World J Microbiol Biotechnol (2012) 28:2903–2912
DOI 10.1007/s11274-012-1096-0

SHORT COMMUNICATION

# Antioxidative properties and inhibitory effect of *Bifidobacterium adolescentis* on melanogenesis

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Received: 2 April 2012 / Accepted: 29 May 2012 / Published online: 14 June 2012


**Abstract** Melanin is a dark pigment produced by melanocytes. Tyrosinase is a key enzyme which catalyzes the rate-limiting step of melanogenesis. However, accumulation of melanin leads to various skin hyperpigmentation disorders. To find a novel skin-whitening agent, the antioxidant capacity of *Bifidobacterium adolescentis* culture filtrate and inhibitory effect on melanogenesis were investigated. The antioxidant effects of *B. adolescentis* culture filtrate include 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging capacity, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical cation scavenging activity and reducing power were measured spectrophotometrically. The reducing power is a useful index for the evaluation of potential antioxidants which carry out reduction of ferricyanide to ferrocyanide. Furthermore, the inhibitory effects of the bacterial culture filtrate on mushroom tyrosinase, B16F10 intracellular tyrosinase activity and melanin content were also determined. The results revealed that *B. adolescentis* culture filtrate (2.5, 5.0 and 7.5 %; v/v) effectively scavenged DPPH and ABTS radicals, and lower concentrations of the bacterial culture filtrates (0.5, 1.0 and 1.5 %; v/v) showed potent reducing power in a dose-dependent pattern. Additionally, the bacterial culture filtrate suppressed murine tyrosinase activity and decreased the amount of melanin in a dose-dependent manner. Our results demonstrated that *B. adolescentis* culture filtrate decreases the melanogenesis process of melanoma cells by inhibiting tyrosinase activity, which we suggest may be mediated through its antioxidant activity.

**Keywords** *Bifidobacterium adolescentis* · Tyrosinase · Melanin · Antioxidant

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## Introduction

Free radicals and active oxygen have been recognized as an important factor in the pathogenesis of several human diseases such as aging, atherosclerosis and cancer (Halliwell et al. 1992). It is reported that ultraviolet (UV) exposure generates reactive oxygen species (ROS) and excessive ROS are causally associated with several skin disorders as well as hyperpigmentation (Yasui and Sakurai 2003). The major type of ROS produced on the skin surface is $^1O_2$, which is generated by a photosensitizing reaction with UVA and porphyrins from normal bacterial flora living on the skin (Ryu et al. 2009). Singlet oxygen ($^1O_2$) is oxidized to cholesterol, squalene and to unsaturated acyl residues in the sebum to yield lipid hydroperoxide. Many antioxidants display depigmentation properties by interfering with lipid peroxidation of melanocyte membranes and increase the intracellular glutathione content (del Marmol et al. 1993). Intracellular glutathione has been reported to act as an antioxidant that determines the expression of a melanin-based signal (Galván and Alonso-Alvarez 2008). Hence, antioxidants may play an important role in regulation of melanin production.

Melanin is responsible for skin color and also plays an important role in protecting the skin against UV light injury. However, it is reported that overproduction and

accumulation of melanin may result in several skin syndromes such as freckles, melasma, age spots and other hyperpigmentation diseases (Briganti et al. 2003). Tyrosinase is the rate-limiting enzyme in the steps of melanin biosynthesis pathway, in which L-tyrosine is hydroxylated to L-Dopa, and L-Dopa is further oxidized to the corresponding *o*-quinone (Seo et al. 2003). It is reported that plant extracts or compounds with redox properties may show depigmentation effects by interacting with *o*-quinones, and then restrict the oxidative polymerization of melanin intermediates, or with copper ions at the active site of tyrosinase. Hence, many redox agents can inhibit epidermal melanogenesis either directly or indirectly by scavenging ROS generated in the skin following UV exposure (Karg et al. 1993). In addition, it has been evidenced that other enzymes such as tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2) also contribute to the production of melanin (Hearing and Jimenez 1987; Tsukamoto et al. 1992; Jimenez-Cervantes et al. 1994). Recently, it has been shown that other melanogenesis inhibitors have been increasingly used in skin care cosmetics for the prevention of hyperpigmentation (Funasaka et al. 2000). Furthermore, melanogenesis is reported to produce hydrogen peroxide ($H_2O_2$) and other ROS, which puts the melanocytes under high-grade oxidative stress. It is reported that ROS scavengers and inhibitors of ROS generation may down-regulate UV-induced melanogenesis (Yamakoshi et al. 2003). Therefore, antioxidants such as ascorbic derivatives and reduced glutathione (GSH) have been applied as inhibitory agents of melanogenesis (Imokawa 1989; Kumano et al. 1998). Interestingly, there are two reports about the antioxidant capacity of probiotc *Bifidobacteria* (Huang et al. 2011a, b), which indicates the dermatological potential of *Bifidobacteria*.

