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Evaluating the Cytotoxic Effects of Ethanol and *n*-Hexane Extracts from Black Cumin Seeds (*Nigella sativa*) on B16F10 Mouse Melanoma Cells : A Preliminary Investigation into Vitiligo Treatment

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ABSTRACT

Black cumin (*Nigella sativa*) is a herbal plant that has been cultivated locally in Indonesia and is traditionally used for various diseases. Thymoquinone, one of the main components, is rich in biological activity. In several countries, topical application of its oil on human skin with vitiligo can stimulate skin repigmentation with minimal side effects. This study aims to determine the viability of B16F10 melanoma mouse cells against ethanol and n-hexane extracts of black cumin seeds through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The ethanol extract (EE) yield was 14.306%, and the *n*-hexane extract (NHE) was 7.442%. Phytochemical screening of EE detected flavonoids, alkaloids, saponins, and steroids, and High-performance liquid chromatography (HPLC) detected 0.040% thymoquinone. The MTT test showed cell viability was >100% from EE at all treatment concentrations, namely 0.75-100 ppm and only 0.75-6.25 ppm for NHE. In conclusion, this study indicates that 96% EE of *Nigella sativa* is less toxic than NHE on B16F10 mouse melanoma cells and has potential as an alternative treatment for vitiligo, which needs to be proven in further research.

1. Introduction

Black cumin (*Nigella sativa*) is an herbal plant from the Ranunculaceae family found worldwide (Eid 2017). In Indonesia, black cumin is an introduced plant and has been cultivated locally (Mardisiwi 2018). Black cumin seeds (BCS) have been traditionally used for various diseases such as asthma, bronchitis, diabetes, hypertension, rheumatism, obesity, cancer, and others. It has also been used to treat various skin diseases such as acne, burns, and ulcers, as an anti-inflammatory for skin inflammation, and to stimulate skin pigmentation through melanin synthesis (Eid 2017). Using local BCS oil for skin problems is a traditional practice used for generations in Indonesia. The biological activity of BCS is mainly caused by thymoquinone (TQ), one of the major components in its essential and fixed oils. Thymoquinone is known for its low toxicity and potential as an anti-inflammatory, antihistamine, antimicrobial, and anticancer (Eid 2017; Aini 2018). Phytochemical studies show differences in the content of local Indonesian BCS and imported BCS from Somalia. Gas chromatography-mass spectrometry (GC-MS) showed that only eight chemical compounds were detected in local BCS, while 18 were detected in imported BCS (Setiana and Wiryowidagdo 2010). In several countries, topical application of BCS oil has been studied *in vivo* on human skin with vitiligo to induce melanin synthesis, leading to skin repigmentation with good results and

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minimal side effects (Ghorbanibirgani *et al*. 2014; Sarac *et al*. 2019; Elzahrani *et al*. 2020).

Vitiligo is a skin depigmentation disease caused by selective damage to melanocyte cells, resulting in an inability to produce melanin, characterized by chalky white spots on the skin, mucosa, and hair. Vitiligo does not cause high mortality or morbidity, but the psychosocial effects are severe, and patients often feel hopeless. Topical treatments available in Indonesia for vitiligo are currently limited and not widely available. Vitiligo treatment lasts years, and the medicines must be rotated to remain safe and effective (Jacoeb 2021). The length of treatment, the limited period of drug use, and the limitations of medicines in Indonesia are essential reasons to look for alternative agents that are safe and effective for the long term and are easily and cheaply available in Indonesia. Current research on the potential of local Indonesian BCS extract for inducing melanin synthesis remains limited. Therefore, this study will contribute valuable insights into the use of BCS as an alternative medicine for vitiligo in Indonesia.

