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Involvement of Intracellular Calcium and MAPK in Aloe Emodin Induced Anti-Proliferative in MCF-7

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Aloe emodin, an anthraquinone of *Aloe barbadensis* Miller has been shown to have more cytotoxic effect in different kinds of human cancer cell lines compared to normal. Accordingly, we found it to selectively inhibit the proliferation of oestrogen-receptor-positive-(ER+)-breast cancer cells, MCF-7; but not controls cells, MCF-10A. However, its precise mechanism is not well understood. Several studies have shown that there is evidence of increased intracellular calcium (Ca^{2+}), both at early and late stage of apoptosis which associated with the down-regulation of ERK1/2 proliferative pathway. Therefore, we aim to elucidate the involvement of intracellular Ca^{2+} in aloe emodin induced apoptosis on MCF-7. Apoptotic morphological changes were observed under fluorescence microscope. The involvement of cytoplasmic Ca^{2+} and MAPKs were investigated using Fluo-4 intracellular Ca^{2+} imaging and QuantiGene 2.0 Plex assay, respectively. IC_{50} of aloe emodin (80 μM) at 72 hours incubation was used. Data were evaluated using the one-way or two-way ANOVA tests. Our results indicated that aloe emodin at IC50 80μM induced apoptosis on MCF-7 through the association of intracellular Ca^{2+} signalling. This observation include a significant increased ($p<0.05$) in Ca^{2+} after treatment with aloe emodin compared to untreated control. Furthermore, the downregulation of H-Ras, MEK1/2 and ERK1/2 expression also observed suggesting the involvement of MAPK pathway.

Key words: Aloe emodin, proliferation, intracellular Ca^{2+}, MCF-7 cells

Calcium ion (Ca^{2+}) acts as a second messenger in the regulation of cell cycle, differentiation and apoptosis; and ultimately determining cell's fate (Yang *et al.* 2010). Cellular Ca^{2+} circulation are compartmentalised into extracellular space, cytoplasm, endoplasmic reticulum and mitochondria by their respective membranes which incorporate specific Ca^{2+} channels and binding proteins necessary for Ca^{2+} signalling. Alteration of the intracellular Ca^{2+} concentration, $(Ca^{2+})i$, can lead to cell apoptosis. Hence, there should be an equilibrium in the mechanisms of cell proliferation and apoptosis, otherwise a defect occurs during the processes may lead to development of cancer.

The transformation of a normal cell into a cancer cell is associated with a major re-arrangement of Ca^{2+} pumps, Na/Ca exchangers and Ca^{2+} channels, which leads to the enhanced proliferation and impaired ability of cells to die (Capiod *et al.* 2007). The apoptotic activity is induced when Ca^{2+} enters cells from the extracellular via plasma membrane after treatment with Ca^{2+} ionophores or is mobilised from the endoplasmic reticulum using thapsigargin, an inhibitor of the specific endoplasmic reticulum Ca^{2+} pump (Mattson and Chan, 2003; Lipskaia *et al*. 2009). Interestingly, lung cancer has been characterised by altered expression of specific Ca^{2+} channels and Ca^{2+}-binding proteins. In addition, in the story of drug resistance of lung cancer, homeostasis of the endoplasmic reticulum of Ca^{2+} has been shown to be disturbed (Yang *et al*. 2010).

Recently, one of the Ca^{2+} channel/pump that is expressed in the breast, SERCA3 has been proven to undergo significant changes during the tumorigenesis which further proven Ca^{2+} deregulation is playing a roles in breast cancer development (Papp & Brouland 2011). One of the diagnostic criteria of breast cancer is the feature of calcification which can be diagnosed through mammographic screening of the breast (Tse *et al.* 2008). This calcification could be as a result of intracellular Ca^{2+} deregulation. Therefore,

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by understanding the mechanisms of apoptosis in breast cancer cells, including their Ca^{2+}-dependency, open a strategy for developing a new anti-cancer agent in breast cancer treatment (Roberts & Der 2007). The involvement of mitogen-activated protein kinase (MAPK) pathways in cancer has been widely studied and suggested to play an important role in regulating cell proliferation. Furthermore, more evidence demonstrated that the involvement of MAPK pathways in transmitting distinct cellular effects in different cell types and more complicated in the story of tumorigenesis. Regulation of intracellular calcium involves activation of MAPK pathway in normal and cancer cells, suggesting intracellular calcium level is influenced by MAPK activity (Park *et al*. 2011)

