

## CHAPTER III

### DISCUSSION

Within the scope of a phytochemical investigation of the native herb *Erycibe subspicata* Wall.(Convolvulaceae) attention had been paid to the main composition of this plant, which had been obtained by extraction and isolation of the stem part, using a method described in the experimental section.

The separation process of the individual substance was based on the use of adsorption chromatographic method and gel filtration. The adsorbent for the gel filtration used was the Sephadex LH-20.

There are two main types of Sephadex gel being used in the field of phytochemistry. Those are Sephadex gel G-series and LH-20. Sephadex gel G-series designed to separate mixtures predominantly on the basis of molecular size (when aqueous solvents are used), the larger molecules being eluted first. G-series sephadex gels behave chromatographically like cellulose but with higher capacity due to regular particle size. Sephadex LH-20 is designed specifically for use with organic solvents, and operates in both modes. Since it produces residue-free eluent, LH-20 is ideally suited to final clean-up of the natural compounds which are not easily clean-up by any other chemical means (Markham, K.R., 1982).

The compound ES-1 was identified as the known simple

coumarin called scopoletin which had been isolated from various plant sources as listed in Table III

Table III  
The occurrence of scopoletin in plants

| Plant                              | Family         | reference      |
|------------------------------------|----------------|----------------|
| <i>Nerium odorum</i> (lvs)         | Apocynaceae    | Gibbs, 1974    |
| <i>Convolvulus</i> spp.            | Convolvulaceae | Gibbs, 1974    |
| <i>Ipomoea</i> spp.                | Convolvulaceae | Gibbs, 1974    |
| <i>Weigela floribunda</i>          | Caprifoliaceae | Gibbs, 1974    |
| <i>W. florida</i>                  | Caprifoliaceae | Gibbs, 1974    |
| <i>Artemisia santolinifolia</i>    | Compositae     | Banerjee, 1985 |
| <i>Succisa pratensis</i>           | Dispacaceae    | Gibbs, 1974    |
| <i>Diospyros maritima</i> (bk)     | Ebenaceae      | Gibbs, 1974    |
| <i>Avena sativa</i> (rt)           | Gramineae      | Gibbs, 1974    |
| <i>Baptisia lecontei</i> (lvs)     | Leguminosae    | Gibbs, 1974    |
| <i>Gelsemium sempervirens</i> (rt) | Loganiaceae    | Gibbs, 1974    |
| <i>Dendrobium thyrsiflorum</i>     | Orchidaceae    | Gibbs, 1974    |
| <i>Prunus serotina</i> (bk)        | Rosaceae       | Gibbs, 1974    |
| <i>Casimiroa edulis</i> (bk)       | Rutaceae       | Gibbs, 1974    |
| <i>Skimmia laureola</i> (bk)       | Rutaceae       | Gibbs, 1974    |
| <i>Amyris liguna</i> (lvs)         | Rutaceae       | Laguna, 1985   |
| <i>Atropa belladonna</i>           | Solanaceae     | Gibbs, 1974    |
| <i>Fabiana</i> spp.                | Solanaceae     | Gibbs, 1974    |
| <i>Mandragora</i> spp.             | Solanaceae     | Gibbs, 1974    |
| <i>Nicotiana</i> spp.              | Solanaceae     | Gibbs, 1974    |

| Plant                           | Family         | reference     |
|---------------------------------|----------------|---------------|
| <i>Physochlaina physaloides</i> | Solanaceae     | Daandai, 1988 |
| <i>Scopolia spp.</i>            | Solanaceae     | Gibbs, 1974   |
| <i>Zygophyllum fabago</i>       | Zygophyllaceae | Gibbs, 1974   |

(lvs = leaves, bk = bark, rt = root)

The origin of the ES-1's major peaks in the mass spectrum can be rationalised through sequential eliminations of methyl radicals and carbon monoxide molecules. Carbon monoxide is a highly stable neutral particle, therefore, the most abundant ion in the mass spectrum of coumarin is formed by the loss of carbon monoxide from the molecular ion.

The mass spectral study of scopoletin (see fig. I, page 36) revealed the molecular ion at  $m/z$  192 and the base peak also at  $m/z$  192 m.u.

The peak at  $m/z$  164 was arised by expulsion of carbon monoxide from the lactone carbonyl group rather than from the phenolic substituent. The peak at  $m/z$  177 corresponded to the emission of a methyl radical to provide the conjugated oxonium ion. The peak at  $m/z$  149 could be described by two path ways. Firstly, it came from the expulsion of carbon monoxide, which is highly stable neutral particle. Secondly, from the loss of a methyl radical to form another conjugated oxonium ion. The peak at  $m/z$  121 corresponded to the loss of another carbon monoxide from the carbonyl group of oxonium ion.