*Bifidobacteria* are Gram-positive obligate anaerobes and are considered to promote a healthy host intestinal tract because of their beneficial effects including reduction of harmful bacteria and toxic compounds, regulation of the environment of the intestine, modulation of immune response, and anticancer activity (Hooper and Gordon 2001; Femia et al. 2002; Ouwehand et al. 2002). Therefore, the healthy and nutritional benefits of orally administered Bifidobacteria species have garnered an increasing amount of attention (Gilliland 1990). Recently, numerous human clinical trials suggested that probiotic supplementation might be useful in the treatment of atopic dermatitis and dry skin (Kalliomaki et al. 2001; 2003; 2007; Rautava and Isolauri 2002; Puch et al. 2008). Moreover, some reports have demonstrated that certain probiotic bacterial cytoplasmic extracts from *Lactobacillus johnsonii*, *L. casei*, *L. plantarum* and *L. acidophilus,* have anti-microbial and anti-adhesion properties when applied to cutaneous and mucous surfaces (Ouwehand et al. 2003; Rodrigues et al. 2005). Interestingly, we have found two *Bifidobacterium* species such as *B. bifidum* (Huang et al. 2011b) and *B. infantis* (Huang et al. 2011a) that exhibited both antimelanogenic and antioxidant activities. *B. adolescentis* has been reported to show anti-proliferative effects on human colon cancer cell lines (Gibson and Wang 1994; Kim et al. 2008; Lee et al. 2008). However, there is no report about application of *B. adolescentis* culture filtrate for the potential use in skin care cosmetics.

In the present study, we examined the inhibitory effects of *B. adolescentis* culture filtrate on melanogenesis by assessing its effects on the mushroom tyrosinase activity, tyrosinase activity, and melanin content in B16F10 murine melanoma cells. Moreover, we looked into the inhibitory effect of the culture filtrate on melanogenesis associated with its antioxidant properties by assessing its antioxidant and free radical scavenging activities.

## Materials and methods

### Culturing and filtrate preparation

The *B. adolescentis* strain (BCRC 14658) was obtained from the Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute, Hsinchu, Taiwan. The bacterial strain was cultivated in Man Rogosa Sharp (MRS) broth (Difco, Detroit, MI, USA) supplemented with 0.4 g $l^{-1}$ cysteine-HCl (Sigma Chemical Co, Saint Louis, MO, USA) anaerobically overnight at 37 °C in an atmosphere-controlled anaerobic system (ThermoForma, Model 1025, Marietta, USA) that was continuously sparged with a mixture of 10 % carbon dioxide, 10 % hydrogen and 80 % nitrogen (Toyogas, Taichung, Taiwan). Before experimental use, bacterial strain was subcultured twice in cysteine-containing MRS. Thereafter, 1 % (v/v) of each culture was transferred to 50 ml cysteine-containing MRS culture medium and incubated anaerobically at 37 °C for 48 h. After incubation, cells were removed by centrifugation 10,000×*g* for 10 min at 4 °C and supernatant was subsequently passed through a cellulose acetate filter (0.45 μm) to get culture filtrate.

### Cell culture and cell viability assay

To evaluate the effect of *B. adolescentis* culture filtrate on proliferation of B16F10 cells, and confirm the inhibitory effects of the bacterial culture filtrate on melanogenesis in B16F10 cells were not resulted from cell death, cell viability assay was carried out in triplicate. B16F10 cells (ATCC CRL-6475; BCRC60031) were obtained from the

Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute, Hsinchu, Taiwan and were cultured in Dulbecco's modification of Eagle's medium (DMEM) with 10 % fetal bovine serum (FBS; Gibco, Langley, OK, USA) and 100 I.U/50 μg/ml of penicillin/streptomycin (Sigma Chemical Co, Saint Louis, MO, USA) in a humidified atmosphere containing 5 % $CO_2$ in air at 37 °C. The cell viability assay of B16F10 was performed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Tada et al. 1986). Briefly, $1 \times 10^4$ cells/well was seeded into a 96-well plate. The cells were exposed to *B. adolescentis* culture filtrate (1.0, 2.5, 5.0, 7.5 and 10 %; v/v) for 24 h, then MTT solutions were added to each well. The insoluble derivative of MTT produced by intracellular dehydrogenase was solubilized with ethanol-DMSO (1:1 mixture solution). The absorbance of each well at 570 nm was read by using a microplate reader. The amount of MTT in bacterial culture filtrate treated group was compared to that of control group. The higher relative amount of MTT measured indicated the culture filtrate is not cytotoxic to the B16F10 cells.

