

NOVEL APPLICATION OF CULTURED ROOTS OF MOUNTAIN GINSENG (*PANAX GINSENG* MEYER)  
AND GINSENOSE RE AS SAFE ANTIMELANOGENIC COSMECEUTICAL COMPONENTS

Zuly Elizabeth Jiménez-Pérez<sup>1</sup>, Yeon-Ju Kim<sup>2\*</sup>, Veronica Castro-Aceituno<sup>2</sup>, Ramya Mathiyalagan<sup>1</sup>,  
Josua Markus<sup>1</sup>, Sungeun Ahn<sup>2</sup>, Shakina Yesmin Simu<sup>1</sup>, Deok-Chun Yang<sup>2\*</sup>

<sup>1</sup>Department of Biotechnology and Ginseng Bank, <sup>2</sup>Department of Oriental Medicine Biotechnology,  
College of Life Sciences, Kyung Hee University, Yongin, Republic of Korea

\*Corresponding Author Email: [dcyang@khu.ac.kr](mailto:dcyang@khu.ac.kr), [yeonjukim@khu.ac.kr](mailto:yeonjukim@khu.ac.kr)

**Article History**

Received: 18, Nov. 2016

Revised Received: 16, Feb. 2017

Accepted: 17, Feb. 2017

Published Online: 01, Oct. 2017

**Abstract**

**Background:** Mountain ginseng (*Panax ginseng* Meyer), which belongs to Araliaceae family, grows naturally in the mountains of Korea. It is highly valued owing to its enhanced pharmacology effects such as immunostimulating, antioxidant, anti-cancer and antiaging activity. An alternative to accessing the sparse mountain ginseng therapy benefits is by tissue-cultured roots of mountain ginseng. The aim of this study is to evaluate the effect of water extract of cultured roots of mountain ginseng (CRMG) and specifically its major compound ginsenoside Re (Re) on melanin synthesis in  $\alpha$ -MSH-stimulated mouse melanoma B16BL6 cells (B16).

**Materials and Methods:** Cell cytotoxicity was evaluated through a comparative study using normal human dermal fibroblast (HDF) and B16. Then,  $\alpha$ -MSH-stimulated B16 cells were analyzed, using melanin and tyrosinase activity assay. Tyrosinase gene expression was evaluated through reverse transcription polymerase chain reaction analysis and quantitative PCR analysis. Finally, an *in silico* docking study was performed.

**Results:** The study demonstrated that CRMG and Re were non-toxic compounds and significantly reduced tyrosinase activity and melanin content in B16 cells. Re decreased the mRNA expression of tyrosinase and other melanin synthesis-related genes in B16 cells. In addition, *in silico* docking studies showed that Re had stronger interaction with tyrosinase compared to control drug arbutin due to its higher binding affinity.

**Conclusion:** Taken together, our results suggest that CRMG and Re possess potential anti-melanogenic activities and may be used as antimelanogenic cosmeceutical agents.

**Keywords:** *Panax ginseng*; cultured root of mountain ginseng; ginsenoside Re; Tyrosinase; Docking.

**List of abbreviations:** CRMG: culture roots of mountain ginseng, Re: ginsenoside Re,  $\alpha$ -MSH: alpha-Melanocyte-stimulating hormone, B16: murine melanoma B16BL6 cells, HDF: human dermal fibroblast cells, TYR: tyrosinase, TRP-1: tyrosinase-related protein 1, TRP-2: tyrosinase-related protein 2, MITF: microphthalmia-associated transcription factor, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, DMEM: dulbecco's modified eagle medium high glucose pyruvate, FBS: fetal bovine serum, HPLC: high-performance liquid chromatography, ADMET: absorption, distribution, metabolism, excretion, and toxicity, PASS: Prediction of Activity Spectra for Substances, DMSO: dimethyl sulfoxide.

**Introduction**

Melanocytes are responsible for producing melanin in melanosomes tissues which determine skin and hair color (Su et al., 2013). Melanin acts as the primary protection factor of the skin from ultraviolet damage by absorbing ultraviolet sunlight and removing reactive oxygen species. However, excessive production of melanin can create severe skin ailments including patches on the skin, ephelis, and melasma (Briganti et al., 2003). Tyrosinase gene family, which is composed of tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2), has an essential function in regulating melanogenesis pathway. Furthermore, microphthalmia-associated transcription factor (MITF) is crucial for the

regulation of melanocyte proliferation, melanogenesis and major regulator of tyrosinase and the related enzymes transcriptions factors (Park et al., 2013). One study demonstrated that utilization of cosmeceuticals with anti-tyrosinase compounds can result in lightening of skin (Kim and Uyama, 2005). Generally, commercially available skin whitening agents are associated with undesirable side effects such as local irritation, contact dermatitis, ochronosis, atrophy and skin cancer (Draelos, 2007). In lieu of conventional chemical-based whitening agents, special interest has been directed to non-artificial, botanical products.

The root of mountain ginseng (*Panax ginseng* Meyer) is well-known due to its enhanced pharmacological activities. However, its expensive price tag and scarcity have made it relatively inaccessible for potential users. Cultured roots from mountain ginseng tissue have emerged as alternatives for larger and cheaper production of ginseng roots.

