A THESIS

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Molecular Docking Studies of Phlorotannins from Brown Algae with Biological Activities

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CONTENTS

국문초록

플로로탄닌은 폴리페놀의 일종으로서 해양 폴리페놀이라고 불리고 있으며, 해조류 중 갈조류에 다량의 함유되어 있는 물질이다. 이런 플로로탄닌의 구조는 기본적으로 아로마틱 또는 페놀릭 링을 가지고 있으며 이를 통하여 다양한 생리활성이 보고 되어지고 있다. 이 연구에서는 갈조류에서 분리된 플로로탄닌에 대한 구조를 분석하였으며 또한, 이 플로로탄닌의 멜라닌생합성, 당뇨, 고혈압에 관련된 효소에 대한 억제 활성을 측정하였다. 그리고 활성을 비교 분석하는 방법으로서 molecular docking 방법을 이용하였으며, 이 방법은 효소의 활성 부위에 플로로탄닌이 어떤 결합 작용을 통하여 효소 활성을 억제하는지 비교 분석하였다.

1. 해조류의 한 종류인 갈조류로부터 플로로탄닌을 분리하여 멜라닌 생합성과정에 미치는 영향을 측정하였다. 분리된 플로로탄닌 모두 멜라닌 생합성 과정을 크게 억제 하는 것을 확인 할 수 있었으며, 특히, 새로운 플로로탄닌인 Octaphlorethol A (OPA)는 매우 높은 억제 활성을 나타내었다. 또한, molecular docking이라는 방법을 통하여 멜라닌 생합성에 관련된 타이로신에이즈에 대한 플로로탄닌의 molecular docking 바인딩을 통하여 활성 및 작용에 기전에 대하여 비교 분석하였다.

2. 당뇨에 관련된 효소로, 장내에서 다당을 단당으로 분해시켜 혈관 내로 당의 흡수를 도와주는 역할을 하는 효소인 알파-글루코시데이즈에 대한 플로로탄닌의 효소 저해 활성을 측정하였다. 디에콜과 OPA는 기존의 알파-글루코시데이즈 효소 저해제로서 사용되어지는 아카보즈보다 높은 활성을 나타내었다. 이를 molecular docking을 통하여 플로로탄닌이 알파-글루코시데이즈에 미치는 영향을 비교 분석하였다.

3. 고혈압에 관련된 효소로, 안지오텐신 I 을 활성하시켜 안지오텐신 II의 작용을 유도하고, 브래디키닌을 불활성화시킴으로써 고혈압을 유도하는 안지오텐신 I 전환 효소에 대한 플로로탄닌의 효소 저해 활성을 측정하였다. 플로로탄닌 중 OPA가 가장 높은 저해 활성을 나타내는 것을 확인 할 수 있었다. Molecular docking을 이용하여 안지오텐신 I 전환 효소의 활성 부위에 플로로탄닌이 어떤 작용을 하는지 비교 분석하였다.

4. 플로로탄닌은 다양한 효소 억제 활성을 나타내었는데, 체내에 존재하는 소화 효소인 트립신과 펩신에 대하여 억제 활성을 정도를 비교하기 위하여 차에 존재하는 카테킨 종류와 함께 비교 분석한 결과 플로로탄닌은 소화 효소에 대하여 카테킨 종류에 비하여 낮은 저해

활성을 보였고 이를 molecular docking을 통하여 비교 분석하였다.

이러한 결과를 종합해 볼 때, 플로로탄닌은 멜라닌 생합성, 당뇨, 고혈압에 관련 된 효소에 대하여 높은 저해 활성을 보여주었으며, 반면에, 체내에 존재하는 효소에 대해서는 낮은 저해 활성을 보여 산업적 용도가 매우 다양할 것이라고 생각되며, 특히 molecular docking을 통하여 비교 분석함으로써 많은 종류의 물질의 다양한 생리활성을 예측 및 작용 기전을 확인 할 수 있을 것이라 판단된다.

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INTRODUCTION

Marine organisms are rich sources of structurally diverse bioactive compounds with various biological activities and their importance as a source of novel bioactive substances is growing rapidly. With marine species comprising approximately a half of the total global biodiversity, the sea offers an enormous resource for novel compounds [1,2]. Among marine organisms, marine algae are rich sources of bioactive compounds with various biological activities [3-4]. Recently, their value as a source of novel bioactive substances has grown rapidly and researchers have revealed that marine algal originated compounds exhibit various biological activities. Although seaweeds are exposed to the adverse environmental conditions such as light and high oxygen concentrations that lead to the formation of free radicals, and other strong oxidizing agents, they do not have any serious photodynamic damage. Thus, it can be known that marine algae are able to generate the bioactive compounds to protect themselves from external factors such as UV radiation, stress and herbivores. Edible marine macro algae or seaweeds are classified into three divisions such as Chorophyta (green algae), Phaeophyta (brown algae), and Rhodophyta (red algae) according to their composition of pigments. For example, the presence of xanthophyll pigment, fucoxanthin, is responsible for the color of brown seaweeds. Edible seaweeds have formed

an important part of the diet of many Far Eastern countries for centuries and demand for ingredients, such as “Kombu” from Laminaria species and “Nori” used in sushi from Porphyra species, have been largely met from cultivation of the seaweeds [5]. Apart from food uses, including their main industrial uses as thickeners and gelling agents, seaweeds are widely used as ingredients in various industries such as cosmeceutical, pharmaceutical, animal feed, and fertilizer industries. In recent years, the seaweeds serve as an important source of bioactive natural substances [6,7]. Moreover, many metabolites, which isolated from marine algae, have shown to possess bioactive effects [8]. Therefore, recently a new trend has been arisen to isolate novel bioactive compounds and constituents from edible seaweeds.

Polyphenols are one of the most common classes of secondary metabolites in terrestrial and marine plants. Although terrestrial and marine polyphenols are similar in some respects, there are fundamental differences in their chemical structure [9]. In general, polyphenols or phenolic compounds have a similar basic structural chemistry including an “aromatic” or “phenolic” ring structure. Phenolic compounds have been associated wth antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals [10,11]. The protective effects of plant polyphenols in biological systems are ascribed to their capacity to transfer electrons to free radicals, chelate metal catalysts, activate antiodixant enzymes and

inhibit oxidase. Polyphenols are classified broadly into two classes; condensed tannins, which are polymeric flavonoids, and hydrolysable tannins, which are derivatives of gallic acid [12]. Phlorotannins, known as marine algal polyphenol, are formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) monomer units and biosynthesized through the acetate-malonate pathway, also known as polyketide pathway. The phlorotannins are highly hydrophilic components with a wide range of molecular sizes ranging between 126 Da and 650 kDa [13]. Marine brown algae accumulate a variety of phloroglucinol-based polyphenols, as phlorotannins of low, intermediate and high molecular weight containing both phenyl and phenoxy units. Based on the means of linkage, phlorotannins can be classified into four subclasses such as fuhalols and phlorethols (phlorotannins with an ether linkage), fucols (with a phenyl linkage), fucophloroethols (with an ether and phenyl linkage), and eckols (with a dibenzodioxin linkage). The isolated and characterized phlorotannins from marine brown algae are compounds (Fig. 1), such as phloroglucinol, eckol, triphlorehol A, dieckol, 2-phloroeckol, fucofuroeckol A, 7-phloroeckol, 6,6'-bieckol, diphlorethohydroxycarmalol, and phlorofucofuroeckol A. Among marine brown algae, *E. cava* is a rich source of phenolic compounds as phlorotannins than other brown algae [14]. However, other brown seaweeds also have been reported for various types of phlorotannins. These phlorotannins help to protect algae from stress conditions and

herbivores. Due to the health beneficial various biological activities of phlorotannins, marine brown algae are known to be a rich source of healthy food.

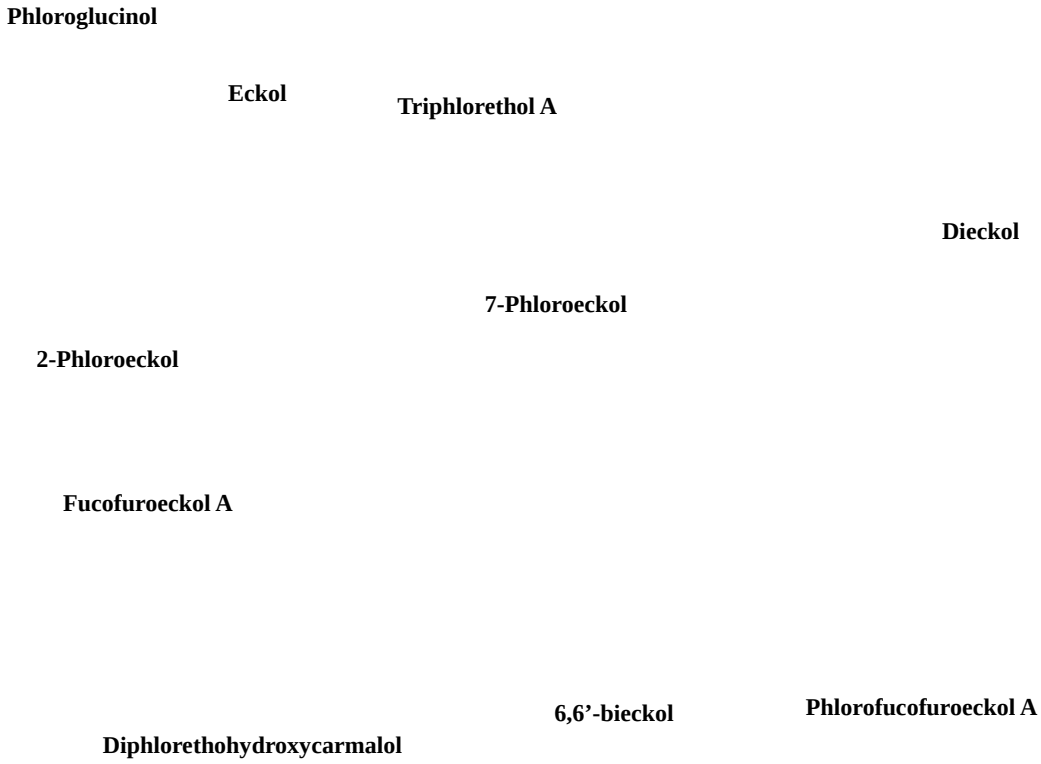

Fig. I. Chemical structure of phlorotannins from marine borwn algae.

Melanin is one of the most widely distributed pigments and is found in bacteria, fungi, plants and animals. It is a heterogeneous polyphenol-like biopolymer with a complex structure and color varying from yellow to black [15]. The color of mammalian skin and hair is determined by a number of factors, the most important of which is the degree and distribution of melanin pigmentation. Melanin is secreted by melanocyte cells distributed in the basal layer of the dermis [16]. The role of melanin is to protect the skin from ultraviolet (UV) damage by absorbing UV sunlight and removing reactive oxygen species (ROS). Various dermatological disorders result in the accumulation of an excessive level of epidermal pigmentation. These hyperpigmented lentigenes include melasma, age spots and sites of actinic damage [17]. Great interest has been shown in the involvement of melanins in malignant melanoma, the most life-threatening skin tumors. The type and amount of melanin synthesized by the melanocyte and its distribution in the surrounding keratinocytes determine the actual color of the skin. The characteristic skin patterns of zebra, giraffes and piebald animals in general are due to this uneven distribution of melanocytes. Melanin is formed through a series of oxidative reactions involving the amino acid tyrosine in the presence of tyrosinase. Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions of melanin synthesis: the hydroxylation of tyrosine by monophenolase action and the oxidation of 3,4-dihydroxyphenylalanine (L-DOPA) to *o*-

dopaquinone by diphenolase action. However, if L-DOPA is an active cofactor, its formation as an intermediate during *o*-dopaquinone production is still controversial. *o*-Dopaquinone is unstable in aqueous solution and rapidly suffers a non-enzymatic cyclization to leukodopachrome, which is further oxidized non enzymatically by another molecule of *o*-dopaquinone to yield dopachrome and one molecule of regenerated L-DOPA [18–20]. Tyrosinase exists widely in plants and animals, and is involved in the formation of melanin pigments [21–23]. In the food industry, tyrosinase is a very important enzyme in controlling the quality and economics of fruits and vegetables [22-24]. Tyrosinase catalyzes the oxidation of phenolic compounds to the corresponding quinones and is responsible for the enzymatic browning of fruits and vegetables. In addition to the undesirable color and flavor, the quinone compounds produced in the browning reaction may irreversibly react with the amino and sulfhydryl groups of proteins. The quinone-protein reaction decreases the digestibility of the protein and the bioavailability of essential amino acids, including lysine and cysteine. Therefore, development of high-performance tyrosinase inhibitors is much needed in the agricultural and food fields. Tyrosinase plays an important role in the developmental and defensive functions of insects. Tyrosinase is involved in melanogenesis, wound healing, parasite encapsulation and sclerotization in insects [25–27]. The development of tyrosinase inhibitors has become an active alternative approach in

controlling insect pests. In addition, tyrosinase inhibitors have become increasingly important for medicinal and cosmetic products that may be used to prevent or treat pigmentation disorders [28, 29].

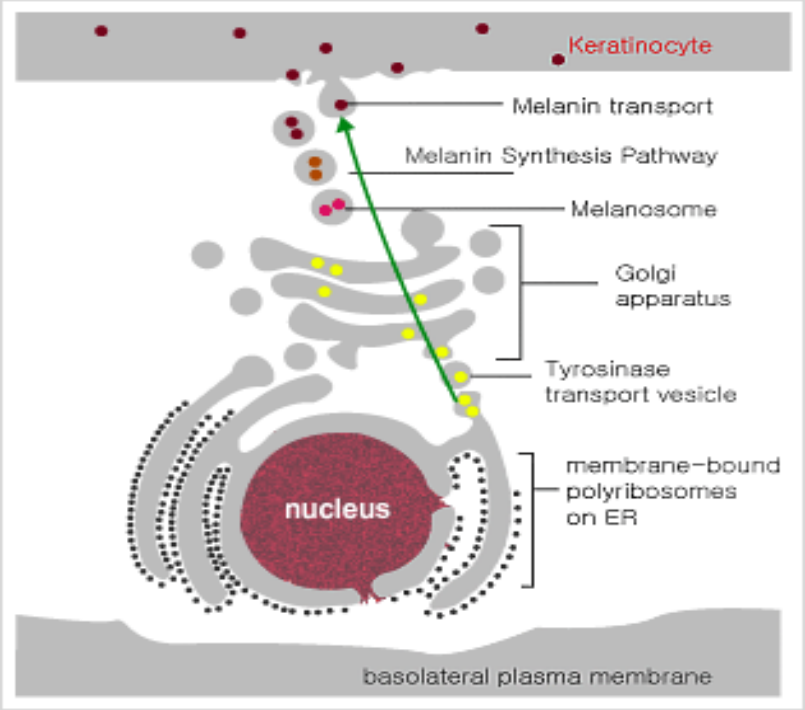

Fig. II. Melaninsynthesis mechanism.

Diabetes mellitus is characterized by abnormal metabolism of glucose, due in part to resistance to the action of insulin in peripheral tissue. The caharacteristic sysptoms are polyuria, polydipsia, and polyuria. Diabetes mellitus is the most serious and chronic disease that is development with an increasing obesity and aging in thhe general population over the world. Diabetes mellitus is a complex disorder that is characterized by hyperglycemia. It is largly classified into insulin-dependent diabetes mellitus (Type I diabete) and non-insulin-dependent diabetes mellitus (Type II diabete). In particular, type II diabetes is an increasing worldwide health problem and is the most common type of diabetes [30]. Hyperglycemia plays an important role in the development type II diabetes and complications associated with the disease such as micro-vascular and macro-vascular disease [31]. Therfore, the effective control of blood glucose level is the key to prevent or reverse diabetic complications and improve the quality of the life in diabetic patients [32]. Currently available therapies for type II diabetes include insulin and various oral antidiabetic drugs such as sulfonylureas, metformin, α-glucosidase inhibitor, and thiazolidinediones. However, these therapies have either limited efficacy or significant mechanism based side effects like hypoglycemia, flatulence, body weight gain of enhancement of gastrointestinal problems. The control of postprandial hyperglycemia has been shown to be important in the treatment of diabetes and the prevention of cardiovascular complications. One of the therapeutic

approaches adopted thus far to ameliorate postprandial hyperglycemia involves the retardation of glucose absorption via the inhibition of carbohydrate-hydrolzyng enzymes including a-glucosidase and a-amylase, in the digestive organs (Fig. III) [33]. The powerful synthetic a-glucosidase and a-ammylase inhibitors, such as acarbose, miglitol, and voglibose, function directly in reducing the sharp increases in glucose levels that occur immediately after food uptake [34-36]. However, the continuous use of those synthetic agents should be limited because those agents may induce side effects such as flatulence, abdominal cramps, vomiting, and diarrhea [37]. Additionally, there have been some reports describing an increased incidence of renal tumors, serious hapatic injury, and acute hepatitis [38,39]. Therefore, a number of studies have been conducted in the search for naturally derived α-glucosidase and α-amylase inhibitors that induce no deleterious side effects [40-42].

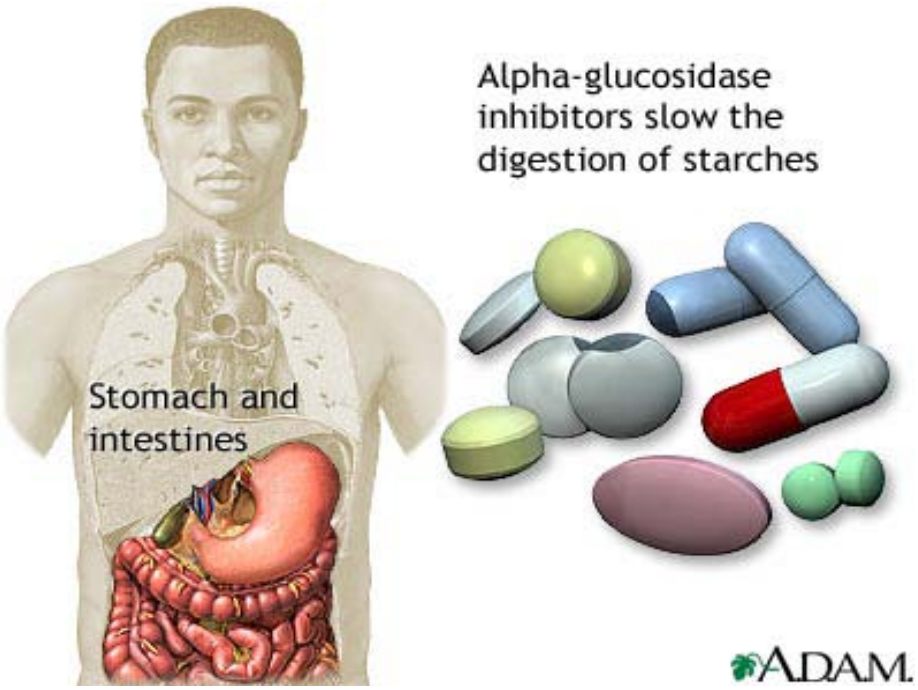

Fig. III. α–Glucosidase inhibitors

Hypertension or high blood pressure is one of the major independent risk factors for cardiovascular diseases [43,44] and it is a major health issue, estimated to be affecting about 20% of the world's adult population [45]. Among processes related to hypertension, angiotensin-I-converting enzyme (ACE) plays an important role in the regulation of blood pressure (Fig. IV). ACE is a dipeptidyl carboxypeptidase (EC. 3.4.15.1) and was originally isolated from horse blood [46]. It plays a crucial role in the regulation of blood pressure as it promotes the conversion of angiotensin-I to the potent vasoconstrictor angiotensin-II as well as inactivates the vasodilator bradykinin, which has a depressor action in the renin-angiotensin system. This potent vasoconstrictor is also involved in the release of a Na-retaining steroid, aldosterone from the adrenal cortex, which has a tendency to increase blood pressure [47]. Inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension. Therefore, in the development of drugs to control high blood pressure, ACE inhibition has become an important activity. Many studies have been attempted in the synthesis of ACE inhibitors such as captopril, enalapril, alcacepril and lisinopril, which are currently used in the treatment of essential hypertension and heart failure in humans [48]. However, these synthetic drugs are believed to have certain side effects such as cough, taste disturbances, skin rashes or angioneurotic edema all of which might be intrinsically linked to synthetic ACE inhibitors [49]. Therefore, the research and

development to find safer, innovative, and economical ACE inhibitors is necessary for the prevention and remedy of hypertension [50,51]. Many research groups have combed for novel ACE inhibitors from natural products [52], microbial sources [53] and food proteins [54].

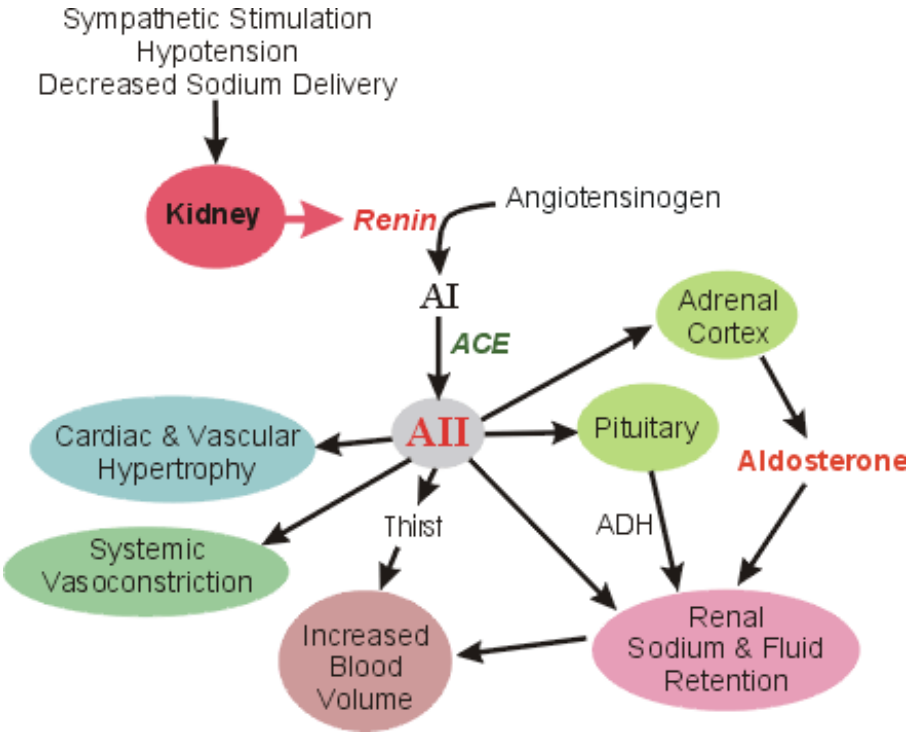

Fig. IV. Angiotensin I converting enzyme (ACE)

The application of computational methods to study the formation of intermolecular complexes has been the subject of intensive research during the last decade. It is widely accepted thet drug activity is obtained through the molecular binding of one molecule (the ligand) to the pocket of another, usually lager, molecule (the receptor), which is commonly a protein. A complex of a protein with a therapeutical drug is shown in Fig. V. In their binding conformations, the molecules exhibit geometric and chemical complementrity, bonth of which are essentidal for successful drug activity. The computational process of searching for a ligand that is able to fit both geometrically and energetically the binding site of a protein is called molecular docking. Absorption, distribution, metabolism, and excretion (ADME) properties of compounds are important in pharmaceutical research. New drug discovery and development are time-consuming, expensive [55] and have a high attrition rate [56]. An evaluation of the reasons for attrition showed that poor pharmacokinetic properties accounted for nearly 40% of drug development failures [57]. Therefore, a substantial effort has been focused on the early estimation of ADME properties. The predicted properties of compounds have been increasingly considered in the design of combinatorial synthetic routes and high-throughput screening experiments and thus, have improved the quality of leading compounds that may enter the development stages [58].

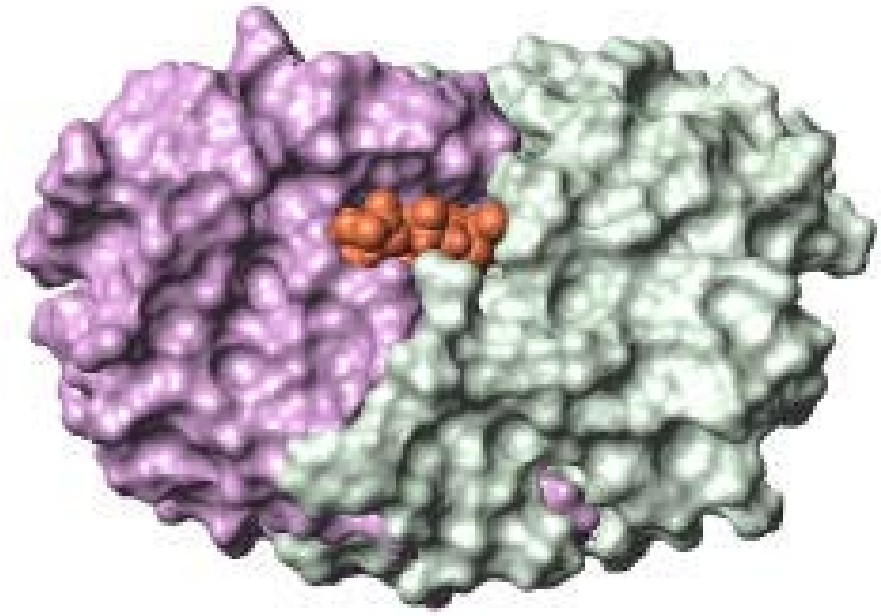

Fig. V. Therapeutic drug molecule (small docked molecule towards the center of the figure) bound to protein receptor (HIV-1 protease). The drug molecule fits tightly in the binding stie and blocks the normal protein function.

Computationally, ADME properties are most often estimated using a quantitative structure-ativity relationship (QSAR) approach [59] that is based on the physic-chemical properties of a compound. Since the 1960s, QSAR approaches have successfully produced many classification and regression models that accurately predict a variety of ADME properties for a diverse array of compound.

Recently, a number of studies have been focused on marine bio-resources. Marine naturl products provide a rich source of chemical diversity that can be used to design and develop new, potentially useful therapeutic agent. In this study, therefore, we tried to isolation and idenfication of active copound from brown algae and examined biological activities of active compounds using molecular docking program which can be developmed as possible medicinal and cosmetic agents for human.

Part Ⅰ.

Molecular docking of phlorotannins isolated from brown algae and their effects on tyrosinase inhibitory activity

Part Ⅰ.

Molecular docking of phlorotannins isolated from brown algae and their effects on tyrosinase inhibitory activity

1. ABSTRACT

In this study, the phlorotannins which were isolated from the brown algae, *Ecklonia cava* and *Ishige foliacea* were examined for its inhibitory effects on melanin synthesis. Tyrosinase inhibitors are important agents for cosmetic products. We therefore examined the inhibitory effects of phlorotoannin isolated from brown algae on mushroom tyrosinase and melanin synthesis, and analyzed its binding modes using the crystal structure of *Bacillus megaterium* tyrosinase (PDB ID: 3NM8). The new tyrosinase inhibitor was identified as an octaphlorethol A (OPA) by the MS data, ^{1}H and ^{13}C NMR. The four phlorotannins (phloroglucinol, eckol, dieckol, and OPA) inhibited mushroom tyrosinase and were more effective as a cellular tyrosinase having melanin reducing activities than the commercial inhibitor, arbutin, in B16F10 melanoma cells, and without apparent cytotoxicity. For further insight, we predicted the 3D structure of tyrosinase and used a docking algorithm to simulate binding between tyrosinase and phlorotannins. These results suggest that phlorotannins has great potential to be further developed as a pharmaceutical and cosmetic agent for use in dermatological disorders associated with melanin.

2. INTRODUCTION

Tyrosinase (monophenol monooxygenase; EC 1. 14. 18. 1) is widely distributed in nature, and is a metalloenzyme oxidase that catalyzes two distinct reactions of melanin synthesis in which L-tyrosine is hydroxylated to 3,4-dihydroxyphenylalanine L-DOPA (monophenolase activity), and the latter is subsequently oxidated to dopaquinone (diphenolase activity) [1].

Melanin helps to protect skin from the damaging ultraviolet radiation of the sun. However, high concentrations of melanin in the skin result in hypopigmentation such as freckles and moles. Tyrosinase is the rate-limiting enzyme in melanin production, which occurs in melanocytes that are located within the basal epidermis [2]. Therefore, the inhibition of tyrosinase is one of the major strategies to treat hyperpigmentation [3]. Safe and effective tyrosinase inhibitors that act to minimize skin pigmentation abnormalities are desired. Despite a large number of tyrosinase inhibitors, only a few of these are used today, as many of them show side effects [4,5]. Well-known tyrosinase inhibitors such as kojic acid, hydroquinone, and 1-phenyl-2-thiourea (PTU) can cause adverse reactions such as dermatitis and skin irritation [6], melanocyte destruction [7], and skin cancer [8]. As a result, it is necessary to search for new candidates that show effective tyrosinase inhibition but are devoid of side effects; thus, metabolites biosynthesized by plants have become promising alternatives to synthetic analogues [9,10].

