Effect of drying methods and solvent extraction on the phenolic compounds of *Gynura pseudochina* (L.) DC. leaf extracts and their anti-psoriatic property

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Abstract

Gynura pseudochina (L.) DC. is a local herb that has been used as an anti-inflammatory agent. To study the utilization of plant phenolics for psoriatic treatment, the effects of the drying process, solvent extraction process and age of the leaves on the total phenolic content (TPC), total flavonoid content (TFC), crude extract and anti-oxidant activity were evaluated. The phenolic composition in the plant extracts was investigated. The most efficient process was applied to obtain a suitable extract for *in vitro* studies. HaCaT cells subjected to non-TNF-α and TNF-α stimulation were utilized to evaluate the cytotoxicity and anti-RelA, anti-RelB and anti-IL-8 properties of the extracts. The results showed that freeze drying and microwave drying preserved high levels of TPC, TFC and anti-oxidant activity. The HPLC results indicated that 50% (v/v) methanol demonstrated a good efficiency for recovering phenolic compounds, specifically chlorogenic acid (CGA), caffeic acid (CA), *p*-coumaric acid (PCA) and rutin (RUT). CA content was increased with microwave drying. Microwave drying and mixed-aged leaves were utilized for further studies. Ethanol was used instead of methanol because the former is a safer solvent for health product application. The LC-MS/MS results revealed that 25% (v/v) and 99.9% (v/v) ethanol extracts of mixed-ages leaves dried using a microwave (MLM) contained phenolic acids, flavonoids, a xanthone derivative, a phenylpropanoid, a phenolic glycoside compound and a glycerol-phospholipid. The MLM extract additionally contained pyrrolizidine alkaloids (PAs) at a low concentration. Co-extraction with 25% and 50% (v/v) ethanol of the MLM extract (EMLM) was performed for recovering each phenolic compound. EMLM extracts and their marker compounds (CGA, CA, RUT and PCA) were not toxic to HaCaT cells subjected to non-TNF-α and TNF-α stimulation, except for CA. The EMLM extracts and certain concentrations of the marker compounds could inhibit the RelB canonical pathway and IL-8 production. All results support the possible application of *G. pseudochina* leaf extracts for psoriasis alleviation.

Keywords: *Gynura pseudochina*; drying process; extraction; phenolic compound; anti-psoriasis

1. Introduction

Thailand has one of the richest biodiversities in the world, which enables the use of its biodiversity in several areas, specifically in terms of traditional medicine from plants. Exports from the herbal market have been continuously increasing (Kamontham and Sorngrung, 2016); however, the industries for herbal products in Thailand have not been growing possibly due to several limitations, including lack of supporting research, quality control and the latest technologies (Daengprasert et al., 2012). For this reason, the study of medicinal plants is challenging, particularly research to develop herbal products with standards of safety, quality and efficacy.

Gynura pseudochina (L.) DC. is a perennial herb in the Asteraceae family, which is distributed throughout tropical regions in South-East Asia and Africa (Vanijajiva, 2009). For a long time, this plant has been used as a traditional medicine in multiple areas. The plant leaves have been reported to possess anti-inflammatory and anti-viral uses (Plant Genetic Conservation Project, 2009). Its tubers have been used against inflammation, haemorrhages and dysentery (Windono et al., 2012). The medicinal properties of this plant, which have been researched and evaluated, include cytotoxic efficiency in leukaemia cells, anti-inflammation in both HeLa cells and human monocytes Siriwatanametanon et al., 2010), alleviation of scorpion envenomation in fibroblast cells (Uawonggul et al., 2006), anti-dengue activity in rats (Moektiwardoyo et al., 2014) and anti-HIV activity (Woradulayapinij et al., 2005). In addition, *G. pseudochina* var. *hispida*, which is a sub-species of *G. pseudochina*, has been reported to exhibit anti-inflammatory (Siriwatanametanon and Heinrich, 2011) and anti-psoriatic activities (Rerknimitr et al., 2016). *G. pseudochina* (L.) DC., a local plant species in Thailand (Vanijajiva, 2009), contains major phenolic compounds similar to those identified in *G. pseudochina* var. hispida (Mongkhonsin et al., 2016).

Psoriasis is a chronic inflammatory skin disorder affecting 2-5% of the world's population (Raychaudhuri et al., 2014). Recent research indicates that responses from immune cells, specifically the activation of T-cells, play an important role in the pathogenesis of psoriasis (Cai et al., 2012; Chen et al, 2016; Johansen, 2016; Diani et al, 2015). When T-cells are activated, multiple inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and interleukin 1-beta (IL-1β), are released (Baliwag et al., 2015), leading to the persistence of the disease. Furthermore, the expression of cytokines, chemokines and enzymes associated with inflammation in all cell types depends on the regulation of nuclear factor kappa B (NF-κB) (Christian et al., 2016).

NF-κB is a transcription factor that plays a fundamental role in controlling the cell cycle, cell proliferation and cell death, as well as regulating the immune system (Hayden and Ghosh et al., 2012). The NF-κB family comprises five related protein transcription factors, including NF-κB1/p50, NF-κB2/p52, RelA/p65, RelB and c-Rel, which are formed as hetero- or homodimers to function. In resting cells, NF-κB is sequestered in the cytoplasm through direct binding with an inhibitor of the kappa B (IκB) family. An initiating signal, such as TNF-α or lipopolysaccharide (LPS), induces activation of the inhibitor kappa B kinase (IKK) complex to liberate the active form of the NF-κB dimer by phosphorylation. The liberated NF-κB dimer is translocated to the nucleus and binds to recognized sites on the DNA sequence to stimulate gene expression (Christian et al., 2016). TNF-α stimulates the activation of NF-κB via two different pathways, the RelA canonical and RelB canonical pathways; thereby, these Rel proteins separately bind with NF-κB1/p50 (Hayden, 2012). In addition, interleukin 8 (IL-8) is a chemokine product from NF-κB regulation. It is released from various cell types, specifically T-cells and keratinocytes, while responding to inflammation (Brandt et al, 2017; Ferran et al, 2010; Wu et al., 2017). IL-8 plays a fundamental role in the recruitment and collection of the immune cells in keratinocytes during the development of psoriasis (Balato et al., 2012). Recently, several drugs have been effective in treating psoriatic patients. However, unpleasant side effects are always present and induce the development of drug resistance after long-term exposure (Mendonça and Burden, 2003; Herman and Herman, 2016). In addition, several psoriatic drugs need to be imported from foreign countries, which increases the cost of these products and leads to difficulty in accessibility for Thai psoriatic patients. Therefore, developing new anti-psoriatic agents with low toxicity remains important and represents an area of research for herbal product development.

