Part I

INTRODUCTION

Steroid drugs, a group of important therapeutic agents used in modern therapy,are composed of the three major classes of drugs namely; - corticosteroids, sex hormones and oral contraceptive drugs. Corticosteroids, originated from adrenal cortex hormones are widely used as antiinflammatory agents to relief the symptom of arthritis, rheumatism, and asthma. Sex hormones, either male or female are used in replacement therapy for deficiency in natural hormones. Testosterone, the most potent of male sex hormones and its derivatives can also be used as anabolic agents. Female sex hormones are widely utilized as oral contraceptive drugs because of their antifertility actions. Furthermore, the steroid drugs may be used as anticancer agents in certain cases of malignancy.

From the analysis done by Farnsworth and his colleague of the National Prescription Audit data which included 1.532 billion prescriptions in the United State in 1973, steroids were the most extensively used drugs among the twelve commonly-encountered pure compounds derived from higher plants e.g., codeine, atropine, reserpine (Beal and Reinhard, 1981). The cost of steroid drugs imported to Thailand in 1984 was 68, 782, 276 baht*.

The commercial production of steroid drugs usually employed the three main processes depending on the starting materials; (i) natural source extraction, (ii) total synthesis, and (iii) partial synthesis.

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* Trade statistics of Thailand, Department of Custom, 1984.

The natural source extraction, suitable for the preparation of conjugated estrogens from urine of pregnant mare, is not frequently used because it is expensive and inefficient method. It requires a large amount of the animal products such as, ovaries, adrenals and urine. The total synthesis method which starts from petroleum, provided about 13% of the world supply (Blundent *et al*, 1975). Because of the high cost of synthetic hormones due to multiple chemical steps and the difficulty in obtaining the active isomers, this method is limited to the production of definite compounds of aromatic and 19-nor steroid compounds like norethisterone, and norgestrol. These led to the use of partial synthesis method from the cheaper sources.

Cholesterol from sheep wool grease, cattle spinal cord and brain are the first materials to be used for the synthesis of steroid hormones by chemical reaction. Deoxycholic and cholic acid,which are bile acids are other starting materials of partial synthesis from animal sources. These animal sources provide about 10% of those used by the steroid industry (Blunden *et al*, 1975).

Phytosterols like stigmasterol, sitosterol or ergosterol become the prominent steroid drug precursors because of the abundant and wide distribution in plant kingdom. The principle sources of stigmasterol are calabar bean (*Physostigma venenosum* Staff) and soybean oil (*Glycine max*, Meril.). Upjohn company in the U.S.A. uses stigmasterol as a starting material for conversion into progesterone. It is estimated that stigmasterol represent about 10% of the total steroid precursors used. Sitosterol, a complex mixture of α, β and γ - sitosterols are easily converted to the intermediate compounds by microbial transforma-

tion. It is used by Searle of the U.S.A. to produce androstenedione and spironolactone. It is also used as precursors for estrogen and progesterone production. Solasodine, a steroidal alkaloid present as basic glycosides in *Solanacea* is introduced as a new and more convenient source of steroid precursors in India, but the amount of solasodine produced commercially is propably small.

Steroidal sapogenins especially diosgenin, are of an economical and versatile precursor of many pharmacological active steroid. The discovery of the degradation method of diosgenin to a number of sex hormones by Marker in 1940 and have led to a marked increase in steroid production from this source it accounts for about 50% of the total steroid drugs output in the world (Asolkar, 1979). Hardman (1969) estimated that over 1,000 tons per annum of diosgenin would be required throughout the world by 1973. On the otherhand, Bammi (1972) estimated that world consumption was somewhere between 250-350 tons. Besides diosgenin, hecogenin, a ketonic steroid sapogenin is also used for the manufacture of corticosteroids and accounts for about 6% of the steroid precursors presently being used by the world pharmaceutical industry (Blunden *et al.*, 1975). Hecogenin is commercially obtained from sisal juice, a by-product of sisal fiber (*Agave sisalana* Perrinc.). The carbonyl group at C_{12} in hecogenin makes it unsuitable for the manufacture of oral contraceptives, however, it presents no problems for corticosteroid synthesis. Smilagenin and its 25 S epimer, steroid sapogenins found in *Smilax*, *Yucca* and *Agave* make a small contribution to the steroid industry. Recently cordylagenin isolated from the leaves of *Cordyline cannifolia* R.Br. and *C. stricta* Endl.; cannigenin, the

25α-epimer of cordylagenin, and brisbagenin, the 3 β-hydroxy epimer of cannigenin from the leaves of *C. cannifolia* R.Br., are found to be easily converted in high yields to desired pregananes and androstanes. Both *C. cannifolia* R.Br. and *C.stricta* Endl., are easily cultivated and are possible economic sources of steroid (Blundent *et al.*,1975).

The tubers of *Dioscorea* species are the major source of diosgenin which was firstly isolated from *D.tokoro* Makino in 1936 by Ueno and Tsukamoto. A diosgenin content of about 3% and moisture free basis, is normally the accepted minimum level for commercial ultilization of the rhizomes. Notably, some species such as *D. friedrichsthalii* R. Knuth from Costa Rica yield up to 6% of diosgenin (Martin *et al.*,1963). However, certain other diosgenin-containing plants may provide small economic quantity of the steroids. Fenugreek, the seeds of *Trigonella foenumgraecum* L. which yield from 0.6 to 1.1% of diosgenin and yamogenin on moisture free basis (Fazli and Hardman,1968) was also suggested as economic source of diosgenin because of the ease of its cultivation. *Kallstroemia pubescens* Dandy, a tropical American plant which was introduced to India as a weed, contains 0.96 to 1.71% of diosgenin in whole plant and 1.78-1.92% from the leaves (Chakravarti *et al.*, 1976). It is an attractive source for numerous reasons such as very pure diosgenin obtained, short cycle and easy cultivation, and easy collection. *Balanites aegyptiaca* Delile fruit from Egyptian origin has also been found to contain as much as 4.08% of diosgenin and yamogenin on dry weight basis (Hardman and Sofowora, 1972) which makes it another promissing source and it is under intense investigation.

