The Promoting Effects of Geniposidic Acid and Aucubin in Eucommia ulmoides Oliver Leaves on Collagen Synthesis

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We have reported that collagen synthesis was stimulated by the administration of a hot water extract from the leaves of Eucommia ulmoides Oliver, Eucommiaceae (Du-Zhong leaves) in false aged model rats. In this paper, we set out to examine the compounds in Du-Zhong leaves that stimulated collagen synthesis in false aged model rats. In experiment 1, a methanol extract of Du-Zhong leaves also stimulated collagen synthesis in aged model rats. An acetone fraction was derived from the methanol extract by silica gel chromatography in experiment 2. The acetone fraction mainly contained iridoides mono-glycosides such as geniposidic acid and aucubin. The administration of geniposidic acid or aucubin stimulated collagen synthesis in aged model rats in experiments 3 and 4 (significance (p<0.05)). The reported pharmacological effects of Du-Zhong leaves, including healing organs and strengthening bone and muscle, are closely related to collagen metabolism. It appears that geniposidic acid and aucubin are the actual compounds in Du-Zhong which caused the effect in our experiments.

Key words Du-Zhong; leaf; geniposidic-acid; aucubin; collagen-synthesis; granuloma

Eucommia ulmoides Oliver (Du-Zhong) is a large deciduous tree which originated in China, and the bark of the tree has been used for natural medicine, as published by Shen Nong Ben Cao Jing3) and Ben Cao Gang Mu.4) Recently, Du-Zhong tea made from Du-Zhong leaves has been consumed habitually as a healthy drink in China, Korea and Japan. Du-Zhong leaves contain compounds similar to those in the bark3–6) and the leaves have the same pharmacological effects as the bark. We have investigated the pharmacological effects of Du-Zhong leaves.7–11) In the previous paper, we reported that collagen synthesis in a false aged model rat was stimulated by the administration of a hot water extract of Du-Zhong leaves.12)

In this paper, we set out to examine the compounds in Du-Zhong leaves that stimulated collagen synthesis in false aged model rats.

MATERIALS AND METHODS

1. Reagents All chemical reagents were reagent grade and were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), unless noted otherwise.

2. Collection and Preservation of Du-Zhong Leaves Approximately 2.7 kg of fresh Du-Zhong leaves were collected in the medicinal herb garden (College of Pharmacy, Nihon University, Funabashi-shi, Chiba, Japan) in September, 1996, and immediately dried at 60 °C for 24 h. Dried Du-Zhong leaves (1 kg) were stored desiccated in the dark.

3. Preparation of Methanol Extract of Du-Zhong Leaves Dried Du-Zhong leaves were ground with a coffee mill, then passed through a sieve (50, 300 mesh, according to Japanese Pharmacopoeia Thirteenth Edition). The powdered leaves were added to 10-fold methanol, and the mixture was placed in an ultrasonic vibrator for 1 h and then filtered. The residue was also extracted in the same manner. The filtrates were gathered and concentrated under 40 °C in vacuo (concentration was done in this condition unless otherwise noted), then lyophilized. The extract was stored at −80 °C.

4. The Fractionation of Methanol Extract of Du-Zhong Leaves Sixty-three grams of a methanol extract of Du-Zhong leaves was dissolved in 10-fold 60% aqueous methanol, then extracted with same volume of n-hexane. The n-hexane layer was concentrated in vacuo, and yielded 10 g of n-hexane fraction. The remaining aqueous methanol layer was concentrated in vacuo, then the residue was applied to the chromatography column with Wako Gel (C-200, 500 g, Wako Pure Chemical Industries, Ltd., Osaka, Japan), then eluted with ethyl acetate (AcOEt, 5 l), acetone (12 l), and methanol (MeOH, 5 l). Each eluted solution was concentrated in vacuo, giving an AcOEt fraction (4 g, yielded a 7%), acetone fraction (15 g, yielded a 24%), and a MeOH fraction (33 g, yielded a 53%). Each fraction was stored at −80 °C until being used for experiment 2.

5. Preparation of False Aged Model Rat False aged model rats13–16) were prepared according to a previous paper.12) Briefly, the preparation of false aged model rats used Wistar/ST male rats aged 6 or 7 weeks (SLC Japan) bred with a low protein diet (6%, Japan Clea, Co., Tokyo, Japan) for 3 weeks. Rats were bred in an environment of 22±1 °C with 12 h light/dark cycles, and were given a 6% protein diet and water ad libitum throughout the experiment. All experiments were done using false aged model rats. The experimental animals were handled in accordance with the Animal Experiment Guidelines established by College of Pharmacy, Nihon University (according to the guidelines of Ministry of Education, Science, Sports and Culture of the Japanese Government).

