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Patchouli Ethanol Extract *Pogostemon cablin* Benth. Against Aging Profiles in Doxorubicin-Induced 3T3-L1 Fibroblast Cell Lines

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ABSTRACT

Objectives: This research aimed to investigate the potential of *Pogostemon cablin* Benth's ethanolic extract (PCEE) as an anti-aging agent.

Methods: We treated 3T3-L1 cells induced by doxorubicin with PCEE and assessed them with antiaging markers such as *GLB-1* gene and telomere length with polymerase chain reaction (PCR), or reactive oxygen species (ROS) accumulation with 2,7-dichlorofluorescein diacetate (DCFHDA).

Results: This study demonstrates that PCEE can inhibit ROS accumulation, decrease the expression level of the senescence-associated β-galactosidase (SA-β-gal) *gene, GLB-1,* and maintain telomere length in doxorubicin-induced 3T3-L1 cells.

Conclusion: These studies reveal that PCEE is a promising antiaging agent. An in vivo approach should be conducted for further research.

1 | Introduction

The rapid development of medical science and technology significantly contributes to the efforts to increase human life expectancy [1]. Global data from the World Health Organization (WHO) indicates that by 2020, the population of elderly individuals aged ≥60 has reached 1.6 billion and is projected to double to 2.1 billion by 2050 [1, 2]. Data from the Central Statistics Agency in 2021 demonstrates that the elderly population in Indonesia constituted 10.7% of the population or 28 million people. This demographic shift warrants attention, considering that aging is a significant risk factor for degenerative diseases. Consequently, WHO, through its global strategy guidelines and action plans, incorporates measurement, monitoring, and research in the field of aging as one of its primary focuses [3].

In general, the accumulating of physical, environmental, and lifestyle factors precipitates aging. Several studies have demonstrated correlations between aging rates, metabolic changes, gene expression patterns, and increased reactive oxygen species (ROS) [4–6]. In contrast, low ROS levels are associated with the extension of the organism's life [7]. Cellular senescence has been established as a significant and a critical stage of aging at the cellular level [8]. In addition to ROS accumulation, several markers of aging include telomere length, telomerase activity, SA-β-gal (β-galactoside hydrolase enzyme, present only in aging

cells and detected at pH6) [9]. High accumulation of ROS leads to damage to cell membranes, organelles, and genetic material, thereby accelerating aging [10]. The *SA-β-gal* gene is utilized to evaluate antiaging candidates in various in vitro, in vivo, and clinical trials [11].

Medicinal plants serve as important sources of antiaging compounds due to their phytochemical properties, such as polyphenols, triterpenes, and sterols, which have been previously reported to possess antioxidant and antiaging potential. One such plant is the patchouli plant. The patchouli plant (*Pogostemon cablin* Benth., PCB) is one of the plants producing essential oils and is widely used in traditional medicine [12]. (Figure 1) This plant is readily cultivated in tropical/subtropical climates. It has branches, stands upright, is perennial (with a lifespan of several years, exceeding 2 years), possesses a distinctive aromatic odor, and belongs to the Lamiaceae plant family [13]. Phytochemicals contained in patchouli plants, including flavonoids, saponins, tannins, and phenols, are extensively associated with their antioxidant capabilities [14–17]. The main component of patchouli oil, PA, has been studied for its antioxidant ability [14–17]. However, studies on the antiaging activity of patchouli plants are still limited to patchouli oil and do not extend to patchouli ethanol extracts [18]. In silico research involving network pharmacology and molecular docking methodologies has been conducted to evaluate the antiaging properties of patchouli plants on human skin. The outcomes of these studies have demonstrated that PCBs exert a significant influence in treating skin aging [19].

