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ACE Inhibitory Lignan Glycosides Isolated from *Eucommia ulmoides* Oliver

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To evaluate a potential possibility of *Eucommia ulmoides* Oliver as a functional food, ACE (angiotensin converting enzyme) inhibitory activities of leaf, bark, stem and 4 compounds isolated from *E. ulmoides* were tested. The 4 compounds were isolated and purified by silica gel column chromatography, thin layer chromatography and reverse phase column chromatography. Compound I was pinoresinol-4,4′-di-O-β-D-glucoside (PG) and compound Ⅱ was dehydrodiconiferyl alcohol 4,γ′-di-*O*-β-*D*-glucopyranoside (DAG) originating from *Eucommial* Cortex. The highest amount of PG was present at raw and roasted bark as 135.13 mg% and 163.67 mg%, and the highest amount of DAG was present at raw and roasted leaf as 117.93 mg% and 133.93 mg% respectively. In an ACE inhibition test, 10 mg/ml of roasted leaf, raw and roasted bark extracts of *E. ulmoides* Oliver were 77.49%, 75.72% and 75.36% respectively, and 10mg/ml of PG and DAG were shown to be 78.51 and 81.20% respectively. IC_{50} values of PG and DAG were 0.6±0.2 and 0.5±0.2 mg/ml respectively.

Key words : *Eucommia ulmoides* Oliver, antihypertension, angiotensin converting enzyme (ACE), pinoresinol-4,4′-di-O-β-D-glucoside (PG), dehydrodiconiferyl alcohol 4,γ′-di-O-β-D-glucopyranoside (DAG)

Introduction

Hypertension is one of the most typical diseases of adulthood, and it is the origin of various circulatory system disorders. As the senior citizen population increases, the incidence of hypertension also tends to increase. Hypertension is one of the three major causes of death along with cancer and heart disorder because when it develops a complication along with cerebral hemorrhage, heart disorder or kidney disorder, it results in a high fatality rate. Causes of hypertension are usually unknown, and this primary (or spontaneous) hypertension requires medicinal therapy [30]. For humans, social and cultural factors contribute to the occurrence of primary hypertension along with environmental factors such as amount of salt in one's diet, physical activity, obesity, and stress [32]. It has been known that renin-angiotensin system plays an important role in the cause of hypertension, with the participation of an enzyme named angiotensin I converting enzyme [EC 3.4.15.1, ACE: peptidyldipeptide hydrolase] [12]. The inactive angiotensin I that exists in living body is converted to angiotensin II as the dipeptide is detached by ACE. Angiotensin II, which has a contraction function of blood vessel walls, inactivates the hypotensive factor bradykinin, and thus causes the blood pressure to rise. In particular, vital regulation functions in herbal medicines and foods are being studied prevalently for they are recognized to be an impediment to ACE [18,23].

As inhibitors of ACE, chemical compounds such as Enalapril® and Captopril® have been developed for usage. However, due to various side effects, naturally induced ACE inhibitors are being demanded [15]. Until now, majority of natural ACE inhibitors are known to be peptides and polyphenolic substances [11,14,39].

Physiologically active substances contained in plants are mostly secondary metabolites, such as flavonoid, alkaloid, coumarin and plant sterol. These physiologically active substances are of the polyphenols commonly found in fruits, grains, and herbs, inducing physiological activity with very small amount [2,13,17]. Especially, plant-originated ACE inhibitors are expected to become useful as a preventive functional food because they exist in the food that we can intake in our daily.

Recent researches regarding natural food resources that show anti-hypertension activity have mostly been about soybean, soybean paste and casein hydrolysates [20,33,37], mackerel and fish proteins, peptides of animal protein including milk casein [8,9], plant resource including tea, garlic [11,14,22] and *Hovenia dulcis* [28], *Rhemannia Radix* [1], *Epimedii herba* [4], *Panax ginseng* [5,7] and various medicinal

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herbs [3,19]. Some others include germinated-buckwheat [26], chitosan oligosaccharides [16], some marine algae including *Ecklonia cava* [15,25], *Pini folium* and *Leonuri herba* [31] and *Phellius ribis* [36].

