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Antioxidant and antiproliferative activities of the four *Hydrocotyle* species from Taiwan

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ABSTRACT. The aim of this study was to examine the possible antioxidant and antiproliferative activities of the ethanol and water extracts of four *Hydrocotyle* species from Taiwan. ABTS radical monocation scavenging, FRAP method, DPPH radical scavenging, reducing power method, total polyphenol content, total flavonoid content, total flavonol content, and inhibition of cancer cell proliferation methods were employed. The results showed that the water extracts of all the samples had higher antioxidant and antiproliferative activities than the ethanol extracts. All tested extracts were weaker than the positive controls (BHT and GSH) in the antioxidant activity. We also found that the water extracts of all the samples had higher content of polyphenol compounds, but lower content of flavonoid compounds than the ethanol extracts. In ABTS radical scavenging assay, the TEAC (trolox equivalent antioxidant capacity) values of the water extracts samples were in descending order: *H. nepalensis* (HN) > *H. setulosa* (HSe) > *H. batrachium* (HB) > *H. sibthorpioides* (HSi). The correlation coefficient (R^2) values of TEAC and total polyphenol content showed a higher correlation (water extracts, R^2=0.934; ethanol extracts, R^2=0.904). The R^2 values of TEAC and total flavonoid content for the water and ethanol extracts were 0.995 and 0.785 respectively. The R^2 values of TEAC and FRAP also showed a higher correlation (water extracts, R^2=0.984; ethanol extracts, R^2=0.971). In HPLC analysis, the chromatograms of the water and ethanol extracts of HN with the highest antioxidant activity were established. Rutin might be an important bioactive compound in HN extracts. The antiproliferative activities of the four *Hydrocotyle* species were studied *in vitro* using human hepatoma Hep3B cells, and the results were consistent with their antioxidant capacities. The water extract of HN had the highest antiproliferative activity with an IC_{50} of 435.88 ± 8.64 μg/mL. The ethanol extracts of HB and HSi had the lowest antiproliferative activities (IC_{50} > 2000 μg/mL) under the experimental conditions. We suggested that HN might be served as a good source of natural antioxidant among the *Hydrocotyle* genus.

Keywords: Antioxidant; Antiproliferative; Free radical; Flavonoid; Hep3B; *Hydrocotyle*; Polyphenol.

Abbreviations: TEAC, trolox equivalent antioxidant capacity; **MTT,** 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide; **GSH,** glutathione reduced form; **BHT,** butylate hydroxyltoluene; **DPPH,** 1, 1-diphenyl-2-picrylhydrazyl; **FRAP,** ferric reducing antioxidant power; **TCA,** trichloroacetic acid; **FBS,** fetal bovine serum; **DMEM,** Dulbecco's modified Eagle medium; **EDTA,** ethylenediamine tetraacetic acid; **IC_{50},** concentration with 50% inhibition; **ABS,** absorbance; **HPLC,** high performance liquid chromatography.

INTRODUCTION

It is commonly accepted that under situations of oxidative stress, reactive oxygen species, such as superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), and peroxyl ($^{\cdot}OOH$, $ROO^{\cdot}$) radicals, were 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, diabetes, and inflammation (Aruoma, 1998; Chen et al., 2006). The use of traditional medicine is comprehensive, and plants were still a large source of natural antioxidants which might serve as leads for the development of novel drugs. Several anti-inflammatory, digestive, anti-necrotic, neuroprotective, and hepatopro-

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tective drugs recently have 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 sources of natural antioxidants and compounds with radical scavenging activity during the last few years, some had been found, such as echinacoside in *Echinaceae* root (Hu and Kitts, 2000), anthocyanin (Espin et al., 2000), phenolic compounds (Rice-Evans et al., 1997), and the extracts of water spinach and sweet potato organs (Huang et al., 2004; Huang et al., 2005).

The *Hydrocotyle* species (Apiaceae family) were the important original plants of Pai-Tsao-Tsa, which was a kind of popular folk drink inherited from ancient time in Taiwan. Pai-Tsao-Tsa made with either traditional Chinese medicine or Taiwan folk medicine has efficacy and beneficial effects to our health (Chiu, 2004). The whole plants of the *Hydrocotyle* species are often used in Taiwan folk medicine for treating common cold, tonsillitis, cephalitis, enteritis, dysentery, zoster, eczema, period pain, hepatitis and jaundice (Chang et al., 2003). Only few studies have confirmed the pharmacological activity of members of the genus *Hydrocotyle*. For example, *H. sibthorpioides* (HSi) could inhibit the growth of transplanted tumors in mice, such as hepatic carcinoma (Hep), sarcoma (S_{180}) and uterine cervical carcinoma (U_{14}). Both *H. leucocephala* Cham. & Schlecht. and HSi have immunomodulatory effects (Ramos et al., 2006; Yu et al., 2007). However, no studies to date had been able to demonstrate the antioxidant effect of the *Hydrocotyle* species. The antitumor effect was also not fully understood, and the pharmacological data for these herbs were incomplete.

The objectives of this work were to investigate the antioxidant and antiproliferative property of crude extracts from *H. batrachium* Hance (HB), *H. nepalensis* Hook (HN), *H. setulosa* Hayata (HSe), and *H. sibthorpioides* Lam. (HSi) in comparison with chemical compounds such as BHT, GSH or rutin and the level of inhibition of the growth of cancer cells in series of *in vitro* tests.

