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Anti-proliperative and antioxidant effects of T*inospora crispa* (Batawali)

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Abstract

***T. crispa* is a well known traditional medicinal plant used in India, Indonesia, Philippines and Malaysia. To date, there are numerous studies on this plant emphasizing it's antioxidant, antidiabetic, antimalarial and cosmetic effects. However, limited informations are available regarding *T. crispa* cytotoxicity potential. The objectives of this study were to determine the cytotoxicity potential and antioxidant activity of different *T. crispa* crude extracts. Viability of cells was measured using the MTT assay on MCF-7, MDA-MB-231, HeLa and 3T3 normal fibroblast cells while antioxidant activity was determined by measuring total flavonoid content, total phenolic content and DPPH free radical scavenging activity. Results obtained showed that the cell viability decreased in a dose-dependent manner in all cancer cells for each extract. The lowest IC_{50} (33.75 ± 4.65 µg/ml) was observed in MCF-7 using the methanol extract. Further more, there were significant differences for total phenolic content ($p<0.01$, df=2, X^2=9.836) and flavonoid content ($p<0.05$, df=0.02, X^2= 7.20) between each extract with the methanol extract having the highest activity for both phenolic (255.33 ± 10.79 mg GAE/g sample) and flavonoid content (9.53 ± 0.50 mg QE/ g sample). The DPPH assay showed that the methanol extract had highest scavenging activity in a dose-dependent manner where the IC_{50} value was 12 µg/ml. *T. crispa* has a dose-dependent antiproliferative activity against many types of cancer cells where the lowest IC_{50} was present in the methanol extract on MCF-7. Further more, methanol crude extract of *T. crispa* had higher total phenolic and flavonoid content and free radical scavenging activity compared to water extract and chloroform extract**.

Key word: *T. crispa*, cytotoxicity, antioxidant

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Introduction

Cancer is characterized by uncontrolled growth of cells that can cause by many factors such as exposure to UV light, certain chemicals [1] and imbalance between oxidant and antioxidant [2]. Novel anticancer agent is one of the important treatments to fight against cancer instead of the surgery and radiotherapy [1].

Malaysia is known for its biodiversity with many plants possessing medicinal value [3]. *T. crispa* is known as batawali or seruntun in Malaysia, bratawali and andawali in Indonesia, makhamukay in Philippines and boraphet in Thailand. It had been used as traditional medicine in rural society to treat fever, cholera, snake bites, rheumatism and fever due to malaria [4]. *T. crispa* has shown to have an antihyperglycemia effect by augmenting the release of insulin [5]. Its antimalarial activity [3], antibacterial [6], anti-inflammatory [7] and anti-oxidant properties [4] are also recorded. Recent study by Chantong et al. [8] showed that *T. crispa* has cytotoxic effect on P19 embryonal carcinoma cells. The cytotoxicity activity of plants might be due to the antioxidant activity or the presence of active compound such as alkaloid. The measurement of different extraction solvents produced valuable information about the antioxidant activity of different type of *T. crispa* crude extracts. This study was designed to measure the cytotoxicity and antioxidant properties in different type of *T. crispa* crude extracts

Materials and Methods

Chemicals.

Folin-Ciocalteu reagent, sodium carbonate anhydrous (NaOH), gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), aluminum chloride hexahydrate ($AlCl_3.6H_20$),

quercetin, dimethyl sulfoxide (DMSO) and tamoxifen were purchased from Sigma Chemical Co. USA. Roswell Park Media Institute (RPMI) 1640, penicillin, streptomycin and foetal bovine serum (FBS) were obtained from PAA, GMBH, (Germany). Methanol and chloroform used were of the highest pure grade (Merck, Germany). 3T3, MCF-7, MDA-MB-231 and HeLa cell line cultures were obtained from the American Type Culture Collection (ATCC).

Plant materials

The raw plant materials (stems) were obtained from a village in Chenor, Pahang, cut into small pieces, dried and ground into powder. The powder was soaked in water, methanol or chloroform overnight at room temperature. The water, methanol and chloroform extractions were filtered and the filtrates were evaporated with a rotary evaporator. Then the dried residues were stored at -80°C until used for cytotoxicity testing and antioxidant determination.

