

SOME CHEMICAL CONSTITUENTS OF
ARTEMISIA HERAN

A Thesis
Presented to
the School of Graduate Studies

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of the Requirmerits for the Degree
Master of Science in Chemistry

by
Hidaat Asghedora
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TABLE OF CONTENTS

	Page
1, Introduction	1
2. Background	6
2.1 Classification of Flavonoids	6
2.2 Extraction	0
2.3 Thin Layer Chromatography	0
2.4 Column Chromatography	8
2.5 Characterization	9
2.6 Physiological Activity of Flavonoids	11
2.6.1 Ultraviolet patterning	XX
2.6.2 Antifungal & Antibacterial Activity	12
2.6.3 Blood Coagulation	12
2.6.4 Metabolism of Vitamin C	12
2.7 Biosynthesis of Flavonoids	13
2.8 Distribution of Flavonoids in the Genus Artemisia	13
2.9 Classification of Terpenoids	10
2.10 extraction	10
2.11 Characterization	10
2.12 Biosynthesis of Terpenoids	19
2.13 Taxonomy and phylogeny in the Genus Artemisia	22

	Page
3, Results and discussion	25
4, Experimental	55
4.1 General Experimental Procedures	55
4.2 Extraction and Purification	5b
4.3 Purification by Caecum Liquid Chromatography	59
4.4 Attempted Methylation of Artemotin	6&
4.5 Oionssay	64,

LIST OF TABLES

	<u>Page</u>
1. Class of Flavonoids	6
2. Color Reactions of Flavonoids in Alkaline Solution	10
3. List of Flavonoids Isolated from Some Artemisin Species	17
4. ^1H NMR of Ar-1 in CDCl ₃	20
5. ^1H NMR of Ar-1 in DMSO-d ₆	30
6. Ozone Induced Shifts for Some OMe Substituents	30
7. Ozone Induced Shifts of the Flavonoids 46, 47, 48, 49	34
8. ^{13}C NMR of Ar-1	35
9. List of Some Plants from which Artemisin has been Isolated	41
10. ^1H NMR of Vulgarin	43
11. ^{13}C NMR of Vulgarin	45
12. ^1H NMR of Ar-3	50
13. ^{13}C NMR of Ar-3	54

LIST OF SCHEMES

	<u>Page</u>
1» Suggested Fragmentation Pattern of the Principal Ions of Ar-1	40
2, Experimental Procedure ~ 1	57
3, Experimental Procedure - 2	58

LIST OF FIGURES

	<u>Page</u>
1. General phenylpropanoid Metabolism	14
2. Biosynthesis of Flavonoids	15
3. Biosynthesis of Terpenoids	20
4. The proposed Biosynthetic Pathways for the Various Structural Classes of Sesqui- terpene Lactones found in the Genus Artemisia	21
5. UV Spectrum of Ar-1	27
6. ¹ H NMR Spectrum of Ar-1 in CDCl ₃	29
7. ¹ H NMR Spectrum of Ar-1 in Benzene	31
8. Proton Decoupled ¹³ C NMR Spectrum of Ar-1	36
9. DEPT Spectrum of Ar-1	37
10. Mass Spectrum of Ar-1	39
11. H,C - COLOC Spectrum of Vulgarin	46
12. IR Spectrum of Ar-3	40
13. proton Decoupled ¹³ C NMR of Ar-3	49
14. ¹ H NMR Spectrum of Ar-3	51
15. DEPT Spectrum of Ar-3	52
16. OFF Resonance Spectrum of Ar-3	53

A B S T R A C T

SOME CHEMICAL CONSTITUENTS OF ARTEMISIA REHMANI

by

Midoat Asghedom

Research Advisor: Dr. Zorhanu Abcgaz

Artemisia rehmanii (1), known by the local name 'Mriti' is an endemic plant to Ethiopia. An *in vitro* antimalarial test of the crude extract gave an IC_{50} of $50 \mu g ml^{-1}$.

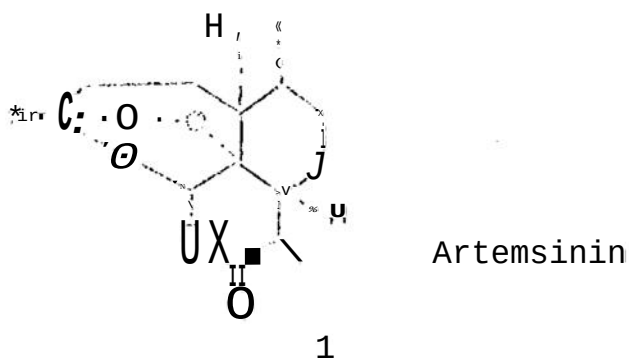
Artemetin and a sesquiterpene lactone were isolated from the aerial parts of the plant, Artemetin was identified by its melting point and spectral data MS, IR, NMR and ^{13}C -NMR. IR, 1H -NMR, ^{13}C -NMR and chemical studies *in vitro* carried out to elucidate the structure of the sesquiterpene. Vulgarin, a Compound that was previously isolated from the same plant was analyzed by two dimensional 1H and ^{13}C -NMR including COLOG spectroscopic techniques.

1. INTRODUCTION

The genus Artemisin is one of the largest and most widely distributed of the nearly sixty genera in the tribe Anthemidae (compositae) (2). It has about 400 species and is found predominantly in the northern temperate region of the world (3,4). This genus has been extensively studied particularly for its sesquiterpene lactone content (5,6). However, isolation of other important class of compounds, e.g. flavonoids (7), coumarins and acetylenes have also been reported (8).

Many species of Artemisia have been reported to show biological activity, Artemisinin, (qinghousu, QHS, arteannuiri) a potent antimalarial drug was isolated from Artemisia annua, a herb that has been used in Chinese folk medicine for treatment of malaria (9). Malaria, one of the most hazardous tropical diseases termed as "the king of diseases" has been the cause of many deaths in the world (10). Though considerable efforts were made to eradicate malaria, there still are 150 million new cases each year. In 1980, it was reported that there were more than 5.5 million cases of malaria in Africa, 3 million in the Indian continent and more than 4 million in E. Asia and China (11). Resistance of Plasmodium species to chemotherapeutic agents such as chloroquine is a serious problem. This unusual sesquiterpene lactone that contains an endonero-

xide moiety showed an inhibitory action against the chloroquine resistant strain Plasmodium falciparum. Treatment of 140 cases of cerebral malaria with QMS has resulted in 128 cure cases (91.4%).

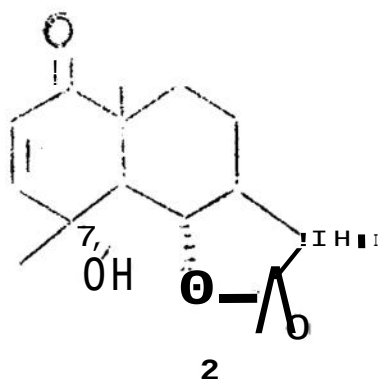


Vulgarin has been isolated from Artemisia rohan, Chiov (1), A. Canarionsis, Lees (12), A. Vulgaris L. (13) A. harrelieri, Besser (14), A. judoica, L (15), A. tauricry Willd (16) and some other Artemisia species. It has been shown to have an oral hypoglycemic agent, preliminary study of the effects produced in the lipid fraction of several organs of rat after administration of vulgarin, showed that it induced some decrease in liver lipid levels (17). It increased the amplitude and decreased the frequency of cardiac contractions when injected i.v, at 1 mg/kg into cats or rabbits. When administered orally at 10 mg/kg to rats, 44% increase in diuresis was observed (18). Both vulgarin and santonin were reported to be powerful central nervous system stimulants, and convulsant poisons in toxic

doses. LD₅₀ of vulgorin was 4.92/mg/kg in mice, 7.94 mg/kg in rats and 14.60 mg/kg in toads (19). However, no antimicrobial activity was exhibited by vulgorin when tested against the following strains of bacteria (20).

- a. Staphylococcus aureus ps 80 - 81 (Gram positive),
- b. Escherichia coli ATCC 12765 (Gram negative),
- c. Bacillus subtilis PCI (Gram positive),
- d. Salmonella enteritidis ATCC 13076 (Gram negative),
- e. Klebsiella pneumoniae ATCC 13883 (Gram negative).

From this data one studies on other sesquiterpenes, it has been reported that antimicrobial activity is likely to be possessed by beta unsubstituted enone system with a five membered ring, as the six membered sesquiterpene lactones like vulgorin, which are chemically less reactive towards Michael addition, were antimicrobially inactive.



Vulgorin

Artemisia eriJentote subspecies tridentota and A. pnniculta have antibacterial activity (21,22). The essential oil of A. monosperma was shown to have insecticidal activity against housefly, cotton leaf worm and the rice weevil (23). Esculetin, 6,7-dimethylether and esculetin 6 and 7-methyl ethers isolated from A. capi-
Haris and A. mescereohmidnna were found to be chlagogic (24,25) as observed in the increase of bile production in dogs.

This paper deals with the study of an endemic plant, Artmisia rohan. It is odorous and grows widely in the Gthio:ian highlands. A bioassay of the crude extract for antimalarial and antibacterial activity was carried out for the first time. In the antimalarial test, based on the inhibition of incorporation of (³H)-thymidine into Plasmodium falciparum (K-1 cblorogquine resistant strain), IC_{50} value of 50 $\mu\text{g ml}^{-1}$ was obtained. Where as for Artemisia annua this value is 3.9 $\mu\text{g ml}^{-1}$ (11). However, no conclusive results were obtained as to whether artemsinin is present or not by TLC analysis, since the extract is made up of a number of complex components. No nntibactsrial activity was shown against the six strains of bacteria tested: S. aureus, Oacillus subtils, E. coli, ohigela soni and Pseudomonas geroginosa. Furthermore Arternetin and a sesejuiterpene lactone were isolated from the aerial parts of the plant for the first time.

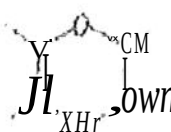
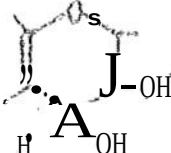
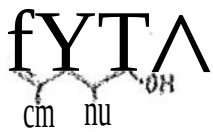
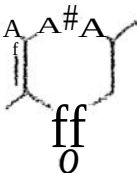
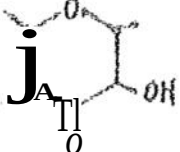
previous studies on the plant include analysis of the essential oil (1) and reinvestigation of the structure of vulgnrin isolated from the chloroform extract (26). However, the quantity of vulgarin and experimental details of its isolation have not been reported. These are now included in this paper. Furthermore the sample is rigorously studied by a heteronuclear shift correlated two-dimensional experiment and the results of our two-dimensional COLOC experiment confirm the orientation of the enone chromophore.

2, BACK GROUND

Classification of Flavonoids

Flavonoids contain a $O=C_3-C_6$ carbon skeleton in which the C_3 unit links two aromatic groups (2, 8). The oxidation state of the C_3 fragment and the ring system it is involved in, form the basis for the classification of flavonoids to the major classes, such as «technically» Flavonones, Flavones etc. The color classes, going from the least to the most oxidised, are given in the table below.

Table I Classes of Flavonoids

Class	Key portion	Example
Isoflavone		
Leucoanthocyanins		
Flavanones		
Flavonols		

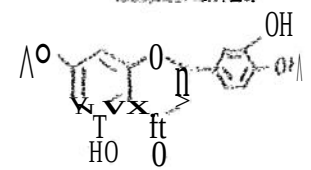
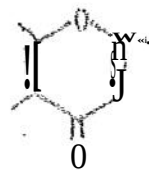
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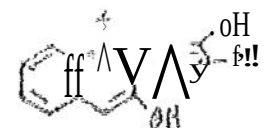
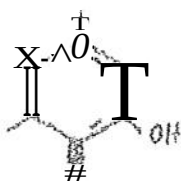
Key portion

Example

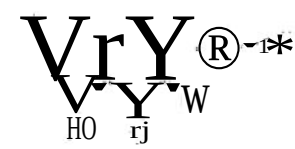
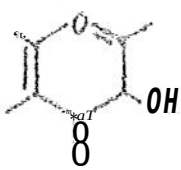
PX ijVD ?Si?S



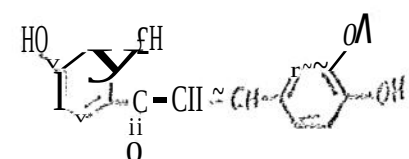
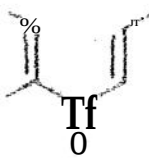
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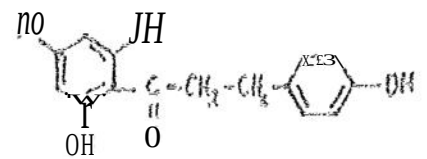
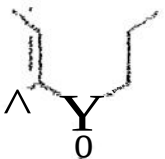
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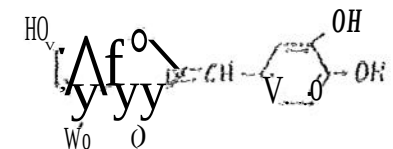
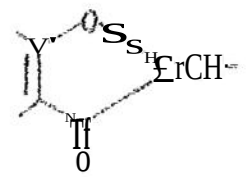
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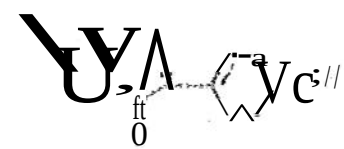
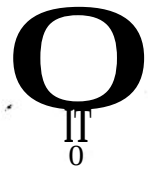
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isoflpvono



Hydroxyl groups are normally present on the aromatic
ibgp or they may be found combined as ffeethoxyl groups or
lycosloop»

2.2 Extraction

In general, the flavonoid compounds of fresh or dessicated plant materials can be extracted by means of ethanol or methanol. The petroleum ether extraction, in some instances is an important step as it removes a number of non-flavonoid constituents such as fats and chlorophyll.

The selective precipitation of flavonoid constituents as lead salts usually using lead acetate is often advantageous (22, 29,30,31). The resulting precipitate may be freed of lead with hydrogen sulfide.

2.3 Thin Layer Chromatography (TLC)

It is suitable for the separation and detection of flavonoids in crude plant extracts. Adsorbents most commonly used for TLC of flavonoids are silica gel, polyamide and cellulose.

Recent reports indicate that high performance thin layer chromatography (HPTLC) can provide much better separations than TLC of complex flavonoid mixture. HPTLC is a development of TLC carried out using small particles, usually about 5 μ m in diameter, all of a closely similar size.

2.4 Column Chromatography

Is a method of choice for the preliminary

purification or for preparative scale separation of larger quantities of flavonoid from crude plant extracts. Packing materials used are polyamide, silica gel.

Other chromatographic methods employed for separation or purification of flavonoids include paper chromatography and reverse phase columns.

