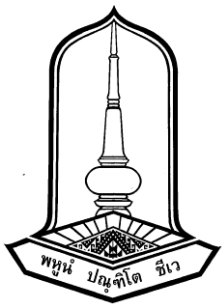

ANTI-INFLAMMATORY EFFECT OF GYNURA PSEUDOCHINA (L.) DC. LEAF EXTRACTS AND PREPARATION OF PROTOTYPIC CREAM FOR PSORIATIC ALLEVIATION

KANNIKA SUKADEETAD

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology at Mahasarakham University

December 2017

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The examining committee has unanimously approved this dissertation, submitted by Miss Kannika Sukadeetad, as a partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology at Mahasarakham University.

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Ms. Kannika Sukadeetad

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ชื่อเรื่อง ฤทธิ์ต้านการอักเสบของสารสกัดใบว่านมหากาฬและการเตรียมครีมต้นแบบเพื่อบรรเทาอาการโรคสะเก็ดเงิน

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บทคัดย่อ

ว่านมหากาฬเป็นพืชสมุนไพรพื้นบ้านที่มีฤทธิ์ต้านการอักเสบ เพื่อศึกษาความเป็นไปได้ในการใช้สารประกอบฟีนอลิกในสารสกัดใบว่านมหากาฬเพื่อเตรียมเป็นครีมต้นแบบบรรเทาอาการจากโรคสะเก็ดเงิน ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของวิธีการทำแห้ง ตัวทำละลายที่ใช้สกัดและอายุของใบพืชต่อปริมาณของสารประกอบฟีนอลิก ฟลาโวนอยด์ สารสกัดหยาบและฤทธิ์ต้านอนุมูลอิสระ ศึกษาองค์ประกอบของสารประกอบฟีนอลิกในสารสกัดหยาบโดยใช้เทคนิค HP-TLC และ HPLC จำแนกชนิดของสารที่เป็นองค์ประกอบด้วยเทคนิค LC-MS/MS วิธีการที่ดีที่สุดจะถูกนำมาใช้ในการเตรียมสารสกัดหยาบเพื่อนำไปใช้ในการทดสอบฤทธิ์ต้านการอักเสบในเซลล์เพาะเลี้ยงและนำไปใช้ในการเตรียมครีมต้นแบบ เซลล์ผิวหนังชนิด HaCaT ซึ่งถูกกระตุ้นและไม่ถูกกระตุ้นด้วยไซโตไคน์ชนิด TNF-α ถูกนำมาใช้ในการทดสอบฤทธิ์ต้านการอักเสบ ศึกษาความเป็นพิษต่อเซลล์โดยใช้เทคนิค MTT ศึกษาฤทธิ์ต้านการทำงานของ RelA และ RelB โดยใช้เทคนิค Immunofluorescence ศึกษาฤทธิ์ต้านการทำงานของ IL-8 และ TSG-6 โดยใช้เทคนิค ELISA เตรียมครีมต้นแบบที่มีส่วนผสมจากใบว่านมหากาฬ รวมทั้งศึกษาความคงตัวทางกายภาพและทางเคมีของครีมต้นแบบที่ระยะเวลาต่าง ๆ

ผลการศึกษาพบว่า 50% เมทานอล สามารถสกัดสารสกัดหยาบได้มากที่สุด ส่งผลให้ได้ปริมาณฟีนอลิกและฟลาโวนอยด์สูงขึ้นตามไปด้วย สารสกัดหยาบที่ได้จากการทำแห้งแบบแช่เยือกแข็งและไมโครเวฟให้ปริมาณสารประกอบฟีนอลิค ฟลาโวนอยด์และฤทธิ์ต้านอนุมูลอิสระสูงที่สุด ปริมาณสารฟีนอลิกและฟลาโวนอยด์ที่สกัดได้มีความสัมพันธ์กับวิธีการทำแห้งและตัวทำละลายที่ใช้สกัด รวมทั้งปริมาณฟีนอลิกและฟลาโวนอยด์ที่สูงขึ้นจะส่งผลให้ฤทธิ์ต้านอนุมูลอิสระสูงขึ้นตามไปด้วย อย่างไรก็ตามไม่พบความสัมพันธ์ของอายุใบพืชกับปัจจัยอื่น ผลการศึกษาองค์ประกอบฟีนอลิกของสารสกัดหยาบพบว่า 50% เมทานอล สามารถสกัดสารประกอบฟีนอลิกอื่นและสารคลอโรจินิก แอซิด คาเฟอิก แอซิด พี-คูมาริก แอซิด และรูทิน ได้ดีที่สุด นอกจากนี้วิธีทำแห้งด้วยไมโครเวฟสามารถเพิ่มปริมาณของคาเฟอิก แอซิด ได้ ดังนั้นใบพืชอายุผสมและวิธีทำแห้งด้วยไมโครเวฟจึงถูกนำมาใช้เพื่อเตรียมสารสกัดหยาบ

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เพื่อทดสอบขั้นตอนต่อไป เนื่องจากตัวทำละลายเมทานอลมีความเป็นพิษซึ่งอาจไม่เหมาะต่อการนำไปใช้สกัดสารเพื่อนำไปใช้ในการเตรียมครีม ดังนั้นตัวทำละลายเอทานอลซึ่งมีขั้วใกล้เคียงกันแต่มีความเป็นพิษต่ำกว่าจึงถูกนำมาใช้แทนเพื่อสกัดสาร ผลการศึกษาการจำแนกชนิดของสารที่เป็นองค์ประกอบพบว่า ใบพืชอายุผสมที่ทำแห้งด้วยวิธีไมโครเวฟ (MLM) ประกอบด้วยกรดฟีนอลิก (คาเฟอิก แอซิด คลอโรจีนิก แอซิด ไดคาเฟโออิลควินิก แอซิดและคาเฟโออิลเมทิลควินิค แอซิด) ฟลาโวนอยด์ (รูทิน แคมเฟอรอลรูทิโนไซด์ เควอซิทิน (+)- เทฟอเพอพูริน, ไฮดรอกซี เมทอกซี เมทิลีนไดออกซีไอโซฟลาโวนและไดไฮดรอกซีฟีนิล ไฮดรอกซี เมทิล เมทิล เพนเทนอิล ไดไฮโดร ไพราโน โครเมน วัน) อนุพันธ์แซนโทน (ไตรไฮดรอกซี เมทิล ไดพรีนิลแซนโทน) ฟีนิลโพรพานอยด์ (ไดไฮดรอกซีซินนาโมอิล ไดไฮดรอกซีฟีนิล เอทานอล) สารประกอบฟีนอลิกไกลโคไซด์ และกลีเซอรอลฟอสโฟลิปิด ผลการศึกษาพบว่าตัวทำละลายเอทานอลที่ความเข้มข้น 25% และ 50% มีความเหมาะสมที่จะนำมาใช้ร่วมกันเพื่อสกัด MLM (EMLM) ให้ได้สารประกอบฟีนอลิกที่เป็นองค์ประกอบมากที่สุด ผลการศึกษาสารฟีนอลิคที่เป็นองค์ประกอบด้วยเทคนิค HPTLC ยืนยันความเหมาะสมของวิธีทำแห้งด้วยไมโครเวฟที่ยังคงให้สารประกอบฟีนอลิกสูง รวมทั้ง MLM มีสารแอลคาลอยด์ประเภทไพโรไรซิดีนเป็นองค์ประกอบในปริมาณน้อย ผลการศึกษาความเป็นพิษต่อเซลล์ผิวหนังพบว่า EMLM และสารบริสุทธิ์มาตรฐานที่พบในสารสกัดหยาบ EMLM ชนิด คลอโรจีนิก แอซิด พี-คูมาริก แอซิด และรูทินนั้นไม่มีความเป็นพิษต่อเซลล์ชนิดที่ถูกกระตุ้นและไม่ถูกกระตุ้นด้วย TNF-α ยกเว้นคาเฟอิกแอซิด ที่พบว่ามีความเป็นพิษต่อเซลล์ทั้งสองชนิด นอกจากนี้พบว่า EMLM และสารบริสุทธิ์มาตรฐานทั้ง 4 ชนิดข้างต้น มีฤทธิ์ยับยั้งการทำงานของ RelB ผ่าน canonical pathway และมีฤทธิ์ยับยั้ง IL-8 ซึ่งฤทธิ์ในการยับยั้งดังกล่าวนั้นมีแนวโน้มขึ้นอยู่กับความเข้มข้นของสาร อีกทั้ง EMLM คลอโรจีนิก แอซิด และพี-คูมาริก แอซิด สามารถลดปริมาณของ TSG-6 ได้ โดยความสามารถในการลดปริมาณของ TSG-6 มีแนวโน้มขึ้นอยู่กับความเข้มข้นของสารอีกด้วย ครีมต้นแบบที่ผสม EMLM ปริมาณ 0.5% และครีมยาพื้นถูกเตรียมขึ้น พบว่าครีมทั้งสองชนิดมีความคงตัวทางกายภาพ สี และค่าพีเอช มากกว่า 30 วัน อย่างไรก็ตาม และสารชนิด คลอโรจีนิก แอซิด คาเฟอิก แอซิด พี-คูมาริก แอซิดและรูทินที่เป็นองค์ประกอบใน EMLM มีความคงตัวอยู่ในครีมมากกว่า 1 ปี ผลการศึกษาครั้งนี้สามารถสนับสนุนการนำสารสกัดใบว่านมหากาฬมาใช้เป็นสารออกฤทธิ์ในผลิตภัณฑ์ครีมเพื่อบรรเทาอาการในผู้ป่วยโรคสะเก็ดเงิน

คำสำคัญ: ว่านมหากาฬ ฤทธิ์ต้านการอักเสบ สารประกอบฟีนอลิก RelA, IL-8, TSG-6

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TITLE Anti-inflammatory effect of *Gynura pseudochina* (L.) DC. leaf extracts and preparation of prototypic cream for psoriatic alleviation

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DEGREE Doctor Philosophy **MAJOR** Biology

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UNIVERSITY Mahasarakham University **YEAR** 2017

ABSTRACT

Gynura pseudochina (L.) DC. is an ethnomedicinal plant that has been used as an anti-inflammatory. To study the possible utilization of plant phenolics for topical cream preparation in psoriatic treatment, the effects of the drying process, extracting solvent and ages of leaves on the total phenolic content (TPC), total flavonoid content (TFC), crude extract and antioxidant activity were investigated. The phenolic compositions in the plant extracts were investigated by HP-TLC and HPLC techniques and were identified by LC-MS/MS. The most efficient process was applied to obtain the valuable extract for cell study and prototypic cream preparation. HaCaT cells followed by non TNF-α and TNF-α stimulation were used as the subject. The cell cytotoxicity was evaluated by MTT assay. The anti-RelA and RelB properties were evaluated by immunofluorescence. In addition, the anti-interleukine 8 (IL-8) and tumor necrosis factor-inducible gene 6 protein (TSG-6) properties were evaluated by ELISA. The *G. pseudochina* leaf extract was used as an active ingredient in the prototypic cream. In addition, the physical and chemical stabilities of the prototypic cream were assessed at different storage times.

The results show that the 50% (v/v) methanol had the most efficient recovery of the crude extracts that lead to the highest recovery of TPC and TFC. The freeze drying and microwave drying processes preserved high levels of TPC, TFC and antioxidant activity. The TPC and TFC had correlations with the drying process and extraction solvent. The higher TPC and TFC levels resulted in higher antioxidant activities. The ages of the leaves had no correlations with the contents. The HPLC results indicated that 50% (v/v) methanol had good efficiency for recovery of each phenolic compound, especially

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the marker compounds including chlorogenic acid (CGA), caffeic acid (CA), p-coumaric acid (PCA) and rutin (RUT). In addition, CA was increased with microwave drying. Therefore, the microwave drying method and mixed-aged leaves were considered as the suitable drying process and plant material. Ethanol was used instead methanol due to a safer product for application. The LC-MS/MS results revealed that the 25% and 100% ethanol extracts of mixed-ages leaves dried with a microwave (MLM) contained phenolic acids (CA, CGA, dicaffeoylquinic acid and 3-O-Caffeoyl-1-O-methylquinic acid), flavonoids (RUT, kaempferol rutinoside, quercetin, (+)-Tephropurpurin, 5-hydroxy-2'-methoxy-6,7-methylenedioxyisoflavone and 2-(2,4-dihy-droxyphenyl)-5-hydroxy-8-methyl-8-(4-methyl-3-penten-1-yl)-2,3-dihydro-4H,8H-pyrano [2,3-f]chromen-4-one), xanthone derivative (1,3,8-Trihydroxy-4-methyl-2,7 diprenylxan-thone), phenylpropa-noid (3,4 dihy-droxycinnamoyl (Z)-2-(3,4-dihydroxyphenyl) ethanol), phenolic glycoside compound (unknown-C-glycoside) and glycerol-phospholipid (1-(9Z-octadece-noyl)-sn-glycero-2,3-cyclic phosphate). Co-extraction with 25% and 50% (v/v) ethanol of MLM (EMLM) was perform for the recovery of each phenolic compound present in the extract. The HP-TLC fingerprints supported the efficiency of microwave drying for phenolic preservation. The MLM also contained pyrrolizidine alkaloids (PAs) at a low concentration. EMLM and their marker compounds were not toxic to HaCaT cells following non TNF-α and TNF-α stimulation, except for CA. The EMLM extracts and four marker compounds (CGA, CA, RUT and PCA) inhibited the RelB canonical pathway and IL-8 production with a trends in a dependent concentration manner. TSG-6 was decreased after treatment with a trends in a dependent concentration manner with HMLM, CGA and PCA. A 0.5% EMLM was incorporated into the prototypic cream. The EMLM cream and base cream were stable in terms of texture, color and pH for more than 30 days. The marker compounds in EMLM, including CGA, CA, RUT and PCA, were stable within the cream for more than a year. These results could support an application of *G. pseudochina* leaf extracts as an active ingredient in a topical product for psoriasis alleviation.

Keywords: *G. pseudochina* (L.) DC., Anti-psoriatic property, Phenolic compound, RelA, IL-8. TSG-6

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List of Abbreviations

1. Standard chemicals

CA	Caffeic acid
CAT	Catechin
CGA	Chlorogenic acid
CUR	Curcumin
EC	Epicatechin
GA	Gallic acid
MCT	Monocrotaline
PCA	P-coumaric acid
PTX	Paclitaxel
RUT	Rutin
VAN	Vanillin

2. Sample codes

FY	Freeze dry, Young leaves
FD	Freeze dry, Developing leaves
FM	Freeze dry, Mature leaves
MY	Microwave dry, Young leaves
MD	Microwave dry, Developing leaves
MM	Microwave dry, Mature leaves
OY	Oven dry, Young leaves
OD	Oven dry, Developing leaves
OM	Oven dry, Mature leaves
MLF	Mixed ages leaves, Freeze dry
MLM	Mixed ages leaves, Microwave dry
MLO	Mixed ages leaves, Oven dry
EMLM	25% and 50% ethanol, Mixed ages leaves, Microwave dry

3. Content values

TPC	Total phenolic content

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TFC	Total flavonoid content
TPAsC	Total pyrrolizidine alkaloid content
CAE	Caffeic acid equivalent
ECE	Epicatechin equivalent
MCTE	Monocrotaline equivalent

4. Proteins and substances

TNF-α	Tumor necrosis factor alpha
IFN-γ	Interferon gramma
NF-κB	Nuclear factor kappaB
IκB	Inhibitor kappaB
IKK	Inhibitor kappaB kinase
IL-8	Interleukin 8
BSA	Bovine serum albumin
GFP	Green fluorescent protein
PAs	Pyrrolizidine alkaloids
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate buffered Saline
DAPI	4',6-diamidino-2-phenylindole
ABC	Avidin-biotin-peroxidase complex
TMB	3,3′,5,5′-Tetramethylbenzidine
HSS	Homospermidine synthase
LPS	Lipopolysaccharide

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CHAPTER 1

INTRODUCTION

1.1 Background

Thailand has a long history of herbal utilization through local wisdom. However, only a few herbal plants have been studied so far and developed into added-value products. The development of herbal products not only provides safe products for patients, but also supports sustainable self-sufficiency in a developing country. In addition, the herbal product industries, especially Small and Medium Enterprises (SMEs), which are the largest part of the national economy, are being pushed to move toward "Thailand 4.0," in response to the long-term national strategy to transform Thailand into a value-based economy.

Gynura pseudochina (L.) DC. or Wan-maha-kan, is a perennial herb in the Asteraceae family that is distributed throughout tropical regions in South East Asia and Africa (Vanijajiva, 2009). There are many reports of its medicinal properties in terms of traditional uses. The plant leaves have reported anti-inflammatory and anti-viral uses (Plant Genetic Conservation Project, 2009). Its tubers have been used against inflammation, hemorrhages and dysentery (Windono et al., 2012). Thus, the medicinal properties of this plant have been researched and evaluated, such as the cytotoxic efficiency in leukemia cells, anti-inflammation in both Hela cells and human monocytes (Siriwatanametanon et al., 2010), anti-envenomation from scorpion in fibroblast cells (Uawonggul et al., 2006), anti-dengue in rat (Moektiwardoyo et al., 2014) and anti-HIV (Woradulayapinij et al., 2005). In addition, *G. pseudochina* var. *hispida*, the most common, which is a sub-species of *G. pseudochina* (L.) DC., has been reported to have anti-inflammation (Siriwatanametanon and Heinrich, 2011) and anti-psoriasis activities (Rerknimitr et al., 2016).

Psoriasis is a chronic inflammatory skin disorder affecting 2 to 5 % of the world's population (Raychaudhuri et al., 2014). Over half a century ago, psoriasis was believed to be a disease caused from abnormal function of the keratinocytes. However, recent research indicates that the responses from immune cells, especially the activation

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of T-cells, plays an important role in the pathogenesis of psoriasis (Cai et al., 2012; Chen et al, 2016; Johansen, 2016; Diani et al, 2015). Basically, T-cells respond to the skin pathogen during acute inflammation. However, the storming of T-cells is also found throughout initiation and maintenance events of psoriasis, which is chronic inflammation. At the time that T-cells are activated, multiple cytokines belonging to inflammation, such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and interleukin 1-beta (IL-1β), are released (Baliwag et al., 2015). Then, those cytokines continuously stimulate keratinocytes as well as other immune cells, such as dendritic cells, to release various cytokines and chemokines leading to the persistence of the disease. Furthermore, the expressions of cytokines, chemokines and enzymes associated with inflammation in all cell types depends on the regulation of a nuclear factor kappa B (NF-κB) (Christian et al., 2016).

NF-κB is the transcription factor that plays a fundamental role in terms of cellular and body levels. At the cellular level, NF-κB plays important roles in regulating the cell cycle, proliferation and cell death; while, at the body level, this transcription factor regulates the activation of 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, that are formed as hetero or homo dimers to function. In resting cells, NF-κB is sequestered in the cytoplasm through direct binding with an inhibitor of the kappaB (IκB) family. An initiating signal, such as TNF-α or lipopoly saccharide (LPS), leads to the activation of the inhibitor kappaB 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 the gene expression (Christian et al., 2016). TNF-α is a central cytokine in the development of inflammation diseases. Stimulating the cells with TNF-α leads to the activation of NF-κB in two different pathways of RelA canonical and RelB canonical so 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 a variety of cell types, especially T-cells and keratinocytes, for responding to inflammation (Brandt et al, 2017; Ferran et al, 2010; Wu et al., 2017). IL-8 plays a fundamental role for recruitment and collection of the immune cells in keratinocytes during the development of psoriasis (Balato et al., 2012). Therefore, the diminishing

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production of IL-8 in psoriatic affecting cells, such as keratinocyte cells, is very important to inhibit the progression of psoriasis. Tumor necrosis factor-inducible gene 6 protein (TSG-6) is an extracellular matrix protein that is secreted for remodeling the cells in the inflammation process (Bayliss et al., 2001). Therefore, TSG-6 can be used as an indicator protein for inflammatory display.

Phenolic compounds are secondary plant metabolites that are produced during development and in response to various conditions (Rao and Ravishankar, 2002). Having the phenol ring contain at least one hydroxyl substituent in their structure allows for the delocalization of the radical electron to the nucleus that promotes the anti-oxidant capacity as the main role of these compounds (Stalikas, 2007). In addition, phenolic compounds can chelate metallic ions to prevent free radical production (Pereira et al., 2009), and they are produced at increased levels in *G. pseudochina* under zinc and cadmium stress (Mongkhonsin et al., 2016). Moreover, the antioxidant capacities of these compounds are responsible for other biological properties (Pereira et al., 2009), including anti-cancer, anti-virus, anti-bacteria and anti-inflammation (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, because they are NF-κB inhibitory compounds (Siriwatanametanon and Heinrich, 2011).

A proper process to recover active phenolic compounds from a plant is an important consideration. Drying processes are necessary for many purposes, such as plant material preservation, volume reduction, decreasing microbial growth and prolonging storage time. However, drying processes have variable effects on phenolic compounds (Chan et al., 2011; Lim et al., 2007; Chan et al., 2009). An appropriate solvent for the recovery of the phenolic compounds depends mostly on the structure of the phenolic composition in the plant materials (Khoddami et al, 2013). In addition, phenolic compounds may change with leaf maturity (Makkar et al, 1988; Makkar et al., 1991; Naz et al., 2013). Pyrrolizidine alkaloids (PAs) are in a class of natural chemicals found in many plant species. Although PAs are present as a mixture with beneficial compounds in plant extracts (Neuman et al., 2015), some alkaloids possess hepatotoxicity and toxicity, depending on the dose and time of intake. PAs can be extracted by ethanol, methanol and methanolic solutions (Mroczek et al., 2006). Many PAs in the *Gynura* genus have been

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reported (Liang and Roeder, 1984; Lin et al., 2011; Qi, 2009; Wiedenfeld, 1982; Roeder, 1996). Methanol can extract senecionine and senkirkine from *G. pseudochina* tubers (Windono et al., 2012). Therefore, these alkaloids should be considered before administration of natural products.

Over 80% of psoriatic patients are affected by mild disease (<10% body surface area) (National Psoriasis Foundation, 2009), which can be remedied by a tropical product. Although topical therapies, such as steroids, anthralin, vitamin A, vitamin D analogues, salicylic acid and coal tar, can treat the disease, unpleasant side effects always present and drug resistance can develop after long term exposure (Mendonça and Burden, 2003; Herman and Herman, 2016). Therefore, achieving new anti-psoriatic agents with low toxicity remains important and represents an area of research activity for herbal product development.

Therefore, this research aims to study the qualitative and quantitative analysis of the phenolic contents and compositions from *G. pseudochina* leaves prepared with different methods in the drying processes, age of leaves and polarity of solvents using high performance thin layer chromatography (HP-TLC) and high performance liquid chromatography (HPLC) techniques, respectively. The moisture removal, dried leaf color, total phenolic content (TPC), total flavonoid content (TFC), total pyrrolizidine alkaloid content (TPAsC) as well as free radical scavenging activity (FRSA) were also determined. In addition, the chemical compositions of the plant extracts were identified by the liquid chromatography mass spectrometry (LC-MS/MS) technique. Moreover, the cytotoxicity of HaCaT cells due to non-TNF-α and TNF-α stimulation were evaluated by MTT assay. As well as the inhibitory properties to RelA and RelB throughout IL-8 and tumor necrosis factor-inducible gene 6 protein (TSG-6) of *Gynura* extracts and their marker compounds on HaCaT cells due to TNF-α stimulation were also assesses by immunofluorescent assay and enzyme-linked immunosorbent assay (ELISA), respectively. The obtained results were evaluated for the first step to develop a prototypic cream for mild psoriasis treatment. Finally, the prototypic cream with our *Gynura* extracts was prepared and studied for physical and chemical properties during storage time.

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1.2 Objectives

1.2.1 Study the qualitative and quantitative analysis of phenolic and flavonoid contents, compositions and the antioxidant activities of *G. pseudochina* leaf extracts. The differentiation of leaf ages, drying methods and solvent extraction was investigated to obtain an optimum condition for highly active phenolic and flavonoid contents.

1.2.2 Study the cytotoxicity of *G. pseudochina* leaf extracts on normal HaCaT cells (non TNF-α stimulated cells).

1.2.3 Study the cytotoxicity of *G. pseudochina* leaf extracts on TNF-α stimulated HaCaT cells.

1.2.4 Study the anti-inflammatory mechanism by NF-κB inhibition of *G. pseudochina* leaf extracts on TNF-α stimulated HaCaT cells by focusing on the inhibition of RelA and RelB.

1.2.5 Study the anti-interleukin-8 property and effect on TSG-6 of *G. pseudochina* leaf extracts on TNF-α stimulated HaCaT cells.

1.2.6 Preparation of prototypic cream and study of the physical, chemical and stable properties of the prototypic cream at different storage times.

1.3 Hypothesis

1.3.1 Total phenolic contents, total flavonoid contents, antioxidant activities and phenolic composition of *G. pseudochina* leaf extracts probably differ in leaf age, drying method and polar solvent extraction.

1.3.2 The cytotoxicity of *G. pseudochina* leaf extracts on normal HaCaT cells are different for each concentration.

1.3.3 The cytotoxicity of *G. pseudochina* leaf extracts on TNF-α stimulated HaCaT cells are different for each concentration.

1.3.4 *G. pseudochina* leaf extracts can inhibit NF-κB performance on RelA canonical pathway (RelA and NF-κB1) or RelB canonical pathway (RelB and NF-κB1) resulting in a decreased release of IL-8 cytokines.

1.3.5 The effect to TSG-6 of *G. pseudochina* leaf extracts on TNF-α stimulated HaCaT cells are different for each concentration.

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1.3.6 Long periods of storage time affect the physical and chemical properties as well as the stability of the prototypic cream.

1.4 Advantages of the study

1.4.1 Preparation methods for *G. pseudochina* leaf extracts will provide optimum conditions for high phenolic and flavonoid contents. In addition, the results could be applied to extract phenolic and flavonoid compounds from plant material in the *Gynura* genus.

1.4.2 The anti-inflammatory mechanism on NF-κB inhibition of *G. pseudochina* leaf extracts will support their application for psoriasis patients.

1.4.3 This research can increase the value of *G. pseudochina* active compounds as an ingredient of a cream for psoriatic alleviation. In addition, this research could promote local Thai herbal utilization for compensation of the chemically synthesized compound usages. Finally, the patients will get a safe topical treatment product from natural compounds with low toxicity.

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CHAPTER 2

LITERATURE REVIEW

2.1 *Gynura pseudochina* (L.) DC.

The genus *Gynura* comprises 44 species and is distributed throughout tropical regions of Africa to South and East Asia and one species in Australia. Although, Southeast Asia is a region that has the highest species diversity of *Gynura*. In Thailand, 10 taxa have been recorded, namely *Gynura procumbens* (Lour.) Merr., *G. calciphila* var. calciphila Kerr, *G. calciphila* var. dissecta F.G. Davies, *G. integrifolia* Gagnep., *G. pseudochina* (L.) DC., *G. nepalensis* DC., *G. hmopaengensis* H. Koyama, *G. cusimbua* (D.Don) S. Moore, *G. bicolor* (Roxb. Ex Willd.) DC. and *Gynura* sp. (Vanijajiva, 2009).

G. pseudochina is distributed from tropical Africa eastward to Sri Lanka, India, Nepal, Bhutan, Myanmar, China, Indonesia and Thailand. It grows on sandy soil, grassland and also dry vertiginous slopes, sandy soil and meadows that are 0-2,600 m in altitude. In addition, it flowers and fruits throughout the year. The local names in Thai are Wan mahakan, Phakkat kop, Phakkat din, Phakkat nok khao and Nat haeng. The taxonomy of the plant species is shown as follows:

Kingdom: Plantae
Division: Spermatophyta
Class: Dicotyledoneae
Order: Asterales
Family: Asteraceae
Genus: *Gynura*
Species: *Gynura pseudochina*

The botanic characteristics of the plants are 10-50 cm high and composed of 1-9 cm diameter subglobose tuber to support an erected stem. Leaves are blades elliptic, ovate or lyrate shaped, 7-40 by 1-12 cm size in basal rosette sparsely pubescent to glabrescent; sparsely pubescent, base truncate or cuneate, apex acute to obtuse, margins

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sinuate or coarsely dentate. Petioles 0.3-3 cm long, exauriculate, sparsely pubescent. Capitula 1-3 in lax corymbs; peduncles 0.5–10 cm long, sparsely pubescent; bracts 3-6, 1-3 mm long; involucres ca. 13 mm long, 7.5-10 mm in diameter; calycular bracts 3-5, 2-6 mm long, pubescent; phyllaries 10-14, 1.5-2 mm broad, somewhat purpletinged, sparsely pubescent. Florets 20-30, red, orange to yellow, 10-13 mm long, exserted part 2.5-4 mm long. Anthers 2-2.5 mm long, anther collars elongate. Style arms 3 mm long. Cypselas 3-4 mm long, brown, sparsely pubescent; carpopodium round, whitish or yellowish; pappus 10-14 mm long, white to dirty-white (Vanijajiva, 2009). Figure 2.1 shows the different parts of *G. pseudochina*, as composed of stem (a), leaf (b), tuber (c) and flower (d).

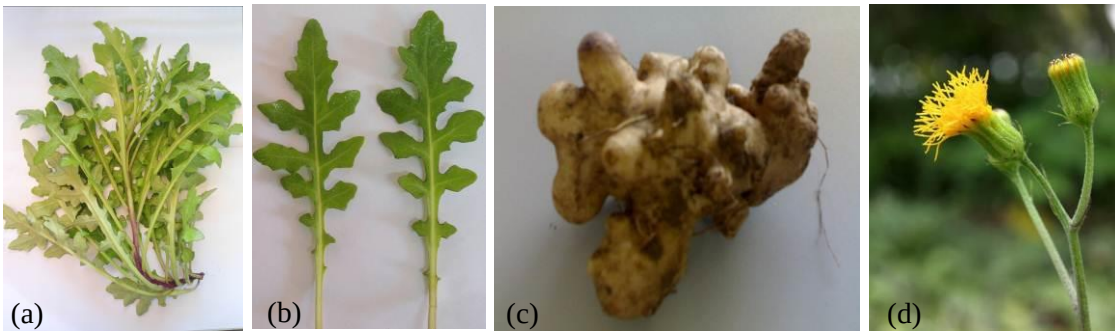

Figure 2.1 Different parts of *G. pseudochina*; (a) stem, (b) leaf, (c) tuber and (d) flower.

Table 2.1 Ethnomedical uses of *Gynura* species.

Species	Usage	References
G. bicolor	Treatment of inflammation, herpes simplex virus, rashes, fever, rheumatism, kidney disease, diabetes migraines, constipation, mellitus, cancer and hypertension	Lu et al., 2012
G. divaricata	Treatment of diabetes, hypertension, herpes, inflammation, cancer, pertussis, bronchitis, pulmonary tuberculosis, sore eye, toothache and rheumatic	Vanijajiva and kundee, 2013 Chen et al., 2009

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Table 2.1 (cont.)

Species	Usage	References
G. japonica	Treatment of muscular skeletal system disorders, injuries and ulcers.	Seow et al., 2011 Weckerle et al., 2009
G. procumbens (Lour.) Merr.	Treatment of cancer, inflammation, rheumatism, viral infections, eruptive fevers, rash, kidney disease, migraines, constipation, hypertension, diabetes, mellitus, anti-hyperlipidemic and anti-hypertensive activities Poultice for diverse skin diseases	Kim et al., 2011 Algariri et al., 2013 Sriwanthana et al., 2007
G. segetum	Treatment of cancer, diabetes and hypertension	Seow et al., 2011
G. pseudochina var. hispida	Treatment of inflammation, viral infections, pain, fever and AIDS	Siriwatanametanon et al., 2010
G. pseudochina (L.) DC.	- Treatment of inflammation, viral infections (herpes), bruises, pimples, breast tumours and sore throat - Relieving hot pain symptoms and fevers	Siriwatanametanon et al., 2010

The plants in the genus *Gynura* are used as herbal folk medicines for many purposes (Table 2.1). Especially, *G. pseudochina* has been reported for its medicinal properties in terms of traditional uses. The fresh leaves are ground with ethanol and masked onto the affected area to remedy an inflammation and virus infection (Plant Genetic Conservation Project, 2009). The tubers are also used against inflammation, hemorrhages and dysentery (Windono et al., 2012). There is some research to evaluate the medicinal properties of this plant. The aqueous extracts from the fresh leaves of *G. pseudochina* were evaluated for anti-envenomation from scorpion in fibroblast. The results revealed a mild efficiency on envenomation activity (Uawonggul et al., 2006). In addition, the dried leaf extracts from ethyl acetate showed ability for inflammation

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remedy, because the extracts had potential on anti-NF-κB on Hela cells stimulated with phorbol-12-myristate-13-acetate (PMA) and anti-PGE-2, IL-6, IL-1β and TNF-α on LPS induced human monocytes (Siriwatanametanon et al., 2010). Likewise, the dried leaf extracts from ethanol had potential anti-thrombocytopenia and anti-anemia activities that belong to the ability on dengue fever treatment (Moektiwardoyo et al., 2014). In addition, *G. pseudochina* var. *hispida*, the most common sub-specie of *G. pseudochina* (L.) DC., has also been reported to have many medicinal properties. Woradulayapinij et al. (2005) found that the water and ethanolic extracts of the fresh leaves of *G. pseudochina* could exhibit the HIV-1 reverse transcriptase inhibitory activity. In addition, the extracts from ethanol had more potential on the activity. The Quercetin 3- rutinoside, 3,5-di-caffeoylquinic acid, 4,5-di-caffeoylquinic acid and 5-monocaffeoylquinic acid obtained from methanolic extracts of dried *G. pseudochina* var. *hispida* leaves exhibited as NF-κB inhibitors on PMA induced Hela cells (Siriwatanametanon and Heinricdh, 2011). Moreover, an ointment containing 1% ethanolic leaf extracts of *G. pseudochina* var. *hispida* was prepared to study the ability on psoriasis treatment with an *in vivo* system. The results revealed that the ointment significantly decreased scaling scores and immunization of phosphorylated NF-κB p65, Ki-67 and epidermal thickness in the patient's lesions after four weeks (Rerknimitr et al., 2016). Therefore, the *G. pseudochina* extracts were strongly evident in anti-inflammation and inflammatory skin disease treatment, especially psoriasis.

2.2 Inflammation

Inflammation is a body's primary response to infection or injury, and it is critical for both innate and adaptive immunity. During infection many cytokines, chemokines, lipid mediators and bioactive amines are secreted by inflammatory associated cells. These secretors immediately trigger, which leads to an increase in blood flow, capillary permeability and recruit additional circulating leukocytes. The response processes to inflammation are determined by the arrival of neutrophils, the phagocytosis of phagocytes to eliminate microbes, the differentiation and proliferation of blood monocytes into macrophages and dendritic cells at the site of inflammation (Lawrence and Fong, 2010). Dendritic cells (DC) bind to foreign antigens and migrate to local lymph nodes to

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stimulate the production of naive T cells and activation of natural killer (NK) cells and/or B cells (Fadilah et al., 2007). Macrophages and DC can recognize a variety of stimulators that lead to the various patterns for recognition receptors (PRR) that are recognized by microbial molecular patterns or obligate intracellular proteins, for example, bacterial lipopolysaccharide (LPS), peptidoglycan (PGN), inflammatory chemokines and cytokines. Leukocytes are accumulated and proliferated during inflammation. However, excessive and prolonged expression of pro-inflammatory mediators could be harmful to the host. Therefore, a variety of negative regulatory mechanisms have evolved to prevent prolonged inflammation. In normal circumstances, the immune system has various mechanisms to resolve the inflammation; however, the inflammatory resolution requires the signaling terminators of pro-inflammatory mediators to clear the inflammatory cells, allowing the restoration of normal tissue function, and a failure of these mechanisms may lead to chronic inflammation and disease (Lawrence and Fong, 2010).

The inflammatory pathway is a complex process depending on many different signaling pathways (Figure 2.2). Inflammatory pathways are basically classified into arachidonic acid (AA)-dependent and AA-independent pathways. Cyclooxygenase (COX), lipooxygenase (LOX) and phospholipase A2 (PLA2) pathways belong to the metabolism of AA, which are considered for AA-dependent. On the other hand, nitric oxide synthase (NOS), NF-kB, peroxisome proliferator activated receptors (PPAR) and NSAID activated gene-1 (NAG-1) are classified as AA-independent. PLA2 is a phoshopholipases enzyme that releases free fatty acids, such as AA from the phospholipid layers of the plasma membrane. AA is then metabolized via either the COX pathway to produce prostaglandins (PGs) and thromboxane A2 or the LOX pathway to produce hydroperoxyeicosateraenoic acids (HETEs) and leukotrienes (LTs). Products from both pathways are key mediators leading to the process of inflammation. Up-regulation of inducible nitric oxide synthase (iNOS) results in overproduction of NO, which serves as a second messenger on inflammation. However, the expression of COX and iNOS are regulated by NF-κB transcription factor (Issa et al., 2006). Therefore, NF-κB is very crucial for regulation of the inflammation process and inflammatory disease treatment.

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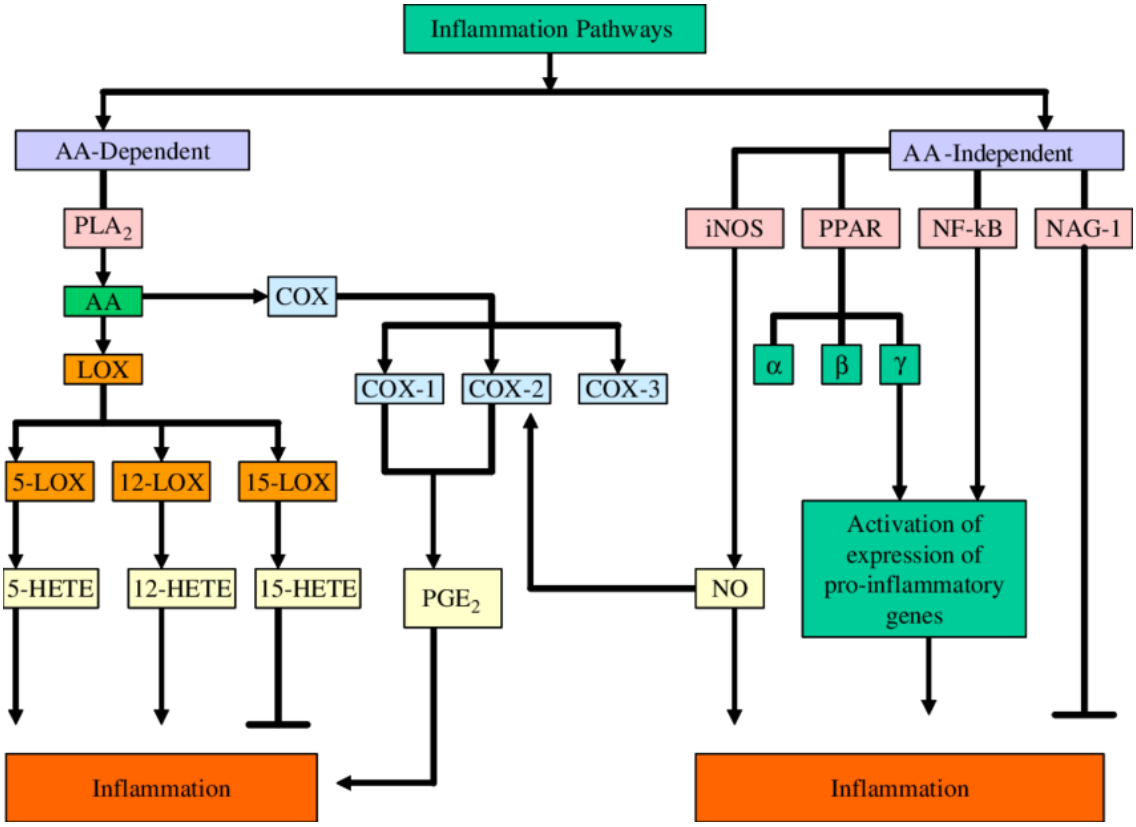

Figure 2.2 Inflammatory cell signaling pathways can be mechanistically classified into arachidonic acid (AA)-dependent and AA independent. 15-hydroperoxyei-cosateraenoic acid (15-HETE) and the gene product of NSAID activated gene-1 (NAG-1) are anti-inflammatory mediators. On the other hand, 5-HETE, 12-HETE, prostaglandin E2 (PGE2) and nitric oxide (NO) are all pro-inflammatory mediators. Peroxisome proliferators activated receptor g (PPARg) and nuclear factor kappa B (NK-κB) activate the expression of pro-inflammatory genes. COX: cyclooxygenase, iNOS: inducible nitric oxide synthase (Issa et al., 2006).

