Endoplasmic reticulum stress-induced apoptotic pathway and mitochondrial dysregulation in HeLa cells treated with dichloromethane extract of *Dillenia suffruticosa*

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**ABSTRACT**

Ethyl acetate and dichloromethane extract of *Dillenia suffruticosa* (EADS and DCMDS, respectively) can be a potential anticancer agent. The effects of EADS and DCMDS on the growth of HeLa cervical cancer cells and the expression of apoptotic-related proteins had been investigated *in vitro*. Cytotoxicity of the extracts toward the cells was determined by 5-diphenyltetrazolium bromide assay, the effects on cell cycle progression and the mode of cell death were analyzed by flow cytometry technique, while the effects on apoptotic-related genes and proteins were evaluated by quantitative real-time polymerase chain reaction, and Western blot and enzyme-linked immunosorbent assay, respectively. Treatment with DCMDS inhibited (*P* < 0.05) proliferation and induced apoptosis in HeLa cells. The expression of cyclin B1 was downregulated that led to G₂/M arrest in the cells after treatment with DCMDS. In summary, DCMDS induced apoptosis in HeLa cells via endoplasmic reticulum stress-induced apoptotic pathway and dysregulation of mitochondria. The data suggest the potential application of DCMDS in the treatment of cervical cancer.

**Key words:** Apoptosis, cell cycle arrest, cervical cancer, *Dillenia suffruticosa*, endoplasmic reticulum stress

**INTRODUCTION**

Cervical cancer ranks as the third most common cause of cancer-related mortality in women.¹¹ Several studies have reported that 99% of human papillomavirus (HPV) infections are associated with cervical cancer with 50–70% and 7–20% of the cases are caused by HPV 16 and HPV 18, respectively.¹⁴ As such, several epidemiological studies have documented the success of prophylactic HPV vaccination program as a preventive measure for cervical cancer but not solely to treat it. Indeed, a significant reduction in the incidence and mortality of cervical cancer does not only rely on screening program or vaccination. Thus, the need to discover a potent and more effective therapy is vital.

Currently, cisplatin is used as a gold standard drug in the management of advanced cervical cancer. Despite the effectiveness, the adverse effects of cisplatin such as hematologic toxicity, neutropenia, anemia, nausea, diarrhea, and emesis have become a major concern.⁷⁻¹⁰ In addition, cisplatin treatment often results in the development of chemoresistance, leading to therapeutic failure.⁹,¹¹ Hence, attentions have been focusing in identifying new agents particularly those present in natural products which have been reported to have a long history for treatment of cancer.

*Dillenia suffruticosa* (Griffith ex Hook. f. and Thomson) Martelli or *Wormia suffruticosa* is indigenous to Malay Archipelago. This plant is traditionally used to treat cancerous growth and fever, for wound healing and to relief rheumatism.¹² D. *suffruticosa* extract also possesses antifungal¹³ and antibacterial properties,¹⁶ and antiviral properties against dengue type 2 virus.¹⁷ From our previous study, ethyl acetate (EADS) and dichloromethane (DCMDS) extract could inhibit the proliferation of HeLa cells and induce apoptosis.¹⁸,¹⁹ In addition, we showed that treatment of HeLa cells with EADS and DCMDS altered the expression of apoptotic-related genes and proteins.¹⁸,¹⁹ Therefore, we aimed to examine the effects of dichloromethane extract of *Dillenia suffruticosa* on the growth and expression of apoptotic-related proteins in HeLa cells.
extract of the root of *D. suffruticosa* exhibited strong cytotoxicity toward breast cancer (MCF-7 and MDA-321) and cervical cancer (HeLa) cell line due to induction of apoptosis and cell cycle arrest.[16–21] However, the actual mechanism underlying apoptosis induced by EADS and DCMDS in HeLa cells was unknown. Hence, this study evaluated the effects of the extracts on cell cycle progression and the expression of intrinsic and extrinsic apoptotic-related genes and proteins in HeLa cells.

**RESULTS AND DISCUSSION**

**Cytotoxicity of ethyl acetate and dichloromethane extract of *Dillenia suffruticosa* via cell cycle arrest and induction of apoptosis**

Ethyl acetate extract of *D. suffruticosa* exerted low toxicity toward HeLa cells at 24 h whereby the IC$_{50}$ was undetectable even at the highest concentration employed (>100 µg/mL). On the other hand, a good drug-response curve was obtained for DCMDS with IC$_{50}$ of 32.98 ± 1.68 µg/mL. DCMDS was most cytotoxic at 72 h (IC$_{50}$ = 11.95 ± 2.07 µg/mL). Based on that, only DCMDS was used for further investigation. Both EADS and DCMDS showed lower IC$_{50}$ values toward the normal cells 3T3 and Vero exhibited compared to HeLa [Table 1].

