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Antioxidant and Antiproliferative Activities of *Crossostephium chinensis* (L.) Makino

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Abstract: *Crossostephium chinensis* (L.) (CC) Makino is a common traditional Chinese medicinal plant used to dehumidify and cure rheumatism and arthralgia. The water and methanol extracts of *C. chinensis* (CCW and CCM) were evaluated for their antioxidant and antiproliferative activities. The antioxidant activities of CC were evaluated by using ABTS radical scavenging, DPPH radical scavenging, nitric oxide scavenging and superoxide scavenging methods. Iron chelating activity, lipid peroxidation, total polyphenol contents, total flavonoid contents and total flavonol contents were also detected. In all the tested models, both CCW and CCM showed their ability to scavenge the free radicals in a does-dependent manner. CCW had higher antioxidant and antiproliferative activities than CCM. In LC-MS-MS analysis, the chromatograms of CCW with good antioxidant activities were established. Rutin might be an important bioactive compound in CCW. The antiproliferative activities of CCW and CCM were also studied *in vitro* by using human hepatoma HepG2 cells. CCW exhibited good antiproliferative activity. These results indicated that CCW might be used as a potential source of natural antioxidants and as an anti-tumor agent.

Keywords: *Crossostephium chinensis*; Antioxidant Activity; Superoxide; Total Phenols; Radical Scavenging; Antiproliferative; HepG2.

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Introduction

The importance of reactive oxygen species (ROS) and free radicals has attracted increasing attention over the past decades. ROS includes free radicals such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals (OH•) and non-free radical species such as H_2O_2, singlet oxygen ($^1O_2$), and various forms of activated oxygen. These molecules exacerbate factors in cellular injury and aging process (Halliwell, 1989).

ROS is continuously produced during normal physiologic events and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. However, they can be removed by antioxidant defense mechanisms. There is a balance between the generation of ROS and the inactivation of ROS by the antioxidant system in organisms. Under pathological conditions, ROS is overproduced and results in oxidative stress. ROS is formed when endogenous antioxidant defense is inadequate. The imbalance between ROS and antioxidant defense mechanisms leads to oxidative modification in cellular membrane or intracellular molecules (Duh *et al.*, 1999). A lot of antioxidants are introduced to minimize actions of ROS. For example, phenolic compounds can trap the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes (Rao *et al.*, 1996).

Crossostephium chinensis (L.) Makino (CC) of Compositae family, is a famous traditional Chinese medicine (TCM) herb in Taiwan. The plant is also named Hai-Fu-Rung, Qi-Ai and Fu-Rung-Ju. Traditionally, it is used for treating colds, measles, rheumatic arthralgia, epigastric pain, bronchitis, pertussis, pyodermas and mastitis (Chang *et al.*, 2003).

The anti-inflammatory effect of flowers of Hai-Fu-Rung's Composite family had been studied (Akihisa *et al.*, 1996). Previous studies of CC had resulted in the isolation of taraxerol, taraxeryl acetate, and taraxerone (Sasaki *et al.*, 1965). Recent chemical studies of the whole plant of CC had isolated 8 compounds such as taraxeryl acetate, taraxerol, alpha-amyrin acetate, beta- amyrin acetate, beta-sitosterol, 3beta-acetoxy-12-ursen-11-one, uracil and 5-O-methyl-myo- inositol (Yang *et al.*, 2008).

To better understand its efficacy, it is necessary to further investigate the biological activities of CC to find out the curative effect through the antioxidant experiments. The objectives of this work were to investigate the antioxidant and antiproliferative activities of CCW and CCM in comparison with chemical compounds such as GSH, BHT or rutin and the level of inhibition of the growth of cancer cells in a series of *in vitro* tests. With this experiment, we hope to help clinicians to better understand its clinical usage.

Materials and Methods

Materials

1, 1-diphenyl-2-picrylhydrazyl (DPPH), potassium peroxodisulfate ($K_2S_2O_8$), tris (hydroxylmethyl) aminomethane, dibutyl-hydroxy toluene (BHT), glutathione reduced form (GSH), phenazine methosulfate (PMS), trichloroacetic acid (TCA), (+)-catechin, Folin-Ciocalteu solution were purchased from Merck (Darmstadt, Germany). Trolox (6-hydroxy-2, 5, 7,8- tetramethychroman-2-car-boxylic acid), ABTS [2,2′-azinobis

(3-ethyl-benzothiazoline)-6- sulfonic acid] diammonium salt, linoleic acid (99%), nitro blue tetrazolium (NBT), sodium carbonate, rutin, ferric chloride, ferrozine (3-(2-pyridyl)-5, 6-bis (4-phenyl-sulfonic acid)-1, 2, 4- triazine) were purchased from Sigma Chemicals Co. (St. Louis, MO). DMEM and FBS were purchased from Gibco BRL Co. (Gaithersburg, MD, USA). The plant materials were collected from Taichung, Taiwan. They were identified and authenticated by Dr. Yuan-Shiun Chang, Professor of Pharmacognosy, Institute of Chinese Pharmaceutical Sciences, China Medical University. A voucher specimen (NO CMU 2007080102B1) had been deposited in the Institute of Chinese Pharmaceutical Sciences, China Medical University, Taichung, Taiwan.

Water Extracts of Plant Materials

As the general procedure, CC (300 g each) was boiled with 1,000 ml water for 1 hour. It was repeated three times. The collected decoction was evaporated to 50 ml. The concentrated extract was frozen at −80°C for one day and then dried on a lyophilizer for two days. The dried CCW obtained was 10.18 g, the percentage of the yield of extract was about 3.13% (Table 1).

CCW stock solution was prepared at the concentration of 2,000 μg/ml in water. From the stock solution, different concentration levels of 0, 125, 250, 500, and 1 000 μg/ml were prepared and used for antioxidant and inhibition of cancer cell proliferation studies.