### Determination of 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) scavenging capacity

The antioxidant activity of *B. adolescentis* culture filtrate was measured in terms of radical scavenging ability using the DPPH assay (Brand-Williams et al. 1995) as modified by Sanchez-Moreno et al. (1998). Bacterial culture filtrates at various concentrations (final concentration 2.5, 5.0, 7.5 %; v/v) were added to 2.9 ml of DPPH (60 μM) solution. When DPPH interacts with an antioxidant that can donate hydrogen, it gets reduced and the resulting decrease in absorbance at 517 nm was recorded using a UV–Vis spectrophotometer (Jasco, V-630, Tokyo, Japan). In this study, vitamin C (50 μM) and vitamin E (50 μM) (Sigma Chemical Co, Saint Louis, MO, USA) were used as positive antioxidant standards, cysteine-containing MRS culture medium was used as a negative control.

### Determination of 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)(ABTS) radical-scavenging activity

The experiments were carried out using an improved ABTS decolorisation assay (Re et al. 1999) and it involves the generation of $ABTS^{+\cdot}$ chromophore by the oxidation of ABTS with potassium persulfate. The ABTS radical cation ($ABTS^{+}$) was produced by reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark for at least 6 h at room temperature before use. Absorbance at 734 nm was measured 10 min after mixing of different concentrations of the *B. adolescentis* culture filtrate (final concentration 2.5, 5.0, 7.5 %; v/v) with 1 ml of $ABTS^{+\cdot}$ solution. The $ABTS^{+\cdot}$ scavenging capacity of the filtrate was compared with that of vitamin C (60 μM) and vitamin E (60 μM), cysteine- containing MRS culture medium was used a negative control. All determinations were carried out in triplicate.

### Determination of reducing power

The reducing potential of the extract was determined according to the method of (Oyaizu 1986). The different concentrations of the bacterial filtrate, vitamin C (44 μM) and vitamin E (44 μM) or cysteine- containing MRS culture medium was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1 % w/v). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10 %; w/v) was added to the mixture, which was then centrifuged for 10 min at 1,000×*g*. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 % w/v), and the absorbance was measured at 700 nm in a UV–Vis spectrophotometer. Higher absorbance of the reaction mixture indicated stronger reducing power. All determinations were carried out in triplicate.

### Assay of mushroom tyrosinase activity

In order to assay the inhibitory effect of *B. adolescentis* culture filtrate on mushroom tyrosinase, dose-dependent inhibition experiments were carried out in triplicate as described previously with a minor modification (Bilodeau et al. 2001). In brief, 10 μl of an aqueous solution of mushroom tyrosinase (500 units) (Sigma Chemical Co, Saint Louis, MO, USA) was added to a 96-well microplate, in a total volume of a 200 μl mixture containing 5 mM L-Dopa solution, 50 mM phosphate buffer (pH 6.8). The assay mixture was incubated at 37 °C for 30 min. Following incubation, the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 490 nm ($OD_{490}$) in a microplate reader.

### Measurement of melanin content

Intracellular melanin content was measured as described by Tsuboi et al. (1998) with some modifications. The B16F10 melanoma cells were treated with 100 nM of α-melanocyte stimulating hormone (α-MSH) (Sigma Chemical Co, Saint Louis, MO, USA) for 24 h, and then the melanin content was determined after treatment with either *B. adolescentis* culture filtrate (final concentration 1.0, 2.5, 5.0, 7.5 %; v/v) or kojic acid (0.2 mM) and arbutin (2 mM) for another

24 h. After treatments, the B16F10 cells were detached by incubation in trypsin/EDTA. The cell number was counted under microscope by trypan blue exclusion assay, the method is based on the principle that live cells possess intact cell membranes that exclude the dye, whereas dead cells do not (Strober 2001). After precipitation, cell pellets containing a known number of cells were solubilized in 1 N NaOH at 60 °C for 60 min. The melanin content was assayed by spectrophotometric analysis at 405 nm absorbance.

### Assays of intracellular tyrosinase activity

Cellular tyrosinase activity was determined as previously described with slight modifications (Yang et al. 2006). Briefly, the B16F10 cells were treated with α-MSH (100 nM) for 24 h, and then the intracellular tyrosinase activity was determined after treatment with various concentrations of *B. adolescentis* culture filtrate (final concentration 1.0, 2.5, 5.0, 7.5 %; v/v) or kojic acid (0.2 mM) and arbutin (2 mM) for another 24 h. After treatments, the cells were washed twice with phosphate-buffered saline and homogenized with 50 mM PBS (pH 7.5) buffer containing 1.0 % Triton X-100 and 0.1 mM PMSF. Intracellular tyrosinase activity was monitored as follows. Cell extracts (100 μl) were mixed with freshly prepared L-Dopa solution (0.1 % in phosphate-buffered saline) and incubated at 37 °C. The absorbance at 490 nm was measured with a microplate reader Gen 5$^{TM}$ (BIO-TEK Instrument, USA) to monitor the production of dopachrome and corrected for auto-oxidation of L-Dopa.

