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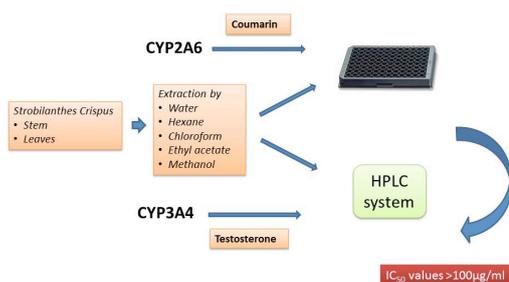
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***In vitro* study of modulatory effects of *Strobilanthes crispus* extracts on human cDNA-expressed cytochrome P450 2A6 (CYP2A6) and CYP3A4**

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Graphical Abstract



Abstract:

Aim: Cytochrome P450 2A6 (CYP2A6) and CYP3A4 play important roles in biotransformation of endogenous substances as well as xenobiotics. *Strobilanthes crispus* (L.) Blume (*S. crispus*) has been found to have anti-cancer activities and it has been suggested that this is due to inhibition of enzymes involved in metabolic activation of procarcinogens. The purpose of this study was to look into the potential inhibitory effects of various extracts (aqueous, hexane, chloroform, ethyl acetate, and methanol) of *S. crispus* from leaf and stem on human cDNA-expressed CYP2A6 and CYP3A4 activities.

Methods: The activity of CYP2A6 was examined via a fluorescence-based 7-hydroxylase coumarin assay. Meanwhile, a high performance liquid chromatography (HPLC)-based testosterone 6 β -hydroxylase assay was performed to assess CYP3A4 activity.

Results: It was shown that none of the extracts from either leaf or stem potently inhibited CYP2A6 or CYP3A4 activities, with 50% inhibitory concentration (IC_{50}) values above 100 μ g/ml.

Conclusion: *S. crispus* is unlikely to display anti-cancer properties through the modulation of CYP2A6 or CYP3A4 activities, but other mechanisms might be involved and merit further investigation. In addition, potential drug-herb interaction occurring between CYP2A6/CYP3A4 substrates and *S. crispus* preparations would probably be limited in effect, but this requires further investigations via *in vivo* animal as well as clinical studies.

Keywords:

CYP2A6; CYP3A4; drug-herb interaction; procarcinogen-activation; *Strobilanthes crispus*

INTRODUCTION

Cytochrome P450s (CYPs) are essential enzymes widely distributed within almost all living organisms. In mammals, they are responsible for biotransformation of a wide spectrum of endogenous compounds including fatty acids, cholesterol, steroids, retinoids, vitamin D derivatives, and bile acids. These enzymes are also involved in the metabolism of numerous xenobiotics such as environmental chemicals, pollutants, natural plant products and medicinal drugs.¹ Among human CYP families, the CYP1, CYP2, and CYP3 families are mainly involved in metabolism of exogenous substances while the CYP4 family contributes to a smaller extent.² Reactive oxygenated intermediates can be formed from parent compounds catalysed by CYPs, and are able to covalently bind to DNA and proteins, leading to carcinogenesis. The damage to DNA or protein by reactive oxygenated intermediates may directly affect various stress-signalling and/or checkpoint-signalling pathways.^{3,4} If this continues for a matter of decades, human carcinogenesis processes are set in motion. In addition to initiating carcinogenesis, CYPs can also be involved in cancer promotion or cancer progression by influencing various signal transduction pathways. Consequently, the cell cycle may be altered and apoptosis or aberrant cell growth may take place.

CYP2A6 accounts for approximately 10% of human microsomal CYP proteins, contributing to the biotransformation of nicotine and cotinine as well as some pharmaceuticals (such as fadrozole, tegafur etc.) and a number of coumarin-type alkaloids.⁵⁻⁶ Attention has been focused on CYP2A6 after nicotine and some tobacco-specific nitrosamines were discovered to be high-affinity substrates for this enzyme. 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), one of the most potent and abundant procarcinogens contained in tobacco smoke, is mainly activated to a mutagenic reactive metabolite via the CYP2A6 metabolic pathway.⁷⁻⁸ Moreover, CYP2A6 genotypes associated with reduced metabolic activity are associated with a lower risk of developing lung cancer.⁹ It has been postulated that employment of a CYP2A6 inhibitor such as methoxsalen could lower cancer risk.¹⁰ CYP3A4, the most abundant CYP isoform in the human liver, is responsible for metabolizing more than 50% of drugs used today, including anti-cancer agents (eg. gefitinib and imatinib). Co-administration of gefitinib with CYP3A4 inhibitor itraconazole resulted in increased exposure to gefitinib.¹¹ Serious adverse reactions to anti-cancer drugs are often reported, and concomitant administration of natural products with CYP3A4-metabolised drugs is one of the major causes.

For example, grapefruit juice reduces dehydrofelodipine/felodipine AUC ratio and increases absolute dehydrofelodipine AUC by inhibiting CYP3A4¹². Moreover, CYP3A4 is able to activate certain procarcinogens, such as aflatoxin B1.¹³⁻¹⁵

Strobilanthes crispus (L.) Blume (*S. crispus*) is a traditional medicinal plant in Malaysia and Indonesia known locally as ‘pecah kaca’ or ‘jin batu’ in Malaysia and ‘pecah beling’ or ‘kecibeling’ in Indonesia. Its leaves have been used to treat various conditions including breast and uterine cancers and gastrointestinal and kidney diseases.¹⁶⁻¹⁷ Crude extracts of this plant have been found to be cytotoxic to human cancer cell lines and protective against chemically-induced hepatocarcinogenesis in rats.¹⁸⁻¹⁹ Administration of *S. crispus* extract also reduced the severity of hepatic necrosis in rats with diethylnitrosamine- and acetylaminofluorene-induced hepatocellular carcinoma and this was suggested to be due to the inhibition of enzymes involved in metabolic activation of the carcinogens.²⁰

This *in vitro* study was designed specifically to look into the modulatory effects of various *S. crispus* extracts on CYP2A6 and CYP3A4 activities, which may serve as one of the mechanisms involved in *S. crispus*'s anti-cancer properties. Additionally, this study was able to provide information to that suggests potential drug-herb interactions.

