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ORIGINAL ARTICLE

Whitening Effects of Marine *Pseudomonas* Extract

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Background: Bacteria associated with marine invertebrates are a rich source of bioactive metabolites. **Objective:** The effects of marine bacteria extracts on pigmentation were investigated to find novel whitening agents. **Methods:** The marine bacteria collected near Gangwha Island in Korea were isolated and extracted using organic solvent. The organic extracts were screened and selected using the cell free tyrosinase activity. The whitening effects of the selected extract were further investigated using cultured melanocytes, cultured skin and *in vivo* zebrafish. The whitening mechanism of the marine extract was also investigated. **Results:** The marine bacterial methylene chloride extract reduced the pigmentation of Melan-a cells, human melanocytes, cultured skin and *in vivo* zebrafish. The decrease in pigmentation was due to the inhibition of tyrosinase activity and the expression of tyrosinase and microphthalmia-associated transcription factor protein. These bacteria were identified as a novel *Pseudomonas* species. **Conclusion:** The methylene chloride extract of marine *pseudomonas* species possesses a whitening effect. Further chemical isolation and characterization of the active compounds from this marine bacterial extract are needed. **(Ann Dermatol 23(2) 144～149, 2011)**

-Keywords-
Marine, Pigmentation, *Pseudomonas*, Whitening

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INTRODUCTION

Hyperpigmentation disorders, including melasma, freckles and senile lentigines, are associated with abnormal accumulation of melanin pigments, and these disorders have a psychosocial and cosmetic impact. Therefore, many efforts have been focused on screening both the already recognized and putative whitening agents. Some natural compounds such as kojic acid, arbutin and hydroquinone have been described for their tyrosinase inhibition properties[1,2]. However, the demand for novel whitening agents is increasing due to the ineffectiveness and unwanted side effects of the existing agents.

In recent years, the ocean has been considered as a rich source of compounds that possess novel structures and biological activity. Biologically active molecules isolated from marine flora and fauna have applications in pharmaceuticals, nutritional supplements, cosmetics and chemicals[3]. Microorganisms associated with marine invertebrates are involved in the production of bioactive molecules[4]. They also have significant medical and industrial applications.

In the present study, we collected marine bacteria near Gangwha Island in Korea and we investigated the whitening effects of marine bacteria extracts to discover novel whitening molecules. The whitening effect of selected bacteria extracts was shown by *in vitro* and *in vivo* systems and the mechanism of the whitening effect was also investigated.

MATERIALS AND METHODS

Isolation and culture of bacteria

Samples from a sandbar near Gangwha Island in Korea were collected using a core sampler. The bacteria from these samples were isolated by extraction using sterilized seawater. The bacterial strains were purified and cultured on seawater complete media (25 g bacto tryptone, 3 g

yeast extract, 3 ml glycerol, 15 g agar in D.W. with 75% added, sterilized, aged sea water, pH 7.0).

Preparation of the organic extracts

The 100 bacterial isolates were inoculated into culture bottles with 1 L seawater complete culture broth. The culture bottles were incubated at 30°C for 5 days in a shaking incubator (170 rpm). After incubation, the bacterial cultures were divided into new four bottles at 250 ml each. The same volume (250 ml) of four organic solvents (hexane, methylene chloride, acetone and methanol) were added to each culture broth. The mixture was vigorously mixed using a shaking incubator at 30°C for 4 hr (170 rpm). The organic phase was recovered in the case of the hexane and methylene chloride added culture broths. The organic phase was evaporated using a rotary evaporator. The dry residues were stored at 4°C for further use. In the case of the acetone-added culture broth, the liquid fraction was recovered using centrifugation and it was completely dried by an evaporator. After this, the dried residue material was dissolved by acetone and we recovered the solution. The solution was then completely evaporated using a rotary evaporator. The dry residues were stored at 4°C for further use. In the case of the methanol-added culture broth, it was also processed in the same manner as the acetone case except for use of methanol. We selected 185 samples based on the sampling site and we applied to our assay system for screening the active compounds.

