Granuloma Maturation in the Rat is Advanced by the Oral Administration of *Eucommia ulmoides* OLIVER Leaf

Yanmei LI,^a Koichi METORI,^b Katsuya KOIKE,^{*a} Fumio KITA,^c Qing-ming CHE,^d Tuneo SATO,^e Wataru SHIRAI,^e and Shushichi TAKAHASHI^f

Biochemistry Laboratory,^a and Chemical Analytical Center,^b College of Pharmacy, Nihon University 7-7-1 Narashino-dai, Funabashi, Chiba 274-8555, Japan, Research and Development Division, SS Pharmaceutical Co., Ltd.,^c 2-12-4 Nihonbashi Hama-cho, Chuo-ku, Tokyo 103, Japan, Department of Traditional Chinese Medicine, School of Pharmaceutical Science, Beijing Medical University,^d Beijing 100083, P. R. China, College of Bioresource Sciences, Nihon University,^e 1866 Kameino, Fujisawa, Kanagawa 252, Japan, and College of Industrial Technology, Nihon University^f 1-2-1 Izumi-cyo, Narashino, Chiba 275-8575, Japan. Received June 11, 1999; accepted September 13, 1999

We have reported that collagen metabolism was improved by the administration of *Eucommia ulmoides* OLIVER leaf. In this paper, we examine the granuloma maturation and deposition of collagen in the granuloma of rats due to the oral administration of this leaf. After 3 weeks of the oral administration, granuloma formation was induced by the formalin soaked filter paper-pellet method. A week later, the developing granuloma was dissected. Granuloma formation was significantly increased due to ingestion of the dried leaf at a dose of 1.8 g/kg of body weight/d. The collagen content in the granuloma was also significantly increased. In the case of the collagen profile, the pepsin-solubilized collagen content and its relative percentage to the total collagen were significantly higher than in the control. Histochemical examination showed that the granuloma tissues were well developed, and displayed many newly synthesized capillary vessels and a greater quantity of fibroblasts and monocytes in the 1.8 g leaf group. High density lipoprotein (HDL)-cholesterol and triglyceride content in the blood plasma were significantly higher than in the control. These results show that granuloma maturation was accelerated and the energy was supplied from fatty acid metabolism. The administration of *Eucommia ulmoides* OLIVER leaf may be effective at speeding up the wound healing process.

Key words *Eucommia ulmoides* OLIVER; leaf; granuloma; maturation; collagen; synthesis

Eucommia ulmoides OLIVER is a large deciduous tree originating in China. The bark of the tree has been used in natural medicine, with the Chinese name Du-Zhong (杜仲), as recorded in Shen Nong Ben Cao Jing (神農本草經)¹⁾ and Ben Cao Gang Mu (本草綱目).²⁾ Recently, tea made from *Eucommia ulmoides* OLIVER leaf has been consumed as a health tonic in China, Korea and Japan. There are articles detailing the chemical components and pharmaceutical effects of the leaf^{3–8)} and bark of *Eucommia ulmoides* OLIVER. It was shown that the leaf contains similar compounds to those in the bark, so the same pharmacological effects are expected from both.^{9–12)} Several pharmaceutical effects are on record in Shen Nong Ben Cao Jing¹⁾ and are related to aging as follows: relieves back pain (Zhi Yao Ji Tong, 治腰脊痛); makes organs healthy (Bu Zhong, 補中); increases one's strength (Yi Jing Qi, 益精氣); makes one's bones and muscles strong (Jian Jin Gu, 堅筋骨); makes one clever (Qiang Zhi, 強志); quickens recovery from fatigue and shows an anti-aging effect when consumed over long time periods (Jiu Fu Qing Shen Nai Lao, 久服輕身耐老). The pharmaceutical effects of Du-Zhong are closely related to protein metabolism, especially that of collagen. It is well known that during the aging process, collagen and non-collagenous protein metabolism is depressed.^{13,14)} Therefore, we suppose Du-Zhong might prevent the aging process by activating protein metabolism. We reported that its pharmacological effect is related to protein metabolism, especially to collagen, and it was shown to improve protein metabolism in false aged model rats due to the oral administration of a hot water extract^{15–23)} and a methanol extract²⁴⁾ of the *Eucommia ulmoides* OLIVER leaf.

