**Dioscorea bulbifera** induced apoptosis through inhibition of ERK 1/2 and activation of JNK signaling pathways in HCT116 human colorectal carcinoma cells

Ahmad Fadhlurrahman Ahmad Hidayat, Chim Kei Chan, Jamaludin Mohamad, Habsah Abdul Kadir

**Abstract**

*Dioscorea bulbifera*, also known as air potato, has been cultivated as food crop mainly in tropical countries in Asia and Australia. The tubers are edible and have often been used in Traditional Chinese Medicine (TCM) and Ayurvedic medicine to treat cancer, diabetes, thyroid disease, and inflammation. This study aimed to investigate the effects of *D. bulbifera* on HCT116 human colorectal carcinoma cells and to unravel the plausible mechanisms underlying its apoptotic effects. The ethanol crude and fractions (hexane, ethyl acetate and water) of *D. bulbifera* were subjected to cell viability MTT assay against various cancer cell lines. The lowest IC50 of the extract and fractions on selected cancer cells were selected for further apoptosis assay and western blot analysis. HCT116 cancer cells were treated with *D. bulbifera* and stained with Annexin/PI or Hoechst 33342/PI for preliminary confirmation of apoptosis. The dissipation of mitochondria membrane potential (MMP) was determined by flow cytometry. The protein expressions of apoptosis-related proteins such as Bcl-2 family, caspases, Fas, PARP, ERK1/2 and JNK were detected by western blot analysis. Moreover, the HCT116 cells were treated with UO126 and SP600125 inhibitors to verify the involvement of ERK1/2 and JNK protein expressions in inducing apoptotic cell death. Based on the result, *D. bulbifera* ethyl acetate fraction (DBEAF) exhibited the most compelling cytotoxicity on HCT116 cells with an IC50 of 37.91 ± 1.30 μg/mL. The induction of apoptosis was confirmed by phosphatidylserine externalization and chromatin condensation. Depolarization of MMP further conferred the induction of apoptosis was through the regulation of Bcl-2 family proteins. Activation of caspase cascades (caspase-3, -9, -8 and -10) was elicited followed by the observation of cleaved PARP accumulation in DBEAF-treated cells. Furthermore, death receptor, Fas was activated upon exposure to DBEAF. Collective apoptotic evidences suggested the involvement of intrinsic and extrinsic pathways by DBEAF in HCT116 cells. Interestingly, the attenuation of ERK1/2 phosphorylation accompanied by the activation of JNK was detected in DBEAF-treated cells. In conclusion, the findings revealed that DBEAF induced apoptosis through intrinsic and extrinsic pathways involving ERK1/2 and JNK.

**1. Introduction**

Colorectal cancer is the most prevalent cancer worldwide and the fourth leading cause of cancer-associated death after lung, breast and prostate cancer [1]. Over decades, a vast array of colorectal cancer treatment was available such as surgery, chemotherapy, radiotherapy, immunotherapy [2] and hormone therapy [3]. However, prolonged exposure of numerous chemotherapeutic agents or radiotherapy may bring severe undesirable side effects to the patient. Therefore, the quests to discover novel approaches for safer and effective treatment of colorectal cancer are needed. Since ancient times, traditional medicinal plants were used in traditional Chinese and Ayurvedic treatments to cure a wide spectrum of diseases. Medicinal plants offer an alternative treatment for colorectal cancer owing to the minimal toxicity and efficacy in resolving drug resistance [4].

Apoptosis cell death has been considered as a promising strategy to target cancer cell growth manifested by cell shrinkage, membrane blebbing, nuclear and chromatin condensation, DNA fragmentation and production of apoptotic bodies. Apoptosis can be mediated by two major pathways, the mitochondrial-mediated (intrinsic) and the death...
receptor-mediated (extrinsic) apoptotic [5]. Various apoptosis-related proteins such as the Bcl-2 family proteins, death receptors and caspases are known to contribute in the regulation of apoptosis. It is well documented that extracellular signal-regulated kinases (ERK1/2) and c-Jun amino-terminal kinase (JNK) are involved in the regulation of cell proliferation and cell death [6]. Activation of ERK1/2 by mitogenic stimuli such as growth factors, promotes the cell growth and survivability of cells [7]. Meanwhile, JNK protein was activated by DNA damage, death-receptor (Fas) or chemotherapeutic drugs that enhance the cell susceptibility towards the initiation of apoptotic cell death [8]. However, previous reports have shown that the modulation of ERK1/2 and JNK may induce dual regulatory effects where it can either promote cell survival or apoptosis [9,10].

