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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文要約：
Dissertation Summary

Chapter 1 General Introduction

The medical functions of plants had been used for rescuing diseases from the very beginning of hum an history. Approximately 5000 years ago, people used medicinal plants' for preparation of drugs.[1]
Some famous ancient pharmacists or doctors like Hippocrates, Galen, Pliny the Elder and others knew willow bark could ease aches and pains and reduce fevers.[2] This method has been used for the trea tment of these conditions in Europe and China and other places like ancient Egypt, Sumer, and Assyr ia.[3,4] The German chemist named Johann Andreas Buchner isolated salicin from the active extract of the bark in 1828.[5] Furthermore, an Italian chemist, Raffaele Piria converted the substance into a sug ar and a second component, which on oxidation became salicylic acid.[6,7] Until nowadays, salicylic ac id is still a popular medicine which is widely used as a food preservative, a bactericidal, and an antis eptic.[8]
In Asia, the Chinese book "Ben cao gang mu," written by Li Shizhen during the Ming dynasty (159 6), treats 365 drugs (dried parts of medicinal plants), many of which are used even nowadays such a s the following: *Rhei rhisoma*, camphor, *Theae folium, Podophyllum*, the great yellow gentian, ginseng, jimson weed, cinnamon bark, and ephedra.[9,10] Since China and japan are close neighbours separated only by a strip of water, the book "Ben cao gang mu" was introduced to Japan in 1604.[11] Nowada ys, the traditional Chinese medicines are still widely used in daily life. In 2015, a Chinese pharmaceu tical scientist, Tu Youyou was rewarded a Nobel Prize in Physiology or Medicine for discovering arte misinin (also known as qinghaosu) and dihydroartemisinin from *Artemisia annua* and using to treat m alaria, which saved millions of lives. Although the medical technology has developed, there are still s ome diseases which could not be rescued. As an abundant resource of medicines, the researches on p lant should be further implemented.
Nowadays, the depletion of the Ozone layer in recent times has raised concerns on the rate of increa se in skin related disorders such as pigmentation and skin cancer. The search for chemical agents whi ch can reduce hyperpigmentation has aroused general interest by pharmacists and cosmetics companies. In the process of pigmentation, Tyrosinase plays an important role in the biosynthesis of melanin.[12] Its best known function is the formation of melanins from l-Tyrosine via l-3,4-dihydroxyphenylalanine (l-DOPA).[13] Tyrosinases (EC 1.14.18.1) (TRs) are enzymes having a binuclear copper center in the catalytic center, which are able to insert oxygen in *ortho*-position of an aromatic ring to an existing hydroxyl group.[14] Furthermore, the subsequent oxidation of the diphenolic product will change into th e corresponding quinone.[15] Thus, the inhibition on Tyrosinase is a core step to avoid pigmentation.[16] There are lots of researches on chemicals involved in Tyrosinase inhibition, however, the side effect s of Rhododendrol in 2013 [17] remind that it is necessary to develop new and safe chemicals.
For another, hypertension is a long term medical condition in which the blood pressure in the arteries is persistently elevated.[18] High blood pressure usually does not cause symptoms.[19] Long term high blood pressure; however, is a major risk factor for coronary artery disease, stroke, heart failure, periph eral vascular disease, vision loss, and chronic kidney disease.[20,21] Angiotensin I-converting enzyme (A CE; EC 3.4.15.1) is a zin-containing carboxy peptidase distributed in many tissues including endotheli

al and epithelial cells as somatic ACE (sACE) and also in germinal cells of testis as germinal ACE (gACE). ACE plays a major role in the regulation of blood pressure by catalyzing formation of poten t vasoconstrictor angiotensin II by cleavage of C-terminal dipeptide His-Leu and inactivation of vasodil ator bradykinin by cleavage of terminal dipeptide Phe-Arg.[22] As a result of this dual role, inhibition of ACE is a key therapeutic target for hypertension. In addition, ACE is also a major drug target for congestive heart failure, myocardial infarction, renal failure, and diabetic nephropathy.[23,24]
In this thesis, a series of studies were therefore set up. In Part I, a study of isolation and identificatio n of Tyrosinase-inhibition active compounds from *Diplomorpha sikokiana* was carried out. And the sy nergistic effect was further estimated while combining with l-ascorbic acid. In Part II, a study of AC E-inhibitor compounds from *Trichosanthes kirilowii* Maxim. Roots (Radix Trichosanthis) was impleme nted. Furthermore, two Gluco-amino acid compounds in *Trichosanthes kirilowii* Maxim. Roots (Radix Trichosanthis) were synthesized and their ACE-inhibition activities were evaluated.