In this experiment, the human cervical cancer cells HeLa demonstrated different response toward EADS and DCMDS. The cytotoxic effects of both extracts were found to be in a time- and dose-dependent manner. The response to the cellular stress depends on many factors such as the cell type, duration, the number and intensity of the new stimuli.[22] This response in turn initiates a number of complex signaling cascade pathways to maintain the cell homeostasis.[23] DCMDS was more cytotoxic than EADS. Cytotoxicity of an extract is very much depending on its phytochemical components. EADS contains saponins, triterpenes, and tannins/polyphenolic while sterols were detected in DCMDS.[21] Plant sterols have been reported to alter the signaling pathways that prevent cell proliferation. [24] Even though EADS and DCMDS were more cytotoxic to the normal cells 3T3 and Vero compared to HeLa, the toxicity of the extracts to the normal cells *in vivo might be different*. This is due to the fact that an *in vitro* system is just evaluating a single cell type, organ, and isolated cells of other organs whereby no interaction and communication between the organs occurs.[25–27] In addition, the natural product has been claimed to exert minimum side effects compared with available synthetic agents to treat cervical cancer.[28]

DNA content analysis shows that there was a significant increase in the G$_2$/M peak of HeLa cells treated with DCMDS (12.5 µg/mL and 25 µg/mL) at 24 h incubation as compared to the control [Figure 1]. The number of cells at G$_2$ and S phase decreased significantly at 24 h (P < 0.05). Decreased percentage of cells indicates that they were not arrested at that particular phase. At 50 µg/mL, DCMDS caused cell cycle arrest at G$_2$/M after 48 and 72 h (P < 0.05) [Figure 1]. The protein analysis shows that cyclin B1 that is involved in the progression of cells from G$_2$ phase to mitosis in cell cycle control was downregulated [Figure 2]. It shows that DCMDS arrested HeLa cells at G$_2$/M. Thus, cell cycle arrest is one of the anticancer properties of the extract.[29]

Based on the Annexin V/FITC analysis, the DCMDS-treated cells (12.5 µg/mL) with high apoptotic index were obviously noted after 48 h, with significant increase in the early (Q2) and late apoptotic (Q3) cells. The number increased with increase in the concentration of the extract. The number of viable cells (Q1) in the treatment of 25 µg/mL DCMDS was very much lower (P < 0.05) as compared to the control cells at 72 h [Figure 3]. The percentage of necrotic cells was considerably low compared with apoptotic cells (P < 0.05). The effects of the treatment were dependent on dose and time. Exposure to a high concentration (50 µg/mL) of DCMDS further increased the rate of apoptosis and suppressed HeLa cells proliferation. A recombinant phosphatidylserine-binding protein, Annexin V interacts with phosphatidyl serine residues (ligand for phagocytes on the surface of the apoptotic cell)[30] and can be used for the detection of apoptosis.[31,32] Low number of viable cells and high percentage of cells at early and late apoptosis following treatment with DCMDS proved that the extract caused the cells to die primarily through apoptosis, not necrosis. It is of the advantage of apoptosis-inducing agent that it will prevent damage to the surrounding normal healthy cells or inflammation.

### Table 1: Cytotoxicity of EADS and DCMDS towards human cell lines as determined by MTT assay. Results are presented as mean±SD of triplicate of three independent experiments.

*Significant different from control at P<0.05. α and β are significantly different

| Cell line | Incubation time (hour) | IC$_{50}$ (µg/mL) |
|-----------|-----------------------|-------------------|
|            | EADS                  | DCMDS             |
| HeLa       | 24                    | >100              | 32.98±1.68         |
|            | 48                    | 67.90±0.88         | 17.37±2.50         |
|            | 72                    | 36.06±2.98         | 11.95±2.07         |
| 3T3        | 24                    | >100              | 10.94±0.44         |
|            | 48                    | 34.86±5.22         | 5.61±0.34          |
|            | 72                    | 25.24±1.76         | 4.85±0.48          |
| Vero       | 24                    | >100              | 23.55±2.28         |
|            | 48                    | 52.61±1.55         | 10.23±1.10         |
|            | 72                    | 41.98±2.05         | 7.77±1.28          |

SD: Standard deviation, EADS: Ethyl acetate, DCMDS: Dichloromethane, MTT: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
Expression of the apoptotic genes and proteins in HeLa cells following treatment with dichloromethane extract of *Dillenia suffruticosa*

**Effects of dichloromethane extract of *Dillenia suffruticosa* on the activity of caspases**

Following exposure to DCMDS (25 µg/mL), the expression of caspase-3, -8, -9, and -12 in HeLa cells was up-regulated at 24 h [Figure 4]. It indicates that caspase-3, -8, -9, and -12 were activated in HeLa cells treated with DCMDS.

