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Article

Antioxidant, Antidiabetic and Cytotoxic Effects of *Eucommia ulmoides* Oliv. Bark *in vitro*

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The aim of this study is to investigate *in vitro* biological properties of *Eucommia ulmoides* Oliv. bark. Ethyl acetate (EtOAc) fraction from aqueous extract of *Eucommia* bark showed strong antioxidant activity of IC_{50} 19.2 μg/m*l* by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay. The *Eucommia* bark extract showed α-glucosidase inhibitory activity and inhibited growth of human liver cancer cell, suggesting its potential biological value of anticancer.

Key words: *antioxidant, cytotoxic activity, Eucommia ulmoides Oliv. bark, α-glucosidase inhibitory activity*

Eucommia ulmoides Oliv. of the Eucommiaceae family is a traditional medicinal material used in east Asia. The *Eucommia* bark was used to strengthen the internal organs, bones, and muscles according to some old famous texts in China.[1] Recently, various biological activities of *Eucommia* bark have been developed or proved by means of *in vitro* and *in vivo* assay. Yen and Hsieh reported that *Eucommia* bark possess inhibitory effects on the oxidative modification of low-density lipoprotein (LDL) induced by Cu^{2+} and on the oxidation of deoxyribose induced by Fe^{3+}-EDTA/H_2O_2/ascorbic acid.[2,3] It was also reported that administration of water extract from *Eucommia* bark alleviated oxidative stress in the erythrocytes in Pb-administered rats.[4] The mechanism of vasorelaxing action of *Eucommia* bark was *in vitro* assayed which accounts for traditional treatment for hypertension.[5]

In this work, antioxidant activity, α-glucosidase inhibition and cytotoxicity against human liver cancer cell of *Eucommia* bark extract were investigated to further develop new biological activities.

Materials and Methods

Plant material. Bark of *Eucommia ulmoides* Oliv. was dried at room temperature in the shade, and then ground to fine powder. About 50 g of sample was macerated in 1 L water for 48 h at room temperature. After filtration, the water extract was evaporated with an Eyela extractor (Japan) at 42°C. Then, 400 m*l* of water extract was sequentially extracted with hexane, dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc).

Antioxidant assay. The antioxidant activity of *Eucommia* bark was tested by DPPH (Sigma, USA) free radical-scavenging and reducing power assay. The DPPH free radical-scavenging assay was carried as described by Kilani *et al.* with some modifications.[6] In brief, an aliquot (1 m*l*) of extract in water with different concentrations (0.05-1 mg/m*l*) were mixed with 1 m*l* of freshly prepared DPPH (65 μM) in MeOH. After incubation for 30 min at room temperature, the absorbance of the reaction mixture was measured photometrically at 517 nm. IC_{50} value is used to assess the antioxidant capability of a sample. Every assay was in triplicate.

The reducing power of extracts was determined as the method of Ordoñez *et al.*[7] Sample solution (1 m*l*) in water was mixed with 2.5 m*l* of 0.2 M (pH 6.6) phosphate buffer and 2.5 m*l* of 1% potassium ferricyanide ($K_3Fe(CN)_6$), then incubated at 50°C for 20 min. Then 2.5 m*l* of 10% trichloroacetic acid (TCA) was added to the mixture, followed by centrifugation at 3,000 rpm for 15 min. The upper layer solution (2.5 m*l*) was mixed with an equal volume of water and 0.5 m*l* of 0.1% ferric chloride ($FeCl_3$) and the absorbance was measured photometrically at 700 nm. The reducing power was expressed as ASE/mg. ASE means that the reducing power of 1 mg sample is equivalent (E) to reducing power of 1 nmol ascorbic acid (AS). Each sample was performed in triplicate.

α-glucosidase inhibitory assay. The reaction mixture contained 0.015 unit α-glucosidase from *Bacillus strearothermophilus* (Sigma, USA) and extract in various concentrations. After 10 min for pre-incubation at 37°C, 100 μ*l* of 3 mM glucopyranoside (pNPG; Sigma, USA) was added to the reaction mixture. The reaction was terminated by adding 750 μ*l* of 0.1 M Na_2CO_3 after 10 min incubation. The optical density was measured at 400 nm.[8] All determinations were in triplicate.

Inhibitory assay of human liver cancer cell. HepG2 cells (Cell Bank, Korea) were cultured in RPMI-1640 medium (Hyclone, USA) containing 2.05 mM L-Glutamine, 10% heat inactivated FBS, 100 units/m*l* of penicillin and 100 μg/m*l* of streptomycin. Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C. For the cytotoxic assay of *Eucommia*

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bark, aqueous extract and its EtOAc fraction were used. 2×10^5 cells were plated onto each well (1 m*l*/well) of 24-well culture plate and cultured for 4 h, then 10 μ*l* sample solutions with various concentrations were added to each culture. 48 h later, cell count was performed.

Results and discussion

Antioxidant activity. Free radicals are involved in a number of diseases due to the oxidative damage to DNA, lipids, and proteins and which can result in failure of cellular functions.[9,10] Dietary intake of antioxidant compounds will reduce oxidative damage.[11] Because of side effects of some synthetic antioxidant compounds, such as butylated hydroxyanisole (BHA),[12] research for safe and natural antioxidants source is attracting more and more attention. DPPH assay evaluates the ability of antioxidants to scavenge free radicals. The present DPPH assays showed each fraction possesses free radical scavenging activities (Table 1). The EtOAc fraction exhibited the highest antioxidant activities with an IC_{50} of 19.2 μg/m*l* (ascorbic acid with an IC_{50} of 2.72 μg/m*l*). The antioxidant activity has been reported to be concomitant with the reducing power[13] and the reducing power property can reduce the oxidized intermediates of lipid peroxidation processes.[7] As shown in Table 2, all the fractions have reducing power and EtOAc fraction took on significantly higher reducing power than the others. Besides bark, however, leaves of *Eucommia* were found to have effective antioxidant activity.[14] These results suggest *Eucommia* can be used to treat some diseases associated with excess free radicals.

