Thesis for the Degree of Master of Science

Phlorotannins isolated from *Ecklonia stolonifera* inhibit tyrosinase activity and melanin production on B16F10 melanoma cells

by

Samsuzzaman

Department of Food and Life Science

The Graduate School

Pukyong National University

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(*Ecklonia stolonifera* 로 부터 분리된 플로로탄닌의 타이로시나아제 저해활성)

Advisor: Prof. Hyeung-Rak Kim

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Samsuzzaman

Approved by:

(Chairman) Jae Sue Choi

(Member) Jae Il Kim

(Member) Hyeung-Rak Kim

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Contents

List of Figures

Figure

Page

List of Tables

Table
Page

Abbreviations

L-DOPA : 3,4-dihydroxyphenylalanine

UV : Ultraviolet

EtOAC : Ethylacetate

ADT : AutoDock Tool

PDB : Protein Data Bank

IC_{50} : Half inhibitory concentration

K_m : Michaeils constant

V_{max} : Maximum velocity

*K*i : Inhibition constant

Ecklonia stolonifera 로 부터 분리된 플로로탄닌의 타이로시나아제 저해활성

Samsuzzaman

부경대학교 대학원 식품생명과학과

요약

멜라닌은 태양 복사로부터 피부를 보호하는 데 필요한 기본 요소이다. 인간의 피부에 멜라닌이 비정상적으로 축적되면 여러 색소 침착 질환이 발생한다. 타이로시나제는 피부의 과 색소 침착을 담당하는 멜라닌 생성을 촉진시키는 주요 조절자 역할을 한다. 본 연 구에서는 곰피로부터 분리된 플로로탄닌이 버섯 타이로시나제, 세포 타이로시나제 및 멜라닌 세포의 멜라닌 생성에 있어서 억제 효과를 하고자하 규명 하 였다. 칼럼 크로마토그래피를 이용하여 곰피 에탄올 추출물의 EtOAc 분획으로부터 7개의 플로로탄닌 화합물을 분리하였다. 그 중 4가지 화합물 인 bieckol, dieckol, phloroglucinol, 2-phloroeckol은 버섯 타이로시나제에 대하여 저해 활성을 보였다. 2-phlororeckol 은 IC_{50} 값이 9.33 ± 0.042 μM 로 가장 높은 저해 활성을 보였고 dieckol 의 IC_{50} 값은 61.81 ± 1.27 μM로 두 번째로 높은 저해활성을 보였다. 또한 kojic acid 의 IC_{50}값은 75.32 ± 4.57 μM 이었다. Lineweaver-Bark plot 의 결과는 2-phloroeckol 과 dieckol이 버섯 타이로시나제에 혼합형 억제제이고 억제 상수 (Ki) 가 각각 6.87 μM과 41.26 μM 로측정되 었다. 킹 분석 에 따르면 버섯 타이로시나제에 대한 2-phloroeckol 과 dieckol 의 결합 에너지 값은 각각 -8.00 Kcal/mol과 -2.6 Kcal/mol이었다. 또한 2-phloroeckol 과 dieckol 은 α-MSH로 자극로니 B16F10 세포의 타이로시나제를 억제하였으며 IC_{50} 값은 각각 72.88 ± 2.09 μM 과 164.66 ± 2.57 μM 이었고 kojic acid 의 IC_{50} 값은 267.06 ± 3.90μM 이었다. 2-phloroeckol 과 dieckol 은 α-MSH로 자극로니 B16F10 흑색종 세포에서 각각 80 μM 과 50 μM 에서 37 %와 21 %의 멜라닌 생성 억제를 보였다. 이러한 결과들은 2-phloroeckol 과 dieckol 이 피부 미백제로 사용될 수 있음을 사사하고 있다.

I. Introduction

Skin pigmentation is the variation in skin color of human depending on the connecting network of genetic, ethnic or physiological characteristics. In body, melanocytes have an important role in pigmentation. These are dendritic cells found in epidermal basal layer of the skin. The quantity of melanocytes is not same in the light and dark color skin. Thus, a smaller number of melanocytes are found in light skin compare to dark skin. Moreover, skin color depends on several parameters such as size, degradation and distribution of melanocytes. Melanin plays a pivotal role in skin pigmentation. It is synthesized in specialized membrane bound organelle called melanosome present in melanocytes, normally, melanin is one of the elements to protect the human skin from ultraviolet radiation (UV) induced damage (Thanigaimalai et al. 2017). However, unusual deposition of melanin in the human skin is associated with several hyperpigmenting diseases, such as freckles, ephelides, senile lentigines and melasma (Slominski et al. 2004). Melanogenesis (Fig. 1) is a process of physiological response of skin to UV light and this process is mainly regulated by tyrosinase and its related proteins (tyrosinase related protein 1 and tyrosinase related protein 2) (Hearing et al. 2011). Any deregulation in tyrosinase function is associated with various skin diseases in mammals and finally, produce cancerous skin cells

(Rao et al. 2013).

Tyrosinase (EC 1.14.18.1), a binuclear copper containing maltifunction monooxygenase, plays an important role in melanin synthesis (Parveen et al. 2010). It is synthesized in melanocytes. Moreover, plants, fungi, bacteria, animal kingdom including humans are the major distributing sites of tyrosinase (Artes et al.1998). Tyrosinase is involved in melanin synthesis by catalyzing two steps reaction : one is conversion of tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) by hydroxylation and another is oxidation of L-DOPA to dopaquinone, and this processes occurred continuously at physiological pH (Cooksey et al. 1997). Quinone derivatives are capable to polymerize and react with amino acids and proteins to form high molecular weight molecules which accelerate the formation of melanin (Vamos-Vigyazo et al. 1981).

Two types of melanin are biosynthesized: the oxidative polymerization of cysteinyldopa is responsible for yellow to red pheomelanin synthesis and the synthesis of eumelanin by oxidative polymerization of 5,6-dihydroxyindoles is responsible for brown to black color (Mapunya et al. 2012 and d'Ischia et al. 2013). In the cytoplasm of melanoma cell, pheomelanin has the ability to absorb free radicals produced from UV light which is the cause of skin demage rather than skin protection (Seo et al. 2003). However, undesirable browning effects of melanin in human skin (Kubo et al. 2000) have encouraged scientists to discover or synthesize new effective tyrosinase inhibitors for use in skin

whitening or its related disorders. To discover the new skin whitening agents, an enormous number of tyrosinase inhibitors, such as kojic acid, arbutin and dihydroxybenzene have been identified via either from natural origin or from synthetic process (Kim et al. 2005). Even though countless efforts have been given to find out potent tyrosinase inhibitors, there are some limitations of existing agents in terms of skin irritation, cost, skin penetration and toxicity (Parvez et al. 2006). Up-to-date, a good number of laboratory synthesized antimelanogenic agents have been reported. The number of skin whitening agents from marine algae are a few. From this background to enrich the skin whitening agents dictionary from marine source, the research was conducted with compounds isolated from ethyl acetate (EtOAc) fraction of ethanolic extract of *E. stolonifera*.

For long time, marine algae have been utilized as a household medicine for the purpose of curing skin related diseases in the Southeastern Europe and Asia (Hoppe et al. 1982). Marine macro algae are classified into three groups: the phaeophyceae (brown algae), the cyanophyceae (blue algae) and the rhodophyceae (red algae). Marine algae are a well established source of phlorotannins (Nwosu et al. 2011). Phlorotannins are derivatives of phloroglucinol (secondary metabolite of brown algae) only produced by brown algae that form polymer complex through different linkage like, phenyl (fucols), ether (fuhalols and phlorethols) and 1,4-dibenzodioxin (eckols) (Lopes et al. 2012). *E. stolonifera* is a brown algae belongs to the family of Laminariaceae. It has been taken as a culinary

dish by Korean people. Pholorotannins, isolated from *E. stolonifera,* are polyphenolic compounds exhibited different biological activities, such as anti-adipogenic (Jung et al. 2014), hepatoprotective (Lee et al. 2012), antioxidant (Kim et al. 2009), anti-inflammatory (Kim et al. 2009) and antidiabetic (Yoon et al. 2008). Previously, the antioxidant (Kim et al. 2009), hepatoprotective (Lee et al. 2012) and anti-inflammatory activities(Kim et al. 2009) of phlorotannins, isolated from *E. stolonifera*, have been studied. Till to date, no research work has been done on anti-melanogenic activity of 2-phloroeckol isolated from *E. stolonifera*. The objective of this study was to investigate the inhibitory effect of phlorotannins on mushroom tyrosinase, cellular tyrosinase and melanin production in α-MSH-stimulated B16F10 melanoma cells.

