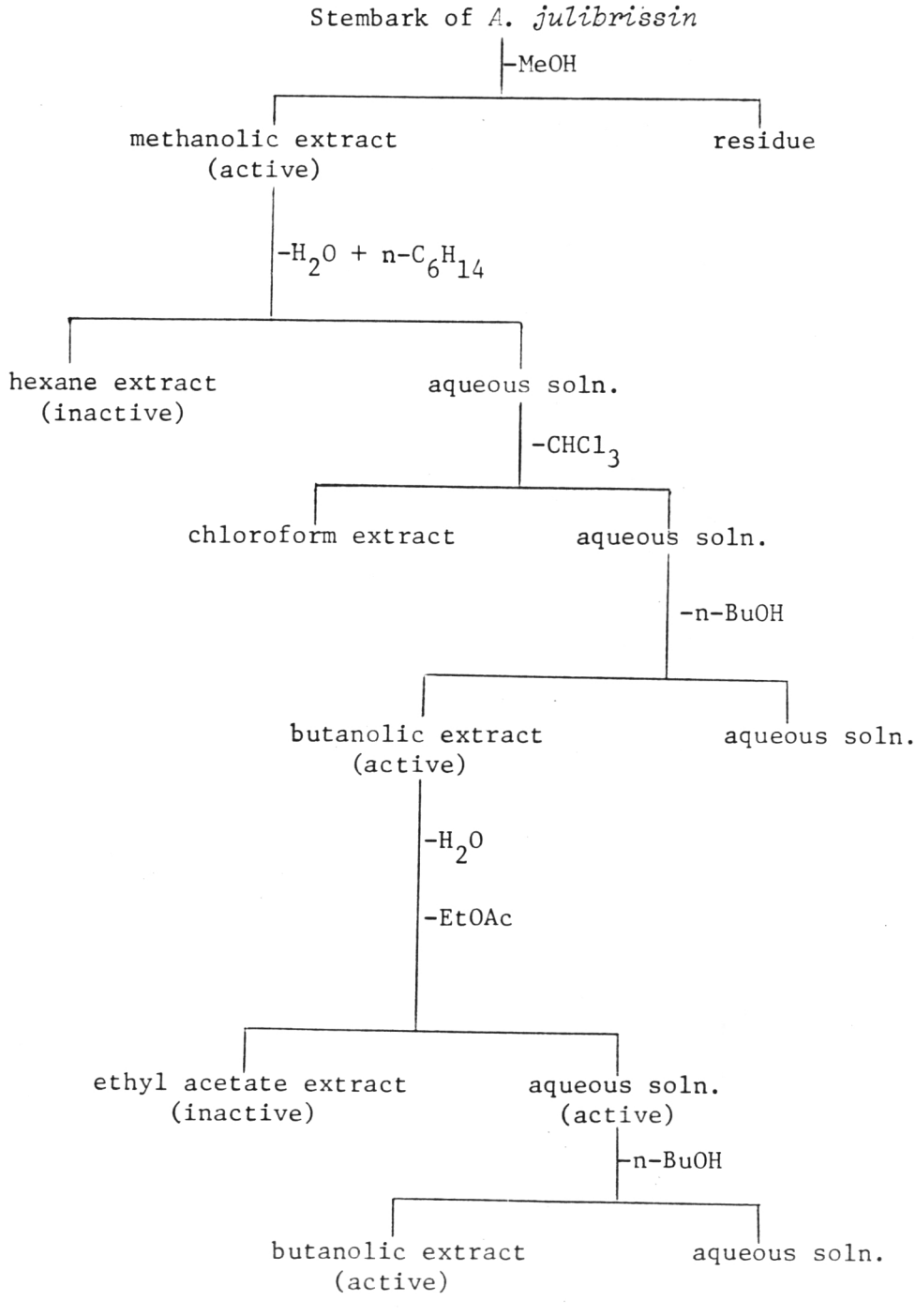
CHAPTER II

RESULTS AND DISCUSSION

One of the main purposes of the presented research consists of isolating the major chemical constituents which have antifertility activity from the stembark of *A. julibrissin*. There is no information in literatures on chemical and pharmacological investigations of this particular part of *A. julibrissin*. Pharmacological tests of the crude methanolic of the stembark and the various fractions obtained from the fractionation procedure shown in Scheme I on next page were carried out in the Natural Products Research Institute at Seoul National University in Seoul. The results of the pharmacological test were encouraging. It was found that the crude methanolic extract of the plant material showed significant uterotonic activity. Crude methanolic extract was further fractionated and several fractions were tested for the uterotonic activity. The results of the pharmacological screening are presented in Scheme I as follow :

Scheme I Pharmacological screening on uterotonic activity



Hexane, chloroform and ethyl acetate fractions did not show any uterotonic activity contrarily to the aqueous and butanolic fractions which were biologically active. Furthermore, the results of the test indicated that the active constituents could be efficiently extracted from aqueous layer by n-butanol.

