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Mal J Nutr 14(2): 173 - 187, 2008

Biological Properties of *Tinospora crispa* (*Akar Patawali*) and Its Antiproliferative Activities on Selected Human Cancer Cell Lines

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ABSTRACT

The antioxidant and anti-proliferative activity of the aqueous crude extract of *Tinospora crispa* stem was investigated. The proximate composition of its stem and leaves was determined. Proximate analysis revealed that *T. crispa* contains - protein: leaves = 4.7%, stem = 1.2%; fat: leaves = 1.5%, stem = 0.43%; carbohydrate: leaves = 11.8%, stem = 19.4%; ash: leaves = 2.7%, stem = 1.1%; moisture: leaves = 79.3%, stem = 77.9%; fibre: leaves = 1.59%, stem = 0.65%; and energy: leaves = 1.59%, stem = 0.65%. The antioxidant activity of the extract prepared at various temperatures and incubation time was evaluated to determine the optimum extraction procedure. Based on DPPH and TBA tests, the preparation of the extract at 60°C for 6 hours was established as the best possible method as it demonstrated the highest inhibition percentage. The extract was tested against brine shrimp to evaluate its toxicity and no significant toxicity was recorded since the IC_{50} value was more than 1000 µg/ml. The extract produced moderate anti-proliferative activity on selected human cancer cell lines (IC_{50} MCF-7: 107 µg/ml, HeLa: 165 µg/ml, Caov-3: 100 µg/ml, and HepG2: 165 µg/ml). The findings from this study suggest that *T. crispa* has the potential to be a source of natural antioxidants and nutrients, besides having a moderate anti-proliferative effect on selected human cancer cell lines.

INTRODUCTION

Increasing evidence suggests that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are implicated in several degenerative diseases like cancer, asthma, arthritis, and cardiovascular problems (Halliwell, 1994). Production of reactive oxidants such as superoxide, hydroxyl radicals and hydrogen peroxide in living cells is an inevitable process of normal oxygen metabolism (Packer, 1995). Peroxidative agents like hydrogen peroxides, free metal cations like iron and copper, and ultraviolet and ionising radiations generate free radicals that have a deleterious effect to the body (De Groot, 1994). The most prominent ill effects are the oxidation of

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phospholipids in the lipid bilayer of cell membrane and side chain modification of proteins rendering the protein dysfunctional and oxidative damage of DNA leading to the dreaded conditions of carcinogenesis as a direct effect of induced mutations (Kinsella *et al.,* 1993). Despite naturally occurring bodily antioxidant systems that are able to control the free radical mediated oxidative damage, under conditions of severe oxidative stress however, cellular defenses do not provide complete protection from the attack of reactive oxidants (Ames, Shigenaga & Hagen, 1993). This phenomenon leads to the onset of oxidative damage related-diseases. On the other hand, the main disadvantage of synthetic antioxidants (butylated hydroxyl anisole and butylated hydroxyl toluene) is their toxicity at fairly high doses, which limit their therapeutic usage (McCormick *et al.,* 1986). Consequently, antioxidants from dietary sources have been recognised as being safer and more effective in the context of their efficiency and non-toxicity (Tsai, Tsai & Ho, 2005).

The intake of herbs and vegetables has been associated with a healthy balance of free radicals/antioxidants status that helps to minimise the oxidative stress in the body and to reduce the risks of cancers and cardiovascular diseases (Kikuzaki *et al.,* 2002). This has been attributed to the presence of various forms of phytochemicals and antioxidants e.g. carotenoids and polyphenol compounds including flavonoids and anthocyanins (Cotelle, 2001).

Tinospora crispa, known by various vernacular names such as 'akar patawali' or 'akar seruntum' is an indigenous plant which grows wild in Malaysia (Noor & Ashcroft, 1989). Traditional folklore attributes the use of its stem to various therapeutic purposes such as treatment for diabetes, hypertension, stimulation of appetite and protection from mosquito bites. Among the Malays, an infusion of the stems is consumed as a vermifuge and a decoction of the whole plant is used as a general tonic. It is also used as an anti-parasitic agent in both humans and domestic animals (Noor *et al.,* 1989; Kongsaktrakoon *et al.,* 1994; Pathak, Zain & Sharma, 1995). Despite its long usage as testified in traditional folklore, the biological properties of *T. crispa* and the scientific evidence of its effects in free-radical mediated diseases such as carcinogenesis is scant. Hence, in the present study, the biological properties of *T. crispa* and the preventive potential of its stem in experimental carcinogenesis in selected human cancer cell-lines were investigated.

MATERIALS AND METHODS

Proximate analysis of stem and leaves

Moisture, ash, crude fat, crude fibre, protein, carbohydrate and moisture content of both leaves and stem sample were determined according to standard methods described by the Association of Official Analytical Chemists (AOAC, 1996).

Moisture content

Percentage dry matter of *T. crispa* extracts was measured using moisture balance.

Ash content

Two grams of *T. crispa* extracts were added to a pre-weighed crucible and weighed, placed in a furnace at 550°C for 4 h, cooled in a desiccator and reweighed. The ash content was determined using Equation 1 (see below).

