ITLE: A PRELIMINARY PHYTOCHEMICAL INVESTIGATION OF CROTON MACROSTACHYUS STEM BARK

BY

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A dissertation submitted in partial fulfilment of requirements for the award of Bachelor of Pharmacy degree.

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JUNE 1986

DEDICATION

To my father and mother, who
through the years, months, weeks
and days; have laboured tirelessly,
to see that I was well prepared and
equipped, to tackle and accomplish
this piece of work.

AND

To those for whom I struggled day
and night, to see that it was done.

ACKNOWLEDGEMENTS

I am agreatly indebted to the following people.-

Dr. Addae - Mensah Ivan.

My supervisor, whose patient guidance, help and constant encouragement went a long way to see the project through, and for critically reading through the report and making useful suggestions.

Muxalughu R. G.

For the technical assistance he offered; especially, for running all the infra - red spectra.

Hamilton O. Orata

My brother-in-law who, without reserve, saw to it, that this script was typed.

Mrs. Mary G. Aguyo

Who did an excellent job in actually typing the script.

CONTENTS

ABSTRACT

During a preliminary investigation of the petroleum ether extract of *C. macrostachyus* stem bark, three compounds were isolated;- lupeol; a compound with a probable terpenoid structure; and an aliphatic compound containing a carbonyl function. In addition, betulin was identified.

An account is given, of the use of various croton species in traditional herbal medicine in Africa, and of the work reported on *croton* genus during 1974 - 1976. Work done on *C. mactrostachyus* uptill 1984 is also given.

INTRODUCTION

The genus *Croton* of the Euphorbiaceae family is widely distributed in many parts of Tropical Africa, from Savanna forests to marshes, rocky stream banks, dry deciduous forests, etc [1]

Some of the Crotons found in E. Africa include *C. dichogamus; C. elliotianus, C. jatrophoides, C. macrostachyus, C. schefferi C. megalocarpus, C. menyhartii, C. polytrichus, C. sylvaticus,* and *C. pseudopulchellus.* [2] These, and several other crotons are used all over Africa, for their medicinal or poisonous effects.

The Maasai of Kenya mix the bark of *C. elliotianus* with curdled milk and use it as a purgative. The seed oil from the kernel of the same plant are purgatives and are also mildly antihelmintic. Small doses of both oil and bark cause diuresis. If appreciably absorbed, the seed and oil cause haemolysis and haemorrhagic spots in tissues; the seed being more poisonous. [3]

In Mbulu (Tanzania) the dried leaves of *C. dichogamus* are burnt for inhalation by, or fumigation of a patient with fever. It is also an excellent remedy for chest ailments and stomach diseases. Chopped roots are added to soup made from goat's meat, and taken as a tonic. [4]

The strongly scented roots of *C. jatrophoides* are used by the Swahili along the E. African coast, for colds and stomach aches. [4]

Various parts of *C. macrostachyus* are used in Kenya, Tanzania and Ethiopia, among other countries. The leaves are boiled and the decoction drunk for coughs. Ash from burnt leaves is also licked for coughs. Juice from fresh leaves is applied onto fresh wounds to hasten blood clotting. Root decoction is used as a purgative and as a taenifuge. Juice from boiled roots is drunk for malaria or venereal diseases. The bark peeled off stems and roots is boiled in water and neonates bathed in the mixture, as a remedy against skin rashes. The seeds and resin of the plant are poisonous. [4]

The oil expressed from seeds of *C. megalocarpus* has been reported on favourably, for medicinal purposes. The Chagga of Tanzania soak the pounded bark in water overnight and drink the extract as an antihelmintic, and for treatment of whooping cough. [4]
The Maasai use the bark decoction mixed with blood, as a tonic. [3]

The root decoction of *C. menyhartii*, is drunk for treatment of influenza and malaria, while roots of *C. polytrichus* are used for headaches and labour pains, by the Iraqw of Tanzania. [4]

C. pseudopulchellus also finds various medicinal uses. Leaves are boiled and applied to the chest for colds by the people along the Kenyan coast. The Nyamwezi, also drink a decoction of roots for asthma. Leaves and twigs are boiled and the juice drunk for gonnorheal and syphilitic sores. An infusion of the leaves is given to cattle as a remedy for anthrax, while the leaves are burnt among crops as an insecticide. [3,4]

C. scheffleri is used as a remedy for miscarriage in Tanzania. The Swazi use the bark of *C. sylvaticus* for gall sickness in cattle, while in Gazaland, the same is used as a fish poison. The Swazi use the root for pleurisy and indigestion. [3] The Digo, found along the Kenyan coast, pound the root of *C. sylvaticus* to make poultices for swellings. A decoction of roots and bark is drunk for tuberculosis, while the leaf decoction is used as a wash for body swellings caused by kwashiokor or tuberculosis. An infusion of the leaves is taken as a purgative. [4]

Croton gratissimus is said to be very poisonous, but despite that, it is used by the Zulu as a carthatic and erruptive irritant. The bark is applied to the chest wall, for any painful respiratory disorders, for intercostal neuralgia and pleurisy. It is also used for dropsy and indigestion. When powdered, it is used as one of the ingredients for a remedy against uterine disorders. The Sotho of Transvaal brush bleeding gums with the charred and powdered bark, while the leaf is used as one of the ingredients for 'smoking' rheumatic patients. [3]

The bitter bark of *C. gubouga* is used as an anti-malarial by Portuguese East Africans, and by natives of Transvaal; while it has also been used as a fish poison in Gazaland and Eastern Transvaal. Both seed and bark cause an intense burning sensation in the throat, salivation, nausea, and slight purgation in man. The Shangana use the seed for purgation, while the Luvale administer an infusion of the root to thin babies to make them fat. [3]

Croton oil from the seed of *C. tiglium* has been used as a very powerful purgative. It is also mildly antihelmintic. The seeds have been used as human poison in W. Africa.

The leaf decoction of *C. zambesicus* is used as a wash for fevers in Sierra Leone and S. Nigeria. It is also drunk for fever, dysentery and convulsion. The roots are used to clear the bowels of waste matter, and seeds find medicinal use in Togo. The Maasai use the plant, together with *Grewia villosa* as an aphrodisiac. [3]

Due to the wide use of plants in this genus, work has been (and is still being) done, on various species, in different parts of the world, to isolate the active principles, determine their structures, and determine their pharmacological activities.

CHAPTER I

WORK DONE ON VARIOUS CROTON SPECIES BETWEEN 1974 - 1976

Croton genus

Evans F.J. and Kinghorn A.D. [5] identified polyhedric diterpene alcohols from the *Croton* and *Euphorbia* genera. The method employed was acetylation of the free alcohol$, followed by TLC separation and colour reactions on silica gel and alumina, with several different solvent mixtures and sprays.

The TLC places used were:

1. Silica gel G with solvent systems S_1 S_2 S_3
2. Silica Gel H with S_4
3. Alumina E with S_5 - S_{11}

Solvent Systems

S_1 Chloroform: Ether (95:5)

S_2 Ether: Ethylacetate: hexane (1:1:1)

S_3 Hexane; Isopropyl alcohol (2:1)

S_4 Chloroform: Ethyl acetate (2:3)

S_5 Toluene: Ethyl acetate: Benzene)95:5:50)

S_6 Chloroform: Acetone: Benzene (95:5:50)

S_7 Chloroform

S_8 Hexane: Ether: Benzene (1:2:1) Eluted three times

S_9 Benzene: Hexane; Ether: Ethylacetate (20:40:15:30) eluted three

S_{10} Chloroform : Ether: Benzene (1:3:3) Eluted three times

S_{11} Ethylacetate: Benzene (1:3) Eluted four times

Reagents for Visualisation

A - 60% w/w sulphuric acid. Plates were heated at 110°C for 15 minutes.

B - 5% w/w vanillin in conc. H_2SO_4

Plates were heated at 110°C for 5 minutes.

C - 1% w/v anisaldehyde and

2% v/v H_2SO_4 in glacial acetic acid

Plates heated at 110°C for 10 minutes

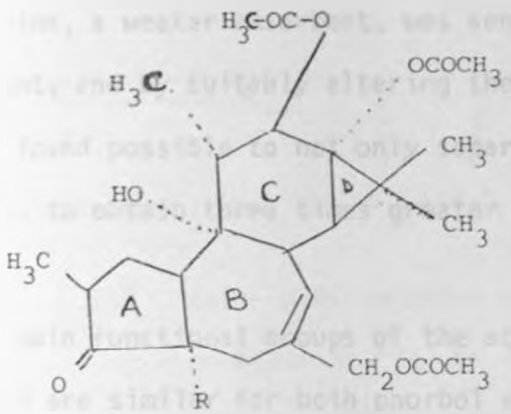
D - Methanol: Sulphuric acid (1:1) - plates heated at 110°C for 15 minutes.

All plates were viewed in daylight and under UV light at 366 nm. The diterpene acetates produced characteristic colours under both conditions, when sprayed with the acid-based sprays indicated. The type of adsorbent used was found to affect the colour produced.

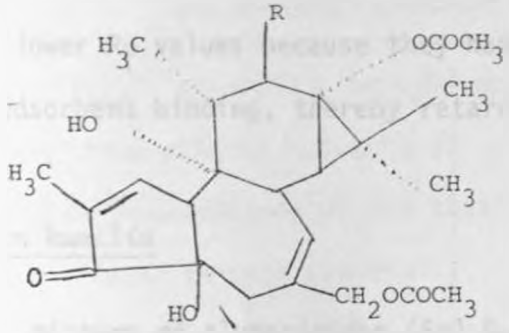
The diterpene acetates were found to fall into two groups:

(i) The phorbol type of lower R_f values

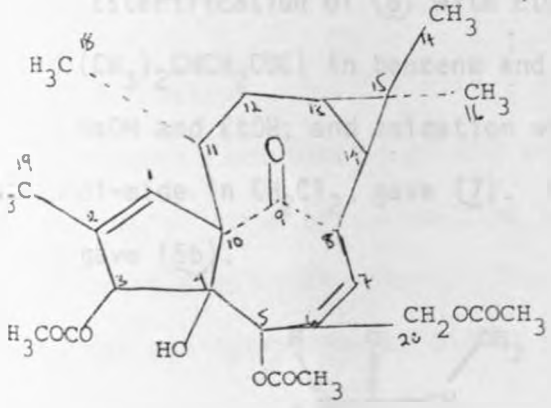
(ii) The ingenol type with higher R_f values



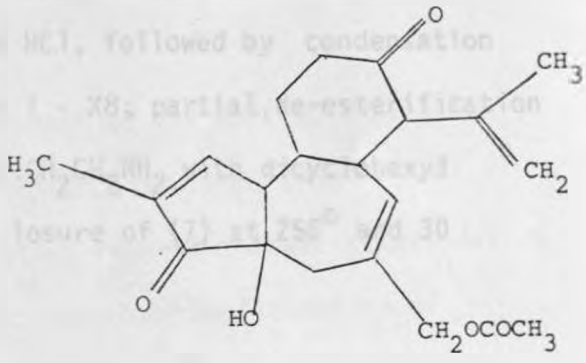
	R	
(1a)	H	- 4 -deoxy - 4α - phorbol triacetate
(1b)	HO	- 4α - phorbol triacetate
(1c)	O-$COCH_3$	- 4α - phorbol tetracetate



	R	
(2a)	$OCOCH_3$	Phorbol triacetate
(2b)	H	12 - deoxy-phorbol diacetate



(3) Ingenol Triacetate



(4) Crotophobolone monoacetate

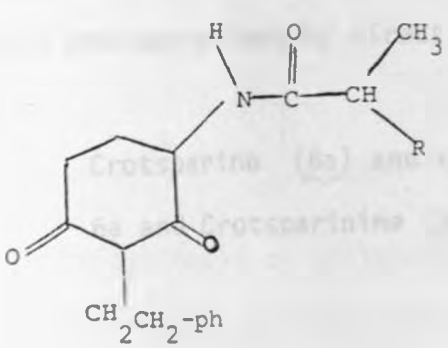
On silica gel, resolution within the phorbol group was found to be poor, an observation characteristic of non-alkaloidal polyfunctional compounds on strong adsorbents.

