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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 shifment 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-inflammator , anti-proliferative, anti-carcinogenic, anti-diabetic, and anti-viral effects.(1)

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 (ΔΨm) 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_2. The HSC-3 cells were detached using a trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) when they achieved 80% confluenc .

Cell Viability Assay

The quantificat on 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 ($5x10^3$/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.

ΔΨM Assay

ΔΨM analysis was conducted in accordance with the previous method.(19,20) Treated cells were collected, resuspended in 20 nM 3,3'-dihexyloxacarbocyanine

iodide ($DiOC_6$), and incubated for 15 minutes. The ΔΨM 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. Briefl , 20 µL sample and 80 µL of sample diluent were put into each well of microplate coated with monoclonal antibody to Bcl-2. Then, 50 µL biotin-conjugate anti-human Bcl-2 monoclonal antibody was added and incubated at room temperature for 2 hours. After washing, 100 µL streptavidin-horseradish peroxidase (HRP) conjugate was added to each well and incubated at room temperature for 1 hour. After washing, 100 µL 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. Briefl , 50 µL of standard or sample were added into each well of anti-tag coated microplate. Then, 50 µL antibody cocktail was added and incubated at room temperature for 1 hour. After washing, 100 µL 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±32) was significantly lower (Mann-Whitney's Post Hoc test, p=0.004) than the ones in the sham group (9,607±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±48; 4,065±170; and 1,112±50 when treated with 1, 10, and 100 µg/mL CCLE, respectively.

CCLE Increased HSC-3 Apoptotic Cells

The percentage of apoptotic HSC-3 cells in doxorubicin group (95.95±2.60%) was significantly higher (Tukey's Post Hoc test, p=0.000) than the ones in the sham group (8.27±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±0.34%, 52.01±1.47%, and 87.07±1.31% when treated with 1, 10, and 100 µg/mL of CCLE, respectively.

CCLE Decreased ΔΨM

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 ΔΨM, marked by the shifment of the peak from 10^4 to 10^3. A low peak-close-to-10^3 was observed in 1 µg/mL CCLE-treated group (red dashed line). A high peak-close-to-10^3 was observed in 10 µg/mL CCLE-treated group (yellow dashed line). Another high peak-close-to-10^3 was observed in 100 µg/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±1.21 ng/mL) was significantly lower (Tukey's Post Hoc test, p=0.000) than the ones in the sham group

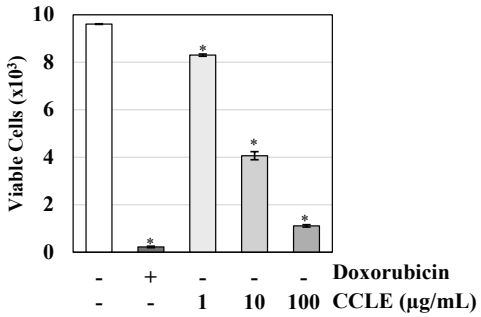

Figure 1. CCLE decreased HSC-3 viable cells in concentration-dependent manner. HSC-3 cells were starved for 12 h, then treated with/without 1 µM Doxorubicin or CCLE in various concentrations for 24 h. Viable cells were measured using MTT assay as mentioned in methods. The data is presented in mean±standard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