*D. bulbifera,* also known as air potato, can be found mostly in Asia, Australia, and Africa. The plant is cultivated as a food crop as the tuber draws great attention within the scientific community to discover new therapeutic properties. Scientific studies have provided mounting evidence of antioxidant, antibacterial, anticancer, anti-diabetic, analgesic and anti-inflammatory activities in *D. bulbifera* [14–20]. Furthermore, the ethyl acetate soluble fraction extracted from *D. bulbifera* has been reported to contain mostly flavonoids such as kaempferol, caryatin, (+)-catechin, quercetin or myricetin. The presence of these chemical constituent may promote towards the anticancer activity. However, to our knowledge, the effects of *D. bulbifera* on human colorectal cancer have not been reported and its underlying mechanisms remain a perplexity. Thus, the aim of the present study was to evaluate the effects of *D. bulbifera* in HCT116 cells and to investigate the underlying cell death mechanisms.

2. Methods

2.1. Cell culture

HCT116, HT-29, MCF-7, Caski, A549 and CDD-18Co cells were purchased from the American Type Culture Collection (ATCC). All cancer cells were cultured in RPMI-1640 medium while normal cells in EMEM medium. All media were supplemented with 10% v/v heat-inactivated fetal bovine serum (Sigma), 1% v/v antibiotic-antimycotic (Thermo). Cells were incubated in a CO2 incubator chamber with 37 °C activated fetal bovine serum (Sigma), 1% v/v antibiotic-antimycotic.

Cancer cells were cultured in RPMI-1640 medium while normal cells in EMEM medium. All media were supplemented with 10% v/v heat-inactivated fetal bovine serum (Sigma), 1% v/v antibiotic-antimycotic (Thermo). Cells were incubated in a CO2 incubator chamber with 37 °C humidity. Cells were observed daily under a light inverted microscope for any sign of contamination or cell growth masses. Once the cell reaches 70 ~ 90% confluence, they were detached using trypLE (gibco) and passage half/quarter of the cells amount into the same or new flasks. For cell count, viable cells were stained using trypan blue and counted using a hemocytometer.

2.2. *D. bulbifera* extraction and fractionations

Fresh tubers of *D. bulbifera* were purchased from a local supplier in Ipoh, Perak, Malaysia in December 2015. The plant was identified by Dr Yong Kien Thai of the Institute of Biological Science, Faculty of Science, University of Malaya, Malaysia with a specimen voucher number: KLU 48652. The tubers were air dried at room temperature for several weeks and ground into powder form using an electric blender. 200 g of the grounded *D. bulbifera* was pooled with 80% ethanol and incubated for 3 days. Soluble ethanol extract was filtered and reduced using a rotary vacuum evaporator (BÜchi) at 40 °C to obtain 5.11 g of *D. bulbifera* ethanol extract (DBEE). The DBEE (2 g) was subjected to fractionation by incubation with hexane for 3 days. Soluble hexane was filtered and reduced using a rotary vacuum evaporator to obtain 0.1 g of *D. bulbifera* hexane fraction (DBHF). Insoluble hexane was further incubated with ethyl acetate and water (1:1 v/v) for 3 days. Ethyl acetate fraction was partitioned, filtered, and reduced using a rotary vacuum evaporator to obtain 0.74 g of *D. bulbifera* ethyl acetate fraction (DBEAF). Meanwhile, the water fraction was lyophilized to obtain 1.3 g of *D. bulbifera* water fraction (DBWF). All sample extracts and fractions were dissolved in DMSO and filtered using 0.22 μm filter prior to the experiments. For cell treatments, all samples were incubated for 24–72 h, and the concentration of DMSO was not exceeding 0.5% v/v in all experiments.

2.3. Measurement of cell viability by MTT assay

The cell viability of selective cancer and normal cells along with treatment with *D. bulbifera* was investigated using the 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyletrazolium bromide (MTT) assay (Sigma). Briefly, 5.0 × 10^5 of all cancer and normal viable cells were seeded into 96-well plates and incubated for 24 h. The cells were pre-treated with or without inhibitor for 1 h, and post-treated with DBEE, DBHF, DBEAF or DBWF (3.12–200 μg/mL) for 24–72 h. MTT solution (5 mg/mL) was added into each well and incubated for 4 h in a dark room. The absorbance readings were measured at 570 nm with reference wavelength 650 nm using a microplate reader (Asys UVM340). The numbers of viable cells were calculated based on the following equation:

\[
\text{Percentage of viable cell} = \left( \frac{\text{absorbance of treated cells}}{\text{absorbance of untreated cells}} \right) \times 100\%.
\]

2.4. Externalization of phosphatidylserine detection by Annexin V and PI staining

Externalization of phosphatidylserine is one of the hallmarks of early onset of apoptosis. HCT116 cells (1.0 × 10^6 cells) were plated in 60 mm² culture dishes, incubated for 24 h and treated with DBEAF at different concentrations (50, 100 and 200 μg/mL) over the duration of 24 h. The cells were harvested, washed twice with PBS, and re-suspended in 1 x Annexin V binding buffer (BD). The cells were stained with Annexin V-fluorescein-isothiocyanate (FITC) (BD) for 10 min followed by propidium iodide (PI) for 5 min in a dark room. 1 x Annexin V binding buffer (BD) was re-suspended into each tube and analyzed by flow cytometry (Accuri C6). The fluorescence intensity was detected in FL1-A (x-axis) and FL2-A channels (y-axis).