Methanol Extracts of Plant Materials

The dried whole herb (100 g each) was macerated with 1,000 ml methanol for 5 days at room temperature. Then it was filtered and extracted two more times with methanol. The methanol extract (3,000 ml) was evaporated to 10 ml and dried in vacuum at 40°C. The obtained CCM was about 69.81 g, the yield of the extract was about 12.98% (Table 1). CCM stock solution was prepared at the concentration of 2,000 μg/ml in methanol. The different concentration of CCM at 0, 125, 250, 500, 1,000 and 2,000 μg/ml, were prepared in methanol and used for antioxidant and inhibition of cancer cell proliferation studies.

Preparation of GSH, BHT and Rutin Stock Solution

GSH, BHT and rutin were used as the standard for the study; the stock solution was prepared at the concentration of 2 000 μg/ml in distilled water, ethanol and methanol respectively. They were prepared freshly and used immediately for the study to avoid spontaneous decomposition. From the stock solution, different concentration of samples at 0, 125, 250, 500, 1,000 and 2,000 μg/ml were prepared in water and methanol and used for antioxidant studies.

ABTS Radical Scavenging

ABTS was dissolved in distilled water to a final concentration of 7 mM, containing 2.45 mM potassium persulphate. This solution was stood at room temperature in dark for 16 hours

to become stable free radical green-blue $ABTS^{+\bullet}$. Then it was diluted with ethanol under 734 nm, until the absorbance of $ABTS^{+\bullet}$ solution was at 0.75 ± 0.05. Different concentration standards of (trolox) 2 μl were added into 20 μl ethanol, followed by 180 μl $ABTS^{+\bullet}$ solution. After the above procedure, the absorbance was measured within one min at 734 nm. The sample solution was detected by the same method. Trolox was used as a reference standard, and results were expressed as TEAC values (mM). These values were obtained from at least three different concentrations of each extract tested in the assay giving a linear response between 20 and 80% of the blank absorbance. Moreover, all analyses were done in triplicate (Re *et al.*, 1999).

Determination of Antioxidant Activity by DPPH Radical Scavenging Ability

An aliquot (20 μl) of CCW or CCM at various concentrations were mixed with 80 μl Tris-HCl buffer (100 mM, pH 7.4) and then with 100 μl of the DPPH in ethanol to a final concentration of 250 μM. The mixture was shaken vigorously and stood 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 in the samples were calculated according to the equation: % decolorization $= [1-(ABS_{sample}/ABS_{control})] \times 100$. IC_{50} value was the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis. A lower IC_{50} value indicated a greater antioxidant activity (Yamaguchi *et al.*, 1998).

Determination of Antioxidant Activity by Nitric Oxide Radical Scavenging Ability

One ml of 25 mM sodium nitroprusside (SNP) in phosphate buffered saline (PBS) was mixed with different concentration of CCW or CCM. After being left at room temperature for 30 min, 0.5 ml reaction solution was added to 0.3 ml of 1% sulfanilamide and 5% H_3PO_4 solution and 0.3 ml 1% N-(1-naphthyl)-ethylenediamine dihydrochloride water solution. These solutions were mixed and the absorbance was recorded at 570 nm (Marcocci *et al.*, 1994).

Determination of Antioxidant Activity by Superoxide Scavenging Ability

Thirty μl sample or standard was added to 30 μl of 300 μM NBT/0.1M PB. The solution was later mixed with 30 μl of 936 μM NADH/0.1M PB and 30 μl of 120 μM PMS/0.1M PB. The mixture was stood at room temperature for 5 min. The absorbance was recorded at 560 nm (Nishikimi *et al.*, 1972).

Determination of Antioxidant Activity by Iron Chelating Activity

One hundred and eighty five μl MeOH was added into 50 μl sample or standard, followed by 5 μl of 2 mM $FeCl_2 4H_2O$. The solutions were mixed and reacted for 30 sec, and then 10 μl

of 5 mM ferrozine solution was added and waited for 10 min. The absorbance was recorded at 562 nm (Benzie and Szeto, 1999).

Determination of Antioxidant Activity by Lipid Peroxidation Assay

Four ml linoleic acid emulsion was mixed with 20 μl sample extract of different concentrations at 40°C in the dark. This mixture was added to 100 μl FTC method solution, 200 μl of 25% TCA and 200 μl of 1% TBA. The solution was maintained at 100°C for 10 min, and then centrifuged at 3,000 rpm for 20 min. The absorbance was recorded at 532 nm (Liegeois *et al.*, 2000).

Determination of Total Polyphenol Content

Twenty μl of each extract (125 μg/ml) was added to 200 μl distilled water and 40 μl of Folin- Ciocalteu reagent. The mixture was allowed at room temperature for 5 min and then 40 μl of 20% 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 polyphenol content was calibrated using the linear equation based on the calibration curve. Total polyphenol content was expressed as mg (+)-catechin equivalent/g dry weight (Ragazzi and Veronese, 1973).

Determination of Total Flavonoid Content

Aliquots of 1.5 ml of extracts were added to equal volumes of a solution of 2% $AlCl_3 \cdot 6H_2O$ (in methanol). The mixture was vigorously shaken, and the absorbance at 430 nm was read after 10 min of incubation. Rutin was used as a standard for the calibration curve. Total flavonoid content was calibrated by using the linear equation based on the calibration curve. Total flavonoid content was expressed as mg rutin equivalent/g dry weight. The dry weight indicated was the sample dry weight (Xu and Chang, 2008).

Determination of Total Flavonol Content

Aliquots of 200 μl of extracts were added to 1 ml of 0.1% *p*-dimethylaminocinnamaldehyde (DMACA) in methanol/HCl (3:1, v/v). The mixture was vigorously shaken, and the absorbance at 640 nm was read after 10 min of incubation. (+)-Catechin was used as a standard for the calibration curve. The total flavonol content was calibrated by using the linear equation based on the calibration curve. The total flavonol content was expressed as mg (+)-catechin equivalent/g dry weight. The dry weight indicated was the sample dry weight (Arnous *et al.*, 2001).

