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BIOACTIVITY OF BLACK CUMIN OIL ON THE SENESCENCE OF HER-2-OVEREXPRESSING BREAST CANCER CELLS

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ABSTRACT

Senescence-induced therapy has been improved to increase its cytotoxicity and reduce the resistance of breast cancer cells to chemotherapy agents. An example of a potential senescence-inducing agent is black cumin oil (BCO) because one of its major compounds, *α*-pinene, can induce senescent cells. This study aims to explore the senescence-inducing activity of BCO in HER2-overexpressing breast cancer cells (MCF7/HER2). The yield obtained from hydro-distillation of BCO was 0.54%, and the main compounds were *p*-cymene (48.03%), dihydrocarveol (11.39%), and *α*-pinene (11.29%). BCO exhibited a moderate cytotoxicity profile indicated by IC_{50}, which was >200 µg/mL in both cell lines. In combination with doxorubicin, BCO did not increase the cytotoxicity of doxorubicin. Moreover, BCO induced senescence by increasing 3% of the senescent cells compared with that of the untreated cells. A combination of BCO and doxorubicin increased the senescent cells by 3%–7% compared with doxorubicin alone. Therefore, the moderate cytotoxicity of BCO could be beneficial to the application of BCO as a chemotherapeutic adjuvant which increases cancer cells senescent and consequently inhibits cell proliferation.

Key words: Chemotherapeutic adjuvant, dihydrocarveol, MCF7/HER2 cell, *Nigella sativa*, *p*-cymene, *α*-pinene

INTRODUCTION

Nigella sativa L. is an annual herbaceous plant, which originated from Southeast Asia. It was used in ancient Egypt, Greece, the Middle East, Africa, and cultivated mainly in countries bordering the Mediterranean Sea. The seed and oil of *N. sativa* L. are extensively used as spices and medicine. Its seed is commonly called black cumin (English) and *Habbah Sawda* or *Habbat el Baraka* (Arabic) translated as "seeds of blessing" (Bourgou *et al*., 2010; Khan *et al*., 2011). The major bioactive compounds in black cumin are terpenes, such as *p*-cymene, γ-terpinene, thymoquinone (TQ), β-pinene, carvacrol, terpinen-4-ol, and longifolene (Bourgou *et al*., 2010). However, the biological properties of essential oils depend on the compounds of the plant which are influenced by where it is planted (Khan *et al*., 2011). Many studies have reported that black cumin essential oil has biological activities, such as anti-bacterial, anti-fungal, anti-cancer, and antioxidant (Bourgou *et al*., 2010). Black cumin has been used as an anti-cancer agent in traditional medicine systems in Saudi Arabia (Unai), India, (Ayurveda; Ind-Bangla), and China (traditional Chinese medicine). The seed and oil of black cumin elicit anti-proliferative effects against several cancer types, including breast cancer (Khan *et al*., 2011).

Black cumin possesses various compounds such as thymoquinone (30-48%), thymohydroquinone, dithymoquinone, *p*-cymene (7 - 15%), carvacrol (6%-12%), 4-terpineol (2%-7%), t-anethol (1%-4%), sesquiterpene longifolene (1% -8%) (Woo *et al*., 2011). Besides, other research stated that black cumin contains cymene (49.48%), *α*-thujene (18.93%), *α*-pinene (5,44%), β-pinene (4.31%), dan γ-terpinene (3.69%) (Harzallah *et al*., 2011). This difference is due to the geographical and climate properties of where black cumin grows. Among many molecular mechanisms underlying the anti-proliferation effect of black cumin, the activity involved in cancer metabolism has yet to be fully explored. Khan *et al*. (2011) found in a review that black cumin oil (BCO), increases the activities of antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase. However, other studies have revealed that TQ and *α*-pinene, compounds found in black cumin, at high concentrations have a pro-oxidant activity and consequently increase reactive oxygen species (ROS) levels; thus, they elicit cytotoxic effects on cancer cells but not on normal cells (Aydin *et al*., 2013; Zubair *et al*., 2013). Interference on cancer metabolism results in cellular senescence, which can be triggered by various extrinsic factors, including conventional chemotherapeutic agents, oxidative stress, and genetic manipulations. This process is also known as an irreversible cell cycle arrest (Childs *et al*., 2014).

