

In Search of an Innovative Agent for Skin Care - Putting an Ancient Herbal Cosmetic Formula on Modern Bioactivity Testing Platforms

Keywords: Qi bai san; Hypopigmentation; Melanogenesis; Topical agent; Whitening; Kojic acid

Abstract

Background: Qi Bai San (QBS) is a traditional Chinese herbal formula used by ancient ladies for healthy skin and whitening. Nevertheless, it contains undesirable animal and toxic herbs, without scientific evidence demonstrating its efficacy.

Objective: This study aims to compare and identify QBS formula with the best efficacy from three different versions of QBS formulations, F1, F2, and F3.

Methods: Cellular melanogenesis and tyrosinase activity assays were used to assess melanin content and tyrosinase activity on α -melanocyte-stimulating hormone (MSH)-induced B16 cells. Collagenase inhibition assay was used to compare the collagenase inhibitory activity. Effects of QBS on melanin production was determined using UV-irradiated Balb/c mice. Transdermal experiment was used to confirm whether QBS could penetrate into the skin. *In vitro* skin toxicity test study was performed to determine whether QBS would cause toxicity to skin cells.

1.4. Results: F1, F2 and F3 dose-dependently reduced α -MSH-induced increase in melanin content and tyrosinase activity, and inhibited collagenase activity. F3 is the simplest formula among all formulations (without animal or toxic herbs), yet demonstrating similar efficacy. Animal study suggested F3 could reduce melanocytes and melanin content in UV-irradiated mice. Further penetration and skin toxicity studies suggested markers from different herbs within F3 could penetrate through the epidermis to exhibit its effects, without causing toxicity to skin cells.

1.5. Conclusion: We showed for the first time that a modified QBS formula exert hypopigmentation and collagenase inhibitory effects, providing *in vitro* and *in vivo* scientific evidence supporting its efficacy on hypopigmentation and healthy skin promotion.

Introduction

Skin, being the most visible organ of the body, serves as the first and outer most organs to provide barrier against foreign pathogen [1]. It also serves as a shield against the harmful effects of ultraviolet radiation emitted by the sun [2]. As we age, we will experience a decrease in the biological activity, regenerative abilities, and adaptation of the skin cells. Skin aging can also be affected by internal factors such as genetics, hormones, nutritional factors, vitamin deficiencies, as well as external factors such as ultraviolet radiation, environmental toxins, smoking, improper care, etc. [3]. These external factors, in particularly ultraviolet radiation, aggressive environments and tobacco smoking can progressively cause damage to the skin's cellular and extracellular structures, thereby resulting

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in wrinkles, sagginess, pigmentation or even neoplastic changes [4]. While these undesirable effects could be minimised with avoidance of strong sunlight and harmful exposures, topical applications of various types of agents have been very common practices

The usage of topical agents, also known as cosmetics for the management of skin conditions and improvement of its outlook is a common practice among different cultures. The word “cosmetics” arises from the Greek “kosmeticos” meaning “adorn”. Since then, any material used for beautification or improvement of appearance is known as cosmetics [5]. This need for adorning the appearance and conditions of the face has been an urge in the human race of different regions and cultures since ancient times, leading to the invention of different remedies which were handed down for generations with unabated enthusiasm.

Herbal medicines have been used as skin-whitening agents since ancient times [6,7].

“Qi Bai San (QBS)” is one of such ancient herbal cosmetic classic especially used by Royal Family members and dignitaries as topical agent [7]. According to Yong Lei Qian Fang written by Zhong Nan Li, a famous Chinese medicine practitioner during the Yuen Dynasty, QBS was traditionally used for the treatment of skin pigmentations and consisted of seven different Chinese herbs [8]. This formula may vary slightly but contain herbs of which all in Chinese language carry the word “White”, signifying its whitening effects. These herbs include *Ampelopsis Radix* (dried root tuber of *Ampelopsis japonica* (Thunb.) Makino), *Atractylodis Macrocephalae Rhizoma* (dried rhizome of *Atractylodes macrocephala* Koidz.),

Paeoniae Alba Radix (dried processed root of *Paeonia lactiflora* Pall.), Bombyx Batryticatus dried body of *Bombyx mori* Linnaeus, or the forth to fifth infected instar larvae (or by artificial inoculation) by *Beauveria bassiana* (Bals.) to death, Angelicae Dahuricae Radix (dried root of *Angelica dahurica* (Fisch. ex Hoffm.) Benth. Et Hook.), Typhonii Rhizoma, Poria (dried sclerotium of *Poria cocos* (Schw.) Wolf), and Tribuli Fructus (dried ripe fruit of *Tribulus terrestris* L.) [8]. Recent scientific studies also supported the notion that the different herbs within QBS are potent on reducing the skin colour of healthy volunteers receiving recreational exposure to sunlight [9]. Laboratory experiments demonstrated that these herbs from QBS could significantly inhibit tyrosinase activity, thereby contributing to their effects in the control of pigmentations [7]. With the several versions of the formula carrying slightly different components, and keeping an open mind on the possible compositions, we chose three formulae of slightly different combinations to be compared in our *in vitro* platform studies to identify the formula with the best cosmetic effects. The most efficacious formula was chosen and tested in our *ex vivo* and *in vivo* platforms to compare its effect with kojic acid alone. The three formulae were: Formula 1, consisting of Poria; Atractylodis Macrocephalae Rhizoma; Angelicae Dahuricae Radix; Paeoniae Alba Radix; Ampelopsis Radix; Bombyx Batryticatus; and Tribuli Fructus; Formula 2, consisting of the same herbs except Bletillae Rhizoma was used to replace Angelicae Dahuricae Radix; and Formula 3, consisting of only 5 herbs of Formula 1 when Tribuli Fructus and Bombyx Batryticatus were removed.

