

RESEARCH ARTICLE

Cardamom Essential Oil Extract Suppress the Progression of Triple-Negative Breast Cancer 4T1 Cell Line

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Abstract

BACKGROUND: Cardamom (*Amomum cardamom*) essential oil (CEO) contains monoterpenes with antioxidant activity and is reported to exhibit anticancer activity against some cancer cell lines. Triple-negative breast cancer (TNBC) has the lowest prognosis among breast cancer types due to its aggressive characteristics. This study was conducted to explore the potency of CEO in inhibiting 4T1 cell proliferation and migration and compared its activity to sappan (*Caesalpinia sappan*) wood extract (CSE).

METHODS: We used the 4T1 cell line as the TNBC cell model and tested the cytotoxicity of CEO by using a trypan blue exclusion assay. We studied the senescence induction ability of CEO using SA- β -Gal assay, the migratory inhibition activity using scratch wound healing assay, and inhibition of matrix metalloproteinase 9 (MMP-9) expression using gelatin zymography.

RESULTS: CEO showed cytotoxicity toward 4T1 cells with the IC₅₀ values of 60 μ g/mL. CEO at $\frac{1}{2}$ IC₅₀ and IC₅₀ concentration significantly increased cell senescence, but CSE did not. CEO at IC₅₀ also reduced cell ability to migrate and also considerably reduced MMP-9 activity. Moreover, these activities related to the inhibition of the cell metastasis process were weaker compared than that of CSE.

CONCLUSION: CEO showed potency as a chemopreventive agent on the TNBC 4T1 cell line model with moderate cytotoxicity. CEO induced 4T1 cell senescence, inhibited cell migration and suppressed MMP-9 expression.

KEYWORDS: *Amomum cardamom*, *Caesalpinia sappan*, 4T1, senescence, cell migration, triple-negative breast cancer

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Introduction

Despite its low prevalence, triple-negative breast cancer (TNBC) has the worst prognosis among breast cancer subtypes.(1-4) The absence of three cancer biomarkers, namely, estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2, leads to a lack of therapeutic targets for TNBC.(3,5) To date, chemotherapy remains the best treatment for TNBC despite its side

effects and accompanying patient discomfort.(3,6,7) The coexistence of side effects in chemotherapy manifests the low selectivity of anticancer agents.(6) Thus, other agents must be discovered to prevent cancer progression and development.

Natural materials can be used as anticancer agents. In Indonesia, cardamom (*Amomum cardamom*) fruit and sappan (*Caesalpinia sappan*) wood are often used as traditional ingredients in a herbal drink.(8,9) This herbal drink can be used for chemoprevention toward cancer

progression.(10) Cardamom essential oil (CEO) contains monoterpenes with antioxidant activity (11,12), such as 1,8-cineol, α -pinene, β -pinene, and α -terpineol (12,13). *In vitro* studies showed that monoterpenes 1,8-cineol and α -pinene inhibited the production of intracellular reactive oxygen species (ROS) in PC12 cells.(14) In addition, the monoterpenes from cardamom exhibited anticancer activity against colon cancer.(13)

Cancer cells have distinct characteristics, as discussed previously.(15) Another study report that cell senescence, microbiome polymorphism, phenotypic plasticity, and epigenetic reprogramming are new dimensions of cancer cells emerging as hallmarks.(16) Senescence is a cellular event occurring naturally as a sign of abnormal ROS level of the cell, leading to apoptosis of the cell.(17,18) This can be one approach for killing cancer cells, including TNBC. On the other hand, it has been mentioned before that TNBC has the lowest prognosis among the different subtypes of cancer. (2) This characteristic is influenced by the highest ability of TNBC to metastasize.(1,3) Using these characteristics as the basis, we explored the potential influence of the CEO on TNBC senescence and cell migration. Our research group reported that sappan wood extract (CSE) decreases cell senescence and inhibits TNBC cancer cell line migration (17,19), manifested by the main contents in sappan wood ethanolic extract, brazilin, and brazilein (20,21). Meanwhile, the activity of CEO on the TNBC cell line, including its senescence induction or metastasis inhibition activity, has yet to be studied.

Therefore, this study was conducted to explore the potential of the CEO to halt TNBC's progression in comparison with CSE. A scientific study on the activity of CEO is warranted as a preliminary investigation in developing CEO as chemopreventive agents. This study focused on the senescence and migration activity and used the 4T1 cell line as the model.