Ecklonia cava and *Ishige foliacea*, kind of brown algae, were abundantly produced around Jeju Island, Korea, and are utilized as a food ingredient, animal feed, fertilizer, and medicine. In addition, brown algae contain variety of compounds including carotenoids, fucoidan, and phlorotannins, which show different biological activities [11-13]. Very recently, some studies have explored the potential cosmetic activities of phlorotannins isolated from brown algae [14]. In our continuing investigation of phlorotannins isolated from *E. cava* and *I. foliacea* were chosen as a target compound for its tyrosinase inhibition activity and reduction of melanin synthesis.

The aim of this study was to evaluate the tyrosinase and melanin synthesis inhibitory activity of phlorotannins isolated from brown algae to see if it can be applied in the cosmetic and pharmaceutical fields.

3. MATERIALS AND METHODS

3.1. Materials

Mushroom tyrosinase, L-tyrosine [3-(4-hydroxyphenol)]-L-alanine(S)-2-amino-3-(4-hydroxyphenol) propionic acid], arbutin [2-hydroxymethyl-6-(4-hydroxyphenoxy)oxane-3, 4, 5-triol], and α-MSH (alpha-melanocyte stimulating hormone) were purchased from sigma

Chemical Co. (St. Louis, MO, USA).

3.2. Extraction and isolation of phlorotannins from *Ecklonia cava*

The brown alga, *E. cava* (Fig.1-1, A.) was collected along the coast of Jeju Island, Korea, between February and April 2009. The sample was washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water and maintained in a medical refrigerator at -20℃. Thereafter, the frozen sample was lyophilized and homogenized with a grinder prior to extraction. The powdered *E. cava* was extracted with 80% aqueous EtOH, and was evaporated under vacuum. The EtOH extract was then partitioned with EtOAc. The EtOAc extract was fractionated via silica column chromatography with stepwise elution of a $CHCl_3$–MeOH mixture (100:1–1:1) to generate separated active fractions. The combined active fraction was then further subjected to a Sephadex LH-20 column (GE Healthcare, USA) saturated with 80% MeOH, and finally purified via reversed-phase HPLC (ThermoFisher Scientific, USA) using a Waters HPLC system equipped with a Waters 996 photodiode array detector and C18 column (J'sphere ODS-H80, 150 x 20 mm, 4 μm; YMC Co.) by stepwise elution with methanol–water gradient (UV range: 230 nm, flow rate: 0.8 ml/min). The purified compounds (phloroglucinol, eckol, and dieckol) were confirmed by comparing their LC/MS, ^{1}H NMR

data to the literature report [15].

Phloroglucinol : LC/MS data (M+, m/z: 126 Calcd. For $C_6H_6O_3$). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.97 (3H, s, OH-1, 3, 5), 5.66 (3H, s, H-2, 4, 5).

Eckol : LC/MS data (M+, m/z: 372.0 Calcd. For $C_{18}H_{12}O_9$). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.54 (2H, s, OH-2, 7), 9.45 (1H, s, OH-4), 9.21 (2h, s, OH-2, 7) 9.16 (2H, s, OH-3', 5'), 6.14 (1H, s, H-3), 5.96 (1H, d, J = 2.8 Hz, H-6), 5.72 (2H, J = 1.7 Hz, H-4'), 5.78 (1H, d, J = 2.8 Hz, H-6), 5.72 (2H, J = 1.7 Hz, H-2-, 6').

Dieckol : LC/MS data (M+, m/z: 742.0 Calcd. For $C_{36}H_{22}O_{18}$). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.71(1H, s, OH-9), 9.61 (1H, s, OH-9''), 9.51 (1H, s, OH-4''), 9.46 (1H, s, OH-4), 9.36 (2H, s, OH-3'', 5''), 9.28 (1H, s, OH-2''), 9.23 (1H, s, OH-2), 9.22 (1H, s, OH-7''), 9.15 (2H, s, OH-3', 5') 6.17 (1H, s, H-3''), 6.14 (1H, s, H-3), 6.02 (1H, d, J = 2.7 Hz, H-8), 5.98 (1H, d, J = 2.7 Hz, H-8''), 5.95 (1H, s, H-2''', 6'''), 5.82 (1H, d, J = 2.7 Hz, H-6), 5.81 (1H, d, J = 2.7Hz, H-6''), 5.80 (1H, t, J = 2.0 Hz, H-4'), 5.78 (2H, d, J = 2.0 Hz, H-2', 6').

The purity of phlorotannins (Fig. 1-1, B.) were >95%, based on the peak area of all components absorbed at each specific wavelength in HPLC analysis. Plorotannin were dissolved in DMSO and used for experiments adjusting the final concentration of DMSO in culture medium to <0.01%.

A

B

Phloroglucinol

Eckol

Dieckol

Fig. 1-1. The photography of a brown alga, *Ecklonia cava* (A) and its phlorotannins (B).

3.3. Extraction and isolation of phlorotannins from *Ishige foliacea*

The brown alga, *I. foliacea* (Fig. 1-2, A.) was collected along the coast of Jeju Island, Korea, between March and June 2010. The powdered, *I. foliacea* was extracted with 80% aqueous EtOH, and was evaporated under vacuum. The EtOH extract was then partitioned with hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc) and water. Since the EtOAc fraction contained the major compound than those of other fractions, the major compound was extracted from EtOAc fraction using a silica column chromatography and finally purified via reversed-phase HPLC (ThermoFisher Scientific, USA) using a Waters HPLC system equipped with a Waters 996 photodiode array detector and C18 column (J'sphere ODS-H80, 150 x 20 mm, 4 μm; YMC Co.) by stepwise elution with methanol–water gradient (UV wavelength: 230 nm, flow rate: 0.8 ml/min) (Fig. 1-2, B.) .

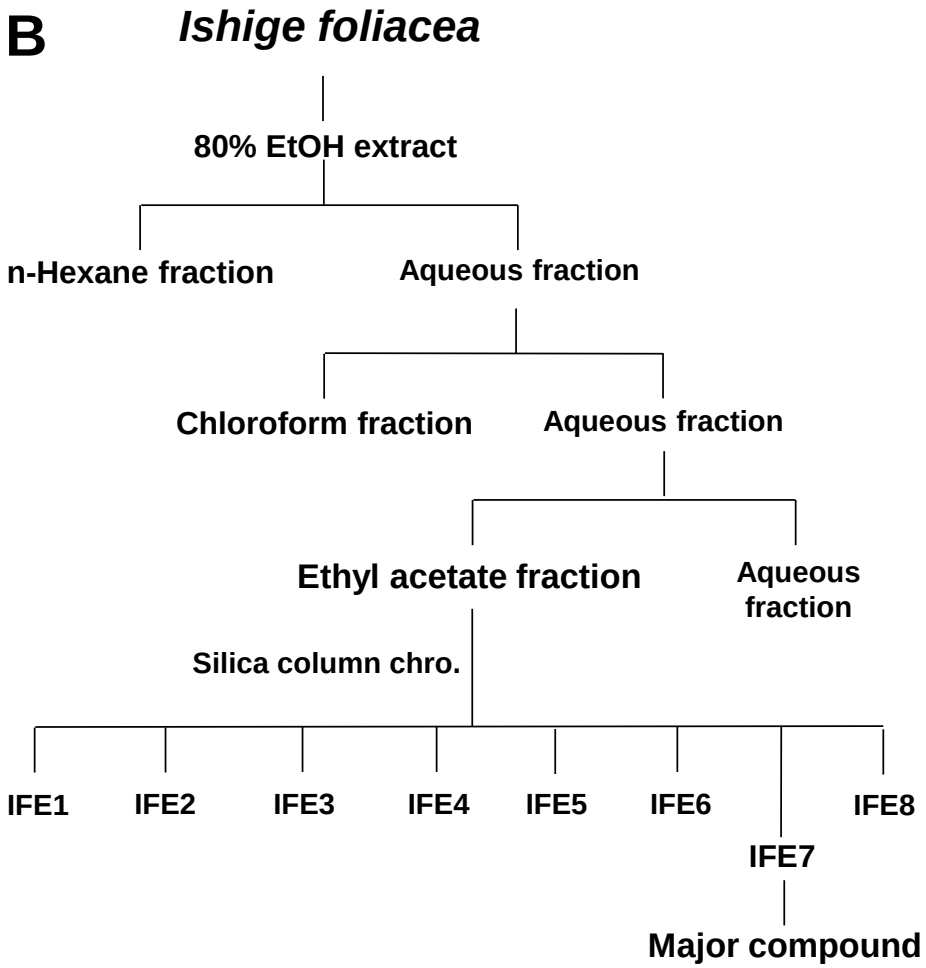

Fig. 1-2. The photography of a brown alga, *Ishige foliacea* (A) and isolation scheme (B) of major compound.

The structure of purified compound was determined by LC/MS data (Fig. 1-3.) and ^{1}H and ^{13}C NMR analysis (Table 1-1. and Fig. 1-4, A and B.).

^{1}H and ^{13}C DEPT and ESI-MS data revealed the molecular formula as $C_{48}H_{34}O_{25}$ at *m/z* 992.19 $[M-2H]^{2-}$, the unsaturated degree is 32 from the 8 aromatic rings, respectively. The ^{1}H spectrum showed the two kinds of typical proton signals consisted of aromatic and phenol protons, and according to the coupling constants of them, all of aromatic protons were assigned as *meta* aromatic protons at 5.57 (1H, d, *J* =2.8 Hz , H-4), 5.58 (1H, d, *J* =2.8 Hz , H-6), 5.59 (1H, d, *J* =2.8 Hz , H-9), 5.59 (1H, d, *J* =2.8 Hz, H-11), 5.68 (1H, d, *J* =1.8 Hz, H-15), 5.68 (1H, d, *J* =1.8 Hz, H-17), 5.71 (1H, d, *J* =1.8 Hz, H-21), 5.72 (1H, d, *J* =1.8 Hz, H-23), 5.85 (1H, d, *J* =1.6 Hz, H-26), 5.84 (1H, d, *J* =1.8 Hz, H-30), 5.94 (1H, d, *J* =1.8 Hz, H-32), 5.94 (1H, d, *J* =1.8 Hz, H-36), 6.15 (1H, d, *J* =1.8 Hz, H-38), 6.15 (1H, d, *J* =1.8 Hz, H-42), 6.16 (1H, d, *J* =1.6 Hz, H-44), 6.01 (1H, d, *J* =1.6 Hz, H-46), and 6.16 (1H, d, *J* =1.6 Hz, H-48), respectively. The other proton signals were elucidated as phenol ones based on the chemical shifts at the lower filed shown as 9.02 (s, OH-1,3), 9.04 (s, OH-5, 27, 29, 33,35), 8.98 (s, OH-8,12), 8.93 (s, OH-14, 18), 8.92 (s, OH-20,24), 9.06 (s, OH-39,41), and 9.07 (s, OH-45, 47). The ^{13}C spectrum showed the 48 carbon atoms elucidated as aromatic carbons including 24 sp^2 quaternary carbon atoms at 153.0 (s, C-1), 153.0 (s, C-3), 156.1 (s, C-5), 152.9 (s, C-8), 151.1 (s, C-10), 152.9 (s, C-12), 151.1 (s, C-14), 154.5 (s, C-16), 151.1

(s, C-18), 151.1 (s, C-20), 151.1 (s, C-24), 154.1 (s, C-25), 154.1 (s, C-22), 151.1 (s, C-27), 151.1 (s, C-29), 154.0(s, C-31), 150.8 (s, C-33), 150.8 (s, C-35), 156.2 (s, C-37), 152.7 (s, C-39), 152.7 (s, C-41), 161.0 (s, C-43), 158.6 (s, C-45), 161.0 (s, C-47), and 8 unsubstitude aromatic carbons at 122.0 (s, C-2), 122.0 (s, C-7), 122.0 (s, C-9), 122.0 (s, C-13), 123.4 (s, C-19), 123.4 (s, C-28), 123.5 (s, C-34), 123.5 (s, C-40), and 16 phenol hydroxyl signals at 94.9 (d, C-6),94.9 (d, C-4), 94.7 (d, C-11), 94.7 (d, C-15), 94.7 (d, C-17), 94.7 (d, C-21), 94.7 (d, C-23), 94.7 (d, C-26), 94.7 (d, C-30), 94.1 (d, C-32), 94.2 (d, C-36), 94.1 (d, C-38), 94.1 (d, C-42), 94.7 (d, C-44), 94.0 (d, C-46), and 94.7 (d, C-47). The comprehensive analyses revealed the major compound as poly-phenols mainly according to the chemical shifts and coupling constants from ^{1}H and ^{13}C NMR data, the phenol hydroxyl signals were assigned completely based on the 2D NMR (HMBC) data. In addition, the major compound was elucidated as a new compound after checking SCI finder database. On the basis of these spectral data, the probable structure of the compound was deduced and tentatively reported as Octaphlorethol A (OPA) (Fig. 1-5.)

The purity of OPA was >95%, based on the peak area of all components absorbed at each specific wavelength in HPLC analysis.

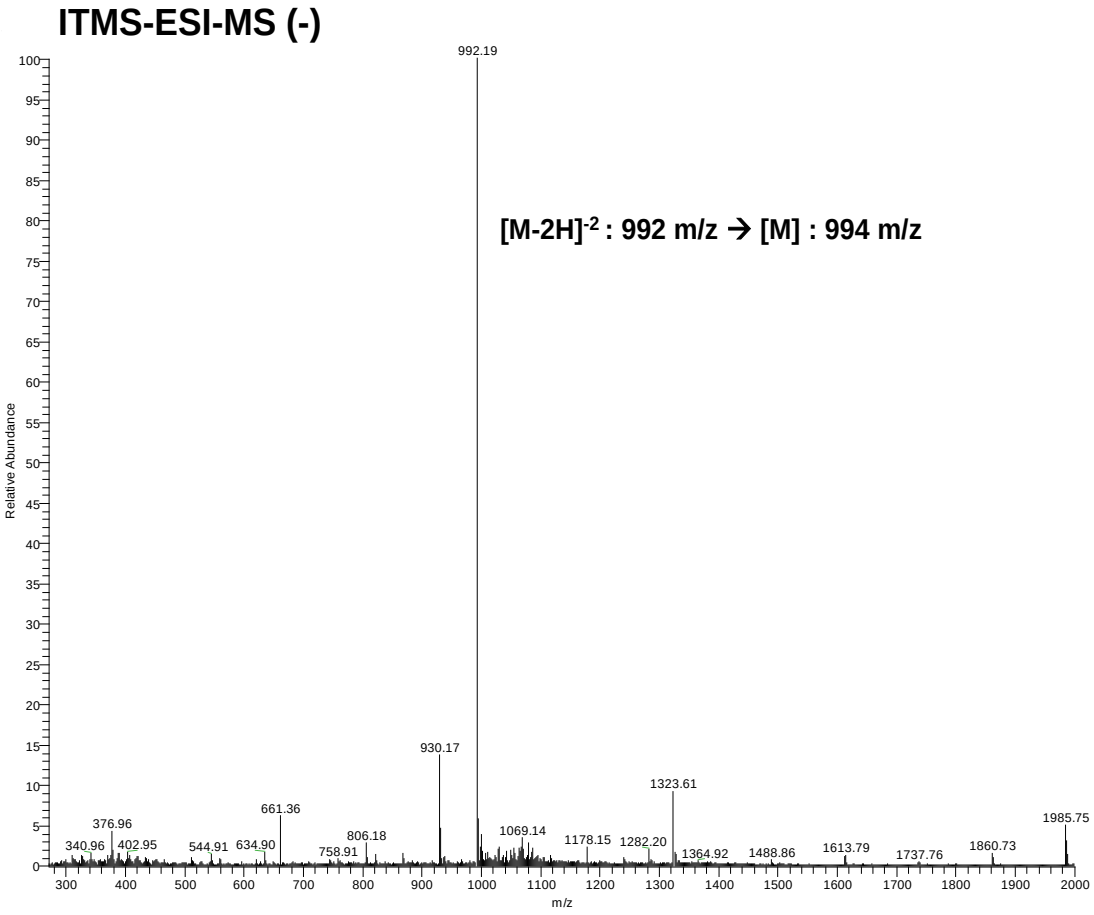

Fig. 1-3. MS spectra of the major compound isolated from *I. foliacea*. The generated in negative ionization mode.

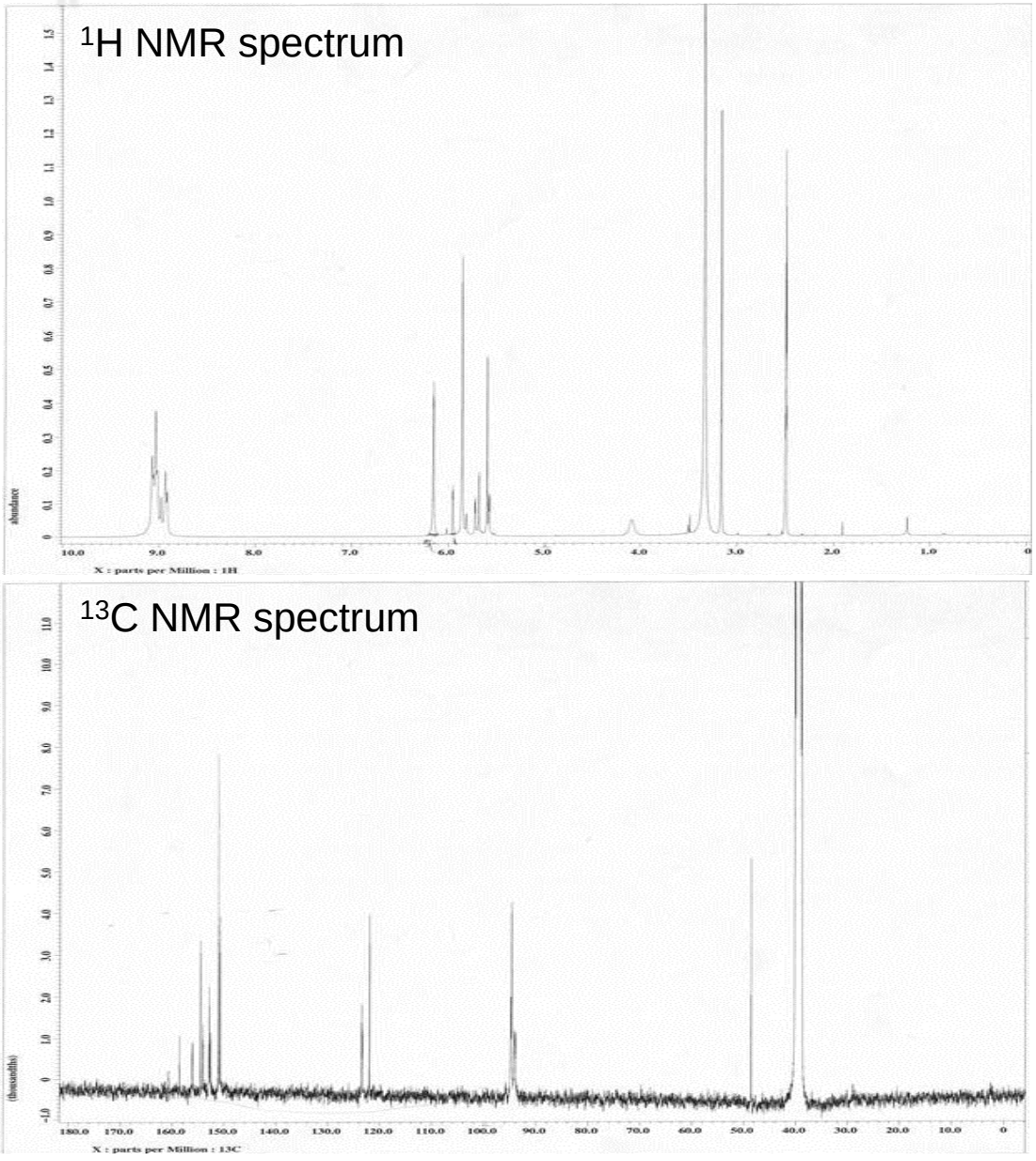

Fig. 1-4. Proton and carbon NMR spectrum of major compound.

Table 1-1. ^{1}H NMR and ^{13}C NMR data of major compound in DMSO-d_6

Position ($C^{\#}$)	δ_H (mult, *J*)	δ_C (mult)	Position ($C^{\#}$)	δ_H (mult, *J*)	δ_C (mult)
1		153.0 (s)	30	5.84 (d, J =1.8 Hz)	94.7 (d)
2		122.0 (s)	31		154.0(s)
3		153.0 (s)	32	5.94 (d, J =1.8 Hz)	94.1 (d)
4	5.57 (d, J =2.8 Hz)	94.9 (d)	33		150.8 (s)
5		156.1 (s)	34		123.5 (s)
6	5.58 (d, J =2.8 Hz)	94.9 (d)	35		150.8 (s)
7		122.0 (s)	36	5.94 (d, J =1.8 Hz)	94.2 (d)
8		152.9 (s)	37		156.2 (s)
9	5.59 (d, J =2.8 Hz)	122.0 (s)	38	6.15 (d, J =1.8 Hz)	94.1 (d)
10		151.1 (s)	39		152.7 (s)
11	5.59 (d, J =2.8 Hz)	94.7 (d)	40		123.5 (s)
12		152.9 (s)	41		152.7 (s)
13		122.0 (s)	42	6.15 (d, J =1.8 Hz)	94.1 (d)
14		151.1 (s)	43		161.0 (s)
15	5.68 (d, J =1.8 Hz)	94.7 (d)	44	6.16 (d, J =1.6 Hz)	94.7 (d)
16		154.5 (s)	45		158.6 (s)
17	5.68 (d, J =1.8 Hz)	94.7 (d)	46	6.01 (d, J =1.6 Hz)	94.0 (d)
18		151.1 (s)	47		161.0 (s)
19		123.4 (s)	48	6.16 (d, J =1.6 Hz)	94.7 (d)
20		151.1 (s)	OH-1,3	9.02 (s)	
21	5.71 (d, J =1.8 Hz)	94.7 (d)	OH-5,27	9.04 (s)	
22		154.1 (s)	OH-29,33	9.04 (s)	
23	5.72 (d, J =1.8 Hz)	94.7 (d)	OH-35	9.04 (s)	
24		151.1 (s)	OH-8,12	8.98 (s)	
25		154.1 (s)	OH-14,18	8.93 (s)	
26	5.85 (d, J =1.6 Hz)	94.7 (d)	OH-20,24	8.92 (s)	
27		151.1 (s)	OH-39,41	9.06 (s)	
28		123.4 (s)	OH-45,47	9.07 (s)	
29		151.1 (s)			

*Recorded in DMSO-d_6 at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR156.2 (s)

Fig. 1-5. Chemical structure of octaphlorethol A (OPA) isolated from brown alga, *Ishige foliacea*.

3.4. Cell culture

B16F10 cells (obtained from the Korea Cell Line Bank) were grown on Dubecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbas, CA, USA) supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL). Cultures were maintained at 37℃ in a 5% CO_2 incubator. B16F10 cells were cultured in 24-well plates for melanin quantification and enzyme activity assays.

3.5. Cell viability

Cell survival was quantified through a colorimetric MTT assay that measured the mitochondrial activity in viable cells. B16F10 cells were seeded (1 x 10^5 cells/mL) together with various concentrations of phlorotannins and incubated for up to 72 h prior to MTT treatment. MTT stock solution (50 μL; 2 mg/mL in PBS) was added to each well to achieve a total reaction volume of 250 μL. After 4 h of incubation, the plates were centrifuged for 10 min at 2000 rpm and the supernatants were aspirated. The formazan crystals in each well were dissolved in DMSO. The amount of purple formazan was assessed by measuring the absorbance at 540 nm.

3.6. Assay for the measurement of inhibitory effects on mushroom tyrosinase

Mushroom was used as the source of tyrosine for the entire study. To begin the assay [16], a 20 μL of an aqueous mushroom tyrosinase solution (1000 units) was added to a 96-well microplate in a 200 μL assay mixture containing 1 mM L-tyrosinase solution and 50 mM phosphate buffer (pH 6.5). the assay mixture was incubated at 25℃ for 30 min. Following incubation, the amount of dopachrome produced in the reaction mixture was determined at 492 nm using microplate reader.

3.7. Assay on cellular tyrosinase activity

Tyrsosinase activity was estimated by measuring the rate of L-DOPA oxidation[18]. Arbutin was used as a positive control. α-MSH, which is known to stimulate melanogenesis, was used at 0.1 μM as the negative control, and its effect on melanogenesis was compared to that of the test sample. Cells were placed in 24-well dishes at a density of 1 x 10^5 cells/mL.B16F10 cells were incubated in the presence or absence of 0.1 μM α-MSH and they were then treated for 72h with various concentrations of dieckol. The cells were washed and lysed in 100 μl of 50 mM sodium phosphate buffer (pH 6.5) containing 1% Triton X-100 and 0.1 mM PMSF (phenyl methyl sulfonyl fluoride), and then frozen -80 ℃ for 30 min. After being thawed and mixed, the cellular extracts were placed in a 96-well plate, and the

absorbance at 492 nm was recorded every 10 min for 1 h at 37℃ using ELISA plate reader.

3.8. Determination of melanogenesis in B16F10 cells

The melanin contents were determined using a modification of the method to the literature report[19]. In the present study, the amount of melanin was used as the index of melanogenesis. B16F10 cells (1 x 10^5 cells/mL) were transferred to 24-well dishes and incubated in the presence or absence of 0.1 μM α-MSH. The cells were then incubated for 72 h with various concentrations of dieckol. The samples were washed with PBS and then dissolved in 100 μL of 1N NaOH. The samples were incubated at 60℃ for 1h and mixed to solubilize the melanin. The amount of melanin contents were assessed by measuring the absorbance at 405 nm.

3.9. In silico docking of tyrosinase and new inhibitor candidate

Molecular docking is an application wherein molecular modeling techniques are used to predict how a protein (enzyme) interacts with small molecules (ligands) [20-22]. The ability of a protein to interact with small molecules plays a major role in the dynamics of that protein, which may enhance or inhibit its biological function. In the present study, we performed docking of phlorotannins into the active site of the mushroom tyrosinase. The

crystal structure of tyrosinase (PDB: 3NM8) (Fig. 1-6) was obtained from the Protein Data Bank (PDB, http://www.pdb.org). We performed the docking studies using CDOCKER in Accelrys Discovery Studio 3.0 (Accelrys, Inc) (Fig. 1-7). We describe the ligand structure of the tyrosinase inhibitor candidate in Fig. 1-1. To prepare for the docking procedure, we performed the following steps: (1) conversion of the 2D structure into 3D structure; (2) calculation of charges; and (3) addition of hydrogen atoms using the CDOCKER docking program.

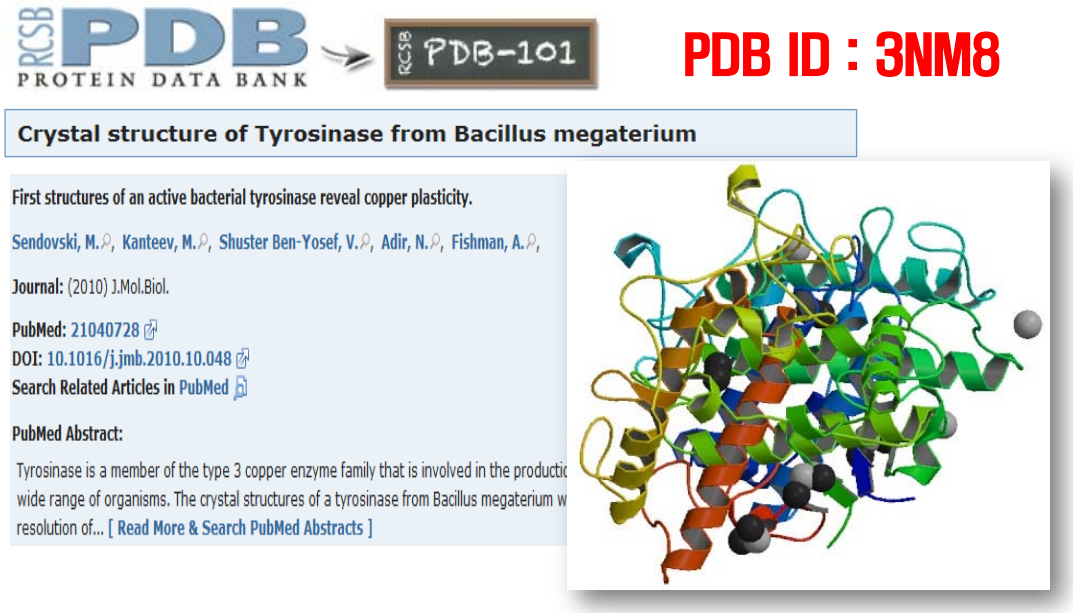

Fig. 1-6. Tyrosinase was obtained from Protein Data Bank (PDB: 3NM8).