Culture and Harvest of Human Hepatoma Cell Line

HepG2 cells were cultured with DMEM with 10% FBS in a T75 flask at 37°C, 5% CO_2, and 90% relative humidity. To cells, HepG2 cells were harvested by washing with PBS buffer

and treated with 4 ml of trypsin-EDTA for 3 min. The reaction was terminated by adding 8 ml of DMEM with 10% FBS. The mixture was then transferred into a tube and centrifuged at 200 g at room temperature for 5 min. After removing the supernatant, cell pellet was resuspended in 4 ml of DMEM with 10% FBS.

MTT Assay for Cell Proliferation

The colorimetric assay for cellular growth and survival was studied previously (Hansen *et al*., 1989). Suspensions of human HepG2 cells (2×10^5 cells/ml) were cultured with or without the test samples (at various concentrations in 10 μl of suspension) in a 96-well microplate (90 μl suspension/well). After 72 hours, 10 μl of MTT solution was added to each well, and the cells were incubated at 37°C for 4 hours. Then, 100 μl of lysis buffer were added to each well, and the cells were incubated at 37°C for 1 hour to dissolve the dark blue crystals. Each well was completely pipetted, and the absorption at 570 nm of formazan product was measured by a microplate reader. Each sample was repeated in order to determine the cell proliferation. The decolorization was plotted against the concentration of CCW and CCM. The IC_{50}, the amount of the sample necessary to decrease 50% of the absorbance of MTT, was calculated.

Analysis of Rutin, CCW and CCM by LC-MS-MS

Moderate amounts of CCW and CCM were weighed and dissolved in water and methanol respectively. At first, the solutions were filtered through 0.45 μm PVDF filters. The LC-MS-MS (Waters 2695 separations module; detector: Waters 996 photodiode array detector; with a ES-D609 mass spectrometer) analysis was carried out as follows: the Waters Cosmosil 5C18-AR-II column (5 μm, 4.6 $\times$ 150 mm) was used with 0.25% methanol as mobile phase A, acetonitrile was used as mobile phase B, and water was used as mobile phase C. The percentage of A: B: C was 20: 20: 60, the gradient elution was run at a flow rate of 0.5 ml/min. The injection volume was 10 μl, and a wavelength of 254 nm was used for detection. Pure compounds, including rutin, were also analyzed under the same conditions, and the retention time was used to identify the flavonoids in the samples.

Statistical Analysis

Experimental results were presented as the mean $\pm$ standard deviation (SD) of three parallel measurements. The statistical analyses were performed by one-way ANOVA, followed by Dunnett's t-test. The difference was considered to be statistically significant when $p < 0.05$.

Results

Extraction Yields

The yields of CCW and CCM are shown in Table 1. The percentage of CCW and CCM were 3.13% and 12.98% respectively.

Table 1. The Yield of Water and Methanol (CCW, CCM) Extracts from *Crossostephium chinensis* (L.) Makino

	Yield (g)*	Extraction (%)
CCW	10.18 ± 0.01	$3.13^{\dagger}$
CCM	69.81 ± 0.02	12.98

*Extracted from air dried materials. Each value is expressed as mean $\pm$ SD ($n = 3$).
$^{\dagger}p < 0.05$ within a column.

Antioxidant Activity Estimated by ABTS Assay

ABTS assay was expressed as a TEAC value. A higher TEAC value indicated that the sample had a stronger antioxidant activity. TEAC values determined from the calibration curve for CCW, CCM and rutin are shown in Table 2. It was observed that CCW (0.46 ± 0.02 mM) had higher antioxidant potencies than CCM (0.15 ± 0.01 mM) while the antioxidant potency of rutin was 4.83 ± 0.65 mM.

DPPH Radical Scavenging

The IC_{50} values of five different concentrations from CCW and CCM are shown in Fig. 1. The IC_{50} value for GSH was 88.11 ± 2.08 μg/ml, for BHT was 67.11 ± 0.76 μg/ml, and for rutin was 10.65 ± 0.05 μg/ml. The IC_{50} value for CCW was 177.40 ± 0.75 μg/ml and for CCM was 1060.88 ± 3.98 μg/ml. This result showed that CCW had a better antioxidant activity in DPPH scavenging ability then CCM.

Nitric Oxide Radical Scavenging

The results from nitric oxide radical scavenging with different concentrations are shown in Fig. 2. The IC_{50} value of GSH was 357.73 ± 0.22 μg/ml, BHT was 380.41 ± 0.12 μg/ml, and rutin was 121.11 ± 0.07 μg/ml. The IC_{50} value of CCW and CCM were more than 2,000 μg/ml. It showed that the nitric oxide scavenging activities were in the order of rutin $>$ GSH $>$ BHT $>$ CCW $\fallingdotseq$ CCM (Fig. 2).

Table 2. Total Antioxidant Activity Determined by TEAC

Sample and Positive Control	TEAC (mM)*
CCW	0.46 ± 0.02
CCM	0.15 ± 0.01
Rutin	4.83 ± 0.65

All values expressed as mean $\pm$ SD of triplicate tests.
*$p < 0.05$ among them when analyzed by ANOVA and Ducan's multiple-range tests.

