# MOLLUSCICIDES AND OTHER COMPOUNDS FROM INDIGENOUS PLANTS

By

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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science

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December 96.

## DECLARATION

I hereby certify that this research is a result of my own investigation which has not already been accepted in substance for any degree and is not being submitted in candidature for any other degree.

Signed :.....

Louis KAYONGA

I hereby certify that this statement is correct.

Signed : ..... 10/2/97.

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December 1996

## Contents

# ACKNOWLEDGEMENTS

Unfortunately, words are not adequate enough to express the deep gratitude one feels from the bottom of one's heart.

Therefore, I feel incapable to express myself in front of the numerous individuals who contributed towards the successful completion of this project.

Nevertheless, a debt of gratitude is due to each of them.

I would like to acknowledge the meticulous supervision of Prof. S.E. Drewes who helped me in various ways : thank you for stimulation, guidance and rigorous criticism that you provided.

This project would not have been at all possible without you. The human relationships established, the confidence and the financial assistance are acknowledged and appreciated.

Thanks go to Umgeni Water Board which was very helpful in helping me financially and special thanks are owed to its Scientific Division for creating a happy working environment.

Grateful acknowledgements are also due to Prof.C.C. Appleton for the documentation provided and for the bioassays performed in his laboratory under his supervision.

A special word of thanks must go to Dr. Tanza E. Clark and to Ms T. Brackenbury whose time and assistance in conducting bioassays, kindness and involvement went beyond what could be required.

I am grateful to the staff of the Chemistry Department who morally supported me during the difficult moments and helped me in researching.

I gratefully acknowledge Mrs Marion Horn for helpful suggestions with respect to my research.

A very special thanks to my colleagues, for knowing how to create an atmosphere full of understanding.

I wish to express my deep gratitude to Mr M.Watson and Mrs Z. Hall for NMR and mass spectra. Your kindness is greatly appreciated.

To Mr. H. Desai and Mr. R. Somaru for their excellent work and help in the laboratory.

To Mr. P. Forder for glassbolowing and his welcoming into his midst as a friend.

To Mr. G.D.Crawley and his staff for technical assistance.

Finally, I'm specially grateful to my wife, Beatrice Kayonga for the patience with typing the script.

To my family: my wife, my daughters Sandrine and Sonia and to my sister Germaine for their continued support and encouragement.

# ABSTRACT

The use of plants with molluscicidal properties is a simple, inexpensive and appropriate technology for focal control of the snail vector.

Isolation and identification of the active constituents is essential for the study of their toxicities and stabilities under field conditions, for dosage purposes, for structure-activity investigations and for effects on snail metabolism or physiology. It is in this context, that an investigation into molluscicides and other compounds from indigenous plants has been carried out.

From *Apodytes dimidiata* subsp. dimidiata, betulinic acid (**53**) genipin (**49**) and 10-acetyl genipin (**58**) have been isolated and the last two have proved to possess molluscicidal activity. Furthermore, 4-ethylcatechol (**60**) isolated from *Gardenia thunbergia* is one of the most powerful naturally-occurring molluscicides.

## PUBLICATIONS

1. Drewes, S.E., Kayonga, L., Clark, T.E., Brackenbury, T.D. and Appleton, C.C. (1996) *J.Nat.Prod.* in press.

2. Brackenbury., T.E., Appleton, C.C. and Kayonga, L. (1996) *S. A. Jnl. Sc.* submitted.

3. Brackenbury, T.E., Appleton, C.C., Drewes, S.E. and Kayonga, L. (1996) *Journal of Medical & Applied Malacology* in press.

## Introduction

Primitive as well as civilised societies have always had an extensive tradition of the use of particular plants and plant preparations both for healing and killing[1]. In the last decade, plant molluscicides have received considerable attention in the search for cheaper alternatives to chemotherapy and synthetic molluscicides in schistosomiasis control. The attraction of a locally grown molluscicidal plant is based on the development of a philosophy of self-reliance and community involvement. It is in this context that an investigation on snail-killing compounds of indigenous plant origin for the control of the tropical infection, schistosomiasis, has been initiated.

## 1.1 Background

Schistosomiasis is a severe, debilitating disease, and most common among rural and agricultural communities living near slow-moving or stagnant water in the tropical and sub-tropical parts of Africa, the Middle East, the Far East, some of the Caribbean Islands and many parts of South America.

There are now 76 countries in which schistosomiasis is endemic, with more than 600 million people at risk of infection and some 200 million infected[2].

Since this estimate was arrived at, the World population has continued to increase with an enlargement, in most endemic areas, in the lower strata of the population pyramid, i.e. an increase in the age-groups known to have the highest prevalence of disease and to be most responsible for transmission[3]. Humans disease-risks i.e. schistosomiasis, are known to increase with an intensification of water related activities[4].

### 1.1.1 Life cycle

Several species of Schistosomes exist and are acquired by human through contact with infected fresh water. The parasites rapidly penetrate the skin, migrate and develop to maturity in the blood vessels of the intestines ("intestinal schistosomiasis") or of the urinary bladder ("urinary schistosomiasis"). The female worms produce eggs which, when numerous, cause disease.

Increased egg deposition leads to a greater probability of serious disease developing. The most eggs are deposited in the body organs. The eggs leave the body in the faeces, or in the urine according to whether it is intestinal or urinary schistosomiasis. If the eggs reach fresh water, an immature form of the parasite, miracidium, hatches and may then enter a particular type of snail in which it is further transformed. After full development in the snail, new larval forms of the parasite, cercariae, escape. These are capable of rapidly penetrating the human skin[5].
This is how schistosomiasis is spread into new areas.

## 1.2 Control of schistosomiasis

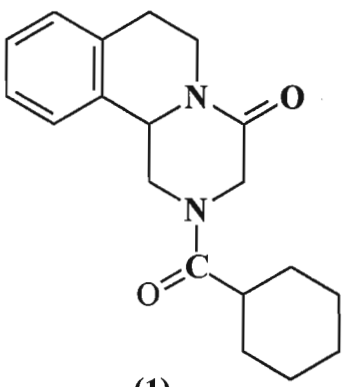
The control and treatment of schistosomiasis has been characterized by an integrated attack against the different stages of the parasite's life cycle:
a) by providing a safe water supply which protects humans from infection by decreasing contact with infected water;
b) by improving sanitation, which protects the snail from infection;
c) by chemotherapy, to kill the adult worms, which offers direct benefit to all infected individuals who receive the treatment;
d) by snail control, to kill both the vector and the larval stages of the parasite, which benefits the community as a whole by halting transmission[6].

a&b) Control by environmental means.

The solution of schistosomiasis problem lies mainly in the provision of adequate water supplies and sanitation in endemic areas. Long term control is therefore based on improvements in basic living conditions and changes in human behaviour.
However, the costs of providing potable water are prohibitively expensive.

c) Treatment.

One of the drugs Praziquantel (**1**) EMD 29810, Embay 8440 "Baltricide", which has a broad spectrum of activity is available and effective against both urinary and intestinal bilharzia and only a single dose is necessary, but it is expensive. Where the basic problem of bad water supplies exists, constant treatment is required, which increases the cost. Chemotherapy will undoubtedly play a vital role in reducing the severity of the disease and contribute significantly to the control of transmission by lowering egg output[7]. However, in most African countries only 20% of inhabitants have access to modern health care facilities and drugs.




**(1)**

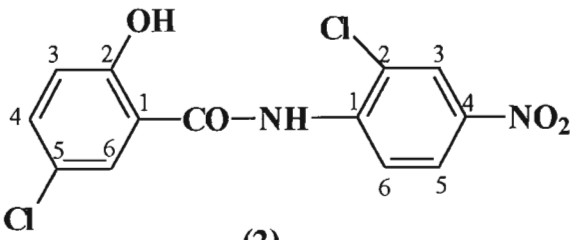
d) Snail control by molluscicides.

The intermediate snail host has been considered to be one of the weakest links in the life cycle of the parasite[8,9]. Destroying the snails which harbour the developing schistosome larvae is one way to interrupt the parasite's life cycle and prevent human infection[5].

The application of molluscicides to water in which snails live is probably the most widely used and considered by many the most effective single measure in control of bilharzia. It is aimed at reducing snail numbers to such a level that transmission no longer occurs.

### 1.2.1 Synthetic molluscicides

Numerous synthetic molluscicides have been used in the control of the intermediate hosts of schistosomiasis. These include : copper sulphate ($CuSO_4$) sodium pentachlorophenate (NaPCP), N-tritylmorpholine (sold under the trade mark Frescon) and Niclosamide (**2**) (tradename Bayluscide)[10,11].




**(2)**

From these, Niclosamide (**2**) has emerged as the only remaining commercially available product, and it is an excellent molluscicide with few disadvantages.

It was first synthesized in 1958[5] and registered for use in South Africa in 1982[12]. Although highly effective, the relatively high cost of this compound, the expenses required to apply it, and the long-term impact of synthetic chemicals on the environment[13] limit its use to endemic countries.

The high cost of control measures has simply deterred control operations and made more urgent the need for development of less costly technology and less toxic substances[14]. Of the others, copper sulphate was too easily removed from solution by organic matter.

Sodium pentachlorophenate was too toxic to man and N-tritylmorpholine, though toxic to adult snails at very low concentration, was too unstable unless the water has a pH of 8.0 or more, and was not toxic to snail eggs.

Several other compounds such as nicotinanilides, organotin and organolead compounds have almost made it to the market without ever being successful on a large scale[15]. No new outstanding, novel, synthetic molluscicide has been developed in the past decade. Industry has claimed that this is due to high development costs and the lack of an assured market[16]. No drug

or chemical molluscicide will be successful, no matter how safe or effective unless it is cheaply available[17].

Finances are one of the most fundamental factors in controlling disease in Third World Countries[5]. The need for less costly control operations and the pioneering research efforts of Dr Akilulu Lemma on Phytolacca dodecandra, commonly known as *Endod* in Ethiopia, has resulted in a surge of interest in plant molluscicides. Plant products as molluscicides are an attractive alternative, not only because many are readily available but the possibility exists that they can be utilised on a "self-help" basis in rural areas[5].

In short, plants offer a wide array of compounds which on extraction may show insecticidal, piscicidal, molluscicidal, medicinal and other biological activities[18]. Plant compounds can either be extracted directly and used in their natural state or serve as models for chemists to synthesize identical compounds or analogues[19]. One of the possible benefits of the use of indigenous plant compounds in their natural state is that they may provide economic advantages which outweight the relatively higher activity of better characterized but more costly synthetics[20].

### 1.2.2 Plants with molluscicidal activity

A very large number of plant extracts has been investigated for molluscicidal activity and guidelines for further screening of numerous species have been outlined[21]. Despite all the data available for plant sources, very little is known about the active principles themselves; only about 80 natural products with recognised molluscicidal activity have been isolated from plants[10].

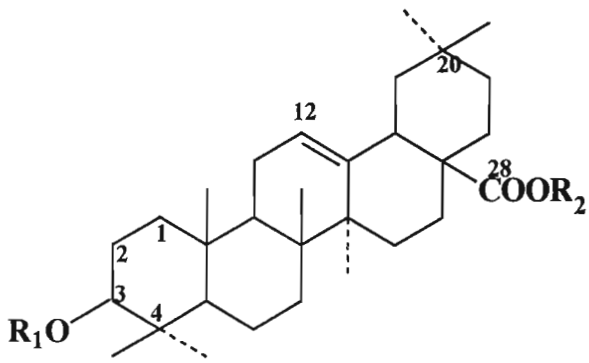
Table 1.Major Classes of Natural products with recognized Molluscicidal activity[5].

| Class of compound | Plant | Family |
|---|---|---|
| Triterpenoid saponins | Phytolacca dodecandra | Phytolaccaceae |
| | Hedera helix | Araliaceae |
| | Lonicera nigra | Caprifoliaceae |
| Spirostanol Saponins | Cornus florida | Cornaceae. |
| | Balanites aegyptica | Balanitaceae. |
| | Asparagus curillus | Liliaceae. |
| Steroid glycoalkaloids | Solanum mammosum | Solanaceae. |
| Diterpenes | Wedelia scaberrima | Asteraceae. |
| | Baccharis trimera | Asteraceae. |
| Sesquiterpenes | Warburgia ugandensis | Canellaceae. |
| | Ambrosia maritima | Asteraceae. |
| | Podachaenium eminens | Asteraceae. |
| Monoterpenes | Genus Lippia | Verbenaceae. |
| Iridoids | Olea europaea | Oleaceae. |
| Naphthoquinones | Diospyros usambarensis | Ebenaceae. |
| Alkenyl phenols | Anacardium occidentale | Anacardiaceae. |
| Chalcones | Polygonum senegalense | Polygonaceae. |
| Flavonoids | Baccharis trimera | Asteraceae. |
| | Polygonum senegalense | Polygonaceae. |
| | Polygonum nodosum | Polygonaceae. |
| Tannins | Acacia nilotica | Fabaceae. |
| Furanocoumarins | Ruta chalepensis | Rutaceae. |
| Isobutylamides | Heliopsis longipes | Asteraceae. |
| | Fagara macrophylla | Rutaceae. |
| Alkaloids | Calpurnia aurea | Fabaceae. |

Triterpenoid Saponins.

The molluscicidal properties of *Phytolacca dodecandra* (*Phytolaccaceae*) berries were discovered by Lemma in 1964 and this plant has subsequently become of great potential importance for the local control of schistosomiasis[5]. The compounds responsible for activity are triterpenoids saponins with $LD_{100}$ values as low as 2ppm.

Compounds **4 - 8** are glycosides of oleanolic acid (**3**).




**(3)**

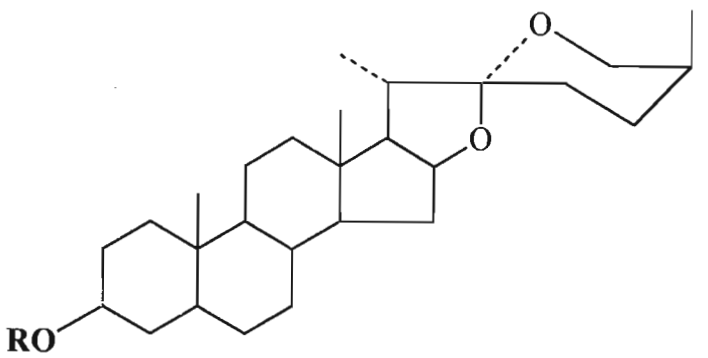
**$R_1 = R_2 = H$ : Oleanolic acid.**

Table 2.Molluscicidal activities of triterpene glycosides.

| Compound | $R^1$ | $R^2$ | Plant | Moll.activity (ppm) |
|---|---|---|---|---|
| **4.** | Glc A- | H | *Lonicera nigra* | 2 ($LD_{100}$) |
| **5.** | $Glc^2$ -Ara- | H | *Lonicera nigra* | 2 ($LD_{100}$) |
| **6.**Lemmatoxin C | Rha $^2Glc^2$-Glc | H | *Phytolacca dodecandra* | 3 ($LD_{90}$) |
| **7.**Oleanoglycotoxin-A | $Glc^4$-Glc<br>\|$^2$<br>Glc | H | *Phytolacca dodecandra* | 6 ($LD_{100}$) |
| **8.**Lemmatoxin | $Glc^4$ -Glc<br>\|$^3$<br>Gal | H | *Phytolacca dodecandra* | 1.5($LD_{90}$) |

Spirostanol Saponins.

The first report on the use of a plant for the control of schistosomiasis involved fruits of the desert palm *Balanites aegyptica (Balanitaceae)*. This Sudanese medicinal plant, which is an effective fish poison, kills both molluscs and cercariae of Schistosomes. A number of other spirostanol glycosides are known to be molluscicides (**9**). Glycosides are known to be molluscicides. Examples are compounds (**10**) & (**11**).



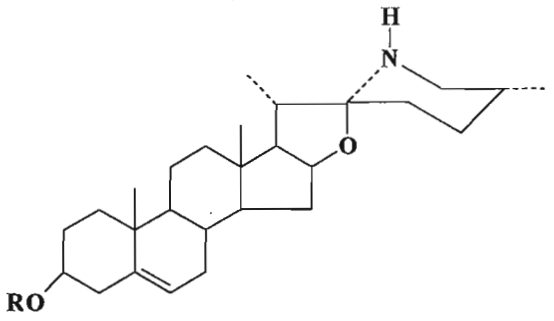

**(9) R = H Sarsapogenin.**

Table 3. Molluscicidal activities of Spirostanol saponins.

| Compound | R | Plant | Moll.activity ppm ( $LD_{100}$) |
|---|---|---|---|
| **10.** | Xyl-$^{2}$Glc- | *Cornus florida* | 6 |
| **11.** | Rha-$^{4}$Glc-<br>$\vert^{2}$<br>Glc | *Asparagus curillus* | 20 |

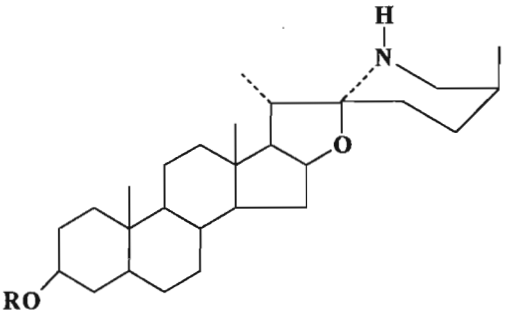
Steroid glycoalkaloids.

Three azaspirostanol saponins, isolated from members of the family Solanaceae are toxic to snails (Table 4)[5].



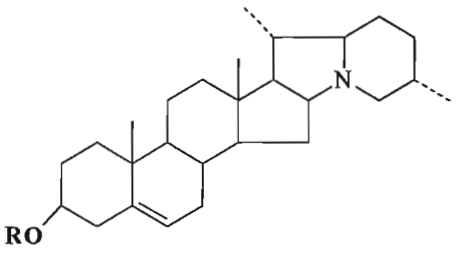

**(12) R = H Solasodine.**

**(A)**




**(13) R = H Tomatidine.**

**(B)**




**(14) R = H Solanidine.**

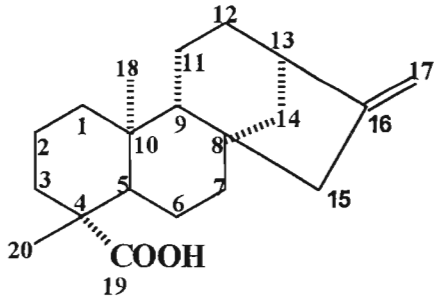
**(C)**

Table 4.Molluscicidal activities of steroid glycoalkaloids.

| Compound | Aglycone | R | Plant | Moll.activity ppm ($LD_{100}$) |
|---|---|---|---|---|
| **15.**Solasonine | A | $Glc^3$-$Gal^2$<br>$\vert^2$<br>Rha | *Solanum mammosum* | 10 |
| **16.** Tomatine | B | $Xyl^3$-$Glc^4$-Gal<br>$\vert^2$<br>Gal | *Lycopersicon esculentum* | 4 |
| **17.** α-Solanine | C | Glc-$^3$Gal<br>$\vert^2$<br>Rha | *Lycopersicon esculentum* | Not recorded. |

Diterpenes.

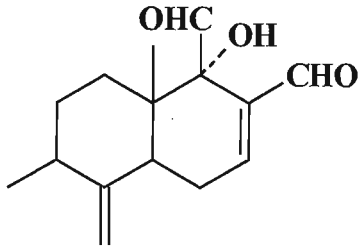
Kaur-16-en-19-oic acid (**18**) was found to be molluscicidal against *Biomphalaria glabrata* but not details of activity were given.



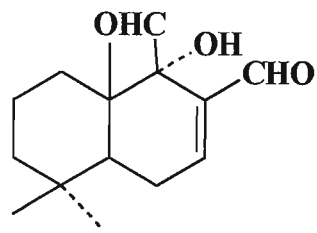

**(18)**

Sesquiterpenes.

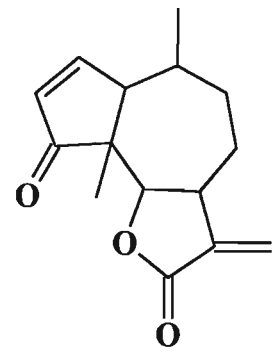
The simple sesquiterpenes muzigadial (**19**) warburganal (**20**) and sesquiterpene lactone ambrosin (**21**) have potent molluscicide properties. Indeed, *Warburgia* species and *Ambrosia* species occur quite frequently in those parts of the World where schistosomiasis is endemic. *Ambrosia maritima* is molluscicidal, ovicidal and harmless to fish. Unfortunately, caution should be excercised in the use of sesquiterpene containing plants, especially those with sesquiterpene lactones, as they are known to be irritating and allergenic to humans[5].




**(19)**



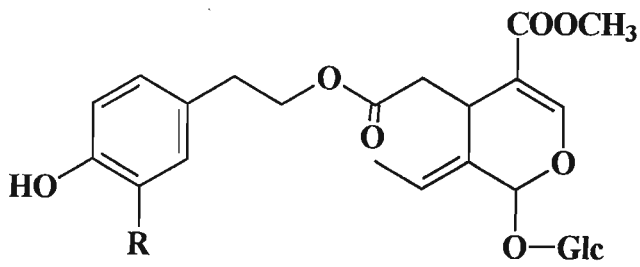

**(20)**




**(21)**

Iridoids.

The iridoid glycosides ligstroside (**22**) and oleuropein (**23**) from the fruits of *Olea europaea* (*Oleaceae*) have been claimed to possess activity against *Biomphalaria glabrata* snails[5].

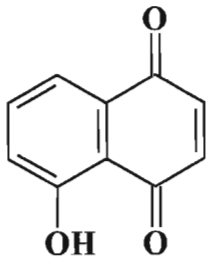



**(22) R = H Ligstroside.**

**(23) R = OH Oleuropein.**

Naphthoquinones.

Simple naphthoquinones juglone (**24**) and plumbagin (**25**) are very effective molluscicides.

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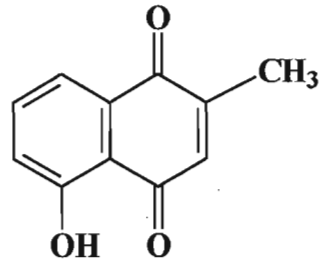
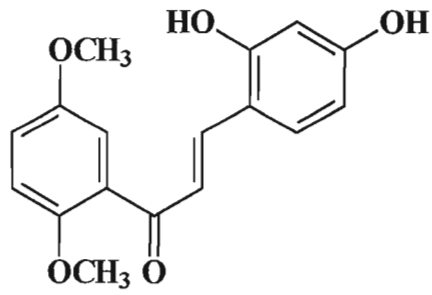



**(25)**

Chalcones.