Eucommia ulmoides Oliver is an arbor that falls under *Eucommial* species. Its place of origin is known to be China, and has been introduced to Korea in 1926. Because *E. ulmoides* is used for medicinal purposes, it is planted as a crop for special uses in farms over the country. It is medicinally effective as a tonic, sedative, alleviant for pain [35] and hypotensive drug [6,34]. It is known to prevent hypertension and scientific researches of some functions have been conducted, but the researches about its effective substances are still incomplete.

In this research, we separated and identified anti-hypertension substances, examined the contents of effective substances in each part of *E. ulmoides* and ACE inhibitory activity to evaluate the usefulness of *E. ulmoides* as a functional food.

Materials and Methods

Preparation of *E. ulmoides* Oliver

The *E. ulmoides* used in this research were provided by the Sancheonggun research fund for medicinal herb development. More specifically, 10 kg of naturally dried *E. ulmoides* such as leaves, bark, and stems were provided and 5 kg of each were roasted in oven prior to research. Samples were categorized as LW; raw leaf, LT; roasted leaf, BW; raw bark, BT; roasted bark, SW; raw stem and ST; roasted stem.

Chemicals and instruments

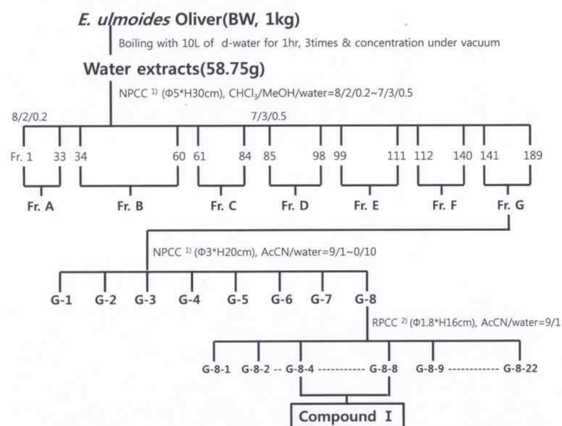
In this research, instruments such as HPLC (Shimadzu LC10-AD, Japan) and microplate reader (BioTek, USA), rotary vacuum evaporator, MPLC, UV-VIS spectrometer (Shimadzu UV-1201, Japan) were used. Reagents and reference standards were all of the special grade, purchased from Sigma or Aldrich co.

Isolation and purification of effective substances

To determine the quantity of anti-hypertensive substances by different parts of *E. ulmoides*, isolation and purification were done as in Fig. 1. To separate the anti-hypertensive substances and its derivatives, as shown in Fig. 1(I) (isolation method started hot water extracts), 1 kg of naturally dried raw bark was boiled with 10 liter of water for 1 hour, and was extracted repeatedly for three times. The concentration under vacuum of these extracts produced 58.75 g of water extract. Next, as shown in Fig. 1(II) (isolation method started methanol extracts), 1 kg of naturally dried raw bark was boiled with 5 liter of methanol for 10 hr, and was repeatedly extracted for three times. The concentration under vacuum of the extracts resulted in 158.75 g of methanol extracts. After isolation and purification using methods such as TLC and column chromatography, four compounds (Compound I~IV) were obtained. Detailed explanation of isolation and purification process is listed along with Fig. 1.

