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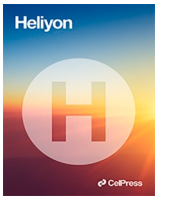

Preparation, development, and scale-up of standardized pentacyclic triterpenoid-rich extract from *Centella asiatica* (L.) Urb. and study of its wound healing activity

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ABSTRACT

This pilot-scale study of an innovative green extraction method shows increased biomarker content in plant extracts. Moreover, green extraction methods decrease the effects on the environment and human health and promote industrial growth. This study optimized the process conditions to obtain a pentacyclic triterpenoid-rich extract (PRE) from *Centella asiatica* (L.) Urb., which contains asiatic acid, madecassic acid, asiaticoside, and madecassoside, and evaluated its biological activities. PRE preparation was scaled up from laboratory to pilot scale. In the pilot scale, a combination of microwave-assisted extraction with an irradiation power of 4 kW and an ultrasonic-assisted extraction at 0.55 kW was used for *C. asiatica* extraction. The total pentacyclic triterpene content was 106.02 mg/g of crude extract. Then, the *C. asiatica* extract was fractionated by a macroporous resin (Diaion® HP-20). The PRE preparation method used 50% and 75% EtOH fractions. This PRE produced a high content of pentacyclic triterpenoids at 681.12 mg/g of crude extract. It presented a high anti-inflammatory effect with an IC_{50} value of 23.88 μg/mL for nitric oxide inhibition and induced wound healing processes (proliferation, migration, and collagen synthesis) in human dermal fibroblast cells. The information gained from this study can advance the industrial extraction of physiologically active substances from various plant sources for use as medicines or components of supplemental food and cosmeceutical products.

1. Introduction

Centella asiatica (L.) Urb., Gotu Kola (common name), or Bua Bok (Thai name) is an important medicinal herbaceous plant in the family Apiaceae [1,2]. It has been used to treat several different systems, including nerves and memory, that is, to help calm and relax an overactive nervous system, as a tonic for the liver, kidneys, and brain, to help stomach aches and stimulate appetite, to refresh and rejuvenate, to promote anti-aging, to stimulate the growth of skin cells and nails, and to heal wounds more quickly [3]. Moreover, its extract has been reported in Chinese, European, German, and Indian pharmacopeias, as well as in the Thai Herbal Pharmacopoeia [4] as helping scar management and wound healing properties [5].

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C. asiatica is currently grown in pantropical nations for use in food, cosmetics, and medicine [6]. It contains various compounds, such as triterpene acids, volatile and fatty oils, alkaloids, glycosides, and flavonoids. The important active markers of *C. asiatica* are the pentacyclic triterpenes asiaticoside (AS), madecassoside (MS), asiatic acid (AA), and madecassic acid (MA), shown in Fig. 1 [3,7]. AS and MS are the glycosides, while AA and MA are the aglycones. These compounds are responsible for the broad therapeutic effects identified in *C. asiatica*. AA and AS actively stimulate collagen synthesis, glycosaminoglycan synthesis, and extracellular matrix accumulation in rats [8]. AS promotes fibroblast proliferation and extracellular matrix synthesis during healing [9,10]. MA and MS show anti-inflammatory activity [3]. Thus, *C. asiatica* has been selected as the active ingredient in several healthcare products, including drugs, dietary supplements, and cosmeceutical products [11–13]. However, the active constituent of *C. asiatica* in the raw material varies due to different factors such as extraction method, solvent for extraction, etc. [14–16]. Therefore, developing a fast and efficient way to prepare a large quantity of high-quality standardized extract with easy-to-control levels of each component will benefit the pharmaceutical, cosmeceutical, and food industries.

Due to the above reasons, the pentacyclic triterpenoid-rich extract (PRE) has been prepared from the *C. asiatica* crude extract. This PRE might be a valuable source of nutraceutical herbal products. In the standardization of the PRE from each plant, the quantity of the different components is often expressed in percentages [17,18]. In a standardized extract, it is easy to reproducibly control the level of each component. Moreover, recent trends in extraction techniques have focused on green extraction methods that can reduce the volume of solvents and energy needed, reduce the number of production steps, use renewable natural materials that are safe, increase the yield of the extract and product quality, and are easily transferred from laboratory experiments to the industrial scale [15,16, 19–26]. At present, the industrial scale uses several methods for preparing the crude extract, such as microwave-assisted extraction (MAE) [27], pressurized hot water flow-through extraction system [28], supercritical carbon dioxide (SC–CO_2) extraction [29], ohmic heating-assisted extraction (OHAE) [30], and ultrasonic-assisted extraction (UAE) [31]. A combination method of SC-CO_2 extraction with MAE pretreatment has been used to increase the yield of oil extracted from *Moringa oleifera* seed [32].

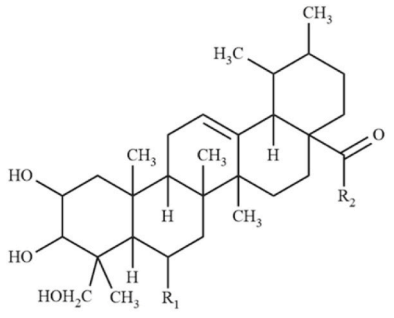
This study developed a new method to prepare PRE modified from the studies of Puttarak and co-workers [15,16]. This previous study used MAE to prepare the *C. asiatica* extract. After extraction, the *C. asiatica* crude extract was repeatedly dissolved with each solvent used as a mobile phase before eluting into the column for preparing the fractionation. This process loses time and energy to drying and dissolving the crude extract. Moreover, the mobile phase used to elute the column cannot precisely calculate its volume. Thin layer chromatography (TLC) must be used to estimate and check the standard components to adjust the volume of each mobile phase. Therefore, a PRE preparation method that uses green extraction concepts should be developed by combining MAE and UAE.

MAE can rapidly heat the sample–solvent mixture, resulting in wide applicability for the rapid extraction of analytes, including thermally unstable substances [33] and reduced extraction time and solvent consumption, potentially simultaneous extraction of multiple samples, and drastically improved sample throughput [34]. The UAE is a rapid and effective extraction method. It uses ultrasound to generate rapid movement in the sample and solvents, resulting in a higher mass transfer speed [25]. A simple one-step fractionation using a macroporous resin (Diaion® HP-20) column was used to prepare the PRE. The pilot scale of the PRE preparation was also performed to establish the utility of the novel green method on the industrial scale. In addition, this study evaluated the activity of PRE following anti-inflammation in mouse macrophage cell lines (RAW264.7), the wound healing process in human dermal fibroblasts (HDF) cells and human keratinocyte lines (HaCaT) cells, and collagen stimulation in HDF cells. PRE was compared with crude extracts, *C. asiatica* extract, and each marker (AA, MA, AS, and MS). The results of this research could support the development of healthcare products for the herbal medicine industry using standardized *C. asiatica* extract or PRE as the active ingredient in the future.

