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'In Vitro Cytotoxicity Assay of *Sauropus androgynus* on Human Mesenchymal Stem Cells'

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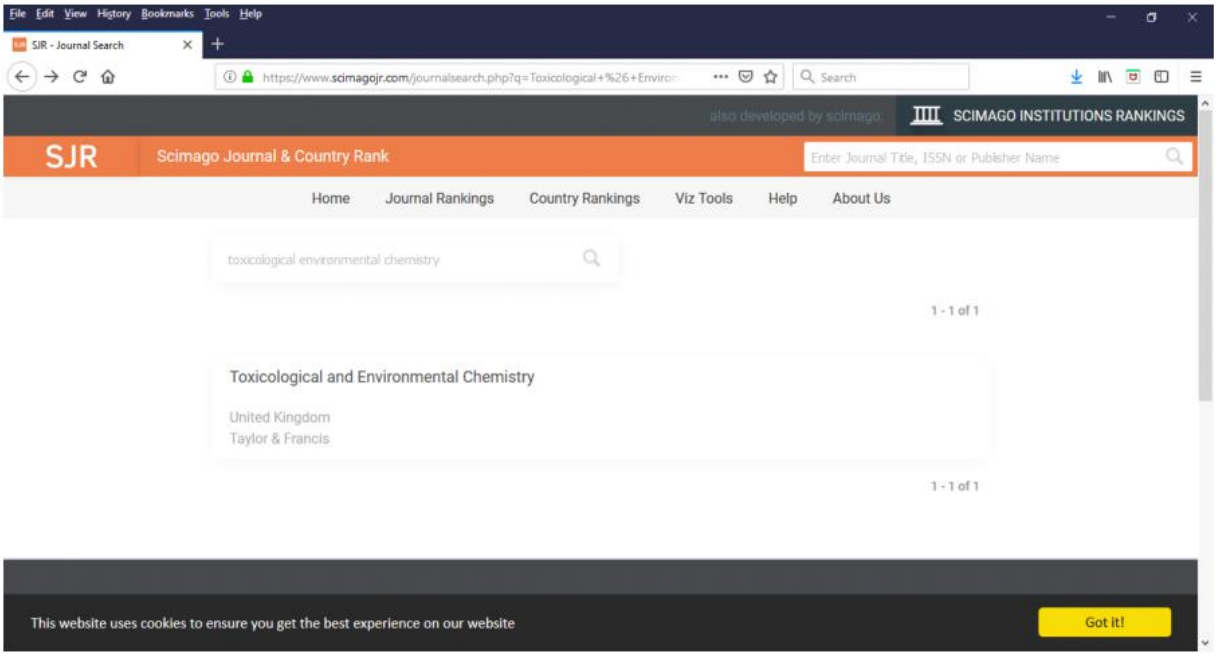

