ISOLATION OF ARISTOLOCHIC ACIDS FROM *ARISTOLOCHIA ALBIDA* (DUCH) AND SOME STUDIES OF THEIR BIOLOGICAL ACTIONS (FAMILY: ARISTOLOCHIACEAE)

BY

OYEBANJO, OMOTAYO OLADIPUPO

(B.Sc. Biochemistry)

A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL,

AHMADU BELLO UNIVERSITY, ZAR1A.

IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF

THE DEGREE OF MASTER OF SCIENCE,

AHMADU BELLO UNIVERSITY, ZAR1A - NIGERIA.

MAY, 2000.

CERTIFICATION

This thesis entitled ISOLATION OF ARISTOLOCHIC ACIDS FROM ARISTOCHIA ALBIDA (DUCH) AND SOME STUDIES OF THEIR BIOLOGICAL ACTIONS by OYEBANJO, Omotayo O. meets the requirements of the regulations governing the award of the degree of MASTER OF SCIENCE of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

..... 20/8/2001
Dr. J.A. Kolawole, B.Sc. (Pharm) Date
M.Sc., Ph.D., MPSN
External Examiner and Head,
Department of Pharm. Chemistry,
University of Jos

..... 20/VIII/2001
Dr. A.K. Haruna, *B.Sc.* (Pharm), M.Sc. Ph.D. (MPSN) Date
MAJOR SUPERVISOR
Department of Pharmaceutical and Medicinal Chemistry,
Faculty of Pharmaceutical Sciences,
Ahmadu Bello University, Zaria

..... 20-8-2001
Prof. M. Ilyas *B.Sc. M.Sc. Ph. D* Date
Department of Pharmaceutical and Medicinal Chemistry,
Faculty of Pharmaceutical Sciences,
Ahmadu Bello University, Zaria.

..... 20/VIII/2001
Dr. A.K. Haruna, *B.Sc.* (Pharm), M.Sc. Ph.D. (MPSN) Date
Head, Department of Pharmaceutical and Medicinal Chemistry,
Faculty of Pharmaceutical Sciences,
Ahmadu Bello University, Zaria

..... 19/9/03
Prof. S.B. Ojo, *B.Sc., M.Sc., ph.D* Date
Dean, Postgraduate School,
Ahmadu Bello University, Zaria

DEDICATION

This project is dedicated to:

THE GLORY OF THE ALMIGHTY GOD [Phil.$_4$[6,13]; Ps.$_1$[1-3]; Prov.$_3$[5]]

My Father, Pa. FRANCIS DOLANIMI OYEBANJO of blessed memory who laid the foundation that ushered in this degree.

My Mother Mrs. CHRISTIANA ADETUTU OYEBANJO who braced up to face the numerous seemingly challenges that raised their ugly heads after the demise of our Dear Father.

My Siblings, Mrs. FUNMILAYO A. SHITTU, SEGUN, ABOLAJI, OMOMWUMI, GBENGA, OLAYEMI, My numerous cousins and BUKKY & BOLA SHITTU.

To my beloved one, Miss COMFORT OLUWAFUNMILAYO OPOWOYE, whose presence in my life has brought the desired difference needed to spur us into greater heights and our glorious destiny: "A Rose of shar'on and the Lily of the Valley" [Amos$_3$[3], Gen$_2$[24-25]; Prov.$_{12}$[4a], 18[22], 31[10-31]].

ACKNOWLEDGEMENT

I wish to express my gratitude to Dr. A. K. Haruna, a renowned scientist and international scholar who designed and supervised this project. He has not only supervised the project thoroughly but he has painstakingly and meticulously exposed me to knowledge beyond this level such that the next stage would be an easy affair for me by his expertise.

In this connection, Dr. A. K Haruna, now the Head of Department of Pharmaceutical and Medicinal Chemistry has become more of a "blood brother" than a supervisor because of his untiring and relentless contributions, criticism, encouragement and his singular midwifery nature through which this degree became a reality; He shall forever be remembered for his good work.

I am indebted to Dr. (Mrs.) M. T. Odunola of this department for her contributions both administratively and in offering scholarly advice to make this project a success. I also like to thank Messers Ilyasu Mohammed Salisu, M. A. Magaji, Francis Obeta, Ahmadu Zubairu and other technical and administrative staff of the Department of Pharmaceutical and Medicinal Chemistry for their assistance.

I am indeed very grateful to Dr, O. Odutola, Head of Department of Pharmacology and Clinical Pharmacy for his fatherly assistance. I also like to thank Messers Iyada Kingsley, Charles Ebute, John Kono and Jonathan Huji and all staff of Department of Pharmacology and Clinical Pharmacy, who helped to carry out the biological work of this project. Mention would be made of Dr. Sanni Ibrahim and Dr. A. S. Agbaji who provided laboratory space and facilities for most of the phytochemical work of this research and for freeze-drying of the venom of *N. nigricollis* respectively at the Department of Biochemistry, A.B.U. and National Research Institute for Chemical Technology, Bassawa, Zaria.

I must admit that I am indebted to a very large number of friends, well-wishers and colleagues who have helped in this endeavour but I hope none will feel slighted by my inability to list every name here. They should be assured that I certainly appreciate their contribution. However, I wish to acknowledge most specially, the special encouragement, care, support and interest of my beloved one, a Vitous woman, is the crown of her husband, Miss COMFORT OLUWAFUNMILAYO OPOWOYE.

Above all, thanks be to Almighty God for seeing me through the thick and thin periods of this work and for fulfilling His promises in my life.

ABSTRACT

Aristolochia albida (Duch) [Family: Aristolochiaceae] is a shrub abundantly available in Nigeria and very much used by the traditional herbalist for a variety of purposes. The plant is used extensively to treat abdominal colic and is also claimed to be beneficial in the treatment and management of snake bites.

The chloroform extract of the rhizomes of the plant gave a mixture of pale yellow micro needle-like fluorescent crystal through chromatographic separation which after crystallization in $CHCl_3MeOH$ (4:1) revealed two spots coded as CA_1 and CA_2. CA_1 and CA_2 were characterised as aristolochic (4,5-methylene dioxy-ll-methoxy-14-nitrophenanthrene-1-carboxylic acid), $C_{17}H_{21}O_7N$ [M^+, 341] and aristoloside (aristolochic acid with glucosyl residue attached to C-9), $C_{23}H_{21}O_{13}N$ [M^+, 519] on the basis of their m.p 191-194 °C and 280 - 283 °C. Spectral analysis (UV, 1R, NMR, MS) and comparison with reference aristolochic acids respectively.

The biological actions of the crude aqueous extract of the plant rhizomes showed a dose-dependent relaxation of the frog rectus abdominis and antagonised the action of acetylcholine on the same tissue in the same manner like atropine. This effect is beneficial in the management of gastrointestinal disorders thus justifying the use of the plant for stomach disorders in traditional medicine. The anti-snake venom studies of rhizomes *of Aristolochia albida* showed that the extract could safely be useful in the treatment of snake bites especially those involving *N. nigricollis,* a cobra snake. The extract (4 mg/ml) antagonised the effective concentration of venom of *N. nigricollis* (0.16 mg/ml) dose dependently and occupy the muscarinic receptors thereby changing the conformation of the receptors thus preventing the snake venom access to these receptors to cause toxic and lethal reactions, which suggests that the extract might consists of agents capable of suppressing the effect of the venom. The biological test on rat phrenic nerve diaphragm revealed that the aqueous extract of the plant reduced slightly the inhibition effect of the venom even though the phrenic nerve diaphragm is a weak tissue in explaining the basis for neurochemical transmission. The use of this plant in traditional medicine is thus justified by the results of these tests in the treatment and management of snakebites.

TABLE OF CONTENTS

		Page
	Title page	i
	Declaration	ii
	Certification	iii
	Dedication	iv
	Acknowledgement	v
	Abstract	vii
	Table of Content	viii
	List of Tables	xii
	List of figures	xiii
	Abbreviations	xv
	CHAPTER ONE: GENERAL: INTRODUCTION	
1.1	Native Medicines from Plant Sources	1
1.2	LITERATURE REVIEW	3
1.2.1	*Aristolochia* Species	3
1.2.2	Morphology of *Aristolochia* species	4
1.2.3	Local uses of *Aristolochia* species	4
1.2.4	Review of the chemical constituent	5
1.2.4.1	Constituents of *Aristolochia* species	5
1.2.5	Chemistry of the major constituents of *Aristolochia* plant	19
1.2.5.1	Nitrophenanthrene derivatives	19
1.2.5.2	Flavonoid and Flavonoid glycosides	27
1.2.5.3	Coumarins	28
1.2.5.4	Volatile oils	28

1.2.6	Pharmacology of *Aristolochia* plants	29
1.2.7	Snakes and effects of their bites	30
1.2.7.1	Snake Venoms	32
1.2.7.2	Serum of specific therapy	33
1.2.7.3	Summary of snake bite treatment	34
1.2.8	Aims and objectives of the project	34
1.2.9	Spectroscopic data of major constituents of Aristolochia plants	35

CHAPTER TWO: MATERIALS AND METHODS

2.1	Materials	45
2.1.1	Chemicals and reagents used	45
2.1.2	Souce of reference sample	45
2.1.3	Instrument used	45
2.2	Methods	45
2.2.1	Extraction procedures	45
2.3.0	Preliminary phytochemical screening	46
2.3.1	Test for carbohydrates	46
2.3.2	Test for flavonoids	47
2.3.3	Test for cardiac glycosides	48
2.3.4	Test for Terpenoids	48
2.3.5	Test for Tannins	48
2.3.6	Test for Saponins	48
2.3.7	Test for Alkaloids	48
2.3.8	Test for Cyanogenic glycosides	49
2.3.9a	Borntrager's Test for Anthracene Derivatives	49

2.3.9b	Free carboxylic Acid compounds	50
2.4.0	Analytical Methods	50
2.4.1	Extraction	50
2.4.2	Column Chromatography	52
2.4.3	Column Chromatography separation of components of fraction A	53
2.4.4	Thin Layer Chromatography	53
2.4.5	Thin Layer Chromatographic Examination of 'CA	54
2.6	Characterization of CA_1 and CA_2	54
2.6.1	Colour test for CA_1 and CA_2	55
2.6.2	Spectra Analysis of CA_1 and CA_2	56
2.6.3	C H-Analysis of $CA_1$and CA_2	56
2.7	Detection of sugars from CA_2	56

CHAPTER THREE: PHYTOCHEMICAL RESULTS AND DISCUSSION

3.1	Preliminary phytochemical screening result	58
3.2	Description of the fraction A	60
3.3	Characterization of CA_1 and CA_2	60
3.3.1	Result of colour tests	61
3.4	Result of spectral Analysis of compounds B and C	61
3.4.1	UV spectra of CA_1 and CA_2	61
3.4.2	IR spectra of CA_1 and CA_2	62
3.4.3	NMR spectra of CA_1 and CA_2	63
3.4.4	Mass spectra of CA_1 and CA_2	65
3.5	C, H Analysis	65
3.6	Discussion	65

3.7	Conclusion	68

CHAPTER FOUR: BIOLOGICAL STUDIES OF *ARISTOLOCHIA ALBIDA*

4.1	Objectives of the study	85
4.2	Materials and methods	85
4.3	Materials	85
4.4	Methods	86
4.4.1	Preparation of isolated tissues from the animal	88
4.4.2	Recording techniques	89
4.5	Results	89
4.5.1	Frog rectus abdominis	89
4.5.2	Rat phrenic nerve diaphragm	90
4.6	Discussion	97
4.6.1 (a)	Frog rectus abdominis	97
4.6.1 (b)	Anti-snake venom studies	98
4.6.2	Rat Phrenic nerve diaphragm	99
4.7	Conclusion	101
	REFERENCES	103

LIST OF TABLES

Table	Title	Page
2a	Extraction scheme of Aristolochic acids	
2b	Column chromatographic separation of components of fraction A	53
3a	Results of the preliminary phytochemical screening	59
3b	^{1}H-NMR and ^{13}C-NMR spectra data of CA_1	63
3c	^{1}H-NMR and ^{13}C-NMR spectral data of CA_2	64
4a	Effect of concentration produced by acetylcholine on frog rectus abdominis	91
4b	Effect of venom of *N. nigricollis* (0.16 µg/ml) in the presence of acetylcholine on frog rectus abdominis	91
4c	Effect of acetylcholine (0.16 µg/ml) in the presence of venom of *N. nigricollis* on frog rectus abdominis	92
4d	Effect of acetylcholine in the presence of extract of *A. albida* (4 mg/ml)on frog rectus abdominis	92
4e	Effect of aqueous extract of *A. albida* on frog rectus abdominis	93
4f	Effect of concurrent administration of venom of *N. nigricollis* (0.16 µg/ml) + extract of *A. albida* (4 mg/ml) and acetylcholine on frog rectus abdominis	93
4g	Effect of acetylcholine and concurrent administration of venom *of N. nigricollis* (0.16 µg/ml) + extract of *A. albida* (4 mg/ml) and on frog rectus abdominis	94
4h	Effect of aqueous extract of *A. albida* (4 mg/ml) on rat phrenic nerve diaphragm	94
4i	Effect of d-tubocurarine on rat phrenic nerve diaphragm	95
4j	Effect of d-tubocurarine (1.6 µg/ml) in the presence of aqueous extract of *A. albida* on rat phrenic nerve diaphragm	95
4k	Effect of venom of *N. nigricollis* (0.128 µg/ml) in the presence of aqueous extract of *A. albida* on rat phrenic nerve diaphragm	96
4l	Effect of venom of *N. nigricollis* on rat phrenic nerve diaphragm	96

LIST OF FIGURES

FIGURE		Page
Fig. 3a	TLC Chromatogram of fraction A	60
Fig. 3b	UV spectrum of CA_1	70
Fig. 3c	UV spectrum of CA_2	71
Fig. 3d	IR spectrum of CA_1	72
Fig. 3e	IR spectrum of CA_2	73
Fig. 3f	1H-NMR spectrum of CA_1	74
Fig. 3g	1H-NMR spectrum of CA_2	75
Fig. 3h	13C-NMR spectrum of CA_1	76
Fig. 3i	13C-NMR spectrum of CA_2	77
Fig. 3j	Mass spectrum fragmentation of CA_1	78
Fig. 3k	Mass spectrum fragmentation of CA_2	79
Fig. 3l	NMR spectral analysis of CA_1	80
Fig. 3m	NMR spectral analysis of CA_2	81
Fig 3n	Fragmentation pattern of CA_1	82
Fig. 3o	Prominent fragmentation pattern of CA_2	83
Fig 3p	Structures of CA_1 and CA_2	84
Fig. 4a	Effect of contraction produced by acetylcholine on frog rectus abdominis	103
Fig. 4b	Effect of venom of *N. nigricollis* (0.16 µg/ml) in the presence of acetylcholine on frog rectus abdominis	104
Fig. 4c	Effect of acetylcholine (0.16 µg/ml) in the presence of venom of *N. nigricollis* on frog rectus abdominis	105

Fig 4d	Effect of acetylcholine in the presence of extract of *A. albida* (4mg/ml) on frog rectus abdominis	106
Fig. 4e	Effect of aqueous extract of *A. albida* on frog rectus abdominis	107
Fig. 4f	Effect of concurrent administration of venom of *N. nigricollis* (0.16 µg/ml) + extract of *A. albida* (4 mg/ml) and acetylcholine on frog rectus abdominis	108
Fig 4g	Effect of acetylcholine and concurrent administration of venom of *N. nigricollis* (0.16 µg/ml) + extract of *A. albida* on frog rectus abdominis	109
Fig. 4h	Effect of aqueous extract of *A. albida* on rat phrenic nerve diaphragm	110
Fig. 4i	Effect of d-tubocurarine on rat phrenic nerve diaphragm	111
Fig. 4j	Effect of d-tubocurarine (1.6 µg/ml) in the presence of aqueous extract of *A. albida* on rat phrenic nerve diaphragm	112
Fig. 4k	Effect of venom of *N. nigricollis* (0.128 µg/ml) in the presence of aqueous extract of A. albida on rat phrenic nerve diaphragm	113
Fig. 4l	Effect of venom of *N. nigricollis* on rat phrenic nerve diaphragm	114
Fig. 4m	Log-Dose response curves for acetylcholine and acetylcholine + extract of *A. albida* on frog rectus abdominis	115
Fig. 4n	Log-Dose % response curves of the extract of *A. albida* on frog rectus abdominis	116

ABBREVIATIONS AND SYMBOLS

Ach	Acetylcholine
E.C.	Effective concentration
C_6H_6	Benzene
B.P.	British Pharmacopoeia
$CaCl_2$	Calcium chloride
$CHCl_3$	Chloroform
C.C.	Column chromatography
Conc.	Concentration
cm^3	Cubic centimeter
°C	Degree Centigrade (Celcius)
$CDCl_3$	Deuterated chloroform
DEPT	Distortionless Enhancement by Polarisation Transfer
$FeCl_3$	Ferric chloride
Fig.	Figure
e.g.	For example
g	gramme
hr	Hour
IR	Infrared
I_2	Iodine
LBR	Liebermann Burchard Reagent
Log	Logarithm
MeOH	Methanol
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
µg	Microgramme

ml	millilitre
m.p.	Melting point
MS	Mass spectrometry
Min.	minutes
nm	nanometer
NMR	Nuclear Magnetic Resonance
N/S	Normal saline solution
HNO_3	Nitric acid
%	Percent
R_f	Retardation factor
RT	Retention time
TLC	Thin-layer Chromatography
UV	Ultra-violet
cm^{-1}	Per centimeter
KCl	Potassium chloride
KOH	Potassium hydroxide
^1H	Proton
r.t.	Room temperature
AcONa	Sodium acetate
NaOH	Sodium hydroxide
Soln.	Solution
H_2SO_4	Sulphuric acid
i.e.	That is
Vm	Venom
Vol.	Volume

CHAPTER ONE

1.0 INTRODUCTION

1.1 NATIVE MEDICINES FROM PLANT SOURCES

Mankind's experience for thousands of years with plants has led him to nurse a notion that herbal remedies being natural products are inherently safer than the potent synthetic drugs which often produce side effects, (Trease and Evans, 1983). This notion conveys a special and popular appeal on the research on medicinal plants by scientists, (Sofowora, 1986).

Most drugs of plants' origin used by medical practitioners are in the form of extracts of the whole plant material or part of it in the view of this, local medicinal plants which show suitable biological effects could be standardized and similarly utilized. Some of the effect elaborated by extracts of plants used in traditional medicine include, antiviral antitumor, antimicrobial, insecticidal and central nervous system effects (Sofowora, 1986).

Many of these herbal or ancient medicines have given cause for detailed investigation. For instance, the fruit of *Momordica charantia* has outstanding importance to the diabetics as it improves glucose tolerance while *Abrus precatorius* seeds have been used as an oral contraceptive. (Trease and Evans, 1983). These interesting pharmacological and clinical effects of plants used in ancient medicine have attracted further research into their untapped potentials or active principles that may allow these materials to be used in western (modern) medicine.

However, such research into medicinal plants for their bioactive compounds is not that a direct simple task. Investigation of a plant may involve the stepwise extraction

procedures including separation and isolation of the constituents; characterization and quantitative evaluation of the isolated constituents having the particular biological effects. The active principle frequently include the alkaloids, glycosides and those of dietary importance such as carbohydrates, fats, proteins and mineral elements. Saponins, steroid, terpenoids, flavonoids and tannins often reported to occur along side with the ones mentioned earlier are invaluable to the pharmacy profession .(Trease and Evans, 1983).

The review of these medicinally useful compounds of plant origin is very essential to establish the potency and side effects of the drugs in response to the growing interest in the alternative system. New drugs from plants are not just a relief of raw materials for the manufacture of known and commonly used drugs, which would provide the basis for some local pharmaceutical industries. Drugs from plant origin known to posses high medicinal values promote the cultivation of such plants which eventually saves the scarce foreign exchange and improves the economy of the country.

One of the major set backs often encountered with drugs of plant source is that, most of the active compounds occur in minute quantity which requires tedious extraction procedures. In order to overcome this limitation the synthetic chemists takes the approach of determining the structure - activity relationship of the isolated, biologically active constituents to synthesise simpler analogues, some of which might be more active and less toxic than the natural products. A good example is the antimalarial drug - quinine, obtained from cinchona bark and its synthetic analogue, chloroquine. Structural modification of pure bioactive molecule of plant origin as with Naloxone (narcotic analgesic antagonist of morphine isolated from *Papavera somniferum* has presented a break through (Trease and Evans, 1983).

It is therefore necessary to review the active chemical constituents of the medicinal plants such as *Aristolochia albida* used in this study with the aim of discovering the prospect for the present and future sources of useful drugs for both local utilization as well as providing alternative modern medicines. Besides in spite of the tremendous advances, certain human ailments have not reached complete remission with these drugs, but evidently some defiant diseases have found complete treatment with bioactive substances of plant source. For example, vincristine and vinblastin, two important alkaloids from *Cathalranthus roseus* have superseded the synthetic antitum or agents in the palliative treatment of certain carcinomas, while reserpine obtained from *Rauwolfia serpentia* still remains an indispensable drug in the management of malignant hypertension (Sofowora, 1982.

1.2 LITERATURE REVIEW

1.2.1 *Aristolochia* Species.

The genus *Aristolochia* represents 350 plant species in the family Aristolochiaceae. Some of these plants include:- *Aristolochia albida, A. indica, A serpentaria, A. bracteala, A. elegans, A. ringens, A reticullata, A. clematitis, A. debilis A. chilensis, A. cymbifera, A. championii*, etc. (Trease and Evans, 1983).

Some of the *Aristolochia* species are native of Brazil introduced into West African gardens. They occur from Senegal to Nigeria, Cameroon, extending to Chad and Angola. Certain species are found in the Southern and Eastern Africa (*Aristolochia bracteata* and *A. densivenia*) (Irvine, 1961).

1.2.2. Morphology of *Aristolochia* species

The prominent species have nearly similar appearance. These species are widely distributed in West Tropical Africa and include *Aristolochia albida, A broacteata, A. elegans, A. gibbosa,* to mention but a few.

Aristolochia plants are climbing shrubs often used as ornamental around houses, it is glabrous in all its parts. The stem is slender broad, sulcate. They have woody base with dark violet or pale leaves with stipules of oil secreting cells as transparent dots in the parenchyma of the leaves. Sometimes the leaf blade is caudate at base. Flowering can occur in June and November. Flowers are hermaphrodite, zygomorphic, often large and showy, solitary, racemose or cymose. The flowers are white and heavily veined purple with yellow centre. The flower tube is pale green in colour and the fruits are capsular or baccate, usually appear like a hanging basket and opening as parachute to release the seeds by splitting the carpels away from each other (Irvine, 1961).

1.2.3 Local Uses of *Aristolochia* plants

The vast distribution of these plants all over the world parallels their local medicinal exploitations. The uses of these plants vary with species and locations, and may suggest antifungal, antibacterial, antidotal and anthelminthic properties of the plants. Although the plants are poisonous most are frequently used as decorative plants.

(a) ***Aristolochia albida***

A cold infusion of the bitter roots is used as a stomatic and tonic. The roots powdered with the seeds of hepidium sativum, gatric, and natron are applied as a poultice to guinea

worm. Dried roots are also used with antron for syphilis. The leaf of the plant can be applied locally to certain painful skin conditions (Dalziel, 1937).

Recently, the ethnopharmacological reports from Northern Nigeria about the use of the rhizomes of this plant against snake bites have been reported. (Haruna *et al.*, 1995).

(b) ***Aristolochia bacteata***

This plant is powerful antidote to snake poison, scorpion bites and as cures for boils and guinea worm (Dalziel, 1937). It is a useful anthleminthic especially in veterinary medicine. The plant is very poisonous but juice of leaf can be applied on leg ulcers or mixed with castor oil for eczema. It is as well valuable in treating scabies. Other species are similarly used but quite a large number of them are decorative plants such as *A elegans, A. gibbosa*, and *A ringens* (Irvine, 1961). However, the leaf and root of *A. densivenus* are used as a sexual stimulant and the leaf rubbed with salt as a cough remedy (Watt and Breyer, 1962).

1.2.4 Review of the chemical constituents

1.2.4.1 Constituents of *Aristolochia* species.

Extensive works have been carried out on *Aristolochia* plants while many chemical constituents were isolated and characterised. Similarly, the active substances of the plants include the nitrophenanthrenes, alkaloids, phenolic esters and ethers, volatile oils and flavonoid class of compounds (Trease and Evans, 1983).

Aristolochia sipho contain cellulose, mineral substances such as phosphates, chloride, carbonates, iron, magnesium, potassium and calcium. A brown fat and an essential oil

were obtained as ether extract while the aqueous extract yielded resins, sucrose, and glucose. The plant was shown also to contain glycosides, aristolochine alkaloids and nitrogenous substances. (Castille, 1921).

Chemical examination of the root of *A. indica* revealed the presence of reducing sugar, allantoin, stigmasterol, **(I)** aristolochine and isoaristolochia acid while subjecting the organic solvent extracts of percolation to steam distillation and recrystallization. The glycerides of palmitic, stearic and linoleic fatty acids were also isolated (Krtishanaswamy, *et al.*, 1935).

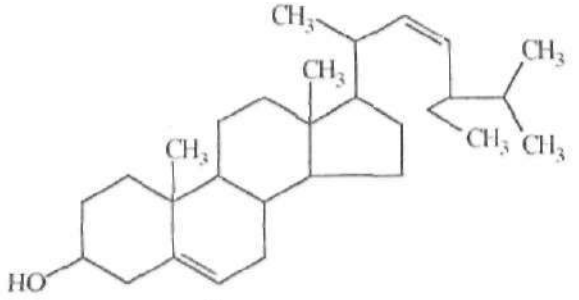
Resonmund and Reichstein (1943) isolated aristolochia acid, (II) and its methyl ester $C_{18}H_{13}O_7N$, **(III)** from *A. sipho*. In an attempt to improve the extractive value of aristolochia acid, **(II)** from various aristolochia plants, Ganshirt (1953) employed column chromatography instead of percolation method. The improved method gave pure crystals of aristolochia acid (II) from *A elematitis, A. serpentaria. A longai, A. maxima* and *A. pandurata*. The identification and determination of aristolochia acid **(III)** was based on solubility melting point, colour reactions, gravimetry and filtration.

Menta *et al.*, (1963) reported the presence of fatty acids such as palmitic, stearic, oleic, myristic and lignoceric acids in *A. bracteata* to confirm the earlier works done by Trishanaswang *et al.*, (1935). Other pure compounds isolated from *A. bracteata* include the β - sitosterol, **(IV)** and aristolochia acid **(II)** in the petroleum extract.

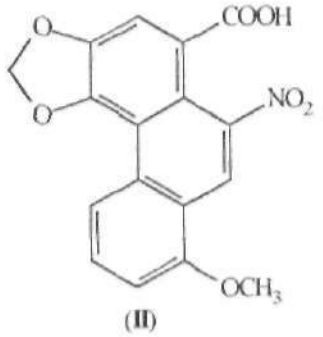
Of much interesting is the presence of flavonoids and related compounds, in *Aristolochia* plants, which are the largest group of naturally occurring phenols. The flavonoidal constituents of *A. sipho* have been studied by ethanol extraction and two dimensional paper chromatography, followed by elution of individual spot which were

identified by fluorescence and colour reactions. Compounds thus identified in the plant were Rutin (quercitin-3-rhamnuglucoside) (**V**), a 3-rhamnoside of kaempferol (**VI**), a 3-monoglycoside (**VIII**), four glycosidic (**X**). Others include an esters of gentistic acid and three unidentified polyphenolic derivatives (Josette, 1965).

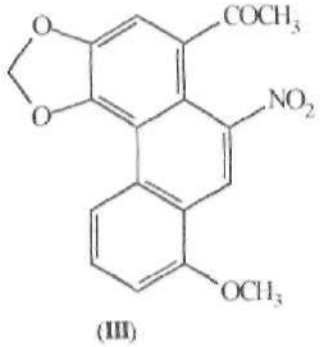
Probably due to the use of gas and thin layer chromatography instead of percolation or column method, aristolochia acid, (**II**) and its derivatives aristoloctam (**X**) and aristolochia acid - C, (**XI**) were obtained from A. rotunda. Sucrose, amino acid L-arginine β-sitosterol, (**IV**) and fatty acids were also identified (Salvatore, *et al.*, 1966). The presence of amino acid in *A rotunda* confirms the earlier work of Grzeyurz (1963) who revealed the presence of serine, glycine, aspartic acid, glutamine, valine, leucine, tyrosine and threonine in *Aristolochia* plant.



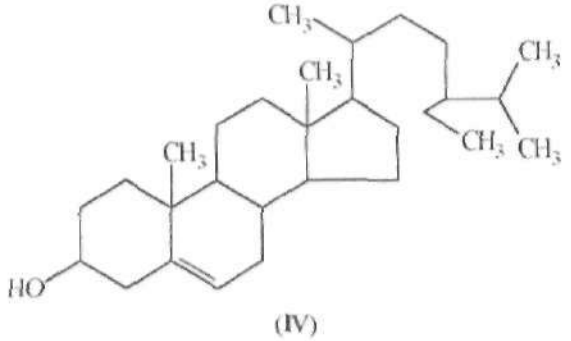
(I)



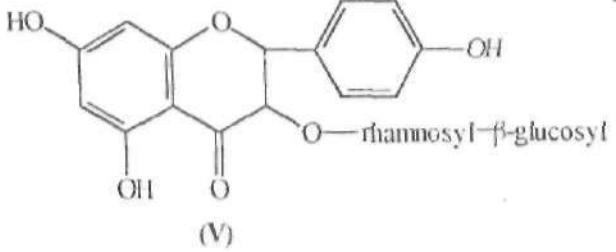
(II)



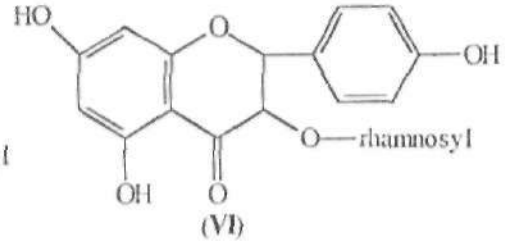
(III)



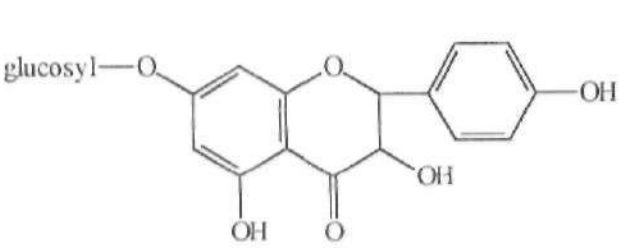
(IV)



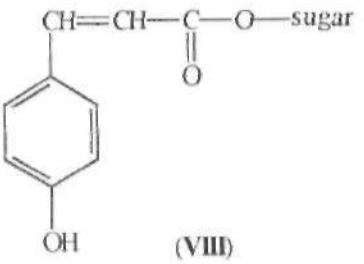
(V)



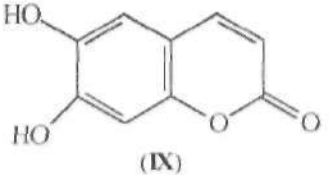
(VI)



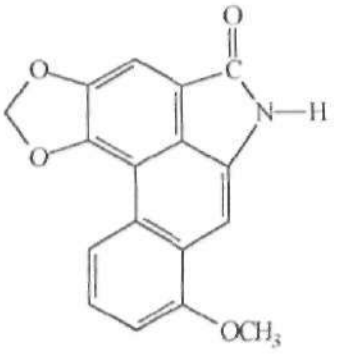
(VII)



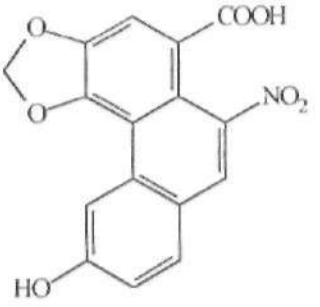
(VIII)



(IX)



(X)



(XI)

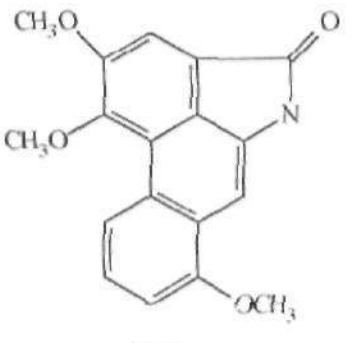
The chemical constituents of *A. taliscana* have been reported to be predominantly alkaloids. Taliscanine (**XII**), along with other semi-synthetic derivatives were obtained from the plant. These derivatives are mainly taliscanine-N-acetate (**XIII**) and methyl taliscanine alkaloids (Herran *et al.*, 1966).

A modified method of extraction and isolation probably made Ruveda and Priestap (1966) to succeed in obtaining two new constituents, such as magnoflorine (**XIV**) and dimethylamine ethyl-phenanthrene (**XV**) from the basic fractions of root extract of A.argentina. Far better yield of aristolochia acid (**II**) can be obtained by extracting with ammonium hydroxide instead of bases sodium hydroxide and sodium carbonate (Shoji, 1968).

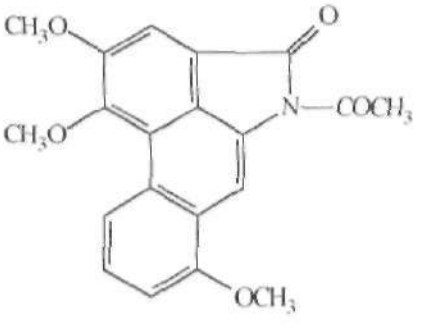
Extraction of *A. griffhi* roots with 5% ammonium hydroxide followed by chromatography on silica gel plates is a useful method for obtaining higher yields of aristolochia acid (**II**) from the plant (Tada *et al.*, 1969). Earlier works of Kupchen and

Merianos (1968) on the isolation and determination of structure of novel derivatives of aristolochia acid, (**III**) from *A. indica* had revealed three new compounds.

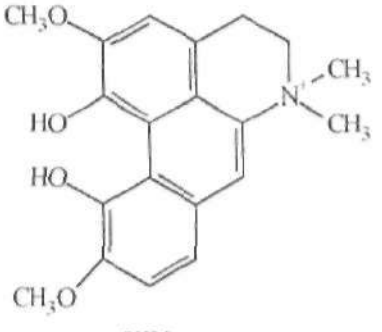
The structures of the compounds were based on the analysis from the NMR spectrum and includes aristolochia - D, (XVI), aristolochia acid D - methylether lactam. (**XVII**), and aristoloctam-β-glucoside. (**XVIII**). Two new sesquiterpene hydrocarbons, ishwarane, (**XIX**), and aristolochene, (**XX**) were shown to be present in *A. indica*. This may be a result of improved distillation techniques used by Govindachari, *et al* (1970). Yet another new compound, argentinine alkaloid, (**XXI**) was identified and isolated from *A. argentina*. The complex structure of argentinine (**XXI**) is based on NMR and UV spectra data and the synthesis of its derivatives (Ruveda and Priestap, 1972). Further works on a argentina led to the identification of aristoloctam compounds (**XXII**) in free states (Priestap *et al.*, 1974) while new saturated sesquiterpenes, 3-oxoishovarane (**XXIII**)[a], have been obtained from *A. debilius* (Nishida, R. and kumazawa, Z.,1973).