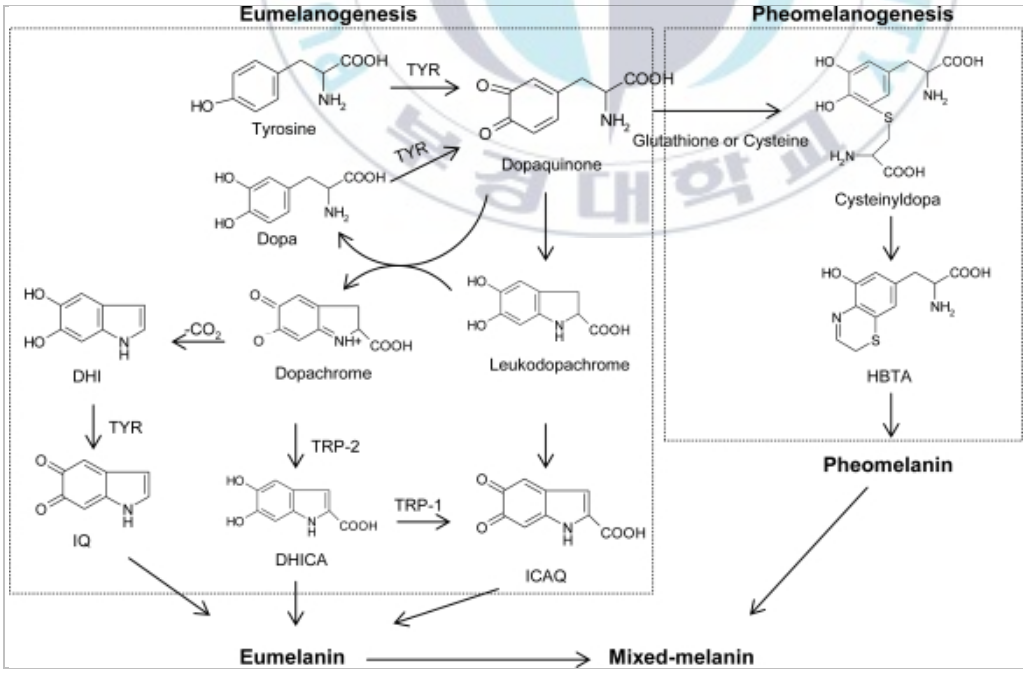


Figure 1. Biosynthesis pathway of melanin formation.

II. Materials and Methods

1. Chemicals and reagents

Mushroom tyrosinase (EC 1.14.18.1), kojic acid, 3,4-dihydroxy-$_{L}$-phenylalanine(L-DOPA), $_{L}$-tyrosine, 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-trtrazolium (MTS), α-melanocyte-stimulating hormone (α-MSH), and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich (St.Luis, MO,USA). Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin mixture, 0.25% trypsin ethylenediamineetraacetate (EDTA) and fetal bovine serum (FBS) were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA). CellTiter 96 AQous one solution cell proliferation assay kit from Promega (Madison, WI, USA). The B16F10 murine melanoma cell line was from American Type Culture Collection (ATCC, Manassas, VA).

2. Isolation of phlorotannins from *Ecklonia stolonifera*

E. stolonifera was was collected from coastal region of Busan, South Korea and deposited with voucher specimen in laboratory (H. R. Kim). Plants were dried by dryer at 50°C for 72 hours and stored at -20°C until used. The isolation procedure of phlorotannin was previously described by Lee et al. 2012. In short, backward process of powder (3 kg) form of *E. stolonifera* was conducted with ethyl alcohol (3x9 L). The partitioning of extract was accomplished with different solvents, yielding the EtOAc (78 g), n-hexane (93 g), n-butanol (87 g) and water (430 g). Among the solvents, highest antioxidant activity of EtOAc fraction was noticed by DPPH scavenging assay. The ethyl acetate fraction of methanolic extract of *E. stolonifera* was subjected to silica gel and hydrophobic chromatographies. Phlorotannins were purified by HPLC and Mass analyzer, and the purity was affirmed >99 %, based on the peak area of specific wavelength in the HPLC analysis.

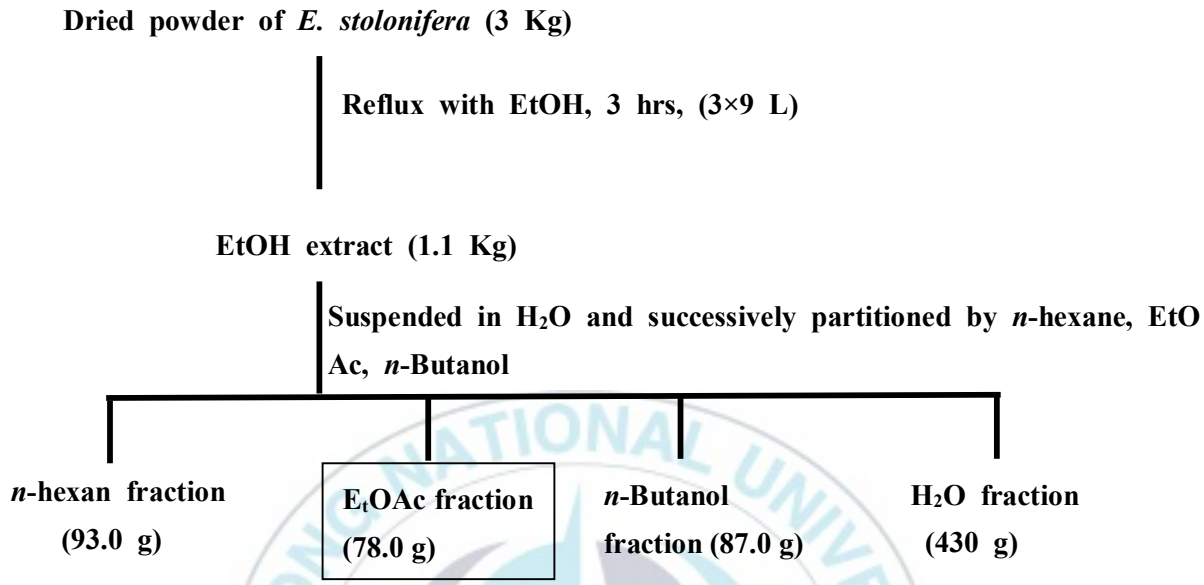


Figure 2. Extraction and fractionation procedure of *E.stolonifera*

3. Mushroom tyrosinase inhibitory activity assay

Inhibitory activity of phlorotannins against mushroom tyrosinase was measured by previously reported method with slight change (Hyun et al. 2008). In 96-well microplate, 10 μL of each compounds at various concentrations was mixed with 170 μL solution containing the ratio 10:10:9 of 1mM L-tyrosine solution, 50 mM phosphate buffer solution and distilled water. After that, mixture was incubated for 10 min at 25°C. Then, 20 μL of mushroom tyrosinase (1000 units/mL) solution, prepared in 50 mM phosphate buffer (pH 6.5), was added to mixture of each well and allowed to incubation for 40 minutes at 25°C. Various concentrations of compounds dissolved in DMSO were diluted with distilled water. Kojic acid was used as a positive control. The absorbance of dopachrome formation was taken at 490 nm. Inhibitory activity of compounds was calculated by the following equation:

Inhibition (%) = 1-[(A-B)/(C-D)] x100

Where, A and B are the absorbance of sample and color control, respectively. C and D are the absorbance of control and blank, accordingly. Sample = The absorbance of mixture with compound, color control = The absorbance of mixture without enzyme, control = The absorbance of mixture without compound, blank = The absorbance of phosphate buffer solution. The inhibitory activity of compounds was expressed as the amount of compound needed to inhibit 50% of tyrosinase activity.

4. Kinetic analysis of 2-phloroeckol and dieckol against mushroom tyrosinase

To determine the inhibition type of 2-phloroeckol and dieckol on mushroom tyrosinase, kinetic analysis was conducted. The type of inhibition was determined by Lineweaver-Burk plots. To obtain Lineweaver-Burk plots, various concentration of 2-phloroeckol (0, 5, 10, and 20 μM), dieckol (10, 20, 40 and 80 μM) and substrate (1, 2 and 4 mM) were used. Method of kinetic experiment was same like as mushroom tyrosinase inhibition assay as previously described in method section, except different substrate concentrations. First, 10 μL of each compound at different concentrations and 20 μL of tyrosinase enzyme (1000U/mL) were mixed in 96-well microplate and incubated for 10 minutes. Then, different concentrations of substrate solution (170 μL) was added to each well of 96-well microplate and incubated for 40 minutes. Enzyme-linked immunosorbent assay (ELISA) reader was used to measure the absorbance at 490 nm. The Lineweaver-Burk plots was also used to determine the maximum velocity (V_{max}) and Michaelis constant (K_m) value. The inhibition constant (K_i) value obtained from Dixon plots.