2. Materials and methods

2.1. Plant materials

The aerial parts of *C. asiatica* were purchased from the local market in Hat-Yai, Songkhla, Thailand. A voucher specimen of this plant was identified and deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand (No. SKP 199 03 01 01). The plant was washed and dried in the hot air oven (50–60 °C) for 18–48 h. The dried plant was then ground with an electric grinder (SK300 Rostfrei, Retsch®, Germany). The plant powder was sifted through a sieve (sieve no. 20, 45, 60,



Name of structure	R1	R2
Asiaticoside	-H	-O-Glu-Glu-Rha
Madecassoside	-OH	-O-Glu-Glu-Rha
Asiatic acid	-H	-OH
Madecassic acid	-OH	-OH

Fig. 1. Chemical structures of the four major compounds in *C. asiatica*.

and 80) to obtain a suitable particle size, weighed, and stored in a desiccator at room temperature (25–30 °C), protected from light until use.

2.2. Chemicals and reagents

Diaion® HP-20 was purchased from Sigma-Aldrich, Germany. Ethanol (EtOH), methanol (MeOH), and acetonitrile (ACN) were purchased from LabScan Asia Co., Thailand. All solvents were used for extraction, isolation, and analytical process of PRE. AA (CAS No. 464-92-6) from Sigma Aldrich, Switzerland, and AS (CAS No. 16830-15-2), MS (CAS No. 34540-22-2), and MA (CAS No. 18449-41-7) from Chengdu Biopurity Phytochemicals Ltd., China, were used as standards in the biological activity testing and analytical process of *C. asiatica* extract and PRE. Dimethyl sulfoxide (DMSO) from Sigma Aldrich, Singapore, was used to prepare the stock solution of each test sample for cell culture assay. Dulbecco's Modified Eagle medium (DMEM, Cat. No. 1IVG1-31600-034), Roswell Park Memorial Institute medium 1640 (RPMI, Cat. No. 1IVG2-21870-076), penicillin (10,000 units/mL) plus streptomycin (10,000 μg/mL) from Gibco® (USA.), and sodium bicarbonate (Loba Chemie PVT. Ltd., India) were used to prepare the medium for cell culture. Fetal bovine serum (FBS, Cat. No. 1IVG3-10270-098, Gibco®, Germany) was used as a growth supplement for cell culture medium. The 0.25% Trypsin-EDTA from Gibco® (USA.) was used for trypsinization of the cell culture. Lipopolysaccharide (LPS) from *Escherichia coli* O55: B5 purified by phenol extraction (LPS, EC No. 297-473-0) for initiating the inflammatory process in RAW264.7 cells was purchased from Sigma Aldrich, USA. The 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT, Cat. No. M6494, Thermo Fisher Scientific Bromide, USA.) was used as a reagent for assessing cell metabolic activity. Indomethacin (Sigma-Aldrich, USA.) was used as a standard for anti-inflammatory activity. Griess reagent for analysis the nitric oxide (NO) production in RAW264.7 cells was prepared by solubilizing the sulfanilamide (Sigma Aldrich, USA.) as 1.0 g in 100 mL of sterile water containing 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine (NEDA) (Sigma Aldrich, USA.). Sircol Soluble Collagen Assay kit (Biocolor, UK.) was used to analyze the collagen production in HDF cells.

2.3. Cell culture

Primary HDF cells (ATCC® PCS-201-012™) were purchased from ATCC®, USA. HaCaT cells were kindly provided by the Medical Science Research and Innovation Institute, Prince of Songkla University, Thailand. RAW264.7 cells (ATCC® TIB-71™) were purchased from ATCC®, USA.

2.4. Preparation of C. asiatica extract

The extraction method was modified from our previous study, including the method and the ratio of solvents in the extraction process, that is, EtOH and water [16]. The study of Puttarak and Panichayupakaranant prepared the *C. asiatica* extract by a single method and didn't optimize the particle size of the *C. asiatica* powder. So, this study optimized the preparation process for preparing *C. asiatica* extraction in a step-by-step manner as follows: (1) particle size, (2) ratio of solvents and (3) extraction procedure. The most appropriate extraction method was the one that provided the highest yield of *C. asiatica* extract and the total content of pentacyclic triterpenes for each parameter.

2.4.1. Optimization of particle size of dried powders

Samples (5 g) of dried *C. asiatica* powder with various particle sizes: (a) < 850 μm (Sieve No. 20), (b) < 355 μm (Sieve No. 45), (c) < 250 μm (Sieve No. 60), and (d) < 180 μm (Sieve No. 80) were separately extracted by MAE three times using a household microwave (Electrolux, Bangkok Thailand) at 900 W for four cycles (1 cycle: 1 min, 30 s power on and 30 min power off) with absolute EtOH (150 mL), filtered with filter paper No.1, and the solutions from each raw powder size were combined. The combined solutions were evaporated to dryness under reduced pressure at 45–55 °C using a rotary evaporator (Heidolph Instruments GmbH & Co.KG., Schwabach, Germany), and their yields were calculated. The total content of pentacyclic triterpenes of the *C. asiatica* extract was further analyzed using high-performance liquid chromatography (HPLC).

2.4.2. Optimization of solvent

The most suitable extraction solvent was determined by using the optimal particle size determined as described in section 2.4.1 and MAE. The solvents used in this step were various ratios of EtOH and water (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100 EtOH:water).

2.4.3. Optimization of extraction procedures

The extraction process was optimized to determine the most suitable method to increase AA, AS, MA, and MS yield and purity in the *C. asiatica* extract. A 5 g sample of dried *C. asiatica* powder with the most suitable particle size was extracted with absolute EtOH (150 mL) by MAE alone, pretreatment with UAE before treatment with MAE, or post-treatment with UAE after treatment with MAE. For the UAE pre- and post-treatment method, the dried *C. asiatica* powder was soaked in absolute EtOH and placed in an ultrasonic bath (Elma Schmidbauer GmbH, Singen, Germany) for 1 h.

2.4.4. Scale-up for preparation of standardized C. asiatica extract

The most suitable extraction method in the laboratory was further evaluated for pilot-scale extraction. In the pilot-scale extraction, 9 kg of dried *C. asiatica* powder was extracted with 90 L of a suitable solvent. MAE was conducted in the industrial microwave (Baan

Innov Co., Ltd., Nakhon Si Thammarat, Thailand) at 4 kW (Fig. 2a) for 2 cycles of 5 min of power and 2 min off. UAE was conducted using an industrial ultrasonic herb extraction machine (Conversant Technology Co., Ltd., Bangkok, Thailand) at 0.55 kW (Fig. 2b) for 15 min. After that, the extract was sucked past a tube in a closed system through a filter (stainless-steel sieve). Then some solvents were evaporated from the initial extract into an industrial evaporator machine tank (Conversant Technology Co., Ltd., Thailand), as shown in Fig. 2c. The industrial evaporator machine adjusted the pressure at −0.8 to −0.9 MPa, the power at 30 kW, and the temperature at 45 °C. The obtained *C. asiatica* extract was further subjected to the fractionation process presented in the next section.

2.5. Preparation of PRE

The PRE was prepared from the *C. asiatica* extract. The pilot-scale crude extract prepared in section 2.4.4 was further subjected to either lab- or pilot-scale fractionation. This fractionation method was modified from a previous study [16]. Fractionation was optimized in a step-by-step manner to determine the most suitable method for preparing PRE as follows (1) preparation of the sample for separation and (2) the ratio of EtOH and water used as the mobile phase. The most appropriate fractionation method provided the highest yield and total content of pentacyclic triterpenes.