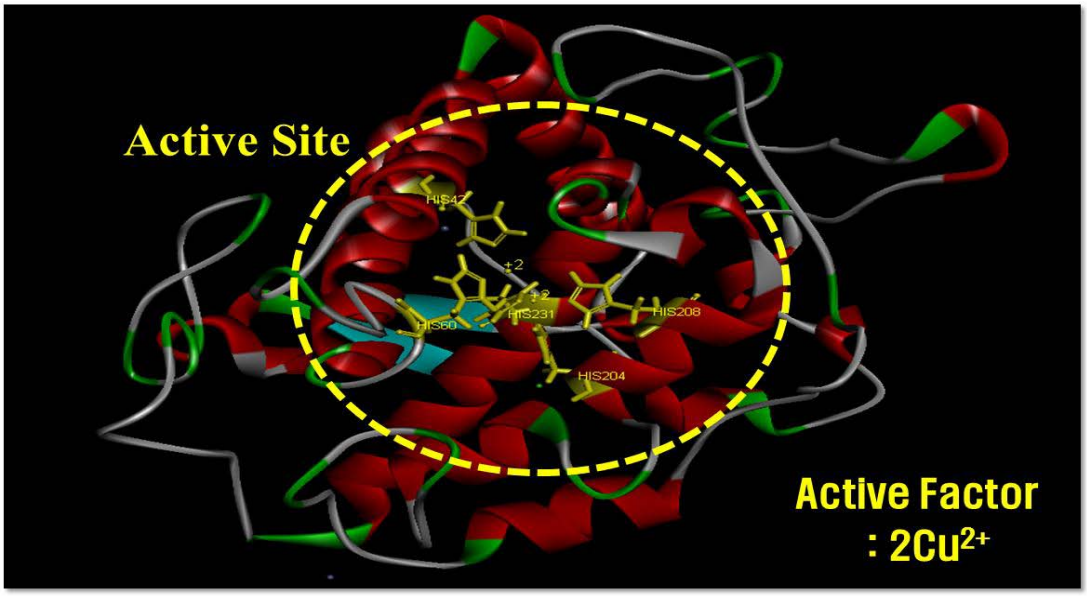

Fig. 1-7. Crystal structure of tyrosinase was obtained from Protein Data Bank (PDB: 3NM8).

4. RESULTS

4.1. Inhibitory activity of phlorotannins against mushroom tyrosinase

The inhibitory effect of phlorotannins against mushroom tyrosinase activity was examined by measuring the hydroxylaytion of L-tyrosine. Arbutin was selected as a positive control compound since its inhibitory activity was already reported [23]. As shown in Fig. 1-8, phlorotannins inhibited tyrosinase activity in a dose-dependent manner (phlroglucinol: 0.5 to 2 mM; eckol: 25 to 100 μM; Dieckol: 40 to 120 μM; octaphlorethol A: 12.5 to 50 μM). Especially, octaphlorethol A (OPA, IC_{50}: 17.2 μM) exhibited strong inhibition against mushroom tyrosinase when compared with phloroglucinol (PG, IC_{50}: 1.43 mM), eckol (EK, IC_{50}: 47.9 μM), dikol (DK, IC_{50}: 20.2 μM) and commercial whitening agent, arbutin (Table 1-2.).

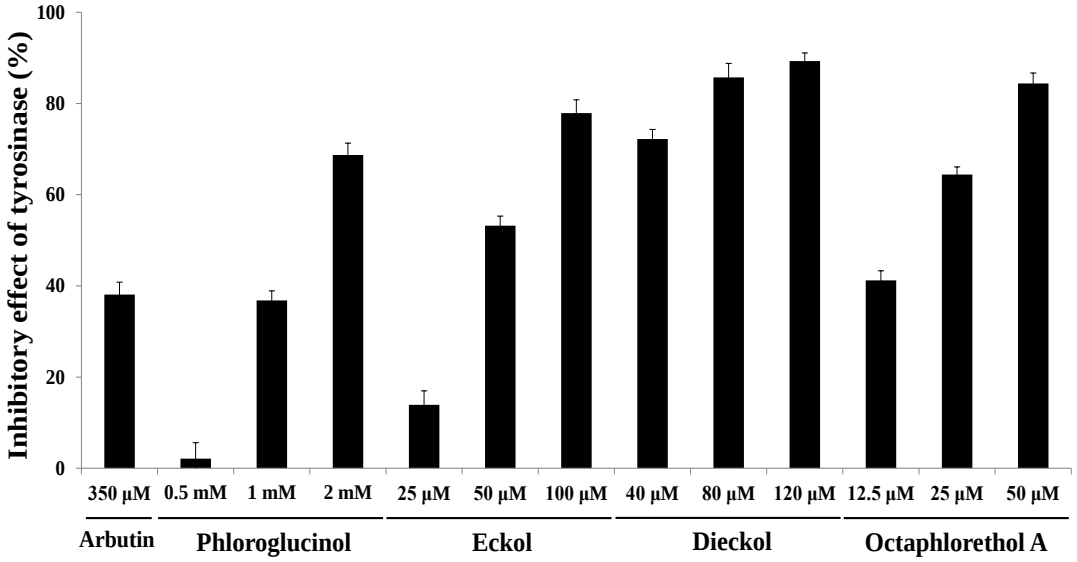

Fig. 1-8. Inhibitory effect of phlorotannins isolated from brown algae against mushroom tyrosinase. L-tyrosine was used as substrate, and arbutin was as positive control.

Table 1-2 Tyrosinase inhibitory activities (IC_{50}) of phlorotannins and arbutin.

Compounds	IC_{50} value
Arbutin	> 350 μM
Phloroglucinol	1.767 mM ± 0.39
Eckol	47.9 μM ± 0.12
Dieckol	20.2 μM ± 0.10
Octaphlorethol A	17.2 μM ± 0.09

*** IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the tyrosinase inhibitory activity. Values are ± SD of three determinations.**

3.3. Cytotoxicity of phlorotannins in B16F10 melanoma cells

An MTT assay was used to investigate if the inhibitor would adversely induce B16F10 melanoma cell death. The samples were treated with various concentrations (phlroglucinol: 0.5 to 2 mM; eckol: 25 to 100 μM; Dieckol: 40 to 120 μM; octaphlorethol A: 12.5 to 50 μM). Arbutin was used as a positive control (350 μM). α-MSH, which is known to stimulate melanogenesis, was used at 0.1 μM as the negative control, and its effect on melanogenesis was compared to that of the test sample. As shown in Fig. 1-9, cells treated with all testing concentrations of phlorotannins exhibited more than 95% of cell viability for up to 72 h. In addition, arbutin and α-MSH exhibited no cytotoxic effects on B16F10 melanoma cells [24].

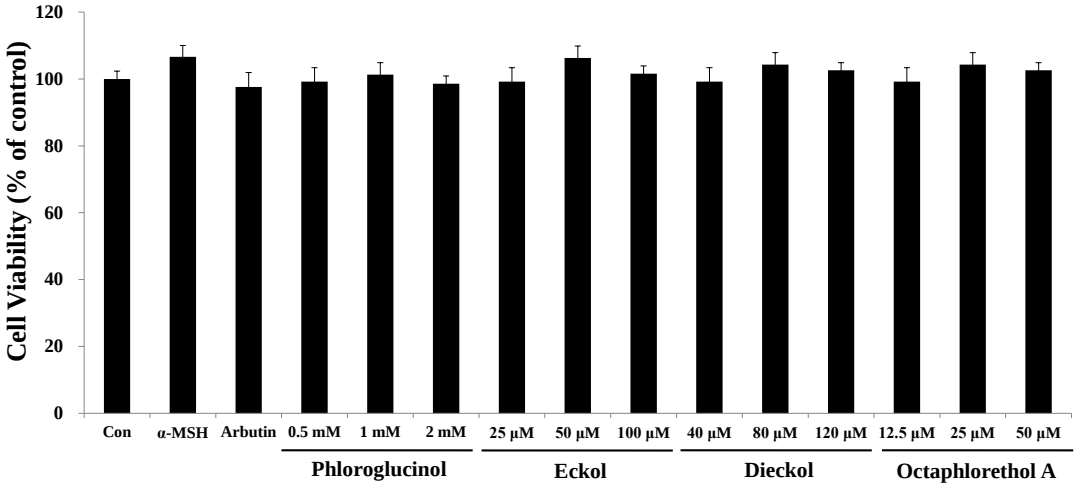

Fig. 1-9. B16F10 cell viability with various concentrations of dieckol. Arbutin was used as melanin and tyrosinase inhibitor at 350 μM and α-MSH was a melanin stimulator at 0.1 μM

3.4. Tyrosinase inhibitory activity of phlorotannins in B16F10 melanoma cells

We examined the effect of phlorotannins in reducing tyrosinase activity in α-MSH stimulated B16F10 melanoma cells. As shown in Fig. 1-10, we found that PG and EK had a similar inhibition effects on B16F10 melanoma cells as upon the mushroom tyrosinase. Upon exposure to 0.1 μM α-MSH alone, the tyrosinase activity of B16F10 cells was significantly increased compared to the controls. To determine the effect of PG and EK on cellular tyrosinase activity, the cells were then exposed to 0.1 μM α-MSH in the presence of PG (0.5 to 2 mM), EK (25 to 100 μM), DK (40 to 120 μM), and OPA (12.5 to 50 μM) or 350 μM arbutin, which are representative tyrosinase inhibitor. The cellular tyrsoinase activities of the OPA, DK, and EK-treated cells were significantly reduced blow the levels of the tyrosinase inhibitor, arbutin-treated cells, however, the PG-treated cells were similar or lower levels reduced tyrosinase activity of arbutin in a same dose.

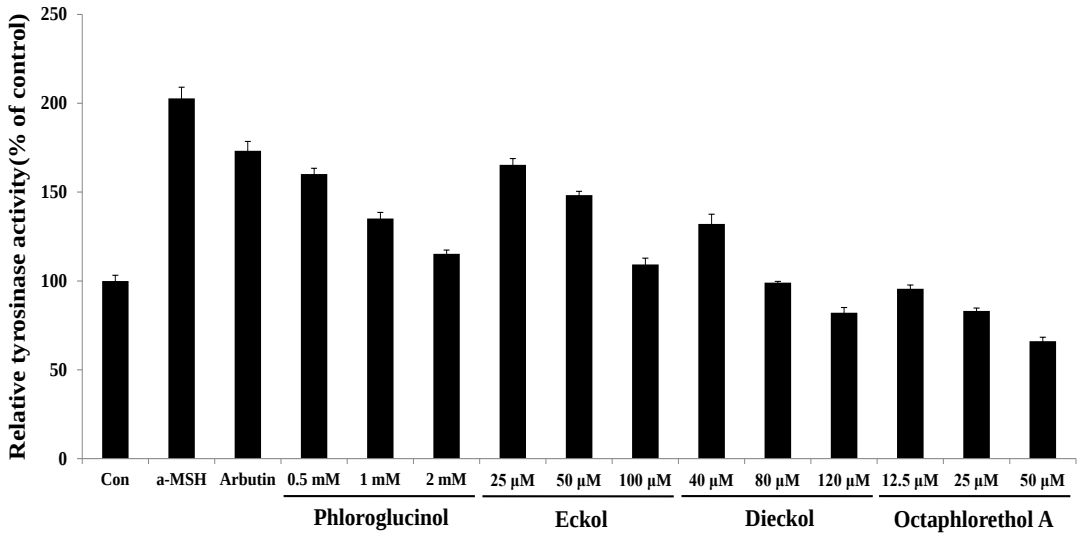

Fig. 1-10. Effect on phlorotannins on cellular tyrosinase activity in B16F10 cells. Cells were exposed to 0.1 μM α-MSH in the presence of different concentration of phlorotannins or 350 μM arbutin of tyrosinase inhibitor. Each percentage value for the treated cells is reported relative to that in the control cells.

3.5. Melanin synthesis of phlorotannins in B16F10 melanoma cells

Melanin formation is the most important factor to determine the mammalian skin color, the inhibition of melanin formation may result in a reduction of skin darkness. To evaluate the inhibitory potency of phlorotannins against melanin synthesis in melanoma B16F10 cells. As shown in Fig. 1-11, the effect of similar levels of phlorotannins and arbtin on melanin production in α-MSH stimulated B16F10 cells. The cellular melanin content of the PG and EK treated cells were significantly reduced below the levels of the arbutin-treated cells, consistent with phlorotannins suppression of melanin synthesis by B16F10 cells on inhibition of cellular tyrosinase activities.

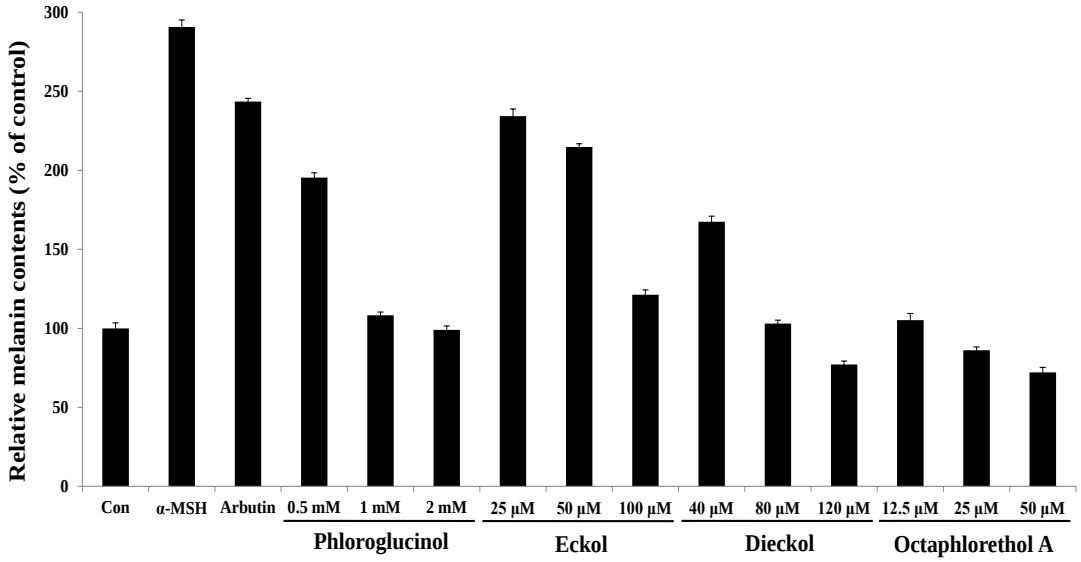

Fig. 1-11. Effect on phlorotannins on cellular melanin synthesis in B16F10 cells. Cells were exposed to 0.1 μM α-MSH in the presence of different concentration of phlorotannins or 350 μM arbutin of melanin inhibitor. Each percentage value for the treated cells is reported relative to that in the control cells.

3.6. In silico docking of tyrosinase

Tyrosinase is a copper-containing enzyme and is widely distributed in nature, and tyrosinase inhibitors that chelate copper or change the shape of the active site of the enzyme show competitive or non-competitive inhibition. Thus, the inhibition mechanism of phlorotannins such as dieckol might involve binding to the active site of mushroom tyrosinase.

We predicted the tertiary structure of tyrosinase and stimulated docking to phlorotannins (PG, Ek, DK, and OPA). Using Discovery Studio 3.0 (DS), we searched for tyrosinase residues that would bind to arbutin and phlorotannins. The docking of the tyrosinase-ligand complexes were well-performed with arbutin and phlorotannins stably posed in the pocket of the tyrosinase by DS 3.0 (Fig. 1-12, 13). As for arbutin and phlorotannins, the binding sites predicted by the 2D program of DS 3.0 (Fig. 1-14) were formed by the following residues:

Arbutin: His208 (pi interaction bond), Gly216 (hydrogen bond), and Asn205 (hydrogen bond).

Phloroglucinol: His208 (pi interaction bond), Gly216 (hydrogen bond), and Asn205 (hydrogen bond).

Eckol: His208 (pi interaction bond), Asn205 (hydrogen bond), and Arg209 (hydrogen bond).

Dieckol: His208 (pi interaction bond), Met215 (hydrogen bond), and Gly46 (hydrogen bond).

Octaphlorethol A: His208 (pi interaction bond), Arg209 (pi interaction bond), Asn205 (hydrogen bond), Glu195 (hydrogen bond), Arg209 (hydrogen bond), Glu141(hydrogen bond), and Gln142 (hydrogen bond).

Moreover, the docking analysis results indicated that the following highest docking binding energy and lowest total binding energy confirmation of the most proposed complex had to be taken into account when using the CDOCKER interaction energy program of DS 3.0: arbutin: 44.01 kcal/mol, phloroglucinol: 15.46 kcal/mol, eckol: 38.26 kcal/mol, dieckol: 70.71 kcal/mol, and octaphlorethol A: 78.36 kcal/mol, and in the calculate program of DS 3.0: arbutin: -111.69 kcal/mol and phloroglucinol: -101.99 kcal/mol, eckol: -115.84 kcal/mol, dieckol: -126.12 kcal/mol, and octaphlorethol A: 237.87 kcal/mol (Table 1-3). As shown in Table 1-3, the tyrosinase binding energy value of eckol, dieckol and octaphlorethol A were more stable and strong than that of phloroglucinol and positive control, arbutin.

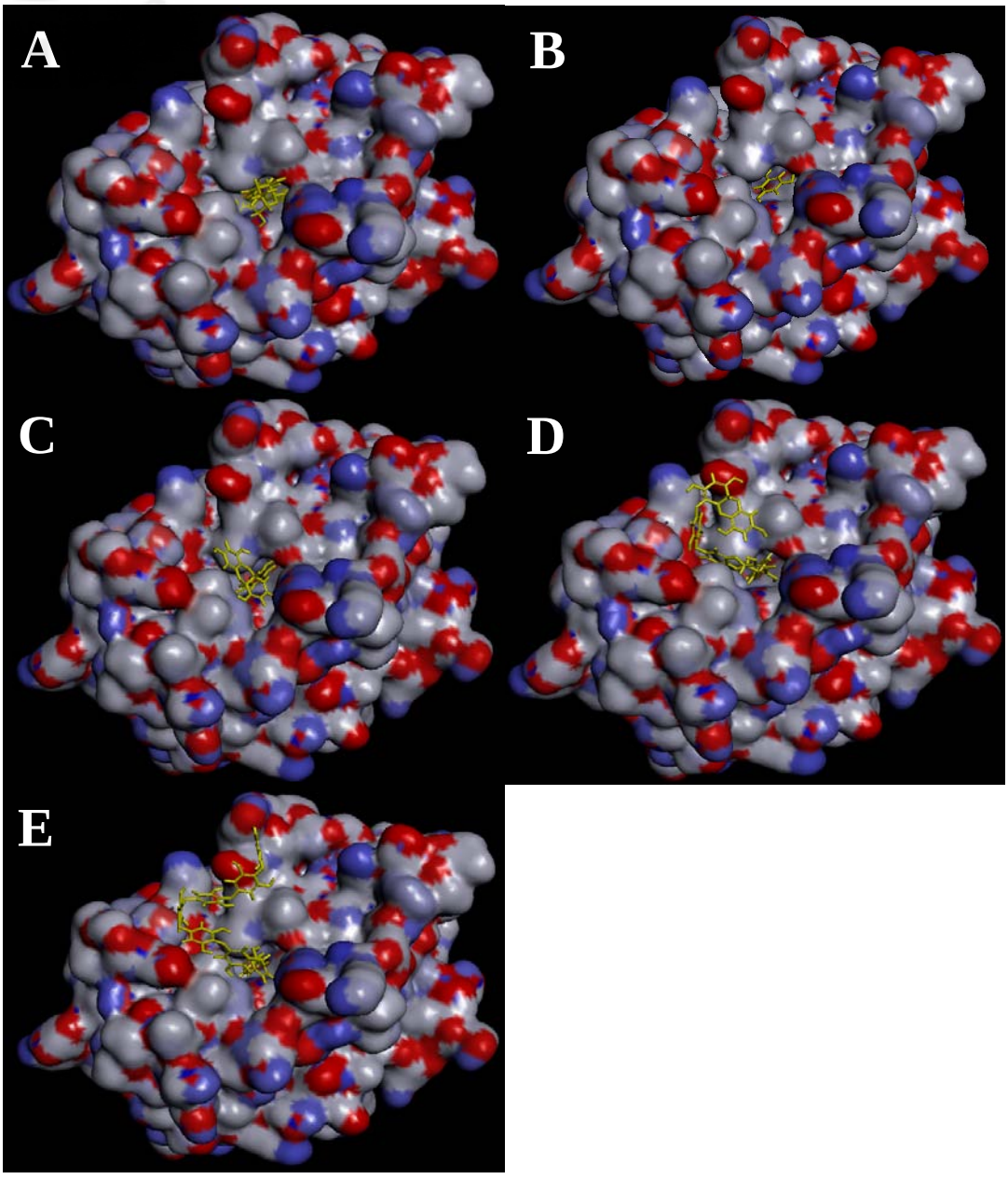

Fig. 1-12. Computational prediction of the structure for tyrosinase and docking simulation with arbutin and phlorotannins. Predicted 3D structure of mushroom tyrosinase 3NM8. Surface model (tyrodinase-ligand complex): A: arbutin, B: phloroglucinol, C: eckol, D: dieckol, E: octaphlorethol A.

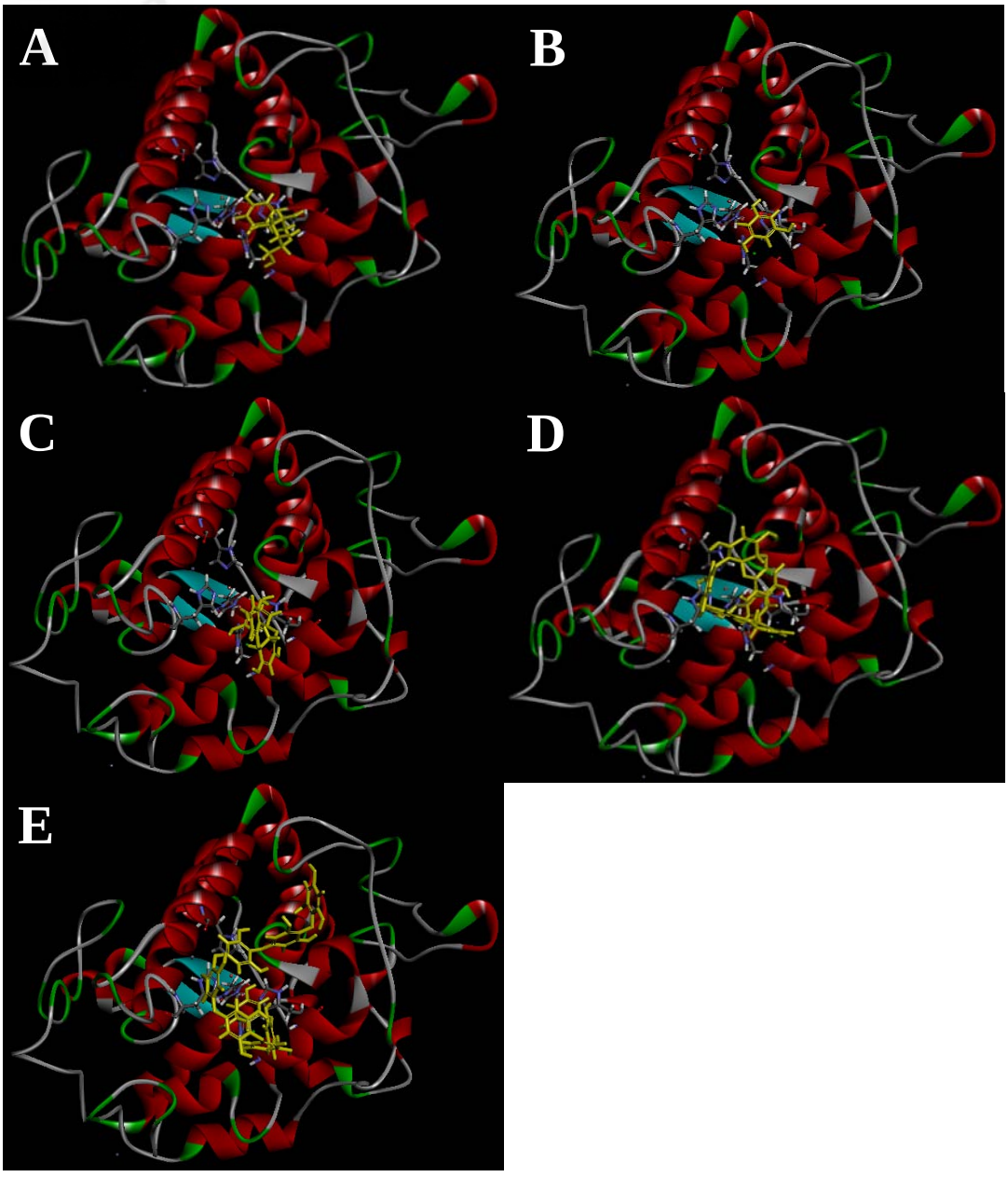

Fig. 1-13. Computational prediction of the structure for tyrosinase and docking simulation with arbutin and phlorotannins. Predicted 3D structure of mushroom tyrosinase 3NM8. Ribbon model (tyrodinase-ligand complex): A: arbutin, B: phloroglucinol, C: eckol, D: dieckol, E: octaphlorethol A.

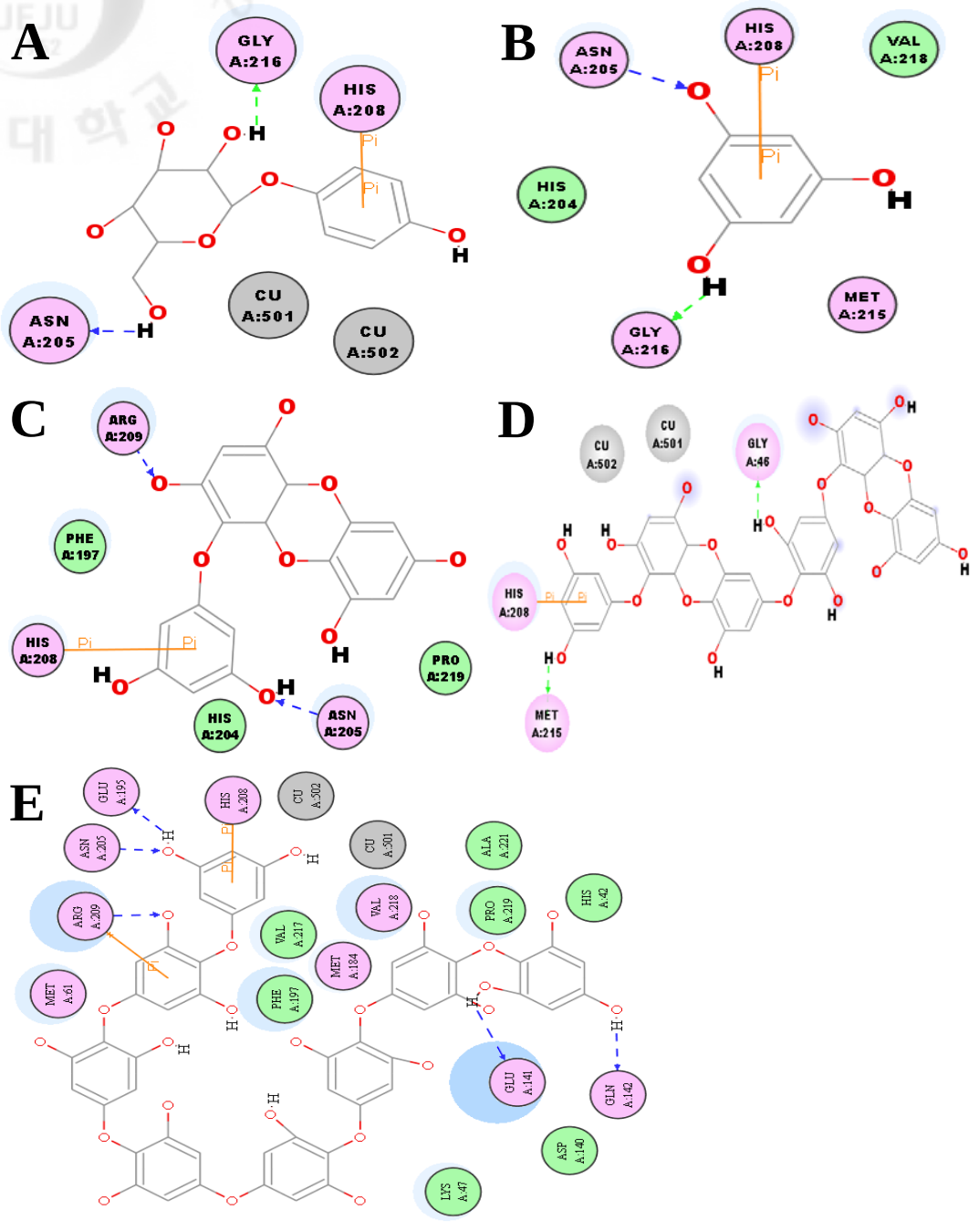

Fig. 1-14. Computational prediction of the structure for tyrosinase and docking simulation with arbutin and phlorotannins. Predicted 3D structure of mushroom tyrosinase 3NM8. 2D program (tyrodinase-ligand complex): A: arbutin, B: phloroglucinol, C: eckol, D: dieckol, E: octaphlorethol A.

Table 1-3 Results of docking experiments of arbutin and phlorotannins with the tyrosinase (PDB ID : 3NM8)

Ligand	Binding Energy (kcal/mol)	CDOCKER Interaction Energy (kcal/mol)
Arbutin	-111.69	44.01
Phloroglucinol	-101.99	15.46
Eckol	-115.84	38.26
Dieckol	-126.12	70.71
Octaphlorethol A	-237.87	78.36

4. DISCUSSION

Melanin pigmentation protects tissues from absorption and dissipation of UV light [25]. And epidermal melanin that is synthesized by melanocytes, resulting from a cascade of enzymatic reactions [26], is responsible for skin darkening. However, overproduction or dysregulation of melanin pigments can cause skin hyperpigmentation disorders such as freckles, melasma, senile lentigo, and age spots [27].

