p-Hydroxybenzoic Acid β-D-Glucosyl Ester and Cimidahurinine with Antimelanogenesis and Antioxidant Effects from *Pyracantha angustifolia* via Bioactivity-Guided Fractionation

Sun-Yup Shim [1], Ye Eun Lee [2], Hwa Young Song [2], Mina Lee [2,*]

[1] Department of Aqualife Science, College of Fisheries and Ocean Sciences, Chonnam National University, 50, Daehak-Ro, Yeosu, Jeonnam 59626, Korea

[2] College of Pharmacy, Sunchon National University, 255 Jungangno, Suncheon-si 57922, Jeonnam, Republic of Korea

(Running title: Antioxidant and Antimelanogenesis Effects of *Pyracantha angustifolia*)

Corresponding author: Mina Lee

College of Pharmacy, Suncheon National University
Tel. 82-61-750-3764
Fax. 82-61-750-3708
E-mail: minalee@sunchon.ac.kr

Abstract: *Pyracantha angustifolia* has been used in traditional medicine to treat a range of diseases of the stomach and improve digestion, blood circulation, diarrhea, dysentery, and hemostasis. This study evaluated bioactivity-guided fractionation as a means to identify therapeutic phytochemicals from *P. angustifolia* that can attenuate melanogenesis and oxidation. Seven compounds with inhibitory effects on melanin production and tyrosinase (TYR) activity, as well as ABTS and DPPH radical scavenging activities, and have not been reported as whitening materials, were isolated from the *n*-butanol fraction from *P. angustifolia* leaves (PAL). Among the seven compounds, *p*-hydroxybenzoic acid β-d-glucosylester (HG), and cimidahurinine (CD) had strong inhibitory effects of melanin production, TYR activity, and ABTS and DPPH radical scavenging activities. Western blot analysis showed that HG and CD suppressed tyrosinase-related protein (TYRP)-1 and TYRP-2 expression. These results suggest that *P. angustifolia* containing active compounds, such as HG and CH, is a potent therapeutic candidate for the development of hypopigmenting agents.

Keywords: *Pyracantha angustifolia, p*-Hydroxybenzoic acid β-D-glucosylester, Cimidahurinine, melanin, tyrosinase, antioxidant

1. Introduction

Melanogenesis involves the production of melanin pigments in melanosomes by melanocytes, which are melanin-producing cells found in the skin, hair follicles, eyes, inner ear, bones, heart, and brain of humans [1-4]. Melanin is important for the prevention of UV-induced DNA damage and binds reactive oxygen species (ROS) that occur because of exposure to ultraviolet (UV) radiation [1,5]. Melanogenesis is enhanced by the activation of tyrosinase, the key enzyme of melanogenesis. Tyrosinase (TYR) is an enzyme that determines the color of mammalian skin and hair in the melanogenesis of mammals and browning of fruits and fungi [1-5].

Tyrosine is hydrozylated to 3,4-dihydroxyphenylalanin (DOPA) in melanin synthesis through TYR as a catalyst, which is a copper-containing enzyme located in melanosomes, is oxidized to dopaquinone by TYR [4]. Mammalian melanogenic enzymes are similar metalloproteins, such as TYR, tyrosinase-related protein 1 (TYRP-1) and TYRP-2. TYRP-1 and TYRP-2 are present in melanosome and play an essential role in catalyzing eumelanin-producing reactions. TYRP-1 increases the eumelanin: pheomelanin ratio and protects against oxidative stress through its peroxidase activity [4]. In contrast, TYRP-2 is an enzyme, dopachrome tautomerase (DCT), which mediates the tautomerization of L-dopachrome (a red melanin precursor) to the colorless dihydroxyindole-2-carboxylic acid (DHICA). In the absence of TYRP-2, L-DOPAchrome is converted to the toxic melanin precursor, dihydroxyindole (DHI), which dramatically affects the properties of the melanins produced [6]. Therefore, melanocytes might act as regulators for the local and global homeostasis of the melanogenic system by controlling TYR, TYRP-1, and TYRP-2. Oxidative stress, which is defined as the production of reactive oxygen species (ROS) and toxic free radicals species, and an imbalance between pro-oxidant and antioxidant homeostasis, is associated with a range of diseases involving immune suppression, cellular aging, DNA damage, and apoptosis [7-9].

Pyracantha angustifolia is a perennial flowering plant in the Rosaceae family that is native to China but has been introduced to North America and Australia. This plant is used as a traditional medicine for the treatment of a range of diseases of the stomach as well as to improve digestion, blood circulation, diarrhea, dysentery, and hemostasis. On the other hand, the physiological properties of *P. angustifolia* have not been studied. In the present study, compounds with whitening effects in α-melanocytes stimulating hormone (MSH)-stimulated B16F10 cells were isolated from *P. angustifolia*, and the anti-melanogenesis and anti-oxidants effects of its compounds were determined.

2. Materials and Methods

2.1. Plant Materials

The leaves, twigs, fruits of *P. angustifolia* were collected from the Nambu Forest inside Seoul National University, Beagwoon Mountain, Gwangyang city, Jeollanam-do, Korea on January 2017. A voucher specimen (SCNUP 22) was deposited in the laboratory of pharmacognosy, College of Pharmacy, Sunchon National University, Suncheon-si, Jeollanam-do, Korea.

2.2. Extraction and Isolation

The dried leaves (PAL), twigs (PAT), and fruits (PAF) of *P. angustifolia* (each 10 g) were pulverized, and their methanol extracts were obtained by sonication at room temperature and concentration. These extracts were used to measure cell viability and bioactivities. Among them, the leaves (859.7 g) with an antimelanogenesis effect without cytotoxicity were extracted three times with 100% ethanol for 2 h with sonication and yielded 146.6 g. This was suspended in distilled water (DW) and partitioned successively with n-hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), and n-butanol, resulting in solid residues weighing 4.9 g, 14.8 g, 16.9 g, and 14.6 g, respectively. The $CHCl_3$ fraction was separated by open liquid chromatography (LC) using a gradient solvent system in the normal phase (NP) LC with 150 g of silica gel and an *n*-hexane:EtOAc ratio of 5:1 into 1:1 to obtain twenty subfractions (C1-20). Compound **3** (1.3 mg, tR 39.1 min) and **5** (1.1 mg, tR 47.5 min) were obtained by separation using a multi-gradient reverse phase (RP) high performance liquid chromatography (HPLC) system (YMC Triart, C_{18}, 250 x 10 mm, H_2O:CH_3CN = 95:5 → 100% CH_3CN, 2 mL/min) from C9 and C10, respectively. C20 was separated using RP-HPLC system (YMC Triart, C_{18}, 250 x 10 mm, H_2O:CH_3CN = 20:80 → 100% CH_3CN, 2 mL/min), and compounds **6** (1.3 mg, tR 20.0 min) and **7** (1.3 mg, tR 17.2 min) were obtained. The EtOAc fraction was separated using a gradient solvent system in open LC with 200 g of silica gel and an $CHCl_3$:MeOH ratio of 10:0 into 100% MeOH to obtain ten subfractions (EA1-10). EA8 was separated with multiple preparative liquid chromatography (MPLC) using a Dispo Pack AT column (SIL-25:40 g, 15 x 25 mm, $CHCl_3$:MeOH = 10:1 → 100% MeOH) to obtain ten subfractions (EA8-1-10). Compound **1** (1.1 mg, tR 25.6 min) was yielded using RP-HPLC system (YMC Triart, C_{18}, 250 x 10 mm, H_2O:CH_3CN = 95:5 → 100% CH_3CN, 2 mL/min) from EA8-4. The n-butanol fraction was subjected to open LC (silica gel 150 g, $CHCl_3$:MeOH = 50:40:1