In aging conditions, cells secrete senescence-associated secretory phenotype (SASP), a marker of inflammation in small quantities (e.g., IL-6). This phenomenon is referred to as chronic inflammation [20, 21]. Many studies have established a correlation between a drug's antioxidant and anti-inflammatory properties and its antiaging effects. For instance, the antioxidant content in the ethanol extract of Officinal breynia cabbage and mustard green gel has been reported to exhibit antiaging properties [14, 16, 17]. Other studies have also demonstrated the association of antioxidant and anti-inflammatory activities of *Clerodendrum cyrtophyllum* turcz and *Tamarindus indica* plants with their antiaging capabilities [22, 23]. Given that many compounds present in patchouli plants are associated with their antioxidant or anti-inflammatory properties, we aims to evaluate the activity of this plant as an antiaging agent in an in vitro study of doxorubicin-induced senescence in 3T3-L1 cell lines. We utilize ethanol extract from the patchouli plant from West Java, one of Indonesia's primary patchouli-producing regions.

2 | Methods

2.1 | Material

The 3T3-L1 fibroblast cell line (ATCC CL-173) was purchased from the American Tissue Culture Collection (ATCC). ROS assay kit DCFHDA (2,7-Dichlorofluorescein diacetate) was a fluorescent redox probe purchased from Sigma Aldrich, USA. Other materials used in this study are doxorubicin hydrochloride (Sigma Aldrich, USA, product number 44583), vitamin E (DL-α-Tocopherol Acetate, SRL, India, product number 59844), total RNA/DNA Mini Kit (Geneaid, New Taipei City, Taiwan), PCR reagent SensiFAST SYBR No-ROX Kit (Meridian Bioscience, USA), dimethylsulfoxide (DMSO, SRL, India, product number 30239), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA), phosphate buffer saline (PBS, Sigma-Aldrich, St. Louis, MO, USA), Roswell Park Memorial Institute 1640 (RPMI 1640, Merck, USA), Fetal bovine serum (FBS, Merck, USA), and 1% penicillin-streptomycin (PS, Merck, USA).

2.2 | Plant Preparation and Extraction

The aerial part of *Pogostemon cablin* Benth. plant was collected from a patchouli plantation in Cianjur, West Java. The taxonomic identification of the plant material was verified using reference books and research articles [15, 24]. To prepare the extract, 189 g of aerial parts of *P. cablin* Benth. were cut down and subjected to maceration in 96% ethanol (48 h, room temperature). The solvent was subsequently removed via evaporation using a rotary evaporator at an optimal temperature of 50°C, and the resulting extract was transferred and placed in a dark vial. To prepare a 100,000 ppm PCEE stock solution, 100 mg of extract was dissolved in 1 mL of DMSO utilizing a vortex mixer. The stock solution was subsequently stored in a light-resistant vial under refrigeration. Upon readiness of the cells for treatment, the stock solution was diluted in RPMI to achieve the specified concentration.

FIGURE 1 | Patchouli plant (Patchouli Cultivation Book. East Java Provincial Plantation Departement, 2013).

2.3 | Cell Culture and Doxorubicin-Stimulated Senescence in 3T3-L1 Cells

3T3-L1 cells were cultured in RPMI 1640 media (Merck, USA), with 10% FBS supplementation (Merck, USA) and 1% penicillin-streptomycin (Merck, USA) at 37°C and 5% CO_2. We used 0.5 μM doxorubicin to stimulate senescence in 3T3-L1 cells during a 24-h incubation period.

2.4 | Treatment of PCEE on Doxorubicin-Stimulated Senescence in 3T3-L1 Cells

Doxorubicin-induced 3T3-L1 cells were co-treated with PCEE at various concentrations (1, 10, 75, or 200 ppm). The cells were incubated for 24 h at 37°C and 5% CO_2. Subsequently, cell morphology, ROS accumulation, and MTT assay were evaluated. For the *GLB-1* gene expression and telomere length experiments, 100 μM DL-α-Tocopherol Acetate (Vit E) was utilized as a positive control.

2.5 | MTT Assay

The 3T3-L1 fibroblast cell line was seeded into a 96-well plate tissue culture at a concentration of 7500 cells/well of a 96-well plate (60%–70% confluence) for 20 h at 37°C in a CO_2 incubator. Cells were stimulated with doxorubicin 0.5 μM for a 24 h incubation to produce 3T3-L1 senescence cells. Then, senescence cells were treated with various concentrations of PCEE (1, 5, 10, 25, 50, 75, 100, 200, 300, 400, and 500 ppm). Senescence cells without ethanol extract treatment or with vitamin E were used as negative and positive controls, respectively. After 24 h of incubation, cell viability was measured with an MTT (Sigma-Aldrich, St. Louis, MO, USA). Optical density was measured at 590 nm with a microplate reader (Thermo Scientific Multiskan EX, USA).