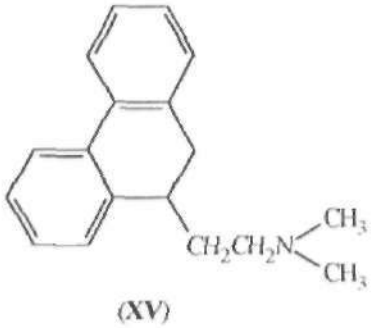
(XII)



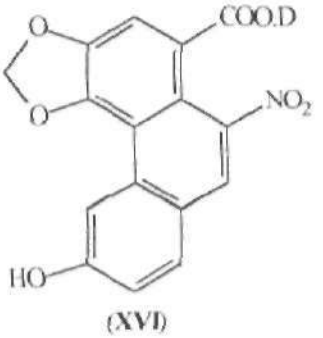
(XIII)



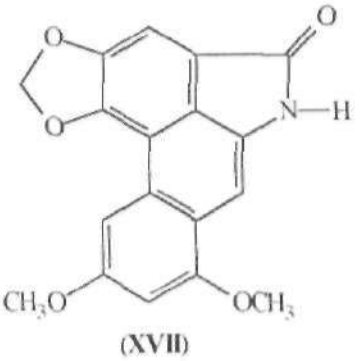
(XIV)



(XV)

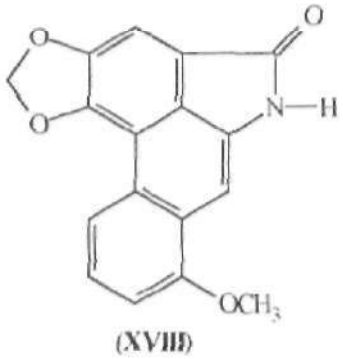


(XVI)

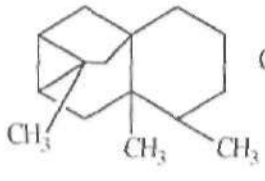


(XVII)

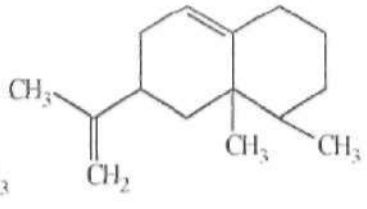
A new tetracyclic sesquiterpene alcohol, ishwarol, (**XXIII**)[b] was obtained from *A. indica* in the earlier works of Govindachari (1971) confirming the result of the work in 1970 on the same plant. Similarly, several workers have identified new constituents *in A. argentina, A esparanzae* and *A. triangularis* in the same year. Priestap *et al.*, (1971) showed that roots of both *A. argentina* and *A esperazae* contain four non-phenolic and two phenolic aristolochic acids. Aristolotam, (**X**) was also identified in *A. argentina.* Multiple extraction of the roots and stems of *A. triangularis* followed by thin layer and column chromatography indicated the presence of saponin, gums, mucilage, reducing sugars, ketoses, tannin, carotenes, β-sitosterol, (**IV**) and alkaloids (Ambros and Desiqueria, 1971).



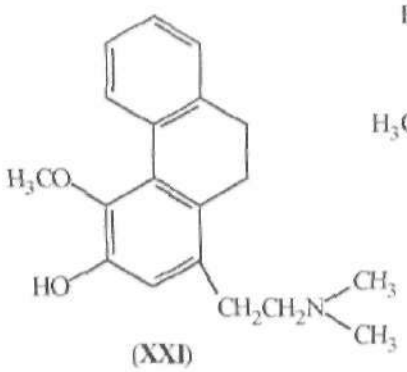
(**XVIII**)



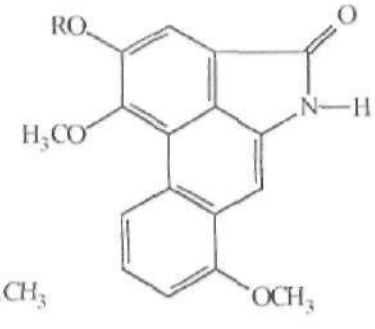
(**XIX**)



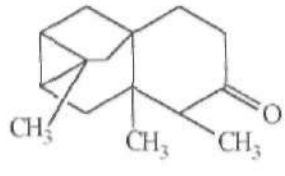
(**XX**)



(**XXI**)



(**XXII**)



(**XXIII**)[a]

Isolation of aristolochic acid (**II**) from roots of A. clematitis has been possible through methanolic extraction of the roots followed by evaporation of the solvent. The

residue of the extract is treated with 15% sodium bicarbonate solution while the crude aristolochic acid, melting point, 170 °C was obtained from the acidified bicarbonate solution. After several recrystallization from dioxane, and finally from methanol, 0.3% yellow needles of aristolochic acid (**II**), melting 280 °C, were isolated. The methyl ester of aristolochic acid (**III**) may be obtained similarly (Podolser and Miceveska, 1972).

Isolation of aristolochia acids in *Aristolochia chilensis* has been carried out. A 1.7 kg sample of air dried powdered plant material was extracted successively with light petroleum and ethanol at room temperature. Concentration of the ethanol extract *in vacuo* afforded a residue, which was partitioned between 21 ml of dilute NH_3 (pH 9) and $CHCl_3$ until fresh $CHCl_3$ extracts appeared colourless. The basic solution was acidified to pH 3 with conc. HCl - ethanol (3:2). The pooled $CHCl_3$ extracts of the acid solution were concentrated under reduced pressure and extracted with 300 ml portions of 5% $NaHCO_3$. This aqueous solution was adjusted to pH 3 with conc. HCl and a greenish - yellow solid was collected by filtration (90 mg). This material showed a single intense chromatographic spot. In systems 1, 2, and 3 and was identified as aristolochia acid I by A. Urzua, *et, al.*, 1982.

Isolation of aristolochia acid **I** and **II**, aristoloctam - **I** and **III** *from Aristolochia ponticum*. A methanolic extract of the roots of *A. ponticum* has yield two aristolochic acid derivatives, three aristoloctams and a 2,3,4,5-substituted furan Lignan. The known compounds aristolochic acid **I** and **II**, aristoloctams -**I** and **II** have been isolated but of the other compounds one is a novel, one had only recently been isolated from *Aristolochia* species and the third is a lignan nectandrin B, not previously isolated from

Aristolochiaceal. The novel compounds are 9-methoxy aristolochic acid **II** and 9 - methoxyaristoloctam I (Peter J. Houghton and Muzaffer Ogutvern, 1991).

Isolation of aristolochic acid, aristolochic acid D, aristolactam-β-D-glucoside and five new compounds from *Aristolochia indica*. Dried roots of *A. indica* were soxhlet extracted successively with petrol, benzene, chloroform and alcohol. The residues from $CHCl_3$ and alcohol extracts were further resolved into acidic and non-acidic parts by extraction with 5% $NaCO_3$ solution. The constituents of the different extracts were purified by repeated chromatography over silica gel columns to isolate aristolochic aicd **(I)**, aristolochia acid D **(XVI)**, aristololactam-β-D-gluycoside, **(XVIII)** (Satyesh Chadra Packrashi, *et al.*, 1977).

Isolation of Aristololactam from *Aristolochia argentina* 4 new aristololactams have been isolated from *A. argentina*. The evidence indicates them to be 10-amino-3-hydroxymethyl-2,4-dimethoxyphenanthrene-1-carboxyulic acid lactam; 10-amino-3-hdyroxymethyl-2,4,6-trimethoxyphenanthrene-1, 3-dicarboxylic acid lactam and 10-amino-2-hdyroxy-4,6-dimethoxyphenanthrene-1,3diocarboxylic acid lactam.

Ground rhizomes of *A. argentina* (24 kg, dry weight) were extracted with boiling petrol and ethanol. The ethanol extracts were evaporated to dryness (3.97 kg), suspended in a mixture of water and diethyl ether and under agitation the pH is adjusted to 9.7 with conc. NH_3. The diethylether phase was separated and the remaining aqueous phase as well as insoluble mateial were further extracted with diethyl ether. The diethylether extracts were combined, extracted water buffered to pH 1.5 and evaporated to dryness. The oily residue (432 g) containing the neutral compounds was submitted to repeated column on silica gel and Al_2O_3 (C_6H_6-$CHCl_3$-MeOH) to give several fractions.

Isolation of aristolochic acids and aristololactams from *Aristolochia auricularia*. A methanolic extract of the roots of *A. auricularia* was found to contain seven known compounds, aristolochic acids I, II, III and IV and aristololactam I, II and III. In edition, four novel compounds, the methyl ester of 3-hydroxy-4-methoxyaristolochic acid II, 9-methroxyaristolactam (**IV**) were isolated and identified (Peter J. Houghton, *et al*., 1984).

A. sipho has been includes among those plants prone to seasonal and age changes in the activity of chlorophylose enzyme present in the plants. The activity of chlorophyllase in conifers such as *Taxus baccata* and in the broad leaved plants, *A. sipho* increase in spring and in the first half of summer. Activity decreases during autumn, winter, and that this chlorophyllose activity parallels the yield of the constituents in the plant (Tsoneva, 1972).

A. manshuriensis contains aristolochic acid (II) while the presence of debilic acid ahs been assumed (Ruecker and Chung, 1975). The compound in A. bracteata exhibiting an insect chemosterilant property was confirmed to be aristolochic acid (II) (Saxena, *et al* 1979). Similar to *A. bracteata*, aristolochic acid (II) has been identified in *A. acuminata* and *A. multiflora* after extraction and purification by suitable methods (Moretti, *et al*., 1979).

The combined work of Nakanishi *et al*., (1982) revealed the presence of another derivative of aristolochic acid, aristoloside (**XXIV**), in *A. manshuriensis*. The previously known derivatives, aristolochic acid (II) and aristolochic acid D were equally isolated. The novel aristoloside (**XXIV**) was shown, from chemical and phytochemical data to be aristolochic acid D-6-*O*-β-glucopyranoside. *A. tuberosa* has been shown to yield two new oxoaporphine alkaloids that have not been isolated previously from any plant of the

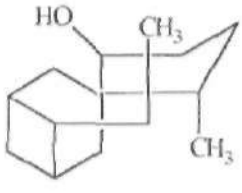
Aristolochiceae. These alkaloids are tuberosinone and tuberosinore-N-β-D-glucoside, and both are 4,5-dioxoaporphine (Zhu Dayuan, *et al.*, 1982).

Chemical studies on *A. mollissima* revealed the presence of aristolochic acid VII and aristolochic acid D- (**XVI**) in the ethanolic extract of the roots of the plant, along with two other unidentified crystal component (HoMingsan, *et al.*, 1982).

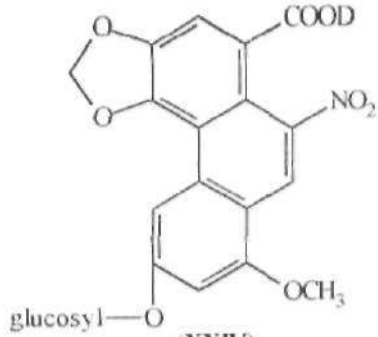
More recent work on *A. argentina* showed the presence of phenanthrene derivatives. Three new phenanathrene derivatives were isolated, and include aristolochic acid Ia (**XXV**), aristolochic acid - C methylester, and aristolochic acid Ia methyl ester.

A. indica had been examined further by Ahari *et al.*, (1983) and confirmed to contain a new phenanthroid lactone, aristololide (**XXVI**) along with steroids and lignans. Other compounds isolated include L-cubedin, h - linocinin and 5α-stigmastane-3,6-dione.

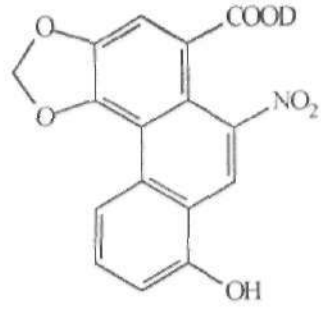
Studies of chemical component of *A. hieterophylla* have been reported. Five crystal components were isolated from the ethanolic extract of the plant. The melting point, colour reaction, R_f values on chromatograms and I.R. spectra revealed one of these components to be allantoin. Other spectroscopic data (UV, NMR, and Mass) were used to identify two other components following appropriate chromatographic development as aristolochic acid A and D. (XVI) (HO Mingsan, *et al.*, 1983).



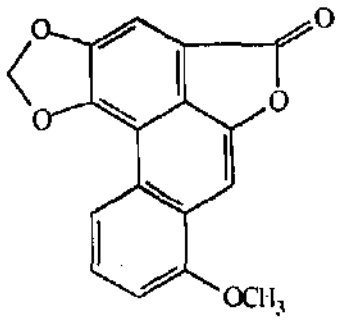
(XXIII)



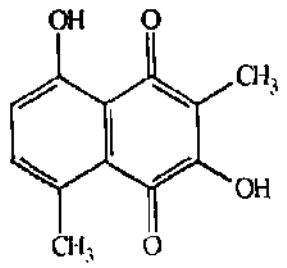
(XXIV)



(XXV)



(XXVI)



(XXVII)

Studies on *A. indica* had led to the isolation of a new naphthoquinone, called aristolindoquinone, **(XXVII)** [Chechen, *et al.*, 1983). Three new *bis*-benzylisoquinoline alkaloids were obtained from the defatted extract of *A gigantea.* These compounds include l-pampulhaimine, L: geraidoamine, and l-pedroamine. Their structures were determined by spectroscopic analysis and preparation of *O*- and *N*-methyl derivatives (Cortes, 1987).

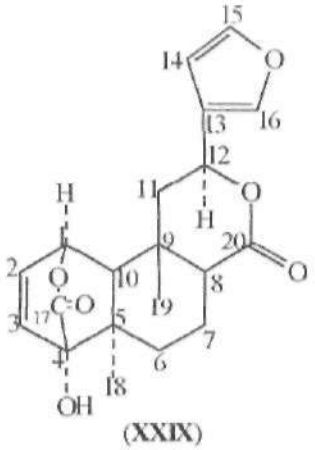
The investigation of *A. brasiliensis* and A. esperanzae afforded two clerodan diterpene derivatives. The three noval ones are rel (5S, SR, 9S, 10R)-2-oxo-ent-3-cleroden-15-oic acid, methyl ester of rel (5S, 5R, 8R, 9S, 10R)-2-oxo-ent-clerod 3,13-dien-15-oic acid and (25, 5R, 8R,9S, 10R)-2-hydroperoxy-ent-3-clerodan-15-oic acid methyl ester (Lopes, *et al.*, 1987).

A. chilensis gave five new 4 eryltetralonhe such as l-aristochilone, L-aristolignone L-aristosynone, L-23 aceto xyaristotetralone and L-2-hydroxyl aristotetralone as well as L-aristotetralone (Urzua and Shamma, 1988).

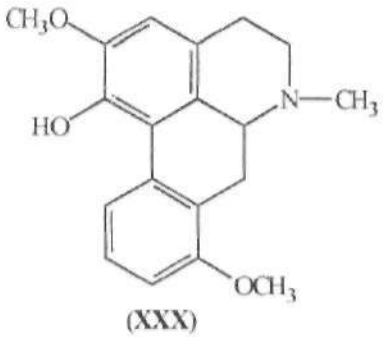
Phytochemical examination of *A. cymbifera, A. esperanzae* and *A. galeata*, led to the isolation of furofuran and dibenzylbutyrolactone type lignans as well as, clerodane and ent-labdane type diterpenes (Lopes and Bozani, 1988). Seasonal variation of 8-methoxyl-3,4-methlenedioxyl-10-nitrophenanthrene 1-carboxylic acid which is equivalent to a

aristolochic acid (II) in *A. bracteata* has been investigated. On feeding uranium labelled l - tyrosine to *A bracteata* in every month the results obtained demonstrate variation in biogeneration magnitude of aristolochia (II) in the plant due to seasonal effect (Sharma, 1988). This observation is similar to the result of the earlier work carried out by Tsoneva (1972) on *A. sipho.*

Chemical studies on *A. albida* in recent times showed the presence of sterol and D-glucose. The air-dried, powder of the rhizomes was deffated with petroleum ether (b.p 60-80 °C) and then extracted with methanol in soxhlet apparatus. The column chromatography (silica gel) of the methanolic extract furnish the diterpene which upon crystallization from chloroform / methanol gave fine colourless needles, m.p. 182 °C. It gave pink turning to violet coloration with Liebermann Burchard reagent (80 ml EtOH + 10 ml AC_2O + l0 ml Conc. H_2SO_4) for terpenoid. The furanoid diterpene lactone was characterised as columbin on the basis of spectral and elemental analysis (A.K. Haruna, *et al.*, 1974, 1996).



(XXIX)



(XXX)

Further studies on *Aristolochic albida* were carried out with the air-dried roots of the plant, collected in early summer (June) from northern Nigeria were defatted with petroleum ether (60 - 80 °C) and then extracted with 95% ethanol at room temperature. The petroleum ether extract was subjected to column chromatography over silica gel (ADH, 60-120 mesh) and on elution with petroleum ether: benzene (1:4) a fraction was separated which when crystallised from chloroform/methanol,. Afforded an amorphous material, m.p. 134° which showed a single spot on TLC (precoated silica gel on plastic, E. Merck, 0.25mm thickness) in different solvent systems. However, this fraction showed well separated three spots on TLC impregnated with 12% $AgNO_3$ (the 12.5% $AgNO_3$ plate was prepared by developing the precoated silica gel plate on plastic in a solution of 12.5% $AgNO_3$ in acetonitrile and drying the plate at room temperature) in a solvent system comprising of benzene: Chloroform (1:1) after developing the chromatogram three times.

All the three spots showed green colour, on TLC after spraying with Liebermann Burchard reagent and subsequently heating the plate at 100° indicating the presence of sterols. The R_f values of the spots (after three times development) were identical with standard β-sitosterol (R_f = 0.49), campesterol (R_f = 0.36) and stigmasterol (R_f = 0.15).

The GC - MS spectrum separated the mixture into three components having retention times (RT) to molecular ion peak (M^+) 25.352 (M^+ 400), 27.300 (M^+ 412) and 30.961 (M^+ 400) which corresponded to campesterol, stigmasterol and β-sitosterol respectively. This further supported the TLC results.

GC/MS	TLC impregnated with 12.5% $AgNO_3$,R_f solvent: PE: C_6H_6 (1:1)	Compound
RT 25.352/M$^+$ 400	0.36	Campesterol
RT 27.300/M$^+$ 412	0.15	Stigmasterol
RT 30.961/M$^+$ 414	0.49	β-sitosterol

The alcoholic extract of the root showed the presence of D(+)-Glucose (R_f = 0.29) (descending paper chromatography, What man No. 1, solvent system: n-butanol: benzone: pyridine. water = 5:1:3:3, aniline hydrogen phthlate spraying reagent, yellow spots were developed by heating the chromatogram at 100 °C in oven) indicating the presence of free sugar in the root: (Haruna *et al.*, 1994).

Isolation of Aristolochic acid I and Ia from *Aristolochia chilensis.* An ethanol extract of the defatted leaves and tender stems was processed to afford an acid fraction (0.025% dry wt) which yielded two crystalline solids identified as aristolochic acids I a and Ia. The identity of the former compound was established by its UV, IR and ^{1}H-NMR spectra, and by high resolution mass spectrometry, as well as by comparison with a reference sample (R_f, IR, mmp).

Aristolochic acid Ia had been isolated previously in minute amounts from the butterfly *Zerynthia polyxena*, an aristolochia feeder like *Bathus archidamas*, its structure was determined by its MS and by diozomethane methylation to aristolochic acid I methyl ester, and no other spectrometric data were published.

Although aristolochic acids have been isolated from the roots of more than twenty high concentrations, the aerial parts which serve as food for papilionid larvae have only been studied with positive results in *A. clematitis* (0.236% crude acids, dry wt basis), *A rotunda* (0.5%), *A acuminata* (0.22%), *A. multiflora* (0.015%), and now *A. chilensis* (0.025%).

Aristolochic acid I is a major component in every species examined but this appear s to be the first report on the presence of aristolochic acid Ia in a plant, in spite of the obvious biogenetic relationship between both compounds (Urzua *et al.*, 1982).

1.2.5 CHEMITRY OF THE MAJOR CONSTITUENT OF A PLANTS

1.2.5.1 Nitrophenanthrene derivatives

Aristolochic acid (**II**) and its derivatives may be the most important nitrophenanthrene compounds in *Aritolochia* plants. Aristolochic acid (**II**) is directly obtained in the plants from isothebaine (**XXX**). Aristolochic acid (**II**) has been characterized as 3,4-methylenedioxy-10-nitrophenanthrene-1-carboxylic acid (Horrisberger, 1971).

Aristolochic acid (**II**) is a yellow, sharp tasting material with a nitro group (Pailer, *et al.*, 1955), melting pt. 275° C (Rose Hmund, 1943). On treatment with dimethylsulphate, aristolochic acid (**II**) fixes two methyl radicals while fusion with potassium hydroxide left a residue which has general properties of anthraquineones (Castille, 1922).

Heating aristolochic acid (**II**) with hydrogen iodine yields methyliodide only in small amount confirming the presence of *O*-methyl group in the compound (Rosenmund

and Reichstein, 1943). Hydrogenation of the optically inactive methylester of aristolochic acid $C_{18}H_{13}O_7N$ yield a bright yellow compound melting at 312 °C. Acetylizable hydroxyl and reactive keto groups were not present while oxidation experiment did not give reliable result following chemical studies of aristolochic acid (**II**). (Rosenumund and Reichstein, 1943).

The derivatives of aristolochic acid (**II**) that have been studied include aristolochic acid - C (**XI**), aristolochic acid D, (**XVI**) and their respective methyl esters. Magnoflorine, (**XIV**) is a related compound but better regarded as a quaternary ammonium compound (Horrisberger, 1971).

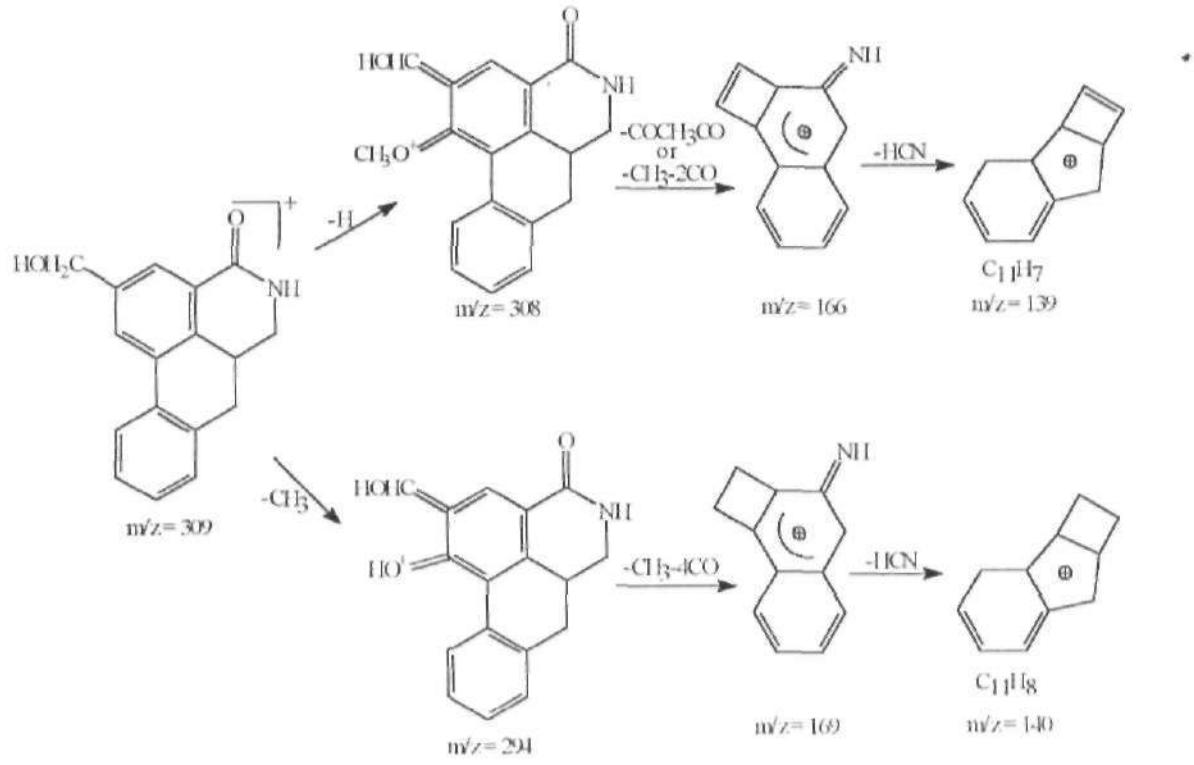
The UV spectrum of the more polar component of the acid fraction of the plant material of *A. chulensis* resembled that of aristolochic acid **I**, but exhibited strong bathochromic shifts upon addition of aqueous alkali, suggesting a phenolic aristolochic acid structure. The ^1H NMR spectrum of this substance in hexadeuteriodimethylsulphoxide showed a two proton singlet at 5.44 ppm corresponding to an aristolochia acid methylenedioxy group, and five signals due to aromatic ring protons which correlate well with those of aristolochic acid **I**. On adding deuterium oxide to the ^1H NMR sample solution of aristolochic acid **Ia**, OCH_2O, C-2, C-7 and C-9 proton resonance were shifted upfield with respect to the rest of the spectrum, presumably due to changes in the solution status of the neighbouring oxygen functions. Although this solvent effect is small, it is sufficient to make the C-9 H signal clearly distinguishable from the upfield component of the C-5 doublet, with which it overlaps.

The fragmentation of the molecular ions of aristolochic acids is characterised by the loss of NO_2, leading generally to the base peak in the MS, followed by successive

elimination of small units such as H, CH_3, the molecular ion peaks of the similarly substituted aristolactams can be observed, as the latter are produced as minor contaminants during the purification of the acids. Aristolochic acid **Ia** fits this scheme, with the molecular ion at m/z = 327, the base peak at M/z = 281 (M^{+}-NO_2), and the aristolactam parent ion at m/z = 279 (A. Urzua, *et al.*, 1982).

Aristoloctam C **II** (**XXXI**), $C_{18}H_{15}NO_4$ ([M-1]$^{+}$, m/z 308) and aristolactam C **III** (**XXXIII**) $C_{19}H_{17}NO_5$ ([M-1]$^{+}$; m/z 338) were found in the neutral fraction of the ethanol extract of the rhizomes accompanying the non-phenolic aristolactams. Examination of the ^{1}H NMR data of aristolactam **CII** and those of reference compounds revealed this compound to be a 2,3,4-trisubstituted aristololactam, two for the substituents being methoxy groups. The third substitutent is a CH_2OH group as indicated by two - proteon singlet at Ca δ5 in the ^{1}H NMR spectrum and a carbon signal at 55.1 (DMSO d_6) which splits to a triplet ($^1J_{CH}$ = 144 Hz) in the fully ^{1}H coupled carbon spectrum. According, aristololactam **II** affords a diacetate (**XXXII**), by treatment with acetic anhydridepyridine. Evidence for the relative location of the CH2OH group and the two mthoxy groups in aristololactam C **II** was mainly inferred from carbon shifts. Since the CH_2OH substituent is expected to slightly protect *ortho* - and *meta* - carbons, the usual 3,4-dioxygenated pattern of aristololactam should be ruled out for aristololactam **CII**. The shifts of the oxyaromatic carbons in this compound are better accommodated in terms of a *meta* relationship between the two oxygenated functions. Consistent with a 2,4-dimethoxy substitution pattern, C-4 resonates at δ155.0 (DMSO - d_6), i.e. ca 55 downfield as compared with C - 4 of 3,4 dioxygenated lactams. Further evidence comes from the methoxy carbon shifts. The OMe - 4 group of aristololactam C **II** resonates at 60.2 (DMSO d_6) i.e. close to the δ

59.4-60.0 range found for this group in several aristololactams. In contrast, the second methoxy group of aristololactam CII appear at δ 61.6; which is quite distinct from the ca $d_6$57 position expected for the OMe - 3 signal and is therefore assigned to C - 2. Thus, aristololactam C **II** can be formulated as 10-amino-3-hydroxymethyl-2,4-dimethoxyphenanthrene-1-carboxylic acid lactam (**XXXI**). The principal cleavages observed in the mass spectrum of aristololactam **CII** are depicted in scheme 1. The mass spectrum typical of aristololactam exhibits very strong ($M^{\cdot}$) peaks, usually the base peak and the principal ions are associated with loss of methyl and carbonyl derived from initial cleavages around the methoxy functions. However, like benzyl alcohol, aristololactam CII shows a preferential loss of a hydrogen atom to give the base peak at m/z 308 followed by elimination of a carboxy group. Expulsion of a hydrogen atom from the CH_2OH group is more pronounced in aristololactam CII than in benzyl alcohol, presumably because the resultant ion is stabilized by the two adjacent methoxy groups. Initial expulsion of methyl may represent an equally like process. The degradation path $M\text{-}2Me - 4CO - 4HCN]^{\cdot}$ can be formulated in which the fission of the CH_2OH group proceeds, like benzyl alcohol, with migration of hydrogen atoms on the aromatic ring system. However, loss of one or two hydrogen atoms also occurs since the fragment ions are usually accompanied by the corresponding -1 and 2 ones. In contrast to typical aristololactams, the $[M]^{\cdot}$ peak of aristololactam CII is not recognizable possibly because of preferred stabilization through loss of one hydrogen atom and also by more facile methyl elimination. Cleavage of one methoxy group with expulsion of methyl radical may be favoured by a hydride transfer from the adjacent CH_2OH group to the ether oxygen in a manner analogous to the behaviour of *O*-methoxybenzyl alcohol. (Urzua, *et al.*, 1982).

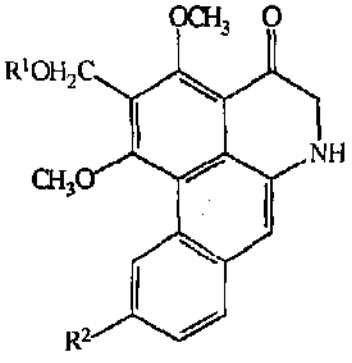


Scheme 1: Mass spectral fragmentation of aristololactam CII

Aristololactam C III (**XXXIII**) *was obtained as a minor component. The presence* of an extra methoxy group located at C 6 was clearly shown by ^{1}H chemical shift data and the partition pattern. Its mass spectrum exhibits the qualitative features expected from the breakdown pattern of aristololactam CII. An additional loss of methyl and carbonyl groups takes place in aristololactam CIII, ion (**XXXIII**) being observed as the main final hydrogen. Spectral data coupled with co-occurrence with aristololactam CII support structure (**XXXIII**) for aristololactam C III.

Two closely related fluorescent compounds were found in the fraction of non-phenolic aristolochic acids. Chemical and spectral evidence showed them to be phenolic carboxyaristololactams. The major compound, aristololactam D II (**XXXIV**) $C_{17}H_{11}NO_5$ ([M]$^+$ m/z 309) could be obtained as the free acid. Attempted methylation with etheral diazomethane was unsuccessful, but prolonged treatment with diethylsulphate potassium

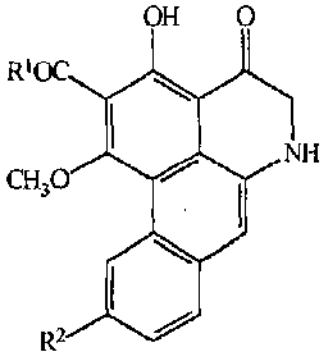
carbonate led to a tri-derivative (**XXXV**), $C_{23}H_{23}NO_5$ ([M]$^+$ m/z 393. ^{1}H NMR analysis showed that the carboxyl and hydroxyl groups and a methoxy group are located at positions 2 - 4 of aristololactam D II. The UV spectrum of (**XXXV**) is closely comparable with that of aristololactam CII. However, aristololactam DII shows a marked displacement of bands towards longer wavelengths. This bathochromic effect may be explained in terms of contribution of *ortho*-quinoid structures due to intermolecular chelation between the OH -2 and the lactam carboxyl, as already known in the case of benzochromone derivatives. The carboxyl group is assigned *ortho* to the hydroxyl because the mass spectrum of aristololactam DII shows a strong dehydration peak at m/z 291. The 2-hydroxy-3-carboxy-4-methoxy substitution pattern in aristololactam DII is further supported by its mass



(**XXXI**) $R^1 = R^2 = H$

(**XXXII**) $R^1 = Ac$; $R^2 = H$

(**XXXIII**) $R^1 = H$; $R^2 = OCH_3$

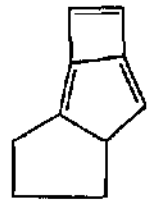


(**XXXIV**) $R^1 = R^2 = H$

(**XXXV**) $R^1 = Et$; $R^2 = H$

(**XXXVI**) $R^1 = H$; $R^2 = OCH_3$

(**XXXVII**) $R^1 = Et$; $R^2 = OCH_3$



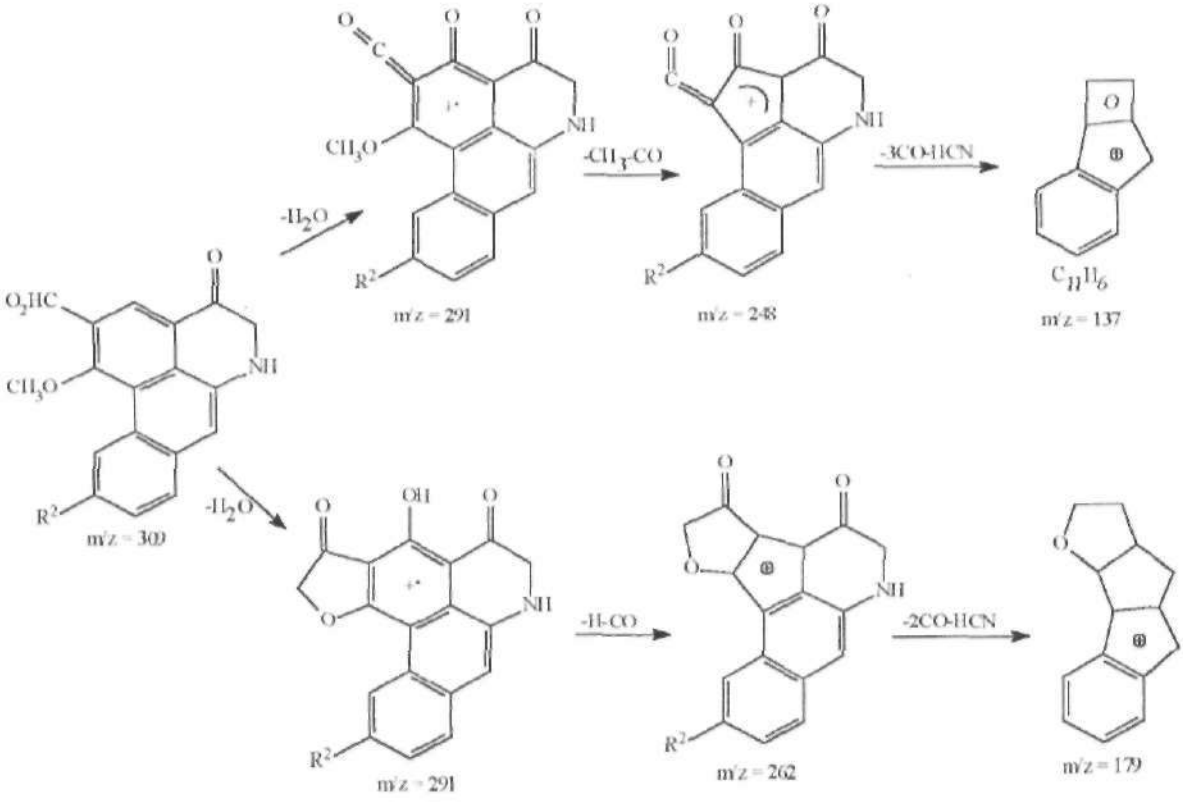
$C_{10}H_6$

m/z = 126

(**XXXVIII**)

spectral fragmentation and carbon shifts. Assignment of the oxyaromatic carbons in compounds (**XXXIV**) and (**XXXV**) was inferred by the similarity of the shifts with those of aristololactam C II, since the carboxyl and CO_2Et groups essentially do not affect resonances of *ortho*- and meter - carbons. The low field position of C-4 is interpreted, as in (**XXXI**) in terms of deshielding because of a *meta*-arrangement of the oxygenated

functions. The signal for the methoxy carbon of aristololactam D II consistently appears at δ 60.0 which is in accord with δ 59.4 - 60.0 found for OMe-4 resonances of known aristololactams. Structural information was also obtained from the fragmentation pattern of aristololactam D II (Scheme 2). This compound behaves like typical *O*-hydroxybenzoic



Scheme 2: Mass spectral fragmentation of aristololactam DII

acids in that it shows a preferential loss of water to give the most abundant fragment species at m/z 291. The primary cleavage of the methoxy group with loss of methol observed in aristololactams, is noted only to a very small extent in aristololactam D II. However, the fragmentation pattern below m/z 291 is very similar to that of the typical aristololactams, the main degradation routes being $[M-H_2O-Me-4COHCN]^+$ and $[M-H_2O-3CO-Me-CO-HCN]^+$ An alternative mode of breakdown in aristololactam DII occurs through dehydration between the carboxyla nd methoxy groups with cyclization to a new

ring, a process also observed in *O*-methoxy benzoic acid. The resulting fragment (m/z 291) then suffer the successive losses of hydrogen, three carboxyls and hydrogen cyanide, as indicated by fragment ions at m/z 290, 262, 234, 06, and 179, respectively. These findings are compatible with a carboxyl group *ortho* located to both hydroxyl and methoxy groups, and support structure (**XXXIV**) for this compound. The mass spectrum of (5) shows the $[M]^{+}$ at m/z 393 (base peak) and important fragments derived from initial cleavages around the ethoxy and CO_2Et groups.