MATERIALS AND METHODS

Materials

BHT, GSH, potassium peroxodisulfate ($K_2S_2O_8$), DPPH, Tris (hydroxylmethyl) aminomethane, trypsin, potassium ferricyanide ($K_3Fe(CN)_6$), TCA, ferric chloride ($FeCl_3$), (+)-catechin, MTT, aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), rutin, 2, 2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), sodium bicarbonate (Na_2CO_3), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu solution and 95% ethanol were purchased from Merck Co. (Santa Ana, CA, USA). FBS was purchased from Gibco BRL Co. (Gaithersburg, MD, USA). DMEM was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Plant materials were collected from Taichung, Nantou, and Hsinchu counties in Taiwan. They were identified and authenticated by Dr. Chao-Lin Kuo, Associate professor and Chairman, Department of Chinese Medicine Recourses, China Medical University, Taichung, Taiwan.

Preparation of the ethanol extracts of plant materials

Dried whole herb (100 g each) was macerated with 1000 mL 95% ethanol for 24 h at room temperature. Filtration and collection of the extract were done three times. Then the ethanol extract (3000 mL) was evaporated to 10 mL and dried in vacuum at 40°C. The dried extract was weighted and dissolved in 95% ethanol (stock 4 mg/mL) and stored in -20°C for further use.

Preparation of the water extracts of plant materials

Dried whole herb (100 g each) was boiled with 1000 mL distilled water for 1 h. Filtration and collection of the extracts were done three times. Resulting decoction (about 1000 mL) was evaporated to 10 mL and dried in vacuum at 50°C. The dried extract was weighted and dissolved in distilled water (stock 4 mg/mL) and stored in -20°C for further use. For each sample, yields were calculated in percentages on the basis of dry weight of the whole herb used (100 g) and the quantity of dry mass obtained after the extraction.

Determination of antioxidant activity by $ABTS^{\cdot+}$ scavenging ability

The $ABTS^{\cdot+}$ scavenging ability was determined according to the method of Re et al. (1999). Aqueous solution of ABTS (7 mM) was oxidized with potassium peroxodisulfate (2.45 mM) for 16 hours in the dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with 95% ethanol to an absorbance of 0.75 ± 0.05 at 734 nm (Beckman UV-Vis spectrophotometer, Model DU640B). An aliquot (20 μL) of each sample (125 μg/mL) was mixed with 180 μL $ABTS^{\cdot+}$ solution and the absorbance was read at 734 nm after 1 min. Trolox was used as a reference standard. A standard curve was constructed for Trolox at 0, 15.625, 31.25, 62.5, 125, 250, 500 μM concentration. The TEAC was expressed as millimolar concentration of trolox solution with the antioxidant equivalent to a 1000 ppm solution of the sample under investigations.

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) assay of the crude extracts was determined according to the method of Benzie and Strain (1996). This assay measured the change in the absorbance at 593 nm owing to the formation of a blue colored Fe^{2+}-tripyridyltriazine compound from colorless oxidized Fe^{3+} form by the action of electron donating antioxidants. To prepare the FRAP reagent, a mixture of 0.1 M acetate buffer (pH 3.6), 10

mM 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), and 20 mM ferric chloride (10:1:1, v/v/v) was made. An aliquot (10 μL) of each sample (125 μg/mL concentration) was mixed with 300 μL FRAP reagent and the absorbance was read at 593 nm after 15 min. A standard curve was constructed for $FeSO_4 \cdot 7H_2O$ at 0, 31.25, 62.5, 125, 250, 500, 1000 μg/mL concentration. In the FRAP assay, the antioxidant efficiency of the sample was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, which represented a one-electron exchange reaction. The results were corrected for dilution and expressed in μmol Fe^{2+}/mg.

Determination of antioxidant activity by DPPH radical scavenging ability

The effect of crude extracts and positive controls (GSH, BHT and rutin) on the DPPH radical was estimated according to the method of Yamaguchi et al. (1998). An aliquot (20 μL) of crude extracts at various concentrations was mixed with 100 mM Tris-HCl buffer (80 μL, 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 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 samples were calculated according to the equation: % decolorization = [1- (ABS_{sample} /$ABS_{control}$)] ×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.

Measurement of reducing power

The reducing power of the crude extracts and positive controls (GSH and BHT) was determined according to the method of Yen and Chen (1995). The samples (0, 31.25, 62.5, 125, 250, 500, and 1000 μg/mL) were 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% 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 an increase in reducing power.

Determination of total polyphenol content

The total polyphenol content of the crude extracts was determined according to the method of Ragazzi and Veronese (1973). 20 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 to stand 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. The total polyphenol content was expressed as mg (+)-catechin equivalent/g dry weight. The dry weight indicated was the sample dry weight.

Determination of total flavonoid content

The total flavonoid content of the crude extracts was determined according to the method of Lamaison and Carnet (1990). 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 the absorbance at 430 nm was read after 10 min of incubation. Rutin was used as a standard for the calibration curve. The total flavonoid content was calibrated using the linear equation based on the calibration curve. The total flavonoid content was expressed as mg rutin equivalent/g dry weight. The dry weight indicated was the sample dry weight.

Determination of total flavonol content

The total flavonol content of the crude extracts was determined according to the method of Arnous et al. (2001). 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 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.

Analysis of rutin, quercetin, HNW, and HNE by HPLC

A moderate amount of the ethanol and water extracts from *H. nepalensis* Hook. (HN) were weighed and dissolved in ethanol and water, respectively. At first, the solutions were filtered through 0.45 μm PVDF filters. The HPLC (Waters 2695 separations module; detector: Waters 996 photodiode array detector) analysis was carried out under the following conditions; the Waters XTerra RP18 column (5 μm, 4.6 × 250 mm) was used with 0.05% phosphate buffer as mobile phase A, and acetonitrile was used as mobile phase B; the gradient elution was run with 30% of solution B at 0 min, 35% of solution B at 30 min at a flow rate of 0.8 mL/min; the injection volume was 10 μL, and a wavelength of 254 nm was used for detection. Pure compounds, including rutin and quercetin, were also analyzed by HPLC with the same conditions, and the retention time was used to identify the flavonoids in the samples.

