ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

FACULTY OF SCIENCE

DEPARTEMENT OF CHEMISTRY

Bioassay Guided Phytochemical Investigation on Roots of *Taverniera Abyssinica* (Dingetegna)

By: Mekuriaw Assefa

Advisor: Ermias Dagne (Professor)

July, 2010

Acknowledgement

I wish to express my heartfelt gratitude to my project advisor Prof. Ermias Dagne for his continuous supervision, encouragement and guidance during the course of this project.

It's also my pleasure to appreciate Prof. Yalemtsehay Mekonnen, Biology Department, Addis Ababa University, for her help and guidance during performing the biological aspect of this project and to Ato Atkilt Esayas for running bioactivity experiments.

I am also grateful to Ato Yoseph Atlaw and Ato Tesfaye Yalew for their boundless assistance. I acknowledge to Wollega University for giving me sponsorship to peruse my MSc and to all research staffs of the Chemistry Department, Addis Ababa University, and especially Prof. Wendimagegn Mammo for his support.

I would like to say big thank you to my families specially Etsubdink Wubalem and Dr. Zeleke G/egziabher. I must say thank you to my friends and my colleagues specially Yihalem Abebe.

Table of Contents

Page

List of Tables **Page**

List of Schemes

List of Figures **Page**

Abstract

The chemical constituents in EtOAc extract of the Ethiopian traditional medicinal plant 'Dingetegna", *Taverniera abyssinica,* were reinvestigated and the claimed antispasmodic activity of them were examined. The chemical constituents were isolated and purified by various chromatographic methods and their structures were elucidated mainly by NMR. The two compounds isolated were identified as 3-hydroxy-9-methoxypterocarpan, a known compound called medicarpin and 3,4-dihydroxypterocarpan (4-hydroxymedicarpin). Both compounds antagonized the histamine induced contraction of guinea pig ileum. Medicarpin was more active than its natural derivative 4-hydroxymedicarpin.

1. Background of the Study

Modern medicine has benefited a lot from traditional medicine in that the latter had provided key leads emanating from folkloric uses of medicinal plants. The recognition, promotion, and development of herbal medicines are highly encouraged by WHO both for its significance in contributing to modern medicine and its socio-economic significance (WHO, **1978**). Plants continue to play a major role in providing prototype molecules for possible development into conventional drugs by the pharmaceutical industry (Fekadu, **2007**; Bekele, **2007**).

The cultivation and use of spices, herbs, medicinal and other essential oil-bearing plants are not new to Ethiopia. It is as old as the crop themselves, and its history can be traced back to the time of Queen Sheba (ca.992 BC). The data obtained from Ethiopian Institute of Biodiversity Conservation and Research shows that more than 800 species of flowering plants in the country are used for medicinal purposes (IBCR, **2001**).

2. *Taverniera abyssinica*

This species belongs to a small genus, *Taverniera* of Leguminosae family and is found in North East Africa and South West Asia. *Taverniera abyssinica* A. Rich is not known to occur elsewhere and even in Ethiopia it is confined to the provinces of Shoa and Tigray. It is sold in many markets of central Ethiopia under the name 'Dingetegna' literally meaning "medicine for sudden illness", and used for the treatment of headache, stomachache and fever. It is commonly administered by chewing the roots and swallowing the juice (Dagne et al., **1987**, Berhanu et al., **1999,** Hedberg, **1989**)

Figure 1. Bundles of 'Dingetegna' bought from market (Photo: Prof. Ermias D., **2010**)

2.1 Previous Studies on Bioassay of *T. abyssinica*

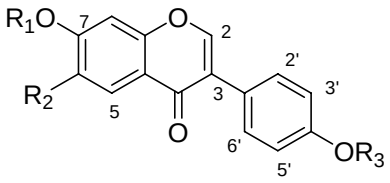
A large number of herbal drugs are reputed to be of excellent medicinal, value, and are used for the treatment of several ailments. In folk medicine, various indigenous drugs are used in single and/or in combined form as drugs with considerable success. (Paula et al., **2003**)

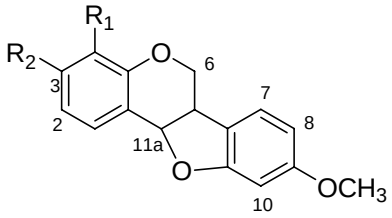
Previous pharmacological works done on *T. abyssinica* have shown that the extracts from the root have antipyretic analgesic and antispasmodic effects (Dagne et al., **1990**).

2.2 Previous Studies on Chemistry of *T. abyssinica*

Previous study on phytochemical investigation of *T. abyssinica* (Dagne et al., **1987**) entitled with "Isoflavonoids from *T. abyssinica*" has reported the isolation of four compounds which were obtained from different chromatographic separations of petroleum ether as well as chloroform extracts of the roots of the plant. These compounds **(Fig. 2)** are classified as isoflavonoids and pterocarpans.

Compounds **1** and **3** were identified as the well known isoflavonoids, formononetin and afrormosin, respectively. Compounds **4** and **6** were distinguished as the pterocarpans and they were characterized as medicarpin and 3,4–dihydroxy–9–methoxypterocarpan, respectively.





1. $R_1 = R_2 = H$, $R_3 = CH_3$

2. $R_1 = CH_3$, $R_2 = R_3 = H$

3. $R_1 = H$, $R_2 = OCH_3$, $R_3 = CH_3$

4. $R_1 = H$, $R_2 = OH$

5. $R_1 = H$, $R_2 = OAc$

6. $R_1 = R_2 = OH$

7. $R_1 = R_2 = OAc$

Figure 2. Compounds isolated from *Taverniera abyssinica.*

The two compounds isolated from *T. abyssinica,* medicarpin (**4**), 4-hydroxymedicarpin (**6**), and also their naturally occurring derivatives found in other plant species have bioactivities such as cytotoxicity (Awale et al., **2008;** Ngamrojanavanich et al., **2007**) stimulative (not general)

(Umehara et al., **2007**), leishmanicidal (Takahashi et al., **2006)**) and antimitotic (Militao et al., **2005**) activities.

In the context of this study, taking into account that the roots of the plant are traditionally used by chewing and swallowing the juice for the treatment of stomach pain acting as an antispasmodic drug the brief explanation of antispasmodic drugs is presented bellow.

3. Antispasmodic Drugs

An antispasmodic (synonym: spasmolytic) is a drug or herb that suppresses muscle spasms.[1] They have been used to treat stomach cramps. Traditionally, they were used to treat stomach ulcers. Most of the drugs used for this purpose as "anticholinergics". The anticholinergic drugs decrease both the movements of the stomach and intestine, and also the secretions of stomach acid and digestive enzymes.[2]

There are two main types of antispasmodic drugs.[3]

1. Antimuscarinics such as dicycloverine, hyoscine, atropine, propantheline.
2. Smooth muscle relaxants such as aleverine, mebeverine and peppermint oil.

Smooth muscle relaxants are used for smooth muscle contraction, especially in tubular organs of the gastrointestinal tract. The effect is to prevent spasms of the stomach, intestine or urinary bladder. Both dicyclomine and hyoscyamine are antispasmodic due to their anticholinergic action.

[1]. http://en.wikipedia.org/wiki/Antispasmodic

[2]. http://medical-dictionary/Antispasmodic+Drug

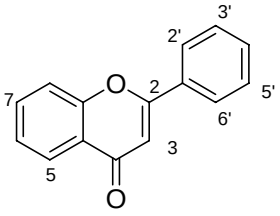
[3]. http://www.patient.co.uk/health/Antispasmodic-Drugs.htm

4. Major Groups of Compounds Isolated from *T. abyssinica*

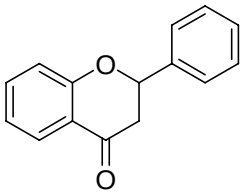
The compounds previously isolated from *T. abyssinica* are classes of natural compounds known as flavonoids. The brief review about flavonoids is presented below.

4.1 Flavonoids

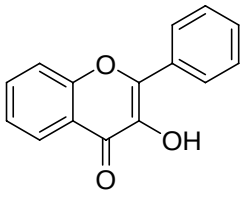
Flavonoids are one of the main groups of natural products. They are a group of naturally occurring phenolic compounds, which occur in different plant parts both in free state and glycosides. Based on the skeleton of flavonoids some subgroups are: flavones, flavonols, isoflavones, chalcones, aurones. (**Fig. 3)** (Dewick, **2001**).



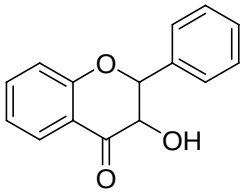
8. Flavone



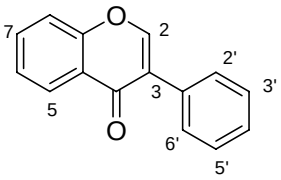
9. Flavanone



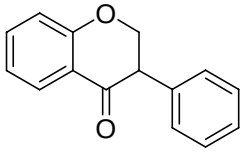
10. Flavanol



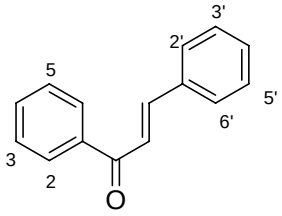
11. Dihydroflavanol



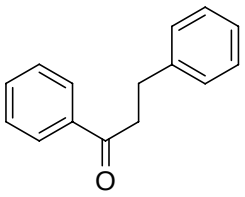
12. Isoflavone



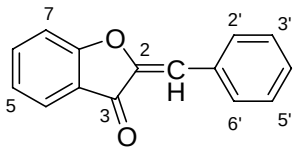
13. Isoflavanone



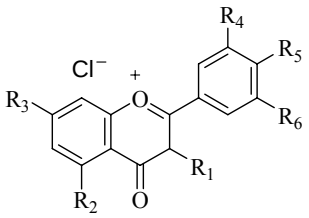
14. Chalcone



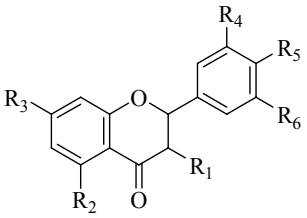
15. Dihydrochalcone



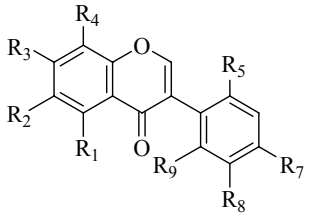
16. Aurone



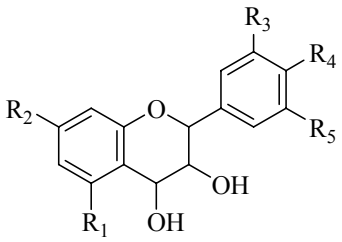
17. Anthocyanidin
$R_1 = R_2 = R_3 = R_4 = R_5 = R_6$ =H, OH or OMe or glycoside / Anthocyanin



18. Derivatives of flavones including glycosides
$R_1 = R_2 = R_3 = R_4 = R_5 = R_6$ =H, OH or OMe or Alkyl or glucoside / glycoside

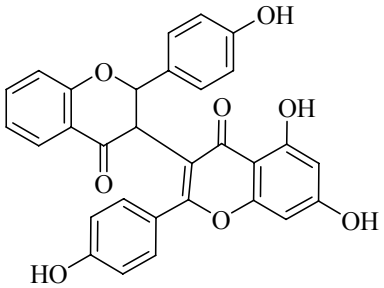


19. Derivatives of isoflavones including glycosides
$R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = R_9$ = H, OH or OMe or Alkyl or glucoside / glycoside

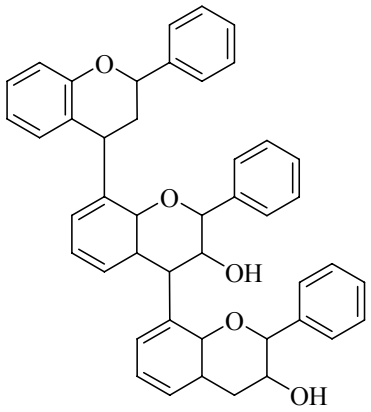


20. Leucocyanidin

$R_1 = R_2 = R_3 = R_4 = R_5$ = H, OH



21. Biflavone



22. Procyanidin

Figure 3. Flavonoids

4.2 Isoflavonoids

The isoflavonoids are structural variants of flavonoids in which the Shikimate-derived aromatic ring has migrated to the adjacent carbon of the hetrocycle. The main reason for understanding biosynthesis of isoflavonoids is the fact that some isoflavonoids are produced in plant tissues as stress metabolites or phytoalexins. The isoflavonoids share a common biosynthetic pathway with the flavonoids as far as chalcone – flavanone intermediates, but then a 1,2–aryl migration **(Scheme 2)** occurs to produce the rearranged 3-phenylchroman skeleton that differentiates isoflavonoids from other flavonoids.

Since pterocarpans are isoflavonoids they share a common biosynthesis pathway of that of flavonoids. The progress of the biosynthesis of flavonoids from primary metabolites can be divided in to a number of phases which are:-

A. Phenyl alanine to cinamic acid

B. Chalcone to flavanone.

C. Flavanone to isoflavone.

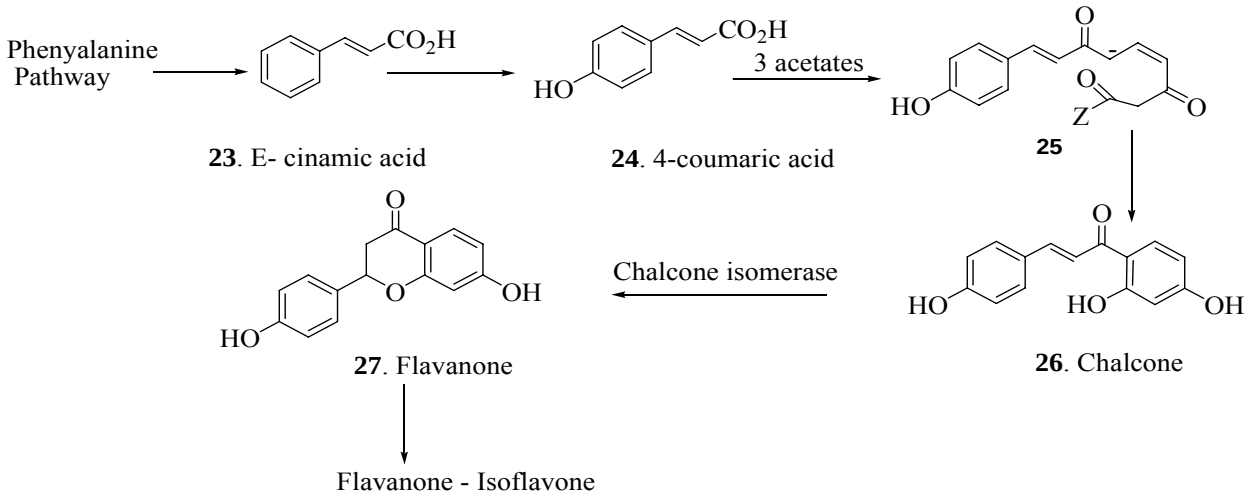
D. Hydroxylation and methoxylation

A) Phenylalanine to cinamic acid

An amino acid phenylalanine is used to produce cinamic acid which is a source of the shikimate pathway derived aromatic ring of flavonoids. The enzyme phenylalanine ammonia lyase (PAL) is used.

