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Antioxidant and antiproliferative activities of water spinach (*Ipomoea aquatica* Forsk) constituents

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Abstract. The aim of this study was to examine possible antioxidant and antiproliferative activities of 95% ethanol or water extract from water spinach (*Ipomoea aquatica* Forsk) organs. DPPH staining, total phenolic compounds, total flavonoid content, DPPH radical, reducing power method, FTC method, and inhibition of cancer cell proliferation were employed. Ethanol extract of stem demonstrated a positive effect in DPPH staining when it was diluted to 6.25 mg dry matter/mL while all other fractions showed no effect at the same dilution. This fraction also had the highest content of the total phenolic compounds, as well as the highest reducing power and FTC activity. Ethanol extract of leaf had the highest amount of flavonoids. Using DPPH colorimetric method, it was found that ethanol extract of stem had the highest radical-scavenging activity, followed by ethanol extract of leaf. The antiproliferative activities of water spinach extracts were studied in vitro using human lymphoma NB4 cells, and the following results were found: water extract of stem had the highest antiproliferative activity with an EC_{50} of 661.40 ± 3.36 μg dry matter/mL, followed by ethanol extract of stem and ethanol extract of leaf. The water extract of leaf had the lowest antiproliferative activity (EC_{50}>1000 μg dry matter/mL) under the experimental conditions.

Keywords: Antioxidant; Antiproliferative; Free radical; Water spinach.

Abbreviations: **BHT**, butylate hydroxyltoluene; **DPPH**, 1,1-diphenyl-2- picryl hydrazyl; **EDTA**, ethylenediamine tetraacetic acid; **GSH**, glutathione; **FBS**, fetal bovine serum; **FTC**, ferric thiocyanate; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **EC_{50}**, dose with 50% efficiency.

Introduction

It is by now commonly accepted that under situations of oxidative stress, reactive oxygen species such as superoxide (O_2^{-}), hydroxyl (OH^{-}), and peroxyl ($^{\cdot}OOH$, $ROO^{\cdot}$) radicals are generated. These reactive oxygen species play an important role in degenerative or pathological processes, such as aging (Burns et al., 2001), cancer, coronary heart disease, Alzheimer's disease (Ames, 1983; Gey, 1990; Smith et al., 1996; Diaz et al., 1997), neurodegenerative disorders, atherosclerosis, cataracts, and inflammation (Aruoma, 1998). The use of traditional medicine is widespread, and plants are still a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or radical scavenging mechanism as part of their activity (Perry et al., 1999; Lin and Huang, 2002; Repetto and Llesuy, 2002). In searching for novel natural antioxidants, some plants have been extensively studied in the past few years for their antioxidant and radical scavenging components. These include echinacoside in *Echinaceae* root (Hu and Kitts, 2000), anthocyanin (Espin et al., 2000), phenolic compounds (Rice-Evans et al., 1997), water extracts of roasted *Cassia tora* (Yen and Chuang, 2000), and whey proteins (Allen and Wrieden, 1982 a,b; Tong et al., 2000).

The aquatic plant water spinach (*Ipomoea aquatica* Forsk) grows wild and is cultivated throughout Southeast Asia and is a widely consumed vegetable in the region. Many of the waters where *I. aquatica* grows serve as recipients for domestic and other types of waste water. Because these waters contain not only nutrients, but often also a wide variety of pollutants, such as heavy metals from various human activities, many people risk poisoning. Water spinach is also supposed to possess an insulin-like activity according to indigenous medicine in Sri Lanka (Malalavidhane et al., 2000). Only a very few scientific studies have been conducted on its medicinal aspects. These include the inhibition of prostaglandin synthesis (Tseng et al., 1992) effects on liver diseases (Badruzzaman and Husain, 1992), constipation (Samuelsson et al., 1992), and hypoglycemic effects (Malalavidhane et al., 2000).

The objectives of this work were to investigate the antioxidant and antiproliferation property of crude extracts from different tissues of water spinach in comparison with chemical compounds such as butylate hydroxyltoluene or reduced glutathione and the level of inhibition of the growth of cancer cells in a series of in vitro tests.

