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Anti-senescence activity of Indonesian black pepper essential oil (*Piper nigrum* L.) on ovarian CHO-K1 and fibroblast NIH-3T3 cells

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ABSTRACT

Background: Cellular senescence or cell aging is a progressive process caused by an increase in free radicals so that cells cannot proliferate. Black pepper essential oil (BPEO) contains terpenoid compounds that perform the antioxidant activity to inhibit cell aging. **Aims:** This study aims to explore the potential of BPEO as an anti-senescence agent to maintain body fitness through *in vitro* methods on ovarian CHO-K1 and fibroblast NIH-3T3 cells. **Subjects and Methods.:** BPEO was obtained by hydrodistillation, then identified the compounds by gas chromatography–mass spectrometry. The cytotoxicity of BPEO was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The senescence was detected by senescence-associated beta-galactosidase assay. Furthermore, the Reactive oxygen species (ROS) level effect was analyzed under 2',7'-dichlorofluorescein diacetate staining assay with flow cytometry. **Results:** The BPEO contains 46 compounds, which the highest concentration compound is β-caryophyllene, as much as 22.60%. Cytotoxic tests indicated that BPEO up to the concentration of 200 μg/mL is not cytotoxic on CHO-K1 and NIH-3T3 cells. Furthermore, the administration of BPEO at concentrations of 50 μg/mL and 100 μg/mL reduces the number of senescent positive cells. This occurs seemingly irrespective of the ROS pathway. **Conclusion:** Overall, this study shows that BPEO contains active compounds that are safe for normal cells and has anti-senescence activities on CHO-K1 and NIH-3T3 cells.

Keywords: Anti-senescence, Chinese hamster ovary cells, essential oil, NIH-3T3 cells, *Piper nigrum* L., reactive oxygen species

INTRODUCTION

Senescence is a physiological process that occurred at the cellular level involving complex signaling pathways.[1] An increase in reactive oxygen species (ROS) level and telomere shortening is an essential factor that can induce senescence with the molecular markers of increasing p53 and p16 expression.[2] In general, the accumulation of ROS levels over the threshold can lead to DNA damage that halts cell proliferation with the senescent phenomenon.[3] Therefore, senescence is also known as a phenomenon by which the cells undergo permanent cell cycle arrest that may occur on any cell in the body, leading to cells or tissues aging and even degeneration.[4] This phenomenon is an unexpected condition and should be avoided by reducing the factors that trigger senescence, such as high energy of light and radicals source of chemicals, and ROS inducing agents or radical containing agents.[5]

Skin tissue is one of the main barriers for the body against agents that can damage the body, both in physical and chemical forms.[6] However, skin tissue is also among those prone to aging due to UV light exposure, which causes increased ROS levels.[7] The skin tissue is composed of fibroblast cells

supported by a matrix formed by connective tissue so that it has a thick, elastic, and robust texture.[8] The occurrence of senescence in fibroblast cells will cause a restructuring of the connective tissue making it no longer tight or saggy.[9] These events are often referred to as aging, characterized by the formation of wrinkles on the skin.[10] Therefore, the protection of fibroblast cells from exposure of senescence inducing agent is necessary to maintain the skin fitness.

We also noted that high ROS can disturb the regeneration of the female reproductive system, especially ovarian cells.[11] The oviduct is constructed by ovarian cells, which function to transmit gametes. Besides, the oviduct is an essential place for fertilization and regulates the initial embryonic development that determines pregnancy success.[12] Senescence in ovarian cells can cause proliferative disorders, resulting in decreased egg production.[13] Consequently, senescence in ovarian cells can reduce fertility and broadly cause a wide range of health problems, particularly for post-menopausal women.[14] Therefore, it is necessary to inhibit aging on ovarian cells to avoid such diseases.