Methods

Sample Preparation

CEO was obtained through the steam-hydro distillation of dried Javanese cardamom (*Amomum cardamom* Soland. Ex Maton.) samples (National Research and Innovation Agency, Tawangmangu, Indonesia). In brief, 1 kg of dried cardamom fruit was distilled for 4 hours. The products of the condensate were separated to obtain the CEO. CSE was prepared by macerating 250 g of *Caesalpinia sappan* heartwood sawdust (National Research and Innovation

Agency) with 1 L of 70% ethanol at room temperature. Maceration was carried out for 24 hours with linear shaking for 6 hours. The macerate was filtered and then concentrated using a rotary evaporator (Buchi, Shanghai, China). The extract was collected after the fixed weight of the extract was obtained, and the extraction yield was then calculated.

Cell Culture

The TNBC cell model, 4T1, was obtained from cell line collection of the Cancer Chemoprevention Research Center (CCRC), Universitas Gadjah Mada, Yogyakarta. The cryopreserved cells were thawed at room temperature until they showed a liquid consistency and then grown on a 10 cm tissue culture dish using Dulbecco's modified eagle medium (Gibco, New York, USA) containing 10% fetal bovine serum (Gibco) and 1.5% penicillin–streptomycin (Gibco).

Trypan Blue Exclusion Assay

CEO and CSE cytotoxicity assays were conducted on 4T1 cells using the trypan blue exclusion method. Cells with 2×10^3 cells/well were grown on 24-well plates (Iwaki Cell Biology, Lardejero, Spain) and treated with serial concentrations of 1-100 $\mu\text{g/mL}$ CSE or CEO. Doxorubicin (Sigma, St. Louis, MO, USA) at 0.1-50 $\mu\text{g/mL}$ was used as a positive control. Incubation was carried out for 24 hours, and viable cells were determined by counting and excluding the cells with trypan blue stain (Sigma) using a hemocytometer. Cell count was replicated in a triple for each well. Cell viability versus concentration cell profiles was statistically processed using linear regression at the 95% confidence level, and IC_{50} values were determined for the CEO and CSE treatments.

Senescence Induction Ability Analysis

A total of 1×10^4 cells/well of 4T1 cells were grown on six-well plates (Iwaki Cell Biology) and treated with the IC_{50} concentrations of CSE and CEO samples. The cells were incubated for 24 hours and then fixed and stained with a dye solution containing X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) (Sigma). Staining was carried out for 72 hours, and subsequent observation was performed using an inverted microscope (Olympus, Tokyo, Japan) at $400\times$ magnification. The stained cells were identified as senescent cells. The number of senescence cells was calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA) with triple replication and then statistically compared using a student *t*-test with Microsoft Office Excel™ software (Microsoft Corporation, Redmond, WA, USA).

Analysis of Cell Migration Inhibitory Activity

4T1 cells with 4×10^3 cells/well were grown on 24-well plates (Iwaki Cell Biology) and treated with the IC_{50} concentrations of CSE and CEO samples. Incubation was carried out until the cells were confluent for 24 hours. The cells were scratched using the yellow tip in a direction parallel to each well. Wound closure was observed at 18 and 42 hours of treatment under an inverted microscope (Olympus, Tokyo, Japan) at $200\times$ magnification, analyzed, and processed using ImageJ software (National Institutes of Health). The wound closure experiment was replicated three times and statistically compared using a student *t*-test with Microsoft Office Excel™ software (Microsoft Corporation).

Analysis of Matrix Metalloproteinase (MMP)-9 Expression

A total of 5×10^5 cells/dish of 4T1 cells were grown on a 6 cm tissue culture dish, treated with the IC_{50} concentrations of CEO and CSE (60 and 9 $\mu\text{g/mL}$, respectively), and incubated for 24 hours. The medium was then collected and normalized with Bradford assay. The same amount of protein concentration was subjected to Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) electrophoresis on a polyacrylamide gel containing gelatin and stained with Coomassie brilliant blue (Sigma). MMP-9 band was observed on a 92 kDa protein weight and then quantified as a band intensity percentage compared with the untreated band on ImageJ software (National Institutes of Health). The experiment was replicated three times. MMP-9 band intensity percentage between each treatment and control with student *t*-test on Microsoft Office Excel™ software (Microsoft Corporation).

