

## Aloe-emodin Induces Apoptosis through the Up-Regulation of Fas in the Human Breast Cancer Cell Line MCF-7

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**Abstract:** Aloe-emodin is a Hydroxyanthraquinone that exists in Aloe Vera leaves and has been known to have anti-tumor effects. Various studies have mentioned the biologic effects of Aloe Vera such as antiviral, antimicrobial and some hepato-protective effects. Recently its antitumor effects have attracted many researchers. Breast cancer is the most common malignancy in women, accounting for 30% of all female cancers and < 1% of all cancer cases in men. Breast cancer also is responsible for 15% of cancer deaths in women. The aim of the present study was to investigate the anti-cancer effect of aloe-emodin on human breast cancer MCF-7 cell line. **Materials and Methods:** MCF-7 cells were cultured in RPMI medium with 10% fetal bovine serum (FBS). The cytotoxic effect of different concentrations (5, 10, 25, 50, and 100  $\mu$ M) of Aloe-emodin on cultured cells were evaluated by MTT assay at different times (24, 48, 72 h). Apoptosis and CD95 (Fas) expression were analyzed by flow-cytometry using an AnnexinV-FITC/PI kit and Fas (CD95) kit according to the manufacturer's protocol. **Results:** Aloe-emodin decreases the viability of MCF-7 cell line in a time and dose dependent manner, so that the most effective concentration of this substance was 100 $\mu$ M and 72h after treatment. According to the data of Fas (CD95) expression and Annexin-PI, the highest apoptosis induction rate was seen in 100 $\mu$ M and 72 h. **Conclusion:** The findings of the present study indicated that Aloe-emodin has some antitumor effects and can be used in the treatment of breast cancer. However, further investigation of its cytotoxic effects against tumor cells, both in vitro and in vivo, is recommended.

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**Key words:** Breast Cancer, Aloe-emodin, Apoptosis, MCF-7.

### Introduction

Anthroquinones, chemical compounds with different functions, have a structure that is related to anthracene. They are synthesized both naturally and industrially and utilized widely in industry and medicine, such that people are in contact with them directly and indirectly [1]. Since anthroquinones are all derived from a parent nucleus, they are alike in most of their activities. However, because they have different substitution groups, intensity of their activities and effects are different. In most activities like antibiotic and anti-cancer properties, there is a connection between the structure and the performance of anthroquinones [2]. Aloe-emodin is a Hydroxyanthraquinone that is found in Aloe Vera

leaves and has antitumor properties [3]. Different biological properties, including antiviral and antimicrobial properties and its protective effects on the liver, of Aloe-emodin have been listed in different studies [4]. It has also been reported that Aloe-emodin has an anti-cancer effect on skin tumors and hepatic and pulmonary cancer cells [5-6]. Aloe-emodin prevents development and proliferation of both type of glia and glioma cells in S phase of the cell cycle [7-8] and damages DNA by producing ROS (Reactive Oxygen Species) in pulmonary cancer cells [3]. It also causes apoptosis in gastric cancer cells by reducing phosphorylation of BID, BH3 and proapoptotic molecules [9]. Breast cancer is the most common malignancy in women worldwide [10] such

