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Inhibitors of Tyrosinase and Melanogenesis from *Galla rhois*

Hyo-Jin Kim, Dong-Il Jang* and Sang-Won Choi†

Dept. of Food Science and Nutrition, Catholic University of Taegu-Hyosung, Hayang 712-702, Korea
**Pacific R & D Center, Kyounggi-Do 456-234, Korea*

Abstract

Previously, a 50% aqueous methanol extract of *Galla rhois* was shown to be the most potent tyrosinase inhibitory activity with an IC_{50}(the concentration causing 50% inhibition of tyrosinase activity) of 0.2mg/ml of 205 crude drug extracts. To isolate tyrosinase inhibitors, the methanol extract was evaporated to a small volume *in vacuo*, and then partitioned stepwise with benzene and ethyl acetate(EtOAc). The EtOAc fraction was solubilized in 10% MeOH solution, and then fractionated successively by Diaion HP-20 and Sephadex LH-20 column chromatography, and preparative HPLC. Three phenolic compounds were isolated, and characterized as gallic acid(GA), methyl gallate(MG) and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose(PGG) by UV, IR, ^{1}H-&^{13}C-NMR, and FAB- MS spectroscopy. PGG(IC_{50}=50μg/ml) showed a considerable inhibitory effect against mushroom tyrosinase, while GA(IC_{50}=1.6mg/ml) and MG(IC_{50}=234μg/ml) did not show an appreciable effect. Meanwhile, MG inhibited greatly melanogenesis in a murine melanocyte cell line, Mel-Ab. MG and PGG showed typical noncompetitive inhibition patterns against mushroom tyrosinase. These results suggest that PGG and MG may be potentially useful as either anti-browning or anti-melanogenic agents in foods and cosmetics.

Key words: *Galla rhois*, tyrosinase inhibitor, melanogenesis inhibitor, gallic acid, methyl gallate, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose

INTRODUCTION

Tyrosinase(EC 1.14.18.1) is a copper-containing enzyme which catalyzes two different reactions : the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine(L-DOPA) and the oxidation of L-DOPA to dopaquinone(1,2). The enzyme was mainly responsible for melanin biosynthesis(melanogenesis) in animals and enzymatic browning(melanosis) in plants(3,4). Therefore, the inhibition of tyrosinase has provided a basis for an *in vitro* method for the screening of anti-browning or anti-hyperpigmentating agents from natural products.

Recently, a number of tyrosinase inhibitors have been isolated and identified from natural and microbial origins (5-10). 4-Hexylresorcinol is used as a functional alternative to sulfites for the control of enzymatic browning in shrimp(11,12). Kojic acid, arbutin, and ascorbic acid derivatives are utilized topically for treatment of local hyperpigmentation in humans such as lentigo, melasma and freckling(13-15). However, the application of other natural tyrosinase inhibitors to foods and cosmetics is somewhat limited due to off-flavors, off-odors, toxicity, and lack of economic feasibility(16,17). Therefore, the development of alternative safe and efficacious tyrosinase inhibitors is needed.

Meanwhile, new screening methods to replace an *in vitro* tyrosinase enzymatic assay have been developed to search for novel melanogenesis inhibitors(18-20). Especially, an *in vitro* cell culture assay using a murine melanocyte cell line, Mel-Ab was recently used to screen out melanogenesis inhibitors(19). Several compounds including methyl gentisate were found to be highly potent inhibitors in this assay, although they were inactive in an enzymatic assay. Thus, there is much attention on the development of novel tyrosinase inhibitors through an *in vitro* cell culture assay in parallel with an enzymatic assay.

In the previous report, we found that among 205 crude drug extracts examined 50% aqueous methanol extract of *Galla rhois* exhibited the most potent tyrosinase inhibitory activity(IC_{50}=0.2mg/ml)(21). *Galla rhois* derived from leaf galls of *Rhus javanica* L. is an important constituent of traditional Chinese medicines used as an anti-diarrhea and astringent agents.

The objective of this study was to isolate and characterize tyrosinase inhibitors from *Galla rhois*, and further

†Corresponding author

to investigate their inhibitory effects on melanin biosynthesis.

