การพัฒนาวิธีวิเคราะห์ใหม่เพื่อหาปริมาณเอเชียติโคไซด์ในบัวบก

นายอริยะ ไชยสวัสดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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ปีการศึกษา 2555

DEVELOPMENT OF A NEW ANALYTICAL METHOD FOR DETERMINATION OF ASIATICOSIDE CONTENT IN *CENTELLA ASIATICA*

Mr. Ariya Chaisawadi

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacognosy
Department of Pharmacognosy and Pharmaceutical Botany
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Thesis Title	DEVELOPMENT OF A NEW ANALYTICAL METHOD FOR DETERMINATION OF ASIATICOSIDE CONTENT IN *CENTELLA ASIATICA*
By	Mr. Ariya Chaisawadi
Field of Study	Pharmacognosy
Thesis Advisor	Associate Professor Wanchai De-Eknamkul, Ph.D.

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อริยะ ไชยสวัสดิ์ : การพัฒนาวิธีวิเคราะห์ใหม่เพื่อหาปริมาณเอเชียติโคไซด์ในบัวบก (DEVELOPMENT OF A NEW ANALYTICAL METHOD FOR DETERMINATION OF ASIATICOSIDE CONTENT IN *CENTELLA ASIATICA*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. วันชัย ดีเอกนามกูล, 74 หน้า.

เอเชียติโคไซด์เป็นสารออกฤทธิ์สำคัญในกลุ่มไตรเทอร์ปีนอยด์ไกลโคไซด์ที่พบในบัวบก เนื่องจากโครงสร้างของสารเอเชียติโคไซด์ขาดหมู่ฟังก์ชันที่ดูดกลืนแสงได้จึงทำให้สารนี้มีการดูดกลืนแสงในช่วงความยาวคลื่นต่ำ ($\lambda \leq 200$ นาโนเมตร) เป็นผลให้การวัดปริมาณสารชนิดนี้ด้วยเทคนิค UV-Vis HPLC ซึ่งเป็นวิธีมาตรฐานเป็นไปได้ยาก ในการศึกษาครั้งนี้จึงได้พัฒนาเทคนิค TLC-Densitometry ขึ้นเพื่อวิเคราะห์ปริมาณสารกลุ่มไตรเทอร์ปีนอยด์ไกลโคไซด์ในสารสกัดบัวบกและผลิตภัณฑ์บัวบกได้อย่างรวดเร็วและมีประสิทธิภาพ โดยเปลี่ยนโครงสร้างของสารเอเชียติโคไซด์ด้วยการทำปฏิกิริยากับ 2-naphthol บนแผ่นซิลิกาเจล เกิดเป็นสารที่สามารถดูดกลืนแสงได้สูงสุดที่ความยาวคลื่น 530 นาโนเมตร ซึ่งสามารถวิเคราะห์ได้ด้วยเทคนิค TLC-densitometry การศึกษานี้ได้เตรียมตัวอย่างสารสกัดหยาบของบัวบกด้วย 80 % (v/v) เมทานอลโดยเทคนิคการสกัดด้วยคลื่นอัลตราโซนิค จากนั้นนำสารสกัดที่ได้มาแยกสารเอเชียติโคไซด์บนแผ่นซิลิกาเจล (silica gel 60 F_{254}) ขนาด10x20 เซนติเมตรด้วยตัวทำละลายผสมของคลอโรฟอร์ม เมทานอลและน้ำในอัตราส่วน 30:15:1.2 โดยปริมาตรจากนั้นนำแผ่น TLC ดังกล่าวไปจุ่มลงในสารละลายกรดของ 2-naphthol อย่างรวดเร็วแล้วให้ความร้อนที่อุณหภูมิ 120 องศาเซลเซียสเป็นเวลา 5 นาที และนำมาวัดการดูดกลืนแสงด้วยเครื่อง TLC-densitometer ที่ความยาวคลื่น 530 นาโนเมตร

เทคนิคที่พัฒนาขึ้นใหม่นี้มีความถูกต้องและความไวในการวิเคราะห์สูง อีกทั้งยังสามารถเทียบเคียงได้กับเทคนิค HPLC ซึ่งเป็นเทคนิคมาตรฐาน โดยมีค่าความเป็นเชิงเส้นในช่วง 100-1000 นาโนกรัมมากกว่า 0.99 ค่าเปอร์เซ็นต์คืนกลับอยู่ในช่วง 98.2–104.3 ค่าเปอร์เซ็นต์ความเบี่ยงเบนมาตรฐานสัมพัทธ์อยู่ในช่วง 1.15-1.9 และเมื่อเปรียบเทียบผลการวิเคราะห์ปริมาณสารสารเอเชียติโคไซด์กับเทคนิค HPLCแล้วพบว่ามีปริมาณสารใกล้เคียงกันในช่วง 99%-101% เทคนิค TLC-densitometry ที่พัฒนาขึ้นใหม่นี้มีความสะดวกรวดเร็วและมีความถูกต้องแม่นยำสูง โดยสามารถวิเคราะห์ตัวอย่าง 18ตัวอย่างพร้อมกันภายในเวลาไม่เกิน 15 นาที

ภาควิชา เภสัชเวทและเภสัชพฤกษศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชเวท ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 .....

5276611633 : MAJOR PHARMACOGNOSY
KEYWORDS : ASIATICOSIDE / TRITERPENOID GLYCOSIDES/ *CENTELLA ASIATICA*/ 2-NAPHTHOL/ TLC-DENSITOMETRIC METHOD

ARIYA CHAISAWADI: DEVELOPMENT OF A NEW ANALYTICAL METHOD FOR DETERMINATION OF ASIATICOSIDE CONTENT IN *CENTELLA ASIATICA*. ADVISOR: ASSOC. PROF. WANCHAI DE-EKNAMKUL, Ph.D., 74 pp.

Asiaticoside, an active triterpene glycoside in *Centella asiatica* L., has weak UV absorption due to the lack of chromophore in its structure, and this causes an ineffective asiaticoside analysis by standard UV-Vis HPLC. In this study, a simple TLC-densitometic method was developed for rapid and effective analysis of the triterpenoid glycosides in *C. asiatica* crude extracts and its commercial products. This new analytical method is based on the derivatization of asiaticoside structure with 2-naphthol acid reagent on a TLC silica gel plate to form a chromophore-containing compound that can be detected by UV-visible based TLC-densitometric analysis (λ_{max} at 530nm). In practice, crude *C. asiatica* extracts were prepared from the plant materials under sonication with 80% (v/v) methanol. The analysis was then performed on a silica gel 60 F_{254} TLC plate (20x10 cm.) with chloroform/methanol/water (30:15:1.2 volume ratio) system as the mobile phase. Densitometric analysis is performed at 530 nm after post-chromatographic derivatization with 2-naphthol sulfuric acid reagent (brownish band for glycoside). This new method showed good accuracy comparable to the UV-HPLC method but is more sensitive and effective. The linear range for the analysis of asiaticoside was 100-1000 ng/band ($r^2 \geq 0.99$) with good precision and accuracy (1.15- 1.9 %RSD, 98-104% recovery) and the values of asiaticoside contents determined by the developed TLC method and HPLC method were well correlated in range 99-101%. The developed densitometric TLC appeared to be simple, accurate, precise and fast as eighteen chromatographic runs could be performed simultaneously per plate within 15 minutes.

Department : Pharmacognosy and Pharmaceutical Botany Student's Signature .....

Field of Study : Pharmacognosy Advisor's Signature .....

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LIST OF ABBREVIATIONS AND UNITS

AUC	= Area under curve
AA	= Asiatic acid
AS	= Asiaticoside
CA	= *Centella asiatica*
cm	= Centrimeter (s)
r^2	= Coefficient of determination
CV	= Coefficient of variation
^oC	= Degree Celsius
et al.	= Et alii
g	= Gram (s)
glu	= glucose
HPLC	= High performance liquid chromatography
hr	= Hour
kg	= Kilogram (s)
l	= Liter (s)
m	= Meter (s)
µg	= Microgram (s)
µl	= Microliter (s)
mg	= Milligram (s)
ml	= Milliliter (s)
mm	= Millimeter (s)
min	= Minute (s)
MA	= Madecassic acid
MS	= Madecassoside
Mw	= Molecular weight
ng	= Nanogram (s)
nm	= Nanometer (s)
NO.	= Number
SD	= Standard deviation
%	= Percent (part per 100), percentage

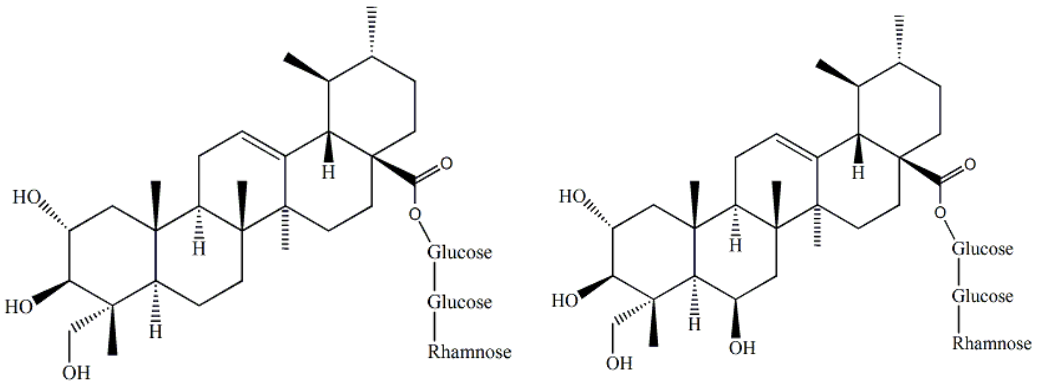
%R	= Percent recovery
rha	= Rhamnose
RSD	= Relative standard deviation
TECA	= Titrated extract of *Centella asiatica*
TLC	= Thin layer chromatography
UV	= Ultraviolet light
v/v	= Volume/volume (concentration)
Vis	= Visible light
λ_{max}	= Wavelength at maximal absorption
w/w	= Weight/weight (concentration)

CHAPTER I

INTRODUCTION

Centella asiatica (Linn.) Urban (CA) is a tropical plant known in various countries as Bua-bok (Thailand), Gotu Kola (India), Asiatic Pennywort and Indian Pennywort. It belongs to the family Apiaceae (Umbelliferae), which can be found in the subtropical and tropical climates of Asia, Africa, North and South America (Brinkhaus *et al*., 1998). According to traditional eastern medicine, CA is widely used to treat various disorders, such as syphilis, hepatitis, diarrhea, stomach ulcers, mental fatigue, epilepsy, fever and asthma (Zheng, 2007). Presently, CA is used as an alternative medicine to treat symptoms of swelling connective tissue such as scleroderma, psoriatic arthritis, and rheumatoid arthritis by European herbalists (Brinkhaus *et al*., 2001).

CA has been reported to contain many compounds, including triterpenoid glycosides (Zhou *et al*., 2011). Asiaticoside (AS), which is a major active compound in CA, is an ursane-type triterpene glycoside (Matsuda *et al*., 2001). It has been reported to have many pharmacological activities, such as wound healing, anti-inflamatory, memory enhancing and immunomodulatory activity. (Zheng, 2007) Because of its potential pharmacological activities and its safety as herbal drinks, CA has been widely used as food supplement, herbal medicine, cosmetic and other finished products. (Uvarajan *et al*., 2012)



Asiaticoside (AS) Madecassoside (MS)

Figure 1 Two major triterpenoid glycosides in *Centella asiatica*

In general, however, scientific evidence on the efficacy of herbs and their active constituents is still limited in Thailand, and in addition, consistency on the quantity of the active principles is inadequate. This may be affected by several factors, such as content variation of biologically active compounds from different locations, time of collection, extraction process, and quality control (Thomas *et al*., 2010). Generally, in the process of herbal drug development, the active compound(s) contained in a herb should be identified and quantitated. Consistency of the presence of the active compounds should also be monitored and controlled during the manufacturing process of the herbal products (Wittawat, 2011). These problems are also happening to the case of CA which contains AS as its active constituent.

In order to identify and quantify AS in the raw materials or products of CA, a simple analytical method should be developed. Chromatographic methods are usually used in separation, purification and quantitation of the natural product extracts. High performance liquid chromatography (HPLC) is the most usable and reliable technique to do the analysis but its time consuming. The method of thin layer chromatography (TLC), on the other hand, is simpler and faster.

For the triterpenoids, various techniques, including titrimetric, colorimetric, TLC and HPLC methods have been developed to determine the triterpenoid glycosides and their aglycones (Oleszek *et al*., 2002). Among these, HPLC is still the only standard analytical method used for the analysis of AS content but it is not so efficient owing to the weak UV absorption of asiaticoside, which was due to the lack of a chromophore in its structure (Oleszek *et al*., 2002). Therefore, the purpose of this research work is to develop a new TLC densitometric technique in order to perform both quantitative and qualitative analysis of AS in various samples. In this study, derivatization of AS with 2-naphthol directly on a TLC silica gel plate was carried out for detection and determination of the triterpenoid glycosides in CA crude extracts using the UV–visible measurement. The developed TLC-densitometric method appeared to be simple, accurate, precise and fast, with at least eighteen samples could be performed simultaneously in a single run per plate within 15 minutes. The newly developed technique can be applied for evaluating the quality of raw materials and finished products of CA as reported here.

CHAPTER II

LITERATURE REVIEW

Figure 2 *Centella asiatica* (Linn.) Urban

1. Centella asiatica: Botanical Description

Centella asiatica (L.) Urban is a cultivated, faintly aromatic, perennial herb in Apiaceae or Umbelliferae family. It is commonly known as Bua-bok (Thai), Gotu Kola (India), Asiatic Pennywort (common name), Mandukparni (Sanskrit), Indian Pennywort (common name), and etc. This plant is usually seen in the subtropical and tropical climates of Asia, Africa, North and South America such as Thailand, Sri Lanka, India etc. (Brinkhaus *et al*., 1998; Auld and Medd, 1992). The plant is a small creeping herb with up to 2 m. long slender stem. It has 2.5 to 6.0 cm thin, brownish-grey roots. Its leaves are 2-5 cm in diameter, kidney shaped with long petioles, arising from the stem nodes (Jamil

et al., 2007). The flowers occurring in July-September are fascicled umbels with 2-5 pale violet flowers (Koh *et al*., 2009). Each umbel has small oval fruits, enclosed within hard pericarp. The aerial part can be harvested all year. (Cambie and Ash, 1994)

2. Chemical Constituents *of Centella asiatica*

C. asiatica is a source of triterpenoids. These triterpenoids usually found in the ursane (R_6, R_7 = methyl) or oleanane (R_7, R_8 = methyl) types which are summarized in Table 1 and Figure 2. Many chemical constituents other than triterpenoids have been reported and which are summarized here in Table 2. Most of the phytochemical studies were performed using leaves and the chemical constituents vary depending on the geographical distribution of the plant (Chong and Aziz, 2011).

