Strobilanthes crispus: A potential anticancer herb for liver and breast

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INTRODUCTION

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- Cytotoxicity properties and mechanism of *Strobilanthes crispus* extract against liver cancer cell line

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Introduction

• Worldwide, cancer causes more deaths than AIDS, tuberculosis, and malaria combined (Garcia et al., 2007)

• In year 2005, cancer killed 7.6 million, 3/4 of whom were in low- and middle-income countries (Coughlin & Ekwueme, 2009)

• By 2015, that number is expected to rise to 9 million and increase further to 11.5 million in 2030 (WHO, 2007)
Liver cancer

- Liver cancer is among the most lethal cancers (five-year survival rates under 11%), which makes it the third most frequent cause of cancer death in men and the sixth in women (American Cancer Society, 2007).

- The meta-analysis from Larson and Wolk (2007) proven that of an increased risk of liver cancer among overweight and obese persons. Their findings indicate that liver cancer may, in part, be prevented by maintaining a healthy body weight.

- More than 80% of hepatocellular carcinomas occur in patients with cirrhosis, and a number of conditions and risk factors have been implicated as the cause of the disease included genetic, virus infection or excessive alcohol consumption (Ahmed and Lobo, 2006).
Breast cancer

- The most common cancer in women at the global level is breast cancer, followed by cancers of the trachea, bronchus and lung, and stomach cancer (WHO, 2004)

- Global burden of breast cancer in women, measured by incidence, mortality and economic costs, is substantial and on the increase (Mackay, Jemal & Parkin, 2006)

- Female breast cancer incidence rates for 2002 vary internationally by more than 25-fold, ranging from 3.9 cases per 100,000 in Mozambique to 101.1 in the United States (American Cancer Society, 2007)
Strobilanthes crispus (L.) Bremek (Acanthaceae)

• Commonly known as “pecah beling” or “pecah kaca” in Malaysia.

• It also known as ‘daun picah beling’ in Jakarta or ‘enyoh kelol’, ‘kecibeling’, or ‘kejibeling’ in Java (Sunarto, 1977).

• Traditionally, S. crispus leaves were boiled with water and the filtrates were used in traditional medicine in Malaysia and Indonesia as antidiabetic, diuretic, antilytic and laxative (Sunarto, 1977).
Figure 1: *Strobilanthes crispus* tree and leaves
• Kusumoto et al., (1992) have been reported that the water extract of *S. crispus* may inhibit 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).

• From our studies showed that the water extract of *S. crispus* leaves are also able to possess anticarcinogenic (Suherman et al., 2004; Fauziah et al., 2005; Asmah et al., 2006b; Abu Bakar et al., 2006a; Iqbal et al., 2010), antidiabetic and antilipidemic (Abu Bakar et al., 2006b; Norfarizan-Hanoon et al., 2009a) as well as wound healing (Norfarizan-Hanoon et al., 2009b) effects.

• These health-giving properties is mainly due to high antioxidant activity and phytochemical constituents especially minerals and vitamins content as well as other component such as catechins, caffeine and tannin (Ismail et al., 2000; Abu Bakar et al., 2004; Asmah et al., 2006a; Liza et al., 2010).
PART 1:
STUDY ON LIVER CANCER
Effect of *Strobilanthes crispus* on Tumour Marker Enzymes and Glutathione During Chemical Hepatocarcinogenesis in the Rat

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**Abstract:** The administration effect of *Strobilanthes crispus* extracts (SC) during hepatocarcinogenesis in rats was studied to investigate the possible cancer suppressive effect of the component existed in the leaves. Hepatocarcinogenesis was induced using Diethylnitrosamine (DEN) (200 mg kg⁻¹) and 2-acetylaminoflourence (AAF) (0.02% w/w). Glycyrrhizin (G), the commercial anticancer drug, was used for comparison. A total of 84 male *Sprague-Dawley* rats were divided into 14 groups viz control (N), DEN/AAF (C), SC1% (NS1), SC2.5% (NS2.5), SC5% (NS5), SC7.5% (NS7.5), DEN/AAF/SC1% (CS1), DEN/AAF/SC2.5% (CS2.5), DEN/AAF/SC5% (CS5), DEN/AAF/SC7.5% (CS7.5), DEN/AAF/G1% (CG1), DEN/AAF/G2.5% (CG2.5), DEN/AAF/G5% (CG5) and DEN/AAF/G7.5% (CG7.5). The effect of SC was investigated by identifying activities of liver and plasma γ-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) and glutathione (GSH) concentrations. Treatment with DEN/AAF caused increase in all enzymes activities when compared to control. The administration of SC (1, 2.5, 5 and 7.5%) to the induced cancer rats decreased the microsomal GGT. T-test showed a significant difference (p<0.05) when the GGT level of all the treatment groups were compared to the control (N) and DEN/AAF (C). The findings suggest that supplementation of SC on DEN/AAF rats reduced the severity of hepatocarcinogenesis by reducing liver GGT and ALP activities and also the levels of GSH.

