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ORIGINAL ARTICLE

Antioxidative and Photocytotoxic Effects of Standardized *Clinacanthus nutans* and *Strobilanthes crispus* Extracts toward HepG2 Liver Cells

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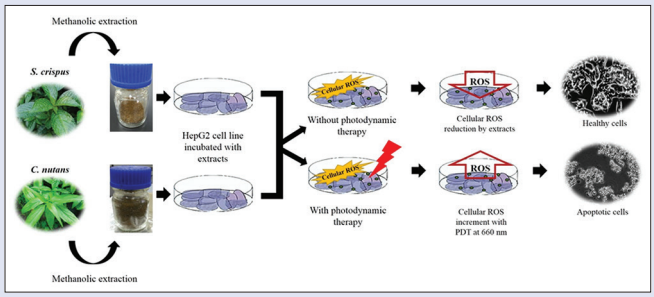
ABSTRACT

Introduction: The methanolic extracts of *Clinacanthus nutans* (CME) and *Strobilanthes crispus* (SME) are used in Malaysia as a complementary and alternative medicine for cancer. **Objective:** The present study aimed to determine the antioxidative and photocytotoxic effects of CME and SME toward liver cancer cells. **Materials and Methods:** Cell-based (2′,7′-dichlorodihydrofluorescein diacetate) and chemical-based (2,2-diphenyl-1-picrylhydrazyl [DPPH]) experiments were utilized to determine the antioxidative properties of both herbal extracts. CME and SME were also tested for their photocytotoxic potentials after photodynamic therapy (PDT). Phytochemical analysis was performed to identify the phytocompounds present in the extracts. **Results:** Both the extracts demonstrated dose-dependent DPPH radical scavenging activities, while SME was found to be a stronger reactive oxygen species scavenger than CME at all concentrations tested on liver cells. Interestingly, on PDT, HepG2 cells treated with SME and CME at non-toxic doses showed a decrease in cellular viability charting half-maximal inhibitory concentration of 13.45 μg/mL and 81.03 μg/mL, respectively. Total phenolic content of SME (36.27 ± 1.31 mg GAE/g extract) was slightly higher than CME (31.76 ± 0.10 mg GAE/g extract). On the contrary, the total flavonoid content of CME (11.32 ± 0.28 mg QE/g extract) was approximately seven times more than SME (1.69 ± 0.03 mg QE/g extract). Phenolic acids, flavonoids, and pheophorbide-a were identified in both extracts. In view of this, these phytocompounds present in CME and SME could lead to the observed beneficial effects. **Conclusion:** CME and SME, especially the latter, are strong antioxidants with photosensitizing potentials that should be further investigated.

Key words: *Clinacanthus nutans,* HepG2, photocytotoxic, photodynamic therapy, reactive oxygen species, *Strobilanthes crispus*

SUMMARY

- Standardized extracts of *Clinacanthus nutans* and *Strobilanthes crispus* exhibited reactive oxygen species scavenging activities in liver cells
- Without light exposure, both plant extracts at concentration up to 500 μg/mL were non-toxic to hepatocellular carcinoma (HepG2) liver cells
- On photodynamic therapy, these herbal extracts demonstrated photocytotoxicity against HepG2 liver cells
- Phenolic acids, flavonoids, and pheophorbide-a were identified in both herbal extracts
- *C. nutans* and *S. crispus* extracts are potential photosensitizing agents with dual functionalities



Abbreviations used: CME: *Clinacanthus nutans* methanolic extract; SME: *Strobilanthes crispus* methanolic extract; DCFH-DA: 2′,7′-Dichlorodihydrofluorescein diacetate; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ROS: Reactive oxygen species; PDT: Photodynamic therapy; DNA: Deoxyribonucleic acid; AIDS: Acquired immune deficiency syndrome; ATCC: American Type Culture Collection; DMEM-F12: Dulbecco's Modified Eagle's Medium: Nutrient Mixture Ham's F-12; MTT: Methylthiazolyldiphenyl-tetrazolium bromide; LED: Light-emitting diode; HPLC: High-performance liquid chromatography; ANOVA: Analysis of variance; NAD(P)H: Nicotinamide adenine dinucleotide phosphate; IC_{50}: Half-maximal inhibitory concentration.

