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## RESEARCH ARTICLE

## *Cosmos caudatus* Leaf Extract Triggers Apoptosis of HSC-3 Cancer Cells by Decreasing Bcl-2 and Increasing Bax

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### Abstract

**BACKGROUND:** Previous studies have demonstrated that *Cosmos caudatus* leaf extract (CCLE) exhibits cytotoxic effects against various types of human cancer. However, the CCLE cytotoxic effect towards oral squamous cell carcinoma (OSCC) cells has not been investigated. Therefore, this study was conducted to evaluate the effect of CCLE towards the viability and apoptosis in human oral squamous carcinoma (HSC)-3 cells.

**METHODS:** HSC-3 cells were treated with various concentrations of CCLE for 24 h. The number of viable HSC-3 cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), meanwhile the apoptotic HSC-3 cells were measured using sub-G1 assay. Mitochondrial membrane potential was measured using flow cytometry. Bcl-2 and Bax protein content of HSC-3 cells were measured using enzyme-linked immunosorbent assay (ELISA).

**RESULTS:** CCLE treatment could decrease the number of HSC-3 viable cells and increase the percentage of HSC-3 apoptotic cells in concentration-dependent manner. In mitochondrial membrane potential assay, CCLE-treated group displayed a peak shiftment from  $10^4$  to  $10^3$ . Bcl-2 protein contents of CCLE-treated group were decrease in concentration-dependent manner, meanwhile Bax protein contents of CCLE-treated group were increase in concentration-dependent manner.

**CONCLUSION:** CCLE could trigger apoptosis in HSC-3 cells by decreasing Bcl-2 protein content and increasing Bax protein content in concentration-dependent manner, leading to intrinsic apoptotic pathway.

**KEYWORDS:** *Cosmos caudatus*, HSC-3, apoptosis, mitochondrial membrane potential, Bcl-2, Bax

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### Introduction

Cancer chemoprevention involves the use of natural substances or synthetic medicines to prevent, slow, or decrease the growth of invasive cancer by inducing apoptosis in cancer cells.(1,2) Apoptosis-inducing natural substances in cancer cells are usually correlated with the presence of flavonoids, which are the most abundant category of phenolic chemicals.(3,4) High flavonoids and

phenolic acid content could be found in the leaves of *Cosmos caudatus*.(5)

Traditionally, *C. caudatus* has been used for enhancing blood circulation, strengthening bones, alleviating fever, improving anti-aging process, and treating infections. (6) In *C. caudatus* plants, there are several phenolic acid such as ferulic acid, cryptochlorogenic acid, caffeic acid, neochlorogenic acid, and chlorogenic acid. Meanwhile, the flavonoids found in *C. caudatus* plants are quercetin glycoside, quercetin, and catechin.(7,8) The main

phytochemical in *C. caudatus* leaves is quercetin, with 51% of the total content.(9) Quercetin is a prevalent polyphenol that is extensively found in nature, frequently found in several plants. These plants component have antioxidant capabilities and a preventive role against the aging process. (10) Quercetin exhibits antioxidative, anti-inflammatory, anti-proliferative, anti-carcinogenic, anti-diabetic, and anti-viral effects.(11)

Apoptosis occurs through two different mechanisms, intrinsic pathway and extrinsic pathway. The intrinsic pathway can occur through mitochondria, while the extrinsic pathway can occur through death receptors on cell surface.(12) Alteration in mitochondrial membrane potential ( $\Delta\Psi_m$ ) is dependent on the proportion of proapoptotic protein (Bax) and antiapoptotic protein (B-cell lymphoma (Bcl)-2), ultimately determining the fate of the cell.(11) Intrinsic apoptosis occurs through cytochrome C release regulation. Bcl-2 protein inhibits Cytochrome C release from mitochondria, meanwhile Bax protein induces the Cytochrome C release. Then, Cytochrome C, apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 form a complex in cytoplasm, called apoptosome, which activates caspase-9. Caspase-9 triggers the activation of caspase-6 and caspase-7, leading to the initiation of apoptosis.(13)