METHODS

Reagents

Isopropyl β-D-1-thiogalactopyranoside (IPTG), Tris-base, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), and glycerol were purchased from Promega (Madison, WI, USA). Terrific Broth media were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Organic solvents were purchased from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MI, USA).

Preparation of *S. crispus* extracts

Fresh leaves and stems of *S. crispus* were collected from Paka, Terengganu, Malaysia. A sample of the plant was deposited at Forest Research Institute Malaysia (voucher specimen number: PID 040114-04). The plant materials were oven dried at 60 °C and then separated into leaves and stems, and subsequently blended into smaller pieces. The fine

pieces of leaves and stems of *S. crispus* were prepared separately in 10 different extracts (5 from leaves and 5 from stems). Water extraction was prepared by mixing the plant materials (50 g) with distilled water (500 mL). The mixture was macerated overnight at room temperature and then maintained at 60 °C for 3 hours. The water extract was then filtered through filter paper. The filtrate was freeze-dried to obtain powder form of the water extract. Approximately 12 g of leaf extract and 9 g of stem extract were obtained by these procedures. Organic solvent extractions were prepared by mixing the plant materials (100 g) with hexane, ethyl acetate, chloroform or methanol (500 mL) and macerating for 3 days at room temperature. The resulting suspensions were filtered and the organic solvents evaporated in a rotary evaporator. The procedures yielded approximately 2 g of leaf extract using hexane as the solvent, 7 g using ethyl acetate, 12 g using chloroform and 10 g using methanol. Extraction from the stems yielded approximately 1.5 g using hexane, 5 g using ethyl acetate, 9 g using chloroform and 5 g using methanol.

Co-expression of CYP2A6 and CYP3A4 with NADPH-CYP reductase in E. coli

Human CYP2A6 and CYP3A4 cDNA were cloned into the bacterial expression vector pCWori+, as detailed in previous articles [²¹⁻²² respectively]. PCW-2A6/pCW-3A4 were subsequently co-transformed with pACYC plasmid containing NADPH-CYP reductase cDNA into *Escherichia coli* DH5 α competent cells to achieve optimum activity. Three-hundred and fifty milliliters Terrific Broth (TB) medium culture was inoculated with 3.5 ml overnight Luria-Bertani (LB) culture of co-transformed bacteria. Induction of CYP expression was initiated by the addition of 1.0 mM IPTG and 0.5 mM-aminolevulinic acid (Δ -ALA) when the optical density of the culture at 600 nm was approximately 0.7. The incubation proceeded for 24 h at 30 °C and 125 rpm in a shaker incubator, after which bacterial cells were harvested and fractionated.²³ Cells were pelleted in Tris-EDTA-sucrose (TES) buffer (15 ml per gram of wet cells). Following lysozyme treatment of the cells (3 mg/ml in Tris buffer; 100 μ l of lysozyme per gram of wet cells) and centrifugation, the spheroplasts were resuspended in a buffer (20 mM potassium phosphate, pH 7.4, containing 500 mM potassium chloride and 20% glycerol, v/v) and stored at -80°C. Spheroplast suspensions were thawed on ice and sonicated in the presence of protease inhibitors (250 μ l per gram of wet cells) and phenylemethanesulfonyl fluoride (PMSF) (1 mM) using an Ultrasonicator (LABSONIC®P, Sartorius AG, Goettingen, Germany). The membranes containing the expressed CYP2A6/CYP3A4 were isolated by ultracentrifugation with a Beckman NVT65 rotor at 37,000 \times

g for 75 minutes at 4 °C. The membrane pellets were subsequently resuspended in 50/50 TES/water and stored at -80 °C until use. A control protein was expressed following the same procedures described above, employing authentic pCWori+ instead of pCW-2A6/3A4.

Coumarin 7-hydroxylase assay

Full CYP2A6 enzyme kinetic activity was assessed by a fluorescence-based coumarin 7-hydroxylase assay following a published protocol.²¹ 0.31-40 μ M of coumarin was incubated and the metabolite 7-hydroxycoumarin was quantified using a Tecan Infinite® 200 Microplate Reader (Tecan, Männerdorf, Switzerland) at the excitation wavelength of 365 nm and emission wavelength of 450 nm, which was used to establish a Michaelis-Menten plot. Absorbance data were exported to Microsoft Excel to acquire pharmacokinetic parameters (K_m and V_{max}).

Testosterone 6 β -hydroxylation assay

The evaluation of the CYP3A4 activity was performed via testosterone 6 β -hydroxylation assay using HPLC according to our published method.²⁴ 1 to 500 μ M testosterone was incubated and the metabolite 6-hydroxytestosterone was measured by an HPLC system equipped with a Gilson 307 pump, a Gilson UV/VIS-152 detector, and an ODS HYPERSIL column (Thermo, 4.6 x 250 mm, 5 μ m). The mobile phase used was 70% methanol/water and was pumped at a flow rate of 1 mL/min at a wavelength of 242 nm. Quantification of metabolites was achieved by comparing the peak area ratio of the metabolite with that of an internal standard, corticosterone.