Over the past years, many natural compounds from herb and fruit extracts have been used in the search for new substances with anti-cancer effects which includes Aloe vera. Aloe emodin (1,8-dihydroxy-3-hydroxymethyl-anthraquinone), a herbal anthraquinone derivative of *Aloe barbadensis* Miller (Aloe vera) has been postulated to efficiently limited the proliferation of cervical cancer cells, HeLa (Wang *et al.* 2013) and hepatoma cancer cells, HCC (Subramaniam *et al*. 2013) through the activation of both apoptotic extrinsic death receptor pathway (Wang *et al*. 2013; Subramaniam *et al*. 2013) and intrinsic mitochondrial pathway ([b]Wang *et al*. 2013). Moreover, studies also indicate that aloe emodin produces less or no cytotoxic effect on several normal cells, including fibroblasts (Pecere *et al*. 2000) and hemopoietic progenitor cells (Pecere *et al*. 2000). Accordingly, we found aloe emodin selectively inhibited the proliferation of oestrogen receptor-positive-(ER^+)-breast cells, MCF-7 but not control cells (Amin *et al.* 2013).

To date, the effects of aloe emodin on the regulation of Ca^{2+} signalling in breast cancer cells proliferation are not well understood. Therefore, this study aims to elucidate the cytoplasmic Ca^{2+} signalling in aloe emodin-suppressed proliferation of MCF-7. We hypothesized aloe emodin inhibits the proliferation of MCF-7 cells through the apoptotic signalling pathway as indicated by the increasing of intracellular Ca^{2+} level. Further understanding and identifying the role of cytoplasmic Ca^{2+} in aloe emodin-treated MCF-7 cells will enable us to understand the basic regulation of its actions.

MATERIALS AND METHODS

Treatment Preparation

Aloe emodin, tamoxifen and thapsigargin respective weights were measured according to the desired concentrations and diluted in dimethyl sulfoxide (DMSO). Each stock solution was filtered (Orange Scientific, Belgium) and stored in aliquots at –20ºC. Final working solutions were prepared by diluting the respective stock solutions with the culture media and Hank's Balanced Salt Solution (HBSS). Tamoxifen was used as positive control for apoptosis study, while thapsigargin used for intracellular Ca^{2+} study. It was reported to increase intracellular Ca^{2+} concentration by cytosolic accumulation of Ca^{2+} (Son *et al.* 2014). The preparations were performed in a sterile tissue culture hood. All the above chemicals were purchased from Sigma Chemical Company, USA.

Cell Culture

MCF-7, an ER^+-breast adenocarcinoma cells was purchased from ATCC (American Type Cell Collection, USA), and derived from metastatic pleural effusion of 69-year-old Caucasian female patient (Debnath *et al.* 2003). Cells were maintained in complete RPMI media, supplemented with 10% foetal bovine serum; and 1% penicillin and streptomycin as monolayer at 80% confluence in T25 flasks, and incubated at 37ºC in 5% CO_2. All the experiments were performed in the tissue culture hood using tissue culture grade chemicals (GIBCO Invitrogen, USA) and disposable equipment (Orange Scientific, Belgium).

Apoptosis Detection of Acridine Orange (AO) and Propidium Iodide (PI) - Stained Cells

Previously, we found aloe emodin, an anthraquinone to inhibit the proliferation of MCF-7 breast cancer cells, with IC_{50} of 80 µM, but not affecting control breast cells, MCF-10A (Amin *et al*. 2013). Contrary, tamoxifen was non-selective to both cells with IC_{50} of 27 µM and 38 µM, respectively (Amin *et al*. 2013). The anti-proliferative mechanism induced by aloe emodin and tamoxifen on MCF-7 cells was determined by morphological assessment using fluorescence staining.

