## Part II

## EXPERIMENTAL

The steroid sapogenins from the rhizomes of *Costus lacerus* Gagn. were investigated in the following steps:- I. Preliminary Examination. II. Extraction and Purification. III. Identification. IV. Quantitative Determination.

### I. PRELIMINARY EXAMINATION.

The chemical constituents of *C. lacerus* Gagn. rhizomes was determined in two categories:- i. systematic qualitative test, ii. detection of saponins by thin-layer chromatography.

#### i. Systematic qualitative test (Ciulei, 1983)

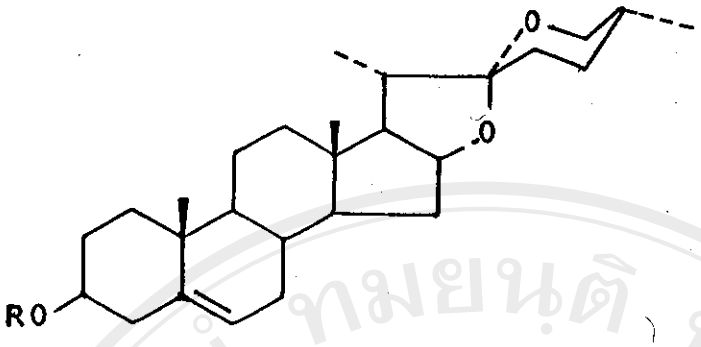
The preliminary determination of plant constituents can be obtained firstly by separation the chemical constituents into three main classes through successive and selective extractions of the plant powder with solvents of different polarities. Plant material reduced to powder, is firstly extracted with lipophilic solvent, e.g., ethyl ether, petroleum ether, benzene, chloroform, etc., followed by the intermediate polar solvent e.g., ethanol or methanol and finally extracted with water which is a strong polar solvent. Therefore, three extractive solutions obtained are:- the ether extract, the alcohol extract and the aqueous extract. The identification of chemical composition in each extract may be determined by means of a qualitative chemical

analysis or color tests. The ether extract will contain the lipophilic compounds including volatile oils, lipids and fatty acids, sterols and triterpenes, carotenoids, basic alkaloids, flavone aglycones, anthraquinone aglycones and coumarins. The ethanol or methanol extract from the defatted plant material may contain the relatively important groups of natural constituents e.g., polyphenol (tannins), reducing compounds, alkaloid salts, polyphenolic glycosides (anthraquinone glycosides and flavone glycosides),cardiac glycosides,steroid and triterpenoid glycosides and anthocyanin. Water will dissolve the hydrophilic constituents from vegetable material such as:- glucides (oses, polyoses), poly uronides, glycosides, and alkaloid salt.

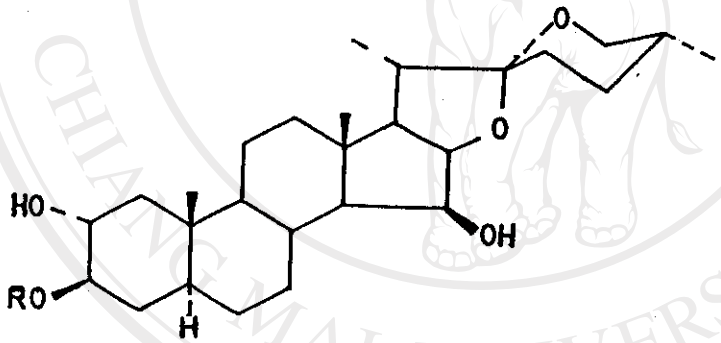
ii. Detection of steroid saponins by thin-layer chromatography

Saponins are plant glycosides of complex alicyclic compounds which have a distinctive properties in aqueous solution, for example, foaming, toxicity toward fish and amphibians, hemolysis and complex formation with cholesterol. Generally saponins are classified into steroid and triterpenoid according to the structural features of aglycone parts (sapogenins). Typical steroid saponins or steroid glycosides, having the afore-mentioned characteristics, are all spirostan-3 -ol derivatives, having a branch-chain oligosaccharide link with the hydroxyl group at C-3 e.g., dioscin(I) and digitonin (II). However a number of a typical spirostanol glycosides which do not show these structural feature has also been discovered. They contain a monosaccharide, or a straight chain oligosaccharide,

Typical saponins with spirostane skeleton

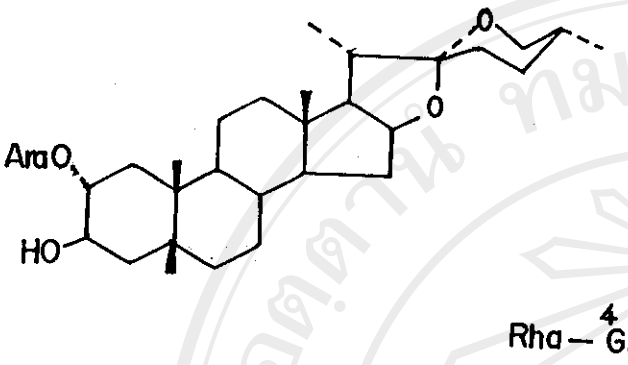



I. dioscin R = Rha $\overset{7}{\underset{4}{<}}$ Glc
Rha

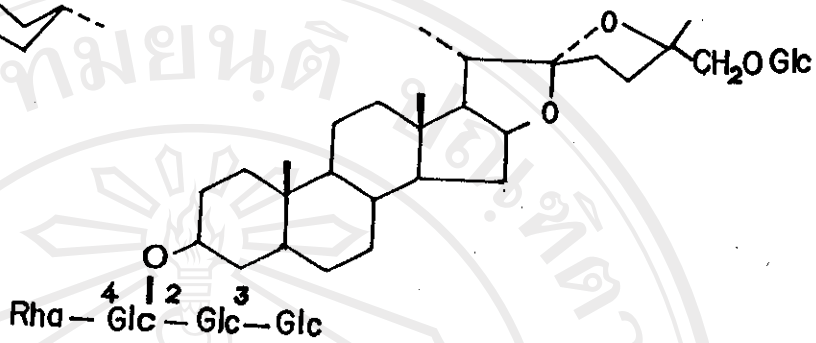



II. digitonin R = Gal $\overset{3}{<}$ Glc $\overset{4}{-}$ Gal
Xyl

Atypical saponins with spirostane skeleton

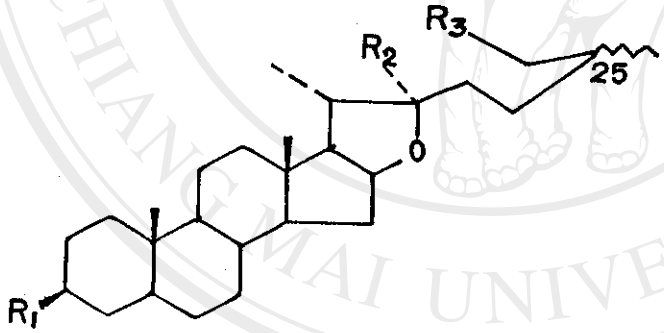



III. yononin




IV. avenacoside B

Furostanol bisglycosides




V. jurubine 5αH, 25R, $R_1 = NH_2$, $R_2 = OH$, $R_3$ = -0-β-D-glc-pyr.

VI. sarsaparilloside 5 β-H, 25 S, $R_1$ = 0-β-D-glc-pyr $^{2}$ β-D-glc, $^{4}$ α-L-rha-pyr, $^{6}$ β-D-glc-pyr

$R_2 = OH$, $R_3$ = -O- -D-glc-pyr

and in some cases, have the sugar moiety linked to a hydroxyl group other than that at C-3, for example, yononin (III), avenacoside B (IV). Moreover, after the discovery of jurubine(V) and sarsaparillosides(VI) in 1966 and 1977 by Schreiber group and Tschesche and his collaborators respectively, the furostanol bisglycosides which have furostanol ring in aglycone part are also regarded as prototype compounds of spirostanol glycosides. All the component monosaccharides of steroid saponins are of the pyranose type with only one exception, the L-arabinofuranose residue found in saponin from *Paris polyphylla* Sm. Oligosaccharides of the sugar moiety consists of two or three kinds of sugar such as D-glucose, D-galactose, L-rhamnose and D-xylose and fork into branches having rhamnose and/or xylose residue at terminals. Digitalose, oleandrose and fucose may also be found. Almost all the structurally a typical glycosides fail to show saponic properties. (Korte and Goto, 1978; Mahato *et al.*,1982.)

Classical methods for the qualitative determination of saponins in plant material are carried out by using an aqueous or alcoholic extracts and tests for saponic properties such as 1) foaming ability, 2) toxicity toward fish, 3) hemolytic activity, 4) molecular complex formation with cholesterol and 5) Lieberman-Burchard reaction. Tests 1-3 are common to both steroid and triterpenoid saponins and test 5 is also positive for sterol glycosides. Besides, some of a typical spirostanol glycosides and furostanol bisglycosides sometimes predominant in plant material are naturally negative to tests 1-4.

Molecular weights of saponins are relatively high, and their molecules differ only slightly in the structure of aglycone part and/or

sugar moiety. They are always accompanied by very polar substances e.g., saccharides and coloring matter. They are hence hygroscopic and not easily crystallized. Therefore, suitable and simple methods for the detection of individual saponins are paper-partition chromatography (PPC), thin layer chromatography (TLC) or other procedures.

The simple and rapid method with high resolution and sensitivity for a small amount of organic compounds initiated by Stahl in 1958 is TLC. The developing solvent systems use in TLC on silica gel for separating steroid saponins according to the differences of the sugar moiety and structural divergencies of the aglycone parts consist of chloroform, methanol and water in the ratio of 65:35:10 or 70:30:10 or 70:30:5. Antimony trichloride (15%), anisaldehyde reagent and 10% $H_2SO_4$ are used as spraying agents for detection of the spots. Anisaldehyde reagent can be used to distinguish steroid saponins (yellow spots in general) from sitosterol glucosides (purple spot) and cardiac glycosides such as digitoxin (dark blue spots)

BuOH-HOAc-water (50:10:40) and BuOH-EtOH-96% $NH_3$ (30:60:50) as solvent and chlorosulfonic acid-HOAc (1:2), 30% $H_2SO_4$ and 1% ceric sulfate in 10% $H_2SO_4$ and cobalt chloride as detectors for free steroid saponins have also been reported. Erhlich's reagent which gave a red colour was found to be useful for the detection of furostanol bis glycosides. (Natori *et al.*; 1981, Stahl, 1969)

## II. EXTRACTION AND PURIFICATION

Sapogenins occur rarely in free state but generally in combined form as glycosides (saponins). The genins may appear to be present in different glycosidal forms e.g., triglucoside such as saponin triosides from *Dioscorea detoidea* Wall. (diosgenin-3-0- β -D-glucopyranosyl (1-4) -8-D-glucopyranoside). Therefore, glycosidic linkage has to be cleaved to liberate the component of monosaccharides (one or more kinds) in addition to non-carbohydrate moiety, and aglycone or sapogenin before the extraction process will carry on. Various methods have been used for the hydrolysis of glycosidic linkage. All these methods can be divided into chemical hydrolysis using acids or some other reagents, and enzymatic hydrolysis using an enzyme occurs along with the saponins in the plant (fermentation) or by fungal enzymes (microbial hydrolysis). Enzymatic and chemical hydrolysis usually yield the same products, though artefacts may be formed by chemical hydrolysis. Acid hydrolysis is the most common method among the chemical methods use for commercial production of sapogenins, while the enzymatic hydrolysis is preferable for structure elucidation of unknown saponins. (Elks. 1971, Singh and Thakur, 1983).

The extraction of sapogenins from fresh or dried plant materials can be carried out in the two alternated ways namely; (i) alcoholic extraction of the glycoside from the plant materials followed by acid hydrolysis (ii) acid hydrolysis of the total plant materials, followed by extraction of the liberated sapogenin with non polar solvents.

The alcoholic extraction employed earlier by most of the diosgenin producing firms in Mexico is no longer in use. In this process, according to Wall *et al.*(1952, b), saponin was extracted from wet plant tissue with 90-95% EtOH, the concentrated extract freed of fat and lipids by extraction with benzene. The saponins was then extracted with butanol from the aqueous phase and the butanol extract was concentrated. The saponins was further hydrolyzed with 4N HCl. The hydrolysis mixture was extracted with benzene to recover the sapogenin for further purification.

This method gives unsatisfactory results due to the extraction of pigment, the formation of gummy material and decomposition of unsaturated sapogenin e.g., diosgenin to spirostadiene (Brain *et al*, 1968., Peal, 1957., and Wall *et al.*, 1952, a). Epimerization of the 25R and 25S forms may also occur in the presence of ethanolic hydrochloric acid. (Shoppe, 1964)

The treatment of the hydrolyzed material with Lime or other alkali is useful for removal of phenolic compounds and other colour impurities. The formation of spirostadiene during the acid hydrolysis of saponin was affected by the acid concentration, duration of heating and the concentration of alcohol present. The higher the concentration of acid, the greater the loss (Peal, 1957). Of the three acids used for hydrolysis; $H_2SO_4$,HBr and HCl,Yamuachi (1959) found $H_2SO_4$ to be the mildest, and show that refluxing dioscin with $4NH_2SO_4$ for 2 hours was the optimum hydrolytic condition for releasing diosgenin from *Dioscorea* tubers.

Acid hydrolysis method derived from Rothrock *et al.* (1957)

released sapogenin from their glycoside by acid hydrolysis *in situ*, and extract the hydrolyzed plant material with a non polar solvent. This method is usually carried out by refluxing the (micropulverized) plant material with mineral acids at a suitable condition in a polar solvent such as water, ethanol or methanol. Hydrochloric acid is more preferable than sulfuric acid. The hydrochloric acid concentrations of 2N to 4N are normally used. The optimum conditions (i.e., concentrations of acid, temperature, and heating time) for hydrolysing each plant material depends on the kinds of plant concerned and the sapogenins present.

The crude hydrolysate obtained by filtration is washed with water and neutralized with alkali solution especially when sulfuric acid is used, otherwise the steroid content will decrease rapidly. The material is dried and exhaustively extracted for about 6-24 hours with hydrocarbon solvent, e.g., petroleum ether, hexane. Blunden *et al.*, (1967) reported that diene formation was minimized to 5-7% of the sapogenin isolated when the hydrolysis was carried out by refluxing the plant material with 2N HCl for 2 hours. Diene formation can be further reduced by aqueous incubation or fermentation of the plant material before refluxing (Hardman *et al.*, 1972) because endogenous enzyme saponase partially or wholly hydrolyzes the saponins to water-insoluble hemi-saponins or saponins which are less susceptible to acid dehydration at $C_3$ during incubation as compared to the water soluble saponins.

Many modifications have been investigated to increase the yield of sapogenins in commercial purposes. The partial hydrolysis

which involved enzymatic hydrolysis, preferably with an *Aspergillus* species before the acid hydrolysis is also prefered for economical extraction. The addition of certain additives like mevalonic acid, geraniol and squalene which are steroid sapogenin precursors or phenoxyacetic acid which is plant growth regulators to the plant material during fermentation was reported to improve the yield of sapogenins (Hardman, 1968, 1969 and 1971).

In the case of screening for the presence of sapogenin in plant samples obtained from cell or tissue culture or from a plant part during breeding program, the method of choice for the isolation of sapogenins in the laboratory is the acid hydrolysis. For rapid screening of plant for sapogenins, TLC or GLC which are convenient and sensitive method can be used. The sapogenin, especially monohydroxy sapogenins may be separated by either adsorption or partition process. The solvents commonly used are of medium polarity:chloroform-95% ethanol 95:1; chloroform-acetone 3:1; n-hexane-ethyl acetate 4:1 on silica gel, silica gel-starch or alumina. The recommended chromatographic procedure (Stahl, 1969) for an unknown mixture in its unchanged form is the use of a relatively polar solvent like methylene dichloride-methanol-formamide (93:6:1), cyclohexane-ethylacetate (50:50) or chloroform-methanol(94:1) on silica gel G to fractionate into groups for further separation. The sapogenins of closed structural relationship such as a pair of C25 epimers, 5$\alpha$, 5$\beta$ and $\Delta^5$ sapogenins, are rather difficult to separate. The modification of the plates and the techniques of development including acetylation of the compounds may be necessary. Partition system of hexane-toluene-ethanol-water (60:30:3:27) can be used to resolve

similagenin (5β, 25α), tigogenin (5α, 25α), diosgenin ($\Delta^5$, 25α), sarsasapogenin (5β, 25β) and neotigogenin (5α, 25β) with difficulty but easily on silver nitrate containing silica gel. The multiple development as six-fold development in n-hexane-ethyl acetate(12:1) is devised for the separation and analysis of monohydroxy saturated sapogenin in *Asperagus*, *Smilax* and *Yucca* species (Blunden and Hardman, 1968). Two dimensional TLC on air-dried silica gel G layer is also used for detection and isolation of sapogenins in plant crude extract of *Cordyline* species (Blunden *et al.*, 1979 and 1981).

The popular reagents for detection of sapogenins are listed below (Stahl, 1969) :-

I. Aromatic aldehyde acids :

1. Anisaldehyde-sulfuric acid
2. Cinnamaldehyde-acetic anhydride-sulfuric acid
3. 4-Hydroxybenzaldehyde-sulfuric acid (Komarowsky reagent)
4. Resorcylaldehyde-sulfuric acid
5. Vanillin-phosphoric acid for steroids
6. Vanillin-sulfuric acid

II. Chlorosulfonic acid-acetic acid.

III. Sulfuric acid.

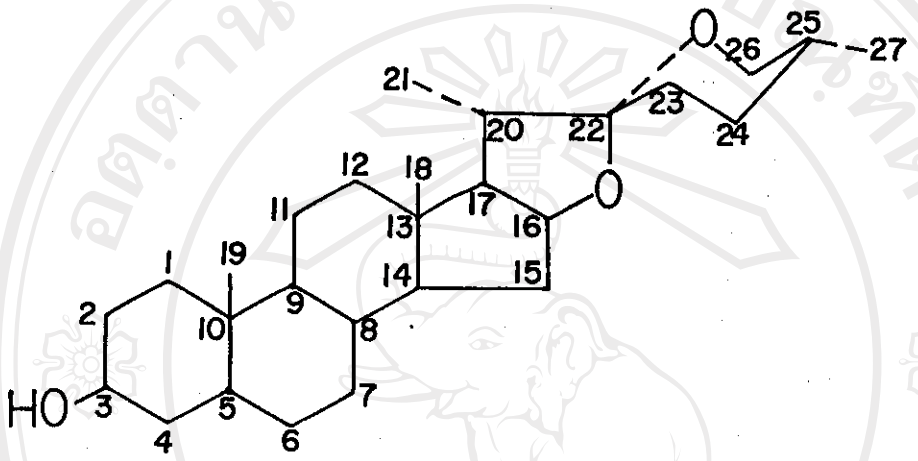
IV. Antimony chlorides.

Extracted sapogenin which may be accompanied with sterol and some fatty oils can be purified by repeated crystallization or by preparative TLC or by column chromatography.