5. Docking simulation in tyrosinase inhibition

Molecular docking was performed to analyze the binding interaction between 2-phloroeckol or dieckol and 3D structure of mushroom tyrosinase. In this study, AutoDockVina 4.2 (ADT), Discovery Studio and chimera software were used for docking simulation because of their wide acceptability (Moustakas et al. 2006). L-tyrosine binding area in *Agaricus bisporus* tyrosinase was used for docking simulation to get better binding results. A crystallographic structure of mushroom tyrosinase was obtained from RCSB protein data bank (PDB: 2y9x) and its structure was edited by removing different chains, solvent and ligand and saved as protein data bank (PDB) file. This PDB file was uploaded into AutoDockVina with fixing X, Y and Z centered as a -10.021, -28.823 and 43.596, respectively. The three dimentional structure of 2-phloroeckol and dieckol were obtained from Chem spider dictionary (http://www.chemspider.com/StructureSearch.aspx) and pubchem, respectively. Addition of hydrogen atoms to compound and charge calculation of compound were accomplished using ChemOffice (http://www.cambridgesoft.com). Discovery studio software was used to identify the possible bond formation between 2-phloroeckol or dieckol and mushroom tyrosinase. Tropolone and arbutin were used for comparison.

6. Cell culture

B16F10 murin melanoma cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Melanoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1 % penicillin-streptomycin at 37°C in humidified condition with 5% CO_2. The medium was changed in every 48 hours.

7. Cellular tyrosinase inhibition assay

The inhibitory activity of phlorotannin on cellular tyrosinase also examined by previously described process (Ohguchi et al. 2003). For cellular tyrosinase inhibition assay, cells were seeded in cell culture plate (100x20 mm) at density of $2x10^5$ cells per well for 24 hours. Then, cells were exposed to α-MSH (200 nm) and incubated for 48 hours. Treated cells were washed twice with cold phosphate buffer saline (PBS) and scrapped to detach the cells from plate. Then, cells were subjected to centrifuge for 5 minutes at 4000 rpm and lysed with 1000 µL of lysis buffer [0.1 M phosphate buffer (pH 6.8), containing 0.1 % Triton (v/v)]. After centrifugation at 11000 xg for 30 minutes, cells supernatant collected as a crude cellular tyrosinase. Inhibitory activity of phlorotannin against cellular tyrosinase was determined using a modified mushroom tyrosinase inhibition assay. Initially, 50 µL of 50 mM phosphate buffer (pH 6.8), 10 µL of compound at different concentrations dissolved in DMSO and 30 µL of crude lysates (58 µg) were added to each well of 96-well microplate and incubated for 10 minutes. Then, reaction was started after adding 10 µL of L-DOPA substrate solution [(0.18 mg /mL, in 50mM phosphate buffer (pH 6.8)] allowing incubation for 40 minutes. The amount of dopachrome formation was observed by measuring absorbance in every 10 minutes at 450 nm using ELISA reader.

8. Cell survival assay

Melanoma cells were cultured in 96-well microplate at a density of $1x10^4$cells per well for 24 hours. After that, 10% FBS containing DMEM replaced by 2% FBS containing DMEM and incubated for 4 hours. Then, cells were exposed to different concentrations of 2-phloroeckol (0, 12.5 , 25 and 50 μM) and dieckol (0, 12.5 , 25 , 50 and 100 μM) with 2% fetal bovine serum for 24 hours. After 24 hours, medium changed with 100 μL of MTS solution 0.5 mg/mL (dissolved in phosphate buffer saline) for 1 hour while control cells were treated with 0.1% DMSO. Enzyme-linked immunosorbent assay (ELISA) reader was used to take absorbance of viable cells at 490 nm.

9. Melanin content inhibition assay

Inhibitory activity assay of 2-phloroeckol and dieckol on melanin production in B16F10 melanoma was conducted by previously described method with slight modification (Hosoi et al. 1985). To determine inhibition of melanin production, B16F10 melanoma cells were seeded ($2x10^4$) in 24-well plate with DMEM at 5% CO_2 atmoshpheric conditions for 24 hours. Then, cells were pretreated with different concentrations of 2-phloroeckol and dieckol for 1 hour, and melanoma cells were stimulated with α-MSH (200 nm) for 72 hours at 37°C. Treated cells were washed twice by phosphate buffer saline (PBS) and harvested with 0.25% trypsin EDTA before centrifugation at 4000 rpm for 5 minutes. Then, cells were lysed with phosphate buffer (pH 6.8) containing 1N NaOH for 40 minutes at 80°C. After centrifuge at 14,000 rpm for 10 minutes, absorbance of the supernatant was measured at 405 nm . The percentage of inhibition expressed as percentage of treated cell compared to untreated cell. Kojic acid was used as positive control.

10. Statistical analysis

Data are expressed as mean ± standard deviation of three individual experiments. The significance of differences were analyzed by one-way analysis of variance (ANOVA). Significant differences were considered at values of $p< 0.05$. Results were analyzed using SPSS (Chicago, USA).

III. Results

1. Preparation and isolation of phlorotannins

Seven phlorotannins, including 2-phloroeckol, dieckol, bieckol, phloroglucinol, eckol, phlorofucofuroeckol A and phlorofucofuroeckol B have been isolated from E_tOA_C fraction of *E. stolonifera*. The structure of phlorotannin was recognized by spectrum data of ^{1}H and ^{13}C NMR as well as by comparison with retention time in HPLC chromatography. The isolated seven phlorotannins structure are shown in Fig. 3.