The apoptotic activity was verified by observing the morphological changes of acridine orange (AO) and propidium iodide (PI)-stained MCF-7 cells treated with aloe emodin under fluorescence microscope. Cells were seeded at 4×10^5 in a 6-well plate (Orange Scientifics, Belgium) and treated with aloe emodin at IC_{50} concentration obtained from previous proliferation study (Amin *et al*. 2013). Both untreated (>0.1% DMSO) and treated cells were incubated in 5% CO_2 at 37ºC for 72 hours. Tamoxifen was used as positive control. Prior to seeding, sterile cover slide was inserted into each well. After washing with phosphate-buffer saline (PBS), 2.0 µL of staining dyes consisting of AO (1 mg/mL) and PI (1 mg/mL) were added. Within 30 minutes, the fluorescence images were captured using fluorescence microscope (Olympus, USA) at 100X magnification before the fluorescence colour starts to fade. A viable cell was identified by the appearance of a large green, intact nucleus

with a well-defined cell membrane whereas a non-viable apoptotic cell appeared as a bright green shrinking cell with a condensed or fragmented nucleus. Its bright green is due to the mixture AO and PI dyes. The occurrence of necrosis in a necrotic non-viable cell with disrupted cell membranes was confirmed by the presence of orange tint stained by PI (Abdel Wahab *et al*. 2009). After another washing, each cover slide was removed from each well and placed into a hemocytometer. Four subgrids on the hemocytometer containing ≥200 to 300 cells were counted to determine the percentages of viable, apoptotic and necrotic cells. The above tissue culture grade chemicals and disposable equipment were purchased from Sigma Chemical Company (USA) and Orange Scientific (Belgium), respectively.

Intracellular Ca^{2+} Imaging

The involvement of Ca^{2+} in the anti-proliferative regulation on MCF-7 was investigated using intracellular Ca^{2+} imaging. 3×10^6 cells were cultured in 35-mm glass bottom dish (MatTek.Corp, USA) for 24 hours. A 5 µM of fluorescent Ca^{2+} indicator (Fluo-4 AM) dye in DMSO and 0.1% pluronic F-127 in HBSS (Invitrogen Gibco, USA) was used as loading solution to measure the intracellular Ca^{2+} concentration. Prior to loading of 2 ml of Fluo-4 AM dye; cells were washed with 2 ml of HBSS for three times. The cells were incubated at 37ºC in 5% CO_2 for 45 minutes. After the incubation time, the loading solution was aspirated gently and the cells were washed again with 2 ml of HBSS for three times. For de-esterification of the dye, cells were incubated with 2 ml of HBSS for 20 minutes in 37ºC in 5% CO_2 to remove the excess fluo-4 AM dye and also to promote hydrolysis of AM-esters. All the above steps were performed in sterile tissue culture laminar flow hood.

Intracellular Ca^{2+} dynamics of live cells imaging were performed using Confocal Laser Scanning Microscope (Leica Microsystems, Germany). Fluo-4 AM will be excited by 488 nm line of an Argon laser with the emission of 516 nm. Relative level of intracellular Ca^{2+} concentration was represented by the brightness of the fluorescent signals. Time scan was started by monitoring the untreated cells that represented basal level of fluo-4 for 10 minutes. Prior to the next recording, 1 µM thapsigargin (in HBSS) was added and was monitored for 1 hour with the exposure time 3 seconds. For aloe emodin (in HBSS), basal record was recorded similar to thapsigargin basal record that was 10 minutes. After aloe emodin treatment at its IC50 concentration, the recording was done for 3 hours with similar 3 seconds exposure time. Offline data analysis was used to measure green channel intensity at each time point to generate the intensity plot over time. For all imaging, it was done at 20X lens magnification at 37ºC and 5% CO_2.

mRNA Expression Analysis

The expression of H-Ras, mitogen-activated protein kinase kinase (MEK)1/2 and extracellular-signal regulated kinase (ERK)1/2 genes of the MAPK proliferative signalling pathways was determined 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, 1×10^6 cells were treated with aloe emodin and tamoxifen at their respective IC50 concentrations for 72 hours. Cell pellets were resuspended and adjusted to approximately 1×10^6 cells per mL in culture media and kept at –20ºC before used. The assay was conducted following the protocol described in QuantiGene 2.0 Plex User Manual.