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INTRODUCTION

Reactive oxygen species (ROS) acts as a double-edged sword in all living beings. Its overproduction could lead to cancer, but if used wisely, ROS could, in fact, facilitate the elimination of this disease. Abnormal accumulation of ROS in normal cell causes tumorigenesis via genetic mutations leading to transformed cells.[1] On the contrary, a sudden increase in ROS on cancer cells exposes them to DNA damage and eventually cell death.[2]

Secondary metabolites from plants such as flavonoids, phenolic acids, and tannins are well-known antioxidants that possess effective

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ROS scavenging activities.[3] Many published works had proven that these phytocompounds could reduce ROS levels *in vitro*[4] as well as *in vivo*.[5] Nevertheless, at higher dosages, plant secondary metabolites could play an adverse role as pro-oxidants that generate ROS instead. The pro-oxidant effect of plant extract is now being harnessed into a beneficial tool for anticancer therapy. It was reported that phytocompounds with pro-oxidant ability were shown to eliminate cancer cells via oxidative DNA strand break.[3] In this study, phytocompounds from herbal plants, *Clinacanthus nutans* and *Strobilanthes crispus,* were investigated for their ROS scavenging and ROS-producing effects.

C. nutans is a species of plant in the Acanthaceae family, also known as "Sabah Snake Grass" or "Belalai Gajah," which commonly found in Malaysia, Thailand, and Indonesia.[6,7] It is widely used as a traditional medicine and is even classified as an important medicinal plant by the Thai Ministry of Public Health.[7] This plant has been used in treatments of inflammation, viral infection, herpes infection, and even cancer.[8]

Other than *C. nutans*, *S. crispus* is also being investigated in this study. *S. crispus* belongs to the same family as *C. nutans* and is known by the names "Pokok Pecah Kaca" or "Pokok Pecah Beling" in Malaysia.[9] It is a type of herbal plant native to countries from Madagascar to Indonesia. *S. crispus* has been used traditionally as antidiabetic, diuretic, antilytic, and laxative. It is proven scientifically to possess high antioxidant activity, anti-AIDS, and anticancer properties.[9]

Importantly, both these plants were found to confer antiproliferative activities toward cancer cells such as hepatocellular carcinoma (HepG2), breast adenocarcinoma (MDA-MB-231), and cervix adenocarcinoma (HeLa).[8-11] However, to date, no photodynamic therapy (PDT) research is conducted using *C. nutans* and *S. crispus* as photosensitizers on cancer cell lines. PDT is a method to confer cytotoxic effect on cancer cells in the presence of a photosensitizer, an oxygen-rich environment, and a light source with appropriate wavelength.[12] The photosensitizer with enough light energy received at the tumor site will produce ROS from oxygen atom-containing molecules, leading to oxidative stress and eventually cell death.[12]

Several Malaysian plants had been reported to demonstrate photosensitizing effects against cancer cells such as *Pentaspadon motleyi*, *Blumea balsamifera*, *Leonurus sibiricus,* and *Curcuma zedoaria*. Chlorophyll catabolites, pheophorbide-a and pheophytin-a, identified from these plants extracts were suspected to confer photocytotoxicity to both HL60 (human promyelocytic leukemia) and K562 (human myelogenous leukemia) cell lines.[13] In this research, we examined the antioxidative and photoinduced cytotoxic properties of *C. nutans* and *S. crispus* based on their abilities to sequester and produce ROS, respectively, in the human hepatocarcinoma HepG2 cell line. Phytochemicals responsible for the observed activities were also identified using high-performance liquid chromatography (HPLC) analyses, and chemical standardization of the extracts was subsequently performed.

MATERIALS AND METHODS

Chemicals

Most chemical reagents used in this research were of analytical grade (except stated) purchased from Sigma-Aldrich and Merck. Standard phenolic compounds, gallic acid, (+)-catechin, chlorogenic acid, caffeic acid, ferulic acid, ellagic acid, quercetin, and kaempferol for phytochemical identification work, were of HPLC grade. Pheophorbide-a was also HPLC grade from Santa Cruz Biotechnology (USA).

Plant materials and extraction

Leaves of *C. nutans* and *S. crispus* were obtained from Seremban TKC Herbal Nursery, Malaysia, on February 2017. Authentication of the plant specimens was made and deposited at the herbarium of the Biodiversity Unit, University Putra Malaysia (UPM), Serdang, Selangor, Malaysia. *C. nutans* and *S. crispus* were given the voucher numbers of SK3266/17 and SK3267/17, respectively. The leaves were dried in a hot-air oven at 40°C before pulverizing them into fine powder. Extraction was carried out with 80% aqueous methanol by cold maceration as described.[14] The methanolic extracts were then filtered through Whatman filter paper No. 1 and concentrated in vacuo at 40°C. Freeze drying was carried out on the organic solvent-free crude extract thereafter. Powdered crude extracts were stored in air-tight containers at 4°C until further use. *C. nutans* and *S. crispus* methanolic extracts (CME and SME) were diluted into desired concentrations for the use in subsequent experiments using DMSO (< 0.05%). The yields of the leaf extracts of *C. nutans* and *S. crispus* were 17.1% and 12.1%, respectively. Percentage was based on the weight of powdered crude extract (g) over dried weight of leaves (g).