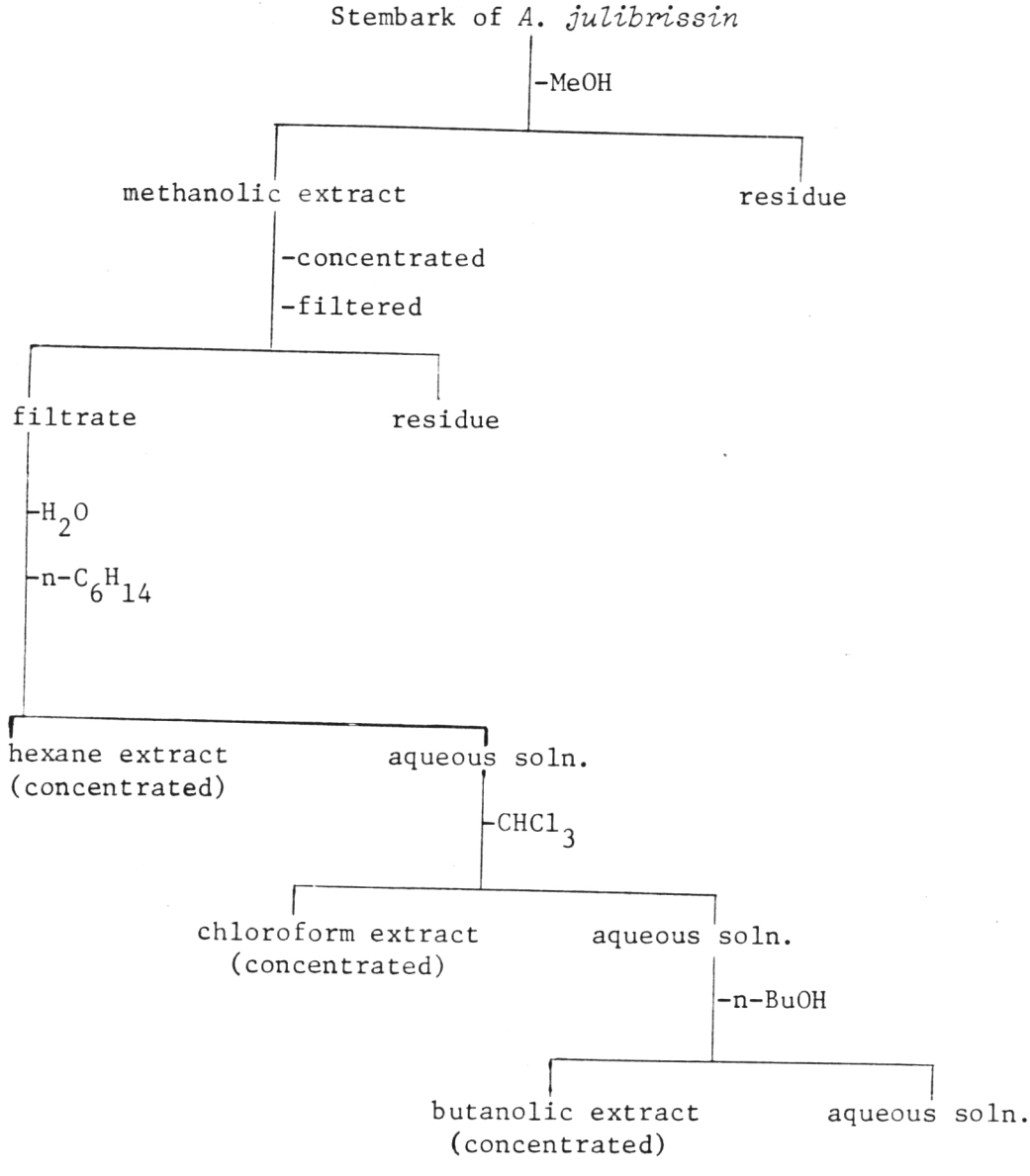
We concentrated our efforts on the extraction, separation and purification as well as structural elucidation of chemical components presented in the butanolic soluble fraction obtained from the fractionation of the aqueous extract with n-butanol because this fraction showed the uterotonic activity according to the results of pharmacological screening. Nevertheless, we paid our attention to the whole chemical processes from the very beginning.

EXTRACTION

We chose methanol as a solvent for extraction of the plant material due to its lower boiling point and cheaper price comparing with ethanol. Furthermore, methanol also dissolves both polar and non-polar compounds. Although methanol is harmful to human being it is less toxic to mice, the tested animal, and produces less interferent effect to the experimental results on utorotonic activity.

The sequence of extraction to seperate the chemical compounds from the methanolic crude product was in the order of increasing polarity from hexane, chloroform and butanol (see Scheme II on next page). It is expected that the compounds from the later fractions are more hydrophilic than those extracted from the previous ones. Nonetheless, since there was no uterotonic activity found in both the hexane and the chloroform fractions, no attempt was made to identify the chemical compounds in these two fractions.

Scheme II Extraction and fractionation procedures



investigated fraction

The results from the extractions with various solvents are shown in Table IV.

