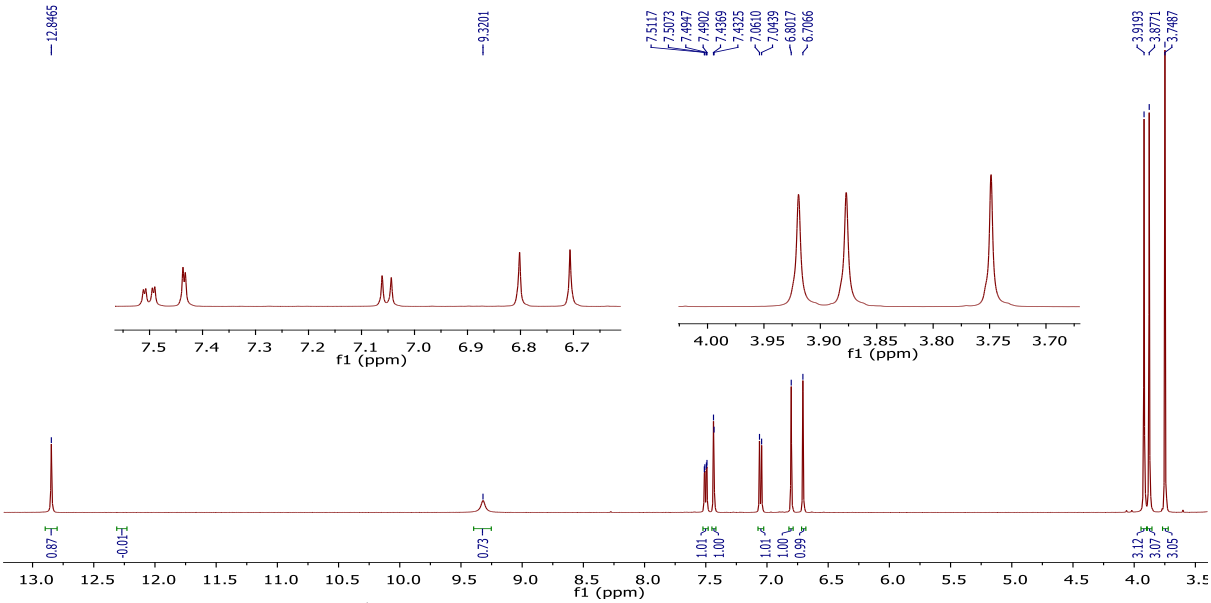


Figure 2: ^{1}H NMR spectrum of eupatorin (DMSO-d_6, 500 MHz).

