Effects of *strobilanthes crispus* extract on the apoptotic pathway of human liver carcinoma cell lines

Susi Endrini[1], Suherman[2], Asmah Rahmat[3], Patimah Ismail[4], Y.H. Taufiq-Yap[5], and Fauziah Othman[4]

[1]*Department of Biochemistry, YARSI University School of Medicine, Jakarta*
[2]*Faculty of Medicine and Health Sciences, University of Muhammadiyah Jakarta*
[3]*Department of Nutrition and Health Sciences*
[4]*Department of Biomedicine, Faculty of Medicine and Health Sciences*
[5]*Department of Chemistry, Faculty of Science and Environmental Study, Universiti Putra Malaysia, 43400, Serdang, Selangor D.E., Malaysia*

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ABSTRACT *Previous study has been shown the potential anticarcinogenic effect of Strobilanthes crispus, a plant native to countries from Madagascar to Indonesia, on human liver cancer (HepG-2) cell lines. The objective of the present study was to investigate the mechanism of anticarcinogenic effect of S.crispus extract through apoptotic pathway. Exposure of HepG-2 cells to S.crispus extract resulted in induction of apoptosis in a dose-dependent manner as measured by Fluoresence Microscopy and Confocal Laser Scanning Microscopy analysis. These findings provide important new insight into the possible molecular mechanisms of the anticancer activity of S.crispus.*

It is now well accepted that apoptosis (programmed cell death), is a physiological phenomenon that plays an important role in the regulation of tissue development and homeostasis. Deregulation of apoptosis has been shown to contribute to the pathogenesis of a number of human diseases including cancer (Woo and Choi, 2005).

Strobilanthes crispus ZII 109 (L) Bremek or *Saricocalix crispus* ZII 109 (L) Bremek (Acanthaceae) plant is native to countries from Madagascar to Indonesia (Sunarto, 1977) and was first authored by Anderson, Thomas (1832-1870) who classified the plant under Spermatophyta (Flowering plants and Gymnosperma) (Brummit and Powell, 1992).

A study in Indonesia found that an infusion of the dried leaves of *S.crispus* has been used as antidiabetic, diuretic, antilytic and laxative. A recent study indicated that the water extract of *S.crispus* contained compounds with very high binding affinity to protein molecules that bind the active site of reverse transcriptase. It inhibits the proliferation of retrovirus; an agent in viral disease such as acquired immune deficiency syndrome (AIDS) and Adult T-cell Leukemia (Kusumoto *et al.*, 1992). *S. crispus* extracts (extracts or isolated components) have previously been shown to possess antitumor activities (Asmah *et al.*, 2006). The present study was performed to investigate the apoptotic pathway by *S.crispus* extract.

MATERIALS AND METHODS

Plant materials and extractions

The leaves of *S. crispus* were harvested at Faculty of Medicine and Health Sciences, UPM, Serdang, Selangor. The herbarium voucher specimen were identified and deposited by Mr. Ahmed Zainuddin from the Department of Botany, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The voucher number of *S.crispus* was AZ-6803.

The extraction methods were obtained from Ali *et al.* (1996) with slight modification. The dried leaves (1 kg) of *S. crispus* were homogenized and soaked in chloroform 100% for a week. The crude extract was then filtered with Whatmann paper No. 4 and evaporated with rotary evaporator. The dried residue was resuspended in DMSO and subjected to TUNEL assay.

Correspondence:
Susi Endrini, SSi, MSc, PhD., Department of Biochemistry YARSI UNIVERSITY School of Medicine, Jakarta, Jalan Let.Jen. Suprapto, Cempaka Putih, Jakarta 10510, Telephone 021-4206674, 4206675, 4206676, Facsimile 021-4244574

Culturing of cells

HepG-2 (Human liver cancer) was obtained from American Type Culture Collection (ATCC, USA). The cells were cultured in Minimum Essential Medium with Earle's salt (Gibco, USA), supplemented with 10% of fetal calf serum, 100IU/ml penicillin and 100µg/ml of streptomycin (Gibco, USA) using 25-cm2 flasks (Nunc, Denmark), in a CO_2 incubator (Sanyo, Japan) at 37°C.

Treatment of cells

The viability of cells was determined with trypan blue. Exponentially growing cells were harvested, counted by using haemocytometer, and diluted with medium, yielding a concentration of $1x10^5$ cells/ml. For the TUNEL assay study, the LabTek Chamber slides were used to grow the cells. The slide chambers were incubated for 24 hours in a 5% CO_2 incubator (Sanyo, Japan) at 37°C. For each cell, the two doses (20 and 30µg/ml) of *S.crispus* extract were used. The other slides were used as a negative and positive control (without treatment). The chamber slides were incubated again in a 5% CO_2 incubator (Sanyo, Japan) at 37°C for 96 hours. Then, the cells in the chamber slides were ready to use.

