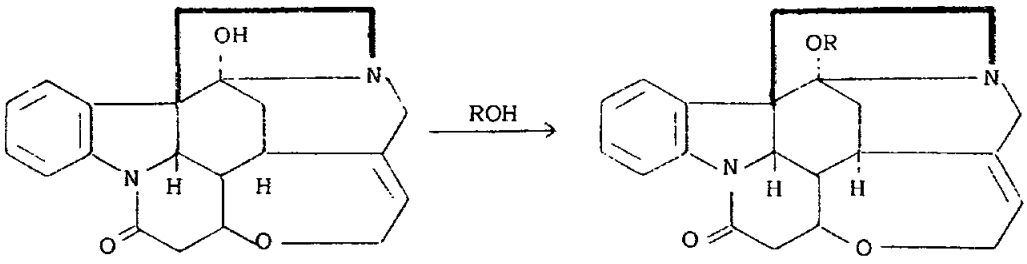
CHAPTER IV

DISCUSSION

Discussion of the Extraction Procedures

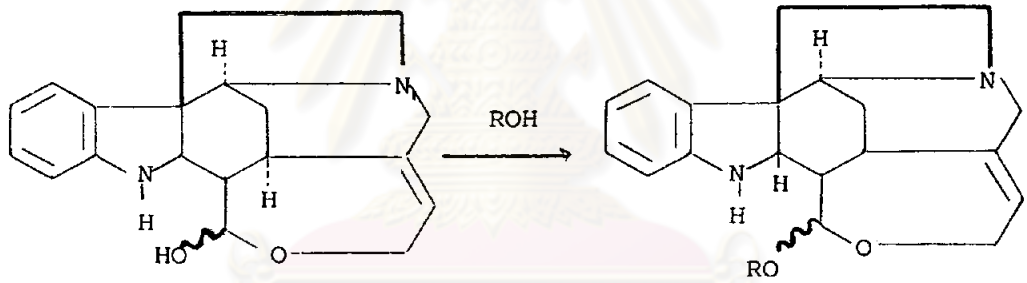
Some anthraquinone glycosides might be hydrolyzed by dilute acid during the extraction to give free aglycones which were considered as artefacts. In the present work, two extraction methods were applied in order to check the artefact formation. The first method was by refluxing with acid ethanol and the other by maceration with ethanol only. Both procedures showed little difference in TLC patterns (Fig. 18-19, pp. 76-77). The acid ethanol extract contained eight aglycones, one of which was lucidin together with three aglycones which were also found in the ethanolic extract. Lucidin (8), a hydrolytic product of lucidin ω-O-β-D-glucoside (Fig. 8, p. 49) which present in large amount in the acid ethanol extract, was not found in the acid-free ethanolic extract. However, ethyl alcohol used in this acid alcohol extraction was also a major cause of an artefact formation. This is because hydrolysis of lucidin ω-O-β-D-glucoside by the acid to give aglycone lucidin which coupled with ethanol to give an ethyl ether of lucidin (Aq-1 or ibericin).

The occurrence of ethyl derivatives of a natural product is very rare and is assumed to be a result of artefact formation. Beke (Beke, 1963) reported the reaction of alcohols, methanol, ethanol, etc. with the carbinolamine moiety of pseudostrychnine (20) as follows :



Pseudostrychnine (20) R=C_2H_5; 3-Ethoxystrychnine (21)

Deyrup *et al.* (Deyrup *et al.*, 1962), have shown that alcohols can also react with Wieland-Gumlich aldehyde (22) in the hemiacetal form to give ether form (23) as follows :



Wieland-Gumlich aldehyde (22) (23)

According to these informations together with the present finding, it is evidence that artefacts may be formed by using alcohols in the process of extraction and isolation of organic compounds from natural products. Research workers must be aware of this fact.

