Differential cellular interaction of *Sutherlandia frutescens* extracts on tumorigenic and non-tumorigenic breast cells

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ABSTRACT

Sutherlandia frutescens (SF) is a traditional African medicinal aid employed for the treatment of various ailments such as inflammation, pulmonary asthma and congestion. The present study was conducted to demonstrate the differential cellular interaction of aqueous SF extracts in a breast adenocarcinoma epithelial cell line (MCF-7) and a non-tumorigenic breast cell line (MCF-12A) by means of polarization-optical differential interference contrast microscopy, crystal violet staining, light microscopy and flow cytometry. Results showed that aqueous SF extracts induced cell death in MCF-7 and MCF-12A via two types of cell death namely apoptosis and autophagy. Effects on proliferation and cytotoxicity were investigated by means of crystal violet staining. The latter indicated that, at a 1/10 dilution, the tumorigenic MCF-7 cell line was more prominently affected when compared to the non-tumorigenic MCF-12A cell line. Apoptosis induction was demonstrated by qualitative and quantitative light microscopy and cell cycle progression studies, while autophagy induction was assessed by an increase in micro-tubule-associated protein light chain 3 (LC3) levels (a specific marker of autophagy). The MCF-7 tumorigenic cells, however, were more susceptible to these extracts when compared to the non-tumorigenic MCF-12A cells. Data obtained contribute towards understanding the differential cellular interaction exerted by aqueous SF extracts in tumorigenic versus non-tumorigenic breast cells. Results will enable researchers to further study cell death mechanisms induced by these aqueous extracts and to identify active compounds for evaluation in anticancer therapy and potential in vivo efficacy.

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1. Introduction

Cancer impacts severely on economic and social development, and thus on human lives. Breast cancer is a major health problem amongst women in South Africa (Vorobiof et al., 2001). According to the National Cancer Registry (2003) it is ranked as the most commonly found cancer among all South African females with the exception of black females where it is ranked as the second most frequently found cancer (Vorobiof et al., 2001; Herbst, 2011). It is estimated that one in six South African men and one in seven South African women will be diagnosed with cancer during their lives. For the past few decades, plant products have been identified to be a valuable source of anticancer drugs since the latter tend to have minimal side effects (Gonzales and Valerio, 2006).

Sutherlandia frutescens (SF), commonly known as the cancer bush, is a Southern African shrub that belongs to the Fabaceae family (Stander et al., 2007). Studies using extracts of this plant have demonstrated that SF possesses pharmacological properties such as antithrombotic (Kee et al., 2008), antibacterial (Katerere and Eloff, 2005), antidiabetic (MacKenzie et al., 2009) and antiproliferative activities (Tai et al., 2004). Recent studies performed in our laboratory (Department of Physiology, University of Pretoria, South Africa) and by other researchers revealed that these extracts inhibit cell growth in cancer cell lines namely the human breast adenocarcinoma cell line (MCF-7, MDA-MB-468), human promyelocyte (HL60), human prostate cancer cell line (DU-145) and esophageal cancer cell line (SNO) (Stander et al., 2009; Tai et al., 2004; Steenkamp and Gouws, 2006; Skerman et al., 2011).

Stander et al. (2009) revealed that exposure of MCF-7 cells to an ethanol extract of SF for a period of 48 h yielded a 50% growth reduction. In addition, the antiproliferative activity of the SF extract was established to be time- and concentration-dependent. MCF-7 cells were found to be more sensitive to ethanol SF extracts when compared to MCF-12A cells, suggesting differential signal transduction exerted by this ethanol extract (Stander et al., 2009). Similarly, a concentration dependent effect of SF extracts on human leukemia (Jurkat) and breast (MCF-7 and MDA-MB-468) tumor cell lines was observed (Tai et al., 2004).

Although SF extracts have been shown to possess antiproliferative properties, several questions still remain with regard to the bioactive chemical compounds that are present in the extracts of SF which are responsible for its anticancer effect (Shaik et al., 2010; Van Wyk, 2012). It has also been shown that extracts of SF contain diverse metabolic characteristics between different geographical locations and furthermore SF samples harvested at different periods at the same location (Albrecht et al., 2012). Samples were categorized in the following manner: A (Garden grown-A at GPS coordinates South 35° 51′ 32.12′; East 18° 39′ 45.69′ with spontaneous appearance in 2010); B (Garden grown-B at GPS coordinates South 35° 51′ 32.12′; East 18° 39′ 45.69′ with continuous growth in 2011) (Albrecht et al., 2012). In an effort to gain a better understanding of the mechanism of action of SF extracts, this study investigated differential in vitro effects of aqueous SF extract A and extract B on a tumorigenic breast cancer cell line and a non-tumorigenic cell line. Data from this study contribute to the unraveling of the mechanism of action that these extracts exert on tumorigenic and non-tumorigenic cells.

2. Materials & methods

2.1. Cell lines

The MCF-7 human breast adenocarcinoma cell line was obtained from Highveld Biological Pty. (Ltd.) (Sandringham, South Africa). The MCF-12A non-tumorigenic epithelial cell line was a gift from Professor Parker (Department of Medical Biochemistry, University of Cape Town, Cape Town, South Africa).

2.2. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, fungizone and trypsin were bought from Highveld Biological (Pty.) Ltd. (Sandringham, South Africa). Ham's F12 medium, Trypan blue, Bouin's fixative, propidium iodide (PI), formaldehyde, methanol, Triton-X100, bovine serum albumin (BSA) and RNase A were purchased

from Sigma-Aldrich Co. (Clubview, South Africa). Fetal calf serum (FCS), sterile cell plates, sterile syringes and filters were purchased from Separations (Pty.) Ltd. (Randburg, Johannesburg, South Africa). Hematoxylin, eosin, ethanol, Entellan® fixative and xylol were purchased from Merck (Munich, Germany). Rabbit polyclonal anti-LC3B conjugated to DyLight 488 was purchased from BIOCOM biotech Pty. (Ltd.) (Clubview, South Africa). All the required reagents of cell culture analytical grade were purchased from Sigma (St. Louis, United States of America) unless otherwise specified.