2.5 Characterization

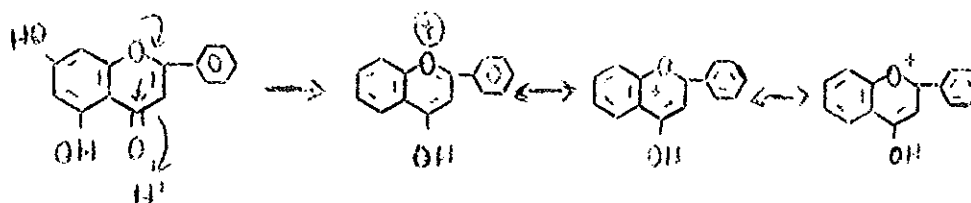
Classically, many different color reactions and solubility properties were used to characterize the different classes of flavonoid pigments.

Exposure of the dried TLC chromatograms to NH_3 fumes causes marked color changes in most of the flavonoid compounds, particularly when the chromatograms, usually paper, are viewed under ultra-violet ($3660 \text{ \AA}$). From this relationship between spot color and flavonoid structure can be made (32). If an aqueous pigment extract is made alkaline various color changes may be observed although the changes in one pigment may mask in another (27).

Table 2: Color Reactions of Flavonoids in Alkaline Solution

Flavonoid	Color change observed
Anthocyanins	Purple-blue
Flavones, flavonols	Yellow
Flavanones	Colorless, becoming orange red (if heated)
Chalcones and aurones	Immediate red-purple
Flavanols	Orange brown

Concentrated sulfuric acid dissolves many flavonoid compounds with the production of colored solutions, the properties of which are often of diagnostic value in structure analysis (29). Flavones and Flavonols dissolve in H_2SO_4 to give intensely yellow solutions which contain the oxonium (flavylium) salts of the following general structure.



Some classes of flavonoids give characteristic colors on reduction with Mg and HCl (Shinoda (1928) test). An ethanolic solution of AlCl_3 (5%) is a specific reagent for flavonoids. For sesquiterpenes this reaction (violet or brown color on the plate, or yellow, brown and green fluorescence under UV light at 366 nm) is evident only 10 - 15 min. after heating at 120°C , while for the flavonoids a yellow or brown fluorescence appears immediately (33).

The above color reactions combined with spectrophotometric methods will make possible a positive identification of a flavonoid in all its structural detail.

2.6 Physiological Activity of Flavonoids

2.6.1 Ultraviolet Patterning

Certain flavonoid types are reported to act as nectar guide components in flowers of various plants (34,35,36). The flavonoids involved in nectar chemistry are yellow flavonols, 2-hydroxyflavones and anthochlors. These compounds absorb UV light and appear dark or "bee purple" to the eye of the pollinators. Nectar guide compounds often occur in association with carotenoids which in reflecting UV and yellow wavelengths provide a back ground against which the nectar guide is visible.

2.6.2 Antifungal and Antibacterial Activity

Flavonoids play a significant role in the protection of plants from infection by fungal and bacterial diseases. Special isoflavonoids are synthesized in the leaf of peas and beans which inhibit the growth of disease causing organisms (37).

Quercetin showed antibacterial activity. At pH 6.5 and in concentrations of 0.075 to 0.1 mg/ml it produced complete inhibition of Staphylococcus aureus, S. albus, Aerobacillus polymixa and Brucella abortus.

2.6.3 Blood Coagulation

Rutin has been used with benefit in the coagulation of blood. It has shown therapeutic effect for certain persons with thrombocytopenic purpura. In rats there was an evidence that rutin tends to increase prothrombin consumption (38).

2.6.4 Metabolism of Vitamin C

Some flavonoids have the ability to inhibit the oxidation of ascorbic acid. It was reported that Guinea Pigs on Scorbuto-genic diet did not develop scurvy when citrin, hesperidin

and dimethylhesperidin were added to the diet (38). It was believed that the co-existence of 2 or 3 free hydroxyls in the ortho positions and the conjugated double bond appear to be responsible for this action (39).

2.7 The Biosynthesis of Flavonoids

All classes of flavonoids are biogenetically closely related, where the first common intermediate is the chalcone. The flavonoids are regarded as being formed by combination of the shikimic acid derived C_6-C_3 fragment with a six carbon unit formed via the linear combination of three acetate units. It has been established that ring A is formed by a head-to-tail condensation of three acetate units and ring B as well as carbon atoms 2,3 and 4 of the heterocyclic ring C arise from phenylalanine (40,41).

2.8 Distribution of Flavones in the Genus Artemisia

Most free aglycones have been reported so far from the family Asteraceae (42). Flavones and flavonols are widely present in Asteraceae with additional substituents in the 6 and/or 8 positions.

Flavonoids are usually present in plants in the water soluble o-glycoside form (40). However, with respect to aglycones, it is likely that in most cases they are formed as artefacts during

Fig.1 General Phegylpropanoid metabolism

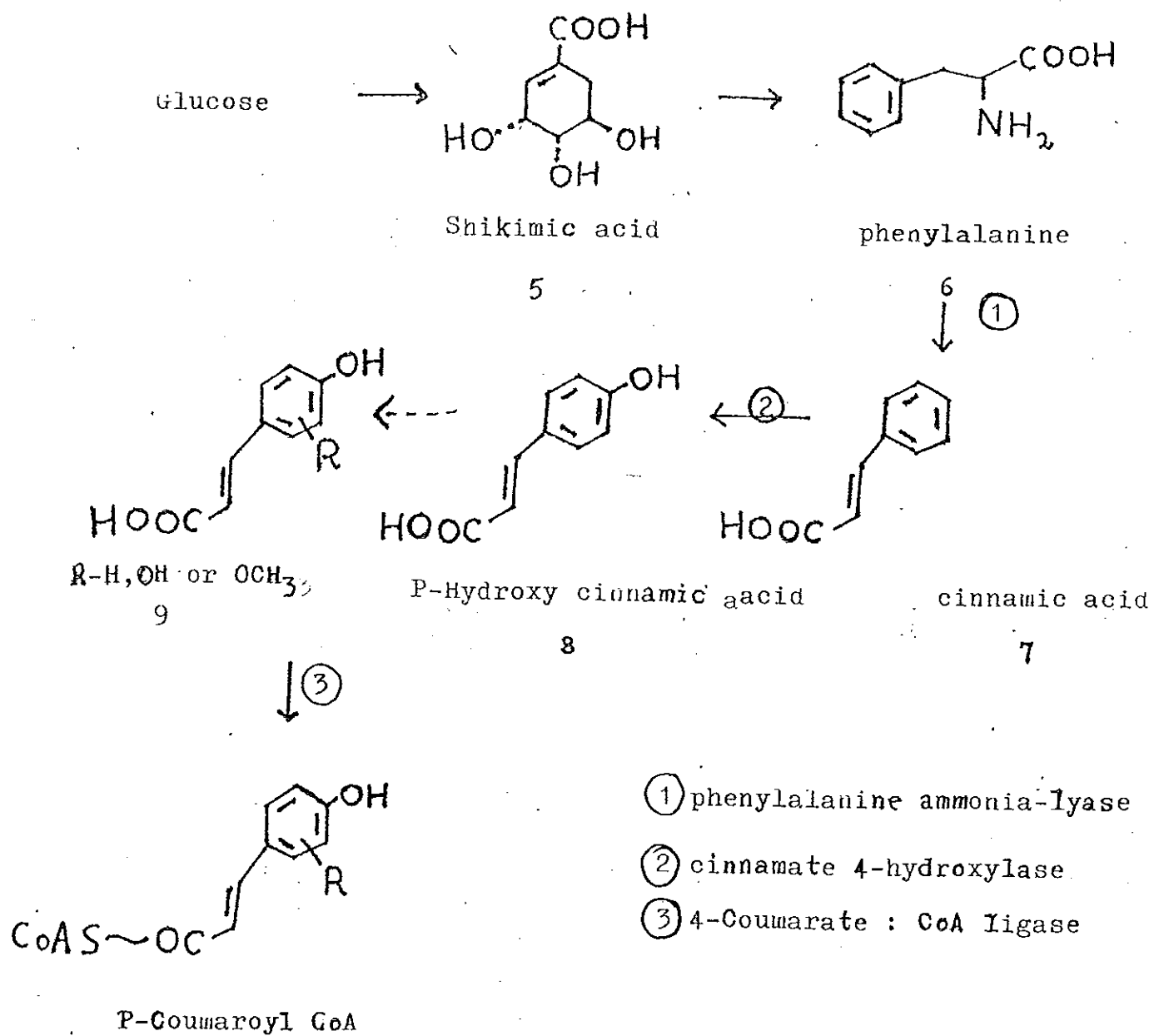
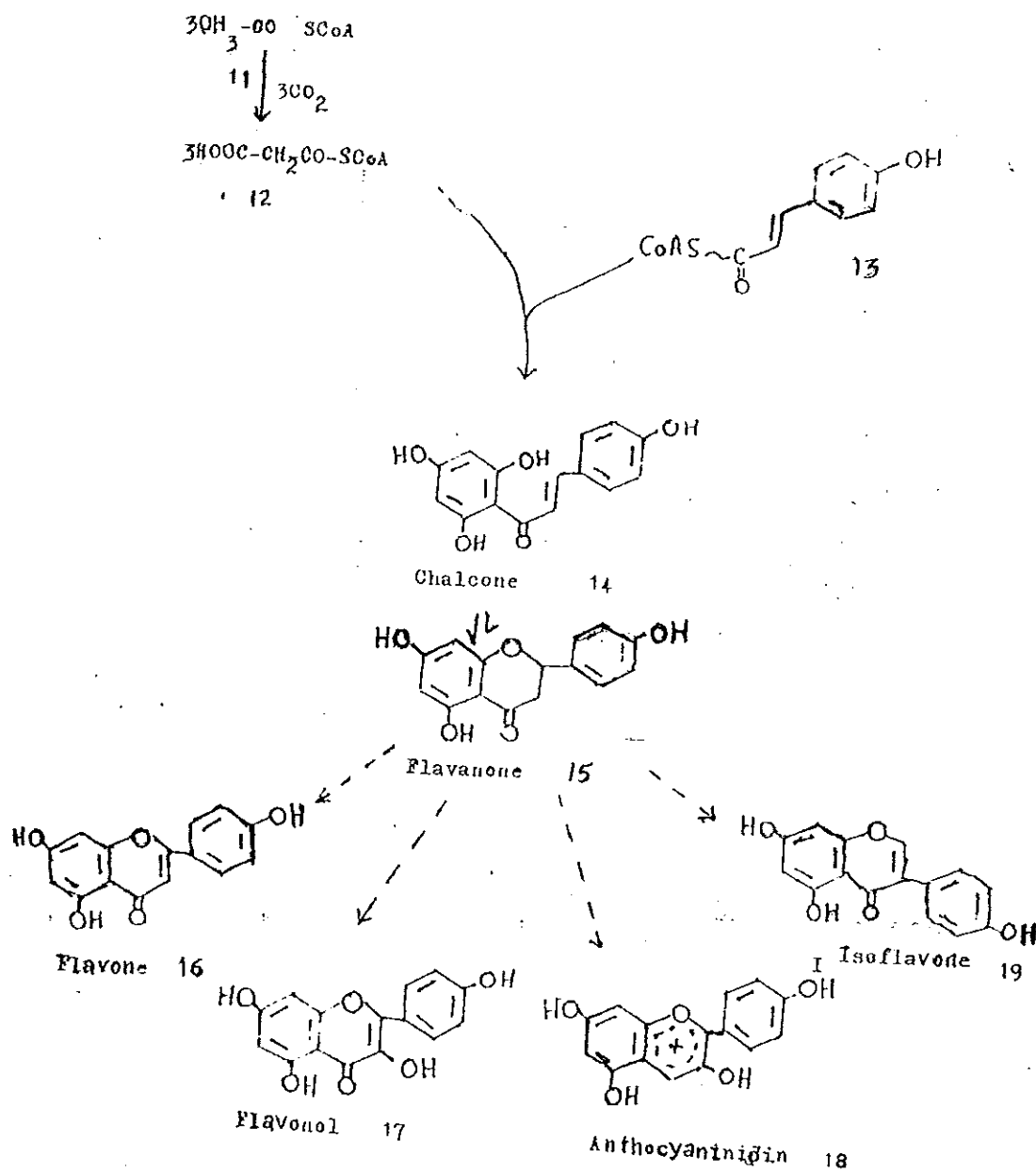


Fig.2 Biosynthesis of Flavonoids



the course of extraction, since the glycosidases in living tissues are very active even in high concentration of organic solvents (43). Where free aglycones are encountered especially highly methylated flavonoids, it is likely that they occur externally, or are associated with secretory structures and are correlated with the production of other lipophilic secondary compounds of terpenoid origin (37). This is most convincingly observed in the Asteraceae, Rutaceae, Betulaceae and the Lamiaceae.

In an attempt to make a correlation between the presence of free aglycones and certain plant structures (44), it was pointed out that in relation with certain metabolic activities some plants develop specialized anatomical structures in which certain products (rich in energy) can be secreted. This is more pronounced especially with plants producing essential oils and possessing the relevant storage structures: oil cells, oil cavities, oil ducts, glandular trichomes. It was concluded that lipophilic flavonoids with a low number of OH groups and/or higher number of methoxy groups are encountered especially in plants with excretion cells or cavities and as constituents of waxy layers. Hydroxy flavonoids with glycosidic moieties can be expected in all plants as vacuolar constituents. Free hydroxy flavonoids are abundant in woody plants occurring together with glycosidic flavonoids.

List of Flavones isolated from some Artemisia Species

Substitution	Trivial name	Plant Source	References
7-Me(G)	Genkwanin	<u>Artemisia capilla-</u> <u>ris aerial parts</u>	45
6-Me(G)	Nepetin (eupafalin)	<u>Artemisia triden-</u> <u>tata ssp. rose-</u> <u>yana</u>	46
6,3'-di-Me	Jaceosidin	<u>Artemisia dracu-</u> <u>unculoides, aerial</u> <u>parts</u>	47
6,7,3'-tri-Me(G)	Cirsilineol (fastigenin) anisomelin	<u>Artemisia mono-</u> <u>sperma</u> <u>Artemisia capillaris</u>	48 45
6,4',5'-tri-Me		<u>Artemisia frigida</u> <u>leaf and stem</u>	49
6,7,8,-tri-Me	Araneol	<u>Artemisia frigidica</u> <u>ana, stem</u>	50
6-Me(G)	6-Methoxy Kaemp- ferol	<u>Artemisia arbuscula</u> <u>whole plants</u>	51
3,6,7-tri-Me	Condulatin	<u>Artemisia 3 spp whole</u> <u>plant</u>	52
3,6,7-tri-Me(G)	Chrysosplenol-O	<u>Artemisia, 3 spp</u> <u>aerial parts</u>	51
3,5'-di-Me		<u>Artemisia monosperma</u> <u>flower head</u>	48
6,7-one	Crismaritin Scrophulein	<u>Artemisia capillaris</u>	45
6,7,3'-OMe	Cirsilineol	<u>Artemisia herba alba</u>	52
3-OMe	Transilutin	<u>Artemisia transilie-</u> <u>nsis</u>	53
3,6-one	Axillarin	<u>Artemisia spp. leaf</u>	51
3,6,7,3'-OMe	Chrysosplenetin	<u>Artemisia cana</u>	54
6,7,5'-OMe		<u>Artemisia capillaries</u>	55

G = occurs in glycosidic combination.