Inhibition studies with S. crispus extracts

Aqueous extracts were dissolved in water and organic solvent extracts were dissolved in DMSO. The percentage of DMSO was kept as 1% (to retain enzyme activity) in all reactions except those involving aqueous extracts. IC_{50} values were determined to estimate the inhibitory potencies for various *S. crispus* extracts (aqueous, chloroform, ethyl acetate, methanol and hexane) from stem and leaf separately. This was achieved by incubating co-expressed CYP2A6 or CYP3A4 and NADPH-CYP reductase proteins (50 μ g per reaction) with coumarin (5 μ M, close to K_m) or testosterone (100 μ M, close to K_m) in the absence (set as control) and presence of various concentrations of *S. crispus* aqueous extract (up to 500 μ g/ml) or organic solvent extracts (up to

100 µg/ml), and subsequently the formation of 7-hydroxy coumarin and 6-hydroxytestosterone were quantified by the assay described in the previous sections. The CYP2A6/CYP3A4 enzyme activities for various concentration of each extract was calculated on the basis of its activity relative to the control reaction (set as 100%).

Data analysis

Enzyme kinetics (K_m and V_{max} values) were ascertained via non-linear regression analysis with Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). IC_{50} values were estimated by non-linear regression analysis with the help of SigmaPlot™ (Version 12.0, Systat Software Inc, USA).

RESULTS

Kinetics of CYP2A6 and CYP3A4 enzyme assays

The plots shown in Figure 1 are consistent with and typical of the Michaelis-Menten kinetic model $\{v=V_{max}[S]/(K_m+[S])\}$, in which, by increasing coumarin concentration, the reaction velocity (v) follows a hyperbolic pattern and reaches the maximum velocity V_{max} . K_m , known as the Michaelis dissociation constant, reflects how well the enzyme and substrate interact. It is the substrate concentration at which the reaction rate is half of its maximum. The K_m and V_{max} values derived from Figures 1A and B were 3.9 ± 1.4 µM (mean \pm std. error) and (1261 ± 135.1) pmol/min/mg protein (mean \pm std. error) for CYP2A6; 86.2 ± 16.3 µM (mean \pm std. error) and (4176.7 ± 200.4) pmol/min/mg protein (mean \pm std. error) for CYP3A4.

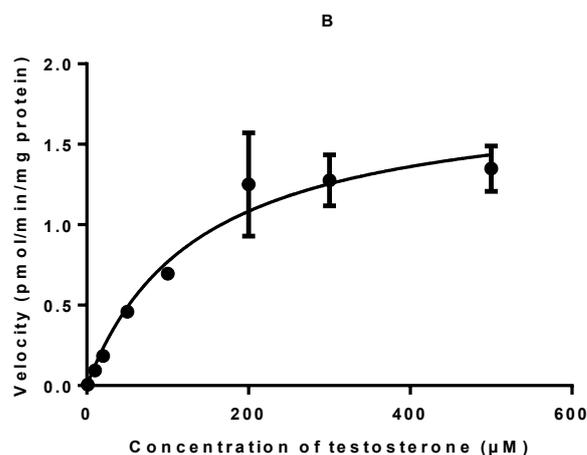


Figure 1: Representative Michaelis–Menten plot of CYP2A6 (A) and CYP3A4 (B) activities versus coumarin and testosterone as substrates. Points are experimentally determined values while solid lines are computer-generated curves of best fit. Each data point was found by taking the mean of 3 experimentally determined values.

Inhibition studies with *S. crispus* extracts

The IC_{50} values determined for the effects of *S. crispus* leaf and stem aqueous extracts on CYP2A6 were 399.1 and 390.9 µg/ml respectively (Figures 2A and 3A). IC_{50} values of all solvent extracts from both leaf (Figure 2B, C, D, and E) and stem (Figure 3B, C, D, and E) samples were more than 100 µg/ml. In the case of CYP3A4, IC_{50} values of leaf and stem extracts were more than 500 µg/ml (aqueous extracts) and 100 µg/ml (solvent extracts) (Figures 4A, B, C, D, and E; Figures 5A, B, C, D, and E). Table 1 shows the percent control activities of various *S. crispus* solvent extracts at a concentration of 100 µg/ml.

Figure 2A

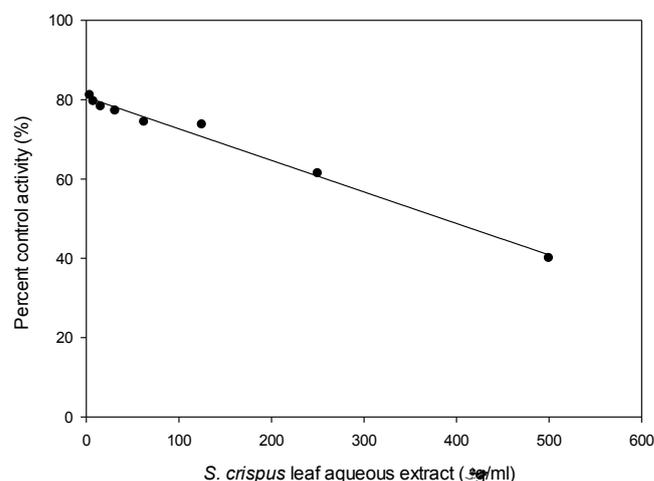
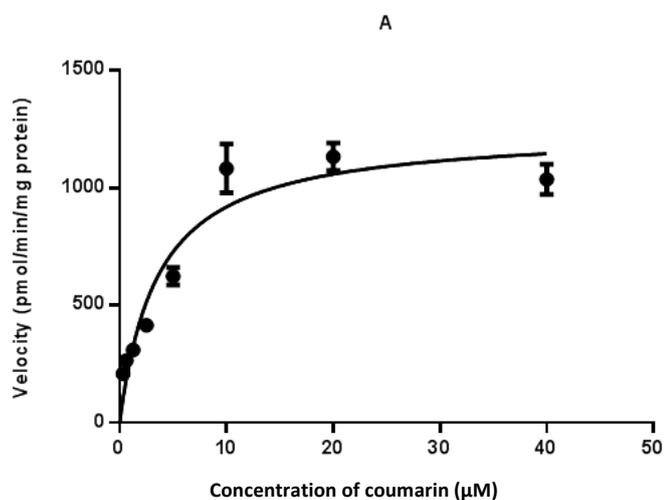