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→ 10:5:1) to obtain ten subfractions (B1-10). B5 subfraction was separated using an open LC system (silica gel 35.7 g, $CHCl_3$:MeOH:H_20 = 50:40:1 → 100% MeOH) and afforded thirteen subfractions (B5-1~13). Compounds **4** (3.2 mg, tR 28.0 min), **8** (1.5 mg, tR 17.7 min), **11** (5.9 mg, tR 29.1 min), **13** (2.3 mg, tR 27.2 min), and **14** (1.2 mg, tR 26.0 min) were isolated using the RP-HPLC system (YMC Triart, C_{18}, 250 x 10 mm, H_2O:CH_3CN = 95:5 → 100% CH_3CN, 2 mL/min) from B5-7. Compound **15** (1.6 mg, tR 33.9 min) was purified using the RP-HPLC system (YMC Triart, C_{18}, 250 x 10 mm, H_2O:CH_3CN = 80:20 → 100% CH_3CN, 2 mL/min) from B5-9. The B5-13 was separated using an RP-HPLC system (YMC Triart, C_{18}, 250 x 10 mm, H_2O:CH_3CN = 95:5 → 100% CH_3CN, 2 mL/min) to yield four compounds, **2** (6.4 mg, tR 34.8 min), **9** (1.1 mg, tR 12.9 min), **10** (3.0 mg, tR 50.0 min), and **12** (1.8 mg, tR 42.0 min).

2.4. Antimelanogenesis Assay

2.4.1. Cell Culture and Viability Assay

The B16F10 mouse melanoma cells were obtained from the Korean Cell Line Bank (KCLB) (Seoul, Korea). The cells were cultured at 37 °C in high glucose DMEM, supplemented with 10% FBS (HyClone, Logan, UT, USA), 100 IU/mL penicillin, and 100 mg/mL streptomycin (HyClone, HyClone, Logan, UT, USA) in a humidified atmosphere containing 5% CO_2 in air. The cytotoxicity of the sample from *P. angustifolia* was assessed by quantitating the cell viabilities in the presence of the sample. The cells (1 $\times 10^4$ cells/well) were seeded and incubated for 24 h in DMEM supplemented with 10% FBS. The culture medium was replaced with serum-free DMEM, and the cells were treated with different concentrations of the compounds with or without α-MSH for 72 h. After removing the serum-free DMEM, the cells were treated with an MTT solution (0.5 mg/mL) and incubated for 4 h. The live cells were determined after the medium was replaced with DMSO and measured at 570 nm on a microplate reader (BioTek Instruments, Winooski, VT, USA).

2.4.2. Melanin Content Assay

The intracellular melanin content was determined using the procedure described elsewhere with minor modifications [10]. Briefly, B16F10 cells were seeded in a 24-well plate at 2×10^4 cells/well and incubated for 24 h in DMEM containing 10% FBS. The cells were pretreated with the sample for 1 h and then stimulated with α-

MSH for 72 h. The cells were washed with PBS, and dissolved in 1N NaOH containing 10% DMSO by boiling at 80 °C for 1 h. The cell lysates were centrifuged at 14,000 rpm for 10 min, and the absorbance of the supernatant was measured at 490 nm. The melanin content was expressed as a percentage of the control. Arbutin (500 μg/mL) was used as a positive control.

2.4.3. Cellular TYR Activity Assay

The cellular TYR activity was measured as the L-DOPA oxidase activity using a previously described method with some modifications [11,12]. The B16F10 cells were seeded and incubated for 24 h in a 24 well plate in the presence or absence of the sample for 72 h. After treatment, the cells were washed with cold PBS and lysed with lysis buffer (0.1 M sodium phosphate buffer, 1% Triton X-100 and 0.1 mM PMSF). The cell lysates were centrifuged at 10,000 rpm for 10 min and the supernatant was used as the cellular TYR solution. The reaction mixture containing 40 μL of cell lysate and 100 μL of L-DOPA (2 mg/mL) was incubated at 37 °C for 1 h and the level of dopachrome formation was measured spectrophotometrically at 490 nm. RIPA buffer was used as the control. The TYR activity was calculated as a percentage of the control, and arbutin (500 μg/mL) was used as a positive control.

2.4.4. Western Blot Analysis

The protein expression of TYRP-1 and TYRP-2 was measured by western blot analysis. The pretreated and stimulated B16F10 cells were washed with cold PBS and harvested using a cell scrapper. The whole cell lysates were extracted with a protein extraction kit (InTRON Biotechnology, Korea). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membrane was blocked with 5% skim milk in a plain buffer (20 mM Tris pH 7.4 and 136 mM NaCl) at RT for 1 h, and incubated with the primary antibodies, TYRP-1 and TYRP-2 (Santa Cruz Biotech, CA, USA), overnight at 4°C. The membrane was then incubated with a 500 fold diluted specific secondary HRP-conjugated antibody (Thermo Fisher Sci. Waltham, MA, USA) at RT for 1 h, and the immunoreactive bands were visualized using an enhanced chemiluminescence ECL assay kit according to the manufacturer's instructions.

2.5. Antioxidative Assay

2.5.1. DPPH Assay

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The radical scavenging effect of 1,1-diphenyl-β-picrylhydrazine (DPPH) (Sigma-Aldrich, Co.) was measured using a modification of the method reported by Blois [13]. A 100 μL sample of 0.2 mM DPPH solution was added to a 100 μL sample on a 96 well plate, mixed for five seconds, and reacted for 30 min after shading the light. The absorbance was measured at 517 nm using a microplate spectrophotometer (Epoch, Biotek Instruments, Inc., VT, USA), and ascorbic acid was used as a positive control.

2.5.2. ABTS Assay

The ABTS ((2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, Sigma-Aldrich, Co.) radical scavenging activity was measured using a modification of the method reported by Proestos [14]. Seven millimoles 2,2'-Azobis (2-aminopropane) dihydrochloride (Sigma-Aldrich, Co.) was mixed with 2.45 mM ABTS and then reacted for 16 h at 23 °C. Fifty microliters of the sample and 100 μL of the ABTS solution were reacted at 23°C for 20 min after adding them to a 96 well plate. The absorbance was measured at 734 nm. Ascorbic acid (Sigma-Aldrich Co.) was used as the positive control.