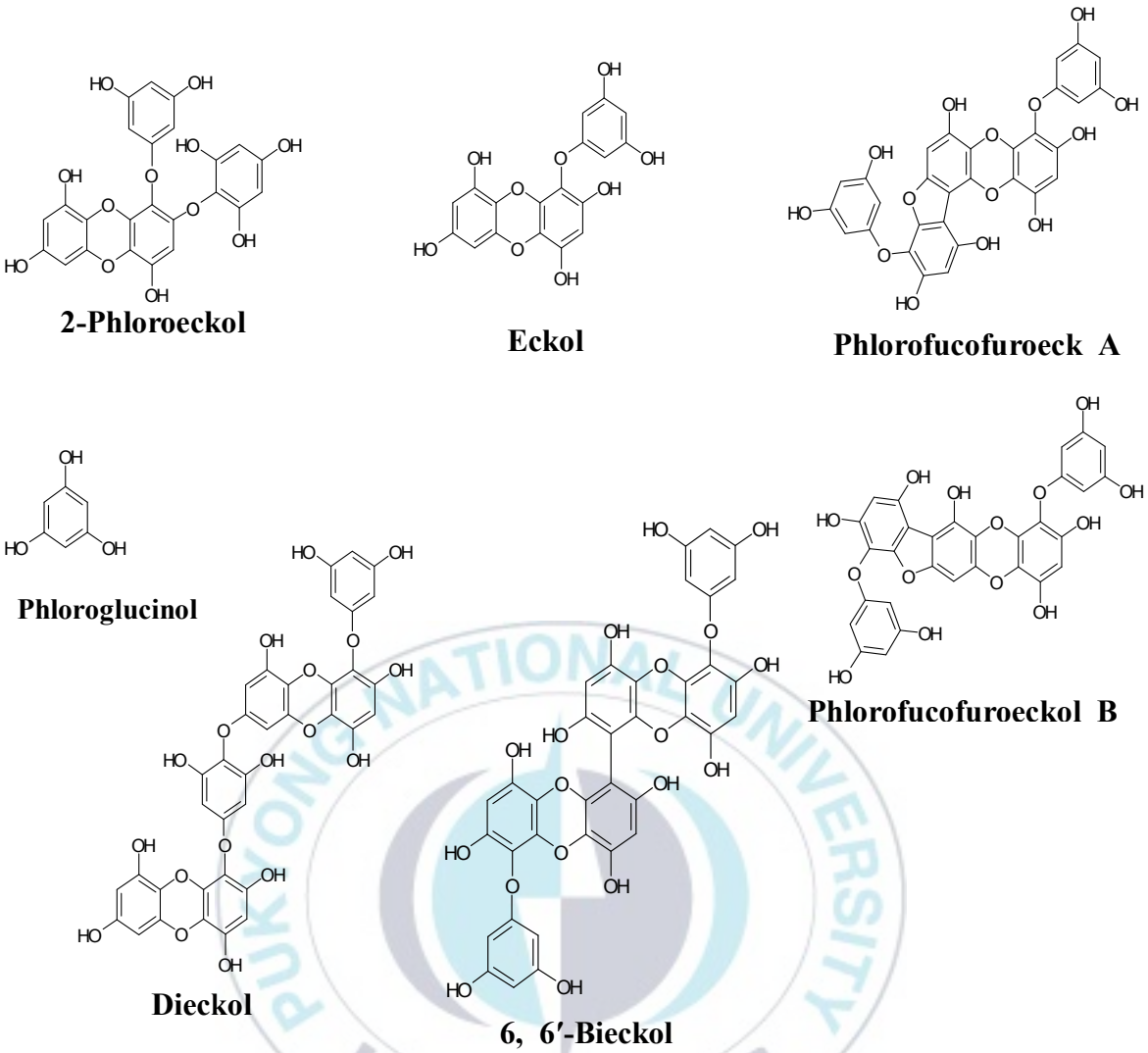


Figure 3. Chemical structure of phlorotannins

2. Mushroom tyrosinase inhibitory activities of phlorotannins

Seven compounds isolated from *E.stolonifera* were assesed the mushroom tyrosinase inhibitory activity. Kojic acid, widely using as a potent tyrosinase inhibitor in cosmetic industry for skin whitening, was used as a positive control. Two of them, bieckol and phloroglucinol exhibited inhibition against mushroom tyrosinase, whereas dieckol showed better inhibitory activity against tyrosinase compare to previous two inhibitors. But 2-phloroeckol showed strong inhibitory activity which was about to nine fold compare to standard inhibitor kojic acid. The IC_{50} value of 2-phloroeckol, dieckol, bieckol, phloroglucinol and kojic acid against mushroom tyrosinase are shown in Table 1. Inhibitory percentage of 2-phloroeckol, dieckol, bieckol, phloroglucinol and kojic acid on mushroom tyrosinase was 64.19 ± 1.76, 39.86 ± 0.66, 29.36 ± 0.79, 19.45 ± 3.07 and 38.85± 0.18, respectively, at 20 μM (Figure 4). The other remaining compounds were not showed their inhibitory activity against mushroom tyrosinase.

Table 1. Inhibitory effects of phlorotannins and kojic acid on mushroom tyroinase activity.

Compounds	**[a]IC_{50} (μM, mean ± SD)**
2-phloroeckol	9.33 ± 0.042
Dieckol	61.81 ± 1.27
Bieckol	83.72 ± 1.25
Phloroglucinol	117.58 ± 1.32
Phlorofucofuroeckol A	> 200
Phlorofucofuroeckol B	> 200
Eckol	> 200
Kojic acid	75.32 ± 4.57

[a]The 50% inhibitory concentration (IC_{50}) values were calculated from log dose inhibition curve.

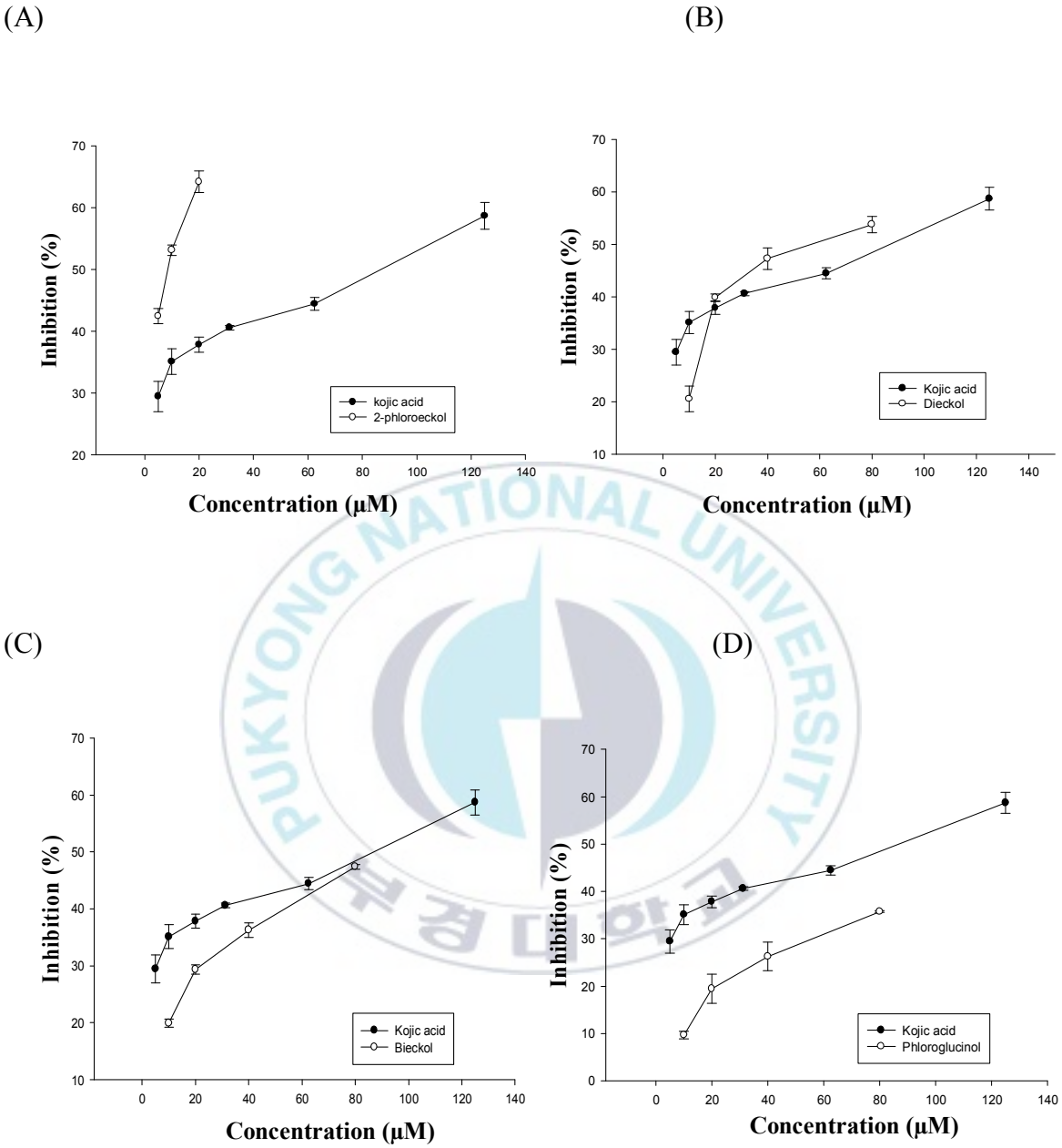


Figure 4. Inhibitory effects of 2-phloroeckol, dieckol, bieckol, phloroglucinol and kojic acid against mushroom tyrosinase. Values are expressed as mean ± SD from three independent experiments.

3. Determination of inhibition type from kinetic analysis

To determine the inhibition type, 2-phloroeckol and dieckol were used for kinetic analysis because of their highest inhibitory activity against mushroom tyrosinase. The inhibitory mode was determined by Lineweaver-Burk plots in the presence of different concentrations of 2-phloroeckol or dieckol and substrates (Fig 5). Kinetic parameters of 2-phloroeckol and dieckol in the presence of different concentrations are shown in Table 2. Straight line with different slopes acquired from 1/[S] versus 1/V plots. In this experiment, different concentrations of 2-phloroeckol and dieckol were facilitated to change the value of V_{max} with altering K_m value (Fig. 5), which suggested that they are a mixed type inhibitor of mushroom tyrosinase. Mixed inhibitors show their inhibitory mechanism by binding not only with free enzyme but also with enzyme-substrate complex (Te-sheng et al. 2009). The estimated km and Vmax values of 2-phloroeckol and dieckol are presented in Table 2. The approximate ki value of 2-phloroeckol and dieckol were estimated to be 6.87 and 4.26 μM, respectively (Table 2).

Table 2. Kinetics parameters of 2-phloroeckol and dieckol against mushroom tyrosinase.

Compound concentrations	**K_m (mM)[a]**	**V_{max} (ΔOD_{490}/min)[b]**	**K_i[c]**
2-phloroeckol			
5 μM	0.71 mM	3.7×10^{-3}	
10 μM	0.90 mM	3.5×10^{-3}	6.87
20μM	μM		
	1.2 mM	2.5×10^{-3}	
Dieckol			
10 μM			
20 μM	0.43 mM	2.5×10^{-3}	
40μM	0.57 mM		1.9×10^{-3}
80 μM	41.26 μm		
	0.64 mM	1.7×10^{-3}	
	0. 87 mM	1.6×10^{-3}	

[a]K_m is the Michaeils constant, [b] V_{max} denotes the maximum velocity and [c]K_i is the inhibitory constant.

