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Induction of cell cycle arrest and apoptosis by betulinic acid-rich fraction from *Dillenia suffruticosa* root in MCF-7 cells involved p53/p21 and mitochondrial signalling pathway

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ABSTRACT

Ethnopharmacological relevance: Dillenia suffruticosa (Family: Dilleniaceae) or commonly known as "*Simpoh air*" in Malaysia, is traditionally used for treatment of cancerous growth including breast cancer. *Aim of the study: D. suffruticosa* root dichloromethane extract (DCM-DS) has been reported to induce G_0/G_1 phase cell cycle arrest and apoptosis in caspase-3 deficient MCF-7 breast cancer cells. The present study was designed to investigate the involvement of p53/p21 and mitochondrial pathway in DCM-DS-treated MCF-7 cells as well as to identify the bioactive compounds responsible for the cytotoxicity of DCM-DS.

Materials and methods: Extraction of *D. suffruticosa* root was performed by the use of sequential solvent procedure. GeXP-based multiplex system was employed to investigate the expression of *p53*, *p21*, *Bax* and *Bcl-2* genes in MCF-7 cells treated with DCM-DS. The protein expression was then determined using Western blot analysis. The bioactive compounds present in DCM-DS were isolated by using column chromatography. The structure of the compounds was elucidated by using nuclear magnetic resonance spectroscopy. The cytotoxicity of the isolated compounds towards MCF-7 cells was evaluated by using MTT assay. The percentage of betulinic acid (BA) in DCM-DS was determined by HPLC analysis.

Results: The expression of p53 was significantly up-regulated at protein level. The expression of p21 at both gene and protein levels was significantly up-regulated upon treatment with DCM-DS, suggesting that the induction of G_0/G_1 phase cell cycle arrest in MCF-7 cells was via p53/p21 pathway. Bcl-2 protein was down-regulated with no change at the mRNA level, postulating that post-translational modification has occurred resulting in the degradation of Bcl-2 protein. Overall, treatment with DCM-DS increased the ratio of Bax/Bcl-2 that drove the cells to undergo apoptosis. 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: DCM-DS induced cell cycle arrest and apoptosis in MCF-7 cells via p53/p21 pathway. In addition, DCM-DS induced apoptosis by increasing the ratio of Bax/Bcl-2. Betulinic acid, which is one of the major compounds, is responsible for the cytotoxicity of the DCM-DS. Therefore, BA can be used as a marker for standardisation of herbal product from *D. suffruticosa*. DCM-DS can also be employed as BA-rich extract from roots of *D. suffruticosa* for the management of breast cancer.

1. Introduction

Plants have long been used for thousands of years in traditional medicine to treat various diseases including cancer. In contrast to the conventional cancer chemotherapy that is based on single molecule-single target approach, being able to target two or more

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pathways at once or a few players in the same pathway would be a more effective therapy for the treatment of cancer. The mixture of phytochemicals in the medicinal plants may have synergistic effect that targets several pathways responsible for cancer development, thus increasing therapeutic efficacy and reducing adverse side effects (Liu and Cheng, 2012; Cao et al., 2011; Yoon et al., 2011). For example, hot water extract of PHY906 from traditional Chinese herbal formulation was used as an adjuvant to chemotherapy for colorectal, liver, and pancreatic cancers (Liu and Cheng, 2012). The successful development of PHY906 has once again inspired the herbal and traditional medicine practitioners to develop evidence-based plant extract for the management of cancer.

Cancer occurs when the balance between cell death and cell proliferation is deregulated. Wild-type p53 tumour suppressor protein has been widely reported to induce cell cycle arrest and apoptosis in cancer cells. The expression of wild-type p53 is mainly up-regulated upon DNA damage by either radiation or anticancer drugs. This protein subsequently up-regulates cell cycle arrest inducer p21 to induce cell cycle arrest at various check-points to repair the damaged DNA. Unrepaired damaged-DNA cells are then targeted for death by apoptosis (Manchado et al., 2012; Taylor and Grabovich, 2009; Essmann et al., 2004). Therefore, anti-cancer agent that can increase the expression level of wild-type p53 offers a promising strategy to inhibit the growth and to induce apoptosis in cancer cells (Antony et al., 2012; Xia et al., 2011).

Apoptosis is an important physiological process essential for normal development and maintenance of tissue homoeostasis. This mode of cell death has been widely studied in the development of anticancer drugs. The advantage of apoptosis-inducing agents for the treatment of cancer is the elimination of tumour cells without causing inflammation (Hou et al., 2005). Numerous reports have implied that mitochondria play a major role in the apoptotic process. Upon treatment with agents that target the mitochondrial such as doxorubicin and UV radiation, the loss of mitochondrial membrane potential through opening of permeability transition pores results in the hyperproduction of reactive oxygen species (ROS) in the cells and apoptosis (Prasad et al., 2011). Bcl-2 family proteins have been reported to maintain cell viability by preventing loss of mitochondrial membrane potential through homo- or heterodimerization. Bax protein has been shown to induce the opening of mitochondrial permeability transition pores whereby Bcl-2 protein inhibits it (Mohan et al., 2012; Ng et al., 2011; Akbas et al., 2005; Timur et al., 2005). Therefore, targeting the mitochondrial pathway is always one of the strategies to eradicate cancer cells.

