PHYTOCHEMICAL STUDIES OF

ARTEMISIA ANNUA L.

THESIS

Presented by

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ABSTRACT

Artemisia annua L. belongs to the tribe *Anthemideae* of the Compositae and comprises some 400 species which are widely distributed, especially in Asia, North America and Europe. A number of species are of medicinal value in traditional medicine and in particular *A. annua* has been used in China for centuries for the treatment of liver disease and malaria. In the search for effective novel antimalarial drugs, the sesquiterpene lactone, artemisinin (qinghaosu, QHS) has been isolated previously from *A. annua*. Artemisinin is active against chloroquine-resistant *Plasmodium falciparum* in the treatment of cerebral malarial. It has been reported that the *in vitro* antimalarial activity of artemisinin and some of its derivatives is markedly enhanced by the presence of methoxylated flavones such as artemetin and casticin. Hence it was of interest to isolate other methoxylated flavones from *A. annua* to ascertain the extent to which such compounds may affect the activity of artemisinin.
Thirty four flavonoids, including six novel flavonoids, four coumarins and two novel chromene compounds have been isolated and identified during the present investigation. Their structure elucidation is based on spectroscopic analyses (UV, 'H NMR and MS). The following compounds were identified and apart from casticin and scopoletin they have not been reported previously as constituents of *A. annua* : casticin, chrysoplenetin, chrysosplenol-D, circilineol, penduletin, eupatorin, axillarin, cirsiliol, tamarixetin, rhamnetin,

quercetin-3-methylether, cirsimaritin, rhamnocitrin, chrysoeriol, apigenin, luteolin, kaempferol, quercetin, isorhamnetin, luteolin-4'-methylether, isokaempferide, quercetagetin-3-methylether, tomentin, astragalin, luteolin-7-glucoside, quercetin-3'-glucoside, isoquercitrin, quercimeritrin, scoparone, fraxetin-8-methylether, and 5,6-dimethoxycoumarin. The following six novel flavonoids and two novel chromenes were also isolated and characterised: quercetagetin-4'-methylether, quercetagetin-3,4'-dimethylether, 5,2',4'-trihydroxy-6,7,5'-trimethoxyflavone, 5,7,8,3'-tetra hydroxy-3,4'-dimethoxyflavone, quercetagetin-3-methylether-4'-glucoside, gossypetin-3-methylether-3'-glucoside, 2,2,6-tri hydroxychromene and 2,2-dihydroxy-6-methoxychromene. Some of them, e.g., casticin, chrysoplenetin, chrysosplenol-D and circilineol have weak antimalarial activity and can potentiate the *in vitro* activity of artemisinin against *Plasmodium falciparum*.

PART 1. INTRODUCTION

1.1. General introduction

The genus *Artemisia* belongs to the tribe *Anthemideae* of the Compositae and comprises some 400 species [1]. A number of species is used for traditional medicine and *Artemisia annua* L. has been used for centuries in the treatment of malaria, low-grade fever, scabies, pruritus, and malignant ulcer. *A. apiacea* Hance was used to treat liver disease and *A. caerulescens* subsp. *gallica* has been used for the treatment of asthma. Many chemical studies of the genus *Artemisia* have been reported and most of the constituents obtained are either flavonoids or sesquiterpene lactones [2]. Artemisinin, a sesquiterpene lactone active against chloroquine-resistant *Plasmodium falciparum* in the treatment of cerebral malaria, was isolated from *A. annua* in 1972 [3]. This compound has been used successfully in several thousands of malaria patients in China, including those with infections of either chloroquine-sensitive or chloroquine-resistant strains of *P. falciparum*. Thus, artemisinin offers promise as a totally new class of antimalarial drug. Interest has focussed recently on the genus and some thirty species have been examined by Chinese researchers but none of these other species yielded extracts with antimalarial activity [4]. American workers have extracted *A. ludoviciana*, *A. vulgaris*, *A. schmiviana*, *A. portia*, *A. arbuscula* and *A. dracunculus*, but none of these species contained artemisinin [5].

Elford et al reported that some methoxylated flavones can enhance the *in vitro* anti *Plasmodium falciparum* activity of artemisinin [6]. The present study was designed to make a detailed phytochemical investigation of *A. annua* and was initiated to isolate other methoxylated flavones, to determine their chemical structures and to find structure-activity relationships.

1.1.1. The genus Artemisia

The genus *Artemisia* is the largest and most widely distributed of ca 60 genera in the tribe Anthemideae of Asteraceae (Compositae). This genus,which has about 400 species, is divided into four subgenera, *Artemisia*, *Seriphidium*, *Tridentatae* and *Dracunculus* [1,7]. Species are distributed in Asia, Europe, America and Africa [7]. The chemical characteristics of the genus are essential oil, coumarins, flavonoids and sesquiterpene lactones. A number of species, for example *A. cina* and *A. maritima*, have been used as anthelmintics particularly for roundworm infestation [8]. The active vermifuge constituents of *Artemisia* species are sesquiterpene lactones and one of these compounds, α-santonin, was official in the British Pharmacopoeia until 1963. Some *Artemisia* species have been used medicinally as bitters, for example *A. absinthum* which is used to flavour the liqueur absinthe but is no longer used in many countries because of its toxicity. Thujone, a bicyclic monoterpene, is the major constituent of the volatile oil of *A. absinthum*. *A. apiacea*

has been used in traditional Chinese medicine for centuries for the treatment of liver disease [9]. A. annua is used to treat malaria, low-grade fevers [10], scabies, pruritus, and malignant ulcers [11]. A sesquiterpene lactone, with an endoperoxide bridge, known as artemisinin (qinghaosu, QHS), has been isolated from the Chinese A. annua and has been shown to be active against chloroquine-resistant P. falciparum.

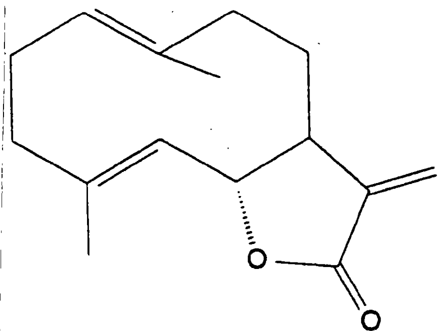
1.1.2. Previous phytochemical studies

Phytochemical studies of the genus Artemisia have been reported for a great number of species. The compounds isolated include essential oil, coumarins and monoterpenes, but mostly they were sesquiterpene lactones and flavonoids.

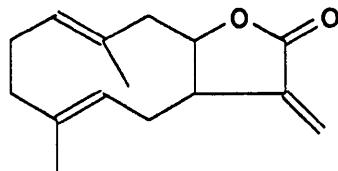
1.1.2.1. Sesquiterpene lactones

There are many species of Artemisia containing sesquiterpene lactones. Yoshioka et al. in 1973 reported some 60 sesquiterpene lactones in about 36 species [12]. The types of sesquiterpene lactones reported include germacranolides, eudesmanolides, guaianolides, pseudoguaianolides [Figure 1] and other skeletal types. Kelsey et al. in 1979 reported 124 sesquiterpene lactones in the genus [13]. These reports are summarised in Appendix 1 . (page 158)
In more recent years, about 74 new sesquiterpene lactones have been isolated from Artemisia species. They include 27 eudesmanolides, 27 guaianolides, 9 glaucolides, 8 germacranolides and 3 other types. They are summarised in Appendix 1.

SESQUITERPENE LACTONES

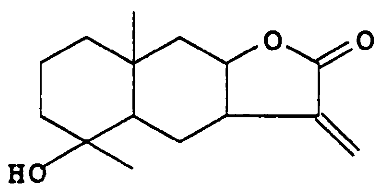


1



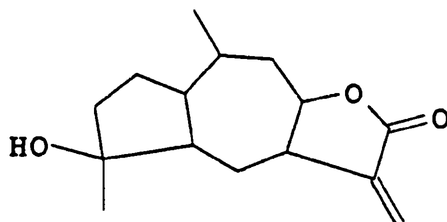
2

Germacranolides



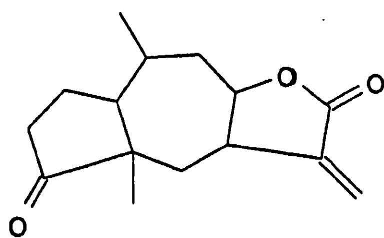
3

Eudesmanolides



4

Guaianolides



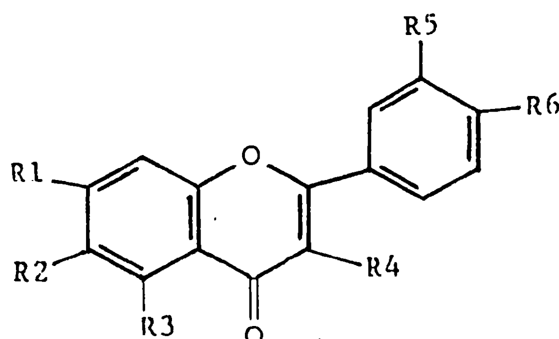
5

Pseudoguaianolides

1.1.2.2. Flavonoids

Flavonoids have been isolated from several species of Artemisia. The first flavone, artemetin, was isolated from A. absinthum in 1962 by Geissman [14]. After that, some 108 flavonoids have been isolated from the genus. The majority of the flavonoids are oxygenated flavones (6) and flavonols (7) (Figure 2) or their glycosides. Six of major flavonoids are shown in Figure 2 and the flavonoids isolated from Artemisia species are summarised in Appendix 2. (page 169)

Figure 2 The major flavonoids of Artemisia species

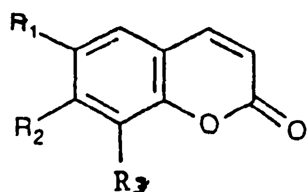


6	Flavone	R1 = R2 = R3 = R4 = R5 = R6 = H
7	Flavonol	R1 = R2 = R3 = R5 = R6 = H R4 = OH
8	Artemetin	R1 = R2 = R4 = R5 = R6 = OCH_3 R3 = OH
9	Casticin	R1 = R2 = R4 = R6 = OCH_3 R3 = R5 = OH
10	Quercetin	R1 = R3 = R4 = R5 = R6 = OH R2 = H
11	Apigenin	R1 = R3 = R6 = OH R2 = R4 = R5 = H
12	Kaempferol	R1 = R3 = R4 = R6 = OH R2 = R5 = H
13	Luteolin	R1 = R3 = R5 = R6 = OH R2 = R4 = H

1.1.2.3. Other types of compounds

Coumarins, chromanones, polyacetylenic derivatives and other types of compounds have also been isolated from *Artemisia* species.
The coumarins including coumarin (14), umbelliferone (15), 7-methoxycoumarin (16), scopoletin (17), isoscopoletin (18), scopolin (19), scoparone (20), 7,8-dimethoxycoumarin (21), and isofraxidin (22) have been isolated from the genus [15,16,17,18] (Figure 3).

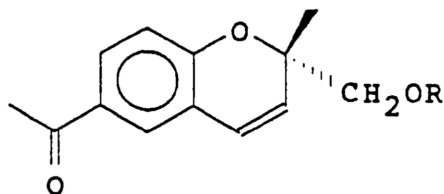
Figure 3 Coumarins isolated from *Artemisia* species



14	Coumarin	R1 = R2 = R3 = H
15	Umbelliferone	R1 = R3 = H R2 = OH
16	7-Methoxycoumarin	R1 = R3 = H R2 = OCH_3
17	Scopoletin	R1 = OCH_3 R2 = OH R3 = H
18	Isoscopoletin	R1 = OH R2 = OCH_3 R3 = H
19	Scopolin	R1 = OCH_3 R2 = OGluco R3 = H
20	Scoparone	R1 = R2 = OCH_3 R3 = H
21	7,8-Dimethoxycoumarin	R1 = H R2 = R3 = OCH_3
22	Isofraxidin	R1 = R2 = OH R3 = OCH_3

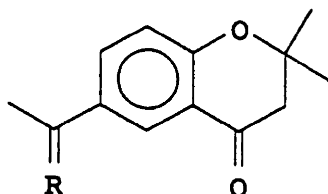
Some chromenes and polyacetylenic derivatives, 6-acetyl-2-methyl-2-hydroxymethylchromenen (23), 6-acetyl-2-methyl-2-acetyl-hydroxymethylchromenen (24), 6-acetyl-2,2-dimethylchromanone (25), 6-(1-hydroxyethyl)-2,2-dimethylchromanone (26), capillene (27), capillin (28),capillarin (29), artepillin-A (30), artepillin-C (31), capillartemisin-B1 (32), have been isolated from Artemisia species [19, 20, 21] (Figure 4).

Figure 4 Chromenes, chromanones and polyacetylenic derivatives isolated from Artemisia species



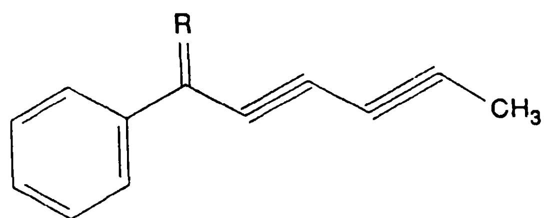
23 6-Acetyl-2-methyl-2-hydroxymethylchromene R = H

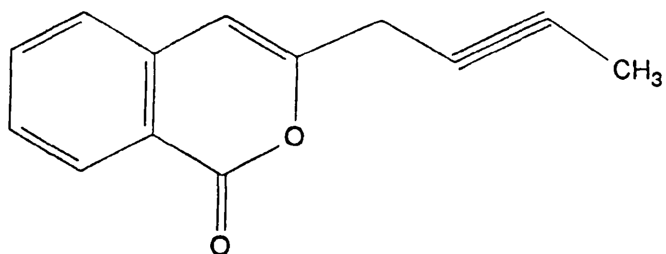
24 6-Acetyl-2-methyl-2-acetyl-hydroxymethylchromene R=Ac



25 6-Acetyl-2,2-dimethylchromanone R = O

26 6-(1-hydroxyethyl)-2,2-dimethylchromanone R = H , OH

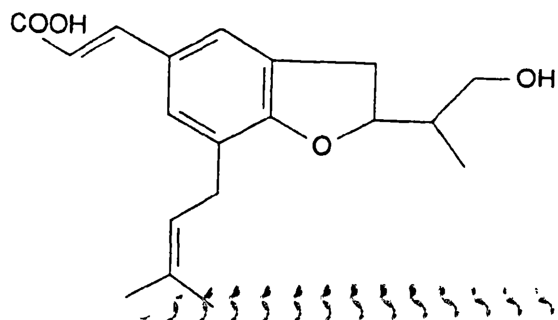


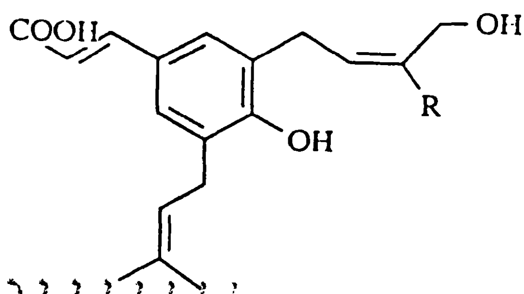


27 Capillene R = 2 H

28 Capillin R = O

29 Capillarin





30 Atepillin-A

31 Artepillin-C R = H

32 Capillartemisin-B-1 R = OH

1.2. Research on _Artemisia annua_

The earliest reported use of extracts of _A. annua_ for medicinal purposes was in the Prescriptions for 52 kinds of diseases that were found in the Mawangdui Han Dynasty tomb dating from 168 BC. _A.annua_ was recommended for the treatment of haemorrhoids [22]. The use of such extracts for fevers, including malarious ones, was first recorded in Zhou Hou Bei Ji Fang (Handbook of Prescriptions for Emergency Treatments) written in AD340 by Ge Hong. The plant was also described by Lishizhen in his famous Ban Cao Gang Mu (Compendium of Materia Medica) in 1596.

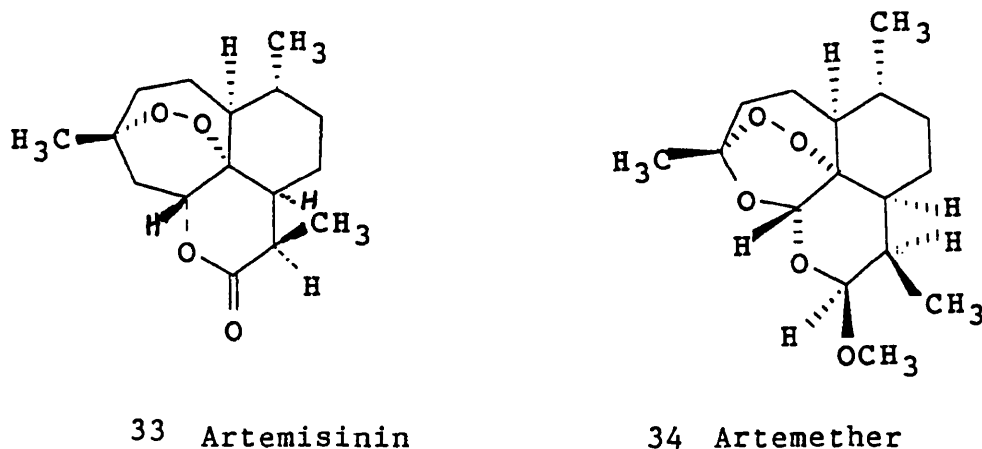
Although the herb has been used for malaria therapy for over a thousand years, the active principle was not isolated and characterised until 1972, when Chinese scientists showed it to be a novel sesquiterpene lactone, named Qinghaosu (artemisinin) (33) [23].

At present, _A. annua_ appears to be the only _Artemisia_ species that contains appreciable amounts of artemisinin.Chinese scientists have reported that extracts from 30 other species of _Artemisia_ did not show antimalarial activity [4] and American scientists have failed to detect artemisinin in _A. arbuscula_, _A. dracunculus_, _A. ludoviciana_, _A. pontica_, _A. schmidviana_ and _A. vulgaris_ [5].

Following its isolation and characterisation, artemisinin and several derivatives have been widely studied by scientists with regard to efficacy in laboratory _in vitro_ malaria models, pharmacology, pharmacokinetics and toxicology. Initial studies

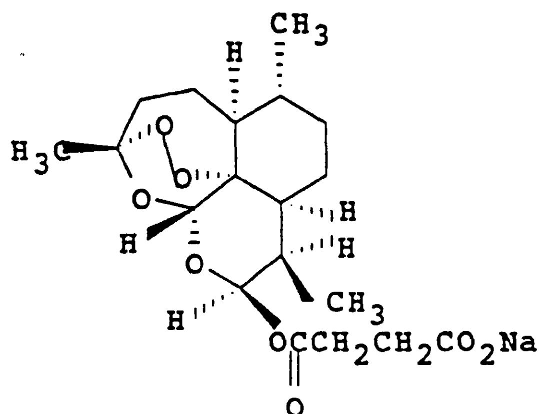
demonstrated the potent blood schizontocidal activity of artemisinin and its derivatives artemether (34) and sodium artesunate (35) against two chloroquine-resistant isolates of P.falciparum from Hainan Island (Figure 5). Artesunate was the most potent compound [24]. Early studies showed that the oral administration of 50 mg kg^{-1} artemisinin daily for three days cleared parasites from the blood of mice infected with P. berghei [25]. The median effective dose (ED 50) was 138.8mg kg^{-1} . Later, studies by these Chinese scientists showed that an oil suspension of artemisinin given intramuscularly was more effective in reducing parasitaemia in mice than a water suspension of the drug given orally or intramuscularly or an oily suspension given orally. The intramuscular administration of this oily suspension was as effective as chloroquine against drug-sensitive parasites [26].

Figure 5 Structures of artemisinin, artemether and sodium artesunate



33 Artemisinin

34 Artemether



35 Sodium artesunate

Electron-microscope studies of malarial parasites treated with artemisinin indicate that the drug damages parasite membranes, but controversy exists concerning which of the parasite membranes are the first to show abnormalities following treatment with the drug. The first morphological changes were observed in the limiting membrane of the food vacuole of trophozoites of P. berghei, followed by swelling of the mitochondrial and nuclear membranes and finally dissolution of the parasites internal structure [27].

Artemisinin was used to treat 2099 malaria patients in Yunnan and Henan provinces and in Hainan Island during 1973-1978 [28]. 588 of these patients were infected with P. falciparum and 1511 with P. vivax. The clinical findings were that body temperature was reduced and parasitaemia was eliminated . All patients were cured as evaluated clinically, the time required for a decline in fever being 24-26 hours in falciparum patients and 20-30 hours in vivax patients. Clearance of P. falciparum

parasites took between 26 and 55 hours and that of P. vivax parasites between 24 and 40 hours. No serious side-effects were reported.

In addition to artemisinin, the following constituents have also been found in A. annua [29-38]:

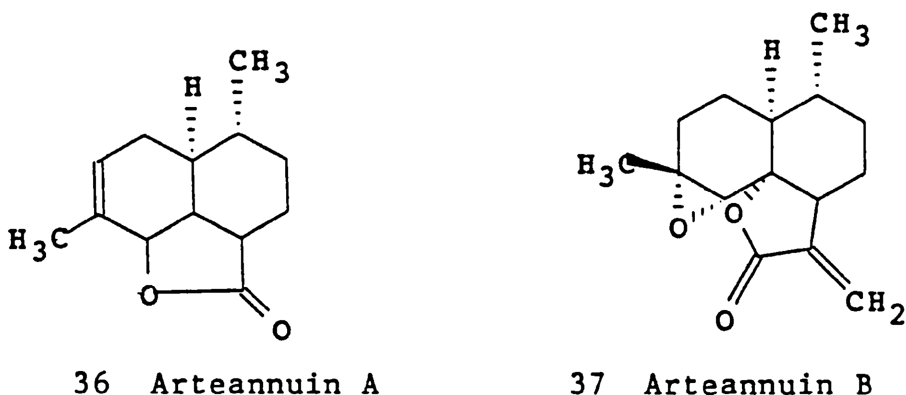
Sesquiterpene lactones: arteannuin A (36), arteannuin B (37),hydroarteannuin (38), artemisic acid (39), deoxyartemisinin (40).

Flavonoids: quercetagetin-6,7,3',4'-tetramethylether (41),5,4'-dihydroxy-3,6,7,3'-tetramethoxyflavone (42), 5,3'-dihydroxy-3,6,7,4'-tetramethoxyflavone (43).

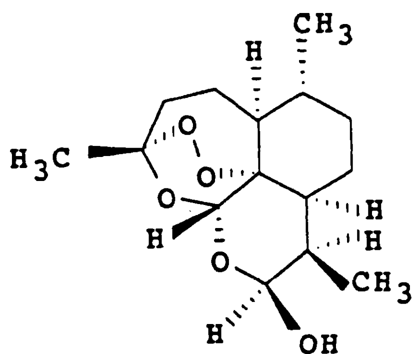
Coumarins: coumarin(14), scopoletin(17), scopolin(19) (Figure 3)

Other compounds: cuminal(44),bornyl acetate(45), cadinene(46), camphene(47), camphor(48), caryophyllene(49), farnesene(50) and 1-β-pinene(51) (Figure 6).

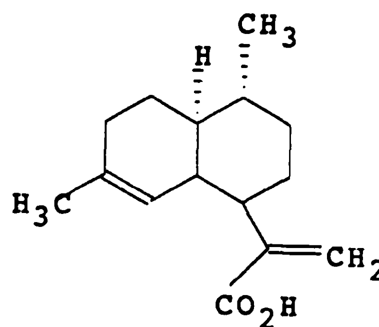
Figure 6 Structures of compounds isolated from A. annua



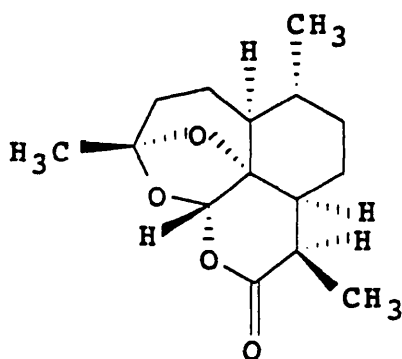
36 Arteannuin A   37 Arteannuin B



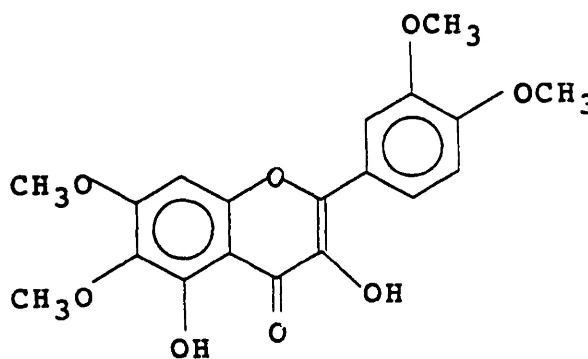
38 DiHydroartemisinin



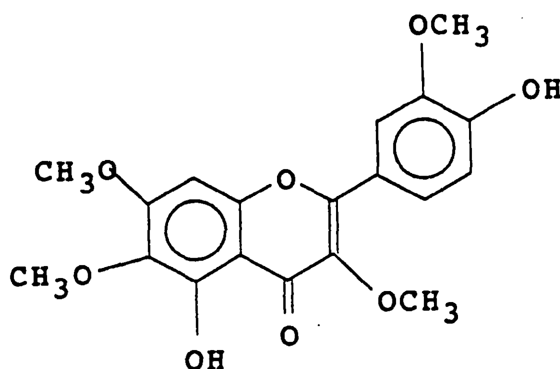
39 Artemisic acid



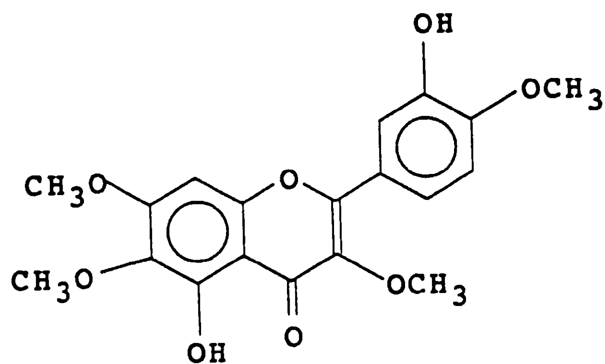
40 Deoxyartemisinin



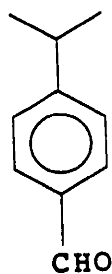
41 Quercetagetin-6,7,
3',4'-tetramethylether



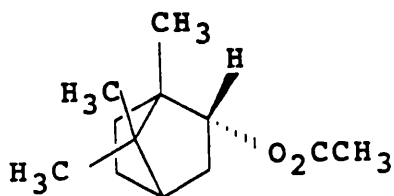
42 5,4'-dihydroxy-3,6,7,3'-tetramethoxyflavone



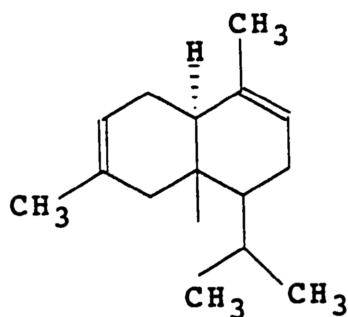
43 5,3'-dihydroxy-3,6,7,4'-tetramethoxyflavone



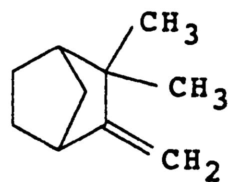
44 Cuminal



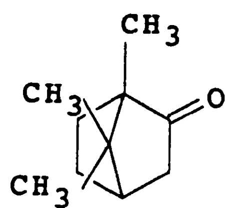
45 Bornyl acetate



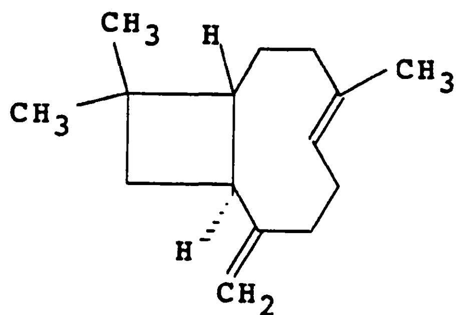
46 Cadinene



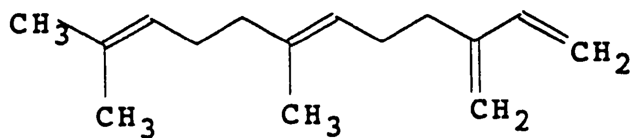
47 Camphene



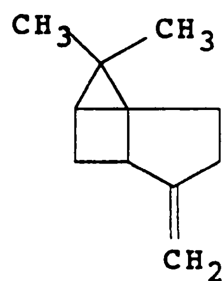
48 Camphor



49 Caryophyllene



50 β-Farnesene



51 1-β-Pinene

1.3. Sesquiterpene lactones

Sesquiterpene lactones have become increasingly of interest to both chemists and biologists, particularly because of their biological activities, e. g., α-santonin is antiparasitic, artemisinin is antimalarial. Most of the naturally-occurring sesquiterpene lactones can be divided into four major skeletal types: germacranolides, eudesmanolides, pseudoguaianolides and guaianolides.

1.3.1. Germacranolides

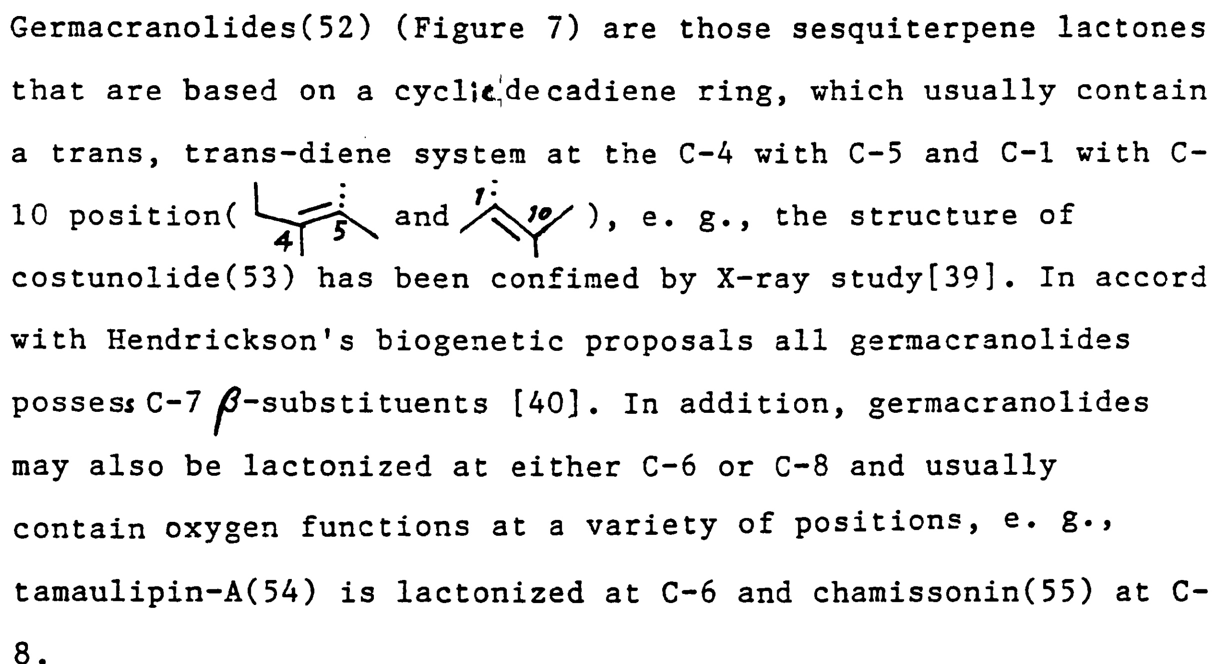
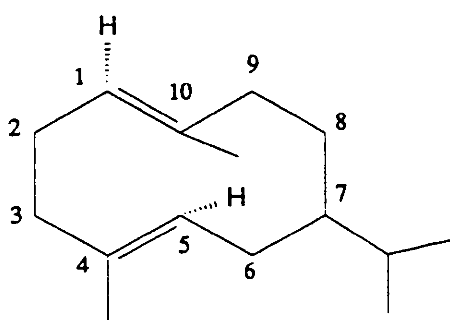
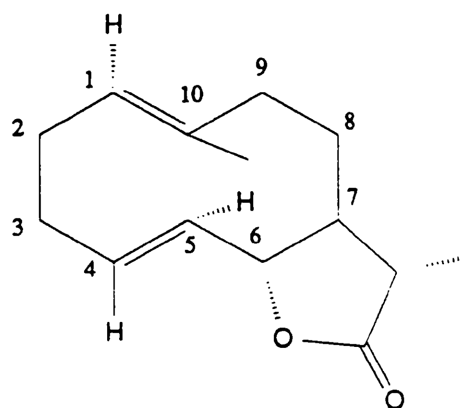
Germacranolides(52) (Figure 7) are those sesquiterpene lactones that are based on a cyclic decadiene ring, which usually contain a trans, trans-diene system at the C-4 with C-5 and C-1 with C-10 position(), e. g., the structure of costunolide(53) has been confimed by X-ray study[39]. In accord with Hendrickson's biogenetic proposals all germacranolides possess C-7 β-substituents [40]. In addition, germacranolides may also be lactonized at either C-6 or C-8 and usually contain oxygen functions at a variety of positions, e. g., tamaulipin-A(54) is lactonized at C-6 and chamissonin(55) at C-8.

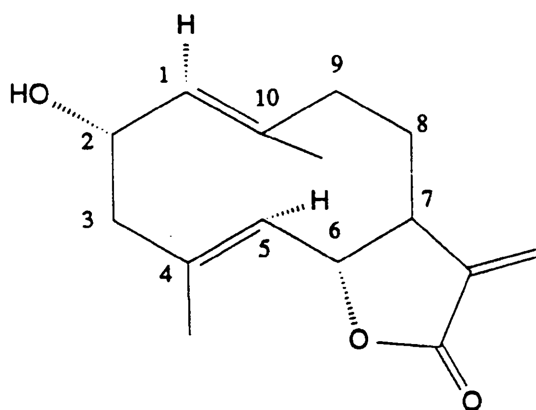
Figure 7 Structures of germacranolides



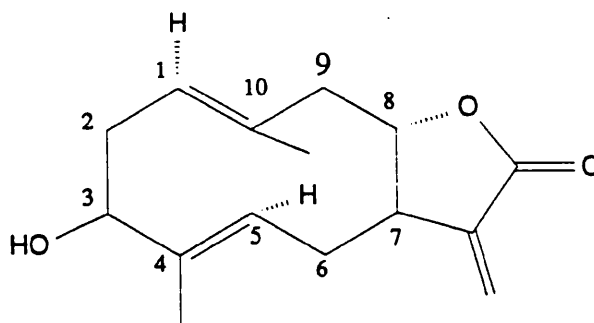
52 germacranolides



53 costunolides



54 tamaulipin-A

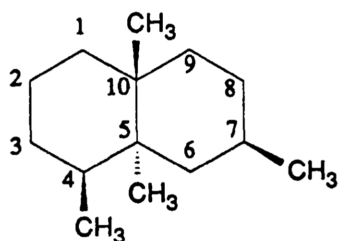


55 chamissonin

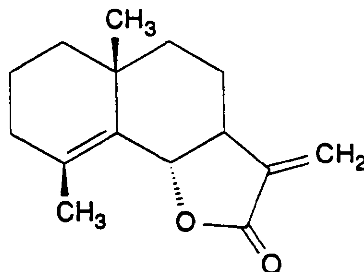
1.3.2. Eudesmanolides

Eudesmanolides(56) (Figure 8) are those sesquiterpene lactones which are based on the eudesmane skeleton. They are lactonized at either C-6 or C-8. Those which are lactonized at C-6 are divided into nonhydroxylated types, e. g., arbusculin-B(57), monohydroxylated types, e.g., douglanine(58), dihydroxylated types, e.g., rothin-B(59), epoxide types, e.g., arbusculin-B epoxide(60), and keto types, e.g., α-santonin(61). Eudesmanolides which are lactonized at C-8 are divided into nonhydroxylated types, e.g.,alantolactone(62), monohydroxylated types, e.g.,telekin(63), dihydroxylated types, e.g., pulchellin-C(64) and keto types, e.g., yomogin(65).

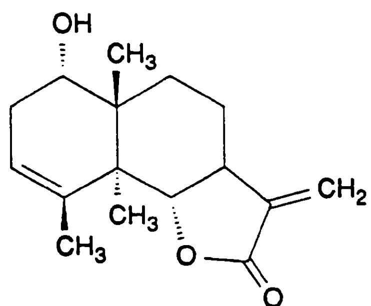
Figure 8 Structures of eudesmanolides



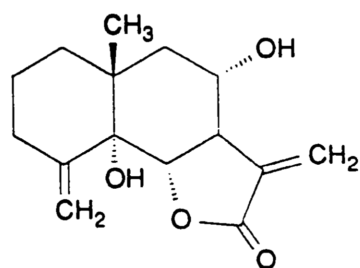
56 eudesmane



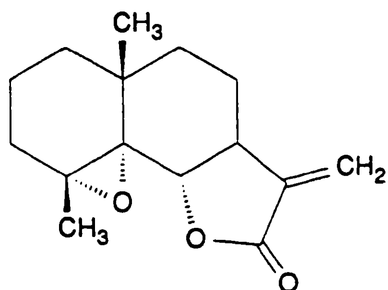
57 arbusculin-B



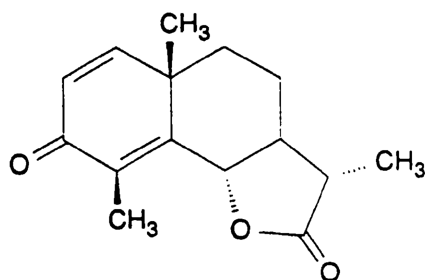
58 douglanine



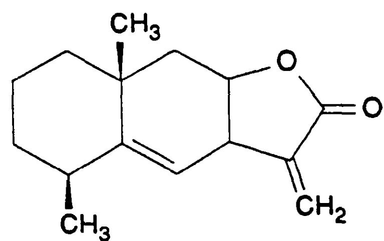
59 rothin-B



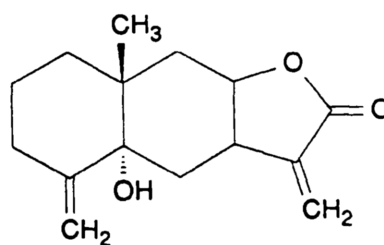
60 arbusculin-B-epoxide



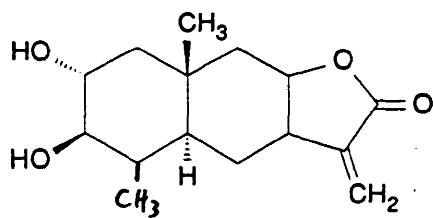
61 α-santonin



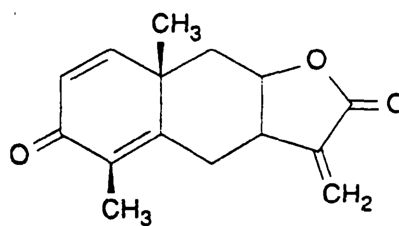
62 alantolactone



63 telekin



64 pulchellin-C



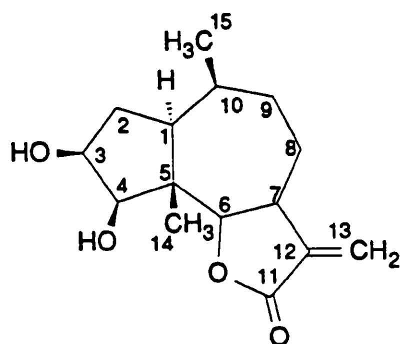
65 yomogin

1.3.3. Pseudoguaianolides

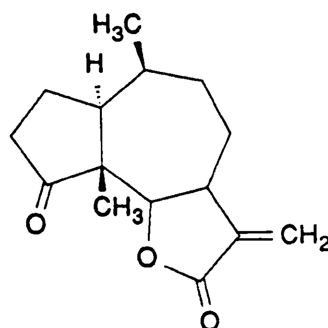
The largest class of sesquiterpene lactones are pseudoguaianolides(Figure 9) which are based upon (or derived from) the 5/7 carbocyclic ring system which contains a methyl group at the C-5 ring junction. The pseudoguaianolides may be lactonized to either C-6 or C-8 and may be cleaved between C-3 and C-4 or between C-4 and C-5. The pseudoguaianolides which are lactonized at C-6 are divided into dihydroxylated types,e.g., ambrosiol(66), keto types, e.g., damsin(67), conjugated cyclopenenone types, e.g., hymenin(68), C-14 hydroxylated types, e.g., incanin(69), C-15 hydroxylated types, e.g., hysterin(70) and anhydro types, e.g., anhydrofranserin(71).

The pseudoguaianolides which are lactonized at C-8 are divided into dihydroxylated types, e.g., cumanin(72), keto types, e.g., peruvin(73), conjugated cyclopenenone types, e.g., aromatin(74), neo-types, nor-types and cleaved pseudoguaianolides,e.g., psilostachyin-C (75).

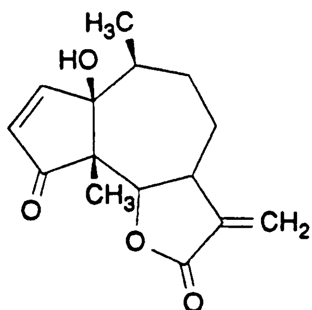
Figure 9 Structures of pseudoguaianolides



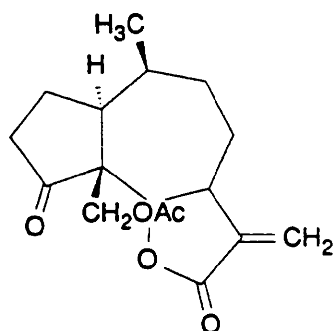
66 ambrosiol



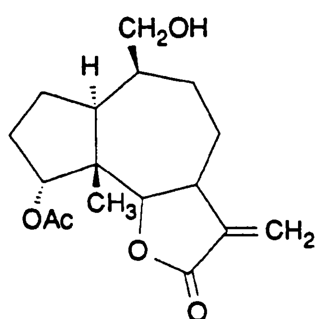
67 damsin



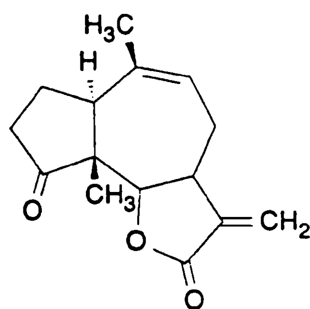
68 hymenin



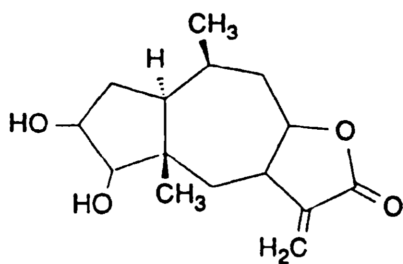
69 incanin



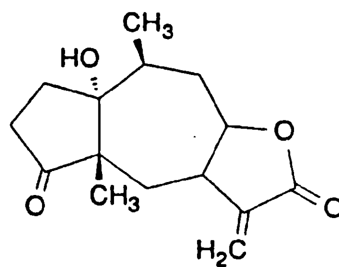
70 hysterin



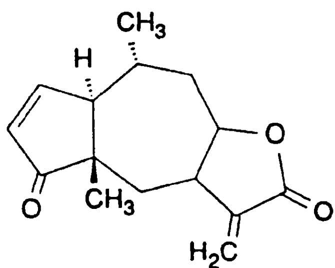
71 anhydrofranserin



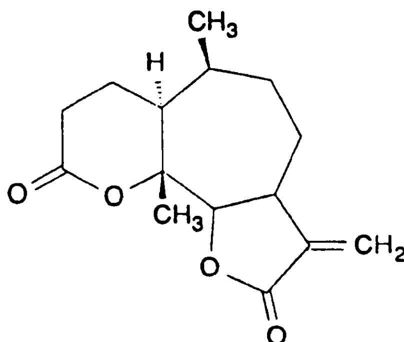
72 cumanin



73 peruvin



74 aromatin

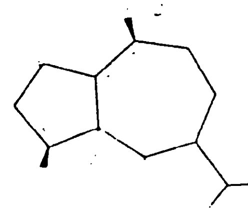


75 psilostachyin-C

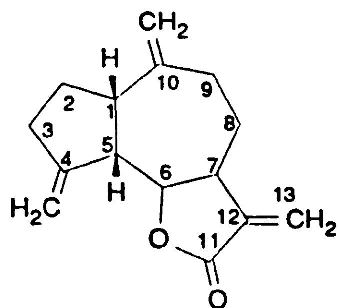
1.3.4. Guaianolides

Guaianolides are those sesquiterpene lactones which are based upon the guaiane skeleton(76)(Figure 10). The guaianolides which are lactonized at C-6 are divided into nonhydroxylated types, e.g., dehydrocostuslactone(77), monohydroxylated types, e.g., artabsin(78), dihydroxylated types, e.g., matricin(79), ketone types, e.g.,parishin-C (80), side chain epoxide types, e.g., euparotin(81) and chloride types, e.g., eupachlorin(82). Other types of guaianolides include C-8 lactonized guaianolides, xanthanolides, cyclopropane, and dimerized guaianolide types.

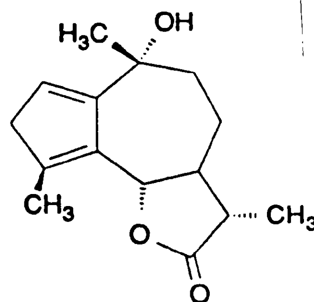
Figure 10 Structures of guaianolides



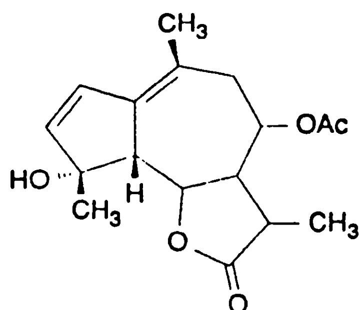
76 guaiane



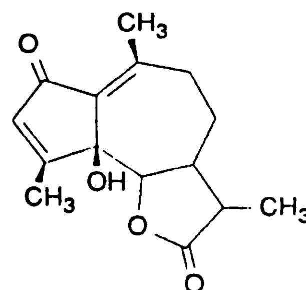
77 dehydrocostulactone



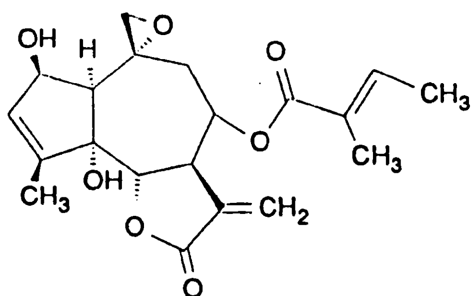
78 artabsin



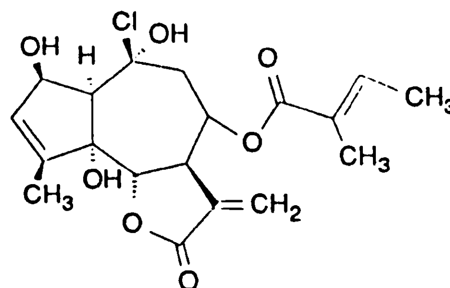
79 matricin



80 parishin-C



81 euparotin

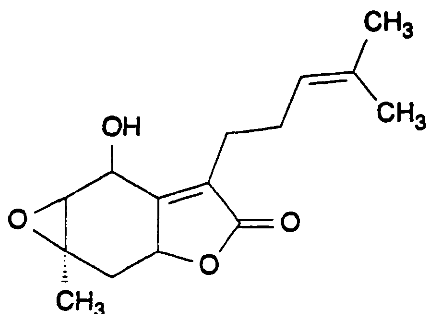


82 eupachlorin

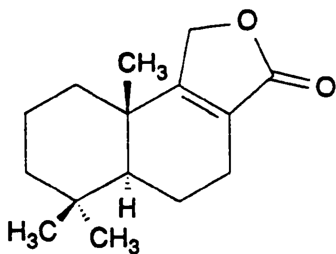
1.3.5. Minor classes of sesquiterpene lactones

Several minor classes of sesquiterpene lactones exist as natural products, e.g., bisabolenolides(83), drimanolides(84), eremophilenolides(85), fukinanolides(86), elemanolides(87), germafurenolides(88), and photo-induced sesquiterpene lactones e.g., photoisabelin(89) (Figure 11).

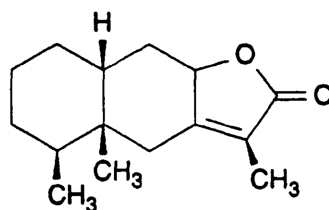
Figure 11 Structures of minor classes of sesquiterpene lactones



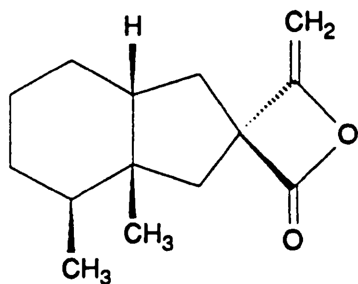
83 bisabolenolides



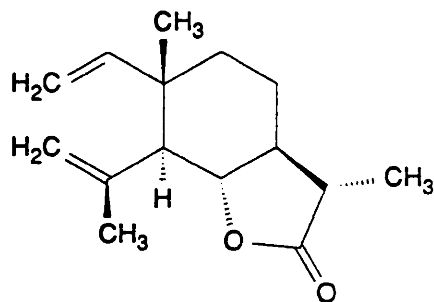
84 drimanolides



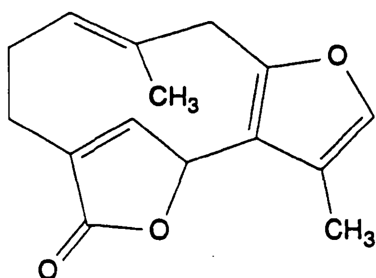
85 eremophilenolides



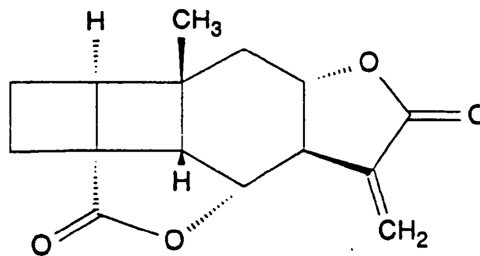
86 fukinanolides



87 elemanolides



88 germafurenolides



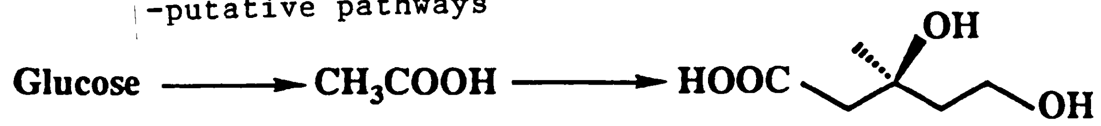
89 photoisabelin

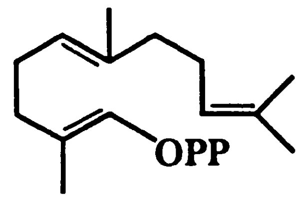
1.3.6. Structure determination of sesquiterpene lactones

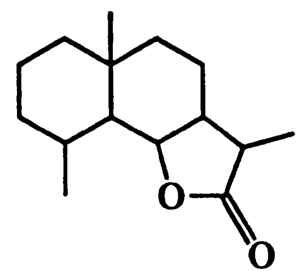
The structure of sesquiterpene lactones have been determined by spectroscopic analysis and by chemical reactions. Known compounds can be identified by direct comparison with an authentic sample or by comparison of their spectral data with those of the literature. The structure of new compounds may be determined mainly by ^{1}H NMR, ^{13}CNMR, MS, IR and X-ray crystallography. Comformation and configuration are determined by special spectroscopic techniques, such as NOE (Nuclear Overhauser Effect) and different temperature NMR analysis, CD and X-ray crystallography[41].

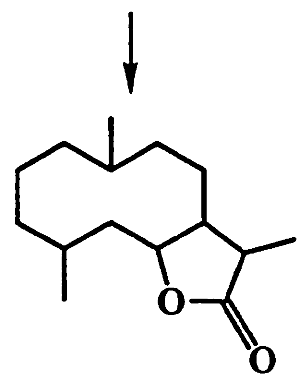
1.3.7. Biosynthetic pathways for sesquiterpene lactones

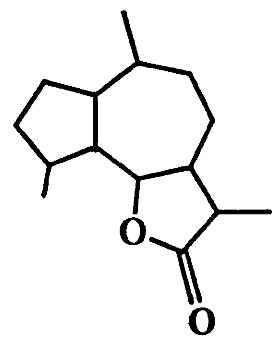
Trans-farnesyl-pyrophosphate, formed from mevalonic acid, is the common intermediate for the biosynthesis of sesquiterpene lactones[42] (Figure 12).

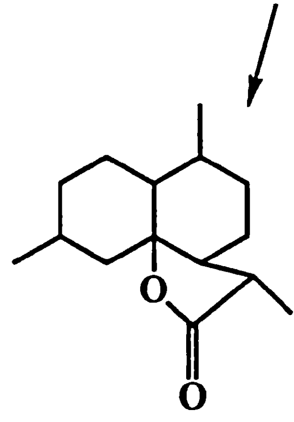


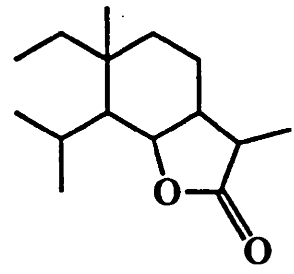


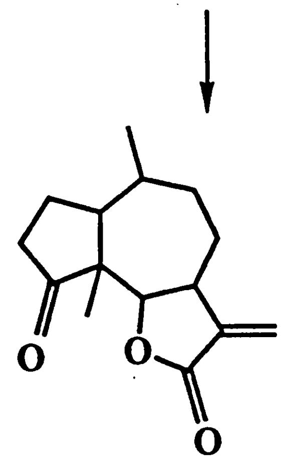










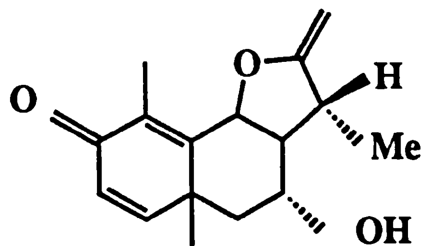


Germacranolides are the biogenetic precursors for all the other types.

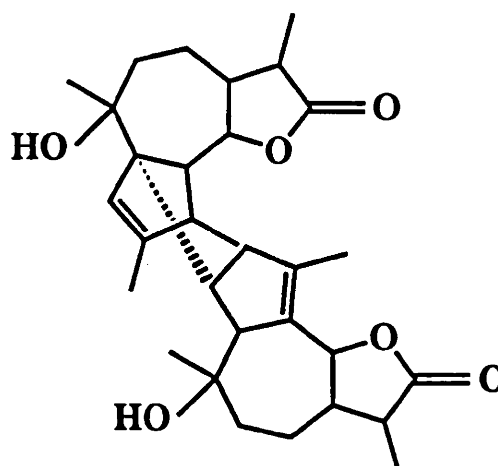
1.3.8. Biological activities of sesquiterpene lactones

Sesquiterpene lactones exhibit a wide range of biological activities, particularly as anthelmintics, antimalarials and anticancers agents (Figure 13) .
α-Santonin(61)(Figure 8) has been used as an anthelmintic, particularly for roundworm infestation, while artemisin(90) and absinthin (91) show similar activity to α-santonin. They were isolated from _A. maritima_ and _A. absinthum_ respectively[43] [44].
Artemisinin, reported from _A. annua_, and its derivatives exhibit a significant antimalarial activity which has been demonstrated clinically for several thousands of patients[45].
Some sesquiterpene lactones have anticancer activity, e.g.,eight sesquiterpene lactones isolated from _Eupatorium rotundifolium_, exhibit anticancer activity, eupatorin(92) being the most potent [46]. Eupaserrin(93) reported from _E. semiserratum_ has anticancer activity[47].
Other sesquiterpene lactones, elephantopin and elephantin from _Elephantopus scaber_[48], gaillaridin from _Gaillardia pulchella_ [49], vernolepin and vernomentin from _Veronica hymenolepis_ [50] have exhibited anticancer activity.
Sesquiterpene lactones show other biological activities,e.g., chamazulene is antiinflammatory [51] and carpesia lactone has sedative activity [52].

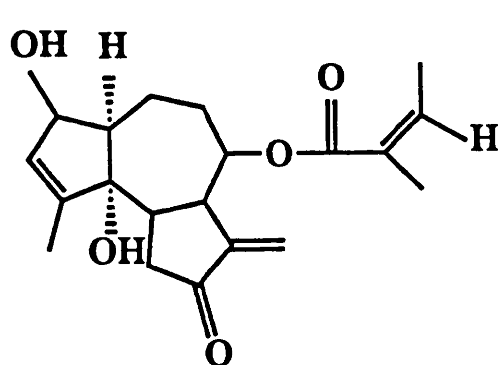
Figure 13 Some biologically active sesquiterpene lactones



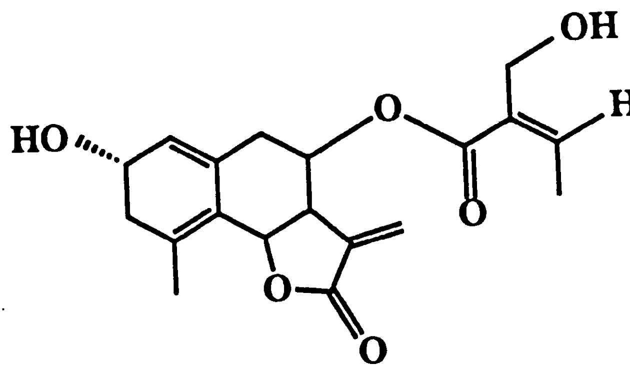
Artemisin 90



Absinthin 91



Eupatorin 92



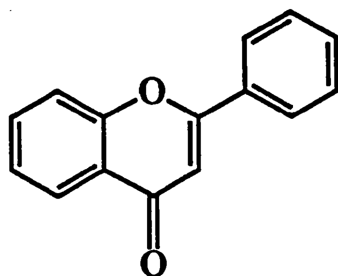
Eupaserrin 93

1.4. Flavonoids

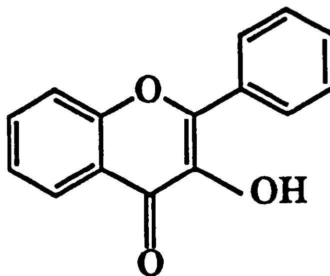
1.4.1. Flavonoid types

The flavonoid constituents are one of the most numerous and wide spread group of natural compounds. They are important to man not only because they contribute to plant colour but also because many members are physiologically active. Many flavonoids have been isolated because of their interesting chemical and biological activities and they have been the subject of books and review articles e.g. [53,54]. Flavonoids exist as different structural types : (Figure 14) flavones(94), flavonols(95), flavanones(96), dihydroflavonols(97), isoflavones (98), isoflavanones(99), flavone glycosides(100), flavonol glycosides(101), chalcones(102), dihydrochalcones(103), C-glycosyl-flavonoids(104), biflavonoids(105), neoflavanoids (106), proanthocyanidins(107), and anthocyanins(108) (Figure 14) [55]. Flavonoids may exist as aglycones or be connected with common sugars, e.g., D-glucose, D-galactose, D-glucuronic acid, L-rhamnose and L-arabinose.

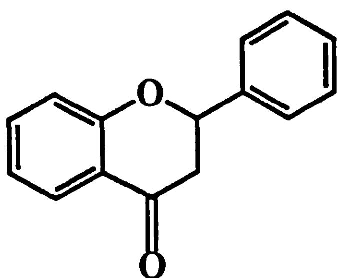
Figure 14 Structures of flavonoids



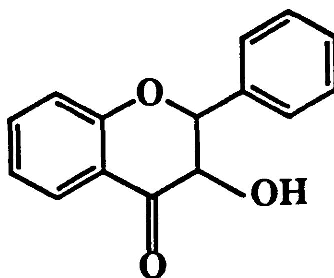
Flavone 94



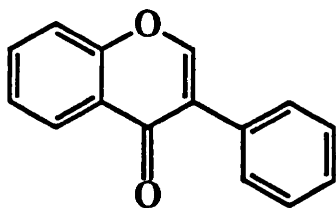
Flavonol 95



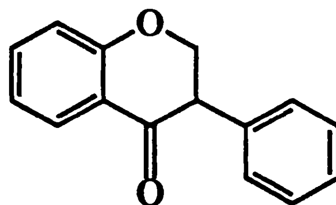
Flavanone 96



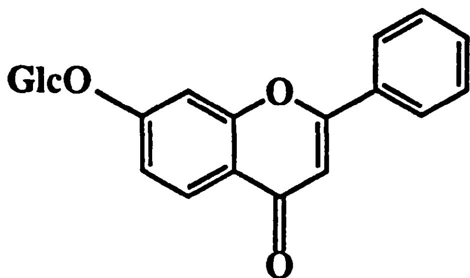
Dihydroflavonol 97



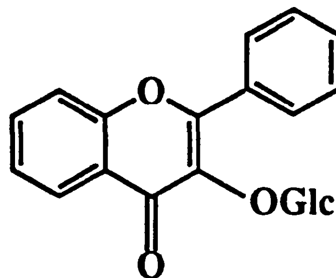
Isoflavone 98



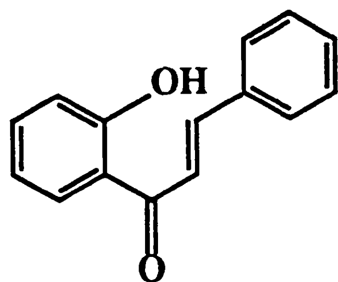
Isoflavanone 99



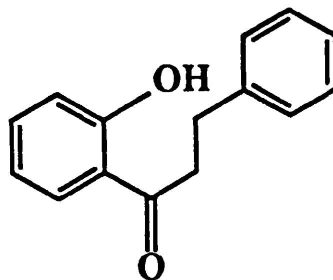
Flavone glycoside 100



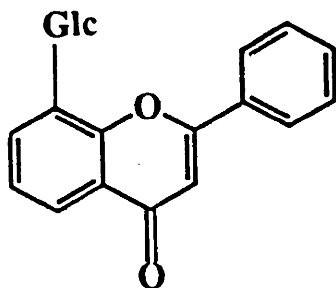
Flavonol glycoside 101



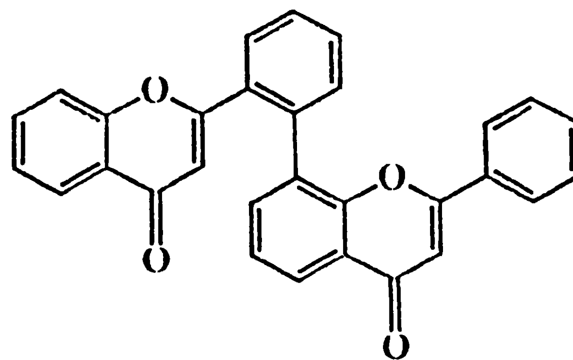
Chalcone 102



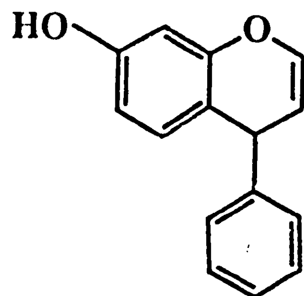
Dihydrochalcone 103



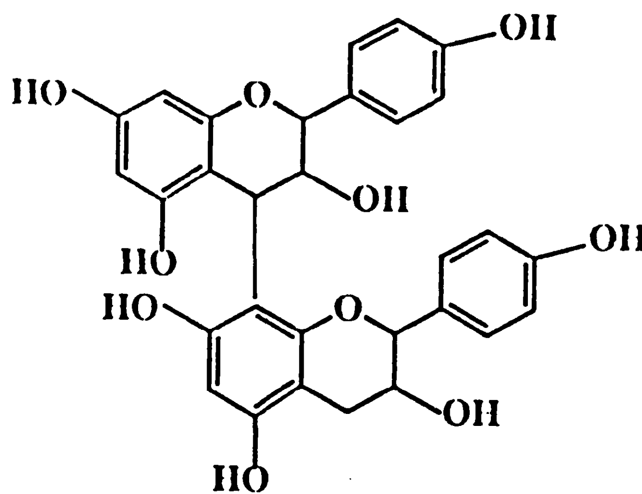
C-glycosyl-flavonoid 104



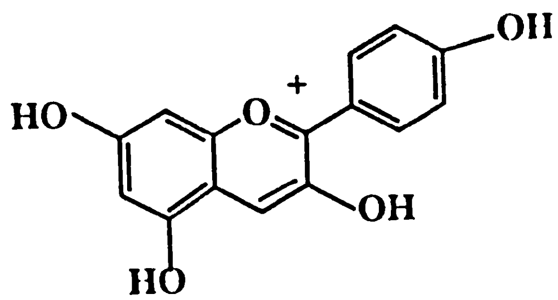
Biflavonoid 105



Neoflavonoid 106



Proanthocyanidin 107

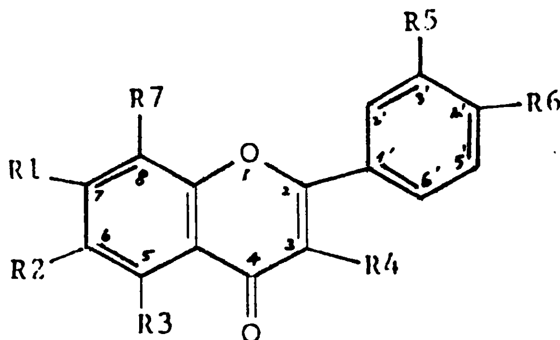


Anthocyanidin 108

1.4.2. Flavones

All flavones, apart from flavone itself, are O-substituted, mainly at C-5, C-7, C-3',and C-4',e.g.,primuletin(106)(5-hydroxyflavone), chrysin(107)(5,7-dihydroxyflavone), apigenin (11)(5,7,4'-trihydroxyflavone), luteolin(13) (5,7,3',4'-tetrahydroxyflavone)(Figure 2) and luteolin-7-methylether(108). More rarely, there may be substitution at C-6 and/or C-8, e.g., cirsiliol(109)(5,3',4'-trihydroxy-6,7-dimethoxyflavone), strobochrysin(110)(6-methyl chrysin) and sideroxylin(111) (5,4',-dihydroxy-7-methoxy-6,8-dimethylflavone) (Figure 15). Flavones have been isolated from many Chinese traditional medicinal plants, e.g., wightin(112) and serpyllin(113) from Andrographis weightiana Arn. which is used for its anti-inflammatory effects [56]. 5,6,4'-Trihydroxyflavone(114) has been isolated from Lonicera japonica Thunb.[57] and 5,6,7-trihydroxyflavone(115) from Scutellaria baicalensis Georgi [58].

