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Nephroprotective effects of cardamom essential oil (*Amomum compactum* Soland. Ex Maton) on kidney cells

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ABSTRACT Many chemotherapeutic agents cause various side effects, including nephrotoxicity. Cardamom essential oil (*Amomum compactum* Soland. ex Maton) contains compounds that exhibit antioxidant activity, such as 1,8-cineole, α-pinene, α-terpineol, and linalool. This study focused on exploring the potency of cardamom essential oil (CEO) as an anti-senescent induced by doxorubicin using the Vero kidney cell line. We first obtained the CEO by steam distillation, then evaluated its cytotoxicity using a trypan blue exclusion assay. Moreover, we performed senescence-associated beta-galactosidase (SA-β-gal) staining and 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) staining to measure the effect of CEO on intracellular ROS level and cell senescence, respectively. Analysis of the compounds with gas chromatography-mass spectrophotometry (GC-MS) revealed seven compounds with significant abundance, namely 1,8-cineole (50.82%), ß-pinene (12.43%), α-terpineol (8.50%), fenchone (4.10%), α-pinene (4.00%), sabinene (3.00%), and linalool (1.98%). The cytotoxicity assay of CEO on Vero cells showed an IC_{50} value of 178 µg/mL. Thus, CEO is considered low cytotoxic for normal kidney cells (>100 µg/mL). Concentrations of 50 and 100 µg/mL CEO reduced the cell senescence induced by doxorubicin. Therefore, CEO has potency as a nephroprotective agent in doxorubicin-induced senescence.

KEYWORDS 1,8-cineole; Cardamom; Reactive Oxygen Species (ROS); Senescence; Vero cells

1. Introduction

Problems related to cancer treatment using chemotherapeutic agents is their toxicity toward normal cells. Side effects of chemotherapy agents can indirectly cause kidney damage (Jia et al. 2015). The use of chemotherapeutic agents is still the primary choice in cancer treatment. One of the chemotherapy agents that often used is doxorubicin. In addition to its effectiveness as an anticancer agent, doxorubicin has dangerous side effect, nephrotoxicity (Ayla et al. 2011). Doxorubicin causes oxidative stress and can increase ROS levels in cancer cells and normal cells (Mobaraki et al. 2017). Doxorubicin threatens health related to physiological functions, especially kidney damage. Therefore, it is necessary to explore chemotherapy companion agents that can inhibit oxidative stress, safe, and do not harm the body. Natural compounds are an option as co-chemotherapy agents because of their abundant availability and ease of finding. Natural ingredients contain various compounds with diverse beneficial physiological activities (Arianingrum et al. 2015).

One natural ingredient often found in Indonesia is Javanese cardamom (*Amomum compactum* Soland. Ex Maton). The community often uses Javanese cardamom as a spice in cooking because of its distinctive aroma of spices. In addition, Javanese cardamom is also used as a room freshener (Fachriyah 2007). The unique smell is inseparable from the content of compounds contained in cardamom. The essential oil of these plants is known to contain a class of monoterpene compounds such as 1,8-cineole, α-pinene, β-pinene, and α-terpineol (Tambunan 2017). Cardamom essential oil is known to have antioxidant activity in DPPH assay with an IC_{50} of 2.22 µg/mL (Pujiarti and Kusumadewi 2020). The antioxidant capacity in cardamom is 85-90%; therefore, it can inhibit lipid peroxidation (Bhatti et al. 2010). *In vitro* studies using H_2O_2-induced PC12 cells revealed that treatment of the monoterpenes 1,8-cineole and α-pinene at doses of 10 and 25 µM showed inhibition on the production of intracellular ROS (Porres-Martínez et al. 2016).

Furthermore, *in vivo* study showed the neuroprotective effect of 1,8-cineole on zebrafish. Administration of 1,8-

cineole at doses of 0.1 mg/mL and 0.01 mg/mL demonstrated 35% and 28% inhibition of copper ion-induced oxidation in zebrafish, respectively (Cho 2012). However, there is no report on the nephroprotective potential of cardamom essential oil. Therefore, this study focuses on observing the potency of cardamom essential oil as an antioxidant to be developed as a nephroprotective agent by using a kidney cell line induced by doxorubicin. The results will provide scientific data to know the potency of cardamom essential oil (CEO) as a nephroprotective agent, particularly its possibility to be an antioxidant and antisenescence. The nephroprotection activity of the CEO will offer its use to reduce the side effects of the chemotherapeutic agent like doxorubicin which cause severe kidney damage.