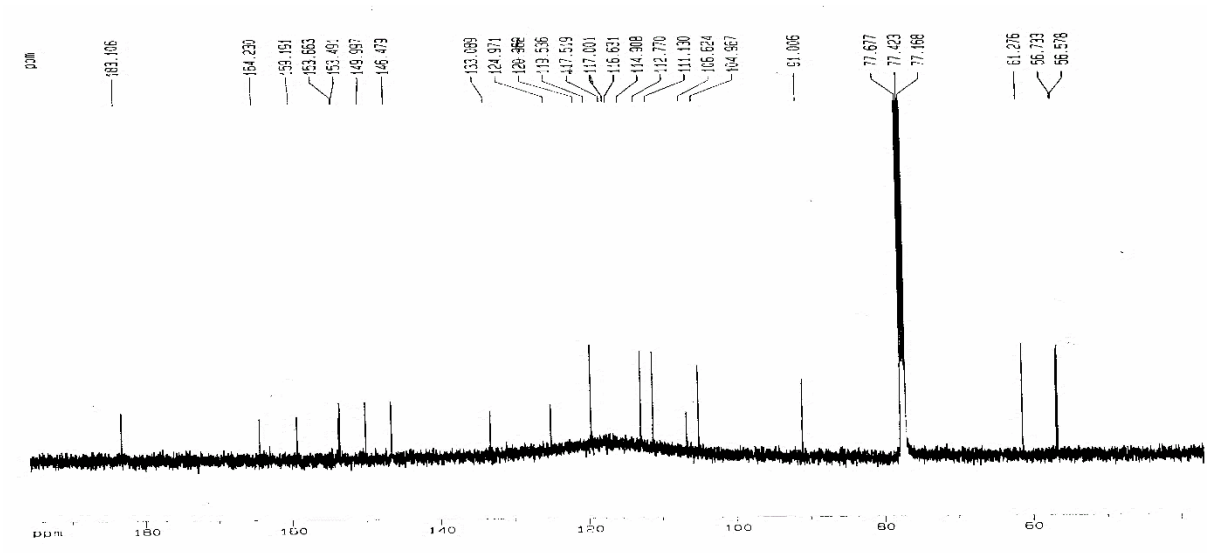


Figure 3: ^{13}C NMR spectrum of eupatorin ($CDCl_3$, 125 MHz

formation of channels by Bcl-2 family. Reactive oxygen species (ROS) generation through mitochondria is another important pathway involved in apoptosis especially in cancers. A moderate increase in ROS leads to cell proliferation and differentiation and potentiate cells for cancer[14,15].

The principal objective of this study was to determine whether eupatorin, as natural non-toxic product, regulate ROS amount and inhibits the growth of colon cancer cells and if this product induce apoptosis through mitochondria by changes in mitochondrial membrane and important apoptotic protein, Bax/Bcl-2 ratio.

MATERIAL AND METHOD

Materials

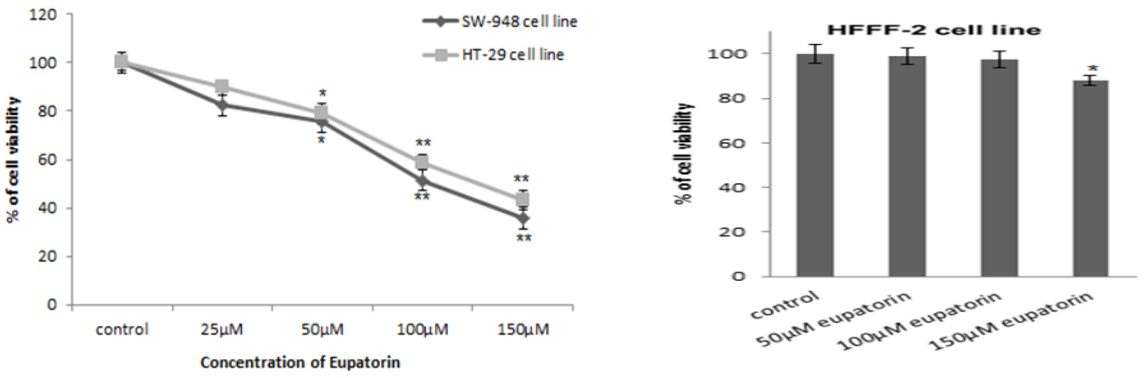
3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide (MTT) and DAPI stain purchase from Sigma Aldrich (MO, USA). Antibody against Bax, Bcl-2, and β-actin were obtained from Cell Signaling Technology. An Electrochemiluminescence (ECL) reagent was bought from (Amersham Bioscience, USA). Polyvinylidene 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).

Plant material

The aerial parts (leaves and flowers) of *Salvia mirzayanii*, were collected from different areas of Iran and identified as described previously[16,17,18].

Isolation and identification of eupatorin

The dried aerial parts of *Salvia mirzayanii* (4.5 kg) was ground and extracted by maceration with *n*-hexane (5×25 L), acetone (5×25 L), and MeOH (5×25 L). Evaporation of the acetone extract afforded 125 g of a dark gummy residue. The extract was separated on a silica gel column (230−400 mesh, 127.0×5.0 cm, 750 g) with a gradient of *n*-hexane−EtOAc (100:0 to 0:100) as eluent, followed by