Costus speciosus Sm., common herb widely distributed throughout

Asia from Philippines, India to New Guinea is also an alternative source of diosgenin in India after Das Gupta and Pandy (1970) who reported that the dried rhizomes of *C. speciosus* Sm. yield 2.1% of pure diosgenin. Nevertheless, chemical analysis of a large number of rhizome samples obtained from various parts of India indicated a wide range in diosgenin content from 0.0-1.8% (Charkravarti *et al.*, 1976) and 0.38 to 1.96% (average pure diosgenin) but some strains, produced as high as 6.10% crude and 4.92% pure diosgenin (Gupta *et al.*, 1979). Po Kya Ban Yi (1980) also reported the diosgenin content of this herb in Burma which ranged from 0.54 to 2.30% (mfb).

The diosgenin content in *C. speciosus* rhizomes was said to be related with altitude, age, and development stage of the plant. In India, the highest yield of diosgenin in rhizomes is in July when the plant is in active stage of growth and flowering has just started. Diosgenin content declines sharply in September when the plant is in full bloom and fruit formation started in the lowest part of the floral heads. There is further decline during September, after which the content again gradually increases up to April or May. As the diosgenin content in rhizomes decrease, its content in seeds increase with the development of the seeds, but when the seeds attain full maturity, there is a gradual decrease in the diosgenin content in rhizomes. (Gupta, *et al.*,1981). The diosgenin content in *C. speciosus* seeds was 2.8% by initial defatting before hydrolysis method and 2.4% by direct hydrolysis method (Singh *et al.*, 1980). Therefore, *C. speciosus* seeds may be an additional source of diosgenin. Prospects for commercial cultivation of *C.speciosus* Sm. as an alternative source of diosgenin in India

has been reported to be good because of many advantages of *C.speciosus* over *Dioscorea* e.g.,short-growing cycle of 6-15 months as compared to about 2-3 years in *Dioscorea* cultivation, ease of cultivation, simple staking, enormous biomass (rhizomes) obtained (Asolkar and Chadha, 1979).

BOTANICAL CHARACTERISTIC OF GENUS COSTUS

The plants in genus *Costus* was previously classified as monocotyledon in the family Zingiberaceae, order Scitaminae. Until 1959, Takhtajan classified these plants into the family Costaceae, order Zingiberales, super order Lilianae, subclass Lilidae. After that in the year 1968, Cronquist classified *Costus* in the family Costaceae, order Zingiberales same as before but in different subclass, Commelinidae. In 1974, Stebbins modified Cronquist's classification of *Costus* by reorganizing Cronquist's subclass Commelinidae as super order Commelinidae. A year later, Dahlgren renamed super order of *Costus* to be Zingiberanae. At present, *Costus* are classified in the family Costaceae, order Zingiberales, super order Zingiberiflorae (Dahlgren and Clifford, 1982).

The important different characteristic of the plant in the family Costaceae is the arrangement of leaves, which are non-distichous instead of distichous as in the family Zingiberaceae. The arrangement is spirally inserted leaves which give the plants their common name Spiral flag. Another characteristic is that the plants in this family contain no oil cells as are found in the family Zingiberaceae (Radford *et al.*,1974).

The Costaceae family contains 4 genera : Costus, Dimerocostus, Monocostus and Tapeinochalos (Dahlgren and Clifford, 1982). The plants in genus Costus consists of about 100-175 species distributed in all tropical areas. Approximately 60 species are in Neotropic of South America, 25 species in Tropical Africa and 5 species in Asia. These plants are mostly bird-pollinated and some of them are grown as the garden plants (Maas, 1979).

Table I.1 The species of some plants in Costus genus and their origins

Species of Costus genus	Origins
C. afer	West Africa
C. cylindricus	West Indies, Central and South America
C. cuspidatus (Nces & Mart.) Maas	Tropical South-East America
C. discolor	Brazil
C. dubius (Af_3) K.Sch.	Tropical West Africa
C. englerianus (*C. unifolinus*)	West Africa, Equatorial Africa, Gabon
C. fridrichsenii, Petersen.	Central America
C. globusus Bl. complex (included)	
C. acanthocephalus K.Sch.	Sumatra
C. chrysocephalus K.Sch.	New Ginea
C. clemensae Ridley	Philippines
C. dhanivalti K. Larsen	Thailand
C. globosus Bl.	Java
C. kingii Baker (*C. globosus* Holttum.var.kingii)	Malay-Peninsula
C. microcephalus K.Sch.	Boneo
C. oligophyllus K.Sch. (*C. kunstleri* Ridle)	Malay-Peninsula
C. ridluji K.Sch. (*C. globosus* Holttum.var.ridluji)	Malay-Peninsula and Thailand
C. suffureus K.Sch.	Celeles
C. tonkinensis Gagnep.	Tonkin
C. velutinus Ridl. (*C. globosus* Holtum.var.velutinus)	Malay-Peninsula
C. igneus, N.E. Brown	Brazil
C. lacerus Gagnep.	China, Thailand, Tibet, Sikkhim, India
C. laevis R. & P.	Tropical America

Table I.1 continued

Species of Costus genus		Origins
C. malorticanus	Wendl.	Costa Rica
C. micranthus	Gagnep.	Martinique
C. musainus	Hort.	Congo, West Africa
C. paradoxus	K.Sch.	Borneo
C. pictus		Mexico
C. pisonis		= *C. spiralis (Jacq)* Roscoe var. spiralis
C. sanguineus		Central America
C. speciosus (Koenig)	J.E. Smith.	Asia, India, Phillippines, Thailand
C. spicatus		West Indies
C. spiralis (Jacq) Roscoe		Equador
C. stenophyllus		Costa Rica
C. zebrinus		West Africa

In Thailand there are 3 species of the Costus genus (Smittinun, 1980 and Maas, 1979) :

1. *Costus globosus* Bl.
2. *C. speciosus* Smith.
3. *C. lacerus* Gagnep.