In breast cancer, HER2 is overexpressed in 20%–30% of primary tumors (Ishikawa *et al*., 2014). Patients with HER2-positive breast cancer are usually treated with trastuzumab, which is a monoclonal humanized antibody. However, only one-third of patients respond to trastuzumab, and a majority of responders experience disease progression after 1 year of treatment; as such,

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patients develop resistance to trastuzumab (Bartsch *et al*., 2007). Besides HER2 inhibitors, anthracyclines such as doxorubicin, daunorubicin, and epirubicin are considered primary drugs for the treatment of breast cancer, including the HER2 subtype. Meta-analyses have revealed that the efficacy of anthracycline is confined to HER-2 subtype cancers (Ishikawa *et al*., 2014).

On the other hand, natural compounds have been considered to be less toxic than chemotherapeutic drugs. Although the efficacy of natural-based alternatives toward cancer cells is weaker than the chemotherapeutic drugs, some meta-analysis studies revealed the potency of herbal medicine as chemotherapeutic adjuvants on breast cancer, nasopharyngeal, and pancreatic cancer (Lin *et al*., 2020). The proposed mechanisms underlie including enhancing efficacy, reducing chemoresistance, and alleviating adverse effects (Lin *et al*., 2020). This study aimed to determine the potential of BCO as a chemotherapeutic adjuvant against HER2-overexpressing breast cancer cells.

MATERIALS AND METHODS

Black cumin oil (BCO) extraction

Black cumin seeds were obtained from and determined its authentication by Technical Implementation Unit Materia Medica, Malang, East Java, Indonesia. The seeds were pounded and subjected to hydro-distillation by using a Clevenger apparatus system to isolate BCO following previously described methods (Benkaci-Ali *et al*., 2007) with slight modifications. Sodium sulfate was added to the isolated oil for up to 24 h to separate traces of water.

Analysis of the phytochemical profile of black cumin oil (BCO)

The phytochemical profile of BCO was identified using gas chromatography-mass spectrometry (GC–MS; QP2010S0-Shimadzu) at the Laboratory of Organic Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada. BCO was dissolved in 96% ethanol and injected into a GC–MS column (Rtx 5 MS column, length = 30 m, diameter = 0.25 mm). BCO was evaporated through heat injection at 300 ºC, and the column temperature was set at 70 ºC. The BCO steam was carried by helium gas to an EI 70 Ev detector to determine its phytochemical component.

Cell culture

A HER2-overexpressing cell line, namely, MCF-7/HER2 (transfected with HER2), which was obtained from Prof. Masashi Kawaichi (Laboratory of Gene Function in Animals, Nara Institute of Technology, Japan) and Prof. Yoshio Inouye (Department of Surgery, Toho University School of Medicine, Japan), was used in this study. MCF-7/HER2 cells were cultured in Dulbecco's modified Eagle's high-glucose medium (Gibco, New York, USA) supplemented with 10% fetal bovine serum (Gibco, New York, USA), 1% penicillin-streptomycin (Gibco, New York, USA), and 0.5% fungizone (Gibco, New York, USA).

Cytotoxicity assay

A cytotoxicity assay was performed using an MTT assay. Cells (2.5×10^4/well) were seeded into 96-well plates until 60–70% confluency was reached. They were treated with 50 and 100 µg/mL BCO alone and in combination with doxorubicin. A sample stock was prepared by diluting BCO in DMSO. The highest DMSO concentration used in the experiment was less than 1%. After 24 h of incubation, the culture medium was removed, and the cells were washed with phosphate-buffered saline (PBS) (Sigma Aldrich, Missouri, USA). Then, an MTT reagent (Biobasic, Markham, Canada) was added to each well and incubated for 4 h. SDS was added and incubated overnight at room temperature. Absorbance was determined with a microplate reader under the wavelength of 595 nm.

