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Evaluation of the extract of traditional Chinese medicine formula Si Ben Cao for skin whitening

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ABSTRACT

Objective: To evaluate the safety, potential benefits and possible mechanisms of the extract of Si Ben Cao (SBC), a traditional Chinese medicine formula composed of four herbs, for skin whitening.
Methods: The reflux method was performed to prepare the extract of SBC. MTT assay was used to evaluate the cytotoxic of SBC extract *in vitro*. Skin irritation test and skin allergy test on rats were performed to evaluate the safety of SBC extract *in vivo*. Intercellular melanin production was analyzed to assess the function of SBC extract for whitening. Afterwards, we analyzed the activity of intracellular tyrosinase, Ultraviolet (UV) absorption, and anti-oxidant activity, which were related to melanin production.
Results: The aqueous extract and ethanol extract of SBC were prepared separately. MTT assay results showed aqueous extract of SBC at a concentration of 100 μg/mL had no inhibitory effect on the proliferation of B16F10 cells even after treatment for 96 h, indicating that aqueous extract of SBC was not cytotoxic. Thus, we conducted subsequent experiments using aqueous extract of SBC. The results on rats showed SBC extract was safe to apply on the skin without causing irritation and allergies. Analysis of melanin content showed SBC extract had inhibitory effect on cellular melanin generation and secretion. Moreover, we found that SBC extract was able to reduce cellular tyrosinase activity, as well as absorb UV light and scavenge DPPH radicals for anti-oxidants.
Conclusion: Our data clearly demonstrated that SBC extract is safe and effective for skincare, and it is suitable for use with the purpose of skin whitening and health benefits.

1. Introduction

Skin is the largest organ in the human body, weighing 3.6 kg and covering an area of 2 m^2. It is the first defense line of the body, protecting us against extreme temperatures, and external physical and biological invasions [1]. In recent years, people have been paying more attention to the health of their skin due to improving living conditions. In Asian countries, fair skin has always been associated with youth, beauty, and health, especially for young women [2]. There is a saying in China that one white hides a hundred ugly things. Thus, a variety of cosmetic skin whitening products are developed to satisfy the desire for fair skin [3].

Skin contains melanin, which is a natural dark pigment produced by melanocytes [4]. Normally, melanin protects the skin from Ultraviolet (UV) damage, but excessive production of melanin leads to hyperpigmentation, which can contribute to a variety of aesthetic problems, including melisma, phelides, and dark spots on the skin [5]. Melanogenesis is the process of melanin synthesis, which is produced in the melanosomes of melanocytes [6]. During melanogenesis, tyrosinase, the enzyme mainly responsible for melanin production, catalyzes the hydroxylation of L-tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine) and the oxidation of L-DOPA to DOPA quinone [7]. Due to this, inhibition of tyrosinase has been regarded as a long-term method of preventing hyperpigmentation [8,9]. In response, cosmetic companies and researchers are engaged in developing new whitening agents that inhibit tyrosinase activity [10].

Recently, scientists have been exploring natural sources like herbs for tyrosinase inhibitors [8,11]. However, it has proven difficult to find compounds that are highly effective, safe, and have low side effects. In China, traditional herbs have been safely used to treat diseases, including skin whitening and skincare, for many years [12,13]. Generally, a

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combination of several herbs is prescribed for use, based on the unique theory of traditional Chinese medicine (TCM), referred to as TCM formula [14]. A formula containing several herbs has more efficacy and low toxicity than a single herb or compound, which is currently attracting the attention of the cosmetic and medical industries [15].

In here, we report on a TCM formula called Si Ben Cao (SBC), made up of four Chinese traditional herbs. The aqueous extract of SBC is effective in inhibiting tyrosinase activity, decreasing melanin generation, and absorption of UV rays, which is much better than herbs alone. Furthermore, it has no irritation and allergy to skin.

2. Materials and methods

2.1. Preparation of SBC extract

All four herbs of SBC were purchased from the PuraPharm Corporation. In order to apply for a patent for the combination of four herbs (SBC formula), we choose not to disclose the names of the herbs, which are represented by A, B, C and D. The four herbs were authenticated by Dr. Sibao Chen based on morphological features. In order to prepare aqueous extracts, all the herbs were soaked in distilled water for 1 h, separately. Then the extract was collected using the reflux method for 2 h according to the method in the publication [16]. After being lyophilized into powder, we got the aqueous extracts of A, B, C, D and SBC respectively. For preparing ethanol extracts, ethanol replaced water and same protocol was performed.