Tyrosinase has long been known to be essential for melanization. In vertebrates, tyrosinase, the enzyme responsible for the initial steps of melanin synthesis, is closely associated with specialized organelles called melanosomes that are found in melanocytes [28]. Although melanin levels vary in the human population, the expression of tyrosinase does not vary significantly among human skin colors, and levels of tyrosinase mRNA were found to be similar in cultured melanocyte systems collected from black and white skin [29].

The inhibition of tyrosinase has been the subject of numerous studies [30, 31], and several inhibitors are used as cosmetic additives and medicinal products in the treatment of hyperpigmentation. A recent ever-growing expansion of the global market demands more new products for depigmentation, cosmeceutical, and skin lighting purposes [32]. The most popular whitening agent is hydroquinone ever since its introduction in 1961, but its use has been curtailed as a cosmetic ingredient because of adverse cutaneous toxicity [30].

Additionally, several other phenolic compounds have been studied as depigmenting agents since their chemical structures are related to tyrosinase inhibitory activity. It has been suggested that the presence of a hydroxylic group and of an electron donator group in the phenol ring is a primary requirement for effective action as an alternative substrate of tyrosinase [31]. In our search for potent tyrosianase inhibitors, we found that phlorotannins has highly effective inhibitory activity against melanin synthesis, which is more potent than the commercial agent, arbutin.

In the present study, we investigated the effect of phlorotannins on tyrosianse inhibition in the anticipation of finding a new effective substance for skin whitening purposes and the prevention of hyperpigmentation, and found that phlorotannins clearly reduced tyrosinase activity in a dose-dependent manner.

To understand the mechanism underlying the interaction between tyrosinase and phlorotannins and to explore their binding mode, a docking study was performed using the CDOCKER function available in Discovery Studio 3.0. These docking studies yielded crucial information concerning the operation of the inhibitor in the binding pocket of tyrosinase. Ligand-enzyme interaction analysis found that the interaction binding residues of Eckol (His208, Asn205, and Arg209), dieckol (His208, Met215 and Gly46), and Octaphlorethol A (His208, Arg209, Asn205, Glu195, Arg209, Glu141, and Gln142) were

more important than those of arbutin (His208, Gly216 and Asn205) in the active site, and were the main contributors to the receptor-ligand interaction. It was observed that for better tyrosinase inhibitory activity, the three amino acid residues mentioned above are in close vicinity to the molecule. The docking simulation supported the non-competitive inhibition observed, as this type of inhibition is generally encountered when there are multiple possible binding sites for an inhibitor.

In conclusion, phlorotannins were identified as a non-competitive inhibitor of mushroom tyrosinase in molecular docking study. The lack of phlorotannins cytotoxicity indicates that the suppression of melanogenesis by phlorotannins can be attributed to the inhibition of murine tyrosinase. These results suggest that phlorotannins has strong de-pigmenting activity without discernible cytotoxicity in B16F10 melanoma cells. Phlorotannins are therefore a promising candidate for the development of safe pharmacological or cosmetic agents that have potent inhibitory effects against tyrosinase activity and melanin synthesis.

Part Ⅱ.

Molecular docking of phlorotannins isolated from brown algae and their effects on α-glucosidase inhibitory activity

Part Ⅱ.

Molecular docking of phlorotanins isolated from brown algae and their effects on α-glucosidase inhibitory activity

1. ABSTRACT

Diabetes mellitus is a most serious and chronic disease whose incidence rates are increasing with incidences of obesity and aging of the general population over the world. In this study, the strong potential α-glucosidase inhibitory effect of active compound isolated from brown algae, *Ecklonia cava* and *Ishige foliacea* were investigated. The active compounds were isolated by silica column chromatography and HPLC methods. New α-glucosidase inhibitors were identified as phloroglucinol, eckol, dieckol, and octaphlorethol A (OPA) by these MS data, ^{1}H and ^{13}C NMR analysis. We analyzed structure-activity relationship of phlorotannins using the crystal structure of human maltose-glucoamylase (PDB ID: 3CTT) by Discovery Studio 3.0. Among the phlorotannins, OPA and dieckol showed to inhibit α-glucosidase were more effective than commercial inhibitor, acarbose. These results suggest that phlorotannins has great potential to be further developed as a pharmaceutical and medicinal food.

2. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by a congenital (DM1) or acquired (DM2) inability to transport glucose from the bloodstream into the cells. Currently, an estimated 150 million people in the worldwide have diabetes and that this increase to 300 million by 2025 [1]. Globally, DM2 accounts for greater than 90% of the cases that prompts every effort in exploring for drug therapeutic agents to stem its progress [2]. Although several drugs for DM2 diabetes exist today, they have drawbacks such as liver toxicity and adverse gastrointestinal symptoms, thereby raising the symptoms and risk factors of heart disease [3].

One of the therapeutic approaches adopted thus far to ameliorate postprandial hyperglycemia involves the retardation of glucose absorption via the inhibition of carbohydrate-hydrolyzing enzymes, such as, α-glucosidase and α-amylase, in the digestive organs [4]. α-Glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) plays an important role in the control of blood glucose levels in the body and is the key enzyme catalyzing the final step in the digestive process of carbohydrates [5]. The powerful synthetic α-glucosidase inhibitors, such as acarbose, miglitol, and voglibose are used widely in clinics to regulate blood glucose levels of patients, although those agents may induce side effects such as flatulence, abdominal cramps, and diarrhea [6,7]. Therefore, a number of studies have been

conducted in the search for naturally derived α-glucosidase inhibitors that induce no deleterious side effects [8,9].

Marine algae are known to generate an abundance of bioactive compound with great potential in the pharmaceuticals, food, and biomedical industries. *Ecklonia cava* and *Ishige foliacea*, are a kind of edible brown algae (Phaeophyta) that has been used as food and medicine in South Korea. In addition, brown algae have a variety of compounds including carotenoid and phlorotannins which showed different biological activities [10,11]. In our continuing investigation of the phlorotannins isolated from *Ecklonia cava* and *Ishige foliacea*, active compound were chosen as the target compound for α -glucosidase inhibition activity.

In this study, we measured phlorotannins isolated from *Ecklonia cava* and *Ishige foliacea*, in an effort to identify a molecule that can inhibit in α -glucosidase activity.

3. MATERIAL AND METHODS

3.1. Materials

p-Nitrophenyl-α-D-glucopyranoside, dimethylsulfoxide, α-glucosidase, bovine serum albumin and sodium azide were purchased from sigma Chemical Co. (St. Louis, MO, USA). All chemicals and reagents used were of analytical and obtained from commercial sources.

3.2. Extraction and isolation of phlorotannins from *Ecklonia cava*

The brown alga, *E. cava* was collected along the coast of Jeju Island, Korea, between February and April 2009. The sample was washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water and maintained in a medical refrigerator at -20℃. Thereafter, the frozen sample was lyophilized and homogenized with a grinder prior to extraction. The powdered *E. cava* was extracted with 80% aqueous EtOH, and was evaporated under vacuum. The EtOH extract was then partitioned with EtOAc. The EtOAc extract was fractionated via silica column chromatography with stepwise elution of a $CHCl_3$–MeOH mixture (100:1–1:1) to generate separated active fractions. The combined active fraction was then further subjected to a Sephadex LH-20 column (GE Healthcare, USA) saturated with 80% MeOH, and finally

purified via reversed-phase HPLC (ThermoFisher Scientific, USA) using a Waters HPLC system equipped with a Waters 996 photodiode array detector and C18 column (J'sphere ODS-H80, 150 x 20 mm, 4 μm; YMC Co.) by stepwise elution with methanol–water gradient (UV range: 230 nm, flow rate: 0.8 ml/min). The purified compounds (phloroglucinol, eckol, and dieckol) were confirmed by comparing their LC/MS, ^{1}H NMR data to the literature report [12].

Phloroglucinol : LC/MS data (M+, m/z: 126 Calcd. For $C_6H_6O_3$). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.97 (3H, s, OH-1, 3, 5), 5.66 (3H, s, H-2, 4, 5).

Eckol : LC/MS data (M+, m/z: 372.0 Calcd. For $C_{18}H_{12}O_9$). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.54 (2H, s, OH-2, 7), 9.45 (1H, s, OH-4), 9.21 (2h, s, OH-2, 7) 9.16 (2H, s, OH-3', 5'), 6.14 (1H, s, H-3), 5.96 (1H, d, J = 2.8 Hz, H-6), 5.72 (2H, J = 1.7 Hz, H-4'), 5.78 (1H, d, J = 2.8 Hz, H-6), 5.72 (2H, J = 1.7 Hz, H-2-, 6').

Dieckol : LC/MS data (M+, m/z: 742.0 Calcd. For $C_{36}H_{22}O_{18}$). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.71(1H, s, OH-9), 9.61 (1H, s, OH-9''), 9.51 (1H, s, OH-4''), 9.46 (1H, s, OH-4), 9.36 (2H, s, OH-3'', 5''), 9.28 (1H, s, OH-2''), 9.23 (1H, s, OH-2), 9.22 (1H, s, OH-7''), 9.15 (2H, s, OH-3', 5') 6.17 (1H, s, H-3''), 6.14 (1H, s, H-3), 6.02 (1H, d, J = 2.7 Hz, H-8), 5.98 (1H, d, J = 2.7 Hz, H-8''), 5.95 (1H, s, H-2''', 6'''), 5.82 (1H, d, J = 2.7 Hz, H-6), 5.81 (1H,

d, J = 2.7Hz, H-6’’), 5.80 (1H, t, J = 2.0 Hz, H-4’), 5.78 (2H, d, J = 2.0 Hz, H-2’, 6’).

The purity of phlorotannins (Fig. 2-1, A) were >95%, based on the peak area of all components absorbed at each specific wavelength in HPLC analysis.

3.3. Extraction and isolation of phlorotannins from *Ishige foliacea*

The brown alga, *I. foliacea* was collected along the coast of Jeju Island, Korea, between March and June 2010. The powdered, *I. foliacea* was extracted with 80% aqueous EtOH, and was evaporated under vacuum. The EtOH extract was then partitioned with hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc) and water. Since the EtOAc fraction contained the major compound than those of other fractions, the major compound was extracted from EtOAc fraction using a silica column chromatography and finally purified via reversed-phase HPLC (ThermoFisher Scientific, USA) using a Waters HPLC system equipped with a Waters 996 photodiode array detector and C18 column (J’sphere ODS-H80, 150 x 20 mm, 4 μm; YMC Co.) by stepwise elution with methanol–water gradient (UV wavelength: 230 nm, flow rate: 0.8 ml/min).

Octaphlorethol A: It was isolated as light yellowish powder. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 5.57 (d, J =2.8 Hz , H-4), 5.58 (d, J =2.8 Hz , H-6), 5.59 (d, J =2.8 Hz , H-9), 5.59 (d, J =2.8 Hz, H-11), 5.68 (d, J =1.8 Hz, H-15), 5.68 (d, J =1.8 Hz, H-17), 5.71 (d, J

=1.8 Hz, H-21), 5.72 (d, *J* =1.8 Hz, H-23), 5.85 (d, *J* =1.6 Hz, H-26), 5.84 (d, *J* =1.8 Hz, H-30), 5.94 (d, *J* =1.8 Hz, H-32), 5.94 (d, *J* =1.8 Hz, H-36) , 6.15 (d, *J* =1.8 Hz, H-38) , 6.15 (d, *J* =1.8 Hz, H-42) , 6.16 (d, *J* =1.6 Hz, H-44) , 6.01 (d, *J* =1.6 Hz, H-46) , 6.16 (d, *J* =1.6 Hz, H-48) , 9.02 (s, OH-1,3) , 9.04 (s, OH-5, 27, 29, 33,35) , 8.98 (s, OH-8,12) , 8.93 (s, OH-14, 18) , 8.92 (s, OH-20,24) , 9.06 (s, OH-39,41) , 9.07 (s, OH-45, 47) ; ^{13}C NMR (DMSO-d_6, 100 MHz) δ_c 153.0 (s, C-1), 122.0 (s, C-2), 153.0 (s, C-3), 94.9 (d, C-4), 156.1 (s, C-5), 94.9 (d, C-6), 122.0 (s, C-7), 152.9 (s, C-8), 122.0 (s, C-9), 151.1 (s, C-10), 94.7 (d, C-11), 152.9 (s, C-12), 122.0 (s, C-13), 151.1 (s, C-14), 94.7 (d, C-15), 154.5 (s, C-16), 94.7 (d, C-17), 151.1 (s, C-18), 123.4 (s, C-19), 151.1 (s, C-20), 94.7 (d, C-21), 154.1 (s, C-22), 94.7 (d, C-23), 151.1 (s, C-24), 154.1 (s, C-25), 94.7 (d, C-26), 151.1 (s, C-27), 123.4 (s, C-28), 151.1 (s, C-29), 94.7 (d, C-30), 154.0(s, C-31), 94.1 (d, C-32), 150.8 (s, C-33), 123.5 (s, C-34), 150.8 (s, C-35), 94.2 (d, C-36), 156.2 (s, C-37), 94.1 (d, C-38), 152.7 (s, C-39), 123.5 (s, C-40), 152.7 (s, C-41), 94.1 (d, C-42), 161.0 (s, C-43), 94.7 (d, C-44), 158.6 (s, C-45), 94.0 (d, C-46), 161.0 (s, C-47), 94.7 (d, C-47). ESI-MS showed the molecular formular as $C_{48}H_{34}O_{25}$ at *m/z* 992.19 $[M-2H]^{2-}$.

The structure of octaphlorethol A (OPA) (Fig. 2-1, B) was determined by LC/MS data and ^{1}H and ^{13}C NMR analysis (Part I.). The purity of OPA was >95%, based on the peak area of all components absorbed at each specific wavelength in HPLC analysis.

Fig. 2-1. Chemical structure of phlorotannins isolated from *Ecklonia cava* and *Ishige f oliacea*. (A): Phloroglucinol, Eckol, and Dieckol; (B): Octaphlorethol A.

3.4. Inhibitory effect of phlorotannins on α –glucosidase

The α-glucosidase inhibitory assay was done by the chromogenic method described by Watanabe et al., [13] using a readily available yeast enzyme. Briefly, yeast α-glucosidase (0.7 U, sigma) was dissolved in 100 mM phosphate buffer (pH 7.0) containing 2 g/l bovine serum albumin and 0.2 g/l NaN_3 and used as an enzyme solution. 5 nM p-Nitrophenyl-α-D-glucopyranoside in the same buffer (pH 7.0) was used as a substrate solution. The 50 μl of enzyme solution and 10 μl of sample dissolved in dimethylsulfoxide were mixed in a micro plate and measured absorbance at 405 nm at zero time. After incubation for 5 min, substrate solution (50 μl) was added and incubated for another 5 min at room temperature. The increase in the absorbance from zero time was measured. Percent inhibitory activity was expressed as 100 minus relative absorbance difference (%) of test compounds to absorbance change of the control where test solution was replaced by carrier solvent.

3.5. In silico docking of α-glucosidase and new inhibitor candidate

Molecular docking is an application wherein molecular modeling techniques are used to predict how a protein (enzyme) interacts with small molecules (ligands) [14,15] The ability of a protein to interact with small molecules plays a major role in the dynamics of that protein, which may enhance or inhibit its biological function. In the present study, we

performed docking of phlorotannins into the active site of the α-glucosidase. The crystal structure of α-glucosidase (PDB: 3CTT) was obtained from the Protein Data Bank (PDB, http://www.pdb.org) (Fig. 2-2). We performed the docking studies using CDOCKER in Accelrys Discovery Studio 3.0 (Accelrys, Inc) (Fig. 2-3). We describe the ligand structure of the α-glucosidase inhibitor candidate in Fig. 3-1. To prepare for the docking procedure, we performed the following steps: (1) conversion of the 2D structure into 3D structure; (2) calculation of charges; and (3) addition of hydrogen atoms using the CDOCKER docking program.

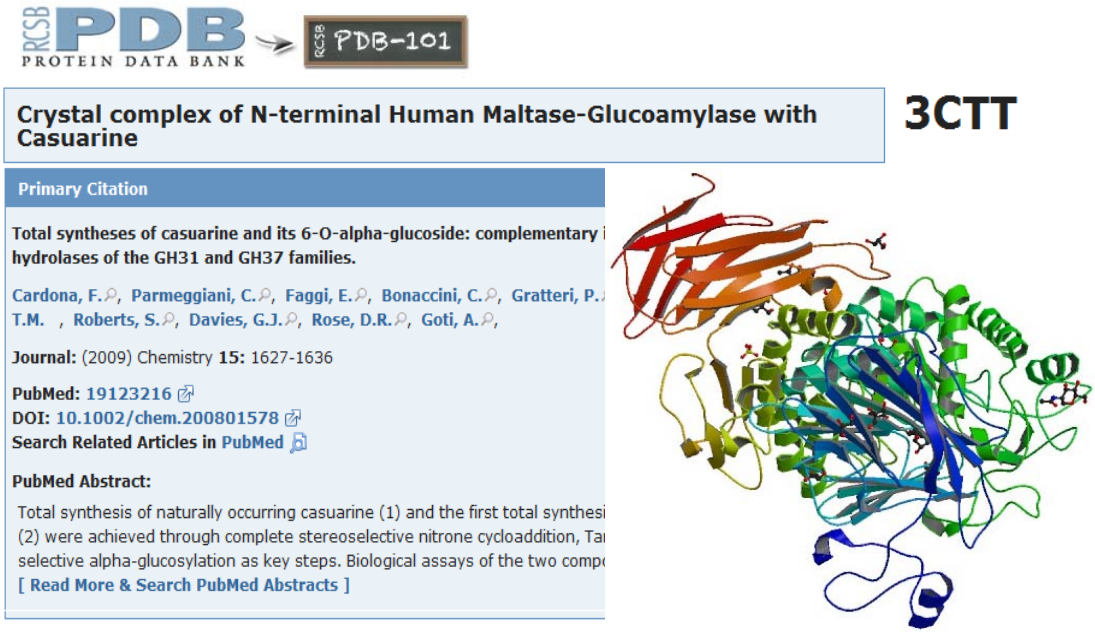

Fig. 2-2. α-Glucosidase was obtained from Protein Data Bank (PDB ID: 3CTT).

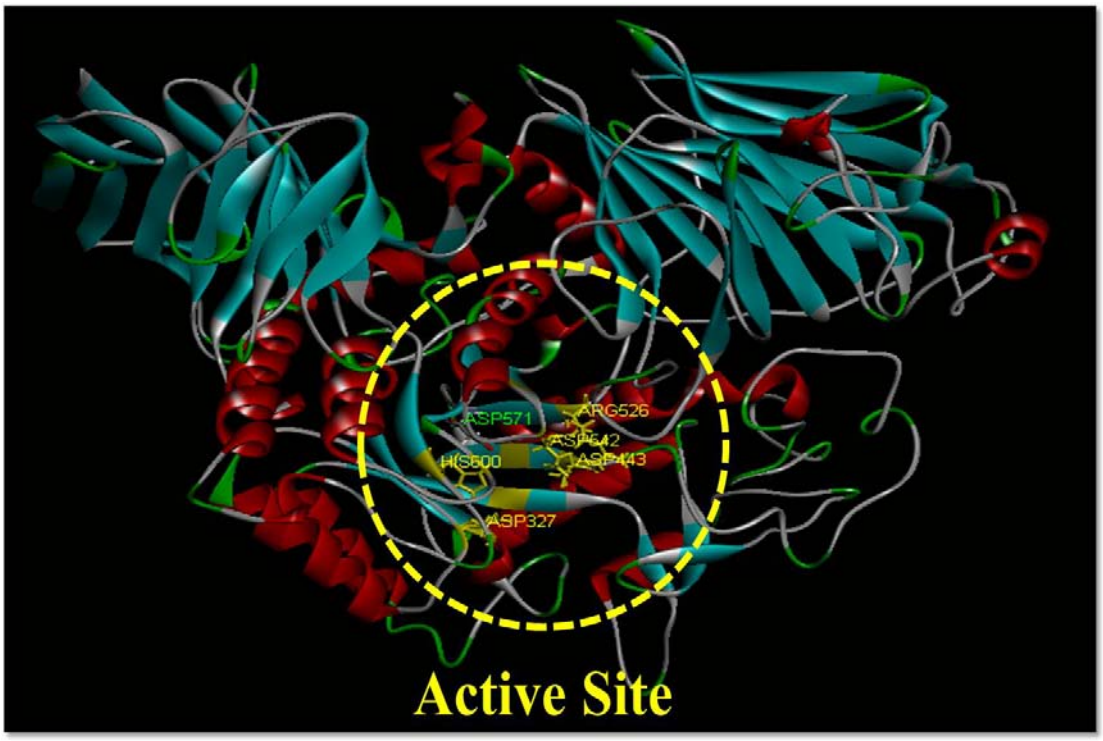

Fig. 2-3. Crystal structure of α-glucosidase was obtained from Protein Data Bank (PDB ID: 3CTT).

4. RESULTS

4.1. Inhibitory activities of phlorotannins against α-glucosidase

The inhibitory effect of phlorotannins against α-glucosidase was determined using p-Nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate. As shown in Fig. 2-4, phlorotannins inhibited α-glucosidase activity in a dose-dependent manner (phlroglucinol: 500 to 1,000 μM; eckol: 250 to 750 μM; Dieckol: 125 to 750 μM; octaphlorethol A: 62.5 to 250 μM). Moreover, the OPA and DK evidenced more effective than that of commercial inhibitor, acarbose even at low concentration (750 μM). IC_{50} values of OPA and DK against α-glucosidase were 110.9 μM and 240 μM, respectively, which was evidenced stronger inhibitory effect than that of acarbose (Table 2-1).

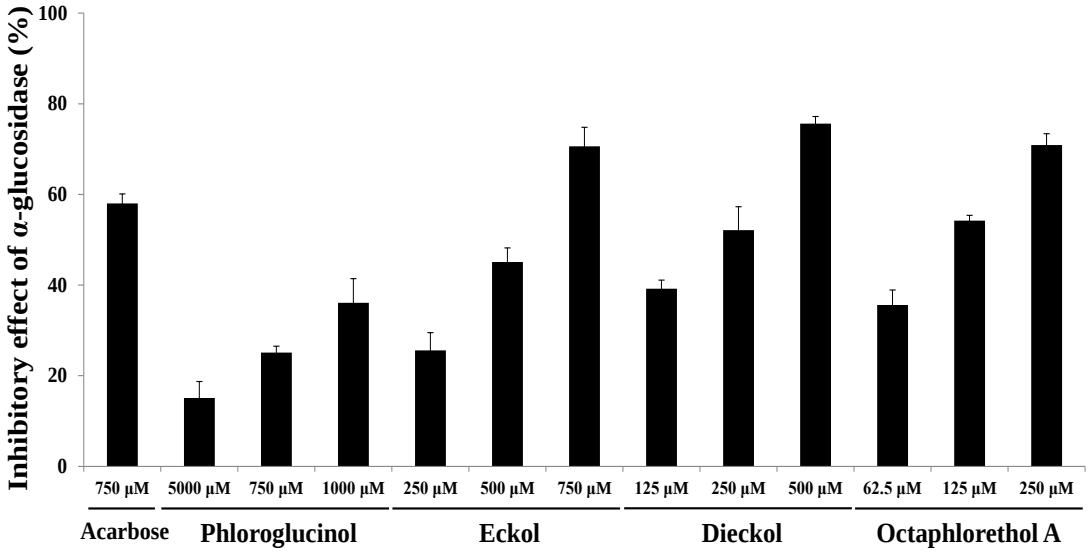

Fig. 2-4. Inhibitory effect of phlorotannins isolated from brown algae against α-glucosidase. Inhibitory effects were determined using pNPG and pNPM as substrates, respectively, and acarbose was employed as a positive control.

Table 2-1 α-Glucosidase inhibitory activities (IC_{50}) of phlorotannins and acarbose.

Compounds	IC_{50} value
Acarbose	290 μM ± 0.31
Phloroglucinol	> 1 mM
Eckol	540 μM ± 0.29
Dieckol	240 μM ± 0.22
Octaphlorethol A	110 μM ± 0.26

*** IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the α-glucosidase inhibitory activity. Values are ± SD of three determinations.**

4.2. In silico docking of α-glucosidase

Docking studies were performed to gain insight into the most probable binding conformation of phlorotannins and to compare with the other known commercial inhibitor, acarbose. α-Glucosidase inhibitors, phlorotannins and acarbose were used for the docking experiments and then for the comparison. The docking of the α-glucosidase-ligand complexes were well performed with acarbose and phlorotannins stably posed in the pocket of the α-glucosidase by DS 3.0 (Fig. 2-5, 6). As for phlorotannins and acarbose, the binding sites predicted by 2D program of DS 3.0 (Fig. 2-7) were formed by residues:

Acarbose: Asn207 (hydrogen bond), Thr205 (hydrogen bond), Asp203 (hydrogen bond), Asp542 (hydrogen bond), Arg526 (hydrogen bond), and Asp443 (hydrogen bond).

Phloroglucinol: Arg526 (hydrogen bond), Asp542 (hydrogen bond), and Asp203 (hydrogen bond).

Eckol: Tyr605 (hydrogen bond), Asp203 (hydrogen bond), Arg526 (hydrogen bond), and Thr205 (hydrogen bond).

Dieckol: Asp443 (hydrogen bond), Arg526 (hydrogen bond), Asn209 (hydrogen bond), and Asn207 (hydrogen bond).

Octaphlorethol A: Lys480 (pi interaction bond), Phe450 (pi interaction bond), Phe575 (pi interaction bond), Arg202 (hydrogen bond), Asp203 (hydrogen bond), Ser448 (hydrogen

bond), Tyr605 (hydrogen bond), Asp542 (hydrogen bond), Arg526 (hydrogen bond), Met444 (hydrogen bond), and His600 (hydrogen bond).

The CDOCKER interaction and binding energy (kacl/mol) evaluated from Discovery Studio 3.0 for phlorotannins and acarbose are CDOCERer interaction energy: Acarbose: 61.20 kcal/mol, phloroglucinol: 18.22 kcal/mol, eckol: 39.26 kcal/mol, dieckol: 54.25 kcal/mol, and octaphlorethol A: 80.97 kcal/mol, and binding energy: Acarbose: -137.29 kcal/mol, phloroglucinol: -64.3 kcal/mol, eckol: -85.4 kcal/mol, dieckol: -129.0 kcal/mol, and octaphlorethol A: -140.98 kcal/mol, respectively (Table 2-2.). OPA showed a higher CDOCKER interaction and binding energy, very likely due to the high number of hydroxyl groups as well as hydrophilic interactions. As OPA resembles the substrate may bind selectively with the active site residues. The binding mode analysis of the OPA with the active site residues provided important information of catalytic site.

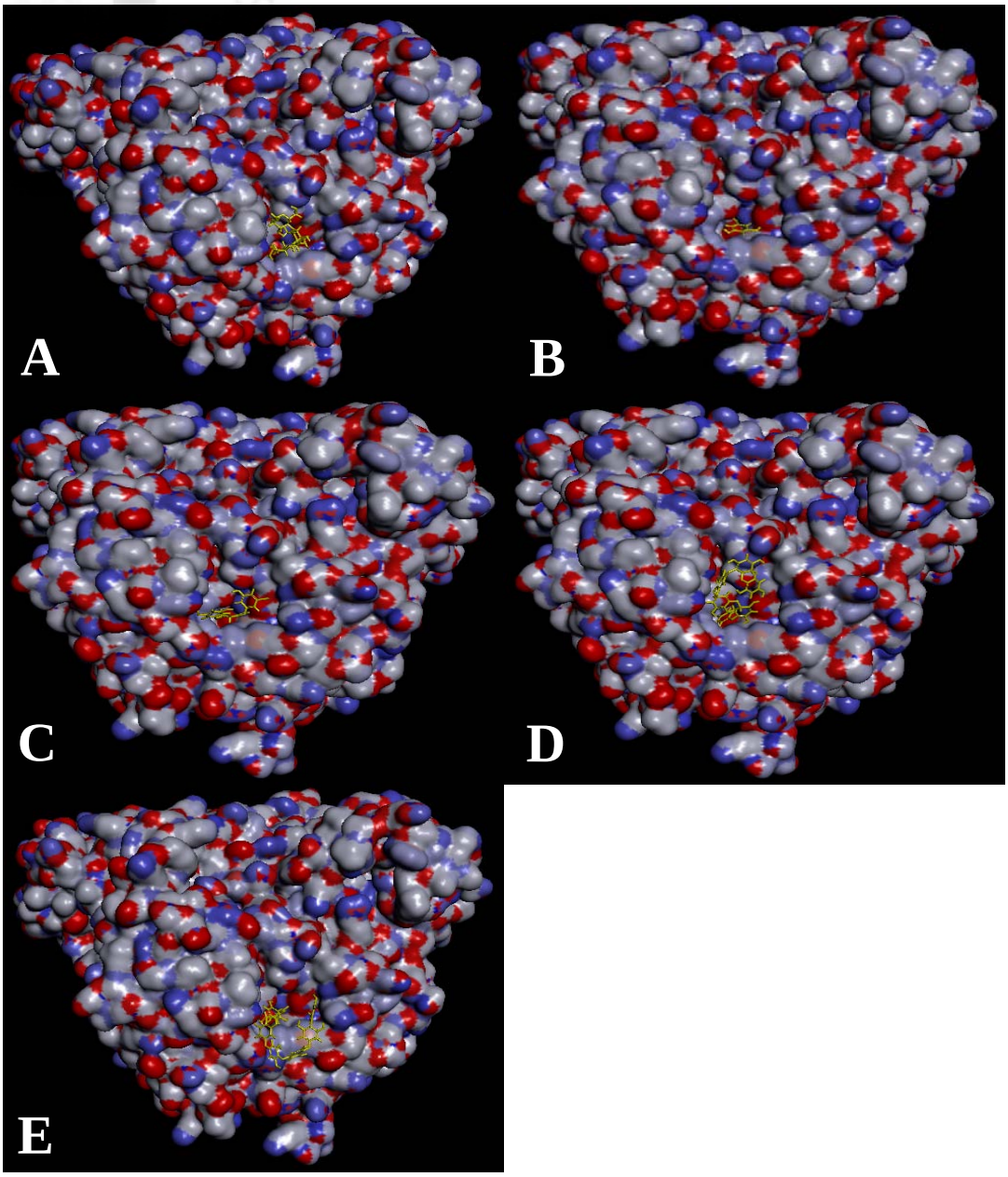

Fig. 2-5. Computational prediction of the structure for α-glucosidase and docking simulation with Acarbose and phlorotannins. Predicted 3D structure of α-glucosidase 3CTT. Surface model (α-glucosidase-ligand complex): A: Acarbose, B: phloroglucinol, C: eckol, D: dieckol, E: octaphlorethol A.