2.6. Statistical Analysis

All measurements were conducted independently, at least in triplicate. Data are expressed as the mean ± SD. The significant differences between the control and EDG and DC groups were determined using a Student's *t*-test at $p < 0.05$.

3. Results

3.1. Anti-melanogenesis and Anti-oxidant Effects of Extract

Hyperpigmentation is the overproduction of melanin, which is a major factor determining skin color and causes visible skin pigmentation disorders, involving albinism, melisma, freckles, moles, and lentigo [15,16]. Therefore, skin whitening agents are applied to treat pigmentation and its related diseases. *P. angustifolia* has been used as traditional medicine for the treatment of various diseases of the stomach as well as to improve digestion, blood circulation, diarrhea, dysentery, and hemostasis. This study investigated the anti-melanogenesis and anti-

oxidant activities of each part of the extracts from *P. angustifolia.* To investigate the anti-melanogenesis activity, melanin production and TYR activity were examined in B16F10 cells. The DPPH and ABTS radical activities were assessed for their anti-oxidant activities. For sample preparation, the dried parts of *P. angustifolia*, such as leaves, twigs, and fruits, were extracted with 100% EtOH. The anti-melanogenesis activities of three extracts were evaluated by studying the inhibition of melanin production and TYR activity in B16F10 cells. Three extracts of the leaves, twigs, and fruits suppressed melanin production at 100 and 250 μg/mL (Figure 1A). Compared to the activity of arbutin, the positive control, the activities were stronger in the order of twigs and leaves. Among the three extracts, the leaf extract inhibited TYR activity most strongly at 100 and 250 μg/mL (Figure 1B). To determine the non-toxic concentration of extracts, its effect on the viability of mouse B16F10 melanoma cells was assessed using an MTT assay. None of the extracts had any cytotoxic effect on B16F10 cells after treatment with 100 and 250 μg/mL for 72 h except 250 μg/mL of the twigs extract (Figure 1C). The subsequent experiments were conducted with the leaf extract with no cytotoxicity. DPPH and ABTS assays were attempted to examine the effects of each part of *P. angustifolia* on the antioxidant activity. All the extracts exhibited strong DPPH radical scavenging activity (Figure E), but PAL and PAT exhibited strong activities on the ABTS radical scavenging activity (Figure 1D).

3.2. Bioactivity-Guided Isolation of Active Phytochemicals from P. angustifolia

The effects of ethanol extracts of PAL, PAT, and PAF on the anti-melanogenesis activities in B16F10 cells were evaluated. Among them, PAL suppressed the melanin contents significantly by 32.4% without cytotoxicity at a concentration of 100 μg/mL (Figure 1A), whereas PAT showed weak cytotoxicity. Therefore, potent PAL was selected for the isolation of active phytochemicals. A large quantity of PAL extract was partitioned with *n*-hexane, $CHCl_3$, EtOAc, *n*-butanol, and aqueous residue, depending on the solvent polarity. All fractions were measured to determine their effects on the inhibition of melanin production at 100 μg/mL (Figure 2A). The *n*-hexane, $CHCl_3$, EtOAc, and *n*-butanol fractions showed 28.4, 35.3, 25.8, and 27.3% inhibition, respectively, without cytotoxicity (Figure 2C). Among them, the *n*-hexane and $CHCl_3$ fractions potently suppressed the TYR activity (Figure 2B). In addition, the *n*-butanol fraction showed the strongest DPPH radical scavenging activity of 69.9% at 100 μg/mL (Figure 2D). With the ABTS scavenging activity, EtOAc and *n*-butanol fractions had higher antioxidant activity than the other fractions (Figure 2C). Therefore, three fractions were used to isolate the bio-active phytochemicals, except for the *n*-hexane fraction that contained an amount of lipid-like phytosterols (Figure

3). Fifteen compounds, four compounds from the $CHCl_3$ fraction, one compound from the EtOAc fraction, and ten compounds from the *n*-butanol fraction were isolated by repeated silica gel column chromatography, MPLC, and RP HPLC. These compounds were identified as (±)-Epicatechin (**1**), cosmosiin (**2**), 9-hydroxyeriobofuran (**3**), 5,7-dihyroxychromone 7-*β*-D-glucoside (**4**), 2-oxopomolic acid (**5**), pomolic acid (6), ursolic acid (**7**), isopropyl *β*-D-glucoside (**8**), arbutin (**9**), p-hydroxybenzoic acid *β*-D-glucosyl ester (**10**), roseoside (**11**), cimidahurinine (**12**), 3-(*β*-D-glucopyranosyloxy)-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone (**13**), dihydrosyringin (**14**), and cinnamoyl glucoside (**15**) by a comparison of the measured spectroscopic (NMR and MS) data with published papers (Figure 4) [16-32].

3.3. Anti-melanogenesis and Anti-oxidant Effects of Seven Compounds Isolated from P. angustifolia Fraction

Among the fifteen phytochemicals, the anti-melanogenesis effects of compounds **1**-**3**, **5**-**7**, **9**, and **11** are reported elsewhere. In particular, arbutin, a representative whitening material, was used as a positive control in this study [33-38]. Except for these compounds, seven compounds isolated from the *n*-butanol fraction were assessed for their anti-melanogenesis and anti-oxidant effects. To examine the anti-melanogenesis activity, the pretreated cells were the melanin production and the TYR activity was measured. As a result, HG and CH showed strong activities in α-MSH-induced melanin production (Figure 5A) and TYR activity (Figure 5B) without cytotoxicity (Figure 5C). The ABTS and DPPH radical scavenging activities were examined to determine the anti-oxidant effects of these compounds. Among these compounds, HG and CH showed strong DPPH (Figure 5E) and ABTS (Figure 5E) scavenging activity, respectively.

3.4. Effects of HG and CD on TYRP-1 and TYRP-2 Expression

TYRP-1 and TYRP-2 are critical enzymes that affect the quantity and quality of melanin [39]. Among the fifteen compounds isolated from PAL, TYRP-1, and TYRP-2, the protein expression of HG and CH, which were not previously known to have an anti-melanogenesis effect, was determined by western blot analysis using the specific antibodies. As shown in Figure 6, HG and CD inhibited the expression of these proteins at 10 and 100 μM (Figure 6).

4. Discussion

The demand for natural pigment reducers from natural products for pharmaceutical and cosmetical applications has increased rapidly [40-41]. Recently, natural products have been considered as an effective source of bioactive compounds in the development of oxidation-reducing and hypopigmenting agents. This study explored potential therapeutic components from natural products. *P. anqustifolia* has been used traditionally for treating various diseases involving the stomach and improves digestion, blood circulation, diarrhea, dysentery, and hemostasis. This study evaluated the applicability of *P. angustifolia* by bioactivity-guided fractionation to find natural therapeutic products against hyperpigmentation.

