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Original Article

Caesalpinia sappan reduces the stemness of breast cancer stem cells involving the elevation of intracellular reactive oxygen species

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Abstract

Background and purpose: Breast cancer stem cells (BCSCs) as a kind of tumor cells are able to regenerate themselves, leading to apoptosis resistance and cancer relapse. It was reported that BCSCs contain lower levels of reactive oxygen species (ROS) associated with stemness capability. *Caesalpinia sappan* has been proposed for its chemopreventive potency against several cancer cells. This study aimed to evaluate the effects of *Caesalpinia sappan* extract (CSE) on cytotoxicity, apoptosis induction, ROS generation, and stemness markers of MDA-MB-231 and its BCSCs.

Experimental approach: *Caesalpinia sappan* was extracted under maceration with methanol. Magnetic-activated cell sorting was used to isolate BCSCs based on CD44+ and CD24- cell surface expression. The MTT test was used to assess the cytotoxic effects of CSE on MDA-MB-231 and BCSCs. Moreover, flow cytometry was used to examine the cell cycle distribution, apoptosis, ROS level, and CD44/CD24 level. Using qRT-PCR, the gene expression of the stemness markers *NANOG, SOX-2, OCT-4*, and *c-MYC* was assessed.

Findings/Results: We found that MDA-MB-231 contains 80% of the BCSCs population, and CSE showed more potent cytotoxicity toward BCSCs than MDA-MB-231. CSE caused apoptosis in MDA-MB-231 and BCSCs cells by increasing the level of ROS. Furthermore, CSE significantly reduced the MDA-MB-231 stemness marker CD44+/CD24- and the mRNA levels of pluripotent markers of cells in BCSCs.

Conclusion and implications: CSE potentially reduces BCSCs stemness, which may be mediated by the elevation of the ROS levels and reduction of the expression levels of stemness transcription.

Keywords: Cancer stem cell; CD44; CD24; MDA-MB-231; ROS; Stemness marker.

INTRODUCTION

Breast cancer stem cells (BCSCs) are a minority population of tumor cells that can self-renew and initiate tumor growth leading to apoptosis resistance (1–3). There is increasing data supporting the existence of BCSCs in breast cancer cells. BCSCs are accountable for cancer initiation, progression, metastasis, relapse, and therapy resistance (4,5). Targeting BCSCs has been considered a promising strategy for treating cancer (6). BCSCs are identified under the specific marker CD44+/CD24- (7). It has been demonstrated that in mice, CD44+ cells are significantly more tumorigenic than CD44- cells (8). Studies have revealed that CD44+ cancer cells have stem cell-like characteristics. They are able to form mammosphere colonies and participate in tumor development and treatment resistance (9,10).

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Recently, *Caesalpinia sappan* extract (CSE) and the bioactive compounds of brazilin and brazilein have been reported for their potential as chemo preventive agents (11,12). CSE has a cytotoxic effect on several cancer cells. Therefore, CSE has been suggested as a potential anticancer agent with the advantage of low side effects (13–15). CSE was also demonstrated to have strong cytotoxic activity on human epidermal growth receptor-2-positive breast cancer cells by blocking NF- κB activation and reducing MMP2 protein levels (16). These activities are mediated by cell cycle inhibition, apoptosis induction, and migration inhibition (15). CSE induces apoptosis by inhibiting reactive oxygen species (ROS) generation and anti-apoptosis protein expression (11,12,16). Moreover, co-administration of brazilin and brazilein with conventional chemotherapeutic drugs may result in stronger anticancer effects at non-toxic quantities of these substances (16). Considering the significance of BCSCs and the need to specifically target this subset of tumor cells, this study aimed to assess how CSE affected the cytotoxicity, cell cycle distribution, apoptosis induction, and ROS production of MDA-MB-231 and its isolated CD44+/CD24- BCSCs.

MATERIALS AND METHODS

Plant and extraction

The *Caesalpinia sappan* L. heartwood was collected from Tawangmangu, Karanganyar Central Java, Indonesia (Latitude 7°40'39.3"S; Longitude 111°08'09.4"E) in April 2021. Then, the plants were identified and verified by a biologist from the Research Center of Pharmaceutical Ingredients and Traditional Medicine, Indonesia. The heartwood of *Caesalpinia sappan* was dried with a circulation at 40 °C for biological analysis, then further dried in an air oven until entirely dehydrated. Based on Tanvir's study (17) with a few minor modifications, the 500 g of *Caesalpinia sappan* powder was extracted separately using the maceration technique with methanol for 72 h (3 cycles). The solutions were further filtered using Whatman no. 1 filter paper and evaporated at 40 ℃ in an IKA HB 10 basic rotating vacuum evaporator under decreased pressure (100 Psi) to obtain the crude extracts. After freeze-drying the crude extract, a dried extract was produced. For further examination, the dried extracts were collected and stored at 4 ℃.

Cell culture

Dulbecco's Modified Eagle's Medium (DMEM)-high glucose (Gibco, USA) was used to sustain MDA-MB-231 (ECACC #92020424). Meanwhile, DMEM F-12 (Gibco, USA) was used to develop the BCSCs. These media were repeatedly supplemented with 150 μg/mL of streptomycin (Gibco, USA), 10% fetal bovine serum (FBS) (Gibco, USA), and 150 IU/mL of penicillin (Gibco, USA). Cells were grown in 5% CO_2 at the temperature of 37°C. The media were changed every two to three days, and cells were subcultured to reach a confluence of 80-90%. Cells with characteristics such as viability > 90%, a passage number of < 10, and reaching the log growth phase were employed for the tests.

BCSCs isolation and validation

Flow cytometry was used to examine BCSCs in MDA-MB-231 cells. BCSCs were isolated from MDA-MB-231 cells using CD44 and CD24 antibodies coupled to magnetic microbeads (Miltenyi Biotec Inc, CA, USA). BCSCs were defined as the population of cells based on the phenotype of CD44+/CD24-. Using anti-CD44 and anti-CD24-biotin coupled anti-biotin microbeads (Miltenyi Biotec Inc, CA, USA #Catalog: 130-095-194), a magnetic-activated cell sorting (MACS) system was used to separate the BCSCs population based on the cell surface expression of CD44 and CD24. Magnetic separation and low dead columns (Miltenyi Biotec Inc, CA, USA #Catalog: 130-095-194) were used for the selection of positive and negative, respectively. Utilizing anti-CD44-FITC and anti-CD24-PE monoclonal antibodies, flow cytometry (BD Biosciences, Franklin Lakes, New Jersey) was used to validate the phenotype of CD44+/CD24-. In addition, the BCSCs population was also confirmed by mammosphere capability assay. Mammospheres derived from the BCSCs were planted into an ultra-low attachment well plate as much as 1×10^5 cells/mL. The number of cell collections (diameter > 50 μm) for each well was morphologically evaluated under a microscope on days 0, 3, and 7.

