PHYTOCHEMICAL AND PHARMACOLOGICAL EXAMINATION OF ENTANDROPHRAGMA ANGOLENSE AND CRYPTOLEPIS SANGUINOLENTA

BY

ADESANWO JULIUS KOLAWOLE
B.Sc. (Hons) Chem. (Ibadan)
M.Sc. Industrial Chemistry
(Ibadan)

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CERTIFICATION

I certify that this work was carried out under my supervision by Adesanwo, Julius Kolawole in the Department of Chemistry, University of Ibadan, Nigeria.

Supervisor

V.C.O. Njar (Ph.D. London)
Senior Lecturer
Department of Chemistry
University of Ibadan
Nigeria.

Prof. D.A. Okorie
Head, Department of Chemistry
University of Ibadan
Nigeria.

DEDICATION

Dedicated to

My Three Natural Products:
IFE, 'SEUN & DAMMY.

ACKNOWLEDGEMENT

I wish to express my profound gratitude to Dr. V.C.O. Njar my supervisor for his invaluable assistance to encouragement, advice and direction without which this project would have been an empty dream. I am grateful to Professor D.A. Okorie, Head of the Department of Chemistry for the encouragement he gave me in standing as my supervisor when Dr. V.C.O. Njar travelled out of the country. My profound gratitude goes also to Dr.(Mrs.) J.M. Makinde and Dr. S.O. Awe both of who supervised the anti-malarial aspect of the project.

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To all staff of this Department I say thank you for assistance in many ways I cannot enumerate for space. I thank all brothers and sisters for their effectual prayers. I also wish to appreciate the love and care of my mother, Mrs. Kehinde Adesanwo and of every member of my extended family.

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Finally, I wish to say a big thank you to my wife, Mrs. O.O. Adesanwo and children for their love and understanding, and for giving me the home base without which this work could not have been a success.

UNTO GOD BE ALL THE GLORY

J.K. Adesanwo

Chemistry Department
University of Ibadan
Ibadan

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ABSTRACT

Dried, pulverized stem bark and the root bark of Entandrophragma angolense were separately extracted with methanol.

The crude methanolic extract of the stem and root bark of E. angolense were subjected to column fractionation and purification. This exercise afforded the isolation of methyl angolensate, 2.26% yield from the stem bark and 0.27% yield from the root bark.

The effect of the crude methanolic stem bark extract was investigated on indomethacin-induced gastric ulcer in rats. Its effect was dose-dependent, doses ranging from 0.4 to 1.6 gkg^{-1} body weight (BW) produced significant effect ($P < 0.05$). At the highest dose used (1.6gkg^{-1} BW), complete inhibition of ulceration occurred. Toxicity study showed that the extract was not toxic when doses ranging from 20-200gkg^{-1} BW was administered to experimental rats.

It was established that methyl angolensate is the major anti-ulcer principle present in the methanolic extract of the stem bark of E. angolensate. Methyl angolensate produced a dose-related inhibition of gastric ulceration induced by indomethacin, 40$mgkg^{-1}$ BW being more effective than 40$mgkg^{-1}$ BW of propanolol. 80$mgkg^{-1}$ BW of methyl angolensate completely inhibited gastric ulceration. Methyl angolensate also significantly reduced gastric acid secretion induced by histamine and carbachol (1.0$mgkg^{-1}$ BW). Thus we confirmed that methyl angolensate produces its anti-ulcer activity through inhibition of gastric acid secretion.

The roots of Cryptolepis sanguinolenta were extracted with methanol. Column fractionation of the methanolic extract afforded the isolation of a new benzocarboline alkaloid labelled (CS-1) melting point 272-274°C.

Spectroscopic analysis of CS-1 including the infra-red (IR), ultra-violet (UV), mass spectrum (MS), proton and ^{13}C-nuclear magnetic spectroscopy were reported.

This new alkaloid exhibited anti-microbial activity on five pathogenic organisms. The in-vivo anti-malarial study of the aqueous extract of the roots of C. sanguinolenta was done on Plasmodium yoeli nigeriensis in mice. Anti-malarial activity of the extract was determined by examining the blood schizontocidal action in established infection using chloroquine as standard drug for comparison. The extract showed a dose-dependent effect against the malarial parasite.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 The Practice of Folk Medicine

From time immemorial, plants have been known to possess medicinal properties. The early man observed that animals and man feeding on certain plants suffered disturbing or fatal reactions and such plants are recognised as poison. Plants that induce vomitting or purging are used in treating food poisoning and indigestion. Often a strong odour or bitter taste attract attention and such plants are used as flavours and others which cause sweating are found to reduce fever (1). Plant parts used in traditional medicine include: the bark, leaves, roots, twig and the flower or seeds. The earliest recorded use of herbal medicine is that of chanlmoogra oil extracted from Hydrocarpus species which are known to be effective in the treatment of leprosy (2). The practice of folk medicine is encouraged especially in black Africa due to the presence of an abundant and diverse vegetation and also due to a shortage of Western drugs.

This use of herbs for the treatment and prevention of diseases is not restricted to any particular region or people. Herbal medicine has been employed for man's benefit in all parts of the world. In Africa, it is of great importance as it has become part of the culture of the people. In India and China, herbal medicine is

very well organised and has been integrated with conventional health care.

Various types of preparation have been made from these plants and used as herbal medicine. Some of these include: concoction, decoction and infusion.

Concoction is a preparation made usually by mixing many ingredients to form a soup or drink.

A decoction is prepared by placing the plant material in cold water (or aqueous alcohol) bringing it to boil, simmering for sometime (usually fifteen minutes to one hour) and then allowing the mixture to stand. The aqueous extract is then decanted or filtered as and when required. Such preparations are often left in pots and heated up daily before use, as a result the extracts get darker in colour.

An infusion is prepared by pouring boiling water on a specified quantity of plant material and allowing the mixture to stand for some minutes.

The herb may also be used without making any preparation for example the stem or root may be chewed. An example is Zanthoxylum zanthoxyloides Waterman (Fagara zanthoryloides) Lam. whose root is used as chewing sticks for control of oral microbial flora (3). Another example is Ageratum conyzoides L. whose leaves are used for dressing wounds and treating ulcers (4).

Scientific investigations have been carried out on some of the herbs used in Africa and the basis of their use for therapeutic purposes have been established and documented while many are yet to be investigated.

1.2 Entandrophragma angolense (Family Meliaceae)

The family Meliaceae contains many species of commercial value and yield various woods known as "Mahogany". The genus Entandrophragma is limited to four West African species and is one of the most common sources of mahogany timber (5). E. angolense is a well known species furnishing valuable timber known as "gedunohor". E. candolei is not common in trade. Its wood is denser than water being locally known as "Sapele wood-sinker". E. cylindricum furnishes the wood known as "Sapele mahogany" much used in the manufacture of plywood. E. utile has a softer timber. Other known species of this genus Entandrophragma include: E. palustre, E. grandifolia; E. bussei and E. caudatum (6,7).

Entandrophragma angolense (Welm CDC) is a gigantic forest tree with decidous leaves and very bitter hard wood bark. It grows to a height of 100-120 feet with a strong trunk of 60-70 feet in circumference (8). The plant flowers between January and February with ripe fruits at the beginning of August. The fruit is

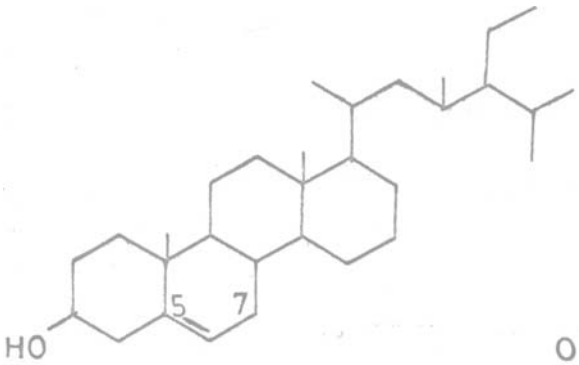
blackish, cylindrical and 5-8 inches long. The seeds are usually 20-30 in number, 4-6 in each cell, red-brown with a long oblong-lanceolate wing attached alternately. The wood is reddish having a cedar-like odour, colour and texture. Compared to Khaya species, the timber is said to be lighter in weight and more coloured.

The plant is known with different names by various localities (8). In Yoruba land, it is known as "Ijebo", in Benin as "gedunohor", in Ibo as "Ngora", in Efik as "Atore" and in Ekoi as "Etori".

1.3 The Phytochemistry of the Genus Entandrophragma

Many scientists have done some work on the genus Entandrophragma. It has been reported (5) that β -sitosterol (1) and either gedunin (2) or methyl angolensate (3) were isolated from authentic specimens of E angolense. According to the report, in no case were both of gedunin (2) and methyl angolensate (3) found in same specimen. β -sitosterol (1) was also reported found in E. candolleii, E. cylindricum and E. utile. Entandrophragmin (4) and Utilin (5) were reported found in E. cylindricum and E. utile (5) respectively.

Adesida and Taylor (9) divided the ten distinct species of the genus Entandrophragma which are confined to Africa into two major groups on the basis of their phytochemistry. According to them,



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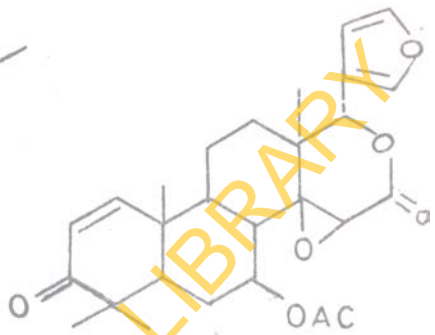

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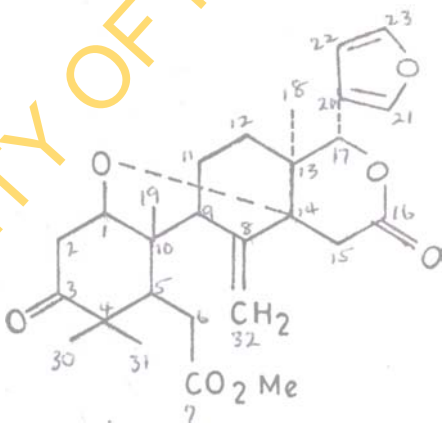

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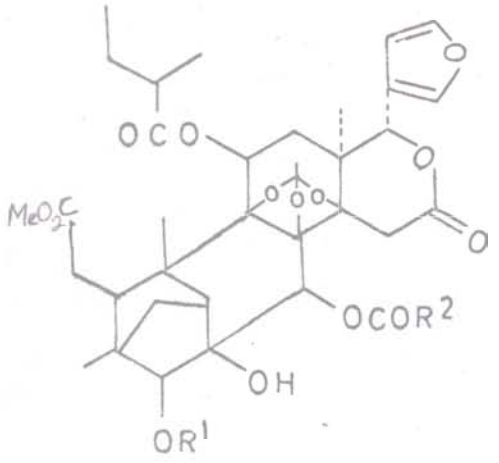
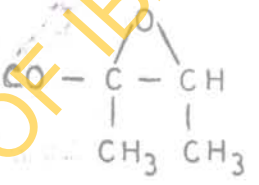
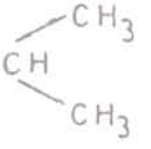
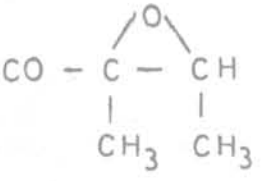
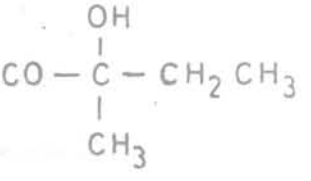
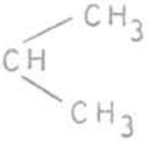
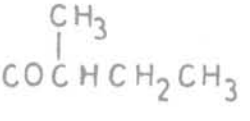
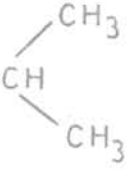


4 R^1 =  R^2 = 

5 R^1 =  $R^2 = CH_3$

6 R^1 =  ; R^2 = 

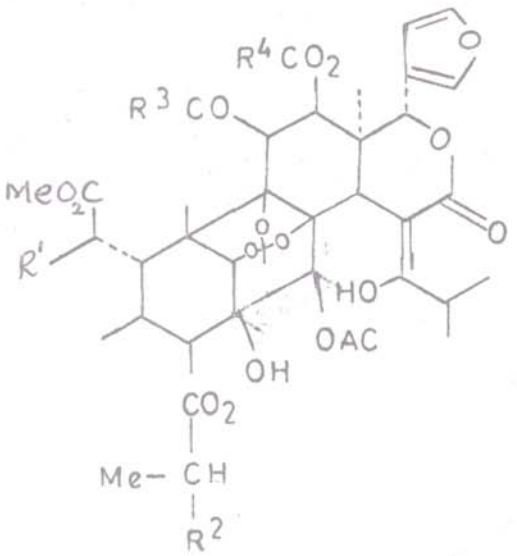
7 R^1 =  R^2 = 

the first group which consisted of E. angolense C.DC., E. delevoyi De Wild, E. excelsum sprague and E. maerophyllum A. Chev. yielded simple meliacins - gedunin (2) and methyl angolensate (3). The other group which consisted of E. brussei Harms ex Engle, E. candollei Harms, E. caudatum Sprague, E. cylindricum Sprague, E. palustre Staner, E. spicatum Sprague and E. utile Sprague, yielded the more complex compounds such as entandrophragmin (4), β - dihydroentandrophragmin (6), candollein (7), utilin (5), bussein (8) and spicata-2 (9). Bevan et al (10) in their paper titled: West African Timbers Part XIX, gave the formula and structure of methyl angolensate (3) isolated from E. angolense as a Ring-B-seco tetranor-tetracyclic triterpene. According to them, its molecular formula $C_{27}H_{34}O_7$ was first proposed by King et al (11).

Bevan etal (10) gave the spectral properties of methyl angolensate thus:

Infrared (IR) data: ($\bar{\nu}_{max}$ cm^{-1}) 1735, 1713, 1655, 1505, 910 and 875.

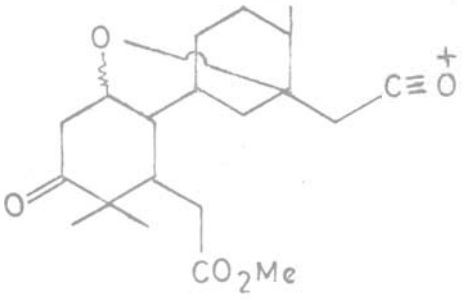
Mass spectral (MS) data m/z 470 (M^+) for $C_{27}H_{34}O_7$ (Found: C, 69.05; H, 7.4. Calculated: C, 68.9; H, 7.3%). Other m/z peaks at 374 and 332 can be accounted for by loss of furfuraldehyde to give compound 10 followed by loss of CH_2CO. Peaks at m/z 411 and 397 correspond to loss of $-CO_2Me$ and $-CH_2CO_2Me$ and peaks at 359 and 299 to loss



8 $R^1 = H$, $R^2 = CH_2CH_3$; $R^3 = R^4 = CH_3$

$R^1 = H$, $R^2 = R^3 = R^4 = CH_3$

9 $R^1 = H$, $R^2 = R^4 = CH_3$; $R^3 = Pr$



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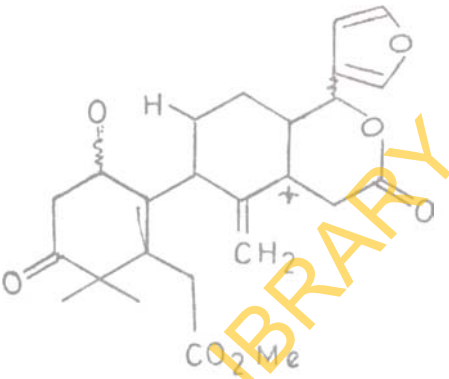

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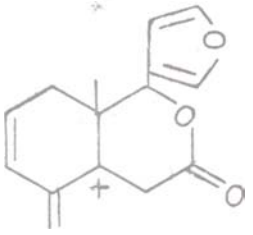

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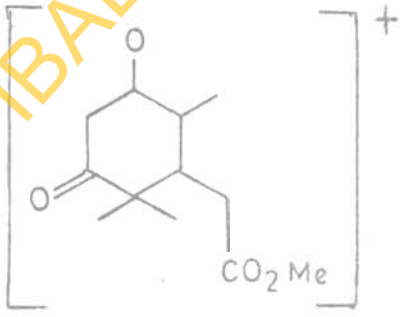

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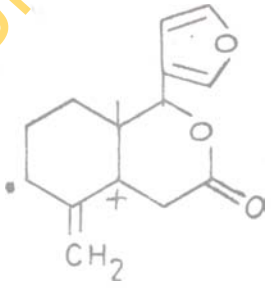

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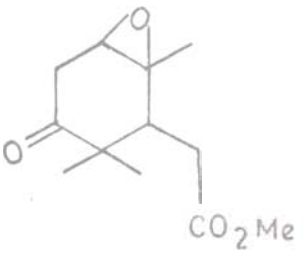


(15)

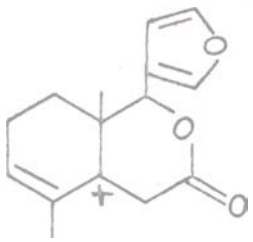
of $-CH_3$, furfuraldehyde, and HCO_2Me. Fission to give 11 and hence to give (a) 12 and 13 (b) 14 and 15 and (c) 16 and 17 account for peaks at m/z 243 and 277, 244 and 226 and 245 and 225 respectively. Loss of furfuraldehyde from 12, 14 and 16 leads to peaks at m/z 147, 148 (18) the base peak and 149. Further loss of CO gives peaks at m/z 119, 120 and 121. Peaks at m/z 209, 210 and 211 can be accounted for by the ions 19, 20 and 21 from which loss of CH_3OH gives peaks at m/z 177, 178 and 179.

The ^{1}H-NMR data and its assignments given by these authors is contained in Table 2.1, page 36. The various assignments were well discussed in their paper.

Balogun and Fetuga (12) in their paper titled: Fatty Acid Composition of Seed Oils from Some Members of the Meliaceae and Combretaceae Families, reported the presence of a high level of monoenoic acids in the seed oil of E. angolense.

1.4 The Pharmacology of E. angolense

In the southwestern part of Nigeria, the stem bark of E. angolense (Ijebo) is widely used as an anti-ulcer. Adelaja (13), a traditional doctor at Ososa town near Ijebu-Ode attested to the use of the bark of E angolense in the treatment of ulcer patients. According to him, the plant is not only used in preventive



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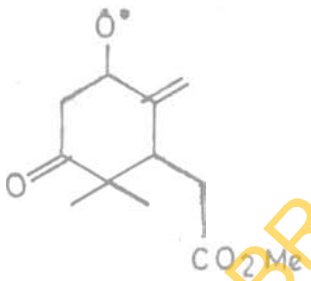

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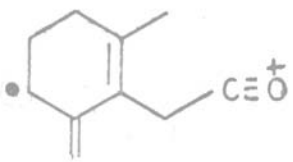

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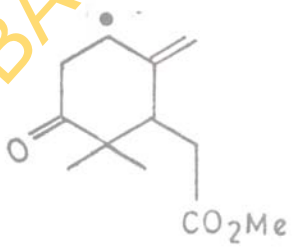

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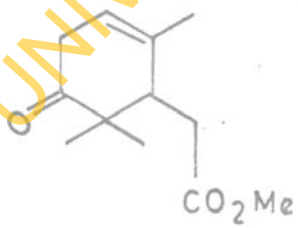

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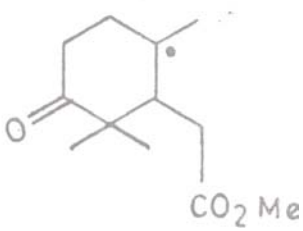


(21)

treatments but also in curative treatments of acute ulcer incidence.

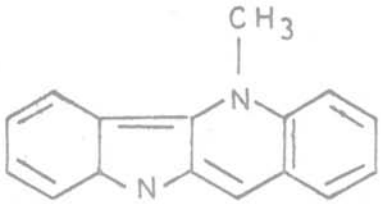
John and Onabanjo (14) reported that E. utile caused a 100% gastroprotection in experimental ethanol-induced gastric ulceration in rats.

1.5 Cryptolepsis sanguinolenta (Family Asclepidaceae)

The plant Cyptolepsis sanguinolenta (lindl) schlecter is native to Tropical West Africa where it grows as shrubs (15). The plant is a twinning and scrambling thin-stemmed shrub with blood-red sap (8). The stem is glaborous and the cut stem and root show bright yellow surfaces. The leaves are thinly herbaceous and rounded which may be between 2.5-7.0cm long. The flowers are bisexual in nature. The seeds are about 12mm long spreading with silky hairs. The endosperm has a straight embryo almost as long as the seed and the cotyledons are flat. The roots are somewhat long and thick but thin at the end. The sap is bitter and characterized by the rapidity with which it turns deep-red on exposure to air.