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Materials and Methods

Materials

BHT, DPPH, EDTA, FTC, MTT, sodium bicarbonate, and Tris (hydroxylmethyl) aminomethane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). FBS, L-glutamine, and RPMI medium 1640 were purchased from Gibco BRL Co. (Gaithersburg, MD, USA). Hydrogen peroxide was purchased from Showa Chemical Co. (Tokyo, Japan). 95% ethanol was purchased from Riedel-deHaen Chemical Co. (Germany).

Plant Material and Sample Preparation

Fresh water spinach (*Ipomoea aquatica* Forsk) was purchased from a local market. One hundred grams of green leaves and veins were cleaned with water and extracted with 500 mL of 95% ethanol or distilled water by stirring at room temperature for 24 h and then filtering through #1 filter paper (Whatman Inc., Hillsboro, OR, USA). The filtrate was concentrated to a powder by freeze dryer (Christ Alpha, Germany) and stored at -20°C. When the experiment was conducted, each plant extract fraction had acquired by the powder (dry matter) a suitable concentration.

Rapid Screening of Antioxidant by Dot-Blot and DPPH Staining

An aliquot (3 μL, 100, 50, 25, 12.5, and 6.25 mg/mL) of a suitable dilution of each plant extract fraction, and GSH (3 μl, 10, 5, 2.5, 12.5 and 6.25 mg/mL) were carefully loaded on a 20 cm × 20 cm TLC layer (silica gel 60 F254; Merck) and dried for 3 min. Drops of each sample were loaded in order of decreasing concentration along the row. The staining of the silica plate was based on the procedure of Soler-Rivas et al. (2000). The sheet bearing the dry spots was placed upside down for 10 sec in a 0.4 mM DPPH solution. The excess solution was then removed with a tissue paper, and the layer was dried with a hair-dryer blowing cold air. The stained silica layer revealed a purple background with white spots at the location of the drops, which showed radical scavenger capacity. The white color intensified according to the amount and nature of radical scavenger present in the sample.

Determination of Total Phenolic Compounds

Total phenolic compounds were determined using the Folin–Ciocalteu method (Ragazzi and Veronese, 1973). One mL of the extract was added to 10.0 mL distilled water and 2.0 mL of Folin–Ciocalteu phenol reagent (Merck-Schuchardt, Hohenbrun, Germany). The mixture was allowed to stand at room temperature for 5 min and then 2.0 mL sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. Catechin was used as a standard for the calibration curve. The phenolic compound contents were calibrated using the linear equation base on the calibration curve. The contents of phenolic compounds were expressed as mg catechin equivalent/g dry weight. The dry weight indicated was water spinach dry weight.

Determination of Total Flavonoid Content

The $AlCl_3$ method (Lamaison and Carnet, 1990) was used for determination of the total flavonoid content of the sample extracts. Aliquots of 1.5 mL of extracts were added to equal volumes of a solution of 2% $AlCl_3 \cdot 6H_2O$ (2 g in 100 mL methanol). The mixture was vigorously shaken, and absorbance at 367 nm was read after 10 min of incubation. Catechin was used as a standard for the calibration curve. The flavonoid contents were calibrated using the linear equation base on the calibration curve. Flavonoid contents were expressed as mg catechin equivalent/g dry weight. The dry weight indicated was water spinach dry weight.

Scavenging Activity Against 1,1-Dipheny-2-Picryl Hydrazyl Radical

The effect of crude extracts on the DPPH radical was estimated according to the method of Yamaguchi et al. (1998). An aliquot of crude extract (20 μL) and GSH (20 μL, 0.04-1.25 mg/mL) was mixed with 100 mM Tris-HCl buffer (120 μL, pH 7.4) and then with 180 μL of the DPPH in ethanol to a final concentration of 250 μM. The mixture was shaken vigorously and left to stand at room temperature for 20 min in the dark. The absorbance at 517 nm of the reaction solution was measured spectrophotometrically. The percentage of DPPH decolorization of the sample was calculated according to the equation: % decolorization= $[1- (ABS_{sample}/ABS_{control})] \times 100$.