B) The Chalcone – Flavanone Phase

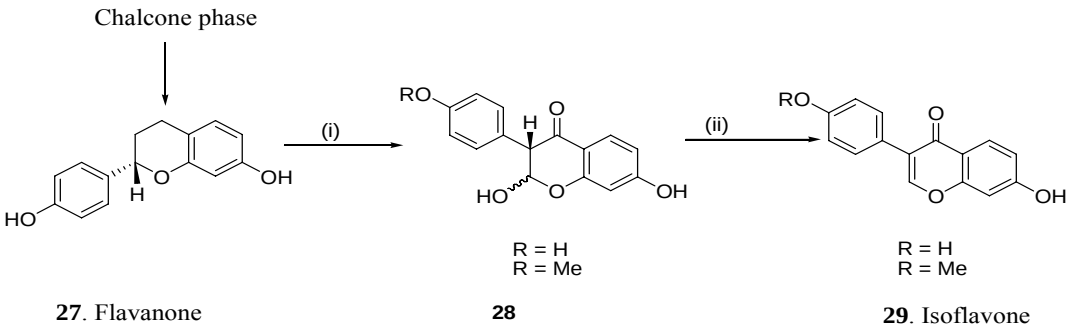
The cinamic acid produced from path A is changed to flavanone through carbon extension.



Scheme 1. The Chalcone – Flavanone Phase

C) The Flavanone - Isoflavone Phase

In this stage the Shikimate-derived aromatic ring has migrated to the adjacent carbon of the hetrocycle. This rearrangement process is brought about by a cytochrome P-450-dependent enzyme requiring NADPH and O_2 *via* intermediate hydroxyisoflavanones. A radical mechanism has been proposed as shown in **Scheme 2** below.

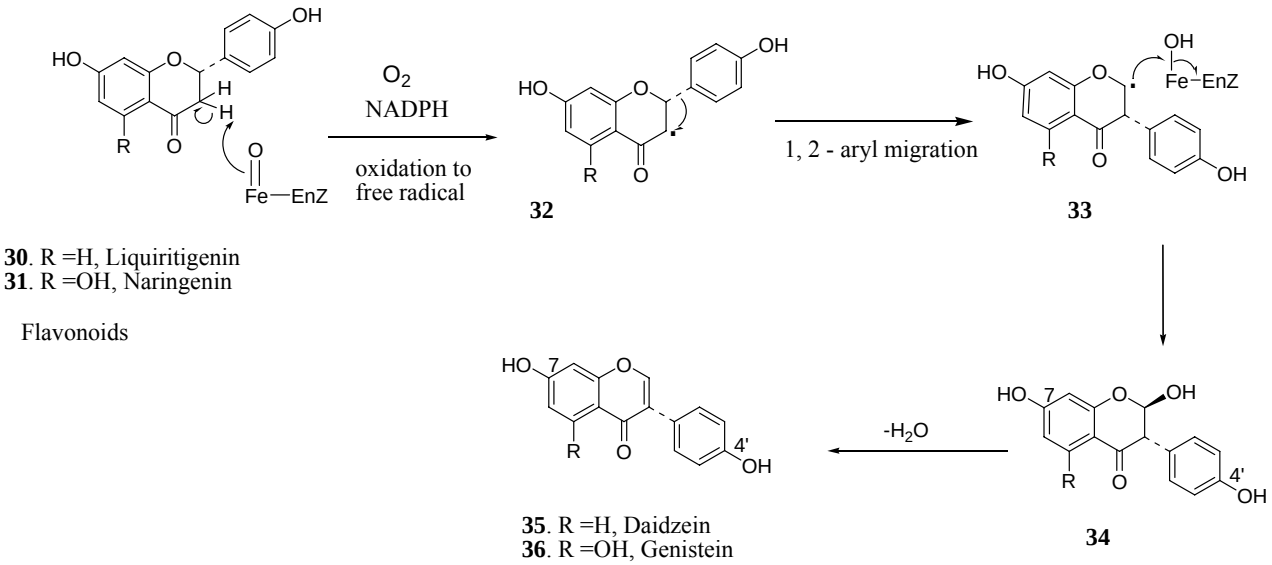


27. Flavanone **28** **29**. Isoflavone

(i) Isoflavone synthase (P-450) enzyme

(ii) Soluble dehydrase enzyme

Scheme 2. Flavanone - Isoflavone Phase



30. R =H, Liquiritigenin
31. R =OH, Naringenin

Flavonoids

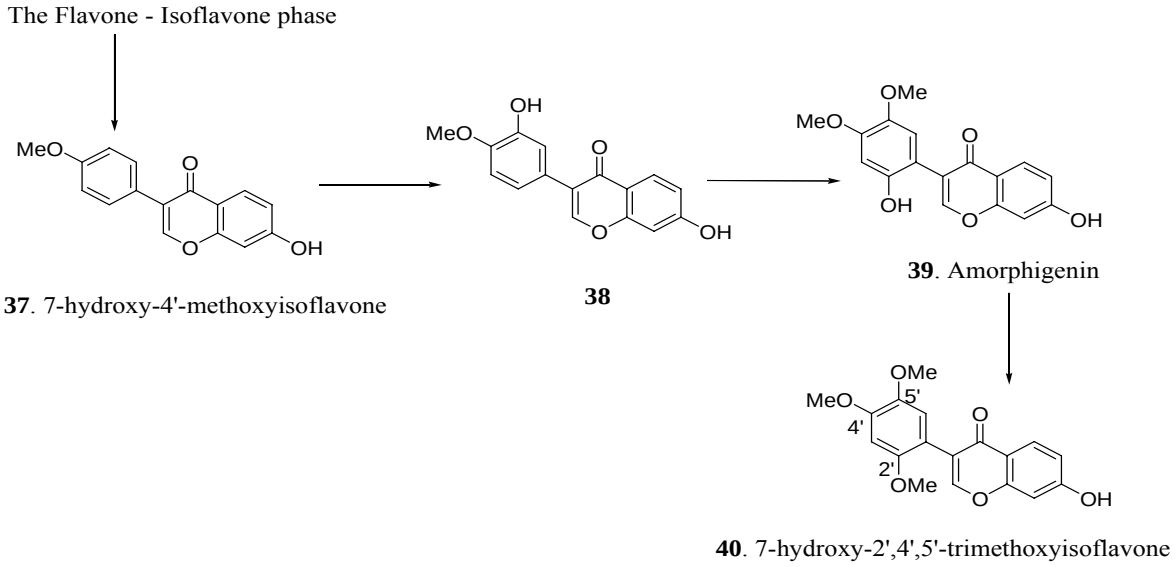
32 **33** **34**

35. R =H, Daidzein
36. R =OH, Genistein

Isoflavonoids

Scheme 3. Mechanism of Flavone - Isoflavone phase.

D) The Hydroxylation - Methoxylation Phase



Scheme 4. The Hydroxylation – Methoxylation phase

4.3 Pterocarpans

Pterocarpans are structural variants of isoflavonoids. They contain a tetracyclic ring system derived from the basic isoflavoinoid skeleton by an ether linkage between the 4 and 2' positions of **12**. The systematic numbering shown in structure **41** rather than that for simple isoflavonoids is used. They are the second largest group of isoflavonoids after the isoflavones. Pterocarpans are subdivided in to pterocarpans (**41),** 6a–hydroxypterocarpans (**42)** and pterocarpenes **(43)**.

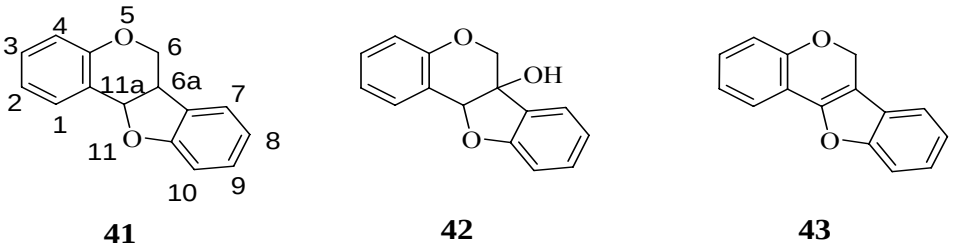
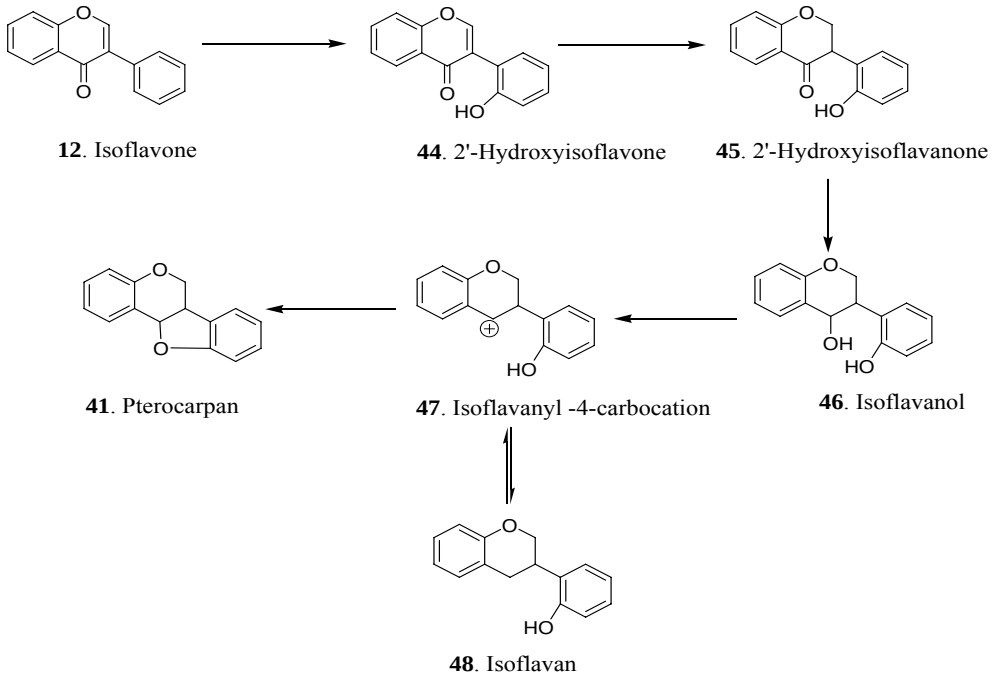


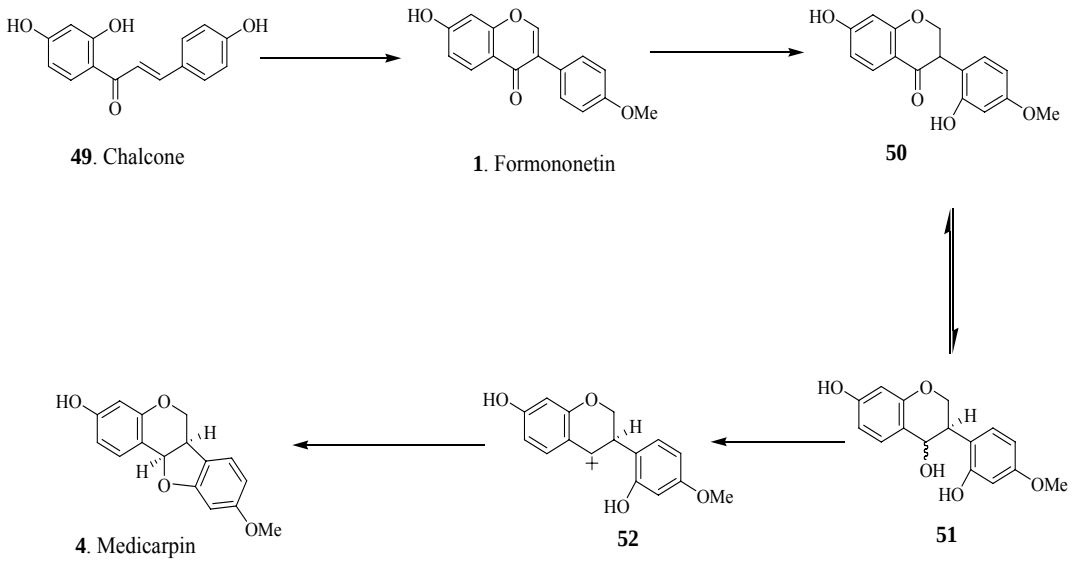
Figure 4. Types of pterocarpans

The majority of natural pterocarpans isolated have arisen from phytoalexin studies, using fungal or abiotically stressed plant tissues. Enzymes catalyzing parts of the pathway can be found at much higher levels of activity during the stress period. **Scheme 5** shows general pathway of

pterocarpan biosynthesis while **Scheme 6** presents biosynthesis of a pterocarpan medicarpin from isoflavone formononetin. (Harborne, **1988**)



Scheme 5. General scheme of pterocarpan biosynthesis



Scheme 6. Biosynthesis of medicarpin

5. Objective of the Study

Previous phytochemical investigations of ‘Dingetegna’ showed that the plant contains natural products which exhibit strong antispasmodic activities. Although these chemical studies conducted on the extracts of the plant led to the isolation and structure elucidation of several natural compounds, the identity of the compounds most responsible for the bioactivities was not known. Therefore, the main objective of this project is to conduct bioassay-guided isolation work on ‘Dingetegna’ roots in order to find out which compounds present in it are responsible for the observed biological activities of the plant.

