行政院國家科學委員會補助專題研究計畫成果報告

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※常見幾種中式飲料在 Caco-2 及 HepG-2 細胞的抗氧化性　※
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計畫類別：■個別型計畫　　□整合型計畫

計畫編號：NSC89－2214－E－041－007－

執行期間：89　年　8月　1日至　90年　7月　31日

計畫主持人：吳明娟

共同主持人：

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- □國際合作研究計畫國外研究報告書一份

執行單位：嘉南藥理學院食品衛生系

中　華　民　國　90　年　10　月　15　日

行政院國家科學委員會專題研究計畫成果報告

常見幾種中式飲料在 Caco-2 及 HepG-2 細胞的抗氧化性

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一、中文摘要

本年度計劃我們針對台灣本土特有的中式飲品包括牛蒡，菊花，蓮花，蓮心，蓮葉及山葡萄的甲醇萃取液進行一系列動物細胞及試管的抗氧化測試。我們發現牛蒡，菊花，蓮花，蓮心的甲醇萃取液對 H_2O_2 所誘導的 HepG-2 細胞的氧化傷害並無顯著的保護作用而蓮葉及山葡萄的甲醇萃取液則有顯著保護效果。試管的抗氧化測試顯示蓮花，蓮心，蓮葉及山葡萄的甲醇萃取液皆可抑制脂肪酸及核酸的氧化，並具 DPPH 自由基，氫氧自由基清除力及還原力。整體而言，抗氧化力由高而低為山葡萄 > 蓮葉 > 蓮心 > 蓮花。

關鍵詞：抗氧化力、山葡萄、蓮葉

Abstract

In this study we investigated the antioxidant activities of several common material used in Taiwanese herbal beverage. We found that the methanol extract of the root and branch of *Ampelopsis brevipedunculata* (Maxim.) Trautv. and leaf of lotus (*Nelumbo nucifera* Gertn.) showed dose-dependent protective effects against oxidative damage in HepG2 cells. In cell-free system, the strong reducing power and scavenging effects on hydroxyl radical and free radicals have also been found in the methanol extracts of *Ampelopsis brevipedunculata* (Maxim.) Trautv. as well as leaf, embryo and flower of lotus. These may partially explain their antioxidant activities against lipid peroxidation and DNA oxidative damage *in vitro*.

Keywords: *Ampelopsis brevipedunculata* (Maxim.) Trautv., *Nelumbo nucifera* Gertn., antioxidant

Introduction

The oxidative deterioration of lipids in foods is responsible for rancidity with a subsequent decrease in dietary quality and safety caused by the formation of potentially toxic compounds (Moure et al., 2001). It is necessary to suppress lipid peroxidation in food in order to preserve flavor, color and nutritive value. The addition of antioxidants to foods is the most effective way for retarding the unpleasant flavor produced by lipid peroxidation.

Many of the antioxidants other than vitamins C, E and β-carotenoids occur as dietary constituents. Researches have demonstrated that strong antioxidant compounds found in fruits (Wang et al., 1996; Kalt et al., 1999). Several studies have analyzed the antioxidant potential of a wide variety of vegetables (Furuta et al., 1997; Gazzani et al., 1998; Hertog et al., 1992; Vinson, et al., 1998). As of beverages, wines have been demonstrated to contain a variety of polyphenolic compounds and strong antioxidant activities (Fogliano et al., 1999; Lapidot et al., 1999). Green and black teas have been extensively studied for antioxidant properties because they can include up to 30% of the dry weight as phenolic compounds (Lin et al, 1998). A number of studies also deal with the antioxidant activity of extracts from herbs, medicinal plants and spices (Duh and Yen, 1997; Jung et al., 1999; Kim et al., 1994).

