CHAPTER III

Isolation of Glycosides and Other Natural Compounds from Plants using Electrolytic Decolourization

1. Glycyrrhizin from *Glycyrrhiza radix* (Licorice)

1.1 Introduction

Glycyrrhiza radix (Licorice) (Figure 1) is a perennial herb native to southern Europe, Asia and the Mediterranean. Licorice is the name applied to the roots and stolons of some *Glycyrrhiza* species and has been used as a crude drug worldwide from ancient times. The herb is extensively cultivated in Russia, Spain, Iran and India. Licorice is one of the most popular and widely consumed herbs in the world. Although many know this herb for its flavoring in candy, licorice contains many health benefits. Ancient cultures on every continent have used licorice, the first recorded use by the Egyptians in the 3rd century BC[1].

Figure 1. *Glycyrrhiza radix* (Licorice)

The Egyptians and the Greeks recognized the licorice herb's benefits in treating coughs and lung disease. Licorice is the second most prescribed herb in China

followed by ginseng. It is suggested for treatment of the spleen, liver and kidney. This plant is also a source of food sweetnener and a colouring agent used for food production in China.[2] Licorice is widely used in Japan and is reported to have benefits in the treatment of chronic hepatitis, especially in the treatment of hepatitis B*. The most common medical use for licorice is for treating upper respiratory ailments including coughs, hoarseness, sore throat, and bronchitis.

Licorice can be as effective as codeine, and safer, when used as a cough suppressant. Rhizomes in licorice have a high mucilage content which, when mixed with water or used in cough drops, sooths irritated mucous membranes. The drug also has an expectorant effect which increases the secretion of the bronchial glands. Licorice is an effective remedy for throat irritations, lung congestion, and bronchitis. Homeopathic use of licorice for gastric irritation dates back to the first century. Rever, a Dutch pharmacist, noted that a proprietary drug containing mainly licorice extract was very popular with patients suffering from stomach condition in his era. They reported that their stomach symtoms were getting much better, and that the effect was more reliable and longer-lasting than with all other medicines they had tried before. The investigations confirmed the usefulness of the drug, and a report was published.[3] The active principle appears to be glycyrrhizic acid. Rarely used alone, licorice is a common component of many herbal teas and is used as a mild laxative, a diuretic, and for the treatment of flatulence. Licorice has also been known to relieve rheumatism and arthritis, regulate low blood sugar, and is effective for Addison's disease. The licorice root extract produces mild estrogenic effects, and it has proven useful in

---

* Description; Hepatitis A (HAV) : HAV is usually not serious. It is transmitted through oral-fecal contact (rimming, eating contaminated raw food). Hepatitis B (HBV) : More than 80% of those with HIV are infected with HBV. The reason is that HBV infects in the same way as HIV infects, that is through body fluids (semen, blood). HBV is likely to be chronic (not curable). Hepatitis C (HCV) : Hemophiliacs and IV drug users (IDU) are the more likely to have HCV than gay/bisexual men. HCV causes serious liver problems (cirrhosis, liver failure). About half of the HCV cases are chronic (not curable). Being HIV+ can make HCV infection more dangerous than it otherwise might be. Hepatitis D (HDV, Delta) HDV is never found unless HBV is also present. HDV is an aggressive disease that is extremely dangerous. Hepatitis G (Non-A Non-B Non-C Hepatitis, NANBNC)

treating symptoms of menopause, regulating menstruation, and relieving menstrual cramps.

The main constituent found in the root is glycyrrhizin. The plant also contains various sugars (14%), starches (30%), flavonoids, saponoids, sterols, amino acids, gums, and essential oil.[1b]

The constituent glycyrrhizin is 50 times sweeter than sugar, making it a widely used ingredient in the food industry. The distinctive flavour of licorice makes it a popular additive to baked confectionaries, liqueurs, ice cream and candies. Licorice is also widely used in other medicines to mask bitter tastes and also to prevent pills from sticking together.

Licorice has also been used in poultices for treatment of dermatitis and skin infections. It helps to open the pores and is used in combination with other cleansing and healing herbs as an emollient. For the common use, licorice is an ingredient in many cough medicines and a popular and well known remedy for bronchial distress. Licorice can have a beneficial effect on gastric disturbances.

The main ingredient in licorice, glycyrrhizin, stimulates the secretion of the adrenal cortex hormone aldosterone[1b] and has also been studied for its anti-viral properties in the treatment of AIDS[4] and may enhance the production of natural killer cells and interferon.[5] In clinical trials in Japan it prevented progression of the HIV virus by inhibiting cell infection and inducing interferon activity. Preliminary evidence on orally administered licorice has also found it to be safe and effective for long-term treatment of HIV infection.[6]

Japanese researchers recently studied the effects of a glycyrrhizin compound, Stronger Neo-Minophagen C (SNMC) in 42 HIV-seropositive hemophiliacs.[7] Participants were randomized to dose regimens of either 100 to 200 ml or 400 to 800 ml administered intravenously daily for the first three weeks and every second day for the following eight weeks. Absolute CD4 counts and CD4/CD8 ratios were unchanged. However, "complete recovery in liver dysfunction," a major problem in HIV-positive hemophiliacs, was reported. The authors conclude that HIV-infected hemophiliacs with impaired immunological ability and liver dysfunction be given prophylactic treatment with SNMC to prevent their conditions from worsening.

At the IX International Conference on AIDS in Berlin, two small, non-randomized studies of glycyrrhizin in asymptomatic HIV-positive individuals suggested some benefits to the treatment.[8-9] However, these studies, both of which were conducted in Japan, are difficult to analyze or draw any conclusions, due to the small size and the extremely limited data that were published.

There are reports of glycyrrhizin causing high blood pressure, water retention, and possibly heart complications when taken in very high doses.[10]

Application of glycyrrhizin derivatives has been reported including glycyrrhetic acid, a pentacyclic triterpenoid obtained by mineral acid hydrolysis of glycyrrhizin present in liquorice root. When glycyrrhetic acid was tested for its oestrogenic activity,[11] it was found that it posses an oestrogenic action when tested on uterine weight of immature mice. The estrogenic action of 5 mg of glycyrrhetic acid corresponds approximately to that of 0.1 microgram of oestradiol. Moreover, glycyrrhetic acid acts synergistically with the female hormone, estradiol, on uterine development when combined together.

Glycyrrhizin also encourages the production of hormones such as hydrocortisone which give it anti-inflammatory properties. Like cortisone it can relieve arthritic and allergy symptoms, without the side effects.[12-13] Also, glycyrrhizin and its aglycone, glycyrrhitinic acid, have anti-ulcerous,[14] anti-viral[15] and interferon-inducing[16] activities. It has been reported that part of the anti-inflammatory activity of glycyrrhizin may be attributable to inhibition of prostaglandin E2 production.[17] Although glycyrrhizin prevents viral growth of several DNA and RNA viruses,[15] including varicella-zoste virus (VZV)[18] and human immuno-deficiency virus [HIV CHTLV/LAV)],[19] its anti-viral mechanism remains to be elucidated.

There are many components in licorice, but the most predominant is glycyrrhizin (**1**) (Figure 2). The other components are saponins, flavonoids, coumarins, sterols, choline, triterpenoids, lignins, amino acids including asparagine, gums, biotin, folic acid, inositol, lecithin, estrogenic substances, pantothenic acid, para-aminobenzoic acid, phosphorous, pentacyclic terpenes, protein, sugar, a yellow dye, and vitamins B1, B2, B3, B6 and E.[20-22] Moreover, glycyrrhizin derivatives, 3-*O*-[β-D-glucuronopyranosyl-(1→2)-β-D-glucuronopyranosyl]-liquiritic acid (**2**), and their derivatives glucosylated at the C-4 position of the terminal glucuronopyranose

with additional one [(**3**) and (**4**)] and two sugar units [(**5**) and (**6**)], repectively[23] were also found in the licorice extract.

Glycyrrhizin derivatives that are commercially available, include dipotassium glycyrrhizinate, glycyrrhetinic acid and stearyl glycyrrhetinate.

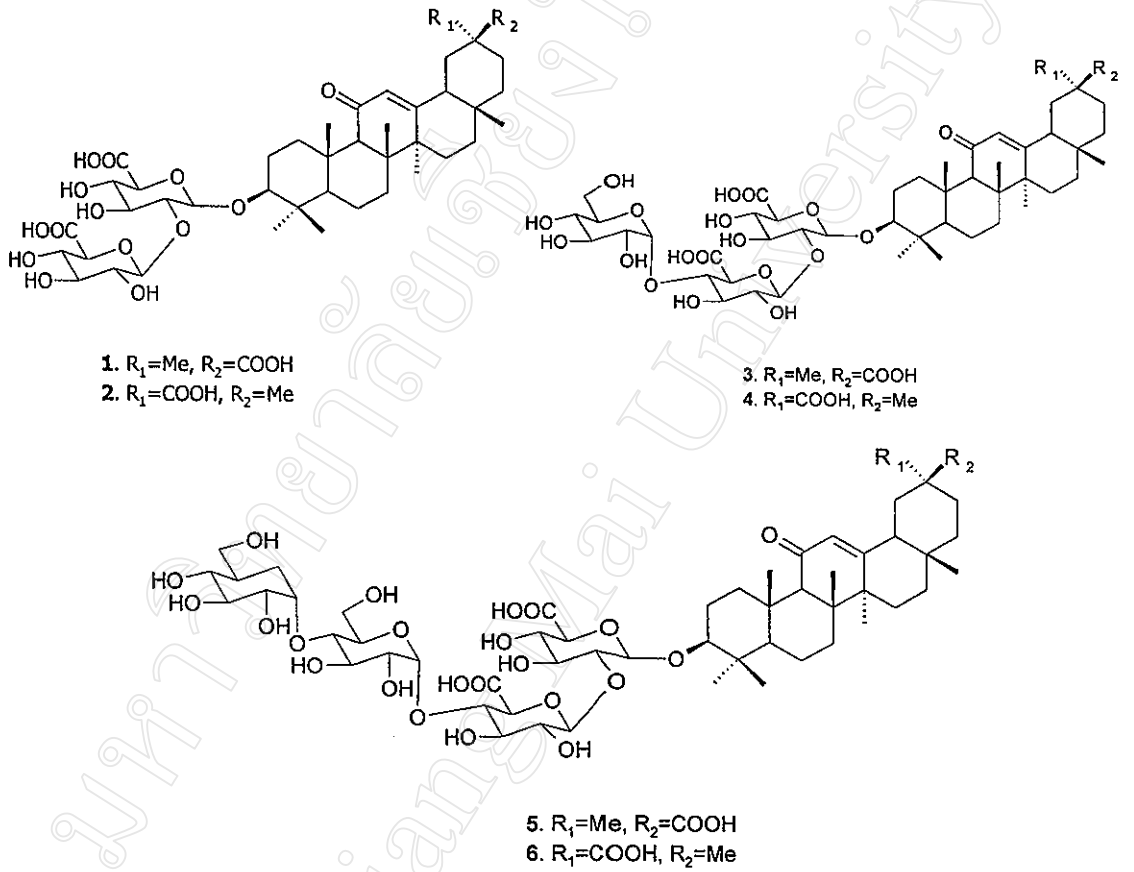


Figure 2. Structures of compounds **1-6:** Glycyrrhizin (**1**), 3-*O*-[*β*-D-glucuronopyranosyl-(1→ 2)–*β*-D-glucuronopyranosyl]liquiritic acid (**2**) , 3-*O*-[α-D-glucopyranosyl-(1→4)–*β*-D-glucuronopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]glycyrrhetinic acid (**3**), 3-*O*-[α-D-glucopyranosyl-(1→4)–*β*-D-glucuronopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-liquiritic acid (**4**), 3-*O*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-*β*-D-glucurono pyranosyl-(1→2)-*β*-D-glucuronopyranosyl]glycyrrhetinic acid (**5**), 3-*O*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-*β*-D-glucuronopyranosyl-(1→2)-*β*-D-glucuronopyranosyl] liquiritic acid (**6**).

1.2 Results and Discussion

Due to the interesting bioactivities of glycyrrhizin, a number of works describing the isolation and purification of glycyrrhizin has appeared in the literature.[24-28] In this chapter, a simple method of isolating glycyrrhizin from licorice is described, using an electrolytic decolourization technique, modified from that of the Adduci *et al.*[29] and Kunihiko *et al.*[30], who used this method for partial purification of a crude aqueous *Stevia* extract.

It was found that after using the electrolytic decolourization technique, glycyrrhizin, from *Glycyrrhiza radix*, was obtained in 2-5 % yield as white crystals after recrystallizing from acetic acid. Identification was carried out mainly by analyzing its chromatographic properties using HPLC.

An HPLC condition for isolation and purification of glycyrrhizin has appeared in the literature which is shown in Figure 3.(GL Science HPLC company)

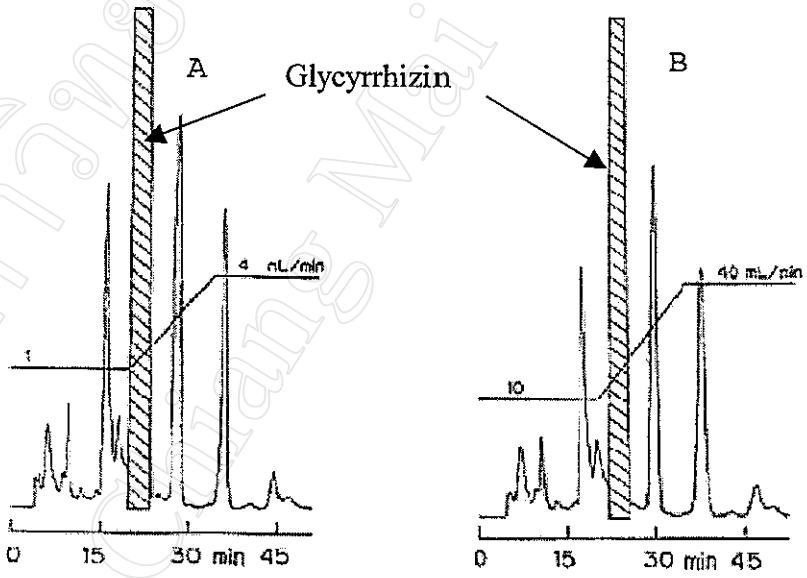


Figure 3. Crude glycyrrhizin analysis chromatogram, data from GL Science HPLC company, Column : Inertsil PREB-ODS 10 μ m; Eluent : CH_3CN/10% CH_3COOH = 35/55; Flow rate : Flow gradient; Detector : UV 254 nm; Column size : A, 250 x 6.0 mm I. D., B, 250 x 20 mm I. D.; Flow rate : A, 1.0 → 4.0 ml/min, B, 10 → 40 ml/min; Sample size : A, 300 μ L, B, 3000 μL.

The HPLC conditions in Figure 3 have been done by scientist from Simmasu (Thailand) company using different samples in this study. An investigation has also been done on the crude licorice extract by HPLC analysis using conditions provided by the HPLC column company (Shodex Asahipak), the results of this are shown in Figure 4.

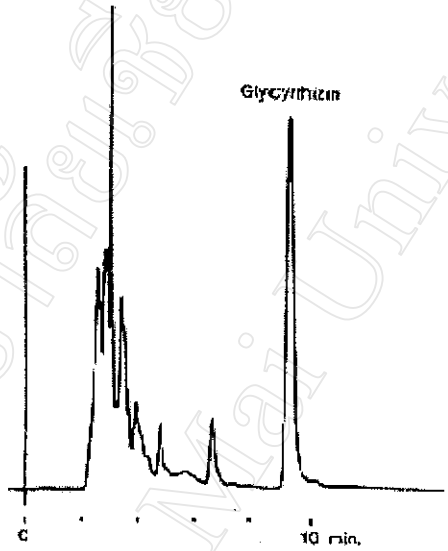


Figure 4. Crude licorice extract without electrolytic decolourization. HPLC operating conditions were: detection wavelength UV 248 nm; column Shodex Asahipak ODP-50 6D, 4.6ID × 150 mm; eluting solvent 0.05% TFA/CH_3CN (62:38) ; flow-rate 1.0 ml/min ; retention time for glycyrrhizin 9.8 min.

However, since only a C-18 HPLC column was available for these experiments, the investigation had been carried out using a new set of HPLC operating conditions. The HPLC analysis of a licorice extract from the electrolysis process and an authentic sample of glycyrrhizin was operated (Figure 5). However, it could be seen that the glycyrrhizin isolated by using electrolytic decolorisation technique still had some impurities (Figure 5b). It was found that the purity of the extracted product after electrocoagulation was 86-88%. However, a further 9-10% of impurities could be eliminated by ion exchange (Figure 5c).