It has been reported that rhizome of *C. speciosus* Smith contains high content of sapogenins especially diosgenin, which is the important precursor for the semisynthesis of the steroid hormones. Since *C. lacerus* Gagn. is very similar to and misidentified as *C. speciosus* Smith. It is suspected to contain the same chemical constituents as *C. speciosus*.

At present there are still no scientific reports about the type and quantity of sapogenins in *C. lacerus* Gagn. The main objective of this research work is to investigate the sapogenins present in the rhizomes of this plant and whether its content is in reasonable amount to be commercial values as precursor for the semisynthesis of steroid hormones.

In order to be sure that the plant used in this study is not misidentified, the details of its botanical characteristics are examined very carefully. The voucher specimen was sent to be identified by the Forest Herbarium (BKF), Royal Forest Department and it has been given collection number of BKF NO 084435.

TAXONOMY

(Essential-field Information)

Family : Costaccae

Genus : Costus

Species : lacerus

Intraspecific taxon : -

Author(s) : Gagnepain

Citation : The Forest Herbarium (BKF), Royal Forest Department

Collector : Miss Supranee Seangsai

Collection no. : BKF No. 084435

Collection Date : September 10, 1986

Locality :

Country : Thailand

Province : Chiang Mai

County : -

Town or Local Reference : Ampor Muang

Remarks : Originally came from Ampor Doi Saket by Dr. V. Ratanapanondh.

Local name : Ueang maai naa

MUNUSCRIPT ORDER OF *COSTUS LACERUS* GAGN.

1. General modifier : tall leafy ornamental herb. (Fig.I.1)
2. Sex of plant : hermaphrodite or monoclinous
3. Plant duration : perennial.
4. Habit : herb.
5. Stem : 1.3 m tall, leafy stem sometimes branched.
6. Roots : horizontal rhizomes with 2-5 cm thick (Fig. I.2).
7. Leaves : see Fig. I.3.
   a. duration : annotinal
   b. structure : simple
   c. arrangement, position : spirally arranged, cauline or ramous
   d. blade : (uppermost leaves excluded) narrowly elliptic to narrowly obovate, 20-40 cm long, 6.5-15 cm wide, round to cordate at the base, acutely to shortly acuminate at the apex, upperside dark brown in herbarium, material, glabrous or nearly so, lower side sparsely to densely puberous to villose.

      sheath : broad, sparsely to densely puberulous to villose, 15-30 mm in diameter.

      Ligule : 1-3 mm long, obliquely rounded, margins strongly decaying into arachnoid fibre.

   e. petiole : up to 10 mm long, densely puberous to villous.
8. Inflorescence : see Fig. I.3 and I.5.
   a. type : spike very dense, cone-like ovoid to broadly ovoid, 4.5-8 cm long (to 15 cm infruit), 4-7 cm wide (to 10 cm in fruit)
   b. position : terminal
   c. peduncle : thick, short connecting to the stem.

d. bract : red, coriaceous to chartaceous, ovate, 20-55 mm long 10-20 (-40) mm wide, glabrous to densely puberulous or villose, often decaying into long, white, arachnoid fibre often glabrescent; apical portion of bract soon disintegrating into fibres.

bracteole : 8-17 mm long, glabrous to densely villose, margin strongly decaying into arachnoid bifres.

e. pedicel : very short.

9. Flowers : see Fig I.4, I.5 and I.6

a. sex : perfect

b. symmetry : equilateral or bilateral (zygomorphic) : epigny

c. calyx or sepal : tubular, red, 20-29 mm long, glabrous to dense -ly villose, mostly bicarinate on the adaxial side, lobes irregular obovate, 9-15 mm long, 5-10 mm wide, apex obtuse, sometimes shortly acuminate, the apical portion (like the bracts) often disintegrating into fibres.

d. corollay petals : white, 50-70 mm long, glabrous or nearly so, tube 50-20 mm long, lobes 35-50 mm long, 10-25 mm wide, labellum; white, bell-shaped tinged with yellow in the middly and toward the apex, broadly obovate when spread out (40-) 60-70 mm long, (50-) 60-90 mm wide.

e. Stamens, anther, filaments : stamen 1 perfect, white, narrowly eliptic, 30-35 mm long, 60- mm wide, anther, 7-18 mm long, filament forming with the connective and oblong petaloid process with the contiguous linear anther cells situated in its middle, laleral staminodes minute or obsolate.

f. carpels, stigma, style, ovary, ovule : carpel stylocarpellous,

stigma; terete with a semiluna foveoli ciliated round the margin, densely puberulous to villose, style; filiform, ovary; 3 locules, ovules many superposed.

g. receptacle (hypanthium) : partly adnate.

h. pedicel : short.

10. Fruits : loculicidal capsules, 3 celled ellipsoid, woody, 15-30 x 10-16 mm longitudinal dehiscing.

11. Seeds : endospermous, obovoid or subglobose, very numerous, black, angled, aril short with axile placentation.

12. Habitat : forests (open forest, evergreen forest, pine forest) or in marshes (500-) 800-1700 (-2000) m altitude.

13. Distribution : China (Yunnan), Thailand, Tibet, Sikkim, and India.

14. Synonyms : Local name; Ueang maainaa.

15. Remarks : This species has always been misidentified as *C. speciosus* Sm. because of its almost woody fruit, but it differs by the following characters :

1. upper side of lamina dark brown;

2. margins of bracts, bracteole, and ligule decaying into arachnoid fibers.