Statistical Analysis

Each experiment was carried out in triplicates and the data obtained were expressed as the mean ± standard deviation. The Statistical Package for Social Sciences (SPSS) version 16.0 was used to analyse the data. The differences between the groups were evaluated using the one-way or two-way ANOVA tests. The results were expressed as mean ± SD. The statistically significance was set at $p<0.05$.

RESULTS

Cell Apoptosis

The anti-proliferative mechanism induced by aloe emodin at its IC_{50} concentration (Amin *et al*. 2013) was determined through morphological evaluation of AO and PI-stained MCF-7 cells observed under fluorescence microscope.

Intracellular Ca^{2+} Dynamics in Aloe Emodin Treated MCF-7 Cells

In the intracellular Ca^{2+} dynamics study, the MCF-7 fluo-4 intensity changes were measured over time for control (untreated), aloe emodin and thapsigargin (positive control) by using confocal laser scanning microscope. For control, intracellular Ca^{2+} dynamics were measured by measuring fluo-4 intensity without any treatment for 60 seconds. The control measurement obtained is reflecting the basal level of the intracellular Ca^{2+} for MCF-7 cells before any stimulation or reaction. After control measurements were obtained, MCF-7 cells were treated either with aloe emodin or thapsigargin for 60 minutes respectively. Thus, the changes in the intracellular Ca^{2+} level of MCF-7 cells after treatments were measured and calculated over control.

As shown in Figure 2a, cytosolic Ca^{2+} measurements of control (untreated) and showing the changes for three

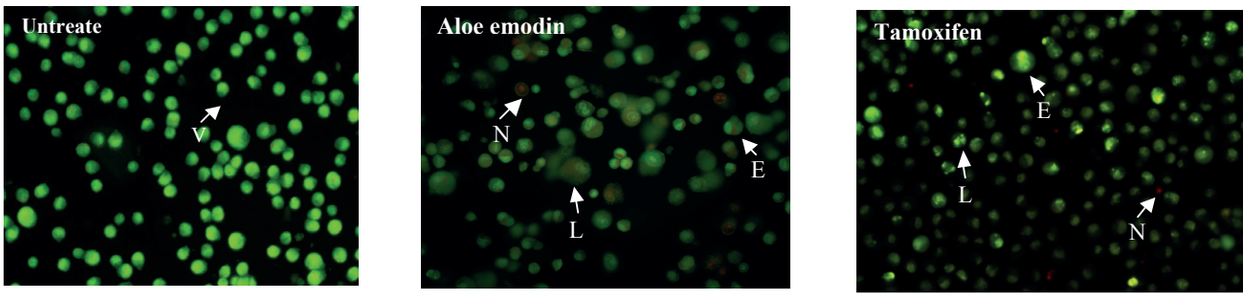

Figure 1a. Images of aloe emodin treatment in MCF-7 cells with distinguished different morphological apoptosis stages.

Note: By applying AO and PI dual staining, percentages of viable (V), early apoptosis (EA), late apoptosis (LA) and necrosis (N) cells were determined and represented by the images above. Apoptosis were characterized by membrane blebbing and fragmented nucleus seen in aloe emodin treatment compared to untreated cells. The percentage of these stages obtained from all three set of experiments was represented in the Figure 1C.

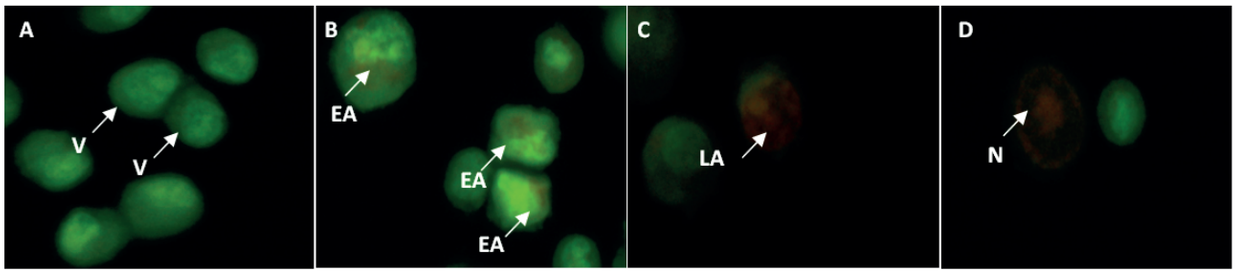

Figure 1b. Enlargement images of aloe emodin- and -tamoxifen treated cells with distinguished different morphological apoptosis stages.