The toxicity of natural or artificial medicine is assessed using cell viability tests; the most used method is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Kamiloglu *et al*. 2020). This study aims to determine the viability of B16F10 melanoma mouse cells against ethanol and n-hexane extracts of BCS through a cytotoxicity test using the MTT method, which serves as preliminary data to further study the melanogenic potential of BCS as an alternative medicine to induce skin repigmentation. The B16 mouse melanoma cell line is commonly used in melanin synthesis assays because their melanogenic mechanisms are similar to human epidermal melanocytes (Kim and Hyun 2022). Ethanol solvent is volatile in nature, making it easier for the subsequent identification process (Abubakar and Haque 2020). Meanwhile, n-hexane is suitable for non-polar secondary metabolites, such as TQ (Cravotto *et al*. 2022).

2. Materials and Methods

2.1. Materials

Black cumin seeds were collected from Karangpandan, Karanganyar, Yogyakarta, Indonesia, and determined by Badan Riset Inovasi Nasional (BRIN), Cibinong, Indonesia. Solvent materials used for extraction were 96% ethanol and n-hexane (Brataco/Indonesia). Thymoquinone 97% High-Performance Liquid Chromatography (HPLC) (Sigma/USA) is used as a standard for HPLC assay. Primate Research Center Animal Studies, IPB University, Bogor, Indonesia, provided mouse melanoma cell culture B16F10 (ATCC CRL 6475/USA). Materials and reagents for cell culture consisted of Dulbecco's Modified Eagle Medium (D-MEM, Gibco/USA), Fetal Bovine Serum 10% (FBS, Hyclone/USA), penicillin-streptomycin 1% (Invitrogen /USA), phosphate buffer saline (PBS, Gibco/USA), trypsin 0.25% (Gibco/USA), dimethylsulfoxide (DMSO, Sigma/USA), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma/USA). All other reagents used met the standards for analytical quality.

2.2. Extraction

A 900 grams of BCS were pulverized into powder and extracted using a simple maceration using 96% ethanol and n-hexane separately as solvents. The ratio of raw material to the solvent used was 1:10. The blend underwent a 72-hour maceration period with daily solvent replacement. The macerated extracts were evaporated using a rotary evaporator at 40°C and 50 rpm until a concentrated extract was obtained (Laksmiani *et al*. 2020). Extracts with more than 10% yield are followed by phytochemical screening and examination of thymoquinone levels using HPLC.

2.3. Phytochemical Screening & Thymoquinone Detection by HPLC

The extract of BCS was subjected to qualitative phytochemical screening tests for secondary metabolite compounds, including flavonoids, alkaloids, tannins, saponins, quinones, steroids, and triterpenoids according to the procedures described by Reji and Rexin (2013). Examination of TQ concentration using HPLC was done according to the procedures described by Noor (2016). The operating conditions used are column Zorbax eclipse C-18(8), mobile phase methanol: water (75:25), flow rate 1 ml/minute, detector UV-Vis, and wavelength 254 nm.

2.4. Viability Test Using The MTT Assay

B16F10 mouse melanoma cells were cultured in D-MEM media using 10% FBS, Penicillin 100 U/ml, and Streptomycin 100 ug/ml) at 37°C and 5% CO_2. The cells were seeded at 5×10^3 cells/ml. The EE and NHE of BCS were used to test the viability of B16F10 mouse melanoma cells by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as developed by Mossmann (Mosmann 1983). Each well

of a 96-well plate was seeded with 5×10^3 cells/ml and incubated overnight. A 100 µL/well of EE and NHE of BCS were added in treatment concentrations of 100 ppm, 50 ppm, 25 ppm, 12.5 ppm, 6.25 ppm, 3 ppm, 1.5 ppm, and 0.75 ppm in triplicate. Untreated cells were included as a control in triplicate. Cells were re-incubated for 48 hours at an incubator temperature of 37°C and 5% CO_2. A 50 ug/well MTT solution was added to each well, and the plates were further incubated at 37°C and 5% CO_2 for 4 hours. The supernatant was discarded, and the formazan crystals formed were dissolved in 100 µL of 96% ethanol. Absorbance reading was taken using a microplate reader at 595 nm. Optical density data obtained from measurements is used to calculate the percentage of cell viability using the following equation (Kamiloglou *et al*. 2020):

$$\% \text{ cell viability} = \frac{\left(\begin{matrix}\text{Sample}\\ \text{absorbance}\end{matrix} - \begin{matrix}\text{Blank}\\ \text{absorbance}\end{matrix}\right)}{\left(\begin{matrix}\text{Control}\\ \text{absorbance}\end{matrix} - \begin{matrix}\text{Blank}\\ \text{absorbance}\end{matrix}\right)} \times 100$$