that it accounts for 30% of all cancers and 15% of cancer-related deaths among women [11]. In Iran breast cancer accounts for 21.4% of all reported cancers. The raw incidence of breast cancer in Iran has been measured to be 22.4 cases out of 100000 women and the existent data indicate that the disease is increasing [12]. In spite of important developments in diagnosing and treating it, breast cancer is one of the most common problems of women. Although a lot of women have survived the disease since last 30 years through effective drugs such as tamoxifen and raloxifene, or anti-tumor drugs of anastrozole along with radiation therapy or adjuvant therapy [13], various weaknesses of these drugs such as recurrence of the disease due to metastasis during first months of treatment, their effects on the metabolism of glucocorticoids, and cancer risk after chemotherapy of breast cancer have caused some limitations for utilizing these drugs [14-15]. This information indicates that preventive and therapeutic strategies are needed [16]. Apoptosis is one of the mechanisms of action of anticancer drugs that kills cancer cells [17]. Apoptosis is the programmed death of cancer cells, which evolves on the opposite direction of cell proliferation and occurs in response to some motivations during the process of morphological differentiation [18]. Any disruption in this process can cause disease [19] which can be due to a reduction in cell death and leads to cancer cell development and growth or autoimmune disorder [20]. Different molecules are involved with apoptosis process. Motivating apoptosis molecules or controlling anti-apoptosis factors depends on cell type and motivation. There are two main apoptosis pathways: exterior pathway or the pathway reliant on death receptors and interior pathway or mitochondrial pathway [21]. Fas(CD95) molecule is one of the proteins that is part of the receptors of the apoptosis exterior pathway. Fas(CD95) is one of the type I membrane proteins with a molecular weight of 45 kDa [22]. Activation of Fas(CD95) occurs by connecting to its antibody or its ligand (FasL) in apoptosis cells or apoptosis susceptible cells [23].

In the present study, cytotoxicity effects, induction of apoptosis, and mechanism of apoptosis induction of Aloe-emodin on breast cancer cells type MCF-7 have been investigated.

#### **Material and method**

Breast cancer cell line (MCF-7) was purchased from Cell Bank, Pasteur Institute of Iran. Aloe-emodin was purchased from Sigma\_Aldrich Co. (St. Louis, MO, USA). It was dissolved in dimethylsulfoxide to a concentration of 10 mM and stored in -20° C until utilization. Roswell Park Memorial Institute (RPMI)1640 and fetal calf serum

(FCS) were obtained from GIBCO Co. (England) (GIBCO/Invitrogen Corp, Carlsbad, CA, USA) and Penicillin-streptomycin was purchased from Roche Co. (Germany). 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), Annexin coloring kits and Fas (CD95) assay kits were purchased from Roche Co. (Germany). In this study we used Elisa (Awareness, USA) and flowcytometry Becton Dickinson Facscalbur (USA).

#### **Cell culture**

MCF-7 cells were cultured in culture medium RPMI-1640 enriched by fetal calf serum (10% volume/volume) and penicillin / streptomycin (1% volume/volume) under controlled conditions of temperature (37 °C) and humidified atmosphere containing CO<sub>2</sub> (5%). These cells grow as a monolayer in the flask. Culture medium replaced three times a week and sterile trypsin-EDTA solution was used for picking up the cells[24].

#### **Cytotoxic Effect of Aloe-emodin was determined by using MTT assay**

MTT assay is a colored assay which is based on the reduction of yellow water-soluble salt 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT) and the formation of dark blue crystals of insoluble formazan in water. Reduction of MTT was done by mitochondrial succinate dehydrogenase and occurs only in living cells. Formazan crystals are soluble in organic solvents such as isopropanol and DMSO that the numbers of living cells which are metabolically active can be determined by measurement of the absorbance of Formazan crystals. The Cells were subcultured into a 96-well plate with 10<sup>4</sup> cells/well, after 24 h and reaching distribution 80%, the supernatant was replaced by various concentrations (5,10, 25, 50 and 100 µM) of aloe-emodin and the cells were maintained in cultured condition for various time periods (24,48 and 72h). Each concentration of the drug replicate in three wells. In each row of wells on each plate along with various concentrations of Aloe-emodin a well as a control group was considered, which includes all materials that was added to the cells except Aloe-emodin. After the time periods, the plate was exit from the incubator, the supernatant was removed and 100 micro liter RPMI medium without  $\alpha$ -Naphthol, accompanied by 10 ml MTT was added to each well. The plates were then incubated at 37 ° C and were kept for 3 to 4 hours. Formazan product is then dissolved by adding 50 ml of solvent dimethyl sulfoxide (DMSO) and the color intensity was measured by ELISA reader at a wavelength of 570 nm[25]. The viability of treated cells with Aloe-emodin was determined in the form of sample absorbance compared to Formazan absorbance in

control group and was shown in the two-dimensional curved (the percent of viability or cell survival relative to Aloe-emodin). The mean level was considered 0.05% as statistical significant.