2. Materials and Methods

2.1. Cardamom essential oil (CEO) preparation

Javanese cardamom (*Amomum compactum* Soland. Ex Maton) was from Rempah Jaya, Lumajang, Indonesia. We ground it to a smaller size and then distilled it with 10 L water for 4 h using a steam distillation apparatus (Imatton Home, Yogyakarta, Indonesia). The oil phase is separated from the aqueous phase and dried by adding some anhydrous sodium sulfate. The yield of CEO was calculated using the following formula (da Costa et al. 2014):

$$\text{yield } (100\%) = \frac{mass\ of\ cardamom\ essential\ oil}{weight\ of\ cardamom\ simplicia} \times 100\% \quad (1)$$

The oil phase was then analyzed using the GC-MS instrument (SHIMADZU, Japan) at Laboratorium Penelitian dan Pengujian Terpadu (LPPT), Universitas Gadjah Mada (UGM) with a helium gas carrier at a temperature of 70-300 °C, column type HP-5MS UI, split flow 50 mL/min, split ratio 50 to obtain the phytochemical profile contained in CEO. The parameter used to measure the sample is retention time.

2.2. Cell culture

Vero cell is a cell line derived from a green monkey kidney. We obtained this from Prof. Masashi Kawaichi, MD., Ph.D. (Nara Institute of Science and Technology, NAIST, Japan). Vero cells were cultured on Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) high glucose, containing 10% FBS (Gibco, USA), 1.5% penicillin-streptomycin (Gibco, USA) at 37 °C and stored in a 5% of CO_2 incubator (Thermo Scientific). The cells will be grown to 80% confluent and then harvested with Trypsin-EDTA (Gibco, USA) to be replanted on the well plate. The passage number for the following experiment was five.

2.3. Cytotoxicity assay

We performed the trypan blue exclusion method to evaluate the cytotoxicity of CEO on Vero cells. The protocol is described previously in (Lestari et al. 2019) with slight modification. Vero cells at 2×10^4 cells/well were grown on 24-well plates and treated with a series of concentrations of CEO (1-500 µg/mL) and doxorubicin (dox) (0.01-10 µM) as the positive control. Samples were dissolved in dimethyl sulfoxide (DMSO), with the final concentration of DMSO in the highest sample concentration was 0.5%. Cells were incubated with samples at 5% of CO_2 incubator, 37 °C for 24 h. After sample incubation, the cells were harvested using trypsin-EDTA and stained with trypan blue. Cells were observed under a microscope, and the viable cells were counted using a Neubauer hemocytometer (Assistant, Germany).

2.4. Senescence-associated β-galactosidase assay

Vero cells (1.5×10^5 cells/well) were grown in a 3.5 cm tissue culture dish (TCD) until 80% confluent. Discard the media in TCD and wash with 1× phosphate-buffered saline (PBS). Cells were then treated with CEO (50 and 100 µg/mL), doxorubicin (100 nM), or both (CEO 50 or 100 µg/mL and doxorubicin 100 nM) for 24 h, whereas the untreated cells were treated with 0.1% DMSO. We added fixation buffer (2% formaldehyde-0.2% glutaraldehyde) and incubated the cells for 20 min at room temperature. The cells were then washed with 1× PBS and stained by adding 1-2 mL of X-Gal solution (0.2% 5-bromo-4-chloro-3-inolyl-β-D-galactoside, 40 mM citric acid/phosphate buffer (pH 6.0), 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ and 2 mM $MgCl_2$) for 72 h (Jenie et al. 2019). Cells were then observed under a microscope (Olympus, Tokyo, Japan) at 400× magnification and documented with a digital camera. In this assay, green cells represent the senescent cells (Debacq-Chainiaux et al. 2009). We calculated the percentage of senescent cells by comparing the number of green-stained cells with the total number of cells, then plotted them in a graphic form along with the concentrations of various treatments.

2.5. Intracellular ROS Assay

Vero cells at 5×10^4 cells/wells were grown on 24-well plates with DMEM culture medium (Gibco, USA) and incubated for 24 h until confluent. Cells were then harvested using trypsin-EDTA and incubated for 3-5 min. Vero cells were inactivated using trypsin-EDTA, stained with 25 µM DCFDA (Sigma), and incubated at 37 °C for 30 min in a 5% of CO_2 incubator. Cells were treated with doxorubicin (dox; Sigma) 100 nM, CEO (50 and 100 µg/mL), or a combination of CEO and dox, then incubated at 37 °C CO_2 5% for 4 h and were analyzed by flow cytometry (Qodria et al. 2022). Debris was excluded, and the cell population was isolated by gating. The data obtained were the average fluorescent intensity, and the fold change between the control and the treated sample was determined.