III. IDENTIFICATION

Steroid sapogenins are a group of C27 compounds having the spi-

rostane skeleton in which the ring A/B juncture is *cis* (5β) or *trans* fused (5α) but the ring B/C, C/D junctures and always *trans* fused. Almost all without exception, they carry a hydroxyl group at C 3, hence they are almost all spirostan-3β-ol derivatives.




The various sapogenins are closely related, variation of natural sapogenin structures arise from (i) configuration at C 5 and C 25, (ii) number, position and configuration of nuclear hydroxyl group, and (iii) the occasional presence of a carbonyl group at C 12 and others.

The configuration at C 5 may be α as tigogenin, β as smilagenin or carry a double bond at C 5-6 as diosgenin. Mixture of C 25 epimers, for examples, sarsasapogenin and smilagenin, yamogenin and diosgenin are of normal occurence and their ratio, one to each other,is dependent upon factors such as morphological part and stage of development(Trease and Evans, 1985). The less stable compounds are called 25 S series (or 25 L, 25 β, normal or neo series) and the more stable compounds are named 25 R series (or 25 D, 25 α, iso series).

Nuclear hydroxyl groups occur at 1,2,3,4,5,6,11,12,15 positions in steroid nucleus and at 23, 24, 25, 27 positions in spiroketal side chain. The classification as mono, di, tri and tetrahydroxy steroid sapogenins may be useful for separation and identification of the compounds. Ketonic compounds as hecogenin which is found in *Agave* species also used as starting material of steroid compounds.

The natural spirostane and their derivatives sapogenin all have the same configuration at C 20 and C 22 (20 S and 22 R). After the discovery of jurubine by Schrieber group in 1966 and Sarsaparilloside by Tschesche and his colleague in 1967,the furostane sapogenin of their aglycone parts which ring F is kept open was thought to be the precursors of spirostane sapogenin.

Melting point and undepressed mixed melting point determination of the test samples, their acetate salts, and the authentic samples can be used for the identification of sapogenins. The presence of sapogenin is mostly confirmed by superimpossible IR spectrum of the purified test sample with that of an authentic one. Besides these, absorption spectra of their sulfuric chromogens are also a useful rapid method for identification. However, the screening method for the mixture of sapogenins in the crude extract of plant sample without purification, the chromatographic procedure of TLC, GLC and HPLC are the convenient and sensitive methods. Compounds are identified by their chromatographic behaviors, e.g., Rf values on TLC, or retention time in GLC and HPLC which can also be used to determine the individual sapogenin content simultaneously. The melting points of the naturally occurring sapogenins and their acetate derivatives as determined by ELK (1971 and 1974) are shown in Table II.1.

Table II.1 Naturally-Occurring Sapogenins

| Name | Source | Configuration $C_{(25)}$ | Configuration $C_{(5)}$ | Structure Substituents OH | Structure Substituents O | Structure Substituents Double bond | Sapogenin M.P. | Sapogenin $[\alpha]_D$ | Acetate M.P. | Acetate $[\alpha]_D$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Sarsasapogenin | Smilax sp.<br>Radix sarsaparilla<br>Yucca schottii (fruit) | S | β | 3β | - | - | 198-199° | -75° | 143-144° | -70° |
| Smilagenin | Radix sarsaparilla<br>Yucca schottii (flowers) | R | β | 3β | - | - | 187-188° | -66° | 150° | -60° |
| Neotigogenin | Chlorogalum pomeridianum<br>Digitalis sp. | S | α | 3β | - | - | 203° | -75° | 179° | -75° |
| Tigogenin | Chlorogalum pomeridianum | R | α | 3β | - | - | 208° | -67° | 206-208° | -74° |
| Yamogenin | Yucca sp.; Agave sp. | S | - | 3β | - | 5 | 201° | -129° | 182° | -119° |
| Diosgenin | Dioscorea sp. | R | - | 3β | - | 5 | 208° | -129° | 199-202° | -121° |
| Rhodeasapogenin | Trillium sp.<br>Rhodea japonica Roth. | S | β | 1β,3β | - | - | 293-295° | -72° | 185-187° | -70.5° |
| Isorhodeasapogenin | Rhodea japonica Roth. | R | β | 1β,3β | - | - | 245-248° | -71° | 205° | -73° |
| Ruscogenin | Ruscus aculeatus L. | R | - | 1β,3β | - | 5 | 205-210° | -127° | 192-194° | -85° |
| Markogenin | Yucca sp. | S | β | 2β,3β | - | - | 257° | -70° | 185-186° | -84° |
| Samogenin | Yucca schottii<br>Samuela carnerosana Trel. | R | β | 2β,3β | - | - | 203-205° | -74° | 198° | -84° |
| Yonogenin | Dioscorea tokoro Makino | R | β | 2β.3α | - | - | 240-243° | -53° | 212° | -29° |
| Neogitogenin | Digitalis purpurea;<br>D. lanata | S | α | 2α.3β | - | - | 248° | - | 218-220° | - |
| Gitogenin | Digitalis sp.;<br>Yucca schottii; Agave sp. | R | α | 2α.3β | - | - | 264-267° | -78° | 242-243° | -97° |
| Lilagenin | Lilium sp. | S | - | 2α,3β | - | 5 | 246° | - | 155° | |
| Yuccagenin | Yucca sp. | R | - | 2α,3β | - | 5 | 248° | -122° | 178° | -139° |

(continued)

Table II.1. (contd.)

| Name | Source | Configuration $C_{(25)}$ | Configuration $C_{(5)}$ | Structure Substituents OH | Structure Substituents O | Structure Substituents Double bond | Sapogenin M.P. | Sapogenin $[\alpha]_D$ | Acetate M.P. | Acetate $[\alpha]_D$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Neochlorogenin | Solanum paniculatum L. | S | α | 3β,6α | - | - | 261-265° | -65° | 200-202° | -51° |
| Chlorogenin | Chlorogalum pomeridianum | R | α | 3β,6α | - | - | 276° | -45° | 155° | -38° |
| Laxogenin | Smilax Sieboldi Miq. | R | α | 3β | 6 | - | 210-212° | -86° | 219-222° | -89° |
| Neonogiragenin | Metanarthecium luteo-viride Maxim. | S | β | 3β,11α | - | - | 128-131° | -75° | 142-144° | -78° |
| Nogiragenin | Metanarthecium luteo-viride Maxim. | R | β | 3β,11α | - | - | 200-201° | -71° | 208-209° | -74° |
| Tamusgenin | Tamus edulis Love | R | - | 3β | 11 | 5 | 180-182° | -75° | 209-213° | -31° |
| Rockogenin | Agave gracilipes Trel. | R | α | 3β,12β | - | - | 216-220° | -63° | 206-209° | -65° |
| Chiapagenin | Dioscorea chiapasensis Matuda | S | - | 3β,12β | - | 5 | 249-251° | -126° | 191-193° | -127° |
| Isochiapagenin | Dioscorea chiapasensis Matuda | R | - | 3β,12β | - | 5 | 236-237° | -121° | 206-207° | -120° |
| Heloniogenin | Heloniopsis orientalis (Thunb.) C., Tanaka | R | - | 3β,12α | - | 5 | 212-213° | -91° | 184-185° | -58° |
| Willagenin | Yucca filijera | S | β | 3β | 12 | - | 166-168° | -5° | 183-185° | -1° |
| Sisalagenin | Agave sisalana Perrine | S | α | 3β | 12 | - | 244-246° | -4.5° | 228-232° | -12° |
| Hecogenin | Agave sp. | R | α | 3β | 12 | - | 268° | -10° | 245° | -1° |
| Hispidogenin | Hechtia texensis Solanum hispidum Pers.; Asparagus umbellatus Link | R | α | 12β | 3 | - | 208° | -46° | 212° | -51° |
| Neobotogenin (= correllogenin) | Dioscorea spiculiflora | S | - | 3β | 12 | 5 | 209-211° | -69° | 213-215° | -67° |
| Botogenin (= gentrogenin) | Dioscorea spiculiflora | R | - | 3β | 12 | 5 | 215-216° | -57° | 227° | -56° |
| 9-Dehydrohecogenin | Agave sp. | R | α | 3β | 12 | 9 | 223-226° | -10° | 215-217° | -9° |

(continued)

| Name | Source | Configuration $C_{(25)}$ | Configuration $C_{(5)}$ | Structure Substituents OH | Structure Substituents O | Structure Substituents Double bond | Sapogenin M.P. | Sapogenin $[\alpha]_D$ | Acetate M.P. | Acetate $[\alpha]_D$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Neodigalogenin | Digitalis purpurea; D. lanata | S | α | 3β,15β | - | - | 223-226° | - | 211-212° | -88° |
| Digalogenin | Digitalis purpurea; D. lanata | R | α | 2β,15β | - | - | 218-219° | -75.5° | 238-239° | -70° |
| Tokorogenin | Dioscorea tokoro | R | β | 1β,2β,3α | - | - | 266-268° | -50° | 255° | -8° |
| Convallagenin-A | Convallaria keisukei | S | β | 1β,3β,5β | - | - | 268-269° | -28° | 208-210° | -78° |
| Diotigenin | Dioscorea tenuipes | S | β | 2β,3α,4β | - | - | - | - | - | - |
| Agapanthagenin | Agapanthus sp. | R | α | 2α,3β,5α | - | - | 285° | - | 298-299° | -101° |
| Magogenin | Maguey cacaya | R | α | 2ξ,3ξ,6ξ | - | - | 284° | - | 214° | - |
| Meragenin | Metanarthecium luteoviride Maxim. | R | β | 2β,3β,11α | - | - | 273-274° | -82° | 250-252° | -77.5° |
| Neomexogenin | Agave roezliana | S | β | 2β,3β | 12 | - | 221-222° | - | 162-164° | - |
| Mexogenin | Yucca schottii; Samuela carnerosana Trel. | R | β | 2β,3β | 12 | - | 237-238° | -6° | 208° | - |
| Agavogenin | Agave huachucensis Baker | R | α | 2α,3β,12ξ | - | - | 242° | -62° | 230° | -98° |
| Neomanogenin | Yucca schottii; Agave striata | S | α | 2α,3β | 12 | - | 242° | - | 222° | - |
| Manogenin | Agave sp.; Manfreda inaculosa Hook | R | α | 2α,3β | 12 | - | 242° | -5° | 259° | -45° |
| Neokammogenin | Dioscorea mexicana | S | - | 2α,3β | 12 | 5 | 230° | - | 203-205° | - |
| Kammogenin | Yucca sp.; Samuela carnerosana Trel. | R | - | 2α,3β | 12 | 5 | 245° | -53° | 259° | -83° |
| 9-Dehydromanogenin | Agave sp. | R | α | 2α,3β | 12 | 9 | 240° | -16° | 263° | -62° |
| Neodigitogenin | Digitalis purpurea | S | α | 2α,3β,15β | - | - | 277-279° | -82° | 229-232° | -114° |
| Digitogenin | Digitalis purpurea; D. lanata | R | α | 2α,3β,15β | - | - | 296° | -80° | 235-236° | -103° |
| Kogagenin | Dioscorea tokoro Makino | R | β | 1β,2β,3α,5β | - | - | 318-322° | -27° | 249-252° | -26° |
| Convallagenin-B | Convallaria keisukei | S | β | 1β,3β,4β,5β | - | - | 277-278° | -43° | 228-230° | -46.5° |
| Kitigenin | Reineckia carnea Kunth. | R | β | 1β,3β,4β,5β | - | - | 298° | -35° | 219-220° | -54° |
| Cacogenin | Maguey cacaya | R | α | 2ξ,3ξ,6ξ | 12 | - | 278° | - | 248° | - |
| Pentologenin | Reineckia carnea Kunth. | R | ξ | 1ξ,2ξ,3ξ 4ξ,5 | - | - | 320° | -54.5° | 165-168° | - |

Table II.1 (contd.)

| Name | Source | Configuration $C_{(25)}$ | Configuration $C_{(5)}$ | Structure Substituents OH | Structure Substituents O | Structure Substituents Double bond | Sapogenin M.P. | Sapogenin $[\alpha]_D$ | Acetate M.P. | Acetate $[\alpha]_D$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Macranthogenin | Helleborus macrantbus | - | β | 3β | - | 25(27) | 162-164° | -69.5° | 138-144° | -63.5° |
| Sceptrumgenin | Isoplexis sceptrum | - | - | 3β | - | 5,25(27) | 182-184° | -122° | 191-193° | -119° |
| (25S)-Ruscogenin | Sansevieria trifasciata | S | - | 1β,3β | - | 5 | 194-196°<br>212-214° | -112° | 182-185° | -88° |
| Neoyonogenin | Dioscorea tenuipes | S | β | 2β,3α | - | - | 198-199° | -64° | 184-187° | -21° |
| 12β-Hydroxy-smilagenin | Yucca gloriosa | R | β | 3β,12β | - | - | 233-235° | -62.3° | 203.5-205° | -54.3° |
| Isoplexigenin-A | Isoplexis sceptrum | R | α | 3β,23S | - | - | 227° | -61° | 194-196° | -60° |
| Isoplexigenin-B | Isoplexis sceptrum | R | - | 3β,23S | - | 5 | 205-207° | -96° | 187-190° | -97° |
| 7-Oxodiosgenin | Tamus edulis Lowe | R | - | 3β | 7 | 5 | 215-220° | -170° | 197-198.5° | -170° |
| Gloriogenin | Yucca gloriosa | R | β | 3β | 12 | - | 188.5-190° | +15° | 217-219° | +12° |
| Torvogenin | Solanum torvum | S | α | 12α | 3 | - | | | | |
| Sisalagenone | Solanum torvum | S | α | - | 3,12 | - | | | | |
| Neotokorogenin | Dioscorea tenuipes | S | β | 1β,2β,3α | - | - | 250° | -55° | 185-190° | -21° |
| Sansevierigenin | Helleborus odorus; H. niger | - | - | 1β,3β,11α | - | 5,25(27) | 236-240° | -87° | 190-193° | -132° |
| Isoplexigenin-C | Sansevieria trifasciata | - | - | 1β,3β,23S | - | 5,25(27) | 234-237° | -125° | - | - |
| Isoplexigenin-D | Isoplexis sceptrum | R | α | 2α,3β,23S | - | - | 272.5-273.5° | -62° | 210-213° | -83° |
| Crestagenin | Isoplexis sceptrum | R | α | 2α,3β,23R | - | - | 280-281° | -74° | 198-200° | -91° |
| Igagenin | Digitalis canariensis | S | α | 2α,3β,27 | - | - | 255-260° | -44° | 196-201° | -94° |
| Asperagenin | Dioscorea tokoro | S | β | 2β,3α,27 | - | - | 253 | -44° | 191-192° | -15° |
| Anosmagenin | Smilax aspera | R | β | 3β,6α,25 | - | - | 264-268° | -136° | 185-186° | -89° |
| 7-Oxotamusgenin | Solanum vespertilio | R | - | 3β,15β,23 | - | 5 | 133-136° | -93° | - | - |
| Lowegenin | Tamus edulis Lowe | R | - | 3β | 7,11 | 5 | 250-253° | -131° | 191-194° | -133° |
| (25S)-25-Hydroxytamusgenin | Tamus edulis Lowe | R | - | 3β,16α | 11 | 5 | 223-226° | -55° | 200-203° | -62° |
| 15-Dehydro-14β-anosmagenin | Tamus edulis Lowe | S | - | 3β,25 | 11 | 5 | 245-247° | -76° | 210-212° | - |
| Dracogenin | Solanum vespertilio | R | j | 3β,23S | 15 | 5 | 139-142° | -75° | - | - |
| Afurigenin | Dracaena draco | - | - | 1β,3β,23S,24S | - | 5,25(27) | 215-217° | -144° | - | - |
| Eduligenin | Tamus edulis Lowe | see text | | | | | 240-243° | -50° | 147-151° | - |
| | Tamus edulis Lowe | see text | | | | | 233-237° | -139° | 187-189° | -125° |

The absorption spectra of sulfururic chromogens of steroidal sapogenins for the identification of these substances were reported by Diaz *et al.* (1952). Walen *et al.*(1954) also used sulfuric acid for the detection and estimation of steroid sapogenins by spectrophotometric method. He found that steroidal sapogenins dissolved in sulfuric acid had a characteristic ultra violet absorption spectra in the 220-400 nm region. The optimum reaction condition suggested by Walen group was the use of 0.1 to 5.0 mg of sapogenins dissolved in 10 ml of 94% $H_2SO_4$ and warmed at $40^{\circ}$ C for 16 hours while Diaz *et al.* (1952) found that the most stable and reproducible chromogens was carried out by heating the sapogenins (approximately 5 mg) in 10 ml of concentrate sulfuric acid at $40^{\circ}$ for one hour and measured the absorption from 320 to 600 nm.

Walen *et al.* (1954) pointed out that a maximum absorption in the region of 270-275 nm of all of the sapogenins was probably due to the spiroketal structure of the E and F rings or to its open derivatives. The peak at 350 nm belongs to the 12 keto sapogenins while the 2,3 dihydroxy sapogenin with a 5-6 double bond has a characteristic peak near 235 nm. Diosgenin (3 OH $\Delta^5$) has a characteristic absorption maxima in the region of 400-600 nm. Saturated, non carbonyl monohydroxy dihydroxy or desoxy sapogenins exhibit a typical absorption maximum near 310 nm except chlorogenin which has a characteristic peak at 330 nm. Many sapogenins have characteristic peaks in the region of 375-600 nm which are useful for their identification. The position of the absorption maxima and minima with log E values for the common steroidal sapogenins reported by Diaz *et al.* (1952 ) and Walen *et al.* (1954) are tabulated in Table II.2 and II.3, respectively.