**Key words:** *Strobilanthes crispus*, GGT, ALP, GSH, hepatocarcinogenesis
Overview of the method

Rats (120-200g)

G1
Control group (Basal diet only)

G2-5
Basal diet + water extracts (1%, 2.5%, 5% & 7.5%)

G6
Cancer (DEN/AAF) with basal diet only

G7-10
Cancer + SC water extracts (1%, 2.5%, 5% & 7.5%)

G11-14
Cancer + glycyrrhizin (1%, 2.5%, 5% & 7.5%)

Histological examinations

Light microscope

Confocal scanning

Transmission electron microscope (TEM)
• Treatment with *S. crispus* extract had no effect on either plasma or liver microsomal GGT activities.
• On the other hand, treatment with DEN/AAF caused a significant increase in plasma and liver GGT activity compared to that of control (p<0.05).
• However, when *S. crispus* (all doses) were supplemented to DEN/AAF treated rats with *S. crispus* or Glycirrhizin caused lowering of ALP activities.
• Rats treated with carcinogen DEN/AAF also caused a significant increased in GSH levels.
• Supplementation of *S. crispus* and G to DEN/AAF decrease GSH values (p<0.05) significantly for all doses and the lowest values was observed on dose 5% of *S. crispus*. 
Figure 2: The electron transmission micrograph illustrated liver cell morphology of normal control rat (N)

a) Arrangement and organization of hepatocytes were normal, nucleus (n) undamaged, 600X.

b) Mitochondria (m), crystal (k) and clear and normal rough surface of reticulum endoplasma (RER), 40,000X.
Figure 3: The electron transmission micrograph illustrated liver cell morphology of rat induced with DEN-AAF (C).

Arrangement of cells were unorganized and membrane cell was unclear: 
a) 6,000X, nucleus (n) enlargement, with shrunken size, nucleolus defragmentation (nl) cytoplasm (s) vacuol.
b) 40,000X, mitochondria (m) enlargement and splited rough surface of reticulum endoplasm (RER), which having serious damaged.
Figure 4: The electron transmission micrograph illustrated liver cell morphology of rat induced with DEN/AAF treated with *S. crispus* 5% (CS5).

a) 6,000X, nucleus and cytoplasm recovered
b) 40,000X, mitochondria (m) rough surface of reticulum endoplasm (RER) recovered as well.
Figure 5: The electron transmission micrograph illustrated liver cell morphology of rat induced with DEN/AAF and treated with glisirhizin 5% (CGL5).

a) 6,000X, nucleus and cytoplasm improved from damage
b) 40,000X, mitochondria (m) rough surface of reticulum endoplasm (RER) was clear and similar to normal cell shape.
Conclusion

- The overall result showed that SC supplementation in the diet of the carcinogen-treated rats lowered the GGT and ALP activities and GSH levels.
- TEM indicated the severe damages at the ultrastructural especially of the nucleus, mitochondria, cell arrangements, RER, Golgi apparatus.
- The severity of neoplastic cells was reduced significantly with treatment of SC and GL.
Cytotoxicity properties and mechanism of *Strobilanthes crispus* extract against liver cancer cell line

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1) Cytotoxicity properties

- Three types of extraction were done to screen for the best cytotoxic properties:
  
  1. Catechin Extraction (Hara, 1994)
  2. Ethanol, Methanol and Chloroform Extraction
  3. Extraction With Solvent of Increasing Polarity

- MTT assay was used to determine the cytotoxic properties

- The $IC_{50}$ values of *S. crispus* against liver cancer cell line was 29.3 µg ml/l for methanolic extract while the chloroform extract showed $IC_{50} = 28$ µg ml/l.
2) Effects of *Sroblanthes crispus* on the expression of *c-myc* oncogene and apoptotic pathway

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR Study):

- Cells (growth in flasks)
  - Treatment
  - Collecting of cells
  - Isolation of mRNA
  - RT-PCR
  - Agarose Gel Electrophoresis
  - DNA Purification
  - Sequencing
Effect of *S. crispus* crude extract on the expression of *c-myC* gene in HepG2 cell line.

PCR products were analysed on a 1.5% agarose gel.