Chapter 2　The evaluation of *Diplomorpha sikokiana*'s Tyrosinase-inhibition activity

Part 1 Isolation and Identification of Tyrosinase-inhibition active compounds from *Diplomorpha sikokiana*

1. Introduction
Thymelaeaceae is a moderately diverse plant family consisting of approximately 50 genera and 500 sp ecies throughout the world.[25] *Diplomorpha sikokiana*, a member of the Thymelaeaceae family is wide ly distributed in Japan from Honshu to Kyushu and Shikoku using for making Japanese paper (Wash i).[26] Its chemical components have been widely studied. These include (2*R*)-hydroxyl-1,5-diphenyl-1-pe ntanone,1,5-diphenyl-1-pentanone, (*S*)-3-hydroxy-1,5-diphenyl-1-pentanone, 3-methoxy-1,5-diphenyl-1-penta none, 1,5-diphenyl-2-penten-1-one, sinapyl alcohol, syringin, syringinoside, (+)-afzelechin, and apiosylski mmin.[27] Herein, we found some other chemicals from *Diplomorpha sikokiana* and, furthermore, the t yrosinase inhibition activity was determined.

2. Material and methods
2.1 Plant's material
Diplomorpha sikokiana was collected from Kochi Prefecture, Japan. The bark of the stern (400 g) was cut into small pieces and extracted with 80% MeOH (4L) for a week at room temperature. After filtration, the filtrate was collected and the residue was extracted by 80% MeOH for 3 hours at 75 ℃ and filtered. The filtrate was collected and the residue was extracted for the third time by 8 0%MeOH for 1 hour at 60 ℃ and filtered. The filtrates were combined and evaporated under pressu re. The extract (35.3 g) was dried and store at 4 ℃.

2.2 Chemicals and reagents
Dimethyl sulfoxide (DMSO) and ether were purchased from JUNSEI (Japan). Methanol, ethyl ace tate (AcOEt), hexane were purchased from Nacalai tesque (Japan). Tyrosinase from mushroom, L-3,4-d ihydroxyphenylalanine (L -DOPA) were obtained from Sigma (St. Louis, MO). Potassium dihydrogen phosphate, di-potassium hydrogen phosphate and Silica C-300 were obtained from Wako Pure Chemic al Industries LTD (Japan). ODS was purchased from FUJI SILYSIA CHEMICAL LTD (Japan).

2.3 Instruments
^{1}H, ^{13}C and 2D NMR spectra were measured on a JEOL JNM-ECX500 spectrometer (^{1}H: 500 MHz and ^{13}C: 125 MHz). Separation and identification of active compounds were performed using SHIMA DZU HPLC system equipped with a SCL – 10Avp controller, a SIL-10ADvp auto-injector, two LC-6 ADvp pumps, a SPD-M20 Avp detector, a CTO-10 ACvp column oven and a FRC-10A friction coll ector. LC - MS was performed using SHIMADZU LC - MS 2010 system. Spectrophotometry was d one by xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, US)

2.4 Isolation and identification
The extracted sample of *Diplomorpha sikokiana* (26g) was dissolved in water and partitioned successi vely with ether (900mL) and ethyl acetate (900mL). The two organic layers were combined and evap orated under reduced pressure. The residue (7.65g) was dissolved in hexane. The hexane solution was subjected to a silica gel column chromatography eluted with AcOEt-Hexane (4L) and MeOH (4L) to

yield Frs.A1 (2.47g, 30:90, v/v) , A2 (1.12g, 50:50, v/v), A3(0.45g, 70:30, v/v), A4 (0.47g, 100% AcOEt) and A5 (1.12g, 100% MeOH). Fr.A2 (1.12g, 50:50, v/v) was applied to an ODS gel column chromatography eluted with MeOH-H_2O (700mL) to yield Frs. B1 (0.14g, 10:90, v/v), B2 (0.13g, 2 0:80, v/v), B3(0.14g, 40:60, v/v) and B4 (0.71g, 100%MeOH). Fr.B1 (0.14g, 10:90, v/v) was further separated via HPLC mentioned in 2.3.
The separation was done by a Cosmosil C_{18} Packed 10 mm × 250 mm column at isocratic elution. The mobile phase was consisted of methanol, acetate acid and water with the ratio at 20: 1: 79 (%, v/v/v) at a flow rate of 3.0 mL/min. The oven's temperature was set at 32 °C and the detection wav elength was at 254 nm.