Previous research has investigated the effects of *C. caudatus* leaf extract (CCLE) in T47D breast cancer cell line (14) and HeLa cervical cancer cell line (15). However, no studies have examined the effects of CCLE on oral squamous cell carcinoma (OSCC) cells, particularly tongue cancer. Moreover, apoptotic mechanism that caused by CCLE is largely unclear and remain to be clarified. Hence, this study investigated the effect of CCLE on cell viability and apoptosis of human oral squamous carcinoma (HSC)-3 cells.

## Methods

### CCLE Preparation

*C. caudatus* leaves were obtained from the Indonesian Medicinal and Aromatic Crops Research Institute in Indonesia. The plant was identified by the Research Center for Plant Conservation and Botanic Garden, Indonesian Institute of Sciences, Indonesia (Identification No. B-1269/IPH.3/KS/X/2020).

The CCLE was acquired with maceration method. *C. caudatus* leaves were finely chopped and dehydrated. The desiccated substance was extracted using 70% ethanol solution, then filtered and evaporated with a rotary

evaporator. The resulting crude CCLE was thereafter stored at a temperature of 4°C.

### HSC-3 Cell Culture

The HSC-3 cell culture was performed using a previously reported method (16), with specific modifications. The HSC-3 cell line was obtained from Sigma-Aldrich (St. Louis, MO, USA). HSC-3 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich) complete medium consisting of 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany) and 50 U/mL penicillin and 50 µg/mL streptomycin (Sigma-Aldrich). The cells were grown in a humidified incubator at 37°C, 5% CO<sub>2</sub>. The HSC-3 cells were detached using a trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) when they achieved 80% confluency.

### Cell Viability Assay

The quantification of viable cells was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, following a previously described protocol.(17) HSC-3 cells were placed in 96-well plates (5x10<sup>3</sup>/well) and treated with/without 1, 10, or 100 µg/mL CCLE or 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) for 24 hours. MTT was added in the treated well (100 µL/well) and incubated for 4 hours. After that, the suspension in each well was removed and dissolved in 100 µL dimethylsulfoxide (DMSO). Formed formazan crystal was measured with a microplate reader (Bio-Rad, Hercules, CA, USA) at OD570. Each experimental group was measured in sextuplicate.

### Sub-G1 Assay

To assess the cytotoxic effects of CCLE, apoptotic HSC-3 cells were quantified using the sub-G1 assay, based on previous method.(18) Treated HSC-3 cells were collected and placed in a hypotonic fluorochrome solution (50 µg/mL of propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan)). Afterwards, the cell suspensions were kept in dark for 30 minutes. The fluorescence of individual nuclei was measured using a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), with a total of 20,000 events recorded.

### $\Delta\Psi_m$ Assay

$\Delta\Psi_m$  analysis was conducted in accordance with the previous method.(19,20) Treated cells were collected, resuspended in 20 nM 3,3'-dihexyloxycarbocyanine



iodide ( $\text{DiOC}_6$ ), and incubated for 15 minutes. The  $\Delta\Psi\text{M}$  measurement was conducted using a FACSCanto II flow cytometer.

### Bcl-2 and Bax Analysis with Enzyme-Linked Immunosorbent Assay (ELISA)

The Bcl-2 protein content was measured using the Bcl-2 Human ELISA kit (Cat. No. ab119506; Abcam, Cambridge, United Kingdom) by following the manufacturer's instruction. Briefly, 20  $\mu\text{L}$  sample and 80  $\mu\text{L}$  of sample diluent were put into each well of microplate coated with monoclonal antibody to Bcl-2. Then, 50  $\mu\text{L}$  biotin-conjugate anti-human Bcl-2 monoclonal antibody was added and incubated at room temperature for 2 hours. After washing, 100  $\mu\text{L}$  streptavidin-horseradish peroxidase (HRP) conjugate was added to each well and incubated at room temperature for 1 hour. After washing, 100  $\mu\text{L}$  3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and incubated at room temperature for 10 minutes. The absorbance of each microplate was read at a wavelength of 450 nm. The detection limit of the kit was  $<0.5$  ng/mL.