The granulation phase belongs to the proliferative stage of the wound healing process and is formed by macrophages,

vascular endothelial cells and fibroblasts. A granuloma contains lymphocytes and creates a protective barrier from infection. The deposition of collagen synthesized by fibroblasts can increase the mechanical tensile strength of a granuloma.²⁵⁾ The deposition of collagen in a granuloma strongly relates to granulation and the wound healing process.

In this paper we examine the deposition of collagen in granulomas and the maturation of granulation and how this is affected by the oral administration of a hot water extract of *Eucommia ulmoides* OLIVER leaf.

MATERIALS AND METHODS

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

Preparation of Hot Water Extract of *Eucommia ulmoides* OLIVER Leaf Dried *Eucommia ulmoides* OLIVER leaves were purchased from Bussan Nakasen Co., Ltd. (Nakasen, Akita, Japan) ground with a coffee mill and passed through a sieve (#50, 300 mesh, according to Japanese Pharmacopoeia Thirteenth Edition). The leaf powder was added to 10-fold distilled water, refluxed 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 under this condition unless otherwise noted), then lyophilized (yield of 27%). The extract was stored at –80 °C until use.

Animal Care Rats were bred in an environment of 22 ± 1 °C with 12 h light/dark cycles, given a commercial diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum* throughout the experiment. The experimental animals

* To whom correspondence should be addressed.

were handled in accordance with the Animal Experiment Guidelines established by College of Pharmacy, Nihon University (according to the guidelines of the Ministry of Education, Science, Sports and Culture of Japan).

Experimental Production of Granuloma with the Formalin Filter Paper-Pellet (FFP) Method This experiment was conducted according to the method of Tanaka²⁶⁾ with slight modifications.²⁴⁾ The skin of the rats (aged 10 weeks) was cut out as follows: 1.5 cm on the back at the point under the *Scapula* and *Os ischii*, symmetrically, under diethyl 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 μ l of 7% aqueous formaldehyde.

Experimental Plan Effect of the Oral Administration of a Hot Water Extract of *Eucommia ulmoides* OLIVER Leaf on Granuloma Formation and Collagen Synthesis in Rats: Twenty-one Wistar/ST male rats aged 7 weeks (197–202 g average body weight (BW)) were divided into 4 groups as follows: group I, control group; group II, 0.6 g leaf group (0.6 g of dried *Eucommia ulmoides* OLIVER leaf/kg of BW/d); group III, 1.2 g leaf group (1.2 g of dried *Eucommia ulmoides* OLIVER leaf/kg of BW/d); group IV, 1.8 g leaf group (1.8 g of dried *Eucommia ulmoides* OLIVER leaf/kg of BW/d). Each group had 5 rats except group I (6 rats). Oral administration dosages were expressed according to the corresponding dried weight of *Eucommia ulmoides* OLIVER leaf in order to easily contrast them. A hot water extract of the *Eucommia ulmoides* OLIVER leaf mixed into the drinking water was administered for 4 weeks. The actual dosages of the hot water extracts were calculated based on the water intake of the day before. After 3 weeks of administration, the rats (aged 10 weeks) were implanted with four pieces of filter paper soaked with 20 μ l of a 7% aqueous formaldehyde, as described in the FFP method. One week after the implantation, the rats were sacrificed by cardiocentesis exsanguination under diethyl ether anesthesia. Ingrown granulomas and organs were carefully dissected and weighed immediately. One of the ingrown granulomas was fixed in 10% neutral formaline solution. The other granulomas and blood plasma were stored at -80°C until ready for the analysis of collagen profile, protein, RNA and DNA content.

Extraction of Protein, RNA, DNA and Collagen in Granuloma Homogenate: The filter paper was carefully removed from the grown granulomas, then three pieces of granuloma were collected, weighed and homogenized with a 10-fold amount of cold distilled water. The obtained homogenate was used for the preparation of protein, RNA, DNA and collagen analysis.

