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Anti-Proliferative and Apoptotic Induction Effect of Elateriospermum Extract on Human Lung Cancer Cell Line A549 †

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† Presented at the The 1st International Electronic Conference on Nutrients—Nutritional and Microbiota Effects on Chronic Disease, 2–15 November 2020; Available online: https://iecn2020.sciforum.net/.

Published: 30 October 2020

Abstract: Natural products derived from plants are used to treat cancer due to fewer side effects compared to standard treatments available for cancer. The second highest cancer that causes death worldwide is lung cancer. Therefore, this study aimed to determine the cytotoxic activity of hot and cold aqueous extract of *E. tapos* seed and shell on human cancer cell line A549 as well as the apoptosis mechanism. The apoptosis mechanisms were evaluated by cell viability (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium reduction assay; MTT assay), Hoechst 33358 staining and detection of reactive oxygen species (ROS) activity. The apoptosis inducing activity was analyzed in set of morphological and biochemical features. Hot aqueous shell extract (SHH) showed an anti-proliferative effect at the IC_{50} of 49.8 ± 0.06 μg/mL correlated with apoptosis induction by increasing the ROS activity by significant ($p < 0.05$) increase of 3.25 folds compared to control. Results suggest that SHH poses an anti-proliferative effect on account of apoptosis through ROS mediated mitochondria mutilation.

Keywords: *Elateriospermum tapos;* lung cancer; apoptosis

1. Introduction

Elateriospermum tapos (*E. tapos*) comes from the Euphorbiaceace family, commonly known as "perah" or "pogoh" nut in Malay, is a large buttresses tree found in primary and secondary forests, mixed dipterocarp forests, and forest edges. In Malaysia, this species can be found in Jengka Forest Reserve, Peninsular Malaysia [1]. *E. tapos* is not classified as a major medicinal as of today. Nonetheless being a tree of ornamental value [2], the different parts of the plant have been used in folk medicine. The white and sticky latex was used in treating cracked soles of the foot [3].

E.tapos has been reported to have phytochemicals such as flavonoid, tannin, and alkaloid that hold therapeutic properties against many diseases. The daily consumption of flavonoids has properties that reduced the risk of cancer [4]. Plant-derived products are widely used as traditional medicine due to less toxicity and fewer side effects [5]. Sita, Thanaset, Prasan, & Auamporn (2018) stated that the compound taraxerane triterpenes found in *E. tapos* was effective against lung cancer (NCI-H187) and breast cancer cell lines and also found evidence for the coumpound as an anti-mycobacterial against Mycobacterium tuberculosis in an in vitro study [6]. Further pharmacological

research has revealed several biological activities such as antioxidant, antitumor, and antimycobacterial [7]. These activities were ascribed by various chemical constituents such as taraxane, triterpenes, flavonoids, acetates, and palmitates [6,7].

Lung cancer is the second highest cancer that cause of death globally after breast cancer [8,9]. The statistics from the year 2012 showed 1.8 million new cases every year, and the mortality rate is 1.6 million worldwide [9]. The standard treatments available for cancer are chemotherapy, radiotherapy, tumor removal via surgery, and immunotherapy [10]. Unfortunately, they are associated with side effects such as nausea, fatigue, appetite loss, and edema. Besides, the 5-year survival rate is still unsatisfying with lung cancer patients [11].

Malaysia, with its broad spectrum of natural biodiversity has been indicated to have more than 6000–7000 higher plant species that have therapeutic or medicinal properties [12]. Based on this fact, it would be an excellent opportunity if we could also utilize the wasted parts that are beneficial to our health which would contribute to economic enhancement. Therefore, the objective of this study was to determine the cytotoxic activity of hot and cold aqueous *E. tapos* seed and shell on human cancer cell line A549 and focused on the apoptosis mechanism.

2. Materials and Methods

2.1. Sample Preparation

The samples were washed and separated to dry in the oven at 40 °C for 3 days. Thereafter, fine powder was obtained from the ground dried samples by using a fine mesh sieve. The fresh samples were kept at −20 °C prior to extraction.