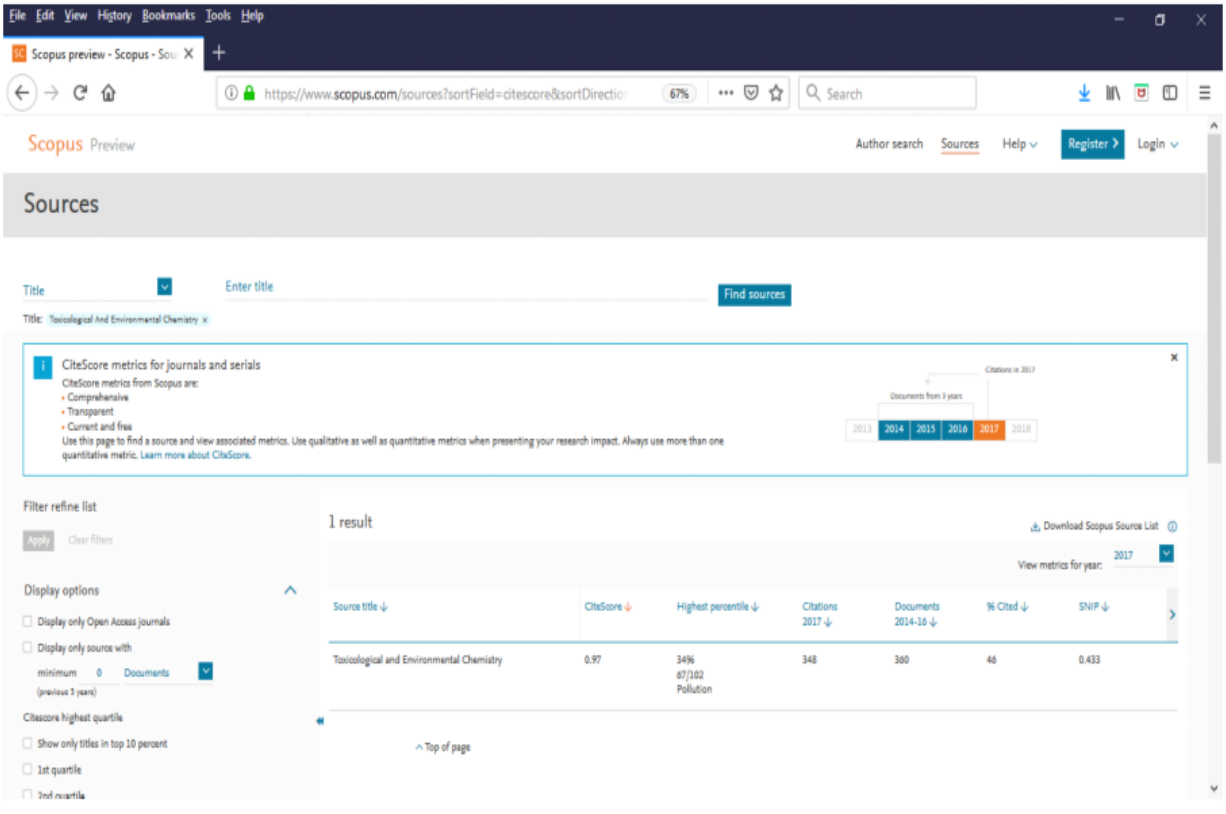

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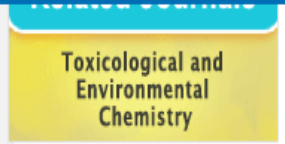

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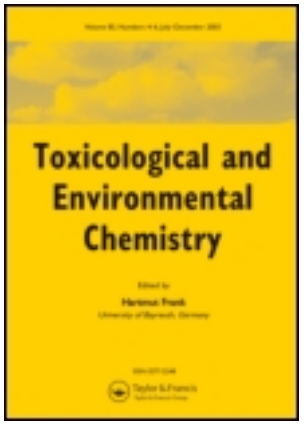

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In vitro cytotoxicity assay of Sauropus androgynus on human mesenchymal stem cells

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In vitro cytotoxicity assay of *Sauropus androgynus* on human mesenchymal stem cells

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Sauropus androgynus is a well-known Indonesian medicinal herb that is used extensively to increase human breast-milk production. However, many studies have also revealed side effects associated with bronchiolitis obliterans in Taiwan and Japan. The present study evaluated the *in vitro* toxic effects of *S. androgynus* on human mesenchymal stem cell culture derived from bone marrow (hMSCs-BM). This is the first report of a cytotoxicity assay of *S. androgynus* extracts from Indonesia. After 72 hours of incubating cell cultures with varying concentrations of extracts (250–2500 mg L^{-1}), cytotoxicity was assayed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and reported in terms of cell viability. The apoptotic effects of the extract were determined by a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) colorimetric assay. The *S. androgynus* methanol extract from East Java, Indonesia, was less cytotoxic to hMSCs-BM with an IC_{50} of 2450 mg L^{-1}, but it could inhibit cell viability via the apoptosis pathway. A sample extract of plants collected near Purwosari had the lowest hMSCs-BM viability percentage (37%), while the extract from plants collected near Surabaya Pusat had a cell viability of 75%. Further studies are required to investigate the metabolites in *S. androgynus* that are highly correlated with its toxic effects.