2.5.1. Sample preparation for separation

The sample preparation was optimized in the lab scale to find the solvent that retained the active ingredients in the stationary phase for as long as feasible for the separation of the active components. Diaion® HP-20 (150 g) was loaded into the column (5 cm × 40 cm) and used as the stationary phase. The *C. asiatica* extract was loaded and eluted in the column at a flow rate of 5 mL/min using various systems: (1) the initial extract or CAE-L-1 (300 mL) and (2) the initial extract mixed with water (ratio 1:3) or CAE-L-2 (1.2 L). After that, each column was eluted with absolute EtOH (1.8 L) (CAE-L-1-A and CAE-L-2-A) to obtain the PRE and subjected to the quantitative HPLC analysis to determine the content of AA, AS, MA, and MS.

2.5.2. The ratio of EtOH to water used as mobile phase system

The most suitable sample system was used to evaluate different mobile phase systems. In addition to elution by absolute EtOH, the separation was also performed in two different mobile phase systems: (1) 1.8 L of 50% EtOH and followed by 2.4 L of 75% EtOH and (2) 2.4 L of 75% EtOH. All fractions were subjected to quantitative HPLC analysis to determine the AA, AS, MA, and MS contents.

2.5.3. Pilot-scale preparation of PRE

The most suitable fractionation method in the lab scale was further evaluated for pilot-scale fractionation. Compared to the lab-scale preparation, the pilot scale was prepared using two methods: (1) the same ratio and elution method for the mobile phase as the lab-scale (CAE-P-(1 or 2)-(A, B or C)-X) and (2) decreasing the volume of the mobile phase to the half the lab-scale but using the same method for elution (CAE-P-(1 or 2)-(A, B or C)–Y). Diaion® HP-20 (4.5 kg) was loaded into the column (12 cm × 120 cm) and used as a stationary phase. *C. asiatica* extract (7.5 L) was prepared following the procedures selected in sections 2.5.1 and 2.5.2. All fractions were subjected to the quantitative HPLC analysis for AA, AS, MA, and MS contents.

2.6. Quantitative HPLC analysis of pentacyclic triterpene content of C. asiatica extract and PRE

The pentacyclic triterpene content was determined using the HPLC method that previously proposed by Puttarak [35]. The method was carried out using Shimadzu® LC-20A series with a quaternary pump, autosampler, and detection by UV at the wavelength of 210 nm. Analytical reverse-phase column (Tosoh TSKgel® ODS-100V, stainless steel column, phase C18, 15 cm × 4.6 mm, 5 μm, 100 °A,

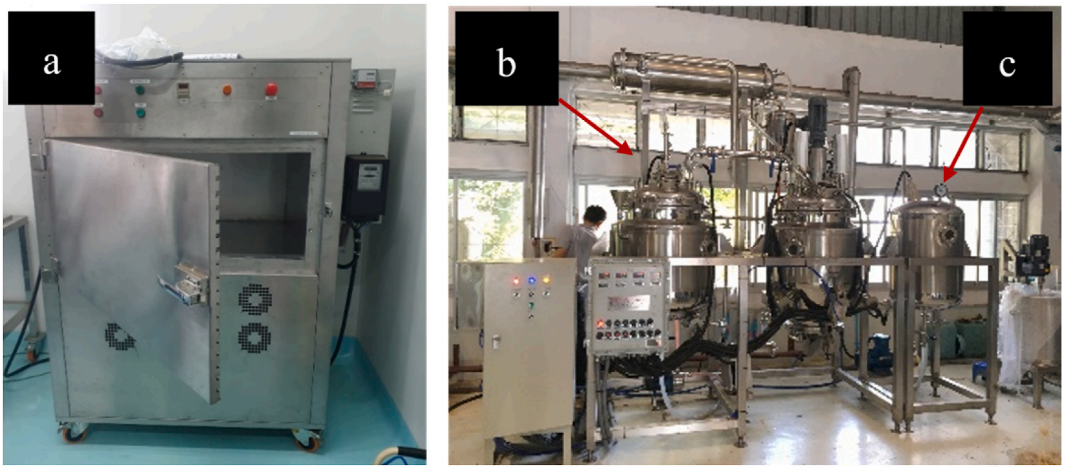


Fig. 2. MAE (a), UAE (b), and evaporator (c) machines.

Tosoh Bioscience®, Germany) was used. The mobile phase consisted of a gradient of ACN:water as follows 0–5 min, 20:80; 5–10 min, 30:70; 10–20 min, 65:35; 20–30 min, 70:30. The flow rate of the mobile phase was set at 1 mL/min. The analyzed samples were dissolved in MeOH and filtered with a 0.45 μm polyvinylidene fluoride membrane. The sample injection volume was 20 μL. The sample analysis was compared with the standard curve of AA, AS, MA, and MS at a concentration between 5 and 500 μg/mL.

2.7. Study on the biological activities

C. asiatica extract and PRE were investigated for their biological activities, i.e., anti-inflammatory activity, cell proliferation, migration assay, and total collagen production content.

2.7.1. Anti-inflammatory activity

NO synthase: The inhibitory effect on NO production by RAW264.7 cells was evaluated using a modified method from the previous study [36]. Briefly, the RAW264.7 cells were cultured in RPMI medium. The cells were harvested with trypsin-EDTA and diluted in a fresh medium. The cells were seeded in 96 well plates with 1×10^6 cells/mL and allowed to incubate at 37 °C for 1 h. After that, the medium was replaced with a fresh medium containing 1 μg/mL of LPS with the samples at various concentrations (1–100 μg/mL), and the cells were further incubated at 37 °C for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using a Griess reagent at a ratio 1:1 by microplate reader at 570 nm. Indomethacin at concentrations 1–100 μg/mL was used as a positive control.

Inhibition (%) and IC_{50} values were calculated. %Inhibition was expressed as [1- (NO release with sample - spontaneous release)/(NO release without sample - spontaneous release)] × 100. The result was determined graphically (N = 3).

2.7.2. In-vitro wound healing assay

Cell proliferation: MTT assay was performed according to the report of Rachpirom and co -workers [36] with some modifications. This method was studied with HDF and HaCaT cells. Both cells at 80–90% confluence were detached with 0.25% trypsin-EDTA with phenol red and seeded in 96-well plates at different densities. HDF cells (2×10^3 cells/well) and HaCaT cells (1×10^4 cells/well) were grown for 24 h in DMEM containing 10% FBS. Cells were then treated with or without the sample in DMEM containing 2%FBS for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that, the solution was removed. Then, 100 μL of DMEM containing 2% FBS and 10% of 5 mg/mL MTT solution was added to each well, and the cells were incubated at 37 °C for 2 h. Subsequently, the medium was gently removed and replaced with 150 μL of DMSO. Samples were incubated for 5 min with shaking to dissolve the precipitate. The absorbance at 570 nm was measured for each well. The optimal densities of treated cells were compared with untreated control cells. The cytotoxicity of samples was considered lower than 80% of cell viability compared to that of the untreated control group. The %proliferation was expressed as $(A_{sample}/A_{control}) \times 100$. When A_{sample} and $A_{control}$ represented the absorbance values determined at 570 nm of sample and control after the reaction, respectively. The tests were carried out in triplicate for each sample.