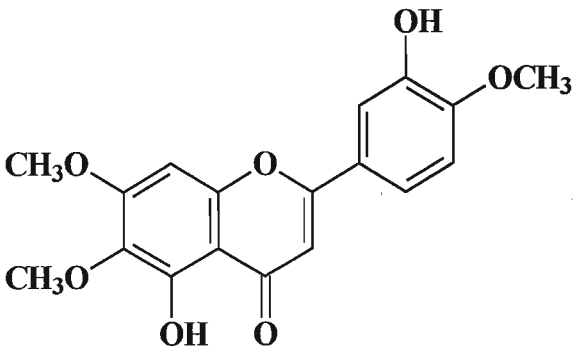
Investigation of the seeds and leaves of *Polygonum senegalense* (*Polygonaceae*) has led to the isolation of 2,4 - dihydroxy - 3', 6'-dimethoxychalcone (**26**) which is active within 6 hours at 40 ppm against snails of the species *Biomphalaria pfeifferi* and *Biomphalaria sudanica*[5].




**(26)**

Flavonoids and rotenoids.

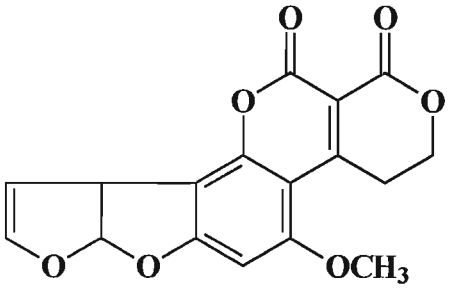
Some flavonoids investigated so far have mollusc-killing activity. Eupatorin (**27**) is marginally active at 100 ppm[5].




**(27)**

Furanocoumarins.

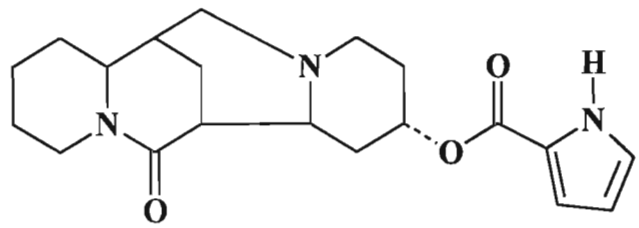
This class of compounds, commonly found in the *Rutaceae*, shows strong snail-killing properties. Aflatoxin $G_1$ (**28**), isolated from mouldy peanuts, is one of the most powerful naturally-occuring molluscicides but at the same time it is a well-known carcinogen.




**(28)**

Alkaloids.

The only example of a molluscicidal alkaloid with any activity whatsoever is the quinolizine alkaloid 2,3-dehydro-0-(2-pyrrolylcarbonyl) virgiline (**29**) from the leaves of *Calpurnia aurea* (*Fabaceae*).




**(29)**

A very large number of plant extracts has been investigated for molluscicidal activity and guidelines for further screening of numerous species have been outlined in the following paragraphs.

When it is considered that more than 20 000 compounds were screened in order to discover the synthetic molluscicide Bayluscide (**2**)[22] the immensity of the effort required in the search for natural, highly active molluscicides can be appreciated. However, the problem is somewhat simplified by the information available from the testing of plant material where the activity is known but the structures of the active components remain to be elucidated. This is essential for the study of their toxicities and stabilities under field conditions, for dosage purposes, for structure-activity investigations and for effects on snail metabolism or physiology[5].

The aims of this thesis are the isolation of molluscicides and other compounds from potentially-active indigenous plants and the structure elucidation of these compounds.

## 1.3 Plants with potential molluscicidal activity

The selection of South African plants with potential molluscicidal activity was conducted in the Department of Zoology and Entomology (University of Natal Pietermaritzburg) by Dr Tanza E. Clark, under the supervision of Professor C.C. Appleton. In the search for new plant-based drugs random selection and mass screening have been the types of programs most widely used[23]. These programs are time-consuming, expensive and have been unproductive in terms of identifying useful drugs for humans. This chapter describes, a brief scientific method of bioassay that can be employed in developing countries to identify plants with potential molluscicidal activity.

### 1.3.1 The selection procedure

The selection procedure employed has been summarised in Fig.1. Since the only information available for all 655 selected species included the plant's distribution and some record of its toxicity, these two criteria were used to reduce the total number of species to a more manageable size[24].

From the 655 species only 150 occurring in Southern Africa (Namibia, Botswana, South Africa, Lesotho and Swaziland) were selected [25].

Further selection was based on information available about their molluscicidal activity when measured against World Health Organisation (WHO) standards. Data were judged positive if a plant extract killed snails at concentrations of 100 ppm or less[24,25], and those not previously tested were included if the literature indicated that a chemical substance or substances of known structure had been detected in, or isolated from the species, or if a chemical substance with predictable molluscicidal effect was present. This left 63 species. The reduced total was still beyond practical screening capabilities. An objective selection to ensure that the final choice would yield species with the greatest potential for use in South Africa was therefore necessary[23].

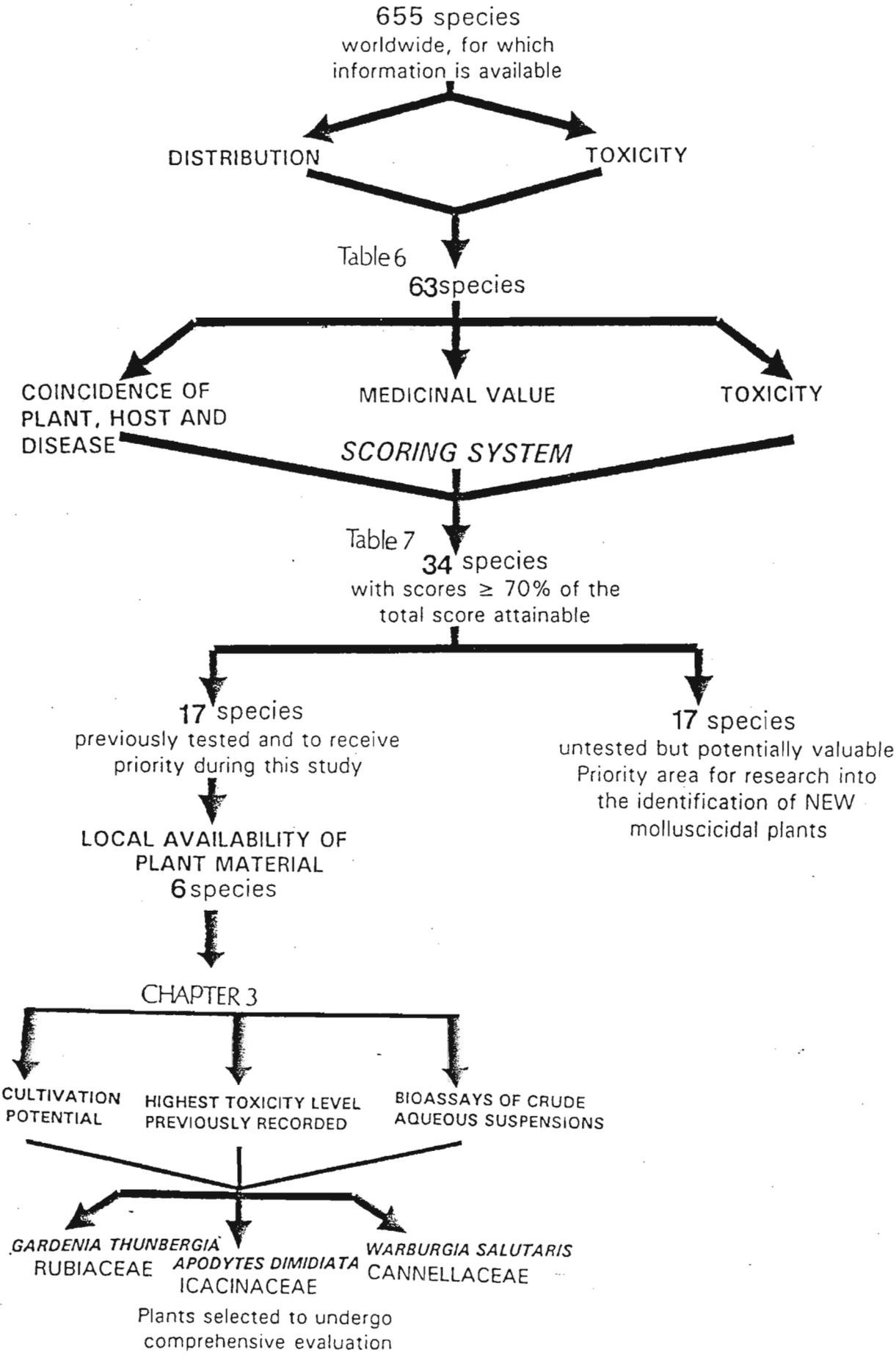



Figure 1: Summary of the procedure followed in selecting candidate South African molluscicidal plants.

### 1.3.2 Desirable characteristics of molluscicidal plants

Information for all species was available for three of eleven characteristics for suitable plant molluscicides, as determined by WHO (1983)[24]. The 11 characteristics are :

| | |
|---|---|
| Toxicity : | High toxicity against target organisms. Low or non toxicity against non-target organisms at molluscicidal concentration. |
| Distribution: | The plant must grow abundantly in areas where schistosomiasis is endemic or be amenable to large-scale cultivation in these areas (an obvious requirement for an indigenous plant to be used as a snail control). |
| Molluscicidal activity levels : | The plant must show molluscicidal activity at levels prescribed by WHO (1983).<br>Crude plant material should have a $LD_{90}$ 100mg/l.<br>Aqueous and solvent extract should have $LD_{90}$<br>20 mg/l |
| Yield : | High yield of molluscicidal material per plant and per unit area of cultivated land. |
| Type of plant: | Perennial rather than annual; reproduce by seeds rather than by tubers; drought resistant for use in arid areas; semiaquatic or aquatic for use directly in snail habitats; high propagation and rapid growth rates with minimum capital and labour input; high adaptability to differing local environmental conditions; high resistance to pests, weeds. |
| Plant parts: | Efforts should be made to test all parts of plants for molluscicidal activity. However, for sustained use in snail control programs emphasis in the case of perennial plants should be placed on berries, fruits, flowers, |

nuts, and regenerating leaves rather than on roots stems and non regenerating bark.

Storage : Molluscicidal material of seasonally producing plants should not lose potency during storage of at least one year.

Extraction : Active principle should be extractable by simple apparatus and commonly-available solvents, preferably water.

Knowledge of plant in endemic area : A good knowledge of growing habit and requirements, toxicity and medicinal properties of plants by local people, is an asset.

Cultural acceptability : Absence of spiritual and ceremonial uses of plants and aversions based on folklore and magic, which might interfere with their use for snail control, is desirable.

Additional uses : Suitability of the same plant parts for other public health, local, domestic or industrial uses.

The list of 14 "tested" species received priority in this investigation for suitable plants. Practicality necessitated the ready availability of material for extraction. Four species, *Warburgia salutaris, Apodytes dimidiata, Gardenia thunbergia* and *Rauvolfia caffra*, were available locally.
Comprehensive evaluation could only be conducted on a maximum of three of these species since it is necessary to avoid complex extraction procedures when developing technology for rural communities. Crude aqueous suspensions of these species, were bioassayed using *Bulinus africanus*[25].

Species were ranked on their toxicity as aqueous suspensions, and according to the highest toxicity level previously recorded in the literature.

Ranks for each plant were summed and the three with the lowest cumulative ranking (i.e. the highest activity) were prioritized. In this manner *Gardenia thunbergia*, *Apodytes dimidiata* and *Warburgia salutaris* were selected for comprehensive evaluation.

Drewes *et al.* and several other researchers have isolated warburganal(**20**) from *Warburgia salutaris* (*Canelaceae*) species and it is active on snails[26]. Thus, the target was reduced to two species, *Gardenia thunbergia* (*Rubiaceae*) and *Apodytes dimidiata* (*Icacinaceae*).

## 1.4 Review of the family Icacinaceae[27]

1.4 *Apodytes dimidiata (Icacinaceae)* (See Fig.2)

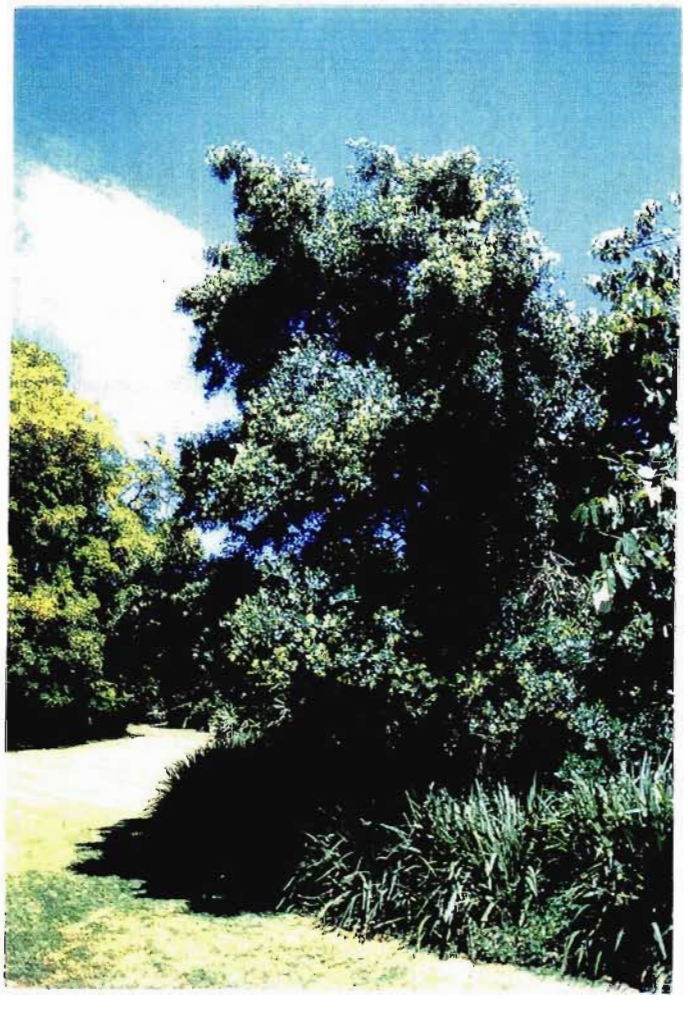


Fig. 2: *Apodytes dimidiata*, National Botanical Garden, Mayor's Walk, Pietermaritzburg.

*Apodytes E.Mey. ex Arn.* is an old World genus of forest trees renowned for their excellent timber and peculiar "pseudoarillate" drupaceous fruit. Its infrageneric taxonomy, however, is still poorly understood, particulary with regard to Madagascar, Indo-Malaysia, Indo-China and China.

Owing to an apparent lack of dependable diagnostic characters, the delimitation of taxonomic entities is generally weak and there is uncertainly as to the exact number of species and infraspecific taxa[28].

Depending on the authority, from two to about seventeen species may be recognized.

In recent years, the trend has been to recognize but one widespread and polymorphic species of the old World tropics, *A. dimidiata E. Mey. ex Arn.Subsp.dimidiata.*

*Apodytes dimidiata (Icacinaceae)* commonly referred as "white pear" is an evergreen tree which occurs along the eastern seaboard from Cape point through Natal northwards and extends an arm into the Transvaal Bushveld. Its distribution therefore roughly coincides with that of the two intermediate host snails in South Africa[29].

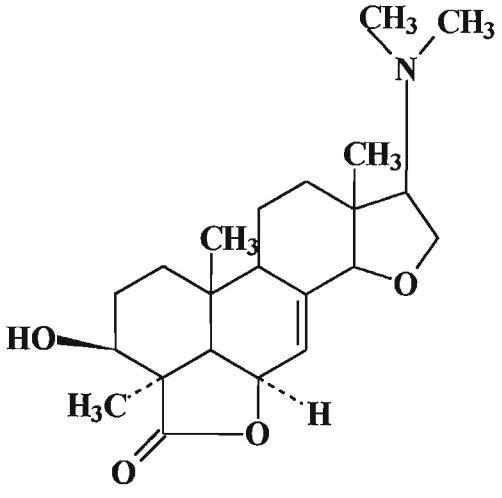
The Zulu use an infusion of the root-bark and other plants as an enema for intestinal parasites.

The leaf is applied by the Luo people in inflammation of the ear. The fruit is inedible and seldom eaten even by birds. The wood is strong, very hard and elastic and has been used in wagon building[16].

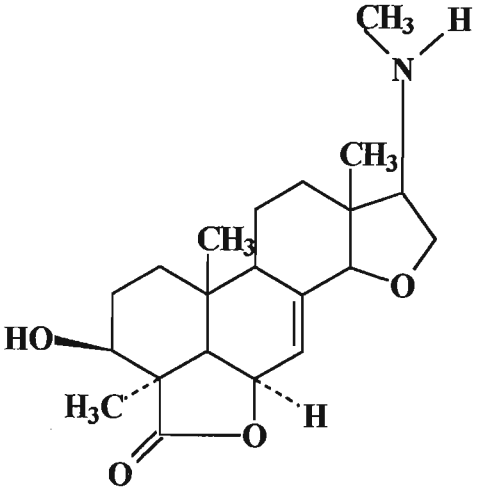
### 1.4.1 Chemical Constituents

Previous studies carried out on the family of *Icacinaceae* has yielded interesting compounds including new diterpenes and diterpene-based alkaloids.

Vanhaelen *et al.*, reported having isolated and identified two new diterpene-based alkaloids from the leaves and roots of *Icacina guesfeldtii*. These were isolated and identified as icaceine (**30**) and N-demethylicaceine (**31**)[30].

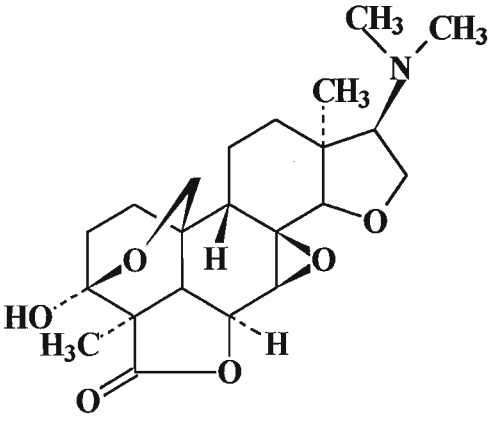



**(30)**



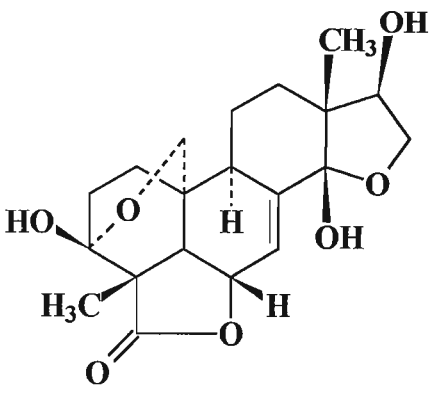

**(31)**

A previous study, by the same researchers[30], had already revealed the presence of icacine (**32**) in the roots of the decoctions of *Icacina claessensis* and *guesfeldtii*. These diterpene lactone structures are related to the pimarane skeleton which occurs in the genus *Icacina*.




**(32)**

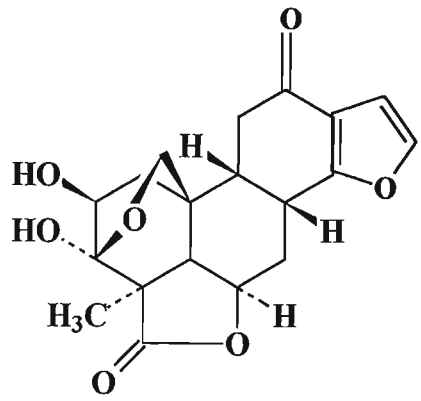
*Icacina guesfeldtii Ascher* is a shrub found in different regions of tropical Africa.
Around Lodja (Zaire), the root decoction is used in popular medicine as an anti-convulsant[31].
Vanhaelen *et al.* continued their study of biologically active constituents by studying *Icacina claessensis*, also used as a popular medicine in Zaire as an anti-convulsant and isolated a diterpene with a similar alkaloidal skeleton. This compound was identified as icacinol (**33**)[31].




**(33)**

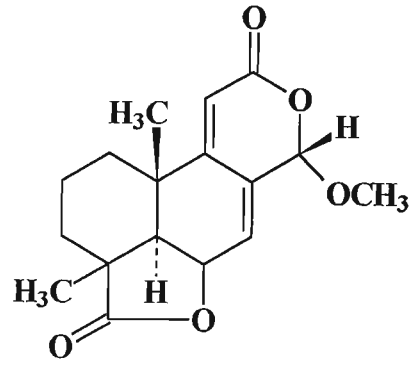
Subsequent study of the species *Icacina mannii* led to the isolation of a new Norditerpene called icacenone (**34**)[32] ($C_{19}$ $H_{20}$ $O_7$). Both icacinol (**33**) and icacenone (**34**) were isolated by Vanhaelen *et al.*[33] in the species *Icacina senegalensis* which is a shrub endemic to Casamance (Senegal). The roots have been used as a starch source in times of famine, although they have been found to be toxic in some cases.

*Icacina mannii* is a shrub endemic to tropical Africa, the root decoctions being used in popular medicine around Kinshasa (Zaire) for treatment of fibrous tumours[32].




**(34)**

Ellestad *et al.* isolated a $C_{17}$ antifungal tetra-Norditerpenoid (**35**) from an unidentified *Acrostalagmus* species[34]. It possesses significant antifungal activity *in vitro* against a number of fungi and *in vivo* against experimental ringworm infections in Guinea pigs.

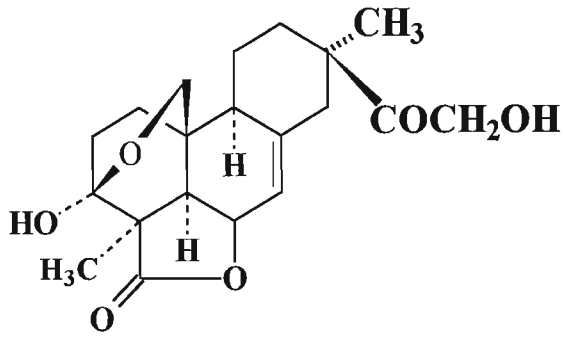



**(35)**

In 1973, *Mussini et al.*[35] isolated a new diterpenoid from the species *Annona coriacea* (*Annonaceae*).

They had previously found 2 new diterpenoids with a clerodane skeleton by extracting the roots of the same species with acetone[35].

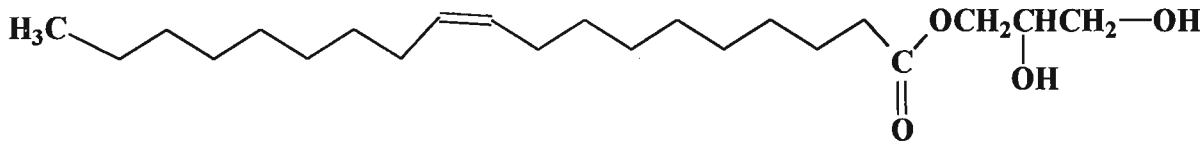
The structure of this new diterpenoid, annonalide (**36**) $C_{20}H_{26}O_6$ is as follow:




**(36)**

Akerman has found glyceryl oleate and glyceryl stearate (**37**)[36] in *Apodytes dimidiata*.

The structure of compound (**37**) is as follow :




**(37)**

Hence, the species *Apodytes dimidiata* belonging to the same family as the above, not only proved to possess molluscicidal activity but also could possibly contain similar products as well as some novel ones. This obviously aroused interest in the species to isolate the active compounds in snails.