A B

Figure 4: Inhibitory effect of eupatorin 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 eupatorin (25, 50, 100 and 150 µM). B) Viability of HFFF-2 normal fibroblastic cell line 24 h after exposure to effective doses of eupatorin (50, 100 and 150 µM) as indicated. *Significantly different from control cells. (*P<0.05, **P<0.01)

increasing concentrations of MeOH (up to 25%) in

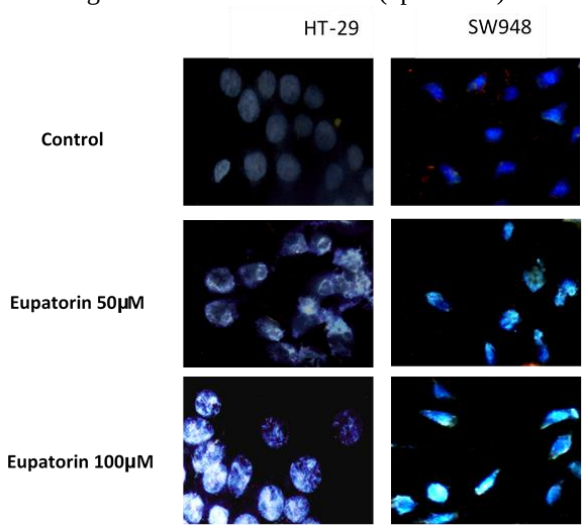


Figure 5: DAPI staining. The cells were exposed 50 and 100 µM eupatorin for 24 hour. The cells were harvested, resuspended in PBS, and incubated with DAPI. The morphological patterns of apoptotic cells are described in the text. All experiments were repeated three times.

EtOAc. Fractions of 250 mL were collected and pooled on the basis of TLC analysis. A total of 30 fractions were obtained. Fraction 16 [4.5 g, eluted with hexane-EtOAc (55:45)] was triturated with Me_2CO to separate an insoluble solid, which was recrystallized from Me_2CO to afford yellowish crystals (350 mg). ^1H- and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker DRX 500 spectrometer, using the residual solvents (DMSO-d_6 and $CDCl_3$) signals as references. These data (Figures 2 and 3) were used to identify the structure of the crystals as 5,3′-dihydroxy-6,7,4'-trimethoxyflavone (eupatorin)[19].

Eupatorin: ^1H NMR (DMSO-d_6) δ: 12.85 (1H, s, OH-5), 9.32 (1H,s, OH-3′), 7.50 (1H, dd, J= 8.5, 2.2 Hz, H-6′), 7.43 (1H, d, J= 2.2 Hz, H-2'), 7.05 (1H, d, J= 8.5 Hz, H-5'), 6.80 (1H, s, H-8), 6.71 (1H, s, H-3), 3.92 (3H, s, OMe-7), 3.88 (3H, s, OMe-4'), 3.75 (3H, s, OMe-6); ^{13}C NMR ($CDCl_3$) δ: 164.2 (C-2), 104.9 (C-3), 183.1 (C-4), 153.7 (C-5), 133.1 (C-6), 159.2 (C-7), 91.0 (C-8), 153.5 (C-9), 106.6 (C-10), 124.9 (C-1′), 112.7 (C-2′), 146.5 (C-3′), 149.9 (C-4′), 111.1 (C-5′), 119.5 (C-6′), 56.2 (OMe-4'), 56.9 (OMe-7), 60.3 (OMe-6).

Cell culture condition

HT-29, SW948 and HFFF-2 cells were purchase from Pasteur cell bank, Iran, Tehran. These cells were grown in RPMI medium with 10% heat inactivated FBS (Fetal Bovine Serum) and penicillin/streptomycin at 37^0 C in 5 %CO2 humified incubator. The medium was changed every 2–3 days and subcultured again when cells population density reached to 70–80% confluence. Cells were seeded at an appropriate density according to each experimental design.

MTT Assays of Cell Viability

Stock solutions of eupatorin were prepared in dimethyl sulfoxide (DMSO). The final concentration of the vehicle in the medium was always 0.1%. Eupatorin (25- 150 µgr/ml), were added to HT-29 and SW948 cell cultures medium for 24 hour. The viability of cells was determined by the MTT assay. Briefly, at appropriated time intervals, 100µL of a 5 mg/ml MTT solution was added to each well. After 3 h incubation, the medium was carefully aspirated and the purple formazan crystals were solubilized with 100 µL DMSO. Optical density was measured at 630 nm in a microplate reader (Bio-Tek, ELX 800, USA). The absorbance of the untreated culture was set at 100%.

Treatment condition

According to MTT result, eupatorin at 50 and 100µg/ml, which are significantly, reduce cell viability, were added to HT-29 and SW948 human colon cancer cell line for 24 hours.