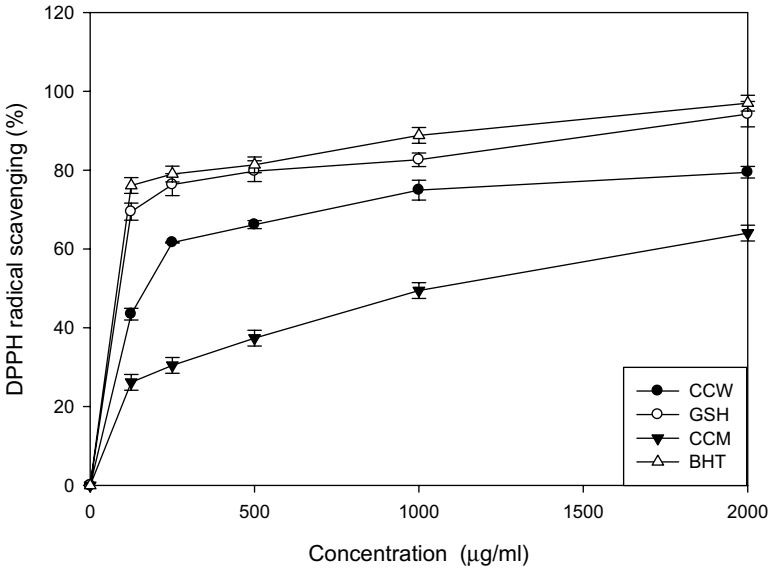


Figure 1. Antioxidant activities of GSH, BHT and CCW, CCM at different concentrations in DPPH radical scavenging. The results are expressed in terms of concentration vs. % scavenging. Each value represents mean ± SD.

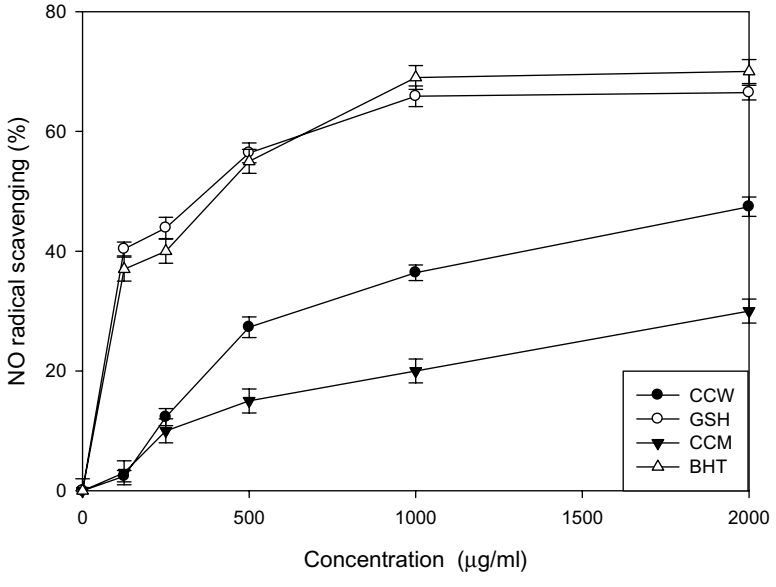


Figure 2. Antioxidant activities of GSH, BHT and CCW, CCM at different concentrations in NO radical scavenging. The results are expressed in terms of concentration vs. % scavenging. Each value represents mean ± SD.

Superoxide Scavenging

Figure 3 shows the results from superoxide scavenging with different concentrations. The IC_{50} value of GSH was $167.46 \pm 1.25\ \mu$g/ml, BHT was $117.7 \pm 0.01\ \mu$g/ml, and rutin was $57.07 \pm 0.12\ \mu$g/ml. It was observed that the IC_{50} value of CCW ($342.51 \pm 1.71\ \mu$g/ml) had higher superoxide scavenging potencies than CCM ($697.98 \pm 0.87\ \mu$g/ml) (Fig. 3).

Iron Chelating Activity

The IC_{50} value for GSH was $180.05 \pm 0.75\ \mu$g/ml, $236.39 \pm 3.33\ \mu$g/ml for BHT, and $98.76 \pm 0.01\ \mu$g/ml for rutin. It was observed that CCW ($386.26 \pm 2.31\ \mu$g/ml) had a higher iron chelating activity than CCM ($> 2{,}000\ \mu$g/ml) (Fig. 4).

Lipid Peroxidation Assay

Lipid peroxidation activities of CCW, CCM and standard compounds were determined by the ferric thiocyanate method in the linoleic acid system (Fig. 5). The IC_{50} value of GSH was $286.15 \pm 0.01\ \mu$g/ml, BHT was $129.69 \pm 0.24\ \mu$g/ml, and rutin was $32.97 \pm 0.13\ \mu$g/ml. The IC_{50} value of CCW was $499.09 \pm 0.02\ \mu$g/ml, and CCM was $591.88 \pm 0.05\ \mu$g/ml. This result suggested that rutin > BHT > GSH > CCW > CCM in the order of lipid peroxidation assay. The antioxidant properties were summarized in Table 4, and the results were normalized and expressed as an IC_{50} value.

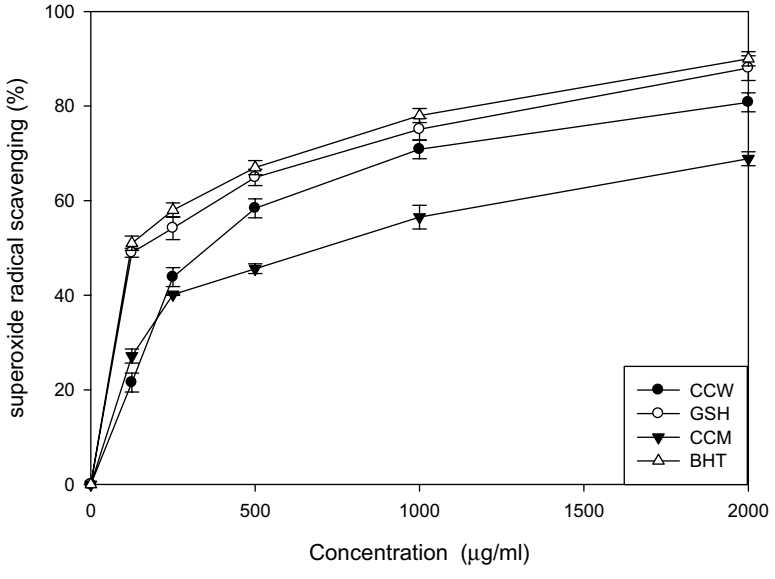


Figure 3. Antioxidant activities of GSH, BHT and CCW, CCM at different concentrations in superoxide radical scavenging. The results are expressed in terms of concentration vs. % scavenging. Each value represents mean ± SD.