Cell viability assay

Using a slightly modified version of the 3-(4,5-dimethyl-2- thiazolyl)-2,5- diphenyl -2H-tetrazolium bromide (MTT) test (18,19), the experiment of cell viability was conducted. In brief, a 96-well plate was seeded at a density of 5×10^3 cells per well and incubated for 24 h at 37°C and 5% CO2. After that, cells were exposed for 24 h by *CSE* (5-500 µg/mL) in triple. Cells that were not treated by the extract were assigned as negative control. Next, cells were cultured for an additional 4 h by 0.5 mg/mL of MTT (Biovision, #Cat K299-1000) dissolved in 100 µL of DMSO and incubated for 15 min. After 4 h a ELISA reader (Biorad iMarkTM Microplate Reader) was used to measure the absorbance at a wavelength of 595 nm. The treated group was compared with the untreated group to convert the absorbance into a percentage of cell viability. The IC_{50} value was calculated using linear regression with an equation of y = Bx + A (x and y represent the concentration and the percentage of viable cells, respectively). The concentration that inhibited 50% of cell growth was defined as the IC_{50} value using the graph's linear equation for the value of y = 50. Three independent replication experiments were performed to evaluate the data.

Cell cycle analysis

MDA-MB-231 cells and BCSCs were seeded individually in 6-well plates at a density of 2×10^5 cells/well for growth in DMEM-high glucose and DMEM F-12 supplemented with 10% FBS, respectively. The CSE was added to the culture medium of cells at concentrations of 5, 10, and 20 µg/mL for 24 h. The cells were harvested using EDTA-free trypsin, washed twice with pre-cooled phosphate buffer saline (PBS), gently added pre-cooled methanol, vortexed, and then left at 4°C for 15 min. Next, the BD Cycletest reagent (BD Biosciences, USA) including 100 µL of propidium iodide (PI) (50 µg/mL) and 10 µL of RNAse A (50 µg/mL) was added to the mixture. Then, each tube gently was mixed and incubated at room temperature in the dark for 30 min. The percentage of cell distribution was calculated using flow cytometry (BD Accuri C6 plus, BD Biosciences, USA). Moreover, the experiment was repeated three times (20).

Apoptosis analysis

To evaluate the effect of dried CSE on apoptosis in MDA-MB-231 cells and BCSCs (20,21) flow cytometry with annexin V/PI staining was utilized. After seeding a 6-well plate with cells (2×10^5 cells/well) and incubating at the temperature of 37 °C adherent cells were exposed to concentrations of 5, 10, and 20 µg/mL of CSE for 24 h, while the control well received a 0.1% DMSO. The cells were harvested and rinsed twice using 1× cooled PBS. The cells were then collected and re-dissolved in a cold 1× binding buffer. Afterward, annexin V and PI (BD Bioscience, #Cat556547) were added and then incubated at room temperature in the dark for 10 min. The assay was performed in three independent experiments, and the data were analyzed in a BD Accuri C6 (BD Biosciences, Franklin Lakes, New Jersey). The percentage of cell death including early apoptosis, late apoptosis, and necrosis was displayed in a bar graph.

ROS level analysis

MDA-MB-231 cells and BCSCs were grown individually in 24-well plates at 1×10^5 cells/well density. The culture media of DMEM-high glucose and DMEM F-12 supplemented with 10% FBS were used for MDA-MB-231 and BCSCs, respectively. The cells were collected through centrifugation, washed with PBS, and then treated with 2′, 7′-dichlorofluorescein diacetate (DCFDA) of 2.5 M in supplemented buffer (10% FBS in PBS) at the temperature of 37°C in the dark for 30 min. The CSE was applied at concentrations of 5, 10, and 20 µg/mL for MDA-MB-231 cells and 1.375, 2.75, and 5.5 µg/mL for BCSCs. The cells were then incubated for 4 h at 37 °C and 5% CO2. In the assay, doxorubicin was utilized as a positive control because of its capacity to increase ROS levels, as was previously documented (15,19,20). Finally, the levels of intracellular ROS were determined by flow cytometry (22).

Stemness marker CD44+/CD24- expression

Flow cytometry was used to determine the effect of *Caesalpinia sappan* on the stemness marker expression of CD44+ and CD24-. After seeding cells into a 6-well plate (2×10^5 cells/well) and incubating at 37 °C, the adherent cells received *CSE* for 24 h, whereas the control well-received 0.1% DMSO. After harvesting the cells fluorescently-conjugated antibodies including anti-CD24-PE (clone ML5; BD Biosciences, Mississauga, ON, Canada) and anti-CD44-FITC (clone IM7; BD Biosciences) were added to the mixture. The BD Accuri C6 flow cytometer was used to evaluate the cells. The fraction of cells with the phenotype of CD44+/CD24- was used to show the data (Fig. 1A).