MATERIALS AND METHODS

Materials and reagents

Galla rhois, produced in Korea, was purchased from local Oriental herbal stores in Taegu, Korea. Column chromatography was performed with Diaion HP-20(Mitsubish Chem., Co., Tokyo, Japan) and Sephadex LH-20(Pharmacia Fine Chemicals, Uppsala, Sweden). Mushroom tyrosinase(EC 1.14.18.1; 2,750units/mg). L-DOPA, kojic acid and L-ascorbic acid were purchased from the Sigma Chemical Co.(St. Louis, MO, U.S.A.). All other chemicals used in this study were of analytical grade.

Extraction and fractionation of tyrosinase inhibitors

Dried and chopped *Galla rhois*(100g) was extracted twice with 80% aqueous MeOH(2L) at room temperature for overnight. The combined extracts were evporated to a small volume *in vacuo*, and the resulting insouble material was removed by filtration through Whatman No. 2 filter paper. The aqueous solution was partitioned stepwise with benzene(500ml×2) and ethyl acetate(500ml ×2), and then evaporated. The EtOAc extract(38.5g) was dissolved in 10% aqueous MeOH, and then applied onto an Diaion HP-20 column(5cm×80cm). The column was washed stepwise with distilled H_2O, MeOH-H_2O(30:70, v/v), MeOH-H_2O(70:30, v/v, 19.1g), and MeOH. A portion of 70% aqueous MeOH fraction(10g) showing strong tyrosinase inhibitory activity was further applied to a Sephadex LH-20 column(2cm×100cm) eluting with MeOH (300ml), MeOH-H_2O(50:50, v/v, 500ml) and MeOH-H_2O-acetone(50:30:20, v/v/v, 300 ml), and MeOH-H_2O-acetone(30:20:50, v/v/v, 300ml) to give four fractions: Fr. I(1.5g), II(238mg), III(4.2g) and IV(1.5g). The tyrosinase inhibitory activity of each fraction was 0, 78.3, 32.1, and 28.8%, respectively, at a concentration of 50μg/ml. A portion(25mg) of Fr. II was further purified by preparative HPLC(Waters Delta Prep 4000, USA) using a Novapak C_{18} column(4cm×25cm×2cartridge) at a flow rate of 10ml/min, monitored at 270nm, using CH_3CN-H_2O-HOAc(20:80:0.1, v/v/v) as a mobile phase to give isolated compounds(**1**, 1mg), (**2**, 6mg) and (**3**, 7mg)(Fig. 1), as off-white amorphous powders.

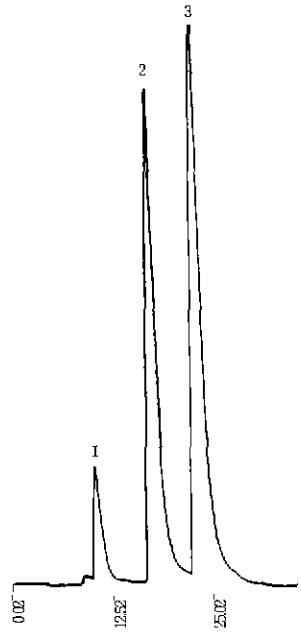

Fig. 1. HPLC chromatography of three phenolic compounds isolated from *Galla rhois*.
HPLC conditions: column, Novapak C_{18}(4cm×25cm ×2 cartridge); solvent, CH_3CN-H_2O-HOAc (20:80:0.5, v/v/v); flow rate, 10ml/min; detector, UV 270nm.

Instrumental analysis

UV-visible absorption spectra in MeOH were recorded on a spectrophotometer(Shinco, S2030, Korea) in MeOH. The IR spectra were taken on an FS 120 HR/FRA infrared spectrophotometer(Bruker, Germany) as KBr discs, and the absorbance frequency was expressed in cm^{-1}. ^1H-NMR(500MHz) and ^{13}C-NMR(125MHz) were taken on a Bruker AMX-500 spectrometer in CD_3OD containing tetramethylsilane(TMS) as an internal standard. The fast atom bombardment mass(FAB-MS) was measured with a JEOL JMS-AX-505 WA spectrometer(Japan Electron and Optics, Tokyo, Japan), using glycerol as a mounting matrix.

