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Evaluation of Cytotoxic, Anti-angiogenic and Antioxidant Properties of Standardized Extracts of *Strobilanthes crispus* Leaves

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Abstract: *Strobilanthes crispus* (Acanthaceae) is widely used for treatment of cancer in the South Asian region. In this study, validation of GC-TOF mass spectrophotometric methods for quantitative determination of phytoconstituents in methanolic and aqueous extracts of *S. crispus* was conducted. The cytotoxicity of standardised methanolic and aqueous extracts of *S. crispus* was assessed against a panel of human cancer cell lines, namely breast carcinoma (MCF7), colon carcinoma (HCT 116), hepatocellular carcinoma (Hep G2), non-small cell lung adenocarcinoma (NCI-H23) and human breast ductal carcinoma (T-47D) cells and one normal colonic fibroblast cell line (CCD-18Co). The cell proliferation assay was performed using tetrazolium (MTT) method. Furthermore, the inhibitory effect of the extracts on angiogenesis was evaluated using *ex vivo* rat aortic ring assay. Finally, the antioxidant properties of both extracts were studied using DPPH free radical, xanthine oxidase activity and β-carotene-linoleate model system. Aqueous extract was found to be nontoxic towards all cell lines used, while the methanolic extract exhibited cytotoxic response towards the T-47D and MCF7 cells. Both the extracts demonstrated detectable anti-angiogenic activity. The extracts displayed very strong inhibitory activity towards xanthine oxidase enzyme, however, they demonstrated moderate antioxidant properties, which is evidenced by the quenching of DPPH free radical and preventing the bleaching of β-carotene by linoleic acid.

Key words: Acanthaceae, tetrazolium, anti-cancer agents, rat aortic ring assay, xanthine oxidase, GC/TOF-MS

INTRODUCTION

Human mortality is increasing drastically due to the different kinds of cancer globally. Therefore, the trend now has been shifted towards the natural medicine, logically based on traditional medicines, in search of better drugs against cancer (Galal *et al*., 1991; Hoffmann *et al*., 1993). Angiogenesis plays a vital role in the growth and metastasis of tumours and several chronic inflammatory diseases including rheumatoid arthritis and proliferative diabetic retinopathy (Folkman, 1995). Since a close relationship between tumor growth and angiogenesis had been clarified, various angiogenic inhibitors for use in cancer treatment have been studied. Therefore, the inhibition of angiogenesis emerges as a potent target for prevention and treatment of cancer (Fadzelly *et al*., 2006).

Strobilanthes crispus L. Bremek (family-Acanthaceae) is traditional medicinal plant native to tropical countries like Madagascar and Malay archipelagos. The plant is believed to have health-giving properties, so locally it is also known as pecah beling. Traditionally the medical practitioners in Malaysia and Indonesia were used to prepare the aqueous extract from leaves of *S. crispus* by boiling them and the different forms of the extract were used as diuretic, antidiabetic, antilytic, laxative and to treat inflammatory diseases and tumors (Fadzelly *et al*., 2006). Several scientific studies have confirmed that infusion of the dried leaves has the antidiabetic, diuretic, antilithic and laxative properties (Perry and Metzger, 1980). Ismail *et al*. (2000) reported that extract of *S. crispus* showed antioxidant activity. The hot aqueous-extract of fermented and unfermented leaves was found to reduce blood glucose in hyperglycemic rats, while unfermented leaves also reduced glucose level in normal rats. Both fermented and unfermented leaves also exhibited improved lipid profiles (Fadzelly *et al*., 2006). Rahmat *et al*. (2006) reported that the methanolic extract displayed modest cytotoxic effect on colon cancer (Caco-2) and human liver cancer (HepG-2).

In this study we developed the validation of GC-TOF mass spectrophotometric methods for quantitative

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determination of phytoconstituents in methanolic and aqueous extracts of *S. crispus*. In order to study the bio-pharmacological properties of methanolic and aqueous extracts of *S. crispus* leaves, we assessed the cytotoxic efficacy the standardized extracts against a panel of human cancer cell lines such as, metastatic breast carcinoma highly invasive breast carcinoma (MCF7), less invasive breast ductal carcinoma cells (T-47D), colon carcinoma (HCT 116), hepatocellular carcinoma (Hep G2) and non-small cell lung adenocarcinoma (NCI-H23) and one normal colonic fibroblast cell line (CCD-18Co). Additionally, the inhibitory effect of methanolic and aqueous extracts on angiogenesis was evaluated using *ex vivo* rat aortic ring assay. Finally, the antioxidant property of the extracts was studied using DPPH free radical, xanthine oxidase and β-carotene-linoleate models.