Melanin is a dark pigment that is synthesized in melanocytes by the oxidation of L-tyrosine from external stimuli, such as UV [40,42,43]. Although the primary role of melanin is the protection of skin tissue from UV irradiation, the excessive production of melanin causes the generation of age spots and freckles [41,44,45]. Considerable efforts have been made to develop products that reduce melanin synthesis for use as whitening constituents [33-38]. TYR is an oxidase that is the rate-limiting enzyme for controlling the production of melanin. The enzyme is a copper-containing enzyme present in plant and animal tissues and is involved mainly in two distinct reactions of melanin synthesis that catalyze the production of melanin and other pigments from tyrosine by oxidation [42]. Oxidative stress, which is defined as the production of toxic free-radical species, and an imbalance between pro-oxidant and anti-oxidant homeostasis, is associated with cellular skin aging [13]. ABTS and DPPH assays used in this study have been used widely to investigate the anti-oxidant capacities of natural products [13]. Both assays are associated mainly with the hydrogen donating or proton radical scavenging capacities of the natural products [13,14]. The hydroxyl radical scavenging properties are involved in two mechanisms, which include the inhibition of hydroxyl radical production from hydrogen peroxide by binding with metal ions and direct single electron transfer to the generated radical. Melanin synthesis involves oxidation reactions, and inhibiting oxidative damage is important in the prevention of hyperpigmentation [47]. Mouse B16F10 skin melanoma cells were used to elucidate the anti-melanogenesis activity. First, the leaves, twigs, and fruits of *P. anqustifolia* were extracted with EtOH, and the levels of melanin production and TYR activity in B16F10 cells were then investigated. These results showed that all extracts of each part inhibited melanin production (Figure 1A) and TYR activity (Figure 1B) at 100 and 250 μg/mL. On the other hand, none of the extracts exhibited cytotoxicity except for the twigs extract at 250 μg/mL (Figure 1C). Three extracts, PAL, PAT, and PAF, were evaluated for their radical scavenging ability as a measure of their anti-oxidant potential. Among

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the three extracts, PAL and PAT exhibited strong ABTS and DPPH radical scavenging activities at 100 and 250 μg/mL (Figure 1D). Subsequent experiments were performed with PAL, which showed the most stable and strong activities on the inhibition of melanin production and TYR activity, as well as on the radical scavenging activities of ABTS and DPPH. The inhibitory effects on melanin production, TYR activity, ABTS, and DPPH radical scavenging activities were assessed by melanogenesis and oxidant activities-guided fractionation to partition the leaf extract using *n*-hexane, ethyl acetate, *n*-butanol, and H_2O (Figures 2). To discover the anti-melanogenesis fraction, the fractions were tested to determine if they could inhibit melanin production and TYR activity in B16F10 cells. Four fractions inhibited melanin production, with the $CHCl_3$ fraction having the strongest inhibition of TYR activity. Moreover, the DPPH and ABTS radical scavenging activities were measured to examine the anti-oxidant fraction. Among the fractions, the EtOAc and n-butanol fractions exhibited strong ABTS and DPPH radical scavenging activities, respectively. Therefore, the $CHCl_3$, EtOAc, and n-butanol fractions were used to isolate the bio-active phytochemicals. Fifteen compounds, four compounds from $CHCl_3$ fraction, one compounds from EtOAc fraction, and ten compounds from *n*-butanol fraction, were isolated and chemical structure was identified (Figure 4). Among the fifteen phytochemicals, the anti-melanogenesis effects of the compounds are reported elsewhere, as representative whitening materials [33-38]. Seven compounds isolated from *n*-butanol fraction were assessed in the present study. To search for anti-melanogenesis and anti-oxidant phytochemicals, this study examined whether seven compounds (**1**-**7**) isolated from the *n*-butanol fraction could inhibit melanin production and TYR activity. Of the seven compounds, compounds **10** and **12** inhibited melanin production (Figure 5A) and TYR activity (Figure 5B) with no cytotoxicity (Figure 5C). These two compounds were identified as *p*-hydroxybenzoic acid β-D-glucosyl ester (HG) and cimidahurinine (CH). To examine the anti-oxidant activity of these two compounds, HG and CH, exhibited strong DPPH and ABTS radical scavenging activity, respectively (Figure 5D). TYR, TYRP-1, and TYRP-2 comprise the TYR family of proteins. TYRP-1 is a melanocyte-specific gene product involved in melanin synthesis and is involved in stabilizing the TYR protein and modulating its catalytic activity. Moreover, TYRP-1 is also involved in maintaining the melanosome structure and affects melanocyte proliferation and melanocyte cell death. TYRP-2 acts as L-DOPAchrome to produce DHICA by eumelanin synthesis [48,49]. Inhibitory effects of HG and CH on TYRP-1 and TYRP-2 expression were investigated by western blot analysis to confirm the inhibitory relationship between melanin production and the anti-oxidant effects. These results showed that HG and CH inhibited TYRP-1 and TYRP-2 at 10 and 100 μM in B16F10 cells (Figure 6). These results suggested that the hypopigmentation of *P. angustifolia* containing HG and CH is due to the inhibition of radicals produced by oxidation. TYRP-1 expression is regulated by the

microphthalmia-associated transcription factor (MITF) [50]. Moreover, the expression of TYR, TYRP-1, and TYRP-2 is induced by cAMP. The biological activity of cAMP is mediated by cAMP-dependent protein kinase A (PKA), which results in the activation of CREB [50]. Further mechanistic studies on the down-regulation of HG and CH in oxidative-stress-induced signaling will be necessary to confirm their potential therapeutic application in the protection of hyperpigmentation.

5. Conclusions

The leaf extract exhibited anti-melanogenesis and anti-oxidant effects. HG and CH, which are phytochemicals isolated from *P. angustifolia* using bioactivity-guided fractionation, suppressed melanin generation and TYR activity, and down-regulated the activation of TYRP-1 and 2 in α-MSH-stimulated B16F10 cells. Moreover, these two compounds exhibited DPPH and ABTS radical scavenging activities. These results suggest that *P. angustifolia*-containing active compounds, such as HG and CH, could be a useful candidate for hypopigmenting agents.

Acknowledgements: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (No. NRF-2017R1C1B2005934).

Conflict of Interest: The authors declare no conflict of interest.

References

1. D'Mello, S.A.; Finlay, G.J.; Baguley, B.C.; Askarian-Amiri, M.E. Signaling pathways in melanogenesis. *Int. J.Mol. Sci.* **2016**, 17, pii:E1144. doi: 10.3390/ijms17071144

2. Bonaventure, J.; Domingues, M.J.; Larue, L. Cellular and molecular mechanisms controlling the migration of melanocytes and melanoma cells. *Pigment Cell Melanoma Res*. **2013**, 26, 316–325.

3. Ogbechie-Godec, O.A.; Elbuluk, N. Melasma: An up-to-date comprehensive review. *Dermatol. Ther. (Heidelb).* **2017**, 7, 305–318.

4. Videira, I.F.; Moura, D.F.; Magina, S. Mechanisms regulating melanogenesis. *An. Bras. Dermatol.* **2013**, 88, 76–83.