#### ***Apoptosis was determined by using flow cytometric assay***

The percentage of apoptotic cells in a treated cell population with drug compared to the negative control cell population was determined by two colors staining of cells, Annexin-V and PI. Thus, after treatment of cells with concentrations of 5, 25 and 100  $\mu\text{mol}$  of Aloe-emodin, the incubation was performed for 72h (at this time, the most effect of the MTT assay was observed), cells were trypsinized, washed with phosphate buffered saline (PBS), centrifuged and 100  $\mu\text{l}$  of binding buffer was added to sediment in 1.5 ml microtube. Then, 5  $\mu\text{l}$  of Annexin and 10  $\mu\text{l}$  of PI were added to microtube. The microtube was moved slowly, the entire contents were mixed together, so that sediment and the other materials were solved readily. In next step, the samples were incubated for 10 min at 25°C in darkness. Finally, cell analyses was done using Coulter Flow Cytometer. Data analysis was performed using the software and recorded points on two-dimensional curved divided into four regions  $Q_1$  to  $Q_4$ . So that, region  $Q_1$  represents necrotic cells with Annexin- V<sup>-</sup> and PI<sup>+</sup> features, region  $Q_2$  represents delayed apoptotic cells or necrotic cells Annexin- V<sup>+</sup> and PI<sup>+</sup> features, region  $Q_3$  represents healthy cells with Annexin- V<sup>-</sup> and PI<sup>-</sup> features and region  $Q_4$  represents young apoptotic cells with Annexin- V<sup>+</sup> and PI<sup>-</sup> features. In order to determine Aloe-emodin role on induction of apoptosis or necrosis, the percentage of cells in each area was calculated by flow cytometry software (FCS Express).

#### ***Evaluation of the expression of Fas (CD95)***

Cells were treated with 5 and 100  $\mu\text{mol}$  Aloe-emodin, incubated for 72h, trypsinized and washed with FCM (PBS+BSA.1%). After centrifuge 100  $\mu\text{l}$  FCM buffer, CD95 and ISO antibodies was added to sediment and incubated for 45 min at 4°C. At last, the cells were washed with FCM buffer and fixed by paraformaldehyde 1%. Fluorochrome was used with ficoeritrin which evaluated in FL2 channel. Cell Analyses was performed using Flow Cytometer (FCS Express).

#### ***Statistical analysis***

The statistical analysis was performed using SPSS software and Excel 2010. P value smaller than 0.05 was considered as statistical significance. Differences between the groups were determined using one-way ANOVA. All post hoc comparisons were made using Tukey's post hoc test.

## **Results**

#### ***Aloe-emodin effect on cell death***

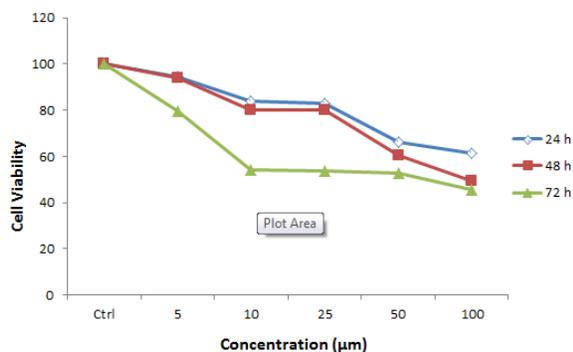
Statistical analysis showed at 24 h incubation period, the percentage of cell death increased following increasing Aloe-emodin dose in MCF-7 cells. As it was shown in figure 1 the percentage of cell death dependant to dose increased in MCF-7 cells at 48h period compared to 24h but this difference isn't significant ( $p=0.62$ ). At 72 h incubation period, cell death had significant increase compared to 24 h ( $p=0.04$ ), but there was no significant difference compared to 48 h ( $p=0.14$ ). Figure 1 show that the percentage of cell death is dependant to dose and time (increased following increasing dose and also passing the time).