2.5. Observation of nuclear morphology changes using Hoechst 33342/PI

The nuclear morphological changes of treated DBEAF in HCT116 cells can be determined by dual staining the cells with Hoechst 33342 and PI dye. Briefly, HCT116 cells (1.0 × 10^6 cells number) were plated in 60 mm² culture dishes and incubated for 24 h. Later, the cells were treated with vehicle DMSO and DBEAF (50, 100, 200 μg/mL) for 24 h. The cells were harvested and washed with PBS twice and stained with Hoechst 33342 (40 μg/mL) for 30 min in a dark room. Changes in cell morphology of DBEAF-treated HCT116 cells were observed and captured under an inverted fluorescence microscope (Leica DM1600B).

2.6. Mitochondria membrane potential analysis

The loss of mitochondrial membrane potential in DBEAF-treated HCT116 cells was analyzed using Mito-ID® Membrane Potential Detection Kit (Enzo Life Science). HCT116 cells (1.0 × 10^6 cells) were plated in 60 mm² culture dishes for 24 h followed by DBEAF treatment at 50, 100 and 200 μg/mL for 24 h. The cells were harvested and washed with 1x Assay buffer and stained with Mito-ID® MP detection reagent for 15 min. The stained cells were then analyzed by Accuri C6 flow cytometry and the fluorescence intensity in the X-axis and the Y-
axis were detected at FL1-A and FL2-A channels respectively.

2.7 Western blot analysis

Protein expressions in DBEAF-treated HCT116 cells were detected by using western blot analysis. HCT116 cells were plated for 24 h, pretreated with or without U0126 Monoethanolamine (5μM) or SP600125 (20 μM) for 1 h, followed by exposure to DBEAF (50, 100 and 200 μg/mL) for 24 h. The cells were lysed using RIPA buffer containing protease inhibitor and phosphatase inhibitor cocktails. The total protein content for each lysate was quantitatively determined using Quick Start Bradford Protein Assay (BIO RAD). 30 μg of each protein sample was separated by 10% SDS PAGE and transferred onto a nitrocellulose membrane followed by blocking in 5% skim milk or BSA for 1 h. Subsequently, the membrane was incubated overnight at 4 °C with primary antibodies PARP, Caspase-3, Cleaved Caspase-3 (Aap175) (5A1E), Caspase-9 (Human Specific), Cleaved Caspase-9 (ASP330), Caspase-10, Caspase-8, Fas (C18C12), Bax (D2E11), Bcl-2 (50E3), Bak (D2D3), Bcl-xl (54H6), p44/42 MAP Kinase (137F5), Phospho-p44/42 MAPK (Thr202/Tyr204)(D13.14.4E), SAPK/JNK (56G8), Phospho-(D2D3), Bcl-xL (54H6), p44/42 MAPK (Thr202/Tyr204)(D13.14.4E), SAPK/JNK (56G8), Phospho-SAPK/JNK (Thr183/Tyr185)(81E11) or GAPDH (D16H11) and fractions (50, 100 and 200 μg/mL) for 24 h. The cells were lysed using RIPA buffer containing 20 μM for 1 h, followed by exposure to DBEAF (50, 100 and 200 μg/mL) for 24 h. Following overnight incubation at 4 °C with primary antibodies PARP, Caspase-3, Cleaved Caspase-3 (Aap175) (5A1E), Caspase-9 (Human Specific), Cleaved Caspase-9 (ASP330), Caspase-10, Caspase-8, Fas (C18C12), Bax (D2E11), Bcl-2 (50E3), Bak (D2D3), Bcl-xl (54H6), p44/42 MAP Kinase (137F5), Phospho-p44/42 MAPK (Thr202/Tyr204)(D13.14.4E), SAPK/JNK (56G8), Phospho-SAPK/JNK (Thr183/Tyr185)(81E11) or GAPDH (D16H11) XP(antibodies were purchased from cell signaling). The membranes were incubated with anti-mouse or rabbit immunoglobulin G-horse-radish peroxidase-conjugated secondary antibody for 1 h at room temperature and detected using enhanced chemiluminescence (ECL) detection kit (BIO RAD). The protein signals were visualized under gel documentation imaging system and quantitatively analyzed using Vilber Lourmart software.

