

Research Article

Biochemical Constituents of *Phaleria macrocarpa* (Leaf) Methanolic Extract Inhibit ROS Production in SH-SY5Y Cells Model

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Background. Reactive oxygen species generation in mammalian cells profoundly affects several critical cellular functions, and the lack of efficient cellular detoxification mechanisms which remove these radicals may lead to several human diseases. Several studies show that ROS is incriminated as destructive agents in the context of the nervous system especially with advance in age leading to neurodegeneration. Current treatments of this disease are not effective and result in several side effects. Thus, the search for alternative medicines is in high demand. Therefore, the aim of this study is to evaluate the reactive oxygen inhibitory effect of *Phaleria macrocarpa* 80% (leaf) extract. **Methods.** The leaf was extracted with 80% methanol. Cytotoxicity studies were carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and ROS inhibitory activities were evaluated using dichlorofluorescein diacetate (DCF-DA) assay in the SH-SY5Y cells model. **Results.** The result revealed ROS inhibitory activities of the crude extract with highly significant differences at $p < 0.001$ between the group that were treated with crude extract only, the group treated with crude extract and exposed to H_2O_2 , and the group exposed to H_2O_2 only as well as the group that were maintained in complete media. Bioactive compounds show the presence of vitexin and isovitexin following the HPLC method. **Conclusion.** High antioxidant activities and low toxicity effect of this crude revealed its high benefit to be used as natural medicine/supplements.

1. Background

Advance in science research especially in the field of mammalian genome has opened new avenues for studying interactions between nutrients and the genome [1]. Recently, it has been discovered that among the common mechanism that enhanced cellular durability, healthful aging shows protection against oxidative stress [2]. However, the most common causes of neuronal cells damage are excessive increase in reactive oxygen species (ROS) along with decreases in normal levels of endogenous antioxidant enzymes

[3]. Hence, the use of natural exogenous antioxidants from plants has been proposed as a unique method for preventing ROS prior to neuronal cells damage [4].

Phaleria macrocarpa also referred to as mahkota dewa or God's crown is an indigenous plant of Indonesia but commonly found in tropical areas of Papua Isl. The plant has been used traditionally in managing several abnormalities such as tumors, diabetes, inflammatory diseases, and diarrhea associated ailments such as cardiovascular diseases, oxidative stress diseases, viral infection, bacterial infection, and fungal infections [5, 6]. Phytochemical studies of

different parts of this plant revealed the presence of several bioactive components in high concentration. These include *mahkoside A*, *dodecanoic acid*, *palmitic acid*, *des-acetyl flavicordin-A*, *flavicordin-A*, *flavicordin-D*, *flavicordin-A* glucoside, ethyl stearate, and lignans sucrose [5]. Mahkoside A (4,4'-dihydroxy-2-methoxybenzophenone-6-O- β -D-glucopyranoside) was initially isolated from this plant leaf along with mangiferin (C-glucosylxantone), kaempferol-3-o- β -D-glucoside, dodecanoic acid, palmitic acid, ethyl stearate, and sucrose [7]. However, studies on the determination of antioxidant potential also revealed the presence of saponins, alkaloids, polyphenolics, phenols, flavonoids, and lignins tannins in high content from leaf and stem bark [8, 9]. Large amounts of icaraside C3, mangiferin, and phalerin gallic acid have been identified and isolated from the fruit extract of this plant [10, 11]. In the seed extract, various constituents such as naringin, quercetin, phorbol esters, des-acetyl flavicordin-A, and 29-norcurcurbitacin have been identified and also isolated [5, 12]. Despite all these medicinal values, *P. macrocarpa* is reported to be poisonous. Oral ulcers, embryo-fetotoxicity, and mild necrosis of proximal convoluted tubules caused by aqua leaf extract of this plant have been documented [13]. Mild hepatic hypertrophy and increase in serum glutamate pyruvate transaminase in guails have also been reported [9, 14, 15].