Cell culture

Melan-a cells (a gift from Dr. Bennett, St George's Hospital Medical School, London, UK) were cultured in RPMI 1640 with 4% fetal bovine serum and 1% antibiotic/antimycotic solution (all from Gibco-BRL, Grand Island, NY, USA). Normal human melanocytes were derived from adult foreskins and they were maintained in F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotic/antimycotic solution, 24 μg/ml 3-isobutyl-1-methylxanthine, 80 nM 12-O-tetradecanoyl phorbor-13-acetate, 1.2 ng/ml basic fibroblast growth factor and 0.1 μg/ml cholera toxin (all from Sigma Chemical Co., St. Louis, Mo, USA). For all the experiments, melanocytes were used at passage 2 or 3 and they were maintained in MCDB-153 (Sigma) containing 4% heat-inactivated FBS, 0.6 ng/ml basic fibroblast growth factor, 5 μg/ml insulin, 1 μg/ml vitamin E and 1 μg/ml transferrin (all from Sigma Chemical Co.).

Skin culture and pigmentation assay

Skin samples were obtained from breast skin during breast surgery after receiving informed consent and the samples were cultured as previously described[5]. The culture dish was filled with Dulbecco's modified Eagle's minimal essential medium supplemented with 10% FBS and 1% penicillin/streptomycin up to the stainless steel grid. The skin specimens were placed on the stainless steel grid and cultured in an incubator at 37°C with 5% CO_2. The medium was changed every three days. The cultured skin was treated with 100 μg/ml of bacteria extract. After 3 days of culture, the specimens were fixed in 4% buffered formaldehyde, embedded in paraffin and stained with hematoxylin and eosin for light microscope examination. Melanin pigments were visualized with Fontana-Masson stain, and this was performed by the usual methods without eosin background staining. The image analysis was done using Image Pro Plus Version 4.5 (Media Cybernetics Co., Silver Spring, MD, USA) and the pigmented area per epidermal area (PA/EA) was measured.

Zebrafish and phenotype-based pigment evaluation

Zebrafish (Danio rerio) embryos of the wild-type strain were produced by paired mating of adult fish. The embryos were maintained in 100 mm^2 petri dishes in embryo media (1 mM $MgSO_4$, 120 mM KH_2PO_4, 74 mM Na_2HPO_4, 1 mM $CaCl_2$, 500 mM KCl, 15 mM NaCl, and 500 mM $NaHCO_3$ in deionized H_2O) at a density of 50 embryos per dish. The bacteria extract (20～100 μg/ml) was treated during a period of 120 hour post-fertilization (hpf) of the zebrafish. Five eggs were randomly distributed into polystyrene plates. The body pigmentation of the zebrafish was observed under a stereomicroscope (Olympus, Tokyo, Japan).

Mushroom tyrosinase inhibition assay

Marine extracts (1～100 μg/ml) were mixed with 1mM tyrosine (Sigma), 34 units/ml mushroom tyrosinase (Sigma) and 0.1 M phosphate buffer (pH 6.8). After incubation at 37°C for 20 min, the optical densities were measured at 470 nm using an enzyme-linked immunosorbent assay reader, Model 680 (Bio-Rad, Hercules, CA, USA).

Cell number, Melanin content and Dopa oxidase activity assay

Melan-a cells were plated at a density of 2×10^5 cells/cm^2 in 60 mm culture dishes. After 1 day of culture, the medium was changed to fresh medium containing different concentrations of bacteria extracts for 3 days. For the melanocytes, the cells were plated at a density of 1.5×10^5 cells/cm^2 in 60 mm culture dishes and they were treated with the samples for 5 days.

The treated cells were counted with a Coulter counter

(Coulter Electronics, Hialcah, FL, USA). The cell cytotoxicity was examined using a trypan blue exclusion assay. After cell counting, 1×10^5 cells were spun down and the supernatant was discarded. The pellet was solubilized in 1 N NaOH and incubated at 37°C for 90 min. The optical densities were measured at 490 nm. The absorbance was compared with a standard curve of synthetic melanin (Sigma). The dopa oxidase activity was assayed spectrophotometrically using L-DOPA as the substrate. The treated cells were solubilized with 1% Triton-X and 10 mM L-DOPA (Sigma). Following 90 min incubation at 37°C, the absorbance was measured at 475 nm.

Western blot analysis of tyrosinase and microphthalmia-associated transcription factor

For Western blot analysis, the Melan-a cells were treated with 100 μg/ml bacteria extract for 5 days and then they were lysed. Ten micrograms of protein per lane were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and the proteins were blotted onto nitrocellulose paper. The blots were incubated with rabbit polyclonal antibodies against tyrosinase (a gift of Dr Hearing, National Institutes of Health, Bethesda, MD, USA) diluted 1:2,000, and this was followed by horseradish peroxidase-conjugated secondary antibodies. A monoclonal antibody for microphthalmia-associated transcription factor (MITF) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) was diluted 1:200. The bound antibodies were detected by using an enhanced chemiluminescence plus kit (Amersham Int., Little Chalfont, UK). To quantify the expression, the intensities of the bands were measured by densitometry using Image-Pro Plus version 4.5 and the intensities are expressed as intensities relative to that of actin.

