Full Length Research Paper

Evaluation of whitening efficacy and safety of the water extract from bamboo shavings

Zhang Jianyou¹,², Gong Jinyan¹, Lu Baiyi¹, Wu Xiaojin¹ and Zhang Ying¹*

¹Department of Food Science and Nutrition, College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310029, Zhejiang Province, PR China.
²College of Biological and Environmental Engineering, Zhejiang University of Technology Hangzhou 310014, Zhejiang Province, PR China.

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This aim of this study was to evaluate the whitening efficacy of the water extract from bamboo shavings (WEBS) in malignant melanoma cells (B-16) of mice. The safety of WEBS was evaluated by the acute oral toxicity test, the repeated skin irritation test and the acute eye irritation test was also evaluated. WEBS showed strong inhibitory effects against the activity of tyrosinase in B-16 cells in a dose dependent manner, and was more potent than arbutin. The melanin content was significantly inhibited by WEBS and the cytotoxicity of WEBS was lower than that of arbutin and hydroquinone. WEBS inhibited mushroom tyrosinase and the maximum was 65.05% at 16 mg/ml with an IC₅₀ of 6 mg/ml. The LD₅₀ was larger than 5000 mg/kg body weight and WEBS was found to be non-toxic and non-irritating.

Key words: Water extract of bamboo shavings, whitening effect, B-16 melanoma cell, safety evaluation.

INTRODUCTION

The appearance of brown-spots on skin, as a consequence of hyperpigmentation, is a very common aesthetic problem (Pawaskar et al., 2007). This skin disorder is a consequence of melanin excess, caused by hyperactivity of melanocytes, which are the cells responsible for skin pigmentation. This disorder can be treated with cosmetically using so-called skin-whitening (also referred to as skin-bleaching) cosmetic products (Briganti et al., 2003), although the most serious cases require medical assistance. These products contain different chemicals called skin-whitening agents (Briganti et al., 2003; Balaguer et al., 2008; Cabanes et al., 1994; Petit and Piérard, 2003).

Tyrosinase is responsible for not only browning in foods, but also melanization in animals. It is important in food and cosmetics to prevent browning and over-production of melanin (Hearing and Tsukamoto, 1991). The tyrosine in skin cells is gradually oxidized into Dopa and Dopaquinone by the action of tyrosinase. This process can be influenced by various environmental factors. Dopaquinone is polymerized into Indo-5,6-quinone through a series of metabolic reaction and finally transformed into skin melanin. Hence, the function of a large number of whitening agents is to interfere with the formation of melanin by inhibiting tyrosinase activity (Yu et al., 2007; Azhar-ul-Haq et al., 2006).

Consumers are cautious about cosmetics made from chemical compounds and cosmeceuticals are preferred (Momtaz et al., 2008). Traditional skin whitening agents such as hydrogen peroxide, aminomercuric chloride and phenolic derivatives can break down melanin rapidly. They are, however, forbidden in many countries, because of the high risks of dermal corrosion, cytotoxicity, and irritability. Current skin whitening agents including arbutin, kojic acid and derivatives, vitamin C and derivatives, and licorice flavonoids are largely natural plants extracts with

*Corresponding author. E-mail: zhjianyou@zjut.edu.cn or yzhang@zju.edu.cn. Tel/Fax: +86 571 88320237 or +86 571 86049803.
good whitening effects and minimal side effects (Yoshikane et al., 1999; Hong et al., 1999; Hansruedi et al., 1999; Curto et al., 1999; Frank et al., 2000).

During the recent years, more attention has been paid to the cosmetics market for skin whitening. There are many recent studies on skin melanin formation, whitening mechanism, whitening effect evaluation, whitening agent selection and production (Khan et al., 2006). Furthermore, various natural tyrosinase inhibitors have been reported (Passi and Nazzaro-Porro, 1981; Mayer, 1987; Khan et al., 2003). These include extracts from leaves and root barks of mulberry, which exhibited high inhibitory effects on 3-(3,4-dihydroxyphenyl)-L-alanine (DOPA) oxidase activity (Lee et al., 2002).