3. apical portion of bracts and calyx very soon decaying and disintegrating in more or less loss fibres (hence the specific epithet "*lacerus*")

4. calyx-lobes obtuse

5. corolla glabrous

16. References : Maas (1979), Ridley (1967), Merill (1968), Hooker (1954), Kirtilcar (1975).

Fig. I.1. *Costus lacerus* Gagn.

Fig. I.2. The rhizomes of *C. lacerus* Gagn.

Fig I.3. The leaves and inflorescence of *C. lacerus* Gagn.

Fig I.4. The flowers of *C. lacerus* Gagn.

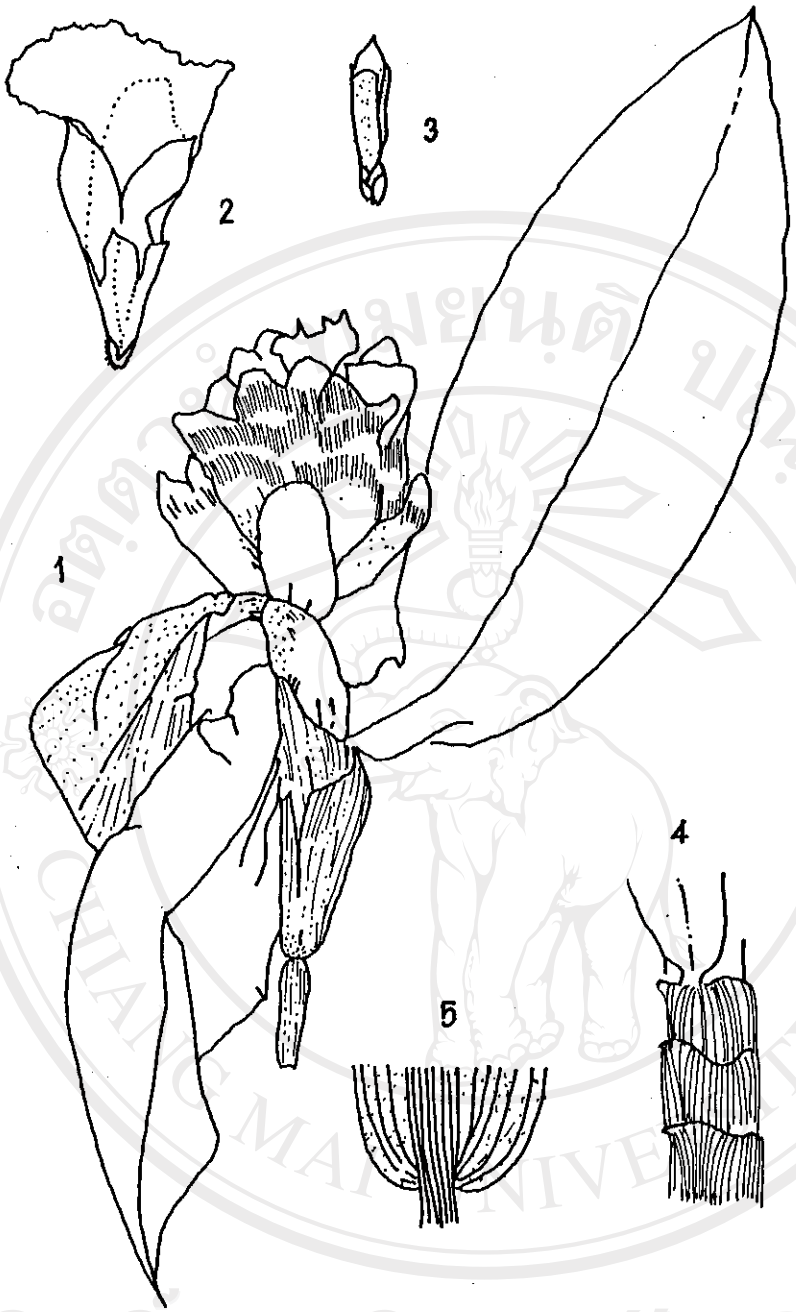


Fig. I.5. *Costus lacerus* Gagn. 1. inflorescence x ½; 2. flower x ½; 3. bracteole, ovary, calyx and corolla bud x ½; 4. leaf-base with petiole, ligule, and sheath x ½; 5. leaf base x ½. (Maas, 1979).