In recent years, scientific discoveries have been disseminated about the efficacy of ginseng extracts and saponins on skin. Previously published reports have demonstrated that saponins possess antimelanogenic activity (Kim, 2015). Ginsenoside F1, a derivative of ginsenoside Rg1, has been found to be effective as skin whitening agent by suppressing tyrosinase (Yoo et al., 2011). *In vitro* studies of ginsenoside Rb1, an aglycone of ginsenoside Rh4, and ethanol extract of ginseng seed have also been shown to have skin whitening effect though inhibition of tyrosinase activity and melanin content (Lim et al., 1999; Jeong et al., 2013; Wang et al., 2014). However, no study has been made to evaluate the anti-melanogenic activities of aqueous CRMG extract and ginsenoside Re. The aim of this study is to evaluate CRMG and its major compound ginsenoside Re for their efficacy as natural whitening therapeutic agents.

## Materials and Methods

Cultured roots of mountain ginseng and ginsenoside Re were acquired from the Ginseng Bank (Kyung Hee University, KO). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin (PS) were purchased from GenDEPOT (TX, USA). 3-(4,5-dimethyl-thiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) was acquired from Life technologies (OR, USA). Arbutin and L-3,4-dihydroxyphenylalanin (L-DOPA) were acquired from Abcam (Cambridge, UK.).  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) was obtained from Sigma (MO, USA).

### Preparation of aqueous extract of cultured roots of mountain ginseng

The CRMG extract was prepared as described previously (Oh et al., 2006) with minor modifications. The CMGR roots (400 g) were washed with water, dried and ground into fine powder. Next, aqueous extract of CRMG roots was extracted by autoclaving with distilled water at 100 °C for 30 min. The resulting extract was centrifuged and the supernatant collected and freeze-dried. The residue was diluted with distilled water and filtered using Whatman filter paper (0.45  $\mu$ m) to obtain filtrate. The total filtrate was stored at -20 °C for further use.

### Analysis of ginseng saponins of cultured roots of mountain ginseng extract by HPLC

The amount of the ginseng saponins in CRMG was determined by HPLC analysis as previously described by Jin et al. (2015). Briefly, HPLC was carried out using an Agilent 1260 system (CA, USA). Ginsenosides were resolved on a column C<sub>18</sub> (Kinetex ID 2.6  $\mu$ m, 50 mm  $\times$  4.6 mm), with H<sub>2</sub>O (solvent A) and acetonitrile (solvent B) at A: B ratios of 83:17, 83:17, 71:29, 60:40, 30:70, 10:90, 10:90, 83:17, and 83:17, with run times of 0, 6, 9, 14, 17, 18.5, 22, 22.5, and 26 min, respectively at a flow rate of 1.0 mL/min and detection wavelength of 203 nm.

### Cell culture

Human dermal fibroblast (HDF) and B16 cell lines were purchased from Korean Cell Line Bank (SEL, KO). Cells were cultivated in DMEM media and complemented with 10 % FBS and 1 % PS at 37 °C, 95 % humidified air and 5 % CO<sub>2</sub> conditions as previous described (Lee et al., 2007; Yoo et al., 2011).

### Cell viability assay

The cytotoxic effect of CRMG and Re on HDF and B16 cells was measured by MTT assay. Both cell lines were seeded at a density of  $1 \times 10^5$  in 96 well plates and cultured for 24 h. At 90 % confluence, cells were treated with various concentrations of CRMG (1-1000  $\mu$ g/mL) and Re (1-300  $\mu$ M) for 72 h. After 3 days of incubation, cells were treated with 10  $\mu$ L of MTT solution and incubated for a period of 3 h at 37 °C. The sample absorbance was measured at 570 nm with a reference wavelength of 630 nm by an ELISA reader (Bio-Tek Instrument, USA).

### Melanin inhibition assay

B16 cells were seeded at a density of  $2 \times 10^5$  cells/well. After 24 h, the cells were treated with CRMG (10-100  $\mu$ g/mL) or Re (10-100  $\mu$ M) in the presence of 100 nM  $\alpha$ -MSH to stimulate melanin production for 72 h. Then, the treated

cells were washed with PBS and harvested by trypsinization. Cell pellet was solubilized in 300  $\mu$ L of 1 N NaOH containing 10 % DMSO at 80°C for 1 h. The relative melanin content was determined by measuring the absorbance at 475 nm by an ELISA reader (Lim et al., 2009). A standard curve for synthetic melanin (0–500  $\mu$ g/mL) was prepared for each experiment. For melanin content analysis, arbutin was maintained as reference standard.

### **Tyrosinase inhibition assay**

The tyrosinase activity was assayed using DOPA oxidase assay previously enunciated (Huang et al., 2013). Arbutin was used as reference standard. B16 cells were seeded at a density of  $4 \times 10^5$  cells/well in six-well plates. After 24 h, the cells were treated with CMRG (10–100  $\mu$ g/mL) or Re (10–100  $\mu$ M) in the presence of 100 nM  $\alpha$ -MSH for 72 h. Then, cells were washed with PBS, harvested by trypsinization and lysed with phosphate buffer (pH 6.9) containing 1 % Triton X-100. Following freeze-thawing at -80 °C for 15 min and storing at room temperature for 10 min, the samples were clarified by centrifugation at  $12,000 \times g$  for a period of 15 min at 4 °C. After centrifugation, 10  $\mu$ L of freshly prepared substrate solution (15 mM L-DOPA in 48 mM pH 7.1 sodium phosphate buffer) was added to 90  $\mu$ L of lysate supernatant and incubated at a 37 °C for 1 h. The absorbance was then measured at 475 nm using an ELISA reader.