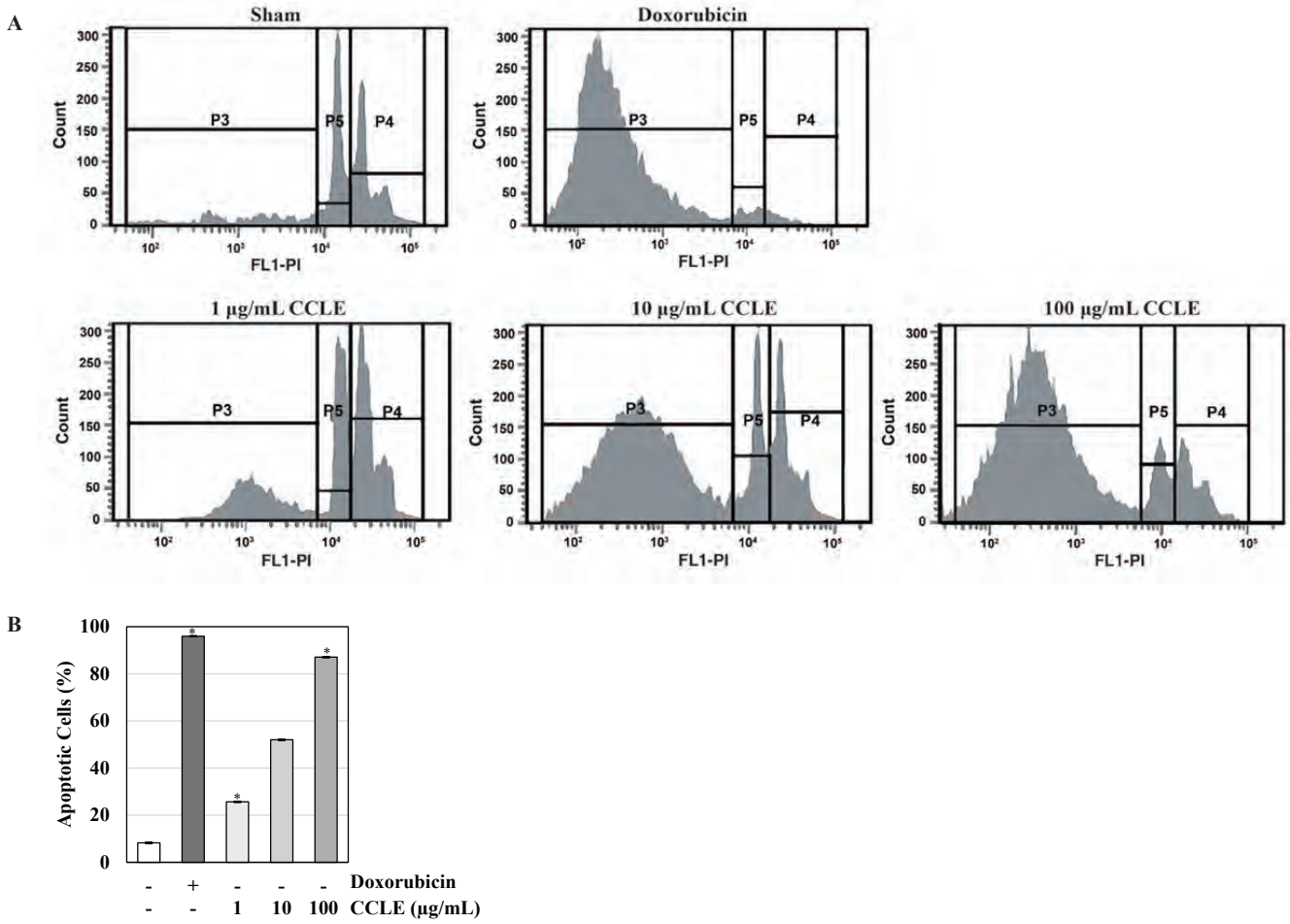

Figure 2. CCLE increased HSC-3 apoptotic cells in concentration-dependent manner. HSC-3 cells were starved for 12 h, then treated with/without 1 µM Doxorubicin or CCLE in various concentrations for 24 h. Apoptotic cells were measured using Sub-G1 test as mentioned in Methods. A: The flow cytometric results. 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

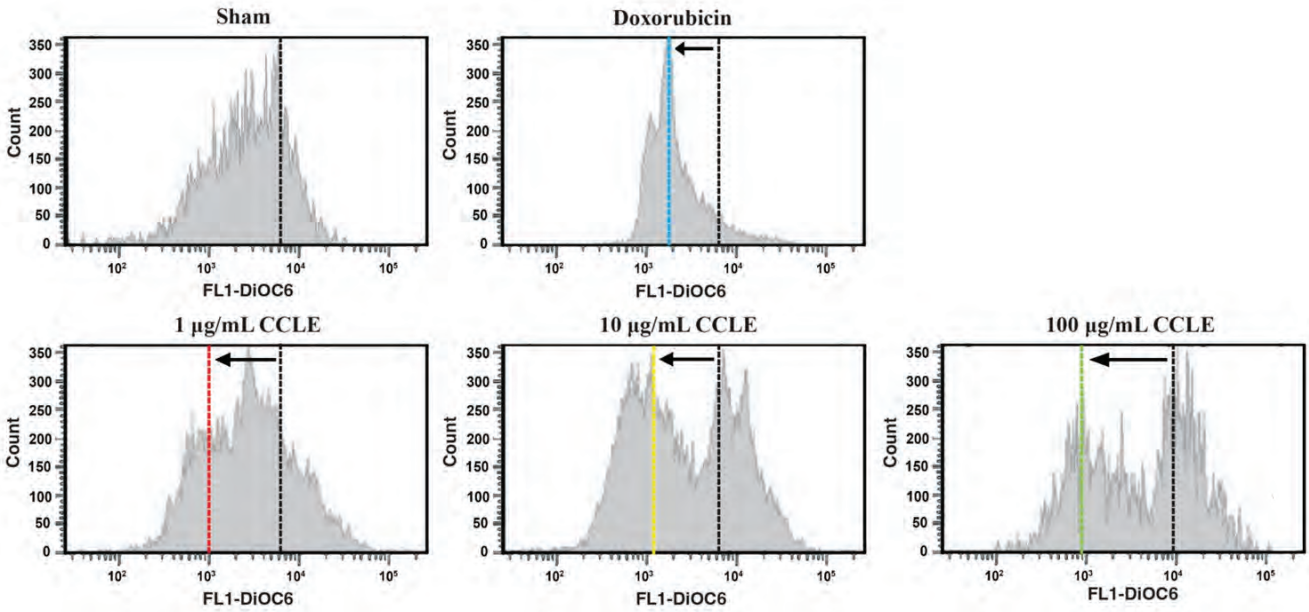

Figure 3. CCLE decreased ΔΨm of HSC-3 cells. HSC-3 cells were starved for 12 h, then treated with/without 1 µM Doxorubicin or CCLE in various concentrations for 12 h. HSC-3 cells were collected, washed, and processed for ΔΨm assay as mentioned in Methods. These experiments were measured in sextuplicate.

cells was 38.39 µg/mL, which was categorized as moderate cytotoxicity (21-200 µg/mL).(21) The IC_{50} of CCLE-induced apoptotic HSC-3 cells was lower than the IC_{50} of CCLE-induced apoptotic T47D cells (344.91 µg/mL) (14), and IC_{50} of CCLE-induced apoptotic HeLa cells (89.90±1.30 µg/mL) (15).

In this study, CCLE-treated group displayed a peak shifment from 104 to 103 (Figure 3). These results showed a ΔΨM, which might cause the release of cytochrome C from mitochondria to the cytosol. Therefore, the ΔΨM 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 efflu 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.(22) ΔΨM has been reported to be associated with Bcl-2 family.(23) 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.(24) Several anti-apoptotic Bcl-2 family

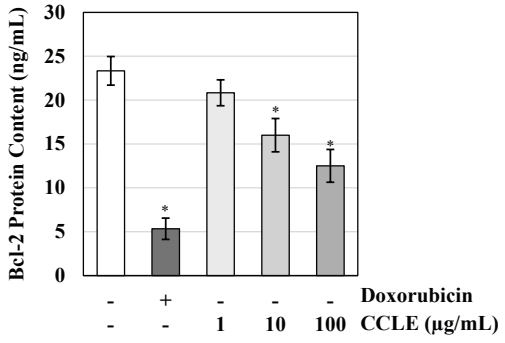

Figure 4. CCLE decreased Bcl-2 protein content of HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 1 µM Doxorubicin or CCLE in various concentrations. Bcl-2 protein content was measured using ELISA as mentioned in methods. *p<0.05 when compared to the sham group.