Culture and harvest of human hepatoma cell line

Hep3B cells were cultured with DMEM with 10% FBS

in a T75 flask at 37°C, 5 % CO_2, and 90% relative humidity. To harvest cells, Hep3B cells were washed with PBS buffer and treated with 4 mL of trypsin-EDTA for 3 min. The reaction was stopped 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 based on Hansen et al. (1989). Suspensions of human Hep3B 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 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 hour 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. Each sample was repeated with the above procedures in order to determine the cell proliferation. The decolorization was plotted against the concentration of the sample extract, and the IC_{50}, which was the amount of the sample necessary to decrease 50% of the absorbance of MTT, was calculated.

Statistical analysis

Experimental results were presented as the mean ± 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 the *p* value was less than 0.05.

RESULTS AND DISCUSSION

Extraction yields

The yields in the water and ethanol extracts of the four *Hydrocotyle* species were given in Table 1. The percentages of the ethanol extract (code as E) yields in descending order were as follows: HSiE (20.26%) > HBE (13.14%) > HNE (12.95%) > HSeE (11.16%). On the other hand, the water extract (code as W) yields in decreasing order were as follows: HSiW (28.55%) > HBW (26.37%) > HSeW (22.68%) > HNW (11.55%).

Antioxidant activity estimated by ABTS assay

ABTS assay are often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants (Katalinic et al., 2006; Chang et al., 2007a, b). 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 interference of compounds, which affected radical formation. This modification made the assay less susceptible to artifacts and prevented overestimation of antioxidant power (Sanchez-Moreno, 2002). The antioxidant sample was added to the reaction medium when the stable absorbance was obtained, and the antioxidant activity was measured in terms of decolorization.

ABTS assay was expressed as TEAC value. Higher TEAC value represented that the sample had a stronger antioxidant activity. TEAC values determined from the calibration curve for the four *Hydrocotyle* species were shown in Figure 1A. Antioxidant activities of the water and ethanol extracts of the four *Hydrocotyle* species were in the following decreasing order: HNW (0.48 ± 0.03 mM) > HSeW (0.40 ± 0.02 mM) > HBW (0.36 ± 0.01 mM) > HSiW (0.23 ± 0.01 mM) > HSeE (O.22 ± 0.00 mM) > HNE (0.17 ± 0.00 mM) > HBE (0.16 ± 0.00 mM) > HSiE (0.08 ± 0.00 mM). Thus, it was observed that the water extracts of all the samples had higher antioxidant potencies than the ethanol extracts, and HNW had the highest activity.

Ferric reducing antioxidant power assay

As shown in Figure 1B, there was a significant difference in total antioxidant power (FRAP) between the samples. The FRAP values varied from 1.55 to 4.02 μmol Fe^{2+}/mg in the water extracts, and 0.51 to 1.29 μmol Fe^{2+}/mg in the ethanol extracts. The FRAP values of the eight samples decreased in the following order: HNW (4.02 ± 0.03 μmol Fe^{2+}/mg) > HSeW (3.43 ± 0.02 μmol Fe^{2+}/mg) > HBW (2.70 ± 0.05 μmol Fe^{2+}/mg) > HSiW (1.55 ± 0.01 μmol Fe^{2+}/mg) > HSeE (1.29 ± 0.07 μmol Fe^{2+}/mg) > HNE (1.06 ± 0.04 μmol Fe^{2+}/mg) > HBE (0.87 ± 0.03 μmol Fe^{2+}/mg) > HSiE (0.51 ± 0.00 μmol Fe^{2+}/mg). Thus, it was

Table 1. The yields in the water and ethanol extracts of four *Hydrocotyle* species.

Species	Water extract yield (% w/w)[a]	Ethanol extract yield (% w/w)
H. batrachium (HB)	26.37%	13.14%
H. nepalensis (HN)	11.55%	12.95%
H. setulosa (HSe)	22.68%	11.16%
H. sibthorpioides (HSi)	28.55%	20.26%

[a]Dried weight basis.

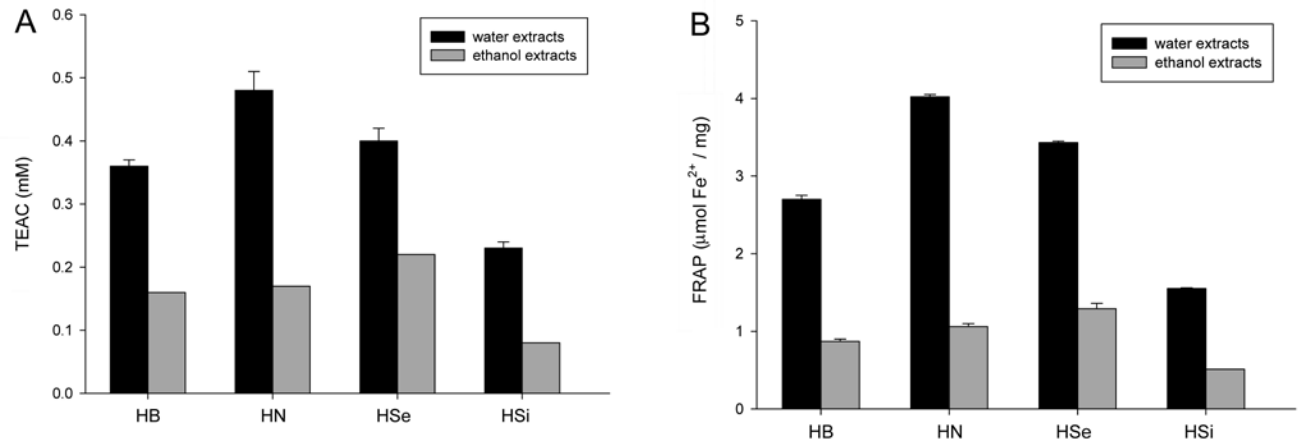

Figure 1. TEAC (A) and FRAP (B) values of the water and ethanol extracts of the four *Hydrocotyle* species. HB: *H. batrachium*; HN: *H. nepalensis*; HSe: *H. setulosa*; HSi: *H. sibthorpioides*. Each value represented mean ± S.D. of three parallel measurements ($P < 0.05$).