The Bax protein content was quantified using the Human Bax SimpleStep ELISAKit (Cat. No. ab199080; Abcam) by following the manufacturer's instruction. Briefly, 50  $\mu\text{L}$  of standard or sample were added into each well of anti-tag coated microplate. Then, 50  $\mu\text{L}$  antibody cocktail was added and incubated at room temperature for 1 hour. After washing, 100  $\mu\text{L}$  TMB was added to each well and incubated in the dark at room temperature for 15 minutes.

### Statistical Analysis

Statistical analysis was carried out using Shapiro-Wilk normality test. The results of a normal data distribution were tested with one-way ANOVA test followed by Tukey's Post Hoc test. Meanwhile, the results of not normal data distribution were tested with Kruskal-Wallis test followed by Mann-Whitney's Post Hoc test.

## Results

### CCLE Decreased HSC-3 Viable Cells

Number of HSC-3 viable cells in doxorubicin group ( $226 \pm 32$ ) was significantly lower (Mann-Whitney's Post Hoc test,  $p=0.004$ ) than the ones in the sham group ( $9,607 \pm 14$ ) (Figure 1). CCLE treatment could decrease the number of HSC-3 viable cells significantly (Kruskal Wallis,  $p=0.000$ ) in concentration-dependent manner. The number

of HSC-3 viable cells were  $8,300 \pm 48$ ;  $4,065 \pm 170$ ; and  $1,112 \pm 50$  when treated with 1, 10, and 100  $\mu\text{g/mL}$  CCLE, respectively.

### CCLE Increased HSC-3 Apoptotic Cells

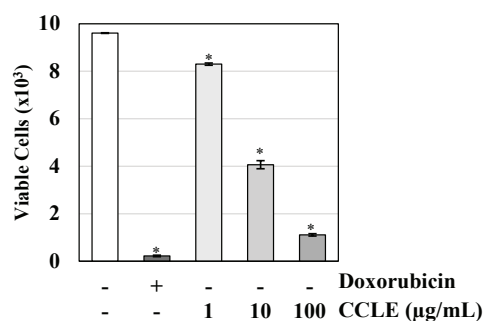
The percentage of apoptotic HSC-3 cells in doxorubicin group ( $95.95 \pm 2.60\%$ ) was significantly higher (Tukey's Post Hoc test,  $p=0.000$ ) than the ones in the sham group ( $8.27 \pm 0.14\%$ ) (Figure 2). CCLE treatment could increase the percentage of HSC-3 apoptotic cells significantly (ANOVA,  $p=0.001$ ) in concentration-dependent manner. The percentage of HSC-3 apoptotic cells were  $25.67 \pm 0.34\%$ ,  $52.01 \pm 1.47\%$ , and  $87.07 \pm 1.31\%$  when treated with 1, 10, and 100  $\mu\text{g/mL}$  of CCLE, respectively.