1.6 The Phytochemistry of C. sanguinolenta

The isolation of an indoquinoline alkaloid cryptolepin (22) from the root of the plant had been reported by many authors (18,19,20). The alkaloid was described as purple in colour (20) and has the



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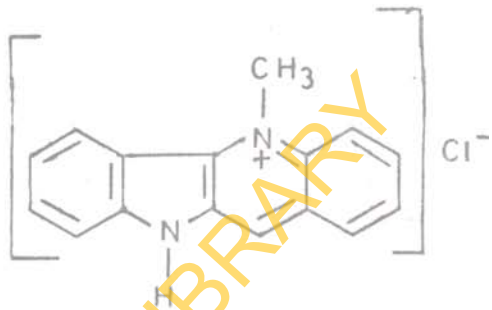

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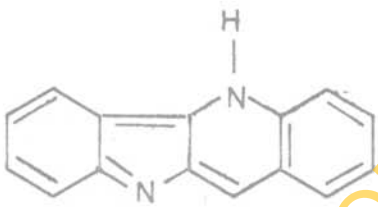

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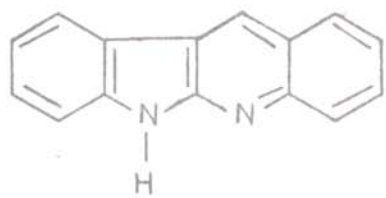

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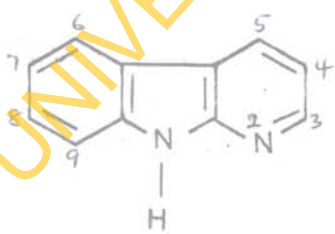

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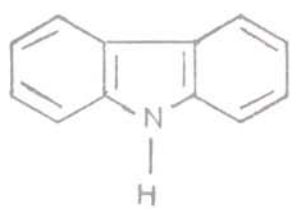


(28)

following properties (19) m.pt. 167-168°C, uv λ_{max} (methanol): 224 (log ϵ 4.11), 246(3.87), 275(4.41), 283(4.43), 355sh(4.02), 370(4.33), 410(3.28), 433(3.29) nm.

λ_{max} (0.01N ethanolic potassium hydroxide): 214 (log ϵ 4.40), 230sh(4.01), 297sh(4.38), 307(4.48), 368(3.50) and 386(4.08) nm.

IR $\bar{\nu}_{max}$ (Potassium bromide), 1631, 1611, 1585, 1505, 1492, 1460, 1400, 1366, 1357, 1330, 1310, 1300, 1275, 1250, 1160, 1150, 1130, 1040, 900, 887, 875, 850 and 750 cm^{-1}.

MS (M^+); m/e: 232(100), 231(12), 217(26), 190(10), 116(14) and 98(15).

NMR (trifluoroacetic acid): δ 5.08(s, 3H N-CH_3), 7.55-8.62 (m 8H aromatic) and 8.95 (s, 1H aromatic) ppm.

Quindoline (25) a yellow coloured alkaloid had also been isolated from an ethanol extract of the roots of C. sanguinolenta (19). Its properties were given as:

UV λ_{max} (methanol) 227 (log ϵ 4.30), 269 sh (4.45), 274 (4.46), 330 sh(3.82) and 345(4.03) nm.

λ_{max} (0.01N ethanolic hydrochloric acid): 224 (log ϵ 4.27), 242sh(3.94), 273(4.36), 280(4.38), 350sh(4.03) and 368(4.26) nm.

IR $\bar{\nu}_{max}$ (potassium bromide): 1632, 1608, 1487, 1457, 1396, 1370, 1333, 1222, 1150, 1140, 1122, 1105, 1000, 875, 865, 845, 837, 813, 754, 745, 738, 710 and 604 cm^{-1},

Mass spectrum (M^+): m/z 218(100%): 217(8); 190(8); 109(12); 95.5(4); 90(3) and 89(5).

Ablordeppey et al (21) also reported the ^{1}H-NMR and ^{13}C-NMR assignments of cryptolepine (Tables 2.6 and 2.8, pages 63-64. In a separate report, Albert et al (22) gave the ^{1}H-NMR and ^{13}C-NMR assignments of cryptolepine as contained in Tables 2.6 and 2.8, page 63-64.

1.7 Pharmacological Properties of C. sanguinolenta

According to Boakye-Yiadon (15) in southern Nigeria, the root of C sanguinolenta is used as a tonic and sometimes in the treatment of rheumatism and urinogenital infections. It was also reported that aqueous extract of the root of the plant possess anti-microbial and anti-bacterial properties against some urinary tract pathogens and wound infections. It was also said to possess anti-inflammatory properties. The study of anti-microbial activity of the aqueous extract of the root of this plant against four urinogenital diseases for which the plant is used by herbalists gave results which justified its use in the treatment of urinary tract infections where Candida albicans, Neisseria gonorrhoeae and Eschesichia coli are the offending pathogens but not against Pseudomonas aeruginosa. It had been earlier reported that the root of C. sanguinolenta is

used along with tamarind fruits as dyestuff to dye goat leather yellow by the Hausas in the northern part of Nigeria (8). The Angolans are also said to use it as yellow dye for leather. Aqueous extract of the root is used in traditional medicine across West Africa for treatment of malaria, wound and urinary tract infections (16). In 1992, Njar V.C.O. (17) reported that in parts of Cross River State of Nigeria, the root extracts of C. sanguinolenta are used as antidotes against most local poisons.

Cryptolepine (22) had been isolated from this plant nearly sixty years ago and had been shown to be hypotensive - causing a marked and prolonged fall in blood pressure in dogs as well as lowering of body temperature (16). Bamgbose and Noamesi (23) also showed that Cryptolepin inhibits carrageen-induced oedema and therefore can be useful in medicine as anti-inflamatory drug in the same way as aspirin or indomethacin, although cryptolepine was found to be a weaker anti-inflamatory agent than indomethacin. Cryptolepine has also been shown to have alpha-adrenoreceptor blocking properties and has the same potency as a drug known as phentolamine

Besides the strong hypothermic effect, cryptolepine reduced considerably the hypotensive effects of adrenaline as well as its renal vasconstrictive effects (24). It was also said to have low toxicity (120g/kg produced death in guinea pigs about 12 hours

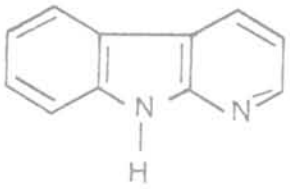
after administration). The hypothermic effect probably explains the use of the plant in traditional medicine against malaria fever but there is no evidence of any plasmodicidal effect for the plant. However it was reported that cryptolepine from the roots of C. sanguinolenta showed weak anti-thrombotic activity. This indoquinoline alkaloid has also been screened for putative antiviral, antifungal and antibacterial activities and found to exhibit prominent activities against the yeast Candida albicans and all Gram-positive bacteria such as Salmonella thyphi, Escherichia coli and Enterobacter aerugenes whereas neither antifungal nor antiviral properties was detected. The alkaloid was reported to be toxic to the host cells in the antiviral in vitro testing system in concentration above 1µg/l. However, cryptolepine hydrochloride (24) has been found to have a wide spectrum of activity against Gram-positive and Gram-negative bacteria as well as Candida albicans (25). Cryptolepine hydrochloride also showed a growth-inhibition effect on Staphylococcus aureus.

1.8 The Benzocarboline Alkaloids

Both cryptolepine (22) and quindoline (25) belong to the benzocarboline group (26). The benzocarbolines are formed from carbolines (27) by fusing on another benzene ring. The

carboline (27) ring system consists of a benzene ring, a pyrole ring and a pyridine ring fused in such a way that the pyrole ring is between the other two which are joined to it through its 2,3- and 4,5- carbon atoms. Carboline (27) ring can also be considered as a carbazole (28) ring in which one of the benzene rings has been replaced by pyridine.

There are four classes of the carboline systems: α-carboline (29), β-carboline (30), γ-carboline (31) and δ-carboline (32) depending on the position of the nitrogen atom in the pyridine ring. Two classes of the benzocarbolines are obtained depending on whether the other benzene ring is fused to the benzene or to the pyridine ring of the carboline. It seems the first will contain twelve possible ring systems, three being derivatives from each of the four carbolines (i.e. possible centres of attachment to the benzene ring are: 6,7-, 7,8- and 8,9- positions). So far only one representative of the benzocarbolines of this class appears to have been described in literature (26) i.e. 6,7-benzo-α-carboline (33). In the second class there are two ring systems derivable from α-carboline: 3,4-benzo-α-carboline (34) and 4,5-benzo-α-carboline (35). From β- as well as γ-carbolines, only one benzocarboline each of this class can be formed. These are: 4,5-benzo-β-carboline (36) and 2,3-benzo-γ-carboline (37) respectively. In δ-benzocarboline, two



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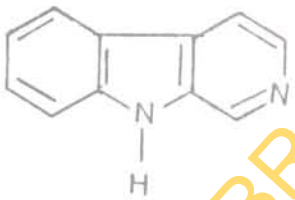

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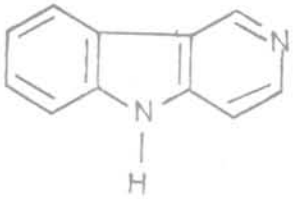

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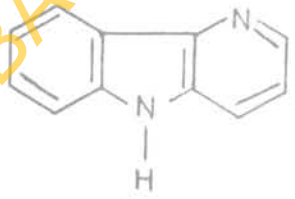

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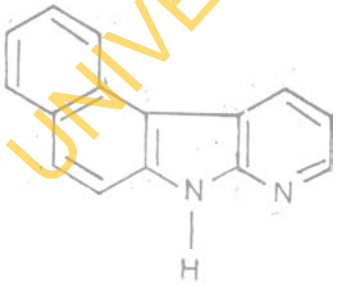

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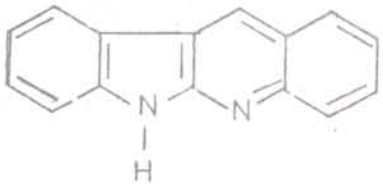

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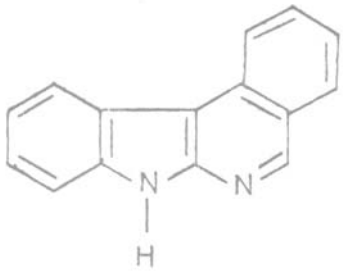

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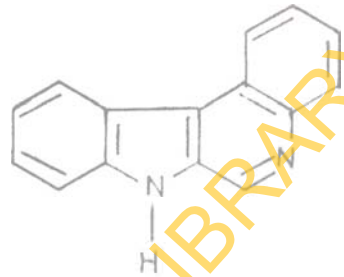

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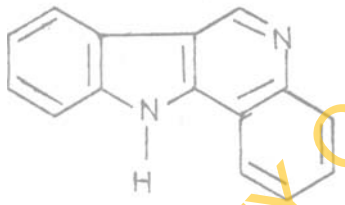

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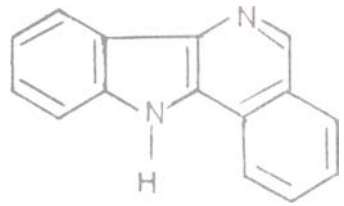

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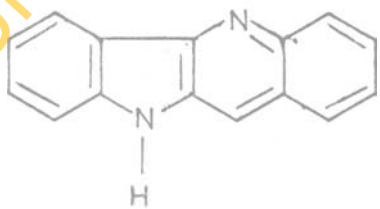

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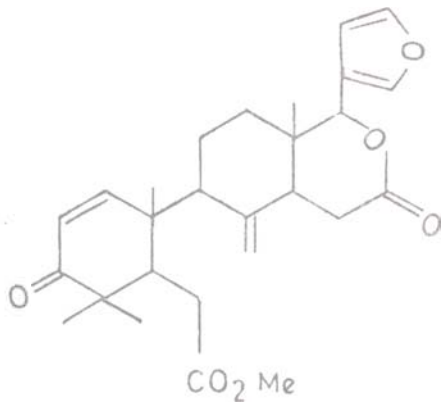


(40)

ring systems are as represented by 2,3-benzo-δ-carboline (38) and 3,4-benzo-δ-carboline (39).

Both quindoline and cryptolepine resemble each other but for the presence of pyridyl N-methyl group in cryptolepine which is absent in quindoline. However quindoline had been obtained from cryptolepine. Selenium dehydrogenation of cryptolepin resulted in loss of the N-methyl group (26) resulting in quindoline.

1.9 Gastric Ulcer

Gastric ulcer is a gastrointestinal disease which usually occur on the lesser curvature. This may be related to the position of the muscle bundles underlying the mucosa (27).

They are usually round but they may be oval, elongated or elliptical. Except in the early stages, gastric ulcers are deep and penetrate the muscularis mucosae. This distinguishes them from superficial erosions which do not extend through the muscularis mucosae (28).

It is estimated that three million persons in the United States are affected annually by peptic ulcer (29). Most series report a male to female ratio of 3:1 or 4:1 (30,31).

Patients with gastric ulcer usually present with abdominal pain and discomfort localised to the epigastric region. The pain is

described as "aching", "nagging", "cramplike" or "dull" and in most patients the pain radiates to the back, the sternum or to the lower abdomen. Food or antacids relieve pain in most patients although in some (25% in a series) food precipitates or aggravates pain (32). Nausea and vomitting may occur in patients with benign peptic ulcer. In few patients vomitting may decrease or relieve pain.

Gastric Ulcer and Gastric Cancer

It is extremely difficult and in most cases impossible to differentiate between gastric ulcer and gastric cancer on the basis of signs or symptoms. Epigastric pain, anorexia, vomitting and bleeding occur with about equal frequency in both diseases. Differentiation is usually made by x-ray, endoscopy with biopsy, cytology and/or response to medical therapy (33).

Medical Therapy

Various forms of medical therapy had been put forward for treatment of peptic ulcer which include: hospitalization, antacids, anticholinergics, dietary restriction and the use of special therapeutic agents.

Hospitalization has been associated with an increased healing rate of gastric ulcer in two studies (34,35). During these studies in-patients were hospitalized for four weeks. Hospitalization for

twelve days does not increase the healing rate of gastric ulcer (36).

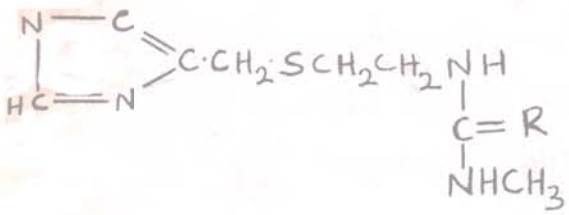
Antacids are prescribed for ulcer patients because they relieve pain and it is hoped that they may hasten healing of the ulcer (37).

Anticholinergics e.g. glycopyrronium bromide (Robinul) are thought to inhibit acid secretion by blocking an acetylcholine receptor on or near the gastric parietal cell (38).

The purpose of dietary management is to eliminate irritating foods and to provide adequate and continuous neutralization of acid. Lenhart introduced the concept of frequent small feedings and through the years, this dietary regimen has been modified to include more milk and cream. Alcohol (ethanol) damages the gastric musocal barrier and may stimulate gastric acid secretion (40). Ulcer patients should therefore be advised not to drink alcohol. Salicylic acid and acetylsalicylate are also known to damage the gastric mucosa, produce mucosal erosions, cause chronic gastric ulcers and predispose to hemorrhage from existing ulcers. Patients with grastric ulcer should therefore be advised not to take aspirin-containing compounds.

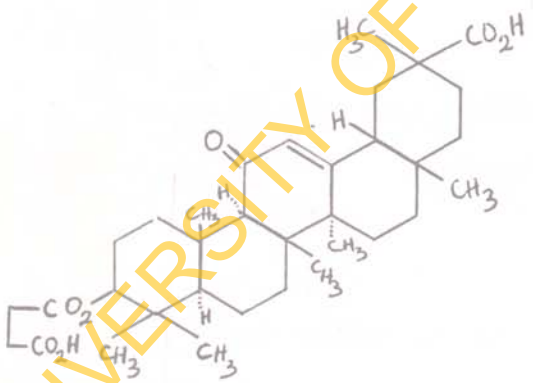
Other special therapeutic agents include: the use of Histamine H_2-receptor antagonists e.g. cimetidine, (41) use of carbenoxolone (42) or Bismuth compounds among others.

Histamine, (43) exerts physiologic and pharmacologic effects by interaction with at least two different receptors: H_1-receptors which



(41) R = N–CN

(44) R = S



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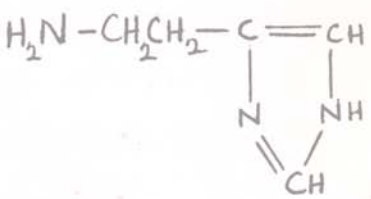


(43)

mediate the action of histamine on smooth muscle of the gut and bronchi and the H_2-receptor which mediate the action of histamine on the gastric parietal cells. Analogues of histamine such as burimamide, metiamide (44) and cimetidine (Tagamet) competitively inhibit the action of histamine on the H_2-receptor. In man these drugs effectively inhibit gastric acid secretion (41).

The development of carbenoxolone can be traced to the use of certain plants or their extracts in folklore medicine as with many other drugs. The treatment of ulcer with carbenoxolone originates from the practice of using Licovice or its extract in treatment of dyspepsia (42,43).

Bismuth-peptide complex compounds differ from bismuth-containing antacids in that they interact with proteins to form protective bismuth complexes rather than in neutralizing gastric acid.

Gastric Ulcer in Experimental Animals

To study gastric ulcer in experimental animals, they are first induced with the ulcer in one of many ways among which include: use of necrotizing agent such as indomethacin, reserpine, ethanol, aspirin, ligation of pylorus and hypothermis restrain stress. Though each of these methods work by different mechanism but

importantly they are all associated with higher values of free gastric acidity.

In hypothermic restraint stress-induced ulcer, the animals were immobilized in restraint cages and placed in a ventilated refrigerator maintained at a temperature of -4°C for two hours (45).

Indomethacin dose of 40mg/kg body weight had been found to be effective at inducing gastric ulcer in experimental animals (46, 47,48).

5mg kg^{-1} body weight of reserpine administered to rats have been found effective in inducing ulcer (49). 1ml of 80% ethanol is also acclaimed effective (14,50) in inducing ulcer.

According to the method of Shay et al (51,52) the technique of ligation of pylorus involves light anaesthesia and care need be taken not to cause bleeding or to occlude blood vessels.

1.10 Malaria

Malaria is a parasitic disease. It is one of the most important diseases affecting the population over wide areas of the world, most especially the tropical and sub-tropical regions (53). It has been regarded as the world's greatest killer ahead of cancer or any of the heart diseases (54).

For years, the causative organism was unknown until 1880 when Alphonse Laveran, a French army surgeon discovered the malaria parasite in human blood and Ross in 1897 observed the sporogenic forms of the parasite in an anopheles mosquito (55).

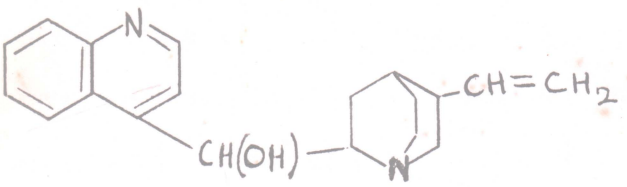
In spite of intensive efforts and programme for drug control and eradication of malaria instituted by the World Health Organization (56), it is still a major cause of mortality among the parasitic diseases that beset man in the warmer parts of the world. The disease has adverse effect on the physical development of the people thereby retarding their social, intellectual and political progress (57). Man has endeavoured to control malaria by eliminating the malaria parasites or by interrupting their transmission by the use of insecticides. At present in Tropical Africa where the disease is endemic, chemotherapy is the main and often the only operationally, administratively and financially feasible method of malaria control especially in rural areas, technical and financial constraints having considerably reduced the use of residual insecticides (58). Consequently, the use of drugs for chemoprophylaxis and treatment has been of particular importance.

Quinine, (45) the active principle of cinchona bark, the first effective antimalaria drug has proved to be such a good suppresant for malaria that it remained the mainstay of antimalarial therapy for centuries (59).

It was a bitter experience of non-availability of quinine from cinchona bark during the World War I that led to organized efforts to develop synthetic antimalarial drugs as substitutes. This led to the development of the first synthetic antimalarial drug, Pamaquine in 1926 by the Germans (60). Mepacrine (46) was synthesized in 1930 and chloroquine in 1934.

Pamaquine is a member of the 8-aminoquinolines (47) which, although was very active against avian plasmodia, was not very active in human malaria and was found toxic in man (61). The most important of these synthetic antimalarial compounds was chloroquine but was initially set aside because of toxicity (62). The loss of Java in 1942 by the Allies during World War II meant loss of supplies of quinine, therefore a programme to exploit synthetic antimalarial drugs was started in United States under the Walter Reed Army Institute of Research (62). The most important antimalarial drug which emerged under this programme was chloroquine (48). But this was only a rediscovery in that it had been earlier synthesized by the Germans in 1934. After the war, synthesis of antimalarial drugs continued and this led to the commercial development of proguanil (49) and pyrimethamine (50) (63).