Wound healing assay: Wound healing assay was assessed using a migration assay. This assay was studied in HDF cells (1×10^5 cells/well in 6 wells plate) that were allowed to grow for 2 days to confluence. At day 0, the medium was removed and gently scraped the confluent monolayer of cells with sterile pipette tips (size 1 mL) producing a tiny linear scratch (care was taken during the scratching process to ensure universal size and distance were made for all samples). Before adding the medium with various treatment solutions at a concentration 10 μg/mL, cells were thoroughly washed with PBS to eliminate cellular debris. Photographic images were obtained at multiple time points, as indicated in the results section. Each condition was photographed in three separate fields. The same field was examined at each time point, allowing the percentage of wound closure caused by infiltration of migratory cells to be quantitated using the ImageJ software [37]. The percentage of wound closure was expressed as 100 × [(Linear distance on day 0 - Linear distance on day 1, 2 or 3)/Linear distance on day 0].

The total collagen production was estimated by total collagen assay kit according to a previous report of Sanguansajapong and co-workers [38]. The Sircol Soluble Collagen Assay kit was used to measure the total collagen in the supernatant. This quantitative dye-binding test for total collagen is intended for the in vitro evaluation of collagens released into the culture medium. In 96-well plates, HDF cells (2×10^3 cells/well) were planted in DMEM with 2% FBS. The cells were cultivated for 24 h before being treated with or without the sample for 48 h. The 100 μL of solution from each well was transferred to a 1.5 mL centrifuge tube at the conclusion of the incubation, and 500 μL of Sircol dye reagent was added to each tube. The combined ingredients were kept and shaken for 30 min at room temperature. The tubes were then centrifuged for 10 min at a speed of 10,000×*g*, and the supernatants were removed. The remaining pellets were washed with EtOH. After that, the alkali reagent (500 μL) was added to dissolve the precipitate. The solutions were put into 96-well plates and read at 540 nm by a spectrophotometer. The standard collagen equivalents (mg/g) were used to compare and express the results.

2.8. Statistical analysis

All experimental values were reported as mean ± SD. Comparison among each group, one-way analysis of variance (one-way ANOVA), was performed with a 95% confidence level (p-value <0.05) using IBM SPSS (version 22) for Windows (SPSS Inc., USA.).

3. Results and discussion

3.1. Development of lab-scale C. asiatica extract preparation

A robust extraction process was developed for the preparation of bioactive-rich *C. asiatica* extract from the aerial part via a three-step approach: optimizing the effects of (1) particle size of the *C. asiatica* crude powder, (2) solvent ratio, and (3) extraction procedure. The extract obtained from each process was examined for the most suitable outcome by evaluating the triterpenoid content (AA, AS, MA, and MS) of the extract. The AA, AS, MA, and MS contents were examined based on linear fits to the HPLC results as follows: $Y = 7042.4X - 10174$ ($r^2 = 0.9999$), $Y = 3617.5X - 4632$ ($r^2 = 0.9999$), $Y = 6575.9X - 7119.8$ ($r^2 = 1$), and $Y = 3387.7X - 12297$ ($r^2 = 0.9999$), respectively. Where Y is the peak area (mAU), X is the sample concentration (mg/g crude extract), and r^2 is the coefficient of determination of each linear regression.

3.1.1. Determination of best particle size for extraction of C. asiatica powder

The *C. asiatica* powders with particle sizes <180 and < 355 μm yielded the highest pentacyclic triterpene content when extracted with absolute EtOH solvent using MAE, followed by those <250 μm and <850 μm (Table 1). A previous report has shown that nano-sized *C. asiatica* powder increased the yield of AA higher than micro-sized powder [39]. The cell tissues of the plant will disintegrate into small sizes. Most active components are found in the inner cytoplasm and cannot be directly exposed during extraction without being liberated from cell walls so that effective components dissolve rapidly [40] (Su et al., 2007). Thus, a smaller powder size could enhance the extraction yield without losing active compounds [41]. The *C. asiatica* powder <355 μm gave a superior result by yielding high total pentacyclic triterpene contents and easily reducing the powder size. Therefore, *C. asiatica* powder with a particle size <355 μm was selected for the next extraction method.

3.1.2. Determination of suitable solvent ratio for C. asiatica extract

This step evaluated the solvent for preparation of the *C. asiatica* extract with the highest total pentacyclic triterpene content. The results showed that higher ratios of EtOH (80%–100%) could significantly increase the crude extract yield and the total pentacyclic triterpene content (Table 2). Moreover, absolute EtOH was suggested as a viable solvent for pentacyclic triterpenes, specifically aglycones [16]. Furthermore, EtOH is preferable due to its lower toxicity, ease of recovery by reduced pressure distillation, and practical use in green extraction methods [42]. Consequently, this study selected absolute EtOH for the next extraction step.

3.1.3. Determination of the extraction procedures

This step evaluated the effect of pre- and post-extraction protocols on the extraction technique using *C. asiatica* powder with size <355 μm and absolute EtOH as the solvent. UAE was used as a pre- or post-treatment method compared with MAE alone. The results showed that post-treatment by UAE could significantly increase the yield of crude extract and total pentacyclic triterpene content (Table 3). This may be attributed to the fact that treatment with microwave energy changes the cell structure due to electromagnetic waves and MAE acceleration. High extraction yield may result from a synergistic combination of two transport phenomena (heat and mass gradients) working in the same direction [43–46]. Moreover, UAE was highly effective in breaking down the dense and hard cell wall structure of the plant and increasing the content of active components and the yield of crude extract [43,44,46–48]. Therefore, MAE-UAE was the most suitable extraction method for *C. asiatica* powder with a size <355 μm. Consequently, this method was used in the next step of this study.

3.1.4. Scale-up for preparation of standardized C. asiatica extract

MAE followed by UAE before the evaporation of the solvent by an industrial evaporator machine was used for the scale-up. The pentacyclic triterpene content in the resulting *C. asiatica* extract is presented in Table 3. Based on the HPLC analysis, the *C. asiatica* extract contained total pentacyclic triterpenes of 106.02 ± 2.30 mg/g crude extract or 10.60%w/w. This value was significantly higher than that obtained at the lab scale due to the power and type of each machine. The *C. asiatica* extract in this pilot-scale study was further fractionated to obtain the PRE and evaluated for its biological activity.

Table 1
The pentacyclic triterpenoid content of *C. asiatica* extract obtained from different particle sizes of dry powder.