## 1.5 Review of the family Rubiaceae

1.5 *Gardenia thunbergia* (*Rubiaceae*) (See Fig. 3)

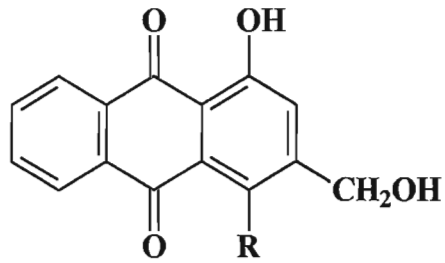
Fig.3: *Gardenia Thunbergia*, National Botanical Garden, Durban.

The species, *Gardenia thunbergia*, belonging to the family Rubiaceae is found growing abundantly along the East Coast of Kwazulu-Natal. It is most frequently found as a small evergreen tree 2 to 5 m tall or a shrub.

*Gardenia thunbergia* was found to possess molluscicidal activity and it was the only plant to approach WHO toxicity standards. The Zulu take an infusion of the root bark of *G.thunbergia thunb*. as an emetic in biliousness. The plant is also used medicinally in Mozambique but no details are available. The Kerewe and the Ha use an infusion of the root externally and internally in syphilis and the Ha use the plant as an emetic in bilious sicknesses[16]. The root, which is thought to contain tannin is widely used in Africa as a local application to treat leprosy; the leaf as remedy for syphilis; the latex as a purgative. Extracts of the plant have given negative antibiotic tests. Despite the fact that the fruit is woody and very hard, it is eaten by the elephant, by the larger antelopes and by the buffalo. The wood which is yellowish-grey in colour and hard and heavy has been used for tool handles.

### 1.5.1 Chemical constituents

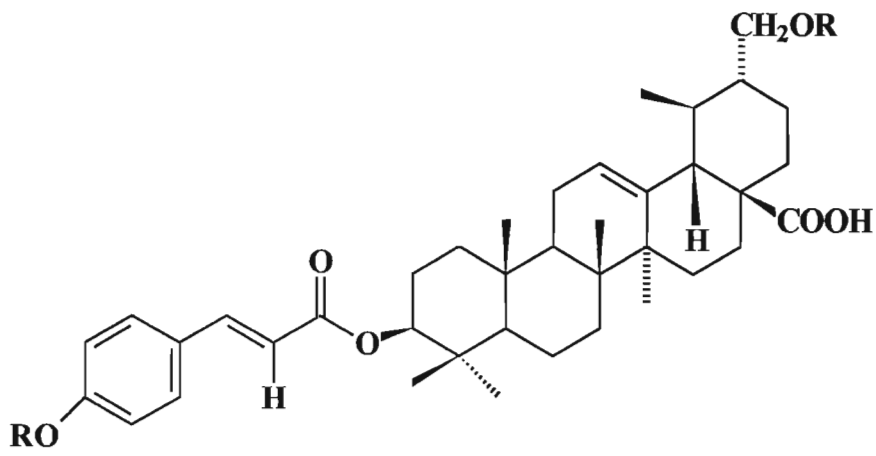
Previous chemical investigations of the plants belonging to the Rubiaceae family revealed a variety of interesting compounds. Imre *et al.* isolated digiferrol (**38**) and ferruginol (**39**) from *Rubia tinctorum* species[37].




**(38) R = OH Digiferrol.**

**(39) R = H Ferruginol.**

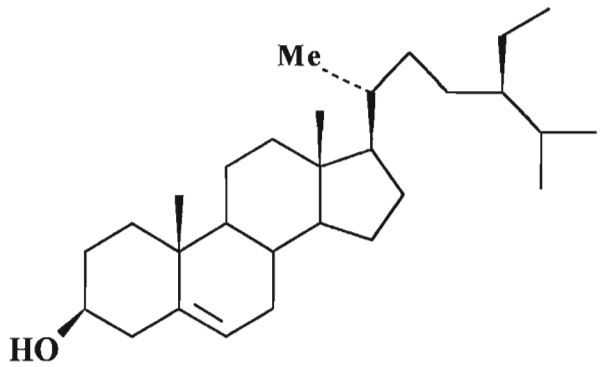
Talapatra *et al.*, isolated two new triterpenes; rubicoumaric acid (**40**) and rubifolic acid (**41**) from *Rubia cordifolia*[38]. This is a small plant growing throughout India in hilly districts. Roots are reported to be used as a tonic and the stems as an antidote for cobra bite and scorpion sting[38].




**(40) R = H Rubicoumaric Acid.**

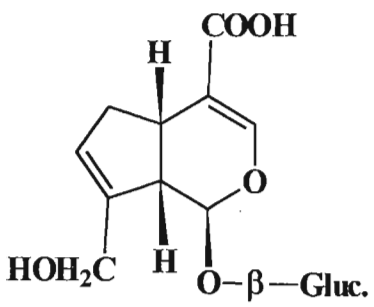
**(41) R = Ac Rubifolic Acid.**

A common steroid constituent , sitosterol (**42**)[39] has been isolated from *Rubia cardifolia L.*

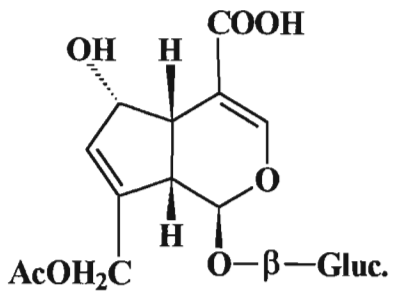



**(42)**

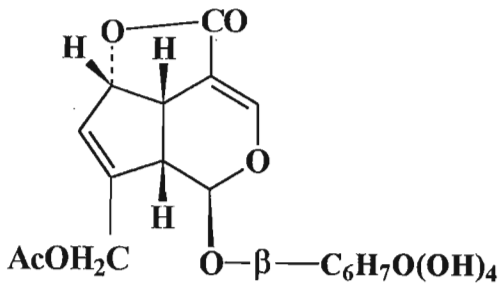
Three new iridoid glycosides have been isolated from plants of the *Rubiaceae* family[40]. Asperulosidic acid (**43**) and 10 - deacetylasperulosidic acid (**44**) are present in small amounts in *Rubia tinctorum* and *Rubia peregrina* along with asperuloside (**45**) as the main constituent. This represents the first isolation of compounds (**43**) and (**44**) from a natural source[40].




**(43)**

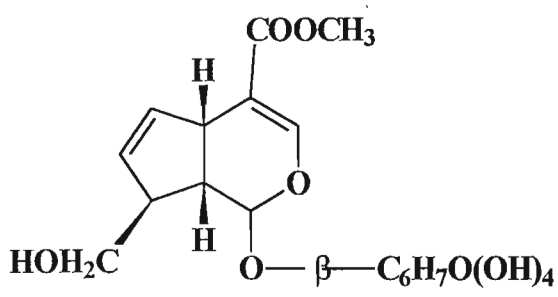



**(44)**



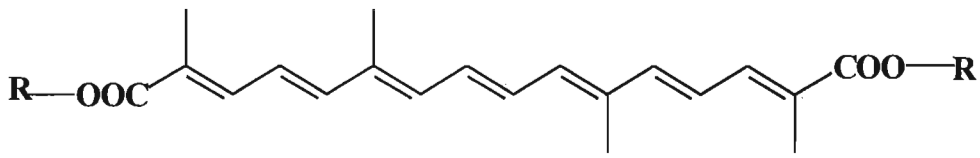

**(45)**

From *Galium mullogo* (*Rubiaceae*), another iridoid, galioside (**46**) has been isolated[40].



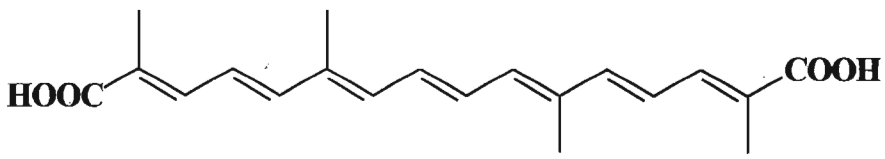

**(46)**

Crocin (**47**)[48] and Crocetin (**48**)[51] were isolated as yellow pigments from *Gardenia jasminoides Ellis* (*Rubiaceae*).



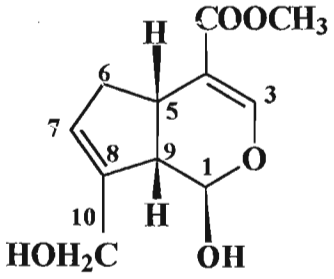

**R = Gentiobiosyl**

**(47)**

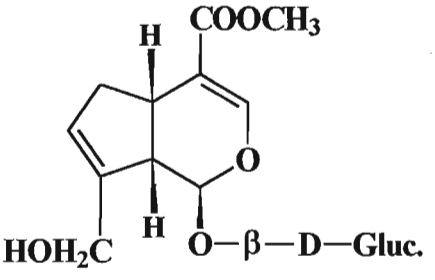



**(48)**

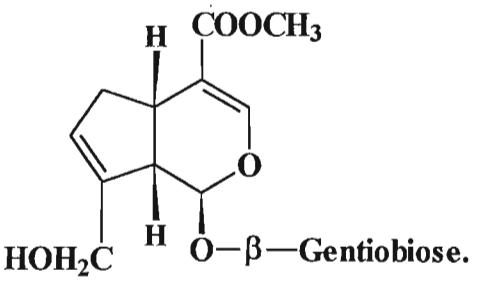
In addition, genipin (**49**) geniposide (**50**) and genipin gentiobioside (**51**) were isolated from the crude extract. Among these iridoid glucosides, geniposide (**50**), the main constituent of *Gardenia fructus* was shown to have diuretic and cholagogic activities[41].




**(49)**

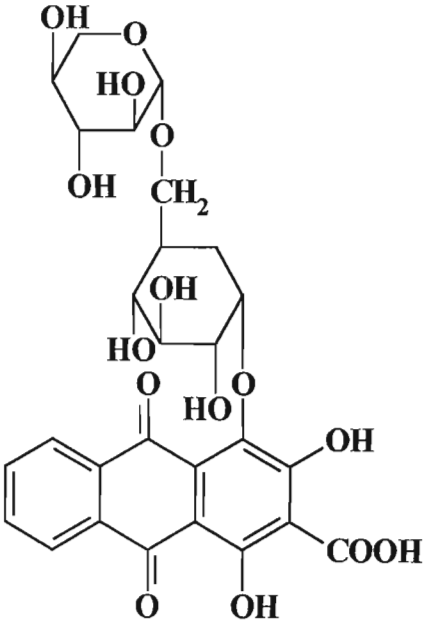



**(50)**




**(51)**

A pigment, galiosin (**52**)[42] has been isolated from *Rubia tinctorum L.* by Hill *et al.*




**(52)**

# CHAPTER 2.

# DISCUSSION OF RESULTS

## 2.1 Extractives from *Apodytes dimidiata* subsp. *dimidiata*

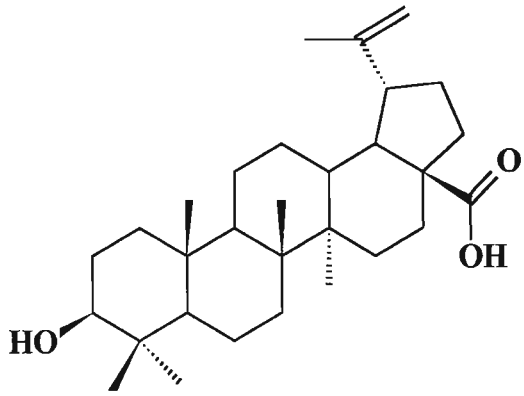
Branches were obtained from *Apodytes dimidiata* trees located in the Hayfields area Pietermaritzburg and the National Botanical Garden, Mayor's Walk, Pietermaritzburg. The source was identified by Dr. Tanza E. Clark of the Zoology Department University of Natal Pietermaritzburg. Voucher specimens were deposited in the Herbarium of the University of Natal Pietermaritzburg. (Sample N° 5 T.E.Clark - 1994).

Branches were debarked and the bark (2.5 kg) was air dried. The air dried bark was then milled to a fine powder. It was extracted using the differential solvent extraction technique[43]. This extraction technique employs the exhaustive extraction of the plant by a series of solvents of increasing polarity. This gives a differential fractionation of the extractives on the basis of their differing polarities.

The procedure followed for the extraction is summarized in Fig.4. Fractions were continually assessed to ensure that activity had not been lost during extraction. The most active fraction was a dichloromethane fraction.

## COMPOUNDS ISOLATED

### 2.1.1 Betulinic acid (53)




**(53)**

Betulinic acid (**53**) was obtained from the ethyl acetate extract after repeated column chromatography using hexane/ethyl acetate (1:1) solvent mixture.

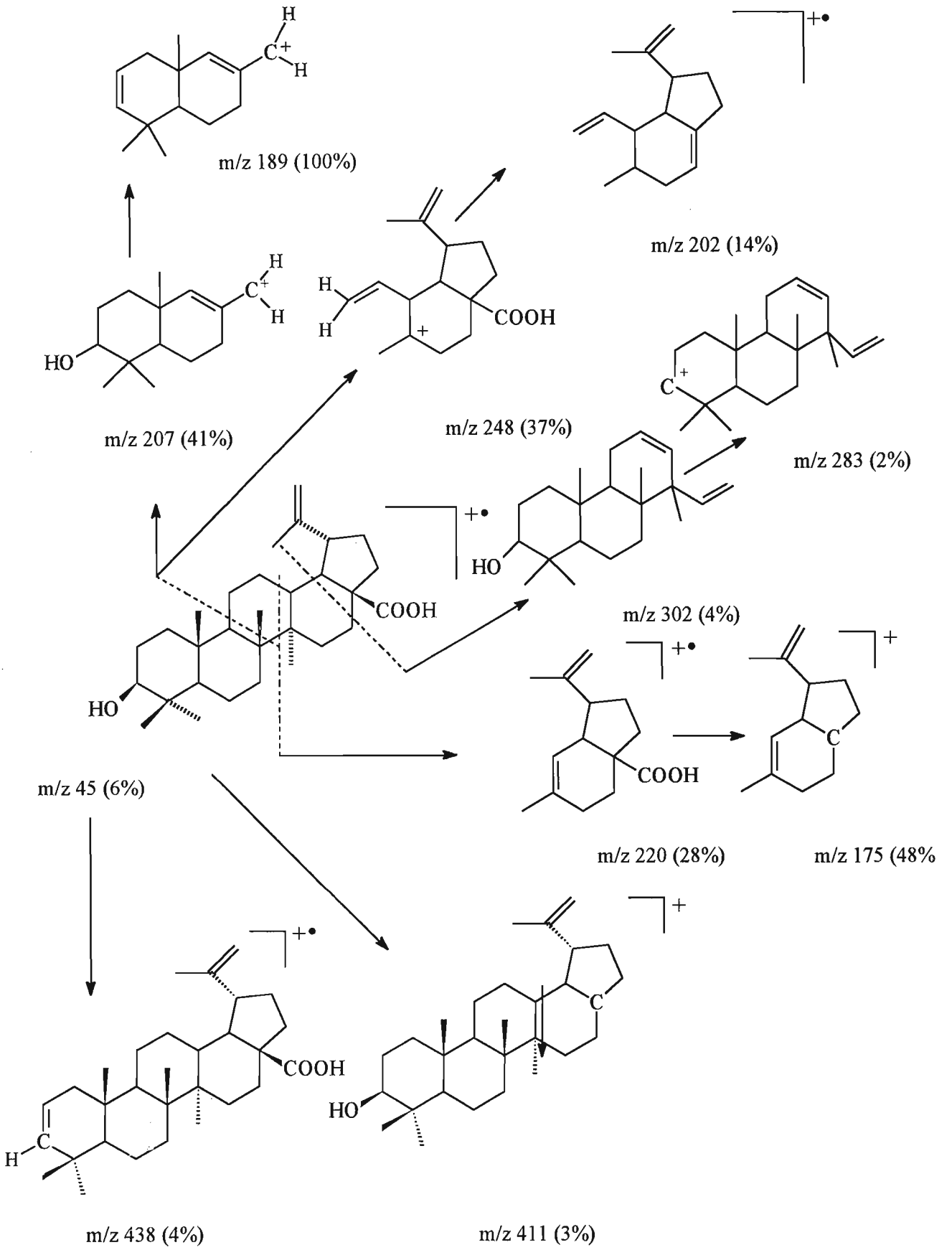
The compound swells in solvents such as ethyl acetate, acetone and methanol. It was obtained pure after recrystallization from methanol to give crystals (207 mg), mp 276°C - 278°C

$[\alpha]_D^{23°C}$ + 6.8° (c.1.02, pyridine)

The fragmentation pattern observed in the mass spectrum strongly suggested the compound to be a lup -20 (29)-ene type triterpene and allowed allocation of the carboxyl group at C-17 and the hydroxyl group in A-ring (Scheme 1)[44].

The mass data of the compound were in fact identical to those of betulinic acid (**53**)[44].

| | |
|---|---|
| m/z calc. | 456.3603 |
| found | 456.3725 |




SCHEME 1 : Mass Fragmantation pattern of Betulinic Acid **(53)**

The fragment ions m/z 438 and m/z 411 are due to the loss of $H_2O$ and angular COOH, respectively.

Cleavage of C-ring between C(12) - C(13) and C(8) - C(14) bonds results in the formation of an ion m/z 220 which on loss of COOH gave rise to a peak at m/z 175. Alternative cleavage of C-ring between C(8) - C(14) and C(9) - C(11) m/z 207, which on losing $H_2O$ gave rise to a peak at m/z 189. The fragment ion m/z 248 also results from C-ring cleavage and then loses a carboxyl group to form a fragment m/z 203 which rearranges and loses a proton to give rise to a peak at m/z 202.

Fragment ions resulting from D-ring cleavage are also observed. Cleavage of the C(13) -C(18) and C(16) - C(17) bonds give rise to a peak at m/z 302 which on losing a hydroxyl group forms an ion at m/z 283.

In $^{13}C$ -NMR (p.62), the spectrum shows the compound to have six methyl groups (δ 14.68, δ 16.61, δ 16.64 ($2CH_3$), δ 19.73 and δ 28.05), an oxymethine (δ 78.34, *d*), a vinylidene group (δ 151.51, ($\underline{C}=CH_2$) and δ 110.23, ($C=\underline{C}H_2$) and an acidic carbonyl (δ 179.07, *s*)[45] . The $^1H$ NMR (p. 61) displays six tertiary methyl groups (δ 1.65, δ 1.07, δ 0.93, δ 0.90, δ 0.85 and δ 0.68) and two vinylidene protons (δ 4.79 (1H, *d*, $J$ = 2.14 Hz) and δ 4.62 (1H, *d*, $J$ = 2.11 Hz) which are long - range coupled to a vinylic methyl group (δ 1.65, 3H, *s*)[44].

The peak at δ 3.34 (2H, *m*) is due to overlapping H-3 and H-19 methine peak and this assignment is strongly confirmed by HETCOR results (p.63) where C-3 (δ 78.34, *d*) and C-19 (δ 49.95, *d*) are coupled to a multiplet at δ 3.34 (2H).

The $^{13}$C-NMR results of the compound are tabulated below :

Comparison of $^{13}$C- NMR of Betulinic acid and the isolated compound

| Carbon | Mashimbye[26] | Present investigation |
|---|---|---|
| 1 | 36.69 | 37.73 |
| 2 | 27.33 | 28.05 |
| 3 | 77.33 | 78.34 |
| 4 | 40.19 | 41.32 |
| 5 | 55.07 | 56.14 |
| 6 | 17.84 | 19.02 |
| 7 | 31.96 | 33.09 |
| 8 | 40.19 | 41.32 |
| 9 | 50.09 | 51.17 |
| 10 | 38.61 | 39.75 |
| 11 | 20.26 | 21.44 |
| 12 | 25.15 | 26.32 |
| 13 | 37.67 | 38.81 |
| 14 | 41.94 | 43.06 |
| 15 | 29.35 | 30.51 |
| 16 | 33.92 | 35.06 |
| 17 | 55.75 | 56.84 |
| 18 | 46.86 | 47.58 |
| 19 | 48.85 | 49.95 |
| 20 | 150.69 | 151.51 |
| 21 | 30.26 | 31.42 |
| 22 | 38.40 | 39.52 |
| 23 | 27.76 | 28.91 |
| 24 | 15.42 | 16.61 |
| 25 | 15.45 | 16.64 |
| 26 | 14.45 | 15.16 |
| 27 | 13.96 | 14.68 |
| 28 | 178.35 | 179.07 |
| 29 | 109.32 | 110.23 |
| 30 | 18.56 | 19.73 |

The chemical shifts of betulinic acid isolated by Mashimbye are very slightly different from the chemical shifts of the isolated compound, largely because of instrument variation.

Physical properties further confirm the compound to be betulinic acid (**53**).

## 2.1.2 Genipin (49)[47]

Two major components, $R_f$ = 0.62 and $R_f$ = 0.26 in [ethyl acetate-hexane (3:2)], were isolated from the $CH_2Cl_2$ extract (78.5 g) after repeated column chromatography using EtOAc-hexane initially as a (1:1) mixture, but gradually increasing the ratio to (3:2). This process afforded genipin (**49**) (140 mg)

Genipin (**49**) was recrystallised from MeOH to give white prisms. mp 120 - 121° C; $[\alpha]_D^{20}$ = + 109° (c.0.06 ,$CHCl_3$), (lit.[47] $[\alpha]_D$ = 135°).

The mass spectrum showed the accurate molar mass of genipin (**49**) to be 226.0836 while the calculated mass was 226.0841. This supported a molecular formula of $C_{11}H_{14}O_5$.

The $^1$H NMR spectrum (p.57) showed a 3-proton singlet at δ 3.72 which is typical of a carbomethoxy group. The proton on C-1 is a doublet at δ 4.78 as anticipated, and the diastereomeric protons on C-10 resonate as a dd at δ 4.35. A one proton singlet occurs at δ 5.88 which is characteristic of HC =C<$CO_2$Me.

The $^{13}$C-NMR spectrum (p.58) showed 8 protonated carbons of which 5 were doublets, 2 were triplets and one was a quartet.

The spectrum had quaternary carbons at δ 168.0, δ 141.9 and at δ 110.7.

There are signals at δ 51.5 indicating the -O$\underline{\underline{C}}H_3$ carbon of the carboxymethyl group,

at δ 96.3 due to a carbon attached to OH and

at δ 61.3 indicating $\underline{\underline{C}}H_2OH$.

It was concluded, from the mass spectrum, that there were 5 oxygen atoms in the molecule : two in the carbomethoxy group (C$\underline{\underline{O}}\underline{\underline{O}}CH_3$), one oxygen in hydroxy group attached to a $CH_2$ ($CH_2O\underline{\underline{H}}$), one oxygen in hydroxy group attached to CH(CH$\underline{\underline{O}}$H)

The remaining oxygen was obviously part of a heterocyclic ring.