Human neuroblastoma (SH-SY5Y) cells were human cells line and have been widely used as *in vitro* models in neuroscience research [16]. The cells can be differentiated following 7-day treatment with retinoic acid and have the ability to be expressed in culture prior to differentiation [17]. However, some scientists recently reported the use of other chemicals such as herbimycin A (herb A), 12-O-tetradecanoyl-phorbol-13 acetate (TPA), dibutyryl cyclic AMP (db AMP), or neurotrophic factors to enhance differentiation of this neuronal cell in another to maintain its viability [18]. Hence, the aim of this study is to evaluate the ROS inhibitory effect of *Phaleria macrocarpa* (leaf) 80% methanol extract in the SH-SY5Y cells model.

2. Materials and Methods

2.1. Plants Collection and Identification. *Phaleria macrocarpa* was collected from Taman Pertanian Universiti (TPU) Universiti Putra Malaysia, Selangor, Malaysia. The plant leaf was authenticated by a botanist at the Institute of Bioscience (IBS), UPM, and voucher number was allocated.

2.2. Plant Extraction. *Phaleria macrocarpa* (leaf) was cleaned, separated, and cut into small pieces with the aid of a anvil pruner (UK). The plant leaf was dried for two weeks at room temperature ($26 \pm 1^\circ\text{C}$) in Biotech 2 Lab UPM and then crushed to semipowder (40–60 mesh) form. A total of 200 g of leaf sample was soaked for 3 days in 1000 mL 80% methanol in flat bottom flasks (Sigma Aldrich, USA). The mixture was shaken daily for three days and kept at 26°C to obtain high crude extract; this procedure was repeated three times. The extract obtained was then filtered with a

Whatman filter paper (1.5 Sigma Aldrich, USA) and concentrated to semisolid form at 42°C with a rotary evaporator (IKA[®] RV 10, USA). The resultant semisolid crude extract obtained was then weighed, transferred into sample bottles, and stored at 4°C until required.

Percentage yield was calculated as the weight of the filtrate divided by the total weight of the ground powder in percentage.

2.3. Plants Sample Dilution and Dose Preparation. Stock solution was prepared by dissolving 100 mg crude extract into 1 mL of 100% DMSO (100 mg/mL). The use of DMSO was to solubilize the crude extract, since the extract is absolutely not soluble in aqua solvent. Preparation of the substocks solution in microliter ($\mu\text{g/mL}$) was done by diluting the stock solution to the concentration of interest using twofold serial dilution with distilled water at eight concentrations (7.81–1000 $\mu\text{g/mL}$) in a 96-well microplate (Sigma Aldrich, USA). DMSO (vehicle) was maintained at 0.1% in all concentrations of the extract.

2.4. Cells Viability Assay. The cytotoxicity test of the crude extract on SH-SY5Y cells was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich), USA. Initially, the cells were seeded in 96-well plates at a density of 1×10^5 cells/well in 150 μL of complete Minimum Essential Media (MEM) and kept in humidified, 5% CO_2 , incubator at 37°C for 24 hours. The seeded cells were kept under observation until 24 hours after incubation. The cells were then exposed to different concentrations of prepared crude extract (7.81–1000 $\mu\text{g/mL}$) dissolved in incomplete MEM and reincubated for another 72 hours in a humidified, 5% CO_2 , incubator at 37°C under frequent observation. Control well used in this experiment includes the blanks which contained only media as well as media with 0.1% DMSO. Preparation of MTT stock solution was carried out by dissolving 5 mg MTT reagent in 1 mL of phosphate buffered saline (PBS). Dilution of the stock solution with PBS at the ratio of 1:10 gives a working solution of 10 μL . The MTT working solution (10 μL) was then transferred to the exposed seeded cells in microplate and kept for 4 hours at humidified, 5% CO_2 , 37°C incubators. Dimethyl sulfoxide (DMSO) was used and replaced the used media that contain crude extract and cells, this is further reincubated for 30 minutes in the laminar flow hood. Absorbance was taken at 570 nm using a microplate reader (Spectra max plus, USA) after dissolving the purple formazan crystal. The same procedure was repeated in evaluation of cytotoxicity effect of H_2O_2 in SH-SY5Y cells to obtained safe dose concentration to be used in ROS protective assay of the crude extract.