MATERIALS AND METHODS

Chemicals, cell culture and reagents: Dulbecco's buffered saline phosphate from Sigma-Aldrich, Germany and trypsin was purchased from Gibco, Life Technology, UK. 1, 1-diphenyl-2-pircryldrazyl (DPPH), sodium bicarbonate, Penicillin/Streptomycin (PS) solution, MTT reagent was purchased from Sigma-Aldrich, Germany. Suramin, amphotericin B, aprotinin, 6-aminocaproic acid, L-glutamine, thrombin and gentamicin were obtained from Sigma-Aldrich, Germany. Fibrinogen was obtained from Calbiochem, USA. Matrigel matrix (10 mg mL^{-1}) was obtained from SABiosciences, USA. M199 was obtained from Gibco BRL. Aprotinin, thrombin (EC 3, 4.21.5), foetal bovine serum, amphotericin B (fungizone), gentamycin, ϵ-aminocaproic acid, phosphate-buffered saline (PBS) and sodium chloride were obtained from Sigma, St Louis, MO. All cancer cell lines used in the study were purchased from American tissue culture collection (Rockvill, MD, USA).

The medium used for HCT 116, T47D and NCI-H23 cell lines was RPMI 1640 (Sigma-Aldrich, Germany) containing 10% heat-inactivated FBS and 1% penicillin/streptomycin. The cell lines CCD-18Co, MCF7 and Hep G2 were cultured in Dulbecco's Modified Eagle Media (Gibco, Life Technology, UK) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Other chemicals used in this study were analytical grade.

Extraction: *S. crispus* leaves were collected from Padang, West Sumatera Indonesia in March 2009. The plants were identified and deposited in herbarium of School of Biology, Universiti Sains Malaysia with voucher number 10931. This research project was conducted in the School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang, Malaysia.

Briefly, the fresh leaves were washed and dried in the oven at 40°C then powdered mechanically. Five gram of the powdered material was macerated with 200 mL of water and 200 mL of methanol in separate flasks at their boiling points for 6 h. After cooling, the mixtures were filtered then the filtrates were dried under vacuum and stored in airtight containers and used for phytochemical investigations.

Analysis by GC-TOF mass spectrometry: Characterisation of the unknown chemical species was performed using a Hewlett-Packard 6890 GC coupled with a LECO Pegasus II time-of-flight mass spectrometer with electron impact ionization in the reflectron mode. Data analysis of peaks observed was carried out using the Chrom TOF mass data analysis software packages. Helium was used as carrier gas at a flow rate of 1 mL min^{-1} using a DB-WAX fused silica capillary column (20 m×0.18 mm, I.D; 0.18 μm film thickness). A 1000 μg mL^{-1} methanolic and aqueous extract of *S. crispus* solution was used for analysis, which was prepared by dissolving 10 mg of the extracts in 10 mL of methanol. The GC open program was set between 50°C to 250°C (5 min hold) at temperature ramping rate of 20°C min^{-1}, using splitless injection with the injector temperature being fixed at 200°C. The ion source temperature was maintained at 180°C with the mass range of detection configured between 50-500 amu. The spectral scan rate was set at 20 spectra/sec and the volume of injected sample maintained at 1 μL. Identification of the compounds was carried out by referring to NIST (National Institute for Standard and Technology) 1998 Mass Spectral Database and the Terpene Essential Oil Library (Korytar *et al*., 2002).

Determination of antioxidant activity

Inhibition of xanthine oxidase activity: Xanthine oxidase inhibitory activity of *S. crispus* extracts were determined according to methods developed by Sweeney *et al*. (2001). The 1.3 mL of buffer phosphate (pH 7.5) and 0.2 mL of 0.2 unit mL^{-1} xanthine oxidase solutions were taken into test tubes containing 0.5 mL of 100 μg mL^{-1} aqueous and methanolic extracts. After 10 min of incubation at room temperature, 1.5 mL of 0.15 mM xanthine substrate solution was added to this mixture. The mixture was incubated for 30 min at room temperature and the reading were taken at 293 nm using UV/VIS spectrophotometer against 0.5 mL methanol, 1.3 mL phosphate buffer (pH 7.5), 0.2 mL xanthine oxidase and 1.5 mL of aqueous as blank. 0.5 mL of methanol, 1.3 mL of phosphate buffer solution (pH 7.5), 0.2 mL of xanthine oxidase and 1.5 mL of xanthine substrate solution were also used as the control. The experiment was carried out in triplicate and the final results averaged between the three data sets. Inhibition percentage was calculated using the formulae:

$$\text{Inhibition (\%)} = \frac{\text{Ac-As}}{\text{Ac} \times 100}$$

where, As is absorbance of sample and Ac is absorbance of control.

