

Cytotoxic effect of γ -sitosterol from Kejibeling (*Strobilanthes crispus*) and its mechanism of action towards *c-myc* gene expression and apoptotic pathway

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Abstrak

Latar belakang: Penelitian ini bertujuan mengetahui efek sitotoksitas γ -sitosterol yang diisolasi dari Kejibeling (*Strobilanthes crispus*), suatu bahan tanaman obat, pada beberapa kultur sel kanker. Mekanisme dari efek yang dilihat kemudian diteliti melalui ekspresi gen penyebab kanker *c-myc* dan jalur apoptosis.

Metode: Penelitian *in vitro* ini menggunakan kultur sel kanker kolon manusia (Caco-2), sel kanker hepar manusia (HepG2), sel kanker payudara tergantung hormon (MCF-7) dan kultur sel normal hepar manusia (Chang Liver). Efek sitotoksik diukur melalui uji MTT melalui penentuan kadar yang membunuh 50% sel (IC_{50}). Mekanisme sitotoksik dipelajari dengan menentukan pengaruh penambahan γ -sitosterol pada ekspresi gen *c-myc* menggunakan metode reverse transcriptase-polymerase chain reaction (RT-PCR). Pengaruh γ -sitosterol melalui jalur apoptosis dipelajari dengan menggunakan asai terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

Hasil: Hasil penelitian menunjukkan bahwa γ -sitosterol bersifat sitotoksik pada sel Caco-2, HepG2 dan MCF-7 dengan nilai IC_{50} masing-masing adalah 8,3; 21,8; dan 28,8 μ g/mL. Tidak didapati nilai IC_{50} dari senyawa ini terhadap kultur sel Chang Liver. Senyawa ini terbukti menginduksi proses apoptosis pada kultur sel kanker kolon manusia dan sel kanker hepar manusia serta mampu menekan ekspresi gen *c-myc* di kedua sel.

Kesimpulan: γ -sitosterol bersifat sitotoksik terhadap kultur sel kanker kolon dan hepar manusia dan efeknya dimediasi oleh penekanan ekspresi gen *c-myc*. Senyawa ini juga menginduksi proses apoptosis pada kedua sel.

Abstract

Background: This study aimed to analyze the cytotoxicity effect of γ -sitosterol isolated from "Kejibeling" (*Strobilanthes crispus*), a medicinal plant, on several cancer cell lines. The mechanisms of the effects were studied through the expression of cancer-caused gene, *c-myc* and apoptotic pathways.

Methods: This *in vitro* study was done using human colon cancer cell lines (Caco-2), liver cancer cell lines (HepG2), hormone-dependent breast cancer cell lines (MCF-7) and the normal liver cell lines (Chang Liver). The cytotoxic effect was measured through MTT assay and the potential cytotoxic value was calculated by determining the toxic concentration which may kill up to 50% of the total cell used (IC_{50}). Meanwhile, the cytotoxic mechanism was studied by determining the effect of adding γ -sitosterol to the *c-myc* gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR). The effect of γ -sitosterol through apoptotic pathway was studied by using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

Results: γ -sitosterol was cytotoxic against Caco-2, HepG2, and MCF-7 with IC_{50} -values of 8.3, 21.8, and 28.8 μ g/mL, respectively. There were no IC_{50} -values obtained from this compound against Chang Liver cell line. This compound induced apoptosis on Caco-2 and HepG2 cell lines and suppressed the *c-myc* genes expression in both cells.

Conclusion: γ -sitosterol was cytotoxic against colon and liver cancer cell lines and the effect was mediated by down-regulation of *c-myc* expression and induction of the apoptotic pathways.