Dillenia suffruticosa (Griffith ex Hook. F. and Thomson) Martelli (Family: Dilleniaceae), commonly known as "*Simpoh air*", is found abundantly in the secondary forest and swampy ground of Malaysia. This plant has been reported to exhibit antimicrobial (Wiart et al., 2004) and antiviral properties (Muliawan, 2008). Our previous studies reported that the root dichloromethane extract of *D. suffruticosa* (DCM-DS) from sequential solvent extraction was the most cytotoxic towards breast cancer cells as compared to hexane, ethyl acetate and methanol extract (Armania et al., 2013a). DCM-DS 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) and protein kinase B (PKB or AKT) at gene and protein level (Foo et al., 2014; Tor et al., 2014; Armania et al., 2013b). To the best of our knowledge, no chemical investigations have been carried out on this *D. suffruticosa*. The present study was designed to investigate the involvement of p53/p21 and mitochondrial pathway in DCM-DS-treated MCF-7 cells, and to identify the bioactive compounds responsible for the cytotoxicity of DCM-DS towards MCF-7 cells.

2. Materials and methods

2.1. Plant material

Fine powder of *D. suffruticosa* was supplied by Primer Herber Sdn. Bhd., Malaysia. The plant's authentication was performed with the parts of the plants (flower, leaf, stem and root) 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 described (Foo et al., 2014). Briefly, 100 g of the powdered root was macerated with 500 mL of hexane (1:5, w/v) (Friedemann Schmidt, Francfort, Germany) for 2 days 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 until the colour disappeared, dried in the oven (40 °C for 24 h) and further macerated with dichloromethane (DCM) (Friedemann Schmidt, Francfort, Germany). The combined DCM extracts were pooled and DCM was removed under reduced pressure (Rotavapor R210, Buchi, Flawil, Switzerland).

2.3. Cell culture

The human MCF-7 breast cancer cell line was purchased from the American Type and Culture Collection (ATCC, Manassas, VA, USA). MCF-7 cells were grown in phenol-red-free 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 20 passage number.

2.4. RNA extraction

Cells were seeded in 6-well plates at 1.3×10^5 cells per well in 3 mL of complete growth culture media. After treatment with DCM-DS for 24 h, the floating cells were collected and the adherent cells were trypsinised to detach the cells. The cells were centrifuged at 100*g* to obtain a pellet and washed twice with PBS. RNA was then isolated from the cells using the Real Genomics Total RNA Extraction Kit (RBCBioscience, Taipei, Taiwan). Briefly, the cells were mixed with 100 μL of lysis buffer, 400 μL of RB buffer and 4 μL of β-mercaptoethanol, and incubated for 5 min on ice. After incubation, 400 μL of 70% ethanol was added. Vigorous pipetting was performed to break any precipitate. The mixture was then transferred to RT column and centrifuged at 1000*g* for 2 min to bind RNA to the column. The RT column was transferred to a new collection tube, washed once with W1 Buffer and 2 times with Wash Buffer. Finally, the RT column was transferred to another new collection tube. RNAse-free water (50 μL) was added into the column matrix to dissolve the RNA. The RT column was centrifuged at 1000*g* for 1 min to elute purified RNA. The RNA concentration and quality were checked by a nano-photometer (Implen, Baxter Avenue, Britain).

2.5. cDNA synthesis

RNA was reverse transcribed with multiplex universal reverse primers (Table 1) according to the GenomeLab™ GeXP Start Kit (Beckman Coulter Inc, CA, USA) from Beckman Coulter protocol with slight modifications. Briefly, 50 ng of RNA (1 μL) from each sample was mixed with 1 μL of KAN^r RNA, 1 μL of reverse transcriptase, 2 μL of multiplex universal reverse primers, 4 μL of

Table 1
Genes used in GeXP multiplex analysis.

Name	Accession number	Forward primer sequence (5′–3′)	Reverse primer sequence (3′–5′)
P53	NM_001126117	**AGGTGACACTATAGAATA**GGGGAGCAGGGCTCA	GGGAGGGGACGGTAAAAA**GGGATATCACTCAGCATG**
P21	NM_000389	**AGGTGACACTATAGAATA**TTAGCAGCGGAACAAGGAGT	ACCTGACAAAAGAGAGCCGAA**GGGATATCACTCAGCATG**
BCL-2	M14745	**AGGTGACACTATAGAATA**ACCACTAATTGCCAAGCACC	TTCTCGTCTGCCTACCTTTTA**GGGATATCACTCAGCATG**
BAX	BC014175	**AGGTGACACTATAGAATA**CCCTTTTGCTTCAGGGTTTC	AACAGCGGGAAAAGATGAAACA**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 normalisation.

[b] Internal control gene.

5X reverse transcription buffer and 11 μL of RNAse-free water. The total volume for each sample was 20 μL. The reverse transcription was performed in a XP Thermal Cycler (Bioer Technology, Hangzhou, China) with the following mode: 48 °C for 1 min; 42 °C for 60 min; 95 °C for 5 min and hold at 4 °C.

2.6. Polymerase chain reaction

Following reverse transcription, polymerase chain reaction (PCR) was performed to amplify the amount of cDNA according to the GenomeLab™ GeXP Start Kit (Beckman Coulter Inc, CA, USA) from Beckman Coulter protocol. cDNA from each sample (9.3 μL) was mixed with 2 μL of 200 nM forward universal primer mixture, 4 μL of 25 mM $MgCl_2$, 4 μL of 5X PCR Master Mix buffer and 0.7 μL of *Taq* polymerase. The total volume for each sample was 20 μL. Amplification conditions consisted of 95 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 70 °C for 1 min. The reactions were performed in a XP Thermal Cycler (Bioer Technology, Hangzhou, China).