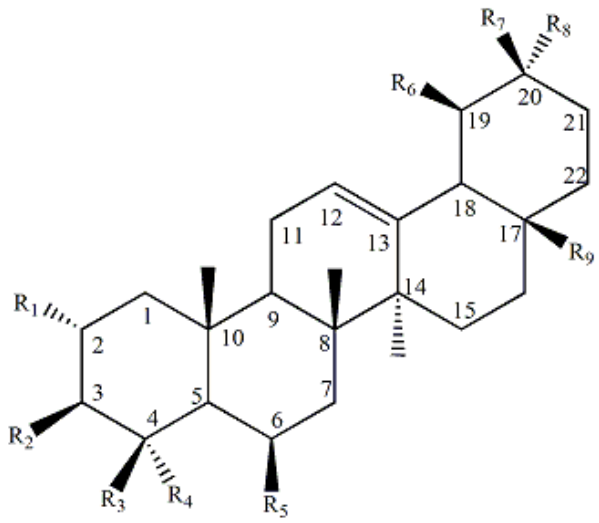
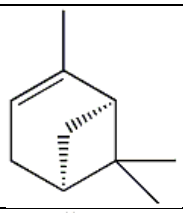
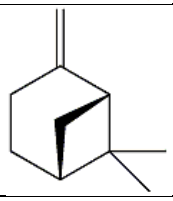
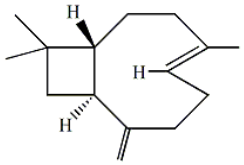
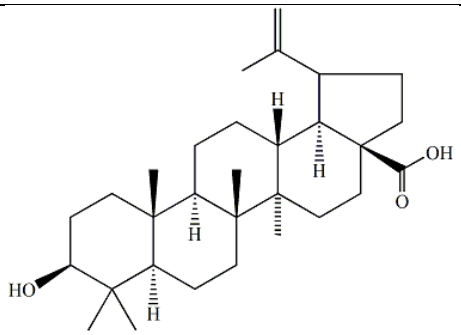
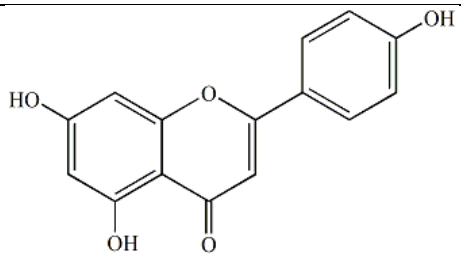


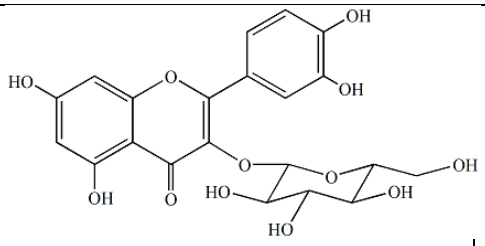
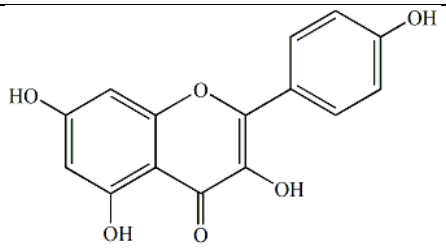
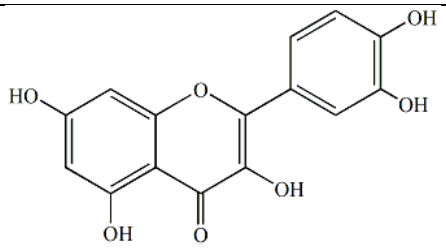
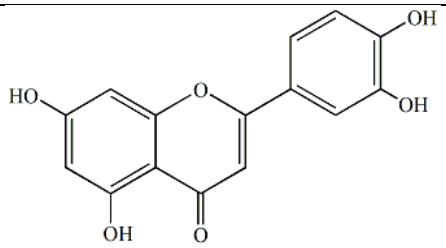
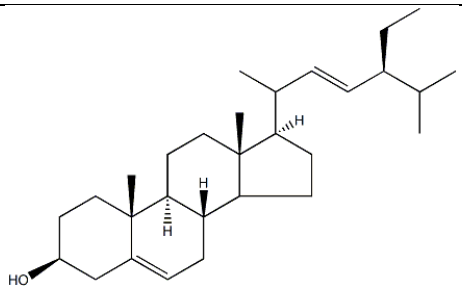
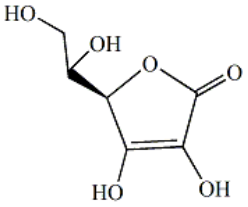
Figure 3 The model of triterpenoids from *C. asiatica*

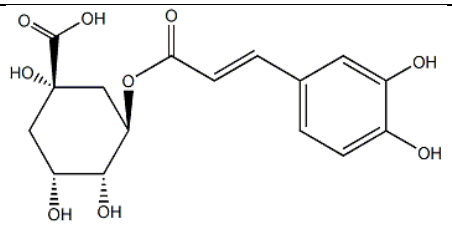
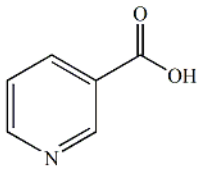
Table 1. Structures of triterpenoids reported in *C. asiatica*

R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R9	Name	Reference
OH	OH	CH_3	CH_2OH	H	CH_3	CH_3	H	COOH	Asiatic acid	Matsuda *et al*., 2001
OH	OH	CH_3	CH_2OH	H	CH_3	CH_3	H	COO-glc-glc-rha	Asiaticoside	Matsuda *et al*., 2001
OH	OH	CH_3	CH_2OH	OH	H	CH_3	CH_3	COO-glc-glc-rha	Asiaticoside B	Schaneberg, 2003
OH	OH	CH_3	CH_2O-Ac	H	CH_3	CH_3	H	COO-glc-glc-rha	Asiaticoside C	Jiang, 2005
OH	OH	CH_3	CH_3	H	CH_3	CH_3	H	COO-glc-glc-rha	Asiaticoside D	
OH	OH	CH_3	CH_2OH	H	CH_3	CH_3	H	COO-glc-glc	Asiaticoside E	
H	OH	CH_3	CH_2OH	H	CH_3	CH_3	H	COO-glc-glc-rha	Asiaticoside F	
OH	OH	CH_3	CH_2OH	OH	CH_3	CH_3	H	CH_2OH	Brahmol	Singh, 1969
OH	OH	CH_3	CH_2OH	OH	CH_3	CH_3	H	COO-glc-glc	Centellasaponin B	Matsuda *et al*., 2001
OH	OH	CH_3	CH_3	OH	CH_3	CH_3	H	COO-glc-glc-rha	Centellasaponin C	
H	OH	H	CH_2OH	OH	CH_3	CH_3	H	COOH	Isothankunic acid	Dutta, 1968
H	OH	H	CH_2OH	OH	CH_3	CH_3	H	COO-glc-glc-rha	Isothankuniside	Singh, 1969
OH	OH	CH_3	CH_2OH	OH	CH_3	CH_3	H	COOH	Madecassic acid	Matsuda *et al*., 2001
OH	OH	CH_3	CH_2OH	OH	CH_3	CH_3	H	COO-glc-glc-rha	Madecassoside	Matsuda *et al*., 2001
OH	OH	CH_3	CH_3	OH	CH_3	CH_3	H	COOH	Madasiatic acid	Matsuda *et al*., 2001
OH	OH	CH_3	CH_2OH	H	CH_3	CH_3	H	$COOCH_3$	Methyl asiatate	Singh, 1969
OH	OH	CH_3	CH_2OH	OH	H	CH_3	CH_3	COOH	Terminolic acid	Schaneberg, 2003

Table 2 Others chemical constituents of *C. asiatica*

Chemical group	Chemical substance	Chemical structure	Reference
Monoterpenoids	α-Pinene ($C_{10}H_{16}$, mw = 136.24)		Zhou *et al*., 2011
	β-Pinene ($C_{10}H_{16}$, mw = 136.24)		Zhou *et al*., 2011
Sesquiterpenoids	β-Caryophyl-lene ($C_{15}H_{24}$, mw = 204.36)		Wogn and Tan, 1994; Zhou *et al*., 2011
Triterpenoids	Betulic acid ($C_{30}H_{48}O_3$, mw = 488.71)		Matsuda *et al*., 2001; Jamil *et al*., 2007; Zhou *et al*., 2011
Flavonoids	Apigenin ($C_{15}H_{10}O_5$, mw = 270.24)		Zhou *et al*., 2011

	Astragalin ($C_{21}H_{20}O_{11}$, mw = 448.37)		Newall *et al*., 1996
	Kaempferol ($C_{15}H_{10}O_6$, mw = 286.24)		Newall *et al*., 1996; Chong NJ and Aziz, 2011;
	Quercetin ($C_{15}H_{10}O_7$, Mw = 302.24)		Newall *et al*., 1996; Zhou *et al*., 2011
	Luteolin ($C_{15}H_{10}O_6$, Mw = 286.24)		Bhandari *et al*., 2007; Zhou *et al*., 2011
Phytosterol	Stigmasterol ($C_{29}H_{48}O$, mw = 412.71)		Ramaswamy *et al*., 1970; Zhou *et al*., 2011
Miscelaneous	Ascorbic acid ($C_6H_8O_6$, mw = 176.13)		Chong and Aziz, 2011; Zhou *et al*., 2011

	Chlorogenic acid ($C_{16}H1_8O_9$, mw = 354.32)		
	Nicotinic acid ($C_6H_5NO_2$, mw = 123.11)		Chong NJ and Aziz, 2011; Zhou *et al*., 2011

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3. Pharmacological Activities

3.1 Wound-Healing and Collagen Enhancing Activity

Wound healing, or cicatrisation, is a process in which the skin or another tissue heals itself after injury. When the wound occurs, the process of wound healing is immediately set in motion. The model of wound healing can be divided into three sequential and overlapping phases for convenient, namely inflammatory, proliferative and remodeling.

A titrated extract of *Centella asiatica* (TECA) is the *C. asiatica* extract which containing Asiatic acid (AA), madecassic acid (MA) and asiaticoside (AS). TECA have been evaluated for their effects in the wound chamber model and characterised by increasing of total protein, dry weight DNA, collagen and uronic acid contents. Peptidic hydroxyproline has also been found to increase, showing an increased remodelling of the collagen matrix in the wound (Maquart *et al*., 1996; Shukla *et al*., 1999). The three purified components of TECA have been shown to be all able to reproduce the effects of the complete drug. The effects of TECA and its individual components have been found to on human foreskin fibroblast monolayer cultures. (Kim *et al*., 1993; Shukla *et al*., 1999)

TECA has been reported to increase the collagen synthesis in a dose-dependent fashion whereas a simultaneous decrease in the specific activity of neosynthesized collagen was observed. AA has been found to be the only component responsible for collagen synthesis stimulation. This effect appears to be independent of the stimulation of collagen synthesis (Tenni *et al*., 1988)

The activity in normal and delayed-type wound healing of AS has been studied using guinea pig punch wounds model. The 0.2% solution of AS was applied topical on punch wounds. The result has been shown that AS solution increased the hydroxyproline, tensile strength, collagen content and improved epithelialisation. Moreover, in delayed type healing studied, the 0.4% solution of AS was applied topical over punch wounds of streptozotocindiabetic rats and has been found to increase hydroxyproline content, tensile strength, collagen content and epithelialisation, thereby facilitating healing. AS has also reported to be orally active at 1 mg/kg dosing and is thought to be the main active constituent of CA. Asaiticoside has been shown to enhanced antioxidant levels at an initial stage of wound healing which can be an important factor in the healing properties of this constituent (Suguna and Sivakumar, 1996). The extract has also shown to protecte skin against radiation injury (Kim et al., 1993). Formulations (ointment, cream and gel) of aqueous extract of CA, when applied topically, twice daily for 24 days on the open wounds in rats was found to increase cellular proliferation and collagen synthesis at the wound site, as evidenced by increase in collagen content and tensile strength (Sunilkumar *et al*., 1998). Alpha CA cream® contains two main components. The first is an extract from the plant *Bulbine frutescens*. This extract has been reported to possesses antibacterial activity and increase hydration under the tape by leaving a layer of fatty vesicles of glycoprotein on the skin surface. The second component is the principal terpenoids extracted from the *Centella asiatica* plant. These include AA, MA, and AS. The most beneficial effect appears to be the stimulation of maturation of the scar by the production of type I collagen and the resulting decrease in the inflammatory reaction and myofibroblast production (Widgerow *et al*., 2000).

CA extract is used effectively in the treatment of keloids, leg ulcers, phlebitis, slow-healing wounds, leprosy, surgical lesions, striae distensae and cellulitis. Although applied frequently to damaged skin, the risk of acquiring contact sensitivity to this plant or its constituents is low. AS, AA and MA have been studied in guinea pigs and found to be very weak sensitizers (Danese *et al*., 1994; Gonzalo and Revenga, 1996).

3.2 Central Nervous System

The alcoholic extract of CA, when given orally to rats and mice treated with phenobarbitone, the result has been shown to significantly prolonged the sleeping time of the animals. In the maximum electroshock-induced convulsion test in rats, it has been found to significantly reduce the duration of individual convulsions. In a behavioral test, it reduces the duration of the immobility phase, sedative, antidepressive and analgesic actions. Intraperitoneal injections of brahmoside and brahminoside have been found to have a CNS-depressant effect in mice and rats (Ramaswamy and Periyasamy, 1970). AA has been patented as a memory enhancer and treatment for dementia (Eun and Lee, 1985).

An extract of *C. asiatica* has been found to increase brain GABA levels (Sairam et al., 2001). In a study on the effects of *C. asiatica* (500 mg capsules of dried *C. asiatica*) on mentally-challenged children compared with placebo, children who took the *C. asiatica* capsuled have been shown significantly improvement in co-operation, memory, concentration, attention , vocabulary and social adjustment (Appa *et al*., 1973). Aqueous extract of *C. asiatica* (200 mg/kg for 14 days) has been shown to have the learning and memory enhancing in both shuttle box and step through paradigms in rats. All doses of aqueous extracts (100, 200 and 300 mg/kg) increase the number of avoidances in shuttle box and prolonged the step through latency in step through apparatus in a dose dependent manner, while only two doses 200 and 300 mg/kg of aqueous extract shows significant increase in the step down latency in the step down apparatus and transfer latency (TL) in

elevated plus maze (Veerendra and Gupta, 2002). A two-compartment passive avoidance task test with rats showed an improvement in 24 hours retention.

Assessment of the turnover of biogenic amines (norepinephrine, dopamine and serotonin) have shown significant reductions of these amines and their metabolites in the brain following oral administration of a fresh juice (1 ml = 0.38 g fresh leaves), at a dose of 0.18 g/kg for 15 days. The decrease of amine levels is correlated to improved learning and memory in rats (Veerendra and Gupta, 2003).

Triterpenoids (active compounds in CA) have been shown to soothe anxiety and boost mental function in mice. A recent study has found that people who took CA are less likely to be started by a novel noise (a potential indicator) than those who take placebo. Although the results of this study are promising, the dose used in this study is extremely high, making it difficult to draw any conclusions about how CA might be used by people with anxiety. CA significantly attenuates the peak acoustic startle response (ASR) amplitude 30 and 60 minutes after treatment. CA has no significant effect on self-rated mood, heart rate, or blood pressure. These preliminary findings have suggested that Gotu Kola has anxiolytic activity in humans as revealed by the ASR (Leung *et al*., 1996).