Table IV Extraction of the stembrak of *A. julibrissin*

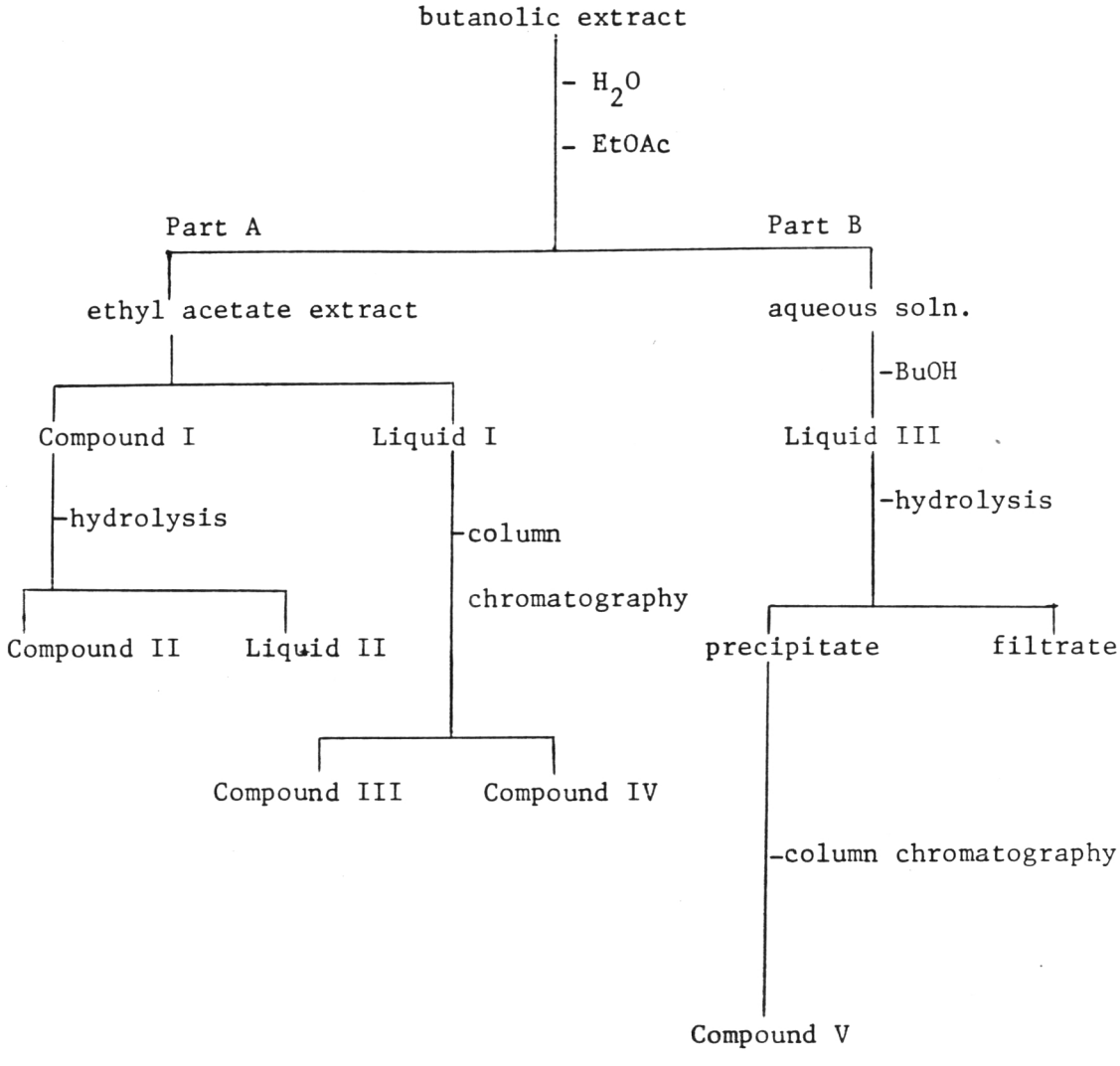
Solvent (b.p.)	color of the crude products	extracted weight(g) from dried plant 5.4 kg	percentage
MeOH (64.7°C)	dark-green	759.8	14.07
n-Hexane (69°C)	greenish-yellow	89.0	1.65
$CHCl_3$ (58°C)	yellow	116.6	2.16
n-BuOH (117°C)	dark brown (syrup)	373.0	6.91

It is worth to note that the colors of the extracted crude products were different from one fraction to another.

STRUCTURAL ELUCIDATION OF COMPOUND I

Compound I was crystallized from the ethyl acetate fraction of the butanolic extract (see Scheme III on next page). Recrystallization from either methanol or ethyl acetate gave colorless needles, m.p. 278-281°C (yield 54 mg) which were identical in TLC with α-spinasteryl-D-glucoside (Rf 0.23 in MeOH-$CHCl_3$-7% HOAc 25:8:5 v./v and 0.29 in MeOH-$CHCl_3$-H_2O 52:25:8 v./v.).

Scheme III Isolation of Compounds I, II, III, IV and V.



According to the literature the above melting point is identical with that of α-spinasteryl-D-glucoside[27]. Compound I gave no depression in mixed m.p. with authentic sample of α-spinasteryl-D-glucoside. This strongly support the hypothesis that both compounds have the same chemical structure.

Compound I gave positive Liebermann-Burchard and Molisch tests which indicated the presence of a steroidal or triterpenoidal nucleus with a sugar attached to it. The IR spectrum of Compound I (Fig. I) shows several characteristic absorption bands which can be assigned as follows:

Absorption peaks (cm^{-1})	Assignments
790, 820, 840	Trisubstituted double bond possibly between C-7 and C-8.
965	Trans-disubstituted double bond at C-22 and C-23
1000 - 1100	glycosidic bond (C_3-O)
1350	CH_3 group
1440	CH_2 group
2280	C-H stretching
3330	O-H peak

Compound I was then hydrolyzed in order to seperate the aglycone from its sugar by treating with 5% methanolic HCl.

Acidic hydrolysis gave Compound II, m.p. 166-167°C and mother liquor designated as Liquid II.

According to the literature, m.p. of Compound II is identical with α-spinasterol. Furthermore, GLC analysis proved that Compound II had the same retention time as an authentic sample of α-spinasterol. GLC analysis also revealed that Compound II contained traces of $^{7}\Delta$-stigmastenol[29,30] (see Fig. II).

The mass spectrum of Compound II (Fig. III) showes the relatively high intensity of the molecular ion peak at m/e 412 which corresponds to its mass of the sterol, the other significant peaks[29] in the high mass range are at m/e 397 ($M^{+}-CH_3$), 369 ($M^{+}-C_3H_7$), 273 ($M^{+}-C_{10}H_{19}$ side chain), base peak 271 (M^{+}-side chain-2H), 256 (M^{+}-side chain-OH) and 255 (256-H). The mass spectrum of Compound II was found to be superimposable with the one of α-spinasterol[29] proving clearly that Compound II was in fact α-spinasterol.

