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Dillenia suffruticosa dichloromethane root extract induced apoptosis towards MDA-MB-231 triple-negative breast cancer cells

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ABSTRACT

Ethnopharmacological relevance: Dillenia suffruticosa is traditionally used for treatment of cancerous growth including breast cancer in Malaysia.
Aim of the study: Dillenia suffruticosa is a well-known medicinal plant in Malaysia for the treatment of cancer. Nevertheless, no study has been reported the cytotoxicity of this plant towards MDA-MB-231 triple-negative breast cancer cells. The present study was designed to investigate the mode of cell death and signalling pathways of MDA-MB-231 cells treated with dichloromethane *Dillenia suffruticosa* root extract (DCM-DS).
Methods: Extraction of *Dillenia suffruticosa* root was performed by the use of sequential solvent procedure. The cytotoxicity of DCM-DS was determined by using MTT assay. The mode of cell death was evaluated by using an inverted light microscope and flow cytometry analysis using Annexin-V/PI. Cell cycle analysis and measurement of reactive oxygen species level were performed by using flow cytometry. The cells were treated with DCM-DS and antioxidants α-tocopherol or ascorbic acid to evaluate the involvement of ROS in the cytotoxicity of DCM-DS. Effect of DCM-DS on the expression of antioxidant, apoptotic, growth, survival genes and proteins were analysed by using GeXP-based multiplex system and Western blot, respectively. The cytotoxicity of compounds isolated from DCM-DS was evaluated towards MDA-MB-231 cells using MTT assay.
Results: DCM-DS induced apoptosis, G_2/M phase cell cycle arrest and oxidative stress in MDA-MB-231 cells. The induction of apoptosis in MDA-MB-231 cells by DCM-DS is possibly due to the activation of pro-apoptotic JNK1 and down-regulation of anti-apoptotic ERK1, which in turn down-regulates anti-apoptotic BCL-2 to increase the BAX/BCL-2 ratio to initiate the mitochondrial apoptotic pathway. The cell cycle arrest in DCM-DS-treated MDA-MB-231 cells is possibly via p53-independent but p21-dependent pathway. A total of 3 triterpene compounds were isolated from DCM-DS. Betulinic acid appears to be the most major and most cytotoxic compound in DCM-DS.
Conclusion: The data suggest the potential application of DCM-DS in the treatment of triple-negative breast cancer.

1. Introduction

Breast cancer is a complex and heterogeneous disease that has been molecularly classified into 5 major subtypes: normal-like, hormone receptor-positive Luminal A (LA) and B (LB), human epidermal growth receptor 2 (HER2) and basal-like (BL) breast cancers (Curigliano, 2012). Normal-like breast cancer shows a gene signature similar to that of normal breast tissue; LA expresses oestrogen receptor (ER) and/or progesterone receptor (PR); LB expresses HER2, ER and/or PR; BL is defined by the absence of ER, PR and HER2 expression, and is therefore also identified as triple-negative breast cancer (TNBC) (Shah and Allegrucci, 2012; De Laurentiis et al., 2010; Kang et al., 2008).

TNBC patients are exclusively treated with conventional cytotoxic chemotherapy as there is no targeted treatment for it up-to-date. In addition, the patients are unresponsive to endocrine therapies or HER2-targeted agents (Stebbing and Ellis, 2012). TNBC is always of the greatest clinical challenge because the tumours are

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prevalent in women under 50 years old, associated with the worst prognosis, metastasis to lung and brain, and relapse always occur (Dent et al., 2007). Although TNBC is accounting for approximately 15% of all breast cancer cases, TNBC has a higher mortality rate (death within the first 3–5 years of diagnosis) (Yadav et al., 2013; De Laurentiis et al., 2010). Therefore, there is an urgent to search for new agents to target the TNBC such as the use of evidence-based herbal medicines. The mixture of active compounds in the herbal medicines may exhibit synergetic effect on cancer cells by targeting on multiple signalling pathways or a few players in the same pathway thus overcome the natural or acquired drug resistance in cancer cells. This strategy was in parallel to the guideline as approved by US Food and Drug Administration (FDA) in 2011 which outlines a path towards developing combination therapy (Dolgin, 2011).

Dillenia suffruticosa (Griffith ex Hook. F. and Thomson) Martelli (Family: Dilleniaceae) has been used by the Malaysian for the treatment of cancerous growth (Ahmad and Holdsworth, 1995). Our previous studies reported that the root dichloromethane and ethyl acetate extract of *Dillenia suffruticosa* from sequential solvent extraction induced G_0/G_1 phase cell cycle arrest and apoptosis towards human caspase-3 deficient MCF-7 breast cancer cells by modulating the expression of mitogen-activated protein kinases (MAPKs) at gene and protein level (Foo et al., 2014; Tor et al., 2014). Nevertheless, the mode of cell death of MDA-MB-231 cells treated with DCM-DS remains to be elucidated. Therefore, the current study was carried out to investigate the mode of cell death, cell cycle profile and signalling pathways of DCM-DS-treated human MDA-MB-231 TNBC cells.

2. Materials and methods

2.1. Plant materials

Dillenia suffruticosa was supplied by Primer Herber Sdn. Bhd., Malaysia. The plant's authentication was performed at the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia, Malaysia (voucher specimen number SK1937/11).

2.2. Preparation of plant extract

DCM-DS was prepared as previously reported with some modification (Foo et al., 2014). Briefly, 100 g of the powdered root was macerated with 500 mL of hexane (1:5, w/v) (Francfort, Germany) for 1 day at room temperature with occasional shaking at 200 rpm (IKA KS 260 basic, IKA, Staufen, Germany). The mixture was then centrifuged at 2000 × *g* for 5 min. The supernatant was filtered through Whatman filter paper No. 1. The residue was re-extracted twice, dried under the fume hood and further macerated with dichloromethane (DCM) (Francfort, Germany) in a condition as specified for hexane extract. The DCM extract solutions were pooled and DCM was removed under reduced pressure (Rotavapor R210, Buchi, Flawil, Switzerland).

2.3. Cell culture

The human MDA-MB-231 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231 cells were grown in RPMI 1640 with L-glutamine (Nacalai Tesque, Kyoto, Japan), supplemented with 10% foetal bovine serum (FBS) (PAA, Pasching, Austria) and 1% penicillin–streptomycin (PAA, Pasching, Austria). The cells used for each experiment were of less than 10 passage number.

2.4. Determination of cytotoxicity

MDA-MB-231 cells were seeded in 96-well plates (5000 cells/well) and treated with DCM-DS for 24, 48 and 72 h. Control cells treated with 0.1% DMSO alone (vehicle) were also included. Following incubation, 20 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (PhytoTechnology Laboratories, Kansas, USA) (5 mg/mL in PBS) was then added and the plate was incubated for 3 h. After removing the excess MTT, the purple formazan crystals formed were dissolved by DMSO (150 μL). The absorbance was then measured at 570 nm and a reference wavelength of 630 nm by using ELx800™ Absorbance Microplate Reader (BioTek Instruments Inc., Vermont, USA).