Senescence-associated β-galactosidase assay

Senescence-associated β-galactosidase (SA β-galactosidase) staining was performed as previously described. MCF-7/HER2 cells were seeded at a density of 2.5×10^4 cells/well in a six-well plate. When the cells were in the log phase growth and 60–70% confluent, they were stained with SA-β-galactosidase 24 h after seeding to minimize false-positive staining. Before SA-β-gal staining, the cells were washed twice with 1× PBS and fixed with 2% formaldehyde–0.2% glutaraldehyde (Merck, Darmstadt, Germany) for 10 min. They have washed again with 1× PBS and incubated in a CO_2-free staining solution containing 0.2% 5-bromo-4-chloro-3-inolyl-β-D-galactoside (Sigma Aldrich, Missouri, USA), 40 mm citric acid/phosphate buffer (pH 6.0), 5 mm $K_4Fe(CN)_6$ (Sigma Aldrich, Missouri, USA), 5 mm $K_3Fe(CN)_6$ (Sigma Aldrich, Missouri, USA), and 2 mm $MgCl_2$ overnight at 37 ºC. Then, they were observed under an inverted microscope (100× magnification) (Debacq-Chainiaux *et al*., 2009).

RESULTS

Isolation of black cumin oil (BCO) and identification of the phytochemical components

Light-yellow BCO was obtained with a yield of 0.54%. The GC–MS analysis of the phytochemical components of BCO revealed 17 compounds (Table 1). The major compounds in BCO were *p*-cymene (48.03%), dihydrocarveol (11.39%), and α-pinene (11.29%). Other components were detected at less than 5% (Figure 1). Our finding differed from previous results (Woo *et al*., 2011) and this may be because the composition of phytochemical components in essential oil is influenced by climatic and geographical factors in areas where plants are grown (Sangwan *et al*., 2001). The climatic and geographical factors have a wide influence on the components of essential oil. These factors cover the light intensity, water availability, altitude and proximal latitude of the growing site, the soil conditions including pH and soil structure. All of those factors influence the plant metabolism and eventually the essential oil component as reviewed by Barra (2009).

Table 1. Compounds of black cumin oil (BCO) were analyzed through gas chromatography-mass spectrometry

Compound	Retention time (minute)	Abundance (%)	Anti-cancer activity	Reference
α-Pinene	9,722	11.29	Inhibit cancer cell growth, induce apoptosis, and elevate G2/M arrest	(Zhao et al., 2018) (Hou et al., 2019) (Xu et al., 2018)
1,3,6-Octariene	9,986	3.03	-	-
Sabiene	11,500	1.81	-	-
β-Pinene	11,678	4.55	Elicit a synergistic effect with paclitaxel	(Zhang et al., 2015)
α-Terpinene	13,163	0.57	Inhibit cancer cell growth and induce apoptosis	(Assmann et al., 2018)
p-Cymene	13.540	48.03	Exhibit antinociceptive property on oncologic pain and act as selective anti-cancer candidates	(Santos et al., 2019) (Lenis-Rojas et al., 2018)
D-Limonene	13,653	5.43	Induce apoptosis and autophagy and elicit a synergistic effect with docetaxel	(Yu et al., 2018) (Shah et al., 2018) (Rabi and Bishayee, 2009)
ϒ-Terpinene	14,686	2.48	Decrease the viability of breast cancer cells and induce apoptosis	(Assmann et al., 2018)
trans-β-Terpinyl pentanoate	15,994	2.02	-	-
Dihydrocarveol	16.808	11.39	-	-
Terpineol-4	18,986	0.81	-	-
Bornyl acetate	22,153	0.31	Elicit a synergistic effect with 5-fluorouracil	(Li and Wang, 2016) (Shahwar et al., 2015)
α-Copaene	23,983	0.38	Exhibit anti-leukemic, breast cancer, and colon cancer properties	(Ding et al., 2018)
α-Longipinene	24,147	1.34	-	-
Copaene	24,856	0.85	Exhibit anti-leukemic, breast cancer, and colon cancer properties	(Ding et al., 2018)
Aromadendrene	25,895	4.98	Induce apoptosis	(Pavithra et al., 2018)
β-Caryophyllene	26,124	0.74	Induce apoptosis and exhibit anti-metastasis property	(Pavithra et al., 2018) (Wang et al., 2018) Dahham et al., 2015