2.3 Psoriasis

Psoriasis is a chronic inflammatory skin disorder that affects 2% to 3% of the world's population. The disease is characterized by clinical performance including red and heavily scaly skin. The most common form of the disease is chronic plaque psoriasis (Figure 2.3) that are infiltrated from many types of immune cells, which lead to hyperproliferation and abnormal differentiation of keratinocytes (Karam et al., 2014).

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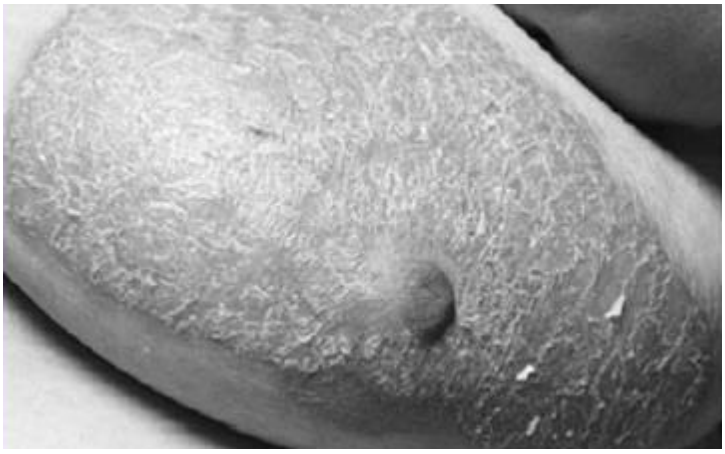

Figure 2.3 Chronic plaque psoriasis (Rerknimitr et al., 2016).

A network of different cytokines including tumor necrosis factor-alpha (TNF-α), interferon-γ (IFN-γ), vascular epidermal growth factor (VEGF), keratinocyte growth factor (KGF) and various types of interleukin (IL) implicats in the initiation and persistence of the disease (Figure 2.4). TNF-α and IFN-γ are over secreted from T lymphocytes (Tc) to stimulate the keratinocyte cells (KC). The stimulated keratinocyte cells can release IL-8 for attraction of T-lymphocytes and neutrophils into the affected area. In addition, the stimulated keratinocyte cells also release VEGF to stimulate the process of angiogenesis and release cathelicidin to stimulate the maturation of dendritic cells (DC). The IL-23 released from stimulated keratinocyte cells can activate the liberation of IL-17 and IL-22 from T-helper 17 (Th17) cells that leads to the positive feedback loop for persistence of the disease (Cesare et al., 2009). Furthermore, the expression of chemokine and cytokine in the disease are regulated by the activation of the transcription factor NF-κB (Figure 2.5).

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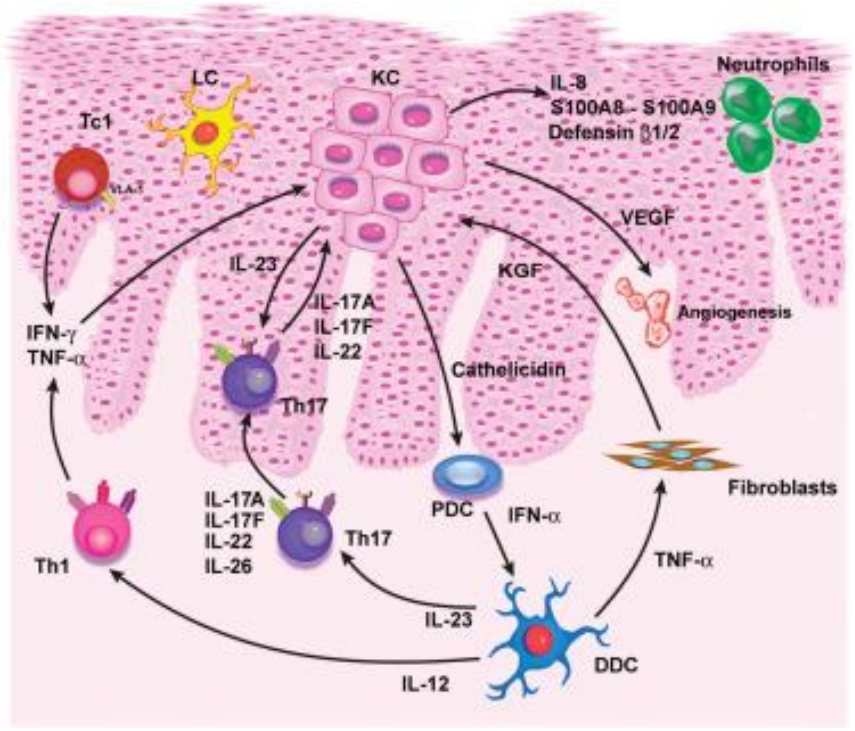

Figure 2.4 Pathogenesis process of psoriasis (Cesare et al., 2009).

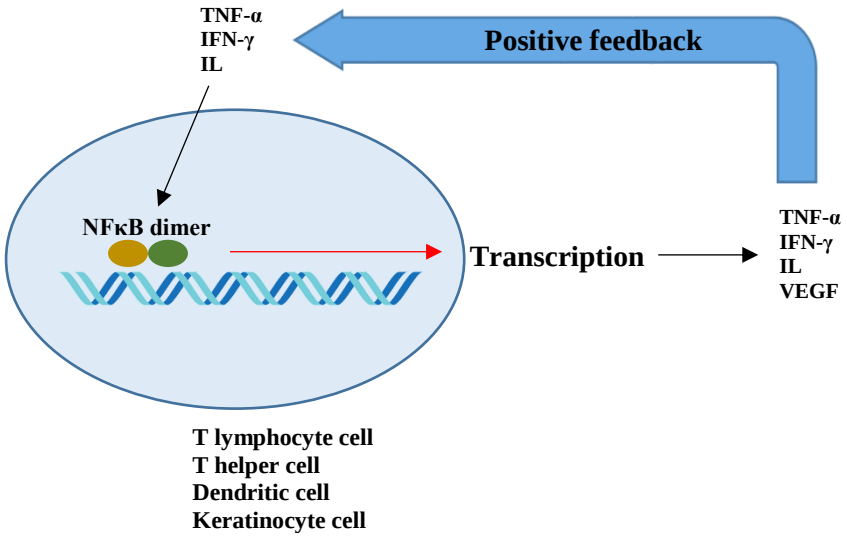

Figure 2.5 Role of nuclear transcription factor kappa B (NF-κB) (Adapted from Hayden and Ghosh, 2012; Cesare et al., 2009).

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2.3.1 Nuclear factor kappa B (NF-κB)

NF-κB is a protein transcription factor that regulates inflammation and other complex biological processes. It is composed of a family of proteins, including NF-κB1 (p105), NF-κB2 (p100), RelA (p65), RelB and c-Rel. The first two of them require a process for transformation into p50 and p52. All NF-κB proteins have a Rel homology domain (RHD) responsible for DNA binding and dimerization (Mowla et al., 2013) (Figure 2.6). NF-κB are formed as an either hetero or homo dimer to function. In resting cells, NF-κB is sequestered in the cytoplasm through direct binding with the inhibitor kappaB (IκB) family. An initiating signal, such as TNF-α or LPS, leads to the activation of the inhibitor kappaB 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 a recognized site on the DNA sequence to stimulate the gene expression (Christian et al., 2016).

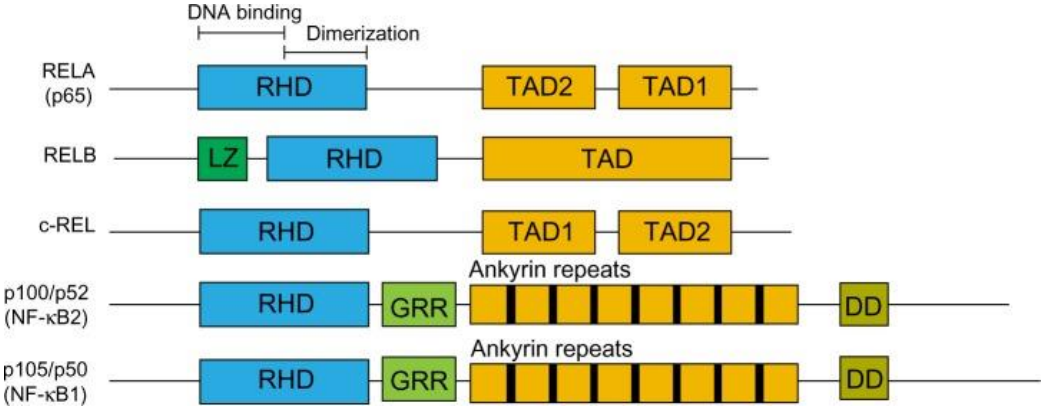

Figure 2.6 NF-κB protein family. All family members share RHD, composed of DNA-binding region and dimerization region. RELA, RELB and c-REL have transactivation domain (TAD). P100 and p105 precursor proteins are processed by proteosome to mature p52 and p50, respectively (Mowla et al., 2013).

The NF-κB are activated via different two pathways, including the canonical and non-canonical pathways (Figure 2.7). The canonical pathway is triggered by microbial products and pro-inflammatory cytokines, such as LPS, TNFα, IL-1 and CpG, that lead to the activation of RelA and RelB containing complexes. A non-canonical pathway is activated by the TNF-family cytokines-lymphotoxin β, receptor (LTβR) and CD40

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activator of NF-κB ligand but not TNFα, resulting in activation of RelB/p52 complexes. These pathways are characterized by the differential requirement for IKK subunits. The IKK complex consists of two kinase subunits of IKKα (IKK1) and IKKβ (IKK2) and a regulatory subunit IKKγ (NEMO). IKKβ regulates activation of the canonical pathway through phosphorylation of IκBs and requires the IKKγ subunit but not IKKα. IKKα is required for activation of the alternative pathway through phosphorylation and processing of p100, which is the precursor for p52, and this is independent of both IKKβ and IKKγ (Hayden, 2012).

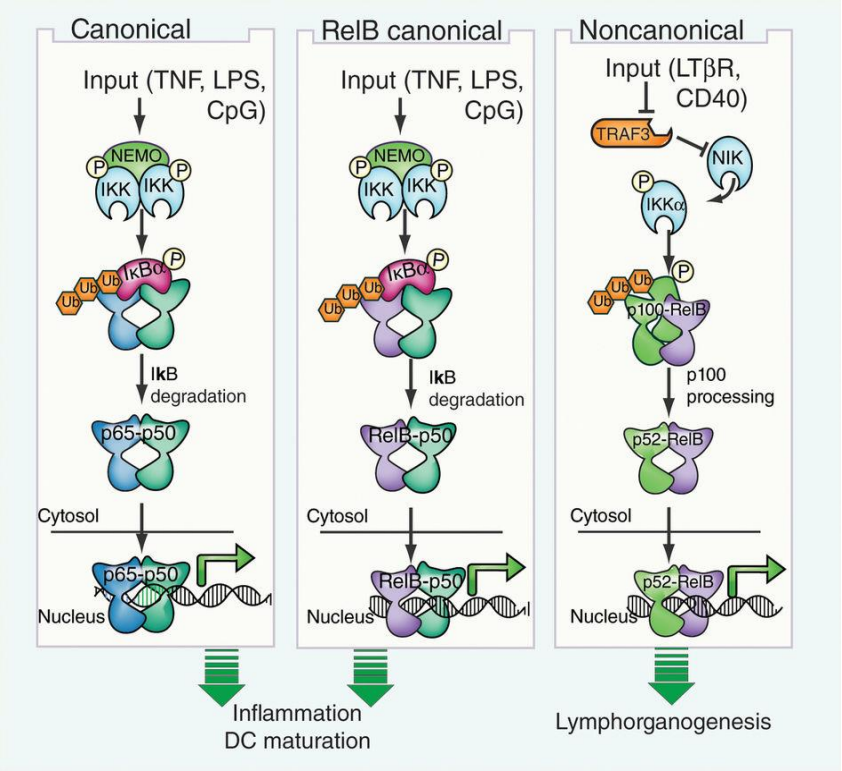

Figure 2.7 Illustrates canonical, RelB canonical and non-canonical pathways for NF-κB activation (Hayden, 2012).

2.3.2 Inflammatory mediators

Interleukin 8 (IL-8) is a chemokine that plays a role to attract T-lymphocytes and neutrophil into the affected area of the inflammation processes. Its production depends on a functional of promoter associated proteins including NFκB nuclear transcription factor, AP-1 activator protein and C/EBP enhancer binding protein (Elliott

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et al., 2001). A great number of cells types, including lymphocytes, monocytes/macrophages, fibroblasts, keratinocytes and endothelial cells, can produce IL-8 through a variety of stimulators, including IL-1, tumor necrosis factor and tumor promoters. However, only RelA can bind with the κB site on the promoter region in Jurkat T cells for IL-8 production (Kunsch and Rosen, 1993). In addition, IL-8 is greatly found in psoriatic skin (Sticherling et al., 1999). There are a number reports for IL-8 inhibitory properties from plant phenolics. The ethanolic extracts of Edelweiss (*Leontopodium alpinum* Cass.), which contains leontopodic acid, chlorogenic acid and 3,5-dicaffeoylquinic acid as the main compounds, was potent in anti- IL-8 production on primary human keratinocytes under TNF-α and IFN-γ combine stimulation (Daniela et al, 2012). Caffeic acid in the water extract of *Ixeris dentate* could inhibit the production of IL-8 in HaCaT and human mast cells (HMC-1) via MAPKs phosphorylation inhibition (Jeon et al, 2015). In addition, the polyphenolic extract from green tea and its main compounds, including epigallocatechin gallate and epicatechin gallate, showed the inhibitory properties to IL-8 in bronchial epithelial cells under IL-1β stimulation (Kim et al, 2006).

IL-17 is a cytokine whose gene was first isolated from a rat-mouse T cell hybridoma in 1993. The current pathogenic model in psoriasis highlights the role of T helper 17 (Th-17) that is a source for interleukin 17 (IL-17) secretion (Balato et al, 2012). IL-17 is a proinflammatory cytokine that is produced from T-cells as well as several immune cells including lymphoid tissue inducer cells, natural killer, natural killer T cells, macrophages and Paneth cells (Jin and Dong, 2013). T helper cells are classically divided into Th1, Th2 and Th17. It is demonstrated that Th17 cells play a role in the protection from both extracellular and intracellular agents. Th17 cells are differentiated from CD4+ T cells via growth factor (TGF)-β and interleukin (IL)-6 stimulation that leads to the expression of IL-17. The Il-17 family consists of IL-17A-F. IL-17A plays a role in neutrophil recruitment host defense and immuno-inflammatory pathology, which is secreted mainly from Th17, Treg cells, NK cells, mast cells and neutrophils. IL-17A and the IL-17F bind to the same receptor, however the influence of IL-17A on gene regulation is much stronger. The function of IL-17B, IL-17C and IL-17D is poorly defined. IL-17E limits the Th17 development and promotes the Th2 cytokines (Wasilewska et al., 2016).

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Tumor necrosis factor-inducible gene 6 protein (TSG-6) is a glycoprotein that has a crucial role in extracellular matrix remodeling, leukocyte attachment and cell proliferation. It is released from a variety of cell types including chondrocytes, fibroblasts, monocytes and vascular smooth muscle cells in response to growth factors and mediators in inflammatory processes. In addition, TSG-6 is induced to secrete via a number of stimulators, including TNF-α, IL-1β, LPS, epidermal growth factor, fibroblast growth factor-1 and transforming growth factor-β1 (Bayliss et al, 2001). TSG-6 can bind with various partners, including the serum glycoprotein inter-alpha-inhibitor (IαI), the growth factor bone morphogenetic protein-2 (BMP-2) and the extracellular matrix protein fibronectin, as well as glycosaminoglycans (GAGs), such as hyaluronan and heparan sulphate (HS), that lead to its multifunctional role on therapeutic potential in inflammation processes (Birchenough, 2014). There are two modules in its structure that are a link module and CUB module. The link module comprises triple-stranded antiparallel b-sheets and two a-helices arranged around a hydrophobic core that serves for binding to various partners. However, the CUB module's function is still unclear (Milner and Day, 2003).

2.3.3 Current treatment

More than 80% of psoriatic patients are affected with mild to moderate disease (<10% body surface area), which could be remediated by topical treatment and phototherapy (National Psoriasis Foundation, 2009). The conventionally tropical treatments for psoriasis are reviewed by Mendonca and Burden (2003). Vitamin D can inhibit the proliferation and differentiation of keratinocytes as well as decrease IL-8 production. Calcipotriol is a synthetic analogue of vitamin D that is commercially available as a topical product in Europe and the United States, which can bind to the same site as vitamin D receptors, but it is 100 times less affected to Ca^{2+} metabolism due to its rapid local metabolism. In addition, steroids are a common tropical treatment to remedy a mild to moderate psoriasis on affected areas around the face, flexures and genitals. Dithranol can up-production the IL-10 receptor. Dithranol is preferred for use in short contact of not more than 60 min to prevent irritation due to it absorbing quickly by psoriatic skin. Likewise, coal tars are used for inhibition of DNA synthesis and cell mitosis. In addition, retinoids are a compound in the class of vitamin A, which modulates

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keratinocyte proliferation and differentiation. However, the compounds above have potential for psoriasis treatment, but the side effects to the patients are shown in Table 2.2. In consideration, steroids are usually applied to remedy psoriasis patients, and they are mostly available in hospitals and drug stores in Thailand.

Table 2.2 Various side effects of current treatments.

Drugs	Side effects
Calcipotriol	Hypercalcemia, irritation
Corticosteroids	Thin skin, suppressed kidney function
Coal tar	Carcinogen, staining of skin, folliculitis,
Dithranol	Irritation
Retinoids	Irritation

Data from Mendonca and Burden (2003)

2.3.4 Active compounds from plants for anti-psoriasis

Vascular plants can syntheses a multitude of organic phytochemicals, referred to as "secondary metabolites". These molecules are involved in a variety of roles in the life span of plants, ranging from structural ones to protection (Figure 2.8).

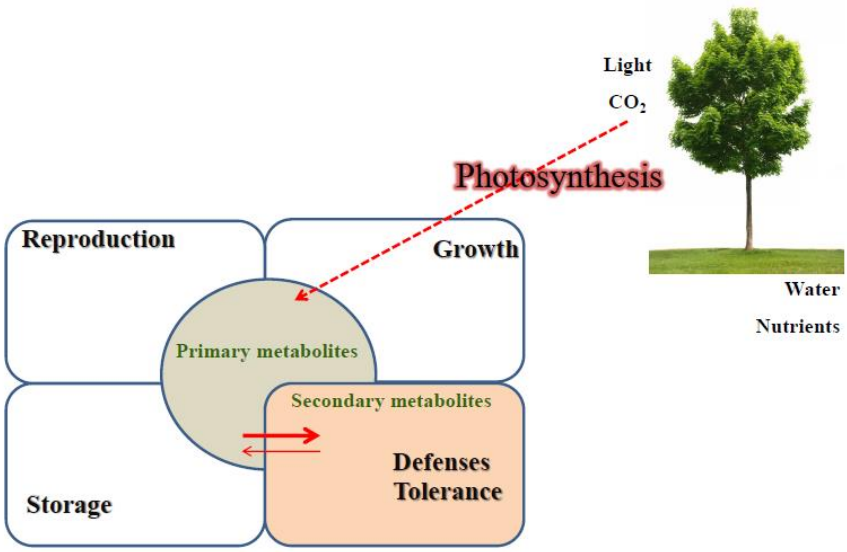

Figure 2.8 Role of secondary metabolites in plants.

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Plants are valuable sources of a wide range for secondary metabolites, which are widely used and exceptional for pharmaceutical advancement. Worldwide, a large number of clinically useful prescription drugs are derived from plants (Table 2.3). Surveys of plant medicinal usage on different regions have shown an increase. Even today, more than 70-95% of the world's population relies on plants for traditional medicine (Mabona et al., 2013).

Table 2.3 Plant-derived pharmaceuticals.

Active compounds	Use	Plant species	References
Ajmalicine	Antihypertensive	*Cath. roseus*	Rao and Ravishankar, 2002
Artemisinin	Antimalarial	*Artemisia annua*	
Berberine	Intestinal ailment	*C. japonica*	
Camptothecin	Antitumour	*Camptotheca acuminata*	
Colchicine	Antitumour	*Colchium autumnale*	
Digoxin	Heart stimulant	*Di. Lanata*	
Diosgenin	Steroidal precursor	*Dioscorea deltoidea*	
Ginsenosides	Health tonic	*Panax ginseng*	Rao and Ravishankar, 2002
Morphine	Sedative	*P. somniferum*	
Podophyllotoxin	Anti-tumour	*Podophyllum petalum*	
Quinine	Anti-malarial	*Cinchon. ledgeriana*	
Sanguinarine	Anti-plaque	*Sanguinaria canadensis*	
Shikonin	Anti-bacterial	*L. erythrorhizon*	
Taxol	Anti-cancer	*Taxus brevifolia*	
Vincristine	Anti-leukemic	*Cath. roseus*	
Vinblastine	Anti-leukemic	*Cath. roseus*	
Rutin	Anti-nociceptive	*Excoecaria agallocha*	Selvaraj et al., 2014
Rutin	Anti-proliferation	*Dimorphandra mollis* Bent	Santos et al., 2011
Caffeic acid	Cerebral ischemia	*Erigeron breviscapus* (vant.)	Chai et al., 2013

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Table 2.3 (cont.)

Active compounds	Use	Plant species	References
Caffeic acid	Anti-inflammation	*Rhodiola sacra*	Jung et al., 2008
Caffeic acid	Anti- inflammation	*Baccharis dracunculifolia* DC	Figueiredo-Rinhel et al., 2013
Caffeic acid	Anti-tumour	*Echinacea purpurea* L.	Tsai et al., 2012

In a few past decades, active compounds for antipsoriasis have had their effects studied. For instance, tests for 60 Chinese medicinal substances revealed that the roots of *Rubia cordifolia*, Realgar and the rhizomes of *Coptis chinensis* extracted with 80% aqueous ethanol possess significant anti-proliferative properties against *in vitro* epidermal keratinocytes on cultured HaCaT cells with IC_{50} values being 1.4, 6.6 and 23.4 μg ml^{-1}, respectively. *R. cordifolia* contains a series of anthraquinone pigments, including purpurin, alizarin rubiadin, munjistin and pseudopurpurin. In addition, triterpene rubifolic acid, rubicoumaric acid, rubiatriol, oleanolic acid acetate as well as sitosterol are also present in this plant (Tse et al., 2006). Gelmini et al. (2013) presented that the oleoresin from *Copaifera langsdorffii* Desf. well established anti-psoriatic properties by oral intake and topical application to psoriatic patients. The oleoresin purified fraction, which contains diterpene acids, diterpenes and sesquiterpenes, reduced the release of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, in a dose-range of 0.1-10 μM. Thai medicinal plants for anti-psoriasis have been explored and reported. Sampson et al. (2001) studied the effects of *Centella asiatica* (Bua-bok, in Thai) and its constituent terpenoids for anti-hyperproliferation of keratinocytes in *in vivo* system, and reported that the two triterpenoid glycosides of madecassoside and asiaticoside could inhibit keratinocytes replication with their IC_{50} values of 8.6 ± 0.1 μM and 8.4 ± 0.6 μM, respectively. Cannabinoid receptors are presented in human skin and that anandamide, an endogenous cannabinoid receptor ligand, inhibits epidermal keratinocyte differentiation. The four constituent cannabinoids of cannabinoids Δ-9 tetrahydrocannabinol, cannabidiol, cannabinol and cannabigerol obtained from *Cannabis sativa* (Kan-chong, in

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Thai) inhibited keratinocyte proliferation in a concentration-dependent manner (Wilkinson and Williamson, 2007). *Curcuma longa* (Khing, in Thai) and *Annona squamosal* (Noi-nhaa, in Thai) had a molecular role in suppressing psoriasis via regulation of NF-κB signaling biomarkers (Saelee et al., 2007). Similarly, indirubin is a major active component of indigo naturalis from *Baphicacanthus cusia* (Nees) Bremek (Hom, in Thai) for the treatment of nail psoriasis (Lin et al., 2014).

Many researchers have reported that phenolic compounds and flavonoids have effects on psoriatic lessons. García-Pérez et al. (2010) investigated the effect of Canadian bark phenolic extract on anti-proliferative properties of normal, lesional and non-lesional psoriatic keratinocytes. They found that the crude extract of black spruce bark obtained from hot water extraction showed a low toxicity on normal human keratinocytes as well as an adequate chemical reactivity towards antiproliferative properties. Procyanidins and monomeric catechins were the main active polyphenols contained in the standardized pine bark extract, and the antiproliferative activity of proanthocyanidins was related to their ability to induce cell cycle arrest and apoptosis in cancer cells (Lizarraga et al., 2007). Polyphenolic from the ethyl acetate fraction of a *Picea mariana* bark extract had inhibitory effects on cytokines, chemokines, adhesion molecules, nitric oxide and prostaglandins produced by keratinocytes under TNF-α activation through down-regulating the NF-κB pathway (García-Pérez et al., 2014). NF-κB plays an important role in immune and inflammatory responses as well as in protection against apoptotic cell death through the regulation of the expression of an exceptionally large number of genes, such as those encoding IL-6, IL-8, fractalkine, elafin, vascular endothelial growth factor (VEGFC), inter-cellular, adhesion molecule, inducible nitric oxide synthase or immune receptors. Caffeic acid derivative and rutin from *G. pseudochina* (L.) var. hispida Thv. showed potential for *in vitro* NF-κB inhibitory activity (Siriwatanametanon and Heinrich, 2011). In which, caffeic acid and rutin have been used in topically formulated ingredients for psoriasis treatment (Priebe et al, 2013; Musthaba et al., 2011).

2.4 Phenolic compounds

Phenolic compounds are a group of plant compounds that are synthesized during plant development in response to various conditions, including infection, wounding and

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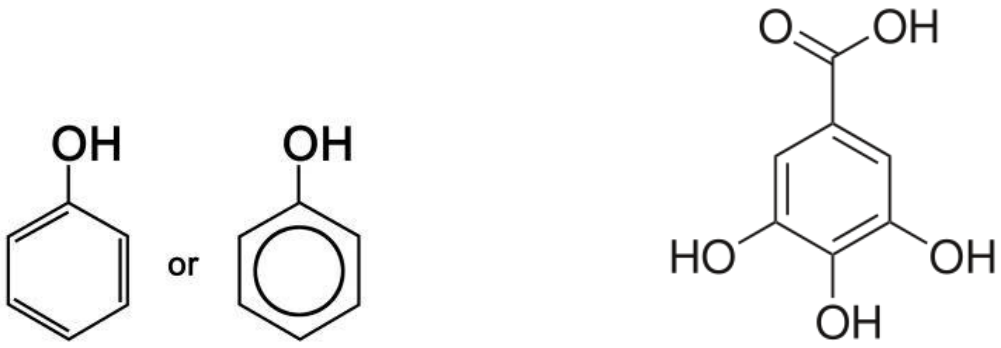
UV radiation (Rao and Ravishankar, 2002). Phenolic acids are produced throughout all stages of plant maturity for many functions. They possess various numbers of biological properties. However, postharvest and extraction processes are very important to derive active phenolic and flavonoid compounds for pharmaceutical application.

2.4.1 Phenolic and flavonoid structures

Over 8,000 compounds in plants belonging to the group of "phenolics". Phenolic acids are the compounds that are composed of one phenol, which is a basic structural of an aromatic ring (Figure 2.9) (Stalikas et al., 2007). The plant phenolic compounds comprise simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, up to hydrolysable and condensed tannins, lignans and lignins.



Phenol

Phenolic acid

Figure 2.9 Chemical structures of phenol and phenolic acid (Stalikas et al., 2007).

The natural phenolic acids comprise two distinctive groups: the hydroxycinnamic and hydroxybenzoic structures (Figure 2.9) . Caffeic, p-coumaric, vanillic, ferulic and protocatechuic are basically present in all plants (Stalikas et al., 2007) . Compounds that are composed of at least two phenol rings are referred to flavonoids, and they have a basic structure arranged in three rings (C6–C3–C6) (Figure 2.10). Their structural variation emanates from the degree and pattern of hydroxylation, methoxylation, prenylation or glycosylation (Agati et al., 2012).

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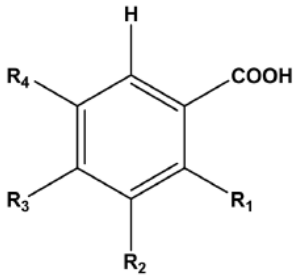
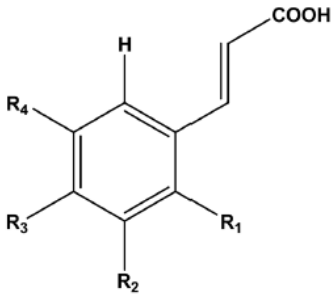


Hydroxybenzoic Acids

Name	R_1	R_2	R_3	R_4
Benzoic acid	H	H	H	H
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
Gentisic acid	OH	H	H	OH
Veratric acid	H	OCH_3	OCH_3	H
Salicylic acid	OH	H	H	H



Hydroxycinnamic Acids

Name	R_1	R_2	R_3	R_4
Cinnamic acid	H	H	H	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	H	H
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H

Figure 2.10 Chemical structures of some phenolic acids and their functional substituents (Stalikas et al., 2007).

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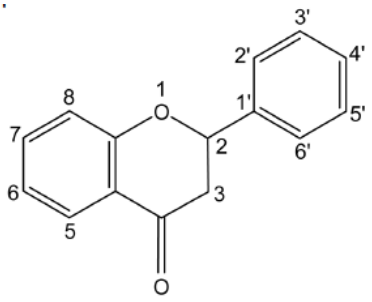
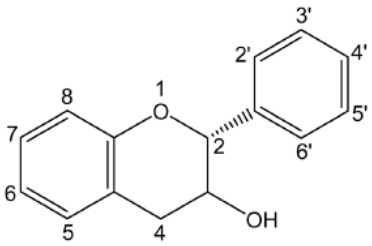


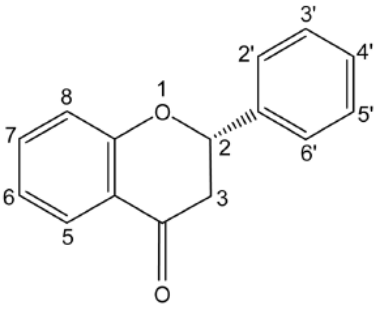
Flavones

Position Compound	5	7	3′	4′
Apigenin	OH	OH	-	OH
Luteolin	OH	OH	OH	OH
Chrysin	OH	OH	–	–



Flavan-3-ols

Position Compound	3	5	7	3′	4′	5′
(+)-Catechin	βOH	OH	OH	OH	OH	–
(−)-Epicatechin	αOH	OH	OH	OH	OH	-
(−)-Epigallocatechin	αOH	OH	OH	OH	OH	OH



Flavanones

Position Compound	5	7	3′	4′
Naringenin	OH	OH	-	OH
Naringin	OH	*O*-Rha-Glu	–	OH
Hesperetin	OH	OH	OH	OCH_3
Hesperidin	OH	*O*-Rha-Glu	OH	OCH_3

Figure 2.11 Chemical structures of flavonoids and their functional substituents (Stalikas et al., 2007).

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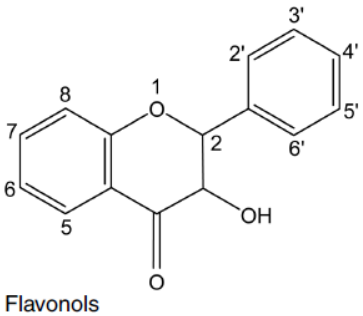
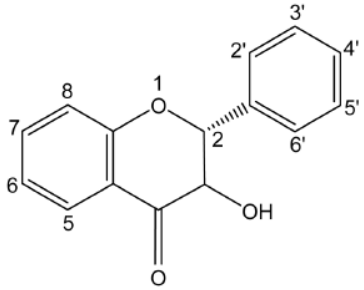


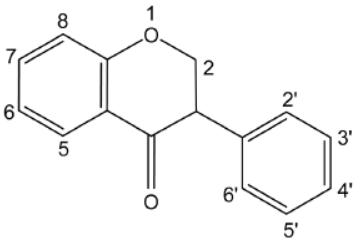
Flavonols

Position Compound	5	7	3′	4′	5′
Quercetin	OH	OH	OH	OH	–
Kaempferol	OH	OH	–	OH	–
Galangin	OH	OH	–	–	–
Fisetin	–	OH	OH	OH	–
Myricetin	OH	OH	OH	OH	OH



Flavanonol

Position Compound	5	7	3′	4′
Taxifolin	OH	OH	OH	OH



Isoflavones

Position Compound	5	7	4′
Genistein	OH	OH	OH
Genistin	OH	*O*-Glu	OH
Daidzein	–	OH	OH
Daidzin	–	*O*-Glu	OH
Ononin	OH	*O*-Glu	CH_3

Figure 2.11 (cont.)

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2.4.2 Sources and role of phenolics and flavonoids in plants

Insoluble phenolics are present around plant cell walls, while soluble phenolics are localized within the vacuoles in plant cytoplasm (Cheynier et al., 2013). The common phenolic acids found in all plants and plant derived foods are cinnamic and benzoic acid derivatives. In addition, most plant phenolic acids are bound to ester, ether or acetal bonds to cellulose, proteins, lignin flavonoids, glucose and terpenes, and the minor fractions are present in the free acid (Ozdal et al., 2013). The diversity of bound phenolic acids contributes to the complexity of the phenolic acids analysis (Cheynier et al., 2013). Flavonoids are polyphenolics that are the most common pigments, next to chlorophyll and carotenoids. They have roles in various functions in plants and ecology. Flavones, flavonols and anthocyanidins act as attractive colors for pollination from insects. In addition, catechins and other flavanols play a role in insect protection in plants (Stalikas et al., 2007).

2.4.3 Biological activities of some phenolics and flavonoids

Phenolics and flavonoids function as antioxidants through chelating with transition metals, such as Cu and Fe, that leads to the prevention of properties in Fenton reactions that can generate high concentrations of hydroxyl radicals. The results from *in vitro* and *in vivo* studies show that they have a diverse ability on phamaceuticals, including prevention of atherosclerotic damage via inhibition of the LDL oxidation. The phenolics also diminish the development of various kinds of tumors, such as cutaneous, mammary, colonic and pulmonary. In addition, phenolics can inhibit *Staphylococcus aureus* and its enterotoxin B production, reduce growth of *Salmonella enteritidis* and inhibit germination and the consequent development of *Bacillus cereus* spores. They also have antiviral activity against the syncytial virus, which affects the human respiratory system (Tripoli et al., 2005).

Flavonoids have abilities on many sides in biology, but the best property of almost all flavonoids is as antioxidants. The antioxidant activities of flavonoids belong to the arrangement of functional groups around the nuclear structure. The B ring of hydroxyl is the most important to determine the scavenging radicals due to its ability on electron donation to hydroxyl, peroxyl and peroxynitrite radicals for the stabilization of radical structures. Flavonoids including catechin, apigenin, quercetin, naringenin, rutin and

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venoruton have hapatoprotective activities. In addition, apigenin, galangin, flavone, flavonol, isoflavones, flavanones and chalcones are reported as bactericidal. In addition, hesperidin, apigenin, luteolin and quercetin have been reported to possess anti-inflammatory and analgesic effects. Moreover, fruits and vegetables containing flavonoids are cancer chemopreventive agents that can inhibit carcinogenesis via both development and hormonal activities in the initiation stage (Kumar and Pandey, 2013).

Chlorogenic acids are formed the compounds in a group of cinnamic acid with quinic acid via esterification. Caffeic acid is a cinnamic acid that is often used as a substrate for chlarogenic acid synthesis that leads to the production of caffeoylquinic acids and dicaffeoylquinic acids as the main natural compounds (Farah et al., 2008). Chlorogenic acid is wildly reported for anti-oxidant and anti-inflammatory properties. It has a potent inhibition on NO production, COX-2 and iNOS, without any cytotoxicity on RAW 264.7 macrophage cells under LPS stimulation. In addition, chlorogenic acid can diminish the production of pro-inflammatory cytokines, including IL-1β and TNF-α, and other inflammation-related markers, such as IL-6. Additionally, and it inhibits the nuclear translocation of NF-κB (Hwang et al., 2014).

Caffeic acid (3,4-dihydroxycinnamic acid) and its derivatives are good substrates of phenolics, which are widely distributed in medicinal plant (fruits, vegetables, wine and olive oil, among others), and they are present in human plasma according to a diet dependent concentration (Buzzi et al., 2009). Varieties of potential pharmacological effects from *in vitro* studies and in animal models of caffeic acid and its derivatives have been reported. Caffeic acid is a tocopherol protectant in low-density lipoprotein (LDL). In addition, its conjugates chlorogenic and caftaric acids that have more powerful antioxidants in a number of different systems (Gülçin, 2006; Wang and Yang, 2011). Caffeic acid possesses anti-inflammatory activity through inhibition of nuclear transcription factor-κB (NF-κB) (Jung et al., 2008; Park et al., 2014) and neutrophils immunomodulatory activity controls (Figueiredo-Rinhel et al., 2013). Caffeic acid has a potent inhibitory effect on proliferative and cancer (Tsai et al., 2012; Sanderson et al., 2013), such as forestomach carcinogenesis, tongue neoplasms (squamous cell papilloma and carcinoma) and preneoplastic lesions (hyperplasia and dysplasia) (Li et al., 2012). In addition, caffeic acid has been reported to have potential antiviral activity, such as anti-hepatitis B virus (Wang et al., 2009) and anti-influenza viruses (Xie et al.,2013).

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Rutin, also called rutoside, quercetin-3 - O-rutinoside and sophorin, is a bioflavonoid usually present in substantial amounts in the plant kingdom. A lot of studies have reported the amazing physiological and pharmacological properties of rutin in mammalian systems, both *in vitro* and *in vivo*. Most of the biological activities are anti-inflammation, anti-asthma, anti-oxidant, anti-microbial and anti-tumor (Santos et al., 2011) and anti-nociceptive (Selvaraj et al., 2014). Interestingly, the stability of rutin against oxidation was found to be higher than aglycone and quercetin. Due to the antioxidantive capacity of rutin, it is also widely used in pharmaceutical, nutraceutical and cosmeceutical industries as a stabilizer, preservative and natural colorant. It is often used in combination with vitamin C since rutin is a bio-flavonoid that is essential for the absorption of vitamin C and acts as an antioxidizer. The human body cannot produce bioflavonoids and rutin, but can be supplied through the diet. Hence, rutin is used not only for prolonging the shelf life of products, but also enriching the nutritional value of products.

2.4.4 Sample preparation and extraction for phenolics and flavonoids

Sample preparation is very important to achieve phenolic recovery. Plant leaves exhibited significantly higher phenolic contents than other parts (Abeysiri et al., 2013; Fernando et al., 2013 and Vilela et al., 2014). In postharvest processes, different drying methods of plant leaves also affect the phenolic and flavonoid contents. In general, solid plant materials are usually subjected to milling, grinding and homogenization, which may be preceded by freeze-drying, air-drying, oven drying or microwave drying (Stalikas, 2007). For example, the highest phenolic contents of *Thunbergia laurifolia* leaves were extracted from the freeze drying method followed by microwave, oven and freeze withering methods with values of 3850±127, 3080±202, 1800±57 and 488±44 mg GAE/100 g, respectively (Chan et al., 2011). The freeze drying method with marionberries, strawberries and corn preserved higher levels of phenolic contents in comparison with air drying (Asami et al., 2003). Freeze drying the leaves of *Alpinia zerumbet* and *Etlingera elatior* resulted in higher gains in phenolic contents than microwave drying, oven drying and sun drying (Chan et al., 2009).

Variations in total phenolic contents between plant leaves of different ages were also reported by several researchers. Developing leaf of *Thunbergia laurifolia* had

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the highest phenolic contents of 513±8 mg GAE/100 g, followed by young and mature leaves with values of 407±11 and 298±9 mg GAE/100 g, respectively (Chan et al., 2011). In contrast, young leaves of two cultivars of bayberry (*Myrica rubra* Sieb. et Zucc.), namely Biqi and Dongkui, recorded the highest levels of total phenolics with values of 194mg/g and 196mg/g, respectively (Yang et al., 2011). Young leaves of five species from the *Zizyphus* genus possessed high total phenolic and total flavonoid contents with values of 19.0 to 28.2 mg GAE/g and 38.1 to 61.8 mg CE/g, respectively (Naz et al., 2013).