Particle Size	Yield	Pentacyclic triterpene content (mg/g crude extract)				
	(%)	AA	MA	AS	MS	Total
≤850 mm	12.62 ± 0.12^{c}	11.26 ± 2.47^{b}	7.29 ± 0.61^{c}	16.07 ± 1.23^{a}	10.79 ± 0.71^{b}	45.40 ± 2.32^{c}
≤355 mm	13.14 ± 0.33^{b}	15.45 ± 0.90^{a}	25.78 ± 0.93^{a}	9.50 ± 0.56^{c}	21.27 ± 0.75^{a}	72.00 ± 0.41^{a}
≤250 mm	14.33 ± 0.73^{ab}	13.25 ± 0.89^{ab}	23.81 ± 0.27^{b}	8.01 ± 0.21^{c}	21.19 ± 0.49^{a}	66.26 ± 1.34^{b}
≤180 mm	15.38 ± 0.60^{a}	16.50 ± 0.25^{a}	24.95 ± 0.26^{ab}	12.24 ± 0.42^{b}	19.64 ± 1.25^{a}	73.33 ± 1.10^{a}

Note: Results were represented as the mean ± SD of three independent determinations ($N = 3$). Values in the column followed by different letter superscripts are significantly different ($p < 0.05$), and values having the same superscript are not statistically significant.

Table 2
The pentacyclic triterpenes of *C. asiatica* extract obtained from different solvents.

The ratio of solvent between EtOH and water	Yield (%)	Pentacyclic triterpene content (mg/g crude extract)				
		AA	MA	AS	MS	Total
100:0	14.14 ± 1.43^{d}	14.52 ± 0.04^{a}	25.36 ± 0.04^{a}	8.77 ± 0.23^{a}	22.47 ± 0.30^{a}	71.13 ± 0.36^{a}
80:20	29.41 ± 1.57^{c}	4.97 ± 0.14^{b}	12.56 ± 0.34^{b}	7.70 ± 0.13^{b}	22.12 ± 0.40^{a}	47.35 ± 0.84^{b}
60:40	33.07 ± 2.72^{b}	0.60 ± 0.19^{d}	2.57 ± 0.12^{d}	6.88 ± 0.69^{c}	16.78 ± 0.92^{b}	26.95 ± 1.40^{d}
40:60	16.68 ± 0.56^{d}	1.77 ± 0.13^{c}	4.97 ± 0.15^{c}	7.32 ± 0.17^{bc}	17.11 ± 0.48^{b}	31.16 ± 0.66^{c}
20:80	35.88 ± 2.42^{a}	0.00 ± 0.02^{e}	0.00 ± 0.03^{e}	1.98 ± 0.72^{d}	15.06 ± 0.75^{c}	17.04 ± 0.96^{e}
0:100	34.02 ± 0.57^{ab}	0.00 ± 0.04^{e}	0.00 ± 0.03^{e}	0.00 ± 0.08^{e}	0.00 ± 0.50^{d}	0.00 ± 0.13^{f}

Note: Results were represented as the mean ± SD of three independent determinations ($N = 3$). Values in the column followed by different letter superscripts are significantly different ($p < 0.05$), and values having the same superscript are not statistically significant.

Table 3
The pentacyclic triterpene content of *C. asiatica* extract obtained from pre-/post-extraction methods by UAE in both laboratory- and pilot-scales.

Method	Yield	Pentacyclic triterpene content (mg/g crude extract)				
	(%)	AA	MA	AS	MS	Total
Lab-scale						
MAE	14.14 ± 1.43^{b}	14.52 ± 0.04^{c}	25.36 ± 0.04^{c}	8.77 ± 0.23^{d}	22.47 ± 0.30^{b}	71.13 ± 0.36^{c}
UAE-MAE	14.53 ± 1.25^{b}	14.88 ± 1.42^{c}	27.60 ± 2.27^{c}	12.28 ± 0.11^{c}	10.30 ± 0.80^{d}	64.77 ± 3.76^{d}
MAE-UAE	20.48 ± 0.76^{a}	20.71 ± 0.20^{b}	41.82 ± 0.19^{a}	15.40 ± 0.29^{b}	14.31 ± 1.11^{c}	92.23 ± 1.08^{b}
Pilot-scale						
MAE-UAE	17.94	21.85 ± 0.54^{a}	37.01 ± 0.21^{b}	21.96 ± 2.35^{a}	25.19 ± 1.50^{a}	106.02 ± 2.30^{a}

Note: Results were represented as the mean ± SD of three independent determinations ($N = 3$). Values in the column followed by different letter superscripts are significantly different ($p < 0.05$), and values having the same superscript are not statistically significant.

3.2. Lab-scale preparation of PRE

The fractionation process to obtain the PRE was also investigated. The fractionation process used at the lab scale was divided into two steps.

3.2.1. Sample preparation for separation

The 1:3 diluted *C. asiatica* extract sustained the active compound in the stationary phase longer than the undiluted *C. asiatica* extract, which can be seen from the fraction of each method that eluted from the column. The best method had a low or non-detectable amount of the active components in the first fraction (CAE-L-1 or CAE-L-2) and presented high amounts of the active components in the fraction further eluted by absolute EtOH (CAE-L-1-A or CAE-L-2-A), as shown in Table 4. The active component content was high in the second fraction of the diluted *C. asiatica* extract (CAE-L-2-A). In addition, further study was performed by concentrating the initial extract with a rotary evaporator to half the initial volume before adding the water at the same ratio (1:3). This decreased the elution time of the sample from the column from 6 to 2 h, and the active components remained at the same level compared to CAE-L-2, as shown in Table 4. This fractionation from the concentrated extract was called CAE-L-2^{C}. Consequently, this method was used in the

Table 4
Pentacyclic triterpenes contents of PRE from different sample preparations and different mobile phase systems for separation.

Fraction of purification methods	Separation time (At a flow rate 5 mL/min)	Pentacyclic triterpene content (mg/g fraction)				
		AA	MA	AS	MS	Total
CAE-L-1	1 h	0.27 ± 0.04^{c}	4.01 ± 0.19^{d}	2.95 ± 0.17^{c}	5.73 ± 0.12^{d}	12.95 ± 0.45^{c}
CAE-L-1-A	12 h	19.38 ± 0.07^{a}	28.93 ± 0.12^{c}	6.87 ± 0.12^{b}	16.85 ± 0.21^{c}	72.02 ± 0.35^{b}
CAE-L-2	6 h	0.00 ± 0.00^{c}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}
CAE-L-2-A	12 h	18.67 ± 0.07^{a}	37.49 ± 0.28^{a}	12.79 ± 0.23^{a}	21.12 ± 0.26^{b}	90.06 ± 0.11^{a}
CAE-L-2^{C}	2 h	0.00 ± 0.00^{c}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}
CAE-L-2^{C}-A	12 h	16.48 ± 0.08^{b}	33.29 ± 0.16^{b}	11.45 ± 0.13^{a}	25.96 ± 0.22^{a}	87.18 ± 0.90^{a}
CAE-L-2^{C}	2 h	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
CAE-L-2^{C}–B-1	6 h	53.44 ± 0.58^{a}	101.11 ± 1.11^{a}	59.99 ± 3.60^{b}	120.12 ± 0.45^{b}	334.66 ± 4.43^{a}
CAE-L-2^{C}–B-2	8 h	15.13 ± 0.84^{c}	53.13 ± 1.62^{b}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	67.31 ± 2.42^{d}
CAE-L-2^{C}	2 h	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
CAE-L-2^{C}–C	8 h	1.87 ± 0.11^{d}	4.98 ± 0.12^{d}	120.19 ± 1.52^{a}	172.73 ± 0.22^{a}	299.77 ± 1.26^{b}

Note: Results were represented as the mean ± SD of three independent determinations ($N = 3$). Values in the column followed by different letter superscripts are significantly different ($p < 0.05$), and values having the same superscript are not statistically significant.

subsequent step of the study.