DAPI staining

Briefly, HT-29 and SW948 cells were seeded in a 6 well plate at $5x10^5$ cells/ plate and after pre incubation time cells were treated as describe previously. After 24 h, the cells were harvested and washed three times with phosphate buffered saline (PBS) and were adjusted to a density of 10^6 cells/ml of PBS. DAPI solution (1mgr/ml) was added to the cell suspension in a final concentration of 100µg/ml. Cellular morphology was evaluated by fluorescence microscope (Zeiss, Germany).

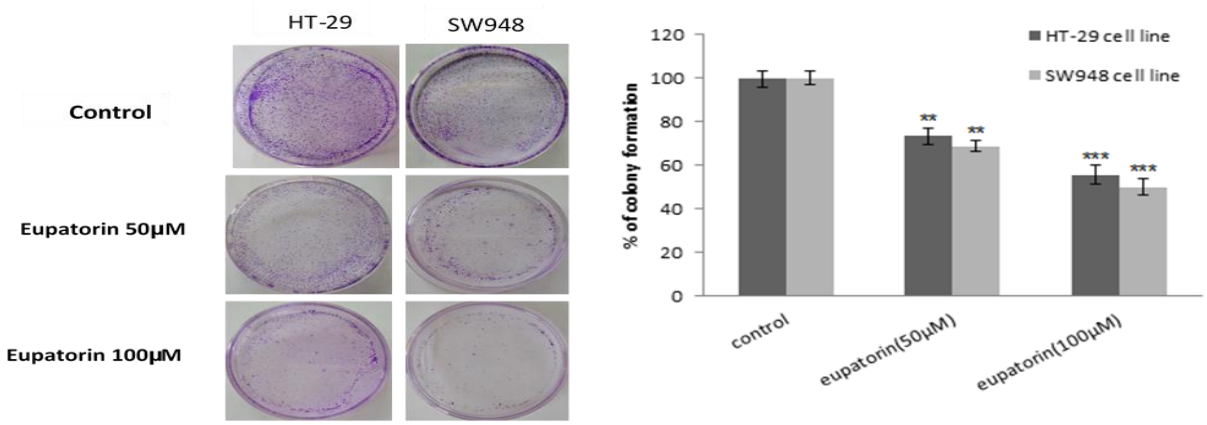


Figure 6: Eupatorin repressed clonal formation of HT-29 and SW948 cells. (A) Morphological analysis and B) Quantities analysis of inhibitory effect of 50 and 100 µM eupatorin for 24 hour. About 1×10^3 HT-29 and SW948 cells were seeded onto the 100 mm plates after the indicated treatment and changed for fresh medium every 3 days. *Significantly different from control cells. (**P<0.01 and ***P<0.001).

Table 1: ROS levels in HT-29 and SW948 cells treated with 50 and 100 µM of eupatorin 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	402.33±3.53	168.44±3.42
Eupatorin 50µM	598.32±3.26*	287.65±2.45*
Eupatorin 100µM	784.25 ±4.27**	454.12 ±3.23**

Table 2: MMP (rhodamin123 fluorescence) levels in HT-29 and SW948 cells treated with 50 and 100 µM of eupatorin 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	459.54 ±4.27	558.54±3.41
Eupatorin 50µM	678.54±3.32*	789.54±2.98*
Eupatorin 100µM	934.66±2.55**	998.54±3.2**

Colony formation

To evaluated the ability of colony formation in HT-29 and SW948 cell lines, these cells were seeded in a 6 well plate at $1x10^3$cells/plate. After cells attached, treatment were added as describe previously. The cells medium were altered every three days until 10 days. A colony is defined as a cluster of at least 50 cells. At the end, cells were fixed by paraformaldehyde for 20 minute and then staining with crystal violet[20].