2.5. Determination of apoptosis by Annexin V/PI staining

Experiment was carried out according to the manufacturer's instructions (Annexin V-FITC kit, eBioscience, Vienna, Austria) with slight modification. Cells were seeded in 6-well plates at 1.3×10^5 cells per well, incubated for 24 h and treated with DCM-DS. Following incubation, the floating and adherent cells were collected. Cells were then counted and a volume of media containing 1×10^5 cells was centrifuged at 100 × *g* to obtain a pellet. Next, 195 μL of 1X assay buffer and 5 μL of Annexin V-fluorescein isothiocyanate (FITC) were added to the pellet. After 10 min incubation at room temperature in the dark, 300 μL of 1X assay buffer and 10 μL of PI (20 μg/mL) were added. Samples were analysed immediately by FACSCalibur flow cytometer (Becton Dickinson, CA, USA). For each sample, 10,000 events were collected. The results were analysed by using FlowJo software.

2.6. Cell cycle analysis

Cells were seeded in 25 cm^2 tissue culture flasks at 2×10^5 cells in 5 mL of complete culture growth media, incubated for 24 h and treated with DCM-DS. Following incubation, the floating and adherent cells were collected. Cells were then washed twice with PBS and resuspended in 70% ethanol at −20 °C overnight. Prior to analysis, the cells were washed once with PBS, suspended in 425 μL of PBS, 25 μL of propidium iodide (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) and 50 μL of RNaseA (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA), and incubated on ice for 20 min. The DNA content of 10,000 cells was analysed by FACSCalibur flow cytometer (Becton Dickinson, CA, USA). The population of cells in each cell-cycle phase was determined by using the ModFit LT software.

2.7. Measurement of intracellular reactive oxygen species in DCM-DS-treated cells

Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO, USA) was used to measure intracellular reactive oxygen species (ROS) in DCM-DS-treated MDA-MB-231 cells. Cells were seeded in 6-well plates at 1.3×10^5 cells per well, incubated for 24 h and pre-treated with 10 μM DCFH-DA for 1 h. After removing the excess DCFH-DA, the cells were washed twice with PBS and further treated with DCM-DS for 3 h. Both floating and adherent cells were collected and analysed immediately by FACSCalibur flow cytometer (Becton Dickinson, CA, USA). For each sample, 10,000 events were collected. The results were analysed by using FlowJo software.

2.8. Evaluation of antioxidants on DCM-DS-induced cell death in MDA-MB-231 cells

MDA-MB-231 cells were seeded in 96-well plates (5000 cells/well) and incubated at 37 °C (5% CO_2 and 95% air) for 24 h. The

Table 1
Apoptotic, growth and survival genes and their respective primers used in GeXP multiplex analysis.

Gene	Accession Number	Forward Primer Sequence (5′-3′)	Reverse Primer Sequence (3′-5′)
JNK	NM_139046	**AGGTGACACTATAGAATA**CAGAAGCTCCACCACCAAAGAT	CACGTCGTCACTAGTTACCGA**GGGATATCACTCAGCATG**
ERK1/2	NM_002745	**AGGTGACACTATAGAATA**GGAGCAGTATTACGACCCGA	TGACCTGCACGAGTCTGTAGA**GGGATATCACTCAGCATG**
BAX	BC014175	**AGGTGACACTATAGAATA**CCCTTTTGCTTCAGGGTTTC	AACAGCGGGAAAAGATGAAACA**GGGATATCACTCAGCATG**
BCL-2	M14745	**AGGTGACACTATAGAATA**ACCACTAATTGCCAAGCACC	TTCTCGTCTGCCTACCTTTTA**GGGATATCACTCAGCATG**
P53	NM_001126117	**AGGTGACACTATAGAATA**GGGGAGCAGGGCTCA	GGGAGGGGACGGTAAAAA**GGGATATCACTCAGCATG**
P21	NM_000389	**AGGTGACACTATAGAATA**TTAGCAGCGGAACAAGGAGT	ACCTGACAAAAGAGAGCCGAA**GGGATATCACTCAGCATG**
18SRNA[a]	M10098	**AGGTGACACTATAGAATA**GGAGTGGAGCCTGCGGCTTAA	TTGCTCTGAGACCGTACGATA**GGGATATCACTCAGCATG**
ACTB[a]	NM_001101	**AGGTGACACTATAGAATA**GATCATTGCTCCTCCTGAGC	CTACTCTAACCGTACCGAAAA**GGGATATCACTCAGCATG**
GAPDH[a]	NM_002046	**AGGTGACACTATAGAATA**AAGGTGAAGGTCGGAGTCAA	GTAGAAGGTCCTCGCTCTAGA**GGGATATCACTCAGCATG**
Kan(r)[b]		**AGGTGACACTATAGAATA**ATCATCAGCATTGCATTCGATTCCTGTTTG	CTACAACCTGCTCAGCCTTAA**GGGATATCACTCAGCATG**

Underlined and bolded sequence represents universal tag sequence.

[a] Gene used for normalization.
[b] Internal control gene.

cells were then treated with DCM-DS or co-treated with 50 μM α-tocopherol or vitamin C (Sigma-Aldrich, St. Louis, MO, USA) for 24 and 48 h. The MTT protocol was then performed as described in Section 2.4.

2.9. Analysis of expression of apoptotic, growth and survival genes

Gene expression study was carried out as previously described (Foo et al., 2015). Briefly, cells were seeded in 6-well plates at 1.3×10^5 cells per well and treated with DCM-DS for 24 h. Both floating and adherent cells were collected. RNA was then isolated using the Real Genomics Total RNA Extraction Kit (RBCBioscience, Taipei, Taiwan). Reverse transcription to cDNA and polymerase chain reaction (PCR) to amplify the amount of cDNA were performed according to the GenomeLab™ GeXP Start Kit from Beckman Coulter protocol. PCR product was analysed on a GeXP genetic analysis system (S. Kraemer Boulevard, USA).

Fold change of the expression of the gene was normalised against *beta-actin*. The studied genes were listed in Table 1.

2.10. Analysis of expression of apoptotic, growth and survival proteins

Primary rabbit antibodies anti-ERK1 (ab32537), anti-p-ERK1 (phospho T202) (ab47310), anti-JNK1 (ab10664), anti-p-JNK1 (Phospho T183) (ab47337), anti-BCL-2 (ab7973) and anti-p21 (ab7960) were purchased from ABCAM (Cambridge, MA, USA). Primary mouse antibodies anti-BAX (N-20: sc-493), anti-p53 (DO-1: sc-126) and anti-beta-actin (sc-47778) were purchased from Santa Cruz Biotechnology (CA, USA). Horseradish peroxidase-conjugated anti-rabbit (ab6721) and anti-mouse (sc-2005) secondary antibodies were purchased from ABCAM (Cambridge, MA, USA) and Santa Cruz Biotechnology (CA, USA), respectively.