- M, 100 bp DNA ladder marker
- lane 1, HepG2 control (untreated)
- lane 2, HepG2 treated with 20 µg mL⁻¹ *S. crispus* extract
- lane 3, HepG2 treated with 30 µg mL⁻¹ *S. crispus* extract

*C-myC* gene at lane 1 and 2 was 218 bp in length. The gene expression was not observed in lane 3.
Apoptotic DNA Ladder

• The apoptotic DNA Ladder Kit is designed for the purification of nucleic acids from culture cells to detect the typical DNA ladder, which is the hallmark of apoptotic cells. DNA can be applied to the agarose gel directly after its elution from the column.

Cells

↓

Isopropanol

↓

DNA

↓

Gel Electrophoresis

• From the apoptotic study, the clear DNA-ladders were visible, when DNA were isolated out of HepG2 cell treated with crude extracts S. crispus.
DNA Ladder assayed with the Apoptotic DNA Ladder kit.

Lane identification:
- M, DNA mix Ladder
- 1, HepG2 untreated;
- 2, HepG2 treated with henna oil
- 3, HepG2 treated $\gamma$-sitosterol
- 4, HepG2 treated henna crude extract
- 5, HepG2 treated *S. crispus* crude extract.
TUNNEL ASSAY

Cells (growth in slides)

⇒ PBS

⇒ Triton X-100

⇒ TdT-Nucleotide Mix addition

⇒ Incubation 37°C, 60 min

⇒ 2xSSC

⇒ 12-dUTP Fluorescein

⇒ PI-Staining and Anti Fade Addition

⇒ Analyse under Fluorescence Microscope and CLSM
The TUNEL assay staining revealed apoptotic cells with intensely yellow fluorescence of PI-FITC. The common feature are condensation of chromatin, fragmentation of DNA and apoptotic bodies.

• Fluorescence micrograph of HepG2 treated with 20 μg/ml (A) and 30μg/ml (B) *Strobilanthes crispus* extract.
• Many condensed nucleus were observed.
• Confocal micrograph of HepG2 cells treated with 30 μg/ml *S. crispus* extract.
• Condensed nucleus, prominent cell shrinkage, and apoptotic bodies.
  • A, mixture of PI and FITC labeled apoptotic cell as yellow intensely.
  • B, FITC labeled for apoptotic cell only.
  • C, PI labeled for both apoptotic and non-apoptotic cells.
Conclusion

This present study verify that *S. crispus* extracts and also their active constituents e.g., sitosterol and essential oil were induced apoptosis and suppressed the *c-myc* overexpression in liver carcinoma cell lines.
PART 2:
STUDY ON BREAST CANCER
SC induced cell death in MCF-7 cells

- Membrane
  - Acridine orange/propidium iodide staining
  - Annexin V-FITC/propidium iodine staining

- Mitochondrial
  - RNAse/propidium iodide staining
  - TUNEL assay
  - DNA ladder

- Cytoplasm
  - Cytochrome c release
  - Caspase 3 activity
  - Caspase 8 & 9
AO/PI staining: Induction of apoptosis in MCF7 cells

Cells at the concentrations of $1 \times 10^5$ cell ml$^{-1}$ were stained with 10µ of Acriderine Orange and Propidium Iodide at concentration of 10µg/mL. Slides were viewed under florescence microscopy (Optiphot 2, Nikon, Japan). Two hundred randomly selected cells were scored for each sample from three independent experiments to detect indications of apoptotic and necrotic cells. Cells were scored as positive based on visual evidence of green fluorescence with characteristics of cell shrinkage, nuclear condensation, and/or the presence of membrane-bound apoptotic bodies. Cells appearing as either red or both green and red fluorescence were considered as necrotic.
Treated and untreated cells at the concentration of $1 \times 10^6$ cells per ml were resuspended in binding buffer and stained with 5µl of both Annexin V FIT-C and Propidium Iodide from [BD Pharmingen Apoptosis Detection Kit I (Cat. No.556547)]. Cells were incubated for 15 minutes at room temperature in the dark before addition of 400 µl binding buffer. Annexin V-FITC and Propidium iodide particle fluorescence was analysed with logarithmic amplifiers set for four log decades. 10,000 events were collected for Annexin V-FITC/PI analysis. Untreated population is used to define the basal level of apoptotic and dead cells.
Cells at the concentration of $1 \times 10^6$ cells per ml were collected and resuspended in 4 ml of -20°C absolute ethanol was added to fix cells followed by centrifugation. The pellet was resuspended again in 1 ml of PBS. 100 µl of 200 µg/ml; DNase-free, Rnase A was added and the cells were left to incubate for 30 minutes. 100 µl of 1 mg/ml propidium iodide (light sensitive) was added to the cells and incubated for another 5-10 minutes at room temperature.
Harvested cells at $10^6$ were fixed with 1% (w/v) paraformaldehyde in PBS for one hour before underwent series of washing with cold PBS. 70% (v/v) ice cold ethanol was added and placed on ice for 30 mins. Cells were stored in 70% (v/v) ethanol at −20°C until use. For analysis, DNA labeling reagent was added and incubated in 37°C water bath for one hour before adding antibody staining solution. Incubation of FITC-labeled anti-BrdU Antibody Solution was performed in dark prior flow cytometry analysis.
DNA fragments detected in MCF7 cells