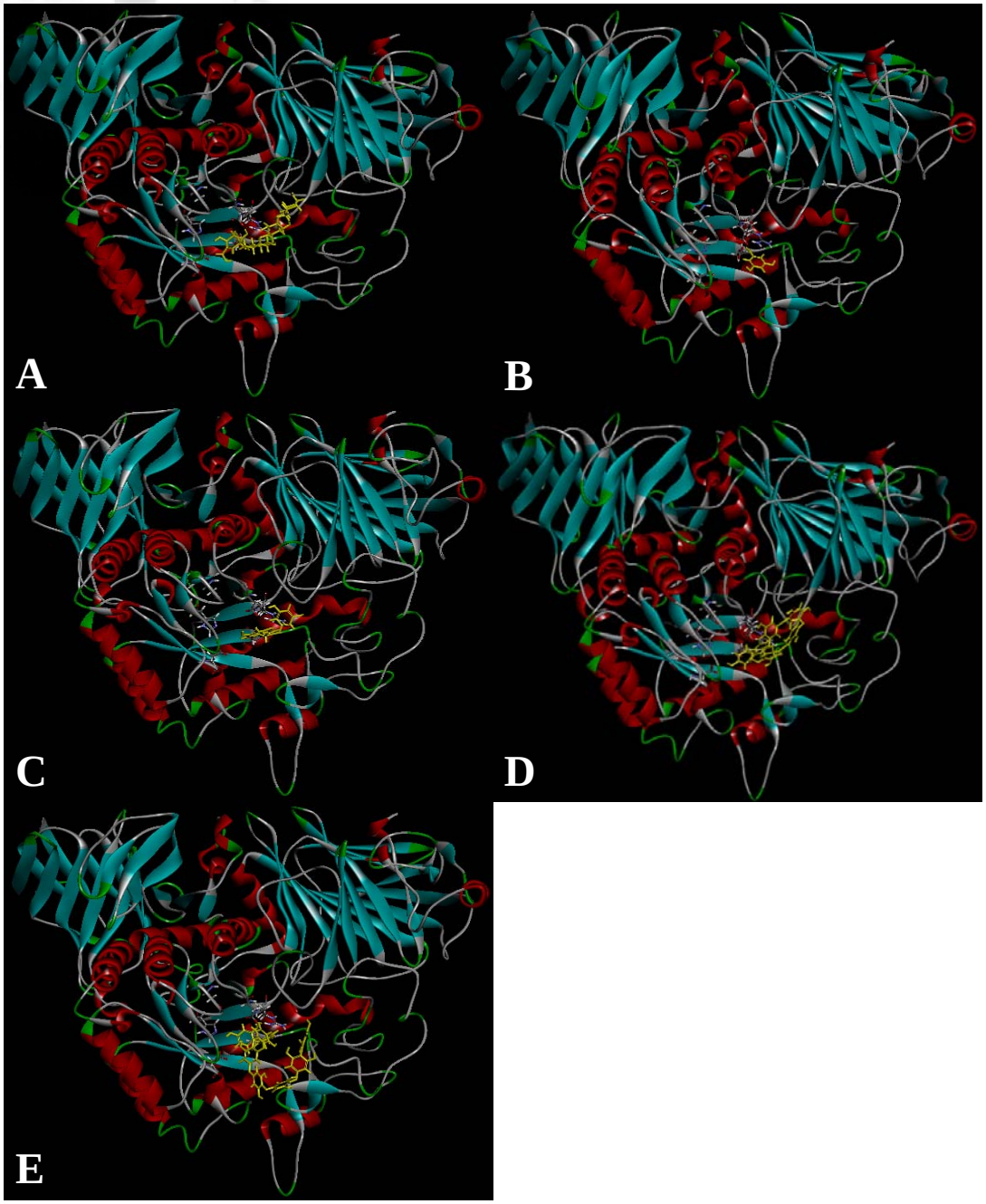

Fig. 2-6. Computational prediction of the structure for α-glucosidase and docking simulation with Acarbose and phlorotannins. Predicted 3D structure of α-glucosidase 3CTT. Ribbon model (α-glucosidase-ligand complex): A: Acarbose, B: phloroglucinol, C: eckol, D: dieckol, E: octaphlorethol A.

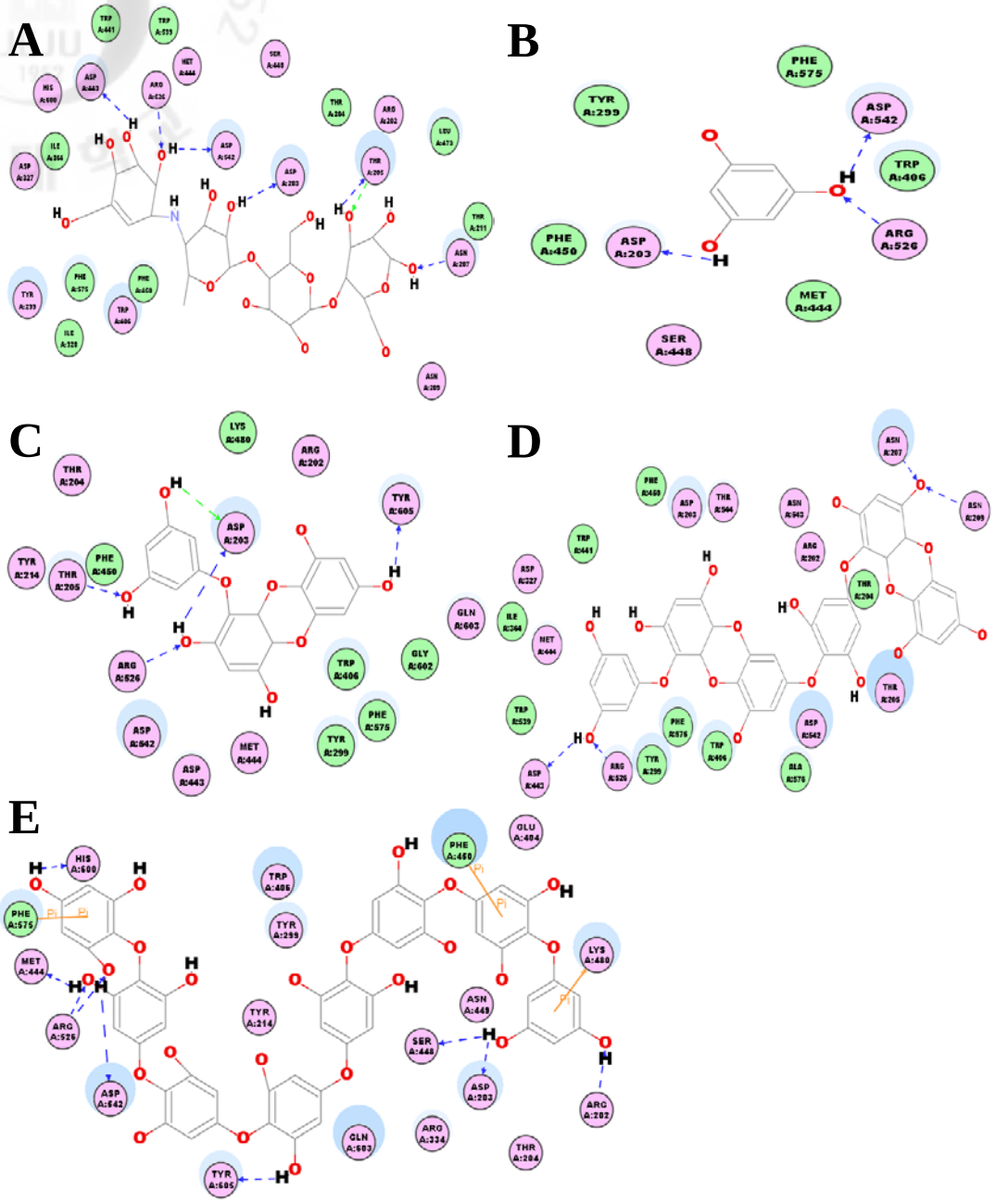

Fig. 2-7. Computational prediction of the structure for α-glucosidase and docking simulation with Acarbose and phlorotannins. Predicted 3D structure of α-glucosidase 3CTT. 2D program (α-glucosidase-ligand complex): A: Acarbose, B: phloroglucinol, C: eckol, D: dieckol, E: octaphlorethol A.

Table 2-2 Results of docking experiments of acarbose and phlorotannins with the α-glucosidase (PDB ID : 3CTT).

Ligand	Binding Energy (kcal/mol)	CDOCKER Interaction Energy (kcal/mol)
Acarbose	-137.29	61.20
Phloroglucinol	-64.3	18.22
Eckol	-85.4	39.26
Dieckol	-129.0	54.25
Octaphlorethol A	-140.98	80.97

5. DISCUSSION

Postprandial hyperglycemia is a primary risk factor in the development of Type 2 diabetes. α-Glucosidase inhibitors that reduce postprandial hyperglycemia has a key role in the treatment of Type 2 pre-diabetic states and also have the potential to reduce the progression of diabetes [16]. Several α-glucosidase inhibitors, including acarbose, vogilbose, a miglitol, are currently being used [17]. The inhibition of their activity in the human digestive tract is regarded as an effective method for the control of diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes. However, α-glucosidase inhibitors exhibit side effects such as abdominal discomfort, bloating, flatulence, and diarrhea. Thus, several natural resources have been investigated with respect for suppression of glucose production from carbohydrate in the gut or glucose absorption from the intestine [8]. Therefore, effective and nontoxic inhibitors of α-gucosidase have long been sought.

In this study, the active compounds, phloroglucinol, eckol, dieckol, and octaphlorethol A (OPA) were isolated from *Ecklonia cava* and *Ishige foliacea* and evaluated the inhibitory effects of phlortannin against α-glucosidase to elucidate the possible use of phlorotannins as an anti-hyperglycemic agent. Among the phlorotannins, dieckol and octaphlorethol A exhibited stronger inhibitory activity against α-gucosidase than that of the commercial

carbohydrate digestive enzyme inhibitor, acarbose.

Since various phenolic compounds have been generally accepted as anti-oxidant agents, it has been shown that the activity of α-glucosidase is effectively inhibited by flavonoids, such as naringenin, kaempferol, luteolin, apigenin and epigallocatechin gallate [18], indicating that polyphenolic compounds are able to inhibit the activities of carbohydrate digestive enzymes, due to their ability to bind with proteins [19]. Moreover, the results of several studies have been demonstrated that the hydroxyl groups in polyphenolic compounds may, therefore, have an important role in promoting inhibitory activity [20].

To understand the mechanism underlying the interaction between α-glucosidase and phlorotannins, and to explore their binding mode, a docking study was performed using the CDOCKER function available in Discoevery studio 3.0. These docking studies yielded crucial information concerning the operation of the inhibitors in the binding pocket of α-glucosidase. Ligand-enzyme interaction analysis shows that Asp542, Arg526, Asp443, Asp327, and His600 are the most important residue present at the active site and are the main contributors to the receptor-ligand interaction. It has been observed that, for better α-glucosidase inhibitory activity, three amino acid residues mentioned above are in close vicinity to the molecule.

In conclusion, the α-glucosidase inhibitory compound isolated from brown algae has been

identified as dieckol and octaphlorethol A. This is the first report of α-glucosidase inhibitory new compound, OPA isolated from *I. foliacea*. Phlorotannins has prominent inhibitory effect against this enzyme that may provide a way to regulate the carbon source, such as starch, in the fermentation process. Phlorotannins aer promising candidates for the development of safe pharmacological or functional food for diabetes, and also can be applied in other therapeutic fields.

Part Ⅲ.

Molecular docking of phlorotannins isolatd from brown algae and their effects on angiotensin-I converting enzyme (ACE) inhibitory activities

Part Ⅲ.

Molecular docking of phlorotannins isolated from brown algae and their effects on angiotensin-I converting enzyme inhibitory activities

1. ABSTRACT

Inhibition of angiotensin I converting enzyme (ACE) activity is the most common mechanism underlying the lowering of blood pressure. In this study, the strong potential angiotensin I converting enzyme (ACE) inhibitory effect of active compounds isolated from brown algae, *Ecklonia cava* and *Ishige foliacea* were investigated. The active compounds were isolated by silica column chromatography and HPLC methods. New ACE inhibitors were identified as phloroglucinol, eckol, dieckol, and octaphlorethol A (OPA) by these MS data, ^{1}H and ^{13}C NMR analysis. We analyzed structure-activity relationship of phlorotannins using the crystal structure of human maltose-glucoamylase (PDB ID: 1O86) by Discovery Studio 3.0. These results suggest that phlorotannins has great potential to be further developed as a pharmaceutical and medicinal food.

2. INTRODUCTION

Hypertension is one of the major risk factors for the development of cardiovascular diseases, including stroke, and may also play a role in the development of vascular cognitive impairment and vascular dementia [1,2]. Angiotensin I converting enzyme (EC 3.4.15.1; ACE), which plays an important role in the rennin-angiotensin system, is a carboxyl-terminal dipeptidyl exopeptidase that catalyzes the conversion of angiotensin I to angiotensin II [3-6]. Specially, ACE converts an inactive form of angiotensin I, a decapeptide, to the potent vasoconstrictor angiotensin II, an octapeptide, to the potent. In addition, since ACE is a multifunctional enzyme, it also catalyzes the degradation of bradykinin, which is a known vasodilator [4,7]. Therefore, inhibition of ACE activity leads to a decrease in the concentration of angiotensin II and increase the level of bradykinin, resulting in reduced blood pressure [8]. ACE inhibitor has been used extensively in therapeutic strategies for the prevention and treatment of hypertension, and the literature regarding ACE inhibitory compounds is also rather extensive. Since the discovery of an ACE inhibitor in snake venom, several synthetic ACE inhibitors have been developmed, including alacepril, captopril, benazepril, fosinopril, ramipril, and zofenopril, all of which are currently extensively used in the treatment of essential hypertension and heart failure in humans. However, these synthetic ACE inhibitors are belived to exert certain side effects, including cough, taste disturbances,

and skin rashes. Therefore, the development of ACE inhibitors from natural products has become a major area of research.

Marine organisms are a rich source of structurally novel and biologically active metabolites. Secondary or primary metabolites produced by these organisms may potentially be bioactive compounds of interest in the food and medicinal industries. The worldwide demand is growing for seaweeds as useful resource for food ingredients and processed foods. Therefore, algae are a very interesting natural source of new compounds with biological activities that could be used as functional ingredients. *Ecklonia cava* and *Ishige foliacea,* are a kind of edible brown algae (Phaeophyta) that has been used as food and medicine in South Korea. In addition, brown algae have a variety of compounds including carotenoid and phlorotannins which showed different biological activities. In our continuing investigation of the phlorotannins isolated from *Ecklonia cava* and *Ishige foliacea,* active compound were chosen as the target compound for angiotensin I converting enzyme inhibition activity.

In this study, we measured phlorotannins isolated from *Ecklonia cava* and *Ishige foliacea*, in an effort to identify a molecule that can inhibit in angiotensin I converting enzyme activity.

3. MATERIAL AND METHODS

3.1. Materials

Angiotesin I converting enzyme (from rabbit lung) (ACE) and N-Hippuryl-His-Leu tetrahydrate (HHL) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All chemicals and reagents used were of analytical and obtained from commercial sources.

3.2. Extraction and isolation of phlorotannins from brown algae

Phlorotannins, phloroglucinol, eckol, dieckol, and octaphlorethol A isolated as previously described (Part I, II, and III). Brieflym the dried algae were extracted three times with 80% EtOH and filtered. The filterates were then evaporated at 40 ℃ to obtain the EtOH extract, which were suspended on distilled water, and partitioned with ethyl acetate. The ethyl acetate fraction was subjected to silica gel and sephadex LH-20 coumn chromatography. The phlorotannins were finally purified by HPLC, and the structure of phlrotannin was identified by comparing the NMR spectral data with those in existing literature.

Phloroglucinol : LC/MS data (M+, m/z: 126 Calcd. For $C_6H_6O_3$). ^1H NMR (400 MHz, DMSO-d6) δ 8.97 (3H, s, OH-1, 3, 5), 5.66 (3H, s, H-2, 4, 5).

Eckol : LC/MS data (M+, m/z: 372.0 Calcd. For $C_{18}H_{12}O_9$). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.54 (2H, s, OH-2, 7), 9.45 (1H, s, OH-4), 9.21 (2h, s, OH-2, 7) 9.16 (2H, s, OH-3', 5'), 6.14 (1H, s, H-3), 5.96 (1H, d, J = 2.8 Hz, H-6), 5.72 (2H, J = 1.7 Hz, H-4'), 5.78 (1H, d, J = 2.8 Hz, H-6), 5.72 (2H, J = 1.7 Hz, H-2-, 6').

Dieckol : LC/MS data (M+, m/z: 742.0 Calcd. For $C_{36}H_{22}O_{18}$). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.71(1H, s, OH-9), 9.61 (1H, s, OH-9''), 9.51 (1H, s, OH-4''), 9.46 (1H, s, OH-4), 9.36 (2H, s, OH-3'', 5''), 9.28 (1H, s, OH-2''), 9.23 (1H, s, OH-2), 9.22 (1H, s, OH-7''), 9.15 (2H, s, OH-3', 5') 6.17 (1H, s, H-3''), 6.14 (1H, s, H-3), 6.02 (1H, d, J = 2.7 Hz, H-8), 5.98 (1H, d, J = 2.7 Hz, H-8''), 5.95 (1H, s, H-2''', 6'''), 5.82 (1H, d, J = 2.7 Hz, H-6), 5.81 (1H, d, J = 2.7Hz, H-6''), 5.80 (1H, t, J = 2.0 Hz, H-4'), 5.78 (2H, d, J = 2.0 Hz, H-2', 6').

Octaphlorethol A: LC/MS data (M+, m/z: 944.0 Calcd. For $C_{48}H_{34}O_{25}$). ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 5.57 (d, *J* =2.8 Hz，H-4), 5.58 (d, *J* =2.8 Hz，H-6), 5.59 (d, *J* =2.8 Hz，H-9), 5.59 (d, *J* =2.8 Hz, H-11), 5.68 (d, *J* =1.8 Hz, H-15), 5.68 (d, *J* =1.8 Hz, H-17), 5.71 (d, *J* =1.8 Hz, H-21), 5.72 (d, *J* =1.8 Hz, H-23), 5.85 (d, *J* =1.6 Hz, H-26), 5.84 (d, *J* =1.8 Hz, H-30), 5.94 (d, *J* =1.8 Hz, H-32), 5.94 (d, *J* =1.8 Hz, H-36)，6.15 (d, *J* =1.8 Hz, H-38)，6.15 (d, *J* =1.8 Hz, H-42)，6.16 (d, *J* =1.6 Hz, H-44)，6.01 (d, *J* =1.6 Hz, H-46)，6.16 (d, *J* =1.6 Hz, H-48)，9.02 (s, OH-1,3)，9.04 (s, OH-5, 27, 29, 33,35)，8.98 (s, OH-8,12)，8.93 (s, OH-14, 18)，8.92 (s, OH-20,24)，9.06 (s, OH-39,41)，9.07 (s, OH-45, 47).

The purity of phlorotannins (Fig. 4-1) were >95%, based on the peak area of all components absorbed at each specific wavelength in HPLC analysis.

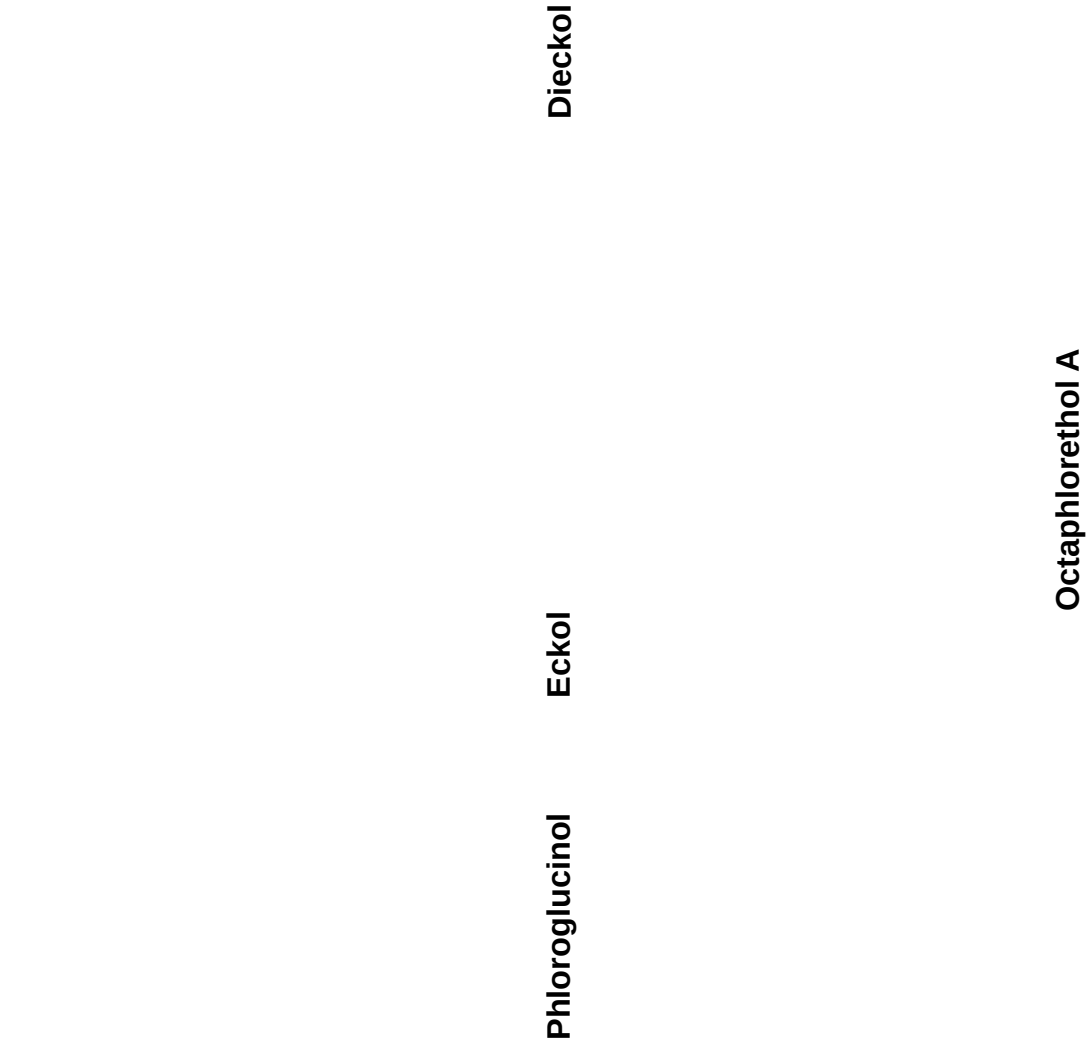

Fig. 3-1. Chemical structure of phlorotannins isolated from brown algae

3.4. Inhibitory effect of phlorotannins on angiotensin I converting enzyme (ACE)

ACE inhibitory activity was determined according to the methods of Cushman and Cheung with slight modifications. For each assay, 50 μl of sample solution with 50 μl of ACE solution (25mU/ml) were pre-incubated at 37℃ for 10 min, after which the mixture was subsequently incubated with 100 μl of substrate (25 mM N-Hippuryl-His-Leu tetrahydrate in 50 mM sodium borate buffer containing 500 mM NaCl at pH 8.3) at the same temperature for 60 min. The reaction was terminated by adding 250 μl of 1M HCl. After that, the resulting hippuric acid was extracted with 500 μl of ethyl acetate. After centrifugation at 4,000 rpm for 10 min, 200 μl of the supernatant was transferred into a glass tube and dried in a dry oven at 80℃ for 1h. The residue was dissolved in 1 ml of distilled water, and the absorbance was measured at 228 nm using an UV-spectrophotometer (Biochrom Ltd., Cambridge, CB4, OFJ, England). The extent of inhibition was calculated as follows.

% inhibition = [(Ac-As)/(Ac-Ab)] X 100

Ac - Absorbance of control solution

As – Absorbance of sample solution

Ab – Absorbance of blank solution

The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of ACE inhibitory activity.

3.5. In silico docking of ACE and new inhibitor candidate

Molecular docking is an application wherein molecular modeling techniques are used to predict how a protein (enzyme) interacts with small molecules (ligands). The ability of a protein to interact with small molecules plays a major role in the dynamics of that protein, which may enhance or inhibit its biological function. In the present study, we performed docking of phlorotannins into the active site of the angiotensin I converting enzyme (ACE). The crystal structure of angiotensin I converting enzyme (ACE) (PDB: 1O86) was obtained from the Protein Data Bank (PDB, http://www.pdb.org) (Fig. 3-2). We performed the docking studies using CDOCKER in Accelrys Discovery Studio 3.0 (Accelrys, Inc) (Fig. 3-3). We describe the ligand structure of the angiotensin I converting enzyme inhibitor candidate in Fig. 3-1. To prepare for the docking procedure, we performed the following steps: (1) conversion of the 2D structure into 3D structure; (2) calculation of charges; and (3) addition of hydrogen atoms using the CDOCKER docking program.

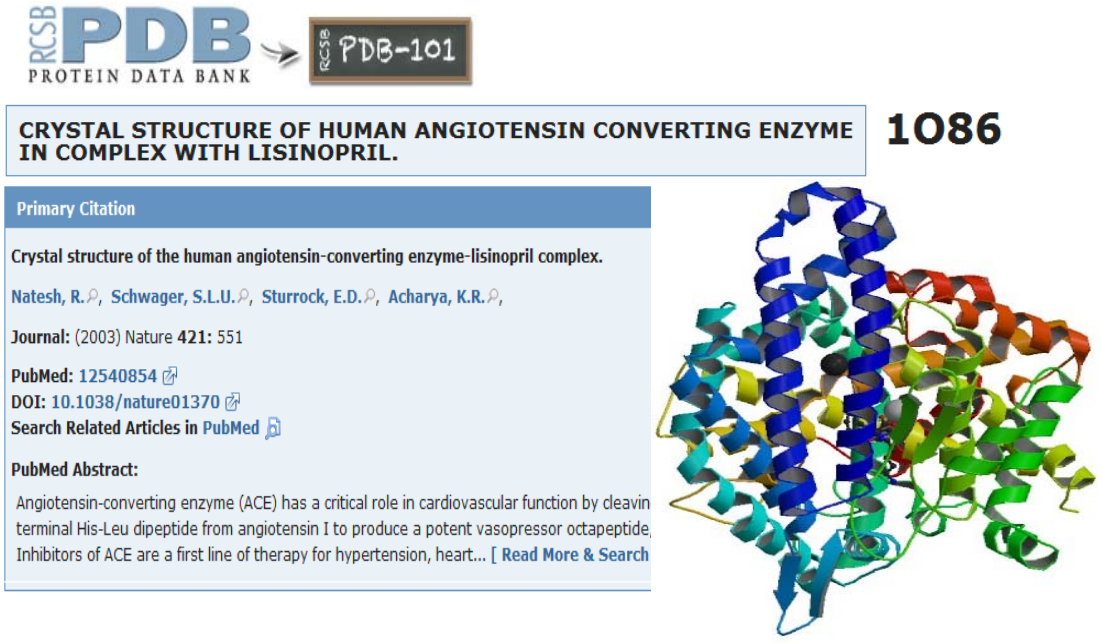

Fig. 3-2. Angiotensin-I converting enzyme (ACE) was obtained from Protein Data Bank (PDB ID: 1O86).