**Involvement of poly (adenosine diphosphate-ribose) polymerase-1 and NF-κB in dichloromethane extract of *Dillenia suffruticosa*-induced apoptosis**

The expression level of poly (adenosine diphosphate-ribose) polymerase-1 (PARP-1) and NF-κB was positively correlated with the rate of DCMDS-induced apoptosis [Figures 2 and 5]. Increasing the concentration of DCMDS resulted in increasing level of PARP-1 cleavage activity and the expression level of NF-κB [Figures 2 and 5].

**Involvement of p53, Bcl-2, and Bax in dichloromethane extract of *Dillenia suffruticosa*-induced apoptosis**

The expression of p53 was lower in the control compared to the treated HeLa cells. The expression of p53 protein significantly increased at 12.5 µg/mL and 25 µg/mL of DCMDS compared with the control (*P < 0.05*) after 24 and 48 h. In contrast, the expression of anti-apoptotic Bcl-2 significantly decreased following treatment with DCMDS at 48 h [Figure 6]. The expression of Bax increased [Figure 2] p53-mediated apoptosis eliminated both types of cells with damaged DNA or an aberrant cell cycle. Based on the data, it is evident that the expression level of p53 increased with decreased expression of Bcl-2 following treatment with DCMDS. It is known that p53 regulates a set of anti-apoptotic genes including members of the Bcl-2 family. Bax and IGF-BP3 are the genes that are regulated by p53 which could influence the decision to proceed to the apoptotic pathway or not. On the other hand, Bcl-2 is a proto-oncogene that protects permanent cells from apoptosis. Bax binds to Bcl-2 and...
Relative expression was calculated by the optical density ratio of protein control. Protein bands were quantified by densitometric analysis.

- To gain more insights into the molecular events pathway, gene expression analysis was performed. DCMDS was found to upregulate the expression of a key indicator of ER stress responses, glucose-regulated protein 78/binding immunoglobulin protein (GRP78/BiP) and its downstream transcription factor CCAAT/enhancer binding protein (C/EBP)-homologous protein or growth and DNA damage (GADD)-inducible transcription factor 153 (CHOP/GADD153). From the quantitative real-time polymerase chain reaction (PCR) analysis, GRP78 mRNA level increased up to 4 folds in HeLa cells after treatment with DCMDS, while CHOP mRNA level increased dramatically up to 13 folds [Figure 6]. Activating transcription factor 4 (ATF4) level increased, which was accompanied by an increase in the X-box binding protein 1 (XBP1) mRNA splicing. The spliced form of the XBP-1 mRNA was detected at low concentration (12.5 µg/mL) of DCMDS treatment but decreased at higher concentration (25 µg/mL). Furthermore, calreticulin (CRT) mRNA was also up-regulated. However, ER chaperone GRP58 was downregulated as the concentration of DCMDS increased [Figures 2 and 6].

**Dichloromethane extract of Dillenia suffruticosa induced apoptosis via endoplasmic reticulum stress pathway**

To gain more insights into the molecular events of DCMDS-induced apoptosis in the endoplasmic reticulum (ER), gene expression analysis was performed. DCMDS was found to increase the expression of GRP78/BiP and its downstream transcription factor CHOP/GADD153. From the quantitative real-time polymerase chain reaction (PCR) analysis, GRP78 mRNA level increased up to 4 folds in HeLa cells after treatment with DCMDS, while CHOP mRNA level increased dramatically up to 13 folds [Figure 6]. Activating transcription factor 4 (ATF4) level increased, which was accompanied by an increase in the X-box binding protein 1 (XBP1) mRNA splicing. The spliced form of the XBP-1 mRNA was detected at low concentration (12.5 µg/mL) of DCMDS treatment but decreased at higher concentration (25 µg/mL). Furthermore, calreticulin (CRT) mRNA was also up-regulated. However, ER chaperone GRP58 was downregulated as the concentration of DCMDS increased [Figures 2 and 6].