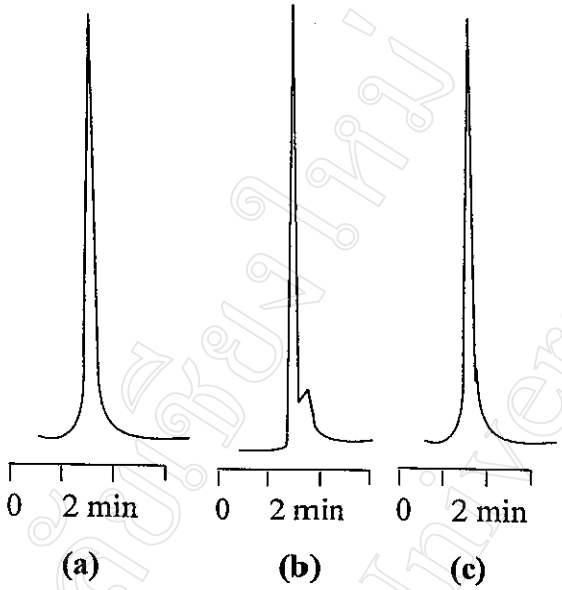
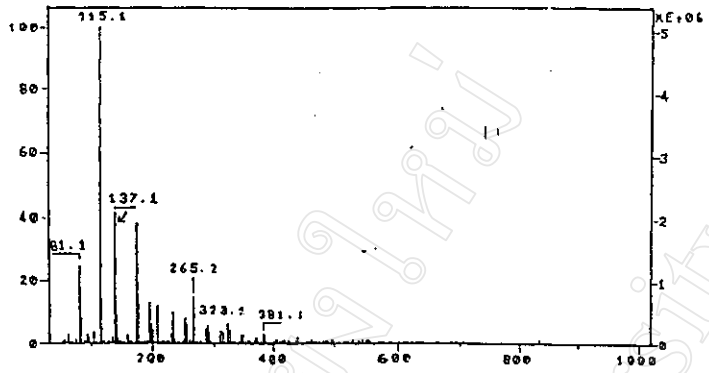
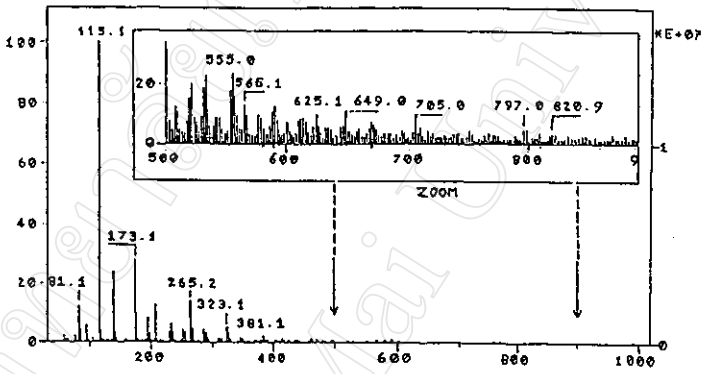


Figure 5. (a). Standard glycyrrhizin (b). Isolated glycyrrhizin after electrolytic decolourization (small peak is impurity) (c). Isolated glycyrrhizin after electrolytic decolourization and resin ion-exchange (small peak is impurity). HPLC conditions; detection wavelength 248 nm; column Inertsil (C18) ODS-3, 4.6ID × 150 mm; eluting solvent CH_3CN : H_2O (65:35) ; flow-rate 0.5 ml/min ; retention time for glycyrrhizin 2.6 min.

The molecular formula of the extracted product was established as $C_{42}H_{62}O_{16}$ [$(M+H)^+$, *m/z* 823 and $(M-H)^+$, *m/z* 821] by high-resolution fast-atom bombardment (FAB) mass spectrometry, identical to that of authentic glycyrrhizin (Figure 6).



a. FAB/MS Positive LR : Mode CI +VE+ LMR BSCAN (EXP) UP LR



b. FAB/MS Positive LR : Mode CI +VE+ LMR BSCAN (EXP) UP LR

Figure 6. a. Mass spectrum of extracted product, b. Mass spectrum of standard sample.

This isolation method of glycyrrhizin is safe and simple, having an important advantage of forgoing the use of expensive and toxic organic solvents and reagents, as well as using a minimum number of expendable common chemicals namely sodium chloride (for electrolysis), hydrochloric acid and sodium hydroxide (for resin regeneration). The ordinary aluminium sheets used as electrodes in the electrolysis step can be reused for a few successive operations, during which they are slowly worn out and finally replaced.

1.3 Experimental

Plant material.

Licorice roots were collected in Chiang Mai, and identified by the Department of Biology of Chiang Mai University, where a specimen is deposited at its herbarium.

Glycyrrhizin.

A standard sample of glycyrrhizin was purchased from Merck as the ammonium salt form as a pale yellow powder. It was converted to the acid form by passing through a column of a mixed-bed ion-exchange resin (Amberlite MB-1) using water as eluent.

Hot Water Extraction.

Water (1,000 ml) at 90-100 °C was used to extract 50 g of the dried roots for 30 min, whereupon, after one coarse filtration, 900 ml of aqueous extract was obtained.

Decolourization by Electrolysis.

A direct current (0.5 ampere, 22 volt) was passed for 2 hours *via* two aluminium plate electrodes (dimension 30 x 10 x 0.05 cm) immersed 3 cm apart in the aqueous extract in a glass tank (34 x 31 x 21 cm), into which 1.8 g of NaCl had been added. The resulting mixture was then filtered. The aqueous solution (760 ml) obtained was subjected to a second electrolysis for 1 1/2 hour under the same condition. After filtration, a clear pale yellow solution (630 ml) was obtained, which, after evaporation to dryness, gave a yellow solid. Recrystallization from glacial acetic acid gave a pale yellow solid (1.2-2.76 g, 2-5% yield). Analysis by HPLC indicated a glycyrrhizin content of 86-88% (indicated using the peak areas and assuming the impurity had the same UV extinction coefficient at 248 nm as glycyrrhizin) (Figure 5b).

Decolourization and Demineralisation by Ion Exchange.

In another experiment, the doubly-electrolysed solution, after filtration, was passed through a column of a mix-bed ion-exchange resin (Amberlite MB-1), and the resulting clear, colourless eluate of conductivity < 50 $\mu S\ cm^{-1}$ was collected. The volume of the mixed resin required to achieve this was 1 liter. After evaporation to dryness, the eluate gave a pale yellow solid and recrystallization in glacial acetic acid

gave a white solid (1.2g, 2% yield). Analysis by HPLC indicated a glycyrrhizin content of 95-98% (indicated using the peak areas and assuming the impurity had the same UV extinction coefficient at 248 nm as glycyrrhizin) (Figure 5c).

Operating conditions for HPLC were : detection wavelength 248 nm; column Inertsil (C18)ODS-3, 4.6ID × 150 mm; eluting solvent CH_3CN : H_2O (65:35) ; flow-rate 0.5 ml/min ; retention time for glycyrrhizin 2.6 min.

Operation for MS used the particle bombardment method, FAB (Fast Atom Bombardment) using glycerol as a matrix. (FAB/MS Finnigan MAT 90. LSIMS = 20 kV. Cs^+ as the particle beam.)

FAB-MS m/z (% relative intensity) : Extracted product, 81 (25), 115 (100), 137 (42), 173 (38), 265 (18), 323 (8), 381 (5). Standard sample, 81 (15), 115 (100), 137 (25), 173 (29), 265 (18), 323 (5), 381 (3).

1.4 References

1. a. W. J. Craig, *J. Am. Diet. Assoc.*, 1997, **97**, 10.
   b. http: // www.go-symmetry.com/licorice.htm
2. S. Foster and Y. Chongxi, *Herbal emissaries,* Healing Arts Press Company, Rochester, 1972.
3. J. G. G. Borst, S. P. ten Holt, L. A, de Vries, J. A. Molhuysen, *Lancet*, 1953, **264**, 657.
4. E. De Clercq, *Med. Res. Rev.*, 2000, **20**, 323.
5. M. Ito, A. Sato, K. Hirabayashi, *Antivir. Res.,* 1988, **10**, 289.
6. K. Mori, et al, *J. Naturopathic Medicine*, 1993, **4**, 2-9.
7. I. Hattori, S. Ikematsu, A. Koito, *et al*, *Antivir. Res.*, 1989, **11**, 255–62.
8. B. Scott-Harland, *GMHC: Treatment issue*, 1993/94, 7.
9. J. S. James, AIDS Treatment News, http://www.aids.org/immunet/atn.nsf/page/a-181-04.
10. http://63.75.36.151/education/alter/Glycyrrhizin.html.
11. S. Korsia, *IHITTG.*, 1992, **7**, 3.
12. A. Sharaf, N. Gomaa, M. H. A. El Gamal, *Nat. Res. Cent.*, 1975, **16**, 245.
13. R. S. H. Finney, G. F. Somers, *J. Pharmacol.,* 1959, **10**, 613.

14. Y. Fujisawa, M. Sakamoto, M. Matsushita, T. Fujita, K. Nishioka., *Microbiol. Immunol.,* 2000, **44**, 799.

15. R. Doll, I. D. Hill, C. Hutton, D. L. Underwood, *Lancet.,* 1979, **11**, 793.

16. R. Pompei, O. Flore, M. A. Marccialis, A. Pani, B. Loddo, *Nature*, 1979, **281**, 689.

17. N. Abe, T. Ebina, N. Ishida, *Microbiol. Immunol.,* 1982, **26**, 535.

18. K. Ohuchi, Y. Kamada, L. Levine, S. Tsurufuji, *Prostaglandin and Medicine.*, 1981, **7**, 457.

19. M. Bada, S. Shigeta, *Antiviral Res.*, 1987, **7**, 99.

20. M. Ito, H. Nakashima, M. Bada, R. Pauwels, E. De Clercq, S. Shigeta, N. Yamamoto, *Antiviral Res.,* 1987, **7**, 127.

21. M. T. Murray, *The Healing Power of Herbs, Prima Publishing*, 2nd ed., Rocklin, California, 1995, pp.228-239.

22. O. Penelope, *The Complete Medicinal Herbal, Dorling Kindersley*, New York, 1993, p. 65.

23. J. F. Balch, A. Phyllis, *Prescription for Nutritional Healing*, Avery Publishing Group, New York, 2nd ed., 1997, p.73.

24. Hong-Min Liu, N. Sugimoto, T. Akiyama, T Maitani, *J. Agric. Food Chem.*, 2000, **48**, 6044.

25. C. Karrer, *Helv. Chim. Acta.,* 1921, **4**, 100.

26. H. Nishizawa, S. Okimura, Y. Watanabe, Y. Abe, *Chem. Pharm. Bull.*, 1991, **39**, 969.

27. H. Nishizawa, S. Okimura, Y. Abe, *Analytical Science*, 1992, **8**, 367.

28. H. Nishizawa, T. Shoji, S. Okimura, Y. Abe, *Analytical Science*, 1995, **11**, 109.

29. J. Adduci, D. Buddhasukh, B. Ternai, *J. Sci. Soc. Thailand.,* 1987, **13**, 179.

30. M. Kunihiko, M. Seiji, M. Yasuo, *Patent Japan Kokai Tokyo Koho*, 1979, JP 7990199; 17 July 1979, 4pp; through *Chem.Abstr.* 91: 173681e.

2. D-Pinitol from *Cassia siamea* Lamk

2.1 Introduction

Cassia siamea Lamk. (Synonyms : *Senna siamea* Lamk, C. *florida Vahl.*; *Senna sumatrana*, Roxb.) (Figure 1) is a non-nitrogen-fixing leguminous tree in the subfamily Caesalpinoideae of the family Leguminosae.[1] It has been widely planted in many Southeast Asian countries for erosion control, windbreaks, shelterbelts, fuelwood, and polewood. It is known as "Kheelek" in Thai. It is a good ornamental tree for planting along roadsides, and it is also used in alley cropping, intercropping, and as hedge rows. It is planted as a shade tree in cocoa, coffee, and tea plantations. It is commonly called Thailand shower, minjiri, or kassod and has many regional names.[2] It is native to South and Southeast Asia, from Thailand and Myanmar to Malaysia, India, Sri Lanka, and Bangladesh.[3] It has been cultivated worldwide and is naturalised in many locations.[4]

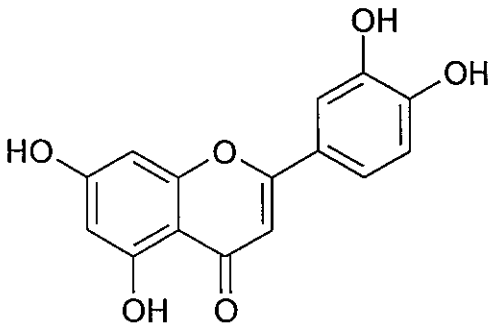
Figure 1. *Cassia siamea* Lamk.

Cassia siamea Lamk is normally a medium sized evergreen tree attaining 5 m height.[2] It has a dense, evergreen, irregular, spreading crown, a crooked stem, and smooth, grayish bark that is slightly fissured longitudinally. Its young branches have fine hairs. The leaves are pinnately compound with an even leaf arrangement of 7-10 pairs of ovate-oblong leaflets, 7-8 cm long and 1-2 cm wide. Its flowers are yellow, with in large terminal panicles that are often 30 cm long. The flowering period is

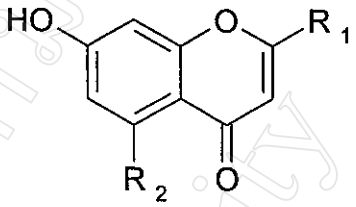
long, and flowers may often be found at various seasons.[6] The fruit is a flat pod 15-25 cm long, thickened at both sutures, containing many seeds.[4,5]

The heartwood is dark, almost black, with yellow streaks that is suitable for furniture, turnery and cabinet work. The leaves and pods are browsed by ruminants but highly toxic to pigs and possibly to other monogastrics. The heartwood has laxative properties and is used for a variety of ailments, of blood forming organs, genito-urinary tract, also for herpes and rhinitis. The leaves are said to be poisonous.[13]

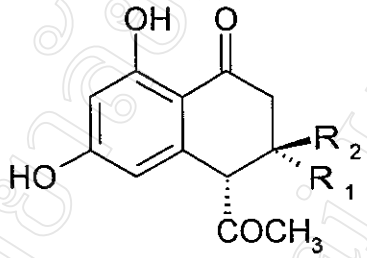
The leaf has been reported to contain anthraquinones, alkaloids, flavonoids, chromones, and terpenoids.[7,8] Polysaccharides also have been isolated from seed endosperms of *Cassia siamea* Lamk.[9] The flowers contain β-sorbiterol, lupeol, and sucrose.[10] The flowers also have been found to contain D-pinitol, and four compounds, m.p. 205-6°, 207-8°, 212-14°, and 216-17°, of which the first three are phenolic in nature.[10] The flowers also contain glucose, fructose, traces of arabinose in the free form, and two oligosaccharides, one consisting of glucose and arabinose while the second consisted of glucose, xylose, arabinose, and glucuronic acid.[10] The trunk bark of this plant contains β-sitosterol, lupeol, and oleanolic acid and considerable amounts of sucrose.[11] It also contains luteolin (**1**) (Figure 2), and four other compounds (**2-5**), one compound being identified as cassia chromone (5-acetonyl-7-hydroxy-2-methylchromone) (**2**). Three other compounds were new, and they were identified as 5-acetonyl-7-hydroxy-2-hydroxymethyl-chromone (**3**), 4-(*trans*)-acetyl-3,6,8-trihydroxy-3-methyldihydronaphthalenone (**4**), and 4-(*cis*)-acetyl-3,6,8-trihydroxy-3-methyldihydro- naphthalenone (**5**).[12]



1



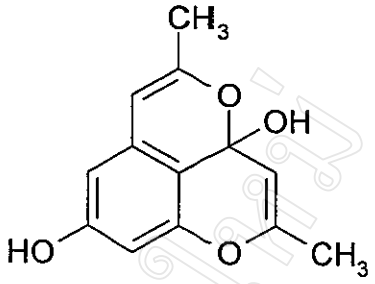
2 $R_1=CH_3$, $R_2=CH_2COCH_3$
3 $R_1=CH_2OH$, $R_2=CH_2COCH_3$



4 $R_1=CH_3$, $R_2=OH$
5 $R_1=OH$, $R_2=CH_3$

Figure 2. Structures of compounds (**1**)-(**5**), luteolin (**1**), cassia chromone (5-acetonyl-7-hydroxy-2-methylchromone) (**2**), 5-acetonyl-7-hydroxy-2-hydroxymethyl-chromone (**3**), 4- (*trans*)-acetyl-3,6,8-trihydroxy-3-methyldihydronaphthalenone (**4**), and 4-(*cis*)-acetyl-3,6,8- trihydroxy- 3-methyldihydronaphthalenone (**5**).

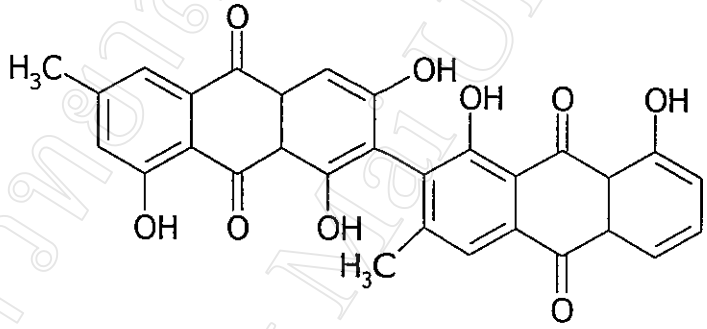
Barakol[14,15], 3a,4-dihydro-3a,8-dihydroxy-2,5-dimethyl-1,4-dioxaphenalene (**6**), is also found in this plant. It is an active chemical in the alcoholic extract of *Cassia siamea*. It was shown to be a dopamine agonist and have possible serotonergic antagonist properties.[16]



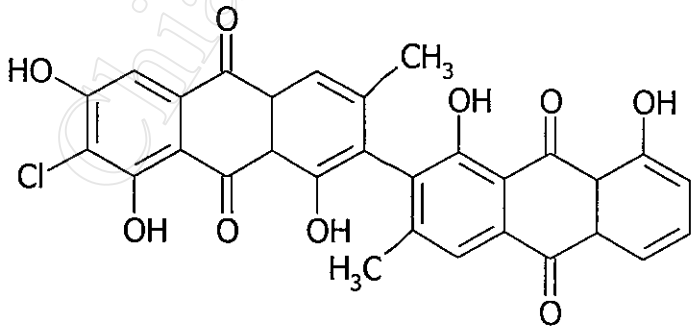
6

Figure 3. Barakol

The isolation of two bianthraquinones, 1,1',3,8,8'-pentahydroxy-3',6 dimethyl[2,2' bianthracene]-9,9',10,10'-tetrone (**7**) and 7-chloro-1,1',6,8,8'-pentahydroxy-3,3'-dimethyl[2,2'-bianthracene]-9,9',10,10'-tetrone (**8**), from the root bark of *Cassia siamea* has also been reported.[13]



7



8

Figure 4. Structures of compounds (7) and (8)

The water soluble compounds, among which include pinitol (Figure 5), are the most interesting as far as the electrolytic decolourization isolation method is concerned.