The essential oil black pepper (*Piper nigrum* L.) performs antioxidant and antimicrobial activities.[15-17] Antioxidants are widely used to prevent deterioration of oxidizable products such as food, cosmetics, and pharmaceuticals.[15,16,18] Black pepper essential oil (BPEO) contains terpenoid compounds such as β-caryophyllene, limonene, β-pinene, and sabinene.[19-21] The main content of β-caryophyllene in essential oils of black pepper has antioxidant activity better than butyl hydroxy anisole and butyl hydroxy toluene.[19] β-caryophyllene also found in other essential oil producing herbs such as *Alpinia galanga* with less abundant compared to black paper. However, the non-polar extract of *A. galanga* which contain β-caryophyllene exhibited antioxidant activity by reducing ROS generation of fibroblast cells.[22] Moreover, this extract also performed anti-senescent activity on NIH-3T3 cells.[22] Besides, black pepper extract is also not cytotoxic to CHO-K1 cells.[23] Therefore, this study focuses on the exploring the potential activity of black pepper essential oil as anticellular senescence against fibroblast and ovarian cells in correlation with oxidative stress condition.

SUBJECTS AND METHODS

BPEO Preparation

Black pepper fruits (*P. nigrum* L.) were obtained from the Center of Materia Medica Batu, Malang, Indonesia, and have been identified and the voucher has been collected as herbarium in the center. Seven hundred grams of fruits were mechanically ground and distilled using hydrodistillation for 4 h in the Clevenger apparatus. Then, the essential oil was given sodium sulfate anhydrous to take the spent of water. All the essential oil were collected in vials and stored at 4° before use.

Cell Culture

The CHO-K1 ovarian normal cells (ATCC® CCL-61TM) and fibroblast normal cells NIH-3T3 (ATCC® CRL-1658) were gained from Professor Masashi Kawaichi, Nara Institute of Science and Technology, Japan. The CHO-K1 cells were cultured in Roswell Park Memorial Institute, while the NIH-3T3 cells cultured in Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum 10% and 1.5% penicillin-streptomycin at 37°C in 5% CO_2 humidified atmosphere and 95% air in a CO_2 incubator. Cells were passed twice/week under sterile conditions.

MTT Assay

Cells were seeded into each well of 96-well plates at concentration 9×10^4 cells/mL and treated with various concentrations of BPEO and in combination with doxorubicin (Sigma) for 24 h. After incubation, the media were discharged, and cells were washed with phosphate-buffered saline (PBS) 1×, then added the MTT reagent (Sigma) and incubated for 4 h. SDS was added and incubated overnight, and then, the absorbances were measured on a microplate reader (Bio-Rad) under $\lambda = 595$ nm.

Senescence-Associated Beta-Galactosidase (SA-β-Gal) Senescence-based Assay

Cells (2×10^5 cells/well) were seeded into each well of the 6-well plates, incubated for 24 h. Cells were washed with PBS 1× twice. Then, a fixation buffer is added, waiting for a while, then washed using PBS 1× once. Further, 1–2 mL X-Gal solution was added, incubated at 37°C. Cells were monitored after 72 h under a microscope (CKX-41 Olympus) using 200×. The appearance of the blue cell is the galactosidase-positive cells as senescent cell representation.[24]

2',7'-Dichlorofluorescein Diacetate (DCFDA) Staining ROS-based Assay

The cells were seeded in 24-well plates for 24 h. After that, the cells are collected and put into a sterile microtube in the supplemented buffer. Then, 10 mM DCFDA (Sigma) was added and incubated for 30 min in a CO_2 incubator. Furthermore, CHO-K1 and NIH-3T3 cells were treated with BPEO in a single and combination with doxorubicin during 4 h. The DCF fluorescence of at least 2500 cells was detected and analyzed by flow cytometry (BD Accuri C6 flow cytometer) to an emission wavelength at 525 nm and excitation wavelength at 488 nm.

Gas Chromatography–Mass Spectrometry (GC–MS) Assay

Analyses were carried out on a Shimadzu single quadrupole GCMS-QP2010 Ultra GC–MS. The compounds were separated on a QP2010 capillary column (30 m × 0.25 mm × 0.25 film). The splitless injection was used for both MD–HS-SPME and conventional HS-SPME samples. The column oven temperature was programmed from an initial temperature set at 40°C for 10 min, increased to 180°C at a rate of 2.5°C/min, and then maintained at a speed of 20 min. The injection temperature and ion source temperature were 250 and 230°C, respectively. Then, the helium gas was used for the carrier with a flow rate of 0.55 mL/min. The ionizing energy was 70 eV. All data were obtained by collecting the full scan mass spectra within the scan range of 28–600 m/z. After that, the spectra were identified using the Wiley mass spectral library.