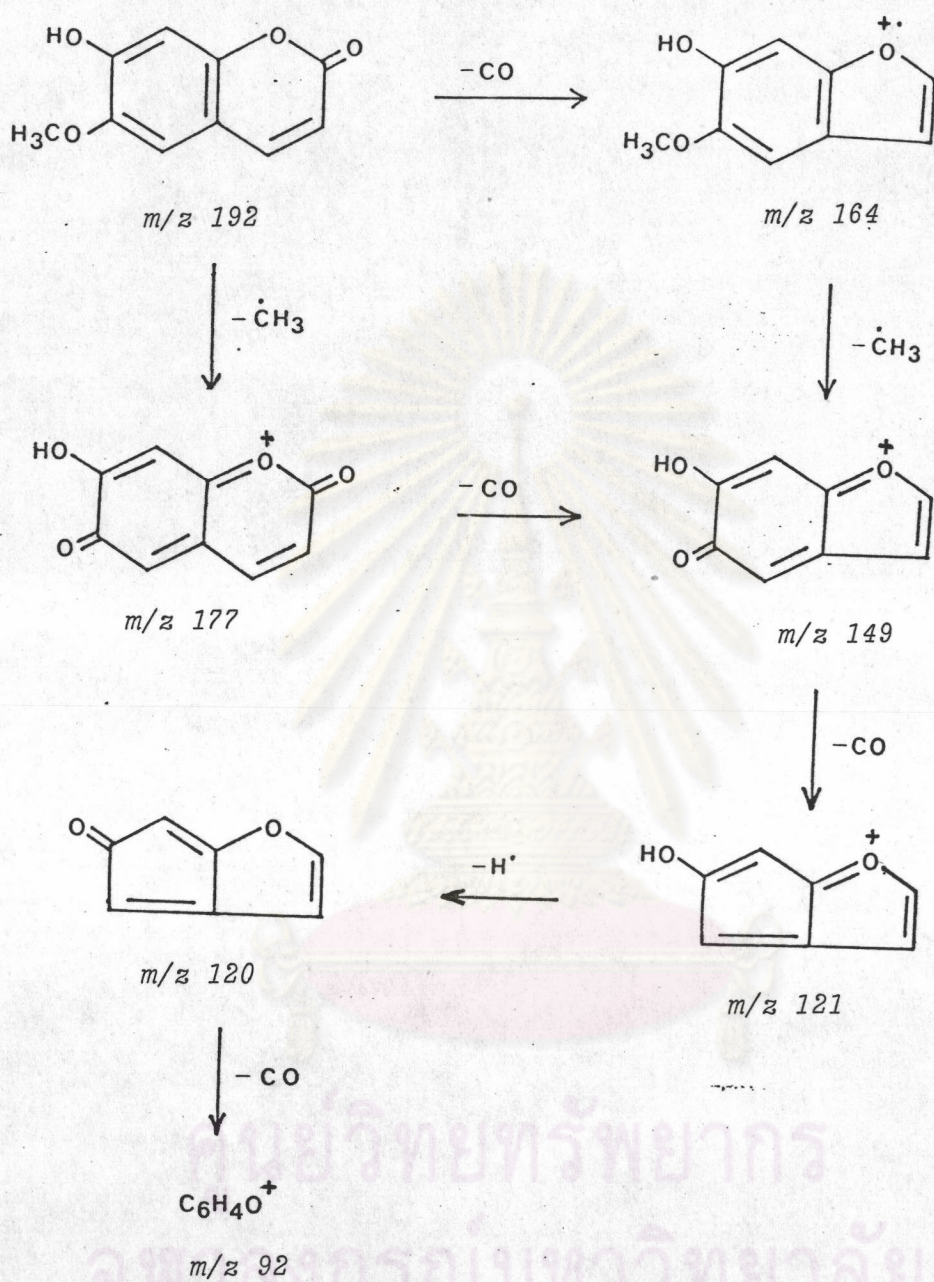


Figure I

Mass fragmentaion pattern of ES-1



Finally, the latter ion decomposed further by consecutive loss of one hydrogen and carbon monoxide to give the  $m/z$  92 ion.

The IR spectrum of ES-1 (see fig.7, page 77) in KBr disc showed a vibration of free OH stretching at  $3600\text{ cm}^{-1}$  and a larger hydrogen bonded peak at  $3345\text{ cm}^{-1}$ . The C-H asymmetric and symmetric stretch occurred at  $3040\text{ cm}^{-1}$  and  $3000\text{ cm}^{-1}$  indicated that the aromatic is presented. An absorption at  $1720\text{ cm}^{-1}$  could be assigned to a lactone carbonyl group. Further, the absorption at  $760$  and  $729\text{ cm}^{-1}$  are characteristic of the C-H out-of-plane bending vibrations of a substituted side phenyl ring. The absorptions at  $862$  and  $820\text{ cm}^{-1}$  are due to the C-H out-of-plane bending vibrations of the pyrone ring.

Its  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 90 MHz, Table V page 42, Fig. 5, page 75) revealed a singlet due to an aromatic methoxy group at  $\delta$  3.989. A pair of doublets ( $\delta$  6.264 and  $\delta$  7.646  $J=9.5\text{ Hz}$ ) revealed the *cis* protons coupling of the H-3 and H-4 of the unsubstituted pyrone ring. This feature is taken as a firm indication of the presence of a coumarin nucleus (Stack and Mazurck, 1972). A pair of singlets ( $\delta$  6.952 and  $\delta$  6.848) corresponded to *para*-positioned aromatic protons for H-5 and H-8, respectively. The positions of the H-5 and H-8 of this compound were assigned by comparisons of the chemical shifts of these protons with those of several other compounds of defined structures. The spectrum also showed a singlet at 6.182 ppm assignable to a phenolic hy-

droxy group.

The carbon-shift determination of the ES-1, portrayed in table VI, p.43, was found on an analysis of ES-1 aided by the values of carbons with those of other coumarins of previously established structures.

The  $^{13}\text{C}$  spectrum is given in Fig.9, page 79 and revealed a lactone carbonyl peak at 161.2 ppm (C-2), four carbons, each of which contained one attached proton, four quaternary carbon, and, finally, one methyl of the methoxyl group at 56.868 ppm. One of the carbon signal in the benzene region was deshielded (152 ppm) and therefore presumably attached to oxygen. This signal was designated to carbon position 8A. Carbon 5A was unique, while C-3 and C-4 were expectedly the lowest and highest field methines, respectively. C-3 of the coumarin skeleton had a strong shielding effect from the -OH group at C-7. This charge transmission onto a vinylogous para carbon in a neighbouring ring can be noted also in other aromatic bicyclic compounds. Thus, the C-3 (111.768 ppm) appeared at the higher field than C-4 (144.281 ppm).

The benzenoid methines (C-5, C-6, C-7, C-8) were differentiated by the fingerprint method. The high field position of C-8 ( $\delta$  99.976) indicated it to be associated with a carbon ortho to an oxy substituent (Wenkert et al, 1978). This phenomenon would cause the electron crowd at the C-8, hence, more shielding effect is observed, i.e., value

of C-8 appeared at the higher field.