Note: A viable (V) cell appeared as green with intact nucleus (A). Early apoptosis (EA) features were seen as bright green colour due to the intercalation of AO and PI in the nucleus (B). Late apoptosis (LA) were observed as visible red/ orange colour with the generation of the highest PI intensity emission (C). Necrotic cells were seen with disrupted cell membranes and the presence of orange tint stained by PI. All cells were treated for 48 hours. The results shown were representative of three independent experiments.

experiments and the average from them which indicating the basal level of the intracellular Ca^{2+}. After aloe emodin were added, the peak of increase in fluo-4 intensity over time were observed approximately after 10 minutes of aloe emodin addition and then gradually decreased till the basal level. In this study thapsigargin is used as positive control, since it was reported to induce increased in intracellular Ca^{2+} concentration by cytosolic accumulation of Ca^{2+} (Son *et al*. 2014).

After thapsigargin addition, the peak of the fluo-4 intensity changes rapidly increases after approximately 2 minutes post thapsigargin addition. The peak of the fluo-4 intensity which represents the changes of the cytosolic Ca^{2+} dynamic obtained for control, aloe emodin and thapsigargin were analysed and graph was plotted against control, aloe emodin and thapsigargin treatment (Figure 2b). Overall, the results indicate the treatment of MCF-7 cells shows a statistically significance change in cytoplasmic Ca^{2+} peak after treatment with aloe emodin (mean ± SD: 10.38 ± 1.02; $p<0.05$, n = 3) in comparison to untreated cells, control (5.55 ± 0.35). Thapsigargin treatment shows an expected increase in cytoplasmic Ca^{2+} intensity (25.39 ± 0.59; $p<0.05$, n = 3).

Involvement of MAPK Signalling Genes in Aloe Emodin Anti-Proliferative Effects in MCF-7

The expression of MAPK signalling cascade genes were measured using QuantiGene 2.0 Plex assay. *Hypoxanthine-guanine phosphoribosyltransferase* (HPRT) was used as a housekeeping gene, and significant differences were only seen after normalising with HPRT. Figure 3 shows, all the target genes ; H-Ras (a), MEK1/2 (b) and ERK1/2 (c) were down regulated significantly by 50.1±8.3% ($p<0.05$, n =

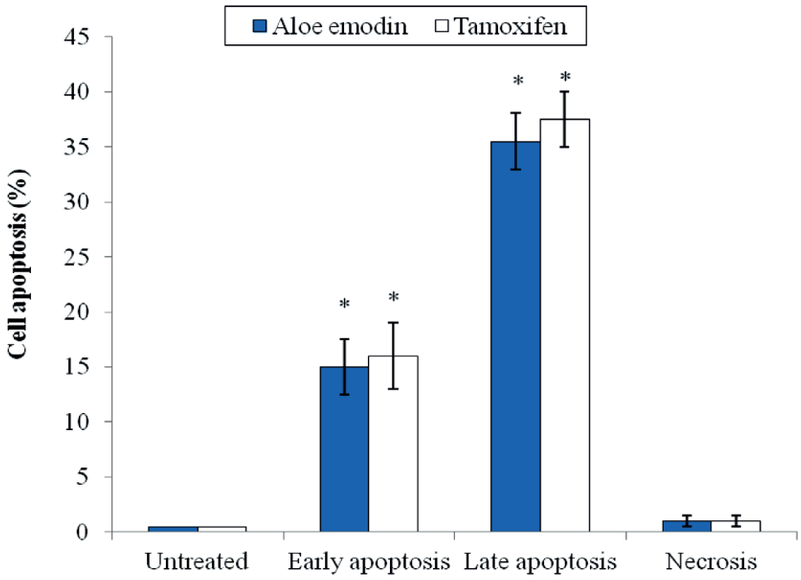

Figure 1c. The percentages of apoptosis induced by aloe emodin on MCF-7.