also observed that the water extracts of all the samples had higher antioxidant potencies than the ethanol extracts, and HNW had the highest activity.

Both FRAP and TEAC assay were used to estimate the total antioxidant power because they were quick and simple to perform, and the reaction was reproducible and linearly related to the molar concentration of the antioxidants (Benzie et al., 1999). FRAP assay was initially developed to assay the plasma antioxidant capacity, but could also be used to measure the antioxidant capacity of a wide rage of biological samples, pure compounds, fruits, wines, and animal tissues (Katalinic et al., 2006).

The correlation coefficients (R^2) of FRAP and TEAC assay of the water and ethanol extracts of the four *Hydrocotyle* species were shown in Figure 2A and 2B. Relationship between FRAP and TEAC assay of the water extracts was shown in Figure 2A (R^2=0.984, $p < 0.001$), and that of the ethanol extracts was shown in Figure 2B (R^2=0.971, $p < 0.001$). The R^2 values of FRAP and TEAC assay showed higher correlation. The higher the FRAP activity, the higher the TEAC activity of the samples.

Scavenging activity against 1,1-diphenyl-2-picrylhydrazyl radical

The relatively stable organic radical DPPH 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 or water extract of plants. DPPH radical was scavenged by antioxidants through the donation of a proton forming the reduced DPPH. The color changed from purple to yellow after the reduction, which could be quantified by its decrease of the absorbance at wavelength 517 nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition. Table 2 showed the IC_{50} values for the radical-scavenging activity of the different extract fractions of the four *Hydrocotyle* species, GSH, BHT, and rutin using the DPPH colorimetric method. It was found that HNW had the lowest IC_{50} value (84.20 ± 1.84 μg/mL), followed by HSeW (117.01 ± 1.73 μg/mL), HBW (178.99 ± 1.87 μg/mL), HSeE (197.87 ± 2.65 μg/mL), HNE (314.51 ± 1.04 μg/mL), HSiW (375.96 ± 2.37 μg/mL), HBE (403.31 ± 0.64 μg/mL), and HSiE (919.47 ± 1.31 μg/mL). The eight extract fractions showed signifi-

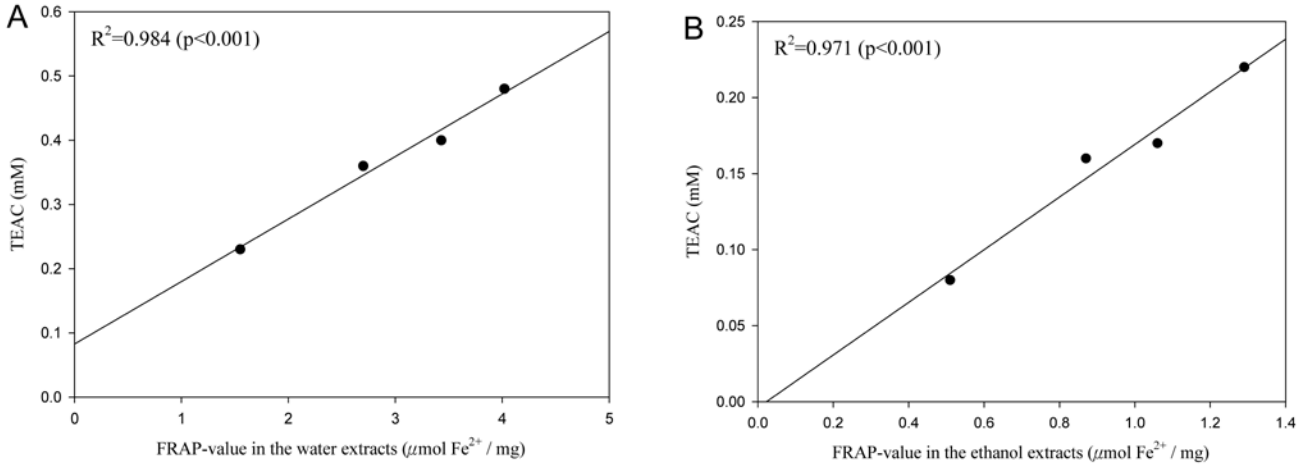

Figure 2. Correlation coefficients (R^2) of TEAC and FRAP in the water (A) and ethanol (B) extracts of the four *Hydrocotyle* species.

Table 2. IC_{50} values of the water and ethanol extracts of four *Hydrocotyle* species in DPPH radical scavenging activity[a].