Phenolic compounds are secondary plant metabolites that are produced during development and in response to various conditions (Rao and Ravishankar, 2002). Their structures strongly promote anti-oxidant activity (Stalikas, 2007). In addition, phenolic compounds can chelate metallic ions to prevent free radical production (Pereira et al., 2009) and are produced at increased levels in *G. pseudochina* under zinc and cadmium stress (Mongkhonsin et al., 2016). Moreover, these compounds present various biological activities including anti-cancer, anti-viral, anti-bacterial and anti-inflammatory activities (Kumar and Pandey, 2013). Quercetin 3-rutinoside, 3,5-di-caffeoylquinic acid, 4,5-di-caffeoylquinic acid and 5-mono caffeoylquinic acid from *G. pseudochina* var. *hispida* leaf extracts have anti-inflammatory properties; anti-NF-κB inhibition by these compounds has been reported (Siriwatanametanon and Heinrich, 2011).

An appropriate process for recovering active phenolic compounds from a plant is an important consideration. Phenolic compound recovery is variable, depending on the drying method (Chan et al., 2011; Lim et al., 2007; Chan et al., 2009), solvent extraction (Khoddami et al, 2013) and age of the leaves (Makkar et al, 1988; Makkar et al., 1991; Naz et al., 2013). Pyrrolizidine alkaloids (PAs) are natural chemicals, which have been identified in several plant species and are present as a mixture with beneficial compounds in plant extracts. Some of these alkaloids possess hepatotoxicity and toxicity, depending on the dose and time of intake (Neuman et al., 2015). Several PAs have been reported in the *Gynura* genus (Liang and Roeder, 1983; Lin et al., 2011; Qi et al., 2009; Wiedenfeld, 1982; Roeder, 1996; Windono et al., 2012). Therefore, these alkaloids should be considered before administration of natural products.

This study aims to conduct qualitative and quantitative analysis of the phenolic contents and compositions in *G. pseudochina* leaves prepared using different methods with respect to the drying process, age of the leaves and polarity of the solvents and using high-performance liquid chromatography (HPLC) techniques. Additionally, the extent of moisture removal, colour of the dried leaves, TPC, TFC, total pyrrolizidine alkaloid content (TPAsC) and free radical scavenging activity (FRSA) were determined. The chemical compositions of the plant extracts were identified using liquid chromatography mass spectrometry (LC-MS/MS). Moreover, the cytotoxicity of HaCaT cells following non-TNF-α and TNF-α stimulation was evaluated using an MTT assay. Additionally, immunofluorescence assays and enzyme-linked immunosorbent assays (ELISAs) were used to assess the inhibitory properties of *Gynura* extracts against RelA, RelB and IL-8 and to evaluate their marker compounds in HaCaT cells following TNF-α stimulation, respectively. The results from this study could provide a suitable preparatory process to obtain active compounds from *G. pseudochina* and lead to the first step in developing the utilization of this plant for skin health products.

2. Materials and Methods

2.1 Plant culture and plant materials

A voucher specimen of *G. pseudochina* (KKU No. 28875) was deposited at the Herbarium of Khon Kaen University (KKU), Thailand. Plantlets of *G. pseudochina* from a tissue culture system were cultivated in the Koeng Sub-district, Mueang District, Maha Sarakham Province, Thailand (16° 12’51” N, 103° 17’72” E) for three months per crop. Sandy loam soil was adjusted by amendment with manure and fertilizer, and the soil pH was 6.5±1.5. The weather during the

daytime in January 2014-April 2016 was 35±5°C, 20000±10000 lux light and 70±20% humidity. The shoots of the plants were harvested and washed with an excess of running tap water and then separated into young, developing and mature leaves. The leaf ages were estimated by leaf length and leaf position on the stem as young leaves (leaf length < 15 cm, the first to tenth leaves from the apical bud), developing leaves (leaf length 16-20 cm, the eleventh to twentieth leaves from the apical bud) and mature leaves (leaf length >20 cm, leaves after the twentieth leaf from the apical bud) (Fig. A1). For the freeze-drying process, liquid nitrogen was used to pre-freeze the leaves before being lyophilized overnight at -40°C, 0.5 psi in a freeze dryer (Heto Power Dry PL3000, Thermo Fisher Scientific, Japan). Microwave drying was performed using a digital microwave (Samsung J7EV, Malaysia) at 600 watts for 8 min. For the oven drying process, the leaves were dried in a hot air oven (RI 53 Binder, Germany) at 60°C for 48 h. All dry samples were ground into a homogeneous powder before being sampled for colour measurement using a bench top colorimeter spectrophotometer (ColorFlex EZ HunterLab, USA). The ground samples were preserved in a closed dark container with silica absorbent for further studies.

2.2 Plant extraction

An extraction was performed with a Soxhlet apparatus and heating mantle (MS-EAM M-TOP, Indonesia). The ratio of solid to liquid was 1:100 (w/v). A 2 g sample of each dried powder was loaded in a cellulose thimble (33 mm x 80 mm) (Whatman, GE Healthcare, UK) and extracted with 200 ml of each solvent.

The first step, serial extraction with various solvents, was conducted using various drying methods (freeze drying and microwave and oven drying) for different leaf ages (young, developing and mature leaves). A dried leaf sample was defatted with 95% (v/v) hexane (30 min per cycle) for five cycles and continuously extracted with 99.5% (v/v) ethyl acetate (35 min per cycle), 99.9% (v/v) ethanol (40 min per cycle) and 50% (v/v) methanol (50 min per cycle) for 10 cycles per solvent. Every fraction was filtered through Whatman no. 4 paper before being concentrated by a rotary vacuum evaporator. The concentrated extract was prepared up to a 25 ml final volume and kept in an amber glass bottle with a tight stopper at -20ºC until further analysis. The concentrated extract was analysed for weight of the crude extract, TPC, TFC, FRSA and HPLC. In addition, the mixed-age leaves dried through microwave-, oven- and freeze-drying processes were investigated via HP-TLC and TPAsC analysis. The extracts of the mixed-age leaves were obtained by defatting with hexane and continuous extraction for seven cycles per solvent of 50% (v/v) ethanol and 25% (v/v) ethanol, respectively.

The methanol residue in the crude extract must be considered; therefore, the plant extractions with various ethanolic concentrations were investigated to obtain an optimum condition. The microwave-dried sample of mixed-age leaves was defatted with hexane before a separate 10-cycle extraction with 25% (v/v) ethanol (60 min per cycle), 50% (v/v) ethanol (50 min per cycle), 75% (v/v) ethanol (40 min per cycle) or 99.9% (v/v) ethanol (40 min per cycle). Every ethanolic leaf extract was compared in terms of TPC, TFC, FRSA and HPLC chromatogram.