Keywords: *in vitro* cytotoxicity; *Sauropus androgynus*; human mesenchymal stem cells; apoptosis; safety assessment

Introduction

Sauropus androgynus (L.) Merr. (family: Euphorbiaceae), commonly known as "katuk," "sweet shoot leaves," "star gooseberry," or "pak wanban," is a small perennial shrub between 0.7 and 1.3 m tall that is often found growing wild in many areas of Southeast Asia. The dark green leaves are between 2–6 cm long and 1.5–3 cm wide, have nutritive value, and contain biologically active constituents such as vitamins (e.g., α-carotene, β-carotene, vitamin C, and vitamin E), phytosteroids, phenolic compounds, quercetin, and kaempferol (Agil 2000; Ching and Mohamed 2001; Miean and Mohamed 2001; Sripanidkulchai, Homhual, and Pocknapo 2005; Yu et al. 2006; Benjapak, Swatsitang, and Tanpanich 2008; Yang, Lin, and Kuo 2008; Andarwulan et al. 2010). The leaves of *S. androgynus* have traditionally been used in Malaysia, Thailand, and Indonesia as food (Benjapak, Swatsitang, and Tanpanich 2008) and as an herbal supplement for increasing human breast-milk production (Bermawie 2004). The leaves of *S. androgynus* also possess medicinal properties, containing antioxidants, antiobesity, and antibacterial agents

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(Rahmat et al. 2003; Yu et al. 2006; Benjapak, Swatsitang, and Tanpanich 2008; Gothandam, Aishwarya, and Karthikeyan 2010; Paul and Anto 2011).

Although *S. androgynus* is used as food and as an herbal supplement, many studies have revealed side effects of this plant in Taiwan and Japan. Cases of temporary insomnia, difficulty in breathing, or death after the ingestion of *S. androgynus* have been reported to the National Poison Center of Taiwan. The main histopathological finding was constrictive bronchiolitis obliterans (Lin et al. 1996; Chang et al. 1998; Oonakahara et al. 2005).

The occurrence of side effects from herbal supplements makes controlled toxicological studies necessary. An early assessment of the toxic effects of *S. androgynus* on humans can be performed by *in vitro* cytotoxicity screening on human mesenchymal stem cells derived from bone marrow (hMSCs-BM). The unique characteristics of human stem cells, including their capacity for self-renewal, their plasticity in generating various cell types, and their human origin, make them attractive for toxicity screening studies of metals, industrial chemicals, or synthetic drugs (Gioacchino et al. 2008; Peters et al. 2008; Lee et al. 2011).

The aim of the present study is to evaluate the *in vitro* toxic effects of *S. androgynus* on hMSCs-BM. To the best of our knowledge, this is the first report of a safety assessment of *S. androgynus* with these cells. The strategy we used may provide a new framework for toxicological testing of herbal products.

Materials and methods

Materials

Fresh leaves of *S. androgynus* were obtained from six different areas of East Java Province, Indonesia (7.9392° South, 112.9528° East), including Batu (B), Bojonegoro (BJ), Purwodadi (PWD), Purwosari (PWS), Surabaya (SB/ST/SP), and Trenggalek (T). All samples were authenticated by the Centre of Information and Development of Traditional Medicine, at the Faculty of Pharmacy, located at the University of Surabaya in East Java, Indonesia. All chemicals and materials for cell culture, unless otherwise indicated, were obtained from Sigma-Aldrich (St. Louis, MO, USA). The hMSCs-BM were obtained from the Laboratory of Stem Cells at the Institute of Tropical Disease, Airlangga University, Indonesia. Alpha minimum essential medium (MEM) supplemented with fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco (Paisley, UK). Phosphate buffered saline (PBS), trypsine, camptothecin, dimethyl sulfoxide (DMSO), formaldehyde, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and an apoptosis assay kit (ApoDIRECT *In Situ* DNA Fragmentation Assay Kit, Biovision, Milpitas, CA, USA) were used for this study.