Discussion of Chemical Structures

The present work had led to the isolation of an anthraquinone glycoside Aq-4 and three anthraquinone aglycones, Aq-1, Aq-2 and Aq-3. The isolation process of anthraquinone glycoside Aq-4 was by crystallization from the ethanolic extract, while of anthraquinone aglycones

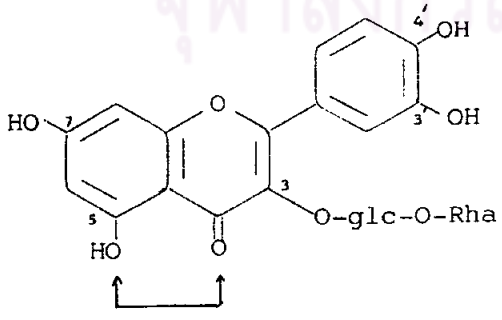
Aq-1, Aq-2 and Aq-3 was based on short column chromatographic technique, which was devised and pioneered by Rigby and Hunt in 1967.

Aq-4

The structure of Aq-4 was elucidated by the ultraviolet, infrared, nuclear magnetic resonance and mass spectroscopy. Acid hydrolysis of Aq-4 gave an aglycone which corresponded chromatographically with the authentic lucidin (supplied by Dr. Yasui Yohko). The sugar part was identified as glucose by using HPLC and by TLC comparing with authentic sugars. (Fig. 20-23, pp. 78-81)

From the above reasons, the elucidation of the structure of Aq-4 would be based on the structure of lucidin.

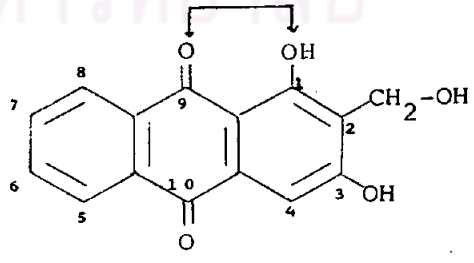
The solution of Aq-4 in methanol exhibited maximum absorptions in ultraviolet and visible region at 315, 341, 348, 400 and 422 nm. The bathochromic shift from 315 to 336 nm when aluminium chloride was added to the methanolic solution of Aq-4 probably was an indication of the 1-hydroxyl or 4-hydroxyl function in the molecule as shown in the structure of lucidin (8).



$AlCl_3$ induced ultraviolet and visible bathochromic shift

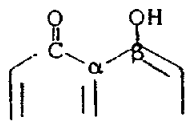
Rutin (24)

$AlCl_3$ induced ultraviolet and visible bathochromic shift



Lucidin (8)

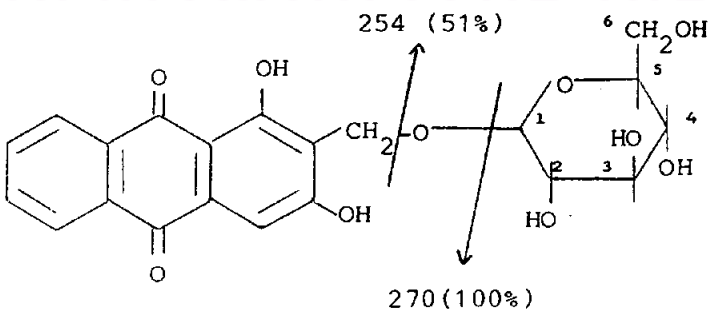
The idea of using the aluminium chloride to induce bathochromic shift when a carboxyl function accompanied by a β-hydroxyl function was adopted from the same situation which arised from a 5-



hydroxy flavonoid such as rutin (24) (Harborne, 1973). The bathochromic shift from 341 to 358 nm when aluminium chloride and sodium acetate were added to the methanolic solution of Aq-4, was also an indication of the 3-hydroxyl in the molecule. So the sugar of this glycoside could only be attached at the remaining C-2 position in the form of glucosidic linkage ($R-CH_2O$-glucose) where R was anthraquinone part.

The IR spectrum of Aq-4 showed OH-stretching at ν_{max} 3380 cm^{-1} and the carboxyl stretching at ν_{max} 1680 cm^{-1} and ν_{max} 1620 cm^{-1}. The band at 1280, 1080 and 1040 cm^{-1} indicated the presence of ether linkage.