Keywords: apoptosis, *c-myc* gene expression, cytotoxic, RT-PCR, *Strobilanthes crispus*, TUNEL assay

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Cancer is the second most common cause of death in the US, exceeded only by heart disease, accounting for nearly 1 of every 4 deaths.¹ In 2014, about 585,720 Americans are expected to die from cancer or almost 1,600 people per day. In Indonesia, cancer has become the 7th cause of death based on a national survey in 2007, accounting for 5.7% of all mortality. Data from the population-based cancer registry in Jakarta Province showed the leading cancers among females in 2005-2007 are breast cancer, cervical cancer, ovarian cancer, colorectal cancer and among males are bronchus and lung cancer, colorectal cancer, liver cancer, pharyngeal cancer, and prostate cancer.²

Historically, natural products have served as a rich source of lead compounds for drug development against a wide array of biological targets, including various forms of cancer. Indonesia is rich in natural resources, especially medicinal plants. One of them is Kejibeling (*Strobilanthes crispus*). *Strobilanthes crispus* ZII 109 (L) Bremek or *Saricocalix crispus* ZII 109 (L) Bremek (Acanthaceae) plant is a native to countries from Madagascar to Indonesia,³ and was first quoted by Anderson. Thomas classified the plant under Spermatophyta (Flowering plants and Gymnosperma).⁴ A study in Indonesia found that an infusion of the dried leaves of *S. crispus* has been used as antidiabetic, diuretic, antilytic and laxative agents. A recent study indicated that water extract of *S. crispus* contains compounds with very high binding affinity to protein molecules that bind the active part 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.⁵ Previous studies reported that the chloroform extract of *S. crispus* has been shown to be cytotoxic against human colon cancer cell lines (Caco-2) and human liver cancer cell lines (HepG2).⁶

The present work was aimed to analyze the cytotoxicity effect of the g-sitosterol isolated from Kejibeling (*S. crispus*) on several cancer cell lines. The mechanism of the effects was also studied through the expression of cancer-caused gene *c-myc* and apoptotic pathways.

METHODS

Plant material

The leaves of *S. crispus* was harvested at the 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.

Preparation of extract

The extraction method was obtained from Ali, et al⁷ with slight modification. The dried leaves (5 kg) of *S. crispus* was 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 fractionation and the isolation of active compounds was performed using solvent extraction method with different polarity.

Culturing of cells

HepG-2, Caco-2, MCF-7 and Chang Liver cell lines were obtained from American Type Culture Collection (ATCC, USA). The medium for HepG-2 and Chang liver were Minimum Essential Medium with Earle's salt (Gibco, USA). While Caco-2 and MCF-7 were grown in Dulbecco's Modified Eagle medium (Gibco, USA). The cells were cultured in their own medium supplemented with 10% of fetal calf serum, 100 IU/mL penicillin and 100 mg/mL of streptomycin (Gibco, USA) using 25-cm² flasks (Nunc, Denmark), in a CO₂ incubator (Sanyo, Japan) at 37°C.

MTT assay

The viability of cells was determined with trypan blue. Exponentially growing cells were harvested, counted with hemocytometer, and diluted with medium, yielding a concentration of 1 x 10⁵ cells mL⁻¹. From this cell suspension, 100 µL was pipetted onto 96 well microtiter plates (Nunc, Denmark) then incubated for 24 h in 5% CO₂ incubator (Sanyo, Japan) at 37°C. The diluted range of test extracts was as follow 0.468, 0.937, 1.875, 3.750, 7.5, 15 and 30 µg mL⁻¹. After addition of the extract samples, new medium was added to make up the final volume of 200 µL each well. The plate was incubated in 5% CO₂ incubator (Sanyo, Japan) at 37°C for 24 and 48 h. Then, 20 µL of MTT reagent (Roche, USA) was added onto each well. This and was incubated again for 4 h (Sanyo, Japan) at 37°C. After incubation, 200 µL solubilization solution (Roche, USA) was added onto each well. The cell was then left overnight at 37°C, 5% CO₂ incubator.