Bamboo shavings (Caulis bamfusae in taeniam), the intermediate layer of the stems of Bambusa tuloides Munro, Sinocalomel beecheysana var. pubescens P.F. Li or Phyllostachys nigra (Lodd.). Munro var. henonis (Mitt.) Stapf ex Rendle, are perennial plants of the family Gramineae (Zhang et al., 2004). In recent years, numerous bio-active components have been found in bamboo shavings including triterpenoids, saponins and sterols. The safety of the triterpenoid-rich extract (EBS) and polyphenol-rich ethanol extract (EEBS) from bamboo shavings have been evaluated (Zhang et al., 2004; Gong et al., 2010). The results show that EBS has excellent anti-fatigue, antihyperlipidemic and antihypertensive activities (Zhang et al., 2006; Jiao et al., 2007). WEBS, a water-phase extract from bamboo shavings obtained through supercritical carbon dioxide extraction, also contains numerous biologically active components such as triterpenoids, flavonoids, anthraquinones and phenolic acids. It is water soluble, yellow or light yellow in color, possesses the aroma of bamboo and is very stable for a period of two years storage.

B-16 melanoma cells are produced by mutation of normal melanocytes. Their biochemical metabolism is similar to that of normal human melanocytes especially with respect to its melanogenesis function. It is widely used in skin studies, because growing primary skin melanocytes in cell culture is difficult (Maeda et al., 2010). The results show that EBS has excellent anti-fatigue, antihyperlipidemic and antihypertensive activities (Zhang et al., 2006; Jiao et al., 2007). WEBS, a water-phase extract from bamboo shavings obtained through supercritical carbon dioxide extraction, also contains numerous biologically active components such as triterpenoids, flavonoids, anthraquinones and phenolic acids. It is water soluble, yellow or light yellow in color, possesses the aroma of bamboo and is very stable for a period of two years storage.

**Materials and Methods**

WEBS was prepared in our laboratory by supercritical carbon dioxide extraction from bamboo shavings and the concentration was 32 mg/ml. Arbutin (purity>98%) and hydroquinone were purchased from Shelligene Biotech Co., Ltd (Shanghai). All compounds were dissolved in DMSO with sterile filtration and stock solutions of compounds were prepared and stored in the dark at -20°C until used. Mice melanomas cell (B-16) was purchased from the Institute of Biochemistry and Cell Biology, SIBS, CAS (Nanjing). RPMI1640 medium and fetal calf serum were obtained from Gibco (US). MTT, L-DOPA, Tyrosinase, SDC and Trypsin were purchased from Sigma-aldrich (US). DMSO and trypsin were purchased from Sinopharm Chemical Reagent Co., Ltd (shanghai).

**Components analysis of WEBS**

Total phenolic content of WEBS was determined by the Folin-Ciocalteu method (Meda et al., 2005). The total flavonoid content was determined according to the aluminum chloride colorimetric method as described by Chang et al. (2002). The triterpenoid content of WEBS was determined by colorimetry using ginsenoside as a standard and the phenol-sulfuric acid method was chosen to determine its total sugar.

**Cell culture and treatment**

B-16 cells were cultured and treated according to the methods described by Zhi-Ming Hu et al. (2009) with some modifications. B-16 is routinely cultured in complete RPMI1640 medium with 10% heat-inactivated fetal calf serum at 37°C with 5% CO₂ and the cell incubums size was 3×10⁶ cells/L once every 3 days.

**Inhibition of mushroom tyrosinase activity**

Inhibition of tyrosinase activity was determined according to the method described by Matsuda et al. (1994) with some modifications. The reaction solution was prepared and mixed according to Table 1 and heated in a water bath at 28°C. Reactions were incubated for 5 min, the absorbance value was determined at 475 nm, and the inhibition rate was \( \frac{1-(A_{3}-A_{4})}{(A_{1}-A_{2})} \times 100\% \) with PBS blank control.

**B-16 cell viability assay**

B16 cells (2×10⁴) were seeded into each well of a 96-well plate after 0.25% trypsin dissociation. The cells were allowed to attach to the plates at 37°C for 24 h with 5% CO₂. At this point, the cultures were supplemented with WEBS at the specific concentrations in triplicate with hydroquinone and arbutin as positive controls. Complete RPMI1640 medium with appropriate compound was immediately added into the wells and left on for 48 h. The treated cells were labeled with MTT reagent for 3 h. The formazan precipitates were dissolved by DMSO and measured by absorbance at 475 nm in a ELISA reader with a reference wavelength of 650 nm (Zheng et al., 2001).