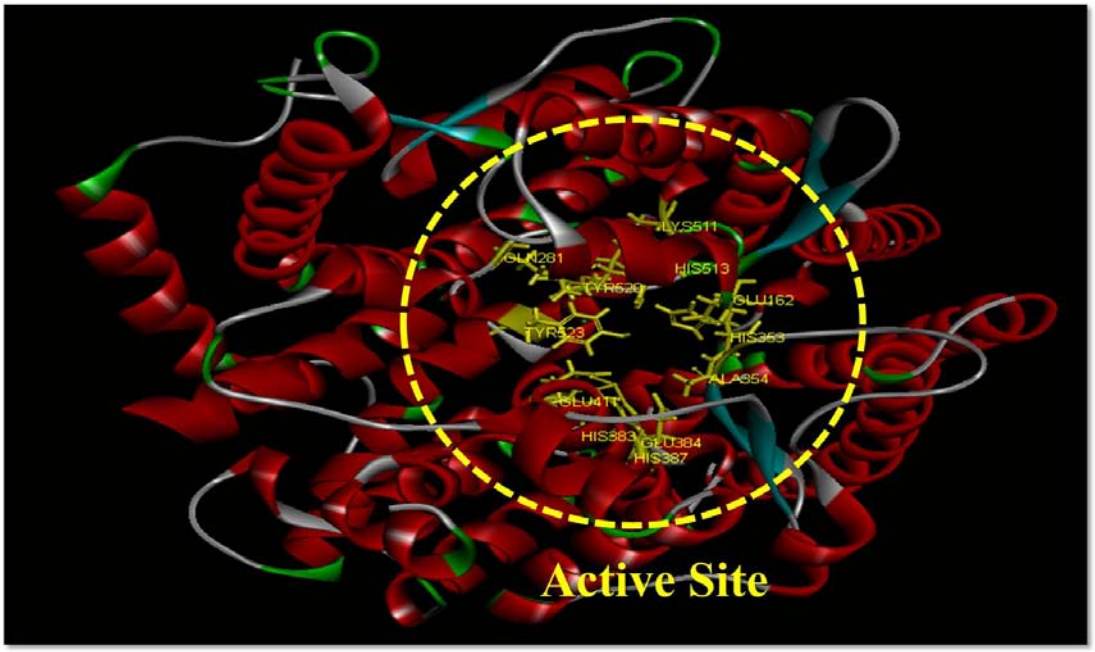

Fig. 3-3. Crystal structure of angiotensin-I converting enzyme (ACE) was obtained from Protein Data Bank (PDB ID: 1O86).

4. RESULTS

4.1. Inhibitory activity of phlorotannins against angiotensin I convertin enzyme (ACE)

The inhibitory effect of phlorotannins against angiotensin I converting enzyme (ACE) activity was examined using N-Hippuryl-His-Leu tetrahydrate (HHL) as a substrate. As shown in Fig. 3-4, among the phlorotannins, eckol, dieckol, and octaphlorethol A inhibited ACE activity in a dose-dependent manner (eckol: 0.65 to 2.6 mM; dieckol: 150 to 600 μM; octaphlorethol A: 125 to 375 μM). Especially, octaphlorethol A (OPA, IC_{50}: 220 μM) exhibited strong inhibition against ACE when compared with phlorotannins, phloroglucinol (PG, IC_{50}: >5 mM), eckol (EK, IC_{50}: 1.3 mM), and dikol (DK, IC_{50}: 315 μM) (Table 3-1.).

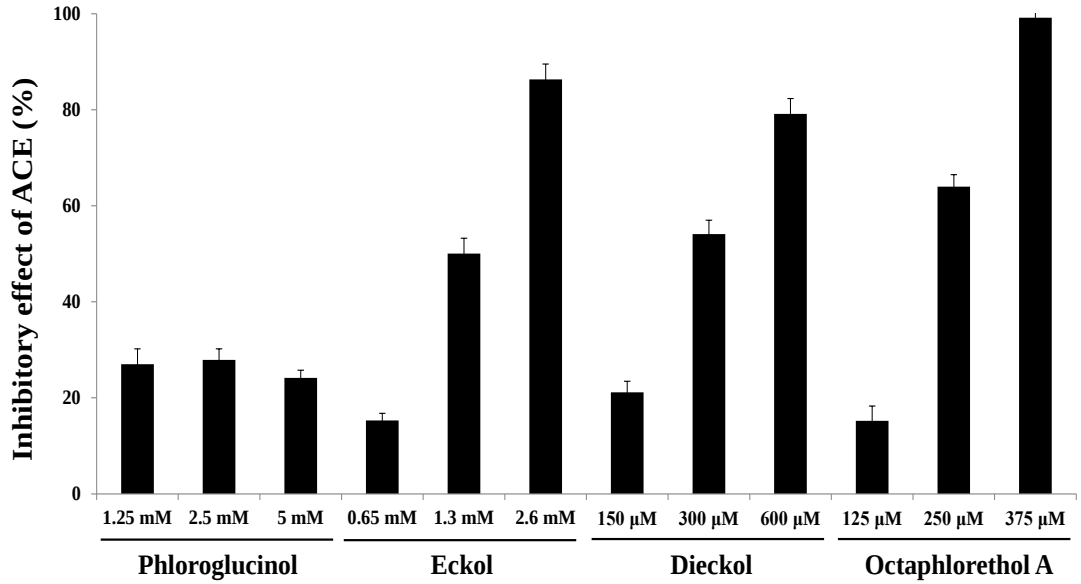

Fig. 3-4. Inhibitory effect of phlorotannins isolated from brown algae on angiotensin I converting enzyme. Inhibitory effects were determined using N-Hippuryl-His-Leu tetrahydrate (HHL) as a substrate. Values are expressed as means ± S.E. in triplicate experiments.

Table 3-1 ACE inhibitory activities (IC_{50}) of phlorotannins.

Phlorotannin	IC_{50} value
Phloroglucinol	> 5 mM
Eckol	1.3 mM ± 0.32
Dieckol	315 μM ± 0.21
Octaphlorethol A	220 μM ± 0.25

*** IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE inhibitory activity. Values are ± SD of three determinations.**

4.2. In silico docking of angiotensin I converting enzyme (ACE)

In this work, we attempt to elucidate how phlorotannins exert theit antihupertensive effects. This was accomplished by automated molecular docking of the phlorotannins at the ACE catalytic site in the presence of amio acid residues, and by analyzing the position, type and energy of the interactions. The three-dimentional structure of native-human ACE was imported from the Protein Data Bank (PDB: 1O86). The structures of ligands (phlrotannin) were generated with Accelrys Discovery Studio 3.0 software and energy minimized with the CHARMm program using steepst descent and conjugate gradient techniques. Before the docking procedure, water molecules were removed from the protein-crystal structure.

Automated molecular docking studies of the phlorotannins at the ACE-binding site were performed with the CDOCKER tool of DS3.0 software, in the presence of amino acid residures. The software Accelrys Discovery Studio 3.0 was used to identify the hydrogen bonds, and the hydrophilic, hydrophobic and electrostatic interactions between residues at the ACE active site and the phlorotannnin poses. Based on ACE's three-dimentional structure, the possible ACE active sites were obtained via a binding site procedure. According to ACEs catalytic mechanism and relevant experimental reports, ACE's active site was identified. The site contains 12 amino acid residues: Tyr520, His513, Tyr523, Lys511, Gln281, Glu411, His353, Glu162, Ala354, His383, Glu384, and His387. The

docking study of the phlorotannins at the ACE catalytic site in the presence of the amio acid residues showed a best pose with a binding energy value in Table 3-2.

ACE inhibitors, phlorotannins were used for the docking experiments and then for the comparison. The docking of the ACE-ligand complexes was well performed with phlorotannins stably posed in the pocket of the ACE by DS 3.0 (Fig. 3-5). As for phlorotannins, the binding sites predicted by 2D program of DS 3.0 (Fig. 3-6) were formed by residues:

Phloroglucinol: His513 (hydrogen bond), Tyr523 (hydrogen bond), and Glu384 (hydrogen bond).

Eckol: His513 (hydrogen bond), Glu384 (hydrogen bond), Ala354 (hydrogen bond), Asp377 (hydrogen bond), and Gln281 (hydrogen bond).

Dieckol: Lys511 (pi interaction bond), Glu376 (hydrogen bond), Glu162 (hydrogen bond), Asp377 (hydrogen bond), His513 (hydrogen bond), Glu411 (hydrogen bond), Asp358 (hydrogen bond), Tyr360 (hydrogen bond), and Glu384 (hydrogen bond).

Octaphlorethol A: His387 (pi interaction bond), Agr522 (pi interaction bond), His383 (pi interaction bond), Lys511 (pi interaction bond), Tyr523 (pi interaction bond), Glu376 (hydrogen bond), Glu162 (hydrogen bond), Cys370 (hydrogen bond), Asp377 (hydrogen bond), Tyr520 (hydrogen bond), Glu411 (hydrogen bond), Glu403 (hydrogen bond), and

Arg522 (hydrogen bond).

The CDOCKER interaction and binding energy (kacl/mol) evaluated from Discovery Studio 3.0 for phlorotannins are CDOCERer interaction energy: phloroglucinol: 28.02 kcal/mol, eckol: 51.84 kcal/mol, dieckol: 94.08 kcal/mol, and octaphlorethol A: 124.55 kcal/mol, and binding energy: phloroglucinol: -106.17 kcal/mol, eckol: -110.48 kcal/mol, dieckol: -206.75 kcal/mol, and octaphlorethol A: -362.16 kcal/mol, respectively (Table 3-2.). OPA showed a higher CDOCKER interaction and binding energy, very likely due to the high number of hydroxyl groups as well as hydrophilic interactions. As OPA resembles the substrate may bind selectively with the active site residues. The binding mode analysis of the OPA with the active site residues provided important information of catalytic site.

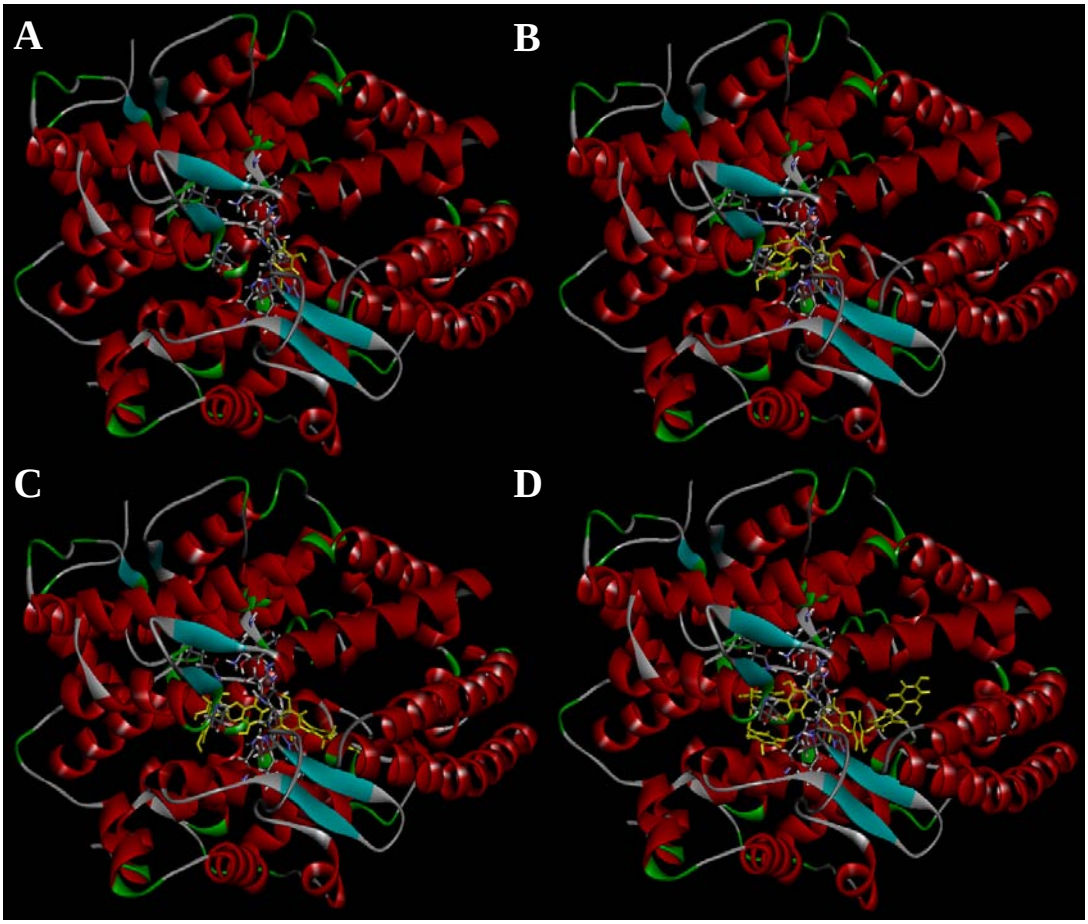

Fig. 3-5. Computational prediction of the structure for ACE and docking simulation with phlorotannins. Predicted 3D structure of ACE 1O86. Ribbon model (ACE-ligand complex): A: phloroglucinol, B: eckol, C: dieckol, D: octaphlorethol A.

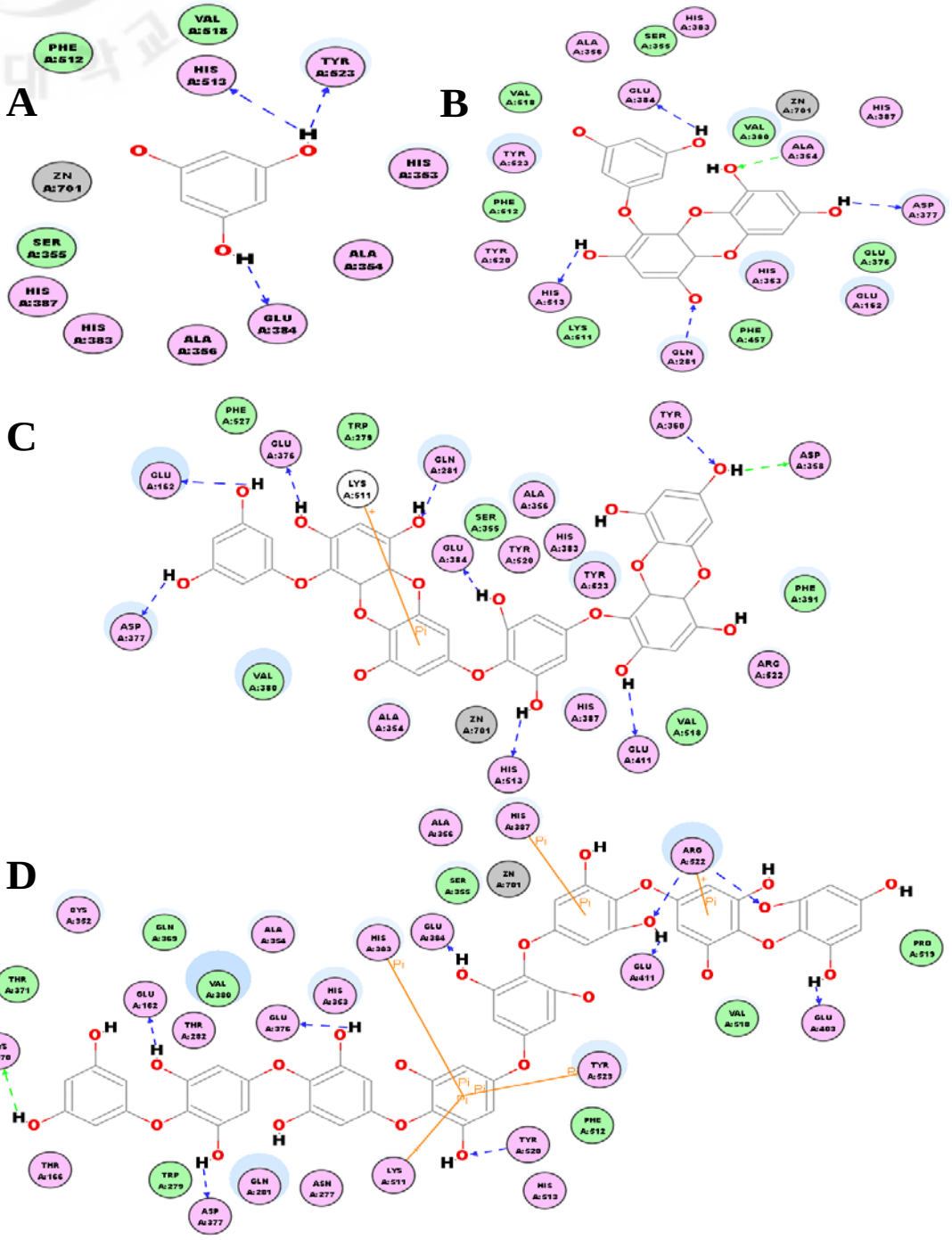

Fig. 3-6. Computational prediction of the structure for ACE and docking simulation with phlorotannins. Predicted 3D structure of ACE 1O86. 2D program (ACE-ligand complex): A: phloroglucinol, B: eckol, C: dieckol, D: octaphlorethol A.

Table 3-2 Results of docking experiments of phlorotannins with the angiotensin I converting enzyme (PDB ID : 1O86).

Ligand	Binding Energy (kcal/mol)	CDOCK Interaction Energy (kcal/mol)
Phloroglucinol	-106.17	28.02
Eckol	-110.48	51.84
Dieckol	-206.75	94.08
Octaphlorethol A	-362.16	124.55

5. DISCUSSION

Polyphenols are secondary metabolites very widely distributed in plants [9]. It is well known that solvents with different polarities can extract different classes of compounds [10]. Extractable polyphenols can be extracted from plants by using solvents such as water, methanol ethanol, acetone, ether, or their mixtures. Naturally occurring polyphenols are known to have numerous biological activities [11]. Further, various extractants can be used to release soluble pholyphenols from plant materials. According to previous experiments, it was also reported that water extract of *E. cava* has considerable ACE inhibitory activity (around 36%) at a concentration of 1 mg/ml [12]. Based on the results of the current study, we found that ethanol extract of *E. cava* enhanced ACE inhibition and was superior compared to water extract. It could have been that ethanol increased the TPC by inhibiting interactions between tannins and proteins during extraction [13] or even by breaking hydrogen bonds between tannin-protein complexes [14]. It is well established that brown algae contain polyphenolic compounds. Polyphenols have become the focus of intense research due to their perceived beneficial effects on health [15,16]. Phlorotannins are systematically grouped according to the bond type between the phloroglucinol units (diphenyl ethers or biphenyls) as well as the presence of additional hydroxyl groups [17]. They are secondary metabolites with a wide range of molecular sizes and an astringent taste,

and they are able to bind to metal ions and precipitate proteins [18]. Based on the increasing evidence of the importance of phlorotannins, this is the first study to demonstrate ACE inhibitory activity of phlorotannins of brown algae. Marine algae produce a great variety of secondary metabolites possessing different skeletal types and biological activities [19,20]. Accordingly, brown algae contains a variety of compounds, including carotenoids, fucoidans, and phlorotannins, showing different biological activities [21,16]. Furthermore, Ahn *et al.* [22] reported that phenolic secondary metabolites from brown algae have many biological activities. ACE is a carboxyl-terminal dipeptidyl exopeptidase that catalyzes the conversion of angiotensin I to angiotensin II in the rennin angiotensin system [23]. Since all enzymes are proteins, inhibition of ACE may be closely associated with the protein-binding abilities of phlorotannins, which are characteristic of all tannins. Furthermore, it has been well described that tannins have the ability to form strong complexes with proteins, either reversibly by hydrogen bonding through peptide or amide linkages or irreversibly by covalent condensations [24]. Therefore, we can suggest that the inhibition was due to the reduced efficiency of ACE after binding with phlorotannins from brown algae.

Part IV.

Molecular docking of phlorotannins isolatd from brown algae and their effects on digestive enzyme effects

Part IV.

Molecular docking of phlorotannins isolated from brown algae and their effects on digestive enzyme effects

1. ABSTRACT

Marine polyphenol, phlorotannins possess many beneficial properties, such reducing the risk of cancer and heart disease, and acting s natural antioxidants for the food industry. At the same time, polyphenol might inhibit digestive enzymes and reduced food digestibility. To explore this possible antinutritional property, the effects of phlorotannins on the inhibitor activity of two typicl digestive enzymes were investigated. The phlorotannins, such as phloroglucinol, eckol, dieckol, and octaphlorethol A were isolated by silica column chromatography and HPLC methods. We analyzed structure-activity relationship of phlorotannins using the crystal structure of trypsin (PDB ID: 1XVO) and pepsin (PDB ID: 1QRP) by Discovery Studio 3.0. These results suggest that phlorotannins has great potential to be further developed as a pharmaceutical and medicinal food.

2. INTRODUCTION

Polyphenols are one of the most common classes of secondary metabolites in terrestrial and marine plants. Although terrestrial and marine polyphenols are similar in some respects, there are fundamental differences in their chemical structure [1]. In general, polyphenols or phenolic compounds have a similar basic structural chemistry including an “aromatic” or “phenolic” ring structure. Phenolic compounds have been associated wth antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals [2,3]. The protective effects of plant polyphenols in biological systems are ascribed to their capacity to transfer electrons to free radicals, chelate metal catalysts, activate antiodixant enzymes and inhibit oxidase. Polyphenols are classified broadly into two classes; condensed tannins, which are polymeric flavonoids, and hydrolysable tannins, which are derivatives of gallic acid [4]. Phlorotannins, known as marine algal polyphenol, are formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) monomer units and biosynthesized through the acetate-malonate pathway, also known as polyketide pathway. The phlorotannins are highly hydrophilic components with a wide range of molecular sizes ranging between 126 Da and 650 kDa [5]. Marine brown algae accumulate a variety of phloroglucinol-based polyphenols, as phlorotannins of low, intermediate and high molecular weight containing both phenyl and phenoxy units. Based on the means of linkage, phlorotannins can be classified into four

subclasses such as fuhalols and phlorethols (phlorotannins with an ether linkage), fucols (with a phenyl linkage), fucophloroethols (with an ether and phenyl linkage), and eckols (with a dibenzodioxin linkage). The isolated and characterized phlorotannins from marine brown algae are compounds, such as phloroglucinol, eckol, triphlorehol A, dieckol, 2-phloroeckol, fucofuroeckol A, 7-phloroeckol, 6,6'-bieckol, diphlorethohydroxycarmalol, and phlorofucofuroeckol A.. These phlorotannins help to protect algae from stress conditions and herbivores. Due to the health beneficial various biological activities of phlorotannins, marine brown algae are known to be a rich source of healthy food.

On thither hand, phlorotannins an important role in protein precipitation and enzyme inhibition, through forming various complexes [6,7]. It is known that most polyphenols, such as tannic acid, gallotannin, catechin and proanthocyanidin, can react with proteins, which should result in the formation of sediment and haze. In food industry this phenomenon occurs frequently in the production of beverages, such as beer, fruit and vegetable juices, leading to low uality products. Phlorotannins should inevitably result in the change of enzyme molecular configuration and lead to the loss of catalytic activity. Many enzymes, such as tyrosianse, peroxidase, trypsin [8], decarboxylase [9], squalene epoxidase [10] and ribonuclease [11], were found to be denatured by tea polyphenols. So it colud be speculated that phlorotannins should bind digestive enzymes and therby reduce food digestibility, when

excessively. Usually, marine products were considered to be a safe natural product. However, it might atc as an antinutritional factor, in terms of the inhibition of enzymes, when ingested in excess. To minimize the antinutritional effects and make full use of marine products, phlorotannins in food industry, knowledge of the interaction between phlorotannins and digestive enzymes is desirable. In this study, phlorotannins were isolated from brown algae and their inhibitory effects on two typical digestieve enzymes were investigated. Although the effects were assayed in vitro, the results of this work should be relevant to the human body.

3. MATERIAL AND METHODS

3.1. Materials

Protein protease such as pepsin and trypsin and Abumin were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). 30% Acrylamide/Bis solution (29:1) were purchased from bio-rad Laboratories, Inc. (USA). Ammonium persulfate (APS) and TEMED were purchase from Amresco Inc. (USA). All chemicals and reagents used were of analytical and obtained from commercial sources.

3.2. Extraction and isolation of phlorotannins from brown algae

Phlorotannins, phloroglucinol, eckol, dieckol, and octaphlorethol A isolated as previously described (Part I, II, and III). Brieflym the dried algae were extracted three times with 80% EtOH and filtered. The filterates were then evaporated at 40 ℃ to obtain the EtOH extract, which were suspended on distilled water, and partitioned with ethyl acetate. The ethyl acetate fraction was subjected to silica gel and sephadex LH-20 coumn chromatography. The phlorotannins were finally purified by HPLC, and the structure of phlrotannin was identified by comparing the NMR spectral data with those in existing literature [12, Part I].

Phloroglucinol : LC/MS data (M+, m/z: 126 Calcd. For $C_6H_6O_3$). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.97 (3H, s, OH-1, 3, 5), 5.66 (3H, s, H-2, 4, 5).

Eckol : LC/MS data (M+, m/z: 372.0 Calcd. For $C_{18}H_{12}O_9$). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.54 (2H, s, OH-2, 7), 9.45 (1H, s, OH-4), 9.21 (2h, s, OH-2, 7) 9.16 (2H, s, OH-3', 5'), 6.14 (1H, s, H-3), 5.96 (1H, d, J = 2.8 Hz, H-6), 5.72 (2H, J = 1.7 Hz, H-4'), 5.78 (1H, d, J = 2.8 Hz, H-6), 5.72 (2H, J = 1.7 Hz, H-2-, 6').

Dieckol : LC/MS data (M+, m/z: 742.0 Calcd. For $C_{36}H_{22}O_{18}$). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.71(1H, s, OH-9), 9.61 (1H, s, OH-9''), 9.51 (1H, s, OH-4''), 9.46 (1H, s, OH-4), 9.36 (2H, s, OH-3'', 5''), 9.28 (1H, s, OH-2''), 9.23 (1H, s, OH-2), 9.22 (1H, s, OH-7''), 9.15

(2H, s, OH-3', 5') 6.17 (1H, s, H-3''), 6.14 (1H, s, H-3), 6.02 (1H, d, J = 2.7 Hz, H-8), 5.98 (1H, d, J = 2.7 Hz, H-8''), 5.95 (1H, s, H-2''', 6'''), 5.82 (1H, d, J = 2.7 Hz, H-6), 5.81 (1H, d, J = 2.7Hz, H-6''), 5.80 (1H, t, J = 2.0 Hz, H-4'), 5.78 (2H, d, J = 2.0 Hz, H-2', 6').

Octaphlorethol A: LC/MS data (M+, m/z: 944.0 Calcd. For $C_{48}H_{34}O_{25}$). ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 5.57 (d, *J* =2.8 Hz，H-4), 5.58 (d, *J* =2.8 Hz，H-6), 5.59 (d, *J* =2.8 Hz，H-9), 5.59 (d, *J* =2.8 Hz, H-11), 5.68 (d, *J* =1.8 Hz, H-15), 5.68 (d, *J* =1.8 Hz, H-17), 5.71 (d, *J* =1.8 Hz, H-21), 5.72 (d, *J* =1.8 Hz, H-23), 5.85 (d, *J* =1.6 Hz, H-26), 5.84 (d, *J* =1.8 Hz, H-30), 5.94 (d, *J* =1.8 Hz, H-32), 5.94 (d, *J* =1.8 Hz, H-36)，6.15 (d, *J* =1.8 Hz, H-38)，6.15 (d, *J* =1.8 Hz, H-42)，6.16 (d, *J* =1.6 Hz, H-44)，6.01 (d, *J* =1.6 Hz, H-46)，6.16 (d, *J* =1.6 Hz, H-48)，9.02 (s, OH-1,3)，9.04 (s, OH-5, 27, 29, 33,35)，8.98 (s, OH-8,12)，8.93 (s, OH-14, 18)，8.92 (s, OH-20,24)，9.06 (s, OH-39,41)，9.07 (s, OH-45, 47).

The purity of phlorotannins (Fig. 4-1) were >95%, based on the peak area of all components absorbed at each specific wavelength in HPLC analysis.

Fig. 4-1. Chemical structure of phlorotannins isolated from brown algae

3.3. Inhibitory effects of Phlorotannins on digestive enzyme, pepsin and trypsin

Activity of enzymes was determined using the colormetric method based on the reaction of the albumin degradation product complexed with the Folin-Ciocaltue phenol reagent (Merck) [13]. The reagent corresponds in its composition to molybdate-tung-stenate reagent and to phenol reagent stock solution for the determination of pspsin activity. The reaction solutions were composed of the following reagents added to the test tubes in the following sequence: 1ml of sunstrate (1% of albumin in redistilled water), 1 ml of restilled water, and 1 ml of test sample, and 1ml of enzyme (pepsin solution in 1 N HCl or trypsin solution in 0.001 N HCl). The mixtures were incubated at 40 for 5 min, and then the reaction was discontinued through the addition of 2 ml of 5% (w/v) trichloroactiv acid (TCA). Subsequntly the soltion were centrifuged to separate precipate from supernatant. Then 1 ml of the supernatant was incubated with 5 ml of 0.2 mol Na_2CO_3/l of aqueous solution, and 1 ml of the Folin-Ciocalteu phenol reagent (diluted with redistilled water in the proportion 1:5. v/v) at 40 for 20 min. Successively the absorbance was measured at 625 nm with the use of spectrometer.