2.6 | ROS Accumulation Assay

DCFHDA BioReagent was utilized to evaluate the accumulation of ROS in the cells. A fluorescent microscope (Olympus, BX51, Japan) was employed to observe the ROS staining outcomes. The microscope was equipped with a Snapscan VNIR Hyperspectral Imaging Camera (Scientific Imaging Inc., part number FPRFV0003A, USA) that was connected to a computer and used with Case Data Manager 6.0 software at a magnification of 100×.

2.7 | DNA/RNA Isolation and PCR Analysis

After intervention with doxorubicin and PCEE, DNA/RNA was isolated using Geneaid's Total RNA/DNA Mini Kit (Cultured Cell). The RNA extraction procedure follows Geneaid's DNA/RNA Mini Kit (Cultured Cell) guidelines, New Taipei City, Taiwan. Then, mRNA expression was examined with PCR SensiFAST SYBR No-ROX Kit (Meridian Bioscience USA) 2-step cycling at 95°C for 2 min for polymerase activation and 5 s for denaturation, then 60–65°C for 15–30 s for annealing/extension, using AriaMx (Agilent, G8830A, USA) Real-time PCR System. Data were analyzed by the $2^{-\Delta\Delta C_t}$ method, using the housekeeping gene, *GAPDH*, as the internal control. The primers are described in Table 1.

2.8 | Agarose Electrophoresis Gel Examination PCR

Gel electrophoresis experiments were performed using 1.5% agarose gels and 6 × 6 cm mini-PCR rigs (Gelato, QP-1600-01, minipcr bio, Cambridge, MA, USA) with a default gel volume of 20 mL. The agarose was dissolved by microwaving for 60 s on high in the same electrophoresis buffer used to cover the gel in the rig. The gels were stained with 1 μL Gel Green Nucleic Acid Stain 10,000 × in water (minipcr bio, Cambridge, MA, USA). After pouring the melted agarose into a plastic tray lined with tape on both ends, the gels were allowed to solidify at room temperature for at least 30 min. Allowing less time to solidify increased the chance of band artifacts, likely due to the agarose near the wells not being completely set. Electrophoresis was performed until the front edge of the tracking dye migrated to approximately 70% down the gel. Electrophoresis was conducted at 75 V in TBE buffer for 30 min. Image visualization was achieved using UV blue light with the Gelato, QP-1600-01, mini-PCR rigs (minipcr bio, Cambridge, MA, USA).

2.9 | Statistical Analysis

Each experiment was performed at least three times, and the results were presented as the mean of ±SD. One-way analysis of variance (ANOVA) followed by Tukey's test was used compare the differences between means. Statistical significances were considered at $p < 0.050$.

3 | Results

3.1 | Inhibitory Effects of PCEE Against Doxorubicin-Induced ROS in 3T3-L1 Cells

According to the MTT assay, PCEE exhibits an IC_{50} value of 336.593 ppm. The viability of 3T3-L1 cells remains at 100% up to a concentration of 200 ppm after treatment, indicating that

TABLE 1 | Primer sequences used for PCR analysis.

Gene	Primer
GLB1[a]	F: 5′TCCCACTGAACACTGAGGC3′ R: 5′GGAGTATGAGGTCCGAAGAAT 3′
GAPDH[b]	F: 5′-CAAGATCATCAGCAATGCCTCC-3′ R: 5′-GCCATCACGCCACAGTTTCC-3′
Telomere[c]	F: 5′CGGTTTGTTTGGGTT TGGGTTTGGGTTTGGGTTTGGGTT3′ R: 5′GGCTTGCCTTACCC TTACCCTTACCCTTACCCTTACCCT3′

[a]Liu et al. [25].
[b]Aslan et al. [26].
[c]O'Callaghan and Fenech [27].

the concentration range of 0–200 ppm is suitable for the next investigation (Figure 2A). This result is in accordance with the morphological appearance of PCEE-induced 3T3-L1 cells. We used an inverted microscope to analyze the cell morphology. Following treatment with PCEE at concentrations exceeding 200, specifically at 200 or 300 ppm, morphological alterations were observed in the cells, characterized by an increase in cellular size (PCEE 200 ppm), the presence of rounded-up cells, and a decrease in cell number (PCEE 300 ppm), relative to the control (Figure 2B).