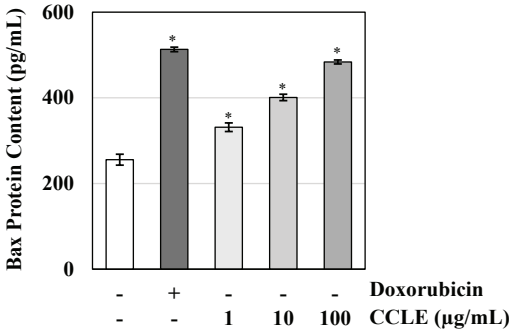

Figure 5. CCLE increased Bax protein content of HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 1 µM Doxorubicin or CCLE in various concentrations. Bax protein content was measured using ELISA as mentioned in methods. *p<0.05 when compared to the sham group.

members 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) ΔΨM 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 ΔΨM 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 µg/mL, indicating moderate cytotoxicity.

Authors Contribution

FS and AYD were involved in concepting 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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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 shifment from 10^2 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

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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_2. 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 ($5x10^3$/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.

ΔΨM Assay

ΔΨM analysis was conducted in accordance with the previous method.(19,20) Treated cells were collected, resuspended in 20 nM 3,3'-dihexyloxacarbocyanine

iodide ($DiOC_6$), and incubated for 15 minutes. The ΔΨM 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 µL sample and 80 µL of sample diluent were put into each well of microplate coated with monoclonal antibody to Bcl-2. Then, 50 µL biotin-conjugate anti-human Bcl-2 monoclonal antibody was added and incubated at room temperature for 2 hours. After washing, 100 µL streptavidin-horseradish peroxidase (HRP) conjugate was added to each well and incubated at room temperature for 1 hour. After washing, 100 µL 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 µL of standard or sample were added into each well of anti-tag coated microplate. Then, 50 µL antibody cocktail was added and incubated at room temperature for 1 hour. After washing, 100 µL 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±32) was significantly lower (Mann-Whitney's Post Hoc test, p=0.004) than the ones in the sham group (9,607±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±48; 4,065±170; and 1,112±50 when treated with 1, 10, and 100 µg/mL CCLE, respectively.

CCLE Increased HSC-3 Apoptotic Cells

The percentage of apoptotic HSC-3 cells in doxorubicin group (95.95±2.60%) was significantly higher (Tukey's Post Hoc test, p=0.000) than the ones in the sham group (8.27±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±0.34%, 52.01±1.47%, and 87.07±1.31% when treated with 1, 10, and 100 µg/mL of CCLE, respectively.

CCLE Decreased ΔΨM

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 ΔΨM, marked by the shifment of the peak from 10^4 to 10^3. A low peak-close-to-10^3 was observed in 1 µg/mL CCLE-treated group (red dashed line). A high peak-close-to-10^3 was observed in 10 µg/mL CCLE-treated group (yellow dashed line). Another high peak-close-to-10^3 was observed in 100 µg/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±1.21 ng/mL) was significantly lower (Tukey's Post Hoc test, p=0.000) than the ones in the sham group

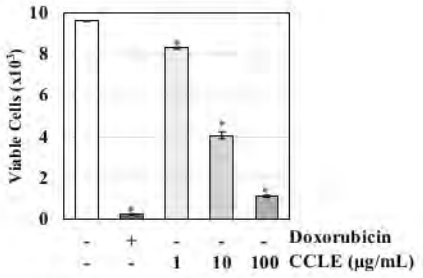

Figure 1. CCLE decreased HSC-3 viable cells in concentration-dependent manner. HSC-3 cells were starved for 12 h, then treated with/without 1 µM Doxorubicin or CCLE in various concentrations for 24 h. Viable cells were measured using MTT assay as mentioned in methods. The data is presented in mean±standard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

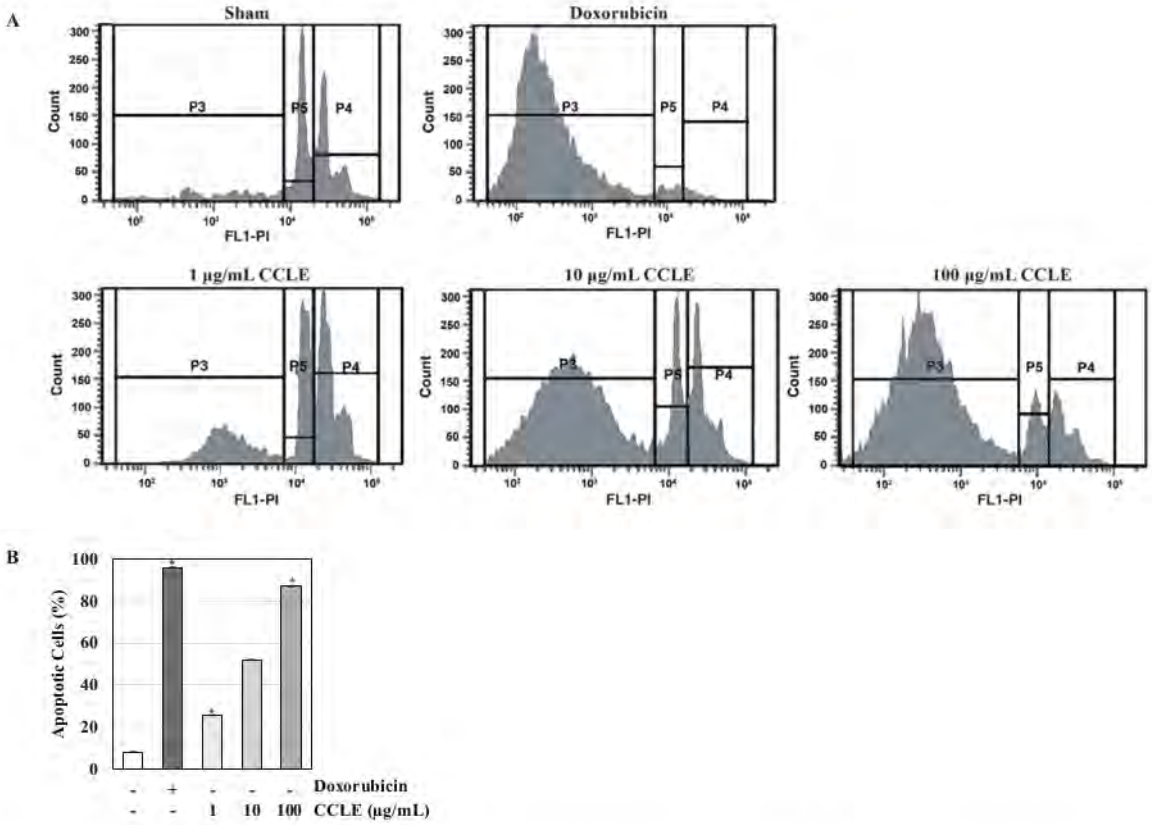

Figure 2. CCLE increased HSC-3 apoptotic cells in concentration-dependent manner. HSC-3 cells were starved for 12 h, then treated with/without 1 µM Doxorubicin or CCLE in various concentrations for 24 h. Apoptotic cells were measured using sub-G1 test as mentioned in Methods. A: The flow cytometric results. 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