2.5 Tyrosinase inhibition
For the tyrosinase inhibition activity experiment, the extracted sample was dissolved in DMSO to for m a 1 mL solution which was equivalent to 10 mg of original plant's sample. Tyrosinase inhibitory activity was determined by a spectrophotometric method which was mentioned by Chan et al.[28] using a modified dopachrome method with L -DOPA as the substrate. The reaction mixture contained: pho sphate buffer (70 μL, 0.1 M, pH 6.8); inhibitor (2 μL) dissolved in DMSO; tyrosinase aqueous soluti on (30 μL, 71.5 units/mL). The reference solution was prepared with 2 μL of DMSO instead of inhi bitor. After adding tyrosinase solution, the reaction mixture was incubated at 25 ℃ for 5 min. After i ncubation, half of both inhibitor containing samples and reference samples were selected to be blank s amples and reference blank samples which were added by 30 μL of water instead of 30 μL 10mM of L-DOPA. Thirty microliter of 10mM of L-DOPA was added to the rest of samples and the 96-wel l microplate was incubated at 25 ℃ for 2 min. The absorbance was measured at 475 nm. Results w ere compared with a positive control and a blank containing DMSO in place of the sample solution and H_2O in place of L-DOPA.

3. Conclusion
DDPA has a tyrosinase-inhibition activity which have been characterized already [32,33]. In our study, it is the first time to reveal that *Diplomorpha sikokiana* has a tyrosinase Inhibition activity and DDPA, as the main active compound, is also the first time to be isolated from *Diplomorpha sikokiana*. Since DDPA is destroyed easily during separation, our results also demonstrate that caffeic acid and syringic acid can stabilize DDPA to some extent. This research acts as a reference for DDPA's application in cosmetic field. Since DDPA can be destroyed easily during evaporation, it is also suggested that DDPA should be accompanied by stabilizing agents on application.

Part 2 The evaluation of the synergistic effect of 3-(2,4-dihydroxyphenyl)propionic acid and L-As corbic acid on tyrosinase-inhibition

1. Introduction
3-(2,4-dihydroxyphenyl)propionic acid (DDPA) has a tyrosinase-inhibition activity which has been characterized already.[32,33] L-ascorbic acid (Vitamin C) is also widely used in cosmetics or food-supplements for its antioxidant and tyrosinase inhibitor activities.[34] In this study, we evaluated the synergistic effect of L-ascorbic acid and DDPA on tyrosinase inhibition.

2. Materials and Chemicals
DDPA was purchased from Fluka (Japan, Purity ≥ 95.0%). L-ascorbic acid was purchased from Wako Pure Chemical Industries(Japan, Purity 99.6%) Dimethyl sulfoxide (DMSO) was purchased from JUNSEI (Japan). Tyrosinase from mushroom, 3,4-dihydroxy-L-phenylalanine (L-DOPA) were obtained from Sigma (St. Louis, MO, Purity 98%). Potassium dihydrogen phosphate and di-potassium hydrogen phosphate were obtained from Wako Pure Chemical Industries LTD (Japan). xMark™ Microplate Absorbance Spectrophotometer was made by Bio-Rad, US.
The stock solutions of DDPA and L-ascorbic acid were prepared by dissolving their reference standards in

DMSO and the final concentrations were 1.0 mg/mL, respectively. All solutions were protected from light and stored at 4 ℃.

3. Method

For the tyrosinase inhibition activity experiment, the samples' solutions were diluted in DMSO to form a series of concentrations. tyrosinase inhibitory activity was determined by a spectrophotometric method which was described by Chan et al. [28], using a modified dopachrome method with L-DOPA as the substrate. The reaction mixture contained: phosphate buffer (70 μL, 0.1 M, pH 6.8); inhibitor (2 μL) dissolved in DMSO; tyrosinase aqueous solution (30 μL,71.5 units/mL). The reference solution was prepared with 2 μL of DMSO instead of inhibitor. After adding tyrosinase solution, the reaction mixture was incubated at 25 ℃ for 5 min. After incubation, half of both samples with inhibitor and reference samples were selected to be blank samples and reference blank samples which were added by 30 μL of water instead of 30 μL 10mM of L-DOPA. 30 μL 10mM of L-DOPA was added to the rest of samples and the 96-well microplate was incubated at 25 ℃ for 2 min. The absorbance was measured at 475 nm. Results were compared with a positive control and a blank containing DMSO in place of the sample solution and H_2O in place of L-DOPA.

