Skin permeation and comparative evaluation of gentisic acid ester derivatives as skin-lightening agents

Nan Shun Ma[1], Marion Abraham[2], Myeong Jun Choi[3], Jung Sun Kim[4]*

[1]College of Pharmacy, Pusan National University, Busan 609-735, South Korea
[2]Department of Marine Chemistry, Leibniz Institute of Baltic Sea Research, 18119 Rostock, Germany
[3]Phytos Co., Gyeonggi University, Suwon, Gyeonggi-do 443-760, South Korea
[4]Division of Health Sciences, Dongseo University, Busan 617-716, South Korea
*Correspondence: jsk@gdsu.dongseo.ac.kr

Gentisic acid (GA) derivatives are known to exhibit inhibitory activity against tyrosinase. Skin permeation of five GA derivatives have been evaluated and correlated with their tyrosinase inhibition and cytotoxicity. All compounds tested were stable at 37 °C up to 12 h. Skin permeation study in mice showed the highest permeation rate for the methyl derivative, MG (232.0033 µg/cm²/h) over GA (172.0852 µg/cm²/h), followed by the ethyl derivative, EG (182.9242 µg/cm²/h). Although all the esters tested were more cytotoxic to melanocytes than GA, MG was the least cytotoxic among the five esters. MG and EG also showed similar mushroom tyrosinase inhibition to gentisic acid (IC_{50} 176 µg/mL). MG showed the highest skin permeation rate among the GA derivatives tested, and was considered a promising candidate together with EG based on the tyrosinase inhibition and melanocyte cytotoxicity.

Key words: Gentisic acid – Methyl gentisate – Ethyl gentisate – Skin permeation – Tyrosinase inhibition – Melanocyte.

Melanin pigments are widespread in the animal kingdom. In humans, they are responsible for the color of skin, hair and iris of the eyes. They are produced by melanocytes that are located in the bottom of the epidermis. Melanin is synthesized when induced by UV radiation to prevent damages from environmental influences. However, it can be the cause of pigmentary disorders as in the case of freckles, melasma or senile lentigines which are considered serious aesthetic problems [1]. Genetic variants within the genes involved in skin pigmentation are reported to be determinants of several skin cancers [2]. For these reasons, developing drugs that can down regulate the synthesis of melanin is necessary.

Tyrosinase is the key enzyme of melanogenisis and could be an effective target enzyme for developing skin-lightening agents [3]. Tyrosinase catalyzes the oxidation of the amino acid L-tyrosine to L-3-(3,4-dihydroxyphenyl)-L-alanine (L-dopa) and subsequently to L-dopaquinone. In the absence of cysteine, L-dopaquinone is converted to cyclodopa and subsequently to eumelanin, giving rise to brown and black pigmentation. In the presence of cysteine, L-dopaquinone is converted to 5-S-cysteinyldopa and finally to pheomelanin that yields in amber and red pigments. The ratio of eumelanin to pheomelanin depends on the availability of cysteine in the melanocytes [1]. Tyrosinase is a copper metalloenzyme and it is assumed that tyrosinase inhibiting compounds chelate the copper and therefore block the active site of this enzyme [3, 4].

There are many compounds that have been identified to inhibit tyrosinase from natural products [5]. Hydroquinone, ascorbic acid, kojic acid, arbutin, gentisic acid and its esters have been reported as skin-lightening cosmetic products [6-10]. The vast search for potential tyrosinase inhibitors have accumulated into a huge number of compounds for use in food and cosmetics derived from natural and synthetic sources [11]. Among these, hydroquinones tend to exhibit very efficient tyrosinase inhibiting activities although they could be highly cytotoxic or mutagenic. However, gentisic acid, a natural product from the root of Gentiana, is known to be a safe and mild agent for treating cutaneous hyperpigmentation disorders including melasma and UV-induced ephelides [12]. The alkyl esters of gentisic acid were reported to be good inhibitors of melanogenesis, showing tyrosinase inhibiting activities and mutagenicity to some extent, among which the methyl gentisate was reported as a promising candidate as a skin-lightening agent [10].

Although skin-whitening agents are applied to and target the skin, studies on these agents are limited to *in vitro* chemical studies, not placing much emphasis on how these compounds could actually penetrate and deposit into the skin. Bian *et al*. has reported the development of a matrix-type formulation for topical delivery of GA into melanocytes where the effect of drug concentrations, enhancers and various adhesives on the permeation rate and skin deposition of GA were investigated [13]. Because melanocytes locate to the bottom layer of the epidermis, a skin-lightening effective agent has to be able to permeate through the skin in order to exert its inhibiting effect within the melanocytes. The potential as a skin-lightening topical drug does not solely rely on the compound's tyrosinase inhibiting activity, but also on its cytotoxicity and structural parameters that are predominantly lipophilicity for sufficient skin permeation and water solubility for its formulation.

In the current study alkyl side chains were added to GA with anticipation of enhancement of skin permeation efficiency. Herein we report on a skin permeation analysis of five gentisic acid esters harboring alkyl side chains of different length in comparison to the gentisic acid as parent compound. Furthermore, tyrosinase inhibition and cytotoxicity to Melan A cells have been studied and correlated to structural parameters in a structure-activity-relationship analysis.