Extraction is the main step for the recovery and isolation of bioactive phytochemicals from plant materials before analysis. The achievement from phenolic extraction depended on various factors, including the chemical nature in the plant materials, the extraction method, the size of the sample particle as well as the presence of interfering substances. The necessary steps may be performed to remove unwanted substances, such as waxes, fats, terpenes and chlorophylls. Solid-liquid extraction is the most commonly used method before analysis of the phenolic compounds from plant samples. In addition, soxhlet extraction is frequently used to isolate flavonoids from solid samples (Stalikas, 2007). Solvents commonly applied in the extraction are alcohols (methanol, ethanol), acetone, diethyl ether and ethyl acetate. Very polar phenolic acids, such as benzoic and cinnamic acids, could be extracted completely with a mixture of alcohol-water. Less polar solvents, such as dichloromethane, chloroform, hexane and benzene are more suitable to extract the nonpolar compounds, like waxes, oils, sterols and chlorophyll from the plant materials. Several studies showed that polyphenol contents are obtained diversely from different solvent polarities (Stalikas, 2007). For instance, absolute methanol was suitable for the extraction of *Limoniastrum monopetalum* leaf polyphenols (Trabelsi et al., 2010). To recover *Phoenix dactylifera* L. total phenolics, 70% acetone was more efficient than 88% ethanol, 50% methanol, water and absolute ethanol (Kchaou et al., 2013). The 50% ethanol could extract phenolics with a higher yield than ethyl acetate, absolute methanol and water (Lou et al., 2014). Other factors, such as pH, temperature, sample to solvent volume ratio and the number and time performance, also contribute to the final phenolic content. Extractions should be repeated two to three times, and the extract fractions are combined before evaporation, concentration and/or purification depending on the purpose of its application.

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2.5 Pyrrolizidine alkaloids (PAs)

Pyrrolizidine alkaloids (PAs) are a large group of natural toxins, which are produced by a variety of plant families, in about 3% of the world's flowering plants. PAs are represented by more than 350 individual heterocyclic compounds and have a common basic structure composed of one of the four necine bases of platynecine, retronecine, heliotridine and otonecine (Bolechová et al., 2015). PAs are esters of hydroxylated 1-methylpyrrolizidines and can be present as either the tertiary base or N-oxide form. Those compounds containing a 1, 2-double bond in their pyrrolizidine structures are known to be genotoxic, carcinogenic and hepatotoxic in humans and animals (Oplatowska et al., 2014). Pas' hepatotoxicity, which has been long recognized, is predominantly observed after exposure to the following plant families of Boraginaceae (*Heliotropium* sp., *Trichodesma* sp., *Symphytum* sp. [Comfrey]), Asteraceae (*Senecio* sp. [Bush Teas], *Eupatorium* sp), *Crotalaria* sp. (Leguminosae), Greater Celandine (*Chelidonium majus*) and Ariaceae (*Castilleja* sp.) (Neuman et al., 2015). Traditional medicines composed of herbs often contain a complex mixture of both beneficial and toxic PAs. Acute poisoning due to the use of traditional medicines is a serious problem that resulted in numerous fatalities. The various PAs in the *Gynura* genus have been reported in the two-past decades. For instance, senecionine and seneciphylline were isolated from the tubers of *G. segetum* (Liang and Roeder, 1983; Lin et al., 2011), as well as seneciphylline, senecionine, seneciphylline and seneciphyllinine, N-oxide was isolated from the whole plant (Qi, 2009). In addition, gynuramine and acetylgynuramine were found in *G. scandens* (Wiedenfeld, 1982), while intergrrimine and usaramine were identified from *G. divaricata* (Roeder, 1996). Moreover, senecionine and senkirkine were also isolated from *G. pseudochina* (L.) DC. (Windono et al., 2012). Therefore, the concentration of PAs in plant derived products are regulated to safe levels by the associated agencies. The United Kingdom Medicines and Healthcare Product Regulation Agency regulates PAs in herbal products to 1 mg/day (16.7 µg/kg/day for a 60 kg of body weigh) for two weeks or to 0.1 mg/day (1.67 µg/kg/day) for long-term (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).

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2.6 Topical cream for skin diseases

Creams are semisolid emulsions. They are often composed of oil and water phases. Oil-in-water (o/w) emulsions are advantageous over water-washable, while water-in-oil (w/o) emulsions are useful to prevent water loss from the skin. The o/w emulsions can clean the skin, because the hydrophobic property can access the lipid bilayer in our skin. The active ingredients are often dissolved in one or both phases depending on their solubilizing properties. The w/o creams are better at spreading and more suitable for inflamed tissue. On the other hand, o/w creams (vanishing creams) rub into the skin. However, the evaporation of the continuous phase can increase the concentration of a water-soluble drug in the adhering film that leads to enhance the drug across the stratum corneum because of a concentration gradient between the cream and skin (Mehta, 2004). In addition, consumers usually choose a o/w cream due to its less greasy feeling. Prototyping activities are performed in bench-scale experiments to prepare a prototype of a product to test the product's properties. All property tests require an amount of the prototypic cream that is enough for each test. The results from the prototype test will be used for making the next prototype (Cheng et al., 2009).

The physical stability of the finished cream is very important for the cream quality. A cream that presents a coalescence of the internal phase, creaming, phase inversion and changes in the apparent characters, including odor, color and other physical properties, are defined as instable physical properties. The presenting of any flocculation and resultant creaming leadings to total coalescence of the internal phase. Therefore, flocculation, creaming, coalescence and breaking are the major phenomena that are considered as physical instabilities of the cream (Bajaj et al., 2012).

Several conditions during storage, such as temperature, light, oxygen and moisture through the intrinsic factors within the cream formulation may affect the active compound concentration via many chemical reactions that lead to a reduction in the drug efficiency. In addition, a destructure of the drug may lead to the formation of toxic products. A number of chemical reactions for drug degradation have been determined by the structural characteristics of the drug, which may occur via hydrolysis, dehydration, isomerization and racemization, decarboxylation and elimination, oxidation, photodegradation, drug-excipients and drug-drug interactions. In addition, oxygen may

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contribute to the chemical properties of the unsaturated oil components via stimulating the oxidation reaction. Adding vitamin C or other anti-oxidant agents can provide the reduction and the absence of unsaturated oil oxidation presents a mild change in color, texture and rancidity in the formulation (Sarkar et al., 2011; Bajaj et al., 2012).

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CHAPTER 3

MATERAILS AND METHODS

This research was divided into three parts of (i) study of the qualitative and quantitative analysis of phenolic and flavonoid compounds from *G. pseudochina* leaf extracts, under variations in leaf age, drying process, and polarity of solvents; (ii) *in vitro* assay of *G. pseudochina* leaf extracts on the toxicology and anti-inflammatory properties with inhibiting NF-kappa B activation on non-TNF-α stimulating and TNF-α stimulating HaCaT cells; and (iii) preparation of a prototypic cream from *G. pseudochina* leaf extracts and then the cream formulas were tested in physical and chemical stability tests at different storage times. Research diagrams of the three parts are shown in Figures 3.1, 3.2, and 3.3, respectively.

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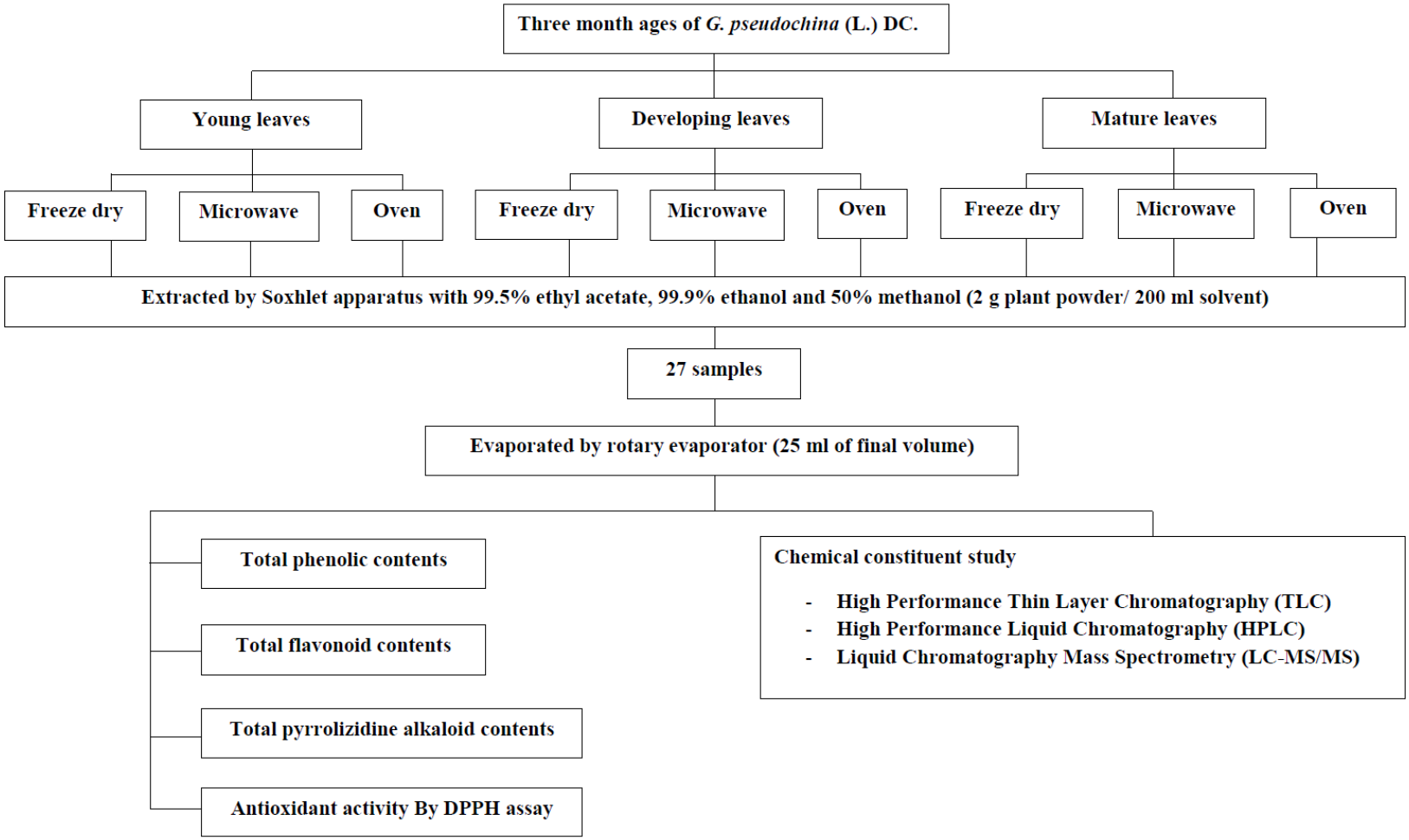

Figure 3.1 Research diagram for study by qualitative and quantitative analysis of phenolic compounds from *G. pseudochina* leaf extracts prepared from variations in leaf age, drying process, and polarity of solvent.

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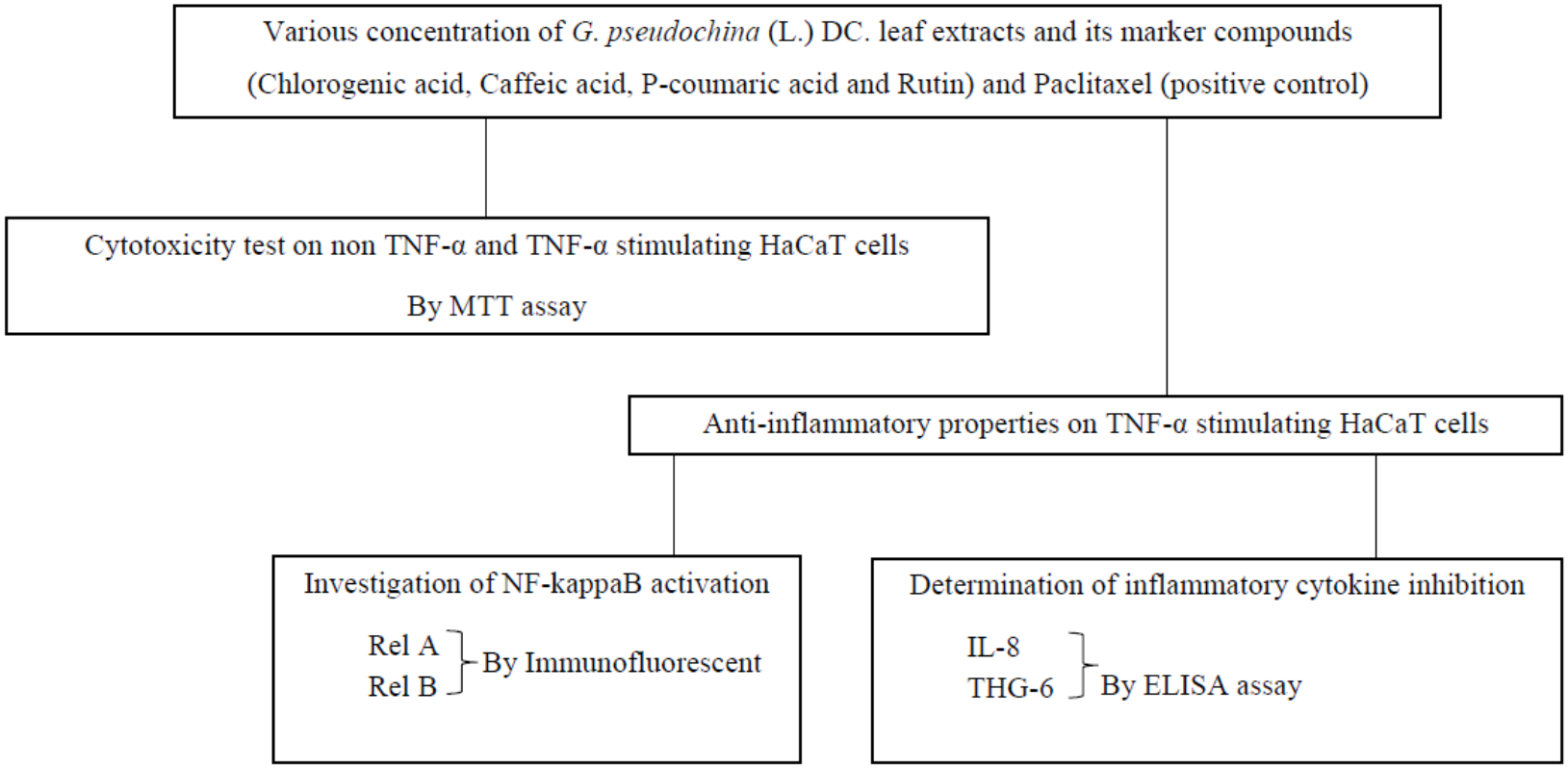

Figure 3.2 Research diagram for study of cytotoxicity test and anti-inflammatory property on non-TNF-α and TNF-α stimulating HaCaT cells of *G. pseudochina* leaf extracts.

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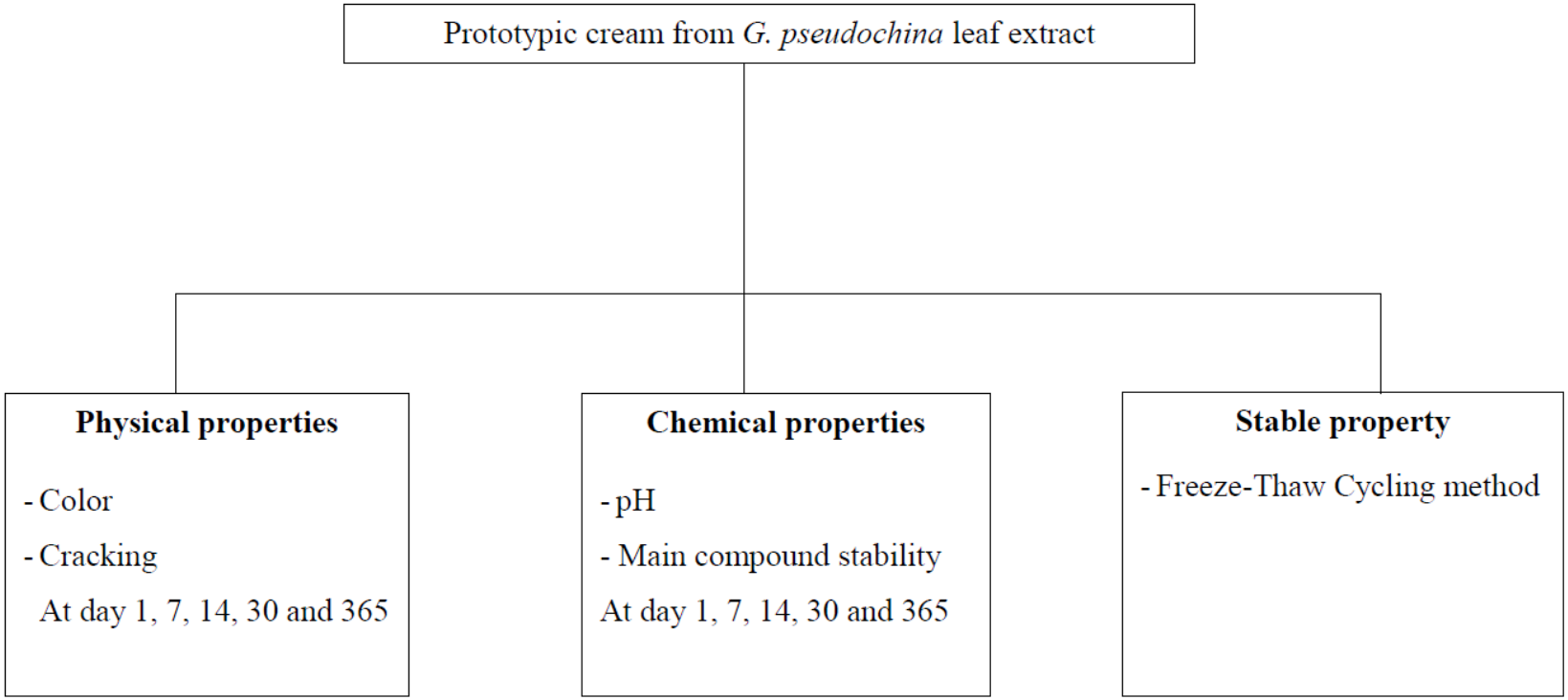

Figure 3.3 Research diagram for study of physical, chemical, and stable properties of prototypic cream from *G. pseudochina* leaf extract.

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3.1 Equipment

3.1.1 High-performance liquid chromatography (Shimadzu SIL-10AD, Japan)

3.1.2 Lyophilizer (Freeze-Dry System Model 77530, Japan)

3.1.3 UV-visible spectrometer (Agilent 8453 UV-Vis, USA)

3.1.4 Soxhlet extraction set (FAVORITE Borosilicate Glass 3.3)

3.1.5 Rotary evaporator (BUCHI Rotavapor R-215, Switzerland)

3.1.6 Microplate reader (VersaMax ELISA, USA)

3.1.7 Micropipettes (NICHIRYU EX, Japan)

3.1.8 Heating mantle (WiZard SAF500, Thailand)

3.1.9 96-well plate (Costar 3599, USA)

3.1.10 Microwave (DAEVOO KOR 86D7, Malesia)

3.1.11 Hot air oven (Red Line Binder, Germany)

3.1.12 Mixer (UZUSIO VTX-3000L, Japan)

3.1.13 Freezer (SANYO, Thailand)

3.1.14 CO_2 Incubator (Thermo Scientific, USA)

3.3.15 Biosafety Cabinet Class II (Lab Gard, USA)

3.3.16 Fluorescent microscope (Life Technologies, UK)

3.3.17 HPTLC system (CAMAG, Muttenz, Switzerland)

3.3.18 Spectrophotometer (HunterLab ColorFlex EZ, USA)

3.3.19 InertSustain C18 column (GL Science, UK)

3.3.20 LC-MS/MS (Agilent Technologies, Waldbronn, Germany)

3.3.21 Luna C18(2) column (Phenomenex, USA)

3.3.22 Guard column (4.6 mm x 10 mm, 5 µm) (VetiSepTM UPS C-18, Thailand)

3.2 Chemical reagents

3.2.1 Solvents

95% Hexane analytical grade (MERCK, Germany)

99.9% Ethanol analytical grade (MERCK, Germany)

99.8% Methanol analytical grade (MERCK, Germany)

99.5% Ethyl acetate analytical grade (FISHER, UK)

99.9% Dichloromethane (MERCK, Germany)

Acetic acid (MERCK, Germany)

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Formic acid (MERCK, Germany)

3.2.2 Reference compounds

Gallic acid ($C_7H_6O_5 \cdot H_2O$) (Sigma-Aldrich, China)

Myricetin ($C_{15}H_{10}O_8$) (Fluka, France)

Caffeic acid ($C_9H_8O_4$) (Sigma-Aldrich, Switzerland)

Catechin ($C_{15}H_{14}O_6$) (Fluka, Switzerland)

Vanillin ($C_8H_8O_3$) (Carlo Erba, France)

Quercetin ($C_{15}H_{10}O_7$) (Fluka, Switzerland)

Kaempferol ($C_{15}H_{10}O_6$) (Fluka, Germany)

Naringenin ($C_{15}H_{12}0_5$) (Sigma-Aldrich, England)

Epicatechin ($C_{15}H_{14}O_6$) (Fluka, Germany)

Rutin ($C_{27}H_{30}O_{16}$) (MERCK, England)

Chlorogenic acid (Sigma-Aldrich, Switzerland)

p-coumaric acid (Sigma-Aldrich, Switzerland)

Curcumin (Sigma-Aldrich, Switzerland)

Paclitaxel (Sigma-Aldrich, Switzerland)

3.2.3. Chemicals

2,2 diphenyl-1-picrylhydrazyl (DPPH) (Fluka, USA)

Aluminum chloride ($AlCl_3$) (Ajax Finechem, Austria)

Folin-Ciocalteu reagent (Carlo Erba, France)

Sodium nitrite ($NaNO_2$) (Ajax, New Zealand)

Sodium Hydroxide (NaOH) (Ajax, New Zealand)

Sodium carbonate (Na_2CO_3) (Fluka, Switzerland)

3,4,5-dimethylthiazol-2-yl-2,5-diphynyltetrazolium bromide (Sigma-Aldrich, Switzerland)

DMEM (GibcoTM ThermoFisher Scientific, UK)

Phosphate Buffer Saline (GibcoTM ThermoFisher Scientific, UK)

Fetal Bovine Serum (GibcoTM ThermoFisher Scientific, UK)

Penicillin-Streptomycin (GibcoTM ThermoFisher Scientific, UK)

DMSO (Dimethyl sulfoxide) (Santa CruzBiotechnology, Inc, Canada)

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Trypsin (GibcoTM ThermoFisher Scientific, USK)

Tumor nuclear factor-α (PeproTech, USA)

Human TSG-6 ELISA kit (RayBio, Georgia)

Human IL-8 ELISA kit (GeneTex, USA)

Human IL-17 ELISA kit (Cytoscrren, Belgium)

Monocrotaline (Sigma-Aldrich, Switzerland)

Triton X-100 (GeneTex, USA)

RelA (Ser529) polyclonal antibody, Alexa Fluor350 (Bioss antibody, USA)

RelB (Ser573) polyclonal antibody, Alexa Fluor488 (Bioss antibody, USA)

Cyto nuclerstain (Bioss antibody, USA)

Poly ethylene glycol 100 (Sigma-Aldrich, Switzerland)

Ethanolamine diphenyl borate (Sigma-Aldrich, Switzerland)

p-dimethyl alaminobenzaldehyde (Sigma-Aldrich, Switzerland)

3.3 Methods and analysis

3.3.1 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 at 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. Weather during the daytime during January 2014-April 2016 was 35±5°C, 20000±10000 lux light, 70±20% humidity. The shoot parts of 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 co-estimated by leaf length and leaf position on stem as young leaves (leaf length < 15 cm, first to tenth leaves from apical bud), developing leaves (leaf length 16-20 cm, eleventh to twentieth leaves from apical bud), and mature leaves (leaf length >20 cm, more than twentieth leaves from apical bud). For freeze drying process, liquid nitrogen was used to prefreeze 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 carried out by a digital

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microwave (Samsung J7EV, Malaysia) at 600 watts for 8 min. For oven drying process, the leaves were dried in a hot air oven (RI 53 Binder, Germany) at 60ºC for 48 h. All dry samples were ground into homogeneous a powder, then they were sampled for color measurement by a bench top Colorimeter spectrophotometer (ColorFlex EZ HunterLab, USA). The ground samples were preserved in a closed dark container with silica absorber for further study.

3.3.2 Plant extraction

An extraction was carried out 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 *G. pseudochina* extracts were summarized, coded, and analysis purpose indicated in Table 3.1.

The first step, serial extraction with various solvents was studied with various drying samples (freeze dry, microwave oven) of different leaf ages (young, developing, mature leaves). A dried leaf sample was defatted with 95% (v/v) hexane (30 min per cycle) for five cycles, then 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. A concentrated extract was made up to a 25 ml final volume before being kept in an amber glass bottle with tight stopper at -20ºC until analysis. A concentrated extract was analyzed for weight of the crude extract, total phenolic content (TPC), total flavonoid content (TFC), free radical scavenging activity (FRSA), and high-performance liquid chromatography (HPLC). In addition, the mixed age leaves dried by a microwave, oven, and freeze-drying processes were investigated for high-performance thin layer liquid chromatography (HP-TLC) and total pyrrolizidine alkaloid content (TPAsC) analysis. The extracts of the mixed age leaves were carried out by defatting with hexane, then 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

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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 removed the chlorophyll with 99.9% (v/v) ethanol for two cycles. Then, the samples were serial extracted with 50% (v/v) and 25% (v/v) ethanol, respectively, 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 study the cell cytotoxicity and to evaluate its effects on RelA and RelB inhibition, TSG-6 inhibition, and IL-8 inhibition. In addition, EMLM was an ingredient in our prototypic cream formula.

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Table 3.1 Samples codes for *G. pseudochina* extracts prepared from various drying processes, leaf ages, extraction processes and analysis purposes.

Sample code	Drying process and leaf age	Extraction process	Fraction and analysis
FY	Freeze dry, young leaves	(i) 95% (v/v) hexane, (ii) 99.9% (v/v) ethyl acetate, (iii) 99.9% (v/v) ethanol, and (iv) 50% (v/v) ethanol	Fraction: (ii), (iii), (iv) Analysis: TPC, TFC, FRSA, HPLC
FD	Freeze dry, developing leaves		
FM	Freeze dry, mature leaves		
MY	Microwave dry, young leaves		
MD	Microwave dry, developing leaves		
MM	Microwave dry, mature leaves		
OY	Oven dry, young leaves		
OD	Oven dry, developing leaves		
OM	Oven dry, mature leaves		
MLF	Freeze dry, mixed ages leaves	(i) 95% (v/v) hexane, (ii) 99.9% (v/v) ethanol, (iii) 50% (v/v) ethanol, and (iv) 25% (v/v) ethanol	Fraction: (iii), (iv) Analysis: TPAsC, HP-TLC
MLM	Microwave dry, mixed ages leaves		
MLO	Oven dry, mixed ages leaves		
EMLM	Microwave dry, mixed ages leaves	Dried crude extract of MLM collecting from (iii) plus (iv)	*in vivo* assay: Cytotoxicity, RelA, RelB, IL-8, TSG-6 Ingredient for prototypic cream

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Table 3.1 (cont.)

Sample code	Drying process	Extraction process	Fraction and analysis
25% EtOH	Microwave dry, mixed ages leaves	Defatted with hexane, Extraction with 99.9% (v/v) ethanol	Analysis: TPC, TFC, FRSA, HPLC
50% EtOH		Defatted with hexane, Extraction with 50% (v/v) ethanol	
75% EtOH		Defatted with hexane, Extraction with 75% (v/v) ethanol	
100% EtOH		Defatted with hexane, Extraction with 100% (v/v) ethanol	

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3.3.3 Total phenolic content determination

The total phenolic content (TPC) determination was performed 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. Then, 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). 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).

3.3.4 Total flavonoid content determination

The total flavonoid content (TFC) determination 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. Then, 30 µl of 5% (w/v) $NaNO_2$ was added and mixed. The mixture was stood in the dark for 5 min before having 60 µl of 10% (w/v) $AlCl_3$ added. 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 value were expressed in terms of EC equivalent (µmol ECE/ g dry weight).

3.3.5 Free radical scavenging activity determination

Free radical scavenging activity determinations (FRSA) were 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 equation:

Free radical scavenging activity (%) = $(A_0 - A_i)/A_0 \times 100$

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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.

3.3.6 Total pyrrolizidine alkaloid determination

The total pyrrolizidine alkaloid determination (TPAsT) was measure 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. Then, 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 Erhlich's reagent (1g of p-dimethyl alaminobenzaldehyde 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 then the absorbance of the magenta color (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.).

3.3.7 HPLC (High performance liquid chromatography) analysis

The phenolic compounds in the extracts were investigated 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 20 µl of a sample was applied. The mobile phase was the 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 at 1 ml/min and the column temperature was 40°C. The gradient profile is shown in Table 3.2. 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

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compounds. CGA, CA, and RUT, which were confirmed by the RT of LC-MS, were measured for quantitative analysis using the external standard methods. The same HPLC conditions were performed with various concentrations for the preparation of the calibration curves.

Table 3.2 Gradient conditions of mobile phase for HPLC.

Time (min.)	Solvent A	Solvent B
0	100	0
5	90	10
10	80	20
15	70	30
20	60	40
30	50	50
35-40	100	0

Validation of HPLC method was concerned in this study. The analytical curve of each standard was drawn between the concentration and the peak area of the phenolic compounds over a wide concentration range (5 and 100 µg/ml). The linearity of a curve was obtained from correlation coefficient of regression line. The blank data with a nonzero standard deviation presenting was analyzed to get the values of Limit of Detection (LOD) and Limit of Quantitation (LOQ). LOD is presented in terms of a concentration corresponding to the sample blank value plus three standard deviation, and LOQ is a concentration corresponding to the sample blank value plus ten standard deviations as shown in the equations (3.1) and (3.2) (Shrivastava and Gupta, 2011)

$$LOD = \bar{x}_{blank} + 3SD_{blank} \quad (3.1)$$

$$LOQ = \bar{x}_{blank} + 10SD_{blank} \quad (3.2)$$

Where $\bar{x}$ is the mean concentration value of the blank and SD is standard deviation value of the blank.

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The LOD and LOQ for each marker compound (CGA, CA, PCA and RUT) were analysed from methanol, which was represented as matrix applied, and also base cream extracted with methanol. The quantitative of the marker compounds in all extracts were obtained from 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.

3.3.8 HP-TLC (High Performance Thin Layer Chromatography) analysis

The investigation of phenolic compounds contained in the plant extracts was carried out by a HPTLC system (CAMAG, Muttenz, Switzerland) consisting of 100 ml syringes on a sample applicator connected to a nitrogen tank, twin trough chamber 20 x10 cm, TLC Plate Heater, and TLC visualizer linked to visionCATS software. Each plant extract and standard compound were prepared at 30 mg/ml. The resultant samples were filtered through a 0.22 µm pore size filter (Corning Inc., Corning, NY, USA) before being operated on a silica 60F 254 on aluminum sheet (10×20 cm) (Merck, Darmstadt, Germany) under the following conditions: syringe delivery speed, 10 s/µl and injection volumes, 4 µl for plant extract and 2 µl for 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. Natural product reagent I (1% (w/v) ethanolamine diphenyl borate in methanol) and 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. The band identification was performed by comparison with four standards of chlorogenic acid (CGA), caffeic acid (CA), p-coumaric acid (PCA), and rutin (RUT).

3.3.9 Liquid Chromatography coupled with Mass Spectrometry (LC-MS/MS) analysis

The main components that could not be identified by HPLC and our chemical standards were determined by LC-MS/MS, with quadrupole-time of flight (QTOF) mass

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analyzers. 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 the solvent flow rate of 500 µl/min. The mobile phase was the gradient elution between water (solvent A) and acetonitrile (solvent B), and both contained 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 system was carried out by an QTOF 6540 UHD accurate mass. The condition for 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. Agilent MassHunter qualitative Analysis Software B06.0 (Agilent Technologies, CA, USA) was applied for the data analysis.

3.3.10 Cell cytotoxicity

The plant extracts were dissolved in 50% (v/v) dimethyl sulfoxide (DMSO) to give 50 mg/ml as 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 Medium/High glucose (DMEM/HG) (GibcoTM ThermoFisher Scientific, USA) with 10% (v/v) fetal 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 air atmosphere. At 80% confluence, 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 5×10^4 cells/ml and allowed to grow for 12 h before being pretreated 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 standards. Paclitaxel (PTX) of 0.3, 0.6, 1.3, 2.5, and 5.0 µg/ml was also used as a positive control. The DMSO contained in the medium were controlled to be less than 1%. After 24 h of

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exposition, the medium was aspirated, the cells were washed twice with PBS and a solution of 110 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at a final concentration of 0.5 mg/ml was added during 2 h. Then, the MTT solution was removed and washed with 0.01M phosphate buffer 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 then 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 equation (3.3):

$$\text{Cell survival rate (\%)} = (OD_{Sample} - OD_{Blank})/(OD_{Control} - OD_{Blank}) * 100\% \quad (3.3)$$

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

3.3.11 RelA and RelB inhibition

The HaCaT cells were cultured in DMEM/HG without phenol red (GibcoTM ThermoFisher Scientific, USA) with the same condition as above. The confluence cells were dissociated with trypsin and plated into 12-well plates at 5×10^4 cells/ml for 12 h before pre-treatment with 50 ng/ml TNF-α for 12 h. The TNF-α stimulation cells were treated with different concentrations of the samples that were determined following the IC_{50} and half of IC_{50} values from the cytotoxicity on TNF-α stimulating cells as follows: 750 and 375 µg/ml for 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. Cells were fixed with 2 ml of methanol and acetone (1:1) at 4°C for 10 min. Then, 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). Cells were incubated with antibodies overnight (1:100) following anti-phospho-RelA (Ser529) conjugated with DAPI and anti-phospho-RelB (Ser573) conjugated with GFP. Then counterstain the nucleus with Texa Red for 5 min. Cells were washed with PBS two times to remove unbound dye before

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being observed under an EVOS FL Cell Imaging System (Life Technologies, UK) at 40x. The cell culture medium after treatment of each sample from this experiment was collected to measure the IL-8 contents by ELISA Kit (GeneTex International Corp., UK). A 100 µl amount of each sample was added into pre-coat 96-well plates, sealed the plate with a cover, and incubated at 37°C for 90 min before discarding the plate contents and blotted the plate onto paper. Then 100 µl of biotinylated anti-human IL-8 antibody was added into each well and incubate the plate at 37°C for 60 min before washing three times 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 five time with PBS. Add 90 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) color developing agent in to each well and incubate at 37°C for 20 min before added 100 µl of stop solution. The absorbance at 450 nm was read within 30 min after exposure to the TMB stop solution. The measurement was compared to an IL-8 standard curve prepared with 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 pg/ml.

3.3.12 Interleukin 8 (IL-8) inhibition

The cell culture medium after treatment of each sample from the RelA and RelB study was collected to measure the IL-8 contents by ELISA Kit. Bring all reagents and samples to room temperature (18-25ºC) before use. Then 100 µl of each sample was added into the pre-coated 96-well plates, sealed the plate with the cover, and incubated at 37°C for 90 min before discarded the plate contents and blotted the plate onto paper. A 100 µl sample of biotinylated anti-human IL-8 antibody was added into each well and incubate the plate at 37°C for 60 min before washing three times 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 five time with PBS. Add 90 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) color developing agent in to each well and incubate at 37°C for 20 min before adding 100 µl of stop solution. The absorbance at 450 nm was read within 30 min after exposure to the TMB stop solution. Measurements were compared to an IL-8 standard curve prepared with 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 pg/ml.

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3.3.13 Tumor necrosis factor-inducible gene 6 protein (TSG-6) inhibition

The cell culture medium after treatment of each sample from the RelA and RelB study was collected to measure the TSG-6 contents by ELISA Kit. Bring all reagents and samples to room temperature (18-25ºC) before use. A 100 µl amount of each standard and sample was added into pre-coated 96-well plates before covering and incubating for 2.5 h at room temperature with gentle shaking. The solution in each well was discarded then each well was washed four times with 300 µl of 1x wash solution. The plate was invert and blotted against clean paper towels. A 100 µl sample of 1x biotinylated antibody was added into each well before incubation for 1 h at room temperature with gentle shaking. The solution in each well was discarded before washing four times with 300 µl of 1x wash solution. A 100 µl sample of Streptavidin solution was added to each well before incubation for 45 min at room temperature with gentle shaking. The solution was discarded before washing four times with 300 µl of 1x wash solution. A 100 µl sample of TMB was added to each well before incubating for 30 min at room temperature in the dark with gentle shaking. A 50 µl sample of stop solution was added to each well before recording the absorbance at 450 nm immediately. Measurements were compared to an TSG-6 standard curve prepared with 0.21, 0.51, 1.28, 3.2, 8, and 20 ng/ml.

3.3.14 Prototypic cream preparation

In this study, an oil in water (O/W) cream was prepared. All ingredients (Table 3.3) were mixed well by adding the oily phase to the aqueous phase with continuous agitation. The oily phase consisted of hydrogenated polydecene, glyceryl stearate, ceteary alcohol, dimethicone, and ceteareth-20. The aqueous phase consisted of aqua (water), xanthan gum dissolved in 1,3 propanediol, and disodium EDTA. Both phases were heated to 75±5°C. Then the oil phase was added to the aqueous phase drop by drop. Stirring was continued at 3000 rpm by a mechanical mixer for about 20 min until the oily phase was added completely. After complete addition of the oily phase, the speed of the mixer was reduced to 1000 rpm for homogenization, and the mixture was cooled until the temperature was 40°C before adding phenoxyethanol, glyceryl laurate, and the *G. pseudochina* extract dissolved in 1,3 propanediol. The finished cream was allowed to cool before packing into packages with 5 g in each and kept at 4°C for further study.

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Table 3.3 Prototypic cream formulation.

Ingredients	Cream base (%w/w)	EMLM cream (%w/w)	Function	Source
Aqua (water)	68.50	68.00	Solvent	Water
Hydrogenated polydecene	10.00	10.00	Emollient	Synthetic
1,3 propanediol	8.00	8.00	Solubilizer	Corn derived
Glyceryl stearate	6.40	6.40	Emulsifier	Palm derived
Ceteary alcohol	2.90	2.90	Emulsifier	palm derived
Dimethicone	1.50	1.50	Emollient	Synthetic
Ceteareth-20	0.90	0.90	Emulsifier	Palm derived
Phenoxyethanol	0.80	0.80	Preservative	Synthetic
PEG-100 stearate	0.60	0.60	Emulsifier	Synthetic
EMLM extract	-	0.50	Anti-inflammation	Plant
Xanthan gum	0.20	0.20	Thickener	Natural derived
Disodium EDTA	0.10	0.10	Chelating agent	Synthetic
Glyceryl laurate	0.10	0.10	Preservative	Synthetic

3.3.15 Cream stability test

The finished creams were submitted to physical and chemical stability testing, and they were evaluated at days 1, 7, 14, 30, and 365 after a freeze thaw cycle. For physical stability, the texture was observed for coalescence of the internal phase and phase inversion. The color of the cream was measured by a colorimeter, which reported the values of lightness (L*), red (a*), and yellow (b*). A 0.1 g sample of the cream was dissolved in 10 ml of distilled water, before being centrifuged and measured for a pH value from the supernatant (Gidwani et al., 2010). The quantity of each marker compound was evaluated by HPLC. A 1 g sample of cream was dissolved in 5 ml of 50% (v/v) ethanol and sonicated for 1 h before being centrifuged at 3000 rpm for 5 min. The supernatant was collected and filtered before analysis. In the case of the stability test, each cream sample was kept at 45ºC for 48 h and was continuously kept at 4ºC for 48 h. The physical and chemical stability tests were repeated six times for texture, color, pH, and freeze-thaw cycling.

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3.3.16 Statistical analysis

The data were expressed as the means and standard deviations (SD). The analysis was performed using 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). Pearson correlation coefficients were determined to compare the correlations between the variations.

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CHARPTER 4

RESULTS

4.1 Dried leaf color and moisture removal

The moisture removal in terms of percentage difference from the drying processes and leaf ages are shown in Table 4.1. The results indicated that freeze drying, microwave, and oven drying processes of all leaf ages were similar in the moisture removal. The freeze drying preserved the green color of the leaves (Figure 4.1). The thermal drying method of microwave and oven decreased the lightness (L^*) and yellow colour (b^*) of the *G. pseudochina* leaves (Table 4.1).