### CCLE Decreased $\Delta\Psi\text{M}$

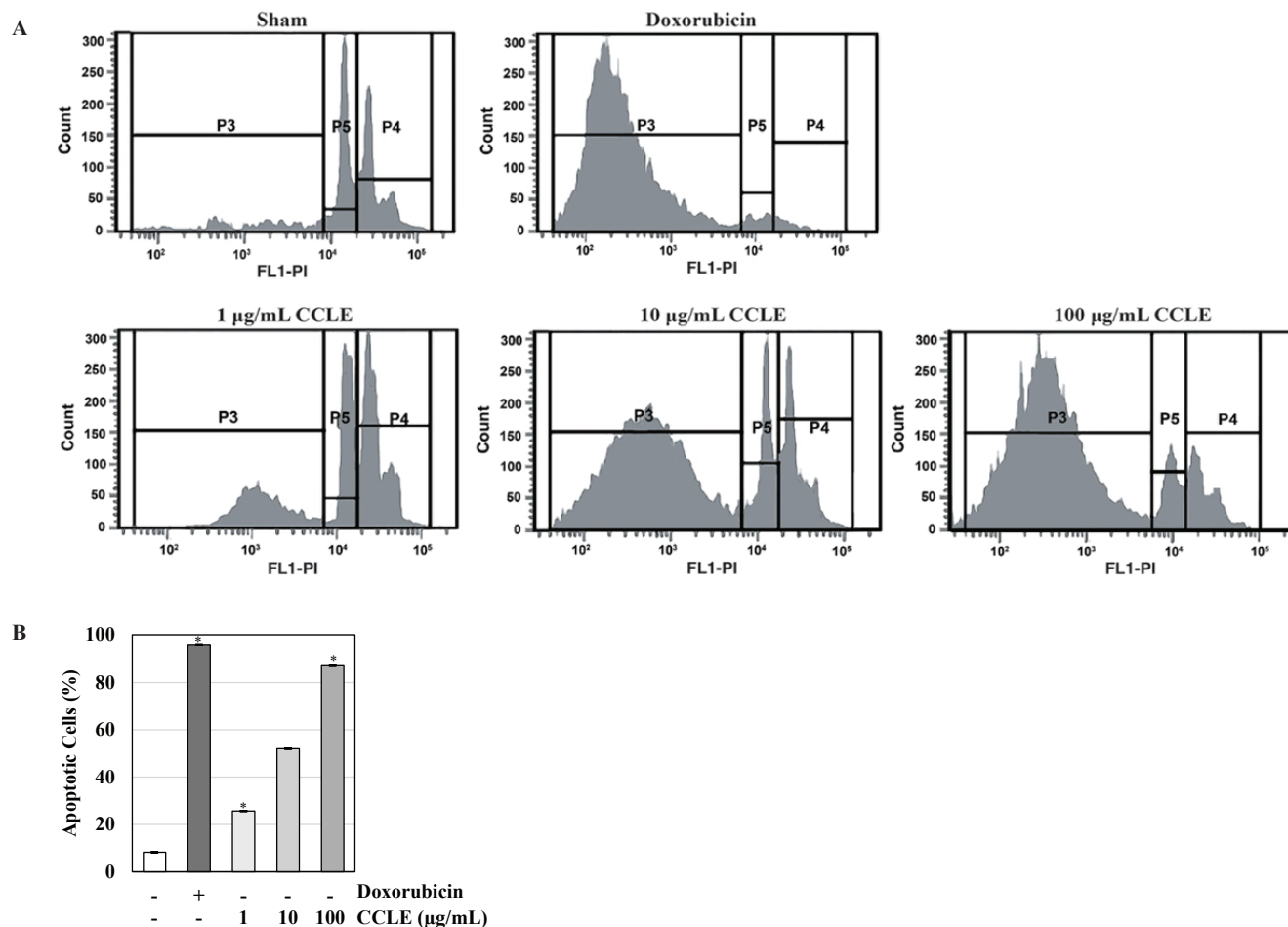
In the sham group, a peak-close-to- $10^4$  at the x-axis (black dashed line) was observed (Figure 3). Meanwhile, a peak-close-to- $10^3$  (blue dashed line) was observed in the doxorubicin-treated group. These results suggested a decrease of  $\Delta\Psi\text{M}$ , marked by the shiftment of the peak from  $10^4$  to  $10^3$ . A low peak-close-to- $10^3$  was observed in 1  $\mu\text{g/mL}$  CCLE-treated group (red dashed line). A high peak-close-to- $10^3$  was observed in 10  $\mu\text{g/mL}$  CCLE-treated group (yellow dashed line). Another high peak-close-to- $10^3$  was observed in 100  $\mu\text{g/mL}$  CCLE-treated group (green dashed line) as well.