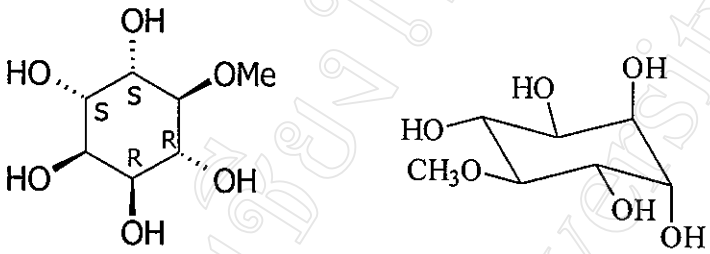


Figure 5. D-Pinitol, (D- Inositol, 3-O-methyl)

Pinitol is a polyol that occurs naturally in alfalfa (*Medicago sativa*), pine-tree shoots and soybeans (*Glycine max*) and other legumes.[17] It is found in many plants and is a major component in soybean plants. It is named "pinitol" merely because it was first isolated from a pine tree species.[17] It is widely distributed in plants.[17] This sweetener imparts no calories and may even positively influence blood-sugar metabolism.[17]

Recent *in vitro* and *in vivo* studies have shown that the oral and injectable forms of pinitol enhance the action of insulin and therefore the uptake of glucose in immature rat muscle cells and in mice with type I-like (but not type II) diabetes.[17]

However, to date, the only published human study was not definitive. The study tested the effects of an oral dose of 9.1 mg/pound body weight/day of soy-derived pinitol.[18] In the trial, conducted at Washington University School of Medicine in St. Louis, 22 subjects who were obese and had mild type II diabetes or impaired blood-sugar control were given doses of pinitol for 28 days. Although pinitol demonstrated no adverse effects, it performed no better than the placebo in improving the actions of insulin in carbohydrate or fat metabolism.

Pinitol belongs to a family of compounds called inositols. Pinitol is released from phospholipids in the cell membrane and then transported into cells where the

conversion of glucose to energy and glycogen synthesis occurs.[19] Recent clinical trials have indicated that this relatively new and excellent supplement has a lot of potential for athletes and individuals involved in a fitness routine. People involved in fitness may be interested in this supplement, since it has been shown to decrease stored fat and decrease elevated blood sugar levels.[19]

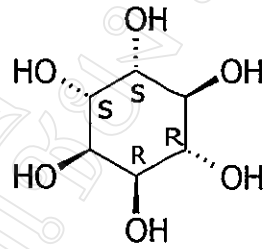


Figure 6. Structure of an inositol

Pinitol, has been shown to improve glucose metabolism by lowering blood glucose levels to 15%-20% on average, improving both the uptake and utilization of glucose in the cells.[20] Pinitol acts similarly to insulin via its ability to promote glucose transport and glycogen synthesis, allowing the body's insulin levels to gradually fall, decreasing insulin resistance. Once insulin sensitivity is restored, the body can then work more efficiently to improve endurance, and increase energy for people who are determined to transform their bodies.[20]

Pinitol was tested to assess the toxicity in rats following a single dose administration. Mortality was not observed in any male or female rats at any dose level. Under the conditions of the acute toxicity assay described in the report of the study, pinitol did not induce toxic effects and was concluded to be totally nontoxic.[21]

Pinitol was isolated from several plants[22-28] which are usually extracted with organic solvents and subjected to chromatographic purification but no separation has been reported from the leaves of *Cassia siamea* Lamk,[29] especially by the decolourization technique. This chapter demonstrates that electrocoagulation technique can be a valuable method for isolation of certain water-soluble natural compounds such as pinitol.

2.2 Results and Discussion

Using the electrolytic decolourization technique on the crude aqueous extract of the dried leaves from *Cassia siamea* Lamk, D-pinitol, was obtained in 1 % yield as white crystals after recrystallizing from MeOH. Characterization was carried out mainly by analyzing its spectroscopic properties. The IR spectrum exhibited a strong broad hydroxyl group (3406 cm^{-1}) absorption band. The ^{1}H-NMR spectra indicated the presence of five hydroxyl groups at δ 4.72, 4.63, 4.52, 4.48 and 4.35, which was confirmed by the HMQC technique (Figure 7): no cross-peaks appeared in this part of the spectrum. The ^{1}H-NMR spectrum showed only one methoxy group at δ 3.45.

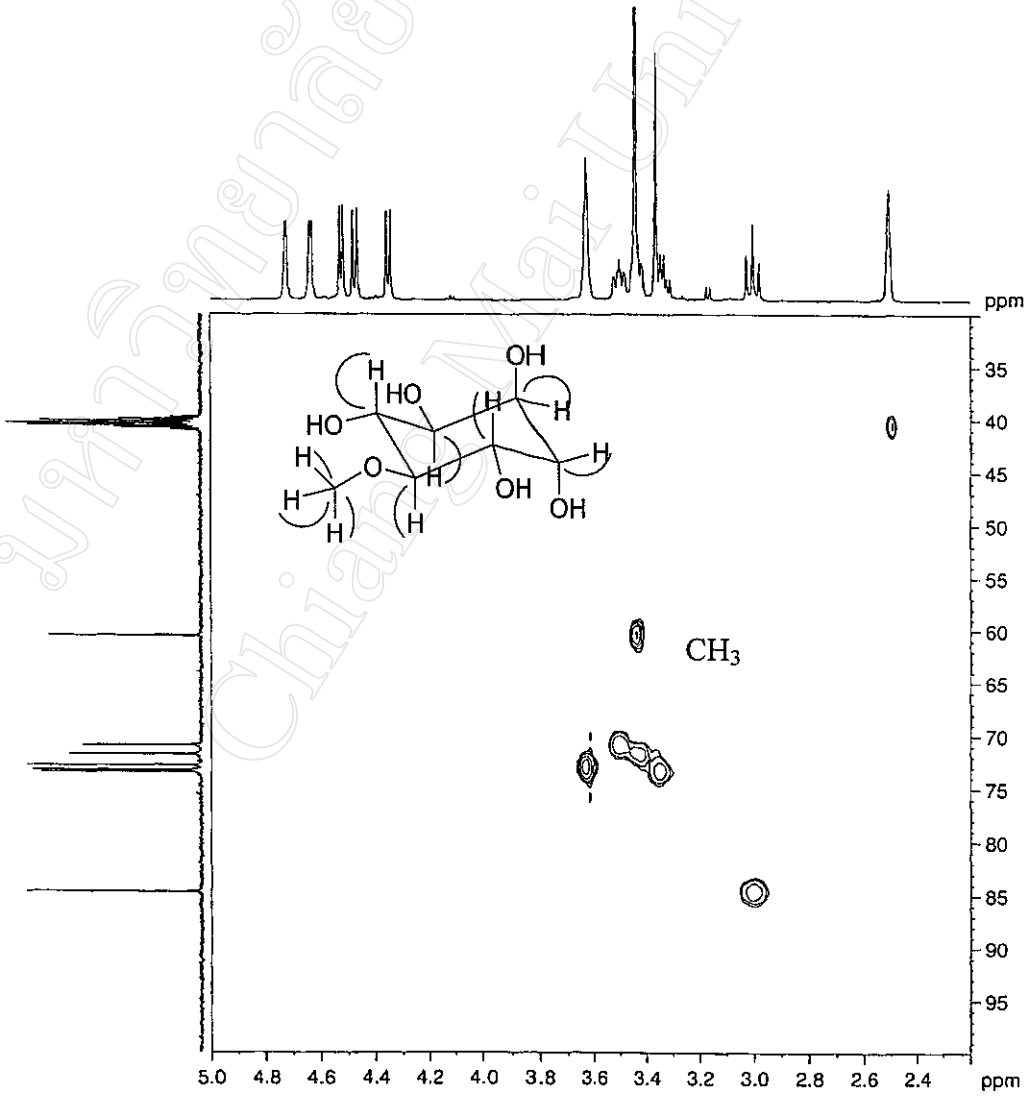


Figure 7. The 400 MHz HMQC spectrum of pinitol (DMSO)

The ^{13}C-NMR spectrum (Figure 9) afforded 7 lines, indicating no symmetrical carbons within the molecule. The ^{13}C-NMR and the DEPT 135° spectra also showed the presence of seven protonated carbon signals that were either CH_3 and CH resonance being positive, with no CH_2 signals on negative peaks. The type of carbons in the pinitol molecule were identified as shown in Table 1. DEPT-90° showed only one methyl group, with only one peak disappearing, which supported the ^{1}H-NMR spectral data for one methoxy group (see ^{1}H, ^{13}C on Figure 8, 10 and also ^{1}H-NMR COSY, Figure 11). The GC-MS (CI) gave *m/z* 194 as M^+ and *m/z* 87 as the base peak. The molecular formula of the extracted product was also established as $C_7H_{14}O_6$ by CHO analysis. The experimental melting point (183-185 °C) was nearly identical to the literature value (185-186 °C).[2-7] This isolation method of D-Pinitol was found to be simple, having an important advantage of forgoing the use of expensive and toxic organic solvents and reagents.

Table 1. The 100 MHz ^{13}C NMR (DMSO) spectral data of pinitol

Line	δ_C (ppm)	Type of carbon	Assignment
1	84.8	CH	C-6
2	73.5	CH	C-5
3	73.2	CH	C-4
4	72.8	CH	C-1
5	71.8	CH	C-3
6	70.8	CH	C-2
7	60.5	CH_3	CH_3

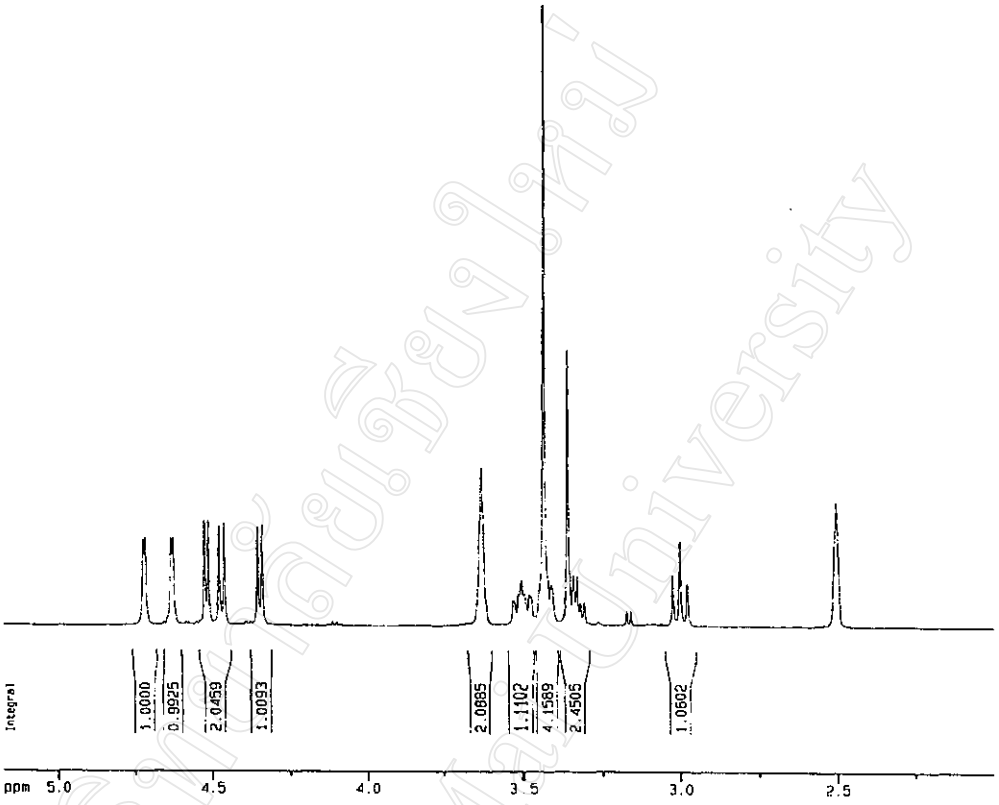


Figure 8. The 400 MHz ^{1}H-NMR spectrum of pinitol (DMSO)

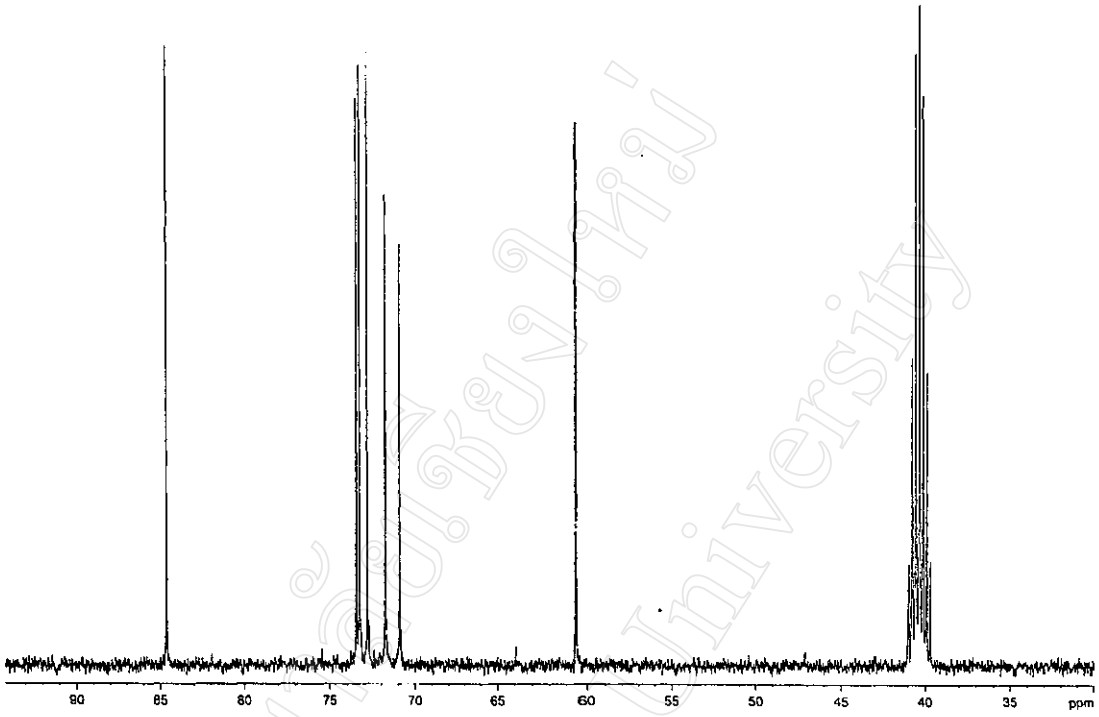


Figure 9. The 100 MHz ^{13}C-NMR spectrum of pinitol (DMSO)

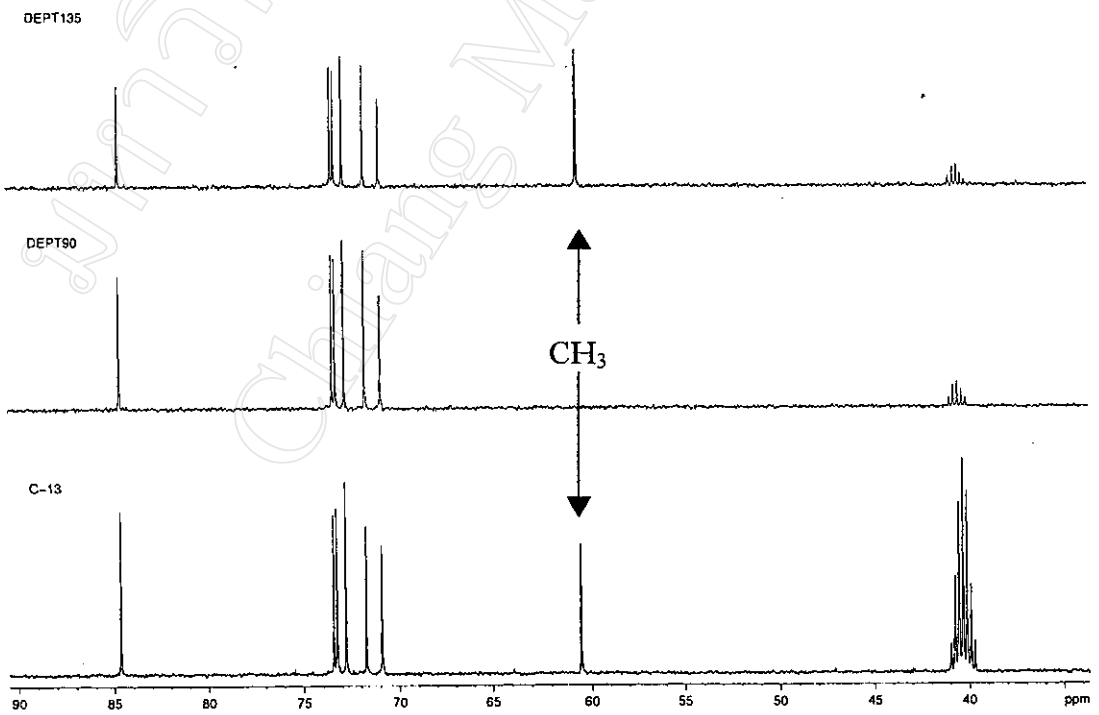


Figure 10. The 100 MHz DEPT spectrum of pinitol (DMSO)

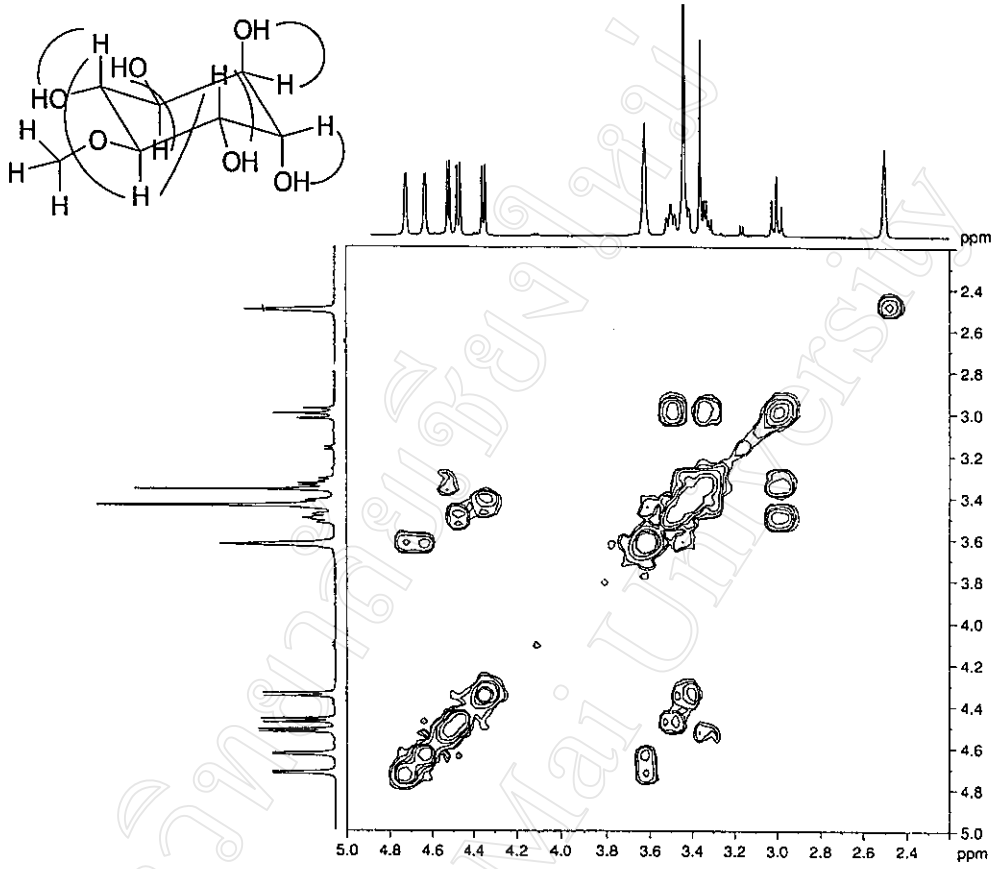


Figure 11. The 400 MHz COSY spectrum of pinitol (DMSO)

2.3 Experimental

Instrumentation.— ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) experiments, including the COSY, HMQC, and DEPT techniques, were carried out on a Bruker AM 400 spectrometer in DMSO solution ; IR spectrum was recorded on a Perkin Elmer FT-IR spectrometer ; Mass spectrum was recorded on a Perkin-Elmer GC-MS(CI) ; Elemental analysis was carried out on a Perkin Elmer Elemental analyzer 2400.