**Endoplasmic reticulum stress** is an organelle that serves many functions including calcium homeostasis, cellular protein quality control and the transport of synthesized proteins to the Golgi apparatus. ER stress happens when ER function is disrupted that subsequently causes accumulation of unfolded proteins in the ER. It triggers the unfolded protein response (UPR) to reduce the excessive protein loading which signals temporary inhibition of protein translation, degradation of misfolded proteins, and the induction of molecular chaperones and folding enzymes to increase the ER capacity of protein folding and degradation.

In our study, DCMDS caused upregulation of GRP78/BiP and its downstream transcription factor CHOP or growth-arrest and DNA-damage-inducible gene 153 (GADD153) (CHOP/GADD153) [Figure 5]. CHOP, c-Jun NH2-terminal kinase (JNK), and caspases have been implicated in mediating apoptotic signals in response to ER stress. During prolonged ER stress, CHOP is one of the most highly up-regulated genes. All three UPR signaling pathways (PKR-like ER kinase [PERK], inositol-requiring transmembrane kinase [IRE1], and ATF6) are involved in inducing CHOP transcription, although the PERK pathway is essential. Conversely, overexpression of CHOP promotes apoptosis in response to ER stress caused by thapsigargin and increases cellular reactive oxygen species, which most likely contribute to ER stress-associated cell death. In response to ER stress, IRE1 alpha has been found to recruit the adaptor protein TRAF2 to the ER membrane. This recruitment is regulated by c-Jun NH2-terminal inhibitory kinase, which has been reported to interact with both IRE1a and TRAF2. The IRE1alpha/TRAF2 complex then recruits apoptosis signal-regulating kinase 1 (ASK1), causing activation of ASK1 and the downstream JNK pathway. JNK phosphorylates Bcl-2 and BH3-only protein, initiating mitochondria-mediated apoptosis. Together, these results suggest that the ER stress sends signals to the mitochondria via the regulation of Bcl-2 proteins by both CHOP and JNK. Activation of JNK pathway could lead to FasL induction and cell death. Activation of FasL followed by caspase-8 can initiate the extrinsic pathway. In this study, NF-κB was also activated, promoting DCMDS-mediated induction of Fas or FasL and thus increasing the level of apoptosis in HeLa cells. NF-κB acts as a pro-apoptotic factor in this context. This indicates that the extrinsic pathway is involved in DCMDS-induced apoptosis.
The ER stress-induced apoptosis also involves caspases. In mice, pro-caspase-12 is localized on the cytoplasmic side of the ER and is cleaved and activated specifically by ER stress. Calpains, a family of calcium-dependent cysteine proteases, have been shown to play a role in caspase-12 activation[46] and calpain-deficient MEFs have reduced ER stress-induced caspase-12 activation and are resistant to ER stress-associated apoptosis[47].

GRP58 might play a role in DCMDS-induced ER stress-induced apoptosis. Following treatment with DCMDS, the rate of apoptosis increased with downregulation of GRP58 but upregulation of other ER stress proteins. Our findings were in concordance with previous reports that upregulation of GRP78 will cause downregulation of (GRP58).[48] The expression level of ER chaperone GRP58 reduced in a dose-dependent
Overexpression of GRP58 is associated with the ER stress due to disruption of the calcium homeostasis, hypoxia and virus or bacterial infection. In human ovarian cancer, prostate cancer, breast cancer, lung cancer, uterine cancer, and stomach cancer, GRP58 level was enhanced when compared with normal tissues of similar origin. However, downregulation of GRP58 expression has also been documented in gastric cancer, esophageal cancer, cervical carcinoma and renal cell carcinoma. A few studies showed the association of GRP58 with the inhibition of cancer cell proliferation. Knock-down of GRP58 was associated with the inhibition of breast cancer cell proliferation and melanoma cells. Downregulation of GRP58 was associated with poor prognosis of early stage cervical cancer. A recent study showed that this stress-responsive protein which is activated on glucose deprivation modulates invasiveness of cervical cancer. In their investigation, knockdown of GRP58 in HeLa cells led to decreased cell invasiveness and inhibition of lung metastasis in a xenograft mouse model.