Table II.2 Absorption Spectra of Sulfuric Acid Chromogens of Steroidal Sapogenins.

| Sapogenin | | | Color with $H_2SO_4$ | Absorption Spectrum in $H_2SO_4$ (320-600 m ) | |
|---|---|---|---|---|---|
| No. | New Nomenclature (ref.4) | Old Nomenclature | | Max (log e) | Min (log e) |
| 1 | 22-Isoallospirostan | Desoxytigogenin | Colorless | No max. | |
| 2 | 22-Isoallospirostan-3β-ol | Tigogenin | Yellow | 394 (3.50) | 375 (3.44) |
| 3 | 22-Isoallospirostan-3-one | Tigogenone | Colorless | 453 (1.98) | 438 (1.92) |
| 4 | 22-Isospirostan-3α-ol | Epismilagenin | Light yellow | 390 (3.52) | 383 (3.50) |
| 5 | 22-Isospirostan-3-one | Smilagenone | Light yelloq | 424 (2.78) | 390 (2.63) |
| 6 | Spirostan-3β-ol | Sarsasapogenin | Yellow | 398 (3.44) | 380 (3.39) |
| 7 | $\Delta^5$-Spirosten-3β-ol | Yammogenin | Orange | 412 (4.13), 512 (3.40) | 364 (3.78), 457 (3.38) |
| 8 | $\Delta^5$-22-Isospirosten-3β-ol | Diosgenin | Orange | 334 (3.86), 412 (4.11), 512 (3.52) | 366 (3.77), 458 (3.36) |
| 9 | $\Delta^4$-22-Isospirosten-3-one | -Diosgenone | Yellow | 459 (1.93), 568 (1.70) | 438 (1.87), 545 (1.66) |
| 10 | $\Delta^7$-22-Isoallospirosten-3β-ol | - | Yellow | 412 (4.23), 470 (3.27) | 364 (3.91), 460 (3.26) |
| 11 | $\Delta^{8\ 14}$-22-Isoallospirosten-3β-ol | - | Orange | 411 (4.21, 469 (3.34) | 363 (3.90), 462 (3.32) |
| 12 | $\Delta^{9\ 11}$-22-Isoallospirosten-3β-ol | - | Yellow | 410 (4.01), 472 (3.22) | 376 (3.84), 460 (3.20) |
| 13 | 22-Isoallospirostan-2α, 3β-diol | Gitogenin | Purple | 399 (3.09) | 386 (3.07) |
| 14 | 22-Isoallospirostan-2α, 3α-diol | - | Colorless | 493 (2.44 | 485 (2.43) |
| 15 | 22-Isoallospirostan-2β, 3α-diol | - | Colorless | 384 (3.07) | 382 (3.06) |
| 16 | 22-Isoallospirostan-3β, 6α-diol | Chlorogenin | Yellow | 330 (3.98), 400 (3.56) 470 (3.11) | 320 (3.95), 378 (3.50), 456 (3.07) |
| 17 | $\Delta^5$-22-Isospirostene-2α, 3β-diol | Yuccagenin | Purple | 345 (3.67), 404 (3.91) | 321 (3.60), 362 (3.55) |

| No | Sapogenin | | Color with $H_2SO_4$ | Absorption Spectrum in $H_2SO_4$ (320-600 m) | |
|---|---|---|---|---|---|
| | New Nomenclature (ref.4) | Old Nomenclature | | Max (log e) | Min (log e) |
| 18 | 22-Isospirostan-2, 3-diol (?) | Samogenin | Colorless | 342 (3.10), 398 (2.83), 494 (2.14) | 320 (3.07), 385 (2.82), 452 (2.06) |
| 19 | 22-Isoallospirostan-3β-ol -12-one | Hecogenin | Yellow | 351 (3.86), 396 (4.20) | 335 (3.83), 358 (3.85) |
| 20 | $\Delta^{9\ 11}$ -22-Isoallospiros-ten-3β-ol-12-one | 9-Dehydroheco-genin | Pink | 388 (4.18), 514 (2.95) | 320 (3.81), 478 (2.91) |
| 21 | 22-Isospirostan-2,3-diol-12-one (?) | Mexogenin | Colorless | 347 (3.62), 394 (3.31), 468 (2.82) | 320 (3.34), 378 (3.28), 440 (2.81) |
| 22 | 22-Isoallospirostan-2α, 3β-diol-12-one | Mannogenin | Light purple | 346 (3.77), 470 (2.78) | 320 (3.58), 440 (2.72) |
| 23 | $\Delta^5$-22-Isospirostene-2α, 3β-diol-12-one | Kammogenin | Purple | 346 (3.65), 474 (2.82), 554 (2.81) | 320 (3.41), 428 (2.72), 530 (2.75) |
| 24 | Cholestane-3β,26-diol-16, 22-dione | Dihydrokrypto-genin | Yellow | 380 (4.00) | 352 (3.75) |
| 25 | $\Delta^5$-Cholestene-3β,26-diol-16, 22-dione | Kryptogenin | Orange | 384 (4.02), 484 (2.89) | 338 (3.88), 462 (2.87) |
| 26 | $\Delta^{5\ 10\ 23}$ Fesadien-3β, 26 diol022-one | Fesogenin | Orange | 363 (3.95), 414 (4.07) | 320 (3.61), 382 (3.80) |

Table II.3 Wave Length Positions and Intensities of Absorption Maxima of Sulfuric Acid Chromogens of Steroidal Sapogenins

| Sapogenin | Absorption Maxima, M | $\log_e{}^a$ at Corresponding Maxima |
|---|---|---|
| Chlorogenin | 270,330,415 | 3.96,4.00,3.74 |
| Diosgenin | 271,415,514 | 3.99,4.06,3.64 |
| Desoxydiosgenin | 271,312 | 4.00,3.39 |
| Gitogenin | 272,308 | 4.08,4.11 |
| Hecogenin | 276,350,396 | 4.06,4.10,4.24 |
| Desoxyhecogenin | 268,348,394 | 3.97,3.98,3.61 |
| Hecogenone | 269,347 | 3.95,3.89 |
| Kammogenin | 233,272,349 | 4.11,4.02,3.89 |
| Kryptogenin | 280,383 | 3.77,4.06 |
| Manogenin | 276,348,400,468 | 4.00,4.04,3.69,3.44 |
| Markogenin | 270,308 | 4.00,3.90 |
| Rockogenin | 273,379 | 3.85,4.07 |
| Samogenin | 270,308 | 4.01,3.91 |
| Sarsasapogenin | 271,310 | 3.98,3.85 |
| Desoxysarsasapogenin | 272,808 | 3.97,3.92 |
| Sarsasapogenone | 267,310 | 4.01,3.76 |
| Smilagenin | 272,312 | 3.98,3.90 |
| Desoxysmilagenin | 273,308 | 4.00,3.94 |
| Smilagenone | 268,310 | 4.06,3.86 |
| Tigogenin | 270,312 | 3.94,3.88 |
| Desoxytigogenin | 274,296 | 3.99,4.03 |
| Yuccagenin | 240,268,405 | 4.11,4.00,3.83 |
| Cholesterol | 308,416 | 3.95,3.58 |

a =Logarithm of molecular absorptivity.

The IR absorption spectra of thirty five steroid sapogenins and derivatives has been investigated by Jones *et al.* (1953). He observes the several strong band characteristics of the spiroketal chain between 875-1350 $cm^{-1}$ while 3 hydroxy steroid sapogenins shows a character of 3-OH between 1000 and 1050 $cm^{-1}$ superimposed on the side chain absorption. The introduction of additional oxygen containing groups into ring B, C and D of the steroid nucleus induces minor but significant changes in the spectra as the 12 keto group was associated with increased absorption spectra near 1040 and 1074 $cm^{-1}$.

The characteristic band in the IR spectra of the spirostane ring and hence of sapogenins are near 980, 915, 900, 865 and/or 840 $cm^{-1}$. IR spectra with high resolution in the 3100-2750 $cm^{-1}$ region are sensitive to structural changes in the carbon skeleton and could possibly be used to distinguish the 25 D and 25 L configurations, to detect opening of E and F rings and to determine the configuration of the methyl group in the 20 position (Smith and Eddy, 1959).

Unknown sapogenin acetates could also be readily identified by means of IR spectrum by the following procedure as suggested by Eddy *et al.* (1953) :

1. Examine the four absorption bands near 860,900,920 and 980 $cm^{-1}$. If any one of these is missing, the substance is not a true steroidal sapogenin with F ring.

2. If the band occurs at 850,900,920 and 986 $cm^{-1}$ with 920 band stronger than 900 band, it is a sapogenin with normal series (25β). If the four bands occur at 865,900,920 and 981 $cm^{-1}$ with the 900 band stronger than the 920 band, the substance is a sapogenin with iso- configuration of the F ring (25 R).

3. Determine whether it is the acetate of a mono or dihydroxy sapogenins by examining the acetate bands near 1240 and 1740 $cm^{-1}$.

4. Mono and dihydroxy sapogenins may be distinguished from each other by examining the detail characteristic of the finger print like $\Delta^5$ ethylenic bond of diosgenin which shows the prominent band at 813 and 838 $cm^{-1}$.

5. Confirm the identification by cnomparing with authentic sample.

## IV. QUANTITATIVE DETERMINATION OF SAPOGENINS

Several methods are available for the determination of steroid sapogenins from plant material such as gravimetric, spectrophotometric, gas-liquid chromatography, densitometric thin-layer chromatography and IR spectrometric method. All these methods have already been discussed in Part I.

High performance liquid chromatography which is a rapid, selective, highly sensitive and widely used in separation techniques for many compounds because suitable conditions such as stationary and mobile phases can be optimized for various compounds. To separate sapogenins, this technique is also used. The sapogenins are identified by their chromatographic characters and retention times and also quantitatively estimated by comparing their peak areas with the reference standard solutions at the some carefully controlled conditions. As sapogenins do not exhibit any UV absorption that would be helpful for detection with UV detector at 210 and 254 nm, a differential refractometer detector are always used. However, for acetate derivatives of sapogenin, UV detector can also be used.

Diosgenin, the most important and versatile starting material

for the production of steroid drugs was determined either by normal phase system or by reverse phase system. Mahato *et al.* (1981) used μ porasil column which was very polar, fully porous silica material column and the mixture of light petroleum (BP.60-80° C) : isopropanol (12:1) as mobile phase to determine diosgenin from *Dioscorea* spp., *Costus speciosus* Sm. and *Kallstroemia pubescens* Dandy, while Tal and Goldberg (1981) used Micropak MCH 10 (RP-C 18 column) which was monomeric layer of octadecylsilane permanently bond to LiChrosorb Si 60 column, with 80% acetonitrile, 10% methanol and 7% chloroform as mobile phase to determine diosgenin content from cell culture of *D. detoidea* Wall. The comparative conditions of these two methods have been previously discussed in Part I.

In this study, the reverse phase HPLC system proposed by Tal and Goldberg was also investigated and the experimental conditions were modified for the separation, identification and quantitative estimation of the sapogenin content in the rhizomes of *Costus lacerus* Gagn.

Apparatus

1. Continuous extraction apparatus (Soxhlet apparatus), capacity 500 ml and 5 liters (Excello)
2. Rotatory evaporator (Buchi).
3. Glass plates, 10x20 cm, 20x20 cm.
4. Layer casting apparatus (Camag).
5. TLC and P-TLC tanks (Camag).
6. Spotting apparatus (Camag Linomat III).
7. Iodine tank for detection.
8. UV lamp for TLC detection (Chromato-Vue).

9. Melting point instrument (Metler FP 61).

10. Double beam spectrophotometer : UVIDEC 650 (Jasco) with 1.0 cm matched quartz cells.

11. Infra-red spectrophotometer : UNICAM sp 1000.

12. Water bath maintained at $40^{\circ}$ C.

13. High Performance Liquid Chromatography equipment (Water Associates) which composed of :

- Solvent Delivery System Model 6000A,
- Universal Liquid Chromatographic Injector Model U6K,
- Differential Refractometer Detector Model R401,
- OmniScribe B 5000 Strip Chart Recorder Model B 5217-1AI.

14. LC column μ Bondapak C 18 (3.9 mmID. x30 cm in length).

15. Ultrasonic bath (Bransonic$^{R}$ 221).

16. Sample clarification kit for organic solvent with Fluoropore$^{R}$ filter, pore size 0.5μ m (FHLP 01300).

17. Solvent clarification kit with Fluoropore$^{R}$ filter, pore size 0.5 μm. (FHUP 04700).

18. Microliter$^{R}$ syringe (capacity 10 μl).

Chemicals and reagents

1. Reagents for preliminary examinations (Appendix A).

2. Visualizing agents for TLC (Appendix B).

3. Hexane (BP. $69.0^{\circ}$ C, density 0.662, $C_2H_4$ (FW=86.9) (Baker Analyzed reagent)

4. 2N hydrochloric acid.

5. 10% Ammonia solution.

6. Silica gel 60 G, GF254 for TLC and silica gel 60 P for P-TLC.

7. n-Hexane, AR (May & Baker).

8. Ethyl acetate, AR (Hopkins and William).

9. Benzene, AR (Merck).

10. Silver nitrate, AR (May and Baker).

11. Dichloromethane, AR (BDH).

12. Acetone, AR (May and Baker).

13. Formamide, AR (BDH).

14. Methanol, AR (May and Baker).

15. Cyclohexane, AR (BDH).

16. Melting point standard benzoic acid (Metler ME 18555, MP. 124.4$^{o}$ C).

17. Diosgenin (Sigma Chemical Company) D-1634.

18. Hecogenin (Sigma Chemical Company) H-2261.

19. Tigogenin (Sigma Chemical Company) T-2626.

20. Potassium bromide for infra-red spectroscopy (BDH).

21. Sulfuric acid, AR (Riedel).

22. Methanol, HPLC grade (Carlo-Erba).

23. Acetonitrile, HPLC grade (Baker Analyzed Reagent).

24. Chloroform, HPLC grade (Carlo-Erba).

Procedure :-

I. PRELIMINARY EXAMINATIONS

I.1 Systematic qualitative tests

Fresh rhizomes of *C. lacerus* Gagn. which had been previously washed and cleaned with water were sliced into small pieces and dried in the oven at 40$^{o}$ C for 48-60 hours or until they were completely dried. Then they were reduced to powder and sifted through seive No.40.

An amount of 10-25 grams of sifted vegetable powder was extracted with ethyl ether and followed by ethanol in a continuous extraction apparatus (Soxhlet apparatus) until it was completely exhausted. The ether and alcohol extracts obtained were filtered through filter paper (Whatman No.1), and concentrated to volume of about 40-50 ml. The vegetable product which had been extracted with ethyl ether and ethanol was dried and finally extracted with warm water in a conical flask for 20 minutes. The aqueous extract obtained was also filtered and concentrated to a volume of 50 ml.

All of the extracts were identified according to chemical constituents as follows :-

I.1.1 Determination of the ether extract

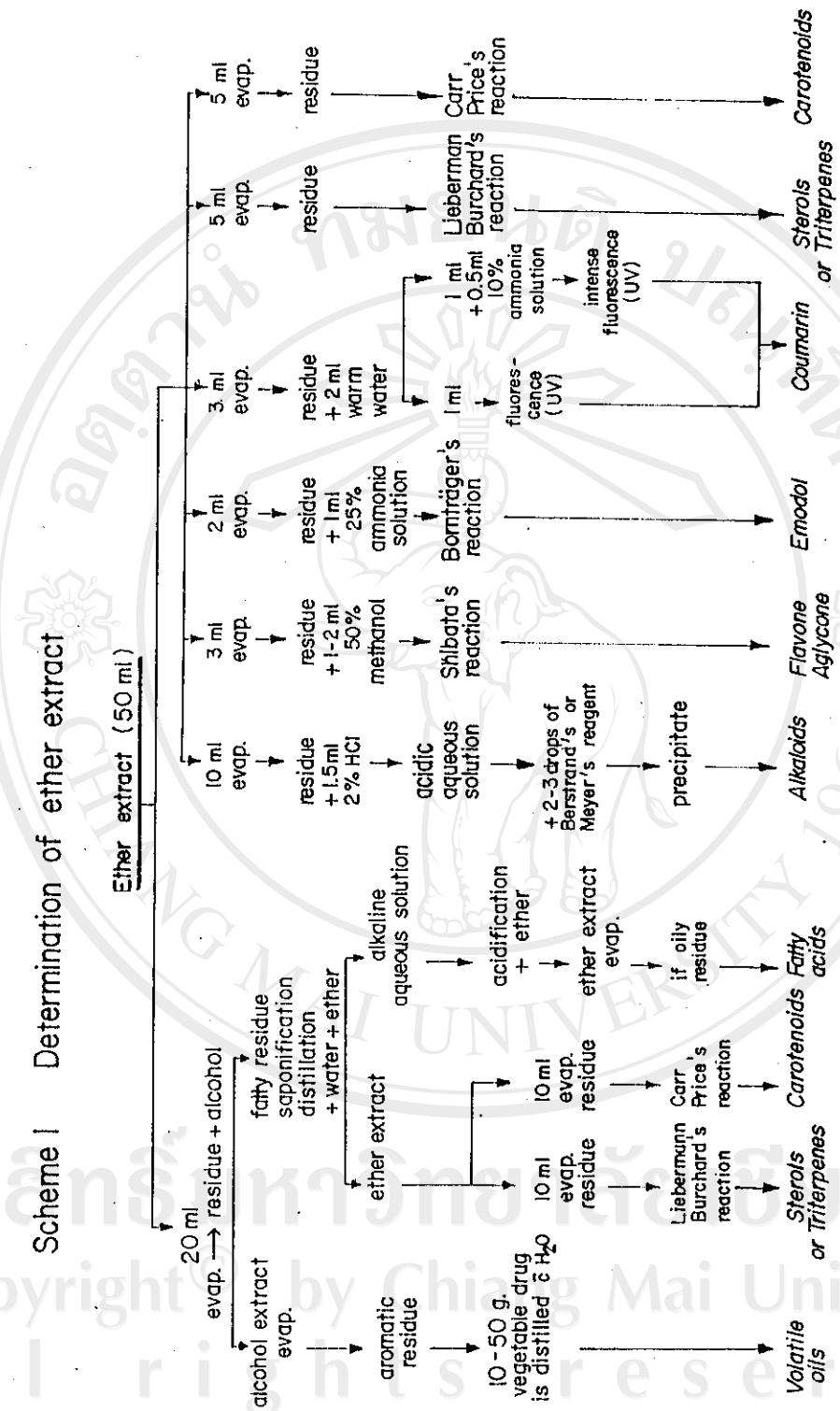
The ether extract was identified for non-polar constituents as follows (Scheme I) :

I.1.1.1. Identification of volatile oils and fatty acids.

The ether extract (10-20 ml) was placed in a flask and evaporated to dryness. If the residue had a pleasant odor, it was then dissolved in a small amount of alcohol and repeated elutions were performed. One part of the alcoholic solution was evaporated to dryness. If the vegetable product contained some volatile oils, the residue should have a characteristic odor. In such a case, the qualitative and quantitative determination of volatile oil in vegetable product could be confirmed by means of Neo-cleavenger apparatus, TLC, or GLC.

Within the alcoholic elution, besides the volatile oil and basic alkaloids, free aglycones might also be found while the fatty substances were usually recovered in the residue. Therefore, the rest of the alco-

Scheme I Determination of ether extract



holic solutions might be served to identified these constituents using the methods described in I.1.1.2.-I.1.1.6.

The residue obtained after an alcoholic elution was used to identify the fatty substances by saponification method. Potassium hydroxide in alcoholic solution (10 ml of 0.5 N) was added to the residue and refluxed on the boiling water bath until the oil drops on liquid surface were disappeared (1-2 hours). The alcohol was distilled off and the residue was redissolved in hot distilled water (15-20 ml). The solution was transferred to a separatory funnel. The residue in the flask was firstly washed with a small amount of hot distilled water and the solution was transferred to the same separatory funnel. The residue was washed several times with ether. These ether solutions were also transferred with agitation to the same separatory funnel that contained the alkaline aqueous solution because of an excess potassium hydroxide added initially. The ether extract could also be used to identify for sterols, triterpenes and carotenoids as described in I.1.1.6 and I.1.1.7. The alkaline aqueous solutions was used to identify higher fatty acids.

The alkaline aqueous solution was acidified with concentrated HCl to pH 3-4. Under these conditions, the fatty acids were released from their alkaline salts and the solution became opalescent. The fatty acids were extracted by shaking the solution repeatedly with a small amount of ether two or three times in a separatory funnel. The ether solutions collected were evaporated to dryness. If the residue was oily, fatty acids were present.

The rest of 30 ml of ether extract was used to identify basic alkaloids,free aglycones,carotenoids and coumarins that might be present.