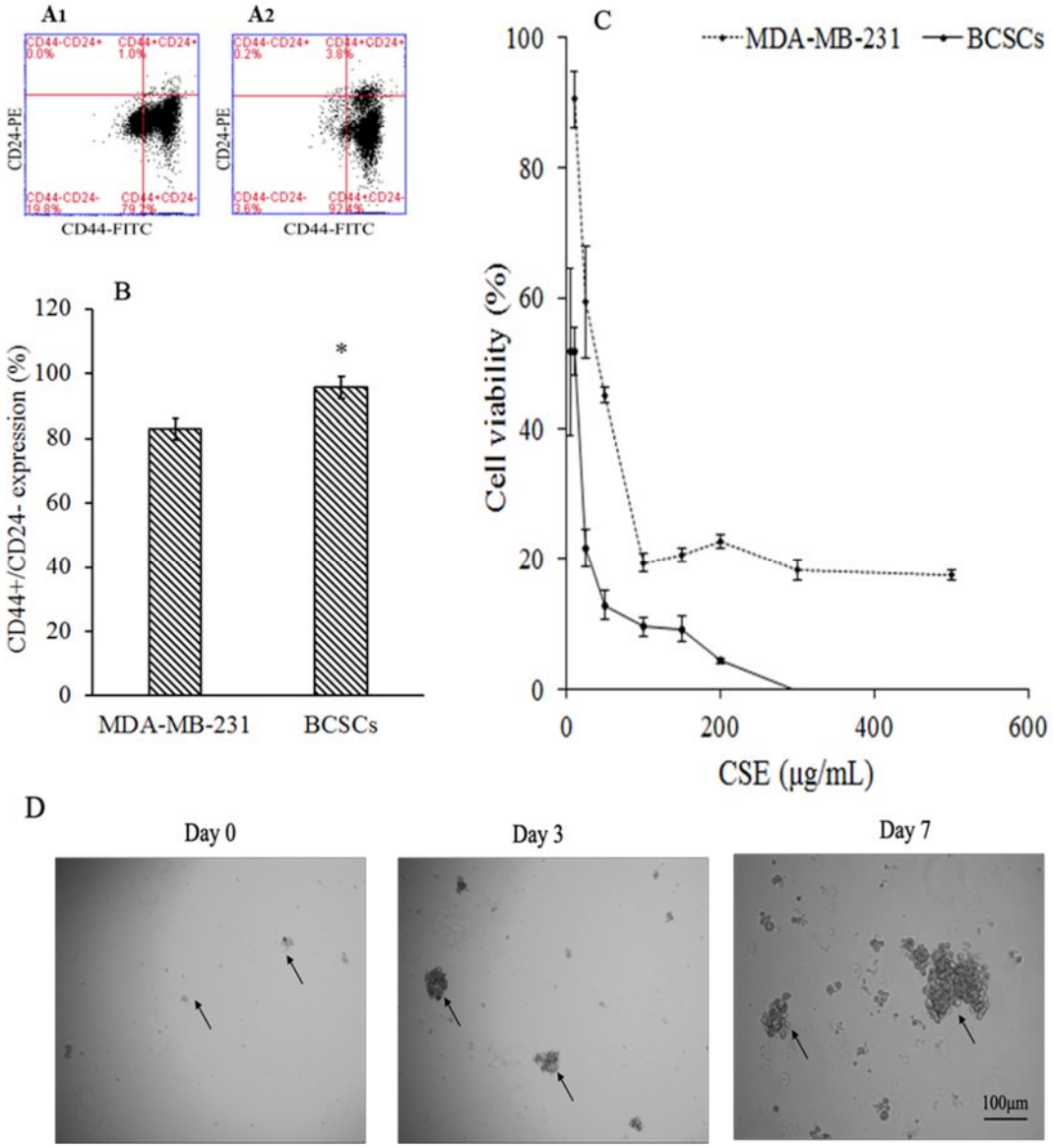


Fig. 1. Stemness marker CD44+/CD24- expression. (A_1) and (A_2) Characterization of BCSCs by surface markers CD44+/CD24- under flow cytometry detection; (B) the percentage of the surface marker on MDA-MB-231 cells. and BCSCs; (C) cytotoxic effect of CSE on MDA-MB-231 cells and BCSCs for 24 h; (D) representative images of mammosphere formation assay on days 0, 3, and 7 after CD44+/CD24- isolation (100× magnification). The bar and graph represent the mean ± SD of three independent experiments with at least 3 replicates. **P* < 0.05 represents a significant difference with MDA-MB-231 cells. BCSCs, Breast cancer stem cells; CSE, *Caesalpinia sappan* extract.

Nanog homeobox (NANOG), SRY-box transcription factor 2 (SOX2), Octamer-binding transcription factor-4 (OCT4), and c-MYC proto-oncogene (c-MYC) gene expression analysis

The total RNA derived from the cell culture of BCSCs was extracted using TRIzol in accordance with the manufacturer's instructions (Invitrogen, Shanghai, China). The first-stranded cDNA was created using Super-Script II (Invitrogen, Massachusetts, USA) and 1 ng of total RNA. In real-time PCR equipment (PCR max Eco 48), SYBR No-ROX Green I dye (SMOBIO Technology Corp, Hsinchu, Taiwan) was used for reverse transcription. The mRNA levels of the *NANOG, SOX2, OCT4*, and *c-MYC* genes were then analyzed using the corresponding primers (Table 1). The thermocycler was set up under the following conditions: a 10 min initial step at 95 °C, 50 cycles at 95 °C, 15 s for each cycle, and 1 min at 60 °C. The cycle threshold (Ct) was used to record the gene expression. Data were obtained using Eco Software v5.0 (Illumina Inc, San Diego, CA, USA), and analyzed by the method of $2^{-\Delta\Delta}$ Ct method (Livak method). Also, each reaction was carried out three times.

***Phytochemical screening of* CSE**

In this study, the CSE was qualitatively analyzed for the presence of flavonoids, alkaloids, tannins, steroids, terpenoids, and saponins. The flavonoids were tested using Wilstater's test as described in the previous studies (23,24). Furthermore, the tannin presence was examined whether the black or blue color was performed after reacting the *CSE* with 1% ferric chloride (25). Both terpenoids and steroids were detected using the Liebermann-Burchard test. In brief, the dried CSE (100 mg) was dissolved in chloroform and shaken. After adding a few drops of acetic anhydride, the mixture was indirectly boiled and cooled, quickly. Then, the mixture was added with 2 mL of concentrated H_2SO_4. Triterpenoids were characterized by the emergence of deep red, whereas steroids were distinguished by forming a brown ring at the intersection of two layers, and the upper layer became green (26). For detecting the presence of saponins 500 mg of CSE was mixed with 10 mL of distilled water. After shaking the mixture was heated in a water bath for five min. The presence of saponins was confirmed by continuing the formation of foam (26).

Total flavonoid content of CSE

The aluminium chloride colorimetric technique (27) with modification was utilized to estimate the total flavonoid concentration using quercetin as the reference. A calibration curve for quercetin was produced in the range of 200-700 µg/mL. In brief, test tubes, each containing 0.5 mL of extract or standard, were prepared. Each of the following was added and blended: 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, 1.5 mL of 80% methanol, and 2.8 mL of distilled water.

A similar procedure was conducted to prepare the blank, wherein 0.5 mL of distilled water was used to substitute the sample or standard, and a specific quantity of aluminium chloride was replaced with water. All tubes were incubated at room temperature for 30 min, and the absorbance was read at the wavelength of 415 nm. The flavonoid content was reported as the milligrams of quercetin equivalent (QE)/gram of dried extract (28).

Total phenolic content of CSE

The Folin-Ciocalteu method was used to calculate the total phenolic content. Briefly, 1.0 mL of Folin-Ciocalteu reagent (Merck) and 100 µL of the sample or standard were mixed. After 5 min 1.0 mL of Na_2CO_3 (Merck) (7.5% w/v) was added to the mixture. The mixture was then incubated at room temperature in the dark for 90 min. Spectrophotometry at λ = 725 nm was used to read the absorbance. Gallic acid (Sigma Aldrich) was assigned as the standard, and its standard curve was applied to calculate the total phenolic content. The total phenolic content was expressed as the milligrams of gallic acid equivalents (GAE)/gram of dried extract (29).

Table 1. Primer for qRT-PCR reaction.