2.8. LCMS-QTOF

The LCMS system consisted of an Agilent Technologies 6550 iFunnel – QTOF coupled to 1200 HPLC system. Chromatographic separation was carried out using an Agilent Zorbax Eclipse Plus C18 column Rapid Solution (4.6 x 100 mm, 3.5 μM). 5 μg of DBEAF was dissolved in LCMS grade methanol to obtained 5 ppm of DBEAF and sterile filtered (0.2 μM) into the Agilent cap vial. The mobile phase solvents are: solvent A is 0.1% (v/v) formic acid in distilled water and solvent B is 0.1% (v/v) formic acid in acetonitrile. The solvent was eluted at 0.5 mL/min flow rate for a total of 30 min run time. DBEAF was injected at 5 μl in 24 °C through C18 column at 22 °C in front and end sides. The elution gradient is: 0 min (90% A,10% B), 1 min (90% A,10% B), 20 min (50% A, 50% B), 24 min (50% A, 50% B), 25 min (90% A, 10% B), 30 min (90% A, 10% B). Scan segment was carried out at negative (-) modes. Identification of compounds was equitized using Agilent Masshunter Workstation software and compared from libraries such as AM_PCDL or Metlin_AM_PCDL library.

2.9. Statistical analysis

All experiments were performed triplicate and data was expressed in mean ± standard error (S.E.). A significant difference between control and treatment groups was assessed respectively using one-way ANOVA followed by Dunnett’s test with p values < 0.05 considered as statistically significant.

3. Results

3.1. Cytotoxic effect of D. bulbifera extract and fractions on different cancer cell lines

Human colorectal carcinoma (HCT116), human colorectal adenocarcinoma (HT-29), human lung carcinoma (A549), human breast carcinoma (MCF-7), human cervix epidermoid carcinoma (Ca Ski) and human colon normal (CCD-18Co) were treated with D. bulbifera extract and fractions (50, 100 and 200 μg/mL) for 72 h followed by the determination of cell viability via MTT assay. The IC50 values of each extract and fractions are summarized in Table 1. Based on the present results, DBEAF significantly inhibited the survivability of HCT116, HT-29 and A549 cells (Fig. 1) in a dose-dependent manner. DBEAF exhibited the most potently towards HCT116 cells among all the cancer cell lines. In addition, DBEAF treatment decreased cell viability of HCT116 cells in a time-dependent manner, where the IC50 values marked at 163.59 ± 1.56, 88.49 ± 2.36 and 37.91 ± 1.30 μg/mL at 24, 48 and 72 h respectively. In contrast, DBEAF treatment did not exert any cytotoxic effect towards the normal colon CCD-18Co cells.

3.2. Externalization of phosphatidylserine by DBEAF-treated HCT116 cells

Plasma membrane asymmetry presented the earliest stage of apoptosis, where the embedded phosphatidylserine (PS) located in plasma membrane became externalized [21]. Annexin V-FITC had a higher affinity in binding to exposed PS on the apoptotic cell surface, while PI was impermeable to live and apoptotic cells, but permeable to dead cells [22]. Therefore, the dual staining of both fluorescien can differentiate between early and late apoptosis by flow cytometry analysis. Based on the results, exposure of DBEAF to HCT116 cells led to a gradual increase of PS externalization in a dose-dependent manner (Fig. 2A). This was evidenced by the total Annexin V-positive cells increase to 7.00 ± 0.81, 48.44 ± 0.80 and 50.57 ± 0.65% at 50, 100 and 200 μg/mL of DBEAF, respectively (Fig. 2B). Thus, the results reveal the apoptotic effects of DBEAF in HCT116 cells were based on the externalization of phosphatidylserine.

3.3. Nuclear morphological alterations by DBEAF

To further validate the induction of apoptosis by DBEAF, the nuclear morphological changes of HCT116 cells were stained with Hoechst 33342 and PI. Hoechst 33342 is relatively smaller size molecules in comparison to PI, which can easily pass through the cell membrane and stain the nuclei. In early onset of apoptosis, the DNA in the nuclei starts to break down and form chromatin condensation while the plasma membrane integrity remains contact. In other words, the accumulation of Hoechst 33342 fluorescence (bright blue color) shows the early events of apoptosis. Progression of apoptosis leads to the breakdown of the plasma membrane where PI can slip through and stain the DNA. During late apoptosis, the cells will be stained with Hoechst 33342 and PI, which is shown as purple in color. As shown in Fig. 3, treatment with DBEAF in HCT116 cells significantly increased the induction of early and late apoptosis in a dose-dependent manner, where the cells displayed features such as chromatin condensation, cell shrinkage and DNA fragmentation.

3.4. Mitochondrial membrane potential depolarization by DBEAF

Dissipation of mitochondrial membrane potential (MMP) is an early
event of apoptosis and suggests the initiating signal of the intrinsic pathway [23]. The loss of MMP was assessed by a cationic dual-emission dye that emits green fluorescence in the cytosol and orange fluorescence in the mitochondria. Viable cells will display as orange fluorescent aggregates while apoptotic cells as green fluorescent monomer. As shown in Fig. 4A, the untreated cells was shown to have an abundance of orange fluorescent aggregates that remained in the mitochondria. Meanwhile, treatment with DBEAF released orange fluorescence aggregates into the cytosol and formed green fluorescent monomers. The total green fluorescent monomers increased to 16.00 ± (12.90), 57.70 ± (15.90) and 73.95 ± (4.05) % with increasing DBEAF concentrations of 50, 100 and 200 μg/mL, respectively (Fig. 4B). These findings suggested that treatment of DBEAF induced apoptosis in HCT116 cells by promoting the loss of MMP.