I.1.1.2. Identification of basic alkaloids

The ether extract (10 ml) was evaporated to dryness. The residue was dissolved in 1.5 ml of 2% HCl. The solution was then divided equally into three separated test tubes. The first tube contained 0.5 ml acidic aqueous sample solution was used as reference. A few drops of Bertrand's reagent or Mayer's reagent (or other alkaloidal precipitating agents) were added to the second and the third ones. An opalescence or precipitates (yellowish white) indicated the presence of alkaloids.

I.1.1.3. Identification of coumarins

The ether extract (3 ml) was evaporated to dryness. The residue was dissolved in hot water. After cooling, the solution was divided equally into two test tubes. The first sample tube was used as reference and the second sample tube was made alkaline with 0.5 ml of diluted ammonia solution. The occurrence of an intense fluorescence under UV light indicated the presence of coumarin derivatives. The presence of these substances could be confirmed by the Feigl's reaction.

I.1.1.4. Identification of flavone aglycones(Shibata's reaction or Cyanidin test).

The ether extract (3 ml) was evaporated to dryness. The residue was dissolved in 1.2 ml of 50% hot methanol. Metallic magnesium and 4-5 drops of concentration HCl were added. A red or orange colour indicated the presence of flavone aglycones.

I.1.1.5. Identification of anthraquinone aglycones. (Borntrager's reaction)

The ether extract (3 ml) was transferred to a test tube and 1

ml of 25% ammonia solution or 10% sodium hydroxide solution was added and shaken. A red colour indicated the presence of anthraquinone glycosides.

I.1.1.6. Identification of sterols and triterpene (Lieberman-Burchard's reaction)

The ether extract (10 ml) was evaporated to dryness. The residue was dissolved in 0.5 ml of acetic anhydride and subsequently, 0.5 ml of chloroform was added. The solution was transferred into a dry test tube and by means of a pipette, concentrated $H_2SO_4$ (1-2 ml) was carefully added at the bottom of the tube. At the contact zone of the two liquids, a brownish red or violet ring was formed and the supernatant layer became green or violet denoting the presence of sterols and triterpenes, respectively.

I.1.1.7. Identification of carotenoids. (Carr Price's reaction)

The ether extract (10 ml) was evaporated to dryness. A few drops of saturated solution of antimony trichloride in chloroform (Carr Price's reagent) was added to the residue. The pigments were firstly blue and later became red. With the addition of concentrated $H_2SO_4$ to this solution, carotenoids usually developed into deep blue or bluish green.

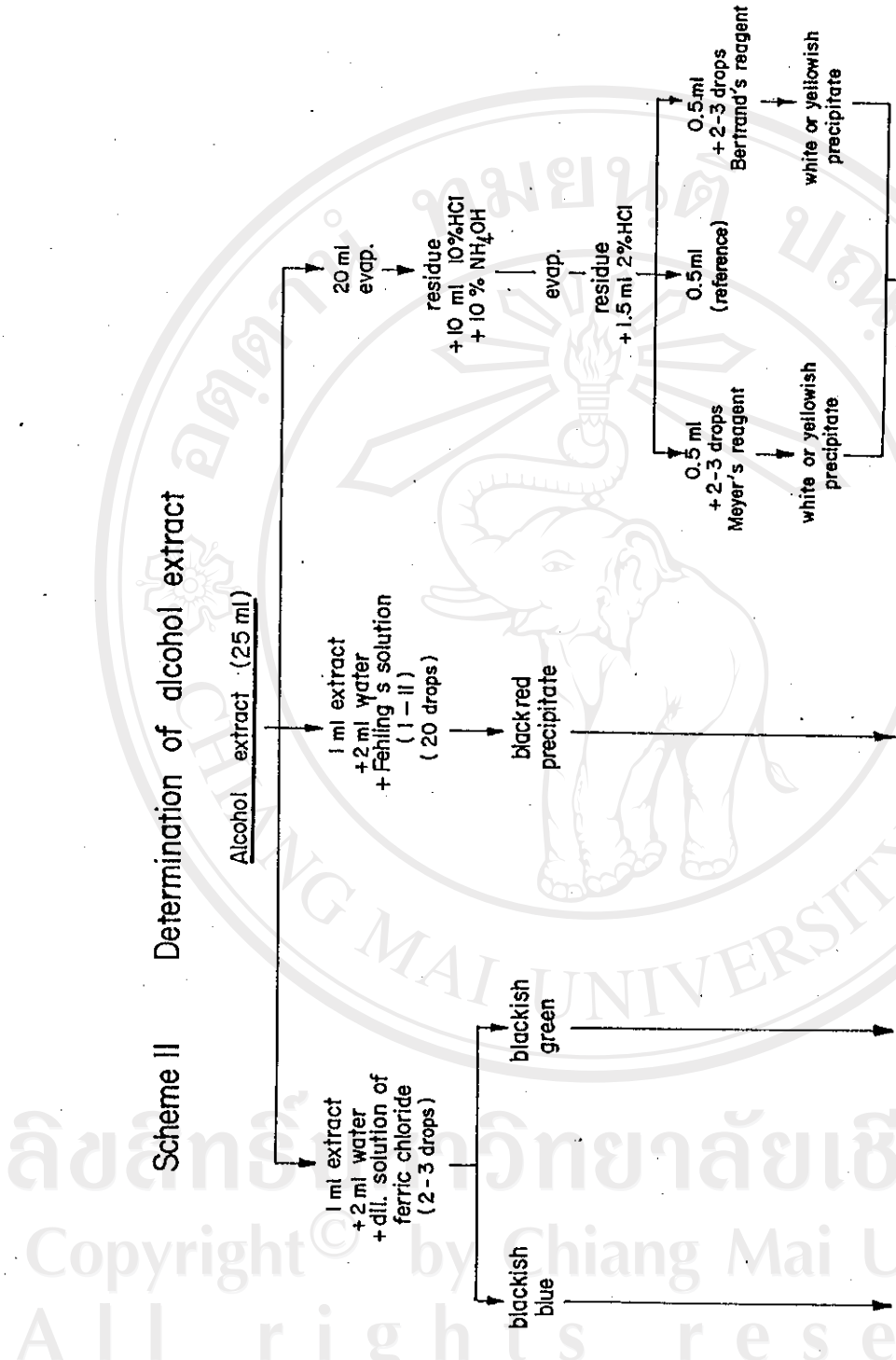
I.1.2. Determination of the alcohol extract.

The alcohol extract was identified for natural constituents of intermediate polarity as illustrated in Scheme II and follows:-

I.1.2.1. Identification of tannins.

The alcohol extract (0.5-1 ml) was diluted with 1-2 ml of water. A few drops of the diluted solution of ferric chloride was added. The

Scheme II Determination of alcohol extract



occurrence of blackish blue colour showed the presence of gallic tannins and a blackish green colour indicated catechol tannins. When the extract contained both types of tannins, separation of the two could be done by adding Styassny's reagent to extract and the solution was boiled under reflux. Under these conditions, the catechol tannin was condensed into red precipitates and subsequently filtered. The solution thus obtained was neutralized with sodium acetate and some drops of ferric chloride solution added. A deep blue colour denoted the presence of gallic tannins.

I.1.2.2. Identification of reducing compounds.

The alcohol extract (0.5-1 ml) was diluted with 1-2 ml of water. Fehling's solutions I and II (0.5-1 ml each) were added and heated. Brick-red precipitates indicated the presence of reducing compounds.

I.1.2.3. Identification of alkaloidal salts.

The alcohol extract (20 ml) was evaporated on a boiling water bath or hot sand bath. Diluted HCl (5-10 ml) was added to the residue. The alkaloids (if present) became salts of mineral acid and were precipitated as bases with diluted ammonia solution at pH 8-9 and then extracted with non-polar solvent such as ether or chloroform. The ether or chloroform solution was evaporated to dryness and identified for basic alkaloids as described in I.1.1.2.

I.1.3. Determination of the hydrolyzed alcohol extract.

To 25 ml of alcohol extract, 15 ml of dilute HCl was added and refluxed for 30 minutes. During the hydrolysis, the solution became opalescent due to the precipitating aglycones obtained by the cleavage

of the glycosides. After cooling, the solution was extracted three times in a separatory funnel with ethyl ether (10-21 ml). The ether extracts were combined (30-36 ml) and dehydrated with anhydrous sodium sulfate, resulting in an ether and an aqueous solutions. The ether solution was used to identify aglycone part of flavone glycosides, anthraquinone glycosides, sterols and triterpenes, and coumarins as described previously for ether extract and to identify cardenolides, aglycones of cardiac glycosides (3.1 below). An acidic aqueous solution was used to identify anthocyanidin glycodise as described in I.1.3.2 below (See Scheme III).

I.1.3.1. Identification of cardenolides (Keddee's reaction)

The ether extract (4 ml) was evaporated to dryness. The residue was dissolved in 1-2 ml of methanol. One or two milliliters of 1N potassium hydroxide in alcoholic solution and a few drops of 3,5-dinitrobenzoic solution (Keddee's reagent) were added. By heating, a disappearance of violet colour indicated the presence of cardenolides.

I.1.3.2. Identification of anthocyanosides(anthocyanin pigment).

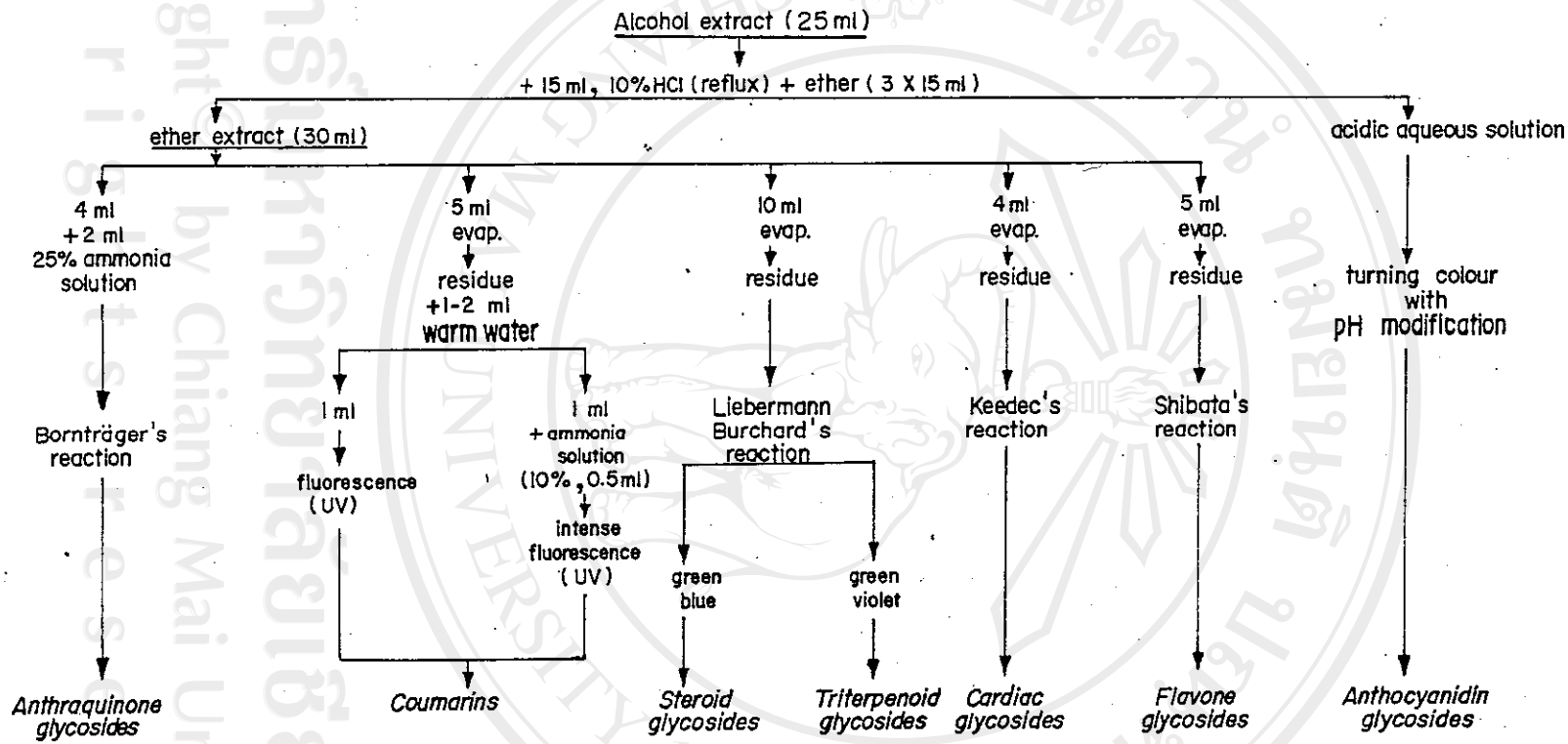
If the acidic solution in I.1.3 was red and turn to violet at neutral pH or turn to green or blue in an alkaline medium, anthocyanins were present.

I.1.4. Determination of the aqueous extract

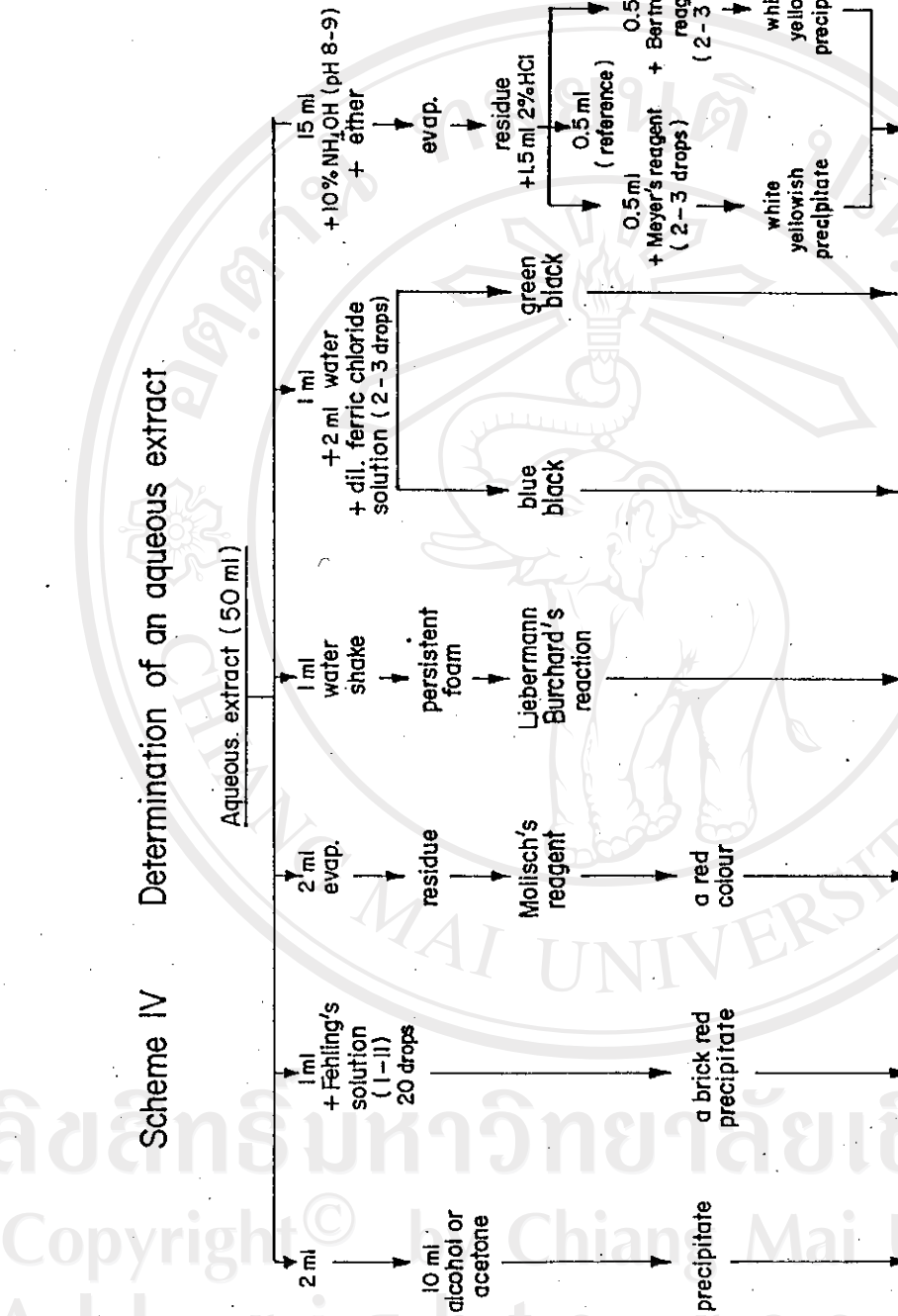
The aqueous extract was identified for polar substances as follows (see Scheme IV)

I.1.4.1. Identification of polyuronides (pectin, mucilages, gums)

Scheme III Determination of hydrolyzed alcohol extract



Scheme IV Determination of an aqueous extract



Two milliliters of the aqueous extract was added dropwise to a test tube containing 10 ml of alcohol or acetone. If thick precipitates were formed, they could be separated by filtration or centrifugation and washed with alcohol, then stained with specific staining reagents e.g., hematoxylin, toluidine blue, methyline blue, etc. The occurrence of a violet or blue precipitate denoted the presence of mucilages.

I.1.4.2 Identification of glucides (oses and polyoses).

The aqueous extract (2 ml) was evaporated to dryness,concentrate $H_2SO_4$ (2-3 drops) was added to the residue and allowed to stand for 3-5 minutes. By dehydration, furfural (from pentoses) or hydroxymethyl furfural (from hexoses) was formed. A few drops of alcoholic solution saturated with thymol (Molisch's reagent) was then added. The occurrence of red colour denoted the presence of glucides. To approximately 1 ml of the aqueous extract, which had been decanted and diluted with distilled water, a few drops of Lugol's solution was added. A blue colour denoted the presence of starch.

I.1.4.3 Identification of saponins

The aqueous extract (1 ml) was shaken with 1 ml of water for 15 minutes in a 1.6 cm diameter test tube. The occurrence of a foam column at least 1 cm in height which persisted for a minimum of 15 minutes indicated the presence of saponins. Then the Liebermann-Burchard's reaction and the hemolysis test were used to confirm their presence.

Hemolysis test* was carried out by suspending 2 ml of red blood

---

* ชมรมพฤกษเคมีและคณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล. 2523. การสัมมนาเชิงปฏิบัติการพฤกษเคมี ครั้งที่ 2. 176.

cells each in two test tubes containing equal volumes of normal saline solution. Normal saline solution (5 ml) was added to the first tube to use as reference and the aqueous extract was added to the second. The appearance of clear red solution due to hemolysis in the second tube indicated the presence of saponins.

I.1.4.4. Identification of alkaloidal salts.

The aqueous extract (15 ml) was made alkaline with diluted ammonia solution to pH 8-9 and extracted with a non polar solvent. The identification of alkaloidal salts was carried out as described in I.1.2.3.

Identification of tannins and reducing compounds in aqueous extract was carried out as described in I.1.2.1 and I.1.2.2, respectively using 1 ml of aqueous solution for each test.

The rest of the aqueous extract could also be hydrolyzed and used to identify various constituents the same methods described for hydrolyzed alcohol extract (I.1.3.).