Further evidence, is revealed by COSY and long range HETCOR results. From COSY (correlation spectroscopy) results ( p.59) the following couplings are observed:

i) H - 5 (δ 3.22) is coupled to H- 6b (δ 2.90) and coupled to H - 9 (δ 2.54) and again coupled to H - 6a ( δ 2.10).

ii) H - 6a (δ 2.10) is coupled to H - 6b (δ 2.90),

iii) H - 1 (δ 4.81) is coupled to H - 9 (δ 2.54)

iv) H - 7 (δ 5.88) is coupled to H - 10 (δ 4.28) and to

H - 6a (δ 2.10) and to H - 6b (δ 2.90).

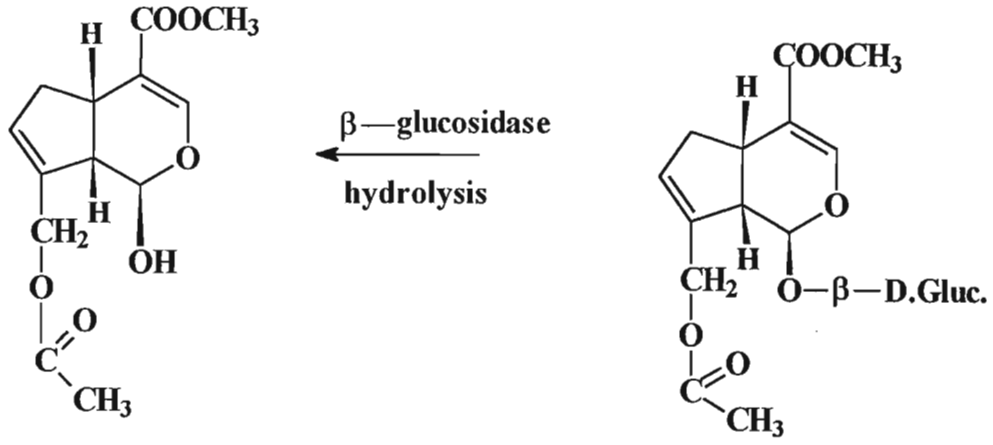
v) H - 3 (δ 7.52) is coupled to H - 5 (δ 3.22).

These results are supported by the long range Hetcor spectrum.(p.60)

Thus, all information gathered, give an Iridoid compound : genipin (**49**).

The significance of this finding lies not so much in the fact that genipin has been identified for the first time as one of the major constituents of the bark and leaves of *Apodytes dimidiata*, but in the discovery that it was indeed an active molluscicide. The only other work on *Apodytes dimidiata* by Fourie and his coworkers[29] had concluded that the extractives from this tree have molluscicidal properties but the active agent was never isolated or identified.

### 2.1.3 10 - Acetyl genipin (58)




**(58)** **(59)**

10 - Acetyl genipin (**58**), with the higher $R_f$ = 0.62 in (ethyl acetate-hexane (3:2), was isolated from the $CH_2Cl_2$ extract (78.5 g) after repeated column chromatography on silica gel 60 (Merck Art No 9385) as a white syrup (176 mg) $[\alpha]_D^{20°}$ = +22.6 ° (c.0.06, $CHCl_3$) The mass spectrum showed the accurate molar mass of 10 - acetyl genipin (**58**) was 268.0947 while the calculated mass for $C_{13}H_{16}O_6$ is 268.0939.

To our knowledge this represents the first isolation of genipin acetate from a natural source. There is only one previous reference in the literature to this compound.

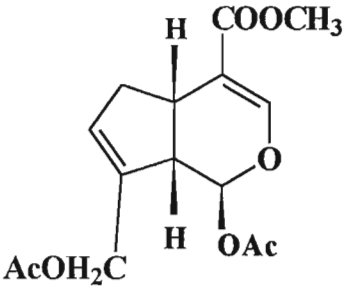
Takeda *et al.*, report the hydrolysis of 10 - acetyl geniposide (**59**)[48] with β- glucosidase which gives the aglycone (**58**) $C_{13}H_{16}O_6$. These authors record an abbreviated NMR spectrum.

The $^1H$ NMR (p.66) spectrum displays a singlet at δ 2.11 assignable to the acetyl group at C-10. The two 1 proton doublets at δ 4.70 and δ 4.95, $J$ = 12.9 Hz are characteristic of the $CH_2OAc$ group. Location of the acetyl group at C-10 in (**58**) was based on the observation that the methylene protons on C-10 in (**58**) are shifted downfield from 4.28 in (**49**) to 4.82 (**58**).

The spectroscopic data $^1H$ NMR & $^{13}C$ -NMR (p.49) is closely allied to that of genipin (**49**) (p.48), so that structure determination was not difficult.

### 2.1.4 Genipin diacetate (55)

As a final proof of structure the isolated genipin (**49**) was converted to its diacetate.



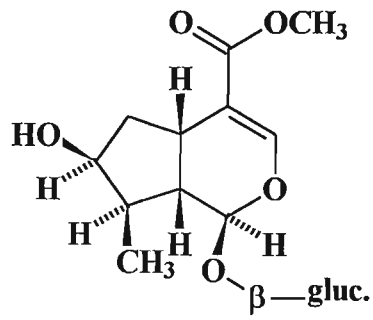

**(55)**

Genipin (65 mg) was acetylated with pyridine / acetic anhydride[49] to give the diacetate derivative as a non crystalline material. Identification of the protons for this compound was facilitated by comparison with Genipin (**49**). Proton and $^{13}C$ data of the diacetate are on p.49. $^1H$ and $^{13}C$ - NMR spectra are shown on pp. 64 & 65.

**There is no reference in the literature to the pure genipin (49) and genipin 10-acetate (58) behaving as a molluscicide. In this investigation it was found that genipin (49) and its monoacetate derivative are active at concentrations comparable to activity levels of plant molluscicides currently under field investigation.** Bioassays have been conducted in the Zoology & Enthomology Department, University of Natal, Pietermaritzburg by Dr.Tanza E. Clark and Ms. T. Brackenbury under the supervision of Prof. C.C. Appleton. Results are summarized in Table 7 (p. 54).

### 2.1.5 The Chemistry of Genipin (49)

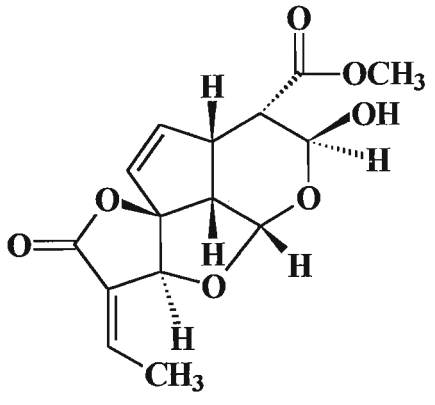
Genipin belongs to the class of compounds known as Iridoids. Iridoids are a group of bitter tasting monoterpenoid lactones, which are widely distributed in angiosperms, being found in about 70 families grouped in some 13 orders. They are characterized by or based on a cyclopentanodihydropyran ring system. Iridoids, which derive from geranyl pyrophosphate, are currently receiving much attention. The "proper" iridoids are lactone derivatives, often with glucose attached to the hydroxyl group of the lactone ring. A typical iridoid glucoside is loganin (**54**) $C_{17}H_{26}O_{10}$ which occurs for example in *Strychnos nux-vomica* fruit to the extent of 4 - 5% dry weight.



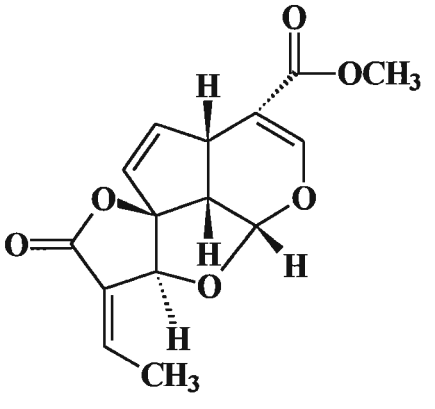

**(54)**

The aglucones are usually highly unstable and disintegrate (after hydrolysis with acid,) into a blue or black polymer. Herbarium plants containing iridoid glucosides also often turn dark during drying. This dark-blue discoloration is the reason why iridoids were originally called "**pseudoindicans**".

However, some "proper" iridoids occur in plants as stable aglycones, e.g. genipin (**49**) allamandin (**56**) and plumericin (**57**)[50].



(**56**)



(**57**)

Iridoids are also known to possess a number of pharmacological properties such as antimicrobial, hypotensive, analgesic, antiphlogistic, sedative, laxative and antitumour[51].

Oshima *et al.*, reported on the anticomplementary activity of iridoid derivatives isolated from the bark of *Eucommia ulmoides* bark[52].

It was found that genipin (**49**) showed the strongest anticomplementary activity.

From these observations, it was concluded that the hemiacetal moiety plays an important role in the manifestation of the anticomplementary activity of iridoids as had previously been reported also for their antimicrobial and antitumour activities[51].

Genipin (**49**) obtained from Gardenia fruits (*Gardenia jasminoides*) is used for making blue pigments and as a crosslinking agent for proteins. Gardenia fruits contain geniposide (**50**) which undergoes hydrolysis with β -glucosidase to yield genipin (**49**) which reacts with amino-acids to form a blue pigment[53].

Ueda *et al.*, report the anti-tumour-promoting activity of genipin (**49**)[54].

Extracts from catkins of *Garrya elliptica* inhibit the growth of wheat embryos. The components responsible for this activity are the iridoids geniposidic acid and geniposide together with their aglucones.The former are less active that the latter[55].

## 2.2 Extractive from *Gardenia thunbergia* (Rubiaceae)

Extraction of *Gardenia thunbergia* leaves using the usual differential extraction with organic solvents afforded little or no useful materials. It was, however, discovered that the crude aqueous extract of the leaves had a surprisingly high molluscicidal activity. It was for this reason, that a somewhat unusual procedure was followed: leaves were first water-extracted and thereafter the aqueous layer was partitioned against ethylacetate. By this procedure several grams of organic extract could be obtained. Purification of this material provided a real challenge as the most active component (detected on the plates by its red colouration with the standard anisaldehyde/sulphuric acid reagent (dip reagent), was present in only very small quantities and it proved difficult to obtain it in pure form. Two major compounds were obtained. One with $R_f$= 0.45 and another one with $R_f$ = 0.26 in EtOAc-hexane (1:1).These compounds labelled as $GT_1$ and $GT_2$ were highly active as molluscicides. (See table 5).

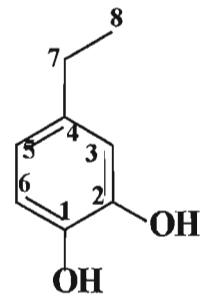
| $GT_1$ | | $GT_2$ | |
|---|---|---|---|
| Conc. ppm | % Mortality | Conc.ppm | % Mortality |
| 1 | 0 | 3 | 0 |
| 3 | 10 | 4.15 | 5 |
| 5 | 90 | 5 | 25 |
| 10 | 85 | 7 | 35 |
| | | 10 | 100 |
| $LD_{50}$ - 4.8 ppm (2.48 - 9.29) | | $LD_{50}$ - 6.45 ppm (5.94 - 6.94) | |
| $LD_{90}$ - 9.48 ppm (4.9 - 18.35) | | $LD_{90}$ - 8.73 ppm (7.86 - 9.56) | |

Table 5: Lethal dosages for $GT_1$ & $GT_2$ isolated from *Gardenia thunbergia.*

Mean percentage mortality of *Bulinus africanus* when exposed to compounds isolated from *Gardenia thunbergia.*

**Compound Identified in *Gardenia thunbergia***

### 2.2.1. 4 - Ethylcatechol (60)




**(60)**

4 - Ethylcatechol (**60**) was finally obtained after separation on a chromatotron plate (4mm) using EtOAc-hexane (2:3) as a solvent mixture. After purification by column chromatography, a pure material (129 mg) was obtained.

The mass spectrum of the compound was identical with that 4-ethylcatechol (**60**).

HR - MS $[M]^+$ 138.0688, calcd. for $C_8H_{10}O_2$ 138.0681.

4 - Ethylcatechol (**60**) was also acetylated by pyridine - acetic anhydride to give the diacetate derivative in order to further confirm its identity.

HR - MS $[M]^+$ 222.0899, calcd. for $C_{12}H_{14}O_4$ 222.0892.

$^{1}H$ - and $^{13}C$ - NMR spectra data of (**60**) ($CDCl_3$ as solvent) are shown in table 6.

4 - Ethylcatechol (**60**)

| Atom | $^{13}C$ | $^{1}H$ |
|---|---|---|
| 1 | 141.2 (*s*) | 7.22 (*d*, $J$ = 10.99 Hz) |
| 2 | 143.6 (*s*) | 6.71 (*m*) |
| 3 | 115.4 (*d*) | -- |
| 4 | 137.6 (*s*) | -- |
| 5 | 121.1 (*d*) | -- |
| 6 | 115.8 (*d*) | -- |
| $-CH_2$ | 28.0 (*t*) | 2.44 (*q*, $J$ = 7.5 Hz) |
| $-CH_3$ | 15.6 (*q*) | 1.13 (*t*, $J$ = 7.6 Hz) |

Table 6 : $^{13}C$ - NMR and $^{1}H$ spectra data of 4 - ethylcatechol.(**60**).

The expanded COSY spectrum of (**60**) (p.76) indicates, with some degree of certainty, that the aromatic protons are part of an ABC system, in keeping with the 4-ethylcatechol structure.

The examination of the available literature showed that ethyl catechol is a product from lignin. Thus, hydrogenation of lignin affords, amongst others, 3-methylcatechol, 4-ethlycatechol and 4-propylcatechol. These phenols were separated from tar acids by precipitation with lead acetate[62].

Comparison of $^{13}C$ - NMR observed for (**60**) and $^{13}C$ - NMR predicted by commercial computer programme.

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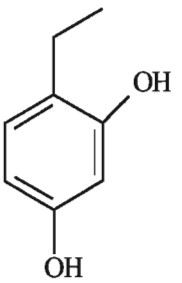
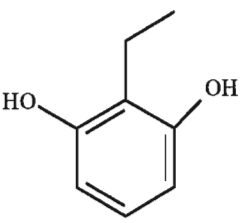
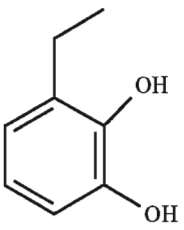
| Atom | 4-Ethylcatechol (**60**) observed | 4-Ethylcatechol predicted | Other isomers predicted | | |
|---|---|---|---|---|---|
| | | |  |  |  |
| 1 | 141.2 | 141.7 | 155.9 | 138.1 | 144.4 |
| 2 | 143.6 | 144.4 | 102.8 | 114.6 | 143.9 |
| 3 | 115.4 | 116.5 | 158.1 | 158.1 | 128.8 |
| 4 | 137.6 | 134.2 | 120.0 | 108.2 | 121.9 |
| 5 | 121.1 | 121.9 | 130.7 | 128.5 | 122.4 |
| 6 | 113.8 | 117.0 | 108.2 | 108.2 | 114.3 |
| 7 | 28.0 | 28.9 | 22.4 | 16.2 | 22.7 |
| 8 | 15.6 | 16.1 | 16.4 | 16.7 | 16.4 |

The chemical shifts of 4-ethylcatechol (**60**) observed are closer to those predicted for 4-ethylcatechol than any other isomers. This shows that the only possibility is 4-ethylcatechol and it is further supported by COSY spectra on p. 76. The compound is clearly still not entirely pure but on p.76a the relevant peaks and their analysis are shown. The shaded peaks are part of an ABC system thus proving the pattern of substitution.

The obvious next step would have been the synthesis of 4-ethylcatechol but time constraints did not permit this.

# CHAPTER THREE

## 3. EXPERIMENTAL

General Method

The silica gel (Merk 60, 230-400 mesh) chromatography columns were run using the flash chromatographic technique of *Still et al.*[56]. This involved the use of different size columns ranging from 2 cm to 5 cm in diameter. Subsequent separation was effected on the Chromatotron 7924T-01 (Centrifugal thin layer chromatography).

Preparative tlc was also used in some cases. All separations were monitored by tlc using precoated Kieselgel 60 $F_{254}$ Merck plastic sheets cut into small strips.

Melting points (MP) were recorded on a Kofler micro hot-stage apparatus and are uncorrected. Optical rotations were measured on a Perkin Elmer 241 Polarimeter.

Infrared spectra (IR) were run on a Shimadzu spectrophotometer (FTIR-4300) using KBr disks. Mass spectra (MS) were obtained with a Hewlett Packard gas chromatographic-mass spectrometer (HP5988A) and a Varian MAT high resolution mass spectrometer.

$^{1}H$ NMR and $^{13}C$ NMR were obtained on a Varian Gemini 200 instrument. All spectra were recorded in $CDCl_3$ as solvent unless otherwise stated and TMS was used as internal standard.

## 3.1 Chemical Investigation of *Apodytes dimidiata* subsp.*dimidiata*

### 3.1.1 Plant Material

*Apodytes dimidiata* subsp. *dimidiata* stem bark and other parts of the plant were collected from trees growing in the Hayfields area of Pietermaritzburg and in the National Botanical Garden, Mayor's Walk, Pietermaritzburg.

Voucher specimens identified by Dr. Tanza E.Clark of the Zoology & Enthomology Department, University of Natal, Pietermaritzburg, were deposited in the Herbarium of the University of Natal, Pietermaritzburg. (Sample No 5 T.E.Clark -1994).

### 3.1.2 Extraction

Bark (2.5 kg) was obtained from mature branches and leaves (3.2 kg) were also collected. After air drying, the bark was milled and differential solvent extraction carried out.

This extraction technique employs the exhaustive extraction of the plant by a series of solvents of increasing polarity. This gives a differential fractionation of the extractives on the basis of their differing polarities.

The procedure followed for the extraction is illustrated below :

Plant Material

(Air-dried)

↓

Differential Solvent extraction

↓

Hexane

↓

Dichloromethane

↓

Ethyl acetate

↓

Ethanol

Figure 4: Differenticial solvent extraction technique.

The average extraction period for each solvent was 72 hours by which time the extraction appeared to be complete.

Bioassays for molluscicidal activity were done for all extracts. The $CH_2Cl_2$ extract (bark) was the most active fraction and also contained the largest number of components in the highest concentration. Thus the $CH_2Cl_2$ extract (11 g) was redissolved in a small volume of EtOAc and

this fraction was filtered through a short silica gel column eluting with MeOH. This separation removed all impurities that caused streaking.

The fraction obtained from this separation showed (tlc) the presence of 8 components of which three at $R_f$ 0.74, $R_f$ 0.62 and $R_f$ 0.26 [EtOAc-hexane (3:2)] were major components. These components could be separated using a suitable solvent system.

### 3.1.3 Isolation of betulinic acid (53)

A portion of the filtered fraction (20.3g) was subsequently separated by column chromatography. It was dissolved in EtOAc and transferred into a silica gel column packed as a slurry. EtOAc-hexane (1:1) solvent mixture was used as eluent and 15ml fractions were collected. Fractions 9-37 were combined and the solvent removed to give impure material (14.22 g).

The above procedure was repeated and the solvent removed to give pure material (1.73 g).

It was noticed that the material swells in solvents such as ethyl acetate, acetone and methanol. Recrystaliization from methanol afforded white feather-like crystals (207 mg).

This compound was insoluble in solvents like acetone, benzene, chloroform, dichloromethane, methanol, toluene and dimethylsulfoxide, even in water.

It was soluble in pyridine. mp.276 - 278° C, lit.[46,58] mp 275 - 278°C, $[\alpha]_D^{23°C}$= + 6.8° , (c.1.02, pyridine), lit.[46,58] $[\alpha]_D^{20}$ = + 7.9 (c.0.057, pyridine). m/z found 456.3725 calculated for $C_{30}H_{48}O_3$ 456.3603.

MS M/Z (rel.int.): 456 (6%), 438 (4%), 411 (3%), 302 (4%), 283 (2.5%), 248 (37%), 220 (28%), 207 (4.1%), 203 (40%), 202(14%), 190 (40%), 189 (100%), 175 (48%), 174 (11%).
$^1$H NMR (pyridine - $d_6$): δ = 0.69 (3H, *s*), 0.83 (3H, *s*), 0.88 (3H, *s*), 0.92 (3H, *s*), 1.03 (3H, *s*), 1.63 (3H, *s*), 3.34 (2H, *m*), 4.65 (1H, *d*, *J* = 2.11 Hz), 4.81 (1H, *d*, *J* = 2.11 Hz);

$^{13}$C-NMR (pyridine - $d_6$) : δ = 37.73 (*t*, C-1), 28.05 (*t*, C-2),78.34 (*d*, C-3), 41.32 (*s*, C-4), 56.14 (*d*, C-5), 19.02 (*t*, C-6), 33.09 (*t*, C-7), 41.32 (*s*, C-8), 51.17 (*d*, C-9), 39.75 (*s*, C-10), 21.44 (*t*, C-11), 26.32 (*t*, C-12), 38.81 (*d*, C-13), 43.06 (*s*, C-14), 30.51 (*t*, C-15), 35.06 (*t*, C-16), 56.84 (*s*, C-17), 47.58 (*d*, C-18), 49.95 (*s*, C-19), 151.51 (*s*, C-20), 31.42 (*t*, C-21), 39.52 (*t*, C-22), 29.91 (*q*, C-23), 16.61 (*q*, C-24), 16.64 (*q*, C-25), 15.16 (*q*, C-26), 14.68 (*q*, C-27), 179.07 (*s*, C-28), 110,23 (*d*, C-29), 19.73 (*q*, C-30).

### 3.1.4 Isolation of genipin (49)

The $CH_2Cl_2$ fraction (78.5 g) was filtered through a short plug of silica gel using MeOH as eluent. A portion of this material (1.38 g) was subsequently separated on a slurry-packed column eluting with EtOAc-hexane, initially as a (1:1) mixture, but gradually increasing the ratio to (3:2) 60 fractions were collected of 15 ml each. Fractions (38 - 57) were combined and the solvent removed to give impure material (457 mg). The procedure as described above was repeated and the solvent removed to give pure material (140 mg) giving one spot on tlc. Recrystallization in MeOH afforded white prisms, mp 120 - 121°C, $[\alpha]_D^{20}$ = + 109° (c.0.06 , $CHCl_3$) (lit.[47], +135°)
$^1$H NMR ($CDCl_3$ 200 MHZ) δ = 2.10 (1H, *m*, H-6a), 2.54 (1H, *dd*, *J* = 9.5, 8.6 Hz, H-9) 2.90 (1H, *m*, H-6b), 3.22 (1H, *m*, H-5), 3.72 (3H, *s*,-$CO_2$Me), 4.28 (1H, *d*, *J* = 13.4 Hz, -$CH_2$OH), 4.35 (1H, *d*, *J* = 13.4 Hz, -$CH_2$OH), 4.81 (1H, *d*, *J* = 8.6 Hz, H-1), 5.88 (1H, *bs*, H-7), 7.52 (1H, *s*, H-3)
$^{13}$C-NMR ($CDCl_3$, 50 MHz) : δ = 36.7 (*d*, C-5), 39.0 (*t*, C-6), 48.1(*d*, C-9), 51.4 (*q*, $CO_2$Me), 61.3 (*t*, $CH_2$OH), 96.3 (*d*, C-1), 110.7 (*s*, C-4), 131.0 (*d*, C-7), 141.9 (*s*, C-8), 152.5 (*d*, C-3), 168.0 (*s*, $\underline{C}O_2$Me).