To prove that ROS accumulation in cells was solely induced by doxorubicin and not by PCEE, we conducted a ROS accumulation assay at various concentrations of PCEE. The results indicate that at a concentration starting from 100 ppm, PCEE induced 2′, 7′-dichlorofluorescein (DCF) formation (Figure 3A). Therefore, we used PCEE 75 ppm for further analysis.

This study employed a 0.5 μM dose of doxorubicin on 3T3-L1 cells for a 24-h incubation. At the specific dosage, doxorubicin was capable of inducing ROS accumulation without causing cellular toxicity (Figures 3B and 4). It is well established that cellular senescence is closely linked to ROS production, which ultimately results in oxidative stress. Notably, PCEE suppressed ROS accumulation in 3T3-L1 induced by doxorubicin (Figure 3B). Further analysis utilizing ImageJ, a software to quantify the accumulation of ROS fluorescence intensity, showed that the administration of PCEE significantly reduced the accumulation of ROS. The fluorescent intensity quantification for doxorubicin alone was 220.977, whereas, with the addition of PCEE, this value decreased to 169.817 (Figure S1).

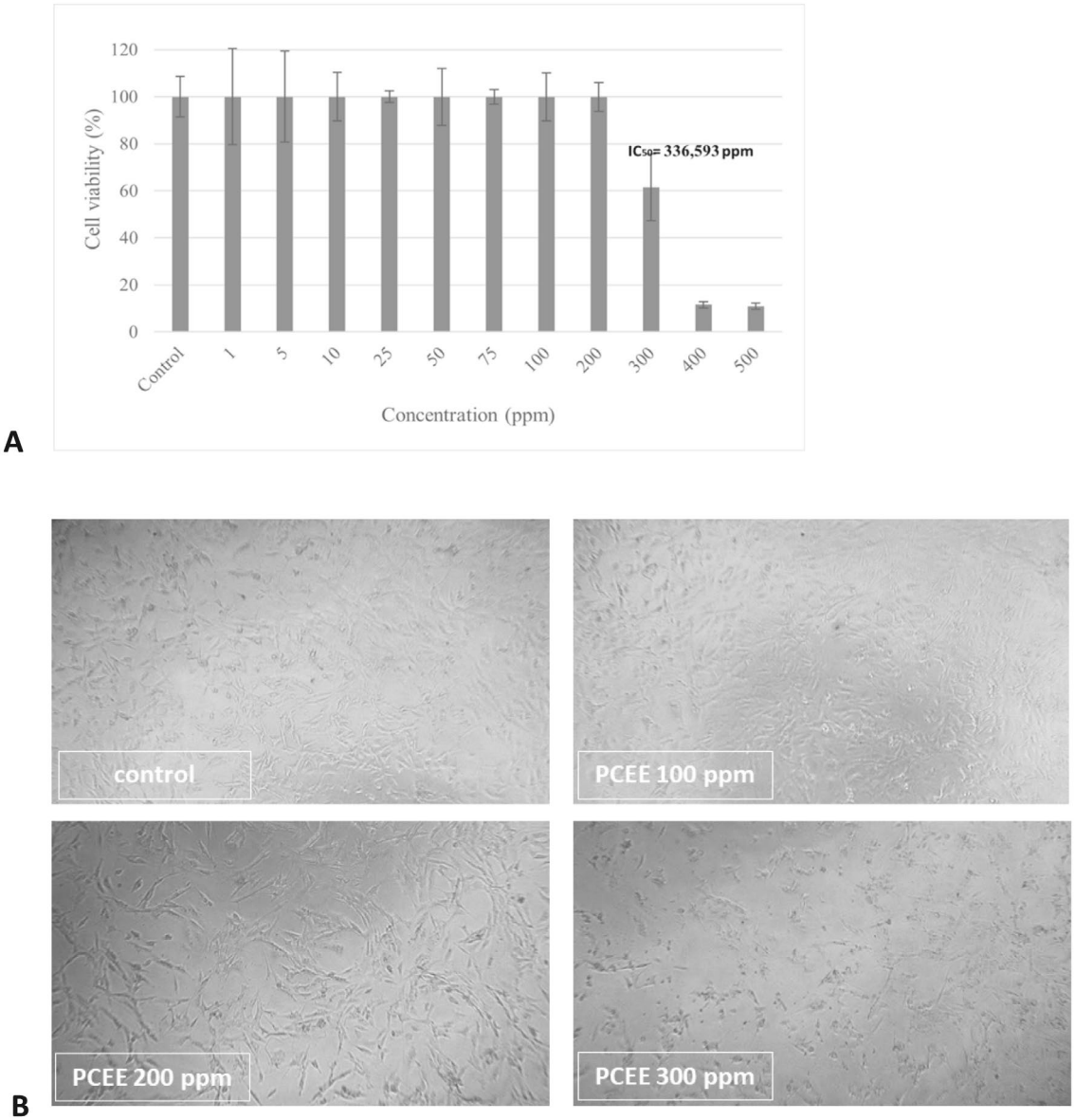

FIGURE 2 | Toxicity assay of PCEE. (A) Relative cell viability as affected by 24 h treatment in complete medium with PCEE 1,5,10,25,50,75,100 ,200,300,400, or 500 ppm or the solvent (control). Cell viability was then evaluated using an MTT assay and expressed as a percentage of untreated cells. Values represent the mean ± SD of triplicate experiments. (B) Cell morphology after treatment with PCEE. An inverted microscope evaluated the cell morphology, showing 40 × magnification. 3T3-L1 cells were treated with 100, 200, or 300 ppm PCEE for 24 h.

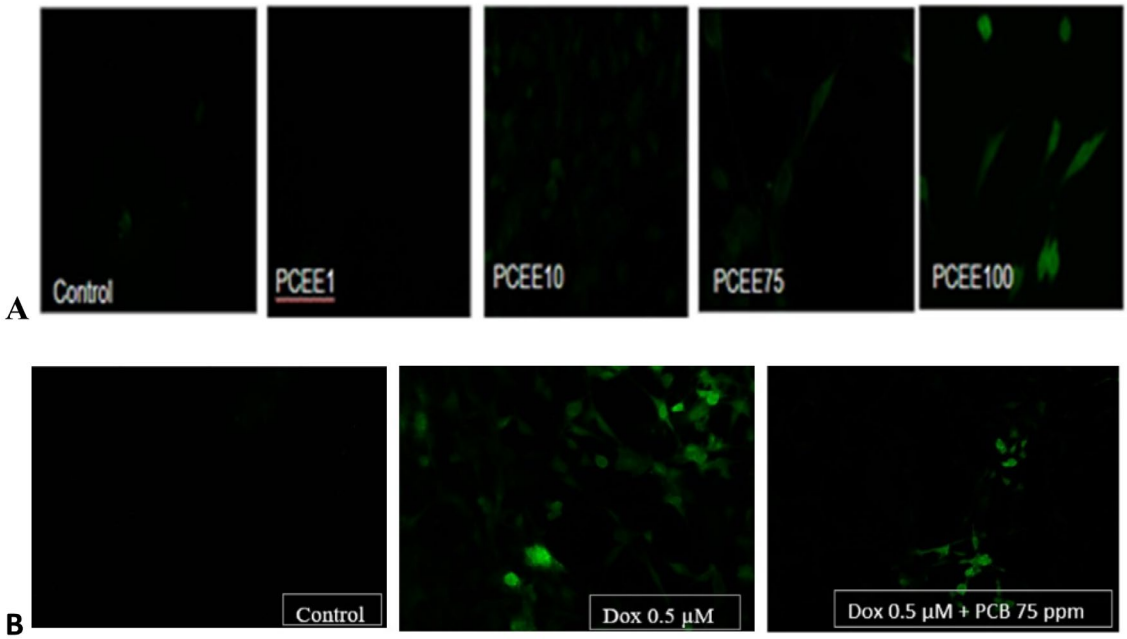

FIGURE 3 | ROS assay PCEE. (A) 3T3-L1 cells were treated or left untreated with various concentrations of PCEE for 24 h. After the PCEE treatment, DCFHDA BioReagent was added and incubated with the cells in the dark for 30 min before measuring the fluorescence using fluorescent microscopy, 40× magnification. (B) PCEE suppresses the accumulation of ROS. 3T3-L1 cells were treated in a control medium, doxorubicin 0.5 μM, and doxorubicin 0.5 μM + PCEE 75 ppm.