I. MATERIALS AND METHODS

1. Materials

All chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, United States) and were reagent grade or better. Gibco RPMI media for maintenance of Murin Melan-A cells were purchased from Invitrogen (Seoul, South Korea). Silica gel plates (Silicage l60F254) used for thin layer chromatography and silica gel (Merck 60) for column chromatography were purchased from Merck Co. (Darmstadt, Germany). Melting points were determined with the Buchi M-530 capillary melting point apparatus. Infrared spectral data (IR) were obtained on a Mattson 3000 Fourier Transform spectrometer and are reported in cm^{-1}. Proton (^1H NMR) nuclear magnetic resonances were recorded on a Varian Gemini-300 Fourier Transform spectrometer. The NMR spectra (300 MHz) were recorded in the deuterated solvent indicated with chemical shifts reported in δ units downfield from tetramethylsilane (TMS). Coupling constants are reported in hertz (Hz). Ultraviolet spectra were recorded on an

Optizen 2120 UV/Vis spectrometer. HPLC analyses were performed with a Young-lin ACME HPLC equipped with a Young-lin S930D pump and Young-lin UV 730D detector.

2. Synthesis of gentisic acid esters

The gentisic acid esters were synthesized as described by Curto *et al*. [10]. The methyl, ethyl, *n*-propyl, *i*-propyl and *n*-butyl esters of gentisic acid were prepared by refluxing gentisic acid in an excess (1 g, 6.49 mmol) of the corresponding alcohols for 1-2 days using *p*-toluenesulfonic acid as a catalyst. The course of the reaction was followed by thin layer chromatography (TLC). The products were purified by evaporation of the solvent, extraction with ethyl acetate, water and small aliquots of aqueous sodium bicarbonate until no gentisic acid was visible in the organic phase by TLC. The organic layer was dried over sodium sulfate and evaporated in vacuum. The structures of the gentisic acid analogues were verified by melting point (m.p.) determination, ^{1}H NMR as well as IR spectroscopy.

3. HPLC analysis and quantification

For chromatographic separation, 20 μL of the gentisic acid esters were separated on an RP-18 column (Lichrospher 125 × 4 mm, 5 μm particle size, Merck, Germany) at ambient temperature at a flow rate of 1 mL/min. Acetonitrile and water containing 2 % (v/v) phosphoric acid have been used as mobile phase in different ratios for separation of the gentisic acid esters (gentisic acid (GA) 10:90, gentisic acid methyl ester (MG) 25:75, gentisic acid ethyl ester (EG) 30:70, gentisic acid propyl ester (PG) and gentisic acid isopropyl ester (IPG) 40:60, gentisic acid butyl ester (BG) 45:55). The mobile phase was filtered through a membrane filter (47 mm, 0.2 μm, Satorius Co., Germany) and degassed in an 8510 Branson ultrasonicator prior use. For detection the variable wavelength detector was set at 239 nm. For quantification different concentrations of each gentisic acid ester was injected and separated as described above. The peak area of each gentisic acid ester was plotted against the respective concentrations to obtain the following calibration curves: GA, $y = 9.895x + 0.621$, $r^2 = 0.999661$; MG, $y = 51.609x + 22.044$, $r^2 = 0.999688$; EG, $y = 46.741x - 30.272$, $r^2 = 0.998451$; PG, $y = 41.55x - 15.706$, $r^2 = 0.999968$; IPG, $y = 42.073x - 8.697$, $r^2 = 0.999995$; BG, $y = 76.194x - 9.574$, $r^2 = 0.999971$.

4. Determination of capacity factors

Gentisic acid esters were dissolved in methanol to a final concentration of 20 μg/mL and separated by HPLC as described above. The capacity factor k' of each gentisic acid ester was calculated as $k' = (t_R - t_0)/t_0$, with t_R as the compound's retention time and t_0 the retention time of the mobile phase. The capacity factor analysis was done in triplicates.

5. Determination of water solubility

To determine the water solubility of the gentisic acid esters, excess amounts of each compound was added to 1 mL of water. The solution was placed in a shaking water bath for 24 h at 37 °C to reach equilibrium. After filtering through a Minisart KC4 filter (0.45 μm, Satorius, Germany), the filtrate was appropriately diluted with methanol and analyzed by HPLC as described above.

6. Temperature stability analysis

Solutions containing 2.5 μg/mL of GA, MG, EG, IPG, PG and 1.25 μg/mL of BG were placed in a shaking incubator at 37 °C. At predetermined time intervals, 100 μL samples were drawn and the concentration of the gentisic acid esters were determined by HPLC as described above. The relative percentage of the remaining compound was calculated with the initial concentration considered as 100 %.

7. Preparation of hairless mouse skin

The animals used for the skin permeation studies were five week old male ICR hairless mice obtained from Orient (Kyounggi, Korea). The animals received standard laboratory chow and had free access to water before the experiment. They were sacrificed by ether right before the experiment, and full-thickness skin was surgically removed. The skin specimen were cut into appropriate sizes after carefully removing subcutaneous fat and washed with normal saline.