### Statistical analysis

Statistical analysis of the experimental data points was performed by ANOVA test, which was used for comparison of measured data using SAS 9.0 statistical software (SAS Institute Inc., Cary, NC, USA). All experiments were carried out in triplicate and the results were presented as mean ± SD of three separate experiments. Differences were considered as statistically significant at $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

## Results and discussion

### 2,2-diphenyl-1-picryl-hydrazyl (DPPH) scavenging capacity assay

Human skin is exposed to UV and environmental oxidizing pollutants and is a preferred target of oxidative stress. It has been reported that UV irradiation induces the formation of ROS in cutaneous tissue provoking toxic changes such as lipid peroxidation and enzyme inactivation (Emerit 1992). To elucidate the potential antioxidant activity of *B. adolescentis* culture filtrate, we measured the DPPH radical scavenging activity, ABTS radical cation scavenging activity and reducing power of the bacterial culture filtrate. The DPPH radical scavenging activity of *B. adolescentis* culture filtrate was increased in a dose-dependent manner as shown in Fig 1a. The radical scavenging activity was 54.6 ± 1.79, 65.1 ± 2.3, 88 ± 0.6 % for 2.5, 5.0 and 7.5 % of bacterial culture filtrate, respectively. The DPPH radical scavenging activity of vitamin C (50 μM) was 43.5 ± 1.1 % and that of vitamin E (50 μM) was 35.9 ± 1.7 %. Most importantly, the *B. adolescentis* culture filtrate display much stronger activity than that of vitamin C or vitamin E. The results shown in Fig 1a indicated that the culture filtrate display DPPH free radical scavenging activity in a dose-dependent manner. The steric accessibility of DPPH radical is a major determinant of the assay reaction, since some small molecules that have better access to the radical site could show higher antioxidant capacity. Besides, many large antioxidant chemicals that react quickly with peroxyl radicals may react slowly in DPPH assay (Huang et al. 2012). Additionally, the spectrophotometric analysis can be affected by the chemical structure of test compounds that absorb at the wavelength of determination or by the turbidity of the sample.

### 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical-scavenging activity assay

The results shown in Fig. 1b depicted the ABTS-scavenging capacity of various concentrations of *B. adolescentis* culture filtrate. The radical scavenging activity for 2.5, 5.0 and 7.5 % of bacterial culture filtrate was 56.5 ± 0.33, 61.9 ± 1.6, 81.5 ± 1.5 in percentage (%), respectively. Meanwhile, the radical scavenging activity of vitamin C (60 μM) was 97.1 ± 0.34 (%) and that of vitamin E (60 μM) was 46.4 ± 6.4 (%). The different relative radical scavenging capacity of *B. adolescentis* culture filtrate against different testing radicals may be due to the mechanisms involved in the antioxidant and radical reactions. Moreover, the stoichiometry of reactions between the antioxidant compounds in the bacterial filtrate and the radicals may be responsible for the difference in the radical scavenging potential. Additionally, the stereoselectivity of the radicals may also affect the capacity of the bacterial filtrate to quench different radicals.

### Measurements of reducing power

The reducing power of *B. adolescentis* culture filtrate increased steadily with the increasing concentration

