

# *Strobilanthes crispus* Extract Induces Apoptosis through Enhanced Caspases Activities in Cervical Cancer Cells

Yen Hoong Chong, Rhun Yian Koh, Anna Pick Kiong Ling, Soi Moi Chye, and Mei Yeng Yew

**Abstract**— *Strobilanthes crispus* extract was found to exert cytotoxicity on several cancer cell lines and able to reduce chemically-induced hepatocarcinogenesis in rats. In this study, the cytotoxicity of *S. crispus* extracts that isolated from leaves and stem by hexane, chloroform, ethyl acetate, methanol and water solvent was determined and its possible underlying apoptotic mechanism was further investigated in HeLa cell line. MTT analysis demonstrated that only hexane-stem extract portrayed cytotoxic effects on HeLa cells with IC<sub>50</sub> of 160 µg/ml. Chloroform extract showed possible cell inhibition trend while other extracts showed little or no cytotoxic effects. Hexane-stem extract was further analysed in this study where it was found to induce apoptosis, which was confirmed with obvious morphological changes in HeLa cells accompanied by sub-G<sub>1</sub> peak detection in cell cycle analysis by flow cytometry. Hexane-stem extract did not induce cell cycle arrest at any phases based on data shown. High level of caspase-3/7 activity detected indicated the involvement of caspase-3/7 activation in the apoptogenic effect induced by hexane-stem extract. Activity of caspase-8 and caspase-9 were found not significant in this study. More studies could be carried out to further elucidate the apoptotic mechanism clearly. Overall findings showed that *S. crispus* acted as apoptotic inducer which could be used as potential anticancer agent in the future.

**Keywords**— Apoptosis, caspase, cervical cancer, *Strobilanthes crispus*

## I. INTRODUCTION

CANCER is one of the leading causes of medically certified deaths due to its profound complex nature. Recently, cervical cancer becomes a highlighted health concern among female population. Current treatment modalities for cancer revolve chemotherapy, surgery or radiation, but it is still tough to foresee dramatic increase in cancer remission rate. The intolerable pain, side effects sufferings and development of chemotherapy resistance had driven more patient to resolve in using natural products as alternatives [1] with trials demonstrated that natural products usage improves

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and complements health condition of patients. There has been a global trend in using natural products to treat cancer. To date, natural products had always be a promising source for breakthrough discovery and development of chemotherapeutic agents.

*Strobilanthes crispus* is native to countries throughout Madagascar regions to Malay Archipelago, traditionally known as pecah kaca (Malay), daun picah beling (Jakarta) or kejibeling (Java). The plant has been used widely in folk medicine preparations as antidiabetic, diuretic, antilytic, laxative, antioxidant, anti-HIV or anti-leukimic agent [2]-[5]. In addition, *S. crispus* is also in the limelight for anticancer properties. Previous study reported that crude methanolic extract of *S. crispus* was cytotoxic against liver (HepG-2), colon (Caco-2) and breast (MDA-MB-231) cancer cell lines, while chloroform extract was cytotoxic to HepG-2 and Caco-2 only [6]. No cytotoxicity against Chang liver cell line was seen [6]. The results were endorsed by Endrini *et al.* with further investigation on cytotoxic mechanism that involved down-regulation of oncogene, c-myc expression [7]. Recent study revealed detectable anti-angiogenic activity in *S. crispus* methanolic extract with exhibited cytotoxic response towards MCF-7 [8]. The dichloromethane extract of *S. crispus* was reported to induce cell deaths in breast (MCF-7, MDA-MB-231) and prostate (PC-3, DU-145) cancer cell lines and was comparatively shown to be more potent than some anticancer drugs [1]. Meanwhile, *in vivo* studies concluded that *S. crispus* extract acted as chemopreventive agent with reduced chemically-induced hepatocarcinogenesis in rats [9], [10].

By taking into consideration that cervical cancer remained as new interest in *S. crispus* research, present study also aimed at comparing cytotoxic effects of stem and leaf extracts from *S. crispus* by using various solvents. Apoptosis is a physiological process which is regulated by caspases (cysteine proteinases) to selectively eliminate seriously damaged cells by self-destruction in order to maintain genomic integrity without inducing inflammation [11]. Emerging evidences regarded apoptosis induction as the ideal way to kill cancer cells had aroused magnificent interest in search of apoptotic inducer and targets underlying mechanism [12]. Thus, this overall study aimed to investigate the involvement of caspases in apoptogenic effect of *S. crispus* extracts in cervical cancer cells (HeLa).