The microwave-dried and mixed-age leaves were used to prepare the crude extract for HaCaT cell studies. The plant leaves were defatted with hexane for five cycles, and the chlorophyll was removed using 99.9% (v/v) ethanol for two cycles. The samples were serial extracted with 50% (v/v) and 25% (v/v) ethanol for seven cycles of each solvent. The dried crude extracts collected from the 50% (v/v) and 25% (v/v) ethanol fractions were pooled together (EMLM) before analysis. The EMLM and chemical standards were applied to evaluate the cytotoxicity and the effects of the extract on the inhibition of RelA, RelB and IL-8.

2.3 TPC, TFC and FRSA determination

TPC was determined using a modified Folin-Ciocalteu method (Cicco et al., 2009). A 100 µl sample of an extract was mixed with 500 µl of 10% (v/v) Folin-Ciocalteu reagent. The mixture was placed in the dark for 3 min before the addition of 400 µl of 7.5% (w/v) Na_2CO_3. The mixture was incubated in the dark for 30 min, and the absorbance was determined at 731 nm using a UV-visible spectrometer (Beckman Coulter DU 730 Life Science, USA). The measurement was compared to a standard curve prepared with 10, 20, 40, 60, 80 and 100 mg/l of CA. The TPC value was expressed in terms of a CA equivalent (µmol CAE/ g dry weight).

TFC was measured using a colorimetric assay (Yoo et al., 2008). In brief, 500 µl of deionized water and 100 µl of an extract were added to a 1.5-ml microtube. Next, 30 µl of 5% (w/v) $NaNO_2$ was added and mixed. The mixture was kept in the dark for 5 min before addition of 60 µl of 10% (w/v) $AlCl_3$. After mixing and standing for 6 min, 200 µl of 1 M NaOH and 110 µl of deionized water were added to the mixture and mixed. After 5 min in the dark, the absorbance was measured immediately at 510 nm. The measurement was compared to a standard curve prepared with 10, 20, 40, 80 and 100 mg/l of epicatechin (EC). The TFC values were expressed in terms of an EC equivalent (µmol ECE/ g dry weight).

FRSA was evaluated based on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical method (Brand-Williams et al., 1995). In brief, 20 µl of different concentrations of each sample were mixed with 180 µl of 80 µM DPPH solution on a 96-well plate. The mixture was placed in

the dark for 30 min. Then, the absorbance was read at 515 nm using a microplate reader. The anti-oxidant activity of the extract was calculated using the following Eq. (1):

$$\text{Free radical scavenging activity (\%)} = \{(A_0 - A_i)/A_0\} \times 100 \quad (1)$$

where A_0 and A_i are absorbance values for the blank and test samples, respectively. The IC_{50} (the concentration of the sample required to inhibit 50% of the radicals) was calculated from the inhibition curve and compared with the IC_{50} of ascorbic acid.

2.4 TPAsC determination

TPAsC was measured using a colorimetric assay with some modifications. In brief, 50 µl of an extract was pipetted to a 1.5-ml microtube and heated for 5 min or until the solvent was evaporated completely. Next, 50 µl of natural product reagent I (1% (w/v) ethanolamine diphenyl borate in methanol) and 50 µl of natural product reagent II (5% (v/v) PEG-100 in ethanol) were added (Pothier, 2000). After 5 min of heating, 100 µl of 1% (w/v) modified Ehrlich's reagent (1 g of p-dimethylaminobenzaldehyde in 100 ml ethanol mixed with 15 ml of HCl) (Kone and Kande, 2012) was added and mixed thoroughly. The mixture was left to stand for 10 min, following which the absorbance of the magenta colour (purple-red) was read at 565 nm using a spectrophotometer. The measurement was compared to a standard curve prepared with 2.5, 5, 10 and 20 mg/l of monocrotaline (MCT). The TPAsC value was expressed in terms of MCT equivalent (µmol MCTE g^{-1} dry wt.).

2.5 HP-TLC analysis

The phenolic compounds contained in the plant extracts were analysed using an HPTLC system (CAMAG, Muttenz, Switzerland) consisting of 100-ml syringes on a sample applicator connected to a nitrogen tank, 20 x10 cm twin trough chamber, TLC Plate Heater and TLC visualizer linked to the visionCATS software. Each plant extract and standard compound were prepared at a concentration of 30 mg/ml. The resultant samples were filtered through a 0.22-µm pore size filter (Corning Inc., Corning, NY, USA) before being analysed on a silica 60F 254 aluminium sheet (10×20 cm) (Merck, Darmstadt, Germany) under the following conditions: syringe delivery speed, 10 s/µl; injection volumes, 4 µl for the plant extract and 2 µl for the standard; band width, 8 mm; and distance from bottom, 8 mm. The HPTLC plates were developed in AcOEt: CH_2Cl_2: H_2O: HCO_2: CH_3C_2H (65:16:7:6:6; v/v/v/v) using an automatic developing chamber after being saturated with the same mobile phase for 5 min at room temperature. The

distance of the chromatogram run was 70 mm from the sample start point. The natural product reagent I and the natural product reagent II (5% (v/v) PEG-100 in ethanol) were applied to derive the plate after mobile phase development (Pothier, 2000). The finished plate was then visualized under a UV light at 254 and 365 nm. Band identification was performed by comparison with four standards: chlorogenic acid (CGA), caffeic acid (CA), p-coumaric acid (PCA) and rutin (RUT).

2.6 HPLC and LC-MS/MS analysis

The phenolic compounds in the extracts were analysed by HPLC with a C18 guard column (4.6 mm x 10 mm, 5 µm) (VetiSepTM UPS C-18, Thailand) and a C-18 reversed-phase column (4.6 mm x 250 mm, 5 µm) (GL Science Lab InertSustain C-18, Japan). Each extract was filtered through a 0.22-µm nylon filter (Whatman, GE Healthcare, UK), before applying 20 µl of the sample. The mobile phase consisted of a gradient elution between 3% (v/v) acetic acid in water (solvent A) and 99.9% (v/v) methanol (solvent B) (Zuo et al., 2002), with a flow rate of 1 ml/min and a column temperature of 40°C. The HPLC chromatogram was detected at 280 nm for both phenolic acids and flavonoids with a UV-diode array detector (SPD-M20A, Shimadzu, Japan). The reference chemicals were CGA, CA, PCA RUT, gallic acid (GA), catechin (CAT), epicatechin (EC) and vanillin (VAN). Peak identification was performed by comparing the retention time (RT) with the standard compounds. CGA, CA and RUT, which were confirmed by the RT in LC-MS, were subjected to quantitative analysis using the external standard method. The same HPLC conditions were used with various concentrations for preparing the calibration curves.