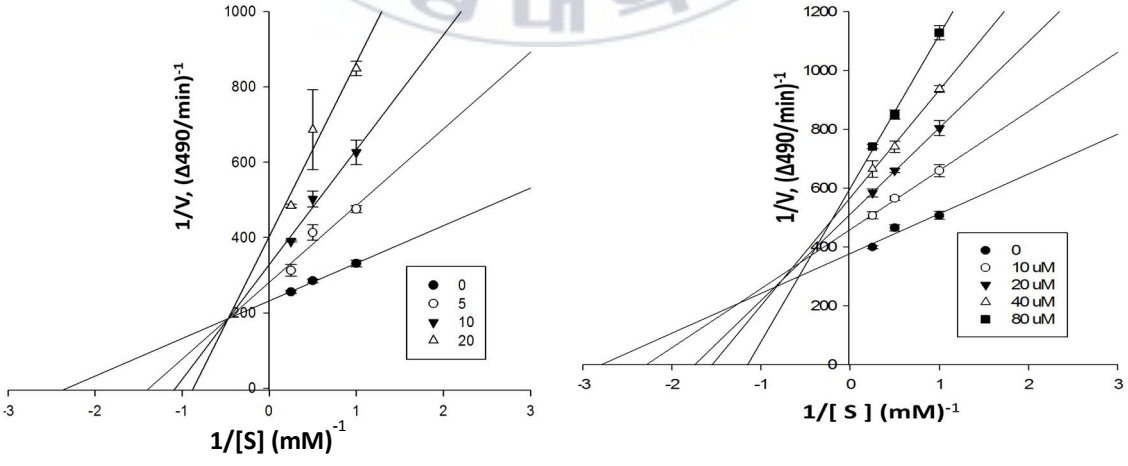


Figure 5. Lineweaver-Burk plots of 2-phloroeckol and dieckol for mushroom tyrosinase.

Activity was measured in the presence of different concentrations of 2-phloroeckol (5, 10 and 20 μM) and that of dieckol (10-80 μM). L-tyrosinase (1-4 mM) used as a substrate.

4. Docking Analysis

In the next, molecular simulation study was conducted to predict accurate interaction between 2-phloroeckol or dickol and mushroom tyrosinase. Some inevitable terms such as binding affinity, hydrogen bond and amino acid residues interactions are exclusively needed for successful docking. Docking affinity score, hydrogen bond, van der walls bond, Pi-Alkyl bond, Pi-Pi stacked bond and other bond formation residues are shown in Table 3 and figure (7, 8).

4.1 Docking simulation with 2-phloroeckol

The docking results and binding sites of 2-phloroeckol to crystal structure of mushroom tyrosinase are represented in Table 3 and figure 7A. The binding affinity score of 2-phloroeckol, arbutin and tropolone against mushroom tyrosinase was shown in Table3. The binding energy values are expressed as Kcal/mol. 2-phloroeckol (Fig. 6A) binds at approximately same binding region of mushroom tyrosinase as tropolone did. 2-phloroeckol formed four hydrogen bonds with different amino acid residues of tyrosinase, while arbutin and tropolone formed one hydrogen bond with amino acid residue of tyrosinase (Fig. 8A & 8B). The oxygen atom of hydroxyl group presented in structure was predicted to responsible for hydrogen bond interaction. The attachment of benzene ring with hydroxyl group at C-13 position of 2-phloroeckol was considered as most responsible for inhibitory

activity. Therefore, tyrosinase residues VAL283 and ALA286 were formed Pi-Alkyl bond with 2-phloroeckol, and the residues HIS263 and PHE264 were found to form Pi-Pi stacked bond. The tyrosinase residues HIS259, ASN260, HIS85, HIS61, SER282, GLY281, HIS279, PHE264 (Table 3) were formed van der walls bond to 2-phloroeckol, which strengthen the binding interaction between tyrosinase and compound. The hydroxyl groups presented in 2-phloroeckol were involved in interaction with amino acid residues of tyrosinase.

4.2 Docking simulation with Dieckol

According to figure. 7B, dieckol formed one hydrogen bond with the residue of mushroom tyrosinase. It also binds near the same binding area of tyrosinase as tropolone (Fig. 6B). The binding affinity of dieckol to tyrosinase was shown in Table 3. The tyrosinase residues VAL283, ALA286, VAL248 and PRO284 (Fig. 7B) were formed Pi-Alkyl bond to dieckol whereas the residue VAL248 to arbutin (Fig 8A), and the residue HIS263 formed Pi-Pi stacked bond. The oxygen atom of hydroxyl group presented in structure was predicted for hydrogen bonding. The other residues such as SER282, MET280, HIS244, HIS285, HIS61, GLU256, GLY281, ASN260, PHE264, ARG268, GLY86 and PHE90 (Table 3) were found to form van der walls bond that later stabilize the ligand-protein interaction.

At a glance, 2-phloroeckol interacted with MET280, HIS263, ASN260, HIS244, GLU322, ALA286 and VAL283, whereas tropolone interacted with HIS263, VAL283 and ALA286 in the active site of tyrosinase. But arbutin interacted with MET280 and VAL248. As like 2-phloroeckol, dieckol showed inhibitory activity against mushroom tyrosinase by binding with HIS263, VAL283, ALA286, VAL248 and ASN81 in the active site of tyrosinase. Therefore, these predicted interactions of 2-phloroeckol and dieckol with tyrosinase may contribute in the inhibition of tyrosinase activity.

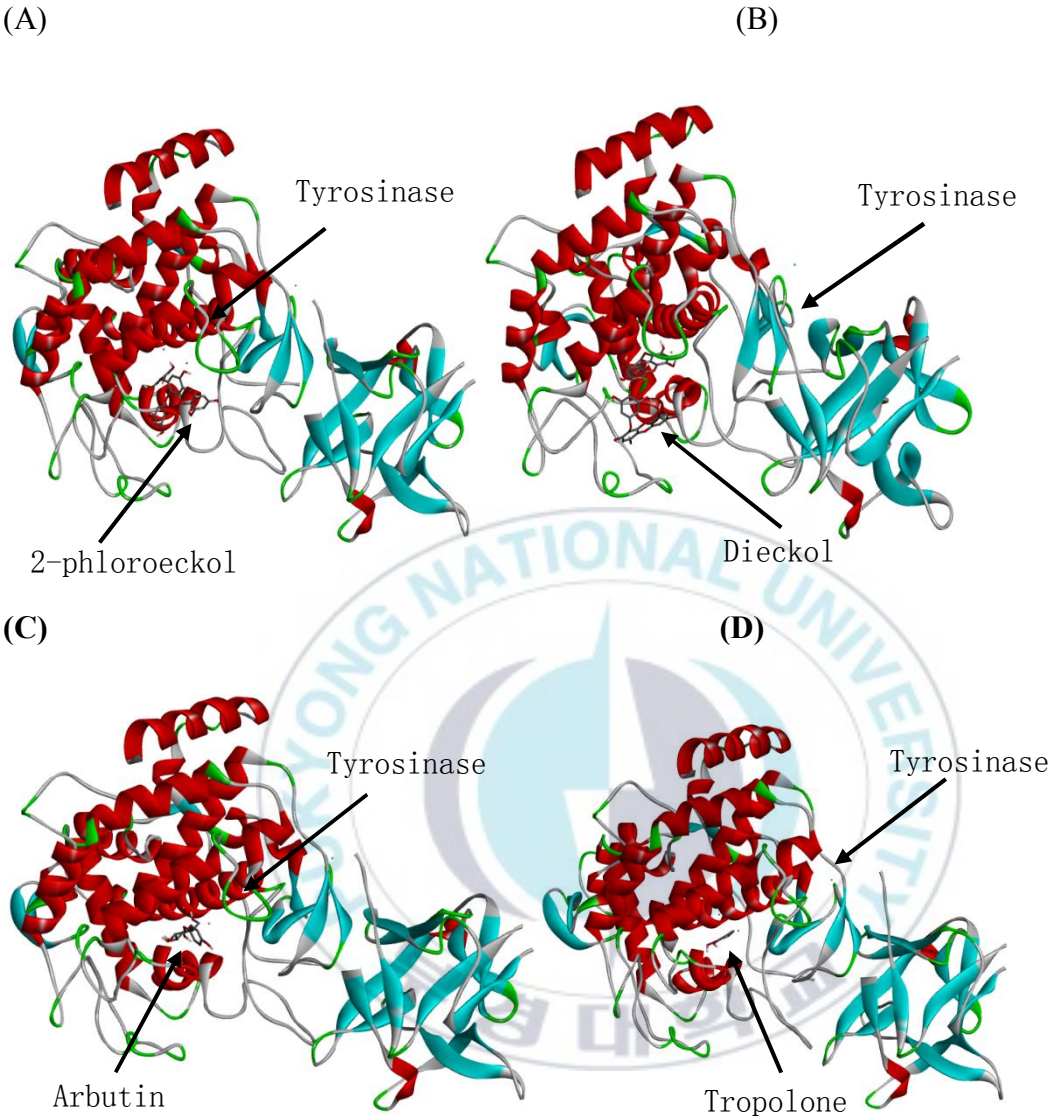


Figure 6. 3D crystal structure and binding position of molecular docking of 2-phloroeckol, dieckol , arbutin and tropolone against mushroom tyrosinase.