Preparation of the methanol extract

Leaves of *S. androgynus* were collected, washed free of dirt, wiped dry, and stored at −80°C until used. Five hundred milligrams of mature leaves were ground in a mortar and pestle and 5.0 mL methanol was added. The mixture was homogenized by vortexing and sonication for 20 minutes. The mixture was then centrifuged at 3000 g for 1 minute and filtered. The sample extract was evaporated with nitrogen gas and diluted with DMSO before being added to the cell cultures.

Mesenchymal stem cell culture

The hMSCs-BM were cultured in α-MEM supplemented with 20% FBS, 100 U mL^{-1} penicillin, and 100 mg L^{-1} streptomycin at 37°C in a humidified incubator containing 5% CO_2. The culture medium was removed and replaced with fresh medium twice a week. All experiments in this study used hMSCs-BM of the 5th–8th passage.

In vitro toxicity test

The *in vitro* toxicity assay with hMSCs-BM was performed by obtaining the IC_{50} value of five groups: a negative control (DMSO 1% solution), a positive control (*camptothecin* 10 mg L^{-1}), and five concentrations of *S. androgynus* methanol extract (250 mg L^{-1}, 750 mg L^{-1}, 1375 mg L^{-1}, 2000 mg L^{-1}, and 2500 mg L^{-1}). Treated and untreated hMSCs-BM were incubated for 0–72 hours.

MTT assay

Cells were plated at a density of 1.5×10^3 cells per well into 96-well plates, treated with an *S. androgynus* extract for 0–72 hours, and incubated with MTT dye for 4 hours. The supernatant was then removed and DMSO was added. Formation of formazan was detected by measuring optical density at 595 nm using a VMax Kinetic ELISA Microplate Reader (Molecular Devices, CA, USA).

Assay of apoptosis

The apoptosis assay was initiated with cell fixation by adding 1% formaldehyde in PBS and then washing and resuspending in PBS. Fixed cells were stained using an ApoDIRECT *In Situ* DNA Fragmentation Assay Kit, the staining solution of which contained terminal deoxynucleotidyl transferase (TdT) reaction buffer, TdT enzyme, and FITC-dUTP. After incubating in the dark for 30 minutes, the stained cells were analyzed by fluorescence microscope.

Calculation of IC_{50} value

The percentage of cell viability was expressed as the mean ± standard deviation ($n = 3$) unless otherwise specified. Relative standard deviations and statistical significance, one-way ANOVA and Duncan *post hoc* honest significance difference tests were provided, where appropriate, for all data collected. $P < 0.05$ was considered as indication of significance. The IC_{50} values of the *S. androgynus* extracts on the hMSCs-BM were calculated using the software GraphPad Prism® 5 ver. 5.04 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

S. androgynus leaves were collected over two months (June and July, 2010) from six areas in East Java, including Surabaya, Trenggalek, Bojonegoro, Purwodadi, Purwosari, and Batu, as summarized in Table 1. All sampling areas had different temperature (24.1–35.4°C) and humidity (31–82%) ranges.

Table 1. Sampling locations, dates and conditions of *Sauropus androgynus* from East Java.

Location	Coordinate	Date	Code	Altitude* (m)	Temperature (°C)	Relative humidity (%)
Surabaya	7° 14′ 57″ South, 112° 45′ 3″ East	12 July 2010	ST	0	29.3–30.0	73–74
			SP	0	25.7–33.7	47–74
			SB	0	27.8–30.5	50–74
Bojonegoro	7° 9′ 0″ South, 111° 52′ 0″ East	16 July 2010	BJ I	50	24.8–30.0	52–66
			BJ II	50	32.8–33.6	54–55
			BJ III	80	32.1–35.4	48–57
Trenggalek	8° 2′ 52″ South, 111° 42′ 31″ East	26 July 2010	T I	120	24.1–33.3	31–82
			T II	120	24.1–33.3	31–82
Purwodadi	7° 48′ 7″ South, 112° 44′ 10″ East	17 June 2010	PWD	320	29.1–33.5	64–73
Purwosari	7° 46′ 13″ South, 112° 44′ 28″ East	17 June 2010	PWS	220	29.7–32.3	66–69
Batu	7° 52′ 12″ South, 112° 31′ 42″ East	13 July 2010	B I	840	25.7–33.7	39–74
			B II	840	25.7–34.0	39–74

*Relative altitude compared to Surabaya.

