Effects of extraction methods on the flavonoid and phenolic contents and anti-aging properties of Rhyncholaeliocattleya Haw Yuan Beauty extracts

Rittipun Rungruang\textsuperscript{a,*}, Tasanee Panichakul\textsuperscript{a}, Wittawat Rattanathavorn\textsuperscript{a}, Nattapon Kaisangsri\textsuperscript{b}, Orapin Kerdchoechuen\textsuperscript{c}, Natta Laohakunjit\textsuperscript{c}, Louis Kuoping Chao\textsuperscript{d}

\textsuperscript{a} Department of Cosmetic Science, Faculty of Science and Technology, Suan Dusit University, Bangkok 10700 Thailand
\textsuperscript{b} Pilot Plant Development and Training Institute, King Mongkut’s University of Technology Thonburi (Bangkhunthien Campus), Bangkok 10150 Thailand
\textsuperscript{c} Division of Biochemical Technology, School of Bioresources and Technology, King Mongkut’s University of Technology Thonburi (Bangkhunthien Campus), Bangkok 10150 Thailand
\textsuperscript{d} Department of Cosmeceutics, China Medical University, Taichung 40402 Taiwan

\textsuperscript{*}Corresponding author, e-mail: rittipun_run@dusit.ac.th

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ABSTRACT: Rhyncholaeliocattleya Haw Yuan Beauty (Rlc. Haw Yuan Beauty) is a traditional orchid in tropical and subtropical Asian regions, which has attracted attention for its fragrant flowers with diverse colors. This study aimed to investigate the effects of different extraction methods on the phenolic profile, total flavonoid and cyanidin-3-O-glucoside contents, and anti-aging properties of Rlc. Haw Yuan Beauty crude extracts. The crude extracts were obtained using solvent, ultrasonic, and supercritical carbon dioxide fluid extraction methods. The major phenolic compounds including gallic, chlorogenic, and caffeic acids were present in all the extracts. The extract obtained with solvent extraction showed the highest total flavonoid content, whereas that obtained with supercritical fluid extraction yielded the highest cyanidin-3-O-glucoside content. Additionally, the different extraction methods had a significant influence on the antioxidant, anti-elastase, and anti-collagenase activities (\(p < 0.05\)) of Rlc. Haw Yuan Beauty extracts. The crude extracts obtained from the ultrasonic and supercritical carbon dioxide fluid extraction methods were not cytotoxic to human skin fibroblasts at any of the tested concentrations (31–500 \(\mu\)g/ml). Thus, the crude extracts from Rlc. Haw Yuan Beauty could be a safe and active ingredient in the production of skincare products in the cosmetic industry for human use.

KEYWORDS: phenolic compounds, cyanidin-3-O-glucoside, Rhyncholaeliocattleya Haw Yuan Beauty, antioxidant activity, anti-aging