Note: This graph was generated from all three sets of experiments. As shown in the graph above, approximately 15.0±5.5% of early (EA) and 35.5 ± 5.6% of late stages of apoptosis (LA) were clearly seen in aloe emodin treatment on MCF-7 compared to untreated cells ($p<0.05$, n = 3). Similarly, tamoxifen induced about 16.0 ± 3.0% of early (EA; $p<0.05$, n = 3) and 37.5 ± 2.5% ($p<0.05$, n = 3) of late stages of apoptosis (LA) in MCF-7. Only 1.0 ± 0.5% of necrosis was seen in both treatments. *Significant as compared to untreated cells; $p<0.05$.

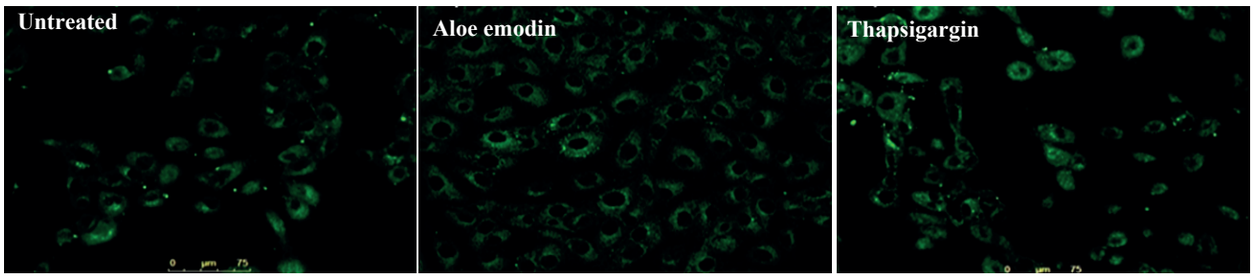

Figure 2a. The live intracellular Ca^{2+} imaging of aloe emodin treatment on MCF-7 (a) and its mean peak levels of fluo-4 intensity (b) as compared to untreated cells.

Note: Aloe emodin, at its IC50 concentration stimulates the uptake of Ca^{2+} as seen by the live imaging of MCF-7 cells loaded with fluo-4AM as compared to untreated cells The results above was presented as Fluo-4 intensity changes over time which is proportional with the cytosolic Ca^{2+} changes upon aloe emodin and thapsigargin addition (Figure 2b).

3), 9.6 ± 4.8% ($p<0.05$, n = 3) and 11.5 ± 6.2% ($p<0.05$, n = 3), respectively in aloe emodin treatment. Similarly, in tamoxifen, the expression of all those genes were downregulated by 28.1 ± 9.5% ($p<0.05$, n = 3), 25.7 ± 6.8% ($p<0.05$, n = 3) and 18.9 ± 8.9% ($p<0.05$, n = 3), respectively. It was interesting to note that using IC_{50} as reference, aloe emodin was more efficient compared to tamoxifen in H-Ras down regulation.

DISCUSSION

Aloe emodin is a natural active compound derived from hydroxyanthraquinone extracted from Aloe vera or known as *Aloe barbadensis* Miller's. For several years, an extensively research done to discover the medicinal properties aloe emodin and among the important findings showed that aloe emodin exhibits (Tanaka *et al.* 2006),

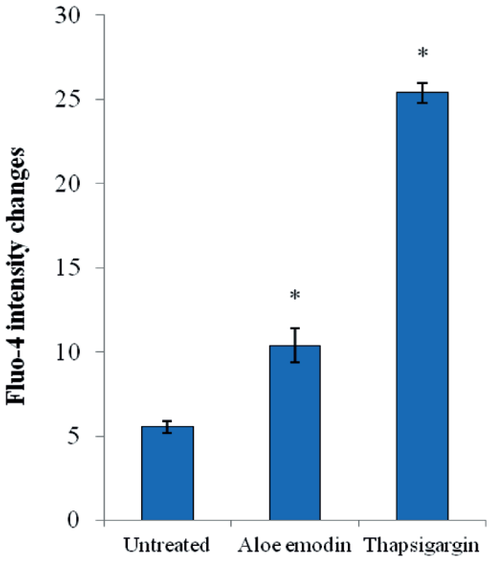

Figure 2b. The peak level of Ca^{2+} changes after aloe emodin and thapsigargin treatment from the curve was presented as mean ± SD with p<0.05.