#### ***Aloe-emodin effect on apoptosis***

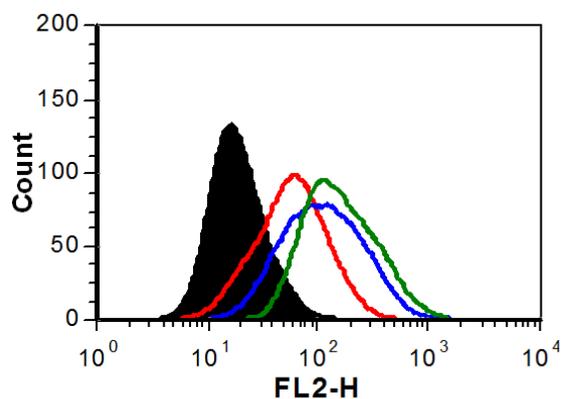
Within apoptosis or programmed cell death, phosphatidylserine is transferred from inner to outer surface of membrane. VAnnexin<sup>-</sup> binds to phosphatidylserine in outer surface and can be detected by flow cytometry. The PI also binds to DNA fragmentation of dead cells and can be detected by flow cytometry. The percentage of apoptotic cells at three concentration of Aloe-emodin along with control group (which indicates cells without Aloe-emodin effect) was shown in figure 2. As seen in figure 2, the percentage of living cells (region  $Q_3$ ) in the control group is 80% higher than the other groups. The percentage of young apoptotic cells (region  $Q_4$ ) (that are in early stages of apoptosis) increased from 31% at a concentration of 5  $\mu\text{mol}$  and 34% at 25  $\mu\text{mol}$  of Aloe-emodin to 37% at a concentration of 100  $\mu\text{mol}$ . The percentage of delayed apoptotic cells or necrotic ( $Q_2$ ) and also the cells in end stages of apoptosis increased following enhancement in Aloe-emodin concentration, such that it increased from 12% to 38%. As shown in figure 2, increase in Aloe-emodin concentration cause increase in apoptotic cells in region  $Q_4$ . It demonstrates that apoptosis of the cells in a dose-dependent manner has been more.

#### ***Aloe-emodin effect on the expression of Fas (CD95)***

Figure 3 show the expression of Fas (CD95) in MCF-7 cells after 72 h when the cells treated with concentrations of 5 and 100 mol of Aloe-emodin. As shown in this Figure, Fas expression levels has been increased compared to the control (red histogram) and the expression of Fas at the concentration of 100  $\mu\text{mol}$  (green histogram) is higher than the concentration of 5  $\mu\text{mol}$  (blue histogram). Expression of Fas (CD95) was also correlated with increased concentrations of Aloe-emodin.



**Figure 1:** The percentage of MCF-7 cells death in incubation for 24, 48 and 72h with various concentration of Aloe-emodin in control group: (without treatment of the cells with Aloe-emodin).



**Figure 3:** Aloe-emodin effect on the expression of Fas (CD95). Black histogram (isotype antibody): cells number in basic state. Red histogram: the expression level of CD95 without Aloe-emodin effect. Blue histogram: the expression level of CD95 at the concentration of 5 µmol of Aloe-emodin. Green histogram: the expression level of CD95 at the concentration of 100 µmol of Aloe-emodin.