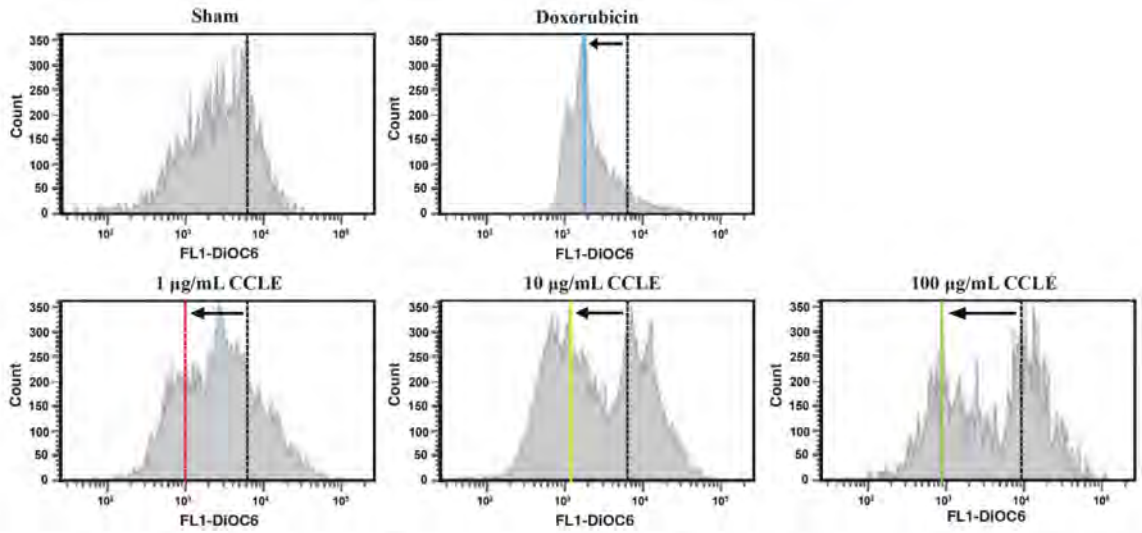

Figure 3. CCLE decreased ΔΨm of HSC-3 cells. HSC-3 cells were starved for 12 h, then treated with/without 1 µM Doxorubicin or CCLE in various concentrations for 12 h. HSC-3 cells were collected, washed, and processed for ΔΨm assay as mentioned in Methods. These experiments were measured in sextuplicate.

cells was 38.39 µg/mL, which was categorized as moderate cytotoxicity (21-200 µg/mL).(21) The IC_{50} of CCLE-induced apoptotic HSC-3 cells was lower than the IC_{50} of CCLE-induced apoptotic T47D cells (344.91 µg/mL) (14), and IC_{50} of CCLE-induced apoptotic HeLa cells (89.90±1.30 µg/mL) (15).

In this study, CCLE-treated group displayed a peak shifment from 104 to 103 (Figure 3). These results showed a ΔΨM, which might cause the release of cytochrome C from mitochondria to the cytosol. Therefore, the ΔΨM is closely correlated with the occurrence of the intrinsic (mitochondria-mediated) apoptotic pathway. Protein 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.(22) ΔΨM has been reported to be associated with Bcl-2 family.(23) 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.(24) Several anti-apoptotic Bcl-2 family

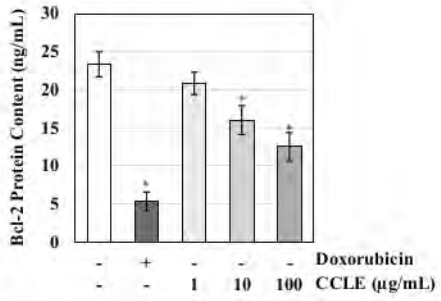

Figure 4. CCLE decreased Bcl-2 protein content of HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 1 µM Doxorubicin or CCLE in various concentrations. Bcl-2 protein content was measured using ELISA as mentioned in methods. *p<0.05 when compared to the sham group.

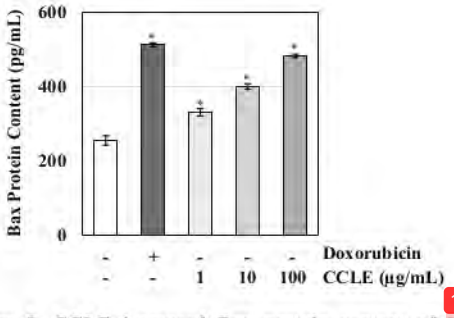

Figure 5. CCLE increased Bax protein content of HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 1 µM Doxorubicin or CCLE in various concentrations. Bax protein content was measured using ELISA as mentioned in methods. *p<0.05 when compared to the sham group.

members 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) ΔΨM 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 ΔΨM 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 µg/mL, indicating moderate cytotoxicity.

Authors Contribution

FS and AYD were involved in concepting 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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Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] M2024176 Editor Decision - Revisions Required (Round 1)

Secretariat of InaBJ <secretariatinabj@gmail.com> Tue, Jun 25, 2024 at 3:36 PM
To: ferry@trisakti.ac.id

Dear Authors,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "***Cosmos caudatus* Leaf Extract Triggers Apoptosis of HSC-3 Cancer Cells by Decreasing Bcl-2 and Increasing Bax**".