ANALYSIS OF LIQUID II

Hydrolysis of Compound I in 5% methanolic HCl gave Liquid II which contained carbohydrate components. Under above conditions glycosidation of sugar might occur. Therefore, the hydrolysis of glycosidic linkage by refluxing Liquid II with 50% aqueous HCl had to be done before further TLC and GLC analyses

of the carbohydrates present.

The sole carbohydrate found in Liquid II was identified as D-glucose by comparisons with authentic samples of D-glucose, raffinose and others in TLC (Fig. IV, V).

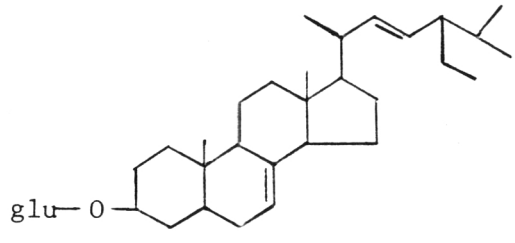
This finding by TLC was further confirmed by the results of GLC analysis which was carried out as follows:

D-glucose, as a polyhydroxy compound cannot be analyzed directly by GLC, it has to be converted into a volatile derivative in which hydroxyl groups are blocked. Thus demethylated Liquid II and a standard sample of D-glucose were trimethylsilylated[28] under the same experimental conditions and then the resulting derivatives were analysed by GLC (Fig. VI).

Gas chromatogram of D-glucose shows two peaks due to the presence of α-and β-anomers of trimethylsilyl derivatives. These two similar peaks having the same retention times (R_t) are also found in the gas chromatogram of the trimethylsilylated Liquid II. Furthermore, expected signal enhancement was observed upon mixed trimethylsilyl derivatives of the tested sample and D-glucose standard.

The results of GLC analysis and TLC examination indicated the presence of D-glucose in Liquid II. We clearly demonstrated that the acidic hydrolysis of Compound I gave

α-spinasterol and D-glucose. Consequently, we can conclude that Compound I has a structure of α-spinasteryl-D-glucoside. The β-configuration of the glycosidic linkage has been established[27] earlier from the value of corresponding coupling constant in ^1H NMR spectrum of α-spinasteryl-D-glucoside



α-spinasteryl-D-glucoside

Incidentally 8 mg of Compound III was isolated from the Liquid I by column chromatography (see Scheme III page 15). This compound has the same m.p. 278-281°C as Compound I and gave no depression in mixing up with Compound I. They also have the same R_f's values (0.23 in $CHCl_3$-MeOH-7% HOAC 25:8:5 v./v., 0.29 in MeOH-$CHCl_3$-H_2O 52:25:8 v./v.). Furthermore, their IR spectra are superimposable. Present data indicate that Compound III and I are identical and have the structure of α-spinasteryl-D-glucoside as we already demonstrated.

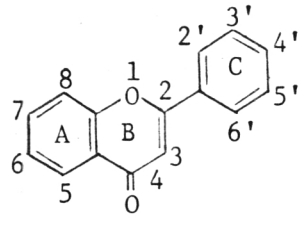
This result is not surprising, since Compound I was crystallized from ethyl acetate fraction and Compound III was isolated by column chromatography of the remaining mother liquors (Liquid I).

STRUCTURAL ELUCIDATION OF COMPOUND IV

Compound IV was isolated from Liquid I by chromatographic techniques. Its purification proved to be difficult. Only after several column, PTLC and PC separations Compound IV could be crystallized to yield the analytically pure sample. Finally, Compound IV m.p. 314-316°C was crystallized from methanol. The yield was 81.4 mg.

On TLC plate examined under UV light it appeared as a light blue fluorescent spot (R_f 0.26 in MeOH-$CHCl_3$-H_2O 52:25:8 v./v. and 0.17 in $CHCl_3$-MeOH-7% HOAC 25:8:5 v./v.) which turns into bright yellow in the presence of ammonia vapour or after spraying with 50% H_2SO_4. The results of TLC examinations indicated the possibility of flavone skeleton for Compound IV.

Literature survey of melting points of flavonoids revealed some difficulty in using these data for the identification of unknown compounds. For example : 7,3',4'-trihydroxyflavone, two different m.p.'s 331-332°C and 318-322°C were reported by the same group of authors[31,32]



flavone skeleton

UV spectroscopy is particulary applicable to flavones because of the direct conjugation of both the A and B rings to the carbonyl group (see the flavone structure above). The UV spectra of Compound IV are shown in Fig. VII and Fig. VIII and listed in Table V and Table VI along with literature data for 7,3',4'-trihydroxyflavone.