2.2. Cell culture

B16F10 cells were purchased from American Type Culture Collection (Manassas, USA). Cells were cultured in DMEM supplemented with 10% FBS in a humidified atmosphere containing 5% CO_2 and 95% air at 37 ˚C. The medium was changed every three days, and cells were passaged using 0.05% trypsin/EDTA.

2.3. MTT assay

The effect of SBC extract on proliferation and viabilities of B16F10 cells was determinated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method according to the report [17]. In generally, 5×10^3 cells were seed in each well of a 96-well plate. After 24 h, cells were exposed to extracts at the concentration of 100 μg/mL for 24, 48, 72 and 96 h. Kojic acid (KA) was used as a positive control and it was added into cells at the concentration of 100 μM [18]. The optical density (O.D.) values for untreated group were set as 100% viability.

2.4. Measurement of melanin production

B16F10 cells were seeded at 1.5×10^4 cells per well in a 12-well plate and stimulated with α-MSH (100 nM) [19], while SBC and four herbs extracts were added at the concentration of 100 μg/mL, separately. When the treatment time achieved at 96 h, melanin production was measured according to the report [20]. The culture mediums treated with different extracts were directly measured at 475 nm to determinate the content of extracellular melanin secretion. For intracellular melanin production, cells were washed with PBS and lysed with NaOH (1 M) containing 10% DMSO for 2 h at 80 ˚C. After that, cell lysates were centrifuged and supernatants were measured at 475 nm. The melanin content was represented as percentage of the vehicle.

2.5. Intracellular tyrosinase activity assay

Intracellular tyrosinase activity was measured according to a method in the publication [21]. Following the seeding of B16F10 cells at a density of 1.5×10^4 cells per well in a 12-well plate and stimulating with α-MSH (100 nM), SBC and four herbs extracts were added at the concentration of 100 μg/mL, separately. When the treatment time achieved at 96 h, cells were ruptured by freezing and thawing. Then cell lysates were collected by centrifugation. After protein concentrations of lysates were determinate by Pierce™ BCA Protein Assay (Thermo Scientific), the concentrations of all lysates were adjusted to be the same. Then, cell lysates were mixed with L-DOPA (0.1% in PBS), incubated at 37 ˚C for 30 min, and the absorbance was measured at 475 nm. The tyrosinase activity was represented as percentage of the vehicle.

2.6. Western blot analysis

B16F10 cells were seeded at 3×10^4 cells per well in a 6-well plate and were stimulated with α-MSH (100 nM). After 24 h, cells were exposed to extracts at the concentration of 100 μg/mL for 96 h. Then whole cell lysates were collected and suspended in lysis buffers according to the report [22]. Following centrifugation at 13,500 rpm for 15 min at 4 ˚C, total protein concentration was measured by Pierce™ BCA Protein Assay (Thermo Scientific), and 10 to 25 μg of protein was separated on 10% SDS-PAGE and transferred to PVDF membranes. After blocking (5% skim milk powder in TBST) for 1 h at room temperature, the membrane was then incubated with tyrosinase antibody (Beyotime, AF8283) overnight at 4 ˚C. The membrane was incubated with secondary antibody for 1 h at room temperature. All antibodies were diluted in TBST containing 5% dry milk. The immune-reactive proteins were detected by enhanced chemiluminescence (ECL) using X-ray film and ECL reagent.

2.7. DPPH radical scavenging activity

In accordance with the report [23], DPPH was used for assessing the free radical scavenging activity. The various concentration of herb extracts at 20 μL were added to 180 μL of 250 μM DPPH solution. The reaction was incubated at 25 ˚C for 30 min in the dark. Then, optical density (O.D.) values were measured at 517 nm on a microplate reader, and the O.D. for untreated group were set as 100% viability.

2.8. UV absorption assay

UV absorption assay was performed according to the publication [24]. SBC and four herbs extracts were dissolved in aqueous solutions, then UV absorption spectra was analyzed on a UV spectrophotometer at room temperature using a quartz cuvette as the holder.