4. Conclusion

Since L-ascorbic acid has been found to have several functions widely applied in cosmetics and medicines, it is recommended that further research can be carried out also on its synergistic effect with DDPA in the area of cosmetics and medicines.

Chapter 3 Isolation and Synthesis of Gluco-amino acid compounds in *Trichosanthes kirilowii* Maxim. Roots (Radix Trichosanthis) and the evaluation of their Angiotensin I-converting Enzyme Inhibitory Activity

1.Introduction

The Chinese medicinal herb *Trichosanthes kirilowii* Maxim. Roots (Radix Trichosanthis), which has been used clinically in China to treat hydatidiform moles, trophoblastic carcinomas, and ectopic pregnancies, and to interrupt early and midtrimester pregnancies[37-38]. There are many components existing in *Trichosanthes kirilowii* Maxim. Roots such as Trichosanthin[39], methyl palmitate, palmitic acid, suberic acid, α-spinasterol, stigmast-7-en-3-β-ol,α-spinasterol, 3-*O*-β-D-glucopyranoside, stigmast-7-en-3-β-ol, 3-*O*-β-D-glucopyranoside, bryonolic acid, cucurbitacin B, cucurbitacin D, vomifoliol, ethyl alpha-L-arabinofranoside and D-glucose. The bitter taste of Trichosanthes Root was considered to be due to cucurbitacin B and D[40]. Concerning the components' bioactivities, there are many reports concerned about trichosanthin and its bioactivity such as anti-HIV and anti-tumor functions[41-43] , immunoregulatory functions, exhibiting inhibitory effects on human lymphoproliferative responses with no cytolysis of immunocytes[44-45], and inducing interleukin (IL)-4 gene expression[46]. However, there are few researches about the activities of Angiotensin I-converting enzyme (ACE) inhibition.

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) is a zin-containing carboxy peptidase distributed in many tissues including endothelial and epithelial cells as somatic ACE (sACE) and also in germinal cells of testis as germinal ACE (gACE). ACE plays a major role in the regulation of blood pressure by catalyzing formation of potent vasoconstrictor angiotensin II by cleavage of C-terminal dipeptide His-Leu and inactivation of vasodilator bradykinin by cleavage of terminal dipeptide Phe-Arg.[47] As a result of this dual role, inhibition of ACE is a key therapeutic target for hypertension. In addition, ACE is also a major drug target for congestive heart failure, myocardial infarction, renal failure, and diabetic nephropathy.[48-49]

Many peptides are described as ACE-inhibitors which contain several amino acids[50-61]. Among those amino acids, the interaction fragments with ACE are difficult to judge. In this paper, we separated some Glyco-amino acids (M.W. < 500) from Radix Trichosanthis and applied on ACE to evaluate their ACE's inhibition activities. Through this research, we separated and synthesized some Glyco-amino acids showed a high inhibition activity and their value of IC_{50} was also evaluated.

2. Materials and Methods

2.1 Plant's Material

Radix Trichosanthis was purchased from TongRenTang (Guangzhou, China). Radix Trichosanthis (20 g) was cut into small pieces and extracted with distilled water (200 mL, 1:10, w/v) by ultrasonic extraction for 60 min. After filtrating, the filtrates were evaporated under pressure. The residue (5.24 g) was dried and stored at 4 ℃.

2.2 Chemicals and reagents

D-(+)-Glucose, methanol, acetonitrile were purchased from NACALAI TESQUE, INC. L-(+)-Arginine and L-citrulline were purchased from TOKYO INDUSTRY CO., LTD. Malonic acid and deuterium oxide were purchased from Wako Pure Chemical Industries, Ltd. ACE Kit – WST(100 tests) was purchased from Dojindo Molecular Technologies, Inc. Float-A-Lyzer® G2(500-1000 Daltons) was purchased from Spectrum Laboratories Inc. Ultrapure water from the "Nanopure" water purification system (YAMATO Scientific Co., Ltd. Japan) was used throughout the study.