Cell line and culture condition

The human HepG2 cells, were obtained from the American Type Culture Collection (ATCC). HepG2 from passage 61st to 70th was used for assay. It was maintained in DMEM-F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% of Penicillin-Streptomycin (Gibco, USA) at 37°C in a humidified incubator with 5% CO_2.

Cytotoxicity assay of *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts on HepG2 liver cells using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

The cell viability was determined by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[10] After respective treatment, the culture medium of cells was replaced with fresh medium without fetal bovine serum (FBS). During removal of medium, cells were rinsed with 1X PBS before addition of new medium. Ten microliters of 5 mg/mL of MTT (Sigma, USA) solution in 1X PBS was then added into each well followed by incubation at 37°C for 3 h. After incubation, 100 µL of 10% sodium dodecyl sulfate (SDS) in 0.01 M of HCl was pipetted in and left for 18 h. Absorbance at 570 nm was then determined by microplate reader (Infinite 200 PRO, Tecan, Switzerland). The absorbance values obtained were deducted against the blank absorbance (medium containing only MTT and solubilizing solvent).

Antioxidative potentials of *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts

2′,7′-Dichlorodihydrofluorescein diacetate assay was performed on HepG2 cells (2×10^5 cells/well) treated with CME and SME. Positive control used in this experiment was *tert*-Butyl hydroperoxide. Fluorescence readings were measured using microplate reader (Infinite 200 Pro, Tecan) (λ_{ex} = 485 nm and λ_{em} = 530 nm).[15] 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of the herbal extracts were also investigated. The absorbance remaining after the reaction was then measured using spectrophotometer (ultraviolet 0281, Hitachi, Japan) at 517 nm.[16]

Photodynamic therapy of *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts on HepG2 liver cells

PDT protocol was slightly modified from previous research.[17] HepG2 cells were seeded in 96 well tissue culture-treated plates at a density of 5×10^4 cells per well. The cells were washed with 100 µL of 1X PBS,

followed by addition of CME or SME extracts at various concentrations. After the incubation period of 2 h, the HepG2 cell line was exposed for 10 min to a portable PDT device with LED light source of peak wavelength 660 nm, a fluence rate of 28.40 mW/cm^2, and a total light dose of 17.04 J/cm^2. On irradiation, the cells were incubated again at 37°C for 24 h prior to MTT assay. Photo-independence cytotoxicity assay was also conducted on the extract-treated and nontreated cells. In addition, the effects of different light exposure durations (5 min, 10 min, and 20 min) were investigated as well.

Cellular morphological observation

Hepatocellular carcinoma cell line (HepG2) was seeded in 60 mm dishes until 70% confluency was reached. The cells were then treated with 3.0 mL of 50 µg/mL of CME and SME, respectively, for 2 h, followed by PDT. The cell morphological changes were photographically captured by inverted contrasting microscope (Leica DM IL, Germany) and compared with cells without extract treatment. Furthermore, observations were also made on treated and nontreated cells without PDT for further evaluation.

Phytochemical screening of *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts

CME and SME were analyzed for the presence of tannins, phenolic compounds, flavonoids, saponins, terpenoids, cardiac glycosides, fixed oil, and also alkaloids.[18] The total phenolic and flavonoid contents of CME and SME were determined using Folin–Ciocalteu assay[19] and aluminum chloride colorimetric method,[19] respectively. HPLC analysis was performed with Agilent HPLC (1260 Infinity) (Santa Clara, USA) to identify the bioactive compounds in the extracts. The C_{18} column (5.0 µm, 4.6 mm inner diameter × 250 mm) was utilized with flow rate of 1.0 mL/min and injection volume of 1 µL. All chromatographic operations were carried out at ambient temperature based on methods described for phenolic compounds[20] and pheophorbide-a.[21] The presence of these compounds was validated by comparing their retention time with chemical standards.

Statistical analysis

Statistical analysis of data was as follows: prior to analysis, the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way analysis of variance was followed by a Tukey test when variances were homogeneous and by the Tamhane test when variances were not homogeneous. Data from cytotoxicity assay was also subjected to probit analysis in which probit-transformed cytotoxicity was regressed against the $\log_{10}$-transformed dose allowing, when statistically significant, half-maximal inhibitory concentration (IC_{50}) values, and slope estimation. Each data point was expressed as mean ± standard deviation with $n = 4$. Means with a common letter are significantly similar, $P < 0.05$. In all these analyses, IBM SPSS Statistics version 21.0 (Armonk, NY, USA) was used.