Figure 15 Structures of some flavones



Primuletin 106 R1=R2=R4=R5=R6=R7=H R3=OH

Chrysin 107 R1=R3=OH R2=R4=R5=R6=R7=H

Luteolin 108 $R1=OCH_3$ $R2=R4=R7=H$ $R3=R5=R6=OH$

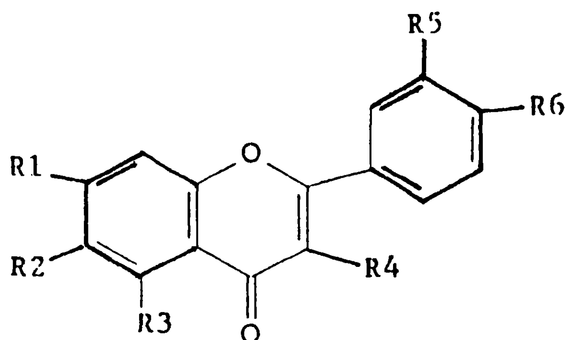
Cirsiliol 109 $R1=R2=OCH_3$ $R3=R5=R6=OH$ $R4=R7=H$

Strobochrysin 110 $R1=R3=OH$ $R2=CH_3$ $R4=R5=R6=R7=H$

Sideroxylin 111 $R1=OCH_3$ $R2=R7=CH_3$ $R3=R6=OH$ $R4=R5=H$

5,6,4'-Trihydroxyflavone 114 $R1=R4=R5=R7=H$ $R2=R3=R6=OH$

5,6,7-Trihydroxyflavone 115 $R1=R2=R3=OH$ $R4=R5=R6=R7=H$



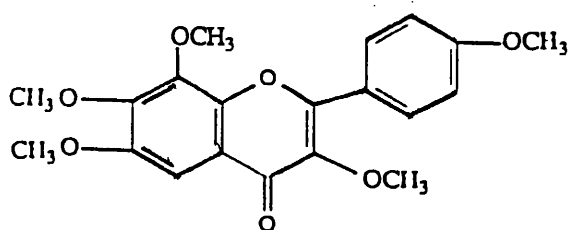
Wightin 112 $R1=R2=R4=OCH_3$ $R3=R5=OH$ $R6=H$

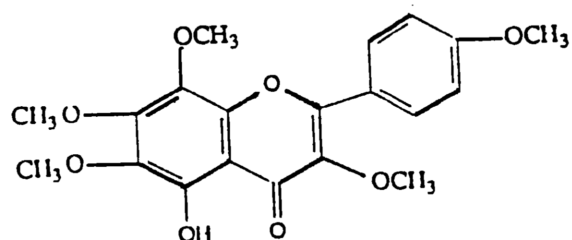
Serpyllin 113 $R1=R2=R4=R5=R6=OCH_3$ $R3=OH$

1.4.3. Flavonols

Flavonols especially kaempferol(12) and quercetin(10)(Figure 2) ,are widely distributed in plants. Most flavonols contain oxygen substituents in an analogous manner to the flavones(for example see Figure 16).Flavonols have been isolated from many Chinese traditional medicinal plants, e.g., auranetin(116) and 5-hydroxyauranetin(117) from Citrus aurantium[59], isoanhydroicaritin(118) and nor-anhydroicartin(119) from Sophora flavescens Ait.[60].

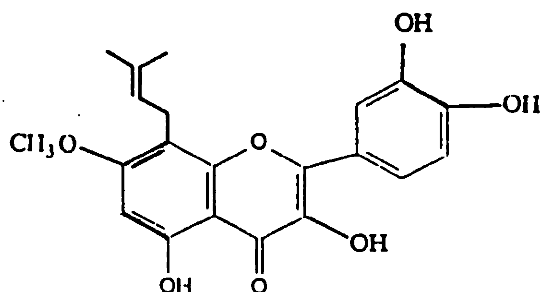
Figure 16 Structures of some flavonols

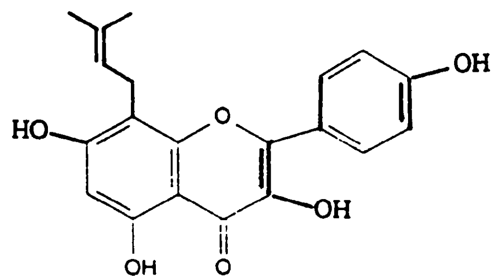




Auranetin 116

5-hydroxyauranetin 117





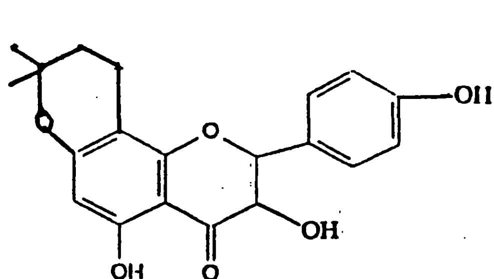
Isoanhydroicaritin 118

Nor-anhydroicartin 119

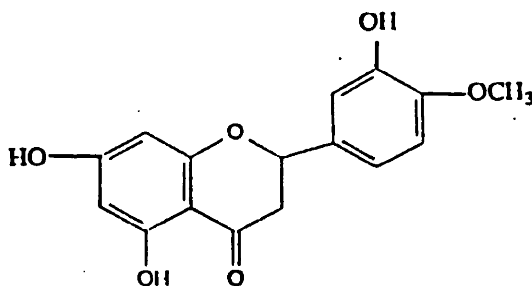
1.4.4. Flavanones and dihydroflavonols

Flavanones are based upon 2-phenyl-benzopyran-4-one. The parent compound is not known to be naturally-occurring. The simplest plant flavanone has a hydroxy group at C-7.
Dihydroflavonols, often called 3-hydroxyflavanones or flavanonols are based upon 2-phenyl-3-hydroxybenzo-pyran-4-one. Dihydroflavonols have two asymmetric carbons at C-2 and C-3. Some Chinese traditional medicinal plants contain flavanones and dihydroflavonols (Figure 17) e.g., phellamuretin (120) from *Phellodendron amurense* Rupr.[61], hesperetin (121) from *Citrus fusca* Lour. and citromitin (122) from *C. mitis*. *Glycyrrhiza uralensis* Fisch. and *G. glabra* L. also contain flavanone constituents [62] and silybin (123) and silydianin (124) were isolated from *Silybum marianum* Gaertn.[63].

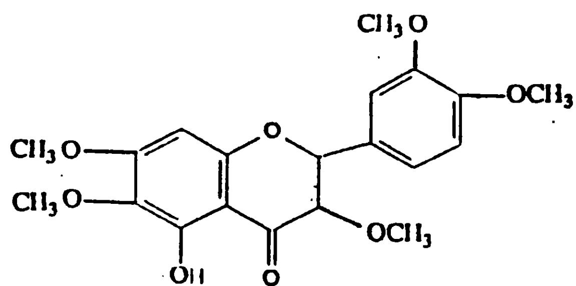
Figure 17 Structures of some flavanones and dihydroflavonols



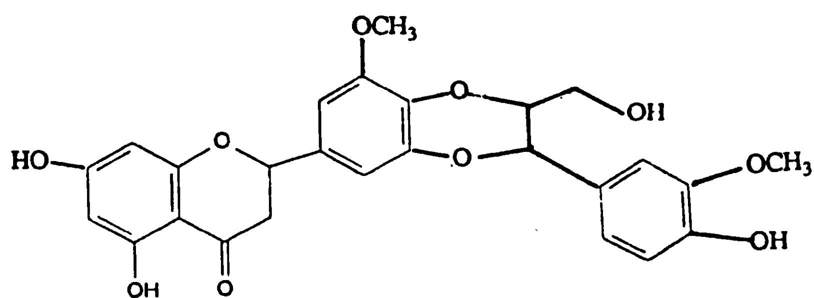
Phellamuretin 120



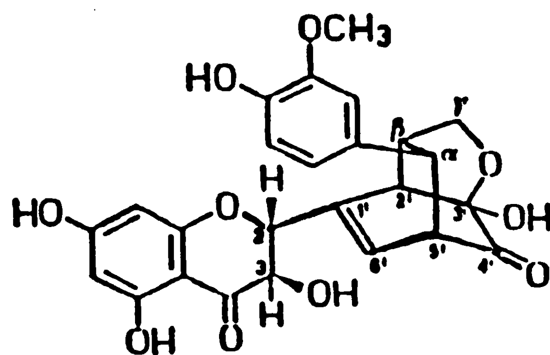
Hesperetin 121



Citromitin 122



Silybin 123

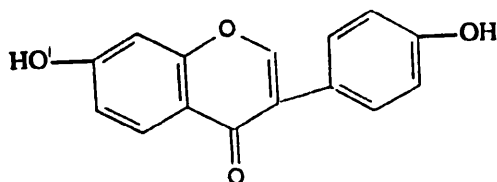


Silydianin 124

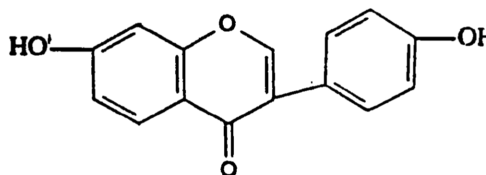
1.4.5. Isoflavones and isoflavanone

The isoflavone structure is different from the other types of flavonoids, being based upon 3-phenyl-benzopyren-4-one. Isoflavones can be readily distinguished from flavones and isoflavanones by UV and NMR spectroscopy.The simple isoflavones have intense absorption at 255-275 nm and generally a less intense band or inflection at 310-330 nm. The low intensity of absorption of the second band of the isoflavones is a valuable diagnostic feature. The NMR signal of the olefinic proton at C-2 in isoflavones appears as a characteristic down field singlet at about 7.8 ppm in DMSO-d6 as compared to about 6.7ppm for the C-3 proton in flavone. The impact of NMR on structure determination is most evident in the complex isoflavones. The number of isoflavanones known in nature is not great , but several have been isolated from Chinese traditional medicinal plants (Figure 18), e.g., daidzein (125) and puerarin (126) from Pueraria lobata (Willd.) Ohwi. [64] and ferreirin (127) and homoferreirin (128) from Astragalus membranaceus Bunge [65].

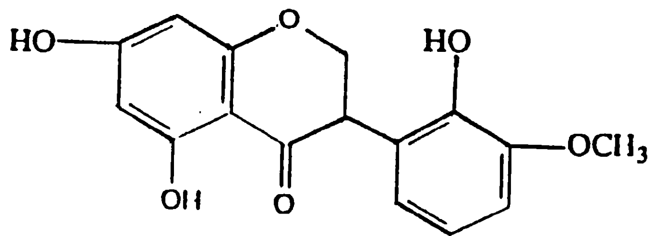
Figure 18 Structures of some isoflavones and isoflavanones



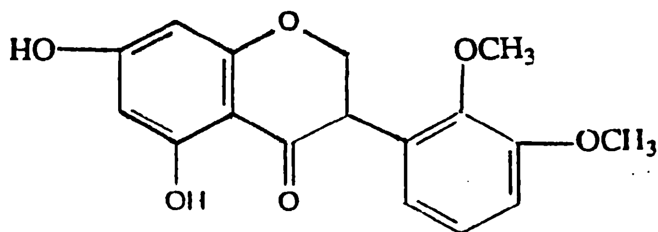
Daidzein 125



Puerarin 126



Ferreirin 127



Homoferreirin 128

1.4.6. Flavonoid glycosides

A vast number of different glycosides has been reported in plants.Their sructural variation is due both to the nature of sugar residues and their position of attachment through hydroxyl groups to the flavone or flavonol nucleus. The glycosides may be classified according to the number and type of sugars present. The outline of present knowledge about the flavone and flavonol glycosides and the relevant data have been tabulated in the chapter on Flavone and Flavonol glycosides in " The Flavonoids " [66, 67, 68].Apigenin and luteolin glucosides are very common flavone glycosides and kaempferol and quercetin glycosides are common flavonol glycosides which have been isolated from numerous species of plants.
The common sugars found in O-monoglycosides are : D-glucose, D-galactose, D-glucuronic acid, D-xylose, L-rhamnose and L-arabinose. Most of them occur also as components of di- and trisaccharides. They are generally present in the pyranose form and only arabinose is known to occur in both furano- and pyrano- forms. Glucose, galactose, glucuronic acid and xylose are usually β-linked to the aglycone hydroxyl; rhamnose and arabinose are normally α-linked. However, there are reports of quercetin-3-β-arabinoside [69] and quercetin-7-α-galactoside. Apiose and allose are uncommon sugars in monoglycosides, e.g., only one apioside, 6-hydroxyluteolin-7-apioside, has been reported [70]. Allose containing monoglycosides reported include kaempferol-3-alloside [71], apigenin-7,4'-bisalloside, apigenin-7-(4", 6"-diacetylalloside)-4'-alloside

[72].Galacturonic acid has been reported in the forms of apigenin-7-galacturonide, quercetin-3-galacturonide and tricin-7-(2"-rhamnosyl)-galacturonide [73].
Of the disaccharides based on glucose (Glc), sophorose (2-O-β-D-glucosyl-D-glucose) is the most common. Disaccharides with two galactose (Gal), two arabinose or two glucuronic acid residues are also known. The most frequently occurring disaccharide composed of two different sugars is rutinose (6-O--L-rhamnosyl-D-glucose), while the -1→2 isomer neohesperidose and -1→3 isomer rungiose are less common. The new disaccharides are listed in [67,68].Some of the more commonly found glycosides are:Gal (1→4) Glc, Gal (1→6) Glc, Rha (1→6) Gal, Rha (1→2) Gal, Gal(1→4) Rha, Glc(1→6) Gal, Glc(1→2) Gal, Glu(1→4) Rha, Glu(1→3) Rha, Gal(1→4) Gal and Gal(1→6) Gal.
Apiose is also found in diglycosides as apigenin-7-apiosyl-glucoside, kaempferol-3-apiosylglucoside and quercetin-3-apiosylglucoside[74]. Allose has been found in disaccharides as chrysoeriol and isoscutellarein-2-allosylglucoside[75], isoscutellarein and 8-hydroxyluteolin-4'-methylether-7-(6"-acetylallosyl(1→2)glucoside)[76] and isoscutellarein-7-allosyl-(1→2)glucoside[77].

New oligosaccharides have been reported, particularly in association with kaempferol and quercetin, e.g., the linear trisaccharides sorborose[Glc(1→6)Glc(1→4)Glc], sophorotriose [Glc(1→2)Glc(1→2)Glc], primflasine[Glc(1-→4)Ara(1→2)Ara] and rhamninose[Rha(1→4)Rha(1→6)Gal]. An

increasing number of branched trisaccharides have been found, particularly in Soya bean (Glycine max), which is a very rich source of kaempferol and quercetin-3-triglycosides.Four such trisaccharides ,2^{G}-glucosylrutinose,2^{G}-rhamnosylrutinose,2^{G}-glucosylgentiobiose and 2^{G}-rhamnosylgentiobiose, have been reported from Soya bean[78].

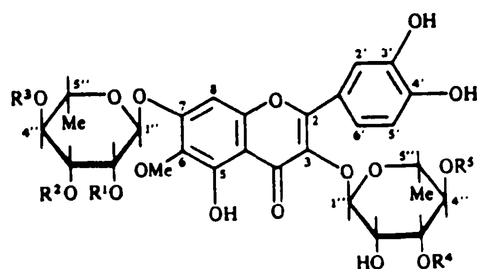
Markham discovered the first flavone-polysaccharide, 8-methoxyluteolin, chemically bonded to water soluble polysaccharide of hemicellulose type and containing about 18 sugar residues[79]. The polysaccharide is linked glycosidically via galacturonic acid to the 7- and 4'-hydroxyls of the flavones.

In recent years, attention has been given to the importance of glycosylation in relation to the function of flavones and flavonols in plants. Free flavones and flavonols are potentially toxic to living cells and inhibit many enzymic activities[80]. Glycosylation must be an essential protective device to prevent cytoplasmic damage. Another protective technique is the location of the flavonoids in the cell vacuole. While glycosylation is most frequent with the fully hydroxylated compounds such as kaempferol and quercetin, it also occurs with many of their methylethers.

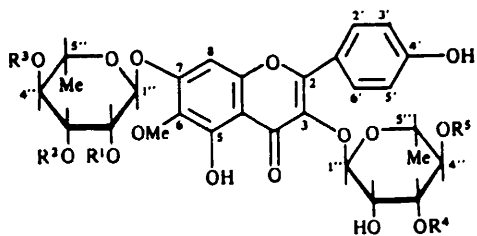
Some acetylated flavonoid glycosides have been reported from plants. On the study of Kalanchoe gracilis Hance, we have reported 9 novel acetylated flavone glycosides. They are 3''' -O-acetyl-patuletin-3,7-di-O-rhamnoside (129), 4''' -O-acetyl-patuletin-3,7-di-O-rhamnoside (130), 3''' ,4''' -O-diacetyl-

patuletin-3,7-di-O-rhamnoside(131), 3‴,4"-O-diacetyl-patuletin-3,7-di-O-rhamnoside(132), 3",3‴-O-diacetyl-patuletin-3,7-di-O-rhamnoside(133), 3‴,4‴,4"-O-triacetyl-patuletin-3,7-di-O-rhamnoside(134), 2‴,4‴,4"-O-triacetyl-patuletin-3,7-di-O-rhamnoside(135), 4‴-O-acetyl-eupafolin-3,7-di-O-rhamnoside(136), and 3",3‴-O-diacetyl-eupafolin-3,7-di-O-rhamnoside(137) [81,82] (Figure 19).

Figure 19 Structures of acetylated flavone glycosides isolated from *Kalanchoe gracilis* Hance



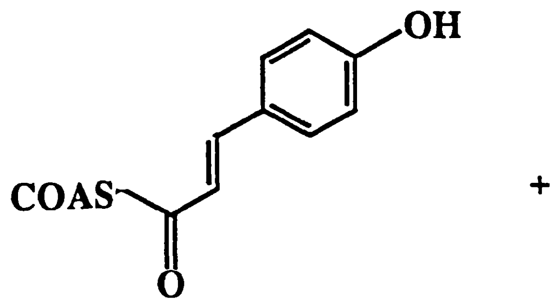
	R1	R2	R3	R4	R5
129	H	Ac	H	H	H
130	H	H	Ac	H	H
131	H	Ac	Ac	H	H
132	H	Ac	H	H	Ac
133	H	Ac	H	Ac	H
134	H	Ac	Ac	H	Ac
135	Ac	H	Ac	H	Ac



	R1	R2	R3	R4	R5
136	H	H	Ac	H	H
137	H	Ac	H	Ac	H

1.4.7. Biosynthesis of flavonoids

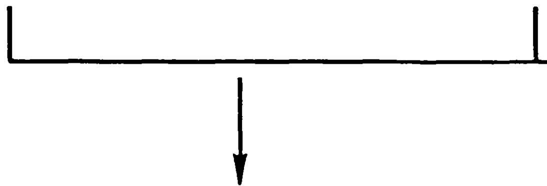
A number of reviews on flavonoid biosynthesis has been reported [83-87]. All classes of flavonoids are biosynthetically closely related, with a chalcone being the first common intermediate. The formation of chalcone is common to all flavonoids and the chalcone isomers are the central intermediate in the synthesis of flavonoids[88]. More recent investigations at the enzymic level have largely confirmed the previous hypothetical steps and the essential steps of the pathway of the main flavonoid classes have been elucidated. The origins of flavonoid precursors and the individual reactions leading to the various flavonoid classes may be demonstrated in the scheme as shown in Figure 20.

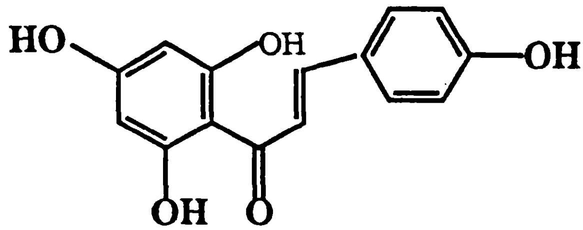


+

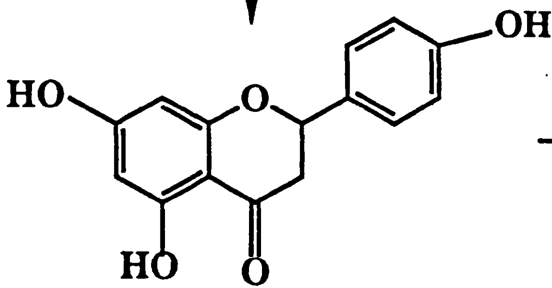
Malonyl-CoA

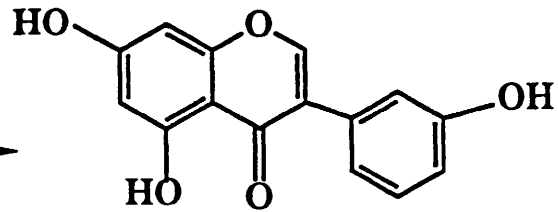
4-Coumaroyl-CoA



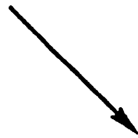


Chalcone

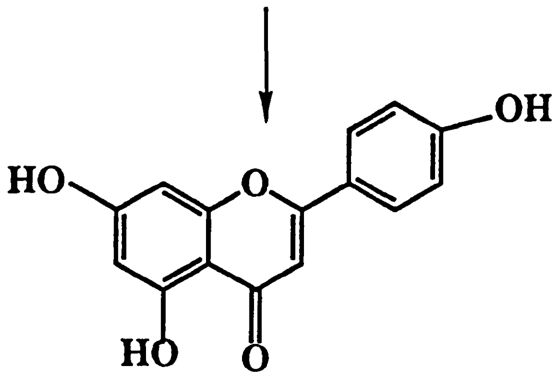




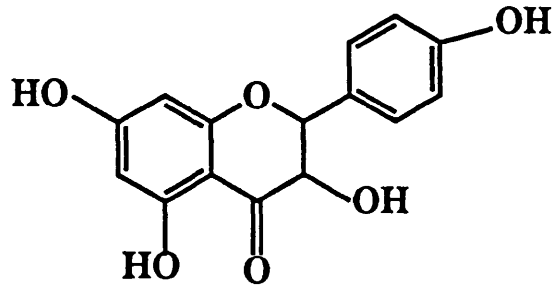
Isoflavone



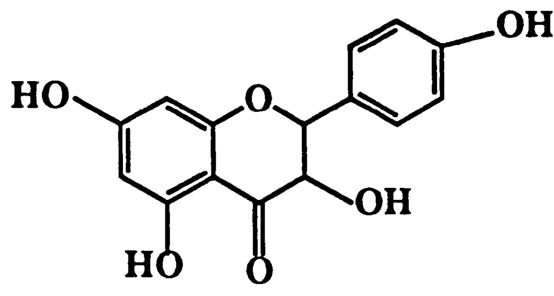
Dihydroflavone



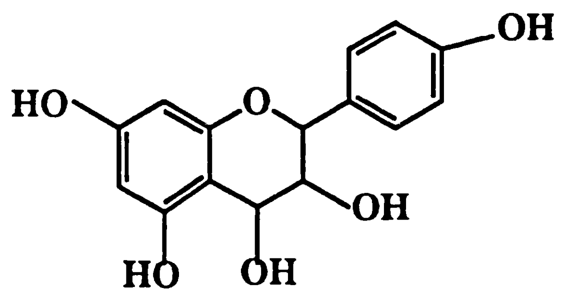
Flavone



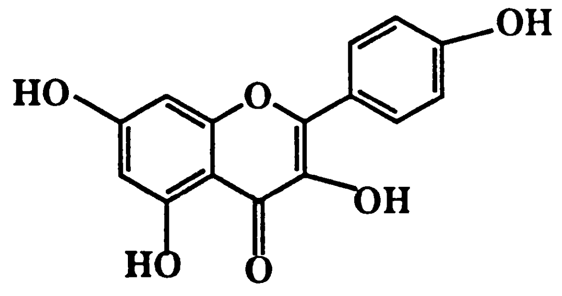
Dihydroflavonol



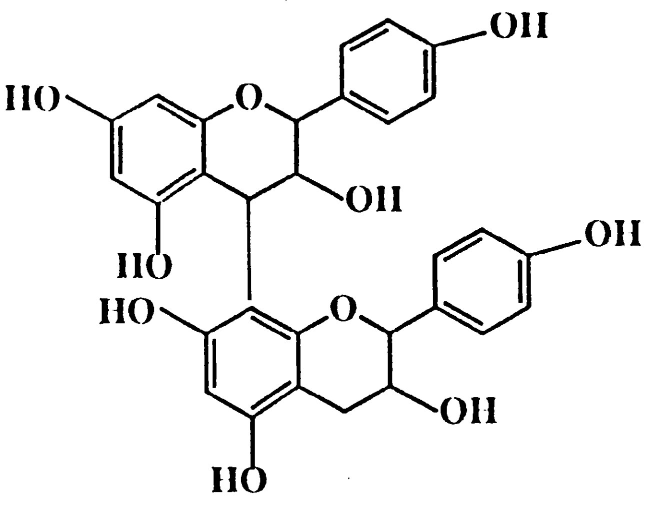
Dihydroflavonol



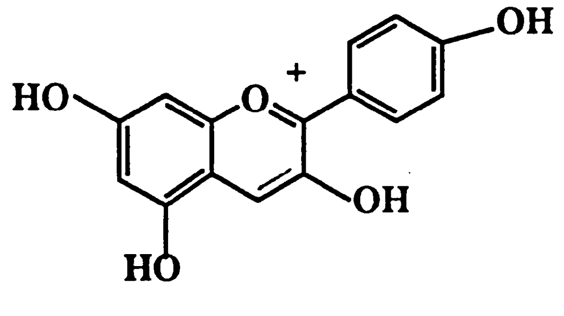
Leucopelargonidin



Flavonol



Proanthocyanidin



Anthocyanidin

1.4.8. Biologically active flavonoids from medicinal plants

Plant flavonoids have been extensively studied and are reported to possess wide spread biological actities. The following are some examples, described in recent reviews, of the various biological effects of flavonoids[89-92].

1.4.8.1, Antitumour activity

It has been reported that using an <u>in vitro</u> assay for the study of tumour invasion, the flavonoid (+)-catechin inhibits invasion. The antiinvasive activity of (+)-catechin can be related to its binding to laminin. (+)-Catechin indeed abrogates cell adhesion to and spreading on laminin substrates, and could in this way inhibit invasion [93]. Flavone acetic acid showed antitumour activity, it was selected through an analogue programme following the test of the diethylamino-2-ethylester of flavone acetic acid. It has minimal activity against murine leukaemia cell lines[94] and would have been discarded if tested only on this first line screening model. Nevertheless, it has demonstrated an original antitumour activity against a broad spectrum of solid tumours when further tested in preclinical screening [95]. Flavone acetic acid showed excellent activity against the colon 38 murine solid tumour which is resistant to most clinical effective cytotoxic agents [96].

1.4.8.2. Anti-inflammatory effects

Flavonoids exert profound effects on cells. A major action of

flavonoids is concerned with fatty acid mobilisation and metabolism. Phospholipase A2 is primarily responsible for the hydrolysis and release of arachidonic acid from membrane phospholipids which is then metabolised via the lipoxygenase pathway to leucotrienes or by cycloxygenase to prostaglandins. These metabolites of arachidonic acid are proinflammatory [97]. Quercetin, rutin and silybin principally inhibit the 5-lipoxygenase pathway [98], while catechin, epicatechin, apigenin and kaempferol predominantly inhibit cycloxygenase activity [99].Other flavonoids e.g., luteolin and morin are active against the enzymes of both prostaglandin synthesis and leucotriene synthesis [100].Animal models, i.e., carrageenin induced oedema, formaldehyde-induced arthritis and granulation tissue formation by cotton pellet implantation in albino rats, are used in the study of antiinflammatory action and have produced the following results: Taxifolin showed activities similar to that of hydrocortisone; gossypin was found to be as effective as phenylbutazone; brazillin, haematoxylin, nepitrin, hypolaetin-8-glycoside, apigenin dimethylether, fisetin and sophoricoside all displayed various degrees of anti-inflammatory activity. _Chamomilla recutita_ is a well-known anti-inflammatory herb for topical use. Among the five chamomile flavonoids which were tested in croton oil-induced inflammation in the mouse ear assay, apigenin and luteolin appeared to be the most active compounds, having similar potency to indomethacin [101].

1.4.8.3. Effects on circulatory system

Flavonoids have been investigated for their action on some aspects of the complex blood-vessel wall interactions and have been reviewed by Beretz and Cazenave [102]. Rutin derivatives have been used in circulatory diseases as veinotonic and vasculoprotector agents, increasing capillary resistance [103]. Important in this area is the effect of flavonoids on blood platelet aggregation. Platelets adhere to components of the subendothelium of the blood vessel walls and their function may be inhibited by stimulating adenylate cyclase which synthesises C-AMP or by inhibiting phosphodiesterases which catabolises the cyclic nucleotides. Flavonoids are effective inhibitors of phosphodieesterases and have also been found to potentiate the action of prostaglandin -I , the most effective inhibitor of platelet function synthesised by the vessel wall. Damaged blood vessels also produce thromboxin which is a platelet aggregating factor and platelets contain the aggregation activating enzymes that ozygenate arachidonic acid[104]. Thus being multi-target inhibitors of the interaction of platelets with the blood vessel wall, flavonoids are of potential interest as antihemorrhagic principles in the traditional hemostatic medicine Biota orientalis [105]. Baicalein and baicalin have been reported to prolong the clotting time of fibrinogen by thrombin [106].

1.4.8.4. Antihepatotoxic activity

The first demonstration that certain flavonoids exert antihepatotoxic activity came from Hahn, et al. who investigated the flavanolignan silybin in some liver damage models [107]. To a great extent the search for liver-protective agents has been promoted since then and progress in this field has been reviewed by Wagner [108]. Silybin,silandrin, silymorin and silyherin exerted strong inhibitory activity on CCl_4 and galactosamine-induced cytotoxicity in primary cultured rat hepatocytes [109]. Kiso et al. screened traditional oriental drugs for liver protection and succeeded in isolating the active flavonoids, capillarisin, arcapillin, quercetin and isorhamnetin from Artemisia capillaris [110]. (+) Catechin (catergen) is a commercially available drug which has been used in Europe since 1976 for the treatment of liver disease. Its protective activity against hepatic steatosis, necrosis and inflammation induced in animals by various hepatotoxic agents as well as its effectiveness in clinical trials in viral hepatitis have been further studied [111].

1.4.8.5. Antiviral activity

Many studies have been published on the antiviral activity of flavonoids under in vitro and in vivo conditions [112]. Recently, the antiviral properties of some flavonoids was further surveyed by Vlietinck, et al.[113]. Ishitsuka, et al. found 5,4'-dihydroxy-3,7,3'-trimethoxyflavone which was isolated from the Chinese traditional herb Agastache rugosa

Kartz, to be highly active in tissue cultures against all picorna viruses except mengovirus [114]. Vanhoof, et al. found several derivatives of 3-methyl-quercetin and 3-methyl-kaempferol isolated from some species to possess pronounced antiviral properties against the picornaviruses [115]. The structure-activity study of antirhinovirus natural occurring flavonoids such as axillarin, chrysosplenol-B and C was reported by Tsushiya, et al [116]. It was suggested that both 3-methoxyl and 5-hydroxy group of the flavone skeleton are necessary for specific antirhinovirus activity; however, some of the most active compounds are the halogenated flavans, e.g., 4,6-dichloflavan [117].

In searching antiviral drugs, we have screened 300 Chinese traditional medicinal plants. Some flavonoids possess antiviral properties against reverse transcriptase, e.g., ellagic acid and gallic acid are antiviral constituents of _Punica granatum_ [118].

1.4.8.6. Other biological activities of flavonoids

Nobiletin, the main flavonoid from _Citrus aurantium_ was shown to have anti-allergic effect by its significant inhibitory action on histamine release from rat peritoneal mast cells [119].

The methoxyflavonnoids cirsilineol, thymonin and 8-methoxycirsilineol from _Thymus vulgaris_ were showed to inhibit smooth muscle activity [120].

The unusual compound 5,2'-dihydroxy-6,7,8,6'-tetramethoxyflavone isolated from the roots of _Scutellaria baicalensis_ exhibits cytotoxic activity [121]. Indeed, many of the 6-methoxyflavones are particularly cytotoxic, an area which has been reviewed by Edwards, et al. [122]. 3',4'-Dihydroxy-5,6,7,8-tetramethoxyflavone was found to be a potent inhibitor of lens aldose reductase, an important enzyme in the pathogenesis of sugar cataract [123]. The 3,6-dimethyl derivatives of 6-hydroxykaempferol were also reported to be the lens aldose inhibitor in the Paraguayan medicinal plant _Acanthospermum australe_ [124].
Elford,et al. have reported that the methoxylated flavones artemetin and casticin have weak antimalarial activity and can markedly enhance the activity of artemisinin [6].

1.5. Methods of isolation and identification of flavonoids

Methods of the isolation and identification of flavonoids have developed in the past thirty years and HPLC and ^{13}C NMR have been especially successful for separation and identification [125].

1.5.1. Chromatographic methods

Chromatographic techniques for the separation and detection of flavonoids in crude plant extracts have been well established. Column, thin-layer and high performance liquid chromatography are used to provide pure compounds in sufficient quantity for structural determination [126]

1.5.1.1 Thin-layer chromatography (TLC)

Thin-layer chromatography is commonly used for the detection and separation of flavonoids. The adsorbents, solvent systems and spray regents for TLC of flavonoids have been previously reviewed and summarised by Markham [127]. Mostly, TLC has been used to detect the purity of compounds and to compare unknown compounds with reference compounds. Different adsorbents have been used for different flavonoids. TLC using silica gel is useful for the separation of flavone-O-glycosides, flavone-C-glycosides and flavonol-O-glycosides.Solvents containing water are often used to reduce the activity of adsorbents. TLC on cellulose is used for the separation of flavonoid glycosides, especially for comparison of compounds before and after hydrolysis; 50% HOAC or 15% HOAC are used as solvents. Polyamide TLC is especially valuable for distinguishing the various hydroxylated flavones, flavonols as well as methylethers of both flavones and flavonols. Useful solvents for analytical and preparative scale TLC on polyamide are toluene-petroleum ether (b.p. 100-140)-MeCOEt-MeOH(30:60:5:5),(30:60:10:5), toluene-MeCOEt-MeOH(60:25:15), $CHCl_3$-MeOH(9:1) and $CHCl_3$-Me_2CO-MeOH(20:5:1). Flavonoid glycosides have been separated on polyamide with the following solvent systems: H_2O-EtOH-MeCOEt-acetylacetone(65:15:15:5), H_2O nBuOH-acetone-acetic acid(16:2:2:1), nitromethane-MeOH(3:4), MeOH-H_2O-acetic acid(90:5:5), H_2O-MeCOEt-MeOH-2,4-pentanedione (13:3:3:1) and $CHCl_3$-MeCOEt-MeOH-H_2O either as (60:30:5:1) or as (40:20:5:1).

1.5.1.2. High performance thin-layer chromatography(HPTLC)

High performance TLC is a development of TLC carried out using very small particles(5μm). It requires very small samples and provides rapid separation. An application has been reported by Hiermann and Kartning who separated flavonoids on HPTLC silica gel plates, using benzene-EtOAC-formic acid(40:10:5) for aglycones and acetone-MeCOEt-formic acid(50:35:5) for glycosides.Reverse phase HPTLC on RP-18, RP-2 RP-8 has also been applied to the separation of flavonoids.

1.5.1.3. Column chromatography

Column chromatography, which is used for the isolation of flavonoids from crude plant extracts, has been discussed in depth in a number of articles [128]. Adsorbents commonly used include silica gel, kieselguhr,magnesium silicate, polyamide, polyclar,sephadex and ion exchange resins. The adsorbents of choice are generally polyamide, silica gel and cellulose. Polyamide commercially available are mainly of the perlon-type (polycaprolactone), nylon-type(polyhexamethylenediamine adipate) or polyclar-type(polyvinylpyrrolidone, PVP). Polyamide column chromatography is suitable for the separation of all types of flavonoids. Flavones and flavonols have been separated with solvent systems similar to those recommended for TLC. Different glycosides have been isolated by gradient elution with MeOH-water mixtures.
Silica gel has been used frequently for the separation of flavonoids. It is also useful for the more polar flavonoids

simply by deactivation through the addition of water. Good separation for flavonoid glycosides was obtained with $CHCl_3$-$MeOH$-H_2O(80:20:1) or in the proportions(65:20:2) and (80:18:2). Markham [129] has discussed the advantages of sephadex gel in the isolation of flavonols. Sephadex G-10, G-25 and LH-20 are the most widely used gel types. On sephadex LH-20, flavonol glycosides have separated, using $MeOH$-H_2O(different ratios).

1.5.1.4. High-performance liquid chromatography (HPLC)

High-performance liquid chromatography has proven to be one of the most useful methods for separating complex mixtures of natural products. It is now almost a standard procedure for the accurate determination of the amount of flavonol glycosides in crude plant extracts [130]. Harborne has compared HPLC to other methods of separation and identification of flavonoids [131].
It is important to choose an appropriate column; for example, silica gel is suitable for the separation of non-polar or weakly polar flavonoid aglycones. HPLC using Lichrosorb Si-60 as an adsorbent and a mixture of heptane-propan-2-ol(60:40) as eluent was found to be a very efficient method for the separation of polymethoxylated flavones [132]. A range of flavonoid acetates has been separated and determined on Lichrosorb Si-60 using four solvent systems by Galensa and Herrmann [133].
The most commonly used column for HPLC are of the reverse-phase type. Reverse-phase columns are prepared by bonding organosilane molecules, e.g., octadecyl-trichlorosilane, to

hydroxyl groups of silica gel type. In reverse-phase systems, the stationary phase is less polar than the mobile phase; thus, highly polar solutes possess shorter retention times than less polar solutes. Glycosides will be eluted first, followed by aglycones in the order of decreasing polarities. In practice, numerous types of packed columns possessing a high degree of reproducibility are commercially available. Wehrli estimated that 80% of separations are on octadecylsilyl bonded phase columns, called C18 columns [134].

The commonly used solvent systems for flavonoid glycosides are acetonitrile-H_2O mixtures and MeOH-H_2O mixtures containing small amounts of acetic acid. These mobile phases are suitable for use with UV detection and can easily be employed in gradient systems for complex separations. Depending on the components to be separated, it may be advantageous to replace C18 by C8 groups. Flavonoids may be detected after HPLC separation by the UV light at 254 or 280nm.

1.5.1.5. Other methods

Besides the methods mentioned, some others have been used in flavonoids studies. Centrifugal thin-layer chromatography is a method to increase the separation speed by acceleration of the flow-rate of the mobile phase using centrifugal force. It has been used for the separation of xanthones and some flavones.Droplet counter-current chromatography(DCCC) is a very efficient, all-liquid separation method carried out by passing droplets of a mobile phase through columns of surrounding

stationary liquid phase . The problem of the irreversible adsorption of solutes onto the solid stationary phase can be avoided in DCCC. The technique is particularly useful for separation of polar compounds, especially glycosides, which are difficult to isolate [135].

Paper chromatography has been used as a general procedure for routinely screening plant tissue in order to determine the pattern of flavonoids present. The flavonoid data obtained from these chromatograms has been used for classification of flavonoid types and for comparing the chemistry of different species within genera [136].

Gas-liquid chromatography(GLC) provides both qualitative and quantitative analysis. Flavonoids are converted to their trimethylsilyl ether derivatives before being subjected to GLC. The combined GC-MS method was used to the determination of new flavonoid triglycosides [137].

Flash chromatography is a simple and efficient adsorption chromatography especially indicated for a quick separation with rather low resolution of crude plant extracts. It is often used in combination with centrifugal TLC, open column chromatography on polyamide and on sephadex LH-20 and with low pressure liquid chromatography [138]. The use of one ideal chromatographic method mentioned above is seldom satisfactory for the separation of both large and small quantities of complex mixtures. Good results can only be obtained by a combination of several methods which are often complementary.

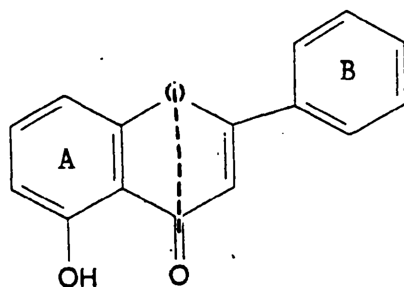
1.5.2. Spectroscopic methods

Spectroscopic methods have been successfully used for the structural analysis of flavonoids. The commonly used spectroscopic methods for structural analysis of flavonoids are UV, MS, 'HNMR and ^{13}CNMR.

1.5.2.1. UV spectroscopy

UV spectrocopy has played an important role in the structural analysis of flavonoids. In general, flavones and flavonols in MeOH exhibit two major absorption peaks in the region 240-400nm. These peaks are referred to as Band 1(300-380nm) and Band 2 (240-280 nm) (Figure 21).

Figure 21 UV absorption of flavonoids



A-ring Benzoyl		B-ring Cinnamoyl	
Band 2	240-280nm	Band 1	300-380nm

Band 1 is considered to be associated with absorption due to the B-ring cinnamoyl system and Band 2 with absorption involving the A-ring benzoyl system. Band 1 gives information about the type of flavonoid as well as its oxidation pattern in B-ring. Flavones exhibit Band 1 at 304-350 nm, whereas for flavonols, it occurs at 352-385 nm. For flavonols with substitution at C-3, the general shape of the curve as well as the ranges of Band 1 (328-357 nm) approach those of flavones [139].

Increasing hydroxylation of A-ring in flavones and flavonols produces a notable bathochromic shift in Band 2 and a small effect on Band 1. The presence or absence of the H-bonded 5-hydroxyl group has a marked effect on both Band 1 and Band 2 in the UV spectra of flavones. When the 5-hydroxyl group is absent from a flavone or flavonol both Bands appear at shorter wave length than for the 5-hydroxylated equivalent (3-10 nm in Band 1 and 6-17 nm in Band 2) [140].

On increasing the oxygenation of the B-ring of flavones and flavonols, a bathochromic shift in Band 1 occurs with each additional oxygen function. On the other hand, Band 2 appears as one peak at about 270 nm in compounds with monosubstituted B-ring, but as two peaks or one peak at about 258 nm plus a shoulder at about 272 nm when a di- or tri-O-substituted B-ring is present [141].

It is well known that structural information may be obtained from the changes in UV characteristic after addition of a series of shift reagents. The effects of these diagnostic

reagents in the UV spectra in flavones and flavonols are as follows : The addition of NaOMe to flavones and flavonols in MeOH produces a large bathochromic shift of Band 1 (40-65 nm) without a decrease in intensity if a free 4'-hydroxyl group is present. Although flavones without 4'-hydroxyl group also produce a bathochromic shift of 50-60 nm in Band 1, the intensity is decreased [142].

The UV spectra of flavones and flavonols containing a free 7-hydroxyl group usually exhibit a diagnostic 5-20 nm bathochromic shift of Band 2 in the presence of NaOAC. The flavones and flavonols which possess a 4'-hydroxyl group and no free 3- or 7-hydroxyl groups usually show a pronounced shoulder on the long wavelength side of Band 1 in the presence of NaOAc. When Band 1 in NaOAc spectrum is the same as, or appears at longer wavelength than Band 1 in the NaOMe spectrum, the flavonoid contains a 7-O-substitution [143].
Flavones and flavonols containing a B-ring ortho-dihydroxyl group show a consistent bathochromic shift of Band 1 (12-30 nm) in the presence of NaOAC/H_3BO_3.
A-ring orthodihydroxyl groups at C-6, 7 or C-7, 8 in flavonoids also exhibit a bathochromic shift of Band 1 (5-10 nm) by the effect of NaOAC/H_3BO_3 [144].
The presence of an ortho-dihydroxyl group in the B-ring of flavones and flavonols can be also detected by comparison of the spectrum of the flavonoid in the presence of $AlCl_3$ with that obtained in $AlCl_3$/HCl. A hypsochromic shift of 30-40 nm is obtained in Band 1 of the $AlCl_3$ spectrum on the addition of

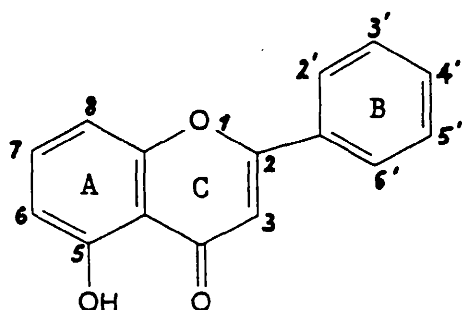
acid. However, a hypsochromic shift of only 20 nm appears in the case of flavonoids having adjacent hydroxyl groups present in the B-ring. The addition of acid to a methanolic solution of a flavone or flavonol which already contains $AlCl_3$ disrupts bonding between $AlCl_3$ and ortho-dihydroxyl groups; therefore, any shift remaining in Band 1 or Band 2 relative to the methanol spectrum will be due to the presence of free 3- and/or 5-hydroxyl groups in the flavone. The bathochromic shifts of Band 1 are in the range 35-55 nm for 5-hydroxyflavones and 3-substituted flavonols; whereas the shift is around 60 nm for 3-hydroxyflavones and in the range of 50-60 nm for 3,5-dihydroxyflavones [145].

For flavones and 3-O-substituted flavonols, possessing hydroxyl group at C-5 and a methoxyl group at C-6, the bathochromic shift of Band 1 obtained by addition of $AlCl_3$/HCl relative to the methanol spectrum is only about 20 nm, whereas with a methoxyl group at C-8 the shift is about 45 nm [146]. This allows the discrimination of 6-O-substituted from 8-O-substituted compounds. $AlCl_3$/HCl produces a large shift of 55-75 nm of Band 1 relative to the methanol spectrum for flavones and 3-O-substituted flavonols with a free hydroxyl group at C-8 [147].

UV spectra of flavonoids exhibit very useful data for identifying type of flavonoid, substitution of A-ring and B-ring and the relationship of substituent groups.

1.5.2.2. 'H NMR spectroscopy

'H NMR spectroscopy is invaluable for the structure analysis of flavonoids [148].Some flavonoid aglycones are sufficiently soluble in the commonly used solvents ($CDCl_3$ or CCl_4) for direct analysis. However, most naturally occurring flavonoids, including all of the flavonoid glycosides, are insoluble in these solvents and hexadeuteriodimethyl sulfoxide (DMSO-d6) is used as a solvent. The chemical shifts (in δ values) of A,B,C-rings and sugar protons are described briefly as follows [149]:



The protons of the A-ring at C-6 and C-8 in flavonoids which contain the common 5,7-dihydroxylation pattern give rise to two doublets (J=2.5 Hz) in the range δ 6.4-6.9 ppm. The H-6 doublet consistently occurs at higher field than H-8, and glycosylation of the hydroxyl group at C-7 causes the signals for both H-8 and H-6 to be shifted downfield [150]. In flavanones and dihydroflavonols which contain the 5,7-dihydroxy substitution pattern, the signals for C-6 and C-8 protons appear at higher field than in the corresponding flavones and flavonols. In the region of the signals for C-6 and C-8 protons of flavones, the only other proton signal which may occur is that of the C-3 proton which appears as a singlet at 6.3-6.9 ppm. [151].

In the spectrum of a compound with an unsubstituted 5-hydroxyl group, the signal for the C-3 proton singlet is shifted

downfield (0.15 ppm) while the C-8 proton signal is shifted upfield by about 0.15 ppm. The signal of the C-6 proton is almost unaffected. Some 6- and 8-C-glycosyl flavones can be distinguished after conversion to acetate derivatives in which the signal for the C-6 proton in acetylated 8-C-glycosyl flavones appears in region of δ 6.5-6.7 ppm while the C-8 proton signal in acetylated 6-C-glycosyl flavones is at δ 7.25-7.40 ppm [152].

The signals for the protons of the B-ring appear in the region of δ 6.5-7.9 ppm. The signal pattern is characteristic for the substitution pattern. If the B-ring is oxygenated at C-4', a typical four peaks pattern of two doublets (J=8.5 Hz) is observed. The signal for C-3' and C-5' protons always appears at upper field in the range δ 6.65-7.1 ppm while that of C-2' and C-6' falls at lower field (δ 7.1-8.1 ppm). In 3',4'-dioxygenated flavonoids, the C-5' proton appears as a doublet signal at δ 6.7-7.1 ppm (J=8.5 Hz) and the C-2' and C-6' proton signal usually occurs at δ 7.2-7.9 ppm. The C-2' proton signal is usually at slightly higher field than the C-6' proton signal in flavonoids containing the 4'-methoxyl group. These compounds give a complex multiplet, usually two peaks for the C-2',C-5' and C-6' protons in the region δ 6.7-7.1 ppm. In flavonoids having the 3',4',5'-trioxygenation pattern, the C-2' and C-6' proton signals usually overlap in the region δ 6.7-7.5 ppm. Methylation or glycosylation of the 3'- or 5'-hydroxyl, results in these protons appearing as distinct doublets (J=2 Hz)[153].

The C-ring protons have considerable variation in their

chemical shifts which is dependent upon the oxidation level of the flavonoid. The C-3 proton in a flavone gives a sharp signal near to δ 6.3 ppm. On the other hand , the C-2 proton in isoflavones, which is in the β position to the C-4 function, occurs at δ 7.6-7.8 ppm in CCl_4 , 7.8-8.1 ppm in $CDCl_3$ or 8.5-8.7 ppm in DMSO-d6. The signal for the C-2 proton of flavanones appears as a quartet (J trans=11 Hz, J cis=5 Hz) as a result of the coupling of the C-2 proton with the two C-3 protons. The C-3 protons couple with each other (J=17 Hz) in addition to their interaction with the C-2 proton and thus give rise to two overlapping quartets near δ 2.8 ppm. In naturally occuring dihydroflavonols, the C-2 proton signal occurs as a doublet (J=11 Hz) near δ 4.9 ppm, while the C-3 proton appears further upfield at about δ 4.3 ppm. Glycosylation of the 3-hydroxyl causes a downfield shift of both the C-2 and C-3 proton signals [154].

In flavonoids, methoxyl proton signals usually appear at δ 3.5-4.1 ppm, while most aromatic acetoxyl proton signals occur at δ 2.30-2.50 ppm [155].

In flavonoid glycosides, the chemical shifts of the sugar protons occur at δ 3.3-3.9 ppm with the exception of the C-1" proton of the sugar which can give some information regarding the site of glycosylation and on occasion, the nature of the sugar. In flavonol 3-O-glycosides, the C-1" proton signal appears downfield at about δ 5.7-6.0 ppm, whereas when a sugar is on the C-4',C-5 or C-7, the C-1" proton, the signal appears in the upperfield region δ 4.8-5.2 ppm. Glucose commonly forms

a β-linkage and the C-1" proton which has diaxial coupling with the C-2" proton usually appear as a doublet (J=7 Hz), while in flavonoid 7-O-glycosides, the C-1" proton which experiences a different electronic environment gives a complex multiplet [156].

In the naturally occurring α-linked rhamnosides, the diequatorial coupling between C-1" and C-2" protons gives rise to a coupling constant of only 2 Hz. In both 3- and 7-O-rhamnosides, the C-1" proton signal occurs at δ 5.0-5.3 ppm. The signal for the rhamnose methyl group which occurs as a doublet (J=6.5 Hz) or multiplet at δ 0.8-1.2 ppm is also a useful distinguishing feature [157].

Therefore, ^{1}H NMR plays an important role in the analysis of flavonoid structures. It can show the type of flavonoid, the pattern of substitution of A and B-rings and the groups of substitution. In flavonoid glycosides, it can exhibit the type of sugar and its connection with the aglycone.

1.5.2.3. ^{13}C NMR Spectroscopy

^{13}C NMR is a useful method for the structure analysis of flavonoids, especially for their glycosides. In the chapter "Carbon-13 NMR spectroscopy of flavonoids" [158], in "The Flavonoids ", a valuable reference of 125 flavonoid spectra is listed. The interpretation of spectra and the usefulness of the technique are summarised as follows [159].

In general , the different types of flavonoid aglycone are not distinguishable on the basis of the aromatic carbon resonances

alone, but the chemical shifts for the central-carbon unit (C-ring) are often quite distinctive. The C-2, C-3 and carbonyl C-4 resonances of flavones appear at δ 160.5-165 ppm, 103-111.8 ppm and 176.3-194 ppm,respectively. The C-2, C-3 and C-4 resonances of flavonols appear at δ 145-150 ppm, 136-139 ppm and 172-177 ppm respectively . In the presence of H-bonding to a C-5-hydroxyl group, the signal of C-4 moves downfield to about δ 182 ppm.

When a C-3-hydroxyl is present as well as a C-5-hydroxyl, the resonance returns to about δ 176 ppm, but with only a C-3-hydroxyl, the resonance appears at about δ 171-173 ppm.

The marked substituent effects on rings A and C by the presence of C-3 and C-5-hydroxyl groups is different from that for aromatic systems. Introduction of a C-3-hydroxyl shifts the C-3 signal more than 33 ppm and the ortho effect on C-2 produces a shift of more than 17 ppm. The introduction of a C-5-hydroxyl causes a downfield shift of about 31 ppm in the C-5 signal and the C-4 resonance is downfield by about 6 ppm, with the C-8 signal shifting upfield by 11 ppm.

^{13}C NMR offers a non-destructive method of studying the sugar moieties of flavonoid glycosides, becouse of the distinctive resonances of the sugar carbons. The chemical shifts of different sugars are readily distinguishable from one another. In flavonol-O-monoglycosides, the C-1 of sugar which is linked via a hemiacetal bond to the aglycone produces resonance to a lower field and the extent of the downfield shift (4-6 ppm) depends very much on the environment of the phenolic hydroxyl

group. The presence of oxygen substituents in both ortho-positions appears to shift the C-1 signal of the sugar downfield to about δ 107 ppm. Other sugar carbon resonances are little affected by glycosylation. Thus the effect of glycosylation of the C-7-hydroxyl on the C-7 signal is an upfield shift and this is accompanied by downfield shifts of about 1 ppm in the ortho-related C-6 and C-8 signals and 1.7 ppm in the para- related C-10 signal.

Glycosylation of the C-3 and C-5 hydroxyls, as expected, produces unusual effects. With a C-3 hydroxyl, although the upfield shift of the C-3 signal is of the expected order (2ppm), the downfield shift of the ortho-related C-2 signal at 9.2 ppm is especially pronounced. Glycosylation of the C-5 hydroxyl in luteolin has a marked effect on the resonance of all A- and B-ring carbons [160].

In flavonol-O-diglycosides, the site at which a second sugar is attached to the sugar of the flavonoid mono-O-glycoside gives significant information in ^{13}C NMR spectra. It has been established that glycosylation of sugar hydroxyls produces a sizeable downfield shift in the resonances of the hydroxylated carbon and upfield shift in the resonances of adjacent carbons. The data for diglycosides have also been used with some success in defining the glycoside structures of flavonoid tri- and tetra-glycosides [161].

Thus in a flavonoid-O-diglucoside, C-1 of the first glucose will normally resonate in the range of δ 100-102.5 ppm, whereas the C-1 of the terminal glucose will resonate at about

104 ppm. Likewise with rhamnose, the C-1 resonance occurs at about 98.8 ppm in 7-O-rhamnose but in rutinosides and neohesperidosides it occurs at about δ 100.6 ppm. In acylated glycosides, the site of acylation is evidenced by a downfield shift of the acylated carbon and in upfield shifts of the signals due to adjacent carbons [162].

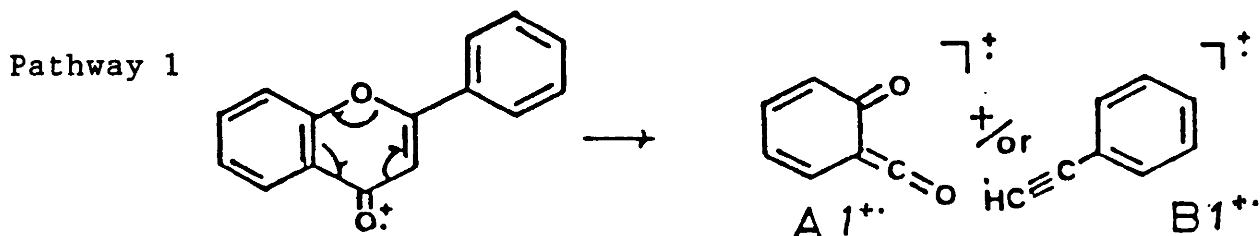
1.5.2.4 Mass spectroscopy

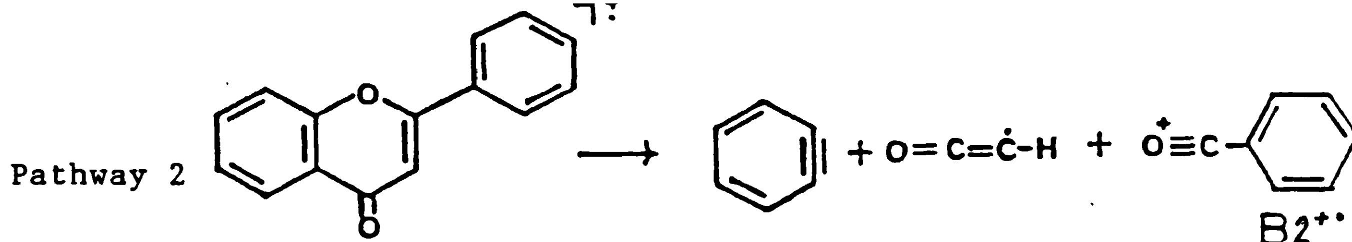
Electron impact (EI) mass spectroscopy has been applied successfully to all classes of flavonoid aglycones and to a number of different types of glycosides [163].

Most flavonoid aglycones give an intense peak for the molecular ion (M^+) (base peak). However, the molecular ion of flavonoid glycosides is rarely observed and even that of permethylated or peracetylated derivatives gives a peak of low intensity. In addition to the molecular ion, the aglycones usually afford a major peak for $[M-H]^+$ and when methoxylated $[M-CH_3]^+$. The most useful fragmentations are those which involve cleavage of intact A- and B-ring fragments. Two common fragmentation patterns are characterized by pathway 1 and pathway 2 [164].

Figure 22 Mass spectral fragmentation pathways of flavonoid aglycones

Pathway 1





Flavones give molecular ions as their base peaks with other major peaks corresponding to $[M-H]^+$, $[M-CO]^+$, A1 , $[A1-CO]^+$, B1 and B2. Substitution in the A-ring can be detected by examning the m/z value for the A1 fragment and similarly, the m/z value for the B-ring fragments can pinpoint substitution in the ring. Flavones with four or more hydroxyl and methoxyl group often give moderately intense A1 and B1 fragments [165].

Most flavonol aglycones give the molecular ion as the base peak. However, other ions including $[M-H]^+$, $[M-CH_3]^+$, $[M-CH_3-CO]^+$ and weak fragments which correspond to A1 and B2 can provide considerable structural information. The process which leads to a $[M-CH_3]^+$ ion is a major fragmentation pathway for flavonols with either 6-OCH_3 or 8-OCH_3 substituents. $[M-CH_3-CO]^+$ ion is primarily derived by a concerted loss of CO and a methyl radical from either 3-methoxyflavonols or 6-methoxyflavonols. $[A1+H]^+$ is one of the most important A-ring ion from flavonols and corresponds to a fragment derived by pathway 1 combined with a hydrogen transfer. B2 and its fragment (loss of CO) are the diagnostic fragments from B-ring through pathway 2 [166]. Discrimination between substitution at C-6 and C-8 is a problem which has received special attention. In 6-methoxyflavones and 6-methoxyflavonols, the M^+ peak is the base peak, while 8-methoxyflavones and 8-methoxyflavonols $[M-15]^+$ is the base peak.For 6-methoxycompounds, the relative intensity of $[M-18]^+$ is greater than 10%, whilst in 8-methoxycompounds it is lower.

A $[M-43]^+$ peak of less than 30% intensity might be indicative of underivatized 8-methoxy and /or 3-methoxyflavonols,whereas a $[M-43]^+$ peak of more than 30% is indicative for underivatized 6-methoxyflavones or flavonols [167].

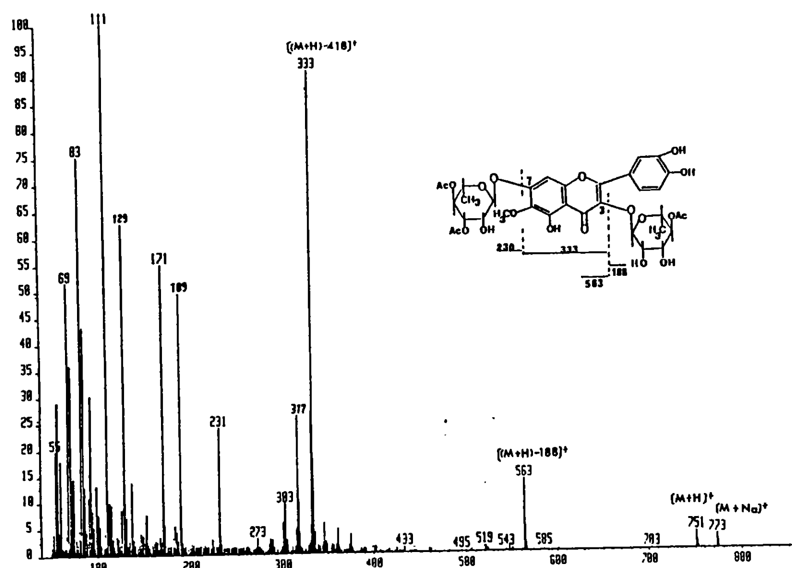
Mass spectrometry, using soft ionization techniques, is a very helpful tool in the structure elucidation of flavonoid glycosides. These recently developed techniques have the advantages that preliminary derivatization is not required and that much information can be obtained with only a few micrograms of sample. The application of field desorption (FD) techniques to the study of flavonoids has been used recently [168]. Other soft ionization methods have been developed including desorption chemical ionization (DCI) which uses a probe consisting of an electrically heated tungsten wire introduced into the chemical ionization source [169].

Another technique is fast atomic bombardment (FAB) mass spectroscopy for use with neutral atoms. In the FAB method, the sample is solubilized in a polar matrix (e.g., glycerol or thioglycerol) and deposited on a copper target which is bombarded with energized atoms inducing desorption and ionization. FAB-MS has the ability to provide useful structural information for flavonoids and flavonoid glycosides. It is not only possible to obtain strong molecular ions without derivatization, but also fragmentation patterns indicative of the location of the glycosidic group. We have reported [81,82] that ten new novel acetylated di-O-rhamnose flavone glycosides have been identified by FAB-MS for obtaining stronger molecular

ions and indication of the location of the acetylation group at two rhamnoses. For example, FAB-MS of 3''',4''',4''-O-triacetylpatuletin 3,7-di-O-rhamnoside showed the molecular ion at m/z 751 $(M+H)^+$, indicating a compound with three acetyl groups on the two rhamnoses. The major fragmentation pattern showed loss of monoacetyl rhamnose from the molecular ion to give an ion at m/z 563 and subsequent loss of diacetylrhamnose to give an ion at m/z 333 which showed that the aglycone was substituted with three hydroxyl groups and two methoxyl groups [170].

Glucuronides are very polar compounds and it is difficult to obtain their mass spectra, e.g., quercetin-3-O-glucuronide was studied by DCI and FAB methods but no molecular ion was observed in the DCI spectrum. However, in the FAB spectrum, $[M+Na]^+$ (m/z 501), $[M+H]^+$ (m/z 479) and $[(M+H)-176]^+$ (m/z 303) signals were observed [171]. Therefore, FAB-MS is particularly useful for determining the molecular ions of very polar flavonoid glycosides.

FIGURE 23 FAB-MS OF 3''', 4''', 4''-O-TRIACETYLPATULETIN 3,7-DI-O-RHAMNOSIDE



1.6. Aims and objectives

Artemisinin has been shown to be the active constituent of the neutral fraction of the ether extract of *Artemisia annua*, but the active compounds of the dilute alcohol extract successfully used in clinical trials has not been elucidated [172]. Elford et al. have reported that some methoxylated flavones can enhance the *in vitro* activity of artemisinin against *Plasmodium falciparum*. The constituents of *A. annua* have been further examined in order to determine whether there are compounds other than artemisinin, which have antimalarial activity and whether there are other flavonoids which are capable of enhancing the *in vitro* activity of artemisinin against *P. falciparum*.

PART 2 EXPERIMENTAL

2.1. Materials

2.1.1 Plant materials

Fresh plant material of A. annua was collected in Dong Beiwong, 20 km west of Beijing in August 1987. Sample were authenticated by Professor W.Lian(IMPLAD Beijing) and a voucher specimen is deposited in the herbarium, IMPLAD, Beijing (NO. Lian 1987-8)

2.1.2. Chromatographic materials

Materials	Source
Polyamide 11F 254 (TLC plates)	Merck
Silica gel 60F 254 (TLC plates)	Merck
Polyclar AT	Graf LtD (UK)
Sephadex LH-20	Pharmacia LtD (Uppsala sweden)

2.1.3. Solvents and Miscellaneous chemicals

Solvents	Source
0.880 ammonia solution	BDH
Aluminium chloride	BDH
Boric acid	Hopkin and williams LtD chadwell Heath, Essex
n-Butanol	M and B
Butanone	BDH
Chloroform	BDH
Ethyl acetate	BDH
Hydrochloric acid	M and B

Methanol	BDH
Petroleum ether (40-60°c)	BDH
Sodium acetate	Hopkin and williams LtD
Sodium methoxide (25% in MeOH)	Aldrich Chemical Co. Gillingham, Dorset

2.2. Apparatus

2.2.1. Thin-layer chromatography

Silica gel plates and polyamide plates (20x20 cm) were prepared, using a Jobling Laboratory Division Moving Spreader.The chromatograms were visualized under a spectralight ultraviolet lamp at 254nm and 366nm.

2.2.2. Column chromatography

Column chromagraphy was carried out in glass columns of different size, filled with silica gel, polyclar or sephadex LH-20.

2.2.3. Spectroscopy

A. Ultra-violet spectroscopy

UV spectra were recorded on a Perkin-Elmer 402 Ultra-violet-visible spectrophotometer.

B. Proton nuclear magnetic resonance spectroscopy

'H NMR spectra were obtained on a Brucker WP80 SY80 MHz or on a Brucker WM 250 MHz spectrometer.

C. Mass spectrometry

Electron impact MS: EIMS spectra were recorded on a VG

Analytical Ltd ZAB IF spectrometer.

2.3. Methods

2.3.1. Extraction and fraction procedures

The fresh aerial parts of A. annua (19kg) was extracted by heating with ethanol for 2h. The concentrated extract (970g) was partitioned sequentially from H_2O into the following solvents which were concentrated to the weights given : n-Hexane (172g); $CHCl_3$ (224g); EtOAC (21.2g) and n-BuOH (288g). Flavonoids from n-hexane, $CHCl_3$ and EtOAC fractions were chromatographed on polyclar AT using a $CHCl_3$-MeOH gradient from 100% $CHCl_3$ to 100% MeOH. Compounds were further purified, where necessary, using silica gel columns in the same solvent system. Sephadex LH-20 was used for the preparation of compounds for spectral analysis.

2.3.2. Chromatographic methods

A. Thin-layer chromatography

TLC plates: Polyamide and silica gel plates were routinely used to monitor each fraction obtained from column chromatography to check the flavonoid components.

Solvents: The following solvent systems were used depending upon the polarity of the compounds.

1. $CHCl_3$: MeOH (9:1)
2. $CHCl_3$: MeOH : Butanone (90:10:10)
3. $CHCl_3$: MeOH : Butanone : Acetone (30:10:5:1)

Visualisation : The developed chromatograms were viewed under UV 254nm and 366nm with and without fuming ammonia vapour.

B. Column chromatography

For the preparation of columns, polyclar AT was soaked in chloroform overnight before being packed into a glass column and was left running with solvent overnight before applying the extract. Extracts were mixed with coarse silica gel before packing the column. Elution was started with chloroform, followed by adding methanol and gradually increasing the concentration of methanol to 100%. The flavonoid-containing bands were monitored under UV 366nm and appropriate bands were collected. Some fractions were rechromatographed on a silica gel column, using the same solvent system. A small sephadex LH-20 column was used for a final purification of all compounds prior to spectral analysis.

2.3.3. Spectroscopic methods

A. UV spectroscopy

The UV spectra were recorded in methanol using the following standard shift reagents [173].

Sodium methoxide (NaOMe) : Three drops of 1%(V/V) NaOMe was added to the solution of compound under investigation.

Aluminium chloride ($AlCl_3$) : Six drops of 5% $AlCl_3$ in methanol solution were added to the solution under investigation.

Aluminium chloride/Hydrochloric acid ($AlCl_3$/HCl) : After the $AlCl_3$ spectra was obtained, three drops of 50% (V/V) conc. HCl were added to the solution and the spectra recorded again.

Sodium acetate (NaOAc) : Anhydrous NaOAc was added to the solution under investigation until about 0.5cm layer of precipitate appeared in the bottom of the solution.