Note: Aloe emodin shows statistically significant cytosolic Ca^{2+} measurement with 10.38 ± 1.02 (p = 0.002, n = 3) in comparison to untreated cells, control (5.55 ± 0.35, n = 3). For thapsigargin treatment, it shows an expected increase in cytoplasmic Ca^{2+} intensity of 25.39 ± 0.59 (p = 0.006, n = 3). *Significant as compared to untreated cells at p<0.05.

anti-oxidant (Yu *et al*. 2009), anti-inflammatory (Da *et al*. 2011) and anti-cancer (Ahirwar & Jain 2011) activities. In the study of cancer, aloe emodin has been shown to inhibit cell proliferation and decrease cell viability. This anti-proliferative property was reported in various cancer cell lines such as hepatoma (Kuo *et al*. 2002), bladder (Lin *et al*. 2006), cervical (Guo *et al*. 2007), oral (Xiao *et al*. 2007), stomach (Guo *et al*. 2008) and lung (Lee *et al*. 2010).

However, its effect on ER^+-breast cancer cells is not well known. Our previous data had demonstrated anti-proliferation effect of aloe emodin on ER^+ breast cancer cells, MCF-7; while no effects was observed in normal breast cells, MCF10A up to the concentration of 150 µM (Amin *et al*. 2013). This indicates aloe emodin is a selective compound that exhibited cytotoxic effect only in cancer cell, suggesting its potential as a good candidate for chemotherapeutic/chemopreventive agent. Similarly, aloe emodin had selectively caused the apoptotic effect on neuroectodermal tumour cells and not for the normal cells (Pecere *et al*. 2000). Furthermore, our findings from morphological assessment on MCF-7 (Figure 1) were concordance with other apoptosis study on different types of cancer cells (Wang *et al*. 2013; Wang *et al*. 2013).

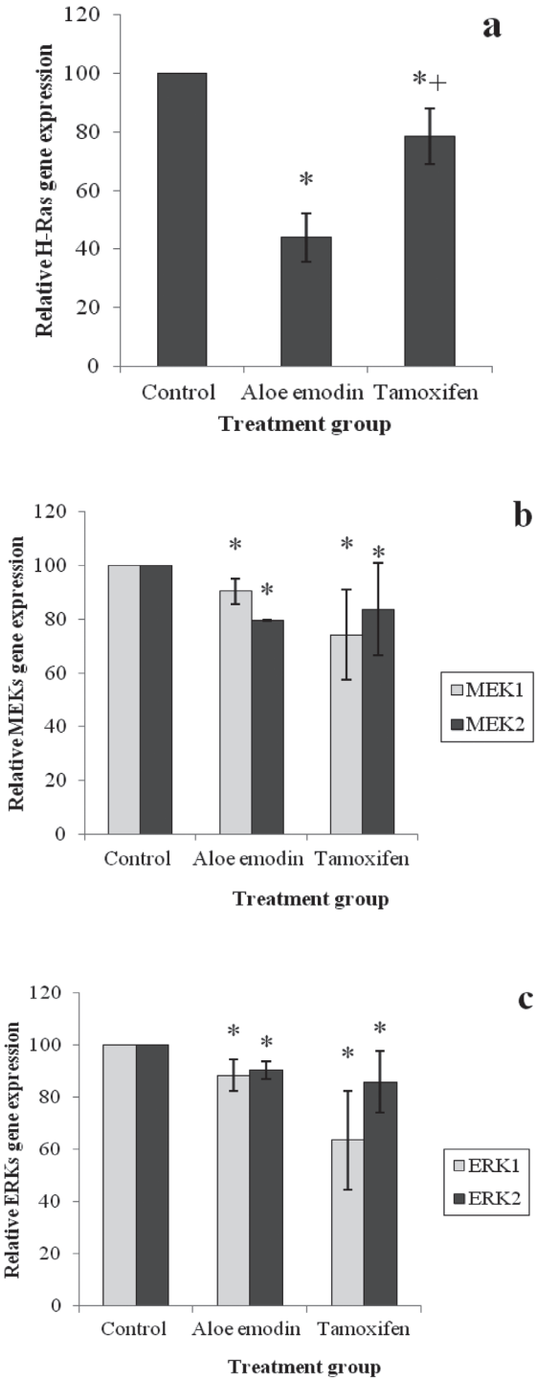

Figure 3. H-Ras (a), MEK1/2 (b) and ERK1/2 (c) genes expression of MCF-7 after treatment with aloe emodin and tamoxifen.