6. Experimental Production of Granuloma in the Formalin-Filter Paper Pellet (FFP) Method This experiment was conducted according to the method of Tanaka17) with slight modification.12) The skin of false aged model rats (aged 9 or 10 weeks) were cut out as follows: 1.5 cm on the back at the point under the Scapula and Os ischii, symmetrically, under ethyl ether anesthesia. Granulomas were induced by the subcutaneous implantation of four pieces of filter
paper (Toyo filter paper # 126, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) soaked with 20 μl of a 7% aqueous formaldehyde. One week after the implantation, rats were sacrificed by cardiocentesis exsanguination under ethyl ether anesthesia. The pieces with ingrown granulomas and other organs were carefully dissected and weighed immediately. Granulomas were stored at −80°C until hydroxyproline contents were analyzed.

7. Experimental Plans Experiment 1: Effect of Granuloma Formation and Collagen Synthesis in False Aged Model Rats by Administration of Methanol Extract of Du-Zhong Leaves: Fifteen Wistar/ST male rats aged 7 weeks (209 g average body weight (BW)) were divided into 3 groups as follows: Group 1-I, control group bred only with a 6% protein diet; Group 1-II, methanol extract group (1.8 g of dried Du-Zhong leaves/kg of BW/d); Group 1-III, methanol extract group (2.7 g of dried Du-Zhong leaves/kg of BW/d), and each group had 5 rats. The administration dosages were expressed according to the corresponding dried weight of the Du-Zhong leaves in order to contrast them, easily. A methanol extract of Du-Zhong leaves mixed in a 6% protein diet was administered for 4 weeks. The actual dosages of the methanol extracts of Du-Zhong leaves to false aged model rats were calculated based on the feed intake of the day before. After 3 weeks administration of methanol extract of Du-Zhong leaves, the false aged model rats (aged 10 weeks) were implanted with four pieces of filter paper soaked with 20 μl of a 7% aqueous formaldehyde, as described for the FFP method. One week after the implantation, rats were sacrificed by cardiocentesis exsanguination under ethyl ether anesthesia. The pieces with ingrown granulomas and other organs were carefully dissected and weighed immediately. Granulomas were stored at −80°C until analysis of the hydroxyproline content.

Experiment 2: Effect of Granuloma Formation and Collagen Synthesis in False Aged Model Rats by Administration of n-Hexane Fraction, AcOEt Fraction, Acetone Fraction or MeOH Fraction of Methanol Extract of Du-Zhong Leaves: Forty-five Wistar/ST male rats aged 7 weeks (206 g average BW) were divided into 9 groups as follows: Group 2-I, control group bred only with a 6% protein diet; Group 2-II, n-hexane fraction group (1.5 g of dried Du-Zhong leaves/kg of BW/d); Group 2-III, n-hexane fraction group (3.1 g of dried Du-Zhong leaves/kg of BW/d); Group 2-IV, AcOEt fraction group (1.5 g of dried Du-Zhong leaves/kg of BW/d); Group 2-V, AcOEt fraction group (2.9 g of dried Du-Zhong leaves/kg of BW/d); Group 2-VI, acetone group (1.5 g of dried Du-Zhong leaves/kg of BW/d); Group 2-VII, acetone fraction group (3.0 g of dried Du-Zhong leaves/kg of BW/d); Group 2-VIII, MeOH fraction group (1.5 g of dried Du-Zhong leaves/kg of BW/d); Group 2-IX, MeOH fraction group (3.0 g of dried Du-Zhong leaves/kg of BW/d), and each group had 5 rats. The administration dosages were also expressed in the corresponding dried weight of Du-Zhong leaves. The administration of each fraction and the granuloma formation were done as described in experiment 1.