## II. METHODOLOGY

### A. Plant collection and preparation plant extracts

Fresh plant materials (*Strobilanthes crispus*) were collected from Terengganu, oven-dried at 40 °C and separated into leaves and stems. These dried materials were then grounded into powder correspondingly. A plant sample was deposited at Forest Research Institute Malaysia (sample number: PID040114-04) and the botanical identity was authenticated.

For organic solvents extraction, dry samples (100 g) were mixed with hexane (500 ml) and left in dark for three days. Resulting suspension were then filtered. Collected filtrate was evaporated and dried overnight. Procedures were repeated by using solvents of increasing polarity (chloroform, ethyl acetate and methanol) in stepwise manner.

For water extraction, dry samples (100 g) were mixed in distilled water (500 ml), macerated overnight and incubated for 3 hours at 60 °C. Resulting suspension was filtered and freeze-dried.

### B. Cell Culture

Cervical cancer cell line (HeLa) was purchased from American Type Culture Collection (ATCC), USA and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, UK) supplemented with 10% fetal bovine serum (GIBCO, South America), 100 units/ml penicillin and 100 µg/ml streptomycin (GIBCO, USA) in 5% CO<sub>2</sub> humidified atmosphere at 37 °C.

### C. Evaluation of cellular toxicity

MTT assay was used to evaluate cytotoxic effects of plant extracts on HeLa. The cells were seeded at density of 3,000 per well in 96-well plates then treated with various concentrations (12.5, 25, 50, 100 and 200 µg/ml) of plant extracts at 37 °C for 72 hours. All treatments or negative control (with media only) were performed in triplicate. Cells were subsequently incubated with 10 µl of MTT (5 mg/ml) (Biobasic, Canada) for 4 hours. Medium was then aspirated and followed by dimethyl sulfoxide (DMSO) (100 µl) addition. Absorbance was read at 570 nm wavelength by plate reader (Opsys MR, DYNEX, USA). Results were interpreted as percentage of cell viability [OD<sub>sample</sub>/OD<sub>control</sub> x 100%]. Extract concentration that inhibited 50% of cell viability, IC<sub>50</sub> was determined through interpolation from dose-response curve. The plant extracts which exhibited IC<sub>50</sub> were subjected to cell apoptosis determination.

### D. Microscopic cell morphology observation

After cell treated with plant extract at IC<sub>50</sub> for 72 hours, cell morphological changes were examined under inverted microscope and micrographs were taken with camera attached (Nikon T1-SAM, Japan).

### E. Cell cycle analysis

After cell treated with plant extract at IC<sub>50</sub> for 72 hours, cells were stained with propidium iodide, incubated for 15 minutes in dark then analyzed by flow cytometry (FACScan,

USA) to determine cell apoptosis.

### F. Measurement of caspases activities

After cell treated with plant extract at IC<sub>50</sub> for 72 hours, caspase-3/7 (Promega, USA), caspase-8 and caspase-9 (Calbiochem, Germany) activities were detected by caspase detection kits respectively. Caspases activites were evaluated by quantifying fluorescent intensity using plate reader (TECAN, Switzerland).

### G. Statistical analysis

All data were presented as mean ± standard deviation (SD) and subjected to analysis of variance (ANOVA) by using SPSS 16.0. P-value < 0.05 was considered statistically significant.

## III. RESULTS

### A. Cytotoxic effect of *S. crispus* extracts in HeLa cells

MTT analysis (Fig. 1) showed that most *S. crispus* extracts from stem and leaf showed little or no cytotoxic effect against HeLa within tested concentration range, except for hexane-stem extract. Hexane-stem extract inhibited HeLa cells in a dose-dependent manner, showing cytotoxicity with IC<sub>50</sub> (160 ± 10.01 µg/ml) whilst chloroform-stem extract showed possible cell inhibition trend from graph.

### B. Morphological changes in HeLa cells after *S. crispus* treatment

HeLa cells treated with hexane-stem extract for 72 hours (Fig. 2B, 2C) showed hallmark features of apoptosis which were manifested as cell shrinkage with irregular shape, nuclear condensation, cell membrane blebbing accompanied by apoptotic bodies and subsequently losing contact with neighbouring cells then lastly floated into medium as round death cells.

