Aloe Emodin Induces Apoptosis in ER+-breast Cancer Cells; MCF-7 through IGF-1R Signalling Pathway

(Aloe Emodin Mengaruh Apoptosis dalam Sel Kanser Payu Dara ER+; MCF-7 melalui Tapak Jalan Pengisyaratan IGF-1R)

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ABSTRACT

Two-third of breast cancer patients expressed estrogen receptors (ER)s and received endocrine treatment with established anti-estrogens such as tamoxifen. But the action and acquired resistance during treatment are largely unknown. In contrary, phytochemicals are more selective and less cytotoxic to normal cells. Accordingly, we found aloe emodin, an anthraquinone to inhibit the proliferation of ER+-breast cancer cells, MCF-7 with IC_{50} of 80 µM, but not affecting control breast cells, MCF-10A. Tamoxifen was non-selective to both cells with IC_{50} of 27 and 38 µM, respectively. Thus, we aimed to investigate the anti-proliferative mechanism of aloe emodin on MCF-7 and its underlying signalling compared to tamoxifen. Cells were treated separately with aloe emodin and tamoxifen at respective IC_{50} for 72 h. Apoptosis was determined using Annexin V-FITC/PI staining. The expression of insulin-like growth factor-1 receptor (IGF-1R), insulin-like growth factor binding protein (IGFBP)-2 and B-raf gene was investigated using QuantiGene 2.0 Plex assay. Paired-student t-test and ANOVA test were used to compare between untreated and treated cells on the measured parameters. Each treatment was conducted in triplicate and repeated three times. Significance was set at p<0.05. The presences of early and late apoptosis in MCF-7 were seen in both treatments. All target genes were down regulated. The anti-proliferation effect of aloe emodin on MCF-7 is similar with tamoxifen which mediates inhibition of IGF-1R signalling pathway. This suggests aloe emodin as a potential anti-cancer agent to be used in combined anti-estrogen therapy to enhance its efficacy in ER+-breast cancer treatment.

Keywords: Aloe emodin; apoptosis; IGF-1R; MCF-7

ABSTRAK

Dua-pertiga pesakit kanser payu dara mengekspresi reseptor estrogen (RE) dan menerima rawatan endokrin dengan anti-estrogen seperti tamoksifen. Tetapi, tindakan dan kerentanan perolehan terhadap rawatan ini masih belum difahami. Sebaliknya, fito-kimia didapati lebih selektif dan kurang memberi kesan toksik kepada sel normal. Selanjutnya, kajian kami mendapati aloe emodin, sejenis antrakuinon berjaya merencat proliferasi sel kanser payu dara ER+, MCF-7 dengan IC_{50} 80 µM, tanpa memberi kesan toksik kepada sel payu dara normal, MCF-10A. Sebaliknya, tamoksifen adalah tidak selektif kepada kedua-dua sel dengan IC_{50} masing-masing 27 dan 38 µM. Oleh itu, penyelidikan ini dijalankan untuk mengkaji mekanisme anti-proliferasi dan laluan pengisyaratan transduksi aloe emodin terhadap MCF-7 berbanding dengan tamoksifen. Sel diaruhkan dengan aloe emodin dan tamoksifen secara berasingan pada IC_{50} selama 72 jam. Apoptosis ditentukan dengan ujian Annexin V-FITC/PI pewarnaan. Pengekspresan gen-gen faktor tumbesaran-1 reseptor (IGF-1R) seperti-insulin, faktor tumbesaran pengikat protein (IGFBP)-2 seperti-insulin dan B-raf ditentukan menggunakan asai Plex QuantiGene 2.0. Ujian-T pelajar-berpasangan dan ANOVA digunakan untuk membandingkan antara parameter sel teraruh dan kawalan. Setiap perlakuan dijalankan sebanyak tiga kali dan diulang tiga kali. Tahap signifikan ditetapkan pada p<0.05. Tanda-tanda awal dan akhir apoptosis di MCF-7 diperhatikan pada kedua-dua aruhan. Ketiga-tiga gen diekspreskan kurang daripada paras normal. Kesan anti-proliferasi aloe emodin terhadap MCF-7 adalah bersamaan dengan tamoksifen iaitu melalui perencatan tapak jalan pengisyaratan tranduksi IGF-1R. Kajian ini mencadangkan aloe emodin berpotensi sebagai agen anti-kanser yang boleh digunakan sebagai rawatan gabungan dengan terapi anti-estrogen yang sedia ada, bertujuan untuk meningkatkan efikasi rawatan tersebut.