TUNEL assay

The TUNEL assay was carried out by using Apoptosis Detection System, Fluorescein (Promega, USA).

Sample were analysed immediately under a fluorescence microscope using a standard fluorescein filter set to view the green fluorescence of fluorescein (FITC) at 520 ± 20nm and red fluorescence of propidium iodide (PI) at >620nm. Confocal Laser Scanning Microscope (CLSM) was also used to obtain the better results.

RESULTS

Figure 1 and 2 showed the morphology of HepG2 cells treated with 20 and 30µg/ml *S.crispus* extract respectively by using Fluorescence microscope. The TUNEL assay staining revealed apoptotic cells with intensely yellow fluorescence of PI-FITC. The features were condensation of chromatin, fragmentation of DNA and apoptotic bodies. Clearer results were obtained by using Confocal Laser Scanning Microscope. Many apoptotic bodies were observed in HepG2 cells treated with 30ug/ml *S.crispus* extract (Figure 3). Mixture of PI and FITC labelled for apoptotic cell as yellow intensely (Figure 3A). Figure 3B and 3C showed the FITC labelled for apoptotic cell only, whereas PI labelled for both apoptotic and non-apoptotic cells.

DISCUSSION

Recent studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents including cisplatin, cytarabine, camptothecin, amsacrine, etoposide and teniposide (Kolfschoten *et al.,* 2002; Solary *et al.,* 2001). There is an accumulating evidence that the efficacy of antitumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis (Clary *et al.,* 1998; Lane, 1993; Clarke *et al.,* 1993). Apoptosis, a physiological mode of cell death, is characterized by reduced cell volume, condensed chromatin in the nucleus, formation of internucleosomal DNA fragmentation and loss of membrane integrity, and generation of apoptotic bodies (Nicoletii *et al.,* 1991; Evans and Dive, 1993).

The methods used to assess DNA strand breaks are based on labelling/staining the cellular DNA. The labelled/stained DNA is subsequently analysed by fluorescence microscopy or Confocal Laser Scanning microscopy for the better results. Extensive DNA degradation is a characteristic event, which often occurs, in the early stages of apoptosis. Cleavage of the DNA may yield double-stranded, low molecular weight DNA fragments (mono- and oligonucleosomes) as well as single strand breaks ("nicks) in high molecular weight DNA. Those DNA strand breaks can be detected by enzymatic labelling of the free 3-OH termini with modified nucleotides. Suitable labelling enzymes include terminal deoxynucleotidyl transferase (end labelling). Terminal deoxynucleotidyl transferase (TdT) is able to label blunt ends of double stranded DNA break independent of a template. The end-labeling method has also been termed TUNEL (TdT-mediated X-dUTP nick end labeling, (Bortner, 1995). The TUNEL reaction is more specific for apoptosis and may be helpful to differentiate cellular apoptosis and necrosis (Gold, 1994).

The present results clearly demonstrate that *S.crispus* extract induces apoptosis in human liver carcinoma HepG-2 cells, which appears to account for its anti-proliferating activity. The induction of apoptosis by *S.crispus* extract was conformed by characteristic morphological changes.

CONCLUSION

Our present findings indicated that *S.crispus* extract potently suppresses proliferation of HepG-2 cells by induction of apoptosis.

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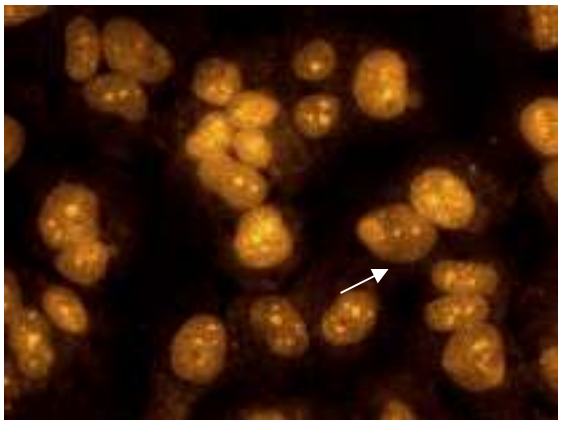

Figure 1. Fluoresence micrograph of HepG2 treated with 20 µg/ml *Strobilanthes crispus* extract. Many condensed nucleus were observed (white arrow). Magnification X40

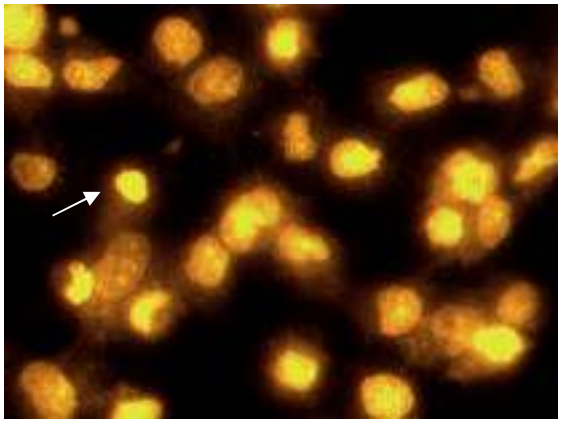

Figure 2. Fluoresence micrograph of HepG2 treated with 30 µg/ml *Strobilanthes crispus* extract. Many condensed nucleus were observed (white arrow). Magnification X400.