Fat content

Crude fat content was determined using the Soxhlet method. One hundred and fifty millilitres of petroleum ether was poured over 5 g of *T. crispa* extracts in an extraction

Equation 1: (#dvk#@#+zhljkw#ri#dvk2zhljkw#ri#vdpsoh,#{#433

thimble. The thimble was placed in a pre-weighed beaker covered with anti-bumping cotton and placed in a Soxhlet for 8 h, after which the beaker was dried in an oven, cooled and reweighed. The fat content of each sample was calculated using Equation 2 (see below).

Protein content

Crude protein content was determined using the Kjeldahl method. Ten grams of *T. crispa* extract sample was digested in 15 ml of sulphuric acid in the presence of 2 kjeltec Ck catalyst tablets by placing in a turbosog fume scrubber for 1 h. Digestion was complete on production of a clear, coloured solution. After digestion, samples were analysed for nitrogen content by placing digested material into a Vapodest 33 distilling unit. The digested sample was then titrated against standard (0.1 M) hydrochloric acid until a change of colour occurred. Nitrogen content was calculated using Equation 3 (see below).

The crude protein content was then calculated using Equation 4 (see below).

Crude fibre

Crude fibre was determined using fat-free samples. Five grams of *T. crispa* extract was placed in a fibre bag, boiled with 360 ml of 0.128 M sulphuric acid for 3 min and then later with 360 ml of 0.313 M hydrochloric acid for a further 30 min. Fibre bags were washed once with hot distilled water and then once with 0.1 M hydrochloric acid and twice more with hot distilled water. They were then patted dry and dried in an oven at 100°C for 4 h, desiccated, cooled and weighed. Later they were ashed in a furnace at 550°C for 6 h, desiccated, cooled and reweighed. Crude fibre content was determined using Equation 5 (see below).

Carbohydrate

Carbohydrate content was determined using Equation 6 (see below).

Preparation of aqueous crude extract

Fresh stems of *T. crispa* were collected from Universiti Putra Malaysia (UPM) after being identified and confirmed by a plant taxonomist. A voucher specimen was deposited in the Institute of Bioscience, UPM (SK015). The stems were cut into small pieces, dried and pulverised. Ten percent of *T. crispa* aqueous crude extract of the stem was prepared by soaking 100 g of the powdered stem in 900 ml distilled water and incubated in a shaking water bath at various temperatures and time settings: 20°C for 24 h, 40°C/12 h, 60°C/6 h, 80°C/3 h and 100°C/15 min. Once filtered, the filtrates were freeze dried and kept at -20°C until used. The variation in temperature and incubation time was proposed with the goal of obtaining the optimum yield of potential biological compounds from the extract since no data on the optimisation of the extraction procedure of this plant has been reported.

Equation 2: #(#fuxgh#idw#@#+zhljkw#ri#gulhg#ehdnhu#.#idw,#D#+#zhljkw#ri#gulhg#ehdnhu#.#judpv#/#{#433
#Zhljkw#ri#vdpsoh

Equation 3: ##Q#+(,##@#47134#{#+po#wlwudwg#iru#vdpsoh#0#po#wlwudwg#iru#eodqn,#{#prdvwxuh#ri#dflg#/#{#433
#Zhljkw#ri#vdpsoh

Equation 4: ##Surwhlq#+(,#@#Q##{##9=58#+surwhlq#idfwru#vshflilf#wr#vdpsoh,

Equation 5: (#fuxgh#ileuh#@#+ehdnhu#.#uhvlgxh#zhljkw#D#ileuh#edj#zhljkw,#D#+ehdnhu#.#dvk#zhljkw,#/#{#433
Vdpsoh#zhljkw

Equation 6: ##(#ri#Fduerk|gudwh#@#433#D#+(#prlvwxuh#.#(#idw#.#(#surwhlq#.#(#ileuh#.#(#dvk,

Antioxidant activity of *T. crispa* extract in *vitro*

The optimisation of *T. crispa* extraction procedure was verified via its antioxidant acitivity. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and the Thiobarbituric Acid (TBA) Test were used, in which Vitamin C and BHT acted as the standard.

1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

The scavenging activity of DPPH free radicals of *T. crispa* extract was determined according to the method reported by Gyamfi (1999) with minor modification. Fifty microlitres of the *T. crispa* extract in methanol, yielding 100 µg/ml in each reaction, was mixed with 1 ml of 0.1 mM DPPH in methanol solution and 450 µl of 50 mM Tris-HCl buffer (pH 7.4). Methanol (50 µl) only was used as the experimental control. After 30 min of incubation at room temperature, the reduction in the number of DPPH free radicals was measured by reading the absorbance at 517 nm. BHT and Vitamin C were used as controls. The percentage of inhibition of the sample against DPPH radicals was calculated using Equation 7 (see below).