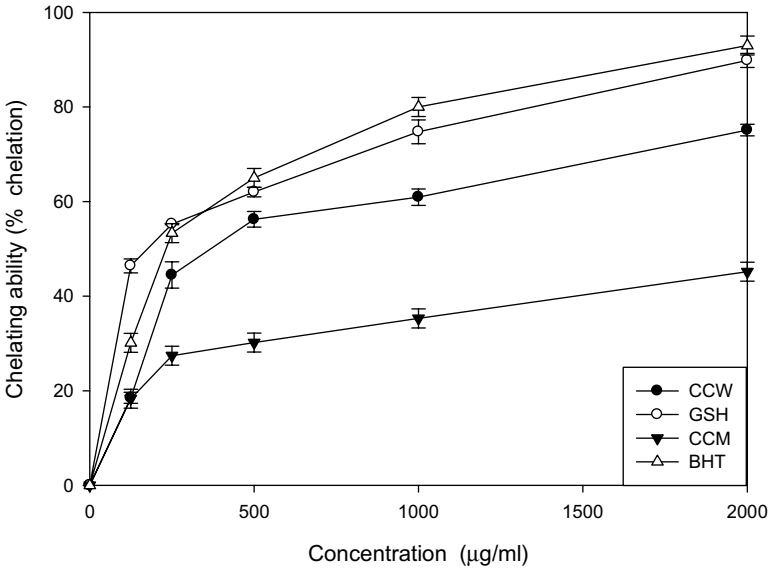


Figure 4. Chelating ability of CCW, CCM, GSH and BHT by spectrophotometric detection of Fe^{3+}–Fe^{2+} transformation. The results are expressed in terms of concentration vs. % chelating. Each value represents mean ± SD.

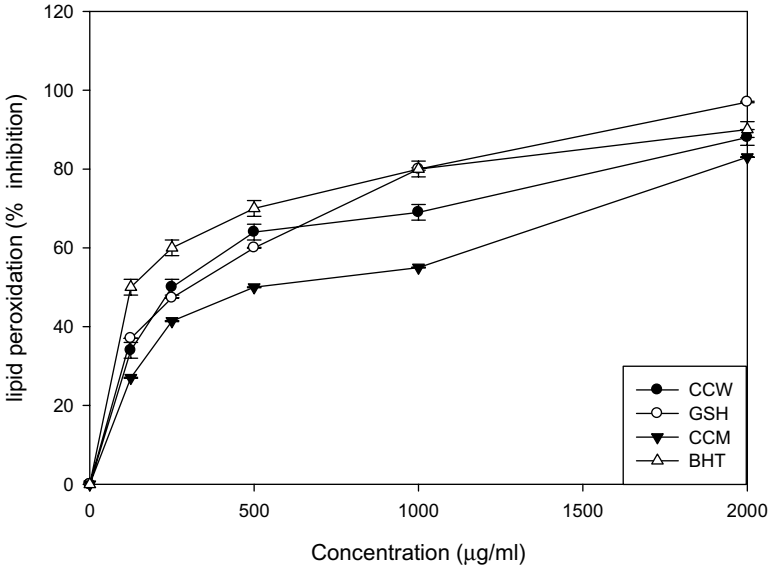


Figure 5. Antioxidant activities of GSH, BHT and CCW, CCM at different concentrations in lipid peroxidation assay. The results are expressed in terms of concentration vs. % inhibition. Each value represents mean ± SD.

Total Polyphenol, Flavonoid, and Flavonol Contents

The total polyphenol, flavonoid, and flavonol contents of CCW and CCM are shown in Table 3. The total polyphenol content was expressed as μg of (+)-catechin equivalent per mg of dry weight. The total polyphenol content of CCW was 145.57 ± 0.11 μg Ce/mg, while CCM was 89.97 ± 1.02 μg Ce/mg; therefore CCW had higher polyphenolic content than CCM.

The total flavonoid content was expressed as μg of (+)-rutin equivalent per mg of dry weight. The total flavonoid content of CCW was 98.27 ± 3.55 μg/mg, CCM was 64.90 ± 0.35 μg/mg, indicating that CCW had higher flavonoid content than CCM.

The total flavonol content was expressed as μg of (+)-catechin equivalent per mg of dry weight. The total flavonol content of CCW was 21.25 ± 0.05 μg/mg, CCM was 15.57 ± 0.11 μg/mg. CCW had higher flavonol content than CCM.

Measurement of Cell Proliferation

The antiproliferative activities of the different concentrations from CCW, CCM and rutin on the the human hepatoma G2 cell line *in vitro* were summarized in Fig. 6 and Table 5. CCW had a better antiproliferative activity than CCM. The IC_{50} for CCW was 436.01 ± 5.64 μg/ml, and was 860.44 ± 0.22 μg/ml for CCM. The IC_{50} of rutin was more than 1,000 μg/ml. It was shown that CC had better antiproliferative activities than rutin under the experimental

Table 3. Contents of Phytochemicals in CCW, CCM*

	Contents	
Phytochemical	**CCW**	**CCM**
Total phenols	$145.57 \pm 0.11^{\dagger}$	$89.97 \pm 1.02^{\dagger}$
Total flavonoids	$98.27 \pm 3.55^{\ddagger}$	$64.90 \pm 0.35^{\ddagger}$
Total flavonols	$21.25 \pm 0.05^{\dagger}$	$15.57 \pm 0.11^{\dagger}$

*All data are expressed as mean $\pm$ SD of triplicate tests. ($p < 0.05$).
†Data expressed in μg (+)-catechin/mg dry weight (μg CE/mg).
‡Data expressed in μg (+)-rutin/mg dry weight (μg rutin/mg).