Our decision is to: **Revisions Required.**

This manuscript is interesting, however based on the peer-reviewers and editors review results, it needs some revisions before it can be published. Please find the manuscript attached to see detailed comments.

Revise this manuscript thoroughly and according to the suggestions before **June 30, 2024**. Mark/highlighted the revised part of the manuscript, so that the editor will notice the changes. You are also obligated to provide a response letter with your response or the answer to reviewers' questions/comments. For an example on how to write a response letter, we also attach a response form template. Hopefully you find it well.

When you are done, you can upload it in: https://inabj.org/index.php/ibj/author/submissionReview/3137, or simply send us an email of your revised manuscript and response letter.

Please let us know when you have received this email. If you have any questions, do not hesitate to contact us. Thank you for your attention. We wish you a nice day.

Best Regards,
--
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Reviewer	:	R1
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No.	Manuscript Components	Yes	No
1.	Does this manuscript present new ideas or results that have not been previously published?	V	
	Notes: Cosmos caudatus is a traditional medicinal plant used widely for culinary and therapeutic purposes.		
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Manuscript Review Form

Reviewer	:	R2
Manuscript #	:	M2024176
Manuscript Title	:	*Cosmos caudatus* Leaf Extract Triggers Apoptosis of HSC-3 Cancer Cells by Decreasing Bcl-2 and Increasing Bax

No.	Manuscript Components	Yes	No
1.	Does this manuscript present new ideas or results that have not been previously published?	√	
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2.	Are the title and abstract of the manuscript appropriate?	√	
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3	Do the title and abstract reflect the study result/content?	√	
	Notes: Abstract reflect the study result/content, but it needs to be revised according to the comments in the manuscript		
4.	Is the significance of the study well explained at the Background?	√	
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	Notes: HSC-3 cells were placed in 96-well plates ($5x10^3$/well) and treated with/without 1, 10, or 100 µg/mL CCLE or 1 µM Doxorubicin. 1) Why was the CCLE concentration range so high, ie 10 dan 100 µg/mL. 2) Why did not use the same concentration units in CCLE (µg/mL) and Doxorubicin (µM)?		
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	Notes: "For cell viability assay, HSC-3 cells were placed in 96-well plates ($5x10^3$/well) and treated with/without 1, 10, or 100 µg/mL CCLE for 24 hours." Results of the present study showed that number of HSC-3 viable cells in the sham group (9,607±14), and in the CCLE group (8,300±48 when treated with 1 µg/mL CCLE). It is important to explain the other conditions that influence the microenvironment of HSC-3 cells during the experiment, so that the number of viable HSC-3 cells can increase significantly within 24 hours of incubation?		
7.	Are all figures and tables necessarily presented?	√	
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8.	Is there a logical flow of argument in the Discussion which elucidate all the presented/obtained data?		√
	Notes: "Since quercetin was the mainly found flavonoids in the leaves of *C. caudatus*[4], the quercetin could have the potential activity in affecting the Bcl-2 and Bax protein contents." Based on the CCL extraction procedure, a crude CCL extract is produced which not only contains flavonoids. It would be better to discuss the influence of each CCLE substances (flavonoids and phenolic acid) on HSC-3 cell apoptosis.		
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Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] M2024176 Editor Decision - Revisions Required (Round 1)

Ferry Sandra <ferry@trisakti.ac.id> Tue, Jun 25, 2024 at 6:00 PM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>

Dear Secretariat of InaBJ,

Please find the revised version of manuscript "***Cosmos caudatus* Leaf Extract Triggers Apoptosis of HSC-3 Cancer Cells by Decreasing Bcl-2 and Increasing Bax**", as well as the response form for reviewer's comments. Comments from reviewers for the first round of the peer-review have been corrected accordingly.

Thank you.

Regards,
Ferry Sandra
[Quoted text hidden]
--
Ferry Sandra, D.D.S., Ph.D.
Head of Medical Research Center
Universitas Trisakti

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

ABSTRACT

Background: Previous studies have demonstrated that *C. 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. The aim of this study was 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 ($\Delta\Psi m$) was measured using flow cytometry. Bcl-2 and Bax protein content of HSC-3 cells were measured using Enzyme-Linked Immunosorbent Assay.

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 ($\Delta\Psi M$) assay, CCLE-treated group displayed a peak shifment 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

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 *C. 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 7, leading to the initiation of apoptosis.[13]

Previous research has investigated the effects of *C. caudatu*s 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 *C. caudatus* leaf extract 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.

MATERIAL AND METHODS

C. caudatus Leaf Extract (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 (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_2. 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 ($5x10^3$/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 OD_{570}. 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.

Mitochondrial Membrane Potential (ΔΨM) Assay

ΔΨM was conducted in accordance with the previous method.[19,20] Treated cells were collected, resuspended in 20 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$), and incubated for 15 minutes. The ΔΨM measurement was conducted using a FACSCanto II flow cytometer.

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

The Bcl-2 protein content was measured using the Bcl-2 Human ELISA kit (#ab119506, Abcam, Cambridge, United Kingdom) by following the manufacturer's instruction. Briefly, 20 µL sample and 80 µL of sample diluent were put into each well of microplate coated with monoclonal antibody to Bcl-2. Then, 50 µL biotin-conjugate anti-human Bcl-2 monoclonal antibody was added and incubated at room temperature for 2 hours. After washing, 100 µL streptavidin-horseradish peroxidase (HRP) conjugate was added to each well and incubated at room temperature for 1 hour. After washing, 100 µL 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 (#ab199080, Abcam) by following the manufacturer's instruction. Briefly, 50 µL of standard or sample were added into each well of anti-tag coated microplate. Then, 50 µL antibody cocktail was added and incubated at room temperature for 1 hour. After washing, 100 µL 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±32) was significantly lower (Mann-Whitney's *post hoc* test, p=0.004) than the ones in the sham group (9,607±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±48; 4,065±170; and 1,112±50 when treated with 1, 10, and 100 µg/mL CCLE, respectively.