RESULTS

Cytotoxicity assay of *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts on HepG2 liver cells using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

Cellular viabilities of HepG2 cell line after 24-h incubation with CME and SME are shown in Figure 1. An increase in cellular proliferation rate was observed when cells were incubated with 125 µg/mL of CME (111.67% ± 4.09%) as compared to untreated control. Overall, no significant toxicity by CME at the concentration range tested (31.25–500 µg/mL) was observed. Similarly, SME at concentrations

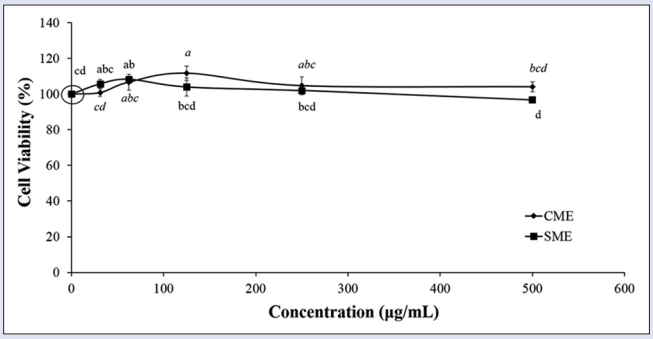


Figure 1: Hepatocellular carcinoma cell viability under various concentrations (31.25, 62.5, 125, 250, and 500 µg/mL) of *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts after 24-h incubation. Each data point was expressed as mean ± standard deviation with $n = 4$. Means with a common letter are significantly similar, $P < 0.05$

31.25 µg/mL to 250 µg/mL showed no significant cell death. Therefore, these two methanolic extracts at the non-toxic concentration range could be further investigated for their other beneficial properties such as antioxidative potentials.

Antioxidative potentials of *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts

The extracts, CME and SME at the non-toxic concentrations (0–500 µg/mL) were then examined for their antioxidative abilities using an *in vitro* liver system. Interestingly, SME was found to be a stronger ROS scavenger than CME at all concentrations evaluated on liver cells. SME at 62.5 µg/mL reduced ROS level (68.27%) twice as effective than CME (40.26%) when compared to untreated control cells. Nevertheless, the ROS-reducing effect of SME plateau from 125 µg/mL upward showing no further significant difference [Figure 2a]. Using a chemical-based antioxidant assay, both the extracts demonstrated dose-dependent DPPH radical scavenging activities. CME and SME at 1000 µg/mL scavenged DPPH radical up to 95%. Based on Figure 2b, SME was a better radical scavenger than CME.

Photodynamic therapy of *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts on HepG2 liver cells

To investigate the photocytotoxicity of CME and SME, the extracts were first incubated with the HepG2 cells for 2 h, followed by PDT treatment at 660 nm for 10 min. Cellular viabilities after treatment with various concentrations of CME and SME with and without PDT were shown in Figure 3a and b, respectively.

C. nutans-treated HepG2 cells without PDT clearly possessed no significant dark toxicity at the concentrations tested ($P < 0.05$). Interestingly, on photoactivation of CME, cell death was observed. The cell viability of HepG2 after PDT decreased simultaneously with increasing concentrations of CME. Cellular viability was lowered to 36% at CME 100 µg/mL after PDT as compared to before light exposure.

Similarly, SME-treated HepG2 without activation by light also showed no significant cell death, but on light activation, the antiproliferative effects of SME were clearly observed. The HepG2 cell viability was found reduced with increment in SME concentrations from 3.125 to 25 µg/mL but the toxicity effect plateau thereafter.

IC_{50} of CME and SME on HepG2 cell lines after 10 min PDT was 76.66 ± 9.71 and 8.51 ± 0.70 µg/mL, respectively. SME conferred