Data Analysis

Data were analyzed using Student's *t*-test. The experimental data were stated as mean ± SD, the significance of differences among the various treated groups was set with *P* values ($^*P < 0.05$; $^{**}P < 0.01$) which were included in each experiment figure.

RESULTS

Cytotoxic Effect of BPEO on CHO-K1 and NIH-3T3 Cells

The study proposed to examine the potential of BPEO as an anti-senescence agent on normal cells. At first, the cytotoxic effect of BPEO was determined using MTT assay toward CHO-K1 and NIH-3T3 cells for evaluating the safety. We used CHO-K1 cells as a model for normal ovarian cells and NIH-3T3 cells as model fibroblast cells representing skin tissue. The cytotoxic effects are evaluated based on the percent of cell viability after treatment for 24 h. We found that BPEO up to 200 µg/mL did not decrease cell viability significantly in both cells [Figure 1]. This result hopefully indicates that BPEO is safe for ovarian cells and fibroblast cells. Hence, BPEO is considered non-cytotoxic and showed that BPEO could maintain normal cell viability.

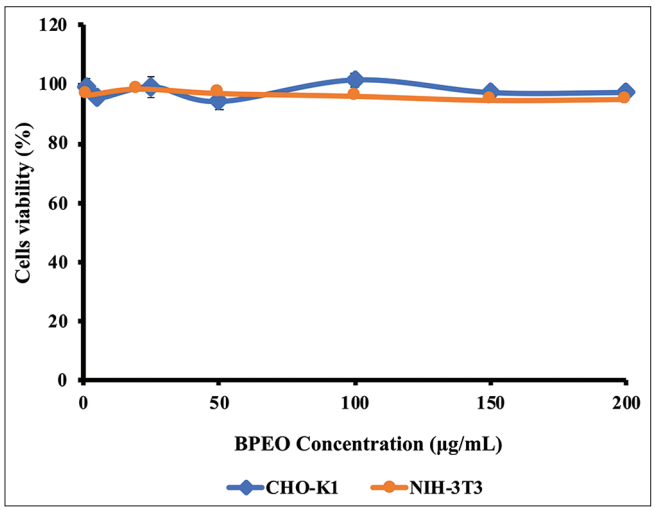

Figure 1: Cytotoxic effect of BPEO on CHO-K1 and NIH-3T3. The cytotoxic effect wasevaluated based on cell viability after treatment with BPEO. CHO-K1 cells (8×10^3 cells/mL) and NIH-3T3 (1×10^4 cells/mL were treated with BPEO 1-200 μg/mL for 24 h then subjected to be evaluated by MTT assay. The administration of BPEO was not cytotoxic in normal ovarian CHOK1 cells and fibroblast NIH-3T3 cells, proven by the trend of cell viability

Anti-senescence Activities of BPEO on CHO-K1 Cells and NIH-3T3 Cells

The SA-β-Gal assay aims to explore the potential anti-senescence of BPEO. In this study, we used doxorubicin as a drug that can induce senescence.[25] The effect of single BPEO at 50 µg/mL and 100 µg/mL does not change the morphologically on CHO-K1 and NIH-3T3 cells. Otherwise, its combination with doxorubicin can change both cells morphologically into blue cells and more prominent than normal cells [Figures 2a and 3a]. After quantified the cells populations showed that the effect of treatment of a single BPEO at 50 µg/mL and 100 µg/mL does not affect the occurrence of senescence significantly on both cells [Figures 2b and 3b], which indicates that BPEO administration is safe for ovarian cells and fibroblast cells. Interestingly, the combination of BPEO and doxorubicin at 50 µg/mL and 100 µg/mL can reduce percentage of the senescent cell to basal conditions ($P < 0.01$) [Figures 2b and 3b]. This result is valid because the doxorubicin can induce the senescence cells significantly than the untreated cells. Therefore, the BPEO demonstrated that it is safe for ovarian and fibroblast cells in a normal condition. In addition it can also reduce the senescence cells, which is shown by the appearance of the cells after induction using doxorubicin.