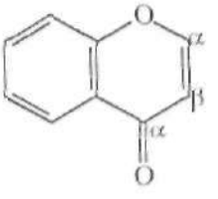
The most favoured cleavage involves loss of ethene from the ethoxy group. The resultant ion, like methyl salicylate, then loses ethanol and a carboxyl group. As indicated by abundant ions at m/z 319, 291 and 276, the main degradation path of (**XXXV**) proceeds thorough successive losses of ethene, ethanol, carboxyl, methyl and carboxyl. Important peaks at m/z 320 and 292 show that loss of an ethoxy group followed by a carboxyl or directly of the whole CO_2Et group from the $[M]^{+}$ or $[M\text{-}C_2H_4]^{+}$. Process already described for methyl salicylate also occur. An initial Machafferty elimination of ethene from the CO_2Et group can also take place giving an acid which, in turn, eliminates the elements of water, as in (**XXXIV**) peaks at m/z 347, 332 and 304 suggest the following decomposition sequences: $[M\text{-}C_{21}H_4\text{-}H_2O\text{-}C_2H_4\text{-}CO]$ and $[M\text{-}C_2H_4\text{-}H_2O\text{-}Me\text{-}CO]^{+}$.

The fragment ion at m/z 349 may be attributed to elimination of acetaldehyde or an equivalent fragment from the CO_2Et group of (**XXXV**), which then loses ethene and carboxyl. Initial loss of an ethyl radical from the ethoxy function originates alternative decomposition paths. $[M\text{-}Et\text{-}C_2H_4\text{-}H_2O\text{-}CO]^{+}$ and $[M\text{-}Et\text{-}C_2H_4\text{-}CH_2O]^{+}$ On the basis of the above evidence, aristololactam D II is formulated as 10-amino-2-hydroxy-4-methoxyphenanthrene-1,3-dicarboxylic acid lactam (**XXIV**).

Aristololactam D III (**XXXVI**) is present in very small amounts and could only be characterized as the N, O-diethyl ethyl ester (**XXXVII**), $C_{24}H_{25}NO_6$ $[M]^+$ m/z 423). Its fragmentation behaviour, which followsw that of (**XXXV**) and its NMR characteristics indicated that aristololactam DIII is the 6 methoxy analogue of aristololactam DII. (A. Urzua, *et al.*, 1982).

1.2.5.2 **Flavonoids and Flavonoid glycosides**

The flavonoids of Artistolochia plants occur mostly as glycosides and are represented by Rutin (**V**), quercitin-7-*O*-glucoside, and glycosides of kaempfore (**VI, VII**). They are phenolic compounds with the benzo-γ-pyrone nucleus. Most of them occur in cell sap and have been reported to be responsible for the yellow colour of plant flowers and fruits (Trease and Evans, 1989).

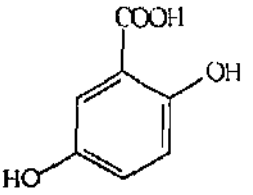


(XXXIX)

Flavonoids of aristololachia are most often *O*-glycosides, which are very soluble in water and alcohol but insoluble in organic solvents. The aglycones or genins are only sparingly soluble in water but readily soluble in ether. Flavonoids generally dissolve in alkali giving yellow solution, which disappears on acidification. The intensity of the yellow colour increases with the number of hydroxyl groups and with increase of pH to alkalinity (Treas and Evans, 1989).

1.2.5.3 Coumarins

Coumarin derivatives and certain aromatic acids have been identified in *Aristolochia* plants. Frequently encountered aromatic acids are *p*-coumaric acid (**VIII**) and gentisic acid. They may occur as ester compounds in the plants.



(XXXX)

Coumarins are chemically benzo-α-pyrone drivatives. Not all coumarins are phenolic but some occur as glycosides. They have been found earlier in sweet cover hay flower and appear to be the prominent constituents of many leguminosae including melilot plant and tanco bears. Ammoniacal solution of coumarins may give a blue, or blue - green or violet fluorescent (Trease and Evans, 1989).

1.2.5.4 Volatile Oils

Volatile oils are ethereal or essential oils present in plants and often evaporate even at warm temperature. They are frequently hydrocarbon compounds but oxygenated derivatives have been encountered. These hydrocarbons are called terpenes. Terpenes consist of isoprenoid unit, 2-methylbuta-1,3-diene. The terpenes of *Aristolochia* plants are the C15 compounds (sequiterpenes), such as ishwarana, (**XIX**) aristolochene (**XX**) and ishwarol (**XXXIII**)[b]. Recently the chemistry of deterpene molecules of Aristolochia plants has been studied. These diterpenes are the derivatives of clerodane (Lopes, *et al.*, 1978).

1.2.6.0 **Pharmacology of Aristolochia plants**.

The most ubiquitous constituent of the *Aristolochia* plant is aristolochic acid (**II**). Many experiments have been carried out on aristolochic acid (**II**) by researchers. These efforts were in response to finding an appreciable medicinal value of this compound. However, some of the animal experiments with aristolochic acid (**II**) demonstrated toxic effects.

Aristolochic acid (**II**) causes cardiac and respiratory paralysis in dogs and mice. It induced intestinal inflammation in dogs and haemorrhage nephritis in tested rabbits (Kansha Ryo, 1929). Aristolochic acid (**II**) was reported to inhibit tonic contraction of isolated uterus preparation (Lanello, 1953)

Two antibiotic substances A and B obtained from *Aristolochia* plants showed activity against *Bacillus authracis* and *Micrococcus pyogenes* (Goncalves *et al.*, 1952). Extract from the roots of *Aristolochia* plants was reported to strongly inhibit the cultures of *Staphylococcus aereus* (Mesa . Alicia, *et al.*, 1950). These antimicrobial properties of the plants may explain the suitability of the in treating sores and skin diseases by traditional medical practitioners.

Aristolochia acid (**II**) has been shown to posses a marked cell meitotic activity especially in plants. It has been suggested to that effect, as a substitute to colchicine in plant breeding and other cytological works (Deufes and Ganshirt, 1953).

Many species of aristolochia have been reported to be carcinogenic in animals but demonstrated potential tumor inhibitory property in man (Trease and Evans, 1983)

It has been shown that aristolochic acid (**II**) exhibit chemosterilant effect against certain insects including *Aedes algypti* and *Dysdercus koenigi*. Treated adults of these

insects with aristolochic acid (II) were proved to sterility and unable to lay eggs. This may explain the effectiveness of aristolochic acid (II) in causing mitotic death or behaving as a dorminant lethal mutant (Saxene, *et al.*, 1979).

It has been shown recently that a furanoid diterpene isolated from *Aristolochia albida* Duch (Aristolochiaceae), which was characterized as columbin exhibited anti-snake venom activities against two species of snake from Northern Nigeria, *Naja nigricollis* (spitting cobra) and *Bitis arietans* (puff - adder) (Haruna, *et al.*, 1995).

1.2.7 Snakes and effects of their bites

The subject of snakes and their venoms is of special interest to people of all tropical countries, as the number of deaths from snake bites reported from such countries is as high as 40,000 annually (WHO, 1963). Deaths from snake venom poisoning occur mostly in india, the countries of southern eastern Asia, Formosa Australia, Africa, West Indices and the tropical belt of North and South America, and the inhabitants of these regions have contributed much to the understanding of the cause of death and mode of action of the venoms. Snake bite is largely a problem of remote rural populace, where the major victims are often plantation workers and farmers, which often than not they resort to traditional herbalists instead of orthodox practitioner which are in accessible for their treatment and management.

Basically in the literature, there are five families of venomous snakes comprising of more than 250 species. These families of venomous snakes are discussed briefly below.

i) **Elapidae**

Asia and Africa, the most popular and famous snakes is in the family Elapidae. They include Asian *Naja naja naja* (cobras) , African *Noja nigricollis* (spitting cobra), this is a black necked cobra typical of the savannah. It has two way defence strategy. It can spit into the eye of victims or it can bite. *Dendroaspis polylepsis* (mambas) occurs mostly in the typical forest and lives on trees and tend to bite on the head area, Bungarus caeruleus and Dendroaspsis (common kraits). Elapids are generally longer snakes with short non - lunged front fangs.

ii) **Colubridae**

Colubridae is the largest of all snakes families but most species under this family are harmless with exception of two notorious species that are clearly known for their blood poisoning envenomatron the *Dispholidus typhus* (boomslang) and *Helotornis kirtlondu* (bird snake). These are indigenous to Africa. They are non hinged back fanged snakes

iii) **Crotolidae**

These are poisonous snakes of North and South America pit vipers (rattlesnakes). No true viper occurs in the Americas Africa has none of the rattler type.

iv) **Viperidae**

Among all the snake families, viperidae is highly distributed in Africa, Europe and middle East. They included the viperidae, the typical vipers and adders of the world. Vipers are relatively shorter and thicker snakes than others. They possess long hinged front fangs. The many example is the *Vipes ruselli, Echis cerinatus* ocellatus (carpet

viper), *Bitis arietans* (puff adder) and the crotalinae. In the viperine snakes (typified by adders) blood poisons appear to predominate in the venom.

v) **Hydrophidae**

These are distributed in indo-pacific West Coast of America. They are venomous and their bites are rare and limited to people engaged in fishing and marine expenditure. Their envemomation is reported to be most toxic and induces paralysis.

1.2.7.1 **Snake venoms**

Snake venoms are extremely complex chemical substances, being made up of several proteins that produce a variety of effects. Venoms include nerve and blood affecting and also digestive elements, the first usually affects the nerves, particularly those controlling locomotion and respiration, the second contains a number of agents that breakdown the lining of the blood vessels, destroy the red corpuscles and tissue cells, cause the blood to clot in the blood vessel or prevent cotting the digestive agents breakdown body tissue. The venom of any one snake contains a mixture of most or all these compounds in varying proportions and the effect this depend on which agent prodominates. Some venoms have complementary properties by possessing both procoagulant and anticoagulant actions. Some snake venoms contain compound that causes vasoconstrictions like sarofotoxins which mimies potent endogenous peptides while others cause local pains, swelling and necrosis.

Investigations into snake venom chemistry revealed enzymes non-enzymic substances and metalloproteins. Specific chemical compounds have also been isolated from the snake venoms, having both pre-and post synaphic activities. The post synaptic

toxic attach to the acetylcholine (Ach) receptor site, giving a myathenic like syndrome, while the presynaptic toxins exhibit phospholipase A activity (Tu, 1987). α-bungarotoxin is a post synaptic toxin that attach to the Ach receptor on the motor end plate. Acetylcholnesterases reverse the effects of α-bungarotoxins but not those of pre-synaphic poisons.

1.2.7.2 **Serum or Specific Therapy**

It is generally accepted that antivene serum is the only sound and reliable form of treatment for snake bite and its administration in adequate quantities should always be resorted to as soon as possible after bite. Antivenom potency stems in the ability to detoxify venom proteins thereby ensuring their elimination from the system.

Antivenoms are prepared by hyperimmunising horses or other animals like sheep as in the recent case of lalarrel (1993a) and Theakson (1994). The immunigens stimulate the production of hypermmune serum which is harvested and suitably precipitated and then digested using enzymes pepsin or paparin which is also recently used (Theakson, 1994). The final product separated by extensive chromatographic separations and purification are concentrated and standardised. The antivenoms can either be lyophillised on prepared in thermolabile liquid form to be stored with uninterrupted cold chain at temperature ranges of 2 – 8 °C with shelf life of about 3 years.

Anti snake bite (Antivenene) serums are produced in monovalent bivalent and polyvalent forms, but of these three types the cost named has the most popular appeal. Antisnake venoms are highly specific. Monovalent serums are prepared using venom of a single snakes, bivalent serum are prepared using venoms of two snakes while polyvalent

serums meet those frequent cases where the snake responsible for the bite has not been identified it includes all venomous snakes found in a given region.

1.2.7.3 **Summary of Snake Bite Treatment.**

(1) Ligature round the nearest single bone above or between the bite and the trunk.

(2) Incise and apply suction to the fang wounds, which should also be thoroughly disinfected.

(3) Serum must be injected as soon as possible near the bite to control swelling and sloughing particularly after adder bites.

(4) Inject serum subcutaneously or intramuscularly on other parts of the body, or intravenously and intraperitoneally.

(5) Administer blood transfusions if there are any signs of pulmonary collapse.

(6) Antihistamines for possible serum sensitivity

(7) Antibiotics and injections of tetanus and gas gangrene antitoxins.

(8) Glucose saline infusions to restore the viscosity of the blood.

(9) Close and unrelexed observation for symptoms of relapse and further injections of serum, if necessary.

1.2.8 Aims and Objectives of the project

Aristolochia albida Duch (Aristolochiaceae) is a perennial climbing shrub commonly found in Nigeria and very much used by the traditional herbalist for a variety of functions. The plant is used extensively to treat abdominal colic and is also claimed to be beneficial in the treatment and management of snake bites.

The present study attempts to carry out the phytochemical screening of this plant which would involve the isolation of the various constituents and their characterization especially of active principles. The study also hopes to ascertain the claimed biological actions of the plant against snake bites.

1.2.9 **Spectroscopic data of Major Constituents of *Aristolochia* Plants.**

a) **Aristololactam Ia**



$C_{16}H_9O_4$; M.w. 279

m.p. >350° (CH_3COOH)

UV (EtOH) 212 (4.38), 242 (4,54), 249 (4.51) 258 (4,55), 291 (4.18), 301 (4.15), 330 (4.00) 400 (3.98).

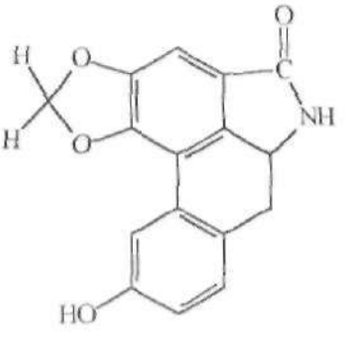
IR (KBr) 3175, 1642, 1276, 1053, 933, 806 740.

MS (eims): 279[M^+] (100), 278 (21.4) 251 (16.5) 250 (13.4) 223 (245), 222 (105), 221 (9.2) 195 (23.1) 193 (15.3), 168 (12.6), 167 (9.8) 166 (14.9), 165 (8.3), 164 (21.9), 140 (8.8) 139 (53.7), 138(14.4), 137(11.8), 111(10.7)

NMR. (DMSO-d_6)

Source: ARISTOLUCHIACEA *A. argentina*

b) **Aristololactam IIIa**



$C_{16}H_9O_4N$; M.w. 279

m.p. > 350° (CH_3COOH)

UV (EtOH): 212 (4.46), 236 (4.47), 255 (4.377), 266(4.45) 280(4.41), 296(4.26), 333(4.04), 3489(4.04) 398(3.98)

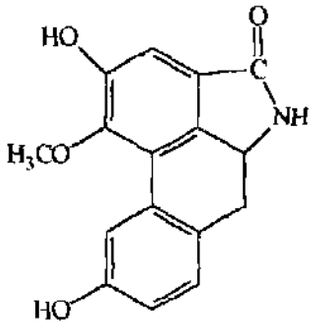
IR (KBr): 3311, 1653, 1`366, 1314, 1270, 1044, 870, 889.

M.S. (eims) 279[M^+] (100), 223 (13.6), 193(8.0), 168 (8.9) 166 (8.7), 164 (11.4), 149 (14.7), 139 (28.4)

NMR: (DMSO - d_6)

Source: **ARISTOLOCHIACEAE**: *A. agentina.*

c) **Aristololactam - AII**



$C_{16}H_9O_4N$; M.w. 265.2676

m.p. 271° (CH_3COOH)

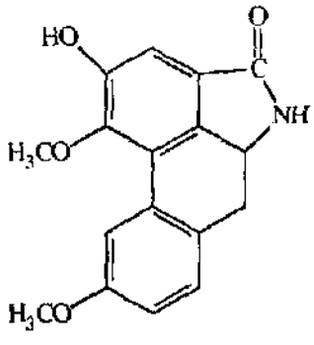
UV (EtOH): 209(4.39), 235 (4.57), 264(4.40), 277 (4.47) 287 (4.47), 317 (3.97), 394 (3.81), 384 (3.81)

IR (KBr): 3356, 3268, 1709, 1295

NMR: (DMSO-d_6)

Source: **ARISTOLOCHIACEAE**: *Aristolochia argentina indica.*

d) **Aristololactam AIII**



$C_{17}H_{13}O_4N$; M.w. 295.2940

m.p. 275° (CH_3COOH)

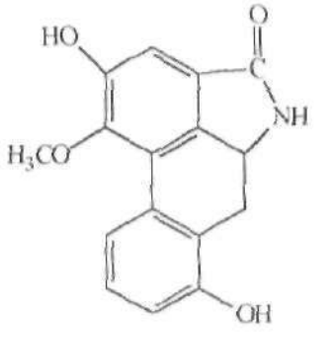
UV (EtOH): 216(4.34), 238(4.58), 253(4.42), 280(4.36), 292(4.36), 322 (3.96), 401 (3.84)

IR (KBr): 3378, 3226, 1684, 1164

NMR: (DMSO - d_6)

Source: **ARISTOLOCHIACEAE**: *Aristolochia argentina*

c) **Aristololactam A Ia**

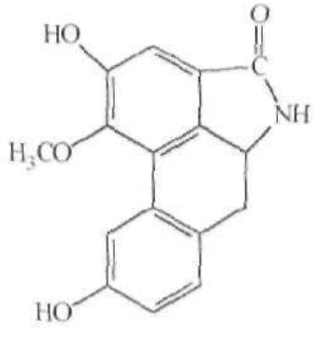


$C_{16}H_{11}O_4N$; M.w. 281

m.p. > 350° (CH_3COOH)

MS (eims) 281[M]$^+$ (100), 266 (58.1), 238 (33.6) 182 (22.2)

D) **Aristololactam - AIIIa**



$C_{16}H_{11}O_4N$; M.w. 281

m.p. > 350° (CH_3COOH)

UV (EtOH): 212 (4.39), 237 (4.53), 252 (4.38), 260 (4.37) 279 (4.27), 292 (4.28), 321(4.08), 402(3.87)

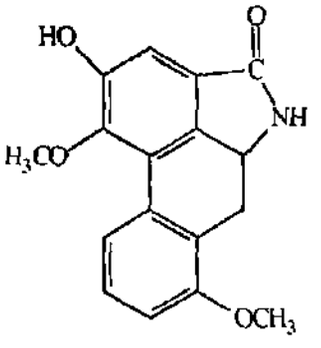
IR (KBr): 3106, 1689, 1292, 1209, 984

MS (eims) 291[M^+] (100), 267 (145), 266 (88.6), 238 (41.3), 182 (40.8) 155 (13.5), 153 (11.0), 126(12.4)

NMR: (DMSO - d_6)

Source: **ARISTOLOCHIACEAE**: *A. argentina*

g) **Aristololactam BI** (TALISCANINE)



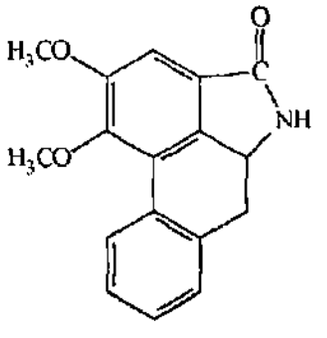
$C_{18}H_{15}O_4N$; M.w. 309

m.p. 272 - 273°

Source: **ARISTOLOCHIACEAE**: *Aristolochia taliсana*

h) **Aristololactam - B - II** (cepharanone - B)

10-amino -3,4-dimethoxyphenanthrene-1-carboxylic acid lactam



$C_{17}H_{13}O_3N$; M.w. 279.2946

m.p. 247 - 250° (n-BuOH)

264 – 265° (acetone)

UV (EtOH) 232 (4.43), 263 (4.43), 276 (4.48), 287 (4.48) 319 (4.00), 396 (3.96)

IR (KBr): 3226, 1724, 1383

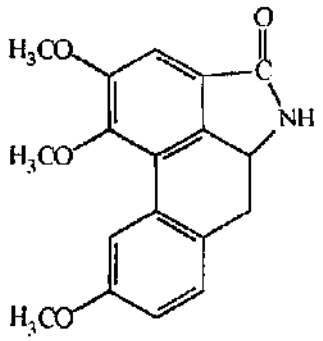
MS. (DMSO -d_6)

Sources: **ANNONACEAE**: *Schefferomitra subaequalis*

ARISTOLOCHIACEAE: *Aristolochia argentina*

MENINSPERMACEAE: *Stephania cepharanta*

i) **Aristololactan B III**



$C_{18}H_{15}O_4N$; M.w. 309.3208

m.p. 225" (n-PrOH-n-BuOH)

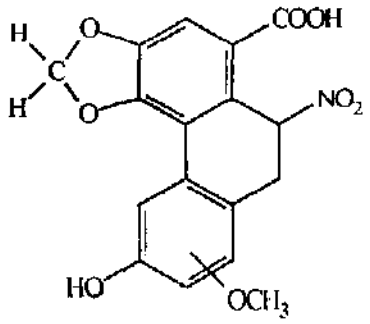
UV (EtOH): 236 (4.53), 255 (4.38), 262 (4.40), 281 (4.30) 293 (4.33), 313 (4.01), 321 (4.01), 399 (3.50).

IR (KBr): 3236, 1730, 1381, 11244

NMR: (DMSO -d_6)

Source: **ARISTOLOCHIACEAE**. *Aristolochia argentina.*

j) **Aristolochia Acid B**



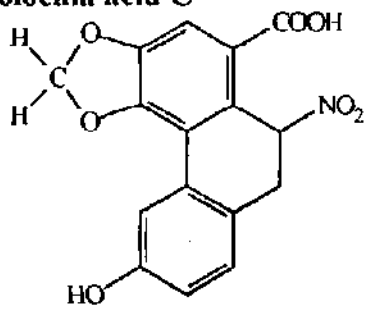
$C_{17}H_{12}O_8N$; M.w. 358..2837

m.p. 275 - 276° (acetone)

IR (M101): 3484, 1684, 1527, 1351`cm^{-1}

Source: **ARISTOLOCHIACEAE**: *Aristolochia debilis* and *frangchi.*

k) **Aristolochia acid C**



$C_{17}H_{12}O_8N$; M.w. 358..2837

m.p. 275 - 276° (acetone)

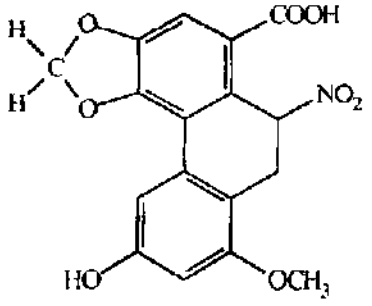
UV (EtOH): 221, 255, 283, 312, 389 nm

MS: 327, 293, 281, 279, 253, 241, 225, 195, 179, 167, 139.

NMR: (DMSO - d_6)

Sources: **ARISTOLOCHIACEAE**: *Aristolochia debilis, fangchi rotunda, watsonii*

l) **Aristolochia Acid D**



$C_{17}H_{12}O_8N$; M.w. 357.2758

(Me ester); 371.3026

m.p. 254 - 259° (DMF-H_2O)

254 – 259 (DMF-n-PrOH)

(Me ester) 243 – 250° dec.

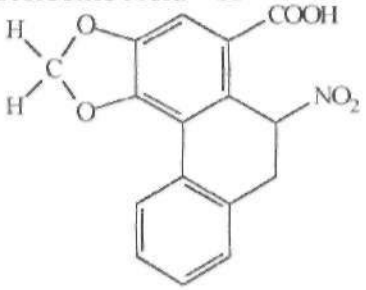
UV (EtOH): 220(4.47), 242(4.58), 252(4.58), 292(4.14) 325(4.05)

IR (Nujol): 3390, 1689, 1520, 1351, 1253, 1176, 1047, 1010, 935, 922.

NMR (DMSO -d_6).

Source: **ARISTOLOCHIACEAE**: *Aristolochia acuminata, argentina, climatitis, esparanzae, manshuriensis multiflora.*

m) **Aristolochic Acid - II**



$C_{16}H_9O_6N$; M.w. 311.2502

(Me ester); 325.2770

m.p. 269 - 271° dec.

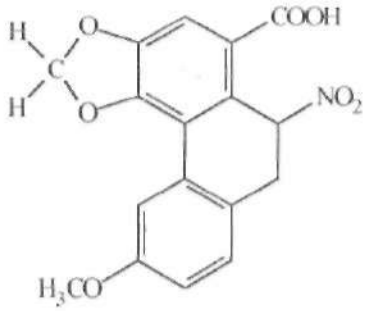
UV: 251 (4.51), 297 (4.15), 353 (3.65)

IR (Me ester) KBr 1718, 1515, 1548

MS (Me ester): 325, 294, 279 (100), 264, 250, 236 208, 150.

Sources: **ARISTOLOCHIACAEA**: *Aristolochia argentina clematitis, debiliks, esperanazae, longa, manshuriensis, rotunda, sipho, moupinensis.*

n) **Artistolochic Acid - III**



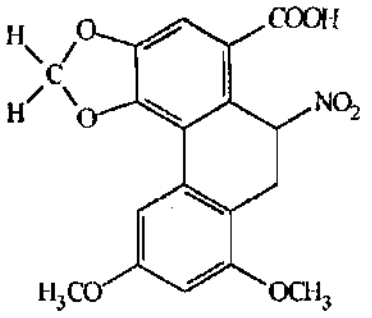
$C_{17}H_{11}O_7N$; M.w. 341.2764

(Me ester); 355.3032

m.p. (Me ester) 270 - 272° acetone.

Sources: **ARISTOLOCHIACEAE**: *Aristolochia argentina, clematitis, esparanzae.*

o) **Aristolochic acid - IV**



$C_{18}H_{13}O_8N$; M.w. 371.3026

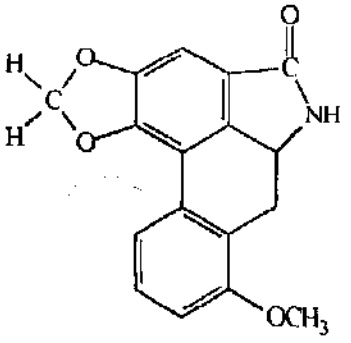
(Me ester); 385.3294

m.p. (Me ester) 238 - 240° (CH_2Cl_2-C_6H_6)

MS (Me ester) 385, 354, 310 (100), 268,

Sources: **ARISTOLOCHIACEAE**: *Aristolochia argentina, clematitis, esparazae, manshuriensis.*

p) **Aristololactan (ARISTOLACTAM, Aristolactam D)**



$C_{17}H_{11}O_4N$; M.w. 293.2782

m.p. 315 - 317° (sublimes at 300°)

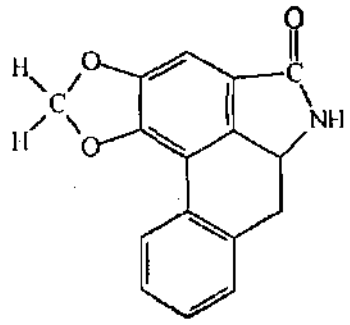
UV (EtOH): 241 (4.50), 250(4.47), 259 (4.56), 291 (416) 300 (4.15).

IR (Nujol): 3175, 1692

NMR: (DMSO - d_6)

Sources: **ARISTOLOCHIACEAE**; *Aristolochia argentina, debilis, fangchi, indica, rotunda.*

q) Aristolactam - II [CEPHARANONE - A]



$C_{16}H_9O_3N$; M.w. 263

m.p. 297- 298° (n-PrOH)

UV (EtOH) nm (log ε): 264 (4.38), 276 (4.46), 287 (4.43) 327 (3.87), 339 (3.86), 374(3.79), 391(3.79).

IR cm^{-1} (KBr): 3148, 1706, 1686, 1377, 1266 1166, 1044.

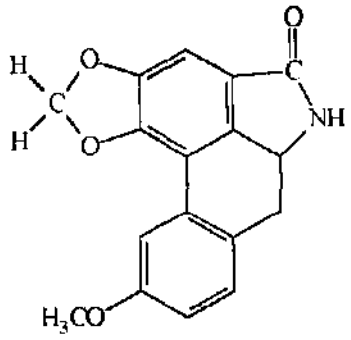
MS (eims): 263 [M^+] (100), 262 (II), 235(6), 207(8) 179(II), 178(8), 177(14), 152(12), 151(6), 150(17),131.5(9), 131(13).

NMR: (DMSO -d_6)

Sources: **ARISTOLOCHIACEAE**: *A argentina, indica*

MENISPERMACEAE: *Stephania cepharantha.*

r) Aristolactam III



$C_{17}H_{11}O_4N$; M.w. 293

m.p. 297- 304° (CH_3COOH)

UV (EtOH) nm (Logε) 237 (4.49), 254 (435), 267 (4.45) 281 (4.48) 294 (4.33), 334 (4.06), 348 (4.07) 396 (3.92)

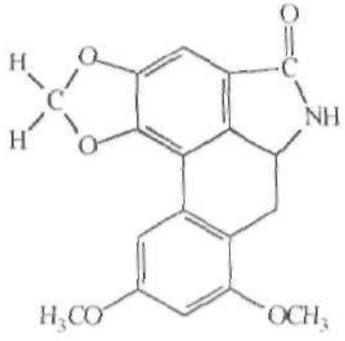
IR (KBr): 3195, 1702, 1376, 1047, 852

MS (eims) 293[M^+] (100), 278 (73), 350(22)

NMR (DMSO - d_6)

Source: **ARISTOLOCHIACEAE**; *A. argentina*

s) **Aristololactam, IV**



$C_{18}H_{13}O_5N$; M.w. 323

m.p. 345° (CH_3COOH)

UV (EtOH): 240 (4.45), 263 (4.37), 291 (4.06), 302 (4.04) 336 (3.84), 346 (3.85) 410 (3.80).

IR (KBr): 3226, 1689, 1626, 1377, 1366,. 1337 1284, 1264, 1047

M.S. (eims) 323 [M^+] (100), 308 (58), 293 (10), 280 (22), 278 (9), 265 (41), 250 (65) 237(14) 161.5(17).

NMR (DMSO-d_6)

Source: **ARISTOLOCHIACEAE**: *A. argentina.*

CHAPTER TWO

2.0 MATERIALS AND METHOD

2.1 Material

2.1.1 Chemicals and Reagents used

All chemicals were obtained from B.D.H. chemicals limited, Poole, England, and distilled before use. Silica gel (TLC and column) was supplied by E Merck Darmstadt, Germany.

2.1.2 Source of reference sample

The reference aristolochic acids was kindly given in the Department of Pharmaceutical and Medicinal Chemistry, A.B.U. Zaria.

2.1.3 Instrument Used

Melting point (m.p.) Gallenkamp Capillary Melting point Apparatus Ultraviolet light (254 and 366 nm) Ultraviolet products Insurance Infrared spectroscopy Perkin Elmer Infrared spectrophotometer (710B).

Mass spectroscopy Prospec Electron Ionizatrion (E.I) Nuclear Magnetic Resonance (NMR). Varian spectrometer (400 MHz).

2.2.0 METHODS

2.2.1 Extraction Procedures

a) **Collection and Drying of the plant materials**

The rhizomes of the plant *Aristolochia albida* from the family Aristolochiaceae

was collected from the matured plant at LIKORO VILLAGE along Zaria - Kano road in the month of August, 1998. The plant was authenticated at the herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria and a herbarium sample was deposited.

The plant material was dried in air under shade for many weeks, crushed and then pounded into powder.

2.3.0 PRELIMINARY PHYTOCHEMICAL SCREENING.

A small portion of the methanolic extract (ME) was used to test for the presence of the underlying constituents of the plant (20.0g).