MS m/z (rel.int.) 226 $[M]^+$ (20), 208(27), 190(22), 180(48), 176(77), 162(49), 148(100), 120(73), 91(49), 78(47). HR - MS$[M]^+$ 226.0836 calculated for $C_{11}H_{14}O_5$ 226.0841.

### 3.1.5 Genipin 1,10 - diacetate (55)

Genipin (**49**) (65 mg) was acetylated by pyridine-acetic anhydride[49] method to give the diacetate derivative as a non crystalline material (41mg). $R_f$ 0.62 [EtOAc-hexane (1:1)]

$^1$H NMR ($CDCl_3$ 200 MHz) : δ = 2.09 (1H, *m*, H-6a), 2.85 (1H, *dd*, *J* = 8.15, 6.68 Hz H-9), 3.3 (1H, *m*, H-5), 3.74 (3H, *s*,-$CO_2$Me), 4.10 (1H, *dd*, *J* = 7.14 Hz $CH_2$OAc), 4.62 (1H, *dd*, *J* = 13.55 Hz $CH_2$OAc), 5.85 (1H, *bs*, H-7), 7.27 (1H, *s*, H-3)

$^{13}$C-NMR ($CDCl_3$, 50 MHz): δ = 20.8 (*s*, $OCOCH_3$), 20.9 (*s*, $OCOCH_3$), 34.6 (*d*, C-5), 38.6 (*t*, C-6), 45.1 (*d*, C-9), 51.3 (*q*, $CO_2$Me), 61.7 (*t*, $CH_2$OAc), 91.7 (*d*, C-1), 111.2 (*s*, C-4), 132.9 (*d*, C-7), 136.5 (*s*, C-8), 151.6 (*d*, C-3), 167.2 (*s*, $CO_2$Me), 169.3 (*q*, $CH_2OCOCH_3$), 170.6 (*q*, $OCOCH_3$).

### 3.1.6 Isolation of 10 - acetyl genepin (58)

From the 60 fractions of 15 ml each collected previously, fractions 19 - 35 were combined and the solvent was removed under vacuum to give a white syrup 10-acetyl genipin (58 mg) with higher $R_f$. 0.62 [EtOAc-hexane (1:1)]. Compounds **55** and **58** have the same $R_f$

$[\alpha]_D^{20} = +22.6^\circ$ (c.0.06,$CHCl_3$)(lit.[48], $+48.2^0$).

$^1$H NMR ($CDCl_3$, 200 MHz): δ = 2.05 (1H, *m*, H-6a), 2.11 (3H, *s*, $OCOCH_3$), 2.52 (1H, *m*, H-9), 2.91 (1H, *m*, H-6b), 3.19 (1H ,*m*, H-5), 3.77 (3H, *s*, $CO_2$Me), 4.79 (1H, *d*, *J* = 8.6 Hz H-1), 4.70 (1H, *d*, *J* = 12.9 Hz, $CH_2$OAc), 4.95 (1H, *d*, *J* = 12.9Hz $CH_2$OAc), 5.95 (1H, *s*, H-7), 7.53 (1H, *s*, H-3)

$^{13}$C-NMR (DMSO-$d_6$, 50 MHz): δ = 20.7 (*q*, OCOMe), 35.7 (*d*, C-5), 38.3 (*t*, C-6), 46.9 (*d*, C-9), 51.0 (*q*, $CO_2$Me), 62.2 (*t*, $CH_2$OAc), 96.0 (*d*, C-1), 109.8 (*s*, C-4), 129.0 (*d*, C-7), 138.9 (*s*, C-8), 152.8 (*d*, C-3), 167.1 (*s*, $CO_2$Me), 170.1 (*s*, OCOMe).

MS m/z (rel.int.): 268 $[M]^+$ (1.0), 155(2), 127(4), 99(13), 85(45) HR-MS $[M]^+$ 268.0947 calculated for $C_{13}H_{16}0_6$ 268.0939.

## 3.2 Chemical Investigation of *Gardenia thunbergia (Rubiaceae)*

### 3.2.1 Plant material

Leaves of *Gardenia thunbergia* were collected from a tree growing in National Botanical Garden, Durban. The dried material (1.2 kg) was milled to a fine powder before extraction.

### 3.2.2 Extraction

Leaves (5.6 kg) were obtained from *Gardenia thunbergia* trees located in the National Botanical Garden Durban. The identification was done by Dr. Tanza E.Clark , and voucher specimens were deposited in the Herbarium of the University of Natal Pietermaritzburg. (Sample n° 1 T.E.Clark - 1994).

The air dried leaves (1.2 kg) were milled to a fine powder. This was extracted in water (5 l) for 7 days. A sufficient amount of EtOAc was added to the aqueous solution (500 ml) for two phases to separate. EtOAc was removed by distillation and the process was repeated a number of times to give (2.37 g) of ethyl acetate extract. The EtOAc extract was purified using a short silica gel column. By this procedure 1.43 g was obtained and was subsequently separated on a chromatotron plate (4mm) using EtOAc-hexane (2:3) as eluent. The polarity was increased stepwise to (1:1) EtOAc-hexane .

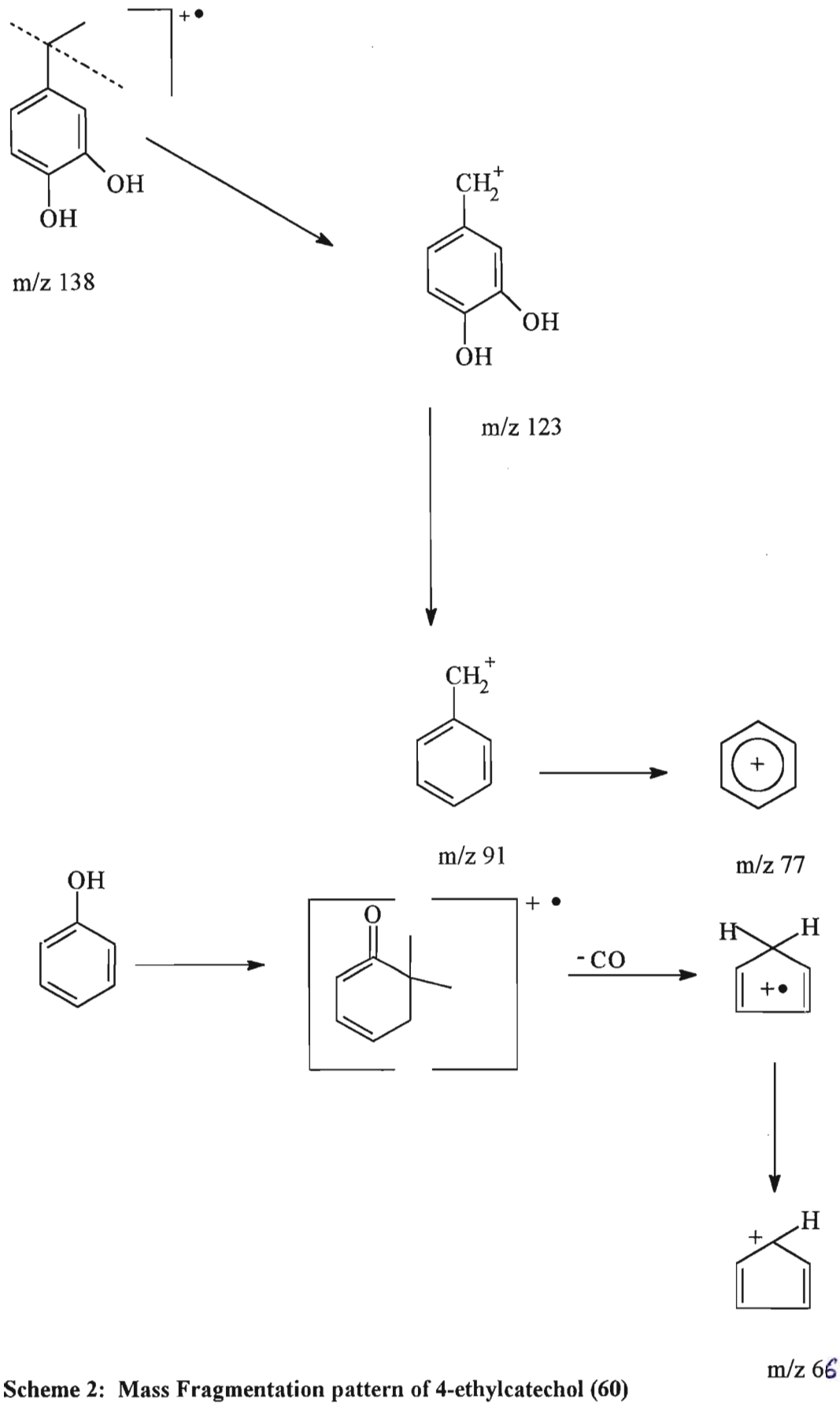
### 3.2.3 Isolation of 4 - ethylcatechol (60)

The EtOAc fraction (2.37 g) was purified using a short silica gel column to provide 1.38 g which was subsequently separated on a chromatotron plate (4 mm) elucting with EtOAc-hexane initially as a (2:3) mixture, but gradually increasing the ratio to (1:1). A total of 18 fractions was collected and the solvent removed to give impure material (338 mg). The procedure was repeated to give a non crystalline pure material (129 mg). It migrated as one red spot on tlc with the standard dip reagent.

4-Ethylcatechol (**60**) proved difficult to identify because of the complexity of the "purified" material.

At one stage, the compound was contaminated by a higher molecule weight impurity at m/z 194 which initially confused the identification process. The most abundant ion in the HR-MS is at m/z 138. The mass fragmentation pattern of 4-ethlycatechol (**60**) is shown on p.52.

$^{1}H$ - and $^{13}C$ - NMR spectra data are shown in table 6 and $^{1}H$ - and $^{13}C$ - NMR spectra are shown on p.71 - 74.




**Scheme 2: Mass Fragmentation pattern of 4-ethylcatechol (60)**

# CHAPTER FOUR

## 4.1 RESULTS FOR MOLLUSCICIDAL ACTIVITY

The results of this investigation are the product of a collaborative effort between the Department of Zoology and Entomology (U.N.P.) and the Department of Chemistry and Chemical Technology. (U.N.P.)

All bioassays were conducted by Dr. Tanza E.Clark and Ms. T. Brackenbury under the supervision of Prof. C.C. Appleton.

*Bulinus africanus*, identification confirmed by Dr D.S. Brown (Natural History Museum, London), the intermediate snail host of *Schistosoma haematobium* ,was used as a target snail.

Solutions of the compound to be tested were made up in aged dechlorinated tap water, on a weight to volume basis (mg/l or ppm). Where a pure compound was oily, solutions were made up volume for volume (ml/l).

Accordingly, the concentrations were converted to mg/l or ppm for comparative purposes.

Snails were exposed in groups of 5 in glass jars containing 400 ml of test solution. Sufficient space was left for snails to leave the solutions should they be irritated. The exposure period of 24- hours was followed by a 24- hours recovery period. The number of replicates and range of test concentrations used was dependant on the quantity of compound and the number of snails available. A minimum of 3 replicates were completed for all test concentrations. Snails were considered dead if no muscular movement could be elicited by mechanical prodding of the head-foot. All tests were carried out at a constant temperature of 23° C ± 1° and light conditions were controlled.

$LD_{50s}$ and $LD_{90s}$ were calculated for active compounds using Fieller's method as implemented in the Genstat 5.0 program[59].

### 4.1.1 Molluscicidal Activity for *Apodytes dimidiata (Icacinaceae)*

The results of exploratory bioassays for molluscicidal activity following solvent extraction showed that all fractions (hexane, dichloromethane, ethyl acetate and ethanol), had some activity between 500 and 1000 ppm. The most active fraction was a dichloromethane bark extract. The broad spectrum of activity detected for all plant parts indicated the active compound(s) are widely distributed throughout the whole plant.

On the basis of these results a fresh dichloromethane extract was further purified and two new molluscicidal compounds were isolated : genipin (**49**) and 10-acetyl genipin (**58**)

The results are summarised in the table below.

Mean percentage mortality ( ± SD) of *Bulinus africanus*

| Genipin (**49**) | | 10-Acetyl genipin (**58**) | |
|---|---|---|---|
| Conc. ppm | % Mortality | Conc. ppm | % Mortality |
| 40 | 100 | 35.27 | 100 |
| 20 | 30 | 23.35 | 30 |
| 15 | 20 | 10 | 10 |
| 10 | 10 | 7 | 0 |
| 8 | 0 | | |
| $LD_{50}$ = 25.27 ppm. (23.57 - 27.11) | | $LD_{50}$ = 21.72 ppm. (15.48 - 29.96) | |
| $LD_{90}$ = 32.57 ppm. (29.96 - 34.96) | | $LD_{90}$ = 39.40 ppm. (27.11 - 56.82) | |

Table 7: Lethal dosages for genipin and 10-acetyl genipin from *Apodytes dimidiata.*

### 4.1.2 Molluscicidal activity for *Gardenia thunbergia (Rubiaceae)*

An aqueous solution of *Gardenia thunbergia* leaves showed molluscicidal activity close to the activity levels recommended by WHO [60,61].

A fresh aqueous solution was extracted with EtOAc and bioassays proved that the active compounds had been transferred into the EtOAc extract.

Further purification of the EtOAc extract allowed the isolation of a new molluscicidal compound $GT_1$ identified as 4-ethylcatechol (**60**). The identification of $GT_2$ has not been completed because of time constraints.

The results are summarised in table 8.

4-Ethlycatechol (**60**)

| Conc. ppm | % Mortality |
|---|---|
| 1 | 0 |
| 3 | 10 |
| 5 | 90 |
| 10 | 85 |

$LD_{50}$ - 4.8 ppm (2.48 - 9.29)

$LD_{90}$ - 9.48 ppm (4.9 - 18.35)

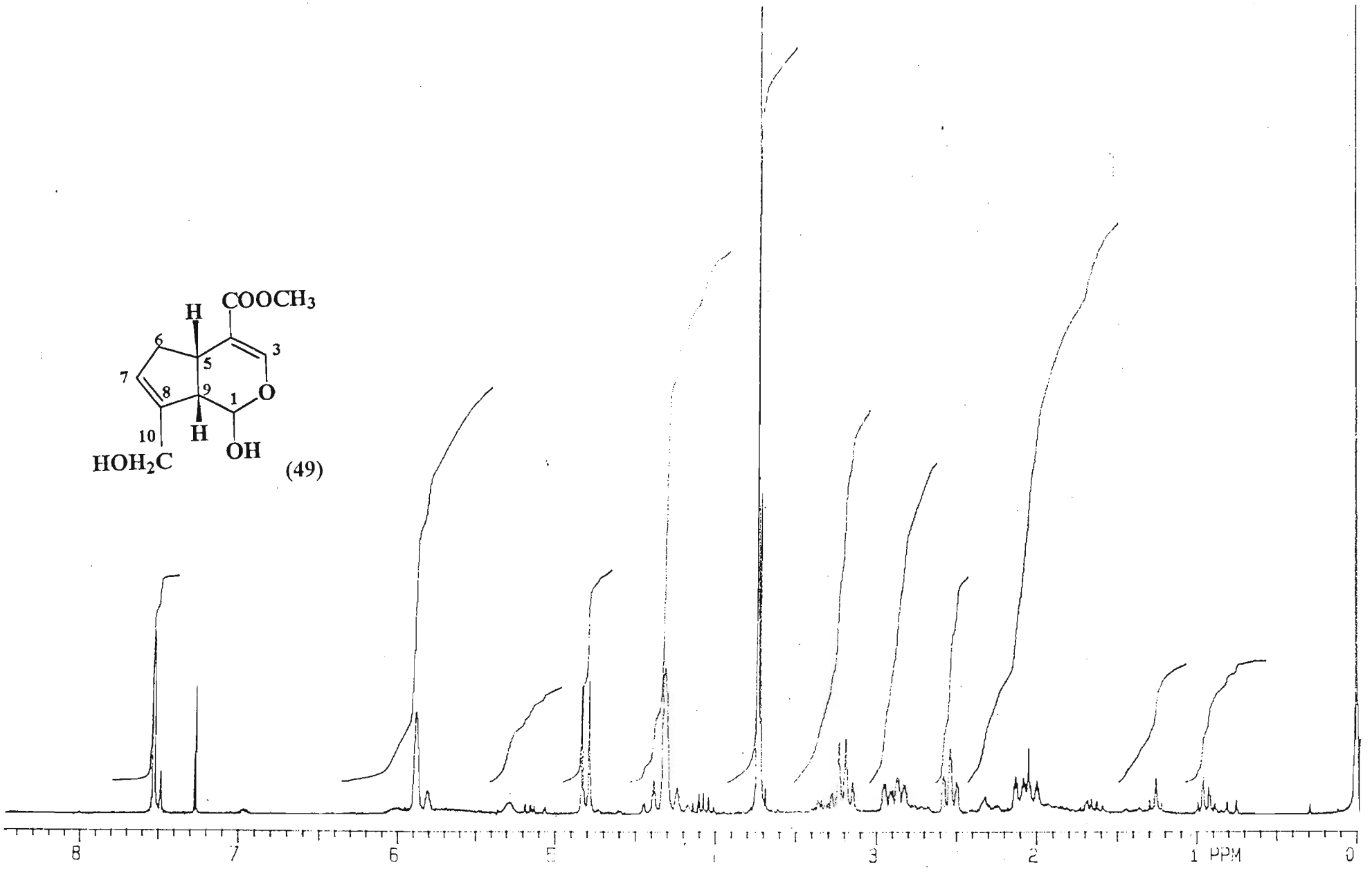
Table 8: Lethal dosages for 4-ethlycatechol from *Gardenia thunbergia*.

## Conclusion

While chemotherapy will undoubtedly play a vital role in reducing the severity of schistosomiasis, and assist in the control of transmission via environmental contamination, it is considered that snail control by the application of molluscicides will continue to play an important complementary part in intervention measures. Every effort should therefore be made to explore the possibility of using low cost natural materials of local origin, if they can be feasibly developed. Care must be taken to investigate the mammalian toxicity of these natural products before successful snail eradication schemes can be considered. A constant search for new classes of molluscicidal natural products is essential, so that problems of selectivity and low activity can be overcome. In addition, as wide a range as possible of structurally-related compounds should be isolated or synthesized for structure-activity and mode-of-action studies. Only by understanding the mechanisms of action can newer, more effecient molluscicides be designed.

It is precisely into this scenario that the two compounds isolated in this work, genipin and 4-ethylcatechol fit. Both originate from plant origins and both have potential as plant molluscicides. Genipin, which occurs in reasonably high concentrations in the leaves of *Apodytes dimidiata* , requires further extensive testing as a "natural" molluscicide present in trees that can be cultivated on stream banks of bilharzia infected areas.

**4-Ethylcatechol, a potent molluscicide is present only in trace amounts in *Gardenia thunbergia*. However, its synthesis is relatively simple and further tests on this compound as well as on analogues related to it, provide a daunting challenge for the future.**

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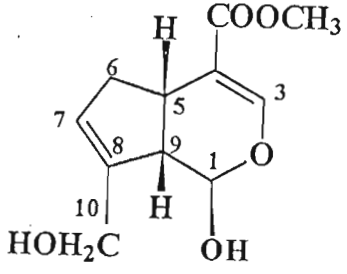
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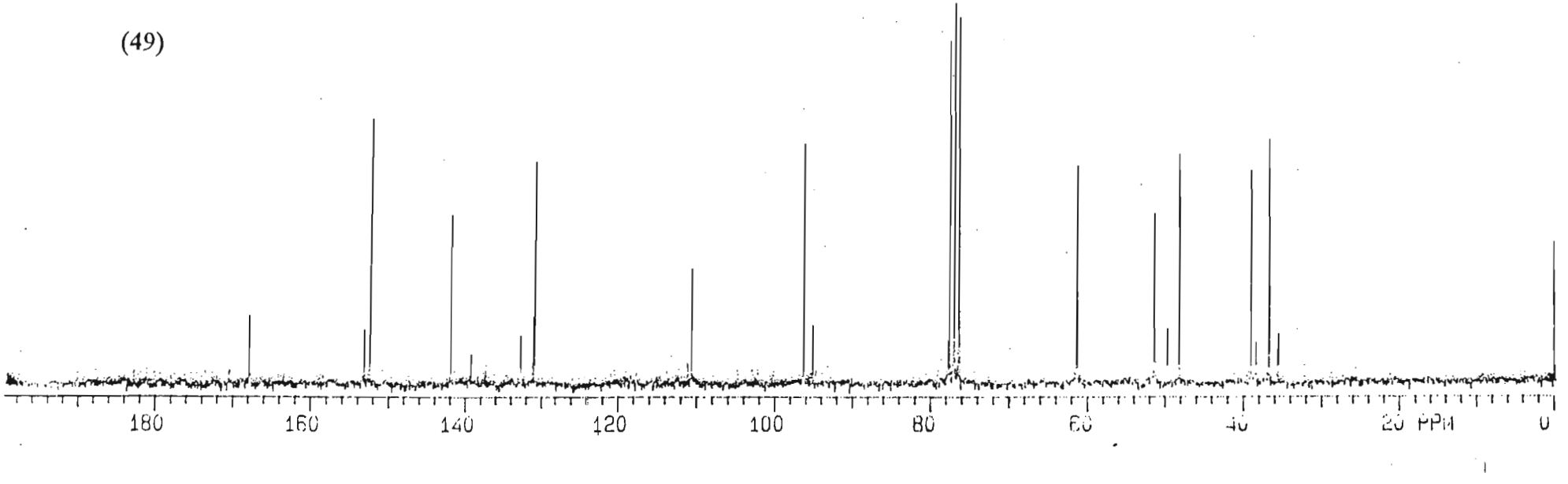
OH