Validation of the HPLC method was conducted in this study. The analytical curve of each standard was prepared between the concentration and the peak area of the phenolic compounds over a wide concentration range (5 and 100 µg/ml). The linearity of the curve was obtained from the correlation coefficient of the regression line. The blank data with a non-zero standard deviation was analysed to obtain the values of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD is presented in terms of a concentration corresponding to the sample blank value plus three standard deviations, and the LOQ is a concentration corresponding to the sample blank value plus ten standard deviations, as shown in the following Eq. (2) and (3), respectively (Shrivastava and Gupta, 2011):

$$\mathrm{LOD} = X_{\mathrm{blank}} + (3 \times SD_{\mathrm{blank}}) \quad (2)$$

$$\mathrm{LOQ} = X_{\mathrm{blank}} + (10 \times SD_{\mathrm{blank}}) \quad (3)$$

where *X* is the mean concentration value of the blank and *SD* is the standard deviation value of the blank.

The LOD and LOQ for each marker compound (CGA, CA, PCA and RUT) were analysed in methanol, which was represented as the applied matrix. The quantitative analysis of the marker compounds in all extracts was performed using an analytical curve with high linearity ($r^2 > 0.99$). The LOD values of CGA, CA, PCA and RUT were 1.64 µg/ml, 0.36 µg/ml, 0.16 µg/ml and 0.02 µg/ml, respectively. The LOQ values for CGA, CA, PCA and RUT were 1.81 µg/ml, 0.43 µg/ml, 0.24 µg/ml and 0.46 µg/ml, respectively.

The major components that could not be identified by HPLC and the chemical standards were determined by LC-MS/MS, with quadrupole-time of flight (QTOF) mass analysers. The LC-QTOF-MS/MS analysis was performed on an Agilent HPLC 1260 series coupled with a QTOF 6540 UHD accurate mass (Agilent Technologies, Waldbronn, Germany). The separation of the sample solution was performed on a Luna C18(2) 150 x 4.6 mm, 5 µm (Phenomenex, USA). A 5 µl sample of each filtrated extract was injected into the LC system with a solvent flow rate of 500 µl/min. The mobile phase consisted of a gradient elution between water (solvent A) and acetonitrile (solvent B), both containing 0.1% v/v formic acid. The linear gradient elution was 5% to 95% for solvent B at 35 min and post-run for 5 min, and the column temperature was controlled at 35°C. The mass analysis was performed using a QTOF 6540 UHD accurate mass. The conditions for the negative ESI source were drying gas (N_2) flow rate 10 l/min, drying gas temperature 350°C, nebulizer 30 psig, fragmentor 100 V, capillary voltage 3500 V and scan spectra from *m/z* 100-1500 amu. The auto MS/MS for the fragmentation was set with collision energies of 10, 20 and 40 V. The Agilent MassHunter qualitative Analysis Software B06.0 (Agilent Technologies, CA, USA) was applied for data analysis.

2.7 Cell cytotoxicity

The plant extracts were dissolved in 50% (v/v) dimethyl sulfoxide (DMSO) to obtain a concentration of 50 mg/ml for the stock solutions. The resultant extracts were filtered through a 0.2-µm pore size filter (Corning Inc., Corning, NY, USA). HaCaT cells (Cell Line Service, Heidelberg, Germany) were cultured in Dulbecco's modified Eagle's medium/high glucose (DMEM/HG) (GibcoTM ThermoFisher Scientific, USA) with 10% (v/v) foetal bovine serum, 62.5 µg/ml penicillin and 100 µg/ml streptomycin. The cell cultures were incubated at 37°C in a 5% CO_2 atmosphere. At 80% confluence, the cells were dissociated with 3 ml of 5% (w/v) of trypsin for 15 min. The detached cells were plated into 96-well plates at a density of 5×10^4 cells/ml and allowed to grow for 12 h before being pre-treated with or without 50 ng/ml TNF-α for 12 h.

The cells with or without TNF-α pretreatment were treated with different concentrations of the MLM extracts (15.7, 31.3, 62.5, 125, 250, 500 and 1000 µg/ml) for 24 h. Four marker compounds, CGA (37.5, 75, 150, 300 and 600 µg/ml), CA (15.6, 31.3, 62.5, 125 and 350 µg/ml), RUT (62.5, 125, 250, 500 and 1000 µg/ml) and PCA (312.5, 625, 1250, 2500 and 5000 µg/ml), were used as the standards. Paclitaxel (PTX) at 0.3, 0.6, 1.3, 2.5 and 5.0 µg/ml was used as a positive control. The DMSO contained in the medium was controlled to less than 1%. After 24 h of exposition, the medium was aspirated, the cells were washed twice with PBS, and 110 µl of a solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at a final concentration of 0.5 mg/ml was added over 2 h. Next, the MTT solution was removed and washed with 0.01 M phosphate-buffered saline (PBS) before being replaced with 100 µl of DMSO to dissolve the crystals of dark-blue formazan. The absorbance was read at 540 nm (Tse et al., 2006). The cytotoxicity to HaCaT cells was expressed as an IC_{50} value. Cell survival (%) was identified as the fraction of cells that were alive relative to the control for each point and calculated by the following Eq. (4):

$$\text{Cell survival rate (\%)} = \{(OD_{Sample} - OD_{Blank})/(OD_{Control} - OD_{Blank})\} \times 100 \quad (4)$$

The IC_{50} is the concentration of the sample required for 50% cell survival, as calculated from the cell survival curve.

2.8 RelA, RelB and IL-8 inhibition

The HaCaT cells were cultured in DMEM/HG without phenol red (GibcoTM, ThermoFisher Scientific, USA) under the same conditions as above. The confluent cells were dissociated with trypsin and plated into 12-well plates at 5×10^4 cells/ml for 12 h before pretreatment with 50 ng/ml TNF-α for 12 h. The TNF-α-stimulated cells were treated with different concentrations of samples that were determined based on the IC_{50} and half IC_{50} values obtained from the cytotoxicity studies on TNF-α-stimulated cells as follows: 750 and 375 µg/ml for the MLM extracts; 280 and 140 µg/ml for CGA; 60 and 30 µg/ml for CA; 1500 and 750 µg/ml for RUT; 2800 and 1400 µg/ml for PCA; and 50 µg/ml of curcumin (CUR) as a positive control. After 24 h of treatment, the medium was removed and washed with PBS twice. The cells were fixed with 2 ml of methanol and acetone (1:1) at 4°C for 10 min. The cell membrane was permeabilized with 0.1% (v/v) of Triton X-100 for 10 min before washing with PBS and blocking with 1% (w/v) bovine serum albumin (BSA) for 1 h (Shukla et al., 2015). The cells were incubated with antibodies overnight (1:100) following anti-phospho-RelA (Ser529) conjugation with DAPI