INTRODUCTION

Orchidaceae is the largest family of flowering plants with over 850 genera and 35,000 species [1]. Rlc. Haw Yuan Beauty is a commercially viable orchid derived from Taiwan, a hybrid cross of Rlc. Haw Yuan Moon and C. Mari’s Song and is a valuable ornamental plant because of the diverse colors of its flowers. This orchid blooms with terrace-shaped, magenta colored flowers with a strong floral scent, approximately 3 times per year. Phytochemicals have bioactivities towards animal biochemistry and metabolism and are widely studied for their health benefits. Alkaloids, flavonoids, carotenoids, anthocyanins, and sterols are the major phytochemicals [2]. The consumption of orchids rich in antioxidants can prevent degenerative and chronic diseases [3]. Previous studies revealed that Rlc. Beauty Girl ‘KOVA’ (KOVA) flowers contained flavonoids, anthocyanins, \(\alpha\)-carotene, and lutein [4], and had high antioxidant activity. Cymbidium spp. contains phenanthrenes while Dendrobium crepidatum extract was found to possess antimicrobial activity with an IC\(_{50}\) of 73.90 \(\mu\)g/ml [5]. The above bioactive compounds have been isolated from orchids and have been used in the cosmetic, pharmaceutical, and food industries for their antibacterial and antioxidative properties [6]. Tadokoro et al [7] reported that skincare products containing 5% orchid extract from Brassica cattleya showed skin-whitening effects on melasma in women. Moreover, Vanda coerulea Griff. ex Lindl. and V. teres
(Roxb.) Lindl. extracts have shown anti-aging and skin-moisturizing potential [8]. The content of orchid phenolic compounds and flavonoids is affected by several factors including ecological conditions, harvest time, and extraction techniques. Several techniques including conventional and innovative methods could be used to isolate bioactive compounds from plants using various organic solvents such as ethanol, methanol, and acetone [9, 10]. In a previous study, the ethanol extract of Commelina nudiflora L. showed high antioxidant and antimicrobial activities [11]. In addition, the 70% ethanol extract of Himantoglossum robertianum contained flavones and flavan-3-ols showing antioxidant and elastase and collagenase inhibitory activities [12]. However, solvent extraction cannot extract active ingredients in their entirety. Therefore, ultrasonic and supercritical fluid extraction methods have been applied to improve the extraction efficiency. The flavone C-glycosides including vicenin-2, vicenin-3, schaftoside, vitexin, and isovitexin were found in Cymbidium kanran after ultrasound-assisted extraction with 70% methanol as a co-solvent [13]. Additionally, miquelianin was detected in Cuphea glutinosa extracts using ultrasound-assisted extraction with 38% ethanol as a co-solvent [14]. However, solvent waste reduction and excess energy consumption for solvent removal are concerns limiting the applicability of this method; thus, supercritical carbon dioxide has been considered useful for bioactive extraction [15]. According to Giménez-Rota et al [16], the ethanolic extracts of Lavandula luisier using the supercritical method contained an adequate amount of rosmarinic acid. The supercritical extraction conditions used were 35 MPa pressure at 45 °C for 150 min, which have been applied to obtain vasilic acid extract from vanilla pods. Despite the potential of Rlc. Haw Yuan Beauty orchid, little information is available on the antioxidant and cytotoxic activities of its flavonoid and phenol contents, which are the major compounds found in orchids [17]. This study aimed to investigate the effects of different extraction methods on the phenolic content, antioxidant activities, cytotoxicity, and anti-aging properties of the crude extracts of Rlc. Haw Yuan Beauty.

MATERIALS AND METHODS

Materials

Rlc. Haw Yuan Beauty flowers were collected in January from Bangkok Flowers Centre Co., Ltd., Thailand. The orchid flowers were air-dried, ground, and stored in a refrigerator until required for experimental analyses.

Solvent extraction (SE)

Rlc. Haw Yuan Beauty flowers were ground using a mortar, and 100 g of the powder was treated with 400 ml of 70% EtOH, according to the method of Molnar et al [18] with some modifications. Ethanolic extraction was performed using a shaker at 120 rpm and 25 ± 2 °C for 24 h. After the extraction, the mixture was filtered through Whatman No. 1 filter paper. The supernatant was removed using a rotary evaporator at 50 °C, 150 rpm. The percentage yield of the crude extract was then calculated based on the dry weight using the formula:

\[
\% \text{ Yield of extract} = \frac{\text{Weight of extract (g)}}{\text{Weight of dried plant (g)}} \times 100
\]

Ultrasonic-assisted liquid extraction (USE)

Dried Rlc. Haw Yuan Beauty flowers (100 g) were extracted with 400 ml of 70% EtOH and sonicated (40 kHz) for 2 h. Extraction was carried out using an ultrasonic cleaner bath (GT SONIC D-series, Shenzhen, PRC) with a designed ultrasonic power (50–150 W) with a sonication time of 2 h at extraction temperature between 25–35 °C [19]. The mixture was filtered, and the percentage yield of the crude extract was calculated as described above.

Supercritical fluid extraction (SFE)

SFE extracts were obtained using a Laboratory Scale SFE (model SFE-500F-2-C50, Thar Technology, Pittsburgh, USA), and 30 g of the dry flower was loaded into the extraction chamber. Extraction was carried out with 70% ethanol as a co-solvent at 40 °C and 200 bar. The total extraction duration was 100 min with a CO\textsubscript{2} flow rate of 10 g/min. A rotary evaporator was used to remove water from the crude extract at 50 °C and 150 rpm [20], and the percentage yield of the crude extract was subsequently calculated.

