CHAPTER II

LITERATURE REVIEW

Centella asiatica (L.) Urb., the synonyms *Hydrocotyle asiatica* (L.) is known as Bua-bok, Gotu Kola, Asiatic Pennywort, Indian Pennywort, Indian Water Navelwort, Mandukparni, etc. It is a cultivated plant in the family Apiaceae or Umbelliferae that creeping subtropical and topical climates of Asia, Africa, North and South America such as Thailand, Indonesia, Sri Lanka, India, and China **(1-3)**.

Figure 2.1 Picture of *Centella asiatica* (L.) Urb.

2.1.Botanical description

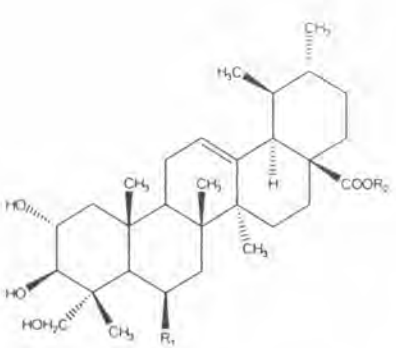
A slender trailing herb. Stems long, prostrate, emerging from the leaf-axils of a vertical rootstock, filiform, often reddish, with long internodes and rooting at the nodes; leaves thin, long-petioled, several from the rootstock and 1-3 from each node

of the stems, 1.3-6.3 cm diameter, orbicular reniform, more or less cupped, entire, crenate or lobulate, glabrous; petioles very variable in length, 7.5-15 cm long or more, channelled; stipules short, adnate to the petioles forming a sheathing base **(4-6)**.

2.2.Chemical constituents

2.2.1 Triterpenes

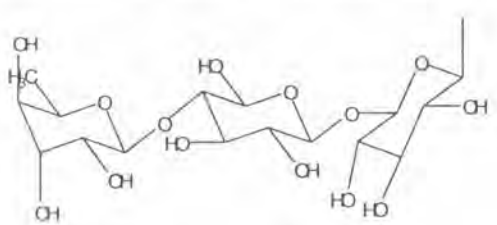
The major principles in CA are the triterpenes asiatic acid and madecassic acid, and their derived triterpene ester glycosides, asiaticoside and madecassoside **(6, 9-12)**.



•Triterpenes (ursane type)
R_2 = H

– R_1 = H , Asiatic acid ($C_{30}H_{48}O_5$)
– R_1 = OH , Madecassic acid ($C_{30}H_{48}O_6$)

R_2 =



(glucose-glucose-rhamnose)
– R_1 = H , Asiaticoside($C_{48}H_{78}O_{19}$)
– R_1 = OH , Madecassoside ($C_{48}H_{78}O_{20}$)

Figure 2.2 The structure of major triterpenes from *Centella asiatica* (L.) Urb.

2.2.2 Essential oil

The aerial parts of CA contains 0.1% of essential oil which compose of 80% sesquiterpenoids such as β-caryophyllene, α-humulene and germacrene-D, elemene and bicycloelemene, trans-farnesene **(13)**.

2.2.3 Flavone derivatives

Kaempferol-3-glucoside and quercentin-3-glucoside have been found **(14)**.

2.2.4 Phytosterols

Stigmasterol, sitosterol have been found **(15)**.

2.2.5 Amino acids

Free amino acids found in the leaf and stem are glutamate, serine and alanine. Amino acids mostly found in the root are aspartate, glutamate, serine, threonine, alanine, lysine, histidine and amino butylate **(4, 14)**.

2.3 Medicinal uses

2.3.1 Uses supported by clinical data

Treatment of wounds, burns, and ulcerous skin aliments, and prevention of keloid and hypertrophic scars **(1-2)**. The extracts of CA have been employed to treat second- and third-degree burns. Extracts have been used topically to accelerate healing, particularly in cases of chronic postsurgical and post-trauma wounds **(1-2)**. Extracts have been administered orally to treat stress induced stomach and duodenal ulcers **(3)**.

The extracts of CA, determined as triterpenes have been found to improve nervous disorders as followed; dementia, cognition enhancer, improvement of learning and memory, soothe anxiety and boost mental function **(6)**.

2.3.2 Uses described in pharmacopoeias and in traditional systems of medicine

CA is reported to be used in the treatment of leprous ulcers and venous disorders. Studies suggest that extracts of CA cause regression of inflammatory infiltration of the liver in cirrhosis patients. Further experimentation is needed to confirm these findings **(4-6)**.