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
OCT4	TATGCACAACGAGAGGATTTTG	TGTGTCCCAGGCTTCTTTATTT
SOX2	CACCTACAGCATGTCCTACTCG	GGTTTTCTCCATGCTGTTTCTT
NANOG	TCAATGATAGATTTCAGAGACAG	GGGTAGGTAGGTGCTGAGGC
c-MYC	CTGAGACAGATCAGCAACAACC	TCTTTTATGCCCAAGTCCAAT

Statistical analysis

Statistical analysis was accomplished by the software of SPSS 26.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± SD, n = 3; and analyzed using One-way ANOVA followed by the Least Significant Difference (LSD) test. *P*-value ≤ 0.05 was considered statistically significant.

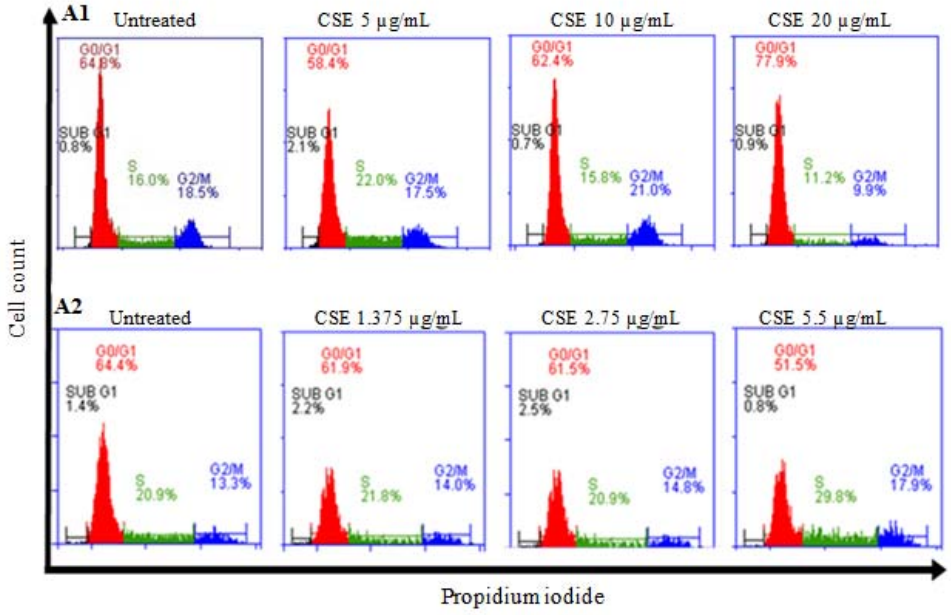
RESULTS

CD44+/CD24- expression profile in BCSCs isolated from MDA-MB-231 and cytotoxicity of CSE against MDA-MB-231 cells and BCSCs

The BCSCs population was isolated from MDA-MB-231 cells by magnetic cell sorting based on the expression of CD44+/CD24-. The purities of the isolated BCSCs and MDA-MB-231 were 95.63 ± 3.47%, and 82.93 ± 3.23%, respectively, expressing CD44 and the lack of CD24 expression (Fig. 1A and B). The high levels of CD44 expression have been linked to cancer development, while the low levels of CD24 expression have been linked to non-differentiated cells (7). The cytotoxic effect of CSE on MDA-MB-231 and BCSCs was analyzed using an MTT assay. Various concentrations of CSE were used for 24 h treatment. In the presence of CSE, the growth of BCSCs was inhibited in a dose-dependent manner with IC_{50} value on MDA-MB-231 and BCSCs of 40 µg/mL and 11 µg/mL, respectively (Fig. 1C). In the mammosphere formation assay, the BCSCs population was shown to induce mammospheres formation *in vitro* in a time-dependent manner (Fig. 1D).

CSE-induced cell cycle arrest on MDA-MB-231 and BCSCs

The effect of CSE on cell cycle distribution in MDA-MB-231 and BCSCs was investigated to explore the mechanism behind CSE cytotoxicity. In MDA-MB-231, 0.5 ± 0.26% of untreated cells were in the Sub G1 phase, 66.00 ± 1.06% in the G0/G1 phase, 15.93 ± 0.06% in the S phase, 17.63 ± 0.81 in the G2/M phase. In untreated BCSCs, 1.23 ± 0.20% were in the Sub G1 phase, 63.56 ± 0.95% in G0/G1 phase, 21.39 ± 0.42% in the S phase, and 13.81 ± 0.70% in the G2/M phase (Fig. 2A). CSE induced the accumulation of MDA-MB-231 in the G0/G1 phase in a dose-dependent manner ($P < 0.05$; Fig. 2B). CSE caused cell cycle arrest in BCSCs at the S and G2/M phases (Fig. 2C).



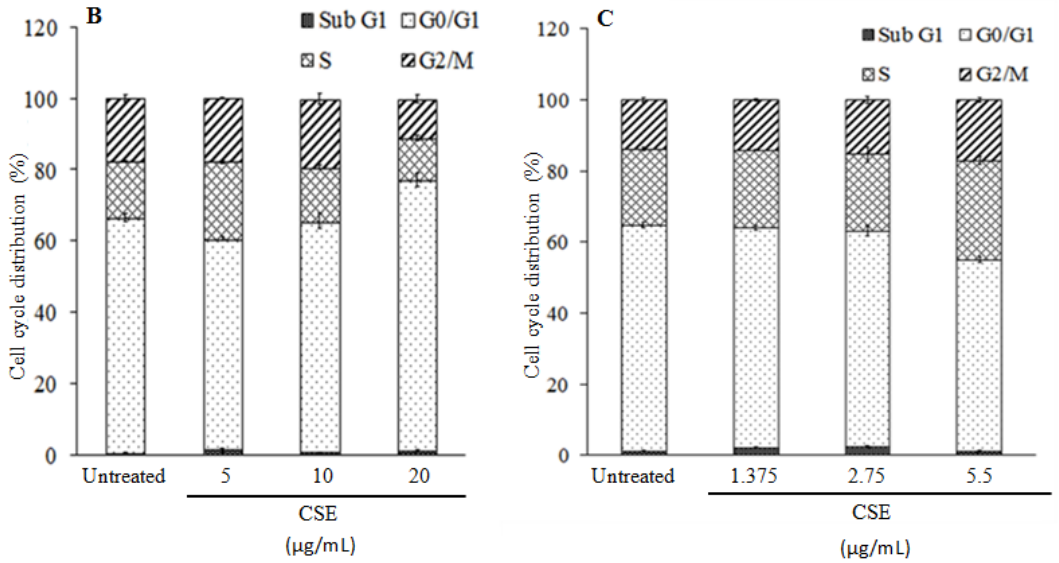


Fig. 2. Cell cycle distribution of CSE on MDA-MB-231 and BCSCs cells. (A_1) and (A_2) The flow cytometry profile of the treatments.in cells of MDA-MB-231 and BCSCs, respectively; (B) and (C) the percentage cell cycle distribution of each phase under different concentration treatments of CSE for 24 h on MDA-MB-231 cells and BCSCs, respectively. The columns represent the mean ± SD of three independent experiments with at least 3 replicates. BCSCs, Breast cancer stem cells; CSE, *Caesalpinia sappan* extract.