3.3 Antiulcerogenic Activity

The antiulcerogenic activity of the fresh juice of CA has been studied against ethanol-, aspirin-, cold restraint stress- and pyloric ligation-induced gastric ulcers in rats. When given orally at doses of 200 and 600 mg/kg twice daily for 5 days, the drug shown significant protection against all the above experimental ulcer models. This effect has been thought to be due to the strengthening of mucosal defensive factors. Oral administrations of CA extract (0.05, 0.25 and 0.50 g/kg) before ethanol administration significantly inhibited gastric lesion formation (by 58-82%) and decrease mucosal myeloperoxidase (MPO) activity in a dose-dependent manner. It prevents gastric mucosal lesions by strengthening the mucosal barrier and reducing the damaging effects of free

radicals (Cheng and Koo, 2000). Extract of CA inhibits significantly gastric ulceration induced by cold and restraint stress (CRS) in Charles-Foster rats, Antiulcer activity of plant extracts have been compared with famotidine (H2-antagonist) and sodium valproate (anti-epileptic). The plant extract, farmotidine and sodium valproate showed a dose dependent reduction of gastric ulceration.

CA extracts have been shown the dose dependent activity of increased the brain GABA level. Pretreatment with specific GABA-antagonist (bicuculline methiodide) at the dose level of 0.5 mg/kg intramuscularly (im) was found to reverse the antiulcerogenic activity of both plant extract and sodium valproate. Bicuculline as such does not induce gastric ulceration in normal rats (Chatterjee and Chakraborty, 1996).

3.4 Spasmolytic Activity

Mokkhasmit *et al*. (1971) reported that CA alcoholic extract solution at a concentration of 10 mg/ml showed the spasmolytic activity on isolated ileum of guinea pig.

3.5 Antitubercular Activity

An injection of 0.5 ml of a 4% solution of hydroxyasiaticoside, which is derivative of AS, was given in guinea pigs that inoculated 15 days previously with tubercle bacillus. The results have been show the reduction of the number of tubercular lesions in the liver, lungs, nerve ganglions and spleen, and decreased the volume of the spleen over that of untreated control animals, thereby displaying antitubercular activity. AS has been reported to be active against *Mycobacterium tuberculosis*, *Bacillus leprae* and *Entamoeba histolytica* (Oliver, 1986).

3.6 Antimicrobial Activity

AS solution (10 mg/ml) has been shown the antibacterial activity against both Gram positive and Gram negative bacteria (*Pseudomonas pyocyaneus; Trichoderma mentagrophytes*) (Ray and Majumdar, 1976). The hexane and ethyl acetate extracts of CA was studied using the disc diffusion method by detected the growth inhibition of Gram positive *Bacillus subtilis* and Gram negative *Pseudomonas aeruginosa, P. cichorii* and *Escherichia coli*. (Escop and Phytotherapy, 2003). The liposomal AS was reported to be more sensitive for *Mycobacterium tuberculosis* and *M. leprae* than the free asiatocoside solution (Fugh- Berman, 2003).

3.7 Antiviral Activity

The alcoholic extract and water extract of CA have been shown the antiviral activity against Herpes simplex type II virus (Zheng, 1989). The aqueous extract of CA was reported to have antiviral activity against type 2 Herpes simplex virus (Escop and Phytotherapy, 2003).

3.8 Antilarval Activity

A triterpenoid glycoside in CA (3-O-[α-L-arabinopyranosyl] 2α,3β,6β,23α-tetrahydroxyurs-12-ene-28 oic acid) has been reported for activity against larvae of *Spilarctia oblique* (Shukla *et al*., 2000).

3.9 Immunomodulatory Activity

The alcoholic extract of CA has been shown stimulating effect on the reticuloendothelial system (RES) in mice and an in vitro study of the aqueous extract demonstrated a positive effect on both the classic and alternative pathways of

complement activation (Labadie *et al*., 1989). One study involving 13 female animals with scleroderma found that CA decreased joint pain, skin hardening, and improved finger movement. The usage of madecassol (AS) in tablet, ointment and powered form was found to be efficacious in the treatment of chronic or subchronic systemic scleroderma with limited skin involvement and inprogressive and/or advanced focal scleroderma (Guseva, 1998). Methanolic extract of CA has been reported for increasing the phagocytic index and total WBC in mice (Jayathirtha and Mishra, 2004). The water extract of CA significantly increased the production and proliferation of IL-2 and TNF-α in human peripheral blood mononuclear cells (PBMCs) but ethanol extract had inhibitory effect (Punturee *et al*., 2005). The methanol extract of CA and AA showedinhibitory effect on three major cDNA, which expressed human cytochrome P450 isoforms (Pan *et al*., 2010).

3.10 Venous Insufficiency and Varicose Veins

CA has been found to effective in the treatment of venous insufficiency and has been shown to reduce ankle edema, foot swelling and capillary filtration in rats and to improve microcirculatory parameters (Incandela *et al*., 2001; Cesarone *et al*., 2001).

When blood vessels lose their elasticity, blood pools in the legs and fluid leaks out of the blood vessels, lead to legs swelling (venous insufficiency). In a study of 94 people with venous insufficiency, those who took CA reported a significant improvement in symptoms compared to those who took placebo. In another study of people with varicose veins, ultrasound examination revealed improvements in the vascular tone of those who took CA (Pointel *et al*., 1987). Another double-blind study with 40 patients suffering chronic venous insufficiency found that CA extract (60 mg/kg for 30 days) significantly improved ankle circumference, vascular tone and leg volume compared with baseline (Monteverde *et al*., 1987).

3.11 Cardioprotective Activity

The variation of capillary filtration rate (CFR), ankle circumference (AC), and ankle edema (AE) was evaluated in three groups of patients with venous hypertension (ambulatory venous pressure greater than 42 mmHg) and in a group of healthy subjects before and after treatment for four weeks with Total Triterpenic Fraction of *Centella asiatica* (TTFCA), a venoactive drug acting on the microcirculation and on capillary permeability. Each group was treated with TTFCA in various dose three times per day. Group A (20 patients) was treated with TTFCA 60 mg; Group B (20 patients) was treated with 30 mg; Group C (12 patients) was treated with placebo; and Group D (10 normal subjects) was treated with TTFCA 60 mg in an open study. Capillary filtration rate was observed by venous occlusion plethysmography, ankle edema by a new system called AECT (Ankle edema coin tester). Subjective symptoms of venous hypertension were assessed by an analogue scale line considering four symptoms which are swelling sensation, restless lower extremity, pain and cramps, and tiredness. CFR, AC, and AE were significantly higher in patients in comparison with normal subjects. After four weeks of TTFCA treatment there was a significant decrease of the abnormally increased CFR, AC, and AECT time in patients. This was also greater in the higher dose group. No significant change was observed in the placebo group and in normal subjects treated with TTFCA. Symptoms were also significantly improved in the two groups treated with the active drug according to the dose. (De Sanctis *et al*., 2001). In another double-blind clinical trial involving 87 patients with chronic venous hypertensive microangiopathy, two dosage forms of CATF (30 mg/day and 60 mg/day) were applied for 60 days and no unwanted effects were observed (Cesarone *et al*., 2001; Arpaia *et al*., 1990). The results also confirmed the efficacy of CATF in a dose-dependent manner. The effects of the CATFs on enzymes involved in mucopolysaccharide metabolism supported the hypothesis that the extract acts on basic metabolism in the connective tissue of the vascular wall (Babu *et al*., 1995). The levels of basal serum uronic acid and enzymes involved in mucopolysaccharide metabolism (β-glycuronidase, β-Nacetylglucosaminidase, and arylsulfatase) were elevated in patients with varicose veins,

indicating an increased mucopolysaccharide turnover. After treatment (60 mg/day for three months) the above enzyme levels fell progressively. In a study of people with heart disease and high blood pressure, those who took Abana, the Ayurveda herbal mixture containing CA, experienced a significant reduction in diastolic blood pressure compared to those who took placebo. Further studies are needed to determine CA alone, some other herb in the Ayuvedic minter, or the particular combination of all the herbs in the remedy is responsible for the beneficial effect.

3.12 Radioprotective Activity

Aqueous extract of *C. asiatica* showed radioprotective effect greater than standard drug (Ondansetron) against conditioned taste aversion (behavioural perturbation) induced by ^{60}Co-γ irradiation at low dose 2Gy in male rats (Shobi and Goel, 2001). Administration of 100 mg/kg of body weight of aqueous extract of *C. asiatica*, just 1 hour before irradiation with 8Gy ^{60}Co- γ rays was found to be most radioprotective in mouse (Sharma and Sharma, 2002), and 100mg/kg of body weight of the powdered extract of *C. asiatica* were found to be effective against modified ^{60}Co-γ irradiation induced damage in the mouse liver (Sharma and Sharma, 2005). 70% ethanol extract of *C. asiatica* reduced radiation-induced damage to DNA (Joy and Nair, 2009). MicroRNA (miRNA) expression profiling analysis was used to evaluate the protective effects of *C. asiatica* against ultraviolet B damage in human keratinocytes, and disclosed that miRNAs with altered expression functionally related with cell proliferation and inhibition of apoptosis may prevent the skin damage (An *et al*., 2012).

3.13 Antitumor Properties

A crude extract and partially purified extract of CA have been reported for the cytotoxicity on different tumor cell line models. However, no cytotoxic effects were

detected against normal cell lines. The oral administration of those extracts delayed the progress of solid and ascites tumors in mice (Lin *et al*., 2002).

3.14 Hepatitis

The clinical study in patients with jaundice has been reported the improvement of all patients using TECA. The results showed the improvement of chronic hepatic disorders in 5 of 12 clients after TECA treated (Yen *et al*., 2001).

4 Traditional and Modern Use

The leaves and stems of the CA plant are widely used to treat a variety of illness, particularly in traditional eastern medicine. Historically, CA has been used to treat syphilis, wound healing, hepatitis, stomach ulcers, diarrhea, mental fatigue, epilepsy, fever and asthma. Nowadays, American and European herbalists use CA for disorders that cause connective tissue swelling, such as scleroderma, psoriatic arthritis (arthritis occurring in conjunction with psoriasis), and rheumatoid arthritis. Recent studies confirm the efficacy of some traditional uses and also suggest the possibility of new application for CA, such as lowering high blood pressure, treating varicose veins (pooling of blood in the veins, normally in the legs), enhancing memory and intelligence, soothing anxiety and rapid wound healing (Brinkhaus *et al*., 2001).

5. Overview of the Analytical Method

There are several methods for qualitative and quantitative determination of active constituents of CA. Titrimetric method was conventionally used to determine the triterpene acids (AA and MA) and glycosides (AS and MS). This method can determine only the terpene acids content. For determination of glycosides, sample must be

derivertized by hydrolysis with acids before titration. This method lacks precision and accuracy and also is a non-selective, non-specific technique (Inamdar, 1996).

Presently, HPLC method has become a method of choice for analysis of herbal medicine. Inamdar (1996) determined AA, MA, AS and MS in the crude plant extracts and preparations using C18 column and acetonitrile-water gradient system as mobile phase. Arunya (1997) reported a reversed-phase gradient HPLC for separation of AS, MA and AA in preparations. R.K. Verma (1999) used 1% trifluoroacetic acid: methanol (30:70) as mobile phase for determination of AS in crude extract. For preparation, P. Morganti (1999) reported acetonitrile-water gradient reversed-phase HPLC that was used to determinate AA, MA and AS in transdermal patch. But HPLC method is not so efficient owing to the weak UV absorption of AS, since the compound lacks chromophore in its structure. Moreover, for analysis of natural crude extract, the pre-column clean-up process is still necessary in order to maintain the column (Oleszek *et al*., 2002).

Colorimetric is a method for quantitative and qualitative determination of colored compounds by measuring its absorbance of a specific wavelength of light. A colorimetric method is used to determine the glycosides by reaction with anthrone reagent. Anthrone reagent tests for the presence of glycosides by hydrolyzing polysaccharides to monosaccharide then forming a color compound in the presence of the monosaccharide. The complex will vary in color from green to black depending upon the amount of glycosides present. This method is non-selective, non-specific (Arunya, 1997).

TLC–densitometry, thin-layer chromatography (TLC) method in combination with TLC scanner, is method using thin layer of adsorbent to separate mixture compounds then analyse the quantity and quality of each separated compounds by exploiting their fluorescence under UV irradiation. Nowadays densitometric TLC technique is used for qualitative and quantitative determination of active constituents of crude drug and herbal products. Many protocols were developed to determine various active compounds such as artemisinin (Thongchai, 2002), glycyrrhizin (Singh *et al*., 2009), iridoid-glycoside (Yadev *et al*., 2008), flavonoids (Bhandari *et al*., 2007), alkaloids (Seiber *et al*., 2007) and

carbohydrate (Malinowska *et al*., 2010). For AS determination, densitometric technique was developed by using TLC plate [silica gel GF254 and hexane:ethylacetate: diethylamine (8:2:0.2)] for qualitative determination in CA (Standard of ASEAN Herbal Medicine., 1993). The system of chloroform:methanol:water (15:7:1) was used to seperate AA, MA, terminolic acid, AS and MS and then detected with 0.2% anthrone reagent for qualitative determination in crude extract (Kongthong, 2004). For quantitative determination of active constiuents of CA, chloroform:methanol:water (40:30:4) was reported as developing solvent used for isolation of AS and MS. The developed plate was sprayed with 10% sulfuric acid in ethanol and heated at 110 °C for 10 min and finally scanned with HPTLC densitometer (Tanwarat *et al*., 2003).

CHAPTER III

MATERIALS AND METHODS

1. Chemicals and Equipments

1.1 Chemicals

AS standard (95.0%) and MS standard (95.0%) were purchased from Quangxi Chemicals Inc., China. 2-Naphthol was purchased from Merck (Damstadt, Germany). Potassium dihydrogen phosphate (KH_2PO_4), p -anisaldehyde, dipotassium hydrogen phosphate (K_2HPO_4), sodium bicarbonate and sodium hydroxide were obtained from Sigma Chemical Company (St, Loius, Mo., USA).

All organic solvents employed throughout this work were all reagent grade or better from LAB-SCAN Analytical Sciences (Dublin, Ireland) and Merck (Damstadt, Germany). Water used in this study was triple distilled water.

The 2-naphthol sulfuric acid reagent was prepared with cooling in ice bath. Five grams of 2-naphthol was dissolved in ethanol (33 ml). Then concentrated sulfuric acid (20 ml), ethanol (127 ml) and water (13 ml) were added respectively. The solution is then stirred vigorously to ensure homogeneity. The reagent was stored in cool place. 2.5% (v/v) p-anisaldehyde acid reagent was prepared by adding p-anisaldehyde (3.7 ml) into ethanol (135 ml). Then concentrated sulfuric acid (5 ml) and glacial acetic acid (1.5 ml) were added respectively. Both reagents were freshly prepared before use.

1.2 Equipment

Thin-layer chromatography plates, TLC aluminum sheet silica gel 60 F_{254} (0.25 mm thicknesses) were purchased from Merck (Damstadt, Germany). The plates were developed using various solvent systems in CAMAG glass twin-through chamber (20 x 20 cm.).

Densitometric technique was performed using CAMAG TLC system which included a CAMAG Linomat 5 automatic sample spotter, CAMAG TLC scanner 3 and a winCATS software version 1.4.4 (Linomat, Muttenz, Switzerland).

High performance liquid chromatography (HPLC) was performed by using Agilent 1120 Compact LC with variable-wavelength UV-VIS detector (Agilent Technology, Santa Clara, USA). The data were recorded and analyzed by Agilent EZChrom Elite Compact software version 3.3.2. The analytical separation was performed by using a reverse-phase C-18 stainless-steel column (Agilent ZORBAX-Eclipse plus C-18, 300 mm x 4.6 mm, particle size 5µm).