and anti-phospho-RelB (Ser573) conjugation with GFP. The nucleus was counterstained with Texas Red for 5 min. The cells were washed with PBS twice to remove unbound dye before being observed under an EVOS FL Cell Imaging System (Life Technologies, UK) at 40x. The cell culture medium was collected after treatment of each sample from this experiment to measure the IL-8 content using an ELISA Kit (GeneTex International Corp., UK). Next, 100 µl of each sample was added into pre-coated 96-well plates, the plate sealed with a cover, and incubated at 37°C for 90 min, following which the contents of the plate were discarded, and the plate was blotted onto paper. Next, 100 µl of biotinylated anti-human IL-8 antibody was added into each well, and the plate was incubated at 37°C for 60 min before washing it thrice with PBS. A 100 µl sample of avidin-biotin-peroxidase complex (ABC) working solution was added and incubated at 37°C for 30 min before washing the samples five times with PBS. Next, 90 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) colour developing agent was added to each well and incubated at 37°C for 20 min before adding 100 µl of the stop solution. The absorbance was read at 450 nm within 30 min after exposure to the TMB stop solution. The measurement was compared to an IL-8 standard curve prepared using concentrations of 7.8, 15.6, 31.3, 62.5, 125, 250 and 500 pg/ml.

2.9 Statistical analysis

The data were expressed as the means and standard deviations (*SD*) from three replicated values. The analysis was performed using the SPSS statistical software (SPSS 14, SPSS Inc., IL, USA). The analysis of variance (ANOVA) was significantly determined for differences between the means under Scheffe's test and Duncan's new multiple range test (DMRT). The Spearman correlation coefficients were determined to compare the correlations between the variations.

3. Results and discussion

3.1 Dried leaf colour and moisture removal

The moisture removal in terms of the percentage difference among the different drying processes and leaf ages are shown in Table 1. The results indicated that freeze drying and microwave and oven drying for all leaf ages demonstrated a similar extent of moisture removal. *G. pseudochina* is a semi-succulent plant with the fresh weight presenting a moisture content of approximately 90-95% (Perera, 2014). The freeze drying preserved the green colour of the leaves (Fig. A2). The thermal drying method using a microwave and oven decreased the lightness (*L**)

and yellow colour (*b**) of the *G. pseudochina* leaves (Table 1), which might be caused by the non-enzymatic browning reaction from thermal drying to induce darkening of the dried plant material (Vega-Gálvez et al, 2009). In addition, for oven drying, moderate temperatures of 50-70°C for a prolonged time were sufficient for major changes in the pigments of the plants, such as β-carotene (Youssef and Mokhtar, 2014) and chlorophylls (Roshanak et al., 2016).

3.2 TPC, TFC, FRSA and crude content of plant extracts

G. pseudochina leaves prepared from different drying processes and leaf ages were used for serial extraction with 99.5% (v/v) ethyl acetate, 99.9% (v/v) ethanol and 50% methanol. The results demonstrated that the TPC and TFC were more efficiently recovered from the freeze-dried and microwave-dried samples (Fig. 1a-1d). The leaf extracts obtained from the ethyl acetate fraction tended to contain higher TPC and TFC than the ethanol and 50% methanol fractions (Fig. 1a and 1c). However, use of the 50% methanol solvent resulted in crude extracts with the highest yield among all samples (Fig. 1e), which led to the highest recovery of the TPC and TFC from the 50% methanol solvent with respect to the plant dried weight term (Fig. 1b and 1d). In addition, Fig. 1f shows the FRSA of the leaf extracts as IC_{50} values. A low IC_{50} value indicates a high anti-oxidant activity. The leaf extracts obtained from freeze drying and microwaving distinctly exhibited lower IC_{50} values than the crude extracts from the oven samples. Serial extraction was performed in this study; therefore, the summary of TPC, TFC and crude contents from the fractions of ethyl acetate, ethanol and 50% methanol are presented in Table 2. TPC and TFC were recovered with a higher yield in freeze-dried and microwave-dried samples in all leaf ages. Interaction plots between different factors included leaf ages, drying processes and polarity of solvents, affecting the TPC, TFC and crude contents, as shown in Fig. A3. Co-factors of the leaf age and drying process and the polarity of the solvents and drying process in plant leaf preparation led to interactions on TPC, TFC and crude content recoveries that may be additive, antagonist and synergistic. The effects of each factor on TPC, TFC and crude content recoveries was evaluated by the values of sum of square (*SS*) (data not shown) that revealed that the critical factors were ordered in the following manner: polarity of solvents> drying processes>>leaf ages. The Spearman's correlation coefficient (*r*) are shown in Table A1. The statistical analysis indicated that the drying processes had a negative correlation (r = -0.716 and - 0.582) with TPC and TFC, respectively, in the order of freeze dry, microwave and oven. The drying processes had a positive correlation (r = 0.542) with IC_{50}, which implied a decreasing FRSA. In addition, the solvents in the order of ethyl acetate, ethanol and 50% methanol exhibited a positive correlation with TFC (r = 0.402) and crude content (r = 0.764). In addition, TPC demonstrated a strong positive

correlation to TFC (r = 0.926) and a moderately positive correlation to the crude content (r = 0.583); however, TPC exhibited a strong negative correlation to IC_{50} (r = -0.853). TFC exhibited a moderately positive correlation with crude contents (r = 0.596) but demonstrated a strong negative correlation to IC_{50} (r = -0.857). In particular, the crude content caused high TPC and TFC values that lead to a low IC_{50}. The different leaf ages did not significantly correlate with TPC, TFC, IC_{50} and crude content.

The appropriate solvent for extraction of phenolic compounds from each plant depends primarily on the nature of the sample material and the properties of the phenolic structure and polarity (Khoddami et al, 2013). Very polar phenolic compounds could be extracted completely with a mixture of solvents, alcohol and water (Stalikas, 2007). This result indicated that *G. pseudochina* leaves contained high polar phenolic compounds. The freeze-drying method has been applied to dehydrate the plant materials. Phenolic compounds strongly relate to anti-oxidant properties because their structures promote the delocalization of radical electrons to the nucleus (Pereira et al., 2009; Stalikas, 2007). The freeze-drying method can lead to high efficiency of phenolic extraction because the freezing process causes ice crystals inside the plant cells that result in a greater rupturing of the plant cell structure. Fast freezing with liquid nitrogen before drying in this study promotes separation and provides very little cell collapse and damage (Voda et al., 2012), which could support solvent access and phenolic extraction from plant materials. On the other hand, microwave radiation suddenly increased the temperature and pressure inside the cells, led to a quick rupture of the cells and provided more sample surface (Li et al., 2012). The oven drying process decreased phenolic compounds distinctly because of a high temperature and longer drying time in the conventional oven process, resulting in the destruction of plant compounds (Vadivambal and Jayas, 2007).