Thiobarbituric acid (TBA) test

The TBA test of *T. crispa* extract was determined by using the method of Ottolenghi (1959) with slight modification. Two milliliters of 20% trichloroacetic acid and 2 ml of 0.67% 2-thiobarbituric acid were added to 1 ml of sample solution. The mixture was placed in a boiling water bath and after cooling was centrifuged at 3000 rpm for 20 min. Absorbance of supernatant was measured at 552 nm. The percentage of inhibition was calculated using Equation 8 (see below).

Brine shrimp lethality test

In vitro toxicity of *T. crispa* stem extract was assessed using the brine shrimp lethality test (BSLT) as suggested by Meyer *et al*. (1982) with a minor modification. The BSLT analysis was conducted into two phases involving a low concentration of the extract (phase 1) and an extremly high concentration of the extract (phase 2). Briefly, 10 brine shrimps were placed into a well of a 24-well plate containing 800ìl of salt water. 200ìl of *T. crispa* stock solution (concentrations of 100 µg/ml, 200 µg/ml, 500 µg /ml, 2.5 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/ml) were added into each well making up the final volume of 1 ml in each well. After a 24-h incubation period, the mortality of the animals was observed using a stereo microscope and the number of brine shrimps which survived was counted as percentage of the total animals. All experimental assays were prepared in triplicates.

Antiproliferative study

Treatment of cells

This study was carried out to determine the anti-proliferative potential of *T. crispa* extract on selected human cancer cell lines namely liver (HepG2), cervix (HeLa), breast (MCF-7), and ovarian (Caov-3). Normal cell line, human umbilical vein endothelial cell (HUVEC) was used as comparison. All cells were purchased from American Type Culture Collection (ATCC). All cells were plated in 96-well microtitre plates.

Equation 7: (#Lqklelwlrq#@# +Devruedqfh#ri#frqwuro#D#Devruedqfh#ri#whvw#vdpsoh,# {#433
Devruedqfh#ri#frqwuro

Equation 8: (#Lqklelwlrq#@# +Devruedqfh#ri#frqwuro#D#Devruedqfh#ri#whvw#vdpsoh,# {#433
Devruedqfh#ri#frqwuro

Table 1. Proximate analysis (protein, fat, carbohydrate, ash, moisture and total dietary fibre) of *T. crispa* leaves and stem

	Shufhqwdjh#+(,#e|#zhljkw	
	Ohdyhv	*Vwhp*
Surwhlq	71: ± 313<9	415 ± 31379
Idw	418 ± 31399	3176 ± 31399
Fduerk|gudwh	441; ± 31375	4<17 ± 31355
Dvk	51: ± 31364	414 ± 313<4
Prlvwxuh	:<16 ± 3137;	::1< ± 313:;
Wrwdo#glhwdu|#ilehuh	418< ± 3138:	3198 ± 3139:
N2fdo2433#hqhuj|	418< ± 3138<	3198 ± 3134<

Extract solution with the concentration of 200 μg/ml was prepared by dissolving 1.0 mg of *T. crispa* extract into 5 ml of deionized water. The stock was filtered using 0.2 μm filters. Next, the stock was serially diluted with RPMI 1640 media into the desired concentration and M200 media was used for HUVEC. The cells were treated with serial concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 and 200 μg/ml respectively. The treatment was done in triplicate. The control was prepared by adding 200 μl of medium alone into the control wells. The 96-well microtitre plate was then incubated in a CO_2 incubator for 72 h. Cisplatin and tamoxifen were used as the control drugs.

MTT assays

MTT assay was used to determine the cell viability as suggested by Mossmann (1983). Each concentration of *T. crispa* ranging from 10 to 200 ìg/ml was added into the 96 well plates containing cancerous cells. The treatment periods were set at 24, 48 and 72 h respectively. Twenty (20) microlitres of MTT solution was added to every well. Then the plate was incubated in a CO_2 incubator at 37°C for 4 h. Following incubation, the medium was discarded and 100 µL of dimethyl sulphoxide (DMSO) was added to each well to dissolve crystals. The plate was transferred to a plate reader and absorbance was read at 570 nm wave length (see Equation 9 below).

Statistical analysis

Data were expressed as mean ± SEM of triplicate samples. Statistical analysis was performed using One-way ANOVA whereas Tukey post hoc LSD was governed for multiple group comparison. In all cases, $p<0.05$ was considered significant.

RESULTS

Proximate analysis of stem and leaves

The results of proximate analysis which are presented in Table 1 show that *T. crispa* has high water content; 79.3% in leaves and 77.9% in stem. The leaves sample contains more protein compared to the stem. Fat, ash, total dietary fibre calories are found in very low quantities in both the leaves and stem samples. The stem contains a higher carbohydrate content (19.4%) than the leaves (11.8%).