### C. Cell cycle analysis on HeLa cell after *S. crispus* treatment

Cell cycle analysis demonstrated cell distribution in each phases of cell cycle, where cell accumulation in sub-G<sub>1</sub> phase is recognized as apoptotic cells. Hexane-stem extract was found to induce cell apoptosis as sub-G<sub>1</sub> peak was detected in graph (Fig. 3B). Increased cell percentage (0.25% to 6.10%) in sub-G<sub>1</sub> phase accompanied by decreased in G<sub>0</sub>/G<sub>1</sub> phase in treated HeLa was noted. There was also slight increase (3.06% to 5.08%) in S phase and a consequent decrease in G<sub>2</sub>/M phase. The changes in G<sub>0</sub>/G<sub>1</sub> phase were not significant.

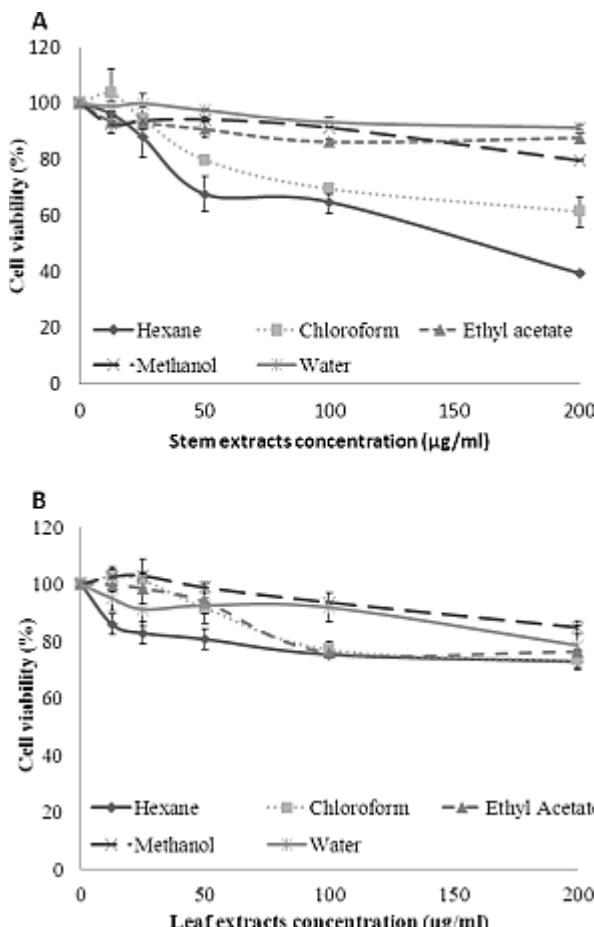


Fig. 1 MTT assay showing the cytotoxic effect of *S. crispus* extracts in HeLa cells. HeLa cells were treated with hexane, chloroform, ethyl acetate, methanol and water extract from (A) stem and (B) leaf respectively, at concentrations 12.5, 25, 50, 100 and 200  $\mu\text{g}/\text{ml}$  for 72 hours. Data were presented in mean  $\pm$  SD.

#### D. Caspase-3/7, caspase-8 and caspase-9 activities in HeLa cells after *S. crispus* treatment

Caspase-3/7 activity was increased significantly in treated HeLa cells as compared to control ( $p < 0.05$ , Fig. 5). In contrast, changes in caspase-8 and caspase-9 were not significant as compared to control although decrease in caspase-8 and slight increase in caspase-9 activity were noted.

#### IV. DISCUSSION

Many active compounds isolated from plant sources were reported to possess anticancer properties. Apoptosis induction is regarded as preferred cancer approach where apoptotic mechanism ideally targeted in pharmaceutical development of anticancer drugs. This study aimed to investigate the cytotoxic effects and its possible apoptotic mechanism induced by *S. crispus* extracts on HeLa.

The cytotoxicity of *S. crispus* extracts on HeLa was evaluated by MTT assay based on percentage of cell viability. Only hexane-stem extract was found cytotoxic against HeLa, showing cell proliferation inhibition in a concentration-dependent manner. Chloroform-stem extract showed possible cell proliferation inhibition trend while other extracts showed

no significant cytotoxic effect on HeLa. The reported constituents that isolated from *S. crispus* leaves which shown to be cytotoxic (apoptotic inducer) [13] include verbacoside, some phenolic acids, tannin, saponin and steroids ( $\beta$ -sitosterol and stigmasterol) [6]. Ironically, no leaf extract was found cytotoxic against HeLa as compared to stem extracts. The active constituents in stem were unknown and yet to be revealed, thus results suggested that there are unknown key compounds in stem which absent in leaf that exerted cytotoxicity on HeLa cells. It might also due to the synergism of various compounds in stem extract that brought effect.