Glucosidase inhibitory effect. Diabetes mellitus is a serious disease associated with many complications, and the incidence of this disease is increasing rapidly.[15,16] Diabetes may be caused by disorders of carbohydrate uptake.[17] Inhibitors of α-glucosidase limit the absorption of dietary carbohydrates and suppress postprandial hyperglycemia.[16] Therefore, glucosidase inhibitors can be used to treat diabetes. The result of this study showed all the fractions were capable of inhibiting α-glucosidase inhibitory activity (Fig. 1). The EtOAc fraction had the highest inhibitory activity, with inhibitory rate of 60.7% and 45.3% at content of 100 and 50 μg/m*l*, respectively, which means some compounds in EtOAc fraction may contribute mostly to the glucosidase inhibitory activity of *Eucommia* bark and they will be associated with their promising medicinal value of treating diabetes. Lee *et al.* reported *Eucommia* leaves showed antidiabetic activity by feeding streptozotocin-induced diabetic rats.[18] Considering these above results, we suggest that antidiabetic activity of *Eucommia* is partly due to its glucosidase inhibitory activity.

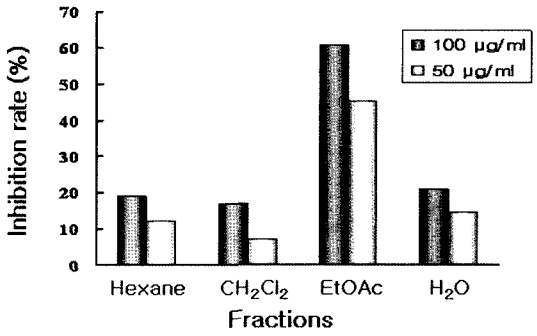

Fig. 1 Inhibitory effect on α-glucosidase activity after treatment with different fractions from *Eucommia* bark aqueous extract. Values are expressed as a mean of three determinations. 50 μg/m*l* acarbose was used as positive control (inhibition rate = 91%).

Cytotoxic activity. The aqueous extract of *Eucommia* bark and its EtOAc fraction were used to test cytotoxic activity. As shown in Table 3, when treatment of aqueous extract >200 μg/

Table 1. DPPH free radical scavenging activity of different fractions from *Eucommia* bark aqueous extract expressed as IC_{50}

Fractions	IC_{50} (μg/m*l*)
Hexane	134.0
CH_2Cl_2	63.6
EtOAc	19.2
H_2O	150.7

Values are expressed as a mean of three determinations. IC_{50}: the concentration (μg/m*l*) of sample that causes 50% loss of DPPH activity. Ascorbic acid was used as a positive control (IC_{50} = 2.72 μg/m*l*)

Table 2. Reducing power of different fractions from *Eucommia* bark aqueous extract expressed as ASE

Fractions	Reducing power (ASE)
Hexane	82.5
CH_2Cl_2	193.0
EtOAc	631.7
H_2O	109.4

Values are expressed as a mean of three determinations. ASE: the reducing power of 1 mg sample is equivalent to reducing power of 1 nmol ascorbic acid.

Table 3. The dose-dependent growth inhibition on HepG2 cancer cell after treatment with crude aqueous extract and its EtOAc fraction from *Eucommia* bark

Dose (μg/m*l*)	Cell number from different treatment ($\times 10^5$)	
	Aqueous extract	EtOAc fraction
400	1.36 ± 0.34[a]	0.24 ± 0.21[a]
200	2.38 ± 0.16[b]	0.46 ± 0.20[a]
100	3.59 ± 0.59	1.54 ± 0.76[c]
50	3.81 ± 0.34	3.42 ± 0.56
0	4.24 ± 0.54[d]	

Values are expressed as mean ± SD, n = 4. Paclitaxel (0.5 μg/m*l*) (sigma, USA) was used as positive control (1.21 ± 0.07). [a]$p < 0.001$, when compared with negative control. [b]$p < 0.01$, when compared with negative control. [c]$p < 0.05$, when compared with negative control. [d]H_2O was used as negative control.

m*l* and EtOAc fraction >100 μg/m*l*, compared with control, showed a significant inhibitory effect on proliferation of liver cancer cell. The result also showed that EtOAc fraction at 100 μg/m*l* had significantly higher cytotoxic activity than aqueous extract at 200 μg/m*l* ($p < 0.05$). Hsieh and Yen reported leaf extract of *Eucommia* has inhibitory effect on oxidative damage in biomolecues, which means drinking of *Eucommia* tea (leaf extract) over a long period time may have anticancer potential.[2] Some results suggest that water extract of *Eucommia* leaves may contribute to the prevention of oxidative gastric injury that precedes carcinogenesis.[19] Our result, however, showed *Eucommia* extract exhibited direct inhibitory activity of human liver cancer cell, so some compounds of *Eucommia* are thought to be potential anticancer materials. Furthermore, EtOAc fraction was found to exhibit higher cytotoxic activity than aqueous extract. Because oxidative stress is involved in pathological process leading to development of cancer[20] and EtOAc fraction (IC_{50}= 19.2 μg/m*l*) showed higher antioxidant activity than aqueous extract (IC_{50}= 125 μg/m*l* in our previous study), we suggest the inhibitory action of *Eucommia* extract on HepG2 cell growth is related to its antioxidant activity to some extent.

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