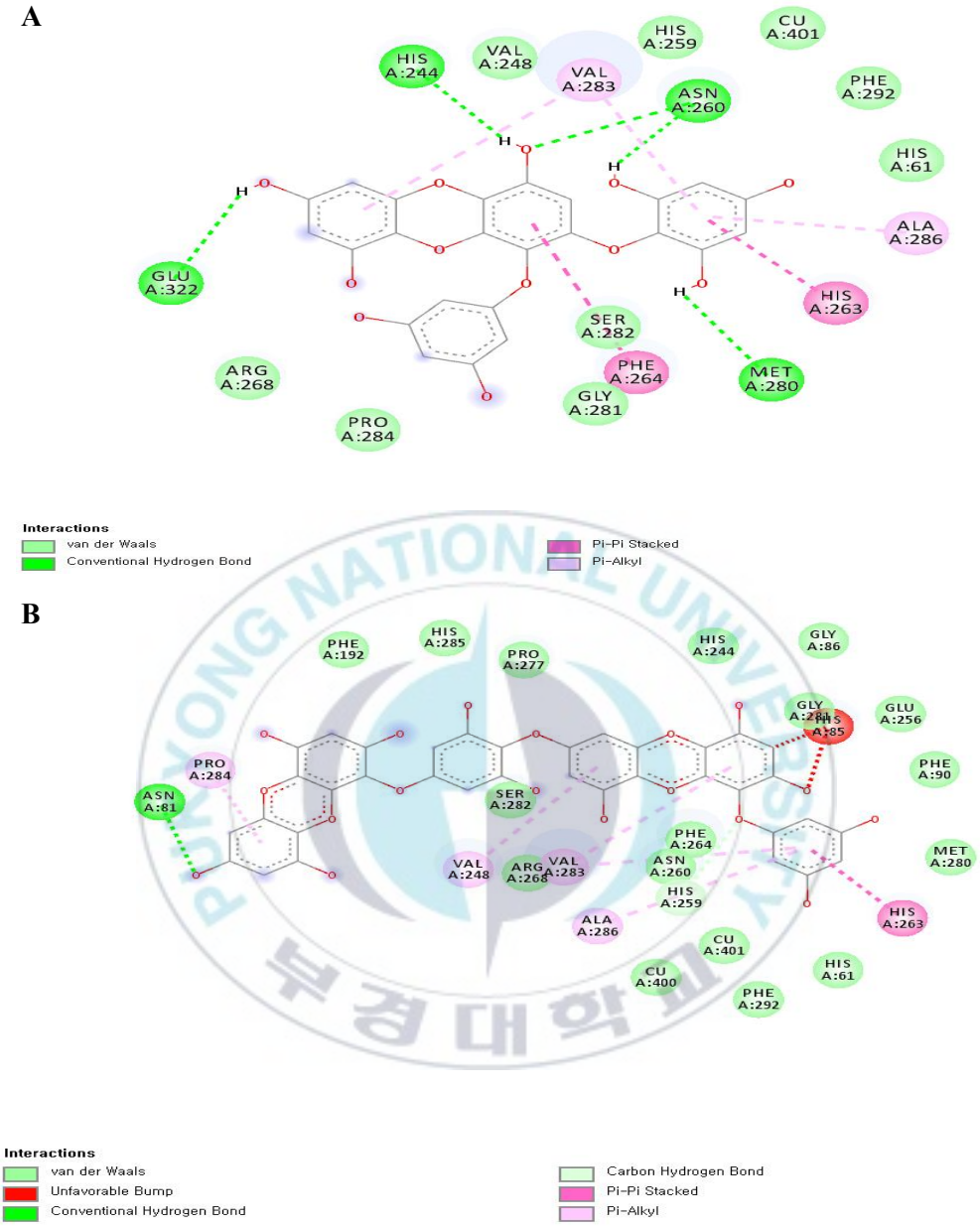
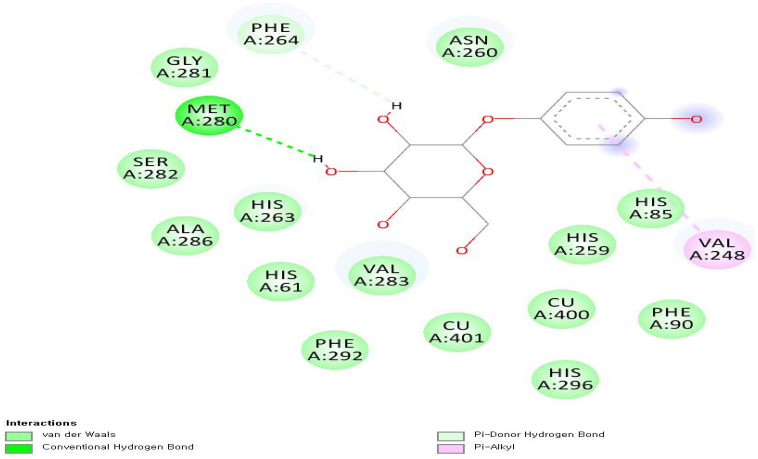


Figure 7. 2D crystal structure of molecular docking of 2-phloroeckol and dieckol against mushroom tyrosinase.

A



B

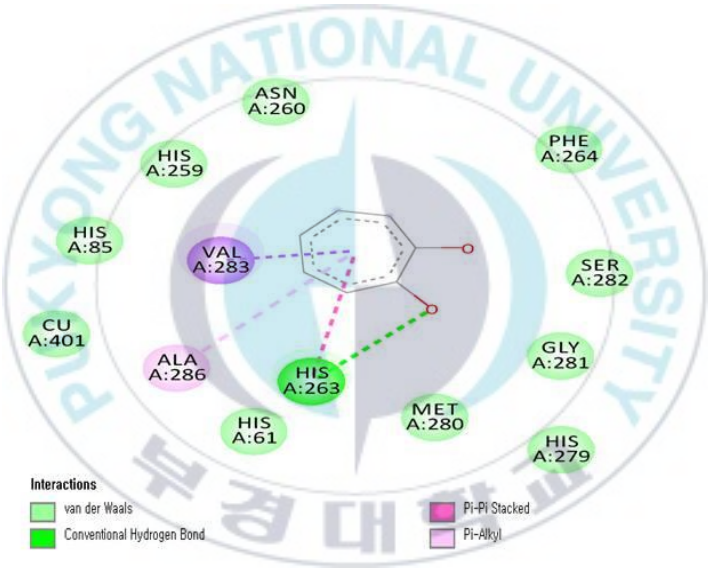


Figure 8. 2D crystal structure of molecular docking of arbutin and tropolone against mushroom tyrosinase.

Table 3. Docking affinity score and possible bond formation of 2-phloroeckol, dieckol and known inhibitors to mushroom tyrosinase active sites.

Compound	**Binding affinity (Kcal/mol)[a]**	**H-bond interecting Residues**	**Other bond interecting residues**
2-phloroeckol	-8.0	GLU322, HIS244, ASN260, MET280 GLY281	VAL283, ALA286, HIS263, PHE264 PHE264, HIS259, HIS61, VAL248 SER282, PHE292, ARG268, PRO284.
Dieckol	-2.6	HIS263, ASN81	VAL283, ALA286, VAL248, PRO284, SER282, MET280, HIS244, HIS61, GLU256, GLY281, PRO277, GLY86,PHE90,ASN260.
Tropolone	-6.0	ASN260	VAL283, ALA286, MET280,
Arbutin	-6.1	HIS263	HIS179, HIS85, HIS259, SER282 SER282, GLY281, HIS279.
		MET280	VAL283, ALA286, HIS263, SER282, HIS259, ASN260, HIS85, HIS61, GLY281, PHE264, HIS296, PHE292.

[a]Binding affinity indicates that the binding capacity of compound for the active site of tyrosinase.

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5. Cellular tyrosinase inhibitory activities of 2-phloroeckol and dieckol

To confirm the inhibition of cellular tyrosinase, the effects of 2-phloroeckol and dieckol on cell free crude tyrosinase were tested. Among the tested compounds, 2-phloroeckol gave highest and dieckol showed second highest inhibitory effects on crude cellular tyrosinase with the IC_{50} value of 72.88± 2.09 and 164.66 ± 2.57 μM, respectively, whereas the value of kojic acid was 267.06 ± 3.90 (Table 3). In case of percent of inhibition, 2-phloroeckol displayed highest inhibition 20.46 % at 20 μM (Fig. 9). As shown in figure 9, Percent of inhibition increased with increasing concentrations of 2-pholoreckol. This results indicated that 2–phloroeckol and dieckol have inhibitory activity on cellular tyrosinase. The other remaining phlorotannins had no activity on cellular tyrosinase (Table 3).

Table 4. Inhibitory effects of phlorotannins and kojic acid on cellular tyrosinase.