### **Reverse transcription polymerase chain reaction (RT-PCR) analysis and quantitative PCR analysis**

Total RNA was extracted using Trizol reagents solution (Sigma, MO, USA). Reverse transcription and cDNA amplification were carried with 2  $\mu$ g of total RNA using Thermo Scientific cDNA synthesis kit (Onebio, LT, EU) according to the manufacturer's instructions. The cDNA obtained was amplified with the following primers: MITF forward 5'- CGGGATGCCTTGTTTA TGGT-3' and reverse 5'TGCCTCTGAGCTTGCTGTAT-3', tyrosinase forward 5'- ATGGGTCAA CACCCATGTTT-3' and reverse 5'-GGCAAATCCTTC CAGTGTGT-3', TRP-1 forward 5'- GTA CTGGTGAGCAGCTCTGT-3' and reverse 5'- CCAG GCTTCTCGGTCATCTT-3', TRP-2 forward 5'CTTGGCTCACTCCTTCCTGA-3' and reverse 5'TTGCTCTGCGGTTAGGAAGA-3'. The reaction was cycled 25 times for 30 s at 95 °C, 30 s at 58 °C and 60 s at 72 °C for tyrosinase, MITF and TIRP-1 and cycled 25 times for 40 s at 94 °C, 40 s at 60 °C and 90 s at 72 °C for TIRP-2. The amplified RT-PCR products were analyzed on 0.8 % agarose gels, visualized by ethidium bromide staining and photographed under ultraviolet light. The quantitative real-time PCR was performed using an R-Corbett Rotor-Gene Model 6000 (Mortlake, Australia), with SYBR Green qPCR Super Mix UDG kit (Invitrogen, CA, USA). The relative expression levels of the target genes against the reference gene (GAPDH) were calculated using the delta cycle threshold (Ct) method (Scheffe et al., 2006).

### **Ligand Preparation**

Ginsenoside Re was selected as ligand molecule in our study and its 2D structure was obtained from our own *Panax ginseng* saponins database. Next, the 2D structure was converted to 3D structure using Discovery Studio 3.5 visualizer. Arbutin structure was obtained from the PubChem compound online data base. All atomic coordinates of the downloaded structure were converted to .pdb format using free on line OpenBabel software (Geldenhuis et al., 2006).

### **Protein preparation and molecular docking study**

The 3D structure of tyrosinase from *Agaricus bisporus* (PDB ID: 2Y9X) was retrieved from protein data bank. Chimera program was used to modify 2Y9X, as by deleting solvent, adding charge, and replacing an incomplete side chain. (Mu et al., 2013). In this study, we used arbutin as control drug. Finally, molecular docking study was performed with Autodock Vina.

### **ADMET and PASS descriptors prediction**

The ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties of Re were determined as described previously (Sathishkumar et al., 2013). Pharmacological properties such as solubility level, serum protein binding, skin permeability, CYP2D6 inhibition probability, octanol/water partition coefficient were measured. Considering toxicity prediction as an important task in natural product research, we also predicted hepatotoxicity descriptors for ginsenoside Re using ADMET module accessible from discovery studio program (DS 3.5). The computational program PASS (Prediction of Activity Spectra for Substances) was used for predicting possible biological activity based on chemical structure (Lagunin et al., 2010). This method produced a list of biological activities from the ginsenoside Re structure, along with probability of activity (Pa) and probability of inactivity (Pi).

## Statistical analysis

All data are presented as mean  $\pm$  standard deviation (S.D.). All experiments were independently performed thrice. Statistical analysis of the data was performed using ANOVA test. Statistical significance was assigned at  $p < 0.05$ .

## Results

### Detection of ginseng saponins using high performance liquid chromatography analysis

Ginsenoside	Rg1	Re	Rf	Rh1	Rg2	Rb1	Rc	Rb2	Rd	Ck	Rh2
mg/g	1.37	15.22	0.13	0.14	0.29	3.72	1.35	1.14	0.41	0.44	0.22

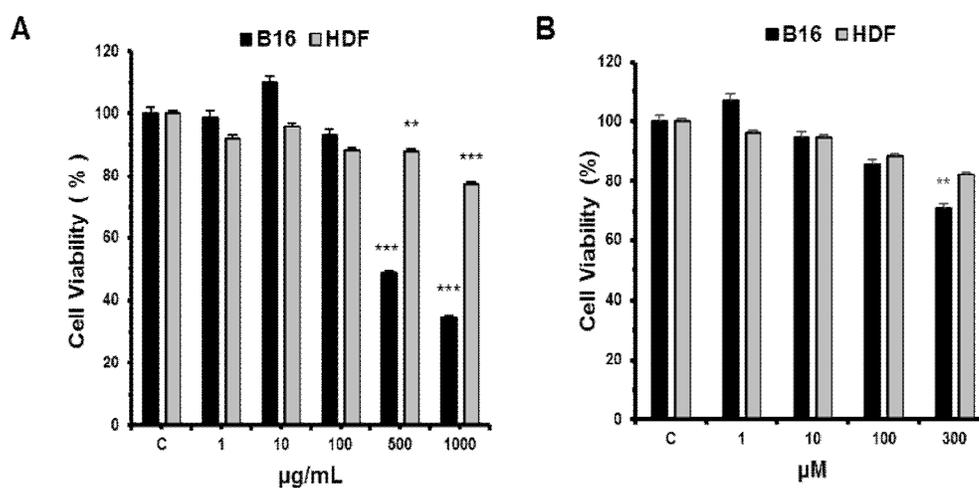
The ginsenosides content of CRMG was determined by HPLC. The HPLC profile of the CRMG extract revealed that ginsenosides Re, Rb1, Rg1, Rc and Rb2 to be the predominant saponins (Table 1). Ginsenoside Re was found to be the most abundant ginsenoside in the CRMG with a quantity of 15.22 mg/g.