Kata kunci: Aloe emodin; apoptosis; IGF-1R; MCF-7

INTRODUCTION

Breast cancer is the most frequent type of cancer (18.1%) with an occurrence of 32.1% among Malaysian women as compared with other type of cancers (Dahlui et al. 2011). Its incidence and death among women remain a major concern not only for Asian countries but also worldwide. Currently,

tamoxifen served as primarily systemic action for ER⁺-breast cancer patients. Unfortunately, its effectiveness is limited as advanced stage patients' progresses to a resistant phenotype (Dorssers et al. 2001). Despite its significant contribution in clinical setting, almost all patients with meta-static stage ultimately relapsed from their disease and are fatal (Ring & Dowsett 2004), thus emphasizing the importance of searching for a new chemotherapy regimens. Recently, aloe emodin has been recommended as a potential natural source of chemotherapeutic agents due to its cytotoxicity on several cancer cells, such as prostate (Lin et al. 2006), colon (Suboj et al. 2012) and hepatoma (Lu et al. 2007). The capacity of aloe emodin to induce apoptosis in these cells through several mechanisms has been well documented. Although there is a significant relationship between apoptosis and aloe emodin, its induction in ER⁺-breast cancer cells has not been identified. Our findings have shown that unlike tamoxifen, aloe emodin selectively inhibited the proliferation of ER⁺-breast cancer cells, MCF-7 in a time and dose dependent manner (Amin et al. 2013), without affecting normal breast cells, MCF-10A. However the mechanism underlying the anti-cancer activity remains unclear.

The insulin-like growth factor (IGF)-2/insulin-like growth factor-1 receptor (IGF-1R) signalling pathway has been suggested to play a causal role in anti-estrogen resistance of breast cancer cells (Zhang et al. 2011). IGF-1R belongs to the receptor tyrosine kinase family. Upon binding of IGFs to their receptors, a series of phosphorylation events occurs. IGFs are positively regulated by 17β-estradiol, progesterone, prolactin and growth hormone for both normal breast development and breast cancer progression (Felice et al. 2013). The 17β-estradiol-ERα complex binds and activates IGF-1R directly. Vice versa, IGF signalling enhances ERα activation in human breast cancer cells by phosphorylation (Yu et al. 2013). 17β-estradiol and IGF-1R synergistically enhances S phase entry in breast tumor cells (Yu et al. 2013).

Tamoxifen directly competes with 17β-estradiol for ER binding in ER⁺-breast cancer cells (Surmacz 2000). It inhibits IGFs/IGF-1R-dependent growth through the down-regulation of IGF-1R (Chan et al. 2001), insulin-like growth factor binding protein (IGFBP)-2 (Juncker-Jensen et al. 2006) and plasma IGF-1 in breast cancer patients (Campbell et al. 2001). IGF-1R expression is up regulated in tamoxifen-resistant breast cancer cells (Massarweh et al. 2008). In normal circulation and extracellular surrounding, IGFs bind with high affinity to IGFBP-1 and -2. IGFBP-2 is highly expressed in tamoxifen-resistant breast cancer cells (Juncker-Jensen et al. 2006). However, not many studies have been done on the interaction of IGFs and their IGFBPs, particularly in pathogenesis of cancer. Autophosphorylation of IGF-1R results in the activation of Ras/Braf/ERKs of the mitogen-activated protein kinase (MAPK) proliferative signalling cascade in ER⁺-breast epithelial cells (Zhang et al. 2011). B-raf, a serine-threonine-specific protein kinase is activated downstream of the small G-protein, Ras. It subsequently activates the MAPK extracellular

signal-regulated kinases (MEKs) and extracellular signal-regulated kinases (ERKs) of MAPK cascade (Holderfield et al. 2013). ERKs signalling pathway is crucial in transmitting proliferative signals generated by cell surface receptors and cytoplasmic signalling components into the nucleus (Holderfield et al. 2013; Zhang et al. 2011). B-raf mutation has been reported in 50-70% of human melanomas (Stones et al. 2013). Its expression is increased in acquired tamoxifen-resistant cells leading to agonist activity of tamoxifen (Jalili et al. 2012).