Alumina, a weaker adsorbent, was senstive to both molecular shape and weight, and by suitably altering the composition of eluting solvent, it was found possible to not only separate ingenol and phorbol types, but also, to obtain three times greater resolution within the phorbol types.

The main functional groups of the acetates are situated in rings A and B which are similar for both phorbol and ingenol types, except that ingenol triacetate has an acetate group at C-3 instead of keto, and lacks an α- OH gp at C-10, (Junction between rings A and B). This explains the difference in migration characteristics. Thus, phorbol type of diterpene acetates have lower R_f values because they have more OH groups that provide sites for adsorbent binding, thereby retarding their movement.

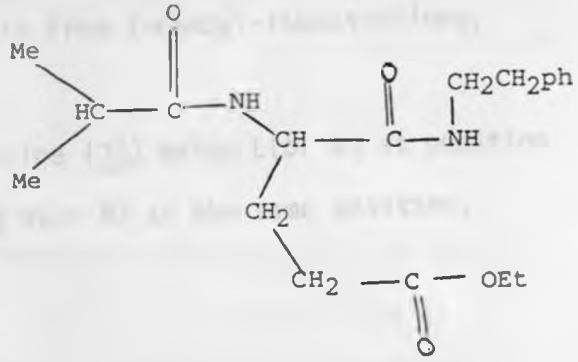
Croton humilis

A 1:1 mixture of glutarimides (5a) R = me, (5b) R = Et was isolated from *C. humilis*, and (5b), was prepared in 5 steps from L -glutamic acid (6) [6]. Esterification of (6) with EtOH and HCl, followed by condensation with $(CH_3)_2CHCH_2COCl$ in benzene and Dowex 1 - X8; partial de-esterification with NaOH and EtOH; and amidation with ph.$CH_2CH_2NH_2$ with dicyclohexyl carbodi-mide in CH_2Cl_2, gave (7). Ring closure of (7) at 250° and 30 mmHg gave (5b).



(5a) R = Me

(5b) R = Et



(7)

Croton sparsiflorus

D. S. Bhakuni, Sheo Satish and M. M. Dhar [7] isolated

(i) three pro-aporphine bases provisionally designated crotsparine (8a) mp 193° - 194°, N-methyl crotsparine (8b) mp 223° - 225°C and N,O dimethyl crotsparine (8c) mp 125° - 127°C;

(ii) two dihydro pro-aporphines. Crotsparinine (9a) mp 184° - 185°C and N-methyl crotsparinine (9b) mp 160° - 161°C;

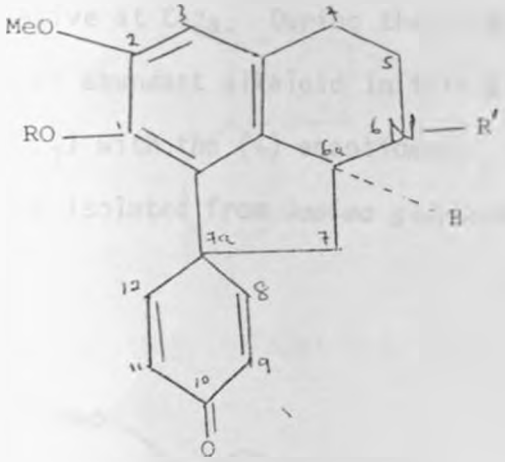
(iii) sparsiflorine (10) mp 229° - 231°C;

from ethanol extracts of *C. sparsiflorus*. The relative concentrations of the bases isolated from this plant were found to vary seasonally.

N-methyl crotsparine (8b) is of biological interest as a hypotensive agent.

D. S. Bhakuni *et al* [8] give a full account of the work leading to structural elucidation of the above compounds isolated from c. sparsiflorus, and also give information regarding other alkaloidal constituents of the plant. They report absolute configuration of these pro-aporphines by direct synthesis from 1-benzyl-isoquinolines, as -

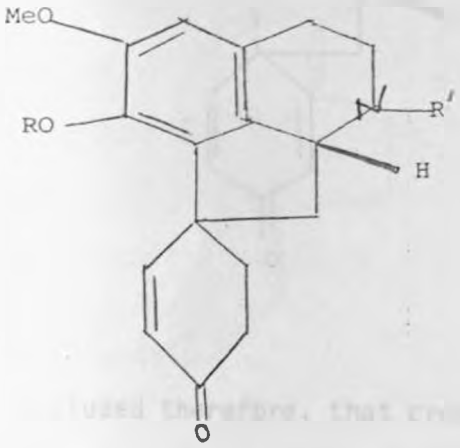
Crotsparine (8a) and sparsiflorine (10) being L(or s) at position 6a and Crotsparinine (9a) being D(or R) at the same position.



(8a) R = R^1 Crotsparine

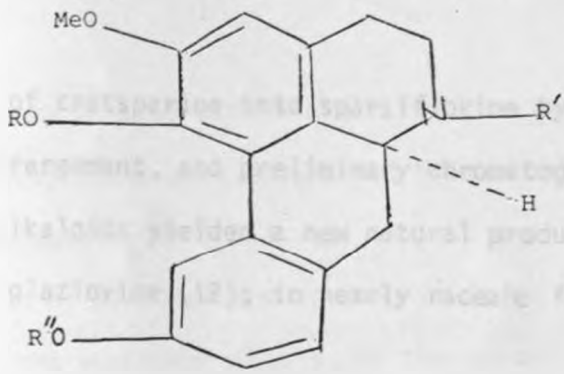
(8b) R = H; R^1 = Me. N-methyl Crotsparine

(8c) R = R^1 = Me. N,O-Dimethyl Crotsparine



(9a) R = R′ = H Crotsparinine

(9b) R = H; R′ = Me, N-methyl Crotsparinine

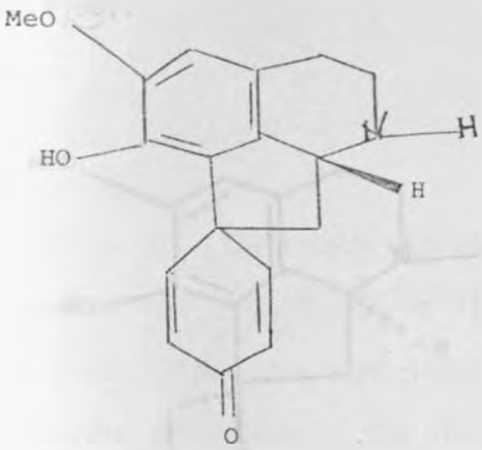


(10) R = R′ = R″ = H Sparsiflorine

The biosynthesis of crotsparine, crotsparinine and sparsiflorine in the same plant was studied by Bhakuni D. S. *et al* [9], using racemic triturated, coclaurine and norcoclaurine. Tyrosine and coclaurine were shown to be the precursors of all the three alkaloids.

Cesare Casagrande *et al* [10] studied these alkaloids further, to try and ascertain the configuration of crotsparinine and its N-methyl derivative at C-7a. During their study, they observed that crotsparine, the most abundant alkaloid in this plant extract was identical (tlc, ir in $CHCL_3$) with the (+) enantiomer, glaziovine (11) which they had earlier isolated from *Ocotea glaziovii;* and whose $[\alpha]_D$ was $^{+}135^{o}$.

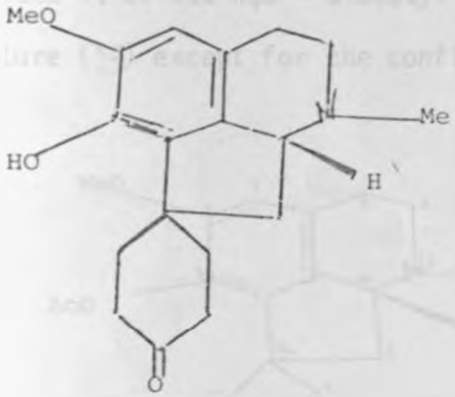
$[\alpha]_D$ of crotsparine was found to be -32^{o}



(11) glaziovine

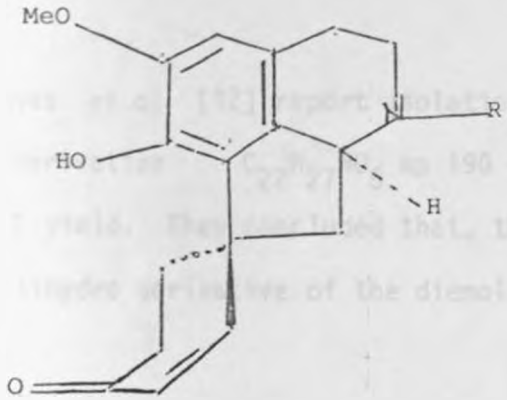
They concluded therefore, that crotsparine occured as (±) and (-) forms; a situation they had already encountered with glaziovine in *O. glaziovii*

Conversion of crotsparine into sparsiflorine by acid catalysed dienone-phenol rearrangement, and preliminary chromatographic separation of the remaining alkaloids yielded a new natural product in *C. sparsiflorus*, tetrahydro-glaziovine (12); in nearly racemic form. $[\alpha]_D + 2^{o}$



(12) Tetrahydroglaziovine

In addition, they isolated isocrotsparinine (13a) and its N-methyl derivative (13b).