Fig. 1. Cytotoxic effect of ethyl acetate fraction of D. bulbifera against various cancer cell lines. (A) Bar chart illustrates the percentage of cell viability of different cell lines after treatment with different concentrations of DBEAF for 72 h. (B) Bar chart illustrates the percentage of cell viability of HCT116 cells when treated with DBEAF at 24, 48, and 72 h. All data represented as mean ± S.E of three independent experiments (n = 9). Asterisks indicate significantly different value from control (*p < 0.05).

Fig. 2. The externalization of phosphatidylserine in HCT116 cells upon exposure to DBEAF. (A) The flow cytometric analysis shows HCT116 cells were treated with different concentrations of DBEAF (50, 100 and 200 μg/mL). (B) The bar chart shows the total Annexin V positive cells. The data expressed as mean ± S.E from three individual experiments. Asterisk indicates significantly different value from control (*p < 0.05).
3.5. Effect of DBEAF on the expression of Bcl-2 family members

The disruption of MMP is closely associated with the regulation of Bcl-2 family member proteins. Thus, the modulation of Bcl-xL, Bcl-2, Bax and Bak was investigated via western blot analysis. Based on the present results, treatment of DBEAF significantly upregulated the pro-apoptotic Bax and Bak protein expressions and markedly down-regulated the anti-apoptotic Bcl-2 and Bcl-xL proteins expressions in a dose-dependent manner (Fig. 5A and B). In addition, the ratio of Bax/Bcl-2 significantly increased by 1.58 ± 0.03, 2.31 ± 0.11 and 2.62 ± 0.18 folds at 50, 100 and 200 μg/mL of DBEAF, respectively (Fig. 5C). Similarly, the ratio of Bak/Bcl-xL also elevated by 1.27 ± 0.08, 1.56 ± 0.14 and 3.21 ± 0.43 at 50, 100 and 200 μg/mL of DBEAF, respectively (Fig. 5D). These results clearly suggested that DBEAF induced dysregulation of the Bcl-2 family proteins and enhanced the apoptotic effect in HCT116 cells.

3.6. Induction of apoptosis-related proteins by DBEAF

The apoptotic pathway can be characterized both by extrinsic and intrinsic pathways. To further investigate the involvement of apoptosis-related protein expressions such as PARP, caspases (caspase-3, -8, -9 and -10) and death receptor (Fas), all proteins were investigated by using western blot analysis. The current results demonstrated that DBEAF significantly downregulated the expression of procaspase -8, -9, -10 and -3. In addition, the data also showed that DBEAF-treated cells significantly reduced PARP protein expression to 0.97 ± 0.45, 0.89 ± 0.22 and 0.77 ± 0.13, respectively. Accordingly, the expression of cleaved caspase-3 and -9 markedly increased by 2.89 ± 0.02 and 2.03 ± 0.02 folds, respectively at 200 μg/mL of DBEAF. Furthermore, DBEAF also resulted in a significant increase of death receptor, Fas expression in a dose-dependent manner (Fig. 6A and B). Therefore, these collective findings suggested that DBEAF induced apoptosis involved both extrinsic and intrinsic pathways.

3.7. Inhibition of ERK1/2 signaling protein in DBEAF-treated HCT116 cells

ERK1/2 signaling transduction plays imperative roles in promoting survival, proliferation, and apoptosis in the cells. Towards investigating the modulation of ERK1/2 protein, we have a comprehensive understanding of DBEAF induced apoptosis mechanism towards HCT116 cells. Based on the present western blot results, exposure of DBEAF significantly inhibited phosphorylation of ERK1/2 in HCT116 cells compared to vehicle (Fig. 7A and B). The analysis revealed that both phospho ERK1/2 at 44 and 42kDa decreased in a dose-dependent manner. Furthermore, the correlation of ERK1/2 inhibition and apoptosis in DBEAF-treated cells were investigated by using a specific inhibitor UO126 monoethanolate. As shown in Fig. 7C, treatment of UO and DBEAF, further decrease HCT116 cells viability when compare to DBEAF treatment alone. After HCT116 cells were pre-treated with

![Fig. 3. Nuclear morphological alterations of HCT116 cells treated with or without DBEAF. Cells were double stained with Hoechst 33,342 and PI followed by detection using fluorescence microscope. Magnification: 400 ×. Arrow 1: chromatin condensation, 2: cell shrinkage, 3: DNA fragmentation, 4: late apoptosis.](image-url)
**Fig. 4.** Depolarization of MMP in HCT116 cells after treated with DBEAF. (A) The flow cytometric analysis illustrates the loss of mitochondrial membrane potential in a dose-dependent manner. (B) The bar chart reveals the total of green fluorescents positive cells. The data expressed as mean ± S.E from three individual experiments. Asterisk indicates significantly different value from control (*p < 0.05).