### CCLE Decreased Bcl-2 Protein Content of HSC-3 Cells

Bcl-2 protein content of HSC-3 cells in doxorubicin group ( $5.33 \pm 1.21$  ng/mL) was significantly lower (Tukey's Post Hoc test,  $p=0.000$ ) than the ones in the sham group



**Figure 1. CCLE decreased HSC-3 viable cells in concentration-dependent manner.** HSC-3 cells were starved for 12 h, then treated with/without 1  $\mu\text{M}$  Doxorubicin or CCLE in various concentrations for 24 h. Viable cells were measured using MTT assay as mentioned in methods. The data is presented in mean  $\pm$  standard deviation ( $n=6$ ). \* $p<0.05$  when compared to the sham group.



**Figure 2. CCLE increased HSC-3 apoptotic cells in concentration-dependent manner.** HSC-3 cells were starved for 12 h, then treated with/without 1 µM Doxorubicin or CCLE in various concentrations for 24 h. Apoptotic cells were measured using Sub-G1 test as mentioned in Methods. A: The flow cytometric results. B: The percentage of HSC-3 apoptotic cells. The data is presented in mean±standard deviation (n=6). \* $p < 0.05$  when compared to the sham group.

(23.33±1.63 ng/mL) (Figure 4). Bcl-2 protein contents of 1, 10 and 100 µg/mL CCLE-treated group were significantly decrease (ANOVA,  $p=0.000$ ) in concentration-dependent manner. Bcl-2 protein content of 1 µg/mL CCLE-treated group (20.83±1.47 ng/mL) was not significantly different (Tukey's Post Hoc test,  $p=0.092$ ) than the ones in the sham group, meanwhile Bcl-2 protein contents of 10 µg/mL CCLE-treated group (16.00±1.90 ng/mL) and 100 µg/mL CCLE-treated group (12.50±1.87 ng/mL) were significantly different (Tukey's Post Hoc test,  $p=0.0000$ ) than the ones in the sham group.

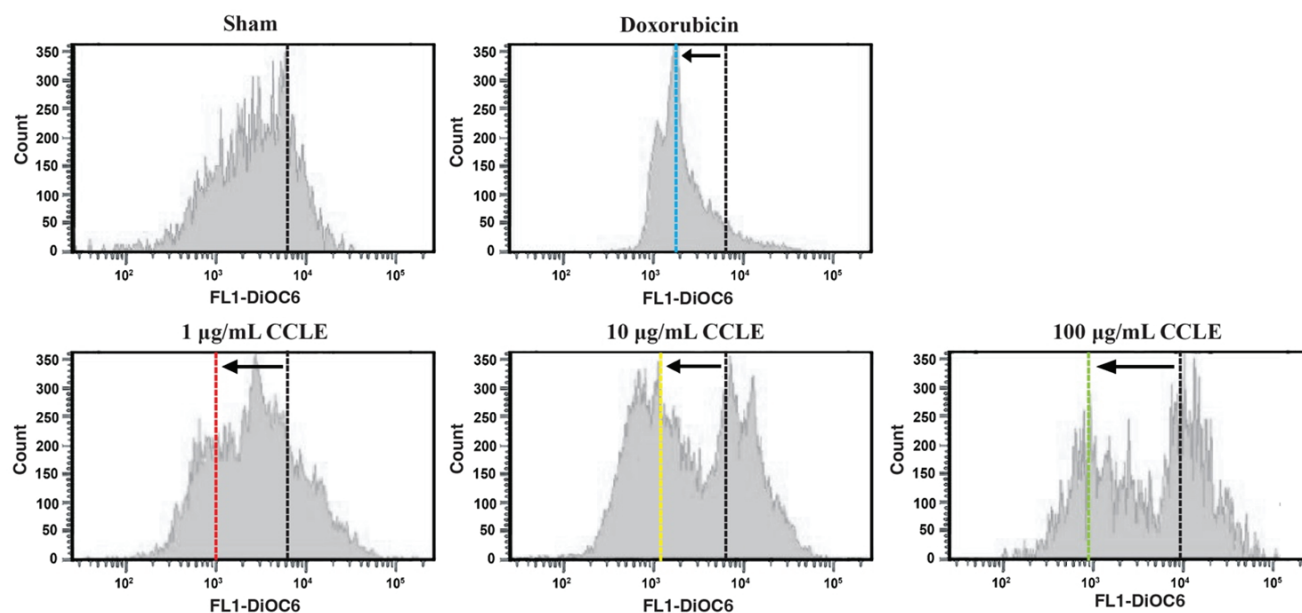
#### CCLE Increased Bax Protein Content of HSC-3 Cells