3.2.2. The ratio of EtOH and water as a mobile phase system

The effect of the mobile phase systems used to separate the aglycone and glycoside from the *C. asiatica* extract on the content of total pentacyclic triterpenes was evaluated. The results showed that the aglycones and glycosides in CAE-L-2^C–B could be separated, and this preparation showed a high amount of pentacyclic triterpenes, as shown in Table 5. Mixing the CAE-L-2^C–B-1 and CAE-L-2^C–B-2 fractions showed a higher pentacyclic triterpene content than CAE-L-2^C–C about 2 times. Moreover, the CAE-L-2^C–B column also helped separate the aglycones and glycosides. In addition, the amount of pentacyclic triterpenes obtained was equivalent to the previous extraction method [15,16,35], but this approach reduced the number of steps of the initial extract preparation and TLC determination.

3.3. Pilot-scale preparation of PRE

The *C. asiatica* extract from section 3.2 was used to prepare the PRE in the pilot-scale study using two mobile volumes. In the first study, the elution was started with 100 L of 50% EtOH (CAE-P-2^C–B-X-1) followed by 140 L of 75% EtOH (CAE-P-2^C–B-X-2). These volumes were the same ratio as in the lab-scale preparation. In the second study, the volume of each mobile phase was decreased to half: 50 L of 50% EtOH (CAE-P-2^C–B–Y-1) and 70 L of 75% EtOH (CAE-P-2^C–B–Y-2). The results showed that the CAE-P-2^C–B–Y method yielded a high pentacyclic triterpene content and used less mobile phase than the CAE-P-2^C–B-X method, as shown in Table 5. The CAE-P-2^C–B–Y-1 had a glycoside content of 256.87 mg/g fraction, the CAE-P-2^C–B–Y-2 had an aglycone content of 424.25 mg/g fraction, and the total pentacyclic triterpene content from CAE-P-2^C–B–Y method was 681.12 mg/g fraction, or 68.11%w/w. Thus, for the pilot-scale preparation of PRE, the CAE-P-2^C–B–Y condition should be used as the fractionation method because the volume of solvent and time needed for elution was half that of CAE-P-2^C–B-X. Moreover, this approach easily separates aglycone and glycoside, which is useful in some specific applications.

3.4. Biological activities

The biological activities of PRE were evaluated against four parameters: anti-inflammation in RAW264.7 cells, proliferation in HDF and HaCaT cells, migration in HDF cells, and collagen stimulation in HDF cells. Moreover, the results compared the activity of the PRE, *C. asiatica* extract, and the biomarker compounds (AA, AS, MA, and MS).

3.4.1. Anti-inflammatory activity

The anti-inflammatory activity of the *C. asiatica* extract, the PRE, and the markers of *C. asiatica* on lipopolysaccharide (LPS)-induced NO production in RAW264.7 cells is shown in Fig. 3. The PRE showed higher activity than the *C. asiatica* extract due to the concentration of AA and MA in the PRE. AA has shown inhibited LPS-induced NO and prostaglandin E2 (PGE_2) production [49]. MA has been shown to inhibit the LPS-induced expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) at the protein and mRNA levels in RAW264.7 cells [50]. Ju and co-workers have previously reported that *C. asiatica* extracts phytosome (5, 10, and 20 μg/mL) and potently inhibited LPS (1 μg/mL)-induced NO production as well as iNOS and COX-2 expression in RAW264.7 cells [51].

3.4.2. Proliferation and migration in HDF cells

Fibroblast proliferation and migration are important steps in wound healing for cell regeneration. The proliferation and migration activity of *C. asiatica* extract, the PRE, and the marker compounds were evaluated in HDF cells.

The PRE showed higher activity than *C. asiatica* extract for all tested concentrations (1–100 μg/mL) due to the concentration of AS and MS in the PRE, as shown in Fig. 4. AS has been observed as a bioactive compound for promoting the proliferation in wound healing process [52].

The migration test results indicated that the PRE increased the rate of cell migration significantly compared to *C. asiatica* extract. The PRE showed higher migration than the *C. asiatica* extract at 12, 18, and 24 h because the concentrations of AS and MS in PRE were higher than those in the *C. asiatica* extract, as shown in Fig. 5. AS has been reported as a bioactive compound for the migration in the wound healing process [6]. The migration of untreated cells was significantly less than those treated with *C. asiatica* extract and PRE.

Table 5
Pentacyclic triterpene contents of PRE from different mobile phase systems (in pilot-scale).

Fraction of purification methods	Yield (%)	Pentacyclic triterpene content (mg/g fraction)				
		AA	MA	AS	MS	Total
CAE-P-2^C–B-X-1	58.14	0.00 ± 0.00^c	55.38 ± 0.63^b	63.14 ± 2.71^b	112.4 ± 1.28^b	230.93 ± 4.56^c
CAE-P-2^C–B-X-2	22.20	328.53 ± 2.26^a	25.93 ± 0.80^d	5.36 ± 0.11^c	0.69 ± 0.07^d	360.51 ± 3.00^b
CAE-P-2^C–B–Y-1	49.86	0.00 ± 0.00^c	37.55 ± 0.37^c	76.76 ± 0.48^a	143.59 ± 3.51^a	256.87 ± 3.76^c
CAE-P-2^C–B–Y-2	32.40	162.36 ± 0.12^b	257.78 ± 0.72^a	0.54 ± 0.01^d	3.63 ± 0.24^d	424.25 ± 0.82^a

Note: Results were represented as the mean ± SD of three independent determinations ($N = 3$). Values in the column followed by different letter superscripts are significantly different ($p < 0.05$), and values having the same superscript are not statistically significant.

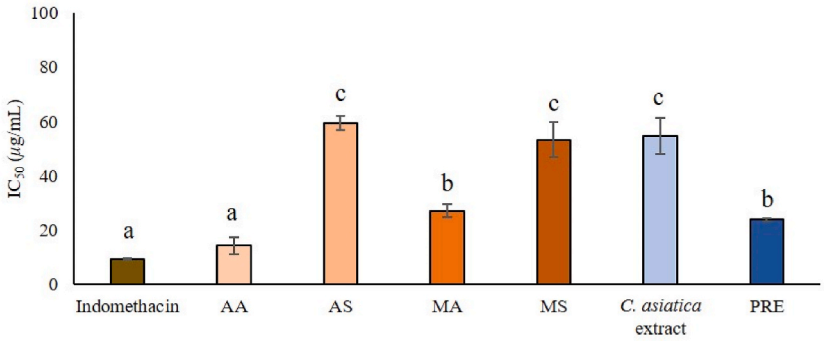


Fig. 3. NO inhibitory activity of the PRE, the marker of *C. asiatica*, and *C. asiatica* extract in RAW264.7 cells. (a–c) are significantly different ($p < 0.05$).