Preparation of stock solution

For cell culture, 5 mg of each dry residue extract was dissolve in 5 ml DMSO to produce 1 mg/ml stock solution of each extract and further diluted with RPMI 1640 to produce a working solution of 100 µg/ml. For determination of the total antioxidant activity, 5 mg of each dry residue extract was dissolve in 5 ml methanol to produce 1 mg/ml working solution of each extract.

Cell culture** and **cytotoxicity test using MTT

MCF-7, MDA-MB-231, HeLa and 3T3 cell lines were cultured in RPMI 1640 supplemented with 10% of FBS, 100 IU/ml of penicillin and 100 µg/ml of streptomycin using 75-cm^2 flasks, in humidified incubator with 5% CO_2 and 95% air at 37°C. Cells were harvested when it reached 70% confluent and 1 x 10^5/ml were plated onto a 96-well plate and incubated overnight to allow the cells to attach to the well. The medium was discarded and 100 µl of the working solution, which was diluted with the medium to yield the final concentrations of the test solutions ranging from 10 - 100 µg/ml were added into the well and incubated for 72 hours. As a control, cells were cultured in 100 µl of medium, while for positive control cells were cultured in the presence of tamoxifen which the concentrations were similar to extracts. All treatments were done in replicates of six. Viability of the cells was determined using MTT assay according to manufacturer's instructions (Roche Diagnostic). The results were recorded as IC_{50} value which is the concentration of sample that inhibits 50% of tumour cells growth.

Determination of total phenolic content

The total phenolic content of *T. crispa* extract was determined by using Folin-Ciocalteu method [9] where 200 µl of samples (1 mg/ml) were added into the test tube followed by 1.5 ml of Folin-Ciocalteu's reagent (diluted 10 times) and allowed to stand at 22°C for 5 minutes in the dark. Then 1.5 ml of NaOH (6% w/v) were added and were incubated for 90 minutes before absorbance were measuring at 725 nm. Total phenolic content was measured as gallic acid equivalents (GAE) in 1 g material.

Determination of total flavonoid content

The total flavonoid content was determined using the Dowd method [10]. Briefly, 1 ml of 2 % $AlCl_3.6H_20$ in methanol was mixed with the same volume of the extract solution (1 mg/ml). Then, the solution was allowed to stand at the room temperature in the dark for 15 minutes before absorbance was measured at 430 nm using spectrophotometer against blank sample consisting of methanol. The total flavonoid content was determined using standard curve with quercetin (mg/ml) as the standard. Total flavonoid content was expresses as mg of quercetin equivalents (CE) in 1 g of extract.

DPPH free radical scavenging assay

The DPPH method [11] was followed by adding 100 µl different concentrations of the *T. crispa* extracts ranging 10 - 100 µg/ml (in triplicate) to 5 µl of DPPH (5 mg/2 ml methanol). DPPH solution was then allowed to stand for 20 min before absorbance was measured at 517 nm. Spectrophotometric measurements was made using methanol as blank. Antioxidant activity was expressed as IC_{50} (inhibitory concentration in mg/ml of plant material necessary to reduce the absorbance of DPPH by 50%). Vitamin C was used as positive control.

Statistical analysis

Data were expressed as mean ± standard deviation. Kruskal Wallis test was used to compare mean of each group. Probability levels of less than 0.05 were taken as statistical significance.

Results

Cell viability

MCF-7

Fig. 1 shows the MCF-7 cell viability on various *T. crispa* extracts (water, methanol and chloroform) and tamoxifen as control. The cell viability was decreased in dose-dependent manner where the IC_{50} were 42.75 ± 4.61 µg/ml, 33.75 ± 4.65 µg/ml and 38.90 ± 3.21 µg/ml for water, methanol and chloroform extracts, respectively.