Plant material.— *Cassia siamea* Lamk leaves were collected in Chiang Mai, and identified by the Department of Biology of Chiang Mai University, where a specimen was deposited at its herbarium.

Hot Water Extraction. —Water (1,000 ml) at 90-100 °C was used to extract 50 g of the dried leaves for 30 min, whereupon, after one coarse filtration, 900 ml of aqueous extract was obtained.

Decolourization by Electrolysis. —A direct current (0.5 ampere, 22 volt) was passed for 2 hours *via* two aluminium plate electrodes of suitable dimensions (10 x 30 cm) immersed 3 cm apart in the aqueous extract in a glass tank (34 x 31 x 21 cm), into which 1.8 g of NaCl had been added. The resulting mixture was then filtered. The aqueous solution (760 ml) obtained was subjected to a second electrolysis for 1 hour and 30 minutes under the same condition. After filtration, a clear pale yellow solution (630 ml) was obtained.

Demineralisation by Ion Exchange. —The doubly-electrolysed solution, after filtration, was passed through a column of a mix-bed ion-exchange resin (Amberlite MB-1), and the resulting clear, colourless aqueous solution of conductivity < 50 μS cm^{-1} was collected. The minimum volume of the mixed resin required to achieve this was 1 liter. After evaporation to dryness, the eluate gave a colorless liquid which crystallized in methanol as a white solid (0.5g, 1 % yield).

D-Pinitol: white solid (MeOH), mp. 183-185° C (lit.[7] mp. 185-186° C) ; $[\alpha]^{23}_D$ +69° (c, 1.3 in H_2O) (lit[4], $[\alpha]^{20}_D$ +60° (c, 6.5 in H_2O)) ; IR (KBr) ν_{max} 3406, 2907, 1460, 1120, 1069, 690 cm^{-1} ; MS *m/z* (% relative intersity) 116 (6), 103 (7), 87 (100), 73 (92), 60 (20), 45 (20) ; ^{1}H-NMR (400 MHz, DMSO) δ 3.05 (1H, t, 3-CH), 3.34 (1H, t, CH), 3.45 (3H, s, OCH_3), 3.42-3.46 (1H, m, CH), 3.51 (1H, t, CH), 3.63 (2H, s (broad), CH), 4.35, 4.48, 4.52, 4.63, 4.72 (1H, d, OH) ; ^{13}C-NMR (100 MHz, DMSO) δ 60.5 (CH_3), 70.8 (C-2), 71.8 (C-3), 72.8 (C-1), 73.2 (C-4), 73.5 (C-5), 84.8 (C-6).

2.4 References

1. http://www.winrock.org/forestry/factpub/FACTSH/S_siamea.html
2. Forestry/Fuelwood Research and Development Project (F/FRED), *Growing Multipurpose Trees on Small Farms*, 1994, Module 9: Species Fact Sheets, 2nd ed., Winrock Intemational, Bangkok, Thailand, , 1994, p. 127.

3. M. S. Khan, M. K. Alam, *Homestead flora of Bangladesh*, Bangladesh Agricultural Research Council, Farmgate, Dhaka, Bangladesh, 1996, p. 275.
4. R. C. Gutteridge, *Plant Resources of South-East Asia*, I. Faridah Hanum, L.J.G. van der Maesen, eds., No. I 1. Auxiliary plants, Backhuys Publishers, Leiden, the Netherlands. 1997, pp. 232-36.
5. M. Jensen, *Trees Commonly Cultivated in Southeast Asia - an illustrated field guide,* RAP Publication:1995/38, FAO, Bangkok, Thailand. 1995, p. 93.
6. R. S. Troup, *The Silviculture of Indian Trees*, vol. 11,Clarendon press, Oxford, 1921, p. 372.
7. H. Wagner, S. M. El-Sayyad, O. Seligmann, V. M. Chari, *Planta Med.* 1978, **33**, 258.
8. K. M. Biswas, H. Mallik, *Phytochem,* 1986, **25**, 1727.
9. V. P. Kapoor, M. Milas, F. R. Taravel, M. Rinaudo, *Food Hydrocolloids*, 1996, **10**, 167.
10. I. P. Varshney, R. Pal, *Indian J. Pharm.*, 1978, **40**, 15.
11. I. P. Varshney, R. Pal, *J. Indian Chem. Soc.*, 1977, **54**, 548.
12. K. Ingkaninan, A. P. Ijzerman, R. Verpoorte, *J. Nat. Prod.,* 2000, **63**, 315.
13. a. J. Koyama, I. Morita, K. Tagahara, M. Aqil, *Phytochem*, 2001, 56, 849.
    b. http://www.fao.org/ag/AGP/AGPC/doc/Gbase/DATA/Pf000378.HTM
14. W. Thongsaard, S. Chainakul, G. W. Bennett, C. A. Marsden, *J. Pharm. Biomed. Anal.,* 2001, **25**, 853.
15. W. Gritsanapan, B. Tantisewie, V. Jirawongse, *J. Sci. Soc. Thailand.*, 1984, **10**, 189.
16. P. Tongroach, P. Jantaraypta, Proceedings of the 1st JSPS-NRCT Joint Seminar in Pharmaceutical Sciences; Advance in Research on Pharmacologicaly Active Substances from Natural sources. Chiang Mai, Thailand, 1992, p OP21.
17. S. H. Bates, R. B. Jones, C. J. Bailey, *Br. J. Pharmacol.*, 2000, **130**, 1944.
18. A. M. Davis, S. Klein, M. Christiansen, M. K. Hellerstein, J. K. Horowitz, R. E. Jr, Ostlund, *Diabetes Care*, 2000, **23**, 1000.
19. a. C. Narayanan, *Current Science*, 1987, **56**, 139.

b. http://www.bodylogix.com/products/dpinitol.cfm

20. R. S. Ochs, In The Effect Of Pinitol On The Key Aspects Of Carbohydrate Metabolism In Isolated Muscle Tissue And Its Interaction With Insulin. St. John's University (http://www.bodylogix.com/products/dpinitol.cfm).

21. Minneapolis MN Performing Laboratory: MA BioServices, Rockville, MD: July 14, 1998 (http://www.bodylogix.com/products/dpinitol.cfm).

22. D. N. Mondal, B. R. Barik, S. Maiti, *J. Indian. Chem. Soc.* 1993, **70**, 7.

23. L. Pistelli, A. Bertoli, I. Giachi, A. Manunta, *J. Nat. Prod.*, 1998, **61**, 1404.

24. M. John, G. B. Nelson, G. B. Paul, *Industrial Crops and Products,* 1998, **8**, 159.

25. A. Hans, H. Harald, R. Melchior, *Phytochemistry*, 1996, **41**, 907.

26. P. Luisa, P. Stefania, B. Alessandra, P. Donatella, *Phytochem*, 1996, **42**, 1455.

27. M. K. Tsung, A. L. Cadance, C. N. Terry, *Phytochem*, 1997, **45**, 29.

28. M. X. L. Lucia, H. Eberhard, *Phytochem*, 1997, **45**, 431.

29. W. Hidebert, M. E. Samia, S. Otto, C. Mohan, *J. Medicinal Plant Research*, 1978, **33**, 258.

3. Asiaticoside from *Centella asiatica*

3.1 Introduction

Centella asiatica (Figure 1) is found in the marshy areas throughout India and Ceylon. It is also found in subtropical Africa, South America, southern parts of America and also widespread in Southeast Asia.[1]

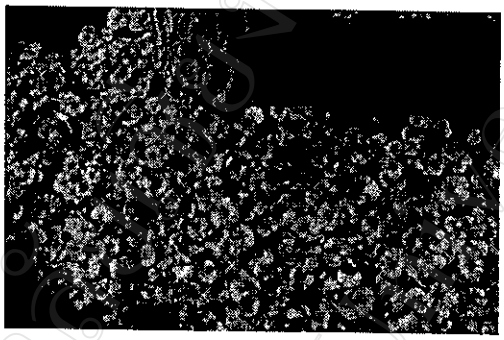


Figure 1. *Centella Asiatica*

The leaves of *Centella asiatica* are used as a vegetable in some Asian countries. In Malaysia, they are steamed and eaten with rice. They are rich in carotenoids, vitamins B and C. The leaves are fed to cows to increase milk yields and also to poultry and rabbits in the Hawaii islands. An ethanolic extract (80 %) of the plant showed the presence of a number of free amino acids.[2]

The plant is valued in indigenous medicine for treatment of leprosy and skin diseases, also for improving memory.[3] In pharmacological, clinical trials it has been found to improve the power of concentration, general ability and behaviour of mentally retarted children. The plant is used as an antidote to cholera.[3] A cold poultice of the fresh herb is used as an external application in rheumatism, elephantiasis and hydrocele. For treating leprosy and other skin diseases it is given as an ointment or dusting powder. Compound proprietory preparations of the plant have been patented for cosmetic use in France.[3] Internally it has been valued as a tonic, is used in bronchitis, asthma, gastric catarrh, leucorrhoea, kidney troubles, urethritis and dropsy. A decoction of very young shoots is given for haemorrhoids.[3]

A syrup of the leaves with ginger and black pepper is taken for cough. The leaf juice with palm jaggery is given to women as a tonic after delivery. The leaf juice is

rubbed on the forehead to cure sever headaches. Mixed with bath water, it is used in eczema. The leaf extract is used in the preparation of a medicated oil for bone fracture. The leaves are diuretic. Pills prepared from the paste of leaves of the plant, *Ocimum Sanctum* Linn and Black pepper are used in the treatment of intermittent fever.[4]

Asiaticoside, a main glycoside in *Centella asiatica,* is useful in the treatment of leprosy and certain types of tuberculosis. Madecassol, an extract of the plant containing the madecassic acid, asiatic acid, asiaticoside accelerates cictrization and grafting of wounds. Madecassoside and madecassic acid also show anti-inflammatory activity. A crude extract of the plant and the glycoside, isothankuniside (asiaticoside) and its derivative methyl-5-hydroxy-3, 6-diketo-23 (or 24)-nor-urs-12-ene-28-oate (BK) exhibit anti-fertilising activity in female mice. An alcoholic extract of the plant showed anti-spasmodic activity on acetylcholine induced contractions of rat ileum, which was attributed to the glycosides. Each of the glycosides (brahmoside, brahminoside, asiaticoside, glycoside D and E) present in the extract is active, the most potent being the new glycoside E.[5]

Amino acid study of the plant indicated that in the leaf, petiole and stolon, the percentage of glutamate and serine is greater compared to other amino acids. The roots are also rich in amino acids, especially in aspartate, glutamate, serine, threonine, alanine, lysine, histidine and amino butyrate.[6]

The leaves are fried and taken for their cooling property. A decoction of the boiled plants is drunk to ease a sore throat. Raw leaves are eaten or a plant decoction is drunk to treat hypertension. Boiled leaves are eaten to prevent or cure urinary tract infections and the growth of kidney stones. Plant decoction taken thrice daily cures skin eruptions. The unfiltered juice of the plant has been found to be particularly efficacious against syphilis and scrofula. The plant has antispasmodic effect. The herbal drug is a powerful stimulant of circulatory system, its action chiefly affecting the cessels of skin and mucous membrane; it is useful in the treatment of some heart diseases. The expressed juice of the plant has shown antifertility activity in experimental animals. The plant also has insecticidal properties.[7]

The plant has shown anticonvulsive activity, beside producing significant alterations in the neurochemistry of the brain. In comparative clinical and instrumental

trial against a placebo, the plant extract was found to improve venous disorders of lower limbs. The clinical trials demonstrated that the herbal drug possess an Ayurvedic medhya rasyana effect (brain invigorating).[7] It was found that the extract increases the intelligence quotient in mentally retarted children. In a clinical trial of 'Mental' (BR-16A), an Ayurvedic drug having *Centella asiatica* as one of the main ingredients, marked improvement is seen in children with behavioural problems. The extract of fresh plant significantly inhibits gastric ulceration induced by cold restraint stress (CRU) in rats.

The plant is greatly valued for its asiaticoside content in leaves. Asiaticoside, a glucoside, has shown encouraging results in the treatment of leprosy. This active principle dissolves the waxy covering of *Bacillus leprae*, so that the casual organism becomes very fragile and maybe easily destroyed.[8]

Asiaticoside is not found in the Sri Lankan variety of *C. asiatica* but a related compound, centelloside, and triterpenic acids, centoic and centellic acids have been reported. It has been shown that, depending on the habitat, the saponins can be of two types, the more common one containing asiaticoside and madecassoside and the less common one showing the additional presence of arabinose in the saponins thus forming brahmoside and brahminoside. The sapogenins and the flavonoid componenets were the same in both varieties.[8-11]

It has not been reported on the isolation of glycoside in this plant using electrocoagulation. The major glycosides of *C. asiatica* is asiaticoside, O-6-deoxy-α-L-mannopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(2α,3β,4α)-2,3,23-trihydroxyurs-12-en-28-oate, a triterpenoid trisaccharide. Asiaticoside is also shown to have a good antioxidant activity [12-13] and antitubercular properties.[14] Many studies have reported methods for its analysis and separation.[15-19] However, there are no reports which use electrocoagulation as a means to separate the compound. This chapter describes the isolation of asiaticoside from *Centella asiatica* by electrocoagulation in order to demonstrate that the technique can be a valuable method for the isolation of certain water-soluble natural compounds.

3.2 Results and Discussion

In this study, electrocoagulation, using sacrificial iron electrodes, has been examined on the aqueous ethanol extracts of *C. asiatica*. Pure asiaticoside, was obtained in 0.025 % yield as white amorphous solid after recrystallising from MeOH-water. Characterisation was carried out mainly by analyzing its spectroscopic properties. It was analyzed for $C_{48}H_{78}O_{19}$ by its ^{13}C NMR data as well as from the ES (positive ion mode)-mass spectrum data at *m/z* 959 $[M]^+$ and 797 $[M\text{-}Rha]^+$. The ^1H and ^{13}C-NMR spectra displayed resonances due to the six methyl signals (four singlets, a broad singlet and a doublet) indicating the present of a Δ^{12}-ursene skeleton. The sugar part of the ^1H NMR spectrum (Figure 5) showed, beside other signals, the three doublets for the anomeric three protons (δ 5.29 *d*, *J*=7.9Hz; 4.84, *d*, *J*=1.5 Hz; 4.37, *d*, *J*=7.9 Hz) suggesting that it contains three monosaccharides with many overlapping multiplets which presented an unambiguous assignment even by performing an ordinary COSY experiment(Figure 4). However, the 2D TOCSY experiment allowed an easy identification of the three sugar spin systems. The sequence of three sugar chains was deduced unequivocally from the HMBC and TOCSY information, as illustrated in Figure 2, 3 and 4.