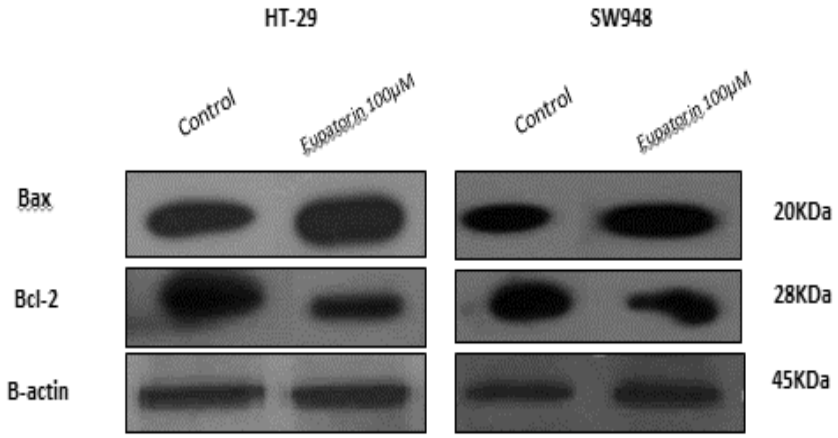
Measurement of Intracellular ROS

The fluorescent probe 20, 70-dichlorofluorescein diacetate (DCF-DA) was used to monitor intracellular accumulation of ROS. Briefly, the DCFH-DA solution 10µM was added to the suspension of the cells ($1x10^6$/ ml) followed by eupatorin treatment. Then the mixture was incubated at 37^0C for 1 h. Cells were then washed twice with PBS and finally, the fluorescence intensity was measured by Varian Cary Eclipse spectrofluorometer with excitation and emission wavelengths of 485 and 530 nm, respectively.

Measurement of the mitochondrial membrane potential (MMP)

Rhodamin 123 (Rh123) used to estimate MMP by fluorescent dye, as described previously [21]. After treatment, HT-29 and SW948 cells were incubated for 30 min at $37^{\circ C}$ with PBS containing 5 µM Rh123. After being washed 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 of 485 and 530 nm, respectively.

Western blotting



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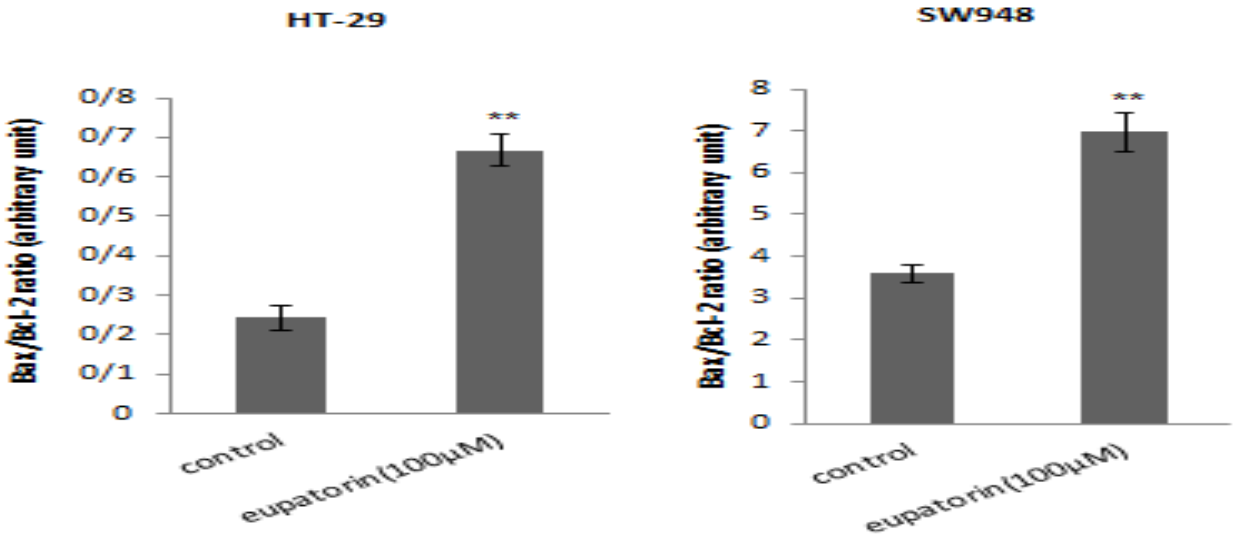

B

Figure 7: Bax and BCL-2 levels in HT-29 and SW948 cells treated with eupatorin. A) HT-29 and SW948 cells were treated with eupatorin (100m 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).