6. Results and Discussion

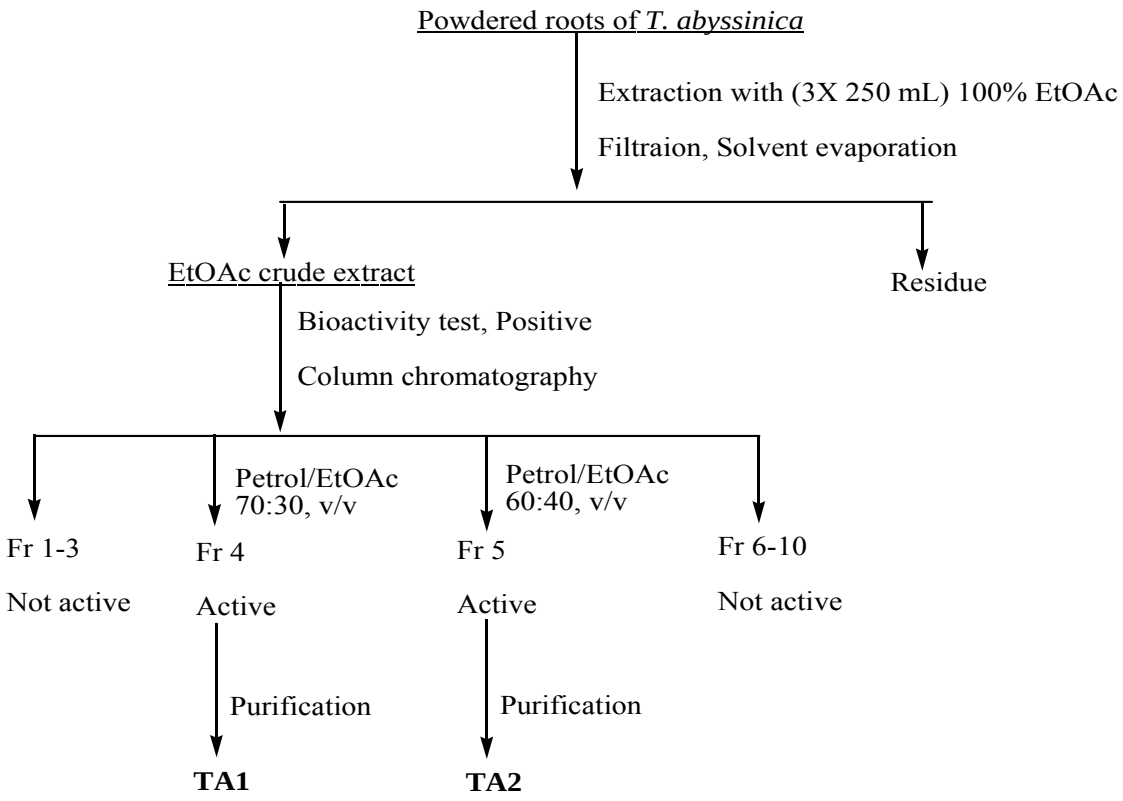
6.1 Bioassay Guided Fractionation

Bioassay guided phytochemical study of medicinal plants is a systematic process which is mainly based on bioactivity of aqueous or organic solvent extracts of the plant material. It is used for investigating principal constituents that are responsible for claimed healing effect of the plants and it also gives pharmacological basis for the ethnomedical uses of the plant.

Crude plant extracts are submitted to different bioassays for rapid estimation of their bioactivity. These bioassays should be specific, sensitive, and simple to perform, robust, economical and preferably suitable for automation because they serve as a guide during the isolation process. The extracts of interest are then fractionated with the help of various chromatographic methods and all the fractions continuing to exhibit activity are carried through further isolation and purification until pure active principles are obtained. (Satyajit., **2006**).

Studies in isolated organ are a useful tool to evaluate the pharmacological activity of a drug in the receptors, channels and enzymes of a tissue. Recently, the use of *in vitro* techniques have increased in the area of ethnopharmacology to evaluate the effects of plant derived extracts and molecules. The growing use of this technique by the international scientific community is due to the fact that it is cheap, requires fewer animals in comparison with *in vivo* models, and permits the evaluation of the pharmacological activity of a great variety of extracts and molecules of vegetal origin in a short period of time. (Jorge et al., **2007**)

The powdered roots of *T. abyssinica* (190 g) were extracted with 100% EtOAc. After filtration, solvents were evaporated under vacuum (40ºC). Column chromatography of the crude extract was done by using Petrol/EtOAc as a solvent and ten fractions were collected (**Table 1**). Antispasmodic test was done for the fractions and two fractions obtained by Petrol/EtOAc (70:30, v/v) and (60:40, v/v) gave positive result for the test. Evaporation of the solvent of the two active fractions *in vacuo* at 40ºC and further purification were used to obtain **TA1** and **TA2** for characterization.



The flowchart above shows the bioassay directed fractionation of 'Dingetegna'. It was done by examining the antispasmodic activity of the crude EtOAc extract from the roots of the plant and the fractions which were obtained from the column chromatography of the crude extract. Different spectroscopic data were used to identify and characterize the two active components **TA1** and **TA2.** As discussed below the characterization of two compounds ensures that **TA1** and **TA2** were medicarpin (**4**) and 4-hydroxymedicarpin (**6**) and they are among the compounds which were previously isolated from this plant under phytochemical investigation (**Fig. 2**)

Table 1. Column chromatography of crude extract

Petrol: EtOAc % (v/v)	100:0	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	100:0
Fractions	1	2	3	4	5	6	7	8	9	10

6.2 Characterization of TA1 and TA2

Two compounds **TA1** and **TA2** were isolated from the EtOAc extract of *T. abyssinica* roots. Different types of instrumental analysis such as 1D and 2D NMR, MS and UV-VIS were carried out in order to identify the isolated compounds. The characterization is presented independently for the two compounds as follows.

6.2.1 Characterization of TA1 as Medicarpin (4)

TA1 was obtained from column chromatography of crude EtOAc extract which was eluted by the solvent system Petrol/EtOAc (70:30, v/v). It is green and gummy which is soluble in MeOH and $CHCl_3$. It gave red color when sprayed with 1% vanillin – H_2SO_4 reagent in TLC analysis. Its maximum UV absorption was (λ_{MeOH} = 286 nm) and optical activity, $[\alpha]_{589}^{21}$ = -90 (in $CHCl_3$).

The high resolution mass spectrometer (HRMS) data (Appendix 12) shows **TA1** has a molecular formula $C_{16}H_{14}O_4$ which was consistent with that of medicarpin and the parent ion peak [M^+ - 1] was m/z 269. The peak m/z 254 [M^+ - 15] was due to loss of -CH_3 radical.

The ^{1}H NMR (400 MHz) spectrum (Appendix 1) of **TA1** in $CDCl_3$ suggested the presence of six aromatic signals at δ 7.40 (1H, d, J = 8.4 Hz), δ 7.15 (1H, d, J = 8.6 Hz), δ 6.58 (1H, dd, J = 8.4, 2.3 Hz), δ 6.48 (1H, d, J = 8.6, 2.4 Hz), δ 6.46 (1H, d, J = 2.4 Hz) and δ 6.44 (1H, d, J = 2.3 Hz). The spectrum also showed one doublet at δ 5.52 integrating for one proton and three resonances at δ 4.26 (1H, dd, J = 10.7, 4.9 Hz), δ 3.79 (3H, s) and δ 3.64 (1H, t, J = 10.7 Hz) corresponding to protons attached to a carbon bearing an electronegative atom oxygen the multi plate at δ 3.55 which was integrated for one hydrogen atom is because of a benzylic proton.

The ^{13}C NMR (Appendix 2) and DEPT–135 (Appendix 3) spectra of **TA1** in $CDCl_3$ indicate it has 16 carbon atoms. There are six quaternary and six CH aromatic carbon atoms. Three resonances are due to oxygenated methine, methlene and methyl carbons, respectively. In addition the peak at δ 39.5 ppm is because of methine carbon atom.

The ^{1}H and ^{13}C NMR data of **TA1** are quite similar to the previously reported data of medicarpin (**4**) which was isolated and identified from the same plant material (Dagne et al., **1987**). The chemical shifts of the carbon and proton atoms of **TA1** are compared with literature data of medicarpin in Table 2.

Table 2: ^{1}H and ^{13}C NMR spectral data of compound **TA1** compared with literature data of medicarpin (Dagne et al., **1987**).

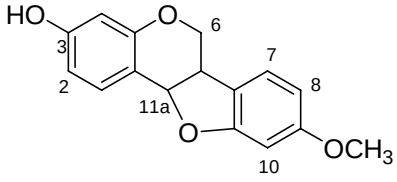
No	Observed NMR data for **TA1**		Previously reported data on medicarpin	
	$\delta\ ^{13}C$	$\delta\ ^{1}H$	$\delta\ ^{13}C$	$\delta\ ^{1}H$
1	132.2	7.40 (1H, d, J = 8.4 Hz)	132.2	7.37 (d, J = 7.8 Hz)
2	109.8	6.58 (1H, dd, J = 8.4, 2.3 Hz)	109.7	6.53 (dd, J = 7.8, 2.3 Hz)
3	157.2	---	157.0	---
4	103.7	6.44 (1H, d, J = 2.3 Hz)	103.7	6.39 (d, J = 2.3 Hz)
4a	156.7	---	156.7	---
6	66.5	H_a: 4.26 (1H, dd, J = 10.7, 4.9 Hz) H_b: 3.64 (1H, t, J = 10.7 Hz)	66.5	H_a: 4.21 (dd, J = 10, 4.5 Hz) H_b: 3.6 (t, J = 10 Hz)
6a	39.5	3.55 (1H, m),	39.5	3.55 (ddd)
6b	119.2	---	119.1	---
7	124.8	7.15 (1H, d, J = 8.6 Hz)	124.7	7.11 (d, J = 8.4 Hz)
8	106.4	6.48 (1H, dd, J = 8.6, 2.4 Hz)	106.4	6.43 (dd, J = 8.4, 2.3 Hz)
9	161.2	---	161.1	---
10	96.9	6.46 (1H, d, J = 2.4)	96.9	6.42 brs
10a	160.7	---	160.7	---
11a	78.6	5.52 (1H, d, J = 6.7 Hz)	78.5	5.48 (d, J = 6.7 Hz)
11b	112.4	---	112.7	---
OCH_3	55.5	3.79 (3H, s)	55.5	3.75

The corresponding correlations between proton and carbon atoms in the proposed structure are observed from COSY and HMBC NMR (Appendix 4 and 5, respectively) data in Table 5.

Table 3: COSY ($^{1}H \rightarrow {}^{1}H$) and HMBC ($^{1}H \rightarrow {}^{13}C$) NMR correlation data for **TA1**

C. No	^{13}C δ (ppm)	^{1}H δ (ppm)	COSY ($^{1}H \rightarrow {}^{1}H$)	HMBC ($^{1}H \rightarrow {}^{13}C$)
1	132.2	7.40	$H^{1} \rightarrow {}^{2}H$	$H^{1} \rightarrow$ 3, 11a, 4a
2	109.8	6.58	$H^{2} \rightarrow {}^{1}H$	$H^{2} \rightarrow$ 4, 11b
4	103.7	6.44	---	$H^{4} \rightarrow$ 2, 11b, 4a
6	66.5	H_a: 4.26	$H_a \rightarrow H^{6a}$, $H_a \rightarrow H_b$	$H_{eq} \rightarrow$ 4a, 6a, 6b, 11a
		H_b: 3.64	$H_b \rightarrow H^{6a}$, $H_a \rightarrow H_a$	$H_{ax} \rightarrow$ 4a, 6a, 6b, 11a
6a	39.5	3.55	$H^{6a} \rightarrow H^{11a}$, $H^{6a} \rightarrow H_a$ $H^{6a} \rightarrow H_b$	$H^{6a} \rightarrow$ 10a, 6, 6b
7	124.8	7.15	$H^{7} \rightarrow {}^{8}H$	$H^{7} \rightarrow$ 6a, 9, 10a
8	96.9	6.48	$H^{8} \rightarrow {}^{7}H$	$H^{8} \rightarrow$ 6b, 10
10	106.4	6.46	---	$H^{10} \rightarrow$ 8, 11a
11a	78.6	5.52	$H^{11a} \rightarrow {}^{6a}H$	$H^{11a} \rightarrow$ 1,4a, 6, 11b
OMe	55.5	3.79	---	$H_{OMe} \rightarrow$ 9

The 1D, COSY and Hetronuclear Multiple Bond Correlation (HMBC) NMR data of **TA1** are in good agreement with the proposed structure. Hence, **TA1** is medicarpin (**4)**.



4. Medicarpin

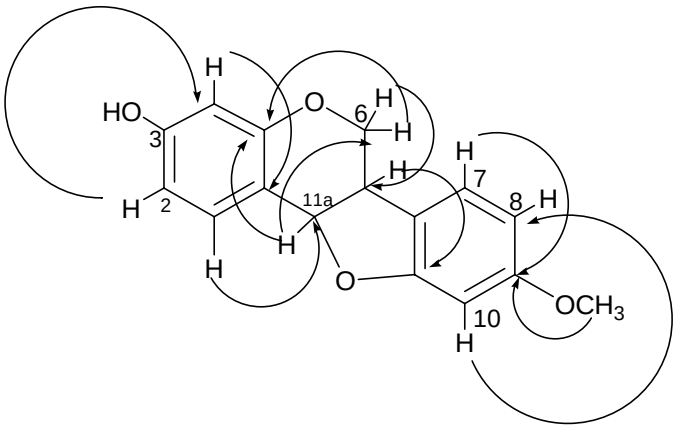


Figure 5. Medicarpin and selected HMBC correlations.

6.2.2 Characterization of TA2 as 4-hydroxymedicarpin (6)

TA2 was obtained from column chromatography of crude EtOAc extract which was eluted by the solvent system Petrol/EtOAc (60:40). Further purification of the compound was done by subsequent recrystallization and passing it through Sephadex LH-20.

This compound is brown and gummy which is soluble in MeOH and $CHCl_3$. It gave red color when derivetized with 1% vanillin – H_2SO_4 reagent in TLC analysis. Its maximum UV absorption was (λ_{MeOH} = 285 nm) and optical activity, $[\alpha]_{589}^{21}$ = -85 (in $CHCl_3$).

The high resolution mass spectrometer (HRMS) data (Appendix 13) shows **TA2** has a molecular formula $C_{16}H_{14}O_5$ which was consistent with that of 4-hydroxymedicarpin and the parent ion peak [M^+ - 1] was m/z 285. The peak m/z 255 [M^+ - 15] was due to loss of -OCH_3 radical.

The similarity between few ^1H and ^{13}C chemical shifts of **TA1** and **TA2** (**Table 2** and **3**) indicates that the two compounds have similar structures. Thus the spectral data of **TA2** was also compared with the data from the literature and it is quite similar with the previously reported data of 4-Hydroxymedicarpin or 3,4-Dihydroxy-9-methoxypterocarpan (**6**). The comparison is presented in Table 4.

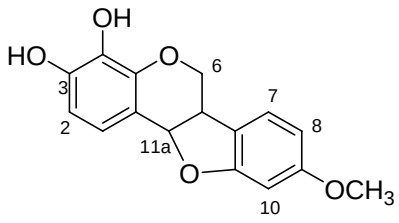
The corresponding correlations between protons and carbon atoms of **TA2** obtained from COSY HSQC and HMBC NMR experiment (Appendix 9, 10 and 11 respectively) are presented in Table 5 and the analysis confirms that **TA2** is 4-Hydroxymedicarpin (**6**).

Table 4. ^{1}H and ^{13}C NMR spectral data of compound **TA2** compared with literature data of 4-hydroxymedicarpin.