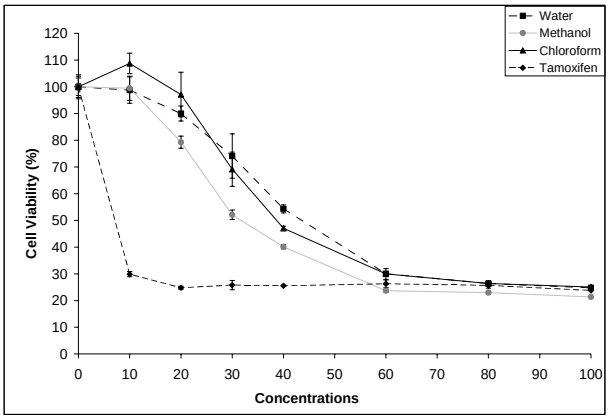

Figure 1*. Percentage of cell viability of MCF-7 cancer cell line against different concentrations of T. crispa extracts (water, methanol and chloroform) and tamoxifen.*

MDA-MB-231

Fig. 2 shows the MDA-MB-231 cell viability on various *T. crispa* extracts (water, methanol and chloroform) and tamoxifen as control. The cell viability was decreased in dose-dependent manner where the IC_{50} were 46.88 ± 1.75 µg/ml, 44.83 ± 1.21 µg/ml and 51.25 ± 3.62 µg/ml for water, methanol and chloroform extracts, respectively.

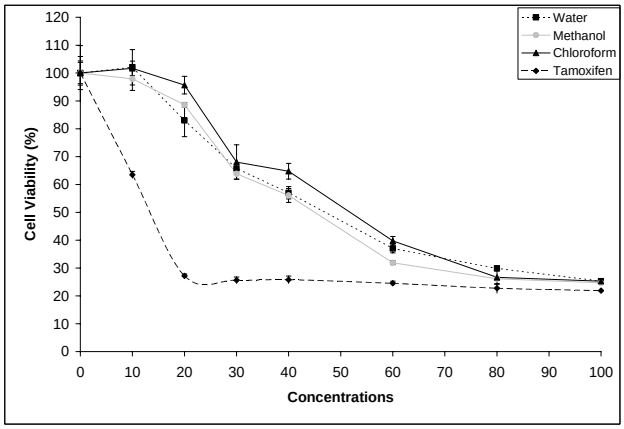

Figure 2. *Percentage of cell viability of MDA-MB-231 cancer cell line against different concentrations of T. crispa extracts (water, methanol and chloroform) and tamoxifen.*

HeLa

Fig. 3 shows the MDA-MB-231 cell viability on various *T. crispa* extracts (water, methanol and chloroform) and tamoxifen as control. The cell viability was decreased in dose-dependent manner where the IC_{50} were 53.83 ± 1.47 µg/ml, 52.5 ± 1.14 µg/ml and 46.13 ± 2.81µg/ml for water, methanol and chloroform extracts, respectively

3T3

Fig. 4 shows the MDA-MB-231 cell viability on various *T. crispa* extracts (water, methanol and chloroform) and

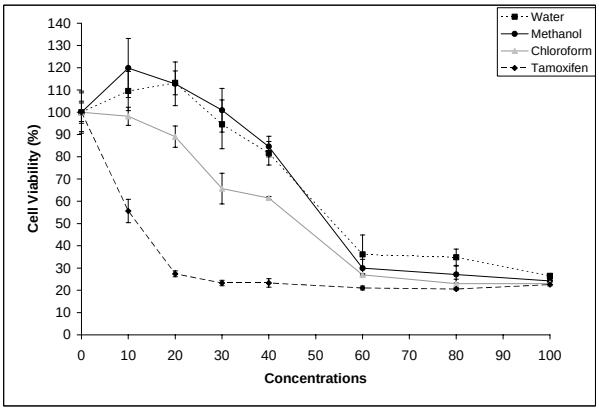

Figure 3. *Percentage of cell viability of HeLa cell line against different concentrations of T. crispa extracts (water, methanol and chloroform) and tamoxifen.*