Determination of the total flavonoid content in the extract

The total flavonoid content in the crude extracts was quantified using the aluminum chloride colorimeter method with some modifications [21]. The crude extract (30 µl of 1 mg/ml) was incubated in a 96-well plate, and a mixture of NaNO\textsubscript{2} (1:5 w/v) dissolved in 100 µl of methanol was added and incubated for 6 min. Subsequently, 15 µl of AlCl\textsubscript{3} (1:10 w/v) was added to the mixture and allowed
to incubate for 6 min with a final addition of 55 µl of 1 M NaOH for 10 min at room temperature (approximately 25 ± 2°C). Thereafter, the absorbance of the reaction mixture was measured at 510 nm using a microplate reader (Biochrom EZ Read 2000, Cambridge, UK). Quercetin was used as a standard flavonoid for the preparation of the calibration curve, and total flavonoid content was reported as quercetin equivalents (mg QE/g extract).

**Separation and quantification of cyanidin-3-O-glucoside**

A 100 µl sample (20 mg/ml) was injected into an HPLC apparatus (Agilent Technology Inc., Palo Alto, CA, USA) equipped with an Agilent Eclipse XBD-C18 reverse-phase (4.6 mm × 150 mm, 5 µm) and a UV-visible diode-array detector. An isocratic method using 0.1% trifluoroacetic acid in Milli-Q water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 ml/min for 25 min was used for the chromatographic separation. Peak areas were identified by comparing the retention times with those of cyanidin-3-O-glucoside as an authentic standard. The absorbance was measured at 530 nm.

**Determination of phenolic compounds**

Phenolic compounds in the crude extracts were analyzed using HPLC and the C18 reverse-phase column as mentioned above. The analysis was carried out using a gradient elution of 0.3 M orthophosphoric acid as solvent A and methanol as solvent B (60:40 v/v) at 0 min. This was increased to 100% B from 10 to 15 min at a flow rate of 0.4 ml/min, for 25 min was used for the chromatographic separation. Peak areas were identified by comparing the retention times with those of cyanidin-3-O-glucoside as an authentic standard. The absorbance was measured at 530 nm.

**Evaluation of the antioxidant activity**

**Reduction of DPPH radicals**

The DPPH* (2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl) radical scavenging activity was evaluated according to Brand-Williams et al [22]. The crude extract was diluted with ethanol to prepare a dilution of the concentrations (0.001953–1 mg/ml). An aliquot of 50 µl of the sample and 150 µl of 0.16 mM DPPH solution in the ethanolic crude extract was mixed in a 96-well plate and incubated in the dark at room temperature for 30 min. The absorbance of the solution was measured at 515 nm, and L-ascorbic acid and Trolox were used as controls. The radical scavenging activity was calculated using the following equation:

\[
\% \text{Scavenging} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100
\]

where \( A_{\text{blank}} \) is the absorbance of the blank and \( A_{\text{sample}} \) is the absorbance of the crude extract. The results were expressed as IC\(_{50}\) values (mg/ml) (50 DPPH* scavenging effect concentration).

**ABTS (2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity**

The reaction mixture contained 7 mM ABTS\(^{++}\) and 2.45 mM potassium persulfate, and the mixture was incubated in the dark at room temperature for 12–16 h. The reaction was monitored at 734 nm until a stable absorbance of 0.70 ± 0.05 nm was obtained [23]. Forty microliters of crude extracts were mixed with 160 µl ABTS\(^{++}\) solution, and the absorbance of the resulting solution was measured after 6 min at 734 nm. The radical scavenging activity was calculated as stated above.