3.3 Phenolic composition by HPLC

The HPLC profiles of the extracts from various leaf ages dried with freeze drying and microwave and oven drying processes are shown in Fig. 2. Four peaks at the retention times of 18.6, 20.2, 24.8 and 29.8 min were identified by LC-MS/MS as CGA, CA, PCA and RUT. The normalized HPLC chromatogram confirmed that the leaf extracts from the 50% methanol fractions contained several phenolic compounds, more than those in the ethyl acetate and ethanol fractions. In addition, the 50% methanol fractions of the freeze dried and the microwave samples contained higher CGA, CA and RUT amounts than those in the oven samples. Moreover, the CA was recovered increasingly from the microwave samples in 50% methanol. In addition, the normalized HPLC chromatograms and relative intensities of the 50% methanol fractions of

various leaf ages were compared for each drying process, as shown in Fig. A4. The HPLC comparison indicated the high efficiency of the freeze drying and microwave drying processes for post-harvest phenolic recovery. Additionally, there were changes in the peak heights of CGA and CA. Leaf maturity might decrease CGA and increase CA.

Likewise, the drying processes affected the phenolic compounds in several plant materials (Heck et al., 2008; Cerretani et al., 2009; Bey et al., 2016). In addition, leaf maturity might decrease CGA and increase CA in the 50% methanol extract. CGA is implicated in plant responses to multiple biotic stresses, and CGA was produced increasingly for plant resistance to pests (Leiss et al., 2009). In addition, the amount of CGA decreased during leaf maturity because the compound was used for the synthesis of cell-wall-bound phenolic polymers, such as lignins (Aerts and Baumann, 1994; Khoddami et al., 2013). The lignin content increased with the maturity of the plant (Rencoret et al., 2011; Cesarino et al., 2012). CA is an intermediate in lignification, and it presents as caffeoyl shikimic acid, caffealdehyde and caffeoyl alcohol through the shikimic pathway (Weng et al., 2010). Therefore, CA production might increase during leaf maturity. Microwave radiation affected the reorientation of the hydrogen bond that lead to liberating free phenolic compounds from the bound phenolic compounds (Lewicka et al., 2015; Xu et al., 2007). For this reason, free CA might be liberated from the bound compounds, such as lignin, when stimulated by microwave energy. In addition, microwave radiation could exhibit the formation of reactive free radicals to produce oxidation products in plant cells (Cerretani et al., 2009). This might lead to rapid production of anti-oxidant compounds such as free CA from bound phenolics. Prolonged exposure to high temperature during the oven drying process destroyed the phenolic compounds and changed the structure of the non-extractable phenolic compounds (Pinelo et al., 2005; Xu et al., 2007; Honest et al., 2016). In this study, both freeze drying and microwave drying could retain the high phenolic compounds from the *G. pseudochina* leaves, specifically CGA, CA and RUT. However, microwave drying provided more advantages than freeze drying for operation, in terms of cost, ease of use and shorter time (Vadivambal and Jayas, 2007). In addition, the microwave-dried *G. pseudochina* leaves provided a high quality and quantity of phenolic compounds and increased CA content. Therefore, the mixed-age leaves dried with a microwave (MLM) and 50% methanol extraction were considered suitable processes to recover the marker compounds from *G. pseudochina* leaves.

Although our previous results showed that extraction using 50% methanol exhibited the highest efficiency, methanol residue in the crude extract must be considered. Therefore, plant extractions were investigated with various ethanolic concentrations to extract the MLM, as shown in Fig A5 and Table 3. The results indicated that 25% ethanol was suitable for the recovery of

TPC, crude extract, CGA and CA. In addition, 50% and 75% ethanol were suitable for the recovery of TFC, CGA and RUT. Therefore, the extraction with both 50% and 25% ethanol was considered as an appropriate process to obtain the phenolic and marker compounds from the MLM extract.

3.4 Phenolic identification by LC-MS/MS

LC-ESI-QTOF-MS/MS was applied to characterize the unknown compounds in the MLM extracts. According to Fig. A5, the HPLC chromatograms of the 25% and 100% ethanol extracts show different marker peaks. Their LC-ESI-QTOF base peak chromatograms (BPC) are presented in Fig. 3. Table 4 shows the mass spectra with characteristic fragmentation patterns obtained in the negative ionization mode. The peak nos. 4, 8 and 9 were identified as CGA, CA and RUT by comparison of the retention times and mass spectra with data from the reference compounds. The results indicated that the ethanolic extracts of MLM contained various groups of phenolic compounds including phenolic acids (CA, CGA, dicaffeoylquinic acid and 3-O-caffeoyl-1-O-methylquinic acid), flavonoids (RUT and kaempferol rutinoside, quercetin, (+)-tephropurpurin, 5-hydroxy-2'-methoxy-6,7-methylenedioxyisoflavone and 2-(2,4-dihydroxyphenyl)-5-hydroxy-8-methyl -8-(4-methyl-3-penten-1-yl)-2,3-dihydro-4H,8H-pyrano [2,3-f]chromen-4-one), a xanthone derivative (1,3,8-trihydroxy-4-methyl-2,7 diprenylxanthone), a phenylpropanoid (3,4 dihydroxycinnamoyl (Z)-2-(3,4-dihydroxyphenyl) ethanol), a phenolic glycoside compound (unknown-C-glycoside) and a glycerol-phospholipid (1-(9Z-octadecenoyl)-sn-glycero-2,3-cyclic phosphate). Caffeoyl quinic acid, which is an ester of caffeic acid and (-)-quinic acid, was identified as a marker compound in the *Gynura* species. Different isomers of caffeoyl quinic acid were found in butanolic extracts of *G. bicolor* and *G. divaricata* extracts including trans-5-O-caffeoylquinic acid, cis-3-O-caffeoylquinic acid, trans-3-O-caffeoyl quinic acid and cis-4-O-caffeoylquinic acid (Chen et al., 2014a; Chen et al., 2015). The 5-O-caffeoyl-D-quinic acid was found in the ethanolic extract of *G. procumbens* (Jarikasem et al., 2013). In addition, caffeic acid is the crucial phenolic for the formation of plant lignin (Weng and Chapple, 2010). Quercetin rutinoside, which is the glycoside between the flavonol and the disaccharide rutinose, was found in the methanolic extract of *G. pseudochina* (Mongkhonsin et al, 2016), ethanolic extract of *G. divaricate* (Wan et al, 2011), butanolic extracts of *G. bicolor* and *G. divaricata* extracts (Chen et al, 2015) and methanolic extract of *G. pseudochina* (Siriwatanametanon and Heinrich, 2011). Dicaffeoyl quinic acid, which is the isomer containing two molecules of caffeic acid, was present as various isomers. The three isomers trans-3,4-dicaffeoyl-quinic acid, trans-3,5-dicaffeoylquinic acid and trans-4,5-dicaffeoylquinic acid were