The compound ES-2 was identified as the known simple coumarin glycoside called scopolin which had been isolated from various plant sources as listed in Table IV.

Table IV  
The occurrence of scopolin in plants

| Plant                           | Family         | Reference     |
|---------------------------------|----------------|---------------|
| <i>Nerium odorum</i>            | Apocynaceae    | Gibbs, 1974   |
| <i>Convolvulus spp.</i>         | Convolvulaceae | Gibbs, 1974   |
| <i>Ipomoea spp.</i>             | Convolvulaceae | Gibbs, 1974   |
| <i>Artemisia spp.</i>           | Compositae     | Gibbs, 1974   |
| <i>Baptisia lecontei</i> (lvs)  | Leguminosae    | Gibbs, 1974   |
| <i>Gelsemium sempervirens</i>   | Loganiaceae    | Gibbs, 1974   |
| <i>Murraya exotica</i> (fl.)    | Rutaceae       | Gibbs, 1974   |
| <i>Atropa spp.</i>              | Solanaceae     | Gibbs, 1974   |
| <i>Mandragora spp.</i>          | Solanaceae     | Gibbs, 1974   |
| <i>Physochlaina physaloides</i> | Solanaceae     | Daandai, 1988 |
| <i>Scopolia spp.</i>            | Solanaceae     | Gibbs, 1974   |

(lvs = leaves, fl. = flower)

The mass spectral study of ES-2 showed the molecular ion at  $m/z$  354 and the base peak at  $m/z$  192 which corresponded to the loss of a glucosyl group. The other peaks at  $m/z$  177, 164, 149 and 121 corresponded to the formation of fragments by the mechanism suggested for the scopoletin

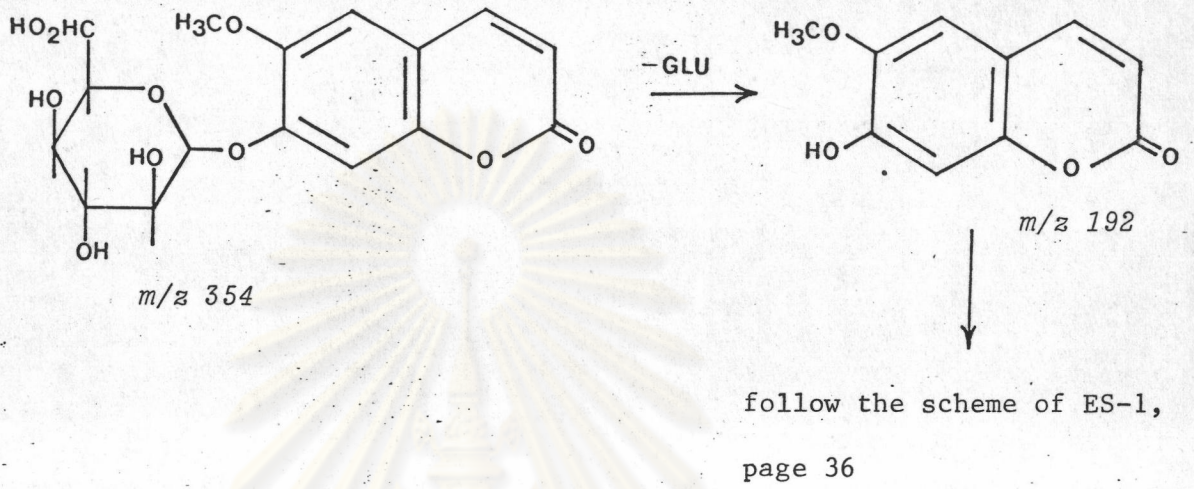


Figure II

Mass fragmentation pattern of ES-2

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(ES-1 compound) (see Fig.II page 40).

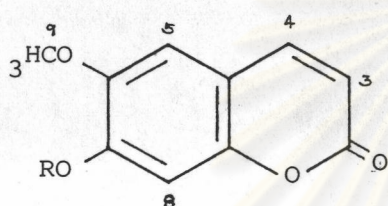
Both the  $^1\text{H}$  - NMR and  $^{13}\text{C}$  - NMR spectra of ES-2 were quite similar to that of ES-1, except for the signals arising from the glycoside group in ES-2 ( See table V, VI, page 42,43).

Compound ES-2 gave on hydrolysis an aglycone  $\text{C}_{10}\text{H}_8\text{O}_3$  m.p.200°C (Scopoletin) and glucose. Conventional acetylation of ES-2 showed the proton decoupled and off-resonance  $^{13}\text{C}$  spectra in Fig.14, page 84 . The quartet at  $\delta$  20.586 ppm revealed the signal for methyl of acetylated group. The rest of signals shown were similar to those of the scopoletin, apart from the signal at C-7 which appeared at 142.749 ppm, on the acetylated  $^{13}\text{C}$  spectrum, and at 146.542 ppm, on the  $^{13}\text{C}$  spectrum of ES-2. This markedly difference ( $\delta$  146.542 - 142.749 = 3.793 ppm) meant that the acetylated group had displaced the O-glucosidic or OH linkage at C-7 of the aglycone. The nature of intersugar glycosidic linkage was deduced to be the  $\beta$ -glucosidic linkage by comparison of the  $^1\text{H}$  - NMR spectra ( $\delta$  5.02 - 4.82 ppm) and the coupling constant ( $J=19.68$  Hz) of several other compounds of defined structure whose signal patterns of the sugar moiety were identical.

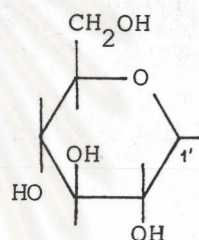
Compounds ES-1 and ES-2 which are apparently the main constituent were identified to be the known called scopoletin and scopolin, respectively. These compounds are distributed limitedly in plant kingdom, mostly found in the

Solanaceae and Convolvulaceae. All the coumarins originate biogenetically from cinnamic acid. The sequence of the biosynthesis and their physiological actions are fully discussed and well documented.