2.3. Plant extract preparation

The SF extract is a complex mixture of flavonoids, triterpenes, saponins and plant steroids (Albrecht et al., 2012). Leaves and small twigs of SF were obtained from Dr Carl Albrecht (Cancer Association of South Africa). Samples were categorized in the following manner: A (Garden grown-A at GPS coordinates South 35° 51′ 32.12′; East 18° 39′ 45.69′ with spontaneous appearance in 2010); B (Garden grown-B at GPS coordinates South 35° 51′ 32.12′; East 18° 39′ 45.69′ with continuous growth in 2011) (Albrecht et al., 2012). Half a gram (0.5 g) of samples A and B was weighed respectively, autoclaved, mixed with 50 ml of boiled distilled water and subsequently extracted over a period of 15 h at room temperature. After extraction, the supernatant was transferred to a 50 ml tube and centrifuged at 2500 rpm for 10 min to remove any debris. The supernatant was filtered thrice with a 0.22 μm filter to obtain a purified 0.01 g/ml stock solution (Stander et al., 2009; Vorster et al., 2012).

2.4. Cell culture

Cells were propagated in DMEM supplemented with 10% heat inactivated FCS and 100 U/ml penicillin G, 100 μg/ml streptomycin and 250 μg/l fungizone at 37 °C in a humidified atmosphere containing 5% CO_2 and left overnight for attachment. MCF-12A cells were cultured in a medium containing a 1:1 mixture of DMEM and Ham's-F12 medium, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin,
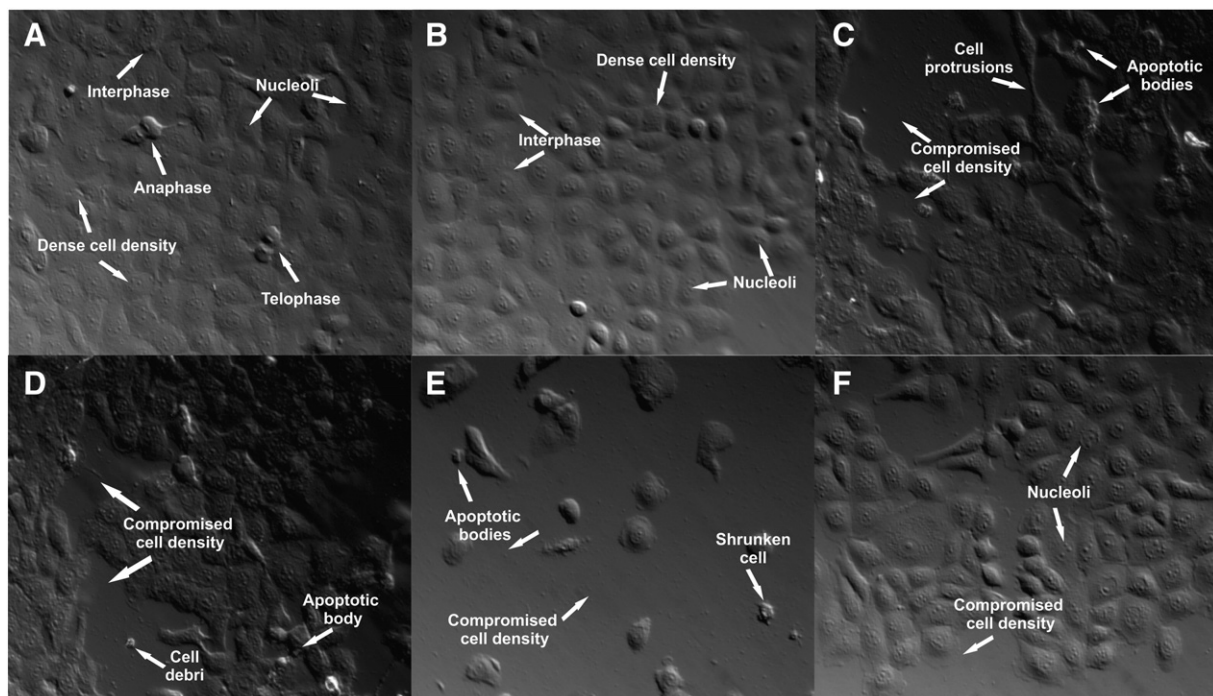

Fig. 1.1. Effects on morphology is demonstrated using PlasDIC on MCF-7 cells. PlasDIC images of MCF-7 cells propagated in growth medium (A), 1/10 dilution of water (B), 1/10 dilution of extract A (C), 1/10 dilution of extract B (D), actinomycin D-treated (E) and tamoxifen-treated (F) cells after 48 h. Cells treated with extract A & extract B displayed a compromised cell density as features of apoptosis, including the presence of shrunken cells, hypercondensed chromatin and apoptotic bodies (Fig. 1.1C & D).
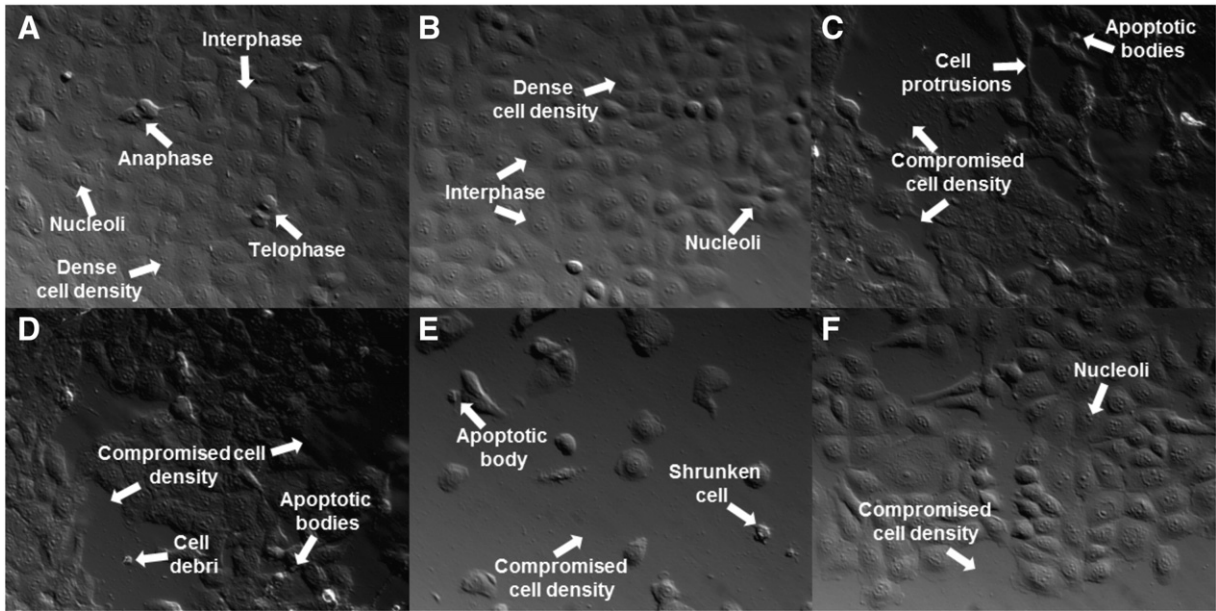
Fig. 1.2. Effects on morphology is demonstrated using PlasDIC on MCF-12A cells. PlasDIC images of MCF-12A cells propagated in growth medium (A), 1/10 dilution of water (B), 1/10 dilution of extract A (C), 1/10 dilution of extract B (D), actinomycin-D-treated (E) and tamoxifen-treated (F) cells following 48 h of exposure. Cell density of extract A- and extract B-treated cells was decreased (Fig. 1.2C & D). Characteristics of apoptosis (shrunken cells, apoptotic bodies) and of autophagy (vacuoles) were observed in actinomycin D- and tamoxifen-treated cells, respectively (Fig. 1.2E & F).