Results

Extraction Yield

We performed steam distillation and maceration to obtain CEO and CSE, respectively. The steam distillation of cardamom fruit yielded 3.6% of CEO from the dried sample. Meanwhile, sappan wood maceration yielded 7.2 grams of CSE, 7.2 % w/w from dried fruit. The prepared extracts of CEO and CSE were then used for *in vitro* experiments.

Cytotoxicity Activity of CEO in 4T1 Cells

We performed a trypan blue exclusion test to determine the cytotoxicity of CEO and CSE in the TNBC cell model, 4T1, with Doxorubicin as the positive control. At 24 hours incubation, based on the trypan blue staining results, the IC_{50} value of CSE was 9 $\mu\text{g/mL}$, whereas CEO was 60 $\mu\text{g/mL}$ (Figure 1). This data showed that the cytotoxicity potency of CEO was weaker than CSE. As a positive control, Doxorubicin showed IC_{50} of 5 $\mu\text{g/mL}$.

The moderate cytotoxicity of CEO may be due to its ability to inhibit cell proliferation by inducing cell senescence. Cell senescence halts cell progression, but cells were still viable under trypan blue exclusion assay. Therefore, to explore the senescence induction ability of both CEO and CSE, we then perform an SA- β -Gal assay.

Senescence Induction Activity of CEO in 4T1 Cells

The result of the SA- β -gal assay showed the senescence induction ability of CEO 4T1 cells compared to CSE (Figure 2). CEO significantly increased the number of senescent

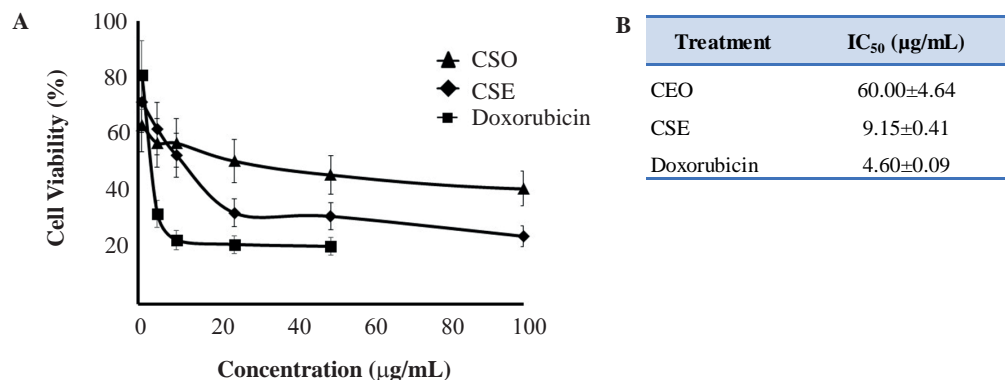


Figure 1. Cytotoxicity of CEO and CSE in 4T1 cells. The cytotoxicity of samples was assessed using the trypan blue exclusion test, and the IC_{50} value was calculated using linear regression on a 95-99% confidence level. A: The graph indicates the viability profile of 4T1 cells after CEO, CSE, and Doxorubicin treatments. B: Comparative table of CEO, CSE, and Doxorubicin IC_{50} values in cell 4T1. Data are shown as mean \pm SD of three independent experiments.