Measurement of Reducing Power

The reducing power of the crude extracts GSH and BHT were determined according to the method of Yen and Chen (1995). The crude extract (0, 5, 10, 20, 40, 60, 80, and 100 mg/mL) GSH or BHT was mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, after which an equal volume of 1% trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 5,000 *g* for 10 min. The upper layer of the solution was mixed with distilled water and 0.1% $FeCl_3$ with a ratio of 1 : 1 : 2, and the absorbance at 700 nm was measured. Increased absorbance of the reaction mixture indicated increased reducing power.

Determination of Antioxidant Activity by the Ferric Thiocyanate Method

The FTC method was adopted from Osawa and Namiki (1981). Samples (20 mg/mL) dissolved in 4 mL of 95% (w/v) ethanol were mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 mL), 0.05 M phosphate buffer pH 7.0 (8 mL) and distilled water (3.9 mL) and kept in screw-cap containers at 40°C in the dark. To 0.1 mL of this solution was then added 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was

measured, and it was measured again every 24 h until the time when the absorbance of the control reached the maximum value. The percent inhibition of linoleic acid peroxidation was calculated as: (%) inhibition = 100 - [(absorbance increase of the sample/absorbance increase of the control) × 100]. All tests were run in duplicate, and analyses of all samples were run in triplicate and averaged.

MTT Assay for Cell Proliferation

The NB4 cell line was isolated from long-term cultures of leukemia blast cells on bone-marrow stromal fibroblasts, as reported by Lanotte et al. (1991). NB4 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS in a humidified atmosphere of 5% carbon dioxide. The cells were subcultured every third day, with cell density in cultures kept below 5×10^5 cells/mL.

The colorimetric assay for cellular growth and survival was based on Hansen et al. (1989). Suspensions of human histolytic lymphoma NB4 monocytes (2×10^5 cells/mL) were cultured with or without test samples (at various concentrations in 10 µL of suspension) in a 96-well microplate (90 µL suspension/well). After 72 h, 10 µL of MTT solution was added to each well, and the cells were incubated at 37°C for 4 h. Then, 100 µL of lysis buffer were added to each well, and the cells were again incubated at 37°C for 1 h to dissolve the dark blue crystals. Each well was completely pipetted, and then the absorption at 570 nm of formazan product was measured using a microplate reader. At least three repeats for each sample were used to determine the cell proliferation. The decolorization was plotted against the concentration of the sample extract, and the EC_{50}, the amount of sample necessary to decrease 50% of the absorbance of MTT, was calculated.

Statistical Analysis

Means of triplicates were measured. Student's *t* test was used for comparison between two treatments. A difference was considered to be statistically significant when $p<0.05$.

Results and Discussion

Rapid Screening of Antioxidant by Dot-Blot and DPPH Staining

To make a semi-quantitative visualization possible, different extract fractions from water spinach were detected in the TLC plates by the DPPH staining method. For the rapid screening each diluted sample was applied as a dot on a TLC plate that was then stained with DPPH solution (Figure 1). The appearance of white in the spots has potential value for the indirect evaluation of the different extracts from water spinach in the dot plot (Soler-Rivas et al., 2000, Chang et al., 2002). The method is typically based on the inhibition of the accumulation of oxidized products since the generation of free radicals is inhibited by the addition of antioxidants.

When different extracts from water spinach were analyzed, white spots with strong intensity appeared quickly up to the dilution of 6.25 mg dry matter/mL for ethanol extract of stem (the final amount in the spot: 18.75 µg dry matter), 12.5 dry matter mg/mL for ethanol extract of leaf (final amount: 37.5 µg dry matter), 25 mg dry matter/mL for water extract of stem (final amount: 75 µg dry matter), 50 mg/mL for water extract of leaf. The reduced glutathione was used as a positive control.