10 μg/ml insulin and 500 ng/ml hydrocortisone, in addition with 10% heat inactivated FCS, 100 U/ml penicillin G, 100 μg/ml streptomycin and fungizone (250 μg/l) at 37 °C in a humidified atmosphere containing 5% CO_2 and left overnight for attachment. Cells propagated in a growth medium, cells treated with the same volume of distilled water used to expose cells with 1/10 dilution of extract A and extract B respectively (v/v), as well as positive controls for apoptosis (0.1 μg/ml actinomycin D) and autophagy (tamoxifen (20 μM)) were included for each experiment as controls. A 1/10 dilution of extract A and extract B and an exposure period of 48 h were selected. These parameters were chosen based on our cell proliferation studies where at this time and dosage extracts significantly decreased cell proliferation of both cell lines, with the tumorigenic MCF-7 cells being more affected when compared to the non-tumorigenic MCF12A cells.

2.5. Polarization-optical differential interference contrast

Polarization-optical differential interference contrast (PlasDIC) is a polarization-optical transmitted light differential interference contrast method. Unlike conventional DIC, linearly polarized light is only generated after the objective (Wehner, 2003). PlasDIC allows for high quality imaging of cells and detection of morphological characteristics for apoptosis and autophagy. Cells were seeded at 500 000 cells per well in 6-well plates. After a 24 h incubation period at 37 °C to allow for cell adherence, cells were exposed to extracts A and B respectively including appropriate controls as previously described. Evaluation was conducted with a Zeiss inverted Axiovert CFL40 microscope equipped with a PlasDIC filter and photomicrographs were taken with a Zeiss Axiovert MRm monochrome camera (Carl Zeiss MicroImaging, Inc., NY, USA).

2.6. Crystal violet staining

Crystal violet is a method used to determine the number of cells by staining the deoxyribonucleic acid (DNA). Crystal violet staining allows for the quantification of cell numbers in monolayer cultures as a function of the absorbance of the dye taken up by the cells (Gillies et al., 1986). Exponentially growing cells were seeded in 96-well tissue culture plates at a cell density of 5000 cells per well. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h to

allow for attachment. Subsequently, the medium was discarded and cells were exposed to the both SF extracts at a dilution series of 1/10, 1/20, 1/40, and 1/80 for 48 h at 37 °C. Vehicle-treated controls consisting of 1/10 water in growth medium were also included. Cells were fixed with 100 μl of 1% gluteraldehyde (incubation for 15 min at room temperature). Subsequently the gluteraldehyde was discarded and cells were stained using 100 μl 0.1% crystal violet (incubated at room temperature for 30 min). Thereafter the crystal violet solution was discarded and the 96-well plate was submersed under running water. Crystal violet was solubilized using 200 μl 0.2% Triton X-100 and incubated at room temperature for 30 min. Solution (100 μl) was transferred to a new microtiter plate. Afterwards, the absorbance was determined at 570 nm using an EL_x800 Universal Microplate Reader
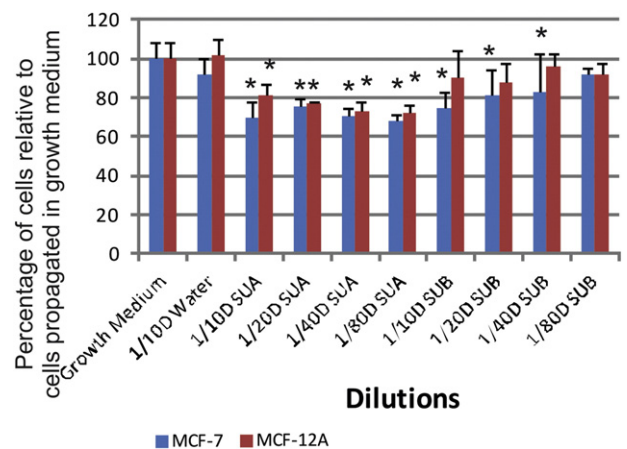

Fig. 2. Crystal violet staining demonstrating effects on cell numbers. Crystal violet staining was conducted to demonstrate the effects of SF extracts A and B on cell growth and cytotoxicity at a dilution series of 1/10, 1/20, 1/40, and 1/80 for 48 h at 37 °C. Vehicle-treated controls consisting of 1/10 water in growth medium were also included. Cell growth is expressed as a percentage of cells propagated in growth medium. Both extracts decreased cell growth significantly with extract A possessing slightly stronger antiproliferative activity. A dilution of 1/10 is optimal for both extracts since the tumorigenic cell line was more affected when compared to the non-tumorigenic breast cell line. An asterisk (*) indicates a statistically significant P-value of <0.05 when compared to cells propagated in growth medium.

available from Bio-Tek Instruments Inc. (Vermont, United States of America).