(49)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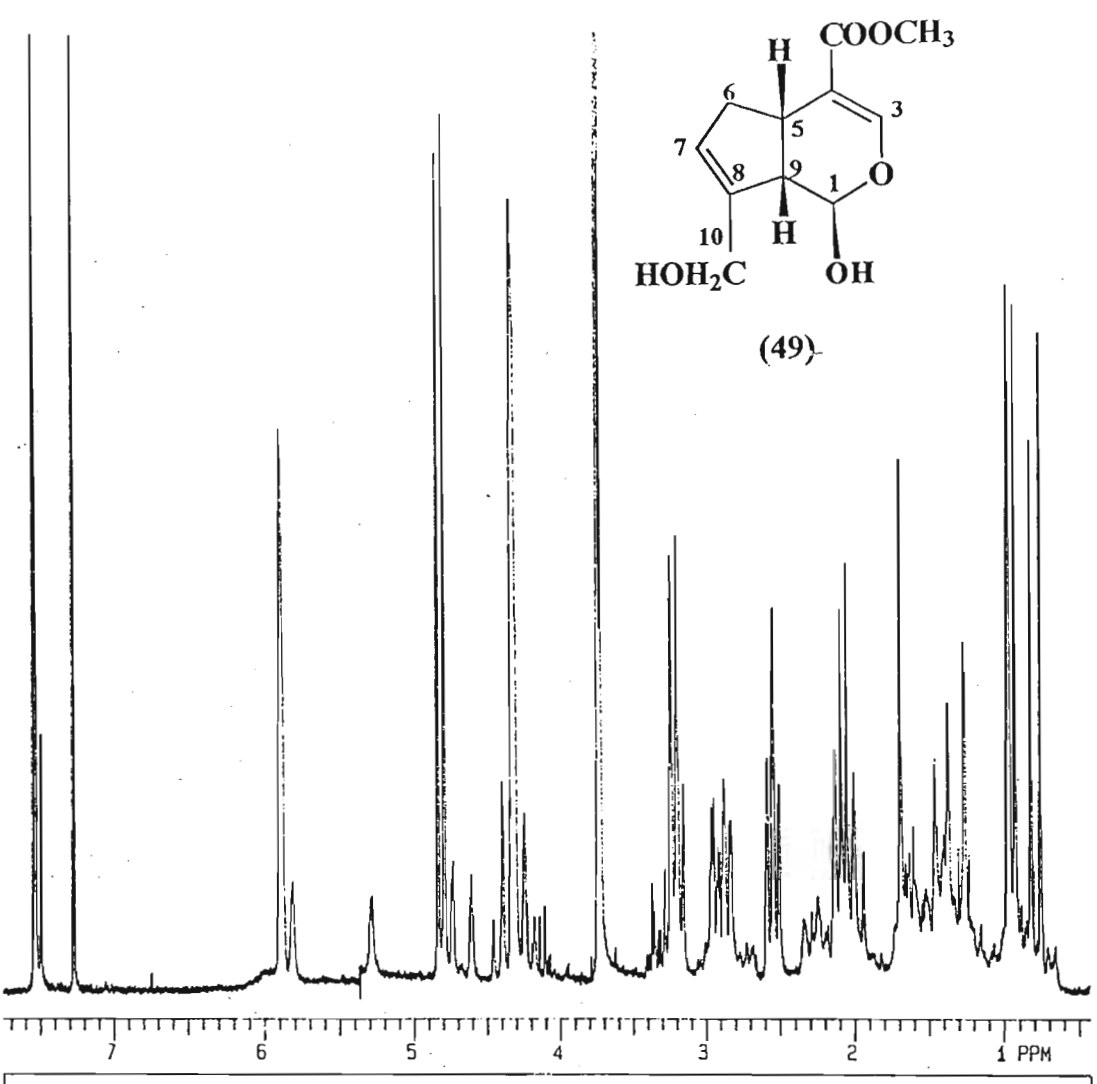


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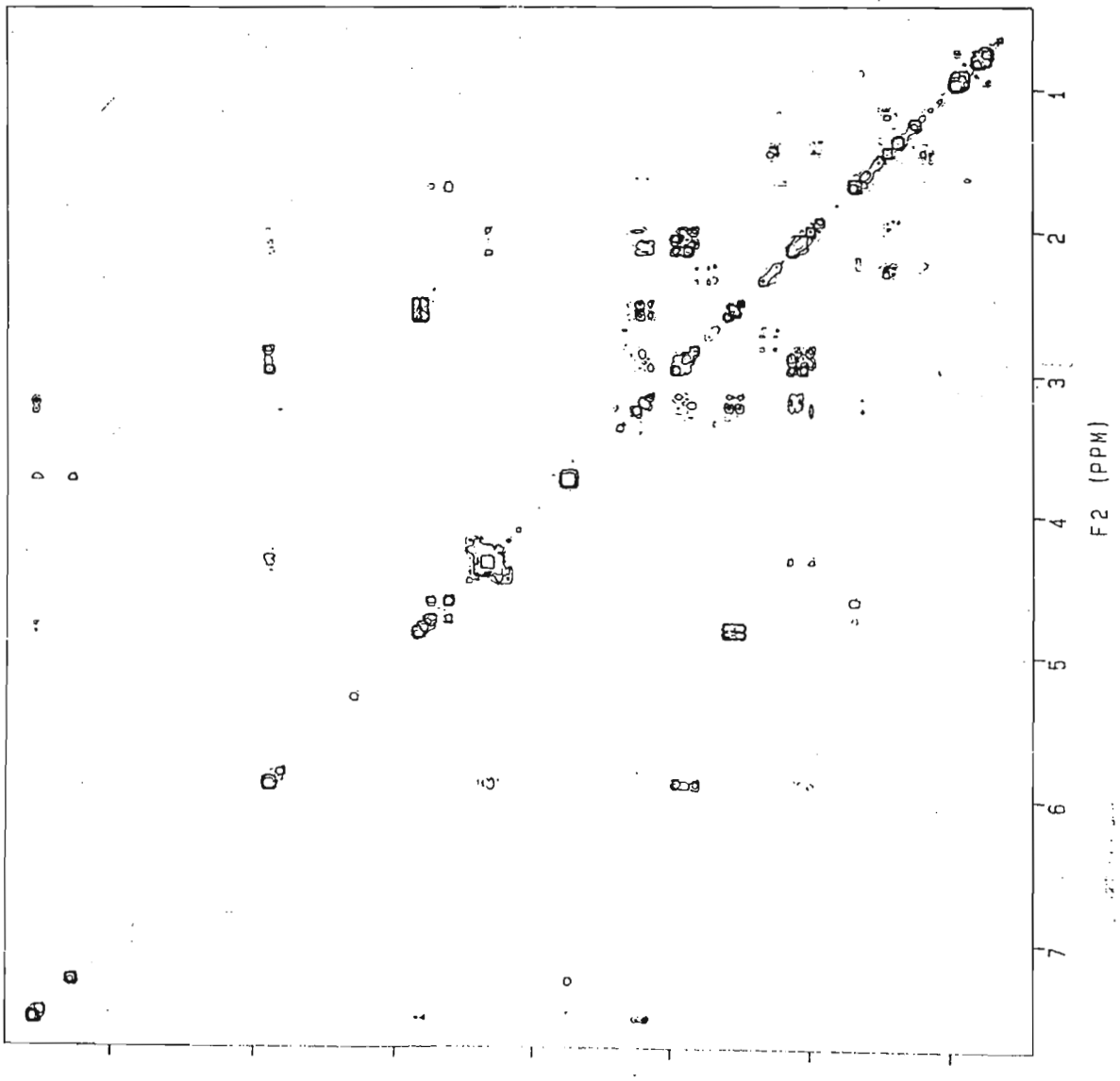

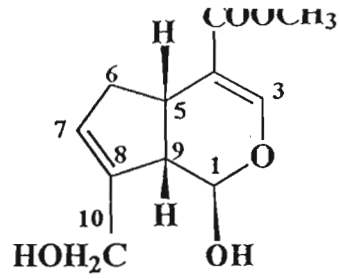
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OH

(49)


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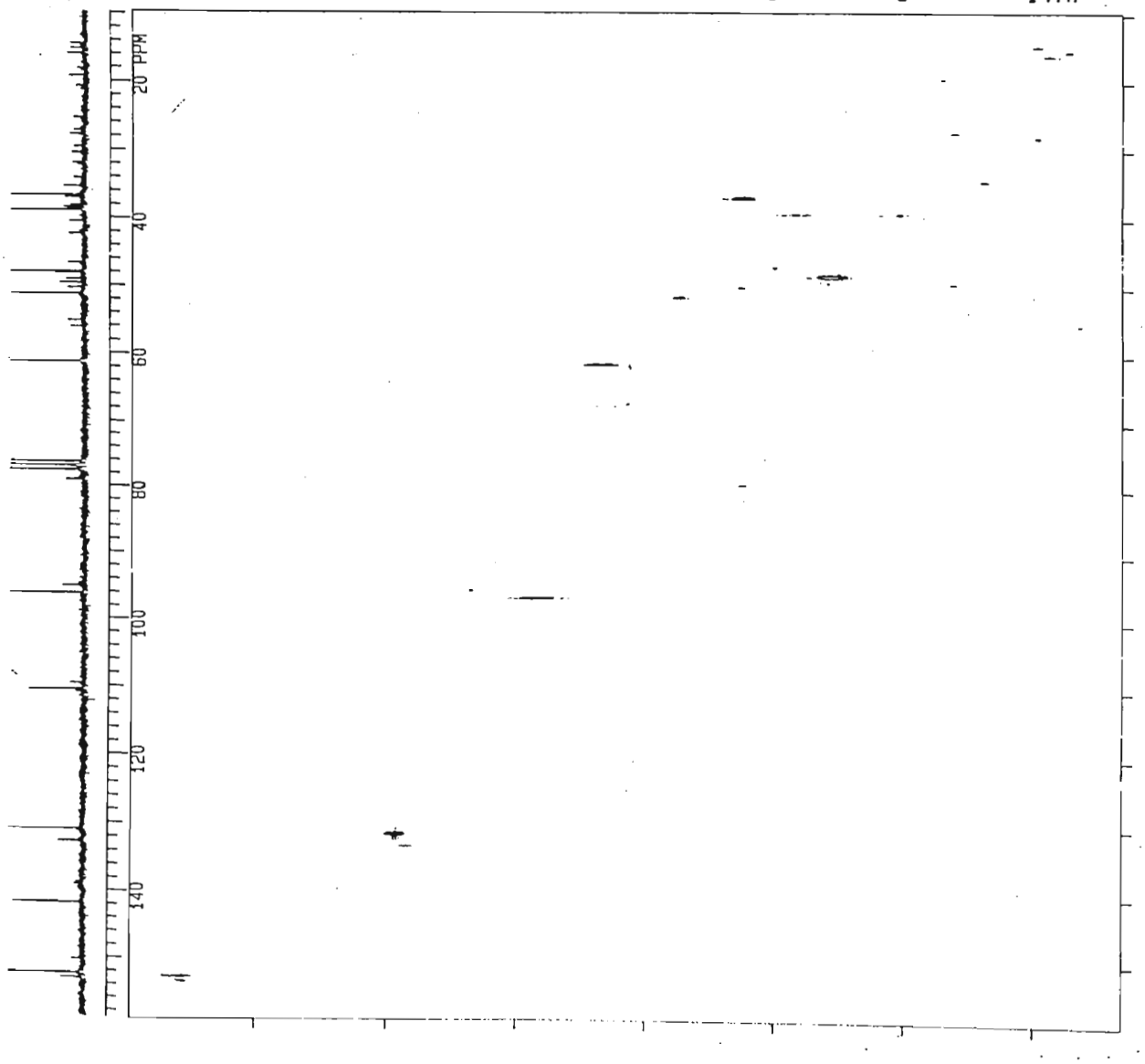
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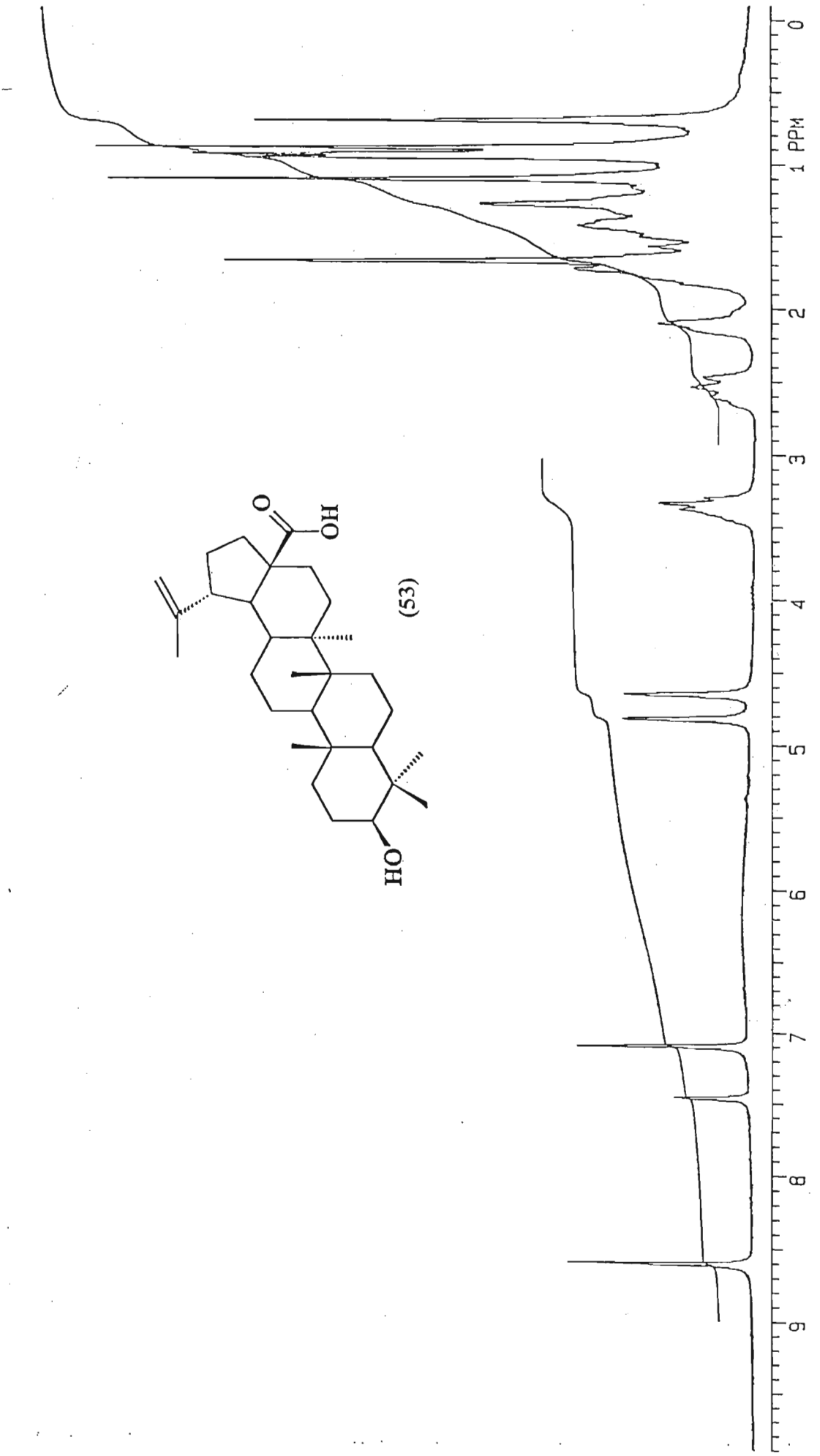
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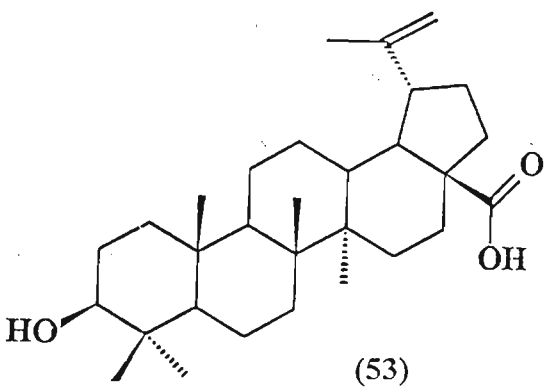
7
8
9

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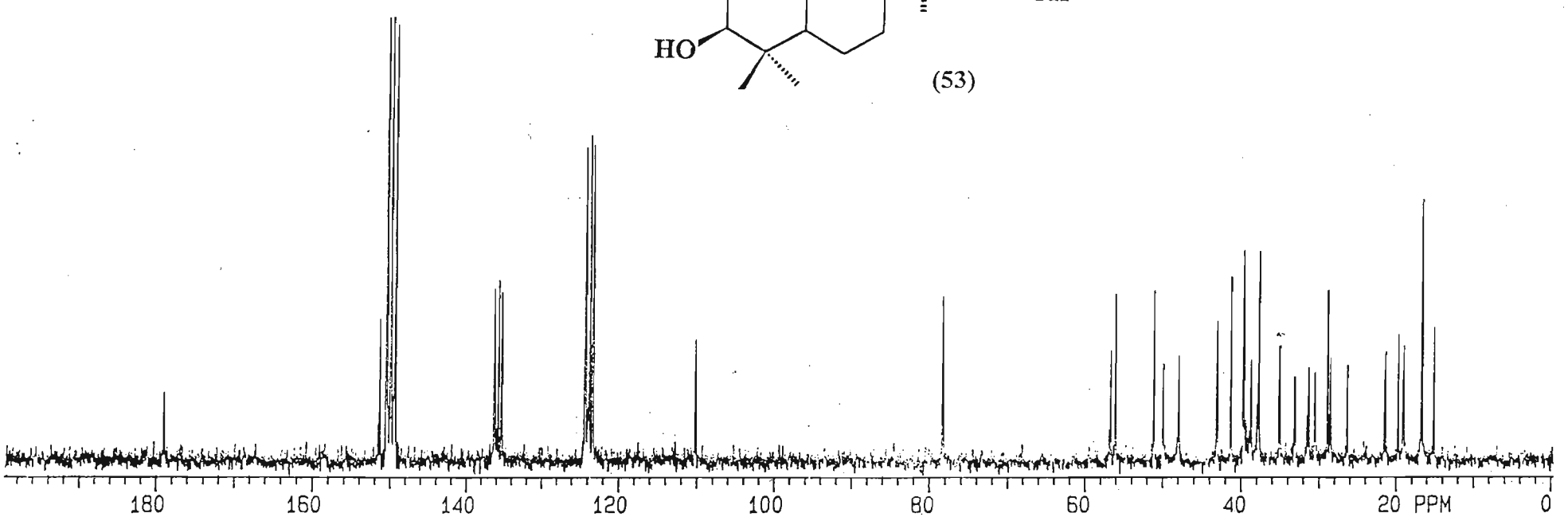

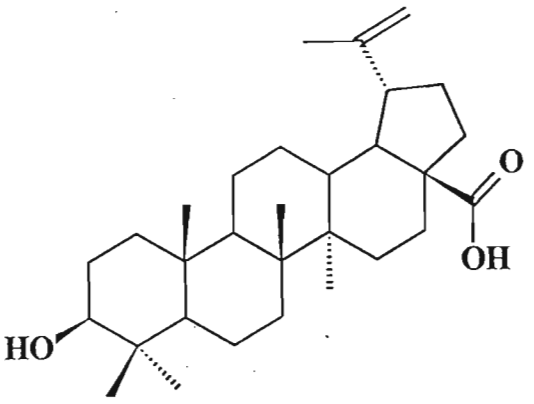


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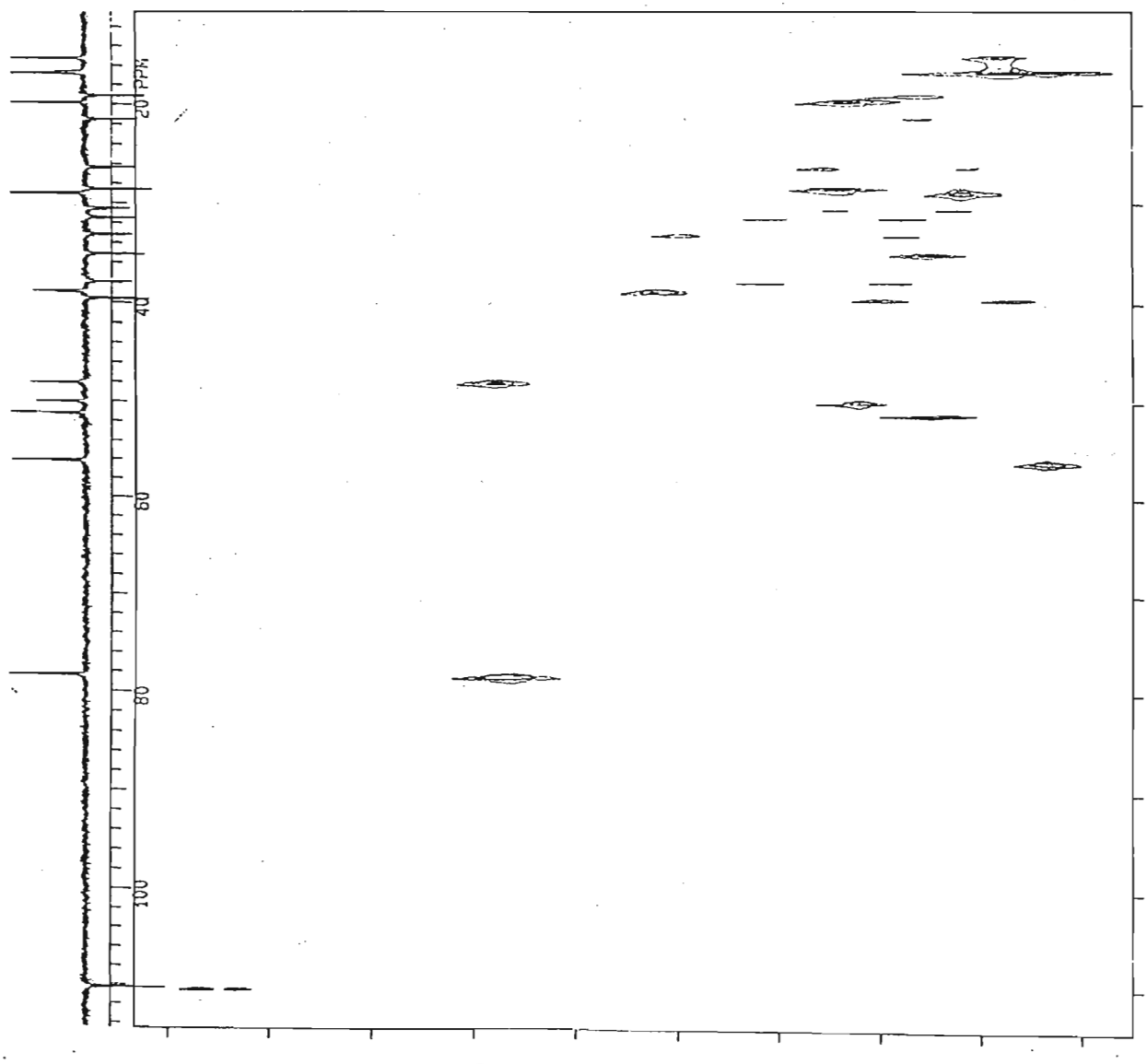