2.9 Classification of Terpenoids

Terpenoids are classified broadly into six major classes based on the number of "isopentenyl" groups they are derived from. Monoterpenes have two sesquiterpenes, three; diterpenes, four; ses- terpenes, five; triterpenes, six; and tetraterpe- nes, eight such units.

The most important class of terpenoids found in the family compositae (asteraceae) is the ses- quiterpenoids. A recent review of sesquiterpenoid lactones list 924 naturally occurring compounds (56).

2.10 Extraction

The low molecular weight terpenes are usua- lly separated by simple distillation or steam di- stillation. Contamination by volatile esters can be removed by saponification. Though there is no general method of isolation applicable to all, a large number of them are non-polar, so that they can be separated by non polar solvents such as petroleum ether, benzene or ether. This is usually followed by purification by column chromatography on Si-gel or alumina.

2.11 Characterization

Most compounds could be detected by spray

reagents, such as vanillin sulfuric acid. However, there is no single test which will distinguish terpenoids as a group from all other plant constituents.

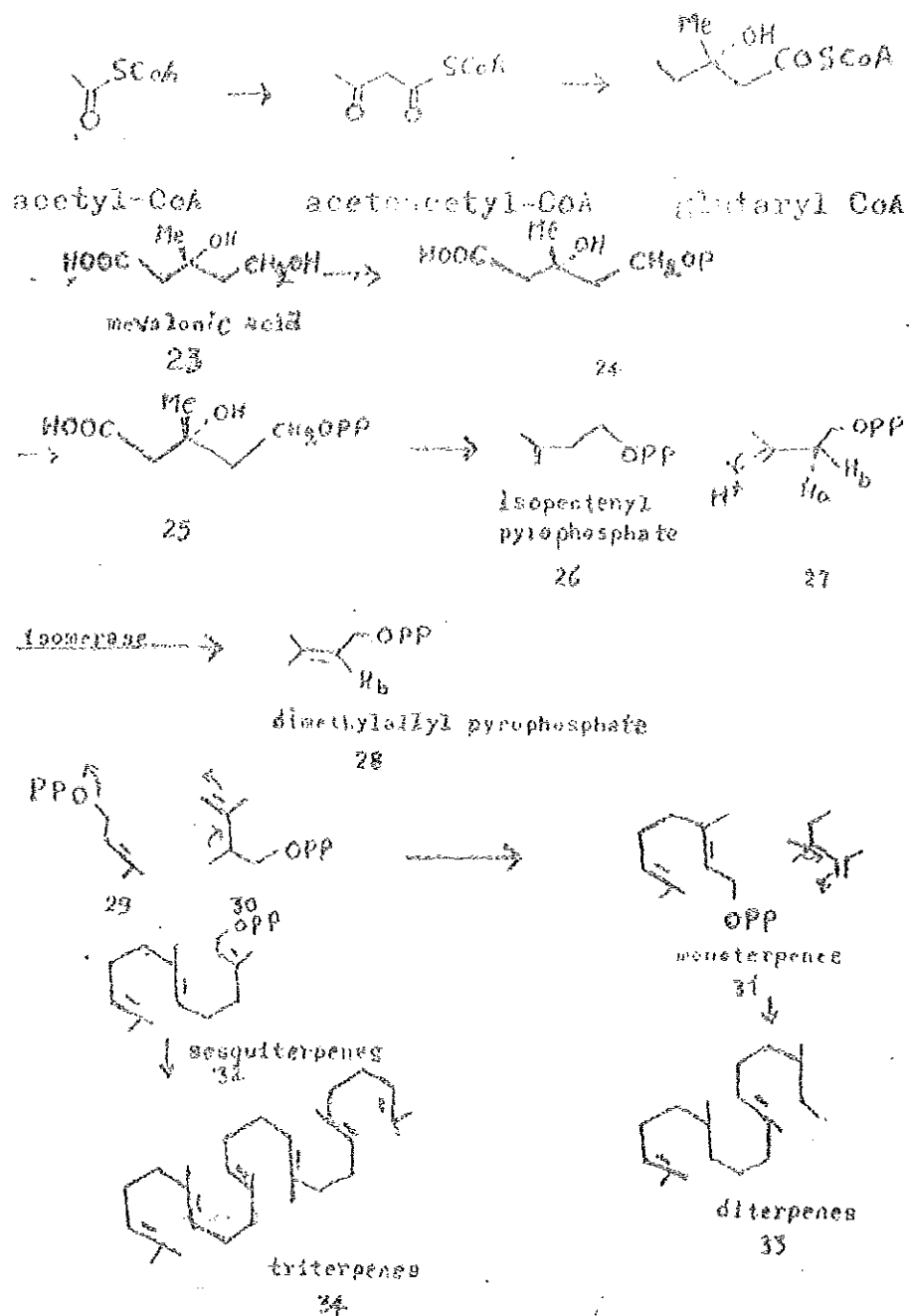
Application of spectroscopic techniques viz- IR, ^1H and ^{13}C NMR are often used in conjunction with Biogenetic considerations to elucidate structures.

The use of labelled substrates especially with ^{13}C and the analysis of the resultant enrichment and coupling patterns of terpenoid metabolites have enabled the determinations of various skeletons.

2.12 Biosynthesis of Terpenoids

Isopentenyl pyrophosphate is the key intermediate in the biosynthesis of terpenoids. It is biosynthesized from acetyl coenzyme - A via mevalonic acid (see Fig. 3).

Fig. 3 Biosynthesis of Terpenoids



The genus Artemisia has been of interest for its sesquiterpene lactone content (5,6). The following biosynthetic pathway was proposed for the various classes of sesquiterpene lactones isolated from the genus Artemisia (57).

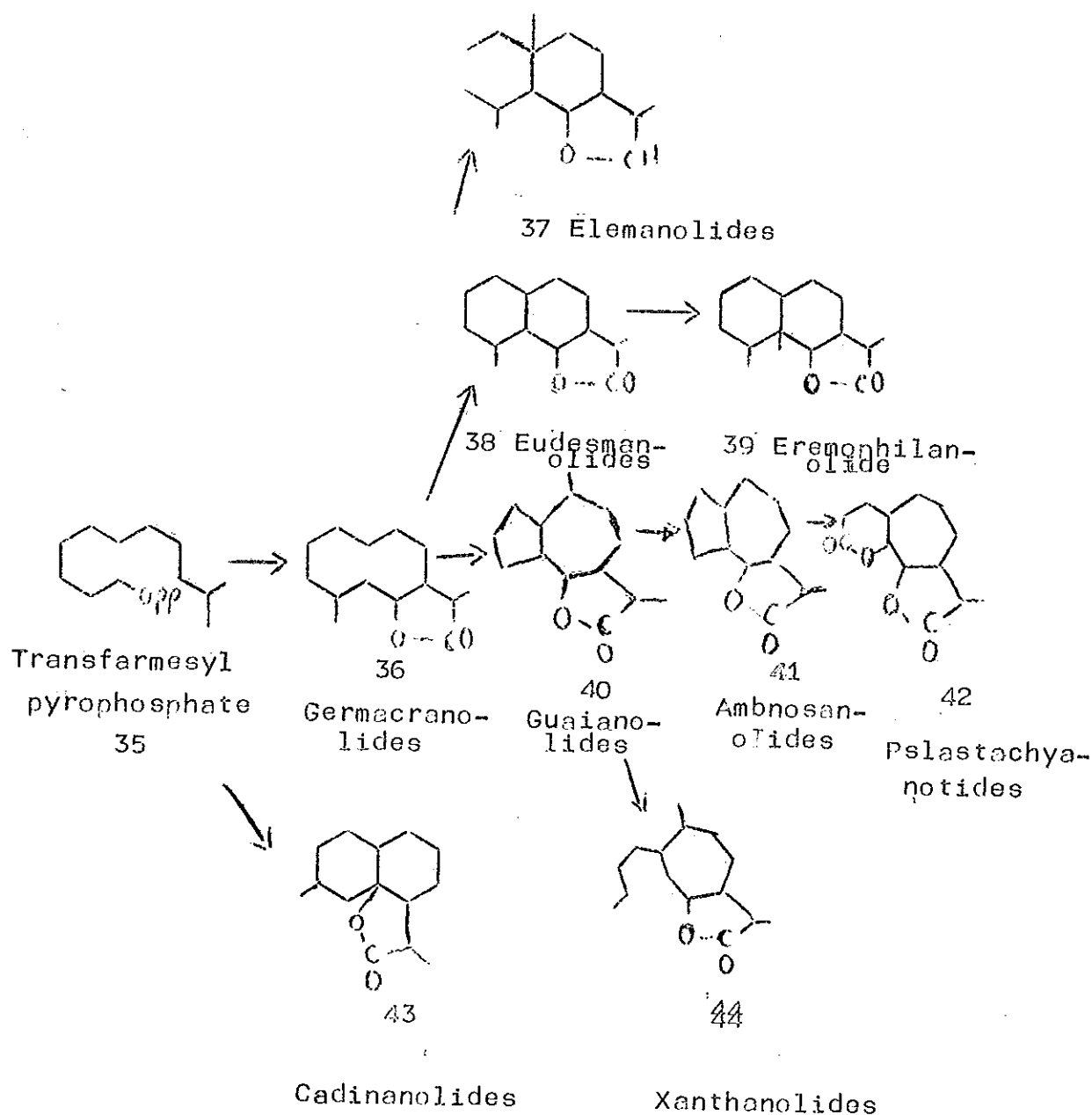


Fig. 4. The proposed biosynthetic pathways for the various structural classes of sesquiterpene lactones

found in the genus *Artemisia*. (Class marked with an asterisk have not yet been discovered in this genus).

2.13 Taxonomy and Phylogeny in the Genus *Artemisia*

In relation to its sesquiterpene lactone content, the genus *Artemisia* is classified into different sections, the one usually adopted is that proposed by Besser and Decandolle in which four sections are described (58). Classification is made on the basis of the differences in floral characters. Besser's classification of *Artemisia* was as follows:-

A. Head heterogamous, marginal flowers pistillate.

1. Abrotanum - central flowers fertile, their achenes normally developed. Receptacle not hairy.
2. Absinthium - central flowers fertile, their achenes normally developed. Receptacle long-hairy.
3. Dracunculus - Central flowers sterile, their achenes aborted.
3. 4. Serphidium - Heads homogamous, marginal flowers absent.

The classification made by Besser was later modified in a review made on the phylogeny of

the four sections (59). It was concluded that, the section Abrotanum and Absinthium could be combined into the single subgenus Artemisia. The only difference between the two being the presence or absence of hair on the receptacle. Otherwise as far as the chemical data is concerned there are several sesquiterpene lactones common to both and if the compounds are not identical, their biosynthesis is closely related. The section Tridentatae was found to produce guaianolides that are identical or structurally related to the sesquiterpene lactones of Abrotanum. Hence, it was suggested that the section Tridentatae originated from Abrotanum.

1. Section Abrotanum: Species in this section produce most structurally diversified and biosynthetically advanced sesquiterpene lactones in the genus. The most advanced compounds isolated from A. vulgaris are the psilostachyin and psilostachyin-C.
2. Section Absinthium: They are reported to produce germacranolides, eudesmanolides with a majority of species capable of producing guaianolides.
3. Section Seriphidium: Most species within this section produce eudesmanolides, although a few germacranolides, guaianolides and the single elemanolide have been reported.
4. Section Tridentatae: Produce only germacranolides, but most can synthesize the more advanced guaianolides and eudesmanolides. The matricarin compounds

isolated from subgenus Serphidium and Artemisia are very abundant in the species of Tridentatae.

5. Dracunculus: Information available on this genus is restricted to two species only from which sesquiterpene lactones are reported. More information is needed to make any generalization about the types of characteristic compounds present in the section.

3. RESULTS AND DISCUSSION

The crude methanolic extract of the aerial parts of A. rehan was tested for antibacterial and antimalarial activity. No inhibitory effect was observed with the following test organisms: Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Shigella soni and pseudomonas aeruginosa. The test method used for antimalarial activity was by assessing the ability to inhibit the incorporation of (^3H) - thymidine into Plasmodium falciparum (K-1 chloroquine resistant strain). The IC_{50} value of the crude extract was found to be $50 \mu\text{g ml}^{-1}$.

The aerial parts of A. rehan were extracted with aqueous methanol, the organic phase removed, and the aqueous layer was partitioned between a series of organic solvents. Artemetin (Ar-1) and vulgarin (Ar-2) were isolated from the chloroform extract. In another experiment when the methanol extract was extracted with organic solvents, the chloroform extract afforded Ar-1, Ar-2 and a sesquiterpene lactone Ar-3. The structure of Ar-1 was confirmed by its melting points and spectral analysis: MS, IR, ^1H and ^{13}C -NMR. Vulgarin, had been previously isolated from the same plant, and a reinvestigation of its structure was done by spectroscopic and X-ray studies (24). We have carried out two dimensional ^1H and ^{13}C -NMR studies and heteronuclear

COLOC experiments to confirm the site of the enone group. UV, IR, ^1H and ^{13}C -NMR analysis together with chemical studies were used in order to elucidate the structure of Ar-3.

Ar-1 was obtained as yellow needles mp. $157 - 159^\circ\text{C}$, Lit. $158 - 159^\circ\text{C}$ (60,61). HRMS exhibited a molecular ion peak M^+/Z 388 (100%) for $\text{C}_{20}\text{H}_{20}\text{O}_8$ and the relative abundance of the fragments: $387 (M-H)^+$ (29%), $373 (M-Me)^+$ (60%) and $345 (M-COMe)^+$ (10%). UV $\lambda_{\text{max}}^{\text{nm}}$: 256, 272, 344; NaOAc 255 (Sh), 273, 341. IR $\nu_{\text{max}}^{\text{cm}^{-1}}$: 3500 cm^{-1} (chelated hydroxyl), 1760 cm^{-1} (phenyl ketone) and phenyl absorption at 1600 cm^{-1} . In the ^1H -NMR spectrum, a one proton singlet at 6.55 is typical for H-8 in 6-methoxy flavones (62). One proton signals each at 7.72 (s) and 7.80 (d) can be assigned to the C2' and C6' protons as C2' is ortho substituted. The signal at 12.65 (s, 1H) corresponds to the chelated hydrogen of the OH group at C5. 4.00 (s) 9H, 3.96 (s) 3H and 3.9 (s) 3H is assigned to five OMe groups, while a one proton doublet at 7.0 is assigned to C5'-H.