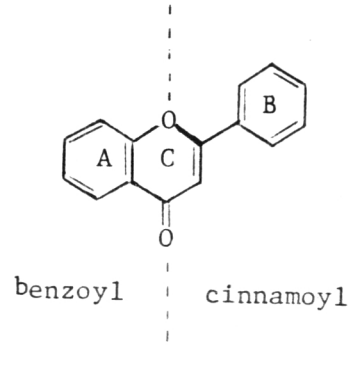
Table V UV spectra of Compound IV in methanol and 7,3',4'-trihydroxyflavone from literature[33]

Solvent	λ max. of Compound IV (nm)	λ max. of the flavone from literature (nm)
MeOH	239, 255 (sh.), 312, 345	235, 250 (sh.) 309, 343
MeOH/NaOMe	259, 318, 340, 408	256,313(sh.),338(sh.)395
MeOH/NaOAc	255, 315 (sh.), 375	255, 310, 373
MeOH/NaOAc/H_3BO_3	259, 308, 372	258, (sh.), 306, 360
MeOH/$AlCl_3$	239, 261 (sh.), 305, 377	234, (sh.) 305, 371, 458
MeOH/$AlCl_3$/HCl	239, 356, 309, 347, 420	235, (sh.), 254 (sh.), 307, 340, 409

Table VI UV spectra of Compound IV in ethanol and 7,3',4'-trihydroxyflavone from literature.[35]

Solvent	λ max of Compound IV (nm)	λ max of the flavone from literature (nm)
EtOH	235, 256(sh.), 314(sh.), 346	237, 255, 314, 344
	346	
EtOH/NaOH	258, 354(sh.), 403	258, 408
EtOH/NaOAc	252, 272(sh.), 310(sh.), 356	254, 267
EtOH/NaOAc/H_3BO_3	258, 310(sh.), 375	370
EtOH/$AlCl_3$	no shift	no shift
EtOH/$AlCl_3$/HCl	no shift	no shift

Generally, the spectra of flavones and flavonoids in methanol exhibit two major absorption peaks in the region 240-400 nm. These two peaks are commonly referred to as Band I (usually 300-380 nm) and Band II (usually 240-280 nm) (see Fig. VII). Band I is considered to be associated with absorption due to the B-ring, cinnamoyl system, and Band II with the absoraption involving the A-ring, benzoyl system.



UV INDICATION OF COMPOUND IV AS A FLAVONE[36]

It had been reported that the position of Band I distinguished between flavones and flavonols as shown in Table VII.[36]

Table VII Band I in the UV spectra of flavones and flavonols.

Flavonoid Type	No. of compounds examined	Range of Band I (nm)
Flavones	50	294 - 352
Flavonols (3-hydroxyl substituted)	26	330 - 359
Flavonols (free-3-hydroxyl)	27	354 - 387

Since the UV spectra of Compound IV in methanol showed its peaks of Band I at 312 and 345 (see Table V) it indicated that Compound IV was likely to be a flavone rather than a flavonol

THE UV SPECTRA IN THE PRESENCE OF NaOMe

Sodium methoxide is a strong base and ionizes to some extent all hydroxyl groups on the flavonoids nucleus. The addition of NaOMe to flavones in MeOH usually produces bathochromic shifts in all absorption band. However, a large bathochromic shift of Band I of about 45-65 nm without a decrease in intensity is diagnostic for the presence of a free 4'-hydroxyl group.

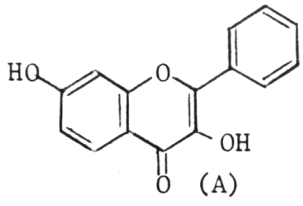
Upon the addition of NaOMe to the methanolic solution of Compound IV a bathochromic shift from 345 to 408 (a difference of 63 nm) was produced (see Table V and Fig. VII), without changes in band intensity, indicating the presence of 4'-OH group in Compound IV.

THE UV SPECTRA IN THE PRESENCE OF NaOAc

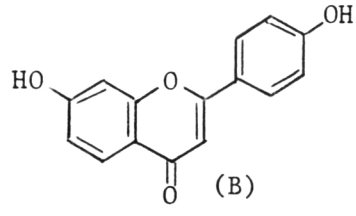
Sodium acetate is a weaker base than NaOMe and, as such, ionizes only the more acidic hydroxyl groups in flavones, i.e., the 3-, 7- and 4'-hydroxyl groups. Because ionization of the 7-hydroxyl group mainly effects Band II (whereas ionization of the 3- and/or 4'-hydroxyl groups mainly affects Band I), NaOAc is a particularly useful diagnostic reagent for the specific detection of 7-hydroxyl groups.

According to Table V and Fig. VII, the presence of NaOAc in Compound IV did effect the UV absorption in both Band I (from 239 to 255, a difference of 16 nm) and Band II (from 345 to 375, a difference of 30 nm). The bathochromic shift in Band II indicates the presence of 7-OH. The presence of either one or both, 3-OH and 4'-OH can be implicated from the changes in position and intensity of Band I.

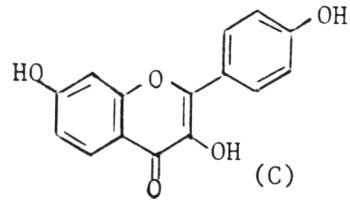
On the basis of the present UV data for Compound IV, three possible structures (A), (B), and (C) have to be taken onto account.



(A)



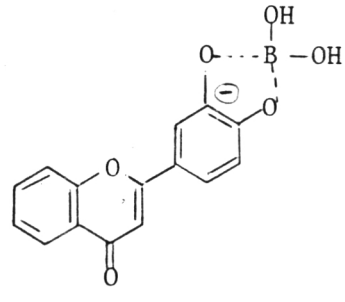
(B)



(C)

THE UV SPECTRA IN THE PRESENCE OF NaOAc and H_3BO_3

In the presence of NaOAc, boric acid will chelate with ortho-dihydroxyl groups at all location on the flavonoid nucleus. Such complexes are probably of the type below.