The mass spectrum of anthraquinone glycoside Aq-4 showed the base peak at *m/z* 270. The molecular ion peak was not observed which is typical for many glycosides. However, the base peak ion showed the presence of lucidin (*m/z* 270). The fragmentation of Aq-4 is recognized as the major ions recorded in the mass spectrum as follows: (Fig. 6)



m/z 448 lucidin

Fig. 6 The fragmentation of Aq-4

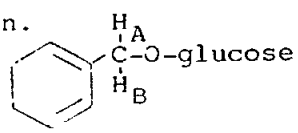
Since glucose and lucidin were obtained from the acid hydrolysis, the true molecular weight of Aq-4 should be 448 amu.

The structure of Aq-4 was established chiefly by the assignment of the 270 MHz proton NMR spectrum. There were five aromatic protons observed in the aromatic region of proton NMR spectrum of Aq-4. This also indicated that the remaining three aromatic proton of Aq-4 were substituted.

The two carboxyl function at C-9, C-10 would induced more downfield chemical shift of C-5 and C-8 than the C-6 and C-7 protons because of the lesser distance of C-5 and C-8 protons from the carbonyl function. Two protons of C-5 and C-8 were deshield and appeared downfield as multiplets at δ 8.23 - 8.26 ppm while other two protons (C_6-H and C_7-H) appeared as multiplets at δ 7.93 - 7.96 ppm.

About nine sugar protons were observed in the proton NMR spectrum ranging from δ 2.6 ppm to δ 5.2 ppm. A proton doublets ($\underline{J}$= 7.9 Hz) resonated at δ 5.12 ppm is designated as the anomeric C_1'-proton of glucose. The rather high coupling constant (7.9 Hz) indicated that the proton probably occupied the β-position in the glucose molecule (the α-proton has $\underline{J} \approx$ 3-4 Hz).

The appearance of two protons δ 4.62 (dd) indicated the presence of nonequivalent methylene (-$\overset{H_A}{\underset{H_B}{C}}$-) geminal protons, this probably because of the connection of methylene protons with the sugar molecule by an ether linkage. The coupling constant 10 Hz was also an indication of the geminal coupling of the nonequivalent H_A and H_B in the following glycoside function.



The singlet at δ 7.49 ppm was assigned as the aromatic C_4-proton of the anthraquinone. The doublets at δ 4.15 ppm was assigned as H-5' in the sugar molecule and unclarified coupling at δ 3.94 was assigned as H-3'. The multiplets at δ 3.28 - 3.39 ppm was assigned as H-4' and at δ 2.98 - 3.08 ppm was assigned as $\underline{CH}_2OH$ in the sugar molecule.

The ^{13}C NMR spectral data (Fig. 7, p. 49) of the Aq-4 were compared with those of lucidin and glucose (in parentheses).

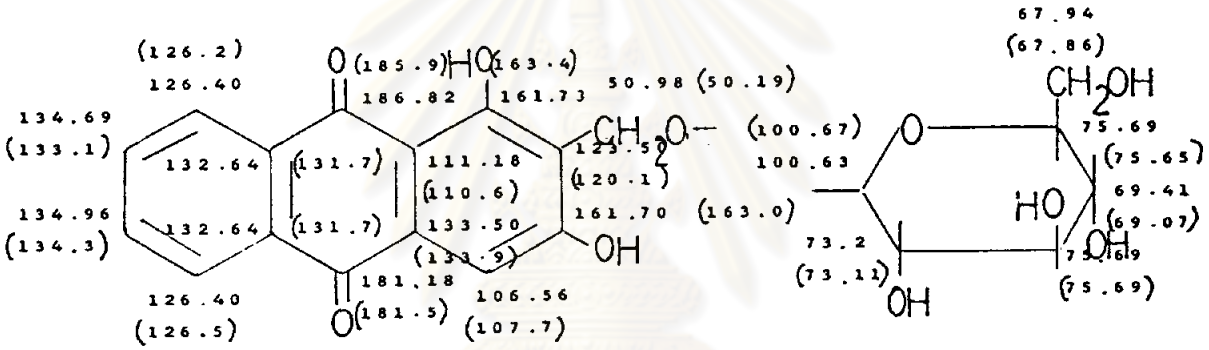


Fig. 7 ^{13}C nuclear magnetic resonance assignment of Aq-4

From the above reasons, it could be concluded that the structure of a probably new anthraquinone glycoside Aq-4 is lucidin ω-O-β-D-glucoside. (Fig. 8)