Overall planning of investigation

To explore the cosmetic effects of the ancient formula which claimed whitening and smoothening effects, it is envisaged that the following procedures be taken:

- i. Proper authentication of the selected herbs;
- ii. Three formulae of slightly different herbal combination will be prepared to be tested in the same platforms to achieve comparative effects;
- iii. Whitening effects will be studied using melanocyte *in vitro* cultures together with collagenase enzyme inhibition tests and tyrosinase activity assay;
- iv. Skin penetration is essential for the topical application of any topical agent; hence *ex vivo* tests using porcine skin and diffusion cell system are to be performed;
- v. Finally, *in vivo* experiments using artificially induced skin melanin pigmentations in C57Bl/6 mice and topical applications of the formulae will be performed to compare the cosmetic effects;
- vi. The final outcomes would reveal a favourable formula and evidences of its cosmetic effects.

Materials and Methods

Herbal materials authentication and preparation

Herbal material authentication Raw herbal material of Atractylodis Macrocephalae Rhizoma, Poria, Angelicae Dahuricae Radix, Paeoniae Alba Radix, Ampelopsis Radix, Bombyx Batryticatus, Tribuli Fructus, and Bletillae Rhizoma were purchased from a renowned supplier in Hong Kong. All herbs were chemically authenticated

using Thin Layer Chromatography (TLC) in accordance to Chinese Pharmacopoeia (CP) [10]. Upon chemical authentication, herbarium voucher specimens of Atractylodis Macrocephalae Rhizoma (2015-3453), Poria (2015-3454), Angelicae Dahuricae Radix (2015-3457), Paeoniae.

Alba Radix (2015-3455), Ampelopsis Radix (2015-3456), Bombyx Batryticatus (2015-3459), Tribuli Fructus (2015-3458) and Bletillae Rhizoma (2015-3460) were deposited at the museum of the Institute of Chinese Medicine at the Chinese University of Hong Kong (CUHK).

Herbal extract preparation

Extractions of herbs were performed following the traditional practice of herbal extraction. For each formula, all raw herbs were mixed in the same ratio and extracted twice by boiling under reflux at 100 °C using 10x distilled water. The aqueous extracts were then combined and filtered using cotton wool and concentrated under reduced pressure at 60 °C. The concentrated extracts were freeze-dried and the yield were recorded. All the extracts were stored in desiccators at room temperature before use.

In vitro cell culture experiments

Cell culture: The B16 melanoma cell line was obtained from the American Type Culture Collection CRL-6322 (USA). Cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Gibco, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin in a 10% CO₂ humidified atmosphere at 37 °C. Cells grown to 80% confluence in T75 culture flasks were trypsinized and seeded into 12-, 24- or 96-well culture plates for experiments.

Cell viability assay: Briefly, using 96-well plate, B16 cells were seeded at 4 x 10³ cells/well. Cells were then treated with various concentrations of aqueous herbal extracts in distilled water (0 - 1 mg/ml), with or without 10 nM α-Melanocyte-Stimulating Hormone (MSH)-induction for 72 hrs. The relative amount of viable cells were determined by measuring the reduction of MTT dye in live cells to blue formazan crystals at optical density at 540 nm (Sigma-Aldrich, USA).

Cellular melanogenesis assay: The amount of melanin present in α-Melanocyte-Stimulating Hormone (MSH)-induced melanoma was used as an index for melanogenesis in the present study. B16 cells were seeded onto 24-well plate at the density of 5 x 10⁴ cells/well. Cells were incubated with 10 nM α-MSH for 72 hrs with or without different concentrations of aqueous herbal extracts in distilled water (0 - 1 mg/ml). Cells were washed with Phosphate Buffered Saline (PBS), followed by trypsinization. Cells were solubilized in 200 µl of 1 N NaOH containing 10% Dimethyl Sulfoxide (DMSO) at 80 °C for 1 hr. The absorbance of all samples were measured at 490 nm [11,12]. 2.5 nM kojic acid was used as the positive control.

Cellular tyrosinase activity assay: For cellular tyrosinase activity measurement, cellular tyrosinase activity was assayed in terms of DOPA oxidase activity. B16 cells were seeded onto 6-well plate at the density of 1.2 x 10⁴ cells/well. Cells were incubated with 10 nM α-MSH for 72 hrs with or without different concentrations of aqueous herbal extracts prepared using distilled water (0 - 1 mg/

ml). Cells were sonicated with phosphate buffer (pH 6.8) containing 1 mM Phenylmethanesulfonyl Fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA.), followed by centrifugation at 10,000 x g. Cell lysate were mixed with 5 mM L-DOPA (Sigma-Aldrich, St. Louis, MO, USA.), followed by incubation at 37 °C for 1 hr, and absorbance was measured spectrophotometrically at 475 nm [13]. 2.5 nM kojic acid was used as the positive control.

Collagenase activity assay: Collagenase inhibition was determined using the commercially available Collagenase Activity Assay Kit (Colorimetric) (Abcam, UK). Test samples were added to the reaction mixture, followed by addition of 0.35 unit/ml collagenase. Enzymatic activity was assessed by measuring the reduction at A345 spectrophotometrically for 20 mins. In our assay, 50 µM EGCG was used as positive control as previously described [14].