Preparation of Protein, RNA and DNA: All operations were performed in a cold room at 5°C . Protein, RNA and DNA were prepared according to Schneider²⁷⁾ with slight modifications. One milliliter of the homogenate was added to 111 μ l of cold 20% aqueous perchloric acid (PCA), the reaction mixture was well mixed, stood for 10 min, and then was centrifuged (15000 rpm, 20 min, the centrifugation was done under the same condition, unless noted otherwise). The precipitate was washed twice with 1 ml of cold 2% PCA and then added to 1 ml of cold 0.3 M aqueous NaOH. The reaction mixture was incubated at 37°C for 1 h. A portion of the solution was stored separately for the quantitative determina-

tion of protein. Then, 100 μ l of cold 20% PCA was added to the solution and centrifuged. The precipitate was washed once with 1 ml of cold 2% PCA. The supernatants were collected and used for the quantitative determination of RNA. The precipitate was added to 1 ml of cold 8% PCA. The reaction mixture was heated at 70°C for 45 min, and then centrifuged. The precipitate was washed once with 900 μ l of cold 2% PCA. The supernatants were collected and used for the quantitative determination of DNA. Obtained protein, RNA and DNA fractions were stored at -80°C until analysis.

Preparation of Collagen: All operations were performed in a cold room at 5°C . Native collagen was prepared according to the method of Sato²⁸⁾ with slight modifications. Two milliliters of the homogenate was centrifuged. The obtained precipitate was added to 15 ml of cold 0.1 M NaOH. The reaction mixture was stirred for 1 d and centrifuged. This alkaline-extraction was repeated 4 times. The final precipitate was washed once with 10 ml of cold distilled water, and then added to 15 ml of cold 0.5 M aqueous acetic acid. The reaction mixture was stirred for 2 d and centrifuged. The precipitate was again extracted with cold 0.5 M aqueous acetic acid. The supernatants were collected, lyophilized and then used for the quantitative determination of acid-soluble collagen (ASC). The precipitate was added to 10 ml of cold 0.5 M aqueous acetic acid containing 0.01% pepsin (Sigma Chemical Co., St. Louis, MO, USA). The reaction mixture was stirred for 2 d and centrifuged. The supernatant and precipitate were lyophilized separately and used for the quantitative determination of pepsin-solubilized collagen (PSC) and insoluble collagen (ISC), respectively. The total collagen content in the granuloma was calculated by the sum of ASC, PSC and ISC.

Determination of Hydroxyproline Content This portion was conducted according to the method of Prockop²⁹⁾ with slight modifications.²⁴⁾ ASC, PSC and ISC fractions, prepared from rat granulomas and blood plasma, (1 ml was lyophilized) were added to 2 ml of 6 N HCl, and then hydrolyzed at 110°C for 24 h. After neutralization with aqueous KOH, hydroxyproline (Hyp) 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. Ehrlich's reagent was rapidly stirred in for the final color reaction, then the optical absorption was determined at 560 nm. Collagen content was expressed by Hyp content, since Hyp specifically exists in collagen.

Determination of Protein, RNA and DNA Amounts Protein content was determined by Lowry's method³⁰⁾ using bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as the standard. The RNA content was assayed by the orcinol method³¹⁾ using RNA derived from yeast (Boehringer Mannheim Gm bH, Mannheim, Germany) as the standard. The DNA content was assayed by the diphenylamine method³²⁾ using DNA from calf thymus (Sigma Chemical Co., St. Louis, MO, U.S.A.) as the standard.

Determination of Chemical Compound Amounts in Plasma The analysis of the amount of chemical compounds was entrusted to Teijin Bio Laboratories, Inc. (Tokyo, Japan).

Light Microscopic Examination The granulomas in the control and the 1.8 g leaf group were fixed in 10% neutral

Fig. 1. Development of Granuloma in Rats Showing the Effect of the Oral Administration of a Hot Water Extract of *Eucommia ulmoides* OLIVER Leaf

Each bar shows the mean $\pm$ S.E. of five rats, except for the control (six rats). * Denotes significance at the $p < 0.05$ level; values are compared with each control value using the *U*-test (Mann-Whitney). The administration dosages are expressed in the corresponding weight of dried *Eucommia ulmoides* OLIVER leaf.