Effect of BPEO on ROS Level in CHO-K1 and NIH-3T3 Cells

This test aims to ensure whether ROS plays a role in decreasing senescence. ROS are by products released through metabolic processes.[26] ROS accumulation that exceeds the acceptable

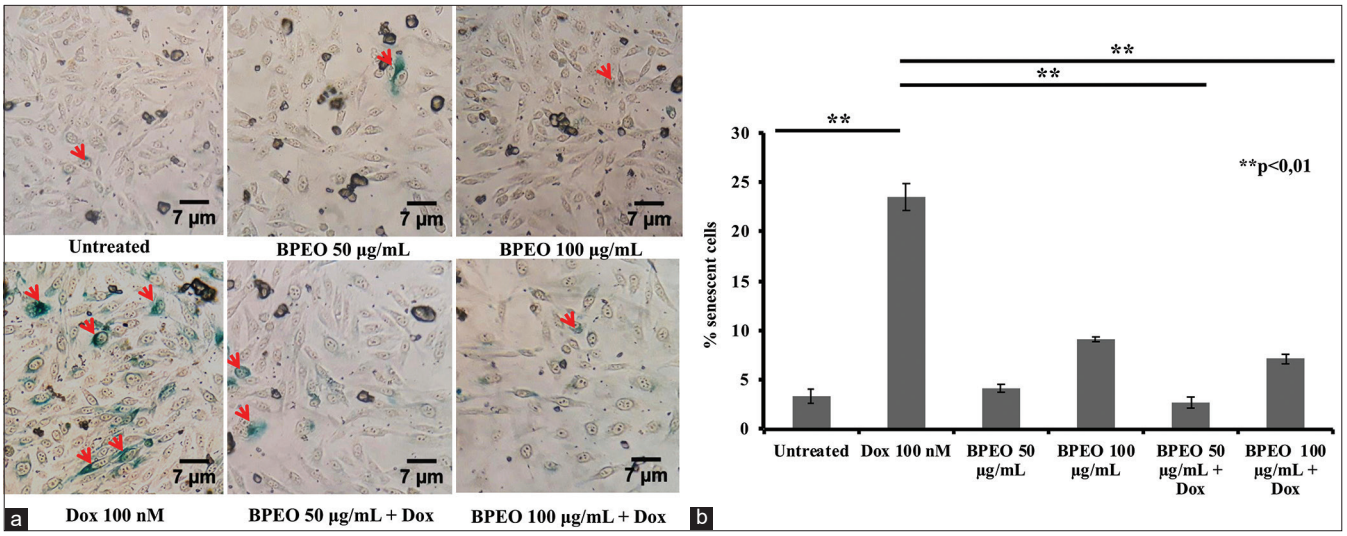

Figure 2: Senescence induction following BPEO treatment on CHO-K1 cells. Senescence cells were analyzed using an SA-βgalactosidase staining assay. CHO-K1 cells as a normal model (15×10^4 cells/mL) were treated with BPEO 50 and 100 μg/mL, doxorubicin (100 nM) combination for 24 h, then subjected to β-galactosidase staining. The percentages of senescent cells (β- galactosidase-positive cells) were calculated (n = 3). (A) The morphology of cells after 72 hours staining under an inverted microscope with magnification 200x (B) The percentage of senescent cells after treatment