DPPH free radical scavenging activity: DPPH free radical scavenging activity of extracts was determined according to Akowuah *et al.* (2005) and Abdille *et al.* (2005) with some modifications. Solutions of 100, 200, 400, 600 and 800 μL of 1000 μg mL^{-1} extracts were placed in separate tubes and the amount was made up to 1 mL with methanol. To this mixture, 2 mL of 0.1 mM DPPH (1,1-Diphenyl-2-picrylhydrazyl) was added. After 60 min of incubation at room temperature, absorbance was measured at 517 nm using spectrometer UV/VIS Lambda 45, against methanol as the blank. Volume of 1, 2, 4, 10, 20, 40, 60 and 80 μL of 500 μg mL^{-1} gallic acid, ascorbic acid, quercetin and Butylated Hydroxyl Anisole (BHA) were used as references and 5 mL of 0.1 mM DPPH was used as the reagent. Two control solutions containing 1 mL of methanol and 2 mL of 0.1 mM DPPH for *S. crispus* were used as reference compounds. The experiments were carried out in triplicate. Free radical scavenging activity of the extracts and effective concentration 50% (EC_{50}) were determined according to the following formulae:

$$\text{Free radical scavenging activity (\%)} = \frac{\text{Ac-As}}{\text{Ac}} \times 100$$

where, As is absorbance of sample and Ac is absorbance of control.

Antioxidant assay using β-carotene-linoleate model system: Antioxidant assay using β-carotene-linoleate model system of *S. crispus* extracts was analyzed according to Abdille *et al.* (2005) with modifications. 0.2 mg of β-carotene was added to 0.2 mL chloroform, 20 mg of linoleic acid and 200 mg of Tween-40 (polyoxyethylene sorbitan monopalmitate). Chloroform was removed under vacuum at 40°C and the resulting mixture was diluted vigorously with 10 mL aqueous. To this emulsion, 40 mL of oxygenated aqueous was added. 4 mL aliquots of the emulsion were pipetted into separate test tubes containing 0.2 mL of 200 ppm extracts, quercetin, BHA and BHT in ethanol. Quercetin, BHA and BHT were used for comparative purposes. A control containing 0.2 mL of ethanol and 4 mL of the above emulsion was prepared. The test tubes were placed at 50°C in an aqueous bath and the absorbance was measured at 470 nm (absorbance at zero time, t = 0) using UV/VIS Lambda 45 spectrophotometer. The next absorbance was measured after 120 min of incubation. A blank with a similar mixture was prepared without the presence of β-carotene. All analyses were carried out in triplicate. The Antioxidant Activity (AA) was evaluated using the following formula:

$$AA = \frac{1-(A_0-A_t)}{A_0^{\circ}-A_t^{\circ}} \times 100$$

where A_0 and A_0° are the absorbance value measured at zero time of the incubation for test sample and control, respectively. A_t and A_t° are the absorbance values were measured after incubation for test sample and control, respectively. The results were expressed in percentage (%) of bleaching inhibition of β-carotene.

Evaluation of cytotoxicity activity: The MTT viability assay was performed with slight modifications as previously described by Mosmann (1983). In brief, cells were seeded at 5000 cell density per well for each 96-well plates in 100 μL medium. After an overnight incubation, 100 μL of culture medium containing test substance were added into each well to make the final concentration of 150, 100, 80, 60, 40, 20, 10 and 5 μg mL^{-1} with 0.01% DMSO in each well. For positive control, the cells were treated with 600 ng mL^{-1} of vincristine. The untreated cells that received only the medium in 0.01% DMSO were used as negative control. All the cells were treated for 72 h. Each experiment was done thrice with four replicates for each concentration. MTT was first prepared as a stock solution of 5 mg mL^{-1} in phosphate buffer saline and was filtered using 0.22 μm syringe filter. At the end of the treatment period (72 h), 20 μL of MTT solution was added to each well. After 4 h incubation at 37°C, the medium was removed and 200 μL of DMSO was added to the well to dissolve the formazon crystal. After 5 min of shaking, the optical density was recorded using Multiskan Ascent plate reader (Thermo Scientific, Waltham) at 570 nm for absorbance and 650 nm as reference filter.

Angiogenesis study: Rat aortic ring explant cultures were prepared by modification of protocols previously described (Brown *et al.*, 1996; Zhu *et al.*, 2000). Aortic rings were prepared from male Sprague Dawley rats. Aortas were sectioned into 1 mm-long cross sections, rinsed several times with Hanks Balanced Salt Solution containing 2.5 μg mL^{-1} amphotericin B (Sigma, St. Louis. MO). The assay was performed in a 48-well tissue culture plate (Coster Corning, USA). 500 μL of 3 mg mL^{-1} fibrinogen (Calbiochem, USA) in serum free M199 growth medium (Gibco, UK) was added to each well with 5 mg mL^{-1} of aprotinin (Sigma-Aldrich, Germany) to prevent fibrinolysis of the vessel fragments. Each tissue section was placed in the center of the well and 15 μL of thrombin (50 NIH U mL^{-1}) (Sigma-Aldrich, Germany) in 0.15 M NaCl. Immediately after embedding