Identification of chemical structure

The Identification of chemical structure of separated substances was processed using instruments such as NMR spec-



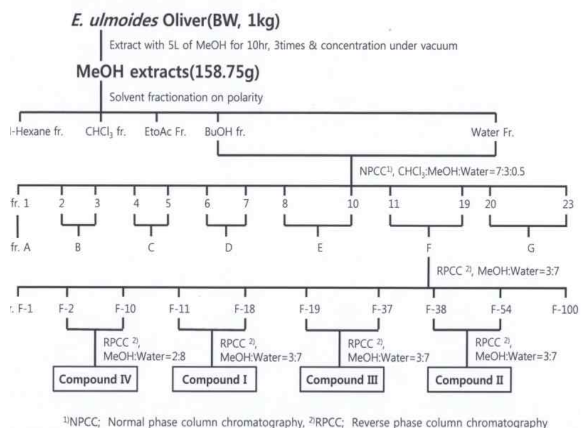


Fig. 1. The isolation procedures of effective substances from water extracts (I) and methanol extracts (II) in *Eucommia ulmoides* Oliver.

troscopy, UV/VIS spectrophotometer, GC-MS, LC-MS, element analyzer, and FT-IR.

Analysis of effective substances

Using HPLC, we examined the contents of effective substances in different parts of *E. ulmoides*. The process of filtrating 10 g of grinded *E. ulmoides* from different parts with 100 ml of distilled water was repeated three times to gather samples. After completely concentrating the water extracts, we dissolved the extract in 10 ml of distilled water again, filtrated by using 0.2 μm membrane filter, and then analyzed using HPLC.

The column used in the analysis was ODS-C18 (Φ3.9×300 mm), mobile phase was acetonitrile / water=70 / 30, detector was UV 254 nm, flow rate was 1 ml/min, and injection volume was 10 μl.

Assay for ACE inhibitory activity

ACE inhibitory activity was measured by the method of Cushman and Cheung [7] with slight modifications implemented by Kim et al. [21]. ACE crude enzyme solution was obtained from 1 g of rabbit lung acetone powder (Sigma, USA), with 20 ml of 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl and placed at 4°C for 2 hr and then by taking the supernatants after centrifugation at 4,000 rpm for 40 min. 25 mg of HHL (hippuryl-histidyl-leucine) was dissolved in 5 ml of 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl. A 200 μl of HHL solution was mixed with 80 μl of sample solution followed by pre-incubation for 5 min at 37°C. The reaction was started by adding of 100 μl of crude ACE solution, and the reaction mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 300 μl of 1 M HCl, and the liberated hippuric acid was extracted with 1 ml of ethyl acetate. After centrifugation (5,000 rpm, 10 min), 0.5 ml of the supernatants was transferred into a test tube and evaporated at room temperature for 2 hr under vacuum. The dried hippuric acid was dissolved in 2 ml of distilled water, and the absorbance was measured at 228 nm using an UV/VIS spectrophotometer (UV-1201, Shimadzu, Japan).

ACE inhibitory activities were calculated using the formula below. The ACE inhibitory effects were compared with each sample and positive controls, Captopril® (*Dongkwang pharm.* co.) and Enalapril® (*Boryoung pharm.* co.). All experiments of this study were performed in triplicate.

$$ACE\ inhibition(\%) = \frac{(C-S)}{(C-B)} \times 100$$

C; control absorbance, S; sample absorbance B; blank absorbance

Statistical analysis

All statistical analyses were performed using one way analysis of variance (ANOVA) followed by Duncan's multiple range test. All values were expressed as mean±SD (n=3) and $P<0.05$ was considered statistically significant.

Results and Discussion

Chemical structure of effective substances

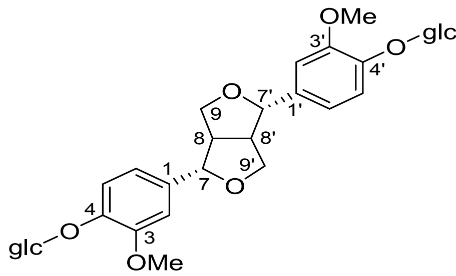
Among the four substances that were isolated from *E. ulmoides*, the chemical structure of compound I and II were analyzed using NMR, MS, and UV/VIS spectrometer. Compound I was found out to be $C_{32}H_{43}O_{16}$, lignan glycoside of M.W. 683, and Compound II was found out as $C_{32}H_{42}O_{16}$, lignan glycoside of M.W. 682 (Fig. 2).