2.7. Gene multiplex data analysis

PCR product (l μL) was mixed with 38.5 μL of sample loading solution along with 0.5 μL of DNA Size Standard 400 (GenomeLab GeXP Start Kit, Beckman Coulter, Inc) and analysed on a GeXP genetic analysis system (S.Kraemer Boulevard, USA). The GeXPS system was used to separate PCR products based on size by capillary gel electrophoresis and to measure their dye signal strength in arbitrary units (A.U.) of optical fluorescence, defined as the fluorescent signal minus background. The data were then analysed using the Fragment Analysis module of the GeXP system software and imported into the analysis module of eXpress Profiler software. Fold change was normalised against beta actin.

2.8. Western blot analysis

Bovine serum albumen (BSA) and Chemi-Lumi One L were purchased from Nacalai Tesque (Kyoto, Japan). Phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor cocktails were purchased from Calbiochem (San Diego, CA, USA). Sodium dodecyl sulphate (SDS), Triton-X 100, Tris-base, glycine, acrylamide, bisacrylamide, ammonium persulfate (APS), tetramethylethylenediamine (TEMED), 10% Tween-20, Bradford Reagent, 2-mercaptoethanol, extra thick blotting paper and pre-stained protein marker were purchased from Bio-Rad (California, USA). Immobilon-FL polyvinylidene fluoride (PVDF) membrane with 0.45 μm pore size was purchased from Millipore (Bedford, MA, USA). Rabbit anti-p21 (ab7960) and anti-Bcl-2 (ab7973) primary antibodies were purchased from ABCAM (Cambridge, MA, USA). Mouse anti-Bax (N-20: sc-493), anti-p53 (DO-1: sc-126) and anti-beta-actin (sc-47778) primary antibodies 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.

MCF-7 cells were seeded in 75 cm^2 tissue culture flasks at 800,000 cells per flask with 15 mL of complete growth culture media and incubated for 24 h. After treatment with DCM-DS for 24 and 48 h, the floating cells were collected in a 50 mL centrifuge tube and washed twice with cold PBS. The adherent cells were also washed twice with cold PBS, lysed with 200 μL of cold lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% SDS (w/v), 1% Triton-X 100 (v/v), 0.5% sodium deoxycolate (w/v)). PMSF (1 mM), protease inhibitor cocktails (10 μL/mL of lysis buffer) and phosphatase inhibitor cocktails (10 μL/mL of lysis buffer) were added to the cold lysis buffer prior to lysis of the cells. The flasks were then kept on ice for 5 min with occasionally swirling the flasks for uniform spreading of the lysis buffer. The lysate was gathered at one side using a cell scraper, collected with 1 mL pipette, pooled with the floating cells, transferred to a microcentrifuge tube and centrifuged at 14,000*g* for 10 min to pellet the cell debris. The clarified supernatant was then collected and stored at −80 °C. The protein concentration quantification was performed by using Bradford Protein Assay (Bradford, 1976).

An equal amount of 10–20 μg of proteins was separated by 12% SDS-PAGE. After electrophoresis, the proteins were transferred to PVDF membrane by semi-dry transfer method, blocked with 3% BSA in 0.1% Tween-20 containing Tris-Buffer Saline (TBS-T) at room temperature (20–25 °C) for 1 h, reacted with anti-p53 (1:1000), anti-p21 (1:10,000), anti-Bcl-2 (1:1000), anti-Bax (1:10,000) and anti-beta-actin (1:10,000) primary antibodies in TBS-T overnight at 4 °C. After washing 3 times with TBS-T at room temperature, 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 in TBS-T for 1 h at room temperature. 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. The protein level was quantified by using the ImageJ software, normalised to beta-actin.

2.9. Isolation of compounds

Analytical grade ethyl acetate (EtOAc), chloroform ($CHCl_3$), acetone, methanol (MeOH) were purchased from Friedemann Schmidt (Francfort, Germany). Cerium (IV) sulphate was purchased from Nacalai Tesque (Kyoto, Japan). Silica gel 60 (0.063–0.200 mm), silica gel 60 (0.040–0.063 mm), thin layer chromatography (TLC) silica gel 60 F_{254} (aluminium sheet and glass plate), deuterated chloroform ($CDCl_3$), deuterated acetone (CD_3COCD_3), deuterated DMSO (C_2D_6OS) were purchased from Merck (Darmstadt, Germany).

The extraction of *D. suffruticosa* root powder was performed as previously described (Foo et al., 2014). Twenty grams (20 g) of

DCM-DS were subjected to silica gel column chromatography and eluted with a combined of *n*-hexane:EtOAc as the solvent system in increasing polarity (9:1 to 6:4 v/v) to give 19 major fractions (DF_{1-19}). Purification of fraction DF_8 (1.1 g) using silica gel column chromatography and eluted with mobile phase consisting of a combination of *n*-hexane:$CHCl_3$:acetone (7:2:1) yielded Compound **1** (50 mg), while purification of fraction DF_{10} (7 g) by using the similar ways as fraction DF_8 gave Compound **2** (1 g). Furthermore, purification of fraction DF_{13} (1.9 g) also using silica gel column chromatography and eluted with a combination of *n*-hexane:$CHCl_3$:EtOAc (4:3:3) as the solvent system yielded Compound **3** (30 mg). The isolated compounds were subjected to nuclear magnetic resonance (NMR) analysis. The proton (^1H) and carbon (^{13}C) NMR spectra were recorded on a Varian Unity Inova 500 MHz spectrometer in deuterated chloroform ($CDCl_3$) or DMSO (C_2D_6OS). HRESI-MS were obtained with a JEOL AccuTOF-T100LP mass spectrometer.