Table 4. IC_{50} Value of CCW, CCM of *Crossostephium chinensis* (L.) Makino in Antioxidant Studies

	IC_{50} Value (μg extract/ml)				
	GSH	**BHT**	**Rutin**	**CCW**	**CCM**
DPPH Radicals	88.11 ± 2.08*	67.11 ± 0.76	10.65 ± 0.05	177.40 ± 0.75	1060.88 ± 3.98
Nitric Oxide Radicals	357.73 ± 0.22	380.41 ± 0.12	121.11 ± 0.07	> 2,000	> 2,000
Superoxide Scavenging	167.46 ± 1.25	117.70 ± 0.01	57.07 ± 0.12	342.51 ± 1.71	697.98 ± 0.87
Ferrous Iron	180.05 ± 0.75	236.39 ± 3.33	98.76 ± 0.01	386.26 ± 2.31	> 2,000
Lipid Peroxidation Assay	286.15 ± 0.01	129.69 ± 0.24	32.97 ± 0.13	499.09 ± 0.02	591.88 ± 0.05

*Each value is expressed as mean $\pm$ SD ($n = 3$). Means with different letters within a row are significantly different ($p < 0.05$).

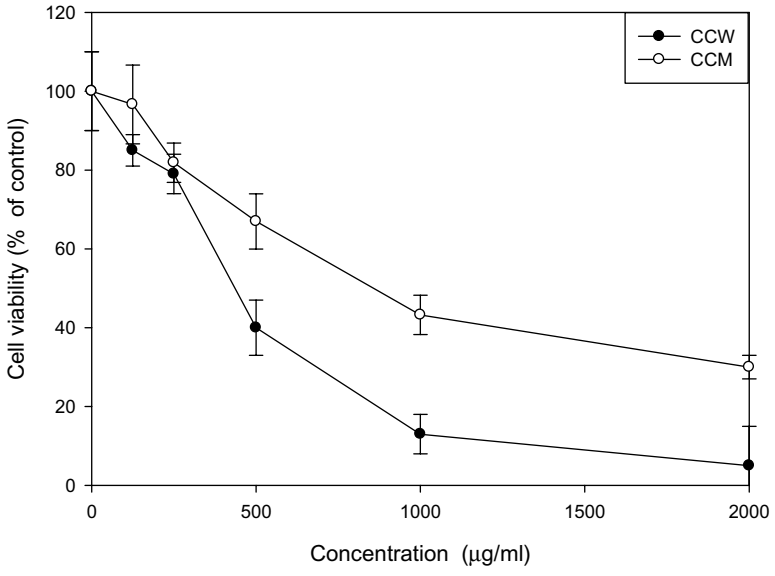


Figure 6. The percentage inhibition of Hep G2 cell proliferation by CCW and CCM at different concentration. Each value represented mean ± SD of three parallel measurements ($p < 0.05$).

Table 5. Effect of CCW and CCM on Inhibiting Hep G2 cell Proliferation*

CCM (μg/ml)	CCM (μg/ml)	Rutin
436.01 ± 5.64	860.44 ± 0.22	> 1,000

*Values represented as mean ± SD of IC_{50} ($n = 3$, $p < 0.05$).

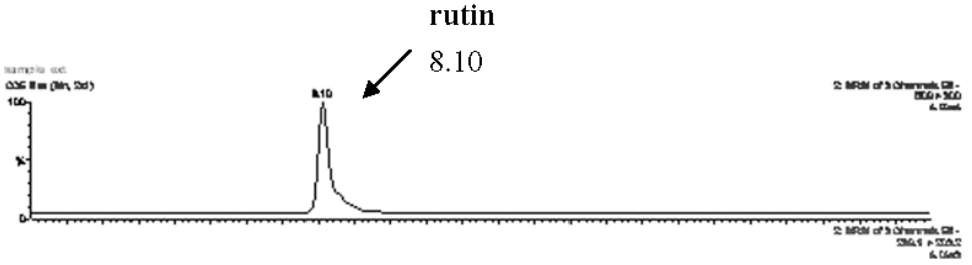
conditions. The antiproliferative activities of CCW and CCM were significantly different from rutin ($p < 0.05$).

Compositional Analysis of Rutin, CCW and CCM by LC-MS-MS

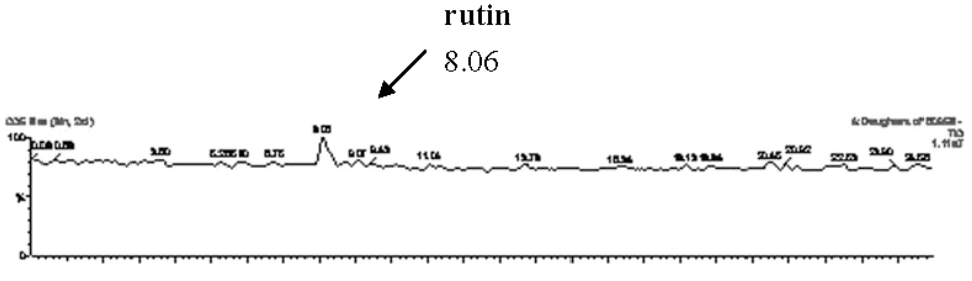
This study indicated that CCW had good antioxidant activities and higher polyphenolic compound content. The prepared CCs and rutin were analyzed by LC-MS-MS and their chromatograms were shown in Fig. 7. It is shown that CCW contained rutin.