Figure 4.1 Dried plant leaves after drying with (a) freeze dry, (b) microwave and (c) oven.

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Table 4.1 Color and moisture removal (%) of *G. pseudochina* leaves with various leaf ages and drying methods.

Drying process	Age of leaf	Moisture removal (%)	Dried leaf color		
			Lightness (*L**)	Red (*a**)	Yellow (*b**)
Freeze dry	Young	92.1±0.2^{b}			
	Developing	92.2±0.1^{ab}	64.43±0.01^{a}	-4.54±0.01^{c}	25.08±0.01^{a}
	Mature	92.6±0.3^{ab}			
Microwave	Young	92.7±0.5^{ab}			
	Developing	92.8±0.3^{ab}	44.75±0.01^{b}	-0.75±0.02^{b}	22.75±0.01^{b}
	Mature	93.1±0.4^{ab}			
Oven	Young	92.8±0.2^{ab}			
	Developing	92.9±0.4^{ab}	37.17±0.02^{c}	2.79±0.02^{a}	18.17±0.01^{c}
	Mature	93.2±0.4^{a}			

The different letter(s) (a-c) in same column are significant differences according to Scheffe's test ($P < 0.05$). Data are given as means ± SD (n=3).

4.2 Total phenolic and flavonoid contents and antioxidant activity 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 strongly recovered from the freeze-dried and microwave-dried samples, as shown in Figures 4.2 and 4.3, respectively. The leaf extracts obtained from the ethyl acetate fraction tend to contain higher TPC and TFC than the ethanol and 50% methanol fractions (Figure 4.2a and 4.2b). However, the 50% methanol solvent could strongly yield the highest crude extracts in all samples (Figure 4.4a). According to the plant dried weight, the highest TPC and TFC were obtained from 50% (v/v) methanol (Figure 4.3a and 4.3b). In addition, Figure 4.4b shows the FRSA of the leaf extracts as IC_{50} values. A low IC_{50} value indicates a high antioxidant 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 4.2. In which, TPC and TFC were more highly recovered from freeze-dried and microwave-dried samples in all leaf ages.

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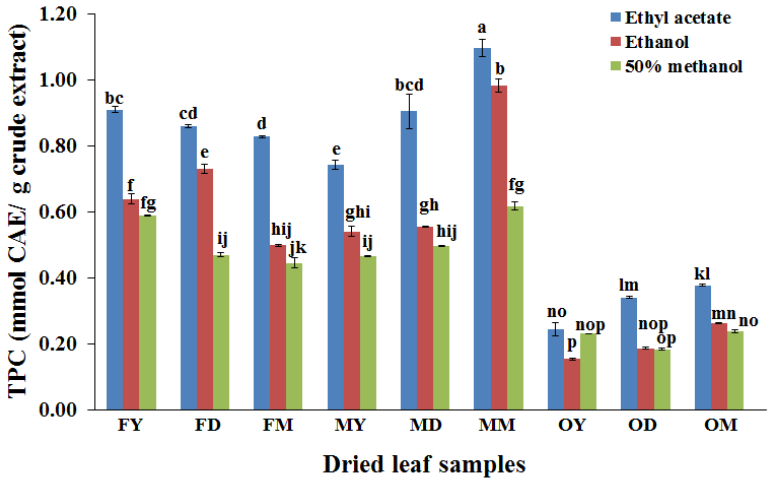
(a)



(b)

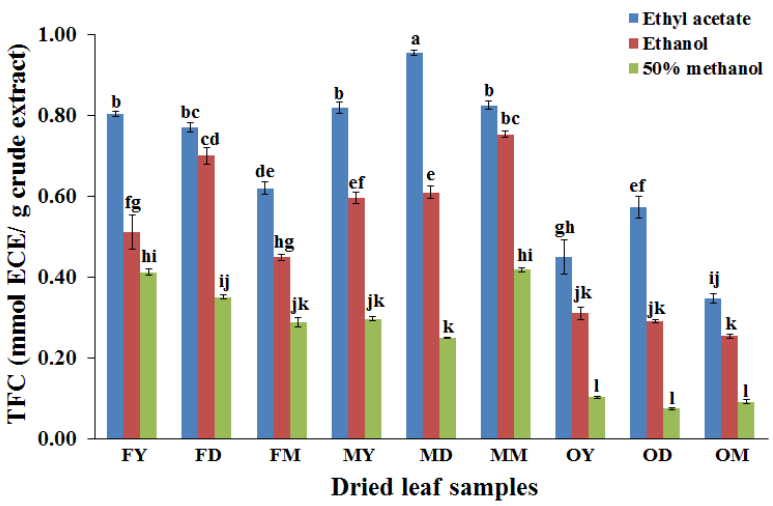


Figure 4.2 TPC (a) and TFC (b) of *G. pseudochina* leaf extracts per g crude extract prepared with different drying processes, leaf ages, and polarity of solvents. Abbreviations of sample names: first letter, F is freeze dry with M is microwave and O is oven; second letter, Y is young leaf, D is developing leaf, and M is mature leaf. Different letter(s) (a-p) are significant differences according to Scheffe's test ($p < 0.05$). Data are given as means ± SD (n=3).

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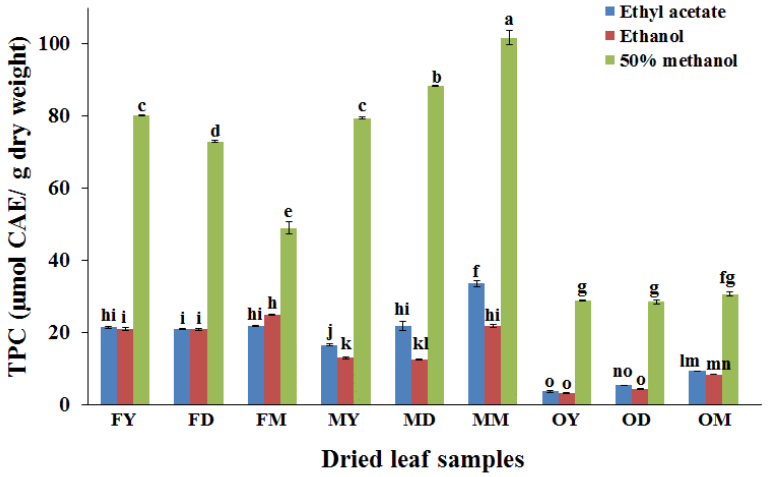
(a)



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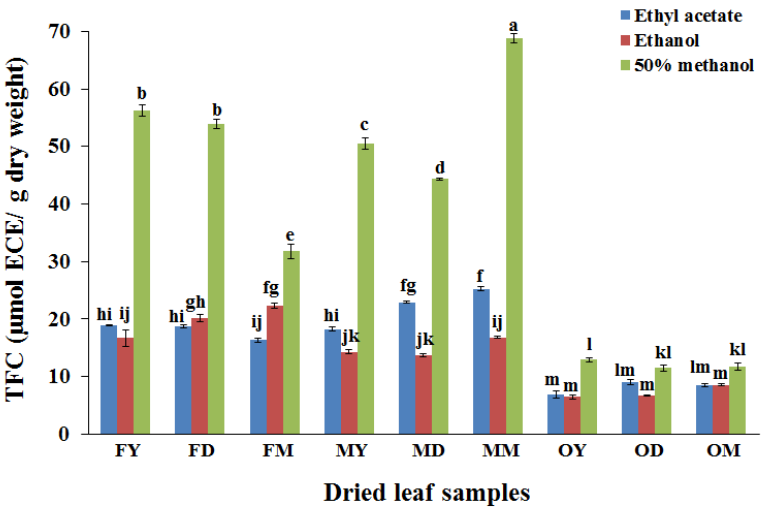

Figure 4.3 TPC (a) and TFC (b) of *G. pseudochina* leaf extracts per g dry weight prepared with different drying processes, leaf ages and polarity of solvents. Abbreviations of sample names: first letter, F is freeze dry with M is microwave and O is oven; second letter, Y is young leaf, D is developing leaf, and M is mature leaf. Different letter(s) (a-o) are significant differences according to Scheffe's test ($p < 0.05$). Data are given as means ± SD (n=3).

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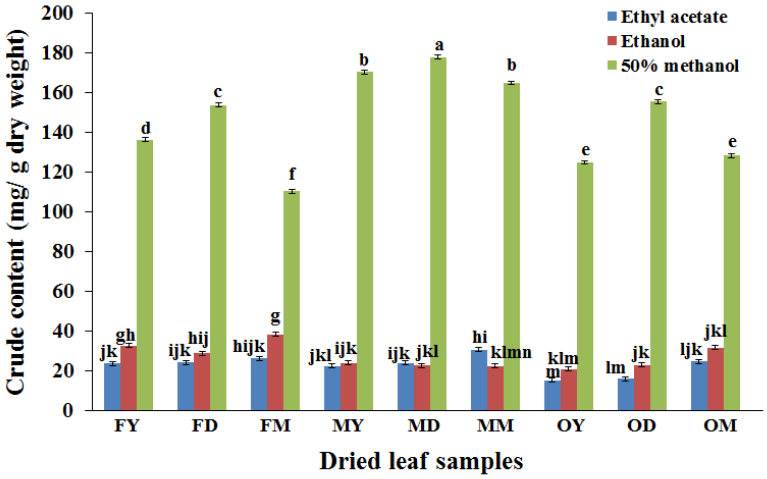
(a)



(b)

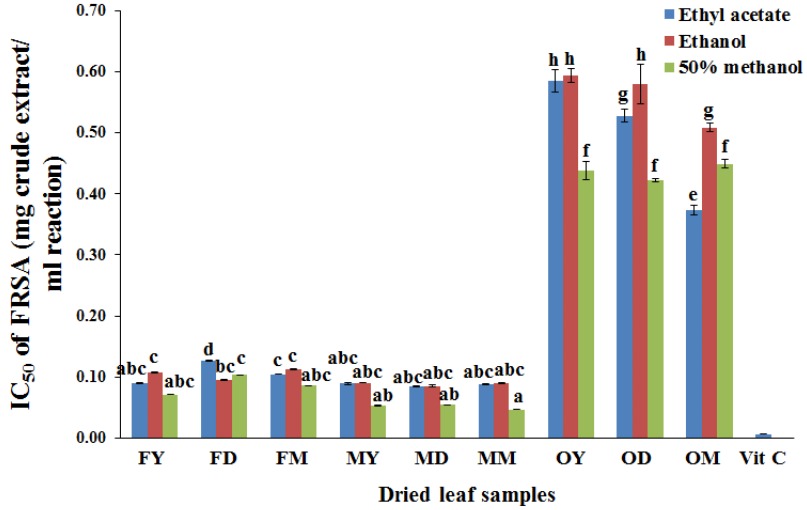


Figure 4.4 Crude content (a) and 50% of FRSA activity (IC_{50}) (b) of *G. pseudochina* leaf extracts prepared with different drying processes, leaf ages, and polarity of solvents. Abbreviations of sample names: first letter, F is freeze dry with M is microwave and O is oven; second letter, Y is young leaf, D is developing leaf, and M is mature leaf. Different letter(s) (a-n) are significant differences according to Scheffe's test ($p < 0.05$). Data are given as means ± SD (n=3).

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Table 4.2 Summary of TPC and TFC in *G. pseudochina* leaf extracts affected by different drying processes and leaf ages.

Drying process	Leaf age	Content/ g dry weight			Content/ g crude extract	
		TPC (μmol CAE)	TFC (μmol ECE)	Crude content (mg)	TPC (mmol CAE)	TFC (mmol ECE)
Freeze dry	Young	122.41±0.83^{b}	91.78±0.61^{b}	192.50±1.08^{cd}	2.14±0.02^{b}	1.73±0.04^{c}
	Developing	114.78±0.22^{c}	92.74±0.64^{b}	206.67±0.36^{b}	2.06±0.00^{bc}	1.82±0.01^{c}
	Mature	95.60±1.81^{d}	70.39±1.26^{d}	186.25±2.86^{cd}	1.77±0.02^{d}	1.36±0.02^{d}
Microwave	Young	108.99±0.25^{c}	83.05±0.97^{c}	216.46±0.72^{a}	1.75±0.00^{d}	1.71±0.01^{c}
	Developing	122.49±1.24^{b}	81.01±0.52^{c}	224.17±4.61^{a}	1.96±0.05^{c}	1.81±0.02^{b}
	Mature	157.18±3.25^{a}	110.85±0.83^{a}	217.71±0.95^{a}	2.70±0.06^{a}	2.00±0.02^{a}
Oven	Young	35.77±0.27^{f}	26.17±0.73^{e}	160.83±3.15^{e}	0.63±0.02^{f}	0.86±0.04^{e}
	Developing	38.14±0.68^{f}	27.19±0.34^{e}	194.17±2.37^{c}	0.71±0.01^{f}	0.94±0.03^{e}
	Mature	48.27±0.69^{e}	28.32±0.57^{e}	184.58±1.57^{d}	0.88±0.01^{e}	0.69±0.01^{f}

Different letter(s) (a-f) in same column are significant differences according to Scheffe's test ($p < 0.05$). Data are given as means ± SD (n=3).

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Spearman's correlation coefficient (*r*) are shown in Table 4.3. The statistical analysis indicated that the drying processes in the order of freeze dry, microwave, and oven had a negative correlation (*r* = -0.716 and - 0.582) with TPC and TFC, respectively. Whereas, 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 had a positive correlation with TFC ($r = 0.402$) and crude content ($r = 0.764$). In addition, TPC was very strongly positively correlated to TFC ($r = 0.926$) and moderately positively correlated to crude content ($r = 0.583$), but TPC was strongly negatively correlated to IC_{50} ($r = -0.853$). TFC was moderately positively correlated with crude contents ($r = 0.596$), but TFC was very strongly negatively correlated to IC_{50} ($r = -0.857$). In particular, the crude content caused high TPC and TFC values that lead to low a IC_{50}. The different leaf ages did not significantly correlate with TPC, TFC, IC_{50}, and crude content.

Table 4.3 Spearman correlation coefficients (*r*) among dying processes, leaf ages, polarity of solvents, TPC, TFC, IC_{50} and crude content.

	Drying process	Leaf age	Solvent	TPC	TFC	IC_{50}	Crude content
Drying process	1	<.0001	<. 0001	-.716**	-.582**	.542**	-.230
Leaf age		1	<.0001	.076	.064	-.023	.181
Solvent			1	.326	.402*	-.223	.764**
TPC				1	.926**	-.853**	.583**
TFC					1	-.857**	.596**
IC_{50}						1	-.418*
Crude content							1

* Correlation is significant at the 0.05 level (2-tailed) according to Spearman's test.

** Correlation is significant at the 0.01 level (2-tailed) according to Spearman's test.

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Only the 50% methanol fractions from every leaf age of each drying process were summarized to obtain the sum total values of TPC, TFC, and crude content (Figure 4.5). The results indicated that the highest crude content was obtained from the microwave-dried leaves. The TPC and TFC of the freeze-dried and microwave-dried extracts were not significantly different in their contents, and they were higher than the contents from the oven dried samples. Therefore, the mixed-age leaves and microwave drying method were applied to prepare *G. pseudochina* leaves for further study.

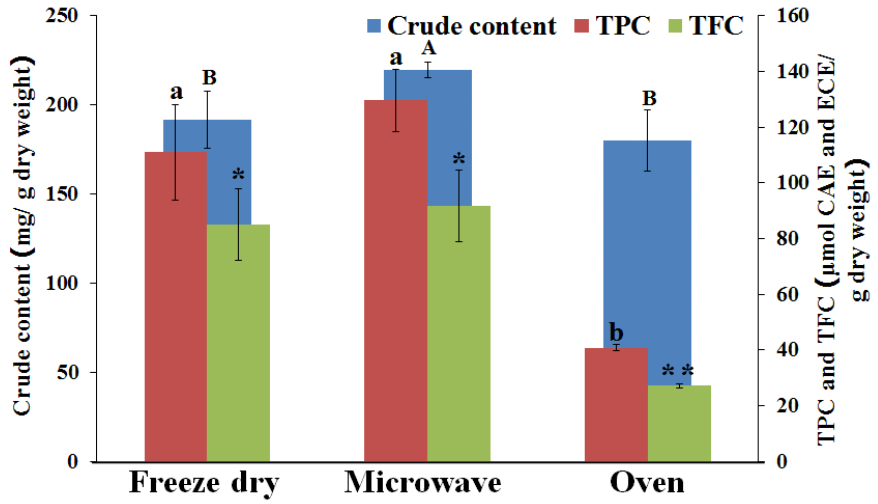

Figure 4.5 Sum totals of crude content, TPC and TFC of *G.pseudochina* leaf extracts of 50% methanol fractions from mixed-age leaves (young, developing, and mature leaves) of each drying process. Different letter(s) (a-b, A-B) and symbols (*, **) are significant differences according to Scheffe's test ($p < 0.05$). Data are given as means ± SD (n=3).

4.3 Phenolic composition by HPLC

The HPLC profiles of the extracts from various leaf ages dried with freeze drying, microwave, and oven drying processes are shown in Figures 4.6 to 4.8, respectively. Four peaks at the retention times of 18.6, 20.2, 24.8, and 29.8 min were identified by LC-MS/MS to CGA, CA, PCA, and RUT. The normalized HPLC

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chromatogram confirmed that the leaf extracts from the 50% methanol fractions strongly contained many phenolic compounds more than the fractions of ethyl acetate and ethanol. In addition, the 50% methanol fractions of the freeze dry and the microwave samples contained higher CGA, CA, and RUT amounts than the oven samples. Moreover, the CA was recovered increasingly from the microwave samples by 50% methanol (Figure 4.7 and 4.9).

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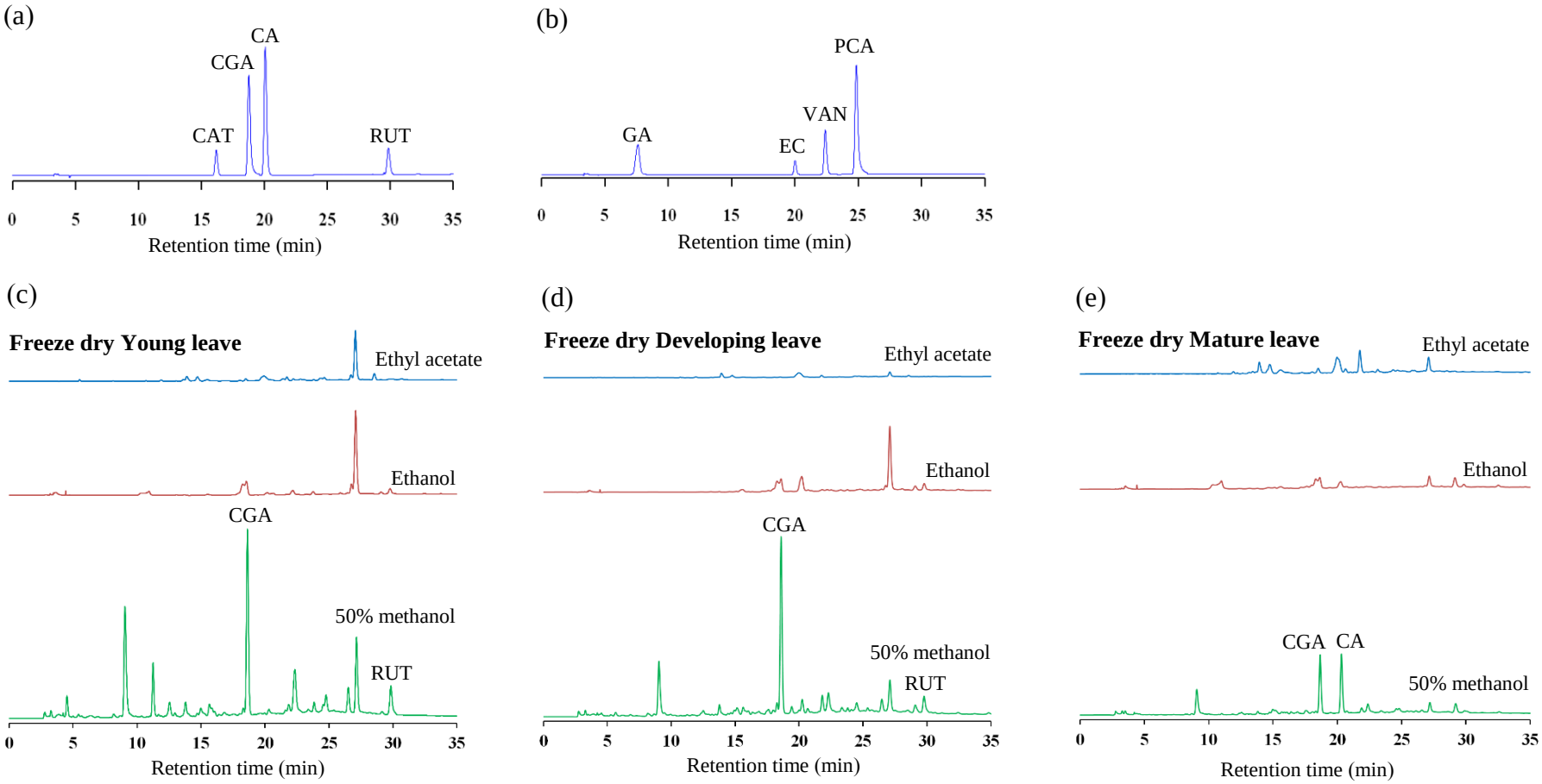

Figure 4.6 Normalized HPLC chromatograms with retention times of (a, b) standards of phenolic compounds and *G. pseudochina* extracts from continuous extracts with ethyl acetate, ethanol, and 50% methanol of the freeze-dried leaves at various leaf ages: (c) young leaves, (d) developing leaves, and (e) mature leaves.

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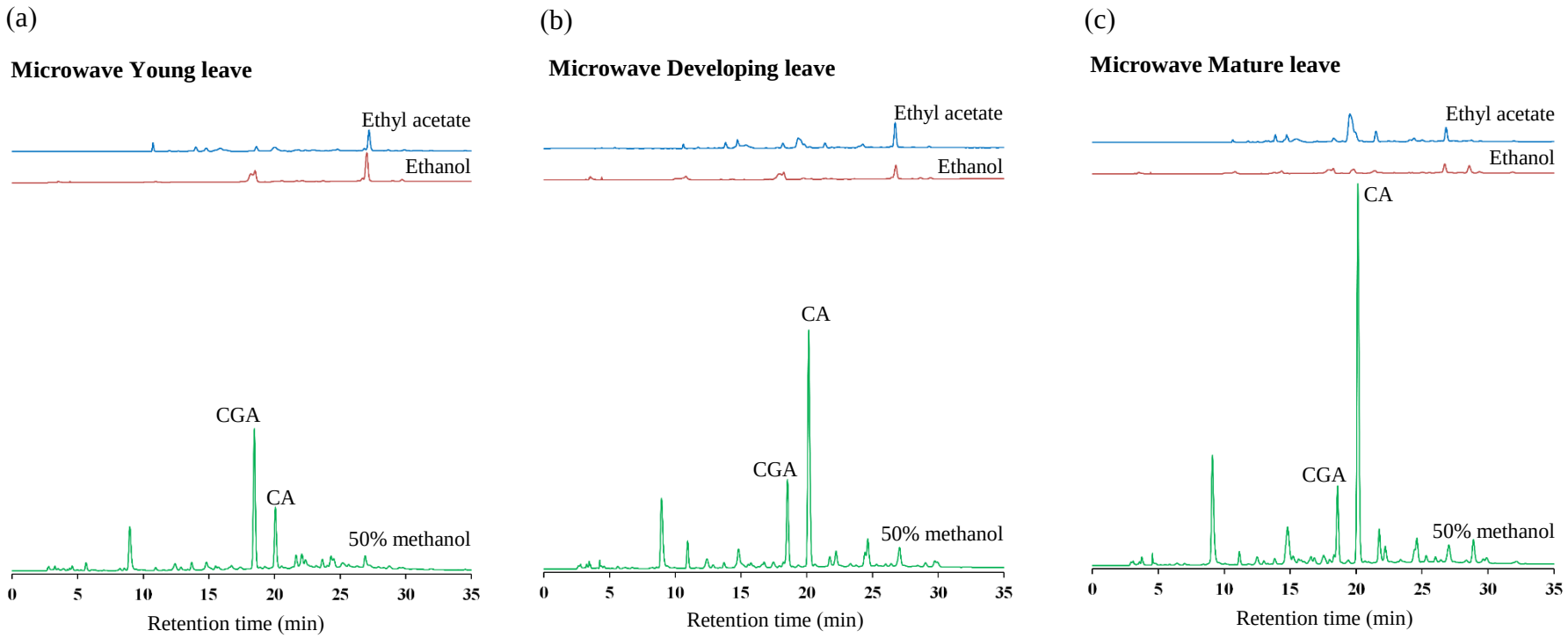

Figure 4.7 Normalized HPLC chromatograms with retention times of *G. pseudochina* extracts from continuous extracts with ethyl acetate, ethanol, and 50% methanol of the microwave-dried leaves at various leaf ages: (a) young leaves, (b) developing leaves, and (c) mature leaves.

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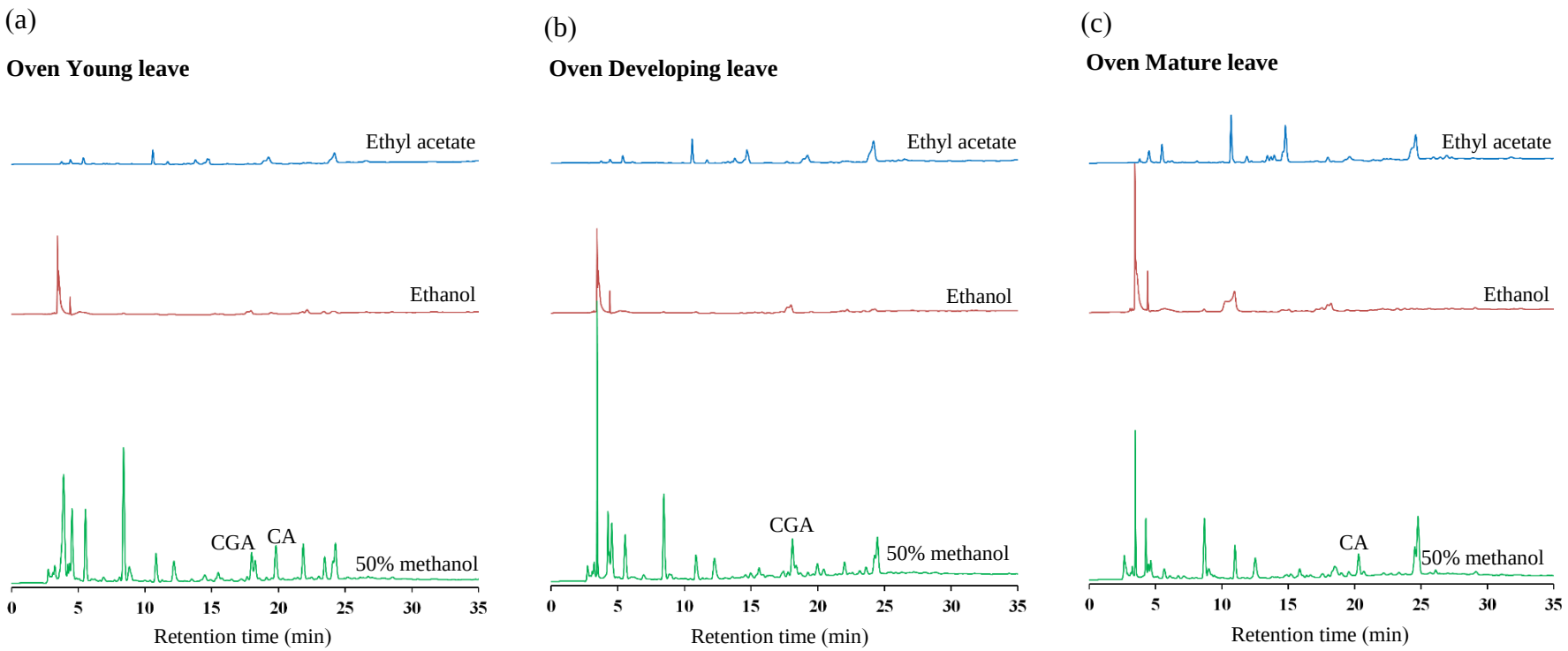

Figure 4.8 Normalized HPLC chromatograms with retention times of *G. pseudochina* leaf extracts from continuous extracts with ethyl acetate, ethanol, and 50% methanol of the oven-dried leaves at various leaf ages: (a) young leaves, (b) developing leaves, and (c) mature leaves.

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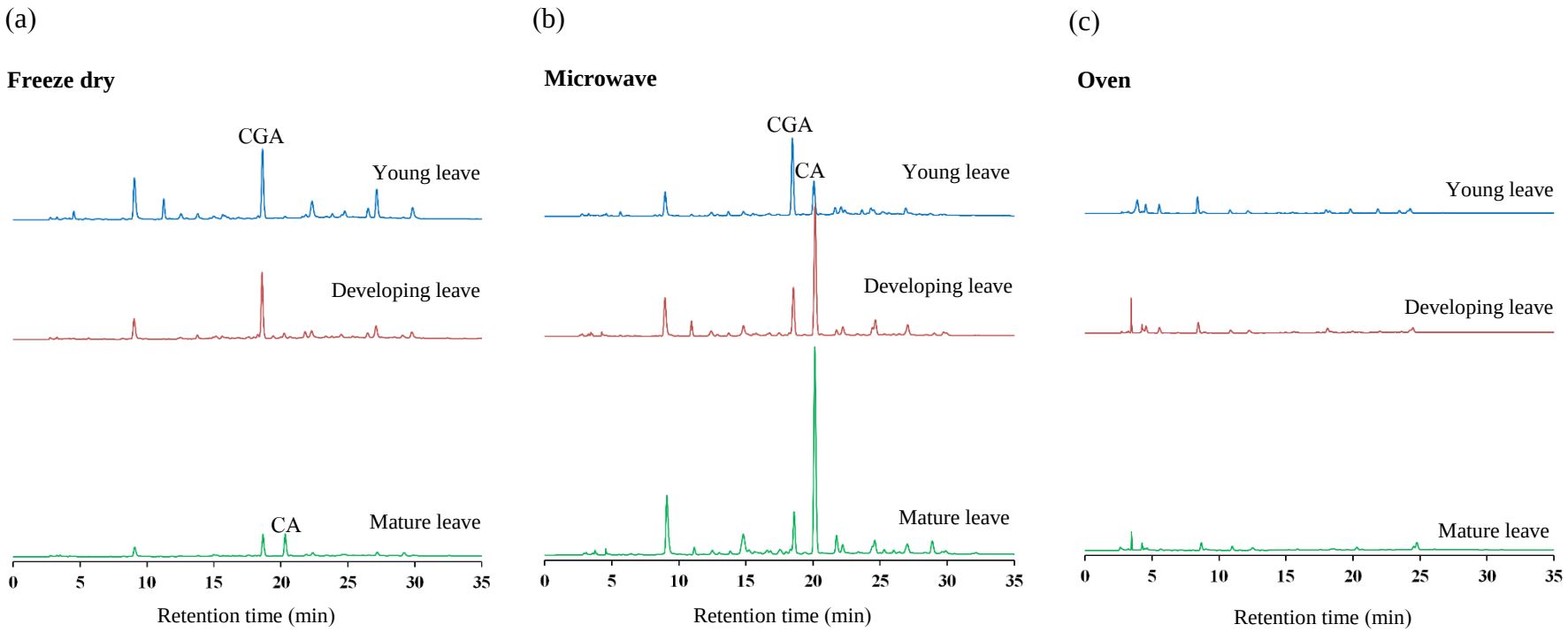

Figure 4.9 Effect of leaf ages on normalized HPLC chromatograms of *G. pseudochina* leaf extacts focusing on only 50% methanol, comparison in each drying process of (a) freeze dry, (b) microwave, and (c) oven.

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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 Figure 4.9. The HPLC comparison indicated the high efficiency of the freeze drying and microwave drying processes for postharvest phenolic recovery. In addition, there were changes in the peak heights of CGA and CA. Leaf maturity might decrease CGA and increase CA.

In addition, the microwave dried *G. psuedochina* leaves also provided a high quality and quantity of phenolic compounds and increased the CA. Therefore, the mixed-age leaves dried with a microwave (MLM) and 50% methanol extraction were considered a suitable processes to recover the marker compounds from *G. pseudochina* leaves.

Although our previous results showed that the 50% methanol was the most efficiency option, methanol residue in the crude extract must be considered. Therefore, the plant extractions with various ethanolic concentrations to extract the MLM was investigated, as shown in Figure 4.10 and Table 4.4. The results indicated that 25% ethanol was suitable for recovery of TPC, crude extract, CGA, and CA. In addition, 50% and 75% ethanol were suitable for recovery of TFC, CGA, and RUT. Therefore, the extraction with both 50% and 25% ethanol was considered as an appropriate process to achieve the phenolic and marker compounds from MLM.

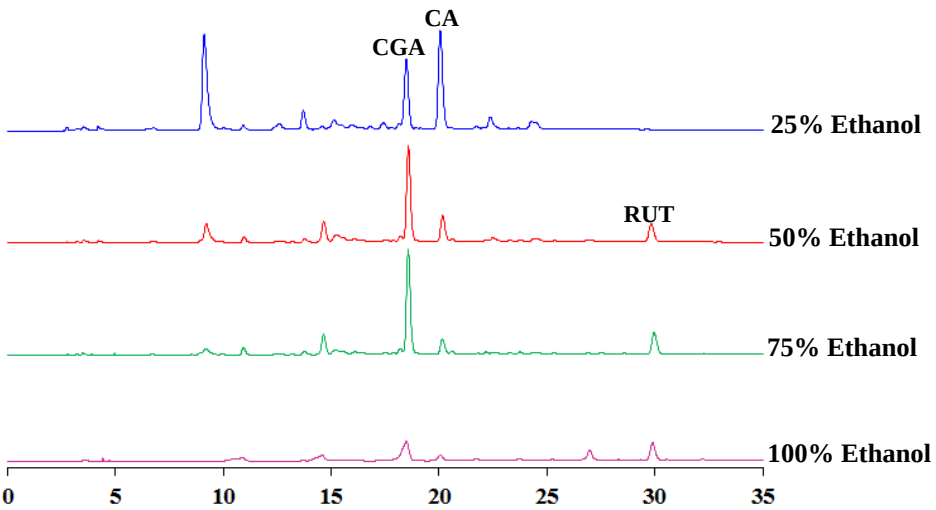

Figure 4.10 Normalized HPLC chromatograms with retention times of MLM extracts from separate extractions with various ethanol concentrations of 25, 50, 75, and 100%.

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Table 4.4 TPC, TFC, 50% DPPH radical scavenging activity (IC_{50}), CGA, CA, and RUT contents of MLM extracts from separate extractions with various ethanol concentrations of 25, 50, 75 and 100%.

Solvent	TPC (µmol CAE/ g dry weight)	TFC (µmol ECE/ g dry weight)	Crude (g crude extract/ g dry weight)	IC_{50} of FRSA (µg crude extract/ ml)	CGA (mg/ g crude extract)	CA (mg/ g crude extract)	RUT (mg/ g crude extract)
25% Ethanol	59.17 ± 6.36^{a}	70.44 ± 6.92^{b}	0.18 ± 0.02^{a}	83.66 ± 2.51^{a}	7.49 ± 0.13^{c}	4.23 ± 0.14^{a}	< LOQ*
50% Ethanol	67.01 ± 5.32^{a}	94.76 ± 4.39^{a}	0.09 ± 0.01^{b}	76.07 ± 8.21^{a}	15.04 ± 0.74^{b}	2.22 ± 0.17^{b}	7.16 ± 0.15^{c}
75% Ethanol	60.28 ± 4.80^{a}	89.26 ± 6.50^{a}	0.09 ± 0.01^{b}	82.58 ± 4.65^{a}	16.85 ± 0.35^{a}	1.33 ± 0.03^{c}	8.69 ± 0.75^{b}
100% Ethanol	27.02 ± 3.97^{b}	20.18 ± 1.20^{c}	0.04 ± 0.00^{c}	102.49 ± 4.51^{b}	5.67 ± 0.14^{d}	1.01 ± 0.04^{c}	15.71 ± 0.25^{a}

Different letter(s) (a-c) in same column are significant differences according to Scheffe's test ($p < 0.05$). Data are given as means ± SD (n=3). * The LOQ of RUT is 0.46 µg/ml.

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4.4 Phenolic identification by LC-MS/MS

The LC-ESI-QTOF-MS/MS was applied to characterize the unknown compounds in the MLM extracts. According to Figure 4.10, 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 Figure 4.11. Table 4.5 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 ethanolic extracts of MLM contained various groups of phenolic compounds included 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-dihy-droxyphenyl)-5-hydroxy-8-methyl -8-(4-methyl-3-penten-1-yl)-2,3-dihydro-4H,8H-pyrano [2,3-f]chromen-4-one), xanthone derivative (1,3,8-Trihydroxy-4-methyl-2,7 diprenylxan-thone), phenylpropanoid (3,4 dihy-droxycinnamoyl (Z)-2-(3,4-dihydroxyphenyl) ethanol), phenolic glycoside compound (unknown-C-glycoside), and glycerol-phospholipid (1-(9Z-octadece-noyl)-sn-glycero-2,3-cyclic phosphate).

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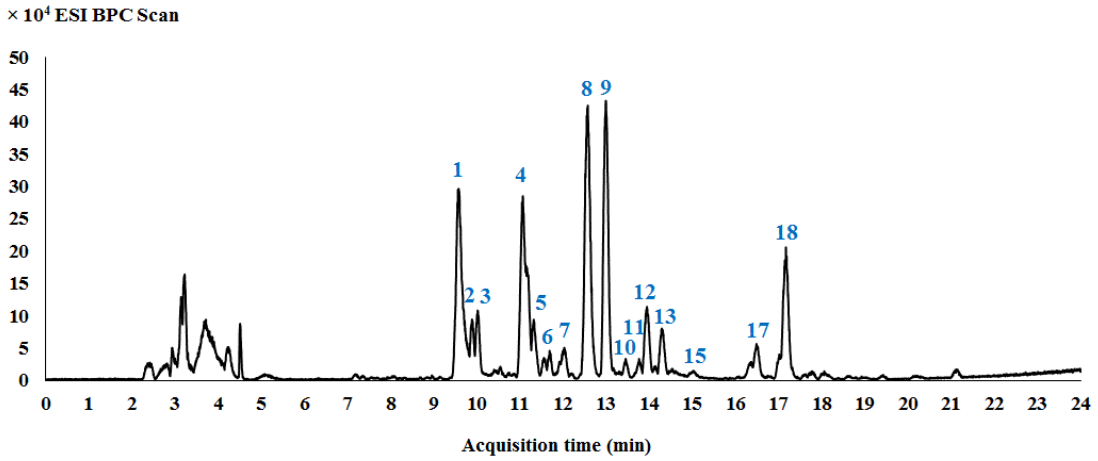
(a)



(b)

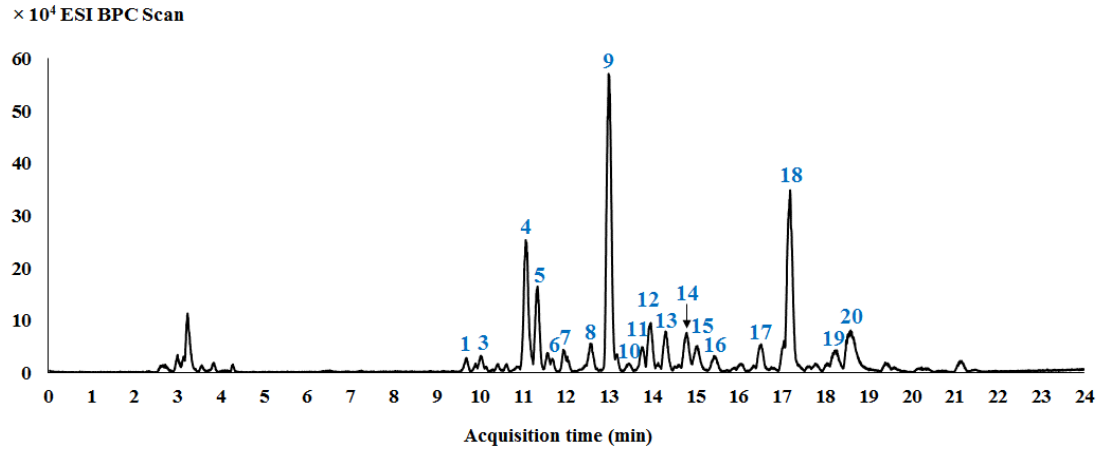


Figure 4.11 LC-ESI base peak chromatograms (BPC) of MLM extracts from separate extractions with (a) 25% ethanol and (b) 100% ethanol. For main peak assignments see Table 4.5.