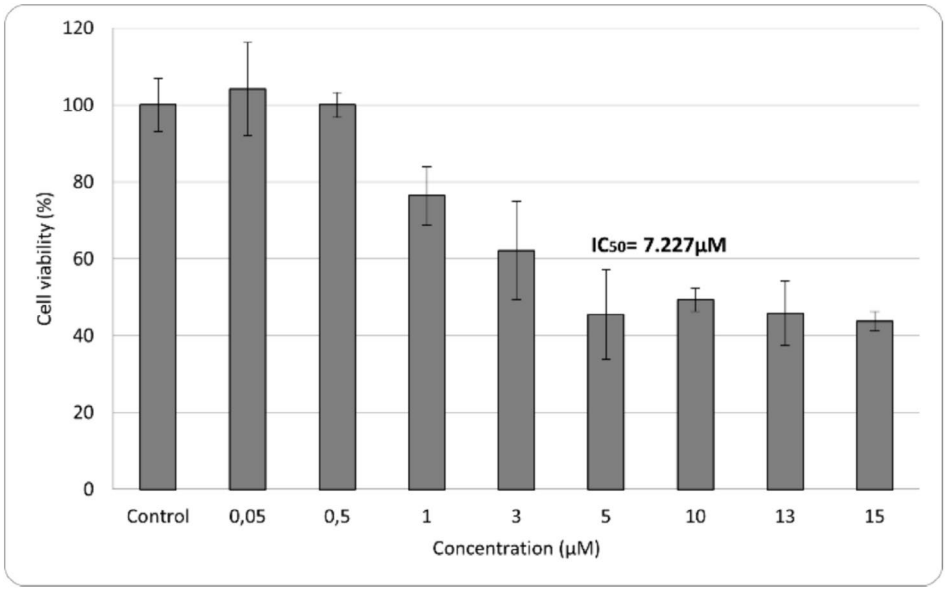

FIGURE 4 | Effect of doxorubicin on cell viability of 3T3-L1 cells. Relative cell viability as affected by 24 h treatment in complete medium with doxorubicin 0.05,0.5,1,3,5,10,13, 15 μM or the solvent (control). Cell viability was then evaluated using an MTT assay and expressed as a percentage of untreated cells. Values represent the mean ± SD of triplicate experiments.

3.2 | PCEE Affects the Decrease of the Senescence-Associated β-Galactosidase Gene in 3T3-L1 Cells Induced by Doxorubicin

Currently, there is a lack of research on patchouli's antiaging potential. In this study, we conducted experiments to investigate PCEE's possible antiaging effect, with a particular focus on SA-β gal and telomere markers. Our findings indicated that PCEE can reduce the expression of the *GLB-1* gene, which encodes the SA-β gal protein, in a manner similar to vitamin E, which served as a positive control (Figure 5).

3.3 | PCEE Successfully Maintained Telomere Length in Doxorubicin-Induced 3T3-L1 Cells

Subsequently, we conducted telomere length analysis utilizing PCR followed by agarose gel electrophoresis. The results indicate

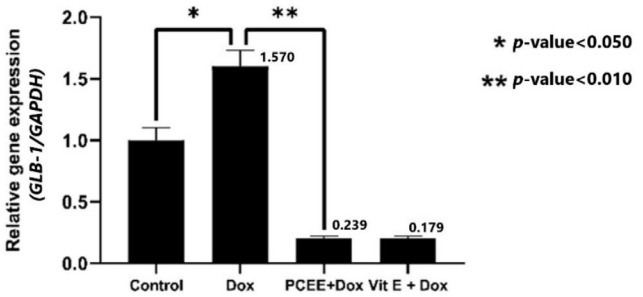

FIGURE 5 | Expression level of the *GLB-1* gene verified by qRT-PCR. *Represents the p value <0.050, **represents the p value <0.010. Data were shown as mean ± SD. The left vertical axis represents gene expression levels verified by qRT-PCR. The horizontal axis represents the treatment group: Control, doxorubicin, PCEE+doxorubicin, and vitamin E + doxorubicin.