Compounds	**[a]IC_{50} (μM, mean ± SD)**
2-phloroeckol	72.88 ± 2.09
Dieckol	164.66 ± 2.57
Bieckol	>200
Phloroglucinol	>200
Phlorofucofuroeckol A	>200
Phlorofucofuroeckol B	>200
Eckol	>200
Kojic acid	267.06± 3.90

[a]The 50% inhibitory concentration (IC_{50}) values were calculated from log dose inhibition curve.

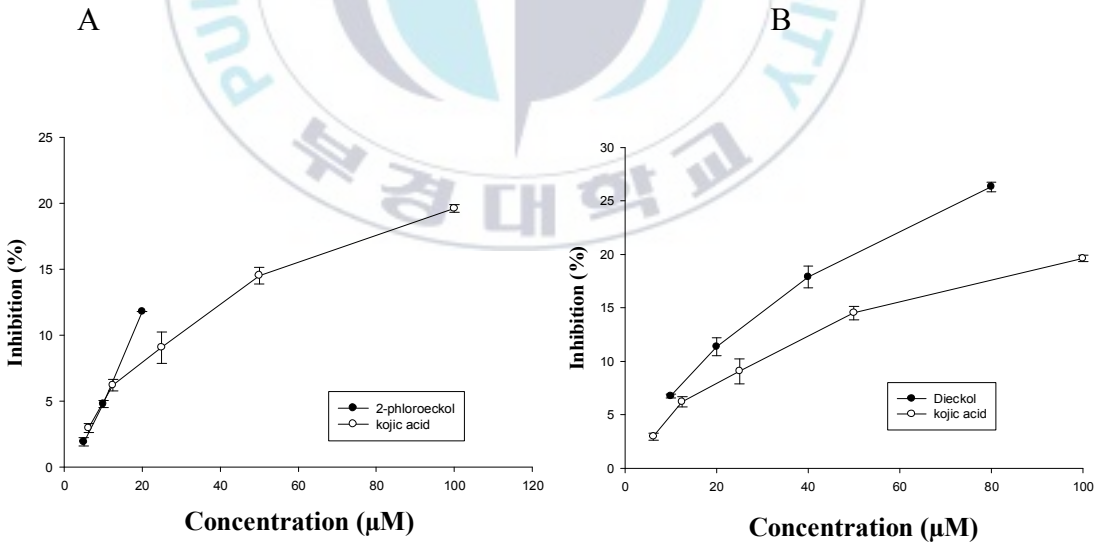


Figure 9. Inhibitory effects of 2-phloroeckol, dieckol and kojic acid against eukaryotic tyrosinase.

6. Cytotoxicity of 2-phloroeckol and dieckol in B16F10 cells

Before measuring the melanin contents, the cytotoxic levels of 2-phloroeckol (0, 12.5, 25 and 50 μM) and dieckol (0, 12.5, 25, 50 and 100 μM) was measured using MTS method on murine B16F10 melanoma cells. After 24 hours of exposure to compounds, Results (Fig. 10) represented no cytotoxic effects on B16F10 melanoma cells up to 50 and 100 μM by 2-phloroeckol and dieckol, respectively. Thus, we used these concentrations of 2-phloroeckol and dieckol for melanin inhibition assay.

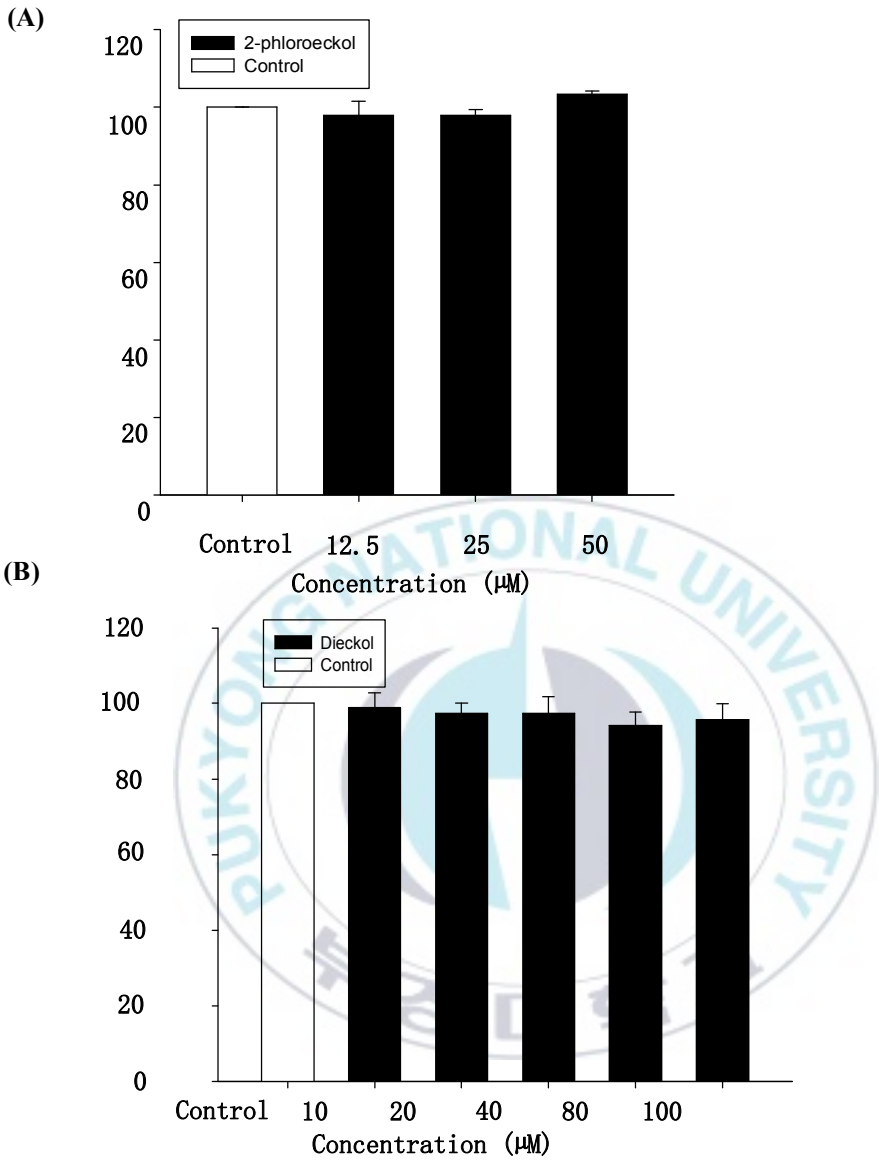
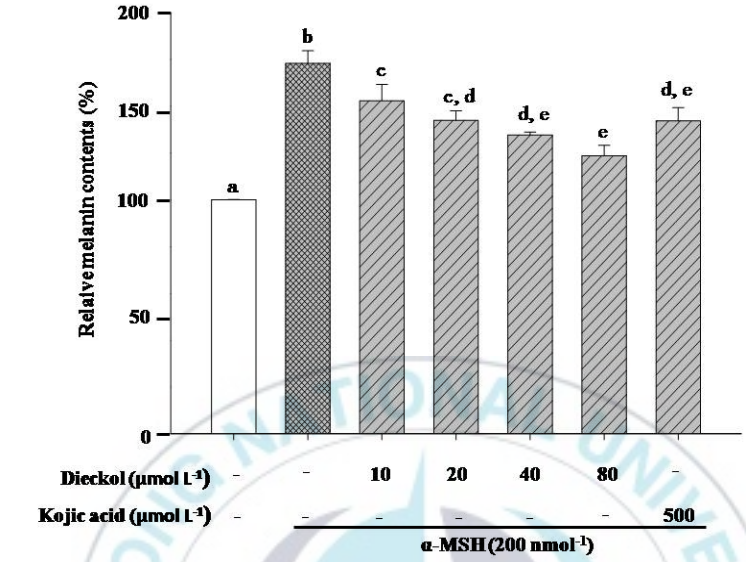


Figure 10. B16F10 melanoma Cells survibility at different concentrations of 2-phloroeckol (A) and dieckol (B). The percentage of viability of cells were expressed compare to the percentage of untreated controls.

7. Inhibition of melanin synthesis by 2-phloroeckol and dieckol

Inhibitory activity of 2-phloroeckol and dieckol on melanin production in α-MSH stimulated B16F10 melanoma cells was measured, because of their inhibitory activity on cellular tyrosinase. Different concentrations of dieckol (0, 10, 20, 40 and 80 μM) and 2-phloroeckol (0, 10, 5, 10, 20 and 50 μM) were used to check the inhibition of melanin production in B16F10 cells. Dieckol inhibited significantly the melanin production in a dose dependent manner displayed in Figure 11A. The melanin inhibition rate of dieckol was approximately 30 % at 40 μM with IC_{50} value of 129 μM, whereas that of kojic acid was 32.17 % at 500 μM with the IC_{50} value of 893 μM under the same conditions. Next, in the same manner 2-phloroeckol also inhibited melanin production (11B), and the inhibition rate of 2-phloroeckol was 21.20 % with the IC_{50} value of 142 μM.