Western blot analysis was carried out as previously documented (Foo et al., 2015). Briefly, MDA-MB-231 cells were seeded into 75 cm^2 tissue culture flasks at 800,000 cells per flask and treated with DCM-DS for 24 and 48 h. Following incubation, the protein lysates were harvested. An equal amount of 10–20 μg of proteins was separated by gel electrophoresis and transferred to PVDF membrane. The membrane was then blocked with 3% BSA in 0.1% Tween-20 containing Tris-Buffer Saline (TBS-T) at room temperature (20–25 °C) for 1 h and reacted with primary antibodies in TBS-T against ERK1 (1:5000), p-ERK1 (1:3000), JNK1 (1:10,000), p-JNK1(1:5000), BAX (1: 10,000), BCL-2 (1:1000), p21 (1: 10,000), p53 (1:1000) or beta-actin (1:10,000). The primary antibodies were either reacted with horseradish peroxidase-conjugated goat anti-rabbit (1:40,000) or goat anti-mouse (1:40,000) secondary antibodies. The protein visualisation was then performed by using Chemi-Lumi One L and ChemiDoc™ MP System (Bio-Rad, Hercules, CA, US) in a dark room.

2.11. Isolation of compounds

The isolation of compounds from DCM-DS was carried out as previously reported (Foo et al., 2015).

2.12. Determination of cytotoxicity of isolated compounds

The stock solution (50 mg/mL) of the isolated compounds was prepared in DMSO. MDA-MB-231 cells were trypsinised and seeded in 96-well flat-bottomed plates with 5000 cells per well in 100 μL of complete growth culture media, followed by incubation at 37 °C (5% CO_2 and 95% air) for 24 h to allow cell attachment. The cells were then treated with the isolated compounds (1.3–50 μg/mL) for 72 h. Control cells treated with 0.1% DMSO alone were also included. The MTT protocol was then performed as described in Section 2.4.

2.13. Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science (SPSS) version 21.0. Data were expressed as mean ± standard deviation (mean ± SD). Results were analysed by one-way analysis of variance (ANOVA), followed by Post Hoc Multiple Comparisons. A difference was considered to be significant at $p < 0.05$.

3. Results

3.1. DCM-DS induced growth inhibition in MDA-MB-231 cells

DCM-DS significantly ($p < 0.05$) reduced the viability of MDA-MB-231 cells in a dose- and time-dependent manner (Fig. 1). The IC_{50} values of DCM-DS towards MDA-MB-231 cell line at 24, 48 and 72 h were 27.3 ± 1.3, 18.7 ± 0.6 and 15.2 ± 1.0 μg/mL, respectively.

Morphological study using inverted light microscope demonstrated that DCM-DS inhibited the proliferation of MDA-MB-231 cells (Fig. 2A and B). The number of cells in the control and the one treated with 12.5 μg/mL of DCM-DS increased from 0 to 72 h. Nevertheless, the growth rate of the cells in the latter was slower than the former. For the cells treated with DCM-DS at 25 μg/mL, growth inhibition was observed. The growth of the cells treated with DCM-DS at 50 μg/mL was completely inhibited with majority of the cells detached from the substratum as early as 24 h. The floating cells demonstrated characteristics of apoptosis such as membrane blebbing, chromatin condensation and formation of

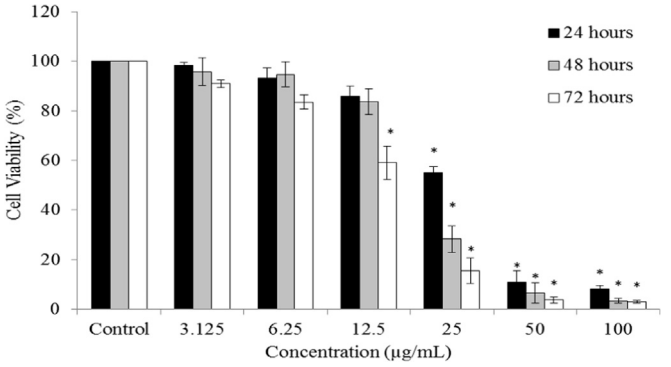


Fig. 1. Effect of DCM-DS on the viability of MDA-MB-231 breast cancer cells as determined by MTT assay. The effect was in a dose- and time-dependent manner. Each data point represents the mean of three independent experiments ± SD. *significantly different from the control ($p < 0.05$).

apoptotic bodies (Fig. 2C).

3.2. DCM-DS induced apoptosis in MDA-MB-231 cells

DCM-DS significantly increased ($p < 0.05$) the percentage of early apoptotic cells (Annexin-V^+/PI^-) as compared to the control (Fig. 3A and B). The percentage of early apoptotic cells for 25 and 50 μg/mL at 48 h was 12.6% and 56.2%, respectively, as compared to the control (8.0%). The percentage of necrotic or secondary necrotic cells (Annexin-V^+/PI^+) for 25 and 50 μg/mL at 48 h was 26.0% and 20.8%, respectively, as compared to the control (2.6%).

3.3. DCM-DS induced G_2/M phase arrest in MDA-MB-231 cells

DCM-DS at 25 μg/mL significantly increased ($p < 0.05$) the G_2/M phase cell population of MDA-MB-231 cells from 14% to 24% at 72 h (Fig. 4). A slight increase in the hypodiploid sub-G_0/G_1 peak (DNA content $< 2n$) was noted. Accumulation of cells ($p < 0.05$) in G_2/M phase and increase in hypodiploid sub-G_0/G_1 peak was also noted as early as 24 h when MDA-MB-231 cells were treated with 50 μg/mL of DCM-DS.

3.4. DCM-DS induced ROS production in MDA-MB-231 cells

The level of ROS in MDA-MB-231 cells treated with DCM-DS at 12.5, 25 and 50 μg/mL was 79%, 57% and 19% ($p < 0.05$), respectively, as compared to the 2% basal level in the untreated control cells ($p < 0.05$) (Fig. 5A).

3.5. ROS partially reduced cell viability in MDA-MB-231 cells

The viability of MDA-MB-231 cells treated with DCM-DS alone at 50 μg/mL for 24 and 48 h was 37% and 18%, respectively. The treatment of 50 μM α-tocopherol with 50 μg/mL of DCM-DS significantly increased ($p < 0.05$) the viability to 46% and 64% at 24 and 48 h, respectively (Fig. 5B). At 25 μg/mL of DCM-DS, co-treatment with α-tocopherol slightly increased ($p < 0.05$) the cell viability from 49% to 58% at 48 h.

3.6. DCM-DS regulated the expression of apoptotic, growth and survival genes

The expression of *ERK1/2* and *P53* was significantly down-regulated ($p < 0.05$) at 50 μg/mL of DCM-DS by approximately 1.6 and 1.8 folds, respectively (Fig. 6A). Treatment of MDA-MB-231 cells with DCM-DS at 25 and 50 μg/mL significantly up-regulated ($p < 0.05$) the expression of *p21* by approximately 2 folds. DCM-DS at 25 and 50 μg/mL also significantly up-regulated ($p < 0.05$) the expression of *BAX* by approximately 1.4–1.6 folds. The treatment at all concentrations of DCM-DS has no effect on the expression of *JNK*, *BCL-2* and ratio of *BAX* to *BCL-2* (Fig. 6B).