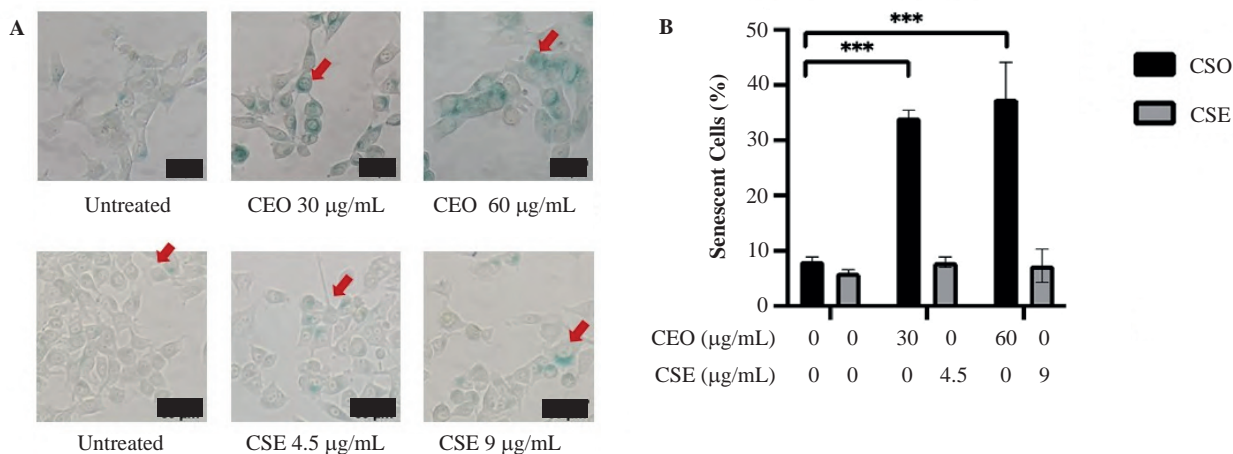


Figure 2. Senescence induction activity of CEO and CSE. The 4T1 cells were treated with 1/2 IC_{50} and IC_{50} concentrations of CEO and CSE for 24 hours then fixed and stained with X-Gal-containing dye. The green-stained cells were the senescent cells observed with an inverted microscope at 400 \times magnification and calculated using ImageJ software. The experiment was replicated three times. The number of senescent cells was compared between each treatment and control and analyzed using a student *t*-test. A: Morphology of senescent cells (red arrows) after CEO and CSE treatments. Black bar: 50 μ m. B: Number of senescent cells after CEO and CSE treatments. Data are shown as mean \pm SD of three independent experiments. *** p <0.001

4T1 cells (p <0.001). Meanwhile, CSE did not considerably increase the number of senescent 4T1 cells (p >0.05). This result showed that CEO induced cell senescence, but CSE did not and supported the potential of CEO as a chemotherapy co-agent by increasing cell senescence levels.

Cell Migration Inhibition of CEO in 4T1 Cells

The TNBC is an aggressive cell that easily migrates and invades its surrounding. We conducted a wound healing test to determine the migration inhibition activity of CEO and compared it to CSE. The results showed that CSE exerted a more significant inhibiting effect on 4T1 cell migration than CEO (Figure 3). CSE significantly inhibited 4T1 wound closure (p <0.001) at 18 and 42 hours compared with that in the untreated cells. Meanwhile, CEO inhibited wound closure only at 42 hours (p <0.001).

MMP-9 Expression Inhibition of CEO in 4T1 Cells

Cancer cells must degrade their extracellular matrix (ECM) to migrate and invade other tissues. Therefore, the cancer cell secretes the protease enzyme, namely MMP, to degrade the ECM. We performed gelatin zymography to determine the MMP-9 level in 4T1 cell culture medium under CEO treatments to manifest the extracellular breakdown in the initiation of the metastasis process of TNBC and compared it to CSE. Gelatin is a specific substrate of MMP-9. We found that CEO significantly inhibited MMP-9 activity in degrading gelatin but had a weaker effect than CSE (Figure 4).

Discussion

We showed that CEO had moderate cytotoxicity on 4T1 cells (Figure 1). Amomum tsaoko, the essential oil of another species of cardamom that has a similar composition to CEO, is moderately cytotoxic against HepG2, Bel-7402, HeLa, and PC3 cells due to its low radical scavenging power.(22) CEO contains 1,8-cineol, β -pinene, α -terpineol, fenchone, α -pinene, sabinene, and linalool as the most dominant ingredients. These compounds are responsible for the chemopreventive activities of CEO in various cell line models, such as its antioxidant, apoptosis induction, and cell proliferation inhibition.(11, 21-26)

We observed that compared with that of the control (untreated cells), the cellular senescence levels in 4T1 cells were significantly increased by CEO (p <0.001) but not by CSE (p >0.05). Cell senescence is an irreversible cell cycle arrest that prompts cells to stop proliferating, thus halting cancer progression.(27) Senescence is a part of the aging process and is induced by various factors, such as high levels of reactive oxygen species, epigenetic changes, and telomere shortening. However, various stimuli can trigger premature senescence, including DNA stress caused by radiotherapy treatment and chemotherapeutic agents.(27) Cell senescence can be directed to proceed to apoptosis under specific treatments. When combined with chemotherapeutic drugs such as Paclitaxel and Vemurafenib, Navitoclax, a Bcl-2 inhibitor, increases cell