2.5. Determination of the Protective Oxidative Stress Test of Plant Extract on SH-SY5Y Cells. Differentiation of seeded SH-SY5Y cells following treatment with 10 μM retinoic acids for 6 days was carried out in the lab and reincubated for

24 hrs in 5% CO₂, 37°C incubators. The cells were then treated with different concentrations of the crude extract (7.81–1000) µg/mL dissolved in serum-free medium for another 24 hr. A 150 mM H₂O₂ was transferred to the microplate containing the seeded cells and reincubated for another 24 hr. The used media that contain H₂O₂ crude extract and death cells were then discarded; cells were washed with phosphate saline (PBS) in dark. A 2'7'-dichlorofluorescein diacetate (DCF-DA) (30 µM) in PBS was then added to each well and reincubated for 30 minutes. Measurement of intracellular reactive oxygen species (ROS) production was taken using microplate reader (Lab Merchant Limited, UK) at fluorescence excitation of 485 nm and 535 nm emission. The concentration of intracellular ROS in the assay was measured indirectly by the percentage increase in fluorescence per well of the 2'7'-dichlorofluorescein diacetate (DCF), formed by the oxidation of 2'7'-dichlorofluorescein diacetate (DCF-DA) with ROS secreted inside the cells. The percentage increase in fluorescence per well was calculated using the following formula:

$$\text{percentage increase in fluorescence} = \frac{F_{t_{30}} - F_{t_0}}{F_{t_0}} \times 100, \quad (1)$$

where Ft₀ is the initial reading and Ft₃₀ is the reading taken after 30 minutes of incubation.

2.6. Identification of Bioactive Compound. Plant bioactive compounds vitexin and isovitexin were used as standard in this experiment. The two standards were prepared at the concentration of 70 to 4.4 µg/mL and 97 to 6.1 µg/mL. To identify the vitexin and isovitexin, crude extract was prepared at the concentration of 1 mg/mL. Phytochemical constituents were then identified using high-performance liquid chromatography (HPLC) and liquid chromatography and mass spectrophotometry (LC/MS).

2.6.1. High-Performance Liquid Chromatography. A HPLC system (Waters, USA) consisting of a 600 pump, an auto-injector, 2998 photodiode array detector of 200 to 500 nm, was set up and used to determine the bioactive compound (vitexin and isovitexin) present in the crude extract. Separation was carried out using 250 × 4.6 mm ODS 3.3 mm column (Inertsil, Japan) thermostated at 40°C. A gradient method with methanol and deionized water was used for the separation. At 0 min, the mobile phase was set at 10% methanol in deionized water and increased to 90% methanol in deionized water for duration of 45 min. The 90% methanol in deionized water was maintained for further 15 min. The peaks were integrated at the wavelength of 337 nm.

2.7. Statistical Analysis. The antioxidant cytotoxicity and hydrogen peroxide scavenging activities results are depicted as the mean ± SD (n = 3). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the Graph Prism (5.0) statistical software to determine the statistical differences observed. All statistical evaluations for the results from

the permeation studies were performed using Student's t-test, and p value < 0.05 was considered for concluding significant difference (n = 3).

3. Results

3.1. Result of Percentage Yield of the Crude Extract. Following extraction of the grounded plant extract in 80% methanol and evaporated to semisolid form with a rotary evaporator at 42°C, the percentage yield obtained was 16.845% w/w.

3.2. Toxicity Assay

3.2.1. Toxicity Assay of Phaleria macrocarpa (Leaf) Extract on SH-SY5Y Cells. Result of the toxicity test of the crude extract on SH-SY5Y cells shows high cells death at a concentration above 125 µg/mL. At 250 µg/mL, 48.53% of the cells survive, 34.61% survive at 500 µg/mL, and 17.61% survive at 1000 µg/mL. There is significant difference at p < 0.001 between the control group and the group that are exposed to 15.6 to 1000 µg/mL. There is no statistical difference between the control group and the group that is exposed to 7.8 µg/mL. Percentage of cells viability (mean ± SD) (n = 3) and lethal dose (LC₅₀) of 155 ± 0.10 µg/mL was calculated (Figure 1).

3.2.2. Toxicity Effect of Hydrogen Peroxide in SH-SY5Y Cells. Result of toxicity test of the H₂O₂ on SH-SY5Y cells shows high cells death at concentration above 150 mM. At 300 mM, 47.11% of the cells survive while only 19.97% survive at 600 mM. There is significant difference at p < 0.001 between the control group and the group that are exposed to 37.5 to 600 mM. There is no statistical difference between the control group and the group that are exposed to 18.8 mM. Percentage of cells viability (mean ± SE) (n = 3) and the safe dose of 150 mM were calculated (Figure 2).