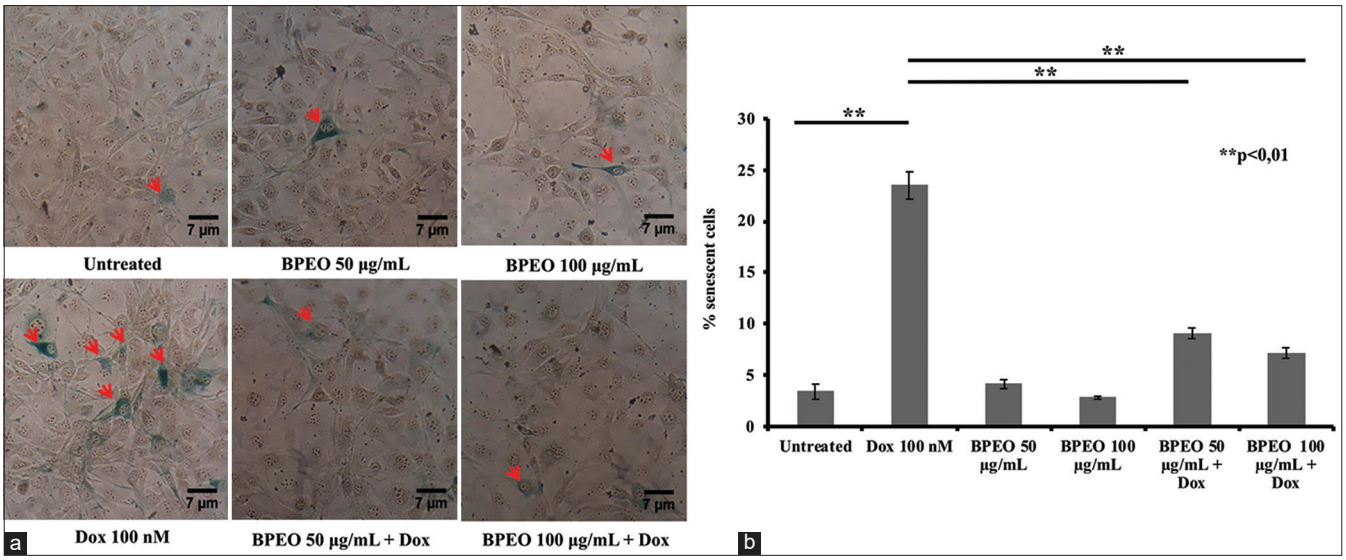

Figure 3: Senescence induction following BPEO treatment on NIH-3T3 cells. Senescence cells were analyzed using an SA-βgalactosidase staining assay. NIH-3T3 cells as a normal model (2×10^5 cells/mL) were treated with BPEO 50 and 100 μg/mL doxorubicin (100 nM), and in combination for 24 h then subjected to β-galactosidase staining. The percentages of senescent cells (β-galactosidase-positive cells) were calculated (n = 3). (A) The morphology of cells after 72 hours staining under an inverted microscope with magnification 200x (B) The percentage of senescent cells after treatment.

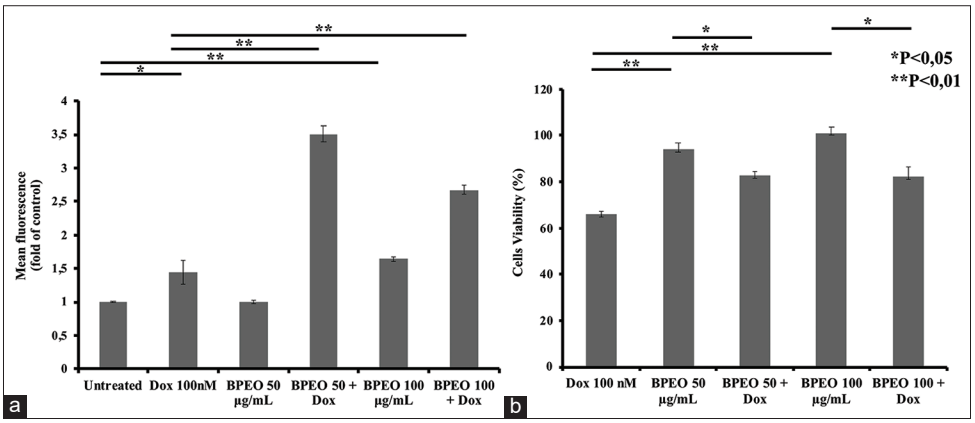

Figure 4: Effect of BPEO on intracellular ROS level and cytotoxic effect on CHO-K1 cells. Total ROS level after single and combination treatment of BPEO with doxorubicin was observed under DCFDA staining assay. (A) CHO-K1 cells (5×10^5 cells/mL) were treated with BPEO 50 and 100 μg/mL, doxorubicin (100 nM), and in combination for 4 h, then subjected to the ROS detection using flow cytometry (n=3). (B) The cytotoxic combination with Dox. The significances between treatment

level by cells can cause DNA damage that proceeds in the aging phase (senescence).[3] We obtained that BPEO increases intracellular ROS levels compared to untreated cells. A similar effect was performed on the cells after being treated with BPEO, and doxorubicin resulted in high intracellular ROS levels compared to doxorubicin as a positive control [Figure 4a]. However, this phenomenon did not resemble the result in cell viability, which means that ROS level did not affect the live cells [Figure 4b]. Thus, it can be inferred that aging in CHO-K1 cells may not occur through the intracellular ROS pathway.