Further, ^1H -NMR spectrum of Ar-1 was run in benzene in order to determine the positions of the methoxyl groups (see Table 5). Our attention has been drawn by data published on benzene induced shifts of OMe resonances in flavones by comparison with OMe resonances in CCl_3 (63). The reported ($\text{CCl}_3 - \text{C}_6\text{H}_6$) for OMe at certain positions are given in

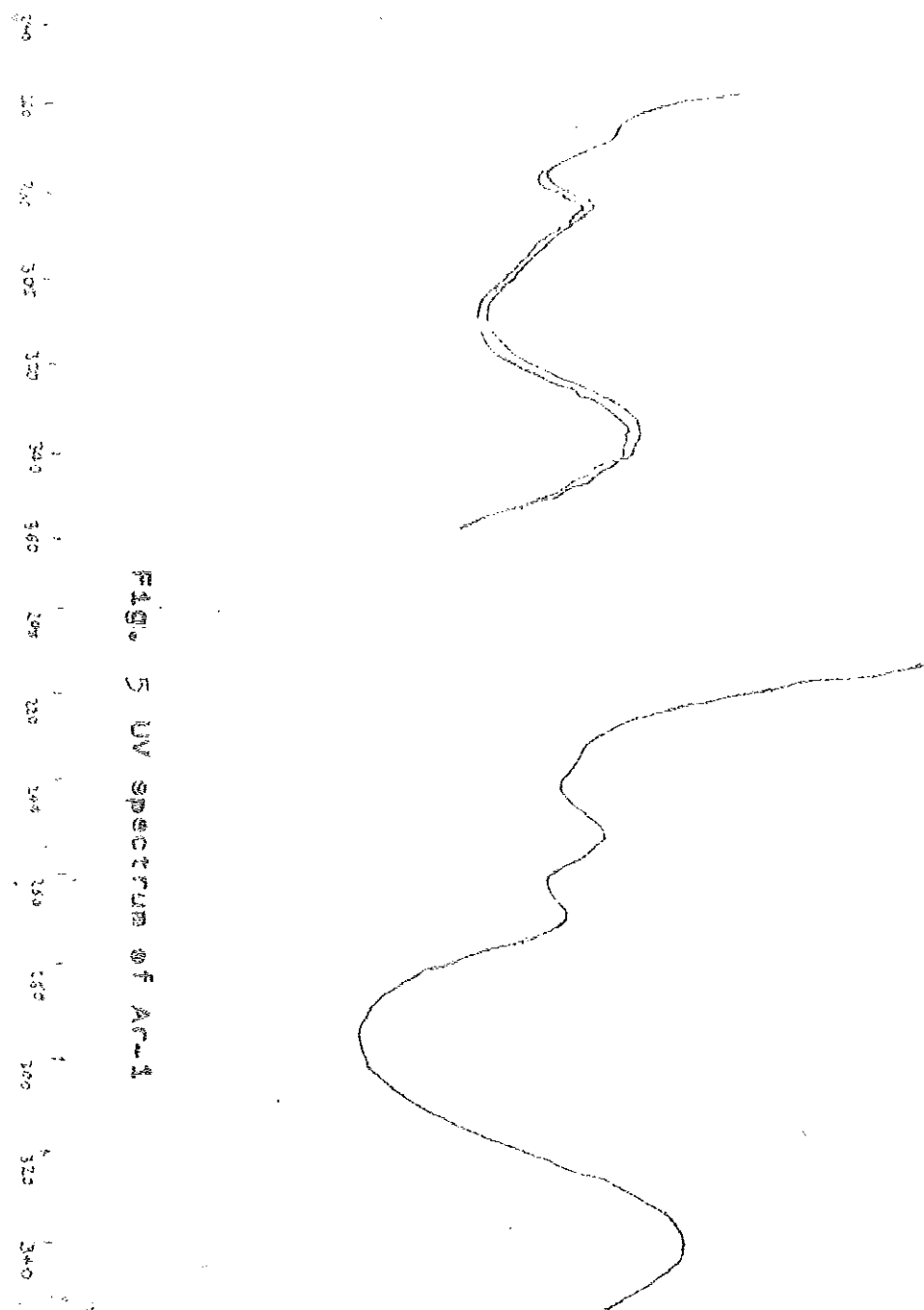


Fig. 5 UV spectrum of Ac-1

Table 4: ^1H NMR data (ppm) of Ar-1 in Chloroform-d at 90 MHz

H	ppm
C2'	7.72 (s) 1H
C5'-H	7.0 (d) 1H
C6'-H	7.8 (d) 1H
C5-OH	12.65 (s) 1H
C8-H	6.55 (s) 1H
C3, 7, 4'-OMe	4.00 (s) 9H
C6 - OMe	3.96 (s) 3H
C3' - OMe	3.9 (s) 3H

Table 6 whereas the methoxy flavones that have been utilized to assign OMe shifts by specific deuteration are given in Table 7. The ^1H NMR spectrum of Ar-1 in benzene exhibited five methoxyl groups resonating at separate shifts. All of the peaks were shifted upfield. However, only three methoxy groups were distinguishable in CDCl_3 .

The benzene induced shifts are accounted for the interaction of benzene with solute molecules and resulting in the formation of charge transfer complex between benzene and aromatic solute molecules (64). Methoxy resonances suffer upfield shifts in benzene. The diamagnetic anisotropy of the disc shaped molecules of benzene in suitable stereochemistry, tend to

10141	5
10151	52984
10161	9.7635
10171	521.1574
10181	12

	NO	FEEDBACK	TIME
1	1129.68	1.47	11.39
2	689.35	2.53	14.22
3	651.42	3.278	19.77
4	585.47	4.1	19.83
5	355.55	5.19	9.32
6	352.52	6.35	48.8
7	347.64	7.79	39.4
8	3.65	8.87	76.8

Fig. 6 ^1H NMR spectrum of Ar-1 in CDCl_3

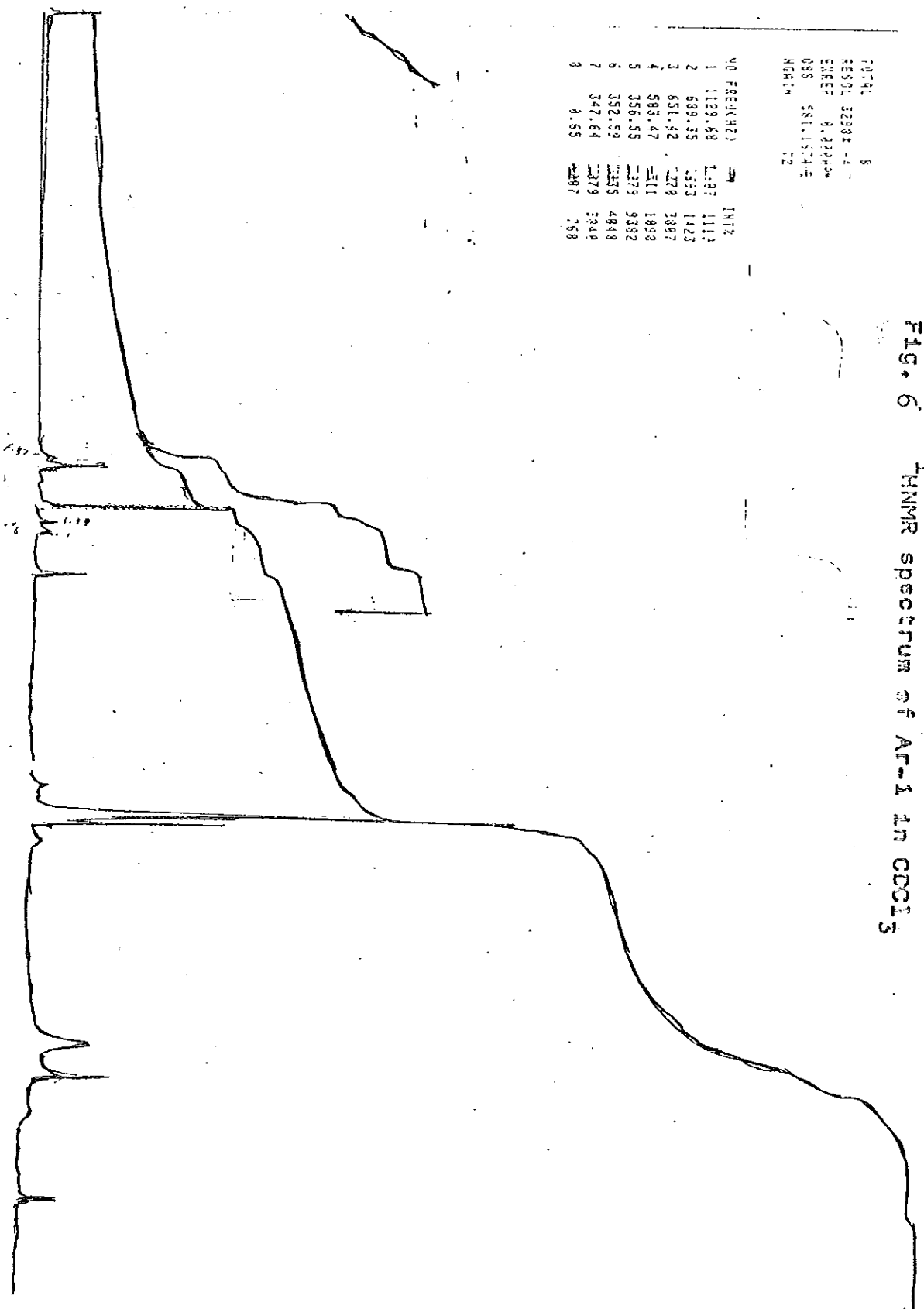


Table 5: ^1H NMR data (ppm) of Ar-1 in C_6D_6 at 90 MHz

OMe	ppm (C_6D_6)
C3-OMe	3.79
C6-OMe	3.92
C7-OMe	3.24
C3 ¹ -OMe	3.55
C4 ¹ -OMe	3.37

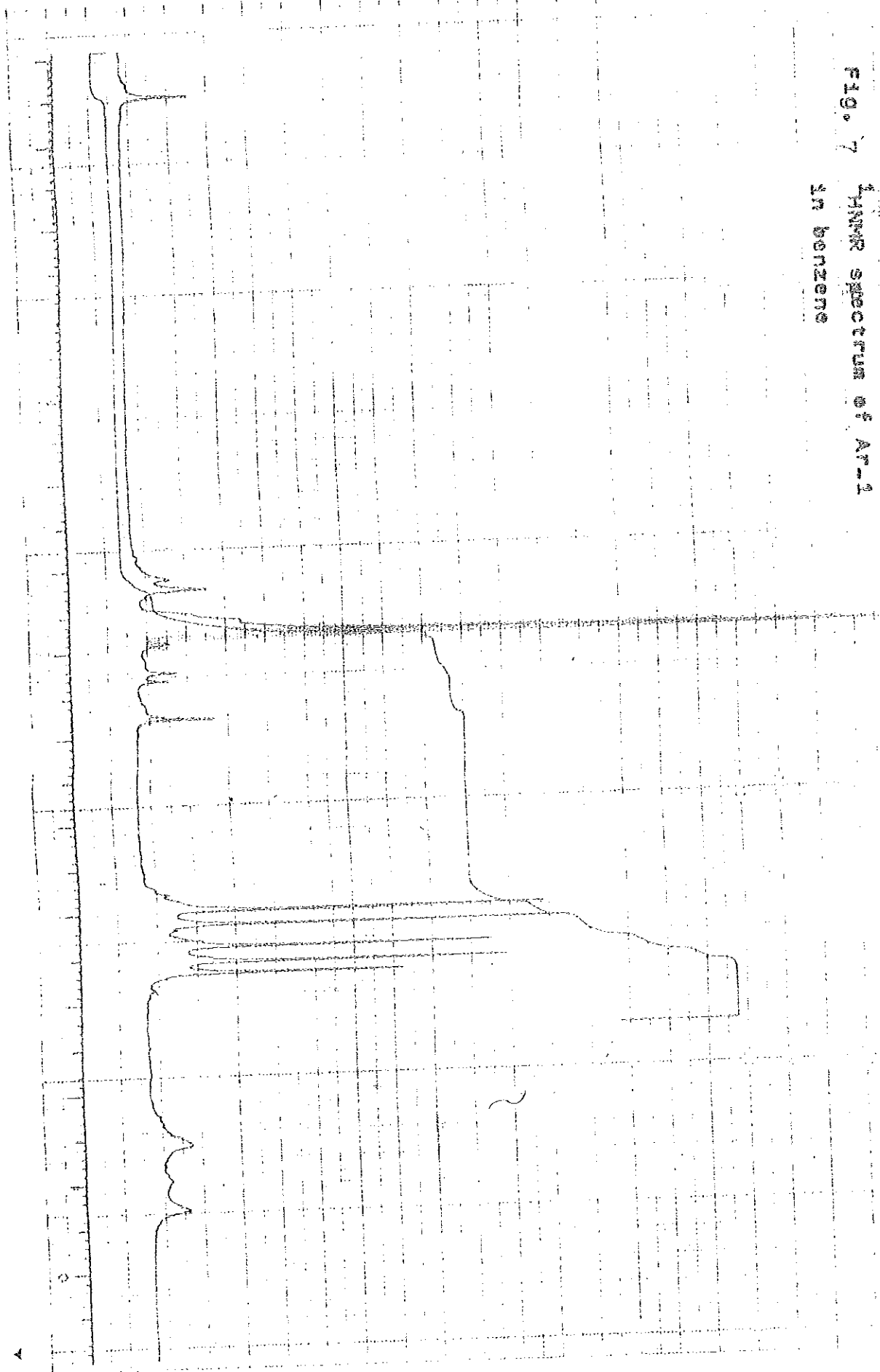
shield the nearby protons. (65,66). When the NMR spectra of flavonoids is examined first in CDCl_3 and then in benzene, the solvent shifts $\Delta = \delta_{\text{CDCl}_3} - \delta_{\text{C}_6\text{H}_6}$ ppm which are observed can be used to assign shifts of the methoxy resonances without much ambiguity.

The reported benzene induced solvent shift values for C3, C5, C7, C2¹ and C4¹, OMe groups in the absence of ortho methoxy or hydroxyl substituents are given in the table below.

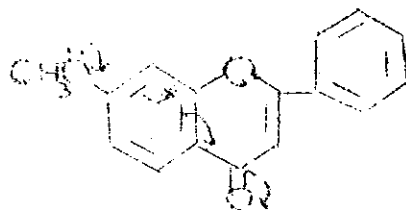
Table 6

Position of OMe	Range of values
C3	-0.07 to + 0.34
C5	+0.43 to + 0.58
C7	+0.54 to + 0.76
C2 ¹	+0.46 to + 0.53
C4 ¹	+0.54 to + 0.71

FIG. 7
 ^1H NMR spectrum of Ar-1
in benzene



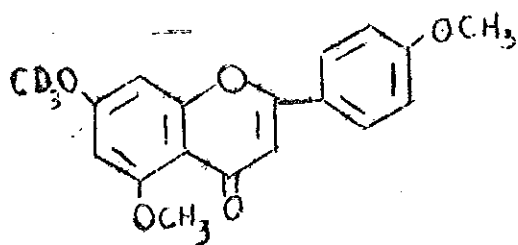
From this data it was reported that, for isolated OMe groups at C5, C7, C2¹ and C4¹ the Δ values are always larger than (0.43 ppm) that of the OMe resonance of anisole. This has been explained due to the ability of the OMe groups to conjugate with the carbonyl group. This will enhance the association of benzene with the oxygen atoms of the OMe whose electron density has now decreased, with a resultant increase in Δ values.