Sodium acetate/Boric acid (NaOAc/H_3BO_3) : After the NaOAc spectra was obtained, another layer of anhydrous H_3BO_3 was added to the solution.

B. NMR spectroscopy

^{1}H NMR spectra were run at 250 MHz respectively. Chemical shifts (δ) were reported in parts per million (PPM) on the scale and were related to tetramethylsilane (TMS). Solvents used were spectroscopic grade deuteriochloroform ($CDCl_3$), tetradeuteriomethanol (CD_3OD) and hexadeuteriodimethylsulphoxide (DMSO-d6).

C. Mass spectrometry

EIMS spectra were recorded by direct inset at 70ev in chloroform at 170-220°C.

PART 3 RESULT AND DISCUSION

3.1 Results

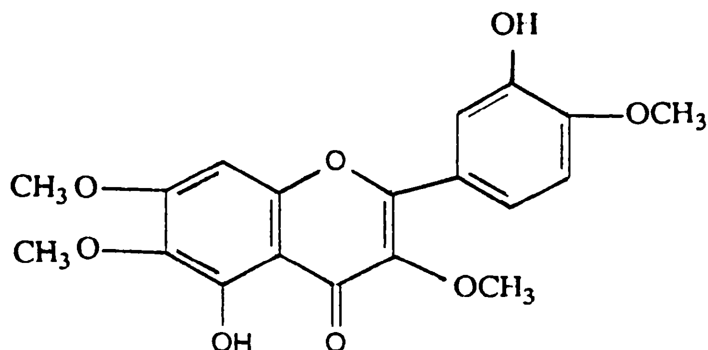
3.1.1. Compounds isolated from Artemisia annua

The ethanolic extract of material was fractionated by the solvent partition method to give the n-hexane, chloroform and ethyl acetate fractions. Polyclar AT column chromatography was used for the separation of individual compounds. Some fractions were rechromatographed on silica gel columns and finally sephadex LH-20 column were used for preparation of compounds for spectral analysis. All separations were monitored by TLC.
A total of forty compounds,twenty seven flavones, seven flavone glycosides, four coumarins and two chromene derivatives, were isolated and their structures elucidated on the basis of spectroscopic analysis (UV,'H NMR and MS). Among the compounds obtained,eight of them are novel compounds, four of them being methoxylated flavones, two being flavone glycosides and the other two chromenes.The thirty known compounds which have not been reported previously from this plant are : chrysoplenetin (12mg),

(0.000063%), chrysosplenol-D (35 mg, 0.000182%), cirsiliol (1mg, 0.000005%), circilineol (2 mg, 0,000010%), penduletin (1 mg, 0.000005%), eupatorin (3 mg, 0.000016%), axillarin (1 mg, 0.000005%), tamarixetin (1 mg, 0.000005%), rhamnetin (1 mg, 0.000005%), quercetin-3-methyl-ether (1 mg, 0.000005%), cirsimaritin (1 mg, 0.000005%), rhamnocitrin (1 mg, 0.000005%), chrysoeriol (2 mg, 0.000010%), apigenin (3 mg, 0.000016%), luteolin (3 mg, 0.000016%), kaempferol (6 mg, 0.000031%), quercetin (6 mg, 0.000031%), isorhamnetin (1 mg, 0.000005%), luteolin-4'-methylether (2 mg, 0.000010%), isokaempferide (2 mg, 0.000010%), quercetagetin-3-methylether (4 mg, 0.000021%), tomentin (2 mg, 0.000010%), astragalin (3 mg, 0.000016%), luteolin-7-glucoside (2 mg, 0.000010%), quercetin-3'-glucoside (25 mg, 0.000132%), isoquercitrin (6 mg, 0.000031%), quercimeritrin (7 mg, 0.000036%), scoparone (2 mg, 0.000010%), fraxetin-8-methylether (1 mg, 0.000005%) and 5,6-dimethoxycoumarin (2 mg, 0.000010%).
Casticin (1 mg, 0.000005%) and scopoletin (4 mg, 0.000021%) have been isolated previously from this plant.

The following six novel flavonoids and two novel chromenes were also isolated and characterised: quercetagetin-4'-methylether (6 mg, 0.000031%), quercetagetin-3,4'-dimethylether (3 mg, 0.000016%), 5,2',4'-trihydroxy-6,7,5'-trimethoxyflavone (2 mg, 0.000010%), 5,7,8,3'-tetrahydroxy-3,4'-dimethoxyflavone (4 mg, 0.000021%), quercetagetin-3-methylether-4'-glucoside (8 mg, 0.000042%), gossypetin-3-methylether-3'-glucoside (3 mg, 0.000016%), 2,2,6-trihydroxychromene (4 mg, 0.000021%) and 2,2-dihydroxy-6-methoxychromene (6 mg, 0.000031%).

3.1.2. Structures and spectral data of compounds isolated



(138)

Casticin (138)

UV (λmax, nm) : MeOH = 254, 270, 346; MeONa = 272, 375

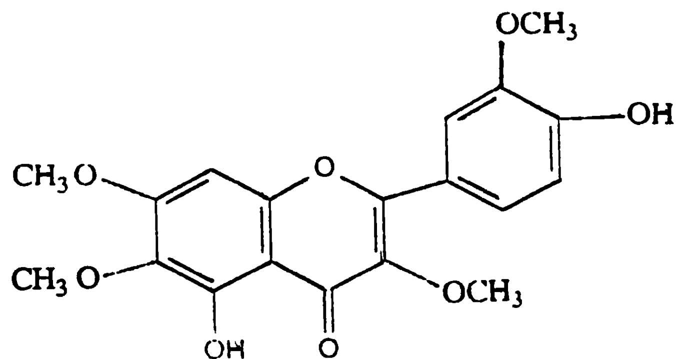
$AlCl_3$ = 265, 374; $AlCl_3$/HCl = 265, 362;

NaOAc = 274, 370; NaOAc/H_3BO_3= 272, 350.

MS, m/z (%), 374 (100), 359 (70), 331 (31), 181 (6), 153 (9)

151 (12) (Figure 29, page 180) [188].

'H NMR ($CDCl_3$), δ 7.69 (1H, d, J=2, H2'), 7.66 (1H, dd, J=9 and J=2, H6'), 7.01 (1H, d, J=9, H5'), 6.52 (1H, s, H8), 3.96 (3H, s, 3-OCH_3), 3.95 (3H, s, 7-OCH_3), 3.92 (3H, s, 4'-OCH_3), 3.80 (3H, s, 6-OCH_3) (Figure 28, page 179) [188].



(139)

Chrysoplenetin (139)

UV (λmax, nm) : MeOH= 256, 272, 350; MeONa= 270, 410;

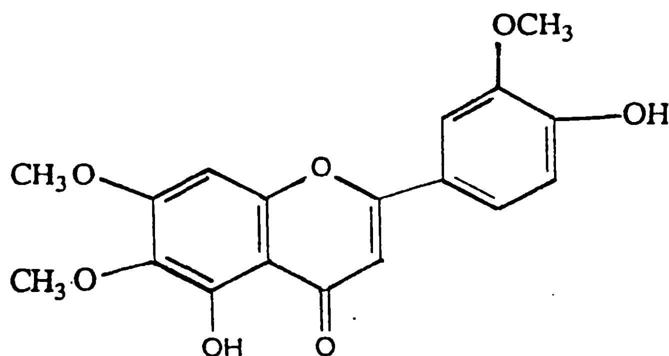
$AlCl_3$= 264,283,380; $AlCl_3$/HCl = 270, 370;

NaOAc = 263, 360; NaOAc / H_3BO_3= 260, 272, 356.

MS, m/z (%), 374 (100), 359 (76), 331 (19), 181 (11), 153 (11), 151 (19), 120 (6), 105 (4) (Figure 33, page 184) [195].

'H NMR ($CDCl_3$), δ 7.71 (1H,d,J=2,H2'), 7.68 (1H,dd,J=9 and J=2,H6'), 7.05 (1H,d,J=9,H5'), 6.51 (1H,s,H8), 3.99 (3H,s,3'-OCH_3), 3.96 (3H,s,7-OCH_3), 3.92 (3H,s,3-OCH_3), 3.86 (3H,s,6-OCH_3) (Figure 32, page 183) [195].

NOE, irradiated at δ 3.96, 6.51 enhancement; irradiated at δ 3.99, 7.71 enhancement.



(140)

Circilineol (140)

UV (λ max,nm) : MeOH= 270, 346; MeONa= 276, 410;

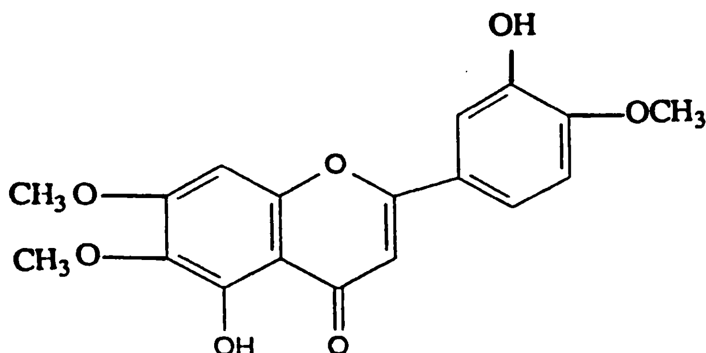
$AlCl_3$= 288,362; $AlCl_3$/HCl= 271, 360;

NaOAc= 276, 352; NaOAc/H_3BO_3= 270,350.

MS, m/z (%), 344 (100), 329 (78), 181 (23), 153 (49), 151 (17),

120 (9), 105 (6).

'H NMR (CD_3OD), δ 7.61 (1H,d,J=2,H2'), 7.45 (1H,dd,J=9 and J=2, H6'), 6.93 (1H,d,J=9,H5'), 6.96 (1H,s,H8), 6.58 (1H,s,H3), 3.94 (3H,s,3'-OCH_3), 3.82 (3H,s,7-OCH_3), 3.81 (3H,s,6-OCH_3).



(141)

Eupatorin (141)

UV (λ max,nm) : MeOH = 274, 344; MeONa = 264, 400;

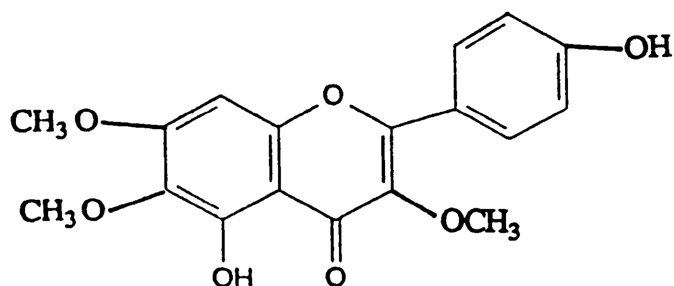
$AlCl_3$ = 290, 374; $AlCl_3$/HCl = 290, 364;

NaOAc = 274, 348; NaOAc/H_3BO_3 = 274, 344.

MS, m/z (%), 344 (100), 329 (91), 181 (22), 153 (49), 151 (16), 105 (8) (Figure 45, page 196) [199].

'H NMR ($CDCl_3$), δ 7.51 (1H,dd,J=9 and J=2,H6'), 7.30 (1H,d,J=2,H2'), 7.01 (1H,d,J=9,H5'), 6.59 (1H,s,H8), 6.53 (1H,s,H3), 4.01 (3H,s,4'-OCH_3), 3.98 (3H,s,7-OCH_3), 3.93 (3H,s, 6-OCH_3) (Figure 44, page 195) [199].

NOE, irradiated at δ 3.98, 6.59 enhancement; irradiated at δ 4.01, 7.01 enhancement; irradiated at δ 6.53, no enhancement.



(142)

Penduletin (142)

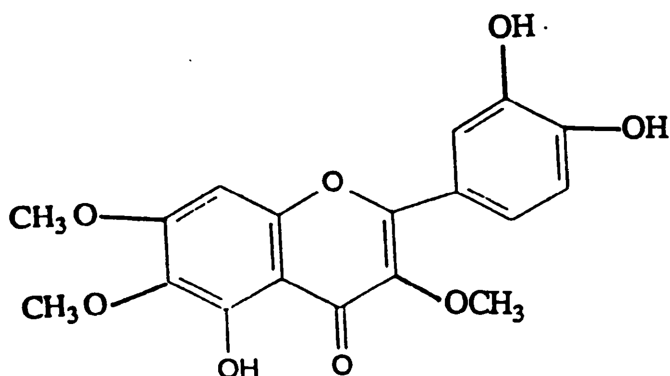
UV (λ max, nm) : MeOH = 271, 342; MeONa = 273, 396;

$AlCl_3$ = 280, 360; $AlCl_3/HCl$ = 280, 360;

NaOAc = 272, 345; $NaOAc/H_3BO_3$ = 272,346.

MS, m/z (%), 344 (100), 329 (66), 301 (31), 286 (17), 181 (17), 167 (11) , 153 (24), 121 (36), 91 (14)(Figure 64, page 215) [190],

'H NMR (CD_3OD), δ 8.01 (2H,d,J=9,H2' and H6'), 7.02 (2H,d,J=9,H3' and H5'), 6.79 (1H,s,H8), 4.08 (3H,s,3-OCH_3), 3.94 (3H,s,7-OCH_3) , 3.78 (3H,s,6-OCH_3)(Figure 65, page 216) [190].



(143)

Chrysosplenol-D (143)

UV (λ max, nm) : MeOH = 260, 352; MeONa = 270, 402;

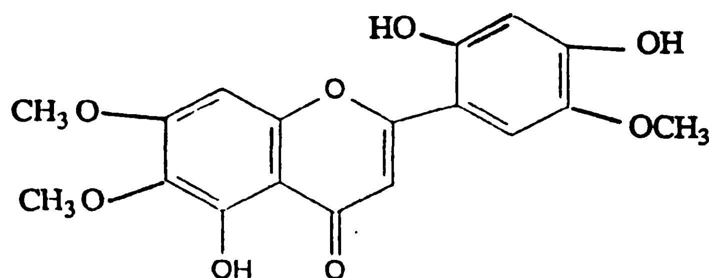
$AlCl_3$ = 280, 440; $AlCl_3/HCl$ = 270,370;

NaOAc = 273, 420; $NaOAc/H_3BO_3$ = 270, 386.

MS, m/z (%), 360 (100), 345 (57), 317 (16), 181 (13), 153 (17), 137 (27), 121 (8)(Figure 35, page 186) [197].

'H NMR ($CDCl_3$), δ 7.70 (1H,d,J=2,H2'), 7.53 (1H,dd,J=9 and J=2,H6'), 6.93 (1H,d,L=9,H5'), 6.54 (1H,s,H8), 3.96 (3H,s,7-OCH_3), 3.89 (3H,s,3-OCH_3), 3.85 (3H,s,6-OCH_3)(Figure 34, page 185) [197].

NOE, irradiated at δ3.96, 6.54 enhancement; irradiated at δ 3.89 and 3.85, no enhancement.



(144)

5,2',4'-trihydroxy-6,7,5'-trimethoxyflavone (144)

UV (λ max. nm) : MeOH = 270, 360; MeONa = 270, 410;

$AlCl_3$ = 280, 398; $AlCl_3/HCl$ = 280, 398;

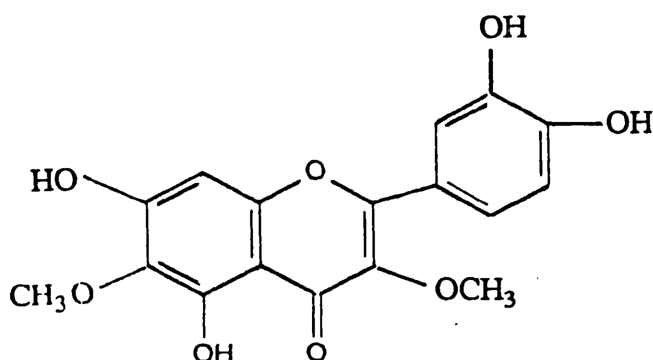
NaOAc = 270, 370; $NaOAc/H_3BO_3$ = 270,368.

MS, m/z (%), 360 (100), 345 (82), 331 (22), 300 (32), 181 (34),

167 (12) , 165 (24), 153 (43), 151 (17), 137 (12)(Figure 99, page 250)

'H NMR (CD_3OD), δ 7.45 (1H,s,H6'), 7.11 (1H,s,H3'), 6.96 (1H,s,H8), 6.57 (1H,s,H3), 3.93 (3H,s,7-OCH_3), 3.81 (3H,s,5'-OCH_3), 3.73 (3H,s,6-OCH_3)(Figure 98, page 249).

NOE, irradiated at δ 3.93, 6.96 enhancement; irradiated at δ 3.81, 7.45 enhancement; irradiated at δ 7.11 and 6.57 no change.



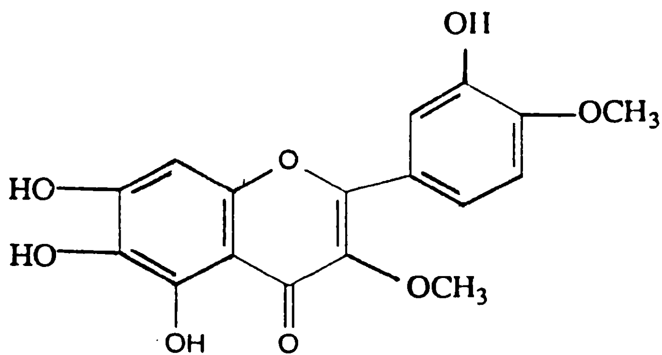
(145)

Axillarin (145)

UV (λ max, nm) : MeOH = 262, 360; MeONa = 266,402;
$AlCl_3$ = 278, 442; $AlCl_3$/HCl = 270,382;
NaOAc = 274, 394; NaOAc/H_3BO_3 = 264, 362.

MS, <u>m/z</u> (%), 346 (100), 331 (42), 303 (21), 187 (14), 139 (16), 137 (21)(Figure 27, page 178)[192].

'H NMR (CD_3OD), δ 7.62 (1H,d,J=2,H2'), 7.55 (1H,dd,J=9 and J=2,H6'), 6.90 (1H,d,J=9,H5'), 6.52 (1H,s,H8), 3.88 (3H,s,3-OCH_3), 3.79 (3H,s,6-OCH_3) (Figure 26, page 177) [192].



(146)

Quercetagetin-3,4'-dimethylether (146)

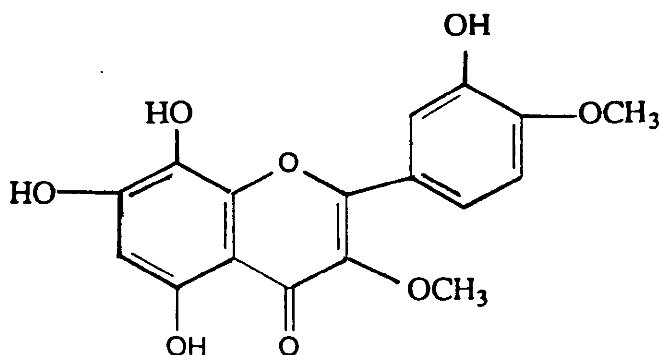
UV (λ max, nm) : MeOH = 258, 370; MeONa = 274, 428;

$AlCl_3$ = 276, 452; $AlCl_3/HCl$ = 270,424;

NaOAc = 270,390; NaOAc/H_3BO_3 = 265, 390.

MS, m/z (%), 346 (100), 303 (86), 164 (19), 153 (10), 137 (27), 121 (11), 109 (9) (Figure 71, page 222).

'H NMR (CD_3OD), δ 7.88 (1H,d,J=2,H2'), 7.75 (1H,dd,J=9 and J=2, H6'), 7.01 (1H,d,J=9,H5'), 6.82 (1H,s,H8), 4.04 (3H,s,4'-OCH_3), 3.89 (3H,s,3-OCH_3) (Figure 70, page 221).



(147)

5,7,8,3'-Tetrahydroxy-3,4'-dimethoxyflavone (147)

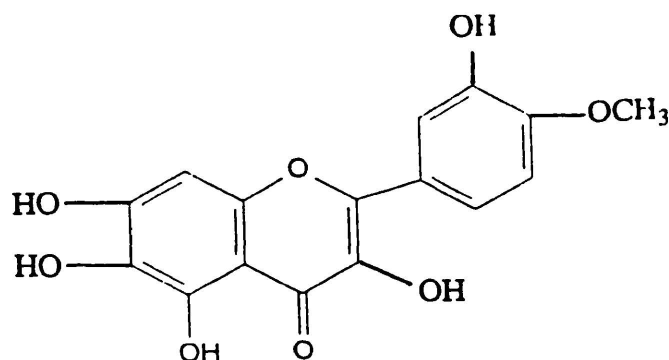
UV (λ max, nm) : MeOH = 260, 370; MeONa = 278,420;

$AlCl_3$ = 270,430; $AlCl_3/HCl$ = 270,430;

NaOAc = 278, 390; $NaOAc/H_3BO_3$ = 260,370.

MS, m/z (%), 346 (100), 328 (43), 303 (87), 173 (12), 151 (17), 135 (6), 120 (8) (Figure 95, page 246).

'H NMR (CD_3OD), δ 7.82 (1H,d,J=2,H2'), 7.72 (1H,dd,J=9 and J=2,H6'), 6.91 (1H,d,J=9,H5'), 6.46 (1H,s,H6), 3.93 (3H,s,4'-OCH_3), 3.86 (3H,s,3-OCH_3) (Figure 94, page 245).



(148)

Quercetagetin-4'-methylether (148)

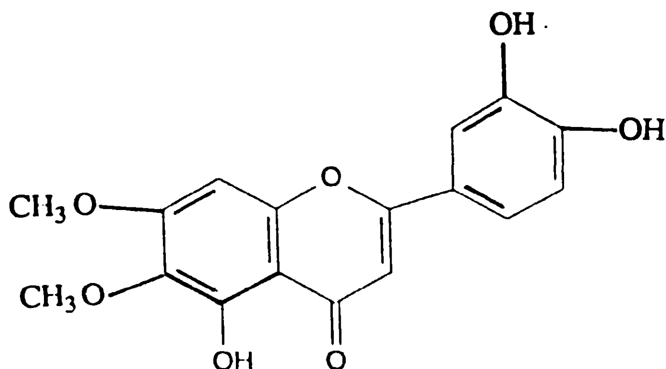
UV (λ max, nm) : MeOH = 266, 368; MeONa = 274,394;

$AlCl_3$ = 284,450; $AlCl_3/HCl$ = 278,398;

NaOAc = 286,398; $NaOAc/H_3BO_3$ = 286, 398.

MS, m/z (%), 332 (100), 149 (37), 136 (51), 121 (23), 109 (31), (Figure 73, page 224).

'H NMR (CD_3OD), δ 7.80 (1H,d,J=2,H2'), 7.65 (1H,dd,J=9 and J=2, H6'), 6.90 (1H,d,J=9,H5'), 6.72 (1H,s,H8), 4.01 (3H,s,4'-OCH_3) (Figure 72, page 223).



Cirsiliol (149) (149)

UV (λmax, nm) : MeOH = 274, 348; MeONa = 276, 400;

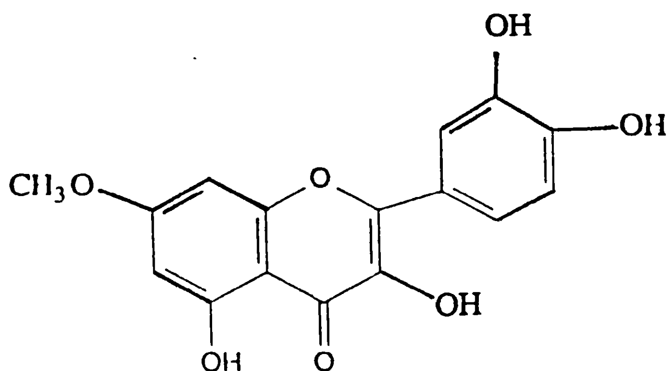
$AlCl_3$ = 276,424; $AlCl_3/HCl$ = 290,368;

NaOAc = 270,390; $NaOAc/H_3BO_3$ = 264,370.

MS, m/z (%), 330 (100), 315 (87), 301 (30), 287 (34), 181 (26), 153 (52), 137 (9), 135 (17)(Figure 37, page 188) [277].

'H NMR (DMSO-d6), δ 7.48 (1H,d,J=2,H2'), 7.44 (1H,dd,J=9 and J=2,H6'), 6.89 (1H,d,J=9,H5'), 6.83 (1H,s,H8), 6.73 (1H,s,H3), 3.93 (3H,s,7-OCH_3), 3.73 (3H,s,6-OCH_3)(Figure 36, page 187) [277].

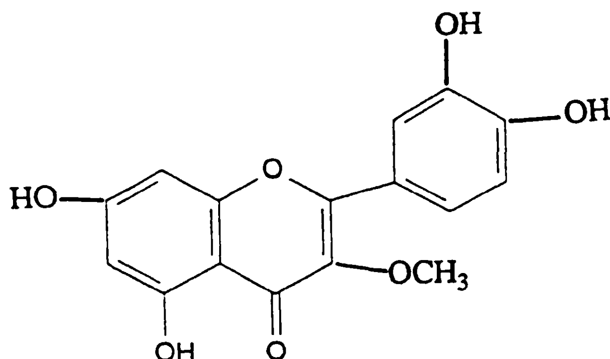
NOE, irradiated at δ 3.93, 6.83 enhancement; irradiated at δ 6.73, nochange.



(150)

Rhamnetin (150)

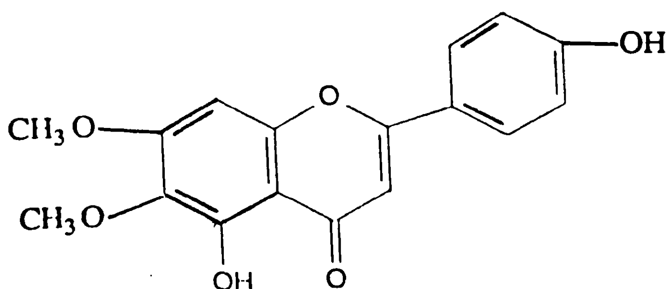
UV (λmax, nm) : MeOH = 257, 370; MeONa = 275, 400;
$AlCl_3$ = 273, 456; $AlCl_3/HCl$ = 268, 428;
NaOAc = 262, 380; $NaOAc/H_3BO_3$ = 262, 386.
MS, m/z (%), 316 (100), 167 (21), 137 (32)(Figure 83, page 234) [183].
'H NMR (DMSO-d6), δ 7.72 (1H,d,J=2,H2'), 7.51 (1H,dd,J=9 and J=2,H6'), 6.90 (1H,d,J=9,H5'), 6.71 (1H,d,J=2,H8), 6.35 (1H,d,J=2,H6), 3.87 (3H,s,7-OCH_3)(Figure 82, page 233) [183].



(151)

Quercetin-3-methylether (151)

UV (λ max, nm) : MeOH = 257, 270, 358; MeONa = 270, 409;
$AlCl_3$ = 275, 445; $AlCl_3/HCl$ = 272,400;
NaOAc = 270, 384; $NaOAc/H_3BO_3$ = 259, 378.
MS, m/z (%), 316 (100), 273 (82), 153 (40), 137 (37).
'H NMR (CD_3OD), δ 7.85 (1H,d,J=2,H2'), 7.72 (1H,dd,J=9 and J=2,H6'), 6.90 (1H,d,J=9,H5'), 6.40 (1H,d,J=2,H8), 6.18 (1H,d,J=2,H6), 3.92 (3H,s,3-OCH_3).



(152)

Cirsimaritin (152)

UV (λ max, nm) : MeOH = 272, 335; MeONa = 275, 390;

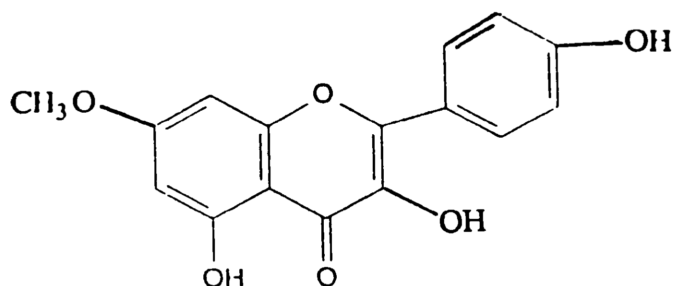
$AlCl_3$ = 275,360; $AlCl_3/HCl$ = 285, 354;

NaOAc = 275, 340; $NaOAc/H_3BO_3$ = 275, 340.

MS, m/z (%), 314 (100), 299 (94), 285 (46), 271 (40), 181 (32) 167 (9), 153 (54), 119 (23)(Figure 39, page 190) [195].

'H NMR (DMSO-d6), δ 7.97 (2H,d,J=9,H2' and H6'), 6.93 (2H,d,J=9,H3' and H5'), 6.92 (1H,s,H8), 6.86 (1H,s,H3), 3.93 (3H,s,7-OCH_3), 3.74 (3H,s,6-OCH_3) (Figure 38, page 189) [195].

NOE, irradiated at δ 3.93, 6.92 enhancement; irradiated at 3.74 and 6.86 no change .



(153)

Rhamnocitrin (153)

UV (λmax, nm) : MeOH = 268, 364; MeONa = 272, 420;

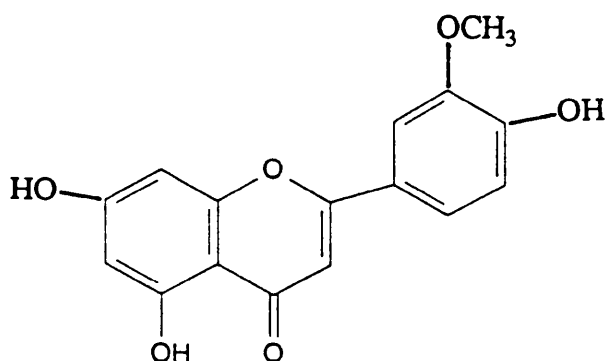
$AlCl_3$ = 270, 350, 420; $AlCl_3$/HCl = 270, 350, 420;

NaOAc = 268, 426; NaOAc/H_3BO_3 = 268, 364.

MS, m/z (%), 300 (100), 257 (11), 167 (12), 150 (19), 121 (34),

(Figure 85, page 236) [187].

^{1}H NMR (CD_3OD), δ 8.12 (2H,d,J=9,H2' and H6'), 7.08 (2H,d,J=9,H3' and H5'), 6.71 (1H,d,J=2,H8), 6.21 (1H,d,J=2,H6), 3.98 (3H,s,7-OCH_3)(Figure 84, page 235) [187].



(154)

Chrysoeriol (154)

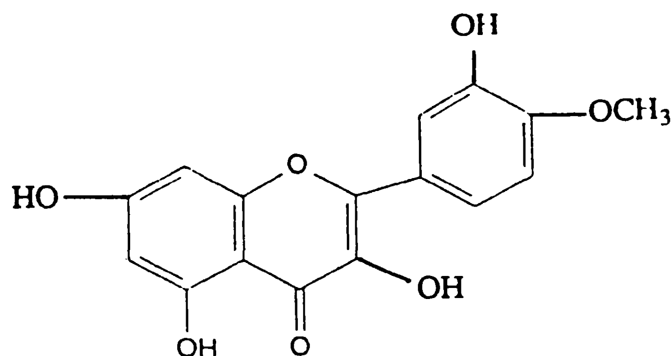
UV (λmax, nm) : MeOH = 270, 351; MeONa = 268, 410;

NaOAc = 274, 380;   NaOAc/H_3BO_3 = 270, 358;

MS, m/z (%), 300 (100), 153 (36), 148 (23), 137 (16), 105 (9),

(Figure 31, page 182) [281].

'H NMR (CD_3OD), δ 7.58 (1H,d,J=2,H2'), 7.54 (1H,dd,J=9 and J=2,H6'), 6.94 (1H,d,J=9,H5'), 6.91 (1H,s,H3), 6.51 (1H,d,J=2,H8), 6.20 (1H,d,J=2,H6), 3.89 (3H,s,3'-OCH_3).
NOE, irradiated at δ 3.89, 7.58 enhancement; irradiated at δ 6.91 , 6.51 and 6.20, no change (Figure 30, page 181) [281].



(155)

Tamarixetin (155)

UV (λ max, nm) : MeOH = 255, 364; MeONa = 274, 404;

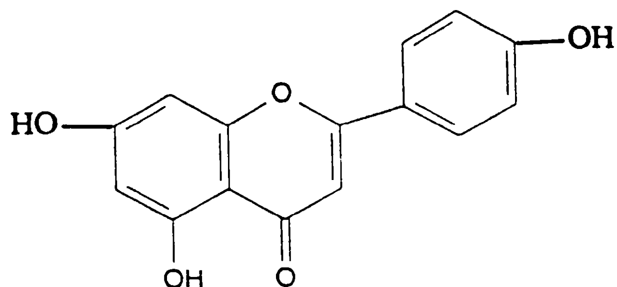
$AlCl_3$ = 270, 350, 435; $AlCl_3$/HCl = 270, 350, 422;

NaOAc = 257, 270, 374;   NaOAc/H_3BO_3 = 262, 376.

MS, m/z (%), 316 (100), 167 (7), 149 (14), 137 (24), 121 (7).

(Figure 91, page 242) [180]

'H NMR (DMSO-d6), δ 7.74 (1H, d, J=2, H2'), 7.56 (1H, d, J=9 and J=2, H6'), 6.88 (1H, d, J=9, H5'), 6.71 (1H, d, J=2, H8), 6.36 (1H, d, J=2, H6), 3.87 (3H, s, 4'-OCH_3).
(Figure 90, page 241) [180].



(156)

Apigenin (156)

UV (λ max, nm) : MeOH = 268, 366; MeONa = 276, 325, 396;

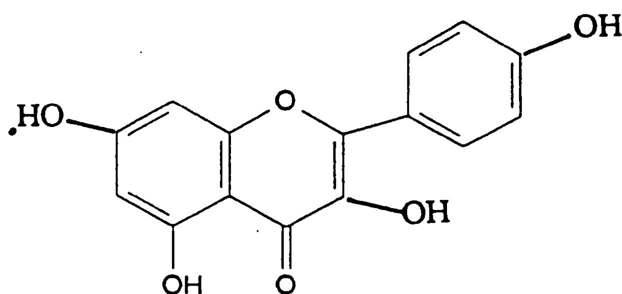
$AlCl_3$ = 276, 348, 390; $AlCl_3/HCl$ = 276, 382;

NaOAc = 274, 376; $NaOAc/H_3BO_3$ = 270, 340.

MS, m/z (%), 270 (100), 252 (26), 153 (62), 137 (76), 121 (57).

(Figure 25, page 176) [281].

'H NMR (DMSO-d6), δ 7.95 (2H, d, J=9, H2' and H6'), 6.95 (2H, d, J=9, H3' and H5'), 6.80 (1H, s, H3), 6.50 (1H, d, J=2, H8), 6.20 (1H, d, J=2, H6)(Figure 24, page 175) [281].



(157)

Kaempferol (157)

UV (λ max, nm) : MeOH = 266, 368; MeONa = 278, 410;

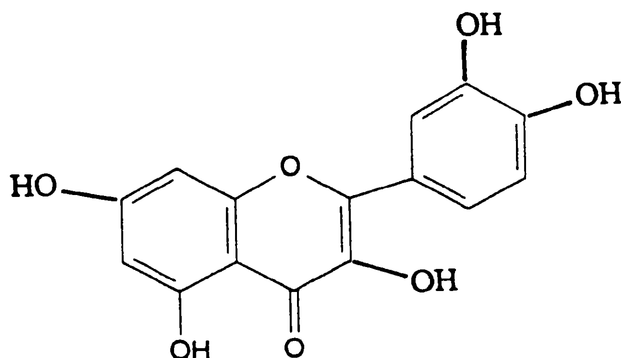
$AlCl_3$ = 268, 420; $AlCl_3/HCl$ = 270, 420;

NaOAc = 274, 390;   NaOAc /H_3BO_3 = 268, 372.

MS, m/z (%), 286 (100), 258 (9), 153 (9), 137 (6), 121 (23).

(Figure 55, page 206) [291].

'H NMR (DMSO-d6), δ 8.05 (2H, d, J=9, H2' and H6'), 6.92 (2H, d, J=9, H3' and H5'), 6.45 (1H, d, J=2,H8), 6.20 (1H,d,J=2,H6). (Figure 54, page 205) [291].



(158)

Quercetin (158)

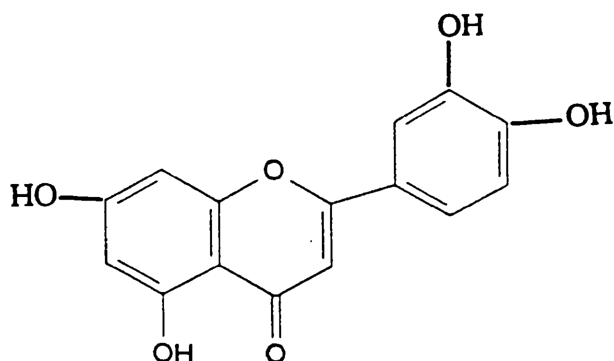
UV (λ max, nm) : MeOH = 256, 372; MeONa = 250, 324;

$AlCl_3$ = 272, 448; $AlCl_3/HCl$ = 266, 426;

NaOAc = 272, 392;   NaOAc/H_3BO_3 = 262, 390.

Ms, m/z (%), 302 (100), 153 (16), 137 (26)(Figure 75, page 226) [293].

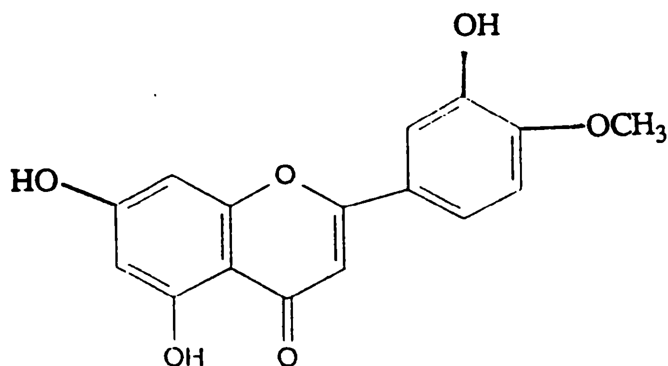
'H NMR (DMSO-d6), δ 7.72 (1H, d, J=2, H2'), 7.50 (1H, d, J=9 and J=2, H6'), 6.85 (1H, d, J=9, H5'), 6.35 (1H, d, J=2,H8), 6.18 (1H, d, J=2, H6)(Figure 74, page 225) [293].



(159)

Luteolin (159)

UV (λ max, nm) : MeOH = 250, 348; MeONa = 266, 400;
$AlCl_3$ = 274, 422; $AlCl_3/HCl$ = 274, 388;
NaOAc = 270, 386; $NaOAc/H_3BO_3$ = 260, 370.
MS, m/z (%), 286 (100), 270 (6), 153 (13)(Figure 61, page 212) [281].
^{1}H NMR (CD_3OD), δ 7.40 (1H, d, J=2, H2'), 7.38 (1H, dd, J=9 and J=2, H6'), 6.90 (1H, d, J=9, H5'), 6.50 (1H, s, H3), 6.41 (1H, d, J=2, H8), 6.20 (1H, d, J=2, H6)(Figure 60, page 211) [281].



(160)

Luteolin-4'-methylether (160)

UV (λ max, nm) : MeOH = 254, 350;   MeONa = 262, 394;

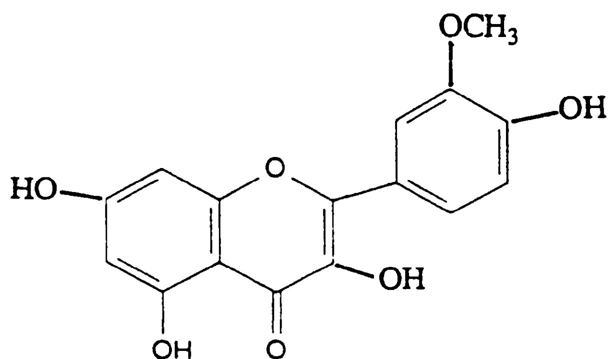
$AlCl_3$ = 274, 430; $AlCl_3/HCl$ = 272, 390;

NaOAc = 260, 402; $NaOAc/H_3BO_3$ = 260, 374.

MS, m/z (%), 300 (100), 151 (8), 149 (21), 137 (7).

(Figure 63, page 214) [179].

^1H NMR (CD_3OD), δ 7.50 (1H, d, J=2, H2'), 7.48 (1H, dd, J=9 and J=2, H6'), 6.90 (1H, d, J=9, H5'), 6.60 (1H, s, H3), 6.45 (1H, d, J=2, H8), 6.20 (1H, d, J=2, H6), 4.02 (3H, s, 4'-OCH_3).
(Figure 62, page 213) [179].



(161)

Isorhamnetin (161)

UV (λ max, nm) : MeOH = 254, 372;   MeONa = 272, 432;

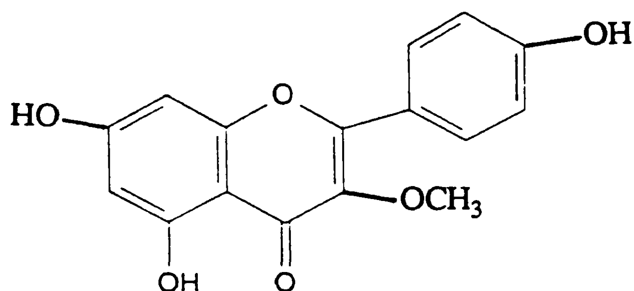
$AlCl_3$ = 264, 430; $AlCl_3/HCl$ = 262, 428;

NaOAc = 274, 394; $NaOAc/H_3BO_3$ = 256, 378.

MS, m/z (%), 316 (100), 301 (11), 153 (16), 151 (11), 137 (6).

(Figure 53, page 204) [174].

^1H NMR (CD_3OD) δ 7.55 (1H, d, J=2, H2'), 7.62 (1H, dd, J=9 and J=2, H6'), 6.85 (1H, d, J=7, H5'), 6.66 (1H, d, J=2, H8), 6.28 (1H, d, J=2, H6), 3.88 (3H, s, 3'-OCH_3).
(Figure 52, page 203) [174].



(162)

Isokaempferide (162)

UV (λ max, nm) : MeOH = 265, 352; MeONa = 270, 390;

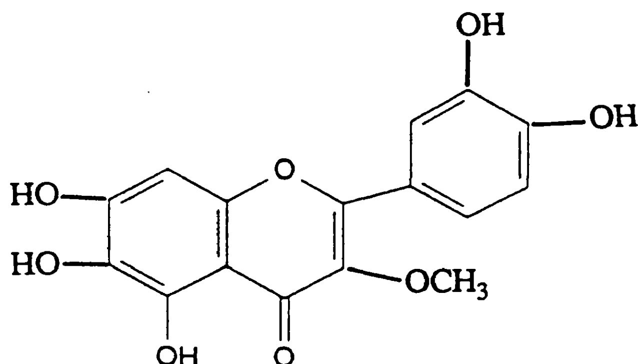
$AlCl_3$ = 272, 400; $AlCl_3$/HCl = 274, 398;

NaOAc = 265, 404; NaOAc/H_3BO_3 = 266, 352.

MS, m/z (%), 300 (100), 257 (64), 153 (11), 121 (34).

(Figure 51, page 202) [175].

^{1}H NMR (CD_3OD), δ 8.02 (2H, d, J=9, H2' and H6'), 6.98 (2H, d, J=9, H3' and H5'), 6.52 (1H, d, J=2, H8), 6.22 (1H, d, J=2, H6), 3.89 (3H, s, 3-OCH_3)(Figure 50, page 201) [175].



(163)

Quercetagetin-3-methylether (163)

UV (λ max, nm) : MeOH = 264, 362; MeONa = 266, 402;

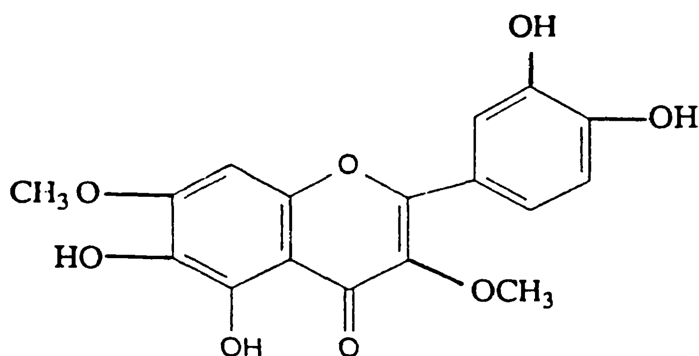
$AlCl_3$ = 276, 440; $AlCl_3/HCl$ = 270, 384;

NaOAc = 274, 396; $NaOAc/H_3BO_3$ = 264, 362.

MS, m/z (%), 332 (100), 289 (87), 153 (5) 151 (7), 137 (19).

(Figure 67, page 218) [284].

'H NMR (CD_3OD) , δ 7.75 (1H, d, J=2, H2'), 7.67 (1H, dd, J=9 and J=2, H6'), 6.85 (1H, d, J=9, H5'), 6.50 (1H, s, H-8), 3.87 (3H, s, 3-OCH_3) (Figure 66, page 217) [284].



(164)

Tomentin (164)

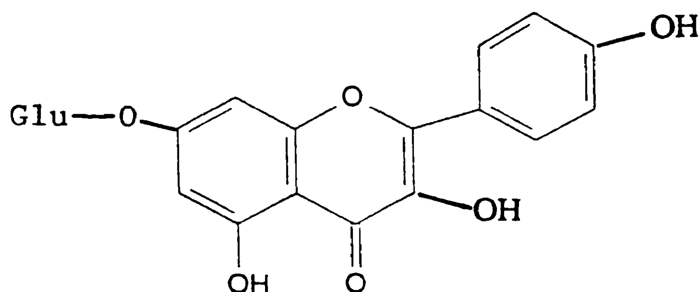
UV (λ max, nm) : MeOH = 262, 354; MeONa = 270, 404;

$AlCl_3$ = 280, 438; $AlCl_3/HCl$ = 270, 370;

NaOAc = 272, 420; $NaOAc/H_3BO_3$ = 270, 386.

MS, m/z (%), 346 (100), 328 (37), 303 (91), 173 (6), 164 (18), 153 (11), 137 (30), 120 (9)(Figure 93, page 244) [176].

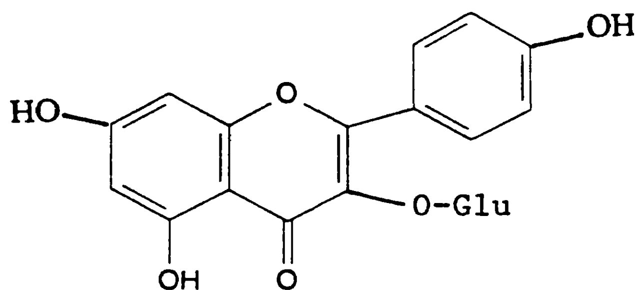
'H NMR (CD_3OD), δ 7.85 (1H, d, J=2, H2'), 7.72 (1H, dd, J=9 and J=2, H6'), 7.02 (1H, d, J=9, H5'), 6.74 (1H, s, H8), 4.01 (3H, s, 3-OCH_3), 3.88 (3H, s, 7-OCH_3)(Figure 92, page 243) [176].



(165)

Astragalin (165)

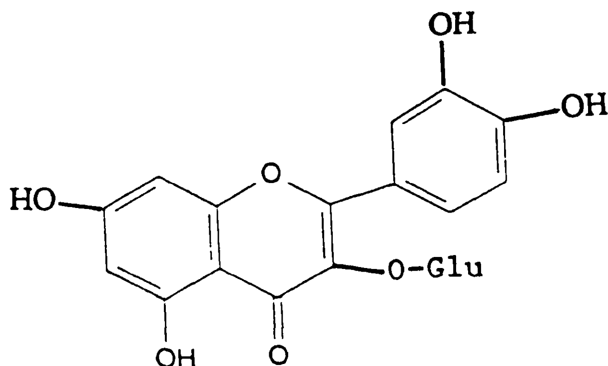
UV (λ max, nm) : MeOH = 265, 356; MeONa = 270, 392;
$AlCl_3$ = 274, 400; $AlCl_3/HCl$ = 274, 398;
NaOAc = 265, 398; $NaOAc/H_3BO_3$ = 265, 354.
MS, m/z (%), 286 (100), 258 (9), 153 (12), 121 (17).
(Figure 57, page 208) [291].
^{1}H NMR (DMSO-d6), δ 8.01 (2H, d, J=9, H2' and H6'), 6.92 (2H, d, J=9, H3' and H5'), 6.49 (1H, d, J=2, H8), 6.20 (1H, d, J=2, H6), 5.32 (1H, d, J=5, glucose H1), 3.55-3.00 (5H, multi-glucose H)(Figure 56, page 207) [291].



(166)

Kaempferol-3-glucoside (166)

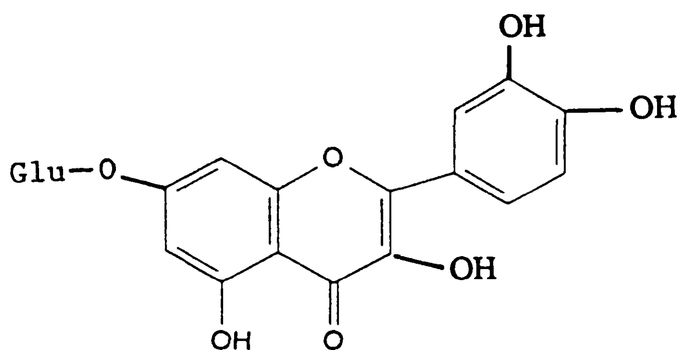
UV (λ max, nm) : MeOH = 254, 350; MeONa = 262, 392;
$AlCl_3$ = 272, 432; $AlCl_3$/HCl = 270, 392;
NaOAc = 262, 400; NaOAc/H_3BO_3 = 260, 372.
MS, m/z (%), 286 (100), 153 (17), 121 (9)(Figure 59, page 210) [178].
^{1}H NMR (DMSO-d6), δ 8.02 (2H, d, J=9, H2' and H6'), 6.92 (2H, d, J=9, H3' and H5'), 6.40 (1H, d, J=2, H8), 6.19 (1H, d, J=2, H6), 5.36 (1H, d, J=5, glucose H1), 3.80-3.00 (5H, multi- glucose H)(Figure 58, page 209) [178].



(167)

Quercetin-3-glucoside (167)

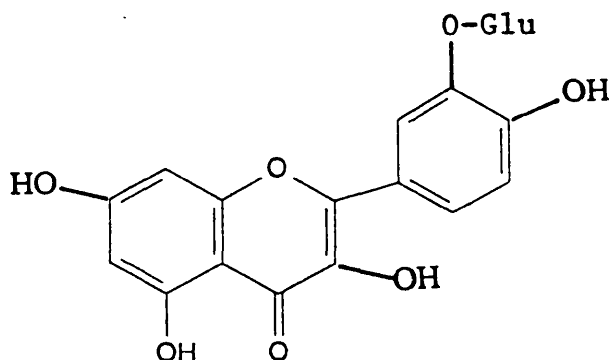
UV (λ max, nm) : MeOH = 256, 350; MeONa = 270, 394;
$AlCl_3$ = 276, 430; $AlCl_3$/HCl = 272, 402;
NaOAc = 272, 374; NaOAc/H_3BO_3 = 260, 368.
MS, m/z (%), 302 (100), 153 (13), 137 (8)(Figure 77, page 228) [291].
^{1}H NMR (CD_3OD), δ 7.85 (1H, d, J=2, H2'), 7.80 (1H, dd, J=9 and J=2, H6'), 6.92 (1H, d, J=9, H5'), 6.70 (1H, d, J=2, H8), 6.40 (1H, d, J=2, H6), 5.12 (1H, d, J=5, glucose H1), 3.95-3.2 (5H, multiplet, glucose H)(Figure 76, page 227) [291].



(168)

Quercetin-7-glucoside (168)

UV (λ max, nm) : MeOH = 256, 372; MeONa = 290, 456;
$AlCl_3$ = 272, 458; $AlCl_3/HCl$ = 268, 424;
NaOAc = 256, 428; $NaOAc/H_3BO_3$ = 256, 386.
MS, m/z (%), 302 (100), 152 (6), 137 (9)(Figure 79, page 230) [181].
'H NMR (CD_3OD), δ 7.82 (1H, d, J=2, H2'), 7.78 (1H, dd, J=9 and J=2, H6'), 6.82 (1H, d, J=9, H5'), 6.40 (1H, d, J=2, H8), 6.20 (1H, d, J=2, H6), 5.05 (1H, d, J=5, glucose H1), 4.05-3.20 (5H, multiplet, glucose H) (Figure 78, page 229) [181].



(169)

Quercetin-3'-glucoside (169)

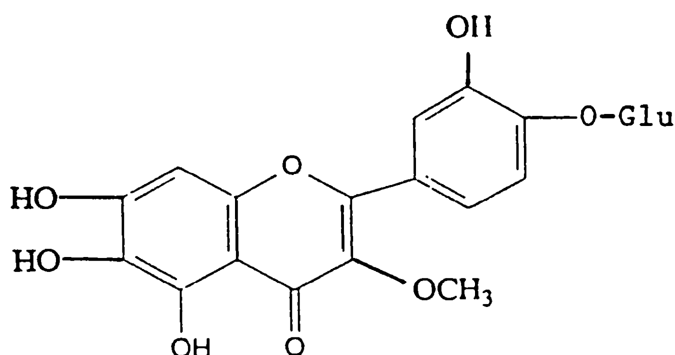
UV (λ max, nm) : MeOH = 254, 370; MeONa = 270, 432;

$AlCl_3$ = 264, 430; $AlCl_3/HCl$ = 262, 428;

NaOAc = 272, 394; $NaOAc/H_3BO_3$ = 256, 378.

MS, m/z (%), 302 (100), 153 (9), 136 (27)(Figure 81, page 232) [182].

'H NMR (CD_3OD) , δ 7.85 (1H, d, J=2, H2'), 7.58 (1H, dd, J=9 and J=2, H6'), 6.85 (1H, d, J=9, H5'), 6.40 (1H, d, J=2, H8), 6.20 (1H, d, J=2, H6), 5.18 (1H, d, J=5, glucose H1), 3.85-3.2 (5H, multiplet, glucose H) (Figure 80, page 231) [182].



(170)

Quercetagetin-3-methylether-4'-glucoside

UV (λ max, nm) : MeOH = 262, 374; MeONa = 278, 398;

$AlCl_3$ = 278, 452; $AlCl_3/HCl$ = 278, 452;

NaOAc = 270, 392; $NaOAc/H_3BO_3$ = 268, 392.

MS, m/z (%), 332 (100), 289 (98), 153 (6), 136 (27), 121 (14). (Figure 69, page 220).

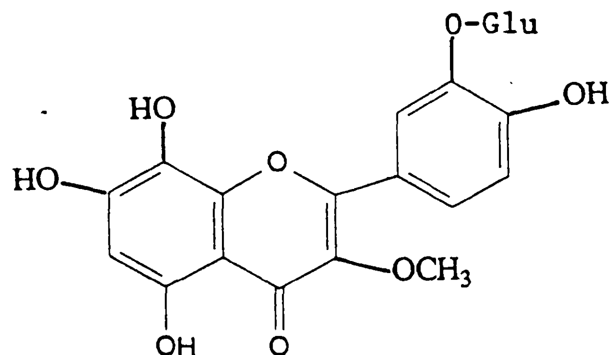
'H NMR (CD_3OD) , δ 7.75 (1H, d, J=2, H2'), 7.65 (1H,dd,J=9 and J=2, H6'), 6.87 (1H, d, J=9, H5'), 6.82 (1H, s, H8), 3.87 (3H, s, 3-OCH_3), 5.10 (1H, d, J=5, glucose H1), 4.0-3.25 (5H, multiplet, glucose H) (Figure 68, page 219).

Hydrolysis: 15% acetic acid heating for 1 hours in boiling water.

TLC: silica gel TLC developed with CHCl3: MeOH (9 : 1). Rf value: patuletin is 0.72, quercetagetin-3-methylether is

0.56, compound 170 is 0.01 and after hydrolysis of compound 170 (170A) is 0.56.

HPLC: ODS2 column. Solvent: MeOH : H20 (72 : 28), 1 ml/min.
Retention time: compound 170 is 1.4 min, quercetagtin-3-methylether 8.5 min, 170 A 8.5 min and patuletin 9 min.



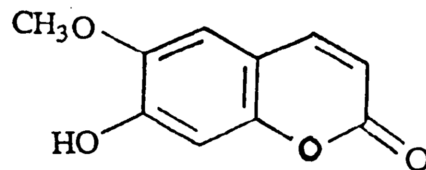
(171)

Gossypetin -3-methylether -3'-glucoside (171)

UV (λ max, nm) : MeOH = 262, 360; MeONa = 275, 412;
$AlCl_3$ = 280, 440; $AlCl_3$/HCl = 278, 370;
NaOAc = 272, 418; NaOAc/H_3BO_3 = 270, 392.

MS , m/z (%), 332 (100), 289 (82), 136 (22), 121 (19), 109 (11) (Figure 49, page 200).

'H NMR (CD_3OD), δ 7.72 (1H, d, J=2, H2'), 7.58 (1H, dd, J=9 and J=2, H6'), 6.88 (1H, d, J=9, H5'), 6.48 (1H, s, H6), 3.87 (3H, s, 3-OCH_3), 5.25 (1H, d, J=5, glucose H1), 3.70-3.20 (5H, multiplet, glucose H) (Figure 48, page 199).

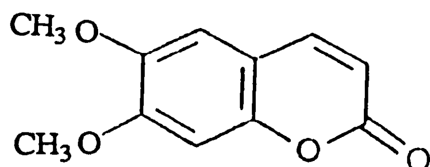


(172)

Scopolin (172)

MS, m/z (%), 192 (100), 177 (73), 164 (31), 149 (54), 121 (23), 69 (57) (Figure 89, page 240).

'H NMR (CD_3OD), δ 7.92 (1H, d, J=10, H4), 7.16 (1H, s, H5), 6.80 (1H, s, H8), 6.21 (1H, d, J=10, H3), 3.96 (3H, 6-OCH_3). (Figure 88, page 239).

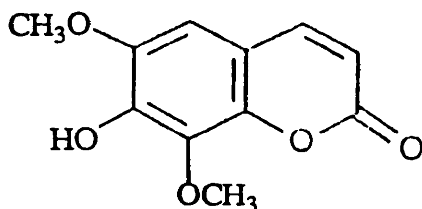


(173)

Scoparone (173)

MS, m/z (%), 206 (100), 191 (41), 178 (17), 163 (29), 135 (18), 107 (17), 69 (28) (Figure 87, page 238).

'H NMR ($CDCl_3$), δ 7.62 (1H, d, J=10, H4), 6.82 (2H, s, H5 and H8), 6.24 (1H, d, J=10, H3), 3.96 (3H, s, 6-OCH_3), 3.94 (3H, s, 7-OCH_3) (Figure 86, page 237).

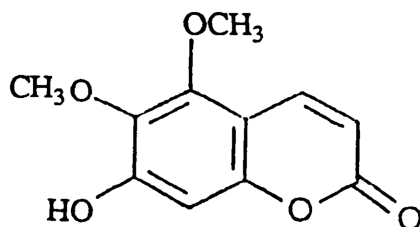


(174)

Fraxetin-8-methylether (174)

MS, m/z (%), 222 (100), 207 (31), 194 (21), 179 (24), 151 (16), 123 (24), 95 (22) (Figure 47, page 198).

'H NMR (CD_3OD), δ 7.92 (1H, d, J=10, H4), 7.01 (1H, s, H5), 6.24 (1H, d, J=10, H3), 3.90 (3H, s, 6-OCH_3), 3.84 (3H, s, 8-OCH_3) (Figure 46, page 197).

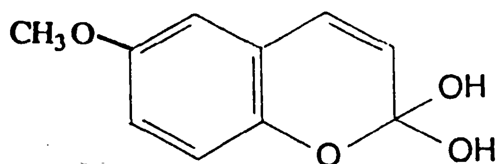


(175)

5,6-Dimethoxy-7-hydroxycoumarin (175)

MS, m/z (%), 222 (100), 207 (41), 194 (20), 179 (36), 151 (14), 123 (20), 95 (20) (Figure 43, page 194).

'H NMR (CD_3OD), δ 8.04 (1H, d, J=10, H4), 6.54 (1H, s, H8), 6.12 (1H, d, J=10, H3), 3.91 (3H, s, 5-OCH_3), 3.80 (3H, s, 6-OCH_3). (Figure 42, page 193).



(176)

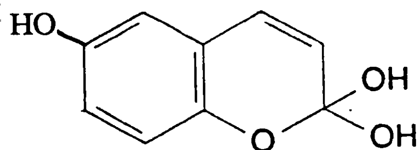
2,2,-Dihydroxy-6-methoxychromene (176)

UV (λ max, nm) : MeOH = 285, 315.

MS, m/z (%), 194 (100), 179 (21), 161 (48), 144 (41), 135 (35), 116 (32), 107 (22) (Figure 41, page 192).

'H NMR (CD_3OD), δ 7.60 (1H, d, J=10, H3), 7.18 (1H, d, J=2, H5), 7.04 (1H, dd, J=6 and J=2, H7), 6.82 (1H, d, J=6, H8), 6.31 (1H, d, J=10, H4), 3.86 (3H, s, 6-OCH_3) (Figure 40, page 191).

NOE, irradiated at δ 3.86, 7.18 and 7.04 enhancement; irradiated at δ 6.31, 7.60 and 7.18 enhancement; at δ 7.04, 3.86 and 6.82 enhancement; at δ 6.82, 7.04 enhancement; at δ 7.18, 3.86 and 6.31 enhancement; irradiated at δ 7.60, 6.31 enhancement.



(177)

2,2,6-Trihydroxychromene (177)

UV (λ max, nm) : MeOH = 282, 314.

MS, m/z (%), 180.0429 (calc. for $C_9H_8O_4$, 180.0423) (64), 163 (27), 136 (100), 110 (19), 89 (58) (Figure 97, page 248).

'H NMR (CD_3OD), δ 7.48 (1H, d, J=10, H3), 7.05 (1H, d, J=2, H5), 6.90 (1H, dd, J=6 and J=2, H7), 6.75 (1H, d, J=6, H8), 6.26 (1H, d, J=10, H4) (Figure 96, page 247).

3.1.3 The contribution of antimalarial activity

Artemisia annua used in traditional Chinese medicine has been investigated as part of the search for novel antimalarial agents. The major constituent responsible for its antimalarial activity is a sesquiterpene lactone , artemisinin and this compound has been found to be particularly active against chloroquine resistant *Plasmodium falciparum* in the treatment of cerebral malaria [2]. It has been reported that the antimalarial activity of artemisinin is markedly enhanceed by the presence of the methoxylated flavones, such as artemetin and casticin [6]. Further investigation of the flavonoids from the $CHCl_3$ extract of this plant yielded 23 methoxylated flavones. The major constituents were chrysosplenol-D , chrysoplenetin, eupatorin, cirxilinol, penduletin, cirsiliol, cirsimaritin and casticin. These compounds and combination of artemisinin with individual flavonoids isolated were examined for antimalarial activity using an assay based on the incorporation of [^{3}H]-hypoxanthine into chloroquine resistant *P. falciparum* [174]. The IC_{50} values for these compounds are given in Table 1 and 2 .

Table 1: The inhibitory activity of artemisinin + flavonoids on P. falciparum growth assayed by [^{3}H]-hypoxanthine incorporation

Compounds tested	Apparent IC_{50} [M $\times 10^{-8}$] + Flavone (5 μM)
Artemisinin	3.3
Artemisinin + eupatorin	3.0
Artemisinin + casticin	2.6
Artemisinin + chrysoplenetin	2.25
Artemisinin + circilineol	1.6
Artemisinin + chrysosplenol-D	1.5

Table 2 : The inhibitory activity of the major <u>A. annua</u> methoxylated flavones on <u>P. falciparum</u> growth assayed by [^{3}H]-hypoxanthine incorporation

Methoxylated flavones	IC_{50} [M $\times 10^{-5}$]
Casticin	2.4
Chrysoplenetin	2.3
Circilineol	3.6
Chrysosplenol-D	3.2
Eupatorin	6.5

3.2. DISCUSSION

3.2.1. Separation and isolation techniques

The flavonoids, particularly those with methoxylated substituents, in this plant, are very similar in structure. Consequently, it was extremely difficult to separate them as single compounds. The strategy for the isolation and purification of these compounds was developed through repeated chromatography on different columns. The separations were achieved by a combination of Polyclar AT, silica gel and Sephadex LH-20 columns.

3.2.2. Structure determination

The identification and structural elucidation of the compounds isolated were based on their UV, 'H NMR and MS analysis. The point of interest in the structure characterisation of flavones is the identification of the substitution within rings A and B, as well as C-3, and as to whether the substitution is hydroxyl or methoxyl. In 'H NMR spectroscopy, the chemical shifts of methoxyl groups (δ 3.5-4.25, sharp singlet peaks) and integration indicated the number of methoxyl groups in the structure. The position of hydroxyl and methoxyl groups was confirmed by MS and UV spectroscopic analysis. The presence of a C-6 methoxyl group was indicated by a strong $[M-15]^{+}$ fragment in the EIMS, whereas a flavonoid with C-3 methoxyl group gave a strong $[M-43]^{+}$ peak. The hydroxyl and methoxyl groups substituted on A or B rings were shown by the fragments A1 or B2 (page 82) in the EIMS. The presence of a C-8

methoxyl group was indicated by the [M-15 $]^+$ fragment being the base peak. The flavonoids presented in this investigation can be divided into several groups by the presence of different types of substituent. MS of one group of flavones, 5-hydroxy-3,6,7-trimethoxyl substituted on A and C rings showed signals for M^+(100%), strong $[M-Me]^+$ more than 55%, $[M-MeCO]^+$ less than 55% , $[A1-Me]^+$ 181 and $[A1-MeCO]^+$ 153 (Table 3). Saleh et al. [275, 277] reported eight whilst Liu and Mabry [287] reported sixteen flavonoids with this substitution pattern from various plant species and all of their MS data are similar to that obtained in the present work.
The MS of other flavonoids with 5-hydroxy-6,7-dimethoxyl but with no C-3 methoxyl group showed a strong $[M-Me]^+$ peak but no/or a weak $[M-MeCO]^+$ peak (less than 40%). This type of flavone exhibits stronger $[A1-Me]^+$ and $[A1-MeCO]^+$ peaks than 5-hydroxy-3,6,7-trimethoxy substituted flavones (Table 4).
The MS of 3-methoxyl substituted but with no C-6 and C-8 methoxyl group flavonoids exhibited only $[M-MeCO]^+$ (more than 64%), but no $[M-Me]^+$ peaks (Table 5).