Generally, flavones containing a B-ring ortho-dihydroxy group show a consistent 12-30 nm bathochromic shift of Band I in the presence of $NaOAc/H_3BO_3$.[36]

Compound IV when mixed with NaOAc and H_3BO_3 exhibited a bathochromic shifts in Band I from 345 to 372 which had a difference of 27 nm (see Table V and Fig. VII) indicating the presence of an ortho-dihydroxyl group. This evidence proved that structure (A) could be eliminated due to its lacking of 4'-hydroxyl

to form an ortho-dihydroxyl group, and that structures (B) and (C) should be added another OH group in ring B to form an ortho-dihydroxyl functional group.

THE UV SPECTRA IN THE PRESENCE OF $AlCl_3$ AND $AlCl_3$/HCl

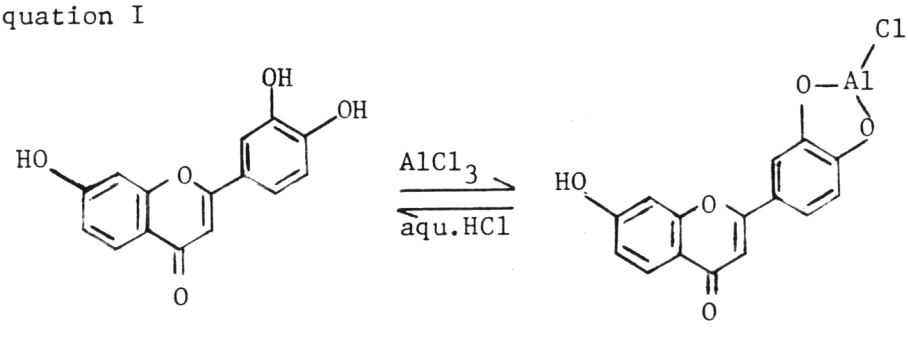
Aluminium chloride generally forms acid labile complexes with flavanoids containing ortho-dihydroxyl system. The structure of these complexes are probably of the types shown in scheme IV on next page. The complexes formed by the reaction of $AlCl_3$ and the ortho-dihydroxyl groups in the A-and B-rings will decompose in the presence of acid. In contrast, the $AlCl_3$ complex between the C-4 keto function and either the 3-or 5-dihydroxyl group is stable in the presence of acid. These phenomena can be illustrated as shown in Scheme IV on next page.

Following to the experimental results in Table V and Fig. VII, the UV spectrum of Compound IV in MeOH on the addition of $AlCl_3$ showed a bathochromic shift of Band I for 32 nm. This shift disappeared when HCl was added, Band I was found at the same wavelength as observed in the absence of diagnostic reagents. This result indicates the decomposition of a corresponding complex and consequently confirms the presence of 3',4'-dihydroxyl groups in Compound IV.

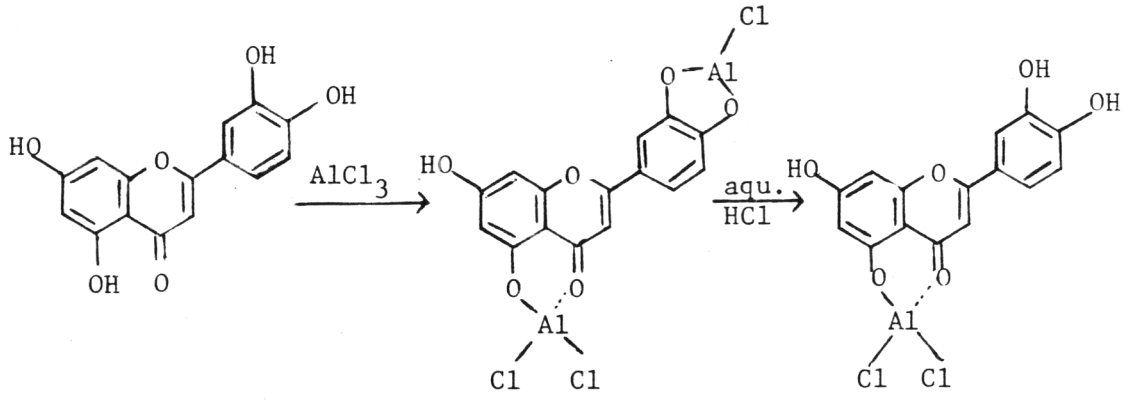
The ensemble of UV data of Compound IV and their comparison with UV spectra of 3',4',7-trihydroxyflavone[31,32,33] (Fig VII, VIII)

Scheme IV $AlCl_3$ complexes with certain flavones and flavonols in the presence or absence of an acid.(36)

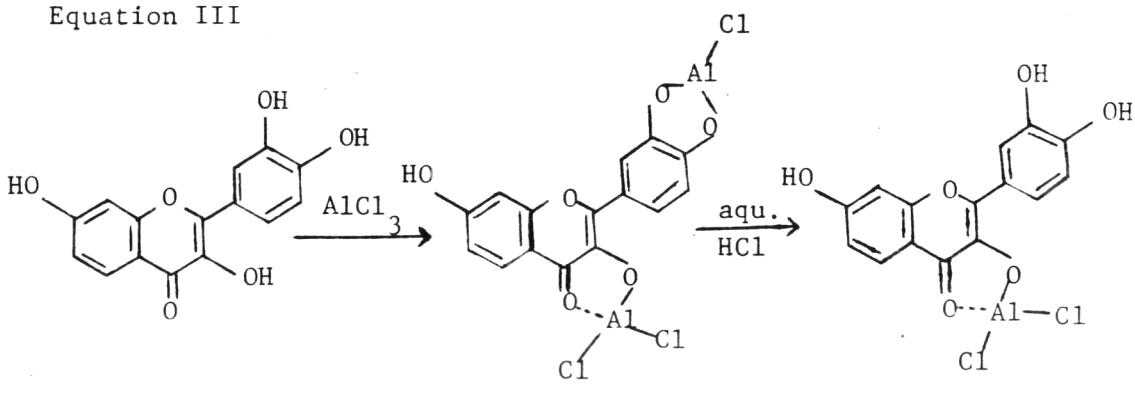
Equation I



Equation II



Equation III



^{1}H NMR spectrum of Compound IV in dimethylsulfoxide is shown in Fig. IX. The values of the chemical shifts, splitting pattern, coupling constants and spectral assignments are listed in Table VIII. The observed splitting pattern is illustrated in Fig. X.

