

Mechanism of Aloe Emodin-Induced Apoptosis in ER⁺-Breast Cancer Cells, MCF-7

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ABSTRACT

The objective of this research is to investigate the regulation of apoptotic associated-genes and proteins expression of aloe emodin on oestrogen receptor (ER)-positive (MCF-7). Oestrogen receptor (ER)-positive (MCF-7) cells were cultured in complete RPMI media. Cells were treated with aloe emodin at its IC₅₀ of 80µM. Maximum treatment time was set for 72 hours in all assays. Both genes and proteins involved in the regulation of apoptosis (Fas, FADD, Caspase-3, Caspase-8, Caspase-9, Bax, Bcl-2, and Cytochrome c) in aloe emodin-treated MCF-7 were determined using Quantigene 2.0 Plex and protein ELISA assays respectively. Aloe emodin, previously reported as anti-cancer agent, was found to act as an apoptotic inducer on MCF-7 cells. In intrinsic apoptosis signalling, Bax, Cytochrome c and Caspase-9 proteins were upregulated (54.11% ± 4.51, 25.17% ± 4.13 and 36.05% ± 11.75); while no change was observed in Bcl-2 protein. Except for Caspase-9, these results are in accordance with gene expression. In extrinsic apoptosis, Fas and Caspase-8 were upregulated (133.82% ± 2.85 and 26.44% ± 2.48), contrary to gene expression. These findings indicate that aloe emodin activates both extrinsic and intrinsic apoptosis pathways. The data suggests (i) aloe emodin has the potential to be a selective apoptotic inducer in ER⁺-breast cancer management; and (ii) the present study could be used as a basis for *in vivo* experiment..

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INTRODUCTION

Breast cancer which accounts for 18.1% of all cancers has a 32.1% rate among Malaysian women compared with other types of cancers (Omar & Tamin, 2011). Approximately two-thirds of breast cancer patients expressed oestrogen receptor (ER), and received endocrine treatment with anti-oestrogens such as tamoxifen, toremifene, raloxifene, and fulvestrant (Clarke et al., 2003; Baumgarten & Frasor, 2012). Despite these therapeutic treatments, the overall incidence rate remains high. (Baumgarten & Frasor, 2012).

Although it is well accepted that cancer refers to uncontrolled cell growth, anti-cancer drugs affect not only cancer cells but also normal active proliferating cells such as bone marrow, gastrointestinal epithelial and dermal papilla of hair follicle. Thus treatment unfortunately worsens the patients' situation as increasing evidences of toxicity have been observed (Thavendiranathan et al., 2013). Besides being non-selective, most of the drugs lead to resistance in cancer patients and have adverse effects when exposed to prolonged periods (Fisher et al., 2001).

Natural sources have been used in traditional remedies to cure many types of diseases such as diabetes, wound healing, osteoarthritis, malaria, skin diseases, cancer and other critical diseases for many years. In fact, phytochemicals and their significant healing properties have been well recognised in the scientific research (Singh, 2007).

Aloe emodin has been recommended as a potential natural chemotherapeutic agent. Aloe is a genus of the widely known species Aloe Vera or also known as Aloe barbadensis Miller. Aloe emodin is the well-known anthraquinone active compound that can be found in some species of Aloe (ElSohly et al., 2004). Accumulative evidences of aloe emodin as anti-cancer agent from in vitro as well in vivo studies were well documented. Unlike tamoxifen, it selectively inhibits proliferation of cancer cells including prostate, neuroectodermal tumour and cervical, without affecting normal cells (Guo et al., 2007; Liu et al., 2012; Pecere et al., 2000).