2.6. Statistical analysis

Statistical analysis was performed using SPSS (v.20.0.0) one-way ANOVA and post-hoc least significant difference (LSD) to determine statistical significance. The test results were expressed in mean±SD or mean±SE.

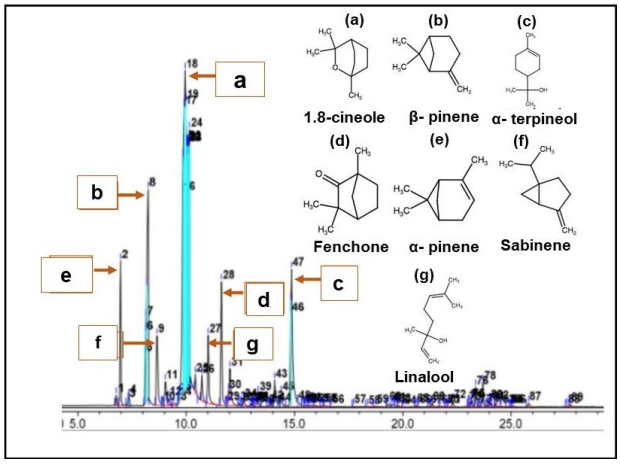
3. Results and Discussion

3.1. Phytochemical profile of cardamom essential oil

Cardamom essential oil (CEO) is often used as a cooking spice because it has a distinctive taste. The unique aroma is inseparable from the compounds contained in cardamom essential oil. We used the steam distillation method to isolate the compound content in CEO. The yield of CEO from steam distillation was 3.6% w/w. CEO is yellowish and smells very similar to eucalyptus oil (Figure 1a). CEO was further identified by GC-MS instrument. The seven most abundant compounds of CEO were obtained (Figure 1b), as shown in Table 1.

The 1,8-cineole probably the most significant role in CEO activity, which is why it smells similar to eucalyptus oil that containing the same compound as cardamom (Sharma and Kaur 2020). Other compounds, including β-pinene, α-terpineol, fenchone, α-pinene, sabinene, and linalool, may have a role in their antioxidant activity (Table 1).

(a)



(b)

FIGURE 1 Phytochemical profile of cardamom essential oil (CEO). The appearance of java cardamom essential oil and its fruit (a), Cardamom essential oil (CEO) was obtained using the steam distillation method and then analyzed using a GC-MS instrument, as shown in the chromatogram profile of CEO (b).

TABLE 1 Most abundance compounds in CEO based on GC-MS analysis.

No	Compound	Peak area (%)	Retention time (min)
1	1,8 Cineole	50.82	9.95
2	ß-Pinene	12.43	8.25
3	α-Terpineol	8.50	14.87
4	Fenchone	4.10	11.63
5	α-Pinene	4.00	6.98
6	Sabinene	3.00	8.15
7	Linalool	1.98	12.02

3.2. Effect of cardamom essential oil on Vero cell viability

We performed a cytotoxicity assay to determine the safe concentration for senescence and ROS assay on Vero cells. We used doxorubicin as a positive control. Doxorubicin has an IC_{50} of 8.5 µM while CEO has an IC_{50} of 178 µg/mL (Figure 2b). Therefore, we categorized CEO as low cytotoxic because it has an IC_{50} value of >100 µg/mL (Prayong et al. 2008).

3.3. Cardamom essential oil (CEO) reduced cell senescence on Vero cells

The cytotoxic assay showed that CEO is relatively safe for normal kidney cells. Therefore, we performed a senescence assay with SA-β-galactosidase assay to see the effect of CEO in rescuing cell senescence induced by a chemotherapy agent, doxorubicin. Cells experiencing senescence are marked in green-stained cells. The fewer stained green cells, the higher the ability of a compound to inhibit cell aging. At a concentration of 100 nM, doxorubicin treatment significantly increased senescent cells compared to untreated cells (Figure 3). CEO at concentrations of 50 and 100 µg/mL significantly inhibited the incidence of senescence ($p < 0.001$) induced by doxorubicin (Figure 3). This result indicated that CEO protects kidney cells from doxorubicin-induced cells aging (senescence).