Figure 2B

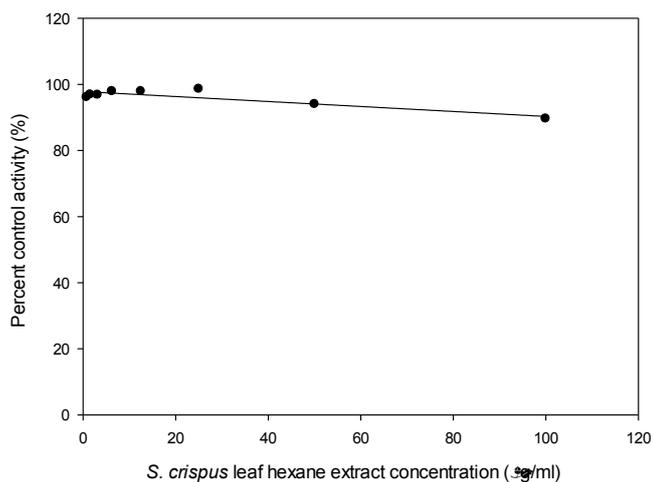


Figure 2D

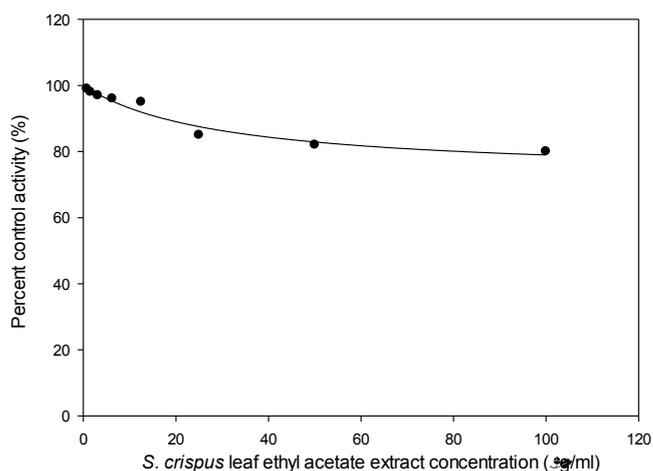


Figure 2C

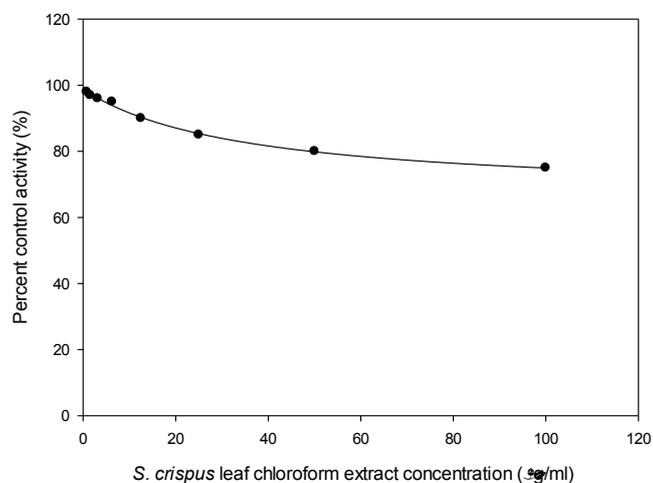


Figure 2E

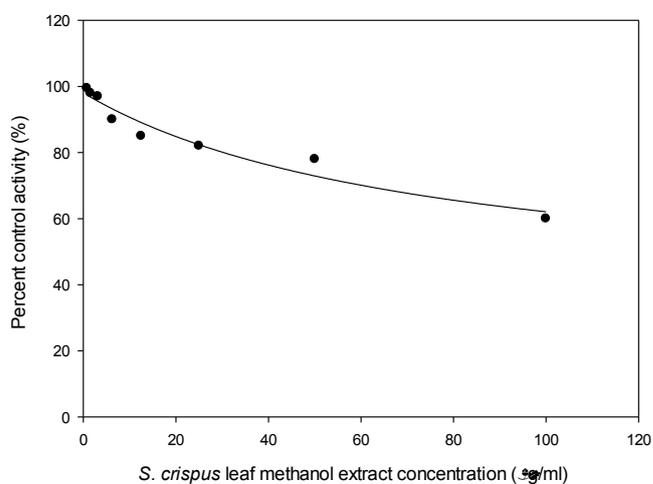


Figure 2. Effects of *S. crispus* leaf extracts on CYP2A6-mediated coumarin 7-hydroxylase activity: aqueous extract (2A), hexane (2B), chloroform (2C), ethyl acetate (2D), and methanol (2E). Points are experimentally determined values, while solid lines are best-fit curves generated by SigmaPlot for Windows Version 12.0. The x axis represents the *S. crispus* extract concentrations (µg/ml), while the y axis represents the percentages of control activity. Data points are the average values of duplicate determinations.