C. No	Observed NMR data of **TA2**		Previously reported data of 4-hydroxymedicarpin	
	^{13}C δ	^{1}H δ (ppm)	^{13}C δ	^{1}H δ (ppm)
1	121.4	6.97 (1H, d, J = 8.6 Hz)	121.8	6.98 (1H, d, J = 8.3 Hz)
2	109.6	6.63 (1H, dd, J = 8.6 Hz)	109.5	6.67 (dd, J = 8.3 Hz)
3	145.0	---	144.4	---
4	132.3	---	131.5	---
4a	143.7	---	143.1	---
6	66.9	H_a: 4.30 (1H, dd, J = 10.7, 4.9 Hz) H_b: 3.65 (1H, t, J = 10.7 Hz)	67.0	H_a: 4.31 (dd, J = 10, 4.8 Hz) H_b: 3.66 (t, J = 10 Hz)
6a	39.5	3.55 (1H, m),	39.7	3.56 (ddd)
6b	118.9	---	118.6	---
7	124.8	7.14 (1H, d, J = 8.6 Hz)	124.8	7.11 (d, J = 8.3 Hz)
8	106.4	6.46 (1H, dd, J = 8.6, 2.3 Hz)	106.7	6.43 (dd, J = 8.3, 2.3 Hz)
9	161.1	---	161.2	---
10	96.9	6.45 (1H, brs)	97.0	6.42 brs
10a	160.7	---	160.5	---
11a	78.7	5.52 (1H, d, J = 6.7 Hz)	78.4	5.51 (d, J = 6.9 Hz)
11b	112.4	---	Not observed	---
OCH_3	55.5	3.75 (3H, s)	55.5	3.75

Table 5: COSY ($^{1}H \rightarrow {}^{1}H$) and HMBC ($^{1}H \rightarrow {}^{13}C$) NMR correlation data for **TA2**, 4-hydroxymedicarpin (**6**)

C. No	^{13}C δ (ppm)	δ ^{1}H (ppm)	COSY ($^{1}H \rightarrow {}^{1}H$)	HMBC ($^{1}H \rightarrow {}^{13}C$)
1	121.4	6.95	$H^{1} \rightarrow H^{2}$	$H^{1} \rightarrow$ 3, 11a, 4a
2	109.6	6.63	$H^{2} \rightarrow H^{1}$	$H^{2} \rightarrow$ 4, 11b
6	66.9	H_a: 4.30	$H_a \rightarrow H_b$, $H_a \rightarrow H^{6a}$	$H_a \rightarrow$ 4a, 6a, 6b, 11a
		H_b: 3.65	$H_a \rightarrow H_a$, $H_b \rightarrow H^{6a}$	$H_b \rightarrow$ 4a, 6a, 6b, 11a
6a	39.5	3.55	$H^{6a} \rightarrow H^{11a}$, $H^{6a} \rightarrow H_a$, $H^{6a} \rightarrow H_b$	$H^{6a} \rightarrow$ 10a, 6, 6b
7	124.8	7.12	$H^{7} \rightarrow H^{8}$	$H^{7} \rightarrow$ 6a, 9, 10a
8	106.4	6.46	$H^{8} \rightarrow H^{7}$	$H^{8} \rightarrow$ 6b, 10
10	96.9	6.45	---	$H^{10} \rightarrow$ 6b, 8, 10a
11a	78.7	5.52	$H^{11a} \rightarrow H^{6a}$	$H^{11a} \rightarrow$ 1,4a, 6, 11b
OMe	55.5	3.79	---	$H_{OMe} \rightarrow$ 9



6. 4-Hydroxymedicarpin

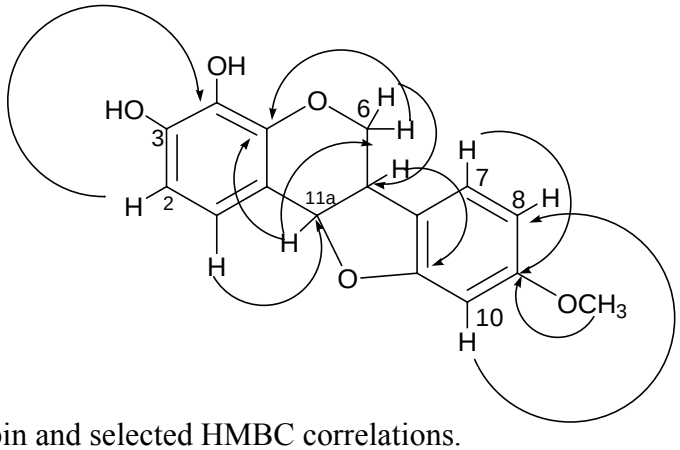


Figure 6. 4-Hydroxymedicarpin and selected HMBC correlations.

6.3 Interpretation of the Bioactivity Test Results

'Dingetegna' is traditionally used to heal stomach pain. The reason behind the claimed healing effect of this plant may be is due to its antispasmodic bioactivity. Those extracts which are active towards the antispasmodic bioactivity test will show the following properties:-

1. Decrease the natural rhythmic contractions of the smooth muscles.
2. Decrease the histamine induced contraction of smooth muscles.

These properties indicate that the extracts that antagonize the contractility of the smooth muscles result in relaxation which may be the reason behind for the antispasmodic effect of the extracts.

Tissue Organ Bath System, the vertical organ bath with its double-walled glass chamber, is the traditional experimental set-up that has been used extensively to investigate the physiology and pharmacology of smooth muscle and other tissue preparations, through measurement of contractile force while bathed in an appropriate physiological buffer at 37°C. The advantages of these studies are that the preparations can be subjected to pharmacological agents while excluding the influence of systemic processes that occur in intact animals. The results of these organ bath systems are generally more consistent and repeatable.

(www.dmt.dk/files/prospects/750tobs_data_sheet_us.pdf)

Experimentally, the responses were recorded isometrically using a Grass FT. 03 strain gauge transducer connected to a Grass Model 7 polygraph. Relaxation can be shown from the reading of the instrument as the peaks which are formed due to natural rhythmic contraction of the tissue and the peak due to the control histamine induced contraction of the tissue are getting decreased after the addition of extracts in to the organ bath in increasing concentration.

Two points worth mentioning here are: the use of control histamine and the stabilization period for the tissue.

- After the tissue is properly mounted in the organ bath it remained there uninterrupted for a period of 30 - 45 min. During this time the tissue will equilibrate and be ready for responding properly for the extract tests.

- Histamine is a biological compound found in the body and is known to induce contractility. This chemical was used as a control to examine the effect of the extract on contractility. But before applying the extract dose response for histamine was established and the standard sub-maximal dose (8 ng/mL organ bath conc.) is taken as control **(Fig. 7 and 9)**. This control was applied at the beginning, in between and at the end so that one can be sure the decline in contractility as time elapses is the effect of the extract only and not the deterioration in the tissue.

Therefore, the antispasmodic activity of extracts and pure isolated compounds from *T. abyssinica* were examined based on the above facts. Figure **8** and **10** show the reading of the instrument for the response of the tissue towards medicarpin (**4**) and 4-hydroxymedicarpin (**6**), respectively, with and without control histamine.

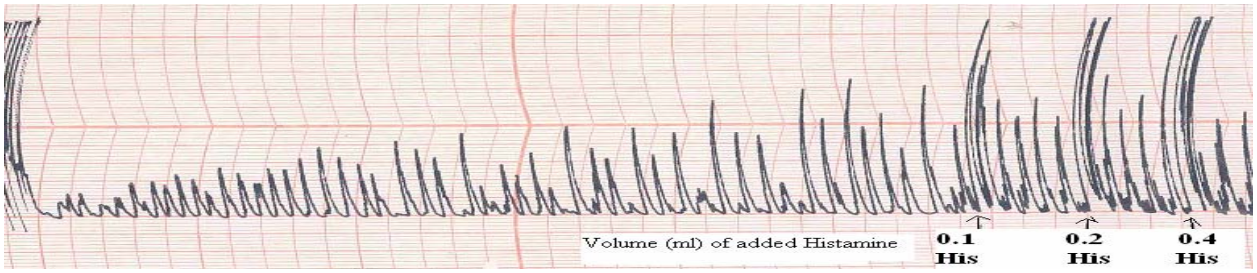


Figure 7. Dose dependent response of guinea pig ileum to histamine. The curves appearing before adding 0.1 mL (4 ng/mL) show the natural rhythmic contraction of the tissue.

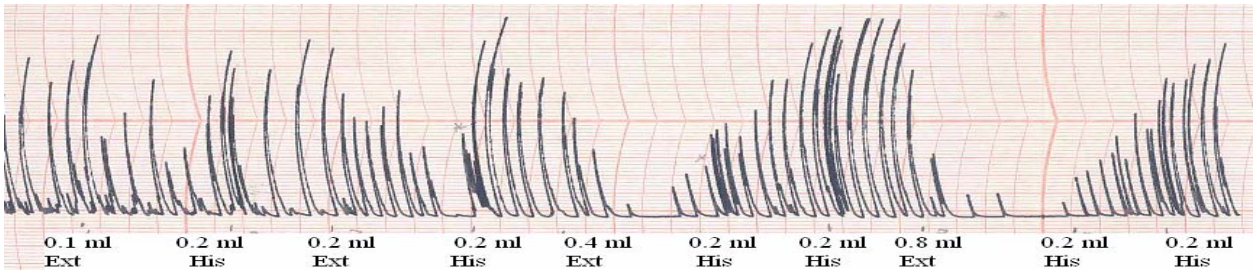


Figure 8. These traces show the response of the guinea pig ileum after adding the extract in increasing concentration. (0.1, 0.2, 0.4 … mL)

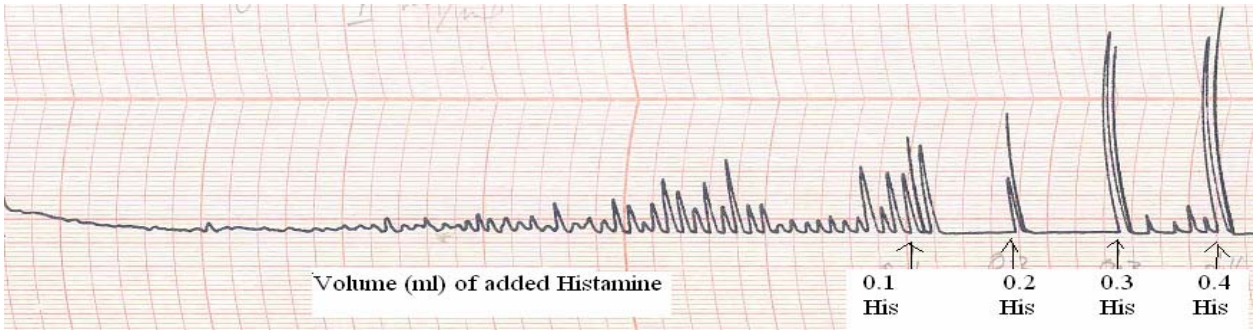


Figure 9. Dose dependent responses of guinea pig ileum to histamine. The curves appearing before adding 0.1 mL (4 ng/mL) show the natural rhythmic contraction of the tissue.

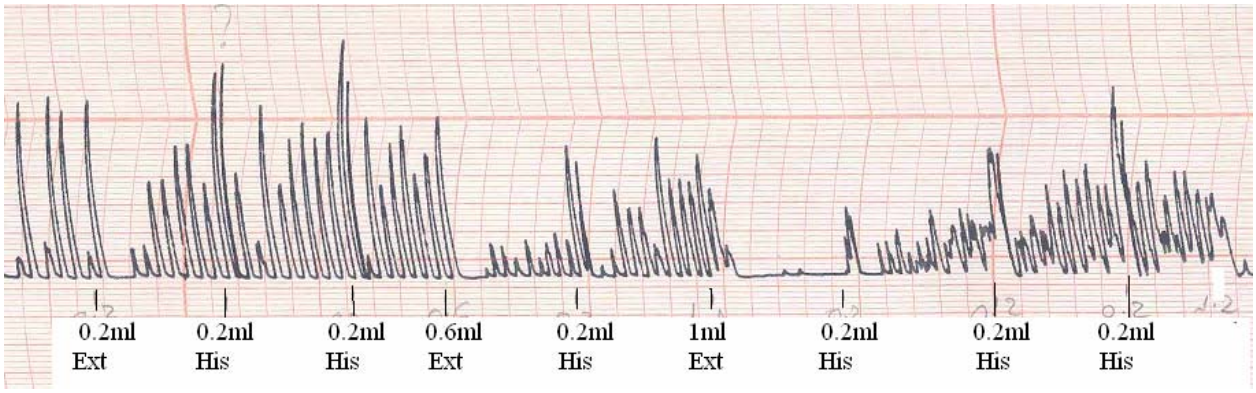


Figure 10. These traces show the response of the guinea pig ileum after adding the extract in increasing concentration. (0.1, 0.2, 0.4 …mL)

As it can be viewed on the figures (**Fig. 8 and 10**) the two active components of *T. abyssinica* extract medicarpin and 4-hydroxymedicarpin show their effects in both ways that is they antagonize the natural rhythmic contraction of the guinea pig ileum as well as the contraction of ileum due to the control histamine.

Table 6. Results of bioactivity test of medicarpin and 4-hydroxymedicarpin.

Compound	Exp't	Volume of extract (mL)	Concentration of extract (µg/mL)	Peak length by histamine, control (cm)	Peak length by control plus extract (cm)	% Control
Medicarpin	1	0.1	12	5.6	4.6	82.14
	2	0.2	24	5.6	2.4	42.85

	3	0.4	48	5.6	1.4	25.00
	4	0.8	96	5.6	0.4	7.14
4-Hydroxymedicarpin	1	0.2	8	4.2	3.8	90.47
	2	0.6	24	4.2	2.5	59.52
	3	1	40	4.2	2.2	52.38
	4	1.2	48	3.4	0.7	20.58

Table 7. Average measurement values.

Sample	Histamine, control	Extract plus control	% Control
Medicarpin	5.6 cm	2.2 cm	39.28
4-Hydroxymedicarpin	4 cm	2.3 cm	55.73

Table 6 shows that, in presence of compounds medicarpin (**4**) and 4-hydroxymedicarpin (**6**) in increasing concentration the rhythmic contractility of the tissue falls as well as the response to histamine decreases in a dose dependent manner. According to Table 7 which shows the average values of the heights which are formed because of the control histamine with and without the compound medicarpin is more active than 4-hydroxymedicarpin. This is because it decreases the control histamine induced contraction to 39.28% while the latter decreases it to 55.73%.