2.5. Data Analysis

The percentage of cell viability was analyzed by determining the linear regression equation to calculate the IC_{50} value. The correlation between absorbance and the concentration of the extracts against mouse melanoma B16F10 cell line using the MTT method was also analyzed. Meanwhile, cell morphology of the B16F10 mouse melanoma cell line after EE and NHE administration at various concentrations was observed using an inverted microscope.

3. Results

3.1. Extraction

The extraction of 450 grams of BCS powder with ethanol produced 64.380 grams of extract (yield 14.306%), while the extraction of 450 grams of BCS powder with n-hexane produced 33.900 grams of extract (yield 7.442%). The EE has more than 10% yield, thus followed by phytochemical screening and examination of thymoquinone levels using HPLC.

3.2. Phytochemical Screening and Examination of Thymoquinone Concentration using HPLC

Phytochemical screening was performed on the EE of BCS, as seen in (Table 1). This screening was carried out to determine the presence of secondary metabolite compounds, including flavonoids, alkaloids, tannins, saponins, quinones, steroids, and triterpenoids.

The results of HPLC analysis described on the chromatogram (Figure 1) showed that the EE of BCS contains 0.040% thymoquinone with a retention time of 3.347 minutes.

3.3. Viability Test Using The MTT Assay

In this study, the MTT assay showed different percentages of cell viability for each EE and NHE at concentrations of 100 ppm to 0.75 ppm (Table 2). The selection of concentrations with cell viability >100% was used to avoid bias and rule out the possibility of melanogenesis inhibition on cell growth. The cells treated with EE of BCS showed higher viability than the control (>100%) at all treatment concentrations, with a tendency to increase viability at lower dose concentrations. The R-square value of IC_{50} EE of BCS was 0.573, meaning that the contribution of concentration to viability was only 57%, and the remaining 43% was not reflected in this model.

Meanwhile, cytotoxicity assays on NHE of BCS showed no cell death starting at a dose of 6.25 ppm, and toxicity decreased at lower dose concentrations. Doses above 6.25 ppm to 100 ppm showed lower viability than the control (<100%). The R-square value of IC_{50} NHE of BCS was 0.990, which means that the contribution of concentration effect on viability was 99%, which is very strong.

The correlation value between EE of BCS concentration and absorbance was -0.250, meaning there was a weak relationship between absorbance and concentration (Table 3). The NHE correlates with absorbance, namely -0.476, suggesting a moderate relationship between absorbance and concentration. The negative values indicate an inverse relationship: the higher the concentration, the lower the absorbance, and vice versa. A decrease in absorbance in treated cells indicates cytotoxicity.

Cell morphology observations on the B16F10 mouse melanoma cell line after EE and NHE administration at various concentrations using an inverted microscope

Table 1. Phytochemical screening of 96% ethanol extract of Black Cumin Seeds

Parameter	Test results
Flavonoids	Positive
Alkaloids	Positive
Tannins	Negative
Saponin	Positive
Quinon	Negative
Steroid	Positive
Triterpenoid	Negative