I.2. Detection of saponin by thin-layer chromotogrphy

I.2.1. Preparation of the test extract. The powder of *C. lacerus* Gagn. rhizomes (5 g) was extracted with ethanol in Soxhlet apparatus and filtered. The extract was evaporated to dryness and dissolved with 2-3 drops of methanol.

I.2.1. Preparation of the plates. Glass plates (10x15 cm) were coated 0.3 mm thick with silica gel 60 G slurry in ratio of absorbent : water; 30:60 g/ml by layer casting apparatus. The plates were dried at room temperature and heated at 105° C for 10 minutes.

I.2.3. Developing solvent systems. The first and second solvent systems consisted of BuOH:HOAc:Water in the ratio of 4:1:2 and $CHCl_3$:MeOH:Water in the ratio of 65:35:10, respectively, were prepared and saturated in the TLC tanks for 1 hour before use.

I.2.4. Detection. After developing the chromatogram, the plates were dried at room temperature for 5 minutes and sprayed with 1. Erhlich's reagent. 2. Vanillin in sulfuric acid. (see appendix B).

## II. EXTRACTION AND PURIFICATION.

### II.1 Acid hydrolysis with preliminary extraction.

#### II.1.1 Preliminary extraction.

The powder of *Costus lacerus* Gagn. rhizomes (933.4 g) was extracted with hexanes to remove the lipid substances and free sapogenin in the Soxhlet apparatus for 24 hours. After the extraction, the residue was dried at room temperature to remove the solvent, weighed and surved for further hydrolysis.

#### II.1.2 Acid hydrolysis.

The acid hydrolysis was carried out by refluxing the residue from previous extraction (II.1.1) with 2N HCl at boiling temperature on a heating mantle for 2 hours. The ratio of the powder material weight to the acid volume was 1:6. After cooling, the content was filtered through Buchner funnel, neutralized with 10% ammonia solution, washed with distilled water and dried at $70^{\circ}$ C in the oven.

II.1.3 Extraction of Sapogenins

The crude hydrolysate material obtained from II.1.2 was exhaustively extracted with hexanes in a Soxhlet apparatus for about 24 hours. The hexanes extract was filtered while hot, concentrated to a volume of 100-150 ml in a rotatory evaporator and chilled. The precipitate of sapogenins obtained was collected by filtering through filter paper. The filtrate was again concentrated to a smaller volume and chilled for two more times, and the second and the third crops of sapogenin were collected.

II.1.4 Detection of sapogenin by thin-layer-chromatography.

The TLC was used to detect the sapogenin present in the crude extract and precipitate of *C. lacerus* Gagn. by using the following system :-

Plates : Layer of silica gel 60 G for TLC with 0.30 mm thickness
: Layer of silica gel 60 $GF_{254}$ for TLC with 0.30 mm thickness

Developing system : n-Hexane-ethyl acetate 4:1
: Benzene

Detection : UV light (short wave)
: Sulfuric acid in methanol.
: Vanillin in sulfuric acid.
: Anisaldehyde in Sulfuric Acid.

II.1.5 Purification of sapogenin by preparative thin-layer chromatography.

Preparation of the plates : Twenty four cleaned glass plates (20x20 cm) were coated with silica gel 60 P for P-TLC with layer casting

apparatus in the ratio of silica : water = 30 g : 75 ml for 0.5 mm thickness. The plates were dried at room temperature for several hours and activated just before used at 110°C for at least one hour.

Preparation of the sample solution : The sample solution was prepared by dissolving the precipitated sapogenin in chloroform to make the final concentration of 2.5%-5% W/V.

Developing solvent system :- The mixture of n-hexane : ethyl acetate, 4:1, 1000 ml was prepared and the P-TLC tank was saturated for one hour before use.

Chromatography and elution :- The sample solution (2 ml) was applied to the P-TLC plates by spotting apparatus (Camag-Linomat III) to make thin uniform band (5-8 mm) in each plate.

The sample plates were developed in P-TLC tank to the height of 10 cm and visualized by $I_2$-staining techniques to detect the band of sapogenins. The silica gel bands which contained sapogenin were scraped and totally placed in a filter paper cone. Chloroform was added on the filter paper cone to elute the sapogenins from the silica gel. The chloroform washing was evaporated and the residue was redissolved in a small quantity of chloroform and methanol then followed by recrystallization.

II.2 Acid hydrolysis without preliminary extraction.

The powder of *C. lacerus* Gagn. rhizomes was hydrolyzed with 2N HCl and extracted for sapogenin as described previously in II.1 without preliminary extraction with n-hexane but the ratio of weight of powder material to volume of acids was 1:18. The precipitates were identified without purification by P-TLC.

Extraction and Purification Scheme

II.1

Sample: *C. lacerus* Gagn rhizomes (aged more than 2 years)

rhizomes powder

↓ preliminary extraction c̄ hexanes

residue dried at room temperature

↓ hydrolyzed c̄ 2N HCl, 2 h (ratio 1:6)

crude hydrolysate powder neutralized c 10% $NH_4OH$ wash c water dried at 70° C

↓ extracted c̄ hexanes

hexane extract concentrated and chilled

↓ filtered

precipitates (MP 207° C)

↓

purification by P-TLC

(compound A)

II.2

Sample: *C. lacerus* Gagn rhizomes (aged 1-2 years)

rhizomes powder

↓ hydrolyzed c̄ 2N HCl 2 h (ratio 1:18)

crude hydrolyzate powder neutralized c̄ 10% $NH_4OH$ washed c̄ water dried at 70° C

↓ extracted c̄ hexanes

hexane extract concentrated and chilled

↓ filtered

precipitates (MP 68° C) (compound B)

III. IDENTIFICATION

III.1 Identity check by TLC of compound A.

The identity of compound A in the test sample previously purified by P-TLC from *C. lacerus* Gagn. was checked against diosgenin using the multiple development and two-dimensional TLC as follows:

1. Plate : Silica gel 60 G containing 2% silver nitrate (0.3 mm thickness)

Solvent system : Dichloromethane : acetone, 49:1

Method of development : Three-folded development

Spraying agent : Sulfuric acid in methanol.

2. Plate : Silica gel 60 G, 0.3 mm thickness (air dried)

Solvent system : Dichloromethane : methanol : formamide, 93:6:1

: Cyclohexane : ethylacetate : water 600:400:1

Method of development : two-dimensional development.

Spraying agent : Sulfuric acid in methanol.

3. Plate : Silica gel 60 G (0.3 mm thickness, air dried)

Solvent system : Dichloromethane : methanol : formamide, 93:6:1

: Cyclohexane : ethylacetate : water, 600:400:1

Method of development : System 1. one folded development.

System 2. two-folded development.

Spraying agent : Sulfuric acid in methanol.

III.2 Melting point determination by Metler FP 61, Melting point instrument.

1. Control test

The temperature accuracy control of the instrument was verified by determining the melting point of benzoic acid supplied with the instrument before use.

2. Melting point determination of the test sample.

The melting points of standard diosgenin, the test sample and the mixtures of them in different ratio were determined with heating rate of $2^{o}$ C/min. The mean of the three values were used.

III.3 Ultraviolet absorption spectra determination.

The test sample and the standard diosgenin, tigogenin and hecogenin (approximately 5.0 mg) were placed separately in test tubes and dissolved with 10 ml of sulfuric acid. The tubes were then immersed in a water bath maintained at $40^{o}$ C for one hour and subsequently were cooled to room temperature. The contents were diluted if necessary with sulfuric acid. The UV absorption spectra of each sample were obtained over the range of 200-600 nm using double beam spectrophotometer (UVIDEC 650, Jasco) with sulfuric acid in 1.0 cm matched quartz cell as a blank.

III.4 Infra-red absorption spectra determination.

The IR absorption spectra of the test sample and standard diosgenin were determined by using potassium bromide disc method with infrared spectrophotometer (UNICAM SP 1000). The pressure for tableting KBr disc was 7 tons/sq. inch with 0.5 mm thickness and 10.0 mm diameter

and the ratio of KBr : test sample was 100:1.

The identity of compound B was determined by using the melting point and IR absorption spectra methods as described for compound A.

## IV. IDENTIFICATION AND QUANTITATIVE DETERMINATION OF SAPOGENINS.

### IV.1 Determination of moisture content.

A 2.0 g sample was accurately weighed and dried on a tared watch glass in an oven at 100° C for 6 hours and reweighed after it had cooled to room temperature in a desiccator containing silica gel. The moisture was the loss in weight. The mean of the two values was used.

### IV.2 Determination of total sapogenin content by gravimetric method.

A 50.0 g sample was accurately weighed in a thimble and extracted with n-hexane in a Soxhlet apparatus for 8 hours. The residue was dried at room temperature to remove the solvent and reweighed, and quantitatively transferred into an erlenmeyer flask. The volume of 2N HCl (18 ml/1 g residue) was added to the residue. The mixture was refluxed at boiling temperature for 2 hours. After cooling, the content was filtered through a Buchner funnel. The hydrolysate material was neutralized with 10% ammonia solution and washed with distilled water and then dried at 70° C in the oven. The crude hydrolysate product was again exhaustively extracted with n-hexane in Soxhlet apparatus for 6 hours. The n-hexane extract was evaporated to dryness. The residue was dried at 100° C to constant weight and weighed. The mean of the two results was used.

### IV.3. Identification of diosgenin and hecogenin by HPLC.

#### IV.3.1 Preparation of sample solution.

A 50.0 g of sample was accurately weighed in a thimble and carried out as described in gravimetric method IV.2. The residue obtained was dissolved in 5 ml of chloroform. One milliliter of this solution was evaporated to dryness, dissolved and diluted to 300 µl with chloroform.

IV.3.2 Preparation of standard solutions.

Each standard diosgenin, tigogenin, and hecogenin was dissolved separately in chloroform to make the final concentrations of 3000 µg/ml solution. The standard mixture solution was prepared by mixing 1 ml of each standard solution together. All the solutions were individually filtered through Fluoropore filter (pore size, 0.5 µm) with the application of sample clarification kit.

IV.3.3. Preparation of mobile phase.

The mobile phase was prepared from the mixture of 83% acetonitrile, 10% methanol and 7% chloroform. All the solvents used were HPLC grade. After mixing, the solvent mixture was filtered through Fluoropore filter (pore size, 0.5 µm) with the application of solvent clarification kit, and degassed by ultrasonic bath for 20 minutes.

IV.3.4 Chromatography.

The HPLC (Water Associates) which composed of Solvent Delivery System Model 6000 A, Universal Liquid Chromatograph Injector Model U6K, and Differential Refractometer Detector Model R401 connected to Omni-Scribe B 5000 Strip Chart Recorder Model B 5217-1AI with µBondapak $C_{18}$ Column (3.9 mm x 30 cm) was optimized with mobile phase for experimental condition according to the manual description.

The experimental condition of analysis was carried out at flow rate of 0.5 ml/min (pressure < 500 psi), attenuation of the detector was 8X, and the chart speed was 1/6 cm/min with the mixture of 83% acetonitrile, 10% methanol and 7% chloroform as mobile phase.

Ten microliters of each standard solution, standard mixture and sample solution were injected into the HPLC column with the aid of Microliter$^{R}$ syringe (10 µl).

IV.4 Quantitative determination of hecogenin and diosgenin by HPLC.

IV.4.1 Preparation of standard solutions

A series of standard solutions of each sapogenins (diosgenin and hecogenin) with the concentrations of 9-30 µg/µl were prepared in chloroform.

IV.4.2 Chromatography

The experimental condition of analysis was carried out at the flow rate of 0.2 ml/min (pressure < 500 psi), the attenuation of the detector at 8X, and the chart speed 0.17 cm/min with the mobile phase as described in III.3.

Ten microliters of each standard solutions were injected into the HPLC column with the aid of Microliter$^{R}$ syringe (10µl). The area of each peak was calculated by multiplying the height of the peak by the width at half-height. The peak areas were plotted against the concentrations of diosgenin and hecogenin. The peak area of each sapogenin was also calculated and the content of each sapogenin were estimated against the standard curves.

RESULTS:

I. Preliminary examinations:

The result of the systematic qualitative test of *C. lacerus* Gagn. rhizomes is shown in Table II.4. The chemical constituents found in the rhizomes were sterols, fatty acids, reducing compounds, anthracene glycoside, anthocyanidine compounds, polyuronides, sugar, starch and saponin, even though the Lieberman-Burchard's Test in aqueous extract was negative but it was positive with hydrolyzed aqueous extract. The catechol tannin and coumarin might also be present.

The presence of saponin in alcoholic extract of *C. lacerus* Gagn. rhizomes confirmed by TLC is shown in Fig. II.1 and II.2. The partition system of BuOH:HOAc:$H_2O$; 4:1:2 detected by Erhlich's reagent showed that at least two compounds of saponin (pink spots) with Rf values of 0.3 and 0.46 were present. The system of $CHCl_2$:MeOH:$H_2O$; 65:35:10, showed two kinds of sterol compounds as violet spots and one component of saponin as green spot with Rf values of 0.77, 0.67 and 0.19 respectively.

Table II.4 The chemical constituents found in *C. lacerus* Gagn. rhizome extract. These results were obtained from systematic qualitative test.

| Chemical Constituents | | Results* |
|---|---|---|
| Ether extract :- | volatile oils | - |
| | sterols and triterpenoids | + |
| | carotenoids | - |
| | fatty acids | + |
| | alkaloids | - |
| | flavone aglycone | - |
| | emodols | - |
| | coumarins | - |
| Alcohol. extract :- | gallic tannins | - |
| | catechol tannins | + (slightly) |
| | reducing compounds | + |
| | alkaloids | - |
| Hydrolyzed alcohol extract :- | anthracene glycosides | + |
| | coumarins | not clear |
| | steroid glycosides triterpene glycosides | + |
| | flavone glycosides | - |
| | anthocyanidine glycosides | + |
| | cardenolides | - |
| Aqueous extract | polyuronides | + (slightly) |
| | reducing compounds | + |
| | oses, polyoses | + |
| | alkaloids | - |
| | saponin 1. foam test | + |
| | 2. Lieberman-Burchard Test | - |
| | 3. hemolysis Test | + (slightly) |
| | gallic tannins | - |
| | catechol tannins | - |

* + = positive result.

\- = negative result.

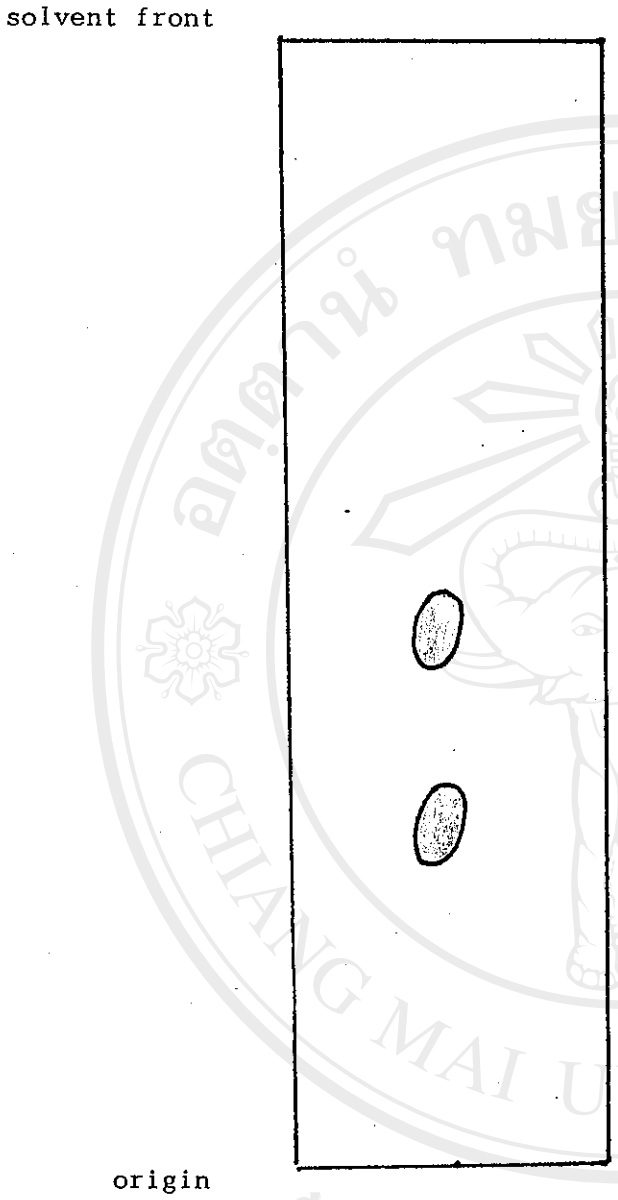



plate : silica gel 60 G 0.3 mm. thickness

developing solvent : BuOH:HOAc :$H_2O$ (4:1:2)

spraying agent : Erhlich's reagent

sample : alcoholic extract of *C. lacerus* Gagn. rhizomes in MeOH.

Fig. II.1 Thin-layer chromatogram of *C. lacerus* Gagn.

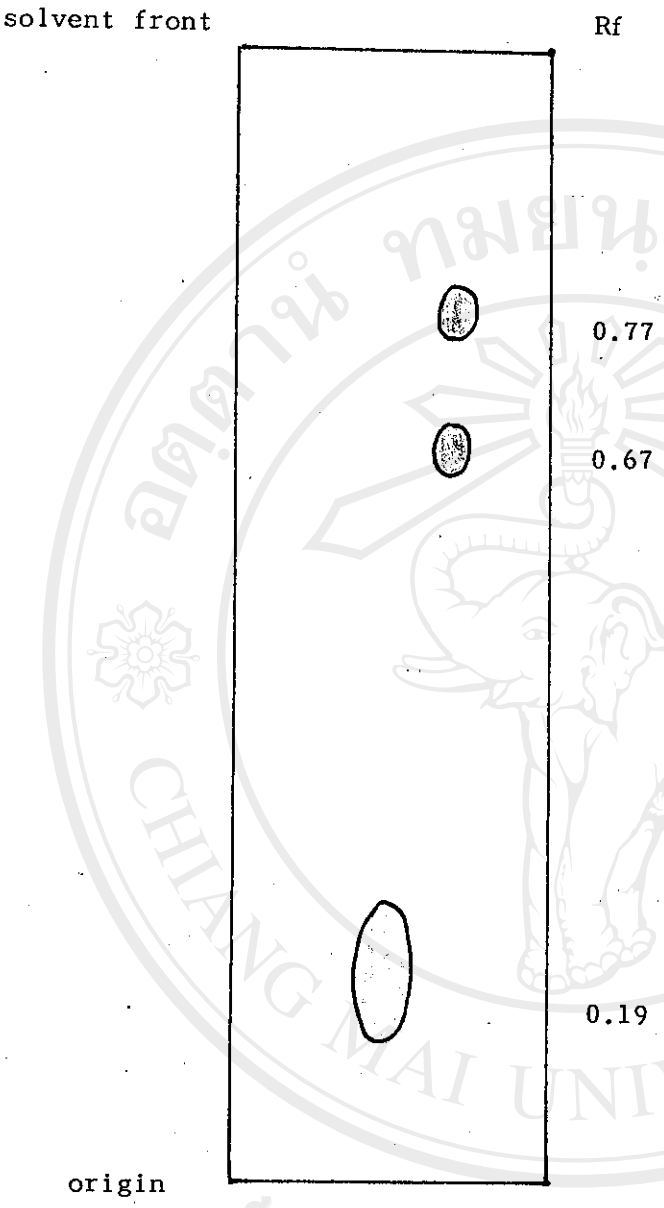



plate : silica gel 60 G
0.3 mm thickness
developing solvent : $CHCl_3$: MeOH: $H_2O$ (65:35:10)
spraying agent : vanillin in sulfuric acid
sample : alcoholic extract of *C. lacerus* Gagn. rhizomes in MeOH.