2.2. Hot and Cold Water Extraction

Decoction method was used to prepare the aqueous extract of samples. For hot water extraction, 100 g of the dried powdered samples were soaked in 1 L of water at 70 °C for 12 h, while the cold water extraction was left for 2 days at room temperature. The samples were filtered using a Whatman filter paper No. 1. Thereafter, the samples were freeze-dried and stored at −20 °C.

2.3. Cells, Reagent, and Antibodies

Human cancer cell line A549 was obtained from ATCC (American Type Culture Collection). The cells were grown in RPMI 1640 medium with 10% FBS (fetal bovine serum) and antibiotic (1% penicillin-streptomycin) at 37 °C in the humidified atmosphere with 5% CO_2 [13].

2.4. Cell Viability Assay

Cell viability was evaluated by using the MTT assay. The cells were plated at 1.0×10^4 cells/well into the 96-well flat bottom plate and incubated for 24, 48, and 72 h in 5% of CO_2 incubator at 37 °C. After 24, 48, and 72 h of incubation, 20 μL of MTT reagent (5 mg/mL) was added to each well for 4 h, and the absorbance was read at 550 nm using a microplate reader. The inhibitory concentration IC_{50} was obtained and tabulated [13].

2.5. Analysis of Apoptosis by Hoechst 33358

Hoechst 33358 staining was used to analyze the morphology of apoptosis cells. The cells were plated at 1.0×10^6 cells per well in a 6-well plate until confluent. Cells were exposed at the IC_{50} of all the extracts for 24 h. After 24 h, the cells were washed twice with cold PBS (phosphate buffer saline), then fixed with cold methanol and acetic acid 3:1 (*v*/*v*) for half an hour in dark, followed by the observation under a fluorescence microscope (Microscope, Olympus DX51; Camera, Olympus DP72) [13].

2.6. Detection of Intracellular Reactive Oxygen Species (ROS)

Intracellular Reactive oxygen species (ROS) was analyzed using a ROS assay kit. Cells were plated at 2.5×10^3 cells/well in a black 96-well plate. Cells were treated with IC_{50} of all the extracts for

24 h. Thereafter, they was incubated with DCFH-DA (Dichloro-dihydro-fluorescein diacetate) (5 μM) at 37 °C for half an hour and exposed to H_2O_2 (0.5 mM) diluted in culture medium for 1 h. After 1 h, the fluorescence of the cells was measured at 480 nm/520 nm (EnSpire Multimode Plate Reader, PerkinElmer) [13].

3. Results

3.1. Inhibitory Effects of E. tapos Extracts on A549 Cell Line

The cells were incubated for 24 h with different concentrations (1000 μg/mL, 800 μg/mL, 600 μg/mL, 400 μg/mL, 200 μg/mL, 100 μg/mL). Cell viability was determined by using MTT assay. As shown in Table 1, the lowest IC_{50} showed was *E. tapos* seed cold aqueous solution (SDC) (28.6 ± 0.18 μg/mL), followed by *E. tapos* seed hot aqueous solution (SDH) (45.8 ± 0.15 μg/mL) and *E. tapos* shell hot aqueous solution (SHH) (49.8 ± 0.06 μg/mL), and the highest IC_{50} showed in *E. tapos* shell cold aqueous solution (SHC) (75.9 ± 0.19 μg/mL). As a positive control (PC), cisplatin was used that showed IC_{50} (17.3 ± 0.01 μg/mL*).* In addition, these extracts showed less toxic effects against the normal cell lines, MRC5 (IC_{50}: > 500 μg/mL; Table 1).

Table 1. Inhibitory concentration IC_{50} of *E. tapos* extracts and cisplatin on A549 and MRC5 cells after 24 h of incubation**.**

	E. tapos	**Solvents**	**Cancer cell line A549 (μg/mL)**	**Normal cell line MRC5 (μg/mL)**
Plant extracts	Seed (SD)	Hot Aqueous (H)	45.8 ± 0.15	>500
		Cold Aqueous (C)	28.6 ± 0.18	>500
	Shell (SH)	Hot Aqueous (H)	49.8 ± 0.06	>500
		Cold Aqueous (C)	75.9 ± 0.19	>500
Standard drug	Cisplatin		17.3 ± 0.01	13.2 ± 0.05

3.2. Effects of E. tapos Extracts on Hoechst 33358 Staining

The morphological changes of nuclei were identified by the fluorescence microscope. The arrows indicated apoptotic cells (Figure 1). The apoptotic cells are recognized by the high fluorescent condensed chromatin. SDH, SDC, and cisplatin showed condensed and small nuclei as compared to SHH, SHC, and control which had normal nuclei.