the vessel fragment in the fibrin gels, 0.5 mL of medium M199 supplemented with 20% HIFCS (Gibco, UK), 0.1% ε-aminocaproic acid (Sigma-Aldrich, Germany), 1% L-Glutamine (Sigma-Aldrich, Germany), 1% amphotericin (Sigma-Aldrich, Germany), 0.6% gentamicin (Sigma-Aldrich, Germany) were added to each well. Methanolic and aqueous extracts at varying concentrations ranged from 6.25 to 100 μg mL^{-1} was added to the complete growth medium. Control cultures received medium without the test substances. Suramin (Sigma-Aldrich, Germany), a well recognized anti-angiogenic agent was used as a positive control (Sahib *et al*., 2009). Cultures were incubated at 37°C for 5 days, in humidified CO_2 and the medium was replaced daily. The magnitude of blood vessel outgrowth on day five of the procedure was quantified as per the technique developed by Nicosia *et al*. (1997) using inverted microscope (Olympus, Japan) supplied with a digital camera (Lieca CCD, Japan) and Leica QWin computerized imaging software.

The experimental work was consistent with guidelines of the USM Committee for Animal Care and received approval from the USM Animal Ethical Committee for the present work (Reference Number USM/PPSF/50 (084) Jld.2).

Statistical analysis: The results of this experiment are presented as Mean±SD of triplicate experiments analyzed using SPSS 12 (SPSS Inc. Chicago, IL). Differences between mean is evaluated by one-way ANOVA and Tukey's-b multiple comparisons at $p<0.05$.

RESULTS

GC - TOF mass spectrometry analysis: Figure 1a shows the results of the GC-TOF MS chromatogram of the

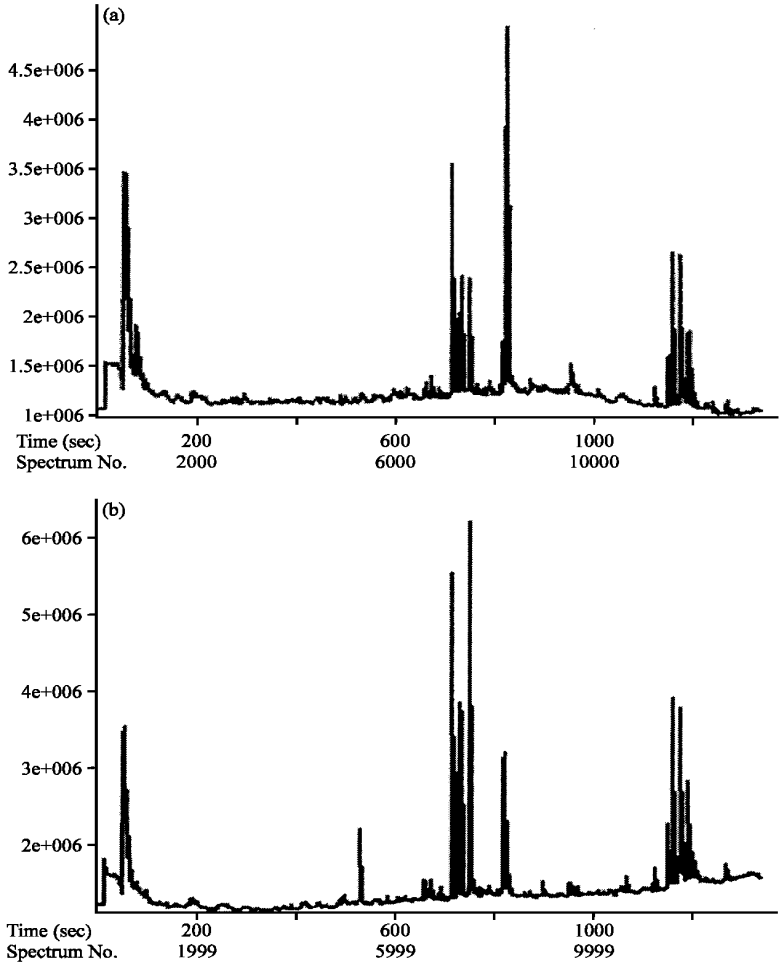

Fig. 1: (a) GC/TOF-MS chromatograms of the methanol extracts of *S. crispus* leaves and (b) GC/TOF-MS chromatograms of aqueous Ew extracts of *S. crispus* leaves