successful isolation and amplification of telomeres. As evidenced by the PCR amplification curve and agarose gel electrophoresis, it is apparent that doxorubicin induces telomere shortening, whereas PCEE effectively maintains telomere length in doxorubicin-induced 3T3-L1 cells. The capacity of PCEE to preserve telomere length is nearly comparable to that of vitamin E (Figure 6).

4 | Discussion

The objective of this study was to investigate the antiaging properties of PCEE in relation to the aging profile of 3T3-L1 cells induced by doxorubicin. To address this aim, experiments were conducted to assess the effects of PCEE on ROS accumulation, senescence-associated β-galactosidase (*GLB-1*) gene expression, and telomere length in doxorubicin-induced 3T3-L1 cells. Initially, we successfully demonstrated that the doxorubicin-induced senescence model in 3T3-L1 cells was characterized by ROS accumulation, increased *GLB-1 gene* expression, and telomere shortening. Subsequently, upon treating cells with PCEE, we observed that PCEE exhibited the capacity to inhibit ROS, downregulate the *GLB-1* gene, and effectively maintain telomere length in doxorubicin-induced 3T3-L1 cells.

The capacity of PCEE to suppress ROS accumulation in this study aligns with other research that has demonstrated the presence of antioxidant properties in the patchouli plant [28, 29]. The main active component of *Pogostemon cablin* Benth., PA, has been shown to exhibit antioxidant properties [15, 29]. Studies have reported that patchouli alcohol can reduce cellular morphology damage and decrease accumulated malondialdehyde (MDA) due to ROS [15, 30]. Patchouli extract has also been found to promote numerous pharmacological activities, including protection against oxidation [15, 31].

Several studies have proposed that the mechanism by which patchouli alcohol provides antiaging and antioxidant effects is through the Nrf2-HO-1 pathway. The HO-1 enzyme has been reported to inhibit inflammation that frequently occurs due to aging by suppressing the transcription factor genes cyclo-oxygenase-2 (COX-2), inducible Nitric Oxide Synthase (iNOS), tumor necrotizing factor alpha (TNF-α), interleukin 6 (IL-6), thereby reducing Prostaglandin E2 (PGE2) and inhibiting inflammation [28, 32].

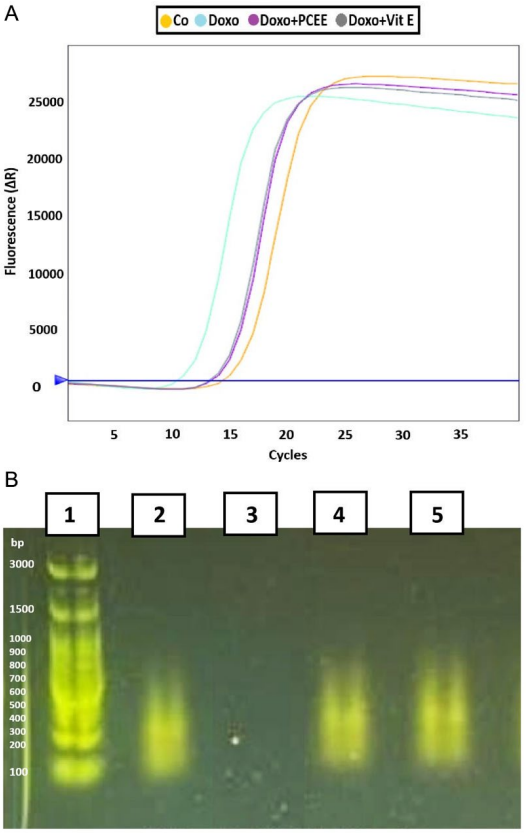

FIGURE 6 | PCR agarose electrophoresis for measuring telomere length. (A) PCR amplification curves. (B) Gel analysis of the PCR product generated from amplification. Lane (2) control medium (3) doxorubicin (4) PCEE and doxorubicin (5) vitamin E and doxorubicin. The size of the DNA band in DNA marker lane (1) is indicated on the left.