3.7. DCM-DS regulated the expression of apoptotic, growth and survival proteins

The expression of total ERK1 was significantly down-regulated ($p < 0.05$) by 1.4, 1.8 and 1.7 folds at the treatment of 12.5, 25, and 50 μg/mL of DCM-DS, respectively, at 24 h (Fig. 7). The expression of phospho-ERK1/2 was significantly up-regulated ($p < 0.05$) by approximately 2 folds at 24 h at all the tested concentrations. Nevertheless, its expression was down-regulated by 1.4 and 2 folds at the treatment of 12.5 and 25 μg/mL of DCM-DS, respectively, at 48 h. The expression of phospho-JNK1 of MDA-MB-231 cells treated with 12.5, 25, and 50 μg/mL of DCM-DS was significantly up-regulated ($p < 0.05$) by 1.6, 2.3 and 1.4 folds, respectively, at 24 h with no effect on the one of total JNK1. The protein expression at the treatment of 50 μg/mL of DCM-DS at 48 h was unable to be analysed due to extensive cell death resulting in low yield of protein lysate.

The expression of p53 of MDA-MB-231 cells treated with 12.5 and 25 μg/mL of DCM-DS was significantly down-regulated ($p < 0.05$) at 48 h by 1.3 and 1.7 folds, respectively (Fig. 8). The expression of p21 of MDA-MB-231 cells was significantly up-regulated ($p < 0.05$) by approximately 8 and 4 folds at 24 and 48 h, respectively, at all the tested concentrations. Treatment of MDA-MB-231 cells with DCM-DS significantly down-regulated ($p < 0.05$) the expression of BCL-2 by approximately 1.5–1.8 folds at 24 and 48 h, at all the tested concentrations. The expression of BAX was not affected by the treatment. The ratio of BAX to BCL-2 significantly increased ($p < 0.05$) by approximately 1.6 folds at 24 and 48 h (Fig. 8).

3.8. Isolated compounds from DCM-DS

Compound **1**, **2** and **3** were identified as katonic acid (Kaneda et al., 1992), betulinic acid (Tadesse et al., 2012; Chatterjee et al., 2000) and koetjapic acid (Kaneda et al., 1992), respectively, as previously reported (Foo et al., 2015).

3.9. Cytotoxicity of the isolated compounds towards MDA-MB-231 cells

Cytotoxicity of Compound **2** towards MDA-MB-231 cells was noted from the concentration as low as 1.6 μg/mL (Fig. 9). Nonetheless, the cytotoxicity of Compound **1** and **3** towards MDA-MB-231 cells was only noted at the concentration above 25 μg/mL. The IC_{50} value of Compound **1**, **2** and **3** towards MDA-MB-231 cells was 29.3 ± 1.5, 4.4 ± 0.5 and 36.3 ± 1.0 μg/mL, respectively.

4. Discussion

Therapeutic properties of plants/herbs against various diseases have long been gathered through series of trial and errors, and documented in the history of medicine. *Dillenia suffruticosa*, commonly known as "*Simpoh air*", is traditionally used for the treatment of cancerous growth (Ahmad and Holdsworth, 1995). Our previous report revealed that fraction of DCM-DS (D/F4 and D/F5) induced apoptosis towards MDA-MB-231 breast cancer cells without the induction of cell cycle arrest (Armania et al., 2013). In contrast, the present study highlighted that crude DCM-DS was found to induce apoptosis and G_2/M phase cell cycle arrest towards MDA-MB-231 cells (Figs. 1–4). The reason for the switch to crude DCM-DS for further investigation in the present study is the

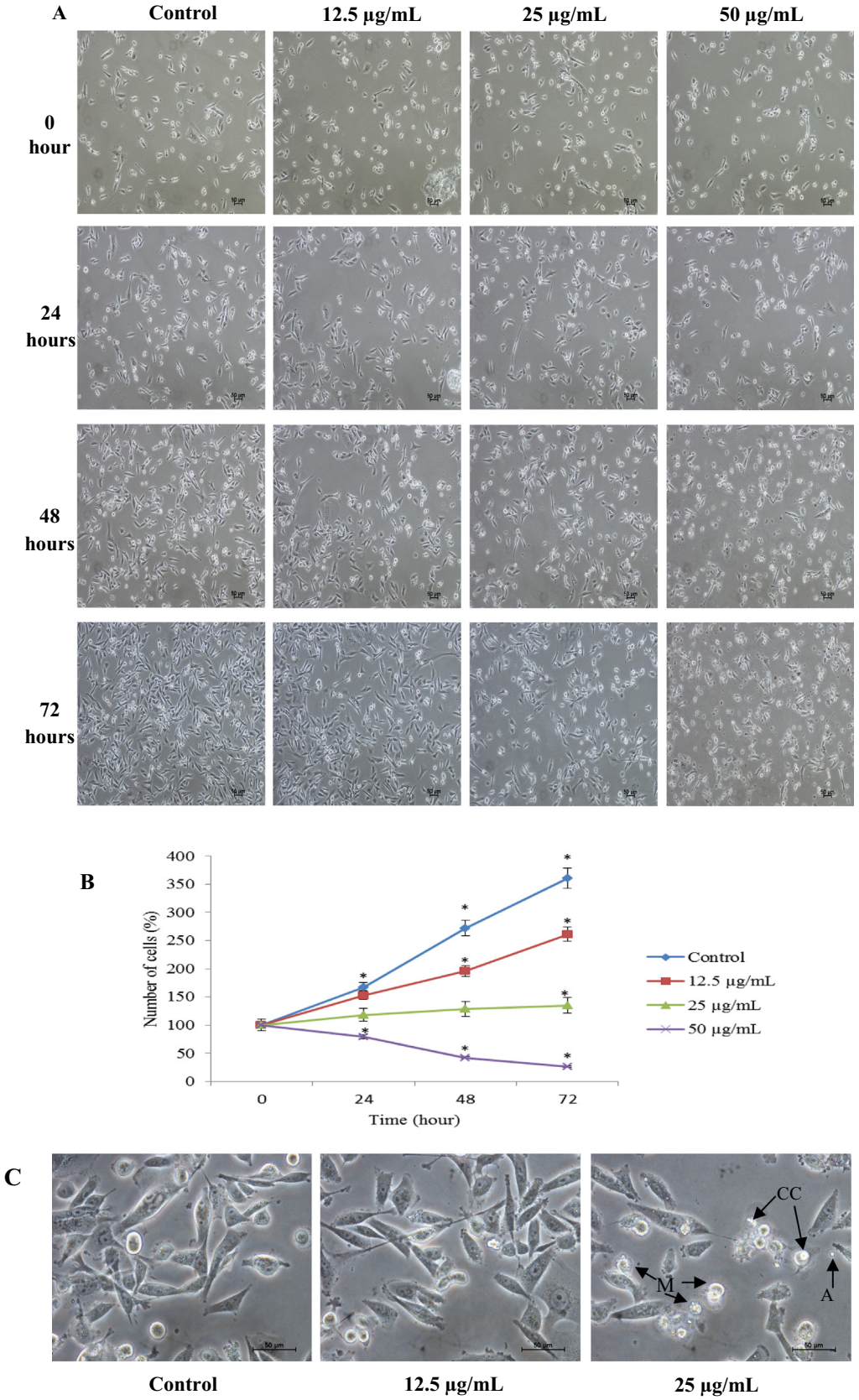
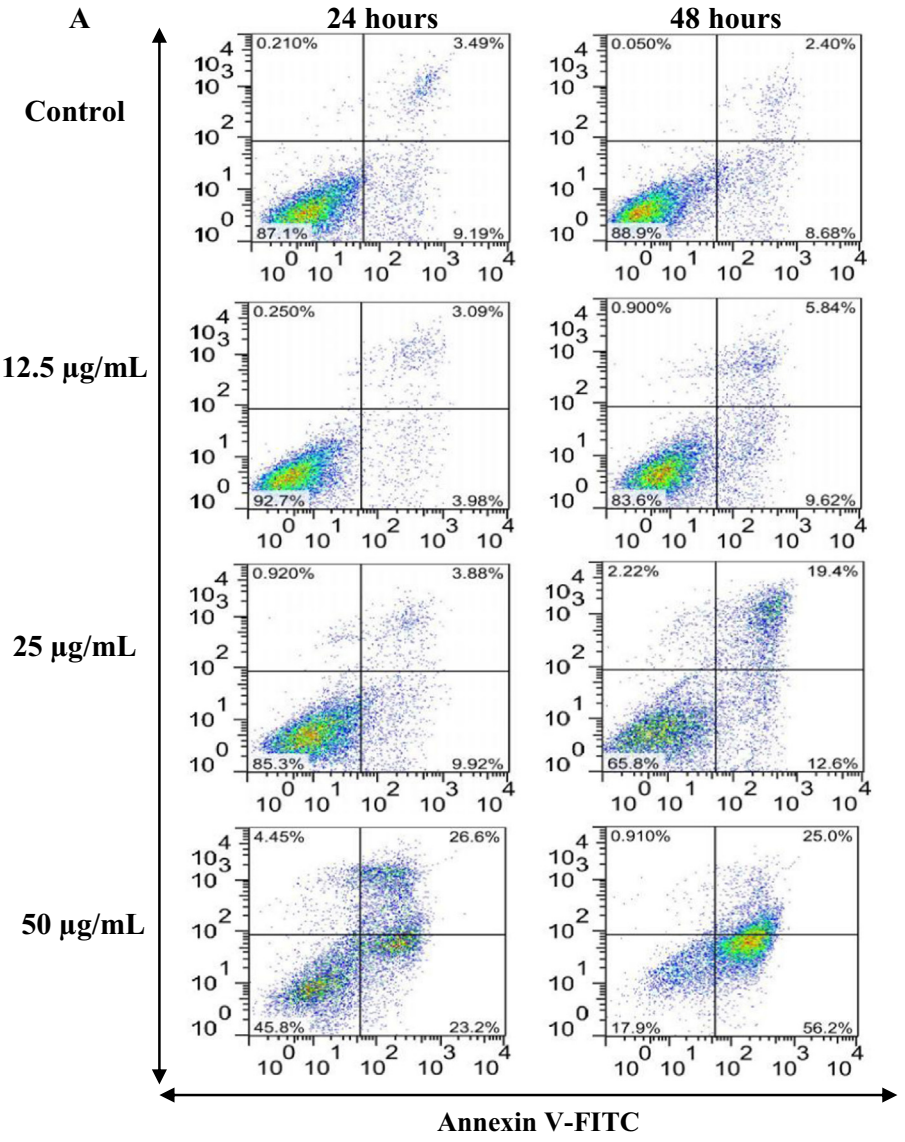