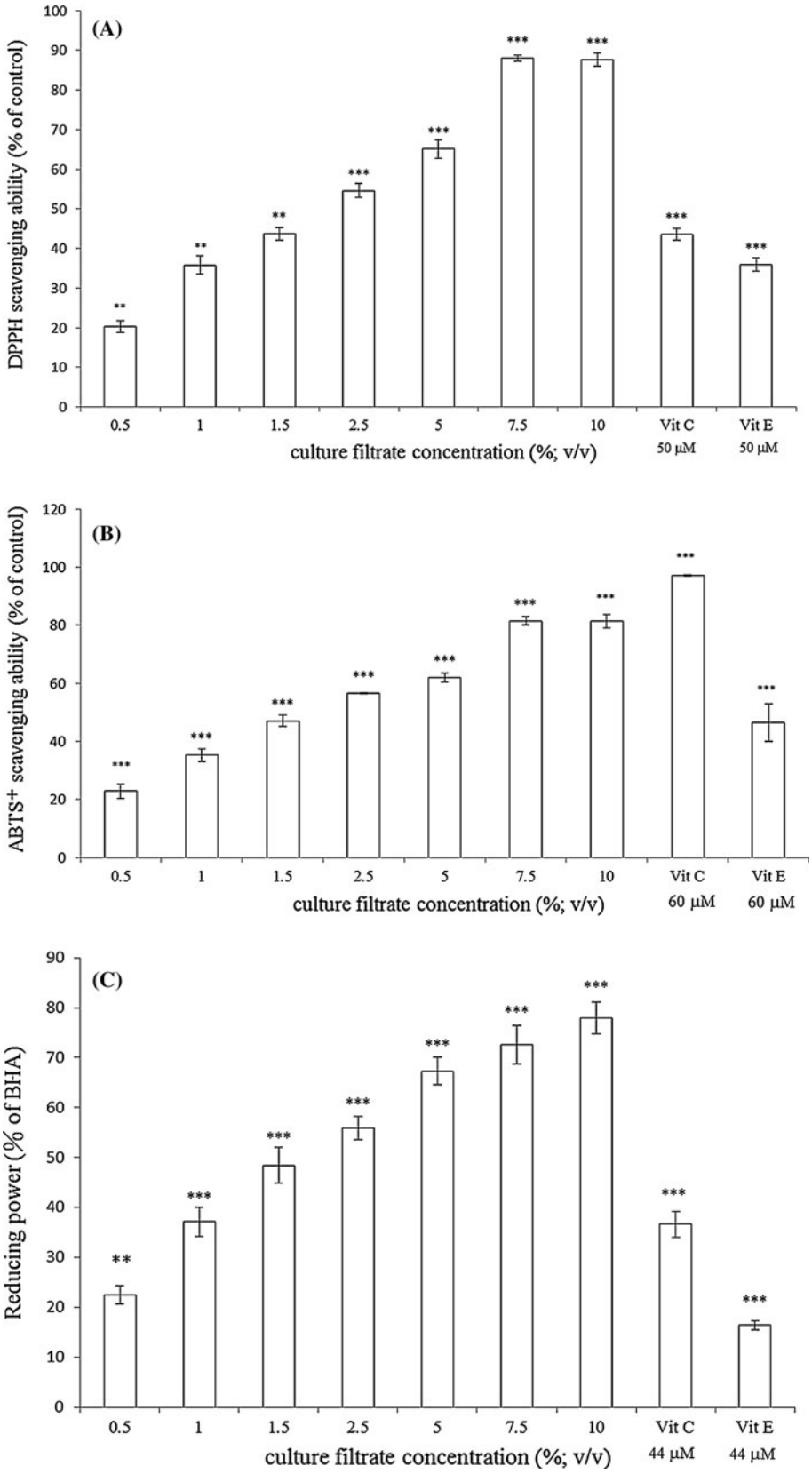


**Fig. 1** Antioxidant capacity of *Bifidobacterium adolescentis* culture filtrate. **a** Determination of 2,2-diphenyl-1-picryl-hydrazyl scavenging capacity. **b** 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical-scavenging activity measurement. **c** Reducing power assay. Vitamin C and vitamin E were used as positive standards in the above assay, the MRS-medium was used as a negative control. Values are represented as percentage of control. Data are mean ± SD of three separate experiments. $^{**}p < 0.01$; $^{***}p < 0.001$

**Table 1** Antioxidant activity of *Bifidobacterium adohscsntis* culture filtrate

| Culture filtrate concentration (%; v/v) | DPPH scavenging ability (% of control) | ABTS—scavenging ability (% of control) | Reducing power (% of BHA) |
|---|---|---|---|
| 0.5 | 20.3 ± 1.57 | 22.8 ± 2.47 | 22.5 ± 1.80 |
| 1.0 | 35.8 ± 2.34 | 35.3 ± 2.21 | 37.1 ± 2.90 |
| 1.5 | 43.7 ± 1.62 | 47.1 ± 1.82 | 48.4 ± 3.60 |
| 2.5 | 54.6 ± 1.79 | 56.5 ± 0.33 | 55.9 ± 2.31 |
| 5.0 | 65.1 ± 2.33 | 61.9 ± 1.60 | 67.3 ± 2.72 |
| 7.5 | 88.0 ± 0.76 | 81.5 ± 1.49 | 72.5 ± 3.84 |
| 10.0 | 87.6 ± 1.64 | 81.4 ± 2.31 | 77.9 ± 3.12 |
| Vit C | 43.5 ± 1.51 | 97.1 ± 0.34 | 36.6 ± 2.53 |
| Vit E | 35.9 ± 1.73 | 46.4 ± 6.40 | 16.4 ± 0.90 |

(Fig. 1c). In this study, the reducing power of bacterial filtrate, vitamin C and vitamin E was compared with that of butylated hydroxyanisole (BHA) (0.03 mg/ml). The reducing power for 0.5, 1.0 and 1.5 % of bacterial culture filtrate was 22.5 ± 1.8, 37.1 ± 2.9, 48.4 ± 3.6 (%), respectively. The reducing power of vitamin C (44 μM) was 36.6 ± 2.53 (%) and that of vitamin E (44 μM) was 16.4 ± 0.9 (%). We also have determined the reducing power of higher concentrations of *B. adolescentis* culture filtrate like 2.5, 5.0 and 7.5 %, higher concentration of the bacterial filtrate show higher reducing power in the system (Table 1).