3.4. The effect of cardamom essential oil (CEO) on intracellular ROS level of Vero cells

One of the doxorubicin mechanisms of cytotoxicity is generating ROS in the cells (Baxter-Holland and Dass 2018). Moreover, high intracellular ROS levels trigger cell senescence, showing aging, disturbed, and damaged cells (Lestari et al. 2019). Based on the cell senescence assay (Figure 3), we observed that doxorubicin induces the incidence of senescent cells. Therefore, we performed a DCFDA staining assay to see the effect of CEO on intracellular ROS levels. In this assay, we used 100 nM doxorubicin (dox) to induce the ROS level (Zulfin et al. 2021), and then we observed the CEO effect at sub IC_{50} concentration on the ROS level after induced by dox. At a concentration of 100 nM, dox is known to increase ROS levels in cancer cells under therapeutic conditions (Hou et al. 2020). Our results showed that treatment of dox-

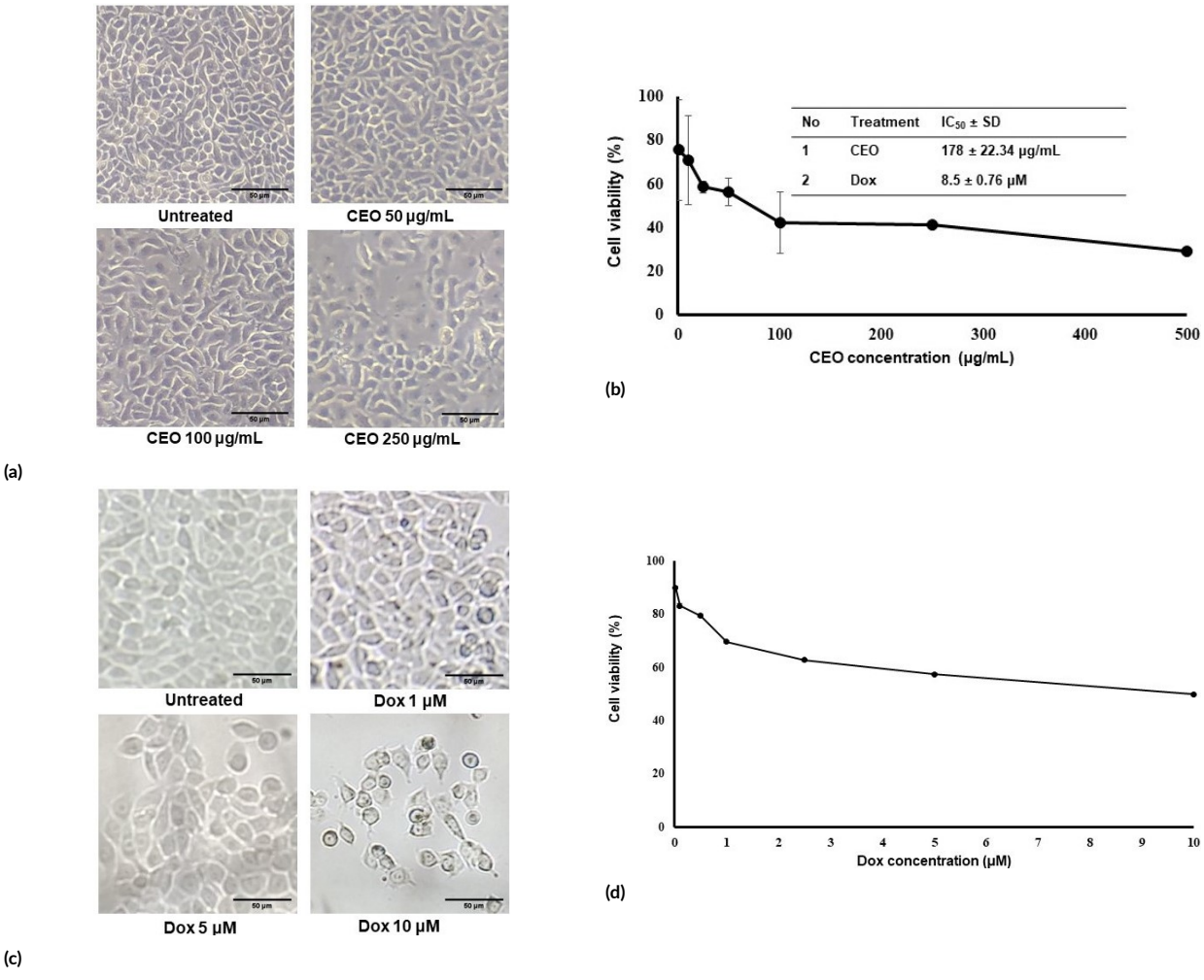


FIGURE 2 Effect of CEO and doxorubicin Vero cell viability. Vero cells were grown at 2×104 cells/well until 80% confluent and then treated with CEO and doxorubicin as described in the Materials and Methods. Trypan blue exclusion assay was conducted to observe the cytotoxicity of CEO on Vero cells. Vero cell viability upon treatment of CEO was observed under a microscope at 400× magnification (a) and doxorubicin (b). The cell viability graph of CEO (c) and doxorubicin (d) was depicted along with their IC_{50} value. The experiment was replicated three times.

orubicin increased ROS levels despite not being significant (Figure 4). Moreover, ½IC_{50} and IC_{50} concentrations of CEO (50 µg/mL and 100 µg/mL) in dox-induced cells caused ROS levels to decrease significantly compared to dox alone treatment ($p < 0.05$ and $p < 0.01$, respectively). Overall, the CEO inhibited the level of ROS induced by dox.