Since chloroquine, proguanil and pyrimethamine were rapidly effective against erythrocytic stages of all species of plasmodium and primaquine (51) was effective against exoerythrocytic stages, the problem



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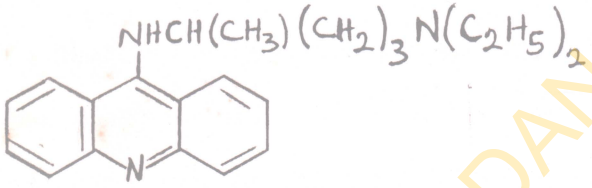

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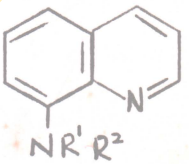
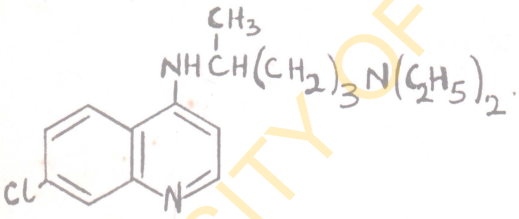


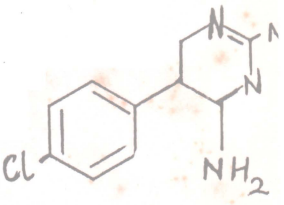
(47)

(47) $R^1 = R^2 = H$



(48)

(51) $R^1 = H$; $R^2 = -\underset{\underset{CH_3}{|}}{CH}(CH_2)_3NH_2$



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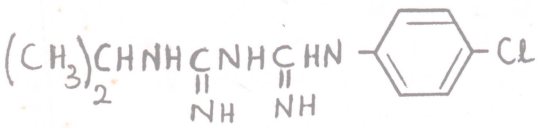


(49)

appeared solved. However, when Plasmodium falciparum resistance to chloroquine was reported in the late 1950s and in addition many strains of malaria parasites were also found resistant to the other available drugs, the need to develop drugs effective against these drug-resistant strains arose (61,64). One the best new drugs is mefloquine, a synthetic compound which is a close analogue of quinine.

This search for new antimalarial drugs has been a continuous one for two reasons: Firstly since the discovery of chloroquine-resistant strain of P. falciparum in 1959 in Latin America, resistant strains to other antimalarial drugs have been found with increasing frequency (65,66). Secondly, there have been series of reports that many of the synthesized antimalarial drugs in the market have adverse side effects (67,68).

The search for more effective and less toxic drugs for malarial management has not been restricted to synthetic efforts of medicinal chemists, the need for intensive screening of plants for bioactive agents has been recognized as a valid approach to the problem (69). This approach is already yielding good dividends; an example of a new antimalarial drug obtained from plant source is Quighaosu (artemisinin). It was obtained from the Chinese medicinal herb Artemisia annua L. Quighaosu has been reported to be active against some strains of chloroquine- resistant P. falciparum.

Aqueous extract of the root of Cryptolepis sanguinolenta is used in traditional medicine across West Africa for the treatment of malaria (16). This claim however is not supported by scientific data and it is not documented in available literatures.

1.11 The Aim of This Study

As reviewed above, E. angolense and C. sanguinolenta are medicinal plants used locally in the treatment of ulcer and malaria respectively; but these claims have not been scientifically verified and documented.

The objective of this research work therefore is to investigate these claims by carrying out the phytochemical and pharmacological studies of these plants to see if they actually possess these properties, thus establishing a rational basis for their use in traditional medicine.

It is also hoped that this work will establish the bioactive principles in these plants responsible for their medicinal properties, thus encouraging local sourcing of drug raw materials and contributing in search for new and more effective drugs in chemotherapy.

Attempts will also be made to determine the mode of action of any such bioactive compound(s) isolated where possible.

CHAPTER TWO

RESULTS AND DISCUSSION

2.1 Extract from Stem Bark of E. angolense

The extraction of 200gm of dried pulverized stem bark of E. angolense afforded 15.0gm of the crude methanolic extract, a 7.5% yield. Chromatographic fractionation of 5.0gm of the crude extract gave 1.51gm of methyl angolensate labelled EA1 (30% yield).

∴ 5.0gm of crude extract gave 1.51g of methyl angolensate

∴ 15gm of crude extract will give (1.51x3)gm of methyl angolensate = 4.53gm.

Thus the percentage yield of methyl angolensage from the stem bark of E. angolensate $= \frac{4.53}{200} \times \frac{100}{1} = 2.26\%$.

2.2 Extract from the Root Bark of E. angolense

450gm of the dried and pulverized root bark of E. angolense afforded 31.0gm of the crude extract (7% yield). Chromatographic fractionation of 5.0gm of the crude extract gave 0.2gm (4% yield) of methyl angolensate.

5.0gm of crude extract gave 0.2g of methyl angolensate,

∴ 31.0gm of crude extract will give $(\frac{0.2}{5.0} \times \frac{31.0}{1})$gm = 1.2gm of methyl angolensate. The percentage yield of methyl angolensate from

the root bark of E. angolense therefore is

$$\frac{1.2}{450} \times \frac{100}{1} = \underline{0.27\%}.$$

Thus the yield of methyl angolensate from the stem bark is higher than the yield from the root bark of E. angolense.

2.3 Spectroscopic Data

Nuclear Magnetic Spectra (NMR)

The ^{1}HNMR of methyl angolensate (EAI) is shown in Fig. 2.1. The assignments are as shown in the experimental section and it is contained in Table 2.1. These data and its assignment are in agreement with those put forward by Bevan et al (10) for the structure of methyl angolensate and Ollis and Ward (80) for the structure of andirobin (40).

The ^{13}C-NMR of methyl angolensate (Fig. 2.2) resolved for 27 carbon atoms whose values are as follows: five methyl carbon signals at: 13.69; 21.42; 21.58; 25.81 and 52.01. Six methylene carbon signals at 23.69, 29.26, 32.62, 33.74, 39.37 and 111.48. Seven methine carbon signals at: 42.86; 49.86; 77.16; 79.53; 109.88; 140.72 and 142.70. Other signals include: The three carbonyl carbon signals at: 169.94: 173.80 and 212.67 and the olefinic quartenary carbon signal at 145.77. The list of assignment of these carbon signals is contained in Table 2.2.

Fig. 2.1: EA-1 1H NMR IN CDCL3 (GHUSSIAN MULT.)

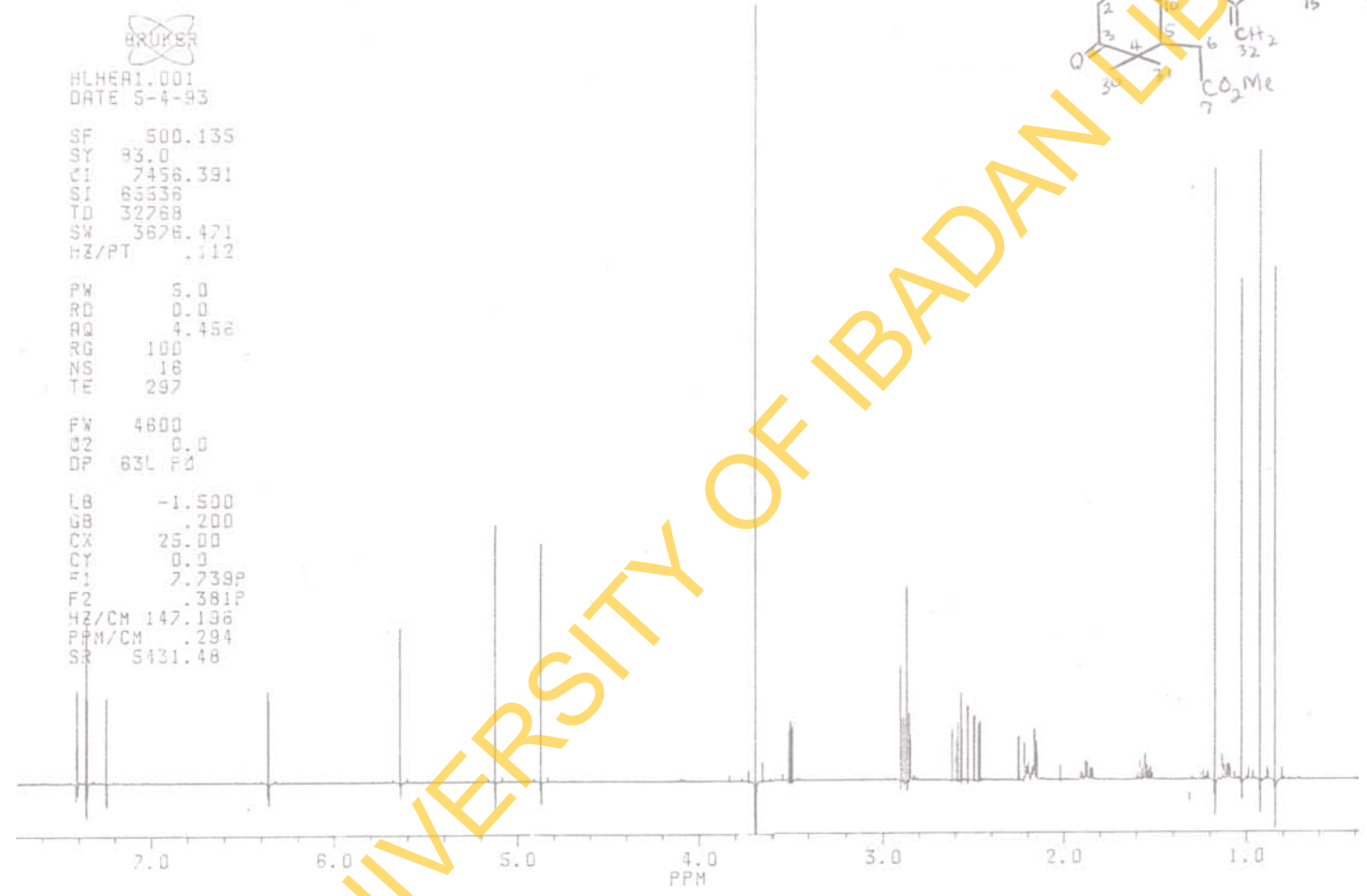


Fig. 2.1(ii): EA-1 1H NMR IN CDCL3 (GAUSSIAN MULT.)

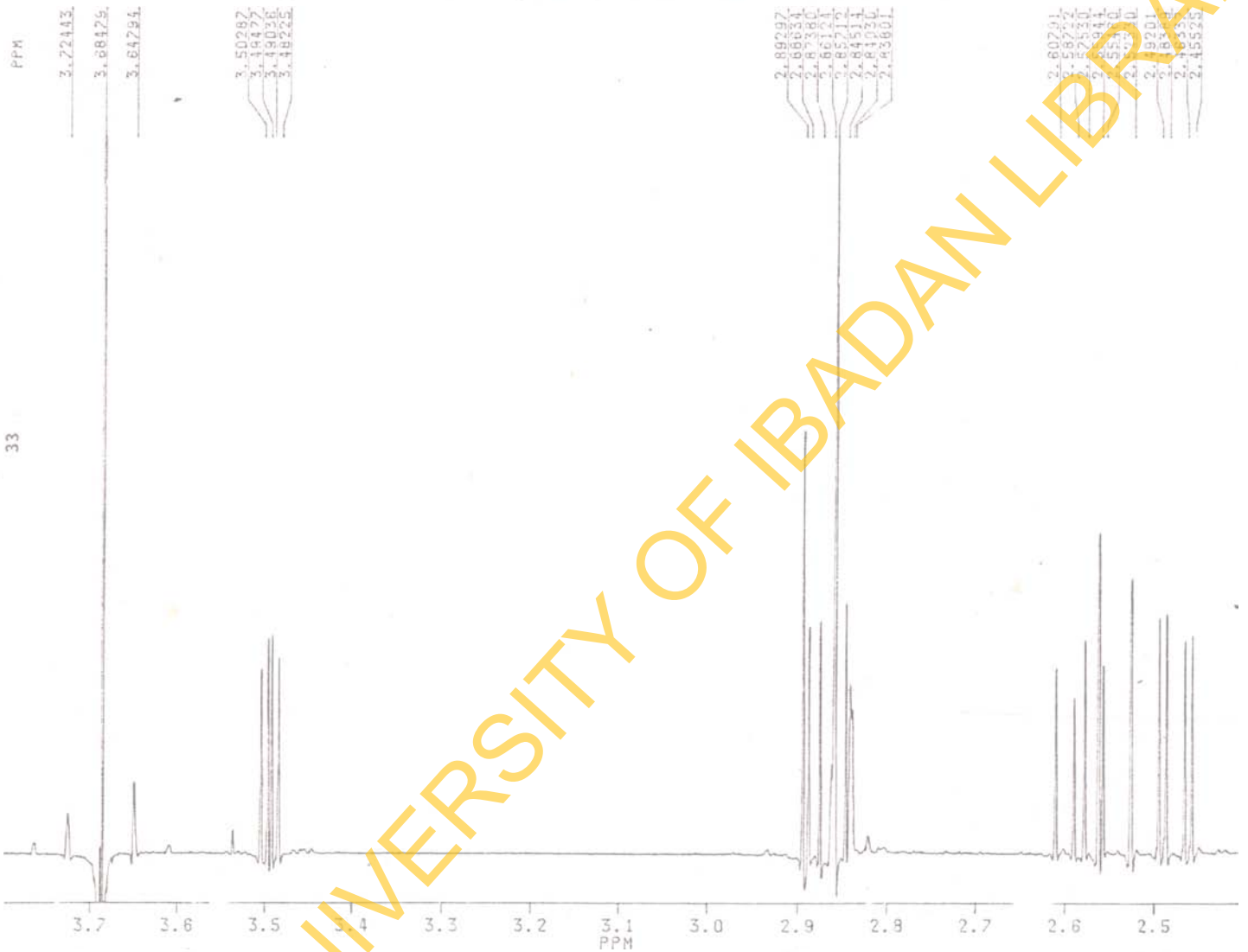


Fig. 2.1(iii): EA-1 1H NMR IN CDCL3 (GAUSSIAN MULT.)

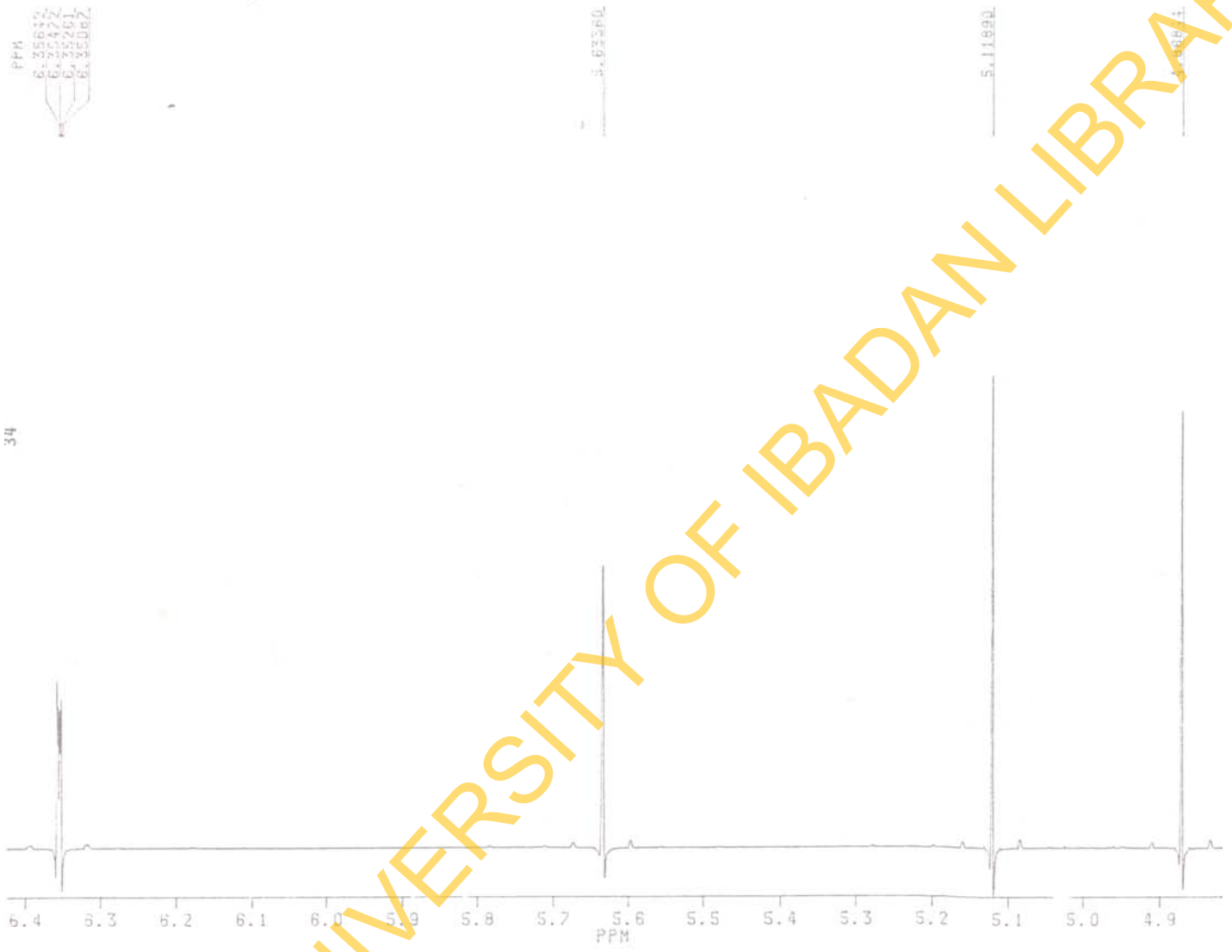


Fig. 2.1(iv): EA-1 1H NMR IN CDCL3 (GAUSSIAN MULT.)