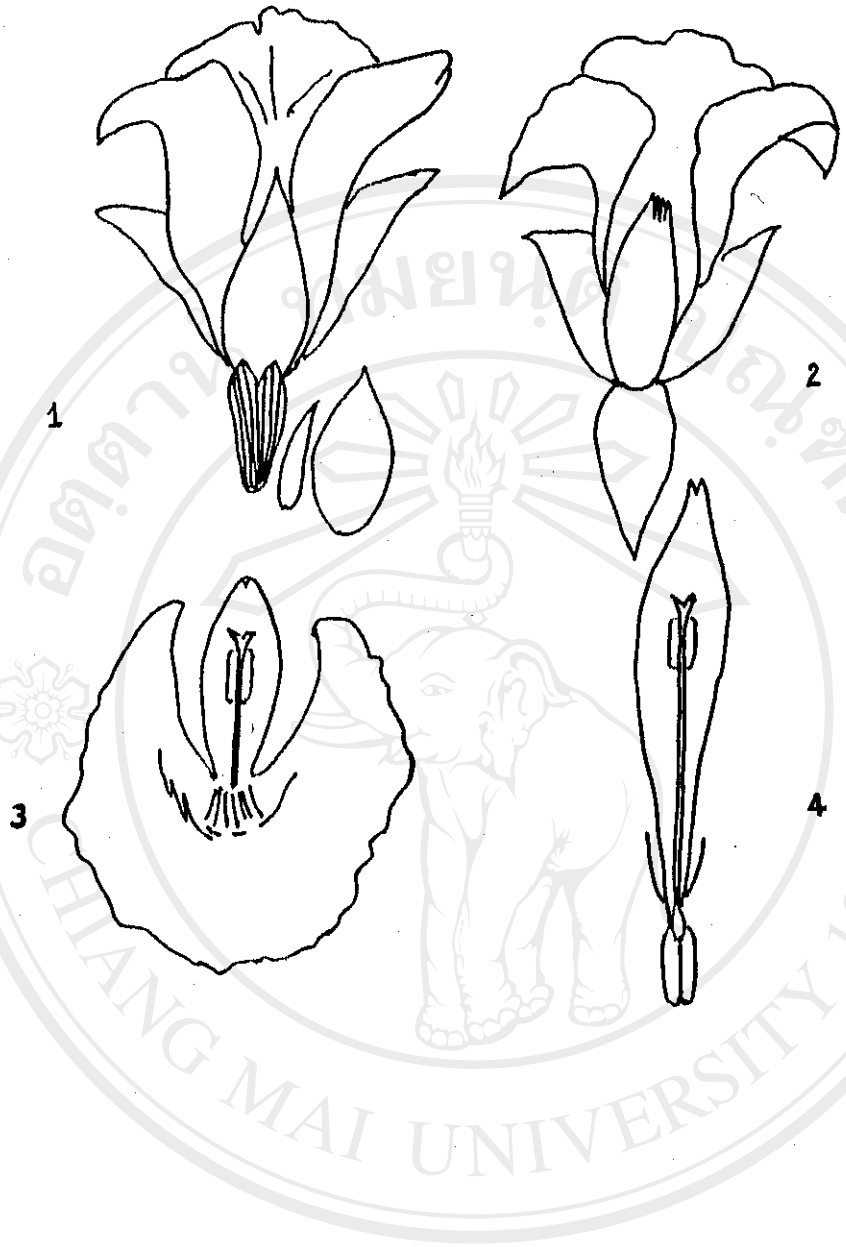


Fig.I.6. *Costus lacerus* Gagn. flower . 1. calyx,corolla x ½; 2. corolla x ½; 3. labellum x ½, 4. stamen and ovary x ½.

QUANTITATIVE ANALYSIS OF STEROID SAPOGENINS

The quantitative analysis of steroid sapogenins in plant material is carried out into 2 steps which are, firstly, extraction of sapogenins from plant material and, secondly, determination of sapogenin content in the extract by chemical or physical method.

As sapogenins occur mainly in nature as saponin glycosides of which sugar molecule (glycone part) is bound to hydroxyl group (-OH) at 3-positon in the aglycone part. It is necessary therefore, to initially hydrolyse the glycosidic linkage in order to liberate sapogenin from saponin glycoside. Sapogenin extraction can be carried out by 2 different methods :

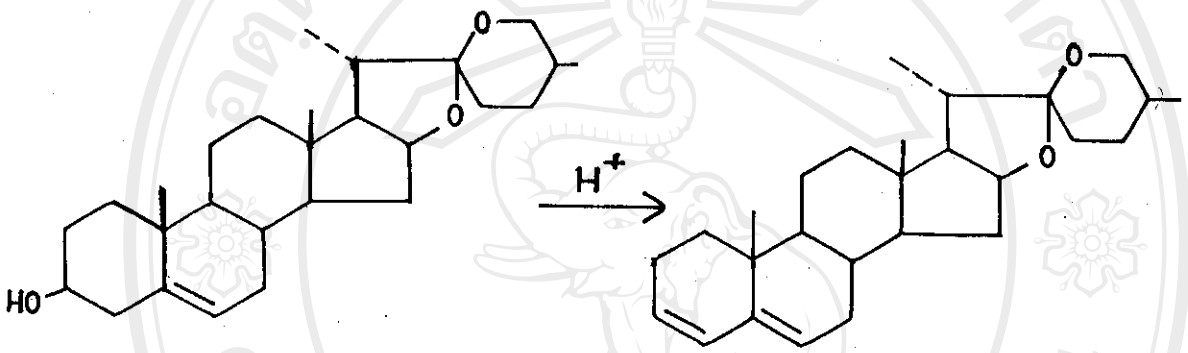
1) Extract saponin from plant material with polar solvent and follow by hydrolysis. Wall *et al*, (1952) extracted saponin from plant material with ethanol and purified the alcoholic extract by extraction with benzene to remove fats and pigments, then hydrolysed saponin ex-extract with 4N HCl.

2) Hydrolyse saponin in plant material by reflux with acid and follow by extraction with non-polar solvent. This extraction method is more favored than the first one. Hydrochloric acid is more commonly used in hydrolysis process than sulphuric acid as it has an advantage over the latter by causing less decomposition of saponin due to oxidation reaction. Various concentration of acid are used such as 2N (Morris *et al*, 1958), 2.5N (Rothrock *et al*, 1957), Selvaraj, 1971), 3N (Rozanski, 1972) and 4N (Wall *et al*, 1952). Ratios of sample weight to volume of acid used vary from 1:5 to 1:30.

Reflux temperature is carried out between 70^{o} to boiling point

of the solvent used and reflux time is generally about 2-4 hours with some exceptional cases such as Fenugreek seeds require 24 hours (Sauvaire and Baccou, 1978).

Acid hydrolysis may cause artefact that is spirosta-3,5-diene which can be 25R or 25S depending on types of saponin as shown in the reaction below (Wall *et al*,1955).