Compound I (Pinoresinol-4,4′-di-*O*-β-*D*-glucoside, PG)

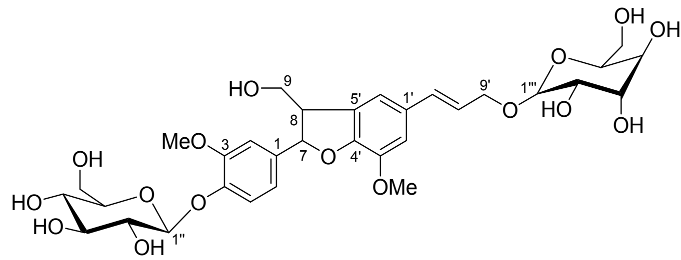
$C_{32}H_{43}O_{16}$, M.W. 683, mp 230°C, ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.11 (2H, m, H-8/H-8′), 3.89 (6H, 3, 3′-OMe), 4.24 (4H, dd, *J*=6.8, 8.8 Hz, H-9/H-9′), 4.75 (2H, d, *J*=4.0 Hz, H-7/H-7′), 4.87 (2H, d, *J*=7.6 Hz, glc H-1′′/H-1′′′), 6.91 (2H, dd, *J*=8.4, 2.0 Hz, H-6/H-6′), 7.02 (2H, d, *J*=1.6 Hz, H-2/H-2′), 7.14 (2H, d, *J*=8.4 Hz, H-5/H-5′); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 55.7 (C-8/C-8′), 56.9 (C-3/C-3′-OMe), 62.6 (C-6′′/C-6′′′), 71.5 (C-4′′/C-4′′′), 72.9 (C-9/C-9′), 75.1 (C-2′′/C-2′′′), 78.0 (C-3′′/C-3′′′), 78.3 (C-5′′/C-5′′′), 87.2 (C-7/C-7′), 103.0 (C-1′′/C-1′′′), 111.8 (C-2/C-2′), 118.2 (C-5/C-5′), 119.9 (C-6/C-6′), 137.6 (C-1/C-1′), 147.7 (C-4/C-4′), 151.1 (C-3/C-3′).

Compound II (Dehydrodiconiferyl alcohol 4,γ′-di-*O*-β-*D*-glucopyranoside, DAG)

$C_{32}H_{42}O_{16}$, M.W. 682, ^{1}H-NMR (400 MHz, CD_3OD) δ: 3.23 (1H, t, *J*=8.0 Hz, H-2′′′), 3.28 (2H, m, H-5′′/H-4′′′), 3.39 (3H, m, H-3′′/H-3′′′/H-4′′), 3.46 (1H, m, H-5′′′), 3.47 (1H, t, *J*=8.0 Hz, H-2′′), 3.68 (1H, dd, *J*=4.0, 12.0 Hz, H-6′′), 3.77 (1H, dd, *J*=7.2, 10.8 Hz, H-9), 3.82 (3H, s, 3-OMe), 3.84 (1H, m, H-9), 3.86 (1H, m, H-6′′), 3.87 (3H, s, 3′-OMe), 3.88 (1H, m, H-6′′′), 4.31 (1H, dd, *J* = 6.4, 13.2 Hz, H-9′), 4.37 (1H, d, *J*=7.6 Hz, H-1′′′), 4.50 (1H, dd, *J*=6.4, 13.2 Hz, H-9′), 4.89 (1H, d, *J*=7.6 Hz, H-1′′), 5.59 (1H, d, *J*=6.0 Hz, H-7), 6.22 (1H, m, H-8′), 6.63 (1H, d, *J*=16.0 Hz, H-7′), 6.92 (1H, dd, *J*=2.0, 8.4 Hz, H-6), 6.96 (2H, s, H-2′/H-6′), 7.02 (1H, s, H-2), 7.15 (1H, d, *J*=8.4 Hz, H-5). ^{13}C-NMR (100 MHz, CD_3OD) δ: 56.860



Pinoresinol-4,4′-di-O-β-D-glucoside (PG) [A]



Dehydrodiconiferyl alcohol 4,γ′-di-O-β-D-glucopyranoside (DAG) [B]

Fig. 2. Chemical structure of Compound Ⅰ [A] and Compound Ⅱ [B] isolated from *E. ulmoides* Oliver.