Equation 9: (#ri#ylddelolw\|###@##Devruedgfh#ri#wkh#wuhdwhg#fhoo#{#433 ##########################Devruedgfh#ri#fdgfu#frqwurc#fhoo

Table 2. Percentage inhibition (IP) of the different temperatures of *T. crispa* aqueous extract, BHT and Vitamin C

Vdpsoh	GSSK#phwkrg	
	Shufhqwdjh lqklelwlrq#+(,	Shufhqwdjh#lqklelwlrq frpsduhg#wr#EKW#+(,
53¹F	991;9#1#3188[g]	5<198#1#3188[g]
73¹F	;71<5#1#419<[d]	45134#1#41:8[d]
93¹F	;81<8#1#3185[d]	431<7#1#3187[d]
;3¹F	::155#1#4136[e]	4;1<5#1#4139[e]
433¹F	::18;#1#5143[e]	4<195#1#514;[e]
Ylw#F	<9169#1#3188[f]	####0
EKW	<9184#1#3137[f]	####0

Note: All tests were conducted in triplicate and the means were used. Values shown are mean ± SEM. Values with the same letter were not significantly different between the samples ($p<0.05$).

Table 3. Percentage inhibition (IP) of the different temperature sof *T. crispa* aqueous extract, BHT and Vitamin C

Vdpsoh	WED#phwkrg	
	Shufhqwdjh#ri dqwlr{lgdqw#+(,	Shufhqwdjh#lqklelwlrq frpsduhg#wr#EKW
53¹F	######0	0
73#¹F	6615< ± 5167[f]	8913;#1#613;[f]
93#¹F	6<153 ± 51<:[f]	7;15<#1#61<5[f]
;3#¹F	5138 ± 41;4[g]	<:15<#1#5173[g]
Ylw#F	:6153 ± 51<:[h]	0
EKW	:81;3 ± 6184	0

Note: All tests were conducted in triplicate and means were used. Values shown are mean ± SEM. Values with the same letter were not significantly different between the samples ($p<0.05$)

Wdeoh#71 #Uhvxow#ri#wkh#sruhqwldo#wr{lf#niihfw#ri#vhohfwhg#frqfhqwudwlrqv#ri# *WL#fulvsd*#xvlqj#wkh#Eulqh#Vkulps dhkdolw|#whvw

H{wudfw	*Skdvh#L#grvh#+ug2po,*			*Skdvh#LL#grvh#+pj2po,*				*LF*$_{83}$[d] *+ug2po,*	*WL#fulvsd#dhkdolw|#FO*$_{83}$[e] *+ug2po,*
WL#fulvsd	433	533	833	518	8	43	53		
Qxpehu#ri#ghdg eulqh#vkulps diwhu#57#k	3243	3243	3243	4243	5243	7243	:243	A4333	A43pj2po

[a] Number of animals dead/number of animals used. LD_{50} was determined from the geometric mean for which 0/10 and 4/10 were found. ($p<0.05$).

[b] In *T. crispa* lethality test, H_2O_2 (IC_{50} = 50 µmol/ml) was used as positive control ($p<0.05$)

Antioxidant activity of *T. crispa* extract *in vitro*

1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

The DPPH assay was utilised to evaluate the ability of antioxidants to scavenge free radicals. As shown in Table 2, the scavenging activities of *T. crispa* extracts, vitamin C and BHT on DPPH radicals were compared. For the extraction at 20°C/24h and 40°C/12h, the percentage of inhibition was 66.86 ± 0.55 and 84.92 ± 1.69 respectively. The percentage of inhibition for the extraction at 60°C/6h demonstrated a significantly high scavenging activity on the DPPH radicals ($p<0.05$) (85.95 ± 0.52) compared to the extract prepared at 20°C/24h. Inversely, the scavenging activity of *T. crispa* extract was significantly lower ($p<0.05$) at high temperature; 80°C/3h (78.22 ± 1.03) and 100°C/15 min (77.58 ±2.10) respectively, compared to 60°C/6h.

Thiobarbituric acid (TBA) test

The percentage of inhibition of *T. crispa* extract for TBA test is shown in Table 3. No significant production of carbonyl compounds was detected for the extract prepared at 20°C/24h. The production of carbonyl compounds was observed for the extraction at 40°C/12h (33.29 ± 2.34 percentage of inhibition) and was higher for 60°C/6h (39.2 ± 2.97). However, the inhibition percentage of the extract prepared at 80°C/3h (2.05 ± 1.81) was significantly lower ($p<0.05$) than its counterpart at 60°C/6h and 40°C/12h.

Brine shrimp lethality test

The results of brine shrimp lethality test (BSLT) *T. crispa* testing are summarised in Table 4. The Phase I-BSLT analysis (with concentrations of 100, 200 and 500 µg/ml respectively) showed no toxic effect exerted by the extract on brine shrimp survival. The Phase II-BSLT analysis (with extreme high concentrations of 2.5, 5, 10, 20 mg/ml) was carried out to determine the toxicological level of *T. crispa* extract on brine shrimp mortality. The results revealed that *T. crispa* extract is not toxic to biological systems as the IC_{50} of the extract was found to be higher than 1000 µg/ml. The value of IC_{50} predicted was 11 mg/ml (Table 4).

Antiproliferative study

Caov-3

The Caov-3 cell viability against the concentration of *T. crispa* extract is illustrated in Figure 1. The IC_{50} value increased from 100µg/ml on first day of treatment to 105µg/ml on second day of treatment. Meanwhile, the value decreased to 80µg/ml on the third day of treatment.