**Evaluation of the cytotoxic activity**

The cytotoxic activity of the extracts was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay [24]. Human skin fibroblast ATCC no. CRL 1947 (CCD-986SK) was cultured in RPMI 1640 medium with 5% fetal calf serum and incubated under 5% CO\(_2\), 95% humidified air, and 37°C. The cells in the exponential phase were harvested and trypsinized with 0.05% trypsin-EDTA. A suspension of 2.5 x 10\(^4\) cells/ml of medium was placed into each well of the 96-well microtiter cell culture plate at 200 µl/well and incubated for 24 h. Extracts were added within a range of 5 cytotoxic concentrations (31, 62, 125, 250, and 500 µg/ml) and incubated for 72 h. Ten microliters of 5 mg/ml MTT were added to each well, and the plate was re-incubated for 4 h. Subsequently, the culture medium was removed, and dimethyl sulfoxide (150 µl) and glycine buffer (pH 10.5; 25 µl) were added to the 96-well plate. The supernatant was gently shaken until the formazan crystals formed dissolved. Finally, the absorbance was measured using a microplate reader (Thermo Scientific, Waltham, MA, USA) at 540 nm, and the percentage of surviving cells was calculated using the following formula:

\[
\% \text{Survival} = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

where \( A_{\text{sample}} \) is the absorbance of the extract and \( A_{\text{control}} \) is the absorbance of the control.
Table 1 Yield, total flavonoid and cyanidin-3-O-glucoside contents of the crude extracts from Rlc. Haw Yuan Beauty.

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>% Yield</th>
<th>Total flavonoid (mg QE/g extract)</th>
<th>Cyanidin-3-O-glucoside (µg/g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE</td>
<td>2.39±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.45±0.009&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.63±0.74&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>USE</td>
<td>4.58±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.01±0.006&lt;sup&gt;b&lt;/sup&gt;</td>
<td>114.69±1.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SFE</td>
<td>1.36±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.68±0.007&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43599.77±10.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Different letters represent significant difference (p < 0.05). Values are expressed as mean ± SD (N = 3).

RESULTS AND DISCUSSION

Extraction yield

The extraction yield obtained by 3 different extraction procedures (SE, USE, and SFE) is summarized in Table 1. The highest yield of 4.58% was obtained from the ultrasonic extraction (USE) method. This result was in concordance with that reported by Hemwimol et al [27] for the extraction of anthraquinones from the roots of *M. citrifolia* using the ultrasonic-assisted ethanol extraction method. Compared with that of the non-sonicated sample, the extraction time was significantly reduced by 75% using the sonicated sample. Sun et al [28] reported that the yield of ultrasonically extracted pectin markedly improved in comparison with the conventional method. This could be explained by the fact that cavitation from ultrasound waves could break down the cell wall, thus allowing the solvent to access the intracellular content, releasing the target compound, and diffusing to the solvent [29]. Therefore, this method has been applied to natural product component extraction processes.

Moreover, Zhang [30] reported that the proficient frequency of ultrasound ranges from 20 to 50 kHz; however, the yield of the crude extracts from Rlc. Haw Yuan Beauty extracted by SE was significantly improved compared to that extracted by the SFE method. This might be because of the combination of the mechanical effects and extraction time in the SE method, which substantially promotes solvent penetration into the cell [31]. Additionally, the SFE method produced the lowest yield (1.36%) with a short duration of extraction and high temperature used. Thus, time and temperature are crucial factors in extraction as long extraction periods enhance the extraction yields [32]. Moreover, the 3 crude extracts obtained via different extraction methods showed similar visual appearance, solidity, stickiness, and magenta color.

Quantitative analysis of the total flavonoid and cyanidin-3-O-glucoside contents

The results showed that the total flavonoid content varied remarkably with the different extraction methods (Table 1). The SE method could be considered advantageous because of the highest flavonoid content (8.45 mg QE/g extract). The flavonoid content of the extract from USE and SFE were 7.01 and 6.68 mg QE/g extract, respectively. The SFE method may have reduced the total flavonoid recovery because of the high extraction temperature. Flavanoids are thermally sensitive and can easily be

Collagenase inhibitory activity

The collagenase inhibitory activity was evaluated *in vitro* with fluorometric assays, according to Pientaweeratch et al [25]. The activity was quantified using the EnzChek® Gelatinase/Collagenase assay Kit. In a 96-well plate, 20 µl of crude extract (1 mg/ml) and 100 µl of collagenase from *Clostridium histolyticum* was added per well with 20 µl of DQ™ collagen 1 (MMP-1) and DQ™ gelatin (MMP-2). The mixture was incubated at 25 °C for 90 min in the dark. The fluorescence intensity was measured at 485 nm (excitation wavelength) and 538 nm (emission wavelength), while epigallocatechin gallate (EGCG) was used as a positive control.