UV-irradiation animal study: Animals and diet: All experiments were carried out in accordance with the guidelines approved by the Animal Research Ethics Committee at the Chinese University of Hong Kong (CUHK) (AEEC approval no.: 16/174/MIS). Female C57Bl/6 mice (8-week old) were supplied by the Laboratory Animal Services Centre, the Chinese University of Hong Kong. All mice were housed in normal standard cages (5 animals per cage) at a constant temperature of 21 °C with a 12-h light-dark cycle. Each standard cage contained aspen as the bedding material. All animals were allowed ad libitum access to diet and water. The UVA and UVB spectra used in this study were produced with a 8W UVB lamp, G8T5E (Sankyo Denki, Japan) and a 15 W UVA lamp, ST-60 (BOYU Aquarium, China), and monitored with a UV light meter UV-340A (Lutron Electronic, Taiwan). To induce pigmentation, female C57Bl/6 mice were subjected to UV irradiation. Briefly, mice were shaved on their dorsal trunk 24 h prior to irradiation with 600 µW/cm² of UVA for 25 minutes each day for 5 consecutive days, then rest for 2 days, followed by irradiation at 600 µW/cm² of UVA for 20 minutes, followed by 1200 µW/cm² (50% UV-A, 50% UV-B) for 5 minutes for another 5 consecutive days. Control mice were shaved and restrained only. To mimic the clinical situation whereby the topical application is given as a cream emulsion, herbal extracts (10%) or kojic acid (1%) were mixed into aqueous cream and applied to the UV-irradiated mice to serve as test group and positive control group, respectively. Aqueous cream alone was applied to the UV-irradiated mice to serve as negative control group. All topical applications were applied to the mice daily for 1 week. After which, all animals were euthanized by cervical dislocation, and ear skin was isolated for further analysis.

Melanocyte counting: The melanocyte count in skin tissues was determined microscopically according to the method of Hiramoto et al. [15]. The isolated skin tissues were soaked in 2 N NaBr solution at 37 °C for 2 hrs. Melanocytes were stained by immersing in 0.1 M phosphate-buffered saline (pH 7.2) containing 0.14% L-DOPA (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 3 hrs and the amount of melanocytes were quantified microscopically [16].

Histological analysis of melanin staining: To identify argentaffin granules and melanin staining within the skin histologically, skins were fixed in 4% paraformaldehyde overnight at room temperature and stained for melanin using a Fontana-Masson staining kit (American Mastertech, Inc. Lodi, CA, USA) according to the manufacturer's instructions. Briefly, sliced skins were stained with ammoniacal silver

solution for 60 min at 60 °C, followed by incubation in 0.1% gold chloride and then in 5% sodium thiosulfate. The tissues were stained with hematoxylin and eosin for morphological analyses.

Localization of tyrosine by immunohistochemistry: Biopsied skin tissues were embedded in paraffin and cut into 3-mm-thick sections. To study the cutaneous expression of pigment markers, the tissue sections were stained and analyzed using a light microscope. The slides were either stained immunohistochemically stained with anti-tyrosine antibody (Abcam, UK) for 2 hrs at room temperature. Specific labelling was detected with a biotin-conjugated anti-rabbit, anti-goat or anti-mouse IgG and avidin-biotin-peroxidase complex (Vector Labs, USA). Immunocytochemistry photographs were assessed by densitometry [17].

Ex vivo diffusion cell experiment

Preparation of porcine ear skin membrane: Porcine ear skin has been widely used for *in vitro* transdermal experiments and has been shown to be the best alternative model for human skin [18]. To prepare the porcine ear skin for transdermal experiment, briefly, fresh porcine ear were purchased from a local butcher. Full thickness of the skin membrane were cut and liberated from the underlying cartilage using a scalpel, with any adhering subcutaneous fat and tissues carefully removed. The skin was then cut into 1.77 cm² sections and stored at -80 °C prior to use [19].

Diffusion cell experiment

The diffusion experiments were carried out using the diffusion cell system as previously described [19]. The diffusion cell system (Taiping Business Mansion, Nanjing, China) comprised of six diffusion cells, diffusion cell drive, and circulating water bath for diffusion cells temperature control. Each diffusion cell consisted of a donor and receiving chamber, with a magnetic stirrer at the bottom to ensure thorough mixing of the solution at the receiving chamber. Phosphate buffered saline (Invitrogen, CA, USA) was used as the diffusion medium at the receiving chamber, with constant stirring at 600 rpm. Temperature was maintained at 37 ± 0.5 °C to ensure all porcine ear skin membranes' temperature were kept at approximately 32 °C throughout the experiment. Prior to use, all porcine ear skin membranes were soaked in pre-warmed PBS for 5 mins to allow hydration of the membranes before the experiment. All porcine ear skin membranes were carefully placed on top of each of the diffusion cells, with dermal side contacting the receiving chambers to avoid the presence of air bubbles between the membranes and buffer solution. Donor chambers were placed on top of the membranes. Samples or PBS (control) were applied to the donor chambers on top of the porcine ear skin membranes. All diffusion cells were clipped tightly using cling wrap and horseshoe clamps to avoid evaporation. Receiver medium was withdrawn after 24 hrs and the sample solution collected was analyzed using the Liquid Chromatography Mass Spectrometry (LCMS). Markers within the skin were extracted by sonication in methanol for 1 hr and subjected to LCMS analysis

Liquid chromatography mass spectrometry (LCMS) analysis: LCMS analysis was performed using the Agilent Liquid chromatography-mass spectrometry LCMS System (Agilent, CA, USA). Sample solution was injected onto a Waters ACQUITY UPLC BEH C18 column (100 x 2.1 mm i.d., particle size 1.7 µm) with Agilent

Waters ACQUITY UPLC BEH C18 1.7 μm guard column (5 x 2.1 mm i.d., particle size 1.7 μm). All solvents were pre-filtered with 0.45 μm Millipore filter disk (Millipore) and de-gassed. A gradient elution was carried out using the following solvent systems: mobile phase A - double distilled water - formic acid (99.9 : 0.1; v/v); mobile phase B - acetonitrile - formic acid (99.9 : 0.1; v/v). A detailed description of the gradient elution system used is shown in Table 1. The flow rate used was 0.5 ml/min. Each sample (5 μl) was injected into the column after filtration through a 0.2 μm filter disk. Identification of the chemical markers was carried out by comparing the retention times of the unknown peaks to those of the standards Table 2. The system was monitored by a computer equipped with the Agilent MassHunter Workstation Software for data collection, integration and analysis.