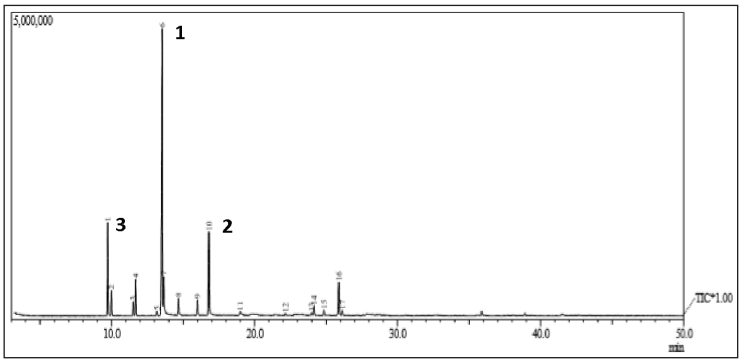

Fig. 1. Phytochemical profile of BCO. The phytochemical profile of BCO was determined through GC–MS. Analysis was carried out using GC–MS with an Rtx 5 MS (50 m long) column and a helium carrier gas. The column temperature was 70 ºC, and the injection temperature was 300 ºC. An ion detector EI 70 Ev was used. The GC–MS chromatogram revealed the following major abundant compounds: 1, *p*-cymene; 2, *dihydrocarveol*; and 3, α-pinene.

Cytotoxicity of black cumin oil (BCO) on HER2-overexpressing breast cancer cells

IC_{50} of BCO was higher than 200 µg/mL (Figure 1), indicating that the potency of BCO against HER2-overexpressing cells is relatively weak (Ellithey *et al*., 2014). Cell morphology analysis did not show any cell shrinkage (Figure 2), suggesting that BCO was weakly cytotoxic and did not induce apoptosis (programmed cell death). As such, other mechanisms, i.e., cellular senescence, involved in halting cell proliferation were investigated.Cellular senescence is an irreversible process of cell cycle arrest that inhibits cell division and proliferation (Childs *et al*., 2015).

Cytotoxic effect of black cumin oil (BCO) combined with doxorubicin on HER2-overexpressing breast cancer cells

Our data (Figure 2) indicated that BCO had a weak cytotoxic effect on HER2-overexpressing breast cancer cells. Then, the cytotoxic effect of the combination of BCO and doxorubicin was observed to verify whether BCO might increase the cytotoxicity of doxorubicin. Doxorubicin, which induces the senescence of cancer cells (Hu & Zhang, 2019), was used as a positive control. The MTT assay showed that BCO did not enhance the cytotoxic effect of doxorubicin (Figure 3). Therefore, BCO alone and in combination with doxorubicin did not affect cancer cell death, but it might be involved in cancer metabolism.

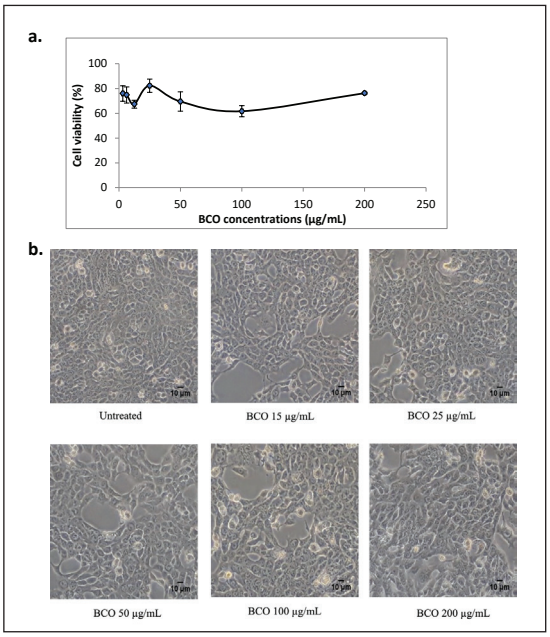

Fig. 2. Cytotoxicity of black cumin oil (BCO) on HER2-overexpressing cells. MCF-7/HER2 cells (2.5×10^4 cells/well) were seeded and incubated as described in the Materials and Methods. They were then treated with 10, 25, 50, 100, and 200 **µg**/mL BCO. After 24 h, the cells were subjected to an MTT assay, and viable cells were measured and compared with untreated cells. IC_{50} was higher than 200 **µg**/mL (a). MCF-7/HER2 cells treated with BCO slightly enlarged in a dose-dependent manner (b). Cells were observed under an inverted microscope with 100× magnification.