Tyrosinase assay

Tyrosinase was assayed according to the previous method(21). The reaction mixture(3ml) containing 1.52 mM L-DOPA, 67mM phosphate buffer(pH 6.8), 90units of mushroom tyrosinase, and the extracts at various concentrations, was incubated at 25°C for 2min. The change in absorbance at 475nm with or without the sample was linear for 2min. The extent of tyrosinase inhibition by the test compounds is expressed as a concentration causing 50% inhibition(IC_{50}). The percentage inhibition of tyrosinase reaction was calculated as follows; Inhibition(%)=

[(A-B)/A×100], where A is an absorbance at 475nm after incubation without test sample, and B is an absorbance at 475nm after incubation with test sample. The results were obtained from the almost concurrent three readings so that the standard deviation(S.D.) was negligible and is not shown in the results.

Assay of melanogenesis

The melanogenesis was assayed by the method of Dooley et al.(19) with a slight modification. The Mel-Ab cell line, a C57BL/6 mouse-derived spontaneously immortalized melanocyte cell line was cultured in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum, 100 nM 12-*O*-tetradecanoylphorbol-13-acetate and 1×10^{-9}M cholera toxin. Mel-Ab cells were incubated at 37°C in an atmosphere of 5% CO_2. Cells were trypsinized with 0.25% trypsin/EDTA and then placed into Corning 24-well plastic culture plates at a density 1×10^5 cells/well. Cells were cultured for 5 days. The media were changed every day with fresh medium except on the fourth day. Thereafter, cells were collected and the quantification of synthesized melanin was compared with vehicle treated. The melanin content of Mel-Ab cells after sample treatment was spectrophotometrically determined after being solubilized with 1N NaOH. Results from duplicate samples were analyzed as percent of control of vehicle-treated Mel-Ab cultures.

RESULTS AND DISCUSSION

Structural elucidation of compound 1, 2 and 3

UV, IR, ^{1}H- & ^{13}C-NMR, and FAB-MS spectral data of compound **1**, **2**, and **3** isolated from *Galla rhois* are shown in Table 1. The UV maximum absorption at 260~275nm, and IR absorption bands at 3400-3410(OH), 1650~1710(C=O), 1400~1620(aromatic) and 1200~1210cm^{-1}(C-O) indicated that three compounds were phenolic compounds with the ester carbonyl group. The ^{1}H- & ^{13}C-NMR spectra of compound **1** and **2**, as measured in CD_3OD, showed the presence of gallic acid and its methyl ester. Meanwhile, the FAB-MS of compound **1** and **2** gave their molecular ion peak [M$^+$] at 170 and 185m/z, respectively. Thus, compound **1** and **2** were determined to be as gallic acid(GA) and methyl gallate(MG), which have been re-

Table 1. UV, IR, NMR and FAB-MS spectral data of gallic acid, methyl gallate and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose(PGG) isolated from *Galla rhois*

	Gallic acid	Methyl gallate	PGG
UV λ_{max}(nm)	264nm(log ε =4 37)	273nm(log ε =4.08)	270nm(log ε =4.62)
IR ν_{max}(cm^{-1})	3413(OH), 1679(C=O) 1429~1616(aromatic) 1211(C-O)	3407(OH), 1680(C=O) 1438~1614(aromatic), 1210(C-O)	3410(0H), 1707(C=O) 1451~1618(aromatic), 1206(C-O)
^{1}H-NMR	7.01(2H, S, H_2 & H_6)	7.05(2H, S, H_2 & H_6)	4.50(2H, m, H_6), 4.61(1H, m, H_5), 5.69(1H, t. 8.0, H_2), 5.72(1H, t, 8.0, H_4), 6.00(1H, t, 8.0, H_3), 6.33(1H, d, 8.0, H_1)
-OCH_3		3.82	
Galloyl ArH			7.01, 7.06, 7.09, 7.16, & 7.22(each 2H,s)
^{13}C-NMR			
Galloyl	147.01(C_3 & C_5), 138.02(C_4), 121.72(C_1), 108.58(C_2 & C_6)	146.48(C_3 & C_5), 139.73(C_4), 121.49(C_1), 110.08(C_2 & C_6)	119.6~120.9($C_{1'}$), 110.3~110.6($C_{2'}$ & $C_{6'}$) 139.9~140.6($C_{4'}$) 146.1~146.4($C_{3'}$ & $C_{5'}$)
Ester C=O	169.71	169.02	167.8, 167.2, 166.9, 166.8 & 166.1
Glucose			93.1(C_1), 72.1(C_2), 74.2(C_3) 69.7(C_4), 74.0(C_5), 63.0(C_6)
FAB-MS(m/z)	170 [M$^-$]	185 [M$^+$]	939 [M$^+$]