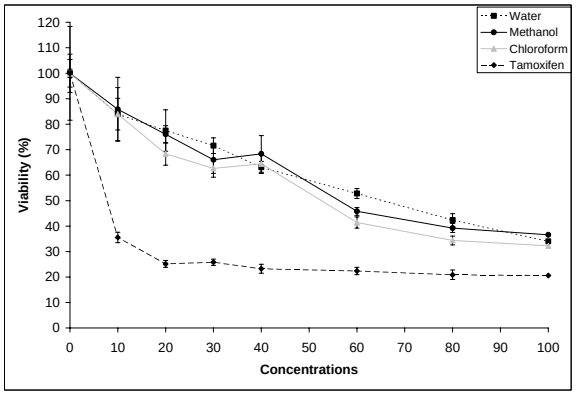

Figure 4. *Percentage of cell viability of 3T3 cell line against different concentrations of T. crispa extracts (water, methanol and chloroform) and tamoxifen.*

tamoxifen as control. The cell viability was decreased in dose-dependent manner where the IC_{50} were 65.50 ± 4.64 µg/ml, 57.50 ± 6.47 µg/ml and 52.58 ± 2.25 µg/ml for water, methanol and chloroform extracts, respectively. The IC_{50} values for 3T3 normal fibroblast cell were higher compared to all cancer cell line for each extract.

Total phenolic content

Table 1 showed the total phenolic contents of water, methanol and chloroform extract of *T. crispa* expressed in milligram gallic acid per gram samples. A significant difference was evident of the total phenolic content for water, methanol and chloroform extracts ($p<0.01$, $df=2$, $X^2=9.836$) where methanol extract poses highest activity (255.33 ± 10.79 mg GAE/ g sample) compared to water and chloroform extracts.

Total flavonoid content

Table 1 shows the total flavonoid content of water, methanol and chloroform extracts of *T. crispa* expressed in milligram quercetin per gram samples. A significant difference of the total phenolic content was evident for

water, methanol and chloroform extracts ($p<0.05$, df=2, X^2=7.20) where methanol extract poses highest activity (9.53 ± 0.50 mg QE/ g sample) compared to water and chloroform extracts.

DPPH free radical scavenging assay

Figure 5 shows the percentage of radical scavenging activity of *T. crispa* extracts and vitamin C as a positive control. The percentage radical scavenging activities were increased with concentrations except for vitamin C which was constant from 10 – 100 ug/ml where the percentage of radical activity nearly 100%. Furthermore, percentage of radical activity for water and methanol extracts increased with concentration, however no IC_{50} values were obtained. Only methanol extract showed IC_{50} values (12 µg/ml) and percentage of radical activity increased to 100% which was similar to vitamin C.

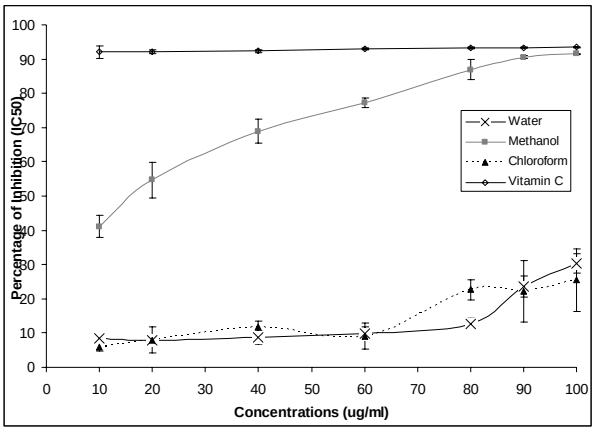

Figure 5. *Percentage of radical scavenging activity by different type of T. crispa extracts and vitamin C as a positive control*

Table 1*: Total phenolic and flavonoid content in 3 different extracts of T. crispa.*

Parameters	Extracts		
	Water	**Methanol**	**Chloroform**
Total Phenolic content (mg GAE/ g sample)	79.00 ± 10.00	255.33 ± 10.79	172.33 ± 22.30*
Total flavonoid (mg QE/ g sample)	2.67 ± 0.15	9.53 ± 0.50	5.38 ± 0.06**

** p< 0.01 Significantly different*
*** p< 0.05 Significantly differen*t

Discussion

In this study, the purpose of testing different types of *T. crispa* extracts was to determine the best extraction method of *T. crispa* that provides the strongest antiproliferative effect against cancer cell lines and antioxidant activity. The antiproliferative activity was measured using MTT assay where it is based on the reduction of a soluble tetrazolium salt by mitochondrial dehydrogenase activity into soluble colored product which could be measured by spectrophotometer. The IC_{50} values obtained from the viability graph were used as parameter for cytotoxicity where it refers to 50% of cells inhibited by the plant extract [13].