2. Preparation of Pure AS from *Centella asiatica*

2.1 Isolation and Purification of AS

CA samples used in this study were purchased from a garden in Nonthaburi province**.** Fresh CA aerial part samples were cleaned by tap water to remove soil and other adulterants, and dried in a hot air oven at 45 °C for 24 hr. Dried samples were ground with electric milling machine. The dried plant powder was stored in sealed plastic bag and kept in dry place at room temperature.

Due to the problems of purity and prices of the commercially available AS, pure AS to be used as a standard was prepared for own use in our laboratory by the method of Byeong-Ryong (1998). The dried powder of CA was sonicated in 80% methanol at 45 °C for 15 minutes and the resulting extract was then filtered by vacuum suction. The filtrate was partitioned with dichloromethane and n-butanol, respectively. The n-butanol layer was collected and partitioned with 0.5% sodium bicarbonate solution and water. N-butanol fraction was collected and concentrated under vacuum by rotary evaporator. Ethyl acetate was added into the concentrated n-butanol extract to salt out the partial purified extract in form of precipitate. The precipitate was then dissolved with ethanol and crystallized using ethyl acetate to give pure AS. The mother liquor was concentrated

under rotary evaporation followed by column chromatography using silica gel as absorbent and eluted with mixtures of dichloromethane: methanol: water (4:3:1) with increasing polarity along with fraction collection. The fraction containing MS was concentrated to dryness to get pure MS.

2.2 Identification and Purity Assay of the Isolated AS

Identification of the isolated AS was performed based on a simple TLC technique. In practice, the solutions of the isolated AS, standard AS; Isolated MS, standard MS and partially purified extracts of CA (100 µg/ml) were all applied on a silica TLC plate (5 µl) and developed by the solvent system of chloroform: methanol: acetic acid: water (15:8:4:2). After that, the TLC plate was sprayed with 2.5% anisaldehyde acid reagent and heated in hot air oven at 110 °C for 5 minutes. The R_f values and color of the analytical spots were compared to those of the standards AS and MS.

Purity of the isolated AS was performed using HPLC. HPLC operation was carried out using Agilent 1120 Compact LC. All the solvents used in HPLC were filtered through a membrane filter and degased using sonication. The chromatographic conditions used included C-18 column (300 x 4.60 mm); 20 µl manually injection of sample solution; isocratic solvent system of 0.05 M phosphate buffer pH 7.1: acetonitrile (8:2); flow rate 1.0 ml/min; wavelength 210 nm. Identification of the isolated AS was carried out by comparing the retention time of the isolated AS and standard AS. The purity of the isolated AS was calculated by comparing the area under curve (AUC) of the isolated AS band on a TLC plate with the standard curve of AS.

3. Development of a New Analytical Method for Quantitative Determination of AS

3.1 Development by TLC Densitometric Method

In order to select a proper chemical reagent for AS visualization on the TLC plate, various chemical compounds were studied. In practice, a solution of AS (100 ug/ml) was spotted (10 µl) onto the silica TLC plate and developed using the solvent system of chloroform: methanol: acetic acid: water (15:8:4:2). Then the TLC plate was dried using hot air dryer. The developed plates were treated with various reagents, including saturated NH_3 vapour, glacial acetic acid, 6% hydrogen peroxide, 10% sulfuric acid, vanillin-sulfuric acid reagent and 2-naphthol-sulfuric acid reagent. Each reagent was studied under various temperatures: room temperature, 70, 120 °C and under various time intervals of 3, 5, 7, 10, 30 and 60 min. After that, the TLC plate was scanned by TLC-densitometer using wavelength scan mode (200-700 nm) to obtain the UV absorption spectrum to see the possibility of being used as a visualizing reagent for AS.

3.2 Optimization of Detecting Wavelength for TLC-Densitometric Analysis of AS

A solution of AS (100µg/ml) was spotted (5 µl) onto a silica TLC plate and developed by the solvent system of chloroform: methanol: acetic acid: water 15:8:4:2. The TLC plate was dried using hot air dryer and dipped into 2-naphthol acid reagent (the preparation of 2-naphthol acid reagent is described in 1.1). After that, the TLC plate was heated at 120 °C for 5 minutes using CAMAG TLC plate heater and the spot of AS was scanned by the TLC-densitometer using the wavelength scan mode (200-700 nm) to obtain its absorption spectrum. Moreover, in order to obtain the UV absorption spectra of non-derivetized AS and 2-naphthol, the solution of AS and 2-naphthol were spotted (5 µl) onto the silica TLC plate without solvent development and then the plate was scanned by TLC-densitometer with wavelength scan mode (200-700 nm).

3.3 Solvent System Optimization for TLC Separation of AS from Other Constituents of CA Crude Extracts

The dried ground sample of CA (500 mg.) was extracted using ultrasonic extraction with 10 ml of 80% MeOH for 15 minutes. After cooling down, the extract was filtered by vacuum suction. The separation of AS from the crude extract components was studied based on the TLC densitometric method. In practice, the solutions of the crude extract, partially purified CA extract and standard AS were spotted (10 µl each) onto the TLC plate and developed using various solvent systems. The developed TLC plate was then dried using hot air dryer then dipped into 2-naphthol acid reagent and heated at 120 °C for 5 minutes using CAMAG TLC plate heater. After that the TLC plate was scanned by TLC-densitometer under wavelength of 530 nm.

4. Validation of the Developed TLC-Densitometric Method

4.1 Preparation of Standard AS and MS solutions

Accurately weighed 10.0 mg of AS or MS was dissolved in 1.0 ml of methanol (10.0 mg/ml of AS or MS). The solution was pipetted 500.0 µl using a micropipette and diluted to 5.0 ml with methanol (1.0 mg/ml of AS or MS). The solution was then diluted sequentially as shown in the Table 3.1 to obtain the AS or MS standard solutions in range 50-500 µg/ml.

Table 3 Preparation of standard AS and MS solutions for TLC method validation

Solution	1	2	3	4	5	6	7	8	9	10
Standard solution (µl)	500	450	400	350	300	250	200	150	100	50
Methanol (µl)	500	550	600	650	700	750	800	850	900	950
Total concentration (µg/ml)	500	450	400	350	300	250	200	150	100	50

4.2 Linearity and Range

Linearity was determined over the range of 100-1000 ng/spot. Ten standard AS solutions with different concentrations were prepared and loaded (2.0 µl each) on to the TLC plate to give spots containing AS from 100 to 1000 ng/spot. Then, the TLC plate was developed by using the solvent system of chloroform: methanol: water (30:15:1.2). The developed TLC plate was dried using hot air dryer and then dipped into 2-naphthol acid reagent and heated at 120 °C for 5 minutes using CAMAG TLC plate heater. After that the TLC plate was scanned by TLC-densitometer under wavelength of 530 nm. A plot of average area under curve (AUC) versus concentration (ng/spot) was obtained as standard curve. Linearity was expressed as the correlation coefficient (r^2).

4.3 Accuracy

Accuracy of the TLC method was determined using the addition method. In practice, three samples of CA partially purified crude extract were prepared and determined for the AS content in each extract. The sample solutions were prepared by dissolving an accurately samples of 50 mg of each CA partially purified crude extract in 100 ml of methanol in a volumetric flask. Then 5 mg of AS standard was spiked into each CA crude extract solutions. Each of these spiked solution were loaded (2.0 µl) on the TLC plate. The TLC plate was developed and analyzed using the new developed method and the percentage recovery of AS was then determined.

4.4 Precision

Precision of the TLC method was evaluated in terms of repeatability and reproducibility, each expressed, respectively, as the standard deviation (SD) and the percent coefficient of variation (% CV) values. Four AS sample solutions were prepared. For each sample solution, 7 separate bands were applied onto the same TLC plate. Then the TLC plate was developed by using the solvent system of chloroform: methanol: water (30:15:1.2) and treated with 2-naphthol acid reagent. After that the TLC plate was

scanned by TLC-densitometer under wavelength of 530 nm. The AUC was recorded and AS content was determined. Data for the study of repeatability were obtained from 7 separate bands on the same plate at the same day, whereas those for reproducibility study were from three plates (7 bands each) taken on 3 different days. The SD and % CV values were calculated for each experiment.

5. Accuracy Comparison with the HPLC Method

Five samples of the partially purified extracts of CA (prepared as described in 2.1) were used as subjects for this study. Each sample solution was prepared by dissolving each unknown extract (10 mg) in methanol (1ml), and then made to 0.1 mg/ml concentration solution by ten-fold dilution with methanol. The solution was then directly injected for HPLC analysis and determined for the AS content. The HPLC operation was carried out using Agilent 1120 Compact LC. A reverse phase C-18 column was used. The chromatographic condition used was an isocratic solvent system of 0.05 M phosphate buffer pH 7.1: acetonitril (8:2); flow rate 1.0 ml/min; wavelength 210 nm.

The same samples of the crude extracts were determined for the AS content using the newly developed TLC densitometric method. An aliquot 2µl of each sample solution (0.1 mg/ml) was spotted onto the silica TLC plate and developed by using the solvent system of chloroform: methanol: water (30:15:1.2). The developed TLC plate was dipped into 2-naphthol acid reagent and heated at 120 °C for 5 minutes using plate heater. After that, the TLC plate was scanned by TLC-densitometer under wavelength of 530 nm. The AS content in the sample solutions was calculated and compared the results with those obtained by HPLC methods.

6. Simultaneous Determination of AS and MS

AS and MS standard solutions were prepared by dissolving accurately weigh 10.0 mg of MS or AS in 10.0 ml of methanol (1.0 mg/ml of AS or MS solution). From this solution, 10 additional standard solutions (50-500µg/ml) were prepared as shown in Table 3.1.

Comparison of AS and MS content over the range of 100-1000 ng/spot was carried out by plotting the calibration curve of the two compounds. Aliquots (2.0 µl each) of the standard solutions of AS and MS (500, 400, 200, 100 and 50 mg/ml) were loaded on to the TLC plate to give spots containing AS or MS 100, 200, 400, 800 and 1000 ng/spot. The TLC plate was developed and analyzed using the new developed method, and a plot of average area under curve (AUC) versus concentration (ng/spot) of each compound was obtained.

7. Analysis of AS and MS in Commercial Products

Three brands of CA capsules (Uthaiprasit, Apaiphubeth,and Thanyaporn), four brands of CA infusions (Pathom-Asok, Apaiphubeth, Thanyaporn, and Lanpang-Ruk) were purchased from drugstores. Aerial part of natural habitat CA was collected from a garden in Lamlukka, Pathumthani province. Freshly CA aerial part sample was cleaned, dried and ground by the protocol shown in 2.1. The dried ground plant sample was stored in sealed plastic bag and kept in dry place at room temperature.

Based on the newly developed TLC densitometric method, the determination of triterpenoid glycosides, AS and MS, in CA commercial products was performed. In practice, five hundred milligrams of CA products or dried aerial part sample was extracted using ultrasonic extraction with 10 ml of 80% MeOH for 15 minutes. The extract was centrifuged and the supernatant part was spotted (3.0 µl) onto the TLC plate. After that, 6 concentrations of standard AS were loaded (2.0 µl) onto the TLC plate to give a calibration curve in range 100-1000 ng/spot. The TLC plate was developed using

the solvent system of chloroform: methanol: water (30:15:1.2) and then treated with 2-naphthol acid reagent. Then TLC plate was scanned by TLC-densitometer under wavelength of 530 nm. Both AS and MS contents in the products samples were determined using AS and MS standard curves and reported in term of % dry weight.

CHAPTER IV

RESULTS

1. Preparation of Pure AS from CA

1.1 Isolation and Purification of AS

Because of the purity problem of the commercially available AS which is in a range from 10%-80% and its high prices, isolation and purification of AS to be used as a standard was carried out in our laboratory as described in Materials and Methods. Starting with 10 kg of fresh aerial part CA sample (approximately 1 kg dry weight) purchased from the market, 20.4 g of partially purified extract was obtained. This partially purified extract was further purified by crystallization to obtain 3.6 g of white powder of pure AS, equivalent to 0.36% of dried weight.

1.2 Identification of AS

Identification of the isolated AS was first performed by a TLC technique using 2.5% anisaldehyde acid as a reagent to detect the spot and R_f value of the compound. As shown in Figure 4, the violet blue spot co-chromatographed with its standard (R_f value of 0.68) appeared after being sprayed with the reagent. Moreover, the identification and purity of AS were also confirmed by the method of HPLC. The retention time of the isolated AS at 2.88 min shown in the HPLC chromatogram corresponded to that of the standard (Figure 5). In terms of purity, the purity of isolated AS was calculated to be 98.80% based on the area under curve (AUC) of the isolated AS solution compared with the standard curve of its standard (correlation coefficient (r^2) of 0.9982). Therefore, the AS preparation showed high purity enough to be used as the standard compound for further studies.

In addition to AS, MS, another major compound in CA, was examined by the same TLC procedure of AS. The isolated MS was, again, detected using 2.5% anisaldehyde acid reagent. As shown in Figure 4, the green spot of isolated MS co-chromatographed with its standard appeared at the Rf value of 0.52 after being treated with the reagent.

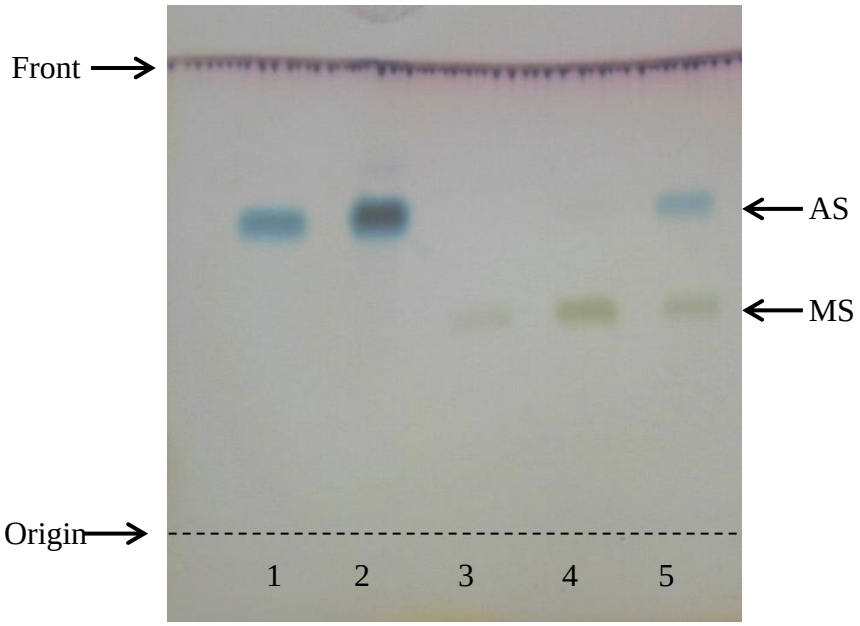

Figure 4 Thin layer chromatography (TLC) of asiaticoside (AS) and madecassoside (MS) after being treated with 2.5% anisaldehyde acid reagent. Lane 1: standard AS, lane 2: isolated AS, lane 3: standard MS, lane 4: isolated MS, lane 5: partially purified extracts of *C. asiatica*

