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 IC₅₀ value was more than 1000 μg/ml. The extract produced moderate anti-proliferative activity on selected human cancer cell lines (IC₅₀ 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...
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; Kongsaatrakoon 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
Biological Properties of T. crispa & Its Antiproliferative Activities on Human Cancer Cell Lines

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: \( \% \text{ crude fat} = \frac{\text{weight of dried beaker + fat} - \text{weight of dried beaker + granules}}{\text{Weight of sample}} \times 100 \)

Equation 3: \( \text{N (\%)} = \frac{14.01 \times (\text{ml titrant for sample} - \text{ml titrant for blank}) \times \text{molarity of acid} \times 100}{\text{Weight of sample}} \)

Equation 4: \( \text{Protein (\%)} = \frac{\text{N}}{6.25} \times \text{protein factor specific to sample} \)

Equation 5: \( \% \text{ crude fibre} = \frac{(\text{beaker + residue weight} - \text{fibre bag weight}) - (\text{beaker + ash weight})}{\text{Sample weight}} \times 100 \)

Equation 6: \( \% \text{ of Carbohydrate} = 100 - (\% \text{ moisture} + \% \text{ fat} + \% \text{ protein} + \% \text{ fibre} + \% \text{ ash}) \)
Antioxidant activity of *T. crispa* extract in *vitro*

The optimisation of *T. crispa* extraction procedure was verified via its antioxidant activity. 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 extremely high concentration of the extract (phase 2). Briefly, 10 brine shrimps were placed into a well of a 24-well plate containing 800 μl of salt water. 200 μl 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.

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Equation 7: 
\[
\text{% Inhibition} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \right) \times 100
\]

Equation 8: 
\[
\text{% Inhibition} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \right) \times 100
\]
Extract solution with the concentration of 200 $\mu$g/ml was prepared by dissolving 1.0 mg of \textit{T. crispa} extract into 5 ml of deionized water. The stock was filtered using 0.2 $\mu$m 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 $\mu$g/ml respectively. The treatment was done in triplicate. The control was prepared by adding 200 $\mu$l 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 \textit{T. crispa} ranging from 10 to 200 $\mu$g/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 $\mu$L 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 \textit{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%).

<table>
<thead>
<tr>
<th></th>
<th>Hotel</th>
<th>Whp</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sanzhiaq</strong></td>
<td>71:</td>
<td>415</td>
</tr>
<tr>
<td><strong>Idw</strong></td>
<td>418</td>
<td>31399</td>
</tr>
<tr>
<td>**Phlvezk</td>
<td>guidek**</td>
<td>411:</td>
</tr>
<tr>
<td><strong>Dvk</strong></td>
<td>51:</td>
<td>31364</td>
</tr>
<tr>
<td><strong>Prlvweah</strong></td>
<td>418:</td>
<td>31389</td>
</tr>
<tr>
<td>**Wvmdh</td>
<td>nudh[</td>
<td>tilenh**</td>
</tr>
<tr>
<td>**N2fek</td>
<td>0433</td>
<td>nwhuj**</td>
</tr>
</tbody>
</table>

% of viability = Absorbance of the treated cell $\times 100$ / Absorbance of cancer control cell
Table 2. Percentage inhibition (IP) of the different temperatures of *T. crispa* aqueous extract, BHT and Vitamin C

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage inhibition (%) compared to BHT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20ºC</td>
<td>66.86 ± 0.55d</td>
</tr>
<tr>
<td>40ºC</td>
<td>84.92 ± 1.69a</td>
</tr>
<tr>
<td>60ºC</td>
<td>85.95 ± 0.52a</td>
</tr>
<tr>
<td>80ºC</td>
<td>78.22 ± 1.03b</td>
</tr>
<tr>
<td>100ºC</td>
<td>77.58 ± 2.10b</td>
</tr>
<tr>
<td>Vit C</td>
<td>96.36 ± 0.55c</td>
</tr>
<tr>
<td>BHT</td>
<td>96.51 ± 0.04c</td>
</tr>
</tbody>
</table>

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 temperatures of *T. crispa* aqueous extract, BHT and Vitamin C

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage of antioxidant (%) compared to BHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>20ºC</td>
<td>--</td>
</tr>
<tr>
<td>40ºC</td>
<td>33.29 ± 2.34c</td>
</tr>
<tr>
<td>60ºC</td>
<td>39.20 ± 2.97c</td>
</tr>
<tr>
<td>80ºC</td>
<td>2.05 ± 1.81d</td>
</tr>
<tr>
<td>Vit C</td>
<td>73.20 ± 2.97e</td>
</tr>
<tr>
<td>BHT</td>
<td>75.80 ± 3.51</td>
</tr>
</tbody>
</table>

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).

Table 4. Results of the potential toxic effect of selected concentrations of *T. crispa* using the Brine Shrimp lethality test

<table>
<thead>
<tr>
<th>Extract Phase I dose (µg/ml)</th>
<th>Phase II dose (mg/ml)</th>
<th>IC50a</th>
<th>CL50b</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. crispa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>500</td>
<td>2.5</td>
</tr>
<tr>
<td>Number of dead</td>
<td>0/10</td>
<td>0/10</td>
<td>2/10</td>
</tr>
<tr>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Note: Number of animals dead/number of animals used. LD50 was determined from the geometric mean for which 0/10 and 4/10 were found. (p<0.05).

b In *T. crispa* lethality test, H2O2 (IC50 = 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 IC50 of the extract was found to be higher than 1000 μg/ml. The value of IC50 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 IC50 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 IC50 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 IC50 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 IC50 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 from 10 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 shows the HUVEC cell viability against the concentration of T. crispa extract. The IC\textsubscript{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 show that 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 alphatocopherol 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°C 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\textsubscript{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*-formylnormuciferine, *N*-acetyl normuciferine, \(\gamma\)-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 significantly different (p<0.05) effects on viability of cell based on day of treatment. However, the IC\textsubscript{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\textsubscript{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.
REFERENCES