Coupling constants(J in Hz) in parentheses

ported to be present in Chinese gallotannin and other plants(22-25). On the other hand, UV and IR absorption bands of compound **3** were similar to those of compound **1**, indicating that compound **3** was also gallic acid with the ester group. Its ^{1}H- & ^{13}C-NMR spectra showed that the presence of glucose core and five gallic acid moieties. Anomeric proton at δ 6.33 was assigned as β-D-glucose based on the vicinal coupling constant with the J value of 8Hz(26). Futhermore, gallic acid was determined to be bonded to the C-1,2,3,4 & 6 hydroxyl groups of glucose by the observation of low-field shifts of protons of glucose, by analogy with the chemical shifts of each hydroxyl group of glucose(27). Meanwhile, the FAB-MS of compound **3** gave its molecular ion peak [M^+] at 939m/z. The fragment ion peak at 765m/z(five galloyl group) [939-174(glucose core)] was observed in the FAB-MS spectrum(data not shown). From these results, compound 3 was assigned to be 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose(PGG, Fig. 2), which has been reported to be present in a range of plants where it appears to act as a key intermediate in the further metabolism of gallic acid(28). The detailed other spectral data of compound **1**, **2**, and **3** on UV, IR, NMR, and FAB-MS are given in Table 1.

Tyrosinase inhibitory activity of three isolated compounds

The tyrosinase inhibitory activities of GA, MG and PGG isolated from *Galla rhois* are shown in Table 2. Three compounds showed a dose-dependent inhibitory effect on the mushroom tyrosinase(data not shown). Among the compounds tested, PGG exhibited the most potent tyrosinase inhibitory activity, followed by MG. and GA, in a descending order. PGG(IC_{50}=50μg/ml) exhibited stronger inhibitory activity than L-ascorbic acid(IC_{50}=70μg/ml), although the activity was weaker than a well-known

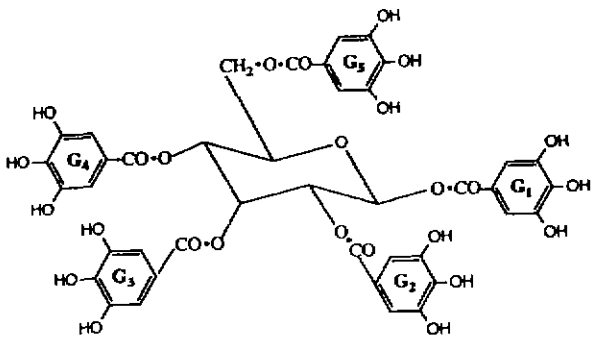

Fig. 2. Chemical structure of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose isolated from *Galla rhois*.

Table 2. Tyrosinase inhibitory activity of three phenolic compounds isolated from *Galla rhois*

Compounds	IC_{50}(μg/ml)[1)]
Gallic acid	>1000
Methyl gallate	214
PGG[2)]	50
L-Ascorbic acid	70
Kojic acid	10

[1)] IC_{50} is the concentration causing 50% inhibition of tyrosinase activity

[2)] 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose

tyrosinase inhibitor, kojic acid(IC_{50}=10μg/ml). MG showed also a moderate inhibitory activity(IC_{50}=234μg/ml), whereas GA did not show an appreciable activity. The result suggest that MG and PGG may be mainly responsible for the strong inhibitory effect of *Galla rhois* extract against mushroom tyrosinase(21). This result is also supported by an earlier report that *Terminalis chebula* Retzius containing PGG as a major tannin compound showed potent tyrosinase inhibitory activity(21,28).