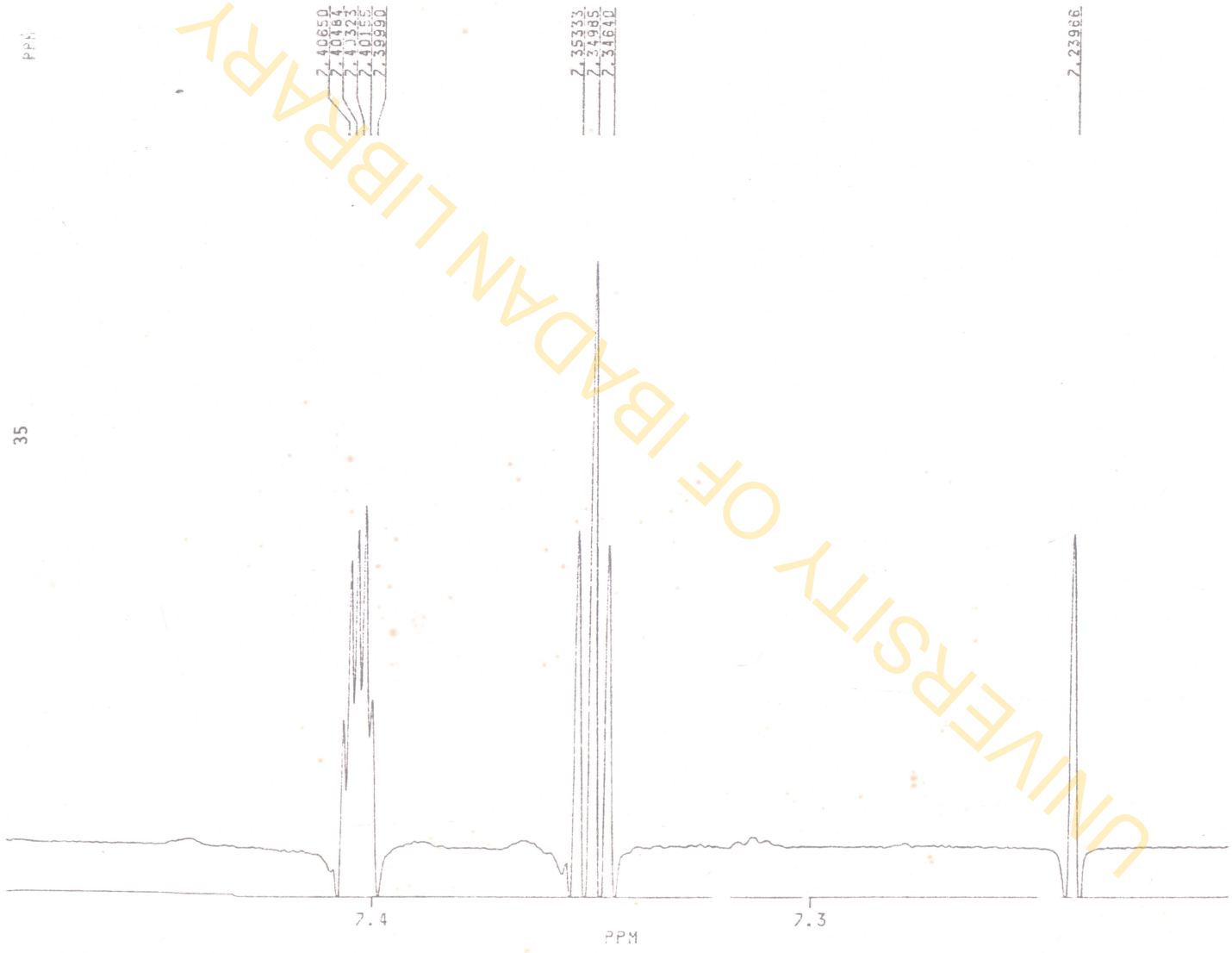


Table 2.1: Proton chemical shifts for methyl angolensate in $CDCl_3$

Position	Chemical Shift δ (ppm) Methylangolensate (10)	EA-1
1	3.54 (1H, q)	3.50 (1H,q J=4.1 H_2)
17	5.64 (1H, S)	5.63 (1H,S)
18	0.88 (3H,S)	0.83 (3H,S)
19	0.95 (3H.S)	0.95 (3H,S)
21	7.35m	7.40 m
22		7.38 dd
23	6.4m	6.35 m
30	1.2 (3H,S)	1.12 (3H,S)
31	1.06 (3H,S)	1.02 (3H,S)
32	5.12 (1H,S)	5.12 (1H,S)
32	4.97 (1H,S)	4.87 (1H,S)
OMe	3.73 (3H,S)	3.68 (3H,S)

Fig. 2.2: EA-1 13C NMR IN CDCL3

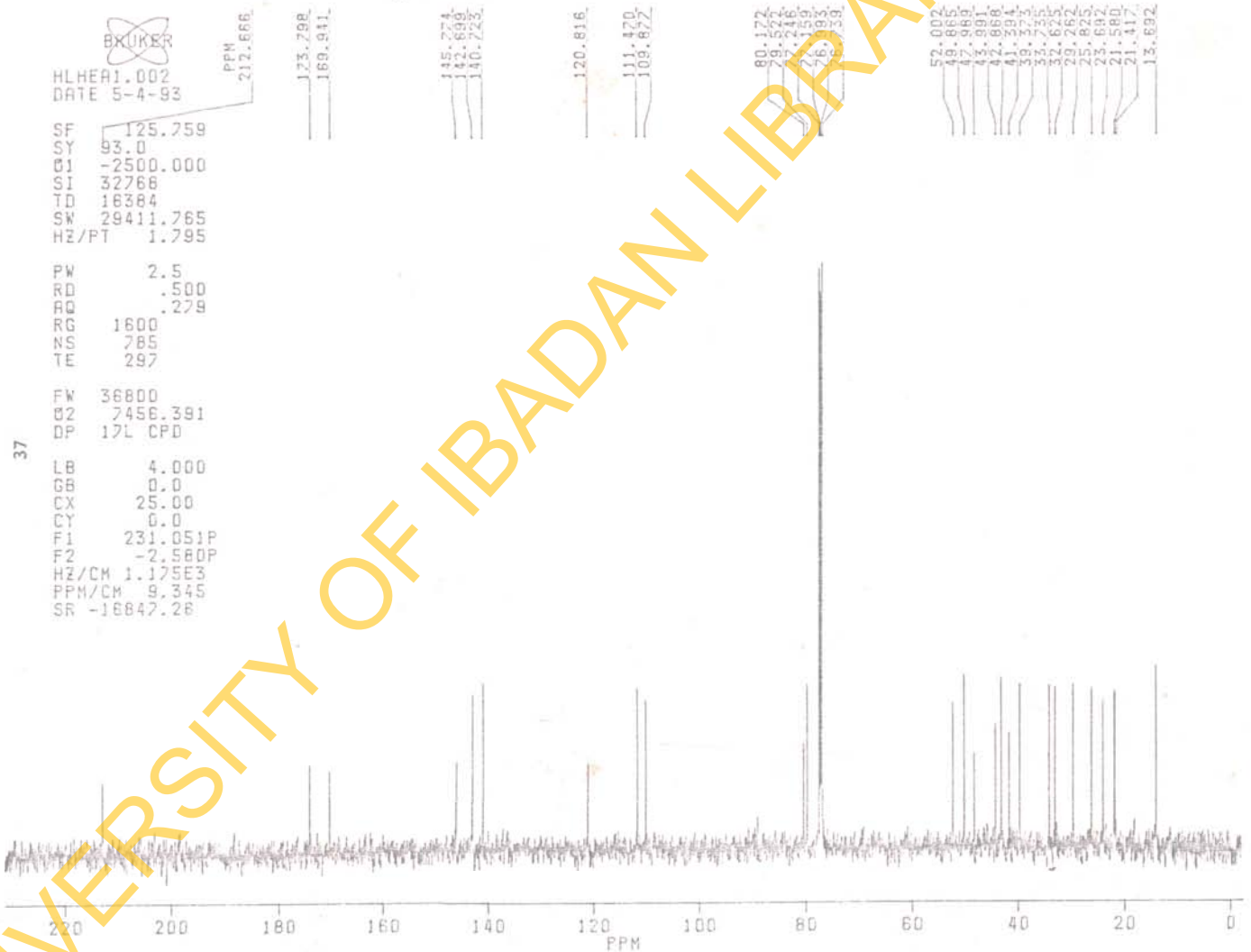


Table 2.2: 13C-NMR data in δ-values from TMS for methyl angolensate (EA-1)

Carbon Position	Chemical Shift (ppm)	Number of attached H.
C-1	77.159	1
C-2	39.372	2
C-3	169.941	0
C-5	42.865	1
C-6	32.624	2
C-7	173.798	0
C-8	145.774	0
C-9	49.861	1
C-11	23.694	2
C-12	29.260	2
C-15	33.735	2
C-16	212.666	0
C-17	79.525	1
C-18	13.688	3
C-19	21.415	3
C-30	21.576	3
C-31	25.814	3
C-32	111.470	2
C-21	109.877	1
C-22	140.723	1
C-23	142.699	1
O-Me	52.008	3

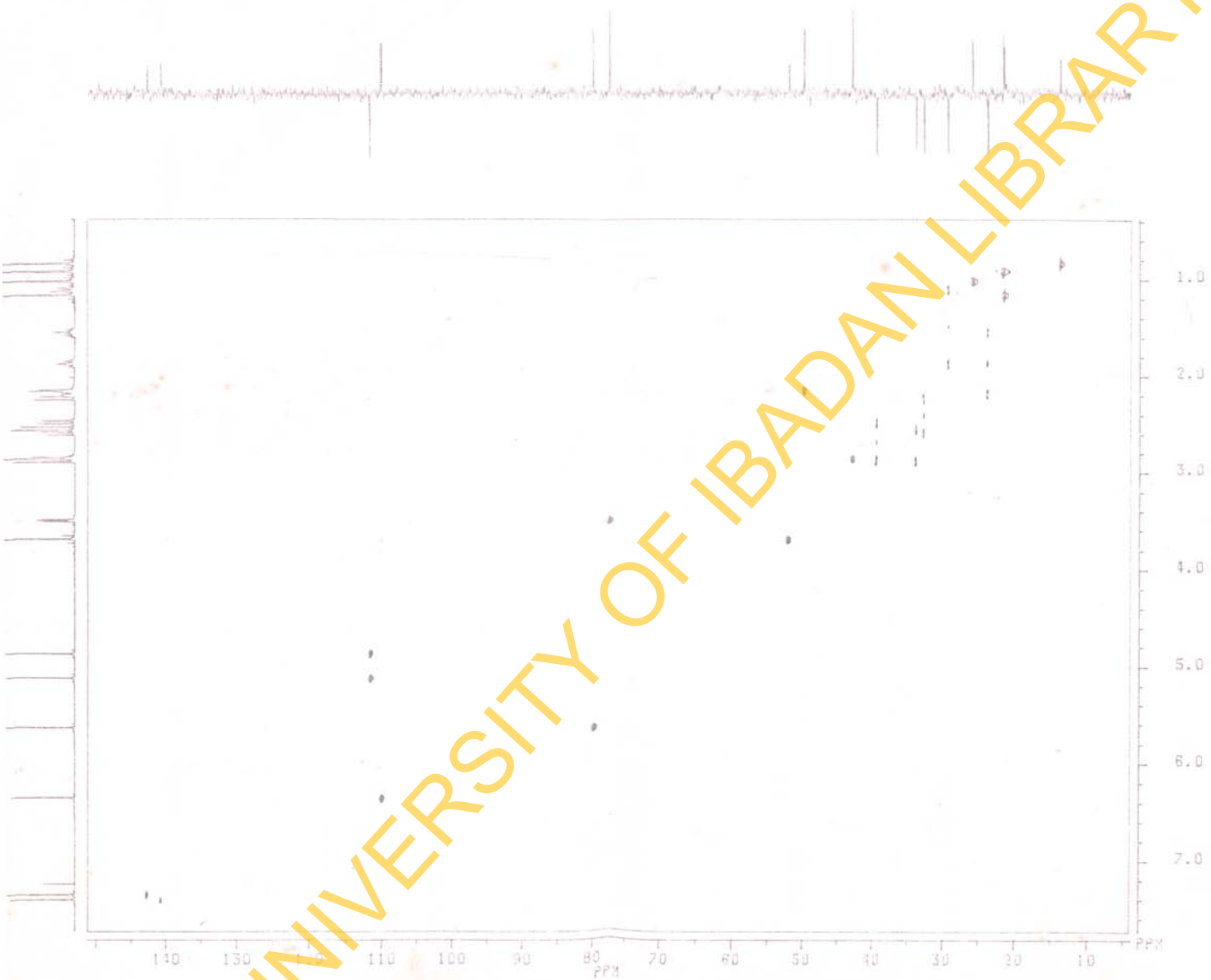


Fig. 2.3: ^{1}H-^{13}C.2D Correlation Spectra of EA-1

The above assignment is on the basis of the chemical shift theory and aided by the $^{1}H-^{13}C$ 2-D chemical shift correlation spectra (Fig. 2.3) and the methyl ($-CH_3$), methylene ($>CH_2$) and methine ($>CH$) spectrum (Fig. 2.4).

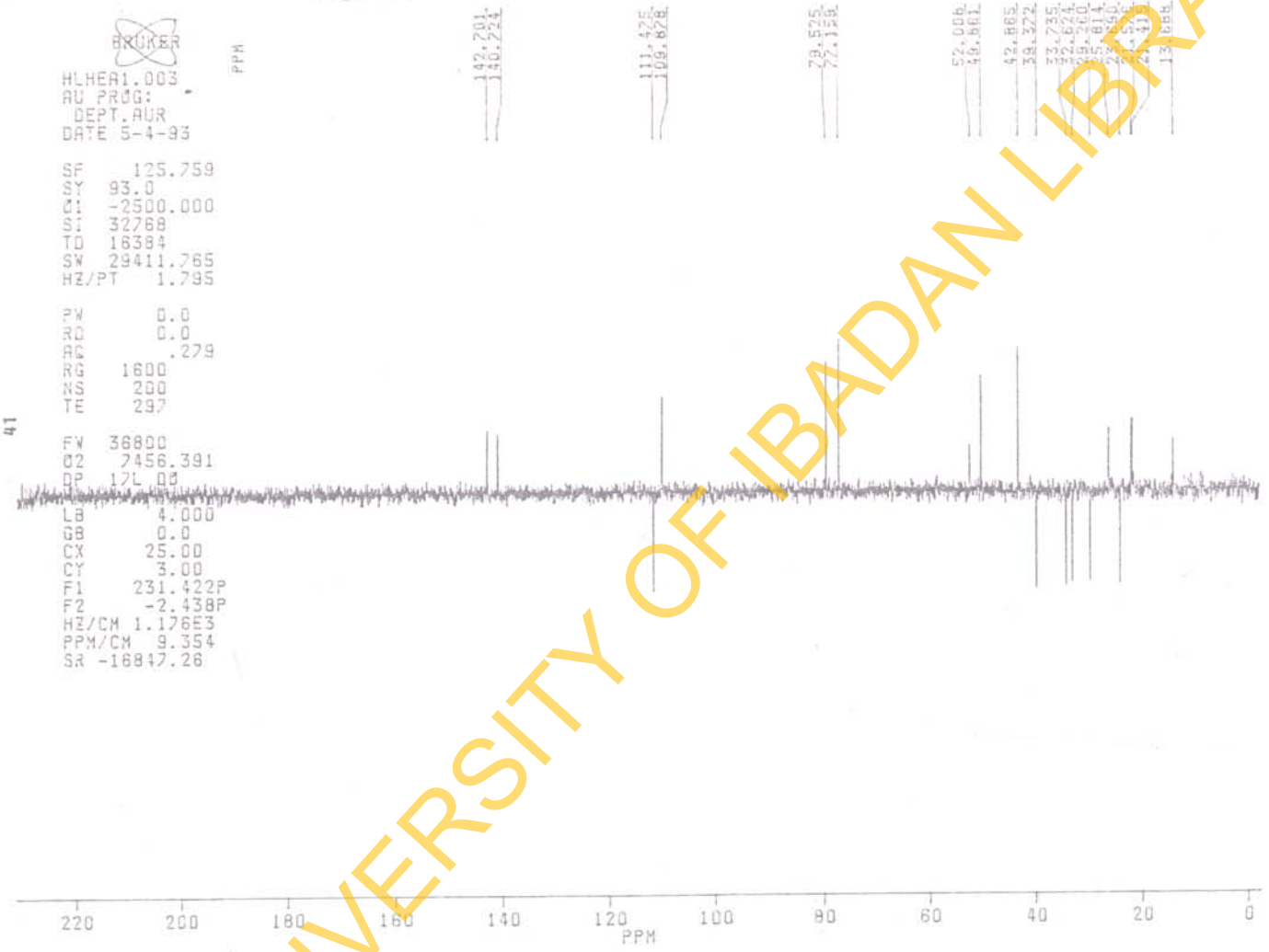
Mass Spectra (MS)

The MS by electron impact (EI) for methyl angolensate (EAI) is shown in Fig. 2.5. The spectrum indicated the molecular ion peak at M^+ 470 (100) which also form the base peak ion. Other prominent fragments observed with the percentage relative abundance shown in parentheses are as follows: 374(11.0); 359(13.0); 332(12.0); 210(24.9); 164(25.7); 148(52.4); 121(12.9); 95(65.7); 69(31.5); 243(13.5); 227(6.4); 244(11.4); 245(5.7), 147(25.1); 149(38.0), 119 (33.7); 120(32.9); 209(18.1); 211(9.5); 177(9.9); 178(7.1) and 179(8.6).

The chemical ionization (CI) mass spectrum in glycerol (Fig.2.6) showed a peak at m/z 471. This is due to the protonated EI molecular ion at m/z 470 i.e. $(M+1)^+$ peak. The MS peaks reported above are identical to those reported and discussed by Bevan et al (10) for the structure of methyl angolensate.

The accurate mass spectrum gave the accurate mass of the molecular ion of methyl angolensate as 470.2307 calculated is 470.5588.

Fig. 2.4: EA-1 13C DEPT CH AND CH3 + CH2 -



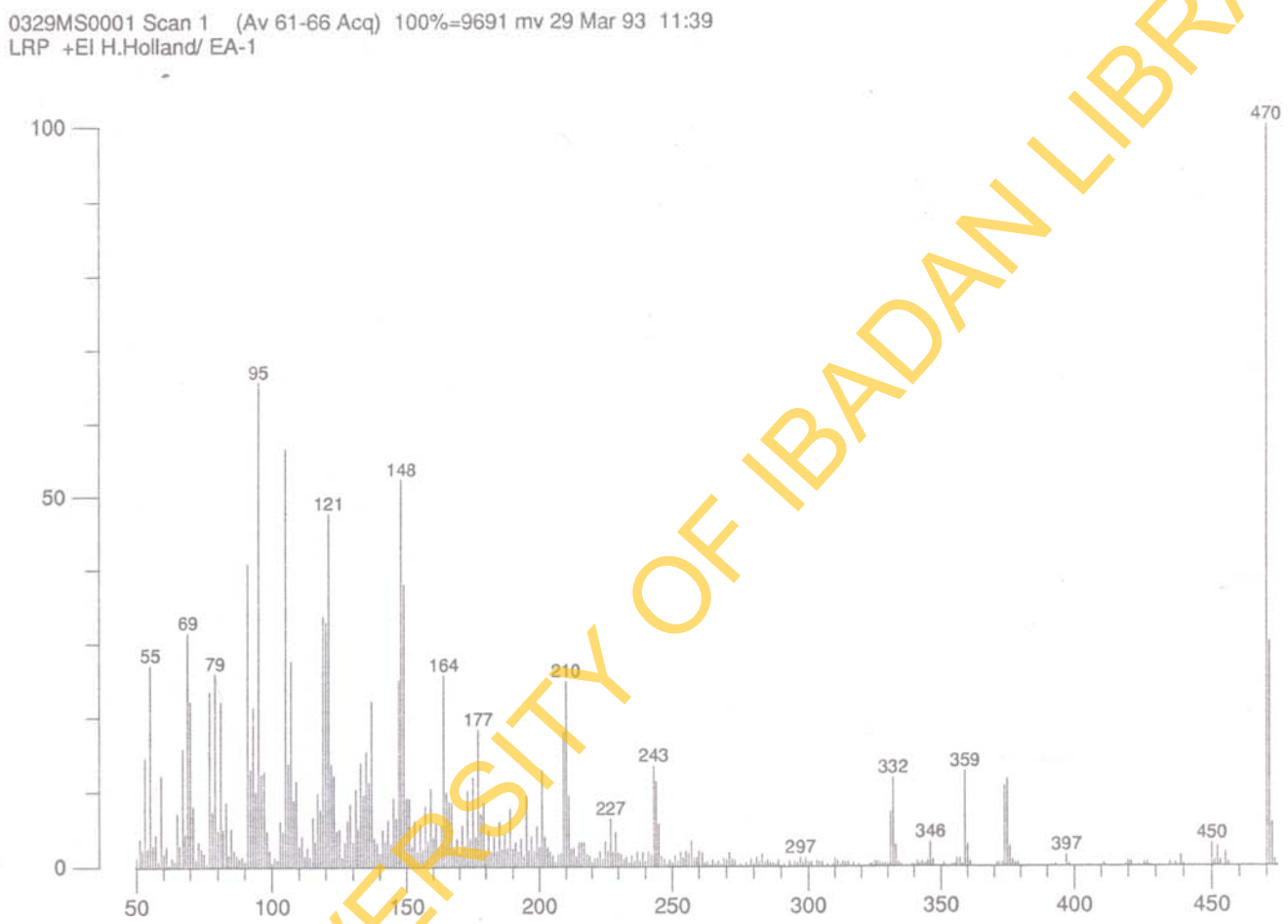


Fig. 2.5: Mass Spectrum of EA-1 (Electron Impact)

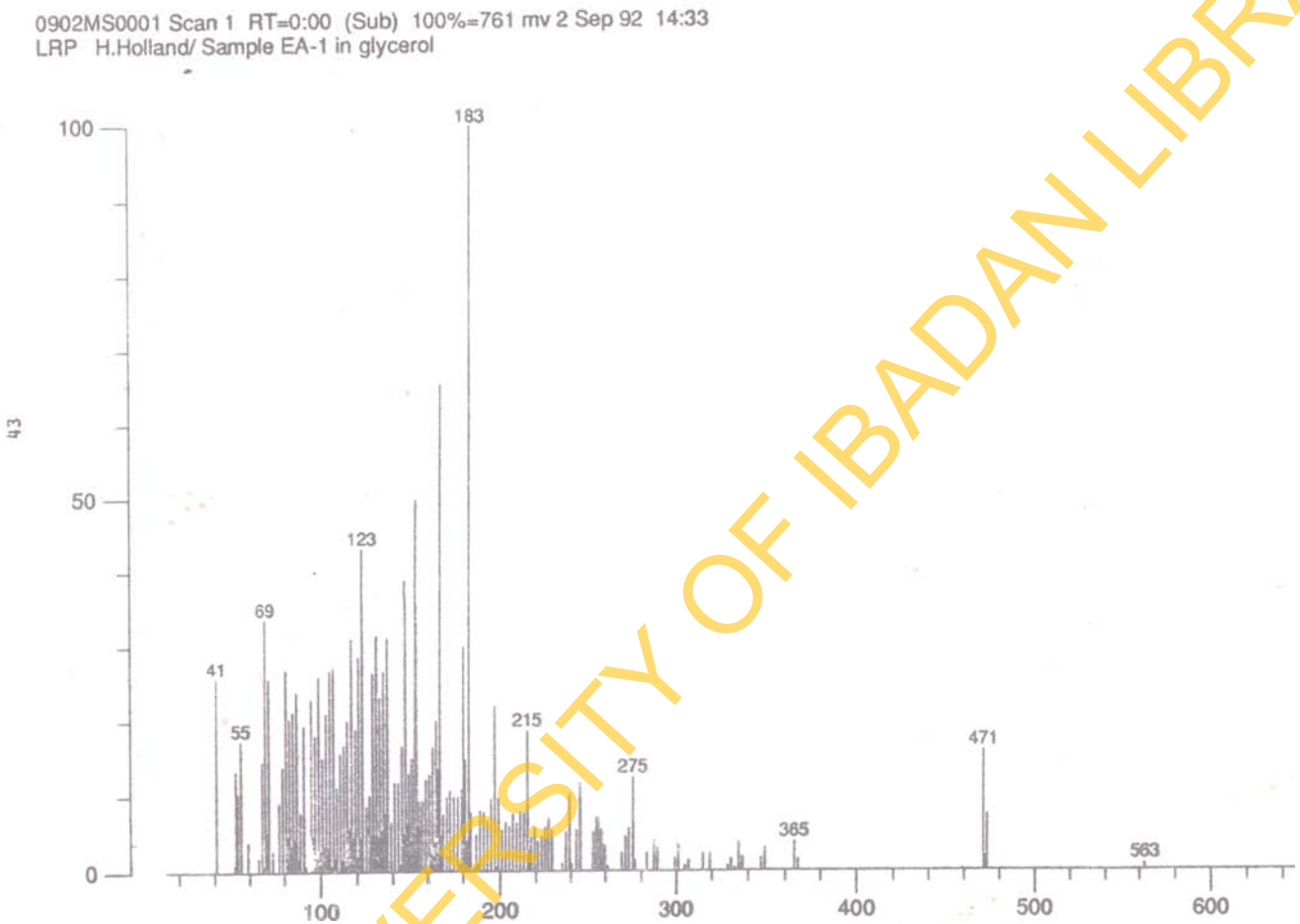


Fig. 2.6: Mass Spectrum of EA-1 (Chemical Ionization)

Elemental Analysis

Elemental analysis of methyl angolensate (Fig. 2.7) gave C = 69.41; H = 7.58 and N = ND (Not Detected). This agrees with that calculated: C = 68.9 and H = 7.3% for $C_{27}H_{34}O_7$. The result of the elemental analysis confirms the absence of nitrogen as shown by the laboratory test. This corrected the impression that the isolated compound EA-1 might be an alkaloid as indicated by a positive Dragendoff's test. This shows that not all compounds that give a positive Drangendoff's test are alkaloids.