After indicated treatment, cells were harvested and the proteins were extracted. At first, the concentration of protein adjusted by Bradford's method[22]. Identical amounts of protein were boiled for 5 min and separated by SDS-PAGE, then transferred onto a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membrane was then blocked with 5% non-fat dry milk in Tris-Buffered-Saline with Tween (TBST) for 1 h at room temperature, and incubated with appropriate 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 extensive 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.

RESULT

Eupatorin inhibits cell proliferation on HT-29 and SW948 human colon cancer but not on normal HFFF-2 cell line

Eupatorin decreased cell viability in both HT-29 and SW948 colon cancer cell line at 50 μM/ml more than 20% and in 100μM/ml more than 40%, for a 24-h exposure time (Fig. 4A). Interestingly, normal HFFF-2 cells were found less sensitive to eupatorin on both HT-29 and SW948 cell lines without any significant different compare to control at mentioned doses (Fig. 4B).

Morphological analyses of apoptosis

To further characterize cell death, we performed DAPI staining. As shown in Fig. 5A and 5B cells treated with eupatorin (50 and 100µg/ml) showed nuclear fragmentation and chromatin condensation. Collectively, these features are characteristic of apoptosis.

Eupatorin treatment reduce colony formation in colon cancer cell lines

In HT-29 and SW948 human colon cancer cell lines, a clonal growth assay confirmed that treatment with 50 and 100 µM/ml of eupatorin, conferred a marked repression of clonal growth. At the most effective dose (100 µM/ml) it was about 66/6% in HT-29 and 73/33% in SW948 and cell line (Fig. 6A and 6B).

Eupatorin increase ROS level in colon cancer cells

To determine whether eupatorin act as pro-oxidant, intracellular ROS level measured. Eupatorin treatment (50 and 100 µM) for 24 hours, increase ROS level in HT-29 human colon cancer cell line about 1.48 and 1.94 fold respectively. The same treatment on SW948 (50 and 100 µM) was increasing ROS level about 1.70 and 2.69 fold respectively (Table 1). It was determined that excessive ROS in cancer cell line lead to cancer cell proliferation and metastasis.

MMP decrease in colon cancer cell lines treated by eupatorin

Decrease in MMP by eupatorin treatment was evaluated by demonstrating in Rh 123 retention in cell. As MMP decrease Rh 123 release and the fluorescence intensity increase. As shown in table 2 MMP significantly decrease in both HT-29 and SW948 colon cancer cell lines that are treated by 50 and 100 µM eupatorin compare to control. Hence, Rh 123 release and the fluorescence intensity in eupatorin treated cells increase.

Eupatorin induce Bax/ Bcl-2 ratio protein expression in human colon carcinoma cell lines

To determine whether eupatorin affects apoptosis pathway through BCL-2 family protein or not, Bax/ Bcl-2 ratio measured by western blotting. As shown in Fig. 7(A and B), treatment of cells with the most effective dose of eupatorin (100 µM/ml) increase Bax/Bcl-2 ratio to 2.74 in HT-29 cell line compare to control. It has been shown in Fig 7(A and B), that in SW948 human colon cell line, eupatorin increase Bax/Bcl-2 ratio about 1.94 fold respectively compare to control.

DISCUSSION

The effective function of natural therapeutic regimens such as vegetable and fruit, to inhibit cancer cell proliferation, metastasis and cell survival with protecting non-tumoral cells has been considered recently. The attractive side of chemopreventive properties of vegetable and fruits are mostly due to minimally toxic or non-toxic feature of their consumption. Flavonoids are one of the most important contributors of human diet and it has been discussed that they are obviously effective in cancer prevention. Since chemotherapy side effects and drug resistance are important obstacle in cancer treatment, especially colon cancer, studied developed recently to cope on these problems[23,24].

In this study, HPLC qualitative analysis, confirmed the presence of a flavonoid, eupatorin, as keys component of *Salvia mirzayanii*. This suggests that the growth-inhibitory effect of this plant extract could be at least partly due to eupatorin flavonoid content[25]. It was shown in this study that eupatorin, at concentrations of 100 µg/mL, effectively inhibited the growth of HT-29 and SW948 colon cancer cell lines. Additionally, identical concentrations of eupatorin exerted no effects on the growth of HFFF-2 human normal fibroblastic cells.