Species and positive controls	Water extract IC_{50} (μg/mL)	Ethanol extract IC_{50} (μg/mL)
H. batrachium (HB)	178.99 ± 1.87	403.31 ± 0.64
H. nepalensis (HN)	84.20 ± 1.84	314.51 ± 1.04
H. setulosa (HSe)	117.01 ± 1.73	197.87 ± 2.65
H. sibthorpioides (HSi)	375.96 ± 2.37	919.47 ± 1.31
GSH	49.63 ± 0.25	N.D.[b]
BHT	N.D.	41.06 ± 0.76
Rutin	N.D.	15.96 ± 0.55

[a] Values represented mean ± S.D. of three parallel measurements (P<0.05).
[b] N.D.: Not detected.

cant differences (p<0.05) in radical-scavenging activity. Observed from the above results, the most active sample was HNW, however, its capacity was still lower than the three positive controls in DPPH assay.

Measurement of Reducing Power

We investigated the reducing capacity of the four *Hydrocotyle* species extracts by measuring the Fe^{3+}-Fe^{2+} conversion. The reducing capacity of a compound may be served 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 prevention 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 the four *Hydrocotyle* species was shown in Figure 3. Both reduced GSH and BHT were used as the positive controls. The different extract fractions from the four *Hydrocotyle* species exhibited a dose-dependent reducing power activity within concentration range of 0, 31.25, 62.5, 125, 250, 500, and 1000 μg/mL. HNW had the highest reducing power, followed by HBW, HSeW, HSeE, HNE≈HBE, HSiW, and HSiE. The water extracts of the four *Hydrocotyle* species had higher reducing power than the ethanol extracts. The different extract fractions showed significant differences (p<0.05) in reducing power.

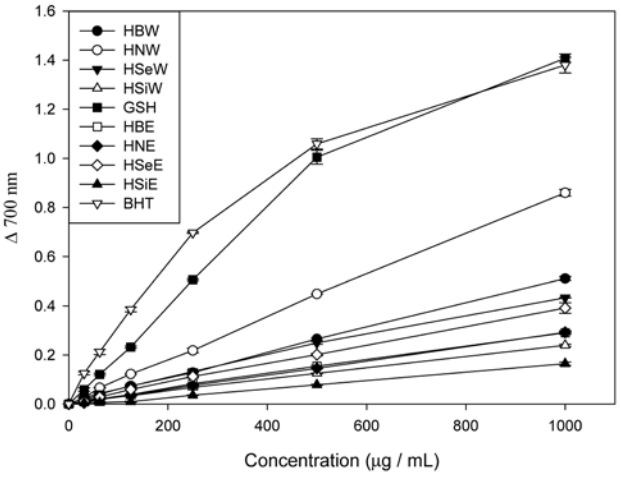

Figure 3. Antioxidant activities of the water and the ethanol extracts (0, 31.25, 62.5, 125, 250, 500, and 1000 μg/mL) of the four *Hydrocotyle* species, as measured by the reducing power method. BHT and GSH were used as the positive controls. Each value represented mean ± S.D. of three parallel measurements (P<0.05).

Total polyphenol, flavonoid, and flavonol content

The total polyphenol, flavonoid, and flavonol content of the four *Hydrocotyle* species were shown in Table 3. The total polyphenol content was expressed as μg of (+)-catechin equivalent per milligram of dry weight. The total polyphenol content of the extracts of the four *Hydrocotyle* species ranged from 34.17 to 115.77 μg CE/mg, and decreased in the following order: HNW > HSeW > HSeE > HBW > HNE > HBE > HSiW > HSiE. The water extracts of the four *Hydrocotyle* species had higher polyphenolic content than the ethanol extracts.

The total flavonoid content was expressed as μg of rutin equivalent per milligram of dry weight. The total flavonoid content of the extracts of the four *Hydrocotyle* species ranged from 15.68 to 86.67 μg RE/mg, and decreased as the following order: HSeE > HNE > HBE > HSiE > HNW> HSeW > HBW > HSiW. The ethanol extracts of the four *Hydrocotyle* species had higher flavonoid content than the water extracts.

The total flavonol content was expressed as μg of (+)-catechin equivalent per milligram of dry weight. The total flavonol content of the extracts of the four *Hydrocotyle* species ranged from 4.10 to 21.09 μg CE/mg, and decreased as the following order: HSeE > HSiE > HNW > HBW > HNE > HSeW > HBE > HSiW. The ethanol extracts of HSe and HSi had higher flavonol content than the water extracts. The ethanol extracts of HB and HN had lower flavonol content than the water extracts.

Table 3. The total polyphenol, flavonoid, and flavonol content in the water and ethanol extracts of four *Hydrocotyle* species[a].

Species	Total polyphenols (μg CE/mg)[b]	Total flavonoids (μg RE/mg)[c]	Total flavonols (μg CE/mg)[b]
Water extract			
H. batrachium (HB)	80.83 ± 0.55	24.81 ± 2.82	16.38 ± 0.07
H. nepalensis (HN)	115.77 ± 1.61	31.48 ± 0.74	18.00 ± 0.37
H. setulosa (HSe)	110.27 ± 0.86	27.05 ± 0.61	11.45 ± 0.35
H. sibthorpioides (HSi)	34.63 ± 0.37	15.68 ± 0.34	4.10 ± 0.14
Ethanol extract			
H. batrachium (HB)	53.93 ± 0.79	47.32 ± 0.56	9.43 ± 0.17
H. nepalensis (HN)	74.53 ± 1.73	66.07 ± 2.65	12.35 ± 0.01
H. setulosa (HSe)	84.47 ± 6.14	86.67 ± 1.17	21.09 ± 0.35
H. sibthorpioides (HSi)	34.17 ± 0.73	41.53 ± 3.71	18.26 ± 0.40

[a]Values represented mean ± S.D. of three parallel measurements.
[b]Data expressed in μg (+)-catechin equivalent / mg dry weight (μg CE/mg).
[c]Data expressed in μg rutin equivalent / mg dry weight (μg RE/mg).