Elastase inhibitory activity

This assay was performed as previously described by Kim et al [26] with slight modifications. Enzymatic inhibition was determined by measuring the intensity of porcine pancreatic elastase (PE, E.C.3.4.21.36) with 1.6 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide as a substrate. For pre-incubation, 20 µl of crude extract and porcine pancreatic elastase were added to 96-well plates and incubated at 25 °C for 15 min. Finally, adjust the total volume to 250 µl with 1.6 mM N-Succinyl-Ala-Ala-Ala-p-nitroanilide and incubated at 25 °C. The absorbance was measured at 381 and 402 nm using a microplate reader, and EGCG was used as a standard.

Statistical analysis

Data were analyzed using the SAS version 9 software (SAS Institute; USA). Statistical analysis was performed using Analysis of Variance (ANOVA), and all the results were expressed as the mean ± standard deviation (SD). Duncan’s Multiple Range Test was used to determine significant differences between means (p < 0.05).

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degraded; according to Marszałek et al [33], they are generally susceptible to thermal degradation in the range of 35–65 °C with pressure higher than 100 MPa during the extraction process. Moreover, the stability of flavonoids is dependent on several factors including pH, time, temperature, and the time/temperature combination [34].

Cyanidin-3-O-glucoside has been found in orchid flowers. According to Duan et al [35], cyanidin-3-O-glucoside from Chinese bayberry was hydrolyzed to cyanidin during extraction process. For all extraction methods, cyanidin-3-O-glucoside was the main flavonoid in Rlc. Haw Yuan Beauty. The results showed that SFE resulted in the highest cyanidin-3-O-glucoside content (43 599.77 µg/g extract) (Table 1). This could be because carbonic acid produced by the solubilization of CO₂ reduced the pH of the water, resulting in more flavylum cations in cyanidin-3-O-glucoside. Thus, their hydrophilic property was increased which promoted the solubility of cyanidin-3-O-glucoside in ethanol [36,37].

**Phenolic profile of the extracts**

The total phenolic content obtained from SE, USE, and SFE was 69.34, 45.23, and 41.11 mg GAE/g extract, respectively (data not shown). The phenolic compounds identified included chlorogenic acid, caffeic acid, myricetin, luteolin, kaempferol, and apigenin (Table 2). Chlorogenic acid was the primary phenolic compound (8042.91 µg/g extract) obtained from the SFE method, which had the lowest extraction yield. The results suggested that the SFE extraction method enhanced the efficiency of the extraction to obtain a higher concentration of chlorogenic acid. Other phenolic compounds such as caffeic acid, myricetin, luteolin, kaempferol, and apigenin were also found using the SE, USE, and SFE methods at low levels. According to Cvetanović et al [38], the optimal temperature for apigenin, luteolin, and kaempferol extraction was 115 °C. However, apigenin was found in USE and SFE owing to its structural differences, and it influenced the solubility of the phenolic compounds.