Table 3 MS data (*m/z*, %) for 5-hydroxy-3,6,7-trimethoxyl substituted flavonoids (Results, page 92)

Compounds	M^+	$[M-Me]^+$	$[M-MeCO]^+$	$[A1-Me]^+$	$[A1-MeCO]^+$
Casticin	374(100)	359(70)	331(36)	181(6)	153(9)
Chrysoplenetin	374(100)	359(76)	331(19)	181(11)	153(11)
Chrysosplenol-D	360(100)	345(57)	317(16)	181(13)	153(17)
Penduletin	344(100)	329(66)	301(31)	181(17)	153(24)

Table 4 MS data (m/z, %) for 5-hydroxy-6,7-dimethoxy substituted flavones without C-3 methoxyl substitution

Compounds	M^+	$[M-Me]^+$	$[M-MeCO]^+$	$[A1-Me]^+$	$[A1-MeCO]^+$
Eupatorin	344(100)	329(78)	-	181(22)	153(49)
5,2',4'-Tri hydroxy-6,7,** 5'-trimethoxy flavone	360(100)	345(82)	-	181(34)	153(43)
Circilineol	344(100)	329(78)	-	181(23)	153(49)
Cirsiliol	330(100)	315(87)	287(24)	181(26)	153(52)
Cirsimaritin	314(100)	299(94)	271(31)	181(32)	153(43)

** New compound

Table 5 MS data (m/z, %) for C-3 methoxyl substituted flavones (page 94 — 120)

Compounds	M^+	$[M-Me]^+$	$[M-MeCO]^+$
Kaempferol-3-methylether	300(100)	-	257(64)
Quercetagetin-methylether	332(100)	-	289(87)
Tomentin	346(100)	-	303(91)
Quercetagetin-3,4'-methylether	346(100) **	-	303(86)
5,7,8,3'-Tetra-hydroxy-3,4'-methoxyflavone	346(100)	-	303(87)
Quercetagetin-3-methylether-4'-glucoside	332(100)* **	-	289(98)
Gossypetin-3-methylether-3'-glucoside	332(100)* **	-	289(82)

*For aglycone **For novel compounds

The MS of flavonoid glycosides with a sugar connected on the A ring showed $[A1-1]^+$ and a peak corresponding to an $[A1-1]^+$ fragment whereas those with a sugar connected on the B ring have a peak corresponding to a $[B2-1]^+$ fragment (Table 6). Quercetagetin-3-methylether showed a B2 fragment at m/z 137 (19%), whereas quercetagetin-3-methylether-4'-glucoside exhibited a fragment at m/z 136 (27%).

Table 6 MS data (m/z, %) diagnostic for A and B ring flavonoid glycosides

Compounds	$[A1-1]^+$	$[B2-1]^+$
Quercetin-7-glucoside	152(6)	
Quercetin-3'-glucoside		136(27)
Quercetagetin-3-methylether -4'-glucoside *		136(27)
Gossypetin-3-methylether -3'-glucoside *		136(22)

*Novel compounds isolated during this investigation

The MS of flavones and flavonols that have one hydroxyl substituent on the B ring showed a m/z 121 fragment whereas those with two hydroxyl substituents on the B ring showed a m/z 137 fragment. If a flavonoid has one hydroxyl and one methoxyl substituent on the B ring, then a m/z 151 fragment is observed in the MS. If two hydroxyl and one methoxyl substituents are on the B ring, then a m/z 167 fragment is observed in the MS.

The 'H NMR spectra of flavonoids isolated from this plant were used in order to assign whether there was substitution at C-3,

C-6 or C-8. Table 7 summarizes the chemical shifts of protons at C-3, C-6 and C-8 of some flavonoids isolated in the current investigation.

Table 7 chemical shifts (δ) assigned to H-3, H-6 and H-8 from 'H NMR spectra of some of the flavonoids isolated from <u>A. annua</u> (see results part, page 94 — 120)

Compounds	Solvent	C-3	C-6	C-8
Apigenin	DMSO-d6	6.80	6.20	6.50
Kaempferol	DMSO-d6		6.20	6.45
Quercetin	DMSO-d6		6.18	6.35
Tamarixetin	DMSO-d6		6.36	6.71
Rhamnetin	DMSO-d6		6.35	6.71
Luteolin	CD_3OD	6.50	6.20	6.41
Luteolin-4'-methylether	CD_3OD	6.60	6.20	6.45
Chrysoeriol	CD_3OD	6.91	6.20	6.51
Isorhamnetin	CD_3OD		6.28	6.66
Isokaempferide	CD_3OD		6.22	6.52
Quercetin-3-methylether	CD_3OD		6.18	6.40
Rhamnocitrin	CD_3OD		6.21	6.71
Quercetagetin-3-methylether	CD_3OD			6.50
Quercetagetin-4'-methylether**	CD_3OD			6.72
Tomentin	CD_3OD			6.74
Quercetagetin-3,4'-methylether**	CD_3OD			6.82

Table 7 (continued)

Compounds	Solvent	C-3	C-6	C-8
5,7,8,3'-Tetrahydroxy-3,4'-dimethoxyflavone**	CD_3OD		6.46	
Quercetagetin-3-methyl ether-4'-glucoside**	CD_3OD			6.82
Gossypetin-3-methyl ether-3'-glucoside**	CD_3OD		6.48	
Axillarin	CD_3OD			6.52
Ciirsimaritin*	CD_3OD	6.86		6.92
Cirsiliol*	CD_3OD	6.73		6.83
5,2',4'-Trihydroxy-* ** 6,7,5'-trimethoxyflavone	CD_3OD	6.57		6.96
Penduletin	CD_3OD			6.79
Circilineol	CD_3OD			6.96
Chrysosplenol-D*	$CDCl_3$			6.54
Eupatorin*	$CDCl_3$			6.59
Chrysoplenetin*	$CDCl_3$			6.51
Casticin	$CDCl_3$			6.52
Kaempferol-3-glucoside	DMSO-d6		6.19	6.40
Kaempferol-7-glucoside	DMSO-d6		6.20	6.49
Quercetin-3-glucoside	CD_3OD		6.40	6.70
Quercetin-7-glucoside	CD_3OD		6.20	6.40
Quercetin-3'-glucoside	CD_3OD		6.20	6.40

* Data proved by NOE ** Novel compounds

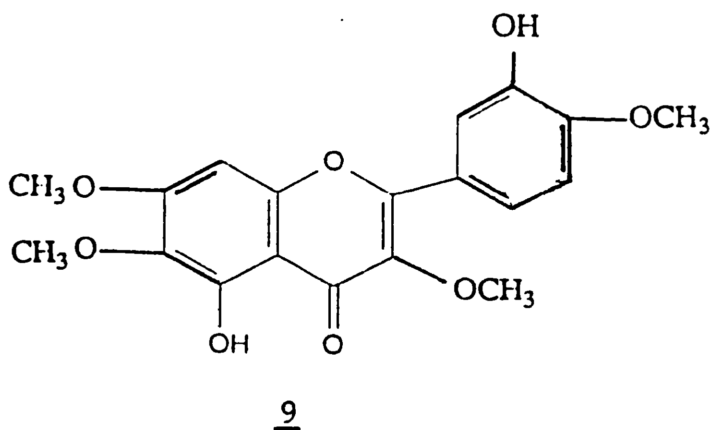
The 'H NMR data in Table 7 shows that signals for C-3 protons occur at δ 6.50-6.91, C-6 at δ 6.18-6.48 and C-8 at δ 6.35-6.96. The signals of 'H NMR determined in $CDCl_3$ are more highfield than those obtained from solution in CD_3OD.

In general, the UV spectra of flavones exhibit Band 1 between 304 and 350 nm, flavonols absorb between 352 and 385 nm. For flavonols with O-substitution at C-3 the general shapes of the curve as well as the ranges of Band 1 (328-357 nm) approach those of flavones. This is as well known as the observation that increasing oxygenation of ring B results in a bathochromic shift of Band 1. Band 2 appears as one peak (at about 270 nm) in compounds with a monosubstituted B ring, but as two peaks or one peak (at about 258 nm) plus a shoulder (at about 272 nm) when a di or tri O-substituted B ring is present. In spectra determined from methanol solutions flavonols containing a C-6 hydroxyl substituent have a hypsochromic shift in Band 1 whilst flavonols with C-8 hydroxylation have a bathochromic shift of 13-16 nm in Band 1 together with an additional peak at 330 nm. The UV absorption curves of these compounds thus are quite characteristic (see page 72).

The UV spectra of flavones and flavonols on the addition of NaOMe produce a large bathochromic shift of Band 1 (40-65 nm) without a decrease in intensity, if a free 4'-hydroxyl group is present. Although flavonoids without a 4'-hydroxyl group also produce a bathochromic shift of 50-60 nm in Band 1, there is a decrease in intensity. The flavones and flavonols containing a free 7-hydroxyl group usually exhibit a diagnostic 5-20 nm

bathochromic shift of Band 2 in the presence of NaOAc.

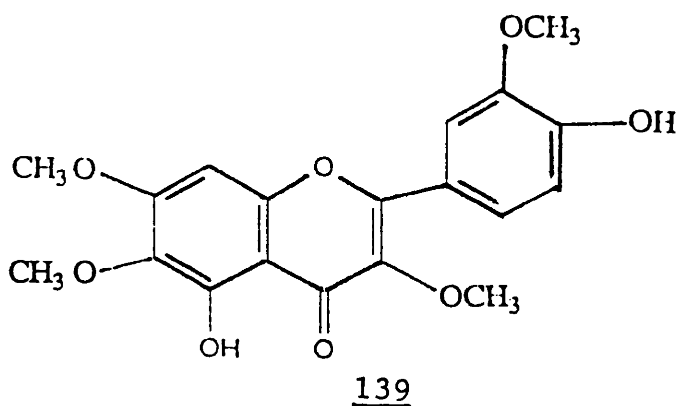
The known compounds isolated in the present investigation were identified by comparison of spectrospic data with literature values and the coumarins were also compared on TLC. The novel compounds were identified by a combination of 'H NMR, MS and UV data.Twenty one flavonoids which are well known constituents of plants (e,g, quercetin, kaempferol and their glycosides) were identified spectroscopically (page 92-120) and are not further discussed here. The following discussion is concerned with those flavonoids which have three substituents in their A ring.



9

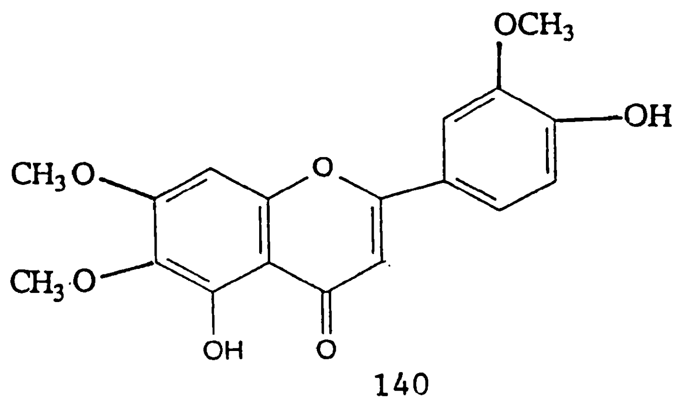
The MS of compound 9 with a M^+ at m/z 374 (100%) suggested a flavone substituted with two hydroxyl and four methoxyl groups.

An ion peak at m/z 359 (70%)[M-15]$^+$, suggested a methoxyl substitution at C-6, and an ion at m/z 331 (36%)[M-43]$^+$, suggested a methoxyl substitution at C-3. Fragment m/z 181 indicated two methoxyl groups on the A ring, whereas the m/z 151 fragment indicated one hydroxyl and one methoxyl on the B ring. The 'H NMR typically exhibited proton signals at δ 7.69 (1H,d,J=2,H2'), 7.66 (1H,dd,J=9 and J=2,H6'), 7.01 (1H,d,J=9,H5') consistent with a B ring having C-3' and C-4' substituents. A singlet at δ 6.52 was commensurate with a proton at C-8. The 'H NMR confirmed the presence of four methoxyl groups with signals at δ 3,96 (3H,s), 3.95 (3H,s), 3.92 (3H,s) and 3.80 (3H,s). The UV data was consistent with a C-5 hydroxyl and no free hydroxyl group at C-7 and the bathochromic shift in MeONa with loss of intensity supported C-4' methoxyl substitution. This compound was identified as 5,3'-dihydroxy-3,6,7,4'-tetramethoxyflavone (Casticin)[188].



139

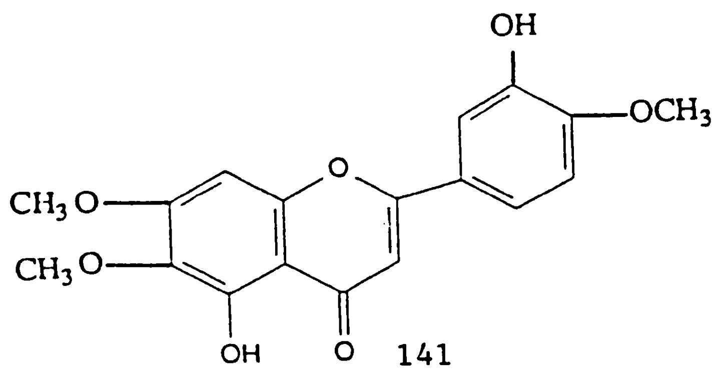
Compound 139 is an isomer of casticin ; it exhibited a similar MS and 'H NMR to casticin. The MS data exhibited peaks at m/z 374 (M^+,100%), 359 (M-15,76%), 331 (M-43,19%), 181 and 151. The 'H NMR showed peaks at δ 7.71 (1H,d,J=2,H2'), 7.68 (1H,dd,J=9 and J=2,H6'), 7.05 (1H,d,J=9,H5'), 6.51 (1H,s,H8), 3.99 (3H,s), 3.96 (3H,s), 3.92 (3H,s) and 3.86 (3H,s). The UV data was consistent with a C-4' hydroxyl with the bathochromic shift in MeONa with increase of intensity. This compound was indicated as 5,4'-dihydroxy-3,6,7,3'-tetramethoxyflavone (Chrysoplenetin)[195].



140

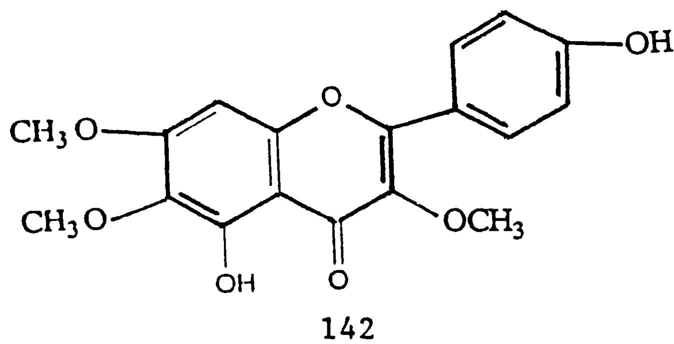
Compound 140 is an isomer of penduletin and eupatorin. The MS of this compound with a peak at 344 (M^+,100%) suggested a flavone substituted with two hydroxyl and three methoxyl

groups. An ion signal at m/z 329 $[M-15]^+$ (78%), suggested a methoxyl substitution at C-6 and fragment 181 indicated one hydroxyl and two methoxyl groups substituted in the A ring and fragment 151 suggested one hydroxyl and one methoxyl groups in B ring. The 'H NMR showed proton peaks at δ 7.61 (1H,d,J=2,H2'), 7.45 (1H,dd,J=9 and J=2,H6') and 6.93 (1H,d,J=9,H5') indicating C-3' and C-4' substitutions. The UV data was consistent with a C-4' hydroxyl with the bathochromic shift in MeONa showing an increase of intensity when compared with a methanol solution. The 'H NMR spectrum with two singlets at δ 6.96 and 6.58 were commensurate with protons at C-8 and C-3. This compound was thus identified as 5,4'-dihydroxy-6,7,3'-trimethoxyflavone (Circilineol)[189].



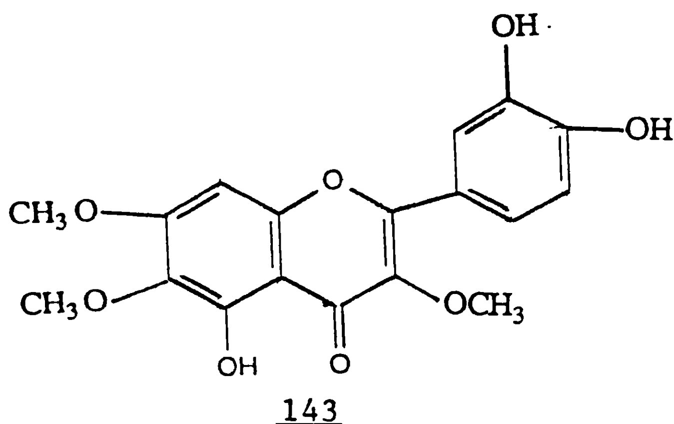
Compound 141 exhibited similar MS and 'H NMR spectra to circilineol (140). The UV spectrum indicated C-4' methoxyl substitution, supported by the bathochromic shift in MeONa with loss of intensity. NOE experiments of 'H NMR confirmed C-4' methoxyl substitution since irradiation at δ 3.98 resulted in enhancement of the signal at δ 7.01; irradiation at δ 7.01

showed enhancement of the signals at δ 3.98 and 7.51 and irradiation at δ 6.52 exhibited no signal enhancement indicating no substitution at C-3. This compound was identified as 5,3'-dihydroxy-6,7,4'-trimethoxyflavone (Eupatorin)[199].



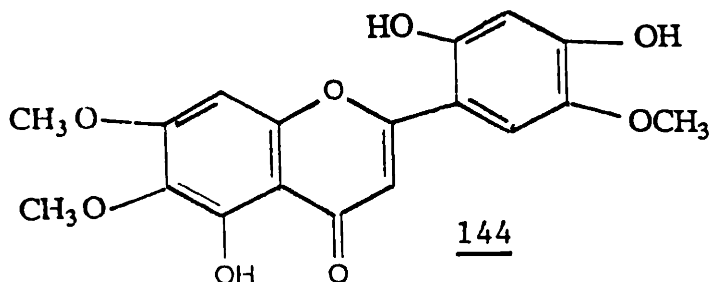
142

The MS data of compound 142 with a peak at m/z 344 (M^+,100%) suggested a flavone substituted with two hydroxyl and three methoxyl groups. It is an isomer of circilineol and eupatorin, but it exhibited a different MS to both of them. The ion signal at m/z 301 (M-43,31%) suggested C-3 methoxyl substitution. Fragment ions at m/z 181 and 121 indicated one hydroxyl and two methoxyl groups substituted in the A ring and one hydroxyl substituted in the B ring. The 'H NMR showed typical C-4' substitution with protons at δ 8.01 (2H,d,J=9,H2',H6'), 7.02 (2H,d,J=9,H3',H5') and a singlet at δ 6.79 was commensurate with a proton at C-8. The 'H NMR confirmed three methoxyl groups in this compound which was identified as 5,4'-dihydroxy-3,6,7-trimethoxyflavone (Penduletin)[190].



143

The MS of compound 143 with a peak at m/z 360 (M^+,100%) suggested a flavone substituted with three hydroxyl and three methoxyl groups. An ion at m/z 345 (M-15,57%) suggested a methoxyl substitution at C-6, and an ion peak at m/z 317 (M-43,16%) suggested a methoxyl at C-3. Fragment ions at m/z 181 and 137 indicated one hydroxyl and two methoxyl groups substituted in the A ring and two hydroxyl groups in the B ring. The 'H NMR spectrum exhibited characteristie proton signals at δ 7.70 (1H,d,J=2,H2'), 7.53 (1H,dd,J=9 and J=2,H6'), 6.93 (1H,d,J=9,H5') consistent with a B ring with C-3' and C-4' substitution. Three methoxyl groups were confirmed by proton signals at δ 3.96(3H,s). 3.89 (3H,s) and 3.85 (3H,s). This compound was identified as 5,3',4'-trihydroxy-3,6,7-trimethoxyflavone (Chrysosplenol-D)[197].



144

MS data of 144 with a peak at m/z 360 (M^+,100%) suggested a flavone with three hydroxyl and three methoxyl groups. An ion

peak at m/z 345 (M-15,82%) was consistent with either a C-6 or C-8 methoxyl. Fragments at m/z 181 and 167 indicated one hydroxyl and two methoxyl groups substituted in the A ring and two hydroxyl and one methoxyl groups substituted in the B ring. The UV data was consistent with hydroxyl substitution at C-5 and C-4' and a C-7 methoxyl. The bathochromic shift in MeONa with increased intensity supported a hydroxyl substitution at C-4'. No shift in the Band 1 in NaOAC compared with the spectrum from MeOH was observed, indicating substitution at C-7 with a methoxyl group. The 'H NMR has three signals at δ 3.93 (3H,s), 3.81 (3H,s) and 3.73 (3H,s) consistent with methoxyl substitution at C-7, C-5' and C-6 or C-8. The spectrum also contained the somewhat unusual pattern of four singlets at δ 6.57, 6.96, 7.11 and 7.45. These were assigned to protons at C-3, C-8, C-3' and C-6' respectively. These assignments were also supported by the NOE data, where irradiation at δ 3.93 (C-7, $-OCH_3$) caused enhancement of the signal at δ 6.96 (H-8 or H-6) and irradiation at δ 3.81 (C-5',$-OCH_3$) gave enhancement at δ 7.45 (H-6'), whilst irradiation at δ 7.11 (H-3') and δ 6.57 (H-3) produced no enhancement of other signals. This compound was therefore identified as either 5,2',4'-trihydroxy-6,7,5'-trimethoxyflavone or 5,2',4'-trihydroxy-7,8,5'-trimethoxyflavone. The MS data suggested C-6 methoxyl substitution with peak at m/z 360 (M^+,100%) and m/z 345 $(M-15)^+$ (82%) and not C-8 methoxyl substitution . If the compound had C-8 methoxyl substitution, then the MS data should have shown the signal at m/z 345 $(M-15)^+$ as the base peak. Comparison of

'H NMR with the literature and for some other flavonoids with 2 or 3 substituents in the A ring are presented on the Table 8. The range of 'H NMR for C-6 is δ 6.14-6.48 and C-8 is δ 6,35-6.96.(see Table 7, page 127 and Table 8, page 138). The 'H NMR data strongly supported the identification of the compound as 5,2',4'-trihydroxy-6,7,5'-trimethoxyflavone, a novel compound.

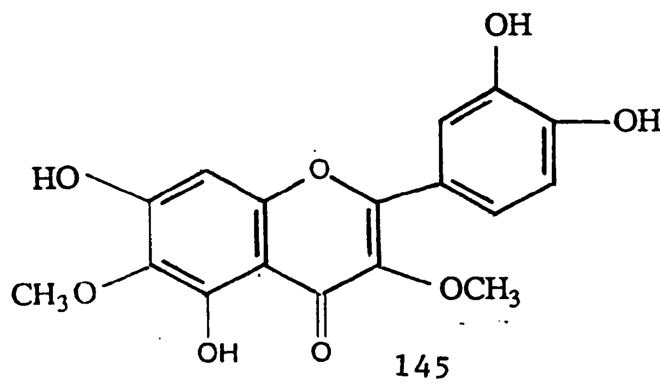
Table 8 'H NMR data for H6 or H8 of flavonoids

Flavonoid	A ring substitution				H signal		Solvent	Reference
	C-5	C-6	C-7	C-8	C-6H	C-8H		
Herbacetin methylether	OH		OCH_3	OCH_3	6.20	-	CD_3OD	200
Isoscutellaretin-4-methylether -8-glucoside	OH		OH	glucose	6.32	-	CD_3OD	200
5,7,8,3'- ** Tetrahydroxy -3,4'-dimethoxyflavone	OH		OH	OH	6.46	-	CD_3OD	
Vitexin-2"-O-glucoside	OH		OH	diglucose	6.37	-	DMSO	194
Vitexin-2"-O-rhamnoside	OH		OH	glucose- -rhamnose	6.39	-	DMSO	194
Hypolaetin -8-glucoside	OH		OH	glucose	6.28	-	DMSO	292
Cytisoside	OH		OH	glucose	6.14	-	DMSO	194
Vitexin	OH		OH	glucose	6.16	-	DMSO	194
Tamarixetin	OH		OH		6.36	6.71	DMSO	180
Rhamnetin	OH		OCH_3		6.35	6.71	DMSO	199
Rhamnocitrin	OH		OCH_3		6.21	6.71	CD_3OD	198
Cirsiliol	OH	OCH_3	OCH_3		-	6.83	CD_3OD	191
Circilineol	OH	OCH_3	OCH_3		-	6.96	CD_3OD	189

Table 8 (continued)

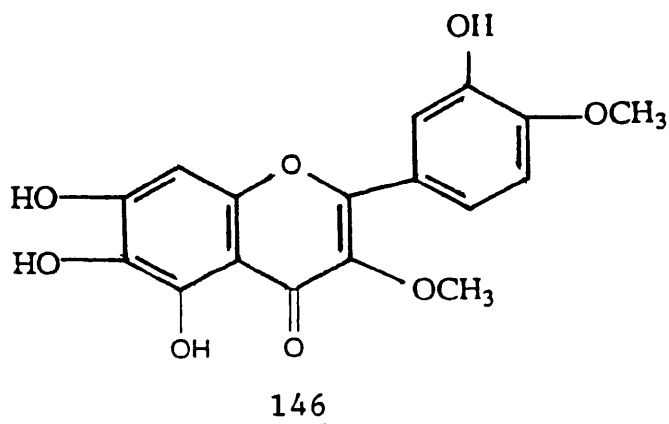
Penduletin	OH	OCH_3	OCH_3	-	6.79	CD_3OD	190
Quercetage-** tin-4'-methy lether	OH	OH	OH	-	6.72	CD_3OD	
Quercetage-** tin-3,4'-di methylether	OH	OH	OH	-	6.82	CD_3OD	
Cirsimaritin	OH	OCH_3	OCH_3	-	6.92	DMSO	195
5,2',4'-Tri** hydroxy-6,7, 5'-trimeth oxyflavone	OH	OCH_3	OCH_3	-	6.96	DMSO	

** The novel compounds isolated from the investigated plant



The MS of compound 145 with a peak at m/z 346 (M^+,100%) suggested a flavone with four hydroxyl and two methoxyl substituents. An ion peak at m/z 331 $(M-15)^+$ (81%) consistent with a C-6 methoxyl substitution and an ion signal at m/z 303 $(M-43)^+$ (71%) suggested a C-3 methoxyl substitution. A fragment

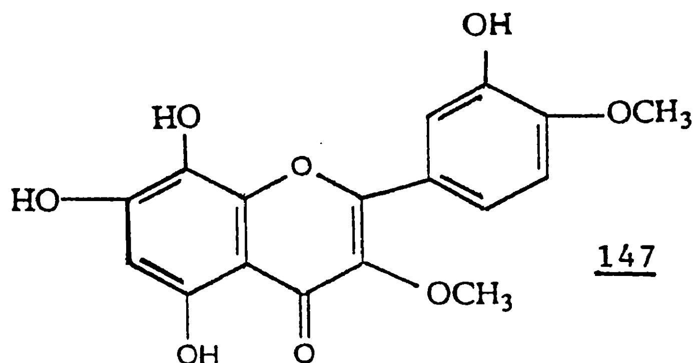
at m/z 137 (41%) indicated a B ring substituted with two hydroxyl groups. The UV data indicated C-5 and C-7 hydroxyl substitution. The 'H NMR confirmed the presence of two methoxyl groups with signals at δ 3.88 (3H,s) and 3.79 (3H,s). The proton signals at δ 7.62 (1H,d,J=2,H2'), 7.55 (1H,dd,J=9 and J=2,H6'), 6.90 (1H,d,J=9,H5') were consistent with C-3' and C4' substitution. The singlet at δ 6.82 (1H,s) was assigned to a proton at C-8. This compound was identified as 5,7,3',4'-tetrahydroxy-3,6-dimethoxyflavone (Axillarin)[192].



146

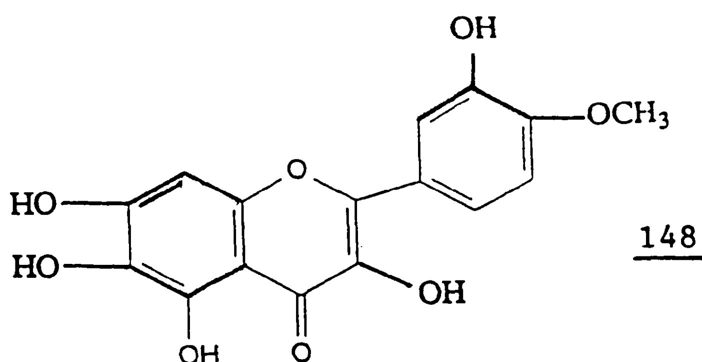
The compound 146 is an isomer of axillarin(145) and eupatolitin (3,5,3',4'-tetrahydroxy-6,7-dimethoxyflavone). The MS with a peak at m/z 346 (M^+,100%) indicates the same number of hydroxyl and methoxyl groups as axillarin and eupatolitin. The MS of axillarin and eupatolitin have peaks corresponding to $(M-15)^+$ [296] which are attributed to the C-6 methoxyl substitution, but this peak is absent from the MS of 146. Eupatolitin showed a typical peak for one hydroxyl and two methoxyl groups in the A ring with a peak at m/z 181 but this signal was not observed in the MS of compound 146. The MS of compound 146 had no peak corresponding to $(M-15)^+$ thus indicating absence of a methoxyl

at C-6 and C-8. An ion peak at m/z 303 $(M-43)^+$ (86%) clearly indicates that a methoxyl substitution is at C-3. The UV data were consistent with C-4' methoxyl substituent due to the bathochromic shift in MeONa and a loss of intensity. A shift of 12 nm was observed for Band 1 on addition of NaOAC in comparison with the spectrum obtained from MeOH solution and this indicated substitution at C-7 with a hydroxyl group. No such shift was noted for eupatolitin [296]. The 'H NMR spectrum exhibited characteristie proton signals at 7.88 (1H, d, J=2, H2'), 7.75 (1H,dd,J=9 and J=2,H6') and 7.01 (1H,d,J=9,H5') consistent with a B ring having C-3' and C-4' substitution. A singlet at δ 6.82 was commensurate with a proton at C-8 (compared to δ 6.46 for C-6 in compound 147). The 'H NMR further confirmed two methoxyl groups with signals at δ 4.04 (3H,s) and 3,89 (3H,s). The NOE experiment also supported the presence of two methoxyl groups at C-4' and C-3. Irradiation at δ 4.04 caused enhancement of the signal at δ 7.01; irradiation at δ 3.89 produced no signal enhancement. Thus compound 146 was identified as quercetagetin-3,4'-dimethylether a novel natural compound.



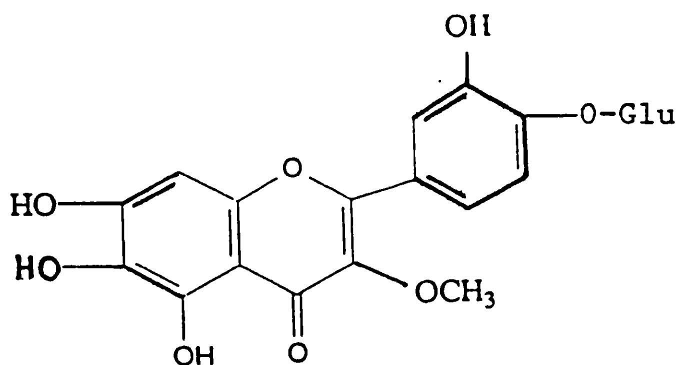
The compound 147 is an isomer of laciniatin(3,5,7,3'-tetrahydroxy-6,4'-dimethoxyflavone) and quercetagetin-3,4'-

dimethylether (146). The MS data with a peak at m/z 346 (M^+, 100%) suggested the same substitution pattern as laciniatin and quercetagetin-3,4'-dimethylether. No peak was observed at $(M-15)^+$ in the MS indicating no C-6 or C-8 methoxyl group substitution. Laciniatin shows a peak at m/z 331 $(M-15)^+$ (48%) indicating C-6 methoxyl substitution [295]. All the data of MS , UV and 'H NMR of 147 were similar to those of 146 except the 'H NMR of 147 showed a signal at δ 6.48 as opposed to δ 6.82 for compound 146. This leads to the proposal that the one hydroxyl in compound 147 was at C-8 rather than at C-6 as in compound 146 (Table 8). Compound 147 was therefore identified as 5,7,8,3'-tetrahydroxy-3,4'-dimethoxyflavone a novel compound.



The MS of compound 148 had a peak at m/z 332 (M^+,100%) which is indicative of a flavone substituted with five hydroxy and one methoxyl groups. The absence of an ion peak at m/z 317 $(M-15)^+$ indicated there was no methoxyl substitution at either C-6 or C-8, and the absence of a peak at m/z 289 $(M-43)^+$ indicated that there was no C-3 methoxyl substitution. The B2 fragment at m/z 151 suggested one hydroxyl and one methoxyl group substitution in the B ring. The UV data showed a Band 2 shift

of 24 nm in NaOAc indicating a free C-7 hydroxyl. The UV data was consistent with a C-4' methoxyl substitution. The bathochromic shift in MeONa with loss of intensity supported a C-4' methoxyl substitution. The 'H NMR exhibited proton signals at δ 7.80 (1H,d,J=2,H2'), 7.65 (1H,dd,J=9 and J=2,H6') and 6.90 (1H,d,J=9,H5') consistent with a B ring containing a C-3' hydroxyl and C-4' methoxyl. A singlet at δ 6.72 was commensurate with a proton at C-8 . The 'H NMR further confirmed the presence of one methoxyl group with a signal at δ 4.01 (3H,s). The NOE experiment supported methoxyl substitution at C-4' when irradiation at δ 4.01 caused enhancement of the signal at δ 6.90 (H5'). The compound was therefore considered to be quercetagetin-4'-methylether, a novel compound.

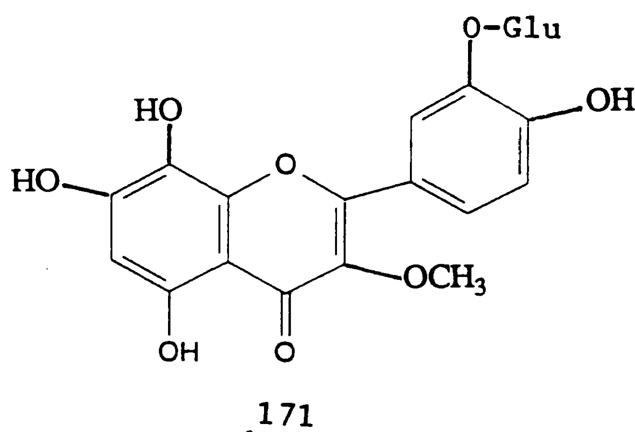


170

The MS data with a base peak at m/z 332 (100%) for compound (170) suggested a flavone with five hydroxyl and one methoxyl group. The absence of an ion peak at m/z 317 (332-15) indicated

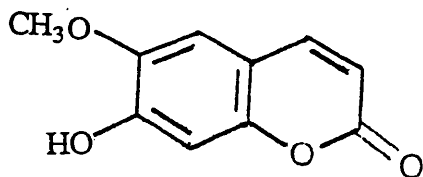
that there was no methoxyl substitution at C-6 or C-8. An ion signal at m/z 289 (332-43)$^{+}$(89%) indicated methoxyl substitution at C-3. The 'H NMR exhibited proton signals at δ 7.75 (1H,d,J=2,H2'), 7.65 (1H,dd,J=9 and J=2,H6'), and 6.87 (1H,d,J=9,H5') consistent with a B ring having C-3' and C-4' substitution. A singlet at δ 6.82 was commensurate with a proton at C-8 and a methoxyl signal at δ 3.87 was assigned a C-3 methoxyl. The UV data showed a Band 2 shift of 8 nm in NaOAc when compared with the MeOH spectrum indicating a free C-7 free hydroxyl. The 'H NMR exhibited glucose signals at δ 5.10 (one proton) and δ 4.0-3.25 (five protons). All data suggested that the compound 170 is quercetagetin-3-methylether glucoside. Comparison was made of the MS of quercetagetin-3-methylether with that of compound 170. Quercetagetin-3-methylether showed an ion peak at m/z 137 (B2+32,21%) whereas compound 170 exhibited an ion signal at m/z 136 (B2+32-1,23%). This fragment suggested that the B ring had C-3' and C-4' oxygenated substitutions, one being a hydroxyl and the other being glucose. The UV data with a bathochromic shift in NaOMe with loss of intensity supported that the glucose was located at C-4'. TLC comparison of 170 before and after hydrolysis was made with quercetagetin-3-methylether, quercetagetin-4'-methyl ether and patuletin (3,5,7,3'4'-pentahydroxy-6-methoxyflavone). Compound 170 showed a different Rf value from patuletin and the aglycone produced after hydrolysis exhibited the same Rf value and showed the same retention time on HPLC with quercetagetin-3-methylether. Hence compound 170 is quercetagetin-3-methyl

ether -4'-glucoside, a novel compound.



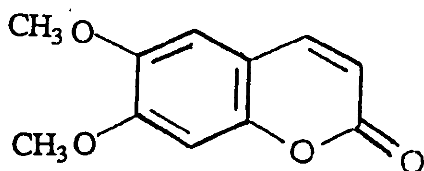
171

The MS data of compound 171 was very similar to that of 170. The 'H NMR exhibited proton signals at δ 7.72, 7.58 and 6.88 suggesting the same B ring substitutions as compound 170. The signal at δ 3.87 was assigned to C-3 methoxyl. A singlet at δ 6.48 was commensurate with a proton at C-6 (comparison δ 6.82 at C-8 for compound 170). The UV data showed a Band 2 shift of 10 nm in NaOAc indicating a free C-7 hydroxyl. An ion peak at m/z 136 (B2+32-1,22%) suggested that the B ring had C-3' and C-4' oxygenated substitution, one being a hydroxyl and the other a glucose. The bathochromic shift in NaOMe with increase of intensity supported a C-4' hydroxyl substitution and glucose at C-3'. The 'H NMR data of the aglycone after hydrolysis of compound 171 was identical with that of gossypetin-3-methylether [294]. Compound 171 was therefore identified as gossypetin-3-methylether-3'-glucoside, a novel compound.



172

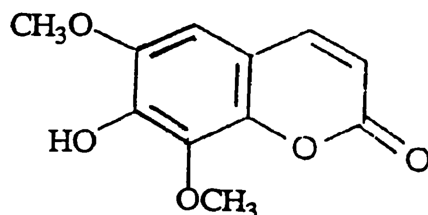
The UV, 'H NMR clearly indicated that compound 172 was a substituted coumarin. The MS with a base peak at m/z 192 (M^+,100%) was indicative of substitution with one hydroxyl and one methoxyl groups. The presence of an ion peak at m/z 177 $(M-15)^+$ and a singlet signal in 'H NMR at δ 3.96 (3H) supported one methoxyl group substitution. The 'H NMR exhibited proton signals δ 7.92(1H,d,J=10,H4), 7.16 (1H,s,H5), 6.80 (1H,s,H8), 6.21 (1H,d,J=10,H3) indicating one methoxyl and one hydroxyl groups substitution at C-6 and C-7. Comparison of TLC and UV with standard sample (Sigma) confirmed the identity of compound 172 as scopolin (6-methoxy-7-hydroxycoumarin).



173

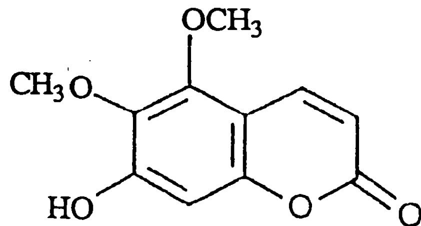
The UV, 'H NMR and MS of compound 173 suggested a close structure to 172. The MS with a peak at m/z 206 (M^+,100%) was indicative

of substitution with two methoxyl groups. The 'H NMR exhibited two methoxyl signals at δ 3.96 (3H,s) and 3.94 (3H,s). The 'H NMR data showed the compound had the same substitution pattern as 172. The compound 173 was therefore considered to be 6,7-dimethoxycoumarin(scoparone).



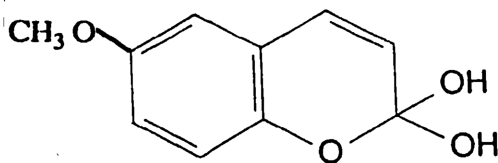
174

The UV, 'H NMR data indicated that compound 174 was a substituted coumarin. The MS with a base peak at m/z 222 (M^+,100%) indicating substitution with one hydroxyl and two methoxyl groups. The 'H NMR confirmed the two methoxyl substitutions with signals at δ 3.90 (3H,s) and 3.84 (3H,s). The 'H NMR spectrum exhibited proton signals at δ 7.92 (1H,d,J=10,H4), 7.01 (1H,s,H5) and 6.24 (1H,d,J=10,H3) indicating one hydroxyl and two methoxyl groups substituted at C-5, C-6, C-7 or C-8. By comparison with a standard sample, compound 174 was determined as 7-hydroxy-6,8-dimethoxycoumarin(fraxetin-8-methylether).



175

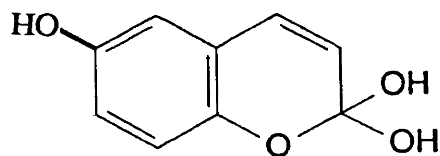
The compound 175 showed a similar MS and 'H NMR data to 174, but the 'H NMR of 175 exhibited a singlet signal at δ 6.54 (1H,s) instead of the peak observed at δ 7.01 (1H,s) in the spectrum of 174. The comparison with a standard sample (sigma) confirmed that 175 was 7-hydroxy-5,6-dimethoxycoumarin.



176

The 'H NMR of compound 176 showed a chromene pattern with proton signals at δ 7.60 (1H,d,J=10), 7.18 (1H,d,J=2), 7.04 (1H,dd,J=2 and J=6), 6.82 (1H,d,J=6) and 6.31 (1H,d,J=10). A methoxyl was indicated with a signal at δ 3.86 (3H,s). The MS data with a M^+ at m/z 194 suggested substitution with one methoxyl and two hydroxyl groups. The 'H NMR data indicated that the methoxyl was substituted at either C-6 or C-7 and that the two hydroxyl groups were substituted at C-2. The 'H NMR data

from NOE experiments confirmed that the two hydroxyl subtituents were at C-2 and that the methoxyl was at C-6. Irradiation at δ 3.86 enhanced the signals at δ 7.18 and 7.04; irradiation at δ 7.04 enhanced the signals at δ 3.86 and 6.82; irradiation at δ 6.31 enhanced the signals at δ 7.60 and 7.18; irradiation at δ 7.18 enhanced the signals at δ 3.86 and 6.31. The results of NOE indicated that the proton at δ 7.18 could be assigned to C-5, because irradiation of the C-4 proton signal at δ 6.31 enhanced the signal at δ 7.18 and the methoxyl at δ 3.86 was at C-6. Compound 176 was therefore considered to be 2,2-dihydroxy-6-methoxychromene.



177

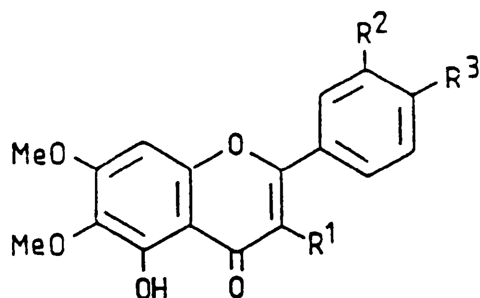
The 'H NMR of compound 177 exhibited similar data with 176, except only for the absence of a signal at δ 3.86 for a methoxyl group. The MS data with a M^+ at m/z 180.0429(calc. for $C_9H_8O_4$,180.0423) suggested three hydroxyl substituents. By analogy with the argument for the structure of 176, compound 177 is considered to be 2,2,2-trihydroxychromene.

3.2.3. Structure-activity relationships for the enhancement of activity of artemisinin

Six methoxylated flavones were assayed by B.C.Elford for their ability to enhance the *in vitro* antiplasmodial activity of artemisinin. These compounds, individual and in combination with artemisinin, were examined for antimalarial activity using an assay based on the incorporation of [^{3}H]-hypoxanthine into chloroquine-resistant *P. falciparum*. The IC_{50} values of these six flavonoids against *P. falciparum* are in the range of 2.3-7.0×10^{-5} M (Table 9). Under the same test conditions artemisinin has an IC_{50} value of 3.3×10^{-8} M and this is reduced to values of 1.5-3.0×10^{-8} M in the presence of 5×10^{-6} M of each of the following flavonoids: artemetin, casticin, chrysoplenetin, chrysosplenol-D, circilineol and eupatorin. At concentrations of 5×10^{-6} M these flavonoids donot exert antiplasmodial activity. Flavonoids have a wide range of biological activities including the inhibition of selected enzymes such as phosphodiesterases, ATP-ases and protein kinases. Artemetin and casticin have the ability to inhibit the influx of L-glutamine and of myo-inositol across host cell membrane in erythrocytes infected with *P. falciparum*. It is not known whether this property is directly relevant to the synergistic effects of these flavonoids on the antiplasmodial activity of artemisinin [6].

Six methoxylated flavones which possess C-5 hydroxyl, C-6, C-7-dimethoxyl and C-3',C-4'-dioxygenated and either C-3 methoxyl or H, enhanced activity of artemisinin.

Table 9 Structure-active relationship of methoxylated flavones isolated from A. annua for their in vitro activity against Plasmodium falciparum* and their potentiating effect on artemisinin



Flavonoid	R1	R2	R3	IC_{50} Flavonoid alone ($M \times 10^{-5}$)	IC_{50} Artemisinin ($M \times 10^{-8}$)+ flavone(5μM)
Artemisinin					3.3
Artemetin**	OMe	OMe	OMe	2.6	2.6
Casticin	OMe	OH	OMe	2.4	2.6
Chrysoplenetin	OMe	OMe	OH	2.3	2.25
Chrysosplenol-D	OMe	OH	OH	3.2	1.5
Circilineol	H	OMe	OH	3.6	1.6
Eupatorin	H	OH	OMe	6.5	3.0

* Inhibition of incorporation of [^{3}H]-hypoxanthine into P. falciparum (multi-drug resistant strain)

** Artemetin isolated from cell cultures of A. annua

3.2.4. Research in the future

There are a number of questions which have not yet been answered in connection with these studies. It is still not known whether the flavonoids themselves have any significant clinical effects as antimalarials. The *in vivo* activity of these flavonoids which potentiate the *in vitro* activity of artemisinin against *P. falciparum* have not been showed to have any clinical significance in potentiating the activity of artemisinin in patients taking traditional medicines containing *Artemisia annua*. Sufficient flavonoids have not been tested biologically in order to ascertain the structural requirements of flavonoids which potentiate the activity of artemisinin against *P. falciparum*. Furthermore , it has not been established whether all of the flavonoids which have potentiating activity on artemisinin are able to inhibit the influx of L-glutamine and of myo-inositol into parasitised erythrocytes. More work should be done in order to answer these questions.

Artemisinin is not readily synthesised and alternative sources are being investigated. One possible alternative is to use plant cell culture techniques. We have reported that four major methoxyflavones, artemetin, chrysoplenetin, chrysosplenol-D and circilineol have been isolated from cell cultures of *A. annua* on agar medium containing Murashige-Skoog basal medium supplemented with 5% sucrose, kinetin ($0.1mgl^{-1}$) and 2,4-dichlorophenoxyacetic acid ($1mgl^{-1}$) [297]. Extracts of cells showed weak activity *in vitro* against *P. falciparum* but

artemesinin was not detected [298]. Different media and different concentrations and types of hormone could be changed so as to investigate the possible production of artemisinin. Chrysosplenol-D (0.1%) and chrysoplenetin (0.035%) which are major methoxylated flavones in whole plants have weak activity *in vitro* against *P. falciparum* and can enhance the activity of artemisinin, (Table 9) and it would be of interest to isolate more of these compounds in order to do clinical trials with artemisinin and its derivatives.

Twenty three methoxylated flavones were isolated but only five of them were assayed for their ability to enhance the activity of artemisinin. The other compounds should be assayed in order to establish structure-activity relationships.

3.3. Conclusions

Scientific studies on herbal drugs used in Chinese medicine started from the isolation of ephedrine from *Ephedra* stems and the discovery of its pharmacological activities. Particularly during the past forty years, studies on medicinal herbs have developed into a search for potential therapeutic agents from traditional medicines. This research has become of worldwide interest. A number of investigations have succeeded in the isolation of active novel compounds and artemisinin from *Artemisia annua* the antimalarial herb is one such example. Artemisinin is a sesquiterpene lactone which is active against chloroquine-resistant *Plasmodium falciparum* in the treatment of cerebral malaria. The compound has been used successfully in several thousands of malaria patients in China, including those with both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* [28]. Thus artemisinin and its derivatives offer promise as a totally new class of antimalarial drug. Subsequently, from 1972 to the present, this drug and its derivatives have been studied in laboratory malarial models, for their pharmacology, pharmacokinetics and their toxicology [26]. Structure-activity relationship studies indicate that the peroxide linkage is essential for antimalarial activity [177].

A number of other *Artemisia* species have been studied chemically and pharmacologically in attempts to find artemisinin or other significant antimalarial compounds, but all of these studies have failed [4,5]. At present, *A. annua*

appears to be the only _Artemisia_ species that contains appreciable amounts of artemisinin.

Total synthesis of artemisinin was achieved with an overall yield of 0.24 % [184], 5% [185] and 37% [186], but such synthesis may not be economical for the large-scale production of artemisinin.

It has been demonstrated that the antimalarial activity of artemisinin and its derivatives is markedly enhanced by the presence of methoxylated flavones such as artemetin and casticin [6]. At the concentrations used these flavones have no antimalarial activity. By contrast the antimalarial activity of chloroquine was unaffected by the presence of these flavones.

Through the detailed phytochemical studies in the present work, a total of forty compounds, twenty seven flavones, seven flavone glycosides, four coumarins and two chromenes have been isolated. Five methoxylated flavones were assayed for their ability to enhance the activity of artemisinin. These compounds and the combination of artemisinin with individual flavones isolated from the plant were examined for antimalarial activity using an assay based on the incorporation of [^{3}H]-hypoxanthine into chloroquine-resistant _P. falciparum_. In our experiments, the apparent IC_{50} for artemisinin was reduced by the presence of some of the methoxylated flavones of _A. annua_, such as, chrysosplenol-D, chrysoplenetin, circilineol and casticin (Table 9, page 151).

Recently, flavonoids have been isolated as active principles of Chinese drug materials and their activities have been studied

to determine whether they are responsible for the therapeutic effects associated with traditional use. Some flavonoids have well documented anti-inflammatory activity e.g. luteolin and quercetin which have been isolated from *A. annua* in the present work. It seems likely that the biologically active flavonoids, luteolin, kaempferol, quercetin, casticin, chrysosplenol-D, chrysoplenetin and circilineol may contribute to the efficacies of some plants including *A. annua* used in traditional medicine. From a chemotaxonomic viewpoint, *A. annua* contains the same major constituents including sesquiterpene lactones and flavonoids as are found in other *Artemisia* species. The sesquiterpene lactones isolated from *A. annua* are of the amorphane type. The major flavonoids of *Artemisia* species are methoxylated flavones, flavonols and their glycosides. The major aglycones of the flavonoid glycosides are quercetin, luteolin, kaempferol apigenin and quercetagetin. *A. annua* yields an interesting series of methoxylated flavonoids, many of which have previously been recorded for other species of *Artemisia*. For example, casticin has been isolated also from *A. judaice* [275]. Circilineol has been isolated previously from *A. ludoriciana*, *A. herba-alba* [189], *A. monosperma* [275] and *A. capillaris* [191], whilst axillarin has been characterized from *A. taurica* [192], *A. incanescens* [193] and *A. ludoviciana* [287] and cirsimaritin from *A. scoparia* [195], *A. mesatlantica* [196] and *A. capillaris* [197]. Rhamnocitrin has been identified from *A. scoparia* [198] and eupatorin from *A. ludoviciana* [199]. The remaining flavonoids not previously isolated from *Artemisia*

spp. are commonly found within members of the tribe *Anthemideae* of the *Asteraceae* [200].

APPENDIX 1. SESQUITERPENE LACTONES ISOLATED FROM THE GENUS *ARTEMISIA* [13]

Section Abrotanum

Species	Compounds	References
A. annua	arteannuin-B	32
	artemisinin	3
A. californica	artecalin	202
A. camphorata	α-santonin	201
A. carruthii	matricin	203
	ludartin	
	11,13-dihydroludartin	
A. douglasiana	arglanine	204
	douglanine	
	ludovicin-B	
	arteglasin-A	
	arteglasin-B	
A. franserioides	artefransin	205
A. incana	deacetylmatricarin	206
A. judaica	tauremisin	207
A. judaica	1-epi-erivanin	264
	1-epi-isoerivanin	
	13-O-desacetyl-eudesma-afraglaucolide	
	13-O-desacetyl-1α-hydroxy-afraglaucolide	
	13-O-desacetyl-1β-hydroxy-afraglaucolide	
	13-O-desacetyl-1α-hydroxy-isoafraglaucolide	
	seco-isoerivanin pseudo acid	

A. klotzschiana	achillin	208
	chrysartemin-A	209
	matricarin	
A. ludoviciana	achillin	208
	ludalbin	209
	douglanine	210
	ludovicin-B	
A. ludoviciana	achillin	260
	parishin-C	
	valgarin	
	artecanin	
	11,13-dihydrodesacetylmatricarin	
	ludovicin-C	
A. mexicana	estafiatin	211
	chrysartemin-A	209
	arglanine	
	douglanine	
	artemorin	212
	armexine	
	α-santonin	213
A. mexicana	tulipinolide	214
	arglanine	
	artemexifolin	
	artexifolin	
A. neo-mexicana	α-santonin	213
A. princeps	yomogin	215
A. stelleriana	1,2-dihydrosantonin	216

A. tilesii	deacetylmatricarin	217
	matricarin	
A. verlotorum	artemorin	218
	verlotorin	
	anhydroverlotorin	
	tauremisin	219
A. vulgaris	psilostachyin	220
	psilostachyin-C	
A. wrightii	α-santonin	213
	Section Absinthium	
A. absinthium	artabsin	221
	absinthin	
	arabsin	
	ketopelenolide-A	
	ketopelenolide-B	
	hydroxypelenolide	
A. anethifolia	ketopelenolide-B	222
A. arborescens	arborescin	223
A. arborescens	3,4,10-trihydroxy-8-acetyloxyguaian -12,6 -olide	266
A. ashurbajevii	hanphyllin	224
	granillin	
A. austriaca	deacetylmatricarin	225
	α-santonin	
A. canariensis	tauremisin	226
	tabarin	

A. caucasica	grossmizin	227
	canin	
A. jacutica	ketopelenolide-B	228
	sieversinin	
A. lanata	achillin	229
	8-hydroxyachillin	
	1,10-epoxyachillin	
	1,10-epoxy-8-hydroxyachillin	
A. lanata	11-epidihydrodentin	267
	6-acetylferulidin	
	carmenin	
	andalucin	
A. montana	neozeoguaianin	268
	ezoyomoginin	
	montanone	
A. rutifolia	rurifolin	257
ex Spreng.	artcaninhydrate	258
	bis-seco-tanapartholide	
	1,9,12-triacetoxybis-abolene	
A. rutifolia	canin	227
A. sieversiana	artabsin	
	absinthin	
	sieversinin	
	globicin	230

Section *Dra-cunculus*

A. afra	artemisia glaucolide	265
	1β-hydroxyafraglaucolide	
	1α-hydroxyafraglaucolide	
	1β-hydroxyisoafraglaucolide	
	eudesmaafraglaucolide	
	12-hydroxy-α-cyperone	
	Ten guaianolides sesquiterpene lactones	
A. agen tea	deacetylargentiolide	262
A. diffusa	1-epi-artemin	270
	1-epi-dehydroisoerivanin	
A. dracunculoides	8-hydroxyarbiglovin	231
A. feddei	himeyoshin	261
A. filifolia	colartin	232
A. frigida	1,10-epoxy-8-hydroxychillin	255
A. hispanica	2-hydroxyartemorin	269
A. gmelinii	8-oxo-nerolidol acetate	
	11-peroxy-8-oxo-9,10-E-dehydronerolide	
	10,11-dihydronerolidol acetate	
	11,13-dihydrosantamarin	
	11-epicolartin	
	1-hydroxy-11-epicolartin	
	1-hydroxy-4,11-diepicolartin	

A. iwayomogi	eudesman-4,11-dien-12,8β-olide	259
	2α-peroxyisoalantolactone	
	3α,5α-dihydroxyisoalantolactone	
	3α-peroxy-5β-hydroxyisoalantolactone	
	3α-peroxy-5α-hydroxyisoalantolactone	
	3α-peroxyeudesma-4,11-dien-12,8β-olide	
	3α-hydroxyeudesma-4,11-dien-12,8β-olide	
	3-oxo-eudesma-4,11-dien-12,8β-olide	
	3α-hydroxy-4α,5-epoxyeudesma-11-en-12,8β-olide	
	3β-hydroxy-4α,5-epoxyeudesma-11-en-12,8β-olide	
	4α-peroxy-eudesma-2,11-dien-12,8β-olide	
	rupicolin-A-8-O-acetate	
	rupicolin-B-8-O-acetate	

Section *Seriphidium*

A. amoena	α-santonin	225
A. balchanorum	costunolide	233
	hydroxycostunolide	
	balchanolide	
	hydroxybalchanolide	
	isobalchanolide	
	balchanin	
A. caerulescens	α-santonin	234
	β-santonin	
	artemin	
	ψ-santonin	
A. caerulescens gallica	artegallin	256

A. cina	α-santonin	234
	artemisin	
	β-santonin	
A. finita	α-santonin	235
	finitin	
A. fragrans	arsubin	236
	taurin	
	stereoisomer of erivanin	
	erivanin	237
A. granatensis	1-keto-6β,7α,11β-H-eudesm-4-en-6,12-olide	238
	1-hydroxy-6β,7α,11β-H-eudesm-4-en-6,12-olide	
A. herba-alba	11,13-dihydrocostunolide	272
	11-epitaurin	
	11,13-dihydrocyclocostunolide	
A. herba-alba	herbolide-A	239
	herbolide-B	
	herbolide-C	
A. herba-alba *herba-alba*	1,8,-dihydroxygermacra-4,10(14)-dien-6,7,11βH-12,6-olide	271
	1-hydroperoxy-8-hydroxygermacra-4,10(14)-dien-6,7,11βH-12,6-olide	
	1-acetoxyeudesm-3en-5,6,7,11βH-12,6-olide	

	8-hydroxygermacra-4,10(14)-dien-6,7,11βH-12,6-olide	
	1-hydroxyeodesm-4(5)-en-5,6,7,11βH-12,6-olide	
	1-hydroxyeodesm-4-en-6,7,11βH-12,6-olide	
	1,8-dihydroxyeudesm-4-en-6,7,11βH-12,6-olide	
	4,5-dihydroxysantolina-1,8-diene	
A. kurramensis	α-santonin	240
	β-santonin	
	lumisantonin	
A. leucodes	deacetoxymatricarin	241
A. maritima	α-santonin	242
	β-santonin	
	artemisin	
	temisin	
	ψ-santonin	
	desoxy-α-santonin	
A. maritima	1-oxo-6,7,11βH-14-methygermacra-4(5)-ene-12,6-olide	263
	1-oxo-6,7,11βH-germacra-4(5),10(14)-dien-12,6-olide	
A. monogyna	mibulactone	243
	monogyna	
	lumisantonin	
A. ramosa	finitin	244
A. santolina	artesin	245
	arsanin	
	arsantin	
A. spicata	santamarine	246

Section *Tridentatae*

Species	Compound	Ref.
A. arbuscula	arbusculin-A	
	arbusculin-B	
	arbusculin-C	
	arbusculin-D	
	arbusculin-E	247
	tatridin-A	
	tatridin-B	248
	badgerin	
	spiciformin	
	deacetylaurenobiolide	
A. bigelovii	arbiglovin	249
A. cana	canin	250
cana	ridentin	
	artecanin	
	artevasin	
A. cana	arbusculin-B	247
viscidula	viscidulin-A	251
	viscidulin-B	
	viscidulin-C	
A. longiloba	longilobol	252
A. nova	cumambrin-A	247
	cumambrin-B	
	8-deoxycumambrin-B	
	novanin	
A. pygmaea	pygmol	252

	cryptomeridiol	
A. rothrockii	rothin-A	247
	rothin-B	
A. tridentata	ridentin	253
	dentatin-B	
	tatridin-A	
	tatridin-B	
	tatridin-C	
	dentatin-A	
	parishin-A	252
	parishin-B	
	parishin-C	
	isophotosantonic lactone	
	artevasin	254
	dehydroleucodin	
	badgerin	248
	spiciformin	
	deacetylaurenobiolide	
	1β-hydroxysant-3-en-6,12-olide	
	1β-hydroxysant-4(14)-en-6,12-olide	
	artecalin	251
	ridentin	
	ridentin-B	
	cumambrin-A	
	cumambrin-B	
	cumambrin-B-oxide	
	rupicolin-A	253

rupicolin-B

rupin-A

rupin-B

colartin

APPENDIX 2 FLAVONOIDS ISOLATED FROM THE GENUS ARTEMISIA

Species	Compounds	References
A. absinthum	artemetin	273
A. arbuscula	6-methoxykaempferol	278
A. campestris glutinosa	pinostrobin	288
	pinocembrin	
	sakuranetin	
	naringenin	
	7-methylaromadendrin	
A. campestris maritima	5,4'-dihydroxy-7,3'-dimethoxyflavone	286
	3,5,4'-trihydroxyflavone	
	5,4'-dihydroxy-6,7-dimethoxyflavone	
	5,8,4'-trihydroxyflavone	
	5,6-dihydroxy-4'-methoxyflavone	
A. cana cana	chrysoplenetin	275
A. capillaris	apigenin-7-methylether	276
A. frigida	5,7,4'-trihydroxy-6,3',5',-trimethoxyflavone	281,282
	quercetagetin-3,6,3',4'-tetramethylether	
	eupatilin	
	jaceosidin	
	hispidulin	
	eupafolin	
	luteolin-7,4'-dimethylether	
	tricin	
	chrysoeriol	

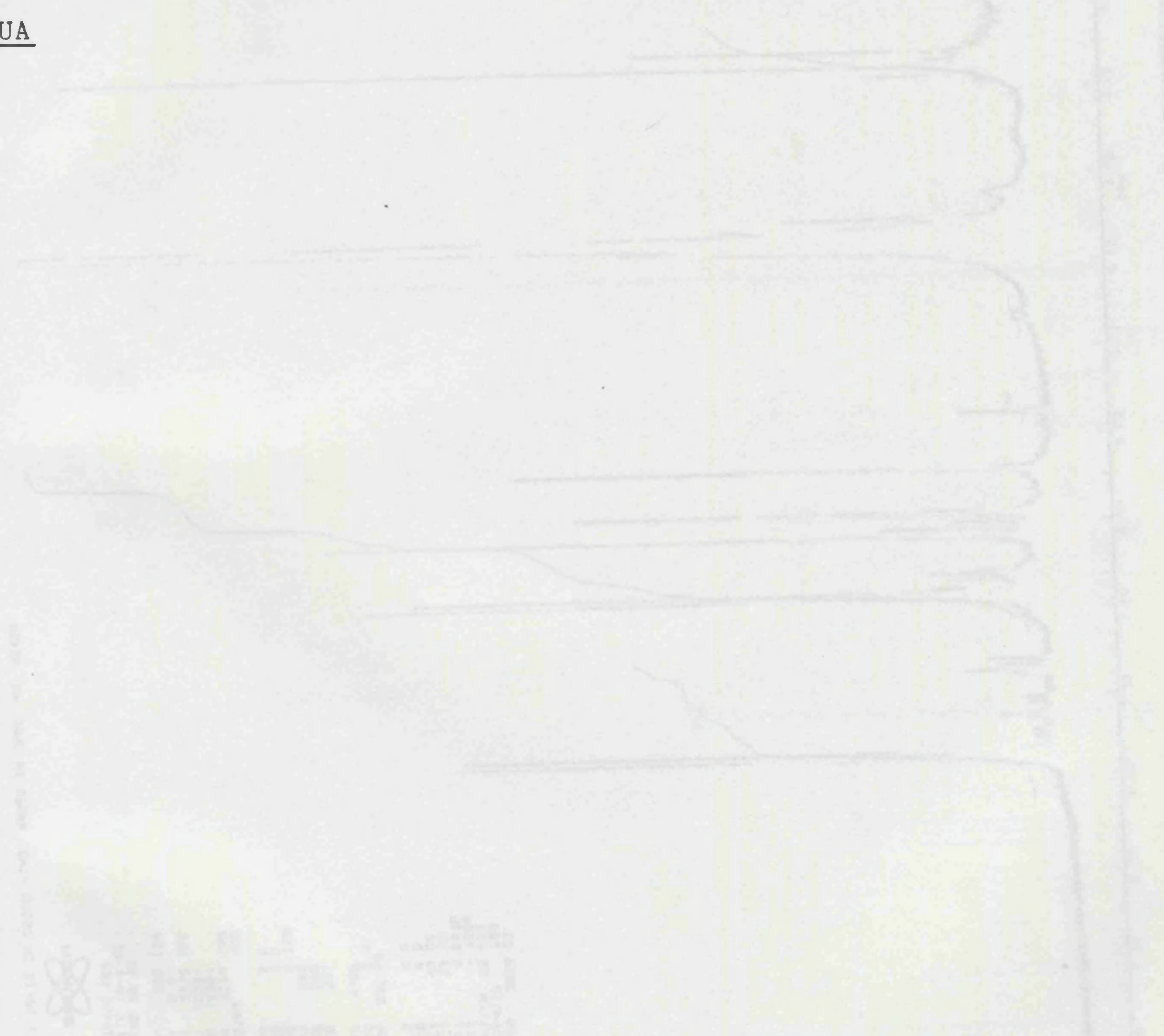
	luteolin	
	luteolin-7-glucoside	
	5,7,3',4'-tetrahydroxy-6,5'-dimethoxyflavone	
A. herba-alba	isovitexin	275
	vicenin-2	
	schaftoside	
	isoschaftoside	
	quercetin-3-glucoside	
	quercetin-3-rutinoside	
	lucenin-2	
	cirsilineol	
A. incanescens	3-methoxyflavone	284
	santin	
	casticin	
	penduletin	
	centaureidin	
	quercetin-3,4'-dimethylether	
	axillarin	
	quercetin-3-methylether	
A. incanescens	isorhmnetin	291
	6-methoxykaempferol	
	kaempferol	
	quercetin	
	kaempferol-3-glucoside	
	isorhamnetin-3-glucoside	
	quercetin-3-glucoside	
	quercetin-3-galactoside	
	kaempferol-3-rutinoside	
	quercetin-3-rutinoside	

A. judicia	apigenin-7-glucoside	275
	apigenin-7-rutinoside	
	apigenin-4'-glucoside	
	apigenin-7-gentiobioside	
	apigenin-7-diglucuroside	
	chryseriol-7-rutinoside	
	chryseriol-7,3-diglucoside	
	luteolin-3'-glucoside	
	luteolin-4'-glucoside	
	luteolin-7-gentiobioside	
	luteolin-7,3'-diglucoside	
	vicenin-2	
	schaftoside	
	isoschaftoside	
	neoschaftoside	
	neoisoschaftoside	
	acacetin	
	pectolinarigenin	
	cirsimaritin	
	jaceosidin	
	eupatilin	
	cirsilineol	
	5,7,3'-trihydroxy-4',5'-dimethoxyflavone	
A. lanata	5-hydroxy-6,7,3',4'-tetramethoxyflavone	285
	artemetin	
	3,5-dihydroxy-7,8,3'4'-tetramethoxyflavone	
A. lanata	5,3'-dihydroxy-3,6,7,4'-tetramethoxyflavone	289

A. ludoviciana ludoviciana	eupatilin	287
	quercetagetin-3,6,3',4'-tetramethyletherflavone	
	5,7-dihydroxy-3,6,8,4'-methoxyflavone	
	luteolin-3,4'-dimethylether	
	jaceosidin	
	5,7,4'-trihydroxy-3,6,-dimethoxyflavone	
	tricin	
	hispidulin	
	chrysoeriol	
	kaempferol-3-methylether	
	apigenin	
	axillarin	
	eupafolin	
	selagin	
	luteolin	
	2'-hydroxy-6-methoxyflavone	
	5,7,3',4'-tetrahydroxy-6,5'-dimethoxyflavone	
A. mesatlantica	4'-methylcirsilineol	290
	cirsilineol	
	cirsimaritin	
	6-methoxytricin	
	tricin	