Furthermore, the effect of intracellular ROS due to exposure to BPEO in NIH-3T3 cells has a different effect, while doxorubicin with a concentration of 100 nM does not significantly improve ROS compared to cell control. Besides, BPEO at concentrations of 50 µg/mL and 100 µg/mL, both singly and combined, did not affect intracellular ROS levels [Figure 5a]. This result confirmed the combination cytotoxic result does not decrease the viability of the cells significantly. This result strengthens the finding that the senescence event in NIH-3T3 cells also does not occur through a decrease in ROS level.

Chemical Composition of BPEO

The distillation process gives the BPEO yield of 0.65% w/w. Based on GC–MS chromatogram profile, BPEO contains 46 active compounds [Figure 6 and Table 1]. The analysis showed that the highest concentration content of BPEO was β-caryophyllene with an abundance of 22.60% supported by a molecular weight of 240 m/z, with 96% similarity level to the database used [Figure 6], followed by trans-anethole (17.56%), 1-limonene (10.82%), 3-carene (9.24%), and β-sabinene (5.75%). In comparison, the other compounds which are contained in BPEO are shown in Table 1.

Table 1: The chemical composition of Black Pepper essential oil from different sources

No.	Components	RI(min)	% peak area
1	1-Methoxy-2-propyl ester of acetic acid	4.414	0.12
2	α-Thujene	5.999	0.54
3	α-Pinene	6.247	3.05
4	Sabinene	7.428	4.88
5	β-Pinene	7.620	5.75
6	Myrcene	7.882	1.50
7	3-Carene	8.604	9.84
8	1,3-Cyclohexadiene	8.861	0.13
9	1-Phellandrene	9.140	0.79
10	1-Limonene	9.303	10.82
11	Octatriene	9.825	0.13
12	1,4-Cyclohexadiene	10.262	0.27
13	Trans-sabinene hydrate	10.728	0.55
14	Terpinolene	11.194	0.28
15	Linalool	11.681	1.29
16	2-Cyclohexen-1-ol	11.817	0.58
17	Terpineol	14.470	1.54
18	3-Cyclohexene	14.983	0.42
19	Trans-Anethole	17.914	17.56
20	Delta-Elemene	19.191	1.02
21	α-Cubebene	19.541	0.18
22	Copaene	20.416	2.14
23	β-Elemene	20.782	1.07
24	α-Gurjunene	21.314	0.13
25	β.-Caryophyllene	21.755	22.66
26	α-Guaiene	22.092	0.33
27	α-Caryophyllene	22.688	1.49
28	Germacrene	23.371	0.47
29	β-Selinene	23.609	1.26
30	α-Selinene	23.781	1.09
31	β-bisabolene	23.985	0.28
32	Delta-Cadinene	24.298	0.70
33	β-Copaen-4	24.437	0.51
34	α-Calacorene	24.959	0.13
35	Cyclohexanemethanol	25.180	0.23
36	Caryophyllene Oxide	26.063	2.22
37	1H-Cycloprop[e] azulen-4-ol, decahydro-1,1,4,7-tetramethyl-	26.225	0.14
38	Humulene oxide	26.763	0.17
39	Spathulenol	27.258	1.63
40	Naphthalenol	27.670	0.50
41	Globulol	28.007	0.23
42	β-carotene	28.565	0.13
43	2,6,10-Cycloundecatrien-1-one	29.686	0.33
44	Phenol, 2-methyl-5-(1,2,2-trimethylcyclopentyl)	31.243	0.51
45	Isopropyl myristate	31.448	0.32
46	Cyclohexane	34.097	0.12

DISCUSSION

In general, our study aims to explore the potential of BPEO as an anti-aging agent to inhibit the aging process, especially in ovarian tissue and skin tissue. The process of aging is correlated with the increasing of ROS level in the cell.[5,7,27] Therefore, to ensure the safety of the material used, at first, a cytotoxic test was carried out on CHO-K1 cells as a model of ovarian tissue and NIH-3T3 cells as a representation of skin tissue. The result displayed that BPEO was not cytotoxic in both cells up to a 200 μg/mL concentration. These results indicated that BPEO is predicted to be safe, especially for ovarian tissue and skin tissue. These data also support the empirical evidence of Asian people who consume BPEO which do not experience poisoning or significant side effects.