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Table 4.5 LC-ESI-QTOF-MS/MS analysis of phenolic compounds from MLM extracts from separate extractions with 25% and 100% ethanol.

Peak no.	RT (min)	ESI-MS m/z		Tentative identification	Formula	Error (ppm)
		[M-H]	MS/MS fragment			
1	9.58	385.04	277.03,204.99,73.02	Unidentified	-	-
2	9.89	353.08	191.05,135.04	Caffeoyl quinic acid isomer1	$C_{16}H_{18}O_9$	1.15
3	10.02	423.15	363.12,113.02	(+)-Tephropurpurin	$C_{24}H_{24}O_7$	-13.37
4	11.08	353.08	191.05,135.04	Caffeoyl quinic acid isomer2 (CGA)*	$C_{16}H_{18}O_9$	1.72
5	11.32	439.18	393.17,163.05,205.06	1,3,8-Trihydroxy-4-methyl-2,7 diprenylxanthone	$C_{24}H_{26}O_5$	-12.09
6	11.68	421.16	341.11,213.04	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	$C_{25}H_{26}O_6$	9.17
7	11.91	353.08	191.05	Caffeoyl quinic acid isomer3	$C_{16}H_{18}O_9$	1.15
8	12.57	179.03	135.04	Caffeic acid (CA)*	$C_9H_8O_4$	8.28
9	12.99	609.14	463.08,300.02,178.99,151.00	Quercetin rutinoside (RUT)*	$C_{27}H_{30}O_{16}$	-1.14
10	13.45	367.10	179.03,135.04,99.01	3-O-Caffeoyl-1-O-methylquinic acid	$C_{17}H_{20}O_9$	6.14
11	13.76	593.15	285.03,327.04,535.21,417.24	Kaempferol rutinoside	$C_{27}H_{30}O_{15}$	-0.35
12	13.94	713.47	677.49,313.06,147.04	Unknown-C-glycoside	$C_{35}H_{70}O_{14}$	-3.51
13	14.29	826.55	790.57,656.96	Unidentified	-	-
14	14.76	515.11	353.08,173.04,179.03	Dicaffeoyl quinic acid isomer1	$C_{25}H_{24}O_{12}$	0.97
15	15.02	515.11	353.08,173.04,179.03	Dicaffeoyl quinic acid isomer2	$C_{25}H_{24}O_{12}$	0.97
16	15.55	313.07	313.07	3,4-Dihydroxycinnamoyl-(Z)-2-(3,4-dihydroxyphenyl) ethanol	$C_{17}H_{14}O_6$	4.99

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17	16.49	463.25	417.24,161.04	1-(9Z-octadecenoyl)-sn-glycero-2,3-cyclic phosphate	$C_{21}H_{39}O_6P$	-13.30
18	17.16	497.21	429.20,249.14,119.0313,59.01	Unidentified	-	-
19	18.17	301.03	273.03, 151.00, 121.02	Quercetin	$C_{15}H_{10}O_7$	4.23
20	18.54	623.11	311.05, 265.04, 147.04, 109.02	5-Hydroxy-2'-methoxy-6,7-methylenedioxyisoflavone	$C_{17}H_{12}O_6$	1.97

* Peaks are compared with standard compounds.

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4.5 Phenolic compound investigation by HP-TLC and pyrrolizidine alkaloid determination

HP-TLC was also performed to compare the phenolic profiles and some separated compounds in the *G. pseudochina* extracts obtained from various drying processes (freeze dry, microwave, and oven) of mixed-age leaves. The continuous extraction following steps of defatting with hexane, reducing chlorophyll with absolute ethanol, and extraction with 50% ethanol and 25% ethanol were applied, and the fractions of 50% and 25% ethanol of each dried sample were compared in the HP-TLC profile. HP-TLC fingerprint profiles were visualized under UV light at 254 nm (Figure 4.12a) and 365 nm (Figure 4.12b). The 25% and 50% ethanol fractions of MLM still presented the high quality phenolic compounds and marker compounds, including chlorogenic acid, caffeic acid, and rutin, when compared with the HP-TLC profiles of MLF. Whereas, MLO clearly exhibited a reduction in phenolic compounds. However, the 50% ethanol fractions still contained some chlorophylls, which were observed as red fluorescence under UV light at 365 nm (Figure 4.12b). In addition, the natural product reagents were applied to define the group of phenolic compounds. After derivatization with the reagents, phenolic acids are a blue color and flavonoids (polyphenolics) are a yellow color. Figure 4.12c presents that the MLF and MLM mostly contained phenolic compounds, and rutin was strongly present as flavonoids in the extracts. Therefore, these HP-TLC profiles supported the efficiency of the microwave drying process to retain the phenolic compounds in *G. pseudochina* leaves.

Total pyrrolizidine alkaloid contents (TPAsC) in our *G. pseudochina* extracts were determined in terms of monocrotaline equivalence, as shown in Table 4.6. The TPAsC were in the range of 0.003±0.002 to 0.684±0.053 mmol monocrotaline equivalence/g leaf extract. The highest TPAsCs were found in the 50% ethanol fractions of MLO. Although the MLM extracts contained the lowest TPAsC, the contents of the PAs in the MLM extracts must be controlled in a safe range when applied in health products.

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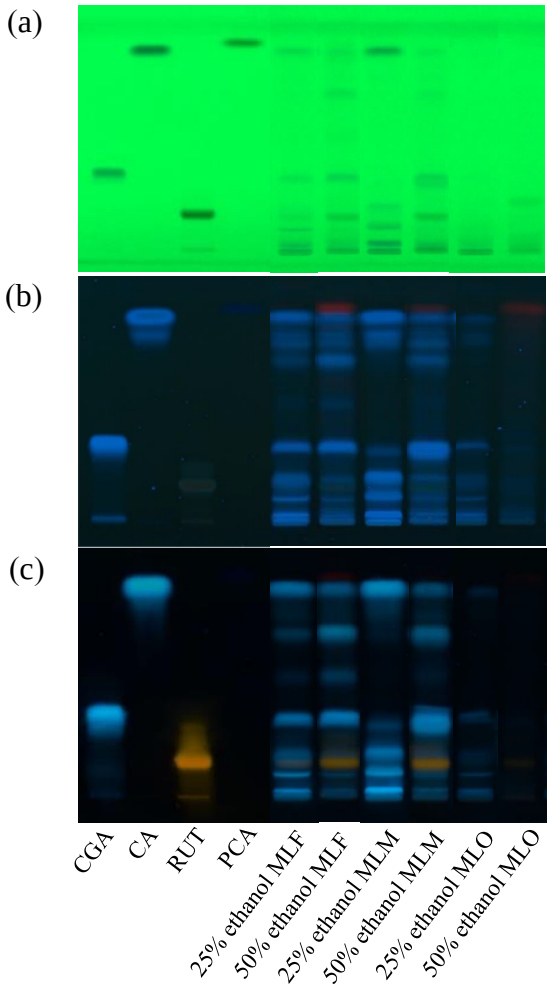

Figure 4.12 HP-TLC fingerprint of *G. pseudochina* leaf extracts obtained from various drying processes (freeze dry, microwave, and oven) of mixed-age leaves and standard phenolic compounds visualized by UV irradiation at (a) 254 nm, (b) 365 nm, and (c) the HP-TLC profiles after derivatization with natural product reagents at 365 nm.

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Table 4.6 Total pyrrolizidine alkaloid content (TPAsC) in *G. pseudochina* leaf extracts prepared from different drying processes (freeze dry, microwave, oven) and continuous extraction by 50% and 25% ethanol.

Solvent fraction and dried leaf sample	mmol MCTE/ g crude extract
25% Ethanol MLF	0.004±0.001[e]
50% Ethanol MLF	0.142±0.015[d]
25% Ethanol MLM	0.003±0.002[e]
50% Ethanol MLM	0.065±0.011[c]
25% Ethanol MLO	0.233±0.038[b]
50% Ethanol MLO	0.684±0.125[a]

Different letter(s) (a-e) in same column are significant differences according to Scheffe's test ($p < 0.05$). Data are given as means ± SD (n=3).

4.6 Cytotoxicity on HaCaT cells of EMLM

The microwave-dried leaf extract obtained from the continuous extraction fractions of both 25% and 50% ethanol of MLM (EMLM) was studied to evaluate the cytotoxicity. The HPLC chromatogram of EMLM and the contents of its marker compounds are shown in Figure 4.13 and Table 4.7, respectively. CGA is the major compound in EMLM among the four marker compounds of CGA, CA, RUT, and PCA (Table 4.7). The cytotoxicity on non-TNF-α and TNF-α stimulated HaCaT cells was studied by applying various concentrations of EMLM and each standard compound. The morphologic changes of HaCaT cells during treatment are shown in Appendix B-4. Their cytotoxicity in terms of IC_{50} values approximated from a linear trendline are presented in Table 4.8. The EMLM extract showed milder toxicity under both conditions of HaCaT cells than other standard chemicals. Among each standard marker, CA exhibited the highest toxicity under both conditions of HaCaT cells, and CGA showed moderate cytotoxicity. Whereas, RUT and PCA were not toxic under both conditions of HaCaT cells due to the high IC_{50} values. Especially, the EMLM extract possessed similar cytotoxicity under both conditions of HaCaT cells. On the other hand, CGA, CA, and PCA showed decreasing toxicity on TNF-α stimulated cells, while RUT showed

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increasing toxicity on TNF-α stimulated cells. In addition, the contents of each marker compound contained in 750 µg of EMLM (approximated from IC_{50} value of EMLM in Table 4.8, which was 744.02 µg/ ml) are shown in Table 4.7. The data implied that the marker compounds composing the EMLM were toxic to TNF-α stimulated cells at lower concentrations than when each pure marker compound was applied (Table 4.8).

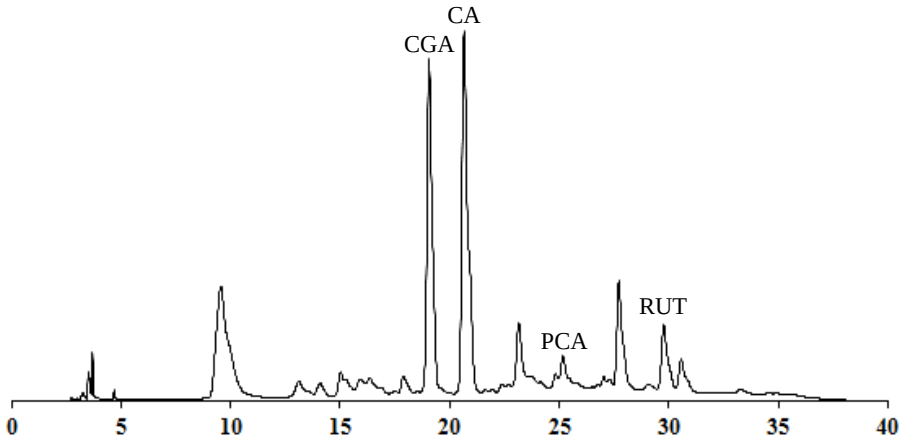

Figure 4.13 HPLC chromatogram of EMLM crude extract, obtained from both 25% and 50% ethanol fractions of MLM.

Table 4.7 Approximate content of each marker compound in EMLM extract.

Compound	Content (%)	Content in EMLM* (µg/ 750 µg crude extract)
CGA	2.04±0.29	15.27±2.14
CA	1.03±0.16	7.75±1.18
RUT	0.86±1.13	6.24±0.85
PCA	0.04±0.11	0.32±0.04

* These approximate data are calculated from each concentration in crude extract.

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Table 4.8 Cytotoxicity of EMLM extract and marker compounds on HaCaT cells, non-stimulated and stimulated by TNF-α.

Samples	Cytotoxicity IC_{50} values (μg/ml)	
	Non TNF-α	TNF-α
EMLM	680.80±23.98^{c*}	744.02±62.2^{c*}
CGA	180.61±7.09^{d*}	284.50±45.76^{d**}
CA	36.18±2.78^{e*}	60.19±1.76^{e**}
RUT	4393.41±436.11^{a*}	1497.99±205.50^{b**}
PCA	1682.78±107.96^{b*}	2840.76±174.12^{a**}
PTX (positive control)	2.69±0.32^{f*}	1.84±0.49^{f*}

Different letter(s) (a-f) in same column are significant differences according to Scheffe's test ($p < 0.05$). Different symbols (*, **) in same column are significant differences according to T-test ($p < 0.05$). Data are given as means ± SD (n=3).

4.7 NF-κB inhibitory properties

The inhibitory property of the EMLM extract and standard chemical compounds to RelA and RelB were observed on TNF-α stimulated HaCaT cells by 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. Figure 4.14 demonstrates that RelA S529 was not active in non-stimulated HaCaT cells. In addition, RelA S529 was slightly active under TNF-α stimulation, which made it difficult for localization of the blue color. Alternatively, RelB S573 was strongly active in 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. For our investigated concentrations, each pure compound of CGA, CA, RUT, and PCA also inhibited the translocation of RelB S573 into the

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nucleus. These results also support that TNF-α could strongly stimulate the NF-κB function through the RelB canonical pathways.

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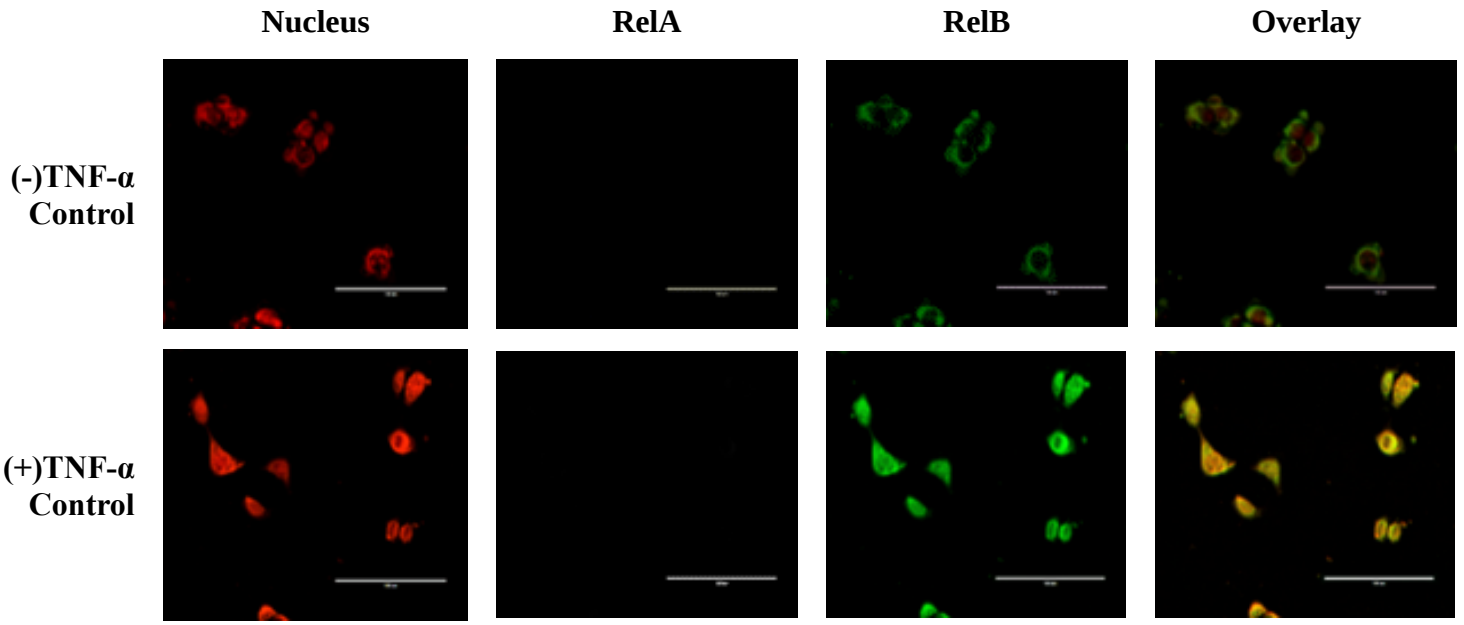

Figure 4.14 Localization of RelA and RelB on HaCaT cells due to TNF-α stimulation. HaCaT cells were pre-treated with 50 ng/ml of TNF-α for 12 h and then treated with EMLM extracts, marker compounds (CGA, CA, PCA, and RUT), and CUR (positive control) for 24 h.

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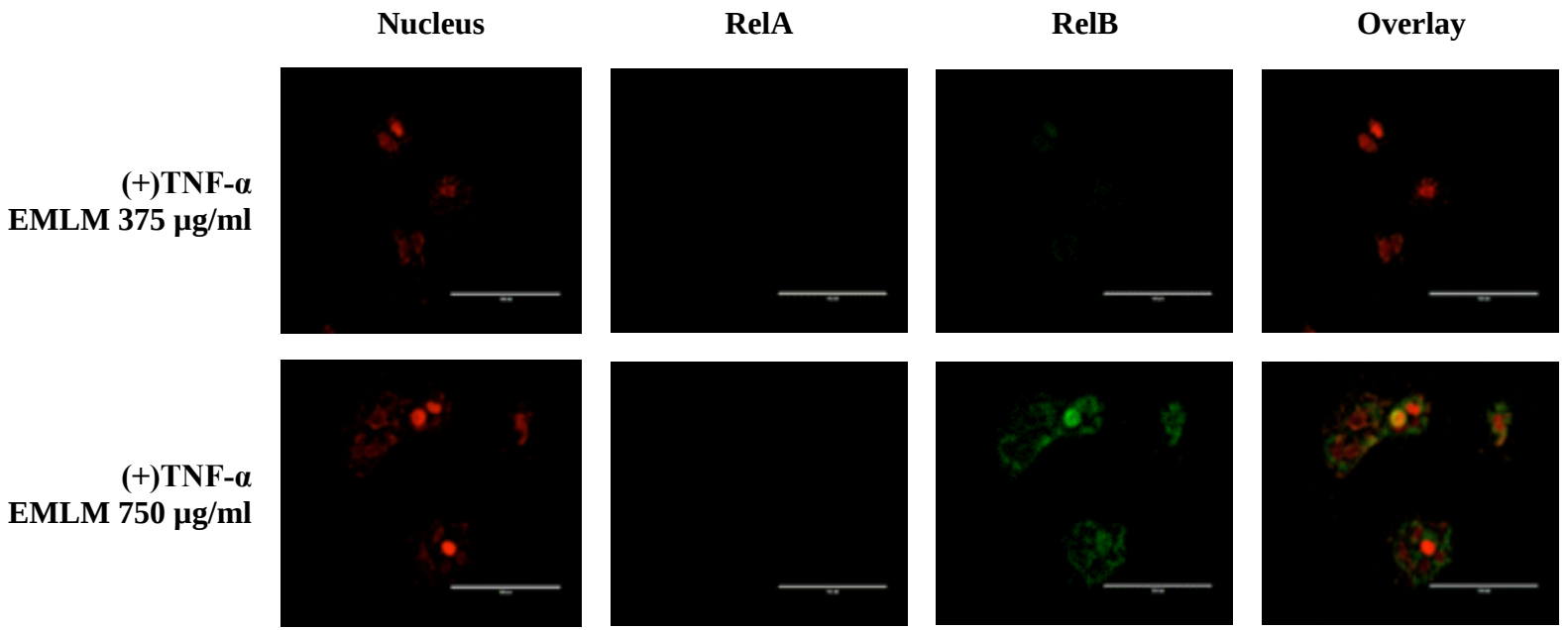

Figure 4.14 (cont.)

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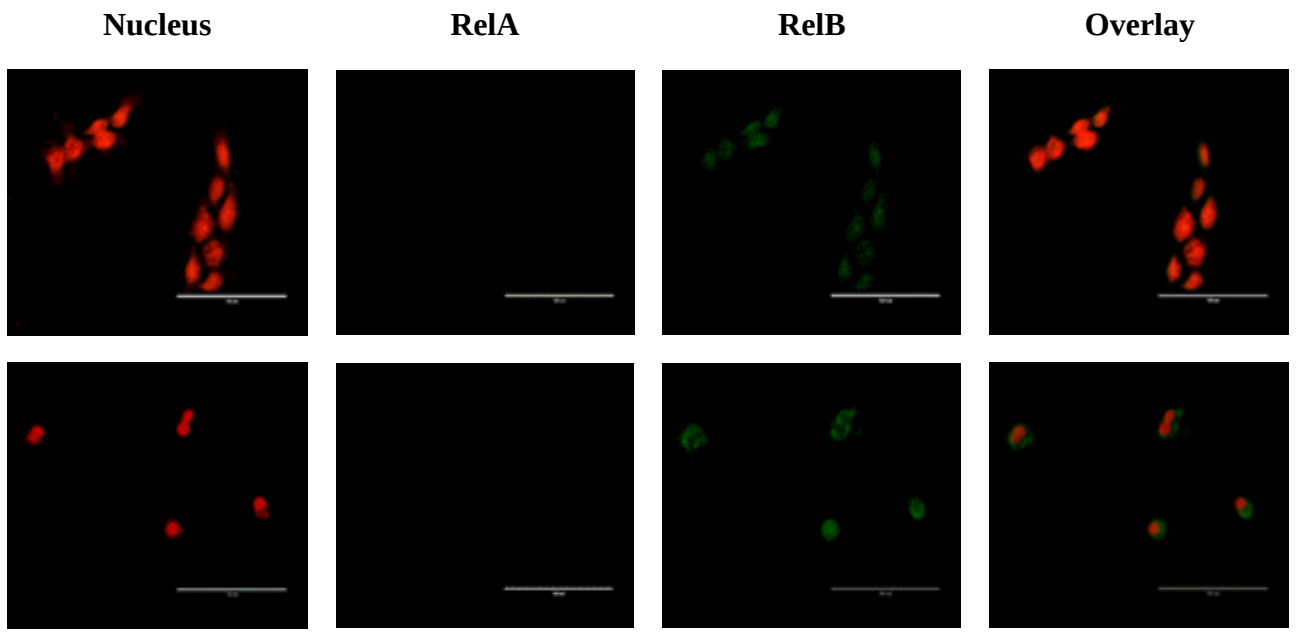

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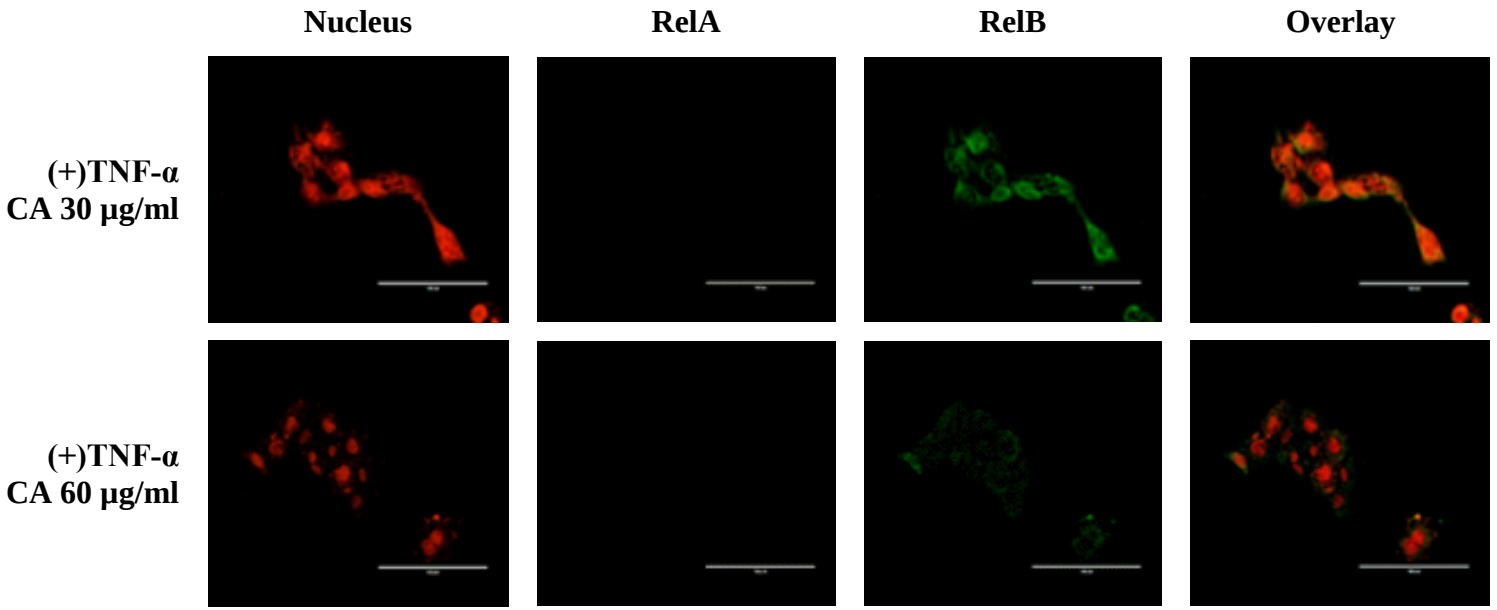

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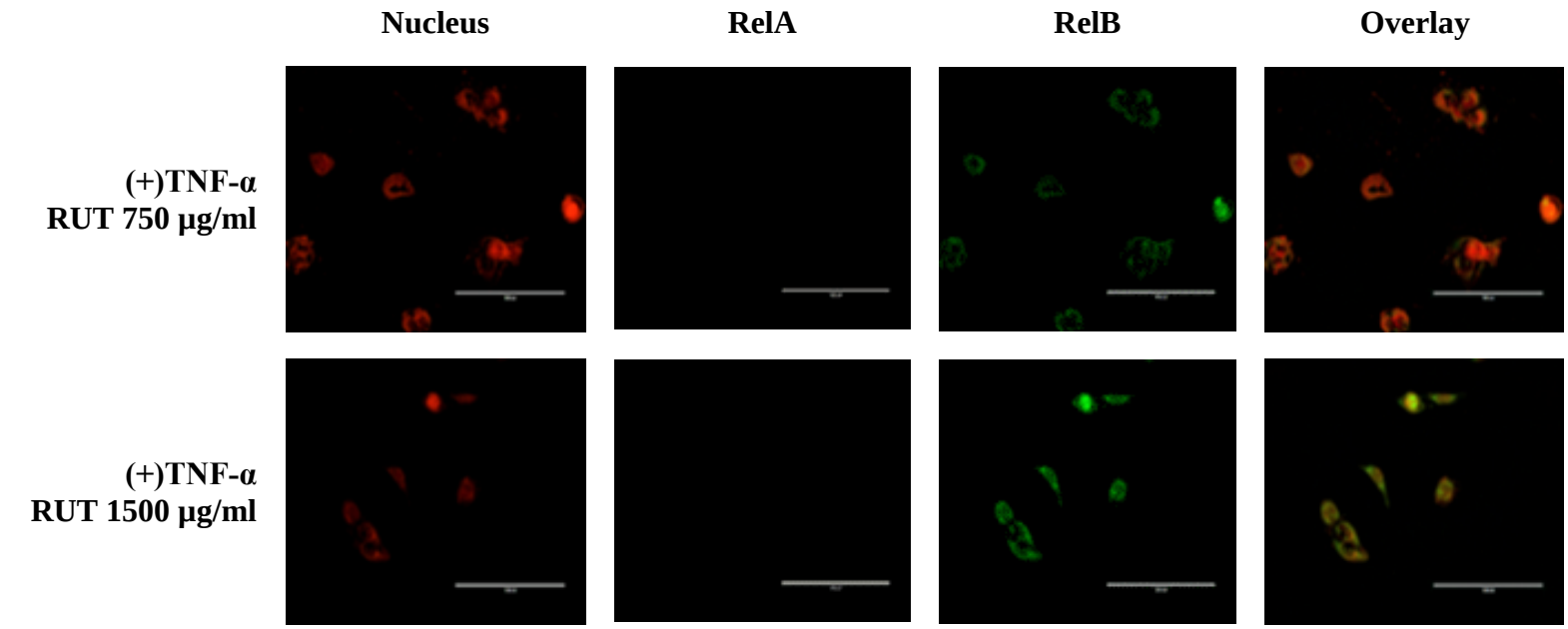

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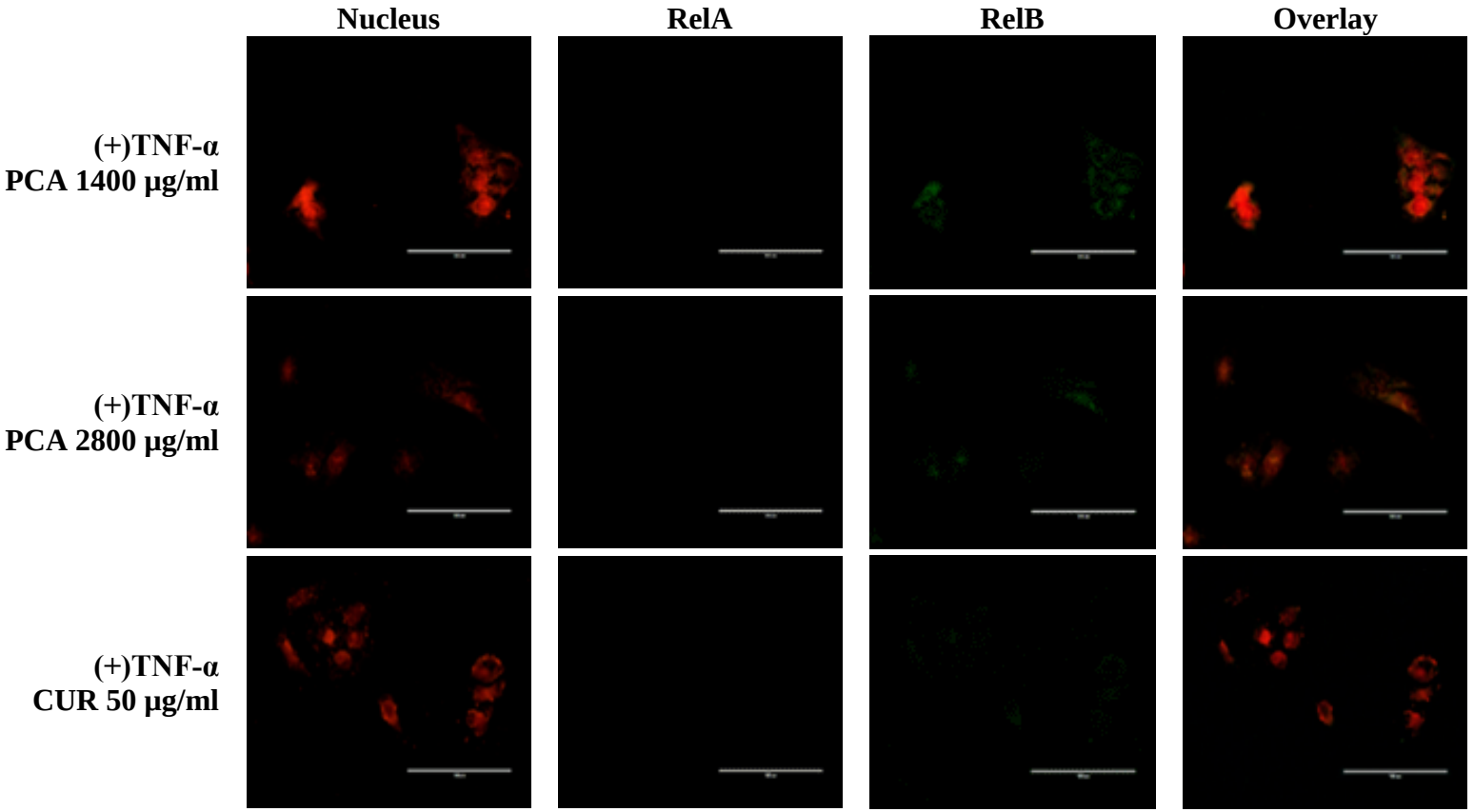

Figure 4.14 (cont.)

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4.8 IL-8 and TSG-6 inhibitory properties by ELISA

The IL-8 and TSG-6 inhibitory properties were evaluated by ELISA, as shown in Table 4.9. IL-8 and TSG-6 were increasingly produced due to the TNF-α stimulation. The 0.7% DMSO contained in the *in vitro* system did not inhibit the protein production. The two concentrations of EMLM, CA, and RUT could inhibit IL-8 production, while only a higher concentration of each CGA and PCA affected the inhibit of IL-8 production. TSG-6 was inhibited by a lower concentration of each EMLM and CGA and a higher concentration of PCA. Whereas, the two concentrations of both CA and RUT did not inhibit TSG-6 production.

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Table 4.9 Interleukin 8 (IL-8) and contents of tumor necrosis factor-inducible gene 6 protein (TSG-6) contents in HaCaT cell lysate after treatment with EMLM and marker compounds.

TNF-α stimulation	Sample for treatment	Concentration (µg/ml)	IL-8 contents (pg/ml)	TSG-6 (pg/ml)
Non TNF-α	Control	-	10.17±2.01^{g}	<0.20*
TNF-α	Control non DMSO	-	342.80±34.62^{a}	412.20±138.73^{a}
	Control 0.7% DMSO	-	322.88±36.03^{ab}	376.10±82.97^{ab}
	EMLM crude extracts	375	133.79±18.18^{ef}	137.96±113.35^{de}
		750	148.82±28.15^{e}	220.64±104.24^{bcde}
	CGA	140	284.82±35.92^{bc}	168.84±31.38^{cde}
		240	90.10±16.39^{f}	321.75±120.39^{abc}
	CA	30	254.60±42.63^{c}	270.03±16.86^{abcd}
		60	87.19±11.76^{f}	234.80±112.66^{bcde}
	RUT	750	115.17±20.50^{ef}	372.37±73.59^{ab}
		1500	93.68±14.33^{f}	394.77±63.99^{ab}
	PCA	1400	369.71±41.43^{a}	249.19±56.15^{abcd}
		2800	201.21±24.38^{d}	139.04±68.44^{de}
	CUR (positive control)	50	14.35±9.78^{g}	73.30±49.15^{ef}

Different letter(s) (a-f) in same column are significant differences according to Duncan's test ($p < 0.05$). Data are given as means ± SD (n=3). * Minimum detectable dose of TSG-6 was determined to be 0.2 pg/ml (RayBio, 2016).

4.9 Physical and chemical properties of prototypic cream

The cream base formula and prototypic cream was supported by TM Cosme Science International Company Limited as shown in Table 3.2, and the quality of their bulk speciation are in Appendix C-2. A 0.5 % amount of EMLM was incorporated into the cream base. The most important consideration for pharmaceutical creams is the stability of the finished product. The chemical stabilities can provide the stability of each compound concentration in the finished cream, especially an active compound that can monitor and pH value. The freeze thaw cycle is part of stability testing to determine if the

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formula is stable. In this study, the coalescence of the internal phase and phase inversion, chromatic parameters, pH value, and main compound stability of the cream base and prototypic EMLM cream were investigated at storage times of 1, 7, 14, 30, and 365 days after preparation and a after freeze thaw cycle. Figure 4.15 (a) to (e) indicates that the cream base and the prototypic EMLM cream did not show the coalescence of the internal phase and phase inversion until 30 days of and after the freeze thaw cycle. However, after the freeze thaw cycle, both creams seemed to lose their humidity due to evaporation of water during the thawing process. Table 4.10 shows the color and pH values of the prototypic cream at various storage times. The color of the cream was presented in terms of CIE L^*, a^*, b^* (CIELAB), for which the three coordinates of CIELAB represent the lightness of the color ($L^* = 0$ yields black and $L^* = 100$ indicates diffuse white; specular white may be higher), its position is between red/magenta and green (a^*, negative values indicate green while positive values indicate magenta), and its position between yellow and blue (b^*, negative values indicate blue and positive values indicate yellow). The results indicated that the cream base stabilized its color properties after more than 30 days, but it was found to be darker and bluer after one year of storage at 4^{o}C. The cream base also became greener after the freeze thaw cycle that might be caused from a chemical reaction between its ingredients under the thermal condition. For our prototypic cream, the EMLM cream was stabile in color for more than 14 days. However, the EMLM cream became greener, darker, and bluer after one year of storage. Some chemical reactions under thermal conditions or evaporation of the water phase might lead to a greener color of the EMLM cream after a freeze thaw cycle. Furthermore, the pHs of the cream base and prototypic EMLM cream at the first day after preparation were 6.16 and 5.30, respectively. The pH values of the cream base did not change at 30 days and after the freeze thaw cycle (6.10±0.06 to 6.25±0.05), but decreased to 5.81 after 365 days of storage time. The pHs of the EMLM cream did not change at 30 days (5.14±0.03 to 5.30±0.09), but decreased after 365 days and after the freeze thaw cycle (5.03±0.03 to 5.07±0.02) (Table 4.10). The HPLC chromatograms and quality of each marker compound in the prototypic EMLM cream are presented in Figure 4.16. The area ratio between the phenoxy ethanol and each marker compound in the EMLM cream at different storage times were considered as internal quality control. Table 4.11 and Table 4.12 show that each marker compound were stable after one year of storage and after the freeze thaw

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cycle. The stability of both physical and chemical properties of the prototypic EMLM cream, especially the contents of the phenolic marker compounds leads to further research in the psoriasis patients and developing a formula to a high content of the *G. pseudochina* extract in the product.

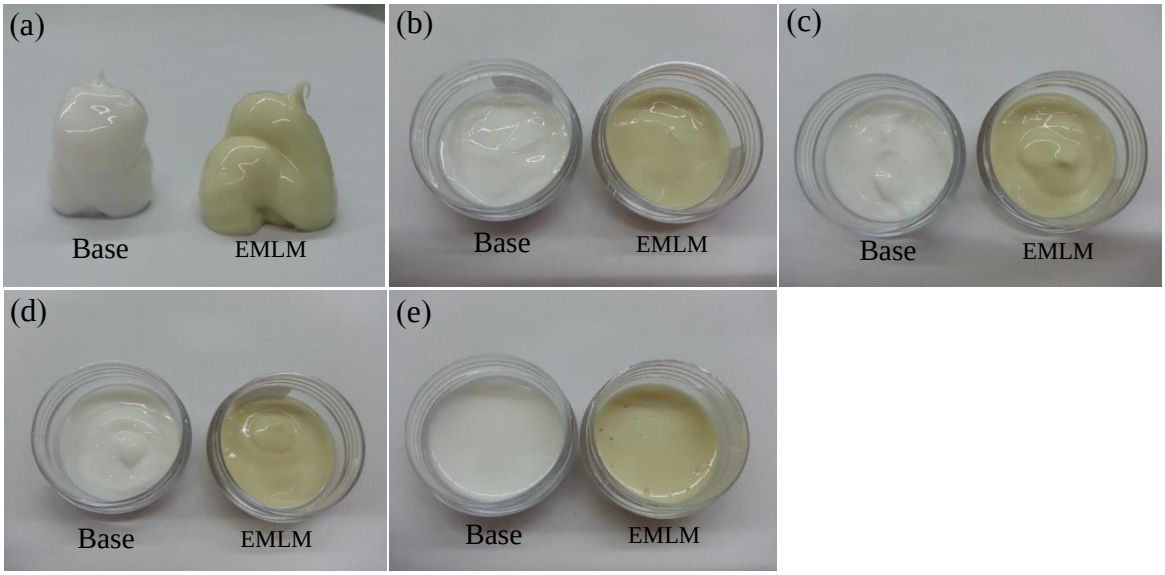

Figure 4.15 Physical appearance of cream base and prototypic EMLM cream at storage times of (a) 1 day, (b) 7 days, (c) 14 days, and (d) 30 days after preparation and (e) after freeze thaw cycle.

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Table 4.10 pH values and chromatic parameters of cream base and prototypic EMLM cream at storage times of 1, 7, 14, 30, and 365 days after preparation and after freeze thaw cycle.