2.10. Determination of cytotoxicity

The stock solution (50 mg/mL) of the isolated compounds was prepared in DMSO (Friedemann Schmidt, Francfort, Germany). MCF-7 cells were trypsinised (trypsin-EDTA (1X), PAA, Pasching, Austria) 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. 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 added into each well and the plate was incubated for 3 h. The excess MTT was then aspirated and the formazan crystals formed were dissolved by 150 μL of DMSO. The absorbance, which was proportional to cell viability, was measured at 570 nm and a reference wavelength of 630 nm by using ELx800™ Absorbance Microplate Reader (BioTek Instruments Inc., Vermont, USA). Cell viability was calculated based on the following equation (Badakhshan et al., 2009):

$$\text{Cell viability}(\%) = \frac{OD_{570-630}\text{Treatment}}{OD_{570-630}\text{Control}} \times 100$$

A graph of percentage of cell viability versus concentration of DCM-DS was plotted, and the concentration of DCM-DS which inhibited 50% of cellular growth as compared to the control (IC_{50} value) was determined (Srisawat et al., 2014; Wang et al., 2014; Latifah et al., 2011).

2.11. Quantification of betulinic acid

Quantification of betulinic acid (BA) in DCM-DS was performed by Alliance high performance liquid chromatography (HPLC) (Waters, USA) according to previously published method with some modifications (Dehelean et al., 2012; Şoica et al., 2012). The chromatography was carried out using a reversed-phase C18 Chromolith column (5 μm, 50 mm × 2 mm) (Merck, Darmstadt, Germany). The mobile phase consisted of A (methanol) and B (0.1% formic acid in water). The elution process was isocratic at 80% A and 20% B for 25 min. The flow rate was maintained at 1 mL/min and the injection volume was 10 μL. The spectral data from the UV detector were collected at 209.7 nm wavelength. The Empower software was used for data processing. The calibration curve of standard BA was constructed by plotting the peak area versus concentration. The linear regression equation was used to calculate the concentration of BA in the DCM-DS. The result was presented as weight/weight percentage using the formula as follow:

$$\frac{\text{Calculated concentration (ppm)}}{\text{Loaded concentration (ppm)}} \times 100$$

2.12. 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 Dunnett's post-hoc test. A difference was considered to be significant at $p < 0.05$.

3. Results

3.1. DCM-DS up-regulated the expression of p21 and Bax genes in MCF-7 cells

Treatment of MCF-7 cells with DCM-DS at 25 and 50 μg/mL significantly up-regulated ($p < 0.05$) the expression of *p21* gene by approximately 2 and 5 folds, respectively (Fig. 1A). The expression of *p53* was unaffected by the treatment at all the tested concentrations. Treatment of MCF-7 cells with DCM-DS at 50 μg/mL significantly ($p < 0.05$) up-regulated the expression of *Bax* gene by approximately 1.4 folds but has no effect on the expression of *Bcl-2* gene at all the tested concentrations of DCM-DS. The ratio of *Bax* to *Bcl-2* was found not affected by the treatment at all the tested concentrations (Fig. 1B).

3.2. DCM-DS increased the protein expression of p53, p21 and ratio of Bax/Bcl-2 in MCF-7 cells

The expression of p53 of MCF-7 cells treated with 25 and 50 μg/mL of DCM-DS for 24 h was significantly up-regulated ($p < 0.05$) by 2.4 and 3.7 folds, respectively (Fig. 2). The expression of p21 was significantly up-regulated ($p < 0.05$) at 24 and 48 h by approximately 4 and 3 folds, respectively, at all the tested concentrations. Treatment of MCF-7 cells with 50 μg/mL of DCM-DS for 48 h significantly up-regulated ($p < 0.05$) the expression of Bax by 1.6 folds. Bcl-2 was significantly down-regulated ($p < 0.05$) by nearly 2 folds after treatment with DCM-DS at 25 and 50 μg/mL for 48 h. The ratio of Bax to Bcl-2 increased by approximately 2 and 3 folds at 25 and 50 μg/mL of DCM-DS, respectively, at 48 h.

3.3. Isolated compounds from DCM-DS

A total of three compounds were successfully isolated from DCM-DS. There are two oleanane triterpenes; katonic acid (**1**) and koetjapic acid (**3**) (Kaneda et al., 1992), and a lupane triterpene; betulinic acid (BA) (**2**) (Tadesse et al., 2012; Chatterjee et al., 2000). The chemical structures of those compounds were characterised based on their ^1H and ^{13}C NMR spectral and comparison with literature. From the result, the main skeleton; olean-12-ene in katonic acid (**1**) was identified from the existence of olefinic carbon signals at δ_C 121.9 (C-12) and 144.4 (C-14), while the attachment of hydroxyl group at C-3 and carboxyl group at C-29 was confirmed from the presence of carbon signals at δ_C 73.8 and 178.0, respectively. This fact was supported by the existence of methine olefinic proton signal at δ_H 5.18 (*t*, *J* = 3.3 Hz, H-12), oxymethine proton signal at δ_H 4.22 (brs, H-3), and seven singlet methyl proton signals at δ_H 0.74–1.13. BA (**2**) was characterised by chemical shift of quaternary and methylene olefinic carbons [δ_C 109.6 (C-29) and 150.3 (C-20), respectively] typical for lupan-20-ene skeleton, hydroxyl group at δ_C 76.8 (C-3) and carboxyl group at δ_C 177.2 (C-28). These signals are in agreement with ^1H NMR spectrum,

which displayed the specific signal for geminal proton signals on an sp^2 carbon at δ_H 4.55 (br d, J=2.0 Hz, H-29a) and 4.68 (br d, J=2.0 Hz, H-29b), oxymethine proton signal at δ_H 2.97 (m, H-3), and six singlet methyl proton signals at δ_H 0.76–1.62. Similar to katonic acid (**1**), the main skeleton; olean-12-en-oic acid of koetjapic acid (**3**) was also identified from the existence of olefinic carbon signals at δ_H 121.7 (C-12) and 144.3 (C-14), and carboxyl group at δ_C 178.0 (C-30). Furthermore, a fissioned ring-A typical for secotriterpene skeleton was characterised from the presence of additional quaternary and methylene olefinic carbons [δ_C 109.6 (C-29) and 150.3 (C-20), respectively] and a carboxyl group [δ_C 174.8 (C-3)] in the ^{13}C NMR spectrum of Compound **3**, and six singlet methyl proton signals at δ_H 0.88 (H-25), 0.97 (H-26), 1.15 (H-27), 0.74 (H-28), 1.07 (H-29) and 1.71 (H-30) in the ^{1}H NMR spectrum of Compound **3**. The structure of isolated compounds is shown in Fig. 3.