Furthermore, the anti-senescence effect was evaluated using the SA-β-Gal assay *in vitro*. The results showed that BPEO in a single treatment did not influence senescence evidence in CHO-K1 and NIH-3T3 cells, but interestingly, after combining with doxorubicin, BPEO was able to significantly reduce the incidence of senescence in both cells ($P < 0.01$). Doxorubicin, an anticancer agent that can damage double-stranded DNA, is used as senescent inducing agent which can increase senescence in the human cardiac and fibroblast cells.[25,28] These results confirm a more detailed profile of the potential of BPEO as an anti-senescence agent, especially in ovarian tissue and skin tissue. We noted that *Cucurbita moschata* and apple extract from Indonesia also have anti-senescence for the anti-aging target which are also recognized as antigenotoxic agent, preventing DNA damage.[29,30] On the other hand, black pepper extract also exhibited antigenotoxic activity.[31] This finding illustrates the possibility that BPEO potentially to be an antigenotoxic agent as well as anti-senescence. In this regard, the potential of BPEO to be an anti-senescence agent may correlate to the decreasing effect of the ROS level.

ROS are metabolic by products that are produced naturally by the body and are influenced by the external environment.[5] ROS are needed by the body to stimulate cell proliferation, but it can become toxic if it reaches the required limit.[3,17,32] To confirm the pathways that might cause cell aging, the DCFDA assay is also carried out at the same concentration as the aging test. The results showed that BPEO generates similar responses of ROS level to untreated cells, which means that BPEO did not affect the level of ROS in ovarian and fibroblast cells. However, the administration of BPEO in combination with doxorubicin can significantly increase ROS levels ($P < 0.05$) in CHO-K1 cells but not in NIH-3T3 cells without significant changes in cell's viability. This phenomenon indicated that the increase of ROS level has not exceeded the threshold that causes the cell to die.[3] Thus, the occurrence of this senescence perhaps does not correlate with intracellular ROS levels. On the other hand, there is a report that increasing ROS up to a specific concentration could maintain cancer cells' proliferation.[33] In contrast, increasing ROS level over the threshold can make cell death. Thus, the ROS concentration in the cell results in a different effect depending on the type of cell, and it is characteristic.[3,34-36] These findings also give insight that the decreasing of senescent evidence on both cell types does not correlate to the ROS status. In this case, BPEO may have other target mechanism to inhibit senescence by chemical stress

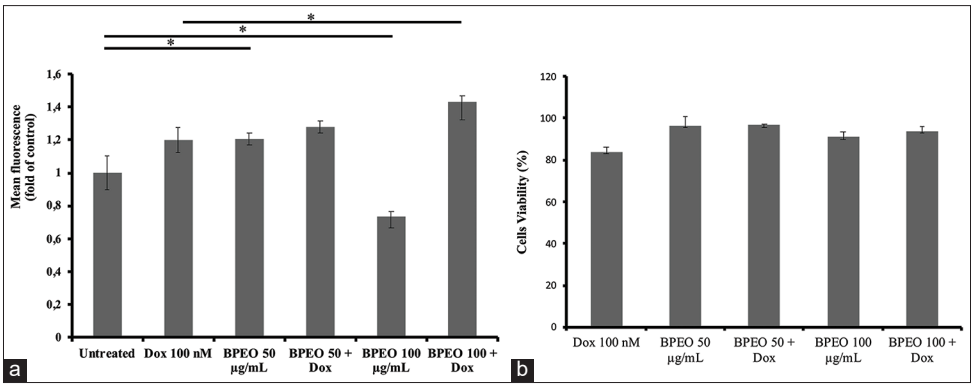

Figure 5: Effect of BPEO on intracellular ROS level and cytotoxic effect on NIH-3T3 cells. Total ROS level after single and combination treatment of BPEO with doxorubicin was observed under DCFDA staining assay. (A) NIH-3T3 cells (5×10^5 cells/mL) were treated with BPEO 50 and 100 μg/mL, doxorubicin (100 nM), and in combination for 4 h, then subjected to the ROS detection using flow cytometry. (n=3). (B) The cytotoxic combination with Dox. The significances between treatment