3.5. Discussion

This study aimed to explore the nephroprotective potency of CEO on kidney cells induced by doxorubicin using the Vero cell line. Nephroprotective is necessary nowadays because senescence problems can cause damage to vital tissues. Doxorubicin treatment caused cytotoxicity against Vero cells (Figure 2) and cell senescence (Figure 3). We explored how a plant commonly used in the community could reduce toxicity to kidney tissue. The focus of this study was to examine the effect of cardamom essential oil (CEO) on doxorubicin-induced Vero cells.

First, we explore the phytochemistry profile of the CEO. CEO contains various compounds that have antioxidant activity and protect cells from oxidative stress. Based on our result, CEO contains 1,8-cineole (50.82%), ß-pinene (12.43%), α-terpineol (8.50%), fenchone (4.10%), α-pinene (4.00%), sabinene (3.00%), and linalool (1.98%). CEO has an aroma similar to eucalyptus oil because it contains the same compound, namely 1,8-cineole (eucalyptol), citronellal, citronellol, citronellyl acetate, p-cymene, eucamalol, limonene, linalool, α-pinene, γ-terpinene, α-terpineol and aromadendrene (Bhandari et al. 2013; Almas et al. 2021). The 1,8-cineole found in eucalyptus oil also has the most abundance compared to other compounds.

The results of the cytotoxicity of CEO on Vero cells showed that up to a concentration of 150 µg/mL, CEO gave low cytotoxic toward Vero cells (Figure 2). This determination is based on the classification related to the cytotoxicity of compounds or materials in vitro, showing that they are low-toxic at concentrations of $IC_{50} > 90$ µg/mL (Prayong et al. 2008). The acute toxicity study in Wistar rats treated with cardamom seed extract at an initial