Finally, the absorbance was read with ELISA reader (LX-800), at 540 nm wavelength.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The isolation of mRNA was performed using the Micro-FastTrack™ 2.0 kit (Invitrogen, USA). The RT-PCR process was carried out using cDNA Cycle kit (Invitrogen, USA). The polymerase Chain Reactions were performed by 30 cycles amplification for 1 minute at 94°C, 2 minutes at 55°C and 3 minutes at 72°C. The PCR products were analyzed by electrophoresis on 1.5% agarose gel.

The sequences of primers were as follows :

c-myc sense :

5'-CAAGAGGCGAAGACACAACGTCT-3'

c-myc antisense :

5'-AACTGTTCTCGTCGTTCCGCAA-3'

Sequencing

The sequencing technique was done on Automatic Sequencer (USA) and the chromatograms were analysed with Chromatos software and blasted to the database in gene bank.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The TUNEL assay was carried out using Apoptosis Detection System, Fluorescein (Promega, USA). Firstly, the cells were fixed by immersing slides in freshly prepared 4% paraformaldehyde solution in PBS (pH 7.4) in a Coplin jar for 25 minutes at 4°C. The cells were then washed by immersing the slides in fresh PBS for 5 minutes at room temperature. The washing step was repeated and cells were permeabilized by immersing the slides in 0.2% Triton X-100 solution in PBS for 5 minutes.

After this, the slides were rinsed by immersing in fresh PBS for 5 minutes at room temperature. The washing step was repeated once again. The excess liquid was removed by tapping the slides. The cells were then covered with 100 µL of equilibration buffer at room temperature for 5-10 minutes. While the cells were equilibrating, the Nucleotide Mix was thawed on ice and sufficient terminal deoxynucleotidyl transferase (TdT) incubation buffer for all experimental reactions were prepared (TdT incubation buffer consists of 45 mL equilibration buffer, 5 mL Nucleotide mix and 1mL TdT enzyme, for each samples). After incubation, the

50 mL TdT incubation buffers were blotted into each cells on a 5 cm² area. The cells were covered with Plastic Coverslips to ensure even distribution of the reagent. The slides were then incubated at 37°C for 60 minutes inside the humidified chamber to allow the tailing reaction to occur. The chamber slides were covered with aluminum foil to protect from direct light. After incubation, the reactions were terminated by immersing the slides in 2X SSC in a Coplin jar for 15 minutes at room temperature. The samples were then washed by immersing the slides in fresh PBS for 5 minutes at room temperature. The washing steps were repeated twice to remove unincorporated fluorescein-12-dUTP. The staining processes were done in a Coplin jar by immersing the slides in 40 mL of propidium iodide solution freshly diluted to 1 mg/mL in PBS for 15 minutes at room temperature in the dark. After that, the samples were washed by immersing the slides in deionized water for 5 minutes at room temperature and repeated twice for a total of 3 washings. Finally, drop of anti-fading solution was added cells and slides were mounted with coverslips, sealed with rubber cement or clear nail polish and let dry for 5-10 minutes. Sample were viewed and analysed immediately under a Confocal Laser Scanning Microscope (CLSM).

RESULTS

γ-sitosterol (Figure 1) was obtained from fraction 97-102 as white needle shaped crystal from the crude chloroform extract of *S. crispus*. After washing with n-hexane and recrystallisation from MeOH, this compound gave a single spot on the TLC plate. The confirmation of structure were done using infra red and mass spectrometry spectrum. The cytotoxic effect of γ-sitosterol has been determined and displayed the strongest cytotoxic effect on colon carcinoma cell lines (Caco-2), liver cancer cell line (HepG2), and hormone-dependent breast cancer cell lines (MCF-7) with IC₅₀ values of 8.3, 21.8, and 28.8 mg/mL, respectively (Figure 2). There were no IC₅₀-values obtained from this compound against Chang Liver cell line.