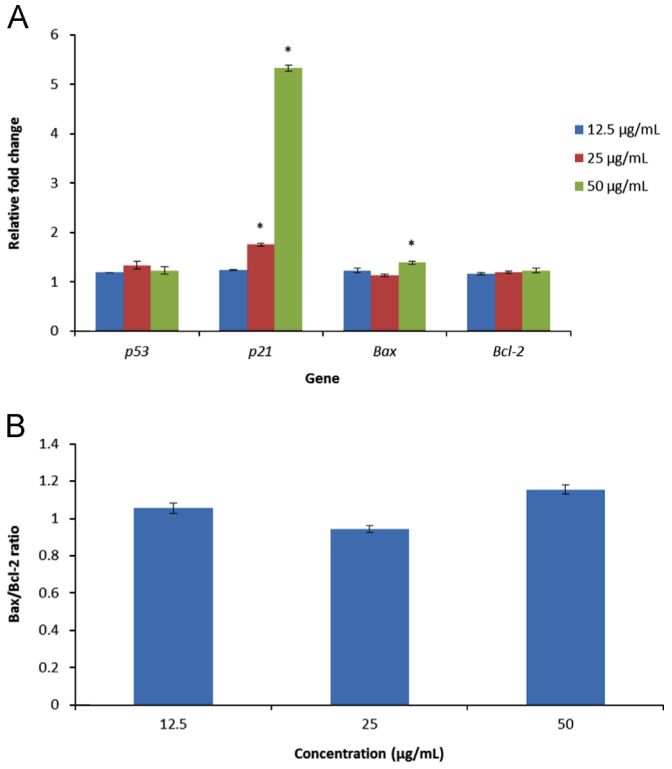

Fig. 1. Expression levels of genes in MCF-7 cells following treatment with DCM-DS. (A) Relative fold change of gene expression in DCM-DS-treated MCF-7 cells at 24 h was normalised against beta-actin and compared to the control. (B) *Bax:Bcl-2* ratio in DCM-DS-treated MCF-7 cells at 24 h. Each data point represents the mean of three independent experiments ± SD. *significantly different from the control ($p < 0.05$).

Katonic acid (**1**)

^{1}H NMR (500 MHz, DMSO-*d*): δ 4.22 (brs, 1H, H-3), 5.18 (t, J= 3.3 Hz, 1H, H-12), 1.07 (s, 3H, H-23), 0.74 (s, 3H, H-24), 0.84 (s, 3H, H-25), 0.89 (s, 3H, H-26), 0.91 (s, 3H, H-27), 0.77 (s, 3H, H-28), 1.13 (s, 3H, H-30). ^{13}C NMR (126 MHz; DMSO-*d*): 32.8 (C-1), 25.7 (C-2), 73.8 (C-3), 38.1 (C-4), 48.1 (C-5), 17.8 (C-6), 32.2 (C-7), 39.5, (C-8), 47.8 (C-9), 37.0 (C-10), 22.9 (C-11), 121.9 (C-12), 144.4 (C-13), 42.4 (C-14), 25.2 (C-15), 26.4 (C-16), 31.6 (C-17), 46.9 (C-18), 41.2 (C-19), 43.2 (C-20), 30.6 (C-21), 36.5 (C-22), 28.7 (C-23), 25.8 (C-24), 15.1 (C-25), 16.5 (C-26), 28.1 (C-27), 28.2 (C-28), 178.0 (C-29), 22.3 (C-30). HRESI-MS *m/z*: $[M]^+$ 456.3605 (Calc. for $C_{30}H_{48}O_3$, 456.3603).

Betulinic acid (**2**)

^{1}H NMR (500 MHz; $CDCl_3$): δ 2.97 (m, 1H, H-3), 2.22 (td, J=3.5; 12.5 Hz, 1H, H-5), 0.65 (s, 3H, H-23), 0.76 (s, 3H, H-24), 0.86 (s, 3H, H-25), 0.86 (s, 3H, H-26), 0.93 (s, 3H, H-27), 4.55 (br d, J=2.0 Hz, 1H, H-29a) and 4.68 (br d, J=2.0 Hz, 1H, H-29b), 1.62 (s, 3H, H-30). ^{13}C NMR (126 MHz; $CDCl_3$): δ 36.3 (C-1), 27.1 (C-2), 76.8 (C-3), 38.5 (C-4), 54.9 (C-5), 18.0 (C-6), 33.9 (C-7), 40.2 (C-8), 49.9 (C-9), 36.7 (C-10), 20.4 (C-11), 25.1 (C-12), 38.2 (C-13), 42.0 (C-14), 29.2 (C-15), 31.7 (C-16), 55.4 (C-17), 46.6 (C-18), 48.5 (C-19), 150.3 (C-20), 30.1 (C-21), 37.5 (C-22), 28.1 (C-23), 15.7 (C-24), 15.8 (C-25), 15.9 (C-26), 14.4 (C-27), 177.2 (C-28), 109.6 (C-29), 18.9 (C-30). HRESI-MS *m/z*: $[M]^+$ 456.3601 (Calc. for $C_{30}H_{48}O_3$, 456.3603).