HepG2

Figure 2 shows HepG2 cell viability against the concentration of *T. crispa* extract. The IC_{50} value for the first day treatment was 165µg/ml, decreasing continuously to 131µg/ml and 60µg/ml respectively on the second day and third day of treatment.

MCF-7

The MCF-7 cell viability against the concentration of *T. crispa* extract is demonstrated in Figure 3. The IC_{50} value was 107µg/ml for the first day of treatment. It was reduced to 91µg/ml on the second day of treatment and further decreased to 60µg/ml on the third day.

HeLa

Figure 4 shows the HeLa cell viability against the concentration of *T. crispa* extract. The IC_{50} value for the first day treatment was 185µg/ml. On the second day, the value declined to 64µg/ml, but increased to 79µg/ml on the third day.

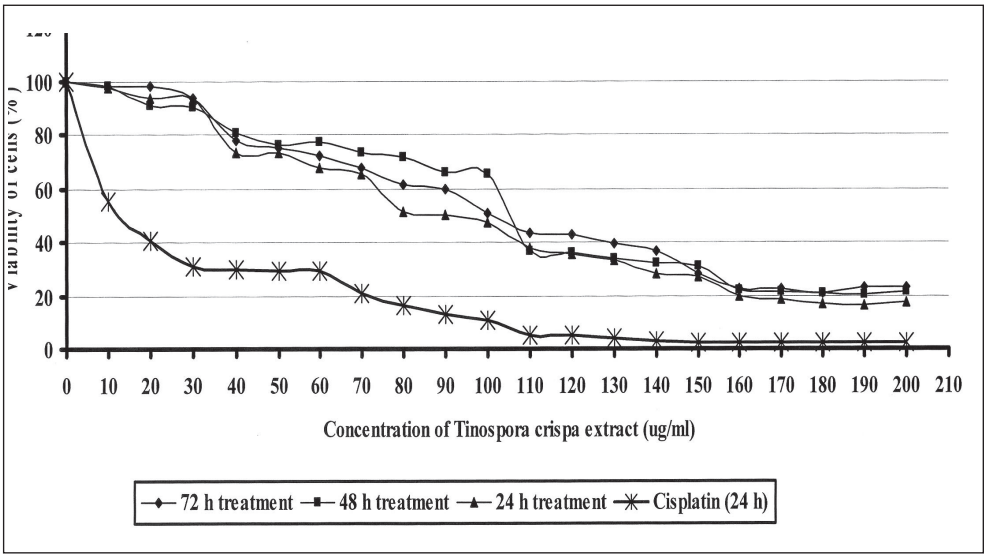

Figure 1. Percentage of viability of Caov-3 against concentration of *T. crispa*.
Note: The viability of Caov-3, ovarian cancer cell lines against treatment of *T. crispa* with different concentrations varying from 10 μg/ml to 200 μg/ml. It also shows the effect of *T. crispa* on Caov-3 with different days of treatment ($p<0.05$)

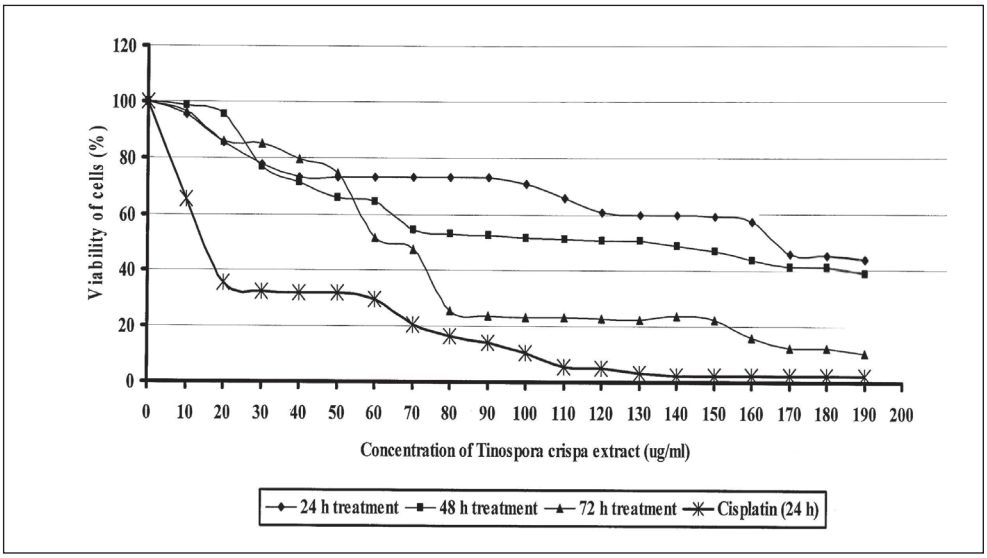

Figure 2. Percentage of viability of HepG2 against concentration of *T. crispa* aqueous extract.
Note: Viability of HepG2, liver cancer cell lines against treatment of *T. crispa* with different concentrations varying from 10 μg/ml to 200 μg/ml. It also shows the effect of *T. crispa* on HepG2 with different days of treatment ($p<0.05$)