2.3.1 Tests for Carbohydrates

a) General Test (Molisch's Test)

Molisch's reagent thymol = 3-hydroxy-4-isopropyltoluene.

3 - 4 drops of the Molisch's reagent was added to a clear solution of the extract dissolved in water followed by the addition of 1 ml conc. sulphuric acid down the side of the test tube (in slanting position), so that the acid formed a layer beneath the aqueous layer. The mixture was allowed to stand for about 2 minutes and then diluted with 5 ml of water. The formation of a red colour at the junction of the two layers was taken as the confirmatory test for carbohydrates (Trease and Evans, 1983).

b) **Test for sugars (Barfoed's Test)**

To a clear solution of the extract in water contained in a test tube, 1ml of

Barfoed's reagent was added and heated on water bath for 2 minutes. A red precipitate of copper (I) oxide, Cu_2O was taken as a positive test for the presence of sugar (Brain & Turner 1973).

c) **Test for reducing sugars (Fehling's Test)**

The clear solution of the extract dissolved in water was heated with 5ml equal volumes of Fehling's solution A and B. The formation of a red precipitate of copper (I) oxide Cu_2O indicated the presence of reducing sugar (Trease and Evans, 1983).

2.3.2 **Test for Flavonoids**

a) **Shinoda's Test**

A little of the extract was dissolved in ethanol warmed and filtered. Three pieces of magnesium turnings were added to the filtrate followed by the addition of a few drops of conc. HCl acid. A faint colour was an indication of the absence of flavonoids instead of a deep orange to red colour (Ventataramon, 1962).

b) **Lead Acetate Test**

A little of the extract was dissolved in 10 ml of water and filtered. To the filtrate 3 drops of lead acetate $Pb(CH_3COO)_2$ solution were added. There was a formation of clear solution instead of coloured precipitate (Brain & Turner, 1975).

c) To another 5ml of water extract 1ml of 20% NaOH was added. A clear solution was formed instead of yellow solution.

2.3.3 Test for Cardiac Glycosides

a) Test for steroidal Nucleus (Salkawoski Test)

A little of the extract was dissolved in 2 ml, of $CHCl_3$ followed by the addition of conc. H_2SO_4 to form a layer. A reddish brown colouration at the interphase indicated the presence of a steroidal nucleus i.e. aglycone portion of the glycoside (Sofowora, 1982).

2.3.4 Test for Terpenoids (Liebermann - Burchard Test)

A little of the extract was dissolved in ethanol. To it 1ml of acetic anhydride was added followed by the addition of conc. H_2SO_4. A colour change from pink to violet was an indication of the presence of terpenoids.

2.3.5 Test for Tannin (Polymers of flavonoids)

A little of the extract was dissolved in water and heated on a water bath. The mixture was filtered and $FeCl_3$ solution added to the filtrate. A dark green precipitate was formed and which was taken as evidence for the presence of Tannins (Trease and Evans. 1983).

2.3.6 Test for Saponins (steroidal glycoside)

A little of the extract was shaken with water in a test tube and frothing which persisted on warming indicated the presence of saponins (Sofowora, 1982).

2.3.7 Test for Alkaloids

A little of the extract was stirred with 5ml of 1% aqueous hydrochloric acid (HCl)

on a water bath and filtered 2 ml of the filtrate was divided into two portions of 1 ml each. The first 1 ml was treated with a few drops of Dragendorff's reagent to give a reddish brown solution instead of a orange red precipitate. To the second 1 ml, Mayer's reagent was added to give a yellowish solution instead of a buff coloured precipitate (Brain and Turner, 1975).

2.3.8. Test for Cyanogenic Glycosides (Brain and Turner, 1975)

About 0.5 g of the powdered rhizome drug was taken in a clean test tube and enough water was added just to cover the powder. A piece of moist sodium picrate paper was suspended above the extract with the aid of a cork. The tube was warmed at about 45 °C for about 1 hr (to cause the release of HCN gas).

The sodium picrate paper remained yellow (instead of turning to brick-red which is positive for the test).

2.3.9 (a) Borntrager's Test for Anthracene Derivatives (Free and combined Anthraquinones) [Trease and Evans, 1983].

About 0.5 g of the powdered rhizome was boiled with 4 ml of 10% HCl for 2 minutes over water bath. The hot mixture was filtered and cooled. The cooled filtrate was then extracted with equal volume of chloroform and the chloroform layer was shaken in a clean test tube with equal volume of 10% ammonia solution.

The aqueous layer (upper layer) was colourless (instead of pink colour, which indicates presence of Anthracene derivatives).

2.3.9 (b) **Free carboxylic Acid Compounds**

About 1.0 g of the coarse powder was socked in about 2 ml of methanol and 1 ml of 15% $NaHCO_3$ solution was added and the solution warmed on water bath. The evolution of colourless gas was noticed.

2.4.0 ANALYTICAL METHODS

2.4.1 Extraction

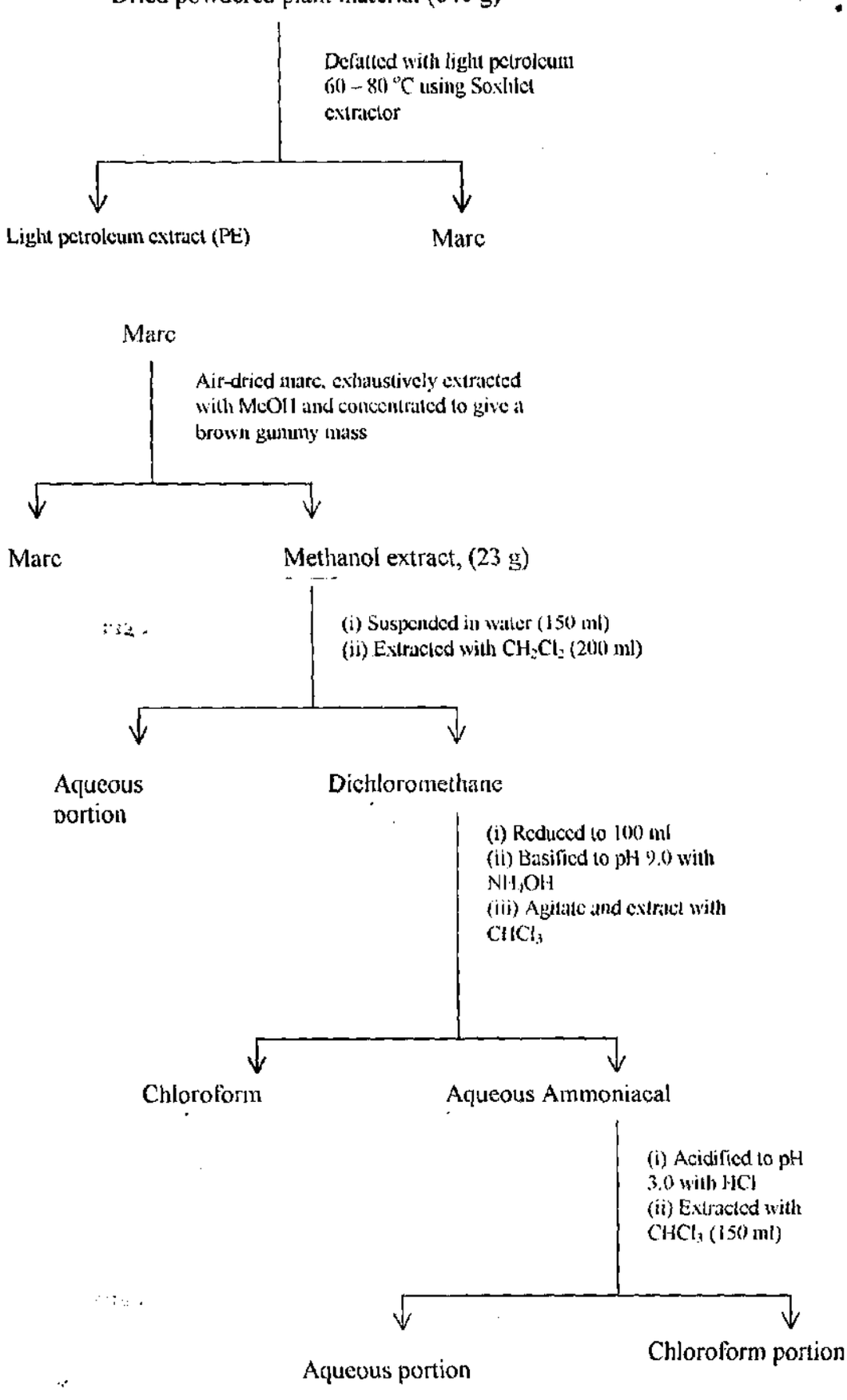
Dried and powdered rhizomes (840 g) of *Aristolochia albida* was defatted with light petroleum (60-80 °C) using soxhlet apparatus. The air-dried marc was extracted exhaustively with methanol. The methanolic extract was evaporated to dryness *in vacuo* to yield a brown gummy mass (23.0 g) which was suspended in 150 ml water and then extracted with (200 ml) dichloromethane. The aqueous portion was concentrated to dryness at reduced pressure (to await further investigations).

The combined dichloromethane portions was reduced to about 100ml and then suspended in dilute NH_4OH (pH about 9.0) and then extracted with chloroform (150 ml).

The aqueous ammoniacal layer was then acidified to pH about 3.0 with dilute HCl (under cold condition) and then extracted with (150 ml) chloroform. The combined chloroform portion was concentrated and then evaporated to dryness *in vacuo* to afford a yellowish reside (2.9 g) which was coded fraction A.

The fraction A was subjected to thin layer chromatographic examination, which reveals' several spots. The fraction A was then passed for column chromatography on silica gel. The above procedure is shown below in Fig 2a.

Fig 2a: Extraction scheme of Aristolochic acids



2.4.2 Column chromatography

The method used for elution was gradient elution technique using sintered glass column (25 x 1.5 cm) packed by slurry method. The adsorbent was silica gel (60-120 mesh).

2.0 g of fraction A was introduced to the top of a well - packed slurry of silica gel column with anti-bumping granules at the surface thereby separating the absorbent from the fraction A. Cotton wool was also placed on top of the extract to prevent interference of the surface on addition of the solvent. The column was then gradiently eluted with eluents (shown in table below) with increasing polarity Benzene (100%), Benzene acetone, acetone 100%, acetone: Methanol. The eluates were collected in 10 ml aliquots and subjected to thin layer chromatography to determine the number of components eluted. Similar aliquots were pooled together, concentrated under reduced pressure and left to crystallise at room temperature. Aliquots 85 - 104 yielded a mixture of yellow fluorescence crystals from Benzene: Acetone and coded 'CA'.

2.4.3 Column chromatographic separation of components of Fraction A: Eluants and Aliquots

Table 2b

Eluent No	Aliquots	Eluants	Ratio	Remarks No. of visible spot	Yield
1	1-6	benzene	100%	-	-
2.	7-12	BZ: acetone	9:1	-	-
3	13-22	"	8:2	1 under UV	trace
4.	23-33	"	7.3	1 under UV	minor
5	34-55	"	6:4	2 under UV	minor
6.	56-78	"	5:5	1 under UV	minor
7.	79.84	"	4:6	1 under UV	minor
8.	85-104	"	2:8	2 under UV	major
9.	205-209	"	1:9	-	-
10.	110-115	Acetone	100%	2 under UV	minor
11.	116-124	Acetone: MeOH	9:1	-	-
12.	125-130	Acetone: MeOH	8:2	-	-

2.4.4 Thin layer chromatography (TLC)

Ascending technique was used and the stationary phase was silica gel G. slurry, which was prepared by shaking silica gel (40 g) in distilled water (80ml) and spread onto the (20 x 10 cm) to a thickness of 0.25 mm. The prepared plates were activated at 110 °C for one hour in the oven.

The spots were applied manually using capillary tubes. The plates were developed at room temperatures in an appropriate solvent system (mobile phase details in the text) using a Shadon chromatank. The developed chromatograms were dried in the air and visualised as follows:

a) viewed under sunlight

b) viewed under ultraviolet light (254 and 366 nm)

2.4.5 Thin layer chromatographic Examination of 'CA'

The aliquots 85 - 104 were evaporated *in vacuo* to yield a mixture of yellow fluorescence solid (600 mg). Thin layer chromatographic examination of this fraction was carried out in different solvent systems revealing two spots in $CHCl_3$ MeOH (4:1) (a major and a minor fraction). The fraction was dissolved in 5 ml of a mixture of $CHCl_3$: MeOH and then applied in form of a band on 1.0mm preparative silica gel plates. The bands on each plate were co-chromatographed with reference aristolochic acid in the same solvent systems and their R_f values noted. The band on equal line with reference aristolochic acid sample was carefull scrapped in a beaker and then filtered with acetone through a funnel packed with cotton wool (to retain the silica gel). The residue was dissolved in methanol by heating on water bath. The filtrate was cooled and kept overnight in a refrigerator at about 4-5 ^{o}C which gave a yellow solid [150 mg]. But recrystallization on different time which afford a pale yellow micro needle-like crystals (30 mg). This was coded CA_1 The other band was scrapped from the silica gel and crystallised in methanol and was treated as a minor compound. This produced yellow amorphous flakes (100 mg). On repeated crystallization, it gave a single fluorescent spot (15 mg). This was coded CA_2.

2.6 CHARACTERIZATION OF CA_1 and CA_2

CA_1 and CA_2 were purified by recrystallization from chloroform., methanol and

methanol respectively and characterised through melting point, co-chromatography with reference sample (synthetic compound) spectral (UV, IR, NMR and MS) and C, H analysis.

(a) Purification and Determination of Melting point of CA_1 and CA_2

CA_1 and CA_2 were recrystallized from chloroform methanol and methanol, filtered and dried in an oven at 50 °C to remove solvent and moisture respectively . The pure crystals were ground into powder and filled into small capillary tube sealed at one end. The capillary tube were fitted into the Gallenkamp capillary melting point. Apparatus to determine the melting points (m.p) of the crystals. Their melting points with synthetic sample were also determined.

b) Solubility Test for CA_1 and CA_2.

The degree of solubility of CA_1 & CA_2 were determined in various solvents such as petroleum ether, benzene, chloroform, acetone, ethyl acetate, methanol and water.

2.6.1 Colour Test for CA_1 and CA_2

About 2 mg of CA_1 and CA_2 were dissolved respectively in 2 ml methanol and used for the following test.

a) Ferric Chloride Test

A few drops of $FeCl_3$ solution was added to the methanolic solutions of CA_1 and CA_2 respectively and the colour change noted.

b) **Sodium Hydroxide Test**

A few drops of aq. NaOH solution was added to the methanolic solutions of CA_1 and CA_2 respectively and the colour change observed.

c) **Shinoda's Test**

Two pieces of magnesium turning and a drop of conc. HCl were added to the solution of CA_1 and CA_2 respectively and the colour change noted.

d) **Sulphuric acid Test**

A few drops of conc. sulphuric acid were added to a little of the solid crystals of CA_1 and CA_2 respectively and the colour change noted.

2.6.2 Spectral Analysis of CA_1 and CA_2

The UV spectra were recorded in ethanol, the IR spectral in KBr. The ^{1}H-NMR and ^{13}C-NMR spectra were run in hexadeuteriodimethylsulphoxide and the mass spectrum was recorded using E.I. (electron ionization) method.

2.6.3 C, H - Analysis

About 0.02 g of CA_1 and CA_2 were completely burnt in a stream of oxygen in the presence of CuO respectively. The resulting water was absorbed into weighed anhydrous magnesium perchlorate and CO_2 into weighed soda lime. Carbon was determined from CO_2 and hydrogen from water (Dazeley, 1979).

2.7 DETECTION OF SUGARS FROM CA_2.

CA_2 was refluxed with 2 M HCl in aqueous methanol on water bath for 2 hrs and

filtered, the filtrate from the hydrolysate was neutralised with silver trioxocarboante (IV), Ag_2CO_3, and filtered. The filtrate was concentrated *in vacuo*. The concentrate (showed a positive test for sugar) was co-chromatographed with reference sugars on paper chromatography (Whatman No. 1). The sample gave a single spot corresponding to glucose.

CHAPTER THREE

3.0 PHYTOCHEMICAL RESULTS AND DISCUSSION

3.1 PRELIMINARY PHYTOCHEMICAL SCREENING RESULT

The dried and powdered rhizome of *Aristolochia albida* (DUCH) was subjected to standard phytochemical procedure which revealed the presence of various chemical constituents. The Table 3 below show the results of the tests performed.

The negative results produced with Borntrager's test and sodium picrate paper test indicate the absence of anthraquinones and cyanogenic glycosides respectively. Alkaloids were found to be absent which shows the absence of aristololactams. Flavones were also indicated to be absent.

The powdered rhizome was also indicated to be rich in terpenes, saponins, sterols and phenolic compounds in addition to free sugars.

The carboxylic acid test was found to be positive which indicates the presence of Aristolochic acids, which are implicated to be the major features of the genus Aristolochiaceae.

Table 3a: Results of the preliminary phytochemical screening

Constituents	Test	Observation	Inference
Carbohydrate			
i) General Test	Molisch's	Red ring	+
ii) Sugar	Barfoed's	Red ppt	+
iii) Reducing sugar	Fehling's	Red ppt	+
Flavonoids	(a) Shinoda's	Faint colour	-
	(b) head acetate	Clear solution	-
	(c) NaOH	Clear solution	-
Cardiac Glycoside		Reddish brown at	
(Steroidal Nuclens)	Salkowski's	interphase	+
Alkaloids	(a) Dragendorff's	Reddish brown solution	-
	(b) Mayor's	Yellowish solution	-
Tannins	$FeCl_3$'s	Dark-green ppt	+
Saponins	frothing	Frothing	+
Terperoid	Liebermann Burchard	Pink to violet	+
Free carboxylic acids	Addition of NaHCO3	Evolution of colourless gas	+
Anthraquinoues	Bontrager's	Colourness solutin	-
Cyanogenic glycosides	Sodium picrate paper test	yellow colour	-

Key: + shows the confirmation of the constituent tested

- shows the absence of the constituent tested.

3.2 DESCRIPTION OF THE FRACTION A

The fraction A on concentration to dryness *in vacuo* afforded a yellow residue 2.9 g It was found to be soluble in $CHCl_3$ and methanol, partially soluble in benzene and acetone.

The TLC ($CHCl_3$: MeOH, 4:1) Chromatogram of fraction A revealed five spots under the UV light (254 and 366 nm).

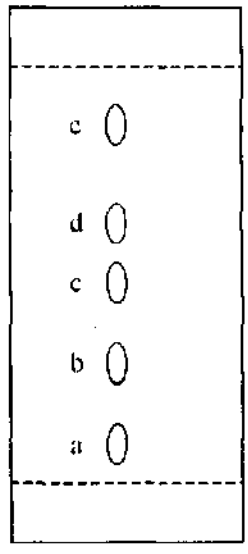


Fig. 3a: TLC Chromatogram of fraction A

3.3 CHARACTERIZATION OF CA_1 and CA_2

a) CA_1 and CA_2 were recrystallized from chloroform : methanol and methanol to give a pale yellow micro needle like crystals, melting point 280-283 °C and a pale yellow fluorescent amorphous flakes, melting point 191-194 °C respectively. The mixed melting point determination of CA_1 also gave the same range 280-283 °C but distinctly different from CA_2.

b) *Solubility of CA_1 and CA_2 in organic solvents*

CA_1 and CA_2 were found to be slightly soluble in petroleum ether, soluble in benzene, chloroform, acetone, methanol and insoluble in water.

c) *Thin layer chromatography of CA_1 and CA_2*

CA_1 was seen as a pale yellow non-fluorescent spot under UV light (2.54 nm) while CA_2 appeared as a single fluorescent spot under UV light (at 254nm and 366nm).

The same observation was made in respect of the reference aristolochic acid with CA_1 and CA_2 also gave the same R_f 0.49 when compared with reference sample in chloroform methanol (4:1)

3.3.1 **Result of Colour Tests**

CA_1 and CA_2 did not give any positive colour test but they appeared as a pale yellow non fluorescent spot and as a single fluorescent yellow spot on TLC plate when viewed under UV light (254 and 366 nm) respectively.

3.4.0 **RESULT OF SPECTRAL ANALYSIS.**

3.4.1 **UV Spectral of CA_1 and CA_2**

The UV spectrum of CA_1 was taken in (EtOH) λ_{max}: 228 (4.10) 261 (4.45), 315 (4.01), 410 (3.63) in nm. The UV λ_{max} absorption bands are typical absorption maxima for polycyclic aroamtic hydrocarbons.

The UV spectrum of CA_2 was also taken in Et(OH) λ_{max}: 266 (4.20), 248(4.58), 256(4.23), 399 (2.61). These UV λ_{max} are also typical absorption maxima for polycyclic aromatic hydrocarbons.

3.4.2 IR spectra of CA_1 and CA_2

The IR spectrum of CA_1 was taken in KBr υ_{max} cm^{-1} 1690, 1517, 1351, 1250, 1044 and 940 cm-1. The absorption at 1692cm^{-1} indicates C = 0 stretching, 940 is typical of methylene dioxy ring - OCH_2O while those of 1351, 1250 and 1044cm^{-1} were absorption bands of C - 0 in ethers, alcohols or esters. The strong absorption at 1517 cm^{-1} corresponds to aryl nitro function.

The IR spectrum of CA_2 was also taken in KBr υ_{max} cm^{-1}: 3380, 1694, 1517, 1355, 1010. The absorption at 3380 cm^{-1} indicates hydroxyl groups in aromatic ring. The other bands are the same with those of CA_1.

3.4.3 NMR Spectra of CA_1 and CA_2

Table 3b: ^{1}H-NMR and ^{13}C-NMR spectral Data of CA_1

^{1}H-NMR			^{13}C-NMR			
H	δ(ppm)	H-H COSY correlated with H	C	δ(ppm)	DEPT	HECTOR Correlated with H
1	10.13 (br s. 1H)	-	1	183.97	C	-
2	-	-	2	150.22	C	-
3	6.84 (s, 1H)	-	3	127.28	CH	3
4	-	-	4	140.71	C	-
5	-	-	5	138.66	C	-
6	-	-	6	119.92	C	-
7	-	-	7	112.67	C	-
8	8.23 (d, 1H, J = 8 Hz)	9	8	108.26	CH	8
9	7.80 (t, 1H, J = 8 Hz)	8, 10	9	102.34	CH	9
10	7.23 (d, 1H, J = 8 Hz)	9	10	111.39	CH	10
11	-	-	11	133.51	C	-
12	-	-	12	101.22	C	-
13	8.42 (s, 1H)	-	13	120.44	C	-
14	-	-	14	132.11	C	-
15	-	-	15	125.52	C	-
16	6.28 (br s, 2H)	-	16	128.12	CH_2	16α, β
17	3.98(s, 3H)	-	17	68.81	Me	17

Table 3c: ^{1}H-NMR and ^{13}C-NMR spectral Data of CA_2

^{1}H-NMR			^{13}C-NMR			
H	δ(ppm)	H-H COSY correlated with H	C	δ(ppm)	DEPT	HECTOR Correlated with H
1	10.21 (s 1H)	-	1	179.12	C	-
2	-	-	2	142.10	C	-
3	7.24 (s, 1H)	-	3	126.45	CH	3
4	-	-	4	144.16	C	-
5	-	-	5	126.52	C	-
6	-	-	6	118.66	C	-
7	-	-	7	114.22	C	-
8	8.44(d, 1H, J = 2.41 Hz)	10	8		CH	8
9		-	9	137.23	C	-
10	7.60 (d, 1H, J = 2.41 Hz)	8	10	124.36	CH	10
11	-	-	11	129.55	C	-
12	-	-	12	99.87	C	-
13	8.61 (s, 1H)	-	13	116.79	C	-
14	-	-	14	133.29	C	-
15	-	-	15	120.56	C	-
16	6.47 (s, 2H)	-	16	131.22	CH_2	16α, β
17	4.11 (s, 3H)	-	17	59.88	Me	17
1′	5.11 (d, J = 8 Hz)		1′	97.20		1′
2′	3.90 (dd, J = 8,9 Hz)		2′	74.12		2′
3′	4.16 (DD, J = 9, 9 Hz)		3′	79.40		3′
4′	4.01 (DD, J = 9, 9.5 Hz)		4′	70.99		4′
5′	3.79 (ddd, J = 2.5, 9.5 Hz)		5′	78.15		5′
6′α	4.45 (dd, J = 2, 11 Hz)		6′	62.64		6′
6β	4.29 (DD, J = 5, 11 Hz)					

3.4.4 Mass Spectra of CA_1 and CA_2

The mass spectrum of CA_1 showed the following peaks Mass (E.I), m/z %): $C_{17}H_{11}O_7N$, 341 (M^+,352), 295 (M^+-OCH_2O or NO_2 100), 294 (M^+-N02-H), 279(M^+-NO_2-H-Me), 278 (M^+-OCH_2O-OH), 251(M^+-H-Me-CO), 248(M^+-OCH_2O-OH-CH_2O).

The mass spectrum of CA_2 showed these peaks in addition to the above showed by CA_1 mass (E.I m/z, 100%): $C_{23}H_{21}O_{13}N$, 519 (M^+) 489(M^+-CH_2O) and 357 [M^+-glucosyl]

3.5.0 C. H ANALYSIS

$C_{17}H_{11}O_7N$: Required C = 59.82%; H = 3.23%

Found C = 59.51%; H = 3.24%

$C_{23}H_{21}O_{13}N$: Required C = 43.18%; H = 4.05%

Found C = 53.17%; H = 4.06%.

3.60. DISCUSSION

The TLC chromatogram of fraction A extract of *A. albida* showed 5 spots when viewed under the UV light. The TLC chromatogram of fraction A revealed under the UV light, two prominent or major spots, C (R_f 0.49; $CHCl_3$, MeOH: 4.1) appeared as a yellow solid while two spots did not appear in iodine and LBR. Spot d gave a positive test for glucose.

The preliminary phytochemical screening showed the presence of carbohydrate, cardiac glycoside (steroidal nucleus), Tannins, saponins Terpenoids free carboxylic acids. The tannins, saponins, steroids and the cardiac glycoside are classified as terpenoids and this plant by the result of phytochemical screening is richly endowed with terpenoids

considering the strong positive result of LBR tests (Robinson, 1967, Harbone, 1973). The result further showed the presence of free carboxylic acid indicating presence of acids in the family Aristolochiaceae.

The literatures also confirmed the strong presence of carboxylic acids in the genus Aristolochia. Carboxylic acids are one of the major constituents earlier reported from species of the genus e.g. *A. clematis, A. rotunda, A. acuminata, A. multifora* and *A .chilensis* (A.urzua, G. Salgado, *et al.*, 1982).

(a) **UV spectra of CA_1 and CA_2.**

The UV (EtOH) λ_{max} of CA_1 absorption at 228 (4.10) 261 (4.50) 315 (4.01), 410 (3.63) in nm are typical absorption maxima for polycyclic aromatic hydrocarbons. There was no shift when dilute NaOH and fused NaOAc were added to the solvent. This confirmed the absence of free hydroxyl group in the ring.

The UV (EtOH) υ_{max} of CA_2 absorption at 226 (4.20) 248 (4.58) 256(4.23), 399 (2.61) nm. These absorptions are typical of polycylic aromatic hydrocarbons. There was shift when dilute NaOH and fused NaOAC were added to the solvent, which confirmed the presence of free hydroxyl group attached to the ring.

(b) **IR spectra of CA_1 and CA_2**

The absorption at 1692 cm^{-1} indicated the presence of carboxyl stretching, 940 cm^{-1} is typical of compounds containing methylenedioxy ring - OCH_2O while those of 1351, 1250 and 1044 cm^{-1} were absorption bands of C = O in ethers in R-O-R′ The strong absorption at 1517 cm^{-1} corresponds to aryl nitro function.

In addition to the above bands, CA_2 has absorption at 3380 cm^{-1} which indicates hdyroxyl group in the aromatic ring.

c) NMR - spectra of CA_1 and CA_2.

^{1}H-NMR signal at δ - 6.28 (br s, 2H) confirmed the presence of methylene dioxy ring absorption at 940cm-1. These signals were characterised as methylenedioxy ring portions. The single proton signal at δ = 10.13 ppm confirmed the IR absorption for the carboxylic group. The relative upfield resonance observed by this proton may be plausibly be due to the methylenedioxy ring slightly opposing and distorting the electrometric effect on the -C=O function of the COOH making this proton to appear at slightly lower frequency than predicted.

The methoxy group attach to C - 11 ensures the assignment of ^{1}H-NMR signal at δ 7.80 (t, J = 8 Hz) to the proton at C-9 coupling with the proton at C - 10 δ = 7.23 (d, 1H; J = 8 Hz) and another proton influence of negative inductive effect by the electronegative atoms of the methylene dioxy ring. All the protons were successfully assigned from the ^{1}H-^{1}H COSY ^{13}C-NMR spectrum HECTOR also assisted in the assignment.

The ^{1}H-NMR data of CA_2 shows in (Table 3c) when compared with compound shown in (Table 3b) showed the disappearance of H - 9 (C-9 quartenary), also supported by the HECTOR analysis. This suggests that the glycosidic linkage was at C - 9. Although C - 1 was also available for such linkage through -OH group, but NMR proton signal at δ 10.21 (br s, 1H) (Carboxylic proton) in the ^{1}H-NMR spectrum of compound C eliminated that possibility. All the aromeric protons were also assigned.

d) **Mass spectrum of CA_1 and CA_2**

The mass spectrum showed the molecular ion peak (M^+) of compound B at m/z 341 (35.2%) corresponding to the formula $C_{17}H_{11}O_7N$. The base peak indicated the presence of nitro group or methylene dioxy group attached to polyclic aromatic hydrocarbons at m/z 295 [M^+ - OCH_2O or NO_2, (46), 100].

A more prominent peak at 279 (M+ - NO2 - H-Me, (62)) was due to loss of nitro function, hydrogen atom and a methyl group in that order from the molecular ion. The peak at m/z 278 [M^+-OCH_2O-OH] was due to loss of methylenedioxy and hydroxyl groups respectively from the molecular ion. The peaks at m/z 251 [M^+-NO_2-H-Me-CO, (90)] accounted for the successive loss of NO_2, H,. Me and C=O groups. The signal at m/z 248 [M^+-OCH_2O-OH-HCOH, (83)] was due to loss of -OCH_2O, OH and formaldehyde from a compound containing methylenedioxy ring.,

In addition to the above MS data, CA_2 showed a mass spectrum having molecular ion peak [M^+] at m/z 519 corresponding to the formula $C_{23}H_{21}O_{13}N$. The major discernible peaks are at m/z 489 [M^+ - OCH_2, (30)] and m/z 357 [M^+-glucosyl, (162)]

3.7 CONCLUSION

The IR spectrum at 940 cm^{-1} indicated the presence of methylenedioxy ring attached to a polycyclic aromatic hydrocarbons has shown by the UV spectrum 228 (4.10), 261 (4.50), 315 (4.01), 410 (3.63) in nm, this was confirmed by ^{1}H-NMR signal at δ = 6.28 (br s, 2H) and ^{13}C NMR spectra. This was further supported by mass spectrum having a base peak at m/z 295 corresponding to [M^+-OCH_2O, (46)]. The presence of ether group in the molecule was supported by IR spectrum at 1351, 1250 and 1044 cm^{-1}. The

IR spectrum further indicates the presence of an aryl nitro function at 1517 cm^{-1} and a strong carbonyl C=O stretching at 1692 cm^{-1} which was confirmed by ^{1}H-NMR signal at δ - 10.13 ppm.

The methoxy group attached to C-11 of the polycyclic aromatic hydrocarbon provided the basis for the assignment of ^{1}H-NMR signal at δ - 7.80 (t, 1H, J = 8 Hz) (aromatic) to a proton at C - 9 coupling with proton at C - 10 δ = 7.23 (d, 1H, J = 8 Hz) and another proton at C-8 δ = 8.23 (d, 1H; J = 8 Hz). The compound CA_1 isolated from *Aristolochia albida* was found to be identical with the reference sample of aristolochic acid I in all respect (same R_f values, co TLC, mixed m.p, superimposible IR) which gave an unambiguous proof for the structure of compound CA_1 shown in Fig 3p.

So also, compound CA_2 to some extent resembles compound CA_1 expect for the attachment of glucosyl residue at C - 9 to the polycyclic aromatic nucleus having UV spectrum absorption at 226 (4.20), 248 (4.58), 256 (4.23), 399 (2.61) in nm. The mass spectrum of compound CA_2 confirmed the presence of glucosyl unit at m/z 357, [M$^+$-glucosyl, (162)]. This compound isolated from Aristolochia albida weas shown to be different from the reference aristolochic acid in all respect and thus characterised as aristoloside, shown in Fig 3p.

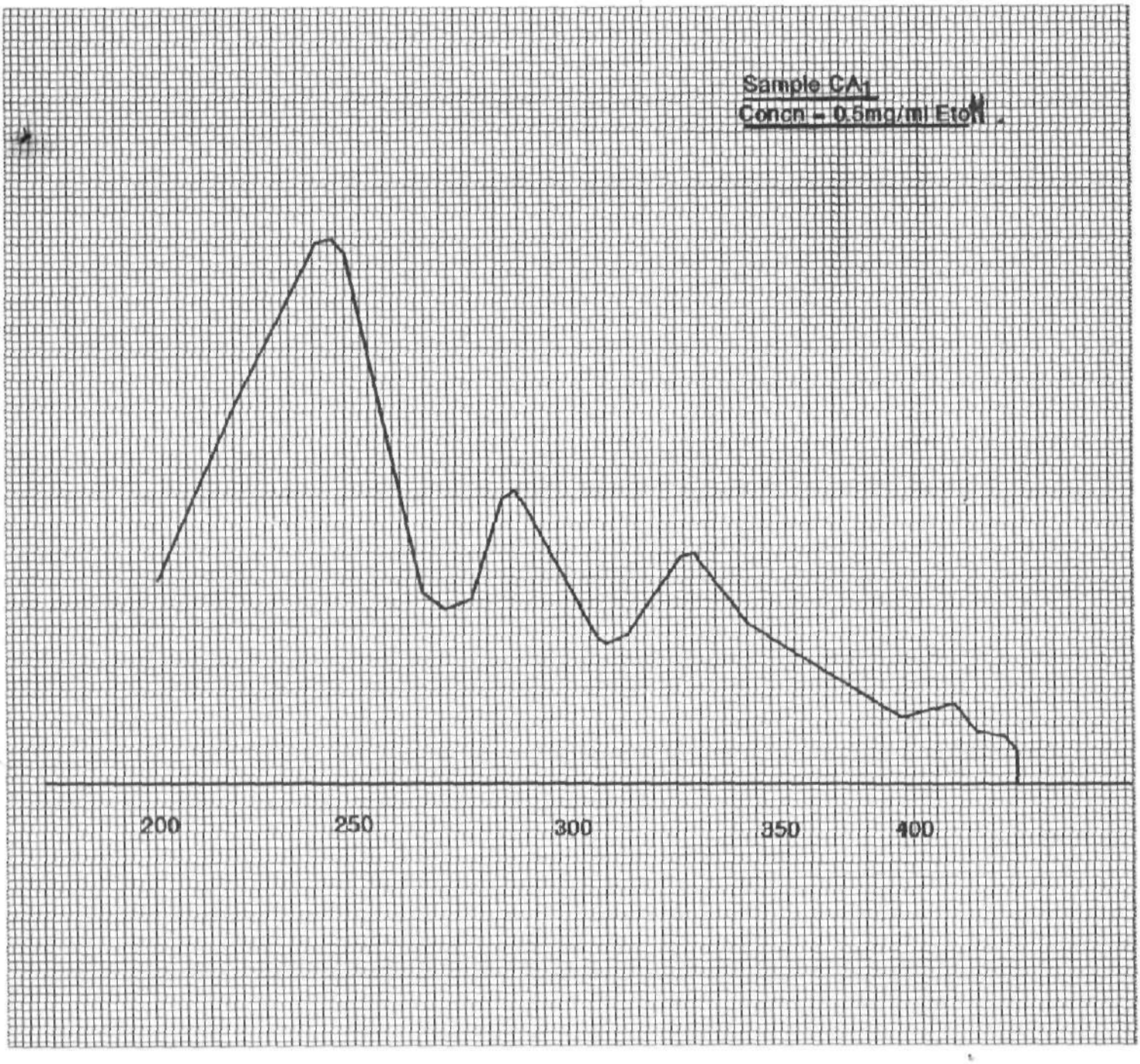


Figure 3b UV Spectrum of CA_1.