Fig. II.2 Thin-layer chromatogram of *C. lacerus* Gagn.

II. Extraction and Purification

The weights of the sample of *C. lacerus* Gagn. rhizome powder and residue obtained in each step from extraction procedures II.1 and II.2 are shown in Table II.5. The yield of crude diosgenin obtained from the extraction procedure II.1 was 0.53% and the yield of pure diosgenin from purification process by P-TLC was 10.31%. In the extraction procedure II.2, the yield of residue was only 0.10%, but it might be due to the differences of sample ages.

Fig. II.3, II.4 and II.5 illustrate the detection of the constituent by TLC in crude extract and the precipitates after the extraction procedure II.1. At least five compounds were present in the crude extract with Rf values of 0.25, 0.35, 0.53, 0.90 and 0.95, respectively in n-hexane-ethyl acetate (4:1), but the precipitates showed only one compound with Rf value of 0.25. The spot which gave the Rf value of 0.25 normally corresponded to monohydroxy sapogenins such as tigogenin, diosgenin and hecogenin and the spot which gave the Rf value of 0.35 also corresponded to sterol compounds (stigmasterd and β-sitosterol). The spots which gave the Rf value of 0.90 and 0.95 might be fatty compounds and spirostadiene which were usually accompanied with sapogenin extraction. Fig.II.6 illustrates the constituents of the crude extract and the precipitates from *C. lacerus* Gagn. rhizomes by extraction procedure II.2 (detected by TLC). At least eleven compounds were present in the crude extract with Rf values of 0.04, 0.10, 0.13, 0.16, 0.21, 0.28, 0.40, 0.9, 0.66, 0.78 and 0.80, respectively, when benzene was used as developing solvent and UV light and $H_2SO_4$ in MeOH were used for detection. The precipitates showed at least two compounds with Rf values of 0.04 and 0.10, respectively.

Table II.5 Determination of the weights and crude sapogenin contents in *C. lacerus* Gagn. rhizome powder by the extraction procedure II.1 and II.2.

| | | |
|---|---|---|
| Procedure II.1 | | |
| Weight of sample before the preliminary extraction | 933.7 | g |
| Weight of residue after the preliminary extraction | 867.6 | g |
| Weight of residue after the hydrolysis | 407.4 | g |
| Weight of precipitates after the sapogenin extraction | | |
| first crop | 2.8164 | g |
| second crop | 0.2270 | g |
| third crop | 1.8921 | g |
| Total weight of crude sapogenin precipitates | 4.9355 | g |
| The yield of crude sapogenin | 0.53 | % |
| Total weight of purified sapogenin after P-TLC | 0.5090 | g |
| The yield of purified diosgenin (from crude sapogenin) | 10.31 | % |
| Procedure II.2 | | |
| Weight of sample before the hydrolysis | 235.00 | g |
| Weight of sample after the hydrolysis | 120.59 | g |
| Weight of precipitates after the sapogenin extraction | 0.2269 | g |
| The yield of crude sapogenin | 0.10 | % |

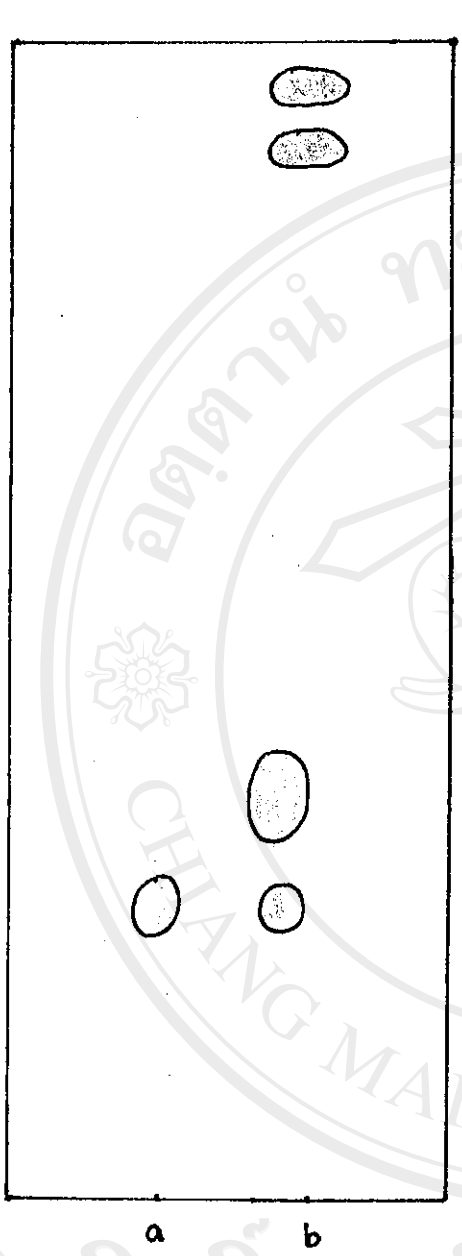



plate : silica gel 60 G,
0.30 mm thickness.
developing solvent : n-hexane:
EtOAc 4:1
spraying agent : $H_2SO_4$ in MeOH
samples :- a. ppt. of *C. lacerus* Gagn. in $CHCl_3$
b. crude extract of *C. lacerus* Gagn. in $CHCl_3$

Fig. II.3. Thin-layer chromatogram of *C. lacerus* Gagn. crude extract and precipitates obtained from extraction procedure II.1.


plate : silica gel 60 G,
0.30 mm thickness.
developing solvent : n-hexane:
EtOAc 4:1
spraying agent : vanillin in
$H_2SO_4$
samples : a. ppt. of *C. lacerus*
Gagn. extract in
$CHCl_3$
b. crude extract of
*C. lacerus* Gagn.
in $CHCl_3$

Fig. II.4. Thin-layer chromtogram of *C. lacerus* Gagn. crude extract and precipitates obtained from extraction procedure II.1.

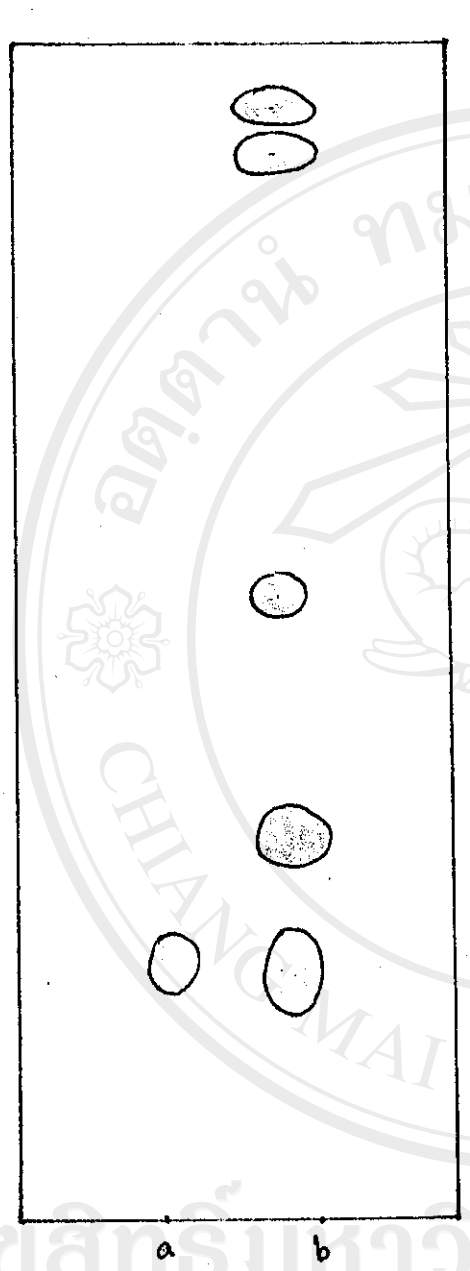



plate : silica gel 60 G, 0.30 mm thickness.
developing solvent : n-hexane: ethyl acetate 4:1
spraying agent : anisaldehyde-$H_2SO_4$
samples : a. ppt. of *C. lacerus* Gagn. extract in $CHCl_3$
b. crude extract of *C. lacerus* Gagn. in $CHCl_3$

Fig. II.5. Thin-layer chromatogram of *C. lacerus* Gagn. crude extract and precipitates obtained from extraction procedure II.1.

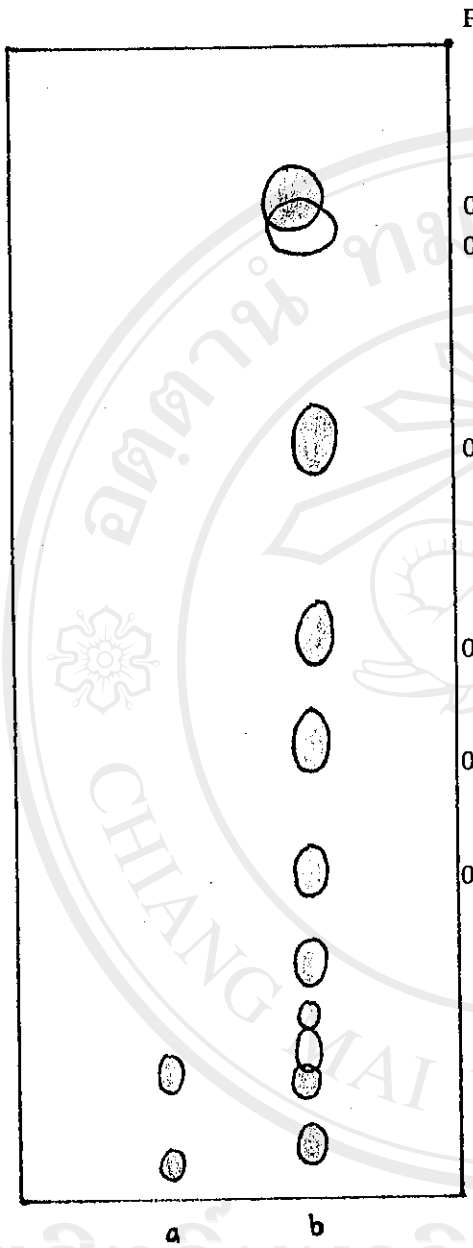



plate : silica gel $GF_{254}$ 0.30 mm thickness.
developing solvent : benzene
detection : UV short wave (no color spot) $H_2SO_4$ in MeOH
samples : a. precipitates of *C. lacerus* Gagn extract in $CHCl_3$
b. crude extract of *C. lacerus* Gagn extract in $CHCl_3$

Fig. II.6. Thin-layer chromatogram of *C. lacerus* Gagn crude extract and precipitates obtained from extraction procedure II.2.

III. Identification of sapogenin.

Nearly white needle like crystal of compound A obtained from P-TLC checked for identity with diosgenin by multiple development and two dimensional development TLC are illustrated in Fig.II.7,II.8 and II.9 The melting points of compound A and authentic diosgenin and the mixed melting point were shown as followed:-

Indicated melting point of standard diosgenin 205.4° C

Actual melting point of standard diosgenin 204.2° ± 0.4° C

Indicated melting point of the compound A 202.0° C

Actual melting point of the compound A 200.8° ± 0.4° C

Mixed melting point of diosgenin and compound A 201.3° C

Actual mixed melting point of diosgenin and compound A 200.1±0.4° C

The UV absorption spectra of diosgenin, tigogenin and hecogenin in sulfuric acid are shown in Fig.II.10a and of compound A is shown in Fig.II.10b. The compound A showed the same characteristic UV absorption peaks as diosgenin at 270, 312 (broad), 415 nm, whereas hecogenin at 290(broad), 397 nm and as tigogenin at 315,396 nm, respectively.

Fig. II.11 shows the IR absorption spectra by KBr disc method of diosgenin and compound A. Compound A showed the same characteristic finger print in the region between 1000-800 $cm^{-1}$ (980, 920, 900, 870 and 840 $cm^{-1}$. The melting point of the white precipitates of compound B obtained from extraction procedure II.2 was 68.0° C and the IR absorption spectra by KBr disc method is shown in Fig.II.12. This compound was not further identified.

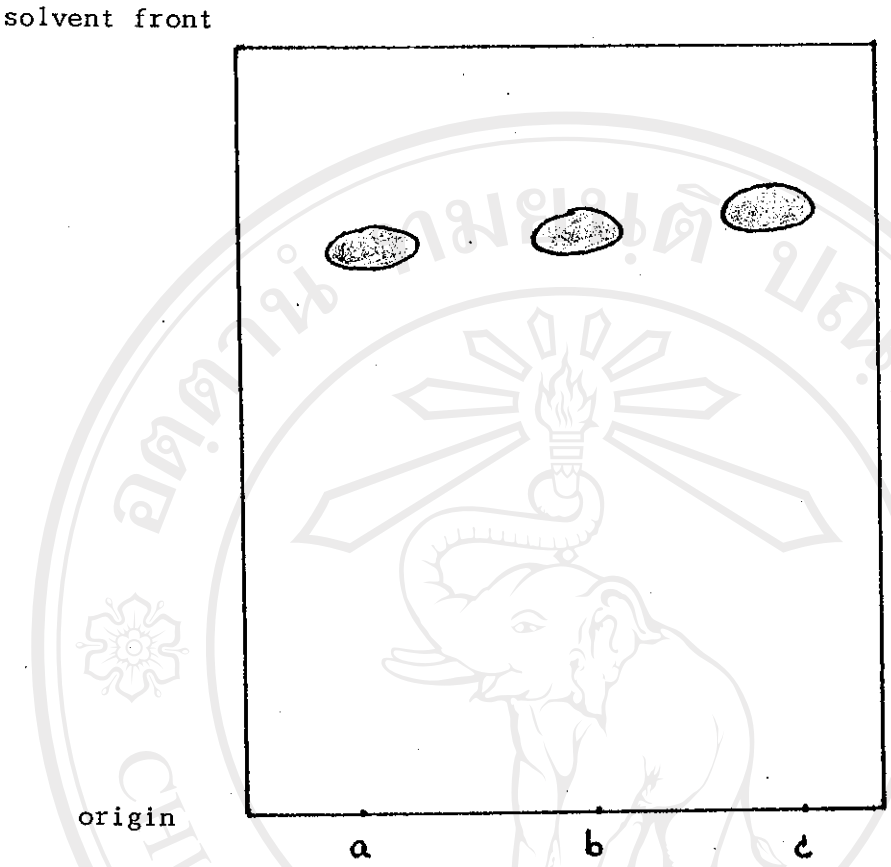



plate : silica gel 60 G containing 2% $AgNO_3$, 0.3 mm thickness

solvent system : dichloromethane : acetone 49:1

method : three fold development

spraying agent : $H_2SO_4$ in MeOH

test sample : a. diosgenin, b. diosgenin + compound A, c. compound A



Fig II.7. The multiple development TLC of the compound A compared with diosgenin (Sigma Chemical Company) D-1630.

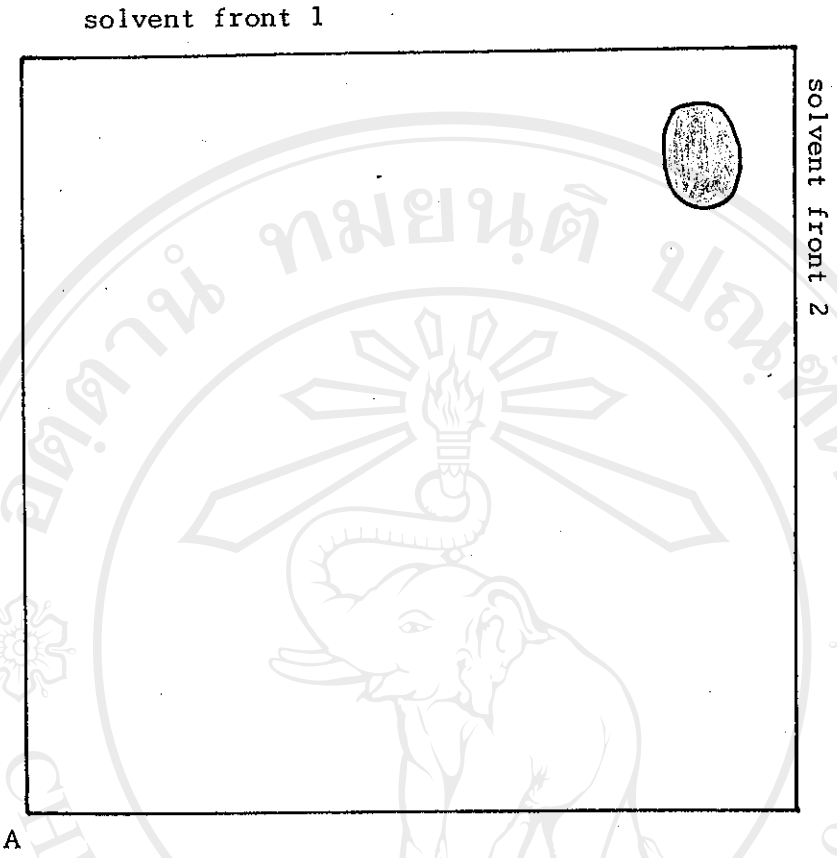



plate : silica gel 60 G, 0.3 mm thickness

solvent system : system 1. dichloromethane : methanol : formamide
93:6:1

system 2. cyclohexane : ethyl acetate : water
600:400:1

method : Two-dimensional development

spraying agent : $H_2SO_4$ in MeOH

A. diosgenin + compound A

Fig II.8. The two dimensional TLC of the compound A combined with diosgenin.

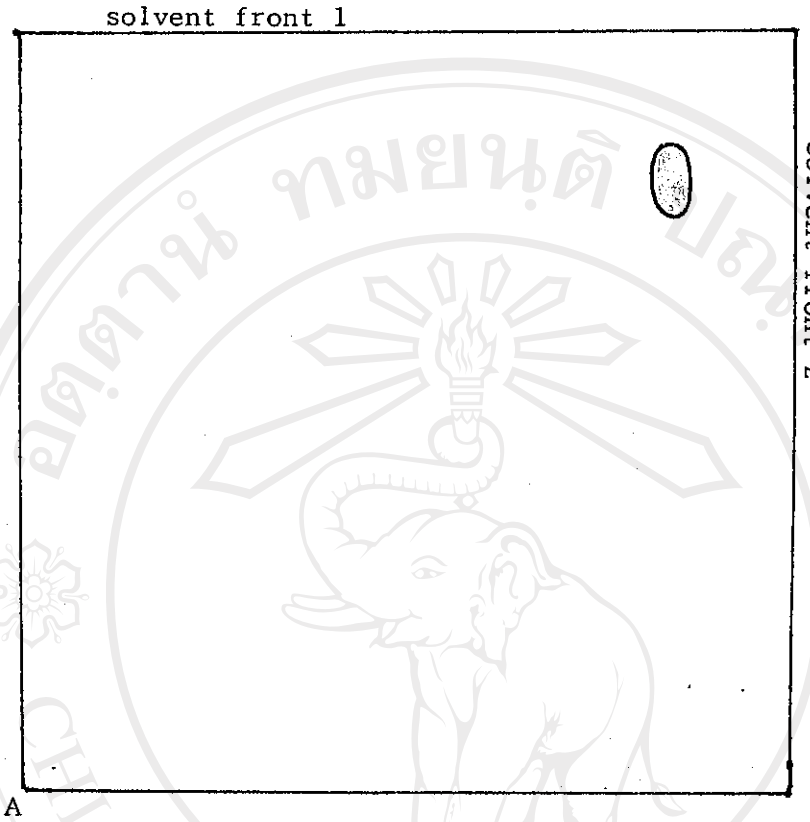



plate : silica gel 60 G, 0.3 mm thickness, air dried

solvent system 1 : dichloromethane : methanol : formamide 93:6:1

cyclohexane : ethyl acetate : water 600:400:1

method : system 1 : simple development

system 2 : two fold development

spraying agent : $H_2SO_4$ in MeOH

A. diosgenin + compound A.

Fig. II.9. The multiple development, two dimensional TLC of the compound A combined with diosgenin.