Bacteria identification

The bacteria were identified using the 16s rDNA sequencing analysis method. PCR-based amplification of the 16S rRNA gene was carried out using the following primers: 27F (5′-AGAGTTTGATCMGGCTCAG), 342R (5′-CACGGATCCCCACTGCTGCSYCCCGTAG), 66F (5′-GTGCTGCAGAACACATGCAAGTCGA), 1100R (5′-GGGTTGCGCTCGTTG), 530F (5′-GTGCCAGCMGCCGCGG), 1525R (5′-AAGGAGGTGWTCCARCC). The complete sequence was aligned using the GenBank BLAST program.

Statistical analysis

Statistical significance was tested with Student's *t*-test.

RESULTS AND DISCUSSION

The mushroom tyrosinase inhibiting effects of the 185 organic extracts were evaluated against a well-known positive control arbutin. Six of the 185 extracts showed strong inhibition of tyrosinase activity with values around 90% as compared to the reference arbutin (97% of tyrosinase inhibition). These were methylene chloride extracts. Further biological investigation of these 6 selected extracts was done using the Melan-a cells and primary human melanocytes. One of the 6 tested extracts dose- dependently inhibited the pigmentation of Melan-a cells as shown by the gross appearance of the cell pellets (Fig. 1A) and the decreased melanin content of the cells (Fig. 1B). As shown in Fig. 1C, the extract dose-dependently reduced the melanin contents in human melanocytes. The whitening effect of the selected bacteria was further shown in cultured skin (Fig. 1D). Image analysis showed a significant decrease in the ratio of PA/EA in the treated skin (88 ± 47.5), as compared to that of the control skin (227 ± 36.6). The Zebrafish model system is a novel alternative to mammalian models with the advantages of physiological relevance[6]. The marine bacterial methylene chloride extract also produced remarkable inhibition on the body pigmentation of zebrafish (Fig. 1E). These results clearly showed that the extract might be introduced as a whitening agent. Further, the bacteria were identified using 16S ribosomal DNA sequences and we found the extract came from an unidentified *Pseudomonas* sp. (Fig. 2). To study the mechanism of inhibitory action of the extract on melanogenesis, we measured the tyrosinase activity and its expression on the treated Melan-a cells. As shown in Fig. 3A, the optical density in the presence of 10, 50 and 100 μg/ml bacteria extract was reduced to 90 ± 4, 66 ± 18 and 58 ± 9% of the control (100%) ($n=5$, $p<0.01$), respectively. This decreased level of tyrosinase activity was accompanied by a parallel decrease in melanin content. These results indicate that the bacteria extract regulates tyrosinase, and it subsequently inhibits melanin synthesis in human melanocytes. The effect of the bacteria extract on cell viability was examined using the crystal violet assay, but no effect was observed at the concentrations used, indicating that the bacteria extract is not cytotoxic to human melanocytes (data not shown). MITF is an important transcriptional regulator in the melanogenic process[7]. To test whether this bacteria regulates the MITF expression, the protein expression of MITF and tyrosinase of the treated Melan-a cells was investigated. The protein expression of MITF was reduced, which was accompanied by a decreased expression of tyrosinase in the Melan-a cells (Fig. 3B, C). This data