Figure 3A

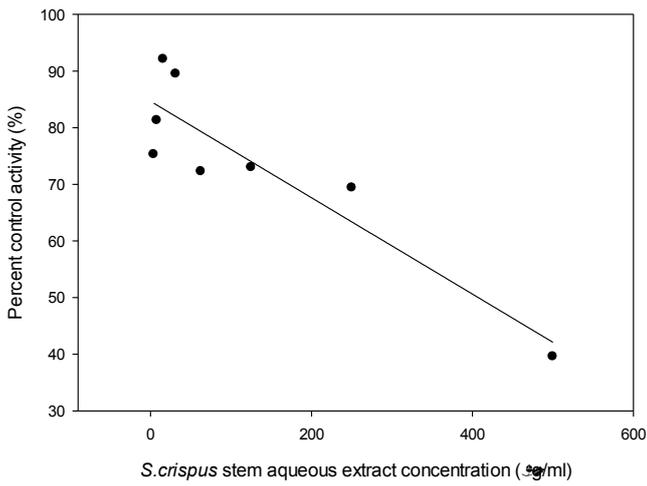


Figure 3B

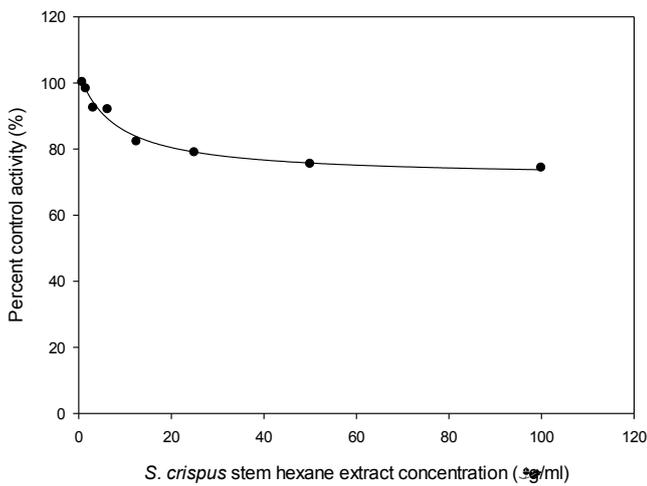


Figure 3C

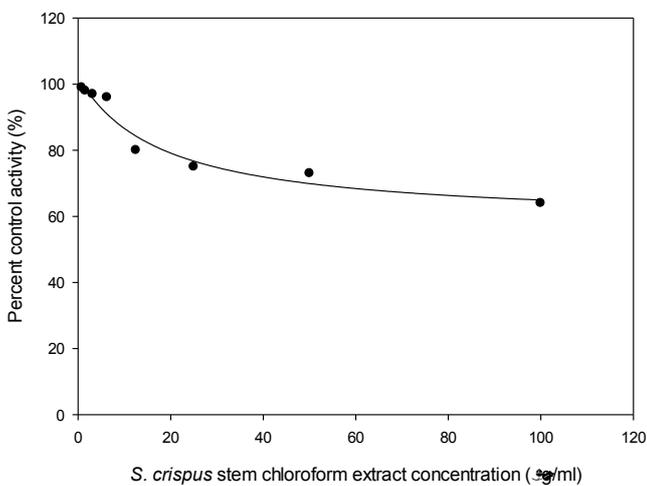


Figure 3D

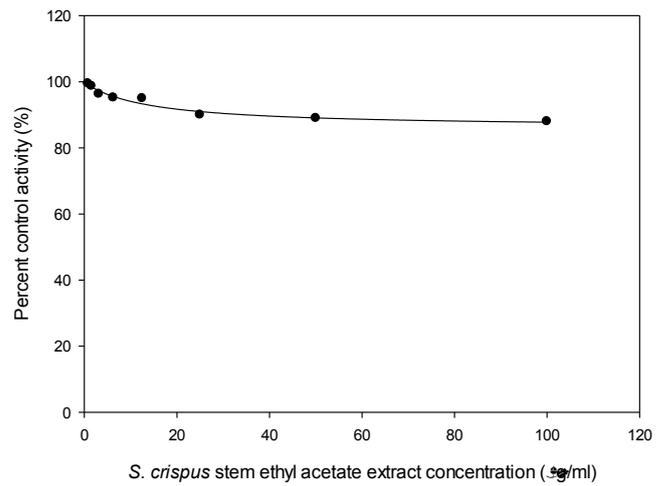


Figure 3E

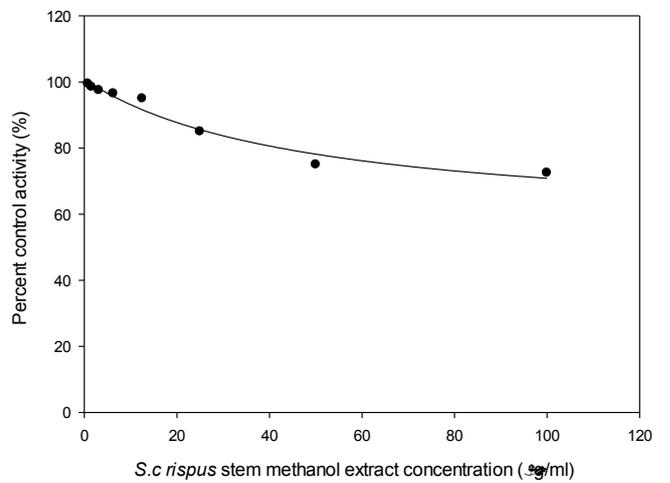


Figure 3. Effects of *S. crispus* leaf extracts on CYP2A6-mediated coumarin 7-hydroxylase activity: aqueous extract (2A), hexane (2B), chloroform (2C), ethyl acetate (2D), and methanol (2E). Data points are experimentally determined values, while solid lines are best-fit curves generated by SigmaPlot for Windows Version 12.0. The x axis represents the *S. crispus* extract concentrations (µg/ml), while the y axis represents the percentages of the control activities. Each data point is the average value of duplicate determinations.