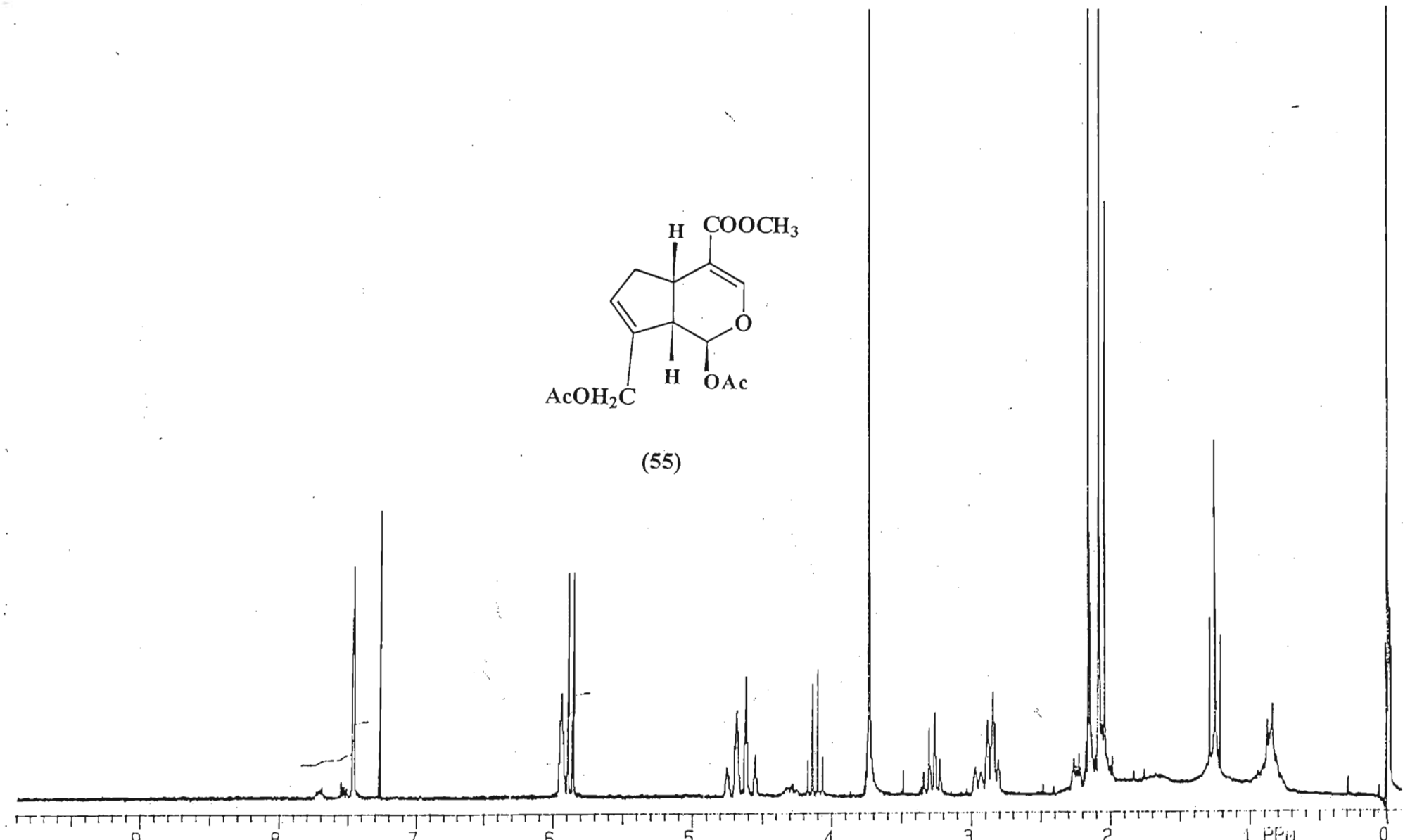


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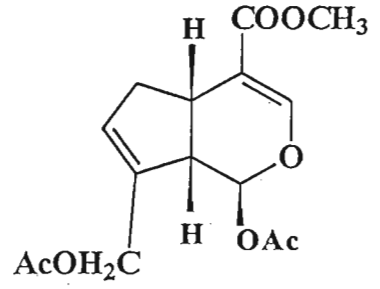


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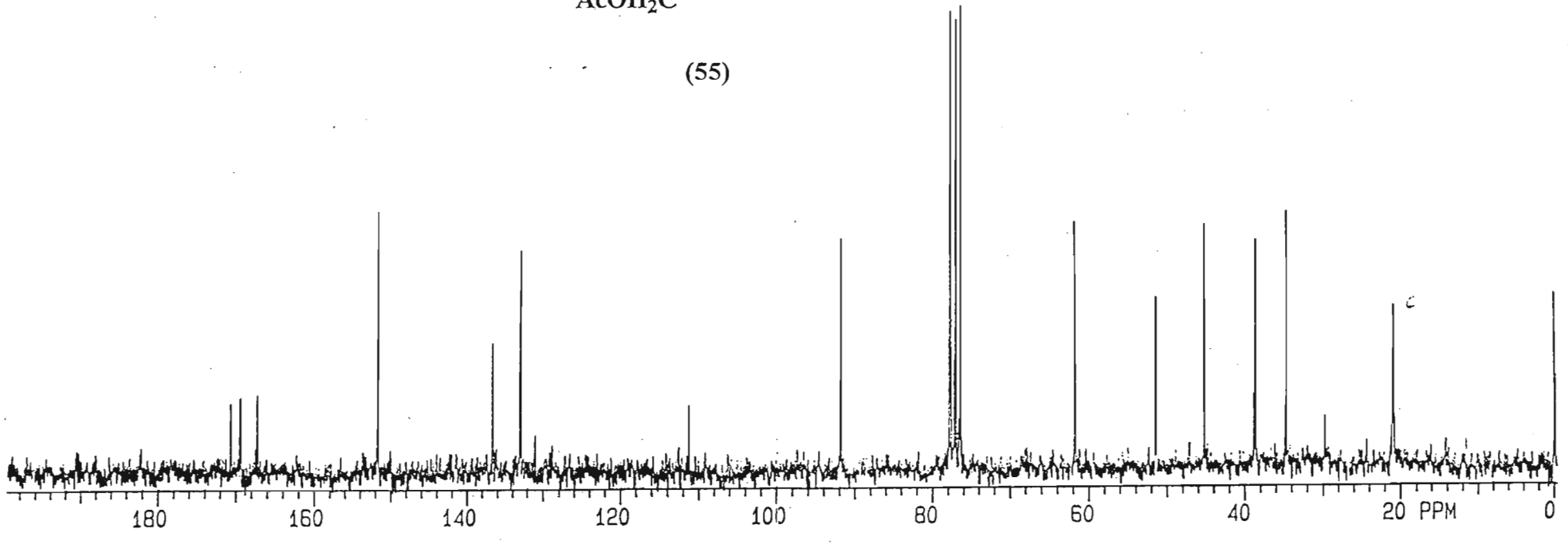

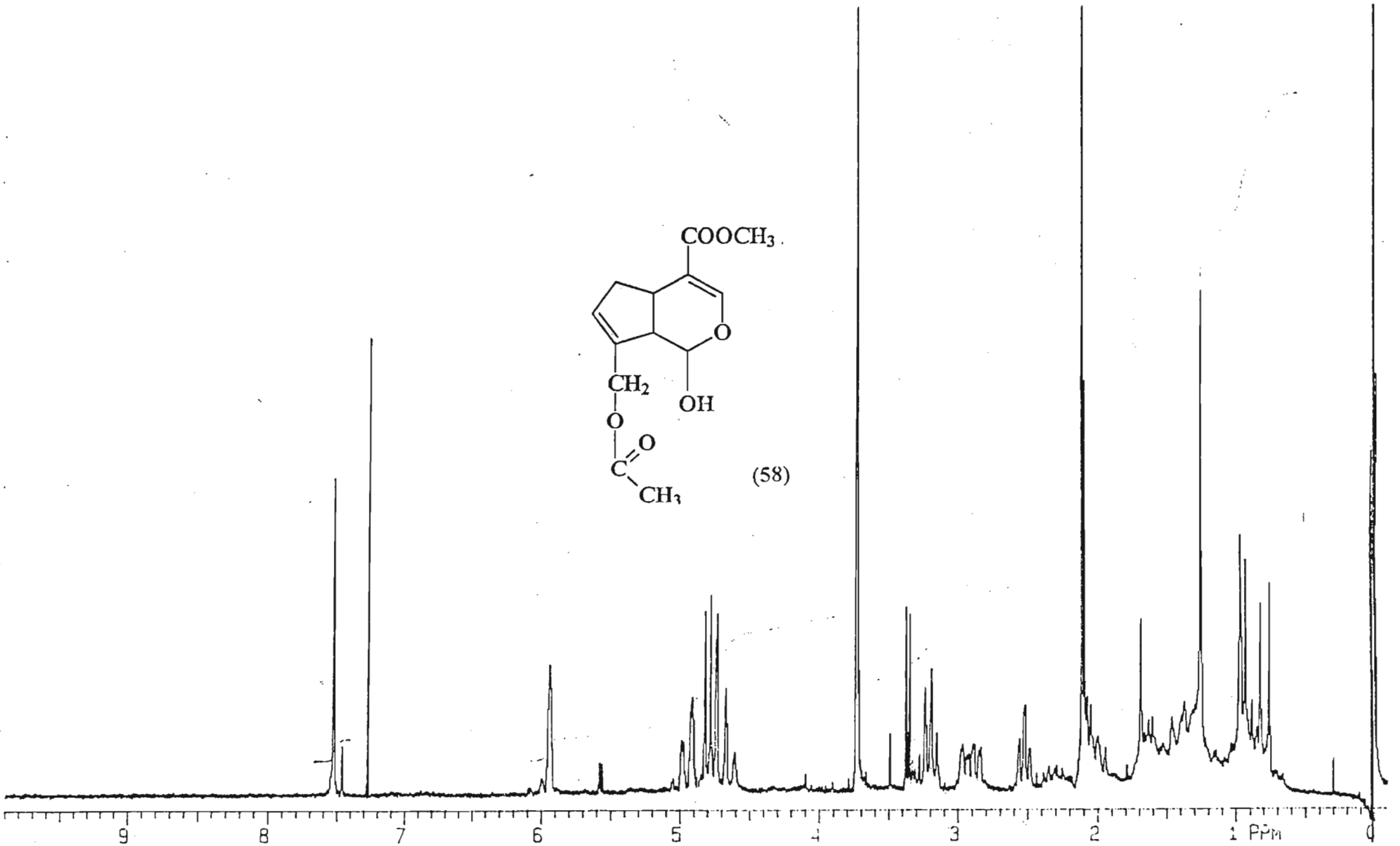


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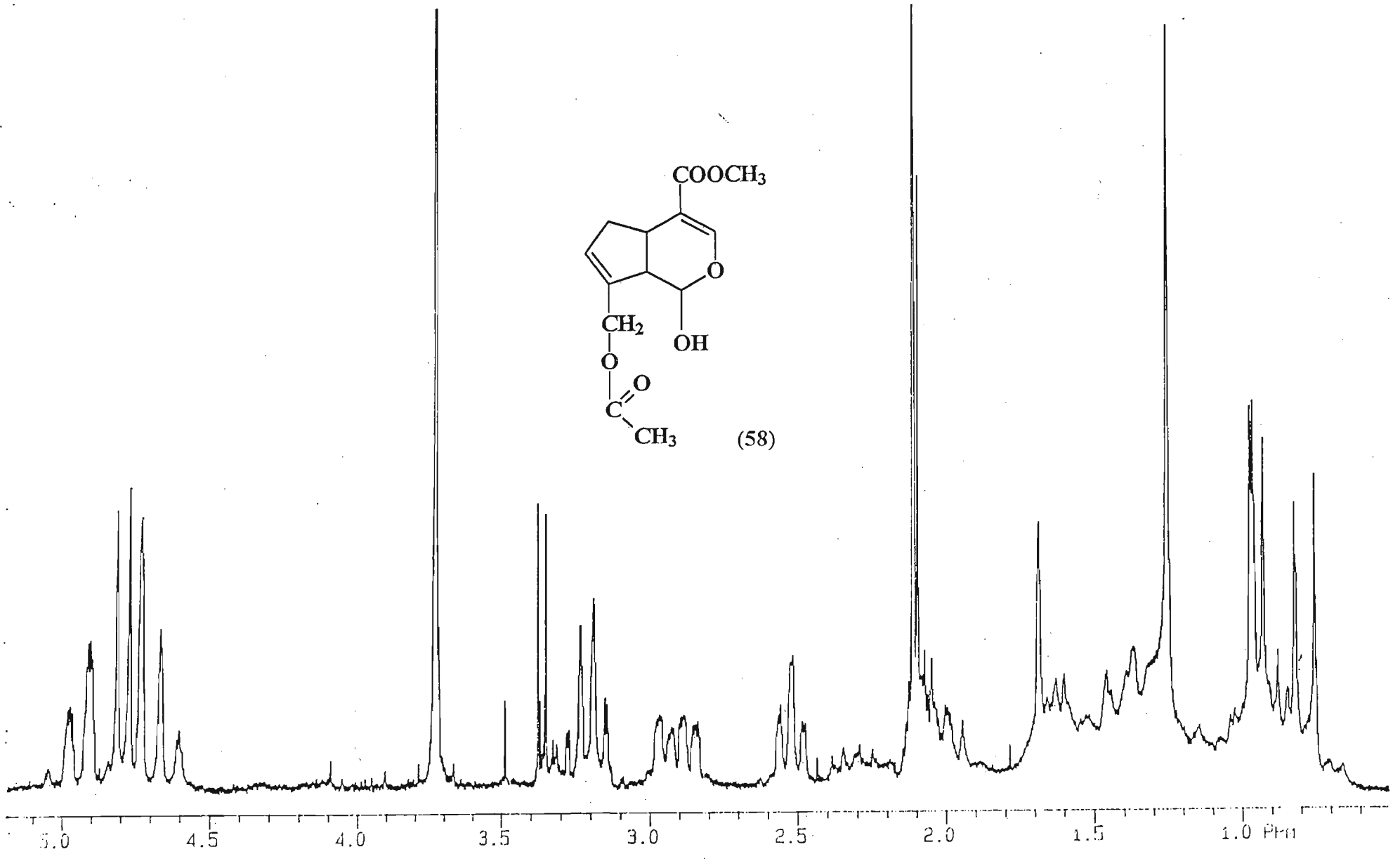


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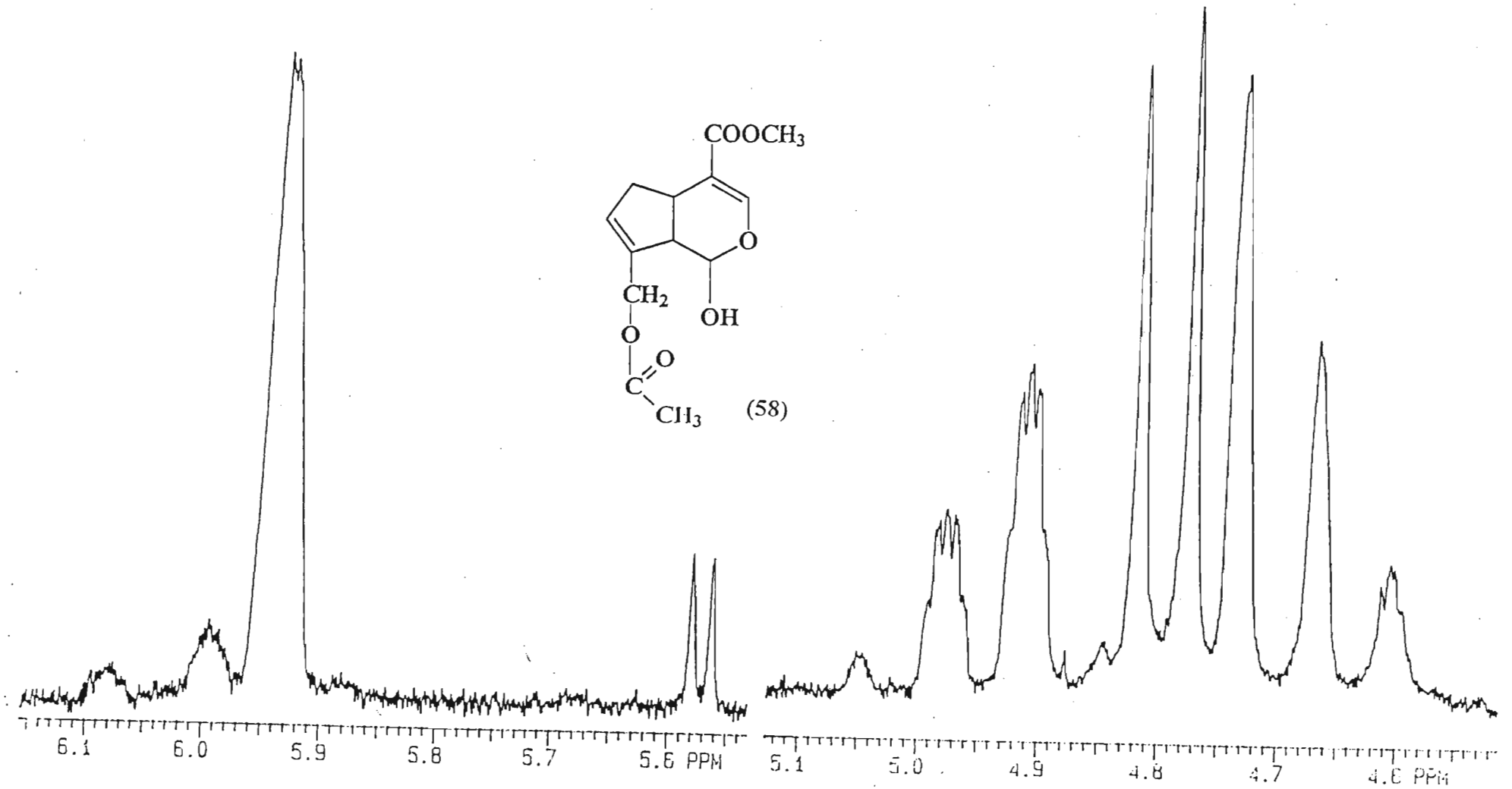


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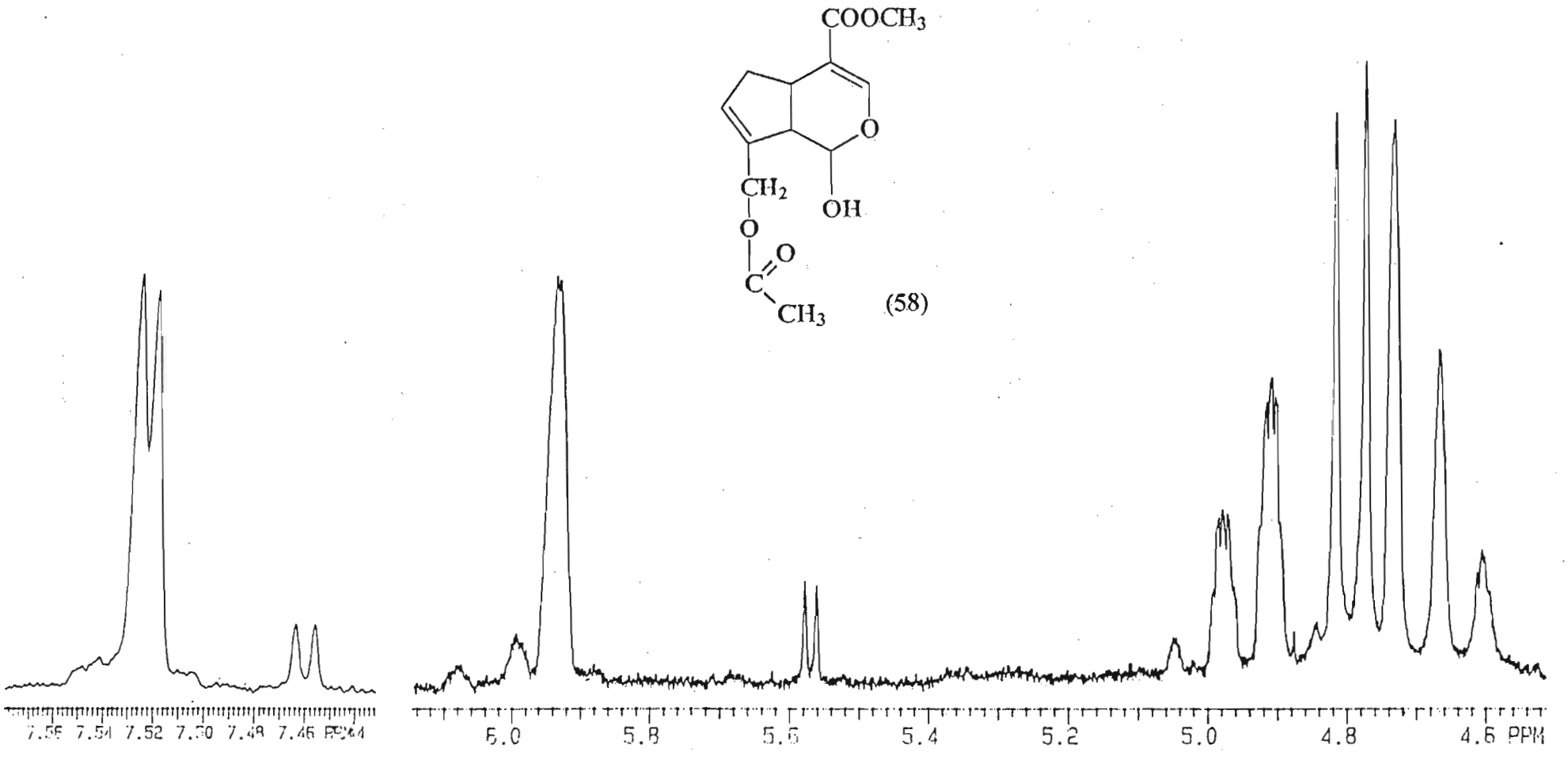
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CH2
OH
O
O
C
CH3
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7.56 7.54 7.52 7.50 7.48 7.46 PPM
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5.6
5.4
5.2
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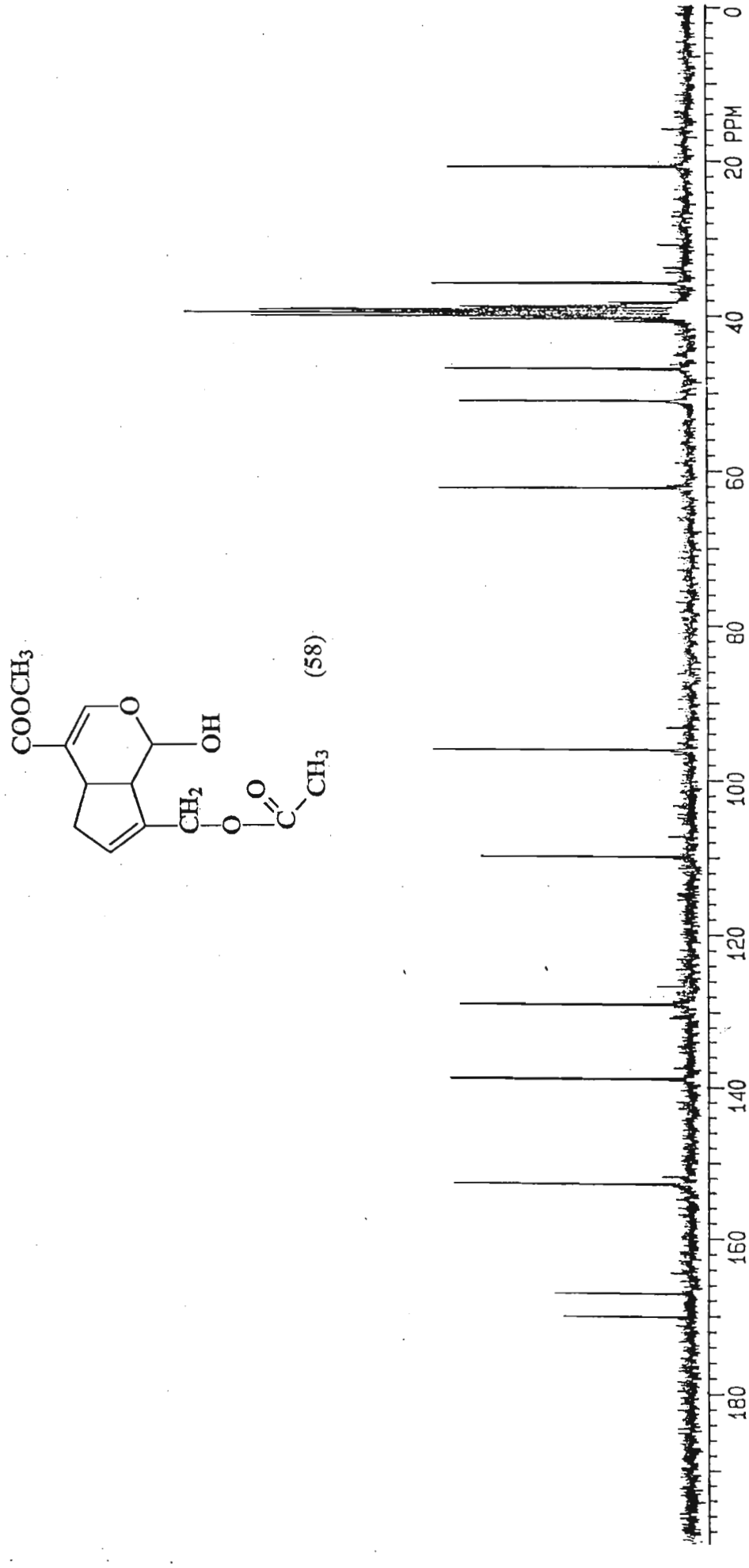
4.8
4.6 PPM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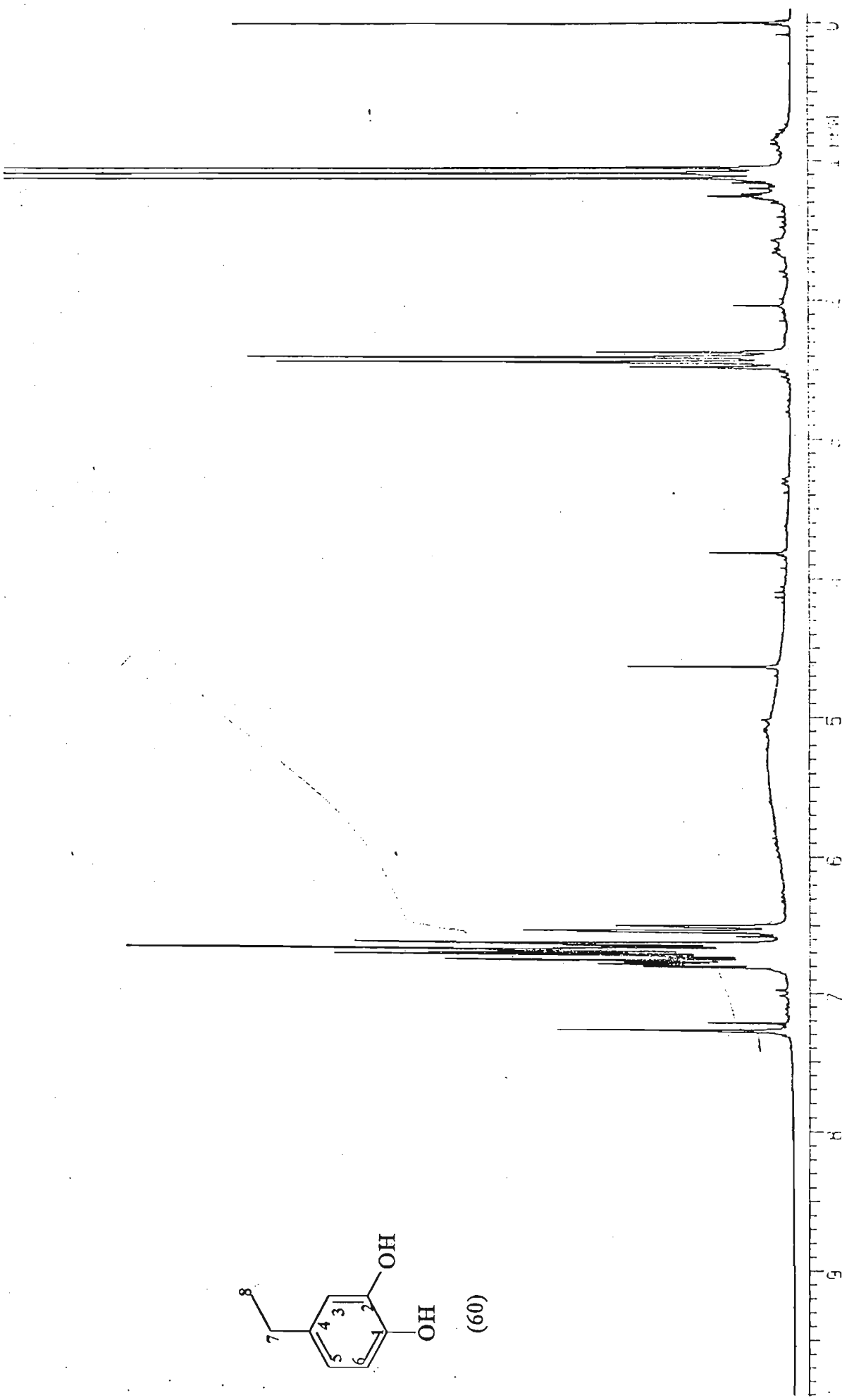