A. monosperma	vicenin-2	275
	lucenin-2	
	lucenin-7-glucoside	
	acacetin-7-rutinoside	
	acacetin-3-glucoside	
	quercetin-3-rutinoside	
	qatuletin-3-rutinoside	
	quercetin-5-glucoside	
	rhamnetin-5-glucoside	
A. monosperma	cirsiliol	277
	5,7,3',4'-tetrahydroxyl-3,5'-dimethoxylflavone	
A. monosperma	5,4'-dihydroxy-6,7-dimethoxy flavone	283
	5,7,4'-trihydroxy-6,3',5'-trimethoxyflavone	
	5-hydroxy-6,7,3',4'-tetramethoxyflavone	
	5,4'-dihydroxy-6,7,3'-trimethoxyflavone	
A. taurica	quercetin-3,6-dimethylether -7-glucoside	280
A. transiliensis	quercetin-3-methylether	274
	quercetin-3-methylether-7-glucoside	
A. tridentata	eupafolin	279
	penduletin	278
	axillarin	
	chrysosplenol-D	

APPENDIX 3 NMR AND MASS SPECTRA OF COMPOUNDS ISOLATED FROM <u>A. ANNUA</u>



A.3.15-12 IN CD3OD -TMS: WM250 1H SPEC. NO. 32420

H32420.001
DATE 19-3-90
TIME 16:23

SF 250.134
SY 0.0
O1 5375.000
SI 16384
TD 16384
SW 3759.398
HZ/PT .459

PW 2.0
RD 0.0
AQ 2.179
RG 200
NS 208
TE 297

FW 4700
O2 0.0
DP 63L PO

LB 0.0
GB 0.0
CX 40.00
CY 23.00
F1 9.601P
F2 -.398P
HZ/CM 62.527
PPM/CM .250
SR 3839.03

FIGURE 24. ^{1}H NMR SPECTRUM OF APIGENIN

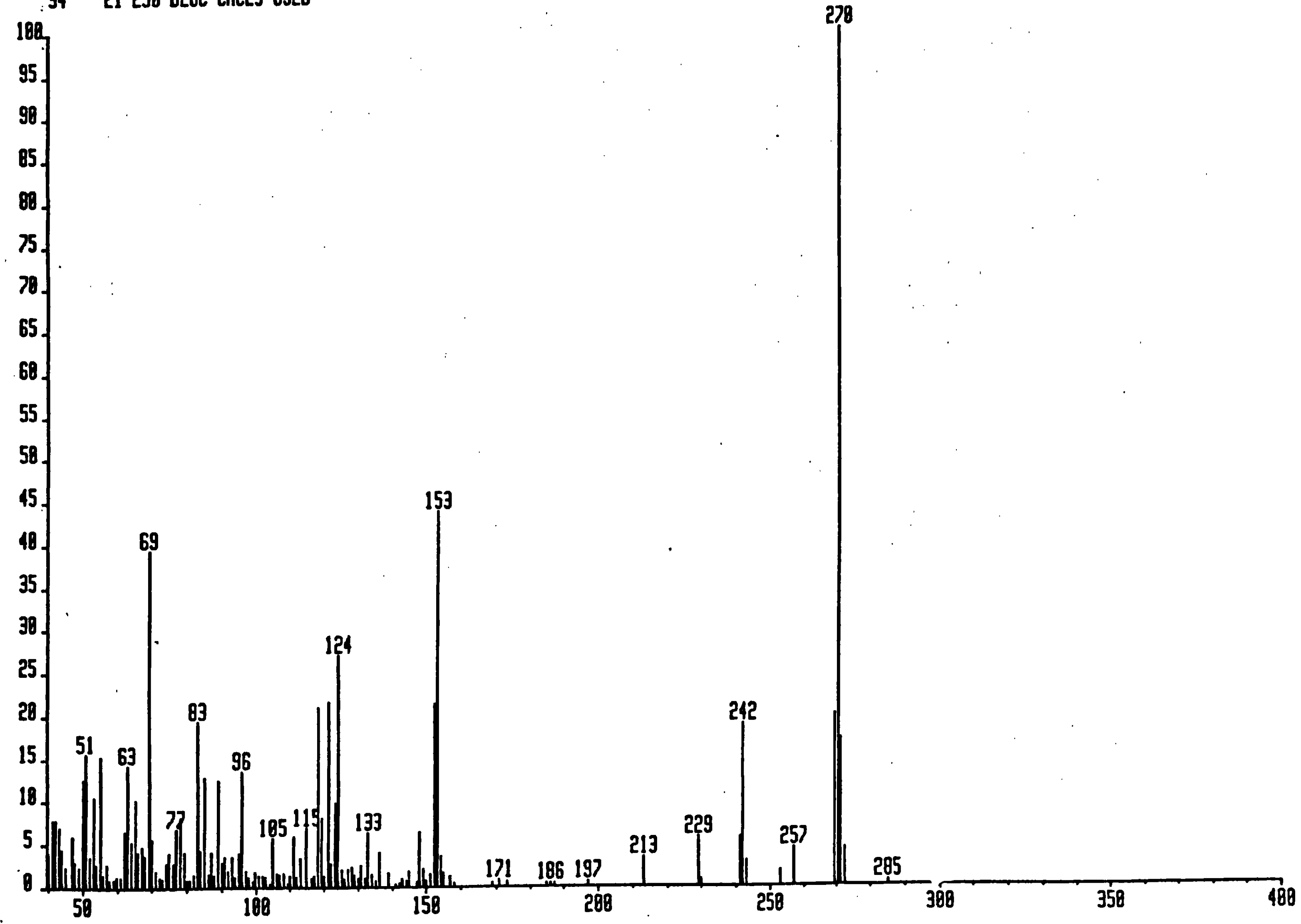


FIGURE 25. MS SPECTRUM OF APIGENIN

FIGURE 26 NMR SPECTRUM OF AXILLARIN

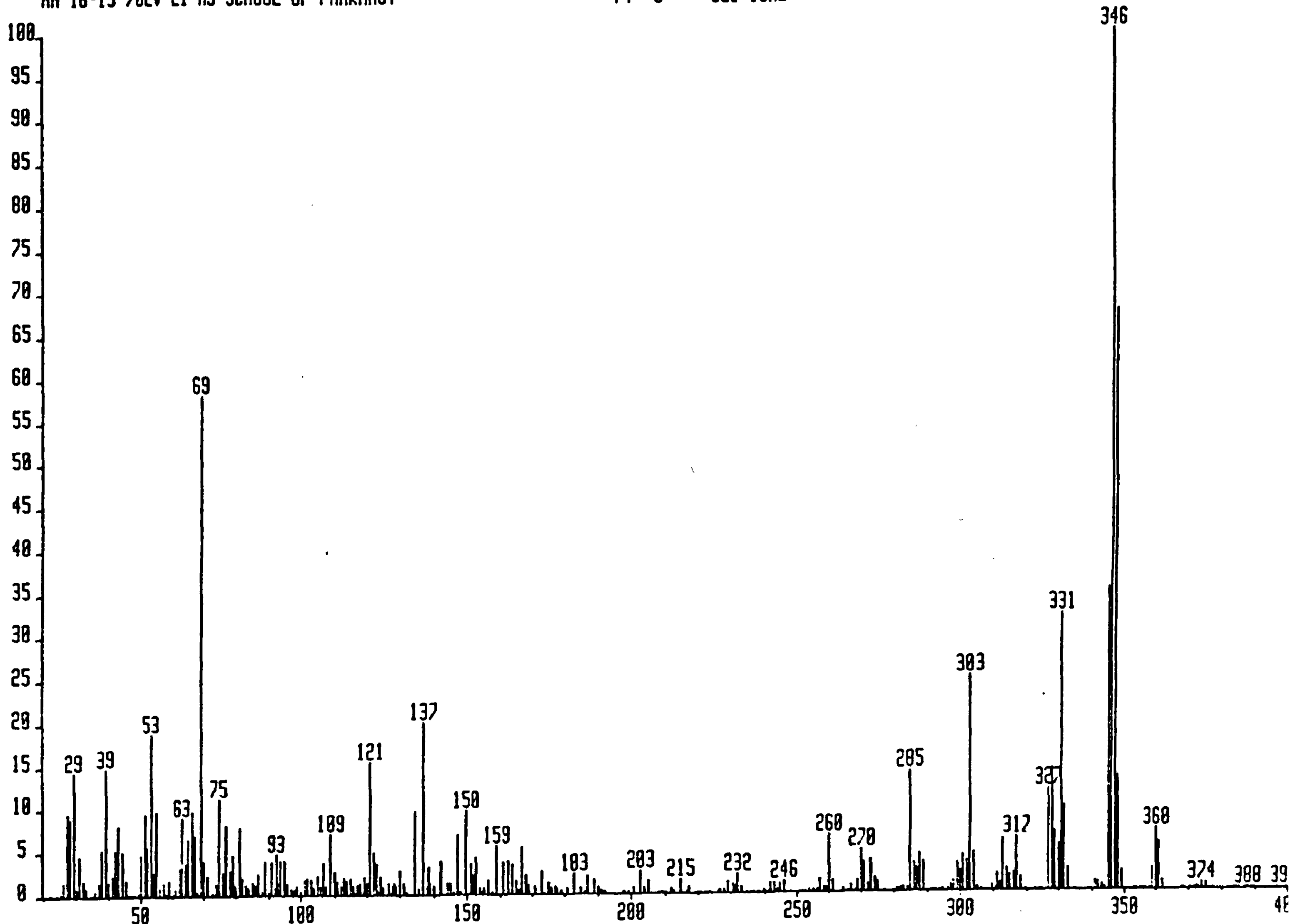


FIGURE 27 MS SPECTRUM OF AXILLARIN

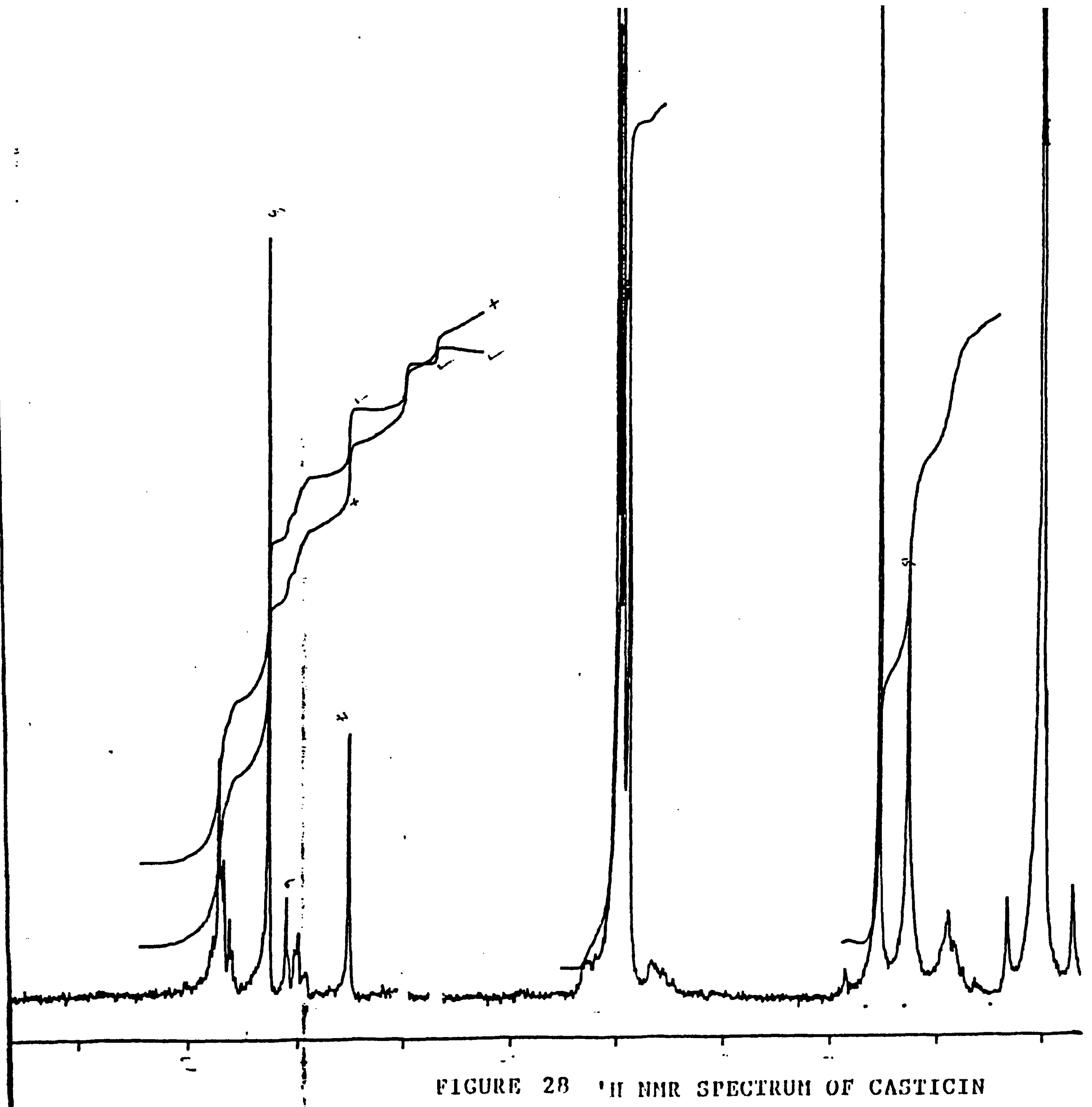


FIGURE 28 ¹H NMR SPECTRUM OF CASTICIN

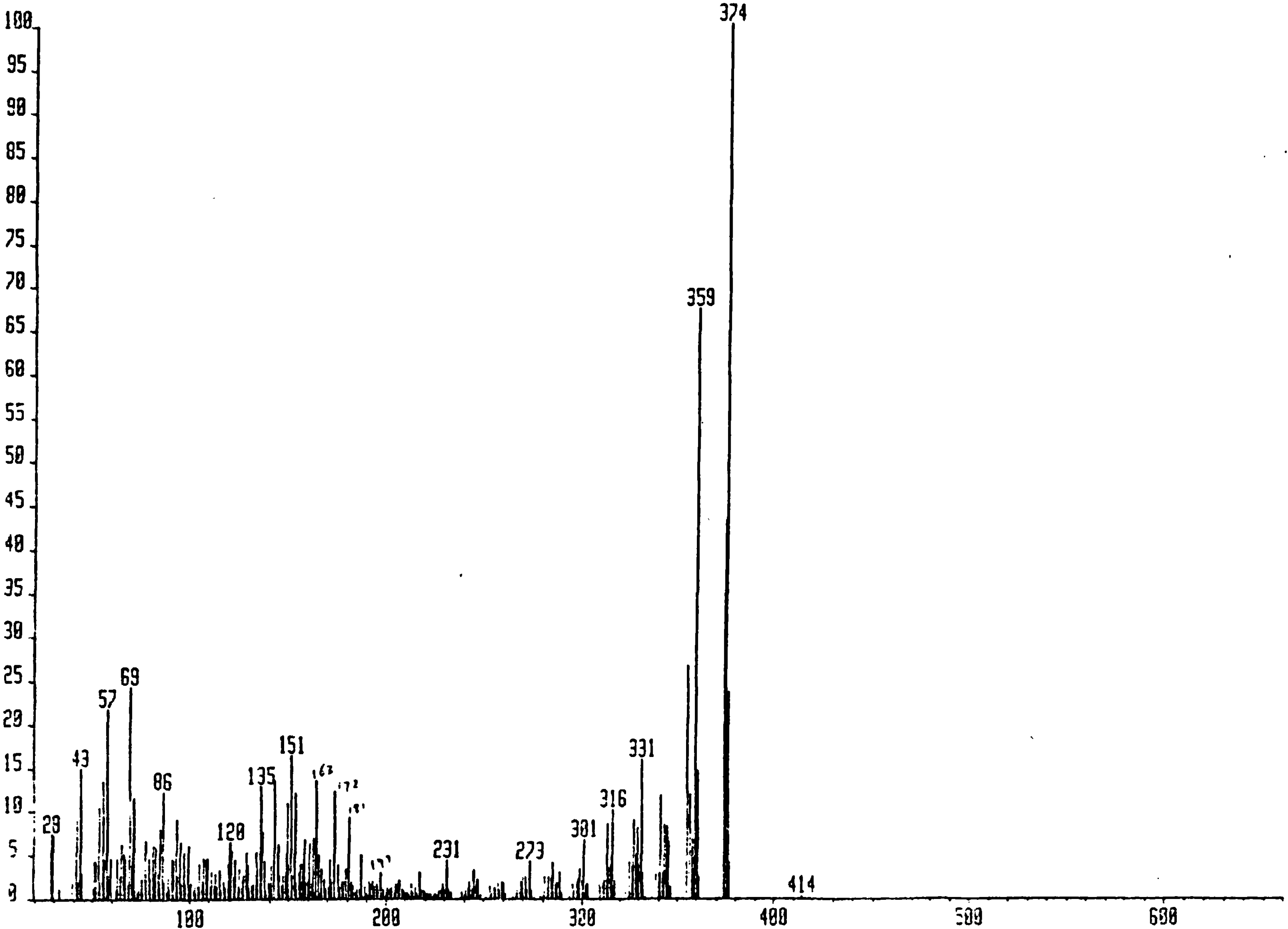


FIGURE 29 MS SPECTRUM OF CASTICIN

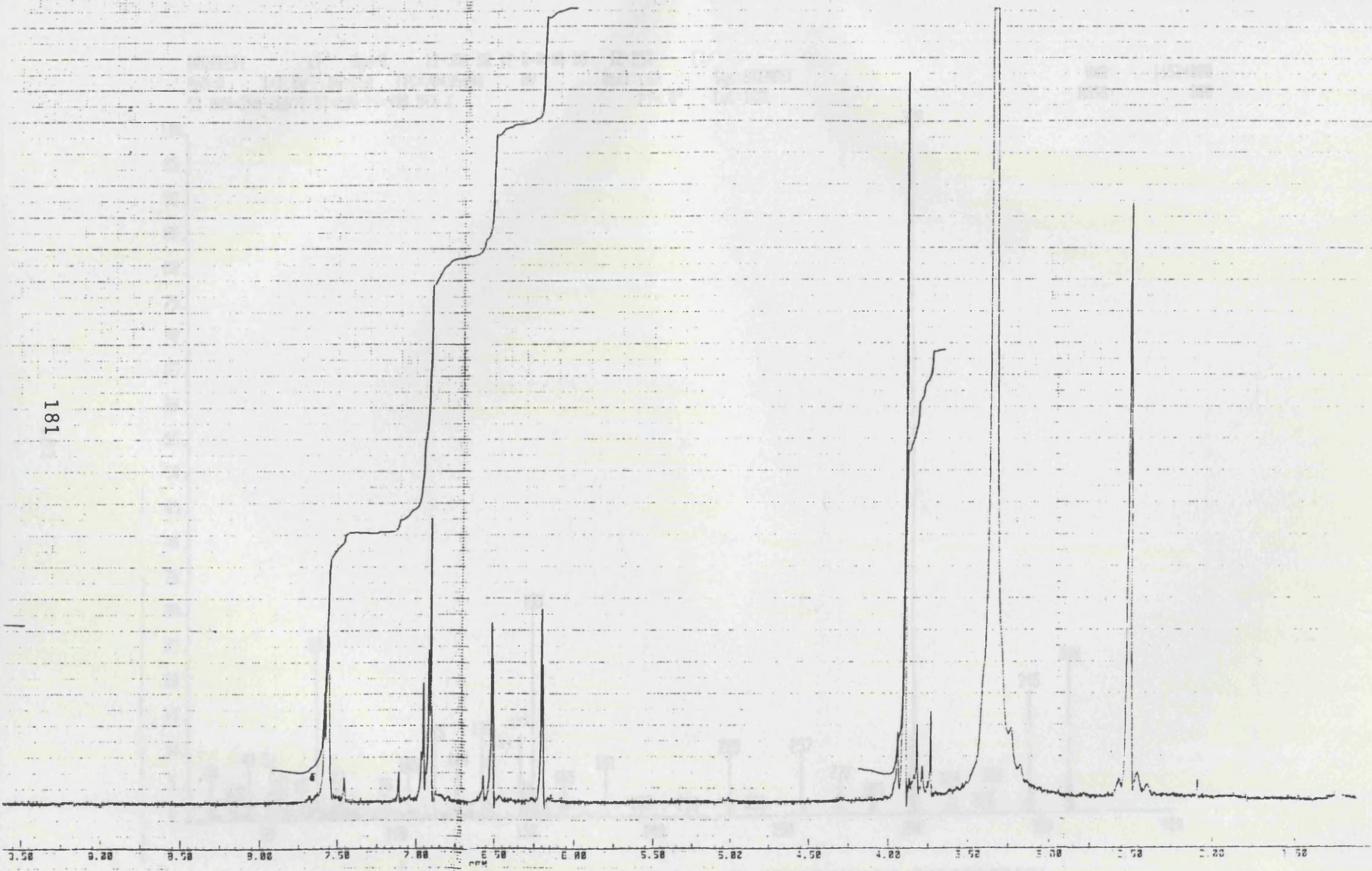


FIGURE 30 'H NMR SPECTRUM OF CHRYSOERIOL

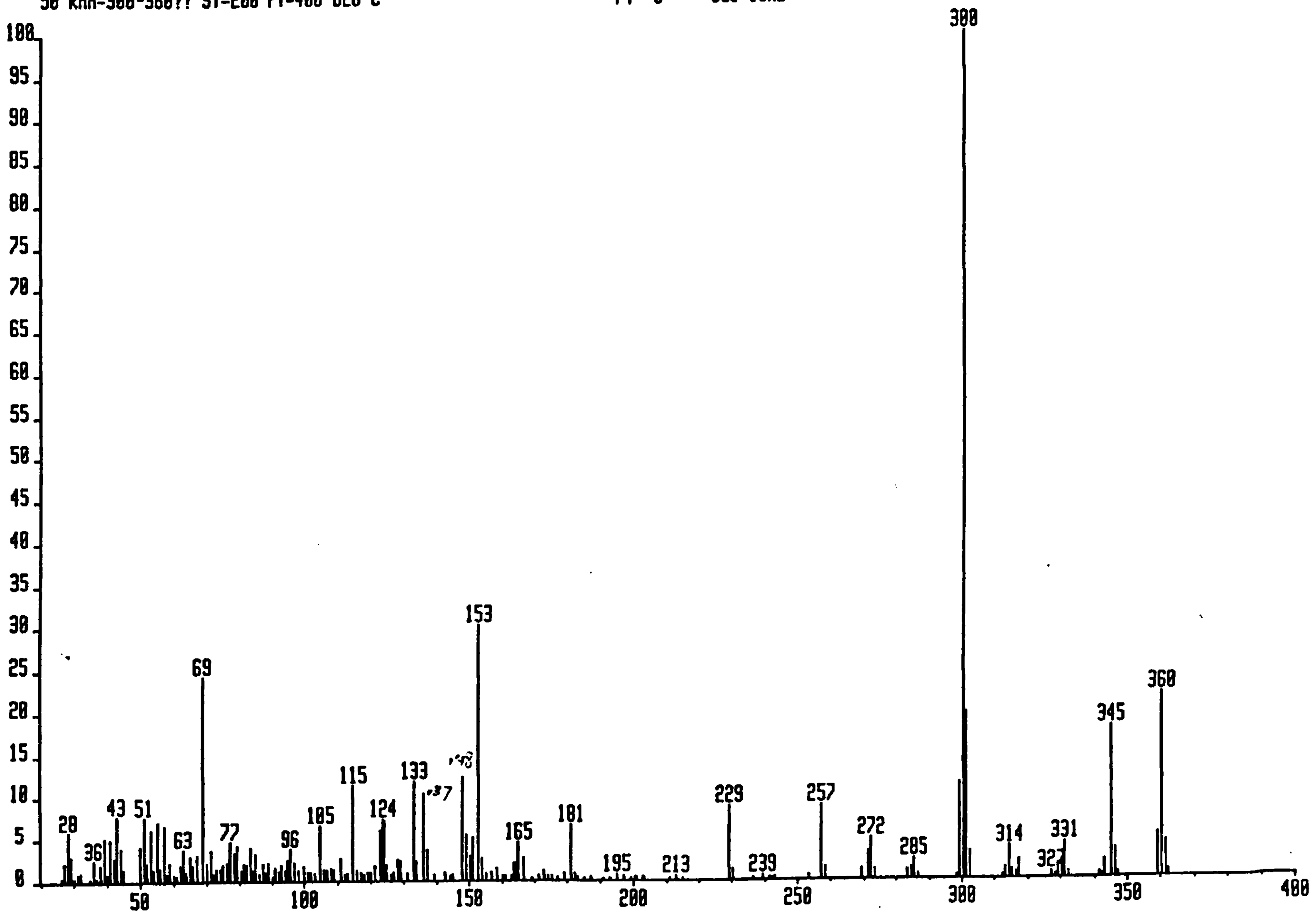


FIGURE 31. MS SPECTRUM OF CHRYSOERIOL

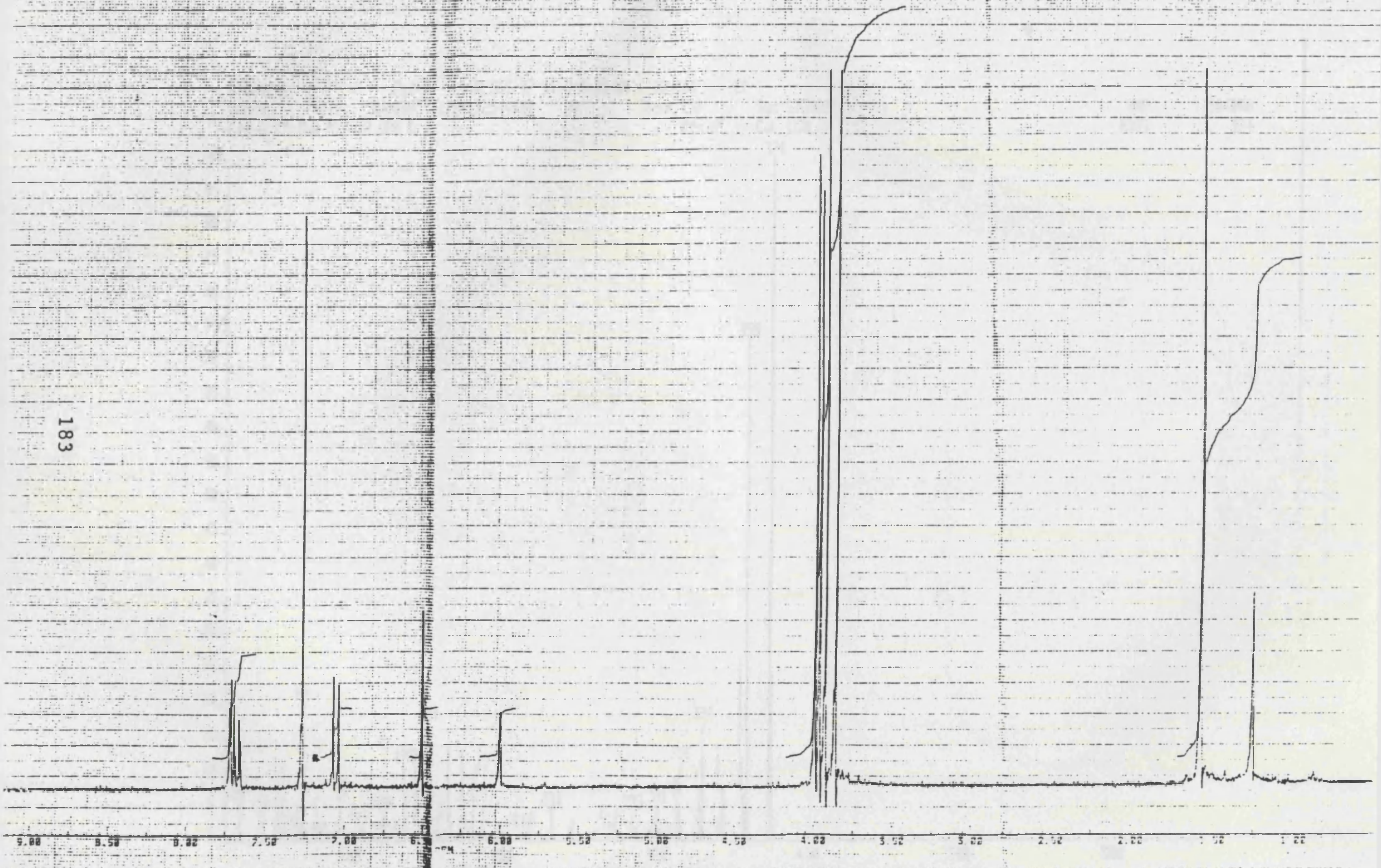


FIGURE 32 NMR SPECTRUM OF CHRYSOPLENETIN

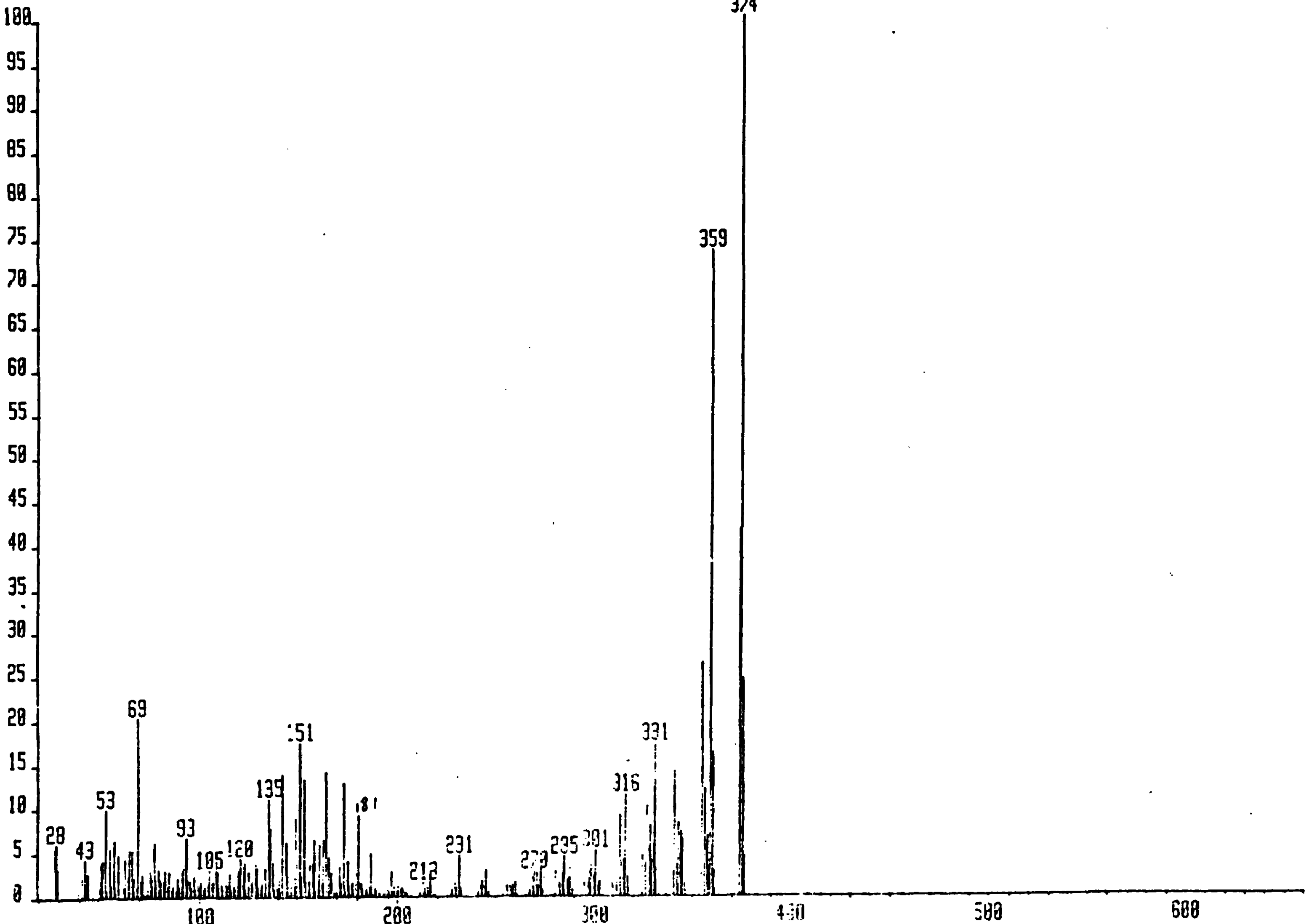


FIGURE 33 MS SPECTRUM OF CHRYSOPLENETIN

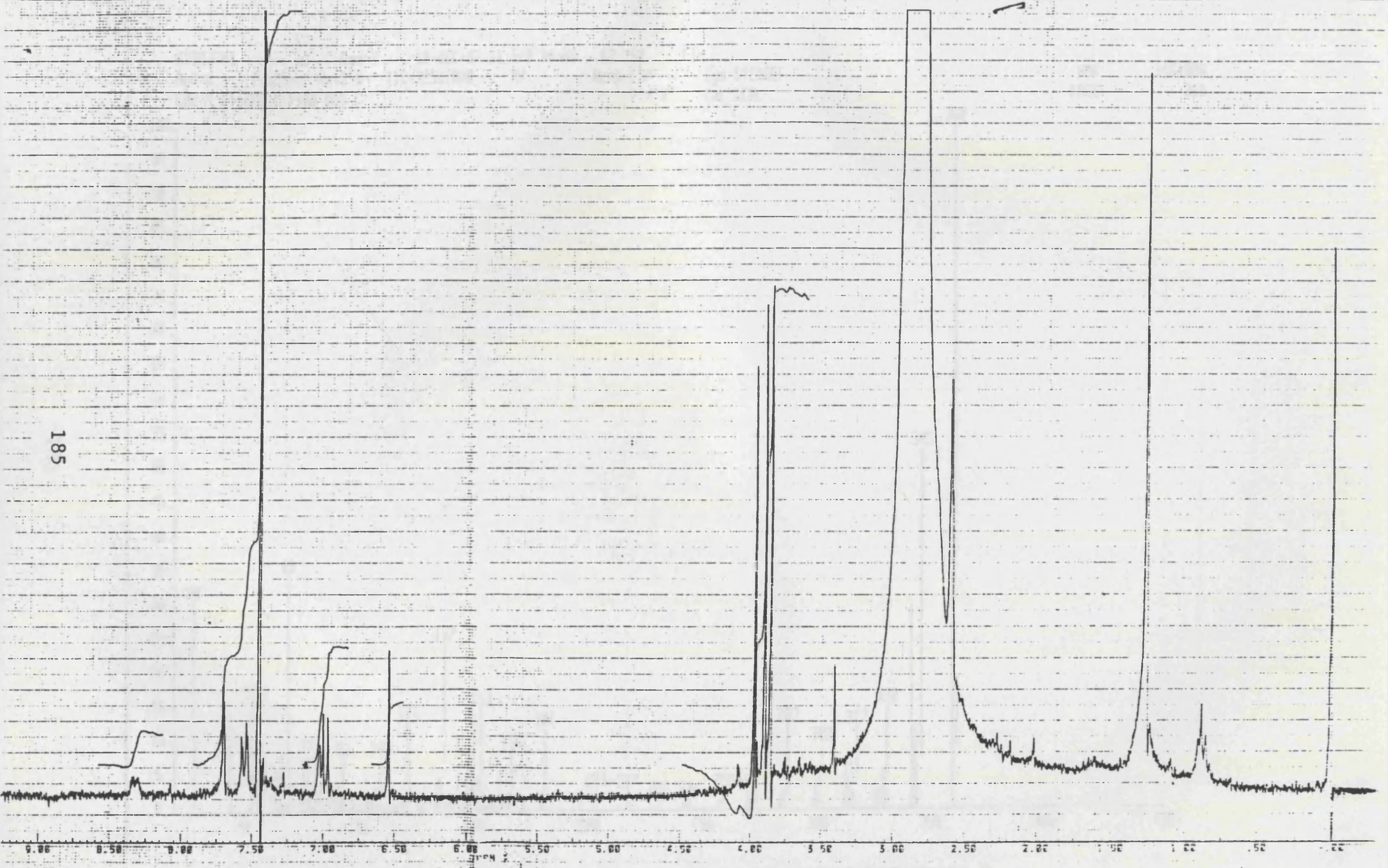


FIGURE 34 NMR SPECTRUM OF CHRYSOSPLENOL-D

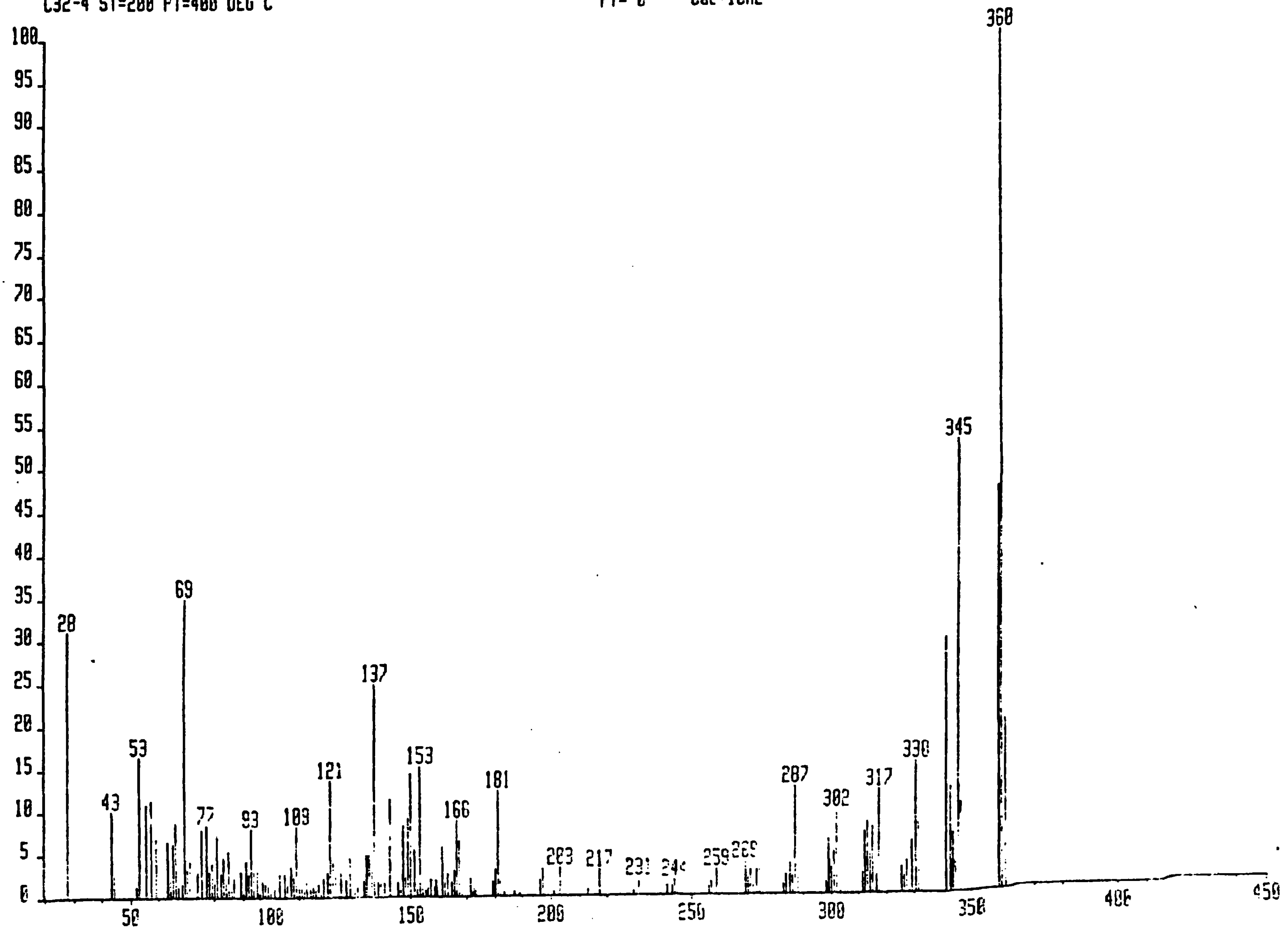


FIGURE 35 MS SPECTRUM OF CHRYSOSPLENOL-D

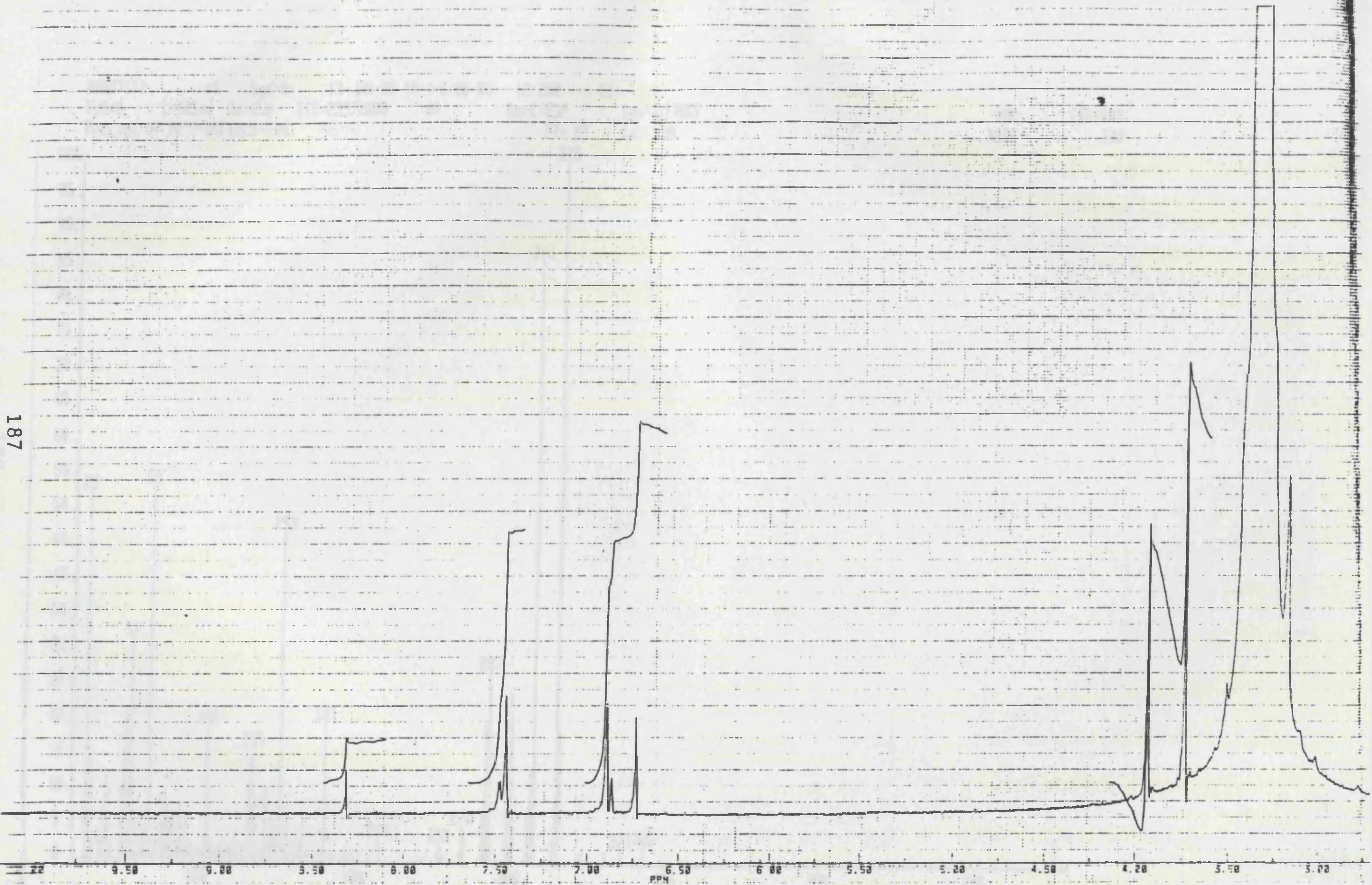


FIGURE 36. ^{1}H NMR SPECTRUM OF CIRSILIOL

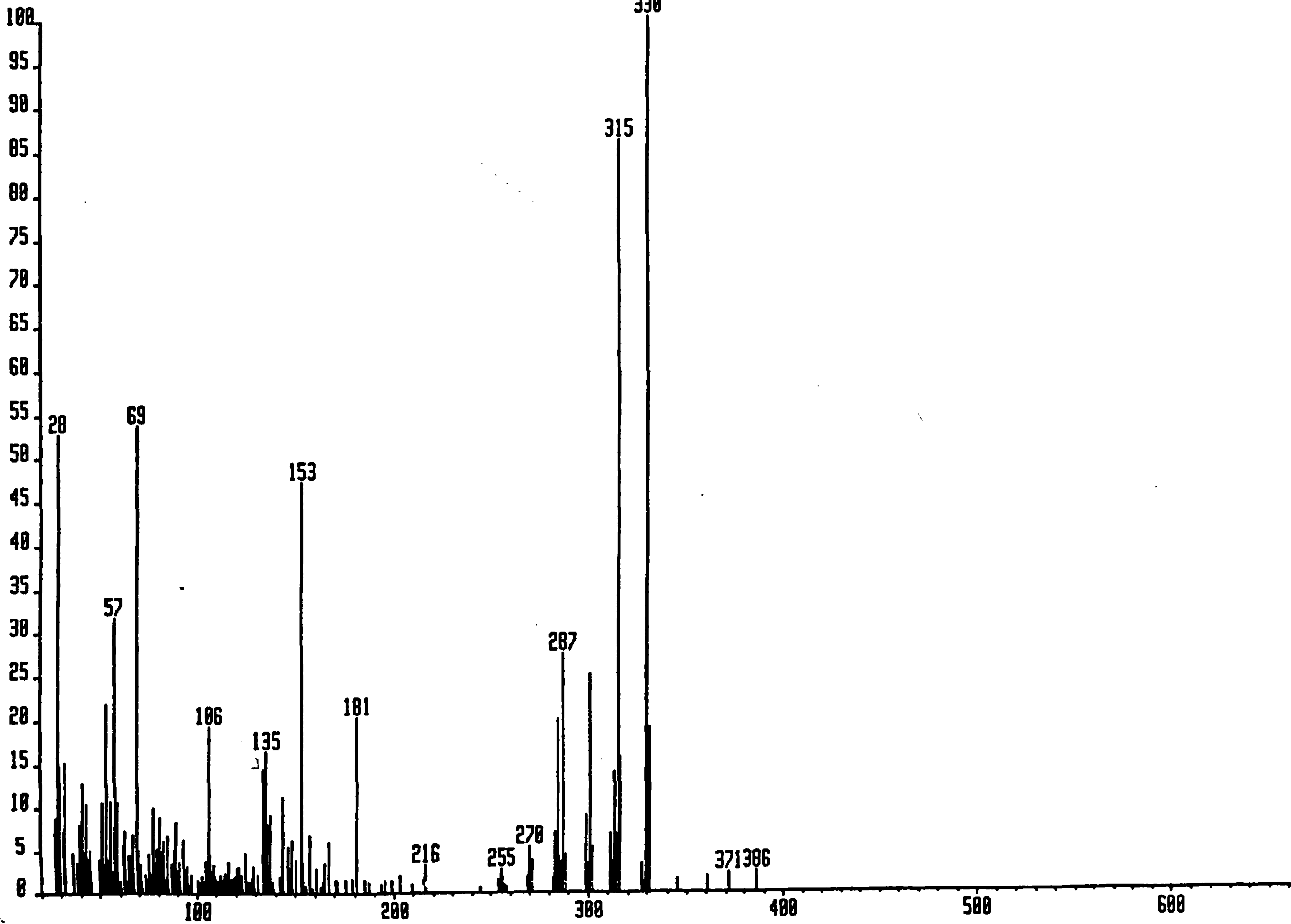


FIGURE 37 MS SPECTRUM OF CIRSILIOL

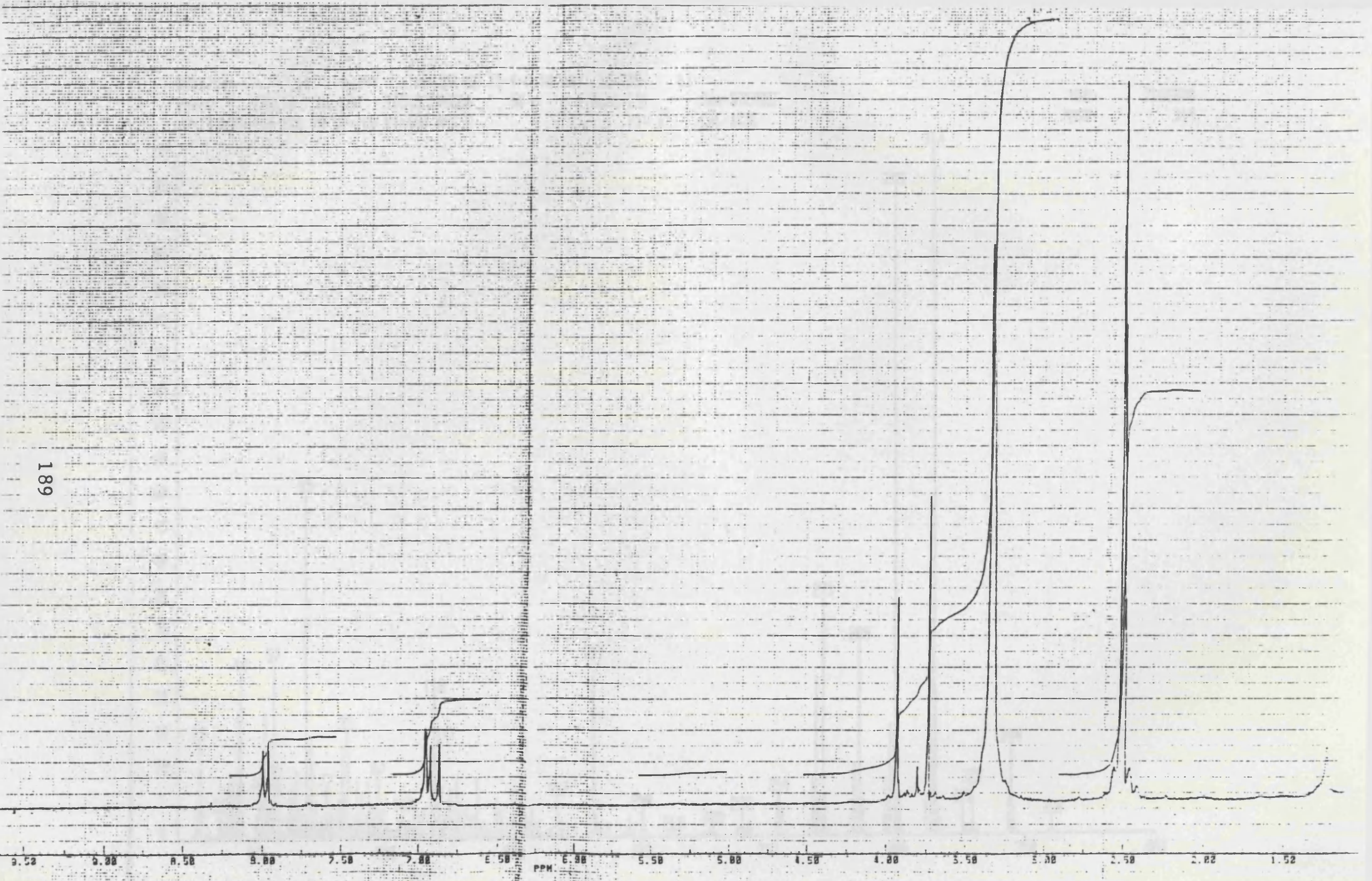


FIGURE 38 'H NMR SPECTRUM OF CIRSIMARITIN