Koetjapic acid (**3**)

^{1}H NMR (500 MHz; DMSO-*d*): δ 5.18 (s, 1H, H-12), 4.84 (s, 1H, H-24a), 4.66 (s, 1H, H-24b), 0.88 (s, 3H, H-25), 0.97 (s, 3H, H-26), 1.15 (s, 3H, H-27), 0.74 (s, 3H, H-28), 1.07 (s, 3H, H-29), 1.71 (s, 3H, H-30). ^{13}C NMR (126 MHz; DMSO-*d*): δ 24.1 (C-1), 33.9 (C-2), 174.8 (C-3), 147.2 (C-4), 49.3 (C-5), 28.1 (C-6), 30.8 (C-7), 39.0 (C-8), 37.2 (C-9), 38.7 (C-10), 23.1 (C-11), 121.7 (C-12),

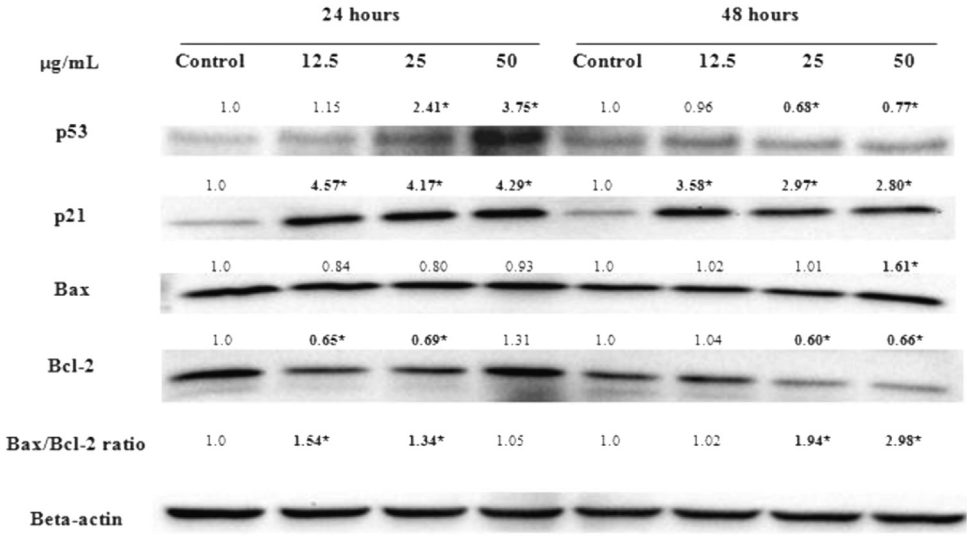

Fig. 2. Expression levels of proteins in MCF-7 cells following treatment with DCM-DS. (A) Western blot analysis of the expression of p53, p21, Bax, Bcl-2 and beta-actin from DCM-DS-treated MCF-7 cells. These figures are from representative experiments carried out at least 3 times. Fold change was normalised against beta-actin and compared to the control. Each data point represents the mean of three independent experiments ± SD. *significantly different from the control ($p < 0.05$).

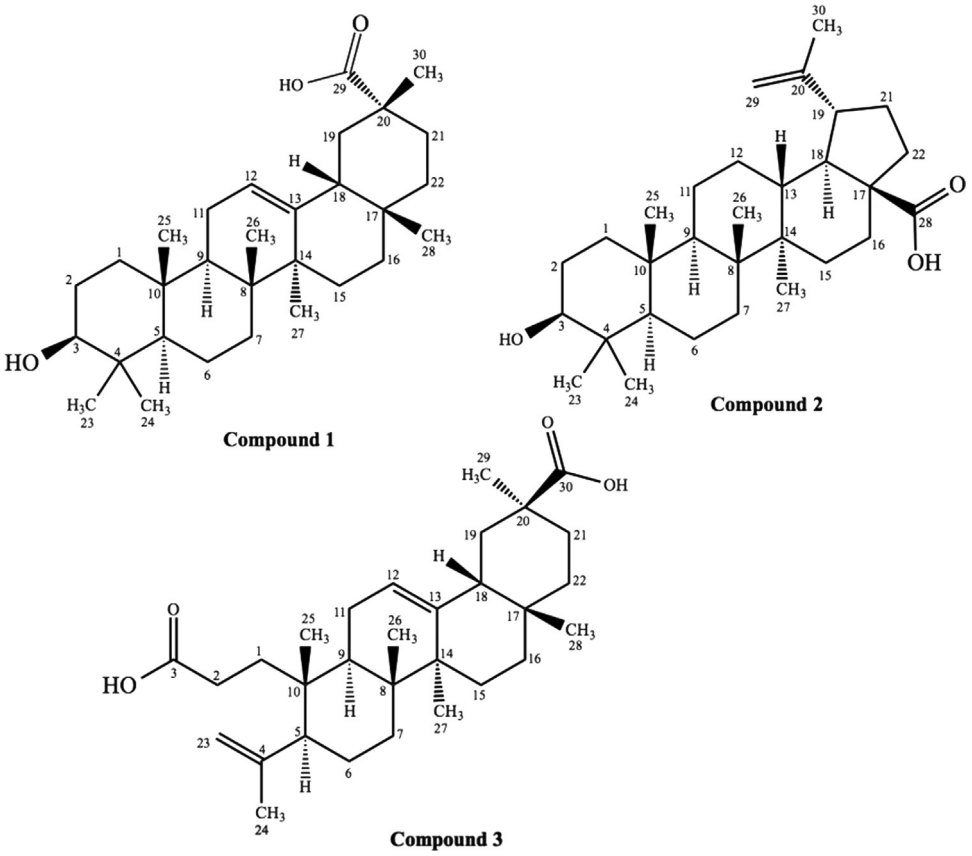

Fig. 3. Chemical structure of isolated compounds from DCM-DS.