5. Miyamura, Y.; Coelho, S.G.; Wolber, R.; Miller, S.A.; Wakamatsu, K.; Zmudzka, B.Z.; Ito, S.; Smuda, C.; Passeron, T.; Choi, W.; Batzer, J.; Yamaguchi, Y.; Beer, J.Z.; Hearing, V.J. Regulation of human skin pigmentation and responses to ultraviolet radiation. *Pigment Cell Melanoma Res*. **2007**, 20, 2-13.

6. Tsukamoto, K.; Jackson, I.J.; Urabe, K.; Montague, P.M.; Hearing, V.J. A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. *EMBO J*. **1992**, 11, 519-526.

7. Epstein, J.H. Photocarcinogenesis, skin cancer, and aging. *J. Am. Acad. Dermatol*. **1983**, 9, 487–502.

8. de Gruijl, F.R.; van Kranen, H.J.; Mullenders, L.H. UV-induced DNA damage, repair, mutations and oncogenic pathways in skin cancer. *J. Photochem. Photobiol*. **2001,** B63, 19–27.

9. Bjelakovic, G.; Nikolova, D.; Gluud, C. Antioxidant supplements and mortality. *Curr. Opin. Clin. Nutr. Metab. Care*. **2014**, 17, 40-44.

10. Yang, S.H.; Tsasakis, A.M.; Tzanakakis, G.; Kim, H.S.; Le, B.; Sifaki, M.; Spandidos, D.A.; Tsukamoto, C.; Chung, G. Soyasaponin Ag inhibits α-MSH-induced melanogenesis in B16F10 melanoma cells via the downregulation of TRP-2. *Int. J. Mol. Med.* **2017**, 40, 631-636.

11. Kwon, M.; Lim, S.J.; Lee, B., Shin, T.; Kim, H.R. Ethanolic extract of sargassum serratifolium inhibits adipogenesis in 3T3-L1 preadipocytes by cell cycle arrest. *J. Appl. Phycol*. **2018**, 30, 559-568.

12. Lin, Y.S.; Chuang, M.T.; Chen, C.H.; Chien, M.Y.; Hou, W.C. Nicotinic acid hydroxamate downregulated the melanin synthesis and tyrosinase activity through activating the MEK/ERK and AKT/GSK3beta signaling pathways, *J. Agric. Food Chem.* **2012**, 60, 4859–4864.

13. Blois, M.S. Antioxidant determinations by the use of a stable free radical. *Nature*. **1958**, 181, 1199-1200.

14. Proestos, C.; Lytoudi, K.; Mavromelanidou, O.; Zoumpoulakis, P.; Sinanoglou, V. Antioxidant Capacity of Selected Plant Extracts and their Essential Oils. *Antioxidants*. **2013**, 2, 11–22.

15. Han, E.; Chang, B.; Kim, D.; Cho, H.; Kim, S. Melanogenesis inhibitory effect of aerial part of Pueraria thunbergiana *in vitro* and *in vivo*, *Arch. Dermatol. Res*. **2015**, 307, 57–72.

16. Denat, L.; Kadekaro, L.M.; Marrot, L.; Leachman, A.S.; Adbel-Malek, A.Z. Melanocytes as instigators and victims of oxidative stress. *J. Investig. Dermatol.* **2014**, 134, 1512-1518.

17. Zhu Yue, Z.; Yulong, W; Zhang Jiaying.; Zhang Nan.; Lei Yuqing.; Gan Zhilin.; Sun Aidong. Isolation and identification of procyanidins in aronia melanocarpa using NMR, LC-IT-TOF/MS/MS and MALDI-TOF MS. *J.Food Sci. Technol.* **2019**, 4, 614-628

18. Peng, Z.F.; Strack, D.; Baumert, A.; Subramaniam, R.; Goh, N.K.; Chia, T.F.; Tan, S.N.; Chia, L.S. Antioxidant Flavonoids from Leaves of Polygonum Hydropiper L. *Phytochemistry.* **2003**, *62*, 219-228.

19. Lin, R.; Chen, M.; Liu, Y.; Lin, Y.; Lu, M.; Hsu, F.; Lee, M. New Whitening Constituents from Taiwan-Native Pyracantha Koidzumii: Structures and Tyrosinase Inhibitory Analysis in Human Epidermal Melanocytes. *Int J Mol* Sci.**2015**, *16,* 28598-28613.

20. Backhouse, N.; Delporte, C.; Negrete, R.; Feliciano, S.S.; López-Pérez, J. Bioactive Phenolic Derivatives from Acaena Splendens Methanol Extract. *Phytother Res.* **2002**, 16, 562-566.

21. Xu, H.; Zeng, F.; Wan, M.; Sim, K. Anti-HIV Triterpene Acids from Geum Japonicum. *Nat. Prod.* **1996**, 59, 643-645.

22. Cheng, J.; Zhang, L.; Cheng, H.; Chiou, C.; Lee, I.; Kuo, Y. Cytotoxic Hexacyclic Triterpene Acids from Euscaphis Japonica. *J. Nat. Prod.* **2010**, 73, 1655-1658.

23. Lee, T.; Juang, S.; Hsu, F.; Wu, C. Triterpene Acids from the Leaves of Planchonella Duclitan (Blanco) Bakhuizan. *J. Chin. Chem. Soc.* **2005**, 52, 1275-1280.

24. Du, X.; Yoshizawa, T.; Shoyama, Y. Butanoic Acid Glucoside Composition of Whole Body and in Vitro Plantlets of Anoectochilus Formosanus. *Phytochemistry* **1998**, 49, 1925-1928.

25. Sugimoto, K.; Nishimura, T.; Nomura, K.; Sugimoto, K.; Kuriki, T. Syntheses of Arbutin-α-Glycosides and a Comparison of their Inhibitory Effects with those of α-Arbutin and Arbutin on Human Tyrosinase. *Chem. Pharm. Bull*. **2003**, 51, 798-801.

26. Shen, Y.; Terazawa, M. Dihydroroseoside, a New Cyclohexanone Glucoside, from the Leaves of Shirakamba (Betula Platyphylla Sukatchev Var. Japonica Hara). *J. Wood Sci*. **2001**, 47, 145-148.

27. Zhou, X.; Yan, L.; Yin, P.; Shi, L.; Zhang, J.; Liu, Y.; Ma, C. Structural Characterisation and Antioxidant Activity Evaluation of Phenolic Compounds from Cold-Pressed Perilla Frutescens Var. Arguta Seed Flour. *Food Chem.* **2014**, 164(1), 150-157.

28. Jiang, Z.; Li, S.; Li, W.; Guo, J.; Tian, K.; Hu, Q.; Huang, X. Phenolic Glycosides from Ficus Tikoua and their Cytotoxic Activities. *Carbohydr. Res*. **2013**, 382, 19-24.

29. Pan H.; Chen B.; Fu L.; Wang M. Chemical constituents from the stems of Dendrobium denneanum (II). *Chin. J. Appl. Environ. Biol*. **2013**, 19, 952-955.