2.7. Hematoxylin and eosin staining

Hematoxylin and eosin staining is a recognized method to demonstrate the morphological effects of compounds on the cell nucleus and cytoplasm and is used to visualize hallmarks of apoptosis (Van Zijl et al., 2008; Stander et al., 2007). Hematoxylin and eosin staining was conducted to observe the morphology of MCF-7 and MCF-12A cells after 48 h of exposure to extract A and extract B. Exponentially growing cells were seeded on heat-sterilized coverslips in 6-well plates at a density of 250 000 cells per well and allowed to attach overnight. Cells were then exposed to 1/10 dilution of extract A and extract B and appropriate controls respectively for 48 h. After incubation, cells were fixed with Bouin's fixative for 30 min. The fixative was discarded and 70% ethanol was added to the coverslips (20 min), rinsed in tap water and subsequently left for 20 min in Mayer's Hemalum. After rinsing with running tap water for 2 min, coverslips were washed with 70% ethanol before being subjected to 1% eosin for 2 min. This was followed by rinsing twice for 5 min with 70% ethanol, 96% ethanol, 100% ethanol and xylol, respectively. Coverslips were then mounted with resin and left to dry before they were analyzed with a Zeiss Axiovert MRc microscope (Zeiss, Göttingen, Germany) to obtain qualitative data. Photos were taken utilizing a Zeiss Axiovert MRc microscope (Zeiss, Oberkochen, Germany). In addition, hematoxylin- and eosin-stained samples were used to determine mitotic indices. Quantitative data for the mitotic indices was acquired by counting 1000 cells on each slide of the biological replicates and expressing the data as the percentage of cells in each phase of mitosis (prophase, metaphase, anaphase and telophase), cells in interphase, cells demonstrating hallmarks of apoptosis and cells displaying any abnormal morphology not associated with apoptosis (Van Zijl et al., 2008). This hematoxylin and eosin staining yielded both qualitative and quantitative information.

2.8. Flow cytometry — cell cycle progression

Flow cytometry using PI staining allows for quantification of cell cycle progression and the presence of apoptosis indicated by the existence of a sub-G_1 phase. Cells were seeded at 500 000 cells per 25 cm^2 flasks and were incubated for 24 h to allow for attachment. Cells were subsequently exposed to 1/10 dilution of extract A and extract B respectively. Controls for both apoptosis and autophagy were included as previously described. After 48 h of incubation, cells were trypsinized and resuspended in 1 ml of growth medium and samples were centrifuged for 5 min at 300 ×g. Supernatant was discarded and cells were resuspended in 200 μl of ice-cold phosphate buffered saline (PBS) containing 0.1% FCS. In order to avoid cell clumping, 4 ml of ice-cold 70% ethanol was added in a dropwise manner while vortexing. Samples were stored at 4 °C for 6 days and centrifuged at 300 ×g for 5 min. Supernatant was removed and cells were resuspended in 1 ml of PBS containing 40 μg/ml PI and 100 μg/ml RNase A. The solution was incubated for 45 min in an incubator (5% CO_2, 37 °C). Fluorescence of PI (measuring relative DNA content per cell) was determined with a fluorescence activated cell sorting (FACS) FC500 System flow cytometer (Beckman Coulter South Africa (Pty.) Ltd.) equipped with an air-cooled argon laser excited at 488 nm. Data from no less than 30 000 cells were analyzed with CXP software (Beckman Coulter South Africa (Pty.) Ltd.). Cell cycle distributions were calculated with Cyflogic 1.2.1 (Perttu Terho & Cyflo Ltd., Finland) by assigning relative DNA content per cell to sub-G_1, G_1, S and G_2/M fractions. Data obtained from the fluorescence channel for red monomers (FL3 Lin) are shown as histograms on the x-axis.

2.9. Flow cytometry — autophagy detection: anti-LC3B staining

Autophagy is a form of programmed cell death where cytoplasmic materials are degraded through lysosomal machinery. The microtubule-associated protein light chain 3 (LC3) is a soluble protein and used as a specific marker to monitor occurrence of this type of cell death. LC3 exists in two forms, LC3-I and its proteolytic derivative LC3-II. LC3-I is found in
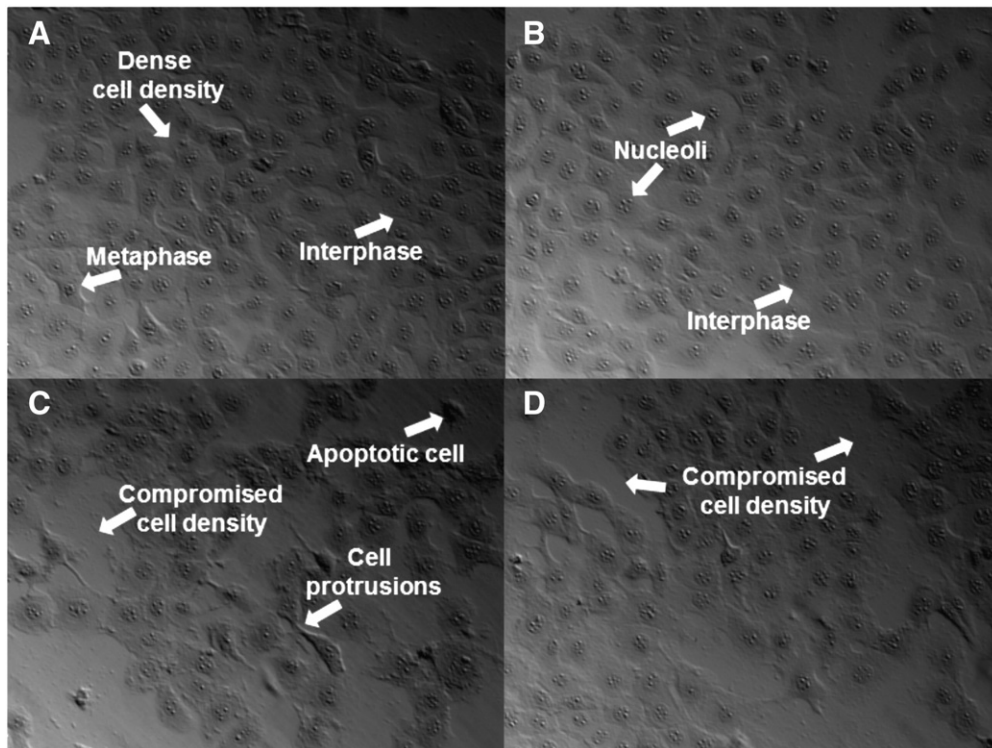