CSE increased apoptosis event of MDA-MB-231 and BCSCs

The effect of CSE was examined on cell apoptosis using flow cytometry analysis with annexin V-PI staining (Fig. 3A). Apoptotic cells were ranged from 9.00 ± 0.95% to 28.63 ± 2.71% in MDA-MB-231 (Fig. 3B) and ranged from 9.43 ± 0.64% to 13.63 ± 1.26% in BCSCs (Fig. 3C). The treatment of MDA-MB-231 and BCSCs with CSE significantly increased the apoptotic cells compared with untreated cells ($P < 0.05$). Furthermore, CSE induced lower apoptosis in the BCSCs than MDA-MB-231. This phenomenon might be triggered by the mechanism of apoptosis resistance in BCSCs.

CSE elevated ROS generation on MDA-MB-231 and BCSCs

Cell viability loss may be triggered by the elevation of ROS. ROS analysis was conducted under the DCFDA staining by fluorescence-activated cell sorting analysis to assess the effect of CSE on intracellular ROS production (Fig. 4A). Doxorubicin, as the positive control, elevated ROS levels in both MDA-MB-231 (Fig. 4B) and BCSCs (Fig. 4C) than untreated cells with higher levels in the BCSCs. A similar trend was observed in the cells treated by CSE. At the same time, CSE increased ROS generation significantly ($P < 0.05$) in both MDA-MB-231 and BCSCs cells in a dose-dependent manner than untreated cells, the ROS levels increased more in BCSCs. These results showed that BCSCs are more sensitive to doxorubicin and CSE treatment, which caused ROS levels elevation.

The effect of CSE on CD44 and CD24 on MDA-MB-231 cells and BCSCs

Recent research has shown that CD44 functions as a signal regulator and an adhesion molecule (7-10). By examining the surface markers CD44+ and CD24-, it was attempted to determine that CSE could have a role in decreasing the stemness of MDA-MB-231 and BCSCs. In the present study, the effect of CSE on CD44 and CD24 was analyzed by flow cytometry (Fig. 5A). CSE with dose of 20 µg/mL decreased the percentage of CD44+/CD24- subpopulations in MDA-MB-231 cells, significantly ($P < 0.05$, Fig. 5B). However, CSE could not decrease considerably CD44+/CD24- subpopulations in BCSCs (Fig. 5C). These results showed that CSE reduced the stemness marker of MDA-MB-231 cells.

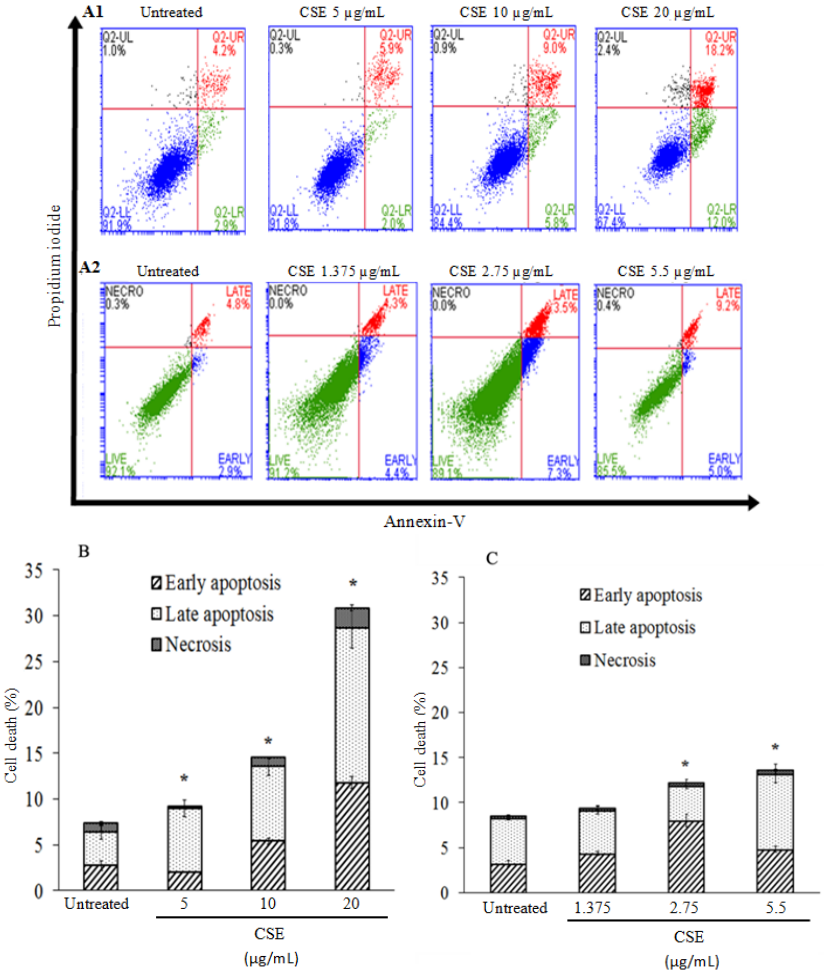
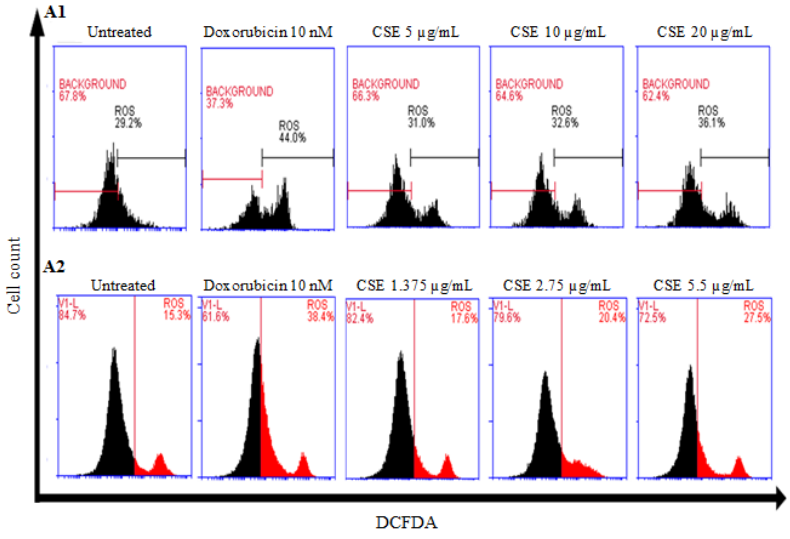


Fig. 3. CSE-induced apoptosis of MDA-MB-231 cells and BCSCs. (A_1) and (A_2) Flow cytometry-in MDA-MB-231 cells and BCSCs, respectively; (B) and (C) The quantification of percentage apoptotic cells under CSE treatment on MDA-MB-231 cells and BCSCs, respectively. The columns represent the mean ± SD of three independent experiments with at least 3 replicates. **P* < 0.05 represents a significant difference with the untreated group. BCSCs, Breast cancer stem cells; CSE, *Caesalpinia sappan* extract.