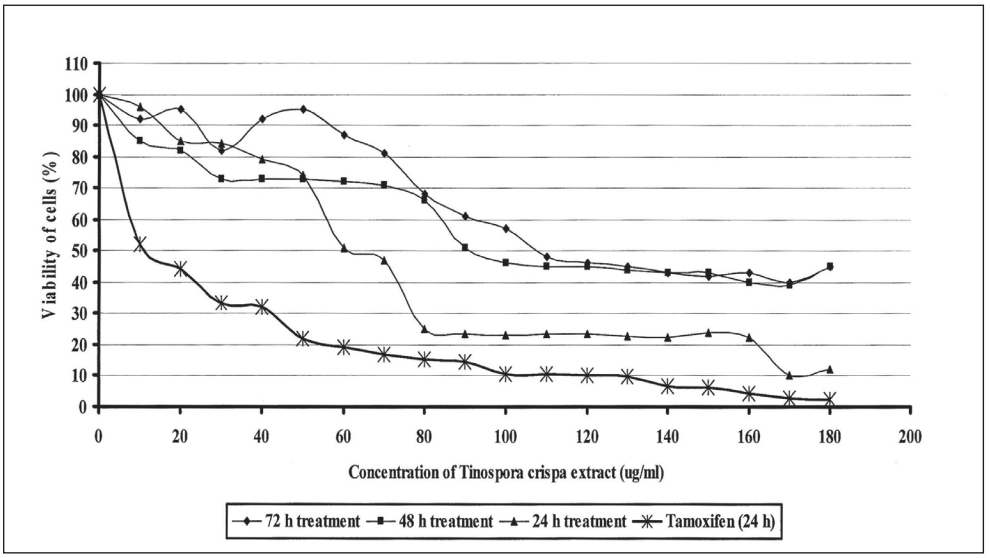

Figure 3. Percentage of viability of MCF-7 cells against concentration of *T. crispa* aqueous extract.
Note: Viability of MCF-7, ovarian cancer cell lines against treatment of *T. crispa* with different concentrations varying from 10 μg/ml to 200 μg/ml. It also shows the effect of *T. crispa* on MCF-7 with different days of treatment ($p<0.05$)

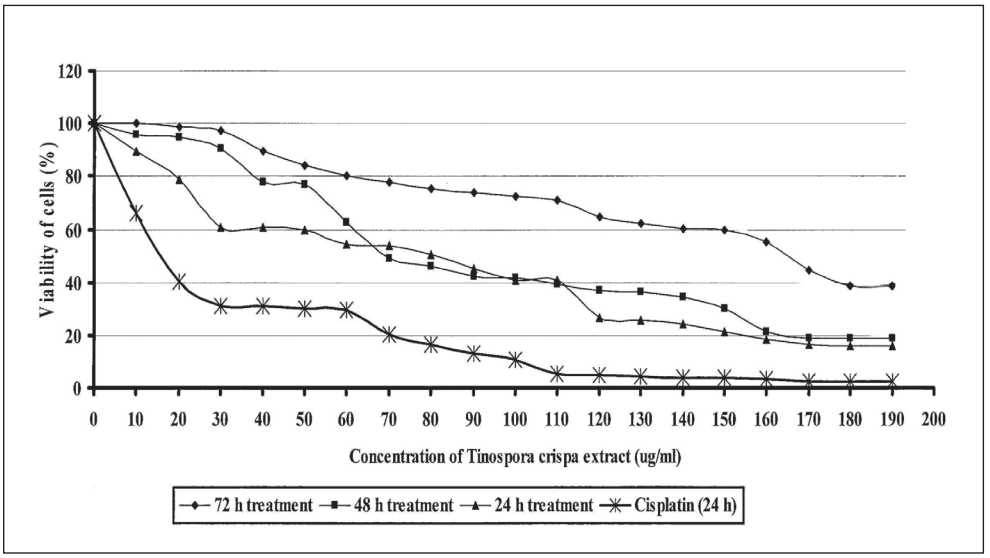

Figure 4. Percentage of viability of HeLa cells against concentration of *T. crispa*.
Note: Viability of HeLa, cervical cancer cell lines against treatment of *T. crispa* with different concentrations varying from10 μg/ml to 200 μg/ml. It also shows the effect of *T. crispa* on HeLa with different days of treatment to find the effectiveness of the herbs against the cancer cell lines ($p<0.05$)