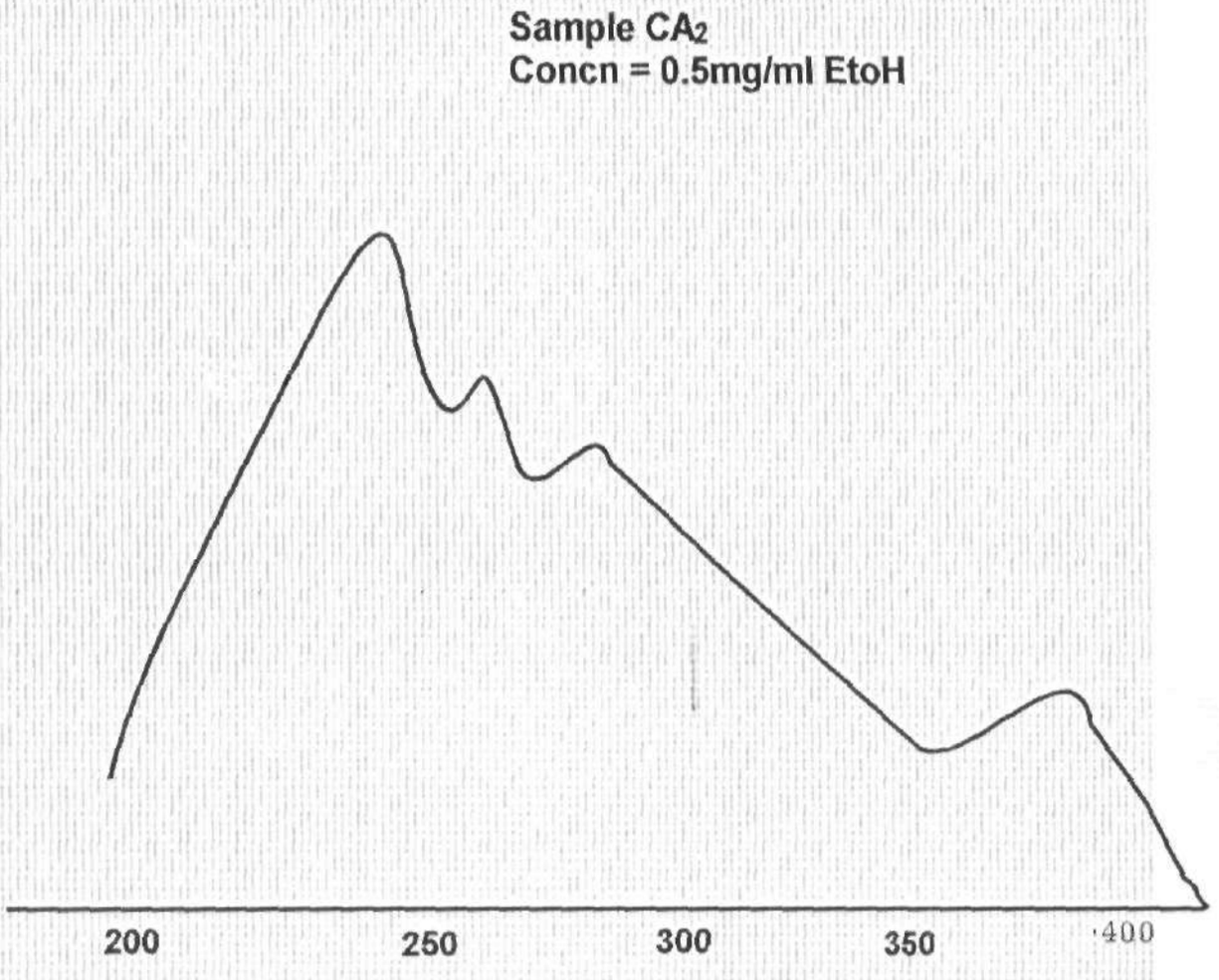


Fig 3c uV Spectrum of C_2A_2

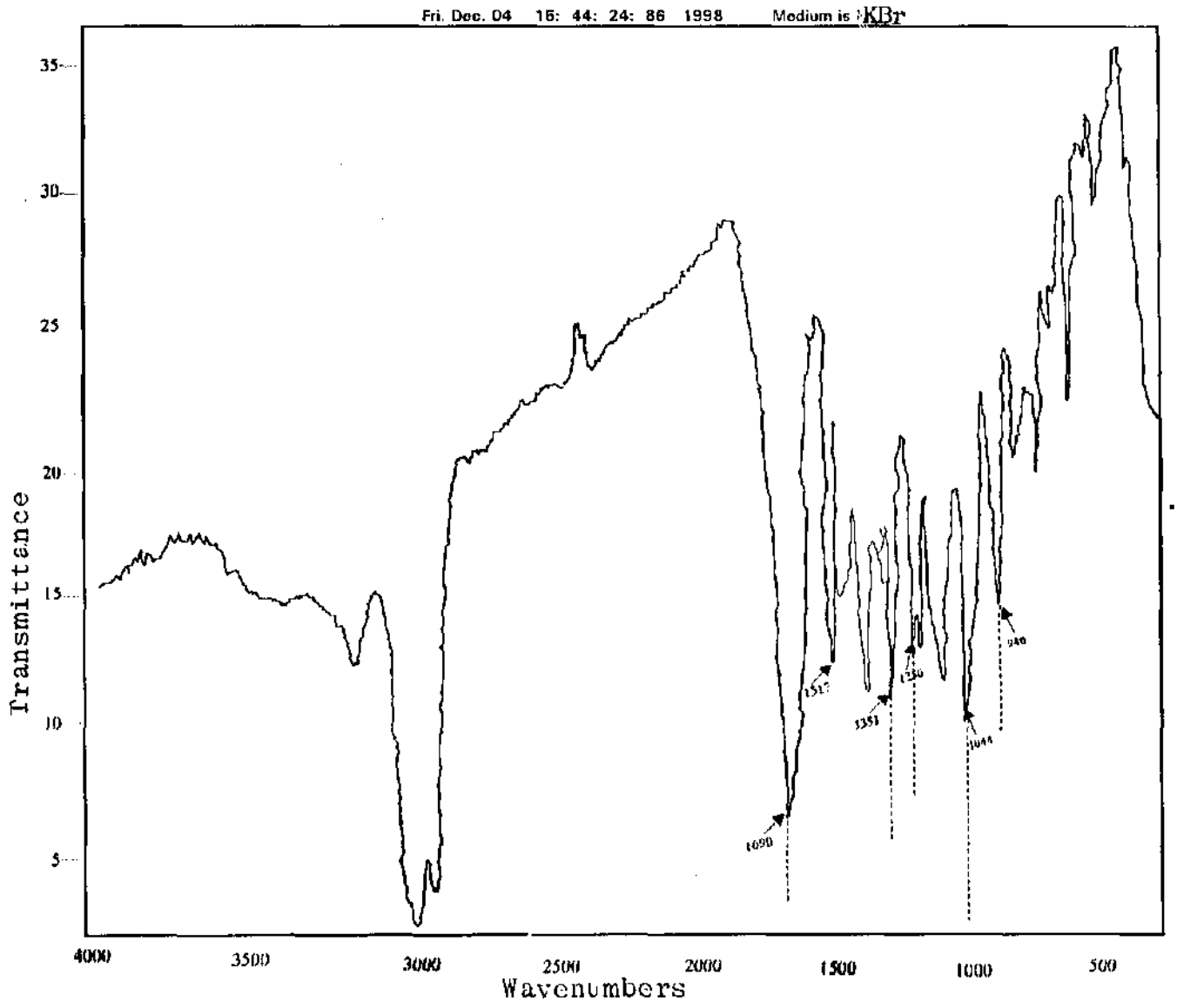


Figure 3d IR Spectrum CA_1

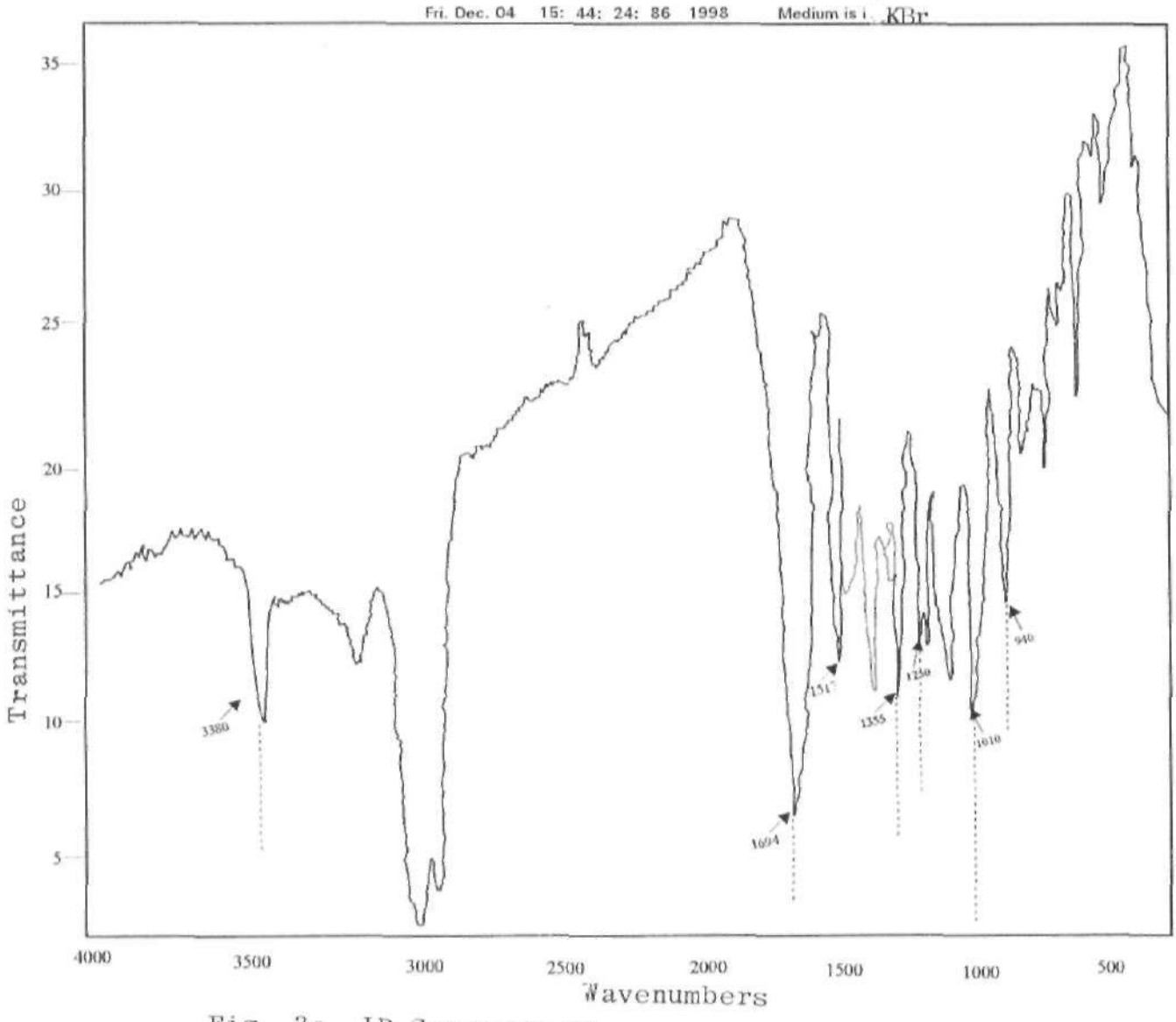
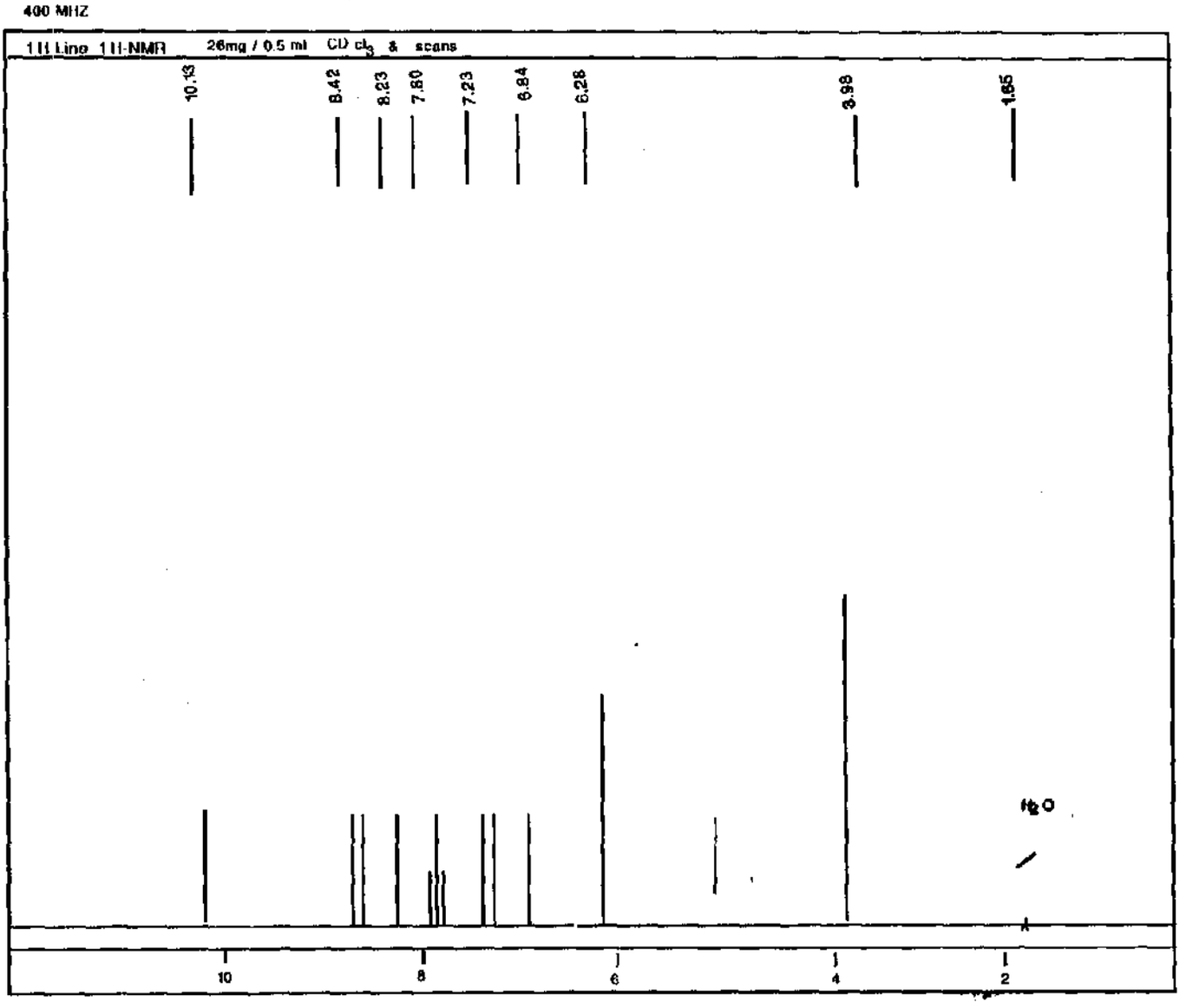


Fig. 3e IR Spectrum CA_2



F Fig. 3g ^{1}HNMR Spectrum CA_{1}

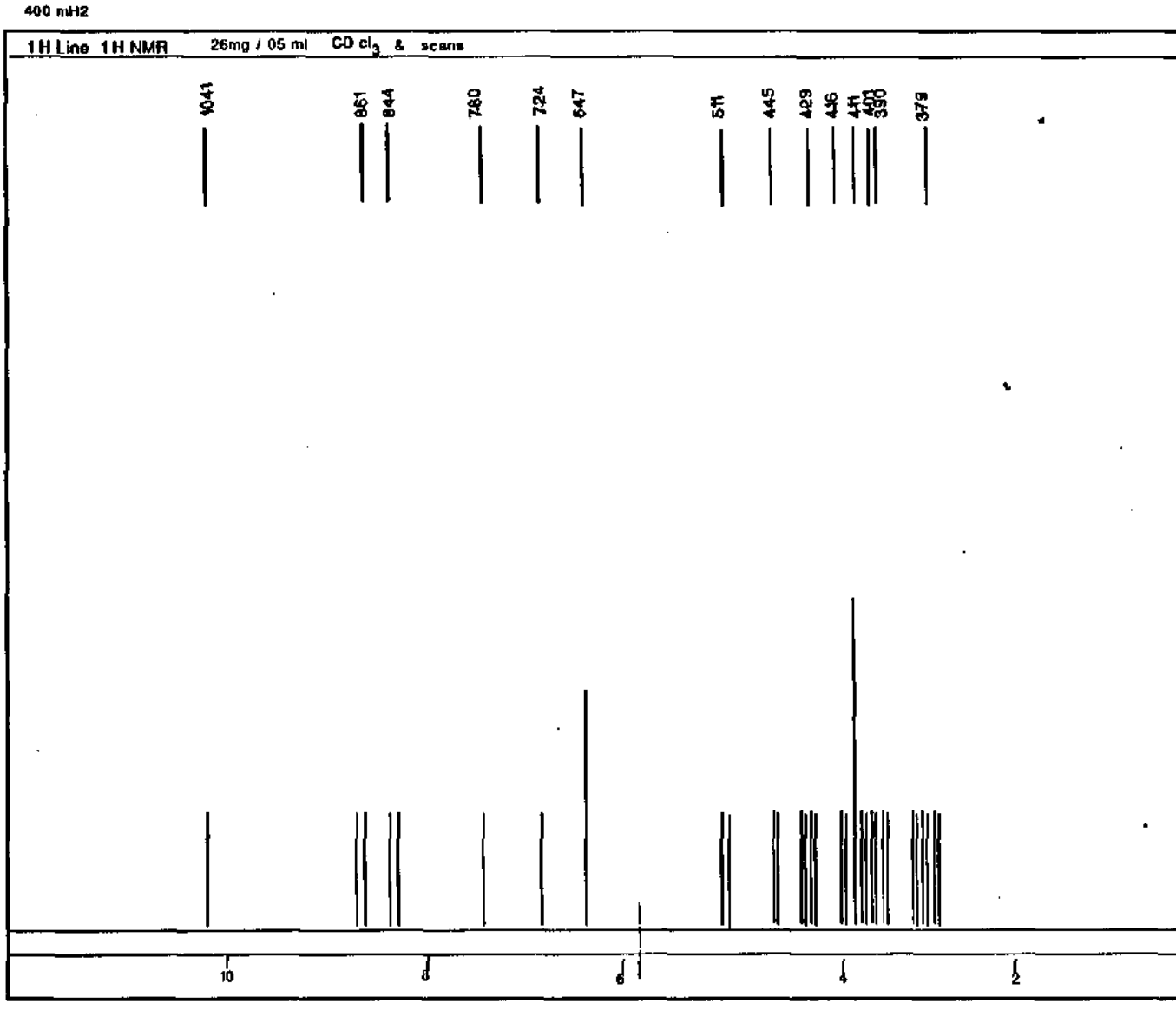


Fig. 3f ^{1}H-NMR Spectrum of CA_2

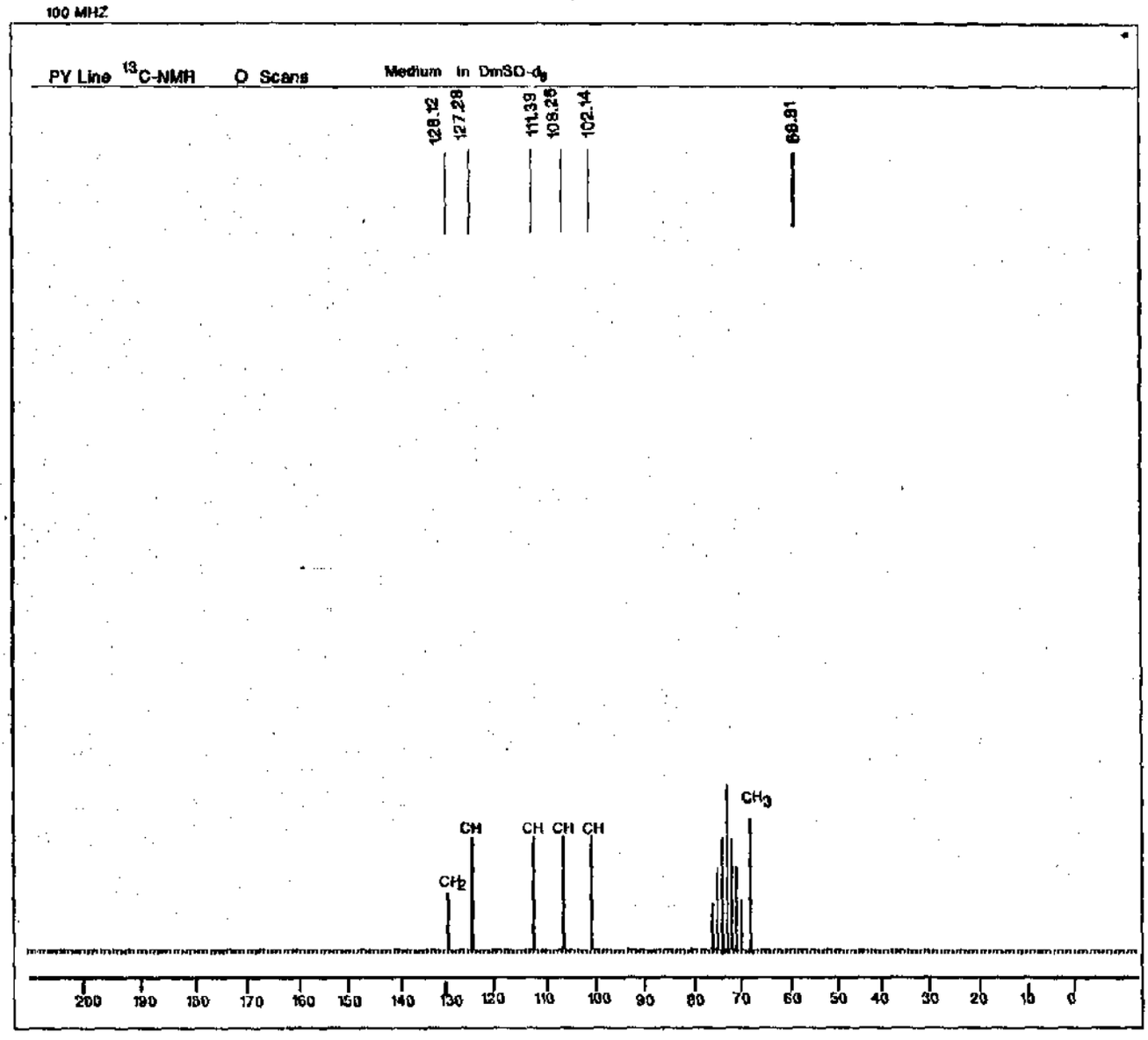


Fig. 3i ^{13}C-NMR Spectrum of CA_1

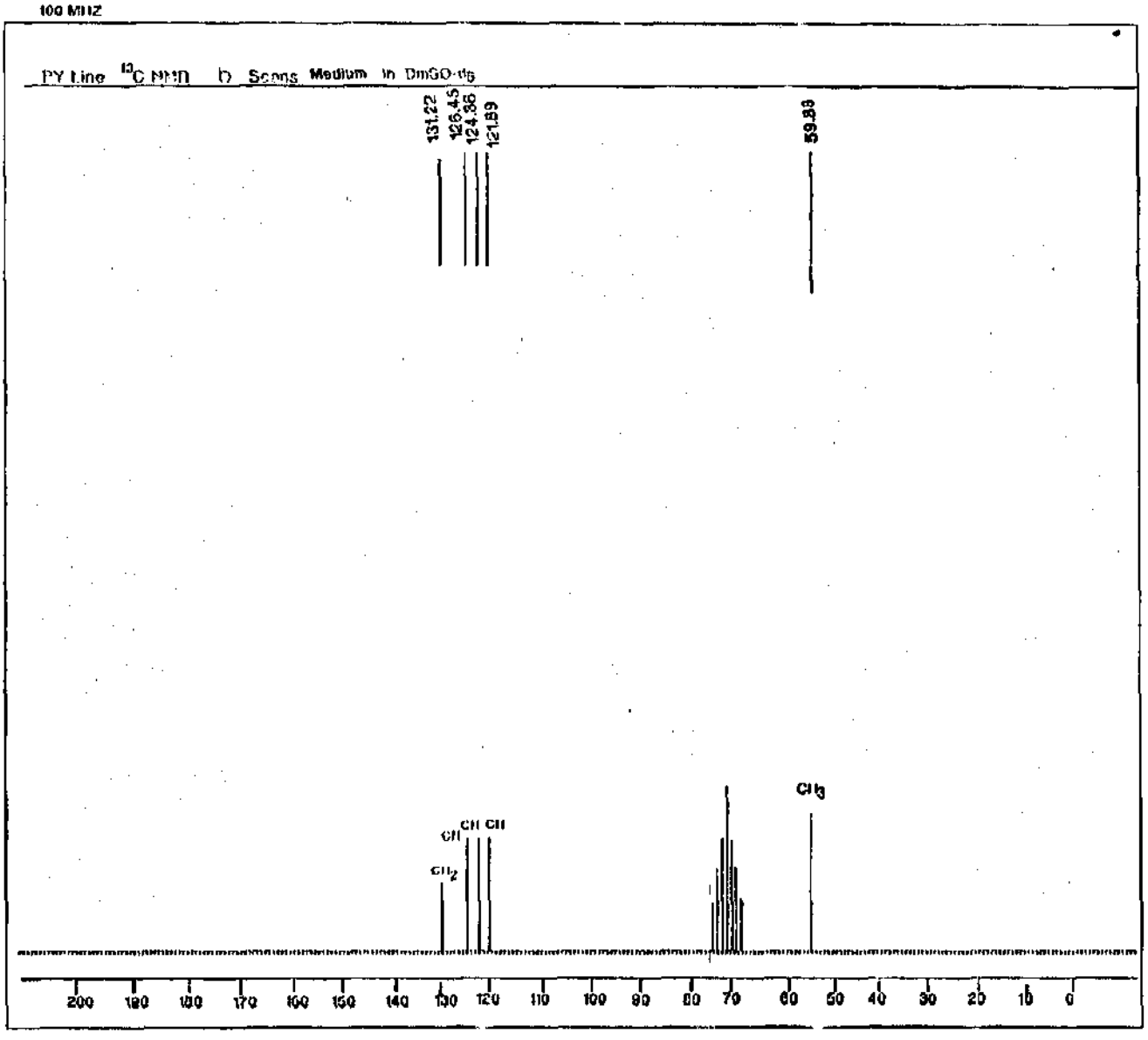


Fig. 3h ^{13}C-NMR Spectrum of CA_2

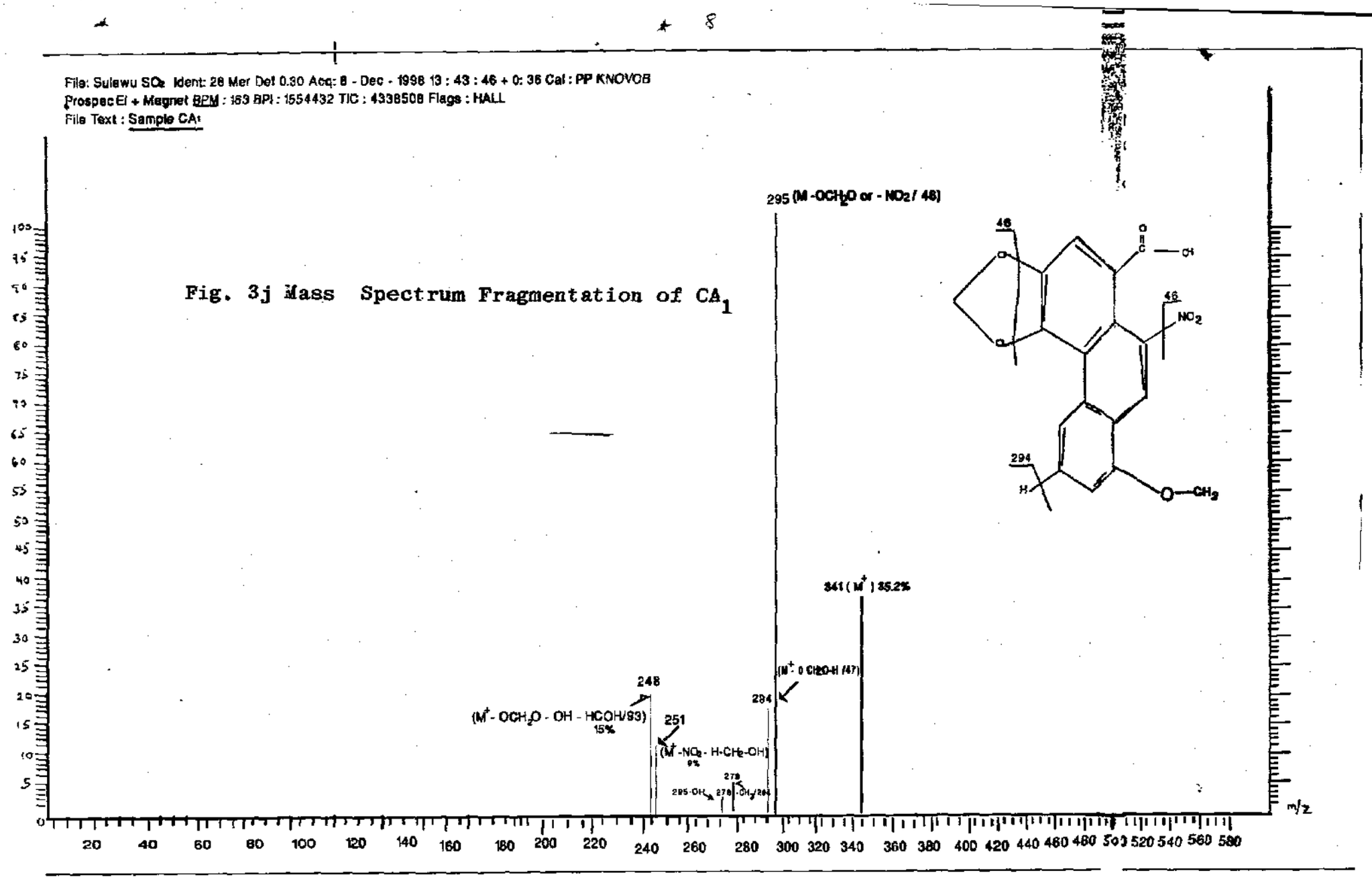


Fig. 3j Mass Spectrum Fragmentation of CA_1

File: Sulewu SQ2 Ident: 28 Mar Def 0.30 Acq: 8 - Dec - 1998 13 : 43 : 45 + 0: 36 Cal : PP KNOVOB
Prospec EI + Magnet BPM : 163 BPI : 1554432 TIC : 4338508 Flags : HALL
File Text : Sample CA2

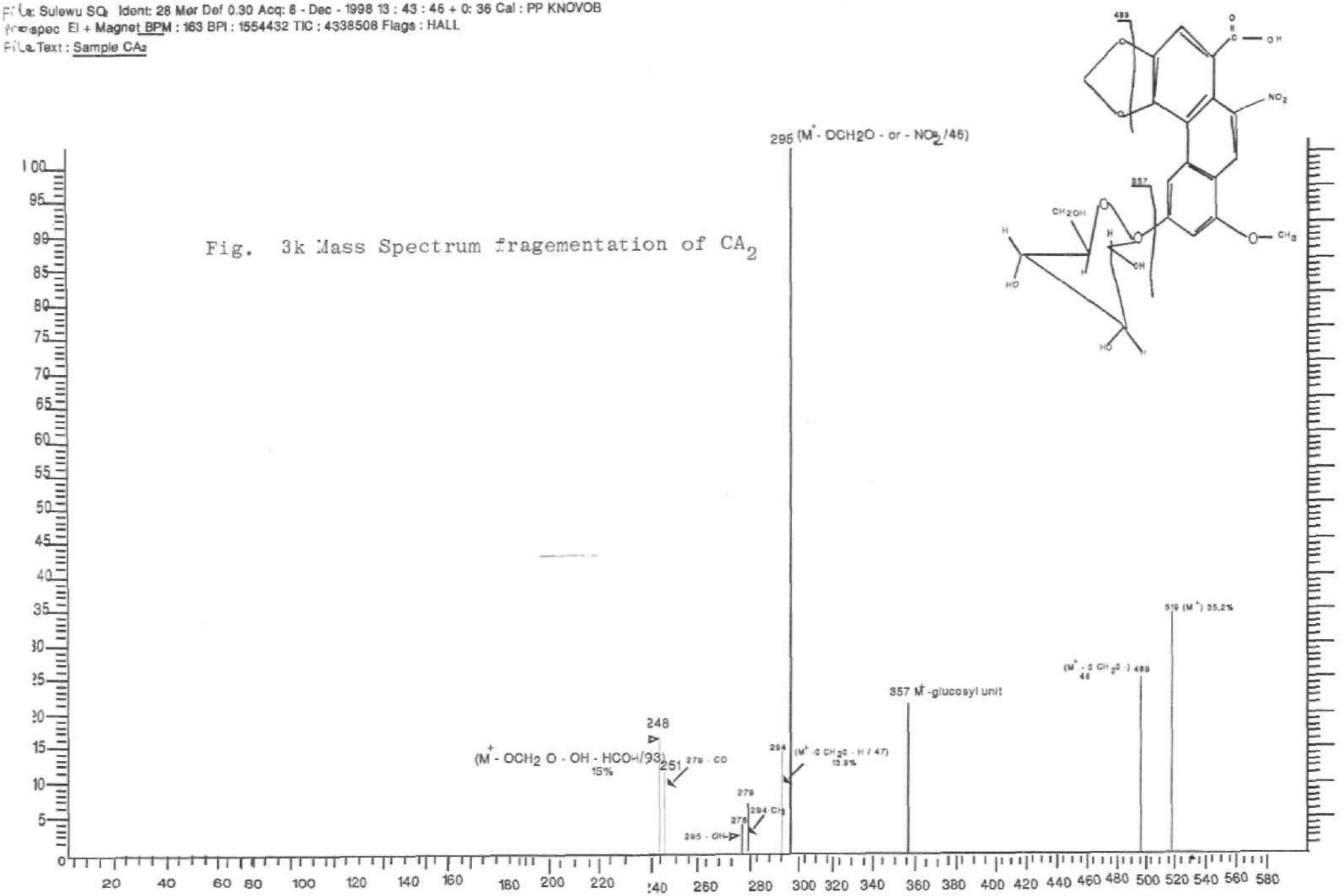
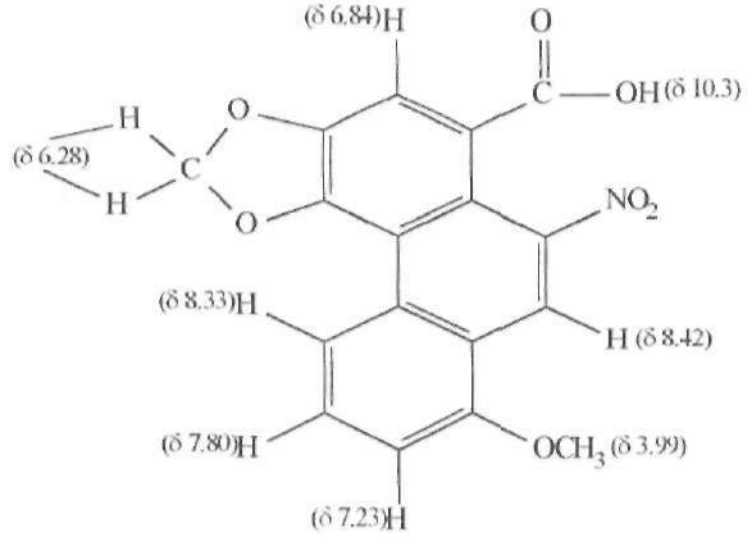