30. Chen Xin.; Zhang Jian.; Liu Ji-Hua.; Yu Bo-Yang. Biotransformation of p-, m-, and o-hydroxybenzoic acids by Panax ginseng hairy root cultures. *J. Mol. Catalysis B: Enzymatic*. **2007,** 54, 72-75.

31. Chen, X.; Zhang, J.; Liu, J.; Yu, B. Biotransformation of p-, m-, and o-Hydroxybenzoic Acids by Panax Ginseng Hairy Root Cultures. *J. Mol. Catal B:* **2008**, 54, 72-75.

32. He, X.; Liu, R.H. Phytochemicals of Apple Peels: Isolation, Structure Elucidation, and their Antiproliferative and Antioxidant Activities. *J. Agric. Food Chem*. **2008**, 56, 9905-9910.

33. Kazuomi Sato.; Masaru Toriyama. Depigmenting Effect of Catechins. *Molecules*. **2009**, 14, 4425-32

34. Seong Zuh-Kyung.; Lee Zuh-Kyung.; Poudel Amrit.; Oh Sei-Ryang.; Lee Hyeong-Kyu. Constituents of Cryptotaenia japonica Inhibit Melanogenesis via CREB- and MAPK-Associated Signaling Pathways in Murine B16 Melanoma Cells. *Molecules* 2016, 21, 1296

35. Lin RD.; Chen MC.; Liu YL.; Lin YT.; Lu MK.; Hsu FL.; Lee MH. "New whitening constituents from Taiwan-aative Pyracantha koidzumii: structures and tyrosinase inhibitory analysis in human epidermal melanocytes." *Int. J. Mol. Sci.* **2015**, 16, 28598-613.

36. Song NY.; Cho JG.; Im DM.; Lee DY.; Wu Qian.; Seo WD.; Kang HC.; Lee YH.; Bae NI. Triterpenoids from Fragaria ananassa calyx and their inhibitory effects on melanogenesis in B16-F10 mouse melanoma cells. *Nat. Prod. Res.* **2013**. 27, 2219–2223

37. Hsu Kai-Di.; Chen Hong-Jhang.; Wang Chi-Shin.; Lum Chi-Chin.; Wu Shu-Pei.; Lin Shin-Ping.; Cheng Kuan-Chen. Antimelanogenic activities of piperlongumine derived from Piper longum on murine B16F10 melanoma cells in vitro and zebrafish embryos in vivo: its molecular mode of depigmenting action. *Sci Rep.* **2016**. 6. 32854.

38. Lim YJ.; Lee EH.; Kang TH.; Ha SK.; Oh MS.; Kim SM.; Yoon TJ.; Gang C.; Park JH.; Kim SY. Inhibitory effects of arbutin on melanin biosynthesis of α-melanocyte stimulating hormone-induced hyperpigmentation in cultured brownish guinea pig skin tissues. *Arch. Pharm. Res.* **2009**. 32. 367–373

39. Yamaguchi, Y.; Brenner, M.; Hearing, V.J. The regulation of skin pigmentation. *J. Biol. Chem*. **2007**, 282, 27557–27561.

40. Brenner, M.; Hearing, V.J. The protective role of melanin against UV damage in human skin. *Photochem. Photobiol*. **2008**, 84, 539-549.

41. Brenner, M.; Berking, C. Principles of skin pigmentation. Biochemistry and regulation of melanogenesis. *Der Hautarzt*. **2010**, 61, 554–560.

42. Korner, A.; Pawelek, J. Mammalian tyrosinase catalases three reactions in the biocynthesis of melanin. *Science*, **1982**, 217, 1163-1165.

43. Hunt, G.; Todd, C.; Cresswell, J.E.; Thody, A.J. Alpha-melanocyte stimulating hormone and its analogue Nle4DPhe7 alpha-MSH affect morphology, tyrosine activity and melanogenesis in cultured human melanocytes. *J. Cell Sci.* **1994**, 107, 205-211.

44. Miyamura, Y.; Coelho, S.G.; Wolber, R.; Miller, S.A.; Wakamatsu, K.; Zmudzka, B.Z.; Ito, S.; Smuda, C.; Passeron, T.; Choi, W.; Batzer, J.; Yamaguchi, Y.; Beer, J.Z.; Hearing, V.J. Regulation of human skin pigmentation and responses to ultraviolet radiation. *Pigment Cell Melanoma Res*. **2007**, 20, 2-13.

45. Yamaguchi, Y.; Brenner, M.; Hearing, V.J. The regulation of skin pigmentation. *J. Biol. Chem.* **2007**, 282, 27557–27561.

46. Manandhar, B.; Wagle, A.; Seong, S.H.; Paudel, P.; Kim, H.R.; Jung, H.A.; Choi, J.S. Phlorotannins with potential anti-tyrosinase and antioxidant activity isolated from the marine seaweed Ecklonia stolonifera. *Antioxidants*, **2019**, 8, 240; doi:10.3390/antiox8080240

47. Yangthong, M.; Hutadilok-Towatana, N.; Phromkunthong, W. Antioxidant activities of four edible seaweeds from the southern coast of Thailand. *Plant Food Human Nutr.* **2009**, 64, 218-223.

48. Fang, D.; Tsuji, Y.; Setaluri, V. Selective down-regulation of tyrosinase family gene TYRP1 by inhibition of the activity of melanocyte transcription factor, MITF. *Nucleic Acids Res.* **2002**, 30, 3096–106.

49. Sarangarajan, R.; Boissy, R.E. Tyrp1 and oculocutaneous albinism type 3. *Pigment Cell Res.* **2001**, 14, 437–444.

50. Yasumoto, K.; Yokoyama, K.; Shibata, K.; Tomita, Y.; Shibahara, S. Microphthalmia-associated transcription factor as a regulator for melanocyte-specific transcription of the human tyrosinase gene. *Mol. Cell. Biol.* **1994**, 14, 8058–8070.

Figure Legends

Figure 1. Anti-melanogenesis and anti-oxidant effects of PAL, PAT and PAF extracts. (A) Melanin content, (B) TYR activity (C) cytotoxicity in B16F10. (D) ABTS and (E) DPPH radical scavenging activities. The datas are expressed as the mean ± SD (n = 3) of three individual experiments. *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, compared with control.

Figure 2. Anti-melanogenesis and anti-oxidant effects of each fraction of PAL. (A) Melanin content, (B) TYR activity (C) cytotoxicity in B16F10. (D) ABTS and (E) DPPH radical scavenging activities. The datas are expressed as the mean ± SD (n = 3) of three individual experiments. *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, compared with control.

Figure 3. Schematic representation of the isolation of HG and CH from PAL using bioactivity-guided fractionation. Bioactivity-guided fractionation of PAL was performed as shown in the schematic representation and resulted in the isolation and identification of HG and CH. Fractionation was guided by assessing the inhibitory effect of HG and CH on melanin content without cytotoxicity and DPPH radical activity at 100 μg/mL. At each level of fractionation, all the fractions generated were tested simultaneously and were compared to the crude extract.