**Table 1.** Content of ginsenosides in CRMG extract

### Effect of CRMG extract and ginsenoside Re on the growth and viability of B16 cells

The CRMG extract did not have cytotoxic effect on B16 cells at concentrations  $\leq 100$   $\mu\text{g/mL}$ , however cells treated with higher concentration of CRMG significantly decreased cell viability ( $\text{IC}_{50} = 672.17$   $\mu\text{g/mL}$ ). Similarly, ginsenoside Re showed no significant cytotoxicity at concentrations  $\leq 100$   $\mu\text{M}$  but was shown to be toxic at 300  $\mu\text{M}$ . To determine whether CRMG extract and Re were toxic to normal cells, HDF cells were treated with CRMG extract and Re with the same concentrations (Fig. 1A and B). CRMG extract affected the viability of HDF cells at concentrations higher than 500  $\mu\text{g/mL}$ . However, HDF cell viability was not affected after treatment with 300  $\mu\text{M}$  of Re.

Therefore, further experiments were carried out at concentration ranging 0-100  $\mu\text{g/mL}$  of CRMG and 0-100  $\mu\text{M}$  of Re.

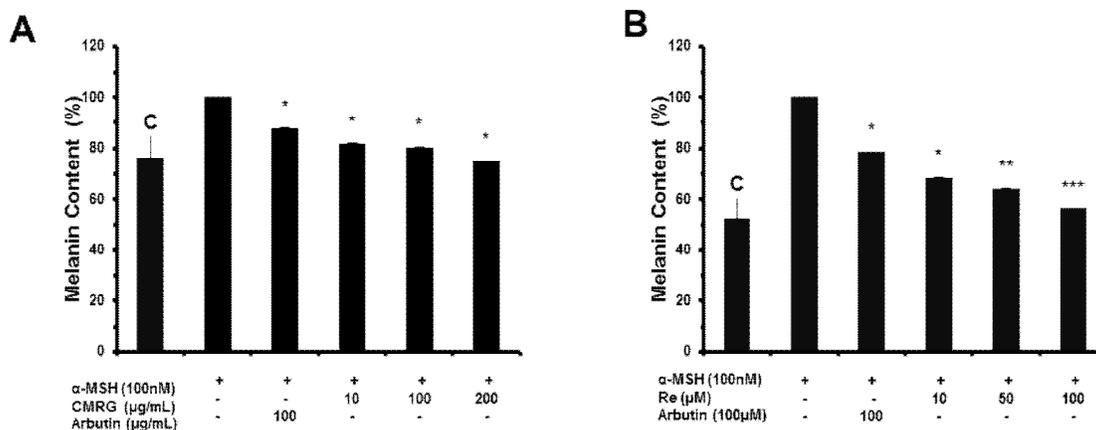


**Figure 1:** Cell viability of B16 and HDF cells after treatment with CRMG (A) and ginsenoside Re (B) in B16 and HDF cells. Results are reported as a percentage of sample-treated control and presented as mean  $\pm$  SD of three different experiments. \* $P < 0.05$  and \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control.

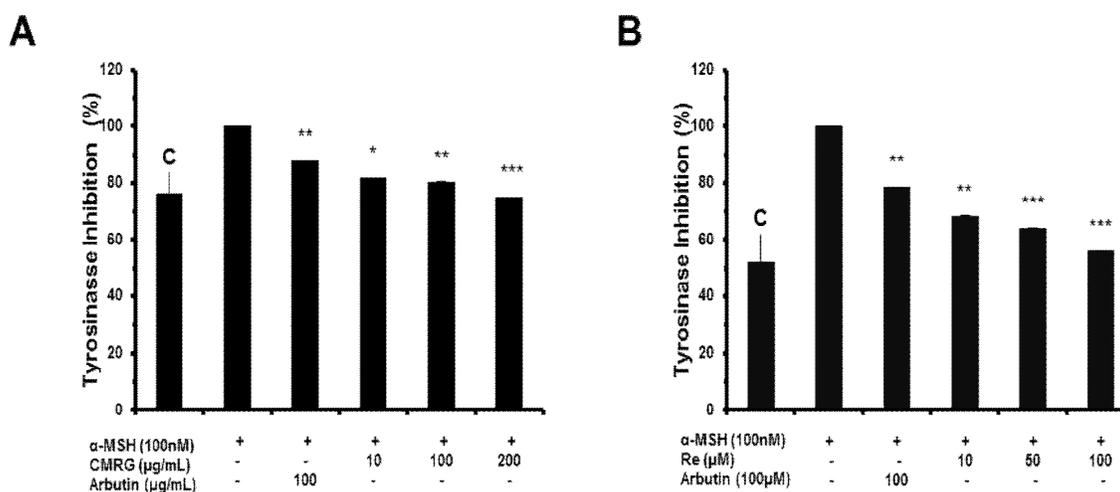
### Measurement of melanin content and tyrosinase activity inhibition

Figure 2A and 2B show the inhibition effect on melanogenesis in B16 cells stimulated with  $\alpha$ -MSH and after 72 h treatment with CRMG extract and Re. B16 cells treated with 100 nM of  $\alpha$ -MSH significantly increased melanin production as opposed to B16 control cell. In addition, CRMG and Re led to a dose-dependent decrease in melanin production in  $\alpha$ -MSH stimulated cells. B16 cells treated with 100  $\mu\text{g/mL}$  of CRMG showed a comparable inhibition to those treated with same concentration of arbutin. However, B16 cells treated with 100  $\mu\text{M}$  of ginsenoside Re showed significant inhibition (\*\* $p < 0.001$ ) compared to that treated with same concentration of arbutin. Cellular tyrosinase activity was significantly increased in B16 cells treated with  $\alpha$ -MSH alone compared to control cells (Fig. 3A and 3B). CRMG and Re also

suppressed tyrosinase in dose dependent manner. Treatments were more effective than arbutin in concentrations >100  $\mu\text{g}/\text{mL}$  of CRMG and >100  $\mu\text{M}$  of ginsenoside Re.