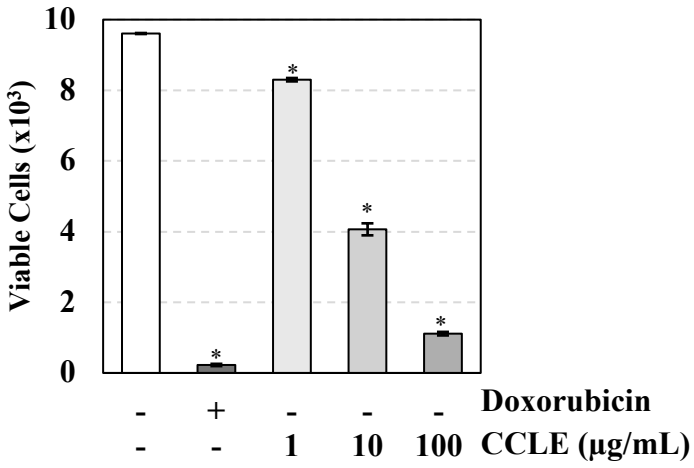

Figure 1. CCLE decreased HSC-3 viable cells in concentration-dependent manner. HSC-3 cells were starved for 12 h, then treated with/without 1 µM Doxorubicin or CCLE in various concentrations for 24 h. Viable cells were measured using MTT assay as mentioned in methods. The data is presented in mean±standard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

CCLE Increased HSC-3 Apoptotic Cells

The percentage of apoptotic HSC-3 cells in doxorubicin group (95.95±2.60%) was significantly higher (Tukey's *post hoc* test, p=0.000) than the ones in the sham group (8.27±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±0.34%, 52.01±1.47%, and 87.07±1.31% when treated with 1, 10, and 100 µg/mL of CCLE, respectively.

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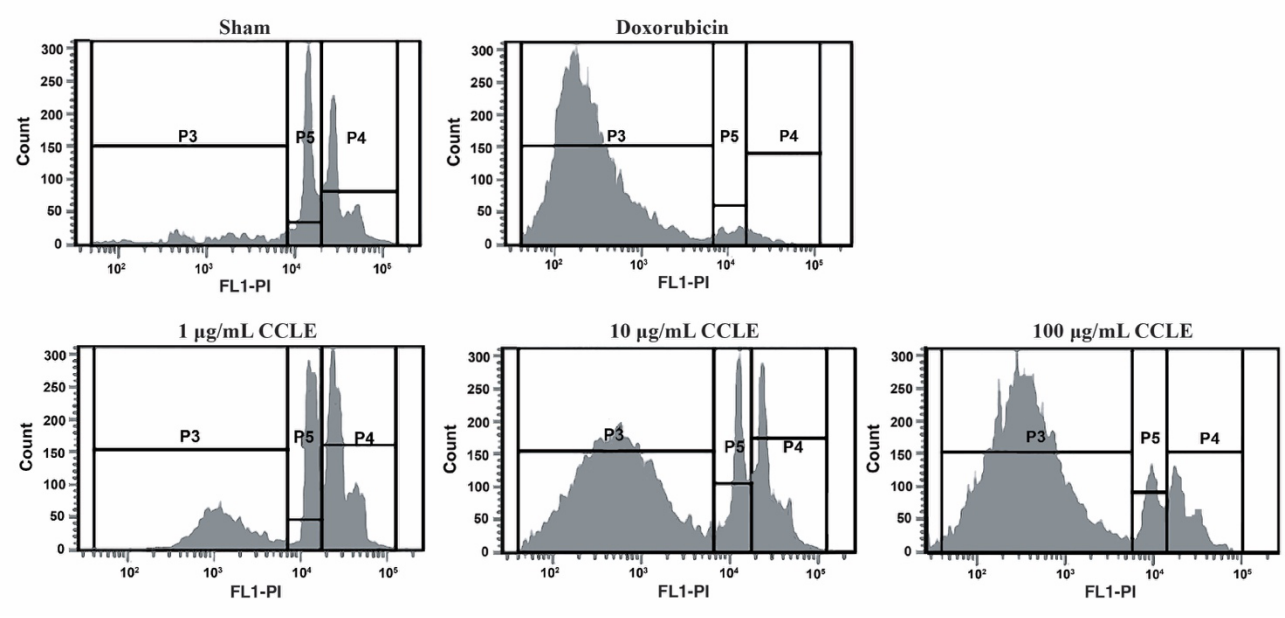

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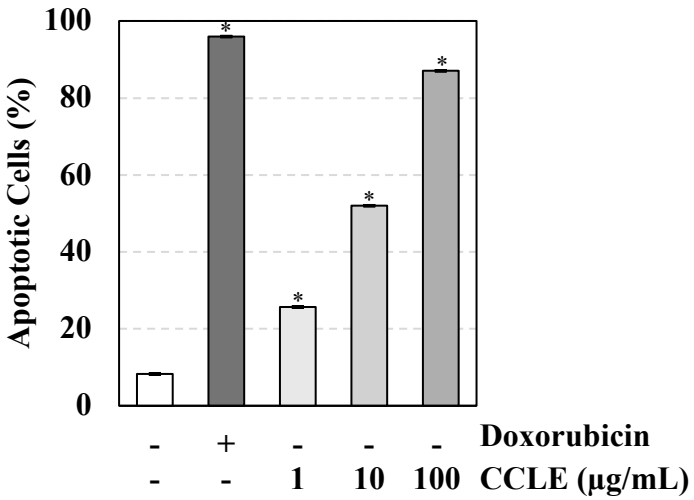

Figure 2. CCLE increased HSC-3 apoptotic cells in concentration-dependent manner. HSC-3 cells were starved for 12 h, then treated with/without 1 µM Doxorubicin or CCLE in various concentrations for 24 h. Apoptotic cells were measured using Sub-G1 test as mentioned in Methods. (A) The flow cytometric 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.

CCLE Decreased ΔΨM

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 ΔΨM, marked by the shifment of the peak from 10^4 to 10^3. A low peak-close-to-10^3 was observed in 1 μg/mL CCLE-treated group (red dashed line). A high peak-close-to-10^3 was observed in 10 μg/mL CCLE-treated group (yellow dashed line). Another high peak-close-to-10^3 was observed in 100 μg/mL CCLE-treated group (green dashed line) as well.