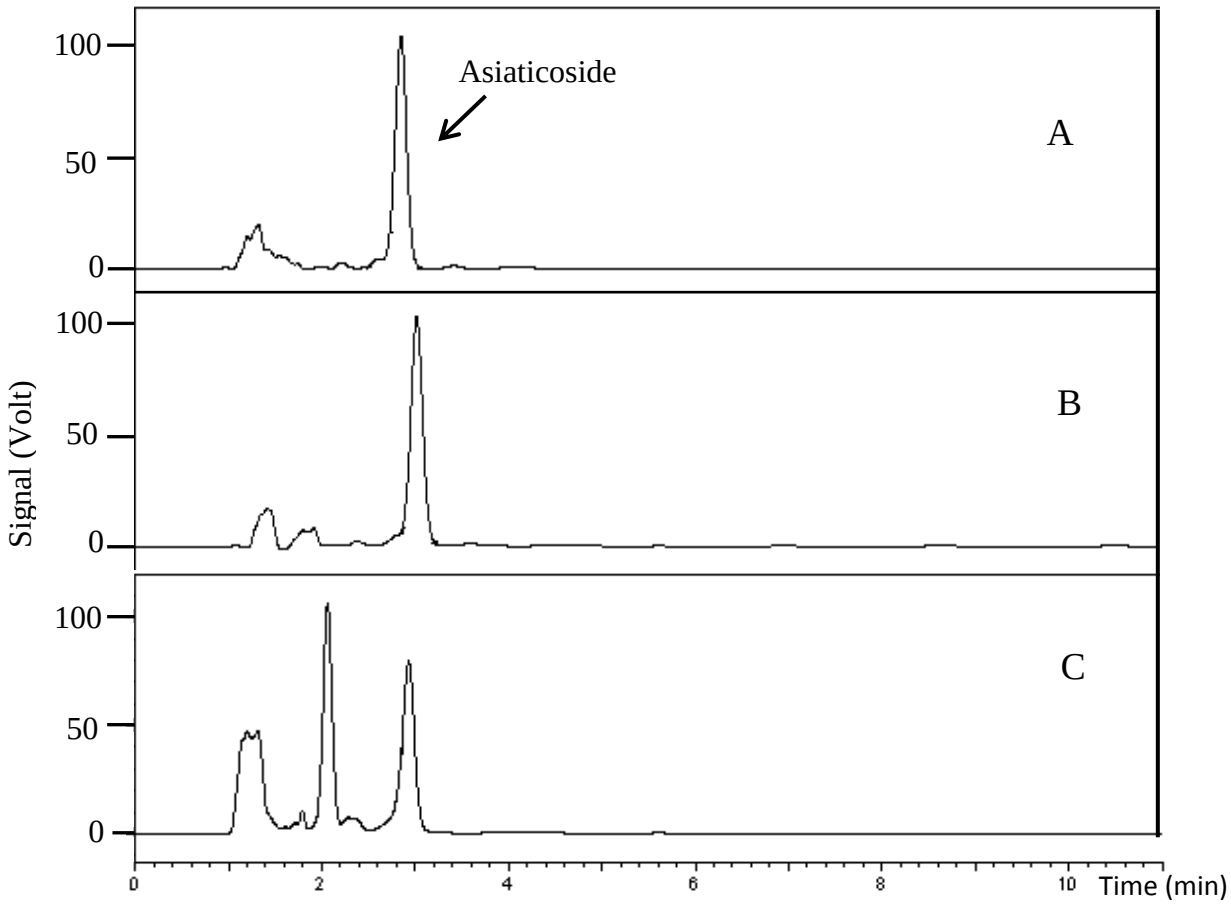

Figure 5 HPLC Chromatograms using a reverse-phase C-18 column and a solvent system of acetonitrile: phosphate buffer pH 7.1 in ratio 2:8. A) AS standard, B) isolated AS, C) partially purified extract of CA

2. Development of a New Analytical Method for Quantitative Determination of AS

2.1 Development of a TLC Densitometric Method

Based on the principle of TLC-densitometry as mentioned before, the lack of a chromophore in AS is a major problem in the detection of this compound. Therefore, a proper chemical reagent used for visualizing this compound was studied. Various chemical compounds for detection of AS on TLC plate were first examined, including NH_3 vapour, glacial acetic acid, 6% hydrogen peroxide, 10% sulfuric acid, vanillin-sulfuric acid reagent and 2-naphthol-sulfuric acid reagent. The obtained visualized spot of AS from each reagent was then analysed by its UV absorption spectrum.

Based on the UV absorption spectrum of each reaction product obtained from the TLC-densitometric scan, many reagents were found to be not suitable for AS detection. Vanillin-sulfuric acid reagent could give a visualized AS band but the color quickly faded. On the other hand, the 2-naphthol acid reagent showed a possibility of being used as a derivatizing reagent because it could convert the invisible AS band into a visible brownish one (Figure 8). Moreover, the intensity of spot chromatogram was stable longer than 1 hour at room temperature after the derivertization, which is long enough for scanning. Therefore, 2-naphthol acid reagent was chosen as the derivertizing reagent for determining the AS content in our developed TLC-densitometric method.

2.2 Optimization of Detecting Wavelengths for TLC-Densitometric Analysis of AS

In order to optimize the detecting wavelength for the TLC densitometric analysis of AS after treating the TLC plate with 2-naphthol reagent, the TLC band of the derivatized AS was scanned using a wavelength-scan mode (200-700 nm) on the TLC-densitometer to generate an absorption spectrum. As shown in Figure 6, the absorbtion spectrum of the derivatized AS appeared to have a single λ_{max} at 530 nm while AS itself showed a λ_{max} at $\leq$200 nm and 2-naphthol at 320 nm. Therefore, after the derivatization,

AS showed its UV absorption spectrum that was completely different from its starting compound (2-naphthol and AS), and thus the wavelength of 530 nm was used throughout to scan TLC plates in order to generating TLC chromatograms for the analysis of AS.

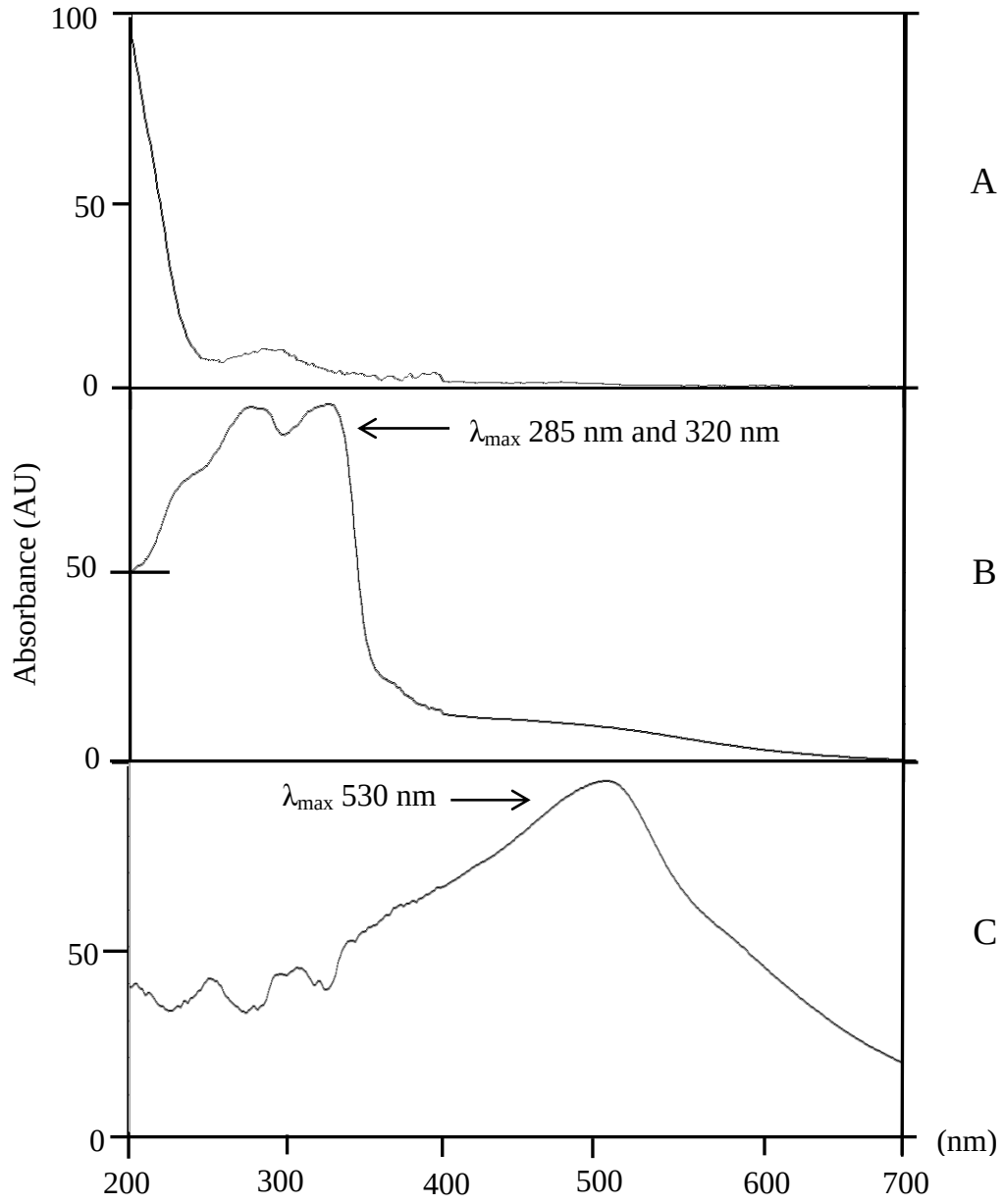

Figure 6 UV-Visible absorption spectra obtained from the TLC -densitometric scans of A) asiaticoside, B) 2-naphthol and C) treated with 2-naphthol acid reagent.

2.3 TLC-System Optimization for Differentiation of AS from Other Constituents of CA Crude Extracts

TLC separation of AS from other constituents in the crude extract of CA was studied using a few solvent systems. These included chloroform: methanol: water (30:15:1.2), hexane: ethyl acetate: diethylamine (8:2:0.2), toluene: ethyl acetate: acetonitrile (7:3:1), and chloroform: methanol: water (40:30:4). The chromatograms obtained from the scanning at 530 nm are shown in Figure 7. It can be seen that the solvent system of hexane: ethyl acetate: diethylamine and toluene: ethyl acetate: acetonitrile could not separate AS from other constituents, whereas the solvent system of chloroform: methanol: water in both ratios could accomplish the separation. However, the solvent system of chloroform: methanol: water (30:15:1.2) showed a better separation of AS than the other solvent system. This system was, therefore, selected for subsequent uses on the TLC separation of CA extracts.

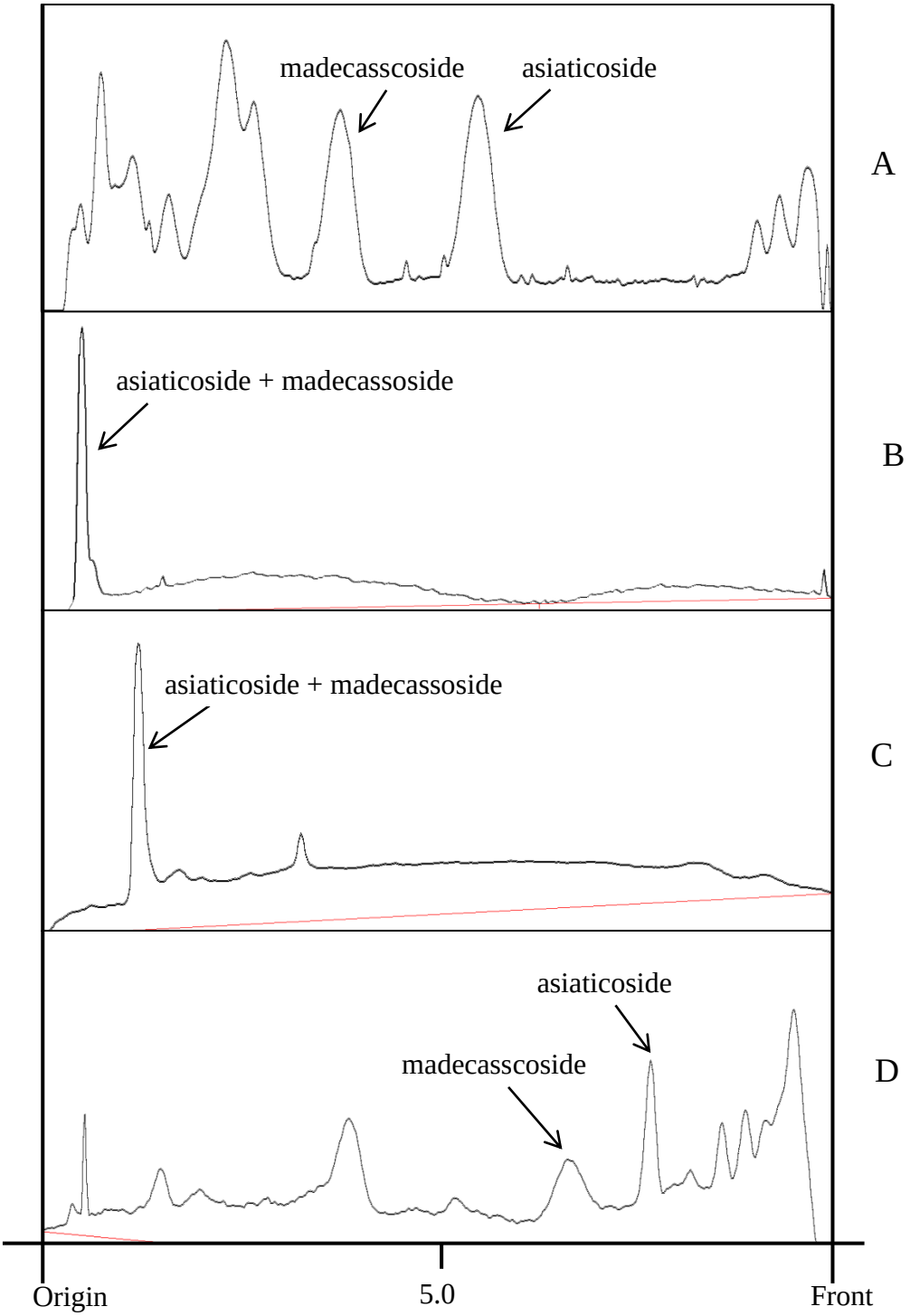

Figure 7 TLC-densitometric chromatograms of CA crude extracts separated by various solvent systems. A) chloroform: methanol: water (30:15:1.2), B) hexane: ethyl acetate: diethylamine (8:2:0.2), C) toluene: ethyl acetate: acetonitrile (7:3:1), D) chloroform: methanol: water (40:30:4).