Table 1. Collagen Profile in Rat Granuloma Due to the Oral Administration of a Hot Water Extract of *Eucommia ulmoides* OLIVER Leaf

	Group I Control	Group IV <i>Eucommia ulmoides</i> OLIVER leaf 1.8 g/kg of BW/d
Total collagen in granuloma (Hyp μ g)	1371 $\pm$ 300	2019 $\pm$ 248*
Collagen profile in granuloma		
ASC (Hyp μ g)	439 $\pm$ 155	558 $\pm$ 96
(Hyp μ g/100 mg of granuloma)	140 $\pm$ 8.8	135 $\pm$ 10
(Relative %) ^a	29.1 $\pm$ 3.6	27.2 $\pm$ 1.6
PSC (Hyp μ g)	693 $\pm$ 147	1216 $\pm$ 173
(Hyp μ g/100 mg of granuloma)	249 $\pm$ 16	295 $\pm$ 9.5*
(Relative %) ^a	50.6 $\pm$ 1.2	59.7 $\pm$ 2.2*
ISC (Hyp μ g)	239 $\pm$ 20	244 $\pm$ 25
(Hyp μ g/100 mg of granuloma)	100 $\pm$ 18	65.9 $\pm$ 14
(Relative %) ^a	20.3 $\pm$ 3.4	13.1 $\pm$ 2.4

Values are mean $\pm$ S.E. of five rats. * Denotes significance at the $p < 0.05$ level; values are compared with each control value using the *U*-test (Mann-Whitney). ^a Represents expression as a percentage of Hyp in granuloma.

formaline and embedded in paraffin. Serial sections with a thickness of 5 μ m were prepared and stained with hematoxylin-eosin.

Statistics The values are expressed as mean $\pm$ S.E. Statistical processing was done using the *U*-test (Mann-Whitney), unless noted otherwise. Differences with a probability of less than 5% were considered to be statistically significant ($p < 0.05$).

RESULTS

Granuloma formation was stimulated by the oral administration of a hot water extract of *Eucommia ulmoides* OLIVER leaf, as shown in Fig. 1. The 1.8 g leaf group showed a significant ($p < 0.05$) increase (39%) in granuloma formation compared with the control group. No significant change was observed in either the 0.6 g or the 1.2 g leaf group.

The deposition of collagen and the collagen profile in rat granulomas are shown in Table 1. Collagen content is ex-

Table 2. Protein, RNA and DNA Content in Rat Granuloma Due to the Oral Administration of a Hot Water Extract of *Eucommia ulmoides* OLIVER Leaf

	Group I Control	Group IV <i>Eucommia ulmoides</i> OLIVER leaf 1.8 g/kg of BW/d
Protein (mg)	9.36 $\pm$ 2.2	12.5 $\pm$ 1.5
(mg/100 mg of granuloma)	8.34 $\pm$ 0.17	8.16 $\pm$ 0.17
RNA (μ g)	245 $\pm$ 60	352 $\pm$ 44
(μ g/100 mg of granuloma)	218 $\pm$ 8.1	230 $\pm$ 4.7
DNA (μ g)	375 $\pm$ 96	481 $\pm$ 67
(μ g/100 mg of granuloma)	328 $\pm$ 3.3	311 $\pm$ 9.8

Values are mean $\pm$ S.E. of five rats. No significant differences are observed.

Table 3. Changes in Rat Blood Plasma Content Due to the Oral Administration of a Hot Water Extract of *Eucommia ulmoides* OLIVER Leaf