Experiment 3: Effect of Granuloma Formation and Collagen Synthesis in False Aged Model Rats by Administration of Geniposidic Acid: Twenty Wistar/ST male rats aged 7 weeks (214 g averaged BW) were divided into 4 groups as follows: Group 3-I, control group bred only with a 6% protein diet; Group 3-II, geniposidic acid group (25 mg/kg of BW/d); Group 3-III, geniposidic acid group (50 mg/kg of BW/d); Group 3-IV, geniposidic acid group (100 mg/kg of BW/d), and each group had 5 rats. Geniposidic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan) was dissolved in water, and was administered to the false aged model rats for 4 weeks. The administration dosages were planned based on the concentration of geniposidic acid (about 2%) in dried Du-Zhong leaves in September.18 Actual geniposidic acid dosages were supplied by water service to give a fixed content per one kg of body weight calculated based on the water intake of the day before. After 3 weeks administration of geniposidic acid, the granuloma formation was done as described in experiment 1.

Experiment 4: Effect of Granuloma Formation and Collagen Synthesis in False Aged Model Rats by Administration of Aucubin: Fifteen Wistar/ST male rats aged 6 weeks (191 g average BW) were divided into 3 groups as follows: Group 4-I, control group bred only with a 6% protein diet; Group 4-II, aucubin group (25 mg/kg of BW/d); Group 4-III, aucubin group (50 mg/kg of BW/d), and each group had 5 rats. Aucubin19 was purified from the methanol extract of Du-Zhong leaves. The administration dosages were planned based on the aucubin concentration (about 2%) in dried Du-Zhong leaves in September.18 The administration of aucubin and the granuloma formation were done as described in experiments 3 and 1, respectively.

8. Determination of Hydroxyproline Contents in Granulomas: This portion was conducted according to the method of Prockop20 with slight modification.21 The filter paper was carefully removed from the grown granuloma. The granuloma was weighed and then lyophilized. Three pieces of the granuloma were added to 5 ml of 6 N HCl, and then hydrolyzed at 110°C for 24 h. After being neutralized by aqueous KOH, hydroxyproline in the reaction mixture was converted to pyrrole derivatives by oxidation with 0.5 M chloramine T (in 2-methoxyethanol) at room temperature. Excess chloramine T was destroyed by adding aqueous sodium thiosulfate. Ehrlisch's reagent was stirred in rapidly for the final color reaction, then the optical absorption was determined at 560 nm. Hydroxyproline contents in granuloma were converted to collagen contents.

9. Statistics: The values were expressed as mean±S.E. Statistical processing was done by analysis of variance ANOVA (Fisher's protected least significant difference (Fisher's PLSD)), unless noted otherwise. Differences with a probability of less than 5% were considered to be statistically significant (p<0.05).

RESULTS: The stimulation of granuloma formation and collagen synthesis by the administration of a methanol extract of Du-Zhong leaves in false aged model rats was tested. In experiment 1, the granuloma weights and collagen synthesis in false aged model rats due to the administration of a methanol extract of Du-Zhong leaves were significantly (p<0.05) higher than in the controls (Fig. 1). The group fed 1.8 g of dried Du-Zhong leaves/kg of BW/d of the methanol extract showed a 69% increase in granuloma formation (p<0.05).
Fig. 1. Development of Granuloma (△) and Hydroxyproline Content in Granuloma (■) with the Administration of Du-Zhong Leaf Methanol Extract

Each bar shows the mean±S.E. of five rats. *p<0.05, compared with the control by ANOVA (Fisher's PLSD). The administration dosages were expressed in the corresponding weight of dried Du-Zhong leaves.

and a 68% increase in collagen synthesis (p<0.05). The group fed 2.7 g of dried Du-Zhong leaves/kg of BW/d of the methanol extract showed a 131% increase in granuloma formation (p<0.05) and a 131% increase in collagen synthesis (p<0.05).

In addition, granuloma formation and collagen synthesis by the administration of n-hexane fraction, AcOEt fraction, acetone fraction or MeOH fraction of the methanol extract of Du-Zhong leaves in false aged model rats was examined. Experiment 2 showed that no change was observed in the granuloma weights and collagen synthesis of false aged model rats with the administration of n-hexane fraction, AcOEt fraction or MeOH fraction of the methanol extract of Du-Zhong leaves compared to the control group (Fig. 2). However, in the acetone fraction group with 3.0 g of dried Du-Zhong leaves/kg of BW/d, a 56% increase in granuloma formation (p<0.05) and a 57% increase in collagen synthesis (p<0.05) was observed (Fig. 2).