(A)



(B)

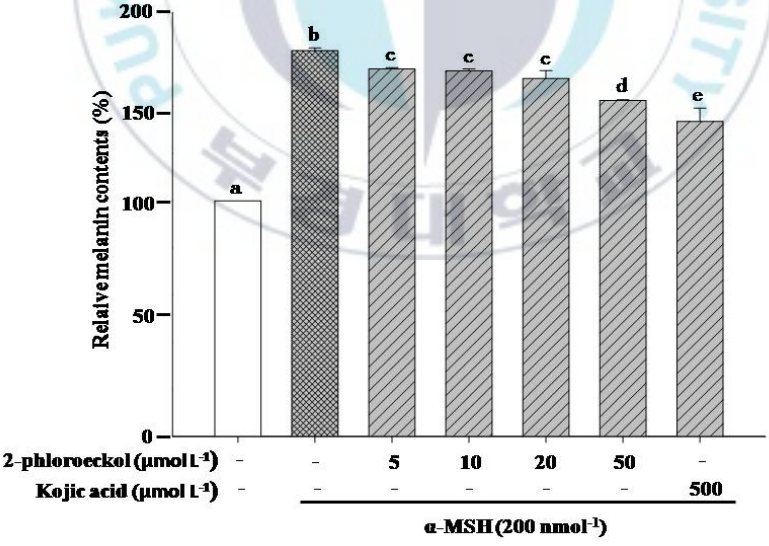


Figure 11. The effect of dieckol (A) and 2-phloroeckol (B) on melanin content in B16F10 melanoma cell. Cells were treated with compounds for 72 hours.

IV. Discussion

Marine algae is considered as plant oriented food because of its rich content of carbohydrate, minerals and protein, and also contain compounds those are capable to act on several disorder (Lordan et al. 2011 & Fitzgerald et al. 2011). Marine algae are widely distributed throughout the globe ranging from cold to warm zone (Mouritsen et al. 2013). It has been reported that Japanese people carrying highest life expectancy compared to other nations due to their consistant consumption of algae (Shimazu et al. 2007). In the last few decades, natural sources especially marine algae are getting preference in terms of using in cosmeceuticals and pharmaceuticals world, because of containing more bioactive components with diverse biological activity. Among them, acceptance of brown algaeis increasing day by day in terms of using in cosmetics sector as a skin whitening agents (Mohamed et al. 2012).

Phlorotannins have been reported for its phenolic characteristics and skin whitening effects (Koivikko et al. 2005). Phenols are the most tyrosinase inhibitor among tyrosinase inhibitors (Harborne et al. 1992). In this regard, the aim of this study was to examine whether phlorotannins isolated from ethanolic extratcs of *Ecklonia stolonifera* have the inhibitory activity on mushroom

tyrosinase or cellular tyrosinase and α-MSH induced melanin production in murine B16F10 melanoma cells. Melanin production capable B16F10 melanoma cell line was used for its melanin producibility. Binding of melanocyte stimulating hormone (MSH) to its receptor MC1R (Melanocortin 1 receptor) on melanoma cells results melanin production up to 100 fold. α-MSH increases intracellular cyclic adenosine monophosphate (cAMP) which is the regulator of melanin formation (Busca et al. 2000). Tyrosinase is a rate limiting catalyzing enzyme in the melanin synthesis, and thus modulating tyrosinase function is the crucial point to get hypopigmentation effect (Ando et al. 2007). In addition to catalytic characteristics of tyrosinase, there are many factors such as tyrosinase maturation and glycosylation as well as melanosome maturation and transfer that could be the target for inhibiting melanin formation. However, direct inhibition of tyrosinase is the first choice of treating pigmentation in skin.

First, the inhibitory effects of phlorotannins on mushroom tyrosinase was checked. Mushroom tyrosinase was used because of its similar characteristics with mammalian tyrosinase (Chang et al. 2012). 2-phloroeckol and dieckol showed higher inhihibitory activity against mushroom tyrosinase. Phloroglucinol and bieckol have less inhibitory activity than previous two, while the other remaining compounds did not show any inhibitory activity. Kojic acid used as positive control. 2-phloroeckol showed about nine fold higher inhibitory effect than kojic acid against mushroom

tyrosinase. Furthermore, the effects of phlorotannins on eukaryotic cellular tyrosinase in cell free system was investigated. B16F10 melanoma cell line was used for preparing crud lysates of cellular tyrosinase. In cellular tyrosinase inhibition assay, L-DOPA (0.1 mg/mL) was used as a substrate, because cellular tyrosinase has a higher turnover number for L-DOPA than L-tyrosine. A previous study showed that melanin production was lower when L-tyrosine was used as substrate compared to L-DOPA. In this study, substrate transformation per min was greater with L-DOPA (Jose et al. 1988) as a substrate. It was noted that only 2-phloroeckol and dieckol have had their inhibitory activity on cellular tyrosinase prepared from B16F10 melanoma cells. Their inhibition in cellular tyrosinase also higher compared to that of kojic acid. To determine the type of inhibitory mechanism of 2-phloroeckol and dieckol, kinetic assay experiment was performed with changing substrate concentration (1, 2 and 4 mM). The inhibitory type of 2-phloroeckol and dieckol was determined by Lineweaver-Burk plots as shown in Figure 6. A group of straight lines with several slope obtained with increasing substrate concentrations which intersected in second quadrant zone, while K_m values changed with changing of V_{max} values. Kinetic analysis demonstrated that 2-phloroeckol and dieckol are mixed type of mushroom tyrosinase inhibitor. The K_i value of kinetic analysis represents stronger binding affinity of compound to either free tyrosinase enzyme or enzyme substrate complex or both. 2-phloroeckol and dieckol contain number of hydroxyl group attached with different phenyl ring. Previously, it was

noticed that compounds with hydroxyl group have potent tyrosinase inhibitiory activity (Kanade et al. 2007). Tyrosinase inhibitors are able to exhibit their inhibitory mechanism by forming chelation with copper ion of enzyme. Three different types of tyrosinase form, such as oxy-, met- and deoxytyrosinase, are involved in pigmentation. Copper ion is centrally located in tyrosinase enzyme containing different active sites. It consists of two nuclear copper sites with three several histidine residues in each. These copper atoms also contain catalytic activity are involved in the conversion of single phenols to diphenols and finally, form ortho-quinones which facilitate the melanin formation (Kang et al. 2004). From this background, docking simulation was conducted to explore the binding site of 2-phloroeckol and dieckol with mushroom tyrosinase tertiary structure. Docking simulation study results revealed that 2-phloroeckol and dieckol did not bind directly with copper atoms, it binds near at the copper binding site. 2-phloroeckol, dieckol, tropolone and arbutin were formed hydrogen, Pi-Pi stacked, Pi-Alkyl and van der waals bond with amino acid residues of mushroom tyrosinase, those are predicted to responsible for tyrosinase inhibitory activity. Therefore, these predicted interaction of 2-phloroeckol and dieckol with residues of tyrosinase may contribute in the inhibition of tyrosinase activity.

Evidences have shown that several tyrosinase inhibitors did not exhibited inhibition on melanin production in B16F10 melanoma cells (Lien et al. 2014). In contrast, Chan et al. reported that hexane

fraction of *Sargassum* polycystum showed less inhibition in mushroom tyrosinase but high inhibition in melanin production (Chan et al. 2011). From that concept, the inhibitory activity of 2-phloroeckol and dieckol on melanin production in B16F10 melanoma cells was checked. 2-phloroeckol had strong inhibitory effects on mushroom and cellular tyrosinase, and dieckol was comparatively less effective in the inhibition of both form of tyrosinase. 2-phloroeckol inhibited melanin production in a dose dependent manner (Figure 11 B), and the inhibition rate of 2-phloroeckol was 21.20 % at 50 μM. Moreover, dieckol inhibited significantly the melanin production in a dose dependent manner displayed in Figure 11 A ($p<0.05$). The inhibition of melanin production dieckol was approximately 30 % at 40 μM, whereas that by kojic acid was 29.12 % at 500 μM.

V. Conclusion

In conclusion, the present study demonstrated that 2-phloroeckol and dieckol isolated from *E.stolonifera* exhibited potent inhibitory activity against mushroom tyrosinase. The results of kinetic study revealed that they are mixed type inhibitor of mushroom tyrosinase. 2-phloroeckol and dieckol also displayed inhibitory effects on mouse cellular tyrosinase. In addition, these compounds showed significant inhibition on α-MSH induced melanin production in B16F10 melanoma cells. Taken together, 2-phloroeckol and dieckol from *E.stolonifera* might be a potent tyrosinase inhibitor in skin whitening sector.

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