**Fig. 5.** Effect of DBEAF on the modulation of Bcl-2 family protein expression in HCT116 cells. Cells were treated with varying concentrations of DBEAF (50, 100 and 200 μg/mL) for 24 h. (A) Presents the western blot band intensity image of Bax, Bcl-2, Bak and Bcl-xL protein expression level. GAPDH was used as loading control. (B) The bar chart shows the relative expression of Bcl-2 family proteins. (C) The bar chart displays the ratio of Bax/Bcl-2. (D) The bar chart displays the ratio of Bak/Bcl-xL. All data expressed as mean ± S.E from three individual experiments. Asterisks indicate significantly different value from control (*p < 0.05).
UO126 for 1 h, phosphorylation of ERK1/2 was decreased to 0.82 ± 0.01 and 0.97 ± 0.01 at 44 and 42 kda, respectively. Additionally, pre-treatment of inhibitor followed by DBEAF further suppressed the phosphorylation of ERK1/2 as compared to the inhibitor alone. As shown in Fig. 7D and E, suppression of ERK1/2 by UO126 potentiated the increase of Bax expression concomitantly with a decrease of Bcl-2 expression upon the treatment of DBEAF. Accordingly, inhibition with UO126 could elevate the proapoptotic effects of DBEAF by enhancing the DBEAF-induced apoptosis through an increase in the ratio of Bax/Bcl-2 along with a significant decrease of procaspase-3 and procaspase-9 as compared to DBEAF treatment alone. Therefore, these results suggested that the inhibitory effect of DBEAF on ERK1/2 was closely correlated with the induction of apoptotic cell death in HCT116 cells.

3.8. Activation of JNK in DBEAF-treated HCT116 cells

The potential role of JNK in the regulation of cell death displays double-edge sword properties, where it can either promote survival or apoptosis towards the cells. Nevertheless, activation of JNK has been closely related to apoptosis induction in several types of cancer cells. As illustrated in Fig. 8A and B, the phosphorylation of JNK was significantly activated in increasing concentrations of DBEAF. Additionally, the correlation between the JNK protein and apoptosis marker was assessed using JNK inhibitor, SP600125. Based on Fig. 8C, treatment with SP600125 and DBEAF increase HCT116 cells viability compared to DBEAF treatment alone. Therefore, these results suggested that the inhibitory effect of DBEAF on ERK1/2 was closely correlated with the induction of apoptotic cell death in HCT116 cells.

3.9. Chemical analysis of DBEAF by using LCMS-QTOF

To further identify the chemical constituent present in DBEAF, the active fraction was analyzed by LCMS-QTOF. Based on Figs. 9 and 10 and Table 2, there were 6 identified compounds that may contribute to the apoptotic effects in HCT1116 cells namely, (1) Apigenin 7-(4''-E-p-coumarylglucoside), (2) (+)Epicatechin, (3) 5'-Butyrylphosphoinosine, (4) Quercitrin, (5) 5,3,4-trihydroxy-3,7-dimethoxyflavone, and (6) Diosbulbin N.

4. Discussion

To date, researchers continue searching for effective naturally derived anticancer agents asstrings of chemotherapy drugs can bring about undesirable side effects. Natural occurring anticancer agents derived from plants or herbs are considered nontoxic and have manifold beneficial effects on human health. Hence, for targeting cancer therapy, complementary and alternative medicine research have been given prime attention. D. bulbifera, commonly known as air potato to the locals, has been reported to possess various ethnomedicinal properties such as anticancer [24] and anti-diabetic [25]. More than that, this plant has been used extensively for the synthesis of metal nanoparticles such as platinum-pladium and was later investigated for its anticancer activities [26]. Hence, as part of our growing interest in ethnomedicinal research, we demonstrated for the first time that D. bulbifera could
effectively inhibit HCT116 colon cancer cells proliferation by inducing apoptotic cell death. We further verified the underlying molecular mechanisms by which D. bulbifera could induce apoptosis, and shows the modulation of ERK1/2 and JNK protein expression.

In the present study, the ethanol extract, hexane, ethyl acetate and water fractions of D. bulbifera were investigated for cytotoxicity on several cancer cell lines. Results revealed that DBEAF significantly inhibited the proliferation of HCT116 human colorectal cancer cells in a dose-dependent manner and hence was selected for further studies. In addition, there is no sign of toxic nature towards the normal colon cells upon exposure of DBEAF, verifying the safety of DBEAF.