7. Conclusions and Recommendation

Two compounds, namely, 3-hydroxy-9-methoxypterocarpan (medicarpin (**4**)), and its naturally occurring derivative 3,4-dihydroxy-9-methoxypterocarpan (4-hydroxymedicarpin (**6**)), have been re-isolated from the plant. The analyses of the results from bioactivity tests confirm both compounds antagonized the histamine-induced contraction of guinea pig ileum. It also shows that medicarpin is the more active compound. These shows that these compounds are probably the ones most responsible for the well known effect of the extract of the plant in combating stomach pain.

Further works such as structure activity relationships and isolation and testing of other minor constituents is recommended.

8. Experimental

8.1 Materials

Mortar and pestle, shaker, sonic bath, rotary evaporator, TLC plate (pre-coated aluminum sheet silica gel 60 F219), UV lamp, column, silica gel, silica gel (70 – 230 mesh), UV-VIS spectrometer, Polarimeter, Grass model 7 polygraph, NMR machine (Bruker Avance 400 MHz NMR spectrometer) were used.

8.2 Plant Material

Bundles of dried roots of *T. abyssinica* ("Dingetegna") were purchased from market in Addis Ababa. This is a well known root and proper authentication was done in previous study (Dagne et al., **1987)**.

8.3 Coding System

TA stands for the genus *Taverniera abyssinica* and the numbers following the letters indicate the position of compounds starting from the highest R_f value in the TLC analysis of the EtOAc crude extract from the roots of the plant material.

8.4 Extraction

The plant material was grounded using mortar and pestle. 135 g of powdered roots of *T. abyssinica* was taken and divided in to two and soaked in 100% EtOAc (2X 250 mL). The suspension was shaken for ½ a day and filtered using suction filtration. After evaporation of the filtrate (45^{o}C) under reduced pressure 4 g (3%) of brown crude EtOAc extract was obtained.

8.5 Isolation of TA1 or medicarpin (4) from crude EtOAc Extract

The crude EtOAc extract (2 g) was adsorbed on 5 g of silica gel and applied on the column which was packed with 60 g of silica. Flash column chromatography was done using the mixture of Petrol and EtOAc as solvent system.

Table 8. Solvent system for CC of crude EtOAc extract

Petrol: EtOAc (%)	100:0	90:10	80:20	80:20	80:20	70:30	70:30	70:30	70:30
Fractions	1	2	3	4	5	6	7	8	9

TLC analysis of fractions from the above column chromatography was done by using solvent Petrol: EtOAc (7:3 and 1:1) for **fr 4 – 9** and fr **6 – 8** respectively **(Fig. 11).**

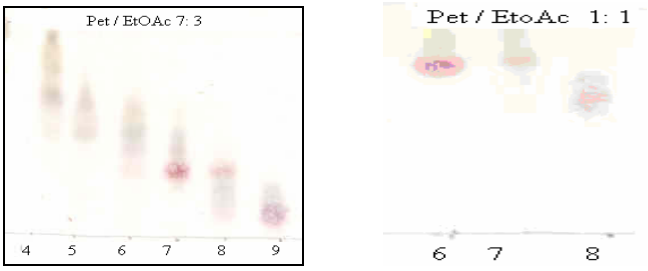


Figure 11. TLC of fractions crude EtOAc extract of 'Dingetegna" (Table 8)

In both TLCs vanillin was used as derivatizing agent. The solvent of the fraction **fr – 6,** which was eluted with Petrol:EtOAc (70:30), was completely evaporated under reduced pressure at 45^{o}C to obtain 70 mg (0.05%) of solid product (**TA1**) which was purified with Sephadex LH-20. The purification was done after washing the Sephadex with MeOH: $CHCl_3$ (1:1) repeatedly in order to have clean and good packing. The same solvent system was used to elute and four fractions (each 50 mL) were collected.

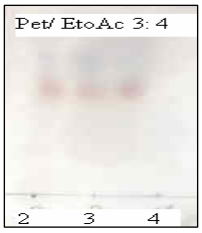


Figure 12. TLC of fractions from Sephadex LH–20.

Fraction **4** in Figure **12** had a better clean spot and its solvent was dried up to dryness (45^{o}C) to obtain pure 14 mg (0.01%) of **TA1.** Instrumental analyses such as NMR, MS and UV were done in order to identify the pure compound. The observed data obtained were compared with the corresponding literature data as a result it was concluded that **TA1** was medicarpin (**4**).

8.5 Isolation of TA2 or 4-hydroxymedicarpin (6) from crude EtOAc Extract

The crude EtOAc extract (2 g) was adsorbed on 5 g of silica gel and applied on the column which was packed with 60 g of silica. The column was eluted with solvent the Petrol:EtOAc (60:40, v/v) and two fractions were collected**.**

In TLC analysis of the collected fractions **(Fig. 13)** Petrol:EtOAc (7:3, v/v) was used as developing solvent and vanillin in conc. H_2SO_4 was used as spraying agent. The solvent of **Fr –2** was evaporated under reduced pressure at 45^{o}C to obtain 50 mg (0.04%) brown solid product (**TA2**).

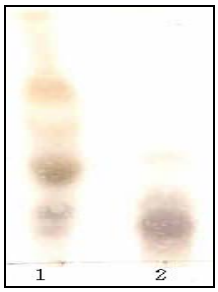
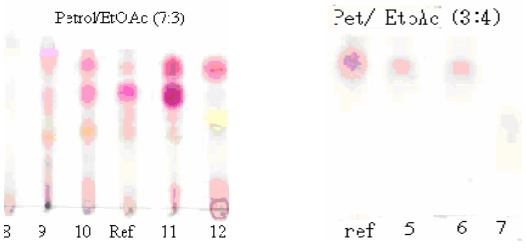


Figure 13. TLC of fractions from CC of crude EtOAc extract

The impure **TA2** was further purified with Sephadex LH-20 with the same procedure as **TA1.** The figure below shows TLCs of the collected fractions from Sephadex in different solvent systems Petrol/etOAc (7:3, v/v) and (3:4, v/v) respectively.



A B

Figure 14. TLC of fractions from Sephadex LH–20 purification of **TA2**

Pure **TA2** was obtained after evaporating the solvent of **fr - 5 (Fig. 12B)** was evaporated under reduced pressure at 45^{o}C to obtain 11 mg (0.008%) of pure **TA2**. Instrumental analyses such as NMR, MS and UV were done in order to identify the pure compound. The observed data obtained were compared with the corresponding literature data as a result it was concluded that **TA2** was 4-hydroxmedicarpin (**6**).

8.7 Bioactivity Test on Medicarpin (4) and 4-Hydroxymedicarpin (6)

Guinea-pigs (300 – 400 g) were used for the study. They have been given a standard diet and tap water. The guinea pigs were killed by a blow to the head and cutting the throat. Every time a tissue required was taken from the abdomen of each animal and cleaned of attached tissues (Susana et al., **2008**). A segment was removed of smooth muscle (2 – 3 cm) from each guinea pig. Tyrode's solution of the following composition (mM), NaCl = 137; KCl = 2.6; MgCl = 1.05; $CaCl_2$ = 0.3; NaH_2PO_4 = 0.04; $NaHCO_3$ = 11.9; glucose = 5.5 was used.

Each individual tissue was set up in a thermo–regulated 25 mL organ bath containing the Tyrode's solution which was maintained at 37^0C and gassed with air. A tension of 1 g was applied to each tissue it was then allowed to equilibrate for at least 30 min before adding histamine (His) or extract. The responses were recorded isometrically using a Grass FT. 03 strain gauge transducer connected to a Grass Model 7 polygraph (Grass instrument Quincy, MA, USA).

Dose response curves of the histamine induced contractions were done for all the tissue preparations and the concentration (8 ng/mL) that effected sub maximal stimulation was taken as the control in each experiment. During each application of histamine, it was removed from the bath after 30 sec contact with the tissue.

Each dose of the extract was applied between 3 minutes interval. It was kept in contact with the tissue for 5 min then control histamine was added up on the extract then both the extract and the control histamine were removed together 30 sec after the addition of histamine. Histamine and extract amounts are expressed as final organ bath concentrations.

9. References

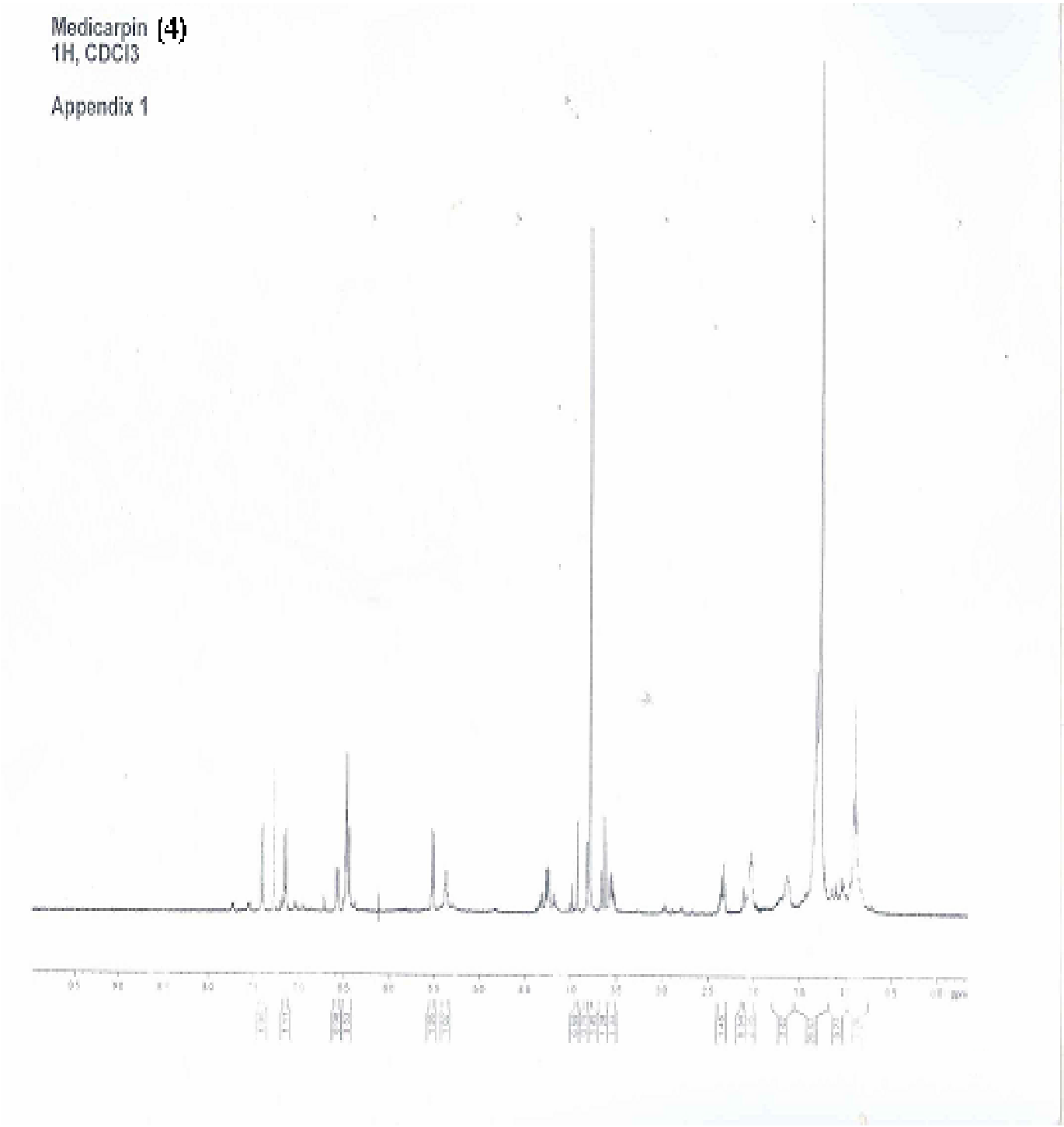
1. Awale S., Li F., Onozuka H., Esumi H., Tezuka Y., Kadota S., **2008**. Constituents of Brazilian red Propolis and their Preferential Cytotoxic Activity against Human Pancreatic PANC-1 Cancer Cell Line in Nutrient-Deprived Condition**.** *Bio. & Med. Chem.*, 16, 181-189.[a]
2. Bekele E., **2007**. Study on Actual Situation of Medicinal Plants in Ethiopia. www.jaicaf.or.jp/publications/ethiopia_ac.pdf [a]
3. Berhanu M. Abegaz., Bonaventure T., Ngadjui, Merhatibeb B., and Ladislaus K. Mdee., **1999.** Novel Natural Products from Marketed Plants of Eastern and Southern Africa. *Pure Appl. Chem*., Vol. 71, No. 6: 919 – 926.[a]
4. Dagne E. Yenesew A, Capasso F., Mascolo N., Pinto A., **1990**. Preliminary Studies on Antipyretic and Analgesic Properties of *Taverniera abyssinica*. *Ethiop. Med. J.* 28: 155-161. [b]
5. Dagne E., Duddeck H., Yenesew A., **1987**. Isoflavonoids from *Taverniera Abyssinica*. *Bull. Chem. Soc. Ethiop*. 1: 36-41.[b]
6. Dewick M.P., **2001**. Medicinal Natural Products A Biosynthetic Approach. 2nd ed. 149 – 156.[b]
7. Fekadu F., **2007**. The Role of Indigenous Medicinal Plants in Ethiopian Healthcare**,** African Renaissance, 1st quarter, 16.[a]
8. Harborne J.B, **1988**. The Flavonoids, Advances in Research since **1980**. 192– 201.[c]
9. Hedberg I., Edwards S., **1989**. *Flora of Ethiopia*. Vol. 3. The National Herbarium, AAU [d]
10. http://en.wikipedia.org/wiki/Antispasmodic [a]
11. http://medical-dictionary/Antispasmodic+Drug [a]
12. Institute of Biodiversity Conservation and Research (IBCR), **2001**.[a] http://www.telecom.net.et/~ibcr/Medicinal%20Plants.htm
13. Jorge H. R., Maurcio P., Oscar G., **2007.** Implementation of the Technique in Isolated Organ Vascular as Tool for the Validation of Medicinal Plants. Study of the Vasodilator Effect of the *S. scutellarioides, Colombia Médica* Vol. 38 Nº 1.[a]
14. Mekonnen, Y., **1999**. Effects of Ethanol Extract of *M. stenopetala* Leaves on Guinea pig and Mouse Smooth Muscle**.** *Phytotherapy Research* 13, 442 – 444.[a]

15. Militao G., Carmen G., Jimenez P. C., Wilke D. V., Pessoa C. F., Maria J C., Lima M. A., Silveira E. R., Odorico de M. M., Costa-Lotufo., Leticia V., **2005**. Antimitotic properties of Pterocarpans Isolated from *Platymiscium floribundum* on Sea Urchin Eggs**.** *Planta Medica*, 71, 683 - 685.[e]
16. Ngamrojanavanich N., Loontaisong A., Pengpreecha S., Cherdshewasart W., Pornpakakul S., Pudhom K., Roengsumran S., Petsom A., **2007**. Cytotoxic Constituents from *Butea superba Roxb*. *J. Ethn. pharm.*, 109, 354-358.[e]
17. Satyajit D., sarker Z., Alexander I., 2006. Natural Products Isolation, 2nd ed, 302 – 305.[b]
18. Susana G., Valeria S., Flavia R., Cristina V., Liliana M., Virginia M., Cristina A., **2008.** Choleretic and Antispasmodic Effects of *Lippia integrifolia* Aqueous Extract. *Rev. Bras. farmacogn.*,18 no.1.[a]
19. Takahashi M., Fuchino H., Sekita S., Satake M., Kiuchi F., **2006.** *In vitro* Leishmanicidal Constituents of *Millettia pendula*. *Chemical and Pharmaceutical Bulletin*, 54, 915-917.[e]
20. Umehara K., Nemoto K., Kimijima K., Matsushita A., Terada E., Monthakantirat O., De-Eknamkul W., Toshio M., Warashina T., Degawa M., Noguchi H., **2007**. Estrogenic Constituents of the Heartwood of *Dalbergia parviflora***.** *Phytochemistry.*, 69, 546-552.[e]
21. World Health Organization, Geneva, **1978**. The Promotion and Development of Traditional Medicine**.** *WHO meeting Technical Report series*, Geneva. [a]
22. www.dmt.dk/files/prospects/750tobs_data_sheet_us.pdf.
    Tissue Organ Bath System. [a]
23. www.patient.co.uk/health/Antispasmodic-Drugs.htm.[a]

Note: Superscripts given at the end of each reference indicate the availability of the documents.