## Cell viability assay

To assess the effect of *B. adolescentis* culture filtrate on cell viability, B16F10 melanoma cells were treated with different concentrations of bacterial culture filtrate (1.0, 2.5, 5.0, 7.5 and 10 %) for 24 h. The MTT assay results indicated that the *B. adolescentis* culture filtrate (1.0–7.5 %) had no inhibitory effect on B16F10 cell proliferation, which means the bacterial culture filtrate is not harmful to cells (Fig. 2). The data shown in Fig. 2 indicated that higher concentration of the culture filtrate seemed to be harmful to the cells. Hence, in B16F10 melanoma cell model, we chose the concentrations of the culture filtrate at the range 1.0–7.5 %.

## Mushroom tyrosinase activity assay

The data shown in Fig. 3a indicated that the mushroom tyrosinase activity was inhibited by higher concentrations of *B. adolescentis* culture filtrate (7.5, 15 and 20 %; v/v), the observed tyrosinase activity for 7.5, 15 and 20 % of bacterial culture filtrate was 98.2 ± 1.3, 59.1 ± 3.9, 20.3 ± 1.9 (%), respectively. On the other hand, the observed tyrosinase activity for kojic acid (0.2 mM) was 45.2 ± 5.5 (%). So far, there is no report about the effect of *B. adolescentis* culture filtrate on melanin production. Figure 3a revealed that higher concentrations (15 and 20 %) of the culture filtrate displayed stronger inhibitory effect on mushroom tyrosinase activity. We also have assayed the effects of lower concentrations of *B. adolescentis* culture filtrate (2.5, 5.0 and 7.5 %; v/v) on mushroom tyrosinase activity. However, lower concentrations of the bacterial filtrate did not show stronger tyrosinase inhibitory activity (Table 2).

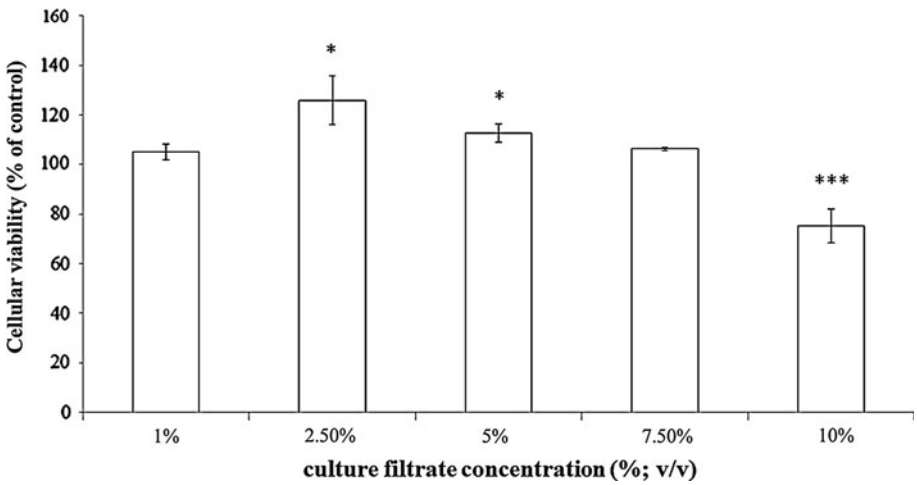


**Fig. 2** Cell viability assay. B16F10 cells were treated with various concentration of bacterial culture filtrate (1.0, 2.5, 5.0, 7.5 and 10 %; v/v) for 24 h and the cell viability was measured by MTT assay. Results are expressed as percentage of cell viability relative to control. Data are presented as mean ± SD. Values are significantly different by comparison with control. *$p < 0.05$; ***$p < 0.001$