In vitro skin toxicity test

Epiderm culture: A commercially available human epidermal equivalent, EpiDerm (EPI-200, MatTek Corporation, Ashland, MA, USA), was used as an *in vitro* model of the epidermis in this study since the general morphology of this model system mimicked that of normal human epidermis. These EpiDerm cultures comprised of human-derived epidermal keratinocytes, which were cultured on standing cell culture inserts (Millipore, Billerica, MA, USA) at the air-liquid interface to form a multilayered, differentiated model of the human epidermis. Upon kit arrival, the EpiDerm cultures were placed in 6-well plates, and pre-conditioned overnight at 37 °C and 5% CO₂. Samples and positive control (5% SDS) were added to the skin inserts the next day and allowed to incubate for 1 hr. After which, all skin inserts were transferred to fresh medium for 24 hrs. All skin inserts were then transferred to fresh plates for MTT assay.

MTT assay - cell viability test: Skin inserts were transferred to fresh plates with pre-filled MTT solution, and allowed to incubate for 3 hrs at 37 °C and 5% CO₂. Upon completion of incubation, all MTT

solution was removed. Skin inserts were transferred to fresh plates and isopropanol was added to each inserts for 2 hrs for formazan extraction, before transferring to 96-well plate for spectrophotometric analysis at 550 nm. Cytotoxicity was expressed as the ratio of the cell viability, per treatment, to the maximum cell viability from the negative control (non-treated skin insert).

Statistical analysis: Data were presented as means \pm SD for all *in vitro* experiments, and means \pm SEM for all *in vivo* experiments. Prism 5 for Window (version 5.0c, GraphPad Software, Inc., USA) was used for statistical analysis. Significant differences among all groups were assessed by one-way ANOVA, followed by Bonferroni's Multiple Comparison Test. A probability of $p < 0.05$ was considered to be statistically significant.

Results

In vitro cell culture experiments

To determine the effects of the herbal extract on hyperpigmentation *in vitro*, cell viability assay was firstly used to determine the maximum non-cytotoxic concentration of the herbal extracts for all 3 herbal formulae. Figure 1a showed the effect of different concentrations of the aqueous herbal extract with or without MSH induction on the viability of the cells. MSH at 10 nM had no significant effect on the viability of B16 cells. There was no significant effect of the aqueous herbal extracts on the viability of the cells at all tested concentrations (0 - 1 mg/ml) Figure 1a, suggesting the aqueous herbal extracts exerted no cytotoxic effect on B16 cells at all concentrations tested (0 - 1 mg/ml) and could be used in further *in vitro* assays.

Cellular melanogenesis assay

The amount of melanin present in α -Melanocyte-Stimulating Hormone (MSH)-induced B16 cells were used as an index for melanogenesis in the present study. Figures 1b showed the effects of the different concentrations of the F1, F2 and F3 aqueous herbal extracts (0 - 1 mg/ml) on MSH-induced melanogenesis. As expected, 10 nM α -MSH induced significant increase on melanin content in B16 cells. 2.5 nM kojic acid significantly reduced this MSH-induced increase on melanin content Figure 1b. All F1, F2, and F3 aqueous herbal extracts dose-dependently reduced the MSH-induced increase on melanin content. There was also no significant difference on the melanin content among the kojic acid and aqueous herbal extract treated groups, suggesting all

3 formulation of the herbal extracts could exert melanin reduction effect that is comparable to the kojic acid treated group. There was however no significant difference among all 3 formulae, although only F3 significantly reduced melanogenesis at the lowest concentration tested (0.25 mg/ml), while F1 or F2 only significantly reduced melanogenesis at 0.5 and 1 mg/ml.

Cellular tyrosinase activity assay

For cellular tyrosinase activity measurement, cellular tyrosinase activity was assayed in terms of DOPA oxidase activity. Figure 1c showed the effects of different concentrations of F1, F2, F3 aqueous herbal extracts on MSH-induced increase in tyrosinase activity. As expected, 10 nM α -MSH induced significant increase on tyrosinase activity in B16 cells. 2.5 nM kojic acid significantly reduced this

Table 1: Gradient elution system used for LCMS analyses.

Time (mins)	Mobile phase A	Mobile phase B
0	95	5
3	95	5
14	42	58
17	0	100
18	0	100
18.5	95	5
20	95	5

Table 2: The different herbs contained within F3 and its corresponding standard markers.