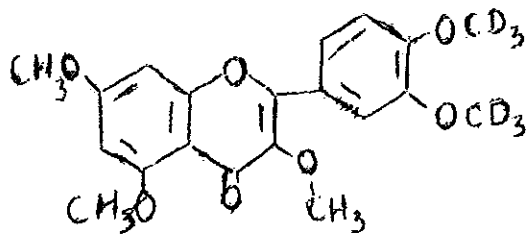
45

The flavones and the reported benzene induced shifts of the OMe resonances by specific deuteration is given below.

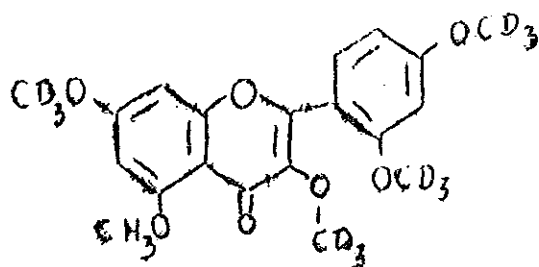
The carbons of Ar-1 were assigned by proton decoupled ¹³C-NMR and DEPT (distortionless enhancement by polarization transfer). An important application of the DEPT pulse train is for the generation of both individual proton coupled and proton decoupled ¹³C



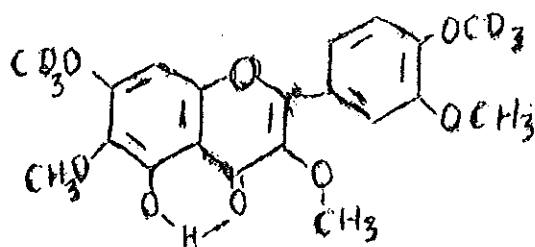
46



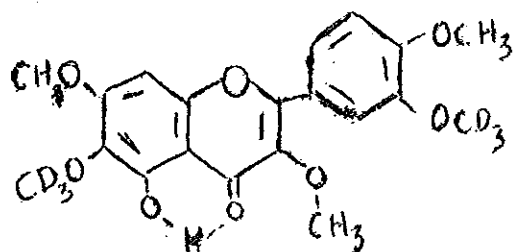
47



48



49a



49b

methine (CH), methylene (CH₂), and methyl (CH₃) sub-spectra. The carbon shift assignment is based on the use of a flavone as a model and additive substituent parameters for benzene. The presence of C6-methoxy

Table 7: Benzene induced shifts of the flavones 46, 47, 48, 49 .

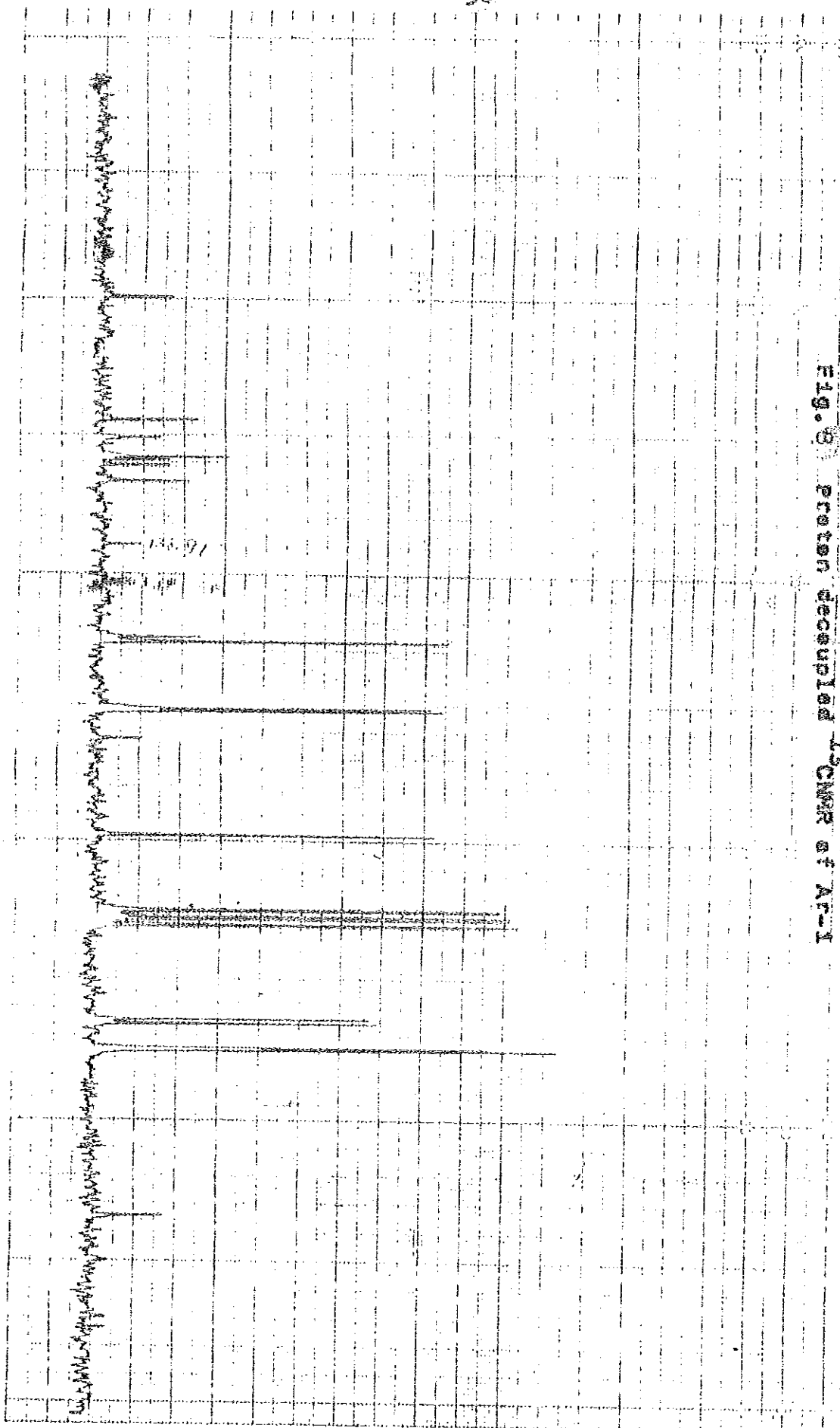
Compound	OMe	ppm CDCl ₃	ppm C ₆ D ₆
46	C5-OMe	3.96	3.35
	C7-OMe	3.90	3.29
	C4'-OMe	3.87	3.25
47	C3-OMe	3.93	3.84
	C5-OMe	3.93	3.37
	C7-OMe	3.93	3.26
	C3'-OMe	3.88	3.52
	C4'-OMe	3.86	3.37
48	C3-OMe	3.84	3.84
	C5-OMe	3.93	3.37
	C7-OMe	3.82	3.17
	C2'-OMe	3.80	3.31
	C4'-OMe	3.77	3.23
49	C3-OMe	4.00	3.78
	C6-OMe	3.95	3.92
	C7-OMe	4.00	3.28
	C3'-OMe	3.89	3.56
	C4'-OMe	4.00	3.40

diortho substituted was supported by the ¹³CNMR spectrum, in which the signal due to the 6-methoxy appeared downfield at 60.95 ppm. Methoxy carbons usually resonate in the range between 55 - 56.5 ppm., however, a further downfield shift between 59.5 and 63.0 ppm is

Table 8: ^{13}C -NMR Chemical Shift of Ar-1 in Chloroform-d

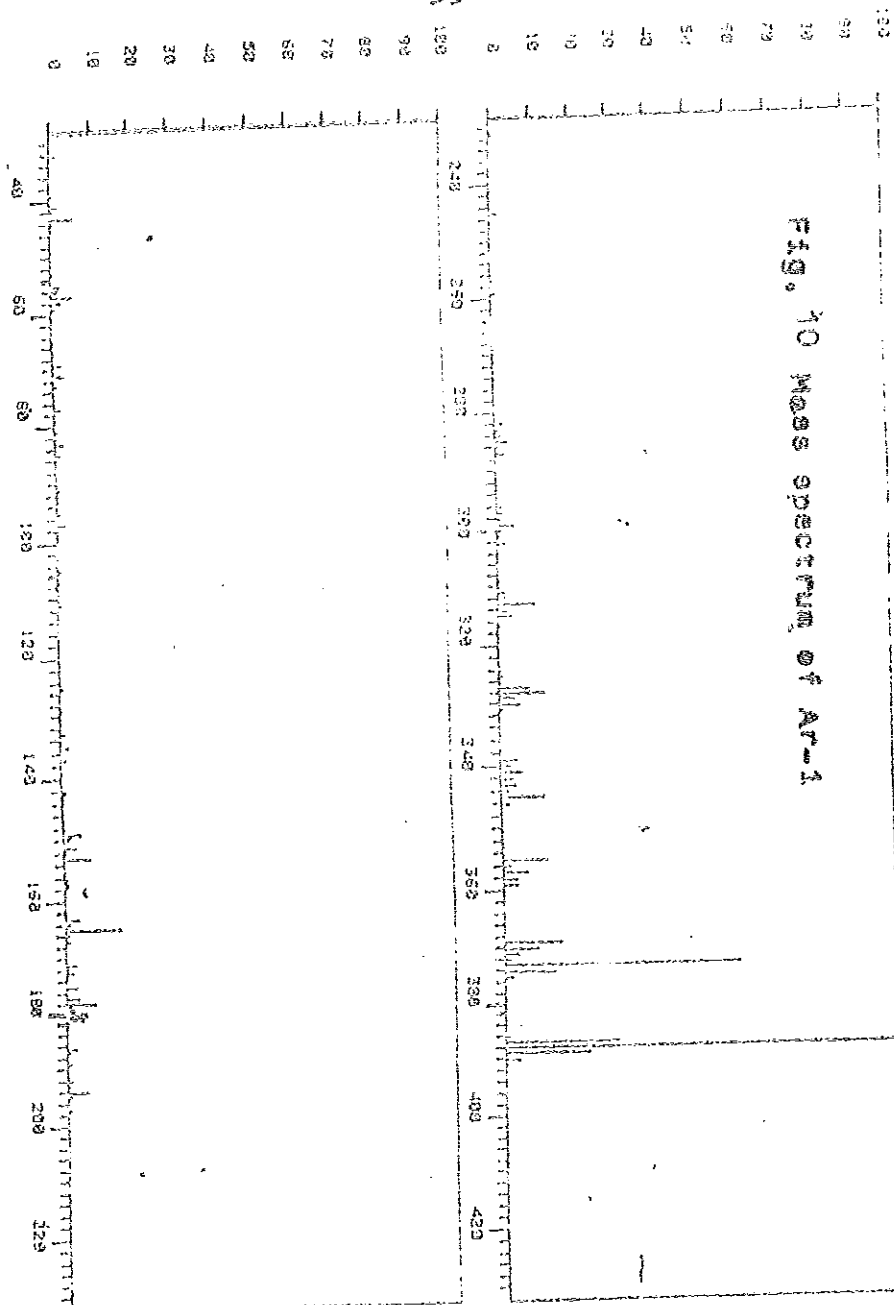
Carbons	(ppm)	Lit	ppm	Calculate	(ppm)
C 2	155.98				
C3	138.91				
C4	178.95				
C4a	106.68			105.9	
C5	152.84				
C6	132.34			132.8	
C7	158.85			156.3	
C8	90.42			93.9	
C8a	152.40			151.4	
C1 ¹	122.98			124.8	
C2 ¹	110.95			112.6	
C3 ¹	148.88			145.8	
C4 ¹	151.34			148.3	
C5 ¹	111.39			115.4	
C6 ¹	122.23			120.3	
C3-OMe	60.25				
C3 ^d -OMe	56.15				
C4 ^d -OMe	56.41				
C6-OMe	60.95				
C7-OMe	56.08				

Fig. 8. proton decoupled ^{13}C NMR of Ar-I

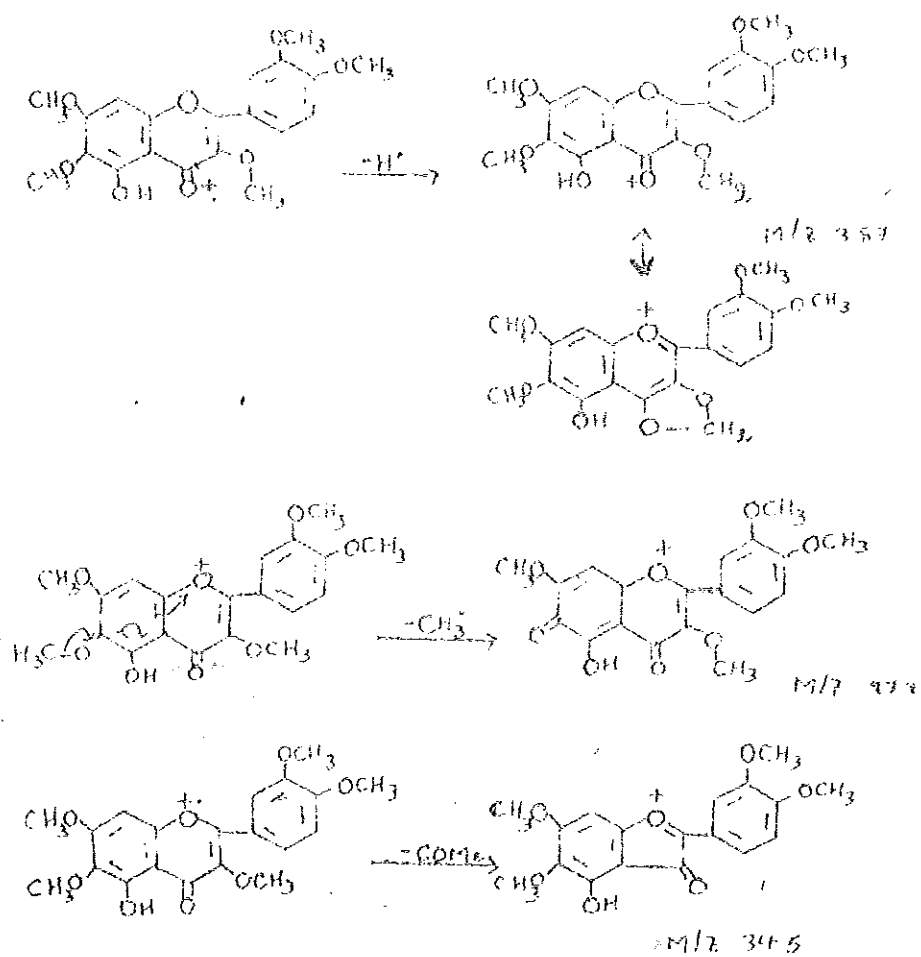


also observed when the methoxy group is diortho substituted by two bulky substituents such as OMe, OH or a ring junction (67). It has been suggested that steric inhibition to resonance between the methoxy oxygen and the aromatic ring are responsible for this (68). It has been reported that maximum overlap of orbitals of the lone pair of electrons in oxygen and the π electrons of the aromatic ring is favored when the methoxy group lies in the plane of the ring. (69).