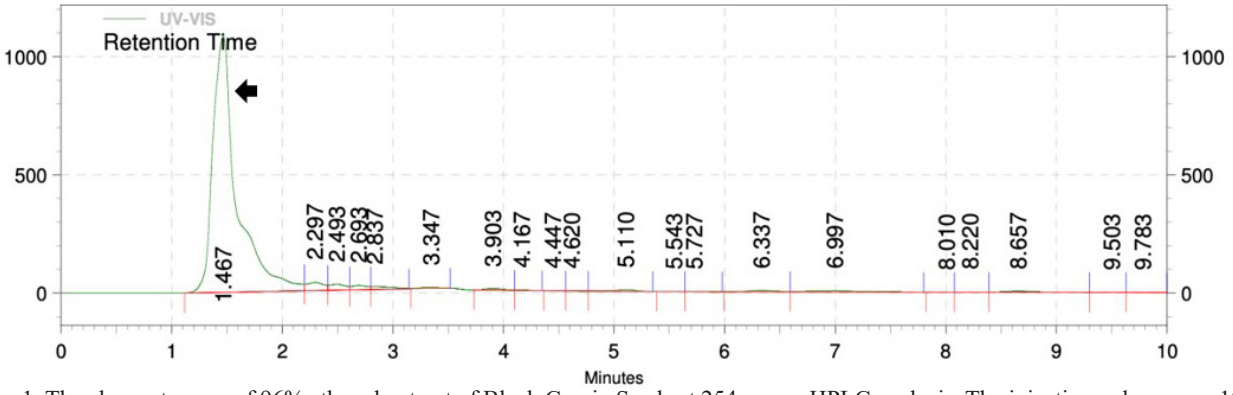

Figure 1. The chromatogram of 96% ethanol extract of Black Cumin Seeds at 254 nm on HPLC analysis. The injection volume was 10µL. ← Thymoquinone

Table 2. Cytotoxic activity and IC_{50} of 96% Ethanol and n-Hexane Extract of Black Cumin Seeds at various concentrations in 48 hours against B16F10 mouse melanoma cells by MTT method. The result is expressed in mean ± SD

Extract concentration (ppm)	96% Ethanol extract cell viability (%)	96% Ethanol extract IC_{50} (ppm)	*n*-hexane extract cell viability (%)	*n*-hexane extract IC_{50} (ppm)
100	231.590±197,209	0.432 ppm (R-square 0.573)	54.700±18,874	19.987 ppm (R-square 0.990)
50	432.380±148,135		34.780±19,729	
25	468.290±57,696		46.310±11,290	
12.5	208.650±24,680		81.500±81,143	
6.25	501.190±36,499		157.680±125,926	
3.125	501.170±17,423		318.250±243,933	
1.5	507.410±10,510		412.750±172,937	
0.75	461.870±47,771		491.700±63,411	
Control	100		100	

Table 3. Correlation between absorbance with ethanol extract (EE) and n-hexane extract (NHE) of Black Cumin Seeds (BCS) at various concentrations

Concentration (%)	EE of BCS		EE of BCS	
	Absorbance	Correlation	Absorbance	Correlation
100	0.395±0.311	-0.250	0.097±0.0350	-0.476
50	0.754±0.220		0.060±0.030	
25	0.824±0.071		0.081±0.018	
12.5	0.368±0.048		0.147±0.149	
6.25	0.887±0.103		0.280±0.232	
3.125	0.884±0.035		0.560±0.443	
1.5	0.896±0.038		0.717±0.252	
0.75	0.817±0.116		0.866±0.096	
Control	0.177		0.177	

(Figures 2 and 3). Morphological changes indicating cell death were seen in cell cultures treated with NHE of BCS and become increasingly evident with higher treatment doses, especially at the highest concentration of 100 pm. These morphological changes were not seen in cell cultures treated with EE of BCS. Cell morphology at EE's highest treatment concentration of 100 ppm still resembled the morphology of control cells.