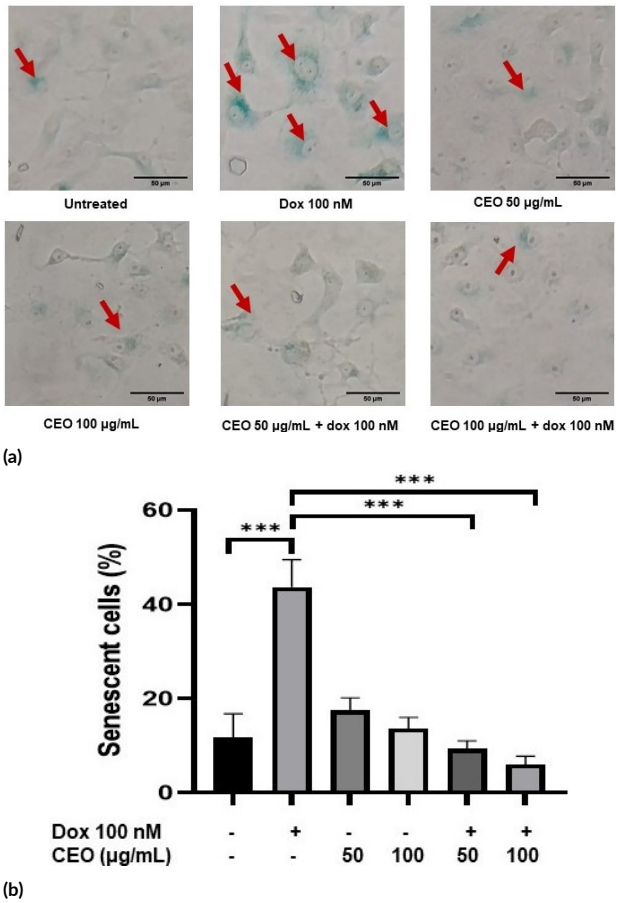


FIGURE 3 Reduction of senescent cells by cardamom essential oil (CEO). Vero cells, 1.5×10^4 cells/well, were grown and treated with doxorubicin or CEO as described in the Materials and Methods. Cells were fixed, stained with X-Gal, incubated for 72 h then observed under a microscope at 400× magnification. Cell morphology that experienced senescence was observed under a microscope (a), and a graph of cells underwent senescence for each treatment (b). The experiment was replicated three times. Significant differences were analyzed using a one-way ANOVA, $^{***}p < 0.001$.

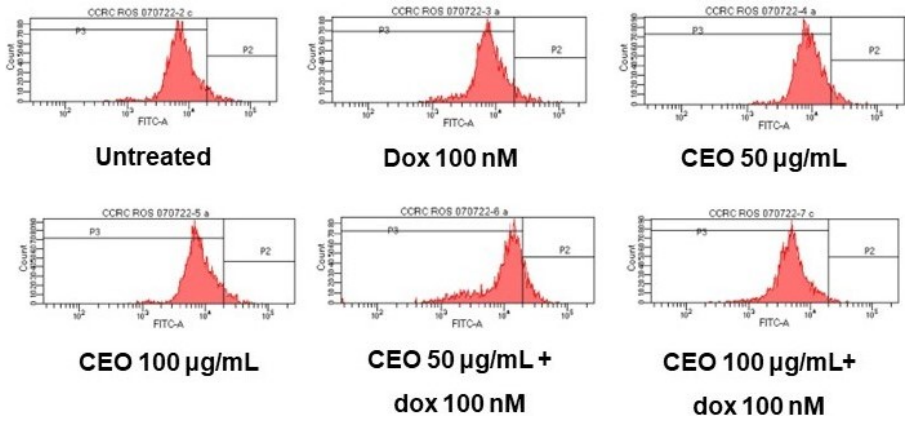
dose of 300 mg/kg BW in the preliminary test followed by a maximum dose of 2,000 mg/kg BW observed for 14 d showed no significant difference in liver damage between the sample treated group and the control group. Based on this study, cardamom is safe and does not harm the body (Yudhani et al. 2020).

Chemotherapy agents, including doxorubicin, are known to have side effects that damage the kidneys (nephrotoxicity) (Mobaraki et al. 2017). Senescence is one of the cells responses toward damage which is define as a response that occurs when cells lose their capacity to reproduce irreversibly (Lestari et al. 2019). Cell senescence occurs naturally or forcibly induced by external agents. Our study proved that CEO reduced the incidence of senescence in kidney cells induced by doxorubicin (Figure 3).

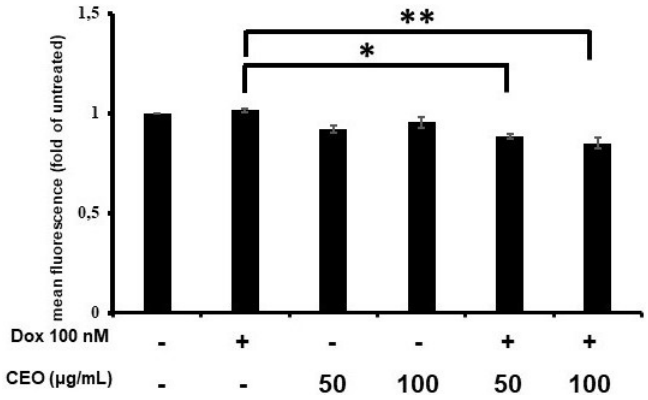
Doxorubicin works through several mechanisms, including inhibition of topoisomerase II enzymes, DNA intercalation, and free radical formation (Taymaz-Nikerel et al. 2018). Free radicals can be formed due to quinone groups possessed by doxorubicin in both normal and cancer cells (Baxter-Holland and Dass 2018). The free radical formation by doxorubicin leads to an increased level of intracellular ROS that are harmful and cause damage to cancer cells or normal cells. Intracellular ROS are associated with senescent events. Increased levels of intracellular ROS that exceed the threshold of cell homeostasis will cause DNA damage, thereby limiting cell replication. Our data showed that cardamom essential oil (CEO) significantly reduced ROS levels in doxorubicin-induced cells (Figure 4). Types of ROS that the DCFDA can detect are hydrogen peroxide (H_2O_2), nitric oxide (NO), oxide anion ($O_{2\text{-}}$), peroxynitrite ($ONOO_{\text{-}}$), hypochlorous acid (HOCl), and hydroxyl radical ($OH_{\text{-}}$) (Wu and Yotnda 2011). Therefore, our study indicated that CEO contains compounds that reduce intracellular ROS, eventually protecting kidney cells from cell senescence induced by doxorubicin.