Name of herbs	Name of markers	Amount ($\mu\text{g/ml}$)
Attractylodis Macrocephalae Rhizoma	Attractylenolide III (AIII)	1.76
Poria	Pachymic acid (PA)	ud
Angelicae Dahuricae Radix	Imperatorin (IP)	ud
	Isoimperatorin (IIP)	ud
Paeoniae Alba Radix	Paeoniflorin (PF)	105.31
Ampelopsis Radix	Gallic acid (GA)	10.62

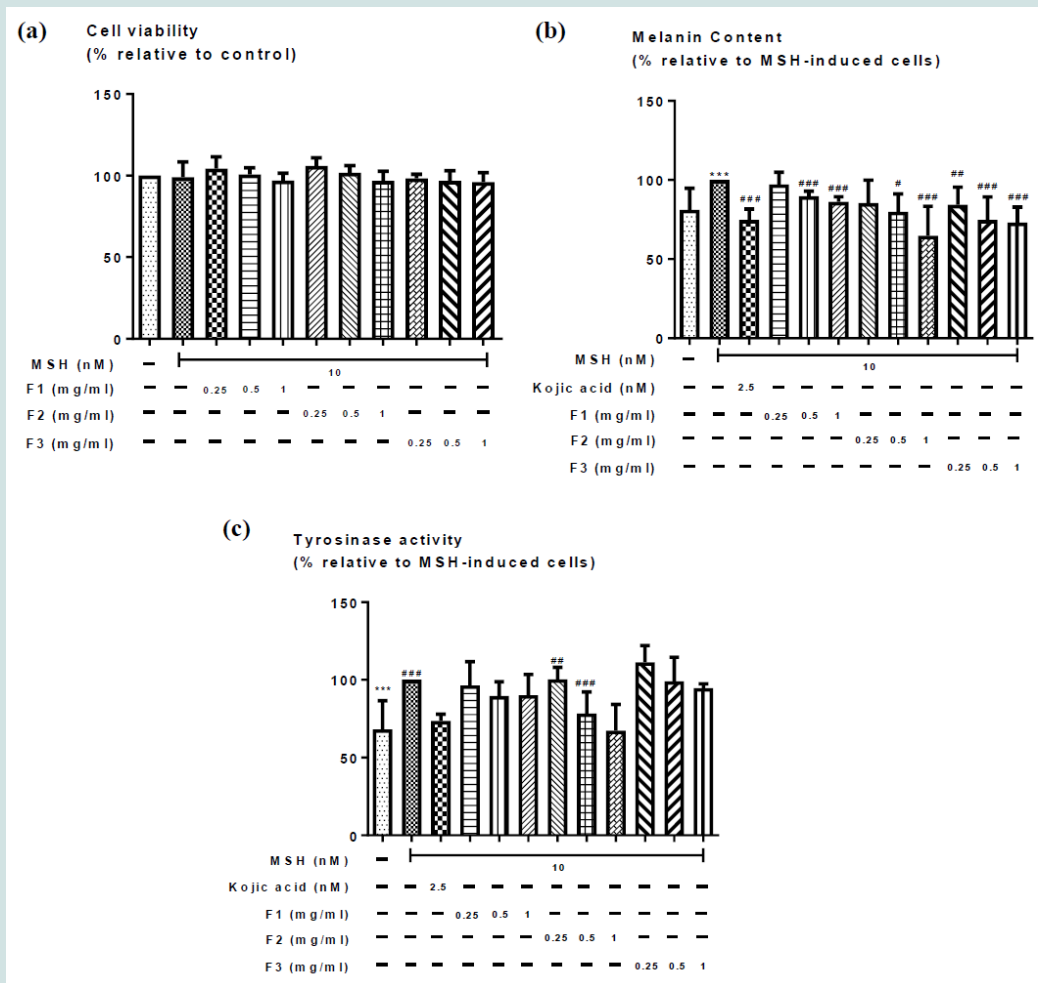


Figure 1: Effects of the different concentrations of F1, F2, F3 aqueous herbal extracts (0 - 1 mg/ml) on (a) cell viability, (b) MSH-induced melanogenesis; and (c) tyrosinase activity in B16 cells. Values represent means ± SD (n = 4 - 10). Significant difference between control group and MSH treatment alone group using Student's t- test: *** p < 0.001. Significant difference among MSH treated groups with or without co-treatment of different formulae extracts (F1, F2 or F3) using one-way ANOVA: # p < 0.05, ## p < 0.01, ### p < 0.001. No significant difference among all 3 formulae.

MSH- induced increase on melanin content Figure 1c. Although all aqueous herbal extracts, could reduce the MSH-induced increase on tyrosinase activity, only F2 had reached statistical significance. There was however no significant difference among all formulae.

Collagenase activity assay

Figures 2 showed the effects of the different concentrations of F1, F2, and F3 aqueous herbal extracts (0 - 1 mg/ml) on the inhibition of collagenase enzyme activity, with negative control demonstrating samples without any enzyme inhibition, and 50 µM EGCG served as the positive control. 50 µM EGCG significantly inhibited collagenase enzyme activity by around 30% Figure 2. F1, F2 and F3 aqueous herbal extracts dose-dependently inhibited collagenase enzyme activity, of which F3 appeared to exert the highest inhibition among all formulae Figure 2. Nonetheless, this difference did not reach statistical significance.