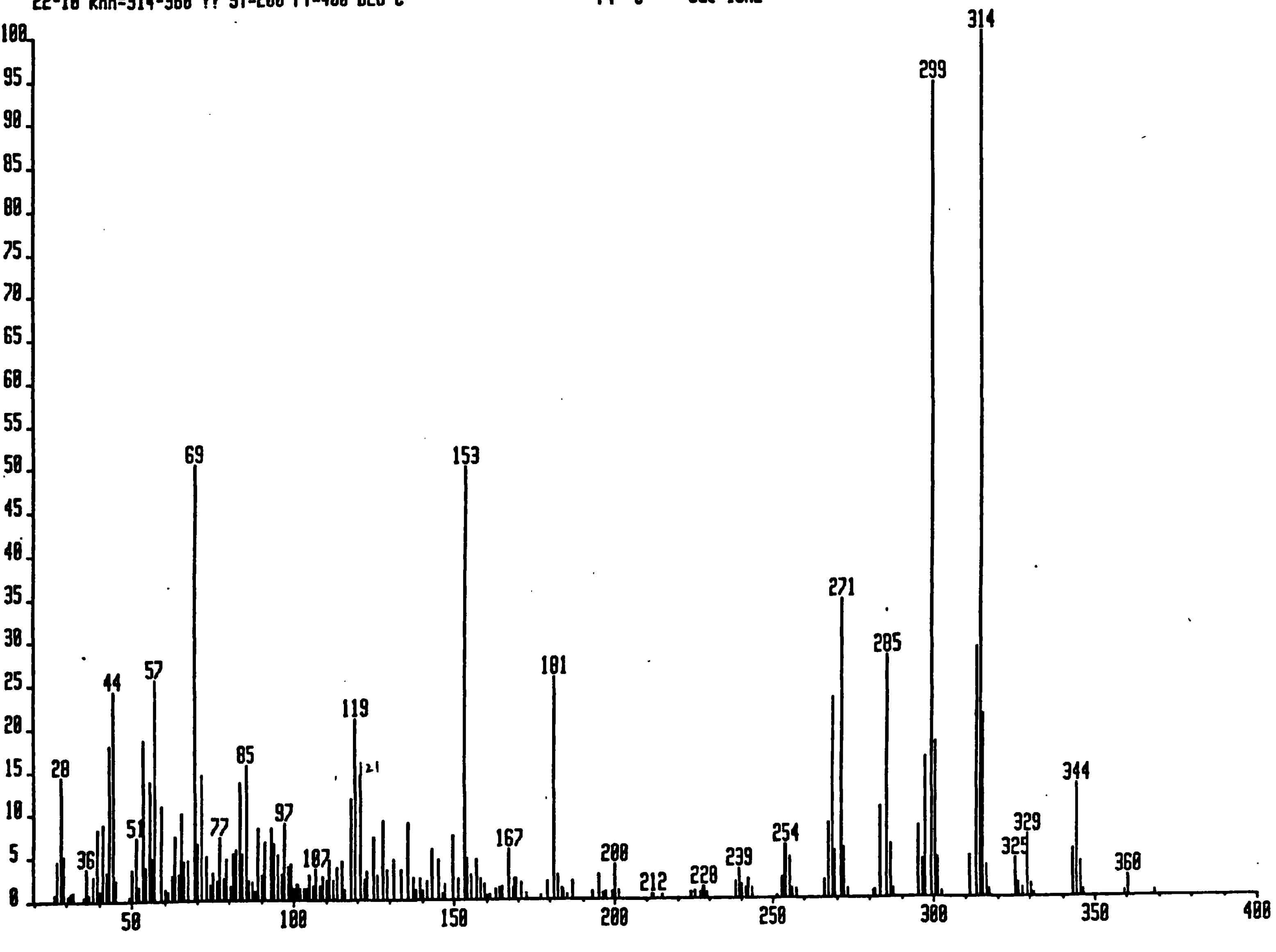


FIGURE 39 MS SPECTRUM OF CIRSIMARITIN

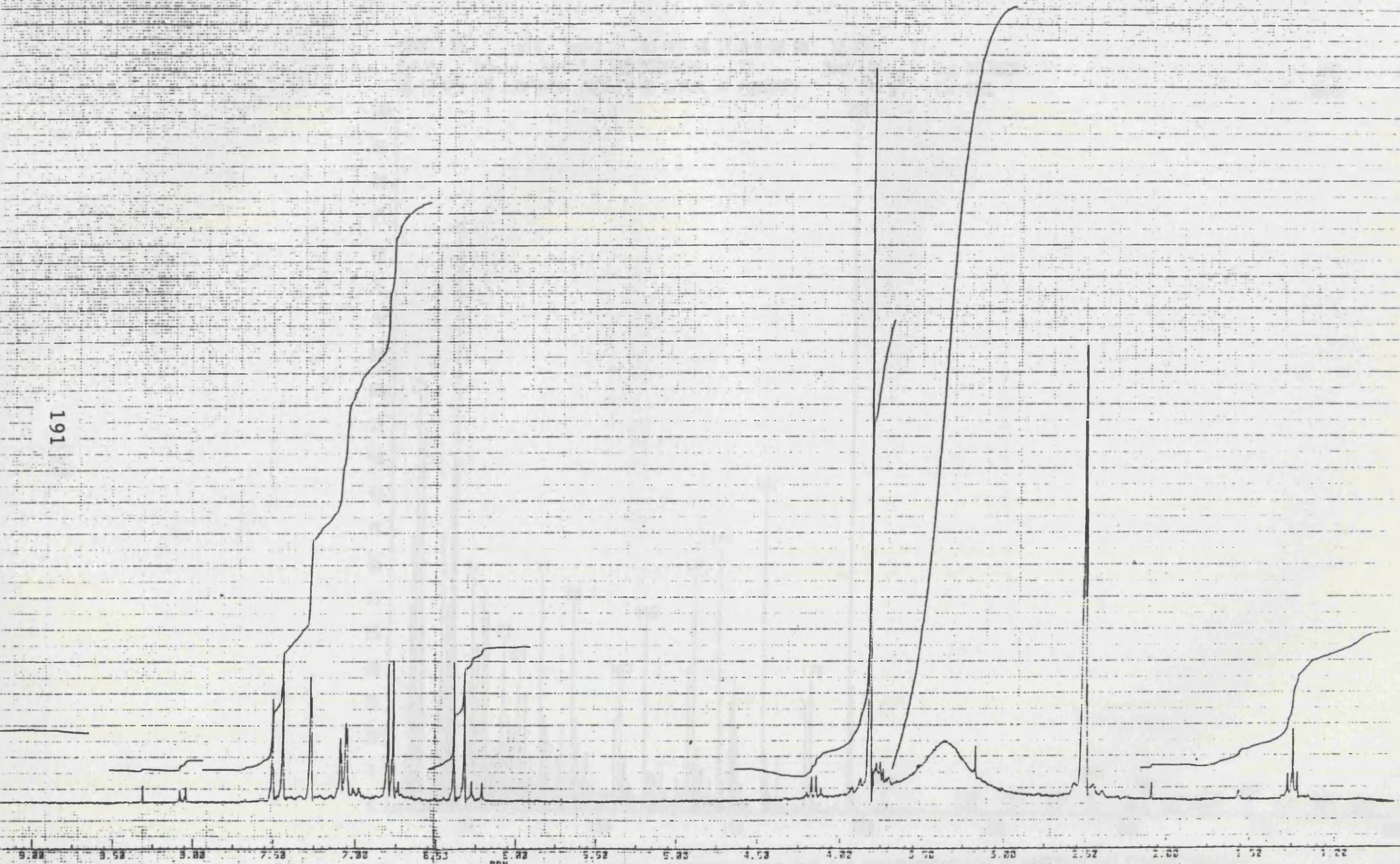


FIGURE 40 NMR SPECTRUM OF 2,2-DIHYDROXY-6-METHOYLCHROMENE

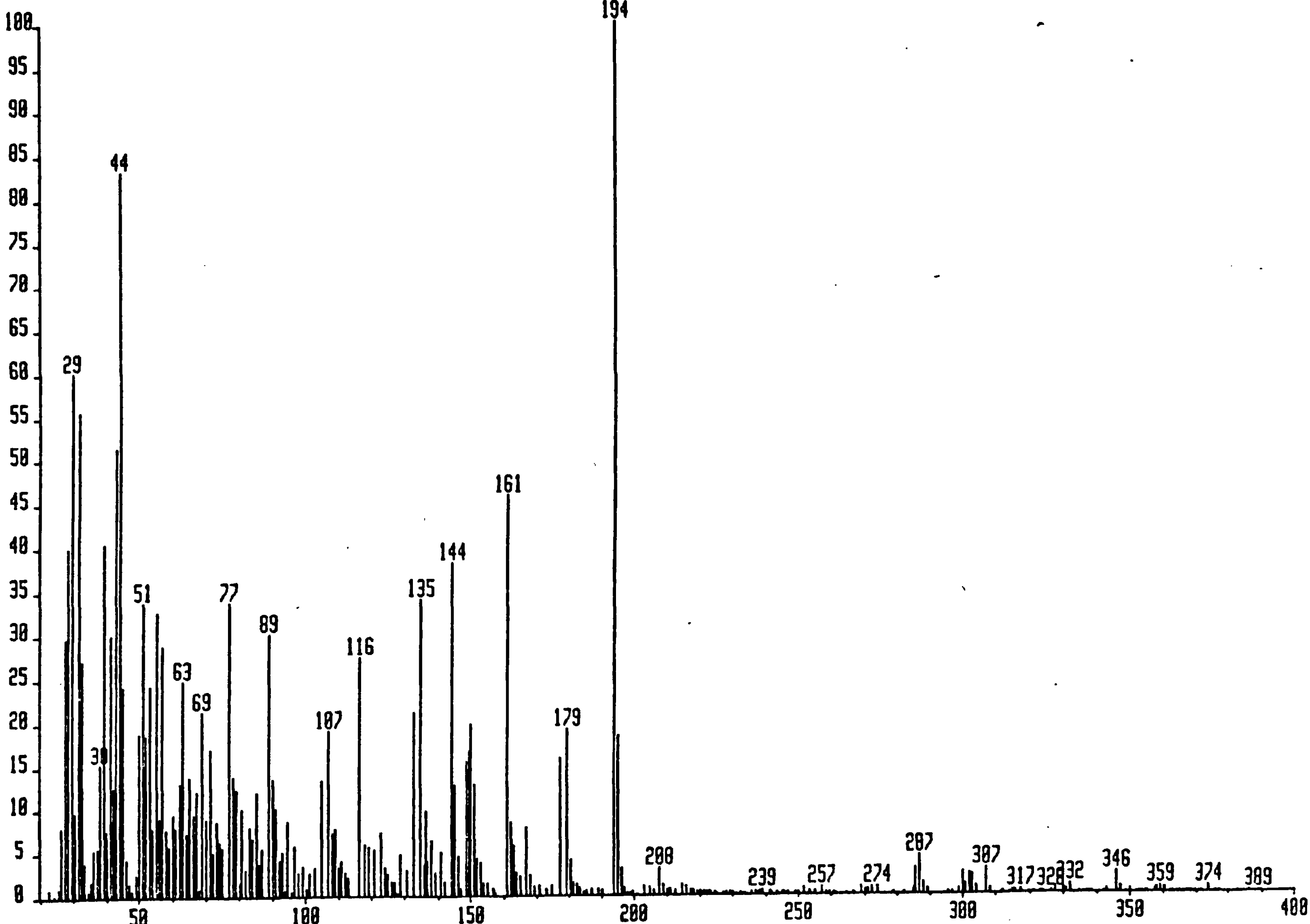


FIGURE 41 MS SPECTRUM OF 2,2-DIHYDROXY-6-METHOXYLCHROMENE

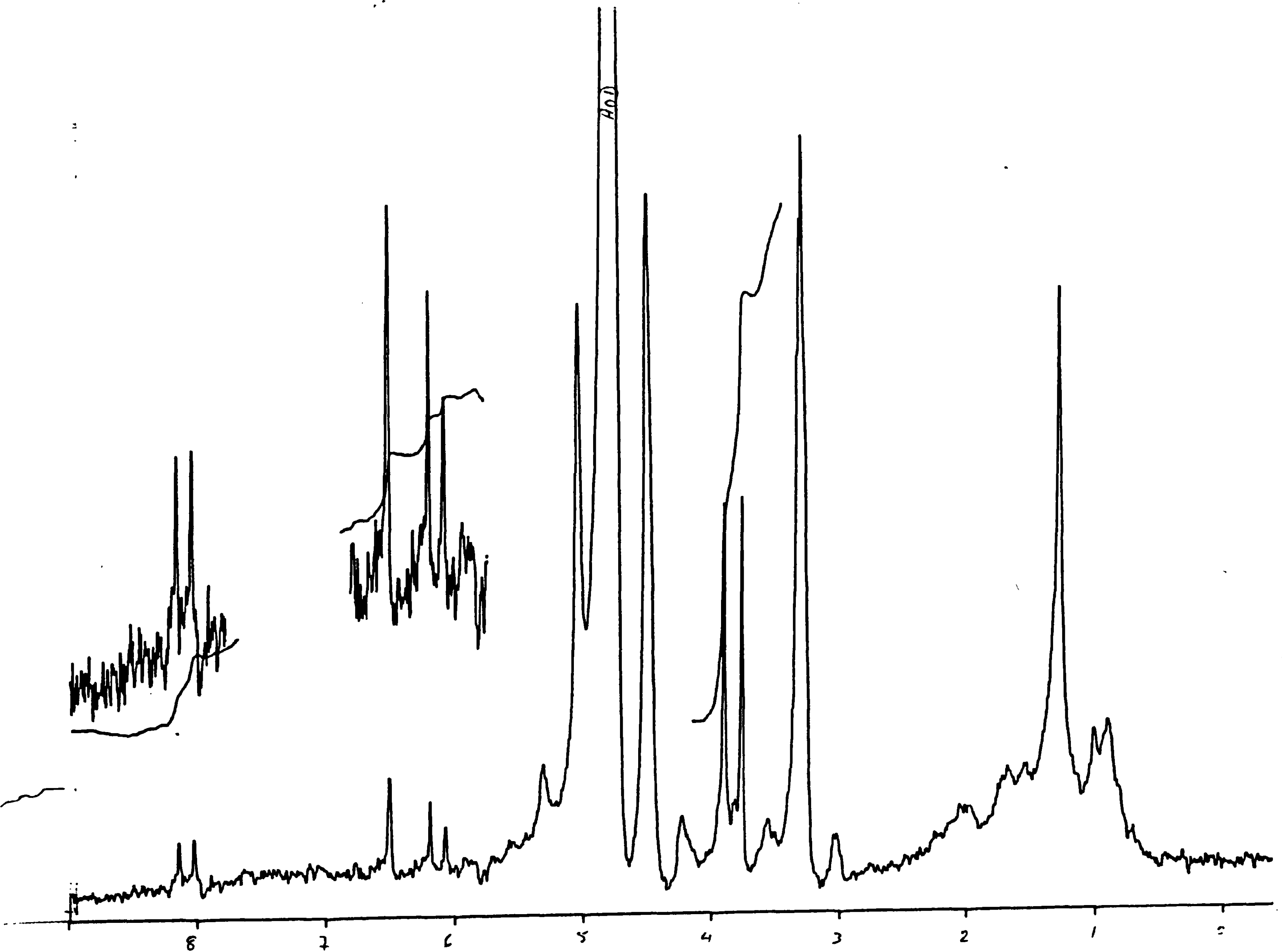


FIGURE 42 NMR SPECTRUM OF 5,6-DIMETHOXYL-7-HYDROXYLCOUMARIN

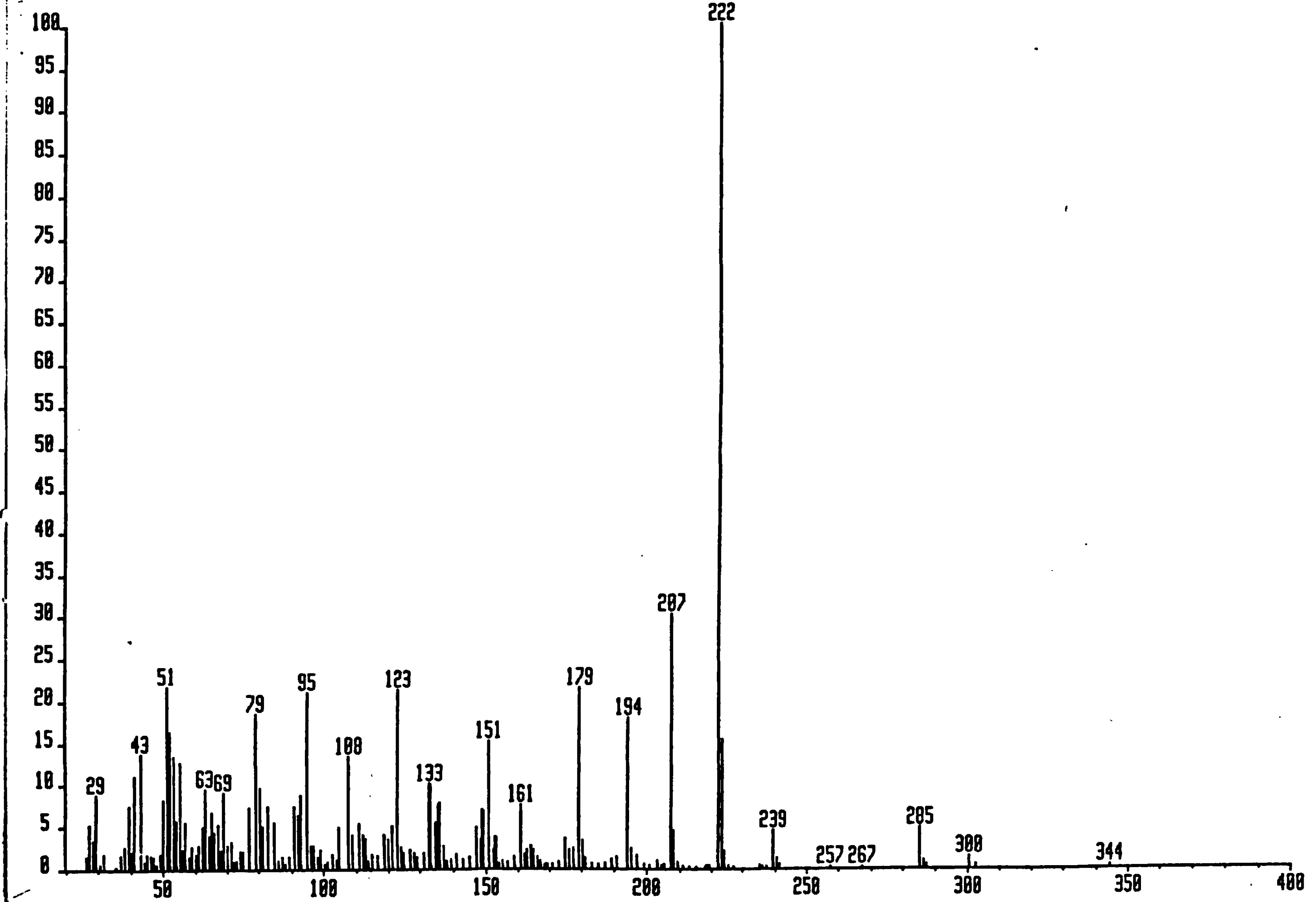


FIGURE 43 MS SPECTRUM OF 5,6-DIMETHOXYL-7-HYDROXYLCOUMARIN

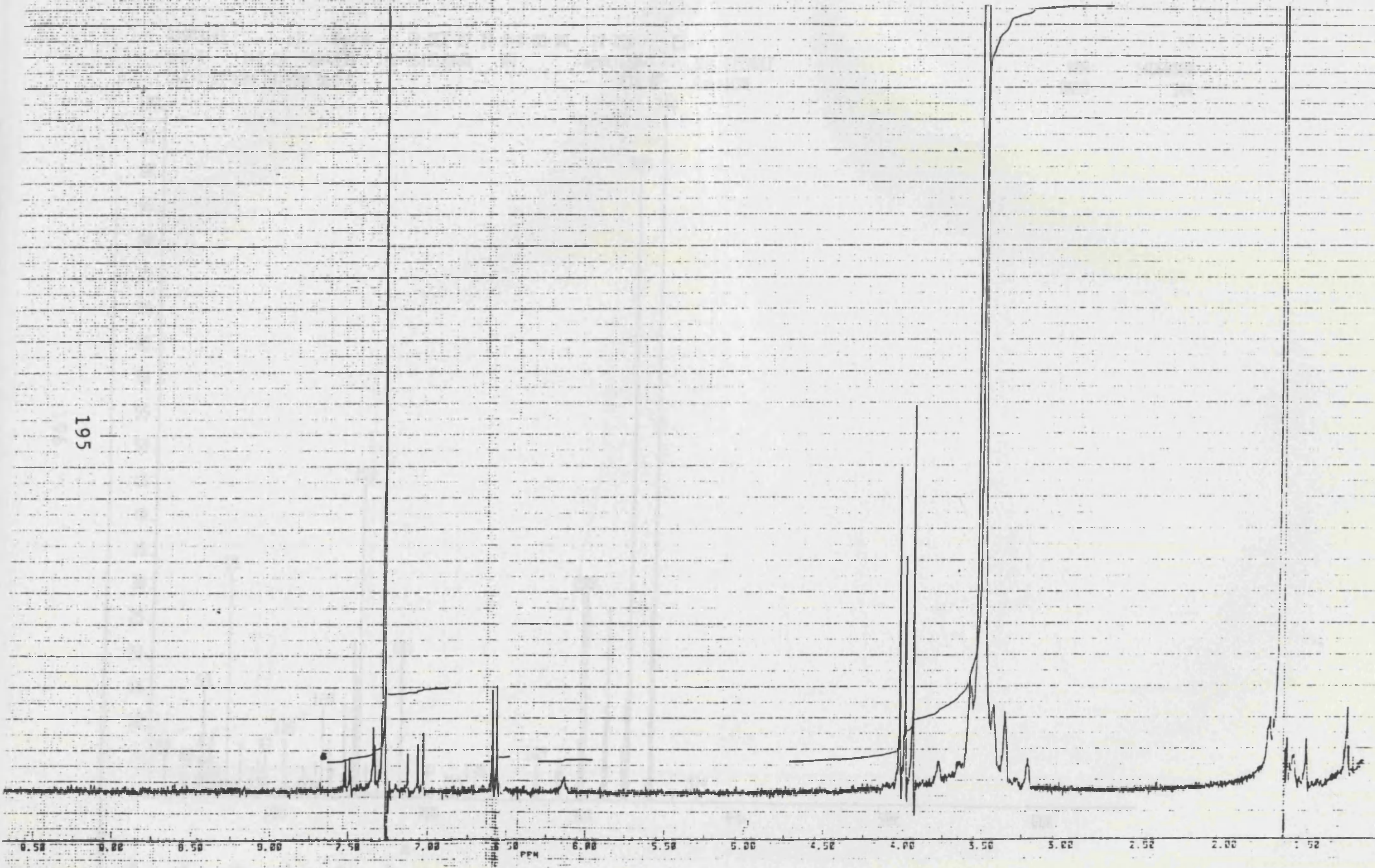


FIGURE 44. NMR SPECTRUM OF EUPATORIN

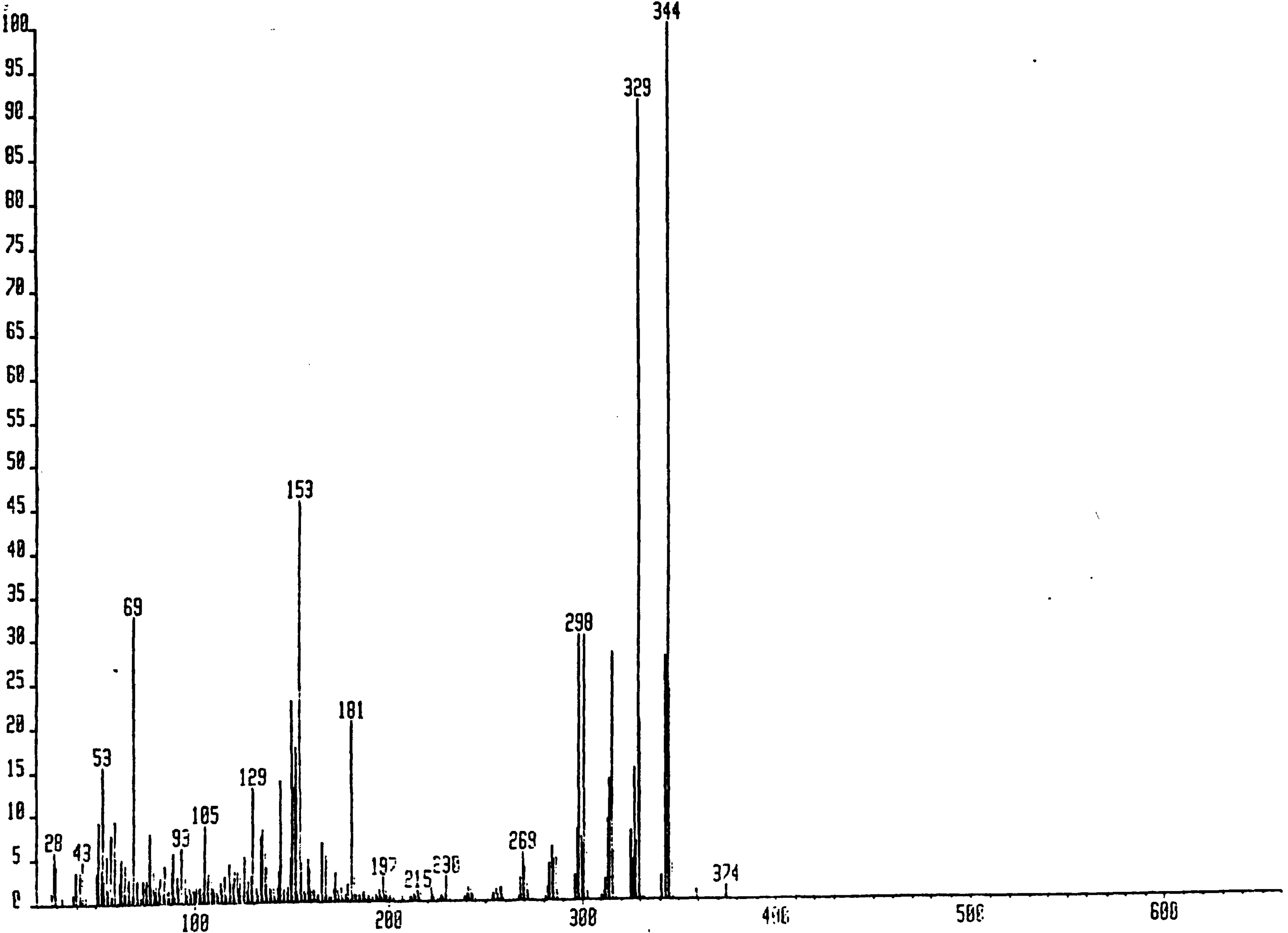


FIGURE 45 MS SPECTRUM OF EUPATORIN

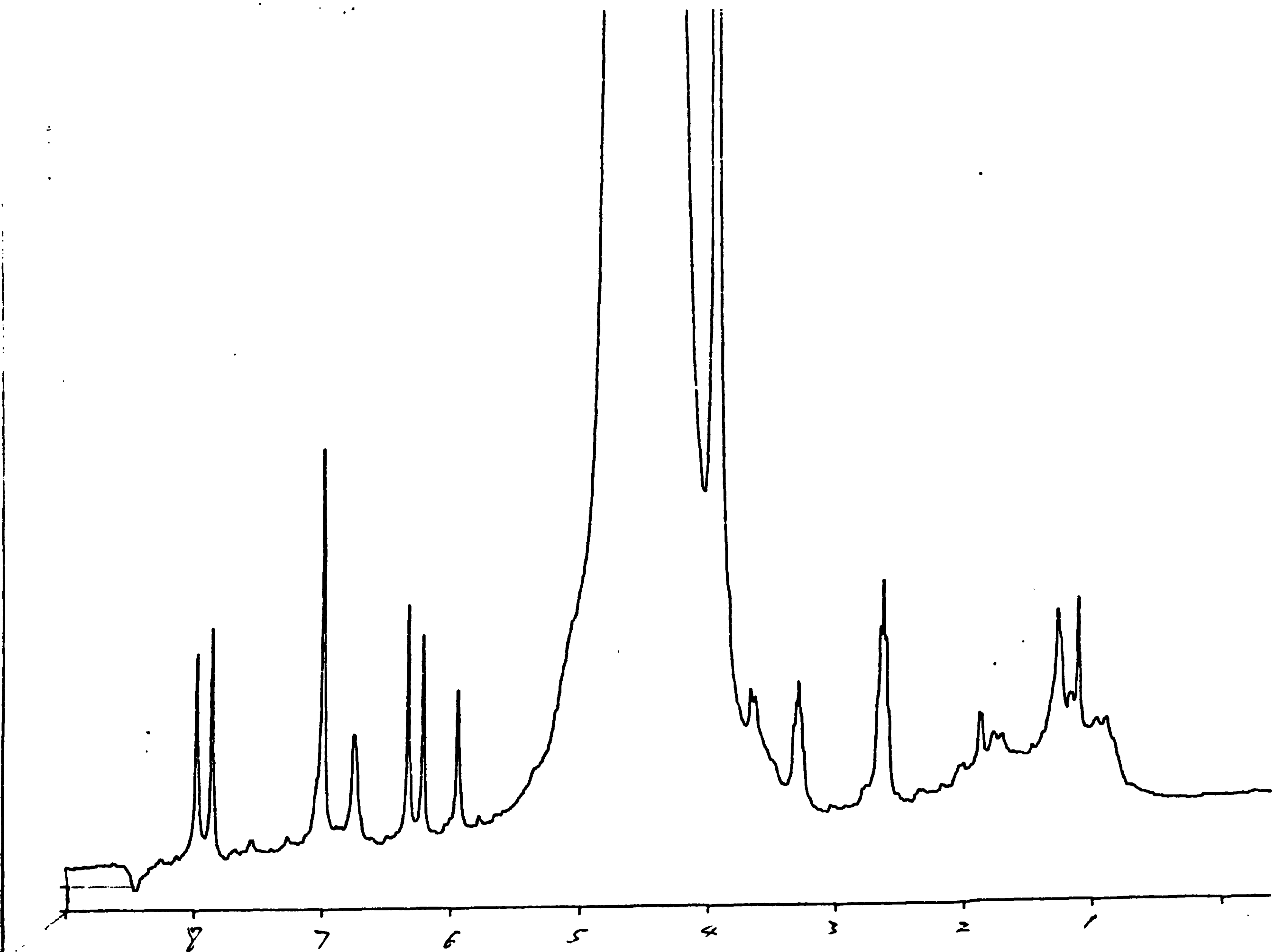


FIGURE 46. NMR SPECTRUM OF FRAXETIN-8-METHYLETHER

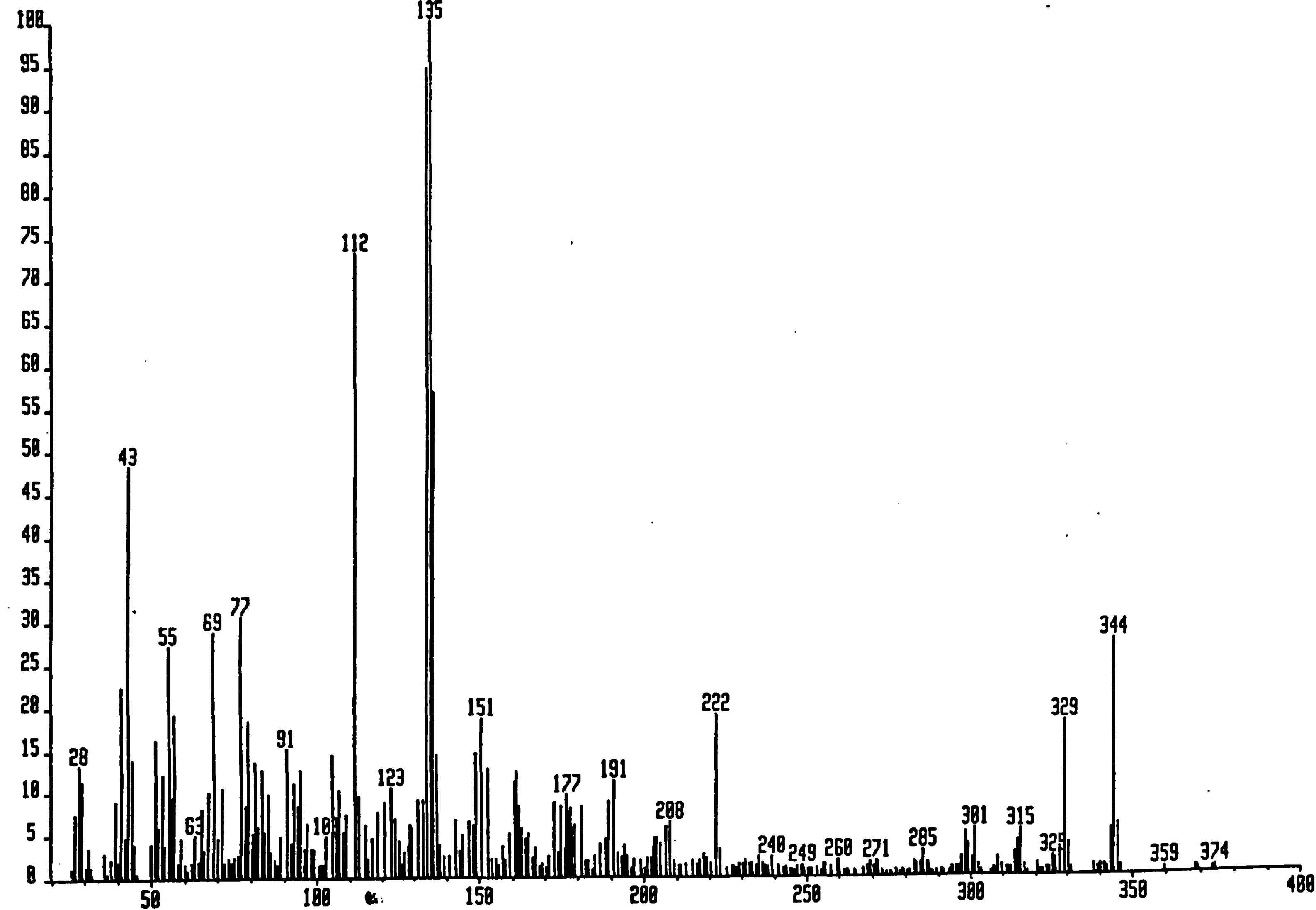


FIGURE 47 MS SPECTRUM OF FRAXETIN-8-METHYLETHER

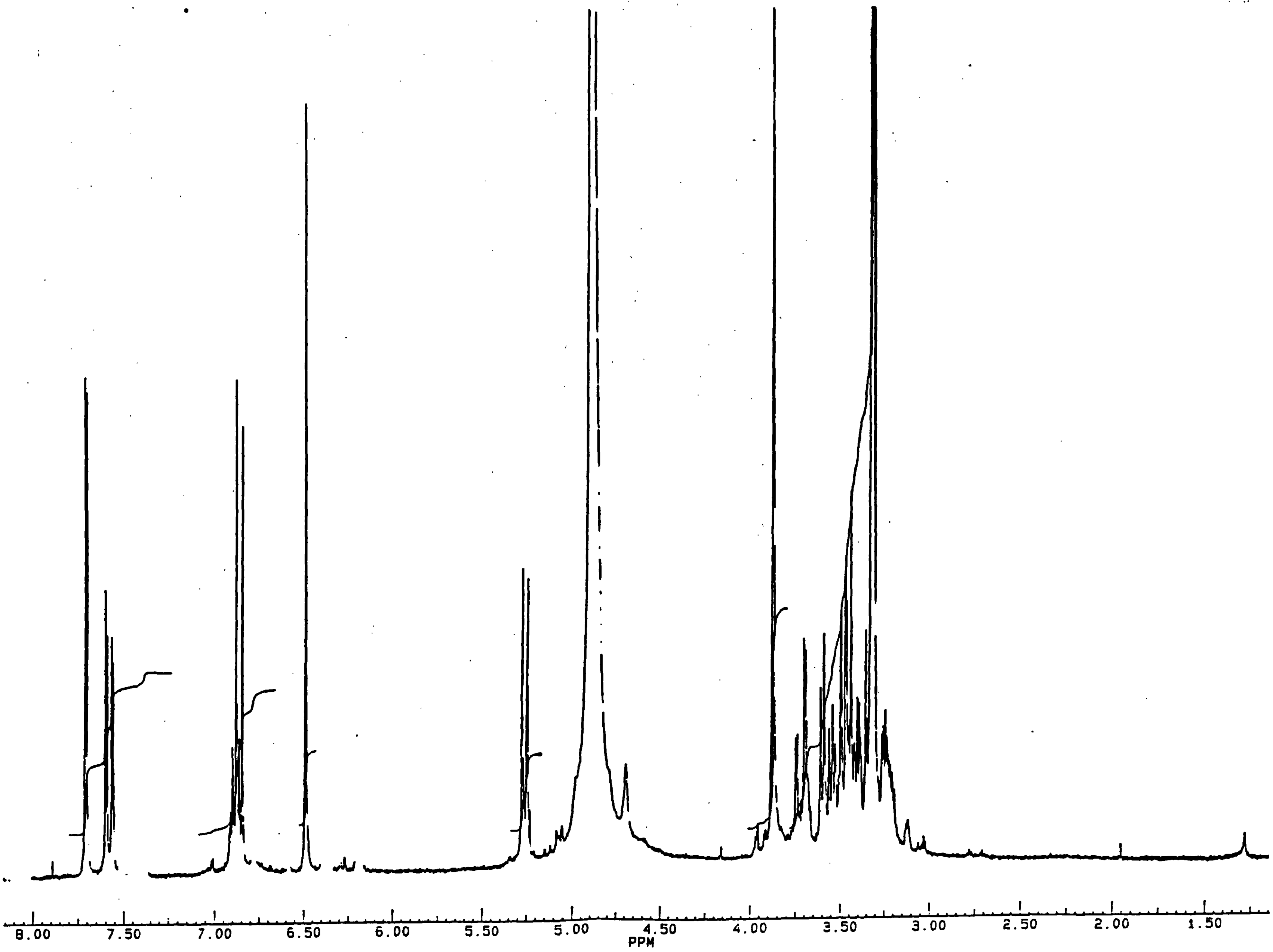


FIGURE 48. ^{1}H NMR SPECTRUM OF GOSSYPETIN-3-METHYLETHER-3'-GLUCOSIDE

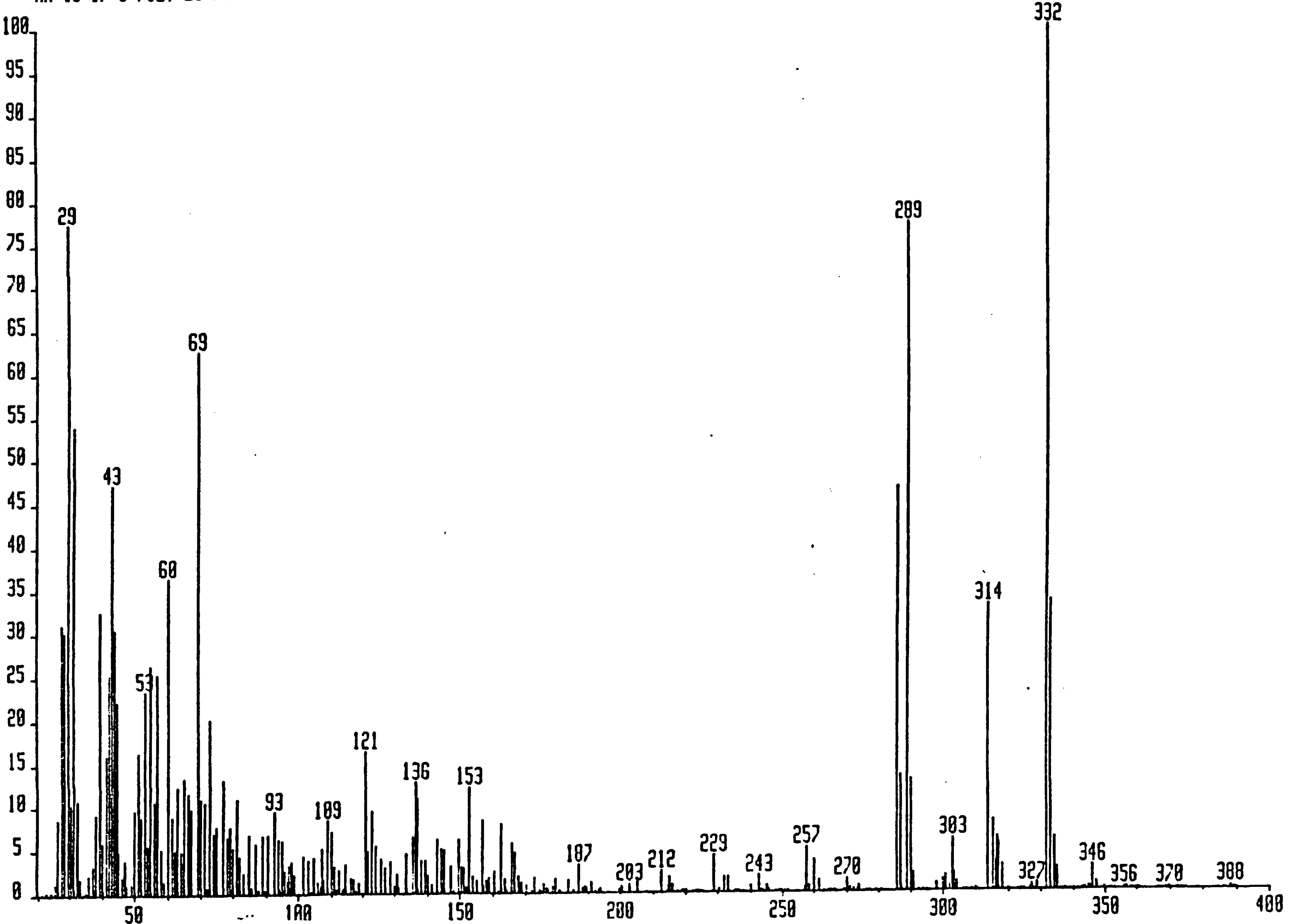


FIGURE 49. MS SPECTRUM OF GOSSYPETIN-3-METHYLETHER-3'-GLUCOSIDE

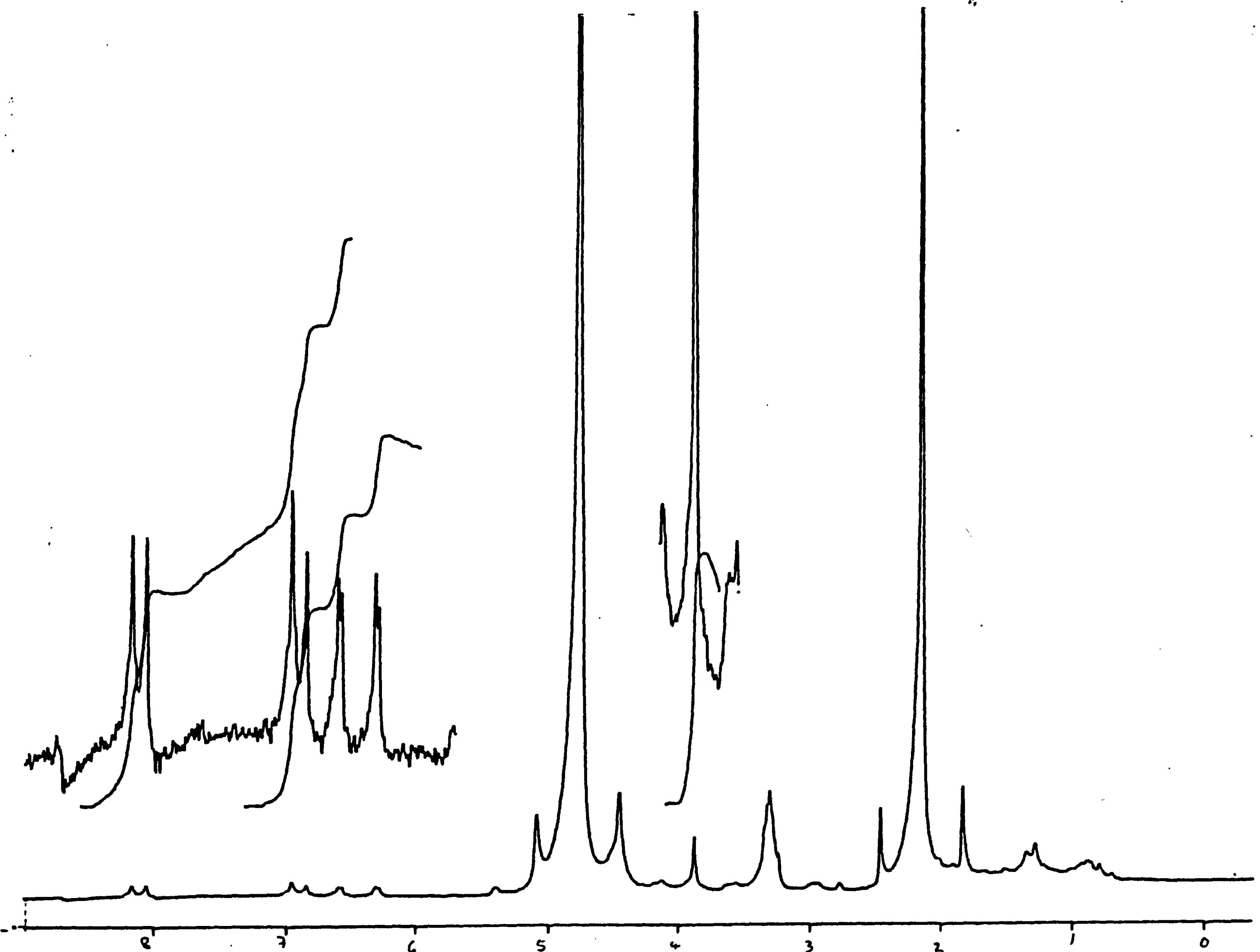


FIGURE 50 'H NMR SPECTRUM OF ISOKAEMPFERIDE

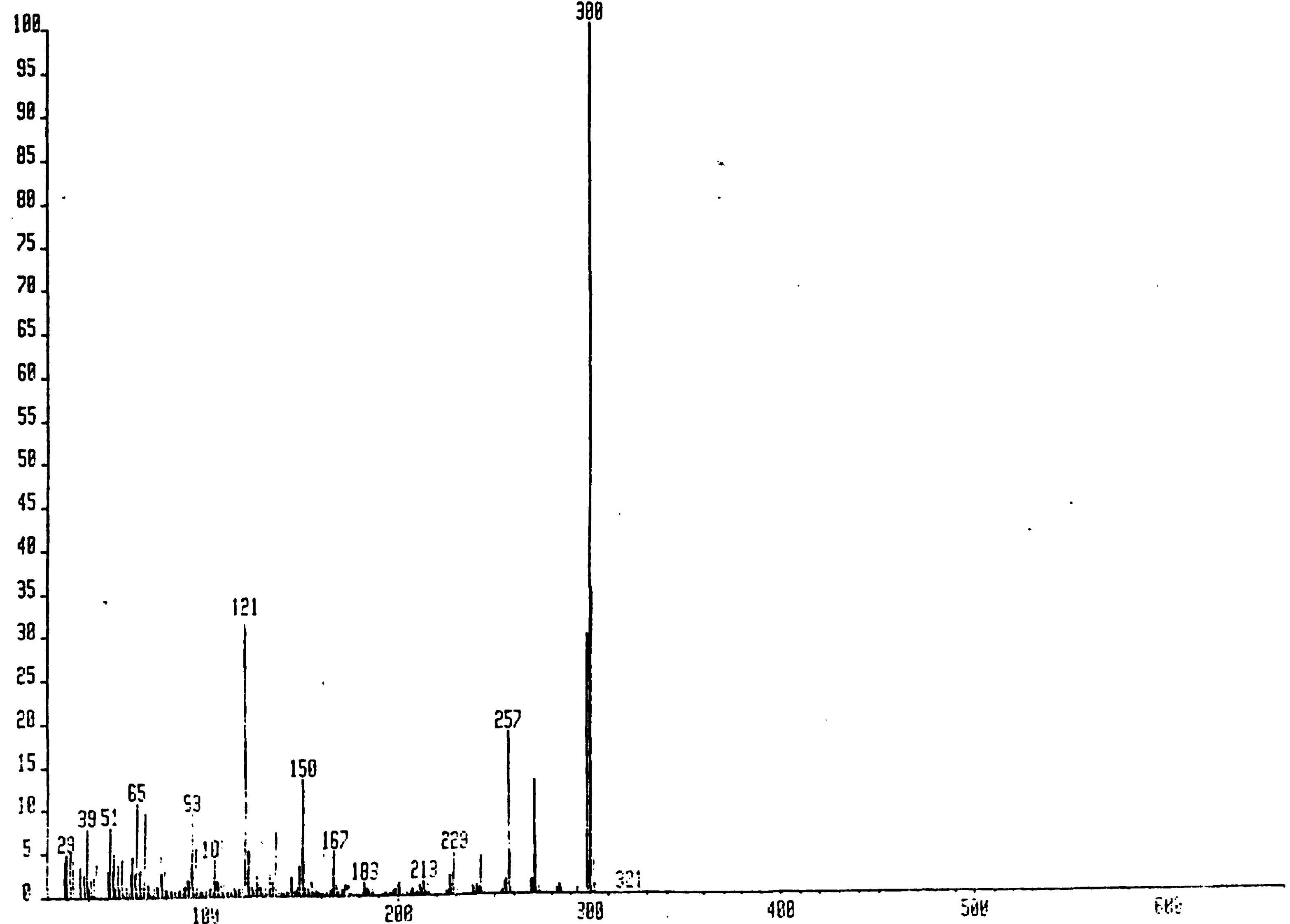


FIGURE 51. MS SPECTRUM OF ISOKAEMPFERIDE

FIGURE 52. 'H NMR SPECTRUM OF ISORHAMNETIN

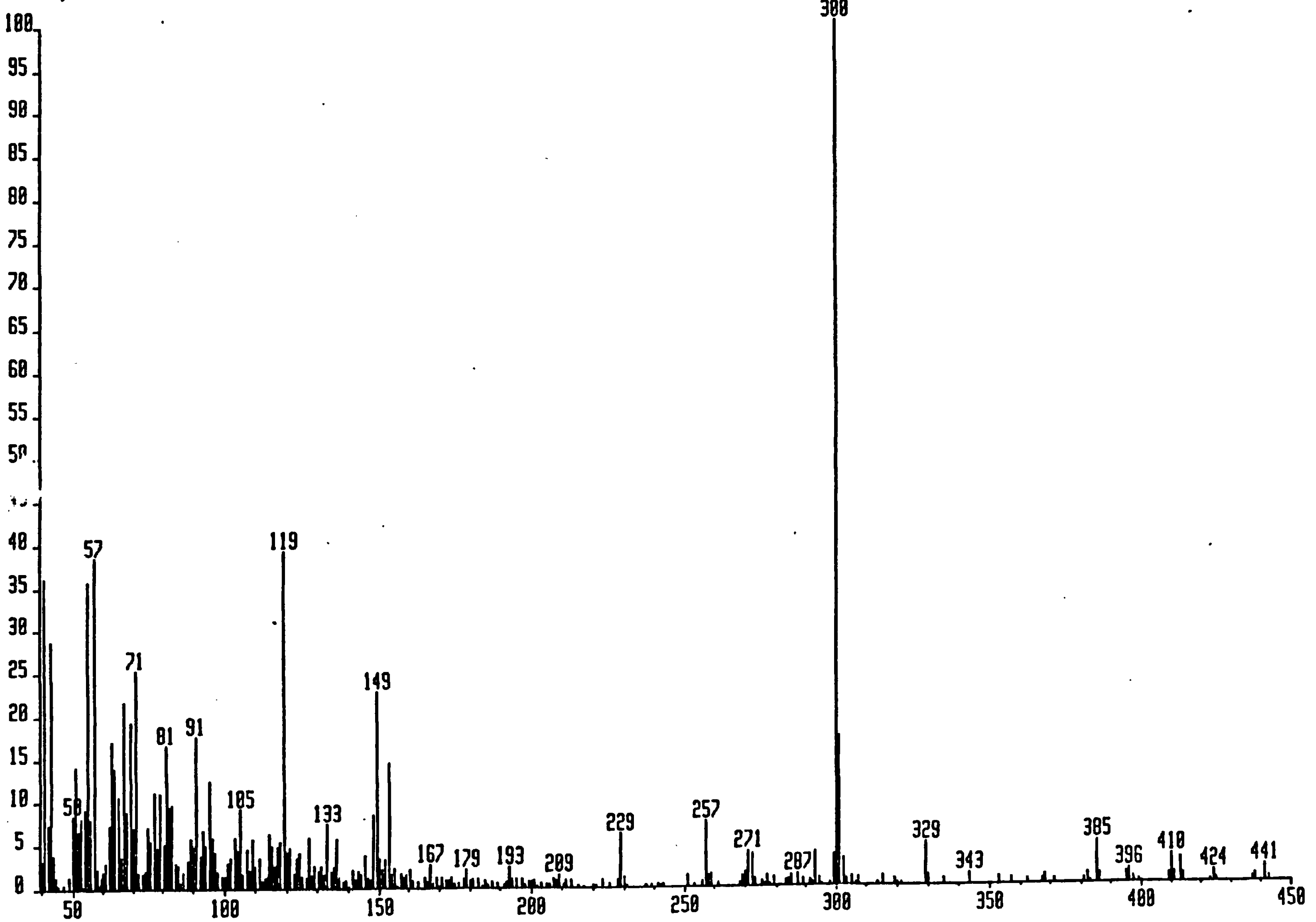


FIGURE 53 MS SPECTRUM OF ISORHAMNETIN

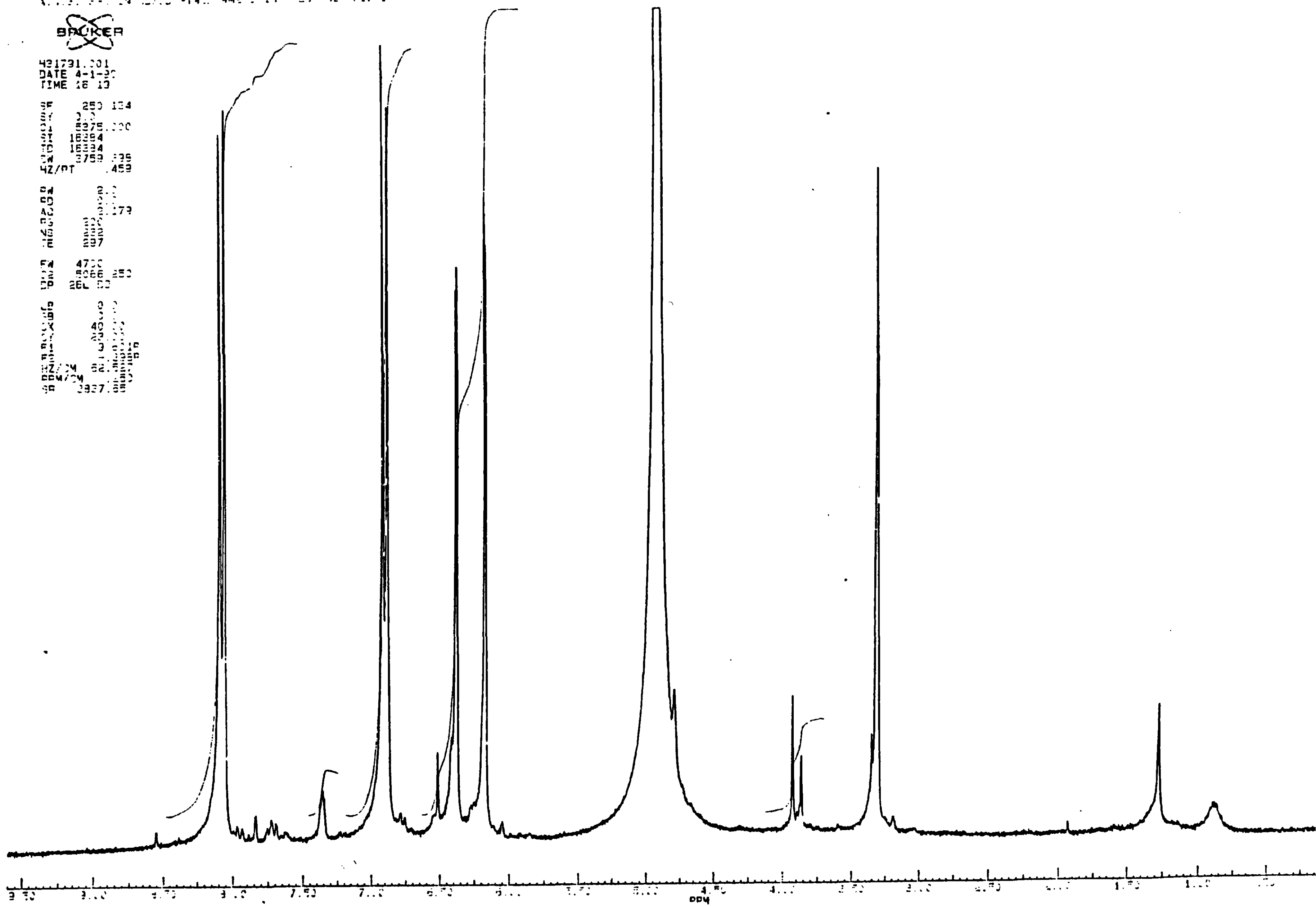


FIGURE 54. 'H NMR SPECTRUM OF KAEMPFEROL

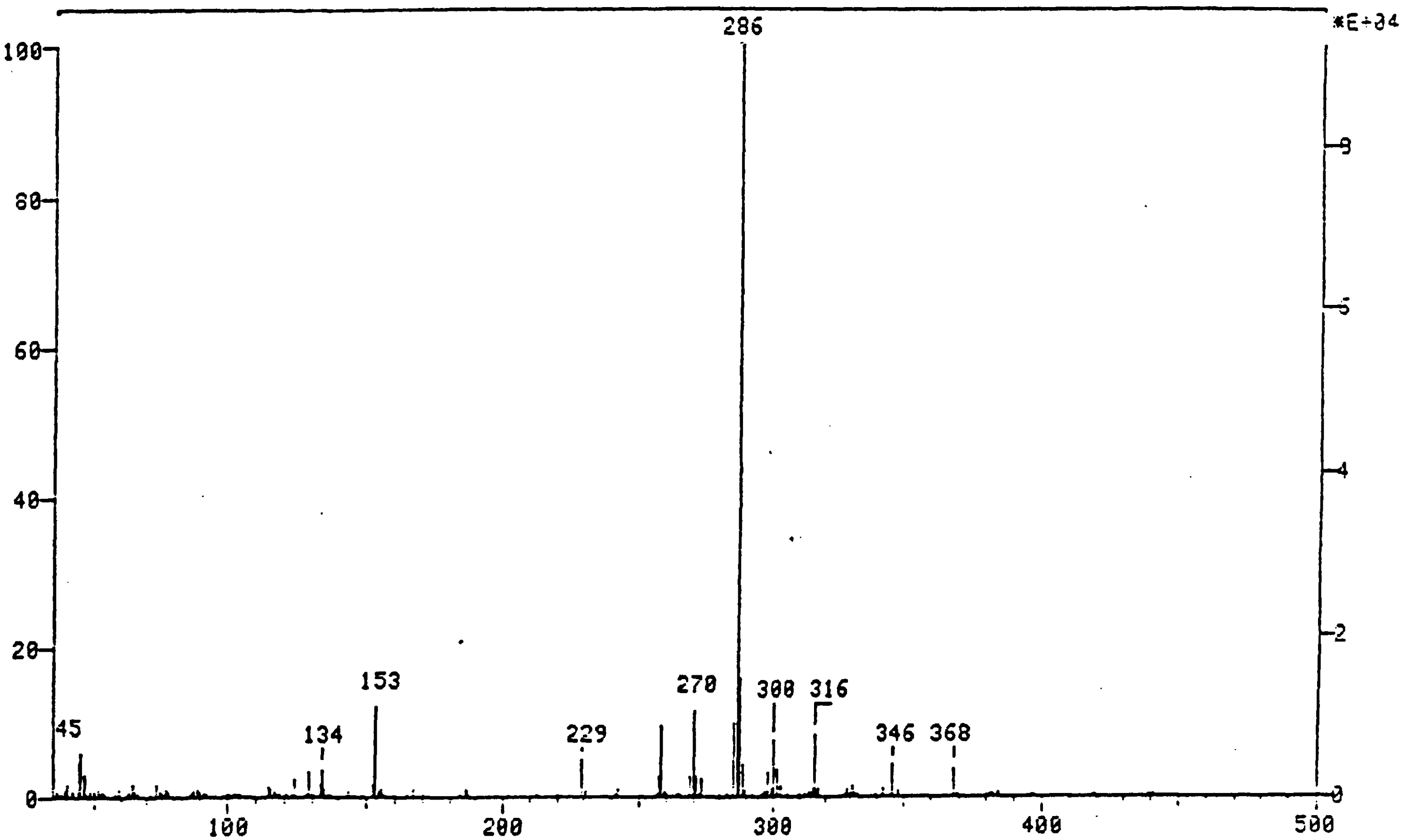
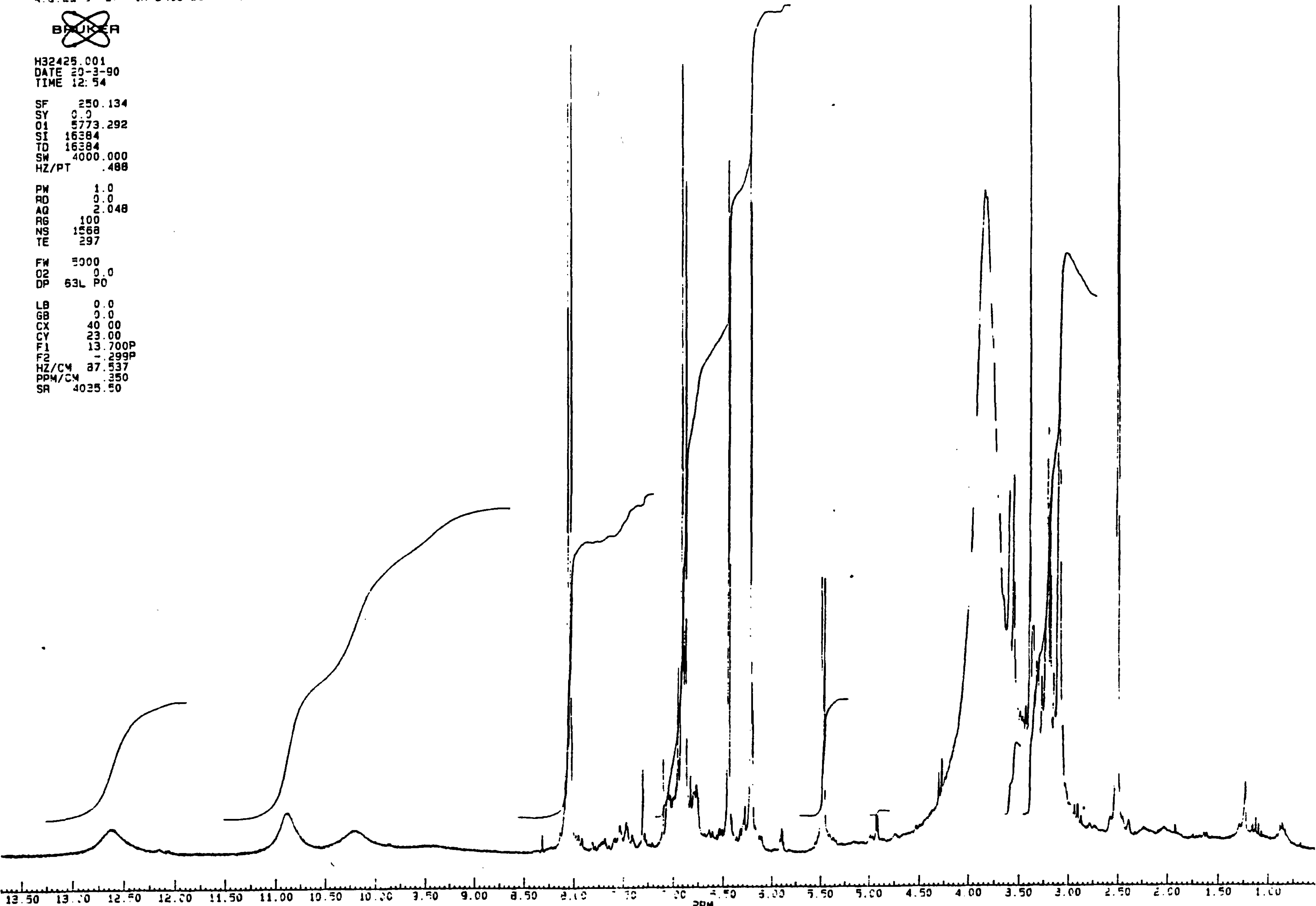


FIGURE 55 MS SPECTRUM OF KAEMPFEROL



FIGUER 56. NMR SPECTRUM OF KAEMPFEROL-3-GLUCOSIDE

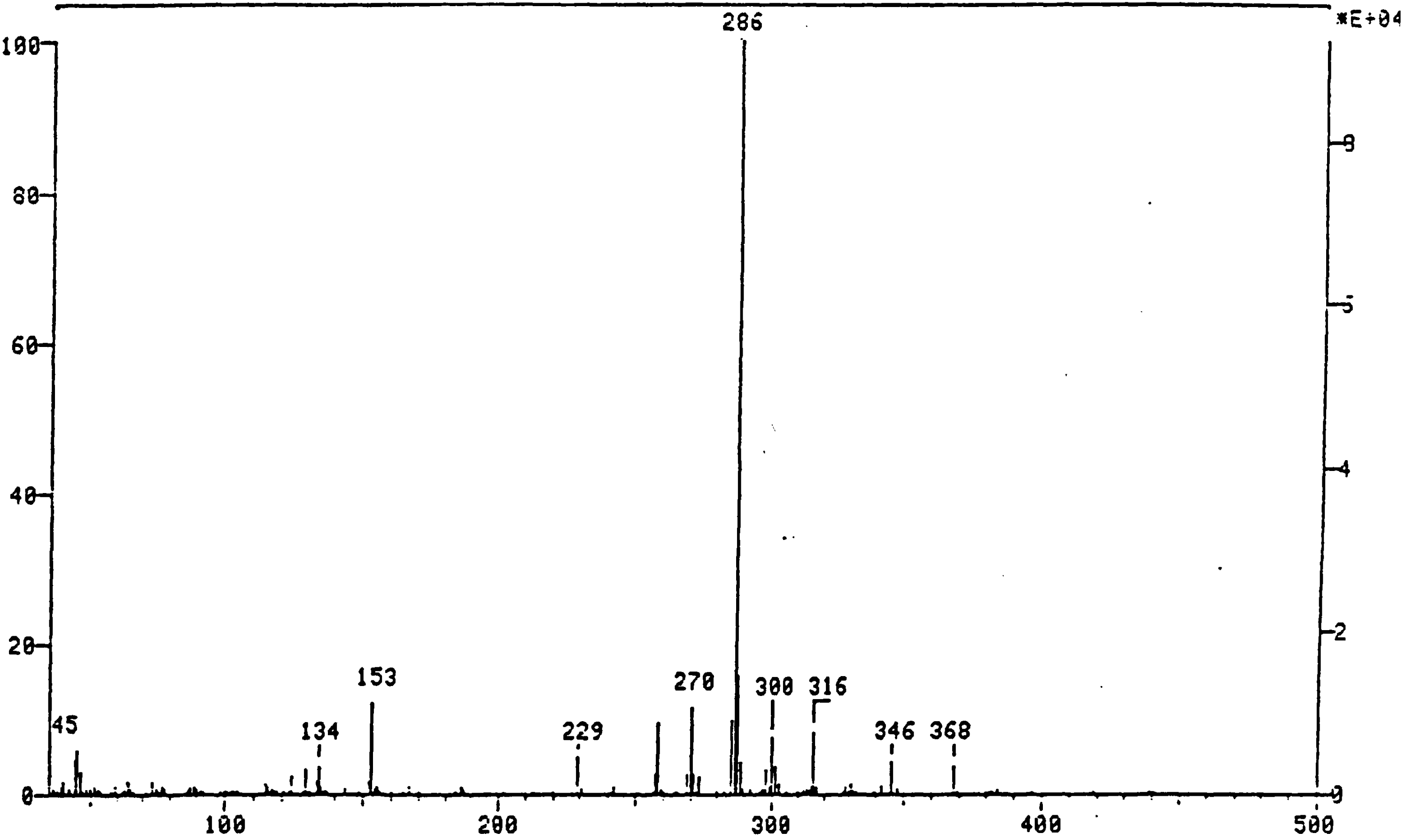


FIGURE 57. MS SPECTRUM OF KAEMPFEROL-3-GLUCOSIDE

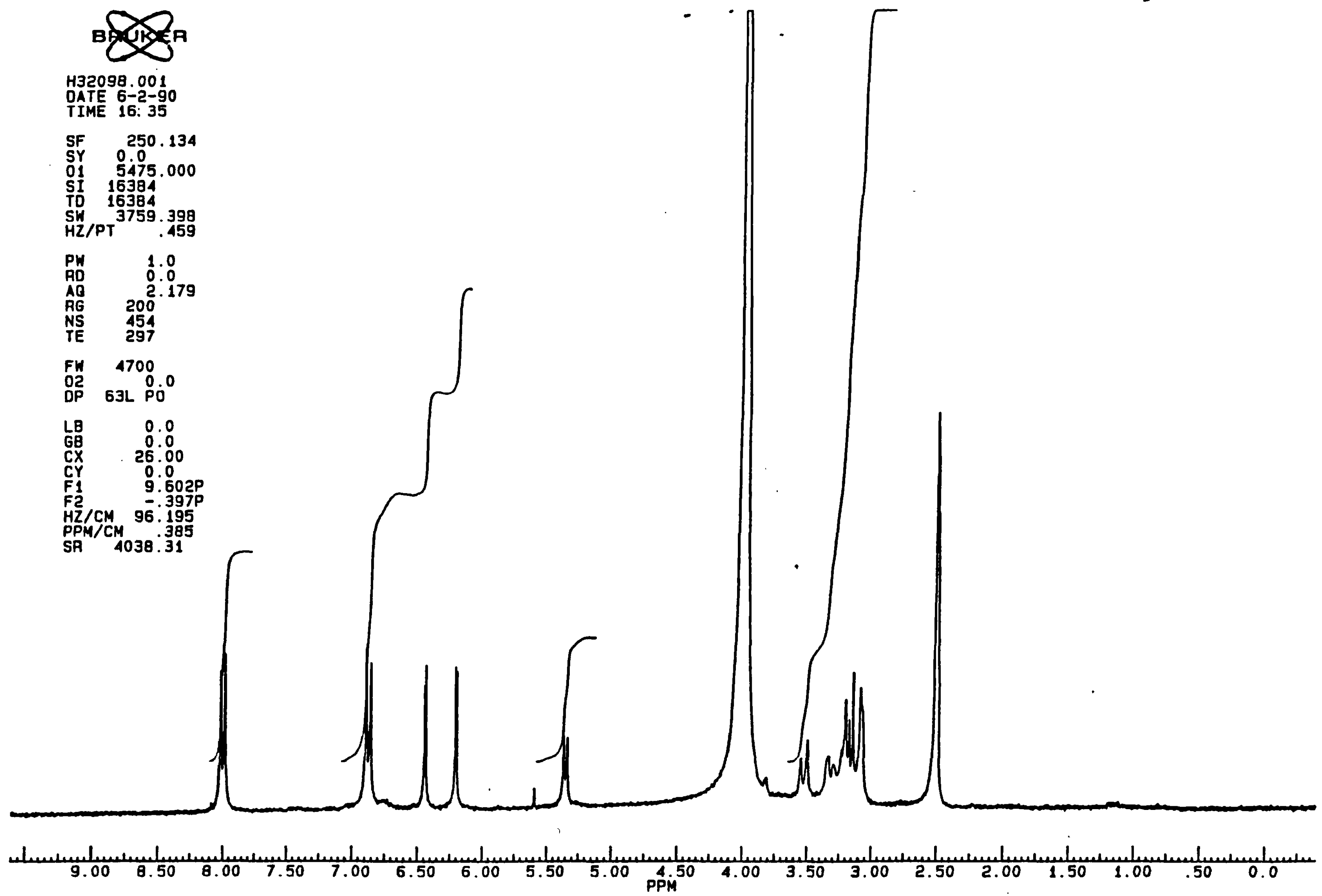


FIGURE 58 ^{1}H NMR SPECTRUM OF KAEMPFEROL-7-GLUCOSIDE

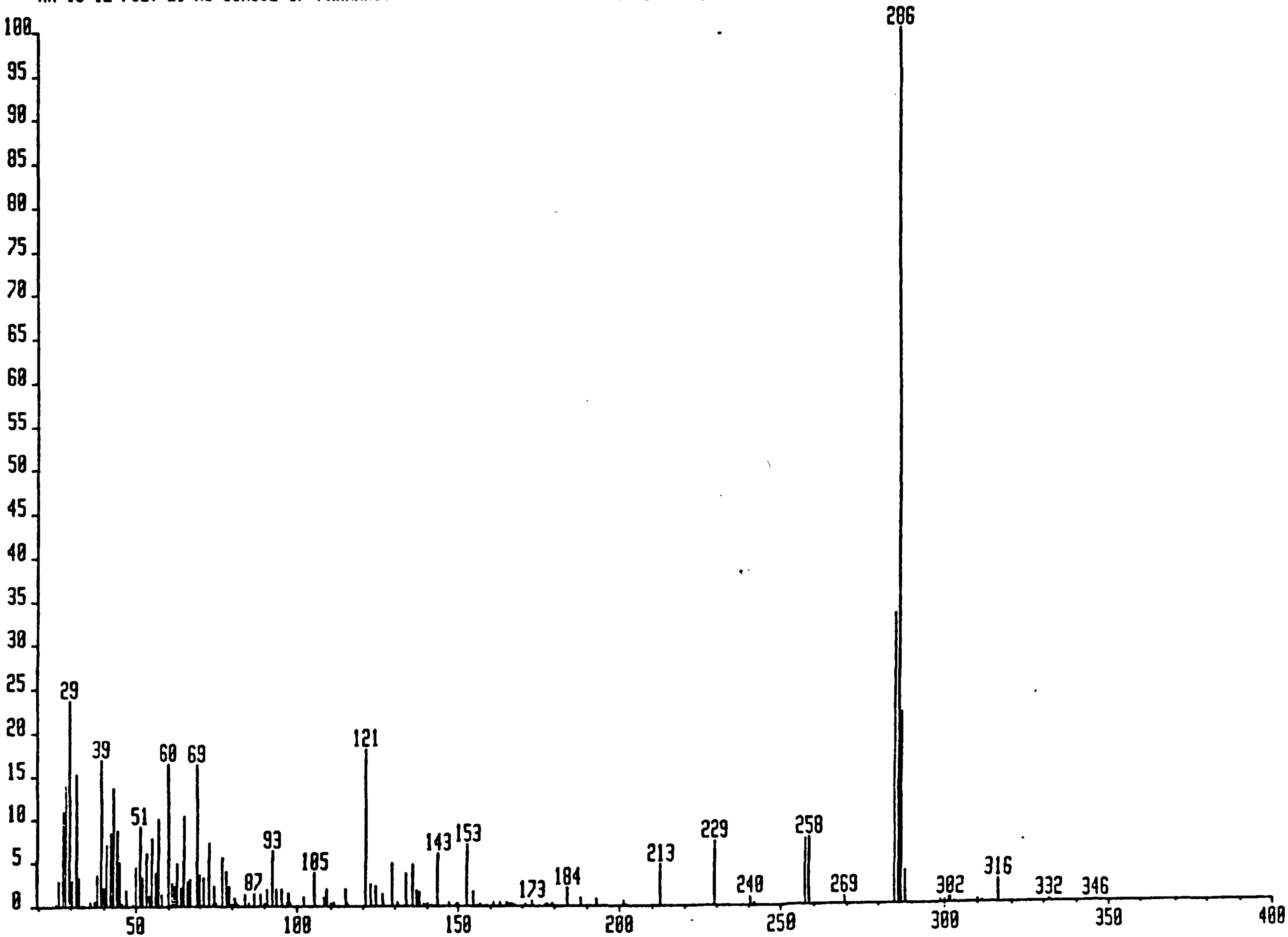


FIGURE 59 MS SPECTRUM OF KAEMPFEROL-7-GLUCOSIDE

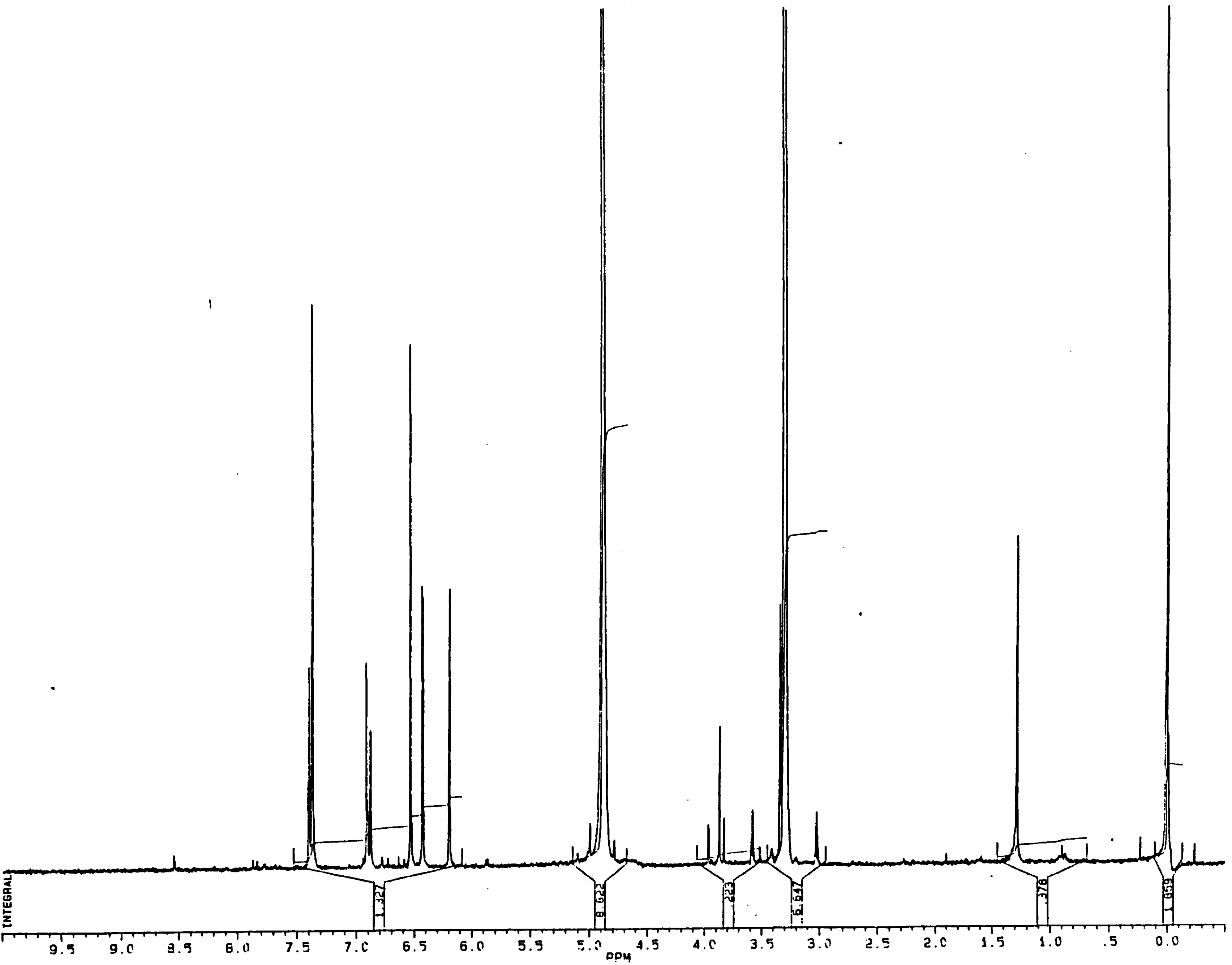


FIGURE 60. ¹H NMR SPECTRUM OF LUTEOLIN

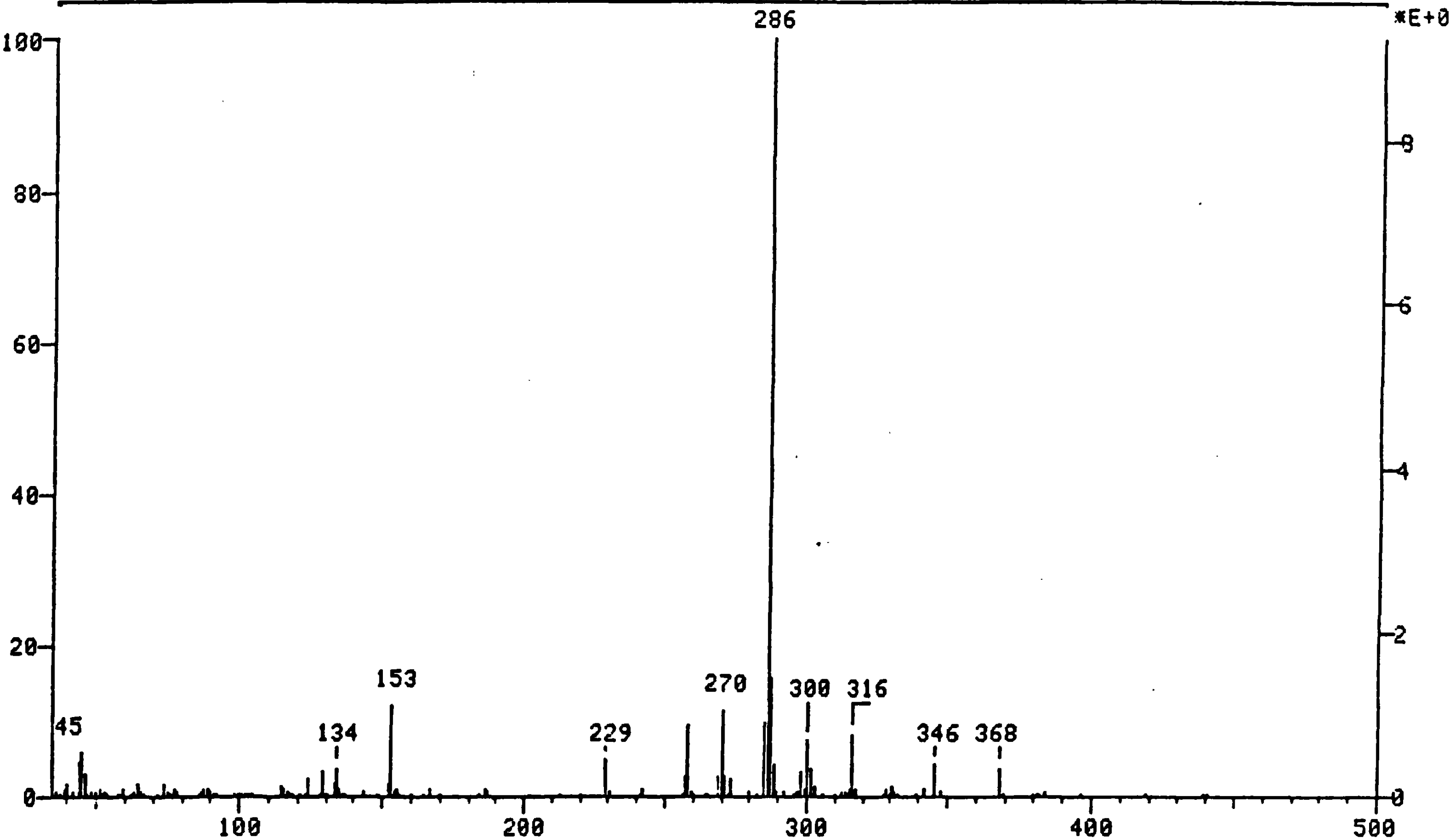


FIGURE 61. MS SPECTRUM OF LUTEOLIN

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H31786.001
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PRESAT.AU
DATE 4-1-90
TIME 14:57