Determination	Group I Control	Group IV <i>Eucommia ulmoides</i> OLIVER leaf 1.8 g/kg of BW/d
Hyp (μ g/ml of plasma)	17.4 $\pm$ 0.8	18.3 $\pm$ 0.7
Protein (g/dl)	5.34 $\pm$ 0.04	5.35 $\pm$ 0.03
A/G ratio	0.758 $\pm$ 0.03	0.755 $\pm$ 0.01
Creatinine (mg/dl)	0.540 $\pm$ 0.04	0.425 $\pm$ 0.03*
GOT (U/l)	110 $\pm$ 7.0	98.0 $\pm$ 4.2
GPT (U/l)	37.8 $\pm$ 3.0	28.0 $\pm$ 3.4*
Glucose (mg/dl)	142 $\pm$ 6.5	149 $\pm$ 7.3
Whole lipid (mg/dl)	194 $\pm$ 16	229 $\pm$ 7.7
Whole cholesterol (mg/dl)	61.4 $\pm$ 4.7	68.5 $\pm$ 3.1
Free cholesterol (mg/dl)	6.00 $\pm$ 0.8	7.00 $\pm$ 1.2
HDL-cholesterol (mg/dl)	39.8 $\pm$ 9.3	57.3 $\pm$ 2.1*
Triglyceride (mg/dl)	9.00 $\pm$ 3.3	24.3 $\pm$ 2.1*
Phospholipid (mg/dl)	92.4 $\pm$ 6.8	102 $\pm$ 2.5

Values are mean $\pm$ S.E. of five rats. * Denotes significance at the $p < 0.05$ level; values are compared with each control value using the *U*-test (Mann-Whitney). A/G ratio means albumin/globulin ratio. GOT and GPT represent glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase, respectively. HDL-cholesterol is high density lipoprotein-cholesterol.

pressed by the Hyp amounts. The 1.8 g leaf group showed a significant ($p < 0.05$) increase, 47%, in Hyp content in the granuloma. Also, the collagen profile in the granuloma showed a significant increase ($p < 0.05$), 18%, in the ratio of PSC per 100 mg of granuloma and in the PSC whole collagen ratio (relative percent). No significant change was observed in the collagen profile of the ASC or the ISC.

Non-collagenous protein, RNA and DNA content of the granulomas are shown in Table 2. No significant differences were detected.

The composition of rat blood plasma is shown in Table 3. In the 1.8 g leaf group significant decreases were observed ($p < 0.05$), by 21% and 26%, respectively, in the amount of creatinine and glutamic-pyruvic transaminase (GPT) activity. In the case of high density lipoprotein-cholesterol (HDL-cholesterol) and triglyceride amounts, significant increases were observed ($p < 0.05$), by 44% and 170%, respectively, in the 1.8 g leaf group.

Histochemical examination showed that the granuloma from both groups were normal because no macrophages were observed in either of them. Contrasted with the control group, granulomas from the 1.8 g leaf group were better developed, with an increase in the numbers of fibroblasts and

Fig. 2. Histochemical Examination of Granulomas Induced by the FFP Method with the Effect of the Oral Administration of a Hot Water Extract of *Eucommia ulmoides* OLIVER Leaf (b and d) and without (Control a and c)

The preparation of serial sections is explained in the text. Sections were stained with hematoxylin-eosin. Magnifications were as follows: a and b, 30 $\times$; c and d, 300 $\times$.

monocytes such as lymphocytes and histiocytes. Also, many newly synthesized capillary vessels were found. (Fig. 2c and d). In addition, many neutrophils infiltrated the implanted filter-paper (Fig. 2a and b).

Body weight, organ weight and organ body weight ratios (g or mg/100 g of BW) are shown in Table 4, but no significant differences were observed.

DISCUSSION

The wound healing process is divided into four phases as follows: exudative, proliferative, fibroblastic and maturation stages.²⁵⁾ Granulation is a characteristic of the second stage of wound healing. Collagen deposition in granulation tissue shows the advance of an early wound healing process and has been described as a result of elevated collagen synthesis.^{33–35)} We reported in a previous paper that the oral administration of a hot water or methanol extract of *Eucommia ulmoides* OLIVER leaf in false aged model rats accelerated collagen synthesis in granulomas.^{17,18,24)} Further investigation showed that the actual chemical compounds involved were aucubin and geniposidic acid.²⁴⁾ In this experiment, granuloma formation in a normal rat was accelerated by the oral administration of the dried leaf at 1.8 g/kg of BW/d. The deposition of collagen in a granuloma depends on the collagen synthesis rate, but also on its degradation rate. In the 1.8 g leaf group, there was a trend toward a higher Hyp content in the rat blood plasma compared to the control (Table 3). The Hyp content in blood reflects the collagen degradation rate in the whole body. It shows that the collagen degradation rate in the 1.8 g leaf group was not suppressed and that the collagen turnover rate tended towards synthesis.