A balance between the cell proliferation and death is of utmost importance in maintaining homeostasis and normal development of the cells [27]. Apoptosis, one of the cell death modalities, is considered as a potential mode in combating cancer by dysregulating the proportion between the cell proliferation and apoptosis. The onset of apoptosis was first described by its morphological hallmarks, where the cells tend to shrink, form membrane blebbing, apoptotic bodies, chromatin condensation and DNA fragmentation [28]. Our findings demonstrated that nuclear morphological changes such as cell shrinkage, DNA fragmentation and chromatin condensation were detected in DBEAF-treated cells via Hoechst 33342 and PI dual staining. Furthermore, the loss of cellular plasma membrane asymmetry is one of the preliminary apoptotic signals, where the embedded phosphatidylserine on the plasma membrane translocated to the outer membrane. After exposure of DBEAF to HCT116 cells, phosphatidylserine was found to translocate from the inner membrane to the outer membrane, which was a biochemical hallmark detected by Annexin V/PI staining assay. Taken together, the biochemical and morphological observations suggested the involvement of apoptosis in HCT116 cells upon treatment of DBEAF.

Intracellular signaling pathway primarily consists of two major apoptosis pathways. The extrinsic pathway involves activation of death receptors (e.g., Fas) located in the plasma membrane, where the signal death ligands (e.g., FasL) resulted in activation of initiator caspase-8 or -10. Subsequently, executioner caspase-3 is triggered leading to amplification of apoptosis signal towards the cells. Consistent with this, our data also revealed that the death receptor Fas was upregulated when treated with increasing concentrations of DBEAF. In addition, activation of caspase-8 may lead to the intrinsic pathway involving changes of mitochondrial integrity. The modulation of intrinsic apoptosis pathway is mainly orchestrated by Bcl-2 family members, which comprised of anti-apoptotic (e.g., Bcl-2 or Bcl-xL) and pro-apoptotic (e.g., Bax or Bak) proteins. The anti-apoptotic proteins prevent cytochrome c release from the mitochondria, while the pro-apoptotic proteins induce the formation of pores on the mitochondrial outer membrane. Notably, it is well documented that the ratio of pro- and anti-apoptotic Bcl-2 family proteins (e.g., Bax/Bcl-2), which modulate the mitochondrial pathway appear to be more predominant in determining the sensitivity to apoptotic stimuli than the expression of each
individual protein (Raisova et al, 2001). In this study, DBEAF was demonstrated to enhance the pro-apoptotic Bax and Bak proteins concomitantly with the reduction of Bcl-2 and Bcl-xL protein expressions in HCT116 cells. Therefore, a shift in the Bax/Bcl-2 and Bak/Bcl-xL ratios allows mitochondrial-mediated apoptosis to occur. Upon loss of MMP, cytochrome c was released, which caused the activation of initiator caspase-9 and executioner caspase-3, followed by the degradation of PARP and eventually leading to apoptosis. This was corroborated with our findings that treatment of DBEAF significantly induced activation of caspase cascades including caspases-3, -9, -10 and -8, resulting in the induction of apoptosis as indicated by the cleavage of PARP. These results strongly demonstrated that DBEAF induced apoptosis through the intrinsic and extrinsic pathways in HCT116 cells.

ERK1/2 and JNK signaling pathways regulate important cellular processes such as cell proliferation, apoptosis, cell cycle, inflammation and autophagy [29]. Several studies have also revealed that inhibition of ERK1/2 alone sufficiently enhanced the induction of apoptosis towards cancer cells. For instance, sunlida induced apoptosis via suppression of ERK1/2 phosphorylation in HCT116 cells [30,31], indicating the inactivation of anti-apoptotic effect of phosphorylated
ERK1/2. Consistent with this, treatment of DBEAF effectively abrogated the ERK1/2 signaling pathway by altering the phosphorylation of ERK1/2. Compelling evidence has been reported that suppression of ERK1/2 signaling triggered inactivation of Bcl-2 and activation of Bax with a concomitant increase of Bax/Bcl-2 ratio, allowing initiation of mitochondrial events, which is required for apoptosis [32]. This event further evoked caspase activation and cleavage of PARP, eventually leading to apoptosis. Trichostation A was found to inhibit ERK1/2 activation and enhance apoptosis via caspase activation in gastric cancer [33]. In accordance with these, our current findings indicated that inhibition of ERK1/2 further enhanced the ratio of Bax/Bcl-2 protein and the activation of caspase-3 and -9 in the presence of UO126 inhibitors prior to DBEAF treatment, suggesting that apoptosis conferred by DBEAF was closely associated with the ERK1/2 signaling pathway.