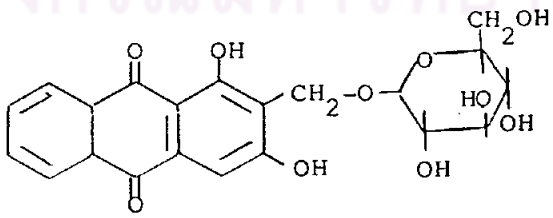


Fig. 8 Structure of lucidin ω-O-β-D-glucoside

Aq-1

Mass spectrum of Aq-1 showed the molecular ion at 298 which could be assumed as the formula $C_{17}H_{14}O_5$. The two fragment ions at *m/z* 252 ($C_{15}H_8O_4$, 100%) and *m/z* 196 ($C_{13}H_8O_2$, 32%) which were lucidin-H_2O and lucidin-(H_2O + 2CO) respectively, were also observed. From the IR spectrum, hydroxyl and ether linkage were recognized.

The structure of Aq-1 was elucidated chiefly by the assignment of the 90 MHz proton and ^{13}C NMR spectra. Preliminary examination of the spectrum in comparison with published proton NMR spectral data for compounds in this series, indicated the presence of hydroxyl protons at δ 9.6 ppm as broad singlet. The ethyl ether function (CH_3-CH_2-O-) was observed in the proton NMR spectrum as a 3-protons triplets at δ 1.26 - 1.42 ppm (CH_3-CH_2-O-) and 2-protons quartets at δ 3.62 - 3.86 ppm (CH_3-CH_2-O-) respectively. The presence of multiplets at δ 7.70 - 7.81 and δ 8.2 - 8.31 ppm indicated the presence of four protons in ring A.

To confirm the chemical structure of Aq-1, the ^{13}C NMR spectral data were compared with those of the literature (in parentheses, Fig. 9) (Itokawa, 1982).

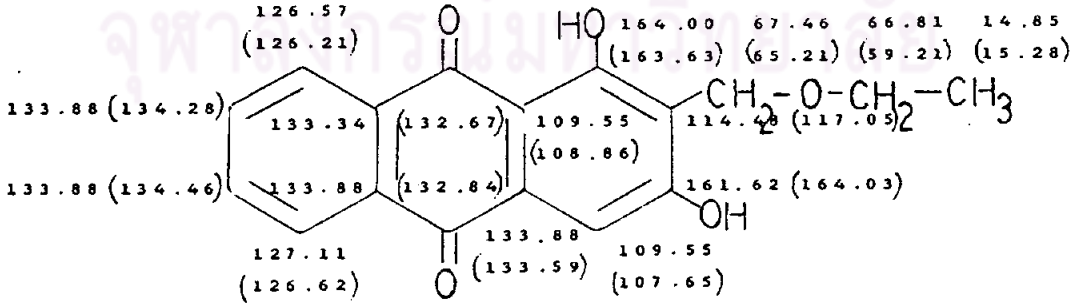


Fig. 9 ^{13}C nuclear magnetic resonance of Aq-1

(the structure of lucidin ω-ethyl ether)

Ethanol used in the extraction procedure could couple with the free hydroxyl group of an anthraquinone such as lucidin to form ethyl derivative of lucidin (lucidin ω-ethyl ether). To confirm this behavior, the anthraquinone was extracted with ethanol, its aglycones contained no lucidin ω-ethyl ether, only lucidin and the others agly aglycones were detected. Lucidin ω-ethyl ether was first isolated from *Rubia tinctorum* Linn. (*R. iberica* Linn.) and was formerly named ibericin (Stikhin *et al.*, 1967). Ibericin was later considered not a naturally occurring anthraquinone because ethanol was used in the isolation procedure.