2.3.3 Uses described in folk medicine, not supported by experimental or clinical data

Therapy of albinism, anaemia, asthma, bronchitis, cellulite, cholera, measles, constipation, dermatitis, diarrhea, dizziness, dysentery, dysmenorrhoea, dysuria, epistaxis, epilepsy, haematemesis, haemorrhoids, hepatitis, hypertension, jaundice, leukorrhoea, nephritis, neuralgia, rheumatism, smallpox, syphilis, toothache, urethritis, and varices; and as an antipyretic, analgesic, anti-inflammatory agent **(6)**.

2.4 Experiment and clinical pharmacology

The pharmacological activity of CA has been proven to be due to several saponin glycosides and their aglycone, including asiaticoside, madecassoside, asiatic acid, madecassic acid **(6)**. *In vitro*, each of these compounds stimulated the production of human collagen I, a protein involved in wound healing. Stimulation of collagen synthesis in foreskin fibroblast monolayer cultures by an extract from CA has also been reported. Asiaticoside accelerated the healing of superficial postsurgical wounds and ulcers by accelerating cicatricial action. Asiaticoside stimulates the epidermis by activating the cells of the malpighian layer in porcine skin, and by keratinization *in vitro*. Topical application of asiaticoside promoted wound healing in rats and significantly increased the tensile strength of newly formed skin. Extracts of CA, and in particular its major triterpene ester glycoside, asiaticoside, are valuable in the treatment of hypertrophic scars and keloids. Asiaticoside has been reported to

decrease fibrosis in wounds, thus preventing new scar formation. The mechanism of action appears to be twofold: by increasing the synthesis of collagen and acidic mucopolysaccharides, and by inhibiting the inflammatory phase of hypertrophic scars and keloids. It has further been proposed that asiaticoside interferes with scar formation by increasing the activity of myofibroblasts and immature collagen **(16)**.

In clinical trials, an extract of CA in a 1% salve or 2% powder accelerated healing of wounds. A formulation containing asiaticoside as the main ingredient healed 64% of soiled wounds and chronic or recurrent atony that was resistant to usual treatment. In an open clinical study, treatment of 20 patients with soiled wounds and chronic or recurrent atony with a galenical formulation containing 89.5% CA healed 64% and produced improvement in another 16% of the lesions studied. Local application of an extract of the drug to second- and third-degree burns expedited healing, prevented the shrinking and swelling caused by infection **(6)**.

Twenty-two patients with chronic infected skin ulcers were treated with a cream containing a 1% extract of CA. After 3 weeks of treatment, 17of the patients were completely healed and the ulcer size in the remaining 5 patients was decreased. Another trial using the same cream preparation demonstrated similar results. A standardized extract of CA was reported to treat ulcus cruris (indolent leg ulcers) effectively in clinical trials. In a double-blind study, no significant effect on healing was observed in patients with ulcus cruris after oral treatment with asiaticoside **(6)**.

Oral administration of CA or asiaticoside and potassium chloride capsules was reported to be as effective as dapsone therapy in patients with leprosy. In a controlled

study of 90 patients with perforated leg lesions owing to leprosy, application of a salve of the plant produced significantly better results than a placebo **(6)**.

Extracts of CA effectively treated stress-induced stomach and duodenal ulcers in humans. Oral administration of CA extracts to rats produced a dose-dependent reduction in stress-induced gastric ulceration, and the antiulcer activity was similar to that of famotidine. The mechanism of action appears to be associated with a central nervous system-depressant activity of CA, owing to an increase in the concentration of GABA (γ-aminobutyric acid) in the brain **(17)**.

Clinical trials of the drug have demonstrated its antiulcer activity after oral administration. Fifteen patients with peptic or duodenal ulcer were treated with a titrated extract of CA (60.0mg/person). Approximately 93% of the patients exhibited a definite improvement in subjective symptoms and 73% of the ulcers were healed as measured by endoscopic and radiological observation **(6)**.

Clinical studies of CA in the treatment of various venous disorders have demonstrated a positive therapeutic effect. In patients suffering from venous insufficiency who were treated with a titrated extract of the drug, venous distension and edema improved significantly, as compared with controls **(6)**.

2.5 Overview of the techniques of isolation of analytes from plant material

Qualitative and quantitative analysis concludes a procedure of sample preparation. The preparation of crude extracts from plants is the starting point for the isolation and purification of chemical constituents present in vegetable tissues.