2.9. Animal study

The animal welfare and all experimental protocols were approved by the Animal Ethic Committee of The Hong Kong Polytechnic University. A total of 35 Specific-Pathogen-Free (SPF) Sprague-Dawley (SD) rats (female 20, male 15, four-month old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The rats were randomly divided to 5 group (4 females and 3 males for each group). During the study, all rats were housed in the standard condition of 23 ± 2 ˚C, 40%–60% humidity and an alternating 12 h light/12 h dark cycle, and all rats were allowed free access to normal diet and distilled water.

2.10. Skin irritation test

The skin irritation test was performed as previously reported [25] and included intact skin group and scratched skin group (female 4 and male 3 for each group). Two sides on the backs of all rats were depilated (3 cm^2/each) using hair removal cream (Veet, French). A day later, both sides of the rats in scratched skin group were scratched using a sandpaper, causing the skin slightly ooze blood. Then the vehicle and SBC extract was applied daily on left side and right side in both groups, respectively, for 7 days. At the last application day, the substances were removed by water after 1 h treatment, then erythema and

edema scoring was performed at 1 h, 24 h, 48 h, 72 h and 7 days. A zero-four scale was used to determine the erythema and edema as follows: zero, no erythema/edema; one, very slight (barely perceptible); two, well defined; three, moderate to severe; and four, severe (beet redness erythema to mild eschar formation/edema raised more than 1 mm and extending beyond the exposure area). The Irritation Index (I.I.) was calculated according to the equation: I.I. = (erythema grades at all-time points + edema grades at all-time points)/ (number of rats).

2.11. Skin allergy test

The skin allergy test was performed as previously reported [26] and rats were randomly divided into 3 groups, including 4 females and 3 males for each group. As the irritation test, all rats were depilated 24 h before the test. Then, the right side of rats was applied dermally with vehicle, positive control (2,4-Dinitrochlorobenzene, 1% in acetone) and SBC extract, respectively, on days 1, 7 and 14. The substances were allowed to stay on the skin for 6 h, then they were removed by water. On day 28, the left side of the rats was treatment once with the same method. After the last treatment, the erythema and edema scoring was performed at 6 h, 24 h, 48 h and 72 h. The erythema was graded by a zero-four scale as follows: zero, no erythema; one, very slight; two, well defined; three, moderate to severe; and four, severe (edematous erythema), while the edema was graded to a zero-three scale as follows: zero, no edema; one, very slight (barely perceptible); two, well defined; three, severe (raised more than 1 mm with clear contours). The allergy Index (A.I.) was calculated as same as I.I. The allergy rate (A.R.) was calculated by the equation: A.R.= (number of allergy rats/ number of total rats) × 100%.

2.12. Statistical analysis

Each experiment was performed at least three times. GraphPad Prism 5.0 software was used for statistical analysis.

3. Results

3.1. Aqueous extract of SBC has no cytotoxicity

Firstly, we separately prepared ethanol and aqueous extracts for SBC formula and individual herbs. To determine the cytotoxicity of extracts, MTT assay was performed to analyze the viability of B16F10 melanoma cells under treatment of extracts. Kojic acid (KA), a skin-lightening agent, was used as a positive control at the concentration of 100 μM [27]. Briefly, cells were cultured in a 96-well plate and treated with different extracts at the concentration of 100 μg/mL for 24 h, 48 h, 72 h and 96 h. The results in Fig. 1 were showed as relative cell viability compared with control. We found that KA significantly inhibited the proliferation of B16F10 cells at the first time point (24 h), and its inhibitory activity was enhanced with time extended. As well as KA, the ethanol extract of A, B, C, D and SBC also showed inhibitory effects. Obviously, the inhibitory effect of SBC ethanol extract was stronger than the ethanol extracts of the four herbs individually. Intriguingly, except for aqueous extract of D showing inhibitory activity in 72 h and 96 h, the aqueous extracts of A, B, C and SBC had no inhibitory effect. These results indicated that aqueous extract of SBC was non-cytotoxic. We therefore conducted subsequent experiments using aqueous extract of SBC, and all extracts mentioned later were aqueous extracts.