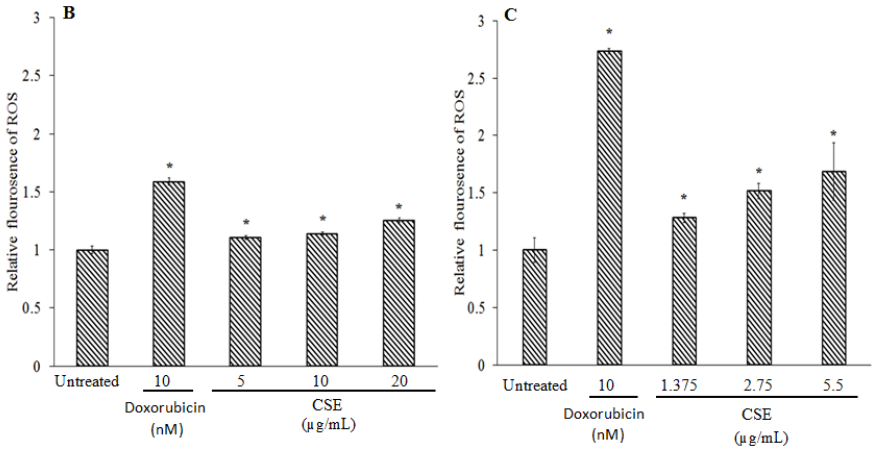


Fig. 4. The effect of CSE-induced ROS generation in MDA-MB-231 and BCSCs cells. (A_1) and (A_2) ROS generation was determined by fluorescence microscopy and FACS flow cytometer after DCFDA staining in MDA-MB-231 and BCSCs cells, respectively; (B) and (C) The percentage of DCFDA stained cell population converted to the fold of change in MDA-MB-231 cells and BCSCs, respectively. The columns represent the mean ± SD of three independent experiments with at least 3 replicates. *$P < 0.05$ represents a significant difference with the untreated group. BCSCs, Breast cancer stem cells; CSE, *Caesalpinia sappan* extract; DCFDA, 2′, 7′-dichlorofluorescein diacetate; ROS, reactive oxygen species; FACS, fluorescence activated cell sorting.

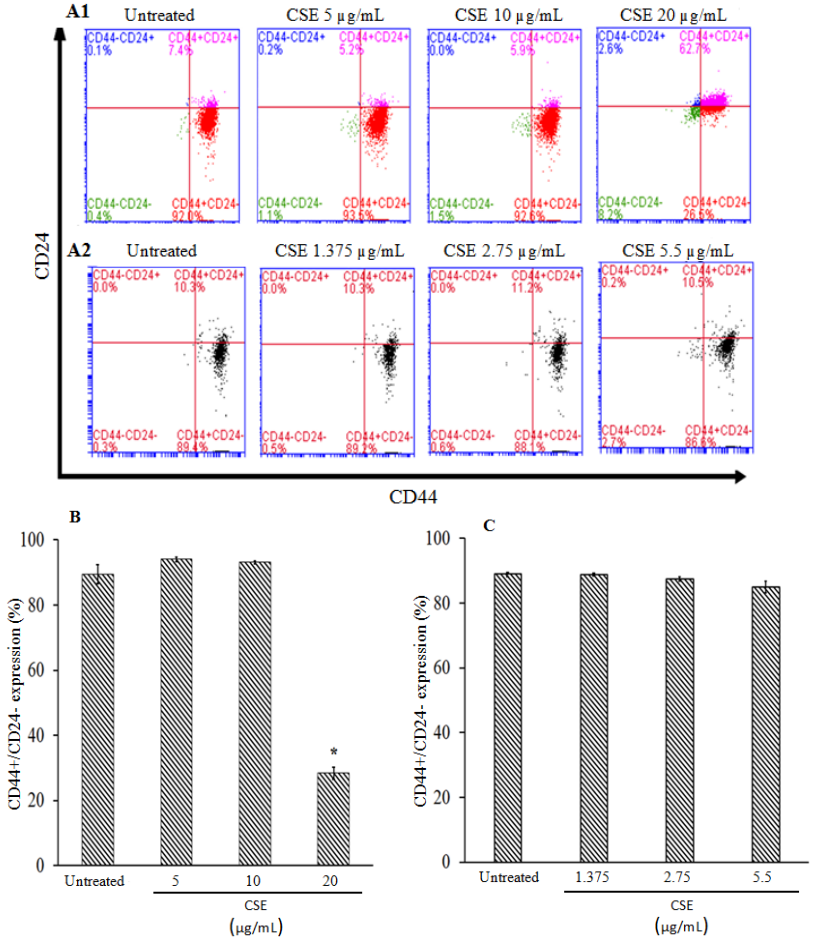


Fig 5. Analysis of CD44 and CD24 surface markers under CSE treatment for 24 h. (A_1) and (A_2) Dot-plot determined by flow cytometry.in MDA-MB-231 cells and BCSCs, respectively; (B) and (C) The percentage of expression CD44+/CD24- subpopulations in MDA-MB-231 cells and BCSCs, respectively. The columns represent the mean ± SD of three independent experiments with at least 3 replicates. *$P < 0.05$ represents a significant difference with the untreated group. BCSCs, Breast cancer stem cells; CSE, *Caesalpinia sappan* extract.

The effect of CSE on *NANOG, OCT4, SOX2,* and *c-MYC* on BCSCs

OCT4, SOX2, c-MYC, and NANOG are proteins from the core pluripotency complex of stem-like cells expressed by BCSCs. The proteins are positively connected with the growth of BCSCs. The expression *of NANOG, SOX2, OCT4,* and *c-MYC* genes decreased in BCSCs after CSE treatment at 1/2 IC_{50}, considerably ($P < 0.05$) (Fig. 6A-D). Although the levels *of SOX-2* and *OCT4* were enhanced by CSE treatment at low concentrations (1/8 IC_{50}), the effect of CSE on the mRNA expression of those transcription factors was dose-dependent. The findings suggest that these genes have a role in the cytotoxicity of CSE on BCSCs.