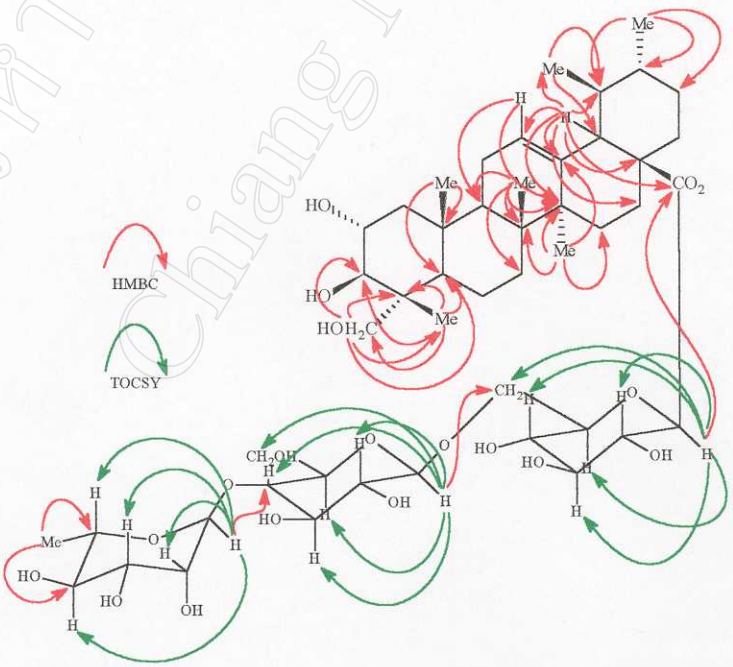


Figure 2. The sequence and linkage position of monosaccharide and triterpenoid established by HMBC and TOCSY experiments.

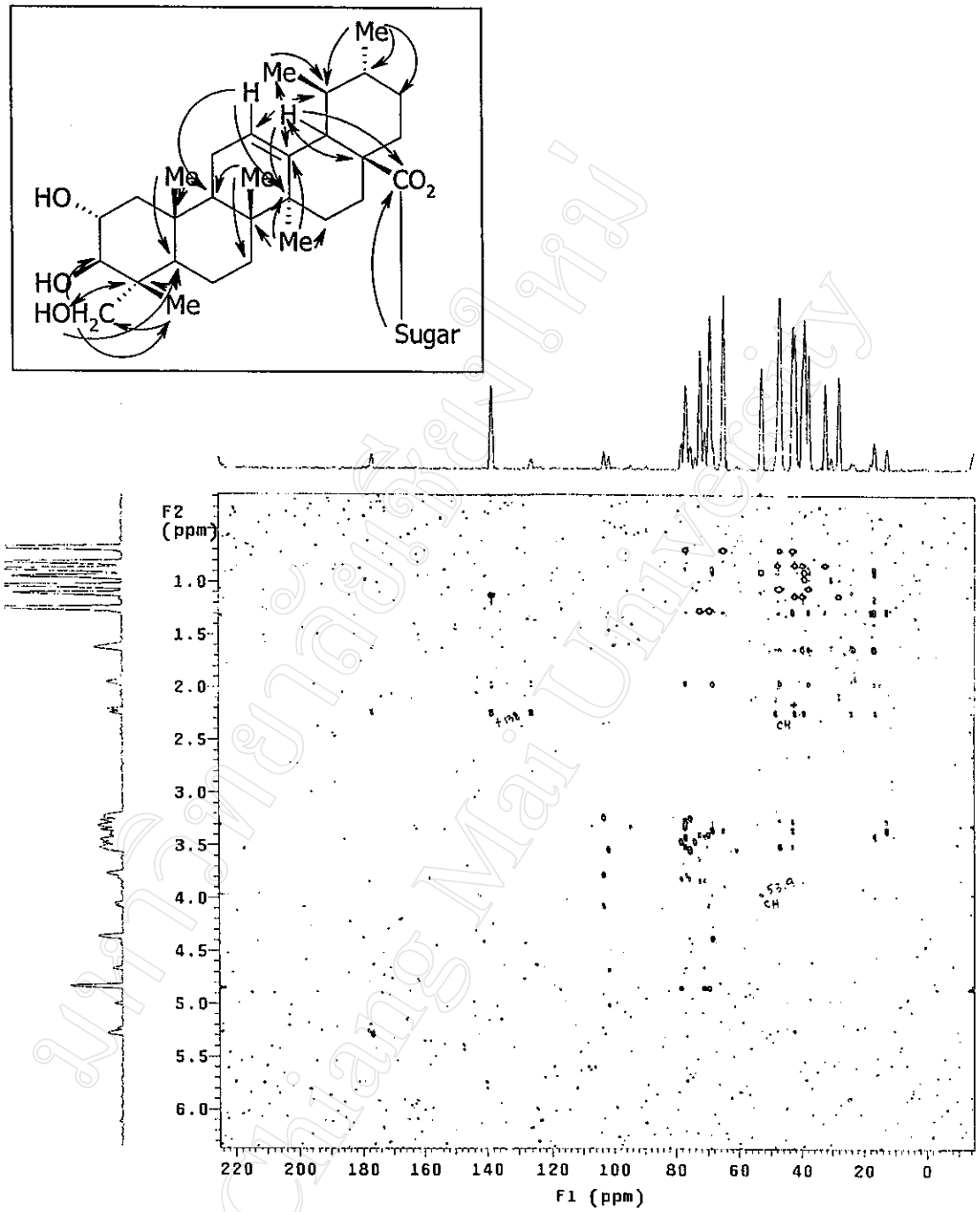


Figure 3. 500 MHz HMBC NMR spectrum for the sequence and linkage position of monosaccharide and triterpenoid (methanol-d_4) of asiaticoside

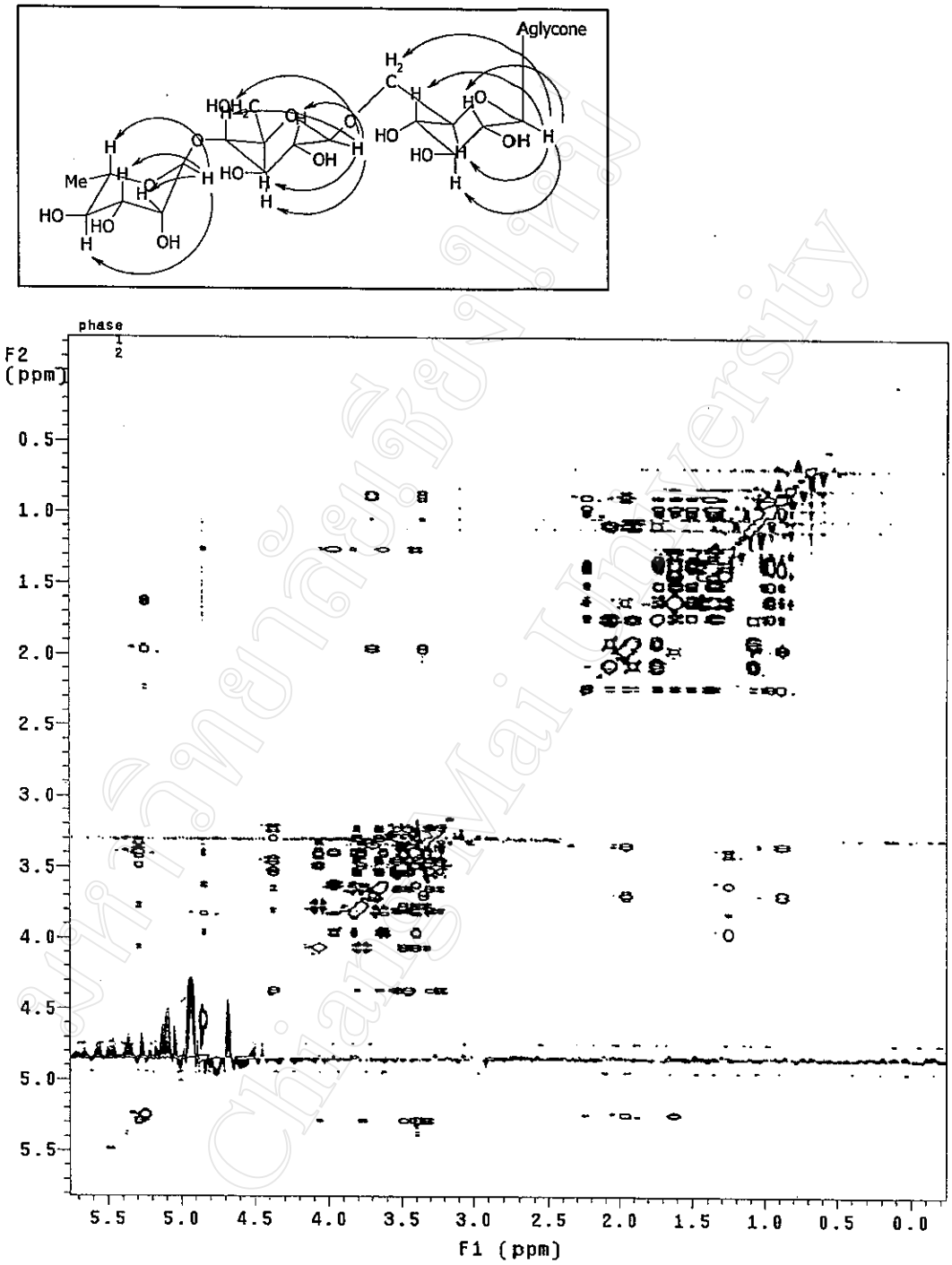


Figure 4. 500 MHz TOCSY NMR spectrum for the sequence and linkage position of monosaccharide and triterpenoid (methanol-d_4) of asiaticoside

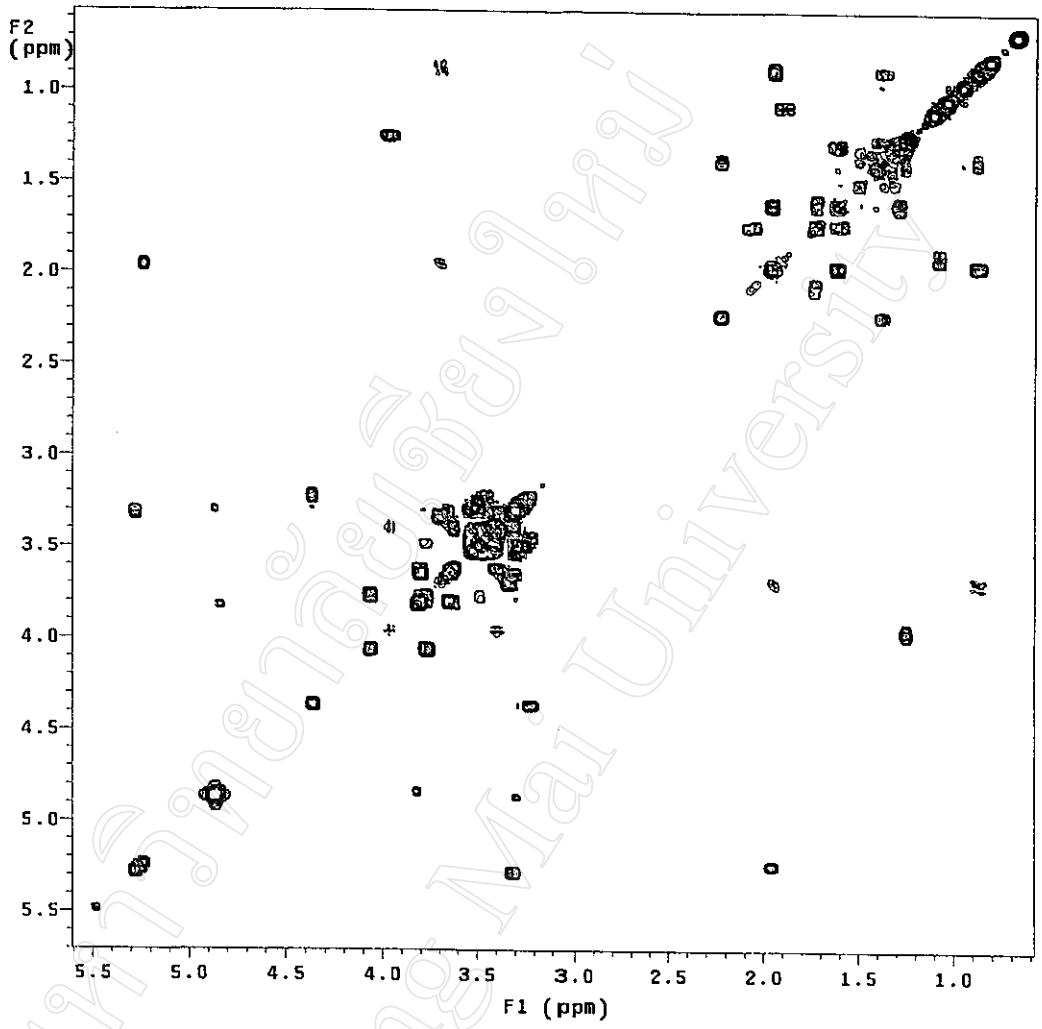


Figure 5. 500 MHz ^{1}H COSY NMR spectrum (methanol-d_4) of asiaticoside.

The glycoside was acetylated with acetic anhydride-pyridine and then flash-chromatographed to give the acetate form, which showed the spectrum of acetate grouping indicating 12 hydroxyl groups in the structure. The ^{13}C NMR (Figure 7) spectrum (Table 1) showed 48 signals, of which 30 were assigned to the triterpenoid moiety, 18 to the saccharide portion units identified from the extensive NMR data obtained. From the HMBC spectrum (Figure 3), the long-range correlation of H-1 (δ 5.29) of Glc' and H-18 (δ 2.23) of aglycone with C-28 (δ 176.7) established that the sugar part was attached to C-17 of the aglycone.

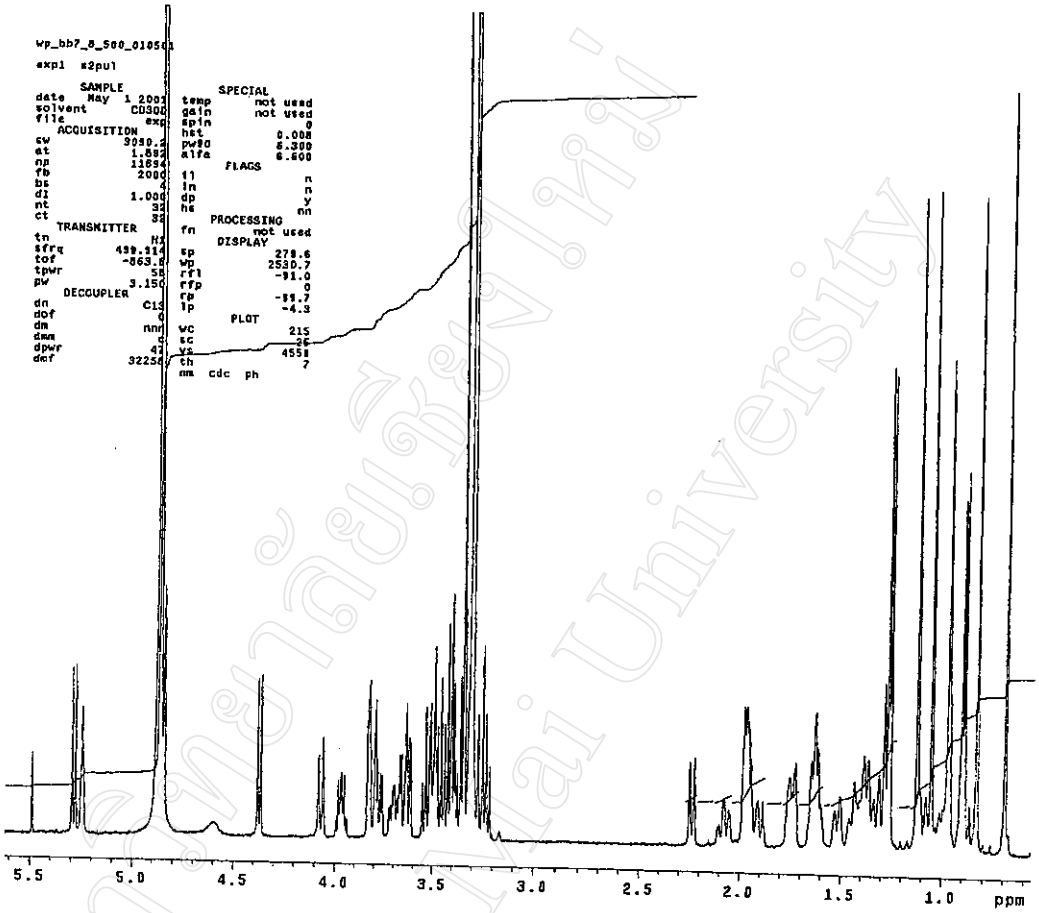


Figure 6. 500 MHz ^{1}H-NMR spectrum (methanol-d_4) of asiaticoside

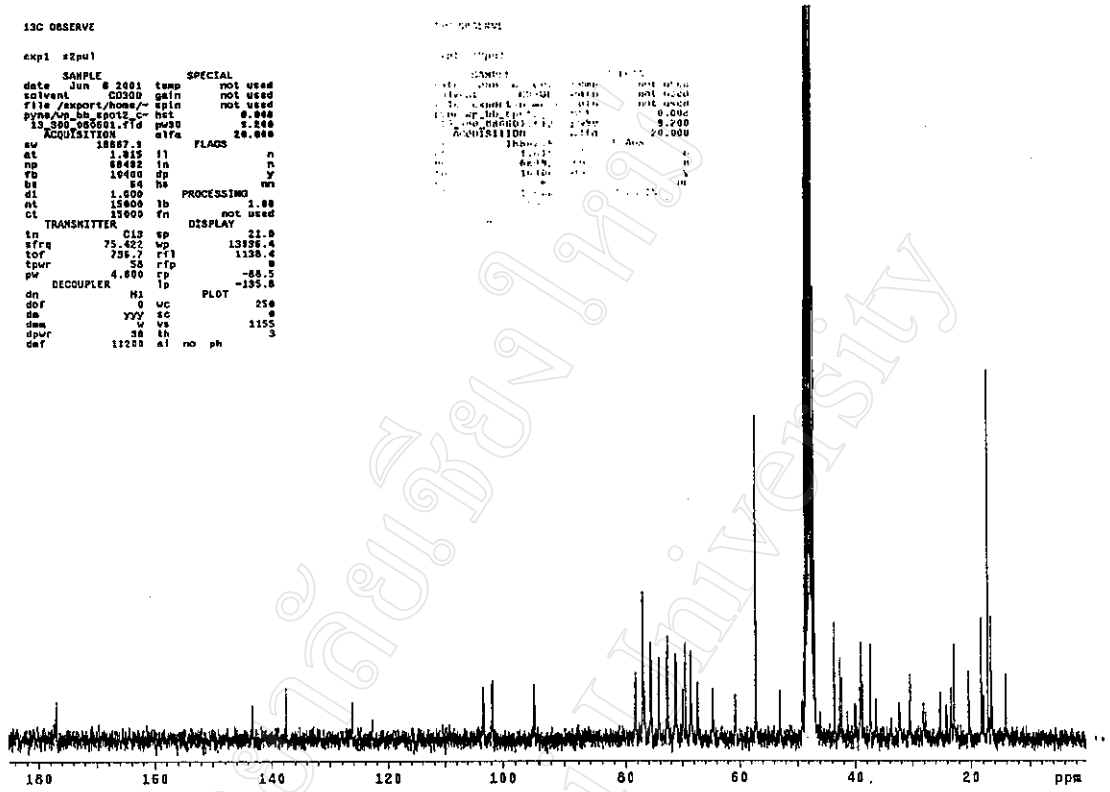


Figure 7. 75 MHz ^{13}C-NMR spectrum (methanol-d_4) of asiaticoside

Table 1. ^{1}H NMR and ^{13}C spectral data of asiaticoside, (in CD_3OD)