Table 4. Changes in Body Weight, Organ Weight, and Organ Body Weight Ratio of Rats Due to the Oral Administration of a Hot Water Extract of *Eucommia ulmoides* OLIVER Leaf

	Group I Control	Group IV <i>Eucommia ulmoides</i> OLIVER leaf 1.8 g/kg of BW/d
Body weight (g)	363 $\pm$ 9.5	364 $\pm$ 4.2
Liver (g)	9.94 $\pm$ 0.61	9.75 $\pm$ 0.33
(g/100 g of BW)	2.95 $\pm$ 0.13	2.92 $\pm$ 0.07
Kidney (g)	2.17 $\pm$ 0.06	2.18 $\pm$ 0.09
(mg/100 g of BW)	646 $\pm$ 17	655 $\pm$ 21
Spleen (mg)	669 $\pm$ 22	625 $\pm$ 27
(mg/100 g of BW)	199 $\pm$ 6.4	187 $\pm$ 7.5
Heart (mg)	982 $\pm$ 28	932 $\pm$ 14
(mg/100 g of BW)	293 $\pm$ 9.2	280 $\pm$ 3.3
Thymus (mg)	596 $\pm$ 50	516 $\pm$ 26
(mg/100 g of BW)	176 $\pm$ 11	155 $\pm$ 8.2
Adrenal (mg)	65.1 $\pm$ 4.3	59.2 $\pm$ 4.4
(mg/100 g of BW)	19.3 $\pm$ 1.1	17.7 $\pm$ 1.2

Values are mean $\pm$ S.E. of six for the control and five rats for group IV. No significant differences were observed.

Collagen fibers located in the outer cellular-matrix have two types of cross-linking that are increased due to maturation and aging. Increasing the amount of cross-links in collagen molecules decreases the molecule's solubility. Miller³⁶⁾ reported that a dilute organic acid solvent, such as 0.5 M aqueous acetic acid, is capable of solubilizing molecules without cross-links, but can also solubilize fibers in which aldimine-containing cross-links are prevalent owing to its lability at an acidic pH. Since the efficacy of a system of 0.5 M aqueous acetic acid containing a pepsin solvent system lies in its abil-

ity to selectively degrade those portions of the native collagen molecule from which cross-links originate, it is the most versatile and widely applicable solvent system for the extraction of native collagen. However, the molecules are recovered with modified nonhelical extremities. The number of cross-links remaining in ISC molecules is higher than in ASC or PSC. This may explain that cross-links in PSC and ISC are due to maturation and aging, respectively. In our experiment (Table 1), no significant change was observed in the ISC content (Hyp μg) in granulomas between the control and the 1.8 g leaf groups, suggesting that the amount of collagen containing cross-links due to aging is almost the same or less than in the control, in spite of the increasing collagen turnover rate. In the case of the PSC amount, the Hyp ($\mu\text{g}/100\text{ mg}$) in the granuloma and the relative percentage of Hyp in the granuloma were significantly increased (Table 1). This shows that the granuloma maturation or wound healing process was accelerated compared to the control group. In the 1.8 g leaf group, the ASC (Hyp μg) in the granuloma was slightly higher, but the Hyp ($\mu\text{g}/100\text{ mg}$) in the granuloma and the relative percentage were lower than in the control (Table 1). Non-collagenous protein, RNA and DNA content in the granuloma were also slightly higher but not significantly different than in the control (Table 2). It seemed that collagen synthesis had reached its peak and collagen maturation had advanced. In blood plasma, the creatinine content and GPT activity were significantly lower and the glutamic-oxaloacetic transaminase (GOT) activity was slightly lower than in the control (Table 3). This shows that the inflammation caused by the FFP was already at a sedative stage at the end of the experimental period in the 1.8 g leaf group.