8. *In vitro* skin permeation analysis

In vitro mouse skin permeation studies of the gentisic acid esters were conducted using Franz diffusion cells at 37 °C. Freshly excised mouse skin was mounted between the donor and receptor cells with the stratum corneum side facing the donor cell. The area of diffusion for all experiments was 2.14 cm^2. The receptor cells were filled with 12 mL of 40 % (v/v) propylene glycol in buffered saline (PBS) to maintain sink conditions. The donor cells contained super-saturated solutions of the respective gentisic acid ester in water and were covered with parafilm. At each predetermined time interval, 1 mL of sample was taken from the receptor cells and refilled with the same volume of fresh receptor solution. Samples were stored at -21 °C until analyzed by HPLC as described above.

9. Determination of mushroom tyrosinase inhibiting activity

To analyze the inhibiting effect of the different gentisic acid esters with respect to mushroom tyrosinase, different concentrations of each compound were tested. Stock solutions containing 1 mg/mL of the respective gentisic acid ester in 10 % (v/v) DMSO were diluted to concentrations of 300, 200, 150, 100, 50 and 10 μg/mL. The test was performed in a 96-well microplate and each test consisted of 20 μL of the respective gentisic acid ester, 80 μL PBS buffer, 50 μL of a 4 mM L-tyrosine solution and 50 μL of a solution containing 100 Units/mL mushroom tyrosinase. The following samples served as control samples: a blank sample without mushroom tyrosinase, a positive control sample without any gentisic acid ester and a negative control sample omitting gentisic acid esters and mushroom tyrosinase. The omitted substances were replaced by appropriate volumes of PBS buffer. The samples were incubated for 1 h at 37 °C and absorbances were determined at 490 nm. The tyrosinase inhibiting activity was calculated as [% inhibition] = [1-(S-B)/(P-N)] × 100 % (S: sample, B: blank, P:positive control, N: negative control). For each gentisic acid ester, the concentration reducing the mushroom tyrosinase activity to 50 % (IC_{50} [μg/mL]) was calculated.

10. Analysis of the cytotoxicity of gentisic acid esters on Murin Melan-A cells

Murin Melan-A cells were maintained according to Bennett *et al*. [14] and cultured on RPMI medium. Cell growth and viability were assayed using the neutral red assay that is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind the supravital dye Neutral Red.

Initially, Murin Melan-A cells were cultured until they approached confluency. Cells were trypsinized and seeded into a fresh culture dish until a cell density of 10^5 cell/mL was reached. Into each well of a 96-well microplate, 150 μL cell suspension were transferred. Cells were further allowed to grow for 24 h after which the growth medium was changed. Fifteen microlitres of the gentisic acid ester solutions was added to the cell suspensions and incubated for four days at darkness. Murin Melan-A cells were washed twice with PBS buffer and dissolved in 1 N NaOH / 10 % (v/v) DMSO. The absorbance was measured spectrophotometrically using a microplate reader at a wavelength of 490 nm. Cytotoxicity of the gentisic acid esters was expressed as IC_{50} that describes the concentration of the gentisic acid esters at which the cell viability was 50 % as compared to non-treated cells.

11. Statistical analysis

Correlation coefficients were obtained by performing a regression analysis using Excel. A *p*-value less than 0.05 was considered to be statistically significant using the Student's t-test for unpaired data. Data were expressed as mean ± standard deviation.

II. RESULTS

1. Synthesis of the gentisic acid esters

The chemical structures of the synthesized derivatives (*Figure 1*) were verified by obtaining their spectral data which were compared with literature values [15].

2,5-dihydroxybenzoic acid methyl ester (MG)
m.p.: 85-87 °C (lit.: 84-86 °C); ^{1}H NMR (CD_3OD) δ: 4.84(s, 3H), 6.80(d, 1H), 6.96(dd, 1H), 7.21 (d, 1H); IR (KBr): 3338.36 cm^{-1} (O-H), 1685.26 cm^{-1} (C=O).

2,5-dihydroxybenzoic acid ethyl ester (EG)
m.p.: 75-76 °C (lit.: 75-77 °C); ^{1}H NMR (CD_3OD) δ: 1.41(t, 3H), 4.37(q, 2H), 6.79(d, 1H), 6.96 (dd, 1H), 7.22 (d,1H); IR (KBr): 3400.82 cm^{-1} (O-H), 1659.24 cm^{-1} (C=O).

2,5-dihydroxybenzoic acid propyl ester (PG)
m.p.: 68-70 °C; ^{1}H NMR (CD_3OD) δ: 1.04 (t, 3H), 1.79 (sex, 2H), 4.27 (t, 2H), 6.80 (d,1H), 6.95 (dd, 1H), 7.24 (d, 1H); IR (KBr): 3390.41 cm^{-1} (O-H), 1669.95 cm^{-1} (C=O).

2,5-dihydroxybenzoic acid isopropyl ester (IPG)
m.p.: 70-72 °C; ^{1}H NMR (CD_3OD) δ: 1.21(d, 6H), 5.09 (m, 1H), 6.63 (d, 1H), 6.81 (dd, 1H), 7.05 (d, 1H); IR (KBr): 3369.65 cm^{-1} (O-H), 1669.65 cm^{-1} (C=O).

2,5-dihydroxybenzoic acid butyl ester (BG)
m.p.: 65-66 °C (lit.: 65-67 °C); ^{1}H NMR (CD_3OD) δ: 1.00 (t, 3H), 1.48 (sex, 2H), 1.76 (quintet, 2H), 4.34 (t, 2H), 6.80 (d,1H), 6.95 (dd, 1H), 7.22 (d, 1H); IR (KBr): 3369.59 cm^{-1} (O-H), 1664.44 cm^{-1} (C=O).