Inhibition of melanogenesis by three isolated compounds

The inhibitory effects of three isolated compounds on melanogenesis in a murine melanocyte cell line, Mel-Ab are presented in Table 3. MG showed a considerable inhibition toward melanogenesis of murine Mel-Ab cells at a concentration of 10ppm, which is not cytotoxic to the cells(data not shown). However, GA, PGG, and kojic acid as positive control did not show appreciable activity at the same concentration. In the previous report, it was found that the methyl ester of several phenols inhibited specifically an *in vitro* synthesis of melanin in Mel-Ab without cytotoxicity(19). Thus, this result suggest that MG may be also used as potential anti-melanogenesis agent in cosmetics. We report here for the first time that

Table 3. Inhibition effects of three phenolic compounds isolated from *Galla rhois* on melanin biosynthesis

Compounds	Melanin content(ng/cell)	
	10ppm	1ppm
Control	0.454	0.454
Gallic acid	0.411	0.576
Methyl gallate	0.295	0.338
PGG	0.501	0.349
Kojic acid	0.489	0.514

Mel-Ab cell with sample of various concentration were incubated at 37℃ for 5 days

MG in *Galla rhois* can be acted as an anti-melanogenesis agent, although MG have already been characterized(24, 25). Further study on the inhibitory effects of MG and PGG in an *in vivo* melanogenesis is now in progress. In addition, the safety test of PGG and MG in foods and skin applications is certainly needed to use as anti-browning or skin-lightening agents.

Inhibition patterns of MG and PGG against mushroom tyrosinase

The type of inhibition exerted by the two principal active compounds, MG and PGG on mushroom tyrosinase was deduced from the Lineweaver-Burk double reciprocal plots(29) of 1/S (L-DOPA concentration) vs 1/v (tyrosinase activity) in the absence and the presence of MG and PGG (Fig. 3). As a result, MG and PGG exhibited typical noncompetitive inhibitions, which may come from its ability to chelate copper at the active site of tyrosinase and to bind a site other than the active site(30). Thus, it is interesting to note that MG and PGG may play an important role in inhibition of melanogenesis and melanosis, respectively. Further screening for other tyrosinase inhibitors from *Galla rhois* is now under investigation.

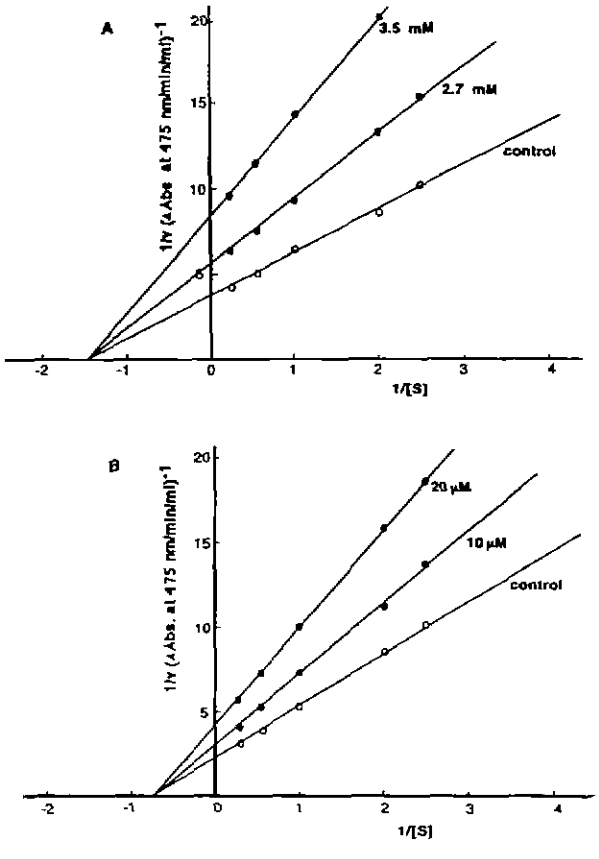

Fig. 3. Lineweaver-Burk plots of mushroom-tyrosinase in the absence(○) or presence(●) of methyl gallate(A) and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose(B).

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