Fig. 2. The population of MDA-MB-231 cells after treatment with DCM-DS as observed under an inverted light microscope. (A) The images were captured on the same spot (100 ×). (B) Changes in number of MDA-MB-231 viable cells treated with DCM-DS at different time point. (C) Close-up view of MDA-MB-231 cells treated with DCM-DS at 48 h (400 ×). Membrane blebbing (M), chromatin condensation (CC) and formation of apoptotic bodies (AB) were observed in DCM-DS-treated MDA-MB-231 cells (arrows). Each data point represents the mean of triplicate ± SD. *significantly different from the control ($p < 0.05$).



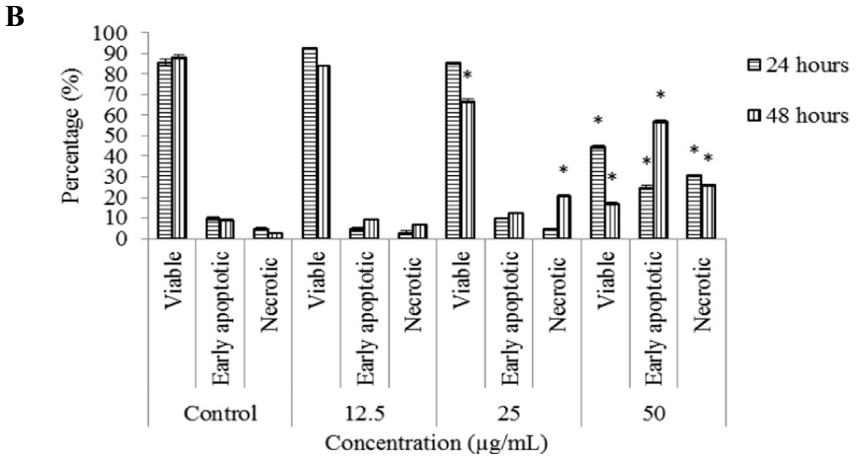


Fig. 3. The percentage of viable, early apoptotic and necrotic/secondary necrotic cells of untreated and DCM-DS-treated MDA-MB-231 cells for 24 and 48 h **as determined by flow cytometry analysis using Annexin-V/PI**. (A) These figures are from representative experiments carried out at least three times. The viable cells was represented by the lower left quadrant (Annexin-V^-/PI^-); the early apoptotic and necrotic/secondary necrotic cells was represented by the lower right (Annexin-V^+/PI^-) and upper (PI^+) quadrant, respectively. (B) DCM-DS induced apoptosis towards MDA-MB-231 cells. Each data point represents the mean of three independent experiments ± SD. *significantly different from the control ($p < 0.05$).