Both flavonoid and flavonol belong to polyphenolic compounds. 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 could 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 measured the oxidation products at the early and final stages of oxidation. The antioxidants had 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 were normally phenolic compounds, which were effective proton donors, including tocopherols, flavonoids, and other organic acids. However, the components which were responsible for the antioxidative activity of the four *Hydrocotyle* species were currently unclear. Therefore, further work must be performed to isolate and identify these components.

Relationship between total antioxidant power and total polyphenol, flavonoid, and flavonol content

The correlation coefficients (R^2) of total antioxidant power (TEAC) and total polyphenol, TEAC and total flavonoid, and TEAC and total flavonol of the water and

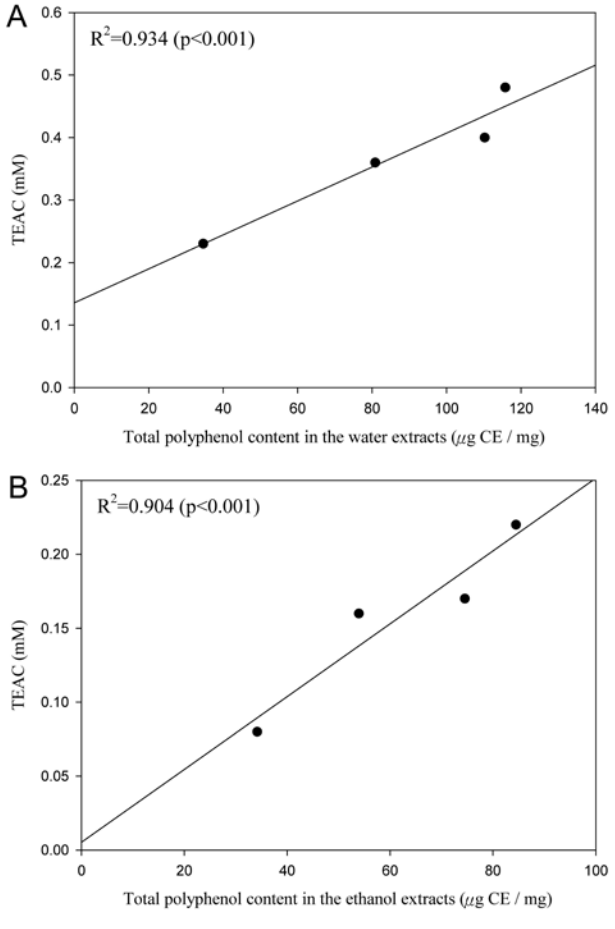

Figure 4. Correlation coefficients (R^2) of TEAC and total polyphenol content in the water (A) and the ethanol (B) extracts of the four *Hydrocotyle* species.

ethanol extracts were shown in Figures 4, 5 and 6. The R^2 values of TEAC and total polyphenol content of the water (Figure 4A) and the ethanol (Figure 4B) extracts were 0.934 and 0.904, respectively. Similarly, R^2 values of TEAC and total flavonoid content of the water (Figure 5A) and the ethanol (Figure 5B) extracts were 0.995 and 0.785 respectively. R^2 values of TEAC and total flavonol content of the water (Figure 6A) and the ethanol (Figure 6B) extracts were 0.766 and 0.008 respectively. Among the above 3 statistics, we could see that there were higher correlation between the TEAC and total polyphenol / total flavonoid. The higher the TEAC activity, the higher the total polyphenol and flavonoid content of the samples.

Compositional analysis of rutin, quercetin, HNW, and HNE by HPLC

The water and ethanol extracts of *H. nepalensis* (HN) were found to have higher antioxidant activities and polyphenolic compounds than most of other samples. HNW and HNE were analyzed by HPLC and their chromatograms were shown in Figure 7. Both HNW and HNE had rutin, but quercetin was not found in the samples. Rutin, a glycoside comprised of the flavonol quercetin and the disaccharide rutinose, widely distributes in plant kingdom, and shows remarkable antioxidant, anti-inflammatory and anticancer activities. It also has relaxing effects on smooth muscles (Liu et al., 2008). In DPPH assay, we found that rutin had much lower IC_{50} value (15.96 ± 0.55 μg/mL) than all the samples. Rutin might be an important component in the antioxidant activity of the HN extracts.

Previous chemical studies of the members of the genus *Hydrocotyle* had resulted in isolation of trans-β-farnesene, α-terpinenes, and thymol methyl ether from *H. sibthorpioides* Lam. and *H. maritime* Honda (Asakawa et al., 1982), quercetin-3-*O*-galactoside from *H. umbellata* L. (Adams et al., 1998), monogalactosyl monoacylglycerol from *H. ramiflora* Maxim. (Kwon et al., 1998), oleane and ursane type glycosides from *H. ranunculoides* Blume (Della Greca et al., 1994a, b) and *H. sibthorpioides* (Matsushita et al., 2004), diacetylene from *H. leucocephala* Cham. & Schlecht. (Ramos et al., 2006). However, there were no chemical studies on *H. batrachium* Hance, *H. nepalensis* Hook. and *H. setulosa* Hayata. There were few reports on the flavonoid compounds of the genus *Hydrocotyle*. Therefore, it is worthy to study the phytochemicals of the genus *Hydrocotyle*.