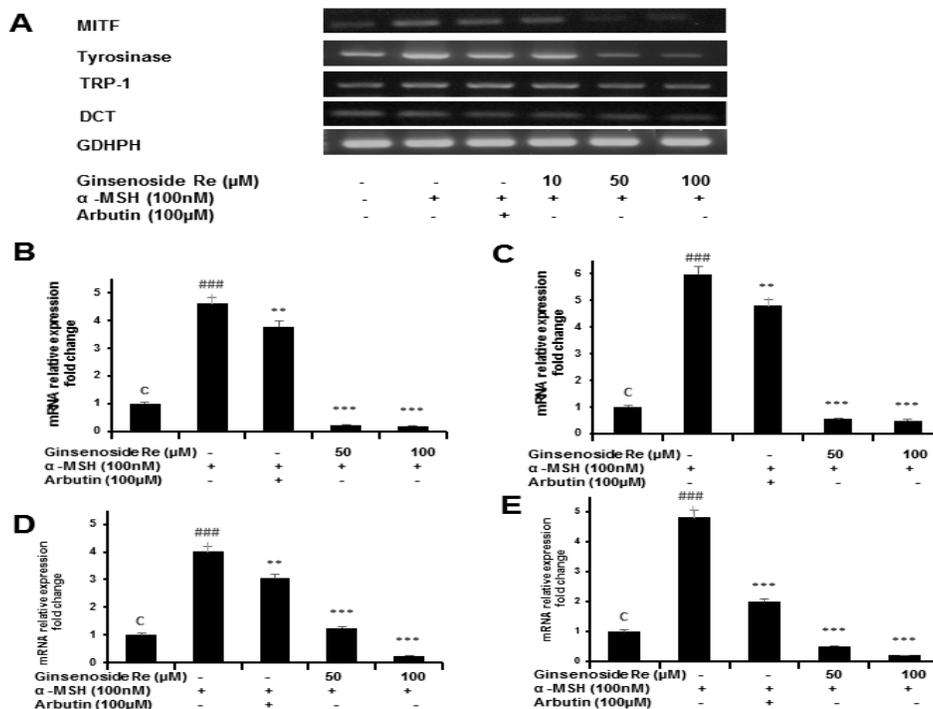
**Figure 2:** Effect on melanin content of CRMG (A) and Re (B). Results are reported as a percentage of  $\alpha$ -MSH – treated control and presented as mean  $\pm$  SD of three different experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs.  $\alpha$ -MSH-treated control.



**Figure 3:** Effect on tyrosinase inhibition activity of CRMG (A) and Re (B). Results are reported as a percentage of  $\alpha$ -MSH – treated control and presented as mean  $\pm$  SD of three different experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs.  $\alpha$ -MSH-treated control.

### RT-PCR analysis

The  $\alpha$ -MSH treatment increased tyrosinase family mRNA expression and its expression could be inhibited in a dose dependent manner by Re (Fig. 4A). B16 cells treated with indicated concentration of Re (50 - 100  $\mu\text{M}$ ) significantly inhibited mRNA expression levels of MITF, tyrosinase, TRP-1 and TRP-2 genes at higher level compared to those cells treated with 100  $\mu\text{M}$  of arbutin (Fig. 4B to 4E). Thus, these results suggested that Re decreased mRNA expression of tyrosinase family leads to suppression of melanogenesis.



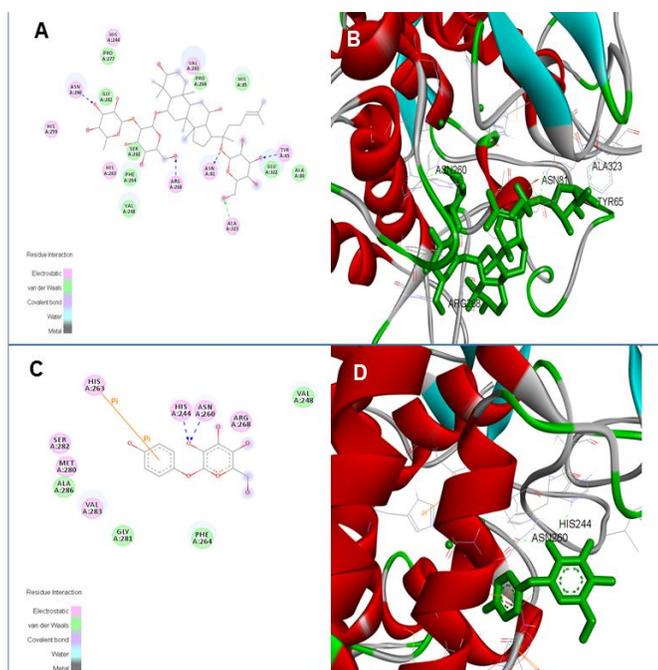
**Figure 4:** Effect of Re on the mRNA expression of melanogenesis-related genes (A). mRNA expression was visualized by RT-PCR. The sizes of amplified gene products were 528 bp for  $\beta$ -actin, 216 bp for MITF (B), 563 bp for tyrosinase (C), 375 bp for TRP-1 (D) and 240 bp for DCT (E). Results are reported as a percentage of  $\alpha$ -MSH – treated control and presented as mean  $\pm$  SD of three different experiments. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs.  $\alpha$ -MSH-treated control and ###P < 0.001 vs. assay control.