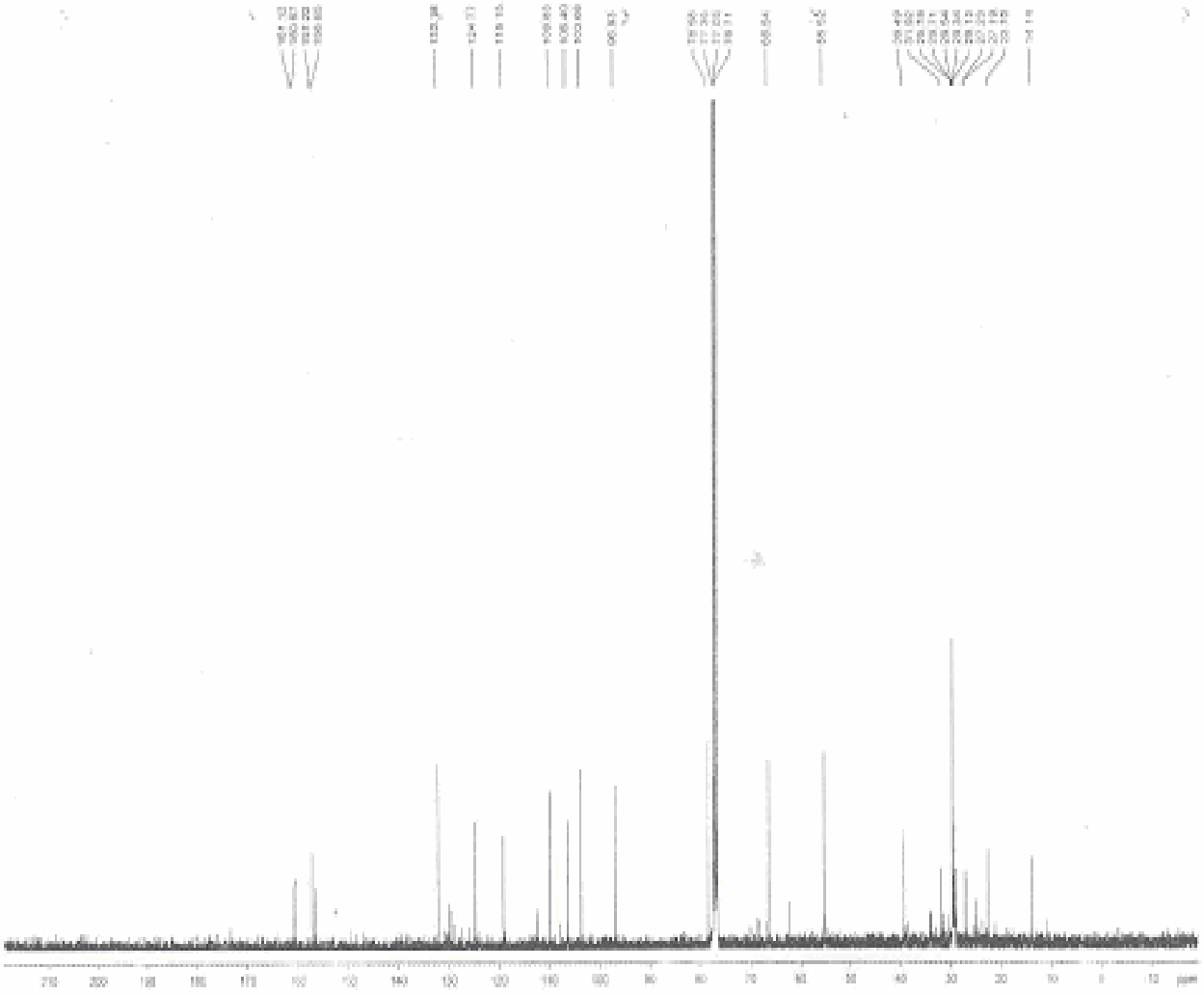
a) Online access
b) Available in Chemical Information center, AAU
c) Science main library, AAU
d) National Herbarium of Ethiopia, , Science faculty, AAU
e) ALNAP database, Chemistry Department, AAU

10. Appendices



Medicarpin (4)
C13, CDCl3

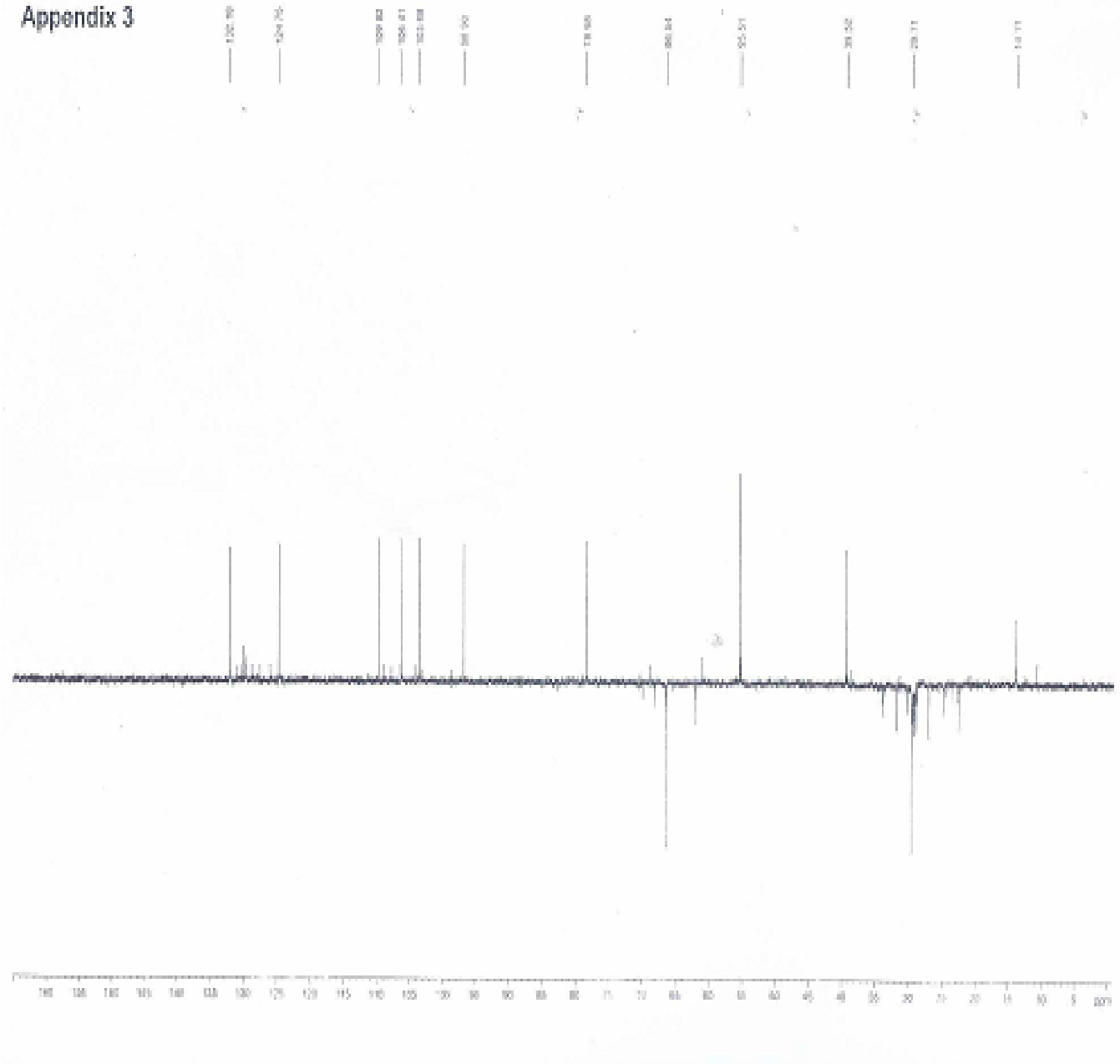
Appendix 2



Medicarpin (4)

