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Antiproliferative Properties and Antioxidant Activity of Various Types of *Strobilanthes crispus* Tea

¹Mohd Fadzelly Abu Bakar, ¹Arnida Hani Teh, ¹Asmah Rahmat,

²Fauziah Othman, ¹Normah Hashim and ²Sharida Fakurazi

¹Department of Nutrition and Health Sciences,

²Department of Biomedical Sciences,

Faculty of Medicine and Health Sciences, Universiti Putra Malaysia,
43400, Serdang, Selangor, Malaysia

Abstract: Regarding to the promising pharmacotherapeutic properties of *Strobilanthes crispus* (Acanthaceae) plant, we report here, the development of nutraceutical herbal tea from *S. crispus* young and old leaves and evaluate the potential antiproliferative properties and antioxidant activity *in vitro*. Unfermented and fermented tea (*Camellia sinensis*) preparation was applied for development of *S. crispus* tea. Antiproliferative properties of *S. crispus* tea extracts were determined by the microculture tetrazolium salt (MTT) assay against human breast cancer cell lines (hormone dependent, MCF-7; non-hormone dependent, MDA-MB-231). The results showed that *S. crispus* tea only inhibit the proliferation of human hormone dependent breast cancer cell lines (MCF-7) but not the non-hormone dependent breast cancer cell lines (MDA-MB-231). The antioxidant activity was determined using FRAP (Ferric Reducing/Antioxidant Power) and DPPH free radical scavenging assay. The results showed that the hot water extract of *S. crispus* tea showed high antioxidant activity especially *S. crispus* unfermented tea from old leaves. But the tea from the leaves of *C. sinensis* displayed better antioxidant activity.

Key words: *Strobilanthes crispus*, tea, antiproliferative, MTT, antioxidant, FRAP, DPPH free radical scavenging

Introduction

Malaysia is a country that rich with many kind of herbs or medicinal plants that usually used in traditional medicine. In Malaysia, over 15,000 species of higher plants were found and about 1200 of these plant species have been reported to have potential pharmaceutical value of which some are being used as herbal medicine (Soepadmo, 1991). Furthermore, throughout the development of human culture, the use of natural products (especially from medicinal plants) has had magical-religious significance and different points of view regarding the concepts of health and disease existed within each culture (Rates, 2001).

One of the herbs that have great potential and is believed to have health-giving properties is pecah beling or *Strobilanthes crispus*. *S. crispus* (Acanthaceae) plant is native to countries from Madagascar to Indonesia (Sunarto, 1977). It is commonly known as daun pecah beling in Jakarta

Corresponding Author: Dr. Asmah Rahmat, Department of Nutrition and Health Sciences,
Faculty of Medicine and Health Sciences, University Putra Malaysia,
43400, Serdang, Selangor, Malaysia
Tel: 603 89468443 Fax: 603 89426769

or enyoh kilo, kecibeling or kejobeling in Java (Sunarto, 1977) as well as pecah kaca or jin batu in Malaysia. Traditionally, the leaves of *S. crispus* were boiled with water and the filtrates were used in traditional medicine in Malaysia and Indonesia as antidiabetic, diuretic, antilytic and laxative. It is also commonly consumed in the form of herbal tea. This plant has many cystoliths of calcium carbonate and an infusion is mildly alkaline (Perry and Metzger, 1980).

A study indicated that the water extract of *S. crispus* inhibits the proliferation of retrovirus; an agent in viral disease such as Acquired Immune Deficiency Syndrome (AIDS) and adult T-cell Leukemia (Kusumoto *et al.*, 1992). Recent studies also showed that the dried leaves of *S. crispus* contained high antioxidant activity and high amount in mineral including potassium (51%), calcium (24%), sodium (24%), iron (1%) and phosphorus (1%) (Ismail *et al.*, 2000). This leaves also contained high amount of vitamin C, B₁, B₂ as well as other composition such as catechins, caffeine and tannin (Ismail *et al.*, 2000). This study was carried out to expand the utilization of tea from *S. crispus* as nutraceutical health product as anticancer and antioxidant supplements.

Materials and Methods

The research was done at Department of Nutrition and Health Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia from June 2003 to June 2004.