Different type of extraction methods contributes different active compounds of *T. crispa* due to polarity of the solvent. Regarding *in vitro* study on anti-malarial activity, chloroform extract of *T. crispa* provided best result compared to methanol extract [3]. Similarly in this study, methanol extract gave the best IC_{50} value compared to water and chloroform extracts in MCF-7 and MDA-MB-231 but not for HeLa cell. Cisplatin and tamoxifen are well established human anticancer drugs and have been used to treat cancer [14]. In this study, we used tamoxifen as a positive control and each *T. crispa* extract showed similar pattern of cell viability curve compared to that of tamoxifen.

Each extract showed that cell viability decreased in a dose-dependent manner in all types of cancer cells and normal cell. Most plant extracts show that their effects depend on time and the dose [15]. However, the IC_{50} obtained for 3T3 normal fibroblast cell was relatively higher than that of MCF-7, MDA-MB-231 and HeLa cells. The IC_{50} of 3T3 appears different from that in some animal cells where *T. crispa* extracts failed to produce any toxic effects even up to a concentration of 1 g/ml [16]. The IC_{50} values in this study were also found to be lower than those reported recently in HepG2, Caov-3, MCF-7 and HeLa cancer cells but not normal HUVEC cell using aqueous extracts [17]. Methanol extract of *T. crispa* againt MDA-MB-231 and MCF-7 had lower IC_{50} which showed the potential antiproliferative effect. The antiproliferative effect of *T. crispa* might be due to its chemical substances especially alkaloids, which are known to have anticancer properties [4].

Total phenolic content of *T. crispa* extracts were measured according to the Folin–Ciocalteu method. This method was widely acceptable for determination of phenolic content. However, this method was not entirely specific for phenolic compound but not all phenolic com-

pounds display the same level of activity in the assay [10]. The total phenolic and flavonoid content of *T. crispa* were higher in methanol compared to water and chloroform extracts. In this study, total amount of phenolic compound for methanol extract was 3.2 folds compared to water extract and 1.5 folds compared to chloroform extract. The difference of total phenolic content may be due to the specificity of Folin-Ciocalteu to the compound in methanol extract compared to water or chloroform extracts.

Flavonoid is plant pigment which has antioxidant activity through scavenging or chelating process and their effects on human nutrition and health are substantial [18]. $AlCl_3$ was used in this measurement because it is specific for total flavonoid which consists of flavanols and flavones compared to the other method for example by using 2,4-dinitrophenlhydrazine which is specific for flavanol only [12]. For total flavonoid content, the value for methanol extract was found to be 3.6 folds compared to water and and 1.8 fold to chloroform extract, respectively. Results showed that methanol extract had higher phenolic and flavonoid contents compared to other study which had reported low association between phenolic and flavonoid content [12].

Antioxidant activity measurement was done using DPPH radical scavenging where principle of the method was based on the ability of the compounds to act as free radical scavengers or hydrogen ion donors [18]. In this study, free radical scavenging activity of the methanol extract was found to be higher compared to water and chloroform extracts. Free radical scavenging activity of the methanol extract was increased from 50% up to 90% on dose-dependent manner and equivalent to vitamin C free radical scavenging activity. It however, showed that *T. crispa* extract has a strong antioxidant activity. In most cases, total phenolic content as determined by Folin-Ciocalteu method was correlated with the antioxidant capacities [19]. Similarly, in this study the methanol extract had shown high level of total phenolic and flavonoid that may contribute to the high level of free radical scavenging activity.