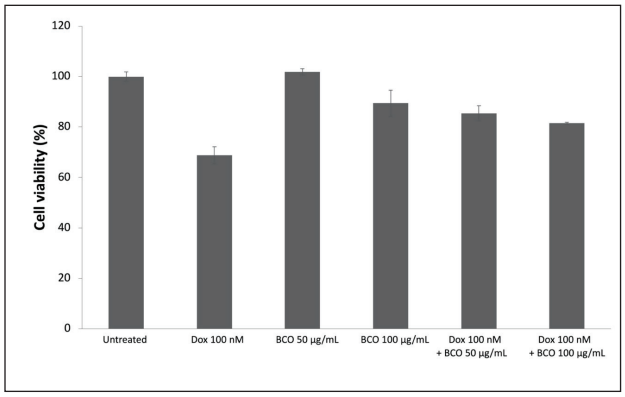

Fig. 3. Cytotoxic effect of black cumin oil (BCO) and doxorubicin (Dox) combination. MCF7/HER2 (2.5×10^4 cells/well) were treated with 50 and 100 **µg**/mL BCO alone and in combination with 100 nM doxorubicin and subjected to an MTT assay.

Effect of black cumin oil (BCO) on the senescence of HER2-overexpressing breast cancer cells

This study showed that BCO alone and in combination with doxorubicin had a weak toxic effect on cancer cells. We then observed whether BCO affected cellular senescence through the SA-β-galactosidase assay. Our results revealed that BCO could induce the senescence of 3% and 7% of MCF7/HER2 cells alone and in combination with doxorubicin, respectively (Figure 4a). Figure 4b illustrates the morphological characteristics of the senescent cells (red arrow).

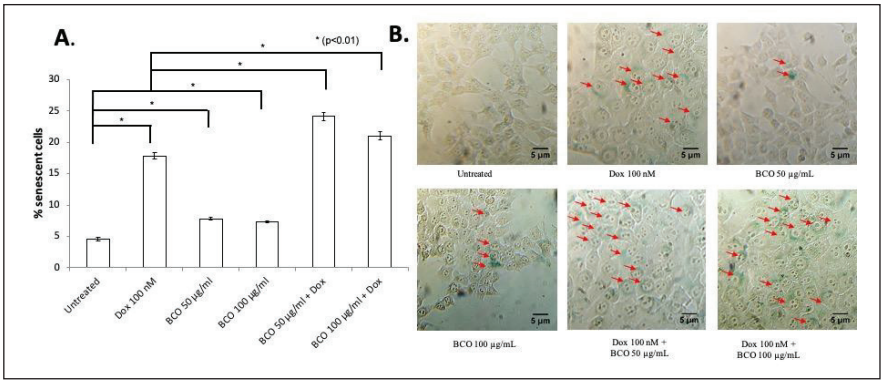

Fig. 4. Profile of MCF7/HER2 senescent cells after the single treatment of black cumin oil (BCO) and in combination with doxorubicin (Dox). (A) MCF7/HER2 cells (5 x 10^5 cells/mL) seeded to 50% confluency and treated with 50 and 100 µg/mL BCO alone and in combination with doxorubicin (100 nM) for 120 h and subjected to β-galactosidase staining. As a positive control, the cells were treated with doxorubicin (100 nM) for 5 days. The β-galactosidase-positive cells percentage, which is called senescent cells, was calculated using ImageJ by two persons to reduce subjectivity. (a) Profile of senescent cell percentage, and (b) morphological characteristics of senescent cells after 120 h of treatment with 50 and 100 µg/mL BCO alone and in combination with doxorubicin (100 nM) observed under an inverted microscope *($p< 0.01$).

DISCUSSION

A well-known prominent therapy for HER2-positive breast cancer is a monoclonal antibody, such as trastuzumab, but this drug is costly, so it is given to patients with late cancer stages. For patients with early cancer stages, chemotherapy drugs, such as lapatinib or doxorubicin, are administered to suppress cancer cell proliferation. However, these drugs cause severe side effects because of their strong cytotoxicity. Alternative regimens and adjuvant agents are then progressively developed from chemical-based, biotechnology-based, immunology-based, and phytopharmacology-based. As black cumin is commonly used among society it also has promising effects on cancer which made it a good candidate for further research. In the present study, the potency of BCO in suppressing the proliferation of HER2-overexpressing breast cancer cells was examined. The black cumin obtained from Java Island, Indonesia, was used, and oil was extracted through hydro-distillation based on methods by Benkaci-Ali *et al*. (2007). Although some of the chemical compounds of BCO obtained in this study differed from those isolated by Benkaci-Ali *et al*. (2007), we confirmed that *p*-cymene was the most abundant compound found in BCO in both studies. Moreover, we did not detect any TQ in our BCO, but Benkaci-Ali *et al*. (2007) reported the presence of approximately 20% TQ in BCO derived from Adrar, Sahara Desert.