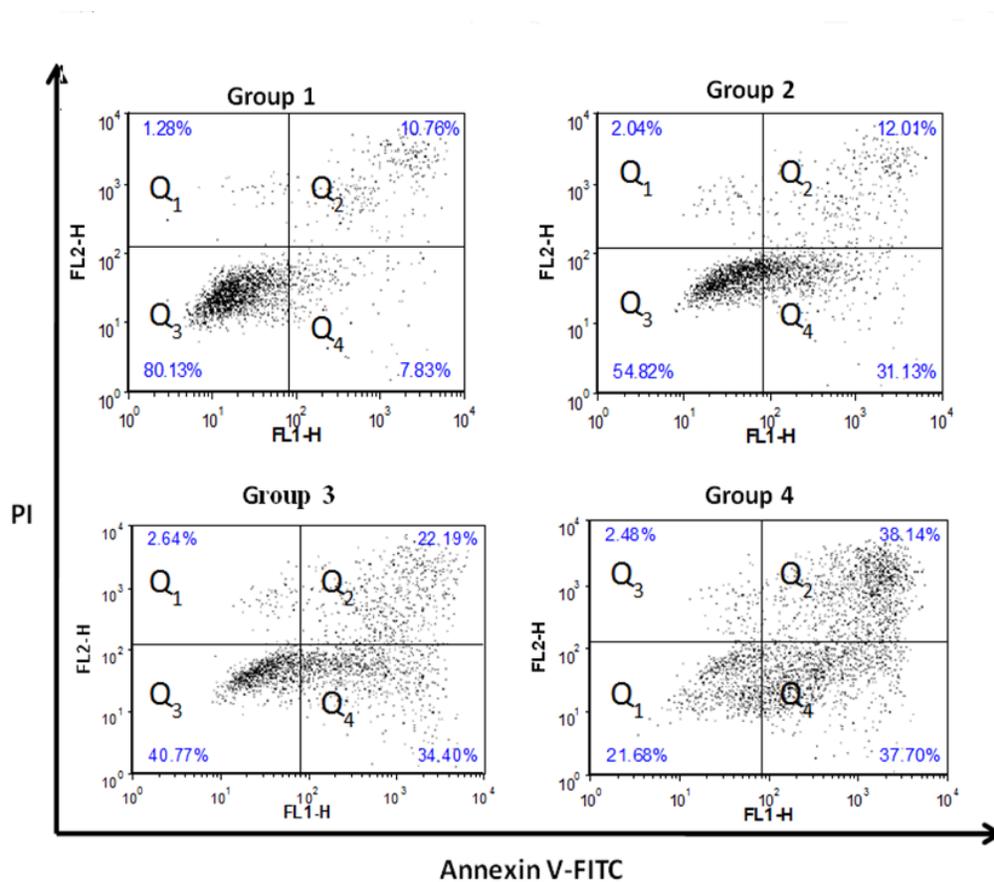
### Discussion

Aloe-emodin is a natural active ingredient that exists in aloe vera leaves. Several studies have indicated its anticancer properties [26]. In the present study anticancer effect of Aloe-emodin and apoptosis induction mechanism on breast cancer cells (MCF-7) were investigated for the first time. Our results revealed that Aloe-emodin can reduce breast cancer cell viability and also induce apoptosis associated with increased expression of Fas (CD95) in these cells. It was known two important pathways for apoptosis. Binding the agents which trigger cell death to receptors such as tumor necrosis factor (TNF $\alpha$ ) on the cell surface can induce apoptosis via the extrinsic pathway. Furthermore, chemotherapeutic agents, genotoxic stress and other agents that trigger cell