4. Discussion

The therapeutic effect of a plant depends on its secondary metabolites; for black cumin, it is mainly isolated from the seeds, consisting of alkaloids, fatty acids, polyphenols, phytosterols, terpenes, terpenoids, and others. Terpenes and terpenoids are the primary secondary metabolites, namely more than 50% (Hossain *et al*. 2021). This study's phytochemical screening of EE

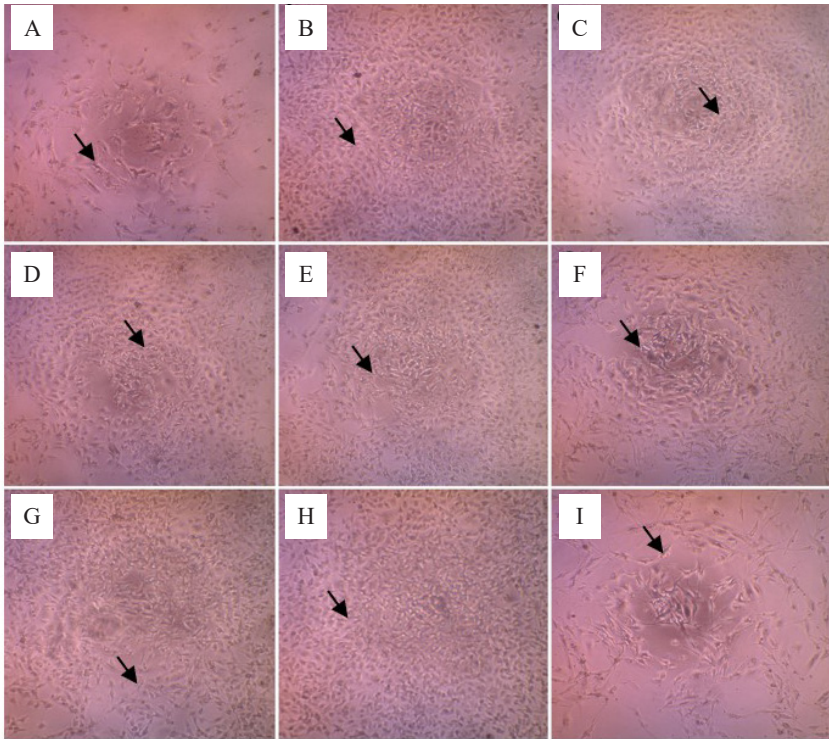

Figure 2. Morphology of mouse melanoma B16F10 cell line treated with EE of BCS (32x magnification). Dose concentrations (A) control, (B) 0.75 ppm, (C) 1.5 ppm, (D) 3 ppm, (E) 6.25 ppm, (F) 12.5 ppm, (G) 25 ppm, (H) 50 ppm, and (I) 100 ppm. Live cells (→)