Storage time (day) of EMLM cream	pH		Chromotic parameters					
	Cream base	EMLM cream	Cream base			EMLM cream		
			*L**	*a**	*b**	*L**	*a**	*b**
1	6.16±0.08^{a}	5.30±0.09^{a}	89.97±0.81^{a}	-0.43±0.06^{a}	0.31±0.03^{a}	84.29±0.31^{a}	-3.19±0.12^{b}	13.57±0.16^{b}
7	6.22±0.07^{a}	5.20±0.01^{ab}	90.06±1.23^{a}	-0.37±0.02^{ab}	0.43±0.03^{a}	84.41±0.96^{a}	-2.86±0.13^{ab}	13.59±0.27^{b}
14	6.15±0.03^{a}	5.14±0.03^{ab}	88.67±0.88^{ab}	-0.44±0.03^{ab}	0.32±0.09^{a}	83.98±0.25^{ab}	-2.79±0.09^{ab}	12.99±0.23^{b}
30	6.25±0.05^{a}	5.18±0.09^{ab}	88.94±0.54^{ab}	-0.47±0.04^{ab}	0.43±0.21^{a}	82.78±0.79^{ab}	-2.39±0.16^{a}	12.24±0.96^{b}
365	5.81±0.03^{b}	5.03±0.03^{b}	85.19±1.56^{b}	-0.51±0.03^{ab}	-0.56±0.29^{b}	77.50±0.55^{c}	-2.47±0.08^{a}	16.58±0.39^{a}
Freeze thaw	6.10±0.06^{a}	5.07±0.02^{b}	89.29±2.23^{ab}	-0.64±0.15^{b}	0.76±0.11^{a}	81.88±1.12^{b}	-2.45±0.30^{a}	13.09±1.79^{b}

Different letter(s) (a-c) in same column are significant differences according to Scheffe's test ($p < 0.05$). Data are given as means ± SD (n=3).

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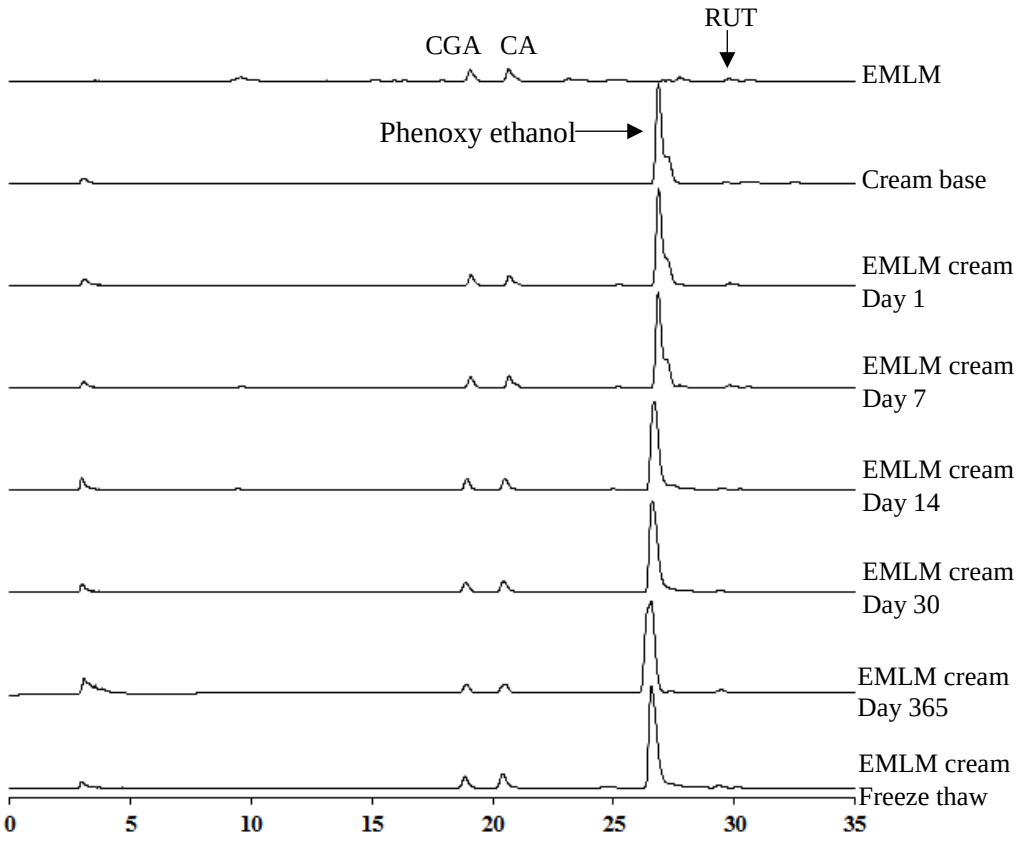

Figure 4.16 HPLC chromatograms of EMLM crude extract, cream base and prototypic EMLM cream at storage times of 1, 7, 14, 30 and 365 days after preparation and after freeze thaw cycle.

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Table 4.11 Ratio of peak areas of each marker compound to phenoxy ethanol in prototypic EMLM cream at storage times of 1, 7, 14, 30 and 365 days after preparation and after freeze-thaw cycle.

Marker compound	Ratio of peak areas (Marker compound/ phenoxy ethanol)					
	Day 1	Day 7	Day 14	Day 30	Day 365	Freeze thaw
CGA	0.084±0.006[a]	0.086±0.002[a]	0.085±0.001[a]	0.085±0.001[a]	0.085±0.002[a]	0.083±0.001[a]
CA	0.093±0.000[a]	0.095±0.001[a]	0.090±0.000[a]	0.086±0.009[a]	0.085±0.002[a]	0.091±0.002[a]
RUT	0.020±0.000[a]	0.020±0.001[a]	0.019±0.000[a]	0.019±0.001[a]	0.019±0.001[a]	0.018±0.002[a]
PCA	0.005±0.000[a]	0.006±0.001[a]	0.005±0.000[a]	0.005±0.000[a]	0.006±0.000[a]	0.005±0.000[a]

Different letter(s) in same row are significant differences according to Scheffe's test ($p < 0.05$). Data are given as means ± SD (n=3).

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Table 4.12 Contents of phenolic and flavonoid marker compounds in prototypic EMLM cream at storage times of 1, 7, 14, 30, and 365 days after preparation and after freeze thaw cycle.

Storage time (day) of EMLM cream	Maker compound (mg/ 100 g cream)			
	CGA	CA	RUT	PCA
1	10.37±1.16[a]	5.07±0.15[a]	4.25±0.20[a]	0.17±0.03[a]
7	10.83±0.03[a]	5.43±0.21[a]	4.42±0.18[a]	0.22±0.05[a]
14	11.44±0.39[a]	5.39±0.10[a]	4.48±0.13[a]	0.18±0.01[a]
30	11.22±0.74[a]	5.10±0.18[a]	4.45±0.12[a]	0.19±0.02[a]
365	9.78±0.20[a]	5.40±0.58[a]	4.32±0.09[a]	0.22±0.01[a]
Freeze-thaw cycle	11.07±0.17[a]	5.37±0.06[a]	4.28±0.44[a]	0.21±0.03[a]
EMLM 0.5 g	10.18±1.43[a]	5.17±0.78[a]	4.32±0.57[a]	0.21±0.03[a]

Different letter(s) in same column are significant differences according to Scheffe's test ($p < 0.05$). Data are given as means ± SD (n=3).

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CHAPTER 5

DISCUSSIONS AND CONCLUSIONS

5.1 Discussions

G. pseudochina or Wan-maha-kan, is a perennial herbal plant that is distributed throughout Thailand (Vanijajiva, 2009). This plant has been used for anti-inflammation and anti-virus by local people for a long time (Plant Genetic Conservation Project, 2009). In 2011, Siriwatanametanon and Heinrich reported that some phenolic compounds isolated from the leaves of *G. pseudochina* var. *hispida* had efficiency on anti-inflammation. In addition, our previous study revealed that the leaf part of *G. pseudochina* gave the highest phenolic compounds among the parts of the tuber and stem (Noisa-nguan, 2009). Therefore, in this study, the plant leaf was selected as a source for obtaining a high content of phenolic compounds. However, a proper process to recover the active phenolic compounds from the plant leaves is an important consideration. In this study we found that the drying methods of freeze drying, microwave and oven could remove 92-93% of the moisture from the plant leaves (Table 4.1) due to *G. pseudochina* being a semi-succulent plant the moisture in the fresh weight is presented as about 90-95% (Perera, 2014). In addition, we found that the thermal drying methods of microwave and oven decreased the lightness (L^*) and yellow colour (b^*) of the *G. pseudochina* leaves (Table 4.1), which might be caused from the non-enzymatic browning reaction from thermal drying to make the darkness of the dried plant material (Vega-Gálvez et al, 2009). Besides, moderate temperatures at 50-70°C for a long time in oven drying were enough for the major changes of pigments in plants, such as β-carotene (Youssef and Mokhtar, 2014) and chlorophylls (Roshanak et al., 2016).

In this study we found that an alcohol-water mixing solvent, 50% methanol had the most efficiency to yield the crude extracts and phenolic compounds from *G. pseudochina* leaves (Figure 4.3 and 4.4). The appropriate solvent for extraction of phenolic compounds from each plant depends mostly on the nature of the sample material and the properties of the phenolic structure and polarity (Khoddami et al, 2013). The very polar phenolic compounds could be extracted completely with a mixture of solvents,

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alcohol, and water (Stalikas, 2007). Our results indicated that *G. pseudochina* leaves contained high polar phenolic compounds, and phenolic compounds were highly extracted from the plant leaves dried with freeze drying and microwave. In addition, the leaf extracts obtained from freeze-dried and microwave-dried samples strongly showed higher content of phenolics and antioxidant activity than the oven-dried sample (Figure 4.3-4.5). Phenolic compounds strongly relate to anti-oxidant properties due to their structures promoting 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 results in a greater rupturing of the plant cell structure. The fast freezing by liquid nitrogen before drying in this study promotes separation and gives 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 that led the cells to rupturing acceleration and provides more sample surface (Li et al., 2012). The oven drying process decreased phenolic compounds distinctly because of high temperature and longer drying time in the conventional oven process resulting in the destruction of plant compounds (Vadivambal and Jayas, 2007).

Many techniques of chromatography were used in this study for different purposes. HPLC was used to investigate the quality and quantity of phenolic composition of plant extracts. HP-TLC was used to investigate the quality of phenolic composition as well as to investigate non-phenolic compounds such as chlorophyll. In addition, LC-MS/MS was used to identify the chemical composition of plant extracts. From the HPLC result, we also found the high efficiency of 50% methanol solvent and drying methods of freeze drying and microwave on phenolic recovery (Figure 4.6-4.9), which might be from the same reasons as we discussed above. Likewise, the drying processes affected the phenolic compounds in many plant materials (Heck et al., 2008; Cerretani et al., 2009; Bey et al., 2016). In addition, the leaf maturity might decrease CGA and increase CA in the 50% methanol extract. CGA is implicated in plant responses to many biotic stresses, and CGA was produced increasingly in 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

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Baumann, 1994; Khoddami et al., 2013). In which, the lignin contents were more produced with the maturity of plants (Rencoret et al., 2011; Cesarino et al., 2012). CA is an intermediate in lignification, and it presents as caffeoly shikimic acid, caffealdehyde, and caffeoly 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, our 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). That might lead to rapid production of anti-oxidant compounds like free CA from bound phenolics. A high temperature for a long time during the oven drying process destroyed the phenolic compounds and changed the structure of the non-extractable phenolic compounds (Pinelo et al., 2005 and Xu et al., 2007; Honest et al., 2016). In this study, both freeze drying and microwave could retain the high phenolic compounds from *G. pseudochina* leaves, especially CGA, CA, and RUT. However, microwave drying provided more advantages for operation in terms of cost, ease of use, and shorter-time than freeze drying (Vadivambal and Jayas, 2007). For this reason, a microwave was considered as a suitable drying method for *G. pseudochina* leaves. In addition, the variation in the leaf ages did not correlate with the crude and phenolic contents as well as anti-oxidant activity (Table 4.3). Therefore, mixed-age leaves of *G. pseudochina* dried with a microwave (MLM) was applied as a bulk leaf material.

Although our results showed high efficiency of the 50% methanol for phenolic recovery, however, the methanol residue in the crude extract must be considered. Therefore, ethanol was used instead of methanol to extract the MLM. The results of the HPLC indicated that 25% ethanol and 100% ethanol were the highest and the lowest polar solvents used in this extraction system, and their extracts covered all peaks of the MLM ethanolic extracts (Figure 4.10). Therefore, they were selected to identify the chemical composition by LC-MS/MS. 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-

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methylenedioxyisoflavone and 2-(2,4-dihy-droxyphenyl)-5-hydroxy-8-methyl -8-(4-methyl-3-penten-1-yl)-2,3-dihydro-4H,8H-pyrano [2,3-f]chromen-4-one), xanthone derivative (1,3,8-Trihydroxy-4-methyl-2,7 diprenylxan-thone) , phenylpropanoid (3 ,4 dihy-droxycinnamoyl (Z)-2 - (3 ,4 - dihydroxyphenyl) ethanol) , phenolic glycoside compound (unknown-C-glycoside), and glycerol-phospholipid (1-(9Z-octadece-noyl)-sn-glycero-2,3-cyclic phosphate) (Table 4.5). The caffeoyl quinic acid, which is an ester of caffeic acid and (-)-quinic acid, was found as a marker compound in *Gynura* species. Different isomers of caffeoly 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 glycosides 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, presented in various isomers. The three isomers of 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) . The 4,5-dicaffeoylquinic acid was obtained from the ethanolic extract of *G. procumbens* (Jarikasem et al., 2013) . In addition, kaempferol rutinoside was isolated from 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 for (+)-tephropurpurin in *Gynura* species. For the most part, this compound is the chalcone flavonoid that has been found as a marker compound in the Genus *Tephrosia* (Chen et al., 2014b) . 1,3,8-Trihydroxy-4-methyl-2,7 diprenylxanthone was found in mangosteen (Yannai, 2012) . In which, various derivatives of xanthone have been reported for many 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, and this compound is an anti-oxidant agent that has

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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 many herbs. It is reported as anti-inflammation due to its potential to inhibit NF-κB/p65 phosphorylation and nuclear translocation (Wu et al., 2017). Quercetin is an abundant flavonoid compound found in a variety of plants (Srivastava et al., 2016). The quercetin isolated from the methanolic extract of *G. pseudochina* showed the most anti-oxidant activity among all isolated compounds (Rivai et al., 2017). In addition, various isomers of hydroxyl methoxy methylene dioxyisoflavone were also isolated from many plant species (Harborne, 1988).

To ensure the suitability of the microwave drying method for preparation of the valuable crude extract, mixed-age leaves of *G. pseudochina* dried with freeze dry (MLF) and oven (MLO) were investigated in the chemical composition by HP-TLC. The result indicated that the 25% and 50% ethanol fractions of MLM still presented high quality phenolic compounds (Figure 4.12). Even though, plants are a large source of many active compounds, however, some toxic compounds from plant extracts are presented as a mixture with beneficial compounds, especially the PAs. Our study found that Pas were still contained in MLM extracts (Table 4.6). Interestingly, oven drying could increase the PAs contents in plant material. The PAs are synthesized from polyamines including putrescine and spermidine that have the homospermidine synthase (HSS) as a key enzyme to produce the N-oxide forms of alkaloids like PAs (Dreger et al., 2009). Polyamines are produced increasingly when plants are stressed (Takahashi and Kakehi, 2009). During a temperature increase to a certain point of 60°C for this study, the HSS enzyme might be over active and respond to high temperature stress. That could lead to a rapid production of the PAs. The United Kingdom Medicines and Healthcare Product Regulation Agency regulates PAs in the herbal products to 1 mg/day (16.7 µg/kg/day for a 60 kg of body weigh) for two weeks or to 0.1 mg/day (1.67 µg/kg/day) for long-term (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).

The crude extracts obtained from the mixed-age leaves dried with a microwave and serial extraction with 25% and 50% ethanol (EMLM) were finally used for the cell study and prototypic cream preparation. The EMLM contained marker compounds of CGA, CA, RUT, and PCA (Figure 4.13). However, the EMLM still contained 95%

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unidentified compounds, approximately (Table 4.7). For the cell study, a HaCaT cell line was selected to use as the subject due to it being a skin keratinocyte cell line that is pretty stable and has been used wildly in *in vitro* studies for skin diseases. The MTT assay is one of the standard methods to evaluate the cytotoxicity via measurement of the insoluble dark blue formazan product that is formed from cleaved MTT by active mitochondria (Slater et al., 1963; Mosmann et al., 1983). In this study, EMLM and some marker compounds (CA and CGA) were toxic to HaCaT cells and both were not under TNF-α and TNF-α stimulation conditions (Table 4.8). Some phenolic compounds could affect cell cytotoxicity. The various metabolism processes in keratinocytes can provide peroxidase, then the phenolics may change to many ROS that can damage mitochondria (Galati et al., 2002).

NF-κB is a transcription factor that controls the production of inflammatory proteins belong to inflammation processes. The over function of NF-κB leads to many chronic and acute inflammatory diseases. Therefore, the inhibition of NF-κB function is one of the targets for treatment of inflammatory disorders. There are two important pathway of NF-κB function in the inflammation process that comprises the RelA and RelB canonical pathways. In this study, we used the anti-phospho RelA and RelB to monitor the localization of the active form of RelA and RelB. In addition, two concentrations of EMLM and each marker compound (CGA, CA, RUT, and PCA) were used to observe the NF-κB inhibitory effect of these compounds under cell survival of more than 50%. However, the results indicated that active RelA529 was not present in both HaCaT cells under non TNF-α stimulated and TNF-α stimulated conditions. Alternatively, some concentrations of EMLM and each marker compound lead to RelB inhibition (Figure 4.14). Actually, RelA is also observed in HaCaT cells, but those with activated RelA may be phosphorylated at another site not S529 (Kang et al., 2008; Feng et al, 2013; Ren et al, 2017). The RelA can be phosphorylated at different sites of serine and methionine in the polypeptide (Christian et al., 2016). The previous studies found RelA S529 in cancer cells upon IL-1β or TNF stimulation (Wang and Baldwin, 1998; Wang and Westerheide, 2000) and monocyte cells under LPS stimulation (Bristow et al., 2008). However, the phosphorylation at S529 in HaCaT keratinocyte cells has not been reported. In addition, *G. pseudochina* var. *hispida* and their marker compounds, included

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rutin and caffeic acid derivatives, possessed an inhibitory effect on the NF-κB on macrophage cells (Siriwatanametanon and Heinrich, 2011).

IL-8 is an inflammatory chemokine that has been produced from keratinocytes to attract various immune cells to migrate to epidermal cells, and lead 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 found to be up-regulated in psoriatic skin more than 80% when compared to normal skin (Baliwag et al., 2015). This result indicated that both the concentration of EMLM, CA, and RUT could inhibit IL-8 production, while only the higher concentration of CGA and PCA could inhibit IL-8 production (Table 4.9). For our result, the inhibition of IL-8 production might be caused from the inhibition of NFκB function. Phenolic compounds have been reported to have anti-IL8 properties. Dihydrocaffeic acid has the potential for IL-8 inhibition on HaCat cells after exposed to UV radiation (Poquet et al., 2008). The polyphenols from green tea show anti-IL8 production in epithelial cells that leads to the ability for an inflammation remedy (Kim et al., 2006). In addition, TSG-6 was inhibited by a lower concentration of EMLM and CGA, and a higher concentration of PCA. While, the two concentrations of CA and RUT applied did not inhibit the TSG-6 production (Table 4.9). The TSG-6 is a secreted glycoprotein that is expressed by a variety of cells in response to the inflammation process, which can protect the tissue from the damaging effects of inflammation (Milner and Day, 2003). TSG-6 can bind to a large number of components in the extracellular matrix, including hyaluronan, heparin, heparan sulfate, thrombospondins-1 and -2, fibronectin, and pentraxin 3. These interactions primarily act to stabilize or remodel the extracellular matrix (Kim et al., 2016). Therefore, the presence of these proteins is implicated in the persistent inflammation process.

The most important consideration for pharmaceutical creams is the stability of the finished product, especially the active compound and pH value (Punam et al., 2014). Our results indicated that the cream base and the prototypic EMLM cream did not show coalescence and phase inversion during 30 days of and after a freeze thaw cycle. However, after a freeze thaw cycle, both creams seem to lose their humidity due to evaporation of water during the thawing process (Figure 4.15). Furthermore, the pHs of the base cream and prototypic EMLM cream at the first day after preparation were 6.16 and 5.30, respectively, so the pHs are in the pH range (4-6) of skin products (Ali and

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Yosipovitch et al., 2013). In addition, the pH values of the cream base and EMLM cream did not change during 30 days and after a freeze thaw cycle, but decreased after one year of storage time. The cream base and the EMLM cream became greener after a freeze thaw cycle, and that might be caused from a chemical reaction between its ingredients under the thermal condition and/or the evaporation of the water phase. In addition, the marker compounds of CGA, CA, RUT, and PCA were stable after one year of storage and after a freeze thaw cycle (Figure 4.11 and 4.12). The stability of both the physical and chemical properties of the prototypic EMLM cream, especially the contents of the phenolic marker compounds, leads to further research in psoriasis patients and developing a formula with a higher content of the *G. pseudochina* extract in the product.

5.2 Conclusions

The recovery of phenolic compounds, antioxidant activity, and crude extracts clearly depended on the extracting solvent and drying process. The use of 50% methanol was the most efficient solvent for crude extract recovery that lead to a high phenolic compound recovery, especially the marker compounds in *G. pseudochina* leaves, including CGA, CA, and RUT. Freeze drying and microwave drying processes distinctly retained high phenolic and marker compounds and antioxidant activity. The ethanol solvent was used instead of methanol due to a safer natural product application. The microwave drying method was considered for further use due to its efficiency for the postharvest *G. pseudochina* leaves, increased CA, and many advantage in the operation process. Mixed-age leaves were also considered as the suitable plant material due to no correlation between leaf ages and the phenolic and crude extract recovery as well as the antioxidant activity. The concentrations of 25% and 50% ethanol were used to co-extract to obtain mostly the phenolic and all marker compounds from the mixed-age leaves dried with a microwave (MLM). The HP-TLC fingerprints revealed CGA, CA, RUT, and some other phenolic compounds as present in the ethanolic extracts of MLM. The MLM extracts also contained the Pas, which should be controlled to safe doses when applied in a health product. The LC-ESI-QTOF-MS/MS indicated that the MLM extracts contained phenolic acid, flavonoid, phenylpropanoid, xanthone, and phenolic glycoside. Therefore, a crude extract obtained via co-extraction with 25% and 50% ethanol from mixed-age

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leaves dried with a microwave (EMLM) was investigated for cell study and prototypic cream preparation.

The EMLM extract and their marker compounds were not toxic to both HaCaT cells due to non-TNF-α and TNF-α stimulation, except with CA. Moreover, the EMLM extract and its marker compounds had efficiency for anti-RelB and anti-IL-8 throughout the decreased TSG-6. Therefore, this result could support the ability of the *G. pseudochina* leaf extract for development as an active ingredient for psoriasis and/or inflammatory diseases alleviation. In addition, 0.5% EMLM was incorporated into the prototypic cream. The physical stabilities of a cream are characterized by the absence of the coalescence of the internal phase and the absence of a phase inversion via the absence of a color change (Punam et al., 2014). The EMLM cream and base cream were stable in terms of texture, color, and pH for more than 30 days of storage time. In addition, the marker compounds in EMLM, including CGA, CA, RUT, and PCA, were stable within the cream for one year. Consequently, this study could support an application of *G. pseudochina*, a local Thai herb, as an herbal ingredient to provide a topical product for psoriatic patients.

5.3 Suggestions

5.2.1 The individual compounds in the *G. pseudochina* leaf extract should be purified and continuously evaluated for the anti-NF-κB property to find other compounds that can act as active agents. An individual pyrrolizidine alkaloid should be identified to improve the safety of the leaf extract utilization.

5.2.2 Other pathways that belong to the inflammation processes and psoriasis pathogenesis should be studied to strongly support the usage of *G. pseudochina* leaf extract for psoriatic alleviation. In addition, the cytotoxicity to other cell types should also be investigated, especially the toxicity to hepatic cells that may be affected by pyrrolizidine alkaloids that were contained in the plant leaf extract as well as the toxicity to fibroblast cells in case of use of the leaf extract as an active ingredient in a topical product.

5.2.3 According to the results obtained from the *in vivo* analysis with the keratinocyte cells, a high concentration of *G. pseudochina* crude extract in a prototypic

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cream should be applied to the base cream. Finally, a patient study for the *in vivo* verification should be studied to evaluate the efficiency of the finished prototypic cream.

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APPENDIXES

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Appendix A

G. pseudochina cultivation and extraction system

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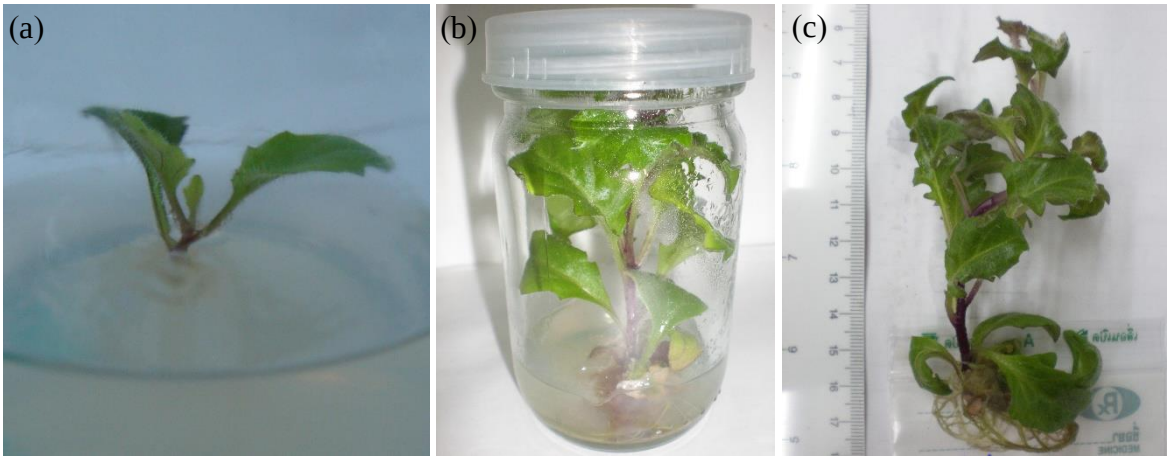
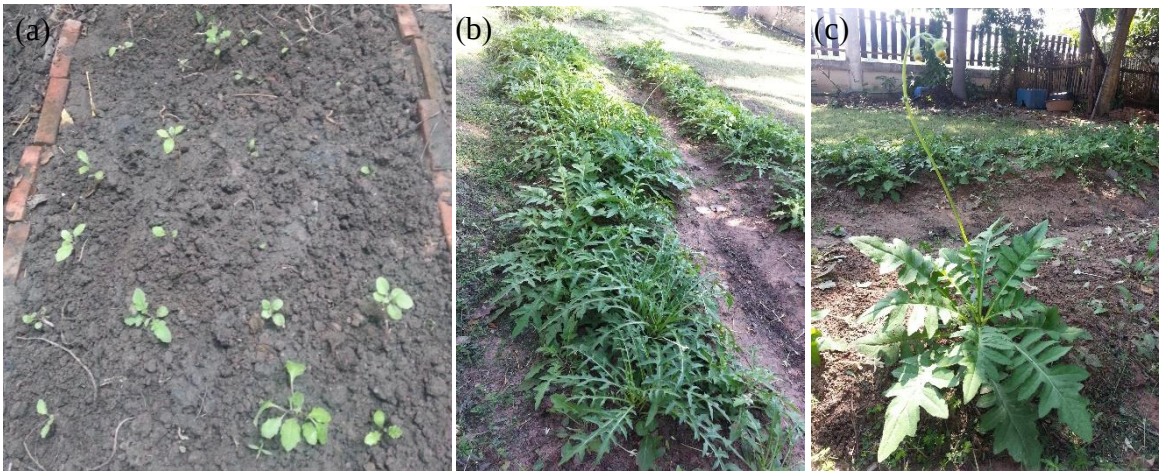
Appendix A-1 Growth of *G. pseudochina* in tissue culture system for (a) one week, (b) two weeks, and (c) plantlet obtained from tissue culture before cultivation in pot.



Appendix A-2 Growth of *G. pseudochina* in soil system for (a) one week, (b) three months, and (c) plant in flowering stage.



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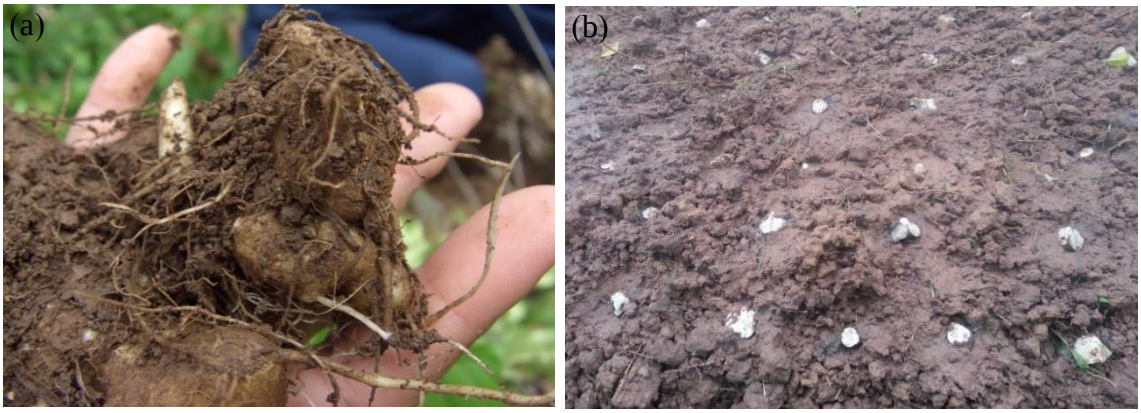
Appendix A-3 Tuber of *G. pseudochina* after harvest of its leaves (a, b) as cultivated in plot to obtain its leaves again in next crop.



Appendix A-4 Fresh leaves of *G. pseudochina* after harvest: (a) young leaves, (b) developing leaves, and (c) mature leaves.

Appendix A-5 Dried leaf samples packed into thimble before extraction by soxhlet apparatus.

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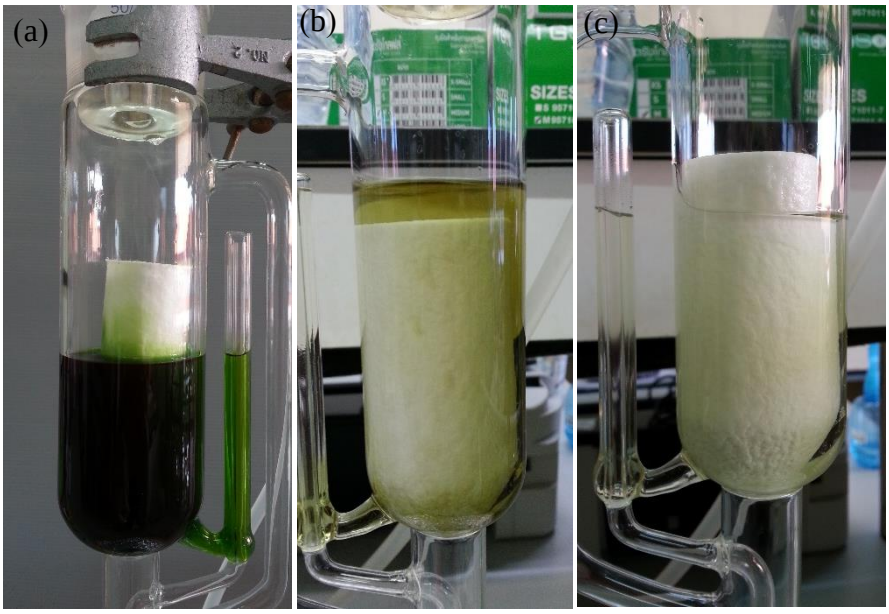
Appendix A-6 Dried leaf sample defatted and removal of chlorophyll with hexane: (a) during extraction with each solvent (b) and last cycle of last solvent that gave clear solvent.



Appendix A-7 Dried leaf sample after extraction with all solvents.

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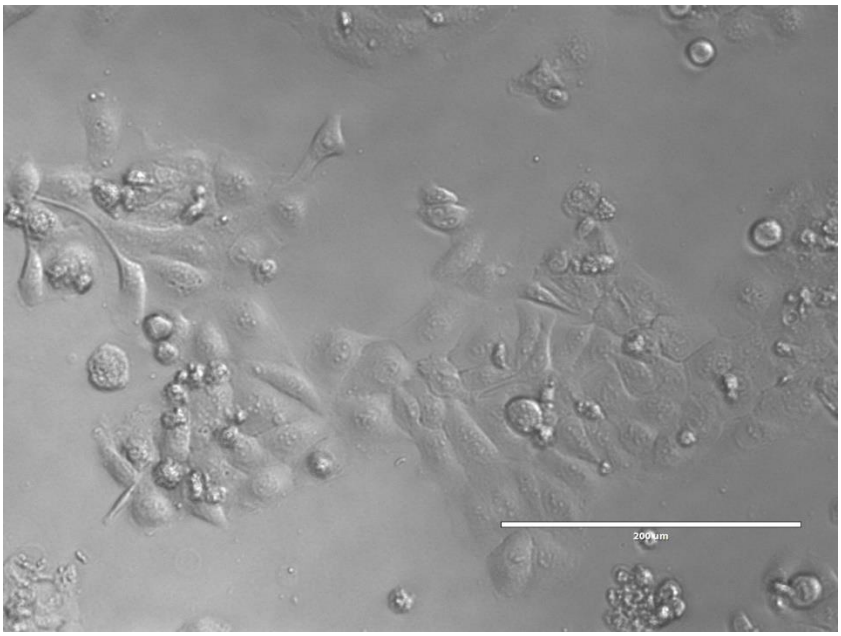
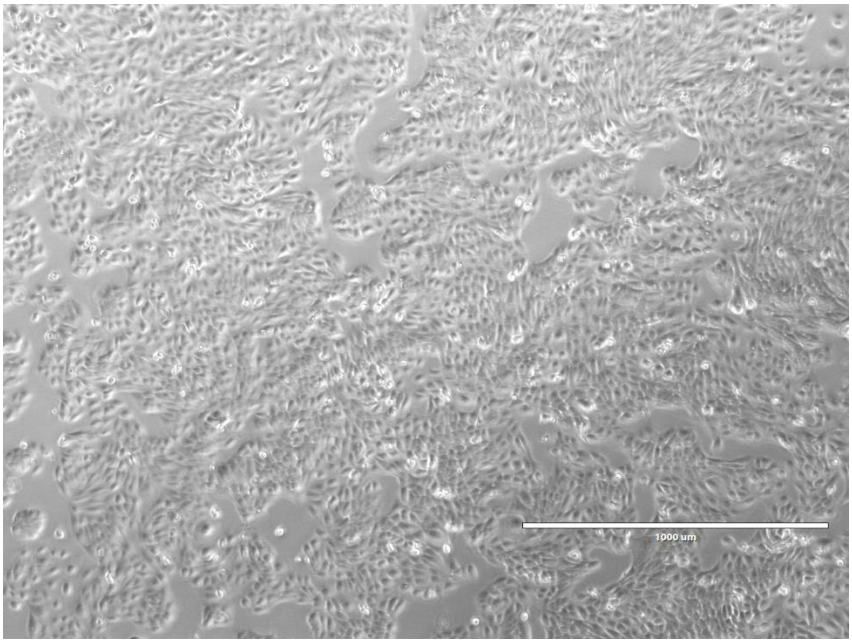
Appendix B

Cell study

Appendix B-1 Appendix B-1 HaCaT cells grown on DMEM medium.



Appendix B-2 80% confluent of HaCaT cells grown in T75 cell culture flask after sub-culture for 96 h.



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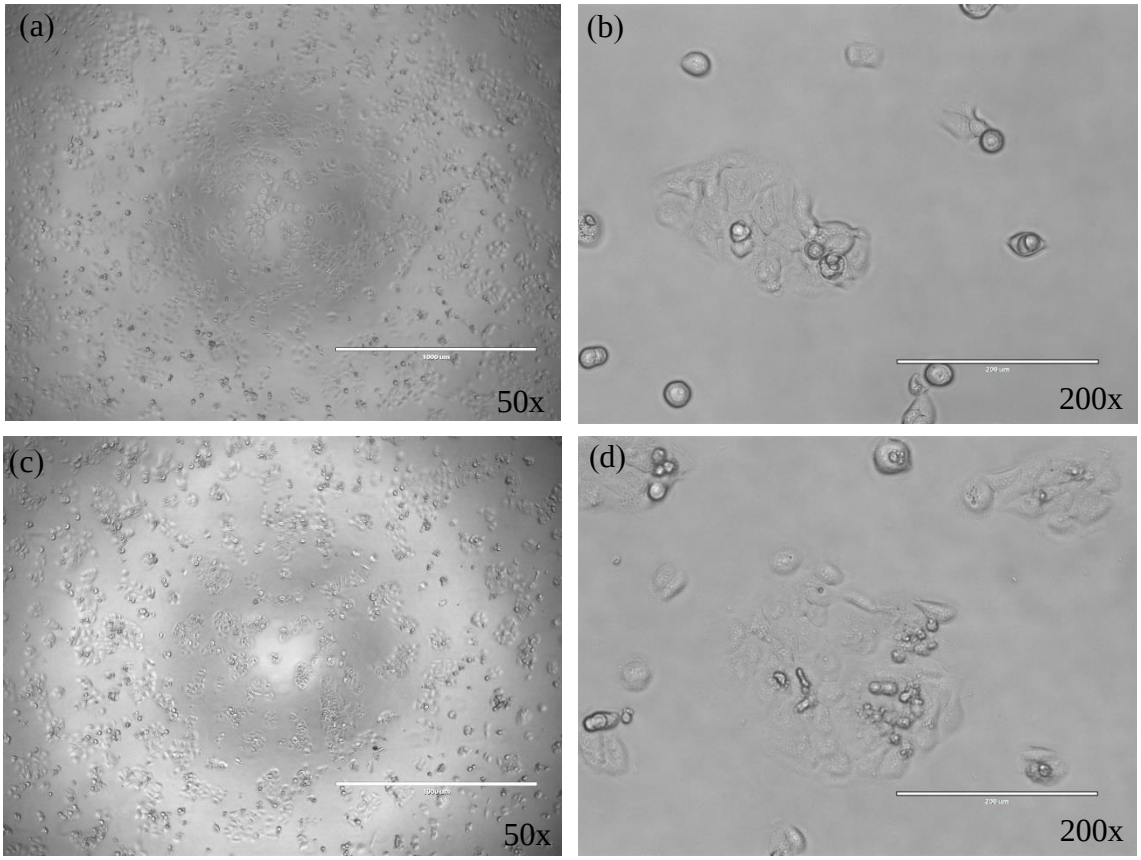
Appendix B-3 HaCaT cells grown in 96-well plate. Cells after sub-culture and growth in DMEM without TNF-α stimulation for 24 h (a). Cells after sub-culture for 12 h and then growth on DMEM containing 50 ng/ml of TNF-α for 12 h (b). These cells were about 50% confluent and were used at each concentration of samples for cell cytotoxicity test.