Fig. 3k Mass Spectrum fragementation of CA_2

NMR SPECTRAL ANALYSIS OF CA_1



^{1}H-NMR (DMSO-d_6)

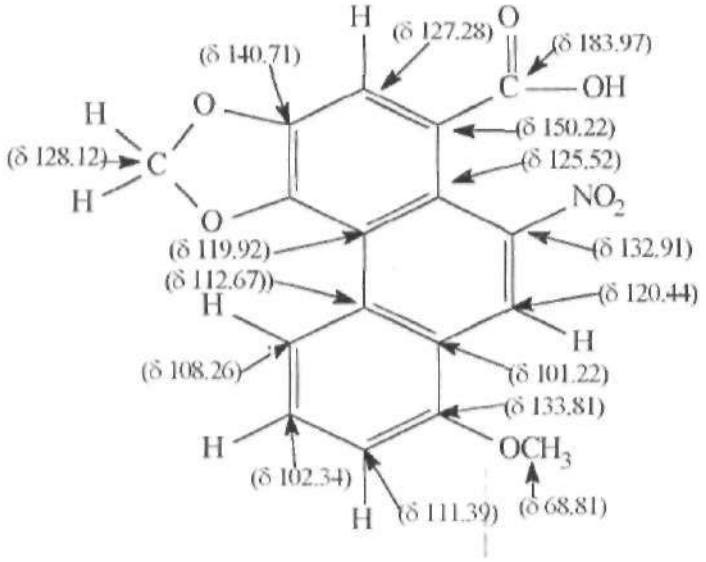
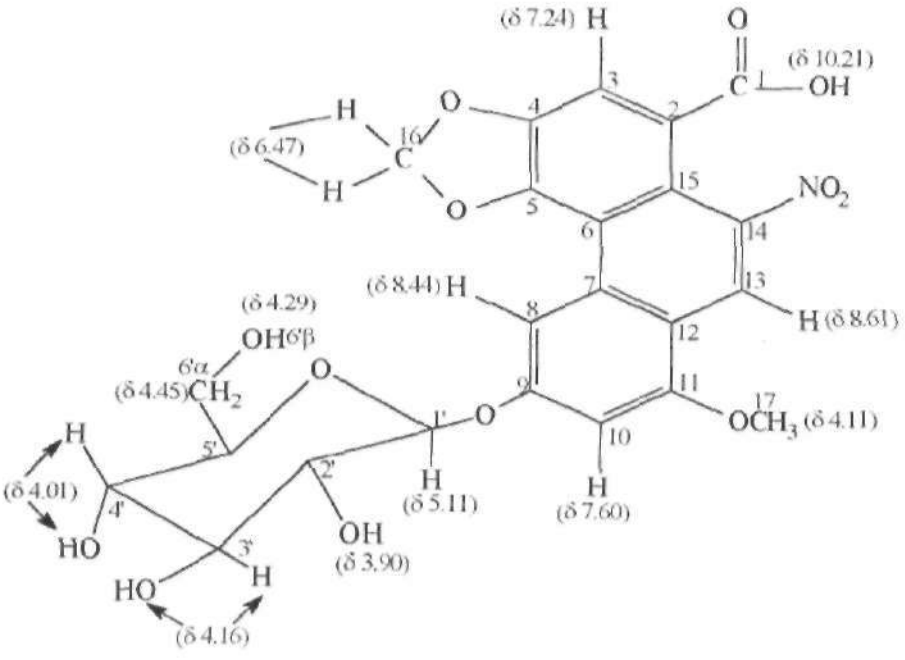
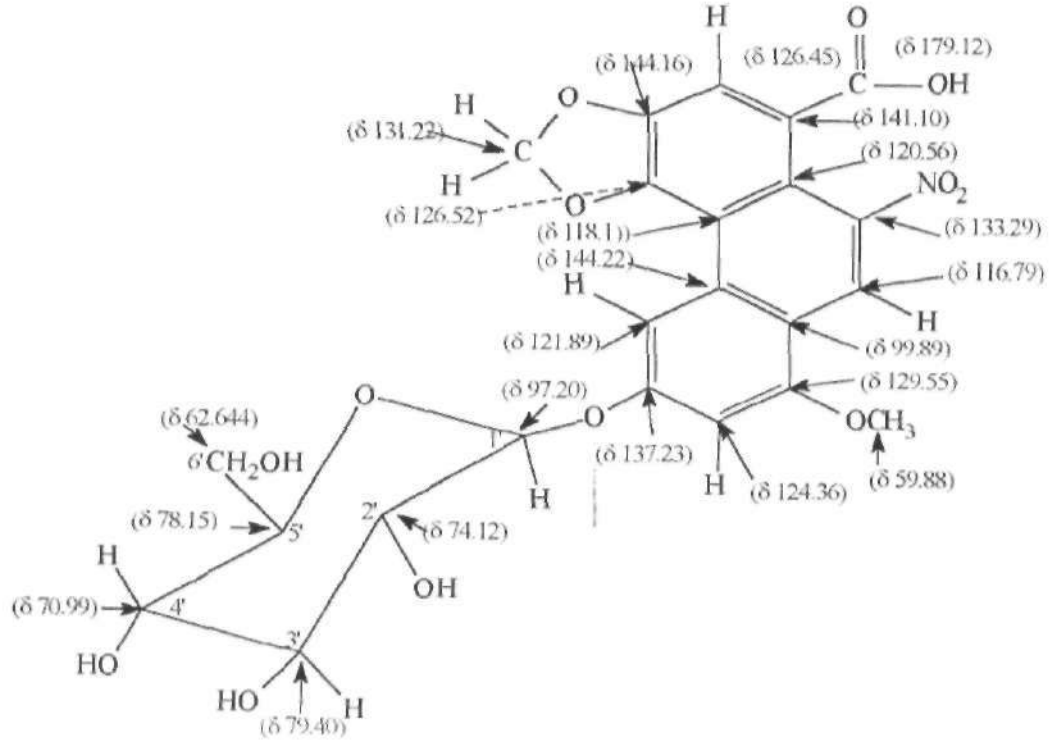


Fig. 3l: NMR Spectral Analysis of CA_1



^{1}H-NMR (DMSO-d_6)



^{13}C-NMR (DMSO-d_6)

Fig. 3m: NMR Spectral Analysis of CA_2

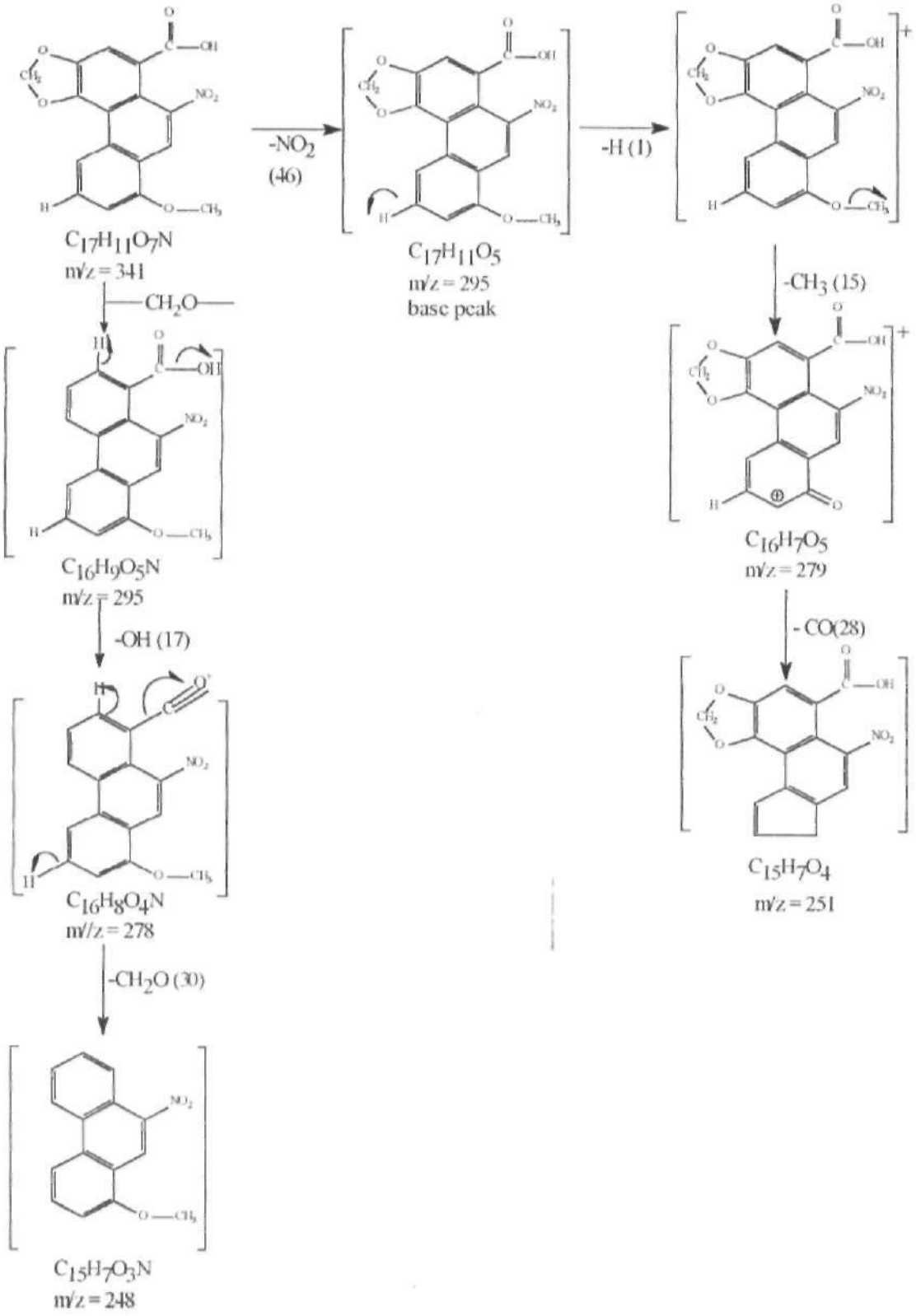


Fig. 3n: Fragmentation pattern of CA_1

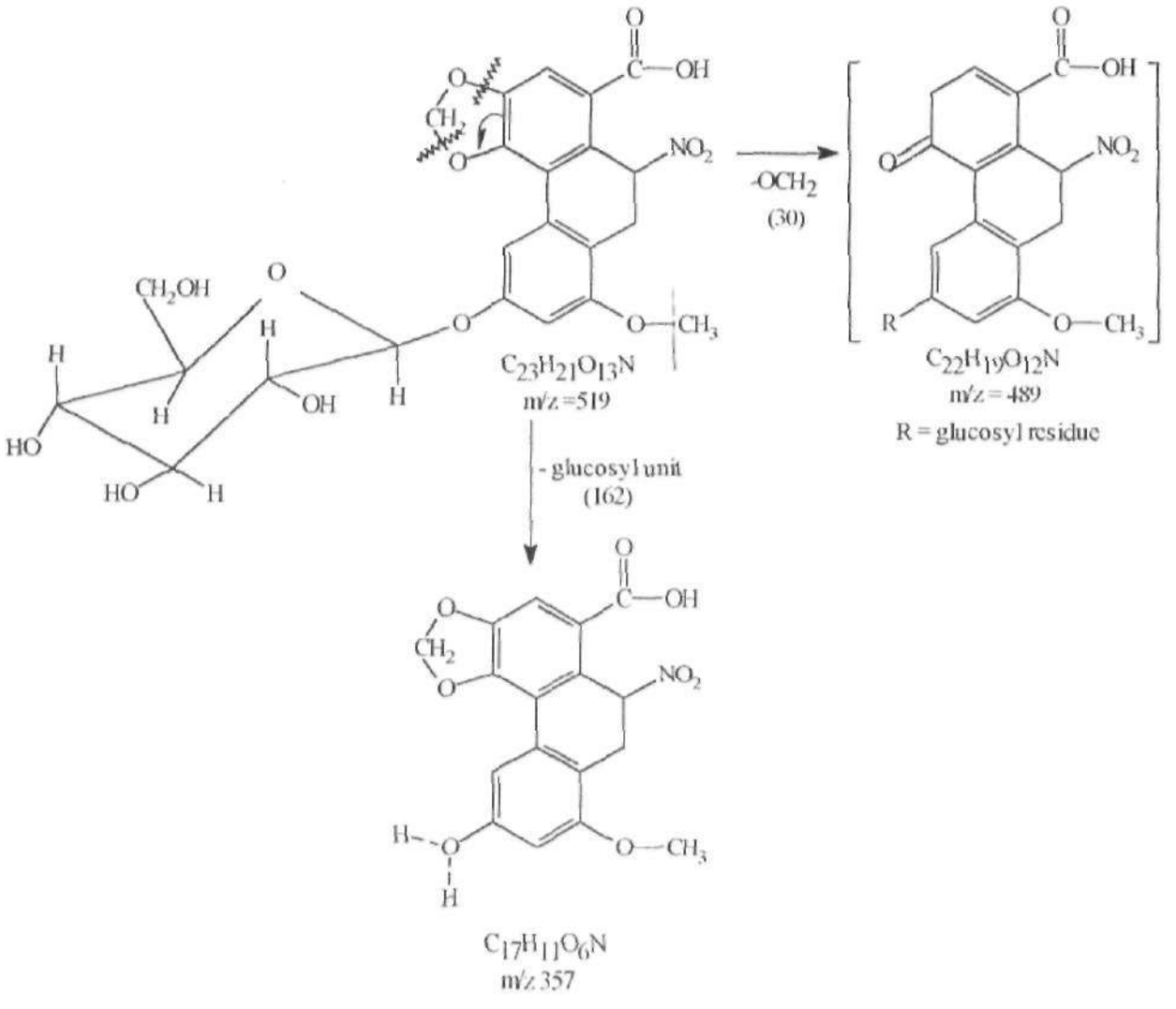
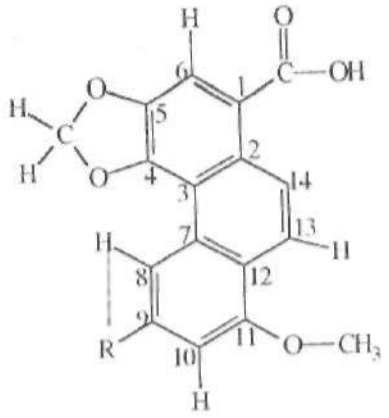
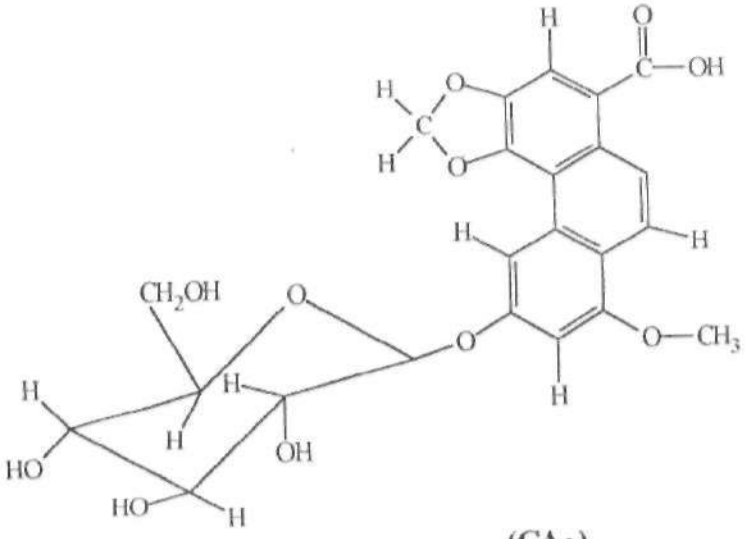


Fig. 3o: shows the prominent fragmentation pattern of CA_2



R = H

(CA_1)



(CA_2)

Fig. 3p shows structures of CA_1 and CA_2

CHAPTER FOUR

4.0 BIOLOGICAL STUDIES OF *ARISTOLOCHIA ALBIDA*

4.1 OBJECTIVE OF THE STUDIES

The objective of this work is to evaluate the biological effect of aqueous extract of the rhizomes of *Aristolochia albida* on isolated rat phrenic nerve diaphragm and frog rectus abdominis muscle in order to establish the basis for inhibitory mechanism of the plant extract and to justify the claimed biological actions of the extract in several gastrol - intestinal disorders, skin diseases and snake bites.

4.2 MATERIALS AND METHODS

4.3 MATERIALS

The test animal adult male rat (weighing 300 g) was purchased from the animal house in the department of pharmacology and clinical pharmacy was bred under standard hygienic and environmental conditions of temperature, relative humidity, light, and fed on standard diet and water (Pfizer feeds, Lagos).

The other test animal, adult frog was caught at Ahmadu Bello University Dam and kept under water for sometimes before sacrificing it.

(a) **Apparatus**

(i) Recording microdynamometer and transducer (Model No. 7050, Ugo Basile, Italy)

(ii) Thermostatically controlled tissue bath (24 ml, 50 ml, 500 ml, 1 litre).

(iii) Perspex chamber

(iv) Dissecting kits.

(b) **Standard Drugs and Sample Used**

The standard drugs and aqueous extract of *Aristolochia albida* were prepared by directly weighing and dissolving the known weight of the drug and solid extract (AFTER evaporation of water) in the required volume of di-ionized water.

The following standard drugs were obtained from BDM, Chemical Ltd., England and concentrations of the stock solutions used for the experiments are indicated below.

Name of compound	Stock solution
(i) Acetylcholine (Ach).....	10 µg/ml
(ii) Tubocuratine (d-tubo).....	100 µg/ml

Three concentrations of stock solution of the aqueous extract were prepared, 1 mg/ml 10 mg/ml and 100 mg/ml respectively.

(b) **Preparation of venom stock solution of *Naja nigricollis***

Venom was obtained from local shake *Naja nigricollis* caught by villagers during hunting., The snake was kept in fully aerated cylindrical gloss and was manually (usually under ether or chloroform anaesthesia) by holding the snake over small beakers covered with thin sheets of polythene. The snake was made to strike the polythene and penetrate it with the fangs just to release the venom into the containers (milking process of Markfarleene, 1967) at the herbarium, Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria.

The yellowish syrup liquid venom was immediately frozen and stored at – 20 °C until used or the venom was freeze dried and stored at 4 °C. Solutions of different concentrations of the venom were prepared in 0.9% (w/v) sodium chloride solution.

c) **Physiological solutions**

The physiological solutions used were

i) Frog ringer

ii) Krebs

d) **Preparation of physiological salt solutions**

	QUANTITIES FOR 10 LITRES	
SALT	FROG - RINGER	KREBS
NaCl	65 g	69 g
KCl (10% solution)	14 ml	35 ml
$MgSO_4$ $7H_2O$(10% solution)	-	29 ml
NaH_2PO_4 $2H_2O$ (10% solution)	1 ml	-
KH_2PO_4 (10% solution)	-	16 ml
D-Glucose	20 g	20 g
$NaHCO_3$	4 g	21g
$CaCl_2$ (Molar)	10.8ml	25.2 ml
10% $CaCl_2$	-	-
$MgCl_2.6H_20$	-	-
Sucrose	-	-
Aerating gas	Air	O_2 + 5% CO_2

e) **The Animal Isolated Tissues**

The following isolated tissues of the rat and frog were used for the experiments:

i) Rat phrenic nerve - diaphragm

ii) Frog rectus abdominis muscle.

4.4 METHODS

4.4.1 Preparation of the isolated tissues from the animals

a) Adult male rat locally bred at the animal house (weights 300 g) in the department of Pharmacology and Clinical Pharmacy, A.B.U., was killed by a blow on the head, its throat was cut and it was left to bleed so as to free the thorax of any blood. The skin was removed from the middle of the chest. The muscles from the chest wall and the ribs was then freed and cut alongside the base of the sternum on both sides of the animal. The upper part of the thorax was removed completely and the phrenic nerve was seen running from the diaphragm right up to the thymus gland. An incision was made in the abdominal wall just below the diaphragm and two cuts were made through the diaphragm and the ribs were left attached to it, towards the spine one from the front and one from the animal's flank. These produced a fan-shaped segment of muscle, whose apex was the tendonous material around the venacava, and which had the roots of the phrenic nerve about 2-4 mm from the apex. The nerve was cut below the thymus and the whole preparation was transferred to a flat dish containing M^{c} Ewens or krebs solution. The preparation was then attached to the electrodes.

The effects of the following drug were compared with that of the aqueous extract of *Aristolochia albida*, acetylcholine, tubocurarine and venon of *Naja nigricollis* of various concentrations.

b) Adult frog from the Dam of Ahmadu Bello University, Zaria was stuned and decapitated and the spinal cord was destroyed. The frog was pinned out on a board (abdomen up). The two rectus skins of the abdomen were removed which exposed the

whole abdomen. The two rectus muscles were clearly seen running each side of the midline from the base of the sternum to the fork. Each piece was divided into two because of their large sizes. Using frog-ringer at room temperature, the muscle was set up. The responses of the following drugs were compared with that of the aqueous extract of *Aristolochia albida*, acetylcholine and venom of *Naja negricollis* of various concentrations.

4.4.2 Recording Techniques

Various concentrations of the standard drugs, venom solutions and the aqueous extract of *Aristolochia albida* were added to the organ bath and the responses recorded isometrically using transducer connected to a recording microdyanometer.

4.5.0 RESULTS

4.5.1 Frog Rectus Abdominis

Acetylcholine (0.04 - 0.16 μg/ml) produced a concentration dependent contraction of the frog rectus abdomins (Table 4a, Fig.4a). *Naja nigricollis* venom (0.08 - 0.40 μg/ml) inhibited the contractions produced by acetlycholine (0.16 μg/ml) (Table 4b). The responses are shown in Fig. 4b and 4c. Venom of N, nigricollis (0.16 - 0.40 μg/ml) produced a slight relaxation effect on frog rectus abdominis (Fig. 4c).

The aqueous extract of *Aristolochia albida* relaxed the frog rectus abodminis slightly (Table 4d, Fig. 4d) and dose dependently (1.6 - 6.4 mg/ml) antagonised the effect of acetaylcholine (0.16 μg/ml) on the frog rectus abdominis (Table 4e, Fig, 4e, Fig. 4d). the antagonizing effect of the extract became more produced following the concurrent

administration of aqueous extract (1.6 - 6.4 mg/ml) and venom of *N. nigricollis* (0.08-0.40 µg/ml) respectively before and after injection of acetylcholine into the organ bath (Table 4f and 4g , fig 4f, fig. 4g). the Log-Dose response curve is represented in Fig 4m.

4.5.2 **Rat Phrenic Nerve Diaphragm**

Naja nigricollis venom in different concentrations (0.016-0.256 µg/ml) of the stock 4 µg/ml caused a sustained twitch tension of rat phrenic nerve diaphragm (Table 4l, fig 4l) In some experiment using different concentration of extract of A. albida (0.8 - 6.4 mg/ml) a slight increase in twitch tension of rat phrenic nerve diaphragm was evident (Table 4h, Fig. h) In a similar experiment, d-tubocurarine of different concentration (0.8 - 6.4 µg/ml) caused a slight decrease in twitch tension of the rat phrenic nerve diaphragm (Table 4l, Fig 4l) when d-tubocurarine of concentration (1.6 µg/ml) was added to the tissue bath containing the extract, an increased twitch tension of the rat phrenic nerve diaphragm was produced over that shown when only the extract was used (Table 4j, Fig 4j). in a like manner, a significant increase in twitch tension was produced when extract of *A. albida* was added to the bath of different venom concentrations of *N. nigricollis* (Table 4k, Fig. 4k) However, when d-tubocurarine was added to the bath in which venom caused a sustained twitch tension, it produced only a small and short lasting increase in the twitch tension.

Table 4a: Effect of contraction produced by acetylcholine on frog rectus abdominis

Vol. of Ach administered (ml)	Conc. (µg/ml)	Contraction Response (mm)	Log. Dose	%Maximum Response
0.1	0.04	12	-1.40	31
0.2	0.08	25	-1.10	66
0.4	0.16	38	-0.80	100

Vol. Of organ bath 25ml

Stock concentration for Ach 10 µg/ml

Table 4b: Effect of venom of *N. nigricollis* (0.16 µg/ml) in the presence of acetylcholine on frog rectus abdominis.

Vol. of Ach administered (ml)	Conc. (µg/ml)	Relaxation Response (mm)	Log - Dose	% Maximum
0.2	0.08	12	-1.10	31
0.4	0.16	24	-0.80	67
0.8	0.32	34	-0.49	89
1.0	0.40	38	-0.40	100

Vol. of organ bath 25 ml

Stock concentration for Ach 10 µg/ml

Stock concentration of *N.nigricollis* venom 4 µg/ml

Table 4c. Effect of acetylcholine (0.16 µg/ml) in the presence of venom *of N. nigricollis* on frog rectus abdominis.

Vol. of venom administered (ml)	Conc. (µg/ml)	Relaxation Response (mm)	Log. Dose	% Maximum
0.2	0.032	10	-1.50	100
0.4	0.064	6	-1.20	60
0.8	0.128	3	-0.90	30
1.0	0.160	1	-0.80	10

Vol. of organ bath 25 ml

Stock concentration for Ach 10 µg/ml

Stock concentration for *N. nigricollis* venom 4 µg/ml

Table 4d: Effect of acetylcholine in the presence of extract of *A. albida* (4 mg/ml) on frog rectus abodminis

Vol. of Ach administered (ml)	Conc. (µg/ml)	Relaxation Response (mm)	Log. Dose	%Maximum Response
0.1	0.04	10	-1.40	63
0.2	0.08	16	-1.10	100
0.4	0.16	11	-0.80	69

Vol of organ bath 25 ml

Stock concentration for extract 100 mg/ml

Stock concentration for Arch 10 µg/ml

Table 4e: Effect of aqueous extract of *A. albida* on frog rectus abdominis

Vol. of Extract administered (ml)	Conc. (mg/ml)	Relaxation Response (ml)	Log. Dose	%Maximum Response
0.4	1.6	0	0.20	0
0.8	3.2	4	0.51	80
1.6	6.4	5	0.81	100

Vol. of organ bath 25ml

Stock concentration for Extract 100 mg/ml

Table 4f: Effect of concurrent administration of venom of *N. nigricollis* (0.16 µg/ml) + Extract of *A. albida* (4 mg/ml) and arch on frog rectus Abdominis

Vol. of Ach administered (ml)	Conc. (µg/ml)	Relaxation Response (mm)	Log. Dose	% Maximum Response
0.2	0.08	5	-1.10	26
0.4	0.16	10	-0.80	53
0.8	0.32	19	-0.49	100

Vol. of organ bath 25 ml

Stock concentration for Extract 100 mg/ml

Stock concentration for *N. nigricollis* venom 4 µg/ml.

Table 4g: Effect of Acetylcholine and concurrent administration of venom of *N. nigricollis* (0.16 µg/ml) + Extract of *A. albida* (4 mg/ml) on frog rectus abdomins

Vol. of Ach administered (ml)	conc. (µg/ml)	Relaxation Response (mm)	Log. Dose	%Maximum
0.2	0.08	12	-1.10	46
0.4	0.16	25	-0.80	96
0.8	0.32	26	-0.49	100

Vol. of organ bath	25ml
Stock concentration for extract	100mg/ml
Stock concentration for N.nigricollis venom	4 µg/ml

Table 4h: Effect of aqueous extract of *A. albida* on rat phrenic nerve diaphragm

Vol of Extract administered (ml)	Conc. (µg/ml)	Twitch Tension Response (mm)	Log. Dose	% Max. Twitch Tension Response
0.2	0.8	6	-0.10	100
0.4	1.6	2	0.20	33
0.8	3.2	5	0.51	83
1.6	6.4	5	0.81	83

Vol. of organ bath	25 ml
Stock concentration for Extract	100 mg/ml

Table 4l: Effect of d-tubocurarine on rat phrenic nerve diaphragm

Vol. Of d-tubocurarine administered (ml)	Conc. (µg/ml)	Twitch Tension Response (mm)	Log. Dose	%Max. Twi Tension Resp
0.2	0.8	2.0	-0.10	50
0.4	1.6	2.0	0.20	50
0.8	3.2	3.0	0.50	75
1.6	6.4	4.0	0.81	100

Vol. of organ bath 25 ml

Stock concentration for d-tubocurarine 100 µg/ml

Table 4j: Effect of d-tubocurarine (1.6 µg/ml) in the presence of aqueous extra

***A. albida* on rat phrenic nerve diaphragm**

Vol. of extract administered (ml)	Conc. (mg/ml)	Twitch Tension Response (mm)	Log. Dose	%Max. Tw Tension Resp
0.2	0.8	6	-0.10	100
0.4	1.6	2	0.20	33
0.8	3.2	4	0.51	67

Vol. of organ bath 25 ml

Stock concentration for Extract 100 mg/ml

Stock concentration for d-tubocurarine 100 µg/ml

Table 4j: Effect of venom of *N. nigricollis* (0.128 µg/ml) in the presence of aqueous of extract of *A. albida* on rat phrenic nerve diaphragm

Vol. of extract administered (ml)	Conc. (mg/ml)	twitch Tension Response (mm)	Log-Dose	%Max. Twitch Tension Response
0.2	0.8	2	-0.10	33
0.4	1.6	5	0.20	83
0.8	3.2	6	0.57	100

Vol of organ bath	25 ml
Stock concentration for extract	100 mg/ml
Stock concentration for N. *nigricollis* venom	4 µg/ml.

Table 4l: Effect of venom of *N. nigricollis* on rat phrenic nerve diaphragm (sustained twitch tension)

Vol. of venom administered (ml)	Conc. (µg/ml)	Sustained Twitch tension Response (mm)	Log. Dose	%Max. Twitch Tension Response
0.2	0.0032	0	-1.50	0
0.4	0.064	0	-1.20	0
0.8	0.128	0	-0.90	0
1.6	0.160	0	-0.80	0

Vol of organ bath	25 ml
Stock concentration of *N. nigricollis* venom	4 µg/ml.

4.6 DISCUSSION

4.6.1(a) Frog Rectus abdomins

The standard drug used was Acetylcholine (Arch) venom of *N. nigricollis* (Vm), and extract of *A. albida* were also used.

Acetylcholine (a quartenary ammonium compound) is an acetyl ester of choline. It serves as a neurotransmitter in a number of organs, including neuromuscular junctiuons, post gangliomic fibres of bath sympathetic and parasympathetic systems (Elliot *et al.*, 1977). The two major actions of acetylcholine on the nerves system are:

i) to stimulate the gauglia , adrenal medulla and skeletal muscle

ii) to stimulate the post ganglionic nerve endings in cardiac muscles, smooth muscles and the glands.

The first action resembles the effect of nicotine and therefore is referred to as the nicotinic effect of acetylcholine, which is elucidated via nicotinic receptors. The second action resembles that of muscerine (an alkaloid from poisonous food stool. *Amante muscarad.* And therefore is referred to as the muscarinis effect of acetylcholine which is elucidated via the muscarinic receptors.

Acetylcholine caused contractions of smooth muscles (Elliot *et al.*, 1977) and in this experiment it produced contraction of the frog rectus abdominis. The aqueous extract of *A. albida* exhibited a dose - dependent relaxation indicating that it has atropine like properties in its modes of action on frog rectus abdominis (Fig. 4a).

The extract of *A. albida* might be mediating muscaric effect similar to atropineor inhibition caused by adrenaline on adrenergic receptors. The extract of *A. albida* dose

dependently inhibited the contraction evolved by acetylcholine. This is similar to atropine in other workers work (A.K Haruna and M. Choudhury, 1995), suggesting that the extract might be mediating its relaxant effect through muscarinic receptors. This effect is beneficial in the management of gastro intestinal disorders thus justifying the use of the drug in stomach disorders in traditional medicine.

4.6.1(b) Anti-Snake Venom Studies

The rhizome of *Aristolochia albida* could safely be useful in the treatment of snake bites particularly those involving *N. nigricollis*. The extract of *A. albida* (4 mg/ml) antagonizes the effective concentration of the venom (0.16 μg/ml) but the antagonizing effect is dose dependent (shown in Fig 4g).

The results shows in (Table 4b and 4c. Fig. 4b and 4c) clearly show the inhibiting effect of venom of *N. nigricollis* on the contractions produced by acetylcholine (0.08-0.4 μg/ml). Thus the inhibitory effect were significant when graded doses of venom of *N. nigricollis* (0.032 - 0.160 μg/ml) was administered against a fixed concentration of acetylcholine (0.16ug/ml) to establish the effective concentration of the venom.