**Antioxidant activity**

In this study, the results of the DPPH• and ABTS•⁺ assays were expressed as the IC₅₀ values, representing the concentration of the extract necessary to decrease the initial absorbance of the DPPH• solution by 50%; lower IC₅₀ values are associated with higher DPPH• radical scavenging activity [39]. The relationship between the extraction method and the antioxidant activity was studied using different extraction methods; these were based on different mechanisms and compared with Trolox and L-ascorbic acid (Table 3). The maximum antioxidant activity was found in the crude extract obtained using the SE method. The IC₅₀ values of the extracts obtained by SE, USE, and SFE were 0.19, 0.21, and 0.26 mg/ml, respectively. The value from the SE method was lower than that of the synthetic antioxidant L-ascorbic acid at 0.24 mg/ml. The result was correlated with the phenolic and flavonoid content owing to the existence of an ortho-dihydroxy structure in the B ring of phenolic compounds, making them effective hydrogen donors. There is a positive correlation between bioactive compounds in the crude extracts and the level of antioxidant activity [40]. It seems that this positive correlation between the antioxidant activity and phenolic content is related to the presence of –OH moieties, which are potent H donors [41]. A slightly different value was observed for ABTS•⁺ radical scavenging. The crude extracts became more active towards ABTS•⁺ radicals with the IC₅₀ values of 0.10, 0.11, and 0.13 mg/ml in SE, USE, and SFE, respectively. However, the antioxidant capacity of the crude extracts from all extraction methods in the ABTS•⁺ assay was lower than that of the L-ascorbic acid. Ethanol as a solvent used in all extraction methods showed the highest ability to extract potential antioxidant-like metabolites. Metabolites with antioxidant properties inhibit the oxidation of other molecules, thereby protecting these molecules against possible damages caused by reactive oxygen species.

**Cytotoxicity**

Human skin fibroblasts (CCD-986SK) were treated with the crude extracts at varying concentrations (31 to 500 µg/ml). The results showed that the crude extracts from the different extraction methods
L-ascorbic acid of some cytotoxic compounds in these extracts. Low concentrations. This indicates the presence affected CCD-986SK fibroblast cell viability even at showed that the crude extract from the SE method cell growth (Fig. 1). Crude extract from the SE method was the most cytotoxic to CCD-986SK fibroblasts; indeed, the SE extract showed the highest method was the most cytotoxic to CCD-986SK fi-

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Gallic acid (µg/g extract)</th>
<th>Chlorogenic acid (µg/g extract)</th>
<th>Caffeic acid (µg/g extract)</th>
<th>Myricetin (µg/g extract)</th>
<th>Luteolin (µg/g extract)</th>
<th>Kaempferol (µg/g extract)</th>
<th>Apigenin (µg/g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE</td>
<td>11053.81 ± 15.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6632.52 ± 12.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>203.36 ± 2.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>567.90 ± 12.93&lt;sup&gt;b&lt;/sup&gt;</td>
<td>92.77 ± 1.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64.27 ± 1.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>USE</td>
<td>8660.00 ± 14.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4890.00 ± 11.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td>130.00 ± 1.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>660.00 ± 7.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.00 ± 1.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.00 ± 1.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28 ± 1.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SFE</td>
<td>2021.90 ± 5.98&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8042.91 ± 12.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86.42 ± 1.77&lt;sup&gt;c&lt;/sup&gt;</td>
<td>383.92 ± 8.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>78.14 ± 1.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58.63 ± 2.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28 ± 1.22&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Different letters represent significant difference (p < 0.05). Values are expressed as mean ± SD (N = 3). ND = not detected.

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Anti-elastase activity (%)</th>
<th>Anti-collagenase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE</td>
<td>89.12 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90.06 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>USE</td>
<td>88.19 ± 0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>89.40 ± 0.17&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SFE</td>
<td>88.08 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>89.03 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Trolox</td>
<td>90.98 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91.98 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>92.77 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>91.42 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Different letters represent significant difference (p < 0.05). Values are expressed as mean ± SD (N = 3).

The highest quantity of bioactive phytoconstituents, flavonoids, and phenolics was present in the extract obtained from the SE method, which may be responsible for the radical-scavenging activity and toxicity [42]. The viability of CCD-986SK fibroblasts was over 90% higher in the crude extract from the SFE method compared to those from the SE and USE methods at 31 µg/ml. In addition, the CCD-986SK fibroblast cell morphology was monitored using inverted fluorescence microscope (Fig. 2). Cells 72 h post-exposure to 500 µg/ml crude extract were compared with DMSO treated cells. This revealed that crude extracts from SE, USE, and SFE were toxic within acceptable limit (cell viability > 80%) [43, 44] and did not affect cell growth. Therefore, cells could be grown continuously under extract treatment conditions.