Plant material

The leaves of *S. crispus* were grown and collected from the herb garden of Faculty of Medicine and Health Science, Universiti Putra Malaysia, Serdang Selangor. The herbarium voucher specimen were identified and deposited by Mr. Ahmed Zainuddin from the Department of Botany, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The voucher number of *S. crispus* was AZ-6803. Two types of *S. crispus* leaves were collected, namely the young leaves (the apex to the 5th leaf) and old leaves (8th leaf to the 12 leaf). The 6th and 7th leaves were excluded as they are considered as intermediate leaves.

Preparation of S. crispus fermented and unfermented tea

The fermented tea preparation was adapted and modified from *Camellia theifera* black tea preparation described by Adisewojo (1982). Unfermented tea preparation was adapted and modified from *Camellia sinensis* green tea preparation (Rasmussen and Rhinehart, 1999). The crude yeild of fermented tea from old and young leaves were approximately 29 and 22%, respectively. In the other hand, the crude yeild for unfermented tea from old and young leaves were approximately 22 and 16%, respectively.

Extraction

The extraction for determination of total antioxidant activity was adapted with slight modification from (1997). Briefly, the tea was extracted by pouring boiling distilled water (25 mL) onto the dry leaves (1 g), followed by stirring with a magnetic stirrer for 30 min and additional sleeping for 30 min at room temperature. The mixture was subsequently filtered (Whatman No. 5) on a Buchner funnel and the filtrate was assayed for antioxidant activity. The tea was cooled to room temperature before further use. For *in vitro* antiproliferative properties, the same extraction method was used, but the filtrate was lyophilized (using freeze dryer) into powder. The dried residue was suspended in DMSO (Sigma) for cytotoxicity assay.

Cytotoxicity study

Culturing of cells

MDA-MB-231 (non-hormone dependent breast cancer) and MCF-7 (hormone dependent breast cancer) cell lines were obtained from American Type Culture Collection (ATCC, USA). MDA-MB-231 cell lines were cultured in low glucose Dulbecco's modified Eagle medium (Gibco, USA) and MCF-7 cell lines were grown in RPMI 1640 medium (Gibco, USA). The cells were cultured in their own medium supplemented with 10% of fetal calf serum, 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ of streptomycin (Gibco, USA) using 25 cm² flasks (Nunc, Denmark), in a CO₂ incubator (Sanyo, Japan) at 37°C.

MTT Assay (Roche Diagnostic, USA)

The viability of cells was determined by staining with trypan blue. Exponentially growing cells were harvested and counted by using a haemocytometer. The specific medium for that particular cell line was used to dilute the cells to a concentration of 1x10⁵ cells mL⁻¹. From this cell suspension, 100 µL was pipetted into a 96 well micro titer plate (Nunc, Denmark) and incubated for 24 h in a 5% CO₂ incubator (Sanyo, Japan) at 37°C. Sample extracts in range of doses were added into the plate. The ranges of the doses were 5, 10, 20, 40, 60, 80 and 100 µg mL⁻¹. After adding the samples extract, new medium were added to make up the final volume of 100 µL in each well. The plate was incubated in a 5% CO₂ incubator (Sanyo, Japan) at 37°C for 72 h. Then, 10 µL of MTT reagent (Roche, USA) was added into each well. This plate was incubated again for 4 h in CO₂ incubator (Sanyo, Japan) at 37°C. Subsequently, 100 µL of solubilization solution (Roche, USA) was added into each well. The cell was then left overnight in 37°C, CO₂ incubator. Finally, the absorbance was read with the ELISA reader (LX-800) at 550 nm.

$$\% \text{ cytotoxicity} = \frac{\text{OD sample (mean)}}{\text{OD control (mean)}} \times 100\%$$

OD = optical density

Antioxidant activity

DPPH free radical scavenging activity

This procedure was followed according to (Ismail and Hong, 2002) with modification. An aliquot of 400 µL of samples or control (distilled water) or commercialized tea were mixed with 1600 µL of 100 mM Tris-HCL buffer (pH 7.4). The mixture was then added with 2 mL of 500 µM 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma) in absolute ethanol. The mixture was shaken vigorously and left to stand at room temperature for 20 min in the dark. The mixture was then measured spectrophotometrically at 517 nm. The free radical scavenging activity was calculated as below:

$$\text{Scavenging effect (\%)} = [1 - \{\text{absorbance of sample/absorbance of control}\}] \times 100$$

Ferric reducing/Antioxidant power (FRAP assay)