The Bax protein content of HSC-3 cells in doxorubicin group (513±5.33 pg/mL) was significantly lower (Tukey's Post Hoc test,  $p=0.000$ ) than the ones in the sham group (255.5±12.79 pg/mL) (Figure 5). Bax protein contents of 1, 10 and 100 µg/mL CCLE-treated group were significantly

increase (ANOVA,  $p=0.000$ ) in concentration-dependent manner. Bax protein content of 1 µg/mL CCLE-treated group (331.33±9.97 pg/mL), 10 µg/mL CCLE-treated group (400.83±7.41 pg/mL) and 100 µg/mL CCLE-treated group (483.67±4.59 pg/mL) were significantly different (Tukey's Post Hoc test,  $p=0.0000$ ) than the ones in the sham group.

## Discussion

In the present study, CCLE showed a cytotoxic effect in HSC-3 cells in concentration-dependent manner. According to the MTT and sub-G1 assay results (Figure 1, Figure 2), viable HSC-3 cells were decreased due to apoptosis induction. These results were in concordance to previous reports showing that CCLE was able to induce apoptosis in the T47D (breast cancer) and HeLa (cervical cancer) cell lines.(14,15)  $IC_{50}$  of CCLE-induced apoptotic HSC-3

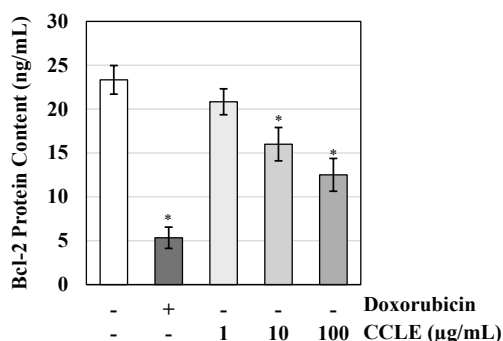


**Figure 3. CCLE decreased  $\Delta\Psi m$  of HSC-3 cells.** HSC-3 cells were starved for 12 h, then treated with/without 1  $\mu M$  Doxorubicin or CCLE in various concentrations for 12 h. HSC-3 cells were collected, washed, and processed for  $\Delta\Psi m$  assay as mentioned in Methods. These experiments were measured in sextuplicate.

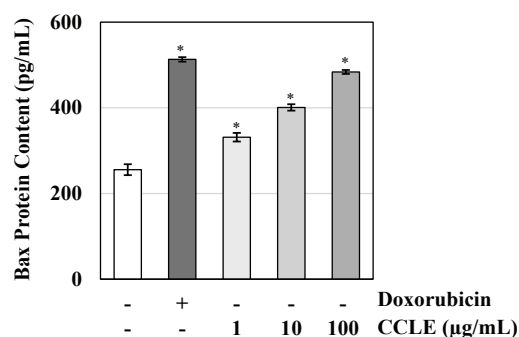
cells was 38.39  $\mu g/mL$ , which was categorized as moderate cytotoxicity (21-200  $\mu g/mL$ ).<sup>(21)</sup> The  $IC_{50}$  of CCLE-induced apoptotic HSC-3 cells was lower than the  $IC_{50}$  of CCLE-induced apoptotic T47D cells (344.91  $\mu g/mL$ )<sup>(14)</sup>, and  $IC_{50}$  of CCLE-induced apoptotic HeLa cells ( $89.90 \pm 1.30$   $\mu g/mL$ )<sup>(15)</sup>.