Extraction and product recovery are the most imperative steps in the evaluation of target molecules from various plant parts. In the last decade there has been an increasing demand for new extraction techniques, amenable to automation, with shortened extraction times and reduced organic solvent consumption-preventing pollution in analytical laboratories and reducing sample preparation costs.

Stirring extraction process using different concentration of solvents have also been used and it was found that the presence of water increase the extraction power of the solvents. Stirring extraction methods have been applied for the extraction of biological active compounds. The extraction of biological active compounds by Soxhlet has been used with several solvents. This method takes a few hours, even more than 24 hours for extraction and large amount of solvents wasted. Soxhlet extraction has also been applied for various compounds from plant materials **(7)**.

Advance techniques in sample preparation such as microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE) and supercritical fluid extraction (SFE) have been developed to apply in plant extraction. The similarity between these techniques is the possibility of working at elevated temperatures and pressures, which drastically improves the speed of the extraction process **(8)**.

Recently, there have been several reports on the application of ultrasonic methods and microwave methods in the extraction of various phytochemicals. These methods required shorter extraction time, less solvents, higher extraction rate and better products with lower costs than other conventional extraction methods.

2.5.1 Ultrasonic-assisted extraction (UAE)

2.5.1.1 Basic principle of UAE

Ultrasounds are waves with frequencies ranging from 16 kHz to 1 GHz, inaudible to humans. Ultrasonic vibrations are the source of energy facilitating the release of some analytes from the sample matrix. The improvement in extraction efficiency due to ultrasound appears at certain values of so-called acoustic pressure. Among the most important phenomena taking place in the acoustic field is: cavitation (generation and collapse of mostly empty cavities), friction at the boundary and interfacial surfaces, and increase in the diffusion rate of analytes **(7-8, 18)**.

The cavitation mechanism involves two types of physical phenomena: the extracting solvent can be diffused through the cell walls of plant tissues and washing out (rinsing) the cell contents once the walls are broken. Both phenomena are significantly affected by ultrasonic irradiation **(7)**.

Cavitation is the most significant phenomenon, because it has a direct effect on a number of phenomena occurring in a liquid subjected to ultrasound. Cavitation involves the formation of pulsating bubbles as a result of strong stretching forces, originating from abrupt local pressure drops. At constant ultrasound intensity, dynamic equilibrium is established between the forming and collapsing bubbles. The process of generation and collapse of the

cavities actively interacts with the liquid/solid boundary surface, thus enhancing the erosion processes of solids **(18)**.

The average time of ultrasonic extraction typically ranges from a few to 30 min, although it can be as long as 70 min. The recoveries obtained during this time are comparable to those obtained after a dozen or so hours of Soxhlet extraction, carried out at the same temperature. The extraction conditions can be optimized with respect to time, polarity and amount of solvent, and the mass and kind of sample. The advantage of this technique is the possibility of extraction of many samples at once in an ultrasonic bath. The extraction is carried out at room temperature, which makes it suitable for the extraction of thermally labile analytes. The need for separation of the extract from the sample following the extraction is a disadvantage of this technique.

2.5.1.2 Applications of UAE

A number of articles have been published dealing with the ultrasonically assisted extraction of different vegetal materials. One of the first citations concerning ultrasonic extraction (1952) was related to hop extraction in an aqueous medium and showed that ultrasonic extraction was comparable with the boiling extraction process. It was shown that during ultrasonic extraction it was possible to save some 30-40% of hops in the production of beer. Several references concerning ultrasonically assisted extraction are summarized in Table 2.1 as followed:

Analyte	Sample matrix	Recovery [%]	Time [min]	References
Alkaloid drugs	Cinchona bark Nux vomica seeds Rauwolfia roots		90 20 15	**(18)**
Cobalamins	Biological samples	94.8-101.1	1	**(19)**
Tartaric and malic acid	Grapes		30	**(20)**
Isoflavones	Soybeans	100	20	**(21)**
Isoflavonoids	Root	83	60	**(22)**
Flavonoids	Plant extract	91.2-95.6	60	**(23)**
Polysaccharides	Buckwheat hulls	147	70	**(24)**
Volatile compounds	Citrus flowers and honey		10	**(25)**
Aroma compounds	Aged brandies	45-113	30	**(26)**
Steroids and triterpenoids	Stems, leaves and flowers		30	**(27)**
Antioxidants	Herbs		60/45	**(28)**
Anticancer (camptothecin)	*Nothapodytes foetida*		30	**(29)**

Table 2.1 Substances isolated using ultrasonic-assisted extraction

Examples of substances isolated by UAE along with extraction time and recoveries are summarized as above; it should be proven to be a powerful tool for the phytopharmaceutical extraction industry. Although ultrasonic can be enhanced the extraction, it is worth noting that when high frequency ultrasound is employed, the extraction yield did not increase significantly however the degradation of the herb constituents was diminished. The appropriate frequencies of ultrasound usually used in UAE were ranging from 16 kHz to 500 kHz **(30-31)**.