Total Phenolic Compounds and Total Flavonoid Content Extracted from Water Spinach

The total phenolic compounds of four different extract fractions were expressed as µmol of catechin equivalent per gram of dry weight (D.W.) (Figure 2). Ethanol extract of stem had the highest phenolic compound content (37.40 ± 1.70 µmol/g D.W.), followed by ethanol extract of leaf (27.60 ± 1.57 µmol/g D.W.) and water extract of leaf (8.70 ± 1.55 µmol/g D.W.). Water extract of stem had the lowest phenolic content (6.10 ± 0.52 µmol/g D.W.). The four extract fractions showed significant differences ($p< 0.05$) in total phenolic compound content.

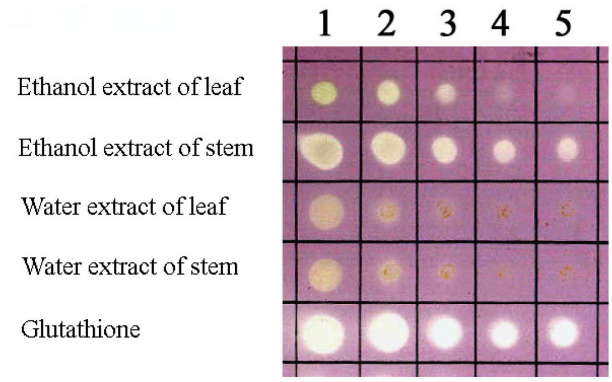

Figure 1. Dot blot assay of different water spinach extracts on a silica sheet stained with a DPPH solution in methanol. Dots from left to right (1, 2, 3, 4, and 5), respectively are: Each 3 µL plant extract (100, 50, 25, 12.5, and 6.25 mg/mL). And each 3 µL GSH (10, 5, 2.5, 12.5 and 6.25 mg/mL).

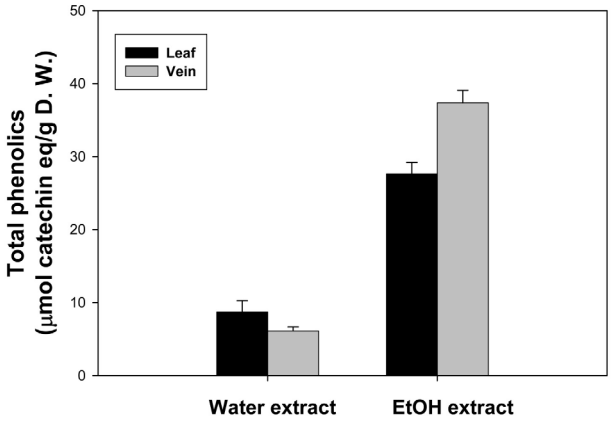

Figure 2. Total phenolic compound content extracted from water spinach. (mean ± SD, n =3).

The total flavonoid compounds of four different extract fractions were expressed also as μmol of catechin equivalent per gram of D.W. (Figure 3). Ethanol extract of leaf had the highest flavonoid content (6.50 ± 0.15 μmol/g D. W.), followed by ethanol extract of stem (4.90 ± 0.09 μmol/g D.W.) and water extract of stem (1.10 ± 0.12 μmol/g D. W.). Water extract of leaf had the lowest flavonoid content (0.10 ± 0.02 μmol/g D.W.). The four extract fractions showed significant differences ($p< 0.05$) in total flavonoid content.

Both ethanol extract of leaf and stem had much higher amounts of both phenolic and flavonoid compounds than water extract fractions. Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity (Yen et al., 1993). The phenolic compounds may contribute directly to antioxidative action (Duh et al., 1999). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when up to 1.0 g is daily ingested from a diet rich in fruits and vegetables (Tanaka et al., 1998). The antioxidative activities observed can be ascribed both to the different mechanisms exerted by different phenolic compounds and to the synergistic effects of different compounds. The antioxidant assay used in this study measures the oxidation products at the early and final stages of oxidation. The antioxidants have different functional properties, such as reactive oxygen species scavenging eg. quercetin and catechin (Hatano et al., 1989), inhibition of the generation of free radicals and chain-breaking activity, e.g. p-coumaric acids (Laranjinha et al., 1995) and metal chelation (Van-Acker et al., 1998). These compounds are normally phenolic compounds, which are effective proton donors, and include tocopherols, flavonoids, and other organic acids.

However, the components responsible for the antioxidative activity of water spinach are currently unclear. Therefore, further work must be performed to isolate and identify these components.