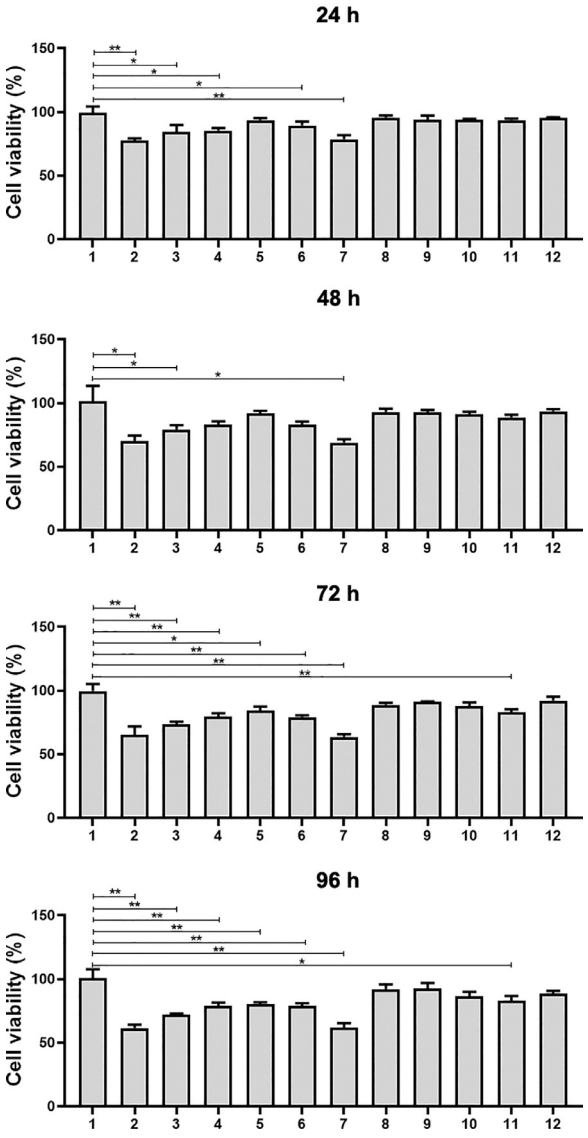

Fig. 1. Cell viabilities were measured by MTT assay at four time points 1. Control; 2. KA; 3-6. ethanol extracts of individual four herbs (A, B, C and D); 7. Ethanol extract of SBC; 8-11. aqueous extracts of individual four herbs (A, B, C and D); 12. aqueous extract of SBC; * $P < 0.05$, ** $P < 0.01$, compared with control group.

3.2. SBC extract has no irritation and allergy to skin

The results of irritation index and allergy index of SBC extract on rats were shown in Table 1 and Table 2, respectively, while the images of them were exhibited in Fig. 2. As shown in Table 1, there were slight ir-

Table 1
Values of the irritation index determined in SD rats (female 4 and male 3/each group).

Group	Treatment	n	average score					Irritation index
			1h	24h	48h	72h	7d	
Intact skin	Vehicle	7	0.43	0.36	0	0	0	0.79
	SBC extract	7	0.14	0.07	0	0	0	0.21
Scratched	Vehicle	7	0.14	0	0	0	0	0.14
skin	SBC extract	7	0.14	0	0	0	0	0.14

Table 2
Values of the allergy index determined in SD rats (female 4 and male 3/each group).

Group	n	Number of allergy rats	6h	24h	48h	72h	Allergy index	Allergy rate (%)
Vehicle	7	0	0	0	0	0	0	0
Positive control	7	7	2	1	0.57	0.29	3.86	100
SBC extract	7	0	0	0	0	0	0	0