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SY 0.0
O1 5375.000
SI 16384
TD 16384
SW 3759.398
HZ/PT .459

PW 5.0
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AQ 2.179
RG 64
NS 361
TE 297

FW 4700
O2 5066.250
DP 26L DO

LB 0.0
GB 0.0
CX 40.00
CY 23.00
F1 13.677P
F2 -1.336P
HZ/CM 93.882
PPM/CM .375
SR 3636.11

13.00 12.00 11.00 10.00 9.00 8.00 7.00 6.00 5.00 4.00 3.00 2.00 1.00 0.0 -1.00
PPM

FIGURE 62. 'H NMR SPECTRUM OF LUTEOLIN-4'-METHYLETHER

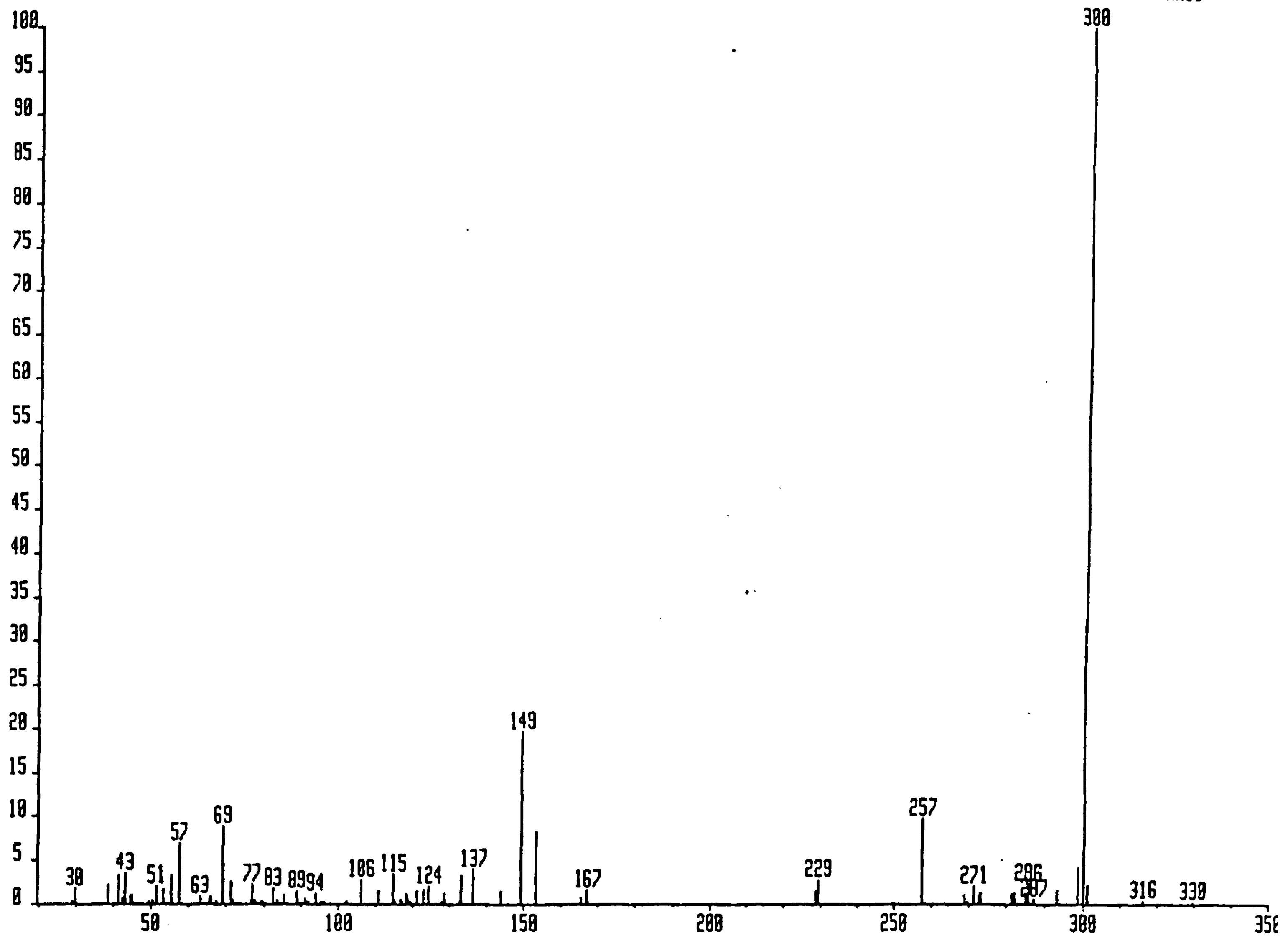

FIGURE 63. MS SPECTRUM OF LUTEOLIN-4'-METHYLETHER

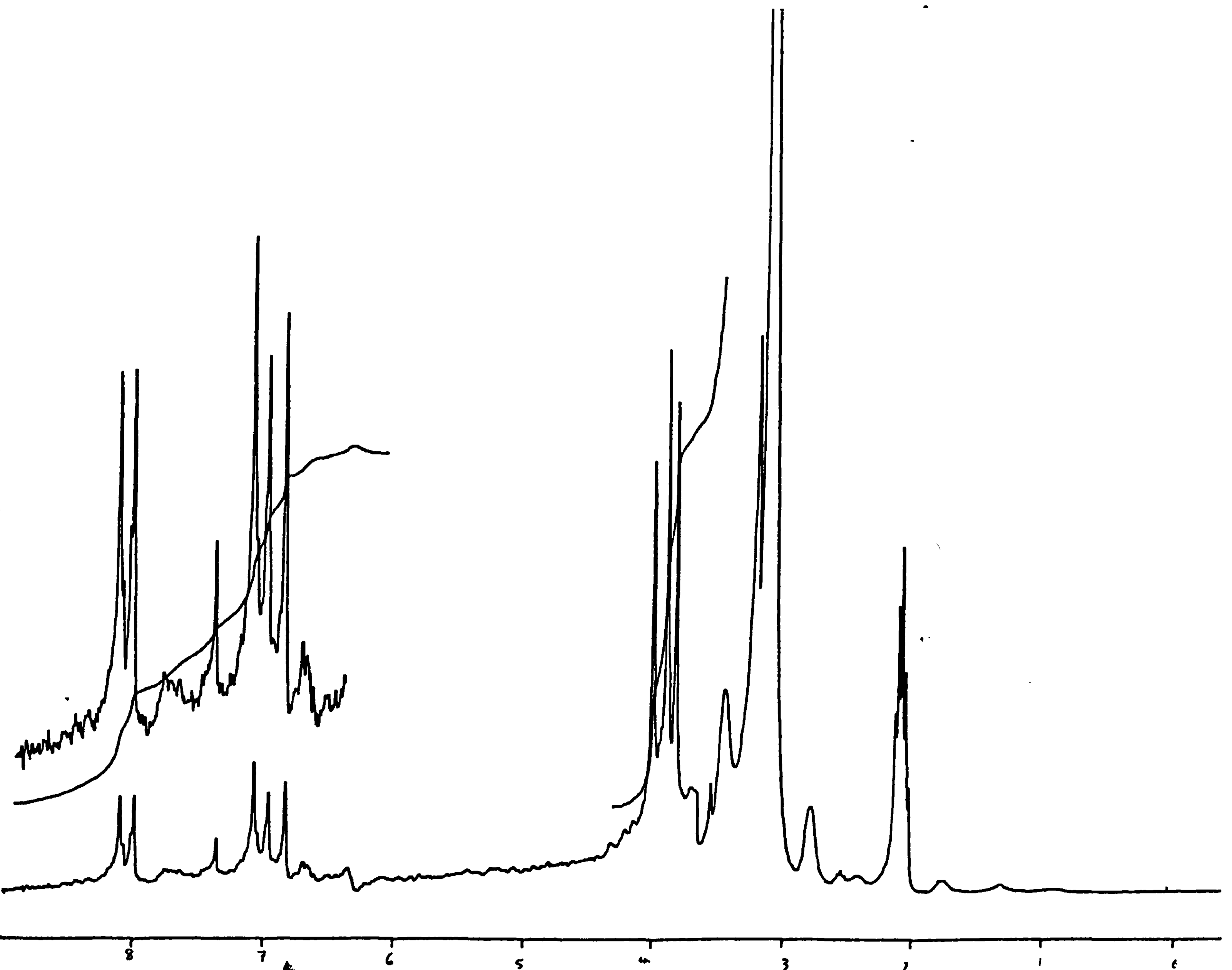

FIGURE 64 NMR SPECTRUM OF PENDULETIN

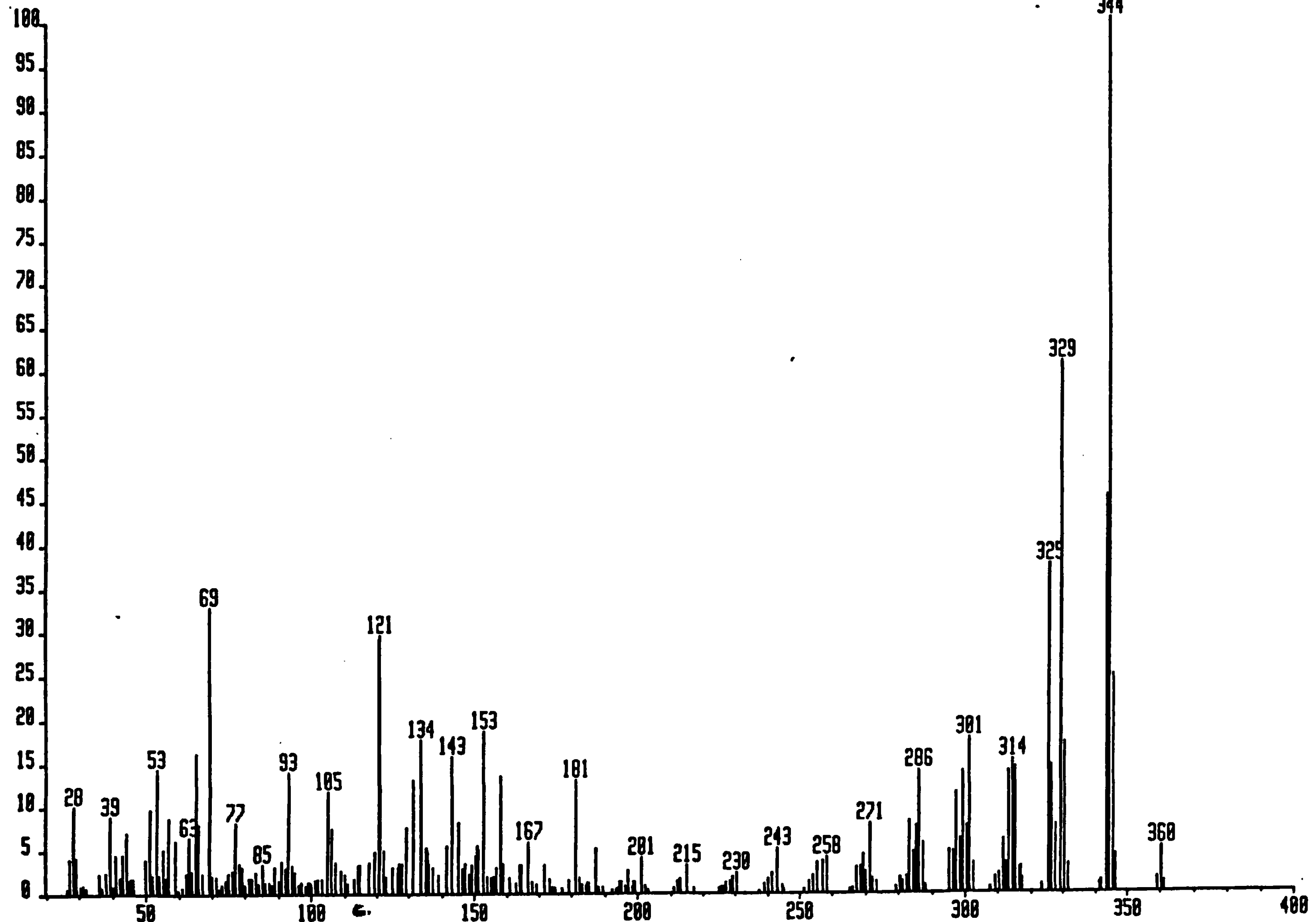

FIGURE 65. MS SPECTRUM OF PENDULETIN

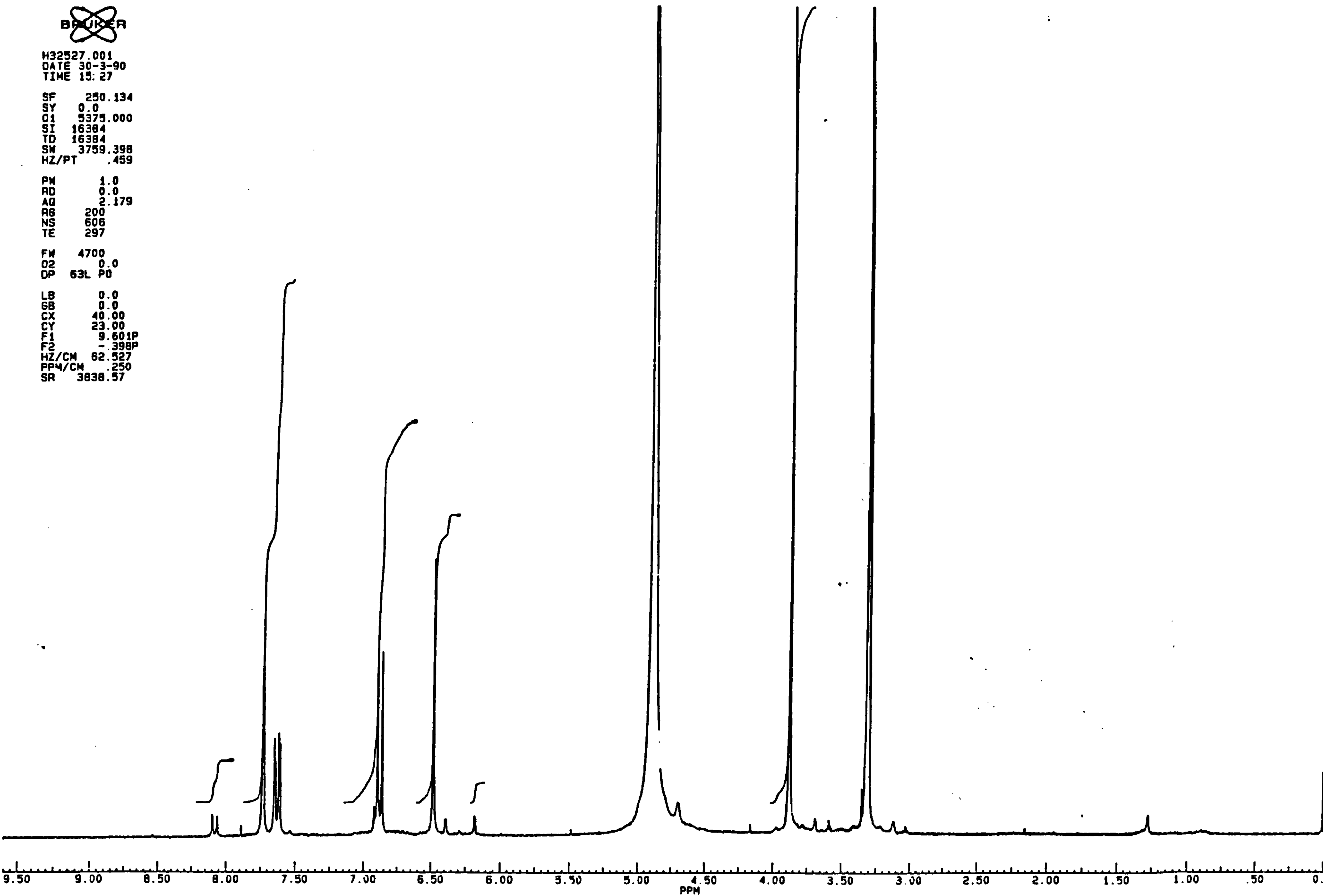

FIGURE 66. 'H NMR SPECTRUM OF QUERCETAGETIN-3-METHYLETHER

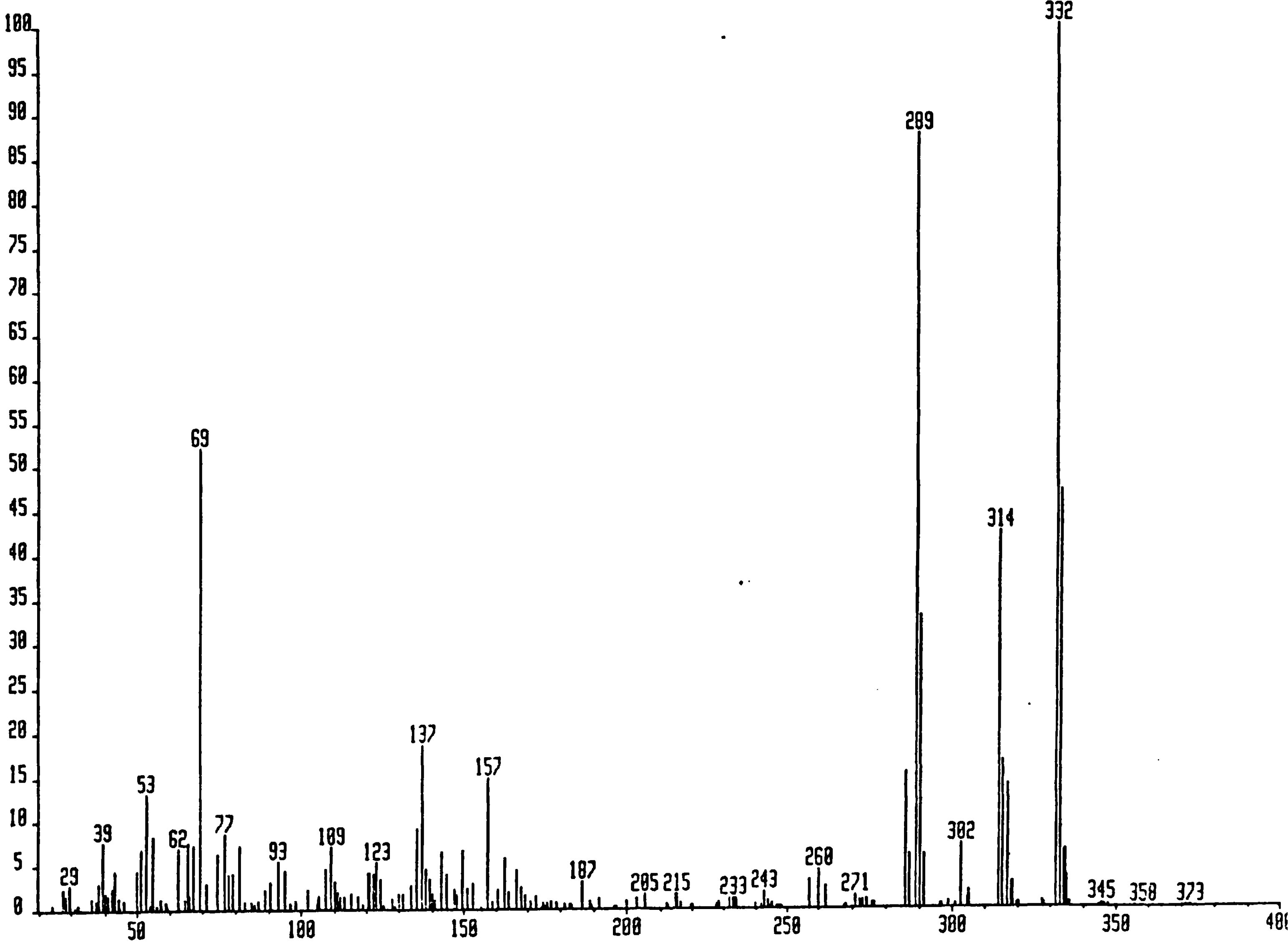

FIGURE 67. MS SPECTRUM OF QUERCETAGETIN-3-METHYLETHER

8.50 8.00 7.50 7.00 6.50 6.00 5.50 5.00 4.50 4.00 3.50 3.00 2.50 2.00 1.50 1.00 .50 0.0
PPM

FIGURE 68 NMR SPECTRUM OF QUERCETAGETIN-3-METHYLETHER-4'-GLUCOSIDE

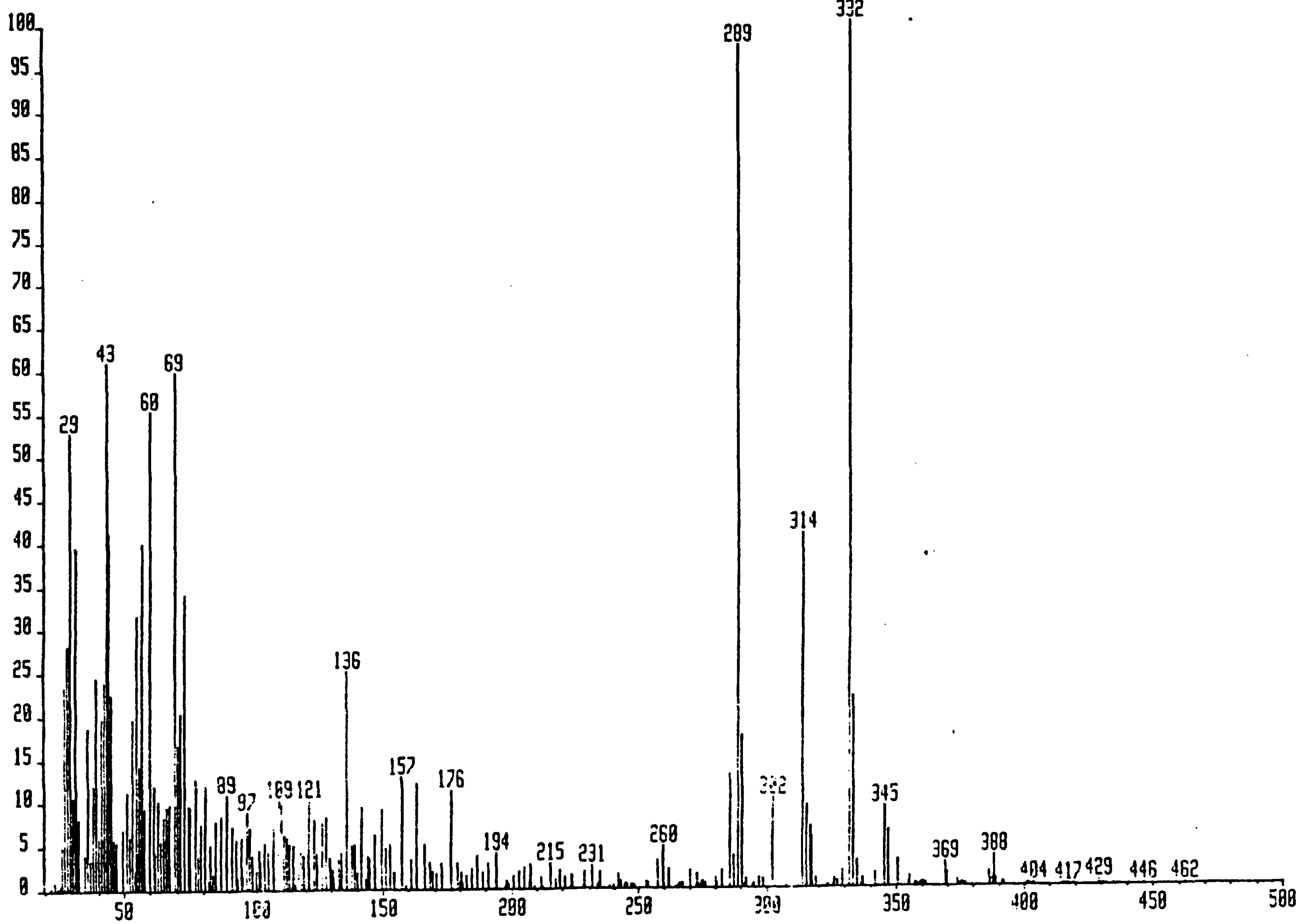

FIGURE 69 MS SPECTRUM OF QUERCETAGETIN-3-METHYLETHER-4'-GLUCOSIDE

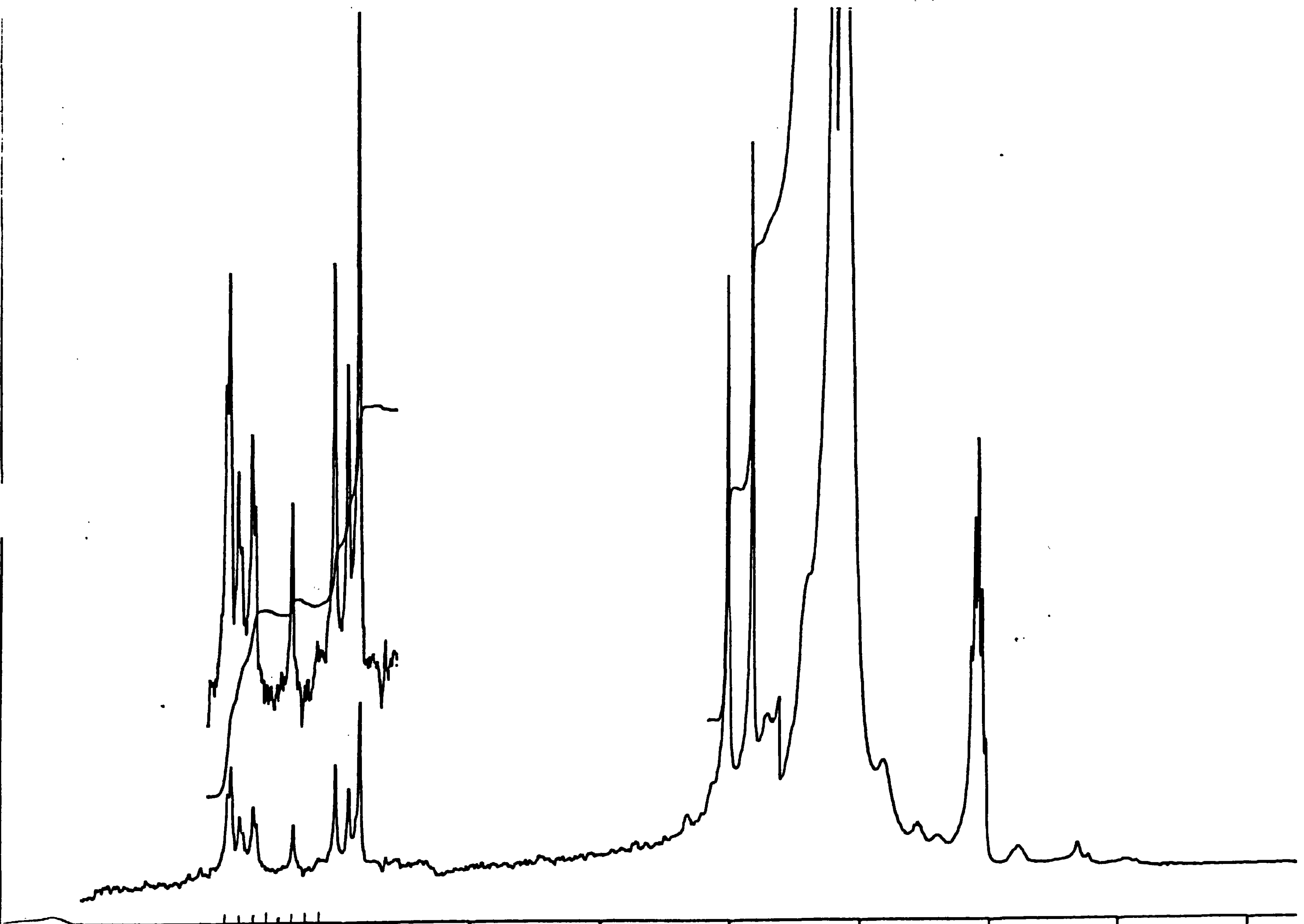

FIGURE 70. NMR SPECTRUM OF QUERCETAGETIN-3',4'-DIMETHYLETHER

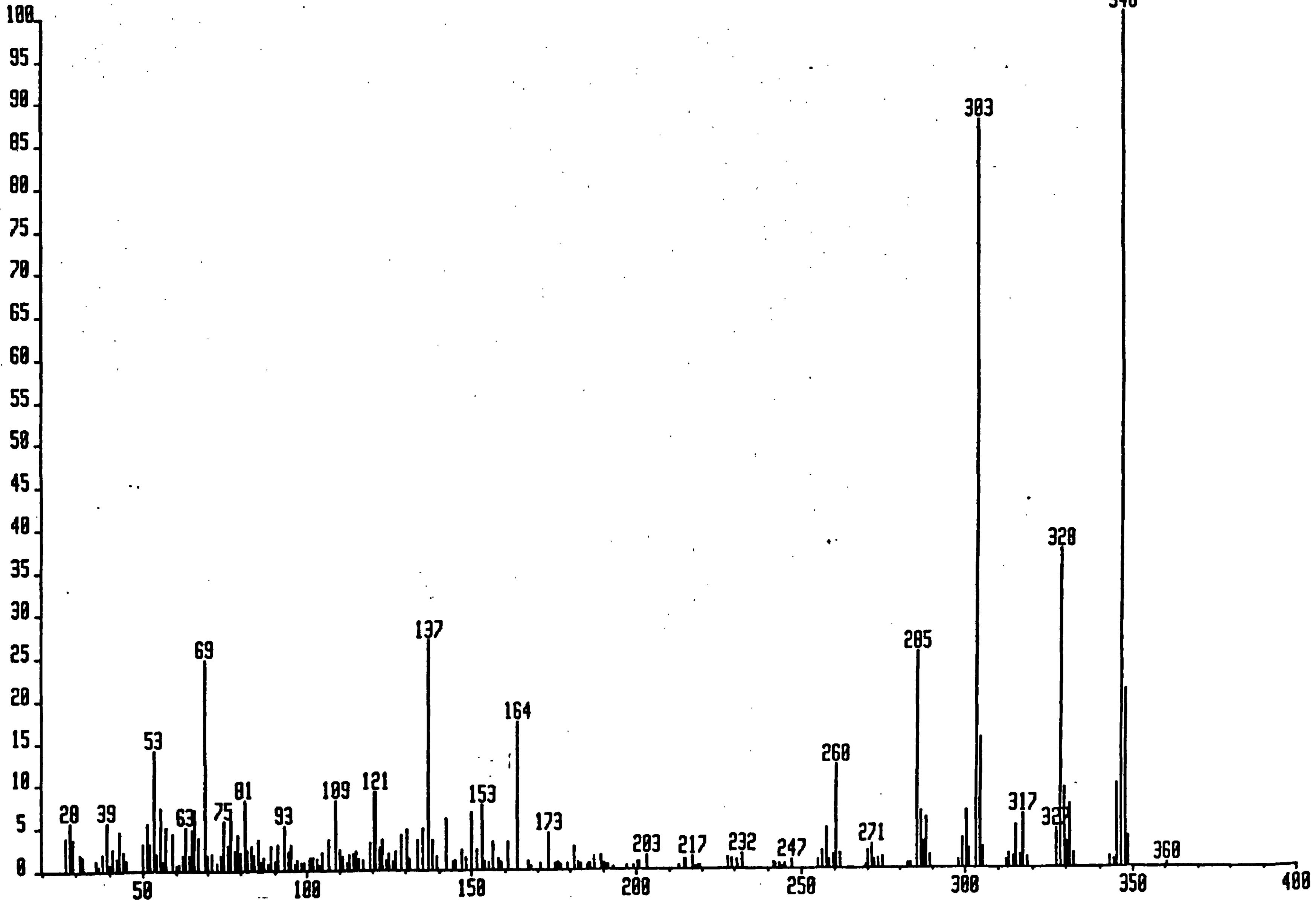

FIGURE 71 MS SPECTRUM OF QUERCETAGETIN-3',4'-DIMETHYLETHER

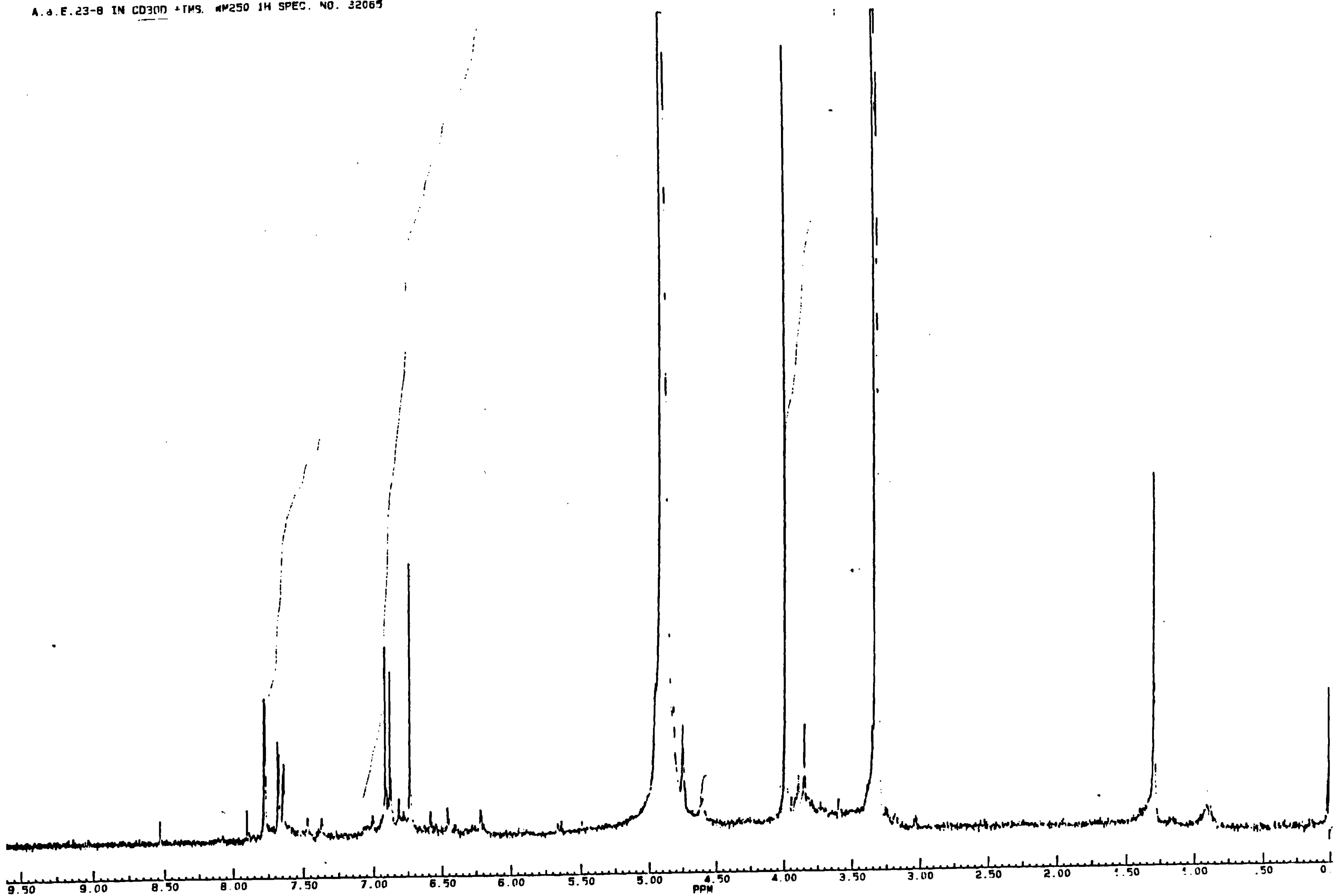

FIGURE 72 . NMR SPECTRUM OF QUERCETAGETIN-4'-METHYLETHER

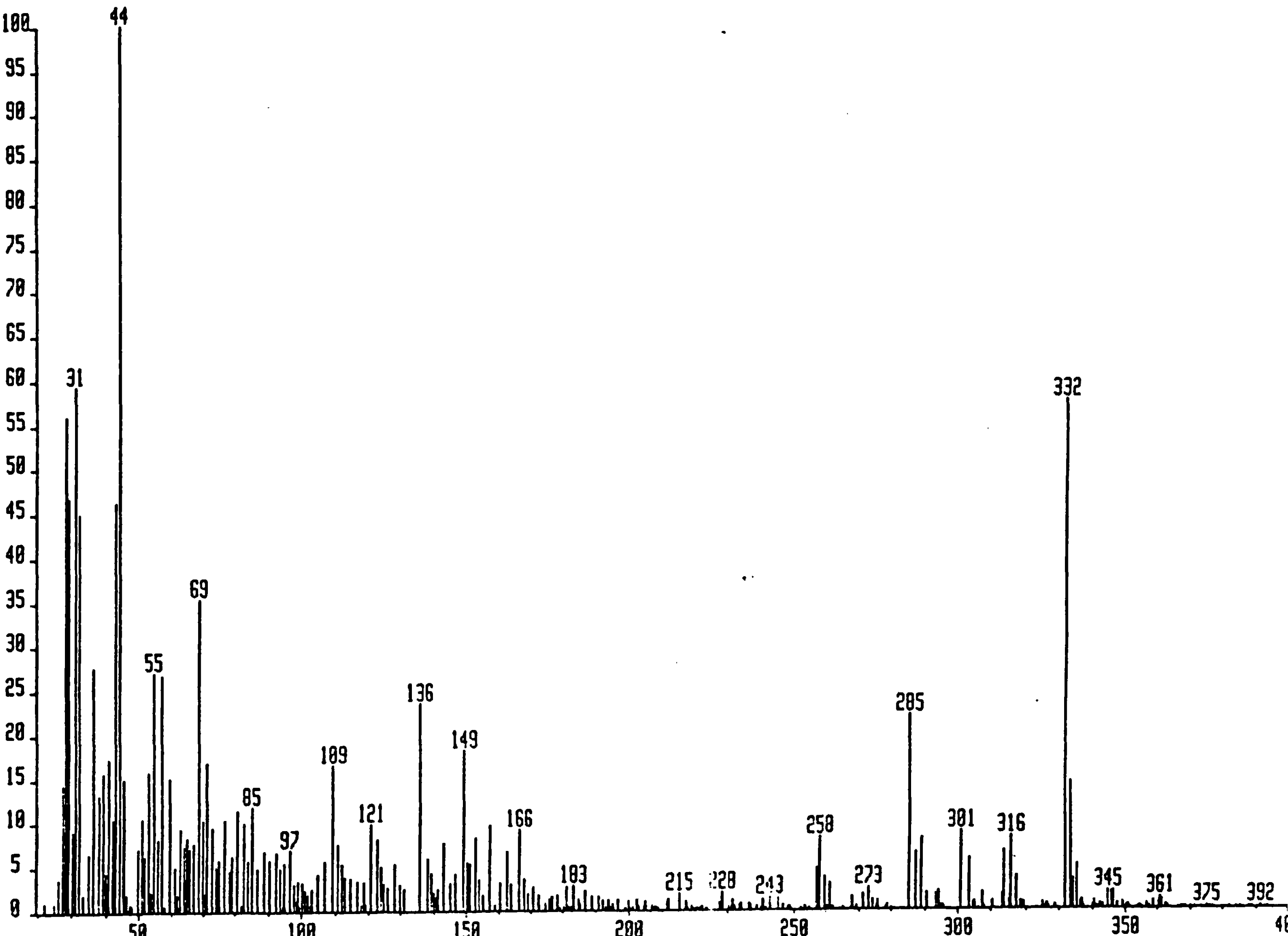

FIGURE 73 MS SPECTRUM OF QUERCETAGETIN-4'-METHYLETHER

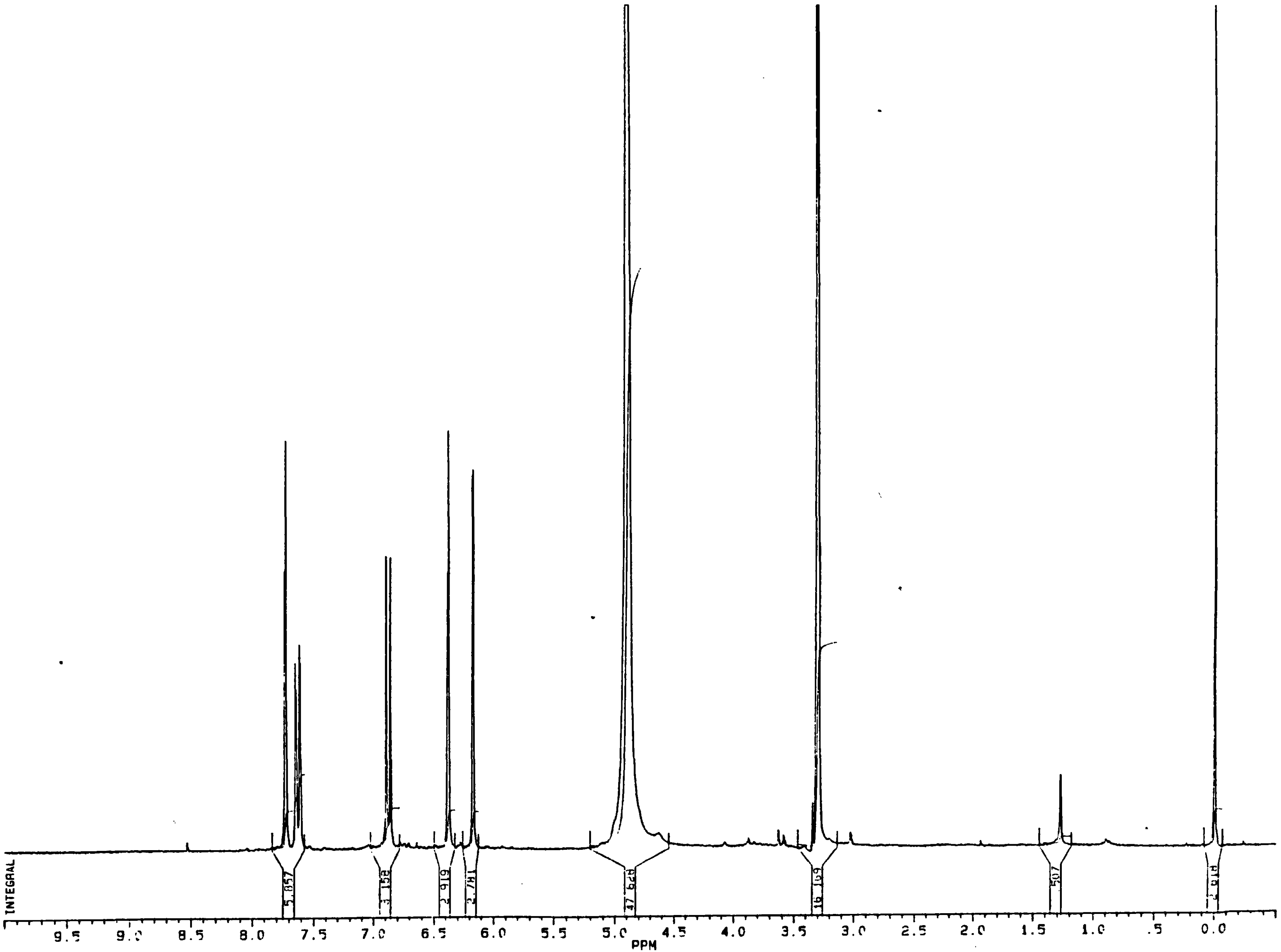

FIGURE 74. ¹H NMR SPECTRUM OF QUERCETIN

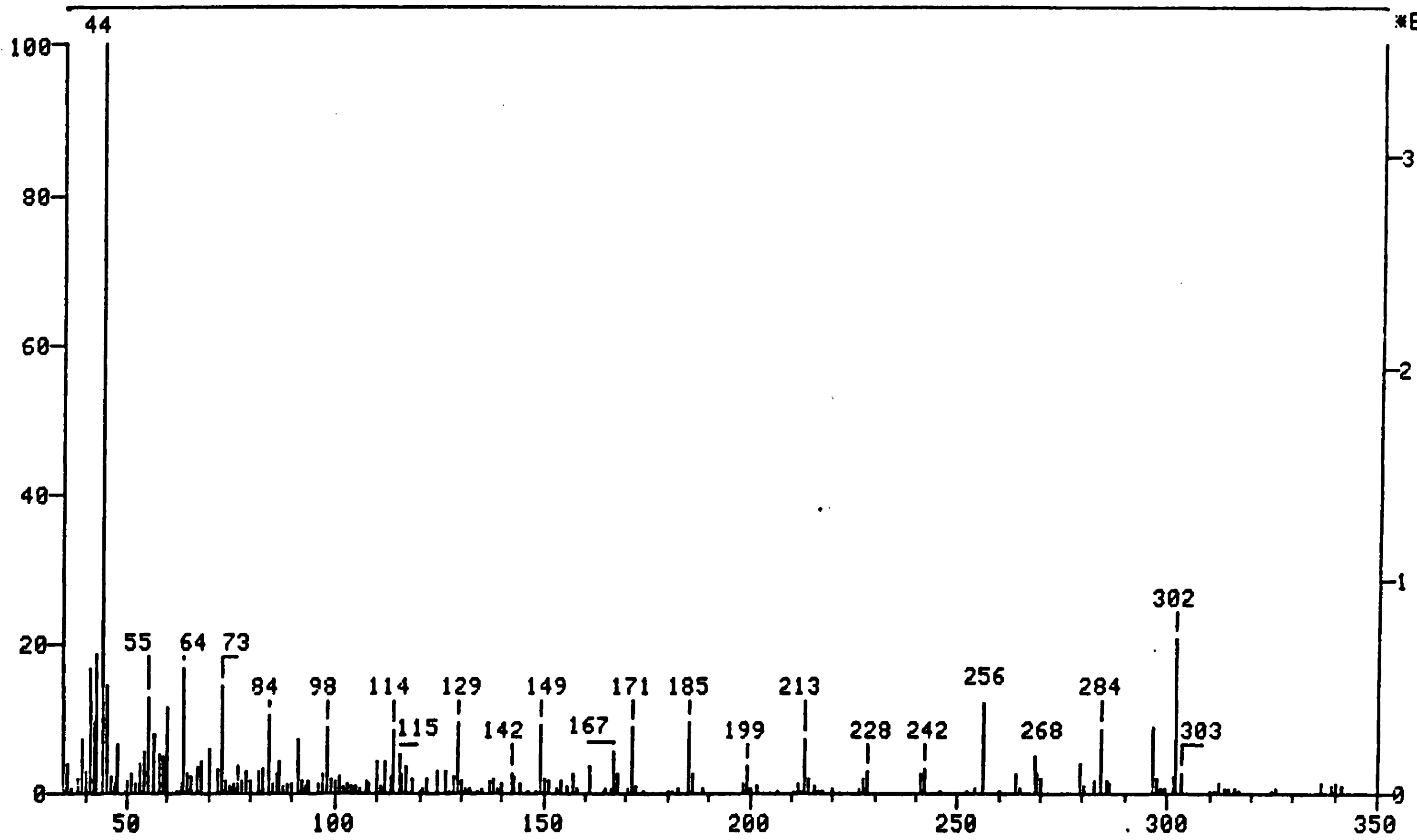

FIGURE 75. MS SPECTRUM OF QUERCETIN

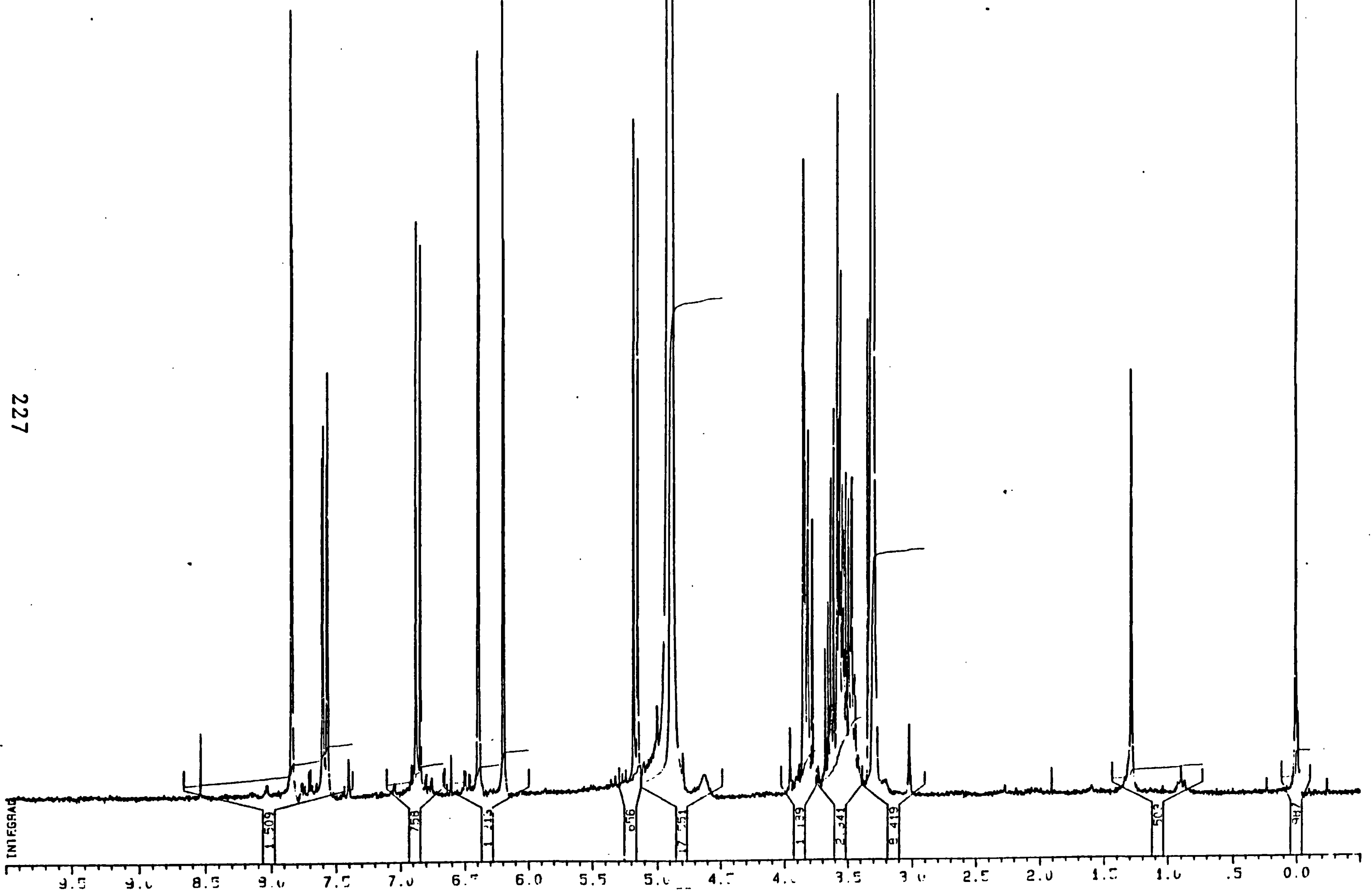

FIGURE 76 SPECTRUM OF QUERCETIN-3-GLUCOSIDE

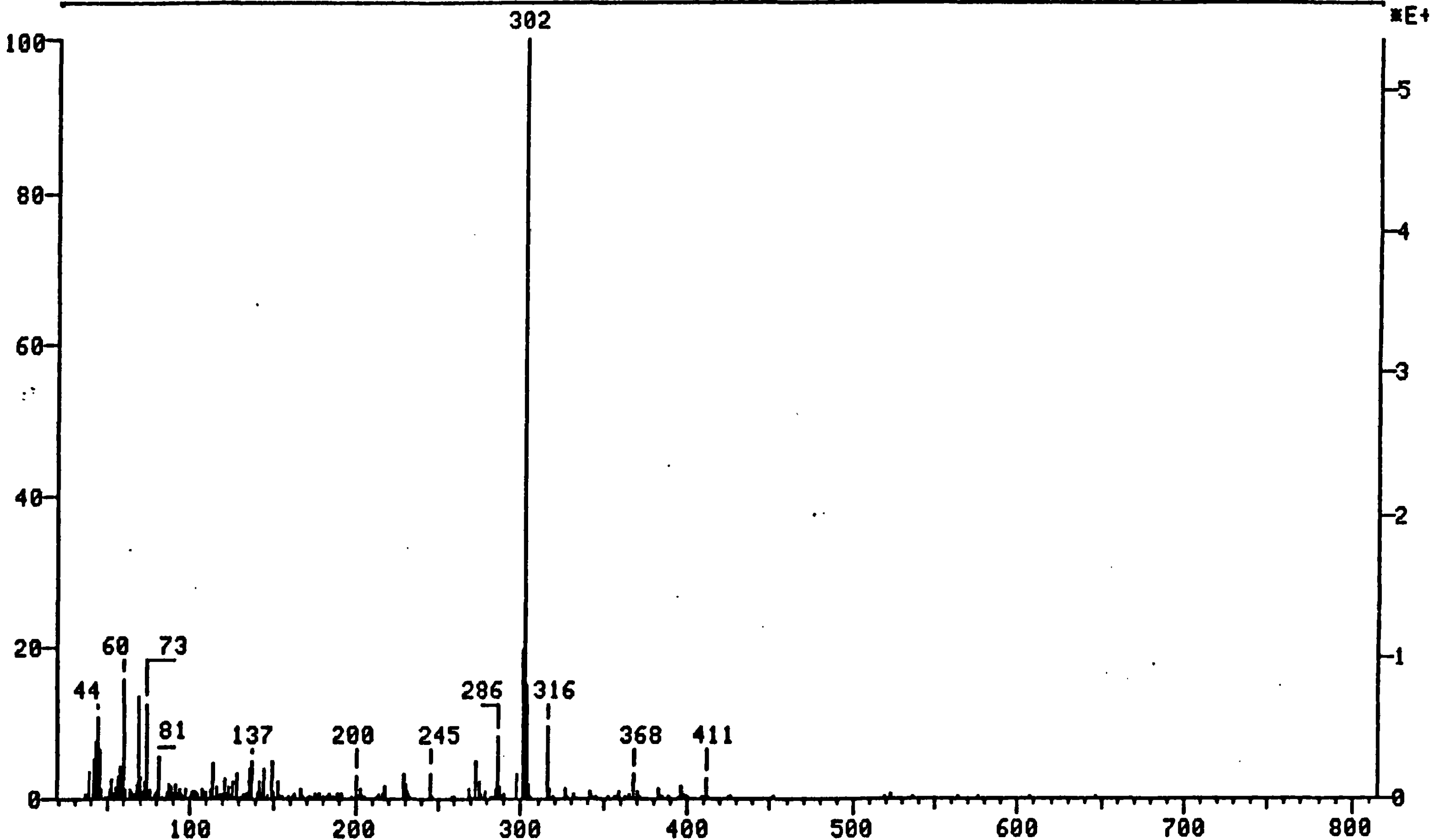

FIGURE 77. MS SPECTRUM OF QUERCETIN-3-GLUCOSIDE

FIGURE 78. ¹H NMR SPECTRUM OF QUERCETIN-7-GLUCOSIDE