Based on our GC-MS analysis (Figure 1), 1,8-cineole is the most abundant compound in CEO. Therefore, we propose that this compound has the most significant role in CEO activities. 1,8-cineole and ß-pinene have cytoprotective activity, inhibit ROS production, and increase endogenous antioxidants (Porres-Martínez et al. 2015; Salehi et al. 2019). The 1,8-cineole has good antioxidant properties known to control oxidative processes through direct inhibition of superoxide and hydroperoxide anions and by increasing superoxide dismutase (SOD) enzymes (Juergens et al. 2018). These compounds suppress the formation of ROS and can protect against DNA damage. Antioxidant enzymes such as SOD, CAT, and GSH-PX are essential to increase cell antioxidants against oxidative stress (Di et al. 2022). In addition, an *in vivo* study showed that eucalyptus essential oil containing 67.85% 1,8-cineole increases SOD activity and decreases malondialdehyde (MDA) content in Ross 308 broiler serum (Mohebodini et al. 2021). Despite the abundance of 1,8-cineole, other compounds in CEO, such as α-terpineol, fenchone, and linalool, also have antioxidant activity and protect cells from oxidative stress (Bicas et al. 2011; Seol et al. 2016; Singh et al. 2020). Moreover, α-pinene and sabinene can affect antioxidant enzymes in the body (Bouzenna et al. 2017; Sharma et al. 2019). This effect may be attributed to CEO's capacity to decrease ROS level.

These results are similar to previous studies on fibroblast cells induced by doxorubicin and sequentially treated with galangal extract or rice bran extract, which showed a decrease in ROS levels and cell senescent (Ahlina et al. 2020; Zulfin et al. 2021). Therefore, the effect given by CEO is expected to be an alternative mechanism for protecting cells from damage. Our study indicated that CEO has the potency to significantly reduce the incidence of cell senescence caused by the chemotherapeutic agent, doxorubicin. The development of the CEO as a co-chemotherapeutic agent is worth exploring more. Its impact as cytoprotective is also worth developing as anti-aging to normal cells. Moreover, the CEO can be developed into various nutraceutical products, including food supplements, cosmetics, and others.



(a)



(b)

FIGURE 4 The effect of cardamom essential oil (CEO) on intracellular ROS level. Vero cells were grown in 5×10^4 cells/well. Cells were harvested, stained with 25 µM DCFDA incubated at 37 °C for 30 min, treated with samples of 100 nM dox or 50 and 100 µg/mL CEO, or a combination of CEO and dox, incubated at 37 °C for 4 h, and analyzed using flowcytometry. The histogram was analyzed (a) and presented as a ROS level graph (b). The experiment was replicated three times. Significant differences were analyzed using a one-way ANOVA, $^*p < 0.05$, $^{**}p < 0.01$.

4. Conclusions

Cardamom essential oil (CEO) has low cytotoxicity against Vero normal kidney cell line. The major compounds of the CEO we obtained in this study were 1,8-cineole (50.82%), ß-pinene (12.43%), and α-terpineol (8.50%). CEO decreased cell senescence and reduced ROS levels in doxorubicin-induced stress oxidative Vero cells. Therefore, CEO has the potential as a nephroprotective agent.

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Authors' contributions

NUH prepared the sample and performed the phytochemical profiling and experiments. AST processed and formatted the data. UMZ conducted the in vitro assay. DUS performed the statistical analysis. RKW contributed to the sample preparation. MI supervised the work and drafted the manuscript. EM designed, organized, and supervised the research. 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.

Competing interests

All of the authors declare no competing interest.

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