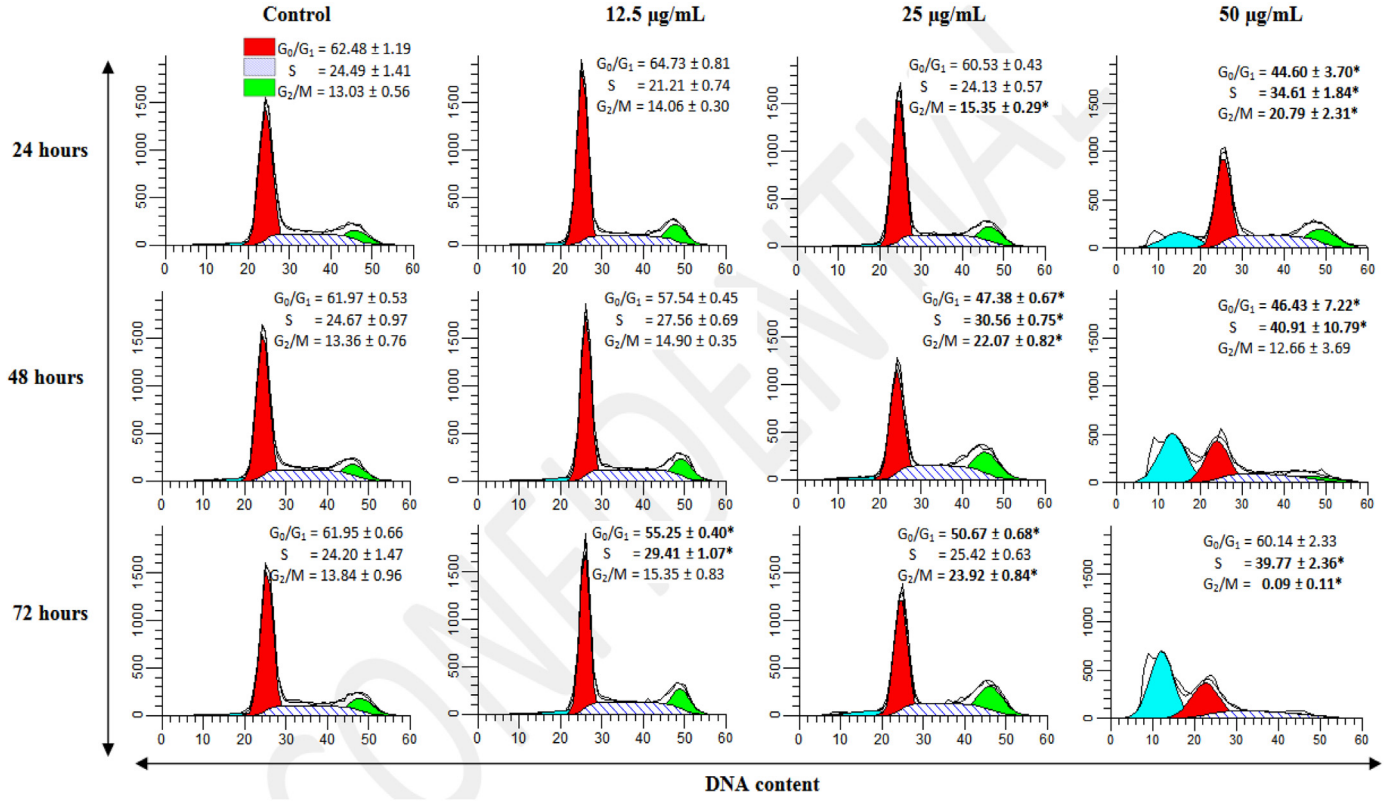


Fig. 4. Cell cycle profile of MDA-MB-231 cells treated with DCM-DS. DCM-DS induced G_2/M phase cell cycle arrest in MDA-MB-231 cells. Each data point represents the mean of three independent experiments ± SD. *significantly different from the control ($p < 0.05$).

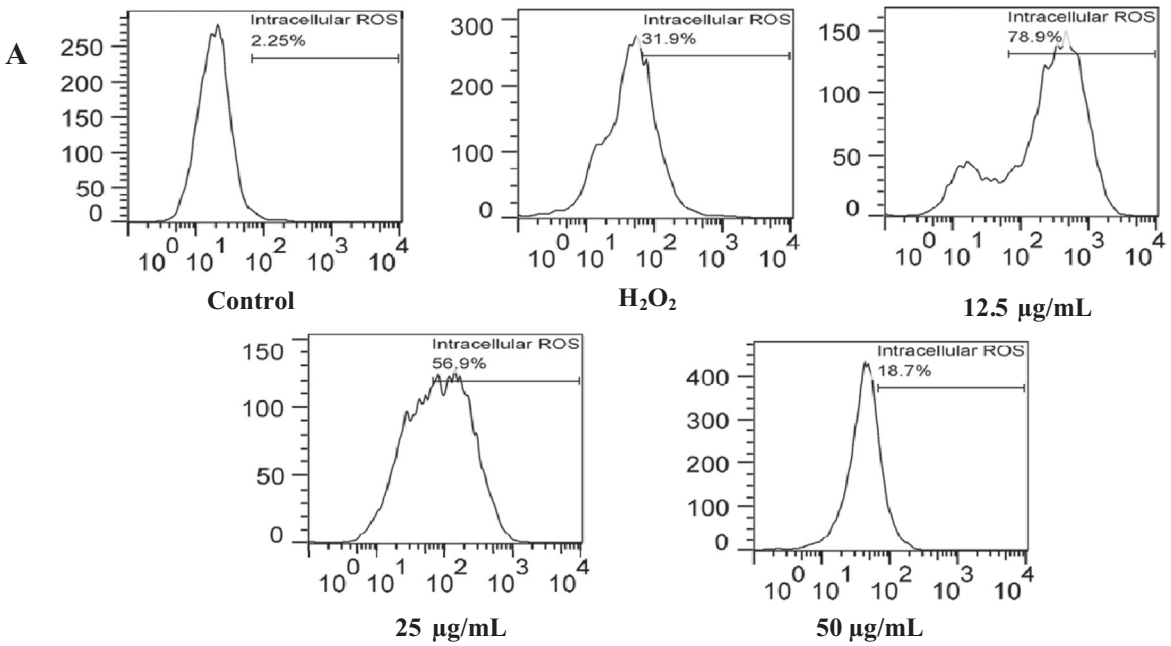
crude DCM-DS seems to have a better profile in terms of toxicity and solubility (Foo et al., 2014; Armania et al., 2013). Other novel findings of the present study include the involvement of ROS in the reduction of viability of MDA-MB-231 cells, shed some light on the apoptosis and cell cycle arrest pathways induced by DCM-DS as well as the identification of bioactive compounds present in DCM-DS.

ROS have long been associated with cancer initiation, progression and metastasis. Ironically, cancer treatment such as chemotherapy and radiotherapy has been reported to induce ROS-dependent cell death in cancer cells (Chua et al., 2009). Therefore, pro-oxidant therapy is another strategy being employed for the treatment of cancer. In the present study, DCM-DS was found to stimulate the production of endogenous ROS in MDA-MB-231 cells that lead to development of oxidative stress (Fig. 5A). Co-treatment of the cells with 50 µg/mL of DCM-DS and α-tocopherol suppressed DCM-DS-induced oxidative stress that reduced the viability of MDA-MB-231 cells (Fig. 5B), indicating that high concentration of the extract induced lipid peroxidation in the cells, resulting in cell membrane damage and cell lysis (Ahmad and Abdullah, 2013). Nevertheless, the suppressive effect of α-tocopherol was not noted at low concentrations of DCM-DS (12.5 and 25 µg/mL) suggesting that the reduced viability of MDA-MB-231 cells at these two concentrations was not due to lipid peroxidation. The mechanism on how the cell viability was reduced remains unclear.