As shown in Figure 1B (a), untreated cells had normal elongated MSC morphology (spindle shape) with round and regular nuclei. In contrast, cells treated with 2500 mg L^{-1} of *S. androgynus* extract had rhomboidal shapes and the cells were separated from each other, as shown in Figure 1B (b). We assumed that cells had lost their adhesion capability and they were in the early stationary phase of growth. In the present study, microscopic observation of cells treated with *S. androgynus* extract (Figure 1B (c)) showed clear morphological signs of cells undergoing apoptosis, such as nuclear condensation.

The inhibitory effect of the extracts was determined by exposing hMSCs-BM to five concentrations of the methanol-extracted constituents in a stepwise manner for 72 hours. The concentration of *S. androgynus* methanol extract that reduced cell survival by 50% (IC_{50}) was determined from a cell survival curve, as seen in Figure 1A. The leaves for the *S. androgynus* methanol extract used to calculate the IC_{50} value were collected from a conservation garden at Batu, East Java (B); this extract reduced cell survival by 50% in the preliminary research. According to the curve of Figure 1A, the IC_{50} value of the *S. androgynus* extract was 2450 mg L^{-1}. Therefore, a 2500 mg L^{-1} extract was used for our cytotoxicity assay.

A one-way ANOVA showed a significant difference ($p < 0.05$) among the cell viability percentages of hMSCs-BM treated with 12 samples of *S. androgynus* from six different geographic areas. The Duncan *post hoc* test revealed that sample of *S. androgynus* from PWS resulted in the lowest cell viability percentage. The results of the cytotoxicity assay of *S. androgynus* methanol extracts from several areas of East Java are summarized in Figure 2. The lowest cell viability percentage (37%) was found in hMSCs-BM treated

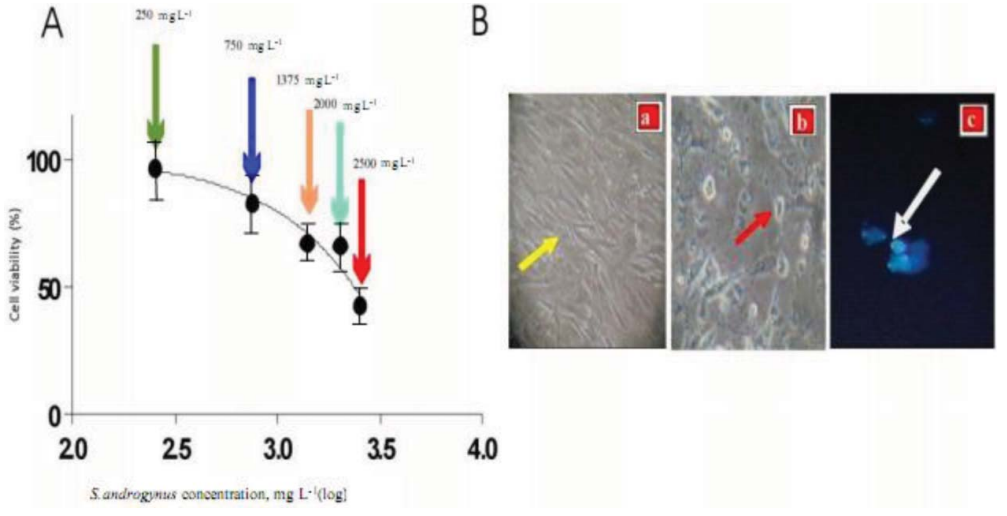

Figure 1. Effects of methanol-extracted and DMSO-dissolved constituents of *Sauropus androgynus* on hMSCs-BM. (A) Cell viability of hMSCs-BM after incubation at five different concentrations of extract constituents. Averages and standard deviations of three experiments. (B) Effect of the methanol-extracted constituents of *S. androgynus* 2500 mg L^{-1} on hMSCs-BM. Cells were detected by light microscopy and fluorescence microscopy (100×): untreated cell cultures (a), cells treated with 2500 mg L^{-1} of *S. androgynus* extract for 72 hours (b), apoptotic cell cultures after treatment with 2500 mg L^{-1} of *S. androgynus* extract for 72 hours (c).