Furthermore, the effect of inhibition was slightly reduced when venom *of N. nigricollis* (0.16 μg/ml) and extract (4 mg/ml) were administered concurrently before and after acetylcholine, thus there was a slight significant increase in the contractions produced by acetylcholine when compared with those shown above when only venom of *Naja nigricollis* was used against Ach. This result is consistent with antagonizing action shown

by the extract on the venom earlier stated and confirmed that the extract might contain metabolic agents capable of detoxifying the venom (antivenom agent).

However, the occupation of the muscarinic receptors by agents of venom of *N. nigricollis* and extract of *A. albida* thereby preventing the arch from eliciting its muscarine activity was also envisaged and thus the displacement of these agents from the muscurinic receptors in Ach dose dependent.

4.6.2 Rat Phrenic nerve - diaphragm

The results of this work showed that venom of *N. nigricollis* cobra snake dispersed throughout the Northern part of the country and particularly common in Katsina state Induces neuromuscular blockade in the rat phrenic nerve diaphragm preparation (sustained twitch tension). The extract of *A. albida* and d-tubocurarine antagonizes the effect of the venom neuromuscular transmission in the rat phrenic nerve diaphragm in different preparations. It is believed that the antagonizing effect of the extract and d-tubocurarine on the blockade produced by the venom in the rat phrenic nerve diaphragm could be due to blockade of miniature and plate potential (e.p.p) and particularly their antagonizing effect on the postsynaptic action of the venom. Thus it can also be inferred that desensitization of the end plate receptors induced by the venom of *Naja nigricollis* could be the main cause of the neuromuscular blockade that it produces.

Snake venom may be a neurotoxin, cardiotoxin, harmorrhagin, or hydrolytic in action (Lecithinosessor phospholiposes). Most viper venoms are chiefly vesculotoxic and gangrene might ensued due to Ischemic thrombosis. Thus cobra venoms are said to be neurotoxic. Bungarotoxin, a neurotoxic agent has been identified in cobra venom which

mimic endotherlins (Warrel, 1993a) and is a postsynaptic toxin that attach to the Ach receptor on the motor and plate.

Most workers assay anti - snake venom activity of elapids (cobra species) by the ability of the test agent to inhibit neurotoxity of the venom (Houghton and Harvey, 1989). This may be due to the fact that Cobra venoms contain neutrotoxic substance identified to be identical with those found in Bungarus species (common kraits) known as α- and β-bungarotoxin, copbrotoxin and toxin alpha. These have curare-like action at neuromuscular junction leading to flaccid - paralysis. The toxin also blocks nerve impulse transmission with Ach (Carey and Wright, 1960, Halstead, 1978, Mintona nd Minton, 1980, Warrel 1987). The respiratory depression was shown to be by blockade of neuron impulse to skeletal muscles (Frimmer, 1969, Hatch, 1977).

In case of extract of *Aristolochia albida* the muscle relaxation shown after its administration suggests a receptor responses because after some snake venomatron the clinical symptoms include among others stresism, ptosis, convulsion, sedation, tissue destruction at the site of the bite, restlessness, as a result of interaction of morphine receptors with some venom constituents and curare like flaccid paralysis due to the interference with Ach muscarinic receptors cobrotoxin, toxin-alpha and other neurotoxin substances.

The extract of *Aristolochia albida* may occupy these receptors competitively with the snake venom still eliciting its mild muscle relaxation action and thereby preventing the snake venom access to these to cause toxic and lethal reactions (competitive antogonism) The result of this present study is consistent with earlier workers on this plant *A. albida* that death from *N. nigricollis* envenonation resulted mainly from neurotoxicity by curare -

like action of cobrotoxin on the respiratory muscles causing flaccid paralysis leading to respiratory depression. A furanoid deterpene an isolate of methanolic extract of this plant was found to interfere with acetylcholine receptors and believed to mediate its actions by antagonising the actions of neurotoxic substances in venom at the acetylcholine receptors sites (A.K. Haruna and M.K. Choudhury, 1995).

It is therefore strongly suggested that since the extract of the plant *A. albida* contain more than one active principles from where a furanoid diterpene was isolated. Other compound might be present which could prove beneficial against snake venoms particularly those involving elapids (e.g. *N. nigricollis*) and this result confirm or support the reports or deductions that the extract act more on the muscarinic receptors.

This present research work proves the protective actions of the *Aristolochia albida* extract against bites of *N. nigricollis* but this could be relied upon to a little extent because it involves only in vitro experiments and experiences have shown that there were some agents which proves beneficial therapeutically *in vitro* studies but fail to potentials their claimed pharmacological actions in *in vivo* studies thus *in vivo* experiments are more imperical. The inhibitory mechanism of action of the extract of *A. albida* against snake venoms could only vividly be adduced if both *in vitro* and *in vivo* experiments are incorporated.

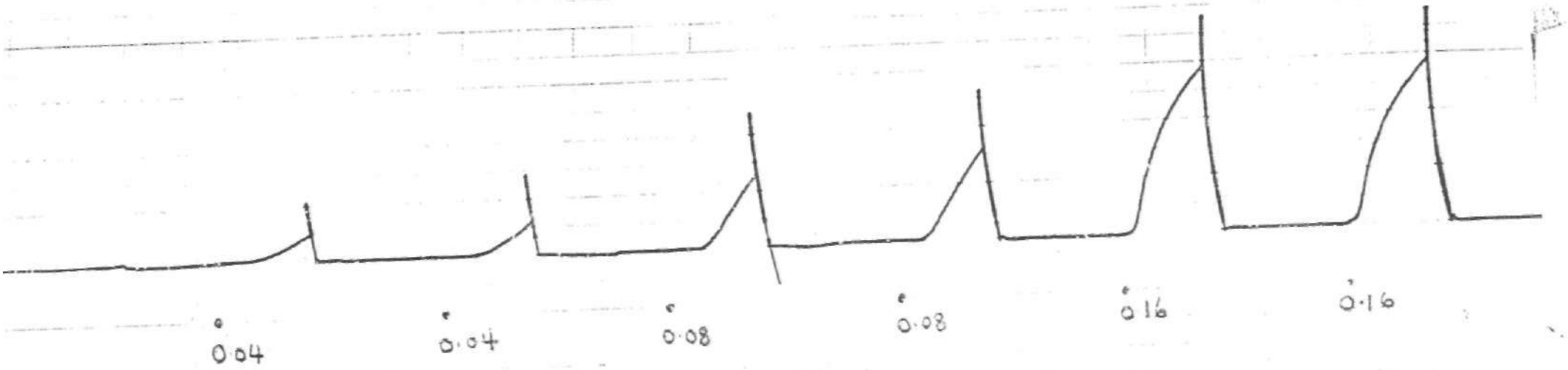
4.7. CONCLUSION

The effects of the aqueous extract of A. albida against bites from *N. nigricollis* on two isolated tissues of an adult mole rat (weigh 300 g) and an adult frog (frog rectus abdominis and rat phrenic nerve diaphragm) were investigated and compared with those of

some standard drugs. The result showed the antagonizing effect of the extract against neuromuscular junction blockade induced by venom of *Naja nigricollis*, a cobra snake dispersed throughout the Northern part of the country and particularly common in Katsina State.

In addition it will be emoneous to conclusively explain the inhibitory mechanism of action of the extract of *Aristolochia albida* against bites from *N. nigricollis* with this result but rather, it provides an avenue to justify the claimed folkloric use by Hausa - Fulani of the Northern part of the country in several gastro-intestinal disorder. Skin diseases and against snake-bites which is supported by the ethnopharmocology report on this plant (Haruna A.K. and Choudhury, M.K., 1995).

Fig. 49 Effect of Contraction produced by acetylcholine on frog rectus abdominis



(Conc in µg/ml)

Fig. 4a Effect of contraction produced by acetylcholine on frog reduce abdominis

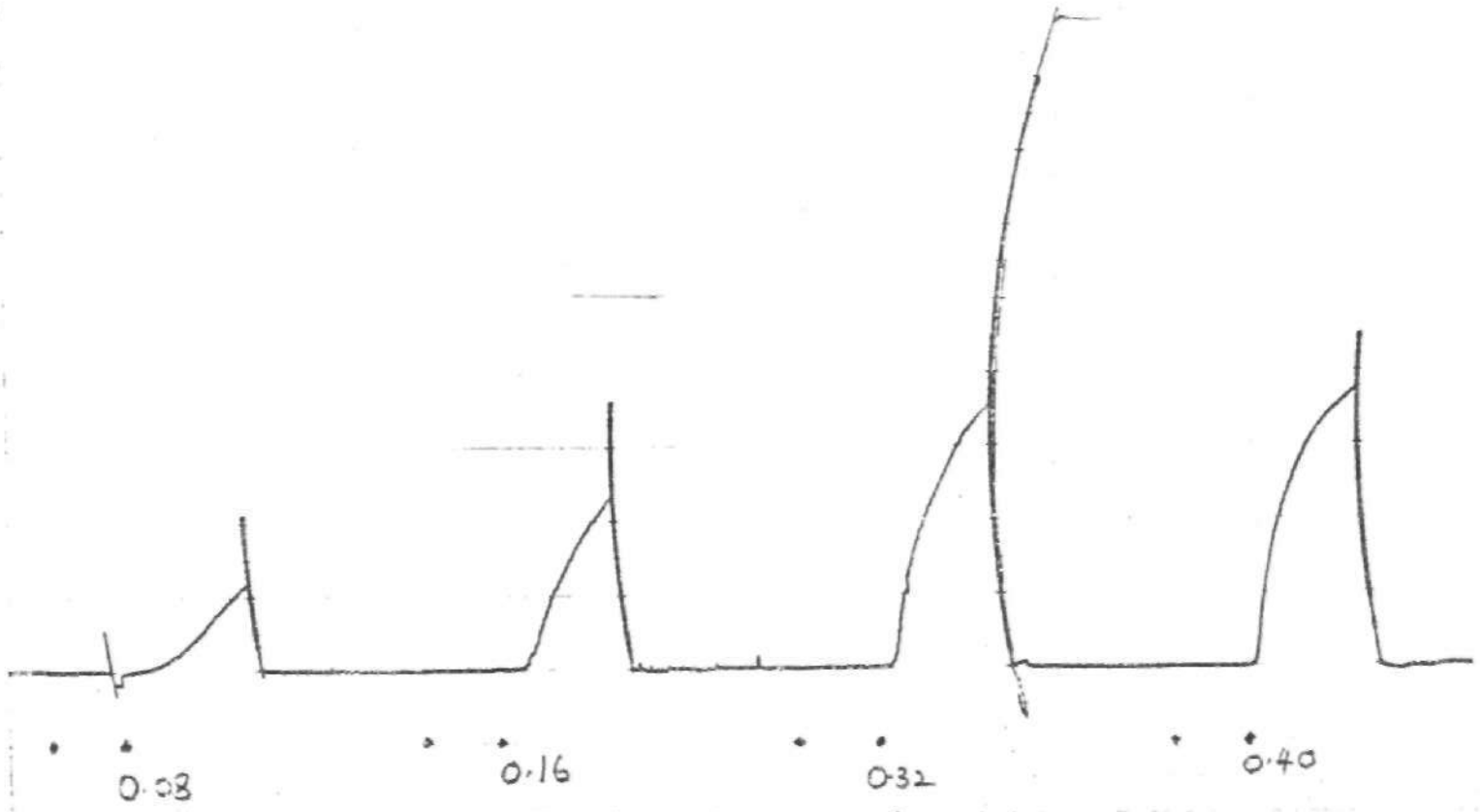
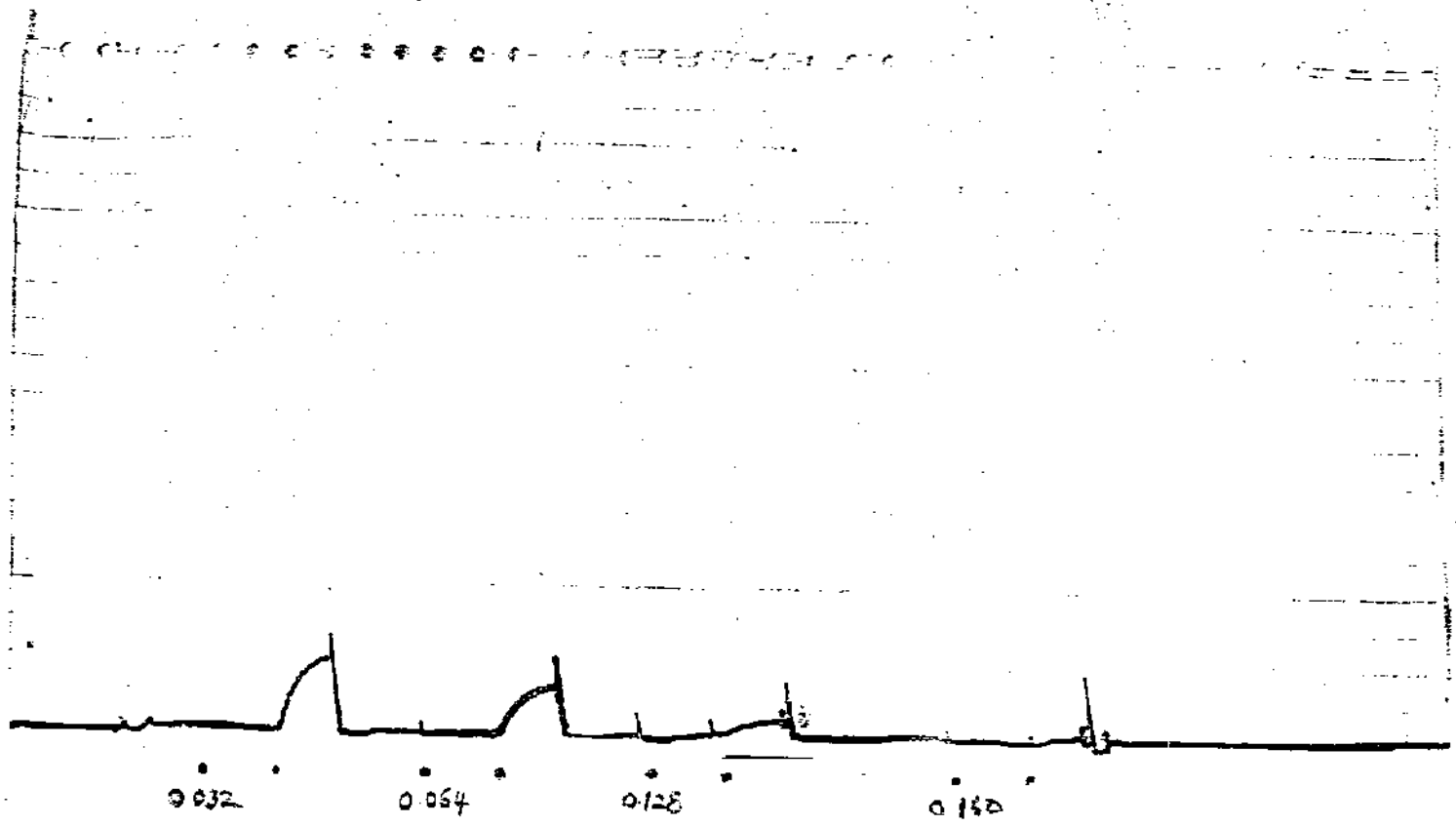


Fig. 4b effect of venom of N. N. nigrocollis (0.16µg/ml) in the presence of acetylcholine on frog rectus abdominis



(Conc. Inμg/ml)

Fig. 4C Effect of Acetylcholine (0.16Ug/ml) in the presence of Venom of N nigricollis on frog rectus abdominis

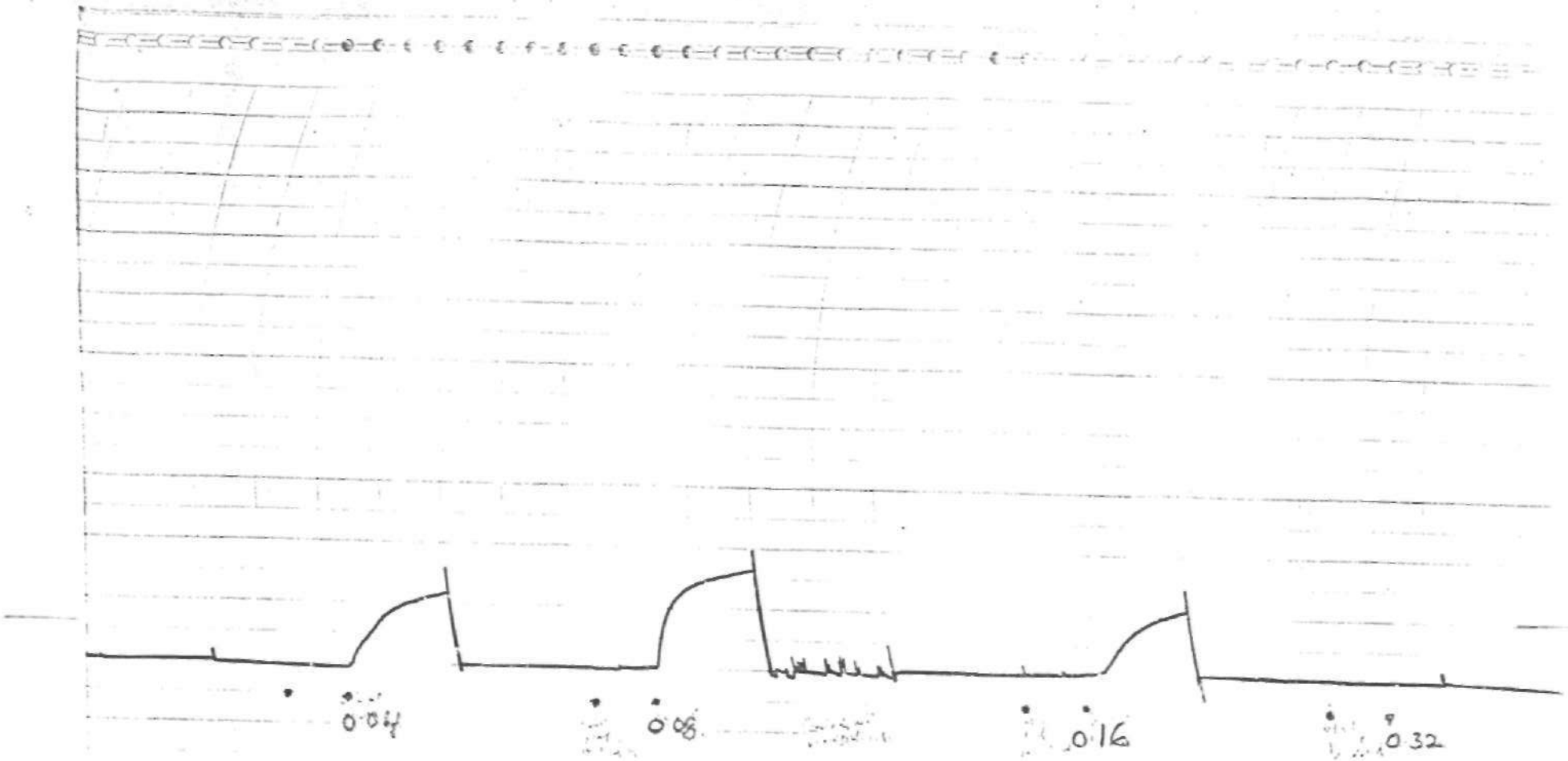


Fig. 4d Effect of Acetylcholine in the presence of aqueous extract (4mg/ml) of A albida on frog rectus abdominis

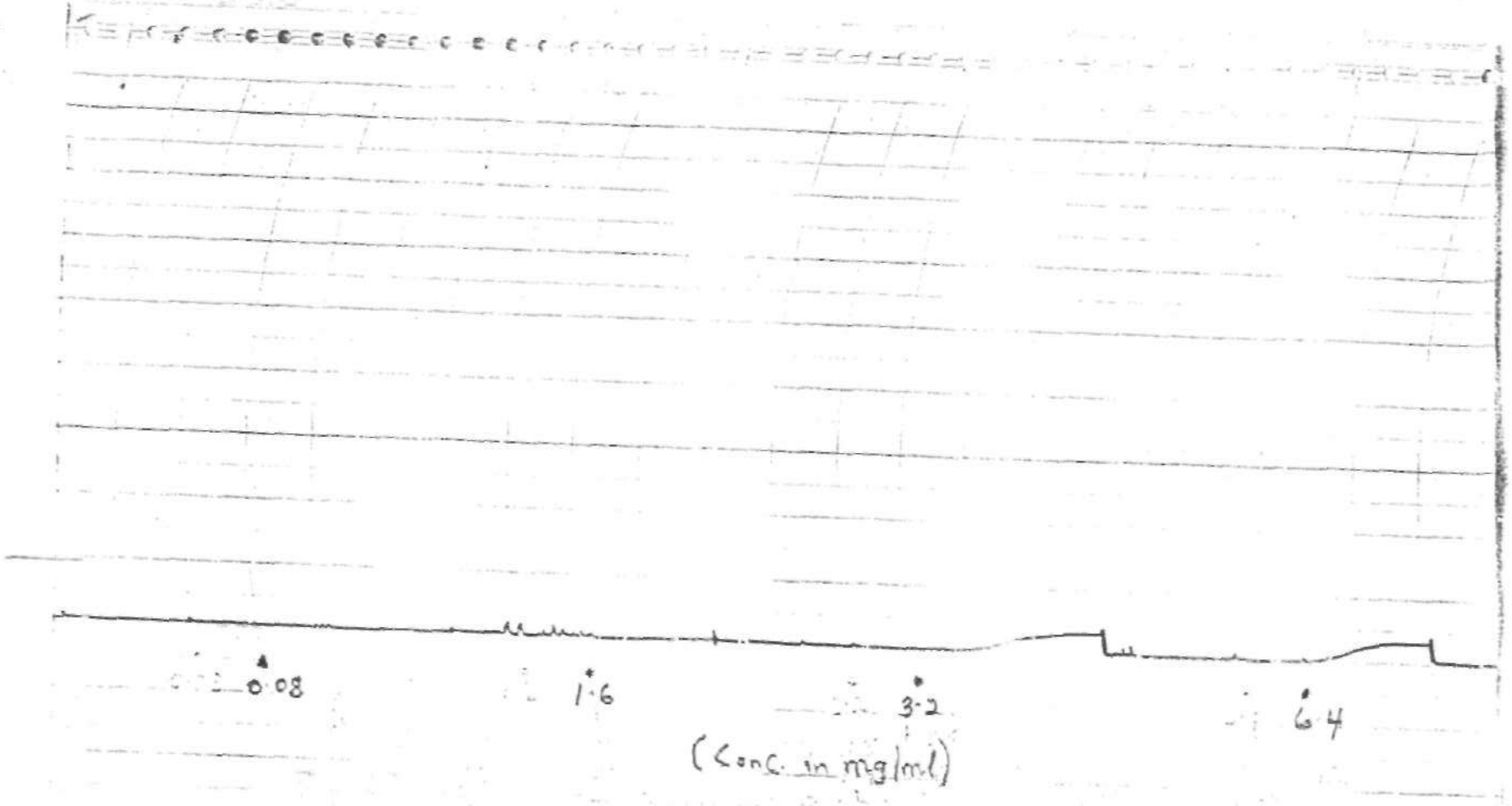


Fig 4e Effect of aqueous extract of A albida on frog rectus abdominis (relaxation).

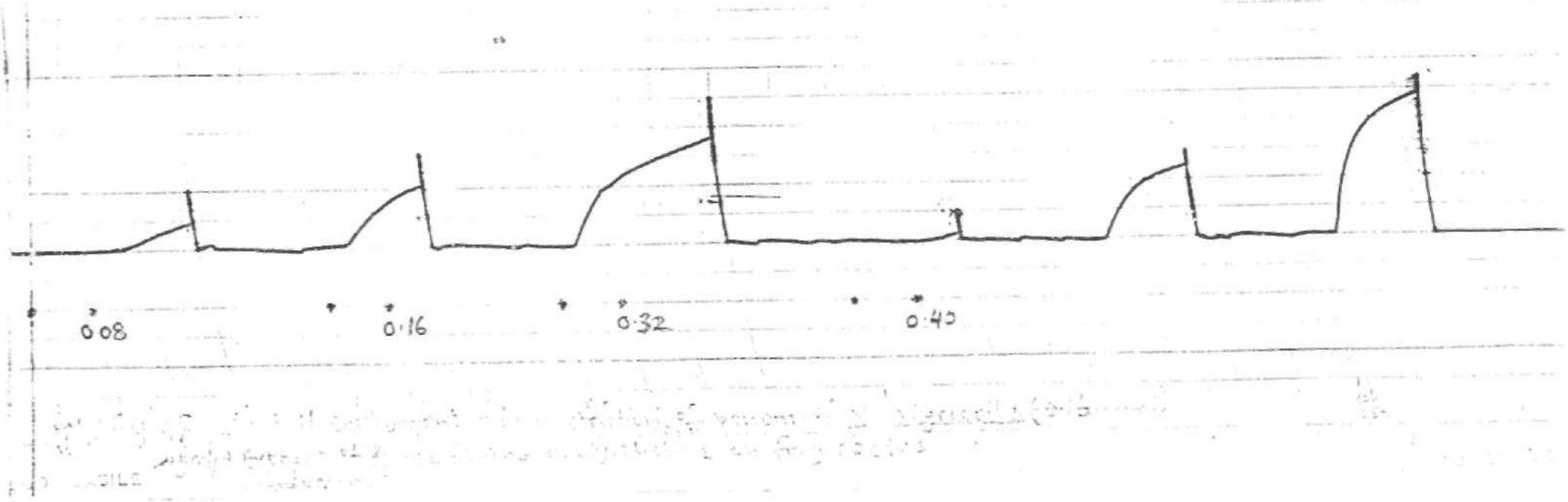
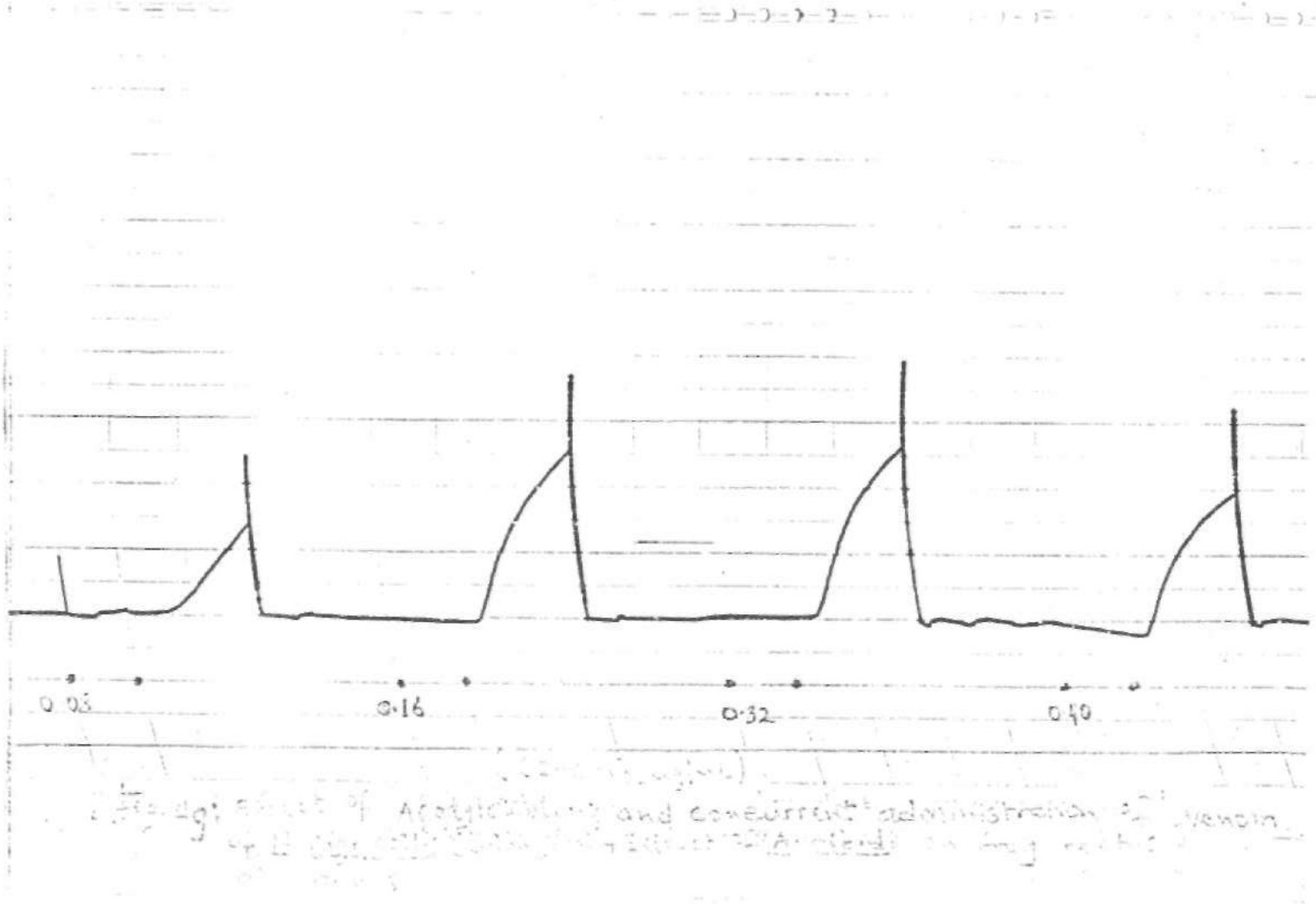


Fig 4f Effect of concurrent administration of Venon of N nigricollis (0.16mg/ml) ug/ml) + Extract of A abida and Acetylcholine on frog rectus abdominis



(conc in ug/ml)

Fig. 4g: Effect of Acetylcholine and concurrent administration of Venom of N nigricollis (0.16 ug/ml) + Extract of A albida on frog rectus abdominis.