^{1}H	^{1}H, δ : ppm	^{13}C	^{13}C, δ : ppm
2	3.67 *ddd* (10/9.5/4)	1	47.3
3	3.34 *d* (9.6)	2	69.4
12	5.24 *br t* (3.4)	3	76.9
18	2.24 *d* (11.0)	4	42.9
19	1.38 *m*	5	47.4
23	3.46 *d* (10.8)/3.26 *d* (10.8)	6	17.8
Me-24	0.71 *s*	7	32.4
Me-25	1.05 *s*	8	39.7
Me-26	0.84 *s*	9	48.3
Me-27	1.13 *s*	10	37.8
Me-29	0.90 *d* (6.6)	11	23.3
Me-30	0.97 *br s*	12	125.7
Glc'		13	138.1
1	5.29 *d* (7.9)	14	42.2
2	3.31 *m*	15	28.0
3	3.39 *m*	16	24.0
4	3.42 *m*	17	48.5
5	3.49 *m*	18	52.9
6a	4.06 *dd* (12.2/1.7)	19	39.0
6b	3.77 *dd* (11.8/5.1)	20	39.2
Glc		21	30.5
1	4.37 *d* (7.9)	22	36.4
2	3.23 *t* (8.4)	23	65.0
3	3.45 *t* (8.8)	24	12.8
4	3.53 *t* (9.0)	25	16.6
5	3.29 *m*	26	16.7
6a	3.80 *br d* (11.5)	27	22.8
6b	3.65 *dd* (12.2/4.1)	28	176.7

Rha		29	16.4
1	4.84 *d* (1.5)	30	20.4
2	3.83 *dd* (3.1/7)	Glc'	
3	3.62 *dd* (9.4/3.4)	1	94.6
4	3.40 *t* (9.4)	2	72.6
5	3.96 *dq* (9.5/6.2)	3	69.9
Me	1.27 *d* (6.1)	4	69.8
		5	76.7
		6	69.4
		Glc	
		1	103.3
		2	74.1
		3	75.5
		4	78.4
		5	75.6
		6	60.7
		Rha	
		1	101.7
		2	71.2
		3	71.0
		4	72.6
		5	69.5
		6	16.6

3.3 Experimental

1D NMR, ^{1}H-NMR (500 MHz), ^{13}C-NMR (75 MHz) and DEPT experiments, including the 2D NMR, COSY, HSQC, HMBC, NOESY, and TOCSY techniques, were carried out on a Varian Mercury 500 NMR spectrometer in CD_3OD solution; mass spectrum was recorded on a VG Quattro LC-MS; melting point was obtained using Gallenkamp hot-stage apparatus.

Extraction and isolation. Fresh leaves of *C. asiatica* (350 g) was extracted with 25% aqueous ethanol (1,000 ml) at 90-100 °C by refluxing for 3 hrs. After one coarse filtration, 900 ml of aqueous extract was obtained. Two iron plates (dimension 30 x 10 x 0.05 cm) were used as electrodes for electrocoagulation of *Centella asiatica* extract. These were dipped 3 cm apart and 9 cm deep into a magnetically-stirred aqueous ethanol extract solution of *Centella asiatica* in a glass jar. Sodium chloride (2 g) was added as an electrolyte. Direct current (0.5 A, 22 V) from a DC power supplier was then passed through the solution *via* the two electrodes. The resulting mixture was then filtered. The aqueous ethanol solution (750 ml) obtained was subjected to a second electrolysis for 30 minutes under the same condition. After filtration, a clear pale yellow solution (600 ml) was obtained. The doubly-electrolysed solution, after filtration, was passed through a column of a mix-bed ion-exchange resin (Amberlite MB-1), and the resulting clear, colourless aqueous solution of conductivity < 50 μS cm^{-1} was collected. The minimum volume of the mixed resin required to achieve this was 1 liter. After evaporation to dryness, the eluate gave a colourless liquid which showed an intense spot and some light spots on the TLC (70% MeOH as eluent, UV 254 nm). The residue was chromatographed over RP18 silica gel column (70% MeOH as eluent) to collect the major component, which easily crystallized in methanol-water as a pure white amorphous solid of asiaticoside (0.025 % yield). Identification was carried out on the basis of the 1D and 2D NMR and mass spectroscopy.

Asiaticoside, Needles mp 229-231°, (lit. [17]: mp 230-232°). ES-MS (Positive ion) *m/z* (rel. int.) : 959 $[M]^+$(29), 797 $[M\text{-}Rha]^+$(797), 635(15), 499(20), 381(30), 359(100), 214(87), 157(40), and 71(45). ^1H NMR, ^{13}C NMR see table 1.

3.4 References

1. http://www.amsar.com/prod12-1.htm
2. V. K. George, J. L. Gnanarethinam, *Curr. Sci.*, 1975, **44**, 790.
3. R. Srivastava, Y.N. Shukla, S. Kumar, *J. Med. Aromat. Plant Sci.*, 1997, **19**, 1049.
4. I. John, *J. Crude. Drug. Res.*, 1984, **22**, 27.

5. a. A.S. Ramaswamy, M. Sirsi, *Indian. J. Pharm.*, 1960, **22**, 34.
b. T. Dutta, N. P.Basu, *Indian J. Exp. Biol.*, 1968, **6**, 181.
c. A. K. A. Chowdhury, I. J. Jahirullah, *J. Bangladesh Acad. Sci.*, 1987, **11**, 75.
6. P. Bagchi, *Herba Hung.*, 1989, **28**, 127.
7. I. Holdsworth, *J. Pharmacogn.*, 1991, 29, 245.
8. S. C. Bhattacharya, *Ind. J. Chem. Soc.*, 1956, **33**, 893.
9. Indian Pharmaceutical codex 1953 (CSIR): P 60-61: Brahmi for improving Memory.
J. R. I. M. 1978: P 37-42: Brahmi for Improving general Mental ability of children. American Journals: Herbalgram 1993, N0. 28, P 32: Mental Function & Gotu Kola by Rob McCaleb.
10. S. Budavari (Ed.), The Merck index : an encyclopedia of chemicals, drugs, and biologicals, 11th ed., centenial ed., Rahway, New Jersey, 1976, p. 131.
11. Scientific Committee, *British Herbal Pharmacoepiea*, Part II, The Association, London, 1976, p. 45.
12. A. hukla, A. M. Rasik and B. N. Dhawan, *Phytother. Res.*, 1999, **13**, 50-54.
13. A. Shukla, A. M. Rasik, G. K. Jain, R. Shankar, D. K. Kulshrestha, B. N. Dhawan, *J. Ethnopharmacol.*, 1999, **65**, 1.
14. P. Boiteau, M. Dureuil, A. Rakoto-Ratsimamanga, *Compt. rend.*, 1949, **228**, 1165.
15. P. K. Inamdar, R. D. Yeole, A. B. Ghogare, N. J. de Souza, *J. Chromatogr. A.*, 1996, **742**, 127.
16. B. Guenther, H. Wagner, *Phytomedicine,* 1996, **3**, 59.
17. C. Castellani, A. Marai, P. Vacchi, *Bull. Chim. Farm.*, 1981, **120**, 570.
18. K. Kim, S. Seo, J. Park, S. Lee, B. Hwang, *Eur. Pat. Appl.* 1998, 9 pp.
CODEN: EPXXDW EP 867447 A1 19980930 CAN 129: 265460 AN 1998: 653723.
19. M. Rerejacque, *Bull. soc. chim. biol.* 1949, **31**, 1510.

4. Mukuroziosíde from *Sapindus rarak* DC

4.1 Introduction

Sapindus rarak DC. (Sapindacea) is a tree native to Southeast Asia. The fruits of this plant are well-known to contain high levels of saponins, and thus have been used as a soap substitute.[1]

Figure 1. ***Sapindus rarak*** DC

Many *Sapindus* species are used ethnomedically, such as *S. emarginata* for the treatment of cholera,[2] *S. laurifolius* as a snake-bite remedy,[3] and *S. mukorossi* for the treatment of epilepsy,[4] worm infestations and rheumatic pains.[5] Alcoholic extracts of the fruits of *S. mukorossi* have been shown in laboratory tests to possess a weak antibacterial activity.[6] The genus *Sapindus* is characterized by the accumulation of glycosides of triterpenes and sesquiterpenes such as mukuroziosíde (Figure 2).[7] Recently, Hamburger *et al.*[8] reported the isolation of four monodesmosidic triterpenoid molluscicidal saponins from *S. rarak*. The isolation of mukuroziosíde from this plant using electrocoagulation has not been reported. This section describes the isolation of a glycoside from *Sapindus rarak* by electrocoagulation in order to demonstrate that the technique can be a valuable method for the isolation of certain water-soluble natural compounds. The fruits of *S. rarak* were obtained in a Thai medicinal plant Market in Chiang Mai for this study.

4.2 Results and discussion

Using the electrocoagulation technique on the crude aqueous extract of the fruits of *Sapindus rarak* DC, mukurozioside IIb, was obtained in 0.27 % (0.2714 g) as white crystals after recrystallizing from MeOH. Glucose and rhamnose, which connect to the aglycone part, were identified in comparison with the literature.[9] The ^{1}H-NMR (300 MHz) (Figure 3) indicated the presence of one $-CH_2OH$ on an olefinic carbon carrying a proton [δ 4.13 (2H, *d*, *J*=7Hz)], one $-CH_2OH$ on an olefinic carbon having no proton [δ 3.92 (2H, s)], three protons, each on an olefinic carbon linked to a methylene group [δ 5.12 (1H, *t*, *J*=5 Hz) and 5.40 (2H, *t*, *J*=5 Hz)] and 5.40 (2H, *t*, *J*=5Hz)] and three methyls, each on an olefinic carbon having no proton [δ 1.68 (3H, s), 1.66 (3H, s) and 1.61 (3H, s)]. The carbon signals (Figure 5 and 6) of mukurozioside were also assigned by comparison with the literture.[9] The assignment of the proton signals due to the sugar moieties of mukurozioside was performed by means of ^{1}H-^{1}H two dimentional correlation spectroscopy (2D COSY) (Figure 4).

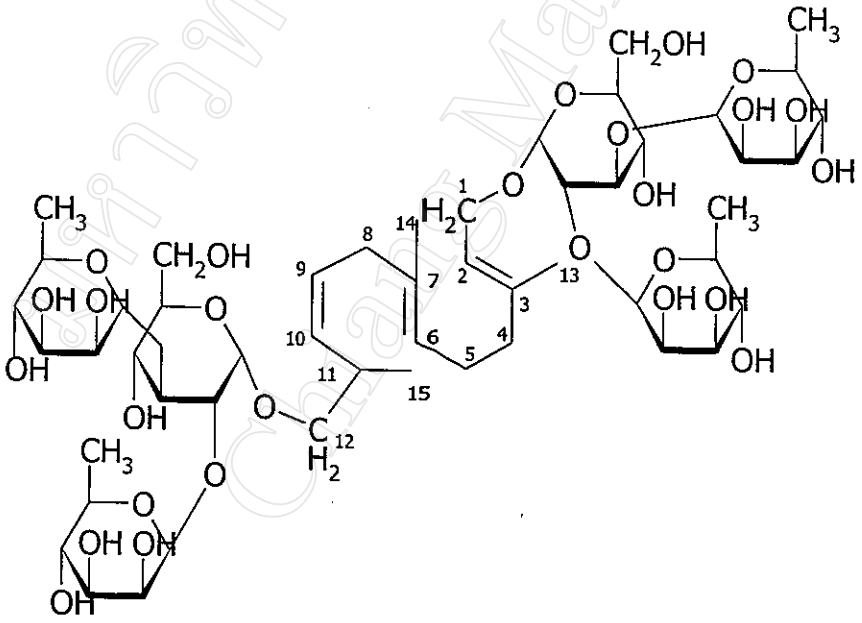


Figure 2. Structure of mukurozioside IIb

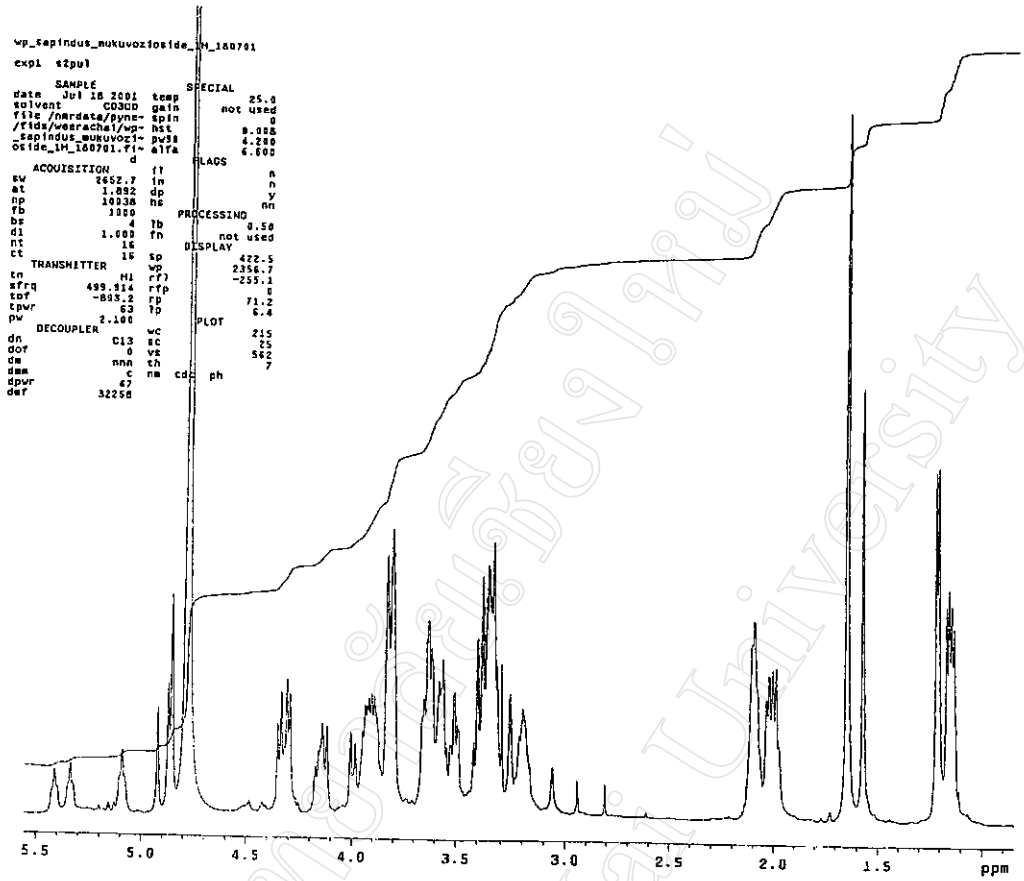
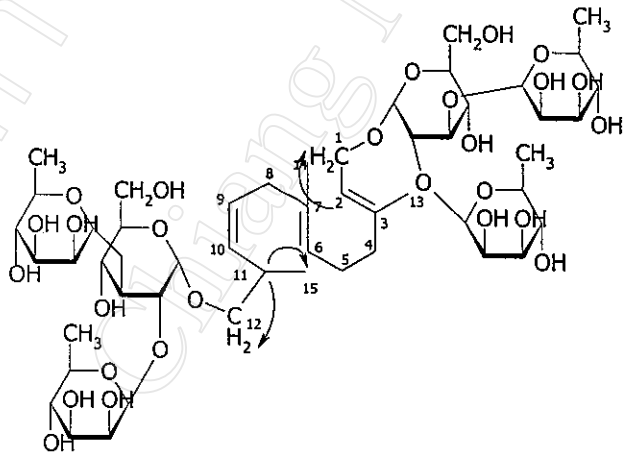