It is obvious that reducing the size of vegetal material particles will increase the number of cells directly exposed to extraction by solvent and thus exposed to ultrasonically induced cavitation. This effect can be utilized by milling the material before extraction. It should be borne in mind however that powerful sonication cab itself serve to mill the vegetal material.

In laboratory scale, ultrasonic-assisted extraction can easily perform using the simple ultrasonic cleaning bath as shown in Figure 2.3

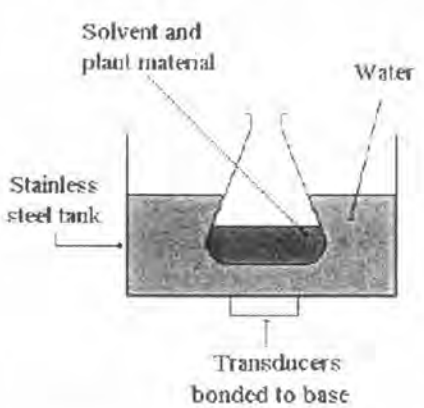


Figure 2.3 Experiment setup for UAE using an ultrasonic bath **(18)**

The experiment as shown in Figure 2.3 applied the ultrasound by indirect method, only small amounts of vegetal material can be extracted, whereas using the direct method such as the ultrasonic probe (horn), large amounts of vegetal material can be employed and scaled up; thus UAE is a versatile technique that can be used both on a small and large scale **(32)**.

2.5.2 Microwave-assisted extraction (MAE)

2.5.2.1 Basic principle of MAE

Microwaves are electromagnetic radiations with a frequency from 0.3 to 300 GHz. Domestic and industrial microwaves generally operate at 2.45 GHz. Microwaves are transmitted as waves, which can penetrate biomaterials and interact with polar molecules such as water in the biomaterials to create heat. Consequently, microwaves can heat a whole material to penetration depth simultaneously **(33)**.

MAE is based on absorption of microwave energy by polar molecules. The energy absorbed is proportional to the dielectric constant of the medium, resulting in rotation of dipoles in an electric field. The hot solvent allows rapid isolation of thermally stable analytes. The efficiency of MAE depends on solvent properties, sample material, the components being extracted, and specifically on the respective dielectric constants. The higher dielectric constant, the more energy is absorbed by the molecules and the

faster the solvent reaches the boiling point. In most cases, the extracting solvent has a high dielectric constant and strongly absorbs microwave radiation **(7-8, 33)**.

There are two types of commercially available MAE systems: closed extraction vessels under controlled pressure and temperature, and focused microwave-assisted extraction (FMAE) using microwave ovens at atmospheric pressure. The closed MAE system is generally used for extraction under drastic conditions such as high extraction temperature. The pressure in the vessel essentially depends on the volume and the boiling point of the solvents. The FMAE system can be operated at a maximum temperature determined by the boiling point of the solvents at atmospheric pressure **(34)**.

Solvent	Dielectric constant [a]	Dipole moment [b]	Boiling point [c] (°C)	Closed-vessel temperature[d](°C)
Acetone	20.7	-	56	164
Acetonitrile	37.5	-	82	194
Ethanol	24.3	1.96	78	164
Hexane	1.89	-	69	-
Methanol	32.6	2.87	65	151
2-Propanol	19.9	1.66	82	145
Water	78.3	2.3	100	-
Hexane-acetone (1:1)	-	-	52	156

[a] Determined at 20 (°C), [b] Determined at 20 (°C),

[c] Determined at 101.4 kPa., [d] Determined at 1207 kPa.

Table 2.2 Physical constants of some solvents commonly used in MAE

Major advantages of MAE include: shortened extraction time, reduced size of extraction apparatus, ease of control of sample heating, reduced amount of solvent used. The limitation of this technique, when applied to extraction of non-polar analytes from non-polar materials, is the need for using solvents with dipole moments greater than zero (*n*-hexane or *iso*-octane can be replaced by dichloromethane or a mixture of acetone and *n*-hexane) (33).