Apoptosis, or programmed cell death, is a normal process. The apoptotic signal is a response to defective cells to commit suicide in a natural way without harming neighbouring and surrounding cells (Johnstone et al., 2002; Elmore, 2007). Thus, deregulation in apoptosis has been implicated in a variety of diseases such as cancer (Brown & Attardi, 2005). Molecular mechanism studies suggest that its anti-cancer property was through the promotion of cell cycle arrest and apoptosis (Chen et al., 2004; Kuo et al., 2002). Although, there is a significant relationship between apoptosis and aloe emodin, its induction on breast cancer cells has not been studied sufficiently. Current studies suggest that aloe emodin reduces the expression of ER α in both time- and dose-dependent manners, thus reducing oestrogen proliferative effect on MCF-7 cells (Huang et al., 2013). In addition, there was evidence that aloe emodin inhibited the activation of ER α even at lower dosage compared to its isomer, emodin (Huang et al., 2013).

In our previous investigation (Amin et al., 2013), aloe emodin inhibited the proliferation of MCF-7 with IC₅₀ of 80 μ M using WST-1 proliferation. No IC₅₀ value was obtained on MDA-MB-231 and MCF-10A, even up to 150 μ M. In contrast, tamoxifen was non-selective to all cells tested. By using the cellular apoptotic assay, our previous findings suggested that the anti-proliferation effect was through activation of apoptosis signalling. The activation of apoptosis was observed through the morphological changes and the increasing percentage of

apoptotic population at 48 and 72 hours treatment using flow-cytometry analyses (Amin et al, 2015, 2016). In the present study we further investigate the underlying molecular mechanism by looking at the regulation of apoptosis associated-genes and protein expressions.

METHOD

Materials

An oestrogen receptor-positive breast cancer cell, MCF-7 was purchased from American Type Cell Collection (Virginia, USA) Complete culture media RPMI, foetal bovine was from GIBCO Invitrogen (GIBCO Invitrogen, California, USA), phosphate buffer saline (PBS), accutase and dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, USA), QuantiGene 2.0 Multiplex Assay was purchased from Affymetrix California, USA. MCF-7. Aloe emodin and tamoxifen from Sigma Chemical Co. (St Louis, USA).

Cell and Treatments

Cells were cultured in complete RPMI media supplemented with 10% foetal bovine serum and 1% of penicillin and streptomycin. The cells were maintained as monolayer up to 70% to 90% confluence in humidified atmosphere of 5% CO₂, at 37°C in T25 and T75 flasks. Aloe emodin is synonym as 1,8-Dihydroxy-3-(hydroxymethyl) anthraquinone, 3-Hydroxymethylchryszazine is in purity of ≥95% as assayed by high performance liquid chromatography (HPLC). Tamoxifen or (Z)-1-(p-Dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene, trans-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-N,N- dimethylethylamine is ≥99% in purity and used as positive control. Aloe emodin was measured and dissolved separately in DMSO to prepare primary stocks of 50 mM. Both primary stock (aloe emodin and tamoxifen) solutions were filtered with a 0.2 µm sterile filter and stored in aliquots at -20°C protected from light. The final working solutions of aloe emodin and tamoxifen were diluted with culture media so that the final concentration of DMSO in cell culture was <0.1%. Tamoxifen, a non-steroidal oestrogen antagonist was used as positive control. All the preparations were performed under sterile conditions.

QuantiGene 2.0 Multiplex Assay

QuantiGene 2.0 Multiplex Assay is based on a series of hybridization method that capture target RNA in samples. Initially, target RNAs were captured by fluorescent microspheres (Capture Beads). Following from this, the target-specific RNA was measured by mixing it with high stringent of cocktails which include Capture Extenders (CEs), Label Extenders (LEs) and Blocker (BLs). They bound to RNA region and selectively capture the target RNA by a series of complex hybridization. The hybridization step was performed overnight at a temperature of 55°C. These resulted in sandwich multifaceted form which includes probe and the target sequence. The final step was the signal amplification and detection of target mRNA. The target mRNA was sequentially hybridized with specific Preamplifier, Amplifier and Label probes. The luminescent signal detected is proportionate to the amount of target mRNA present in the sample. Prior to the experiment, cells were seeded at 1 x 10⁶ into six different T25 flasks in 5% CO₂, at 37°C. Each group was treated separately with aloe emodin (IC₅₀), and non- treated

cells were used as control. After 72 hours of treatment, cells were washed with PBS, detached with accutase and centrifuged at 15,000 rpm for 5 minutes. Cell pellets were re-suspended in complete media, adjusted to 1×10^6 cells/ml and kept in 15 ml tubes at -20°C before used. This assay was conducted following the protocol outlined in QuantiGene 2.0 Plex User Manual (Affymetrix., California, USA). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as housekeeping gene.