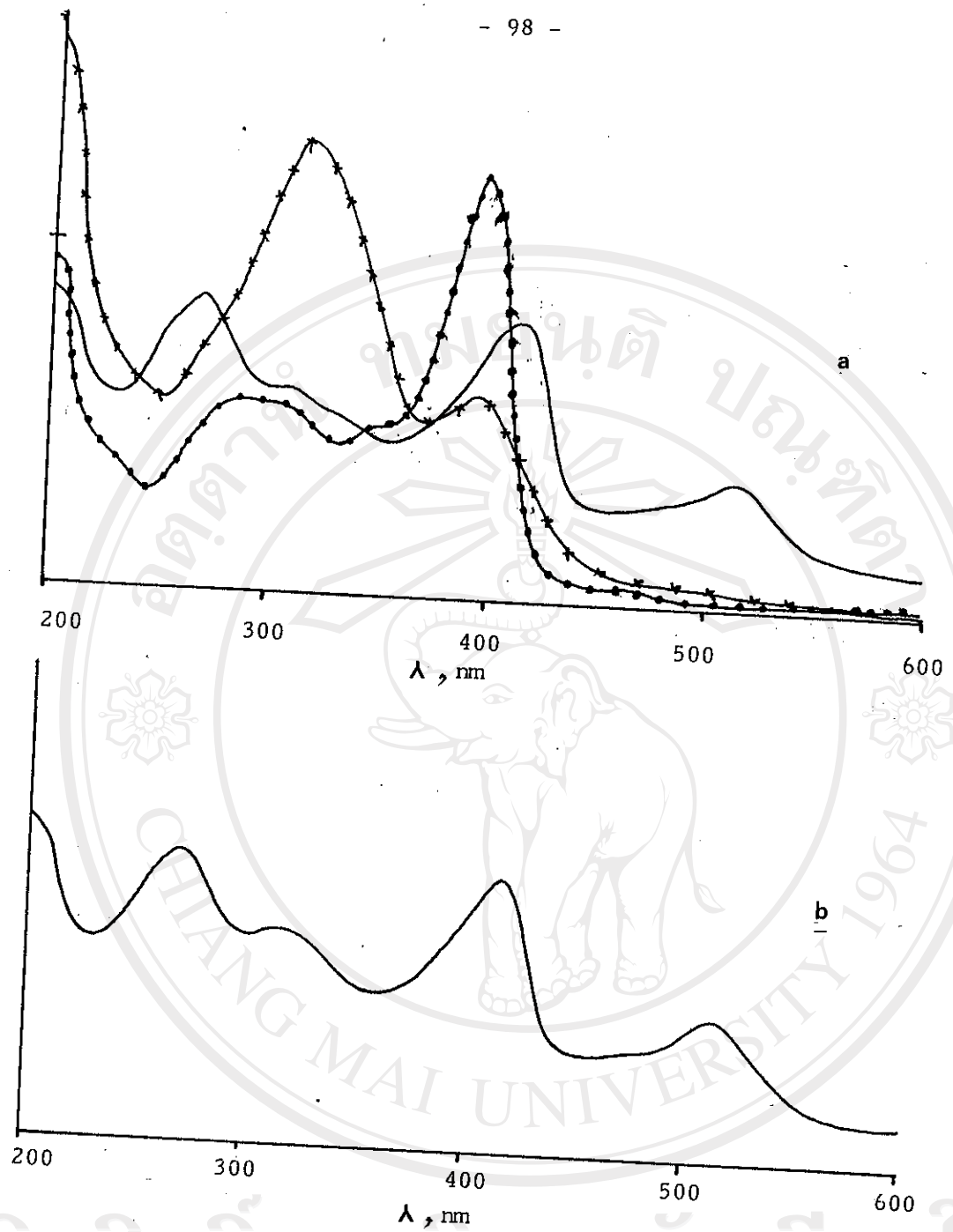
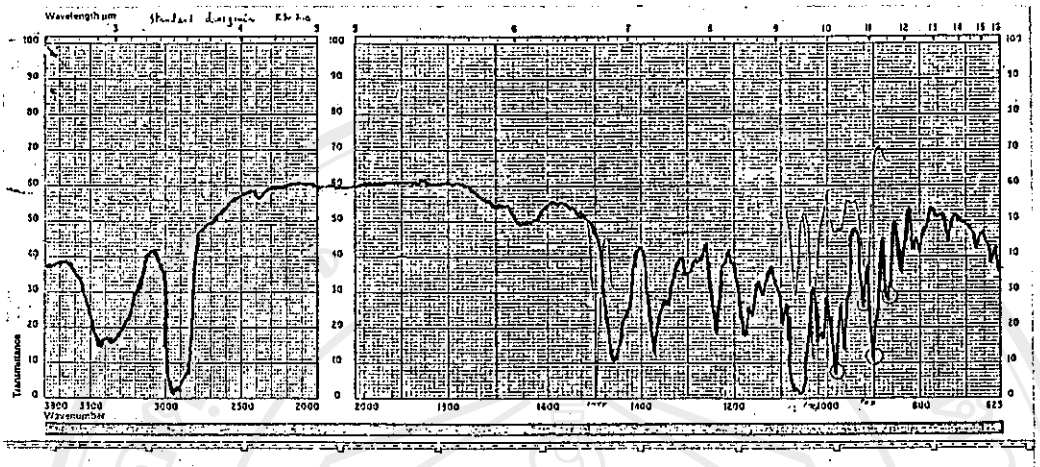
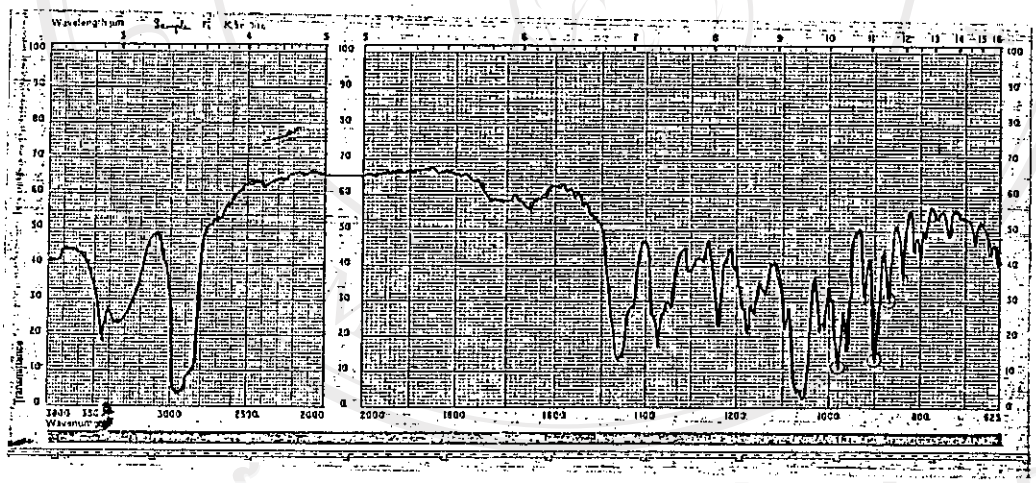



Fig II.10. UV absorption spectra of a. hecogenin —•—•—, tigogenin —×—×—, diosgenin ——; b. compound A.



a



b



Fig II.11. IR absorption spectra of a. diosgenin; b. compound A

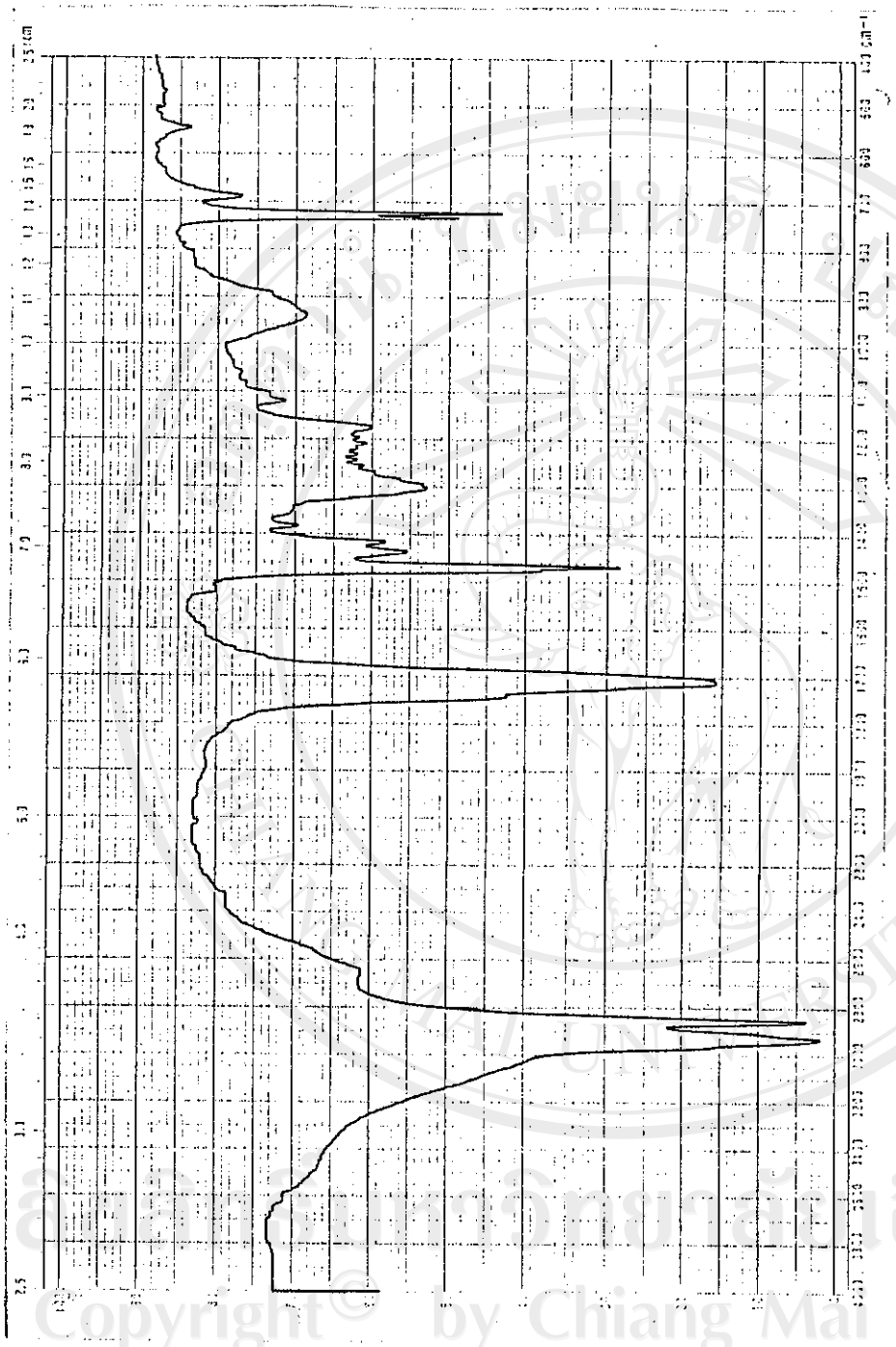


Fig II.12. IR absorption spectra of precipitates obtained from extraction procedure II.1.

IV. Identification and quantitative determination of sapogenins.

The samples used for quantitative determination of sapogenin were the young rhizomes of *C. lacerus* Gagn. aged about 1-2 years and moisture content of about 95% in fresh rhizomes.

The analysis of moisture content of *C. lacerus* Gagn. dried rhizomes powder is shown by Table II.6. The average moisture content was 4.90%. Table II.7 shows the total sapogenin content in *C.lacerus* Gagn. by gravimetric method. The weight of residue was assumed to be total sapogenins with the separation of the precipitates because the quantity of residue was too small. However the content of residue calculated as total sapogenins must be higher than the actual value because the sterols, spirostadiene and lipid substances which always accompanied with sapogenin in the process of its extraction were also included. When the HPLC method was used for identification of sapogenin by comparing the retention time, the characteristic of sapogenins peaks are shown in Fig. II.13. Table II.8 shows the retention time of standard sapogenins used, hecogenin, tigogenin and diosgenin in the system of acetonitrile : methanol : chloroform, 83:10:7 as mobile phase at a flow rate of 0.5 ml/min and a chart speed of 0.25 cm/min. In order to get the optimum condition, the flow rate had been changed to 0.2 ml/min and the chart speed been also been changed to 1/6 cm/min, the typical chromatogram is shown in Fig. II.14. The retention times of standard sapogenins was also changed as such; hecogenin from 8 min to 22 min 12 sec, diosgenin from 11 min 24 sec to 31 min 12 sec and tigogenin from 12 min 24 sec to 34 min 12 sec. Fig.II.15shows the characteristic chromatogram of crude extracts of *C.lacerus* Gagn. rhizomes by extraction pro-

cedure II.1 and II.2 which were carried out with the same mobile phase. There were at least five peaks in the sample chromatogram with retention times of 22.2, 25.8, 31.2, 60.6 and 66.6 min, respectively. There were two peaks in the sample solution of *C. lacerus* Gagn. rhizomes which corresponded to hecogenin (peak 1) and diosgenin (peak 3) with the same retention times of 22 min 12 sec and 11 min 24 sec, respectively. The peaks with retentimes of 60.6 and 66.6 min normally corresponded to sterol compounds. For quantitative determination of hecogenin and diosgenin identified previously, the relationship between the concentrations of hecogenin and diosgenin (from 0.40 µg/10 µl) and their peak areas obtained from the system used are shown in Fig. II.16 and II.17 and Table II.9. When these relationship were plotted, two straight lines were obtained and used as colibration curves for quantitating hecogenin and diosgenin as illustrated in Fig. II.18. Therefore, the contents of hecogenin and diosgenin calculated from the peak areas in sample peak (Fig. II.9) were 0.026% and 0.031%, respectively.

Table II.6 Determination of moisture content of *C. lacerus* Gagn. rhizome

| | I | II |
|---|---|---|
| Weight of sample before drying (g) | 2.1455 | 2.0267 |
| Weight of sample after drying (g) | 2.0430 | 2.9250 |
| Weight loss (g) | 0.1025 | 0.1017 |
| Moisture content (%) | 4.77 | 5.02 |
| Average (%) | 4.90 | |

Table II.7 Determination of total sapogenin content in *C. lacerus* Gagn. rhizomes by Gravimetric method.

| | I | II |
|---|---|---|
| Weight of sample | 50.0000 | 50.0000 g |
| Weight of sample after preliminary extraction | 49.0196 | 49.0058 g |
| Weight of residue | 0.0662 | 0.0708 g |
| % residue | 0.1324 | 0.1416 g |
| Average total sapogenin | 0.1370% | |
| Average total sapogenin as mfb. | 0.1440% | |

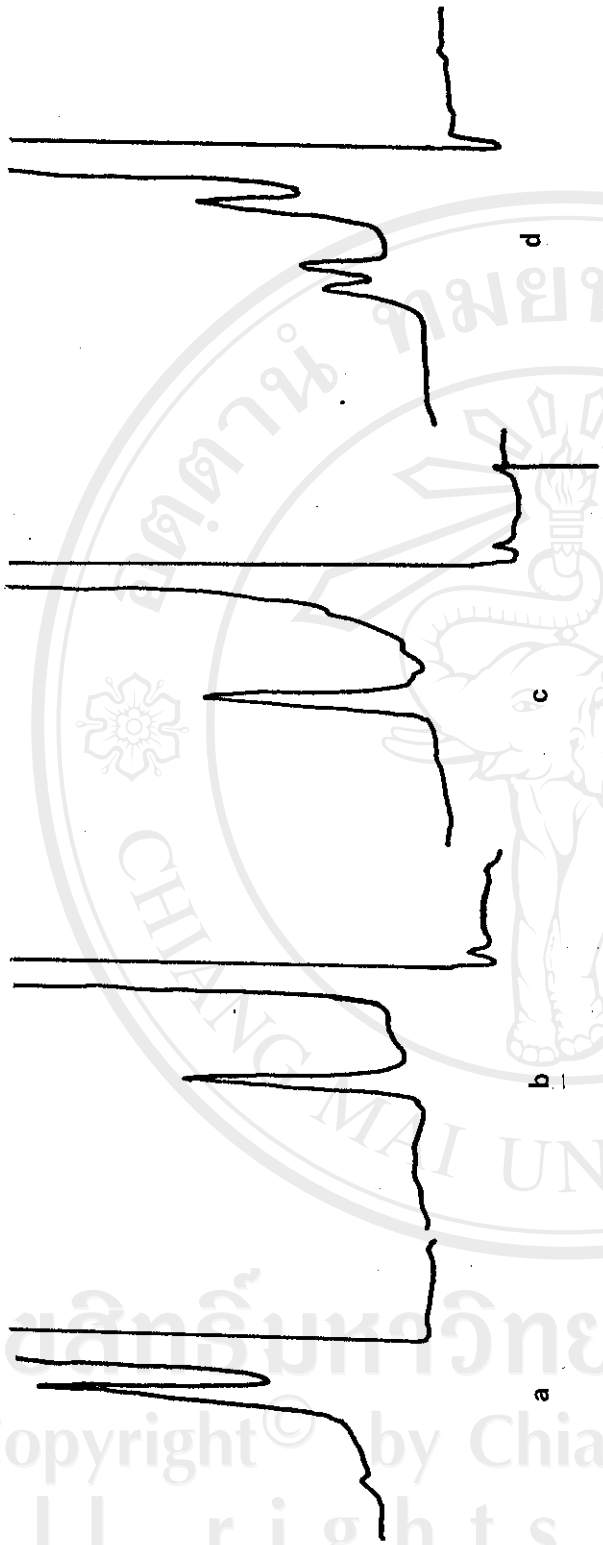



Fig. II.13. Representative chromatogram of (a) standard hecogenin, (b) standard diosgenin, (c) standard tigogenin, and (d) synthetic mixture of (a), (b), and (c). HPLC conditions:acetonetrile :methanol:chloroform; 83:10:7 as mobile phase, flow rate of 0.5 ml/min, chart speed of 0.25cm/min.