### Molecular Docking Study

The main purpose of molecular docking study was to predict the potential binding of Re to mushroom tyrosinase through its binding affinity with specific active site of the selected protein (Table 2). The molecular docking result showed that ginsenoside Re interacted with mushroom tyrosinase residues ASN 81, ASN 260, ARG268, ALA323, and TYR65 (Fig. 5A, B) with a binding affinity of -8.4 kcal/mol. In case of Arbutin, His 244 and ASN 260 residues of mushroom tyrosinase was predicted as the hydrogen bonding interactions (Fig. 5C, D), with binding affinity of -6.4 kcal/mol.

**Table 2.** Molecular interaction results of Mushroom Tyrosinase (PDBID 2w9x) with ginsenoside Re and Arbutin, binding affinity values with key residues.

No.	Compounds	H-Bonding interactions		Amino acid involved in interaction	Docking Energy (kcal/mol)	H-bond distance (Å)
		Ligand	Protein			
1	Arbutin	O---	---HE2	HIS244	-6.4	2.48349
		O---	---OD1	ASN260		3.19587
2	Ginsenoside Re	HH--	---OD1	ASN81	-8.4	2.31153
		O---	--HH12	ARG268		2.19575
		O---	---OD1	ASN260		2.46082
		O---	---OH	TYR65		3.07871
		O---	---O	ALA323		3.06033



**Figure 5:** Predicted structure of *Agaricus bisporus* containing the binuclear copper binding active site (PDB ID 2Y9X). Docking of ginsenoside Re into the active site of mushroom tyrosinase (A- B). Docking of arbutin into the active site of mushroom tyrosinase (C-D).

### ADMET and PASS analysis of ginsenoside Re

The results of predicted ADMET values of ginsenoside Re with acceptable range were described in Table 3. In addition, PASS analysis results indicated that ginsenoside Re supported the probability of active  $P_a > 0.8$  as an anticarcinogenic, immunostimulant, antioxidant, transcription factor inhibitor, G-protein-coupled receptor kinase inhibitor, anti-inflammatory, transcription factor inhibitor, dermatologic, oxidoreductase inhibitor and wound healing agent all directly or indirectly related to melanogenesis.

**Table 3.** ADMET results of selected ginsenoside Re with pharmacokinetic properties

Ginsenoside	Solubility Level	Serum protein binding	Skin Permeability	CYP2D6 inhibition	Hepatotoxicity	Qlog Po/w	Lipinski rule of 5 violations	Jorgensen rule of 3 violations
Re	-2.623	-1.339	-6.383	0.227	0	-1.008	3	2
<b>Acceptable range</b>	(-6.5-0.5)	(-1.5-1.5)	(Kp in cm/hr)	0 is Non-inhibitor and 1 is inhibitor	0 is non-toxic and 1 is toxic	(-0.2 to 6.5)	Maximum is 4	Maximum is 3

### Discussion

The cultured root of mountain ginseng extract and ginsenoside Re were analyzed for their effect on melanogenesis in  $\alpha$ -MSH-treated B16 melanoma cells lines. Firstly, we evaluated the cytotoxicity of CRMG and Re on the growth and viability of melanoma B16 cells and normal HDF cells using MTT assay method. As shown in figure 1A-B, CRMG and Re has no significant cytotoxic effect at 100  $\mu$ g/mL and 100  $\mu$ M respectively. Results indicate that inhibition of melanogenesis by CRMG extract and Re in B16 cells at mentioned concentrations could be attributed to their ability to inhibit tyrosinase

activity rather than to their cytotoxic effects. On the other hand, at higher concentrations CRMG extract and Re were toxic to the normal cells. However, CRMG and Re preferentially inhibited B16 melanoma cells growth. It suggested that CRMG and Re may possess potential anti melanoma activities. Previous studies have reported that mountain ginseng and Re have anti-cancer activity (Oh et al., 2006; Chen et al., 2016) although further studies have yet to be made to confirm this hypothesis.

Application of  $\alpha$ -MSH to B16 cells promotes activation of pre-existing molecules of tyrosinase in the cells by elevating cyclic AMP levels leading to the increase in production of melanin (LEE et al., 1972; Pawelek, 1976). CRMG and ginsenoside Re decreased tyrosinase activity and down-regulated cellular melanin synthesis in B16 cells in a concentration-dependent manner (Fig. 2 and Fig. 3). Ginseng root extracts are rich in phenolic compounds which are capable to inhibit tyrosinase and melanin synthesis, while ginsenosides prevent intracellular increase in reactive oxygen species (Kim and Uyama, 2005; Hwang et al., 2006).