(13a) R = H Isocrotsparinine

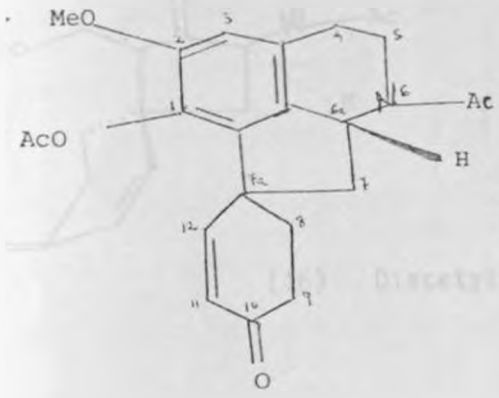
(13b) R = Me N-methyl isocrotsparinine

The structures of isocrotsparinine, its N-methyl derivative and (±) tetrahydroglaziovine were determined from chemical and spectral data. The stereochemistry of (13a) (6a = β H) and its isomer crotsparinine (9a) (6a = α H) were defined as (6aR 7aS) and (6aS 7aS) respectively, indicating the stereospecificity of the biosynthetic process.

Croton linearis

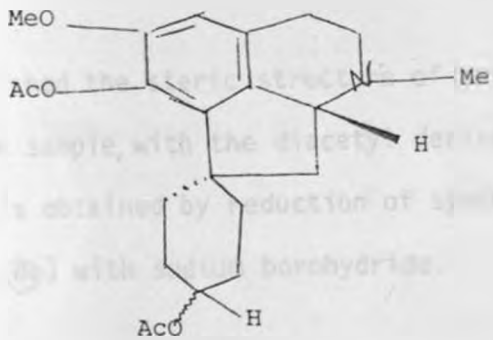
Jacularine and base E, two minor alkaloids of *C. linearis* have been isolated as their acetyl derivatives. J. K. Stuart *et al* [11] report isolation and characterisation of jacularine.

They isolated it as its N,O - diacetyl derivative and established its structure (14) except for the configuration at C-7a.



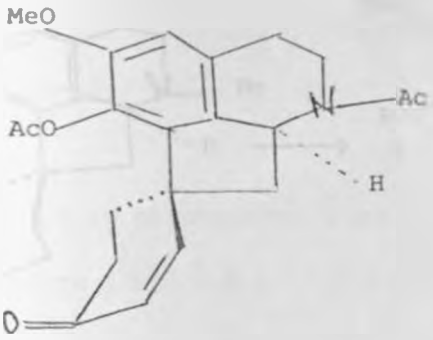
(14) N,O diacetyl jacularine

L. J. Haynes *et al* [12] report isolation of base E as its O,O-diacetyl derivative $C_{22}H_{27}NO_5$ mp 190 - 193°C, from *C. linearis*, in very small yield. They concluded that, the new compound was the 8,9 or 11,12-dihydro derivative of the dienol (15).

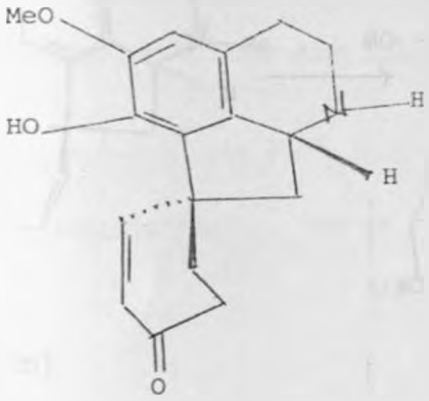


(15)

Casagrande Cesare *et al* [10] observed that nmr and spectral data reported for diacetyljacularine were in accord with those of diacetylisocrotsparinine (16). They concluded, (from spectral and chemical data) that jacularine appeared to be enantiomeric with isocrotsparinine and should thus be represented by (17).



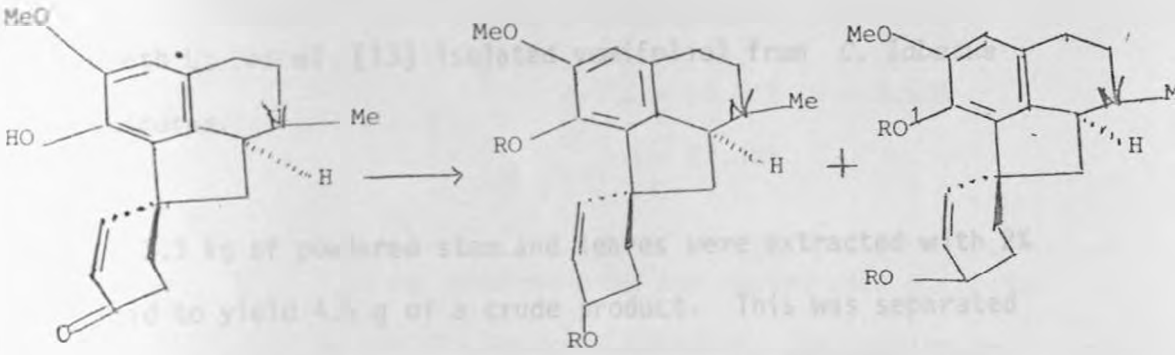
(16) Diacetylisocrotsparinine



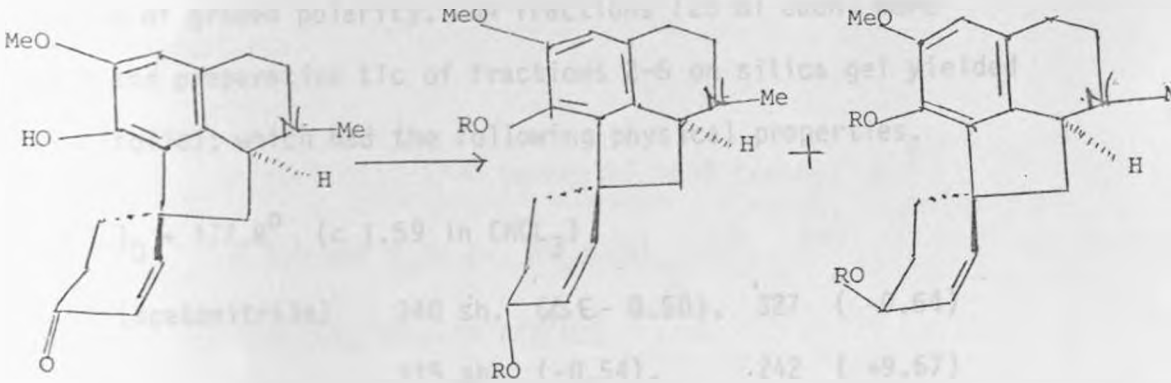
(17) Jacularine

They established the steric structure of base E diacetate by comparing an authentic sample, with the diacetyl derivatives (19b - 22b) of the four alcohols obtained by reduction of synthetic (±) dihydroglaziovines (18a) and (18b) with sodium borohydride.

Base E was found to be identical (except for its optical rotation) with compound (22b), so they proposed structure (23) for base E.



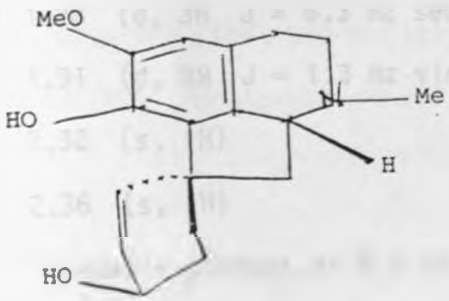
(18a) (19) (20)



(18b) (21) (22)

For structures 19 - 22, R = H for a series

R = Ac for b series



(23) Base E

Croton lobatus, and Croton trinitatus

Stuart Kenneth L; *et al* [13] isolated vomifoliol from *C. lobatus* and *C. trinitatus*.

C. lobatus. 3.3 kg of powdered stem and leaves were extracted with 2% tartaric acid to yield 4.6 g of a crude product. This was separated into phenolic (1.3 g) and non-phenolic fraction with 5% NaOH. The phenolic fraction was chromatographed on Grade II - III alumina using $CHCL_3$- MeOH of graded polarity. 54 fractions (25 ml each) were collected and preparative tlc of fractions 2-6 on silica gel yielded 46 mg vomifoliol; which had the following physical properties.

$[\alpha]_D$ + 177.8° (c 1.59 in $CHCL_3$)

CD (acetonitrile) 340 sh. ($\Delta\epsilon$- 0.50), 327 (-0.64)
315 sh. (-0.54), 242 (+9.67)

UV

EtOH

237 nm (log ϵ 4.05)

max

NMR ($CDCl_3$) showed signals at

δ 1.07, 1.01 (s, 3H each, gem. dimethyls).
1.28 (d, 3H J = 6.3 Hz sec. methyl)
1.91 (d, 3H J = 1.3 Hz vinylic CH_3)
2.30 (s, 1H)
2.36 (s, 1H)

Exchangeable protons at δ 2.64 and 2.86

1 proton at 4.4 (m - CH - OH)

3 olefinic protons between 5.82 - 5.92

Found C-68.53; H- 9.23; O - 22.09; calculated for $C_{13}H_{20}O_3$

C - 69.61% H - 8.99%

O - 21.40%

C. trinitatus. 1.57 kg powdered stem and leaves were extracted with 2% tartaric acid to yield 4.9g crude material. Separation on silica plates with $CHCL_3$ - AcOEt as solvent yielded 52 mg of vomifoliol. Acetylation with Ac_2O - pyridine yielded the mono-acetate with the following properties.

IR 3410 (OH), 1740 (acetate), 1650 (enone) cm^{-1}

NMR δ 1.0 and 1.07 (s, 3H each)

1.31 (d, 3H J = 6.3 Hz)

1.86 (d, 3H J = 1.2 Hz)

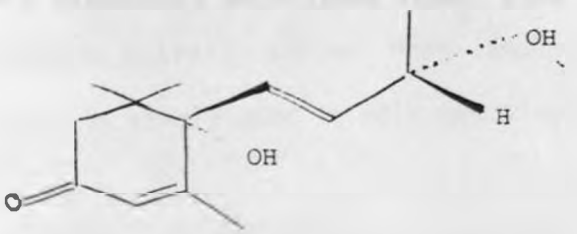
2.03 (s, 3H acetate)

5.35 (m, 1H)

5.73 - 5.78 (3H)

An exchangeable proton at δ 2.30

mp had been earlier determined from vomifoliol, isolated from *Palicaurea* species as 112 - 114°C, and from the mp and spectral data (IR, NMR, CD, UV), it was apparent that vomifoliol was identical with Blumenol A (24) isolated from *Podocarpus blumei*.



(24) Blumenol A.

Vomifoliol had first been isolated from *Rauwolfia vormotoria* and was also isolated from *C. sparsiflorus*. It was concluded that the compound is more widly distributed in plants than previously recognised, and may play a biologically significant role.