(3-OMe), 56.914 (3′-OMe), 62.6 (C-6′′), 64.0 (C-6′′′), 65.1 (C-9), 71.1 (C-9′), 71.5 (C-4′′), 71.8 (C-4′′′), 75.0 (C-2′′), 75.3 (C-2′′′), 78.0 (C-5′′′), 78.1 (C-5′′), 78.267 (C-3′′), 78.313 (C-3′′′), 89.0 (C-7), 102.9 (C-1′′), 103.3 (C-1′′′), 111.3 (C-2), 112.4 (C-6′), 116.8 (C-2′), 118.1 (C-5), 119.5 (C-6), 124.5 (C-8′), 130.2 (C-5′), 132.6 (C-1′), 134.3 (C-7′), 138.2 (C-1), 145.7 (C-3′), 147.8 (C-4), 149.5 (C-4′), 151.1 (C-3).

Contents of effective substances

Table 1 shows the result of analyzing contents of pinoresinol-4,4′-di-O-β-D-glucoside (PG) and dehydrodiconiferyl alcohol 4,γ′-di-O-β-D-glucopyranoside (DAG), separated in this research by using HPLC. In the case of PG, content was 135.12 and 163.94 mg%, for naturally dried bark and roasted bark respectively. This result showed highest amount compared to those of leaves and stems. Naturally dried leaves showed 85.13 mg%, which means that the bark contains 1.59 times that of the leaves. Also, the naturally dried stem showed 70.49 mg%, meaning that the bark contains 1.92 times that of the stems. For roasted bark, it contained 2.35 times that of the roasted leaves (69.19 mg%) and 1.54 times that of roasted stems (106.33 mg%).

Table 1. Pinoresinol-4,4′-di-O-β-D-glucoside (PG) and dehydrodiconiferyl alcohol 4,γ′-di-*O-b-D*-glucopyranoside (DAG) contents in different parts of *E. ulmoides* Oliver analyzed by HPLC

Samples[1)]	Yield of water extracts (%, w/w)	Contents (mg%, w/w)	
		Compound I (PG)	Compound II (DAG)
LW	5.36±0.009[2)c3)]	85.13±0.018[d]	117.43±2.116[b]
LT	5.87±0.030[b]	69.19±0.457[f]	133.93±2.076[a]
BW	5.26±0.011[d]	135.12±0.025[b]	57.79±1.924[d]
BT	6.01±0.011[a]	163.94±0.352[a]	70.77±2.010[c]
SW	5.02±0.014[f]	70.49±0.016[e]	37.86±1.230[e]
ST	5.22±0.024[e]	106.33±0.206[c]	39.95±1.562[e]

[1)]LW; Raw leaf, LT; Roasted leaf, BW; Raw bark, BT; Roasted bark, SW; Raw stem, ST; Roasted stem. [2)]Values are given as mean±SD (n=3). [3)]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

In the case of DAG, naturally dried leaves and roasted leaves showed 117.43 and 133.93 mg% respectively, which were relatively high compared to those of bark and stem. Overall, effective substances were increased by roasting.

Takamura *et al.* [38] said that notable natural substances of green leaves of *E. ulmoides* were geniposidic (2.53%), aucubin (2.99%), and chlorogenic acid (0.16%), and they show higher content in green leaves compared to those of roasted leaves. In this research, PG of roasted leaves decreased in small amount, but in general, PG and DAG both tended to increase through the roasting process.