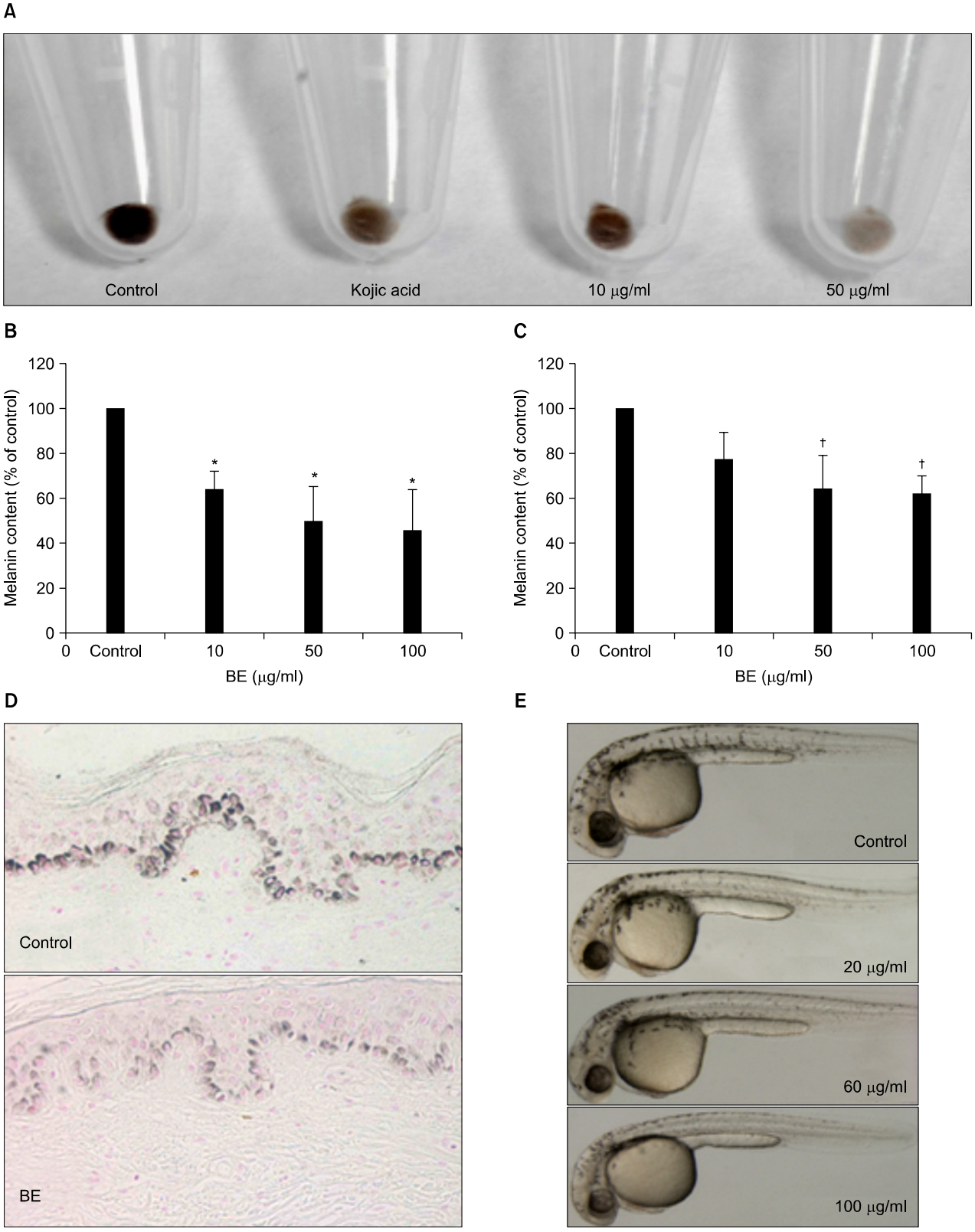

Fig. 1. The bacterial extract (BE) reduced the pigmentation *in vitro* and *in vivo*. The extract dose-dependently inhibited the pigmentation in Melan-a cells as shown by the gross appearance of the cell pellets (A) and the decrease in melanin content (B) of the cells. (C) Melanin content of human melanocytes. The values indicate the mean of five independent experiments±SD. *$p<0.01$, †$p<0.05$. (D) The bacteria extract (100 μg/ml) reduced the basal melanin pigments of cultured human skin as compared to that of the control (Fontana-masson stain, ×200). (E) The effects on the pigmentation of zebrafish observed under the stereomicroscope. Lateral view of the embryos.

Fig. 2. Identification of potential bacteria. The DNA sequencing data revealed that the bacteria belonged to an unidentified *Pseudomonas* sp.

suggests that the bacterial extract decreases melanogenesis through the inhibition of the MITF expression, which was accompanied by a decrease in the expression and activity of tyrosinase in the Melan-a cells.

In conclusion, the present study explored the usefulness of marine *Pseudomonas* as a valuable source of novel skin whitening agents. Our results clearly showed that the methylene chloride extract from the novel *Pseudomonas* sp. possesses a potential skin whitening effect. Further chemical isolation and characterization of the active compounds from this bacterial extract are needed.