The results showed that *c-myc* genes expression (218 bp) were observed in untreated HepG2 and Caco-2 cell lines. Figure 3 and 4 showed the expression of *c-myc* genes in both cell lines and suppression effects of γ-sitosterol on Caco-2 and HepG2 cell lines. The *c-myc* gene was not expressed in both cells treated with 30 mg/mL γ-sitosterol. The confocal micrograph of Caco-2 cell lines treated with 30 mg/mL sitosterol has shown that this compound induced apoptosis (Figure 5).

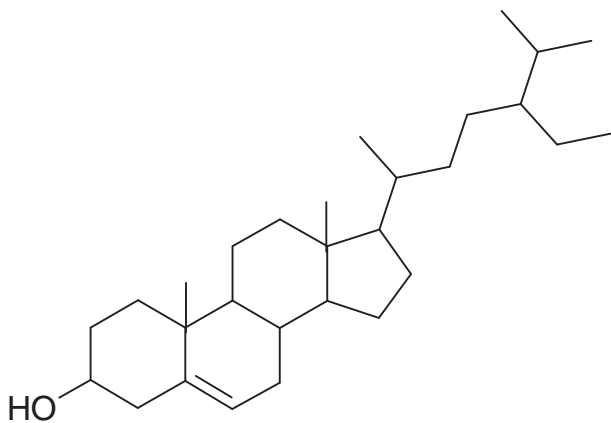


Figure 1. Molecular structure of γ -sitosterol from *S. crispus*

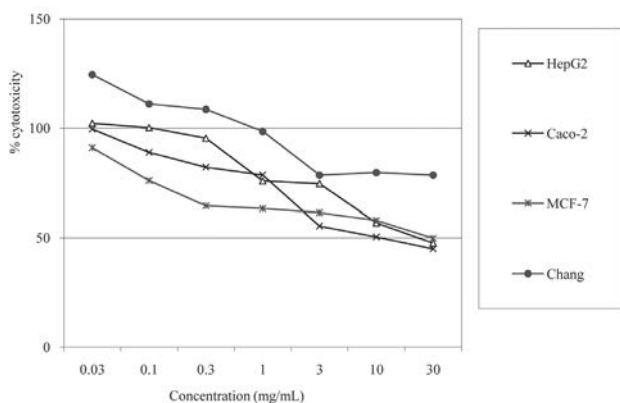


Figure 2. The effect of γ -sitosterol from *S. crispus* on different human cell lines. The protocol applied was similar to the previous one. IC_{50} of 8.3, 21.8, and 28.8 $\mu\text{g/mL}$ were obtained for Caco-2, HepG2 and MCF-7 cell lines, respectively

DISCUSSION

The *c-myc* oncogene contributes to the genesis of many human cancers. Recent insights into its expression and function have led to new cancer therapeutic opportunities.⁸ Many plant extracts have been reported to inhibit cell proliferation through the down regulation of *c-myc* expression.^{9,10} In this study, the *c-myc* expression was suppressed by γ -sitosterol which was isolated from *S. crispus*. To observe the effectiveness of γ -sitosterol in suppressing oncogenes, mRNAs were extracted from the treated cells. Due to instability of RNA and for the PCR purpose, mRNA was converted to cDNA before proceeding to the PCR process. The PCR has been selected as the most suitable technique to amplify the quantity of oncogenes, so that the suppression of oncogenes can be visualized clearly after a gel electrophoresis analysis. Besides, different oncogenes have their own temperature for denaturation, annealing, elongation in a number of cycles to get the best PCR products. Results showed that the effect was