3.3. Preparation of phlorotannins by protease hydrolysates for SDS-PAGE

2 mg of the albumin dissolved in water 10ml and was adjusted pH of protease opmimum

condition (Table 4-1.). And then protease of substrat to enzyme ratio 10:1 was added. Protein hydrolysis enzyme used two proteinases such as pepsin and trypsisn. Hydrolysis was carried out at protease optimum temperature (Table 4-1.). And then 20 μg of phlorotannins from brown algae were added in albumin-enzyme mixture. At the end of the reaction, the hydrolysates were heating at 100 ℃ for 10 min in order to inactivate the enzyme. The hydrolysates were centrifuged at 10,000 rpm for 5 min to spate insoluble and soluble fractions. The soluble phase was used further experiments.

Table 4-1. Optimal hydrolysation conditions of digestive enzyme

Enzyme	Sources	Optimal condition		Reaction Time (h)
		pH	Temperature (℃)	
Pepsin	Porcine gastric mucosa	2.0	37	3
Trypsin	Bovine Pancreases	7.6	37	6

3.4. SDS-PAGE electrophoresis

Sodium dodecyl sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) was performed on the protein hydrolysates using a 12% Tri/HCl gel to characterize the hydrlysates based on their molecular weights (MW). The MW of the hydrlysates was calculated with reference to the migration of SDS-PAGE wide range molecular weight standard. Sample were heated at 100 ℃ for 5 min prior to the electrophoresis run. After electrophoresis, the gels were stained with Bio-Rad Commassie Blue R-250. The bands in the samples were compared with known bands of protein standards.

3.5. In silico docking of digestive enzyme and phlorotannins and tea polyphenol

In the present study, we performed docking of phlorotannins into the active site of the trypsin and pepsin. The crystal structure of trypsin (PDB: 1XVO) and pepsin (PDB: 1QRP) were obtained from the Protein Data Bank (PDB, http://www.pdb.org). We performed the docking studies using CDOCKER in Accelrys Discovery Studio 3.0 (Accelrys, Inc) (Fig. 4-2, 3). We describe the ligand structure of the digestive enzyme inhibitor candidate in Fig. 4-4. To prepare for the docking procedure, we performed the following steps: (1) conversion of the 2D structure into 3D structure; (2) calculation of charges; and (3) addition of hydrogen atoms using the CDOCKER docking program.

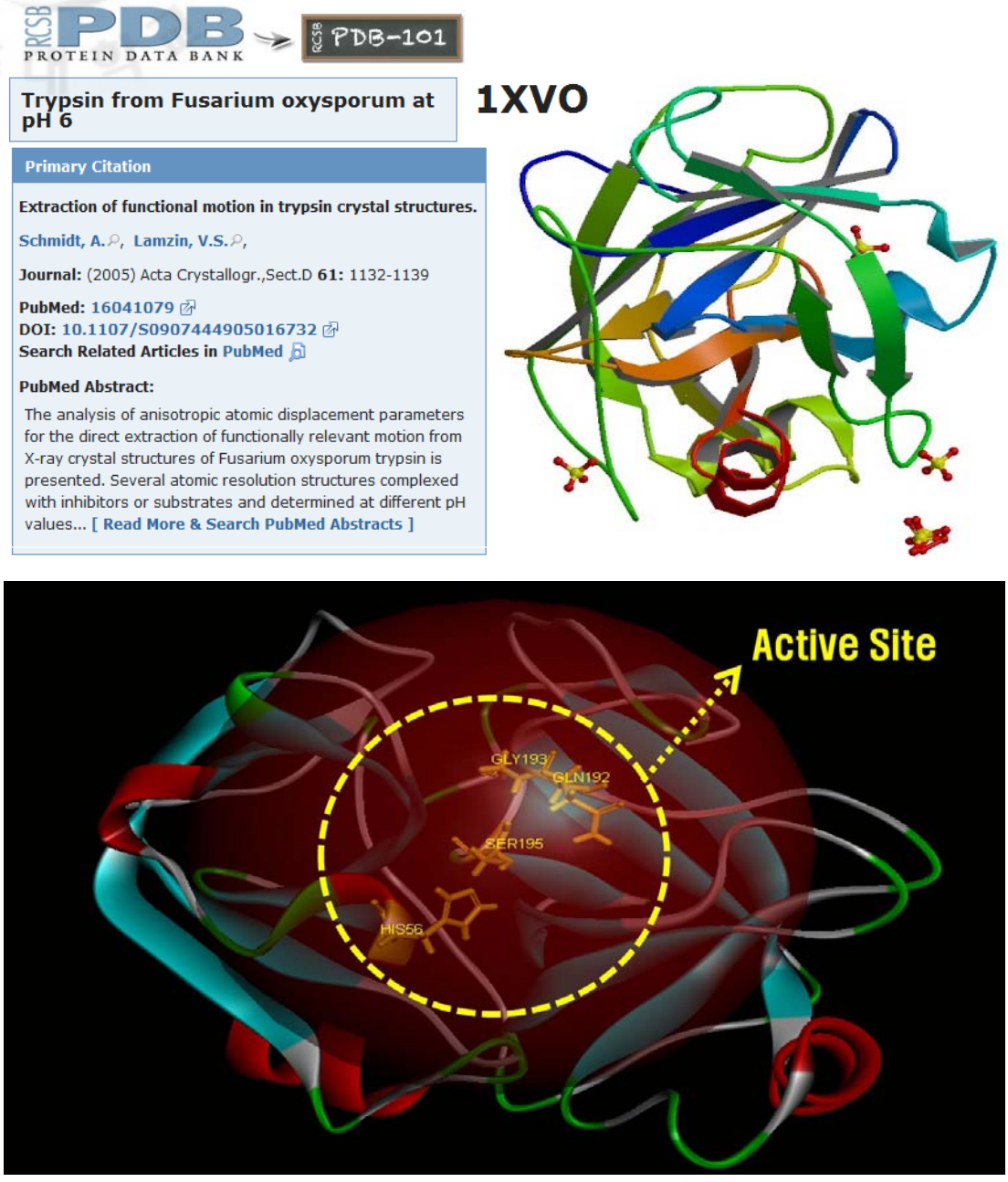

Fig. 4-2. Crystal structure of trypsin was obtained from Protein Data Bank (PDB ID: 1XVO).

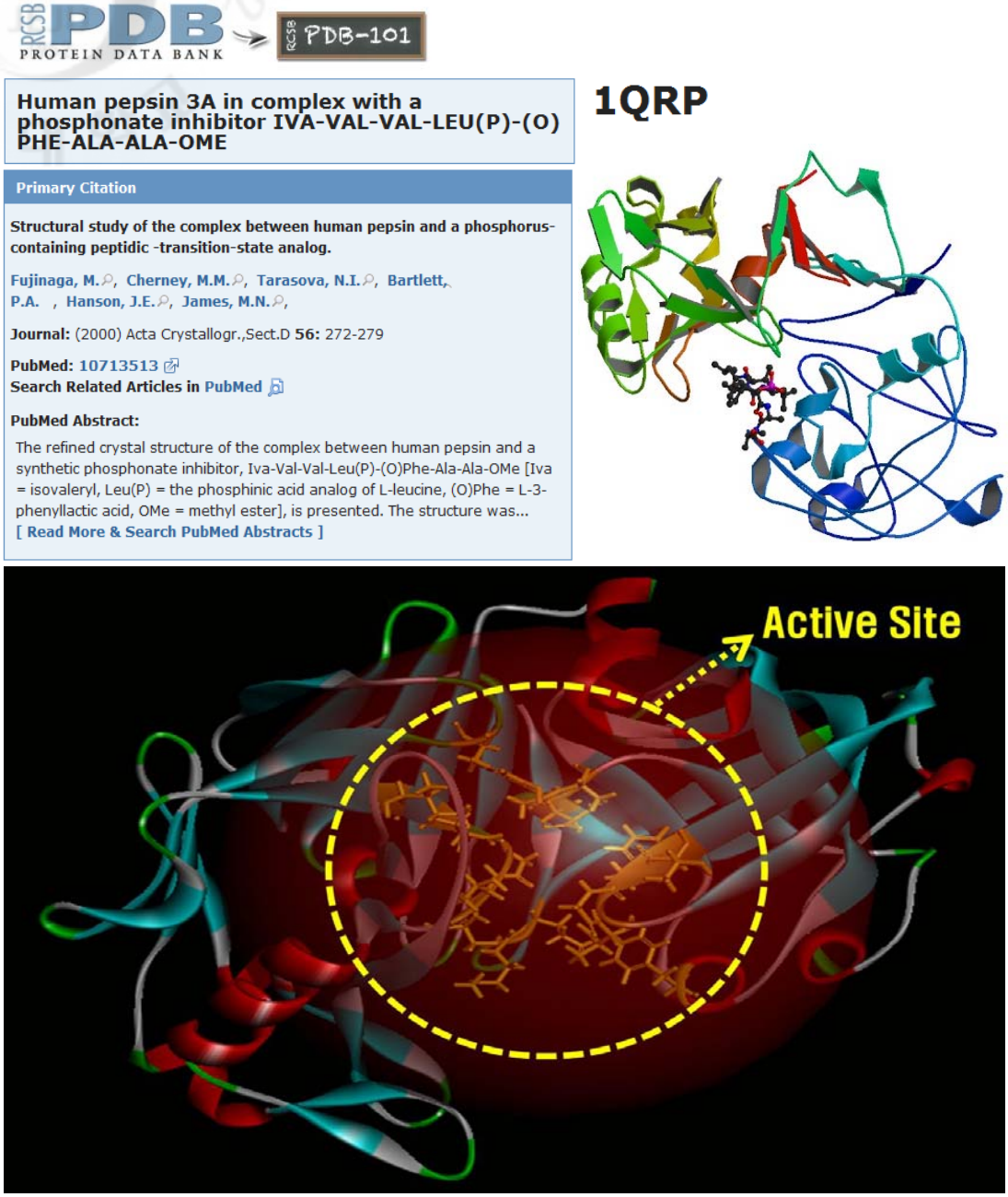

Fig. 4-3. Crystal structure of pepsin was obtained from Protein Data Bank (PDB ID: 1QRP).

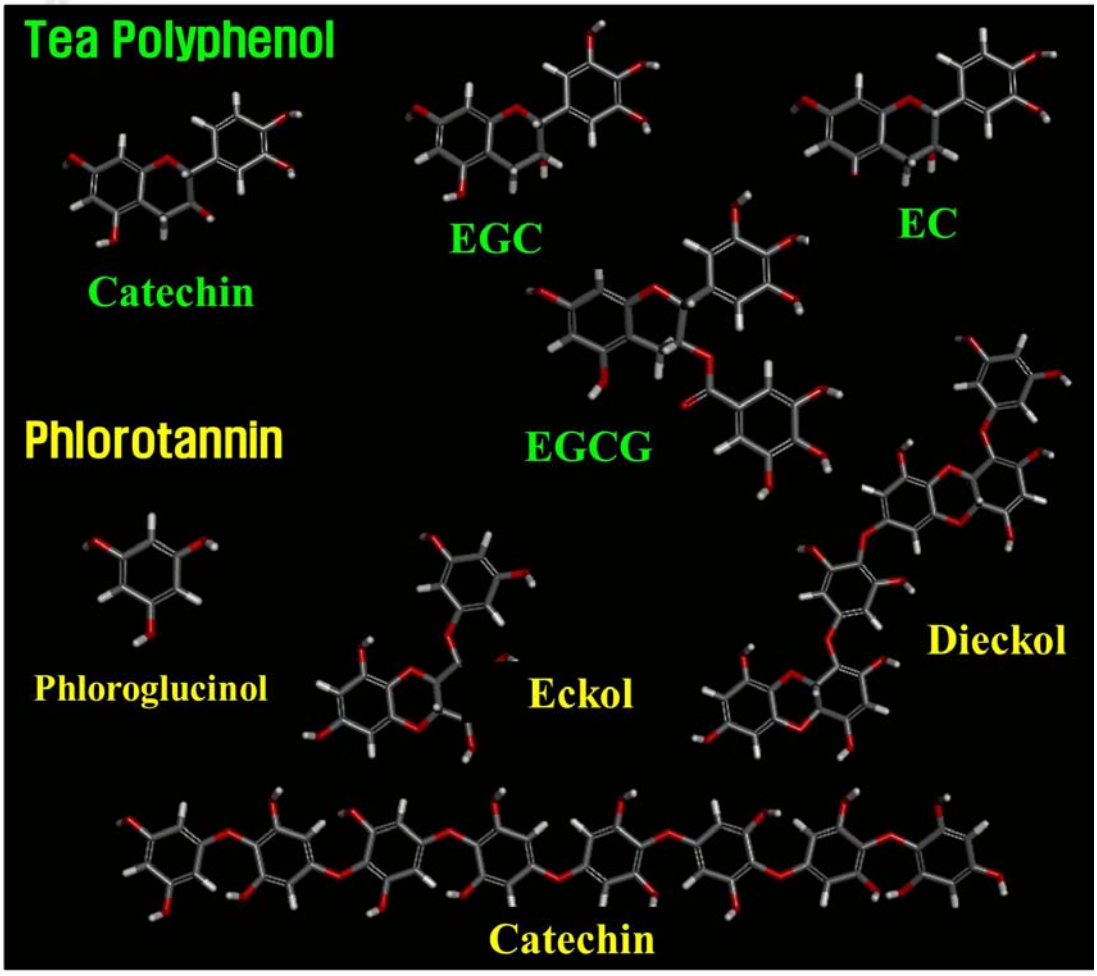

Fig. 4-4. Chemical structure of phlorotannins and tea polyphenol of digestive enzyme inhibitor candidates

4. RESULTS

4.1. Inhibitory effects of phlorotannins on digestive enzyme, trypsin and pepsin

Two typical digestive enzymes including pepsin and trypsin were used to investigate the phlrotoannin-enzye interaction and to explore the potential antinutritional property of phlorotannins. In experiment, phlorotnnin showed different binding ability in vitro with the digestive enzymes employed. As shown in Table 2, the inhibition ratio of pepsin by phlorotnnin was 10.4 (PG), 15.7 (EK), 19.8 (DK), 16.9 (OPA), and trypsin was 7.1 (PG), 10.6 (EK), 13.2 (DK), 19.2 (OPA). Moreover, the phlorotannins evidenced less inhibitory effect than that of tea polyphenol even at the same concentration [14].

Table 4-2. Inhibitory effect of digestive enzymes by phlorotannins.

Inhibition (%)	Trypsin	Pepsin
Phloroglucinol (PG)	10.4	7.1
Eckol (EK)	15.7	10.6
Dieckol (DK)	19.8	13.2
Octaphlorethol A (OPA)	16.9	19.2
Tea polyphenol* (TP)	38	32

*** Tea polyphenol : Reference [14]. Qiang, H., Yuanping, L., Kai, Y.2006.**

4.2. Characterization of trypsin digests SDS-PAGE

Pepsin digests according to differents phlorotannins such as PG, EK, DK, and OPA were characterized using SDS-PAGE electrophoresis (Fig. 4-5). In the results, negative control group, non-treated trypsin group showed one major band of albumin (M.W. 67 kDa). On the other hand, trypsin digest group without phlorotannins showed various band patterns. Also, trypsin digest group with phlorotannins such as PG, EK, DK, and OPA showed various band patterns like negative control. Therefore, phlrotannins don’t influenced on digest enzyme, trypsin.

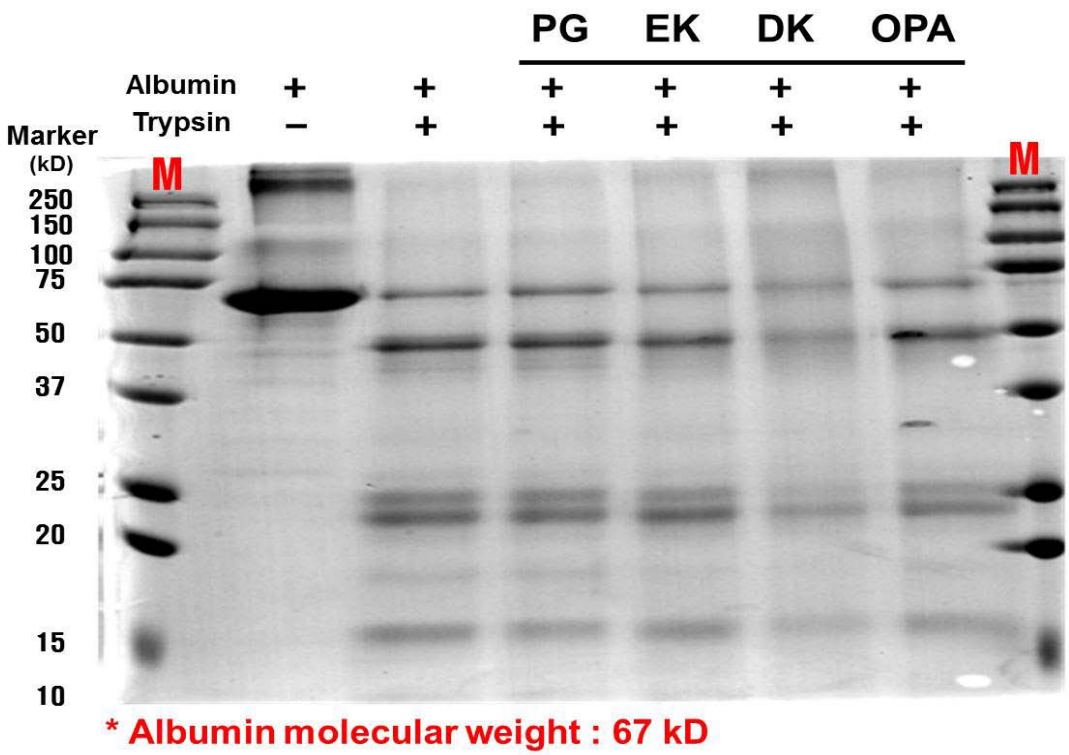

Fig. 4-5. Hydrolysis patterns of trypsin digest of albumin with phlorotannins on 12% SDS-PAGE. M: Marker.

4.3. Characterization of pepsin digests SDS-PAGE

Pepsin digests according to differents phlorotannins were characterized using SDS-PAGE electrophoresis (Fig. 4-6). In the results, negative control group, non-treated pepsin group showed two major band of albumin (M.W. 67 kDa) by acidic hydrolysis (pH 2.0). On the other hand, pepsin digest group without phlorotannins showed number of band patterns of below 10 kDa. Also, pepsin digest group with phlorotannins such as PG, EK, DK, and OPA showed band patterns of below 10 kDa like negative control. Therefore, phlrotannins don’t influenced on digest enzyme, pepsin.

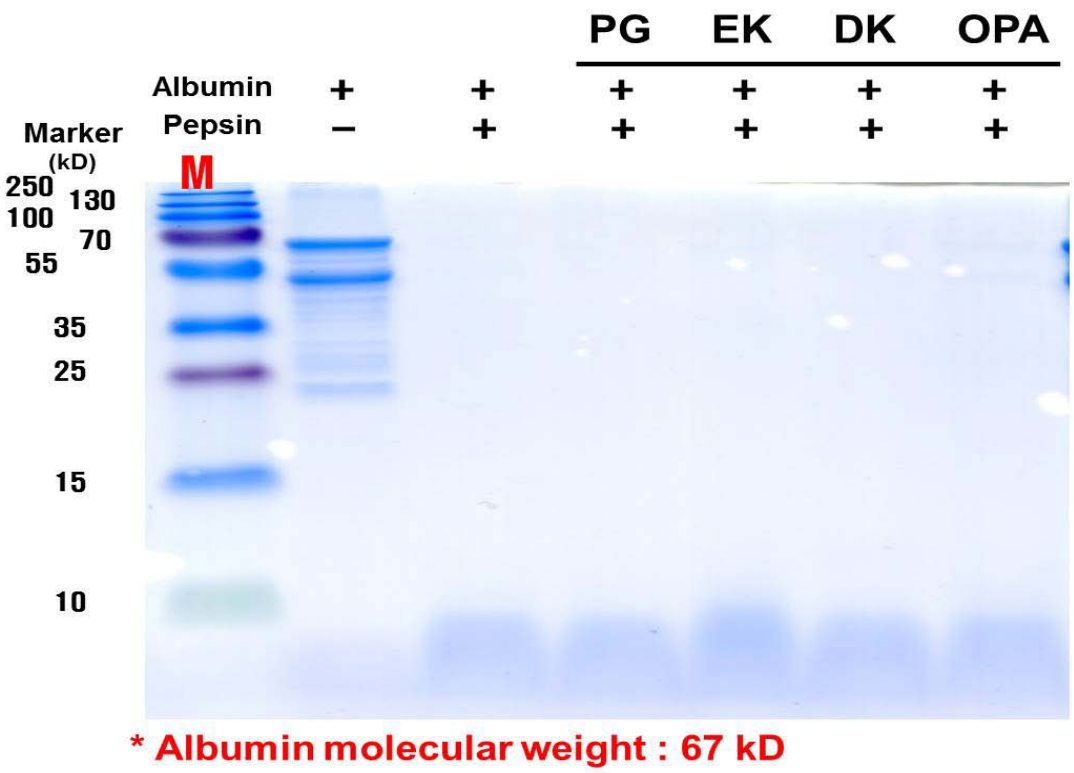

Fig. 4-6. Hydrolysis patterns of pepsin digest of albumin with phlorotannins on 12% SDS-PAGE. M: Marker.

4.4. In silico docking of digest enzymes, trypsin and pepsin

Docking studies were performed to gain insight into the most probable binding conformation of phlorotannins and to compare with phlorotannins. Digestive enzyme inhibitors, phlorotannins were used for the docking experiments and then for the comparison.

Automated molecular docking studies of the phlorotannins at the digestive enzyme-binding site were performed with the CDOCKER tool of Discovery Studio 3.0 software, in the presence of amino acid residures. The software Accelrys Discovery Studio 3.0 was used to identify the hydrogen bonds and the hydrophilic, hydrophobic and electrostatic interactions between residues at the digestive enzyme active site and the phlorotannnins poses. Based on digestive enzyme's three-dimentional structure, the possible digestive enzyme active sites were obtained via a binding site procedure. According to digestiv enzymes catalytic mechanism and relevant experimental reports, digestive enzyme's active site was identified. The digestive enzyme, trypsin active site contains 4 amino acid residues: Gln192, Gly193, Ser195, and His56; and pepsin active stie contains 16 amino acid residues: Tyr189, Ile213, Asp215, Gly34, Asp32, Ser35, Gly217, Thr218, Ser219, Met12, Glu13, Gly76, Thr77, Tyr75, Thr74, Phe11. The docking study of the phlorotannins at the digestive enzyme catalytic site in the presence of the amio acid residues showed a best pose with a binding energy value in Table 4-3 (trypsin) and Table 4-4 (pepsin).

Table 4-3 Results of docking experiments of phlorotannins from brown algae with trypsin (PDB ID : 1XVO).

Docking Mode	Ligand	Binding Energy (kcal/mol)	CDOCK Interaction Energy (kcal/mol)
CDOCKER	Catechin	-45.68	36.03
	EC	-26.86	32.08
	EGC	-36.94	32.37
	EGCG	-148.76	53.40
	Phloroglucinol	-5.78	18.96
	Eckol	-67.57	42.85
	Octaphlorethol A	-23.05	37.24
Flexible CDOCKER	Dieckol	-58.48	51.07

Table 4-4 Results of docking experiments of phlorotannins from brown algae with pepsin (PDB ID : 1QRP).

Docking Mode	Ligand	Binding Energy (kcal/mol)	CDOCK Interaction Energy (kcal/mol)
CDOCKER	Catechin	-127.88	44.07
	EC	-120.24	45.08
	EGC	-86.11	45.11
	EGCG	-100.9	51.25
	Phloroglucinol	-60.78	28.01
	Eckol	-77.71	50.44
	Dieckol	-88.02	74.10
	Octaphlorethol A	-119.11	96.04

5. DISCUSSION

Two typtical digestive enzymes such as trypsin and pepsin were used to investigate the phlorotoannins-enzyme interaction and to explore the potential antinutritional property of phlorotannins. In experiments, phlorotannins showed different bonding ability in silico with the digestive enzymes employed. As shown in Table 4-2, the digestive enzumes inhibition ratios of phlorotannins were under 20%, which inhibitory activities were lower than tea polyphenol, when the phlorotannin concentration was 0.05 mg/ml. There could be a reduction of the digestibilities of proteins, whose hydrolyzation reactions in the gut are enzyme mediated. Phlorotannins might little act as an antinutritional factor, in terms of its potential to inhibit the activities of digestive enzymes.

The main mechanism of phlorotannins-enzymes bonding is considered to be non-covlaent interactions. Phlorotannins contain hydroxyl groups and phenolic groups in their molecular structure. The phenolic groups can form hydroge honds with the polar groups (amide, guanidine, peptide, amino and carboxyl groups) of protein. In other words, the composition and quantity of the polar groups in the enzyme protein will affect the formation and stability of hydrogen bonds between phlorotannins and the enzyme. On the other hand, the phenolic groups in phlorotannins exhibit cetain hydrophobicity, which was well discussed in the

report. With the recognition that there are many hydrophobic amino acids present in enzyme protein, such as proline, phenylalanine and tyrosine, it could be considered that phlorotannins should strongly bind enzymes through hydrophobic association. The occurrence of hydrogen bond and hydrophobic association will change the enzyme molecular configuration, resulting in an impact of the enzyme activities. So, it could be conclused that phlorotannin might little act as an antinutritional factor, in terms of their inhibitory effects on digestive enzymes, which may be due to the cooperative effects of hydrophobic association and hydrogen bond formation between phlorotannin and the enzyme.

Reference (Introduction)

1. Aneiros, A., Carateix, A. Bioactive peptides from marine sources: pharmacological properties and isolation procedures. J. Chromatogr. B. 2004, 803: 41-53.

2. Barrow, C., Shahidi, F. Marine Nutraceuticals and functional foods. USA: CRC Press; 2008.

3. Kim, S.K., Wijesekara, I. Development and biological activities of marine-derived bioactive peptides: a review. J. Functional Foods. 2010, 2: 1-9.

4. Wijesekara, I., Kim, S.K. Angiotensin-I-converting enzyme (ACE) Inhibitors from marine resources. Mar. Drugs. 2010, 8: 1080-1093.

5. Nwosu, F., morris, J., Lund, V.A., Stewart, D., Ross, H.A., McDougall, G.J. Anti-proliferative and potential antidiabetic effects of phenolic-rich extracts from edible marine algae. Food. Chem. 2011, 126: 1006-1012.

6. Plaza, M., Cifuents, A., Ibanez, E. In the search of new functional food ingredients from algae. Trends. Food. Sci. Technol. 2008, 19: 39.

7. Smit, A.J. medicinal and pharmaceutical uses of seaweed natural products: a review. J. Appl. Phycol. 2004, 16: 245-262.

8. Faulkner, D.J. Marine natural products. Nat. Prod. Rep. 2002, 19: 1-48.

9. Shibata, T., Fujimoto, K., Nagayama, K., Yamaguchi, K., Nakamura,T. Inhibitory activity

of brown algal phlorotannins against hyaluronidase. Int. J. Food. Sci. Technol. 2002, 37: 703-709.

10. Rice-Evans, C.A., Muller, N.J., Bolwell, P.G., Barmley, P.M., Pridham, J.B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free. Radical. Res. 1995, 22: 375-383.

11. Jorgensen, L.V., Madsen, H.L., Thomsen, M.K., Dragsted, L.O., Skibsted, L.H. Regulation of phenolic antioxidants from phenoxyl radicals: An ESR and electrochemical study of antioxidant hierarchy. Free. Radical. Res. 1999, 30: 207-220.

12. Haslam, E. plant polyphenols: vegetable tannins revisited. Cambridge, UK: Cambridge University Press.

13. Regan, M.A., Glombitza, K.W. handbook of physiological Methods. Cambridge: Cambridge University Press: 1986, 129-241.

14. Heo, J.J., Park, E.U., Lee, K.W., Jeon, Y.J. Antioxidant activities of enzymatic extracts from brown seaweeds. Biores. Technol. 2005, 96: 1613-1623.

15. Prota, G. progress in the chemistry of melanins and related metabolites. Med. Res. Rev. 1988, 8: 525-56.

16. Spritz, R.A., Hearing, V.J.Jr. Genetic disorders of pigmentation. Adv. Hum. Genet. 1994, 22: 1-45.

17. Frenk, E. Treatment of melasma with depigemting agents. In: Melasma: New Approaches to Treatment, pp. 9-15, matin Dunitz, London.

18. Garcia-Canovas, F., Garcia-Carnoma, F., Vera-Sanchez, J., Iborra-Pastor, J.L., Lozano-Taruel, J.A. The role of pH in the melanin biosynthesis pathway. J. Biol. Chem. 1982, 257: 8738-8744.

19. Rodriguez-Lopez, J.N., Tudela, J., Varon, R., Garcia-Carnova, F. Kinetic study on the effect of pH on the melanin biosynthesis pathway. Biochim. Biophys. Acta. 1991, 1076: 379-386.

20. Cooksey, C.J., Garratt, P.J., Land, E.J., Pavel, S., Ramsden, C.A., Riley, P.A. Evidance of the indirect formation of the catecholic intermediate substrate responsible for the autoactivation kinetics of tyrosinase. J. Biol. Chem. 1997, 272: 26226-26235.

21. Pawelek, J.M., Korner, A.M. The biosynthesis of mammalian melanin. AMSCA. 1982, 70: 136-145.

22. Mayer, A.M. Polyphenol oxidase in plants: recent progress. Phytocehmistry. 1987, 26: 11-20.

23. Whittaker, J.R. In: Food enzymes, structure and mechanism, pp. 271-307, Wong D. (ed.), Champman and Hall, New York.

24. Friedman, M. Food browning and its prevention: an overview. J. Agric. Food. Chem.

1996, 44: 631-653.