Note: Using Hypoxanthine-guanine phosphoribosyl-transferase (HPRT) as a reference, the fold change of each target genes was calculated. Value of 1.00 is indicated as no changes, above 1.00 as up regulated and below 1.00 as down regulated. The results are presented as percentage. *Significant compared to untreated cells at; p<0.05. +Significant compared between aloe emodin and tamoxifen treated groups at; p<0.05

Ca^{2+} is crucial for the regulation cell's normal physiological function and cell survival. It is known that the increase Ca^{2+} in intracellular concentration subsequently leads to apoptotic initiation. Transient changes in intracellular Ca^{2+} following certain anti-cancer agents and radiation lead to apoptosis (Pereira *et al*. 2002). Several studies have shown that increased level of intracellular Ca^{2+} was seen during stages of both early and late apoptotic pathways (Lynch *et al*. 2000; Pinton *et al*. 2008). In lung cancer, altered expression of channels and binding proteins for Ca^{2+} is characterizing features (Yang *et al*. 2010). Likewise, in breast cancer, altered expression in calcium channel is one of the significant changes observed during tumorigenesis (Williams *et al*. 2013).

The influx of Ca^{2+} through its activated channels and the release of Ca^{2+} from the endoplasmic reticulum have been proposed to be apoptogenic (Pinton *et al*. 2006). A study using human papilloma virus-immortalised keratinocytes (HPV-G) has shown that elevation of intracellular Ca^{2+} due to expose to irradiation has caused an overload of Ca^{2+} in the mitochondria, thus leading to a transient loss of membrane potential and production of reactive oxygen species (ROS) (Lyng *et al*. 2006).

Subsequently, ROS leads to the inactivation of Ras/Raf/ERKs of MAPK proliferation pathways and activation of apoptosis in cancer cells (Azzam *et al.* 2002). Since, MAPK is known as important signalling pathways that control important events in cancer cell migration and invasion, we also investigated the expression of MAPK signalling cascade. Our finding suggested that aloe emodin downregulates the expression of H-Ras, MEK1/2 and ERK1/2 in MCF-7 (Figure 3). Similarly, our positive treatment (tamoxifen) shows downregulation of those genes. Accordingly, emodin enhances the cytotoxicity in lung cancer (Su *et al.* 2009) and glioma cells (Harhaji et al., 2007) through the inactivation of both ERK1 and ERK2. Emodin induces loss of mitochondrial membrane potential in neuroblastoma cells by elevation of ROS content and cytoplasmic free Ca^{2+}, leading to apoptosis (Fu *et al*. 2013).

To date, no studies done to directly correlate the effect of aloe emodin with the involvement of the intracellular Ca^{2+} and MAPK signaling in the breast cancer cells. Another different anthraquinone known as emodin, which is an active compound of Rheum palmatum, has been demonstrated to cause transient increase of intracellular Ca^{2+} in smooth muscle cells of rat colon (Ma *et al*. 2003). Our results had revealed the significant involvement of the of intracellular Ca^{2+} signaling as its level was found to be increased in aloe emodin-treated MCF-7 cells compared to control (Figure 2). However, the molecular mechanisms responsible for these processes remain unclear. Thus, from this study, we believe that an elevated level of intracellular Ca^{2+} is involved in inducing apoptosis in aloe emodin-treated ER^{+}-breast cancer cells, MCF-7.

CONCLUSION

Thus, we believe that aloe emodin-induced apoptosis in MCF-7 is mediated by elevated intracellular Ca^{2+} level. Further understanding and identifying of the role of intracellular Ca^{2+} signalling in aloe emodin-treated MCF-7 may provide an early insight on its potential role as an anti-cancer; thus widen its possibilities as a future alternative chemo-preventive agent in the management of breast cancer patients.

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