Infra-red Spectrum (IR)

The IR spectrum of methyl angolensate in potassium bromide (Fig. 2.8) showed the presence of β-substituted furan ring ($\bar{\nu}_{max}$ 1503 and 875 cm^{-1}) δ-lactone ring and ester ($\bar{\nu}_{max}$ 1735 and 1720 cm^{-1} respectively).

The UV spectrum taken in ethanol showed only absorption due to the furan ring. λ_{max} 205.3 nm ($\log\varepsilon$ 3.95) (Fig. 2.9).

Pharmacological Data on E angolense Extract

2.4 Toxicity Study

The toxicity study with the crude bark extract of E. angolense showed that the extract was not toxic when doses ranging

Guelph Chemical Laboratories Ltd.
246 Silvercreek Parkway N., Guelph, Ontario, Canada. N1H 1E7 Tel. (519) 836-2313 Fax (519) 836-3273

April 12, 1993 Our Report No. 34160

H. L. Holland
Department of Chemistry
Brock University
St. Catharines, Ontario
L2S 3A1

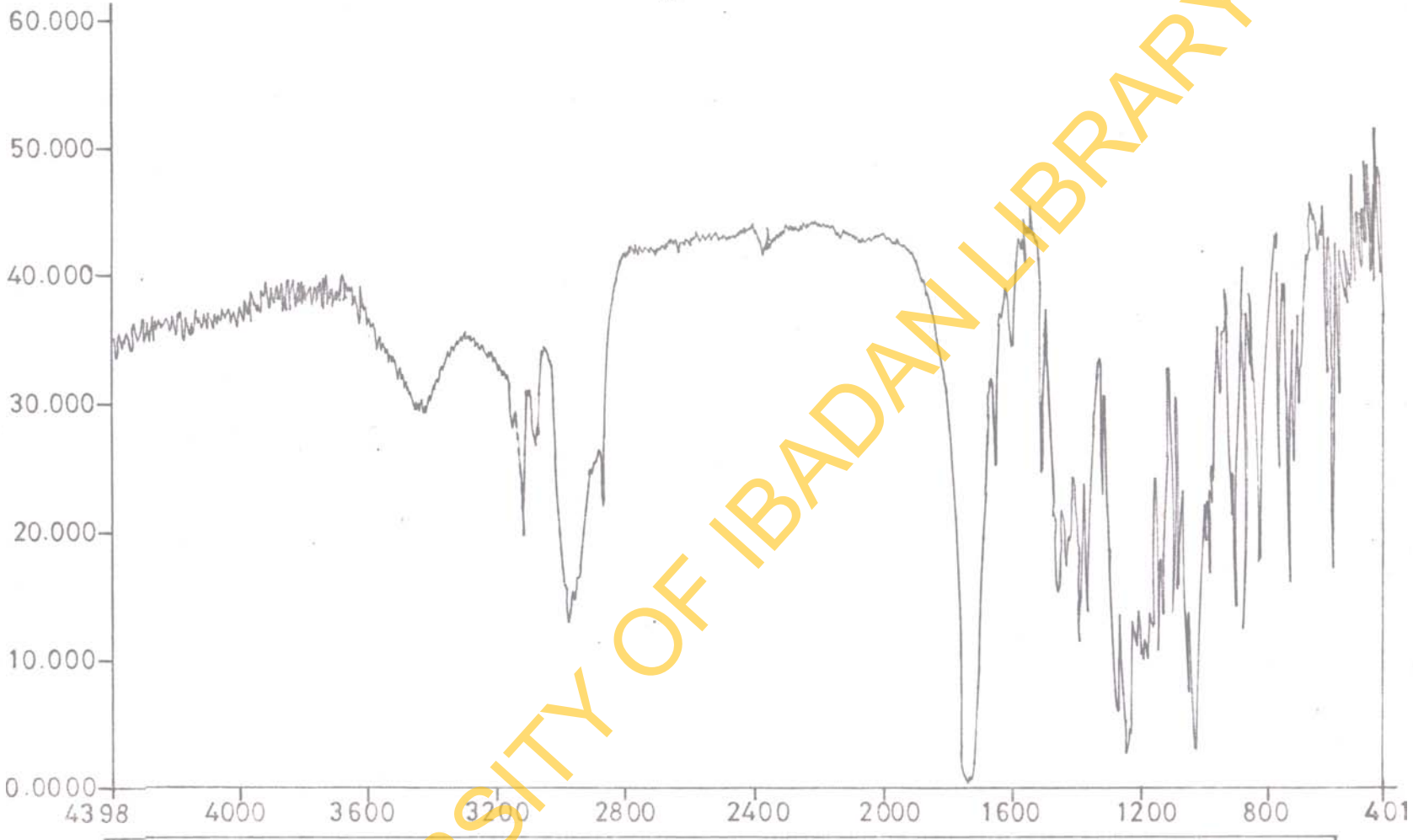
Results of Analysis

	RESULTS		
Sample No.	Carbon %	Hydrogen %	Nitrogen %
EA-1	69.41	7.58	N.D.

N.D. = Not Detected

R. N. Pandey, Ph.D., Queen's
General Manager, Research & Services

Fig. 2.7: Elemental Analysis of EA-1



FILE NAME : EA-1	GAIN : 2	ANALECT FX – 6260
SCANS : 64	DET : TGS	ORD : %T
BKG : 64	RES : 4 CM-1	ABSC : WAVENUMBER
APOD : HAPP-GENZEL	DATE :	TIME :
COMMENT : KBr pellet		

Fig 2.8: Infra-red spectrum of EA-1

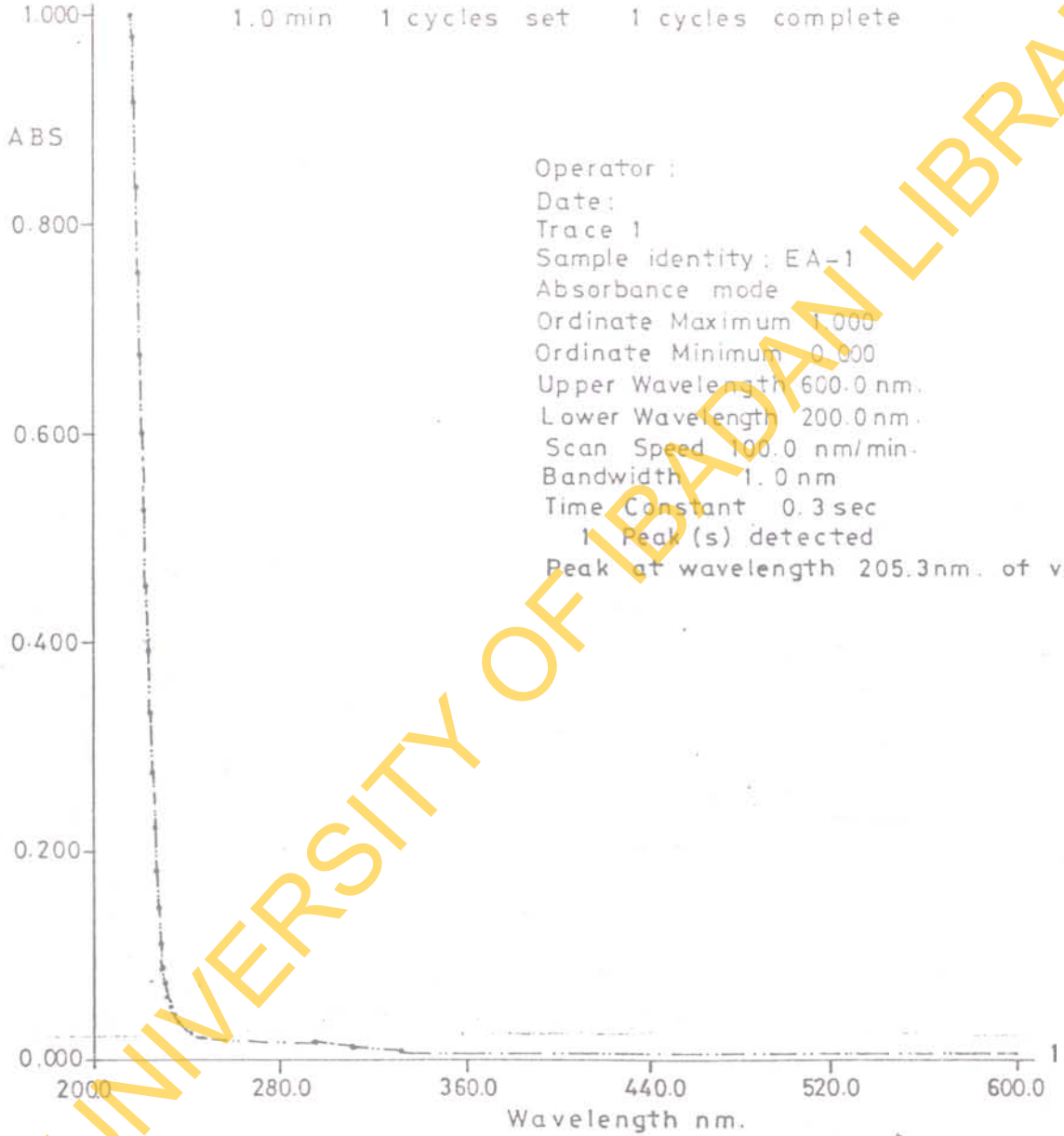


Fig. 2.9: Ultra-violet spectrum of EA-1

from 20-200g kg^{-1} body weight was administered to twenty-five male rats in five groups. The administration of the drug was through the oral route daily for five days.

A dose as high as 200g kg^{-1} neither caused any death nor any observable symptoms. In addition no symptoms of diarrhoea or stereotypic behaviour were observed over the one week period of study.

2.5 Experimental Gastric Lesions

This experiment was performed on both the crude bark extract of E. angolense and the isolated methyl angolensate.

1. Experiment with Crude Extract

In the ulcer studies, indomethacin (40mg kg^{-1}) administered intraperitoneally was effective in inducing acute gastric mucosal damage. This dose has been reported to be effective in inducing gastric ulceration(47,48). The data presented in Fig. 2.10 and Table 2.3 show that the methanol extract of E. angolense produced a dose-dependent gastroprotective effect in indomethacin-induced ulceration in rats. Doses ranging from 400 to 800mg kg^{-1} exerted significant protective effect. Total protection was exerted at a dose of 1,600mg kg^{-1}. The cytoprotection produced by propranolol (40mg kg^{-1}) is lower than that caused by 800mg kg^{-1} of the extract.

Table 2.3: Effect of methanol extract of E. angolense on gastric mucosal lesion induced by indomethacin

Treatment[a]	Ulcer Index[b]	Percent inhibition of ulceration[c] (%)
Control (2% 'Tween' 80*, 2ml kg^{-1}, P.O)	1.8±0.2	-
E. angolense (mg kg^{-1}, P.O)		
200	1.2±0.1	33.3
400	0.8±0.08[d]	55.6
800	0.1±0.02[d]	94.4
1,600	0.0±0.00[d]	100.0
Propranolol (40 mg kg^{-1}, P.O)	0.4±0.07[d]	77.8

[a] Seven animals were used in each test.

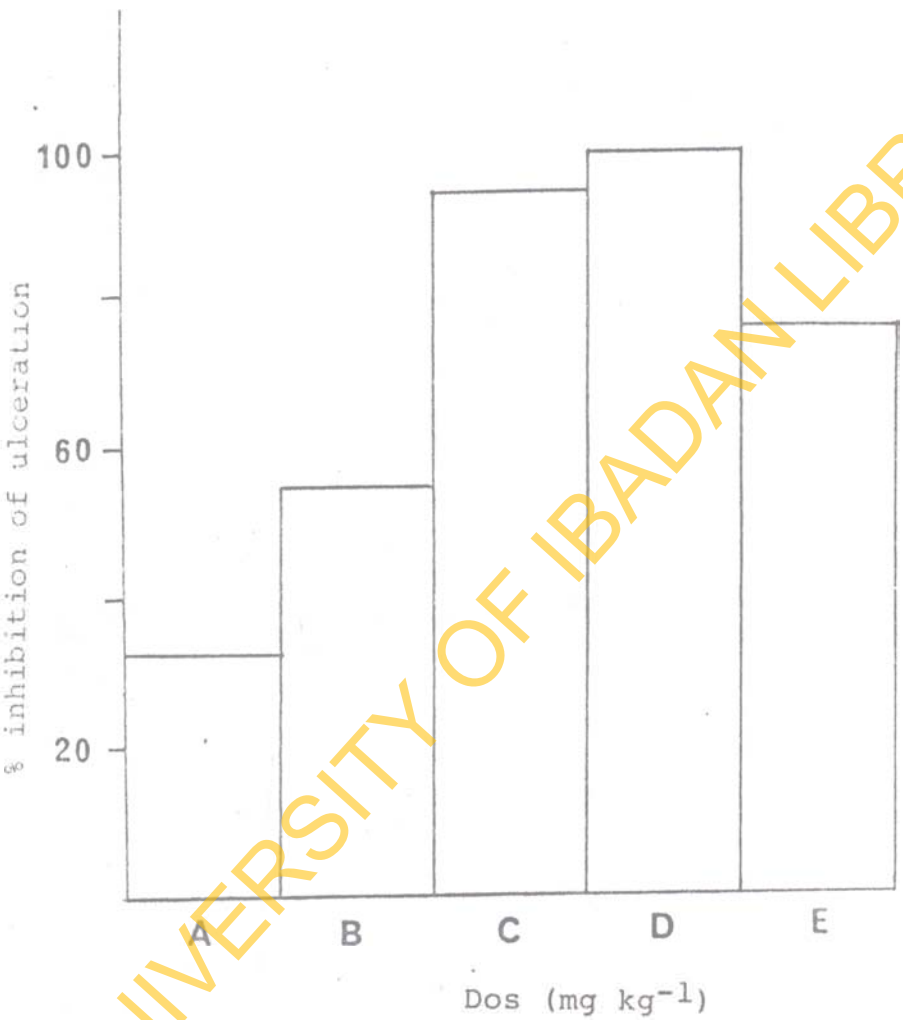
[b] Ulcer index = $\frac{\text{Mean degree of ulceration} \times \text{\% of group ulcerated}}{100}$

[c] % inhibition of ulceration = $\frac{\text{Ulcer index in control} - \text{Ulcer index in test}}{\text{Ulcer index in control}} \times 100$

[d] Significant compared with control ($P < 0.05$).

*'Tween' 80 = Polyoxyethylene Sorbitan mono-oleate.

Figure 2.10: Chart showing the percentage inhibition of ulceration of various doses of extract and propranolol



A = 200 mg kg^{-1} of E. angolense extract.

B = 400 " " " " "

C = 800 " " " " "

D = 1600 " " " " "

E = 40 mg kg^{-1} of propranolol.

The highest dose of the crude extract that was used in the acute toxicity study, and which did not cause any death was 200g kg^{-1}. This dose is over a hundred times that of the extract (1.60g kg^{-1}) which conferred total protection against indomethacin-induced gastric mucosal injury. This is an indication that the extract has a wide margin of safety and that oral administration of the plant extract as it is used traditionally may not have any immediate deleterious effect.

In an earlier study (14) 50g kg^{-1} of the aqueous extract of E. utile, another species of the genus Entandrophragma was reported to cause total protection in alcohol-induced gastric ulceration in mice and rats. In this study, a dose of 1.6g kg^{-1} of E angolense conferred total protection against indomethacin-induced gastric mucosal damage.

2. Experiment with the Isolated Methyl angolensate (EAI)

The results presented in Table 2.4 and Fig. 2.11 show that methyl angolensate (EAI) isolated from E. angolense produced a dose-related gastroprotective action in indomethacin-induced ulceration in rats. A dose of 40mg kg^{-1}BW produced significant protective effect. Total protection was exerted at a dose of 90mg kg^{-1}. The cytoprotection produced by propranolol (40mg kg^{-1}) was lower than that caused by 40mg kg^{-1} of the methyl angolensate.

Table 2.4: **Effect of methyl angolensate (EA-1) on gastric mucosal lesion and total gastric acid content induced by indomethacin**

	Treatment [a]	Ulcer Index[b]	% inhibition of ulceration	Total gastric acid content uEq HCl/100g B.W.
	Control			
A	(2% Tween 80 4ml kg^{-1} B.W.)	2.83±0.62	-	59.7±13.9
	Methylangolensate (mg Kg^{-1} B.W.)			
B	20	1.55±0.06[d]	45.3%	36.0±5.7
C	40	0.60±0.07[d]	78.8%	19.7±5.1
D	80	0.00±0.00[d]	100%	18.1±8.5
E	Propranolol (40 mg kg^{-1} B.W)	1.17±0.62	58%	43.8±9.2

[a] Five animals were used in each test

[b] Ulcer index = $\frac{\text{Mean degree of ulceration} \times \text{\% of group ulcerated}}{100}$

[c] % inhibition of ulceration = $\frac{\text{Ulcer index in control} - \text{Ulcer index in test}}{\text{Ulcer index in control}} \times 100$

[d] Significant compared with control ($P < 0.05$).

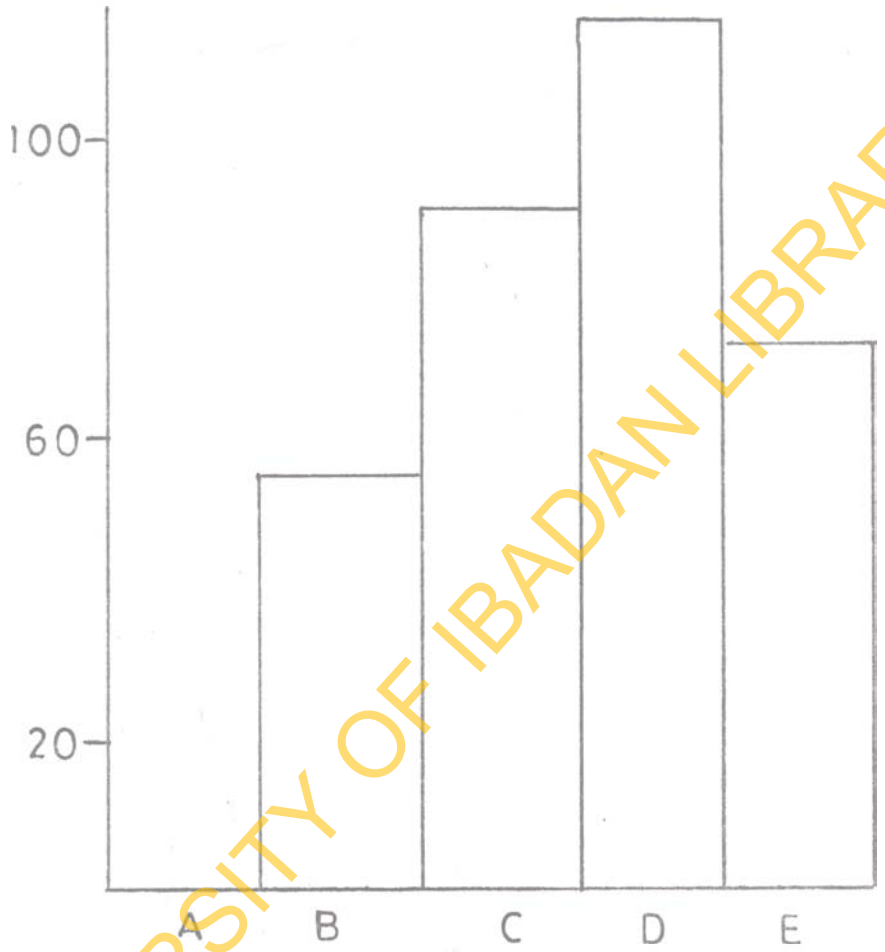


Fig. 2.11: Chart of percentage inhibition of ulceration by EA-1.