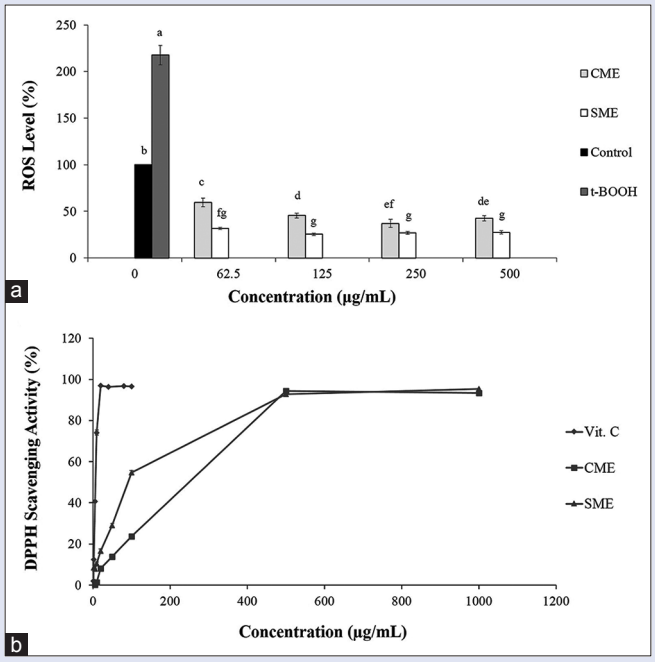


Figure 2: Reactive oxygen species reduction in HepG2 cells upon treatment with various concentrations of *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts as compared to untreated control and the pro-oxidant, *tert*-Butyl hydroperoxide (a). 2,2-Diphenyl-1-picrylhydrazyl radical scavenging potential of *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts (b). Values are expressed as means ± standard deviation with $n = 4$. In reactive oxygen species assay, means with a common letter are significantly similar, $P < 0.05$

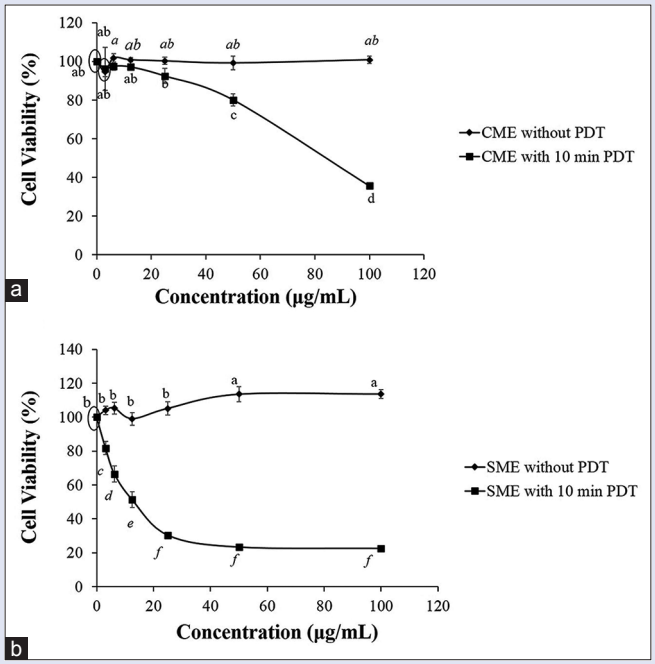


Figure 3: HepG2 cell viability under various concentrations (3.125, 6.25, 12.5, 25, 50, and 100 µg/mL) of *Clinacanthus nutans* methanolic extract (a) and SME (b) with 10 min photodynamic therapy or without photodynamic therapy. Each data point was expressed as mean ± standard deviation with $n = 4$. Means with a common letter are significantly similar, $P < 0.05$

stronger photo-induced cytotoxic effect with IC_{50} eight-fold lower than CME. Extract-treated cells without PDT treatment exhibited IC_{50} more than 500 µg/mL.

The length of PDT treatment was also investigated in this study. Figure 4 illustrates the percentage of HepG2 cell viabilities after treatment with 50 µg/mL of CME and SME at different light irradiation durations. *C. nutans*-treated HepG2 showed a significant reduction in cell viability with increasing PDT exposures. The results indicated that after a 20-min light irradiation period, CME-treated cells possessed lower viability (41.21 ± 2.64%) as compared to CME-treated cells exposed to PDT for 10 min (80.09 ± 3.14%). However, time dependence was not observed in SME-treated HepG2, as there was no significant difference among cells subjected to PDT for 5 min, 10 min, and 20 min.

Hepatocellular carcinoma (HepG2) cell line treated with only CME and SME (at 50 µg/mL) without PDT maintained normal morphology even after 24 h as compared to untreated control [Figure 5]. Moreover, no obvious change in morphology was observed when untreated cells underwent PDT for 10 min, indicating that light alone did not exert cytotoxicity toward the cell line. However, extract-treated cells after PDT showed rounded cellular morphologies, membrane blebbing, cell shrinkage, and appearance of cell debris in the culture.

Phytochemical screening of *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts

Except for alkaloids (Mayer's test and Wagner's test), the phytochemical screening of CME and SME showed the presence of tannins (ferric chloride test), flavonoids (Shinoda test), saponins (foam test), terpenoids (Salkowski's test), and cardiac glycosides (Keller–Kiliani test) [Table S1]. The total phenolic content in SME (36.27 ± 1.31 mg GAE/g extract) was found to be slightly higher than in CME (31.76 ± 0.10 mg GAE/g extract). However, the total flavonoid content in CME (11.32 ± 0.28 mg QE/g extract) was seven times more than in SME (1.69 ± 0.03 mg QE/g extract).