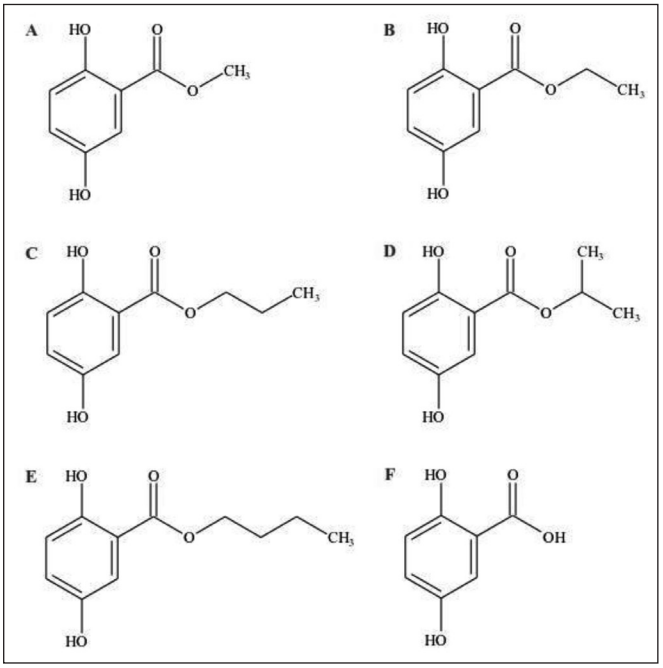

Figure 1 - Chemical structures of synthesized gentisic acid esters. The compounds are: A) gentisic acid methyl ester (MG), B) gentisic acid ethyl ester (EG), C) gentisic acid n-propyl ester (PG), D) gentisic acid iso-propyl ester (IPG), E) gentisic acid n-butyl ester and (BG), F) gentisic acid (GA).

2. Capacity factor, partition coefficient and water solubility of the gentisic acid esters

The capacity factors (k'), partition coefficients (log P) and water solubilities of the analogues are summarized in *Table I*. Partition coefficients which reflect the hydrophobicity of the compounds increased in the order of GA<MG<EG<PG, IPG<BG, with PG and IPG harboring similar hydrophobic characteristics. Experimentally obtained capacity factors correlated well with this hydrophobicity. Linear correlations were obtained for log k' versus log P ($r^2 = 0.9881$, *Figure 2*). As expected, the hydrophobic nature of the gentisic acid derivatives increased with increased carbon number of the esterified side chain.

With the exception of IPG, water solubility of the gentisic acid esters decreased as the carbon number of the esterified side chain increased. Thus, GA and MG exhibited the highest water solubilities with 27.7568 and 21.5873 μg/mL, respectively. Obviously, increase of the side chain length of only one or two methyl groups gave rise to a strong decrease in water solubilities for EG and PG that were 4.3707 and 1.4072 μg/mL, respectively. The water solubility for IPG (1.6722 μg/mL) was similar to that of PG. The lowest water solubility was obtained for BG as 0.3240 μg/mL.

Table I - Partition coefficients (log P), capacity factors (k') and water solubilities of the gentisic acid esters.

Compound	Log P*	Capacity factor (k')	Water solubility (mg/mL)
GA	1.750±0.255	5.9383	27.7568±3.5330
MG	1.827±0.255	3.8870	21.5873±3.8114
EG	2.358±0.255	7.8462	4.3707±0.2276
PG	2.890±0.255	17.4329	1.4072±0.1218
IPG	2.706±0.258	16.4869	1.6722±0.0374
BG	3.421±0.255	38.8012	0.3240±0.0074

*Values from literature (SCIfinder).

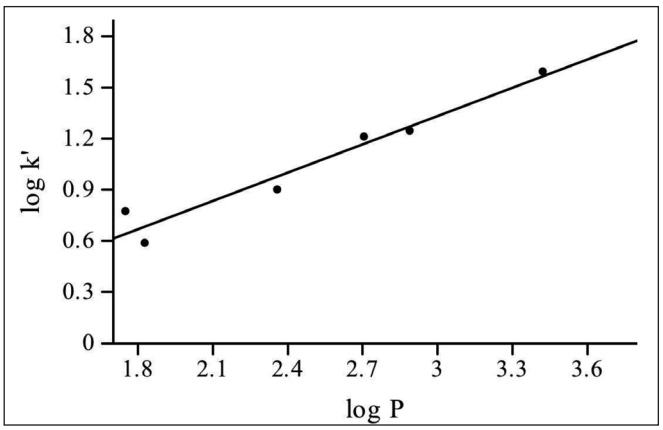

Figure 2 - Regression analysis of capacity factors and partition coefficients. The capacity factors (log k') of the gentisic acid esters were plotted against their partition coefficients (log P). A linear regression ($y = 0.553x - 0.327$, $r^2 = 0.94666$) was obtained.

3. Temperature stability

Stability over a given time period is an important prerequisite of the compounds studied as to eliminate the possibility of degradation during the skin permeation experiments that follow. Temperature stability profiles of the gentisic acid esters were determined over a time range of 12 h at 37 °C (*Figure 3*). No degradation of any gentisic acid analogues tested was observed within this time. Thus, the gentisic acid esters were stable at 37 °C for at least 12 h. Furthermore, no degradation was anticipated during the *in vitro* skin permeation study.