methanolic extract of *S. crispus* and Table 1 reveals the molecular components identified above 1% detection level. The results show that the sample contains 1-heptatriacotanol (1.03%), 3,5-dithiahexanol 5,5-dioxide (1.04%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (3.48%), 3-octadedecyne (9.25%), 7-hexadecenoic acid, methylester (2.70%), 9,12,15-octadecatrienoic acid, methylester, (Z, Z, Z)- (1.21%), 9,12-octadecadienoic acid, methylester (2.24%), α-sitosterol (7.08%), aromadendrene oxide-(2) (1.39%), campesterol (2.63%), hexadecanoic acid, methylester (12.11%), hydrazine carboxamide (2.03%), lupeol (3.60%), monoethanolamine (2.52%), n-propyl acetate (2.20%), octadecanoic acid, methylester (1.14%), phenol,2,4-bis(1,1-dimethylethyl)- (2.60%), phytol (3.78%) and stigmasterol (7.86%).

Figure 1b shows the results of the GC-TOF MS chromatogram of the aqueous extract of *S. crispus* and Table 1 reveals the molecular components identified. The following chemical constituents were identified; 1,1-dimethylamino-1-butene (1.13%), 2,5-dimethoxy-4-(methylsulfonyl)amphetamine (2.55%), 3,5-dithiahexanol 5,5-dioxide (3.09%), benzenemethanol, alpha-(1-aminoethyl)- (1.37%), cyclobutanol (13.56%), dimethyl sulfoxide (1.57%), hexadecanoic acid, methylester (1.84%), hydrazine carboxamide (3.32%), monoethanolamine (5.53%), n-propyl acetate (5.38%) and undecane (2.10%).

Determination of antioxidant activity

Inhibition of xanthine oxidase assay: Both aqueous and methanolic extracts of *S. crispus* displayed a strong inhibitory effect on xanthine oxidase activity as presented in Table 2, at 100 μg mL^{-1} concentration, methanolic extract showed 90.28% and the aqueous extract exhibited about 89.06% xanthine oxidase inhibition.

Table 1: The quantitative estimation of phytochemicals identified in the extracts of *S. crispus* leaves detected using GC-TOF MS

		Extracts			
		Methanolic		Aqueous	
Phytochemicals	Molecular Formula	t_R (sec)	A (%)	t_R (sec)	A (%)
1,1-Dimethylamino-1-butene	$C_6H_{13}N$	NA	NA	81.73	1.13
1-Heptatriacotanol	$C_{37}H_{76}O$	1204.28	1.03	NA	NA
2(1H)Naphthalenone,3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-phenol	$C_{15}H_{22}O$	1179.98	0.81	NA	NA
2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl,(R)-	$C_{11}H_{16}O_2$	535.18	0.70	535.03	0.87
2,4-Bis(dimethylbenzyl)-6-t-butylphenol	$C_{28}H_{34}O$	960.88	0.26	961.03	0.29
2,5-Dimethoxy-4-(methylsulfonyl)amphetamine	$C_{12}H_{19}NO_4S$	NA	NA	56.53	2.55
26-Nor-5-cholesten-3-á-ol-25-one	$C_{26}H_{42}O_2$	1122.68	0.76	NA	NA
2-Pentadecyn-1-ol	$C_{15}H_{28}O$	834.48	0.10	NA	NA
3,5-Dithiahexanol 5,5-dioxide	$C_4H_{10}O_3S_2$	61.08	1.04	61.93	3.09
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	725.78	3.48	NA	NA
3-Octadedecyne	$C_{18}H_{34}$	715.38	9.25	NA	NA
7-Hexadecenoic acid, methylester	$C_{17}H_{32}O_2$	819.68	2.70	NA	NA
9,12,15-Octadecatrienoic acid, methylester, (Z,Z,Z)-	$C_{19}H_{32}O_2$	819.28	1.21	NA	NA
9,12-Octadecadienoic acid, methylester	$C_{19}H_{34}O_2$	817.08	2.24	816.93	0.31
Acetic acid, anhydride with formic acid	$C_3H_4O_3$	58.08	0.74	NA	NA
Alpha-Sitosterol	$C_{29}H_{50}O$	1173.78	7.08	1172.33	0.26
Ammonium acetate	$C_2H_7NO_2$	NA	NA	58.83	1.32
Aromadendrene oxide-(2)	$C_{15}H_{24}O$	1185.78	1.39	NA	NA
Benzenemethanol, alpha-(1-aminoethyl)-phenol	$C_9H_{13}NO$	62.68	0.23	65.13	1.37
Butyrolactone	$C_4H_6O_2$	NA	NA	101.63	0.49
Campesterol	$C_{28}H_{48}O$	1150.68	2.63	NA	NA
Cyclobutanol	C_4H_8O	NA	NA	62.63	13.56
Cyclopentaneundecanoic acid, methylester	$C_{17}H_{32}O_2$	NA	NA	963.23	0.10
Dimethyl sulfoxide	C_2H_6OS	83.38	0.95	82.83	1.57
Hexadecanoic acid, methylester	$C_{17}H_{34}O_2$	752.48	12.11	751.93	1.84
Hydrazine carboxamide	CH_5N_3O	57.48	2.03	58.43	3.32
L-Alanine, ethylester	$C_5H_{11}NO_2$	80.88	0.29	NA	NA
Lupeol	$C_{30}H_{50}O$	1191.38	3.60	NA	NA
Methyl tetradecanoate	$C_{15}H_{30}O_2$	661.18	0.6	661.33	0.19
Monoethanolamine	C_2H_7NO	56.98	2.52	57.83	5.53
Nitrous oxide	N_2O	NA	NA	55.93	21.44
n-Propyl acetate	$C_5H_{10}O_2$	65.98	2.20	66.83	5.38
Octadecanoic acid, methylester	$C_{19}H_{38}O_2$	829.58	1.14	NA	NA
2,4-bis(1,1-dimethylethyl)- Phenol	$C_{14}H_{22}O$	532.08	2.60	NA	NA
Phytol	$C_{20}H_{40}O$	824.38	3.78	NA	NA
Stigmasterol	$C_{29}H_{48}O$	1159.68	7.89	NA	NA
Tetratetracontane	$C_{44}H_{90}$	1121.18	0.53	NA	NA
Tungsten,pentacarbonyl (4,5-diethyl-2,2,3-trimethyl-1-phenyl-1-phospha-2-sila-5-boracyclohex-3-ene-P)- (oc-6-22)-phenol	$C_{21}H_{26}BO_5PSi$	NA	NA	20.13	2.57
Undecane	$C_{11}H_{24}$	194.08	0.70	194.53	2.10
Vitamin E	$C_{29}H_{50}O_2$	1128.88	0.11	NA	NA