2.5.2.2 Applications of MAE

The main advantage of MAE respect to the other techniques is both the considerable reduction in time and smaller solvent consumption, if compared to conventional extraction. In recent years many reports have been published on the application of MAE to the extraction of active compounds from plants.

MAE can extract nutraceutical products from plant sources in a faster manner than conventional solid-liquid extractions. Several references concerning microwave assisted extraction are summarized in Table 2.3 as followed:

Analytes	Matrix	Solvent	Extraction time (min.)	Recovery (%)	Ref.
Camptothecin	*Nothapodytes foetida*	90% methanol	3	-	**(29)**
Triazines	Freshly spiked And aged soils	CH_2Cl_2-MeOH (9:1)	20	89-103	**(35)**
Imidazolinones	Spiked plant tissue	Water	3	97-103	**(36)**
Chloramphenicol	Eggs	ACN: IPA	10 sec.		**(37)**
Sulphamethazine	Swine tissue	MeOH	25 sec.		**(38)**
Taxane	*Taxus* biomass	95% ethanol	10	90	**(39)**
Felodipine	tablets	5%MeOH in ACN	-	100	**(40)**
Puerarin	*Radix puerariae*	-	1	100	**(41)**
Capsaicinoid	*Capsicum* fruit	-	15	95	**(42)**
Artemisinin	*Artemisia annua*	-	12	92.1	**(43)**
Glycyrrhizic acid	*licorice* root	Ethanol-water	4 -5	90	**(44)**
Polyphenols and caffeine	Green tea leaves	-	4	91	**(45)**
Ginsenosides	*ginseng* root	Ethanol-water	15	-	**(46)**
Coumarin	Flower of *Melilotus officinalis*	50%aqueous ethanol	5	-	**(47)**

Table 2.3 MAE applications of organic compounds reported in literature

MAE described as Table 2.3 can be also reduced the solvent consumption, shorter extraction time and greater extraction yield than other extraction methods such as reflux, soxhlet, and UAE **(48)**.

MAE in plant materials was also found that the presence of water in the solvent of methanol had a beneficial effect and allowed faster extractions than with organic solvent alone.

MAE has been considered as a potential alternative to traditional solid-liquid extraction for the extraction of metabolites from plants. MAE is also comparable to other modern extraction techniques such as supercritical fluid extraction (SFE) due to its process simplicity and low cost. By considering economical and practical aspects, MAE is a strong novel extraction technique for the extraction of nutraceuticals **(48-49)**.

However, the efficiency of microwaves can be very poor when either the target compounds or the solvents are non-polar, or when they are volatile. For the extraction of thermo labile compounds, high temperatures may cause the degradation of extracts, thus can be avoided excess temperature that produced by closed MAE system. In this case, the FMAE has been chosen to solve degradation because it can be operated the extraction at the boiling point of the solvents **(34)**.

FMAE was performed in an open cell at a frequency of 2450 MHz. The commercial apparatus such as Soxwave 100 (Prolabo), its schematic diagram is presented in Figure 2.4.

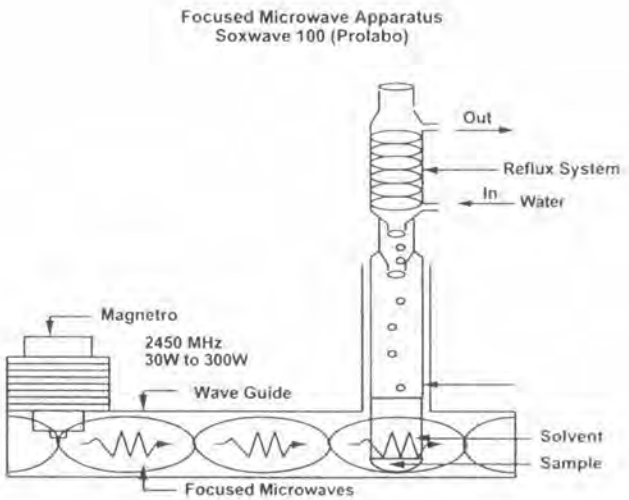


Figure 2.4 Experiment setup for FMAE **(34)**

This method is safe, due to the extraction is performing at atmospheric pressure and no risk of explosion is involved. The temperature of extraction is the boiling point of the solvent. There is no problem of compound degradation by high temperature; thus can be applied to the extraction of many compounds from various matrices **(34, 50)**.

2.5.3 Fractional crystallization-based on separation processes

Crystallization is one of the most powerful purification methods available in industrial applications. Fractional crystallization is a stepwise separation technique that relies upon liquid-solid phase transition and enables multicomponent mixtures to be split into narrow fractions, ultimately leading to top purities of selected components, through the virtue of selectivity found in solid-liquid equilibrium **(51)**.