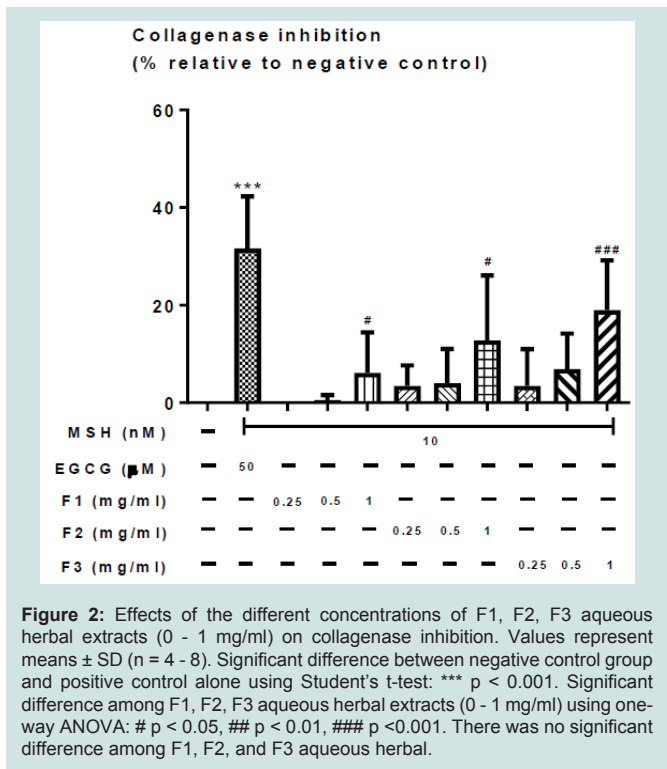
Animal experiment

From all 3 formulae, we had chosen F3 for testing in further

animal experiments since F3 appeared to be effective in both reducing melanogenesis and inhibition of collagenase activity. Figure 3 showed the effect of different topical treatment on melanocyte production in the epidermis of ear skin of mice given UV-irradiation. UV-irradiation significantly increased the number of melanocytes in the epidermis of ear skin in UV-irradiation treated mice Figure 3. These increase were apparently reduced in the epidermis of ear skin of mice given different treatment. However, none of the treatment had reached statistical significance. There was also no significant difference among all treatment groups. These data are also presented as % area and shown in Figure 3.

Similarly, Figure 4 showed the effect of different topical treatment on melanin production in the cross section of ear skin of mice given UV-irradiation. UV-irradiation had significantly increased melanin production as evidence by the increased in the % area of skin that were stained with melanin Figure 4. All treatment significantly reduced the melanin production. There was however no significant effect among all treatment groups.

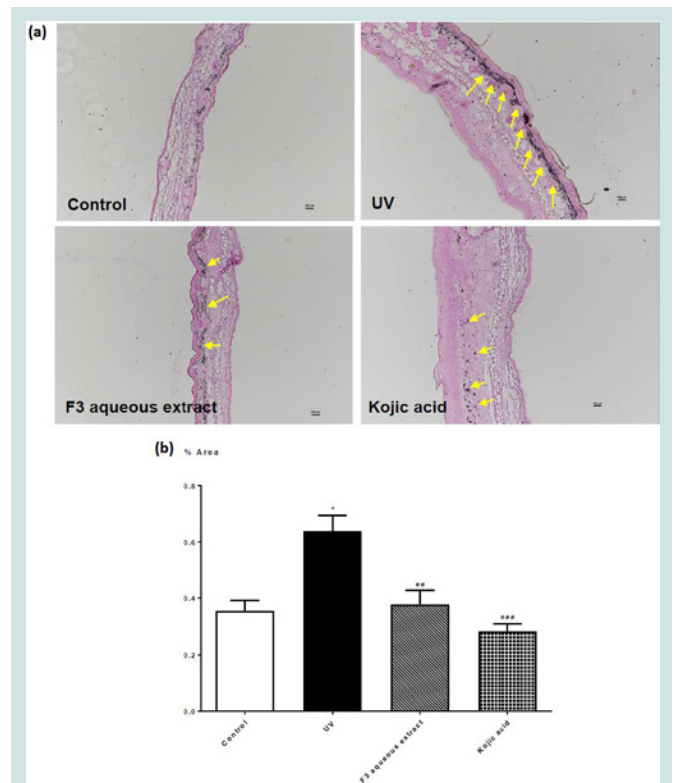
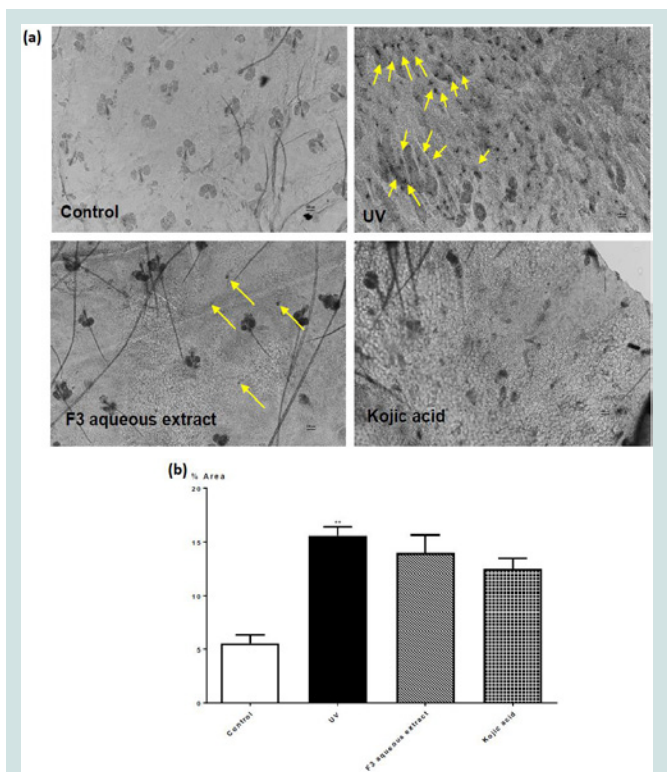
Figure 5 showed the immunohistochemical staining for the



localization of tyrosine in ear skin sections of mice given different topical treatments. Ear skin of mice given UV-irradiation increased % of area of skin that were stained with tyrosine (p < 0.08). F3 aqueous extract exerted a trend to reduce this increased in tyrosine localization which did not reach statistical significance.