3.3. The Effects E. tapos Extracts on ROS on Treated A549 Cell Line

ROS decreases the cell viability with an increase in ROS production that results in cell death. As shown in Figure 2, ROS produced by cisplatin-treated cells increased 1.03-fold as compared to control. However, SDH showed the highest (1.4-fold) ROS released as compared to cisplatin. SHC has the lowest (0.94-fold) ROS, followed by SDC (0.99-fold) and SHH (1.04-fold) as compared to control.

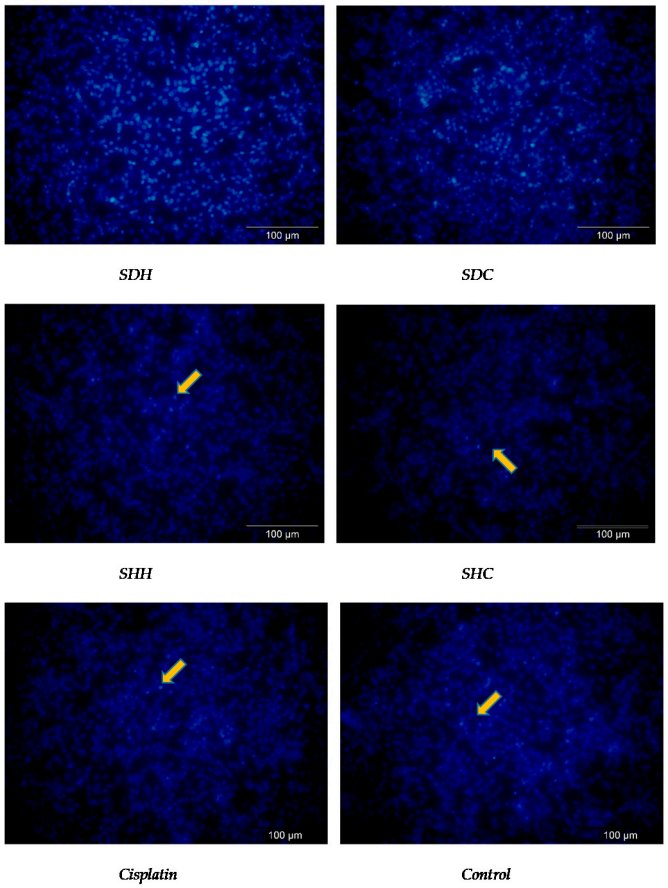

Figure 1. Cell apoptosis morphology observation by Hoechst staining. SDH—Seed Hot Aqueous, SDC—Seed Cold Aqueous, SHH—Shell Hot Aqueous, SHC—Shell Cold Aqueous, Cisplatin, Control—Untreated cells.

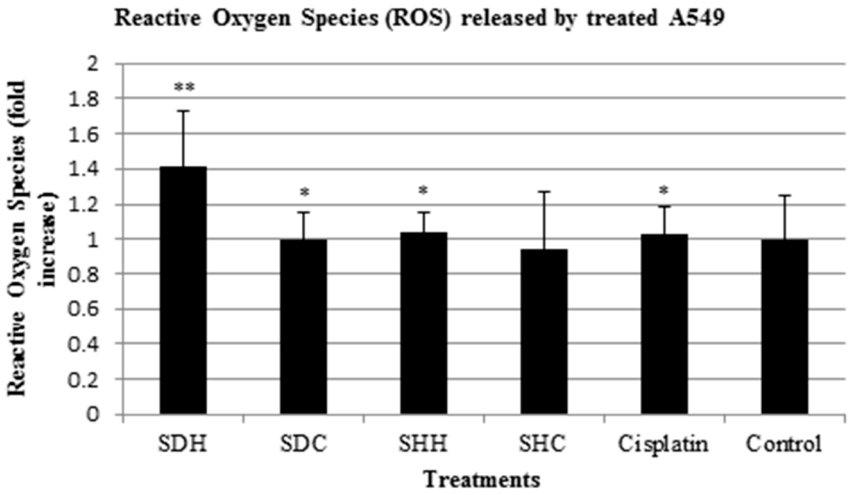

Figure 2. Fold increment of reactive oxygen species (ROS) released from treated A549 cells. Error bar indicates the standard error of the mean of three independent experiments. SDH—Seed Hot Aqueous, SDC—Seed Cold Aqueous, SHH—Shell Hot Aqueous, SHC—Shell Cold Aqueous, Cisplatin—Positive control, Control—Untreated cells. * $p < 0.05$, ** $p < 0.01$ vs. control.