ACE inhibition activities of *E. ulmoides* extracts

The result of analyzing anti-hypertension activity of extracts from different parts is as shown in Fig. 3. The ACE inhibition activities by treated concentration indicated similar activities when treated in 10 mg/ml, 72.70~77.56% for leaves and 75.68~75.73% for bark, while the leaves showed relatively low inhibition activity by 46.80~53.19%.

When treated in 5 mg/ml, there were no indicative differences in leaves and stems, but ACE inhibition activity decreased significantly in bark. As seen from the result, ACE inhibition activities of *E. ulmoides* were high for leaves and bark in 10 mg/ml, and were relatively low for stems. In this case, the data was somewhat inconsistent with the content of PG, which is also one of the most important hypotensive substances of *E. ulmoides*. In leaves of *E. ulmoides*, there are other flavonoids, alkaloids, and glycosides aside from PG [24,29], indicating relatively high anti-hypertension activity. Also, according to reported experiment results of medicinal herbs, the ACE inhibition activities of water extracts of *Hovenia dulcis* and ethanol extracts of

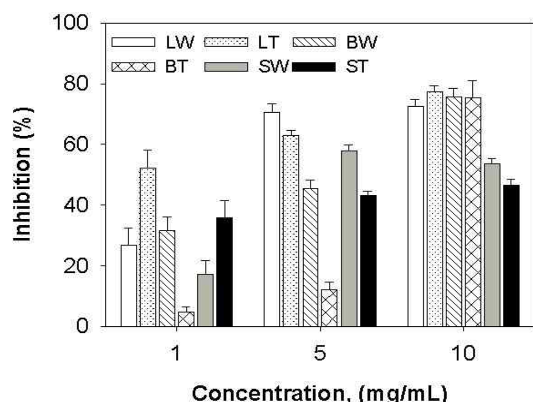


Fig. 3. ACE inhibition effects of extracts from the raw and roasted *E. ulmoides* Oliver leaf, bark and stem. LW; Raw leaf, LT; Roasted leaf, BW; Raw bark, BT; Roasted bark, SW; Raw stem, ST; Roasted stem. Values are given as mean±SD (n=3).

Rehmannia radix were 75.8% at the concentration of 4 mg/ml and 55.24% at the concentration of 1 mg/ml respectively [1,25]. And ACE inhibition activity of *Psoralea corylifolia* was reported as 65.2% at the concentration of 10 mg/ml [9].

ACE inhibition activities of lignan glycosides isolated from *E. ulmoides*

The ACE inhibition activities of four compounds, Compound I (PG), Compound II (DAG), Compound III and Compound IV that were isolated from *E. ulmoides*, were analyzed and compared with two kinds of medication that are currently prescribed and sold on the market. The ACE inhibition activity of the four compounds along with Captopril® (*Dongkwang pharm. co.*) and Enalapril® (*Boryoung pharm. co.*) are compared in Fig. 4.

The concentrations of samples were divided into three categories: 10, 5 and 1 mg/ml. Captopril®, which was used as a positive control, showed 95.20, 85.41, and 75.61% of ACE inhibition activities according to the concentration, and Enalapril® showed a bit higher inhibition activity, of 96.16, 91.15, and 85.29%. Compound I (PG) showed 81.20, 77.82, and 71.94% of inhibition activity by each concentration and the results of Compound II (DAG) were similar, as 78.51, 74.78, and 74.15%. Compound III and IV showed similar inhibition activity, between 67.71 and 85.25%. And the IC_{50} values of PG, DAG, Compound III and IV were indicated as 0.6±0.2, 0.5±0.2, 0.6±0.2 and 0.5±0.2 mg/ml and those of Captopril® and Enalapril® were 0.4±0.06 and 0.2±0.04 mg/ml respectively (Table 2).

The four compounds that were separated from *E. ulmoides* are thought to be glycosides of lignan, and because they have similar structures, the anti-hypertension activities are also expected to be similar.