In this study, CCLE-treated group displayed a peak shiftment from  $10^4$  to  $10^3$  (Figure 3). These results showed a  $\Delta\Psi m$ , which might cause the release of cytochrome C from mitochondria to the cytosol. Therefore, the  $\Delta\Psi m$  is closely correlated with the occurrence of the intrinsic (mitochondria-

mediated) apoptotic pathway. Proteins in intrinsic apoptotic pathway have important role to permeabilize the mitochondrial membranes and allow efflux of apoptotic factors such as cytochrome C. The cytosolic cytochrome C binds to the adaptor protein Apaf-1, which then forms apoptosome and activates caspases, such as caspase-3, -7, and -9.<sup>(22)</sup>  $\Delta\Psi m$  has been reported to be associated with Bcl-2 family.<sup>(23)</sup> The Bcl-2 family conserves Bcl-2 homology (Bh)1-4 structural homology domain, has relation with cell death, that can either inhibit or promote apoptosis.<sup>(24)</sup> Several anti-apoptotic Bcl-2 family members



**Figure 4. CCLE decreased Bcl-2 protein content of HSC-3 cells in concentration-dependent manner.** HSC-3 cells were treated with/without 1  $\mu M$  Doxorubicin or CCLE in various concentrations. Bcl-2 protein content was measured using ELISA as mentioned in methods. \* $p < 0.05$  when compared to the sham group.



**Figure 5. CCLE increased Bax protein content of HSC-3 cells in concentration-dependent manner.** HSC-3 cells were treated with/without 1  $\mu M$  Doxorubicin or CCLE in various concentrations. Bax protein content was measured using ELISA as mentioned in methods. \* $p < 0.05$  when compared to the sham group.



are Bcl-2, Bcl-XL and Mcl-1, meanwhile pro-apoptotic Bcl-2 family members are Bax, Bak, Bok, Bad, Bid, Bim, Noxa, and puma.(25)  $\Delta\Psi$  has been reported to be associated with the ratio between Bax and Bcl-2 proteins.(23) Both Bax (pro-apoptosis) and Bcl-2 (anti-apoptosis) controls the mitochondrial movement during cell death stimulation and influences the immune cells.(25,26)

Results of the present study showed that CCLE-treated group decreased Bcl-2 protein content of HSC-3 cells (Figure 4) and increased Bax protein content of HSC-3 cells (Figure 5) in concentration-dependent manner. Since quercetin was the mainly found flavonoids in the leaves of *C. caudatus* (5), the quercetin could have the potential activity in affecting the Bcl-2 and Bax protein contents. As reported previously in human breast cancer MDA-MB-231 cell line study, quercetin could reduce  $\Delta\Psi$  and decrease the expression of Bcl-2.(27) Further studies should be conducted to confirm the mechanism of intrinsic apoptotic factors including the release of cytochrome C, activation of caspase-3, -7, and -9.

## Conclusion

CCLE could trigger apoptosis in HSC-3 cells by decreasing Bcl-2 protein content and increasing Bax protein content in concentration-dependent manner, leading to intrinsic apoptotic pathway.  $IC_{50}$  value of CCLE in HSC-3 cells was 38.39  $\mu$ g/mL, indicating moderate cytotoxicity.

## Authors Contribution

FS and AYD were involved in conceiving and planning the research, performed the data acquisition/collection, and performed the data analysis. FS and AES drafted the manuscript and designed the figures. FS, MIR, and KHL aided in interpreting the results and gave critical discussion. All authors took parts in giving critical revision of the manuscript.

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