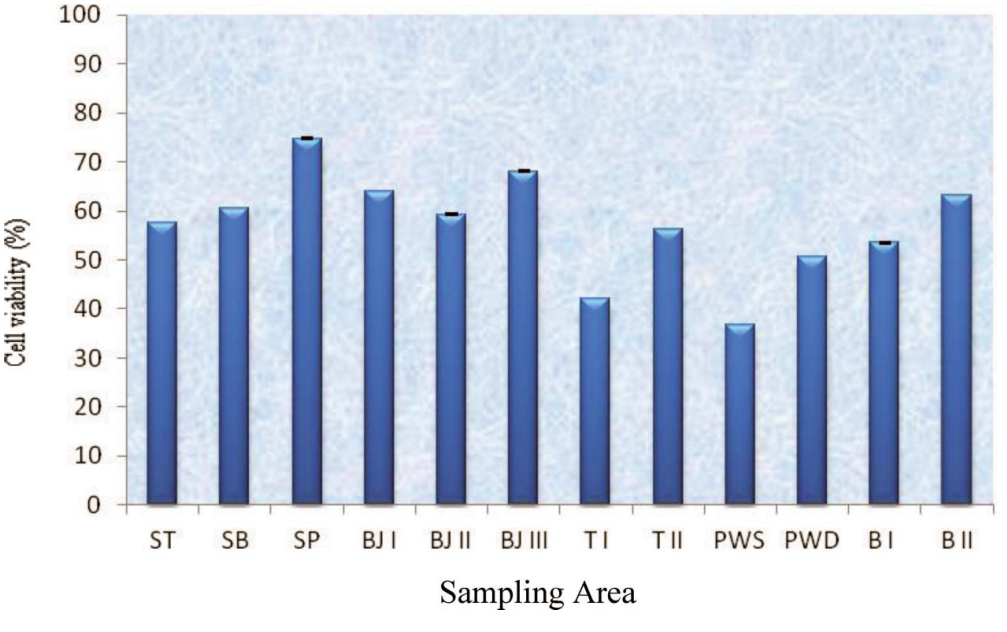

Figure 2. Percentage of viable cells of hMSCs-BM after incubation with *Sauropus androgynus* extract 2500 mg L^{-1} from several areas of East Java, Indonesia. Averages and standard deviations of three experiments: (B) Batu, (BJ) Bojonegoro, (PWD) Purwodadi, (PWS) Purwosari, (SB) West Surabaya, (SP) Center Surabaya, (ST) East Surabaya, (T) Trenggalek.

with sample from PWS, while sample from SP maintained a cell viability of 75%. The capacity of PWS sample to inhibit hMSCs-BM viability was significantly similar to those of samples from T and PWD.

Discussion and conclusion

An early assessment of the toxic effects of *S. androgynus* on humans can be performed by *in vitro* cytotoxicity screening on hMSCs-BM. *In vitro* cytotoxicity screening is an alternative strategy for performing early safety assessments of herbal products. This could be especially useful for studying herbs about which very little regarding safety for humans is known. Although cytotoxicology screenings may be acute, they are not short term. In addition, they are less expensive and time-consuming compared with animal tests (Barile 2008). This type of assay has the capacity to test a large number of compounds in a short period of time with a minimal number of materials. The data can provide information on potential mechanisms of toxicity and subcellular targets. In addition, this assessment strategy can be applied to compounds that produce significant toxicity in animal models and may never be tested in humans because they would never be allowed into clinical trials (McKim 2010).