Phytochemical Screening of the CSE

The phytochemical analysis of the crude methanolic CSE indicated the presence of several secondary metabolites, including flavonoids, tannins, saponins, and terpenoids (Table 2). There were large quantities of flavonoid (203.95 ± 7.79 mg QE/g) and phenol (575.69 ± 12.42 mg GA/g) in CSE.

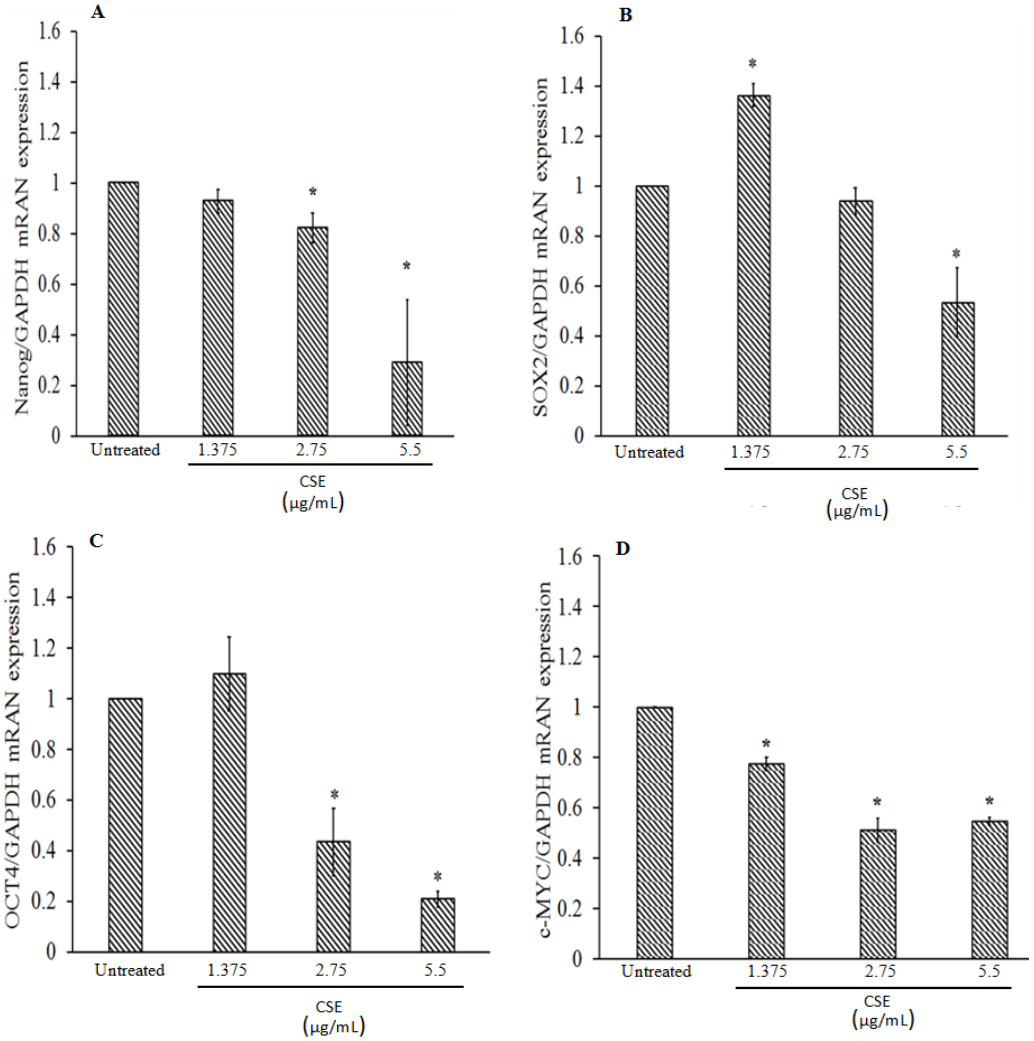


Fig. 6. The gene expression of stemness markers *OCT4, SOX2, NANOG,* and *c-MYC* in BCSCs. qRT-PCR analysis of the expressions of (A) *NANOG*, (B) *SOX2*, (C) *OCT4*, and (D) *c-MYC* in BCSCs cells. Total RNA was extracted and used for reverse transcription and qRT-PCR analysis, as mentioned in materials and methods. Data are expressed as the average ± SD of results obtained from three independent experiments. *$P < 0.05$ represents a significant difference from than untreated group. BCSCs, Breast cancer stem cells; CSE, *Caesalpinia sappan* extract; NANOG, Nanog homeobox; OCT4, octamer-binding transcription factor-4; SOX2, SRY-box transcription factor 2; c-MYC, c-MYC proto-oncogene.

Table 2. Phytochemical screening of *Caesalpinia sappan* extract.

Chemical component	Name of the test	*Caesalpinia sappan*	Total flavonoid (mg QE/g)	Total phenolic (mg GA/g)
Alkaloids	Wagner test	-		
Flavonoids	Wilstater test	+		
Tannins	Braemer's test	+	203.95 ± 7.79	575.69 ± 12.42
Saponins	Forth test	+		
Steroids	Lieberman Burchardt test	-		
Terpenoids	Lieberman Burchardt test	+		

DISCUSSION

The current study investigated the effectiveness of CSE against the population of BCSCs isolated from MDA-MB-231 (cell line of human triple-negative breast cancer (TNBC)). TNBC cell is a subtype of breast cancer characterized as highly aggressive and poorly differentiated (30). The BCSCs, on the other hand, are assumed to be responsible for cancer recurrence and breast cancer resistance to chemotherapy or radiation (30). TNBC cell proliferation and migration have been reported to be inhibited by *Caesalpinia sappan* (15). Therefore, it is interested in exploring the effect of *Caesalpinia sappan* on the population of BCSCs and the stemness characteristics of the cells. According to studies, CD44+ cells displaying a more mesenchymal-like profile frequently enriched in cell proliferation, motility, and angiogenesis genes, and develop tumors in mice (8). In this study, the population of stem cells increased using a magnetic cell sorter with $CD44^+/CD24^-$ as a marker. Based on the sorting, it was revealed that MDA-MB-231 contains 80% of the BCSCs population. After sorting, we got almost 100% of the BCSCs population. This result indicated that most of the population of MDA-MB-231 cells is BCSCs. Interestingly, CSE cytotoxicity toward the BCSCs population was one-fourth of the TNBC (MDA-MB-231) (Fig. 1C), indicating that BCSCs were more sensitive to CSE than MDA-MB-231. The data led to exploring the profile of CSE cytotoxic activity on the BCSCs. When exposed to CSE up to 5.5 µg/mL, BCSCs arrested at the phases of S and G2/M (Fig. 2C), dose-dependently, and apoptosis was increased, significantly (Fig. 3C). However, 20 µg/mL of CSE (IC_{50}) halted the progression of MDA-MB-231cells at G0/G1 phase and induced more apoptosis compared with untreated cells, indicating that MDA-MB-231 and BCSCs gave a slightly different profile of cell cycle upon CSE treatment. Also, it was demonstrated that BCSCs are more resistant to apoptosis. Moreover, the more sensitive BCSCs viability upon CSE treatment compared to MDA-MB-231 (Fig. 1) did not correspond with the induction of apoptotic cells (Fig. 3), indicating a different mechanism for the cytotoxic effect of CSE against BCSCs. In normal epithelial Vero and CHO-K1 cells, the ethanolic CSE did not cause cytotoxicity when treated up to 50 µg/mL, according to our earlier investigation (31).