Dept-135, CDCl3

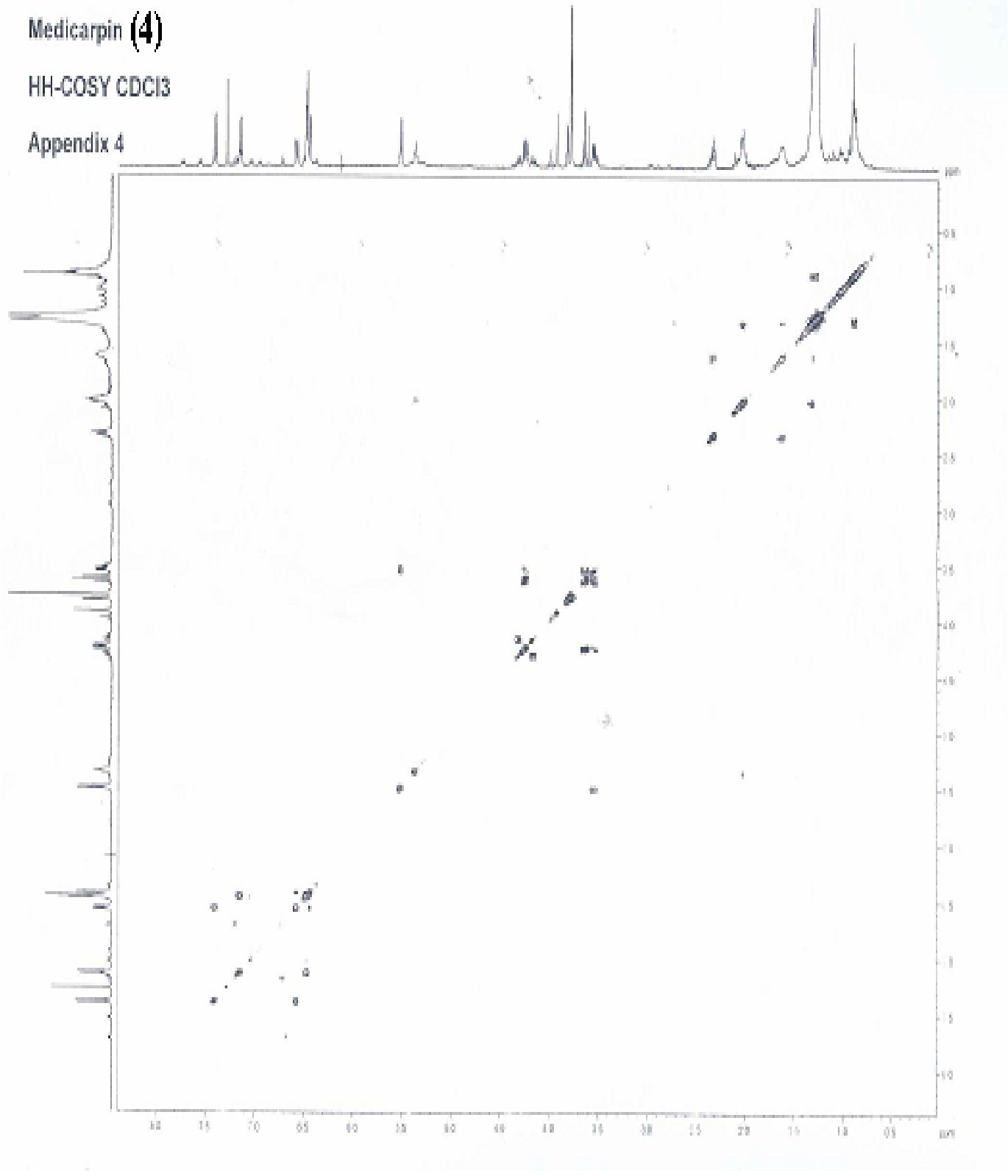
Appendix 3



Medicarpin **(4)**

HH-COSY CDCl3

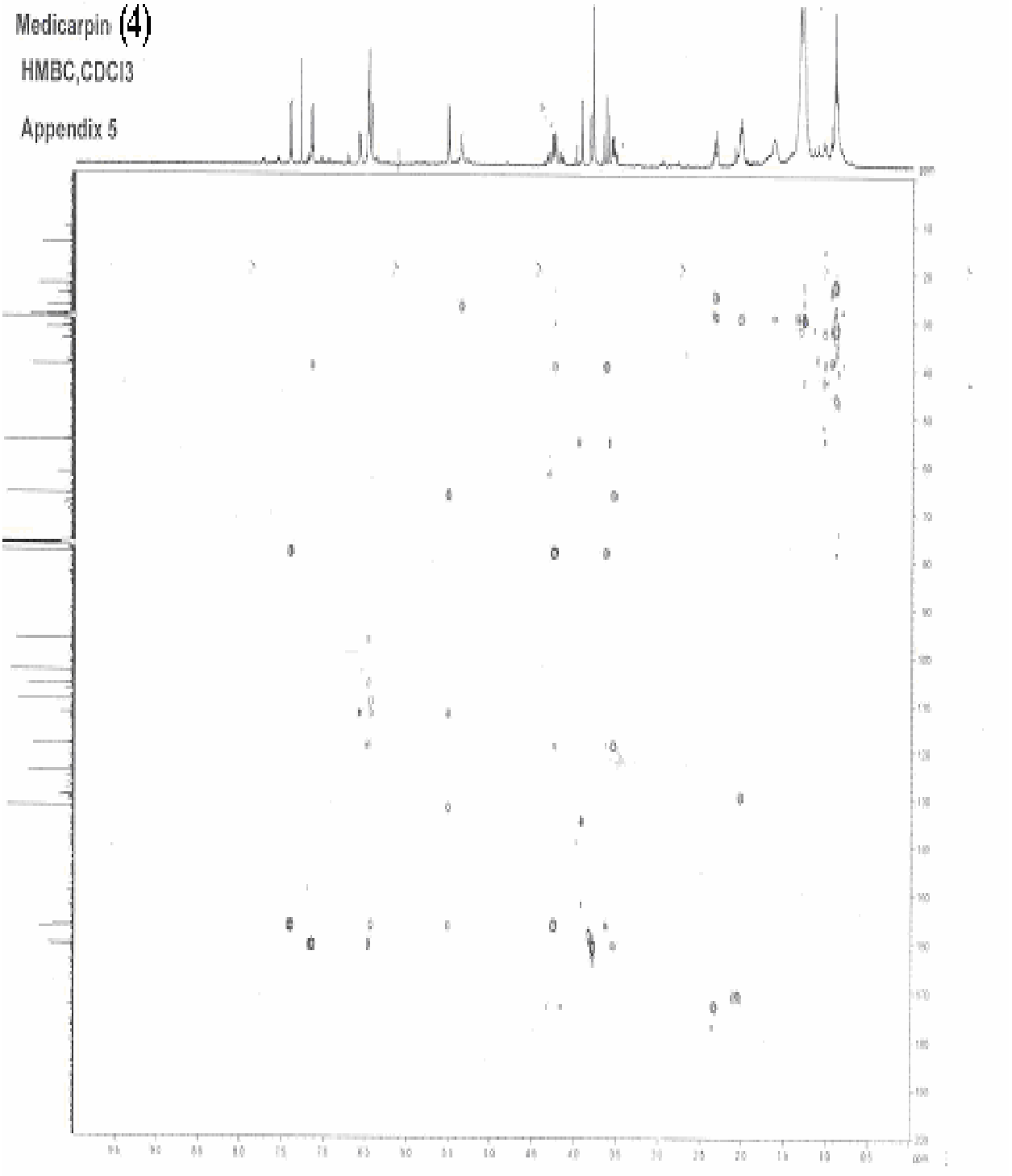
Appendix 4



Medicarpin (4)

HMBC,CDCl3

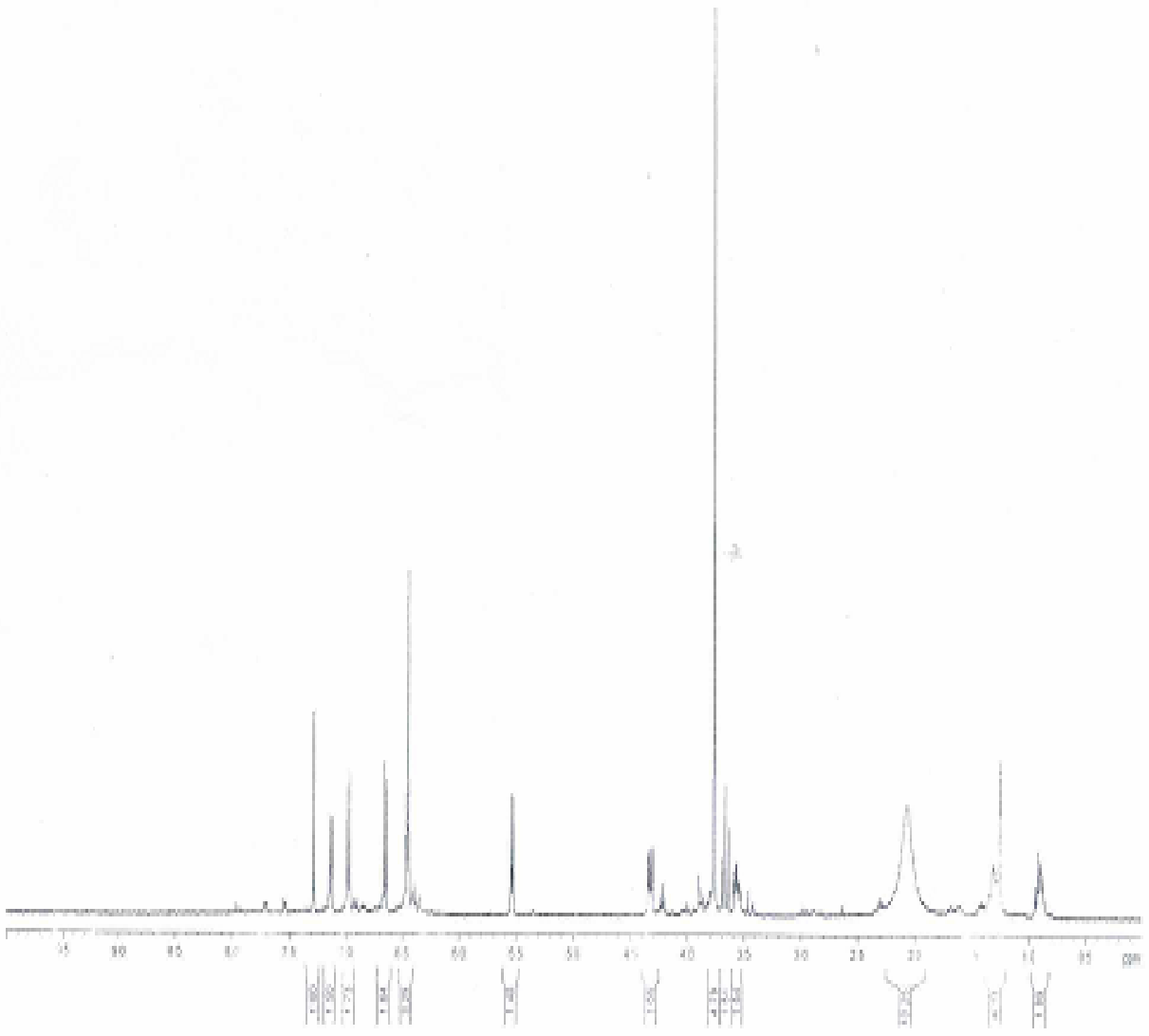
Appendix 5



4-hydroxymedicarpin (6)