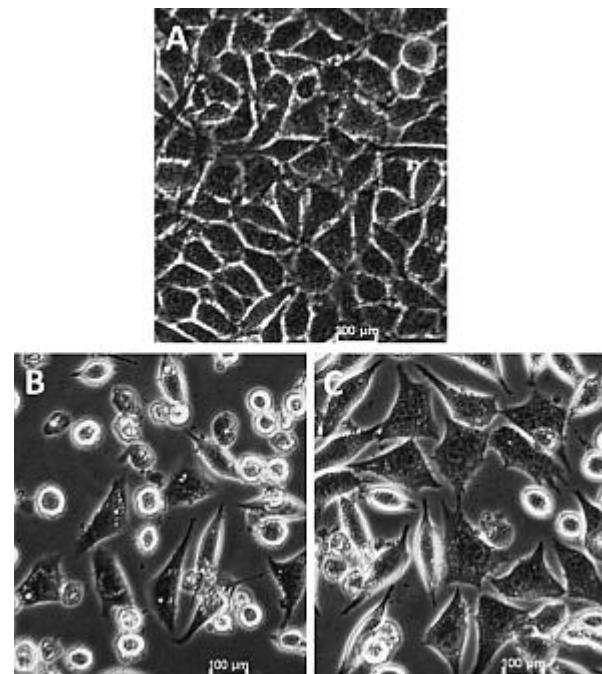


Fig. 2 The morphological changes in HeLa cells by *S. crispus* extracts. (A) Healthy control HeLa. (B, C) HeLa cells treated with hexane-stem extract at 160  $\mu\text{g}/\text{ml}$  for 72 hours, demonstrating reduced number of viable cells with shape distortion, cell shrinkage, cell membrane blebbing and vacuolation.

Meanwhile, hexane extracted lipophilic compounds and this indicated cytotoxic compounds in hexane-stem extract are non-polar. The current results in which water extract was not cytotoxic against HeLa was supportive to previous study by Muslim *et al.* on different cell lines [8].

According to National Cancer Institute, any plant extracts with  $\text{IC}_{50} < 20 \mu\text{g}/\text{ml}$  are cytotoxic against treated cells [14]. High  $\text{IC}_{50}$  value obtained for hexane-stem extract in this study could be affected by different extraction methods used and unknown stability of crude extract that may contribute to loss of cytotoxic activity throughout research period.

Apoptosis or programmed cell death [11] is cellular suicide that characterised by series of biochemical activation events leading to distinctive cell morphology features and ultimate cell death. It is a pivotal protective mechanism in maintaining tissue homeostasis by removing excess, abnormally damaged cells [11], [15]-[17]. This study determined apoptosis event by microscopic observation on cells and cell cycle analysis.

Apparent hallmark morphologies of apoptosis were observed in treated HeLa cells (Fig. 2). Healthy control HeLa cells which were elongated and spindle in shape became distorted after *S. crispus* treatment. Cell shrunken accompanied with membrane blebbing and appearance of apoptotic bodies was observed and this led to decreased number of viable cells.

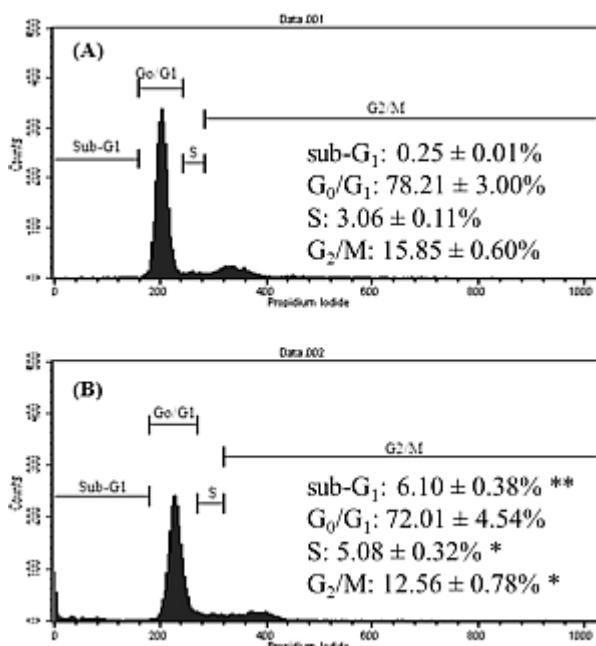


Fig. 3 Cell cycle analysis of (A) control HeLa and (B) treated HeLa with hexane-stem extract at 160 µg/ml for 72 hours. Cell cycle distribution was determined in samples stained with PI and analysed by flow cytometry. Data were presented in mean  $\pm$  SD. \* indicated  $p < 0.05$  and \*\* indicated  $p < 0.01$  when compared to control.