ELISA Assay

Concentrations of 1×10^6 cells were cultured in 1 ml complete media in 6-well plate with 5% CO_2 , at 37°C for 24 hours. Cells in each well were treated separately with aloe emodin. Non-treated cells were used as control. After washing the cells twice with cold PBS, 380 μl of cold RIPA buffer containing Pierce protease inhibitors. The plate was incubated on ice for 5 minutes, swirled gently to ensure uniform spreading of lysis buffer on each well. Each cell lysate was collected using cell scraper and transferred to a 10-ml tube. Each lysate was sonicated at 50% pulse for 1 minute and centrifuged at $14,000 \times g$ for 15 minutes. Standard solution was reconstituted with 1.0 ml of sample diluents and allowed to stand for 15 minutes. A series of standard solutions ranging from S0 to S6 was prepared by two-fold serial dilution of standard stock with its diluents. Using the standard curve, all the samples from different treatment groups were standardized at 3 mg/ml. Concentration of 100 μl of treatment samples and standard were loaded into a pre-coated 96-well microplate.

Statistical Analysis

The Statistical Package for Social Sciences (SPSS) version 16.0 was used to analyse the data. Each experiment was carried out in triplicates and repeated three times. The differences between the groups were evaluated using the one-way ANOVA test.

RESULTS AND DISCUSSION

Effects of Aloe Emodin on Apoptosis Associated-Gene Expressions in MCF-7 cells

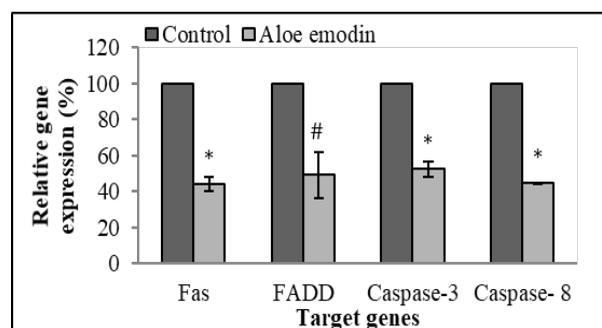


Figure 1. Fas, FADD, Caspase-3 and Caspase-8 gene expressions of ER⁺-breast cancer, MCF-7 cells after treatment with aloe emodin against control (untreated) cells and normalization with HPRT.

Note: *Significant as compared to control (untreated) cells at $p < 0.01$; # Significant as compared to control (untreated) cells at $p < 0.05$

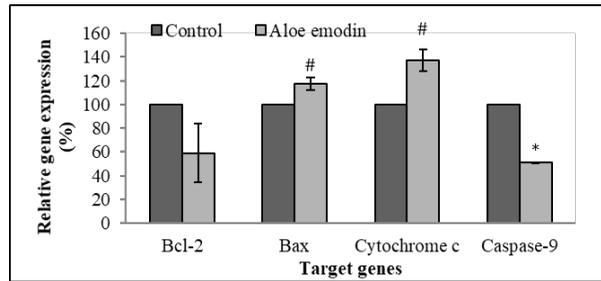


Figure 2. Bcl-2, Bax, Cytochrome c and Caspase-9 gene expressions of ER⁺-breast cancer, MCF-7 cells after treatment with aloe emodin against control (untreated) cells and normalization with HPRT

Note: *Significant as compared to control (untreated) cells at $p < 0.01$; # Significant as compared to control (untreated) cells at $p < 0.05$