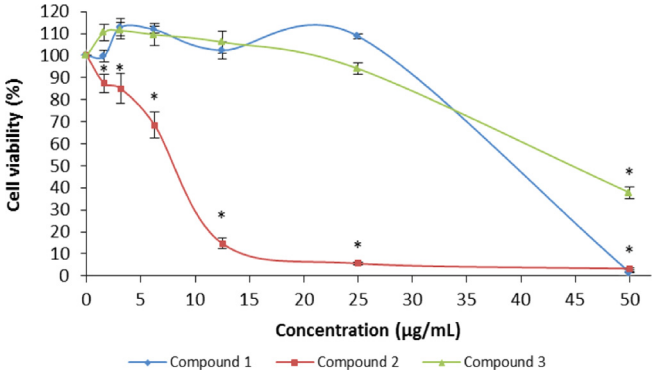

Fig. 4. Effect of Compounds **1**, **2** and **3** on the viability of MCF-7 breast cancer cells at 72 h as determined by MTT assay. Each data point represents the mean of three independent experiments ± SD. *significantly different from the control ($p < 0.05$).

Table 2
Retention time and area under curve of different concentration of standard BA obtained from HPLC.

Standard BA concentration (ppm)	Retention time (min)	Area under curve (AUC)
250	10.441	668,100
500	10.471	1,540,994
750	10.508	2,430,419
1000	10.507	3,098,348

144.3 (C-13), 41.6 (C-14), 25.7 (C-15), 26.4 (C-16), 31.6 (C-17), 47.9 (C-18), 42.4 (C-19), 43.2 (C-20), 30.6 (C-21), 38.0 (C-22), 113.4 (C-23), 23.5 (C-24), 19.4 (C-25), 16.6 (C-26), 25.5 (C-27),

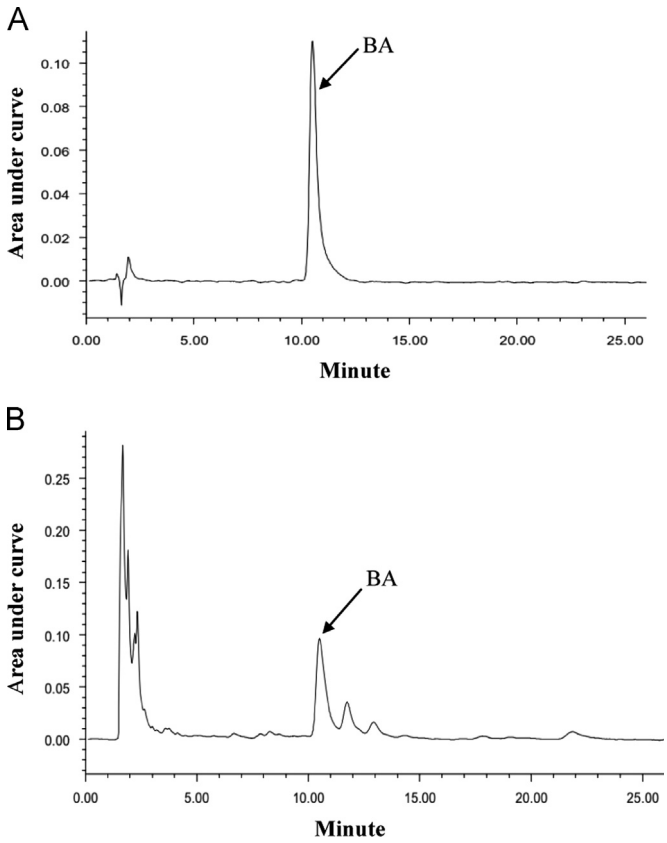

Fig. 5. HPLC profile of standard betulinic acid (A) and DCM-DS (B).

28.2 (C-28/29), 178.0 (C-30). HRESI-MS m/z: $[M]^+$ 470.3392 (Calc. for $C_{30}H_{46}O_4$, 470.3396).

3.4. Cytotoxicity of the isolated compounds towards MCF-7 cells

Compound **2** was cytotoxic towards MCF-7 cells in a dose dependent manner from the concentration as low as 1.6 μg/mL. Nonetheless, the cytotoxicity of Compounds **1** and **3** towards MCF-7 cells was only noted at the concentration above 25 μg/mL (Fig. 4). The IC_{50} value of Compounds **1**, **2** and **3** towards MCF-7 cells was 39.25 ± 0.29, 7.55 ± 0.21 and 44.75 ± 1.17 μg/mL, respectively.

3.5. Percentage of betulinic acid in DCM-DS

The details of the HPLC profile of standard BA are demonstrated in Table 2. A profile with a retention time of 10.482 (± 0.03) was obtained. The standard peaks were clear and sharp with purity of 99% (Fig. 5A). The linear standard curve for standard BA within the concentration range of 250–1000 ppm was $y = 3272.1x - 110577$ ($R^2 = 0.996$). The HPLC profile for DCM-DS is illustrated in Fig. 5B. The yield of BA in DCM-DS was found to be $33 \pm 2.5\%$.