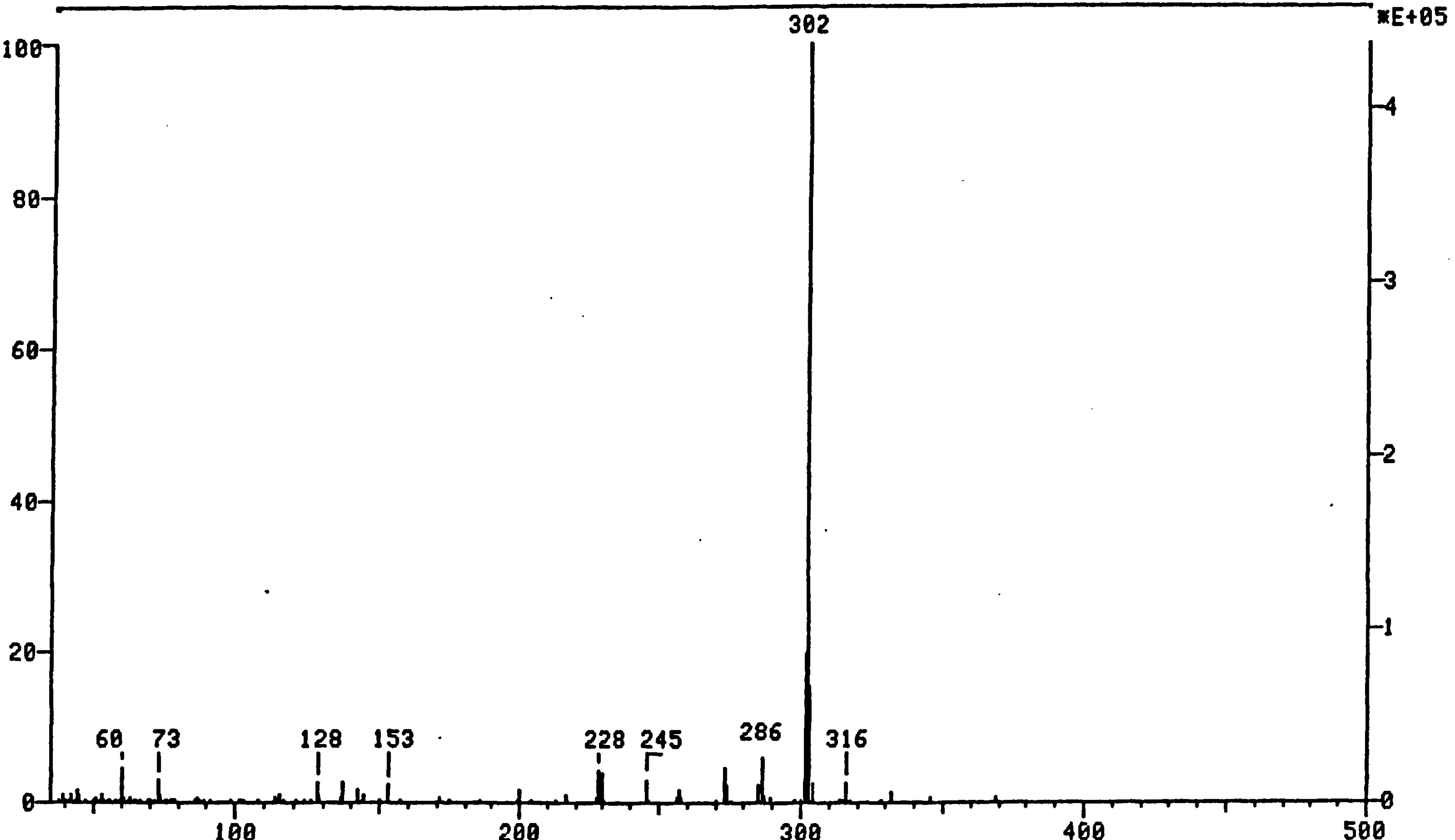

FIGURE 79 MS SPECTRUM OF QUERCETIN-7-GLUCOSIDE

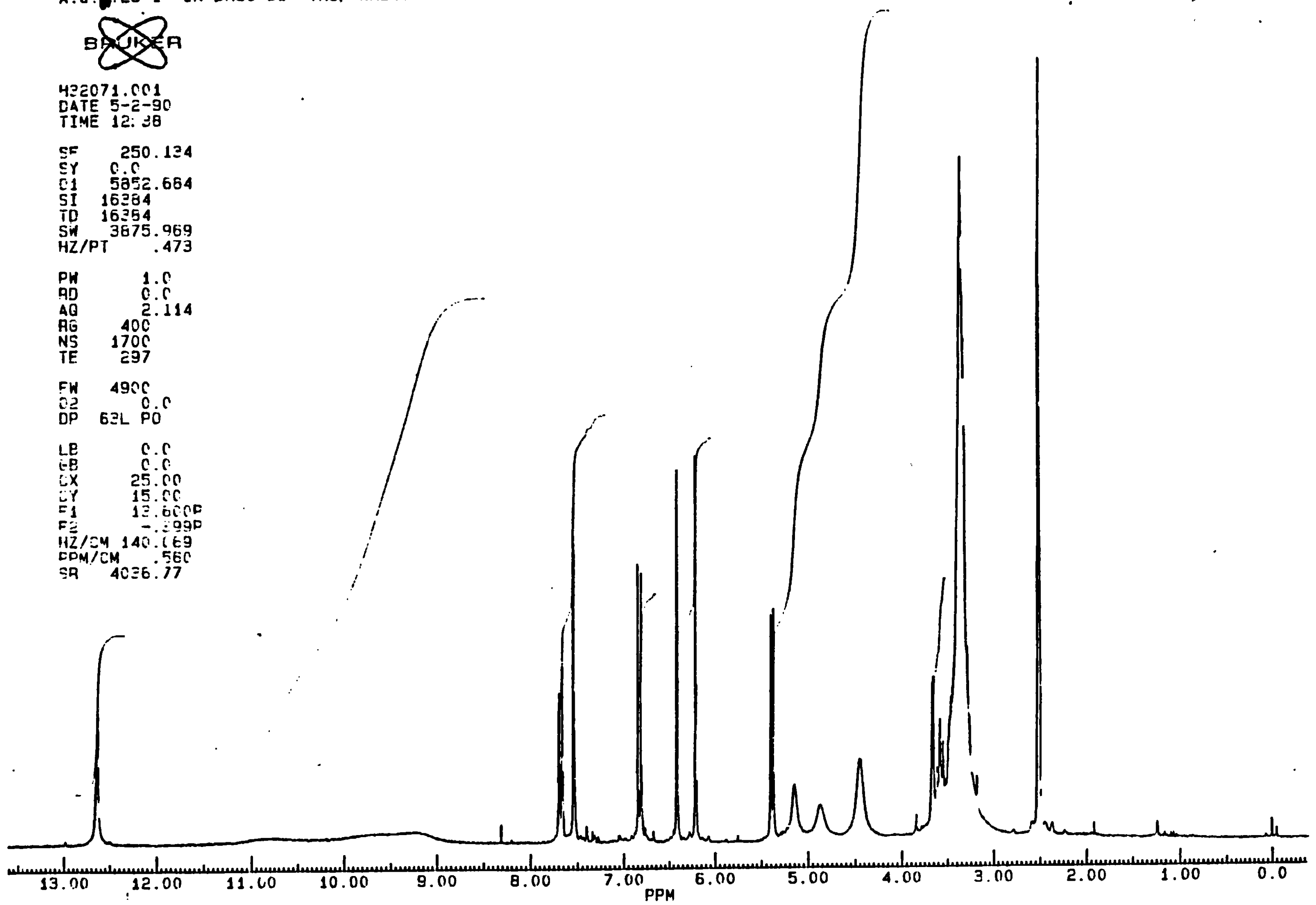

FIGURE 80 ^{1}H NMR SPECTRUM OF QUERCETIN-3'-GLUCOSIDE

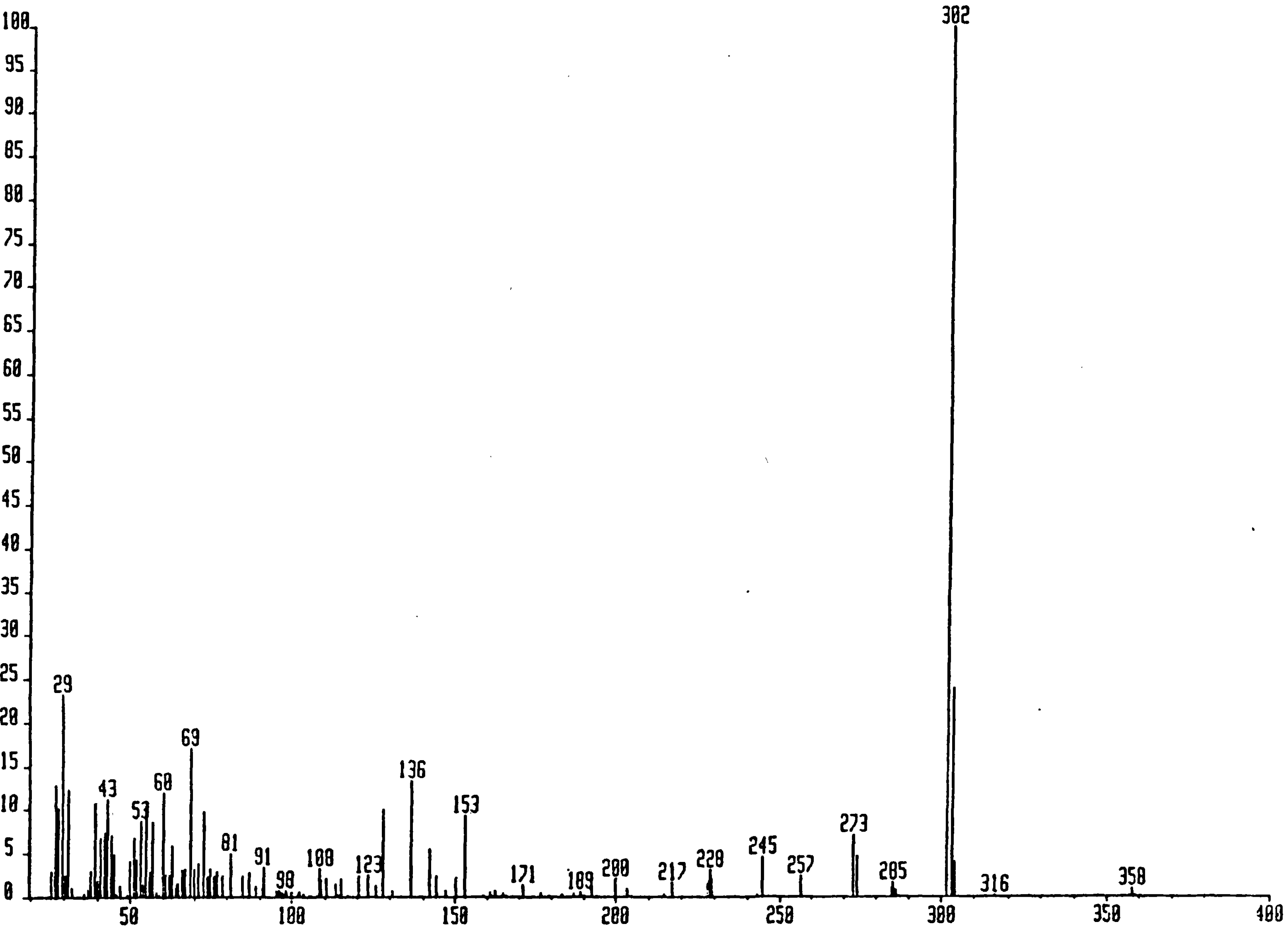

FIGURE 81 MS SPECTRUM OF QUERCETIN-3'-GLUCOSIDE

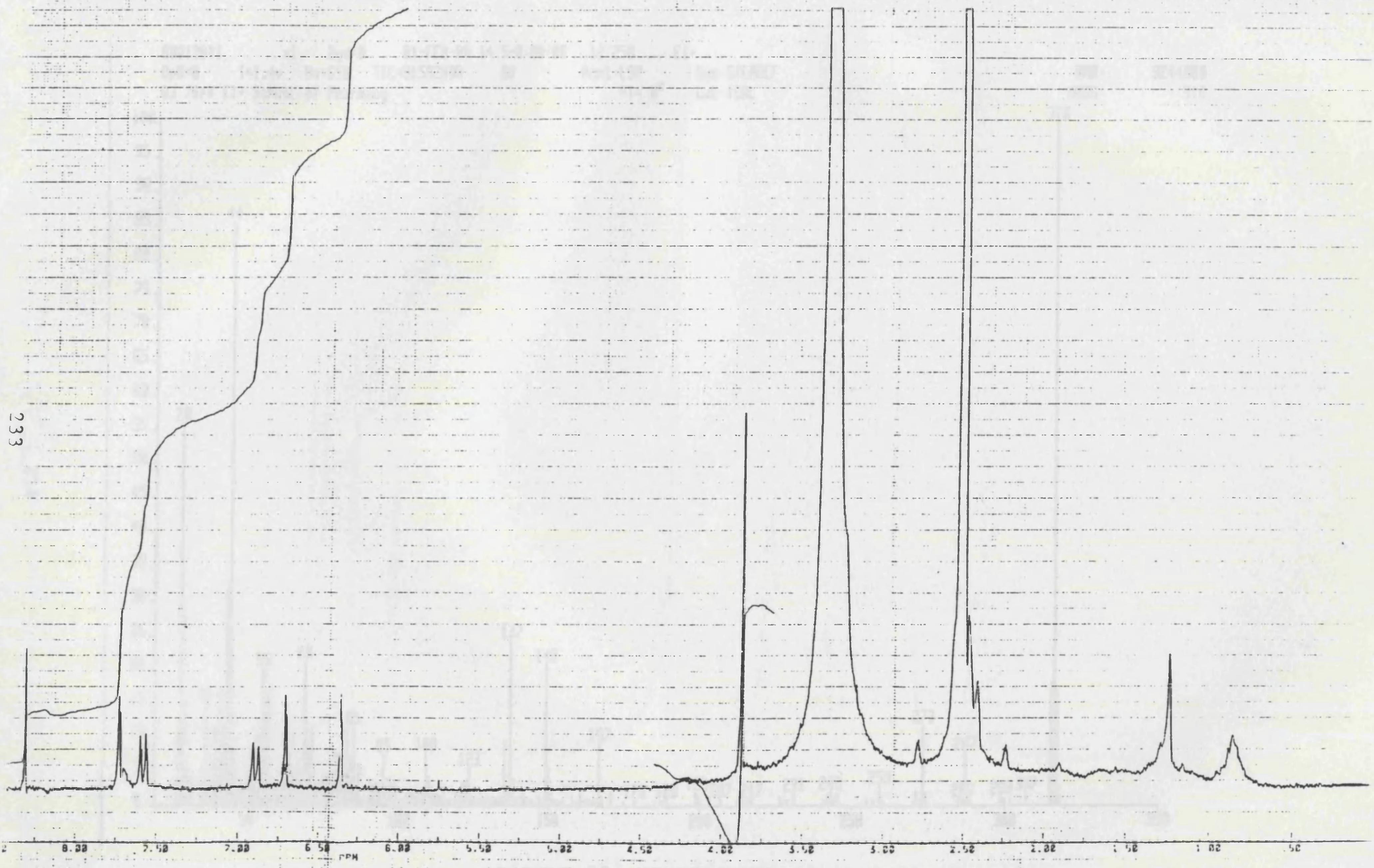

FIGURE 82 'H NMR SPECTRUM OF RHAMNETIN

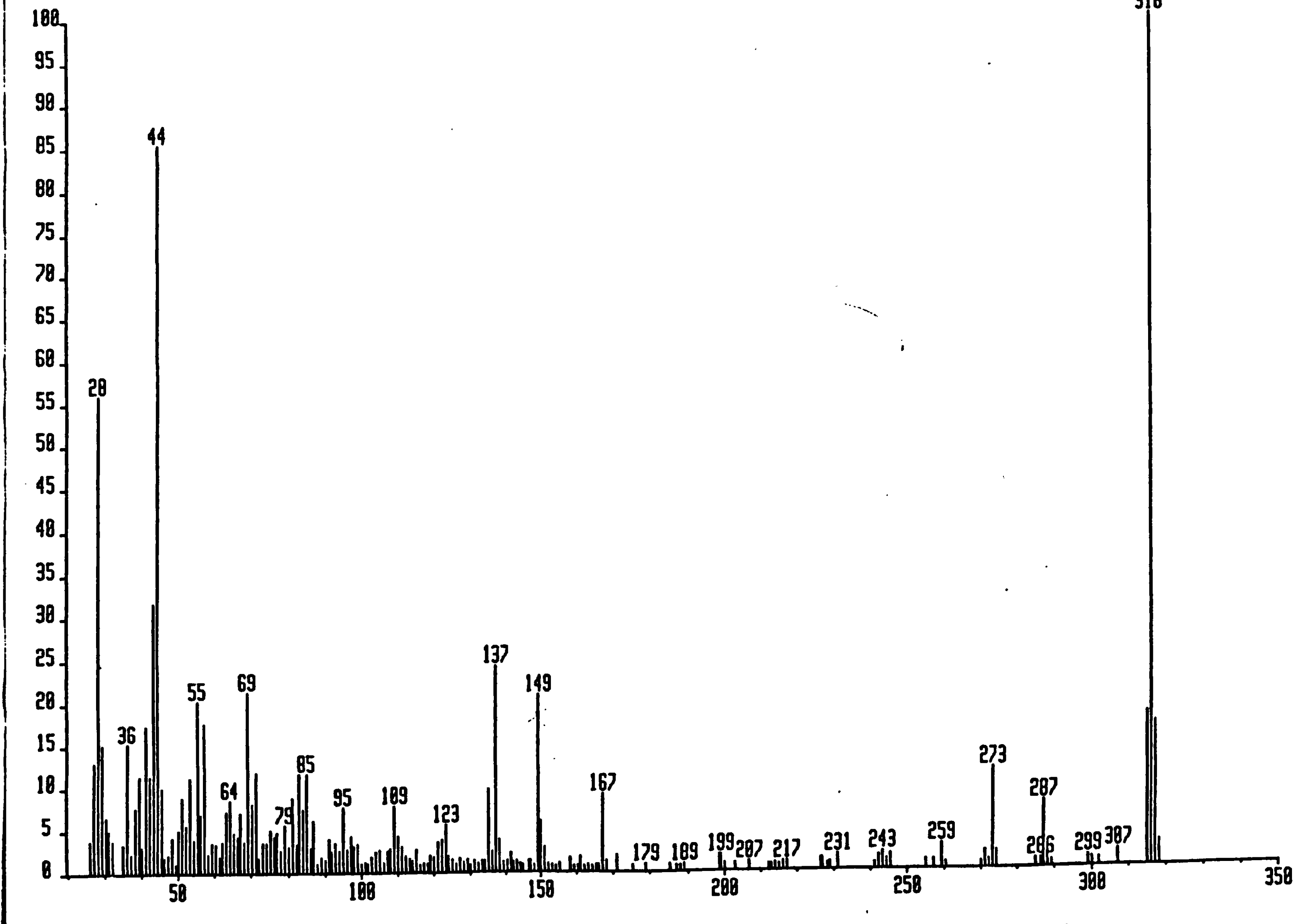

FIGURE 83. MS SPECTRUM OF RHAMNETIN

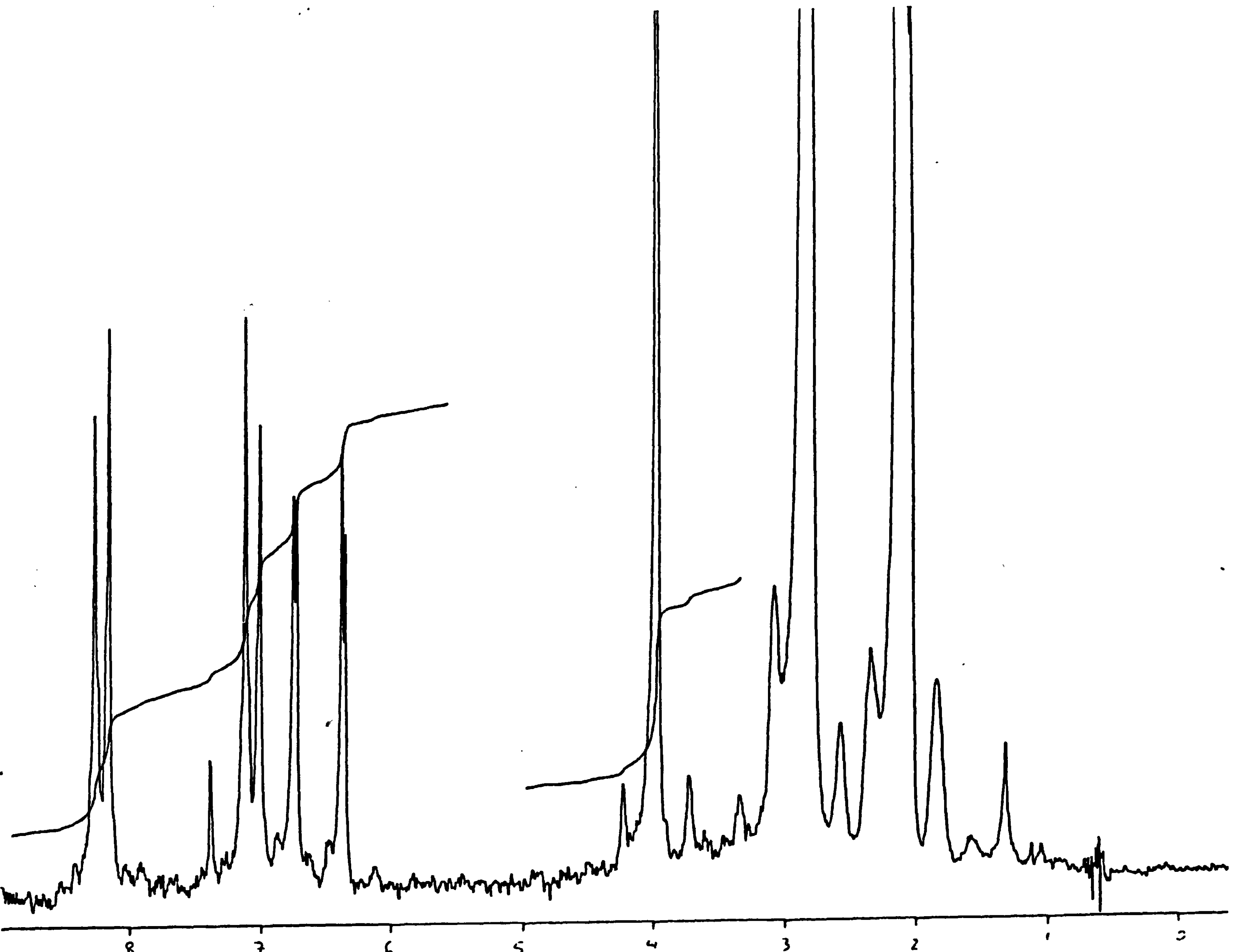

FIGURE 84 'H NMR SPECTRUM OF RHAMNOCITRIN

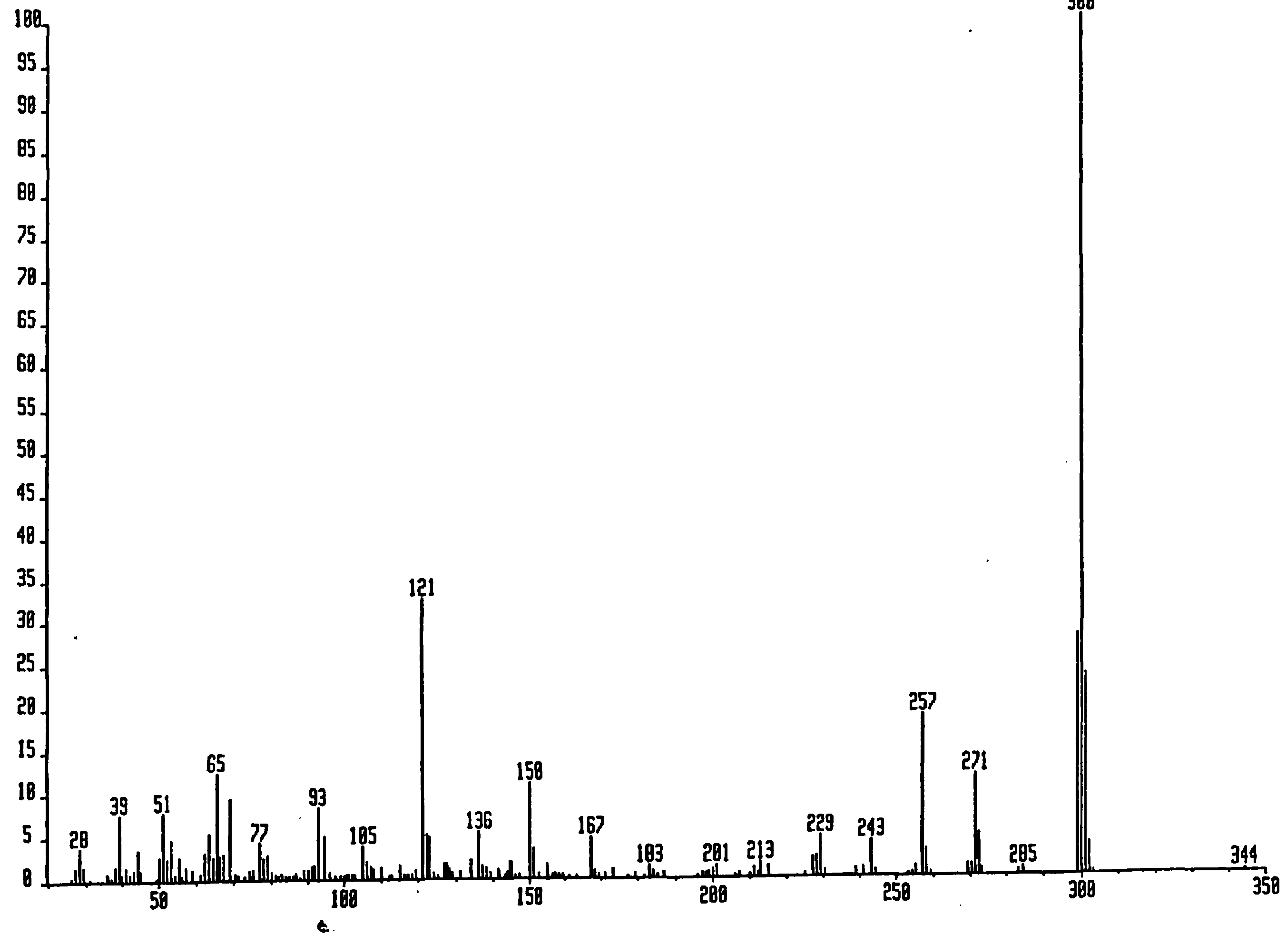

FIGURE 85. MS SPECTRUM OF RHAMNOCITRIN

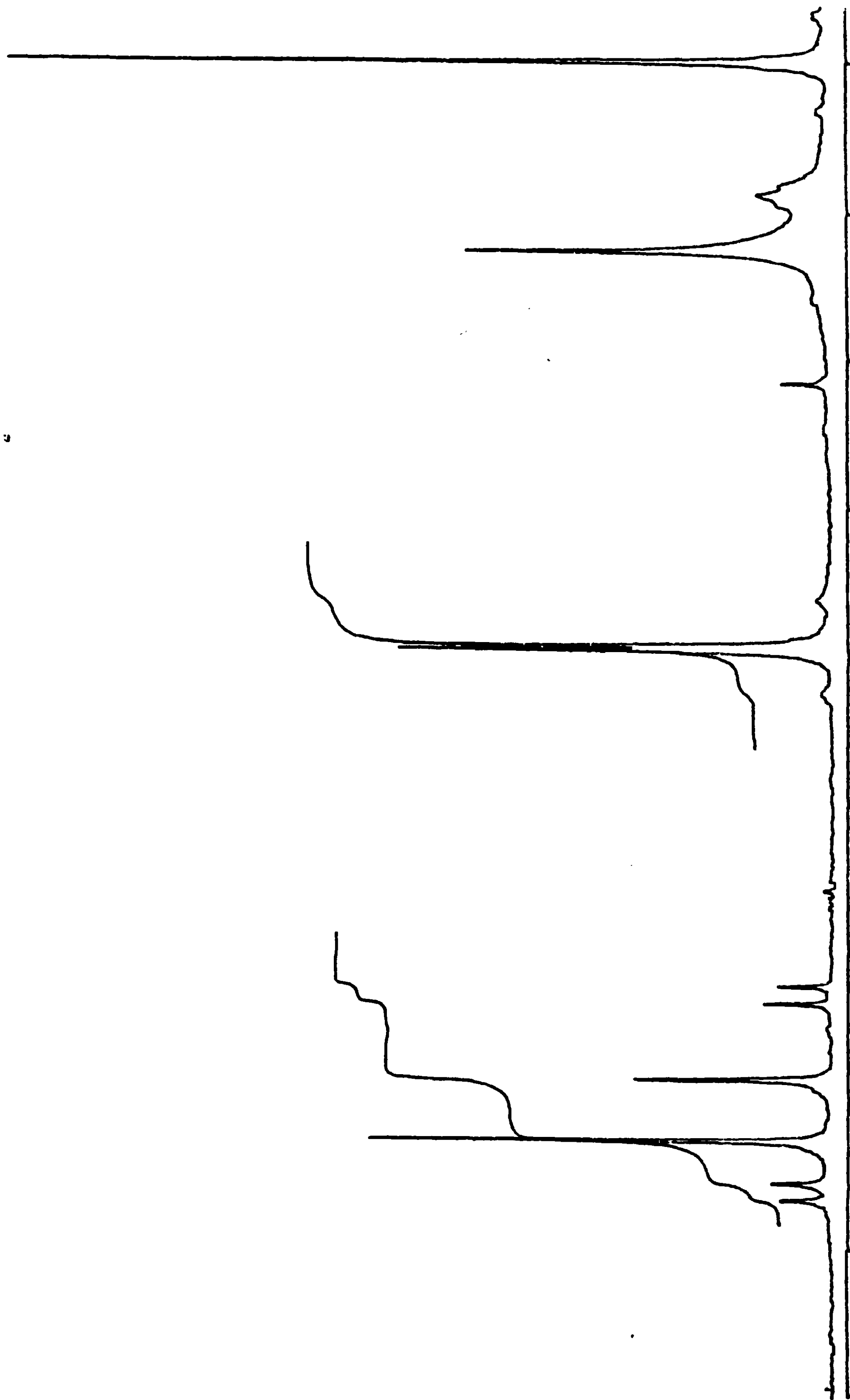

FIGURE 86, NMR SPECTRUM OF SCOPARONE

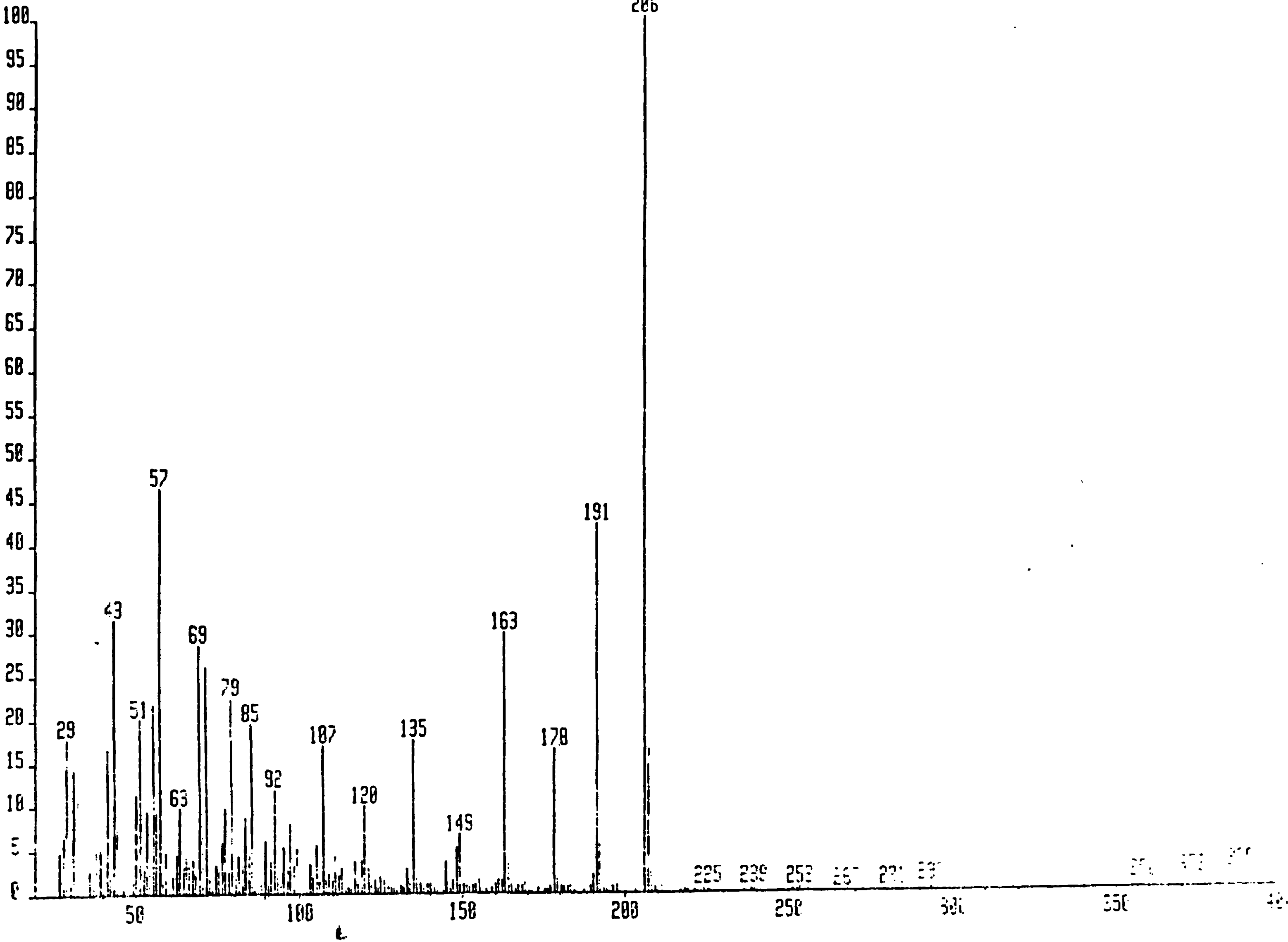

FIGURE 87 | MS SPECTRUM OF SCOPARONE

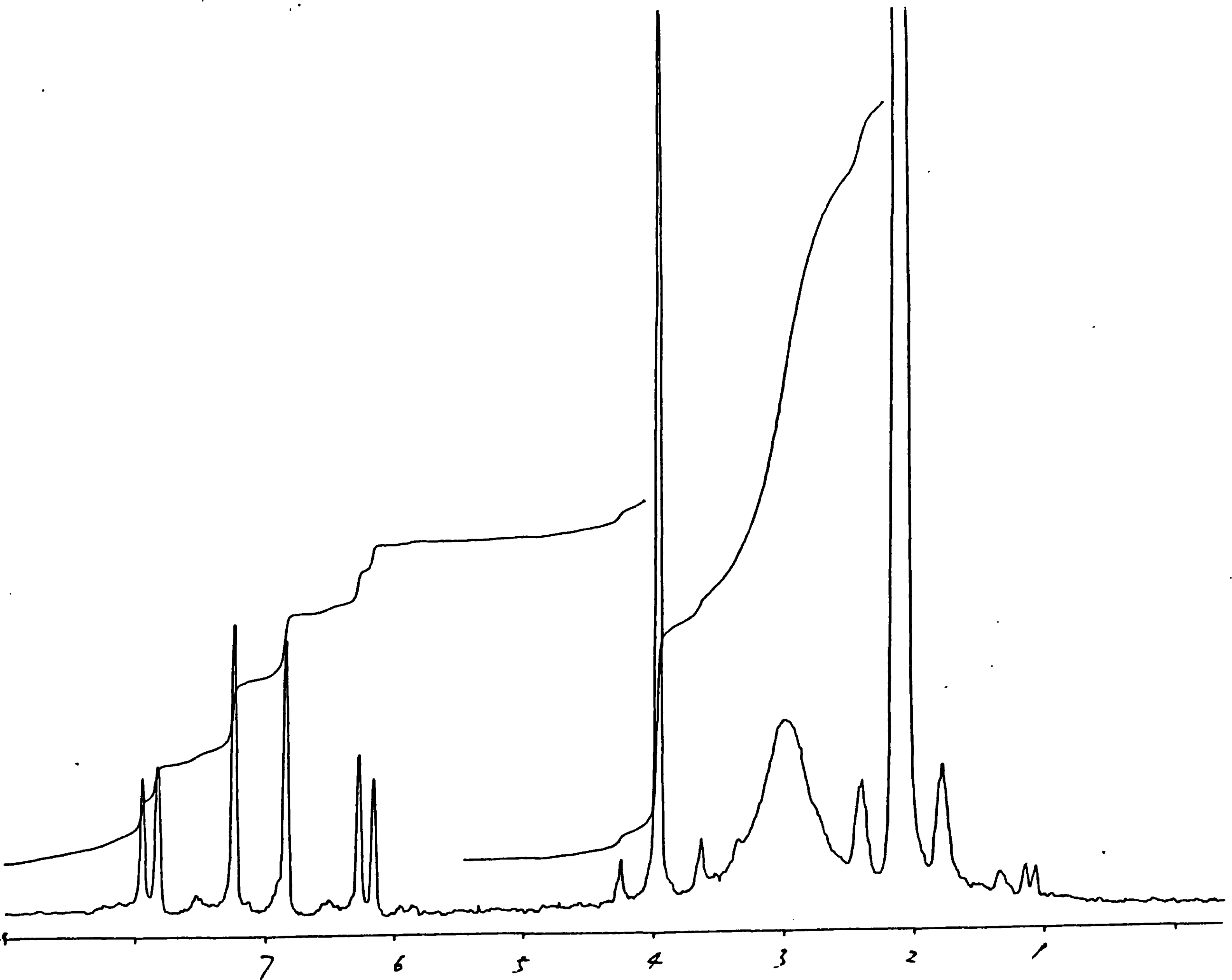

FIGURE 88. NMR SPECTRUM OF SCOPLIN

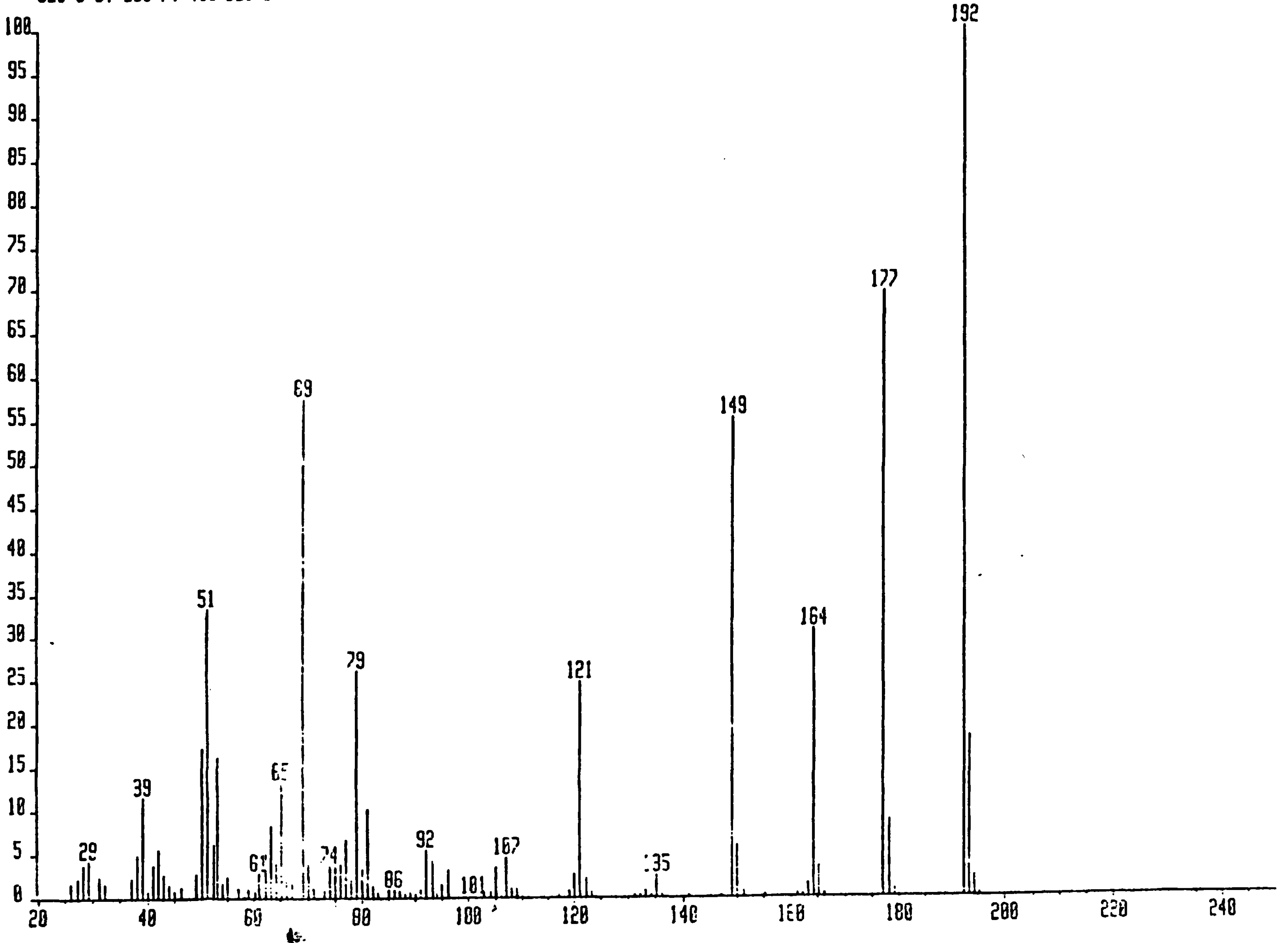

FIGURE 89. MS SPECTRUM OF SCOPLIN

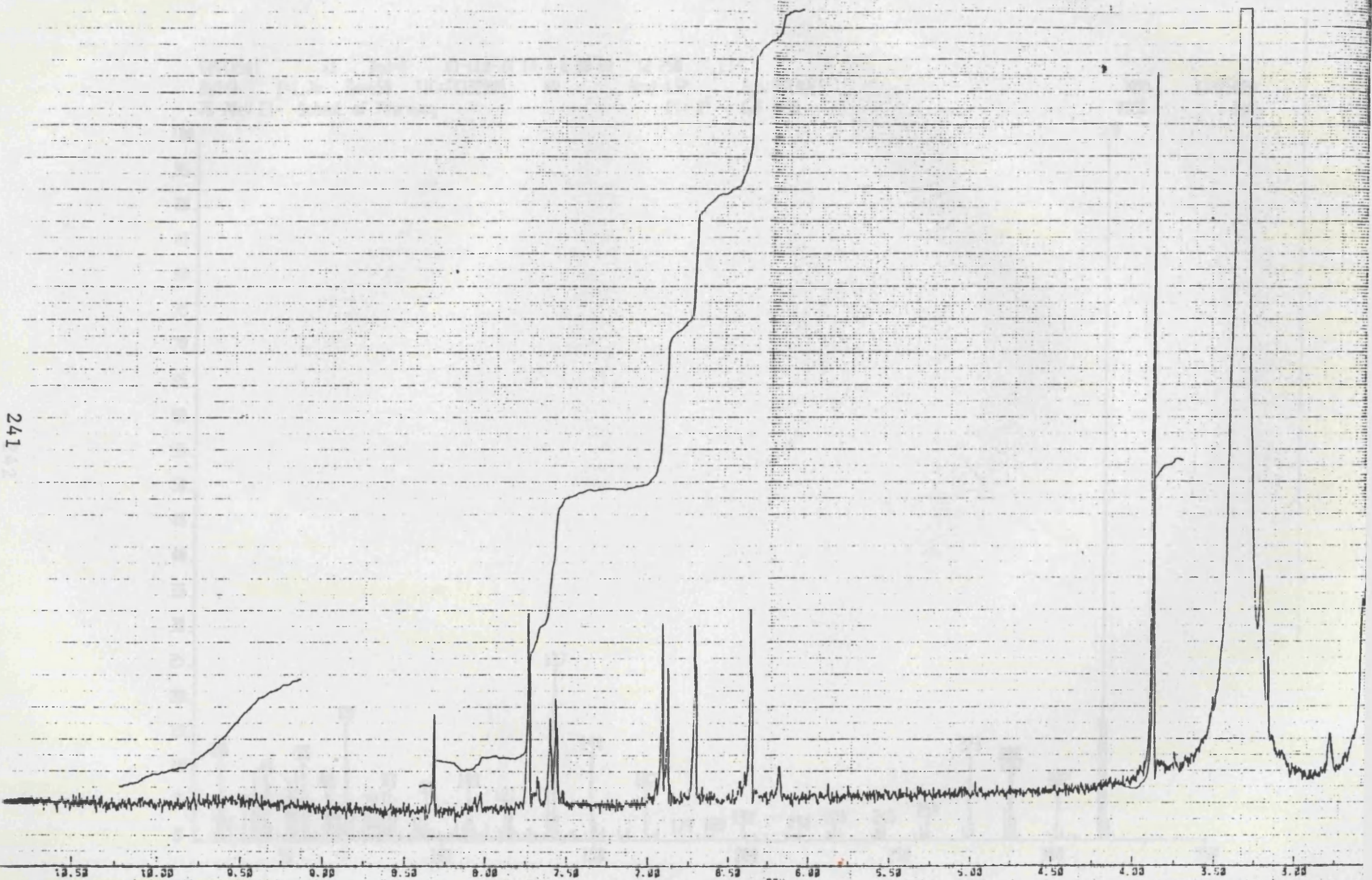

FIGURE 90 'H NMR SPECTRUM OF TAMAXIRITIN

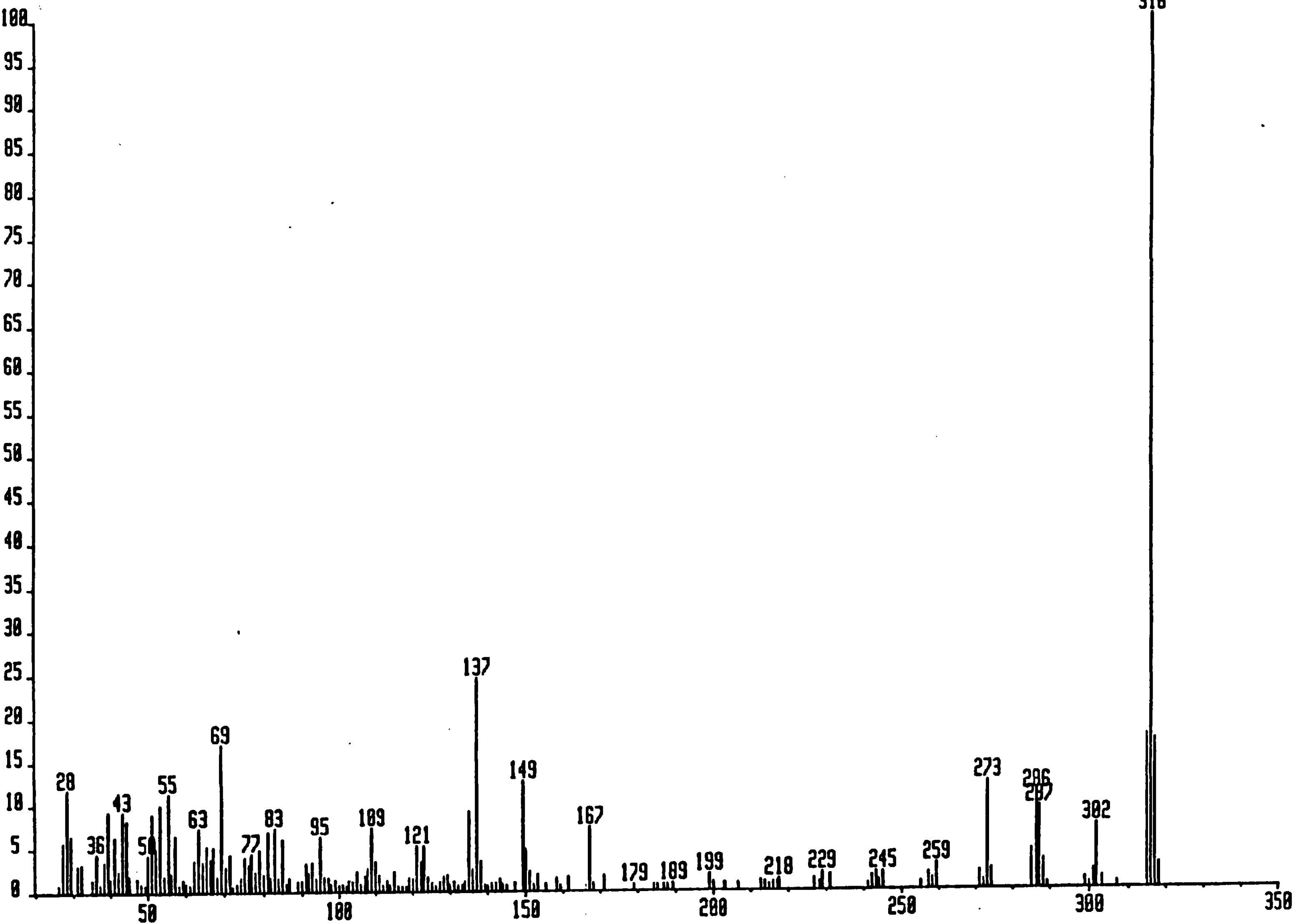

FIGURE 91 MS SPECTRUM OF TAMAXIRITIN

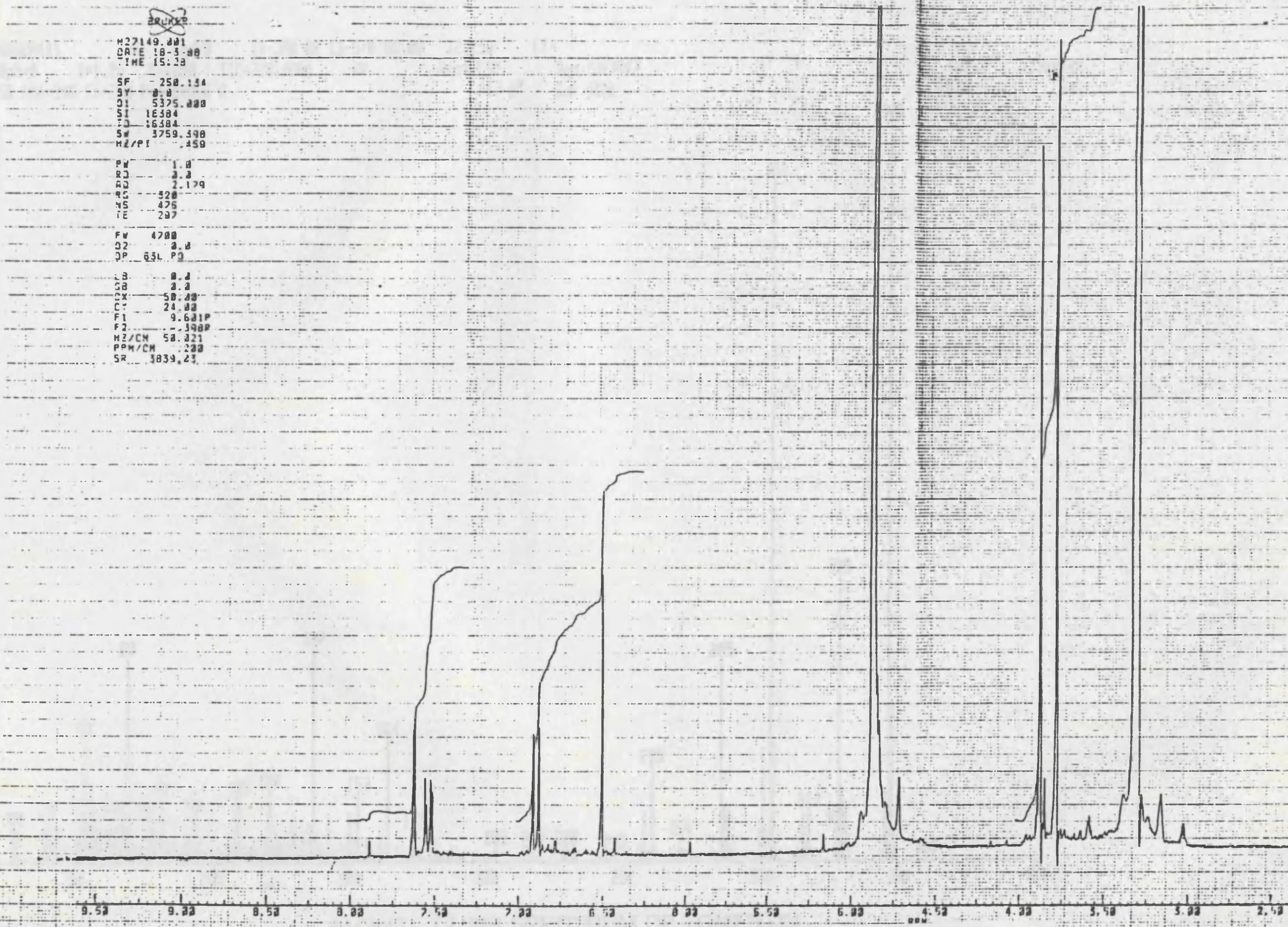

FIGURE 92 NMR SPECTRUM OF TOMENTIN

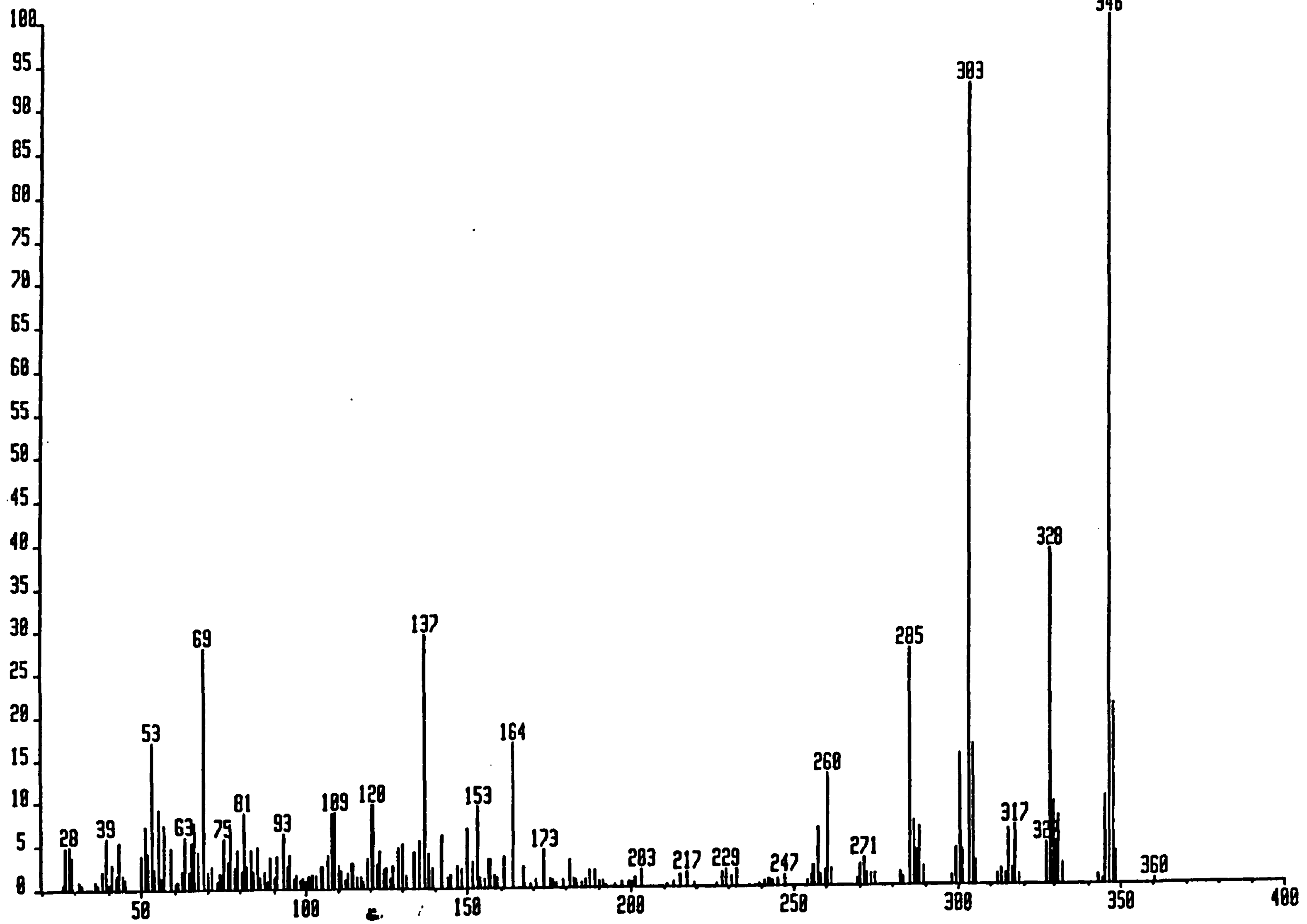

FIGURE 93 MS SPECTRUM OF TOMENTIN

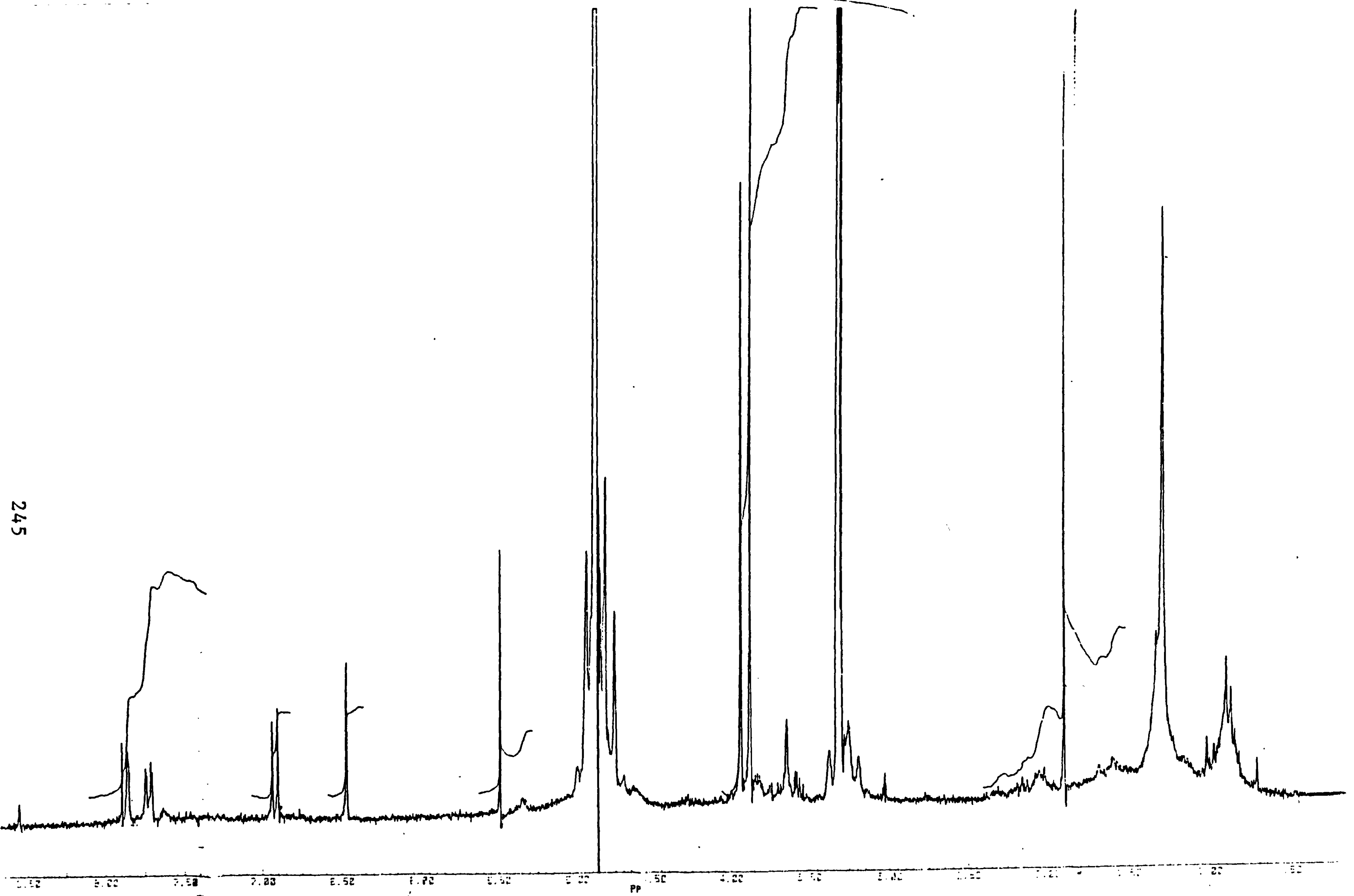

FIGURE 94 NMR SPECTRUM OF 5,7,8,3'-TETRAHYDROXY-3,4'-DIMETHOXYFLAVONE

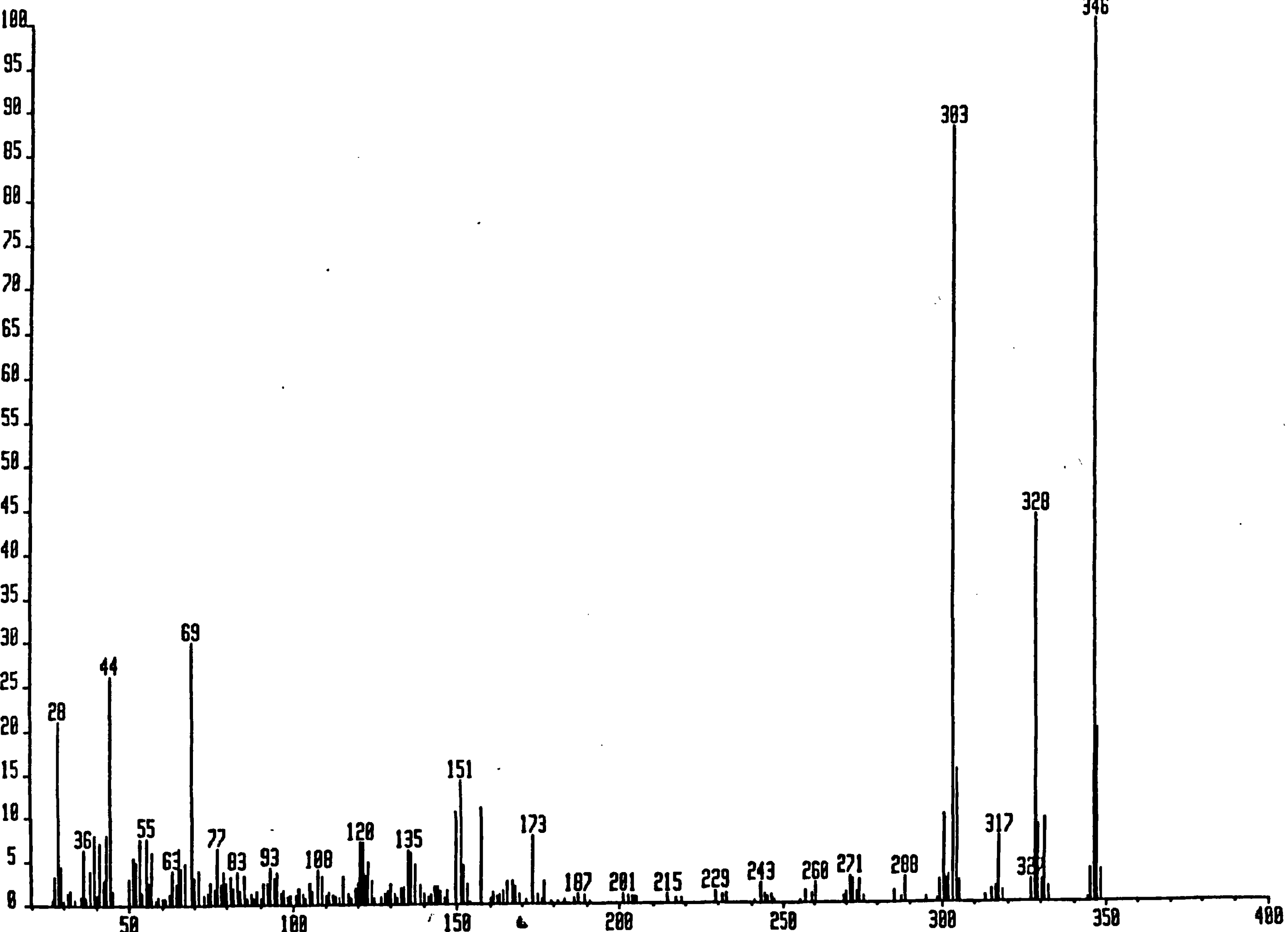

FIGURE. 95 - MS SPECTRUM OF 5,7,8,3'-TETRAHYDROXY-3,4'-DIMETHOXYFLAVONE

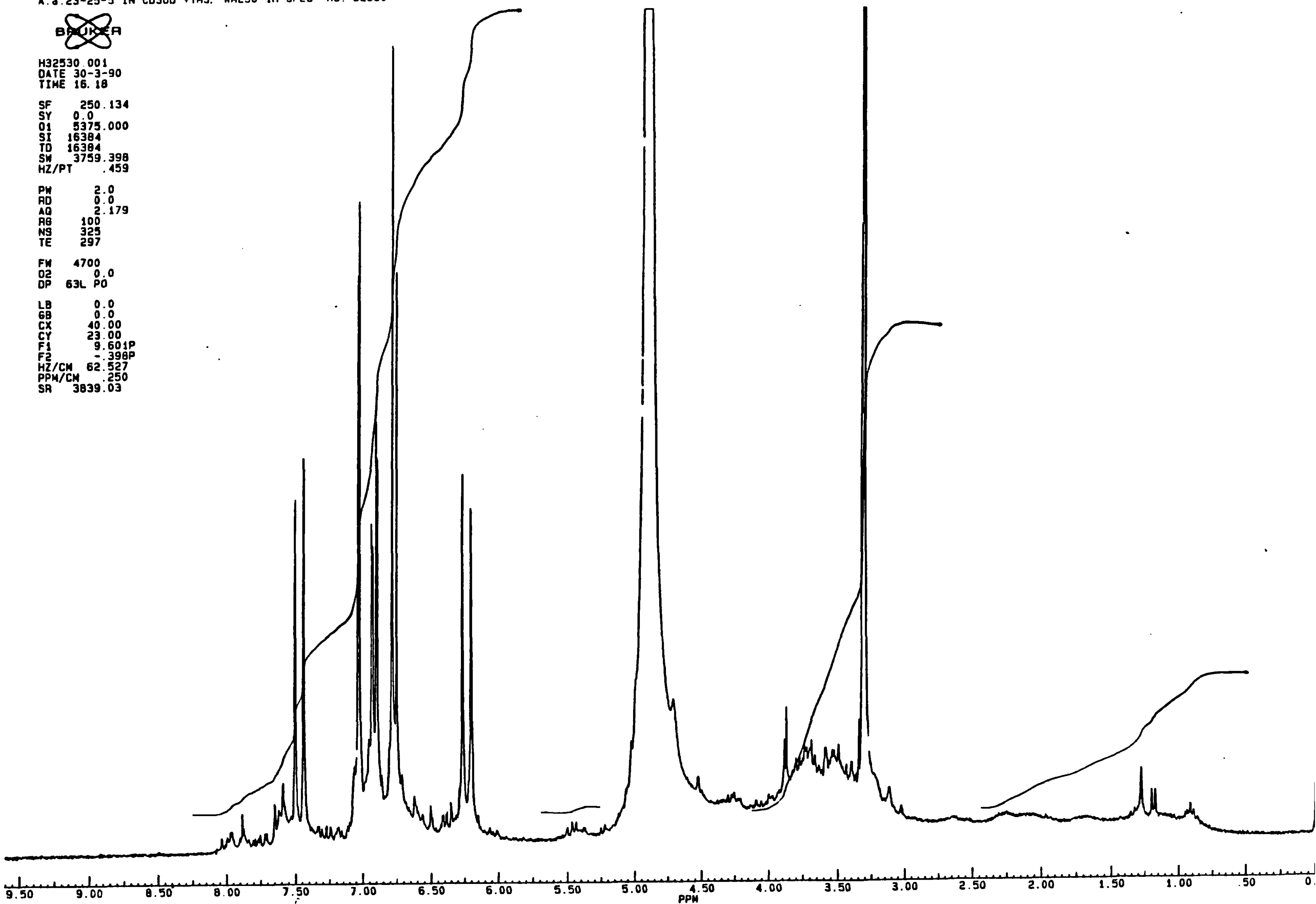

FIGURE 96 NMR SPECTRUM OF 2,2,6-TRIHYDROXYLCHROMENE

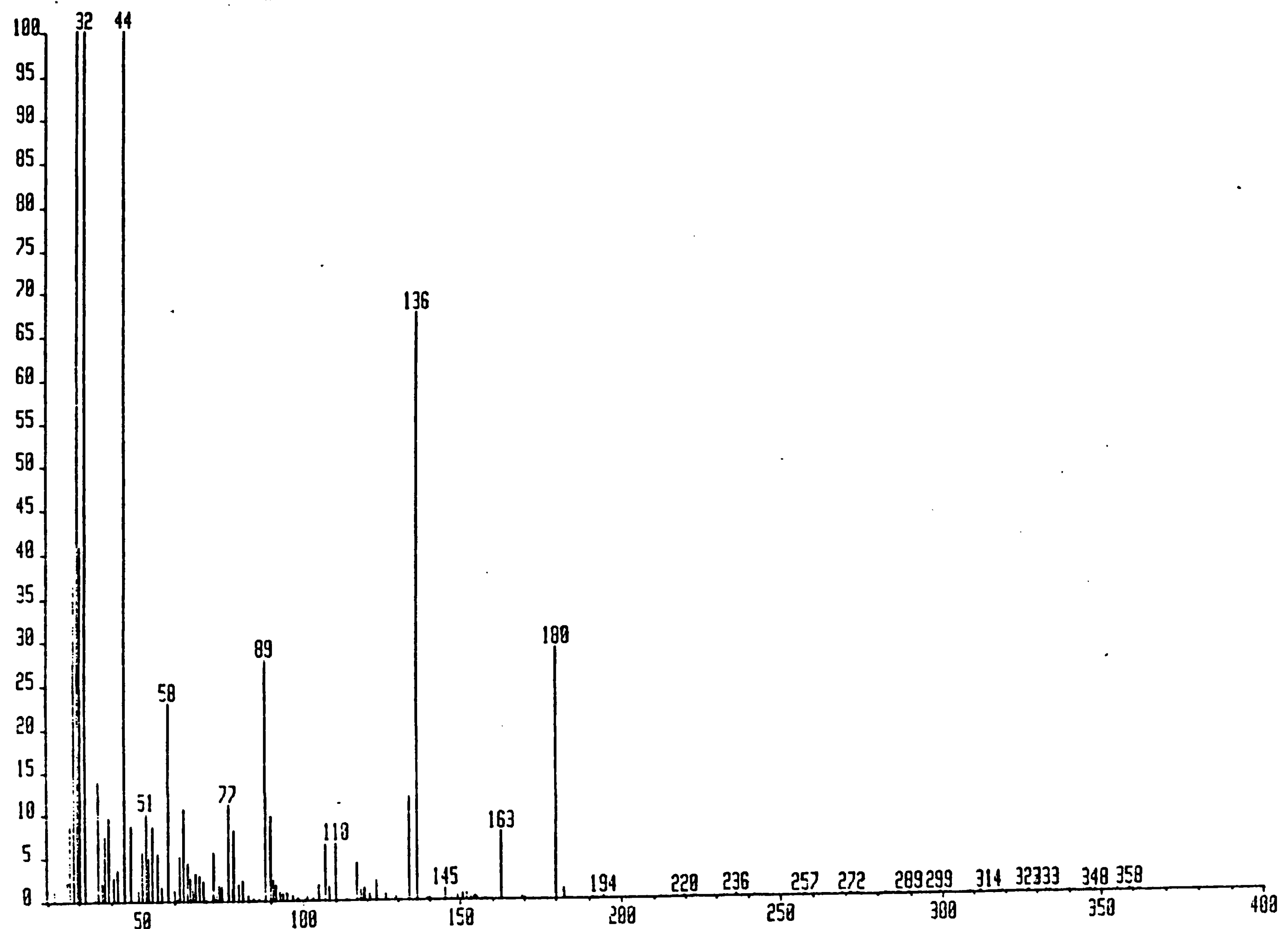

FIGURE 97. MS SPECTRUM OF 2,2,6-TRIHYDROXYLCHROMENE

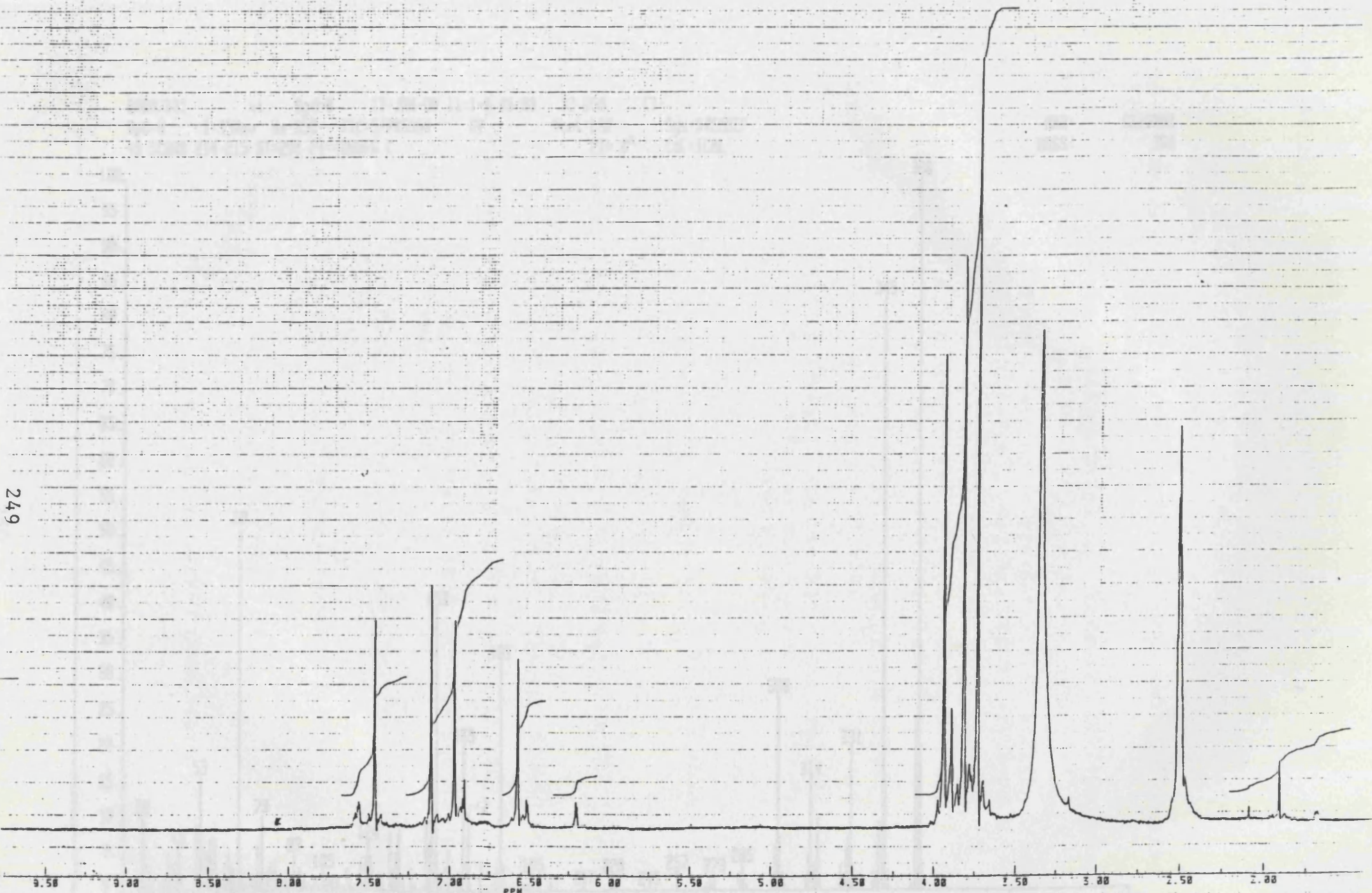

FIGURE 98 ^{1}NMR SPECTRUM OF 5,2',4'-TRIHYDROXY-6,7,5'-TRIMETHOXYFLAVONE

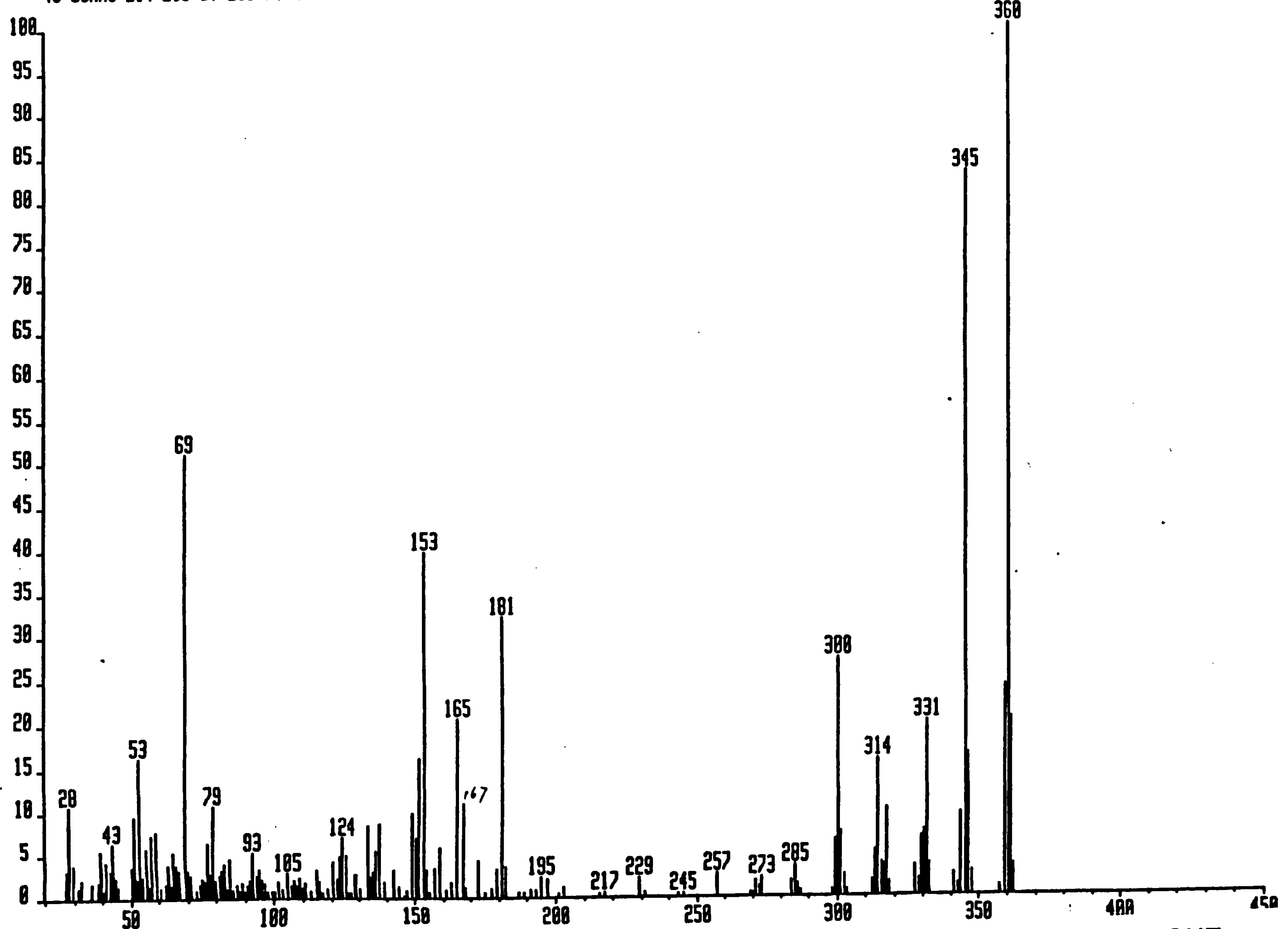

FIGURE 99 MS SPECTRUM OF 5,2',4'-TRIHYDROXY-6,7,5'-TRIMETHOXYFLAVONE

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