NA: Not available

DPPH free radical scavenging activity: Results show that both aqueous and methanolic extracts were able to quench the stable free radical DPPH to yellow-coloured 1,1-diphenyl-2-picrylhydrazyl by its hydrogen donating ability. The scavenging activity of aqueous extract was more than the methanolic extract of *S. crispus* as shown in Table 3. The reference standards, gallic acid, quercetin, BHA and ascorbic acid displayed potent DPPH free radical scavenging activity, their EC_{50} values are tabulated in Table 4.

β-carotene-linoleate model system: The result of antioxidant activity using β-carotene-linoleate model system of methanolic and aqueous extracts of *S. crispus* is depicted in Table 5. The extracts showed weak response in the β-carotene-linoleic acid assay. Whereas, the standard references, BHA and BHT, demonstrated potent activities in this assay system with bleaching inhibition percentage about 95.84 and 95.12, respectively (Table 5).

Table 2: Xanthine oxidase inhibition assay

Groups (n)	Inhibition (%)
Methanolic extract	90.28±0.20*
Aqueous extract	89.06±0.28*

Data is represented in Mean±SD (n =6), *p<0.05.when compare to control

Table 3: DPPH free radical scavenging activity of extracts of *S. crispus* leaves

	Free radical scavenging activity of extracts (%)	
Concentration (μg mL^{-1})	Aqueous	Methanolic
100	1.88±0.67	1.67±0.11
200	3.93±0.78	3.40±0.52
400	8.34±0.96	6.31±0.51
600	13.44±0.91	9.58±0.43
800	17.46±0.26	12.38±0.35

Data is represented in Mean±SD (n = 6)

Table 4: EC_{50} values of reference standards against DPPH free radical scavenging activity

Compound	EC_{50} (μg mL^{-1})
Gallic acid	12.6
Ascorbic acid	25.5
Butylated hydroxyl anisole (BHA)	21.9
Quercetin	15.3

Table 5: Antioxidant activity of using β-carotene linoleate model system

Compounds (n)	Antioxidant activity (%)
Methanolic extract of *S. crispus*	1.510±0.60
Aqueous extract of *S. crispus*	0.910±0.44
Butylated hydroxyl anisole (BHA)	95.84±1.11*
Butylated hydroxyl toluene (BHT)	95.12±1.86

*Data is represented in Mean±S.D (n = 6), *p<0.05. when compare to control

Evaluation of cytotoxicity activity: MTT cell proliferation assay was conducted. Five human cancer cell lines (MCF7, T-47D, HCT 116, Hep G2 and NCI-H23) and one normal colonic fibroblast cell line (CCD-18Co) were exposed to the different concentrations of extracts. However, result showed that, only at their high concentrations the extracts demonstrated cytotoxicity against all cell lines except for the T-47D and MCF7 breast cancer cells. Table 6 depicts the IC_{50} values of the extracts for various cell lines.