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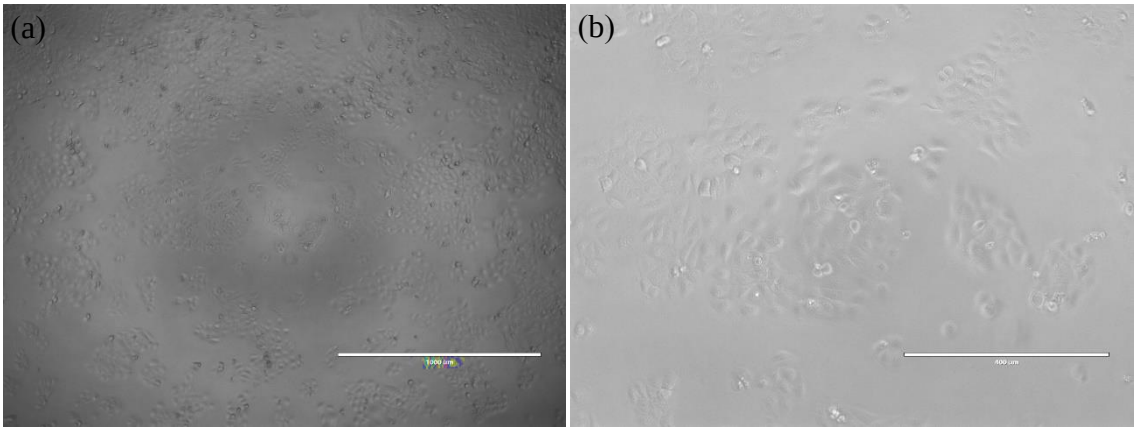
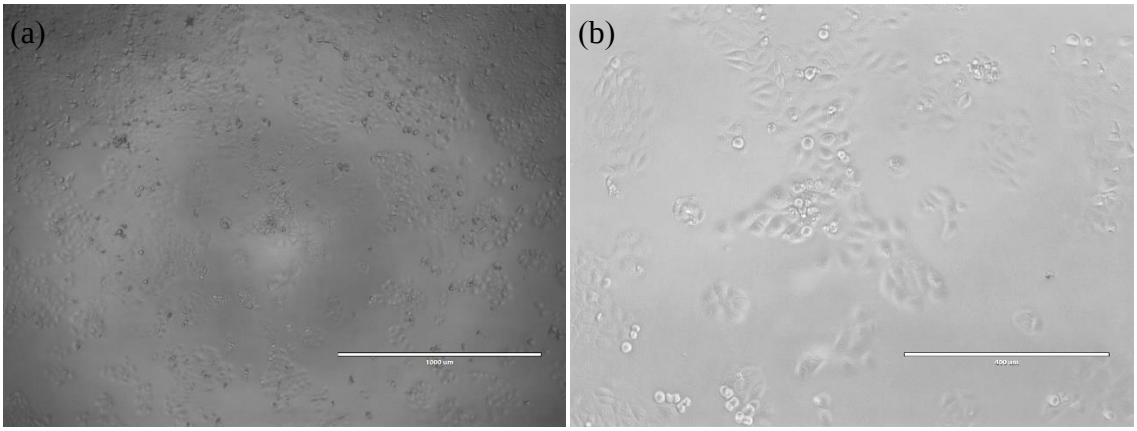
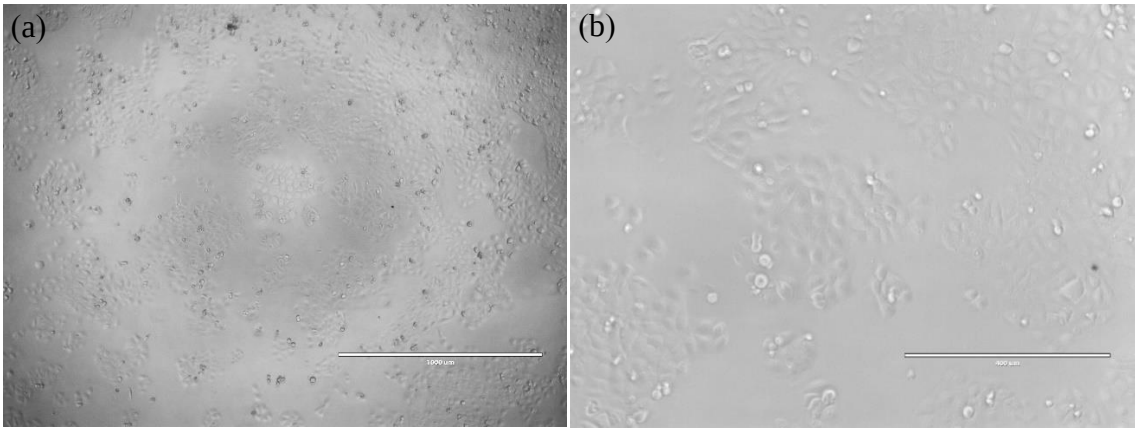
Appendix B-4 Non TNF-α stimulated HaCaT cells after treatment for 24 h. Magnification at 50x (a) and 100x (b).



Control containing 0% DMSO



Control containing 0.13% DMSO



Control containing 0.25% DMSO

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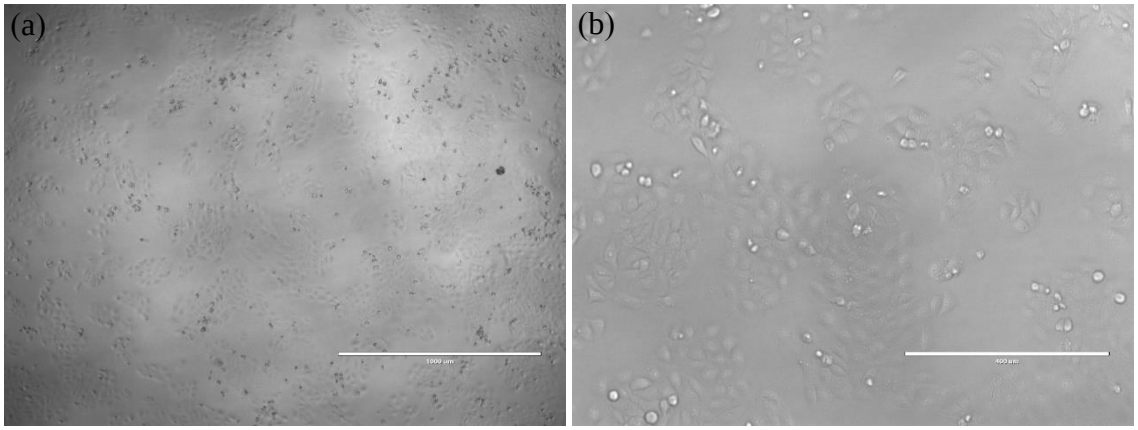
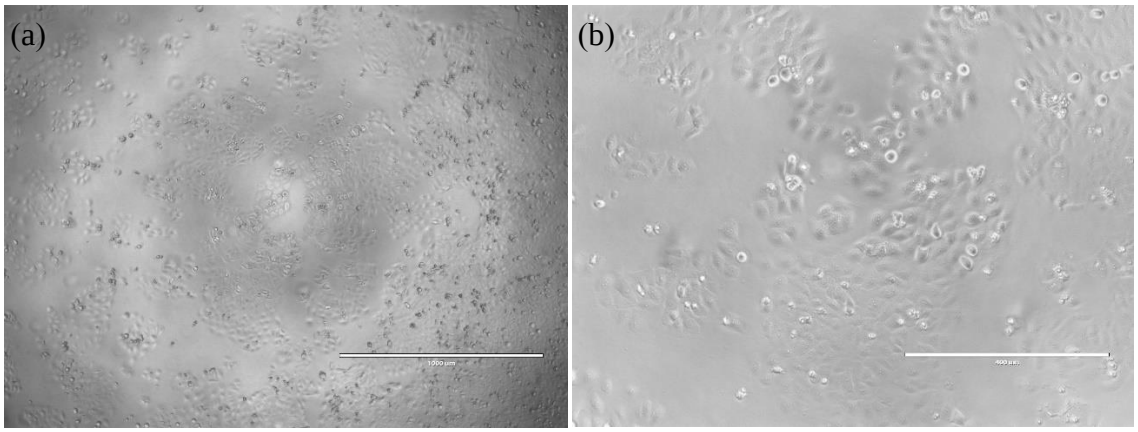
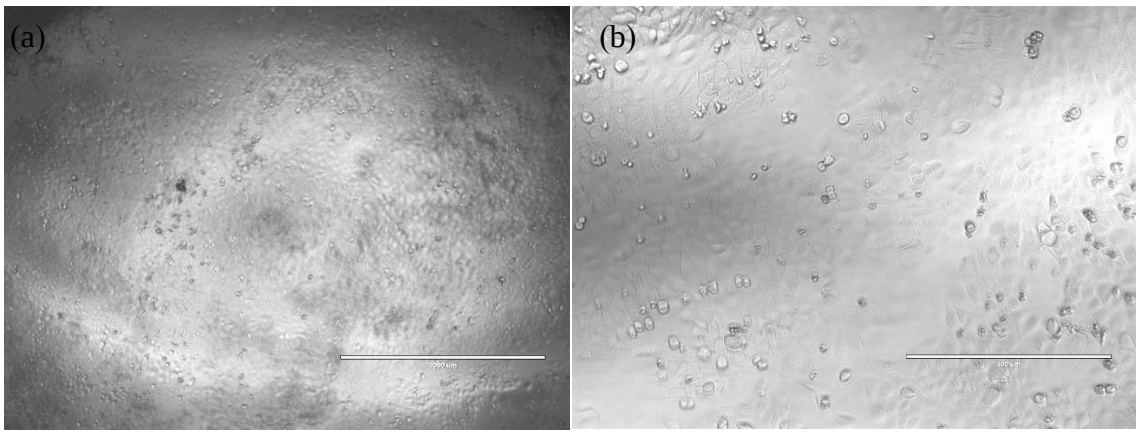


Control containing 0.50% DMSO



Control containing 1 % DMSO



EMLM 31.3 µg/ml

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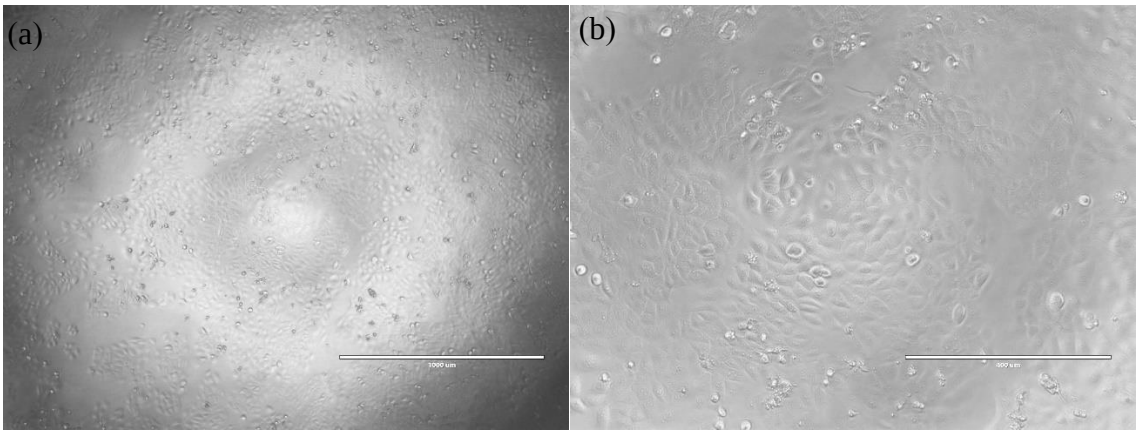
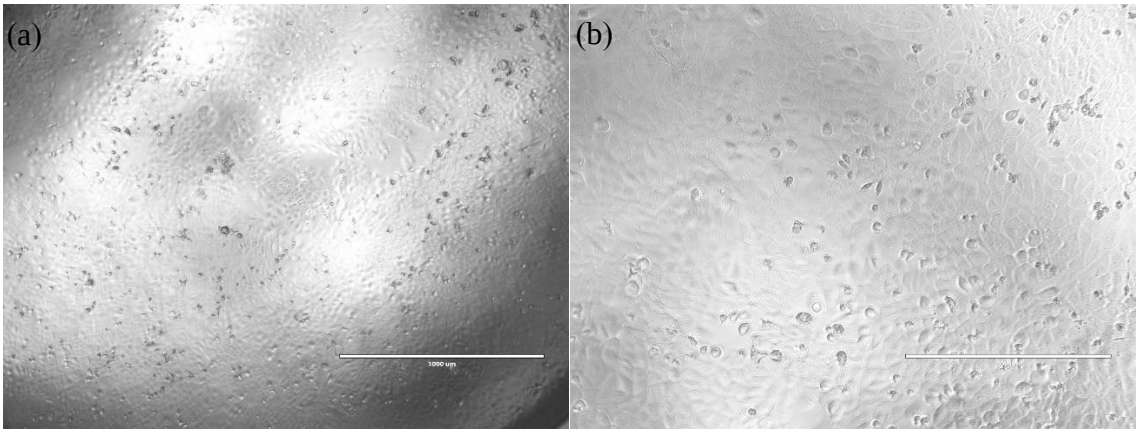
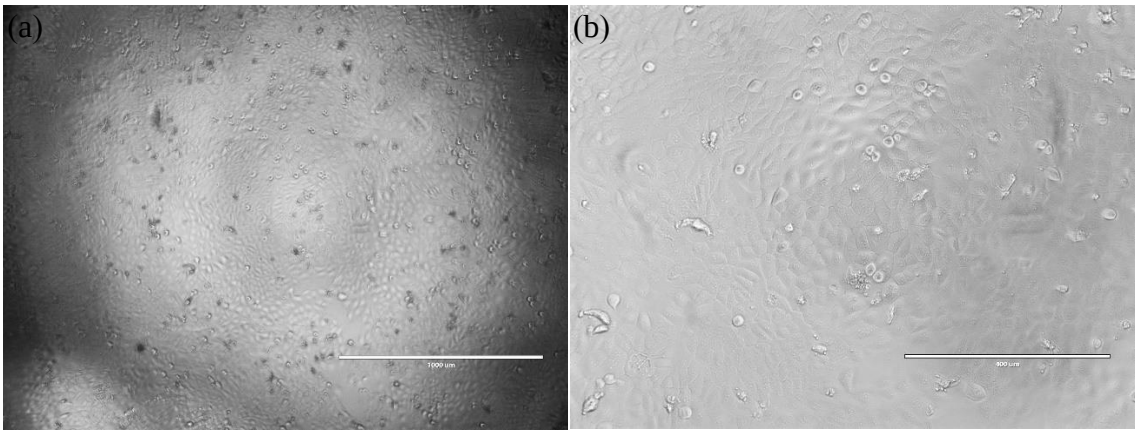


EMLM 62.5 µg/ml



EMLM 125 µg/ml



EMLM 250 µg/ml

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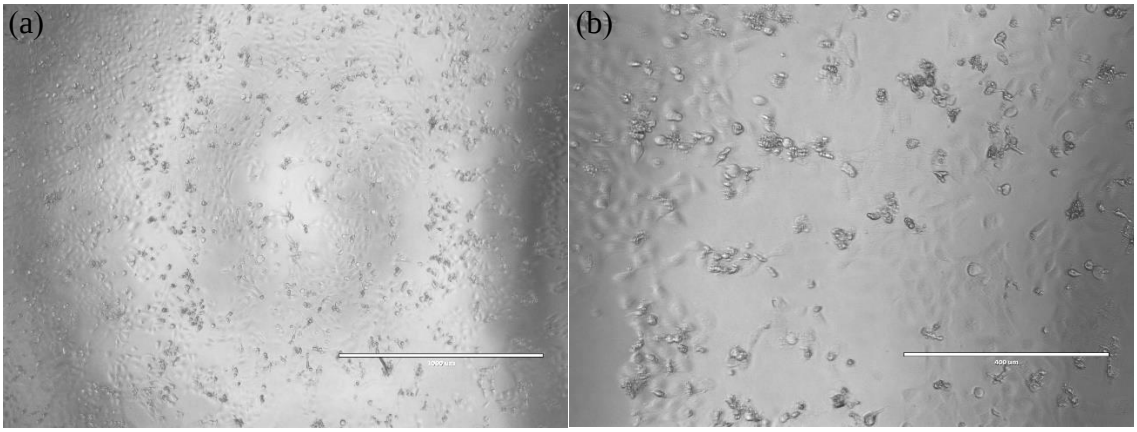

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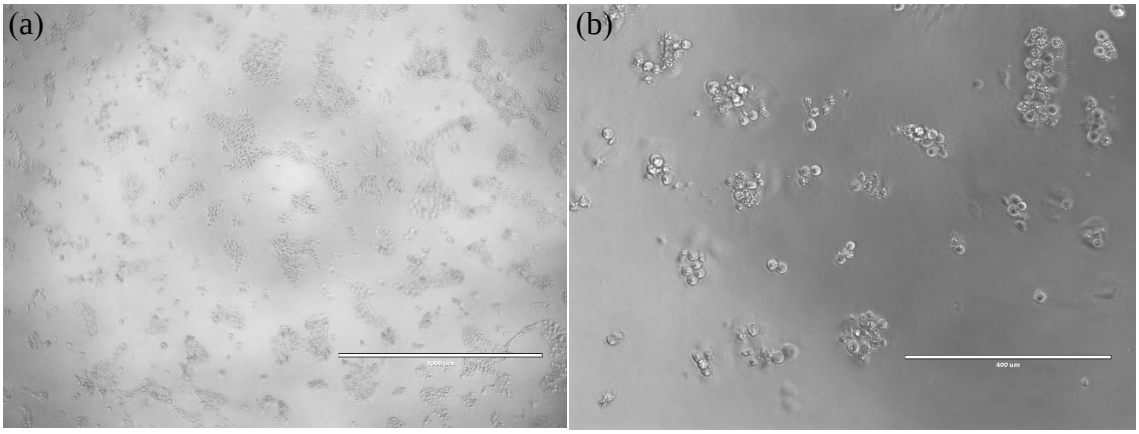
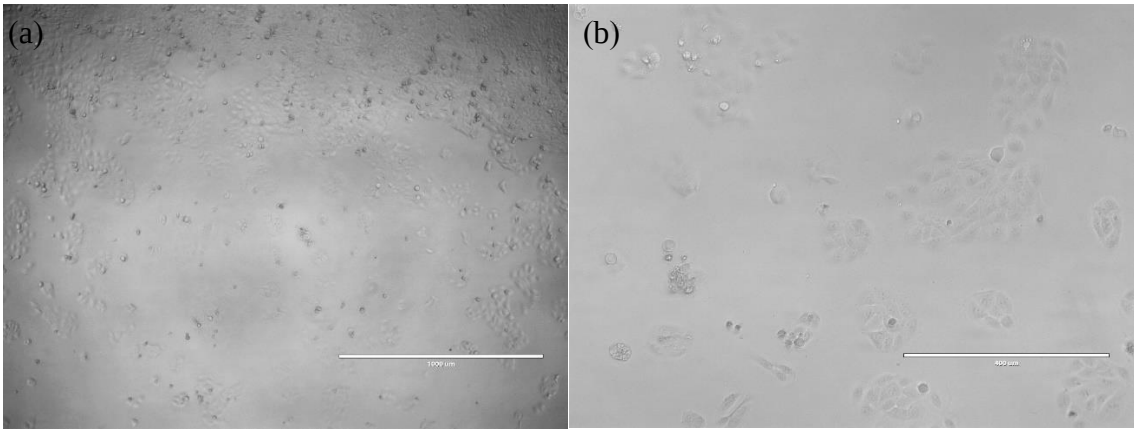


EMLM 1000 µg/ml



CGA 37.5 µg/ml

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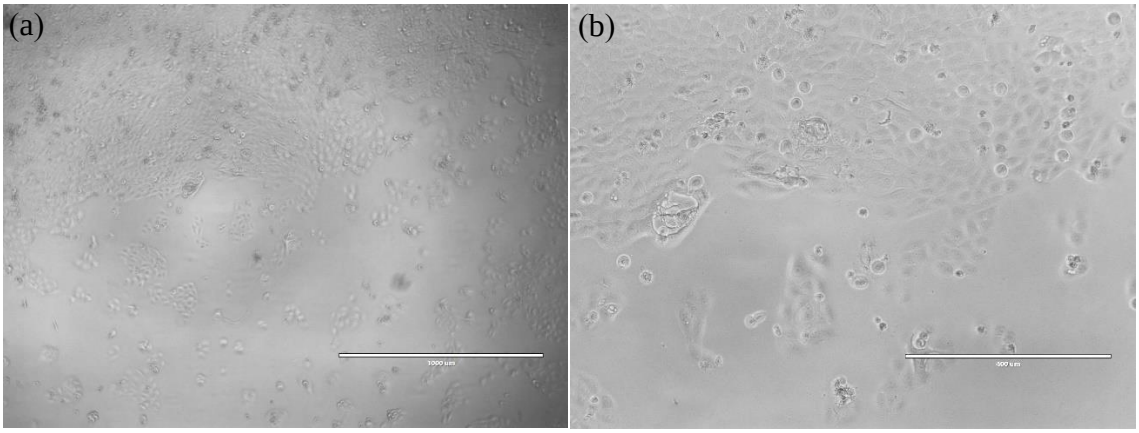
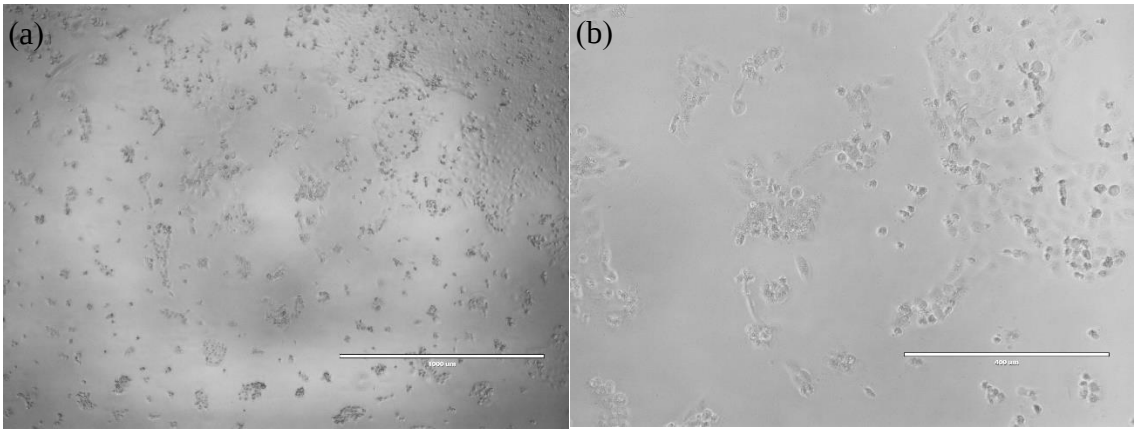
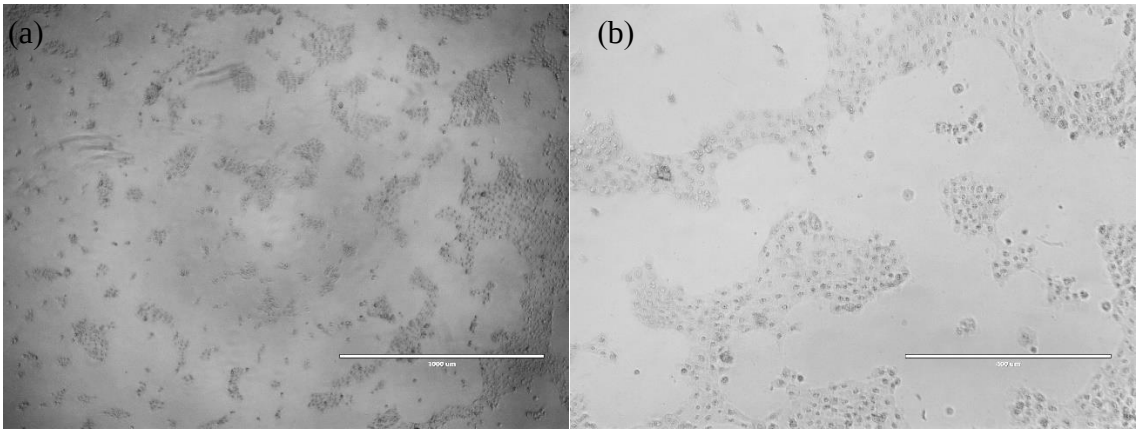


CGA 75 µg/ml



CGA 150 µg/ml



CGA 300 µg/ml

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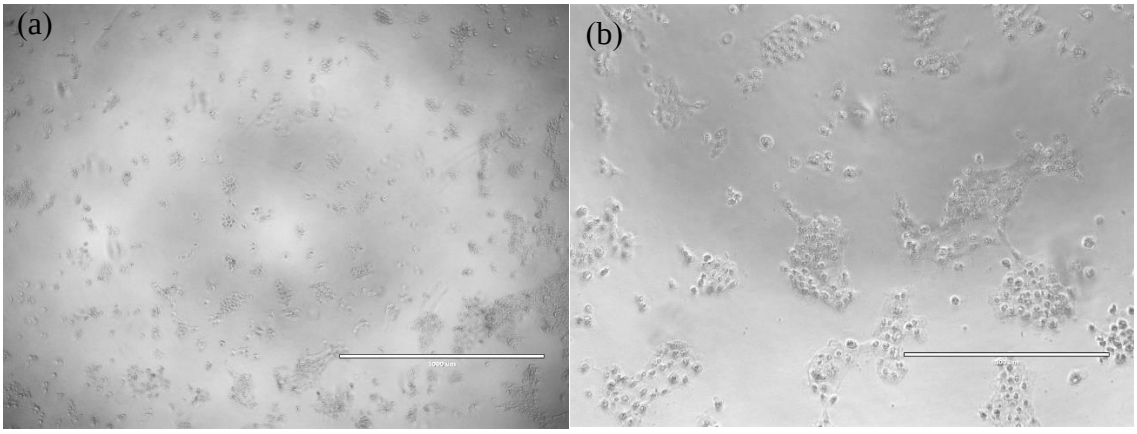
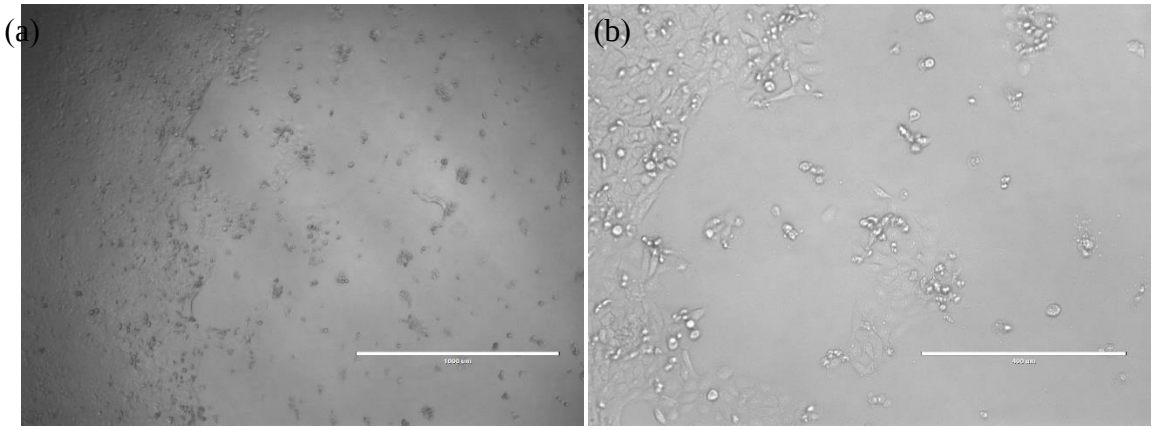
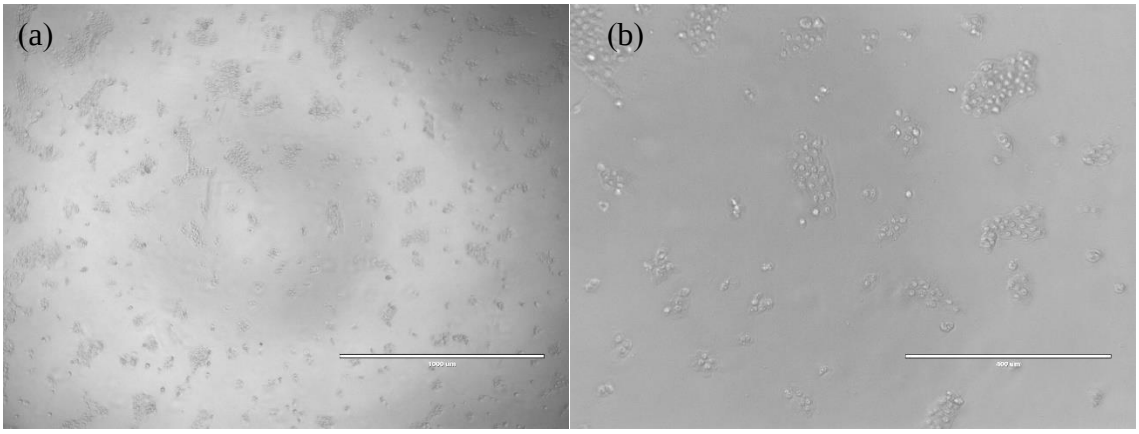


CGA 600 µg/ml



CA 31.3 µg/ml



CA 62.5 µg/ml

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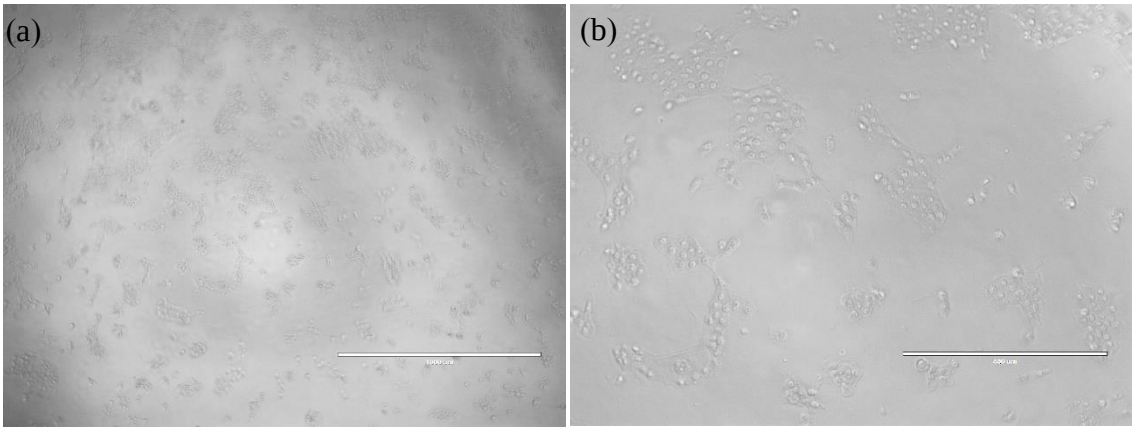
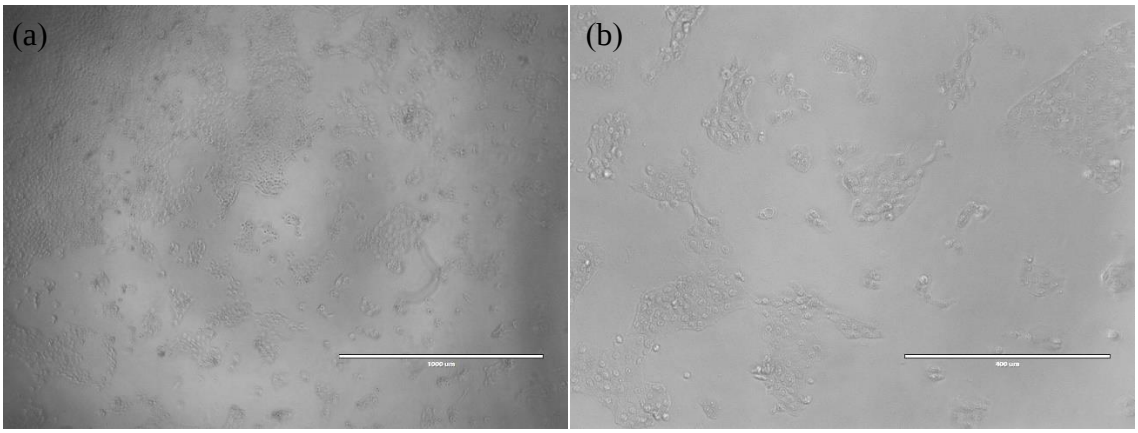
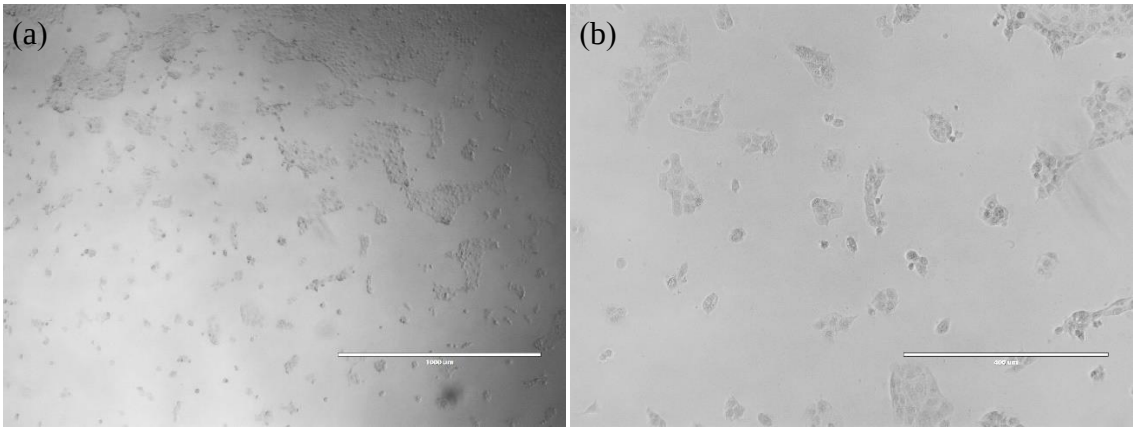


CA 125 µg/ml



CA 250 µg/ml



CA 500 µg/ml

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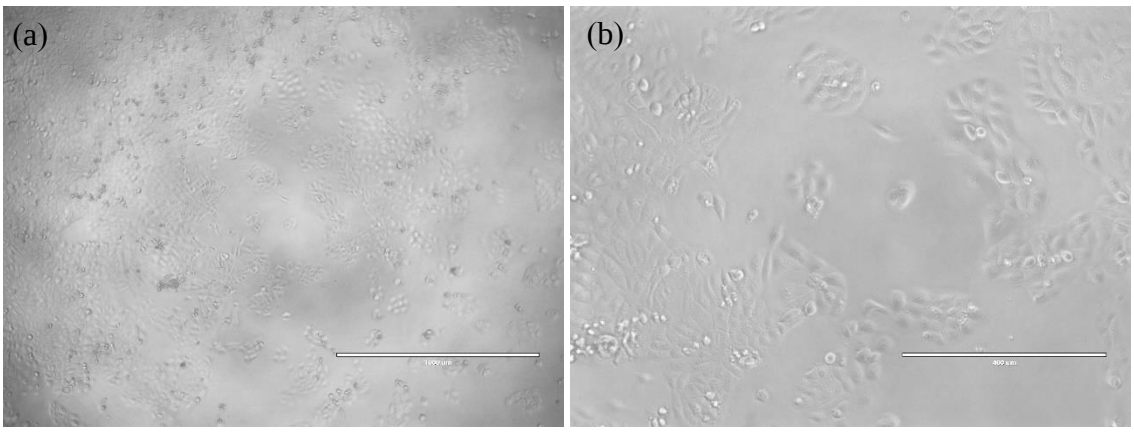
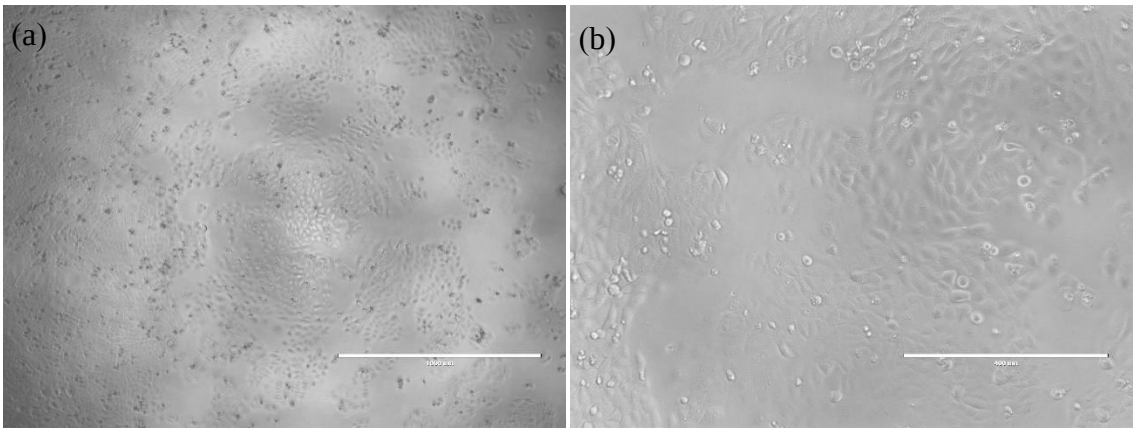
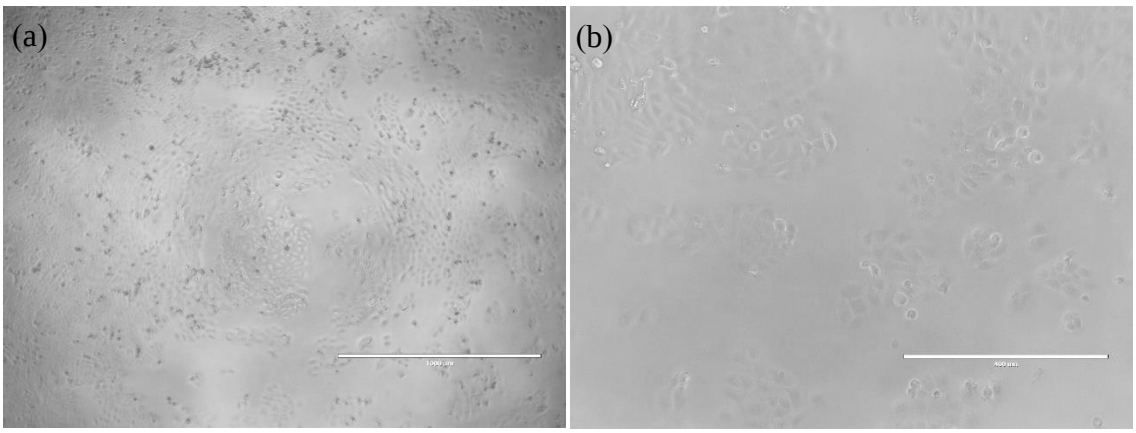


RUT 62.5 µg/ml



RUT 125 µg/ml



RUT 250 µg/ml

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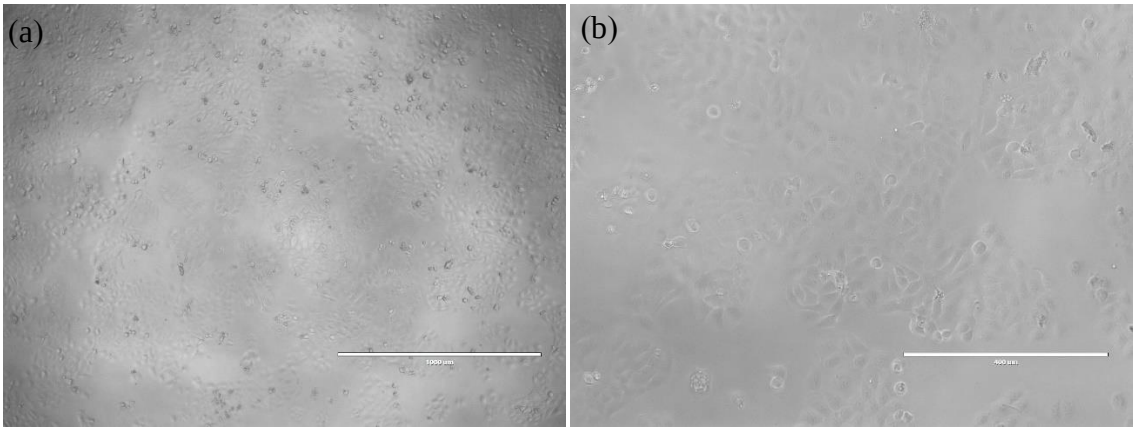
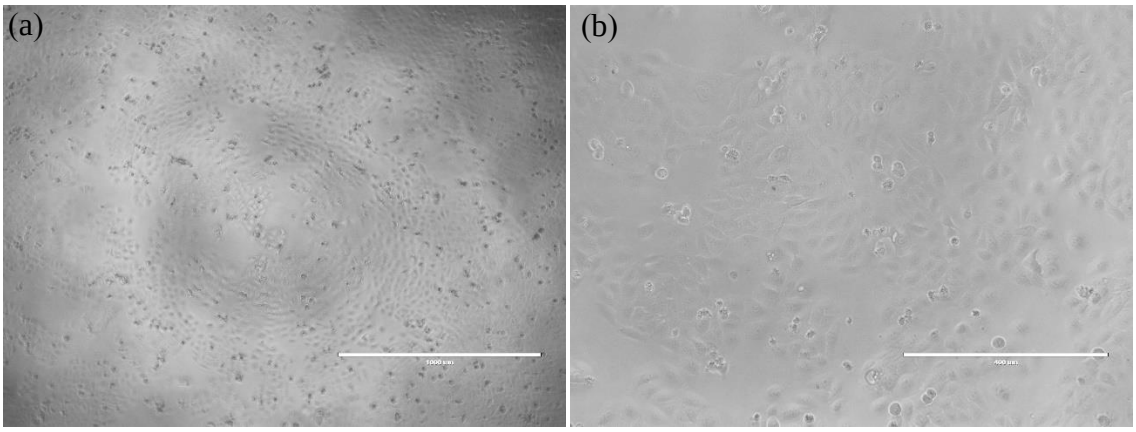
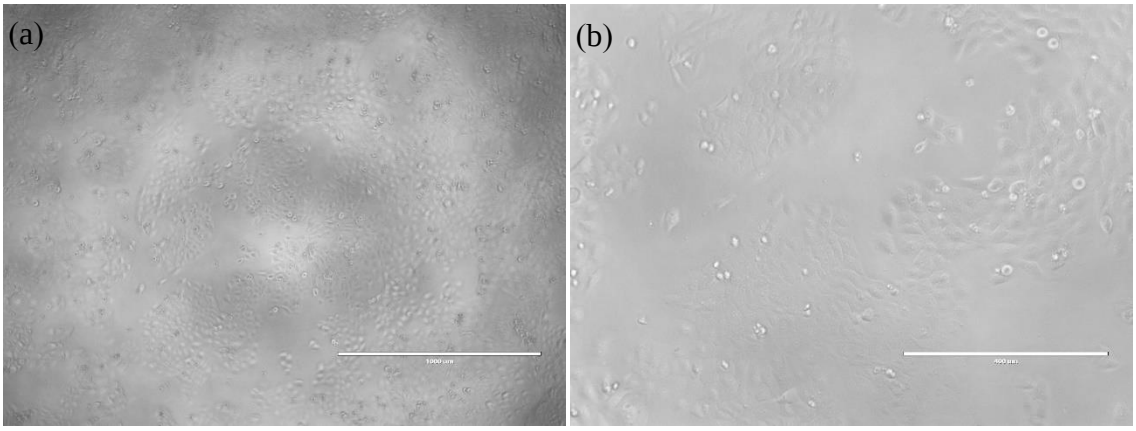