Figure 4. Chemical structures of compounds isolated from PAL.

Figure 5. Anti-melanogenesis and anti-oxidant effects of seven compounds isolated from *n*-

butanol fraction of PAL.

Figure 6. Effects on TYRP-1 and TYRP-2 expression of HG and CH. The datas are expressed as the mean ± SD (n = 3) of three individual experiments.

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Figure 1.

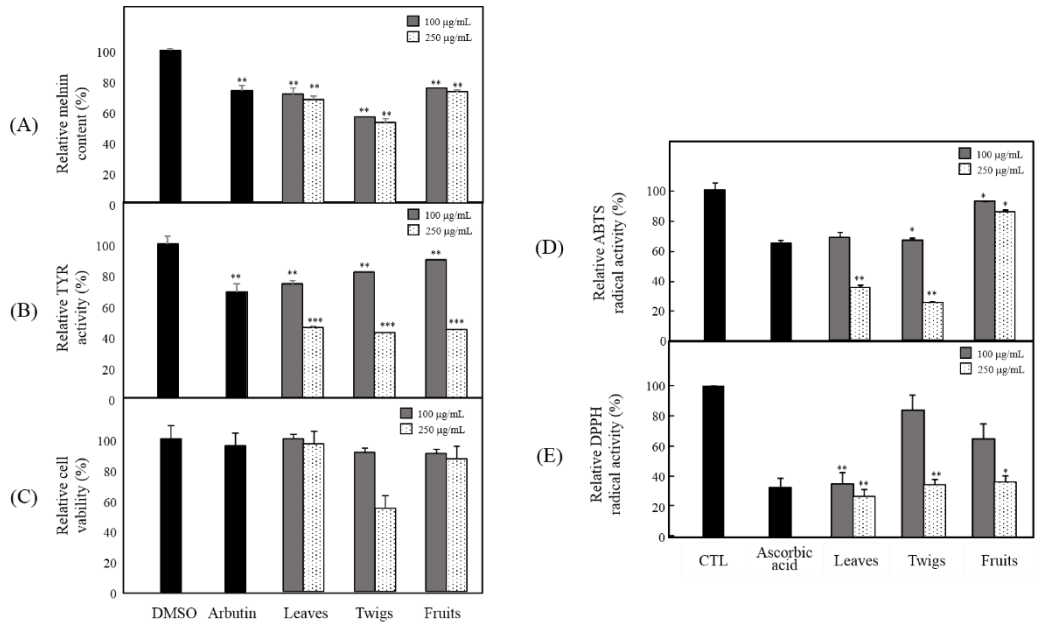

Figure 2.

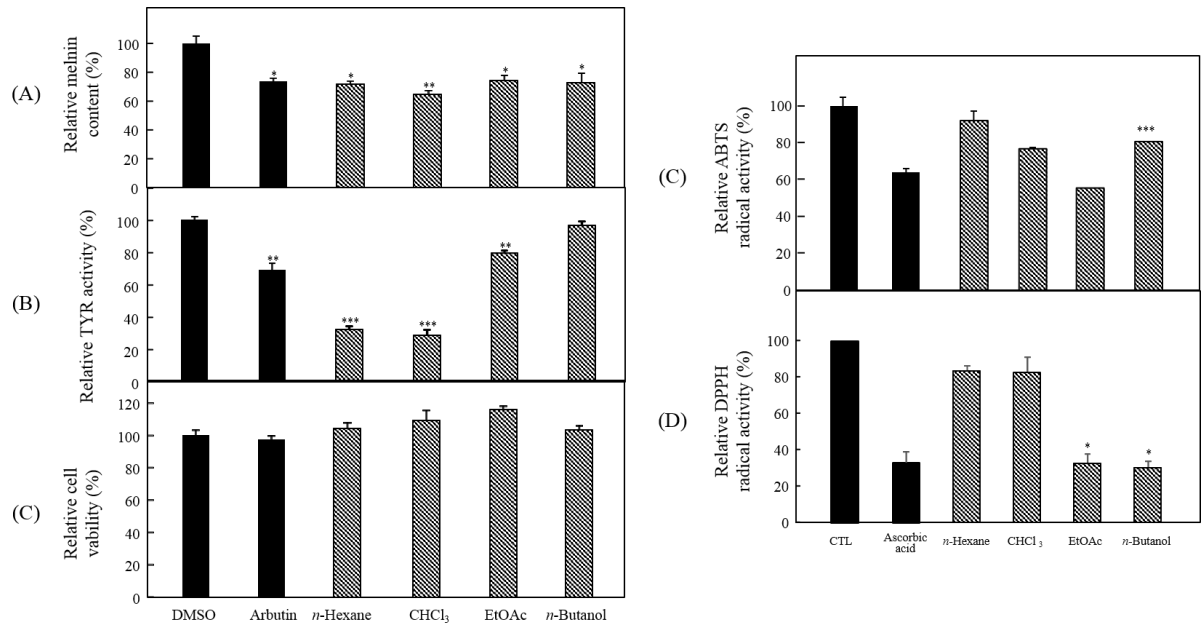

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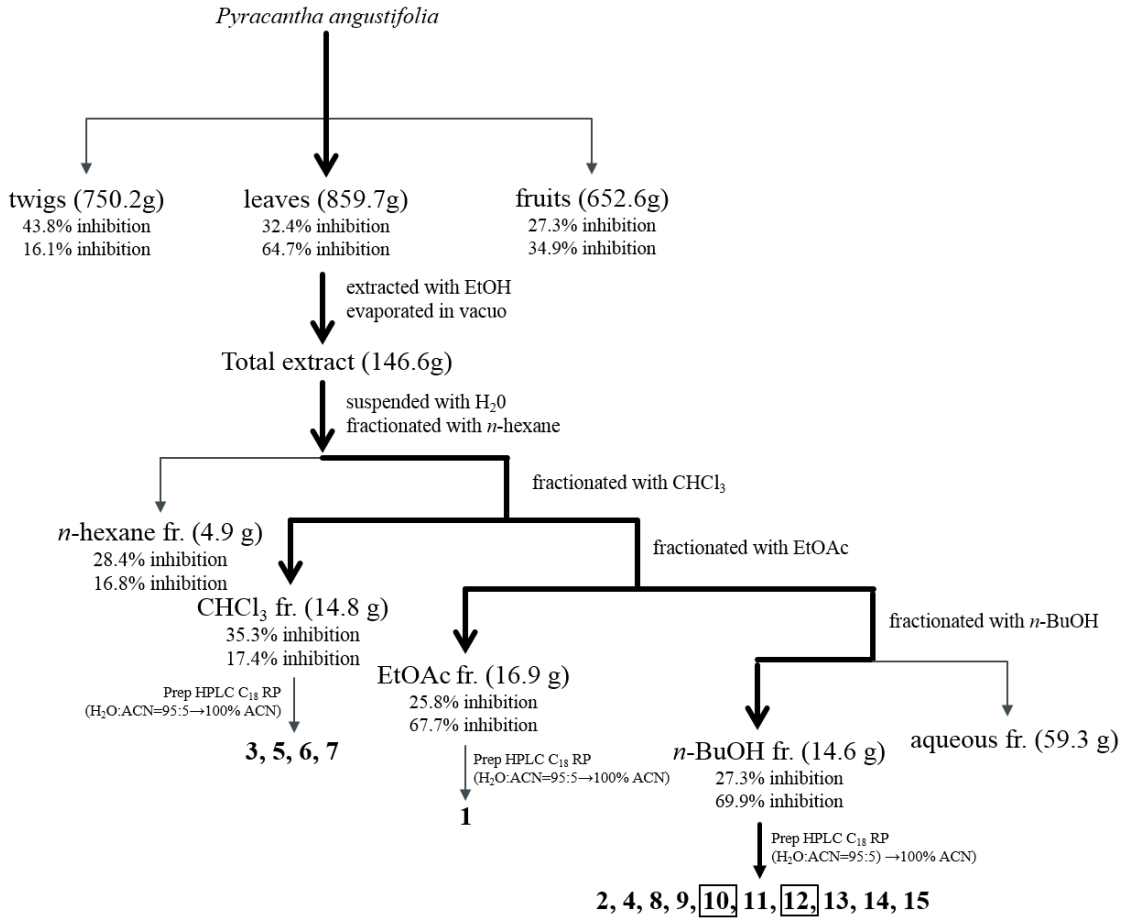
Figure 3.