Effect of extracts on vessel sprout formation from rat aorta: The effect of methanolic and aqueous extracts on angiogenesis was studied using an *ex vivo* rat aortic ring assay. In this assay, the rat aortic endothelium exposed to a three-dimensional matrix containing angiogenic factors switches to a microvascular phenotype generating branching networks of microvessels (Nicosia *et al*., 1992). As shown in Fig. 2, microvessels grew out from the rat

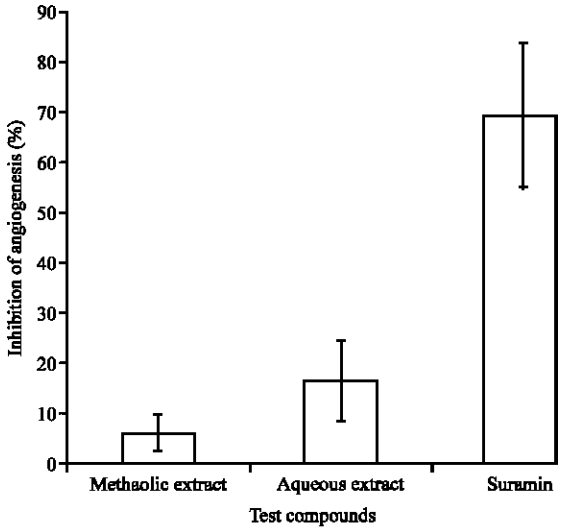

Fig. 2: Graph showing the extent of angiogenesis inhibition by methanolic and aqueous extracts of *S. crispus* with compare to suramin as the positive control

Table 6: IC_{50} value for cytotoxicity on panel of cell lines for *S. crispus* aqueous and methanol extracts

	Cell lines (IC_{50} values are presented in μg mL^{-1})					
Groups (n)	Hep G2	HCT 116	T-47D	NCI-H23	CCD-18Co	MCF7
Methanolic extract	>200	>200	121.53*	>200	>200	160.16*
Aqueous extract	>200	>200	>200	>200	>200	120.7*

Methanol extracts. Data is represented in Mean±SD (n = 6), *p<0.05.when compare to control

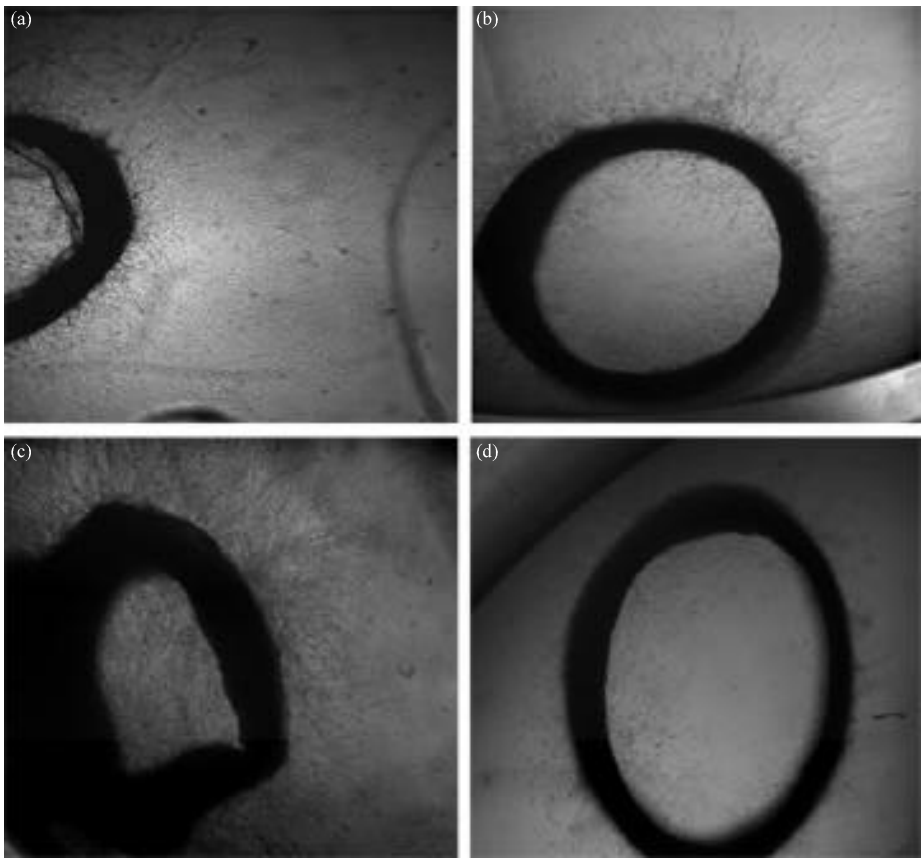

Fig. 3: Effect of *S. crispus* extracts on rat aortic micros vessel growth. (a) Control, (b) aortic ring treated with methanolic extract, (c) aortic ring treated with aqueous extract and (d) aortic ring treated with suramin