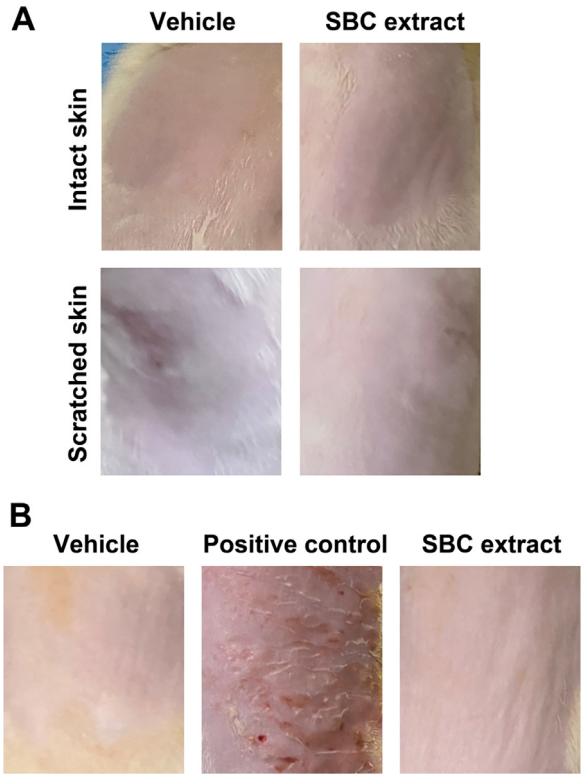

Fig. 2. Images of skin irritation and allergy on SD rats A: image of skin irritation. The vehicle and SBC extract was applied daily on 4-month old SD rats (female 4 and male 3 for each group) after scratching or not for 7days; B: image of skin allergy. The SD rats (4-month old, female 4 and male 3 for each group) was applied dermally with vehicle, positive control (2,4-Dinitrochlorobenzene, 1% in acetone) and SBC extract, respectively, on days 1, 7 and 14.

ritations, no matter in intact skin and scratched skin group treated with or without SBC at 1 h, while the irritation disappeared over time, which suggesting that the irritations might induced by hair removal cream. In the right side, SBC treatment did not cause any irritation in scratched skin rats compared with intact skin rats. The allergy test showed that the positive control of 2,4-Dinitrochlorobenzene showed an obvious moderate allergenicity compared with vehicle group, while SBC extract did not induce any allergy at all-time points (Table 2). The above results demonstrated that the SBC extract was neither irritating or allergenic to skin, suggesting SBC extract was safe enough for application on the skin.

3.3. SBC extract decreases melanin content

Next, we examined melanin formation regulated by SBC extract in B16F10 cells and culture mediums. α-MSH (100 nM) was added to the plates after cells were seeded, followed by the separate addition of extracts from SBC and four individual herbs at the concentration of 100 μg/mL. Melanin content was measured after 96 h of treatment, and it was represented as a percentage of control. Results in Fig. 3A and 3B showed melanin content in cells and in culture medium increased sharply following α-MSH stimulation. As a positive control, KA inhibited the increase of melanin in cells and in culture medium. Compared with KA, SBC extract resulted in more drop of melanin content, both in cells and in culture medium. The results indicated that SBC extract has inhibitory effect on melanin generation and secretion. Interestingly, SBC extract had a greater inhibitory effect than the four extracts taken alone, although they both decreased melanin content. As seen visually in Fig. 3C, cells became extremely black under α-MSH stimulation, while SBC extract-treated cells appear the whitest, except for control cells, which did not receive α-MSH stimulation.

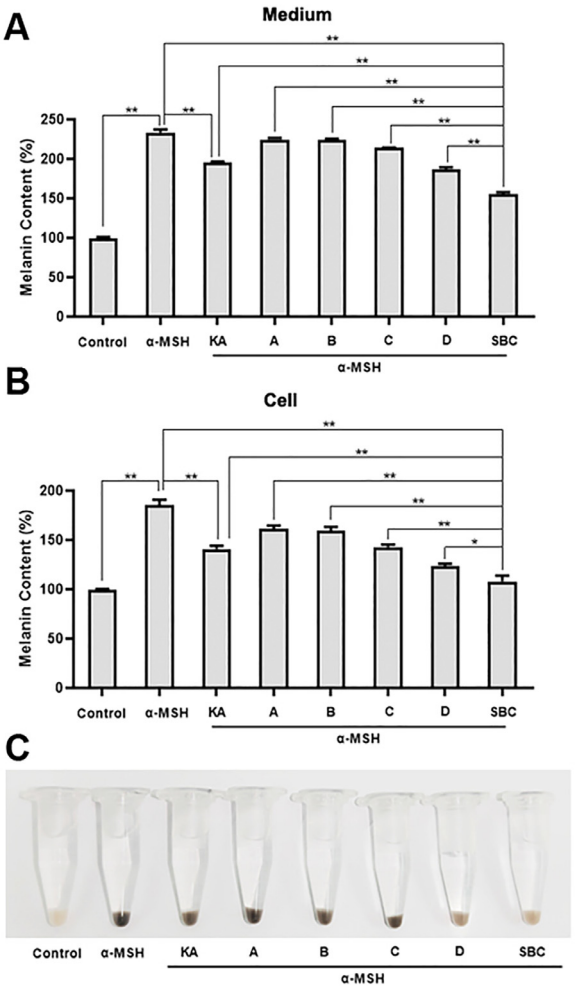

Fig. 3. Inhibition of melanin synthesis by SBC extract A. Melanin content in culture medium was measured after treatment for 96 h, ** $P < 0.01$; B. Melanin content in cells was measured after after treatment for 96 h, ** $P < 0.01$; C. Photo of cell pellets after treatment for 96 h.