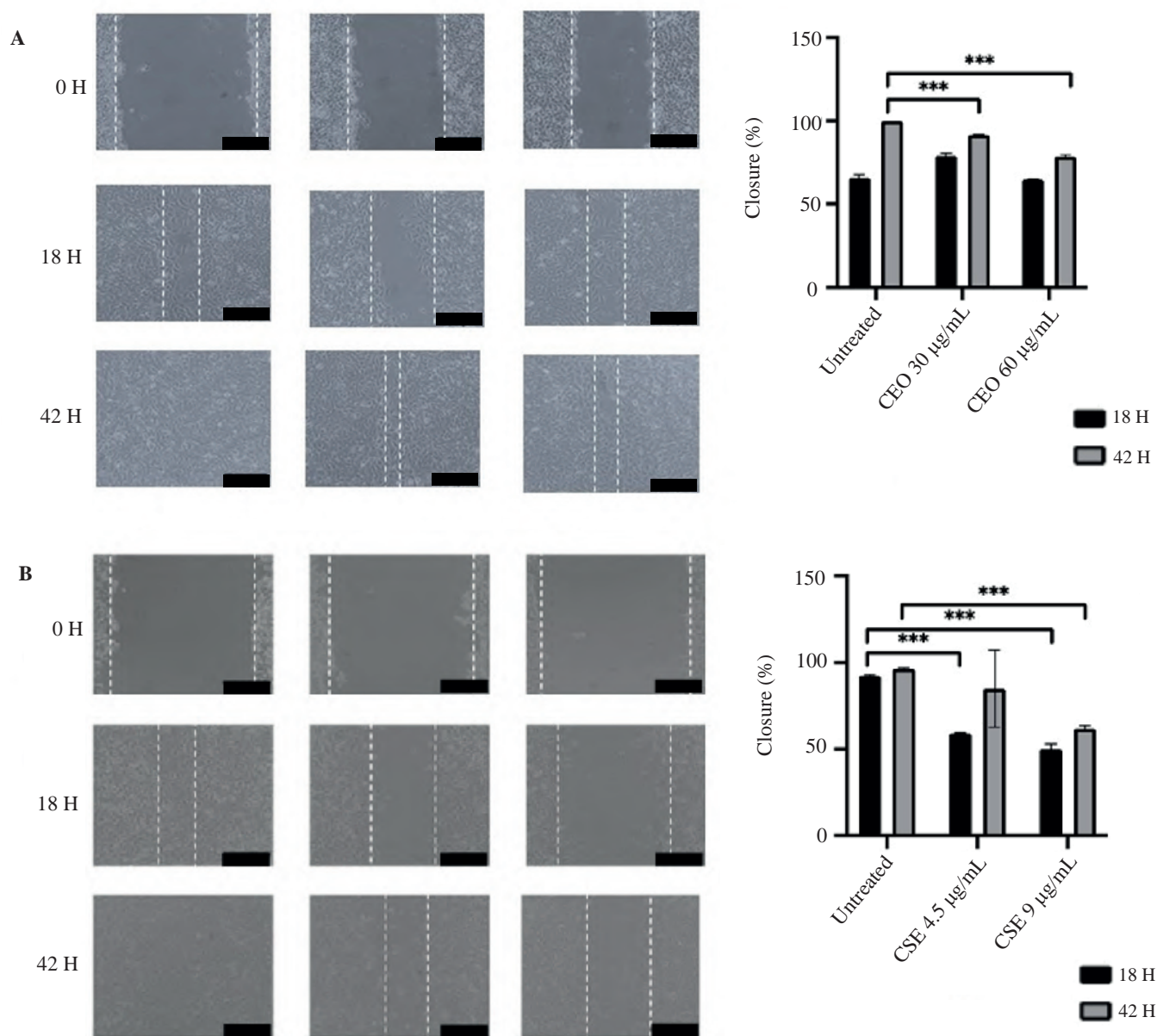


Figure 3. Antimigratory activity of CEO (A) and CSE (B) to 4T1 cells. 1×10^5 4T1 cells/well were grown on a 24-well plate and then scratched with a yellow tip. The cells were then treated with $1/2IC_{50}$ concentrations of CEO and CSE. Wound closure was observed at 0, 18, and 42 hours under an inverted microscope at $200\times$ magnification, examined using ImageJ software and statistically analyzed using a student *t*-test. The experiment was performed three times. *** $p < 0.001$. Black bar: 200 μ m.