Apoptosis was further evidenced by cell cycle analysis that showed the appearance of sub-G<sub>1</sub> peak, thus demonstrating apoptotic cells with fractional DNA content (Fig. 3). Little apoptotic cells found in control HeLa might be due to natural cell death by nutrient depletion in growth media or contact inhibition [18], [19]. Cancer cell proliferation is inhibited by preventing cell division via cell cycle arrest and hence, complementing apoptosis to eliminate cancer cell [18]-[20]. Cell accumulation in S phase suggested S/G<sub>2</sub> phase transition block. Cell cycle arrest at S phase eventually led to the decrease in G<sub>2</sub>/M phase.

Apoptosis is highly regulated by caspase family in a series of cascades system to manage apoptotic signals [11] involving the two major upstream apoptotic pathways: extrinsic pathway (by initiator caspase-2, caspase-8 or caspase-10) and intrinsic pathway (by initiator caspase-9). Both upstream signaling pathways converge downstream to activate final effector caspase mechanism by caspase-3, caspase-6 or caspase-7 that responsible for apoptosis execution to dismantle dying cells [11], [21]. It was cleared that *S. crispus* hexane-stem extract induced apoptosis, hence apoptotic mechanism was further studied in this study. The investigated caspase-3, caspase-7, caspase-8 and caspase-9 are main caspases involved in apoptotic pathways. The caspase-3/7 activity was increased

significantly in treated HeLa as compared to control ( $p < 0.05$ ) indicated enhanced caspase-3/7 activation in apoptosis induced by hexane-stem treatment (Fig. 4). Results obtained were supportive to Yaacob *et al.* that reported *S. crispus* extract induced apoptosis via caspase-3/7 activation in breast and prostate cancer [1]. While for caspase-8 and caspase-9, their changes in treated HeLa were not significant. Low caspase-8 activity showed that the extrinsic pathway might not be activated. Caspase-9 activity barely induced by treatment might be due to influence of activated cyclin B/CDK1 which inhibits caspase-9 activity [22]. Caspase inhibitor assay could be done to help in determining involvement of various caspases in apoptotic pathway [22]. There is a possibility of intrinsic pathway activation (by caspase-2 or caspase-10) as they were not ruled out completely by overall findings in this study. Potential apoptotic role of other caspases were also discussed, demonstrating that apoptosis does not always rely on caspase-8 and caspase-9 [23]. On the other hand, Burkley *et al.* reported that apoptosis might occur without upstream cascades whereby the RGD (arginine-glycine-aspartate)-containing peptides was able to induce apoptosis directly without signals by promoting procaspase-3 auto-activation [24].

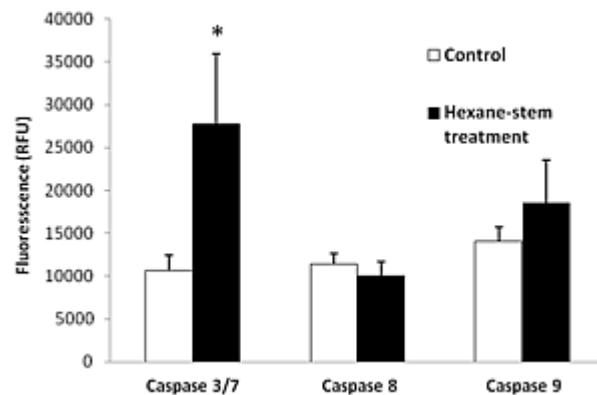


Fig. 4 Detection of caspase-3/7, caspase-8 and caspase-9 activities in treated HeLa cells with hexane-stem extract at 160 µg/ml for 72 hours. The caspases activities were detected by commercial kits. Data were presented in mean  $\pm$  SD. \* indicated  $p < 0.05$  when compared to control.

## V.CONCLUSION

In conclusion, this study clearly demonstrated that hexane-stem extract of *S. crispus* induces apoptosis via enhanced caspase-3/7 activation in HeLa. These findings suggested that *S. crispus* that acted as apoptotic inducer could become a potential anticancer agent in pharmaceutical development. Future prospects of this study involve identification and isolation of active cytotoxic compounds with further elucidation on their molecular structures and mechanism of action. More studies could be carried out to evaluate the cytotoxic effects on more cell lines followed by implementation of *in vivo* studies.

## ACKNOWLEDGMENT

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