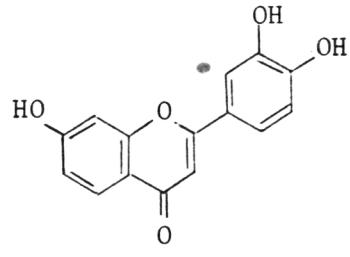
Table VIII ^{1}H NMR data of Compound IV.

Chemical shifts (δ, ppm)	Splitting pattern	Coupling constants J (H_3)	Assignments
			ring A
7.95	d	8	H_5
7.02	d	2	H_8
6.95	dd	2,8	H_6
			ring B
6.95	d	6	$H_{5'}$
7.45	dd	2,6	$H_{6'}$
7.47	d	2	$H_{2'}$
			ring C
6.67	s	-	H_3

The signal of H-6 appears as an exceptionally high sharp peak due to overlapping with signals of protons H-6 and H-5'

The sharp singlet at the highest field at 6.67 can be assigned to H-3 indicating the absence of hydroxyl group at C-3.

^{1}H NMR data of Compound IV are consistent with the proposed structure.



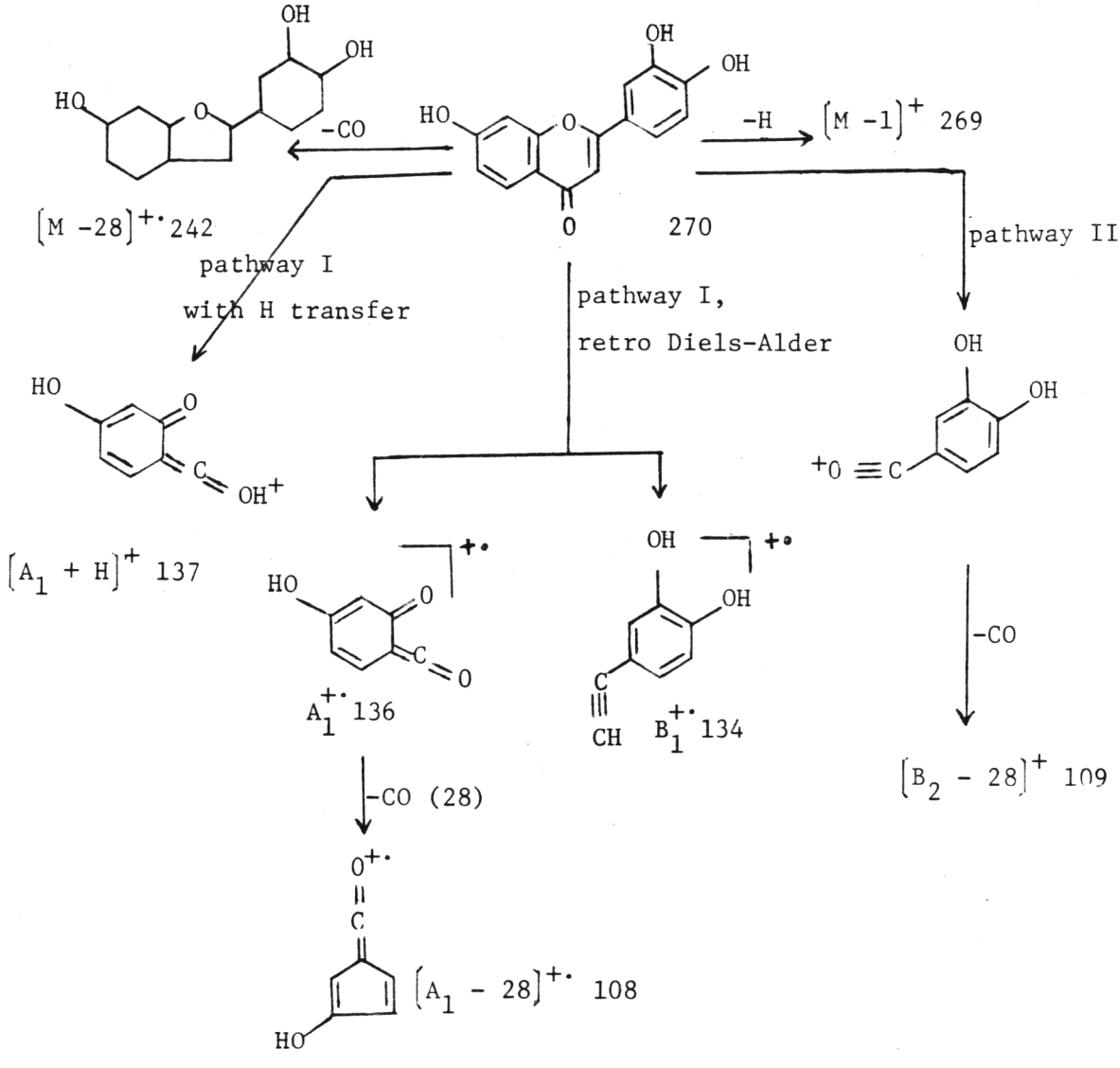
^{13}C-NMR spectrum of Compound IV in d_6-DMSO and the spectral assignments according to literatures[37, 38] were shown in Fig. XI.