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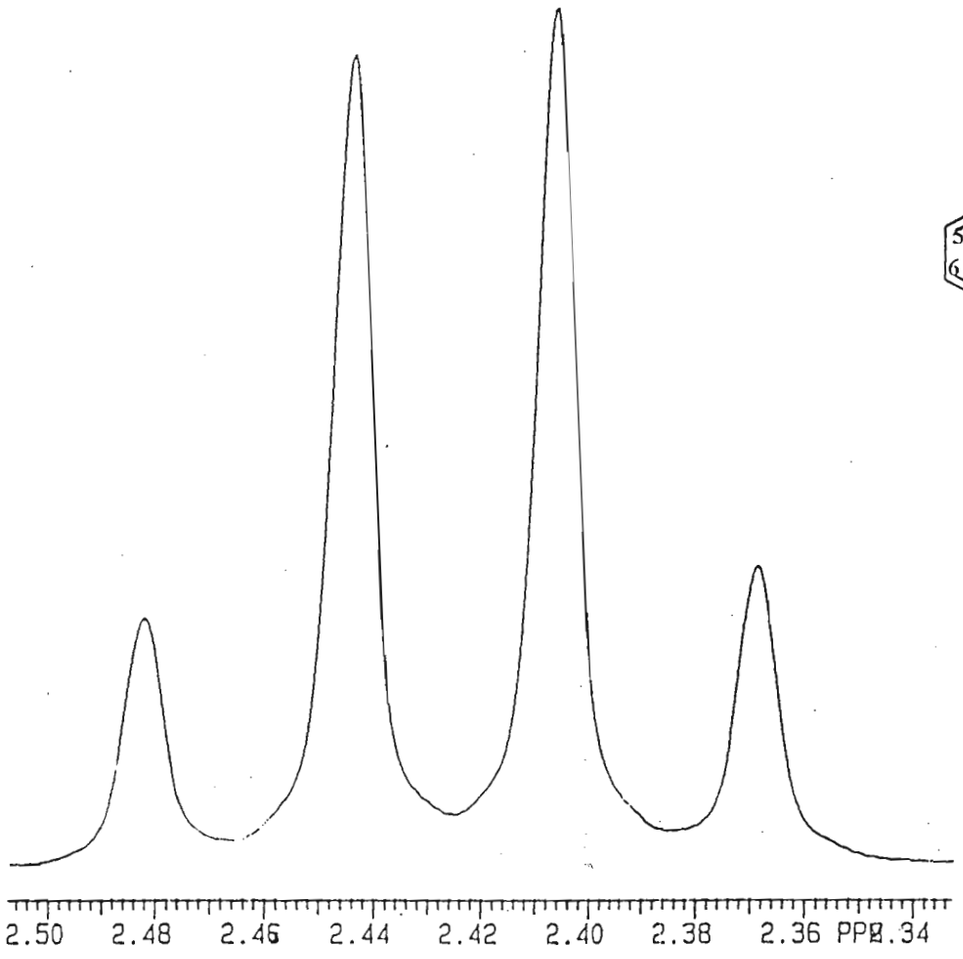


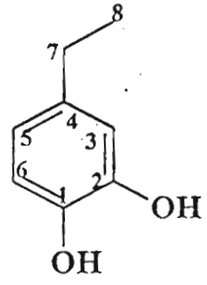


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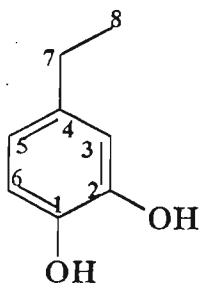


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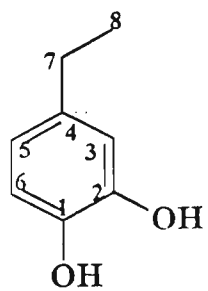


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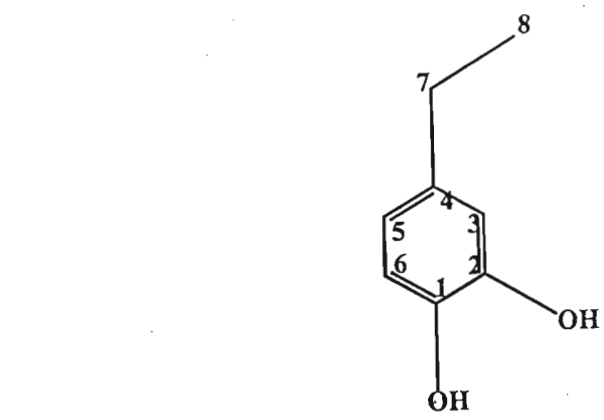


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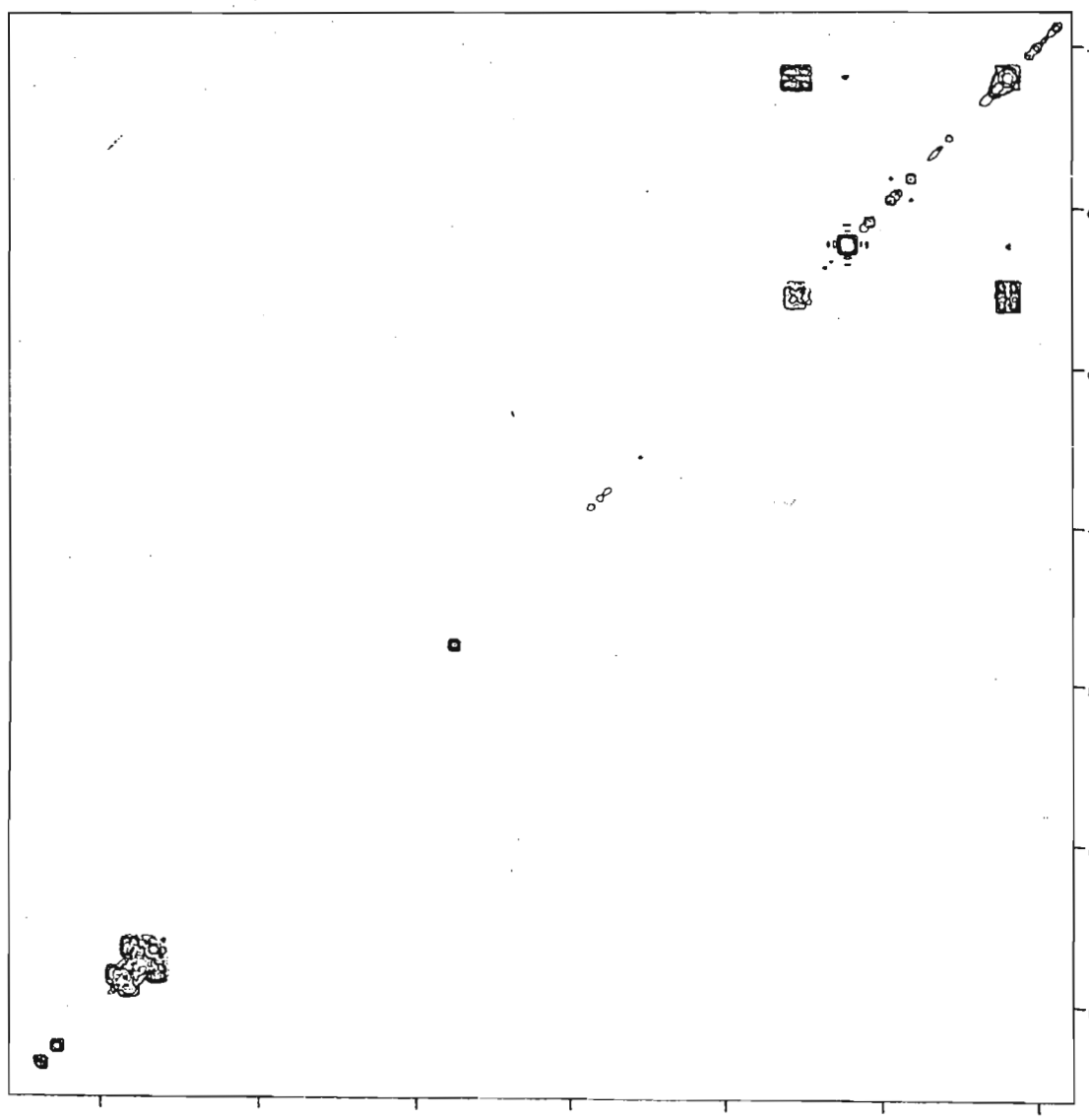

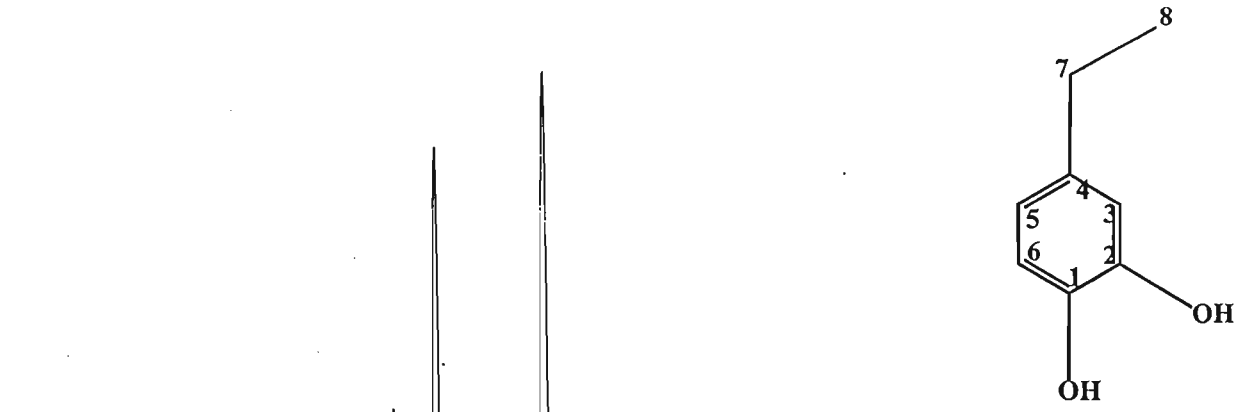


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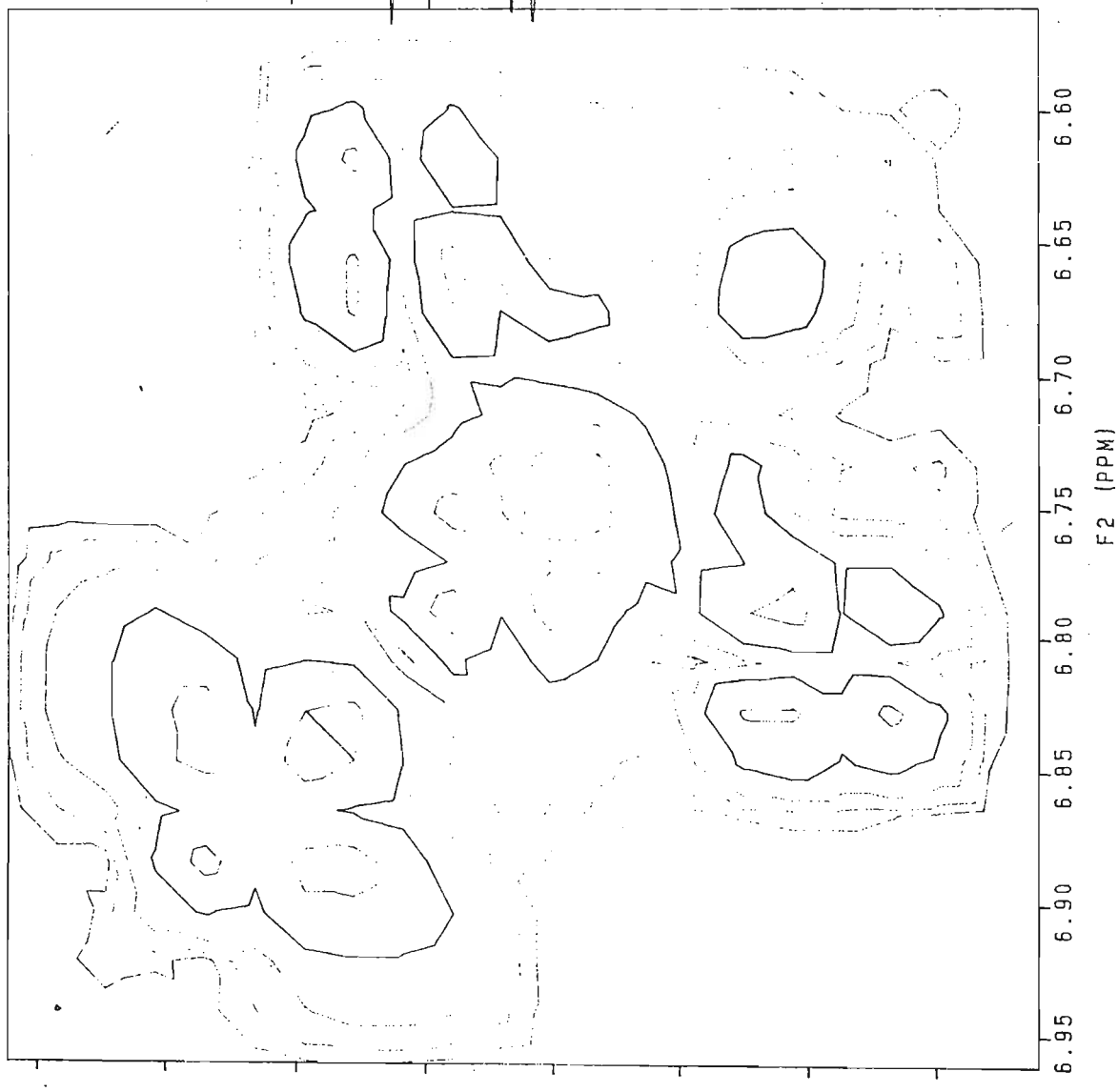

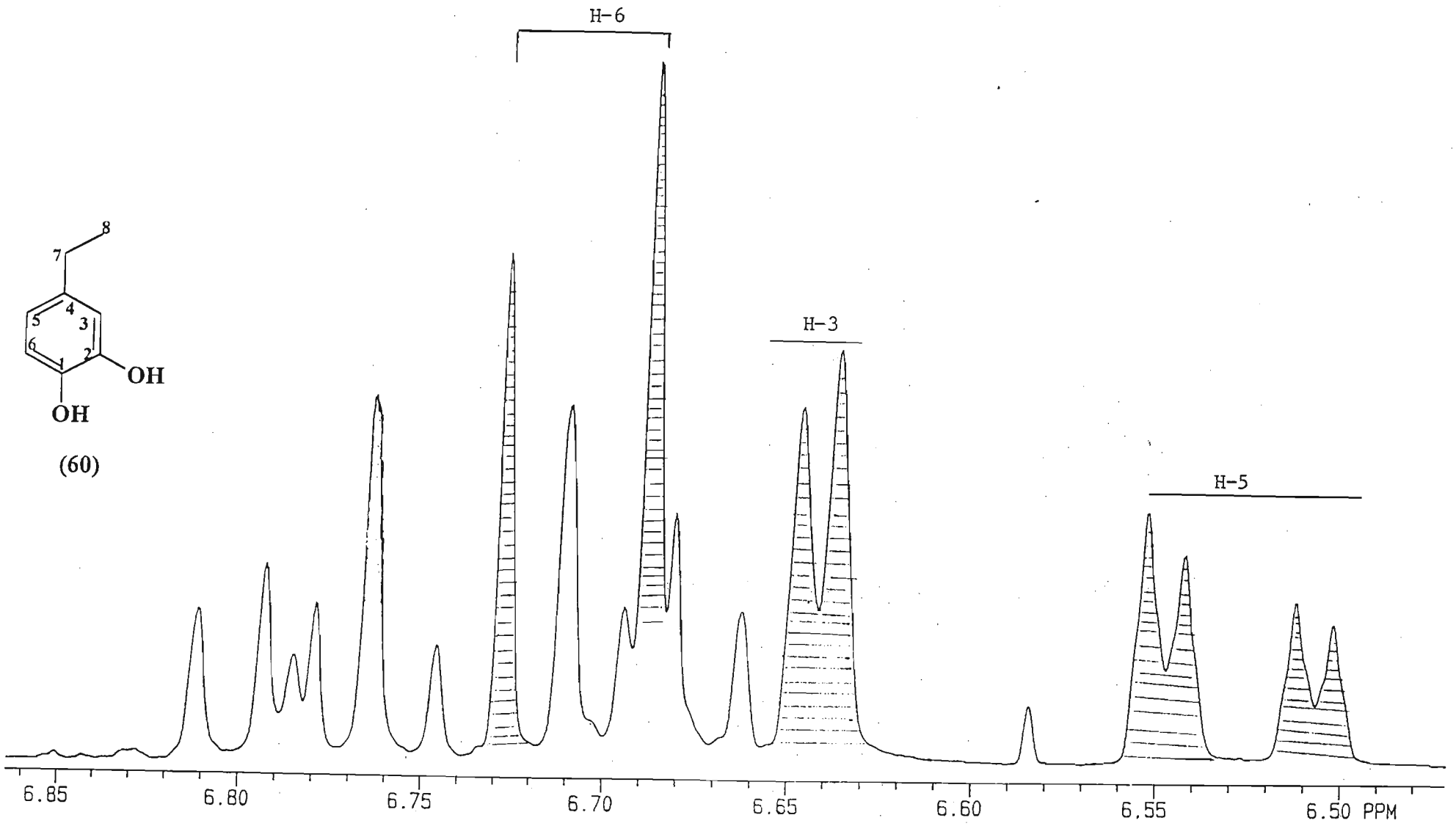


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