The proliferation of fibroblasts and monophytes plays an important role in wound healing, along with newly synthesized capillary vessels, to help supply enough nutrients and oxygen to the injury site.³⁷⁾ These are both more numerous and better observed in the 1.8 g leaf group, and are a possible reason for the better developed granulomas in the 1.8 g leaf group (Fig. 2). Since the proliferation of fibroblasts could lead to an augmentation of collagen synthesis,³⁸⁾ we suppose that the well developed and rapidly matured granulomas induced by *Eucommia ulmoides* OLIVER leaf are caused by the proliferation of fibroblasts, considering our results show no significant changes in DNA or RNA content. In addition, a greater cell reaction was observed in the 1.8 g leaf group, shown by the more numerous neutrophils that had infiltrated the implanted filter-paper indicating that the immune ability of the whole organism was strengthened.

We reported that the oral administration of a hot water extract of *Eucommia ulmoides* OLIVER leaf depressed the fatty acid synthesis in rats fed a high fat and cholesterol diet.¹⁹⁾ In addition, our unpublished data shows that 3-hydroxyacetyl-CoA dehydrogenase (a key enzyme of β -oxidation pathway) activity was accelerated. This indicates that fatty acid metabolism tended towards degradation due to the administration of this leaf, and this may act as an energy source to help develop granuloma formation. Proof is indicated in the blood plasma where HDL-cholesterol and triglyceride contents were dramatically higher than in the control (Table 3).

Finally, in Table 4, the oral administration of a hot water extract of *Eucommia ulmoides* OLIVER leaf had no effect on body weight or organ weight, indicating there are no side ef-

fects of the administration of this dosage of the leaf.

In conclusion, granuloma formation in normal rats was accelerated by the oral administration of a hot water extract of *Eucommia ulmoides* OLIVER leaf. The effective dosage was 1.8 g of dried leaf/kg of BW/d for 4 weeks. Granuloma maturation was advanced because the PSC amount had increased. Fatty acid catabolism was accelerated and these liberated fatty acids may act as an energy source to help develop granuloma formation. No side effects were observed. All this suggests that the oral administration of this leaf might be effective at speeding up the wound healing process.

Acknowledgments We would like to thank Bussan Nakasen Co., Ltd., for the donation of *Eucommia ulmoides* OLIVER leaf and President Hideo Mizukawa at PS Co., Ltd., for the scholarship of Yanmei Li. We likewise thank Mr. C. Alex DePaula at the University of Medicine and Dentistry of New Jersey and Rutgers University for revising our manuscript, especially in English. This paper is developed for the doctoral thesis of Yanmei Li.