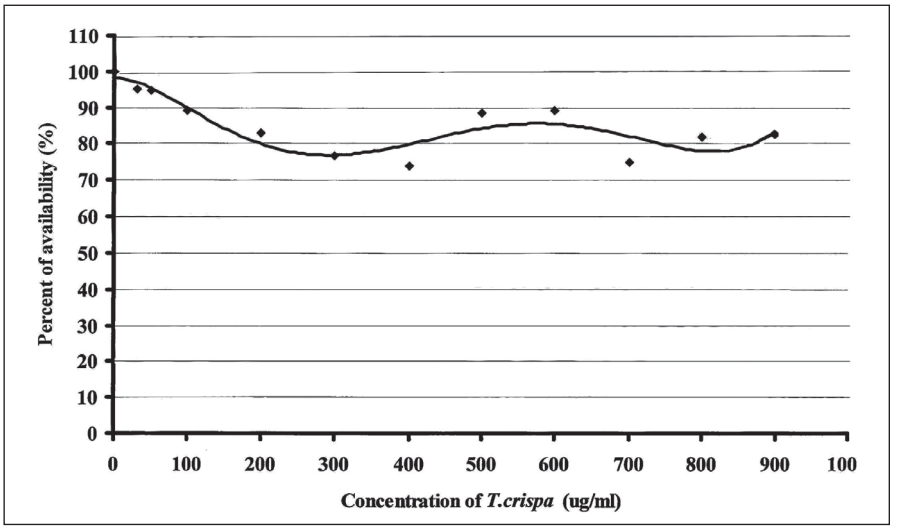

Figure 5. Percentage of viability of HUVEC cells against concentration of *T. crispa* within 72 h incubation.

Note: Viability of HUVEC, normal cell lines against treatment of *T. crispa* with different concentrations varying from 50 μg/ml to 900 μg/ml. It shows *T. crispa* is not toxic to HUVEC cell ($p<0.05$).

HUVEC

Figure 5 shows the HUVEC cell viability against the concentration of *T. crispa* extract. The IC_{50} value for 72 h of treatment was not detected from the curve.

DISCUSSION

A high level of free radicals leads to oxidative stress and induces degenerative disorders such as cancer, cardiovascular problems and neurodegenerative diseases (Yen, Duh & Tsai, 2002). The antioxidant activity of phenolics, on the other hand, is mainly due to their redox properties, which allow them to quench free radicals by acting as reducing agents, hydrogen donors, singlet oxygen quencher and may also have a metal chelating potential (Rice-Evans *et al.*, 1995).

Dietary supplements consisting of antioxidants such as flavonoid and vitamins, may be used to effectively defend body cells from oxidative stress and to maintain human body health in general (Rahman, Biswas & Kirkhan, 2005; Sies *et al* ., 2005). Many phenolic compounds have been reported to possess potent antioxidant activity, anticancer, antimutagenic, antibacterial, antiviral and anti-inflammatory activities to a greater or lesser extent (Chung *et al*., 1998).

The DPPH is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant (Lu & Yeap, 2001). It is believed that the DPPH assay is sensitive to active ingredients at low concentrations. The DPPH scavenging activity has been widely used to evaluate the antiradical activity of various samples (Piao *et al.,* 2004;). Results from this study showthat *T. crispa* extract is able to scavenge DPPH free radicals in a concentration-dependent manner. Similar results were observed from the study by Amorati *et al.* (2003), which governed established antioxidant, BHT and alpha-tocopherol in DPPH analysis. Temperature and incubation periods are important factors involved in the extraction process in order to produce a high antioxidant reading, as has been reported in the extraction of antioxidant from *Rosmarinus officinalis* (Albu *et al.,* 2004). This was also demonstrated in

previous studies whereby pharmacologically active compounds extracted from *Salvia officinalis* increased its efficiency when 60% of the target compounds were extracted within 2 h at ambient temperature (Durling *et al.*, 2007). No prior experimental investigation was done to determine the optimum temperature and incubation time in the preparation of *T. crispa* extract. However, antioxidant compounds in some herbs are likely to be heat labile (David *et al.*, 2007). The processes of steaming, flaking and boiling of plants have been reported to decrease their biological compounds (Bryngelsson *et al.*, 2002).

The variations of antioxidant compounds in plants obtained through several extraction processes could be explained by the different temperature and time prevailing in each case. The reduction in antioxidant activities observed could be due to the effect of high temperatures (more than 100°C) on the reactivity of the polyphenol aromatic rings. High temperatures could promote polymerisation and/or decomposition of the aromatic structure, hindering their quantification with the Folin–Ciocalteu reagent (Granito *et al.*, 2005). Likewise, contact with water at high temperatures could increase the solubility of polyphenols, increasing the losses in the cooking water (Turkmen, Sari, & Velioglu, 2005). This experiment revealed that the optimum parameter for *T. crispa* extraction was at 60^0C with 6 h incubation period as both DPPH and TBA analyses exhibited a significantly high antioxidant ability compared to other settings.

It is believed that antioxidant compounds are major components of antiradical activity measured in tested solutions (Moure *et al.*, 2001). There are epidemiological studies illustrating the relationship between the consumption of products rich in antioxidants and a low incidence of diseases like cancer, coronary heart disease and atherosclerosis (Randhir, Wattem & Shetty, 2004). Apart from compounds with very strong antiradical properties, other ingredients for antioxidant activity, for example, aromatic amino acids and peptides (e.g. glutathione), are scientifically proven to be present in tropical herbs.