Discussion

ABTS assay are often used in evaluating antioxidant power of single compound and complex mixtures of various plants. In this assay, ABTS radical monocation was generated directly in stable form from potassium peroxodisulfate. Generations of radical before the antioxidants were added to prevent the interference of compounds, which affected radical formation. This modification made the assay less susceptible to artifacts and prevented overestimation



(A)



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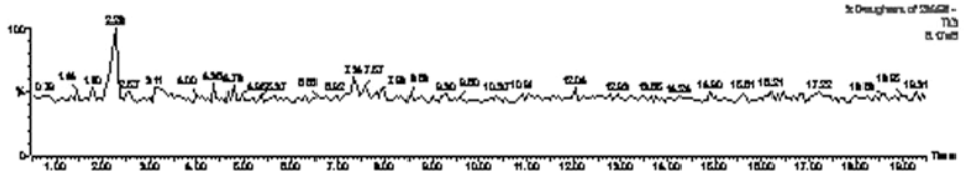
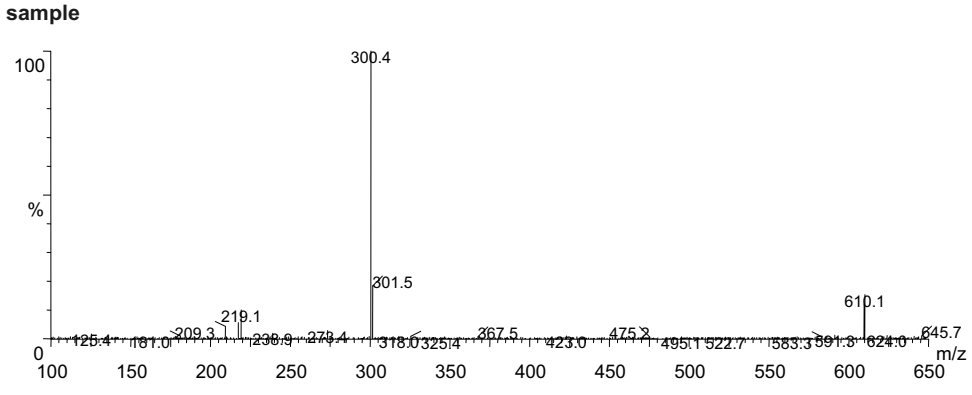
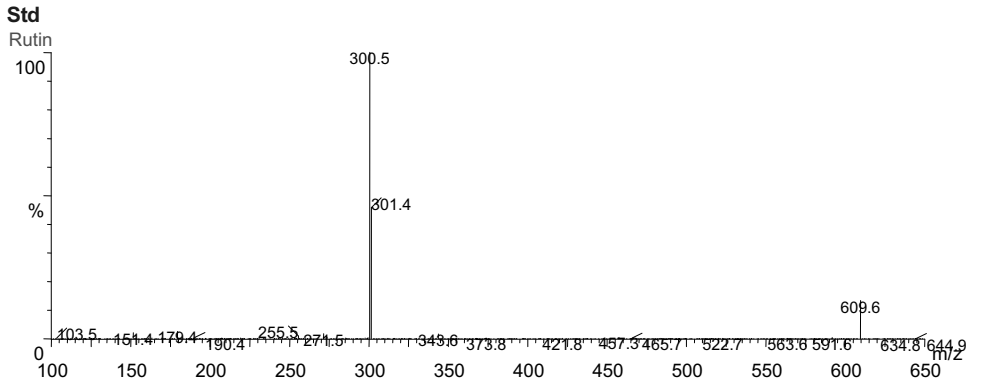


(C)



(D)

Figure 7. The chemical profile of CC was analyzed by LC-MS-MS. (A) Chromatographic patterns from HPLC analysis (254 nm) of rutin. (B) Chromatographic patterns from HPLC analysis (254 nm) of sample CCW. (C) Chromatographic patterns from HPLC analysis (254 nm) of sample CCM. (D) The main product peak (with a retention time of 8.10 min) shown in (A) was then subjected to mass spectrometer. (E) The main product peak, rutin, was subjected to mass spectrometer.



(E)

Figure 7. (*Continued*)

of antioxidant power (Brand-Williams *et al.*, 1995; Long *et al.*, 2000). ABTS assay was expressed as TEAC value. The TEAC values for CCW and CCM indicated that CCW had higher antioxidant potencies than CCM.

DPPH• is used to study free radical scavenging effects of natural materials, such as polyphenolic compounds, anthocyanins and some extracts from TCM. However, a decrease or an absence of absorbance was observed after adding antioxidant reagents (Dorta *et al.*, 2008). The IC_{50} values of five different concentrations from CCW and CCM were determined. It suggested that CCW had a better antioxidant activity in DPPH scavenging ability.

Nitric oxide synthase inhibitor has shown to have beneficial effects on some aspects of inflammation and tissue changes as seen in models in inflammatory bowel disease (Govindarajan *et al.*, 2003; Kirby and Schmidt, 1997). Therefore, we studied the effects of CC on inflammation in mice. The result indicated that CCW was similar to CCM in nitric oxide radical scavenging. Therefore, we may raise the concentrations of the extracts to obtain obviously different results.

The superoxide anion radical (O_2•−) scavenging activity of CCW was better than that of CCM. The IC_{50} values of scavenging activity were increased with the higher concentration. A previous study demonstrated that relatively smaller molecular components (MW $<$ 10,000) contributed to the strong O_2•− scavenging activity (Noda and Mori, 2007). The hydrophilic compounds which led to the strong activity have not been identified. The present study, together with the earlier findings described above strongly suggest that a comprehensive analysis of the chemical compounds in CCW should be carried out in the future.

Iron stimulates lipid peroxidation by the Fenton reaction and accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals which themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidantion. The observed results which demonstrated the marked capacity of CC for iron binding suggested that its reaction as a peroxidation protector may be related to its iron binding capacity. The IC_{50} value showed that CCW had a higher iron chelating activity than CCM. Therefore, ferrous

ions are the most effective and commonly found pro-oxidants in the food system (Yamaguchi *et al.*, 1988; Shimada *et al.*, 1992).