Figure 4A

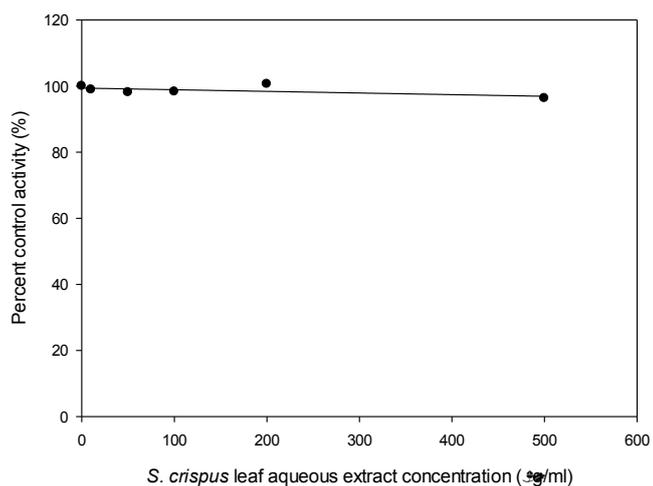


Figure 4D

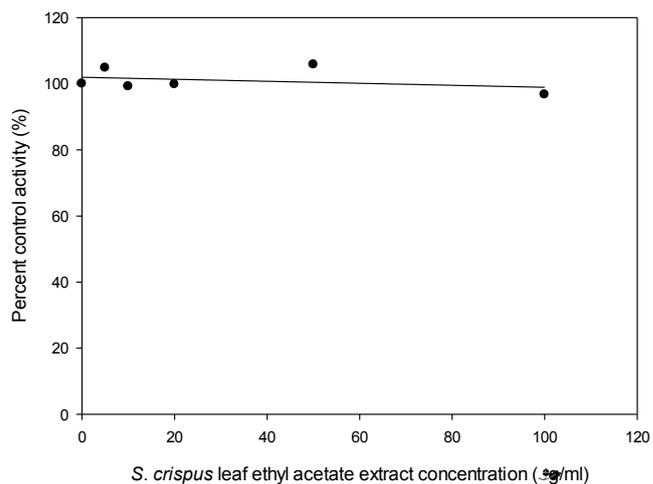


Figure 4B

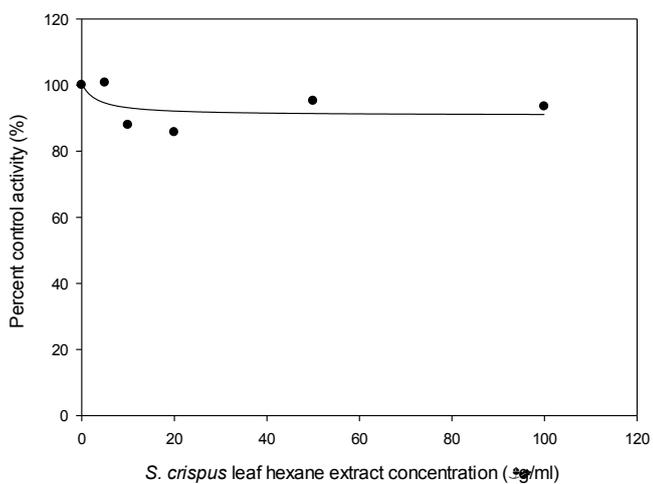


Figure 4E

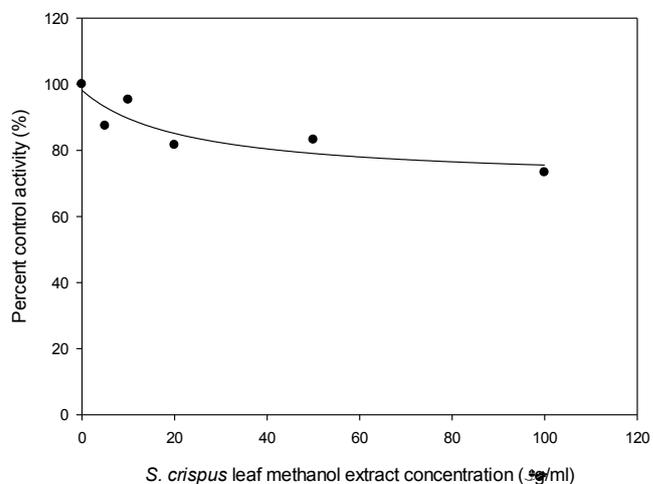


Figure 4C

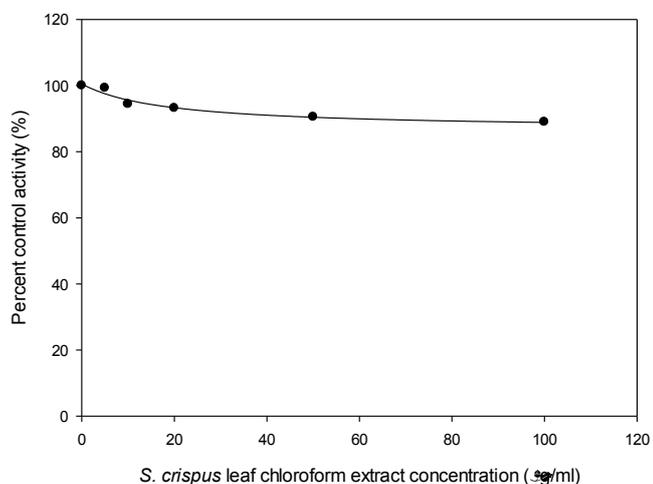


Figure 4. Effects of *S. crispus* leaf extracts on CYP3A4-mediated testosterone 6 β -hydroxylase activity: aqueous extract (2A), hexane (2B), chloroform (2C), ethyl acetate (2D), and methanol (2E). Data points are experimentally determined value, while solid lines are best-fit curves generated by SigmaPlot for Windows Version 12.0. The *x* axis represents the *S. crispus* extract concentrations ($\mu\text{g/ml}$), while the *y* axis represents the percentages of the control activities. Data points are average values of duplicate determinations.