In order for the topical agent to contribute its beneficial effects, it is important to understand whether the said topical agents could penetrate through to contribute its beneficial effects using the transdermal experiment. Chemical markers for the different herbs within the aqueous herbal extract F3 was chosen in accordance to CP recommendations Table 2. Table 2 showed the amount of different markers contained within the different extract before diffusion. Markers for Poria (pachymic acid), and Angelicae Dahuricae Radix (imperatorin and isoimperatorin) were however not detected within the F3 aqueous extracts. Figure 6 showed the penetration profile of the F3 aqueous extract in porcine ear skin after 24 hrs. Upon 24 hours of penetration, the representative markers from the QBS extract including atractylenolide III (marker for Atractylodis Macrocephalae Rhizoma), paeoniflorin (marker for Paoniae Alba Radix), and gallic acid (marker for Ampelopsis Radix) were detected within the skin and receiving chamber after 24 hours of transdermal study, suggested the formula could penetrate through the skin.

Figure 7 showed the effects of the different samples on the



Reconstructed Human Epidermis using the commercially available Epiderm *in vitro* skin toxicity test kit. As expected, positive control SDS showed the lowest% of cell viability. F3 aqueous herbal extract had no significant effect on the cell viability, suggesting the formula exert no toxicity on skin cells.

Discussion

In the present project, we demonstrated the potential beneficial effect of a simplified and modern form of the classical herbal formula Qi Bai San, which contains *Atractylodis Macrocephalae Rhizoma*, *Poria*, *Angelicae Dahuricae Radix*, *Paoniae Alba Radix* and *Ampelopsis Radix* for promotion of healthy skin. Our *in vitro* results suggested that this formula exerted potent effects on the inhibition of melanin production. This was further supported by our *in vivo* results which demonstrated that the herbal formula extract could reduce UV-induced increase in melanin production and trended to reduce dopa production and tyrosinase activity. Taken together, these data suggested the potential of this novel modified form of QBS to be developed as a topical product for the control of hyperpigmentation.

In the study, we had compared the effects of three different versions of QBS formulations. We observed significant effect of all three herbal formula extract on melanin production and tyrosinase activity inhibition *in vitro*, with no significant difference observed among all three formulae. Nonetheless, both F1 and F2 contained

Bombyx Batryticatus which is an undesirable animal herb. *Bombyx Batryticatus* is the dried larva of *Bombyx mori L.* (silkworm of 4-5 instars) infected by *Beauveria bassiana* (Bals.) Vuill. It could often be contaminated with aflatoxin, during the process of storage and transportation [20]. Aflatoxin is a well-known human carcinogen [21, 22].

As part of the authentication and standardization process reported in the Chinese Pharmacopoeia 2015, the quantity of aflatoxin within

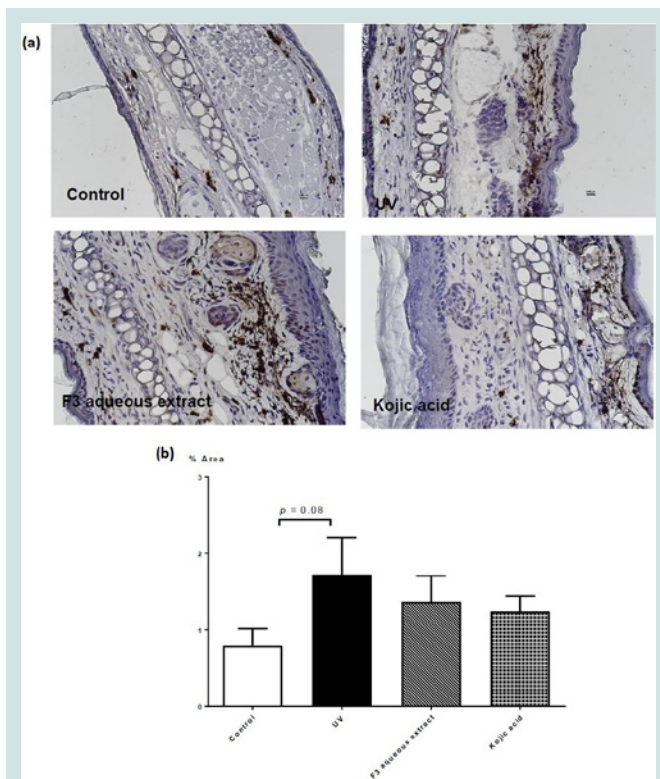


Figure 5: (a) Immunohistochemical staining (x400 magnification) for tyrosinase in ear skin sections of mice given different topical treatments and (b) percentage area of skin section with tyrosine immunohistochemical staining in different groups. Values represent means ± SEM (n = 4 - 8). Significant difference between control and UV-irradiated control group alone using Student's t-test: ** p < 0.01. No significant difference was observed among all UV-irradiation treated groups.

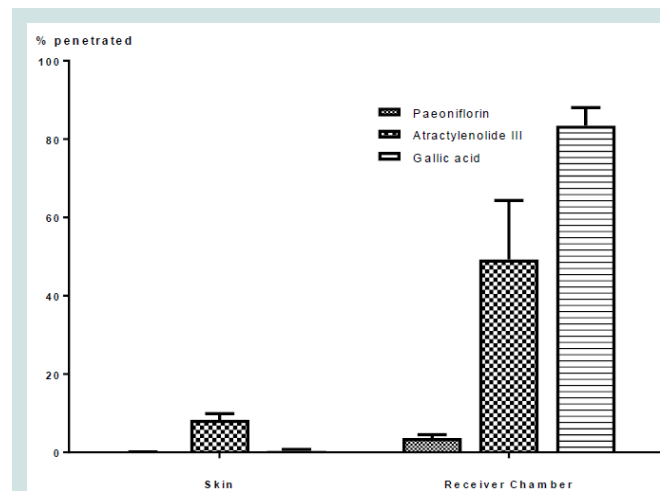


Figure 6: Diffusion performance of F3 Herbal formula extracts (% penetrated) in skin and receiving chamber after 24 hrs.