Ginsenoside Re was found to be the most abundant ginsenoside in the CRMG (Table 1). This result is in accordance to a previously reported study by Kim et al. (2009). Ginsenoside Re is a panaxatriol saponin and is abundantly present in ginseng root and berries. Cho et al. reported that Re has anti-inflammatory and antioxidant effect (Cho et al., 2006). Oxidative stress and inflammatory factors alter the redox state of cell membrane proteins and affect melanocyte homeostasis resulting in melanogenesis (Kim and Uyama, 2005). To better understand the molecular mechanisms of Re-induced inhibitory effect, we further examined the effect of Re on the mRNA expression level of melanogenic transcription genes. It has been reported that MITF down-regulation is related to the suppression of the cyclic AMP-dependent signaling pathway stimulated by  $\alpha$ -MSH in melanoma cells (Huh et al., 2010). However, in the current study, we did not evaluate the inhibitory activity of ginsenoside Re in the cyclic AMP-mediated melanin production. MITF is regarded to be a key regulator of melanogenic markers including TRP-1 and TRP-2 expression, and consequently play an essential role in hypopigmentation as well as melanogenesis. As shown in Fig. 4A-E, Re significantly decreased mRNA expression levels of MITF, tyrosinase, TRP-1 and TRP-2 melanogenic genes. Our results showed that the mRNA expressions of MITF, tyrosinase, TRP-1 and TRP-2 were significantly enhanced in  $\alpha$ -MSH-treated B16 melanoma cells.

However, this  $\alpha$ -MSH stimulatory effect was markedly down-regulated by Re in a dose-dependent manner compared to arbutin treatment. Thus, these results are in accordance with our hypothesis that ginsenoside Re decreases tyrosinase at the transcription level and consequently may reduce melanin content in B16 melanoma cells.

In addition, to understand the mechanism of interaction between tyrosinase and ginsenoside Re and to find their binding energy, an *in-silico* docking study was performed. As given in supplementary Table 3, binding interaction of ginsenoside Re showed high activities and as well as selectivity for the inhibition of tyrosinase. In addition, the binding energies calculated by docking programs between tyrosinase and ginsenoside Re (-8.4 kcal/mol) were higher than to that of arbutin (-6.4 kcal/mol). This result implies that ginsenoside Re interacts more strongly with tyrosinase compared to arbutin. According to the docking analysis, the binding residues interacting with ginsenoside Re were ASN81, ARG268, ASN260, TYR65 and ALA323. These residues could have a key function and effect on the binding affinity with Re. Therefore, ginsenoside Re could inhibit tyrosinase activity by binding the active site of mushroom tyrosinase. Moreover, the ADMET and PASS results showed that ginsenoside Re was non-toxic and showed significant predicted biological activities. Taken together, these results suggested that ginsenoside Re can be used as anti-melanogenic agent.

## Conclusion

Our result indicated that CRMG and Re inhibited melanin content in B16BL6 cell as well as inhibited tyrosinase activity. The anti-melanogenic effects of ginsenoside Re were mediated through the down-regulation of the expression levels of  $\alpha$ -MSH-induced MITF and tyrosinase activity, as well as suppression of mRNA expression levels of melanogenesis factors such as TRP-1 and TRP-2 in B16BL6 melanoma cell line stimulated by  $\alpha$ -MSH. These results suggested that CRMG extract and ginsenoside Re might be used as potential therapeutic agents in cosmetics for the inhibition of melanogenesis.

## Acknowledgments

This research was supported by the grant from Next Generation Biogreen 21 program (SSAC# PJ0120342016), Rural Development Administration, Republic of Korea.

## Author contributions

The authors listed have made the following declarations of contributions. ZEJP, VCA, RM, and SA conceived and designed the experiments. ZEJP, VCA, SA and SYS performed the experiments. ZEJP analyzed the data. ZEJP wrote the manuscript, JM and RM revised the manuscript.