3.4. SBC extract inhibits tyrosinase activity

SBC extract was assessed for its inhibitory effect on tyrosinase activity in B16F10 melanoma cells. In accordance with the protocol in analysis of melanin content, cells were stimulated with α-MSH, fol-

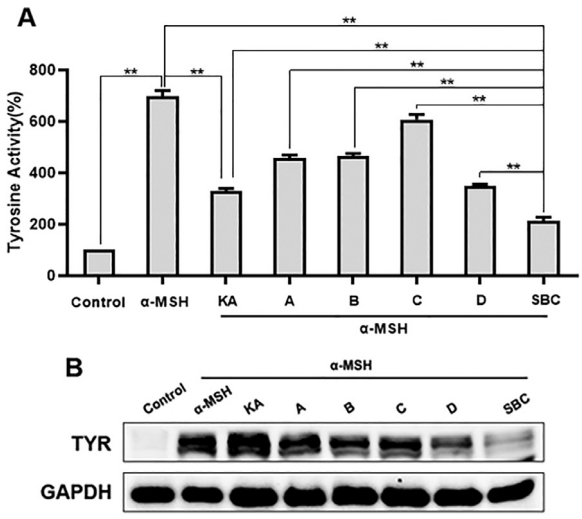

Fig. 4. Inhibition of tyrosinase activity by SBC extract A. Intracellular tyrosinase activity was measured after treatment for 96 h, ** $P < 0.01$; B. The expression of tyrosinase was analyzed by western blot after treatment for 96 h.

lowed by SBC extract and extracts of four herbs at the concentration of 100 μg/mL. KA was also used as a positive control. The activity of cellular tyrosinase was measured after 96 h of treatment, and it was represented as a percentage of the control group [28]. Results in Fig. 4A showed that α-MSH greatly increased intracellular tyrosinase activity to 698.64 ± 21.43%. As a positive control, KA decreased it to 329.25 ± 9.64%. There was a degree of inhibitory activity observed in extracts from four herbs. Interestingly, SBC, as a formula that combined these four herbs, showed stronger effect than KA and four extracts alone, which was 213.60 ± 13.12%. Additionally, western blot assay was used to analyze the expression of tyrosinase protein. Results in Fig. 4B showed that α-MSH stimulation significantly increased the expression of this protein, but KA could not affect this increase. As expected, SBC extract also inhibited the expression of this protein, confirming that SBC extract inhibited tyrosinase activity.

3.5. SBC extract shows Ultraviolet absorption

Ultraviolet (UV) ray is common in life, it can activate melanin synthesis in skin [29]. Therefore, a reagent with skin whitening function is best to absorb UV. The absorption of UV radiation at wavelengths of 200–400 nm was analyzed [30], and we found SBC extract had an obvious ability to absorb UV ray, especially between 200 and 290 nm. With the concentration of SBC extract increased from 62.5 μg/mL to 500 μg/mL, its intensity of UV absorption was also significantly increased (Fig. 5A), confirming that SBC extract had the activity of UV absorption. Subsequently, UV absorption of SBC extract and the four herbs extracts alone were compared as shown in Fig. 5B. There was moderate UV absorption by C and D, but relatively poor UV absorption by A and B. Interestingly, with these four herbs combined into SBC formula, the overall intensity was greatly enhanced. In particular, although only C produced a peak in UV absorption at 250-290 nm, the intensity of this peak regulated by the SBC remained much stronger than that regulated by C alone.