The root and branch of *Ampelopsis brevipedunculata* (Maxim.) Trautv. (abbreviated as AB in the text) has long been used in folk medicine for anti- inflammatory and antihepatotoxic effects in Taiwan and the

aqueous extract is getting popular as a functional beverage. Antiviral (Sun et al., 1986), antimutagenic (Lee and Lin, 1988) and antihepatotoxic activities (Yang et al., 1987; Ohima and Ueno, 1993) were found in the berries of AB. The pharmacological study of the anti-inflammatory and analgesic effects of the methanol extract of root and branch of AB has also been demonstrated (Hsieh et al., 1998). Its active principles are thought to be mainly flavonoids and steroids (Hsieh et al., 1998).

Lotus (*Nelumbo nucifera* Gertn.) is a major economic aqueous plant in Tainan County. Lotus seed and rhizome are mainly consumed as food. However, the rest parts of lotus, including leaf, flower and embryo, were used as beverage or folk medicine. Lotus leaf contains several alkaloids, including nuciferine, roemerine, O-nornuciferine, anonaine, pronuciferine, anneparine, N-methylcoclaurine and N-methylisococlarurine. It is used to disperse body heat during summers and is said to increase essential body energies, in particular those of the defenseive systems (Huang, 1999). The alkaloids of this leaf have a relaxing effect on smooth muscle. Lotus embryo contains various alkaloids, including liensinine, isoliensinine, neferine, lotusine, methylcorpalline, and demethylcoclaurine. Neferine has a vasodilating effect. Liensinine has antihypertensive and antiarrhythmic actions. In addition to house decoration, lotus flower is currently popular for herbal beverage. Although the popularity of lotus is high, the definitive evidence to prove its biological activity is relatively rare.

In attempt to assess the possible antioxidant activity of the flower, embryo and leaf of lotus as well as the root and branch of AB, we conducted a series of experiments in cell culture and cell-free systems. The HepG2 cells have been employed in the study of the cytotoxicity of both well-characterized chemicals and novel clinical hepatotoxins (Dierickx, 1989; Duthie et al., 1995). Our data showed that the methanol extracts of these herbs possessed dose-dependent protective effects against oxidative damage in HepG2 cells. In cell-free system, the strong reducing power and scavenging effects of hydroxyl radical and free radicals have also been found. These may partially explain their antioxidant activities against lipid peroxidation and DNA oxidative damage *in vitro*.

Results and Discussions

1. *The protective effects against oxidative damage in HepG2 cells*

To investigate ROS-induced cytotoxic effects on confluent HepG2 cells, we added increasing amounts of H_2O_2 to the serum-free DMEM medium, and evaluated the cell viability by MTT assays. Viable but not dead cells can reduce a tetrazolium-based compound (MTT) to a blue formazan product, therefore the differences in the amount of formazan formation indicated a variation in the number of live cells. Incubation of the cells in the presence of millimolar concentrations of H_2O_2 resulted in a significant decrease in HepG2. After 18 hour of treatment with 10 mM H_2O_2, we observed more than 90% loss of cell viability. (Figure1).

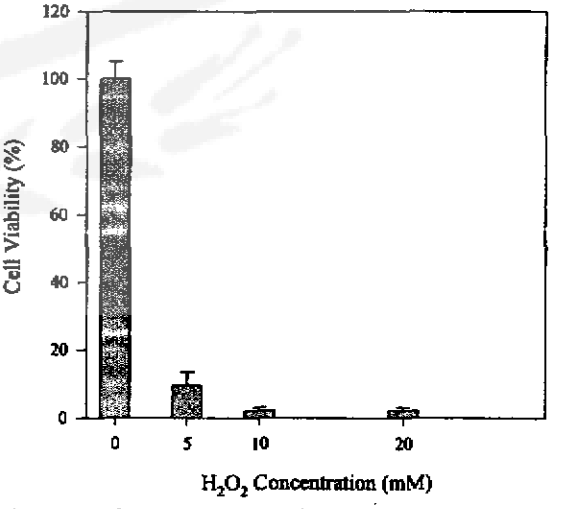

Figure 1. The cytotoxicity of H_2O_2 on HepG2 cell. Cells were cultured in 6-well plates until confluent. Various concentrations of extract and 10mM H_2O_2 containing serum free-MEM was added and cultured for 18 hours. The cell viability was measured in triplicate by MTT assay.