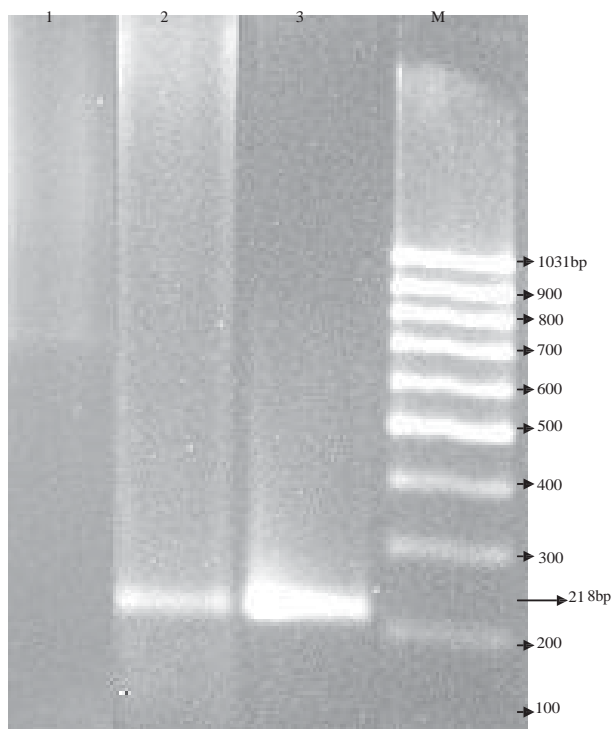


Figure 3. Effect of γ -sitosterol from *S. crispus* on the expression of *c-myc* gene in Caco-2 cell line. PCR products were analysed on a 1.5% agarose gel. M, 100 bp DNA ladder marker; lane 1, Caco-2 treated with 30 $\mu\text{g/mL}$ γ -sitosterol; lane 2, Caco-2 treated with 20 $\mu\text{g/mL}$ γ -sitosterol; lane 3, Caco-2 control (untreated)

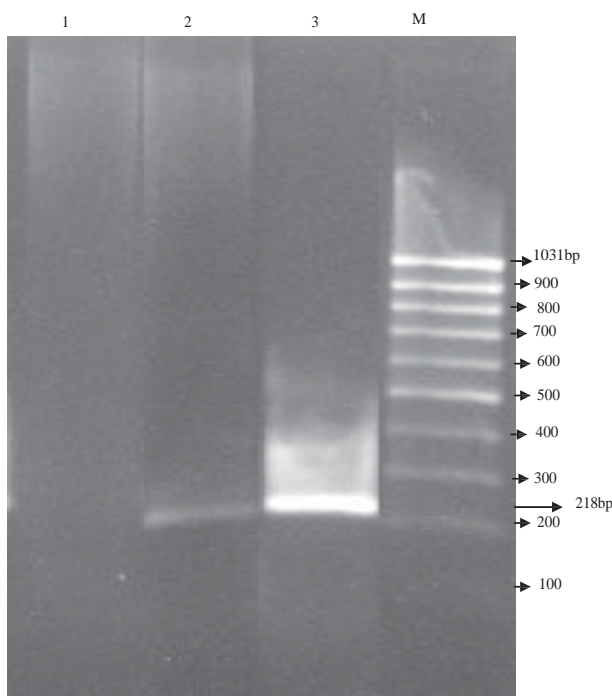


Figure 4. Effect of γ -sitosterol from *S. crispus* on the expression of *c-myc* gene in HepG2 cell line. PCR products were analysed on a 1.5% agarose gel. M, 100 bp DNA ladder marker; lane 1, HepG2 treated with 30 $\mu\text{g/mL}$ γ -sitosterol; lane 2, HepG2 treated with 20 $\mu\text{g/mL}$ γ -sitosterol; lane 3, HepG2 control (untreated). Gene expression was observed in lanes 2 and 3, but not in lane 1