### Collagenase inhibitory activity

Collagenase inhibitory activity was determined by analyzing the inhibition of MMP-1 and MMP-2. Collagen is the most abundant extracellular matrix protein in dermal fibroblasts. It plays a key role in regulating the tensile strength of the skin and connective tissue and in cell division and differentiation [45, 46]. The inhibitory activities of crude extracts from Rlc. Haw Yuan Beauty against MMP-1 and MMP-2 are shown in Table 4. All of the crude extracts from the different extraction methods (SE, USE, and SFE) exhibited significant inhibitory effects on MMP-1 and MMP-2 at greater than 80%. The crude extracts from SE and SFE showed the

Fig. 2 Morphology of human skin normal cells ATCC no. CRL 1947 (CCD-986SK). (A) untreated cells (DMSO-treated); (B) cells treated with SE; (C) cells treated with USE; and (D) cells treated with SFE, at 500 µg/ml. Cell morphology was monitored using inverted fluorescence microscope (Olympus IX71). All images were magnified 4X, and the scale bar was 100 µm.

Exhibited considerable dose-dependent inhibition of cell growth (Fig. 1). Crude extract from the SE method was the most cytotoxic to CCD-986SK fibroblasts; indeed, the SE extract showed the highest percentage of dead cells because of high levels of phenolic and flavonoid compounds. The results showed that the crude extract from the SE method affected CCD-986SK fibroblast cell viability even at low concentrations. This indicates the presence of some cytotoxic compounds in these extracts.

![Images of human skin normal cells](www.scienceasia.org)
highest and lowest MMP-1 and MMP-2 inhibitory activities, respectively. Additionally, all crude extracts showed significant differences compared to EGCG. Collagenases (MMP-1, MMP-8, and MMP-13) and gelatinases (MMP-2 and MMP-9) break down skin collagen, contributing to aging and wrinkling. Over 90% of the collagen in the human body is type I, and MMP-1 is a collagenase that specifically degrades type I collagen. MMP2 is commonly known as gelatinase, which has the potential to degrade both collagen and elastin fiber networks. The crude extracts from Rlc. Haw Yuan Beauty contain bioactive compounds such as flavonoids and phenolic compounds which possess enzyme inhibitory activities, possibly resulting from the hydroxy group attached to the benzene moiety in the polyphenol structure. The compound acts as a hydrogen acceptor or donor to the functional group of the enzyme. This interaction changes the enzyme conformation, which could lead to denaturation. This possibly leads to prolonged skin damage, as collagenase cleaves the peptide bond and breaks down the collagen.

**Elastase inhibitory activity**

Elastase is a proteolytic enzyme that causes degradation of elastin in the extracellular matrix and results in skin aging (sagging and wrinkles). The assay was performed using N-succinyl-Ala-Ala-Ala-\(p\)-nitroanilide as the substrate. The results of the elastase inhibition test are shown in Table 4. Crude extract from SE showed lower elastase inhibition compared with EGCG. It can be proposed here that the hydrogen group in the phenolic compound interacts with the carboxyl group in the elastase active site. Therefore, the enzyme was inhibited which increased skin elasticity and decreased wrinkle formation. However, elastase inhibition by the crude extract obtained with SE had the highest activity, which was related to antioxidant activity as observed with the DPPH and ABTS analyses. Among phenolic compounds, gallic acid could be an effective anti-aging agent by affecting human fibroblasts in wound healing [47]. The anti-elastase properties could be a result of highly polar compounds that are not extracted using the SFE method. Furthermore, the elastase inhibitory activity of the crude extracts from SE, USE, and SFE was significantly different \(p < 0.05\) when compared with that of EGCG.

**CONCLUSION**

Solvent extraction (SE) displayed high efficiency in extracting bioactive compounds including flavonoids and gallic acid. Cyanidin-3-O-glucoside and chlorogenic acid were the major compounds observed in crude extracts from supercritical fluid extraction. The crude extract from SE showed the lowest IC\(_{50}\) values for DPPH* and ABTS** assays. In addition, these crude extracts were also non-toxic to normal CCD-986SK cells in a dose-dependent manner. Moreover, remarkable collagenase and elastase inhibitory activities were observed in crude extracts from the different extraction methods.

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