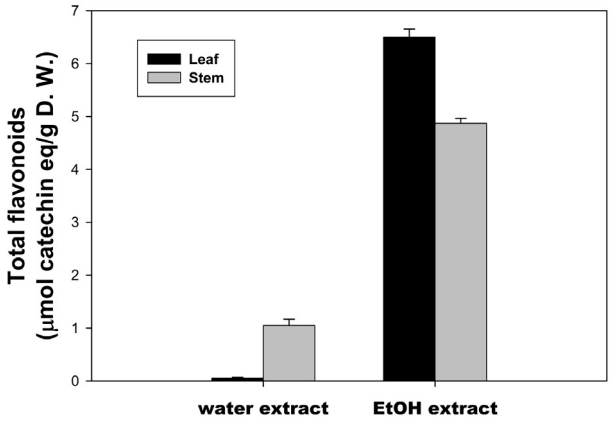

Figure 3. Total flavonoid content extracted from water spinach. (mean ± SD, n =3).

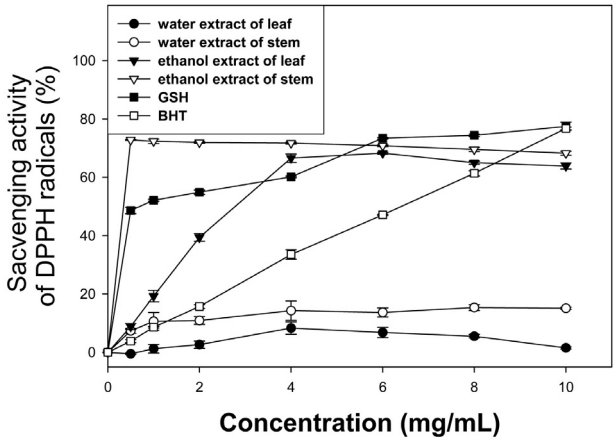

Figure 4. DPPH radical scavenging activity of water spinach extracts. Absorbance values represent means of triplicates of different samples analysed.

Scavenging Activity Against 1,1-Diphenyl-2-Picryl Hydrazyl Radical

The 1,1-diphenyl-2- picryl hydrazyl (DPPH) radical was widely used in the model system to investigate the scavenging activities of several natural compounds such as phenolics and anthocyanins or crude mixtures such as the ethanol extract of plants. DPPH radical is scavenged by antioxidants through the donation of a proton forming the reduced DPPH. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. Radical-scavenging activity increased with increasing percentage of the free_radical inhibition. Figure 4 shows the dose-response curve for the radical-scavenging activity of the different extract fractions of water spinach, GSH and BHT using the DPPH colorimetric method. It was found that in 10 mg dry matter/mL (final concentration) ethanol extract of stem had the highest radical-scavenging activity (68.30 ± 0.66 %), followed by ethanol extract of leaf (63.90 ± 0.06 %), water extract of stem (15.10 ± 0.39 %), and water extract of leaf (0.50 ± 0.37 %). The four extract fractions showed significant differences ($p< 0.05$) in radical-scavenging activity. Ethanol extract of stem (72.80 ± 0.16 %; at a final concentration of 0.5 mg dry matter/mL) and ethanol extract of leaf (68.30 ± 0.34 %; at a final concentration of 6 mg dry matter/mL) have the highest radical-scavenging activity. However, the radical-scavenging activity decreased little as the concentration increased further. The reason is that the interference substance(s) can not donate more protons at critical higher concentrations.

Measurement of Reducing Power

We investigated the reducing capacity of water spinach extracts by measuring the Fe^{3+}-Fe^{2+} conversion. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). The antioxidant activities of putative antioxidants have been attributed to various mechanisms such as pre-

vention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued proton abstraction, and radical scavenging (Diplock, 1997). The reducing power of the different extract fractions from water spinach are shown in Figure 5. Both the reduced GSH and BHT were used as positive controls. The different extract fractions from water spinach exhibited a dose-dependent reducing power activity within concentration range of 0, 0.5, 1, 2, 4, 6, 8, and 10 mg dry matter/mL in the final concentration. Ethanol extract of stem had the highest reducing power, followed by ethanol extract of leaf and water extract of stem. Water extract of leaf had the lowest reducing power. The different extract fractions showed significant differences ($p< 0.05$) in reducing power.