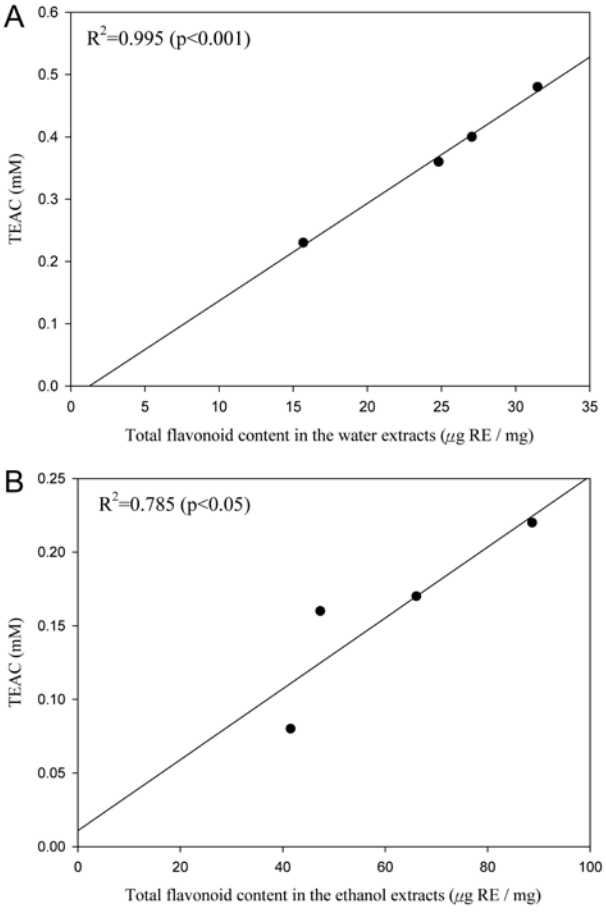

Figure 5. Correlation coefficients (R^2) of TEAC and total flavonoid content in the water (A) and the ethanol (B) extracts of the four *Hydrocotyle* species.

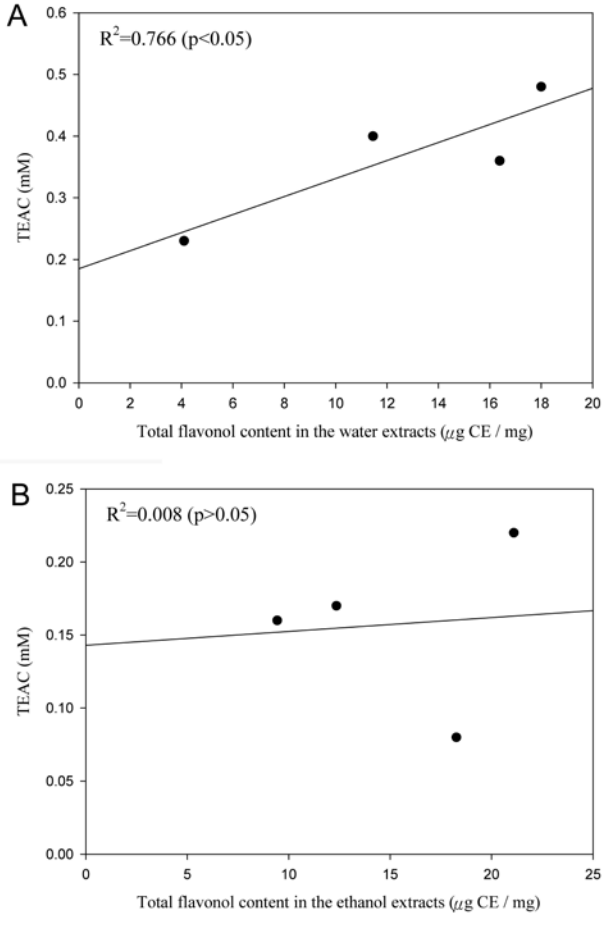

Figure 6. Correlation coefficients (R^2) of TEAC and total flavonol content in the water (A) and the ethanol (B) extracts of the four *Hydrocotyle* species.

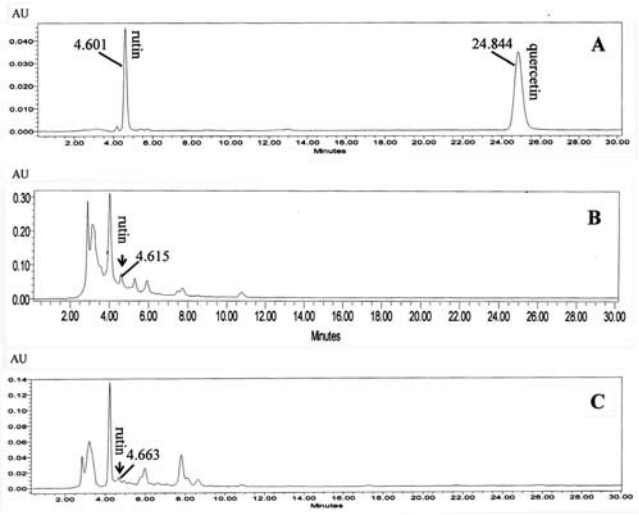

Figure 7. HPLC of HNW and HNE extracts. (A) standards, (B) HNW, and (C) HNE.

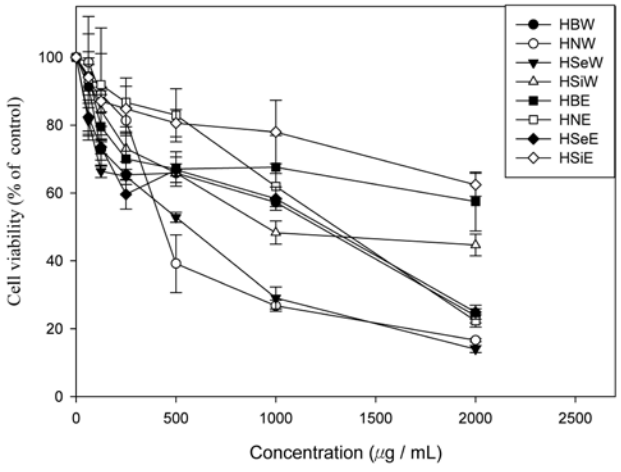

Figure 8. Percent inhibition of 3B cell proliferation by different extracts from the four *Hydrocotyle* species. Each value represented mean ± S.D. of three parallel measurements (P<0.05).