IGF-1R signalling pathway and its key components may be directly involved in anti-estrogen resistance of ER⁺-breast cancer cells and suggested as potential targets in combined anti-estrogen therapy (Zhang et al. 2011). Therefore, the present study was carried out to investigate whether aloe emodin-induced apoptosis in breast cancer is mediated by IGF-1R signalling as reported in tamoxifen.

MATERIALS AND METHODS

CELLS AND TREATMENTS

ER⁺-breast cancer cells, MCF-7 were cultured in complete RPMI 1640 media, supplemented with 10% fetal bovine serum; 1% 10,000 IU penicillin and 10000 ug/mL streptomycin. Non-tumorigenic breast epithelial cells, MCF-10A used as control were maintained in complete DMEM high glucose media, supplemented with 5% horse serum, 20 ng/mL of epidermal growth factor, 0.5 mg/mL of hydrocortisone, 10 μg/mL insulin and 1% of penicillin and streptomycin. Both cells (American Type Cell Collection, USA) were routinely maintained as monolayer in humidified atmosphere of 5% CO_2, at 37°C in T25 flask up to 70-80% of confluence. For treatment, both aloe emodin and tamoxifen stock solutions were dissolved in dimethyl sulfoxide (DMSO) at 500 μM and kept at -20ºC. These stock solutions were diluted in culture media so that the final concentration of DMSO in the culture media was <0.1%. Aloe emodin, tamoxifen and DMSO were purchased from Sigma Chemical Co (USA). The other chemicals used were from GIBCO Invitrogen (USA). T25 flask and disposable equipments were from Orange Scientific (Belgium). All the procedures followed were in accordance with current ethical standards.

VIABILITY ASSAY

Cells were seeded at 4×10^5 in 6-well plates and treated separately with aloe emodin and tamoxifen at respective IC_{50} of 80 and 27 μM up to 72 h. The IC_{50} were obtained from our previous results using MTT proliferation assay (Amin et al. 2013). As no IC_{50} was obtained for aloe emodin treatment in MCF-10A, its IC_{50} for MCF-7 was used. The morphology of cells was observed under Olympus inverted light microscope (Japan) at 100× magnification. Cells were then detached using accutase and stained with trypan blue dye at 10 fold dilution. A viable cell was presented with intact membrane and clear cytoplasm because it

was able to exclude the dye as compared to blue-colored cytoplasm of a non-viable cell. The cells were loaded into a hemocytometer (Marienfeld-Superior, Germany) and counted under Olympus inverted light microscope at 100× magnification. All chemicals used were from GIBCO Invitrogen (USA), while 6-well plate and disposable equipments were from Orange Scientific (Belgium).

APOPTOSIS ASSAY

Cells were treated separately with aloe emodin and tamoxifen at respective IC_{50} concentrations for 72 h. Their effects on cell apoptosis were quantified using AnnexinV-FITC/PI assay kit following the manufacturer's direction (BD Biosciences Pharmingen, USA). Annexin V has the ability to conjugate with phosphatidylserine, a component of membrane phospholipid that was exposed to the extracellular environment of membranes during early stage of apoptosis. Propidium iodide (PI) is a unique dye that was able to penetrate only the cells that have lost their membrane plasma integrity, thus making it as a good indicator during the late stage of apoptosis or necrosis. Both of these fluorescence dyes were measured using Calibur flow cytometry analyses (BD Biosciences Pharmingen, USA).

mRNA EXPRESSION ANALYSIS

The expression of IGF-1R, IGFBP-2 and B-raf was carried out using QuantiGene 2.0 Plex assay (Affymetrix, USA) that employed direct quantification on aloe emodin and tamoxifen-treated against untreated cells. Prior to the experiment, cells were seeded separately at 1×10^6 in T25 flask (Orange Scientific, Belgium) with aloe emodin and tamoxifen at their respective IC_{50} concentrations. After 72 h of incubation, cells were washed with phosphate buffered saline (Sigma Chemical Co, USA), detached and centrifuged. Cell pellets were resuspended and adjusted to approximately 1×10^6 cells per mL in culture media and kept at -20°C before used. The assay was conducted following the protocol described in QuantiGene 2.0 Plex User Manual. Approximately 2×10^4 cells were loaded into each of a 96 well-plate for the detection of the specific-target RNA. Initially, cells were lysed to release the RNA. Capture beads were added to the lysis mixture and bind to the free RNA molecules. Next, the target-specific RNA was measured by mixing it with high stringent of cocktails which include capture extenders, label extenders and blocking probes. They bound to the region of RNA and selectively captured the target RNA by a series of complex hybridization. The hybridization step was carried out overnight at 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 the 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. Target hybridization; signal amplification and detection analysis was conducted at I-DNA Biotechnology (M) Sdn. Bhd., Malaysia).