2.3 Instruments

^{1}H, ^{13}C and 2D NMR spectra were measured on a JEOL JNM-ECX500 spectrometer (^{1}H: 500 MHz and ^{13}C: 125 MHz). Separation was performed using SHIMADZU HPLC system equipped with a SCL-10Avp controller, a SIL-10ADvp auto-injector, two LC- 6ADvp pumps, a SPD-M20 Avp detector, a CTO-10 ACvp column oven and a FRC-10A friction collector. SUNRISE Rainbow RC-R microplate-reader was made by TECAN. LC - MS and LC-MS/MS were performed using Waters ACQUITY UPLC tandem Xevo TQD Detector.

Chromatographic separation was obtained by an Acquity UPLC (Waters) using an Inertsil ODS-4 column (2.1 mm × 150 mm) with a mobile phase of 95% methanol and water (95:5, v/v) at a flow rate of 0.2 mL/min. The column was held at a temperature of 38 ℃. Mass spectrometric detection was performed using a XEVO TQ tandem mass spectrometer (Waters). Electrospray ionization (ESI) in positive mode and daughter ion scan was performed. The optimum conditions were: capillary voltage, 3.8 kV; source temperature, 150 ℃; desolvation temperature, 250 ℃ Cone Voltage, 30 V; Collision Energy, 30 eV; desolvation gas (N2) was delivered at a flow rate of 600 L/h; collision gas (Ar) was flowed at 0.1 mL/min. Analysis was carried out by scan ion monitoring from 150 to 500. The other parameters were fixed as for the tuning file. Data was handled by the MassLynx software v4.1 (Waters).

Chapter 4 General discussion

In Chapter 2, 3-(2,4-dihydroxyphenyl)propionic acid (DDPA), 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid) and 3,4-dihydroxycinnamic acid (caffeic acid) had been isolated from *Diplomorpha sikokiana*. The DDPA has an tyrosinase-inhibition activity which have been characterized already.[32-33] Although there's not specific literature shows syringic acid and caffeic acid have tyrosinase-inhibition activity, the two compounds exist in some extracts which show a tyrosinase-inhibition activity. There's reported that Wine phenolic acid fraction could inhibited tyrosinase activity when concentration was 200 mg/L. The percentage of syringic acid and caffeic acid in the Wine phenolic acid fraction was 9.9 % and 22.3 % respectively.[65] Similarly, the methanol extract of *Melia azedarach*'s fruit at 100 μg/mL demonstrated a 12.20±2.17 % of tyrosinase-inhibition activity. The methanol extract included 1.64 μg/g of syringic acid and 1.33 μg/g of caffeic acid.[66] Though caffeic acid has not specific tyrosinase-inhibition activity, antioxidant activity was elucidated.[67] Therefore, the main compounds of tyrosinase-inhibition should be concluded by DDPA, syringic acid and caffeic acid which acted as co-operation. When the sample including caffeic acid and DDPA and syringic acid was evaporated, the caffeic acid stabilized DDPA and syringic acid from being oxidized in some degree.

Since DDPA can be destroyed easily, we consider whether the activity will increase when we use DDPA and another anti-oxidant chemical together. L-ascorbic acid (Vitamin C) has been widely used in cosmetics and health supplements for a long time because it has been proven to be safe and effective on anti-oxidant. In Part II of Chapter 2, we evaluated the synergistic effect of tyrosinase-inhibition of DDPA and L-ascorbic acid. From the results, we can conclude that the mixture of DDPA and L-ascorbic acid has a significantly increase on tyrosinase-inhibition activity than the two compounds at the same concentrations, respectively. At the same time, the value of IC_{50} decreased either DDPA or L-ascorbic acid. The finding is the first time to reveal that that DDPA and L-ascorbic acid has a synergistic effect on tyrosinase-inhibition activity. Since L-ascorbic acid has several functions which have been widely applied on cosmetic and medical field, the synergistic effect of L-ascorbic acid and DDPA should be worth to investigate and apply further on cosmetic or medicines.

In Chapter 3, a Chinese Traditional Medicine, *Trichosanthes kirilowii* Maxim. Roots (Radix Trichosanthis) had been evaluated for its ACE inhibition activity. Radix Trichosanthis has been used for abortion and cure and treatment of ectopic pregnancies widely in China. In this thesis, we developed its new application on anti-ACE.