Table V  $^1\text{H}$  - chemical shift ( $\delta$ ) of ES-1, ES-2

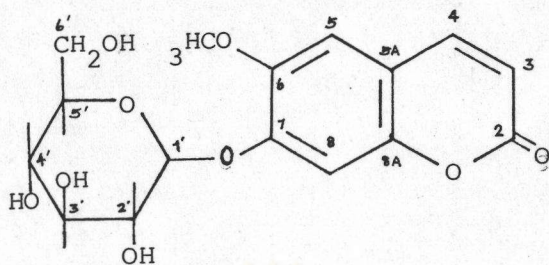


H = R; ES-1



= R; ES-2

| Proton position | Chemical shift |             |
|-----------------|----------------|-------------|
|                 | ES-1           | ES-2        |
| H-3             | 6.264          | 6.300       |
| H-4             | 7.646          | 7.806       |
| H-5             | 6.952          | 7.111       |
| H-8             | 6.848          | 7.029       |
| H-9             | 3.989          | 3.879       |
| H-1'            | -              | 4.900       |
| H-2' - H-6'     | -              | 4.0 - 2.558 |
| OH              | 6.182          |             |

Table VI  $^{13}\text{C}$ -Chemical shift ( $\delta$ ) of ES-1, ES-2

| Carbon position | Chemical shift |         |
|-----------------|----------------|---------|
|                 | ES-1           | ES-2    |
| C-2             | 161.2          | 160.789 |
| C-3             | 111.768        | 112.740 |
| C-4             | 144.281        | 143.562 |
| C-5             | 113.890        | 113.712 |
| C-6             | 149.5          | 149.250 |
| C-7             | 144.288        | 146.542 |
| C-8             | 99.976         | 104.725 |
| C-9             | 56.868         | 56.395  |
| C-5A            | 112.187        | 109.540 |
| C-8A            | 152.00         | 150.225 |
| C-1'            | -              | 100.872 |
| C-2'            | -              | 75.194  |
| C-3'            | -              | 77.000  |
| C-4'            | -              | 69.993  |
| C-5'            | -              | 78.065  |
| C-6'            | -              | 61.325  |



## Biosynthesis of Simple Coumarins

Coumarins are lactones of phenylpropanoid acid, a class which derives from the shikimic acid pathway via the protoaromatic amino acids, phenylalanine and in a few plants, tyrosine. The entire carbon skeleton of the simple coumarins, i.e. those having no rings additional to the benzopyrone nucleus, is phenylalanine derivative. In the number of early biosynthesis experiments on these compounds in plants it was shown that shikimic acid, phenylalanine and *trans*-cinnamic acid are common precursors of this benzopyrone nucleus. Cinnamic acid, however, represents a branch point in the elaboration of coumarin itself and probably other coumarins lacking 7-oxygenation on one hand and the 7-hydroxy coumarins based on umbelliferone on the other the latter group constituting the vast majority of the class. *Ortho*-hydroxylation of *trans*-cinnamic acid leads to coumarin itself, via a light-catalysed *trans-cis* isomerization, and lactone ring formation which can be formally represented as a dehydration (Edwards and Stoker, 1967). The enzyme responsible, which is membranebound, has been obtained from chloroplast. The mechanism involved is not yet known (Goodwin and Mercer, 1983). *Para*-hydroxylation of *trans*-cinnamic acid is a necessary prerequisite for synthesis of the 7-hydroxycoumarins, via *ortho*-hydroxylation and lactonization as before. The *ortho*-hydroxylations of cinnamic and *para*-hydroxycinnamic acids are mediated by different enzymes

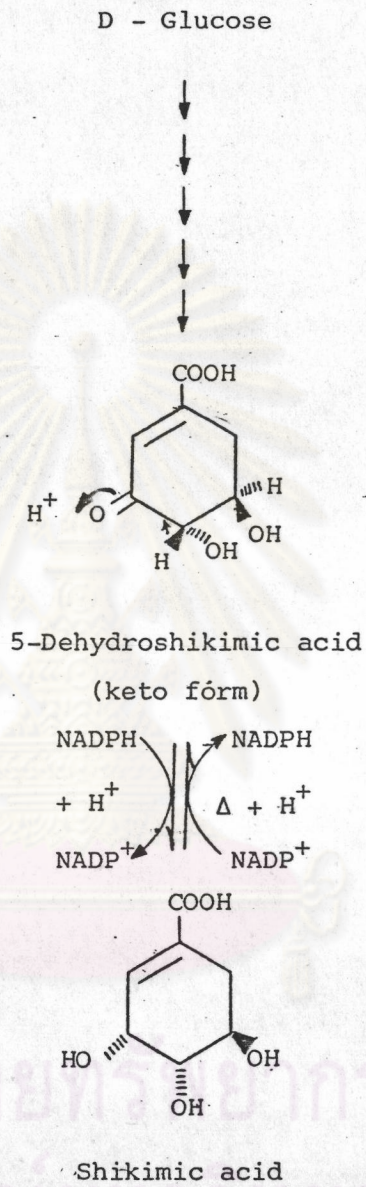


Figure III: Formation of shikimic acid (Goodwin and Mercer, 1983)