Figure 3. 300 MHz ^{1}H-NMR ($CDCl_3$) of mukurozioside



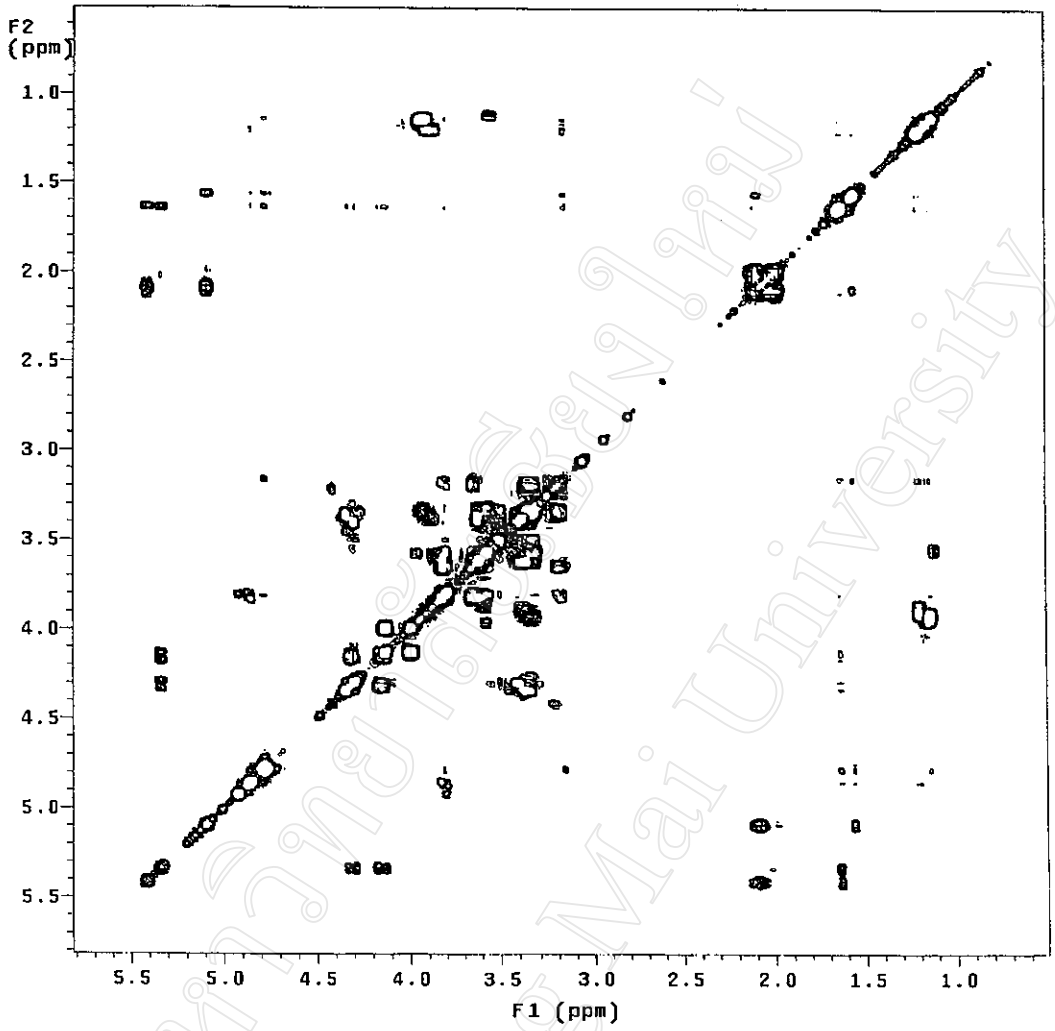


Figure 4. 300 MHz ^{1}H COSY NMR ($CDCl_3$) of mukurozioside

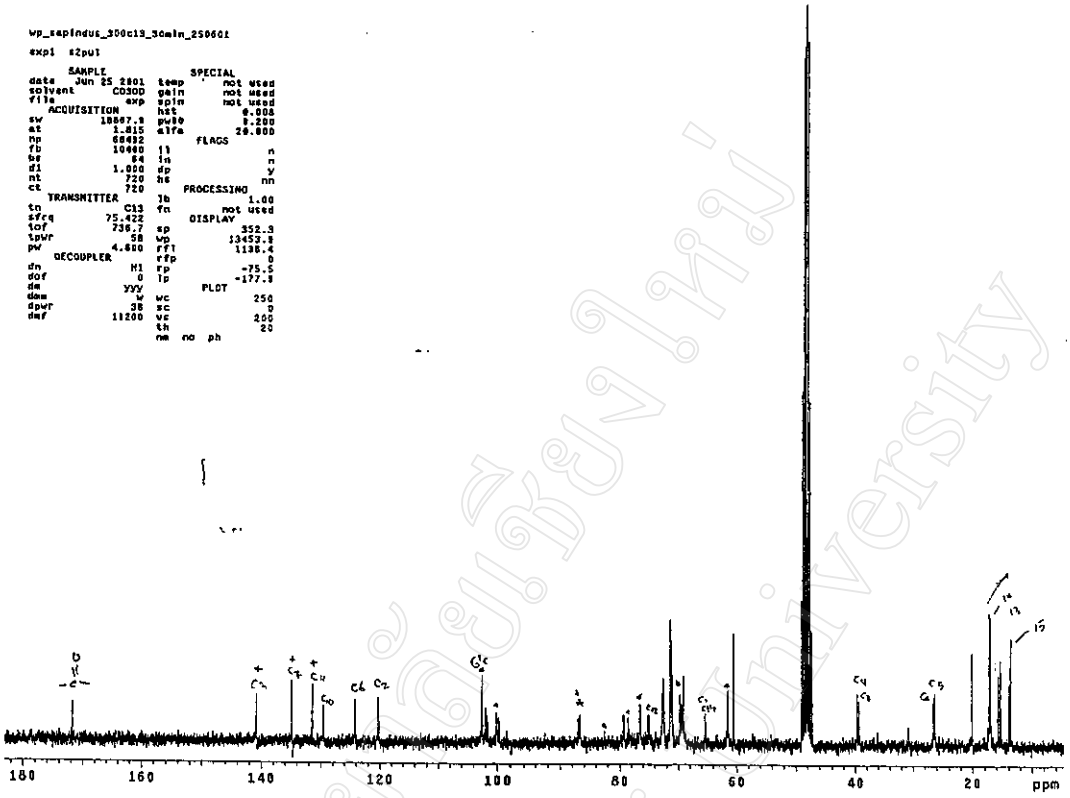


Figure 5. 75 MHz ^{13}C-NMR ($CDCl_3$) of mukurozioside

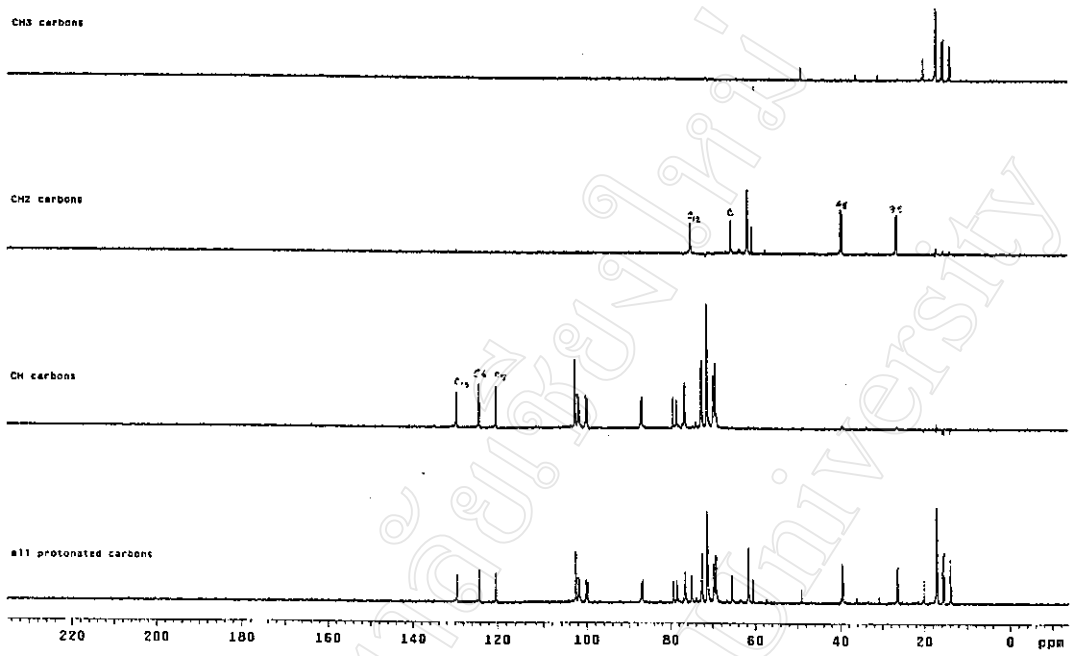


Figure 6. 75 MHz DEPT NMR ($CDCl_3$) of mukurozioside

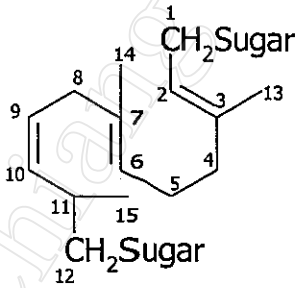


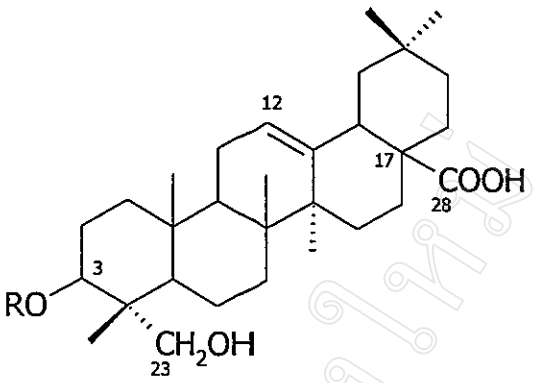
Table 1. ^{13}C-NMR chemical shifts of aglycone moieties of mukurozioside IIb (CD_3OD, TMS as internal standard).

C	^{13}C, δ : ppm
1	65.5
2	120.8
3	141.5
4	39.5
5	26.4
6	124.4
7	136.0
8	39.7
9	26.8
10	129.8
11	132.0
12	76.0
13	15.9
14	15.8
15	13.9

Table 2. ^{13}C-NMR chemical shifts of sugar moieties of mukurozioside IIb (CD_3OD, TMS as internal standard).

C	^{13}C, δ : ppm
Glc-1	103.6
2	80.2
3	87.9
4	70.4
5	77.5
6	62.5
Glc'-1	102.7
2	79.4
3	88.1
4	70.1
5	77.5
6	62.5
1	103.6
2	72.3
Rha 3	72.3
Rha' 4	73.6
5	70.7
6	18.0

In addition, this experiment also gave a compound (0.3 % yield, 0.296 g) which had NMR spectroscopic data similar to that of monodesmoside[9], one of the known glycosides in *Sapindus rarak*. The presence of an acetoxyl group was demonstrated by a three-proton singlet at δ 1.94 and a set of carbon signals at δ 20.7 for the methyl carbon and at δ 170.4 for the carbonyl carbon. However, further structural elucidation of this compound was not continued due to the limitation of time. The spectroscopic data of this compound are shown below (Figure 8).