Some studies have linked ER stress with apoptosis mediated by the mitochondrial mechanisms involving the Bcl-2 family of proteins. During ER stress, Bax and Bak in the ER membrane undergo conformational changes. This allows the release of calcium from the ER to the cytoplasm, which in turn activates m-Calpain. Subsequently, it cleaves and activates procaspase-12. Activated caspase-12 cleaves and activates procaspase-9, which in turn activates downstream caspases, including caspase-3. In addition, calcium released from the ER is taken up by the mitochondria, causing mitochondrial inner membrane depolarization and caspase-8 activation. Cytochrome c is released into the cytoplasm to stimulate the assembly of the apoptosis (consisting of Apaf-1, cytochrome c, ATP, and procaspase-9), activation of procaspase-9, which in turn activates caspase-3, DNA fragmentation, and cell death.

The expression of ER molecule, CRT was also up-regulated in DCMDS-treated HeLa cells. Within ER, CRT together with calnexin and GRP58 serve as a molecular chaperone to ensure proper folding of glycoproteins. It is believed that treatment with DCMDS triggered the activation of the CRT exposure pathway by preapoptotic ER stress molecules and the phosphorylation of the eukaryotic translation initiation factor eIF2alpha by the kinase PERK, followed by caspase-8-mediated proteolysis, and activation of the pro-apoptotic proteins Bax and Bak. It is therefore concluded that DCMDS induced ER stress and caused cell death via apoptosis in HeLa cells.
EXPERIMENTAL

Preparation of ethyl acetate and dichloromethane extracts of Dillenia suffruticosa

The extracts were obtained using a sequential cold solvent extraction method and tested as described previously.[29]

Cell culture

The human cervical adenocarcinoma (HeLa), mouse fibroblast (3T3), and African green monkey kidney epithelial (Vero) cell lines (American Type and Culture Collection [ATCC], Rockville MD, USA) were grown in RPMI-1640 supplemented with 10% (v/v) of fetal bovine serum and 1% of penicillin-streptomycin (100 IU/mL of penicillin and 100 µg/mL of streptomycin) (PA Laboratory, Linz, Austria). The cells were allowed to grow at 37°C with 5% CO₂ atmosphere.

Determination of cytotoxicity of ethyl acetate and dichloromethane extract of Dillenia suffruticosa through induction of apoptosis and cell cycle arrest

Cytotoxicity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay[67] as described previously. Untreated and treated HeLa cells (0.7 × 10⁶ cells/mL) were scraped, washed twice with ice-cold PBS and lysed for 60 min at 4°C with the primary antibody, rabbit anti-PARP, NF-κB, Bax, cyclin B1, mouse anti-GRP58 (Abcam, Cambridge, MA, USA), and anti-β-actin (Santa Cruz, USA) at a dilution of 1:2000. These steps were followed by incubation with horseradish peroxidase-conjugated secondary antibody, rabbit anti-mouse IgG (Santa Cruz, USA) for 60 min at 25°C on rocker (dilution 1:4000). All immunoblots were visualized by enhanced chemiluminescence plus Western blotting detection reagents (Abcam Corporation, USA). Densitometrical quantification of autoradiograms was performed by ImageJ software (version 1.41, National Institutes of Health, Bethesda, MD).

Enzyme-linked immunosorbent assay

Untreated and treated HeLa cells (12.5 µg/mL, and 25 µg/mL) were scraped, washed twice with ice-cold PBS and lysed for 60 min at room temperature (27°C) in lysis buffer (1X) (Invitrogen, USA). The p53 and Bel-2 protein concentration in the extract was estimated using...
the Human p53 Platinum enzyme-linked immunosorbent assay (ELISA) kit and the Human Bel-2 Platinum ELISA kit, respectively (Bender MedSystems GmbH, Vienna, Austria). The protease activity of caspases-3, -8 and -9 was evaluated spectrophotometrically using Colorimetric Assay kit (Gene script, USA) and caspase-12 using the Human Caspase-12 ELISA kit (Cusabio Biotech, China). Experiments were performed in triplicate. The samples extinction values were determined using ELISA plate reader (Biotek, USA) at 450 nm.

**Statistical analysis**

Statistical analysis was performed with General Linear Model (Univariate), Duncan’s multiples range test, and Dunnett’s test using Statistical Package of Social Sciences (SPSS) for Window version 21.0 (SPSS Inc., Chicago, IL, USA). All the data are presented as mean ± standard deviation. A mean difference is considered significant when \( P < 0.05 \).

**CONCLUSIONS**

In short, this study demonstrates that EADS and DCMDs have the potential to be developed into an agent for the treatment of cervical cancer. DCMDs induced cellular stress in HeLa cells by activation of mitochondrial and ER stress signaling pathways that ultimately led to apoptosis. Thus, DCMDs may be a potential source of lead compounds for drug development.

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