**B-16 tyrosinase activity assay**

For the tyrosinase activity assay, B-16 cells were seeded into each well of a six-well plate. The next day, the cells were fed with fresh medium containing the experimental compounds. Two days later, the cells were re-fed with compound-containing fresh medium again. On the 5th day of treatment, the cultures were harvested with trypsin/EDTA. Each cell suspension was centrifuged for 5 min at 1500 g, washed with cold PBS, and then solubilized in 200 ml extraction buffer. Extracts were solubilized at 4°C for 1 h, and aliquots were stored at -20°C until tyrosinase activity was assayed. (Curto et al., 1999).

**Melanin content assay in B-16**

For melanin content assay, B-16 cells were seeded into six-well
plates. The next day, the cells were fed with fresh medium containing compounds incubated for 48 h, 1 ml 0.1 mol/L NaOH (containing 10% DMSO) was added after 0.25% trypsin dissociation. The absorbance at 400 nm was measured after 30 min in water bath at 80°C and the cells were counted concurrently (Nerya et al., 2003).

Determination of lactate dehydrogenase (LDH) in supernatant

LDH is an important indicator of the integrity of the cell membrane and LDH levels in cell supernatant can be determined so as to judge the cytotoxicity of the sample. 200 μl of supernatant and 0.25 ml of substrate buffer were taken and 0.05 ml of coenzyme was added with 0.05 ml of water as the blank. 0.25 ml of 2,4-dinitrophenylhydrazine was added after water bath immersion for 15 min at 37°C. 2.5 ml of 0.4 mol/L NaOH was added for 3 min at room temperature and the absorbance at 400 nm was measured (Bonnekoh et al., 1990).

Changes of cell morphology

Changes were observed in cell morphology after 48 h of growth in culture with an inverted microscope.

Acute oral toxicity test

Ten male and 10 female Kunming mice whose weights between 19 and 22 g were provided by Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The temperature of the feeding room was 20 to 25°C and the relative humidity was 40 to 70%. The feed was provided by Suzhou Shuangshi Laboratory Animal Feed Science Co., Ltd.

After 16 h-fasting of animals, 10 male and 10 female mice were selected according to weight requirement. The experimental animals were treated with WEBS by one-time oral administration. After contamination, the general state was observed, weight change, toxicity symptoms, death situation, etc. of the animals for one week. Postmortem examination was carried out on dead animals and animals were killed and pathologic changes were noted.

Repeated skin irritation test

Two male and 2 female New Zealand white rabbits of common grade whose weights were from 2.5 to 2.8 kg were provided by Shanghai Shengwang Laboratory Animal Breeding Co., Ltd. The temperature of the feeding room was 19 to 26°C and the relative humidity was in the range of 40 to 70%. The feed was provided by Suzhou Shuangshi Laboratory Animal Feed Science Co., Ltd.

The hair on both sides of the spines of animals was cut 24 h before the test. The cutting area measured 3×3 cm, respectively for the left and right sides. During the test, 0.5 ml of WEBS was evenly applied on the left side with distilled water as control on the right side. The application was carried out once a day for successively 14 days. After the second day, application was conducted every day and hairs were cut, the skin reaction was observed 1 h later and the skin irritation reaction was scored according to relevant regulations in the Hygienic Standard for Cosmetics (2002) issued by the Ministry of Health. After completion of the test, the total score for 14 days, the average score for each animal in the 14 days and the average score of each animal on each day respectively, were calculated and the skin irritation intensity was graded.

Acute eye irritation test

With the slightly open left eyelid of New Zealand white rabbits of common grade, 0.1 ml of WEBS was dropped into the conjunctival sac. The upper and lower lids were closed for 1 s passively. The untreated right eye was used as control. 30 s later, physiological saline was used to wash the eye for 30 s, 1, 24, 48, and 72 h, 4 and 7 days after the eye drop. If there was no irritation reaction within 72 h, the observation was terminated. If there was an eye irritation reaction after 7 days, the observation was prolonged to 21 days. Scoring and irritation intensity grading were carried out in accordance with the Hygienic Standard for Cosmetics (2002) issued by the Ministry of Health.

RESULTS AND DISCUSSION

Components of WEBS

The pH, triterpenoid, total sugar, flavonoids and phenolic was 2.97, 3.65, 0.8, 0.69, and 0.465 mg/ml, respectively.