**Disclosure:** The authors declare that they do not have any conflict of interest.

## References

1. Briganti, S., Camera, E. and Picardo, M. (2003). Chemical and instrumental approaches to treat hyperpigmentation. *Pigment. Cell. Res.*, 16: 101 - 110.
2. Chen, X.J., Zhang, X.J., Shui, Y.M., Wan, J.B. and Gao, J.L. (2016). Anticancer Activities of Protopanaxadiol-and Protopanaxatriol-Type Ginsenosides and Their Metabolites. *Evid.Based Complement Altern Med.*, .
3. Cho, W.C., Chung, W.S., Lee, S.K., Leung, A.W., Cheng, C.H. and Yue, K.K. (2006). Ginsenoside Re of Panax ginseng possesses significant antioxidant and antihyperlipidemic efficacies in streptozotocin-induced diabetic rats. *Eur. J. Pharmacol.*, 550: 173 - 179.
4. Draelos, Z.D. (2007). Skin lightening preparations and the hydroquinone controversy. *Dermatol Ther.*, 20: 308 - 313.
5. Geldenhuys, W.J., Gaasch, K.E., Watson M., Allen, D.D. and Van der Schyf, C.J. (2006). Optimizing the use of open-source software applications in drug discovery. *Drug. Discov. today.*, 11: 127 - 132.
6. Huang, Y.C., Liu, K.C., Chiou, Y.L., Yang, C.H., Chen, T.H., Li, T.T. and Liu, L.L. (2013). Fenofibrate suppresses melanogenesis in B16-F10 melanoma cells via activation of the p38 mitogen-activated protein kinase pathway. *Chem.biol. interact.*, 205: 157 - 164.
7. Huh, S., Jung, E., Lee, J., Roh, K., Kim, J.D., Lee, J. and Park, D. (2010). Mechanisms of melanogenesis inhibition by propafenone. *Arch. Dermatol. Res.*, 302 : 561 - 565.
8. Hwang, E.Y., Kong, Y.H., Lee, Y.C., Kim, Y.C., Yoo, K.M., Jo, Y.O. and Choi, S.Y. (2006). Comparison of phenolic compounds contents between white and red ginseng and their inhibitory effect on melanin biosynthesis. *J. Ginseng Res.*, 30: 82 - 87.
9. Jeong, Y.M., Oh, W.K., Tran, T.L., Kim, W.K., Sung, S.H., Bae, K., Lee, S. and Sung, J.H. (2013). Aglycone of Rh4 inhibits melanin synthesis in B16 melanoma cells: possible involvement of the protein kinase A pathway. *Biosci. Biotechnol. Biochem.*, 77: 119 - 125.
10. Jin, Y., Kim, Y.J., Jeon, J.N., Wang, C., Min, J.W., Noh, H.Y. and Yang, D.C. (2015). Effect of white, red and black ginseng on physicochemical properties and ginsenosides. *Plant. Food Hum. Nutr.*, 70: 141 - 145.
11. Kim, K. (2015). Effect of ginseng and ginsenosides on melanogenesis and their mechanism of action. *J. Ginseng Res.*, 39: 1 - 6.
12. Kim, Y.J. and Uyama, H. (2005). Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. *Cell. Mol. Life Sci.*, 62: 1707 -1723.
13. Kim, Y.K., Yoo, D.S., Xu, H., Park, N.I., Kim, H.H., Choi, J.E. and Park, S.U. (2009). Ginsenoside content of berries and roots of three typical Korean ginseng (*Panax ginseng*) cultivars. *Nat. Prod. Commun.*, 4: 903 - 906.
14. Lagunin, A., Filimonov, D. and Poroikov, V. (2010). Multi-targeted natural products evaluation based on biological activity prediction with PASS. *Curr. Pharm. Des.*, 16: 1703 - 1717.
15. Lee, J., Jung, E., Lee, J., Huh, S., Kim, J., Park, M., So, J., Ham, Y., Jung, K. and Hyun, C.G. (2007). Panax ginseng induces human Type I collagen synthesis through activation of Smad signaling. *J. Ethnopharmacol.*, 109: 29 - 34.
16. Lee, T.H., Lee, M.S. and Lu, M.Y. (1972). Effects of  $\alpha$ -MSH on melanogenesis and tyrosinase of B-16 melanoma. *J. Endocrinol.*, 91: 1180 - 1188.
17. Lim, J.Y., Ishiguro, K. and Kubo, I. (1999). Tyrosinase inhibitory p-Coumaric acid from Ginseng leaves. *Phytother. Res.* 13: 371 - 375.
18. Lim, Y.J., Lee, E.H., Kang, T.H., Ha, S.K., Oh, M.S., Kim, S.M., Yoon, T.J., Kang, C., Park, J.H. and Kim, S.Y. (2009). Inhibitory effects of arbutin on melanin biosynthesis of  $\alpha$ -melanocyte stimulating hormone-induced hyperpigmentation in cultured brownish guinea pig skin tissues. *Arch. Pharm. Res.* 32: 367 - 373.
19. Mu, Y., Li, L. and Hu, S.Q. (2013). Molecular inhibitory mechanism of triclinic on tyrosinase. *Spectrochim. Acta Mol. Biomol. Spectrosc.*, 107: 235 - 240.
20. Oh, C.H., Kang, P.S., Kim, J.W., Kwon, J. and Oh, S.H. (2006). Water extracts of cultured mountain ginseng stimulate immune cells and inhibit cancer cell proliferation. *Food Sci. Biotechnol.*, 15: 369 - 373.
21. Park, H., Song, K.H., Jung, P.M., Kim, J.E., Ro, H., Kim, M.Y. and Ma, J.Y. (2013). Inhibitory effect of arctigenin from fructus arctii extract on melanin synthesis via repression of tyrosinase expression. *Evid. Based Complement Alternat Med.*
22. Pawelek, J.M. (1976). Factors regulating growth and pigmentation of melanoma cells. *J. Invest. Dermatol.*, 66: 201 - 209.
23. Sathishkumar, N., Karpagam, V., Sathiyamoorthy, S., Woo, M.J., Kim, Y.J. and Yang, D.C. (2013). Computer-aided identification of EGFR tyrosine kinase inhibitors using ginsenosides from Panax ginseng. *Comput. Biol. Med.*, 43: 786 - 797.

24. Schecke, J.H., Lehmann, K.E., Buschmann, I.R., Unger, T. and Funke-Kaiser, H. (2006). Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's C T difference" formula. *J. Mol. Med.*, 84: 901 - 910.
25. Su, T.R., Lin, J.J., Tsai, C.C., Huang, T.K., Yang, Z.Y., Wu, M.O., Zheng, Y.Q., Su, C.C. and Wu, Y.J. (2013). Inhibition of melanogenesis by gallic acid: Possible involvement of the PI3K/Akt, MEK/ERK and Wnt/ $\beta$ -catenin signaling pathways in B16F10 cells. *Int. J. Mol. Med.* 14: 20443 - 20458.
26. Wang, L., Lu, A.-P., Yu, Z.-L., Wong, R.N., Bian, Z.X., Kwok, H.H., Yue, P.Y.K., Zhou, L.M., Chen, H. and Xu, M. (2014). The melanogenesis-inhibitory effect and the percutaneous formulation of ginsenoside Rb1. *AAPS PharmSciTech.*, 15: 1252 - 1262.
27. Yoo, D. S., Rho, H.S., Lee, Y.G., Yeom, M.H., Kim, D.H., Lee, S.J., Hong, S.Y., Lee, J.H. and Cho, J.Y. (2011). Ginsenoside F1 modulates cellular responses of skin melanoma cells. *J. Ginseng Res.*, 35: 86 - 91.