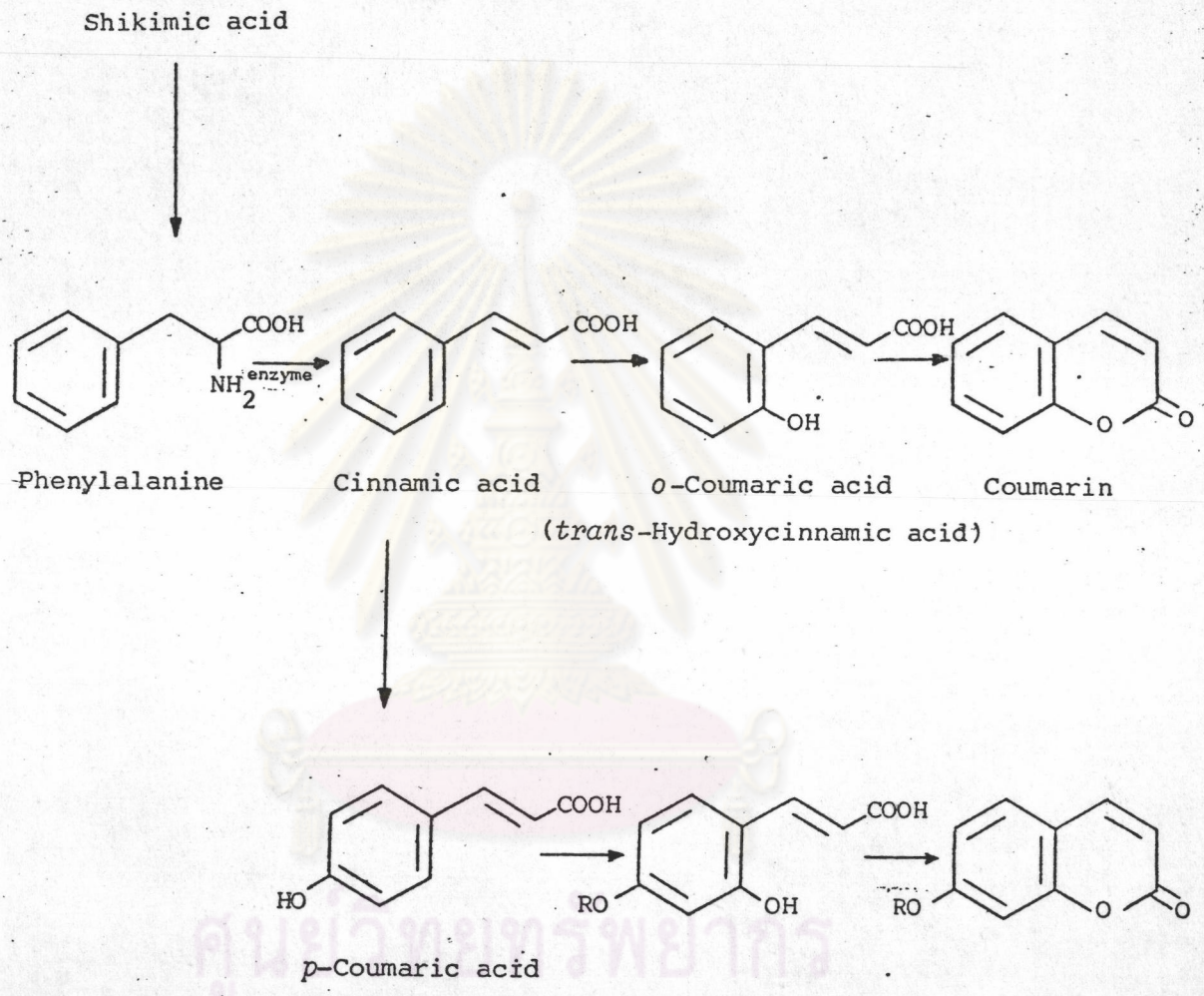


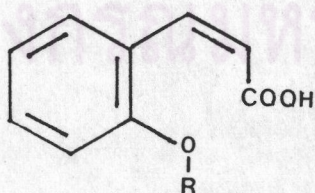
Figure IV: Pathways from *trans*-cinnamic acid to coumarins

(Street and Cockburn, 1972) (Brown, 1979)

(Gestetner and Conn, 1974), which are apparently seldom found together in the same species, so that coexistence of coumarin and the umbelliferone is an exceptional phenomenon (Brown, 1979).

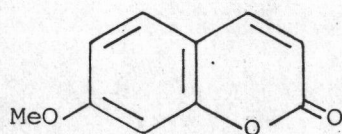
It should be noted that a number of the simple coumarins are artifacts of isolation, and do not occur to any very significant extent in the free form in the intact plant cell. Thus coumarins herniarin, and umbelliferone have all been shown to occur in the form of glycosides of the corresponding *cis*-*o*-hydroxy (I). However, glucosidases are also present which gain excess to these substances upon disruption of the cells, and the liberated aglycones (II), lactonize spontaneously to the free coumarins (Kosuge and Conn, 1961). The pungent odour of coumarin associated with new-mown hay originates in this way (Brown, 1979).

In at least one case in the elaboration of microbial coumarin, novobiocin (III) by *Streptomyces nivosus* Smith, Dietz, Sokolski & Sawage, lactone ring closure is not by dehydration but by an oxidative mechanism (Brown, 1979).

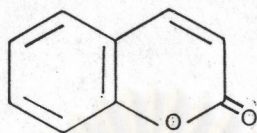


*Cis*-*o*-hydroxycinnamic acid R = glucosyl (I)

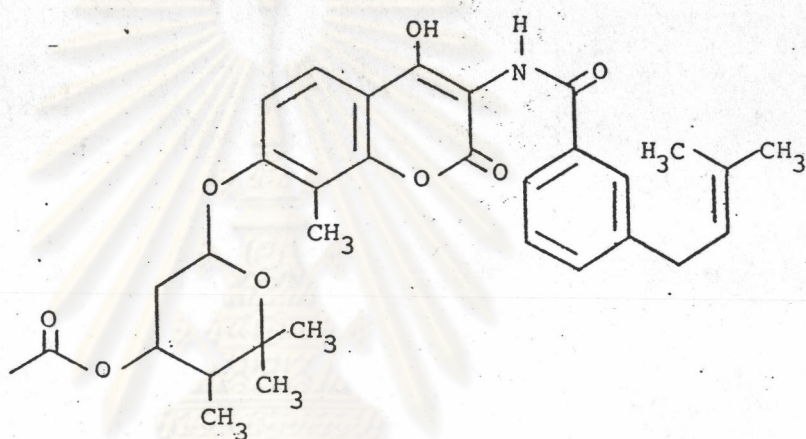
aglycone R = H (II)