This marker was then utilized to verify the protective effect of the methanol extracts against H_2O_2 induced oxidative damage to the hepatoma HepG2 cells. We then coincubated HepG2 cells with 10 mM H_2O_2 and various amounts of methanol extracts,

including those of burdock, flower of *Chrysanthemum morifolium* Ramat, lotus flower, lotus embryo, lotus leaf and AB for 18 hours followed by measuring their viabilities. We found that there was no significant protective effect for burdock, flower of *Chrysanthemum morifolium* Ramat, lotus flower and lotus embryo, while lotus leaf and AB can significantly protect HepG2 cell from oxidative stress. Figures 2 and 3 showed that the protective effects of methanol extracts of lotus leaf and the stem and branch of AB on HepG2 oxidative stress exhibited a dose-dependent manner in tested ranges. Due to the potential cytotoxicity or mutagensis, the tested concentration for lotus leaf is up to 1.8 mg/ml and for AB is up to 0.25 mg/ml. It seemed that AB possessed stronger protective effect than lotus leaf on HepG2 cells system.

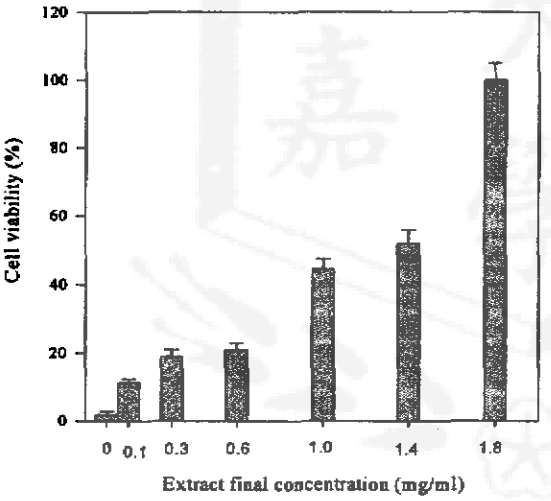

Figure 2. Protective effect against H_2O_2-induced oxidative stress by coincubating HepG2 cells with the methanol extract of lotus leaf.

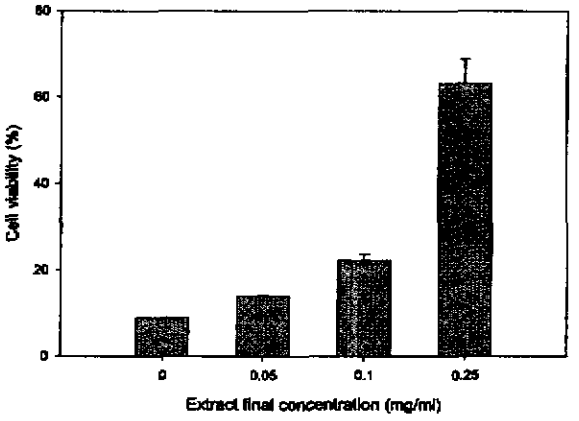

Figure 3. Protective effect against H_2O_2-induced oxidative stress by coincubating HepG2 cells with the methanol extract of AB root and branch.

2. *The scavenging powers against free radical, hydroxyl radical and ferrious ion as well as reducing powers*

To investigate the possible mechanisms for the antioxidant activities of these herbs, we employed a series of tests in cell-free systems. Because the antioxidant mechanism for burdock and flower of *Chrysanthemum morifolium* Ramat have been studied (Duh, 1998; Duh and Yen, 1997). We thus only investigated those were not been published. We found that in the cell-free system, they all exhibited DPPH free radical scavenging activities and the IC_{50s} are summarized in Table 1.The order of scavenging powers against DPPH free radical is lotus leaf > AB > lotus flower ≒ lotus embryo

Table 1. The IC_{50} for scavenging 0.5 mM DPPH

Extract	IC_{50} for DPPH (mg/ml)
Lotus flower	0.28 ±0.06
Lotus embryo	0.30 ±0.03
Lotus leaf	0.0075 ±0.0012
AB	0.025 ±0.003

The scavenging activities against hydroxyl radicals were tested by deoxyribose methods. All four extracts exhibited fair activities (data not shown). The ferrious ion chelating activities were also investigated. The IC_{50} for chelating ferrious ion was listed in Table 2. Lotus flower did not have detectable chelating power. Although the rest possessed weak chelating ability, their correspondent IC_{50}s were beyond the physiological concentration.