Genomic DNA was isolated and precipitated following manufacturers instructions Apoptotic DNA ladder (Roche, USA). 2µg of purified DNA was loaded into the loading pocket of prepared 1% agarose gel. GeneRulerTM 100bp DNA Ladder Plus was loaded as a marker for size comparison. Agarose gel electrophoresis was set at 75V and electrophoresed until Bromophenol blue dye migrated to the end of platform. The agarose gel was destained with water after placed in ethidium bromide solution. Visualization of the DNA was done with an UV illuminator.

Treatment of 72hr ethanolic extract of SC leaves against MCF7 (B). A (i) and A(ii) were DNA marker and MCF7 control cells respectively.
Cytochrome C released in MCF7 cells

Treated cells at the concentration of $10^5$ were lysate by addition of lysis buffer. Human Cytochrome C present in treated cells bound to antibodies adsorbed to the surface of the microwells. 50μl biotin-conjugated antihuman Cytochrome C antibody was added to all wells prior incubation for 2hr at room temperature. Streptavidin-HRP was added to bind the biotin-conjugated anti-human Cytochrome C antibody and further incubated for one hour. 100μl substrate solution reactive with HRP was added to all wells. Coloured products were formed in proportion to the amount of human Cytochrome C present in cells. The reaction is terminated by addition of acid and absorbance is measured at 450 nm.
100μl lysis and reagent were added to treated cells at $1 \times 10^4$ following manufactures protocol. Cleavage caspase substrate generated luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present. Caspase 3/7 activity was measured by the release of p-nitroaniline (pNA) from the labeled substrates Ac-DEVD-pNA which later quantified at 405nm.
Detection of caspase 8 and 9 activity in MCF7 cells

Treated cells at the concentration of $10^5$ were lysed by addition of lysis buffer. Human Caspase-8 and 9 found present in 50μl cell lysate were bound to antibodies adsorbed to the surface of the microwells. 100μl Anti-rabbit-IgG-HRP was added and attached to the detection antibody. Substrate solutions reactive with HRP were added to the wells for the development of coloured formazan formed in proportion to the amount of caspase-8 and 9 present. The reactions were terminated by addition of acid and absorbance was measured at 450 nm.
Protein identification were made using monoclonal antibodies against bax, bcl2 and bcl-XL (Abcam, UK). Secondary FIT-C conjugated anti-mouse IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoreactive proteins were detected with FIT-C. Cytofix-cytoperm solution is used to enable fixation and permeabilization of cells in suspension for flow cytometry analysis.
Involvement of bcl-2 family protein in MCF7 cells

• *S. crispus* did not alter expression proapoptotic protein bax levels but decrease antiapoptotic bcl-2 family protein bcl-2 in MCF-7 cells

• Induction of apoptosis by *S. crispus* extract in MCF-7 cells was found to be transcription independent.

• Exposure of *S. crispus* extract in MCF-7 cells reduced statistical one fold (p<0.05) decrease in anti-apoptotic bcl-2 protein level of at 72 hours but did not induced the expression of bax protein
Protein identification were made using monoclonal antibodies against p53 and XIAP ((BD Pharmigen)). Secondary FIT-C conjugated anti-mouse IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoreactive proteins were detected with FIT-C. Cytofix-cytoperm solution is used to enable fixation and permeabilization of cells in suspension for flow cytometry analysis.
1. IC$_{50}$ for SC extract was found as 30 µg/mL against MCF-7.

2. Presence of subG1 population in MCF-7

3. Detection of apoptotic MCF-7 was also apparent in flow cytometry Annexin V/FIT-C staining and via detection of double or single DNA break strands in TUNEL assay.

4. Mitochondrial activated apoptosis induction by SC in MCF-7 cells was found to involve caspases and release of Cytochrome C into the cytosol, activating initiator and effector caspase 3/7.

5. Upregulation of tumor suppressor p53 protein was detected.

6. However apoptosis induction was found to be p53 transcriptive independent as pro-apoptotic bax and bcl-2 protein were not activated upon activation of apoptosis machinery.
Conclusion

The targeted modulation *Strobilanthes crispus* extracts towards breast cancer cells offered a potential strategy in preventing abnormal cell proliferation and promoting cell death in an *in-vitro* model.


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