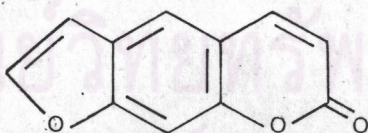
Herniarin (Ia)



Umbelliferone (IIa)



Novobiocin (III)

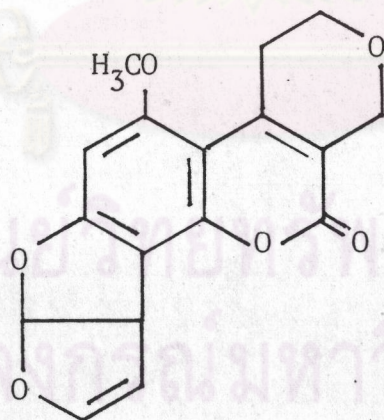


Psoralen (IIIa)



### Physiological Activities of Coumarins

Natural coumarins as a class can not be classified as a pharmacodynamic group, although varied types of activities have been encountered in some of these substances. A more synthetic analogues have been prepared and evaluated for physiological activities found in natural coumarins (Tandon and Rastogi, 1979). The least complex member of the class structurally, known simply as coumarins, is toxic to mammals, another microbial coumarin type is the aflatoxin (IV) elaborated by *Aspergillus flavus* Lk., these metabolites are potent hepatotoxins and are among the most intense carcinogens yet discovered (Ciegler, Detroy & Lillehoj, 1971).



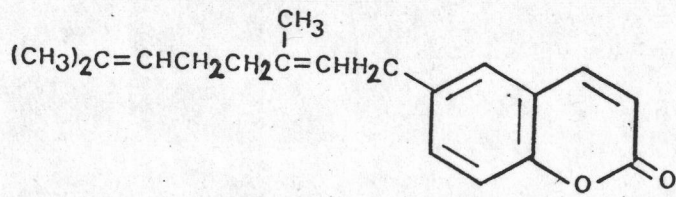
Aflatoxin type (IV)

### 1. Anticarcinogenic activity

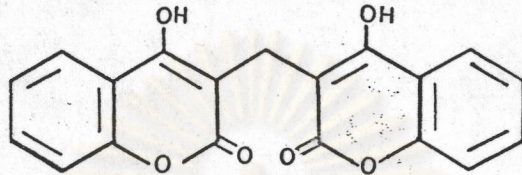
The compounds act as inducers of aryl hydrocarbon hydroxylase (AHH) system and that this increased activity gives protection against neoplasia induced by polycyclic aromatic hydrocarbons (PAH). Further, these inducers of AHH have been found to be present in certain vegetable species and dietary components, some of them have been identified as coumarins, flavones, aromatic isothiocyanates, selenium salts and indoles. Although the mechanism by which these inhibitors of chemical carcinogenesis inhibit PAH-induced neoplasia is not known, the induction of increased AHH activity reflected the formation of phenolic metabolites of PAH. Probably the relative amounts of these metabolites of detoxification, as compared to those leading to the formation of ultimate carcinogenic species, control the response to a particular dose of carcinogen. In these studies, coumarin was found to suppress 7, 12-dimethylbenzanthracene-induced carcinoma in rats (Feuer, Kellen & Kovacs, 1976).

### 2. Antibiotic activity

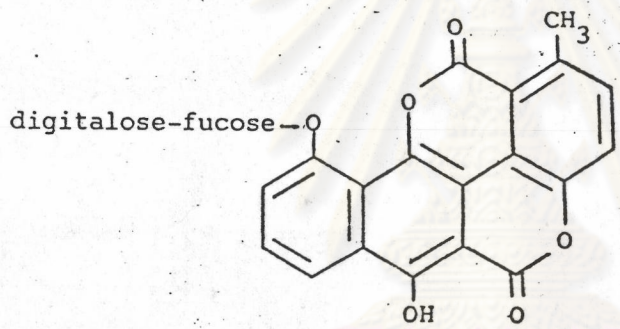
Although coumarin itself has a very low antibacterial activity, other members, such as ammosesinol, ostruthin (V), dicoumarol (VI), novobiocin (III), coumermycin, chartreusin (VII), and athamantin (VIII), exhibited significant activity. Ostruthin and ammosesinol were most active against a wide spectrum of bacteria, e.g. *Staphylococcus aureus* Rosenbach,



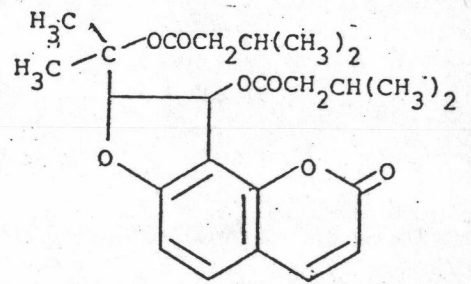
Ostruthin (V)



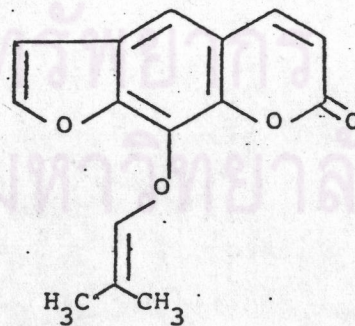
Dicoumarol (VI)



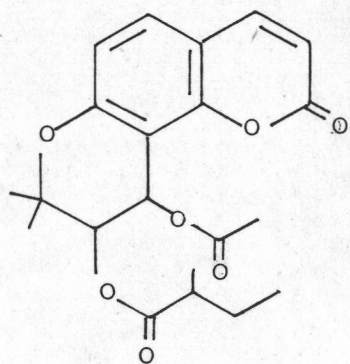
Chartreusin (VII)