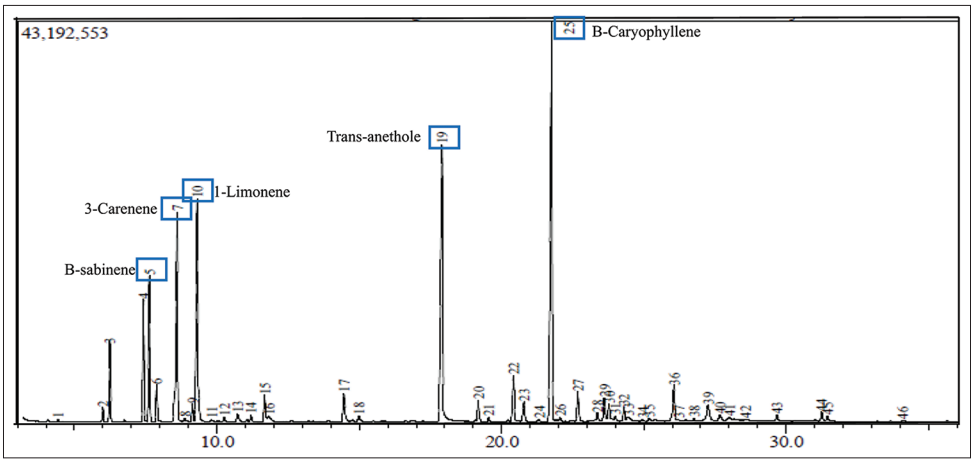

Figure 6: Chromatogram of BPEO chemical compounds. BPEO was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS), then the spectra identified using Wiley mass spectral library.

Table 2: Chemical composition (%) of essential oil of Black pepper

Source	Methods	Chemical compounds (%)	References
India	Hydrodistillation	β-caryophyllene (29.9), limonene (13.2), β-pinene (7.9), sabinene (5.9)	[20]
Bangladesh	Steam distillation	Caryophyllene (18.393), α-Pinene (16.685), D-Limonene (16.168), β-Pinene (13.618)	[26]
India	Steam distillation	3-Carene (32.611), D-Limonene (5.222), β-Pinene (13.204)	[26]
Malaysia	Supercritical carbon dioxide	β-caryophyllene (18.6), limonene (15.0), sabinene (13.2), 3-carene (8.6)	[21]
Bulgaria	Hydrodistillation	β-caryophyllene (20.225), sabinene (18.054), limonene (16.924)	[27]
Khatunganj, India	Steam distillation	Caryophyllene (19.12), limonene (9.74), camphene (8.44)	[20]
Brazil	Steam distillation	Caryophyllene (26.2), o-cymene (5.8), and pinene (5.5)	[28]

rather than inhibiting ROS generation. Therefore, further explorations are needed to elucidate the other mechanisms of BPEO in inhibiting the cell aging process.

The identification of chemical compounds in BPEO confirmed that it contains terpenes, which the primary compound is β-caryophyllene that seems to be the common constituent in black pepper around the world,[18,19,21,37] although the composition of the other compound is varied. Furthermore, some previous research found that β-caryophyllene has potent antioxidant activity, anticancer, and antimicrobial.[38,39] Moreover, black pepper extract has different main content from its essential oil and alkaloid compound. This might occur because the plant's origin and the distillation method used affect the compound content of each plant [Table 2]. This statement reinforced the possibility that the difference in content in each sample is due to the location of growth, plant environment, and the different methods. However, we should clarify whether or not the antioxidant properties of chemical

constituents are the main contributor to the anti-senescence activity because we found that the senescence evidence does not in line with ROS level. In this regard, we may propose that the essential oil of black pepper play a pivotal role as anti-senescence regardless of its antioxidant property and could be used as the standard of the quality of its essential oil product of black pepper for anti-aging product purposes. We also noted that this study is *in vitro* model utilizing respective cell types that should be clarified further under *in vivo* model and using appropriate formula as well.

CONCLUSION

Overall, BPEO is safe and tends to inhibit senescence in normal cells, especially in ovarian and fibroblast cells. However, the phenomena' mechanism should be clarified between the two types of cells, focusing on the relationship between ROS and senescence. Since senescence is closely related to the aging process in cells and BPEO shows to inhibit senescence, it is suggested that BPEO demonstrated the potential effect to inhibit aging and aging-related phenomena.

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