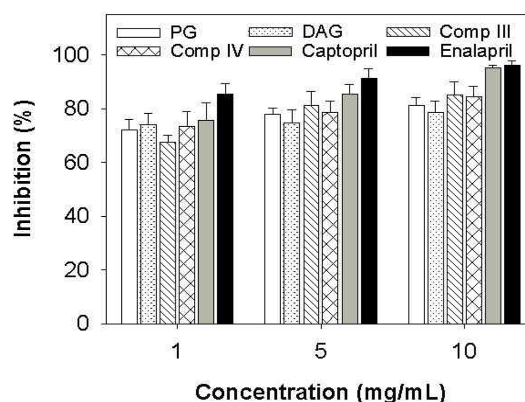


Fig. 4. Comparisons of ACE inhibition activities between positive controls and PG, DAG, compound III & IV isolated from *E. ulmoides* Oliver. Antihypertensive drugs from Boryoung (Captopril®) and Dongkwang (Enalapril®) pharm. Co. Ltd., PG, DAG, Com III & IV were isolated *Eucommia ulmoides* Oliver in this study. Values are given as mean±SD (n=3).

Table 2. IC_{50} values of positive controls and PG, DAG, compound III & IV isolated from *E. ulmoides* Oliver by ACE inhibition test

Samples[1)]	IC_{50} values[2)] (mg/ml)
PG	0.6±0.2
DAG	0.5±0.2
Compound III	0.6±0.2
Compound IV	0.5±0.2
Captopril	0.4±0.06
Enalapril	0.2±0.04

1)PG, DAG, Com III & IV were isolated from *Eucommia ulmoides* Oliver in this study. and antihypertensive drugs from Boryoung (Captopril®) and Dongkwang (Enalapril®) pharm. Co. Ltd.

2)IC_{50} value is the concentration of sample required for 50% inhibition. Each value is expressed as mean±SD (n=3).

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초록 : 두충으로부터 분리한 lignan glycoside의 ACE 활성 억제

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두충(*Eucommia ulmoides* Oliver)으로부터 항고혈압 활성을 가진 유효물질을 분리하였으며, 두충의 잎, 껍질 및 줄기를 건조한 것과, 건조한 후 볶은 것에 대하여 ACE (angiotensin converting enzyme) 저해활성을 실험하였다. 두충으로부터 분리한 유효물질은 pinoresinol-4,4′-di-O-β-D-glucoside (PG)와 dehydrodiconiferyl alcohol 4,γ′-di-O-β-D-glucoside (DAG)였으며, 이들은 각각 껍질과 잎에 많이 함유되어 있었다. 즉 pinoresinol-4,4′-di-O-β-D-glucoside (PG)는 자연 건조한 껍질에 135.12 mg%, 볶은 껍질에 163.94 mg%로 가장 많이 함유되어 있었으며, dehydrodiconiferyl alcohol 4,γ′-di-O-β-D-glucoside (DAG)는 자연 건조한 잎에 117.43 mg%, 볶은 잎에 133.93 mg%로 비교적 많이 함유되어 있었다. ACE 억제활성은 볶은 잎, 말린 껍질 및 볶은 껍질을 10 mg/ml씩 처리하였을 때, 각각 77.49, 75.52 및 75.36%의 억제효과를 나타내었으며, 두충으로부터 분리한 화합물인 pinoresinol-4,4′-di-O-β-D-glucoside (PG)와 dehydrodiconiferyl alcohol 4,γ′-di-O-β-D-glucoside (DAG)를 1∼10 mg/ml 처리하였을 때에는 67.1∼85.25%의 억제활성을 나타내었으며, IC_{50}값은 각각 0.6±0.2, 0.5±0.2 mg/ml 였다. 이것은 PG와 DAG가 두충에서 분리한 천연물임을 감안하면 매우 높은 활성을 가지고 있다고 할 수 있다.