To identify the cytotoxic effects of *S. androgynus*, the inhibitory effects on cell viability of 12 sample extracts from several areas in East Java, Indonesia, were evaluated using hMSCs-BM. This cell type was used for the cytotoxicity assay because hMSCs-BM are adult stem cells that have unique biological properties. Their role in tissue renewal, the early stages of their biochemical processes, and their unique intracellular compartments (Gioacchino et al. 2008), combined with the fact that they are of human origin, make them attractive for use in toxicity screens. Peters et al. (2008) used embryonic stem cells for embryotoxicity tests of several drugs, including 6-aminonicotinamide, valproic acid, boric acid, and penicillin G. Toxicological responses to cadmium and chromium were also observed by Gioacchino et al. (2008), while Lee et al. (2011) observed the hepatotoxicity mechanism of CCl_4 on MSCs-BM.

The cytotoxicity of each sample was expressed as an IC_{50} value. According to the US NCI plant screening program, a plant extract is generally considered to have an active cytotoxic effect if the IC_{50} value, following an incubation of 48–72 hours, is 20 mg L^{-1} (Boik 2001). The high IC_{50} value (2450 mg L^{-1}) of the *S. androgynus* extract showed that it was less cytotoxic to hMSCs-BM. This value was still within the range of the IC_{50} values of the juice of *S. androgynus* leaves applied to Chinese hamster lung cells, which ranged from 1250 to 10,000 mg L^{-1}, as previously reported by Li et al. (2006). This value was higher than the IC_{50} value from another study, because this study used the crude extract from fresh leaves. There are several other papers reporting different toxic effects of *S. androgynus*. Rahmat et al. (2003) found that an ethanol extract of *S. androgynus* from Serdang, Malaysia inhibited proliferation of the breast cancer line MDA-MB-231, with an IC_{50} value of 53 mg L^{-1}. Furthermore, $CHCl_3$, EtOAc, and *n*-BuOH fractions of an *S. androgynus* extract from Southern Taiwan had IC_{50} values of 58, 13, and 144 mg L^{-1}, respectively. Yu, Chen, and Chen (2007) found that *S. androgynus* 300 mg L^{-1} fractions caused potent inhibition of N1H3T3 cell viability. The different toxic effects of *S. androgynus* was assumed to be due to the different environmental conditions where the plants were cultivated or because of their differing genetic material (Yunita and Sulisetiorini 2011).

In addition to inhibitory effects, the mode of cell death induced by the *S. androgynus* extract was investigated. Adding the *S. androgynus* methanol extract to hMSCs-BM led

to sequential morphological changes. Typical condensation of the nucleus and chromatin appeared after cells were treated with a 2500 mg L^{-1} *S. androgynus* methanol extract for 72 hours. This result was similar to the reports from Yu, Chen, and Chen (2007), where N1H3T3 mouse embryonic fibroblast cells, exposed to the *n*-BuOH fraction of *S. androgynus*, led to typical apoptotic chromatin condensation and nuclear fragmentation, detected by Hoechst staining. The observation of apoptosis in hMSCs-BM is an essential step for discovering the mode of cell death because several factors can switch the death pathway between necrosis and apoptosis. Under pathologic conditions, apoptosis and necrosis may often coexist, as previously reported by Leist et al. (1995). Necrosis was related to a bronchiolitis obliterans outbreak after *S. androgynus* consumption in Taiwan (Chang et al. 1998).

The high inhibitory effect of *S. androgynus* extracts from PWS, T, and PWD on hMSCs-BM viability could be attributed to compounds that contribute to cytotoxic effects in these samples. However, the observed toxic effects might be due to synergism between compounds present in the plant extract. The synergism among compounds that contribute to cytotoxic activity depends not only on the concentration of the compounds but also on the structure and interactions between the compounds. Malek et al. (2009) showed differences in cytotoxic effects between crude extracts and isolated compounds against the same cell line.

Further studies are required to investigate the metabolites in *S. androgynus* that are highly correlated with its toxic effects and to study the action mechanism of toxic metabolites. Future research will combine the findings of this study with the results from an assessment of organ-specific toxicity, organizational toxicity and *in vivo* toxicity tests, to obtain a complete understanding of the toxic effects of *S. androgynus* associated with bronchiolitis obliterans in humans.

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