A = control (2% Tween 80 4ml kg^{-1} BW
B = 20mg kg^{-1} BW of methyl angolensate
C = 40mg kg^{-1} BW of methyl angolensate
D = 80mg kg^{-1} BW of methyl angolensate
E = 40mg kg^{-1} BW of propranolol.

This indicates that methyl angolensate is more potent than propranolol in protecting against gastric ulceration induced by indomethacin.

2.6 Total Acidity of Gastric Content

The significant reduction in total intra-gastric acid secretion by methyl angolensate as shown in Table 2.4 strongly suggest that the compound may act by inhibiting the gastric acid secretion by the parietal cells. Excessive gastric acid secretion has long been reported to play a major role in gastric ulceration (81). The secretion of gastric acid is regulated by the autonomic nervous system; the sympathetic nervous system decreasing acid secretion while the parasympathetic nervous system through vagus nerve increasing acid secretion (71).

2.7 Mechanism of Gastric Ulceration by Methyl Angolensate

Tables 2.5a to 2.5c show the effect of methyl angolensate on basal-, histamine- and carbachol-induced gastric acid secretion in male albino rats. This drug reduced basal-, histamine- and carbachol-induced gastric acid secretion indicating that this compound competes with histamine receptors as well as blocking the cholinergic mechanism of gastric acid secretion.

Table 2.5a: Effect of methyl angolensate (40 mgkg^{-1}BW) and histamine (1.0 mgkg^{-1}BW) on gastric acid secretion in male albino rats

	Basal	Methylangolensate	Histamine
	0.2±0.01	0.1±0.01	0.8±0.02
	0.1±0.01	0.1±0.01	0.9±0.02
	0.2±0.01	0.05±0.01	0.9±0.01
	0.3±0.01	0.05±0.01	0.07±0.01
	0.2±0.01	0.1±0.01	-
Mean	0.2±0.01	0.08±0.01	0.83±0.02

Table 2.5b: Combined effect of methyl angolensate (40 $mgkg^{-1}$ BW), histamine (1.0 $mgkg^{-1}$ BW) and methyl angolensate (40 $mgkg^{-1}$) plus histamine (1.0 $mgkg^{-1}$) on gastric acid secretion in male albino rats

	Basal	Methylangolensate	Histamine	Methylangosate Histamine
	0.3±0.02	0.3±0.02	1.0±0.03	0.6±0.02
	0.3±0.02	0.2±0.01	1.20±0.02	0.7±0.02
	0.3±0.02	0.4±0.01	0.90±0.01	0.5±0.01
	0.4±0.02	0.2±0.01	1.20±0.01	0.4±0.01
	0.3±0.02	0.2±0.01	0.8±0.01	0.5±0.01
Mean	0.32±0.02	0.26±0.01	1.02±0.02	0.54±0.02

Table 2.5c: Combined effect of methyl angolensate (40 $mgkg^{-1}$ BW), carbachol (1.0$mgkg^{-1}$ BW) and methyl angolensate (40 $mgkg^{-1}$), plus carbachol (1.0 $mgkg^{-1}$) on gastric acid secretion in male albino rats.

Basal	Methylangosate	Carbachol	Methylangosate Carbachol
0.2±0.01	0.1±0.01	0.9±0.01	0.5±0.02
0.1±0.01	0.1±0.01	0.8±0.01	0.6±0.02
0.2±0.01	0.2±0.01	1.1±0.01	0.7±0.01
0.2±0.01	0.15±0.01	1.2±0.02	0.5±0.02
0.2±0.01	0.1±0.01	0.8±0.01	-
Mean 0.18±0.01	0.13±0.01	0.96±0.01	0.58±0.02

The data presented in this thesis provide further experimental support for the use of the stem bark of E. angolense as an anti-ulcer drug and also established the fact that methyl angolensate is the active principle.

2.8 Phytochemical Data on C. sanguinolenta Extract

Fractionation of 5.0gm of the crude extract using preparative tlc afforded a combined weight of 0.035g (35mg) of CS-1 (since the separated bands of CS-1 and CS-2 had been found identical). This gives a 7% yield of the compound from the crude methanolic extract.

Fractionation of 2.5gm of the crude extract using the neutral alumina-packed column chromatography afforded 0.35gm of CS-1 (13% yield). These results confirm the speculation that there were some binding of the CS-1 compound to the silica gel both in the silica-gel packed column and also in the ptlc purification.

2.9 Spectroscopic Data on CS-1

Nuclear Magnetic Resonance Spectra

The spectral data for CS-2 (Table 2.6a) are identical to those of CS-1 hence they are considered as one with the labelling of CS-1.

The ^{1}H-NMR(δ) spectrum of CS-1 (Fig. 2.12) showed the

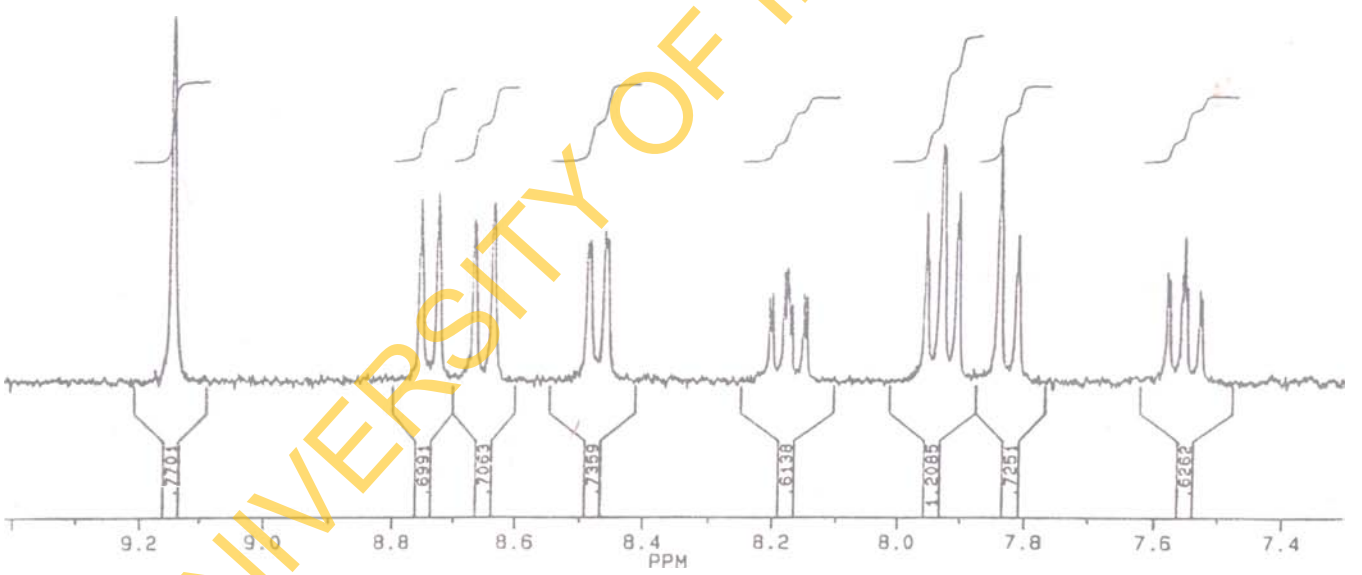
Table 2.6a: **Comparative proton chemical shift for cryptolepine, CS-1 and CS-2 in CD_3OD**

Position	Cryptolepine	Cs- 1	Cs- 2
1	8.02	8.48	8.49
2	7.90	7.93	7.94
3	7.96	8.18	8.19
4	8.15	8.66	8.67
6	8.31	8.75	8.76
7	7.19	7.56	7.57
8	7.96	7.94	7.95
9	7.68	7.83	7.84
11	8.82	9.14	9.16
N-CH_3	*4.88	5.11	5.12

For Cryptolepine

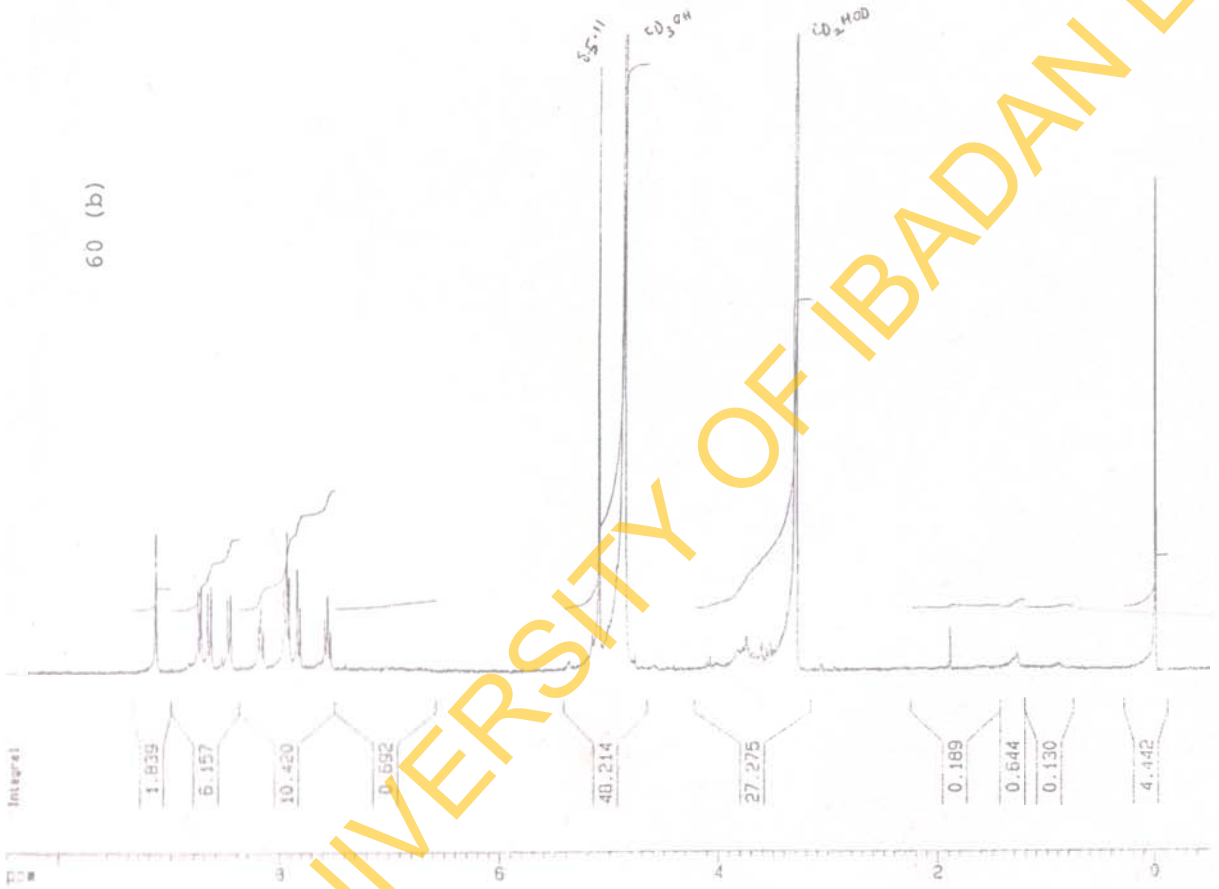
* The N-CH_3 signal was burried under the CD_3OH signal in the spectrum Fig-2.13.

Fig. 2.12: CS-1 1H NMR (CDCl3) (AC-300)



CS-1

Fig. 2.12 b ^{1}H-NMR of CS - 1 IN CD_3OD



Current Data Parameters

NAME	VN-CS-1
EXPNO	10
PROCNO	1

F2 - Acquisition Parameters

Date	930806
Time	17.58
PULPROG	zg45
SOLVENT	MeOH
AQ	2.6542175 sec
FIDRES	0.188381 Hz
DW	81.0 usec
RG	2048
NUCLEUS	1H
HL1	1 dB
D1	1.0000000 sec
P1	7.1 usec
DE	68.0 usec
SFO1	300.1383445 MHz
SWH	6172.86 Hz
TD	32768
NS	20
DS	2

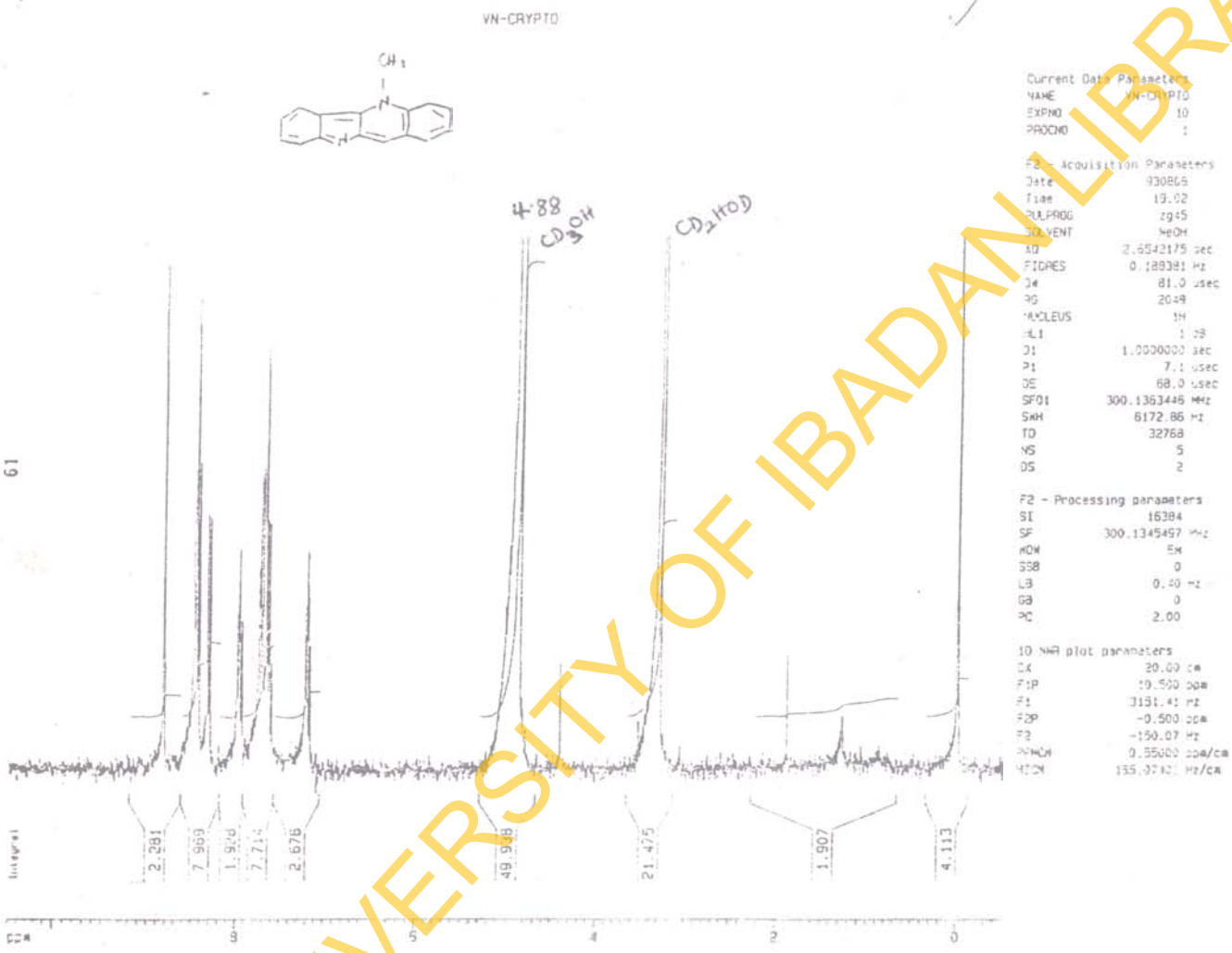
F2 - Processing parameters

SI	16384
SF	300.1345500 MHz
WDW	EM
SSB	0
LB	0.40 Hz
GB	0
PC	2.00

1D NMR plot parameters

CX	20.00 cm
F1P	10.500 ppm
F1	3151.41 Hz
F2P	-0.500 ppm
F2	-150.07 Hz
PPMCM	0.55000 ppm/cm
HZCM	165.07401 Hz/cm

Fig. 2.13: ^{1}H-NMR of Cryptolepine in CD_3OD



following signals: 5.11(3H,s,); 7.56(1H,dd,J 7.1,1.0 Hz); 7.83(1H d J:8.3 Hz); 7.94(2Hdd J:8.4, 8.0 Hz); 8.18(1H m J:6.9; 5.5 Hz); 8.48 (1H d J:8.2, Hz); 8.65(1H d J:9.1); 8.75(1H d J:84, 0.9, Hz); 9.14 (1H S). This data was compared with that of cryptolepin an alkaloid previously isolated from this plant (21,22). However, the ^{1}H-NMR spectrum of CS-1 and that of cryptolepine are not identical as can be seen in Table 2.6b. This difference is too much to be explained as being due to the solvent effect. Furthermore the ^{1}H-NMR spectra of both CS-1 and an authentic sample of cryptolepine (supplied by Dr. H.L. Holland) (Fig. 2.13) was obtained under similar experimental conditions to annul any bias that might be due to solvent effect or instrument. Different results were obtained as shown in Table 2.6a. The ^{1}H-NMR of CS-1 showed the presence of one 3H,S at 5.11 due to $N\text{-}CH_3$ protons; eight aromatic protons multiplet (7.82-8.61) and one aromatic proton singlet at 9.14.

The ^{13}C-NMR of CS-1 (Fig. 2.14) resolved for sixteen carbon atoms having the following values: 40.7, 114.4; 115.4; 118.3; 123.0; 126.2; 126.9; 128.1; 128.4; 131.2; 134.0; 135.2; 135.4; 137.3; 139.9 and 147.9.