HPLC analysis showed the presence of a number of phenolic acids and flavonoids in both extracts, as shown in Table 1. The phenolic acids detected in CME comprised gallic acid, caffeic acid, and ferulic acid while the flavonoids were quercetin and kaempferol. SME was found to contain similar composition but with an additional flavonoid, catechin. Besides phenolic compounds, a chlorophyll derivative, pheophorbide-a, was also elucidated in the extracts. Both CME (0.652 ± 0.122 mg/g of crude extract) and SME (0.405 ± 0.025 mg/g of crude extract) possessed comparable amount of pheophorbide-a. HPLC chromatograms for the extracts and chemical standards were illustrated in Figures S1 and S2.

DISCUSSION

Our study indicated that both CME and SME at concentrations 31.25 µg/mL to 500 µg/mL were non-toxic to HepG2 hepatocarcinoma cells [Figure 1]. Extracts were considered non-toxic when cellular viability was more than 90%.[22] This finding was supported by a recent report stating that the CME showed no significant antiproliferative activity on HepG2 cell lines even at concentration up to 300 µg/mL.[23] Furthermore, SME was mentioned to exhibit pro-proliferative activities on the same cell line at concentration up to 200 µg/mL.[11] Cytotoxicity of natural products was highly influenced by their cellular bioavailability, phytochemical concentration, and interaction which may be protective to some cells and also cause damaging effects to others.[22] The non-toxic nature of the extracts could be due to the extraction solvent utilized, 80% methanol (v/v), which was effective in the extraction of polar compounds such as phenolics that often played the role as antioxidants[24] rather than

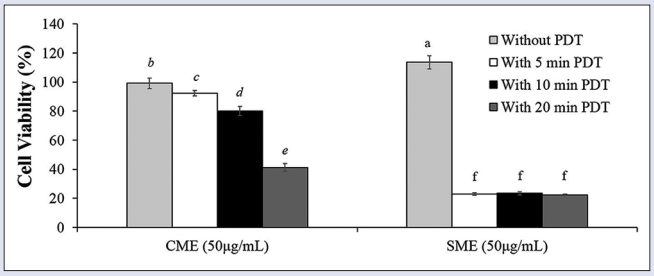


Figure 4: HepG2 cell viability upon treatment with *Clinacanthus nutans* and *Strobilanthes crispus* methanolic extracts, followed by photodynamic therapy at different exposure time (5 min, 10 min, and 20 min). Each data point was expressed as mean ± standard deviation with $n = 4$. Means with a common letter are significantly similar, $P < 0.05$

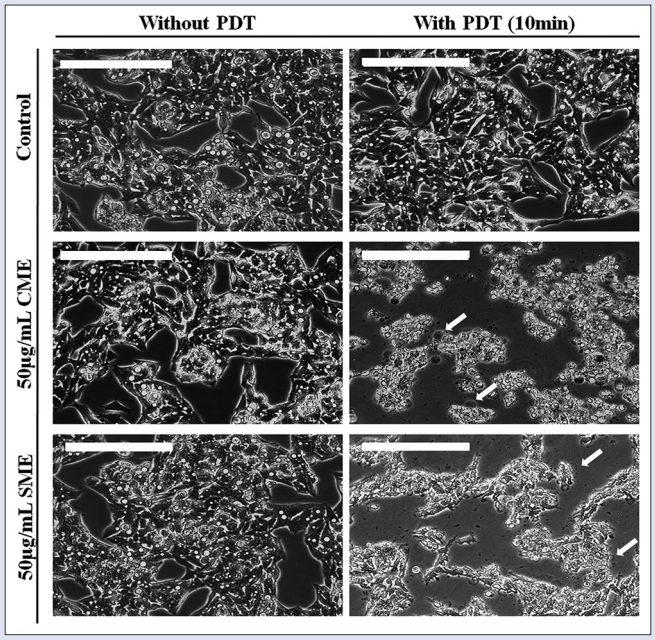


Figure 5: Morphologies of HepG2 cells after incubation with 50 µg/mL of *Clinacanthus nutans* or *Strobilanthes crispus* methanolic extracts with or without photodynamic therapy. Arrows indicated membrane blebbing. Scale Bar: 200µm. (×200)

cytotoxic agents. Therefore, these two methanolic crude extracts at the non-toxic concentration range were further investigated for their other beneficial properties such as antioxidative activities.