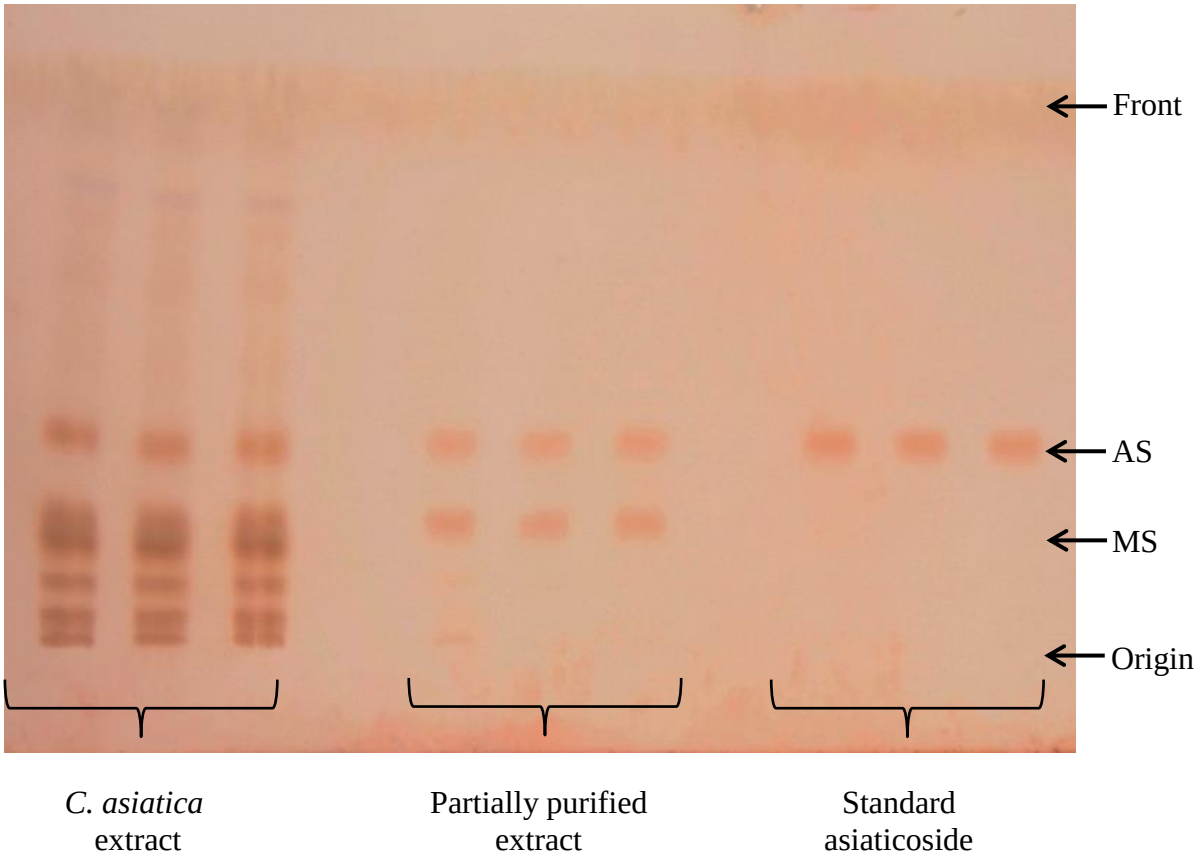

Figure 8 TLC patterns of crude CA extract, partially purified CA extract and standard AS obtained from the newly developed TLC-densitometric method using the solvent system of chloroform: methanol: water (30:15:1.2), then dipped in 2-naphthal reagent and heated at 120ºC for 5 mins before taking the plate picture.

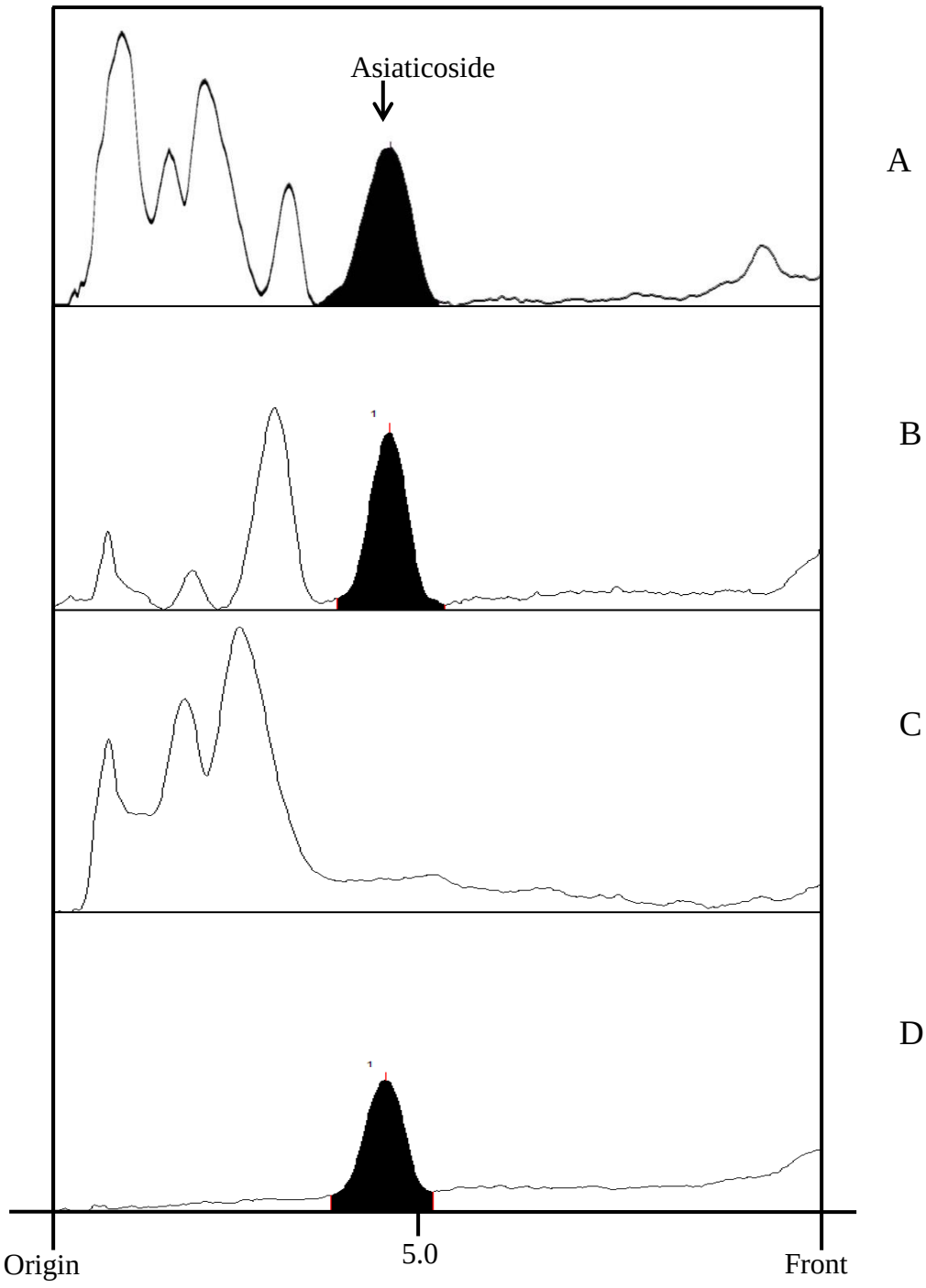

Figure 9 TLC-densitometric chromatograms of some CA crude extracts developed by using the solvent system of chloroform-methanol-water; 30:15:1.2 , A) CA crude extract, B) partially purified extract, C) *Hydrocotyl asiatica* crude extract (adulterant crude drug), D) standard AS .

3. Method Validation of the Developed TLC-Densitometric Method

3.1 TLC Method Validation

The propose of method validation is to demonstrate that the developed method is suitable for use and to ensure that the results obtained are reliable. In this study, the developed TLC densitometric method was validated according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human: Validation of Analytical Procedures Guideline (ICH guideline) with respect to linearity, accuracy and precision.

3.1.1 Linearity and Range

The results obtained from the linearity study shown in Figure 10 suggested that the range of 100-1000 ng AS was good for the validation study because it showed a good linear correlation between AS content versus the peak area.

Detailed study on this quantity range (100-1000 ng/band) was also confirmed by good linear correlation with the acceptable accuracy (96-104 %R) and precision (%RSD $\leq$ 2). The linear equation of the chosen range was as follows: y = 9.7171 xs+916.42. The value of the correlation coefficient (r^2) was 0.9984 which is in the acceptance criteria ($r^2 \geq 0.99$) as shown in Figure 11. This r^2 value showed a good relationship between the standard addition and the amount of analytical found.

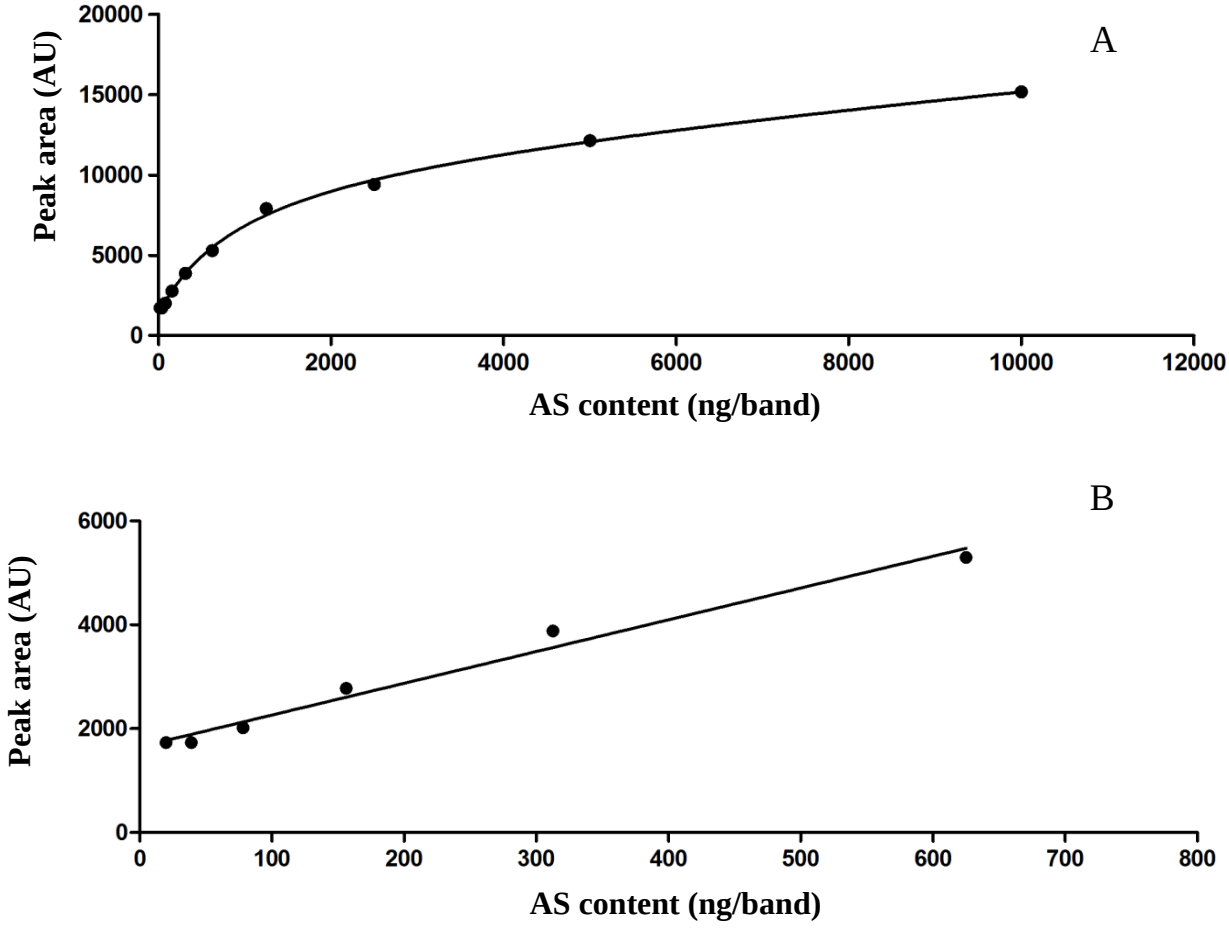

Figure 10 Preliminary studies for the optimal range of AS amounts on a TLC plate that show linearity with the peak areas (scanned at 530 nm). A) Wide range of AS amounts from 20-10,000 ng/band and B) Narrow range of AS amounts from 20-625 ng/band.

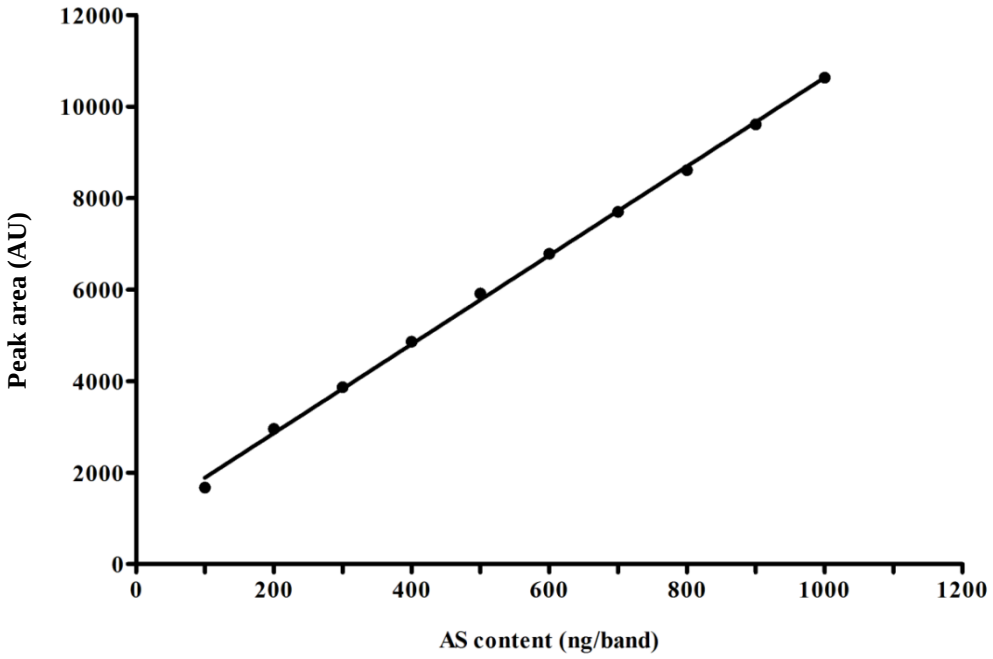

Figure 11 Calibration curve of AS determined by the newly developed TLC densitometric method showing linearity in the range of AS amounts from 100 ng-1000 ng per band.

3.1.2 Accuracy

Accuracy was tested in terms of percentage recovery by adding the certain amount of standard compound in unknown sample. As shown in Table 4, the recovery of AS was found to be in the range 97 - 104%. The accuracy test of this method was in the acceptance criteria (100±4 % R) which showed the closeness of the values between the theoretical value and the value obtained from the analysis.

Table 4 Data showing accuracy of AS measurement by the TLC densitometric method. The values are presented as mean ± SD (obtained from 5 determinations). The theoretical content = true AS content in crude extracts plus spiking content (100ng).

Sample No.	AS content in CA crude extracts (ng/band)	Theoretical content (ng/band)	Experimental content (ng/band)	% Recovery
1	341.0	441.0	443.3	101
2	335.6	435.6	423.0	97
3	452.2	552.2	569.9	104

3.1.3 Precision

The criteria of precision were determined, considering at two levels: repeatability (intra-day precision) and reproducibility (inter-day precision).

Intra-day precision

The value of the coefficient of variation was calculated from seven replications of a sample solution in the same day at various concentrations. As shown in Table 5, the %CV of AS was in the range of 1.2-2.7, with the average %CV of 1.85. This was in the range within the acceptance criteria (%CV $\leq$ 2).

Inter-day precision

The coefficient of variation was calculated from the replications of sample solution for three days at various concentrations. It was found that the %CV of inter-day precision was in range of 0.9-1.9, with the average %CV of 1.5. This was in the range of acceptance criteria (%CV $\leq$ 2) (Table 5).

Table 5 Data showing the intra-day and inter-day precision measurement obtained by the new TLC-densitometric method obtained from 7 determinations for each parameter.