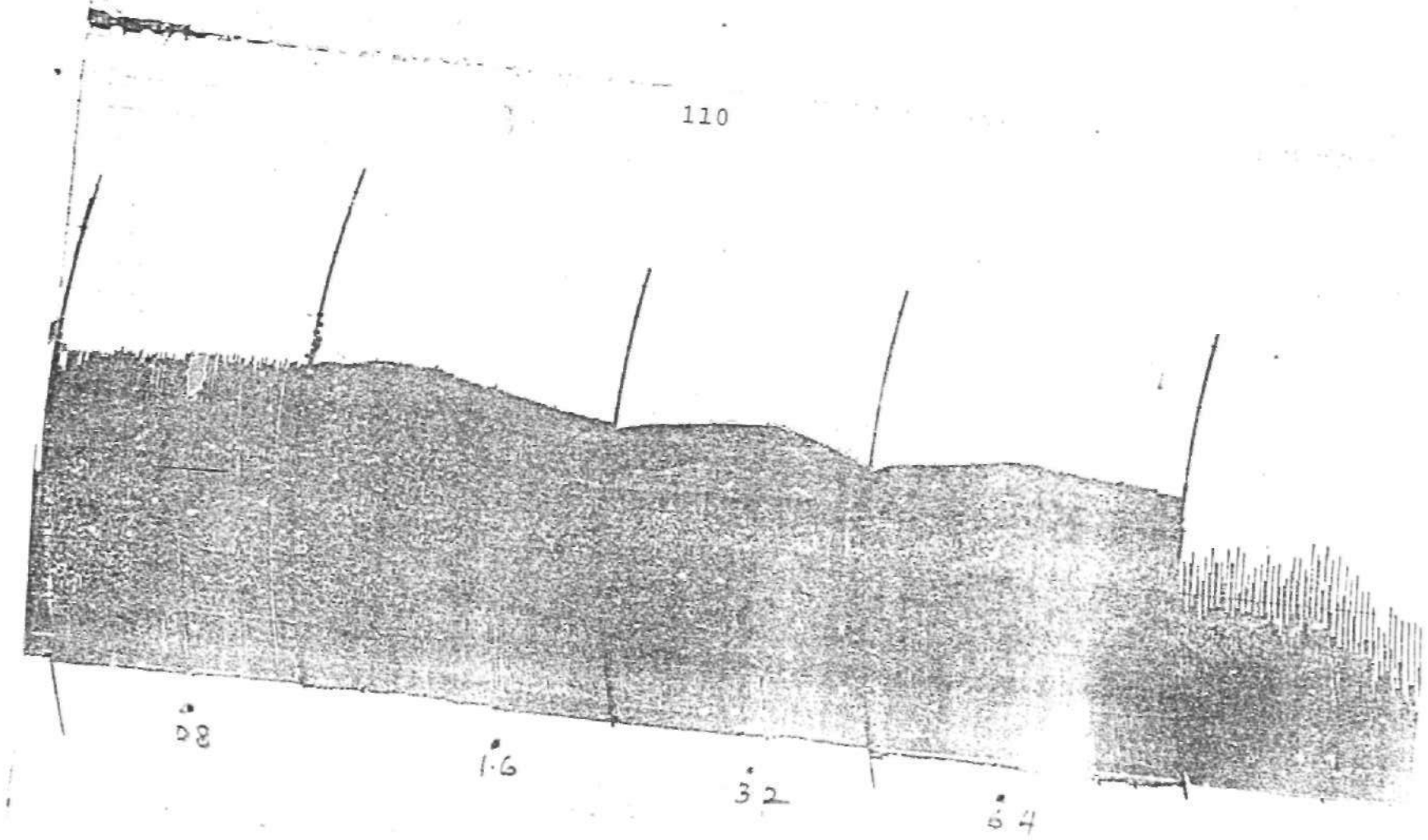
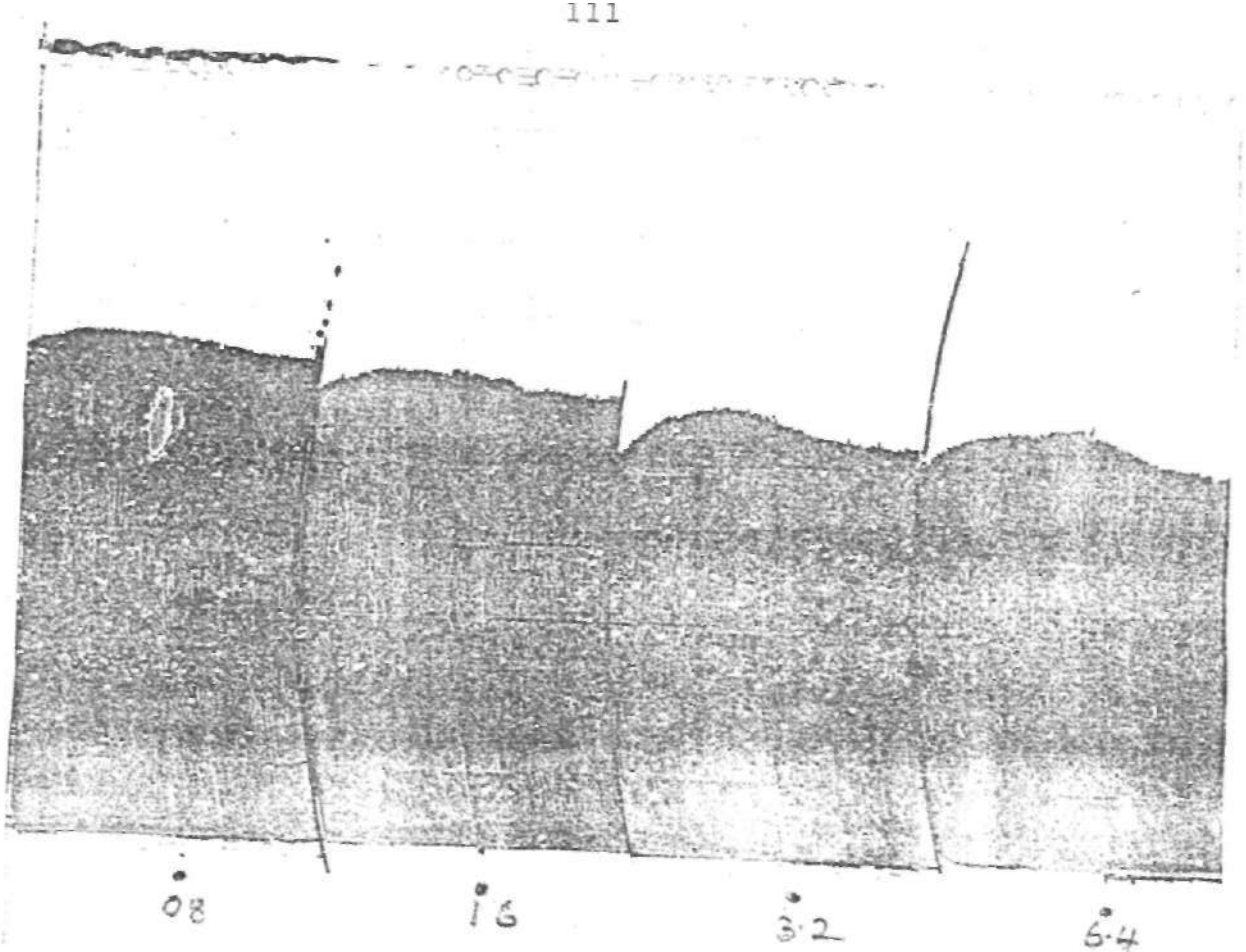
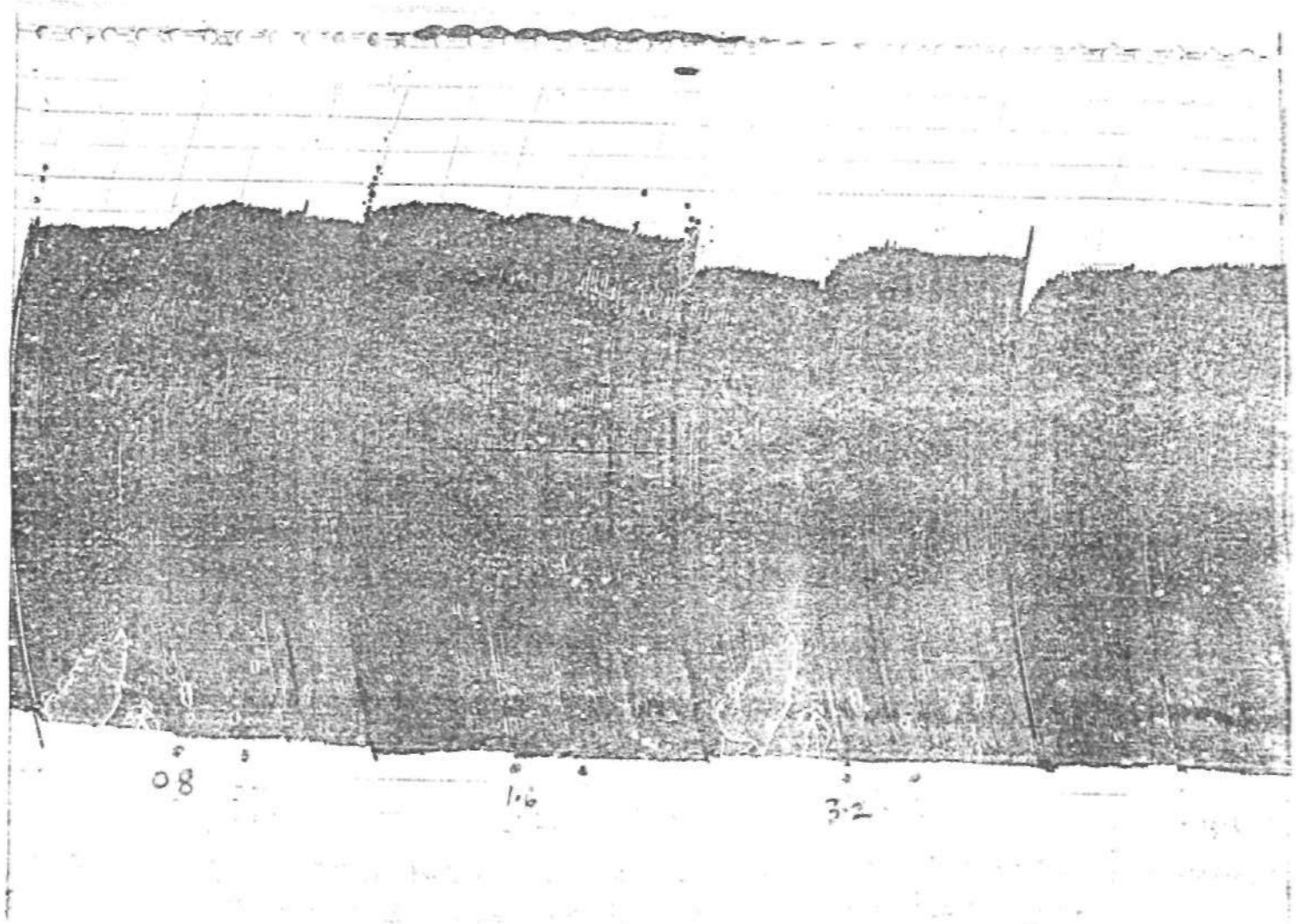


Fig 4h Effect of aqueous extract of A abida (Conc. In mg/ml)
phrenic nerve diaphragm.



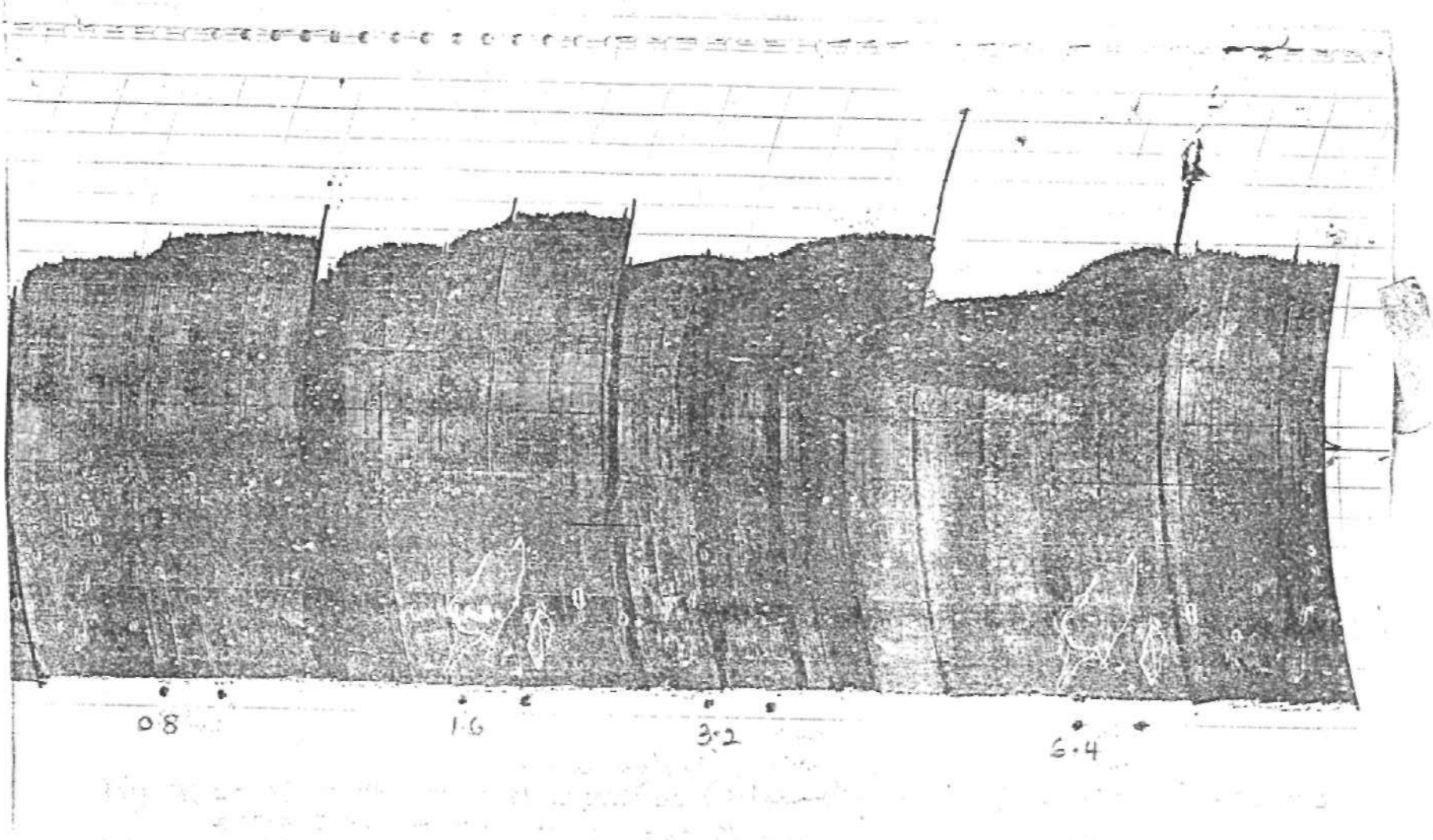
(Conc. in µg/ml)

Fig 4i Effect of d-tubocurarine on rat phrenic nerve diaphragm.



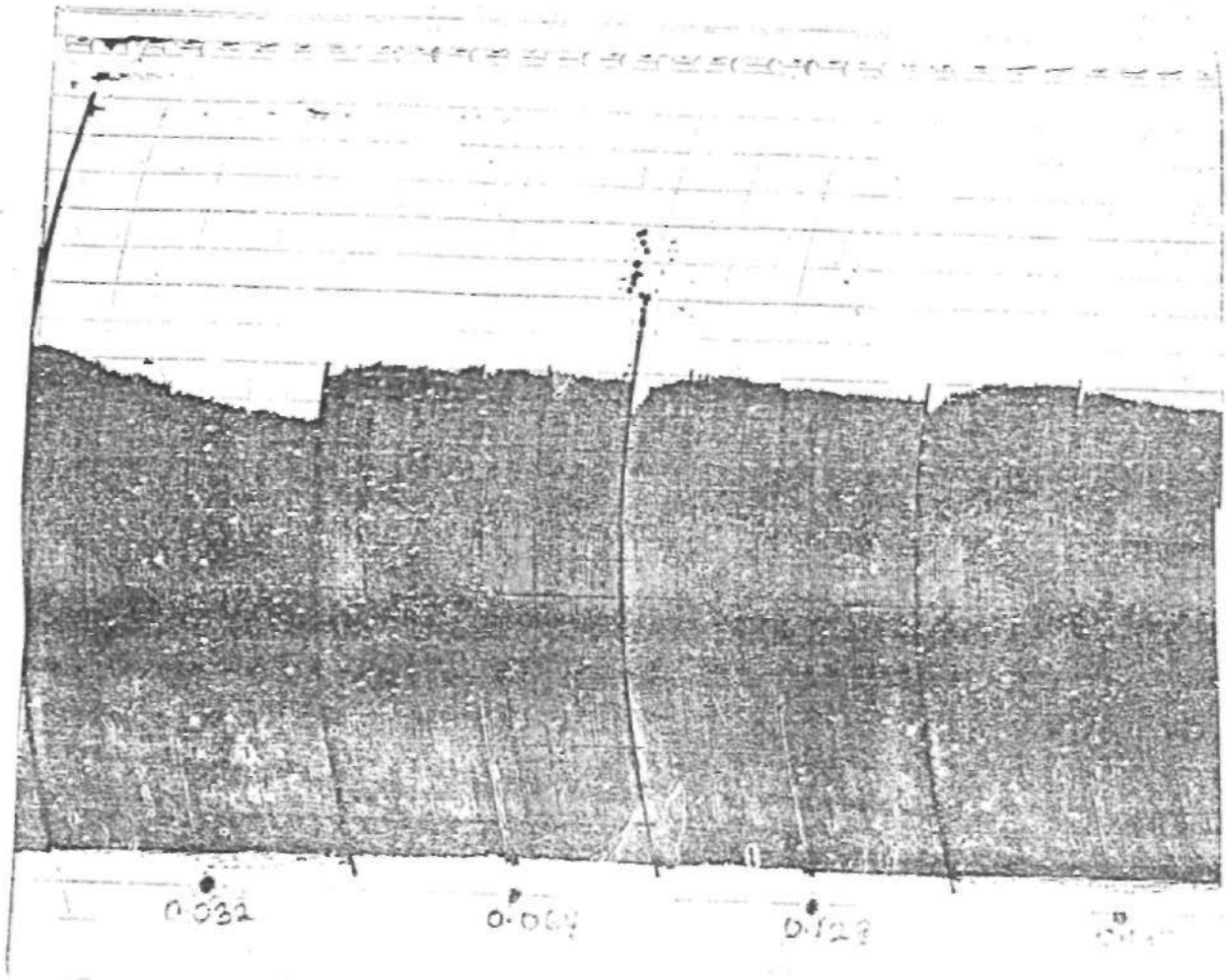
(conc in mg/ml)

Fig. 4j Effect of d-tubocurarine (1.6 ug/ml) in the presence of aqueous extract of A abida on rat phrenic nerve daphragm.



(conc. in mg/ml)

Fig. 4k Effect of Venom of N nigricollis (0.128 ug/ml) in the presence of aqueous extract of A abida on rat phrenic nerve diaphragm..



(Conc. in ug/ml)
Fig 41 Effect of Venom of N nigricollis on rat phrenic nerve diaphragm.

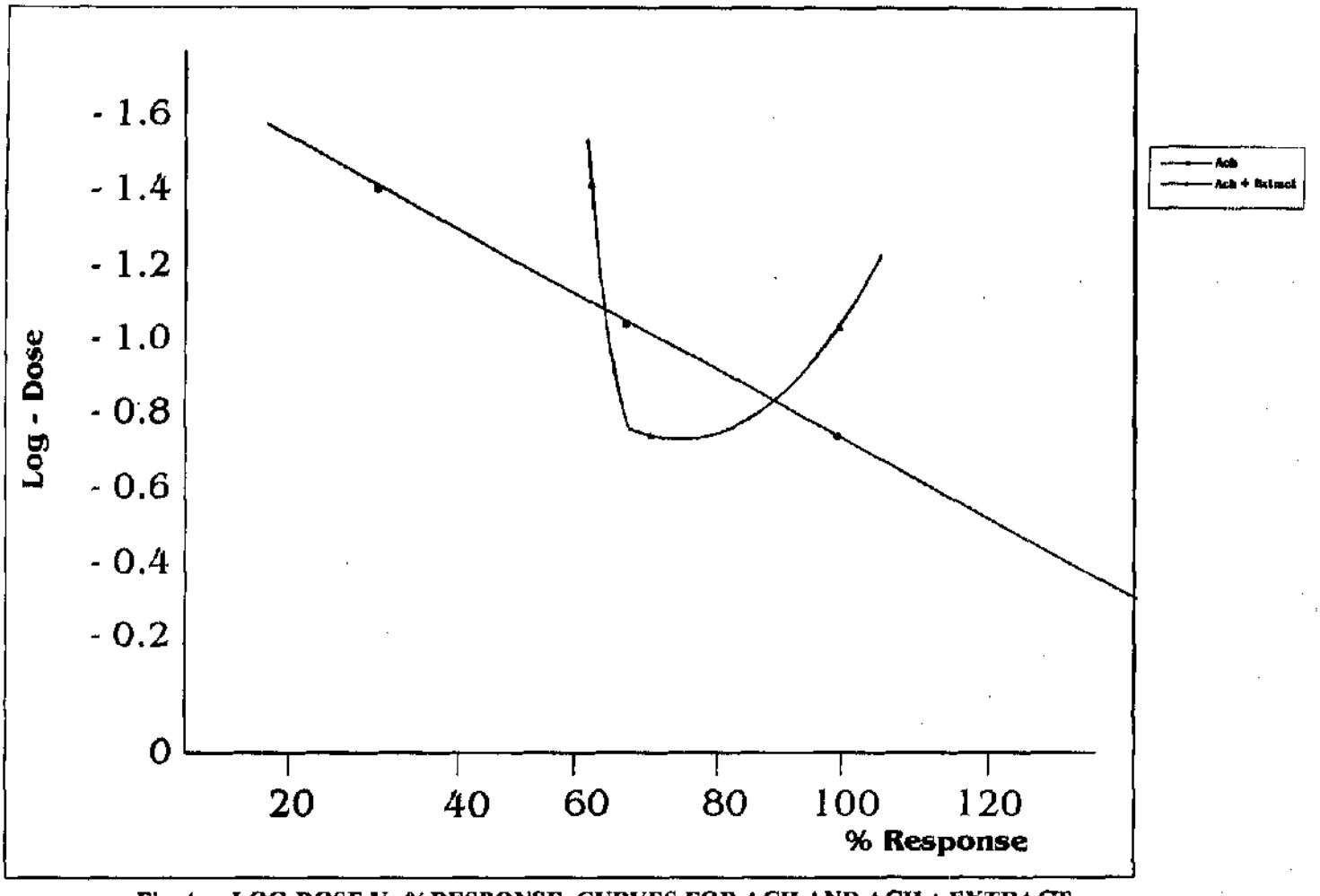


Fig. 4m: LOG-DOSE Vs %RESPONSE CURVES FOR ACH AND ACH + EXTRACT OF A. ALBIDA ON FROG RECTUS ABDOMINIS.

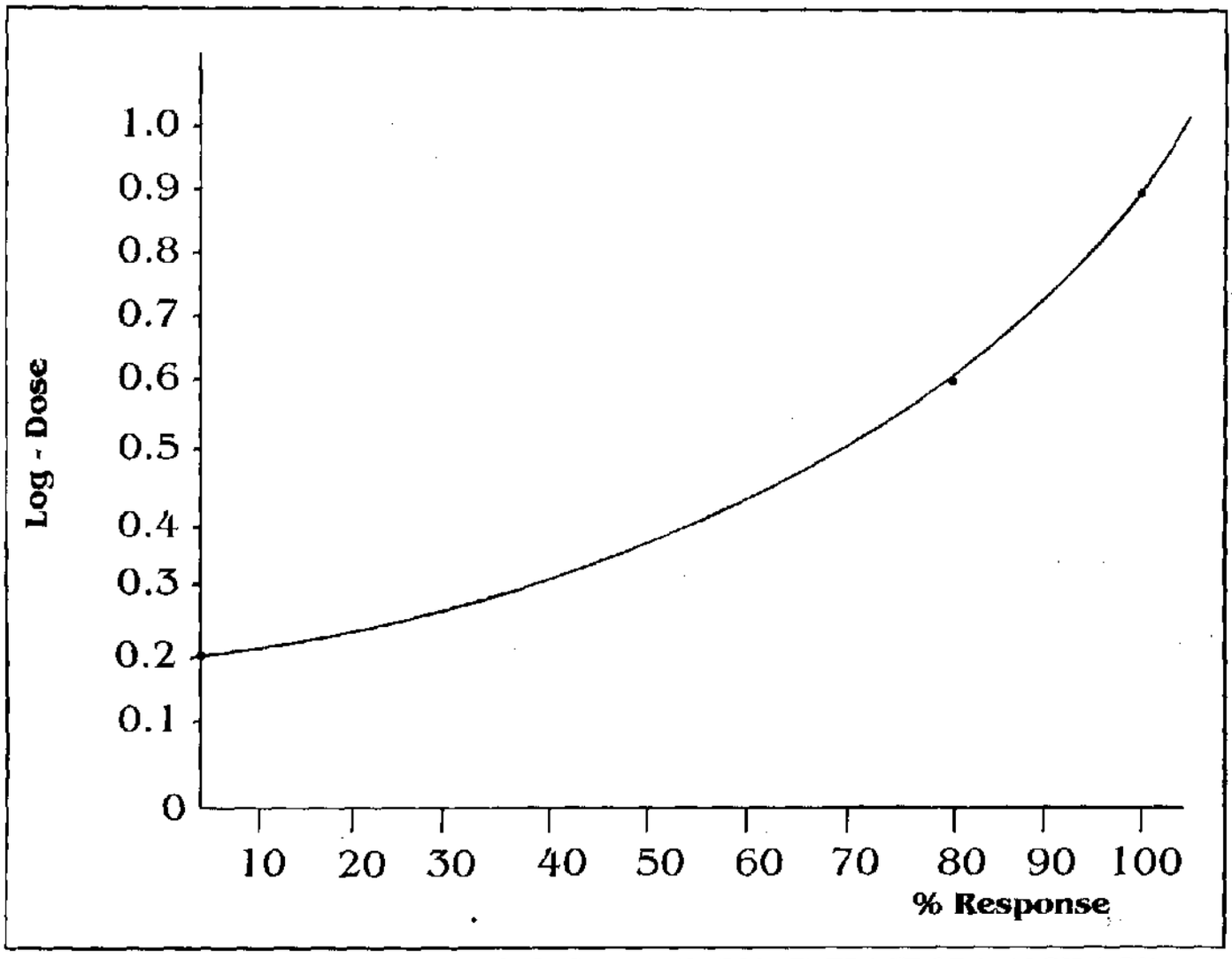


Fig. 4n: LOG-DOSE Vs %RESPONSE CURVE FOR EXTRACT OF A. ALBIDA ON FROG RECTUS ABDOMINIS.

REFERENCES

Achari, B. Bandyopadhyay, S. Saha, C. R. and Pakrashi, S.C. (1983). A phenanthroid Lactone. Steroid and Lignan from *A. indica* . *Heterocycles* **20** (5): 771 - 4.

Achari, B. Chakrabarty, S. and Patrashi, S. (1981). An N-glycoside and steroids from *A. indica*. *Phytochem.*(**6**): 769 - 70.

Ambros, M. L. and De-sequiera, N.A. (1971a) Aristolochic acids in *A. triangularis*. *Rev Brasil farm*, **52** (2): 62 - 3.

Ambros, M. L. and De-sequieram, N. S. (1971b) Phytochemical study of *A. triangularis*. *Rev. Brasil farm*, **52** (3): 125 - 9.

Bhat, M. K. Kasturi, S. and Gowda, T. V. (1991). Structure function relationships among neutrotoxic phospholipases. NN - XIII - PLA 2 from Indian cobra (*Naja naja naja*) and VRV PL - V Russell's Viper (*Viopera resselli*) *Venoms Toxicon*, **29**(1): 97-105.

Brain, K. R. and Turner, T. D. (1975).The practical evaluation of Phytopharmaceuticals Wright scientechnica, Bristol, pp. 152 - 154.

Carabaza, A., Cabre F., Garcia, A.M. Gomex, M. Sanchez, J. Mauleon, D. and Carganico, G. (1993) Inhibition of phospholipase A2 purified from human herniated disc. *Biochem Pharmacol;* 45(3): 783 - 6.

Castille, A. (1921). Constituents of *A. sipho Bull Acad. Roy. Med. Belg.* (**5**) 1: 569-76.

Castille, A. (1922). Aristolochic acid extracted from *A. sipha. J. Pharm. Belg*; **4**: 569 - 571.

Choudhury, M.K. and Haruna, A. K. (1994) Phytochemical investigation of *Aristolochia albida* Duch. *Indian J. Pharm.Sci.*, **56**:230.

Che, C. T. Cordell, G.A. Fong, H.H., S., Evens A. (1983) studies on aristolochia part 2 Aristololin diquinone. A new naphthoquinone from *A. indica* L. *Tetrahedron Lett.* **24** (13) 1333 - 6.

Cortes, D., Dodoun, H; Paiva, R. L. et al (1987) New bisbenzyl isoquinoline alkaloids isolated from leaves of *A. gigantea J. Nat. Prod.*, **50** (5). 910 - 14.

Dalziel, J. M. (1937). Useful plants of Tropical West Africa Crown Agents For Overseas Governments and Administrations, London, P. 316 - 17.

Dazeley, G. H. (1979). Organic chemistry, Cambridge University press, Cambridge , New edn. P. 29.

Elliot, G.R. Edelman, A. M., Bensan, J.F. and Berger P.A. (1977). Indole amines and other Neuroregulators, in psychopharmacology From Theory to practice (Braches, J.D. Berger, P.A., Claranello, R.D. and Elloit, G.R. eds) Oxford Unversity Press London.

Etkins, N. (1990). Indigenisation of pharmaceuticals: Therapeutic transitions in rural Hausa Land. *Social Science and Medicine* **30**:919 - 928.

Finar, I. L. 1975). Organic chemistry. Vol. 2. The fundamental principles 5th Edition pp. 411.

FreuVogel, T. A. (1972) Posionous and Venomous animals of East Africa. *Acta Tropcia.* **29**: 401 - 451.

Ganshirt H. (1953a) Localisation of Aristolochia acid in seeds of *A. clematitis Nativwissenschaften.* **40**: 247.

Ganshirt. H. (1953b) Isolation of aristolochic acid from various Aristolochiaceae and its quantitative determination pharmazic **8**: 584 - 92.

Govindachari,m T.R., Mohammed, P. A and parthasarath, P.C. (1970). Iswarane and aristolochiae, two new sesquiterpene hydrocarbons from *A. indica. Tetrahedron* **26** (2): 615-19.

Grzeyurz, S. (1963). Free amino acids from *A. clematistis. Polania Sect.* DD **18**: 91 - 07.

Harbone, J.B. (1973) Phytochemical Methods A guide to modern Techniques of plant Analysis Chapman and Hall, London, pp. 116, 518.

Harris, G. Dictionary of organic compounds Eyre and Spottiswood publishers Ltd., E & F N spon Ltd., London, Vol. 5, P. 2449.

Haruna, A. K. and Choudhury. M. K. (1995) *In vivo* Anti-snake venom Activity of A furanoid Diterpene from *Aristolochia albida* Duch (Aristolochiaceae) *Indian J. Pharm. Sci.* **57**: 222.

Houghton, P. J. (1990). Africa Med. Plants. Future Research in School of Pharm. Univ. of London.

Houghton, P.J. and Harvey, A. R. (1989) Neurotoxic and cardiotoxic effects of snake venoms on nerve muscle preparation. *Plant Med.* **55**: 273 - 276.

Houghton, P.J. and Ogutveren, M.(19991b) Aristolochia acids and aristoloctams from *A. auriscularia. Phytochem.* **30** (1): 253 – 4.

Hutchinson, J. and Dalziel, J. M. (1937) Flora of West Tropical Africa. The crown agents for the colonies London Vol. I, 2nd edn. P. 695.

Irvine, F. R. (1961) Woody plants of Ghana with special Reference to their uses. Oxford University press London 2nd edition pp. 878.

Jagannandha, K.V.R. Ramachandra, R. L. and suryaharayanamurty, Y. (1959) chemical examination of *A. bracteats. Sci. ind. Res.* (India) 18B: 245-6.

Josette, T. (1965). The flavonoids of leaves young stems, and old stems of *A. sipho. Ann. Sci. Univ. Besancon Botan,*. **3** (2). 12-23.

Krishnaswamy, P. R. Manjuunath, B. L. and Venkata, R. S., (1935) Chemical examination of the roots of *A. indica* Linn. I. *J. Indian Chem Soc.*, **12**: 476 - 85.

Lopes L.M. X. and Bolzani, v. (1988). Lignans and diterpenes of three aristolochia spp. Phytochem. **27** (7): 2265-8.

Lopes, L.M.X., Bokani, V.S. and Trevisan, L. M. V. (1997) Clerodane diterpenes from *Aristolocha* spp. *Phytochem*; **26** (10) 2781 – 4.

Nakanishi, T. Iwasaki, K, nasu, M, Miura I., Yoneda, K. (1982) Aristoloside, an aristolochia acid derived from stems of *A. manshuriensis. Phytochem*, **21** (7): 1759 - 62.

Nashida, R. and Kumazanci, Z. (1973). 3-oxoischwarane a new sesquiterpenic ketone from *A. debilis. Agric. Boil. Chem.* **32** (2): 341.

Nelson, B.K. (1989). Snake envenomation Incidence clinical presentation and Management. *Toxicol Manag. Rev. Medical Toxicology* **4**. 17-31.

Olaniyi, A. A. (1985). Providing local raw materials for the pharmaceutical industries in Nigeria The *Nig. J. Pharm*, **16** (1): 20.

Olaniyi, A. A., and Ogungbamila, F. O. (1991). Experimental pharmaceutical chemistry, Shanesan C.I. Ltd., Ibadan, Nigeria P. 229.

Olaniyi, A. A. and Motoyeshi, S. (1990) comtributions to the phytochemistry of medicinal plants growing in Nigeria as reported in the 1979 - 1990 literature. A preview. *Afr., J. Pharm & Pharm Sci.*, **22** (3) 177.

Osibogun, I.M. Houghton, P. J. Theakston, R.D.G and Laing, G. (1994). *In vivo* protective activity of Schumaniophyton magnificum extracts against cobra venom. *J. Pharm. Sci. and pharm. Pract.* Vol. 2: 75 - 77.

O. Vital Brazil, M.D. Fontana, and N.F. Heluany (1995) Mode of action of the cobral snake Micrurus spixii Venom, at the neuromuscular Junction *Journal of natural Toxins*, **4** (1): 19-32.

Pailer M. Belohav,. L.and Simmonitsch, E. (1955). Constitution of aristolochic acid, a natural plant product with nitro group I *Monatsch Chem.* **86**: 767-80.

Priestap, H.A. (1985b) Two carboxy -a nd two hdyroxylmethyl substituted aristololactams from *A. argentina Phytochem.*, **24** (12): 3035 - 9.

Priestap, H. A. Ruveda, E. A. Macaretts, O. A. and Deulofeu, V. (1971) Aristolochic acids from *A. argentina* and *A. esperanzae Ann. Asoc. Quin Argent*, **59** (3-4): 245 - 50.

Priestap, H. A. Ruveda, E. A., Albonico, S. M. and Deulofeu V (1972). Alkaloids from the roots of *A. argentina. Phytochem.* **24** (12): 3035 –9.

Rang H.P and Dale M. M. (1987) Pharmacology. Longman Group, Singapore, P. 161 - 176

Ruveda, E. A. Priestap, H. A. and Deulofeu, V (1966). Alkaloids and aristolochic acids from *A. argentina An assc. Quin. Argent*, **54**: 234 – 9.

Salvatore, C. O. Domenico, S. and Luciano, M. (1966). Constituents of A. rotunda. *Gazz. Chim Ital.*, **95** (5): 641- 61.

Scheinmann, F. (1970). An introduction to spectroscopic methods for identification of organic compounds. Nuclear Magnetic resonance and infrared spectroscopy. Vol. 1. Pergamon Press, Oxford, p. 169, 181.

Schild, H. O. (1980). Applied Pharmacognosy Churchill Living stone 12th edn, Edinburgh, P. 115, 147.

Shoji, J. (1968) Extractioon of aristolochic acid from *Aristolochia* Japan 7127, 834 (C1 Bold, A16k Co7d). 12 Aug. 1971. Appl. 02 Dec. '68

Sofowora, A. (1982) Medicinal plants and Traditional Medicine in Africa, John Wiley & Sons Ltd.,. Chichester, U. K. p. 142 - 145.

Sofowora, A. (1986) State of Medicinal plants Research in Nigeria Ibadan University press, Ibadan, Nigeria P. 109.

Tada, A. Sose, K; Ohmura I., Shoji J. and Tanoka, O. (1969). Aristolochiaceae plants I: Isolation of aristolochic acid from A. *griffithii.. Shoyakugaku zasshi*, **23** (2): 99-101.

Theakston, R.D.,G. (1994). Study of the potency and safety of a new monospecific f(b) Echis antivenom in the treatment of patients with incoagulable blood after bites by *Echis occellatus* in Nigeria. *Nigeria protocol. Livepool School of Trop. Med. Bull*,. (II).

Tsai, L. H. Yang, L.L. and Chang, C. (1980) Inactivation of formosan snake venoms *in vivo* by aristolochic acid, the chemical component of *Aristolochia radix Taiwan ko Hsueh*, **34** (2): 40-44.

Trease, G. E and Evans W.,C. (1993). A Textbook of Pharmacognosy, 12th edn. Bailliere Tindall. London P. 658 - 692.

Tu, A.T. (1987) Bioxtoxicology of sea snake venoms *Annals of Emergency Medicine* **16**: 1023 - 1028.

Urzua, A. and Shamma, M. (1988). The 4-aryltetralones of *Aristolochia chilensis Nat. prod.* 51 (l): 117 - 21.

Vogel, A. I. (1974) A testbook of practical organic chemistry including qualitative organic Analysis Longman Group Ltd., London, p. 781.

Walter, H. I., Memory, P. F. and Lewis, E. (1977). Plants affecting man's health In: Medicinal Botany J. W. and Sons, New York, London, sydney, pp. 345 - 7.

Warrel, D. A. (1989) snake bites in five constituents. In. Horizons in medicine Bunch C. ed. Balliere Tindall pp. 106 - 114.

Warrel, D. A. (1993a) Snake bite and snake vaenoms. Editorial *Ojm. Quarterly Medical Journal*, 1993.

Warrel, D. A. (1993b) Venomous bites and stings in *Saudi Arabia Saudi Medical Journal*, **14** (3): 196 - 202.

Warrel, D. A. (1993c) Venonomus bite and stings in the Tropical World. Tropical Health. The medical J. of Australia 159(6/20): 773 - 779.

Warrel, D. A. Greenwood, B., M. Davidson, N, M. Ormerol, L. Dand Prentice,, C.R.M. (1976) Necrosis, haemorrhage and complement depletion following bites by the spitting cobra (*Naja nigricollis*) *Quart. J. Med.* **45**: 1 - 22.

Watt. J. M. and Brayer, G. M. (1962) The Medical and poisonous plants of South and Eastern Africa E & S. Livingstone Edinburg. 2nd Edition pp. 100 - 119.

Wilson, C., O. Grisvold O. and Doerge R. F. (1977). Textbook of organic medicinal and pharmaceutical chemistry 7th Edition.

Zhu, D. Wang, B, Huang, B; Xu. R. *et al.*, (1982). Two new 4,5-dioxosaporphine alkaloids isolated from *A. tuberosa*. Heterocycles, 17 (Sept. Issue): 345 - 7.