Our results clearly showed that the solvent used during extraction did affect the total phenolic content, flavonoid and free radical scavenging activity. Methanol extract of buckwheat showed highest antioxidant activity when sequentially extracted with hexane, diethyl ether, ethyl acetate and acetone [20]. Determination of antioxidant activity using different methods and extraction media lead to different observations. A combination of several tests could give a more consistent judgment of the antioxidant activity [20]

Conclusion

In conclusion, *T. crispa* has a dose-dependent antiproliferative activity against many types of cancer cells where the lowest IC_{50} is found to be present in the methanol extract on MDA-MB-231 breast cancer cells. Further more, methanol crude extract of *T. crispa* showed higher total phenolic and flavonoid content and free radical scavenging activity compared to water extract and chloroform extract. Further studies are needed to elucidate the mechanism of cell death caused by *T. crispa* extract.

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Reference

1. Larsen HR. Cancer: Causes, Prevention and Treatment. The International Journal of Alternative and Complementary Medicine 1996; 14: 9-11
2. Kikuzaki H, Hisamoto M, Hirose K, *et al.* Antioxidant properties of ferulic acid and its related compounds. J Agric Food Chem 2002; 50: 2161-2168
3. Nik Rahman NN, Furuta T, Kojima S, *et al*. Antimalarial activity of extracts of Malaysian medicinal plants. J Ethnopharmacol 1999; 64: 249-254
4. Dweck CA, Andawali CJ. (*Tinospora crispa*): A review. 2006. http://www.dweckdata.co.uk/Published_papers/A_List_of_published_papers.htm
5. Noor H, Ashcroft SJ. Pharmacological characterization of the antihyperglycaemic properties of Tinospora crispa extract. J Ethnopharmacol 1998; 62: 7-13
6. Zakaria ZA, Mat Jais AM, Somchit MN, *et al*. The in vitro antibacterial activity of Tinospora crispa extracts. J. Biol. Sci. 2006; 6: 398-401
7. Sulaiman MR, Zakaria ZA, Lihan R. Antinoceptive and anti-inflammatory activities of Tinospora crispa in various animal models. Int J Tropic Sci. 2008; 3: 66-69
8. Chantong B, Kampeera T, Sirimanapong W, *et al*. Antioxidant activity and cytotoxicity of plants commonly used in veterinary medicine. Acta Hort (ISHS) 2008; 786: 91-98
9. Kähkönen MP, Hopia AI, Vuorela HJ, *et al.* Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem 1999; **47**: 3954-3962
10. Meda A, Lamien CE, Romito M, *et al.* Determination of total phenolic, flavonoid and proline contents in bukina fasan honey, as well as their radical scavenging activity. Food Chemistry 2005; 91: 571-577
11. Chan, EWC, Lim YY, Chew, YL. Antioxidant activity of *Camellia sinensis* leaves and tea from a lowland plantation in Malaysia. Food Chemistry 2007; 102: 1214-1222

12. Edrini S, Rahmat A, Ismail P, Hin, TYY. 2002. Anti-carcinogenic properties and antioxidant activityof henna (*Lawsenia Inermis*). J Med Sci. 2002; 2: 194-197
13. Smit HF, Waedenbag HJ, Singh RH, *et al*. Ayurvedic herbals drugs with cytotoxicity activity. J Ethnopharmacol 1995; 47: 75-84
14. Behrens D, Gill JH, Fichtner I. Loss of tumourigenicity of stably ER transfected MCF-7 breast cancer cells. Mollec Cell Endocrinol 2007; 274: 19-29.
15. Shahin SA, Naresh K, Abhinav L, *et al*. Indian Medicainal Herb. Food Res Intl 2008; 41: 1-15.
16. Hartl M, Humpf HU. Toxicity assessment of fumonisins using the brine shrimp (*Artemia salina*) bioassay. J Food Chem Toxicol 2000; 38: 1097-1102.
17. Zulkhairi A, Abdah MA, Kamal NH, *et al*. Biological Properties of *Tinospora crispa* (*Akar Patawali*) and Its antiproliferative activities on selected human cancer cell lines. Mal J Nutr 2008; 14: 173-187
18. Cook NC, Samman S. Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources. . J Nutr Biochem 1996; 7: 66-76
19. Roginsky V, Lissi EA. Review of methods to determine chain breaking antioxidant activity in food. Food Chem. 2005; 92: 235-254
20. Sun T, Chi-Tang H. Antioxidant activities of buckwheat extracts. Food Chemistry 2007; 90: 743-749

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