apoptosis by inducing senescent cells into the apoptosis. (27) A curcumin derivative, pentagamavunon-1 (PGV-1), induces cell senescence and another cell death mechanism, the mitotic catastrophe. (28) Natural compounds also induce cell senescence. Alone or combined with cisplatin, fisetin, a flavonoid abundant in apples, strawberries, and persimmons, can increase apoptosis via a mechanism involving cell growth arrest. (27) Other natural compounds, such as curcumin and piperine, can induce cell senescence in leukemic and TNBC cells, respectively. (29,30) Therefore, CEO may also enhance its chemotherapeutic agent efficacy

against cancer cells, particularly TNBC, by inducing cell senescence. CSE did not induce senescence, possibly due to its high antioxidant activity, which caused a reduction in intracellular ROS levels. Our findings were in line with previous studies showing that CSE and its active compound, brazilin, have high antioxidant and antisenescence activity. (17,31)

Metastasis involves some mechanisms in cancer cells, starting from extracellular matrix disruption by the family of MMPs, cell adhesion, and invasion of a cancer cell on the other tissues, which mainly correlated with cell migration.

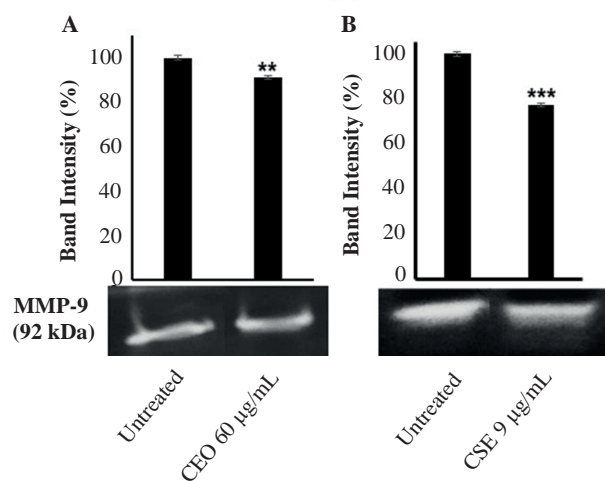


Figure 4. MMP-9 expression regulation of CEO and CSE to 4T1 cells. 2×10^5 4T1 cells/dish were grown on a 6 cm tissue culture dish and treated with IC₅₀ concentrations of CEO (A) and CSE (B). The amount of protein in each treatment was normalized through the Bradford method, and the MMP-9 level was analyzed by gelatin zymography. The MMP-9 band intensity was quantified using ImageJ software and then statistically analyzed using a student *t*-test. The experiment was performed three times. ***p*<0.01; ****p*<0.001.

(19,20) This process continued until the cell reached the second host tissue and completed the metastasis process. (20,31,32) However, extracellular matrix disruption in cancer was regulated by MMP-9.(28,32) The suppression of MMP-9 secretion can inhibit the metastasis initiated by tumor cells.(32) Thus, the antimigration activity of CEO and MMP-9 suppression indicated its potential as an antimetastatic agent. Based on the current study, in terms of cancer migration, CSE showed more significant migration-inhibiting activity, and MMP-9 suppression than CEO. These abilities of CSE have been previously reported and compared with positive control Doxorubicin.(33) Further research on other possible activities of CEO in inhibiting cancer cell proliferation, for example, inducing cancer cell death, is interesting to be explored.

Conclusion

CEO is moderately cytotoxic against 4T1 cells, whereas CSE is strongly cytotoxic. Moreover, CEO, but not CSE, significantly induces cell senescence. CEO also inhibits cancer cell metastasis progression by inhibiting cell migration and MMP-9 expression with weaker potency than CSE.

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Authors Contribution

AST conducted experiments with the CSE sample, analyzed data, and wrote the manuscript. NUH conducted experiments with the CEO sample and prepared the manuscript. DUS performed the statistical analysis. RKW prepared and conducted gelatin zymography experiments. UMZ prepared the 4T1 cell culture. MI supervised the work with the CEO sample. EM designed, organized, and directed the research and the writing flow. RIJ designed and supervised the work, analyzed the data, developed the writing flow, and revised the manuscript. All authors discussed the results and commented on the manuscript.

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