The stimulation of granuloma formation and collagen synthesis by the administration of geniposidic acid in false aged model rats was also tested. In experiment 3, granuloma formation and collagen synthesis in false aged model rats was significantly increased (p<0.05) by the administration of geniposidic acid compared with the control group. The group with 25 mg/kg of BW/d showed significant increases (p<0.05) in granuloma formation, 82%, and collagen synthesis, 79% (Fig. 3). The group with 50 mg/kg of BW/d showed significant increases (p<0.05) in granuloma formation, 53%, and collagen synthesis, 82% (Fig. 3). The group with 100 mg/kg of BW/d showed significant increases (p<0.05) in granuloma formation, 69%, and collagen synthesis, 95% (Fig. 3).

Furthermore, the stimulation of granuloma formation and collagen synthesis by the administration of aucubin in false aged model rats was investigated. In experiment 4, granuloma formation and collagen synthesis in false aged model rats were significantly increased (p<0.05) by the administra-
Table 1. Body Weight and Organ Body Weight Ratio of Rats by the Administration of Geniposidic Acid in Du-Zhong Leaf Methanol Extract

<table>
<thead>
<tr>
<th></th>
<th>Group 3-I control</th>
<th>Group 3-II geniposidic acid 25 mg/kg of BW/d</th>
<th>Group 3-III geniposidic acid 50 mg/kg of BW/d</th>
<th>Group 3-IV geniposidic acid 100 mg/kg of BW/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>269±9.1</td>
<td>279±7.5</td>
<td>271±8.6</td>
<td>271±8.6</td>
</tr>
<tr>
<td>Liver (g/100 g of BW)</td>
<td>3.07±0.11</td>
<td>3.39±0.11</td>
<td>2.97±0.19</td>
<td>3.02±0.13</td>
</tr>
<tr>
<td>Kidney (mg/100 g of BW)</td>
<td>545±19</td>
<td>516±9.0</td>
<td>560±13</td>
<td>572±21</td>
</tr>
<tr>
<td>Spleen (mg/100 g of BW)</td>
<td>163±4.4</td>
<td>167±13</td>
<td>181±9.7</td>
<td>171±7.1</td>
</tr>
<tr>
<td>Heart (mg/100 g of BW)</td>
<td>301±12</td>
<td>282±6.8</td>
<td>286±13</td>
<td>287±4.3</td>
</tr>
<tr>
<td>Thymus (mg/100 g of BW)</td>
<td>174±9.7</td>
<td>181±14</td>
<td>186±16</td>
<td>192±22</td>
</tr>
</tbody>
</table>

Values are mean±S.E. of five rats.

Table 2. Body Weight and Organ Body Weight Ratio of Rats by the Administration of Aucubin in Du-Zhong Leaf Methanol Extract

<table>
<thead>
<tr>
<th></th>
<th>Group 4-I control</th>
<th>Group 4-II aucubin 25 mg/kg of BW/d</th>
<th>Group 4-III aucubin 50 mg/kg of BW/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>260±6.3</td>
<td>262±5.4</td>
<td>255±6.2</td>
</tr>
<tr>
<td>Liver (g/100 g of BW)</td>
<td>3.45±0.14</td>
<td>3.20±0.13</td>
<td>3.20±0.21</td>
</tr>
<tr>
<td>Kidney (mg/100 g of BW)</td>
<td>640±23</td>
<td>610±9.1</td>
<td>598±12</td>
</tr>
<tr>
<td>Spleen (mg/100 g of BW)</td>
<td>207±8.6</td>
<td>182±5.5</td>
<td>206±17</td>
</tr>
<tr>
<td>Heart (mg/100 g of BW)</td>
<td>329±8.3</td>
<td>315±7.5</td>
<td>324±9.5</td>
</tr>
<tr>
<td>Thymus (mg/100 g of BW)</td>
<td>219±16</td>
<td>222±8.9</td>
<td>207±7.6</td>
</tr>
</tbody>
</table>

Values are mean±S.E. of five rats.

The body weight and organ body weight ratio (g or mg/100 g of BW) in false aged model rats by the administration of geniposidic acid was examined. No change was observed compared with the control group in body weight or liver-, kidney-, spleen-, or thymus body weight ratio (g or mg/100 g of BW) of the false aged model rat by the administration of geniposidic acid (25, 50 or 100 mg/kg of BW/d) (Table 1).

The body weight and organ body weight ratio (g or mg/100 g of BW) in false aged model rats by the administration of aucubin was determined. No change was observed compared with the control group in body weight and liver-, kidney-, spleen-, and thymus body weight ratio (g or mg/100 g of BW) of a false aged model rat by the administration of aucubin (25 or 50 mg/kg of BW/d) (Table 2).