3.2.3. Oxidative Stress Assay of Phaleria macrocarpa (Leaf) Extract. Result of ROS inhibitory effect of crude extract on SH-SY5Y cells shows very low ROS inhibition in group exposed to H₂O₂ (150 mM). In this study, ROS inhibition was measured by decrease in absorbance at 485 nm and 535 nm emission using microplate reader. Increase of ROS inhibition was recorded at 62.5–250 µg/mL. At concentration below 62.5 µg/mL, ROS inhibitions severely decrease as indicated by increases in absorbance. At concentration above 250 µg/mL, ROS production is low and the absorbance was also decreased due to low cells viabilities. There is significant difference at p < 0.001 between the control group and the group that are exposed to the crude extract (7.9–1000 µg/mL) followed by exposure to H₂O₂ (150 mM). Percentage of cells viability is shown (mean ± SD) (n = 3) (Figure 3).

3.2.4. High-Performance Liquid Chromatography. Bioactive compounds' in *Phaleria macrocarpa* (leaf) was identified using the HPLC method with vitexin and

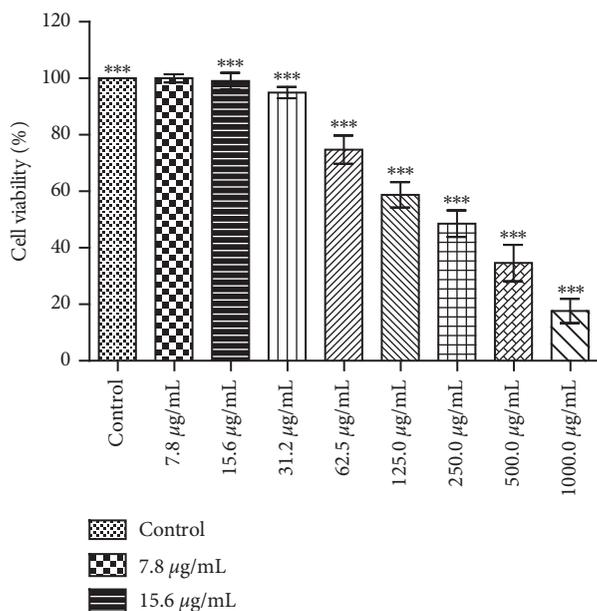


FIGURE 1: Cytotoxicity effect of crude extract on SH-SY5Y cells exposed to different concentration (7.8–1000 µg/mL). The percentage of cells viability is shown versus concentration of the tested crude extract. *** $p < 0.001$ represented significantly different values from control and tested samples. The values represent mean \pm SD from three ($n = 3$) independent experiments.

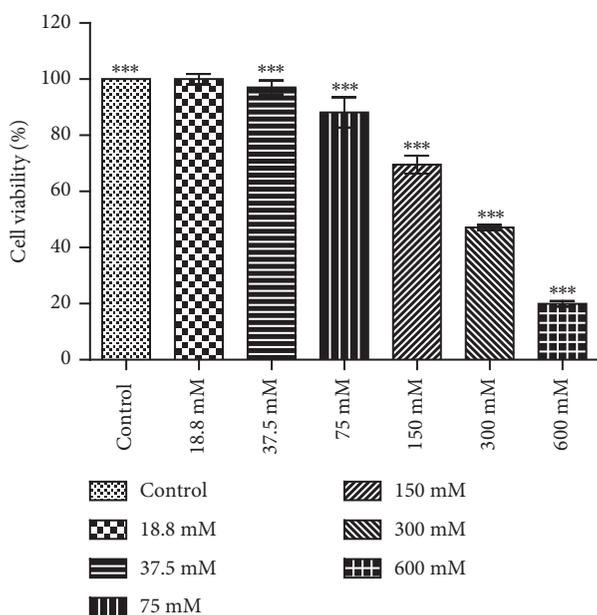


FIGURE 2: Cytotoxicity effect of H₂O₂ on SH-SY5Y cells exposed to different concentration (18.8–300 mM). The percentage of cells viability is shown versus concentration of the tested H₂O₂. *** $p < 0.001$ represented significantly different values from control and tested samples. The values represent mean \pm SD from three ($n = 3$) independent experiments.

isovitexin as standard (Figures 4 and 5). Both peak areas identified in this crude extract were compared with vitexin (retention time 21.834) and isovitexin (retention time 23.002) (Figure 6).