The mass spectrum of Compound IV (Fig. XII) shows the highest intensity (base peak) of molecular ion peak at m/e 270. The other peaks represent m/e 242 (M^+-CO), 137 (RDA+H, A ring$^+$), 134 (B ring$^+$), 108 (A ring$^+$-CO), 28 (CO^+). The mass fragmentation patterns of Compound IV are in perfect agreement with the assigned structure. The principal ion fragments are summerized in Scheme V[36] on next page.

In conclusion, the structural elucidation clearly proved that Compound IV had the structure of 7,3',4'-trihydroxyflavone.

To the best of our knowledge, this is the first example of 7,3',4'-trihydroxyflavone found in the genus of *Albizzia*. However, this compound has been found in other genus and species such as *Ladino clover*[31] *(Trifolium repens)*, Alfalfa,[32] and *Baptisia lecontei*.[34]

Scheme V Mass fragmentation patterns of Compound IV

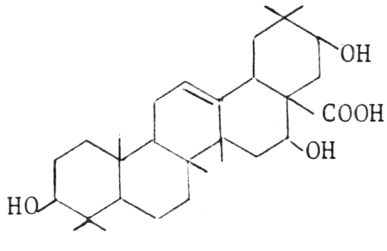


STRUCTURAL ELUCIDATION OF COMPOUND V

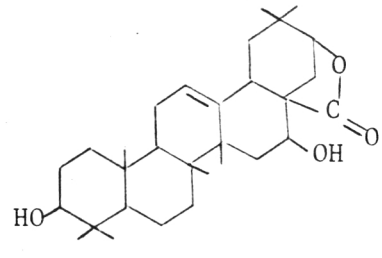
Compound V was isolated from a fraction of the butanolic extract (see Scheme III page 15) designated as Liquid III. Acidic hydrolysis of Liquid III and subsequent column chromatography over silica gel yielded 13 mg of Compound V, m.p. 252-254°C.

Compound V was identified by means of mass spectrum. Fortunately, the mass spectrum of Compound V appears to be superimposable with that of acacic acid lactones.(39) As a matter of fact the lactone structure found in this case was caused by an antifact due to the hydrolysis of the original saponin.(17, 21, 22, 39, 40)

Acacic acid and its lactone have the following structures:



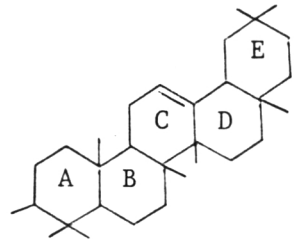
acacic acid



acacic acid lactone

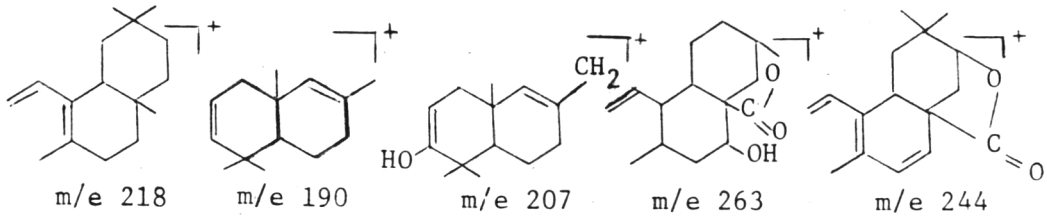
The mass spectrum of Compound V (Fig. XII) showes the molecular ion peaks M^+ at 470 which is in definite agreement with molecular formula $C_{30}H_{46}O_4$. There is a peak at m/e 452 arising out by the loss of one molecule of water. The triterpenoid $^{12}\Delta$-compounds as shown below undergo predominantly

retro Diels-Alder reaction[39] leading to fragments of m/e 218 and m/e 190 (base peak) while the rings A and B lead to the



fragment of m/e 207.

Thus the peaks at m/e 263 and 244 are due to the fragments which are derived from a fragment of m/e 218. Another peak at 175 can be attributed by a substraction of one of methyl groups from the m/e 190 fragment. The principal ions resulted from the fragmentation are shown as follows.[39]



m/e 218 m/e 190 m/e 207 m/e 263 m/e 244

TLC of Liquid III on $CHCl_3$-MeOH-7% HOAC at the ratio of 25:8:5 v./v. as a solvent system revealed the presence at least of 15 components. However, due to the limited amount of Liquid III (100 g of the crude extract), only one compound was isolated successfully by column chromatography over silica gel. Its

acidic hydrolysis produced acacic acid and three sugars, namely glucose, rhamnose and fucose which were identified by comparison with authentic samples on TLC. Thus the isolated compound was identified as a saponin. No attempts were made to locate the positions of the sugar moieties on the saponin molecule. The results of pharmacological screening indicated that the isolated saponin of Compound V showed strong uterotonic activity at a minimum effective concentration (MEC) on the range of 10^{-4}-10^{-5} mol. However, when the saponin was hydrolyzed to its genin, acacic acid, the uterotonic activity was eliminatied. These results indicate the critical importance of the sugar moieties in the saponin molecule on its antifertility activity.

As a matter of fact a flavonoid was isolated from the Liquid III by column chromatography on Sephadex LH-20. Although a complete structural elucidation was not possible to be done due to very limited amount of the compound, we believe that the isolated flavonoid might be rutin. Further chemical and pharmacological studies are needed to complete the analysis of Liquid III which appears to be a very complex mixture according to TLC examination.