A HepG2 cell-based *in vitro* antioxidant system was employed to determine the effects of CME and SME on cellular ROS reduction. This cell line had long been used as an *in vitro* model to study cytoprotective, genotoxic, and antigenotoxic effects of compounds since they retained many of the specialized functions of normal human hepatocytes.[25] Importantly, this model system was proven reliable and well established in the study of the antioxidative properties of dietary compounds.[15]

Results indicated that the basal ROS level was lowered by 70% and 40% after treatment with SME and CME, respectively, at the lowest concentration of 62.5 µg/mL [Figure 2a]. Cellular ROS is generated by various metabolic pathways such as tricarboxylic acid (TCA) cycle and the respiratory pathway taking place in the inner mitochondrial membrane.[26] Excessive production of ROS would harm the cells by causing lipid peroxidation, oxidation of proteins, DNA damage, and enzyme inhibition and even induces cell apoptosis.[1] Plant extracts such as *Carica papaya*,[14] *Tamarindus indica*,[4] and *Ocimum sanctum*[27] had been reported to demonstrate antioxidant capabilities using similar *in vitro* HepG2 model system. These extracts were shown able to elevate the gene expression and activity of antioxidant enzymes to eliminate ROS.

Nevertheless, research done by a group of researchers showed that short-term incubation of papaya leaf fraction did not alter the transcription of Phase II antioxidant genes; heme oxygenase and NAD(P)H:quinone oxidoreductase in HepG2 cells.[14] Since CME and SME were incubated with the cells for only 2 h, it was more likely that the phytocompounds reduced the ROS levels through the radical scavenging mechanism. The radical scavenging effects of CME and SME were further confirmed by DPPH radical scavenging assay. It was found that the scavenging activities of these plants reached 95% when tested at concentration of 1 mg/mL [Figure 2b].

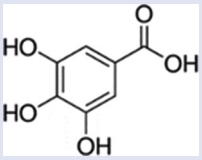
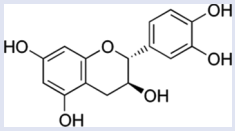
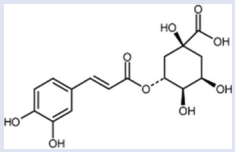
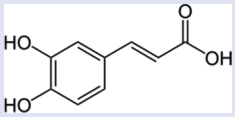
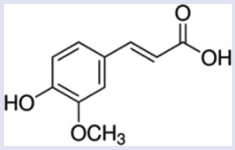
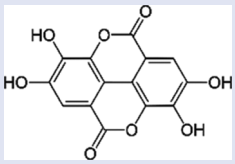
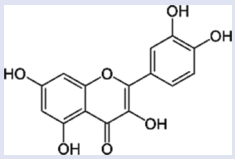
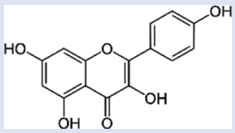
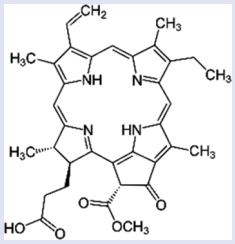
The total phenolic content of SME was only slightly higher than CME. The total flavonoid content of CME, on the other hand, was seven times more than SME. Phenolic compounds elucidated from these extracts were gallic acid, catechin, caffeic acid, ferulic acid, quercetin, and kaempferol. Previous reports mentioned the presence of gallic acid, caffeic acid, and ferulic acid in CME and SME in line with our work.[28,29] Polyphenols were well-known antioxidants with effective radical scavenging activities. Scavenging activity depended on the ability of the phenolic compounds to donate hydrogen or electron, the stability of the resulting antioxidant-derived radicals, and their capability to form chelates with metals.[30] Other research groups also stated the importance of interaction between polyphenols and the cellular membrane to confer the antioxidant abilities. According to this theory, the hydrophobicity of the polyphenols was more crucial than its antiradical activity as these compounds were required to permeate the membrane to influence the antioxidant strength.[31]

Although the total flavonoid content of SME was remarkably lower than CME, this extract exhibited stronger radical scavenging activity in both cell-based and chemical-based assays. This phenomenon could be explained by the fact that there may be other unidentified phytochemicals in SME that indirectly contributed to its high radical-quenching ability. Besides phenolic acids and flavonoids, other phytocompounds reported in *S. crispus* were saponins[29] and tannins.[32]

Since photo-independent cytotoxicity was not observed in HepG2 cells when tested with both CME and SME [Figure 1], PDT could be used to induce the antiproliferative activity of both extracts on these cells. PDT is a promising noninvasive anticancer therapy. PDT makes the use of natural or synthetic photosensitizing agents which can be activated by light at appropriate wavelengths to generate oxidative stress in cancer cells leading to cell death.[12] Research is still ongoing in order to obtain photosensitizers with the least side effects.[33] Plant extracts from *C. nutans* and *S. crispus* possess chlorophyll catabolites that may be isolated for their photosensitizing properties to be utilized further in PDT for anticancer treatment.