STATISTICAL ANALYSIS

Each experiment was carried out in triplicates and the data obtained were expressed as the mean±standard deviation. Statistical comparisons were conducted using paired-student t-test and two way analysis of variance (ANOVA) test and significance was set at $p<0.05$.

RESULTS

CELL VIABILITY EFFECT OF ALOE EMODIN AND TAMOXIFEN ON MCF-7

The effect of aloe emodin and tamoxifen on the number of viable cells in proliferation was examined using trypan blue exclusion test. Using MCF-7 IC_{50} as a reference, aloe emodin was found to inhibit the viability of MCF-7 by 62.86±10.36%, ($p<0.05$, $n=3$) while not affecting MCF-10A. In contrast, tamoxifen was non-selective as it inhibited the viability of both MCF-7 (IC_{50} of 27 µM) and MCF-10A cells (IC_{50} of 38 µM) by 34.55±15.05% and 44.50±11.33% ($p<0.05$, $n=3$), respectively (Figure 1). These results verified our previous findings of aloe emodin potential as a selective anti-proliferative agent to ER+-breast cancer cells, MCF-7 (Amin et al. 2013).
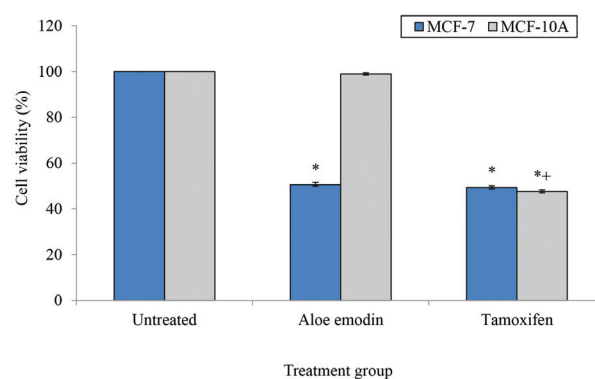
*Significant compared with untreated cells, $p<0.05$; +Significant difference between the effect of aloe emodin on MCF-10A viability compared with tamoxifen, $p<0.05$

FIGURE 1. Aloe emodin and tamoxifen effects on cell viability

CELL APOPTOSIS EFFECT OF ALOE EMODIN AND TAMOXIFEN-TREATED MCF-7

Aloe emodin- and tamoxifen-induced cell death in MCF-7 cells was investigated using Annexin V-FITC/PI staining. The results generated from flow cytometer analysis were presented as scatter plots seen in each graph with four distinguished regions (Figure 2(a)). Cells residing in the upper right (UR) and the lower right (LR) on each side of the scatter plots were indicated as late and early apoptosis, respectively. Figure 2(a) is the representative findings of Annexin V-FITC/PI staining on MCF-7 cells ($n=3$). The

overall percentage of apoptosis obtained from the above assay was calculated by combining the percentage of the all three sets of UR and LR (Figure 2(b)).

The apoptotic effect of aloe emodin on MCF-7 was clearly seen with the increased population on both UR and LR at 72 h of treatment. As indicated in Figure 2(b), aloe emodin and tamoxifen induced apoptosis in MCF-7 by 29.3±0.55% ($p<0.05$, $n=3$) and 30.2±0.56% ($p<0.05$, $n=3$) at 72 h, respectively. It is interesting to note that aloe emodin apoptotic activity was similar to tamoxifen.