Ferric Thiocyanate Method

Low-density lipoprotein (LDL) peroxidation has been reported to contribute to atherosclerosis development (Steinbrecher, 1987). Therefore, delay or prevention of LDL peroxidation is an important function of antioxidants. Figure 6 shows the time-course plots for the antioxidative activity of the different extract fractions from water spinach and BHT using the FTC method. Ethanol extract of stem had the highest antioxidative activity, followed by ethanol extract of leaf and water extract of stem. Water extract of leaf had lower antioxidative activity than H_2O. Hence, water extract of leaf may contain some compounds that can enhance peroxidation. There were significant differences ($p< 0.05$) among the different extract fractions in antioxidative activity.

Measurement of Cell Proliferation

Antiproliferative activities of the different extract fractions from water spinach on the growth of the human lymphoma NB4 cell line in vitro are summarized in Figure 7. Cell proliferation was analyzed 72 h after NB4 cells had been cultured with an extract fraction of 0, 1.25, 2.5, 5, 10, 20, 30, 40, or 50 µg dry matter/mL in the final concentration using the MTT assay. NB4 cell proliferation was inhibited in a dose-dependent manner after exposure to the different extract fractions. The antiproliferative activities of each fraction were expressed as the median EC_{50}, with a lower EC_{50} value indicating a higher antiproliferative activity. Water extract of stem had the highest antiproliferative activity with the lowest EC_{50} of 661.40 ± 3.36 µg dry matter/mL, followed by ethanol extract of stem (717.10 ± 42.37 µg dry matter/mL) and ethanol extract of leaf (EC_{50}>1000 µg dry matter/mL). The water extract of leaf had the lowest antiproliferative activity (EC_{50}>1000 µg dry matter/mL) under the experimental conditions. Significant differences ($p< 0.05$) in antiproliferative activity appeared among the different extract fractions.

The antioxidant activities of the different extract fractions were directly correlated to the total amount of phenolics and flavonoids found in each fraction, but there was no relationship between antioxidant activity and antiproliferative activity. This experiment suggests that the inhibition of tumor cell proliferation in vitro by the water

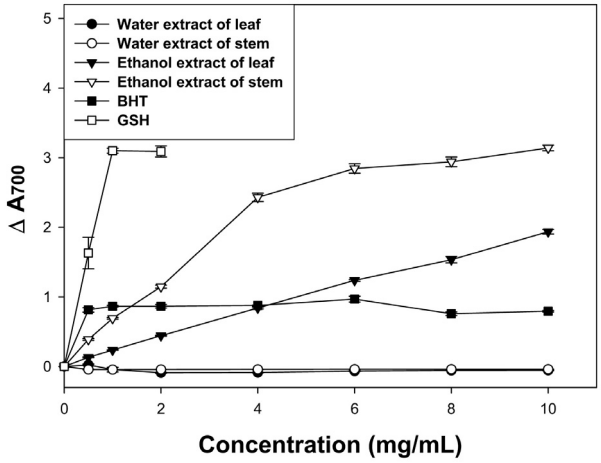

Figure 5. Antioxidative activities of different water spinach extracts as measured by the reducing power method. Absorbance values represent means of triplicates of different samples analysed.

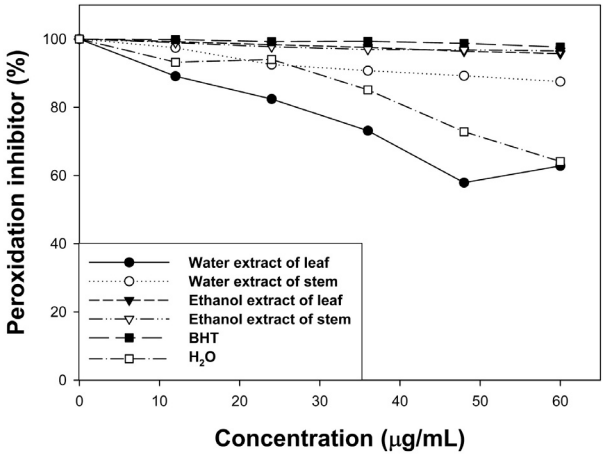

Figure 6. Inhibition of linoleic peroxidation by different water spinach extracts, as measured by the FTC method. Absorbance values represent means of triplicates of different samples analysed.