isolated from the ethyl acetate extract of *G. divaricata* (Chen et al., 2014a). 4,5-Dicaffeoylquinic acid was obtained from the ethanolic extract of *G. procumbens* (Jarikasem et al., 2013). In addition, kaempferol rutinoside was isolated from the butanolic and ethanolic extracts of *G. divaricate* (Chen et al., 2015; Tan et al., 2013; Wan et al, 2011). Moreover, this is the first report that identified (+)-tephropurpurin in the *Gynura* species. For the most part, this compound is the chalcone flavonoid that has been identified as a marker compound in the genus *Tephrosia* (Chen et al., 2014b). 1,3,8-Trihydroxy-4-methyl-2,7 diprenylxanthone was identified in mangosteen (Yannai, 2012), in which various derivatives of xanthone have been reported for several medicinal properties such as anti-inflammation (Pedraza-Chaverri et al., 2008). 3-O-Caffeoyl-1-O-methylquinic acid is a chlorogenic acid derivative, which was first isolated from an ethanolic extract of bamboo; this compound is an anti-oxidant agent with a potential for the prevention of ROS cellular damage (Kweon et al., 2001; 2006). In addition, 3,4 dihydroxycinnamoyl (Z)-2-(3,4-dihydroxyphenyl) ethanol or nepetoidin B is a phenyl-propanoid compound that has been isolated from multiple herbs. It is reported to possess anti-inflammatory activity due to its potential to inhibit NF-κB/p65 phosphorylation and nuclear translocation (Wu et al., 2017). Quercetin is an abundant flavonoid compound observed in various plants (Srivastava et al., 2016). The quercetin isolated from the methanolic extract of *G. pseudochina* exhibited the highest anti-oxidant activity among all isolated compounds (Rivai et al., 2017). In addition, various isomers of hydroxyl methoxy methylene dioxyisoflavone were isolated from several plant species (Harborne, 1980).

3.5 Pyrrolizidine alkaloid determination

Although plants are a large source of several active compounds, some toxic compounds from plant extracts are presented as a mixture with beneficial compounds, specifically the PAs. To ensure the suitability of the microwave drying method for preparation of the valuable crude extract, HP-TLC was used to investigate the chemical composition of mixed-age leaves of *G. pseudochina* dried through freeze drying (MLF) and oven drying (MLO). Fig. A6 illustrates that the MLF and MLM extracts chiefly contained phenolic compounds, and rutin was abundantly present as flavonoids in the extracts. Therefore, these HP-TLC profiles supported the efficiency of the microwave drying process in retaining the phenolic compounds in *G. pseudochina* leaves. Total pyrrolizidine alkaloid content (TPAsC) in the *G. pseudochina* extracts were determined in terms of monocrotaline equivalence, as presented in Table 5. The TPAsC was in the range of 0.003±0.002 to 0.684±0.053 mmol monocrotaline equivalence/g leaf extract. The highest TPAsC was found in the 50% ethanol fractions of the MLO extract. Although the MLM extracts contained

the lowest TPAsC, the content of the PAs in the MLM extracts must be controlled in a safe range when applied in health products. Interestingly, oven drying could increase the PA content in plant material. The PAs are synthesized from polyamines including putrescine and spermidine, with homospermidine synthase (HSS) as a key enzyme to produce the N-oxide forms of alkaloids such as PAs (Dreger et al., 2009). Polyamine production increases when the plants are stressed (Takahashi and Kakehi, 2009). During a temperature increase to 60°C for this study, the HSS enzyme might become over active and respond to high temperature stress, which could lead to a rapid production of the PAs. The United Kingdom Medicines and Healthcare Product Regulation Agency regulates PAs in herbal products to 1 mg/day (16.7 µg/kg/day for 60 kg of body weight) for two weeks or to 0.1 mg/day (1.67 µg/kg/day) for long-term use (Neuman et al., 2015). In addition, the European Medicines Agency restricts the maximum dose to 1 µg for a few years and 0.35 µg for long periods (European Medicines Agency, 2016).

3.6 Cytotoxicity of EMLM extracts on HaCaT cells

The EMLM extract was evaluated for its cytotoxicity. The HPLC chromatogram of the EMLM extract and its marker compounds are presented in Fig. A7 and Table A2, respectively. The cytotoxicity of the extract on non-TNF-α- and TNF-α-stimulated HaCaT cells was evaluated by applying various concentrations of the EMLM extract and each standard compound. The cytotoxicity in terms of IC_{50} values approximated from a linear trendline are presented in Table 6. The EMLM extract showed milder toxicity in HaCaT cells under both conditions than other standard chemicals. Among each standard marker, CA exhibited the highest toxicity in the HaCaT cells under both conditions, and CGA showed moderate cytotoxicity. In contrast, RUT and PCA were not toxic to the HaCaT cells under both conditions due to their high IC_{50} values. The EMLM extract exhibited similar cytotoxicity in the HaCaT cells under both conditions. CGA, CA and PCA showed decreasing toxicity, while RUT showed increasing toxicity, in the TNF-α-stimulated cells. In addition, the content of each marker compound contained in 750 µg of the EMLM extract (approximated from the IC_{50} value of the EMLM extract in Table 6, which was 744.02 µg/ ml) are presented in Table A2. The data implied that the marker compounds composing the EMLM extract were toxic to the TNF-α-stimulated cells at lower concentrations than that when each pure marker compound was applied (Table 6). The MTT assay is a standard method to evaluate cytotoxicity via measurement of the insoluble dark-blue formazan product, which is formed from cleaved MTT by active mitochondria (Slater et al., 1963; Mosmann et al., 1983). In this study, the EMLM extract and certain marker compounds (CA and CGA) were toxic to the HaCaT cells that were not subjected to TNF-α and non-TNF-α stimulation. Some phenolic compounds could

affect cell cytotoxicity. Various metabolic processes in keratinocytes can provide peroxidase, and the phenolics may get converted to multiple ROS that can damage mitochondria (Galati et al., 2002).