Table 2. The IC_{50} for scavenging 0.1 mM ferrious

Extract	IC_{50} for DPPH (mg/ml)
Lotus flower	N/A
Lotus embryo	5.11 ± 0.541
Lotus leaf	13.31 ± 1.62
AB	5.52 ± 0.641

In addition, we tested their reducing powers by potassium ferricyanide method (Oyaisu, 1986). We found that the reducing power is also a dose-dependent manner. The concentrations for 1 unit of A700 are listed in the table 3. The order of reducing power is

AB>lotus leaf > lotus flower ≒ lotus embryo

Table 3. The concentrations for 1 unit of A700

Extract	Concentration for 1A700 (mg/ml)
Lotus flower	0.36 ±0.04
Lotus embryo	0.40 ±0.01
Lotus leaf	0.08 ±0.01
AB	0.05 ±0.006

3. *The antioxidant activities against linoleic acid peroxidation induced by hemoglobin*

The antioxidant acitivities were performed by modified rapid photometric assay (Kuo et al., 1999). The linear response curves were also obtained and the IC_{50}s were listed in the Table 3. The antioxidant activity for linoleic acid peroxidation is AB>lotus leaf > lotus embryo> lotus flower.

Table 4. The IC_{50} for 1 mM linoleic acid peroxidation

Extract	Concentration for 1A700 (mg/ml)
Lotus flower	0.306 ±0.156
Lotus embryo	0.031 ±0.006
Lotus leaf	0.01 ±0.001
AB	0.0075 ±0.0006

The plasmid relaxation assay was used to semi-quantitatively assay the DNA oxidative damage. In the process of DNA damage, supercoiled form DNA was first nicked into open circular form, which was the product of single-strand cleavage of supercoiled DNA. The open circular DNA can be further cleavaged by ROS into linear form, which was the result of double-strand cleavage. Extensive oxidative damage would eventually cause DNA fragmentation and degradation subsequently. As shown in figure 4, H_2O_2, Fe^{+++}, or ascorbate alone only partially damaged DNA and caused a small portion of supercoiled pUC18 DNA, which migrated fastest, nicked to open circular form, which migrated slowest (lanes 2, 3 and 4). However, in the presence of all three reagents together, DNA was almost completely degraded by oxidative damage induced by Fenton reaction as no DNA band shown in lane 5. In the presence of extract, the extent of DNA damage could be significantly diminished. At 1 mg/ml (lane 6), methanol extract of lotus leaves showed the strongest protective effect: a small portion of supercoiled form (about 10% of control, measured by a computing densitometer) was preserved, along with predominantly produced open circular form and tiny amount of linear form, which migrated between supercoiled form and open circular form. When extract concentration decreased, the concurrence of linear form progressively increased and open circular form decreased (lanes 7-9). This indicated that the methanol extract inhibited DNA damage in a dose-dependent manner.

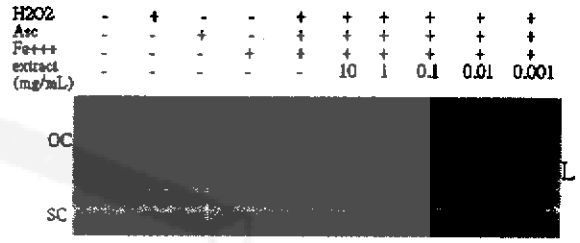

Figure 4. The plasmid relaxation assay for antioxidant activity against DNA oxidation.

In conclusion, we found that among these tested material, the strength of the antioxidant activity is by the order of AB > lotus leaf > lotus embryo> lotus flower although different systems may give a little bit of different results.

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