Figure 5A

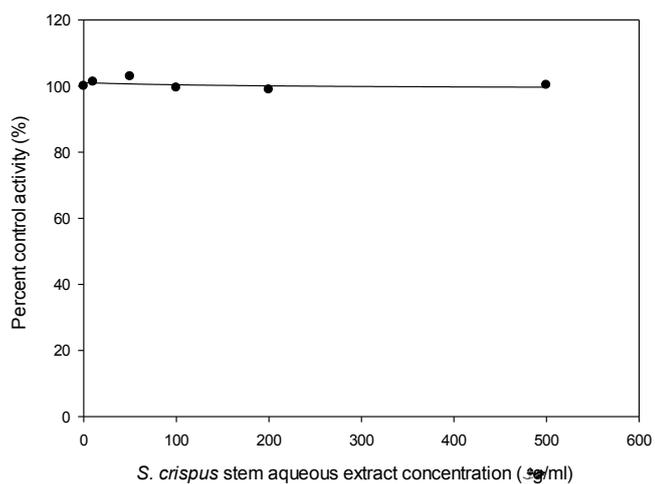


Figure 5B

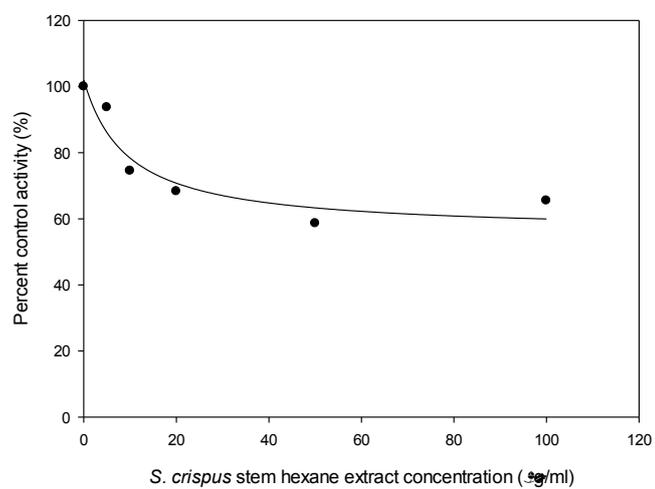


Figure 5C

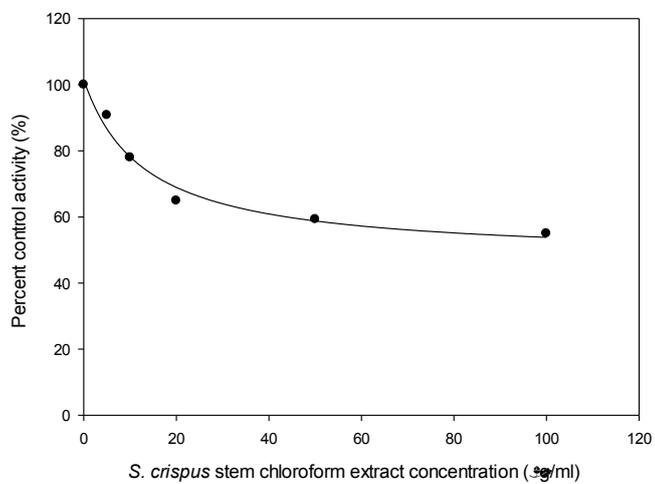


Figure 5D

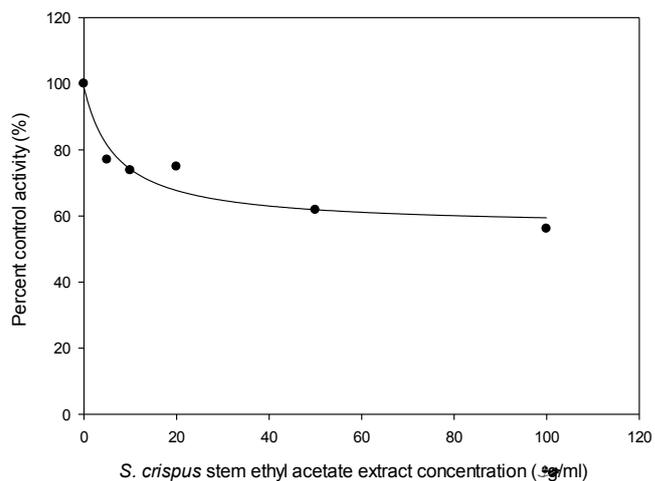


Figure 5E

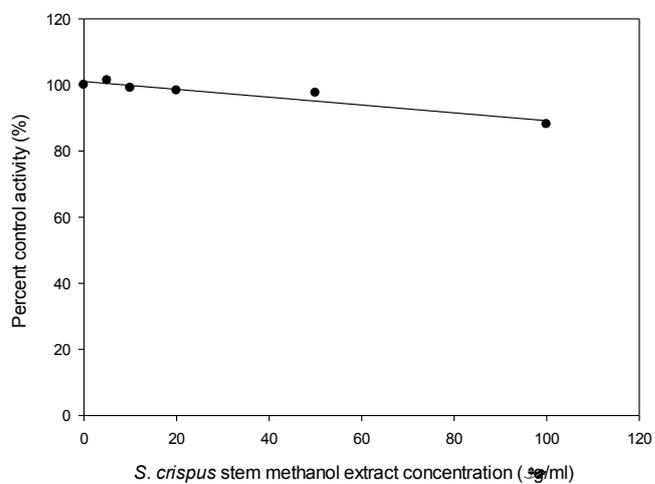


Figure 5. Effects of *S. crispus* stem extracts on CYP3A4-mediated testosterone 6β-hydroxylase activity: aqueous extract (2A), hexane (2B), chloroform (2C), ethyl acetate (2D), and methanol (2E). Data points are experimentally determined values while solid lines are best-fit curves generated by SigmaPlot for Windows Version 12.0. The x axis represents the *S. crispus* extract concentrations (µg/ml), while the y axis represents the percentages of the control activities. Data are the average values of duplicate determinations.