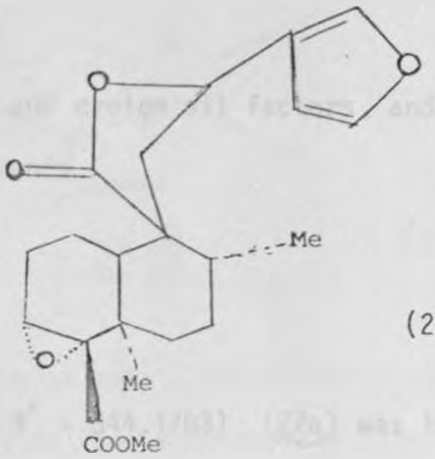
Stuart, Kenneth L. *et al* [14] report a general method for vomifoliol detection. They tested this method on 6 different plants, including *C. linearis*, which had not been previously screened for vomifoliol. They found that *C. linearis* also contained vomifoliol.

The method involves methanol extraction of the plant material in Soxhlet and separation of the mixture obtained on an alumina column, using solvent mixtures of graded and increasing polarity. Preparative tlc is then used to further purify fractions which might contain vomifoliol as judged by tlc monitoring.

Identification is made by tlc comparison with natural or synthetic vomifoliol in 3 solvent systems, and then finally confirmed by a Glc method.

Croton draco

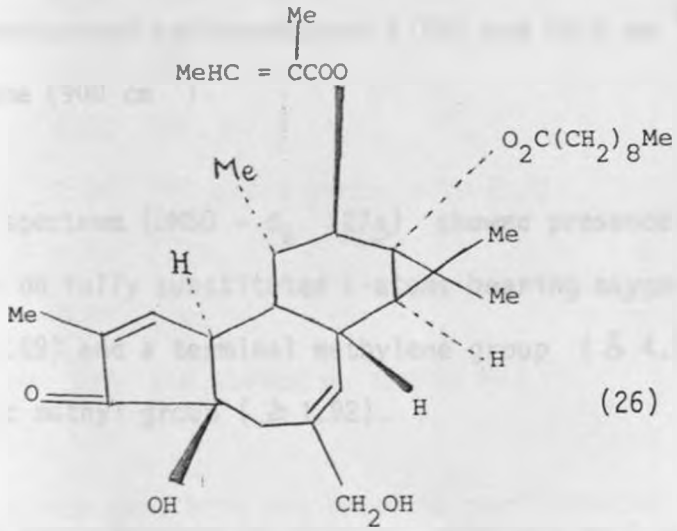
Draconin (25) mp 184^{o} - 6^{o} was isolated from *C. draco* and its structure determined by UV, IR, Mass and NMR spectra. Several derivatives were prepared and their structures determined also. [15]



(25) Draconin

Croton tiglium

Kupchan, S. Morris *et al* [16] isolated anti-leukamic principles from Euphorbiaceae plants, including *C. tiglium*. Systematic fractionation of croton oil extracted from *C. tiglium* led to characterisation of phorbol - 12 - tiglate - 13 - decanoate (26) as an active principle.



(26)

It showed antileukamic activity against P388 lymphocytic leukamia in mice. The plant is widely used in folk medicine for treating cancer.

Irritants and co-carcinogens of *C. tiglium* were also studied by Hecker E., *et al* [17]. They reviewed the fractionation of croton oil,

the chemistry of phorbol and croton oil factors, and the biological activities of these compounds.

Croton corylifolius

Crotofolin A ($C_{20}H_{24}O_5$ M^+ = 344.1703) (27a) was isolated from a benzene extract of *C. corylifolius* [18] as colourless crystals mp 277° - 279° $[\alpha]_D^{25}$ - 20° (EtOH) and λ_{max}^{EtOH} 227 nm ϵ = 23,800

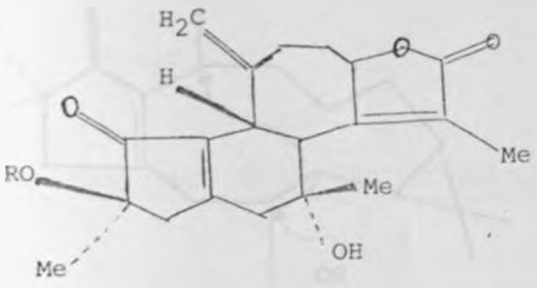
IR (nujol) absorption bands attributed to the following functional groups were observed:-

OH (3465 cm^{-1}); α,β unsaturated γ - lactone (1740 cm^{-1}) α,β - unsaturated cyclopentanone (1705 and 1650 cm^{-1}) exocyclic methylene (900 cm^{-1})

The nmr spectrum (DMSO - d_6 (27a) showed presence of 2 methyl groups located on fully substituted C-atoms bearing oxygen functions, (δ 1.30 and 1.09) and a terminal methylene group (δ 4.50 and 4.84); and one vinylic methyl group (δ 1.92).

In addition, a broad doublet of doublets, centered at δ 5.15 (IH J = 10 and 2 HZ) was assigned to an allylic proton situated on a C-bearing an oxygen function and adjacent to a pair of non-equivalent protons.

The presence of two D_2O exchangeable protons (δ 4.56 and 4.58) indicated that Crotofolin A is a diol.



(27a) R = H crotofolin A

(27b) R = $CH_3C(=O)-$

Treatment of crotofolin A with acetic anhydride in pyridine at 25°C for 18 hours yielded a crystalline mono-acetate (tentatively assigned structure (27b) mp 240° - 243°C, m+ = 386

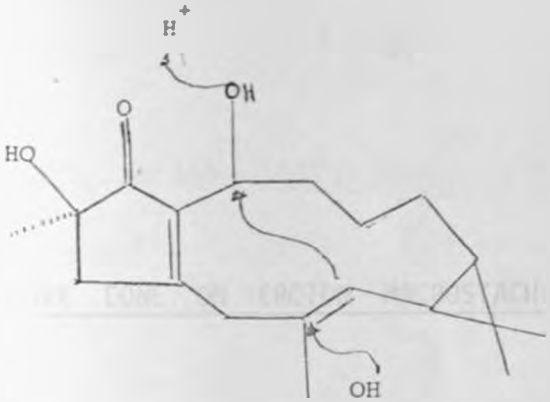
λ_{max}^{EtOH} 225 nm ε = 18 835

ν (KBr) 3560, 1760, 1730, 1715, 1650, 900 cm^{-1}

δ ($CDCL_3$) 1.18 (3H, s)
1.40 (3H, s)
1.90 (3H, s)
2.05 (3H, s)
2.30 (1H exchangeable with D_2O)
4.50 (1H, s)
4.87 (1H, s)
5.14 (1H, broad d, J = 10 Hz)

Determination of the structure and relative configuration of crotofolin A was effected through a single crystal X-ray analysis.

It was suggested that biogenesis of the crotofolane skeleton probably occurred via a transannular closure of a bicyclic precurser such as (28). Compounds embodying the salient skeletal features depicted in (28) have been isolated from Europhobiaceae plants.



(28)

Croton jacobensis

A purified substance (Mol.Wt = 302 empirical formula $C_{20}H_{30}O_2$) from a methanol extract of the stems of *C. jacobensis* showed antimicrobial activity against several genera of bacteria and fungi. [19]

CHAPTER 2

PREVIOUS WORK DONE ON CROTON MACROSTACHYUS

C. macrostachyus is a tree that grows upto 50 feet high. It has white flowers and its fruits which are slightly 3-lobed, are ½-inch in diameter. [2]

It grows in Savannah forests in many parts of tropical Africa, from Guinea to Cameroon in W. Africa, to parts of East and Central Africa, including Kenya. [1]

Its use in folk medicine for treatment in various ailments has been mentioned in the introduction. Following is an account of work that has been done on the plant, upto 1984.

In the course of a continuing search for tumour inhibitors of plant origin, alcoholic extracts of *C. macrostachyus* showed significant inhibitory activity in Lewis lung carcinoma in mice. Crotepoxide, a cyclohexane di-epoxide derivative was found to be the active principle. It was isolated and its structure determined. [20]

Fractionation of the EtOH extract, guided by assay against lewis lung carcinoma revealed that an active principle was concentrated successively in the methanol layer of a 10% aqueous methanol-skellysolve B partition; and in the t-butanol layer of a n-1-butanol-water partition. Further fractionation involving silicic acid chromatography yielded crotepoxide -

$C_{18}H_{18}O_8$, mp 150 - 151°, $[\alpha]_D^{25^\circ}$ + 74° (c 1.70, $CHCL_3$)

λ_{max}^{MeOH} 279 mμ (ε 1050) and 281 mμ (ε - 860);

$\lambda_{max}^{CHCL_3}$ 3,35, 5.71, 5.78, 6.24, 6.31, 6.89, 7.29, 7.89, 8.20, 9.00, 9.60, 10.24 and 11.12 μ

NMR signals (in $CDCL_3$)

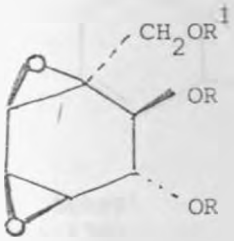
at τ 2.28 (5H, m, aromatic)
4.27 (^{1}H, d Jxy = 9.5 Hz > CHOAc)
5.02 (^{1}H, d,d, Jxy = 9.5 and J_{AY} = 1.5 Hz > CHOAc)
5.42 and 5.75 (2H doublets J = 12.0 Hz CH_2OCOph)
6.32 (1H, d, J_{BC} = 2.5 Hz)
6.56 (1H, d,d. J_{BC} = 2.5 and J_{AB} = 4.0 Hz)
6.90 (1H, d,d, J_{AB} = 4.0 and J_{AY} = 1.5 Hz)
7.88 (3H, s, acetate)
and 7.95 (3H, s, acetate)

Hydrogenation of crotepoxide (29) over platinum dioxide gave a hexahydro derivative, mp 121° - 2°, $[\alpha]_D$ 59°. Saponification of (29) yielded (30) mp 101° - 2°, $[\alpha]_D$ 30° and C_6H_5COOH.

Treatment of (29) with HCL in aqueous methanol for 30 minutes yielded (31) mp 170° - 171°C $[\alpha]_D$ − 4°. Prolonged reaction yielded (32) mp 241° - 2°C $[\alpha]_D$ − 10°; the triacetate mp 217 - 218° and tetracetate mp 153 - 4°C.