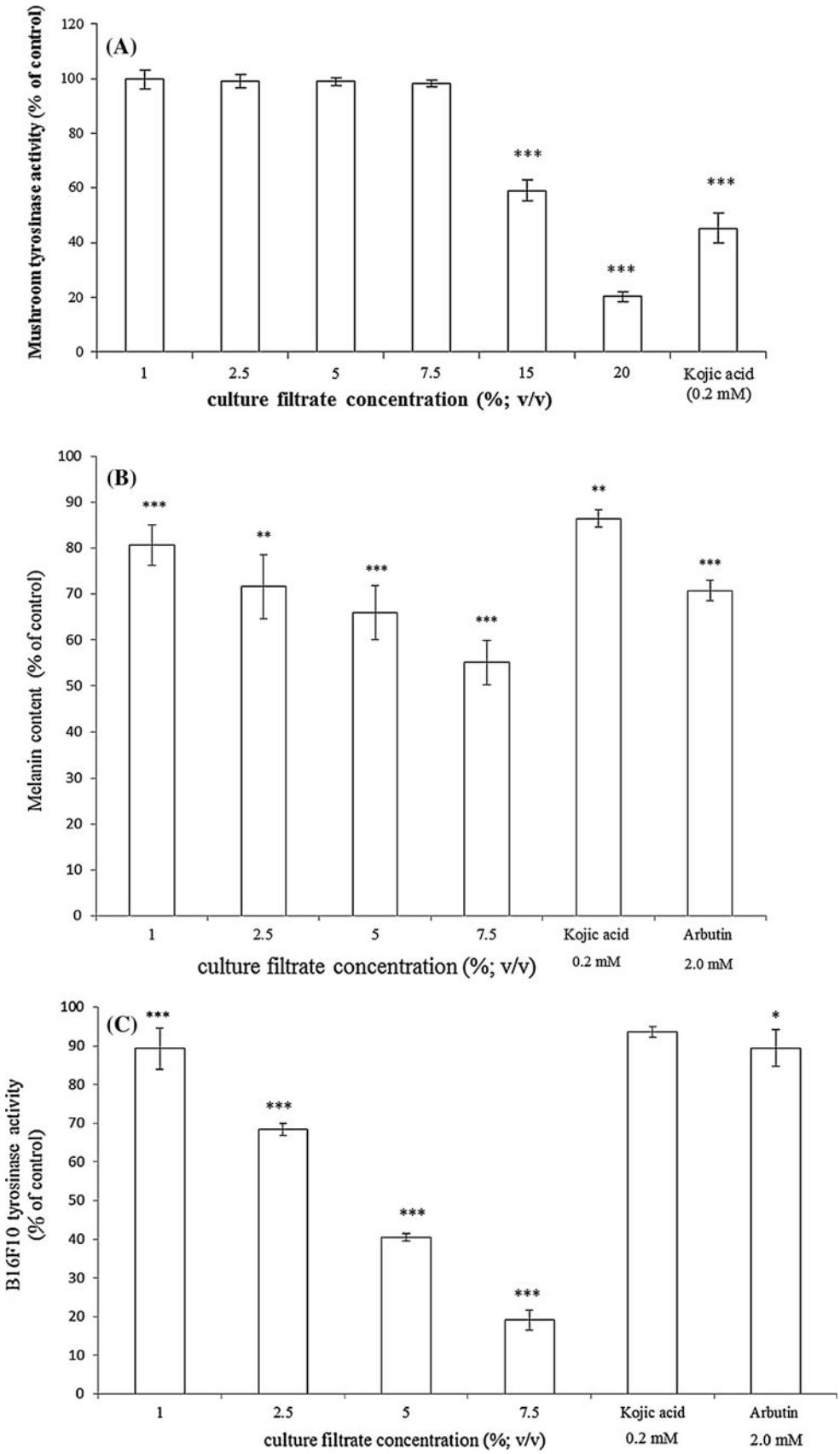


**Fig. 3** Inhibitory effects of *Bifidobacterium adolescentis* culture filtrate on melanogenesis. **a** Mushroom tyrosinase activity assay. **b** B16F10 melanin content determination. **c** B16F10 intracellular tyrosinase activity measurements. Results are represented as percentages of control, and the data are presented as mean ± SD of three separate experiments. Values are significantly different by comparison with the control. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

**Table 2** Anti melanogenesis effects of *Bifidobacterium adolescentis* culture filtrate

| Culture filtrate concentration (%; v/v) | Mushroom tyrosinase activity (% of control) | B16F10 intracellular melanin content (% of control) | B16F10 intracellular tyrosinase activity (% of control) |
|---|---|---|---|
| 1.0 | 99.8 ± 3.52 | 80.6 ± 4.40 | 89.3 ± 5.30 |
| 2.5 | 99.2 ± 2.45 | 71.6 ± 6.90 | 68.4 ± 1.50 |
| 5.0 | 98.9 ± 1.61 | 65.9 ± 5.90 | 40.5 ± 0.90 |
| 7.5 | 98.2 ± 1.30 | 55.1 ± 4.80 | 19.1 ± 2.60 |
| 15 | 59.1 +3.90 | ND | ND |
| 20 | 20.3 ± 1.90 | ND | ND |
| Kojic acid (0.2 mM) | 45.2 ± 5.5 | 86.4 ± 1.90 | 93.6 ± 1.40 |
| Arbutin (2.0 mM) | ND | 70.7 ± 2.20 | 89.4 ± 4.70 |