Apoptosis is regulated by multiple intracellular signal transduction pathways such as MAPKs, p53/p21 and mitochondrial signalling pathways. In this study, the up-regulation of expression of phospho-JNK1 in MDA-MB-231 cells with no change to the one of total JNK1 upon treatment with DCM-DS suggests the involvement of JNK1 pathway in the induction of apoptosis by the extract (Fig. 7). JNK is a stress responsive kinase and its activation has been reported to induce apoptosis in various cancer cells including breast cancer (Singh et al., 2012; Chaudhary et al., 2010). The expression of phospho-ERK1 in MDA-MB-231 cells was initially up-regulated at 24 h postulating that activation of phospho-ERK1 inhibits apoptosis for the survival of the cells (Jokinen et al., 2012). Nevertheless, this protein was then down-regulated at 48 h which may involve in the induction of apoptosis in MDA-MB-231 cells (Ng et al., 2014).

Tumour suppressor p53 and p21 are known to be involved in cell cycle arrest and apoptosis. The up-regulation of wild-type p53 can up-regulate p21 which in turn inhibits cyclin-dependent kinases (CDKs), resulting in G_0/G_1 or G_2/M phase cell cycle arrest (Xin et al., 2013). Mutant p53 has been reported to be responsible for the anticancer drugs resistance (Baker et al., 1989). In the present study, the mutant p53 of MDA-MB-231 cells was down-regulated by DCM-DS (Fig. 8). This is an important finding as mutant p53 in human cancers renders the cells to be more resistant to anticancer drugs. Inhibiting the expression of mutant p53 may offer a promising approach for the treatment of breast cancer (Al-Dhaheri et al., 2013). Treatment with DCM-DS also up-regulated the expression of p21 at both gene (Fig. 6) and protein levels (Fig. 8) in MDA-MB-231 cells, suggesting that the extract induced DNA damage in the cells, which triggers the accumulation of p21, leading to G_0/G_1 and G_2/M phase cell cycle arrest in MDA-MB-231. Since MDA-MB-231 cells possess mutant p53, therefore, the up-regulation of p21 by DCM-DS in the MDA-MB-231 cells was via p53-independent pathway (Al-Dhaheri et al., 2013; Antony et al., 2012).

Treatment with DCM-DS significantly increased the BAX/BCL-2 ratio in MDA-MB-231 cells (Fig. 8). It was noted that down-regulation of BCL-2 was at the protein level, but not at the mRNA level. Similarly, the increment of BAX/BCL-2 ratio was also noted at the protein but not at the gene level. Thereby, it is deduced that the mitochondrial apoptotic pathway was initiated at post-translational level in which BCL-2 protein is modified by either



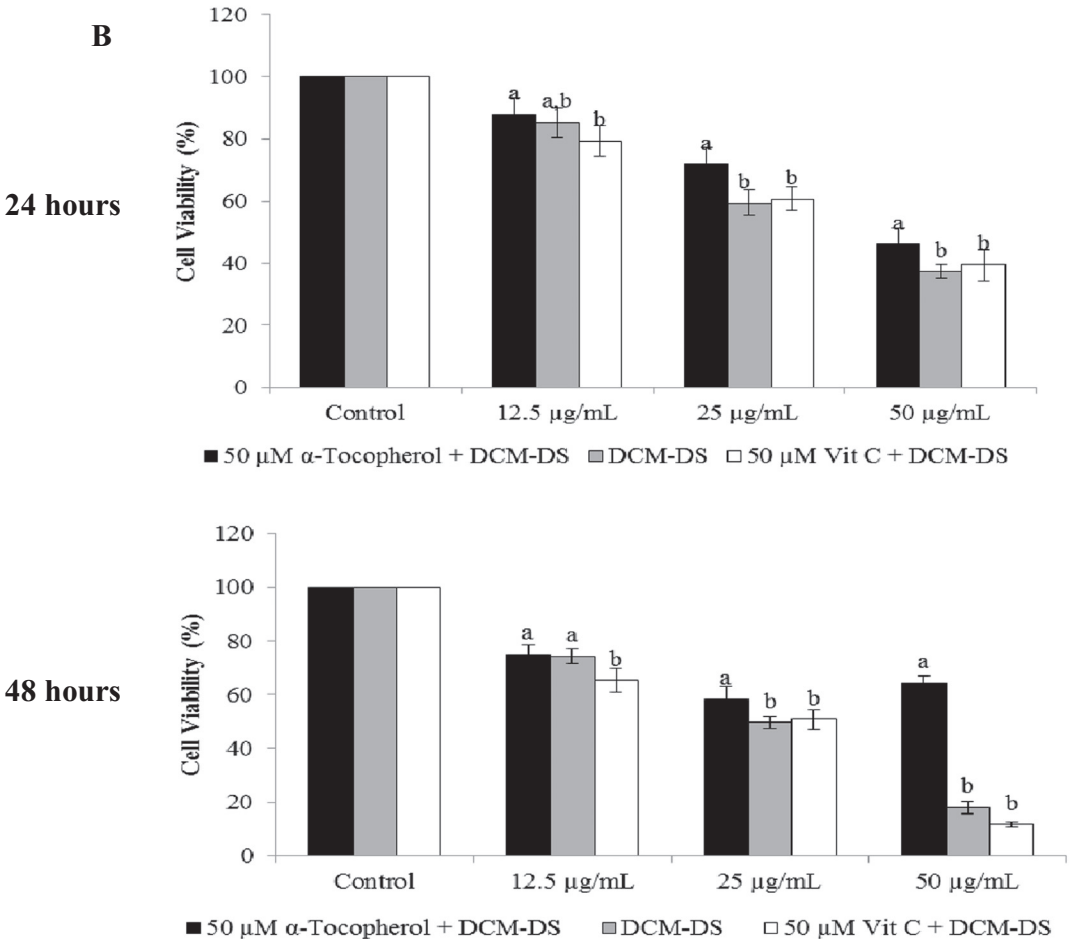


Fig. 5. Determination of the involvement of ROS in the cytotoxicity of DCM-DS in MDA-MB-231 cells. (A) The intracellular ROS level in MDA-MB-231 cells treated with 50 µM H_2O_2 or DCM-DS for 3 h as determined by flow cytometry analysis using DCFH-DA assay. (B) Viability of MDA-MB-231 cells treated with DCM-DS and α-tocopherol or ascorbic acid for 24 and 48 h. Each data point represents the mean of three independent experiments ± SD. a and b in the same concentration at the same time point were considered significant ($p < 0.05$).