Fig. 3.1. Light microscopy micrographs of hematoxylin & eosin stained MCF-7 cells. Hematoxylin & eosin images of MCF-7 cells propagated in growth medium (A), 1/10 dilution of water (B), 1/10 dilution of extract A (C), and 1/10 dilution of extract B (D) following 48 h of exposure. Cell density was compromised and the presence of cell protrusions, as well as apoptotic bodies was observed.
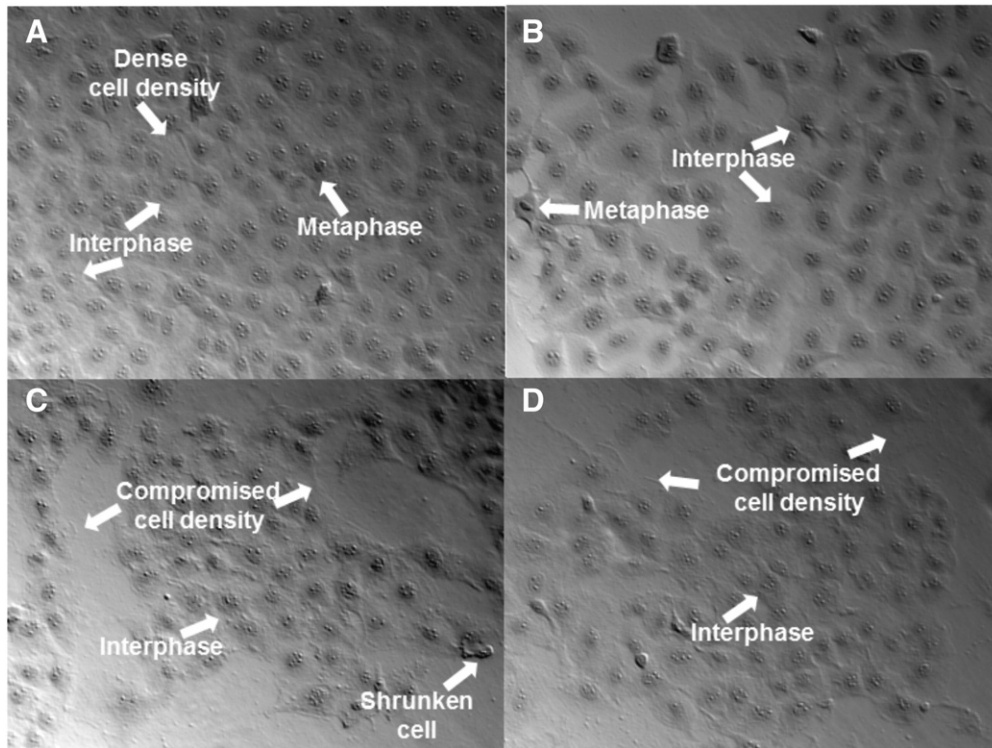
Fig. 3.2. Light microscopy micrographs of hematoxylin & eosin stained MCF-12A cells. Hematoxylin & eosin images of MCF-12A cells propagated in growth medium (A), 1/10 dilution of water (B), 1/10 dilution of extract A (C), and 1/10 dilution of extract B (D) following 48 h of exposure. Extract A- and extract B-treated cells displayed compromised cell density and shrunken cells (Fig. 2.2 C & D).

the cytoplasm whereas LC3-II binds to autophagosomes. The induction of autophagy stimulates the conversion of LC3-I to LC3-II and the upregulation of LC3 expression. Identification of LC3 II by immunofluorescence is a method that is widely used for monitoring autophagy (Mizushima, 2004). The LC3 antibody detection method was utilized to determine whether extracts A & B induce autophagy in MCF-7 and in MCF-12A cells.

Cells were seeded at 500 000 cells per 25 cm^2 flask. After 24 h of attachment, the medium was discarded and cells were exposed to extract A and extract B and to appropriate controls as previously defined. After incubation, cells were trypsinized and washed with ice-cold PBS. Cells were fixed with 0.01% formaldehyde in PBS for 10 min, pelleted and then resuspended in 200 μl PBS. To permeabilize the cells, 1 ml of ice-cold methanol (-20 °C) was added to the solution in a dropwise manner. Cells were pelleted and washed twice with ice-cold PBS. After washing, cells were pelleted, 0.5 ml of the primary antibody cocktail was added and samples were incubated for 2 h at 4 °C in the dark. Following 2 h of incubation, cells were washed trice with a wash buffer (PBS/0.05% Triton/1%BSA) and analyzed with CXP software (Beckman Coulter South Africa (Pty.) Ltd.). Data was analyzed using Cyflogic 1.2.1 software; data from fluorescence channel for green monomers (FL1 Log) was represented as histogram on the x-axis.
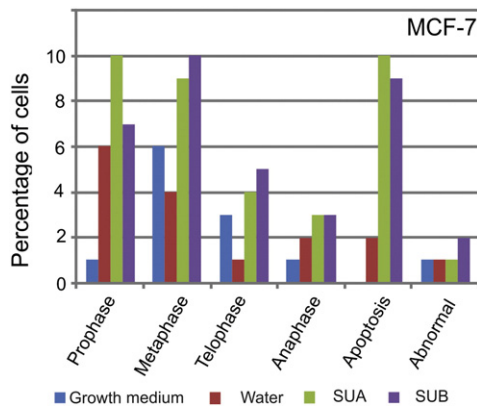

Fig. 3.3. Mitotic indices of MCF-7 cells. Mitotic indices of MCF-7 cells propagated in growth medium, 1/10 water dilution in growth medium and exposed to extract A and extract B. Mitotic indices revealed a slight increase of cells present in apoptosis as demonstrated by hallmarks of apoptosis after exposure to both extracts for 48 h (e.g. apoptotic bodies, cell membrane blebbing and shrunken cells).

Fig. 3.4. Mitotic indices of MCF-12A cells. Mitotic indices of MCF-A cells propagated in growth medium, 1/10 water dilution in growth medium and exposed to extract A and extract B. Mitotic indices revealed a slight increase of cells present in apoptosis as demonstrated by hallmarks of apoptosis after exposure to both extracts for 48 h (e.g. apoptotic bodies, cell membrane blebbing and shrunken cells).
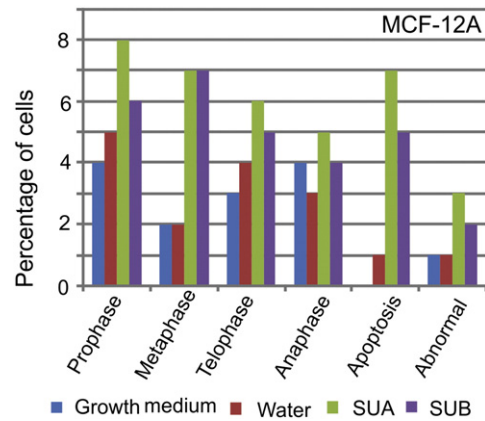
3. Statistics

Qualitative data were provided by PlasDIC images and hematoxylin stained images. Data obtained from independent experiments are shown as the mean ± S.D. Means are presented in bar charts, with T-bars referring to standard deviations. All experiments were conducted in triplicate. Quantitative data for the mitotic indices was acquired by counting 1000 cells on each slide of the biological replicates and expressing the data as the percentage of cells in each phase of mitosis (prophase, metaphase, anaphase and telophase), cells in interphase, cells demonstrating hallmarks of apoptosis and cells displaying any abnormal morphology not associated with apoptosis. In addition, further quantitative data were obtained via cell cycle progression analysis and autophagy detection analysis (anti-LC3B) assays. For flow cytometric data no less than 30 000 events were counted for each sample. Flow cytometric data produced was analyzed using Cyflogic 1.2.1 (Perttu Terho & Cyflo Ltd., Finland).