3.6. SBC extract shows anti-oxidant activity

UV ray can cause free radicals generation in human skin [31]. As a result of protectable mechanism, melanin production is strongly correlated with free radicals [32]. α, α-diphenyl-β-picrylhydrazyl (DPPH), a free radical, is usually be used for the colorimetric evaluation of anti-oxidant radical scavenging activity [33]. In this study, we measured the anti-oxidant activity of the SBC extract using the DPPH assay. According to Fig. 6A, when the concentration of SBC extract was 50 μg/mL, the free radical scavenging rate was up to 49.65 ± 0.57%. Following the concentration increased, the free radical scavenging rate was also increased, confirming that SBC extract had anti-oxidant activity with a dose-dependent manner. In addition, anti-oxidant activities of SBC and the four herbs at the same concentration of 100 μg/mL were compared in Fig. 6B. Consistently, SBC extract also showed much more effective at scavenging DPPH radicals than the four herbs alone.

4. Discussion

In modern society, cosmetics are used to change people's appearance to enhance their beauty and attractiveness, which has been considered an indispensable commodity. Skin whitening products are a major category of cosmetics in the world, especially in eastern Asian countries. In recent years, skin whitening products containing natural ingredients from herbs have gained increasing attention [34,35]. Since TCM formulas have long been safely used to improve complexions, developing skin whitening products based on them is more feasible. Therefore, we evaluated the potential of our SBC formula as a skin whitening agent in light of scientific research.

Because whitening agents are used on the skin, it is the most important that agents need to be safe, non-toxic, and non-irritating to the skin [36]. In our study, to evaluate the safety of SBC extract for skin application, MTT assay was performed that examines the inhibitory effect

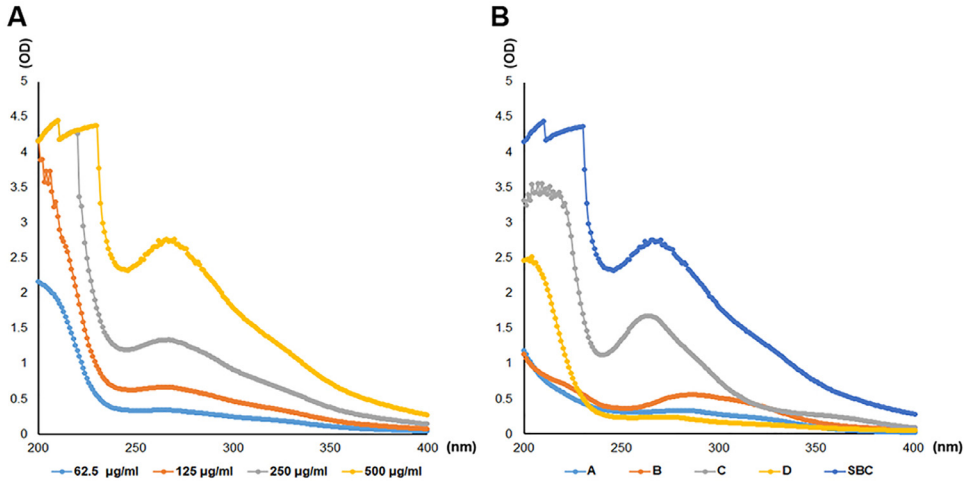

Fig. 5. SBC extract absorbs UV ray A. the UV absorb curve of SBC extract with concentrations from 62.5 μg/mL to 500 μg/mL; B. the UV absorb curve between SBC extract and extracts of individual four herbs with concentration of 500 μg/mL.