It is conceivable that JNK signaling transduction, which was stimulated by stress signal, is involved in the trigger of apoptotic cell death. A growing number of studies have reported the induction of apoptosis in HCT116 cells was activated via JNK phosphorylation. For example, the methanol extracts of Chamaecyparis obtuse leaves and Gefitinib induced HCT116 cells apoptosis via JNK activation [34,35]. Based on our current findings, treatment of DBEAF induced the phosphorylation of JNK in HCT116 cells. It is well established that phosphorylation of JNK regulates a wide variety of downstream targets such as Bcl-2 family proteins and caspases. To investigate the correlation of DBEAF induced apoptosis and activation of JNK, HCT116 cells were pre-administered with JNK biological inhibitor, SP600125 for 1 h. Towards inhibiting phosphorylated JNK, the ratio of Bax/Bcl-2 protein was decreased and the activation of caspase-3 and -9 were reversed when compared to DBEAF treatment alone. These results are in line with previous studies, which have shown that treated Hispolon and SP600125 towards human nasopharyngeal carcinomas cells inhibited apoptosis by attenuating the cleavage of caspase-3 and -9 [36]. Furthermore, treatment of quercetin and SP600125 in CT26 colon carcinoma also further inhibited apoptosis by increasing Bcl-2 and Bcl-xL along with reduction of cleaved caspase-3 and -9 proteins expression when compared to quercetin alone [37]. Therefore, the induction of phosphorylation JNK in HCT116 cells is dependent in DBEAF-induced apoptosis.

The presence of phenol or flavonoids groups in DBEAF might associated to the apoptotic activity in HCT116 cells. Therefore, the LCMS-QTOF analysis technique was exploited to identify the chemical constituent presence in DBEAF. According to the data obtained, there are 6 compounds that was identified such as (1) Apigenin 7-(4'-E-p-coumarylglucoside), (2) (+)Epicatechin, (3) S'-Butyrylphosphonosine, (4) Quercitrin, (5) 5,3,4-trihydroxy-3,7-dimethoxyflavone, and (6) Diosbulbin N. According to the literature, (2) (+)Epicatechin was also

### Table 2

The identification of DBEAF chemical constituents by using a LCMS-QTOF.

<table>
<thead>
<tr>
<th>No</th>
<th>Compounds</th>
<th>Molecular Weight</th>
<th>Molecular Formula</th>
<th>Retention Time</th>
<th>Ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Apigenin 7-(4'-E-p-coumarylglucoside)</td>
<td>578.141</td>
<td>C30H26O12</td>
<td>6.146</td>
<td>(M-H)-</td>
</tr>
<tr>
<td>2</td>
<td>(+)Epicatechin</td>
<td>290.260</td>
<td>C15H14O6</td>
<td>6.819</td>
<td>(M-H)-</td>
</tr>
<tr>
<td>3</td>
<td>S'-Butyrylphosphonosine</td>
<td>418.089</td>
<td>C14 H19 N4 O9 P</td>
<td>10.000</td>
<td>(M + HCOO)-</td>
</tr>
<tr>
<td>4</td>
<td>Quercitrin</td>
<td>448.388</td>
<td>C21 H20 O11</td>
<td>11.088</td>
<td>(M-H)-</td>
</tr>
<tr>
<td>5</td>
<td>5,3,4-trihydroxy-3,7-dimethoxyflavone</td>
<td>330.074</td>
<td>C17 H14 O7</td>
<td>11.422</td>
<td>(M-H)-</td>
</tr>
<tr>
<td>6</td>
<td>Diosbulbin N</td>
<td>362.378</td>
<td>C19 H22 O7</td>
<td>13.201</td>
<td>(M-H)-</td>
</tr>
</tbody>
</table>
found in ethyl acetate fraction of *D. bulbifera* which promotes the anti-tumor effects in JB6 mouse epidermal cells with IC50 of 13.1 µg/ml [38]. In another example, the repeated purified of ethyl acetate fraction of *D. bulbifera* afford two new norclerodane diterpenoids, (6) Diosbulbin N and P which induced cytotoxicity against five human cancer cell lines, HL-60, SMMC-7721, A-549, MCF-7, and SW-480 with IC50 > 40 µg/ml [39]. Thus, we proposed the presence of the bioactive phytochemicals in DBEAF such as (2) and (6) may involve in the apoptotic activity towards HCT116 cells. However, further studies are required to isolate and purify the bioactive compounds which responsible to the anti-tumor activity in HCT116 cells.

In conclusion, this study demonstrated that DBEAF inhibited the growth of HCT116 cells via induction of apoptosis through ERK and JNK signaling pathways. DBEAF executed apoptosis by upregulating the Fas, caspase-10 and -8 protein expression. Subsequently, DBEAF induced the dysfunction of the mitochondria by increasing the ratio of Fas, caspase-10 and -8 protein expression. Subsequently, DBEAF inactivated JNK signaling pathways. DBEAF executed apoptosis by upregulating the growth of HCT116 cells via induction of apoptosis through ERK and JNK signaling pathways. DBEAF executed apoptosis by upregulating the growth of HCT116 cells via induction of apoptosis through ERK and JNK signaling pathways. DBEAF executed apoptosis by upregulating the growth of HCT116 cells via induction of apoptosis through ERK and JNK signaling pathways.

**Conflict of interest**

The authors have no conflicts of interest to declare.

**Acknowledgment**

This study was supported by the Fundamental Research Grant Scheme (FRGS) FP009-2014B from the Ministry of Education, Malaysia.

**References**