Recently, ROS have been targeted due to their role in cancer progression. ROS are physiologically generated from metabolism activities. Therefore, over-proliferated cells like cancer cells generated higher levels of ROS (moderate levels compared to the low level of the normal cells) (32,33). Cancer cells have mechanisms to shift the redox balance to maintain a high level of ROS, such as overexpressing ROS metabolic enzymes (34). On the other hand, cancer stem cells (CSCs) need fewer ROS to keep becoming stem cells (32). The low level of ROS is achieved by keeping ROS production low or enhancing the ROS scavenging system (34). The present study revealed that BCSCs were more sensitive than MDA-MB-231 in the enhancement of the levels of ROS upon treatment of 10 nM doxorubicin, with a 1.6-fold in MDA-MB-231 whereas in BCSCs was 2.7 fold compared to the untreated cells. Moreover, CSE significantly enhanced the ROS levels in MDA-MB-231 cells and the BCSCs (Fig. 4B-C). Similar to doxorubicin treatment, the treatment of CSE at the doses of 5, 10, and 20 µg/mL increased the levels of ROS in BCSCs more than the ones in MDA-MB-231. Increased levels of ROS in CSCs induce cell differentiation, senescence, and apoptosis (35). Although the elevated levels of ROS were lower than the positive control,

doxorubicin, this result explained one of the mechanisms of CSE exerting higher cytotoxicity toward BCSCs over MDA-MB-231. The possibility of CSE in inducing BCSCs differentiation is intriguing to explore. Previously, it was reported that CSE decreased the levels of ROS in murine mammary carcinoma 4T1 cells (4T1) (36). Based on the current study, it was observed the opposite effect of CSE on the cellular levels of ROS in TNBC (MDA-MB-231) and BCSCs. This result may be caused by different characteristics between the two cell lines regarding the CD44 and CD24 expression, in which both proteins are highly expressed in the 4T1 cells (37). In contrast, only CD44 was highly expressed and lacked CD24 expression in the MDA-MB-231 cells (Fig. 1A). Bensimon *et al.* reported that the knockdown of CD24 in CD24+ cells leads to a decrease in cell proliferation rate, the levels of ROS and genomic instability (38). On the contrary, artificial CD24 expression in CD24- cells showed increased levels of those factors (38). Several studies have reported the scavenging mechanisms of ROS in cancer cells and CSCs (35,39). Ding *et al* reported that the upregulation of GST in CSCs contributes to maintaining the low level of ROS (34). Moreover, Larasati *et al*. demonstrated that curcumin binds with ROS-scavenging enzymes, including glutathione-S-transferase *phi* 1, carbonyl reductase 1, glyoxalase 1, and NAD(P)H dehydrogenase [quinone] 1 resulted in elevating the levels of ROS in leukemic cells, K562 (39). The mechanisms of how CSE increases the levels of ROS in MDA-MB-231 and BCSCs may be similar to curcumin. However, it requires further examinations to elucidate.

This study hypothesized that the viability of BCSCs upon treatment of CSE reduced because cells prepared to differentiate; therefore, the present study explored how CSE affects the stemness of the MDA-MB-231 by looking at several stemness markers including CD44+/CD24-, NANOG, SOX2, OCT4, and c-MYC expression. The present study revealed a significant decrease of the CD44+/CD24- in the cells of MDA-MB-231 but not in the BCSCs upon treatment of 1/2 IC_{50} CSE (Fig. 5B and C). Interestingly, the mRNA level of *NANOG, SOX2, OCT4, and c-MYC* decreased in the CSE-treated BCSCs, significantly (Fig. 6A-D). NANOG, SOX2, OCT4, and c-MYC are transcription factors that contribute to the pluripotency of the cells, and their expression elevates in human cancer cells such as breast cancer cells (6,40). Their expression is associated with the increasing stemness of cancer cells, poor differentiation, and decreased apoptosis (40). Therefore, the reduced mRNA level of these transcription factors in the CSE-treated BCSCs indicates that although the surface marker of CSCs, *i.e.*, CD44+/CD24- was not affected by CSE, the reduced mRNA of *NANOG, SOX2, OCT4,* and *c-MYC* upon CSE treatment maybe sensitize BCSCs toward apoptosis, reduce their pluripotency and stemness. These findings still need to be clarified, particularly in a time course, because the cells need time to complete the differentiation process and observe their changes.

In this study, phytochemical profiling of the CSE revealed that CSE contains abundant phenol and flavonoids, along with other components, including tannins, saponins, and terpenoids (Table 2). Brazilin and brazilein are two major isoflavones reported in many studies to inhibit cancer progression in various cancer cells (12,16). Several natural compounds with a phenolic structure, including genistein, curcumin, quercetin, epigallocatechin gallate, and resveratrol, have been reviewed to have potency in targeting CSCs with various mechanisms (30). There are two strategies to target BCSCs: 1. reducing their stemness and self-renewal capacity; 2. inducing their differentiation. The current data just explored the effect of CSE in one aspect, and it is worth performing further studies to investigate the role of CSE in the inducing of CSC differentiation.

CONCLUSION

This study provides new insight into the effects of CSE on the BCSCs and the cells of MDAMB-231. BCSCs were more sensitive to CSE compared to triple-negative breast cancer, MDA-MB-231. CSE increased the levels of ROS and decreased the expression levels of stemness transcription factors in BCSCs. These mechanisms may induce BCSCs to differentiate resulting in apoptosis.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

R.I. Jenie conceptualized and designed the study, analyzed the data, and conceptualized and wrote the manuscript; ND. Amalina conducted the experiments, analyzed the data, and drafted the manuscript; A. Hermawan analyzed the data and designed the part of the study; M. Suzery provided the study materials and data analysis; A. Putra provided study material data interpretation; E. Meiyanto conceptualized the study and provided the manuscript. All authors approved the final version of the manuscript.

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