Aq-2

Aq-2 was isolated and was identified to be hystazarin monomethyl ether of molecular weight 254 ($C_{15}H_{10}O_4$). The chemical structure was shown in Fig. 10.

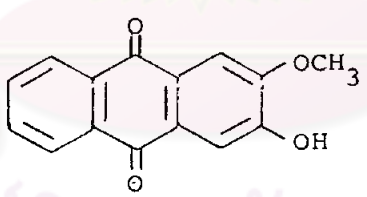


Fig. 10 Structure of hystazarin monomethyl ether

The IR spectrum showed hydroxyl, carbonyl and methyl functions at the wave numbers 3395, 1662, 1624 and 1365 cm^{-1} respectively.

The proton NMR spectral data indicated a hydroxyl proton at δ 9.365 ppm, and the four aromatic protons of the ring A which resonated between δ 7.765 - 7.797 ppm and δ 8.253 - 8.296 ppm as multiplets. The C_1-proton appeared as singlet at δ 7.362 ppm where as the C_4-proton also as a singlet at δ 7.323 ppm. The methoxy group

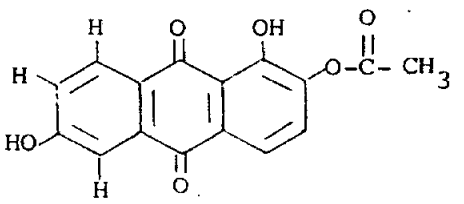
was recognized as a singlet at δ 3.577 ppm. Hystazarin monomethyl ether was first isolated from the root of *Hedyotis umbellata* Linn. (Perkin and Hummel, 1907). This is the first report of the isolation of hystazarin monomethyl ether from Rubiaceous plants.

Aq-3

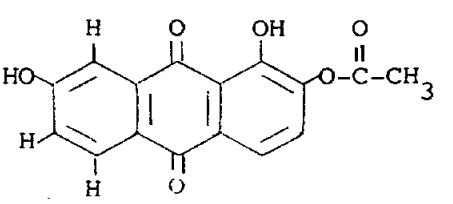
Mass spectrum of Aq-3 gave the molecule ion 298 amu and could give a molecular formula $C_{16}H_{10}O_6$. The ultraviolet and visible absorption spectrum gave λ_{max} at 219, 269, 297, 315 and 411 nm. The bathochromic shift from 411 to 481 nm when aluminium chloride was added into the methanolic solution of Aq-3 was an indication of the 1-hydroxyl function in the molecule. The IR spectrum showed the presence of hydroxyl (3400 cm^{-1}), carbonyl (1640, 1610 cm^{-1}) and ether (1030 cm^{-1})

Proton NMR spectral data indicated 3 protons singlet of $O-\overset{O}{\overset{\|}{C}}-CH_3$ at δ 2.294 ppm and of hydroxyl proton at δ 13.10 ppm. Two sets of doublets centered at δ 7.57 and δ 7.66 (J = 8 Hz) represented the ortho aromatic protons at the position 3 and 4 of ring C. The assignment of the 3 prqton in ring A could not be clarified. This is due to equivalent substitution of either C-6 or C-7 in ring A of the anthraquinone (Aq-3) which would hamper a clear cut assignment of the three unsubstituted protons

Therefore one could only assigned Aq-3 as either 1,2,6-trisubstituted anthraquinone (25), or 1,2,7-trisubstitutted anthraquinone (26).