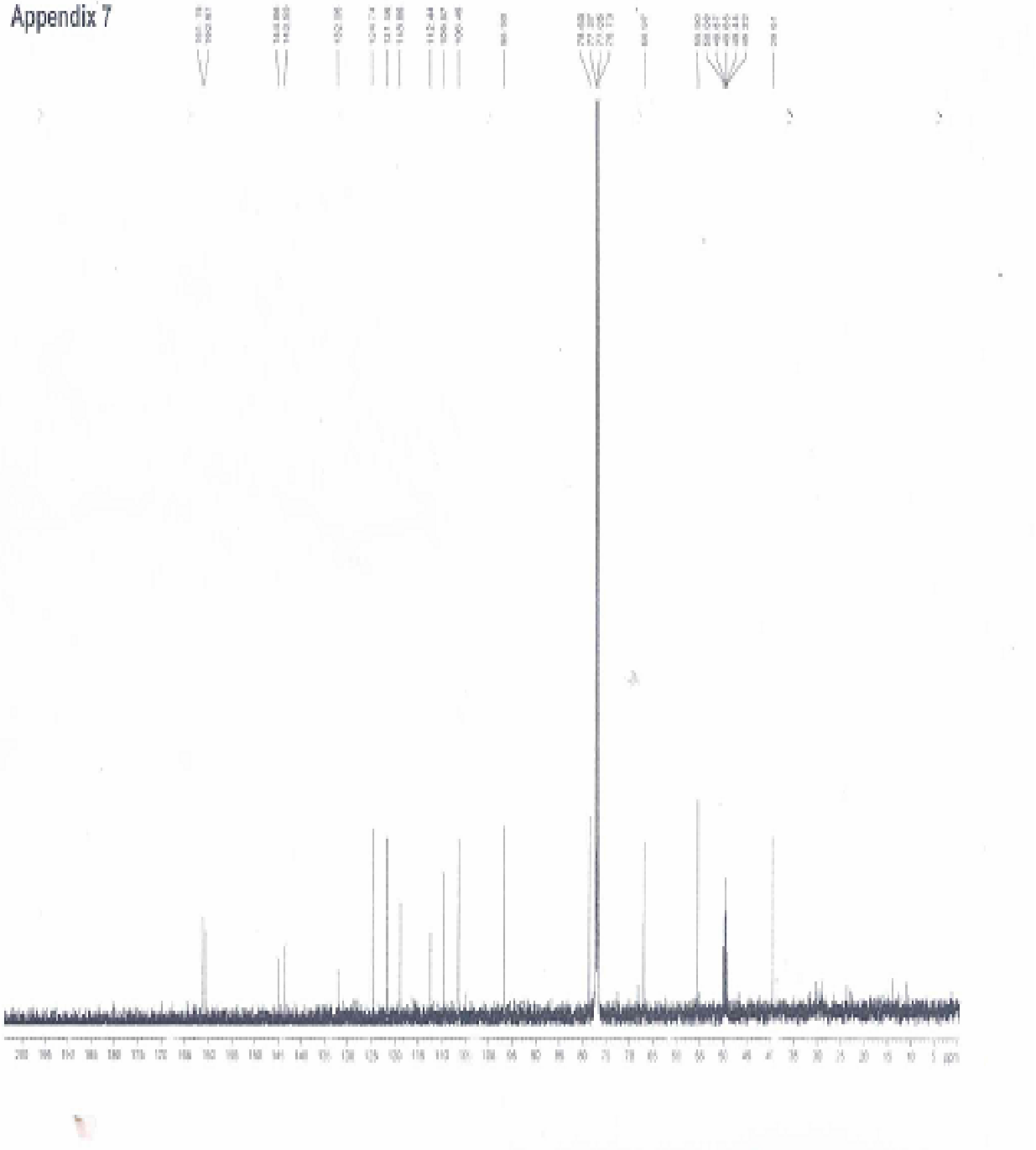
1H, CDCl3

Appendix 6



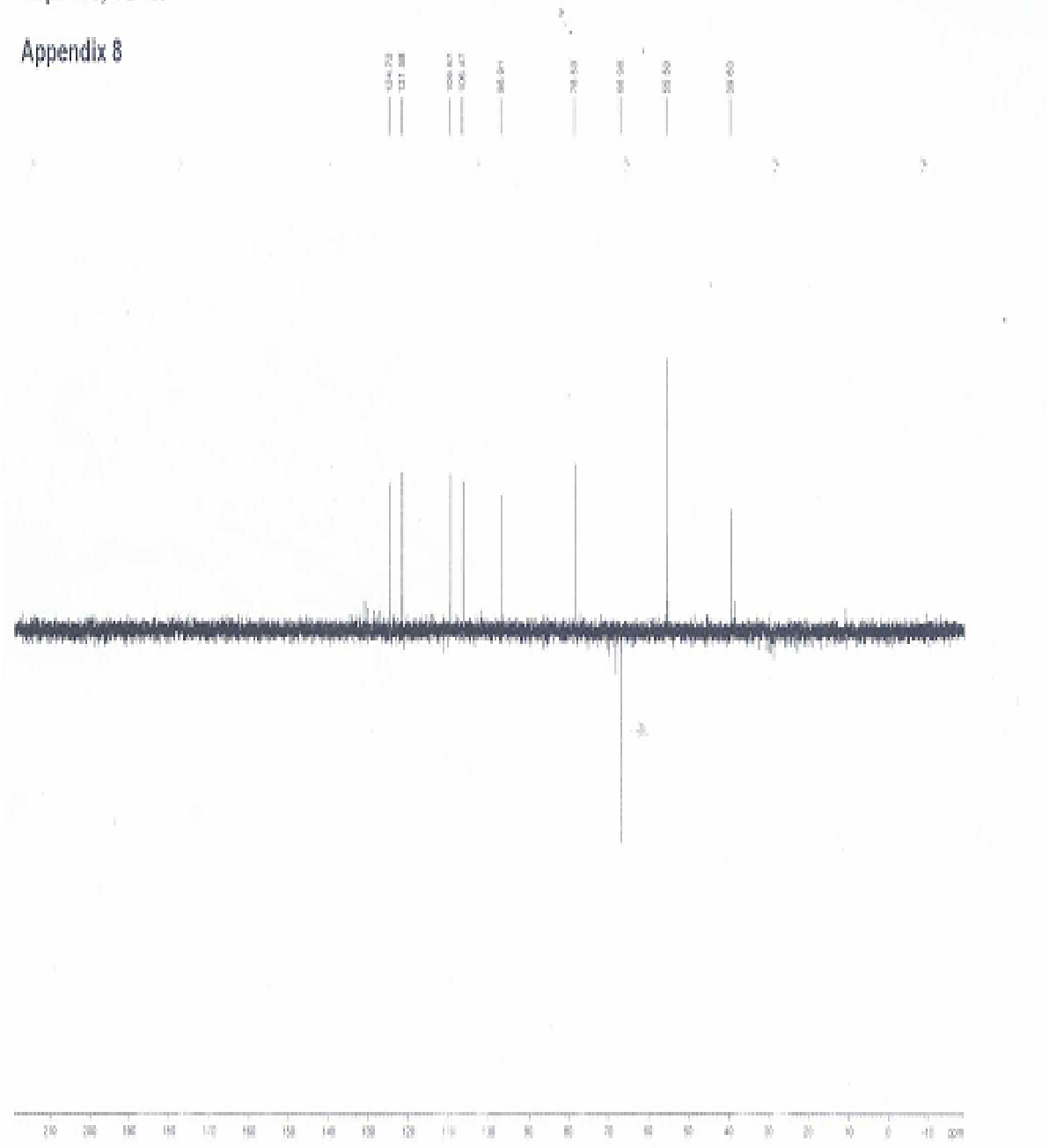
4-hydroxymedicarpin (6)
C13, CDCl3

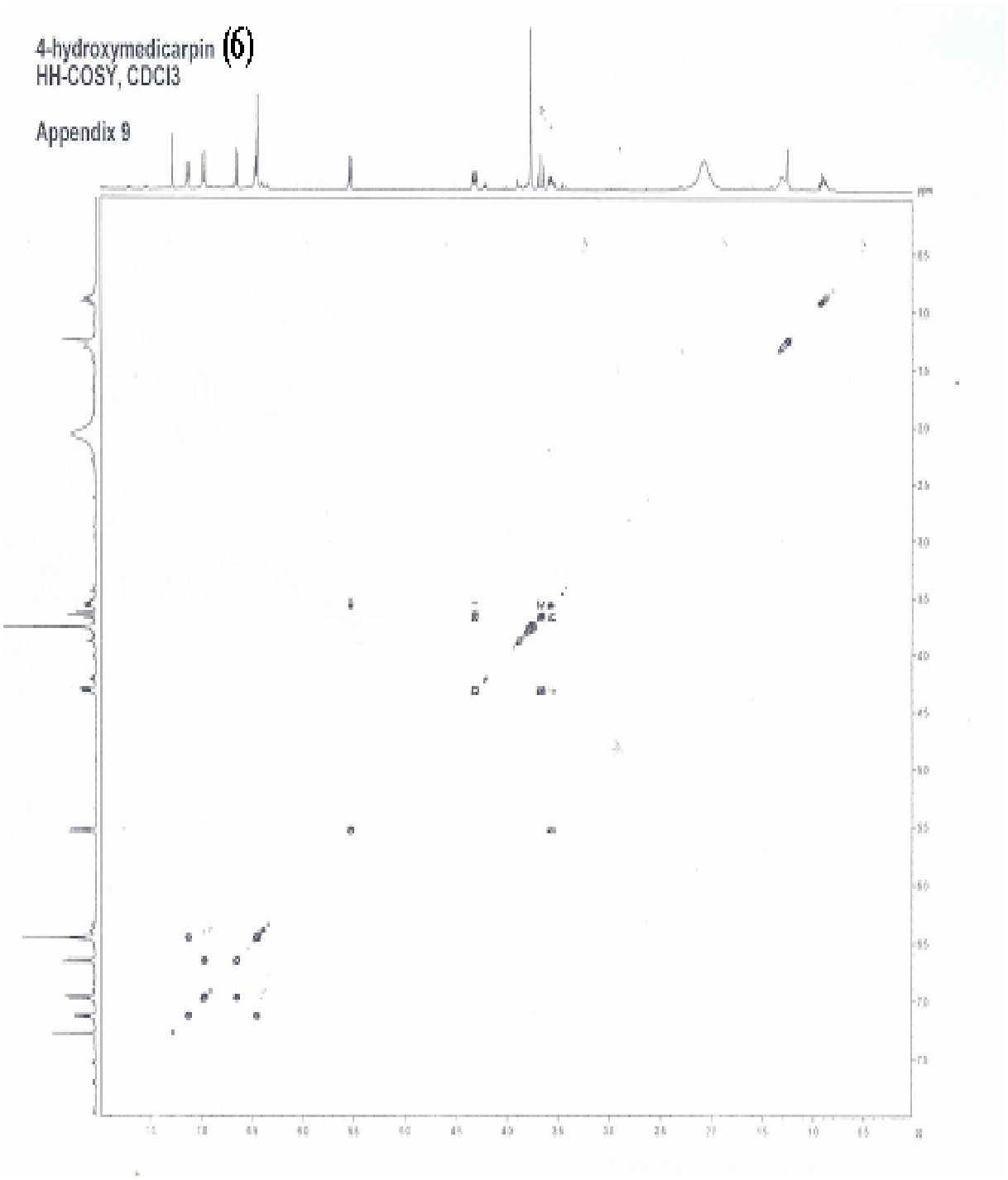
Appendix 7



4-hydroxymedicarpin (6)
Dept-135, CDCl3

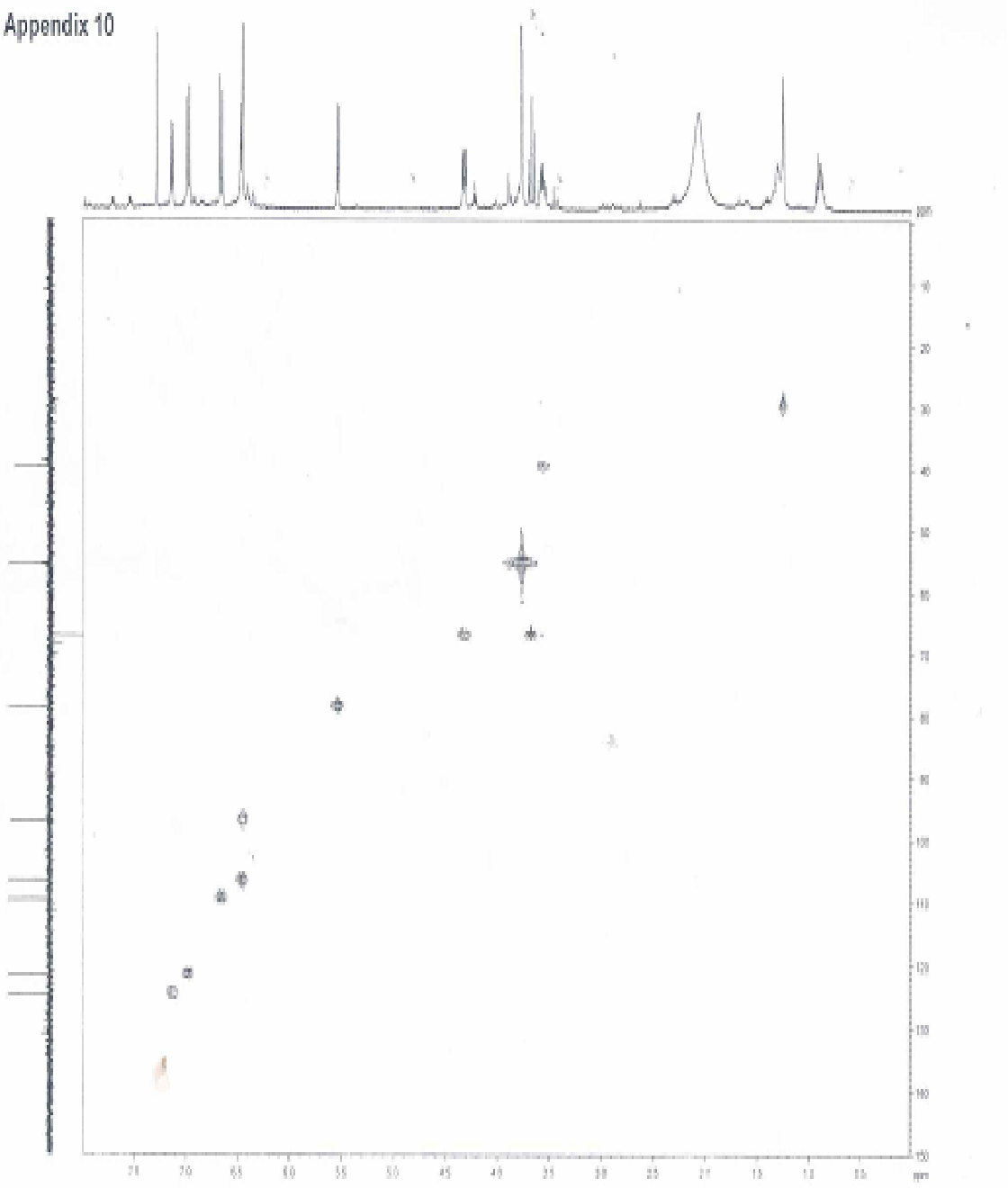
Appendix 8

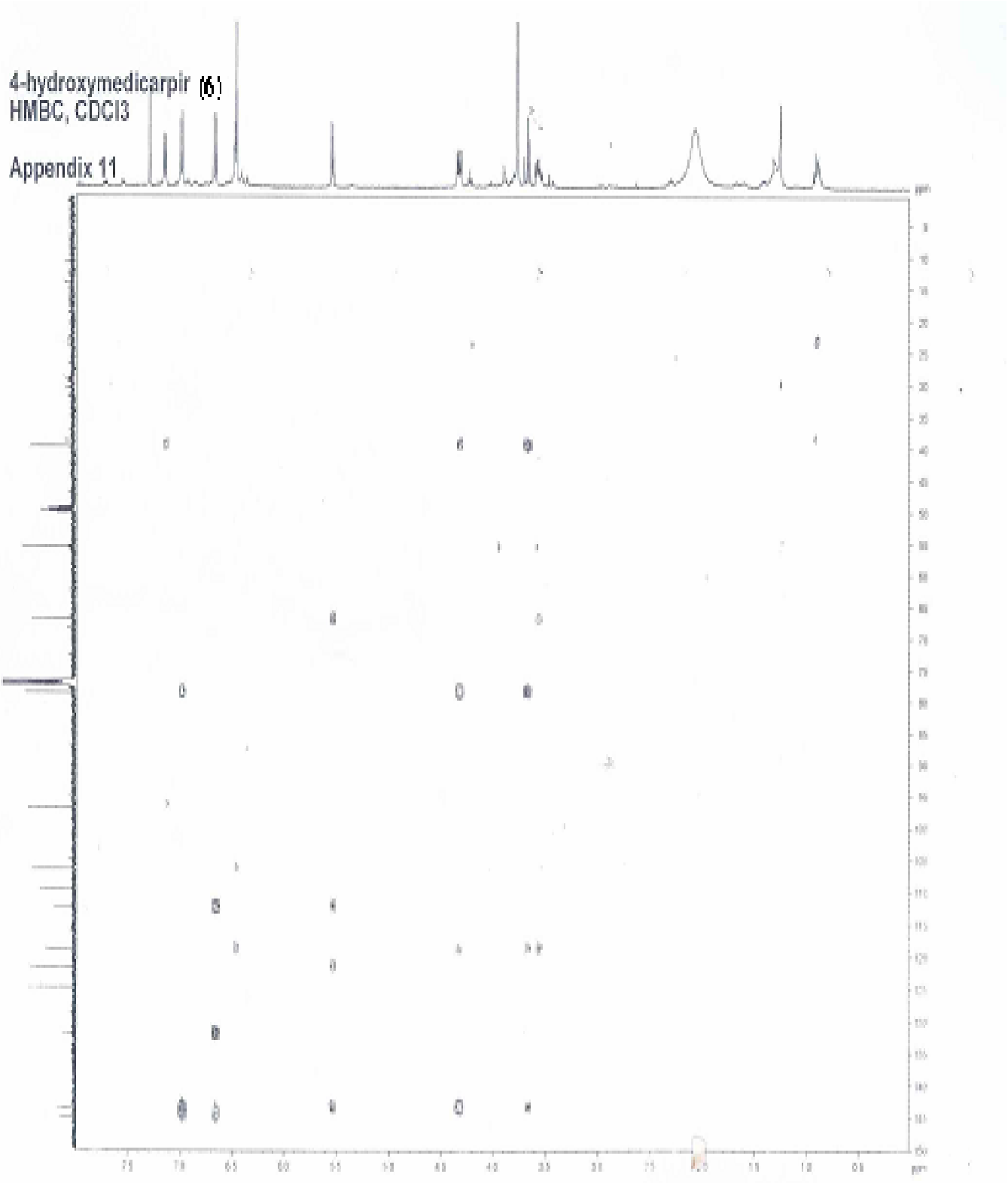



4-hydroxymedicarpin (6)
HH-COSY, CDCl3
Appendix 9

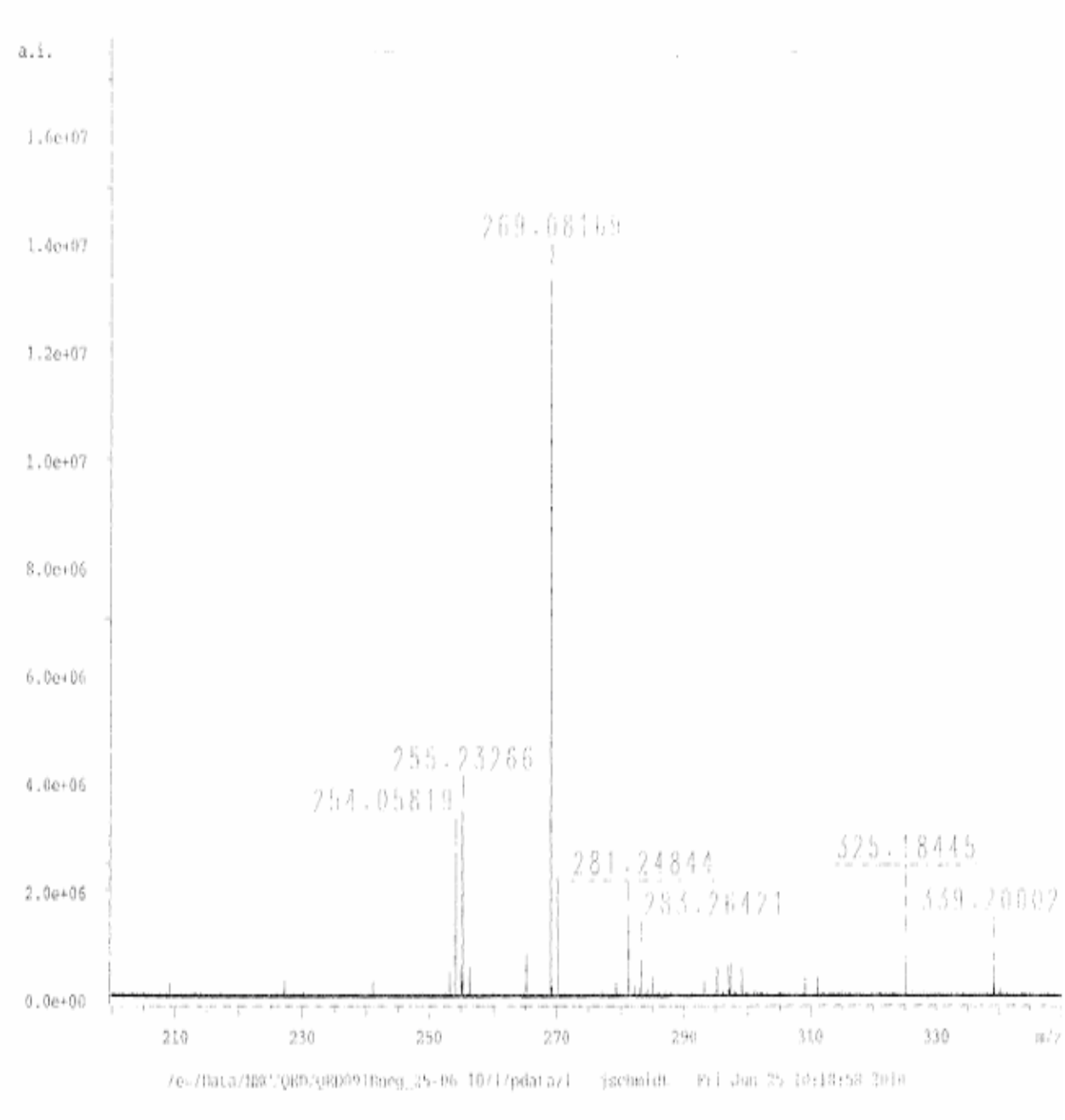
4-hydroxymedicarpin (6)
HSQC , CDCl3

Appendix 10




4-hydroxymedicarpir (6)
HMBC, CDCl3
Appendix 11

Appendix12: HRMS of medicarpin (**4**)



Appendix 13: HRMS of 4-hydroxymedicarpin (**6**)