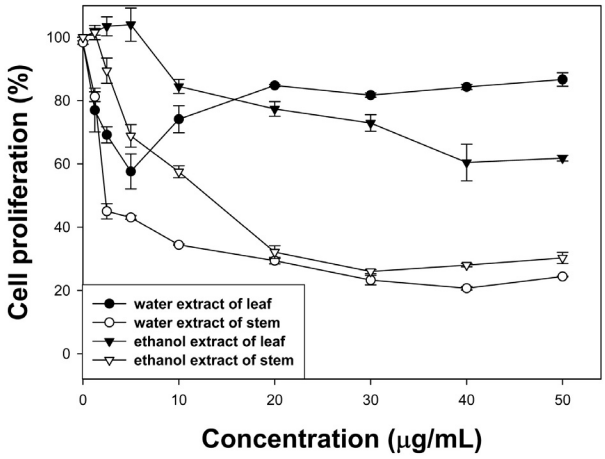

Figure 7. Percent inhibition of NB4 cell proliferation by different extracts from water spinach. Absorbance values represent means of triplicates of different samples analysed.

extract of stem can not be solely explained by the concentration of phenolic/flavonoid compounds. The inhibition of cancer cell proliferation is also attributed to some unknown compound(s) present in the water spinach extracts. Other phytochemicals may play a major role in the antiproliferative activity.

In conclusion, the results from in vitro experiments—including DPPH staining (Figure 1), total phenolic compounds and flavonoid content (Figure 2 and 3), DPPH radical (Figure 4), reducing power method (Figure 5), FTC method (Figure 6), and cell proliferation (Figure 7)—demonstrated that the phytochemicals in water spinach may have a significant effect on antioxidant and anticancer activities. In addition, the antioxidant activity was directly related to the total amount of phenolics and flavonoids found in the water spinach extracts. The additive roles of phytochemicals may contribute significantly to the potent antioxidant activity and the ability to inhibit tumor cell proliferation in vitro. Hence, water spinach can be used as an easy accessible source of natural antioxidants, as a food supplement, or in the pharmaceutical and medical industries. Further work should be performed to isolate and identify the antioxidative or antiproliferative components of water spinach.

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空心菜組成成份具有抗氧化和抗細胞增生的活性

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本研究的目的在分析空心菜（*Ipomoea aquatica* Forsk）不同器官之 95% 乙醇或水的萃取物的抗氧化和抗細胞增生的活性。分析的方法有 DPPH 染色法、總多酚類和黃酮類成份測定、清除 DPPH 自由基之能力、還原力、抑制亞麻油酸過氧化物形成之能力，和抗細胞增殖的活性。在 DPPH 染色法中，當莖之酒精萃取物稀釋到 6.25 mg 乾重/毫升時，仍然具有抗氧化的活性；而在此濃度其它萃取物均已顯示不出抗氧化的活性。在所有的萃取物中，莖之酒精萃取物含有最多的多酚類，表現最高之還原力及 FTC 活性；而在葉子的酒精萃取物中則含有最多的黃酮類成份。在清除 DPPH 自由基之定量時，莖之酒精萃取物表現最佳，其次是葉子的酒精萃取物。在抑制細胞增生方面，使用的細胞為人類白血球 NB4 癌細胞，具有最高的抑制能力者為空心菜莖的水抽液（EC_{50} 為 661.40 ± 3.36 μg dry matter/mL），其次為莖之酒精萃取物和葉子的酒精萃取物。葉子的水萃取物不具有抑制細胞增生的活性 （EC_{50}>1000 μg dry matter/mL）。

關鍵詞：空心菜；抗氧化；抗細胞增生；自由基。