Sample No.	Intra-day precision			Inter-day precision		
	Mean area (AU)	S.D.	% CV	Mean area (AU)	S.D.	% CV
1	11527.6	222.5	1.9	11331.27	220.5	1.9
2	8352.314	96.1	1.2	8320.211	109.6	1.3
3	12654.77	339.2	2.7	12443.46	241.4	1.9
4	8786.629	143.8	1.6	8897.9	84.0	0.9

4. Comparison with the HPLC Analysis

Regarding accuracy and precision, the developed TLC-densitometric method was compared with the standard HPLC-UV method to ensure the reliability of the developed TLC-densitometric method. The comparison was done by determining the AS content of 5 crude extracts samples using both the TLC densitometric and HPLC methods.

The standard curve of AS standard by HPLC techniques is shown in Figure 12. which appeared to have its correlation coefficient (r^2) at 0.9982. Based on this standard curve, the AS contents of the unknown samples are shown in Table 6. It was found that the values of AS content determined by the two techniques were well correlated in range 98.6%-101.3 %. Therefore, the newly developed densitometric technique could replace the HPLC technique in determining the AS contents.

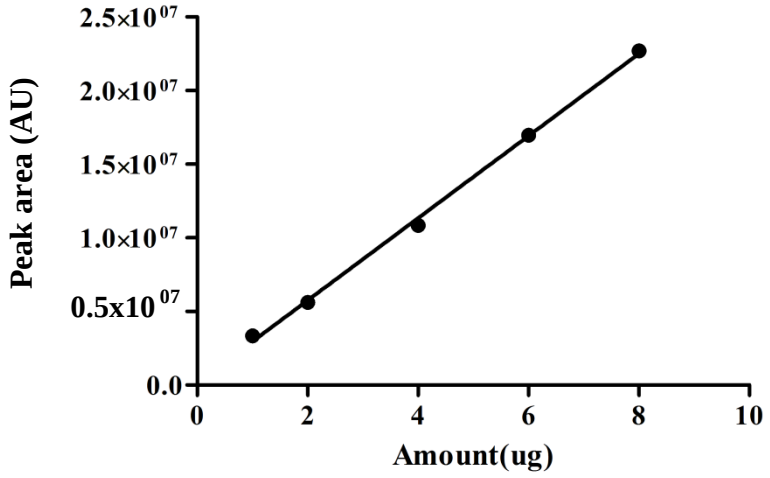

Figure 12 Calibration curve of AS determined by the standard method of HPLC.

Table 6 Comparison of the average AS contents obtained by TLC-densitometric and HPLC methods.

Sample	Average AS content obtained by HPLC method (ng/ml)	AS content obtained by TLC method (ng/ml)	% Comparable
A	341.0	342.1	100.3
B	335.6	333.2	99.3
C	412.2	410.4	99.6
D	358.7	359.6	100.2
E	340.6	342.4	100.6

5. Simultaneous Determination of AS and MS

The quality of *C. asiatica* crude extracts and commercial products are normally reported in terms of total triterpenoid glycosides content which is the total content of AS and MS. MS is another major compound present in CA which is also an ursane type triterpenoid glycoside closely related to AS with no chromophore as previously described in Chapter 2. To test for the possibility of using the newly developed desitometric TLC method to determine the total triterpenoid glycoside contents, a simultaneous determination of AS and MS was studied.

Quantitative analysis of AS and MS contents were carried out by plotting their calibration curves in the same concentration range. The calibration curves of AS and MS are shown in Figures 13 and 14. It can be seen that both standard curves showed the same pattern of concentration range from 100 ng to1000 ng. The linear equations of MS and AS were obtained as the following; $y = 13.321x+1181.2$ for AS and $y = 13.747x+662.19$ for MS. The correlation coefficients (r^2) for MS and AS were 0.9907 and 0.9931 respectively. These results suggested the possibility of using the TLC densitometric method to determine both compounds at the same time in CA.

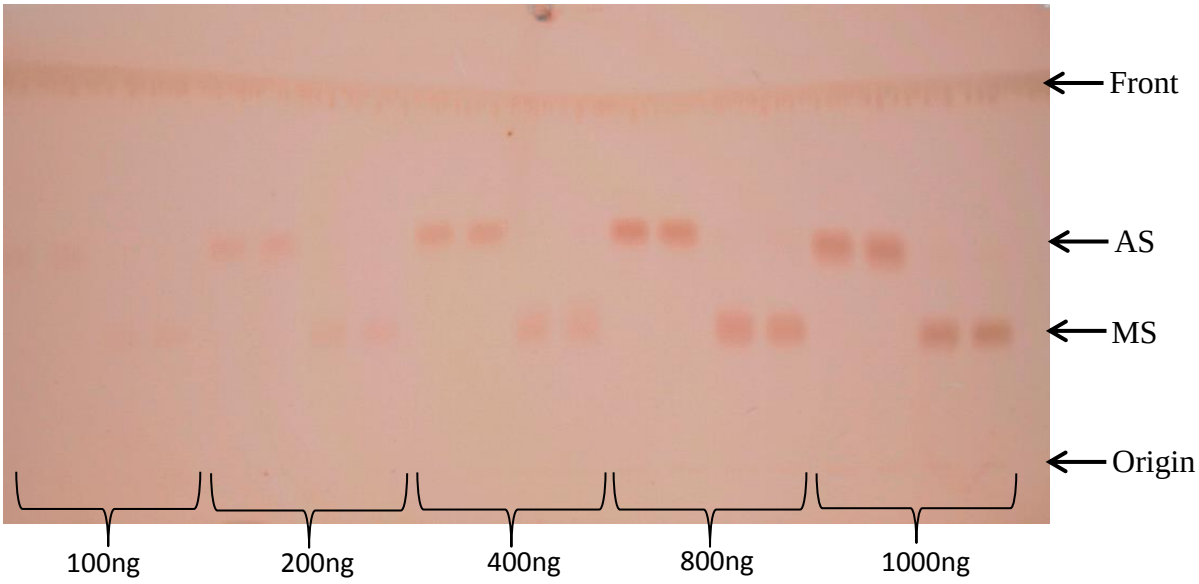

Figure 13 TLC patterns of the standard AS and standard MS in various concentrations in the range of 100 ng-1000 ng per band.

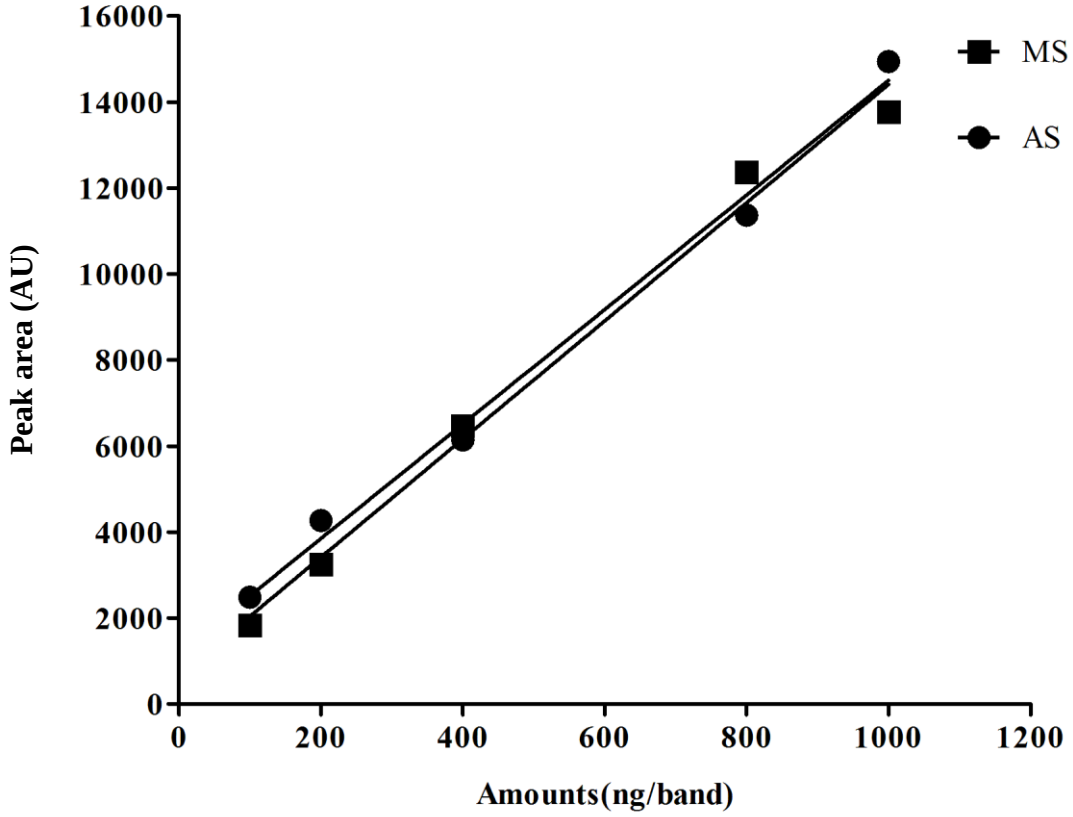

Figure 14 Calibration curves of the standard asiaticoside (AS) and madecassoside (MS) obtained from the newly developed TLC densitometric method.

6. Analysis of AS and MS in Commercial Products

The new TLC densitometric method was then used to evaluate the quality of CA product samples in various brands and dosage forms. Crude extracts of various samples were prepared and analyzed, and the results of the TLC patterns are shown in Figure 15 and the contents of AS, MS and total triterpenoid glycosides are summarized in Table 7.

It can be seen that there were a wide range of AS and MS content in different CA product samples (0.2 % - 0.6 % w/w for AS, 0.2 %-1.0 %w/w for MS). The capsule dosage forms have an average triterpenoids content both AS and MS (0.3 % w/w for AS, 0.4% for MS) lower than the infusion dosage forms (0.5 % w/w for AS, 0.5% for MS). It

should be noted that AS and MS contents of the product samples were lower than those of the natural source of raw material samples (0.7 % w/w for AS, 1.0% for MS).

The total triterpenoid glycosides contents of CA product samples were in range of 0.4% - 1.3% w/w which were lower than the natural source CA (1.7% w/w). The AS: MS ratios of all samples were in range 1:1 – 1:1.5, approximately.

Table 7 AS and MS contents in seven CA products and in the natural dried leaves of CA determined by TLC densitometric method**.**

Product brand	Dosage form	AS and MS content (ng per spot)		AS and MS content (%w/w)		Total triterpenoid glycosides content (%w/w)
		AS	MS	AS	MS	
Uthaiprasit	Capsules	556.2	835.2	0.4%	0.6%	0.9%
Aphaiphubeth	Capsules	310.8	433.8	0.2%	0.3%	0.5%
Thanyaporn	Capsules	495.6	517.6	0.3%	0.3%	0.7%
Pathom asok	Infusions	666.8	681.2	0.4%	0.4%	0.9%
Thanyaporn	Infusions	897.9	1004.4	0.6%	0.7%	1.3%
Aphaiphubeth	Infusions	258.3	296.0	0.2%	0.2%	0.4%
Lampang-Ruk	Infusions	901.0	872.6	0.6%	0.6%	1.2%
Dry leave		1011.8	1494.3	0.7%	1.0%	1.7%

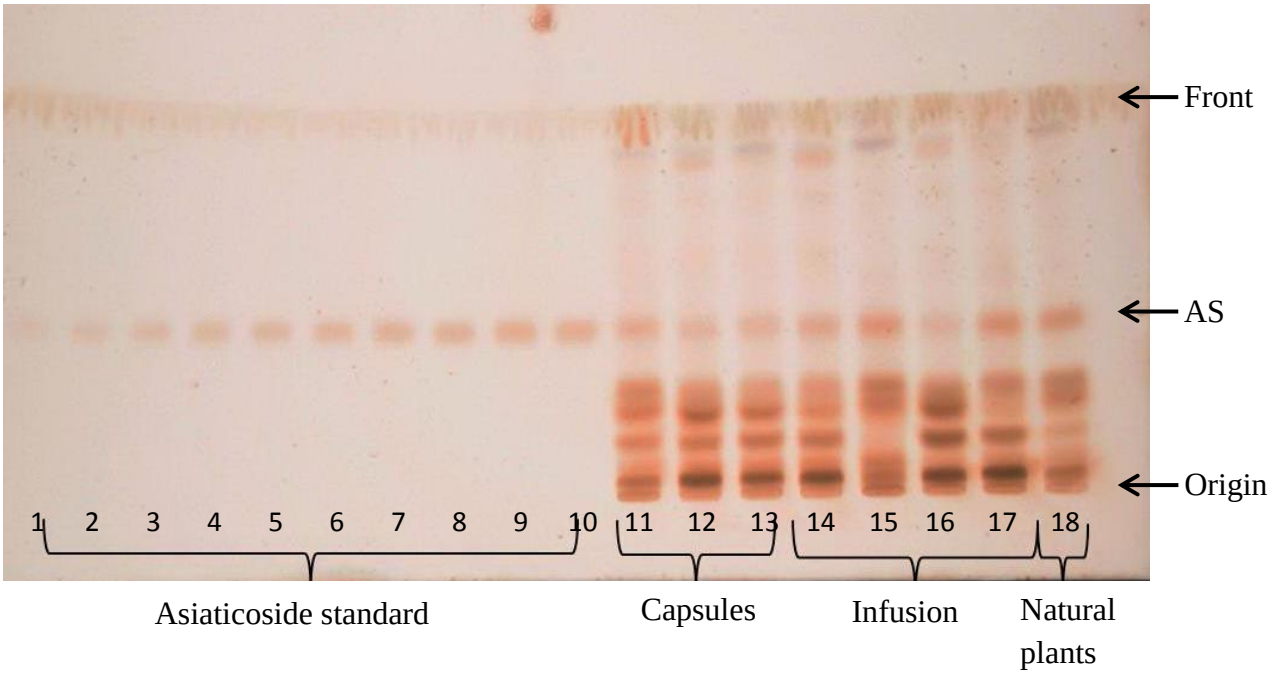

Figure 15 TLC patterns of standard AS (1-10), the crude extracts from CA products of capsules (11-13) and infusion (14-17), and the crude extracts from the CA aerial part samples (18). (Lane 1-10: standard AS amounts of 100 ng -1000 ng, respectively, lane 11: Uthaiprasit, lane 12: Aphaiphubeth, lane 13: Thanyaporn, lane 14: Aphaiphubeth, Lane 15: Pathom asok, lane 16: Aphaiphubeth, lane 17: Lampang-Ruk)

CHAPTER V

DISCUSSION

5.1 Development of a New Analytical Method for Determination of AS

TLC has been used for many years as a primary method for screening plant-based drugs and preparations. It has recently been developed for quantitative purposes to obtain reproducible and reliable results. With a possibility of working on a large number of samples, TLC has its advantages in terms of high reproducibility at low cost. So far, only a few TLC methods for the identification of saponins in plant extracts and pharmaceutical powders have been developed. However, because of the weak UV absorption due to the lack of a chromophore in the basic structure of triterpenoid glycosides, colorimetric determination was the first developed in this study for the evaluation of *Centella asiatica* extracts and preparations (Curl, 1985). Many visualizing reagents for the saponin glycoside were reported, including Liebermann burchard reagent (Atta 1958), Carr-price reagent (Coulson 1958), 10% H_2SO_4 in ethanol (Fenwick, 1981), phosphotungstic acid (Kazerovskis, 1962), 1% $CeSO_4$ in 10% H_2SO_4 (Lutz, 1980) with vanillin and anisaldehyde-based spraying reagents, as the most widely used. However, the intensity of the color formed by using either the anisaldehyde reagent or vanillin reagent decreases rapidly with time. Therefore, these reagents are obviously not suitable for quantitative analysis of AS (Oleszek *et al*., 2002). Nowadays, although HPLC is a method of choice for the determination of the active constituent in CA (as reviewed in Chapter 2.), its use of UV detection is still not efficient owing to the weak UV absorption of AS. In addition, the use of pre-column for a clean-up process is still necessary for maintainance of the column (Oleszek *et al*., 2002). Therefore, in this study, a new technique of TLC-densitometry was developed as a more efficient method for the determination of AS in the crude extracts of CA.