4. Results

4.1. Polarization-optical differential interference contrast

PlasDIC indicated a decrease in the density of MCF-7 (Fig. 1.1) and MCF-12A (Fig. 1.2) cells treated with extract A and extract B respectively after 48 h when compared to cells propagated in growth medium and to vehicle-treated cells. Qualitative images of extract A revealed a more pronounced antiproliferative effect when compared to extract B after 48 h of exposure.

4.2. Crystal violet staining

Dose-dependent studies were conducted with the purpose of evaluating the antiproliferative effects of extract A and extract B on tumorigenic breast epithelial and non-tumorigenic breast cell lines (Fig. 2). Statistical significant antiproliferative activity was demonstrated using
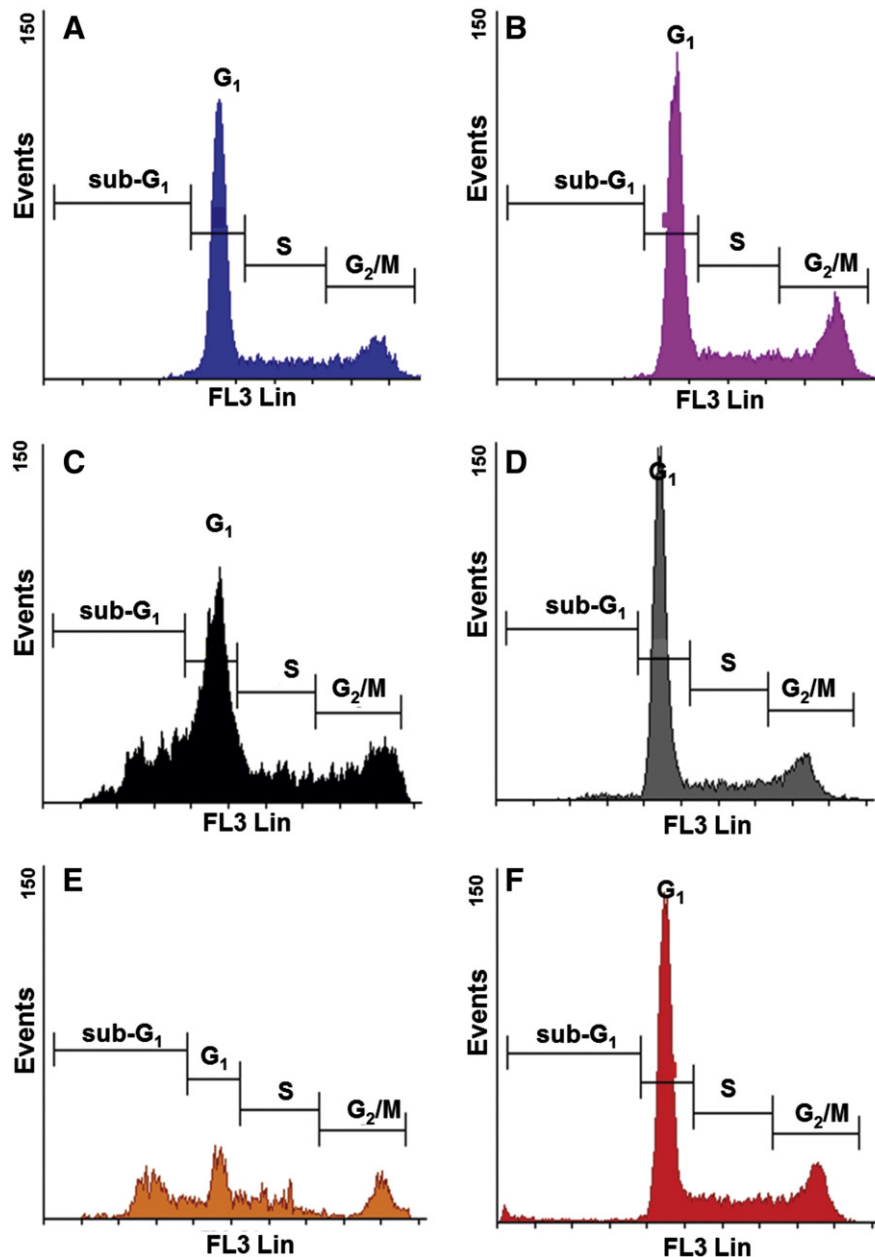

Fig. 4.1. Cell cycle progression of MCF-7 cells. Cell cycle distribution histograms of MCF-7 cells propagated in growth medium (A), vehicle-treated cells (B), 1/10 dilution of extract A-treated (C), and 1/10 dilution of extract B-treated (D), actinomycin D-treated (E), and tamoxifen-treated (F) cells after 48 h of exposure. An increase in the number of cells in sub-G_1 phase was observed in extract A-treated and in actinomycin D-treated cells indicating induction of apoptosis.

extract A at all dilutions decreasing cell growth by more than 20%. Extract B also exerted antiproliferative activity at all doses. However, both extracts possessed optimal antiproliferative activity at 1/10 dilution. However, the latter dilution affected the tumorigenic cell line more prominently.

4.3. Hematoxylin & eosin staining

Hematoxylin & eosin staining of extract A and extract B exposure to MCF-7 (Fig. 3.1) and MCF-12A (Fig. 3.2) cells were performed to investigate in vitro effects on morphology. Extract A and extract B exposures resulted in compromised cell density in both cell lines after 48 h when compared to vehicle-treated cells. Furthermore, the MCF-7 tumorigenic cells were pronouncedly affected when compared to the non-tumorigenic MCF-12A cells. Mitotic indices (Figs. 3.3 and 3.4) revealed a slight increase of cells present in apoptosis as demonstrated by hallmarks of apoptosis after exposure to both extracts for 48 h (e.g. apoptotic bodies, cell membrane blebbing and shrunken cells).