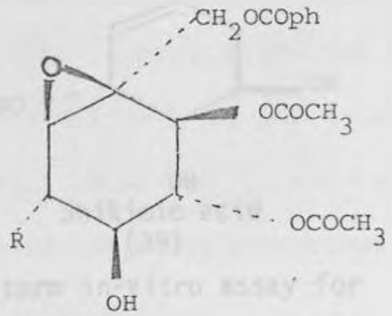
Reaction of crotepoxide with hydrogen iodide in aqueous methanol yielded (33) mp 143 - 144°C $[\alpha]_D$ - 46° (the structure of which was determined by X-ray analysis); and (34) mp 145 - 6°C $[\alpha]_D$ 127°; the triacetate, mp 141 - 2°C $[\alpha]_D$ 151°; and the tetracetate, which was an oily product.

Oxidation of (34) with aq. CrO_3 H_2SO_4 - Me_2CO gave an oily unsaturated ketone; while hydrogenation yielded the saturated ketone mp 117 - 118°C $[\alpha]_D$ - 3°



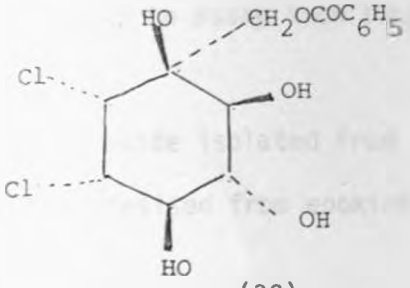
(29) R = Ac R^1 = COC_6H_5

(30) R = R^1 = H

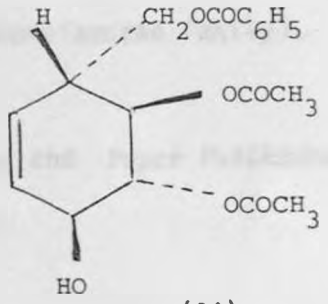


(31) R = Cl

(33) R = I



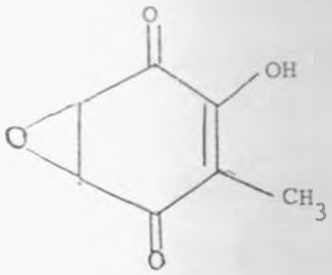
(32)



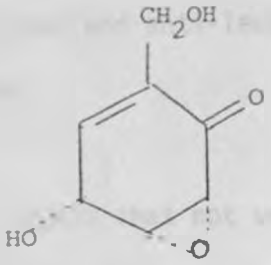
(34)

Crotepoxide is the only one, among a small group of naturally occuring oxygenated cyclohexane derivatives, that possesses diepoxide functionality. This function has been shown to confer tumour inhibiting activity to other classes of synthetic compounds.

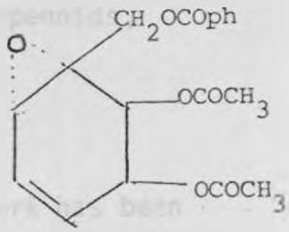
Other members of the naturally occuring oxygenated cyclohexane derivatives include terreic acid (35) [21], epoxydone (36) [22] senepoxyde (37) [23], seneol (38) [23] and shikimic acid (39) [21]



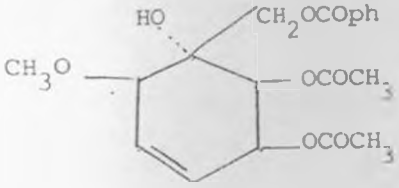
Terreic acid
(35)



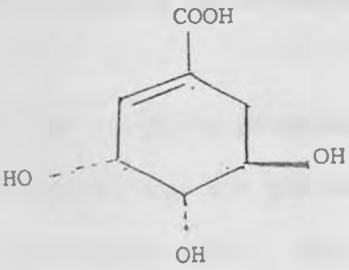
Epoxydone
(36)



Senepoxyde
(37)



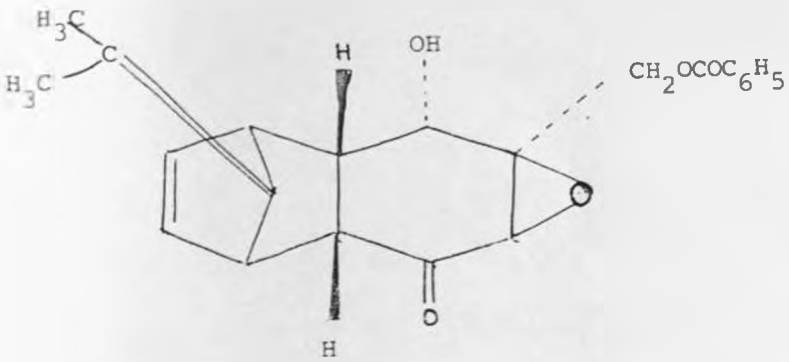
Seneol
(38)



Shikimic acid
(39)

Ho Yohei *et al* [24] carried out a short term in-vitro assay for promoter substances in *C. macrostachyus* and *C. megalocarpus*. They used human lymphoblastoid cells, latently injected with Epstein-Barr virus (used to assay such substances from Euphorbiaeceae family).

dl crotepoxide isolated from *C. macrostachyus* and *Piper Futikadzura* [25] was synthesised from epoxide (40) in 9 steps.



(40) Epoxide

The roots, stem bark and leaves of *C. macrostachyus* probably contain alkaloids and potential anti-ulcer and anti-leukamic diterpenoids, which have not yet been studied.

It is evident from the above report that not very much work has been done on *C. macrostachyus* to date. Also, most of the work reported on crotons has been on species found elsewhere, including a few from W. Africa. East African crotons have received virtually no attention.

This project was thus undertaken, as part of an on-going programme to study the phytochemistry of East African crotons, and the pharmacological activities of their active principles. The stem bark of *C. macrostachyus* was investigated with an aim of extracting and isolating pure compounds, and making an attempt at elucidating their structures.

CHAPTER 3

THE PRESENT INVESTIGATION

RESULTS and DISCUSSION

Column chromatography of the petroleum ether extract of *Croton macrostachyus* stem bark yielded 2 main components which were isolated. A third component was isolated from the mother liquor of the pet-ether extract; and several minor components were observed on tlc, but these were not isolated due to their presence in very minute quantities.

Compound 1 (CMSB.1)

This was obtained from the column as a whitish-brown crystalline solid mp 64^{o} - 66^{o}C. It appeared as a purple spot on tlc (chloroform) with anisaldehyde reagent, indicating a possible terpenoid.

Nothing further was done on it, because attempts at recrystallisation failed.

Compound 2. (CMSB 1.2)

This was a greenish-white crystalline solid, m.p 148^{o} - 172^{o}C. Tlc in chloroform showed it to contain as its major component, a substance suspected to be lupeol, which had been previously isolated from the same plant. [26]

In view of its wide melting range, it was compared on tlc in various solvent systems, with authentic lupeol, to determine whether the main

component corresponded to lupeol.

TABLE 1 - Comparison of Rf values of authentic lupeol with those of CMSB 1.2's main component

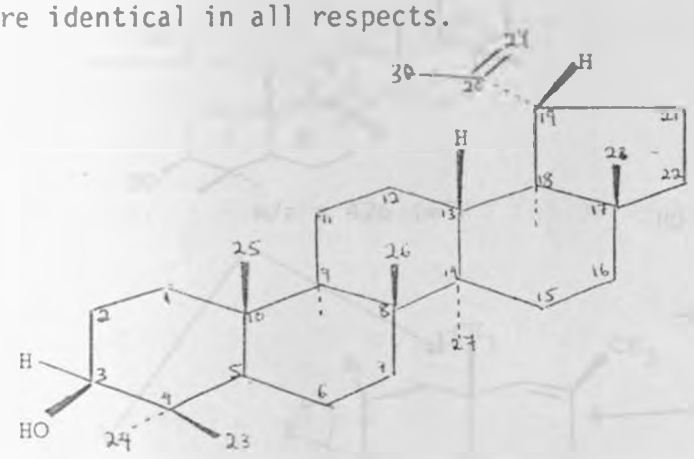
Solvent System	Authentic lupeol	Mixture of lupeol and CMSB 1.2	CMSB 1.2
1. Chloroform	0.69	-	0.67
2. $CHCl_3$: Benzene (1:3)	0.52	-	0.54
3. $CHCl_3$: Benzene (3:1)	0.40	0.36	0.37
4. Benzene	0.34	0.30	0.31
5. Toluene: EtOAc (9:2)	0.61	0.56	0.54
6. Cyclohexane; EtOAc 95 : 2	0.27	0.26	0.23

Tlc thus confirmed this major component to be lupeol, and showed in addition other minor purple spots and a major brick red one.

The IR spectrum of CMSB 1.2 (figure 1a) showed absorption maxima attributable to the following groups.

OH stretching (3150 - 3600 cm^{-1}), C - H stretching of CH_3 2800 - 3050 cm^{-1}) C═C stretching (1600 - 1680 cm^{-1}), C—H bending in CH_2 (1440 - 1480 cm^{-1}) >C—$(CH_3)_2$ (1380 cm^{-1}) O—H bending (1300 and 1360 cm^{-1}); 1040, 1100 - 1110 cm^{-1}, - C—O stretching of >C—OH; C—H bending in disubstituted geminal alkene (875 cm^{-1}).

xcept for an additional peak at about 800 cm^{-1} which is likely due to an impurity, the IR spectra of CMSB 1.2 (fig. 1a) and lupeol (fig. 1b) (41) were identical in all respects.