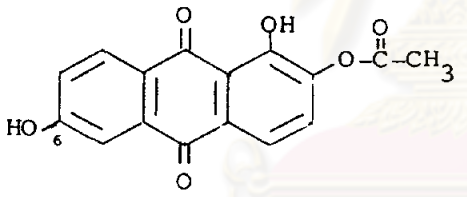
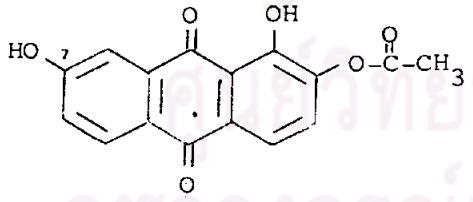
(25)



(26)

Fig. 11 Structure of 1,6/1,7-dihydroxy-2-acetyloxy anthraquinone

Table 2 Assignment of three aromatic protons in ring A of Aq-3

Aq-3 Structure	Proton (δ in ppm)			
	5	6	7	8
 (25)	7.47-7.48	-	7.22-7.26	8.09-8.13
 (26)	8.09-8.13	7.22-7.26	-	7.49-7.48

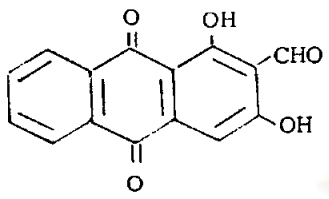
Discussion of Relationship between Biogenesis Pathway of Anthraquinone and Chemotaxonomy

The positions of substitution in anthraquinone molecules, could point to the biogenesis pathway leading to the final anthraquinone. If they were produced *via* acetate-malonate pathway, they would be substitution in ring A and C (Fig. 3, p. 17). It was proved that chrysophanol (6) is produced *via* acetate-malonate pathway in *Rumex alpinus* Linn. and *Rhamnus frangula* Linn. (Leistner and Zenk, 1969). The ring C substituted anthraquinone; alizarin (7), rubiadin (9) and tectoquinone (17) were mostly found to be produced by shikimate-mevalonate pathway (Fig. 5, p. 21), they often occur in the Rubiaceae, subfamily Rubioideae.

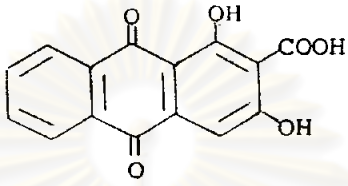
The anthraquinones which were isolated in the present work possessed two types of chemical structures, one of which was substituted in only ring C and the other was substituted in both ring A and C. These evidences lead to the suggestion that the Rubiaceous anthraquinones might be substituted not only in ring C but also in ring A. Usually anthraquinones which occurred in primitive species such as in fungi and lichens seems to be confined to only the acetate-malonate pathway of biogenesis.

Concerning the chomotaxonomic point of view, alizarin and rubiadin are mostly found in *Morinda* species. The presence of lucidin and its derivatives provides more information concerning chemical transformations and constituents in this genus. Lucidin itself can be converted to several anthraquinones. Oxidation with manganese dioxide led to the corresponding aldehyde, nordamnacanthal (27)

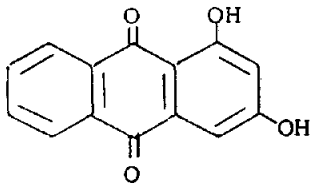
(Ayyangar and Venkataraman, 1956), while treatment with alkaline silver oxide produced the acid, munjistin (28) (Hirose, 1962). Lucidin and nordamnacanthal was also occurred naturally in the same plant (Leistner, 1975).



Nordamnacanthal (27)



Munjistin (28)



Xanthopurpurin (29)

Lucidin (8) has been synthesized from xanthopurpurin (29) by hydroxy methylation with formaldehyde (Ayyangar and Venkataraman, 1956), and from rubiadin diacetate by side chain bromination, followed by traetment with sodium acetate and hydrolysis of the prepared triacetate (Joshi *et al.*, 1955).

Anthraquinones occur in every level of evolution of the plant kingdom. The degree of evolution of plants seem to be related with the anthraquinone types as well as their chemotaxonomic pathways.