4.4. Flow cytometry — cell cycle progression

To provide quantitative data in addition to the qualitative data supplied above, flow cytometry was used to analyze the effects of on cell cycle progression. MCF-7 cells showed an increase in the number of extract A-treated (1/10 dilution) (20.59%) cells in sub-G_1 phase indicative of apoptosis when compared to extract B-treated (2.02%) cells and vehicle-treated cells present in sub-G_1 (0.57%) (Figs. 4.1 and 4.2). Flow cytometry results suggest that extract A statistically significantly ($P < 0.005$) inhibits growth of MCF-7 cells in vitro via apoptosis. Extract A- (4.9%) and extract-B-treated (1.6%) MCF-12A cells demonstrated a statistically insignificant increase in the number of cells in sub-G_1 phase respectively when compared to vehicle-treated cells (3.5%) present in sub-G_1 (Tables 1 and 2).
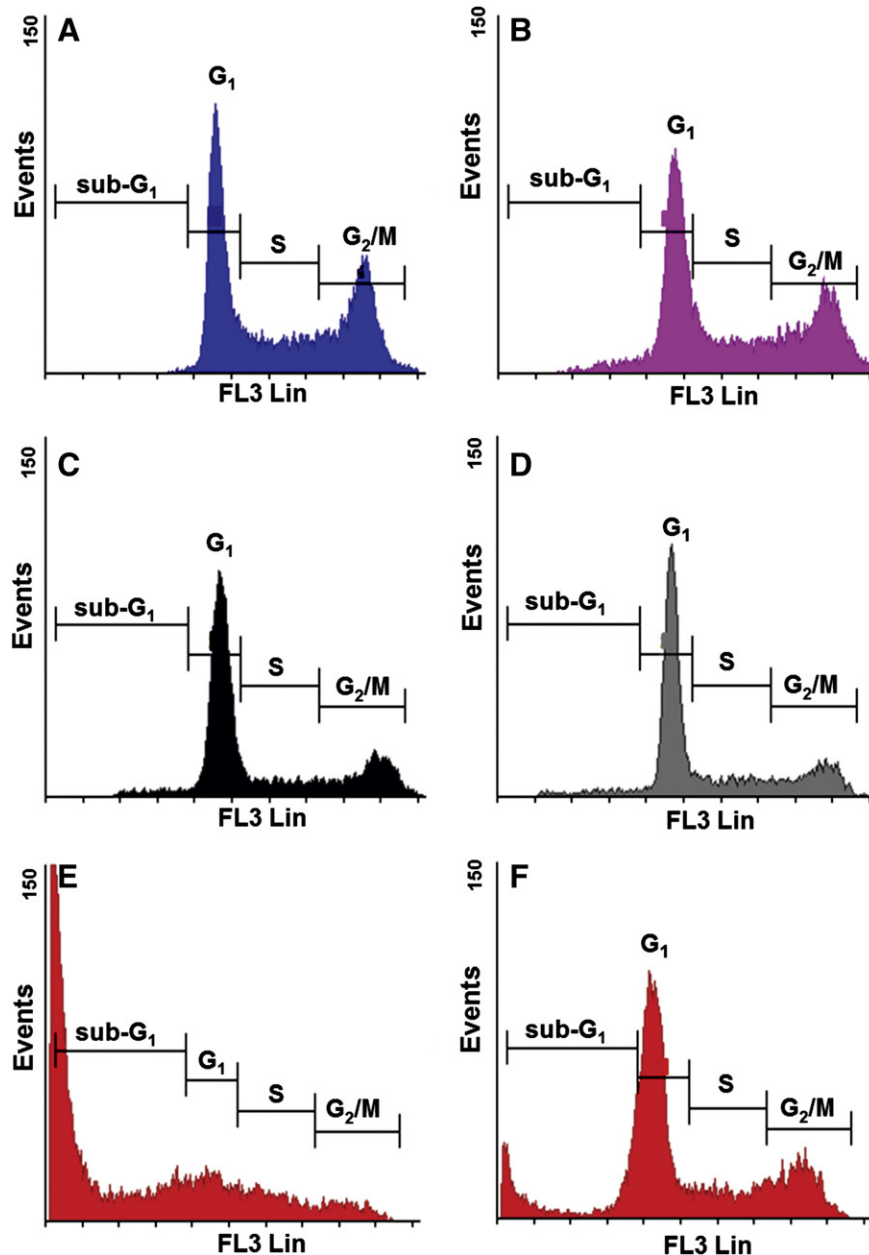

Fig. 4.2. Cell cycle progression of MCF-12A cells. Cell cycle distribution histograms of MCF-12A cells propagated in growth medium (A), vehicle-treated cells (B), 1/10 dilution of extract A-treated (C), 1/10 dilution of extract B-treated (D), actinomycin D-treated (E), and tamoxifen-treated (F) cells after 48 h. The non-tumorigenic MCF-12A cells were less susceptible to the apoptotic influence of extracts A and B respectively when compared to the tumorigenic MCF-7 cells.

Table 1
Percentage of MCF-7 cells in sub-G_1, G_1, S and G_2/M phases as determined by Cyflogic 1.2.1 (Perttu Terho & Cyflo Ltd.).

Phase	Growth medium	Water 1/10D	Extract A	Extract B	Actinomycin D	Tamoxifen
Sub-G_1	0.57	0.19	20.59	2.02	30.31	2.21
G_1	62.73	55.35	45.5	54.18	30.87	58.54
S	15.67	17.07	14.4	16.19	19.93	17.06
G_2/M	20.97	27.17	18.66	24.2	18.43	21.69

4.5. Flow cytometry — autophagy detection: anti-LC3B

Autophagy induction by extract A and extract B in MCF-7 and MCF-12A cells was evaluated by means of a conjugated rabbit polyclonal anti-LC3B antibody assay (Figs. 5.1 and 5.2). As previously mentioned, LC3 is a soluble protein used as a specific marker to demonstrate the induction of autophagy. LC3-I is found in the cytoplasm whereas LC3-II binds to autophagosomes. The induction of autophagy stimulates the conversion of LC3-I to LC3-II and the upregulation of LC3 expression. Thus, LC3 II is a useful indicator when investigating the presence of autophagy. According to the supplier's manual, an increase in mean fluorescence intensity (shift to the right) indicates an increase in the protein levels of LC3. MCF-7 cells treated with both extract A and extract B showed an increase in fluorescence when compared to cells propagated in growth medium. MCF-12A cells treated with extract A and extract B showed a statistically insignificant increase in LC3 levels when compared to control. Results indicate that the tumorigenic MCF-7 cells are more susceptible to the treatment of extract A and extract B when compared to the non-tumorigenic MCF-12A cells.