Blunden *et al.*(1967) estimated that hydrolysis with 2-3N HCl by reflux for 2-4 hours yields no more than 6% of 3,5-diene. Thus the extraction method of sapogenin from plant material to obtain the highest yeild depends on acid concentration and hydrolysis condition. Standardized hydrolysis methods have been proposed and tried to obtain the highest yeild of sapogenin for some plant material. Chaudhuri (1977) studied the optimum condition for quantitative analysis of diosgenin in rhizome of *Dioscorea composita* and *D. floribunda* and found that boiling fresh or dried plant material in 2N HCl in the ratio of 18:1 for 2 hours maximizes the yield of diosgenin. Gandotra *et al*(1977) had also determined the standard method for quantitative analysis of diosgenin from

rhizome of *Costus speciosus*

When the hydrolysis process has completed, the plant material can be neutralized with 5% (Brain *et al*, 1968) or 10% ammonia solution or by washing with water until it is free from acid. The latter method usually takes longer time. Then the neutralized plant material is extracted with non-polar solvent to give sapogenin. Commonly used solvents for monohydroxysapogenin are petroleum ether 30-60°, 40-60°, 60-80° (Rothrock *et al*, 1957), n-hexane (Gupta *et al*, 1979). The less common solvents are benzene, xylene and chloroform (Sanchez *et al*, 1972, Dawidar 1973, Sauvaire and Baccou, 1978). Time required for extraction depends on plant material such as Dioscorea rhizome needs only 3-8 hours while fenugreek seeds take longer time, i.e. 24 hours-7 days (Brain *et al*, 1968). Thus the selection of suitable solvent and period of reflux time affected sapogenin content obtained. Some plant material, such as seeds which is rich in oil, should be defatted before hydrolysis.

Determination of sapogenin content in the extract previously prepared as above can be carried out by various methods: gravimetric method, colorimetry, densitometric thin layer chromatography, infra-red spectrophotometry, preperative thin layer chromatograph-infra-red spectrophotometry, column chromatography infra-red spectrophotometer, gas liquid chromatography and high performance liquid chromatography. Choice of the suitable method depends on the quantity of sample available and the complexity of mixture in the extract.

1. Gravimetric method

Morris *et al.* (1958) used the gravimetric method to determine diosgenin content of *Dioscorea* rhizome. It is simple, uncomplicated and

is also a quantitative isolation method. Its disadvantage is that large sample size (up to 50 g) is required. The sapogenin content assayed by this method is higher than those obtained by the others as some other compounds are also included. Thus this method is suitable for plant materials containing only single sapogenin with high purity such as rhizomes of *Dioscorea* and of *Costus*.

This method can be done as follow : concentrate the extract, refrigerate at a very low temperature and filter the residue. These steps may be repeated until no more residue obtained, dry and weigh the combined residues. The first crop sapogenin is usually more purified than the later crops.

2. Colorimetric method

Many reagents are used to form color complexes with sapogenin. Walens *et al* (1954) used 94% sulphuric acid to produce the color complexes and measured the absorbance at the wavelength range from 220-400 nm depending on the type of sapogenins. For the sapogenin mixture, each sapogenin should be previously separated by column chromatography. Yamakishi and Nakamura (1958), Sofowora and Hardman (1974) and Rishi *et al*(1976) used antimony trichloride in acid solution or in sulfuric acid and formaldehyde, Hiai *et al* (1974) used vanillin and sulphuric acid to form color complexes with sapogenin including other compounds like bile acid, sterol and triterpenoids. Baccou *et al*(1977) determined the total sapogenin content in the extract by using the chemical reaction between anisaldehyde, sulphuric acid and ethyl acetate.

Okanishi and Togami (1969) had modified and improved the method by separating only sapogenin on kiesel gel layer. The sapogenin spot

was scraped out and warmed with anisaldehyde and phosphoric acid then followed by centrifugation. A clear solution thus obtained, was measured the absorbance at the wavelength of 540 nm. This colorimetric method can be used in sample containing sapogenin as low as 0-40 µg.

3. Densitometric Thin Layer Chromatographic Method.

This method can be used to separate 25R and 25S epimers from sterol and dienes. Blunden *et al* (1967) and Blunden and Hardmen (1968) isolated monohydroxy, saturated sapogenin from Asparagus, Similax and Yucca by thin layer chromatography (TLC) using silica gel G as the absorbent and n-hexane : ethyl acetate (12:1,V/V) as the developing solvent. The 6-folded development was performed and then sprayed with antimony trichloride solution in sulphuric acid,3:1 W/W. The absorbance of the spot was measured by means of densitometer.

Brain and Hardman (1968) improved the spotting method for sample, this method was later modified by Lockwood *et al* (1974) using lanosterol as internal standard, in order to reduce the error due to sample spotting, and the sterol content can be determined simultaneously.

4. Infra-red spectrophotometry.

The Infra-red (IR) spectrophotometry is one of the most rapid method for routine determination of sapogenin content in the plant materials. It was firstly introduced by Wall *et al* (1952). The sample was acetylated and then measured its absorbance by scanning at wave number 800-1050 cm^{-1}. Sapogenin has 4 absorption bands at 852, 900, 922 and 987 cm^{-1} which are the characteristic of spirotan structure. Steroid sapogenin which showing more intense absorption band at 900cm^{-1} than that of 922 cm^{-1} indicates the majority of the 25R epimer presented.

On the other hand, if the 900 cm^{-1} band in less intense than 922 cm^{-1} band, the 25S epimer is the major component. However, if the intensity of those bands is nearly the same, the quantity of two epimers is also equally presented. Absorption band at 982-987 cm^{-1} can be used to approximate the sapogenin content, but some errors may occur owing to sterol and triterpenoid sapogenin which also exhibit absorption band near 982 cm^{-1}.

Brain *et al* (1968) improved this method by measuring the absorption band at 915 cm^{-1} and 900 cm^{-1}. By this method the 25S and 25R epimers can be separately determined and the ratio of these two epimers content can be calculated. It is very useful for studying the C 25 epimers ratio which vary in different parts of plant materials and in different seasons.

Jeffereis and Hardman (1972) separated the two epimers which are diosgenin and yamogenin, by using preparative thin-layer chromatography on silica gel G and then analyzed with IR spectrophotometry. The spirosta-3,5-diene, which has the same IR spectrum as sapogenin, is separated by column chromatography before analyzing with IR-spectrophotometry.