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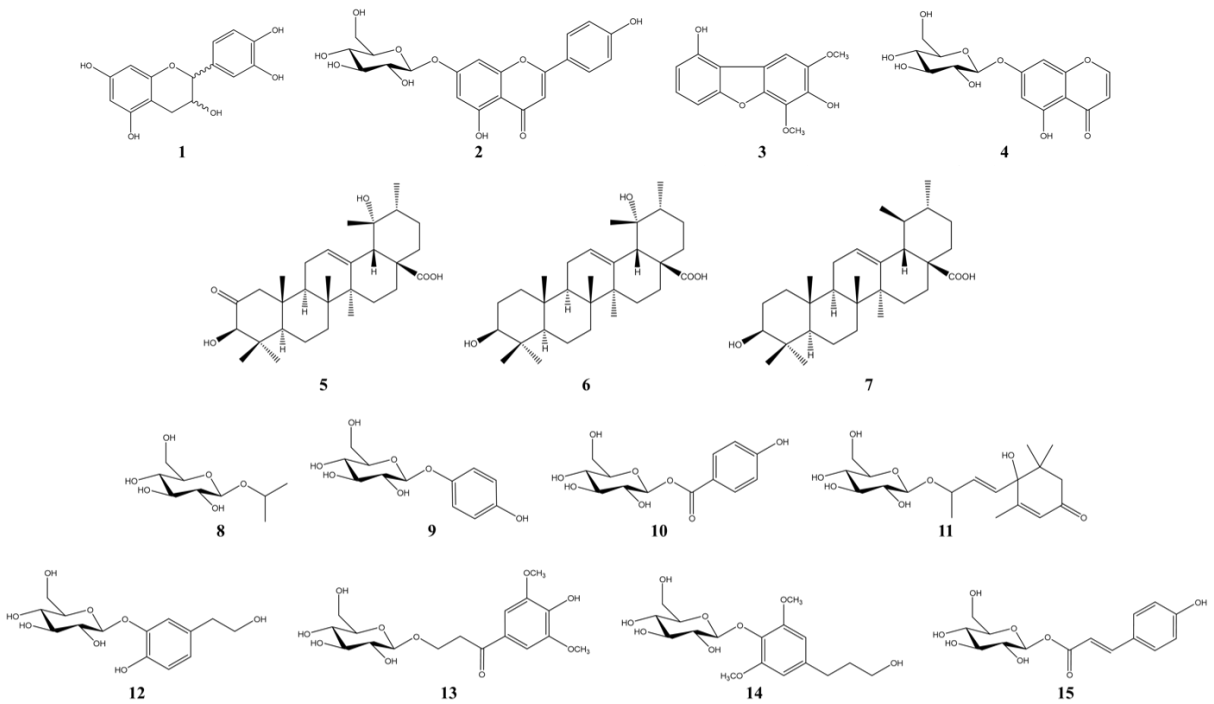

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Figure 5.

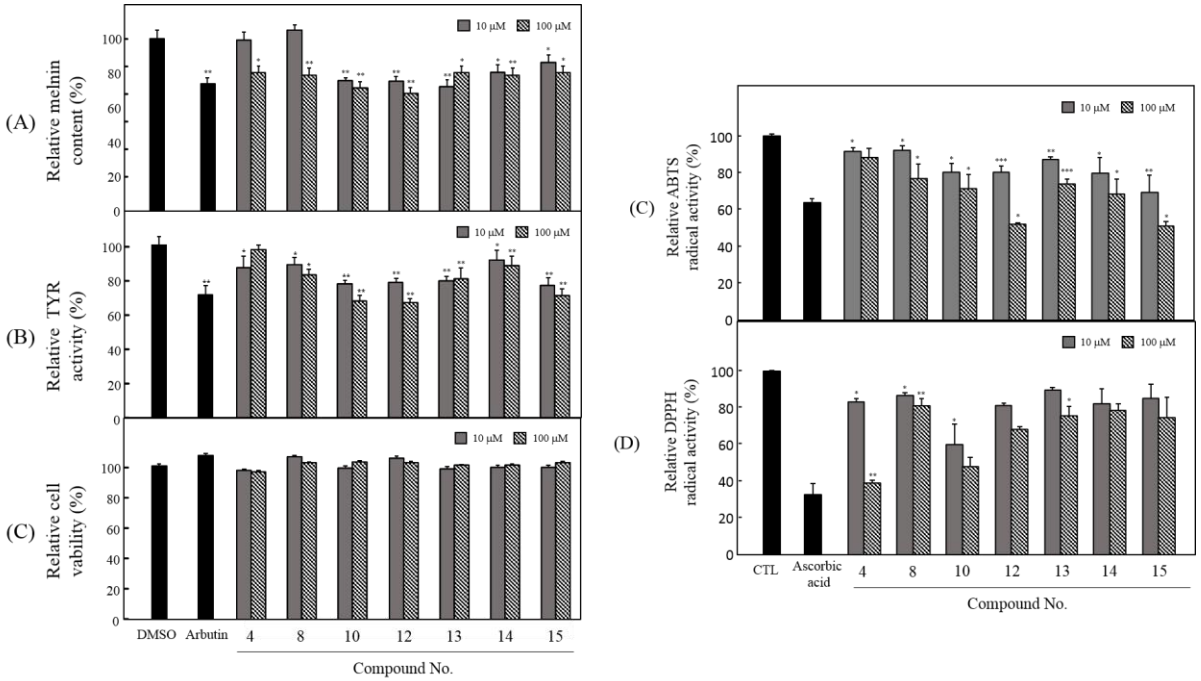

Figure 6.