25. Barrett, F.M. Wound-healing phenoloxidase in larval cuticle of Calpodes ethulius (Lepidoptera: Hesperiidae). Can. J. Zool. 1984, 62: 834-838.

26. Sugumaran, M. Molecular mechanism for culticular sclerotization. Adv. Insect. Physiol. 1988, 21: 179-231.

27. Sugumaran, M. Molecular mechanisms for mammalian melanogenesis comparison with insect cuticular sclerotization. FEBS Lett. 1991, 293: 4-10.

28. Mosher, A.M., Pathak, M.A., Fitzpatrick, T.B. In: Dermatology in general medicine, pp. 205-225, Fitpatrick, T.B., Eisen, A.Z., Wolfff, K., Freedberg, I.M., Austern, K.F. (eds), Mc-Geaw-Hill, New York.

29. Maeda, K., Fukuda, M. In vitro effectiveness of several whitening cosmetic components in human melanocytes. J. Soc. Cosmet. Chem. 1991, 42: 361-368.

30. Zimmet, p., Alberti, K., Shaw, J. Global and societal implications of the diabetes epidemic. Nature. 2001, 414: 782-787.

31. baron, A.D. postprandial hyperglycemia and a-glucosidase inhibitors. Diabetes. Res. Clin. Pract. 1998, 40: 51-55.

32. Defronzo, R.A., Jacot, E., Jequier, E., Maeder, E., Wahren, J., Felber, J.P. The effect of insulin on the disposable of intervenous glucose. Results from indirect calorimetry and

hepatic and femoral venous catheterization. Diabetes. 1981, 30: 1000-10007.

33. Bhandari, M.R., Jong-Anurakkun, N., hong, G., Kawabata, J. a-Glucosidase and a-amylase inhibitory activities of Nepalese medicinal herb Pakhanbhed (Bergenia ciliate, haw.). Food Chem. 2008, 160: 247-252.

34. Saito, N., skai, H., Sekihara, H., Yajima, Y. effect of an a-glucosidase inhibitor (volibose), in combination with sulphonilureas, on glycemic control in type 2 diabetespatients. J. Int. med. Res. 1998, 26: 219-232.

35. Sels, J.P., hujiberts, M.S., Wolffenbuttel, B.H. Miglitol, a new alpha-glucosidase inhibitor. Expert opin. Pharmacother. 1999, 1: 149-156.

36. Stand, E., baumgartl, H.J., Fuchtenbusch, M., Stemplinger, J. Effect of acarbose on additional insulin therapy in type 2 diabetic patients with late failure of sulphonulurea therapy. Diabetes. Obes. Metab. 1999, 1: 215-220.

37. Hanefeld, M. The role of acarbose in the treatment of non-insulin-dependent diabetes mellitus. J. Diabetes. Complicat. 1998, 12: 228-237.

38. Diaz-Gutierrez, F.L., Ladero, J.M., Diaz-Rubio, M. Acarbose-induced acute hepatitis. Am. J. Gastroenterol. 1998, 93: 481-481.

39. Chapentier, G., Riveline, J.P., Varroud-Vial, M. management of drugs affecting blood glucose in diabetic patients with renal failure. Diabetes. Metab. 2000, 26: 73-85.

40. Matsui, T., Tanaka, T., Tamura, S., Toshima, A., Miyata, Y., Tanaka, K., matsumoto, K. Alpha-glucosidase inhibitory profile of actechins and theaflains. J. Agric. Food. Chem. 2007, 55: 99-105.

41. Kim, K.Y., Nam, K.A., kurihara, H., Kim, S.M. Potent a-glucosidase inhibitors purified from the red alga *Grateloupia ellipitica*. Phychem. 2008, 69: 2820-2825.

42. heo, S.J., Hwang, J.Y., Choi, J.I., Han, J.S., Kim, H.J., jeon, Y.J. Diphlorethohydroxycarmalol isolated from Ishige okamurae, a brown algae, a potent a-glucosidase and a-amylase inhibitor, alleviates postprandial hyperglycemia in diabetic mice. Eur. J. Pharmacol. 2009, 615: 252-256.

43. Harris, T., Cook, E.F., Kannel, W., Schatzkin, A., Goldman, L. Blood pressure experience and risk of cardiovascular diseas in the elderly. Hypertention. 1985, 7: 118-124.

44. Kannel, W.B., Higgins, M. Smoking and hypertension as predictors of cardiovascular risk in population studies. J. Hypertens. 1990, 8: 3-8.

45. Alper, A.B., Calhoun, D.A., Oparil, S. Hypertension. In Encyclopedia of life Science; nature publish group: London, UK; 2001, pp.1-8.

46. Skeggs, L.T., kahn, J.R., Shumway, N.P. the preparation and function of the hypertension converting enzyme. J. Exp. Med. 1956, 103: 295-299.

47. Li, G.H., Le, G.W., Shi, Y.H., Shrestha, S. Angiotensin I-converting enzyme inhibitory

peptides derived from food proteins and their physiological and pharmacological effects. Nutr. Res. 2003, 24: 469-486..

48. Ondetti, M.A. Design of specific inhibitors of angiotensin-converting enzyme: New class of orally active antihypertensive agents. Science. 1977, 196: 441-444.

49. Atkinson, A.B., Robertson, J.I.S. Captopril in the treatment of clinical hypertension and cardiac failure. Lancet. 1979, 2: 836-839.

50. Goretta, L.A., Ottaviani, J.I., Keen, C.L., Fraga, C.G. Inhibition of angiotensin converting enzyme (ACE) activity by flavan-3-ols and procyanidins. FEBS Lett. 2003, 555: 597-600.

51. Lee, D.H., Kim, J.H., Park, J.S., Choi, Y.J., Lee, J.S. isolation and characterization of a novel angiotensin-I-converting enzyme inhibitory peptide derived from the edible mushroom *Trichlolma gigaanteum*. Peptides. 2004, 25: 621-627.

52. Maruyama, S., Miyoshi, S., Tanaka, H. Angiotensin-I-converting enzyme inhibitor derived from *Ficus carica*. Agric. Biol. Chem. 1989, 53: 2763-2769.

53. Demain, A.L, Somkuti, G.A., Hunter-Cevera, J.C., Rossmoorem H.W., Novel microbial products for medicine and agriculture. Elsevier Science Publishers: Amsterdam, The Netherlands; 1989.

54. Fujita, H., Yokoyama, K., Yoshikawa, M. Classfication and antihypertensive activity of

angiotensin I-converting enzyme inhibitory peptise derived from food proteins. J. Food. Sci. 2000, 65: 564-569.

55. Wishart, D.S. Improving Early Drug Discovery through ADME meodelling: An Overview, Drug in R&D. 2007, 8: 49.

56. Kola, I., Landis, J. Can the pharmaceutical industry reduce attrition rates?. Nat. Rev. Drug. Discov. 2004, 3: 711-716..

57. Prentis, R.A., Lis, Y., Walker, S.R. Pharmaceutical innovation by the seven UK-owned pharmaceutical companies (1964-1985). Br. J. Clin. Pharmacol. 1988, 25: 387-396.

58. Hodgson, J. ADMET-turning chemicals into drugs. Nat. Biotech. 2001, 19: 722-726.

59. Butina, D., Segall, M.D., Frankcombe, K. Predicting ADME properties in silico: methods and models. Drug Discovery Today. 2002, 7: 83-88.

Reference (Part I)

1. Robb, D.A., Lontie, R, Editor, Copper proteins and Copper enzymes, Vol. 2 CRC Press, Boca Raton, FL, (1984), pp. 207- 240.

2. Espin, J.C., Wichers. H.J. Effect of captopril on mushroom tyrosinase activity in vitro. *Biochim. Biophys. Acta*. 2001, 1544, 289-300.

3. Chen, Q.-X., Kubo, I. Kinetic of mushroom tyrosinase inhibition by quercetin. *J. Agric.*

Food Chem. 2002, 50, 4108-4112.

4. Kariota, A.; Protopappa, A.; Megoulas, N.; Skaltsa, H. *Bioorg. Med. Chem*. 2007, 15, 2708.

5. Okombi, S., Rival, D., Bonnet, S., Mariotte, A.-M., Perrier, E., Boumendjel. A. Analogues of N-hydroxycinnamoylphenalkylamides as inhibitors of human melanocyte-tyrosinase. *Bioorg. Med. Chem. Lett*. 2006, 16, 2252-2255.

6. Tocco, G., Fais, A., Meli, G., Begala, M., Podda, G., Fadda, M.B., Corda, M., Attanasi, O.A., Filippone, P., Berretta, S. PEG-immobilization of cardol and soluble polymer-supported synthesis of some cardol-coumarin derivatives: Preliminary evaluation of their inhibitory activity on mushroom tyrosinase. *Bioorg. Med. Chem. Lett*. 2009, 19, 36-39.

7. Chawla, S., Delong, M.A., Visscher, M.O., Wickett, R.R., Manga, P., Boissy, R.E. Mechanism of tyrosinase inhibition by deoxyarbutin and its second generation derivatives. *Br. J. Dermatol*. 2008, 159, 1267-1274.

8. Zheng, Z.-P., Cheng, K.-W., Zhu, Q., Wang, X.-C., Lin, Z.-X., Wang, M. Tyrosianse inhibitory constituents from the roots of morus nigra: a structure-activity relationship study. *J. Agric. Food Chem*. 2010, 58, 5368-5373.

9. Sirat, H.M., Rezali, M.F., Ujang, Z. Isolation and identification of radical scavenging

and tyrosianse inhibition of polyphenols from *Tibouchina semidecandra* L. *J. Agric. Food Chem*. 2010, 58, 10404-10409.

10. Liu, D., Lan, R., Xin, X.L., Wang, X.J., Su, D.H., Yang, G.W. A new lavandulyl flavonoid from *Sorphora flavescens* Ait. *Chin. Chem. Lett*. 2008, 19, 1453-1455.

11. Kim, K.N., Heo, S.J., Yoon, W.J., Kang, S.M., Ahn, G.N., Yi, T.H., Jeon, Y.J. Fucoxanthin inhibits the inflammatory response by suppressing the activation of NF-kB and MAPKs in lipopolysaccharide-induced RAW264.7 macrophages. *Euro. J. Pharma*. 2010, 649. 369-375.

12. Kang, S.M., Kim, K.N., Lee, S.H., Ahn, K.N., Cha, S.H., Kim, A.R.D.S., Yang, X.D., Kang, M.C., Jeon, Y.J. Anti-inflammatory activity of polysaccharide purified from AMG-assistant extract of *Ecklonia cava* in LPS-stimulated RAW264.7 macrophages. *Carbohydr. Poly*. 2011, 85. 80-85.

13. Lee, S.H., Park, M.H., Heo, S.J., Kang, S.M., Ko, S.C., Han, J.S., Jeon, Y.J. Dieckol isolated from *Ecklonia cava* inhibits a-glucosidase and a-amylase in vitro and alleviates postprandial hyperglycemia in streptozotocin-induced diabetic mice. *Food. Chem. Toxicol*. 2010, 48. 2633-2637.

14. Heo, S.J., Ko, S.C., Cha, S.H., Kang, D.H., Park, H.S., Choi, Y.U., Kim, D.K., Jung, W.K., Jeon, Y.J. Effect of phlrotannin isolated from *Ecklonia cava* on melanogenesis

and their protetive effect against photo-oxidative stress induced by UV-B radiation. *Toxicol. in Vitro*. 2009, 23. 1123-1130.

15. Li, Y., Qian, Z.J., Ryu, B.M., Lee, S.H., Kim, M.M., Kim, S.K. Chemical components and its antioxidant properties in vitro: An edible marine brown alga, *Ecklonia cava*. *Bioorg. Med. Chem*. 2009, 17, 1963-1973.

16. Chiari, M.E.; Ullah, N. *Bioorg. Med. Chem*. 2010, 18, 4042.

17. Chen, Q.X., Song, K.K., Qiu, L., Liu, X.D., Huang, H., Guo, H.Y. Inhibitory effects on mushroom tyrosinase by p-alkoxybenzoic acids. *Food Chem*. 2005, 91, 269-274.

18. Kim, D.S., Kim, S.Y., Chung, J.H., Kim, H.H., Eun, C., Park, K.C. Delayed ERK activation by ceramide reduces melanin synthesis in human melanocytes. *Cell Signal*. 2002, 14, 779-785.

19. Bilodeau, M.L., Greulich, J.D., Hullinger, R.L., Bertolotto, C., Ballotti, R., Andrisani, O.M. BMP-2 stimulates tyrosinase gene expression and melanogensis in differentiated melanocytes. *Cell Res*. 2001, 14, 328-336.

20. Perola, E. Minimizing false positives in kinase virtual screens. *Bioinform.* 2006, 64, 422-435.

21. Gopalakrishnan, V., Aparna, J., Jeevan, M., Ravi, G.R., Desiraju. *J. Chem. Inf. Mod*. 2005, 45, 1101.

22. Fradera, X., Knegtel, R.M.A., Mestres, J. Similarity-driven flexible ligand docking. *Bioinform*. 2000, 40, 623-636.

23. Jang, J.Y., Lee, J.H., Jeong, S.Y., Chung, K.T., Choi, Y.H., Choi, B.T. partially purified *Curcuma longa* inhibits alpha-melanocytes-stimulating hormone-stimulating melanogenesis through extracellular signal-regulated kinase or Akt activation-mediated signaling in B16F10 cells. *Exp. Dermatol*. 2009, 18, 689-694.

24. Arung, E.T., Shimizu, K., Tanaka, H., Kondo, R. 3-Prenyl luteolin, anew prenylated flavone with melanin biosynthesis inhibitory activity from wood of Artocarpus heterophyllus. *Fitoterapia*. 2010, 81, 640-643.

25. Nofsinger, J.B., Liu, Y., Simon, J.D. Aggregation of eumelanin mitigates photogeneration of reactive oxygen species. *Free Radic. Biol. Med*. 2002, 32, 720-730.

26. Tomita, Y., Torinuki, W., Tagami, H. Stimulation of human melanocytes by vitamin D3 possibly mediates skin pigmentation after sun exposure. *J. Invest. Dermatol*. 1988, 90, 882-884.

27. Briganti, S., Camera, E., Picardo, M. Chemical and instrumental approaches to treat hyperpigmentation. *Pigment Cell Res*. 2003, 16, 101-110.

28. Chakrabotry, A.K., Chakraborty, D.P. *Int. J. Biochem*. 1993, 16, 101.

29. Iwata, M., Corn, T., Iwata, S., Everett, M.A., Fuller, B.B. The relationship between

tyrosinase activity and skin color in human foreskins. *J. Invest. Dermatol*. 1990, 95, 9-15.

30. Njoo, M.D., Westerhof, W., Bos, J.D., Bossuyt, P.M. The development of guidelines for the treatment of vitiligo. *Arch. Dermatol*. 1999, 135, 1514-1521.

31. Fenoll, L.G., Rodriguez=Lopez, J.N., Varon, R., Garcia-Ruiz, P.A., Garcia-Canovas, F., Tudela, J. Action mechanism of tyrosinase on meta-and para-hydroxylated monophenols. *J. Biol. Chem*. 2000, 381, 313-320.

32. Gandia-Herrero, F., Jimenez, M., Cabancs, J., Garcia-Carmona, F., Escribano, J. Tyrosinase inhibitory activity of Cucumber compounds: enzymes responsible for browning in Cucumber. *J. Agric. Food Chem*. 2003, 51, 7764-7769.

Reference (Part II)

1. Li, Y., Wen, S., Koda, B.P., Peng, G., Li, G.Q., Yamahara, J., Roufogalis, B.D. 2005. *Punical grantum* flower extract, a potent α-glucosidase inhibitor, improves postprandial hyperglycemia in Zucker diabetic fatty rats. J. Ethnopharmacol. 99, 239-244.

2. Zimmet, P., Alberti, K., Shaw, J. 2001. Global and societal implications of the diabetes epidemic. Nature. 414, 782-787.

3. Tewari, N., Tiwari, V.K., Mishra, R.C., Tripathi, R.P., Srivasrava, A.K., Ahmad, R.,

Srivastava, R., Srivastava, B.S. 2003. Synthesis and bioevaluation glycosyl ureas as alpha-glucosidase inhibitors and their effect on mycobacterium. Bioorg. Med. Chem. 11, 2911-2922.

4. Bharndari, M.R., Jong-Anurakkun, N., Hong, G., Kawabata, J. 2008. α-Glucosidase and α-amylase inhibitory activities of Nepalese medicinal herb parkhanbhed (*Bergenia ciliate* Haw.). Food Chem. 106, 247-252.

5. Kimura, K., Lee, J.H., Lee, I.S., Lee, H.S., Park, K.H., Chiba, S., Kim, D.M. 2004. Two potent competitive inhibitors discriminating alpha-glucosidase family I from family II. Carbohydr. Res. 339, 1035-1040.

6. Hollander, P. 1992. Safety profile of acarbose, an alpha-glucosidase inhibitor. Drugs. 44, 47-53.

7. Hanefeld, M. 1998. The role of acarbose in the treatment of non-insulin-dependent diabetes mellitus. J. Diabetes Complicat. 12, 228-237.

8. Matsui, T., Tanaka, T., Tamura, S., Toshima, A., Miyata, Y., Tanaka, K., Matsumoto, K. 2007. α-glucosidase inhibitory profile of catechins and theaflavins. J. Agric. Food Chem. 55, 99-105.

9. Nishioka, T., Kawabata, J., Aoyama, Y. Baicalein, an α-glucosidase inh ibitor from *Scuellaria baicalensis*. J. Nat. Prod. 61, 1413-1415.

10. Heo, S.J., Ko, S.C., Kang, S.M., Cha, S.H., Lee, S.H., Kang , D.H., Jung, W.K., Affan, A., Oh, C., Jeon, Y.J. 2010. Inhibitory effect of diphlorethohydroxycarmalol on melanogenesis and its protective effect against UV-B radiation-induced cell damage. 48, 1355-1361.

11. Kim, K.N., Heo, S.J., Kang, S.M., Ahn, G., Jeon, Y.J. 2010. Fucoxanthin induces apoptosis in human leukemia HL-60 cells through a ROS-mediated Bcl-xL pathway. Toxicol. In Vitro. 24, 1648-1654.

12. Li, Y., Qian, Z.J., Ryu, B.M., Lee, S.H., Kim, M.M., Kim, S.K. 2009. Chemical components and its antioxidant properties in vitro: An edible marine brown alga, *Ecklonia cava*. Bioorg. Med. Chem. 17, 1963-1973.

13. Watanabe, J., kawabeta, J., Kurihara, H., Niki, R. 1997. Isolation and identification of alpha-glucosidase inhibitors from Tochucha (*Eucommia ulmoides*). Biosci. Biotechnol. Biochem. 61, 177-178.

14. Perola, E. 2006. Minimizing false positives in kinase virtual screens. Prot. Struct. Funct. Bio. 64, 422-435.

15. Gopalakrishnan, B., Aparna, V., Jeevan, J.; Ravi, M., Desiraju, G.R. 2005. A virtual screening approach for thymidine monophosphate kinase inhibitors as antitubercular agents based on docking and pharmacophore models. J. Chem Inf. Model. 45, 1101-

1108.

16. Baron, A.D. 1998. Postprandial hyperglycemia and alpha-glucosidase inhibitors. Diabetes Research and Clinical Practice. 40, 551-555.

17. Kim, Y.M., Wang, M.H., Rhee, H.I. 2004. A novel α-glucosidase inhibitor from pine bark. Carbohydrate research. 339, 715-717.

18. Tadera, K., Minami, Y., Takamatsu, K., Matsuoka, T. 2006. Inhibition of α-glucosidase and α-amylase by flavonoids. Journal of Nutritional Science and Vitaminology. 52, 149-153.

19. Nickavar, B., Abolhasani, L., Izadpanah, H. 2008. α-Amylase inhibitory activities of six Salvia species. Iranian Journal of Pharmaceutical research. 7, 297-303.

20. Kim, K.Y., Nam, K.A., Kurihara, H., Kim, S.M. 2008. Potent α-glucosidase inhibitors purified from the red alga *Grateloupia ellipitica*. Phytochemistry. 69, 2820-2825.

Reference (Part III)

1. Amenta, F., Mignini, F., Rabbia, F., Tomassoni, D., Veglio, F. Protective effect of anti-hypertensive treatment on cognitive function in essential hypertension: Analysis of published clinical data. J. Neurol. Sci. 2002, 147: 203-204.

2. Lee, S.H., Quian, Z.J., Ki, S.K. A novel angiotensin I converting enzyme inhibitory

peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. Food. Chem. 2010,118: 96-102.

3. Jung, W.K., Mendis, E., Je, J.Y., Park, P.J., Son, B.W., Kim, H.C., Choi, Y.K., Kim, S.K. Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats. Food. Chem. 2006, 94: 26-32.

4. Bougatef, A., Nedjar-Arroume, N., Ravallec-Plé, R., Leroy, Y., Guillochon, D., Barkia, A., Nasri, M. Angiotensin I-converting enzyme (ACE) inhibitory activities of Sardinelle (*Sardinella aurita*) by-products protein hydrolysates obtained by treatment with microbial and visceral fish serine proteases. Food. Chem. 2008, 111: 350-356.

5. Park, E.J., Jhon, D.Y. The antioxidant, angiotensin converting enzyme inhibition activity, and phenolic compounds of bamboo shoot extracts. LWT-Food. Sci. Technol. 2010, 4: 655-659.

6. Kim, J.E., Hwang, K., Lee, S.P. ACE inhibitory and hydrolytic enzyme activities in textured vegetable protein in relation to the solid state fermentation period using *Bacillus subtilis* HA. Food. Sci. Biotechnol. 2010, 19: 487-495.

7. Kim, S.K., Byun, H.G., Park, P.J., Shahidi, F. Angiotensin I converting enzyme inhibitory peptides purified from bovine skin gelatin hydrolysate. J. Agric. Food. Chem. 2001, 49:

2992-2997.

8. Lee, J.K., Hong, S., Jeon, J.K., Kim, S.K., Byun, H.G. Purification and characterization of angiotensin I converting enzyme inhibitory peptides from the rotifer, *Brachionus rotundiformis*. Bioresour. Technol. 2009, 100: 5255-5259.

9. Shibata, T., Yamaguchi, K., Nagayama, K., Kawaguchi, S., Nakamura, T. Inhibitory activity of brown algal phlorotannins against glycosidases from the viscera of the turban shell *Turbo cornutus*. Eur. J. Phycol. 2002, 37: 493-500.

10. Xu, M.L., Wang, L., Hu, J.H., Lee, S.K., Wang, M.H. Antioxidant activities and related polyphenolic constituents of the methanol extract fractions from *Broussonetia papyrifera* stem bark and wood. Food. Sci. Biotechnol. 2010, 19: 677-682.

11. Handique, J.G., Baruah, J.B. Polyphenolic compounds: an overview. React. Funct. Polym. 2002, 52: 163-188.

12. Athukorala, Y., Jeon, Y.J. Screening for angiotensin 1-converting enzyme inhibitory activity of *Ecklonia cava*. J. Food. Sci. Nutr. 2005, 10: 134-139.

13. Hagerman, A.E. Extraction of tannin from fresh and preserved leaves. J. Chem. Ecol. 1988, 14: 453-461.

14. Porter, L.J. Condensed tannins. In: Rowe JW, editor. Natural Products of Woody Plants I. Berlin, Germany: Springer-Verlag; 1989. p.651-690.

15. Loganayaki, N., Siddhuraju, P., Manian, S. Antioxidant activity of two traditional Indian vegetables: *Solanum nigrum* L. and *Solanum torvum* L. Food. Sci. Biotechnol. 2010,19: 121-127.

16. Lee, S.H., Ko, S.C., Kang, S.M., Cha, S.H., Ahn, G.N., Um, B.H., Jeon, Y.J. Antioxidative effect of *Ecklonia cava* dried by far infrared radiation drying. Food. Sci. Biotechnol. 2010, 19: 129-135.

17. Glombitza, K.W., Hauperich, S. Phlorotannins from the brown alga *Cystophora torulosa*. Phytochemistry .1997, 46: 735-740.

18. Lüder, U.H., Clayton, M.N. Induction of phlorotannins in the brown macroalga *Ecklonia radiata* (Laminariales, Phaeophyta) in response to simulated herbivory the first microscopic study. Planta. 2004, 218: 928-937.

19. Heo, S.J, Jeon, Y.J. Radical scavenging capacity and cytoprotective effect of enzymatic digests of *Ishige okamurae*. J. Appl. Phycol. 2008, 20: 1087-1095.

20. You, J.S., Sung, M.J., Chang, K.J. Evaluation of 8-week body weight control program including sea tangle (Laminaria japonica) supplementation in Korean female college students. Nutr. Res. Pract. 2009, 3: 307-314.

21. Heo, S.J., Ko, S.C., Cha, S.H., Kang, D.H., Park, H.S., Choi, Y.U., Kim, D., Jung, W.K., Jeon, Y.J. Effect of phlorotannins isolated from *Ecklonia cava* on melanogenesis and

their protective effect against photo-oxidative stress induced by UV-B radiation. Toxicol. In Vitro. 2009, 23: 1123-1130.

22. Ahn, G.N., Kim, K.N., Cha, S.H., Song, C.B., Lee, J., Heo, M.S., Yeo, I.K., Lee, N.H., Jee, Y.H., Kim, J.S., Heu, M.S., Jeon, Y.J. Antioxidant activities of phlorotannins purified from *Ecklonia cava* on free radical scavenging using ESR and H2O2-mediated DNA damage. Eur. Food. Res. Technol. 2007, 226: 71-79.

23. Torruco-Uco, J., Chel-Guerrero, L., Martínez-Ayala, A., Dávila-Ortíz, G., Betancur-Ancona, D. Angiotensin-I converting enzyme inhibitory and antioxidant activities of protein hydrolysates from *Phaseolus lunatus* and *Phaseolus vulgaris* seeds. LWT-Food. Sci. Technol. 2009, 42: 1597-1604.

24. Appel, H.M.. Phenolics in ecological interactions: the importance of oxidation. J. Chem. Ecol. 1993, 19: 1521-1552.

Reference (Part IV)

1. Shibata, T., Fujimoto, K., Nagayama, K., Yamaguchi, K., Nakamura,T. Inhibitory activity of brown algal phlorotannins against hyaluronidase. Int. J. Food. Sci. Technol. 2002, 37: 703-709.

2. Rice-Evans, C.A., Muller, N.J., Bolwell, P.G., Barmley, P.M., Pridham, J.B. The relative

antioxidant activities of plant-derived polyphenolic flavonoids. Free. Radical. Res. 1995, 22: 375-383.

3. Jorgensen, L.V., Madsen, H.L., Thomsen, M.K., Dragsted, L.O., Skibsted, L.H. Regulation of phenolic antioxidants from phenoxyl radicals: An ESR and electrochemical study of antioxidant hierarchy. Free. Radical. Res. 1999, 30: 207-220.

4. Haslam, E. plant polyphenols: vegetable tannins revisited. Cambridge, UK: Cambridge University Press.

5. Regan, M.A., Glombitza, K.W. handbook of physiological Methods. Cambridge: Cambridge University Press: 1986, 129-241.

6. Cartiona, M.S., Cai, Y., Russell, M., Haslam, E. Polyphenol complexation-some thoughts and observations. Phytochem. 1988, 27: 2397-2409.

7. Shi, B., He, X.Q., Haslam, E. Gelatin-polyphenol interaction. J. Am. Laeth. Chemi. Associat. 1994. 104: 98-104.

8. Huang, H.H., Kwok, K.C., Liang, H.H. Effect of tea polyphenols on the activaties of soybean trypsin inhibitors and trypsin. J. Sci. Food. Agric. 2004. 84: 121-126.

9. Bertoldi, M., Gonalvi, M., Voltattorni, C.B. Green tea polyphenols: novel irreversible inhibitors of DOPA decarboxylase. Biochem. Biophysic. Res. Commun. 2001. 284: 90-93.

10. Abe, I., Seki, T., Umehara, K., Miyase, T., Noguchi, H., Sakakibara, J., et al. Green tea polyphenols: novel and potent inhibitors of squalene epoxidase. Biochem. Biophysic. Res. Commun. 2000. 268: 767-771.

11. Ghosh, K.S., maiti, T.K., Dasgupta, D. green tea polyphenols as inhibitor of ribonuclease A. Biochem. Biophysic. Res. Commun. 2004. 325: 807-811.

12 Li, Y., Qian, Z.J., Ryu, B.M., Lee, S.H., Kim, M.M., Kim, S.K. Chemical components and its antioxidant properties in vitro: An edible marine brown alga, *Ecklonia cava*. *Bioorg. Med. Chem*. 2009, 17, 1963-1973.

13. Cam, J.M., Luck, V.A., Eastwood, J.B., De Wandener, H.E., The effect of aluminum by hydroxide orally on calcium, phosphorus, and aluminum metabolism in normal subjects. Clin. Molec. Med. 1976. 51: 407.

14. Qiang, H., Yuanping, L., kai, Y. Effects of tea polyphenol on the activities of α-amaylase, pepsin, trypsin and lipase. Food Chem. 2006, 101: 1178-1182.

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