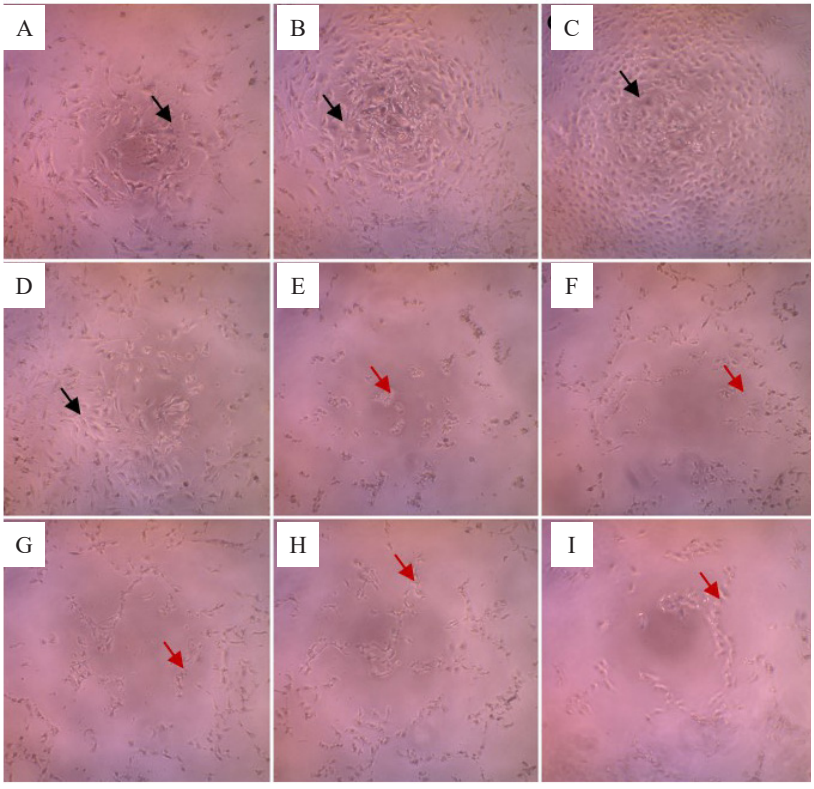

Figure 3. Morphology of mouse melanoma B16F10 cell line treated with NHE of BCS (32x magnification). Dose concentrations (A) control, (B) 0.75 ppm, (C) 1.5 ppm, (D) 3 ppm, (E) 6.25 ppm, (F) 12.5 ppm, (G) 25 ppm, (H) 50 ppm, and (I) 100 ppm. Live cells (→) dead cells (→)

of local BCS detected flavonoids, alkaloids, saponins, and steroids, but tannins, quinones, and triterpenoids were not detected. This is in accordance with the study of Setiana and Wiryowidagdo (2010), Hidayat *et al*. (2022) and Seran *et al*. (2023) which reported that local BCS contained steroids, which were not present in the imports (Setiana and Wiryowidagdo 2010; Hidayat LNR *et al*. 2022; Seran *et al*. 2023). Thymoquinone (TQ), a monoterpene from the terpenoid group, is found abundantly in BCS oil and extract, around 30%-48%. (Khan S *et al*. 2022). The biological activities of TQ in dermatology include anti-inflammatory and antioxidant (Liang 2021), immunomodulatory (Majdalawieh and Fayyad 2015), and triggering melanogenesis (Zaidi *et al*. 2019). In this study, the TQ content in the EE of local BCS was 0.040%, much lower than imported BCS. Geographic location and climate differences can influence the types and percentages of secondary metabolites in BCS (Gharby *et al*. 2015), which may explain the differences in secondary metabolites between local and imported BCS. This difference may have implications for differences in biological activity. Although the main active ingredient of BCS extract is TQ, the biological activity of other secondary metabolites also plays a vital role in determining the final effect on the inhibition or viability of the B16F10 melanoma mouse cells used in this study.

The cytotoxicity test of EE of BCS on B16F10 melanoma mouse cells using the MTT method showed increased viability by 231.590% to 461.870% compared to controls in all treatment doses. This means that EE of BCS at doses up to 100 ppm did not cause toxicity to B16F10 melanoma mouse cells. A percentage higher than 100% demonstrated the potency of the samples in stimulating cell growth. Since the viability of the cells is higher than 100% in all treatment doses, the toxic dose of the EE of BCS is unknown in this study, and a further MTT test should be carried out with a higher treatment dose concentration. In the study of cytotoxicity of EE of BCS on mouse L6 muscle cell lines (L6myc) and human hepatocellular carcinoma (HepG2) cells with the MTT method, cytotoxic effect was seen at doses >500 ppm (Mashayekhi-Sardoo *et al*. 2020). In another study using EE of BCS on isolated rat splenocytes, a concentration of 500 µg/ml significantly decreased the cell viability ($p<0.05$) (Gholamnezhad *et al*. 2015). The NHE of BCS is not toxic (157.680%) to B16F10 mouse melanoma cells starting at a dose of 6.25 ppm and becomes less toxic at lower dose concentrations. However, in the study by (Borgou *et al*. 2021), no significant cytotoxic effect was found from the n-hexane extract of BCS at a 25-200 ppm concentration. In this study, the viability percentage of B16F10 mouse melanoma cells treated with EE and NHE of BCS at various concentrations showed that the cells had a higher viability to EE than NHE at the same treatment dose, suggesting a lower cytotoxicity activity of EE of BCS than NHE of BCS.