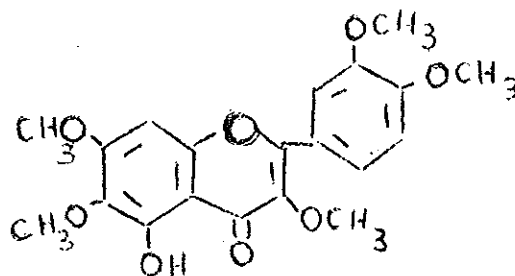
The greater abundance of M^+ as compared to $(M-15)^+$ in that $M^+ > (M-15)^+$ is indicative of 5-hydroxy 6,7 dimethoxy or 5,7, 8 trimethoxy flavones (70). The peak intensity for M^+ being (100%) and 60% for M-15 supports the 5-hydroxy 6,7-dimethoxy substitution pattern in Ar-1. 3 and/or 5-OMe flavones are known to display a $M^+ -1$ ion in their mass spectra (71). This again confirms the 3-OMe substitution of Ar-1 suggested by 1H NMR data mentioned above. On the basis of this the following fragmentation pathways have been suggested for Ar-1.



Scheme I. Suggested fragmentation pattern of the principal ions of Ac-1.



The following structure has been established for Ar-1, from the UV, IR, ^1H -NMR ^{13}C -NMR and MS spectral data. This structure corresponds to a natural product artemetin that has been isolated from many plants in



50

the family Asteraceae, UV, IR ^1H and ^{13}C -NMR data reported for this compound are consistent with our spectral data (1,72,73).

Table 9: List of Some Plants from which Artemetin was Isolated

Plant sources	Reference
<u>Achillea millefolia</u> (Ast)	74
<u>Artemisia absinthium</u> (Ast)	75
<u>Artemisia arborescens</u> (Ast)	76
<u>Blumea arianthes</u> (Ast)	77
<u>Bulmea lacera</u> (ast)	78
<u>Brickellia chlorolepis</u> (Ast)	79
<u>Brickellia cyclindraceae</u> (Ast)	79
<u>Brickellia varnicifolia</u> (Ast)	73
<u>Vitex negundo</u> (verb)	80
<u>Vitex trifolia</u> (verb)	81

Vulgarin

Vulgarin recrystallized as colorless needles m.p.

173 - 175°C. IR absorptions are: 3480 cm^{-1} (OH), 1770 cm^{-1} (γ -lactons) and 1663 cm^{-1} (α,β -unsaturated ketons). The sample was rigorously analyzed by two dimensional NMR techniques based on COSY (correlation spectroscopy) and ^1H - ^{13}C heteronuclear correlation experiments. In the COSY - 45 experiment the homonuclear correlated 2D-NMR spectra displays all of the proton - proton spin couplings on one 2D-plot where as in the heteronuclear correlated 2D-NMR of the ^1H - ^{13}C COSY, ^1H and ^{13}C are related through scalar coupling. The ^1H and ^{13}C chemical shift assignments which are based on these two COSY experiments were compared to the data available and the orientation of the enone chromophore was confirmed by CDLOC (correlation spectroscopy via long range couplings) a two dimensional experiment recently introduced by Kessler and Coworkers (82). This method employs H,C couplings via two and three bonds, ignoring short range couplings, and can be used with advantage for the assignment of quaternary carbon atoms, whose environment is thus determinable. Although NOE spectra show NOE at H-15 and H-14 when H-3 and H-2 are irradiated.

Table 10: ^1H NMR Data (ppm) of Vulgarin in Chloroform-d

H	ppm	Coupling constants
C2-H	5.85 (d)	$J_{2,3} = 9.1$
C3-H	6.57 (d)	$J_{2,3} = 9.1$, weak coupling to H-15
C5-H	2.39 (d)	$J_{5,6} = 11.5$
C6-H	4.13 (dd)	$J_{5,6} = 11.7$, $J_{6,7} = 10.6$
C7-H	1.65 (m)	
C8-H	α : 1.95 (m)	$J_{8e, 8a}^{13}, J_{8a, 7}^{13}, J_{8a, 9a}^3$
	β a: 1.44 (m)	
C9-H	β e: 1.99 (ddd)	$J_{9e, 9a}^{12}, J_{9a, 8a}^3$
	a : 1.54 (m)	
C11-H	2.32 (q)	
C13-H	1.21 (s)	
C14-H	1.17 (s)	
C15-H	1.52 (s)	

irradiated the effects are small due to the relatively large distances. Moreover, the distance differences for 3-H and H-3 with respect H-14 on one or H-15 on the other side is not large enough to allow a structural assignment with careful optimization of the experiment. Thus a method, that enables us to establish proton carbon connectivities over larger distance, i.e. over more than one bond including quaternary carbon atoms is required.

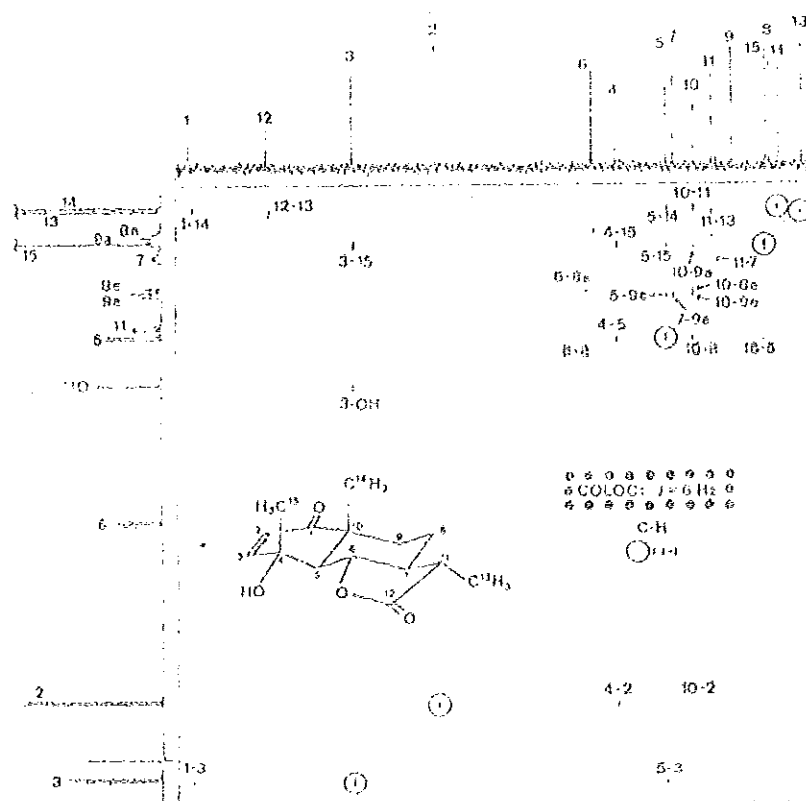
In COLOC experiment the vertical dimension gives ^1H and the horizontal ^{13}C chemical shifts. For each cross peak the first number indicates the carbon, the second the hydrogen involved in the ^{13}C - ^1H coupling. Encircled peaks refer to one bond couplings, (see Fig. 9). In our experiment the pulse sequence was optimized to couplings of 6H_z . In contrast to the heteronuclear correlation spectrum, cross peaks can be observed in the COLOC spectrum even for signals of quaternary carbons. The figure shows, that there are significant cross peaks indicating vicinal couplings.

The inconsistencies in the spectroscopic data of vulgarin that have led to different names of the same compound had been solved by reinvestigation of vulgarin and 4-enivulgarin, an epimer differing only in the configuration at C-4 (26). ^1H and ^{13}C -NMR spectra were unambiguously assigned based on COSY - 90° , ^1H - ^{13}C correlation experiments and X-ray study. Furthermore,

Table 11: ^{13}C -NMR Data of Vulgarin in d-chloroform

C	ppm
1	201.5
2	125.5
3	151.7
4	70.0
5	54.5
6	79.5
7	52.3
8	22.6
9	34.2
10	46.2
11	40.4
12	178.1
13	12.4
14	19.7
15	23.7

the stereochemistry at C-H was established by NOE measurements. However, the NMR techniques mentioned above were not able to establish the orientation of the enone chromophore which is isolated from the rest of the molecule by two quaternary carbon atoms. We have been able to established this by the ^2D - ^{13}C , COLOC correlation

Fig41 ^1H , ^{13}C -COLOC spectrum of vulgarin

^{13}C between C-1 and H-14 C-3 and H-15 C-3 and H-3 as well as C-1 and H-2. However, no such peaks can be observed if the orientation of the enone group is suggested with the carbonyl being at C-3 and double bond between C-1 and C-2.

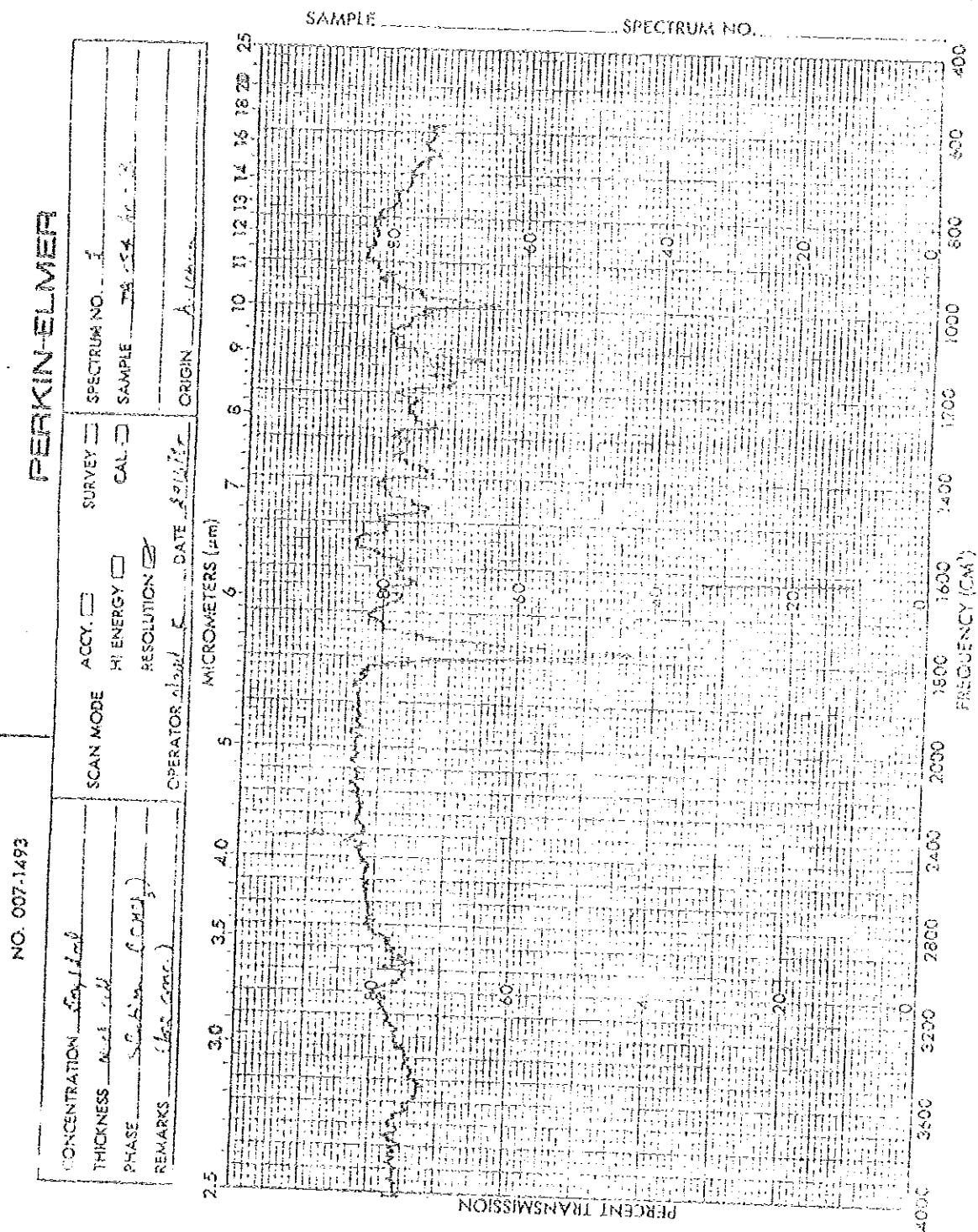
3.1 Structural Elucidation of Ar-3

Ar-3 is a yellow oily substance, a single spot on tlc (benzene - acetone 9:1, CHCl_3 and CHCl_3 :EtOAc 9:1), that turned red when sprayed with vanillin sulfuric acid and heated. With hydroxylamine - ferric chloride the tlc plate turned green like that of vulgarin, a positive test for a lactone. It has $(\alpha)_D^{25} + 9.0^\circ$ (1.5mg/ml CHCl_3) and IR spectrum showed absorption bands at 3550 cm^{-1} (OH) and 1770 cm^{-1} (β -lactone). The proton decoupled ^{13}C -NMR spectrum gave signals for 15 carbons suggesting a sesquiterpene lactone.

Furthermore, the chemical shifts gave definitive information about the functional groups, 179.65 (C=O), 133, 121 (C=C) and 75.3 (-OH). This suggests a sesquiterpene lactone having an OH and one site of unsaturation. From the ^1H -NMR the three proton singlet at 0.8 suggests a C14-H at an angular position for an eudesmanolid (83).