4. Discussion

Globally, various plants have been used to treat many diseases, especially in undeveloped countries. As previously stated, natural products such as plants are used due to less toxicity. *E. tapos* has been used for obesity, anti-hyperlipidemia, and cancer research. It is commonly known as perah that is consumed either in a cooked or fermented way by the people in local villages in Southern Thailand [12]. In the present work, we determined the cytotoxic activity of *E. tapos* seed and shell extracts on human cancer cell line A549 and focused on the apoptosis mechanism. Cisplatin is a chemotherapeutic drug that was used as a positive control.

The cytotoxic effect of extracts on A549 cells was determined by using MTT assay. Based on Table 1, cisplatin revealed the lowest IC_{50} which is 17.3 ± 0.01 μg/mL, indicating it has the highest inhibition towards A549 at a low concentration. The lowest IC_{50} showed among *E. tapos* treated group was SDC (28.6 ± 0.18 μg/mL), followed by SDH (45.8 ± 0.15 μg/mL), SHH (49.8 ± 0.06 μg/mL), and the highest IC_{50} showed in SHC (75.9 ± 0.19 μg/mL). As comparison to the seed skin ethanol extract by Sita, Thanaset, Prasan, & Auamporn (2018) the IC50 of all samples were within the range of 13.26–87.94 μg/mL [6]. This indicates that the killing effect of *E. tapos* samples is in range of the same period of incubation.

In order to further analyze the mechanism of A549 cell death, Hoechst 33358 was used to observe morphological changes on the DNA of the cells. Cell death is easily visible by the chromatin condensation and fragmentation of the DNA, when compared to the even, flat disc-like in non-apoptotic chromatin. Moreover, dead cell also presents uneven morphology which comprises of a cluster of comparatively bright fragments of condensed DNA. Similarly reported in the study by Thiagarajan et al., 2019, the features were observed to reflect on traits seen during the early stage of apoptosis [13].

Confirming the morphological features of the cells, ROS signaling was studied, as the increase in ROS can be due to several events, which include the alteration of mitochondrial lipids. This suggests that the increase in ROS signaling is vital in cell disruption causing apoptosis. A minor ROS dose will result in cell survival while a major ROS dose will trigger apoptosis [14]. Therefore, an increase of ROS in *E.tapos*-treated cells caused mitochondrial dysfunction that led to cancer cell apoptosis by the mitochondrial stress pathway [13].

According to a previous study, *E. tapos* shell has a highest flavonoid content than *E. tapos* seed. Among the extraction, the highest amount of flavonoid was found in SHH [15]. Flavonoid is widely used as cancer chemoprevention and chemotherapy [16]. Flavonoid has the potential to induce apoptosis in human lung cancer cell [16,18]. This finding is correlated with extracts that contain a flavonoid compound causing apoptosis where morphological changes occur, increasing ROS activity [19–21].

5. Conclusions

All the *E. tapos*-treated groups showed the formation of apoptosis. The most effective effects can be seen in SHH-treated A549 cells that correlated with apoptosis induction by increasing ROS activity. Therefore, SHH is effective for lung cancer treatment via ROS activation due to the presence of the highest content of flavonoid.

Author Contributions: S.K.T.: Conceptualization, Methodology, Formal analysis, Writing—original draft preparation. K.V.P.: Data curation, Writing—draft preparation, N.H.S. and K.K.A.K.: Writing—review and editing, H.B.: Supervision, Validation, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This project was funded by Universiti Putra Malaysia (GP-IPM/2016/9470400 and GP-IPS/2018/ 9639700).

Conflicts of Interest: The authors declare no conflict of interest.

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