Measurement of cell proliferation

Antiproliferative activities of the different extract fractions from the four *Hydrocotyle* species on the growth of the human hepatoma 3B cell line *in vitro* were summarized in Figure 8 and Table 4. The cell proliferation was analyzed 72 h after 3B cells had been cultured with an extract fraction of 0, 62.5, 125, 250, 500, 1000, 2000 μg/mL in the final concentration using the MTT assay. 3B 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 IC_{50}. A lower IC_{50} value indicating a higher antiproliferative activity. The water extracts of the four

Table 4. IC_{50} values of the water and ethanol extracts of four *Hydrocotyle* species in inhibiting Hep3B cell proliferation[a].

Species	Water extract IC_{50} (μg/mL)	Ethanol extract IC_{50} (μg/mL)
H. batrachium (HB)	1216.99 ± 1.79	> 2000
H. nepalensis (HN)	435.88 ± 8.64	1301.59 ± 3.38
H. setulosa (HSe)	560.06 ± 1.90	1249.45 ± 3.24
H. sibthorpioides (HSi)	952.34 ± 3.27	> 2000

[a]Values represented mean ± S.D. of three parallel measurements (P<0.05).

Hydrocotyle species had a higher antiproliferative activity than the ethanol extracts. HNW had the highest antiproliferative activity with the lowest EC_{50} of 435.88 ± 8.64 μg/mL, followed by HSeW (560.06 ± 1.90 μg/mL), HSiW (952.34 ± 3.27 μg/mL), HBW (1216.99 ± 1.79 μg/mL), HSeE (1249.45 ± 3.24 μg/mL), HNE (1301.59 ± 3.38 μg/mL). Both HBE and HSiE had the highest IC_{50} values (> 2000 μg/mL), and it was shown that the two samples had the lowest antiproliferative activities 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 polyphenols and flavonoids found in each fraction. Their antiproliferative activities were consistent with their antioxidant activities. This experiment suggested that the antiproliferative activities of the four *Hydrocotyle* species might be also correlated to the total amount of polyphenols and flavonoids found in each fraction.

In conclusion, the results from *in vitro* experiments, including ABTS radical monocation scavenging (Figure 1A), FRAP method (Figure 1B), DPPH radical scavenging (Table 2), reducing power method (Figure 3), total polyphenol content, total flavonoid content and total flavonol content (Table 3), HPLC assay (Figure 7), and inhibition of cancer cell proliferation (Figure 8 and Table 4), demonstrated that the phytochemicals in the four *Hydrocotyle* species might have a significant effect on antioxidant and anticancer activities, which were directly related to the total amount of polyphenols and flavonoids found in the four *Hydrocotyle* species extracts. The additive roles of phytochemicals might contribute significantly to the potent antioxidant activity and the ability to inhibit tumor cell proliferation *in vitro*. Hence, the four *Hydrocotyle* species could be used as an easy 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 the four *Hydrocotyle* species.

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臺灣產四種天胡荽屬植物之抗氧化及抗增生活性

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本實驗針對臺灣產四種天胡荽屬植物之水萃取物及乙醇萃取物進行抗氧化及抗增生活性研究，分析方法包括 ABTS 自由基的清除、FRAP 方法、DPPH 自由基的清除、還原力、總多酚類含量、總類黃酮類含量、總黃酮醇類含量以及癌細胞增生之抑制，結果顯示所有樣品的水萃取物之抗氧化及抗增生活性皆大於乙醇萃取物，但在抗氧化活性方面，所有受測樣品活性皆小於正對照組（BHT 及 GSH）。我們也發現所有樣品的水萃取物之多酚類含量高於乙醇萃取物，但類黃酮類含量則低於乙醇萃取物。在 ABTS 自由基的清除分析中，水萃取物樣品的 TEAC（trolox equivalent antioxidant capacity）值依序遞減為乞食碗＞阿里山天胡荽＞臺灣天胡荽＞天胡荽，TEAC 與總多酚類含量之相關係數 (R^2）顯示較高的關係（水萃取物的 R^2 值為 0.934；乙醇萃取物的 R^2 值為 0.904），而 TEAC 與總類黃酮類含量之相關係數（R^2），在水萃取物及乙醇萃取物分別為 0.995 及 0.785，又 TEAC 與 FRAP 的 R^2 值也顯示較高的關係（水萃取物的 R^2 值為 0.984；乙醇萃取物的 R^2 值為 0.971）。在 HPLC 的分析中，由於乞食碗具有最高的抗氧化活性，其水萃取物及乙醇萃取物已建立指紋圖譜，蘆丁（rutin）發現可能是乞食碗萃取物中的一個重要活性成分。這四種天胡荽屬植物的抗增生活性以人類3B肝癌細胞進行體外研究，結果和它們的抗氧化能力相近，乞食碗的水萃取物具有最高的抗增生活性，其 IC_{50} 為 435.88 ± 8.64 μg/mL，臺灣天胡荽及天胡荽乙醇萃取物在相同實驗條件下，其抗增生活性最差（IC_{50}＞2000 μg/mL）。因此，在天胡荽屬的植物中，乞食碗可能是一個天然抗氧化劑的優良來源。

關鍵詞：抗氧化；抗增生；自由基；類黃酮類；3B 肝癌細胞；天胡荽屬；多酚類。