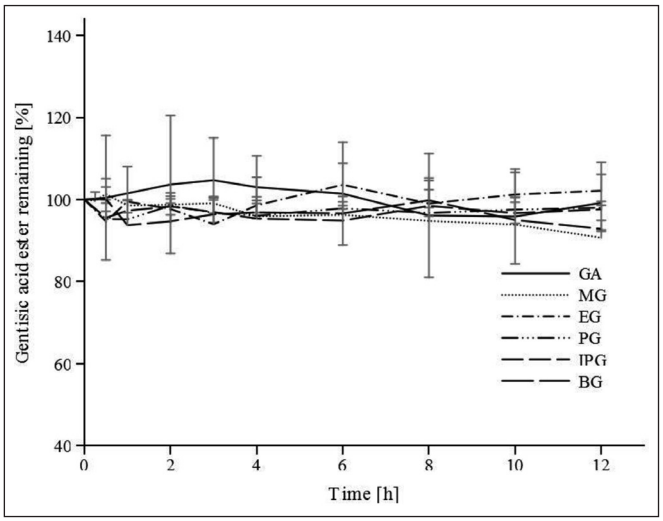

Figure 3 - Temperature stability analysis of the gentisic acid esters. Solutions of GA, MG, EG, IPG, PG and BG were incubated at 37 °C. At predetermined time intervals samples were drawn and the concentration of each gentisic acid ester was determined by HPLC. Percentage of the remaining compound was calculated where the initial concentration was considered as 100 %.

4. Mushroom tyrosinase inhibiting acitivity of the gentisic acid esters

To evaluate the melanogenesis interfering properties of the gentisic acid esters, their mushroom tyrosinase inhibiting activities were determined [16]. The IC_{50} values of mushroom tyrosinase of each gentisic acid ester are presented in *Figure 4*. The calculated IC_{50} data increased in the order of GA > MG, EG > BG > PG > IPG with 176 μg/mL for GA and 500 μg/mL for IPG. The IC_{50} values of MG and EG were very similar with 211 and 215 μg/mL, respectively.

5. Cytotoxicity of the gentisic acid esters

Cytotoxicity of the gentisic acid esters was studied in Murin Melan-A cells. Results are presented in *Figure 5* as relative melanocyte IC_{50} of the analyzed compounds to that of GA, where the IC_{50} of GA is considered as 1. All derivatives used in this study showed IC_{50} values to be 20 % or less than that of GA.

6. *In vitro* skin permeation study

As melanocytes locate to the interphase of epidermis and dermis, the effective compound needs to be able to permeate through skin to exhibit its skin-lightening effect. The *in vitro* skin permeation profiles of the gentisic acid esters are presented in *Figure 6*. Each gentisic acid species permeated through mouse skin with different permeations rates. Permeation rates as well as lag times calculated from the permeation profiles are summarized in *Table II*. Skin permeation rates of the gentisic acid esters decreased in the order MG, EG, GA, IPG,

Table II - Permeation rates and lag times of the gentisic acid esters. Skin permeation rates and lag times were obtained from regression analysis of the permeation profiles of the gentisic acid esters. The permeation rate is defined as the slope of the linear regression while the time interval until the linear region of the profile is set as lag time.

Gentisic acid ester	Permeation rate (μg/cm^2/h)	Lag time (h)
GA	172.0852	2.9302
MG	232.0033	2.6515
EG	182.9242	0.8426
PG	91.7938	0.6455
IPG	130.3660	1.4713
BG	19.5890	1.0235

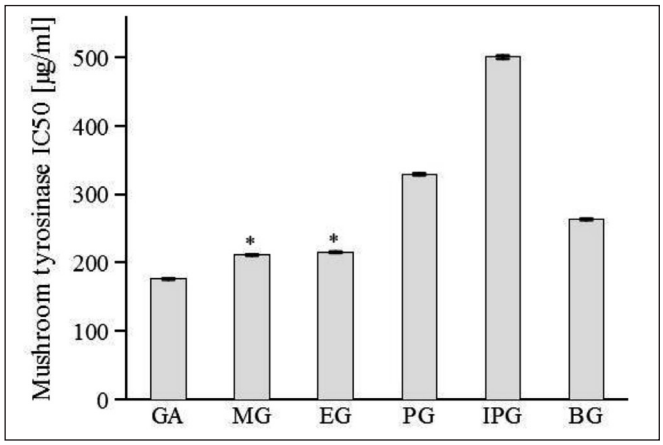

Figure 4 - Mushroom tyrosinase inhibiting activity (IC_{50}) of the gentisic acid esters. Solutions containing different concentrations of the respective gentisic acid esters were analyzed for their mushroom tyrosinase inhibiting activity. The concentration necessary to decrease the mushroom tyrosinase activity to 50 % (IC_{50}) was calculated (*after t test analysis $p < 0.05$).