Haiming Chen et al. and Xiao Ying Chen et al. reported pogostone in *P. cablin* Benth. in Sprague Dawley (SD) rats; the experiment with doses of 10, 20, and 40 mg/kg BW orally has antioxidant effects by increasing super oxide dismutase (SOD), glutathione sulf-hydryl (GSH), catalase (CAT), and decreasing MDA [15, 33, 34]. Liang et al. also reported that the presence of PAO (patchoulene epoxide) in patchouli plants in an experiment on SD rats at oral doses of 10, 20, and 40 mg/kg body weight showed increased activity in enhancing GSH, SOD, and CAT while reducing MDA levels [35].

Our further analysis indicated that PCEE can decrease *GLB-1* gene expression, which codes for SA-β gal protein. We hypothesize that the decrease in senescence β-galactosidase is attributable to the antioxidant properties of PCEE. The antioxidant and anti-inflammatory abilities are often associated with its antiaging properties [14, 16, 17, 28]. Beta-galactosidase is an enzyme that breaks down galactoside into glucose-6-phosphate and adenosine triphosphate (ATP) detected as active in the lysosome organelle at pH4 only in normal cells. Under cellular senescence conditions, galactoside is used as an energy source, resulting in a heightened presence of the beta-galactosidase enzyme within the lysosome. Consequently, its activity can be discerned at pH6, termed as SA-β-gal [36]. As Roberta Ricciarelli et al. reported, the level of SA-β-gal increased in cells with high passage and aging in subjects of human primary skin fibroblasts and significantly decreased when treated with vitamin E [37]. In line with this study, a study conducted by Chen et al. reported that PA inhibits

oxidative stress, thereby improving the quality of aging cartilage by activating the Nrf2/HO-1 pathway [28]. These findings align with our study, which suggests that PCEE functions as an anti-aging agent. Further research, such as examining the expression level of SA-β-gal protein, is needed to confirm our results.

As it has been known from previous research, senescent cells have shorter telomeres compared to young cells. When the telomere reaches a critical shortening length, the cell will no longer divide and enter a state of senescence. Telomeres function to prevent end-to-end fusion between chromosomes and maintain chromosome integrity. During each cell replication, an "end fork replication problem" occurs where the lagging strand DNA shortens by about 8–12 base pairs due to the removal of RNA primer during replication. With telomeres at the end of the chromosome, the loss of base pairs containing genetic information can be prevented [38–40].

Our study demonstrated that PCEE effectively maintains the shortened telomere length caused by doxorubicin in 3T3-L1 cells. As a DNA intercalator, doxorubicin disrupts the telomere DNA replication process, and as a ROS inducer, it also leads to telomere degradation and shorter telomere length [41]. In our study, we observed a similar effect; this shortened telomere length was not visible on agarose electrophoresis.

In conjunction, we postulated that PCEE acts against senescence associated with oxidative stress stimulation. This action is similar to that of vitamin E, which was previously reported [42–44]. Oxidative stress is a significant factor that causes telomere shortening [38, 45, 46]. Telomeric DNA is particularly vulnerable to oxidative damage compared to nontelomeric DNA.

5 | Conclusion

Our study demonstrated that PCEE can inhibit ROS, decrease the *SA-β gal* gene, and maintain telomere length in doxorubicin-induced 3T3-L1 fibroblast cells. These findings corroborate that the patchouli plant is identified as a natural resource of antioxidants. Emerging evidence concerning the bioactivity of PCEE in diverse experimental settings supports that PCEE could be a promising therapeutic or preventive agent for degenerative diseases or aging. Recently, molecular targets of the patchouli plant have been reported in diverse experimental in vitro and in vivo models; however, there is still a small number of human clinical studies in antiaging. Based on the evidence in this study, we propose that PCEE acts against senescence associated with oxidative stress stimulation mechanisms and could be a promising multipotent drug and, thus, a safe natural compound for anti-aging candidates.

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request. The references used to support the findings of this study are included in the article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.