From the results shown in Figure 3, no dark toxicity of either CME and SME could be detected in HepG2 cell line. However, after light exposure, the photocytotoxic effects were clearly observed in CME- and SME-treated HepG2 cells. SME demonstrated an enhanced photocytotoxic effect as compared to CME as its IC_{50} (8.51 ± 0.70 µg/mL) was significantly lower than CME (76.66 ± 9.71 µg/mL). The cytotoxic effects of chloroform and hexane fractions of *S. crispus* without PDT on HepG2 cells had been conducted by Koh *et al.* where the IC_{50} obtained was 175.70 µg/mL and 176.70 µg/mL, respectively.[11] Therefore, light-activated SME was a stronger antiproliferative agent against

Table 1: Phenolic compounds and the chlorophyll derivative identified in *Clinacanthus nutans* methanolic extract and *Strobilanthes crispus* methanolic extract using high-performance liquid chromatography analysis

Chemical standards	Molecular structure	CME	SME
Gallic acid (4.332 min)		√ (4.311 min)	√ (4.340 min)
(+)-catechin (7.962 min)		x	√ (7.954 min)
Chlorogenic acid (9.246 min)		x	x
Caffeic acid (11.891 min)		√ (11.895 min)	√ (11.902 min)
Ferulic acid (16.733 min)		√ (16.827 min)	√ (16.748 min)
Ellagic acid (23.561 min)		x	x
Quercetin (39.617 min)		√ (39.640 min)	√ (39.600 min)
Kaempferol (51.116 min)		√ (51.254 min)	√ (51.094 min)
Pheophorbide-a (14.134 min)		√ (14.244 min) 0.652±0.122 mg/g of crude extract	√ (14.086 min) 0.405±0.025 mg/g of crude extract

Retention times are as stated in parenthesis. √: Present; x: Absent. CME: *Clinacanthus nutans* methanolic extract; SME: *Strobilanthes crispus* methanolic extract

liver cancer cells as proven in our work. However, the photocytotoxic level of CME was not as high as the reported chloroform-extracted *C. nutans* (with no light exposure) which had an IC_{50} value of 25 µg/mL[23] on HepG2 cell lines.

Nevertheless, CME-treated cells showed a time-dependence effect of PDT with higher cell death at longer light exposure [Figure 4]. On the contrary, time dependence was not observed in SME-treated HepG2 cells as there was no significant difference among cells subjected to PDT for 5 min, 10 min, and 20 min. This could be due to the fact that SME at the tested concentration had reached its optimal cell-killing effect at light irradiation of 5 min.

The cellular morphologies of HepG2 at different conditions are illustrated in Figure 5. The control cells with and without PDT showed no obvious changes in morphology. Morphologies of CME- and SME-treated cells also did not differ from the control cells. Interestingly,

extracts treated cells after PDT showed rounded cellular morphologies, membrane blebbing, cell shrinkage, and debris residue in the culture. These morphological changes were described by the previous report as cell apoptosis.[34] Therefore, the mechanisms of cell death by CME and SME through PDT on HepG2 cells were postulated to be by apoptosis.

Various plant extracts such as *Scutellaria barbata*,[35] *Lumnitzera racemosa,* and *Albizia procera*[2] had been scientifically proven to exhibit photocytotoxic activities against many cancer cells; HepG2, mammary cell adenocarcinoma, oral cavity and tongue squamous carcinoma cell lines. The modes of action of these extracts in cell apoptosis were through elevation of ROS level, DNA fragmentation as well as disruption of cell cycle.[2,35] Therefore, we speculated that this chain of events could occur during the treatment of CME and SME upon light activation leading to the observed phenomenon.

Based on the HPLC analysis [Table 1], pheophorbide-a was detected in both CME and SME. Pheophorbide-a had demonstrated its photosensitizing abilities in previous research against cancer cells when exposed to light of wavelength about 660 nm.[12,13] Hence, the photocytotoxic nature of CME and SME could be partly contributed by this compound. Nevertheless, there could be other novel photosensitizers present in these plants. Besides pheophorbide-a, other photosensitizers, for examples, derivatives of pheophorbide-a and pheophorbide-b were found in *Piper penangense* which also conferred similar photocytotoxicity effects on human promyelocytic leukemia (HL60) cell line.[36]

In short, our research was the first to reveal that the CME and SME at non-toxic concentrations were capable of reducing ROS when not irradiated by light. However, when extract-treated cells were exposed with light at wavelength of 660 nm, cell-killing effect was observed on HepG2 liver cancer cells. In view of that, these extracts, especially SME, could be developed into potent photosensitizing agents with dual functionalities under the control of light exposure. Work is now being conducted to identify other photosensitizers involved in this research. In addition, the mechanisms of action of *C. nutans* and *S. crispus* in their anticancer activities are presently under investigation.

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Conflicts of interest

There are no conflicts of interest.

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