Data presented here are average value + SD of at least three replications in each concentration of culture filtrate or dose of compound. *ND* not determined

Determination of B16F10 intracellular melanin content

To determine the inhibitory effect of *B. adolescentis* culture filtrate on melanogenesis, B16F10 melanoma cells stimulated with 100 nM α-MSH for 24 h and cultured in the presence of *B. adolescentis* culture filtrate(1.0, 2.5, 5.0, 7.5 %), kojic acid (0.2 mM) or arbutin (2 mM) for another 24 h. After treatment, the melanin content was 80.6 ± 4.4 (%), 71.6 ± 6.9 (%), 65.9 ± 5.9 (%), 55.1 ± 4.8 (%) for 1.0, 2.5, 5.0 and 7.5 % of bacterial culture filtrate, respectively (Fig. 3b). In addition, the melanin content was 86.4 ± 1.9 % for kojic acid and 70.7 ± 2.2 (%) for arbutin. The results indicated that *B. adolescentis* culture filtrate express more potent inhibitory effect on melanin synthesis in B16F10 melanoma cells than that of kojic acid. Besides, higher concentrations of the bacterial filtrate (5.0 and 7.5 %; v/v) also show stronger inhibitory activity than that of arbutin.

Assay of B16F10 intracellular tyrosinase activity

We assessed the intracellular tyrosinase activity in B16F10 melanoma cells after treatment with. *B. adolescentis* culture filtrate, kojic acid (0.2 mM) or arbutin (2 mM). *B. adolescentis* culture filtrate significantly inhibited α-MSH-induced tyrosinase activity in a dose-dependent pattern (Fig. 3c). After those treatments, the residual B16F10 intracellular tyrosinase activity was 89.3 ± 5.3 (%), 68.4 ± 1.5 (%), 40.5 ± 0.9 (%), 19.1 ± 2.6 (%) for 1.0, 2.5, 5.0 and 7.5 % of bacterial culture filtrate, respectively. Additionally, the intracellular tyrosinase activity 93.6 ± 1.4 % for kojic acid and 89.4 ± 4.7 (%) for arbutin. Interestingly, the results shown in Fig. 3c were in accordance with the results indicated in Fig. 3b, which means *B. adolescentis* culture filtrate inhibit intracellular tyrosinase activity and then decrease the melanin content in a dose-dependent manner in B16F10 melanoma cells (Table 3).

This is the first report about the effect of *B. adolescentis* culture filtrate on melanin production. In the present study, it is found that *B. adolescentis* culture filtrate inhibit intracellular tyrosinase activity significantly in a dose-dependent pattern, the inhibitory effect of the culture filtrate was stronger than that of kojic acid. However, the culture filtrate (1.0–7.5 %; v/v) could not effectively suppressed mushroom tyrosinase activity in the cell-free system (Tables 2, 3). The results suggested that *B. adolescentis* culture filtrate decreased melanin production may be attributed to its inhibitory action upon the signaling pathway regulating tyrosinase activity. To identify the signaling pathway of *B. adolescentis* culture filtrate, we will study the effects of various protein kinase inhibitors on melanogenesis in *B. adolescentis* culture filtrate treated B16F10 melanoma cells. Certainly, we will also analyze the active components in the *B. adolescentis* culture filtrate and try to elucidate the antioxidant and whitening mechanisms.

## Conclusion

In the study, it is found that the antioxidant capacity of *B. adolescentis* culture filtrate scavenged DPPH and ABTS radicals in a dose-dependent manner. The reducing power of the culture filtrate was also increased in a dose-dependent pattern. Furthermore, the bacterial culture filtrate inhibited mushroom tyrosinase and B16F10 intracellular tyrosinase activity, and decreased melanin in a dose-dependent manner. The results revealed that *B. adolescentis* culture filtrate inhibited the melanogenesis process of melanoma cells by inhibiting tyrosinase activity, which we suggest may be mediated through its antioxidant activity.

**Table 3** Regression analysis of dose–response in the antioxidant and antimenogenic assays

| | R square | *p* value |
|---|---|---|
| DPPH scavenging ability assay | 0.8994 | 0.0011 |
| ABTS + scavenging ability assay | 0.8628 | 0.0025 |
| Reducing power assay | 0.8164 | 0.0053 |
| Mushroom tyrosinase activity assay | 0.8983 | 0.0040 |
| B16F10 intracellular melanin content assay | 0.9759 | 0.0121 |
| B16F10 intracellular tyrosinase activity assay | 0.9906 | 0.0047 |

**Acknowledgments** The authors gratefully acknowledge financial support by grants from Ministry of Economic Affairs, ROC. (98-EC-17-A-17-S1-128 and S09900074-603).

**Conflict of interest** None.

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