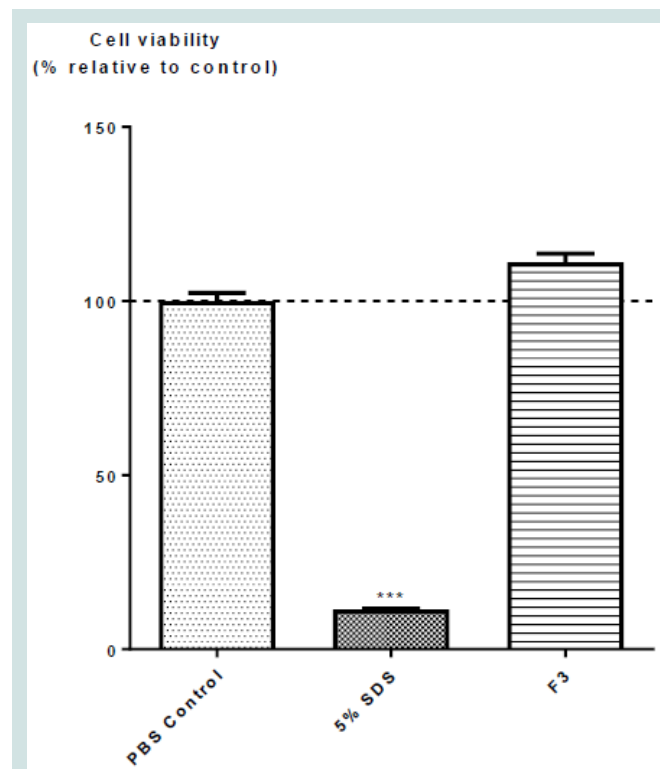


Figure 7: Effects of F3 Herbal formula extracts on skin toxicity. Values represent mean ± SD (n = 4). Significant difference between control and all treatment groups using one-way ANOVA: *** p < 0.001.

Bombyx Batryticatus needs to be closely and routinely monitored [10]. There had also been numerous case reports from different researchers reporting the poisoning incidents due to *Bombyx Batryticatus* [23- 25]. Therefore, the use of *Bombyx Batryticatus* remains controversial. In seeing so, we had therefore chosen F3, the only formula without *Bombyx Batryticatus* for further more thorough *in vivo* and *ex vivo* studies. From our *in vivo* results, there was a trend for an improvement on tyrosinase localization. When comparing the effects between the positive control kojic acid and our herbal formula extracts, we demonstrated that our modified Qi Bai San F3 exerted beneficial effects on hyperpigmentation which appeared comparable or even carried a better trend in potency than kojic acid. Although kojic acid has been proven to be a potent hypopigmentation agent and is used widely by various cosmetic company as agents for the control of pigmentation in the market, increasing concerns are arising since some animal data suggest kojic acid is weakly carcinogenic [26]. Furthermore, the European Commission's Scientific Committee on Consumer Products (SCCP) had also determined that based on a margin of safety calculation, the use of kojic acid at 1.0% in skin care formulations poses a risk to human health due to potential systemic effects. Our modified Qi Bai San, F3 possessing whitening effects comparable to kojic acid and yet not containing kojic acid, is therefore of great advantage.

Topical cosmetic agent demand genuine improvement on skin texture, clearance of pigmentations and maintenance of shininess. Hydroquinone has been used for decades as a hypopigmentation agent. However, since 2001, its use has been banned for cosmetic purposes due to the increasing cases of leukoderma-en-confetti/occupational vitiligo and exogenous ochronosis due to the prolonged use of hydroquinone. Hydroquinone could also possibly lead to DNA damage and mutations, causing carcinogenesis [27]. An appropriate choice could be one that satisfies the demands while maintaining the natural beauty of the facial cover. An ancient cosmetic formula inherited from the Imperial Court of the six century Tang Dynasty is the target of the present study. While the related stories appear convincing, the composition of most herbal natural products is well-known for slow, mild activities. What need to be provided before its proper endorsement would be evidences of its skin protection and safety.

We applied *in vitro* and *in vivo* experiments to demonstrate the de-pigmentation bioactivities of the formula as well as its protection effects of skin texture. When comparing three slightly different combinations of herbs, we prefer the simplest one which excludes animal and toxic items. The herbs chosen are also contained in the Inventory of Existing Cosmetic Ingredients (IECIC) which will facilitate the future commercialization of the finished product. With the intriguing *in vitro* and *in vivo* results, it would be reasonable to move on to the next step to conduct a pilot clinical study comparing the effects of the herbal formula with the commonly used treatment for hypopigmentation clinically such as the kojic acid and hydroquinone groups.

In conclusion, our present results have provided pre-clinical scientific support from *in vitro* to *in vivo* studies for the efficacy of a modified form of the traditional, and yet well-known herbal formula Qi Bai San on UV-induced hyperpigmentation. The beneficial effects of our modified Qi Bai San is comparable to the commercially positive

control of kojic acid. These data provided support for this novel herbal formula to be developed as a cosmetic product for marketing in the near future.

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