In this study, the viability of B16F10 mouse melanoma cells by administering two types of extracts, EE and NHE of BCS, demonstrated different results. This indicates that different solvents may produce secondary metabolite compounds that differ in type, diversity, and percentage. Solvent selection is contingent upon the targeted compounds (Hait 2019), which is TQ in this study. Solvents used in TQ extraction include water, methanol, ethanol, benzene, n-hexane, chloroform, and ether (Iqbal *et al*. 2018; Hait 2019; Rahim *et al*. 2022). Ethanol solvent has higher antibacterial activity than water solvent, easily penetrating cell membranes to extract secondary metabolites from plant material. As a solvent, 96% ethanol has a small water content and a high boiling point. It evaporates quickly, making the subsequent identification process easier (Abubakar and Haque 2020). It was therefore chosen in this study. The n-hexane has been widely used in vegetable oil extraction and is suitable for non-polar secondary metabolites (Cravotto *et al*. 2022). Since TQ is a non-polar terpenoid, n-hexane was chosen as a second solvent in this study to obtain a higher TQ content. The concentration and amount of TQ extracted from BCS are also influenced by the extraction method, such as cold extraction, microwave-assisted extraction, or Soxhlet extraction with methanol and n-hexane solvents (Kiralan *et al*. 2014, Butt *et al*. 2019, Rahim *et al*. 2022). Thymoquinone is hydrophobic, insoluble in water, and easily degraded due to pH, heat, and light (Mostafa *et al*. 2018). Thus, the extraction method used in this study is maceration, which is suitable for medicinal plants that are unstable to temperature.

Cell shape morphology changes are one of the parameters that can be observed when cells undergo toxic conditions, such as the presence of compounds or chemicals (Aisyah *et al*. 2020). Therefore, the cytotoxic activity of BCS extracts against mouse melanoma cells can also be observed by its morphological change. The morphological characteristics of B16 mouse melanoma cell culture are a mixture of spindle-shaped and epithelial-like cells, as seen in controls (Figures 2A and 3A). The

image of cells in culture after administration of various concentrations of substances shows concentration-dependent changes in cell morphology. In general, the higher the dose, the more changes in cell morphology, which may be caused by cytotoxicity. Cytotoxicity in melanoma cells will be characterized by shrinkage and detachment from the surface of the tissue culture site (Ndhundhuma and Abrahamse 2017), reduced cell and nuclear volume, increased dead or floating cells, cytoplasmic condensation, and cell shrinkage indicating cell death (Marvibaigi *et al*. 2016, Kumar *et al*. 2018) which evident in cell cultures given NHE of BCS (Figure 3B-I). Cell shrinkage indicating cell death becomes more evident with the higher treatment dose and is most apparent at the highest treatment dose (Figure 3I). These morphological changes were absent in cell cultures treated with EE of BCS at all treatment doses (Figure 2B-I). This is likely due to the low cytotoxicity activity from EE of BCS against mouse melanoma cells. This observation is consistent with the MTT test of EE of BCS, which showed no cytotoxicity in all treatment doses. Akinwumi *et al.* 2020 reported normal and intact histological architecture of the liver and kidneys after administration of 50 ppm EE of BCS on the liver of female Wistar rats, which is aligned with our study.

In conclusion, the EE of BCS is less toxic to B16F10 mouse melanoma cells than NHE. It has more potential to be used as an alternative treatment for vitiligo. These results serve as preliminary data that support further research on EE of BCS to stimulate melanin synthesis and induce skin pigmentation. A skin repigmentation effect will be obtained if BCS extract can increase the amount of melanin in the skin. The potential to use higher doses with good viability of EE in further study is expected to increase cell growth and its melanogenesis activity in the skin.

Conflict of Interest

The authors declare no conflict of interest.

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