RUT 500 µg/ml



RUT 1000 µg/ml



PCA 312.5 µg/ml

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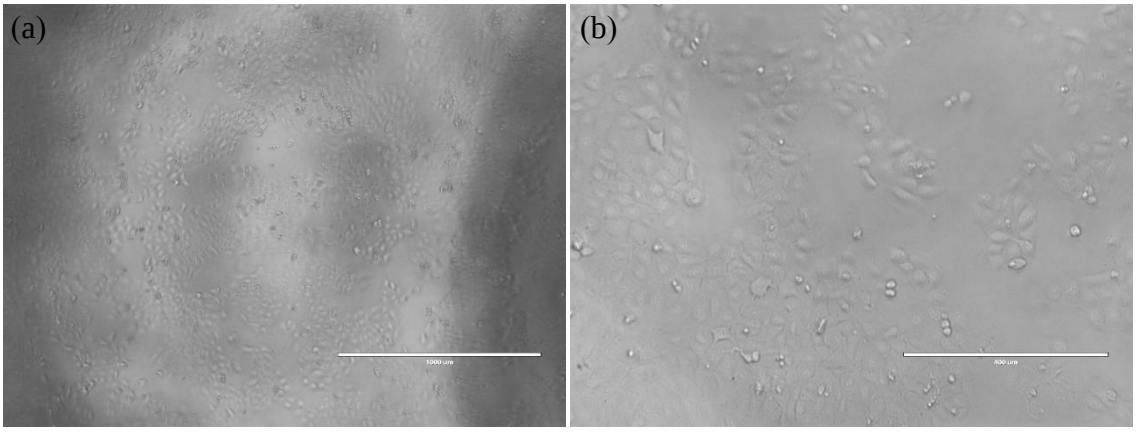
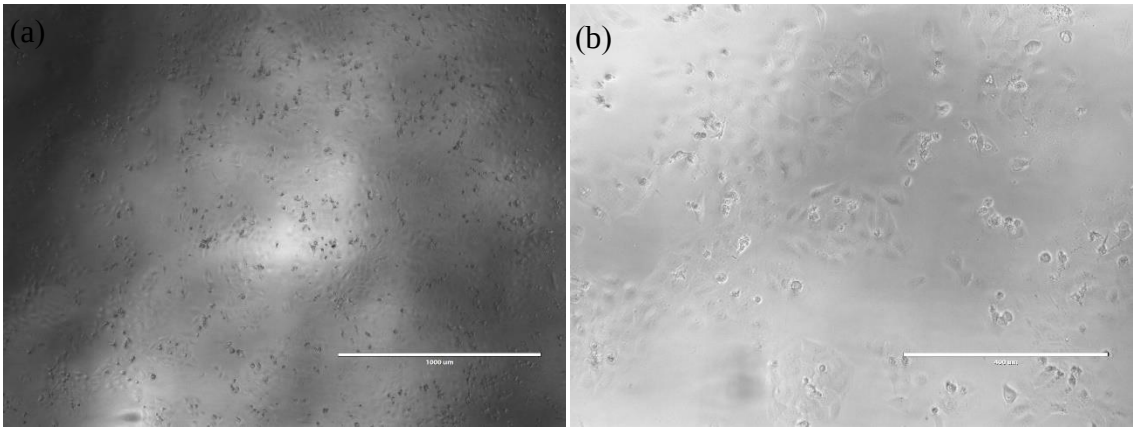
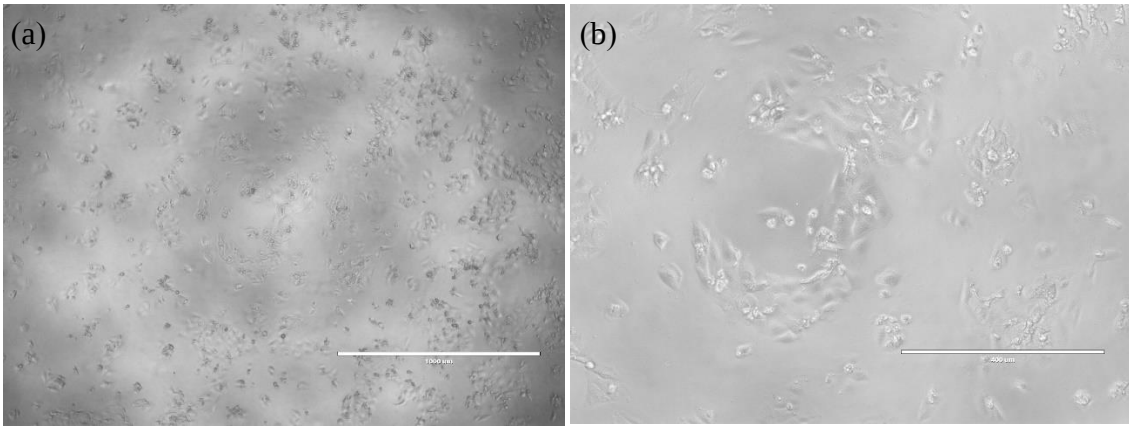


PCA 625 µg/ml



PCA 1250 µg/ml



PCA 2500 µg/ml

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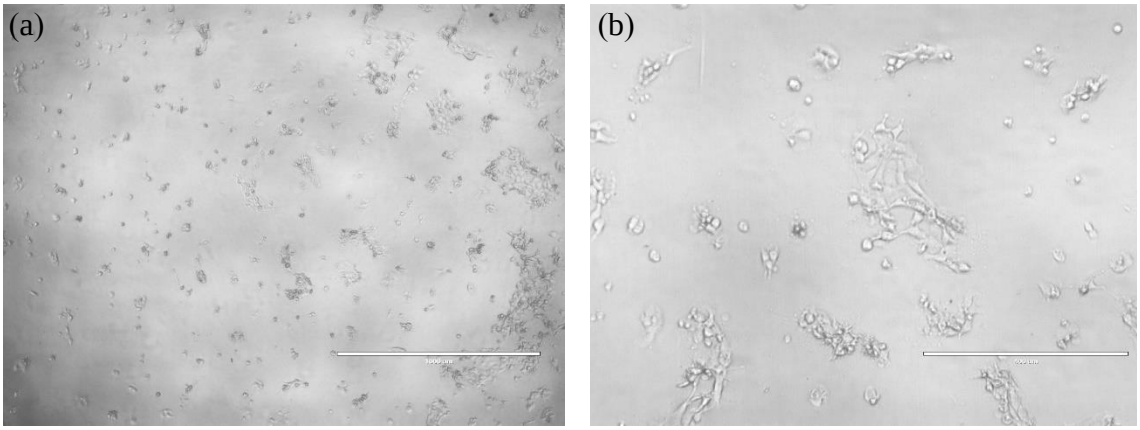
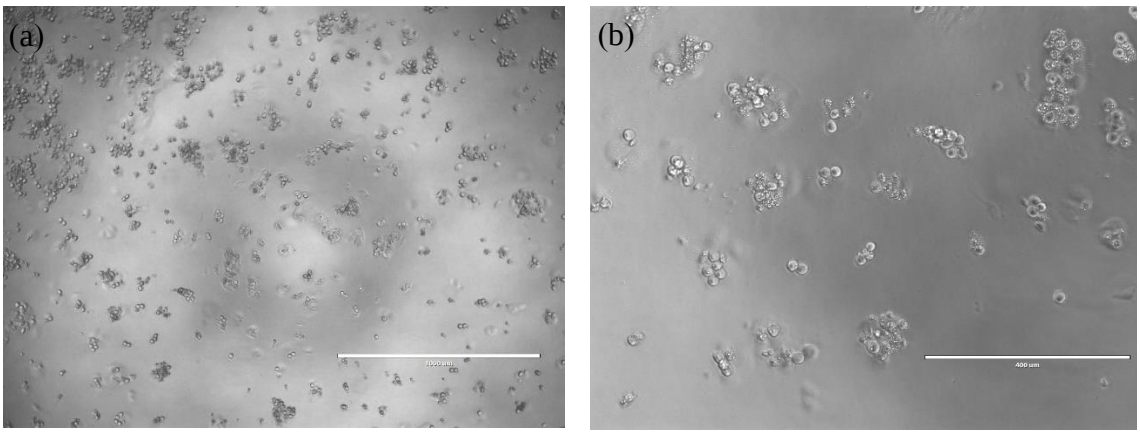
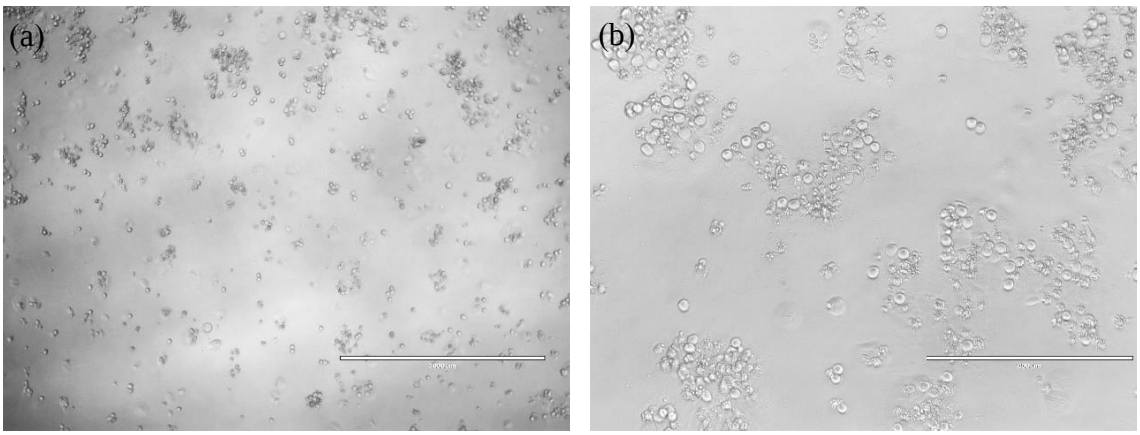


PCA 5000 µg/ml



PTX 0.8 µg/ml



PTX 1.6 µg/ml

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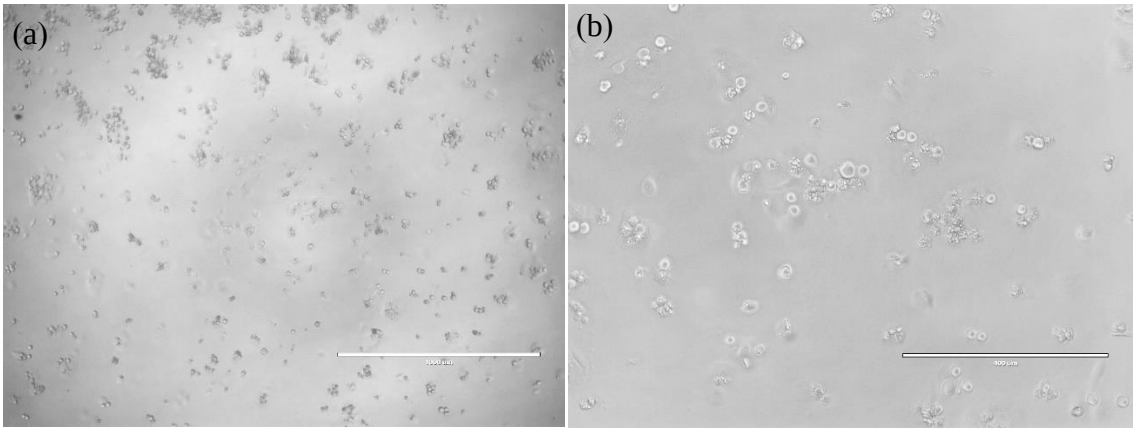
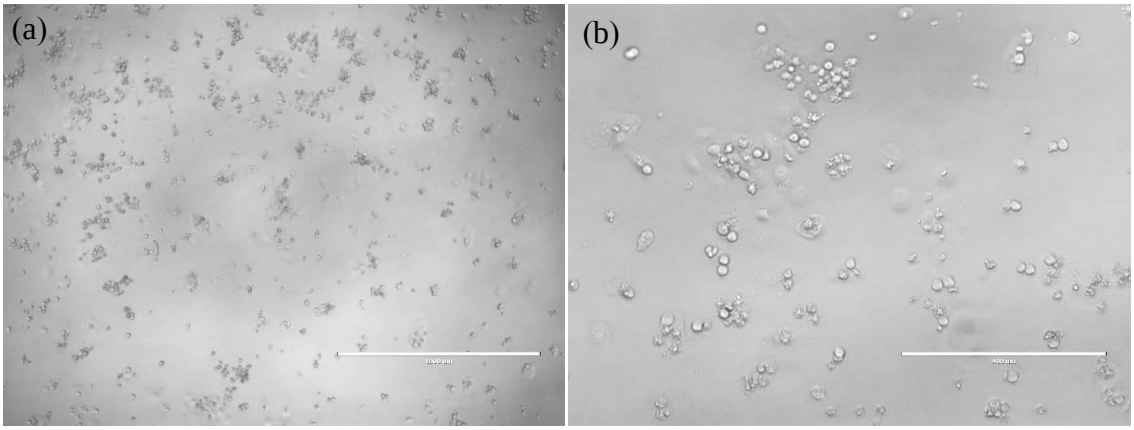
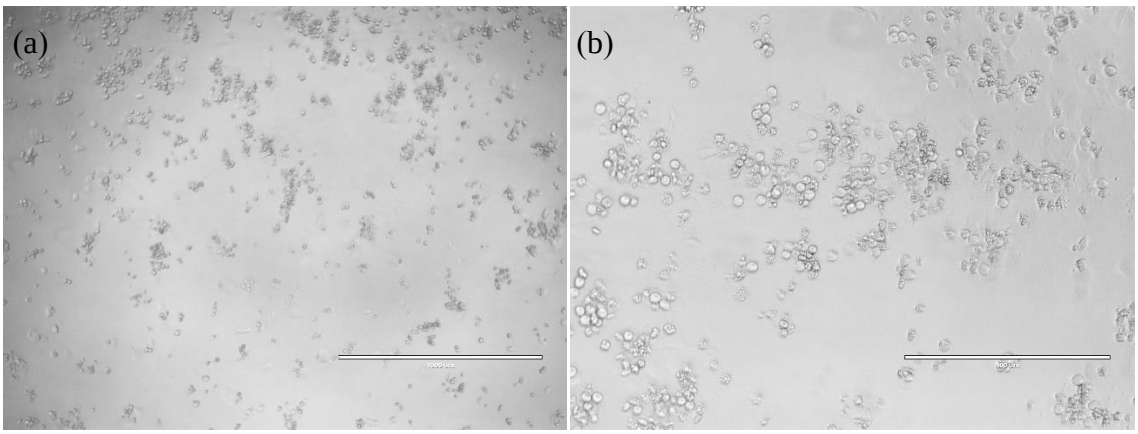


PTX 3.1 µg/ml



PTX 6.3 µg/ml



PTX 12.5 µg/ml

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Appendix C

Cream preparation and finished cream properties

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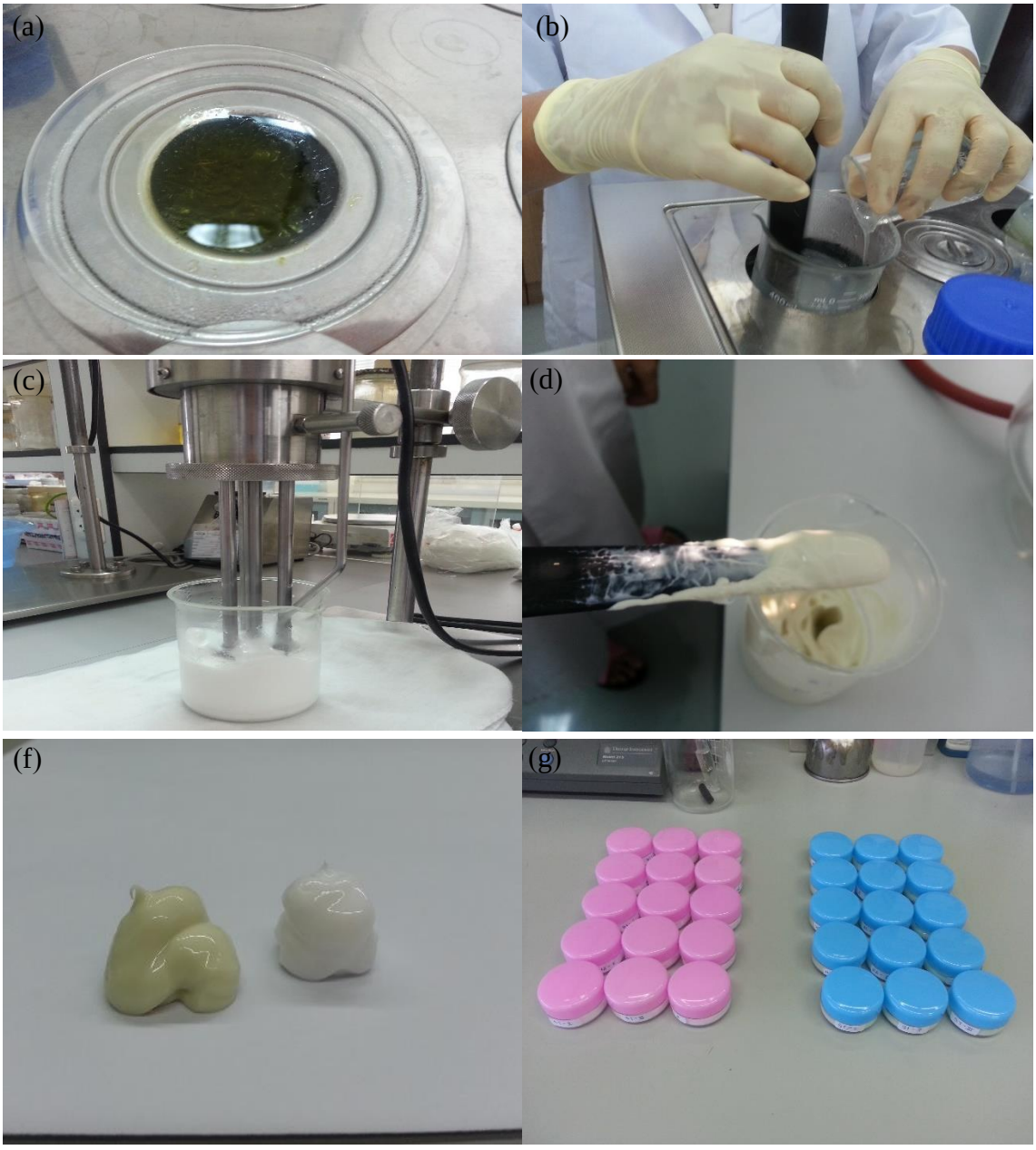
Appendix C-1 Dissolving EMLM extract with propanediol (a), oil phase added into water phase (b), combining phases with homogenizer (c), cream after homogenization and EMLM adding (d), finished cream base and EMLM (e), and packaging of finished cream, which was packed as 5 g each (f).



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Appendix C-2 Speciation of prototypic cream after preparation. All properties were proved by TM Cosme Science Company Limited.

Properties	Speciation	
	Base cream	EMLM cream
Color	White	Yellow
Odor	Typical	Typical
pH	4.5-5.5	4.4-5.0
Viscosity (appearance)	Very viscous	Very viscous
Total plate count (TPC) test	<10	<10
Yeast and Mold test	Absent	Absent
Yield percentage	95-100	95-100
Viscosity value	30,000-35,000 (#4 S6)	30,000-35,000 (#4 S6)

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Appendix D

Statistical analysis

Appendix D-1 One way ANOVA analysis for moisture removal in terms of percentage.

ANOVA

Moisture removal

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.623	8	.453	4.351	.005
Within Groups	1.873	18	.104		
Total	5.496	26			

Scheffe

Processes	N	Subset for alpha = .05	
		1	2
FY	3	92.0333	
FD	3	92.2333	92.2333
FM	3	92.6333	92.6333
MY	3	92.7333	92.7333
MD	3	92.8333	92.8333
OY	3	92.8333	92.8333
OD	3	92.9000	92.9000
MM	3	93.1333	93.1333
OM	3		93.2333
Sig.		.081	.143

Appendix D-2 One way ANOVA analysis for lightness color.

ANOVA

Lightness

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1188.018	2	594.009	3818629.500	<.0001
Within Groups	.001	6	.000		
Total	1188.019	8			

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Appendix D-2 (cont) One way ANOVA analysis for Lightness color.

Scheffe

Drying process	N	Subset for alpha = .05		
		1	2	3
Oven	3	37.1667		
Microwave	3		44.7533	
Freeze dry	3			64.4300
Sig.		1.000	1.000	1.000

Appendix D-3 One way ANOVA analysis for red color.

ANOVA

Lightness

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1188.018	2	594.009	3818629.500	<.000
Within Groups	.001	6	.000		
Total	1188.019	8			

Scheffe

Drying process	N	Subset for alpha = .05		
		1	2	3
Oven	3	37.1667		
Microwave	3		44.7533	
Freeze dry	3			64.4300
Sig.		1.000	1.000	1.000

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Appendix D-4 One way ANOVA analysis for yellow color.

ANOVA

Yellow

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	74.153	2	37.077	370767.000	<.0001
Within Groups	.001	6	.000		
Total	74.154	8			

Scheffe

Drying process	N	Subset for alpha = .05		
		1	2	3
Oven	3	18.1700		
Microwave	3		22.7500	
Freeze dry	3			25.0800
Sig.		1.000	1.000	1.000

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Appendix D-5 One way ANOVA analysis for total phenolic content (TPC) in terms of μmol CAE/ g dry weight.

ANOVA

TPC content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	61749.315	26	2374.974	5870.835	<.0001
Within Groups	21.845	54	.405		
Total	61771.160	80			

Scheffe

Method preparation	N	Subset for alpha = .05														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
OYET	3	3.1														
OYEA	3	3.7														
ODET	3	4.2														
ODEA	3	5.3	5.3													
OMET	3		8.3	8.3												
OMEA	3			9.2	9.2											
MDET	3				12.4	12.4										
MYET	3					12.97										
MYEA	3						16.5									
FDET	3							20.8								

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Appendix D-5 (cont.) One way ANOVA analysis for total phenolic content (TPC) in term of μmol CAE/ g dry weight.

FDEA	3							20.9							
FYET	3							20.9							
FYEA	3							21.3	21.3						
MDEA	3							21.7	21.7						
FMEA	3							21.7	21.7						
MMET	3							21.9	21.9						
FMET	3								24.8						
ODMD	3									28.5					
OYMD	3									28.8					
OMMD	3									30.6	30.6				
MMEA	3										33.5				
FMMD	3											48.9			
FDMD	3												72.9		
MYMD	3													79.4	
FYMD	3													80.1	
MDMD	3														88.2
MMMD	3														101.7
Sig.		.860	.242	1.000	.113	1.000	1.000	1.000	.051	.878	.272	1.000	1.000	1.000	1.000	1.000

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Appendix D-6 One way ANOVA analysis for total phenolic content (TPC) in terms of μmol CAE/ g dry weight.

ANOVA

TPC content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	44611.905	8	5576.488	2916.946	<.0001
Within Groups	34.412	18	1.912		
Total	44646.317	26			

Scheffe

Process	N	Subset for alpha = .05						
		1	2	3	4	5	6	7
OY	3	35.77						
OD	3	38.14						
OM	3		48.27					
FM	3			95.59				
MY	3				108.99			
FD	3					114.78		
FY	3						122.40	
MD	3						122.49	
MM	3							157.18
Sig.		.80	1.00	1.00	1.00	1.00	1.00	1.0

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Appendix D-7 One way ANOVA analysis for total phenolic content (TPC) in terms of μmol CAE/ g crude extract.

ANOVA

TPC content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12.394	8	1.549	1779.943	<.0001
Within Groups	.016	18	.001		
Total	12.409	26			

Scheffe

Process	N	Subset for alpha = .05					
		1	2	3	4	5	6
OY	3	.6300					
OD	3	.7100					
OM	3		.8800				
MY	3			1.7500			
FM	3			1.7700			
MD	3				1.9567		
FD	3				2.0633	2.0633	
FY	3					2.1367	
MM	3						2.6967
Sig.		.270	1.000	.999	.054	.375	1.000

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Appendix D-8 One way ANOVA analysis for total phenolic content (TPC) in terms of μmol CAE/ g dry weight.

ANOVA

TPC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13081.934	2	6540.967	23.211	.001
Within Groups	1690.805	6	281.801		
Total	14772.739	8			

Scheffe

drying method	N	Subset for alpha = .05	
		1	2
Oven	3	40.7267	
Freeze dry	3		110.1067
Microwave	3		129.5533
Sig.		1.000	.420

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Appendix D-9 One way ANOVA analysis for total flavonoid content (TFC) in terms of μmol EPE/ g dry weight.

ANOVA

TFC content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	22773.458	26	875.902	2375.695	<.0001
Within Groups	19.909	54	.369		
Total	22793.367	80			

Scheffe

Method preparation	N	Subset for alpha = .05												
		1	2	3	4	5	6	7	8	9	10	11	12	13
OYET	3	6.44												
ODET	3	6.66												
OYEA	3	6.82												
OMET	3	8.06												
OMEA	3	8.54	8.54											
ODEA	3	9.03	9.03											
ODMD	3		11.49	11.49										
OMMD	3		11.71	11.71										

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Appendix D-9 (cont.) One way ANOVA analysis for total flavonoid content (TFC) in terms of μmol ECE/ g dry weight.

MDET	3			13.72	13.72								
MYET	3			14.31	14.31								
FMEA	3				16.30	16.30							
FYET	3				16.68	16.68							
MMET	3				16.79	16.79							
MYEA	3					18.24	18.24						
FDEA	3					18.70	18.70						
FYEA	3					18.89	18.89						
FDET	3						20.10	20.10					
FMET	3							22.33	22.33				
MDEA	3							22.93	22.93				
MMEA	3								25.24				
FMMD	3									31.74			
MDMD	3										44.35		
MYMD	3											50.49	
FDMD	3												53.93
FYMD	3												56.20
MMMD	3												68.81
Sig.		.431	.083	.247	.114	.431	.954	.242	.190	1.000	1.000	1.000	.716	1.000

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Appendix D-10 One way ANOVA analysis for total flavonoid content (TFC) in terms of μmol ECE/ g dry weight.

ANOVA

TPC content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	25212.726	8	3151.591	5414.829	<.0001
Within Groups	10.477	18	.582		
Total	25223.202	26			

Scheffe

Process	N	Subset for alpha = .05				
		1	2	3	4	5
OY	3	26.1700				
OD	3	27.1900				
OM	3	28.3233				
FM	3		70.3967			
MD	3			81.0033		
MY	3			83.0533		
FY	3				91.7800	
FD	3				92.7400	
MM	3					110.8467
Sig.		.227	1.000	.281	.958	1.000

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Appendix D-11 One way ANOVA analysis for total flavonoid content (TFC) in terms of μmol ECE/ g crude extract.

ANOVA

TFC content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.704	8	.713	1218.403	<0.0001
Within Groups	.011	18	.001		
Total	5.714	26			

Scheffe

Process	N	Subset for alpha = .05					
		1	2	3	4	5	6
OM	3	.6933					
OY	3		.8633				
OD	3		.9367				
FM	3			1.3567			
MY	3				1.7133		
FY	3				1.7233		
MD	3					1.8167	
FD	3					1.8200	
MM	3						1.9967
Sig.		1.000	.161	1.000	1.000	1.000	1.000

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Appendix D-12 One way ANOVA analysis for total flavonoid content (TFC) in terms of μmol ECE/ g dry weight.

ANOVA

TFC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7023.906	2	3511.953	26.239	.001
Within Groups	803.059	6	133.843		
Total	7826.965	8			

Scheffe

Drying method	N	Subset for alpha = .05	
		1	2
Oven	3	27.2267	
Freeze dry	3		79.4433
Microwave	3		91.6367
Sig.		1.000	.479

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Appendix D-13 One way ANOVA analysis for crude content in terms of mg/ g dry weight.

ANOVA

Crude content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	281403.906	26	10823.227	6449.151	<.0001
Within Groups	90.625	54	1.678		
Total	281494.531	80			

Scheffe

Method preparation	N	Subset for alpha = .05												
		1	2	3	4	5	6	7	8	9	10	11	12	13
OYEA	3	15.2												
ODEA	3	15.8	15.8											
OYET	3	20.8	20.8	20.8										
MYEA	3		22.2	22.2	22.2									
MMET	3		22.2	22.2	22.2									
MDET	3		22.5	22.5	22.5									
ODET	3			22.9	22.9									

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Appendix D-13 (cont.) One way ANOVA analysis for crude content in terms of mg/ g dry weight.

MYET	3			23.9	23.9	23.9							
MDEA	3			23.9	23.9	23.9							
FDEA	3			24.1	24.1	24.1							
OMEA	3			24.5	24.5	24.5							
FMEA	3			26.2	26.2	26.2	26.2						
FDET	3				28.7	28.7	28.7						
MMEA	3					30.6	30.6						
OMET	3						31.6	31.6					
FYET	3						32.7	32.7					
FMET	3							38.3					
FMMD	3								110.2				
OYMD	3									124.7			
OMMD	3									128.3			
FYMD	3										136.2		
FDMD	3											153.7	
ODMD	3											155.4	
MMMD	3												164.7
MYMD	3												170.2
MDMD	3												177.7
Sig.		.387	.094	.474	.131	.094	.131	.094	1.000	.989	1.000	1.000	.474	1.000

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Appendix D-14 One way ANOVA analysis for crude content in terms of mg/ g dry weight.

ANOVA

Crude content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9700.068	8	1212.509	217.576	<.0001
Within Groups	100.311	18	5.573		
Total	9800.379	26			

Scheffe

Process	N	Subset for alpha = .05				
		1	2	3	4	5
OY	3	160.8333				
OM	3		184.5867			
FM	3		186.2533	186.2533		
FY	3		192.5033	192.5033		
OD	3			194.1700		
FD	3				206.6700	
MY	3					216.4633
MM	3					217.7100
MD	3					224.1700
Sig.		1.000	.090	.090	1.000	.106

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Appendix D-15 One way ANOVA analysis for crude content in terms of mg/ g dry eight.

ANOVA

Crude content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2618.180	2	1309.090	1643.209	<.0001
Within Groups	4.780	6	.797		
Total	2622.960	8			

Scheffe

Drying method	N	Subset for alpha = .05		
		1	2	3
Freeze dry	3	133.4000		
Oven	3		136.2000	
Microwave	3			170.9000
Sig.		1.000	1.000	1.000

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Appendix D-16 One way ANOVA analysis for IC_{50} of free radical scavenging activity (FRSA) in terms of mg crude/ ml reaction.

ANOVA

Antioxidant activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.183	26	.122	1604.572	<.0001
Within Groups	.004	54	.000		
Total	3.188	80			

Scheffe

Method preparation	N	Subset for alpha = .05							
		1	2	3	4	5	6	7	8
MMMD	3	.0466							
MYMD	3	.0532	.0532						
MDMD	3	.0543	.0543						
FYMD	3	.0714	.0714	.0714					
MDEA	3	.0845	.0845	.0845					
MDET	3	.0855	.0855	.0855					
FMMD	3	.0858	.0858	.0858					
MMEA	3	.0883	.0883	.0883					
MYEA	3	.0892	.0892	.0892					
FYEA	3	.0897	.0897	.0897					
MMET	3	.0899	.0899	.0899					
MYET	3	.0906	.0906	.0906					
FDET	3		.0948	.0948					
FDMD	3			.1034					
FMEA	3			.1047					
FYET	3			.1073					
FMET	3			.1126					
FDEA	3				.1721				
OMEA	3					.3737			

Appendix D-16 (cont.) One way ANOVA analysis for IC_{50} of free radical scavenging activity (FRSA) in terms of mg crude/ ml reaction.

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ODMD	3						.4222		
OYMD	3						.4378		
OMMD	3						.4493		
OMET	3							.5089	
ODEA	3							.5283	
ODET	3								.5801
OYEA	3								.5854
OYET	3								.5941
Sig.		.118	.199	.215	1.000	1.000	.948	1.000	1.000

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Appendix D-17 One way ANOVA analysis for total phenolic content (TPC) in terms of μmol CAE/ g dry weight.

ANOVA

TPC content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2883.932	3	961.311	35.729	<.0001
Within Groups	215.245	8	26.906		
Total	3099.177	11			

Scheffe

Ethanol concentration	N	Subset for alpha = .05	
		1	2
100% EtOH	3	27.0267	
25% EtOH	3		59.1733
75% EtOH	3		60.2767
50% EtOH	3		67.0067
Sig.		1.000	.390

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Appendix D-18 One way ANOVA analysis for total flavonoid content (TFC) in terms of μmol ECE/ g dry weight.

ANOVA

TFC content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10375.545	3	3458.515	124.858	<.0001
Within Groups	221.597	8	27.700		
Total	10597.142	11			

Scheffe

Ethanol concentration	N	Subset for alpha = .05		
		1	2	3
100% EtOH	3	20.1833		
25% EtOH	3		70.4400	
75% EtOH	3			89.2633
50% EtOH	3			94.7533
Sig.		1.000	1.000	.666

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Appendix D-19 One way ANOVA analysis for crude content in terms of mg crude/ g dry weight.

ANOVA

Crude content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.032	3	.011	76.392	<.0001
Within Groups	.001	8	.000		
Total	.034	11			

Scheffe

Ethanol concentration	N	Subset for alpha = .05		
		1	2	3
100% EtOH	3	.0400		
75% EtOH	3		.0867	
50% EtOH	3		.0900	
25% EtOH	3			.1833
Sig.		1.000	.989	1.000

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Appendix D-20 One way ANOVA analysis for IC_{50} of free radical scavenging activity (FRSA) in terms of mg crude/ ml reaction.

ANOVA

IC_{50} of FRSA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1163.291	3	387.764	13.379	.002
Within Groups	231.863	8	28.983		
Total	1395.154	11			

Scheffe

Ethanol conc	N	Subset for alpha = .05	
		1	2
50% EtOH	3	76.0660	
75% EtOH	3	82.5896	
25% EtOH	3	83.6575	
100% EtOH	3		102.4962
Sig.		.443	1.000

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Appendix D-21 One way ANOVA analysis for chlorogenic acid content (CGA) in terms of mg/ g crude extract.

ANOVA

CGA content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	272.866	3	90.955	520.490	<.0001
Within Groups	1.398	8	.175		
Total	274.264	11			

Scheffe

Ethanol concentration	N	Subset for alpha = .05			
		1	2	3	4
100% EtOH	3	5.6733			
25% EtOH	3		7.4860		
50% EtOH	3			15.0373	
75% EtOH	3				16.8487
Sig.		1.000	1.000	1.000	1.000

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Appendix D-22 One way ANOVA analysis for caffeic acid content (CA) in terms of mg/g crude extract.

ANOVA

CA content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	18.852	3	6.284	478.464	<.0001
Within Groups	.105	8	.013		
Total	18.957	11			

Scheffe

Ethanol concentration	N	Subset for alpha = .05		
		1	2	3
100% EtOH	3	1.0120		
75% EtOH	3	1.3333		
50% EtOH	3		2.2227	
25% EtOH	3			4.2300
Sig.		.054	1.000	1.000

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Appendix D-23 One way ANOVA analysis for rutin content (RUT) in terms of mg/ g crude extract.

ANOVA

RUT content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	124.828	2	62.414	288.908	<.0001
Within Groups	1.296	6	.216		
Total	126.124	8			

Scheffe

Ethanol concentration	N	Subset for alpha = .05		
		1	2	3
25% EtOH	3	7.1580		
50% EtOH	3		8.6880	
75% EtOH	3			15.7113
Sig.		1.000	1.000	1.000

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Appendix D-24 One way ANOVA analysis for IC_{50} of cell cytotoxicity on non- TNF-α stimulated HaCaT cells in terms of μg/ml.

ANOVA

IC_{50}

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	43558146.481	5	8711629.296	258.150	<.0001
Within Groups	404956.914	12	33746.409		
Total	43963103.395	17			

Duncan

compound	N	Subset for alpha = .05					
		1	2	3	4	5	6
PTX	3	1.8333					
CA	3		60.1900				
CGA	3			284.5000			
EMLM	3				744.0233		
PCA	3					1497.9933	
RUT	3						2840.7567
Sig.		1.000	1.000	1.000	1.000	1.000	1.000

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Appendix D-25 One way ANOVA analysis for IC_{50} of cell cytotoxicity on TNF-α stimulated HaCaT cells in terms of μg/ml.

ANOVA

IC_{50}

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	43558146.481	5	8711629.296	258.150	<.0001
Within Groups	404956.914	12	33746.409		
Total	43963103.395	17			

Duncan

compound	N	Subset for alpha = .05					
		1	2	3	4	5	6
PTX	3	2.6933					
CA	3		36.1833				
CGA	3			180.6100			
EMLM	3				680.7967		
PCA	3					1682.7833	
RUT	3						4393.4133
Sig.		1.000	1.000	1.000	1.000	1.000	1.000

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Appendix D-26 One way ANOVA analysis for interleukin 8 (IL-8) on TNF-α stimulated HaCaT cells in terms of pg/ml.

ANOVA

IL-8 content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	562444.488	13	43264.961	59.538	<.0001
Within Groups	20346.953	28	726.677		
Total	582791.441	41			

Duncan

Concentration	N	Subset for alpha = .05						
		1	2	3	4	5	6	7
Control non TNFα	3	10.16						
CUR	3	14.33						
CA60	3		87.16					
CGA280	3		90.13					
RUT1500	3		93.66					
RUT750	3		115.16	115.16				
EMLM375	3		133.76	133.76				
EMLM750	3			148.80				
PCA2800	3				201.20			
CA30	3					254.60		
CGA140	3					284.83	284.83	
Control TNFα DMSO	3						322.90	322.90
Control TNFα nonDMSO	3							342.80
PCA1400	3							369.70
Sig.		.851	.067	.159	1.000	.180	.095	.053

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Appendix D-27 One way ANOVA analysis for Tumor necrosis factor-inducible gene 6 protein (TSG-6) on TNF-α stimulated HaCaT cells in terms of pg/ml.

ANOVA

TSG-6 content

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	697601.732	13	53661.672	6.464	<.0001
Within Groups	232440.619	28	8301.451		
Total	930042.351	41			

Duncan

Concentration	N	Subset for alpha = .05					
		1	2	3	4	5	6
Control non TNFα	3	-45.37					
CUR	3	73.30	73.303				
EMLM375	3		137.96	137.96			
PCA2800	3		139.03	139.03			
CGA140	3		168.84	168.84	168.84		
EMLM750	3		220.64	220.64	220.64	220.64	
CA60	3		234.80	234.80	234.80	234.80	
PCA1400	3			249.19	249.19	249.19	249.19
CA30	3			270.03	270.03	270.03	270.03
CGA280	3				321.74	321.74	321.74
RUT750	3					372.37	372.37
Control TNFα DMSO	3					376.10	376.10
RUT1500	3					394.77	394.77
Control TNFα nonDMSO	3						412.20
Sig.		.122	.064	.133	.079	.051	.065

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BIOGRAPHY

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2016	Newton Fund cofounded with Thailand Research Fund (TRF) in the research topic "Anti-inflammatory activity of *Gynura pseudochina* (L.) DC. leaf extracts and development of prototypic cream for psoriatic alleviation"

Research output

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