REFERENCES

- 1) Wei: Wu J., Ting: Sun X. X., Sun F. Y., "Shen Nong Ben Cao Jing (神農本草經)," Shang Wu Yin Shu Guan, Beijing, 1955, p. 43.
- 2) Li S. Z., "Ben Cao Gang Mu (本草綱目)," Ren Min Wei Sheng Chu Ban She, Beijing, 1987, pp. 1986—1987.
- 3) Koike K., Nakamura H., Takahashi S., *J. Trad. Med.*, **15**, 225—227 (1998).
- 4) Hattori M., Che Q.-M., Gewali M. B., Nomura Y., Tezuka Y., Kikuchi T., Namba T., *Shoyakugaku Zasshi*, **42**, 76—80 (1988).
- 5) Namba T., Hattori M., Yie J.-N., Ma Y.-H., Nomura Y., Kaneko S., Kitamura Y., Koizumi T., Katayama K., Lu W., *J. Trad. Med.*, **3**, 89—97 (1986).
- 6) Shimoyama A., Yamadaki M., Nakazawa Y., Yahara S., Nohara T., *Shoyakugaku Zasshi*, **47**, 56—59 (1993).
- 7) Ma Y.-H., Yie J.-N., Hattori M., Kaneko S., Nomura Y., Wakaki K., Kurashige Y., Namba T., *J. Trad. Med.*, **4**, 26—34 (1987).
- 8) Nakazawa Y., *Gekkan Food Chemical*, **9**, 72—78 (1995).
- 9) Deyama T., *Chem. Pharm. Bull.*, **31**, 2993—2997 (1983).
- 10) Deyama T., Ikawa T., Nishibe S., *Chem. Pharm. Bull.*, **33**, 3651—3657 (1985).
- 11) Deyama T., Ikawa T., Kitagawa S., Nishibe S., *Chem. Pharm. Bull.*, **34**, 523—537 (1986).
- 12) Shi C. J., Ravikumar P. R., Huang F.-C., Buckner C. B., Whitlock H., Jr., *J. Am. Chem. Soc.*, **98**, 5412—5413 (1976).
- 13) Singh S. N., Kanungo M. S., *J. Biol. Chem.*, **243**, 4526—4529 (1968).
- 14) Geary S., Florini J. R., *J. Gerontol.*, **27**, 325—332 (1972).
- 15) Koike K., Matsubara S., Iwamoto K., Takahashi S., *Bull. Nihon Univ. Coll. Pharm.*, **37**, 20—25 (1997).
- 16) Metori K., Ohashi S., Takahashi S., Tamura T., *Biol. Pharm. Bull.*, **17**, 917—920 (1994).
- 17) Metori K., Furutsu M., Takahashi S., *Biol. Pharm. Bull.*, **20**, 237—242 (1997).
- 18) Metori K., Tanimoto S., Takahashi S., *Natural Med.*, **52**, 465—469 (1998).
- 19) Nakasa T., Yamaguchi Y., Okinaka Y., Metori K., Takahashi S., *Nippon Nogeikagaku Kaishi*, **69**, 1491—1498 (1995).
- 20) Shioya M., Sato T., Shirai W., Taira T., Takahashi S., *J. Trad. Med.*, **7**, 18—23 (1990).
- 21) Tanimoto S., Koike K., Takahashi S., *Biosci. Biotechnol. Biochem.*, **57**, 325—327 (1993).
- 22) Tanimoto S., Ikuma K., Takahashi S., *Biosci. Biotechnol. Biochem.*, **57**, 205—208 (1993).
- 23) Yokogoshi H., Hayase K., Yamazaki J., Koike K., Takahashi S., *Agric. Biol. Chem.*, **55**, 3133—3134 (1991).
- 24) Li Y., Sato T., Metori K., Koike K., Che Q.-M., Takahashi S., *Biol. Pharm. Bull.*, **21**, 1306—1310 (1998).
- 25) Torikai K., Shioya N., *Orthop. Surg. Traumatol.*, **29**, 367—374

- (1986).
- 26) Tanaka A., Kobayashi F., Miyake T., *Endocrinol. Jpn.*, **7**, 357—364 (1960).
- 27) Schneider W. C., *J. Biol. Chem.*, **164**, 747—751 (1946).
- 28) Sato K., Yoshinaka R., Sato M., Itoh Y., Shimizu Y., *Comp. Biochem. Physiol. B*, **90**, 155—158 (1988).
- 29) Prockop D. J., Udenfriend S., *Anal. Biochem.*, **1**, 228—239 (1960).
- 30) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
- 31) Munro H. N., Fleck A., “Analysis of Tissues and Body Fluids for Nitrogenous Constituents,” ed. by Munro H. N., Academic Press, New York, 1969, pp. 424—525.
- 32) Giles K. W., Myers A., *Nature* (London), **206**, 93 (1965).
- 33) Zeitz M., Ruiz-Torres A., Merker H.-J., *Arch. Dermatol. Res.*, **263**, 207—214 (1978).
- 34) Dunphy J. E., Udupa K. N., *N. Engl. J. Med.*, **253**, 847—851 (1955).
- 35) Barnes M. J., Morton L. F., Bailey A. J., Bennett R. C., *Biochem. Soc. Trans.*, **3**, 917—920 (1975).
- 36) Miller E. J., Rhodes R. K., “Methods in Enzymology,” ed. by Cunningham L. W., Freder D. W., Academic Press, New York, 1982, pp. 33—64.
- 37) Ozaki Y., Sakaguchi I., Tujimura M., Ikeda N., Nakayama M., Kato Y., Suzuki H., Satake M., *Biol. Pharm. Bull.*, **21**, 366—370 (1998).
- 38) Laato M., Kahari V.-M., Niinikoski J., Vuorio E., *Biochem. J.*, **247**, 385—388 (1987).