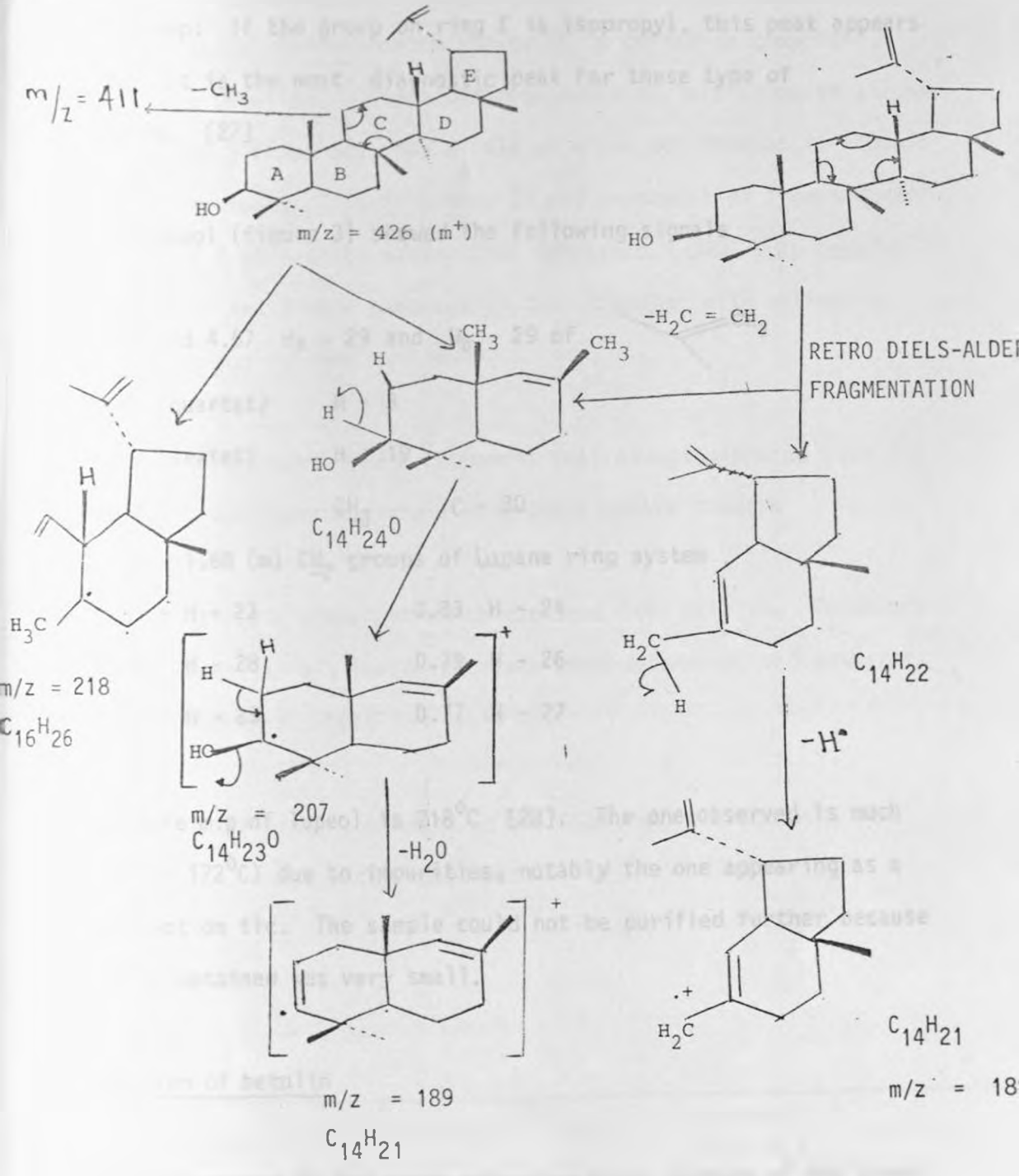
Lupeol

(41)

The mass spectrum of lupeol (fig. 2a) showed the following prominent diagnostic peaks.

M/z	426	(52%) M^+	218 (68%)
	411	(8%) $M^+ - CH_3$	207 (100%)
	315	(Metastable)	189 (82%)

The peaks are a result of the following fragmentations;



The peak at m/z = 41 is due to $CH_3-\overset{+}{C}-CH_2$

The strong peak at 189 is in conformity with the observation that lupane type triterpenoids have an intense peak at 189, irrespective of the nature of substitution in rings A, B, C and D; if ring E bears an

oprenyl group: If the group on ring E is isopropyl, this peak appears
. m/z 191. It is the most diagnostic peak for these type of
-iterpenoids. [27]

he nmr of lupeol (figure 3) showed the following signals

δ 4.69 and 4.57 H_A - 29 and H_B - 29 of 

3.19 (quartet) H - 3

2.38 (sextet) H - 19

1.69 $\underline{CH}_3$ C - 30

1.39 - 1.68 (m) $\underline{CH}_2$ groups of lupane ring system

1.03 - H - 23 0.83 H - 24

0.97 H - 28 0.79 H - 26

0.94 H - 25 0.77 H - 27

The literature m.p of lupeol is 218°C [28]. The one observed is much lower (148° - 172°C) due to impurities, notably the one appearing as a brick red spot on tlc. The sample could not be purified further because the quantity obtained was very small.

Identification of betulin

Column chromatography of the crude petroleum ether extract of the plant material yielded 5 main fractions. Of these, the mother liquors of fractions 3 and 4 were combined, concentrated, and left in the fridge

or 3 weeks at the end of which a brownish sticky material was deposited. lc of this material in chloroform showed it to contain a component, uspected to be betulin. Column chromatography of this brownish sticky aterial yielded 3 fractions; the middle of which was thought to contain etulin in purer form. This (fraction 2) was crystallised from methanol o yield CMSB 3.2 as a white paste-like substance. CMSB 3.2; together vith fractions 1 and 3 were examined on tlc, together with authentic betulin; in various solvent systems.

All three fractions contained a component that always migrated with the solvent front, and appeared purple with anisaldehyde reagent.

They also contained a component which migrated like betulin. R_f values of this component, in all three fractions were determined and compared with those of authentic betulin (Table 2).

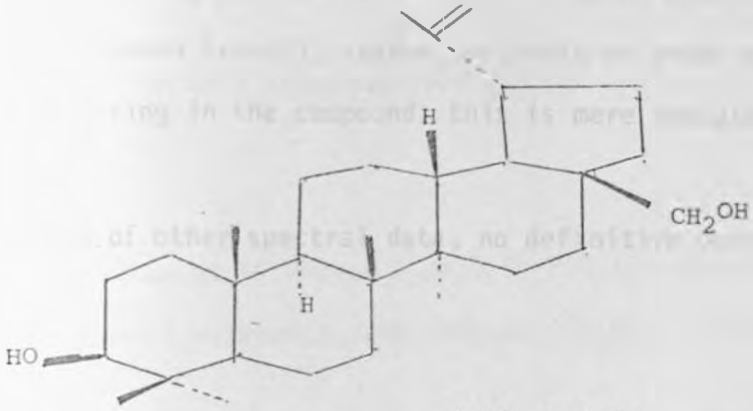
Fraction 1 had one other component in addition, while fraction 3 contained several other minor components .

TABLE 2

Solvent Systems	Fraction 1	CMSB 3.2	Authentic betulin	Fraction 3
1. $CHCl_3$: Ether (1:1)	0.62	0.62	0.61	0.65
2. $CHCl_3$: Benzene (1:5)	0.22	0.18	0.24	0.34
3. Pure $CHCl_3$	0.31	0.20	0.32	0.30
4. Pure Benzene	0.13	0.09	0.14	0.12
5. Benzene: $CHCl_3$ (1:1)	0.17	0.1	0.20	0.17

om R_f values, fraction 1 can be said to contain betulin.

etulin (42) has been previously isolated from the same plant, and has een fully characterised. [26]



(42) Betulin

Compound 3 (CMs.2)

This was obtained from the mother liquor of the crude petroleum ether extract (CMs.1), as a white crystalline solid mp 72^0 - 74^0C.

It gave no colour with anisaldehyde reagent, indicating that it is not a terpenoid.

When the plate was sprayed with aqueous acetic acid, a white spot against a creamish background was formed.

IR spectrum (figure 4) indicated presence of the following functional groups.

CH in CH_3 (2900 - 2920 cm^{-1}); 2800 and 2810 cm^{-1} (CH in CH_2); Carbonyl function (1700 cm^{-1}).

The compound could probably be a long chain aliphatic ester or ketone.

The relatively strong peak at about 720 cm^{-1} could also be attributed to a monosubstituted aromatic system, but with no proof of the presence of an aromatic ring in the compound, this is mere speculation.

In the absence of other spectral data, no definitive conclusion can be drawn.

Work is still being done, to elucidate the structure of this compound.

EXPERIMENTAL

The plant material was collected from Karatina, about 200 km North of Nairobi, at an altitude of about 2000 m, above sea level, around July-August 1985.

Column chromatography was on silica gel 60 (70 - 230 mesh. ASTM).

Analytical tlc was carried out on silica gel 60 GF_{254} as adsorbent. The plates were sprayed with anisaldehyde reagent, and placed in the oven at 110°C for 10 minutes for development of colour.

Anisaldehyde reagent was prepared by mixing the following:

0.5 ml conc. H_2SO_4

0.5 ml anisaldehyde

50 ml glacial acetic acid

melting points were determined on Gallenkamp melting point apparatus,
1 are uncorrected.

spectra were taken with Perkin Elmer Infrared spectrophotometer 727B.

ss spectrum was run on a Finnigan 4000 and MAT 312/ss 200 instrument
70 eV e.i.

1R spectra were done at 90 MHz on a Jeol FX 90Q instrument, in $CDCl_3$,
ith TMS as internal reference, and also at 400 MHz. (Instrument
nknown).

Extraction of the plant material

700g of the dried powdered stem bark of *Croton macrostachyus* were extracted in a soxhlet apparatus using about 3 litres of petroleum ether (Bp 60^{o} - 80^{o}C) for 48 hours.

The extract was concentrated to about 150 ml, was placed in a labelled conical flask and left in the fridge for 2 weeks, at the end of which 6.14 g of crude material, (CMs.1) was deposited. This was filtered off.

The filtrate was concentrated to about 1/4 its volume, was labelled (mother liquor 1) and was left in the fridge for about 4 months.

Column chromatography of CMs.1

4.95 g of CMs.1 was dissolved, (with warming) in the minimum amount of chloroform. This solution was introduced onto a 43 cm column, and was eluted successively with pure benzene; benzene: chloroform (4:1); pure chloroform; and chloroform: methanol (10:1).

The eluate was collected in 15 ml portions and constituents of every 3rd portion monitored by tlc with anisaldehyde reagent.

5 main fractions were obtained. Each was evaporated to dryness and crystallised from aqueous methanol.

Fraction 1

Addition of methanol resulted in a rock-like solid, some of which did not dissolve in excess methanol, even on boiling. This was filtered off, the solution was concentrated and left in the fridge for several days.

56.1 mg of brownish-white crystals (CMSB.1) were obtained, mp 64 - 66^{o}C. Tlc in pure chloroform showed a major purple spot with anisaldehyde reagent; and a few other minor spots. Attempts to recrystallise CMSB.1 were unsuccessful.

The mother liquor from this fraction was concentrated and left in the

fridge for several days. 20.6 mg of CMSB 1.2 were obtained as greenish - whitish crystals. These were recrystallised from methanol to give CMSB 1.2 with m.p 148^{o} - 172^{o}.

In view of its tlc ($CHCL_3$) R_f behaviour, CMSB 1.2 was suspected to be lupeol, so tlc was done in 6 different solvent systems, to compare R_f values with those of authentic lupeol (Table 1) as already indicated in the discussion.

Ir (KBr) (fig. 1a) showed absorption maxima as follows:

3150 - 3600, 2800 - 3050, 1600 - 1680, 1440 - 1480,
1360 - 1385, 1300, 1100 - 1110, 1040, 1010, 875 cm^{-1}

The functional groups attributed to these peaks have been indicated in the discussion.

Fractions 2 - 5

On evaporation to dryness, a viscous yellow liquid was left in each fraction. Crystallisation from methanol deposited small amounts of solids (CMSB.2 - CMSB.5) which on tlc ($CHCl_3$) examination showed mixtures of many components. No further work was done on these solids.