5. Discussion

In this study the in vitro differential cellular interaction of aqueous SF extract A and extract B (Albrecht et al., 2012) was investigated on a breast adenocarcinoma cell line (MCF-7) and a non-tumorigenic breast cell line (MCF-12A). Morphological studies conducted by means of PlasDIC and light microscopy revealed characteristics associated with apoptosis induction including shrunken cells, hypercondensed chromatin and the presence of apoptotic bodies. Reduced cell density was observed in both MCF-7 and MCF-12A cells, with the tumorigenic MCF-7 cell line being more affected. Similarly, Stander et al. (2007) found that there was a significant increase in the number of hypercondensed shrunken in MCF-7 cells and apoptotic bodies after treatment with an ethanol extract of SF. Apoptotic cells were observed in SF-treated esophageal cancer cell line (SNO), Chinese hamster ovary (CHO) cell line as well as in human mammary adenocarcinoma (MDA-MB-468) cell line (Skerman et al., 2011; Chinkwo, 2005; Tai et al., 2004).

To further investigate the differential interaction on cell cycle progression of extract A and extract B on MCF-7 and MCF-12A cells, flow cytometry studies were conducted. Treatment of MCF-7 cells with extract A and extract B caused an increase in the sub-G_1 fraction which is indicative of apoptosis induction. Results were consistent with those of our previously reported data (Stander et al. (2009)). An increase in the sub-G_1 fraction for extract A- (4.9%) and extract B-treated (1.6%) samples were observed. This increase in the sub-G_1 fraction was more pronounced when compared to cells propagated in growth medium

Table 2
Percentage of MCF-12A cells in sub-G_1, G_1, S and G_2/M phases as determined by Cyflogic 1.2.1 (Perttu Terho & Cyflo Ltd., Finland).

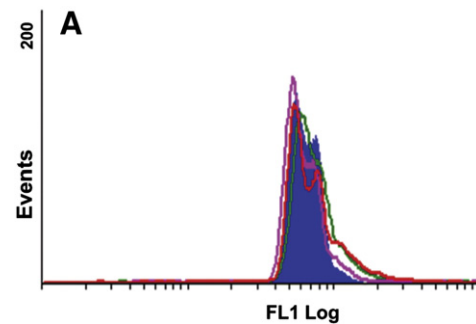| Phase	Growth medium	Water 1/10D	Extract A	Extract B	Actinomycin D	Tamoxifen
Sub-G_1	0.14	3.51	4.9	1.6	56.35	15.16
G_1	43.75	41.72	58.27	68.13	14.28	49.62
S	20.24	21.44	13.63	12.33	12.71	13.9
G_2/M	35.41	31.95	22.48	17.88	6.16	19.35

Fig. 5.1. Autophagy induction in MCF-7 cells. Histogram of mean fluorescence intensity of MCF-7 cells treated with 1/10 dilution of extract A (green histogram), 1/10 dilution of extract B (pink histogram) and tamoxifen (red histogram) overlaid on cells propagated in growth medium (blue histogram) following 48 h of exposure (A). An increase in LC3 levels (shown by a shift to the right) was observed in MCF-7 cells treated with extract A and extract B when compared to the control-treated cells.

(0.14%). Extract A- and extract B-treated MCF-12A cells demonstrated a statistically insignificant increase in the number of cells in sub-G_1 phase respectively when compared to vehicle-treated cells (3.5%). To further substantiate induction of apoptosis, morphological changes were investigated to obtain both qualitative and quantitative data to prove the presence of apoptosis (Van Zijl et al., 2008). Mitotic indices revealed a slight increase of cells present in apoptosis as demonstrated by hallmarks of apoptosis after exposure to both extracts for 48 h (e.g. apoptotic bodies, cell membrane blebbing and shrunken cells).

Additionally it was shown that SF extracts induce another type of cell death together with apoptosis known as autophagy. Autophagy is a mechanism of survival for cells during periods of stress and starvation, but it has been observed that there are times where autophagy can progress to cell death (Tsujimoto and Shimizu, 2005). Studies revealed an increase in LC3 levels in MCF-7 cells treated with extract A and extract B was observed when compared to cell propagated in growth medium. Again, the non-tumorigenic MCF-12A cells treated with extract A and extract B respectively did not show a statistically significant increase in LC3 levels when compared to the control.

The occurrence of both apoptosis and autophagy implies existence of crosstalk among these two types of cell deaths induced by aqueous SF extracts A and B (Vorster et al., 2012; Azad et al., 2009). This in vitro study revealed that both extract A and extract B induce apoptosis and autophagy in the tumorigenic MCF-7 cells leaving non-tumorigenic MCF-12A cells less affected. Our data also confirms the importance of sample collection at specific time intervals. Results from this study demonstrated that the specific time period when harvesting takes place
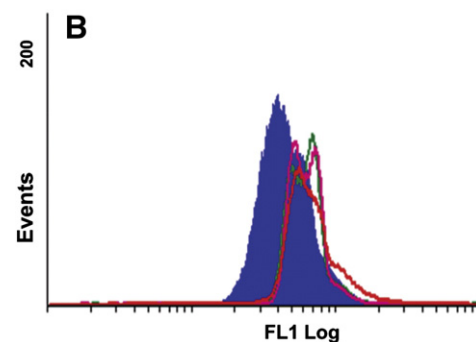

Fig. 5.2. Autophagy induction in MCF-12A cells. Histogram of mean fluorescence intensity of MCF-12A cells treated with 1/10 dilution of extract A (green histogram), 1/10 dilution of extract B (pink histogram) and tamoxifen (red histogram) overlaid on the vehicle-treated cells (blue histogram) following 48 h of exposure (B). Results demonstrated no significant increase in LC3 levels of MCF-12A cells treated with extract A and extract B when compared to control-treated cells.

influences the extract's antiproliferative potential. These extracts may have significantly different chemical constituents and therefore exert differential mechanisms of action in the molecular crosstalk between apoptosis and autophagy. Future studies on effects of various proposed bioactive chemical compounds of the extracts on tumorigenic and non-tumorigenic cells are warranted to further understand the mechanism of action of SF and the potential in vivo efficiency as a cancer treatment.

Acknowledgments

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