In principle, the newly developed, TLC-densitometic method for AS analysis started with the separation of AS on a TLC plate from other constituents present in CA crude extract. The band of separated AS is then derivatized with 2-naphthol reagent by dipping the plate into the solution and heating the plate to form a chromophore-containing compound of AS which allows detection by UV light and scanning to generate a TLC chromatogram.

Our preliminary studies on the derivatization of AS on a silica gel plate were carried out using various chemical reagents. It was found that 2-naphthol reagent was the most appropriate reagent for AS detection. 2-Naphthol acid is a reagent reacting with sugar molecules (Gertrud, 2007) and this presumably with the sugar molecules of AS. The reaction causes the change of the absorbtion spectrum of 2-naphthol with its λ_{max} at 320 nm to the 2-naphthol-derivertized AS with a single λ_{max} at 530 nm (Figure 6). The mechanism involved in the reaction was proposed here following the mechanism of Molisch's test, to occur in 3 steps (Figure 16). First, the strong acid which is sulfuric acid in the reagent causes the hydrolysis of glycosidic bond of AS and cleaves each sugar molecule apart. Second, the hydrolyzed monosaccharides are dehydrated to form furfuryl derivates in the presence of high sulfuric acid concentration followed by the attack of 2-naphthol as a last step. 2-Naphthol acts as a nucleophile to add to the positive polarized carbon of the aldehyde group of the furfural derivative. After an intramolecular proton migration, water is then eliminated and a C-C double bond is formed.

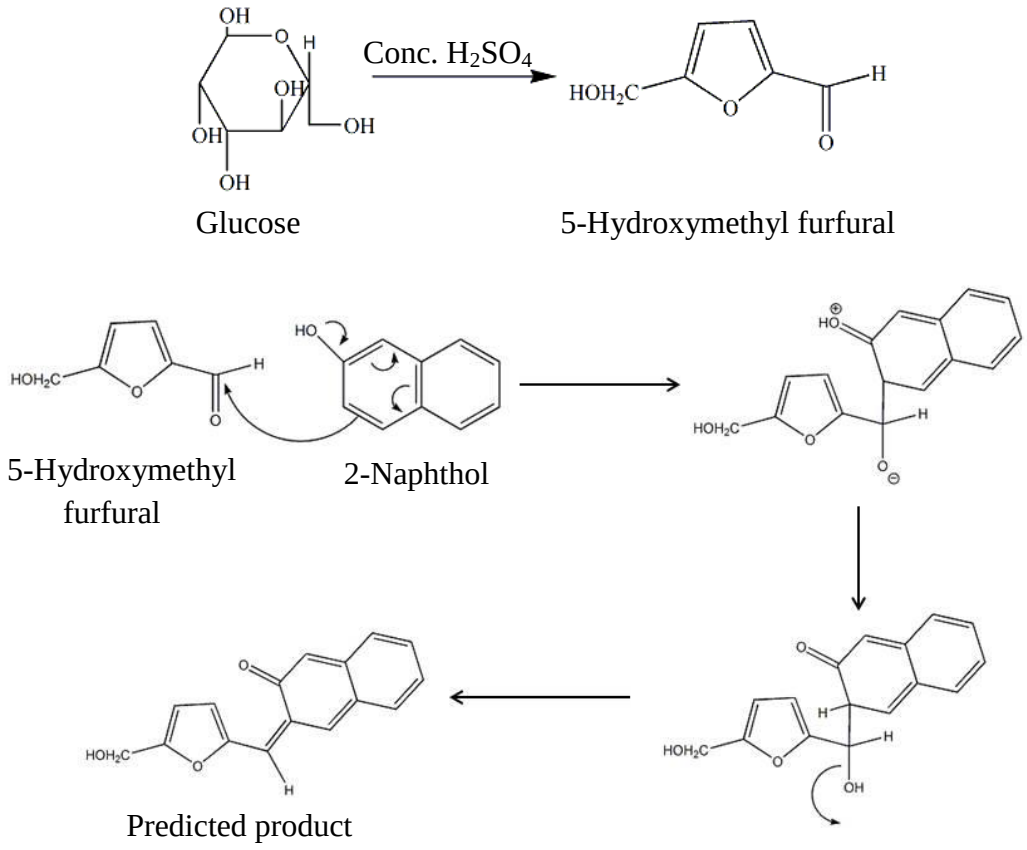

Figure 16 The predicted mechanism of sugar derivetization by 2-naphthol acid reagent.

Regarding the TLC-separation of AS, because AS is a partly polar molecule due to the presence of the trisaccharide (glucose-glucose-rhamnose) in its glycone part, the main solvent component was chosen to be a moderate polar solvent combined with another polar solvent for dissolving both glycone and aglycone part of AS in the TLC develoment. Other solvent components in the solvent system should support the separation of AS from other constituents in the extract, especially MS which is another related terpenoid glycosides also present in CA. Based on this working solvent system, a suitable solvent system was obtained in good results for the separation of AS. Its composition is chloroform: methanol: water (30:15:1.2) which showed good separation of

AS from the other compounds in the crude extracts, as shown by the TLC-densitometric chromatograms (Figure 9).

5.2 Validation of the Developed TLC-Densitometric Method

The reliability of the developed TLC-densitometric method in the analysis of AS was studied by validation of the method. Validation of an analytical procedure is to demonstrate that the method is suitable for its intended purpose before use. According to the guideline of "The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human: Validation of Analytical Procedures" (ICH guideline 2005), accuracy, precision, linearity and range of detection are considered as parameters for the method validation study.

Range of the method is the interval between the upper and lower concentrations or contents of an analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level validation. Our preliminary studies were performed by plotting a graph between a wide range of various amounts of standard AS (in range of 20 ng to 20,000 ng per band) and their signals obtained by the TLC-densitometric method. This was followed by a narrow range of 100-1000 ng which was chosen for the validation study because it showed a good linear correlation (Figure 10).

Linearity is the ability of an analytical method to obtain test results which are directly proportional to the content of a compound of interest in a sample. It is usually evaluated by a plot of signal as a function of analyst concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In this study, the linearity was determined over the range from 100 ng to 1000 ng per band and showed a good linear correlation (r^2 = 0.9984) within the acceptable criteria ($r^2 \geq 0.99$) which represented a good relation between the content of AS and the signal of its band as shown in Figure 11.

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. The accuracy assessment can be determined by several methods. One of them is comparing the results of the method with the results obtained from the standard method. Another method of finding the accuracy value by is analyzing the known concentrations of a certified standard compound, and comparing the results from the validating method with the true value of the standard compound. If no certified standard compound available, we can use recovery technique by spiked an analyte sample (a substance or chemical constituent that is of interest in an analytical procedure) with a known concentration standard by weight or volume, which is used in this study. As shown in Table 4, the percent recovery of AS was found to be within the acceptable range (96-104 % R) of the closeness of agreement between the true value and the value found.

The precision of an analytical procedure is defined as the closeness of agreement between a series of measurements obtained from multiple samplings of the same homogeneous sample. Precision is usually expressed as the variance, relative standard deviation (%RSD) or coefficient of variation (%CV) of a series of measurements. In this study, the coefficients of variation of intra-day and inter-day precisions were found to be within the acceptable range (%CV $\leq$ 2). These results showed a good precision of the new developed method which expressed the closeness of measurements of the new method. Therefore, it should be a reliable method for determination of the AS content.

In summary, the newly developed TLC analytical method was validated in terms of accuracy, precision, linearity and range according to ICH guideline. Satisfactory levels of those parameters were obtained in our studies.

To ensure the reliability of the developed TLC-densitometry, the developed TLC method was compared with the normally used HPLC method. Both methods have shown their AS calibration curves with good linearity in the range of 100 to 1000 ng. Moreover, the values of AS content determined by two techniques were well correlated in the range 99%-101% (Table 4.3). However, one major problem in the analysis of natural derived

samples using the HPLC method is the interference by impurities of the plant extracts which may lessen the efficiency and ultimately deteriorate the separation column by the irreversible adsorption of impurities in the column head. Hence, usually the HPLC method requires sample preparations before the analysis to remove such impurities. However, the sample preparation process may affect the active compounds contents in the samples (Oleszek *et al*., 2002). On the other hand, the sample preparation is not necessary for the TLC-densitometric technique because the nature of the TLC separation is to separate the target compound from other constituents in the crude extract. Another point of advantage of the new TLC-densitometry method is the handling of sample numbers. Eighteen samples could be performed simultaneously in a single run per plate (20x10 cm.) within 15 minutes while the UV-HPLC method takes 5-15 minutes per sample. This could solve the time-spend problem when analyzing a large number of samples. Therefore, the TLC-densitometry is an ideal technique for this study with high accurate, sensitive, reliable and speed for determination of AS content.

5.3 Simultaneous Determination of AS and MS

MS is another major compound present in CA. Its structure differs from AS only one hydroxyl group in the core structure. The quality of CA crude extracts and commercial products are usually reported in terms of total triterpenoid glycosides content which is the total content of both compounds. Therefore, the simultaneous determination was studied to test for the possibility of using the newly developed densitometric TLC method to determine the total triterpenoid glycoside content. The study was performed by plotting the calibration curves of both compounds using the newly developed technique on the same plate. As shown in Figure 14, the results showed very similar patterns of the calibration curve for both compounds, which suggested the possibility of using the newly TLC-densitometric method to determine both compounds simultaneously.

According to the mechanism proposed above, the newly TLC-densitometric method can determine both AS and MS because they both have same sugar pattern in their glycone parts. For each concentration, the same amount of sugar in both compounds

were equally cleaved and reacted with 2-naphthol reagent and same peak areas were obtained. For this reason, this also suggested a possibility of using this method to analyze the other active compounds with sugar moiety including cardiac glycosides, flavonoid glycosides, glycosylated cyclic peptides (Perez-Zuniga *et al*., 2004), cyanogenic glycosides (Vetter, 2000), glucosinolates (Hayes *et al*., 2008), glycosylated macrolides (Tang, 2007), and etc. Further study of using the TLC-densitometric method for determining these high drug potential natural products is necessary and should be useful for improving the quality of the herbal products in the market.

5.4 Analysis of AS and MS in Commercial Products

Using the new TLC densitometric method, determination of AS and MS contents in CA product samples in various brands and dosage forms was performed in this study. The variation of AS contents of each brand might be affected by many factors such as storage environment, source and origins (Thomas *et al*., 2010), production process (Wittawat, 2011), time of harvesting (Jirapan, 2010) and plant nutrition on soil (Somporn *et al*., 2003; Devkota *et al*., 2009).

Moreover, AS and MS contents of the product samples were lower than those of the natural material. It might be the effect of degradation of glycoside due to manufacturing process. The drying process of the raw material may cause the hydrolysis of glycosidic bond in AS structure due to high temperature. Many drying methods were used in herbal industry including sun drying method, hot air drying method, contact drying method and vacuum method. Each method as mentioned above using various ranges of temperature which significantly correlated to the degradation of the active compounds (Wittawat, 2011).

Extraction of the active compounds from the materials is another important step for herbal analysis. The traditional methods of solvent extraction of plant material are based on the correct choice of suitable solvent and the use of heat and agitation to increase the solubility of interesting constituents. Usually, the traditional methods, such as merceration or soxhlet extraction, which have been used for a long time are high time-

consuming and required a large scale of solvent (Luque de Castro & Garcia-Ayuso, 1998). Various extraction methods including ultrasound-assisted extraction (Vinatoru, 2001), microwave-assisted extraction (Kaufmann & Christen, 2002), supercritical fluid extraction (Marr & Gamse, 2000; Lang & Wai, 2001), accelerated solvent extraction, and etc. (Kaufmann & Christen, 2002; Smith, 2002) have been developed for faster and more efficient extraction.

Indirect ultrasonic extraction is inexpensive, simple and efficient comparable to the traditional extraction methods. The advantages of using ultrasonic extraction in solid–liquid extraction are the increasing of extraction yield and faster kinetics. Ultrasound can also reduce the working temperature allowing the extraction of thermo labile compounds. Several reports have shown the effectiveness of using ultrasonic extraction to extract various active constituents of crude drugs and herbal products. Wu et al., (2001) reported that the ultrasonic extraction of ginsenoside, which is saponin glycoside, similar to AS, was about 3 times faster than the traditional soxhlex extraction method. Nuttawan (2013) have also reported that amount of xanthone obtained from ultrasonic extraction in 0.5 hr. is significantly higher than xanthone obtained from soxhlet extraction in 2hr. Ultrasonic extraction is used for extraction of other active compounds including curcuminoids from *Curcuma longa* (Rouhani, 2009), antioxidants from *Rosmarinus officinalis* (Albu *et al*., 2004), steroids from *Chresta spp*. (Schinor, 2004). Hence, indirect ultrasonic extraction was chosen to extract AS from CA products in this study.

Based on the results of this study, the newly analytical method should be useful for the examination the active compounds content in CA products on the drug market. The information gathered this way could be beneficial to the customers as the dose of the active compounds could be adjusted so that maximal therapeutic efficacy is achieved. Moreover, it can be helpful for selection of CA with high AS yield or perform a mapping of AS content of CA in various areas of Thailand, which can help the herbal industry in searching for the high quality of raw material, and therefore leading to greater cost-benefits ratio of using herbal products.

CHAPTER V

CONCLUSION

From this study, the following conclusions can be drawn.

1. A new TLC-densitometric method has been developed successfully for AS analysis. The analysis is performed on a silica gel 60 F_{254} TLC plate (20x10cm.) with chloroform/methanol/water, 30:15:1.2 system as the mobile phase. Densitometric analysis is performed at 530 nm after post-chromatographic derivatization with 2-naphthol sulfuric acid reagent to give brownish bands of the triterpenoid glycosides.

2. The newly developed TLC-densitometric method shows good sensitivity and selectivity for both qualitative and quantitative analysis. The linear range for the analysis of AS is 100-1000 ng/band ($r^2 \geq 0.99$) with good precision and accuracy (1.2- 1.9 %RSD, 98 -104 %). The developed densitometric TLC is to be simple and fast. Eighteen samples could be performed simultaneously in a single run per plate within 15 minutes, and the results are comparable to those obtained by the UV-HPLC method (99 - 101% correlation).

3. The standard curves of standards AS and MS are very close to each other, showing a possibility of using the same standard curve for determining the two glycosides.

4. The new technique of TLC-densitometry can be used for the determination of AS in both the crude extracts of CA and its commercial products. Seven samples of CA products were analyzed for the AS content.

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Poster presentations

1. Chaisawadi, A., De-Eknamkul, W. 2012. Development of a new analytical method for determination of asiaticoside content in *Centella asiatica*. Proceedings of the 29th annual research conference in pharmaceutical sciences, faculty of pharmaceutical sciences, December 6-7, 2012, Bangkok, Thailand.