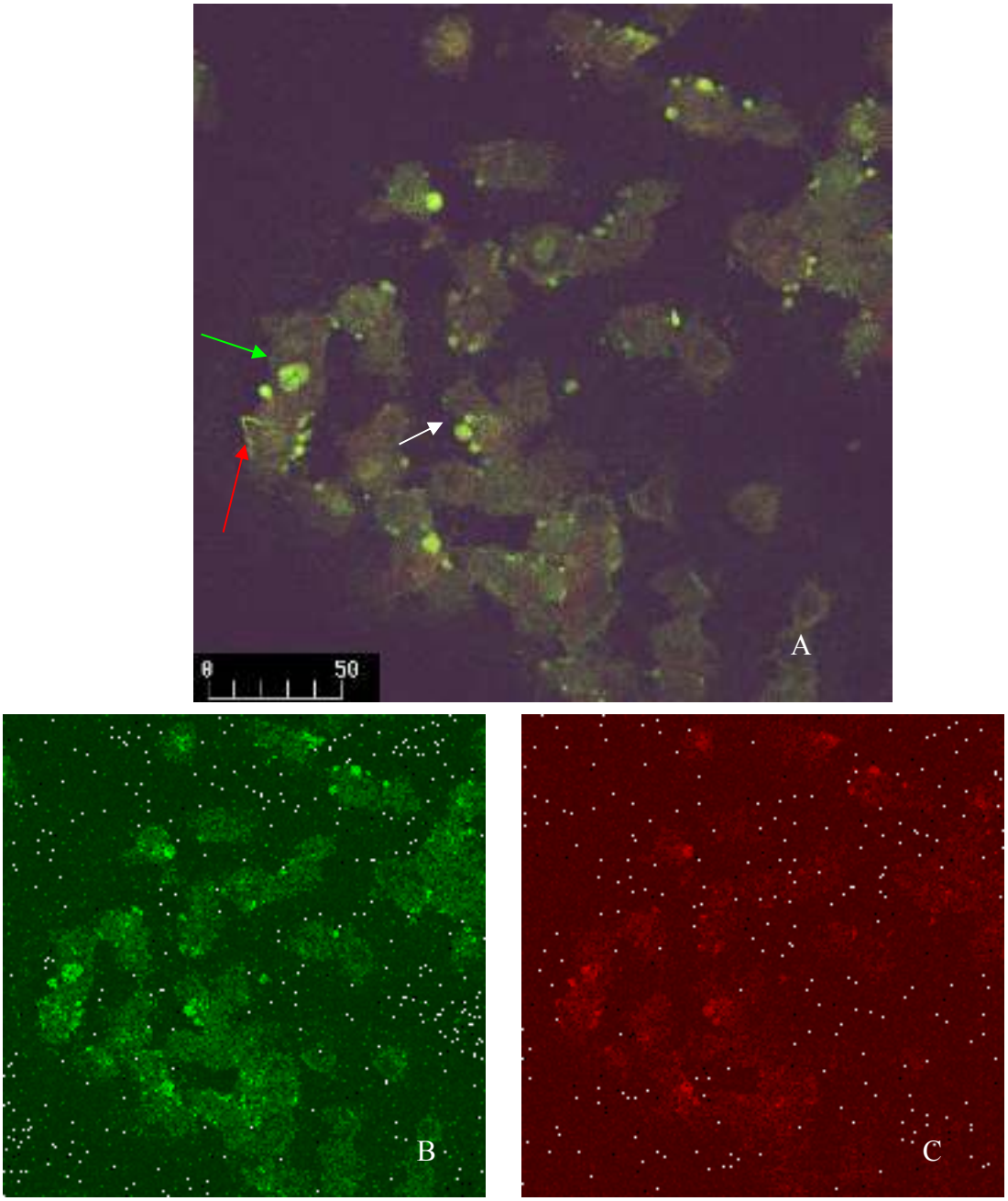

Figure 3: Confocal micrograph of HepG2 cells treated with 30 µg/ml *S. crispus* extract. Condensed nucleus (green arrow), prominent cell shrinkage (red arrow), and apoptotic bodies (white arrow) were observed (white arrow). A, mixture of PI and FITC labelled apoptotic cell as yellow intensely. B. FITC labelled for apoptotic cell only. C. PI labelled for both apoptotic and non-apoptotic cells. Magnification X400.