Effects of WEBS and arbutin on mushroom tyrosinase activity

Tyrosinase catalyzes both the hydroxylation reaction converting tyrosine into DOPA and the oxidation reaction converting DOPA into dopaquinone. This, in turn, leads to polymerization of brown pigments (Olivares et al., 2001; Jang et al., 2002). Thus, tyrosinase inhibitors can be applied to whiten the skin in cosmetics (Katagiri et al., 1998). The amount of enzyme required to increase the absorbance value by 0.001/min was defined as an enzyme activity unit and the activity was 2173.9 IU/mg. The concentration-dependent effects of WEBS, arbutin on mushroom tyrosinase inhibition are as shown in Figure 1. Different concentrations of WEBS showed differential inhibitory effects on mushroom tyrosinase and the maximum inhibition rate was 65.05% at 16 mg/ml, that is, IC_{max} of WEBS was 16 mg/ml. Arbutin, a naturally occurring glucoside of hydroquinone, has been traditionally used in Japan to treat pigmented disorders (Hori et al., 2004; Maeda and Fukuda, 1996). Many studies have demonstrated that arbutin is safe and less cytotoxic as compared to hydroquinone. However, its effectiveness and its depigmenting mechanism have not yet been fully characterized (Hori et al., 2004; Bang et al., 2008a, b). The

<table>
<thead>
<tr>
<th>Item</th>
<th>Reaction solution (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0.10% L-DOPA</td>
<td>2.0</td>
</tr>
<tr>
<td>Whitening agent solution</td>
<td>0</td>
</tr>
<tr>
<td>PBS</td>
<td>8.0</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>2.0</td>
</tr>
<tr>
<td>Total volume</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 1. Composition of reaction solution.
value for arbutin was 68.18% at 1 mg/m. The IC50 for WEBS and arbutin were 6 and 0.35 mg/ml, respectively. These results demonstrate that WEBS can be used as a skin-whitening agent, however, the tyrosinase-inhibiting ability was less in comparison with arbutin.

### Effects of WEBS, arbutin and hydroquinone on the viabilities of B-16

Skin melanocytes can transfer melanin to the epidermal basal cells, leading to excessive darkness and local pigmentation of skin. Therefore, B-16 cell proliferation is an important indicator for judgment of the whitening effect. The effect. The effects of different concentrations of WEBS, arbutin and hydroquinone on cell viability are as shown in Figure 2.

The viability of WEBS treated cells gradually declined with increasing WEBS concentration. The viability of arbutin treated cells decreased slightly, but not to the degree observed with WEBS treatment and it was around 80%. However, the viability of hydroquinone treated cells declined drastically to 12% at the highest concentration. These results suggest that inhibiting B-16 proliferation may be the main reason that hydroquinone has striking whitening effect. WEBS could affect the quantity of melanocytes in a dose-dependent manner and it might be a possible reason for its whitening effect.

### Effects of WEBS, arbutin and hydroquinone on the tyrosinase activity of B-16 cells

Most whitening products act by inhibiting tyrosinase activity or by blocking the oxidation pathway of tyrosine’s generation of melanin, because tyrosine is transformed to Dopa and Dopaquinone under the action of tyrosinase. The effects of WEBS, arbutin and hydroquinone on the tyrosinase activity of B-16 cells are as shown in Figure 3.

The inhibitory effects of WEBS and arbutin on tyrosinase activity in B-16 cells was enhanced significantly with the increasing concentration. The inhibition of hydroquinone on tyrosinase in B-16 cells was relatively strong in comparison to others and was 74.9%. The results were consistent with inhibition of tyrosinase activity in vitro.

### Effects of WEBS, arbutin and hydroquinone on the melanin of B-16 cells

Melanin is produced inside the melanocytes as a mixture of brown-black eumelanin and red-yellow phaeomelanin (Hearing and Tsukamoto, 1991). A decreased susceptibility of subjects with darker skin phototypes to UV-induced photodamage is believed to be due to the filtering ability and radical scavenging capacity of melanin (Kaidbey et al., 1979; Ezzahir, 1989; Kollias et al., 1991). The effects of WEBS, arbutin and hydroquinone on the melanin content in B-16 cells are depicted in Figure 4.

The melanin content of B-16 cells decreased with increasing concentration of WEBS, arbutin and hydroquinone, suggesting that reductions in melanin generation might underlie its whitening efficacy.