5. Gas liquid chromatography (GLC)

Many authorities use this method for the determination of sapogenin content (Cooke, 1970; Dixit and Srivastava, 1977; Fazli and Hardman, 1971; and Rozanski, 1972). This method is more rapid and requires smaller sample size than other methods such as column chromatography and TLC. Various adsorbents can be used such as 2.5% and 2% SE 30 coated on acid-washed DMCS-treated chromosorb G (80-100 mesh)

(Cooke, 1970) or chromosorb (80-100 mesh) coated with 3% SE 30 (Dixit *et al.*, 1977).

6. High Performance Liquid Chromatography (HPLC)

Higgins (1976) introduced the HPLC method for the analysis of the sapogenin content of Agave plants in the form of benzoate ester, using Lichrosorb RP-8 column, and acetonitrile as mobile phase. Mahato *et al* (1981) used HPLC for the determination of diosgenin content in *Kallstroemia pubescens*, *D. prazeri*, *D. floribunda*, *D. detoidea* and *C. speciosus* by using normal phase system. Tal and Goldberg (1981) used this method for the analysis of diosgenin in the lyophilized plant cell sample from *D. deltoidea* by reversed phase system. These methods utilized the refractive index detector since sapogenins do not exhibit any UV absorption characteristics.

The method of Tal and Goldberg (1981) can be used to seperate, hecogenin, tigogenin and diosgenin by decreasing the polarity of solvents. When the ratio of acetonitrile is increased, diosgenin and tigogenin are better separated but the retention time of sterol is also increased. Stigmasterol and cholesterol can also be separated as the retention time of cholesterol is longer than stigmasterol. Quantitative determination of sterol can be done by increasing the flow rate of mobile phase, 15 minutes after the injection of sample from 1 ml/min to 3 ml/min. In addition, Hunter *et al.*(1981) studied the method to isolate sapogenin in the form of acetate salt both in normal and reversed phase systems. Simultaneous quanlitative and quantitative analyses of sapogenin may be done by this method.

The HPLC is a rapid and a simple method, accurate results can

be obtained owing to its high sensitivity. The HPLC is also very versatile since it can be used for isolation, identification, and quantitative analysis simultaneously.

Table I.2 The summarisation of steroidal sapogenin determination (Chaumanochan, 1979)

Name of workers	Sample	Acid hydro-lysis	Refluxing		Extraction solvent and time	Method of quantification	Product of quanti-fication
			Time (h)	Temp.			
Wall *et al*, 1952	Saponins from plant material (general method)	4N HCl+	4	70-80°	10% EtOH in benzene	ppt. with $(AcO)_2$+ IR	SS
Rothman *et al*, 1952	Dioscin from *D. composita*	"	2	75-78°	10% EtOH in benzene	"	"
Rothrock *et al*, 1957	Ground tuber of *D. barbasco*	2N HCl	2	-	Petrol (30-60°) 6 h	gravimetric	D
Morris *et al*, 1958	Fresh sliced tuber of *D. composita*	1.9-2.1 HCl*	3	-	Petrol (30-60°)	gravimetric 8 h	D
Brain *et al*, 1968	Ground Dioscorea tuber, whole fenugreek seed, defatted Balanites seed	2N HCl	2	-	Petrol (40-60°) 24 h	IR	SS
Brain and Hardman, 1968	Ground Dioscorea tuber	2N HCl	2	-	" "	densitometric TLC	SS (D+Y)

+ = In the presence of 2 ml benzene

* = Depending of moisture content

SS = Steroidal sapogenin; D = Diosgenin; Y = Yamogenin; T = Tigogenin; NT = Neotigogenin; YC = Yuccagenin; L = Lilagenin; G = Gitogenin; NG = Neogitogenin

Table I.2 (contd.)

Name of workers	Sample	Acid hydro-lysis	Refluxing		Extraction solvent and time	Method of quantificatio	Product of quanti-fication
			Time (h)	Temp.			
Fazli and Hardman, 1971	Whole fenugreek	2N HCl	2	-	Petrol (40-60°) 2 h to 7 days	TLC + IR; GLC (SE-30)	D and Y, G
Cooke, 1970	Dried sliced or undried minced tuber of Dioscorea	2N HCl	3	-	Petrol (60-80°) 3 h	GLC (2.5% SE-30)	D
Hardman and Jefferies, 1972	Whole seed of fenugreek	2N HCl	2	-	Petrol (40-60°) 24 h	Column Chro. + IR	D and Y
Sanchez *et al*, 1972	Ground tuber of *D. composita*	30% V/V HCl	4	-	$CHCl_3$, 2 h	TLC + Spectro.	D
Rozanski, 1972	Pulverized tuber of	3N HCl+	4	93°	-	GLC (2% SE-30)	D
Dawidar and Fayez, 1972	Saponin from pow-dered fenugreek	4N HCl	3-4	-	Benzene	TLC + Photo.	D + T, G
Dawidar *et al*, 1973	Saponin from pow-dered fenugreek	conc. HCl	2	-	$CHCl_3$	TLC + IR	D + Y, T + NT, G

Table I.2 (contd.)

Name of workers	Sample	Acid hydro-lysis	Refluxing		Extraction solvent and time	Method of quantification	Product of quanti-fication
			Time (h)	Temp.			
Khanna and Jain, 1973	Fenugreek tissue culture	5% V/V HCl in 70% EtOH	4	-	EtOAc	GLC (10% SE-30)	D, T, G
Bohannon *et al*, 1974	Whole fenugreek	2N HCl	2	-	Petrol, 6 h	GLC (3% OV-1)	D
Dixit and Srivastava, 1977	Powdered fenugreek seed	2.5 N HCl	4	-	Petrol(40-60°) 8 h	GLC (3% SE-30)	D
Knight, 1977	Isolated sapogenin from fenugreek seed	-	-	-	-	GLC (3% QF-1)	D,Y,T, NT,YC, L,G,NG
Sauvaire & Baccou, 1978	Defatted powdered fenugreek seed	2.5 N H_2SO_4 in 70% isopro-panol	12	-	$CHCl_3$ until exhausted	Densitometric TLC	D
Chakravarti *et al*, 1976	rhizome of *C. speciosus*	dil HCl 1:6	5	in boi-ling water bath	Petrol(60-80°) 10 h	gravimetric	D

Table I.2 (contd.)