This method was adapted and modified from Benzie and Strain (1996). The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma) solution and 20 mM FeCl₃.6H₂O in a 10:1:1 ratio prior to use and heated to 37°C in water bath. A total of 3.0 mL FRAP reagent was added to a cuvette and blank reading was then taken at 593 nm using spectrophotometer. A total of 100 µL tea extracts and 300 µL distilled water was then added to the cuvette. After addition of the sample to the FRAP reagent, a second reading at 593 nm was performed after 4 min. The change in absorbance after 4 min from the initial blank reading was then compared with standard curve. Standards of known Fe (II) concentrations were run using several concentrations from 100 to 1000 µM. A standard curve was then prepared by plotting the FRAP value of each standard versus its concentration. The FRAP values for the samples were then determined using this standard curve. The final result was expressed as the concentration of antioxidant having a ferric reducing ability.

Data analysis

All determinations were carried out in six replicates and the data were expressed as mean±SEM.

Results

Cytotoxicity

The hot water extract of *S. crispus* unfermented tea from old leaves and *S. crispus* fermented tea from old leaves displayed cytotoxic effect on human hormone dependent breast carcinoma cell lines (MCF-7) with IC₅₀ value of 80.5 and 72.5 µg mL⁻¹, respectively. However, no IC₅₀ values were obtained from MDA-MB-231 cell lines at the concentration tested.

Antioxidant activity

The calibration curve of FeSO₄.7H₂O standard was used to estimate the FRAP value for determination of antioxidant activity (FRAP assay). The capability of tea extracts to scavenge the DPPH radical was calculated by using the following equation: [1-(absorbance of sample/absorbance of control) x 100]. For both experiments, *S. crispus* teas were compared with commercial tea (green and black tea) (Table 1). The results from both experiments for antioxidant activities showed the same trend with green tea possessed the highest antioxidant activity, followed by black tea, *S. crispus* unfermented tea (old leaves), *S. crispus* unfermented tea (young leaves), *S. crispus* fermented tea (old leaves) and *S. crispus* fermented tea (young leaves). The values were 79.56, 74.27, 63.21, 61.22, 27.58 and 12.59%, respectively in DPPH free radical scavenging activity. On the other hand, for FRAP assay, the values were 56.79, 34.30 mmol L⁻¹, 2091.00, 1305.45, 601.88 and 452.94 µmol L⁻¹, respectively (Table 2).

Table 1: IC₅₀ values of *S. crispus* tea extracts against MCF-7 and MDA-MB-231 cell lines

Samples	MCF-7(µg mL ⁻¹)	MDA-MB-231(µg mL ⁻¹)
<i>S. crispus</i> unfermented tea (young)	>100	>100
<i>S. crispus</i> unfermented tea (old)	80.5	>100
<i>S. crispus</i> fermented tea (young)	>100	>100
<i>S. crispus</i> fermented tea (old)	72.5	>100

Table 2: FRAP and DPPH free radical scavenging value

Samples	FRAP value	DPPH free radical scavenging activity (%)
<i>S. crispus</i> unfermented tea (young)	1305.45±36.67 $\mu\text{mol L}^{-1}$	61.22±0.47
<i>S. crispus</i> unfermented tea (old)	2091.00±188.68 $\mu\text{mol L}^{-1}$	63.21±0.72
<i>S. crispus</i> fermented tea (young)	452.94±28.81 $\mu\text{mol L}^{-1}$	12.59±1.06
<i>S. crispus</i> fermented tea (old)	601.83±8.12 $\mu\text{mol L}^{-1}$	27.58±1.83
Green tea (<i>C. sinensis</i> , Sencha)	56.79±0.57 mmol L^{-1}	79.56±0.28
Black tea (<i>C. sinensis</i> , Boh)	34.30±0.22 mmol L^{-1}	74.27±0.07

Data are presented as mean±SEM

Discussion

Tea is an aqueous infusion of dried leaves of the plants *Camellia sinensis* L. (family Theaceae) and is the most popular beverage consumed by human society worldwide, second only to water (Wu and Wei, 2002). The per-capita consumption worldwide of averages 4 fluid ounces per day (Graham, 1992) and approximately 2.5 million of metric tons of dried teas is produced each year, mainly in India, China, Sri Lanka, Turkey, Russia and Japan.