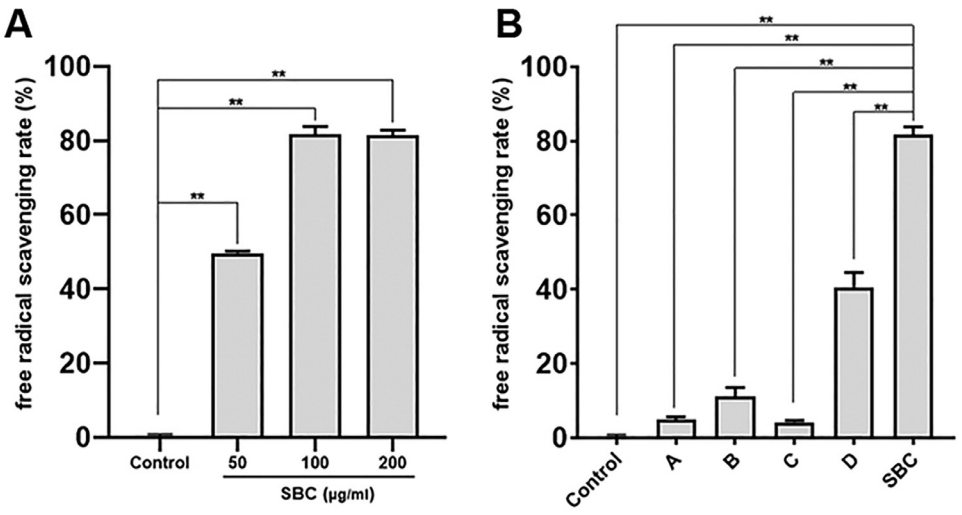

Fig. 6. The OD value was measured in DPPH radical scavenging assay A. The OD value regulated by SBC with the concentrations from 50 μg/mL to 200 μg/mL, ** $P < 0.01$; B. The OD value regulated by SBC extract and extracts of individual four herbs at the concentration of 100 μg/mL, ** $P < 0.01$.

of on B16F10 cells proliferation. We found KA, a skin whitening ingredient that typically has a limited concentration, and ethanol extract of SBC to have moderate inhibitory activity on the proliferation of B16F10 cells. In contrast, aqueous extract of SBC had no effect on B16F10 cells proliferation, even after 96 h of incubation, confirming that aqueous extract of SBC is non-cytotoxic. Thus, instead of ethanol extract of SBC, aqueous extract of SBC was used in our subsequent experiments. It is interesting that aqueous extraction is accordance with TCM practices for using herbs. Furthermore, skin irritation and skin allergy were analyzed on rats. The results of non-irritating and non-allergic on rats confirmed that of non-cytotoxicity, which all suggested that SBC extract is safe for skin application.

Melanin is an important component of skin, and its quantity determines the color of skin [6]. In this study, we examined the content of intercellular and extracellular melanin regulated by SBC extract. Compared with KA, SBC extract showed significantly greater efficacy in reducing both intracellular and extracellular melanin levels. Melanin is synthesized by tyrosinase, an oxidoreductase widely present in skin. For the development of skin whitening products, tyrosinase inhibition is regarded as one of the most effective strategies [37]. According to our study, SBC extract significantly reduced the activity of cellular tyrosinase compared to KA. It is known that KA is a tyrosinase inhibitor, but it does not decrease tyrosinase expression in several B16 cell lines [38]. In contrast to KA, SBC extract showed a strong inhibitory effect on tyrosinase expression, suggesting SBC extract may have different mechanisms for inhibiting tyrosinase activity.

Additionally, melanin synthesis in the skin is also stimulated by external factors such as ultraviolet (UV) rays [39] and free radicals [40]. The UV rays can generate free radicals, and free radicals are involved in the reaction of tyrosinase, which leads to melanin production [41]. More importantly, UV rays can cause more damage to people's skin if exposed for a long period of time. It is therefore better for cosmetic products that have whitening properties to also have UV filter and anti-oxidant functions. According to our study, SBC extract can absorb UV rays with a dose-dependent manner. Further, SBC extract demonstrated a high anti-oxidant capacity via analysis of DPPH radical scavenging activity.

More interestingly, compared with four herbs extracts, SBC extract not only had more inhibitory effect on melanin production and tyrosine activity, but also absorbed more UV rays and scavenged more free radicals. Generally, the aqueous extract of a TCM formula contains a series of active ingredients derived from individual herbs, including polysaccharides, flavonoids, and alkaloids, some of which have been reported to inhibit the activity of tyrosinase, filter UV rays, or reduce oxidative stress [42–44]. Studies in recent years have also shown that combination therapies, like TCM formulas, have better effectiveness and fewer side effects than single drugs [45]. Our study revealed that SBC formula has several advantages over single herbs in preventing hyperpigmentation, and it is not toxic after combining, which is a TCM advantage.

5. Limitations

This study has some limitations. Due to medical ethics and our limited financial sources, we were unable to test the whitening effect of SBC extract directly on the human body. Additionally, functions of SBC extract may be different from its cosmetic products, because cosmetic products usually contain a variety of substrates and added functional ingredients. Further work should address these issues.

6. Conclusions

In summary, our findings indicate that aqueous extract of SBC, a TCM formula, is safe to apply on the skin with no irritation and allergy. And it not only inhibits tyrosinase and melanin production, but also scavenges free radicals and absorbs UV rays. It is suggested that a cosmetic product containing SBC extract could be developed for skin whitening and health benefits.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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