Anthraquinones possessing both ring A and C substitutions are likely to take the acetate-malonate route of biogenesis. This route is observed in most of rather primitive plants ranging from fungi to lichens as well as the more advanced ones such as monocotyledons, apetalae (flowering plants without petal), to the polypetalae (flowering plants with many but separate petals) in order of increasing degree of evolution respectively. (Table 3, p. 57)

The shikimate-mevalonate pathway of biogenesis seems to be confined to the anthraquinone occurred in the sympetalae (flowering plants with united petals) such as Rubiaceous plants especially in the *Morinda* spp. (Table 3, p. 58)

the minor anthraquinones which possess substitutions in both ring A and ring C of the *Morinda* spp. might probably derive from the shikimate-mevalonate biosynthetic anthraquinones in later stages of biosynthesis.

The above suggestion might be useful for the prediction of the type of anthraquinone being isolated.

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จุฬาลงกรณ์มหาวิทยาลัย

Table 3 Relationship between biogenesis pathway of anthraquinones and chemotaxonomy

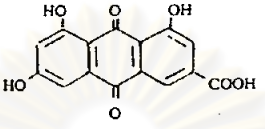
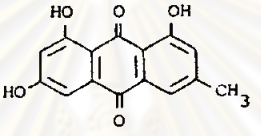
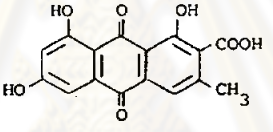
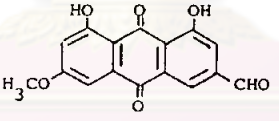
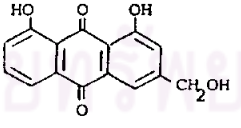
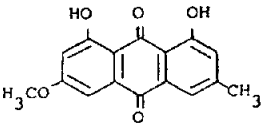
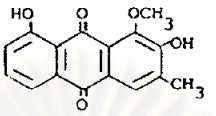
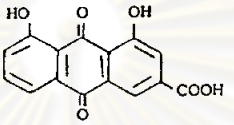
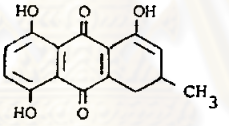
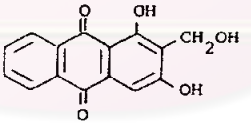
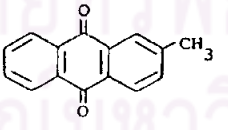
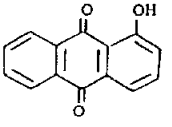
Plant species	Taxonomic position	Anthraquinone	Pathway	Reference
Penicillium cyclopium Westl.	fungus	 Emodic acid	a	Anslow *et al.*, 1940
Aspergillus terreus Thom.	fungus	 Emodin	a	Yamamoto *et al.*, 1968
Nephromopsis endocrocea Asahina	lichen	 Endocrocin	a	Asahina and Fujikawa, 1935
Xanthoria fallax (Hepp.) Arn.	lichen	 Fallacinal	a	Murakami, 1956
Aloe spp.	monocotyledon	 Aloe-emodin	a	Rhude, 1963
Bulbine annua Willd.	monocotyledon	Aloe-emodin	a	Rheede, 1964
Rheum spp.	dicotyledon -apetalae	 Physcion	a	Tsukida *et al.*, 1954

Table 3 (Continued)

Plant species	Taxonomic posotion	Anthraquinone	Pathway	Reference
Cassia spp.	dicotyledon -polypetalae	 Obtusifolin	a	Takido, 1958
Rumex spp.		 Rhein	a	Tsukida *et al.*, 1954
Rhamnus spp.		Aloe-emodin	a	Shibata and Takido, 1952
Ventilago viminalis Hook.		 Helminthosporin	a	Cooke and Johnson, 1963
Morinda spp.	dicotyledon -sympetalae	 Lucidin	b	Burnett and Thomson, 1968
Tectona grandis Linn.		 Tectoquinone	b	Ahluwalia and Seshadri, 1957
Tabebuia avellanedae Lor.ex Griseb.		 1-Hydroxyanthraquinone	b	Burnett and Thomson, 1967

a = acetate-malonate pathway

b = shikimate-mevalonate pathway

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