Fas, FADD, Caspase-3 and Caspase-8 gene expressions were down regulated by $65.91\% \pm 0.93$, $50.83\% \pm 12.77$, $47.42\% \pm 4.31$ and $55.23\% \pm 0.01$ ($p < 0.05$, $n=3$), respectively after being normalized with HPRT (Fig. 1). Based on these findings, it appears that the apoptosis effect of aloe emodin on MCF-7 cells is independent of the extrinsic pathway. In addition, Bax, Cytochrome c gene expressions were upregulated by $17.47\% \pm 5.29$ and $37.29\% \pm 9.37$ ($p < 0.05$, $n=3$), respectively after being normalised with HPRT gene. On the contrary, Caspase-9 was downregulated by $48.79\% \pm 0.17$ ($p < 0.05$, $n=3$) while Bcl-2 shown changes when compared with the control however was not significant. This data suggests that the apoptosis effect of aloe emodin on MCF-7 cells is mitochondrial-dependent. However, more studies on the effect of aloe emodin on the expression associated apoptosis proteins that mediated both intrinsic and extrinsic apoptosis signalling needs to be done to validate gene expression results.

Effects of Aloe Emodin on Apoptosis Associated-Protein Expressions in MCF-7 cells

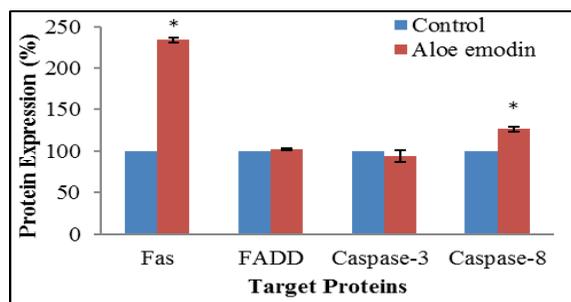


Figure 3. Fas, FADD, Caspase-3 and Caspase-8 protein expressions of MCF-7 cells after treatment with aloe emodin

Note: *Significant as compared to control (untreated) cells at $p < 0.01$

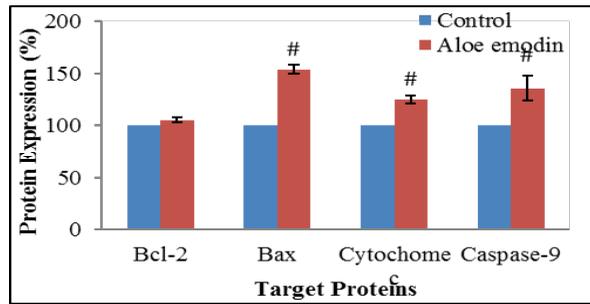


Figure 4. Bcl-2, Bax, Cytochrome c and Caspase-9 protein expressions of MCF-7 cells after treatment with aloe emodin

Note: #Significant as compared to control (untreated) cells at $p < 0.05$

Contrary to gene expression, Fas and Caspase-8 proteins were upregulated by $133.82\% \pm 2.85$ and $26.44\% \pm 2.48$ respectively (Figure 3). This indicates the apoptosis effect of aloe emodin on MCF-7 cells is via the extrinsic pathway. However, FADD and Caspase-3 gene expression results did not agree with their protein expressions. No changes were observed in the expression of FADD and Caspase-3 proteins. Aloe emodin upregulated the expression of Bax, Cytochrome c and Caspase-9 proteins by $54.11\% \pm 4.51$, $25.17\% \pm 4.13$ and $36.05\% \pm 11.75$, respectively. No change was seen in Bcl-2 protein (Figure 4). Except for Caspase-9, these results are in accordance with gene expression profiles. This indicates the apoptosis effect of aloe emodin on MCF-7 cells is through the intrinsic pathway targeting the mitochondrial function.