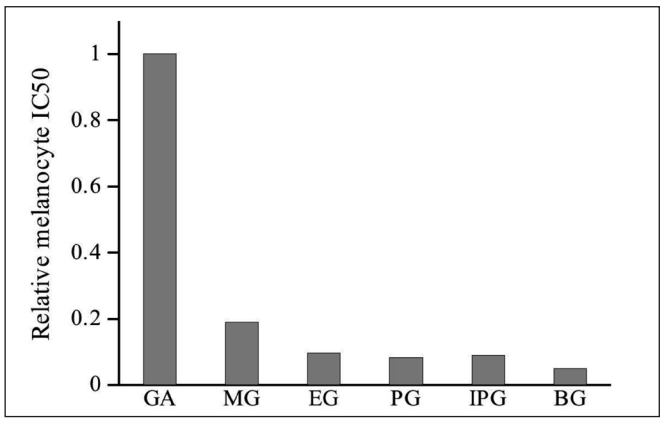

Figure 5 - Melanocyte cytotoxicity of the gentisic acid esters. The data present a relative pattern of the cytotoxic effects of the gentisic acid esters to Murin Melan-A cells. The data are relative IC_{50} values with respect to GA where the IC_{50} of GA is considered as one.

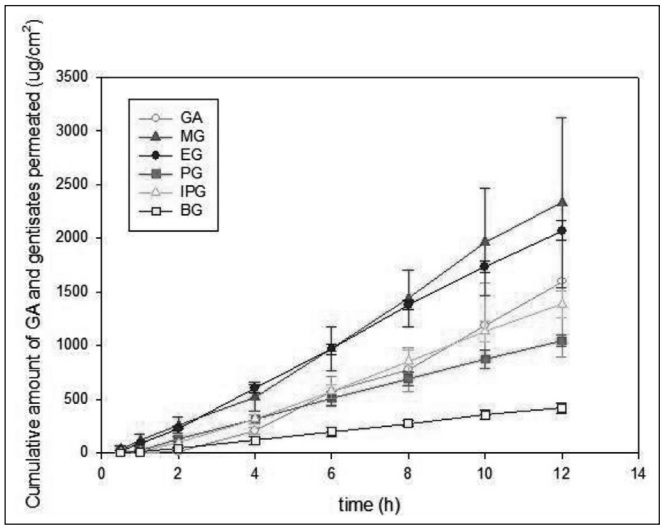

Figure 6 - *In vitro* mouse skin permeation analysis of gentisic acid esters. The analysis was conducted using Franz diffusion cells at 37 °C. The donor cells contained saturated solutions of the respective gentisic acid ester. At predetermined time intervals 1 mL of sample was drawn from the receptor cells and analyzed by HPLC.

PG, BG with very similar skin permeation rates for GA, MG and EG (172.0852, 232.0033 and 182.9242 μg/cm^2/h, respectively).

7. Structure-activity relationship analysis

A structure-activity relationship analysis has been conducted to investigate the influence of the structural parameters' lipophilicity on the gentisic acid esters' efficiency specified by their skin permeation, melanocytotoxicity and tyrosinase inhibition. The data obtained for the mouse skin permeation rates, mushroom tyrosinase IC_{50} and Melan-A IC_{50} have been correlated to their capacity factors (log k') and water solubilities (*Figure 7*).

Correlations were obtained for lipophilicity and skin permeation rates. The log k' data and skin permeation rates correlated linearly ($r^2 = 0.947388$, *Figure 7A*) while for skin permeation rates and water solubility, a square correlation was identified ($r^2 = 0.930391$, *Figure 7B*). Exponential correlations were obtained for the Melan-A IC_{50} data versus log k' ($r^2 = 0.901375$, *Figure 7C*) and water solubilities ($r^2 = 0.855921$, *Figure 7D*), respectively. No correlation was obtained for the mushroom tyrosinase inhibition data and lipophilicity (data not shown).

III. DISCUSSION

Five gentisic acid esters of different alkyl chain lengths have been synthesized and evaluated for their skin permeation in mouse skin. The main objective of the current paper was to determine the relationship of this permeation profile with their respective lipophilicity as specified by capacity factors (log k'), in addition to mushroom tyrosinase inhibition activity and Melan-A cytotoxicity.

Among the gentisic acid esters prepared and tested in this study, MG and EG showed the highest tyrosinase inhibition. Unfortunately, the activities of both MG and EG did not exceed that of the parent GA itself (*Figure 4*). The least tyrosinase inhibition, on the other hand, was shown by IPG. Interestingly, a difference in activity was revealed for PG and IPG even though both compounds had a three carbon chain. The difference in activity between branched and straight-chained alkyl groups have been reported [17]. This implies that inhibition of mushroom tyrosinase also depends to a large extent on steric characteristics [18]. In a mouse melanocyte tyrosinase inhibition by Curto *et al*., the strongest tyrosinase inhibition was shown by EG and MG followed by PG, IPG and BG, where BG showed the highest IC_{50} value [10]. In our study, however, using mushroom tyrosinase, the IC_{50} data for BG was in between those of EG and PG. BG seems to behave differently in mammalian versus plant tyrosinases.

When gentisic acid esters were analyzed for their cytotoxic effects to Murin Melan-A cells, the obtained IC_{50} data were evaluated relative to the IC_{50} of GA, which was set as 1 (*Figure 5*). Similar to the tyrosinase inhibition, none of the derivatives exceeded the IC_{50} of GA, making GA the least cytotoxic. Nevertheless, the relative cytotoxicity pattern for the ester derivatives, MG, EG, PG, IPG and BG, showed MG to be the least cytotoxic, which resembled the results reported by Curto *et al*. [10]. Cytotoxicity correlated with the alkyl chain length of the gentisic acid esters. This is probably due to the increased lipophilicity due to the alkyl groups which facilitates the passage of the drug through the cell membrane and thus resulting in a more efficient uptake by the melanocytes. This can also be explained by the structure-activity relationship analysis in *Figure 7C*.