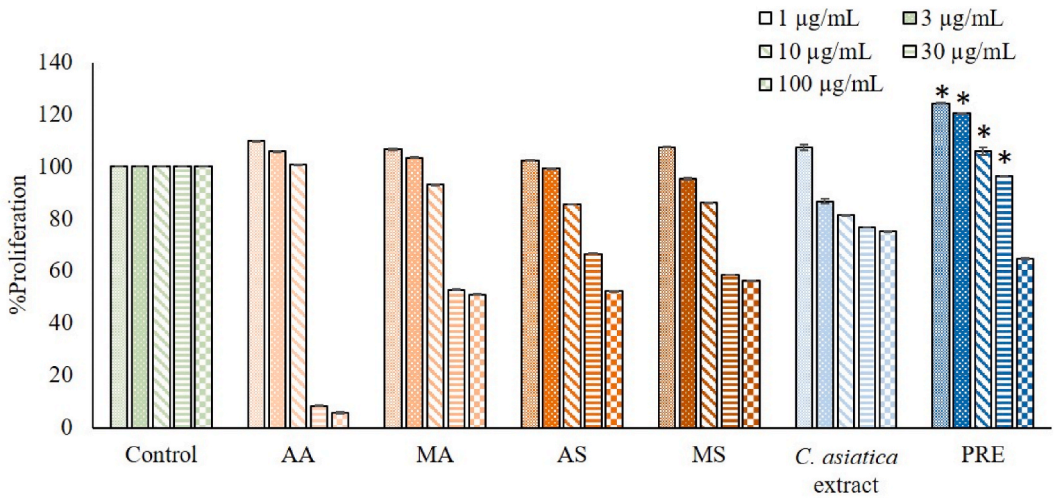


Fig. 4. Proliferation activity of the PRE, the biomarker of *C. asiatica*, and *C. asiatica* extract in HDF cells. The results are shown as means ± SEM. *$p < 0.05$ vs *C. asiatica* extract was considered to be significant. (Control: 0.1% DMSO in DMEM medium contained 2%FBS).

3.4.3. Effects on soluble collagen production in HDF cells

Type I collagen synthesis by HDF cells after 48 h of sample treatment at 10 μg/mL was examined. The production of collagen type I was significantly higher with PRE treatment than with *C. asiatica* extract ($p < 0.05$), as shown in Fig. 6, possibly due to the AS and MS content present in the PRE. Studies by Lu and co-workers, as well as other investigators, have found that AS strongly induced cell-cycle progression, proliferation, and collagen synthesis in dermal fibroblasts [9,52,53]. Wu and co-workers have reported that AS and MS (doses of 3 and 10 μM, respectively) promoted collagen synthesis in human skin fibroblasts [54]. The mixture of pentacyclic triterpenes showed a higher effect on collagen synthesis than cells treated with the individual marker compounds. These results support using highly enriched bioactive fractions rather than single pure compounds to achieve the desired bioactive property. This approach is also less expensive [14,35].

3.4.4. Proliferation in HaCaT cells

Skin comprises three types of cells: keratinocytes, melanocytes, and fibroblasts. In multiple wound healing, transplantation, and cell culture studies, it is shown that HaCaT cells may be used as an in vitro model for investigating the epidermal proliferative effect of active ingredients involved in wound healing [55–57]. The proliferation effects of *C. asiatica* extract, the PRE, and the marker compounds were evaluated on HaCaT cells.

The behavior of HaCaT cells on untreated and treated samples evaluated using MTT assays is presented in Fig. 7. The PRE showed higher activity than the *C. asiatica* extract in concentrations of 1–30 μg/mL due to the concentration of AS and MA in the PRE. AS is known to stimulate the wound healing process, induce increased cell behavior, and enhance monocyte chemoattractant protein-1 (MCP-1) production in HaCaT cells [58–60].

Many previously studied extraction methods focused on preparing and analyzing only one active compound in the extract and used some solvents that are toxic to the environment. This study used a green solvent and a green method for extracting and isolating the PRE. The results showed high pentacyclic triterpene content in the *C. asiatica* extract and PRE. Moreover, preparing extracts by MAE

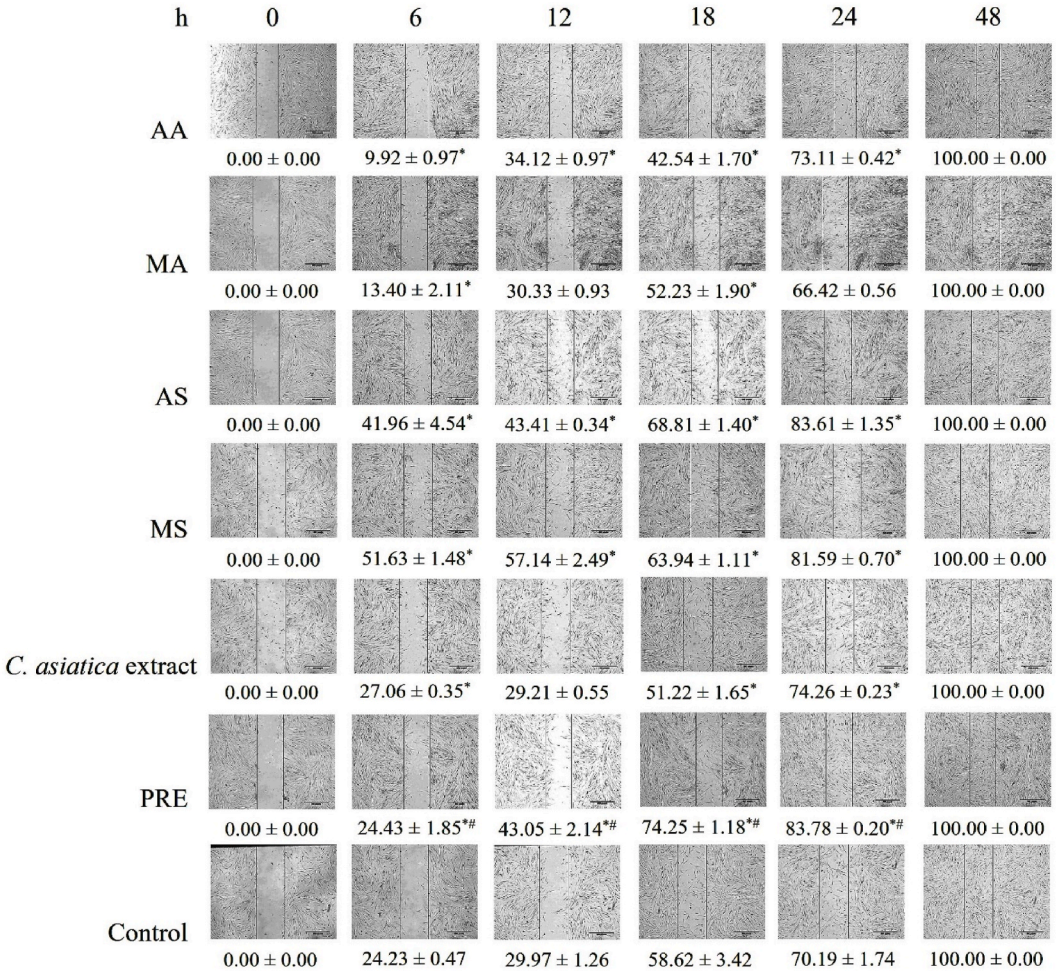


Fig. 5. Effects of the PRE, the biomarker of *C. asiatica*, and *C. asiatica* extract at dose 10 μg/mL on HDF migration. Images were recorded at 0, 6, 12, 18, 24, and 48 h. Quantitative analysis of the migration was calculated by computing software (Control: 0.1% DMSO in DMEM medium contained 2%FBS).