From this information the following structure could be suggested. Based on this structure the

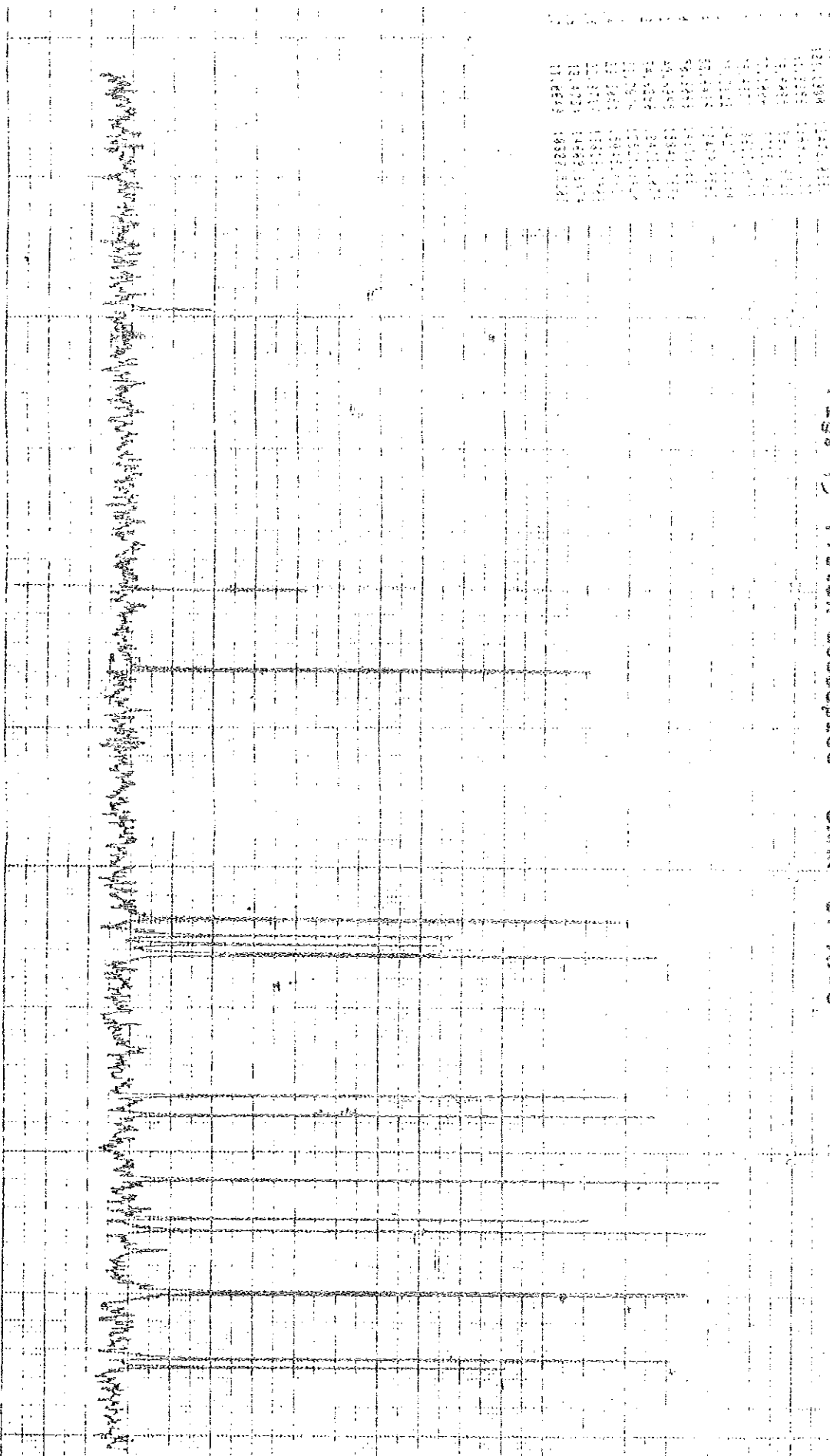
Fig. 12 IR spectrum of Ar-3

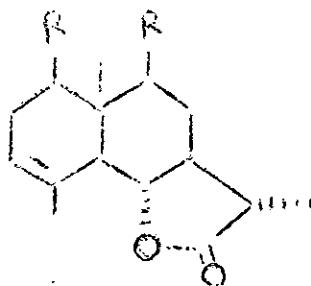


100
90
80
70
60
50
40
30
20
10
0

100
90
80
70
60
50
40
30
20
10
0

Fig. 13 Proton decoupled ^{13}C NMR of Ar-5





R=H, R=OH or 51

R=OH, R'=H

following assignments were made from ^1H NMR data.
 0.8 (s, C10-Me), 1.20 (d, C11-Me), 1.7 (broad s, C4-Me),
 3.60 (dd, C1-H), 3.9 (broad t C6-H) and 5.3 (C3-H).
 This structure was further supported by DEPT spectrum,
 which shows the presence of 6 methine (CH), 3-methylene
 (CH_2) and 3-methyl (CH_3) groups. Information concern-
 ing the multiplicities of ^{13}C signals were obtained
 from proton off - resonance decoupling experiment.

Table 12: Partial ^1H NMR Chemical Shift Data (ppm) in
 Chloroform-d at 90 $^\circ\text{C}$ MHz

H	(ppm)
1	3.6 (dd)
3	5.3 (br.t)
6	3.9 (brt)
13	1.2 (d)
14	0.8 (s)
15	1.7 (br.s)

FIG. 14 ^1H NMR spectrum of Ar-3

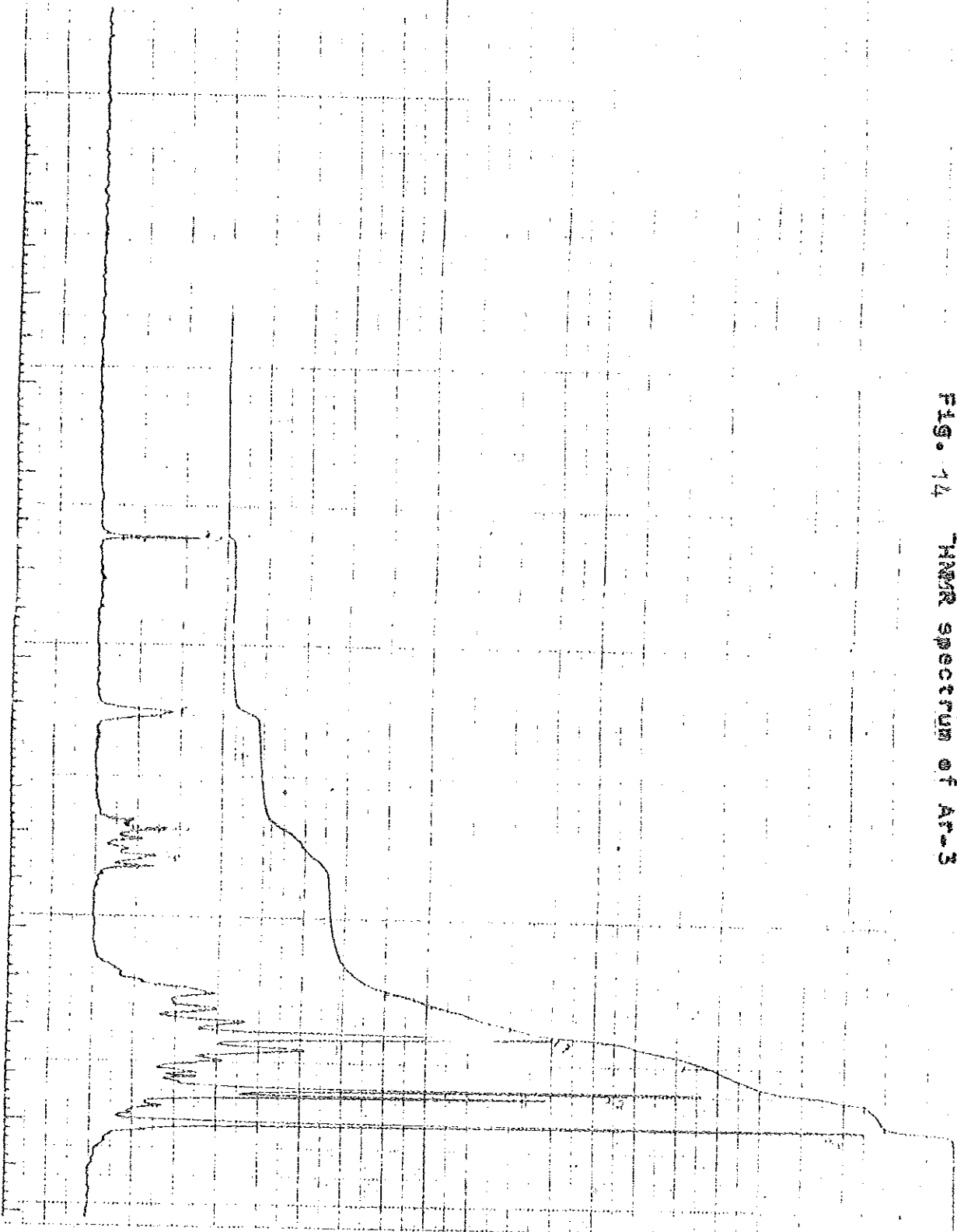
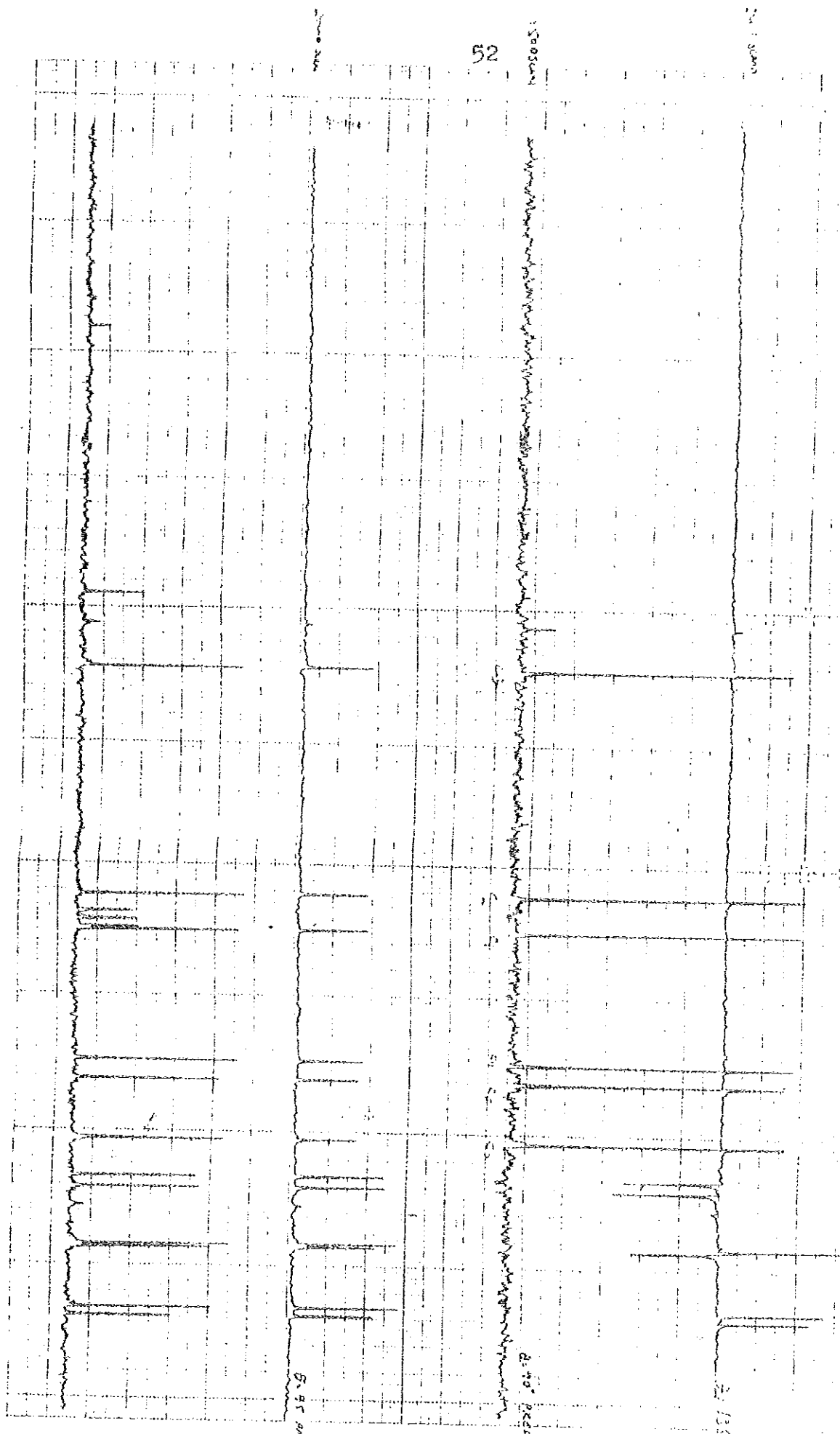
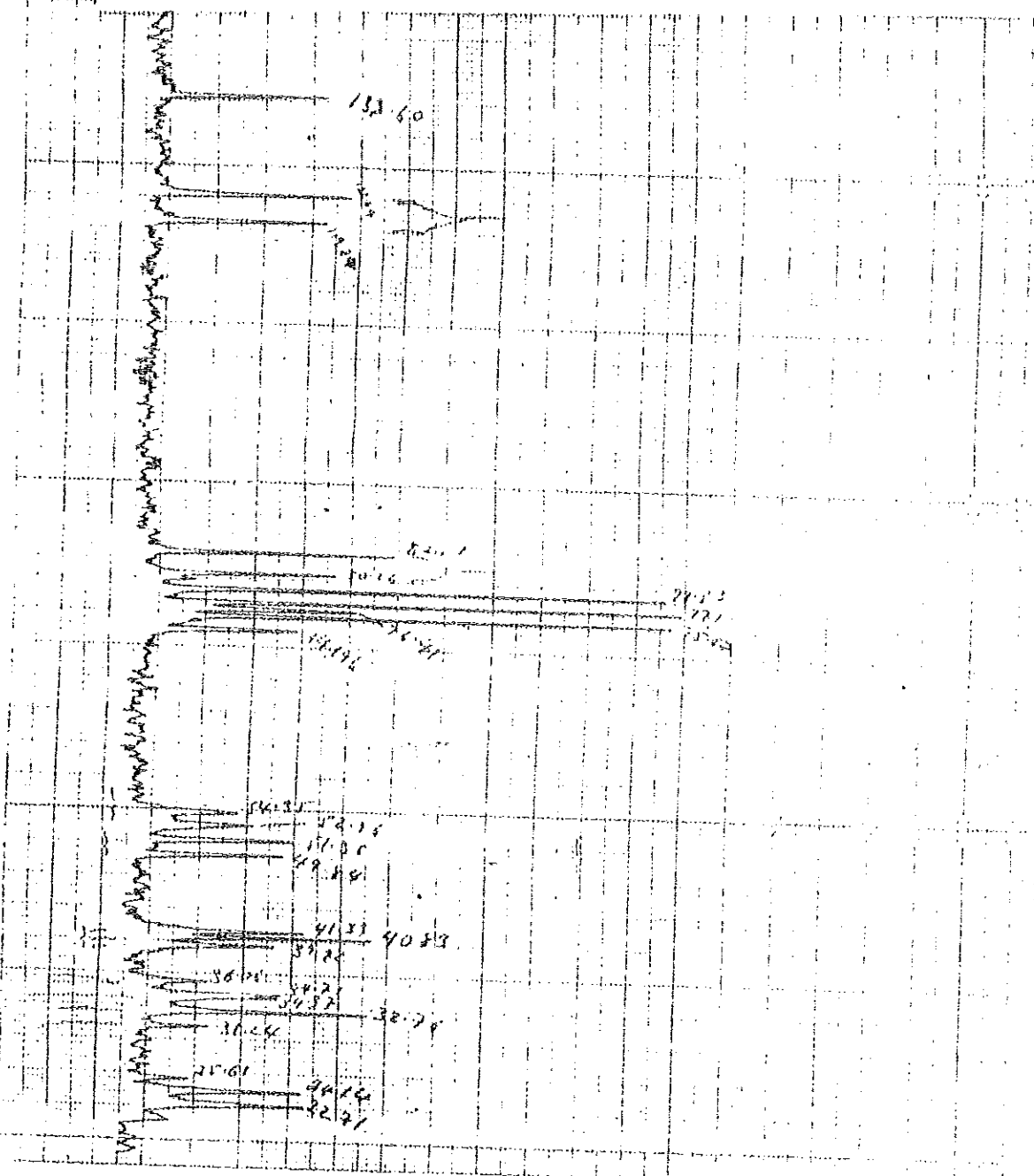
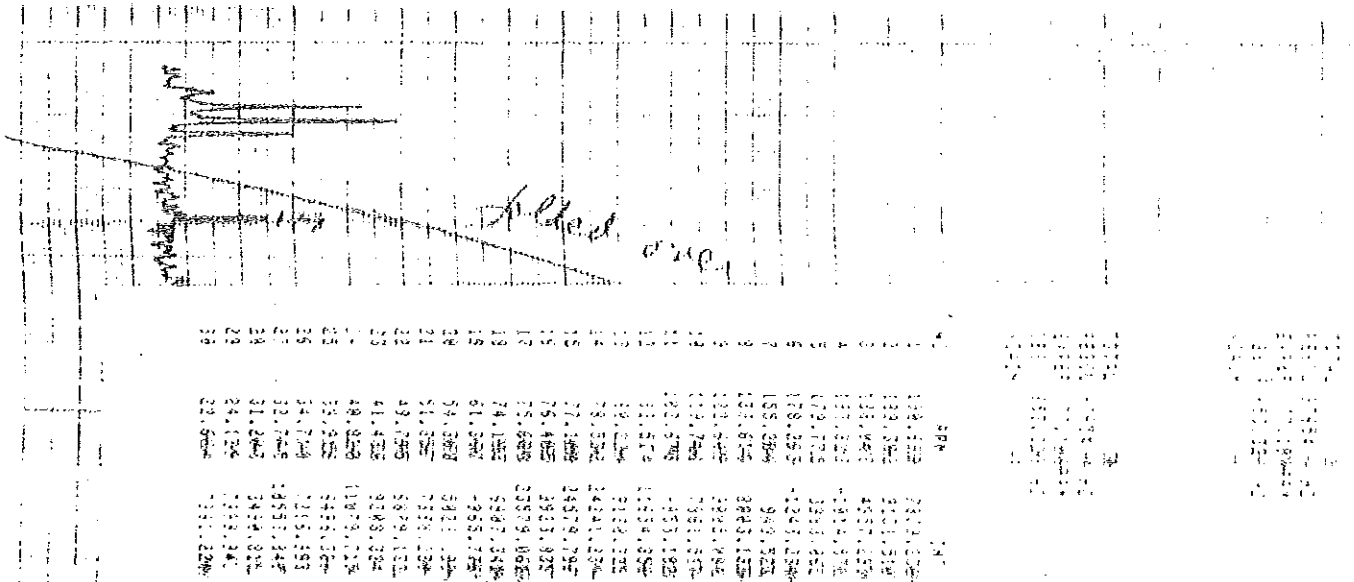