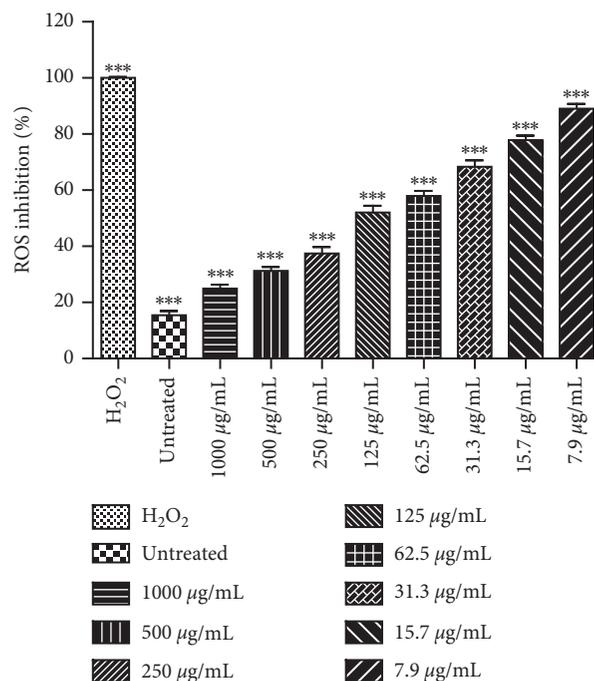


FIGURE 3: Intracellular ROS inhibitory levels were measured by fluorescent probe DCF-DA in SH-SY5Y cells pretreated with extract for 24 hrs and challenge with H₂O₂ (150 mM) to induce ROS production. The values represent mean \pm SD from three independent experiments. *** $p < 0.001$ represented significantly different values from H₂O₂ treated group and the group that are treated with crude extract (7.9–1000 µg/mL) followed by exposure to H₂O₂.

4. Discussion

Methanol, ethanol, and acetone ethyl acetate are commonly used in the extraction of plant bioactive compounds [19, 20]. Literature shows that high yield of phenolic compound was recorded in acetone solvent compared to methanol solvent in an experimental extraction of fruit vegetable [21]. High yield of phenolic compound in methanol leaf extracts compared to acetone and hot water chloroform leaf extracts has also been documented [22]. This may be due to variation in high concentration of polar or nonpolar compounds resulting in increased affinity of one solvent then another to phenolic compounds present in the plant parts. Ethanol and ethanol/water solutions were also reported as one of the best solvent for the extraction of phenolic compound in horseradish roots extraction study [23]. Therefore, considering the high potential of polar solvent in extracting large quantities of phenolic compounds, it is used by traditional herbal practitioners in preparation of decoction or infusion, and 80% methanol was chosen as extraction solving. The result of percentage yield obtained after extraction of 200 g leaf sample with 80% methanol was 16.845% w/w. This result is in agreement with the finding reported by [13] which shows high yield of 11.99% w/w following extraction of *Phaleria macrocarpa* leaf with 80% methanol.

Effect of crude extract on SH-SY5Y cells follows a concentration gradient as there is increase in toxicity effect