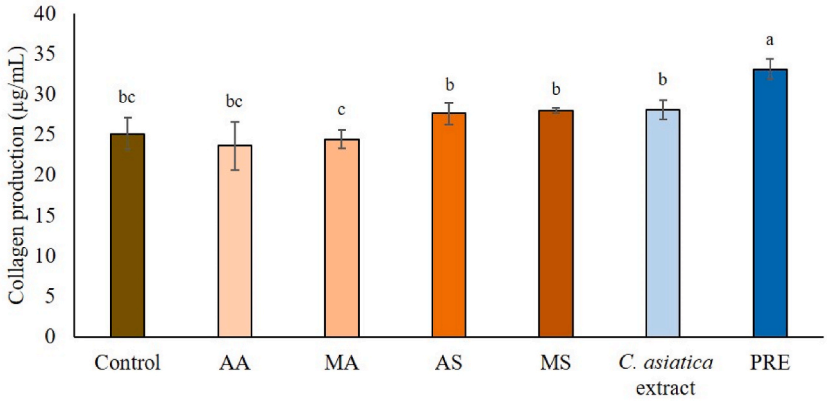


Fig. 6. Collagen type I production of the PRE, the marker of *C. asiatica*, and *C. asiatica* extract in HDF cells. (a–c) are significantly different ($p < 0.05$).

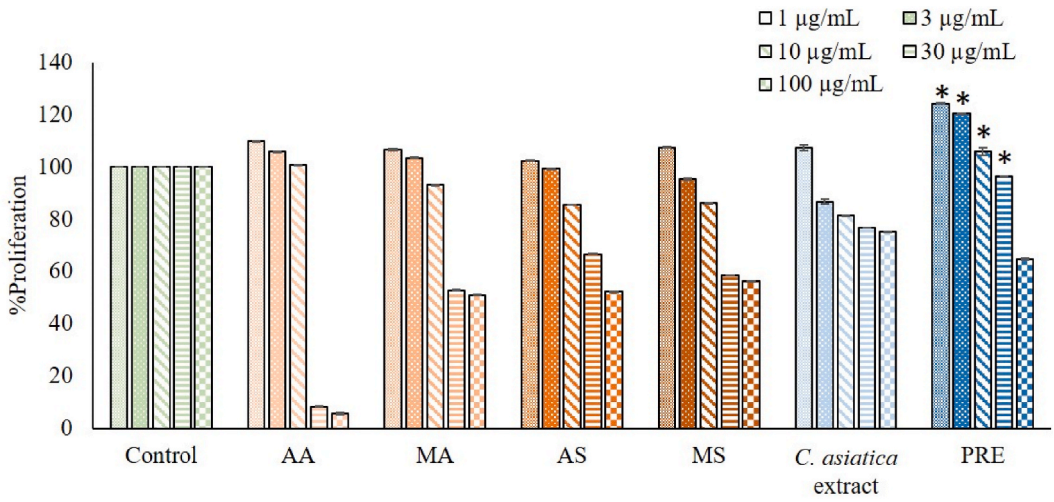


Fig. 7. Proliferation activity of the PRE, the biomarker of *C. asiatica*, and *C. asiatica* extract in HaCaT cells. The results are shown as means ± SEM. $^{*}p < 0.05$ vs *C. asiatica* extract was considered to be significant. (Control: 0.1% DMSO in DMEM medium contained 2%FBS).

and UAE with EtOH improved extraction efficiency, resulting in an increased yield and total pentacyclic triterpene content. Okselni and co-workers reported an extraction technique with the optimal AS yield from *C. asiatica* in the systematic review and meta-analysis results [61]. Our process also decreased the time and energy needed for drying and dissolving the extract and had a high yield of *C. asiatica* extract and PRE, especially for recovering the pentacyclic triterpene mixture (that is, AA, MA, AS, and MS) content at more than 68%w/w. In addition, the AS content was greater than 7%w/w, which is better than the content of AS extracted using only UAE (2.27%w/w) or MAE (2.66%w/w) [43]. Moreover, the PRE showed good anti-inflammatory effects and stimulated collagen production and the healing of wounds.

4. Conclusion

This research presents the first study optimizing *C. asiatica* extraction with combination methods that use a green extraction solvent and a new method for preparing the PRE. The study successfully determined the optimal conditions for high yields of *C. asiatica* extract and high total pentacyclic triterpene content in PRE. The results of this study helped to identify significant factors affecting the extraction yield and total pentacyclic triterpene content in *C. asiatica* extract and PRE. The extraction condition was the most important factor in *C. asiatica* extract preparation. The UAE-MAE extraction method with absolute EtOH solvent and *C. asiatica* particle size <355 µm was the best for preparing *C. asiatica* extract in lab-scale and scale-up experiments. Moreover, this research optimized the method for preparing the PRE by reducing the process of drying and reverse dissolution of the extract before loading it onto the column. The most suitable method for PRE was evaporation to decrease the volume of *C. asiatica* extract to half the initial volume and adding water in a ratio of 1:3. This was then loaded on the column and eluted with 50% EtOH and 75% EtOH. The PRE exhibited a pentacyclic triterpene content greater than 68%, could separate aglycone and glycoside, and showed high anti-inflammation activity in RAW264.7 cells, wound healing in HDF and HaCaT cells, and stimulated collagen production in HDF cells. This research can contribute to the future scale-up of *C. asiatica* extract production for the food, biochemical, and medical industries. As AA, AS, MS, and MA are the main triterpenes in *C. asiatica*, the detection of these bioactive compounds confirmed that this method is suitable for their extraction from the plant with the advantages of high yield, high pentacyclic triterpene content, and a green extraction method. These green and efficient procedures should be a promising option to guide industrial design for producing PRE from plants.

Author contribution statement

Mingkwan Rachpirom: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Wiwat Pichayakorn: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Panupong Puttarak, Ph.D.: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

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Credit authors statement

Mingkwan Rachpirom: Performed the experiments; Writing the original draft; Data curation; Formal Analysis; Investigation; Visualization.

Wiwat Pichayakorn:: Writing - review & editing; Conceptualization; Data curation, Methodology; Resources, Supervision; Validation.

Panupong Puttarak: Writing - review & editing; Conceptualization; Data curation; Formal Analysis; Funding acquisition; Methodology, Resources; Project Administration; Software; Supervision; Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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