Leafy materials, such as tea, are well known as rich source of flavonoids and phenolic acids and are recognized as a major source of flavonoids in the diet (Hertog *et al.*, 1993). The chemical composition of tea is complex: polyphenols, catechins, caffeine, amino acids, carbohydrate, proteins, chlorophyll, volatile compounds, fluoride, minerals and other unidentified compounds (Graham, 1992). Meanwhile, the leaf of *S. crispus* contained chemical composition almost the same like *C. sinensis* such as: polyphenols, catechins, caffeine, tannin, alkaloid, potassium, sodium, calcium, iron, phosphorus, carbohydrate, proteins, vitamin C, B₁, B₂ and fiber (Ismail *et al.*, 2000) as well as other bioactive compounds such as β -sitosterol and stigmasterol (Edrini, 2003).

S. crispus, a well-known herb, possesses diverse biological activities and pharmacological functions including antioxidant, antiproliferative, antimicrobial and antihyperglycemic. It has long been used traditionally to treat diabetes mellitus and related disorder. It is also commonly consumed in the form of herbal tea. Although it has been used widely in the treatment of various ailments, scientific data on this plant is still lacking especially its biological activity. Many studies need to be done before it can be promoted for utilization or be commercialized.

The antiproliferative properties of the hot water extracts were determined by using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT)-based cytotoxicity assay. The principle of this assay is based on the reduction of a soluble tetrazolium salt, by mitochondrial dehydrogenase activity of viable tumor cells, into a soluble colored formazan product, which can be measured spectrophotometrically after dissolution (Edrini *et al.*, 2002). The IC₅₀ value (the concentration causing the 50% inhibition of the cell lines) was used as a parameter for cytotoxicity (Smit *et al.*, 1995).

The antiproliferative properties of *S. crispus* tea might be due to its polyphenol content (Ismail *et al.*, 2000) or its bioactive compounds such as β -sitosterol and stigmasterol (Edrini, 2003). Numerous studies have indicated that green tea, black tea and pure tea polyphenols inhibit both ultravioletlight and chemically-induced tumorigenesis in animals (Yang *et al.*, 2001). Tea polyphenols have demonstrated inhibitory activity during the initiation, promotion and progression stages of carcinogenesis.

A study by Ismail *et al.* (2000) showed that the leaves of *S. crispus* contained a moderate amount of other proximate composition such as catechins, alkaloids, caffeine and tannin, contributing further

to the total antioxidant activity. In this study, two methods were applied for determination of antioxidant activity. Both methods showed same trends with extracts of *S. crispus* tea were found to possess antioxidant activity. Green tea (*C. sinensis*) exhibit the highest scavenging effect, followed by black tea (*C. sinensis*), *S. crispus* unfermented tea (old), *S. crispus* unfermented tea (young), *S. crispus* fermented tea (old) and *S. crispus* fermented tea (young).

The antioxidant activity of *S. crispus* tea might be due to the catechin content since this compound has been found in abundance in *S. crispus* leaves (Ismail *et al.*, 2000). In addition, other polyphenol present in the extract, plus vitamins and mineral might contributed to the antioxidant activity of this tea. Green and black tea from leaves of *C. sinensis* represented an excellent source of antioxidant, especially catechin, with green tea being about five times more potent than black tea (Serafini *et al.*, 1996). However, it was reported that oolong tea (semi-fermented tea), displayed stronger antioxidant activity compared to green tea, suggesting that variable in antioxidant activity of the tea might not be completely come from the catechin content alone (Yen and Chen, 1994).

In *S. crispus* tea, unfermented tea showed a better antioxidant and antiradical activities *in vitro*. This condition may be due to the deterioration of phenolic substances during fermentation in fermented tea. In addition, the old leaves showed a better antioxidant and antiradical properties. The higher antioxidant observed in the old leaves might be due to the accumulation of bioactive compounds including phenolic content in the older or more matured leaf physiology. Thus, utilization of herbal tea from *S. crispus* tea especially from old leaves has the potential for large-scale application as natural antioxidant.

The results showed that *S. crispus* tea exhibit high antioxidant activity as well as anticancer properties by inhibit the proliferation of cancer cell lines *in vitro*. Consumption of *S. crispus* tea could contribute to the additional antioxidant needed to enhance defense system in the body, as well as an additional nutraceutical supplement in breast cancer patients. A more detailed investigation focusing on the mechanism of action and active compounds that involved in their antioxidant and anticancer properties is in progress. Clinical study is also needed to verify the efficacy of this tea in human diseases.

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