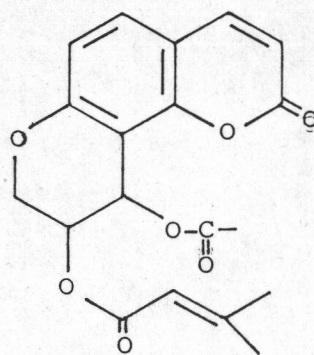
Athamantin (VIII)



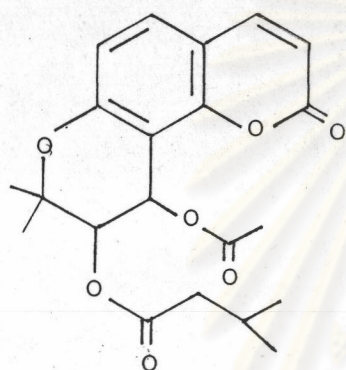
Imperatorin (IX)



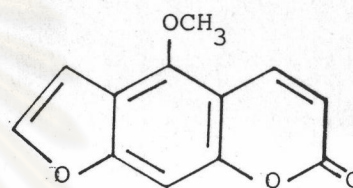
Visnadin (Va)



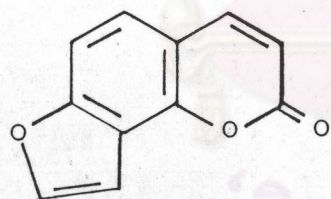
Samidin (VIa)



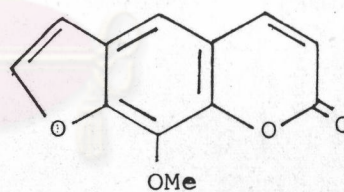
Dihydrosamidin (VIIa)



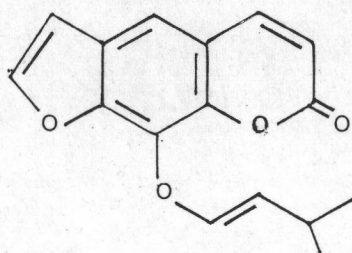
Bergapten (VIIIa)



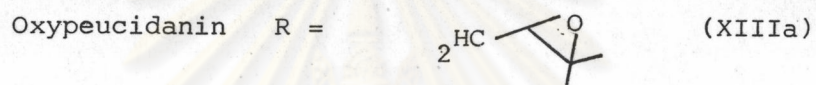
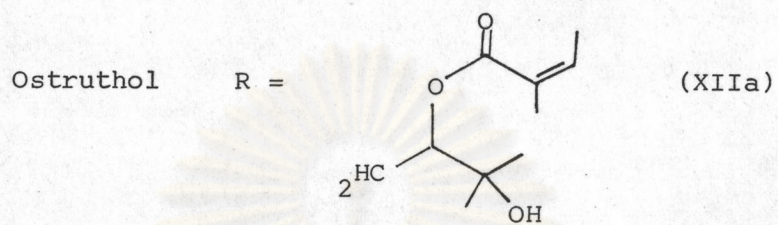
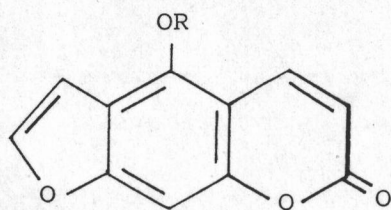
Angelicin (IXa)



Xanthotoxin (Xa)



Imperatorin (XIa)



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*Micrococcus luteus* (Schroeter) Cohn, *Bacillus megatherium* de Bary (Hodak, Jakesova & Dadak, 1967). A number of coumarins have been tested for antifungal activity; and three most effective ones were psoralen (IIIa), imperatorin (IX) and ostruthin (V) (Tandon & Rastogi, 1979).

### 3. Anticoagulant

The coumarin anticoagulants, e.g. dicoumarol (VI), are employed in therapy to depress blood coagulation and the present thrombosis in diseases of coronary artery and related conditions. These compounds acted only *in vivo* by blocking the synthesis of four proteins in the prothrombin complex necessary for the blood coagulation process. The coumarin anticoagulants are absorbed relatively quickly, but their effect is slow and, therefore, acute human toxicity of these compounds was rare, although poisoning may occur in children ingestion large doses (Ingram, 1961).

During fermentation of hay, some microorganisms (Such as *Aspergillus fumigatus* Fres.) transform coumarin into dicoumarol. Such spoiling of hay can be dangerous to animals as dicoumarol is a powerful blood anticoagulant and can cause fatal haemorrhages in cattle that eat it (Manitto, 1981).

### 4. Antispasmodic action

The coumarin pteryxin from *Pteryxia terebinthina* Nutt., and suksdorfin-A from *Lomatium suksdorfii* S. Wats (Willette

& Soine, 1962) were found to exhibit this activity (Tandon & Rastogi, 1979).

#### 5. Vasodilatory action

A number of coumarins have been isolated from Umbelliferae plants which possessed vasodilatory activity. Other active coumarins, are visnadin (Va), samidin (VIa) and dihydrosamidin (VIIa). Visnadin has also been used in therapy for the treatment of angina pectoris under the registered name "Provisimine" (Tandon & Rastogi, 1979).

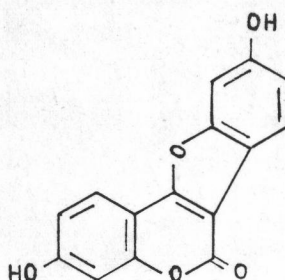
#### 6. Effect on central nervous system

Coumarin itself exerts hypnotic action on frogs, rabbits and mice, but it has not been used therapeutically because of its hepatic toxicity (Tandon & Rastogi, 1979).