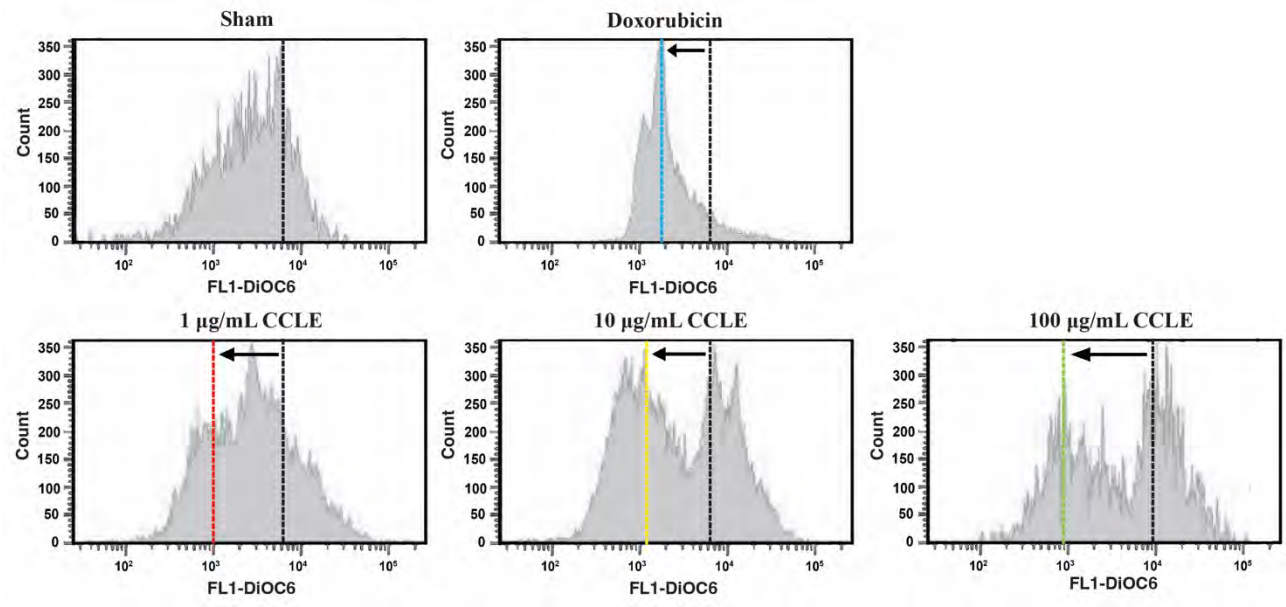

Figure 3. CCLE decreased ΔΨm of HSC-3 cells. HSC-3 cells were starved for 12 h, then treated with/without 1 μM Doxorubicin or CCLE in various concentrations for 12 h. HSC-3 cells were collected, washed, and processed for ΔΨm assay as mentioned in Methods. These experiments were measured in sextuplicate.

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

Bcl-2 protein content of HSC-3 cells in doxorubicin group (5.33±1.21 ng/mL) was significantly lower (Tukey's *post hoc* test, p=0.000) than the ones in 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.

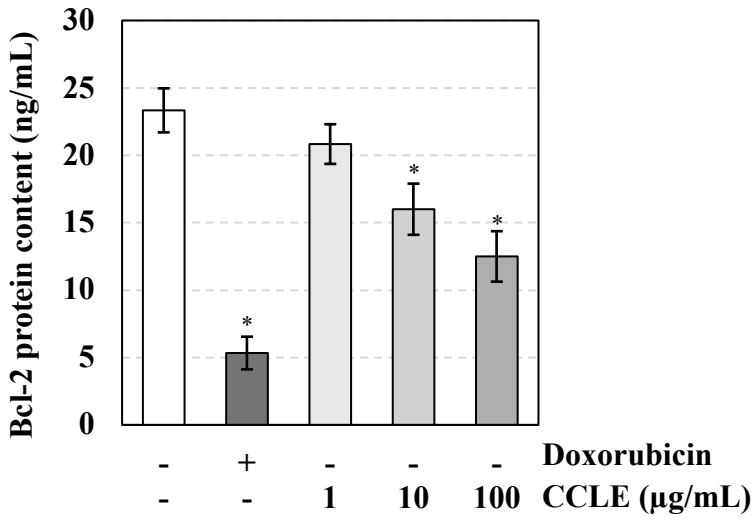

Figure 4. CCLE decreased Bcl-2 protein content of HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 1 μM Doxorubicin or CCLE in various concentrations. Bcl-2 protein content was measured using ELISA as mentioned in methods. *p<0.05 when compared to the sham group.

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.

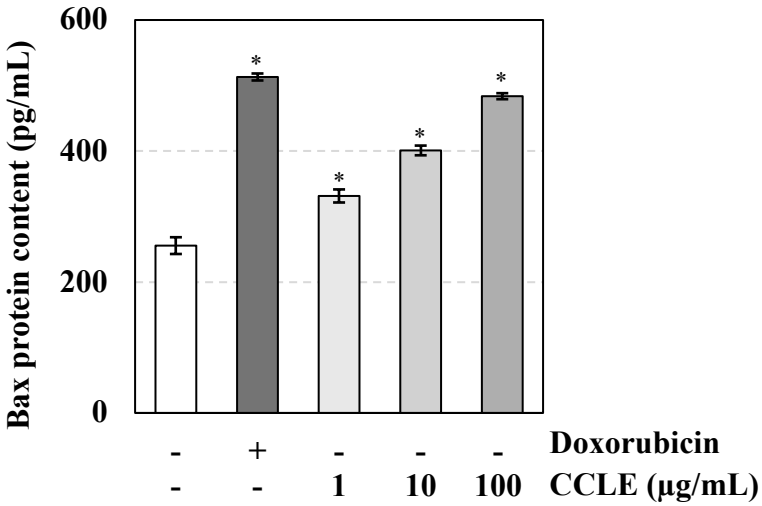

Figure 5. CCLE increased Bax protein content of HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 1 μM Doxorubicin or CCLE in various concentrations. Bax protein content was measured using ELISA as mentioned in methods. *p<0.05 when compared to the sham group.