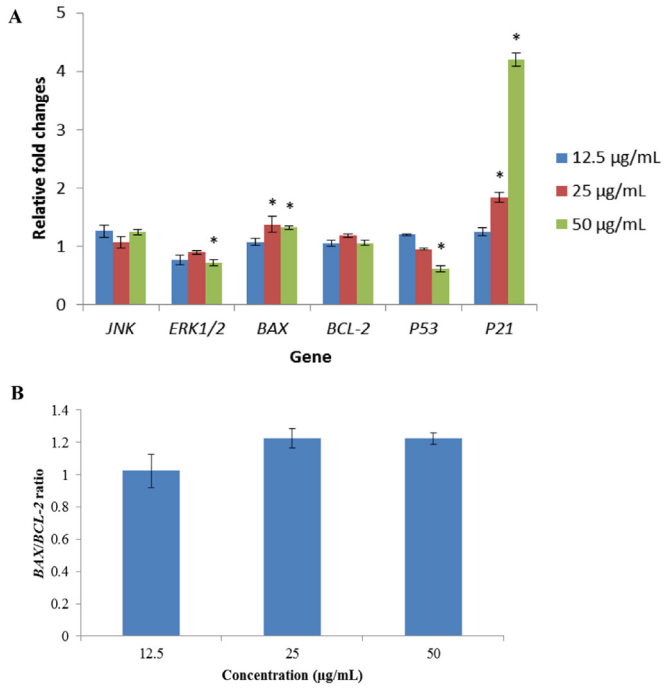


Fig. 6. Effect of DCM-DS on the expression of the apoptotic, growth and survival genes in MDA-MB-231 cells. (A) Relative fold change of the level of gene expression in DCM-DS-treated MDA-MB-231 cells at 24 h which was normalised against beta-actin and compared to the control. (B) *BAX: BCL*-2 ratio in DCM-DS-treated MDA-MB-231 cells at 24 h. Each data point represents the mean of two independent experiments ± SD. *significantly different from the control ($p < 0.05$).

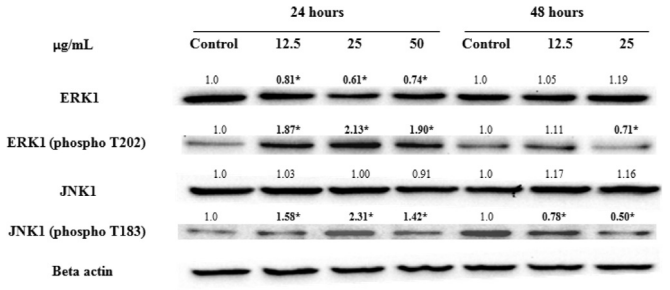


Fig. 7. The expression of ERK1, phospho-ERK1, JNK1 and phospho-JNK1 of DCM-DS-treated MDA-MB-231 cells as determined by Western blot analysis. Figures are from representative experiments carried out at least 3 times. Fold change was normalised against beta-actin and compared to the control. The protein expression at the treatment of 50 µg/mL of DCM-DS at 48 h was unable to be analysed due to extensive cell death. Each data point represents the mean of three independent experiments ± SD. *significantly different from the control ($p < 0.05$).

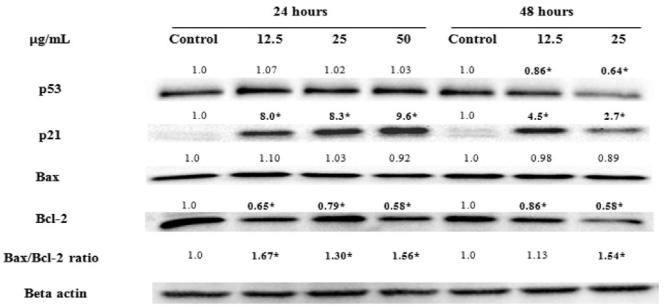


Fig. 8. The expression of p53, p21, BAX, BCL-2 and BAX/BCL-2 ratio of DCM-DS-treated MDA-MB-231 cells as determined by Western blot analysis. Figures are from representative experiments carried out at least 3 times. Fold change was normalised against beta-actin and compared to the control. The protein expression at the treatment of 50 µg/mL of DCM-DS at 48 h was unable to be performed due to extensive cell death. Each data point represents the mean of three independent experiments ± SD. *significantly different from the control ($p < 0.05$).

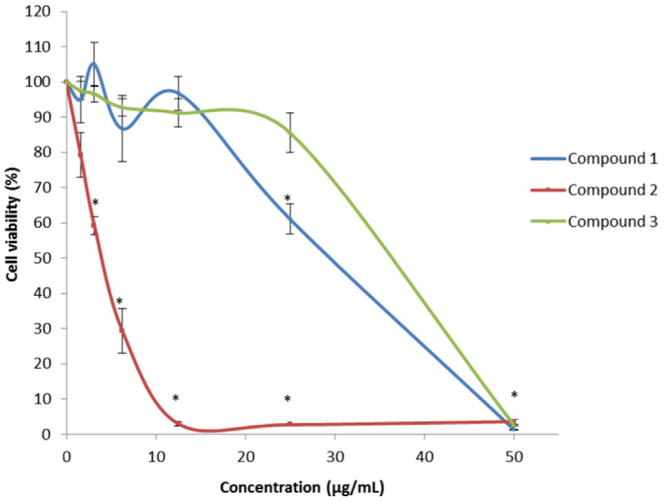


Fig. 9. Effect of Compound 1, 2 and 3 on the viability of MDA-MB-231 breast cancer cells at 72 h as determined by MTT assay. Each data point represents the mean of three independent experiments ± SD. *significantly different from the control ($p < 0.05$).

phosphorylation or ubiquitination via MAPKs resulting in a degradation of BCL-2 protein. Studies have been reporting that inhibition of anti-apoptotic ERK dephosphorylated BCL-2 (Breitschopf et al., 2000) whereby activation of pro-apoptotic JNK phosphorylated BCL-2 (Yanamadala et al., 2007), leading to the degradation of BCL-2. Therefore, the activation of mitochondrial apoptotic pathway in the present study could be attributed to down-regulation of anti-apoptotic phospho-ERK and up-regulation of pro-apoptotic phospho-JNK1 in MDA-MB-231 cells.

Three (3) triterpenes were successfully isolated from DCM-DS. Compound **2**, which was identified as betulinic acid (BA), was the most major and most cytotoxic agent towards the MDA-MB-231 cells (Fig. 9) (Foo et al., 2015). Anti-breast cancer properties of BA have been documented recently (Sun et al., 2013; Wang et al., 2009). BA has been reported to up-regulate p53 and p21 in MCF-7 cells (Wang et al., 2009). In addition, up-regulation and down-regulation of Bax and Bcl-2 proteins, respectively, were also noted in MCF-7 cells treated with BA (Sun et al., 2013). The reported data in those studies are in agreement with our current findings. Therefore, the cytotoxicity of DCM-DS in the present study is largely contributed by BA as the major compound in the extract. Nevertheless, the cytotoxicity may also be due to the synergetic effects of BA with other compounds present in the extract.

5. Conclusion

The induction of apoptosis in MDA-MB-231 cells treated with BA-rich DCM-DS is possibly due to the activation of JNK1 and down-regulation of ERK1, which in turn down-regulates BCL-2 to increase the BAX/BCL-2 ratio to initiate the mitochondrial apoptotic pathway. The down-regulation and up-regulation of mutant p53 and p21, respectively, in MDA-MB-231 cells leading to growth inhibition and apoptosis. This extract can then be employed for the management of breast cancer.

Declaration

There are no conflicts of interest.

Authors' contribution

JBF carried out the study and prepared the manuscript. JBF, AW, YST and NA collected and interpreted the data. NI contributed to GeXP analysis. LSY, YKC and RA contributed to the design and conception of the study and interpretation of data. LSY critically revised manuscript. All authors have read and approved the manuscript for publication.

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