The chemical compounds in BCO depend on their growth origin and extraction method. In this study, *p*-cymene, dihydrocarveol, and α-pinene were obtained as the major components of BCO (Table 1). A high *p*-cymene level in essential oil can contribute to the volatility of Tunisian *N. sativa* species (Bourgou *et al*., 2010). Cultivating black cumin in different altitudes correlates with its content because of varying temperatures (Barra *et al*, 2009). Although altitude and temperature affect the biochemical process of TQ and the production of other secondary metabolites, soil nutrition and many factors may also contribute.

BCO alone and in combination with doxorubicin did not elicit a cytotoxic effect on MCF-7/HER-2 cells (Figure 2 & Figure 3). However, BCO increased the number of senescent cells (Figure 4). The relatively weak cytotoxic activity of BCO in single and combination treatments needs further study, including the cellular uptake of BCO into the cells and an in vivo study to investigate the pharmacokinetic profile of BCO. The activity of BCO in inducing cellular senescence devoid of interfering cell viability also needs to be further examined. Senescent cells are stable viable cells (Childs *et al*., 2014), which explained our result on the MTT assay which showed high viability of BCO-treated cells. One of the major compounds in our BCO was *p*-cymene. This compound was also found as a major component in the essential oil of ripe fruits (RFO) of *Echinophora spinosa* L. and reported to have weak cytotoxicity against human promocytoid cells, U937 (Fraternale *et al*., 2013). Ferraz *et al*. (2013) reported similar results of *p*-cymene, as major components of essential oil of *Lippia gracilis* Schauer. When they tested *p*-cymene against HepG2, K562, and B16-F10 cell lines, it only showed cytotoxicity against B16-F10 cells and not for the other cell lines suggested cell selectivity of *p*-cymene. On the other hand, α-pinene, another major component in our BCO, was reported to modulate signaling pathways that regulate oxidative stress. It increases the phosphorylation of MAPK which activates redox-sensitive transcription factors including Nrf2 and NF-κB leading to increased activity of its downstream. This mechanism can be reversed by an antioxidant, N-acetyl-L-cysteine (NAC) (Jin *et al*., 2010). Senescence induced by BCO may be correlated with cellular stress caused by components in BCO like α-pinene.

Cells' response to stress is regulated by two main axis signaling pathways, p16-Rb and p53-p21, which lead to senescence and apoptosis, respectively. Depending on the degree of the stress, cells will have different responses. Severe stress leads to apoptosis whereas weak or mild stress leads to senescence. However, besides the degree of stress,

the balance between the regulators of senescence and the apoptosis signaling pathway also determines the cellular response. The expression level of p21 is known to regulate both apoptosis and senescence. Cell senescence induced by low concentration of doxorubicin was associated with high expression of p21 and vice versa which leads to cell apoptosis (Childs *et al*., 2014). Studies have revealed that senescence is a prominent solid tumor response to therapy through which cancer cells evade apoptosis and enter a stable and prolonged cell cycle arrest. Furthermore, targeted therapeutics and cancer immunotherapies cause cancer regression by inducing senescence in tumor cells (Qin *et al*., 2018). Senescence-induced cancer therapy is a promising strategy because of its selective function of modulating p53 (Lee & Lee, 2019). Therefore, further studies should be performed to explore BCO activities that modulate proteins or transcription factors involved in cancer cell senescence and to develop BCO potential as a chemotherapeutic adjuvant for cancer.

CONCLUSION

Our observation on black cumin oil (BCO) derived from Java Island, Indonesia revealed that the main constituents were *p*-cymene, dihydrocarveol, and α-pinene. Although BCO had weak cytotoxicity against MCF7/HER2 in single or in combination with doxorubicin, it increased senescent cells and significantly increased senescent cells in doxorubicin-treated cells. The mechanisms underlying that event still need to be clarified but based on these results we suggest the potential of BCO as a chemotherapeutic adjuvant against HER2-overexpressing breast cancer cells.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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