Given that upregulation of proapoptotic factors and suppression of antiapoptotic factors were shared among many colonic epithelial cell differentiation pathways, this mechanism should be modulated in colon cancer proliferative signal. So that apoptosis can be commenced[26]. Mitochondrial dysfunction is an important event in the apoptotic pathway whether it is an early event in apoptosis or is a consequence process[27]. Mitochondria have been shown to be a cell survival regulator and major source of ROS generation, which are both, crucial in apoptosis and cell function. A study proposed that in HCT116 colon tumor cell, loss of mitochondrial membrane potential takes place prior to other apoptosis marker activation[28].

In this study, eupatorin apoptotic pathway triggered from mitochondria by the relative amounts of apoptosis-promoting and apoptosis-inhibiting Bcl-2 proteins in the outer membrane of these organelles, were measured through Bax/Bcl-2 ratios by quantitative Western blotting. The function of Bcl-2 in apoptosis is either through forming rupture to mitochondrial outer membrane or by participating in channel formation in outer membrane of mitochondria[29]. Furthermore, it was observed that Bax has direct impact on mitochondrial membrane potential when it is translocated as a monomer from cytosol to outer membrane of mitochondria and forms an oligomer. Bcl-2 can also bind to Bax and inhibit the formation of Bax-complex, which is another role of Bcl-2 in apoptosis[30,31]. This suggests that not only Bcl-2 have a role on apoptosis pathway solely but also, it can have effect on Bax. As for the importance of Bcl-2 family function in apoptosis it was shown that, a lag time may be present between translocation of Bax and cell death. This will go to prove that releasing directly in subsequent marker of apoptosis is not always followed by activation of proapoptotic family member but may be the result of mitochondrial dysfunction that lead to release in subsequent marker[32,33].

In a different manner, the effect of eupatorin on the amount of ROS was investigated on this study as it was previously discussed whether mitochondrial ROS is initiator, amplifier or Achilles heel in cancer and in apoptosis induction[34,35,36]. Many types of cancer cells express increased amount of ROS, which enhance tumor genesis and lead to metastatic behavior because of accumulation in mutation by induce mutation in oncogenes or tumor-suppressor genes. Eupatorin as a flavonoid could act as either antioxidant or pro-oxidant. However, ROS production by flavonoids as a pro-oxidant in cancer cells possesses dual effect compare to normal cells[37]. Flavonoids acting as a pro-oxidant in cancer cells have

been studied, which has guided us to study whether eupatorin can increase ROS generation in colon cancer cells and lead to cellular damage.

Changes in outer membrane mitochondrial potential are one of the early characteristics of intrinsic apoptotic pathway. The Bcl-2 family proteins regulate the potential of the mitochondrial membrane carefully by forming either homo- or hetero-dimers[38]. In this study, eupatorin was shown to have a significant effect on the levels of MMP and Bcl-2 family proteins. Treatment by eupatorin flavonoid increased the levels of Bax/Bcl-2 ratio in both HT-29 and SW948 human colon cancer cell line. Along with this, a reduction in mitochondrial membrane potential in presence of eupatorin treatment initiated an important and irreversible aspect of apoptosis in colon cancer cell lines[39]. In addition, eupatorin treatment significantly reduces cell growth in colon cancer cell lines and has an influence on ROS generating system. In order to show the importance of ROS induced apoptosis via eupatorin in HT-29 and SW948 colon cancer cells, we measured ROS production using DCFH-DA, which confirmed that this natural flavonoid has cytotoxic effect on these cells through ROS pathways.

Natural therapeutic flavonoids, such as eupatorin, are able to increase apoptosis through Bcl-2 family member and mitochondrial membrane potential[40,41]. So that, the efficacy of treatment in patients of colon cancer is improved, natural products such as eupatorin, which protect non-tumoral cells, can play a vital role if utilized as combination chemotherapies. Most colon cancer patients whose treat with chemotherapies have only temporary responses to treatment, associated with the side effects and drug resistance of the therapies[1,42].

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.

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