Proximate analysis was done to detect the nutrients and minerals existing in the plant. The disease preventive abilities of fruit and vegetables have been attributed to the nutrients present in these dietary sources (Geleijnse *et al.*, 1999). The outcome of the proximate analysis showed that *T. crispa* had high contents of protein, carbohydrate and moisture. Prior studies also confirm that chemical substances in plants including protein, carbohydrate, vitamin and fibre also contribute to the antioxidant capacity (Betancur-Ancona *et al.*, 2004). The plant proteins present in the extract of grass pea seeds and soluble proteins of legume seeds contain compounds of strong antioxidant activity, for example, isoflavones, which are effective peroxyl radical scavengers (Patel *et al.*, 2001). Moreover, soluble proteins from plant are proven to be capable of inhibiting lipid peroxidation in oil-in-water emulsions at pH 7.0 (Anna, Bozena & Malgorzata, 2008).

The amount of ash in *T. crispa* extract was considered low compared to other herbs that were examined by Maisuthisakul, Sirikarn & Pitiporn (2007). There are inverse correlations between ash and antioxidant properties whereby ash contains minerals and heavy metals (including iron) which can act as pro-oxidants (Maisuthisakul *et al.*, 2007). Low ash content indicates that *T. crispa* contains low pro-oxidant substances.

Dietary fibre content is inversely associated with the DPPH radical scavenging activity. The main antioxidant mechanism of dietary fibre is as a metal chelating agent. Another mechanism is free radical scavenging due to some polyphenols which are associated with dietary fibre (Ubando-Rivera, Navarro-Ocaña & Valdivia-López, 2005). However, increased dietary fibre could correspond to a lower polyphenol content resulting in a lower

molecular weight and hence reduced radical scavenging activity which would explain the relationship observed for the *T. crispa* extract.

The brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity (Sam, 1993). A report by Hlywka, Beck & Bulleman (1997) indicated that there is correlation between number of dead shrimps and concentration of extract. *T. crispa* extract produced no toxic effect on animal cells and does not demonstrate any IC_{50} even up to an extreme concentration of 1g/ml. This data is in accordance with the findings by Hartl & Humpf (2000), where there are associations between toxicological level of the herb extracts and the mortality of brine shrimp. Besides that, numerous previous studies done on *T. crispa* in several experimental animals reported no evidence of organ damage. However, there are other factors that are considered as a confounder in this assay and will affect brine shrimp mortality such as lack of oxygen (Hartl & Humpf 2000) and age of the shrimp (Hlywka *et al.*, 1997). The shrimp will barely survive for 72 h alone on their own resource.

Tinospora crispa contains quartenary alkaloid compounds and chemical constituents such as borapetol A, borapetol B, borapetoside A, borapetoside B, tinocrisposide, *N*-formylanondine, *N*-formylnornuciferine, *N*-acetyl nornuciferine, γ-sitosterol, picrotein, tinotubride (Pathak *et al.*, 1995;). All of these chemical substances especially alkaloids, contain anti-cancer properties which can interfere with microtubule function. Alkaloids are widely used in combination with chemotherapy regimens for treating many solid tumours (Rowinsky & Donehower, 1997)

Cisplatin and tamoxifen are well-established human anticancer drugs and have been used to treat cancer disease (Behrens, Gill & Fichtner, 2007). *T. crispa* showed a significant cytotoxicity effect compared to both drugs in all experiments. The findings from this study demonstrate that the effect of *T. crispa* on viability of HepG2, Caov-3, MCF-7 and HeLa cancer cells are dose and time dependent. These findings have been similarly reported where most plant extracts depend on dose and time to demonstrate their effects (Shahin *et al.*, 2008). All cancer cells showed signi-ficantly different ($p<0.05$) effects on viability of cell based on day of treatment. However, the IC_{50} value for Caov-3 throughout the 3 days of treatment was not significantly different ($p<0.05$) which indicates that the extract was not sufficiently potent to kill the cells. On the other hand, this study also found that the treatment of *T. crispa* slowly decreased the viability of normal cell lines (HUVEC) but there was no IC_{50} detected even at the 72 h incubation period. This indicates that *T. crispa* may possess a selective anti-proliferative activity on cancer cell lines.

This study shows that *T. crispa* offers a moderate effect on blocking the proliferation of cancer cells used. The anti-proliferative screening of cancer cells *in vitro* on *T. crispa* aqueous extract provides important preliminary data for the use of its potential anti-neoplastic properties in future studies.

CONCLUSION

T. crispa contains certain nutrients and minerals as shown in the proximate analysis. The antioxidant activity of *T. crispa* extract might be attributed to its effective hydrogen-donating ability and its effectiveness as scavenger of hydrogen peroxide and free radicals. In addition, the extract was not found to be toxic on biological systems and normal cell lines. The results obtained from this study suggest that *T. crispa* could be used as an easily accessible source of natural antioxidants and a possible supplement in the pharmaceutical industry. However, the major components responsible for preventing cancer activities need to be further investigated.

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