death can cause apoptosis via inside or mitochondrial pathway [27]. Death receptors are members of the tumor necrosis factor receptor super family (TNF). When these receptors are stimulated by their respective ligands, can cause activation of caspase and induction of apoptosis. Important inducer of apoptosis in the extracellular of TNF-related to apoptosis inducing ligands is Fas ligand. Fas ligand molecule between various apoptosis induction pathway is an important controlled pathway [28]. Previous studies showed anticancer effect of Aloe-emodin on some cell line viability and that, apoptosis induction associated with increased expression of Fas (CD95). Their findings are consistent with the results of this research. Chiu and colleagues showed that Aloe-emodin can cause apoptosis in tongue cancer cells (SCC-4) which is associated by increased expression of Fas (CD95) and activation of caspase-eight [29]. In this study, we examined the cytotoxic effect of Aloe-emodin on MCF-7 cells using MTT assay. As seen in Figure 1 following increasing the concentration of Aloe-emodin, cell death in a dose- and time-dependent manner has been more. The maximum cell death is at concentration of 100 micromoles after 72 h. In a similar study Guo and colleagues investigated the cytotoxic effect of Aloe-emodin on gastric cancer cells (MGC-803) using MTT assay. They showed that gastric cancer cells viability has been reduced in a dose- and time-dependent manner by treatment with Aloe-emodin [30]. Next, we investigated the apoptosis induction mechanism in MCF-7 cells. For evaluating the apoptosis, we used concentrations of 100 µmol, 5 µmol and 25 µmol of Aloe-emodin which had maximum, minimum and median cytotoxicity respectively and also the maximum cytotoxicity was observed 72h after treatment, so these concentrations of cells were then incubated for 72 hours. Flow cytometric analysis showed that the most of the cells treated with Aloe-emodin have been undergone apoptosis or programmed cell death and very small percentage of the cells was necrotic. Apoptosis of cells in a dose-dependent increased. These findings agree with other studies that Aloe-emodin can trigger apoptosis and inhibit their proliferation. To understand the mechanism of action of Aloe-emodin on MCF-7 cells, we examined the expression of Fas molecules. Our findings indicate an increase in Fas expression which was associated with increased dose of Aloe-emodin. Aloe-emodin likely affects on MCF-7 cell membranes, stimulates death receptors, specifically Fas protein, activates caspase and induces programmed cell death or apoptosis via the Extracellular pathway. Lin and his colleagues found that Aloe-emodin can cause apoptosis via activation of P53, P21, Bax, Fas and Caspase3, prevent the

proliferation of bladder cancer cells and stop cell cycle in G2/M1 phase [31]. Several studies have noted that apoptosis induced by Aloe-emodin is associated with changes in transmembrane gene expression of family bcl2, apoptosis regulators. Aloe-emodin also causes the release of cytochrome C from mitochondria in lung cancer cell in CH27 line. Further observed that Aloe-emodin can induce apoptosis in liver cancer cells (both HPG-2 and HPG-3) which is associated with the expression of p21 and p53 [32].

In another study, Teresa and his colleagues investigated on neuroectodermal tumor cells and showed that Aloe-emodin has no significant difference in either acute or chronic state on a variety of normal cells, but has selectively toxic effect on neuroectodermal tumor cells. However, in order to determine the effects of Aloe-emodin on healthy human cells, more research is needed [5].



**Figure 2:** the effect of various concentration of Aloe-emodin on apoptosis in MCF-7 cells.

**Group 1:** Control group (without treatment of the cells with Aloe-emodin). **Group 2:** Cell treatment with the concentration of 5  $\mu\text{mol}$  of Aloe-emodin. **Group 3:** Cell treatment with the concentration of 25  $\mu\text{mol}$  of Aloe-emodin. **Group 4:** Cell treatment with the concentration of 100  $\mu\text{mol}$  of Aloe-emodin. **region Q1:** Necrotic cells with Annexin-V<sup>-</sup> and PI<sup>+</sup> features, **the region Q2:** apoptotic cells with Annexin-V<sup>+</sup> and PI<sup>+</sup> features, **the region Q3:** healthy cells with Annexin-V<sup>-</sup> and PI<sup>-</sup> features and **the region Q4** represents young apoptotic cells with Annexin-V<sup>+</sup> and PI<sup>-</sup> features.

## Conclusion

The findings of this study showed that Aloe-emodin decreases viability of breast cancer cells (MCF-7). Aloe-emodin can also induce apoptosis in these cells and increase the expression of Fas which is one of the apoptotic extracellular markers. These findings show that Aloe-emodin can be used as an anti cancer drug or as an Adjuvant (it needs more research) for treating breast cancer.

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