Solubility of the compound of interest within the test medium is an important prerequisite for any test system. Therefore, experimentally observed noncytotoxic effects might be partly due to the low solubility of the analyzed substance within the test medium. The considerable differences in the cytotoxic characteristics of GA and the other gentisic acid compounds may be attributable to their different water solubilities (*Table I*). However, GA and MG exhibit almost similar water solubilities (27.7568 and 21.5873 mg/mL, respectively), although the melanocyte IC_{50} of MG is about 20 % that of GA. Furthermore,

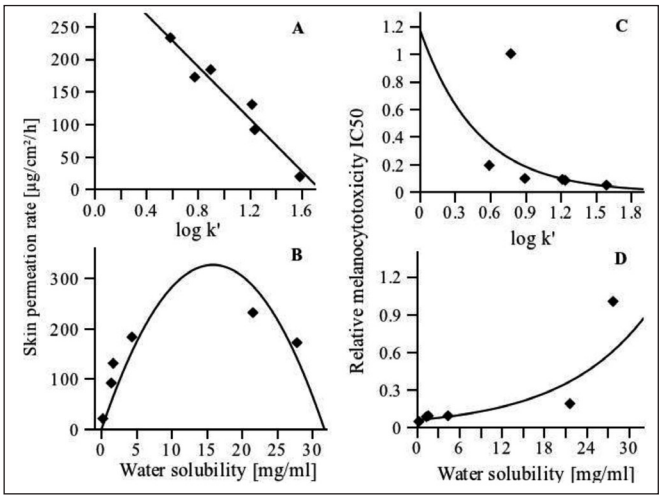

Figure 7 - Structure-activity-relationship study. Regression analysis has been conducted with data obtained for capacity factor (log k') and correlated to the skin permeation rates and relative melano cytotoxicity data. A. Skin permeation rates correlate linearly with capacity factors ($y = -200.193x + 348.506$, $r^2 = 0.947388$). B. A square correlation has been obtained for skin permeation rates and water solubility data ($y = -1.30877x^2 + 41.4231x$, $r^2 = 0.930391$). C. Exponential correlations have been obtained for the melano cytotoxicity data and capacity factors ($\ln(y) = -2.04677x + 0.161804$, $r^2 = 0.491932$). D. For the data obtained for water solubility and melano cytotoxicity ($\ln(y) = 0.0823845x - 2.77338$, $r^2 = 0.855921$).

as can be drawn from the structure-activity relationship analysis, increasing water solubilities and lowering hydrophobicity of the gentisic acid esters did not result in higher cytotoxic effects (*Figure 7C-D*). Therefore, the different cytotoxic effects of the gentisic acid esters may be attributable to further characteristics besides water solubility. An increased hydrophobic nature of the compound might even result in improved transport into the melanocytes and therefore to a higher cytotoxic effect.

Following the assumption that the addition of alkyl side chains of different lengths would influence lipophilicity and hence improve skin permeation, the gentisic acid esters have been analyzed for their efficiency to permeate through mouse skin. As anticipated, increase in hydrophobicity as for MG and EG led to a higher skin permeation rate for these two compounds as compared to GA (*Figure 6, Table II*). Further increase of the hydrophobic nature for PG, IPG and BG did not enhance skin permeation, but resulted in a decreased skin permeation rate for these compounds. Interestingly, the log k' data obtained for IPG and PG are very similar (*Table I*), but IPG permeates through mouse skin notably faster (130.3660 μg/cm^2/h) than PG (91.7938 μg/cm^2/h). Although the skin deposition study was not conducted, it has been reported in previous papers that the amount of drug permeated correlates with the amount of drug deposited [13].

Obviously, the ability of the gentisic acid esters to permeate through mouse skin is not exclusively dependent on hydrophobicity. With increasing number of carbon atoms of the alkyl side chain, the skin permeation characteristics of the gentisic acid esters might be determined by steric effects rather than by their hydrophobic nature. This can be speculated from the structure-activity relationship study, too. Capacity factors and skin permeation data gave rise to a negative linear correlation (*Figure 7A*). It is interesting to note that gentisic acid esters with high water solubilities exhibited high skin permeation rates (*Figure 7B*). Thus, skin permeation does not seem to rely on lipophilic characteristics alone. The skin permeation rates of the gentisic acid esters could be greatly influenced by steric effects rather than their hydrophobic properties stemmed from the number of carbon atoms in the alkyl side chain.

As skin contains esterases, the gentisic acid esters might get metabolized during the skin permeation process to GA which would be the effective compound [19, 20]. Future studies to evaluate the ability of the enzymes to effectively cleave the alkyl chains of the esters are therefore underway.