### Determination of LDH in B-16 supernatant

The effects of WEBS, arbutin and hydroquinone on LDH release of B-16 cells are shown in Table 2. The level of LDH in the supernatant of B-16 cells treated with WEBS and arbutin was relatively high and very little cytotoxicity was observed. The level of LDH in cells treated with greater than 5 mg/ml hydroquinone could not be tested due to the increased cytotoxicity. It was, therefore, presumed that hydroquinone may seriously damage the cell membrane despite its strong whitening effect. Hydroquinone has been used successfully to treat many forms of epidermal hyperpigmentation, such as melasma, freckles and postinflammatory hyperpigmentation, etc. However, the biosafety of hydroquinone as a skin whitening ingredient remains controversial, although it has been in use topically for more than 50 years (Nakajima et al., 1998; Westerhof and Kooyers, 2005; Nordlund et al., 2006; Levitt, 2007).

### The morphology changes of B-16 cells

The morphology of B-16 melanocytes is as shown in Figure 5. The morphology of B-16 blank cells was normal after three days in culture: the supernatant was light yellow and cells were spindle-shaped and mostly had an easily observable cell nucleus. B-16 cells treated with arbutin were round with the cell wall detached and contained irregular spheroplasts. B-16 cells treated with hydroquinone were distributed sparsely with reduced and shortened cell dendrites. It was difficult to form a mutually-maintaining reticular structure so that paracrine could not be carried out through the reticular interconnected structure to achieve common proliferation and differentiation. Concern regarding the side effects of

### Table 2. LDH release of B-16 cells (control group 72 U/L).

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>WEBS (U/L)</th>
<th>Arbutin (U/L)</th>
<th>Hydroquinone (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>165±14</td>
<td>79±19</td>
<td>74±23</td>
</tr>
<tr>
<td>1.25</td>
<td>166±8</td>
<td>90±4</td>
<td>75±9</td>
</tr>
<tr>
<td>2.5</td>
<td>183±12</td>
<td>96±17</td>
<td>81±7</td>
</tr>
<tr>
<td>5.0</td>
<td>248±32</td>
<td>101±11</td>
<td>-</td>
</tr>
<tr>
<td>10.0</td>
<td>267±14</td>
<td>107±35</td>
<td>-</td>
</tr>
<tr>
<td>20.0</td>
<td>278±17</td>
<td>177±39</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3. Results of acute oral toxicity.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Dose (mg/kg)</th>
<th>Number of animals</th>
<th>Number of dead animals</th>
<th>Death rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>5000</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Male</td>
<td>5000</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

LD₅₀: female mice; LD₅₀>5000 mg/kg; Male mice: LD₅₀>5000 mg/kg.

Figure 1. Effect of WEBS and arbutin on mushroom tyrosinase.

Figure 2. Effect of concentration on cell viability.
Figure 3. Effect of WEBS and positive control on tyrosinase in B-16.

Figure 4. Effects of WEBS, arbutin and hydroquinone on the melanin of B-16 cells.

hydroquinone arose primarily, because topical application of hydroquinone may cause exogenous ochronosis, its benzene metabolites in vivo may cause bone marrow toxicity, carcinogenesis with excessive use (Nordlund et al., 2006; Levitt, 2007). The density of B16 cells treated with WEBS declined with less morphology damage, demonstrating that WEBS was safer than arbutin and hydroquinone.
Safety evaluation for WEBS

**Acute oral toxicity test**

The results of acute oral toxicity test are shown in Table 3. All animals in each group were normal with good hair gloss and no toxicity symptoms or death during the test. The LD50 of WEBS to female and male mice was both more than 5000mg/kg and classified as non-toxic.

**Repeated skin irritation test**

The average score for the skin irritation reaction of each animal each day was 0 in the repeated irritation test and there was, therefore, no irritation.

**Acute eye irritation test**

The average score of irritation to eyes of rabbits by WEBS was cornea: 0; iris: 0; conjunctiva: 0. Thus there was no irritation.

Prospect

Since biosynthesis of melanin is an enzymatic biochemical reaction with multiple steps, the inhibition of melanogenesis may be related to other enzymes besides tyrosinase. Further research is required to fully understand the effect of WEBS on other enzymes and functional factors.

ACKNOWLEDGEMENTS

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