The list of specific assignment of these signals and a comparison with those reported for cryptolepine is shown in Table 2.8. The difference in the signals especially due to carbon atoms 2,3,

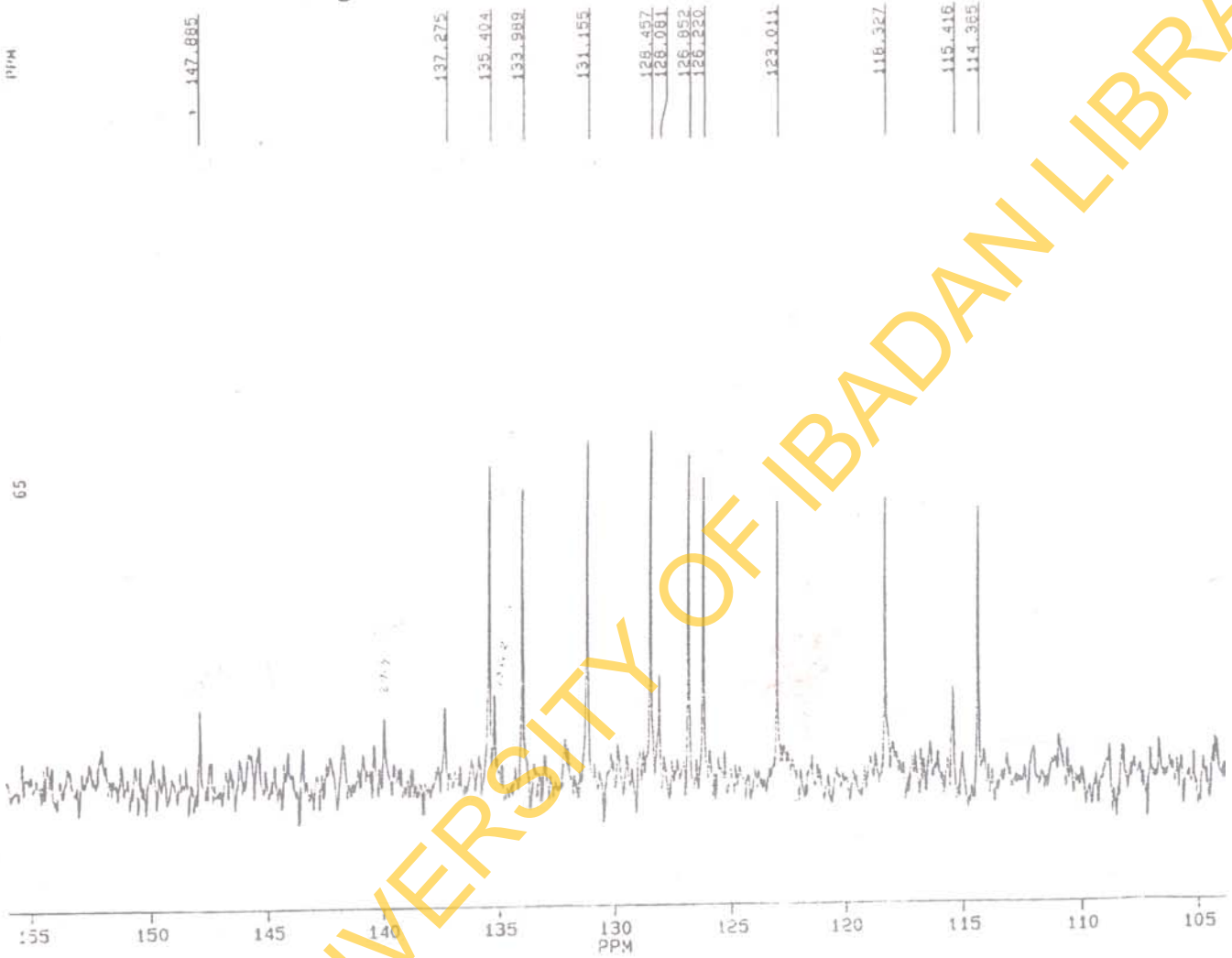
Table 2.6b: Proton chemical shifts for cryptolepine in DMSO, $CDCl_3$, and for CS-1 in CD_3OD

Position	Chemical Shift δ (ppm) DMSO (22)	$CDCl_3$ (21)	CD_3OD for Cs-1
1	8.40,(d,J=8.2)	7.92(d,J=8.0)	8.48(d,J=8.2)
2	7.69(dd,J=7.9,6.6)	7.46(dd,J=8.0,7.5)	7.93(dd,J=7.0,7.9)
3	7.90(dd,J=9.1,6.8)	7.69(dd,J=8.7,7.5)	8.18(m ,J=6.9,5.5)
4	8.53(d,J=9.2)	7.83(d,J=8.7)	8.65(d,J=9.1)
5	8.51(d,J=7.6)	7.75(d,J=8.0)	8.75(d,J=8.4,0.9)
6	7.05(dd,J=8.6,6.8)	6.76(dd,J=8.0,7.2)	7.55(dd,J=7.1,1.0)
7	7.53(dd,J=8.6,6.6)	7.37(dd,J=8.0,7.2)	7.94(t ,J=8.4,8.0)
8	7.66(d,J=8.6)	7.65(d,J=8.0)	7.83(d,J=8.3)
9	8.95, S	8.45, S	9.14, S
$N-CH_3$	4.92, S	4.32, S	5.11, S

Table 2.8: ^{13}C-NMR Data in δ -values for cryptolepine in DMSO, $CDCl_3$ and for CS-1 in CD_3OD

Position	Chemical Shift (ppm) DMSO (22)	$CDCl_3$ (21)	CD_3OD for Cs -1
1	129.6	129.6	131.2
2	123.9	123.4	128.4
3	128.9	128.5	134.0
4	116.6	114.8	118.3
4a	132.8	132.5	137.3
5a	139.0	138.9	135.2
5b	113.8	113.3	115.4
6	125.1	123.5	126.1
7	116.6	117.0	123.0
8	130.4	130.6	135.4
9	119.5	119.8	114.4
9a	160.0	161.0	147.9
10a	144.4	145.0	139.9
11	126.2	126.3	126.2
11a	124.4	124.3	128.1
$N-CH_3$	38.9	37.8	40.7

Fig. 2.14: CS-1 13C NMR IN CD3OD (AC-300)



4a, 5b, 7, 8, 9, 9a and 11a is much. This further indicates that compound CS-1 may not be cryptolepine.

Infra-red Spectrum (Fig. 2.15)

$\bar{\nu}_{max}$ (potassium bromide): 3400, 1640, 1500, 1450, 1380, 1299, 1258, 1158, 1139, 1115, 1035, 905, 860 and 760 cm^{-1}.

The IR spectrum of compound CS-1 exhibited very few bands in the functional group region (4000-1600 cm^{-1}) and many sharp and intense bands in the fingerprint region (1600-660 cm^{-1}). The absorption band between 3500 and 3420 cm^{-1} is strong and broad. This may be attributed to the $N-CH_3$ stretching vibration.

The absorption band at 1640 cm^{-1} can be attributed to the C = N stretching vibration while those observed at 1350, 1299 and 1258 cm^{-1} can be attributed to the C-N stretching vibration in the aryl ring. The strong absorption bands at 1610, 1500 and 1450 cm^{-1} may be attributed to the C=C aromatic ring vibration which is confirmed by the out of plane and substituted aromatic ring absorption bands at 905, 860 and 760 cm^{-1}.

Ultra-Violet Spectrum (Fig. 2.16)

λ_{max} (ethanol): 223, 244, 273, 281.5, 297, 306, 368 and 385nm. The intense absorption suggest the presence of conjugated system in the compound.

Fig. 2.15: Infrared Spectrum of CS-1

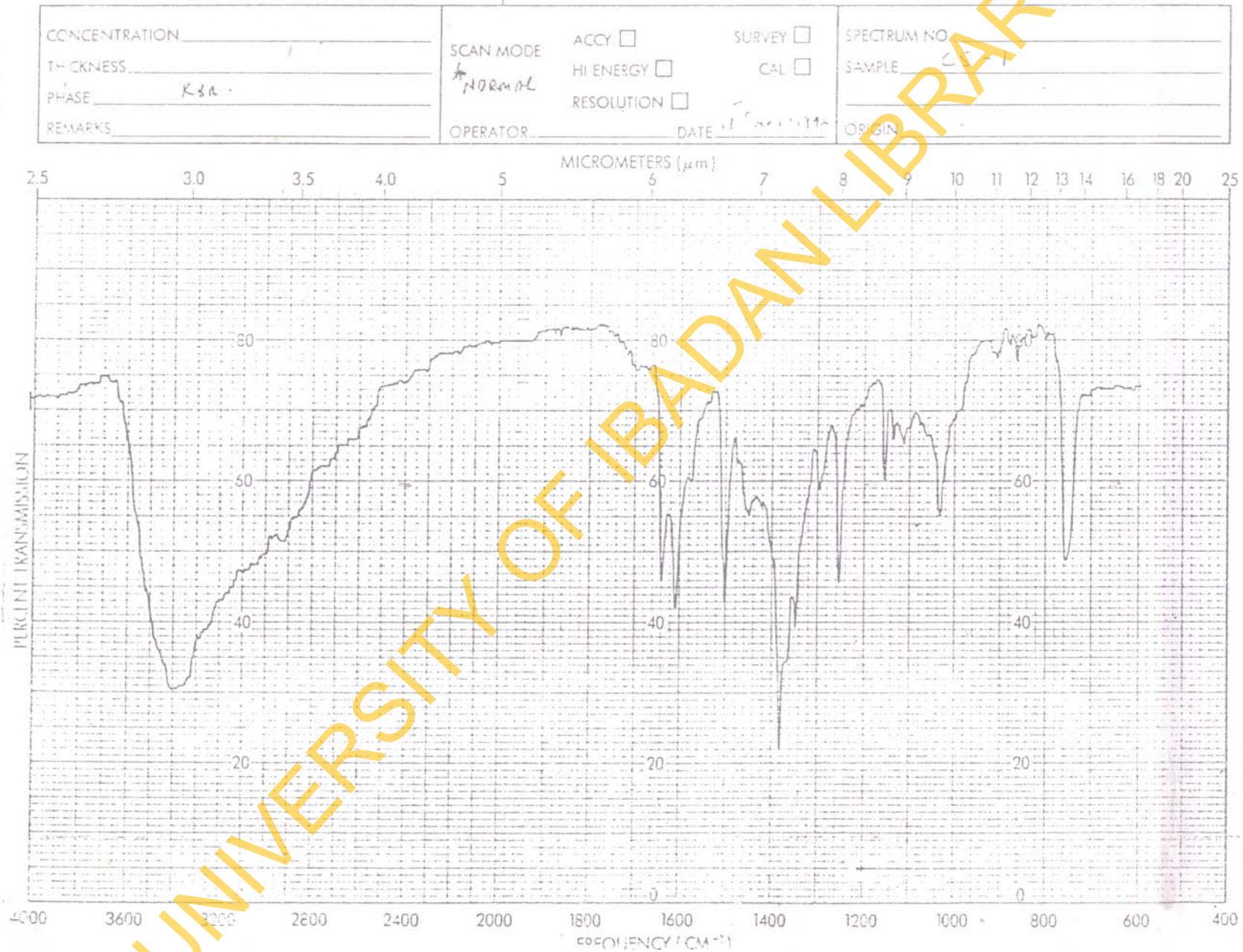
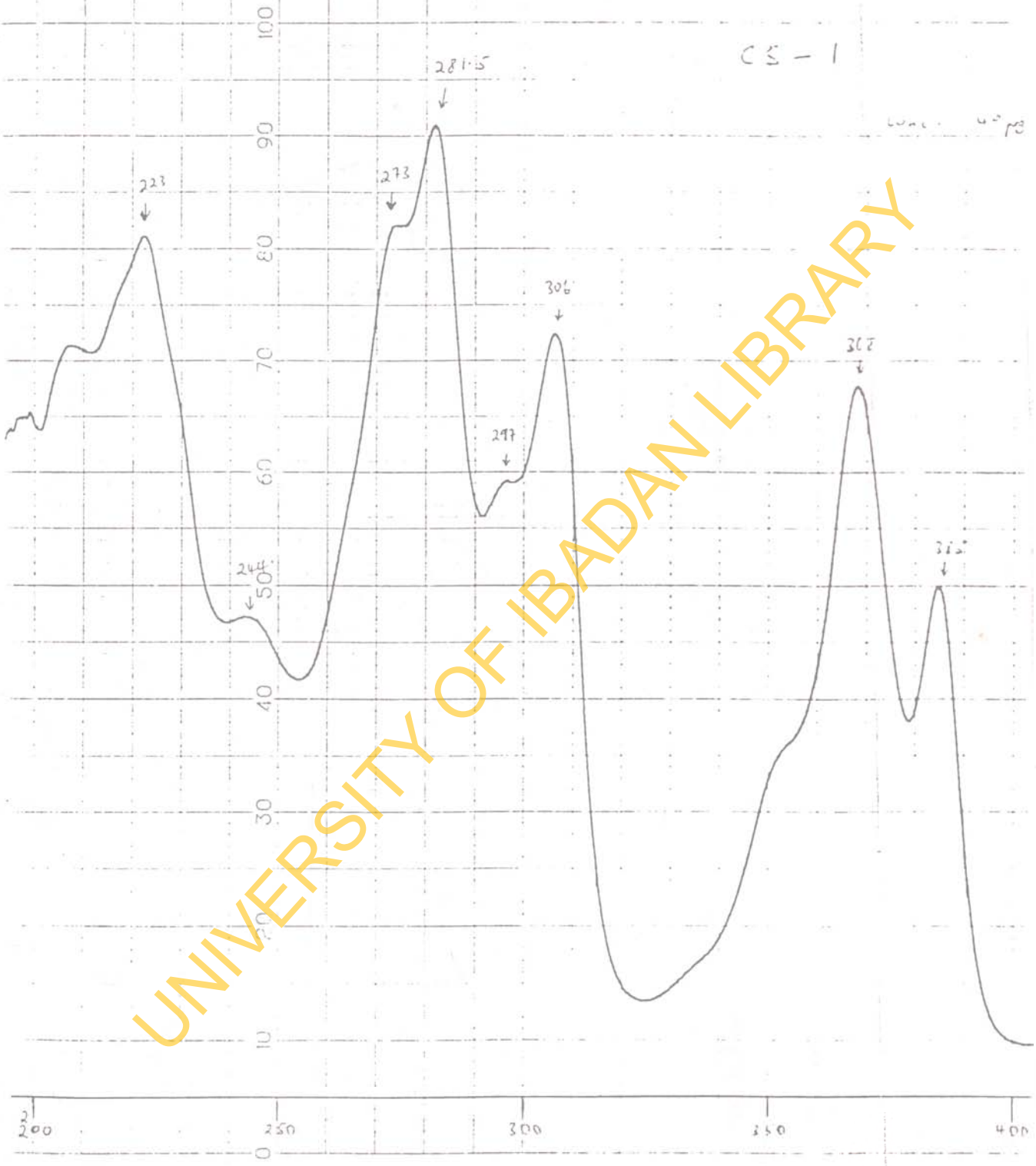


Fig. 2.16: Ultra-violet Spectrum of CS-1



Mass Spectra (MS)

The MS by electron impact (EI) for CS-1 is shown in Fig. 2.17. The spectrum indicated the molecular ion peak at M^+ 232 (100) which also forms the base peak. Other prominent fragments observed with the percentage relative abundance shown in parentheses are as follows: m/z(%); 231(14), 217(26), 190(12), 116(16), 89(16), 69(8) and 60(74).

The chemical ionization MS in glycerol showed the $(M^+ + 1)$ peak at 233(100) as the base peak.

Though the IR, UV and MS data for CS-1 and those reported (19) for cryptolepine are similar, this only shows that the two compounds have identical functional groups, chromophores and equal extent of conjugation. Their NMR data are quite unidentical suggesting that some atoms or group of atoms have different chemical environments in the molecules in each compound.

Apart from the spectroscopic information, the melting point properties of CS-1 and cryptolepine are unidentical. Mpt for CS-1 272-274°C while that reported for cryptolepin is 167-168°C (19).

The fact that CS-1 is an alkaloid was confirmed by the laboratory test for the presence of nitrogen as described in section 3. The appearance of a blue colouration confirms the presence of nitrogen which confirms that CS-1 is truly an alkaloid.

1211MS0004 Scan 1 (Av 9-13 Acq) 100%=3925 mv 11 Dec 92 14:48
LRP +EI H.Holland/ CS-1

CS-1 (EI)

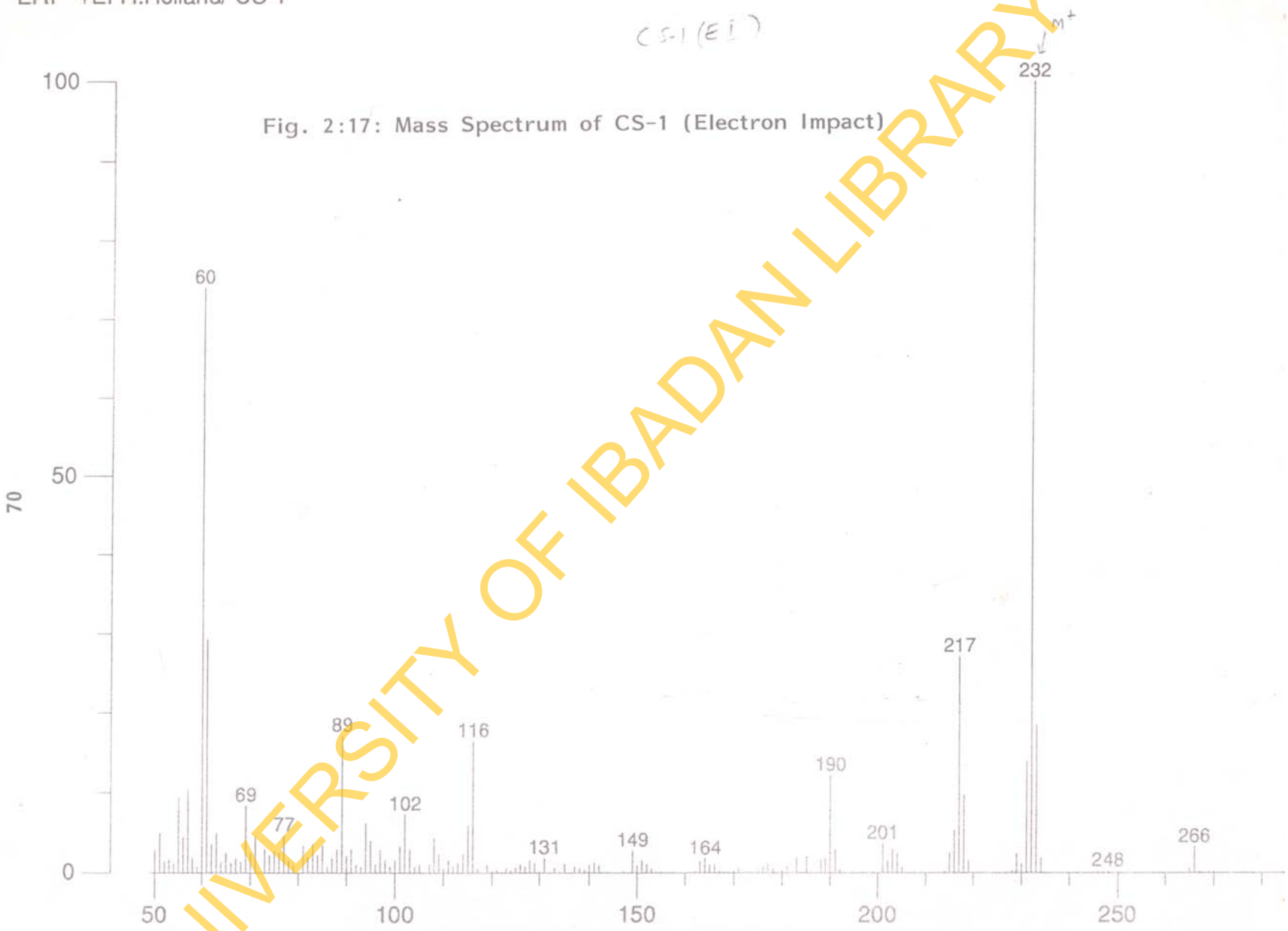
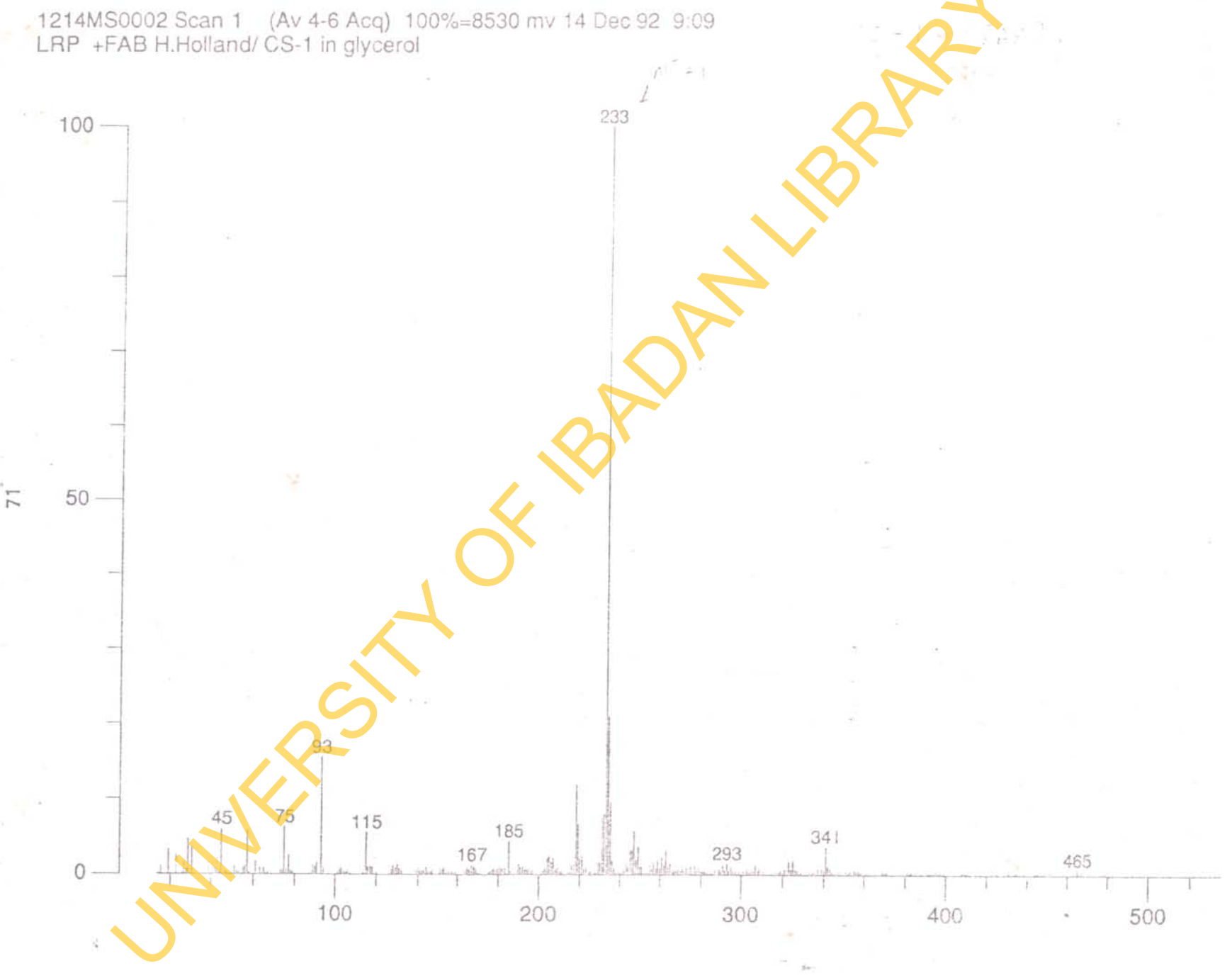