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 and 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 cells was 38.39 μg/mL, which was categorized as moderate cytotoxicity (21-200 μg/mL).[21] The IC_{50} of CCLE-induced apoptotic HSC-3 cells was lower than the IC_{50} of CCLE-induced apoptotic T47D cells (344.91 μg/mL)[14] and IC_{50} of CCLE-induced apoptotic HeLa cells (89.90±1.30 μg/mL)[15].

In this study, CCLE-treated group displayed a peak shifment from 10^4 to 10^3 (Figure 3). These results showed a ΔΨM, which might cause the release of cytochrome C from mitochondria to the cytosol. Therefore, the ΔΨM 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.[22] ΔΨM has been reported to be associated with Bcl-2 family.[23] 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.[24] Several anti-apoptotic Bcl-2 family members are Bcl-2, Bcl-X_L and Mcl-1, meanwhile pro-apoptotic Bcl-2 family members are Bax, Bak, Bok, Bad, Bid, Bim, Noxa, and puma.[25] ΔΨM 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*[4], 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 ΔΨM 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 µg/mL, indicating moderate cytotoxicity.

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310 acts through mitochondrial- and caspase-3-dependent pathways in human breast cancer
311 MDA-MB-231 cells. Hum Exp Toxicol. 2009; 28: 493-503.

Response Form for Reviewer's Comments

Corresponding Author : Ferry Sandra
Manuscript Code : M2024176
Manuscript Ttile : *Cosmos caudatus* Leaf Extract Triggers Apoptosis of HSC-3 Cancer Cells by Decreasing Bcl-2 and Increasing Bax

Reviewer	Comments (Comments/question from reviewer or editor)	Author's Response (Please write your response regarding the comment here)	Line Number (Please write the line number of the said revision)
R1	The title could be better structured and clearer.	Thank you for the suggestion. After doing an extensive literature study, according to us, the title of this study is already clear and reflects the novelty of the research.	
R2	Abstract reflect the study result/content, but it needs to be revised according to the comments in the manuscript.	Thank you for your suggestion, but we could not find any comment in the manuscript. However we assure you that the abstract is already reflect the study result/content.	
R1	The division of test groups can be explained by scientific reasons (in methods).	Thank you. Actually, there was no scientific reason, because the division of test groups were an exploration.	
R2	HSC-3 cells were placed in 96-well plates (5x103/well) and treated with/without 1, 10, or 100 µg/mL CCLE or 1 µM Doxorubicin. 1) Why was the CCLE concentration range so high, ie 10 dan 100 µg/mL. 2) Why did not use the same concentration units in CCLE (µg/mL) and Doxorubicin (µM)?	1) Well, a wide concentration range for CCLE was chosen to thoroughly explore its effects on HSC-3 cells. High concentration range may help in identifying the optimal concentration at which CCLE may significantly affect HSC-3 cells. Therefore, future studies could be conducted based on the IC_{50} of CCLE. 2) The use of µg/mL for CCLE and µM for Doxorubicin was related to the chemical properties and characteristics. CCLE is a plant extract that contains many active ingredients, while Doxorubicin is a chemotherapy drug that has often been reported in various literature with concentrations in µM, which provide a more consistent representation regarding its pharmacological activity.	
R2	"For cell viability assay, HSC-3 cells were placed in 96-well plates (5x103/well) and treated with/without 1, 10, or 100 µg/mL CCLE for 24 hours." Results of the present study showed that number of HSC-3 viable cells in the sham group (9,607±14), and in the CCLE group (8,300±48 when treated with 1 µg/mL CCLE). It is important to explain the other conditions that influence the microenvironment of HSC-3 cells during the experiment, so that the number of viable HSC-3 cells can increase significantly within 24 hours of incubation?	Yes, actually cancer cells that exhibited rapid growth after 24 hours of incubation were a typical of cells proliferation. The cancer cell culture microenvironment in this study used Dulbecco's modified eagle medium (DMEM) supplemented with fetal bovine serum (FBS). These components provided essential nutrients, growth factors, and hormones for the survival and proliferation of HSC-3 cells. The use of DMEM with FBS as a culture medium is standard practice.	

R1	Need additional discussion of several similar studies (in discussion)	Thank you for the suggestion. Actually, in the discussion section, we have discussed several similar studies, such as the T47D (breast cancer), HeLa (cervical cancer), and MDA-MB-231 cell line (breast cancer).	205, 206, 230, 231
R2	"Since quercetin was the mainly found flavonoids in the leaves of C. caudatus4, the quercetin could have the potential activity in affecting the Bcl-2 and Bax protein contents." Based on the CCLE extraction procedure, a crude CCLE extract is produced which not only contains flavonoids. It would be better to discuss the influence of each CCLE substances (flavonoids and phenolic acid) on HSC-3 cell apoptosis.	Thank you. We agree with you, a crude CCLE extract is produced which not only contains flavonoids, but the CCLE seems to contain an abundance of components. Every component deserves a comprehensive investigation. It will be our future study to explore the phytochemicals of CCLE. Thank you for your suggestion.	
R2	In conclusion, it is important to mention the IC50 value of CCLE-induced apoptotic HSC-3 cells.	Yes, IC_{50} value of CCLE in HSC-3 cells is already added in the conclusion. Thank you for your suggestion.	238, 239

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[InaBJ] M2024176 Editor Decision - Manuscript Accepted

1 message

Secretariat of InaBJ <secretariatinabj@gmail.com> Wed, Jun 26, 2024 at 5:11 PM
To: ferry@trisakti.ac.id

Dear Dr. Ferry Sandra,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "***Cosmos caudatus* Leaf Extract Triggers Apoptosis of HSC-3 Cancer Cells by Decreasing Bcl-2 and Increasing Bax**".

Our decision is to: **Accept Manuscript.**

Your manuscript will be sent to our publisher for typesetting and you should receive the proofreading in due course.

Congratulations on your interesting research, and thank you for allowing us to publish this valuable material. Please let us know once you have read this email. We wish you a nice day.

Best Regards,

--

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