ALOE EMODIN AND TAMOXIFEN EFFECT ON IGF-1R, IGFBP-2 AND B-RAF GENE EXPRESSION IN MCF-7

We sought to study the expression of IGF-1R and its downstream genes (IGFBP-2 and B-raf) on both aloe emodin- and tamoxifen-treated MCF-7. Three housekeeping genes were used i.e. hypoxanthine-guanine phosphoribosyltransferase (HPRT), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and β-actin. However significant differences were only seen after normalizing with HPRT. For tamoxifen-treated MCF-7, as expected IGF-1R and its key signalling components (IGFBP-2 and B-raf)

were found to be down regulated. The highest changes was seen in IGFBP-2 (77%; $p<0.05$, $n=3$), followed by IGF-1R (75%; $p<0.05$, $n=3$) and B-raf (42%; $p<0.05$, $n=3$). Aloe emodin-treated cells show similar story, down regulation of IGF-1R and its downstream target genes. As indicated in Figure 3, the highest changes were seen in IGFBP-2 (63%; $p<0.05$, $n=3$), followed by IGF-1R (53%; $p<0.05$, $n=3$), B-raf (41%; $p<0.05$, $n=3$). Interestingly, aloe emodin was as efficient as tamoxifen in down regulating IGFBP-2 and B-raf (Figure 3).

DISCUSSION

Our findings have provided insight view of aloe emodin involvement in transducing apoptosis signalling in ER+-breast cancer cells, MCF-7. Our preliminary study has shown that it has selective cytotoxic effect on MCF-7 with IC_{50} of 80 μM, but without affecting control breast cells, MCF-10A (Amin et al. 2013). The results from trypan blue exclusion test were in concordance with the proliferation assay. Consistently, our results were in accordance with other cancer cells such as prostate (Lin et al. 2006),
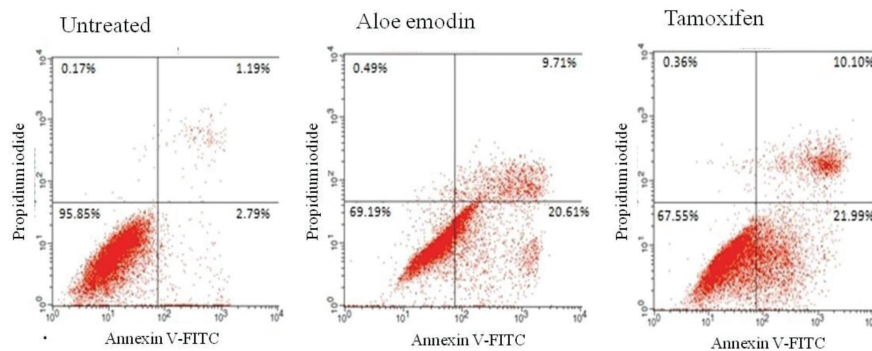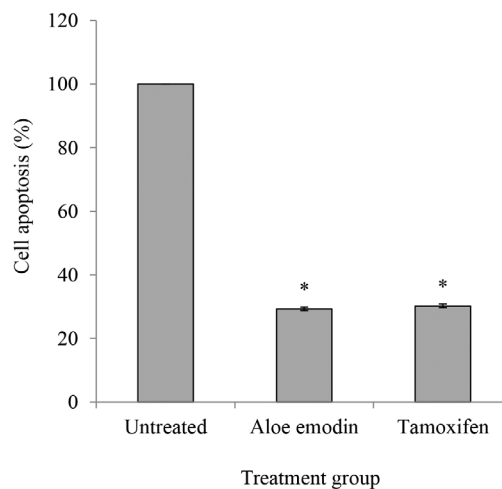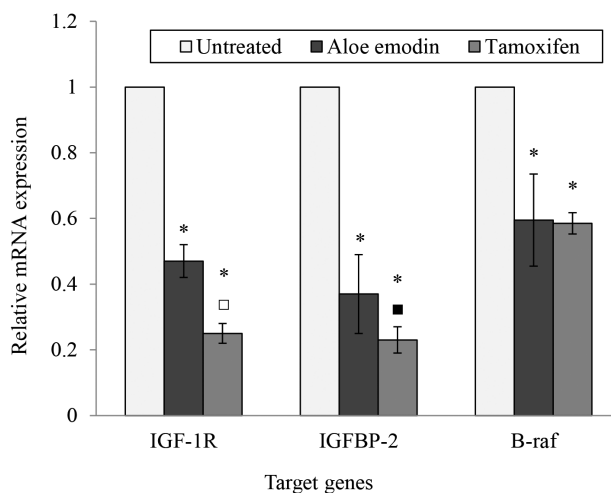

FIGURE 2. (a) PI and Annexin V-FITC staining of MCF-7 cell apoptosis analysed by Calibur flow cytometry