Tlc (chloroform) of the mother liquors showed that fractions 3 and 4 contained a major component in common. This component was purple when sprayed with anisaldehyde reagent, and in view of its tlc R_f behaviour, it was suspected to be betulin.

The 2 fractions were therefore combined, concentrated, and left in the fridge for 3 weeks, at the end of which a brownish sticky material was deposited.

Column chromatography of the brownish sticky material

The material was dissolved in the minimum amount of chloroform and introduced onto a 23 cm column. It was eluted with pure benzene; benzene: chloroform (3:1), (1:1) and (1:3), and pure chloroform.

3 fractions were collected, all were evaporated to dryness.

Fraction 2 was crystallised from methanol to give 17.2 mg of a white paste-like susbstance (CMSB 3.2).

The oily liquids left on evaporation of Fractions 1 and 3 and some of CMSB 3.2 were each dissolved in chloroform, and all three fractions were examined on tlc, in various solvent systems, together with authentic betulin. (Table 2)

Motner liquor 1

At the end of 4 months, a solid had deposited which was filtered off and was triturated several times with acetone. The resulting white residue was filtered and recrystallised three times from methanol to give CMs.2 (91.3 mg) m.p 72^{o} - 74^{o}C.

It gave no colour with anisaldehyde reagent on tlc, but appeared as a wnite spot when the plate was sprayed with aqueous acetic acid.

IR (KBr) ν_{max}

2900 - 2920 (CH of CH_3) 2800 and 2810 (CH in CH_2)

1700 (carbonyl) 1460 (CH deformations of CH_2 and CH_3)

720 (probably CH_2 bending of an aliphatic system) cm^{-1}

As indicated before, work on this compound is still going on.

FIGURE 1a - IR SPECTRUM OF CMSB 1.2.

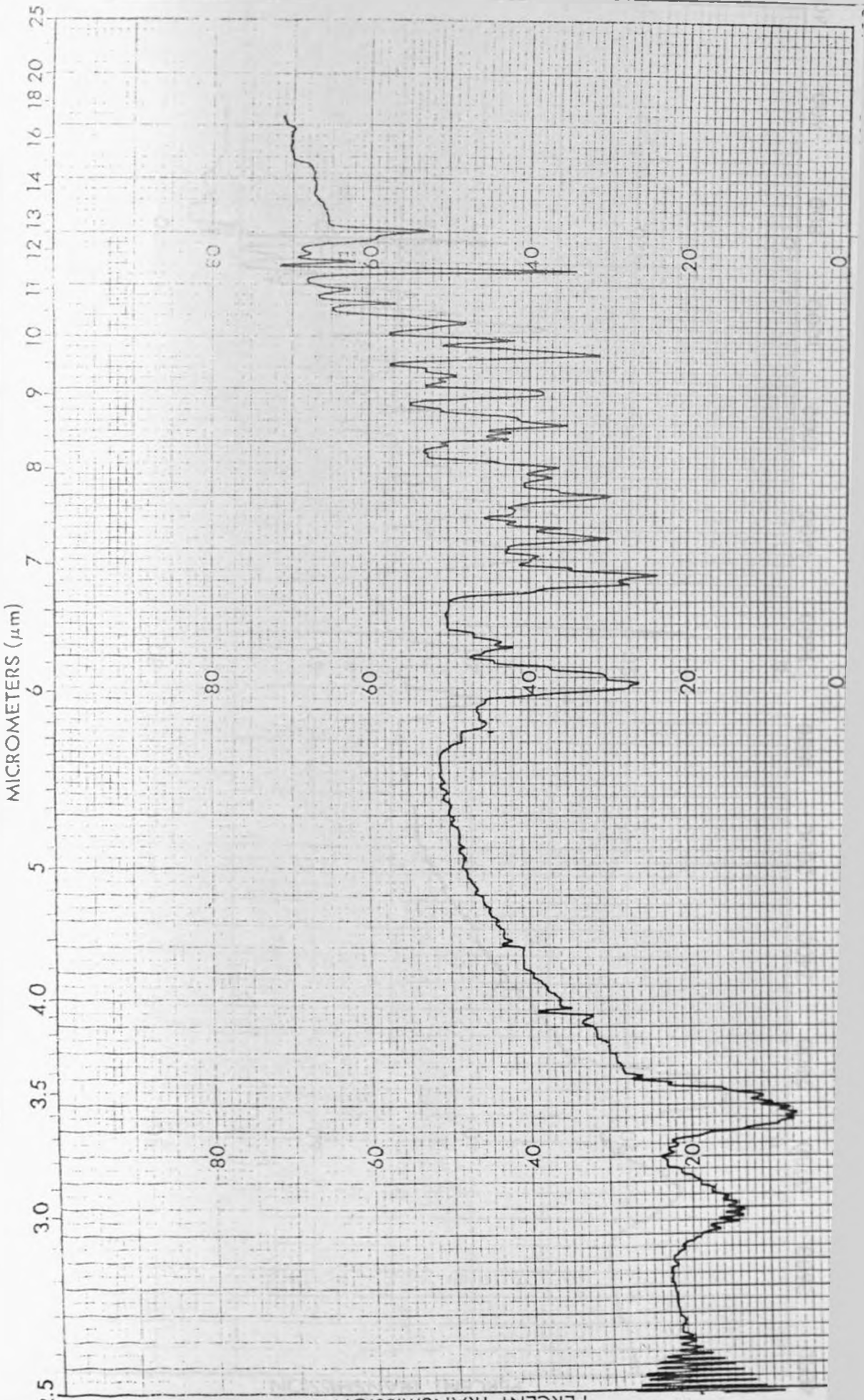
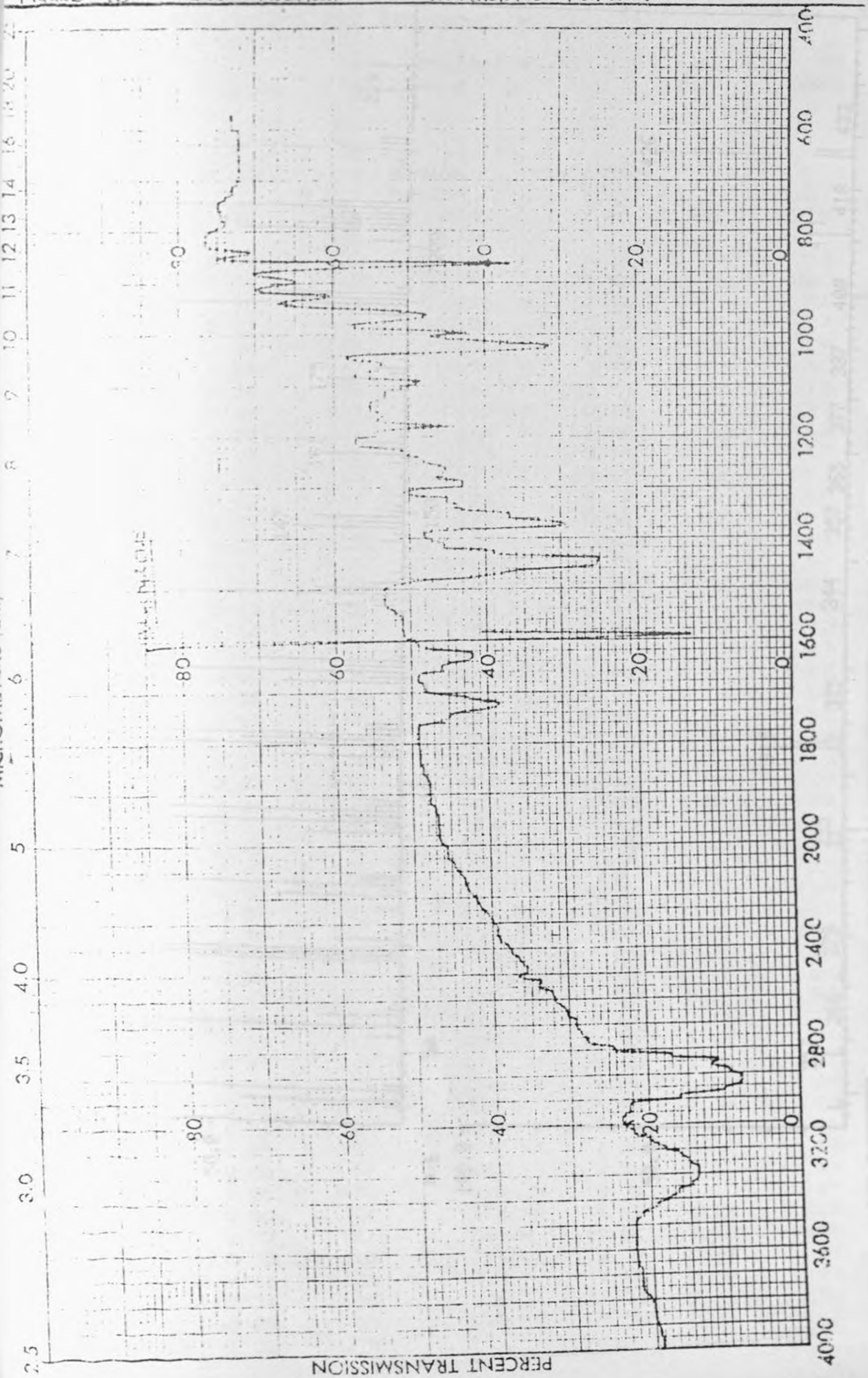


FIGURE 1b - IR SPECTRUM OF AUTHENTIC LUPEOL.