#### 7. Oestrogenic activity

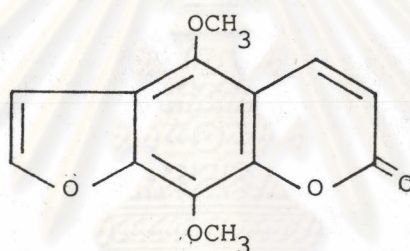
All the coumarins which have been shown to possess this property belong to the group of 3-phenyl coumarins which has been discussed adequately (Krishnaswamy, 1965). It is recognized that the *trans*-stilbene character in these compounds was responsible for oestrogenic properties. Coumestrol (X), one of the most potent compounds occurs in lindo clover and alfalfa (Tandon & Rastogi, 1979).

Coumestrol (X)



### 8. Molluscacidal activity

Some furanocoumarins possess molluscacidal activity. Bergapten (VIIIa) and isopimpinellin (XI) have shown activity similar to those of some of the most potent synthetic compounds used for snail control (Schonberg & Latif, 1954). The application of these coumarins has been considered for the control of reproduction of the snail which is a host animal for the blood fluke responsible for human bilharziasis (Tandon & Rastogi, 1979).

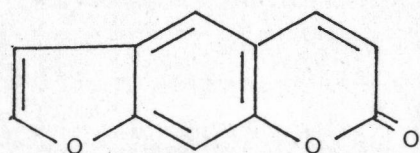


Isopimpinellin (XI)

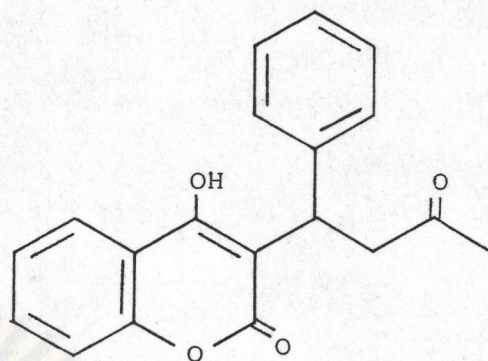
### 9. Miscellaneous activity

Certain plant extracts and juices have been known to increase dermal photosensitivity. Application of these extracts to the skin followed by exposure to sunlight cause erythema and pigmentation. The substances responsible for this action were simple furanocoumarin e.g. psoralen, bergapten, angelicin (IXa), xanthotoxin (Xa), imperatorin (XIa), isopimpinellin (XI), Xanthotoxol (XII), ostruthol, oxypeucedanin (XIIIa) etc. Warfarin (XIII) has been applied as an effective rodenticide.





Xanthotoxol (XII)



Warfarin (XIII)

(Synthetic 4-hydroxycoumarin)

The coumarins of *Mammea americana* Linn. (Crombie, Games, Harkins & Reed, 1972) have been reported to possess insecticidal activity.

Xanthotoxin, a linear furanocoumarin, showed 100% mortality to larvae of armyworm (Berenbaum, 1978).

The medicinal use of *Erycibe subspicata* Wall. is used mainly for the antipyretic in north-east of Thailand. The main compound isolated from this plant is scopoletin and scopolin. These two compounds had been isolated from *Erycibe obtusifolia* Benth in 1984 and the pharmacological actions reported were used for antiinflammatory and analgesia. Zhu et.al reported in 1985 the pharmacological action of the scopoletin which stated that the scopoletin was the prostaglandin synthetase inhibitor and also the antihistamine. The antipyretic action of *Erycibe subspicata* Wall. still need to be further studied by a pharmacologist in order to establish the mechanism which can be used to

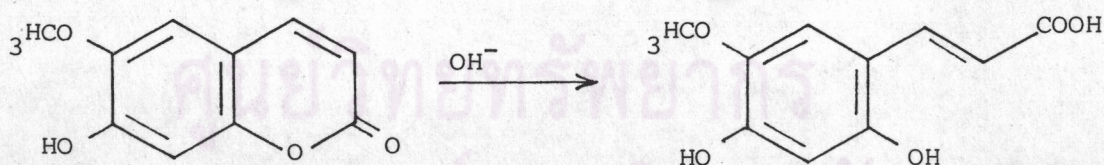


explain the antipyretic action of this plant.

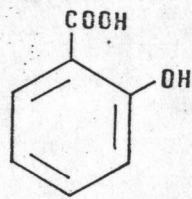
One could make a possible suggestion for the antipyretic action is by using the structure activity relationship of the salicylate group (see Fig.V, page 59).

Salicylates act by virtue of their salicylic acid content. Substitutions on the carboxyl or hydroxyl groups serve only to change the potency or toxicity of the compound. The *ortho* position of the OH is an important feature for the action of salicylate. The efficacy of the different salicylates depends in part on the amount of salicylic anion ( $C_6H_4(OH).COO^-$ ) liberated in the body. (Goodman and Gilman, 1970).

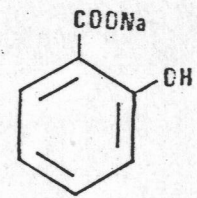
The ring of scopoletin, which is a natural coumarin, in the alkaline environment such as, in the small intestine, is broken to form 2,4-dihydroxy-1,5-methoxy-cinnamic acid.



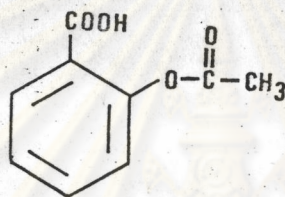
This particular cinnamic acid has all the essential features, the *ortho* position of the OH and the carboxylic acid part, to behave like one of the salicylate family.



Salicylic acid

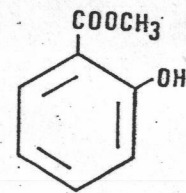


Sodium Salicylate



Aspirin

(Ester of acetic acid)



Methyl Salicylate

Fig. V

Structure of the salicylate group.