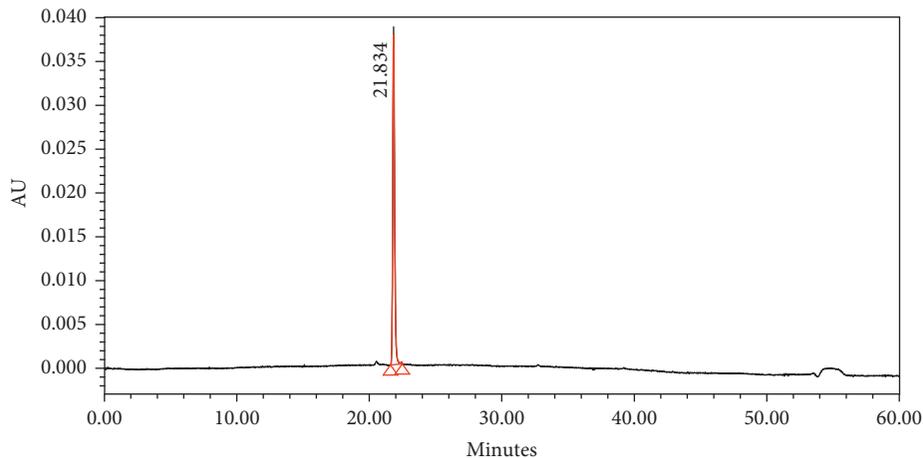


FIGURE 4: Normal-phase HPLC profile of vitexin (standard) identified at a retention time of 21.834 min.

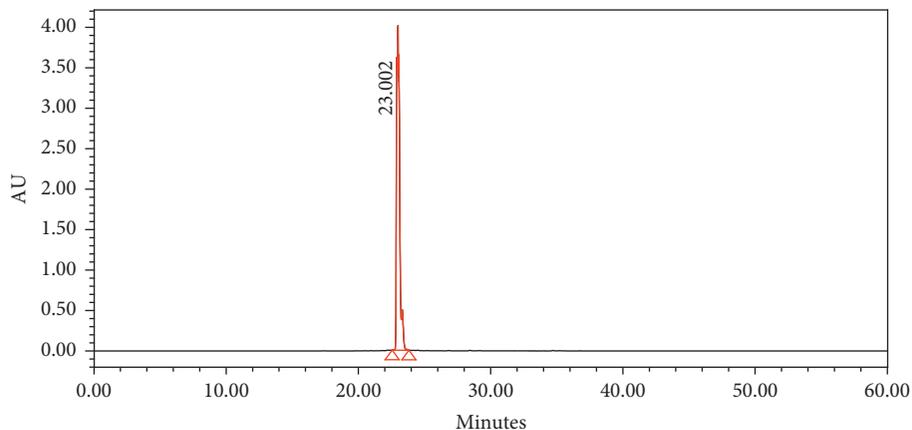


FIGURE 5: Normal-phase HPLC profile of isovitexin (standard) identified at a retention time of 23.002 min.

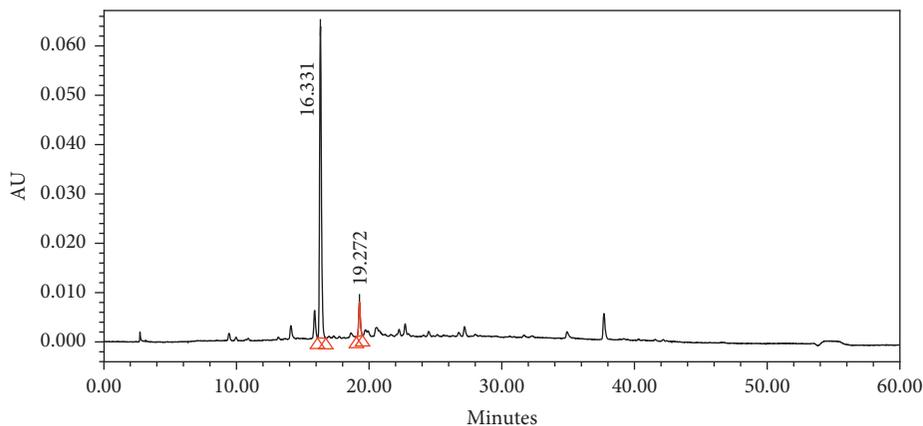


FIGURE 6: Normal-phase HPLC profile of *Phaleria macrocarpa* (leaf) with minute concentration of vitexin and isovitexin identified at a retention time of 21.834 and 23.002 min.

with increase in crude extract concentration. There was significant difference at $p < 0.001$ and $f = 68.334$ between the control group and the groups that were exposed to 15.6–1000 $\mu\text{g/mL}$ crude extract. This is due to decrease in cells viabilities with increase in concentration of the crude extract. This finding is similar to the one documented by