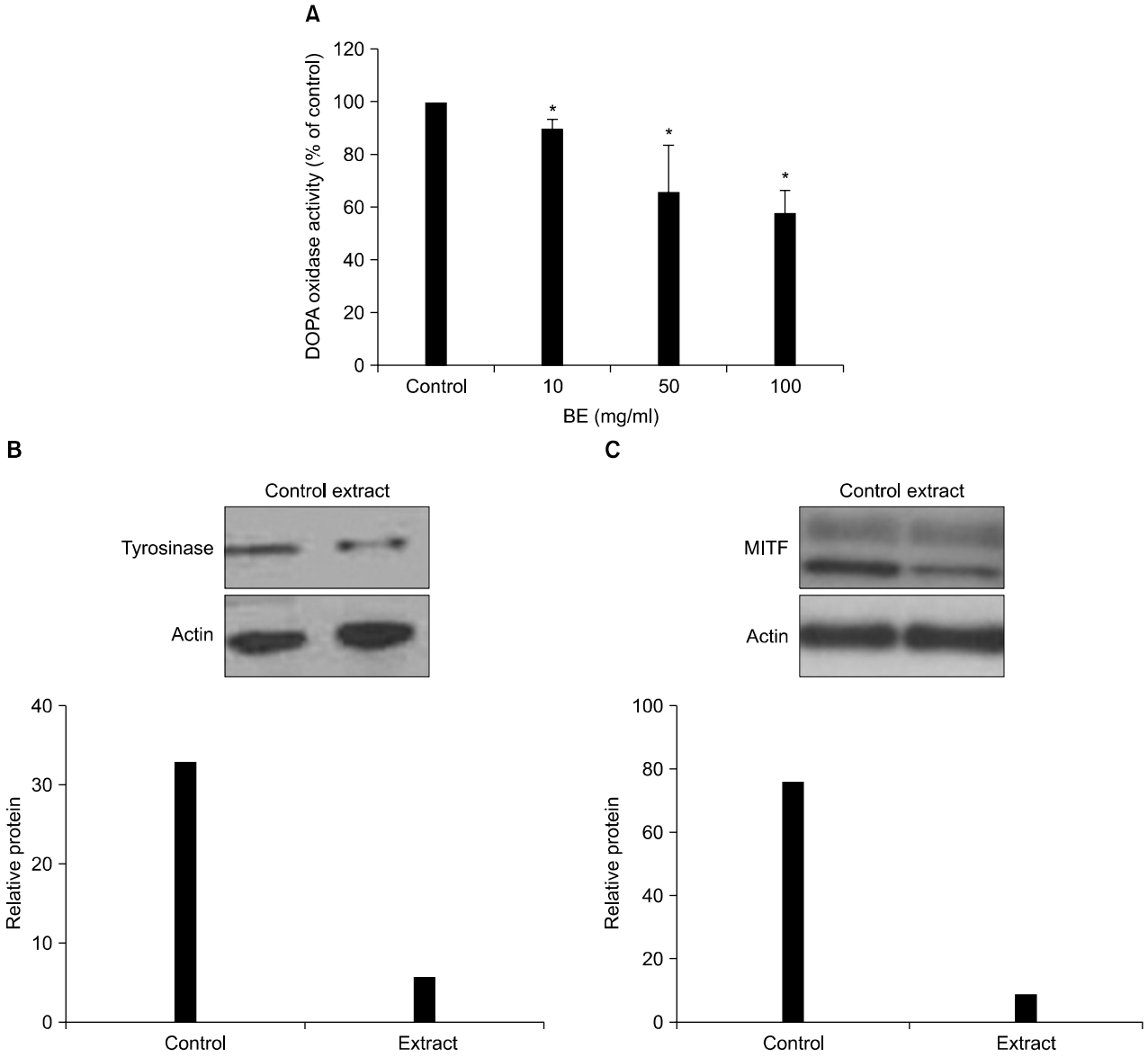

Fig. 3. Effect of the bacterial extract (BE) on the tyrosinase activity and the tyrosinase and MITF expressions. (A) The Melan-a cells were incubated with 10 mM L-DOPA for 90 min and the absorbance was measured at 475 nm. The values indicate the mean of five independent experiments ± SD. $*p < 0.01$. The tyrosinase (B) and MITF (C) protein expressions of the Melan-a cells treated with bacterial extract (50 μg/ml) were determined by western blotting.

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