Name of workers	Sample	Acid hydro-lysis	Refluxing		Extraction solvent and time	Method of quantification	Product of quanti-fication
			Time (h)	Temp.			
Mahato *et al*, 1981	plant material from *Kallstroemia pube-scens*, *D. prazeri*, *D. floribunda*, *D. deltoidea*	2.5 N HCl	4	in boi-ling water bath	Petrol(60-80°) 4 h	hplc (μβora-sil column c̄ RI detector	D
Gupta *et al*, 1981	different parts of *C. speciosus*	2.5 N HCl	4	-	n-hexane	SE 30 tlc c̄ LB reagent	D
Tal and Goldberg, 1981	lyophilized plant cells from culture of *D. diltoidea*	2N HCl	2	-	$CHCl_3$, 3 h	hplc (Micro-pak MCH column c̄ RI detector	D. (includ-ing H, T, stigmas-terd and cholesterol)

Table I.3 THE RELATIVE MERITS AND DEMERITS OF VARIOUS ASSAY PROCEDURES FOR SAPOGENIN.

Merits	Demerits	Remarks
	1. GRAVIMETRIC	
Easy;no sophisticated instrument necessary, except the Meltars balance	Approximate estimation; requires at least 50g of plant material; the whole of the extract or at least most of it should be sapogenin	Suitable for the estimation of diosgenin from *Dioscorea*, *Costus*, *Kallstroemia*, etc.; used by commercial extractors.
	2. COLORIMETRIC	
Only 50mg of the dried plant material required; rapid and accurate, 8 to 10 samples per day can be assayed; no significant interference from phytosterol and limited amounts of fixed oil	Interference from other sapogenins such as pennogenin; 25R-&25S-epimers not estimated separately	Suitable for the estimation of diosgenin from *Dioscorea* or samples from plant survey or breeding programme
	3. GLC	
About 5g of undried plant material adequate; dienes, sterols can be separated	25R- and 25S- epimers not separated	Suitable for the estimation of diosgenin from *Dioscorea* in a breeding programme or in tissue culture
	4. DENSITOMETRIC TLC	
Small amount of plant material adequate; rapid and accurate; can separate diosgenin from dienes and sterols	The pair of 25R- & 25S-sapogenins not easily separable; preparative TLC necessary to separate the epimers	Suitable for the estimation of diosgenin from *Dioscoreas* in a breeding programme or in tissue culture

Merits	Demerits	Remarks
5. IR SPECTROPHOTOMETRY		
1 to 10 g of undried material sufficient; rapid, 100 to 150 samples per week (40 hours); 25R- and 25S-epimers estimated	Cannot estimate 3.5 dienes (of both 25R- & 25S-); interference due to absorption by sterols and fixed oil; yamogenin content highly exaggerated; sample must yield minimum of 50mg of sapogenin	Appropriate for the study of the variation in C-25 epimers in morphological parts and with season for routine screening of steroid sapogenins
6. P-TLC - IR SPECTROPHOTOMETRY		
2.5g of sample sufficient; can estimate D&Y separately and together in almost all proportions; eliminates most of the impurities	If the minor epimer, (generally Y) is only 10% of the sapogenin mixture the amount is too low to be estimated accurately	Suitable for the estimation of diosgenin yamogenin individually and totally with accuracy from fenugreek and *Balanites* seeds
7. COLUMN CHROMATOGRAPHY-IR SPECTROPHOTOMETRY		
Same as P-TLC - IR analysis. Simultaneous isolation of sterols and gitogenin. Column can be used at least 5 times	Does not permit the separation of diosgenin from tigogenin, and yamogenin from neotigogenin; D+Y content also includes the said 5 α & β sapogenins	The only suitable method for the estimation of D/Y individually and totally with near accuracy from fenugreek seed in a breeding programme. The method can be applied to *Balanites* seeds as well

Table I.4 The HPLC System for Quantitative Analysis of Sapogenin in Plants.

	Normal phase system	Reverse phase system
Column	µ Porasil (varian) (30 cm x 3.9 mm. ID)	Micropak MCH-10 (Water Assoc.) (30cm x 4 mm ID)
Mobile phase	light petroleum (60-80°): isopropanol (12:1)	acetonitrile:methanol:$CHCl_3$ (83:10:7)
flow rate	0.8 ml/min (P≃125 Psi)	1 ml/min
Detector	RI detector (attenuation 8)	RI detector (attenuation 2)
recorder speed	1 cm/min	0.25 cm/min
Sensitivity	2.26-0.226 mg/ml (c̄ 20 µl injection)	0-40 g
Retention time of diosgenin	347 ± S sec.	≃ 12 min.
Sample	*Kallstroemia pubescens* *D. floribunda*, *D. deltoidea* *D. prazeri* and *C. speciosus* plant material	lyophilized plant cell from . *detoidea*

Table I.5 The HPLC System for Separation of Sapogenin Acetate (Hunter *et al*, 1981)

	Normal phase system	Reverse phase system
Column	Zorbax SIL (Dupont) (4x250x4.6 mm. ID)	Zorbax DDS (Dupont) (4x250x4.6 mm. ID)
Mobile phase	n-hexane:acetone (99:1)	Acetonitrile:n-hexane: tetrahydrofuran (17:2:1)
flowrali	2.0 ml/min (P.3200 Psi)	1.0 ml/min (P≃1100 Psi)
Detector	UV detector (210 nm, range 0.1)	UV detector 210 nm, range 0.2)
Recorder Speed	10 min/cm.	10 min/cm.
Separation	C 5 and C 25 epimers.	5α and Δ^5 sapogenins.