3.7 NF-κB and IL-8 inhibitory properties

The inhibitory property of the EMLM extract and standard chemical compounds to RelA and RelB were observed on the TNF-α-stimulated HaCaT cells via immunofluorescence assay. The anti-phospho-RelA S529 and anti-phospho RelB S573 were applied to bind with the specific protein. Therefore, the fluorescent signals were presented as the activated forms of RelA (blue) and RelB (green) after phosphorylation at the specific position. Fig. 4 demonstrates that RelA S529 was not active in the non-stimulated HaCaT cells. In addition, RelA S529 was very slightly active under TNF-α stimulation, and localization of the blue colour was difficult to observe. Alternatively, RelB S573 was strongly active in the non-stimulated HaCaT cells, but most of them were sequestered in the cytoplasm. In addition, TNF-α could stimulate the translocation of RelB S573 into the nucleus. Moreover, EMLM extracts could suppress the translocation of RelB S573 into the nucleus. In addition, some concentration of each marker compound CGA, CA, RUT and PCA inhibited the translocation of RelB S573 into the nucleus. In addition, this result indicates that TNF-α could strongly stimulate the NF-κB function through RelB canonical pathways in HaCaT cells. Additionally, RelA was observed in the HaCaT cells; however, activated RelA in these cells may be phosphorylated at another site, not S529 (Kang et al., 2008; Feng et al, 2013; Ren et al, 2006). RelA can be phosphorylated at different serine and methionine sites in the polypeptide (Christian et al., 2016). Previous studies found RelA S529 in cancer cells upon IL-1β or TNF stimulation (Wang and Baldwin, 1998; Wang and Westerheide, 2000) and in monocytes under LPS stimulation (Bristow et al., 2008). However, phosphorylation at S529 in HaCaT keratinocyte cells has not been reported. In addition, *G. pseudochina* var. *hispida* and its marker compounds, including rutin and caffeic acid derivatives, demonstrated an inhibitory effect on NF-κB in macrophage cells (Siriwatanametanon and Heinrich, 2011).

The inhibitory properties of IL-8 were evaluated using ELISA, as presented in Table 7. IL-8 was increasingly produced due to TNF-α stimulation. The 0.7% DMSO contained in the *in vitro* system did not inhibit the protein production. Two concentrations of the EMLM extract, CA and RUT could inhibit IL-8 production, while only the higher concentration of CGA and PC each inhibited IL-8 production. IL-8 is an inflammatory chemokine that has been produced from keratinocytes to attract various immune cells to migrate to epidermal cells, leading to the beginning of an inflammation process and psoriatic disease (Bristow et al., 2008). In addition,

IL-8 production is regulated by the NF-κB transcription factor (Kunsch and Rosen, 1993), and it was observed to be upregulated in psoriatic skin by more than 80% compared to normal skin (Baliwag et al., 2015). The inhibition of IL-8 production might be caused by the inhibition of NFκB function. Phenolic compounds have been reported to exhibit anti-IL8 properties. Dihydrocaffeic acid demonstrates a potential for IL-8 inhibition in HaCat cells after exposure to UV radiation (Poquet et al., 2008). The polyphenols from green tea exhibit anti-IL8 production in epithelial cells, leading to an inflammation remedy (Kim et al., 2006).

4. Conclusions

It is crucial at the beginning to develop a suitable preparation to obtain beneficial products from herbs. This study discussed the strong influence of the extracting solvents and drying process in achieving a high yield of valuable phenolic compounds from *G. pseudochina* leaves. This study provides suitable processes for plant extract preparation through co-extraction with 25% and 50% (w/v) ethanol solvent with microwave drying, which could decrease the cost of operation during application of the natural product in the future. In addition, microwave drying could increase CA content in plant extracts, which might lead to using microwave drying to liberate several free phenolics for increased utilization. Additionally, microwave drying did not affect the increase in PA contents in the plant extracts, which might serve to save the product for further development. However, a small amount of PAs remains present in the microwave-dried and mixed-age leaf sample (MLM) extract, which should be controlled to safe doses when applied in a health product. Furthermore, the extract obtained from co-extraction of the MLM extract with 25% and 50% (v/v) ethanol (EMLM) acted as an anti-RelB and anti-IL-8 agent at a concentration at which more than half of the cells could survive. In this study, CA tends to be the most suitable anti-psoriatic compound among other marker compounds, because it presented anti-RelB and IL-8 activities at a lower concentration. However, a number of unknown compounds are still present in the EMLM extract that may act as anti-psoriatic agents. Therefore, this result could support the potential of the *G. pseudochina* leaf extract for development as an active ingredient for the alleviation of psoriasis and/or inflammatory diseases. However, other pathways involved in inflammatory processes and psoriasis pathogenesis should be studied in the future to strongly support the use of *G. pseudochina* leaf extract for psoriasis alleviation, and an individual pyrrolizidine alkaloid should be identified to improve the safety of leaf extract utilization.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest in this paper.

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Figure captions:

Fig. 1 TPC of *G. pseudochina* leaf extracts per g dry weight (a) and per g crude extract (b), TFC of *G. pseudochina* leaf extracts per g dry weight (c) and per g crude extract (d), crude content (e) and 50% of FRSA activity (IC_{50}) (f) prepared with different drying processes, leaf ages and polarity of solvents. Abbreviations of sample names: first letter, F is freeze dry, M is microwave and O is oven; second letter, Y is young leaf, D is developing leaf and M is mature leaf. Different letter (s) (a-p) are significant differences according to Scheffe's test ($p < 0.05$). Data are presented as the means ± *SD* ($n = 3$).

Fig. 2 Normalized HPLC chromatograms with retention times of (a, b) standards of phenolic compounds, and *G. pseudochina* extracts from continuous extracts with ethyl acetate, ethanol and 50% methanol of (c-e) the freeze-dried leaves, (f-h) microwave-dried leaves and (i-k) oven-dried leaves at various leaf ages.

Fig. 3 LC-ESI base peak chromatograms (BPC) of the MLM extracts from separate extractions with (a) 25% ethanol and (b) 100% ethanol. For major peak assignments, see Table 4.

Fig. 4 Localization of RelA and RelB on HaCaT cells due to TNF-α stimulation. HaCaT cells were pre-treated with 50 ng/ml of TNF-α for 12 h and treated with the EMLM extracts, marker compounds (CGA, CA, PCA and RUT) and CUR (positive control) for 24 h.

Table 1 Colour and moisture removal (%) of *G. pseudochina* leaves with various leaf ages and drying methods.

Table 2 Summary of TPC and TFC in *G. pseudochina* leaf extracts obtained through different drying processes and leaf ages.

Table 3 TPC, TFC, 50% free radical scavenging activity (IC_{50}), CGA, CA and RUT content of the MLM extracts from separate extractions with various ethanol concentrations of 25, 50, 75 and 100%.

Table 4 LC-ESI-QTOF-MS/MS analysis of phenolic compounds from the MLM extracts from separate extractions with 25 and 100% ethanol.

Table 5 Total pyrrolizidine alkaloid content (TPAsC) in *G. pseudochina* leaf extracts prepared from different drying processes (freeze drying and microwave and oven drying) and serial extraction with 25% and 50% ethanol.

Table 6 Cytotoxicity of the EMLM extract and marker compounds on HaCaT cells, non-stimulated and stimulated by TNF-α, and quantity of each marker compound in the EMLM extract.

Table 7 Interleukin 8 (IL-8) content in HaCaT cell lysate after treatment with the EMLM extract and marker compounds.