Table II.8 The retention times of diosgenin, tigogenin, hecogenin and standard mixture by using acetonitrile : methanol : chloroform (83:10:7) as mobile phase, flow rate of 0.5 ml/min and chart speed of 0.25 cm/min.

| Steroidal sapogenin | No. of peak | Retention time min | sec |
|---|---|---|---|
| Diosgenin | 1 | 11 | 36 |
| Tigogenin | 1 | 12 | 24 |
| Hecogenin | 1 | 8 | - |
| Standard mixture | 3 | | |
| | $1^{st}$ peak | 8 | - |
| | $2^{nd}$ peak | 11 | 36 |
| | $3^{rd}$ peak | 12 | 24 |

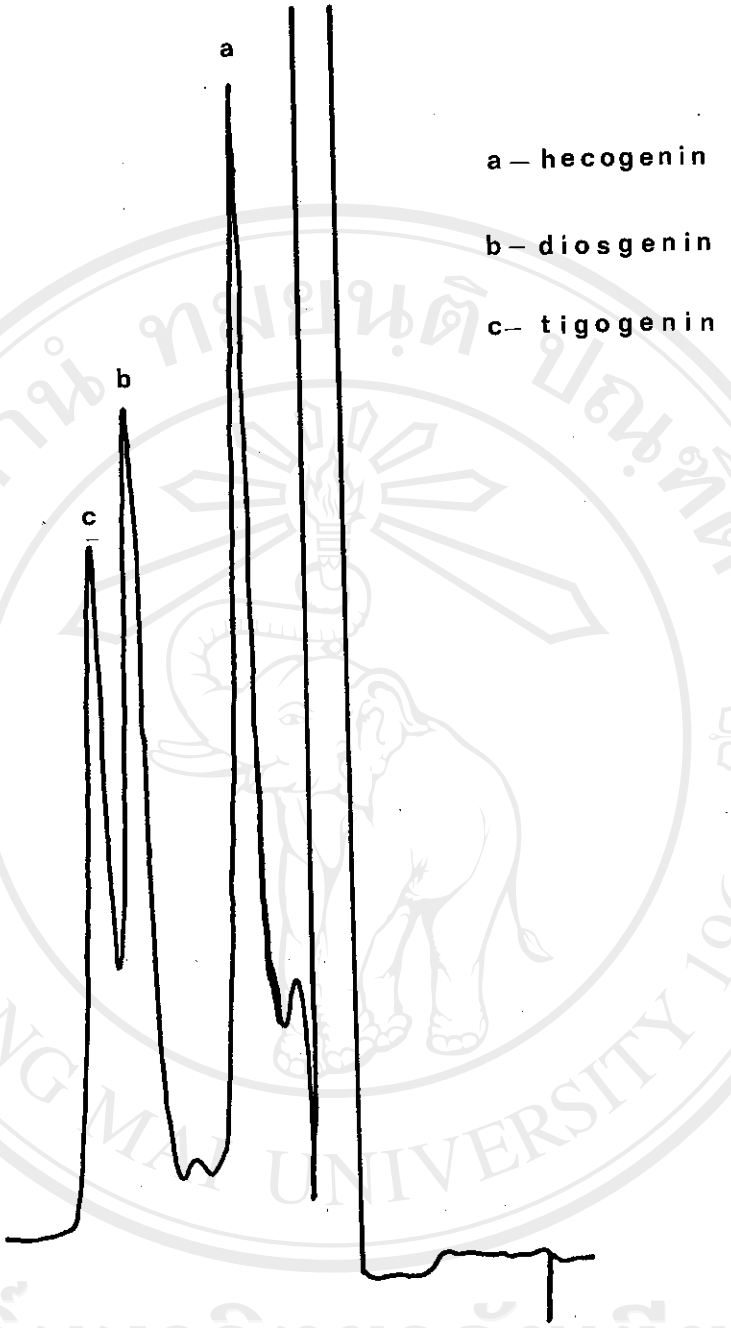



Fig. II.14. Representative chromatogram of synthetic mixture. HPLC conditions : acetonitrile : MeOH : $CHCl_3$; 83:10:7 flow rate of 0.2 ml/min, chart speed of 10/60 cm/min.

a. Flow rate 0.2 ml/min

Chart speed 1/6 cm/min

b. Flow rate 0.5 ml/min

Chart speed 0.25 cm/min

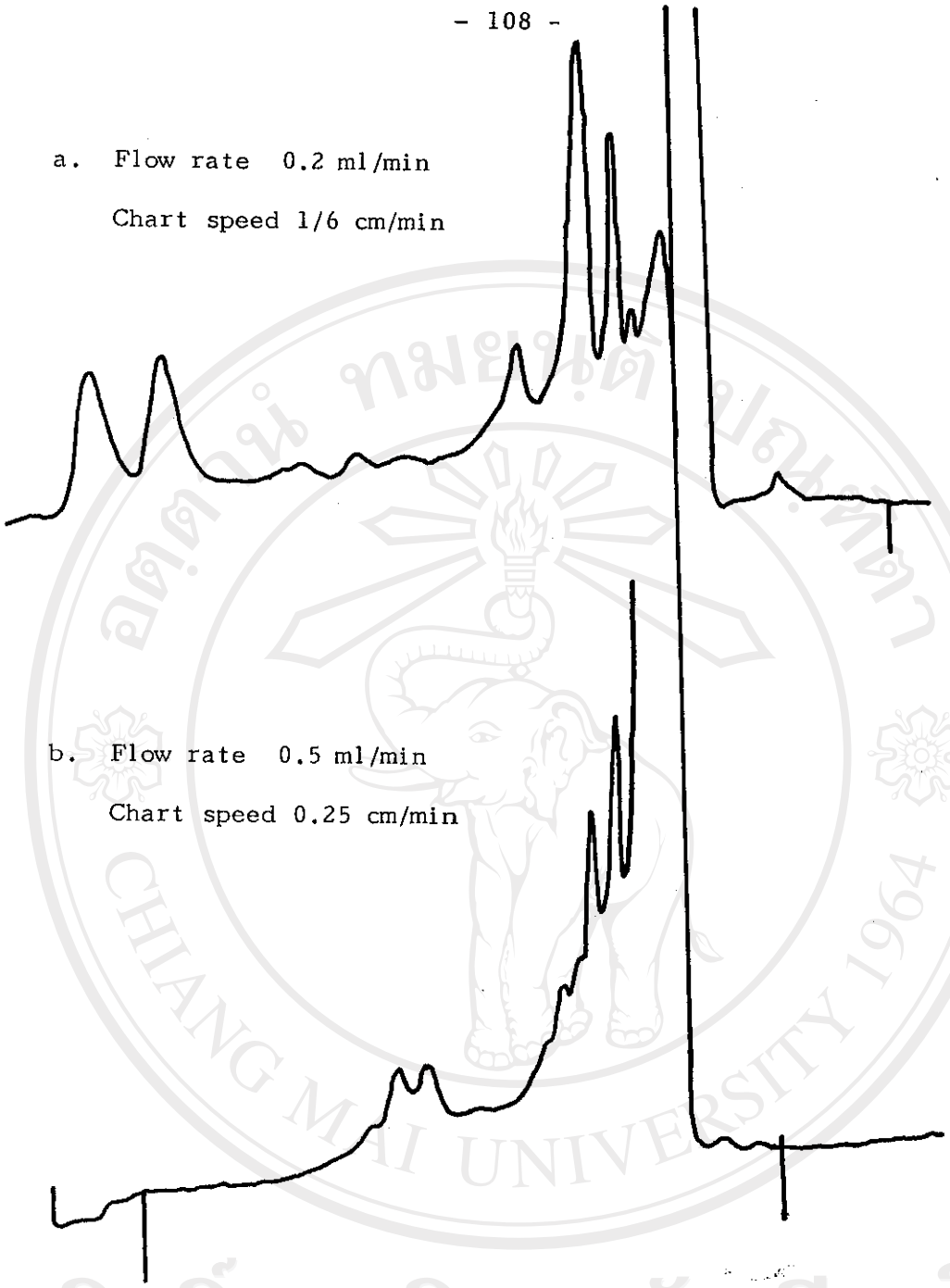


Fig. II.15 Representative chromatogram of *C. lacerus* Gagn. rhizomes extract (a) extraction process II.1, (b) extraction process II.2. HPLC conditions : acetonitrile : MeOH : $CHCl_3$; 83:10:7 as mobile phase,

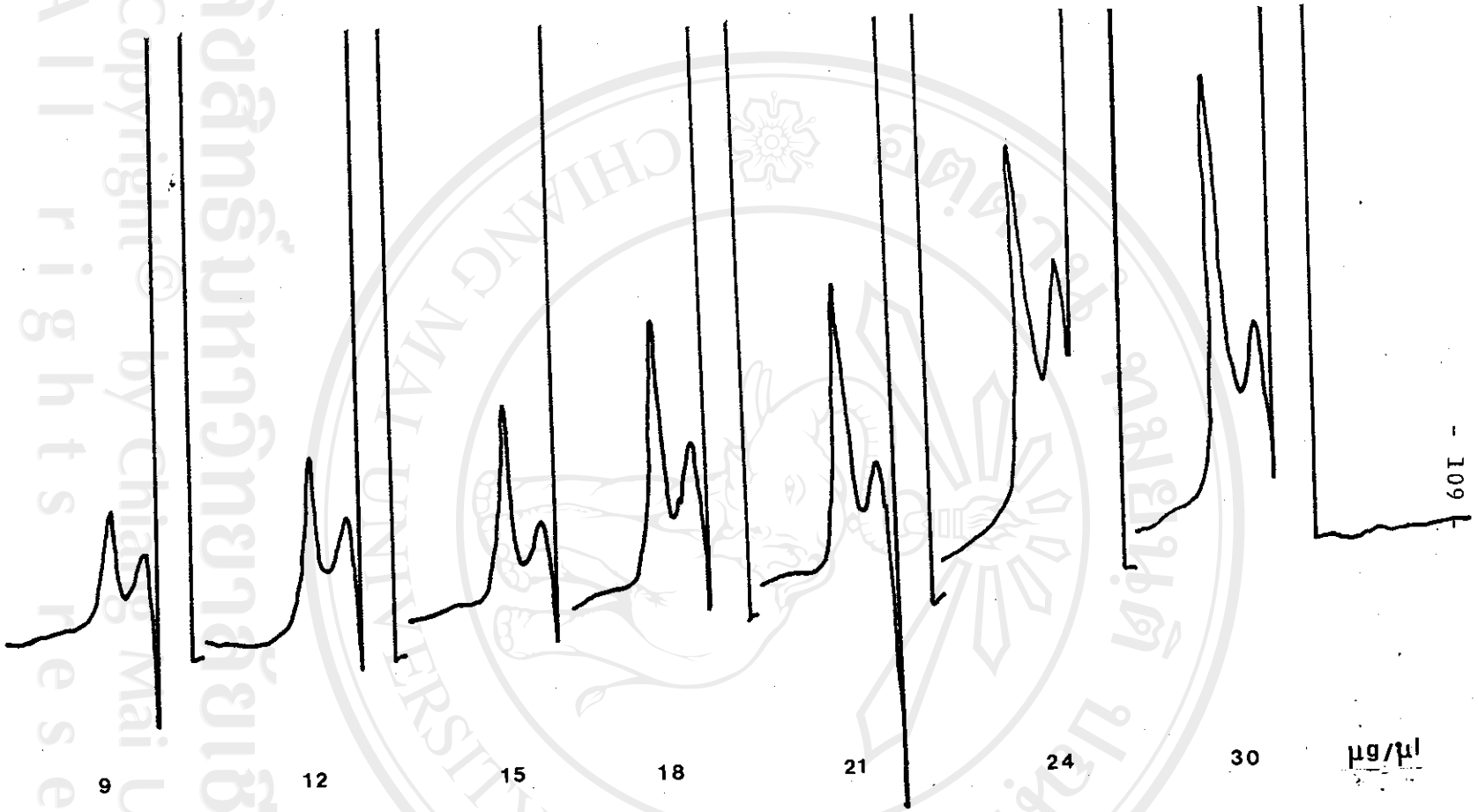



Fig. II.16. Representative chromatogram of standard hecogenin at various concentrations.

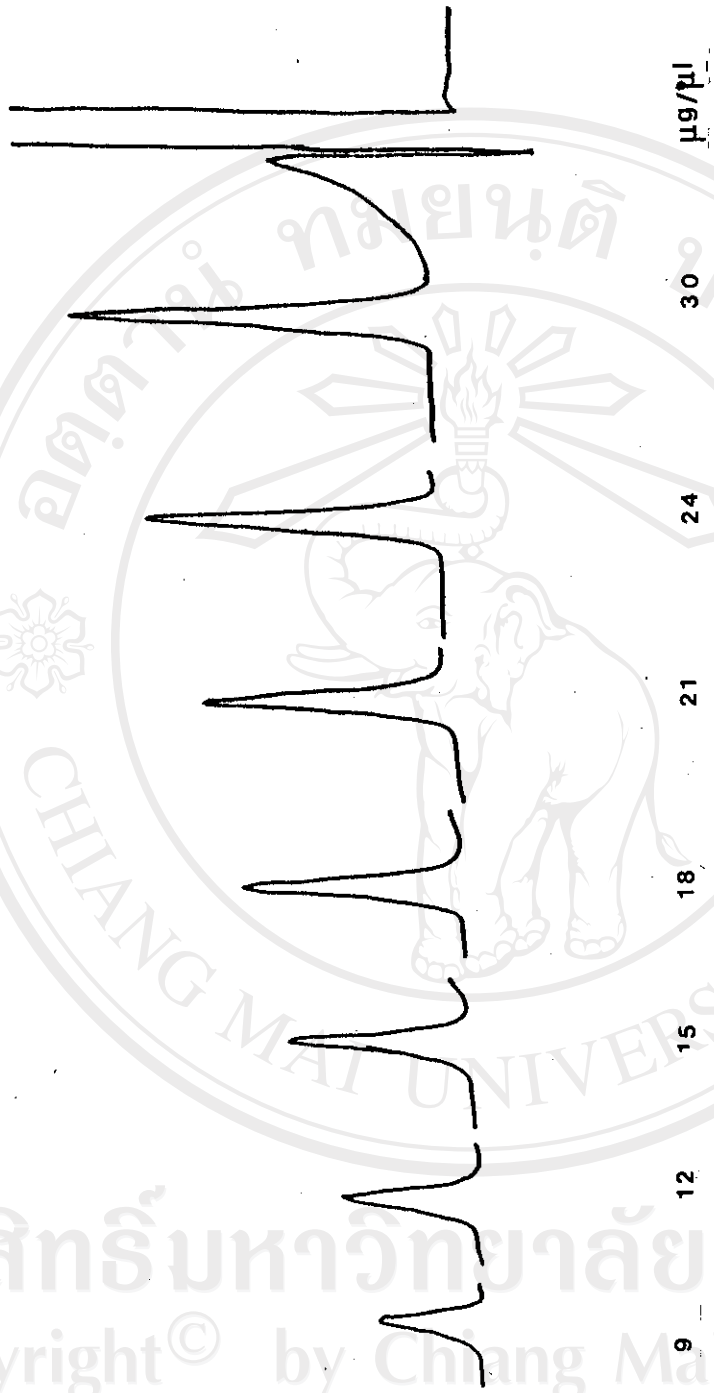



Fig. II.17. Representative chromatogram of standard diosgenin at various concentrations.

Table II.9 Relationship between the concentrations of hecogenin and diosgenin and their peak areas obtained from the HPLC method.

| Hecogenin concentration (µg/µl) | Peak area ($cm^2$) | Diosgenin concentration (µg/µl) | Peak area |
|---|---|---|---|
| 9 | 0.30 | 9 | 0.26 |
| 12 | 0.42 | 12 | 0.34 |
| 15 | 0.52 | 15 | 0.44 |
| 18 | 0.62 | 18 | 0.54 |
| 21 | 0.74 | 21 | 0.60 |
| 24 | 0.84 | 24 | 0.72 |
| 30 | 1.06 | 30 | 0.92 |

The sample peak areas of diosgenin and hecogenin were nearly equal. They were 0.30 $cm^2$.

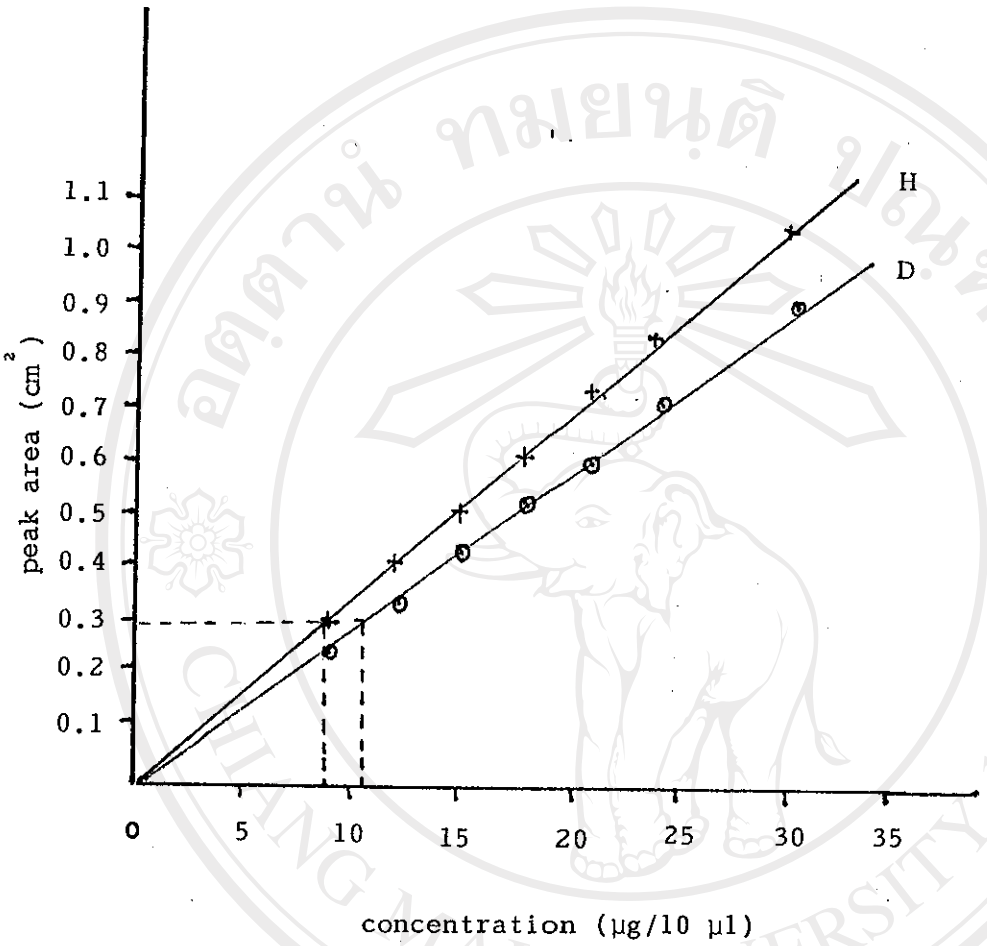





Fig. II.18 Calibration graphs for hecogenin (H) and diosgenin (D).