Table 1. Percent control activity of *S. crispus* solvent extracts at a concentration of 100 µg/ml:

CYP isoform		Solvent	Percent control activity (%)
CYP2A6	Leaf	Hexane	90.6
		Chloroform	74.5
		Ethyl acetate	81.5
		Methanol	61.2
	Stem	Hexane	74.3
		Chloroform	64.8
		Ethyl acetate	84.9
		Methanol	72.5
CYP3A4	Leaf	Hexane	93.5
		Chloroform	89.0
		Ethyl acetate	96.7
		Methanol	73.3
	Stem	Hexane	65.5
		Chloroform	55.1
		Ethyl acetate	54.2
		Methanol	88.1

DISCUSSION

K_m is thought to be relatively consistent from one study to another. The K_m value acquired for CYP2A6-mediated coumarin 7-hydroxylase assay derived from Figure 1A was $3.9 \pm 1.4 \mu\text{M}$ (mean \pm std. error), which fell within the reported range (1.7 - $10.6 \mu\text{M}$).²⁵⁻²⁸ Similarly, the K_m value determined for CYP3A4-mediated testosterone 6β -hydroxylase assay was $86.2 \pm 16.3 \mu\text{M}$ (mean \pm std. error), which was compared with previously reported values (see Figure 1B).^{22, 29-31} The major factors contributing to the slight deviation from values established in previous studies were considered to be different sources of CYP enzymes, incubation conditions, and co-factor variables. In comparison to these small deviations, V_{max} values (1261 ± 135.1 pmol/min/mg CYP2A6 protein; 4176.7 ± 200.4 pmol/min/mg CYP3A4 protein, mean \pm std. error) were shown to have a bigger difference from values established in previous other studies. This is not unusual since the V_{max} value is a function of the expression level of enzyme, which varies based on enzyme concentration and incubation conditions. Because of this, the *in vitro* CYP2A6 and CYP3A4 enzyme systems served as an activity marker for the following inhibitory screening assays.

It has been suggested that one of the anti-cancer mechanisms of *S. crispus* may be the inhibition of CYP isoforms, which are able to bio-activate procarcinogens.³² The initial intention

of this study was to evaluate the inhibitory potencies of various *S. crispus* extracts prepared from both leaf and stem on CYP2A6-coumarin 7-hydroxylation as well as CYP3A4-testosterone 6β -hydroxylation, which might account for the reported anti-cancer properties of this plant, and potentially enable it to be used in cancer prevention.

Aqueous and organic solvents ranging from polar to non-polar (methanol, chloroform, ethyl acetate and hexane) were used to do extractions, to provide a complete screening outcome covering a wide spectrum of active constituents. Leaf and stem samples were collected and processed separately since they are the most commonly consumed portion of *S. crispus*. Nevertheless, none of the extracts demonstrated potent inhibition towards CYP2A6 and CYP3A4 activities, with IC_{50} values more than $100 \mu\text{g/ml}$ (Figure 2-5). It was suggested that the anti-cancer potential of *S. crispus* was unlikely to result from modulation of CYP2A6 or CYP3A4 activity. Hence, alternative mechanisms and pathways must be assumed to play more important roles. For example, *S. crispus* extract has been shown to induce apoptosis in breast cancer cells via mitochondria the dependent p53 pathway.³³ Alternatively, the anti-cancer effect of the plant extract might be mediated by down-regulation of c-myc, an oncogene, in hepatocellular carcinoma.³⁴ In addition, *S. crispus* extract has been found to possess anti-angiogenic properties and hence inhibit cancer cell growth in that way.³⁵ It is advised that other CYP isoforms also be investigated, such as CYP2E1, which is involved in the metabolism of nitrosamine N-nitrosodiethylamine (NDEA) in rats' livers,⁸ or CYP1A2 and CYP1B1, which play roles in colon cancer carcinogenesis- *S. crispus* extract's cytotoxicity to this cancer has been demonstrated.³⁶

Independence of CYP2A6 and CYP3A4 activities from action of *S. crispus* extracts was an encouraging finding in terms of drug-herb interaction assessment. Known to metabolise the chemotherapy drug tegafur to active component 5-fluorouracil, CYP2A6 enzyme is crucial for patients with malignant cancer. This could be gastric cancer, colorectal cancer, head and neck cancer, non-small cell lung cancer, breast cancer, pancreatic cancer or biliary tract cancer.³⁷ The majority of currently prescribed anti-cancer drugs are biotransformed via CYP3A4 pathways, including docetaxel, erlotinib, imatinib, irinotecan, paclitaxel and vinorelbine.³⁸ The use of herbal products is popular among cancer patients.³⁹ Although herbal products are claimed to be natural and health-improving, concomitant use together with anti-cancer drugs potentially leads to drug-herb interaction, which has become one of the safety issues among cancer patients. In patients who wish to consume *S. crispus* herbal infusion for anti-cancer or other health-promoting purposes, it is important that it does not affect anti-cancer agents'

metabolism rate in the liver or the chemotherapy outcome. Found not to affect CYP2A6 or CYP3A4 drug-metabolising activity, *S. crispus* is believed to be safe to take together with drugs metabolized by CYP2A6 and CYP3A4. However, this finding has to be supported with more clinical data. Caution is urged for those taking alternative medicine or herbal remedies. To prevent the possibility of serious adverse reactions due to drug-herb interactions, it is suggested that other CYP isoforms be tested with *S. crispus* extracts including CYP2C9, CYP2D6, and CYP2C19. These CYP enzymes altogether contribute to the metabolism of approximately 40-50% clinically used drugs.

CONCLUSIONS

In vitro enzyme systems have been constructed successfully to be used to evaluate CYP2A6 and CYP3A4 activities. No significant inhibitory effects for aqueous or organic solvent extracts of *S. crispus* plant were discovered in the current study, suggesting that the anti-cancer properties of this plant involve mechanisms other than the inhibition of the CYP2A6 or CYP3A4 pathways. Moreover, co-administration of *S. crispus* products with drugs metabolised by CYP2A6 or CYP3A4 is unlikely to result in drug-herb interactions, which merits more clinical assessments.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no conflict of interest associated with this paper.

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