The crystallization stages are usually accompanied by various techniques, including cooling, evaporation, reactions, changing the quantity of solvent, and using some of the components as pseudosolvents (salting-out) to obtain the desired separation **(51)**.

The use of other agents such as solvents for decreasing the solubility has also awakened interest in method for obtaining separation of some species, a technique known as drowning-out.

Separation and purification of chemical compounds and active compounds from medicinal herbs by fractional crystallization have been presented in many publications as followed;

Shiengthong, Kokpol, and Karntiang **(52)** isolated tetracyclic triterpenes; aglaiondiol and two isomers of aglaitriol from the light petroleum extract of leaves *Aglaia odorata*. The isomers of aglaitriol were separated by fractional crystallization of the triacetates which on hydrolysis gave the epimers.

Holmback and Rasmuson **(53)** studied the size of benzoic acid crystals produced by drowning-out crystallization in semi-batch experiments. It is found that benzoic acid crystals grown from ethanol-water solutions range from needles to platelets. The mean size ranges from 69 to 218 μm. while adding water in saturated ethanol-water solutions.

Qiang Nie, Jingkang Wang, and Qiuxiang Yin **(54)** reported the separation and purification of two isomorphic steroidal pharmaceutical intermediates. The extraction and crystallization process are conducted in

the same crystallizer simultaneously. The two immiscible (partial miscible) liquid phases were achieved by adding water into the toluene-DMF system. The advantage of this method is that two relative pure product can be achieved in a crystallization process.

Compare to other separation and purification processes, the fractional crystallization can be reduced number of stages due to high selectivity, promoted the consistence of separation and reduced thermal degradation due to lower operating temperatures; thus can be operated for large-scale production.

2.6 Overview of the determination of active constituents in CA

Over the last 15 years, several methods for determination of active constituents in CA have been reported in many publications. Inamdar **(10)** determined AS, MS, AA, and MA in the crude plant extracts and preparations by HPLC system using C18 column as the stationary phase and acetonitrile-water gradient system as the mobile phase.

British pharmacopoeia **(5)** established TLC method to identify AS in CA extracts using TLC silica gel G plate as stationary phase, a mixture of 11 volumes of acetic acid, 11 volumes of formic acid, 27 volumes of water and 100 volumes of ethyl acetate as mobile phase. TLC spots were detected with anisaldehyde solution and heated at 100°C to 105 °C. Furthermore, the determination of four active constituents in CA were examined by HPLC

system using C18 column as the stationary phase, a gradient mobile phase of acetonitrile, and water (0.3% phosphoric acid), and UV detection at 200 nm.

Schaneberg et al. **(55)** improved the HPLC qualitative and quantitative method of six triterpenes in CA (raw plant material and preparations). After 50 minutes the six active triterpenes were separated and detected in the methanolic extract at a limit of 0.01 μg/ml. The method uses C18 column as the stationary phase, a gradient mobile phase of water, acetonitrile, and methyl tert-butyl ether, and UV detection at 206 nm.

Aziz et al. **(56)** reported the distribution of AS and MS in 2 types (heavily fringed-leaf margin and smooth-leaf margin) of Malaysian CA. Chemical analysis was performed using gradient HPLC with acetonitrile: water as the mobile phase.

Schieffer **(57)** reported the use of SPE for solving interference problems in the HPLC assay. Anion-exchange SPE is used to remove an interfering compound coeluting with triterpenoid glycosides in CA. The HPLC system was developed from BP (2002) using the gradient mobile phase of 0.3% aqueous phosphoric acid/ acetonitrile.

Several gradient elution methods for determination the active compounds in CA have also been reported. However, this technique is usually suffered from drift and unstable baseline during the chromatograph. Bungon Kongthong **(58)** developed and validated a suitable analytical method for determined AS, MS, AA, and MA in the extracts of CA. An isocratic reversed phase HPLC system comprises of C18 column as the stationary phase, a 29:71

mixture of acetonitrile: phosphate buffer (10mM, pH 7.1) as the mobile phase, and detection with photodiode array at 210 nm. Furthermore, the TLC-densitometer was also developed to determine AS and MS by using silica gel plate GF_{254} as the stationary phase and chloroform: methanol: water (30:15:2) as the developing solvent. The TLC spot was detected with anthrone reagent and scanned with densitometer at wavelength 525 nm.