4. Discussion

Uncontrolled cell division is the hallmark of cancer (Taylor and Grabovich, 2009; Bartek and Lukas, 2001). Numerous studies have reported that the metastatic spread of a cancer cell to a new site does not involve cell cycle regulation (Cunha et al., 2014; Mego et al., 2010; Hunter et al., 2008). However, once at a new site, the cancer cell divides and develops as secondary tumour. Thus, cell division is the foundation of tumour formation. It is for this reason that cell division provides another important target for the treatment of cancer (Prasad and Koch, 2014; Taylor and Grabovich, 2009; Jada et al., 2008).

Our previous study reported that DCM-DS induced G_0/G_1 phase cell cycle arrest and apoptosis in MCF-7 cells (Foo et al., 2014). In the present study, the wild-type tumour suppressor p53 and p21 of MCF-7 cells was up-regulated (Figs. 1 and 2). It is suggested that the up-regulation of wild-type p53 by DCM-DS triggers the accumulation of p21 protein, leading to G_0/G_1 phase cell cycle arrest and apoptosis in MCF-7 cells (Xia et al., 2011; Taylor and Grabovich, 2009; Alkhalaf and El-Mowafy, 2003). p53/p21 involves in the control of cell cycle, apoptosis and maintenance of genomic stability (Antony et al., 2012). Once DNA damage occurs, wild-type p53 is expressed to induce cell cycle arrest at various checkpoints to repair the damaged DNA. Unrepaired damaged-DNA cells are then targeted for death by apoptosis (Taylor and Grabovich, 2009).

Bcl-2 family members have long been reported to play an important role in maintaining cell viability by preventing loss of mitochondrial membrane potential. Overexpression of Bcl-2 protein inhibits apoptosis whereby up-regulation of Bax protein induces apoptosis in the cancer cells (Mohan et al., 2012; Wu et al., 2012; Ng et al., 2011). Outcome of cellular viability/apoptosis is determined not only by the expression of specific pro- and anti-apoptotic genes/proteins, but is dependent on the ratio between pro and anti-apoptotic proteins (Kumar et al., 2013; Tasyriq et al., 2012; Gao and Dou, 2000). In the present study, DCM-DS significantly increased the Bax/Bcl-2 ratio at the protein level (Fig. 2) but not at the gene level (Fig. 1) in MCF-7 cells. It was noted that the increase in Bax/Bcl-2 ratio was mainly due to the down-regulation of Bcl-2 at protein level, but not at the mRNA level. Thus, it is suggested that Bcl-2 protein is post-translationally modified by either phosphorylation or ubiquitination via MAPKs and AKT, resulting in a proteosomal degradation of Bcl-2 protein which increases the BAX/BCL-2 ratio to initiate the mitochondrial apoptotic pathway (Deeb et al., 2014; Liu et al., 2013; Gao and Dou, 2000).

Recent studies reported that down-regulation of anti-apoptotic AKT decreased the expression of Bcl-2 in MCF-7 cells (Imanishi et al., 2011; Bratton et al., 2010; Shankar et al., 2010). 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. Our previous study reported that DCM-DS activated JNK1 and deactivated AKT1 and ERK1/2 in MCF-7 cells (Foo et al., 2014). Putting all these together, the activation of mitochondrial apoptotic pathway in the present study could be attributed to down-regulation of anti-apoptotic AKT and phospho-ERK, and up-regulation of pro-apoptotic phospho-JNK1 in MCF-7 cells.

The investigation of phytochemicals of DCM-DS led to the isolation of 3 triterpenes (Fig. 3). We report here for the first time the presence of BA in *D. suffruticosa* extract. Compound **2**, which was identified as BA, was the most major (33%) and most cytotoxic agent (IC_{50}: 7.55 μg/mL) towards the MCF-7 cells as compared to Compound **1** and **3** (Fig. 4). BA is a pentacyclic triterpenoid that was first isolated from the stem bark of *Zizyphus mauritiana* in 1995 and has been demonstrated to induce apoptosis in various cancer cells including breast, colon, lung, melanoma, neuroblastoma, prostate and leukaemia *in vitro* and *in vivo* (Damle et al., 2013; Reiner et al., 2013; Sun et al., 2013; Mertens-Talcott et al., 2012; Kessler et al., 2007; Tan et al., 2003; Pisha et al., 1995). A study reported that BA up-regulated p53 and p21 in MCF-7 cells (Wang et al., 2009). In addition, BA is also reported to up-regulate and down-regulate Bax and Bcl-2 proteins, respectively, in MCF-7 cells (Sun et al., 2013). The reported data in those studies are in agreement with our current findings. Some vegetal metabolites have similar biological activities. For instance, cruciferous vegetable benzyl isothiocyanate (Xiao et al., 2006) and curcumin obtained from the rhizomes of *Curcuma longa* (Tuorkey, 2014) have been reported to induce p53/p21 and mitochondrial pathway of apoptosis in MCF-7 cells. It is believed that the cytotoxicity of DCM-DS in the present study is largely contributed by BA as the major compound (Fig. 5) 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

DCM-DS induced cell cycle arrest in MCF-7 cells via p53/p21 pathway. In addition, DCM-DS induced apoptosis by increasing the ratio of Bax/Bcl-2 proteins. A total of 3 triterpenes which are the major compounds of DCM-DS were isolated. BA was the most major compound and the most cytotoxic towards MCF-7 cells. Therefore, BA can be used as a marker for standardisation of herbal product from *D. suffruticosa*. DCM-DS can also be employed as BA-rich extract from roots of *D. suffruticosa* for the management of breast cancer.

Conflict of Interest

The authors declare that they have no competing interests.

Authors' contribution

JBF carried out the study and prepared the manuscript. JBF, AW, YST, CWH and AN collected and interpreted the data. NI contributed to GeXP analysis. LSY, SPL, IIS, 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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