*Significant compared to untreated cells, $p<0.05$

FIGURE 2. (b) The percentages of apoptosis induce by aloe emodin and tamoxifen on MCF-7

*Significant compared to untreated cells, $p<0.05$; □Significant difference between the expressions of IGF-1R in aloe emodin treatment group compared to tamoxifen, $p<0.05$; •Significant difference between the expressions of IGFBP-2 in aloe emodin treatment group compared to tamoxifen, $p<0.05$

FIGURE 3. The graph showed the expression of genes in MCF-7 after treatment with aloe emodin and tamoxifen at their respective IC_{50} against untreated cells

hepatoma (Lu et al. 2007) and colon (Suboj et al. 2012). Numerous reports investigating the anti-proliferative effect of aloe emodin have proposed the importance of apoptosis as the mode of death induced in various cancer cells (Lin et al. 2006; Lu et al. 2007; Suboj et al. 2012). However, its action on ER+-breast cells and in comparison with tamoxifen is not well known. Therefore we aimed to compare its effect with tamoxifen on ER+-breast cancer cells, MCF-7. Using Annexin V-FITC/PI staining, we observed the presence early apoptotic and late apoptotic cells after 72 h of aloe emodin and tamoxifen treatments in MCF-7 as compared to untreated cells. Aloe emodin apoptotic activity was similar to tamoxifen.

Even though the relationship between the aloe emodin and apoptosis effect in MCF-7 cells was obvious, little knowledge was available on its underlying signalling pathways. Since its effects on MCF-7 were similar with tamoxifen, we then investigated the pathway that was known to be involved in tamoxifen-induced cell death of MCF-7. Therefore, we investigated the involvement of IGF-1R, IGFBP-2 and B-raf genes in aloe emodin-treated cells as compared with tamoxifen using QuantiGene 2 Plex Assay. This method was favoured compared with a normal real time (RT)-PCR as it did not required total RNA purification or reverse transcription processes. Thus, by applying direct quantification on its biological source, much variability introduced by pre-processing samples can be eliminated (Ariffin 2013).

The IGF system has the potential to prevent apoptosis by promoting the survival-signal. Once activated by IGFs, it stimulated the activation of Ras/Braf/ERKs of MAPK cascade and ultimately proliferation (Zhang et al. 2011). Clinically, IGF-1R is commonly over expressed in primary breast tumors (Foulstone et al. 2013) and phosphorylated in all breast cancer subtypes (Law et al. 2008). Its expression is fundamental in facilitating cell survival and metastasis in breast neoplastic cell lines (Sachdev et al. 2010). IGF-IR and ERα are co-expressed in ER+-breast tumours. Moreover, estrogens stimulate the expression of IGF-IR; while anti-estrogen such as tamoxifen down regulate its signalling (Surmacz 2000). It is up regulated in tamoxifen-resistant breast cancer cells (Massarweh et al. 2008). The elevation of IGFBP-2 expression has also been associated with breast tumor (So et al. 2008). Observation using clinical, *in vivo* and *in vitro* breast cancer samples, have found that the expression of IGFBP-2 was higher comparing to benign samples (So et al. 2008). The expression of both IGFBP-2 and IGF-1R were much related as the exogenous action of IGFBP-2 on ERα in breast cancer cells was via the involvement of IGF-1R and activation of PI3K (Foulstone et al. 2013). Similarly to tamoxifen, we observed that IGF-1R, IGFBP-2 and B-raf gene expressions were down regulated in aloe emodin-treated MCF-7. Thus, we believed that aloe emodin has potential as an inhibitor in the drug therapy targeting through the IGFs pathway. However, further research on mRNA and protein expressions *in vivo* and *in vitro* are required to gain more information on the inter- and intra-relationship of these target genes.

CONCLUSION

In conclusion, we found that aloe emodin selectively inhibited the proliferation ER+-breast cancer cells, MCF-7, but not control breast cells, MCF-10A. In contrary, tamoxifen inhibited both cell proliferations. Interestingly, aloe emodin induced apoptosis in MCF-7 through similar pathway as tamoxifen i.e., down regulation of IGF-1R, IGFBP-2 and B-raf. These findings suggested aloe emodin can be used in combination with tamoxifen to enhance its efficacy in ER+-breast cancer treatment. These findings

might be a good baseline for further investigation in this particular research.

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