aorta in the control (Fig. 3a) when cultured in the medium. The anti-angiogenic activity was quantified by measuring the cell-sprouting area around the aortas exposed to the various concentrations of methanolic extract and the control. The aqueous extract (Fig. 3b) at 100 μg mL^{-1} showed moderate activity (16.67±8.11 %) and methanolic extract (Fig. 3c) exhibited least activity (6.25±3.6 %) at 100 μg mL^{-1} concentration, whereas, the anti-angiogenesis effect of suramin (Fig. 3d) on the sprouting of microvessels from rat aorta was significantly dose dependent ($p<0.05$) and it showed 69.44±14.24% inhibition at 100 μg mL^{-1} concentration.

DISCUSSION

Highly reactive free radicals induce oxidative damage in lipids, proteins and other biomolecules. They are the main responsible elements for numerous degenerative diseases including cancer (Aruoma, 1994; Ray and Husain, 2002). Free radical scavengers function as inhibitors at both initiation and promotion/propagation stages of degenerative diseases and consequently, antioxidants play an important role in the protection of human body against oxidative damage (Govindarajan *et al*., 2005). Antioxidants serve as potent inhibitors of angiogenesis and thus curb the neoplastic processes (Bagchi *et al*., 1997). The consumption of edible plants, fruits and herbs has been proven to play the prophylactic role in a number of diseases in humans and animals. Vegetables, fruits and herbs are rich sources of antioxidants, protease inhibitors and also compounds that might protect the organism against free radical induced injury and diseases (Tapiero *et al*., 2002; Harborne and Williams, 2000).

In the present study, validation of GC-TOF mass spectrophotometric methods for quantitative determination of phytoconstituents in methanolic and aqueous extracts of *S. crispus* was carried out. The result of the mass spectrometric analysis reveals the difference in composition of two different organic solvent extracts of *S. crispus*. The phytol is a decomposition product of chlorophyll (Windholz *et al*., 1976), which is found in the methanolic extracts (3.78 %) however it failed to appear in the aqueous extract. Stigmasterol and α-sitosterol are

found in significant quantities in the methanol extract (stigmasterol, 7.89 %; α-sitosterol, 7.08 %), but none was detected in the aqueous extract. Steroids and lipids are non-polar compounds; hence their solubility is higher in methanol than aqueous. Stigmasterol and sitosterol are widely distributed in the plant kingdom synthesised via the mevalonic acid pathway (Torssell, 1993). Researchers have indicated that stigmasterol and sitosterol may be useful in prevention of certain cancers, including ovarian, prostate, breast and colon cancers and may help to reduce cholesterol level. Rahmat *et al.* (2006) reported that the methanolic extract of *S. crispus* containing these compounds displayed strong cytotoxic activity on colon cancer (Caco-2). Results of the present study appear to be synchronized even with the different human colon carcinoma cell types (HCT 116 and CCD-18Co) used in the study. In addition the methanolic extract found to be significant cytotoxic against human non-small cell lung adenocarcinoma cell (NCI-H23) and human breast ductal carcinoma cell (T-47D). The process of carcinogenesis is a multistep event and oxidative damage can lead to the development of tumours through several distinct pathways. Polyphenols such as quercetin exhibits significant antioxidant, anti-inflammatory and antiproliferative activity. Polyphenols present in plants and because their anti-oxidation ability they have been shown to regulate cell proliferation and induce apoptosis (Chu *et al*., 2002; Sun *et al*., 2002). In the present study, for the first time the anti-angiogenic effect of *S. crispus* extracts is being reported. The rat aortic ring assay showed detectable levels of antiangiogenic properties of methanolic and aqueous extract of *S. crispus* when compared to the control. The antioxidants are well known to have potent anti-angiogenic activity (Losso, 2003). The extracts of *S. crispus* are rich in antioxidant phytosterols, such as α-sitosterol, campesterol, phytol and stigmasterol. Thus the strong antioxidative properties of methanolic and aqueous extract of *S. crispus* are defined by the xanthine oxidase inhibition assay, β-carotene-linoleic acid assay and the DPPH scavenging activity.

CONCLUSIONS

The stark contrast to the findings made by other researchers highlights the importance of standardisation of plant extract to ensure reliability and repeatability of research outcome. The methanolic extract exhibited a moderate level of cytotoxic activity in human adenocarcinoma cell lines. The aqueous extract showed considerable antiangiogenic activity compared control. The cytotoxic and antiangiogenic properties of *S. crispus* may probably due to the presence of potential antioxidants. These findings suggest that *S. crispus* could be a source of promising cytotoxic agents and angiogenesis inhibitors.

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