[24], which revealed increase in mitosis intensity of cells induced with low concentration of *Solanum nigrum* extract. Decrease in mitotic activities was observed in the same cells following increased concentration of the extract. Some of the major factors that contribute to cells death may be attributed to toxic phytochemical constituents. It has been revealed

that high concentration cytokinin-like substance if present in crude extract can induce cytotoxicity effect in cells followed by cells death [25, 26]. Previous studies also show that presence of bioactive compounds such as β -sitosterol, stigmaterol, and taraxeryl acetate cyclopropane in *Hibiscus rosa-sinensis* if used in cells causes decrease in cells viabilities [27]. There is significant difference on cells viability at $p < 0.001$, $f = 133.975$ between the control group and the groups that were exposed to 37.5–600 mM of H_2O_2 . This is because of the cytolytic effect of increase in H_2O_2 concentration on SH-SY5Y cells. It is clearly shown from this experiment that groups exposed to above 150 mM exhibited low viabilities due to increase in toxicity of the agents, leading to cells death as seen in MTT assay. The finding in this study shows those not corresponding to the finding reported by Park et al. who shows 250 mM as the safe dose of H_2O_2 to induce ROS in the SH-SY5Y cells [28]. Variation in result may be due to concentration of the chemical used during study. In this study 30% H_2O_2 was used while there is no specific concentration of H_2O_2 indicated during the experimental study by Park et al.

The result of reactive oxygen species inhibition effect of crude extract on H_2O_2 -induced ROS generation shows high significant difference at $p < 0.001$, $f = 271.923$ among the groups that were exposed to H_2O_2 , groups that were treated with crude extract only, and the group that were treated with crude extract followed by challenge with H_2O_2 . There are no sufficient literatures on the ROS protective effects of *Phaleria macrocarpa* induced by H_2O_2 on SH-SY5Y cells model. It has been reported that diets rich in fruits have vegetables protective effects against all diseases caused by ROS due to the presence of antioxidant bioactive compounds [29]. Presence of bioactive compounds with antioxidant effects in the extract may be responsible for the decrease in fluorescent absorbance. Antioxidant agent within the cells act by neutralized H_2O_2 by converting it to water with reduced oxygen radical to oxidize DCFH to form fluorophore [30].

Bioactive compounds` (vitexin and isovitexin) were identified in this the crude extract using HPLC at retention time 21.834 for vitexin and 23.002 for isovitexin. Medicinal effects of *Phaleria macrocarpa* (leaf) may be due to vitexin and or isovitexin have been reported previously. Few among many studies involved are *in vitro* anti-inflammatory activity [31], antioxidant activity [6], anticancer activity [32], vasorelaxant activity [7], and antimicrobial activity [33]. Increase in therapeutic potential of this crude extract may be due to activities of many compounds besides vitexin and isovitexin. These include the following biological components: saponins, alkaloids, polyphenolics, phenols, flavanoids, and lignans tannins [6, 8]. Even though vitexin and isovitexin may be present at minute concentration in this crude extract, a number of secondary metabolite may be responsible for its high antioxidant medicinal effect.

5. Conclusion and Recommendation

It is concluded that the presence of vitexin and isovitexin in the crude extract contributed to its ROS protective effect in SH-SY5Y cells. This justified the potential of this plant

extract to be used as chemotherapeutic agents. Toxicity screenings of this crude extract on mammal such as mice and rat to reaffirm their toxicity profile are recommended. Antioxidant screening as well as isolation of bioactive compounds such vitexin and isovitexin is strongly recommended.

Data Availability

Data are available with the permission from Dr. Syahida Ahmad and Universiti Putra Malaysia.

Ethical Approval

Ethical approval was provided by the institutional ethics committee of the Universiti Putra Malaysia and is submitted as supplementary document.

Disclosure

The study was a part of postgraduate Ph.D. thesis and is available with the permission from the supervisor and University Management.

Conflicts of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, or publication of this article.

Authors' Contributions

Dr. Hassan Maina Ibrahim carried out the research and wrote the thesis and the manuscript. Dr. Wan Norhamidah Wan Ibrahim was the cosupervisor, participated in design of the proposal, and monitored the project and data analysis. Dr. Ferdaus Binti Mohamat Yusuf was the cosupervisor, participated in design of the proposal, and monitored the project. Dr. Siti Aqlima Ahmad was the cosupervisor, participated in design of the proposal, and monitored the project. Dr. Syahida Ahmad was a major cosupervisor, participated in design of the proposal, monitored the project to conclusion, and financed the research.

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