Fig. 15 DEPT spectrum of Ar-3





59.15: Off resonance spectrum of H4A-3

Table 13: ^{13}C -NMR Chemical Shift Data (ppm) in Chloroform-d 90 MHz

C	ppm
1	75.3 (d)
2	32.8 (t)
3	121.2 (d)
4	133.6 (s)
5	50.6 (d)
6	81.3 (d)
7	53.6 (d)
8	22.9 (t)
9	34.6 (t)
10	40.3 (s)
11	47.7 (d)
12	179.7 (d)
13	12.5 (q)
14	11.1 (q)
15	23.3 (q)

4. EXPERIMENTAL

4.1. General Experimental Procedure:

Melting points were determined on a Thomas Hoover melting point apparatus and are not corrected. UV spectrum were run on UV-Beckman Model 24. Infrared spectra were determined using a Perkin - Elmer model 727B grating spectrophotometer. MS, ^1H -NMR and ^{13}C -NMR were determined by Jeol FX 90Q FT NMR Spectrometers.

Analytical thin layer chromatography were run on a 0.25 mm thick layer of silica gel GF₂₅₄ (Merck). Adsorbent used for column chromatography was silica gel 60₂₅₄ (70 - 230 mesh) Merck.

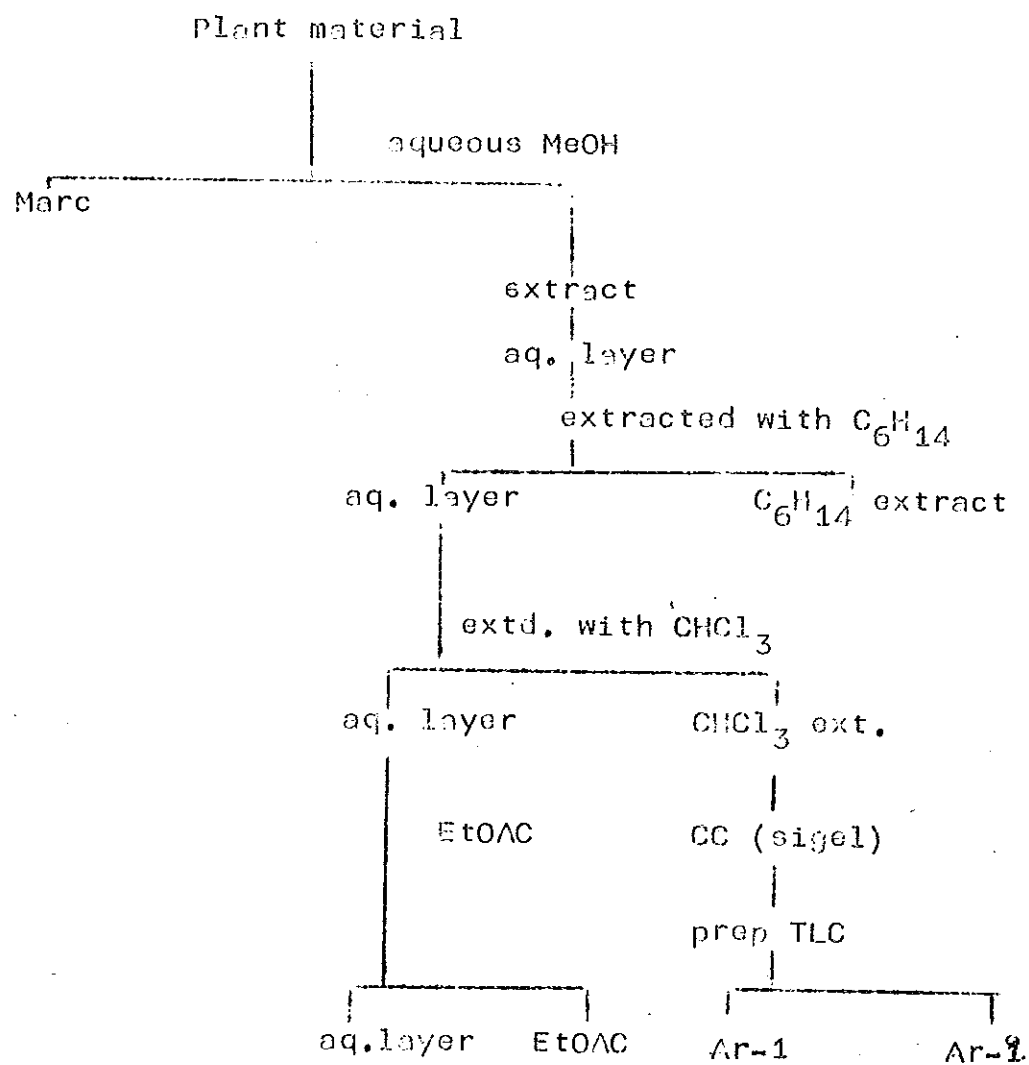
4.2 Extraction and Purification

Plant material was collected from the experimental garden of the Department of Chemistry AAU. This was air dried and powdered prior to extraction. 100 g of the aerial parts was extracted with 4 x 500 ml of 80% and 9 x 500 ml of 50% aqueous MeOH. The combined extracts were concentrated under reduced pressure until only water remained. The aqueous layer was extracted first with hexane followed by chloroform and finally with ethylacetate (73). The material from the chloroform extract (7.5 g) was chromatographed over Si gel column packed in the elution solvent. The column was eluted first with hexane. Polarity of solvent was in-

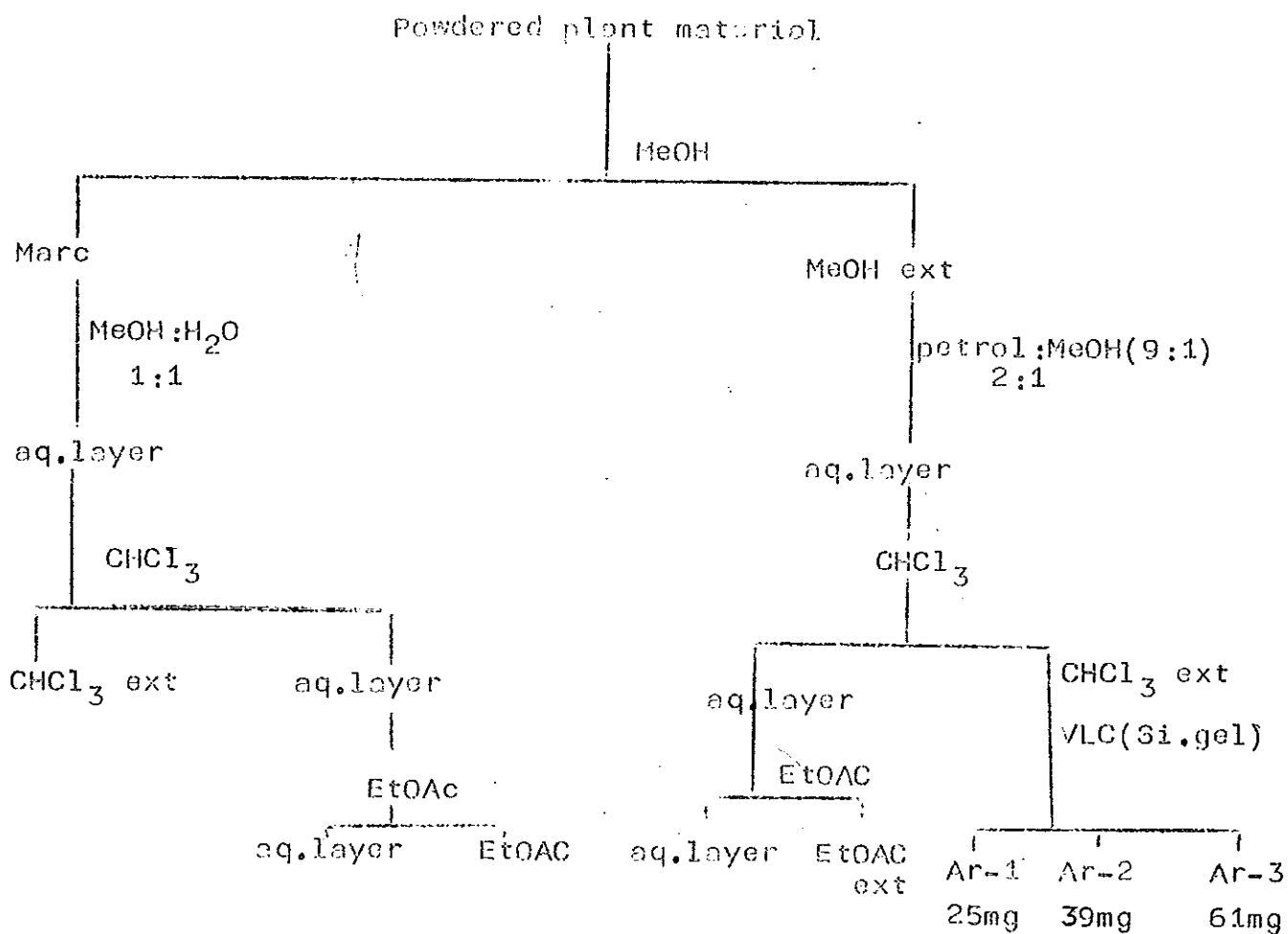
created by adding EtOAc until the column was finally eluted with methanol. Fractions that contained artemetin, vulgarin and two other spots (detection in UV light) were mixed. The mixture was further purified by prep. tlc on Si gel using solvent system benzene-acetone (8:2). 53 mg of artemetin and 41 mg pure vulgarin while 44.1 mg of partially purified vulgarin were obtained. Vulgarin was recrystallized from cold EtOAc.

In another experiment 507.4 g of the dried and powdered aerial parts of the plant was extracted with MeOH at room temperature. 55.8 g of crude extract was further extracted with petroleum ether (50-70°C) - MeOH:H₂O (9:1) mixture in the ratio 2:1 three times to remove fats and chlorophyll. The aqueous layer was then extracted with chloroform followed by EtOAc. The chloroform extract weighed 30.6 g while the EtOAc extract weighed 0.539 g. Marc from the methanol extract was extracted with MeOH-H₂O (1:1) and the aqueous layer was subjected to similar extraction procedures mentioned above.

Scheme 14: Experimental Procedure - 1



Scheme 3: Experimental procedure -2



erial parts of the plant was extracted with MeOH at room temperature. 55.8 g of crude extract was further extracted with petroleum ether (50-70°C) - MeOH:H₂O (9:1) mixture in the ratio 2:1 three times to remove fats and chlorophyll. The aqueous layer was then extracted with chloroform followed by EtOAc. The chloroform extract weighed 30.6 g while the EtOAc extract weighed 0.539 g. More from the methanol extract was extracted with MeOH-H₂O (1:1) and the aqueous layer was subjected to similar extraction procedures mentioned above.

4.3 Purification by Vacuum Liquid Chromatography

10 g of the chloroform extract was applied over 80 g of Si gel (Merck) packed on a sintered glass funnel. Elution started with benzene followed by benzene - acetone mixture (85:15). Artemetin and a sesquiterpene were together with other impurities were eluted with benzene, while vulgarin eluted when polarity was increased. Fractions that contained artemetin and the ternenoid were purified by VIC on 20 g of prep. grade Si gel (Merck). Solvent system used was benzene - chloroform - ethylacetate (5:4:1). Artemetin was purified by chromatography on microcolumn (benzene followed by benzene - acetone) and finally by recrystallization with

MeOH. Like wise, the terpenoid was purified by repeated chromatography on microcolumn (CHCl_3) followed by purification on sephadex LH-20 (CHCl_3 -MeOH) 2:1 and 1:1). Vulgarin was recrystallized from cold EtOAc. Visualization of the spots was made possible by spraying the tlc plates with vanillin - sulfuric acid.

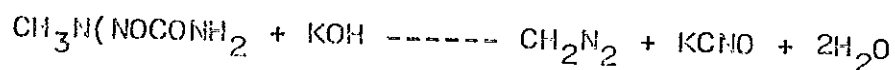
4.4 Attempted Methylation of Artemetin

24.7 mg of the sample was dissolved in a minimum amount of chloroform. To this a few drops Et_2O and some drops of diazomethane was added till the yellow color persisted. The solution was kept on an ice bath in the hood. MgSO_4 was added for drying. Artemetin was recovered unchanged, tlc comparison with an authentic sample (benzene-acetone 9:1 and hexane-acetone 8:2) gave same Rf.

Diazomethane was Prepared as follows:

12 ml 50% (W/V) of KOH (aqueous) was placed in 100 ml erlenmeyer flask placed in an ice bath. To this, 40 ml diethylether was added and also Nitrosomethyl urea with shaking (2 gms).

The color of the ether layer was observed to change from colorless to yellow as the reaction



This was pipetted out and added to the solution

containing the flavonoid.

4.5 Bioassay

Test for antibacterial activity: the crude methanol extract of the dried aerial parts of the plant was tested by the Agar-disc diffusion method. Amount of material used for the test was 300 ug/disc. No antibacterial activity against the test organisms was observed. The following test organisms were used: S. aureus, Bacillus subtilis, E. coli, Shigella soni, Pseudomonas aeruginosa

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