The present findings show the significant downregulation of associated-extrinsic apoptotic genes such as Fas, FADD, Caspase-3 and Caspase-8 after being normalised with both HPRT. Contrary to the above results, Fas and Caspase-8 proteins were unregulated but no changes were observed in FADD and Caspase-3. Based on Fas and Caspase-8 protein expressions, aloe emodin apoptosis effect on MCF-7 cells is suggested to act through the activation of the extrinsic pathway. Chemotherapy drug such as doxorubicin induces Fas/FasL interaction on the surface of tumour cells to activate apoptosis (Mitsiades et al., 2001). Similarly, in vivo and in vitro studies 5-fluorouracil showed to mediate the apoptosis signal through Fas/FasL interaction (Eichhorst et al., 2001). The lack of death receptors and their ligand expressions in leukemic cells after chemotherapy is suggested to be due to the failure of apoptosis and which in turn contributed to poor treatment outcome (Tourneur et al., 2004). In breast cancer treatment, the Fas system was used as a biomarker to evaluate the chemo responsiveness of anthracycline-based adjuvant therapy for type I and II breast cancer patients. It was suggested that the prognostic value of Fas receptor and FasL is strongly associated with the aggressive tumour phenotype that can be correlated with the progression of the disease (Botti et al., 2004). Activation of Fas system by aloe emodin in MCF-7 cells support the suggestion that this agent could be as competitive as other available drugs such doxorubicin and tamoxifen. Similar findings were also observed in different type of human cancer cells such as lung squamous CH27 (Lee et al., 2001), bladder T24 (Lin et al., 2006) and tongue squamous SCC-4 (Chiu et al., 2009) cells.

It also appears that the apoptosis action of aloe emodin on MCF-7 cells is mitochondrial-dependent. Interestingly, the expressions of Bax, Cytochrome c and Caspase-9 proteins were upregulated while no changes were observed for Bcl-2. Thus, it is suggested that aloe emodin apoptosis effect on MCF-7 cells does involve the activation of the intrinsic pathway. Deregulation of Bcl-2 family members is constantly associated with attribution of human malignant diseases. Understanding how they become involved in the regulation of death signal can be useful in designing targeted therapeutic regimen. One of the spectacular achievements of anti-cancer therapies is the direct targeting of Bcl-2 family proteins in mitochondria (Frenzel et al., 2009). Clinical trials using drugs targeting these Bcl-2 family members such as ABT-263, oblimersen sodium GX15-070 and AT-101 are ongoing (Kang & Reynolds, 2009). Figure 5 shows the suggested pathways that illustrate the effect of aloe emodin in inducing the intrinsic and extrinsic apoptosis pathways.

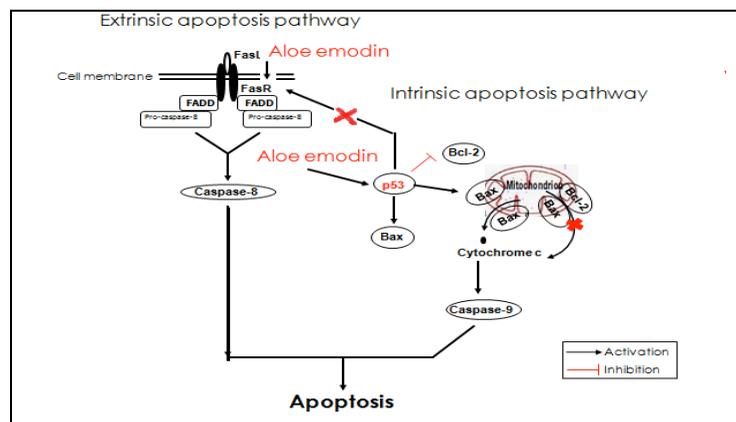


Figure 5. Schematic diagram of the suggested pathways of aloe emodin in inducing the intrinsic and extrinsic apoptosis

It is noted that the gene expression study reported in this work is not in accordance with the protein study. There are three main possible explanations for the poor correlation between mRNA and protein levels in experimental designs (Greenbaum et al., 2003): (i) post-transcriptional mechanisms might affect the regulation at gene and protein levels; (ii) different protein half-lives limited the capacity to directly measure their precise concentrations; and (iii) error and noise involved throughout the mRNA and protein experiment setting. Even with where the latest technology is used, discrepancies were observed between mRNA and protein measurements (Pradet-Balade, 2001; Vasconcelos et al., 2002; Pascal et al., 2008).

CONCLUSION

Our findings suggest aloe emodin to be used in combination with anti-oestrogen therapy in the treatment of cancer. The findings could provide a foundation for in vivo study and widen the possibility for future clinical experiment setting.

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