*

For application in a tyrosinase inhibiting formulation a compound with high tyrosinase inhibiting activity and noncytotoxic effects to melanocytes is desirable. Furthermore, the compound should exhibit effective skin permeation characteristics and sufficient water solubility. We observed that among the compounds tested in this study, skin permeation of the gentisic acid esters increased for MG and EG in comparison to the parent gentisic acid. Moreover, less cytotoxic effects have been observed for gentisic acid compounds with decreased hydrophobicity. On the other hand, EG requires much less drug to result in the same thermodynamic activity as MG and its permeation is considerable to GA with less lag time. The tyrosinase interfering characteristics of the gentisic acid esters were not attributable to any of the structural parameters we analyzed. In summary, MG among the gentisic acid esters showed highest skin permeation rate, and was considered the most promising candidate together with EG based on the tyrosinase inhibition and melanocyte cytotoxicity. To overall assess the suitability of the candidate compound as an effective agent for skin lightening formulations, further studies are underway to enlighten characteristics of the compound with respect to skin metabolism and possible skin irritation effects.

REFERENCES

1. Spritz R.A., Hearing V.J. - Genetic disorders of pigmentation - Adv. Hum. Genet., **22**, 1-44, 1994.
2. Scherer D., Kumar R. - Genetics of pigmentation in skin cancer, a review - Mutation Research/Reviews in Mutation Research, **705** (2), 141-153, 2010.
3. Ito S. - Presidential lecture: a chemist's view of melanogenesis - Pig. Cell. Res., **16**, 230-236, 2003.
4. Fontecave M., Pierre J.L. - Oxidations by copper metalloenzymes and some biomimetic approaches - Coord. Chem. Rev., **170**, 125-140, 1998.
5. Jiang T-F., Liang T-T., Wang Y-H., Zhang W-H., Lv Z-H. - Immobilized capillary tyrosinase microreactor for inhibitor screening in natural extracts by capillary electrophoresis - J. Pharm. Biomed. Anal., **84**, 36-40, 2013.
6. Garcia A., Fulton J.E. - The combination of glycolic acid and hydroquinone or kojic acid for the treatment of melasma and related conditions - Dermatol. Surg., **22**, 443-447, 1996.
7. Kojima S., Yamagucho H., Morita K., Ueno Y., Paolo R. - Inhibitory effect of sodium-5,6-benzylidene ascorbate (SBA) on the elevation of melanin biosynthesis induced by ultraviolet-A (UV-A) light in cultured B-16 melanoma cells - Biol. Pharm. Bull., **18** (8), 1076-1080, 1995.
8. Cabanes J., Chazarra S., Carcia-Carmona F. - Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase - J. Pharm. Pharmacol., **46** (12), 982-985, 1994.
9. Maeda K., Fukuda M., Griffiths C.E., Finkel L.J., Hamilton T.A., Bulengo-Ransby S.M. - Arbutin: mechanism of its depigmenting action in human melanocyte culture - J. Pharmacol. Exp. Ther., **276** (2), 765-769, 1996.
10. Curto E., Kwong C., Hermersdorfer H., Glatt H. - Inhibitors of mammalian melanocyte tyrosinase: *In vitro* comparisons of alkyl esters of gentisic acid with other putative inhibitors - Biochem. Pharmacol., **57**, 663-672, 1999.
11. Chang T.S. - An updated review of tyrosinase inhibitors - Int. J. Mol. Sci., **10**, 2440-2475, 2009.
12. Schved F., Kahn V. - Effect of different isomers of dihydroxybenzoic acids(DBA) on the rate of DL-dopa oxidation by mushroom tyrosinase - Pig. Cell. Res., **5** (2), 58-64, 1992.
13. Bian S., Doh H-J., Zheng J., Kim J.S., Lee C-H., Kim D-D - *In vitro* evaluation of patch formulations for topical delivery of gentisic acid in rats - Eur. J. Pharm. Sci., **18**, 141-147, 2003.
14. Bennett D.C., Cooper, P.J, Hart I.R -A line of non-tumorigenic mouse melanocytes, syngeneic with the B16 melanoma and requiring a tumour promoter for growth. - Int. J. Cancer, **39**, 414-418, 1987.
15. SCIfinder: http://www.cas.org
16. Maddaluno J.F., Faull K.F. - Inhibition of mushroom tyrosinase by 3-amino-L-tyrosine: molecular probing of the active site of the enzyme - Experientia, **44**, 885-887, 1988.
17. Piao L.Z., Park H.R., Park Y.K., Lee S.K., Park J.H., Park M.K. - Mushroom tyrosinase inhibition activity of some chromones. - Chem. Pharm. Bull., **50** (3), 309-311, 2002.
18. Nicols D.E. - Structure-activity relationships of phenethylamine hallucinogens. - J. Pharm. Sci., **70** (8), 839-849, 1981.
19. Prusakiewicz J.J., Ackermann C., Voorman R. - Comparison of skin esterase activities from different species. - Pharm. Res., **23** (7), 1517-1524, 2006.
20. Doh H.J, Cho W-J., Yong C-S., Choi H-G., Kim J.S., Lee C-H., Kim D-D. - Synthesis and evaluation of ketorolac ester prodrugs for transdermal delivery - J. Pharm. Sci., **92** (5), 1008-1017, 2003.

ACKNOWLEDGMENTS

This study was funded by the Dongseo University Frontier Project Fund (2009) and a research grant from the National Research Foundation (KRF-2011-0013898) of Korea.

MANUSCRIPT

Received 6 August 2013, accepted for publication 11 December 2013.