Fe^{3+} ion also produces radicals from peroxides though the rate is 10-fold, which is less than that of Fe^{2+} ion. Fe^{2+} ion is the most powerful pro-oxidant among the various species of metal ions. Ferrozine can quantitatively form complexes with Fe^{2+}. Therefore, the measurement of color reduction allowed the estimation of the metal chelating activity of the coexisting chelator.

Metal chelating capacity was significant because it reduced the concentration of the catalyzing transition metal in lipid peroxidation (Halliwell and Gutteridge, 1984). It was reported that chelating agents were effective as secondary antioxidants because they reduced the redox potential thereby stabilizing the oxidation of the metal ion.

Malondialdehyde formed from the breakdown of polyunsaturated fatty acids served as a convenient index for determining the extent of lipid perxoidation reaction. The antioxidant of CCW, CCM and standards was determined using the ferric thiocyanate method (Mitsuda and Yuasumoto, 1996). The ferric thiocyanate method measuring the amount of peroxide produced during the initial stages of oxidation was the primary product of oxidation. Lipid peroxidation activities of CCW, CCM and standard compounds were determined by the ferric thiocyanate method in the linoleic acid system. This result suggested that CCW > CCM in lipid peroxidation assay. The antioxidant properties assayed herein were summarized, and the results were normalized and expressed as IC_{50} value.

Traditionally, CC has been boiled with hot water extract or with other forms, and thus produces aqueous extract. This study exhibited a good protective activity against free radicals damaging at a concentration level of 100–250 μg/ml from CCW. The result obtained from LCMSMS suggested that CCW contained rutin while CCM did not. In conclusion, these findings indicated that CCW may contain strong antioxidant complex.

The total polyphenol, flavonoid, and flavonol contents in CCW were higher than in CCM. Both flavonoid and flavonol are polyphenolic compounds. Polyphenolic compounds play 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 human beings when up to 1.0 g is absorbed daily from fruits and vegetables (Tanaka *et al.*, 1998). The antioxidative activities observed could be ascribed both to the different mechanisms exerted by different phenolic compounds and to the synergistic effects of different compounds. The antioxidant assays used in this study measured the oxidation products at the early and final stages of oxidation. The antioxidants have different functional properties, such as reactive oxygen species scavenging, e.g. quercetin, rutin, and catechin (Hatano *et al.*, 1989; Liu *et al.*, 2008); 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, including tocopherols, flavonoids, and other organic acids. However, the components which are responsible for the antioxidative activity in CC are still unclear. Therefore, further research must be performed to isolate and identify these components.

The cell proliferation was analyzed 72 hours after Hep G2 cells had been cultured with a different concentration of extract fraction by using the MTT assay. Hep G2 cell proliferation was inhibited in a dose-dependent manner after the exposure to the extract fractions. The antiproliferative activities of each fraction were expressed as the median IC_{50}. CCW had a better antiproliferative activity than CCM.

Assessing antioxidant vitamins and phytochemicals, such as polyphenols, flavonoids, and other bioactive compounds is a tool to explore potential benefits of plant foods on human health, on the basis of the putative role of these components in the prevention of a number of degenerative and age-related disorders (Tesoriere *et al.*, 2007). This study indicated that CCW had good antioxidant activities and higher polyphenolic compounds. The prepared CC under the investigation was analyzed under the same conditions for rutin contents by LCMSMS method. It indicated that CCW contained rutin while CCM did not. The results revealed that CCW contains rutin, possesses higher antioxidant activities in each model tested, including ABTS radical scavenging, DPPH radical scavenging, nitric oxide scavenging, superoxide scavenging methods, iron chelating activity, and lipid peroxidation. CCW showed better antioxidant activity than CCM. Rutin, a glycoside comprised of flavonol quercetin and disaccharide rutinose, is widely distributed in fruits and vegetables and shows remarkable antioxidant, anti-inflammatory and anticancer activities (Guardia *et al.*, 2001; Choi *et al.*, 2007). It also has relaxing effects on smooth muscles (Ahmed *et al.*, 2007; Liu *et al.*, 2008). In DPPH assay, we found that rutin had much lower IC_{50} value (10.65 ± 0.05 μg/ml) than all the other samples. However, rutin had no significant effect on antiproliferative activities in this study, contrarily to the study by Ramos *et al.* (2008) using HepG2 cells, in which different cell culture conditions were used. Nevertheless, in an *in vivo* situation, dietary rutin can still play a role in chemoprevention, since it is known that it can be deglycosylated to form quercetin in the intestine by colon microflora (Kuo, 1996). For this reason, rutin might be an important component in the antioxidant activity of CCW. Previous chemical studies of CC had resulted in the isolation of taraxerol, taraxeryl acetate, and taraxerone (Sasaki *et al.*, 1965). Therefore, it would be worthwhile to look into the phytochemicals of the genus *Crossostephium*.

In conclusion, the results from *in vitro* experiments, including total antioxidant activity assessed by TEAC (Table 2), DPPH radical scavenging (Fig. 1), nitric oxide scavenging (Fig. 2), superoxide scavenging methods (Fig. 3), iron chelating activity (Fig. 4), lipid peroxidation (Fig. 5), total polyphenol content, total flavonoid content and total flavonol content (Table 3), IC_{50} value of CCW and CCM in all antioxidant properties (Table 4), inhibition of cancer cell proliferation (Fig. 6 and Table 5), and LCMSMS assay (Fig. 7), demonstrated that the phytochemicals in CC might have a significant effect on antioxidant and anticancer activities, which are directly related to the total amount of polyphenols and flavonoids found in CCW and CCM. The additive roles of phytochemicals might significantly contribute to the potent antioxidant activity and the ability to inhibit tumor cell proliferation *in vitro*. Hence, CC could be used as an easily accessible source of natural antioxidants in pharmaceutical and medical industries. For this reason, further work should be performed to isolate and identify the antioxidative or antiproliferative components of CC.

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