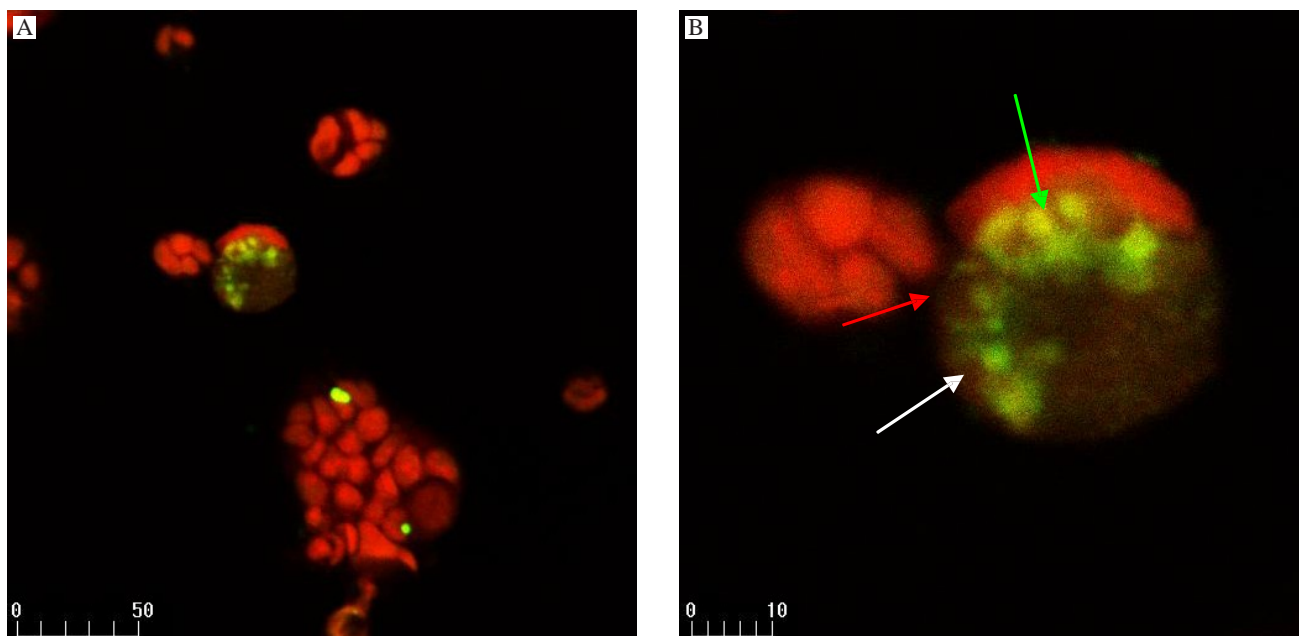


Figure 5. Confocal micrograph of Caco-2 cells treated with 30 µg/mL γ -sitosterol from *S. crispus*. Condensed nucleus (green arrow), prominent cell shrinkage (red arrow), and apoptotic bodies (white arrow). A: mixture of PI and FITC labelled apoptotic cell as yellow intensely. B: zoom of A for observed a single cell (apoptotic bodies). x400 magnification

dose dependent and it seemed to be correlated with the IC_{50} value of each treatment.

Recent studies have demonstrated that apoptosis involved in cell death induced by chemotherapeutic agents including cisplatin, cytarabine, camptothecin, amsacrine, etoposide and teniposide.^{11,12} 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.¹³⁻¹⁵ 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.^{16,17}

The methods used to assess DNA strand breaks are based on labeling/staining the cellular DNA. The labeled/stained DNA is subsequently analysed by fluorescence microscopy or confocal laser scanning microscopy for 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 labeling of the free 3-OH termini with modified nucleotides. Suitable

labeling enzymes include terminal deoxynucleotidyl transferase (end labeling). 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).¹⁸ The TUNEL reaction is more specific for apoptosis and may be helpful to differentiate cellular apoptosis and necrosis.¹⁹ This study demonstrated that γ -sitosterol from *S. crispus* may induced the apoptosis in Caco-2 and HepG2 cell lines. The morphology of chromatin condensation, DNA fragmentation and several apoptotic bodies were found by confocal laser scanning microscope in the treated groups. There were no apoptotic phenomena observed in the untreated group.

In conclusion, our results verifies that γ -sitosterol from *S. crispus* was cytotoxic against colon and liver cancer cell lines and the effect was mediated by down-regulation of *c-myc* expression which induced the apoptotic pathways.

Acknowledgment

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Conflict of interest

The authors affirm no conflict of interest in this study.

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