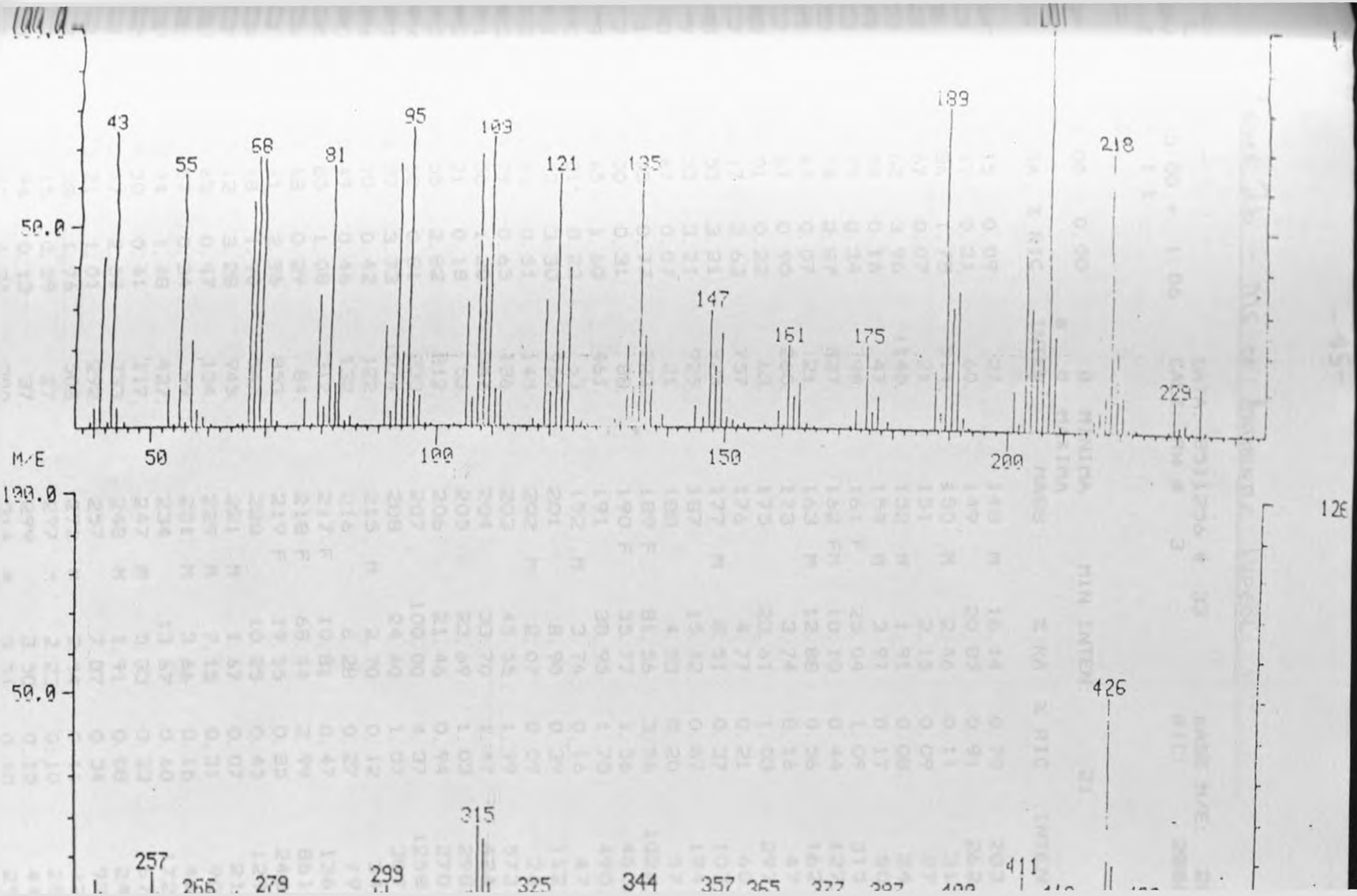

100.0
50.0
M/E
50
100
150
200
43
55
68
81
95
103
121
135
147
161
175
189
218
229
100.0
50.0
257
265
279
299
315
325
344
357
411
426

FIGURE 2b - MS OF AUTHENTIC LUPEOL.

S LIST
25/85 18:13:00 + 1:06
PLE: C.M.P. 1.1
DS. EI SST

DATA: 8511256 # 33
CALI: RW # 3

BASE M/E: 20
RIC: 2880

39 1.00 0.00 0. MINIMA MIN INTEN: 21.
40 # 0 MAXIMA
SS % RA % RIC INTEN.

					MASS		% RA	% RIC	INTEN
39?	M	2.15	0.09	27.	148	M	16.14	0.70	203
40	M	4.77	0.21	60.	149		20.83	0.91	262
41		40.86	1.78	514.	150	M	2.46	0.11	31
42	M	1.67	0.07	21.	151		2.15	0.09	27
43		90.62	3.96	1140.	152	M	1.91	0.08	24
44	M	3.74	0.16	47.	159	M	3.97	0.17	50
53		7.79	0.34	98.	161	F	25.04	1.09	315
55		65.74	2.87	827.	162	FM	10.10	0.44	127
56	M	1.67	0.07	21.	163	M	12.88	0.56	162
57	M	20.67	0.90	260.	173		3.74	0.16	47
58	M	5.01	0.22	63.	175		23.61	1.03	297
67		60.17	2.63	757.	176		4.77	0.21	60
68	F	73.53	3.21	925.	177	M	8.51	0.37	107
69	F	73.53	3.21	925.	187		15.42	0.67	194
70	M	1.67	0.07	21.	188		4.53	0.20	57
71		17.73	0.77	223.	189	F	81.56	3.56	1026
77	M	7.00	0.31	88.	190	F	35.77	1.56	450
79		36.65	1.60	461.	191		38.95	1.70	490
80		5.01	0.22	63	192	M	3.74	0.16	47
81		75.52	3.30	950	201		8.90	0.39	112
82		11.76	0.51	148	202	M	2.07	0.09	26
83	M	14.79	0.65	186.	203		45.55	1.99	573
91		27.58	1.20	347.	204		33.70	1.47	424
92		4.21	0.18	53.	205		23.69	1.03	298
93		64.55	2.82	812.	206		21.46	0.94	270
94		18.52	0.81	233.	207		100.00	4.37	1258
95		85.37	3.73	1074.	208		24.40	1.07	307
96		9.70	0.42	122.	215	M	2.70	0.12	34
97		10.49	0.46	132.	216		6.28	0.27	79
105		24.80	1.08	312.	217	F	10.81	0.47	136
106	F	6.68	0.29	84.	218	F	68.44	2.99	861
107	F	67.81	2.96	853.	219	F	19.55	0.85	246
108	F	43.48	1.90	547.	220		10.25	0.45	129
109	F	75.12	3.28	945.	221	M	1.67	0.07	21.
110		10.65	0.47	134.	229	M	7.15	0.31	90
111	M	7.87	0.34	99.	231	M	3.66	0.16	46
119	F	33.94	1.48	427.	234		13.67	0.60	172
120	F	9.30	0.41	117.	247	M	5.33	0.23	67
121	F	58.27	2.55	733.	248	M	1.91	0.08	24
122		23.21	1.01	292.	257		7.87	0.34	99
123		40.38	1.76	508	272	M	2.54	0.11	32
124	M	2.15	0.09	27.	277	M	2.23	0.10	28
131	M	2.94	0.13	37.	299		3.50	0.15	44
133		23.05	1.01	290.	314	M	2.31	0.10	29
134	F	13.83	0.60	174.	315	FM	18.12	0.79	228
135	F	65.26	2.85	821.	316	F	14.31	0.62	180
136		21.30	0.93	268	317	M	3.26	0.14	41
137	M	10.49	0.46	132.	411		7.71	0.34	97
139	M	5.56	0.24	70.	426		51.75	2.26	651
145	M	5.01	0.22	63	427		9.30	0.41	117
147		30.13	1.32	379					

FIGURE 3 - NMR SPECTRUM OF AUTHENTIC LUPEOL

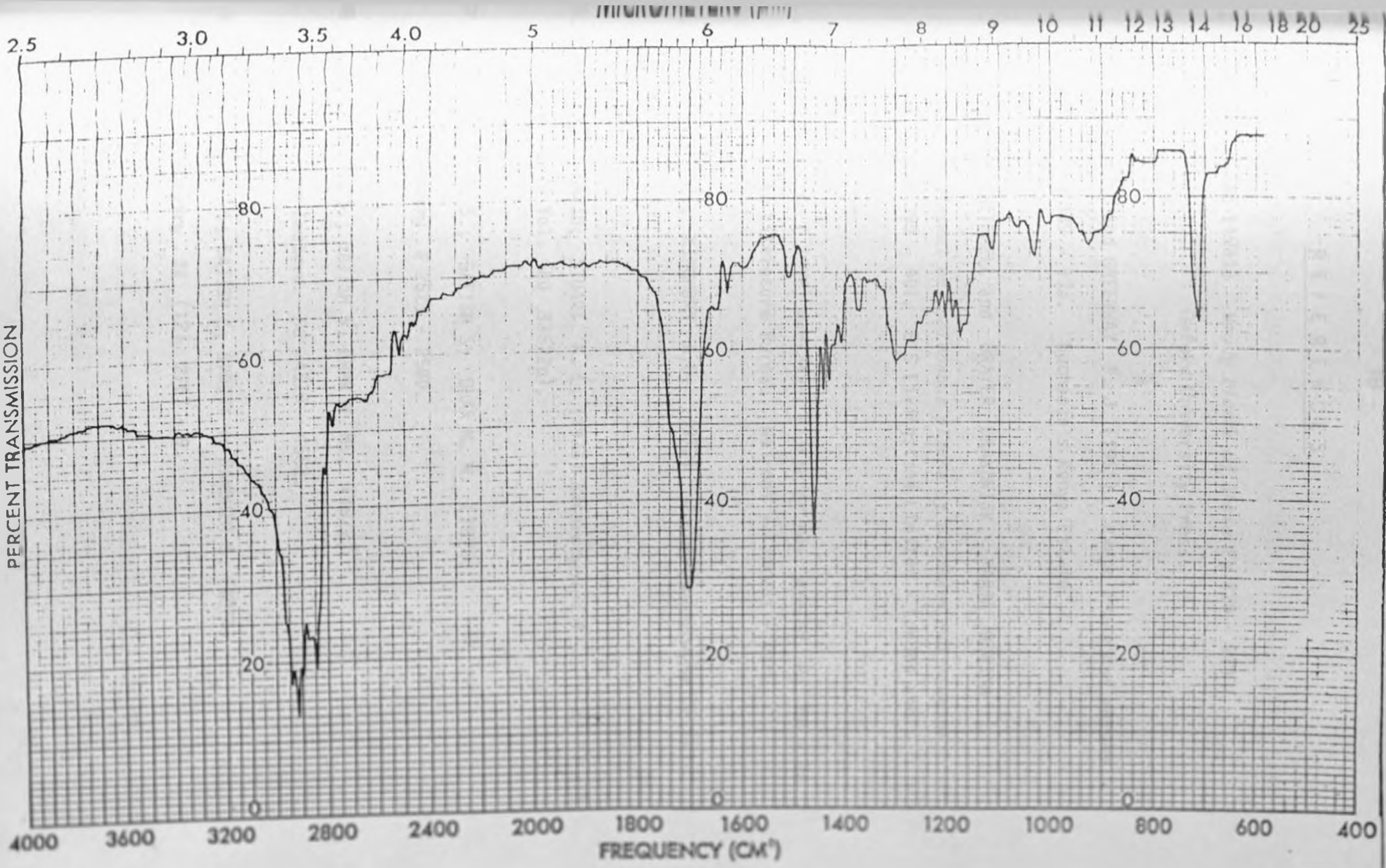


FIGURE 4- IR SPECTRUM OF CMS-2

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