DISCUSSION

Promoting Compounds in Du-Zhong Leaves Stimulated Granuloma Formation and Collagen Synthesis in the False Aged Model Rat

The measurement of collagen synthesis in true young developing rats is not so easy because the protein level in the body is strictly regulated. Collagen synthesis was downregulated in the experimental animals bred with a low protein diet.14–16 This phenomenon was more apparent in younger animals than in the aged.13 When animals showed lower protein synthesis, they acted the same as the aged ones. So we can easily investigate the recovery of the collagen synthesis in false aged model rats by the administration of the compounds. We have reported the promoting effect on granuloma formation and collagen synthesis of a hot water extract of Du-Zhong leaves on false aged model rats.12 In experiment 1, granuloma formation and collagen synthesis were dose dependently, increased by the administration of the methanol extract of Du-Zhong leaves, showing that the methanol extract of Du-Zhong leaves also contains compounds with promoting effects on granuloma formation and collagen synthesis (Fig. 1).

The methanol extracts of Du-Zhong leaves were divided into n-hexane, AcOEt, acetone and MeOH fractions by gel chromatography, then the second experiment was performed. Experiment 2 indicated that the acetone fraction had a strong promoting effect on granuloma formation and collagen synthesis (Fig. 2). On TLC of the acetone fraction of the methanol extract of Du-Zhong leaves, the main discoveries were iridoid mono-glycosides, such as geniposidic acid and aucubin. This indicates that the iridoid mono-glycosides in Du-Zhong leaves might accelerate granuloma formation and collagen synthesis. The n-hexane fraction and AcOEt fraction also showed a tendency to increase the granuloma formation, indicating the possibility of some other effective compounds beside iridoid mono-glycosides in the methanol extract of Du-Zhong leaves.

Several pharmacological effects of geniposidic acid21–23 and aucubin24–27 were reported, but there was no report related to collagen metabolism. Geniposidic acid and aucubin are among the active compounds in Du-Zhong leaves. In experiments 3 and 4, geniposidic acid and aucubin displayed promoting effects on granuloma formation and collagen synthesis in false aged model rats (Figs. 3 and 4). It was shown that geniposidic acid and aucubin are among the active compounds which stimulate granuloma formation and collagen synthesis in Du-Zhong leaves. But, the effect of aucubin is slightly weaker than that of geniposidic acid.

By the administration of geniposidic acid or aucubin, no effect was observed on body weight and organ body weight ratio in false aged model rats (Tables 1 and 2), and no side effects were observed for the dosages used of geniposidic acid or aucubin.

The bark from the Du-Zhong tree has been used as a natural medicine since ancient times.12 It's well known that the bark has many effects: releases back pain (Zhi Yao Ji Tong),
makes organs healthy (Bu Zhong), increases one’s strength (Yi Jing Qi), makes one’s bones and muscles strong (Jian Jin Gu), increases ability to remember (Qiang Zhi), increases recovery from fatigue (Qing Shen) and shows an anti-aging effect (Nai Lao). In addition, the bark causes no side effects (Jiu Fu), and the constituents of the leaves of Du-Zhong were quite similar to those contained in bark. Thus, it is reasonable to predict that the leaves from the Du-Zhong tree may have the same pharmacological effects as the bark.

Many of the medical effects of Du-Zhong are closely related to protein metabolism, especially collagen, such as releases the back pain (Zhi Yao Ji Tong), makes organs healthy (Bu Zhong), makes one’s bones and muscles strong (Jian Jin Gu). By the administration of Du-Zhong leaves, we developed the activation of the protein synthesis rate in the muscle of mice. It has also been reported that the ratio of soluble collagen is increased in the muscle in eel, and that the aged muscle type changed to a younger one. The protein metabolite rate was thus stimulated in organs, bones and muscles by the administration of Du-Zhong leaves. It appears that geniposide acid and aucubin are the actual compounds in Du-Zhong that created the outcome in our experiments.

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This paper is developed for the doctorate thesis of Miss Yanmei Li.

REFERENCES AND NOTES

19) Isolation of aucubin was done as follows. Du-Zhong leaves, 20 g were extracted with methanol according to the text. Methanol extract was suspended in 100 ml water, then filtered. The filtrate was concentrated in vacuo and yielded 3.5 g. Aucubin was purified from this residue with an MCI GEL column (CHP 20P, 100 ml, Mitsubishi Chemical Corporation, Tokyo, Japan) and then with a charcoal column (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (yield 389 mg, 1.9%).