R= -Ara^2-Rha^3-Ara

Ara : Arabinopyranosyl

Figure 7. Structure of monodesmoside

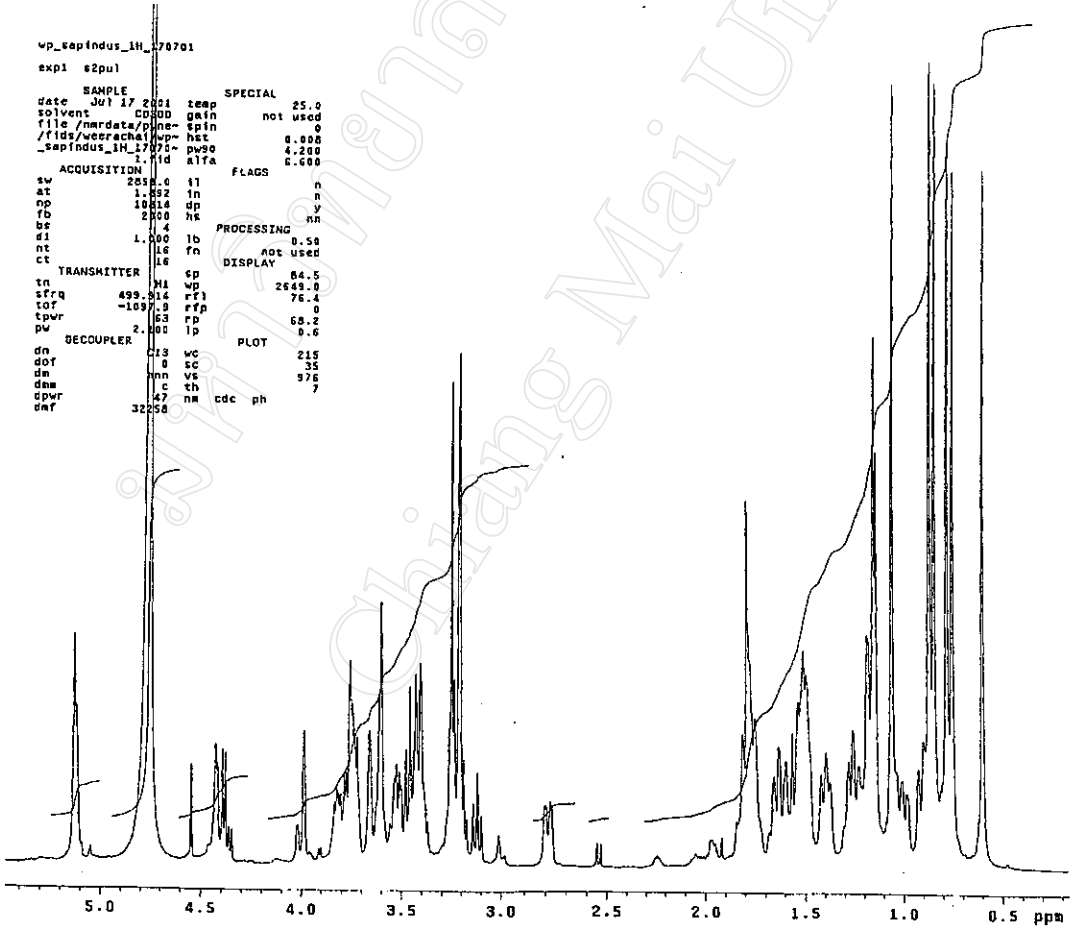


Figure 8. 300 MHz ^{1}H-NMR (CD_3OD) of the unknown compound

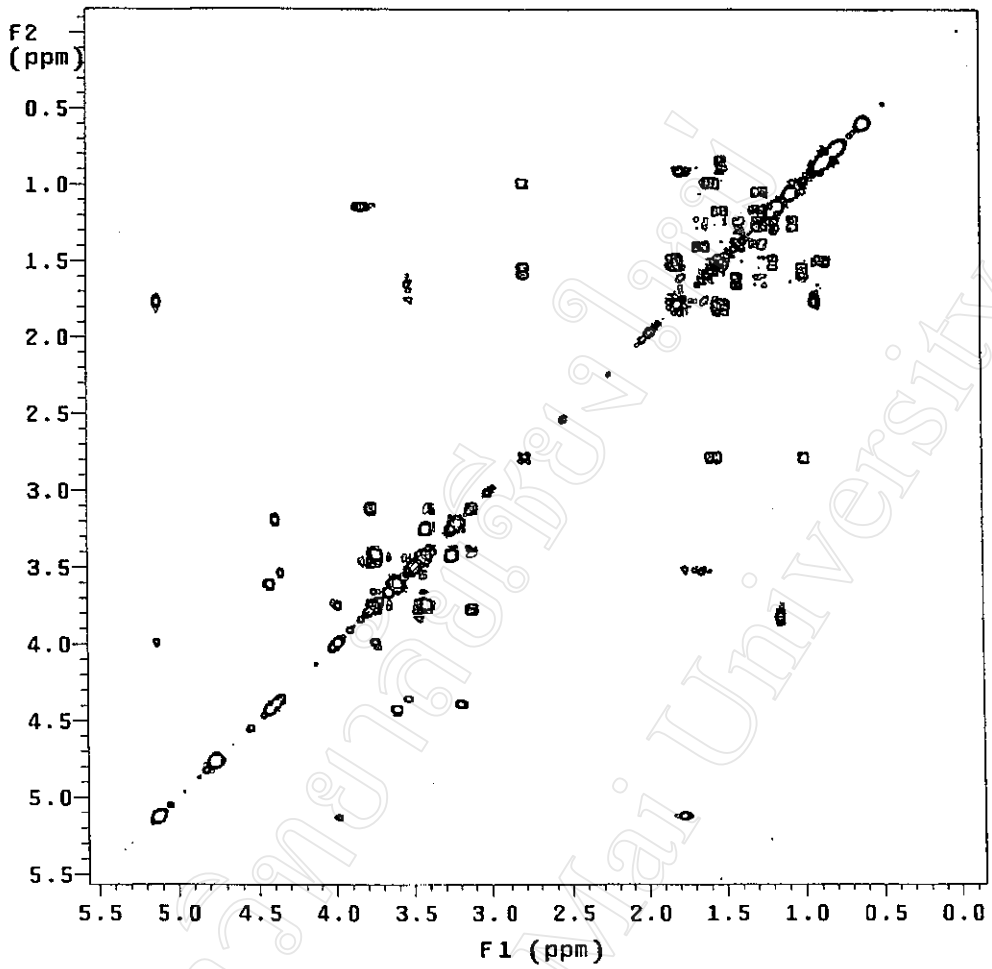


Figure 9. 300 MHz COSY NMR spectrum (CD_3OD) of the unknown compound

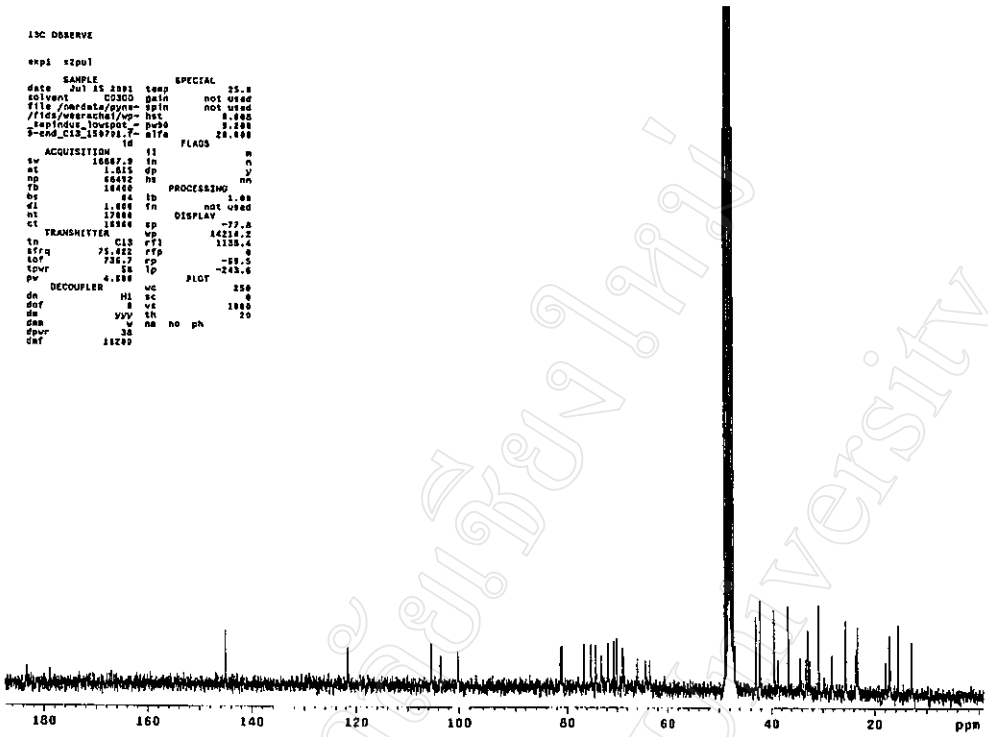


Figure 10. 75 MHz ^{13}C-NMR spectrum (CD_3OD) of the unknown compound

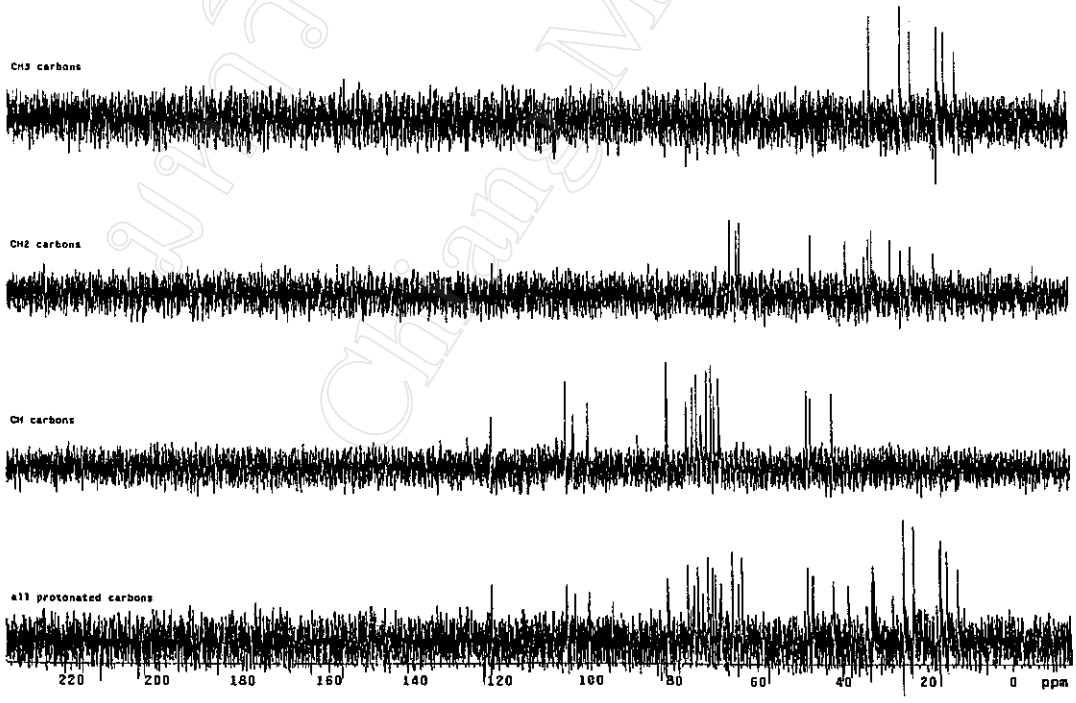


Figure 11. 75 MHz DEPT NMR spectrum (CD_3OD) of the unknown compound

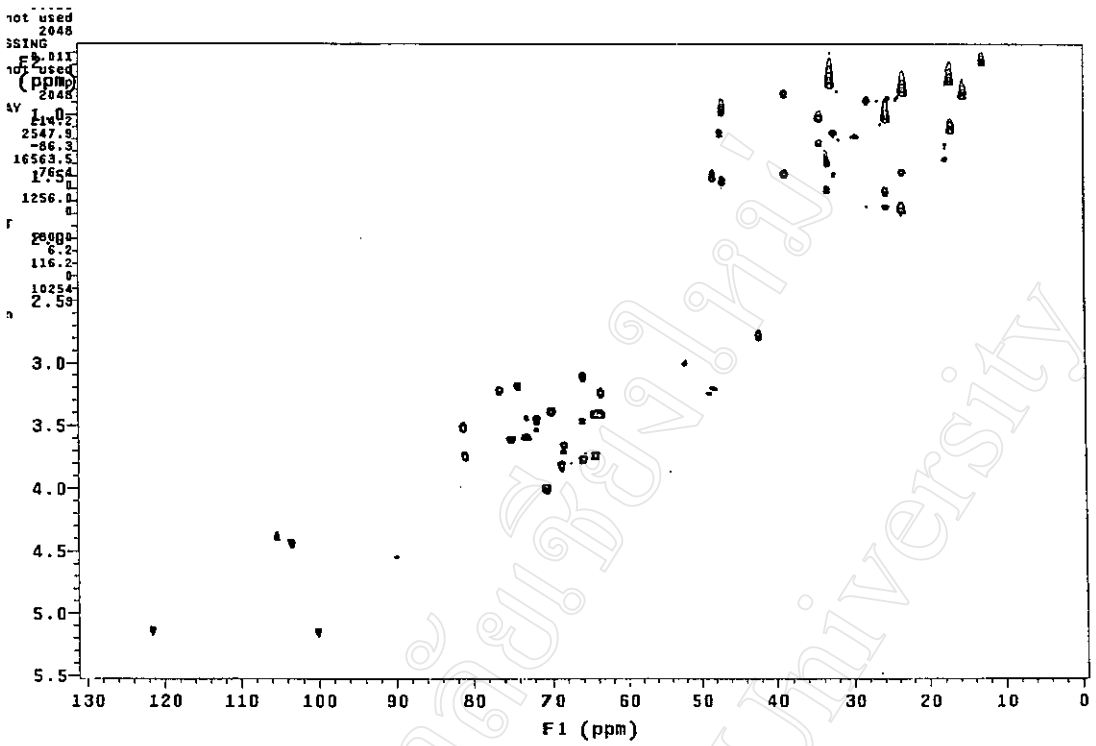


Figure 12. 300 MHz HSQC NMR spectrum (CD_3OD) of the unknown compound

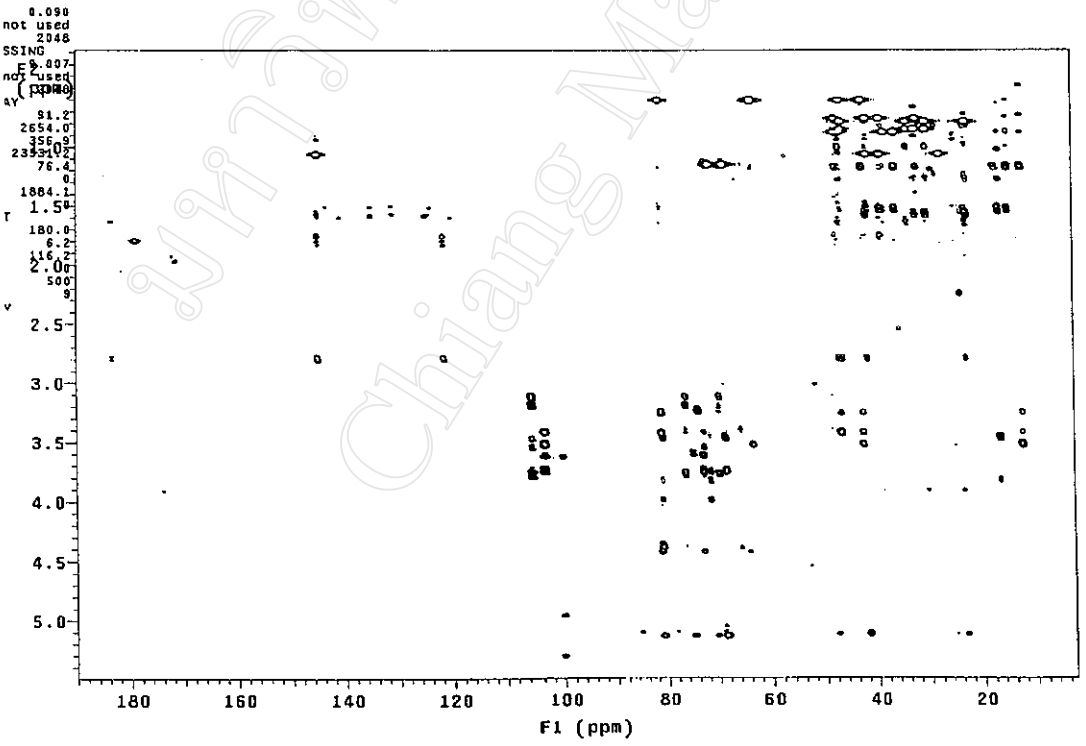


Figure 13. 300 MHz HMBC NMR spectrum (CD_3OD) of the unknown compound

4.3 Experimental

1D NMR, ^{1}H NMR(300 MHz), ^{13}C NMR(100 MHz) and DEPT experiments, including the 2D NMR, COSY, HSQC, HMBC, techniques, were carried out on a Varian Mercury 300 NMR spectrometer in CD_3OD solution.

Extraction and isolation. Sapindus rarak fruits (100g) were extracted with water (1,000 ml) at 90-100 °C by refluxing for 3 hrs, after one coarse filtration, 880 ml of aqueous extract was obtained. Two aluminium plates (dimension 30x10x0.05 cm) were used as electrodes for the electrocoagulation of *S. rarak* extract. These were dipped 3 cm apart and 9 cm deep into a magnetically-stirred aqueous ethanol extract solution of *Sapindus rarak* in a glass jar. Sodium chloride (2 g) was added as an electrolyte. Direct current (0.5 A, 22 V) from a DC power supplier was then passed through the solution *via* the two electrodes. The resulting mixture was then filtered. The aqueous solution (750 ml) obtained was subjected to a second electrolysis for 30 minutes under the same condition. After filtration, a clear pale yellow solution (600 ml) was obtained. The doubly-electrolysed solution, after filtration, was passed through a column of a mix-bed ion-exchange resin (Amberlite MB-1), and the resulting clear, colourless aqueous solution of conductivity < 50 $\mu S\ cm^{-1}$ was collected. The minimum volume of the mixed resin required to achieve this was 1 liter. After evaporation to dryness, the eluate gave a colorless liquid which showed 5 spots on reverse phase TLC (70% MeOH, UV 254 nm). The residue was chromatographed over RP18 silica gel column (70% MeOH as a mobile phase) to collect the major component, which easily crystallized in methanol-water as a pure white amorphous solid of Mukuroziosíde (0.2714 g, 0.27 %). Another unknown compound was also isolated (0.296 g, 0.3 %). Identification was carried out base on the 1D and 2D NMR.

Mukuroziosíde, Colourless glassy solid mp 143-150 °C (lit.[9]: mp 146-148 °C) $[\alpha]_D^{26}$ –65.7° (MeOH; C 1.80)). ^{1}H-NMR see discussion, ^{13}C NMR see table 1.

4.4 References

1. D. J. Mabberley, *The plant Book : A Portable Dictionary of the Higher Plants.* Cambridge University Press, Cambridge, 1993.
2. S. N. Arseculeratne, A. A. L. Gunatilaka and R. G. Panabokke, *J. Ethnopharmacol.*, 1985, **13**, 323.
3. S. D. Sapnis, S. J. Bedi, *Ind. J. For.*, 1983, **6**, 65.
4. M. P. Singh, S. B. Malla, S. B. Rajbhandari, A. Manandhar, *Econ. Bot.*, 1979, **33**, 185.
5. S. P. Jain, H. S. Puri, *J. Ethnopharmacol.*, 1984, **12**, 213.
6. S. A. H. Naovi, M. S. Y. Khan, S. B. Vohora, *Fitoterapia*, 1991, **62**, 221.
7. a. K. Nakayawa, H. Fujino, R. Kasai, O. Tanaka, J. Zhou, *Chem. Pharm. Bull.*, 1986, **34**, 2209;

   b. W. H. Wong, R. Kasai, W. Choshi, Y. Nakagawa, K. Mizutani, K. Ohtani, O. Tanaka, *Phytochem*, 1991, **30**, 2699.

   c. A. Ribeiro, C. L. Zani, T. M. de Almeida Alves, N. M. Mendes, M. Hamburger, K. Hostettmann, *J. Pharmacog.*, 1995, **33**, 177.
8. M. Hamburger, I. Slacanin, K. Hostettmann, W.Dyatmiko and Sutarjadi, *Phytochem. Anal.*, 1992, **3**, 231.
9. W. H. Wong, R. Kasai, W. Choshi, Y. Nakagawa, K. Mizutani, K. Ohtani, O. Tanaka, *Phytochem*, **30**, 1991, 2699.

5. Electrocoagulation study on various plants

5.1 Introduction

The application of the electrocoagulation process to the isolation of pure plant products has been clearly demonstrated in this chapter. Various other plants have also been investigated in order to demonstrate the efficiency and limitation of the electrocoagulation method.

Six other plants were chosen and their aqueous plant extracts were processed using the electrocoagulation procedure. After passing the filtered solution through resins, the samples were examined by TLC analysis. The results are shown in Table 1.

5.2 Result and discussion

Using the electrocoagulation conditions mentioned previously, with aluminium electrodes, a marked decrease in the colour of the crude extract solutions was evident after electrolysis and decolourization by resin. The TLC analysis (the solvent systems as indicated) showed some spots were weaker or disappeared upon comparing the crude extracts before and after electrocoagulation (Table 1). However, the aqueous extract of the fruits of *Hibiscus sabdariffa* could not be purified using electrocoagulation since the solution obtained after electrocoagulation was dark, and did not show any decolourization. This may be due to some chemical transformations such as reductions occuring to certain plant pigments. Therefore, although the electrocoaguation technique proved to be useful in decolourizing some of the aqueous plant extracts, this technique could not be used for all plants.

Table 1. TLC analysis before and after the electrocoagulation method.

Plant name	R_f of components before the electrocoagulation	R_f of components after the electrocoagulation	Solvent system	Detection
Pandanus Amarillyfoius (leaf)	0.31, 0.78, 0.85	0.31(light), 0.78(light), 0.85	Ethyl acetate-methanol-water (100 : 13.5 : 10)	UV 366
Azardirachta indica (leaf)	0.22, 0.51, 0.96	0.22, 0.51(light), 0.96,	Ethyl acetate-formic acid-glacial acetic acid-water (100 : 11 : 11 : 26)	Anisaldehyde-H_2SO_4
Cassia angustifolia (leaf)	0.11, 0.24, 0.32, 0.48, 0.72, 0.87, 0.98	0.11(light), 0.24(light), 0.32(light), 0.48(light), 0.72(light), 0.87(light), 0.98	*n*-proanol-ethyl acetate-water-acetic acid (40 : 40 : 29 : 1)	HNO_3/ KOH
Abrus precatorius (leaf)	0.48, 0.84, 0.88, 0.92	0.48 (disappeared), 0.84, 0.88, 0.92	$CHCl_3$:MeOH : H_2O, (6 : 2 : 1.3)	Vanillin/ H_2SO_4
Hibiscus sabdariffa (fruit)	The crude extract solution showed no decolourized after electrolysis, a dark solution was obtained.			
Thunbergia laurifolia (leaf)	0.04, 0.11, 0.19	0.04(light), 0.11, 0.19	ethyl acetate : formic acid : acetic acid: water, (100 : 11 : 11: 26)	30% H_2SO_4
Ocimum sanctum (leaf)	0.17, 0.97(tail)	0.17, 0.97(tail)(light)	Ethylacetate : formic acid : acetic acid : water, 100 : 11 : 11 : 26	30% H_2SO_4

5.3 Experimental

Hot Water Extraction.

Water (1,000 ml) at 90-100 °C was used to extract 50 g of the dried plants for 30 min followed by filtration.

Decolorisation by Electrolysis.

A direct current (0.5 ampere, 22 volt) was passed for 2 hours *via* two aluminium plate electrodes (dimension 30 x 10 x 0.05 cm) immersed 3 cm apart in the aqueous extract in a glass tank (34 x 31 x 21 cm), into which 2 g/L of NaCl had been added. The resulting mixture was then filtered. The aqueous solution obtained was subjected to a second electrolysis for 1 1/2 hour under the same condition. After filtration, a clear pale yellow solution was obtained.

Decolorisation and Demineralisation by Ion Exchange.

The solution was passed through a column of a mix-bed ion-exchange resin (Amberlite MB-1), to give a clear solution. The minimum volume of the mixed resin required to achieve this was 1 liter. Analysis by TLC indicated components as shown in Table 1.

6. Conclusion

Glycyrrhizin was isolated in 2.5% as a pale yellow solid from *Glycyrrhiza radix* (Licorice). Asiaticoside was isolated in 0.025% as a white amorphous solid from the water-ethanol extract of *Centella asiatica.* Mukurrozioside, a glycoside found in *Sapindus rarak,* was isolated in 0.27% as a white crystal, along with an unknown compound (0.30%) Another natural compound, *D*-pinitol, a polyol, was also isolated in 1.0% as a white crystal from *Cassia siamea* Lamk. In addition, six more plants were investigated. This isolation method is relatively safe and simple, having an important advantage of forgoing the use of expensive and toxic organic solvents and reagents, as well as using a minimum number of expendable common chemicals. However, the method still has an important restriction in that only water or dilute aqueous alcoholic solution can be used as extracting solvents. There is a need to further develop this method to suit specific plants.