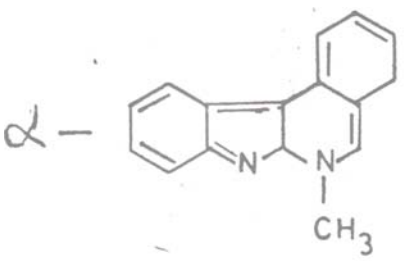
Fig. 2:17: Mass Spectrum of CS-1 (Electron Impact)

Fig. 2.18: Mass Spectrum of CS-1 (Chemical Ionization)

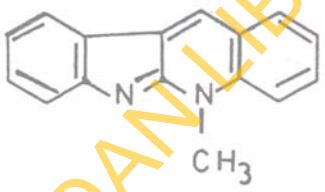


The Structure Proposed for CS-1

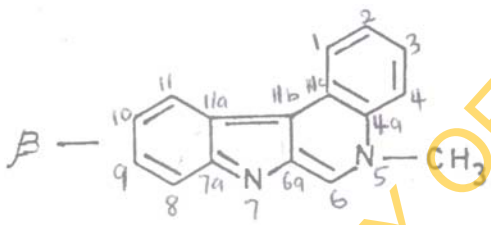
On the basis of the above spectra data and in relation to the structure of cryptolepine, possible structures for compound CS-1 are: 1A-4B all having the same molecular weight;



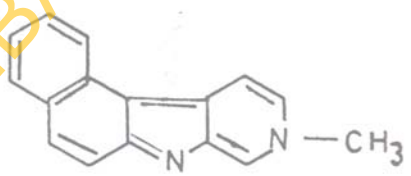
1A



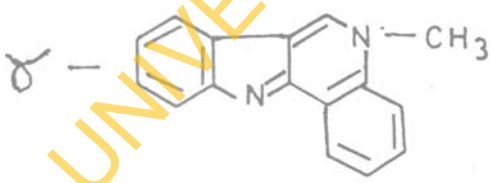
1B



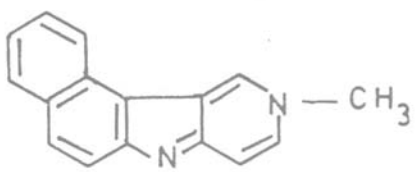
2A



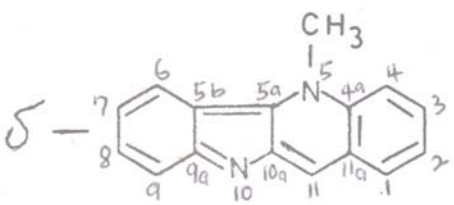
2B



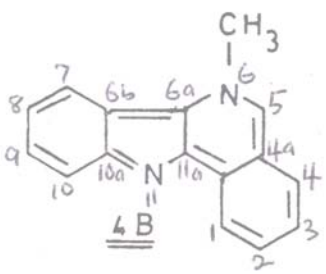
3A



3B



4A (Cryptolepine)



4B

Structures 1A, 1B, 3A and 3B are ruled out on biogenetic grounds since no α- or γ -carbolines are known from natural source.

Structure 4A (cryptolepine) is also ruled out on the basis of melting point, ^{1}H and ^{13}C-NMR differences.

2B is also ruled out by inspection of ^{1}H-NMR of CS-1. In addition it is reported (26) that the β-carboline system can only have a benzo moiety attached at position 4,5 as in 2A.

We are now left with 2A and 4B. The ^{1}H-NMR of CS-1 best fits the structure 2A.

The one proton singlet at 9.14 ppm in ^{1}H-NMR of CS-1 is equivalent to the ^{1}H-11 of cryptolepine which resonates at 8.82 ppm. Based on chemical shift theory, H6 of 2A, should appear down-field relative to H-11 of cryptolepine while H-5 of 4B might have a similar chemical shift value as H-11 of cryptolepine.

On the basis of the above discussion, the structure for compound CS-1 could possibly be that of 2A or 4B.

2.10 Antimalaria Test

Table 2.9: Schizontocidal activity of the aqueous extract of C. sanguinolenta in the 4-day test

Drug	Dose (mg/kg)	Mean % Parasitaemia	Average % Suppression
C. sanguinolenta	200	37.94±6.28	13.49
C. sanguinolenta	100	45.95±7.43	- 4.76
C. sanguinolenta	50	51.10±7.77	-16.50
C. sanguinolenta	25	51.48±6.48	-17.37
Chloroquine	05	3.91±1.44	91.09
Distilled water	-	43.86±6.20	-

The antimalarial activity of methanolic extract of C. sanguinolenta was investigated on early malaria infection, induced by Plasmodium yoeli nigeriensis in mice. Chloroquine served as a reference drug and distilled water served as control.

The 4-day test is a good method of assessing the blood schizontocidal effect of antimalarial compounds. Any compound which causes up to 50% or more suppression of parasitaemia demonstrates that it is effective against the schizont stage of the malaria infection (75,77).

Results shown in Table 2.9 above showed that chloroquine (5mg/kg) produced average percentage suppression of parasitaemia of 93.94. This observation shows that P yoeli nigeriensis parasites used were sensitive to chloroquine and afford easy comparison of the activity of the extract with chloroquine. The dose levels of the extract, 200 mg/kg, 100 mg/kg, 50 mg/kg and 25 mg/kg produced 41.21, 28.79, 20.81 and 20.22 per cent suppression of parasitaemia respectively. This shows that the effect of the extract was dose-dependent.

2.11 Results of the Anti-Microbial Activities

Table 2.10: The effect of methyl angolensate on micro-organisms

Micro-organism	Zone of Inhibition (nm)		
	EA-1	*Ch	**St
Staphylococcus aureus	7.5	22	24
Candida albicans	0	8	0
Escherichia coli	0	17	22

*Ch = Chloramphenicol
**St = Streptomycin

Size of disc is 6.5mm.

As shown in Table 2.10 above, methyl angolensate showed poor activity towards Gram-negative bacteria and the fungus Candida albicans. However, it showed slight activity towards Gram-positive bacteria Staphylococcus aureus. This is in consonance with literature report (82) that the Gram-positive bacteria Staphylococcus aureus is more susceptible to the action of various antibiotics than the Gram-negative bacteria i.e. the response of bacteria to anti-bacterial agents is influenced by their Gram staining properties.

Table 2.11: The effect of CS-1, an extract of C sanguinolenta and cryptolepin "Cp" on micro-organisms

Micro-organism	Zone of Inhibition (mm)				
	CS-1	Cp	T	Ch	S
Staphylococcus aureus	19	16	23.4	21	21
Salmonella paratyphi	07	08	17	19	24
Pseudomonas aeruginosa	11	13	12.5	14	07
Escherichia coli	10	10	16	23	24
Proteus mirabilis	07	06.5	06.5	17	24
Slugella flexheri	15	14	17	15	25
Klebsiella edwardsiella	08	07	16	21	26
Candida albicans	22	19	08	06.5	11

Cp = cryptolepine; T = Tetracycline; Ch = chloramphenicol
S = Streptomycin

Size of disc is 6.0mm.

CS-1 and Cp both have similar pattern of activity towards the organisms. These compounds are quite active towards the gram-positive bacteria Staphylococcus aureus but showed a weak activity towards the Gram-negative bacteria. Activity is lowest against Salmonella paratyphi, Protens mirabilis and Klebsiella edwardsiella. However both compounds showed highest activities against the fungus Candida albicans. In both activities against Staphylococcus aureus and Candida albicans, CS-1 showed greater activity than cryptolepine.

CHAPTER THREE

MATERIALS AND METHODS

All melting points (m.pt.) were taken on a hot stage microscope and were uncorrected. The infrared (IR) spectra were run in Kbr disc with Perkin Elmer 710B spectrophotometer. The Nuclear magnetic resonance (NMR) spectra were taken with a Bruker AM-300 MHz spectrophotometer in deuterochloroform solution (unless otherwise stated) against tetramethylsilane (TMS) as internal standard. The NMR absorptions were quoted in δ-units. Mass spectra (MS) were taken with a Kratos Aspect System instrument. Ultraviolet (UV) spectra were taken in ethanol solution (unless otherwise stated) with a Perkin Femer Lambda-3 instrument.

Neutral alumina Brookman Activity 1, 80-200 mesh (Fischer) and silica gel BDH 60-120 mesh were used for column chromatography.

Thin layer chromatography (tlc) were run on plates made by spreading an aqueous slurry of silica gel in water (1 silica gel : 2 water) on glass plates and drying the plates at about 120°C for at least 1hr. Spots, showing the relative positions of component compound(s) in a sample were detected by leaving the developed plates in a tank of iodine vapour for a few minutes. Plates for preparative thin layer chromatography (ptlc) were made by spreading an aqueous

slurry of silica gel PF 254 or PF 254 + 366 (silica gel: water, 112g: 230cm^3) to a thickness of 1mm on square plates (20x20cm) and drying at 120°C for at least 2 hrs. The chromatoplates were developed in one of the many possible solvent mixtures and the bands for the various components viewed under UV (254/356) lamp.

3.1 Animals

The animals used for the pharmacological tests were (i) male albino Sprague-Dawlen rats, (ii) albino swiss mice weighing between 18 and 22gm each. The animals were obtained from the animal house in Biode building, College of Medicine, University of Ibadan. They were maintained under standard laboratory conditions and were fed normal chew and tap water ad libitum.

3.2 Parasite Strain

The parasite strain used in the antimalarial test was the chloroquine-sensitive strain of Plasmodium yoeli nigeriensis. It was obtained from Nigeria Medical Research Institute, Lagos and maintained in the laboratory at the Department of Pharmacology by the method of serial passage of blood from mouse to mouse as recommended by Brucechwatt (70).

3.3 Plant Materials

The stem and root barks of Entandrophagma angolense were collected from the forest reservation area, Ijebu-Ode, along Ondo-Benin road, Ogun State, Nigeria.

The roots of Cryptolepis sanguinolenta were collected from Ikom in Cross-River State, Nigeria.

These plant materials were authenticated by Dr. (Mrs) Joyce Lowe of the Department of Botany, University of Ibadan.

3.4 Extraction of E angolense Materials

The stem and root bark of E. angolense were air dried and grounded to powder. 200gm of the pulverized stem bark was exhaustively extracted with methanol using the soxhlet's apparatus. The resulting mixture was then evaporated in vacuo by means of rotary evaporator. The brownish residue left was further dried to constant weight of 15g (7.5% yield).

A similar extraction procedure was carried out with 450gm of the dried and pulverized root bark of E. angolense. 31g of dark brown crude methanolic extract (7% yield) was obtained.

3.5 Column chromatography of E angolense stem Bark Extracts

5.0gm of the crude extract was pre-adsorbed on 15g silica gel (60-120 mesh) and loaded on a column (2.5cm internal diameter) already packed with a slurry of silica gel in hexane in a ratio of 25g silica gel to 1gm of sample. The column was eluted with solvent of increasing polarity from 100% hexane through hexane-ethylacetate mixtures to 100% ethylacetate and 10% methanol in ethylacetate. The eluents were collected in 100ml portions. Each portion was concentrated and examined with analytical tlc. Similar fractions were pulled.

A yellow oil was eluted with 10% ethylacetate in hexane which was neglected because it was extremely small for any meaningful work.

Ten fractions eluted with 40-50% ethylacetate (EtOAc) in hexane were found to be identical and fairly pure on analytical tlc. They were pulled to give 1.73gm of a dirty white solid. On recrystallization in 20% hexane in ethylacetate, 1.51gm of a white crystalline solid (30% yield) was obtained. This was labelled compound EA.1 m.pt. 198°C.

It was noted that on tlc compound EA.1 was not fluorescent under UV lamp at both 254 and 366 wavelength. The spot on tlc was indicated by iodine vapour in iodine tank.

When a tlc spot of EA-1 developed with 30% hexane in EtOAc was sprayed with Drangendoff's reagent, the spot was readily indicated by an orange-red stain. Thus indicating that compound EA-1 might be an alkaloid.

To confirm the above suspicion a laboratory test for detecting the presence of nitrogen was carried out thus:

3.6 Test for Nitrogen

6.0mg of the sample EA-1 was added to a piece of sodium metal in an ignition tube and fused effectively. The red-hot tube was covered-up in an evaporating dish containing some distilled water. The cracked tube was crushed and filtered.

To the filterate was added a few crystals of ferrous sulphate, boiled and cooled. Few drops of ferric chloride solution was added and the mixture acidified with dilute H_2SO_4. A yellow colouration resulted. This showed the absence of nitrogen.

3.7 Column Fractionation of the Root Bark Extract of E. angolense

5.0gm of the crude methanolic extract was fractionated by column chromatography as described.

The third fraction collected with hexane was a reddish oil which was too small to work with and so it was neglected.

Two fractions eluted with 30% ethyacetate in hexane gave needle-like crystals in the sample bottle after allowing the concentrated solutions to undergo slow evaporation overnight. The two fractions have similar tlc pattern: each contained four different spots. When pulled and dried, 8.0mg dirty white solid was obtained. Further work on this fraction was suspended because of small sample size.

A set of five fractions eluted with 40-50% ethylacetate in hexane were pulled because they have similar tlc pattern. The fraction was purple in colour and contained a crystalline solid compound. On recrystallization with 80% ethylacetate in hexane, 0.2gm (4% yield) of white crystalline solid was obtained. This compound gave identical properties with compound EA-1 isolated from the stem bark. It melted at 198°C; on tlc, it was not flourescent under UV lamp, both at 254 and 366 wavelength. On tlc, the compound was indicated by an orange-red stain when sprayed with Drangendoff's reagent. It was spotted along with compound EA-1 on the same tlc plate and developed with 30% hexane in EtOAc. They have same Rf value of 0.87. Another tlc plate containing both compounds was developed with dichloromethane-methanol mixture (9:1). They have same Rf value of 0.44.

The test for nitrogen as described above was also carried out with this compound isolated from the root bark extract. A

similar result was obtained. Thus this compound isolated from the root bark was found to be identical with EA-1 isolated from the stem bark.

3.8 Elemental Analysis of EA-1

To further confirm the absence of nitrogen or otherwise, a sample of compound EA-1 was sent for elemental analysis. The result confirmed the absence of nitrogen (Fig. 2.7).

3.9 Spectra Analysis of EA-1

The ir spectrum (Fig. 2.8) gave absorptions at 1735 and 1720 cm^{-1} indicating the presence of δ-lactone ring and ester group respectively. Absorptions at 1503 and 875 cm^{-1} indicate the presence of β-substituted furan ring.

^{1}H-NMR (δ values ppm): 0.83 (3HS , H18); 0.95(3HS, H19); 1.12 (3HS, H30); 1.02(3HS, H31); 3.50(broad 1Hq, H1); 3.68(3HS, -OMe); 5.12(1HS, H32); 4.87(1HS, H32); 5.63(1HS, H17); 6.35(1Hm Furan ^{1}H); 7.40(1Hm, Furan ^{1}H) and 7.38(1Hdd, Furan ^{1}H).

^{13}C-NMR signals include: (ppm); 15.688; 21.415; 21.576; 25.814; 23.692; 29.260; 32.624; 33.735; 39.372; 42.865; 49.861; 52.008; 77.159; 79.525; 109.877; 111.470; 140.723; 142.699; 145.774; 169.941; 173.798 and 212.666.

The MS gave a molecular ion peak m/e 470(M^+) required for $C_{27}H_{34}O_7$ [Found: C = 69.41%, H = 7.58%; Calculated: C = 68.9%, H = 7.3%]. Other major ions are observed at m/e (% relative intensity); 374(11); 359(13); 332(12.0); 210(24.9); 164(25.7); 148(52.4); 121(12.9); 95(65.7); 69(31.5); 243(13.5); 227(6.4); 244(11.4); 245(5.7); 147(25.1); 149(38.0); 119(33.7); 120(32.9); 209(18.1); 211(9.5); 117(9.9); 178(7.1) and 179(8.6).

The above spectra data were found to be identical with that of methyl angolensate, a compound which had already been isolated from the plant (10,11) (Fig. 2.1 and Table 2.1). Thus EA-1 is methyl angolensate.

UV taken in ethanol λ_{max} 205.3um ($\log_{\epsilon}$ 3.95) (Fig. 2.9).

PHARMACOLOGICAL TESTS WITH E. angolense EXTRACTS

3.10 Toxicity Study

Twenty-five male rats (190-220g) divided into 5 equal groups were used. Varying doses of defatted crude extract ranging from 20-200g kg^{-1} were given orally, each as a single dose. The control group received 0.5ml of 2% 'Tween' 80 (the solvent for the extract). Mortality rate within 24 hours period was recorded. All animals were observed for general behaviour over a period of one week.

3.11 Gastric Lesions Experiment with Crude Extract

Male rats (190-220g) of approximately the same age were randomly divided into six groups of seven animals each. One group served as control and received 2% "Tween" 80 (0.4ml); a second group received propanolol (ex Sigma) while the remaining groups received different doses of the extract. All the test animals received approximately the same volume of extract in 2% "Tween" 80. Prior to the start of the experiment, food and water were withdrawn 28 hours and 2 hours respectively. Except the group which received propanolol (40mg kg^{-1}) intraperitoneally (ip), all other groups received their respective drugs orally. One hour after administering propanolol and two hours after administering of 2% "Tween" 80 or extract, indomethacin (40mg kg^{-1}) dissolved in 2% sodium carbonate in water was given intraperitoneally to all animals in all the groups. Four hours laters, the animals were killed by a flow to the head. Their stomachs were opened along the greater curvature and washed with saline. Macroscopic and microscopic examinations of the spots and scoring of gastric ulceration was done according to the method of Elegbede (71) and Zaidi and Mukerji (72).

3.12 Gastric Lesions Experiment with Methyl Angolensate (EA-1)

The experiment was essentially as described above but for some statistical changes. For example, male rats (150-180g) of approximately the same age were randomly divided into five groups of five animals each. The doses of methyl angolensate administered ranges from 20-80mg kg^{-1} BW.

3.13 Measurement of the Total Acidity of Gastric Contents

In the experiment with methyl angolensate, the total acidity of gastric contents were measured in order to examine the mode of action of the drug.

The opened stomach of each rat was washed into a 25ml measuring cylinder with 10ml of distilled water. This gastric content water mixture was then centrifuged at 2500 rpm for 10 min at room temperature. The total acidity of the supernatant was determined by titrating with N/400 sodium hydroxide solution according to the method of Lai (73) to an end point, using phenolphthalein indicator. The total acidity of the gastric content was expressed as µEq/100g BW.

Statistical Analysis: Mean ± S.E. mean of values was calculated. The test of significance was performed using the student's t-test.

3.14 Mode of Action Study - Gastric Acid Secretion

In this experiment, fifteen male albino rats (150-180g) were divided into three equal groups and gastric acid secretion responses to methyl angolensate (40mg kg^{-1}BW), histamine (1.0mg kg^{-1}BW) and carbachol (1.0mg kg^{-1}BW) was carried out according to the method of Ghosh (74).

Normal rat chew was withdrawn from the animals twenty-four hours before the experiment but they were given glucose solution. This was done to reduce the debris in the stomach. The animal was anaesthetised with urethane (0.6ml/100g BW of a 25% solution) given intraperitonally. The anaesthetised rat was tied to the dissecting board. An incision was made in the neck region exposing the trachea which was cannulated to prevent the blockage of the air-way by mucus. A polythene tube of length 11cm and 2cm external diameter was introduced into the esophagus and tied at the neck excluding the vagus. This esophageal tube connects the rat to the Langerdoff's apparatus (Plate 3.1). Another incision was made in the anterior abdominal wall and the stomach exposed. A cut was made in the duodenum through which was inserted another polythene cannula and secured firmly by tying a ligature around the pylorus, care was taken not to include blood vessels with the ligature.

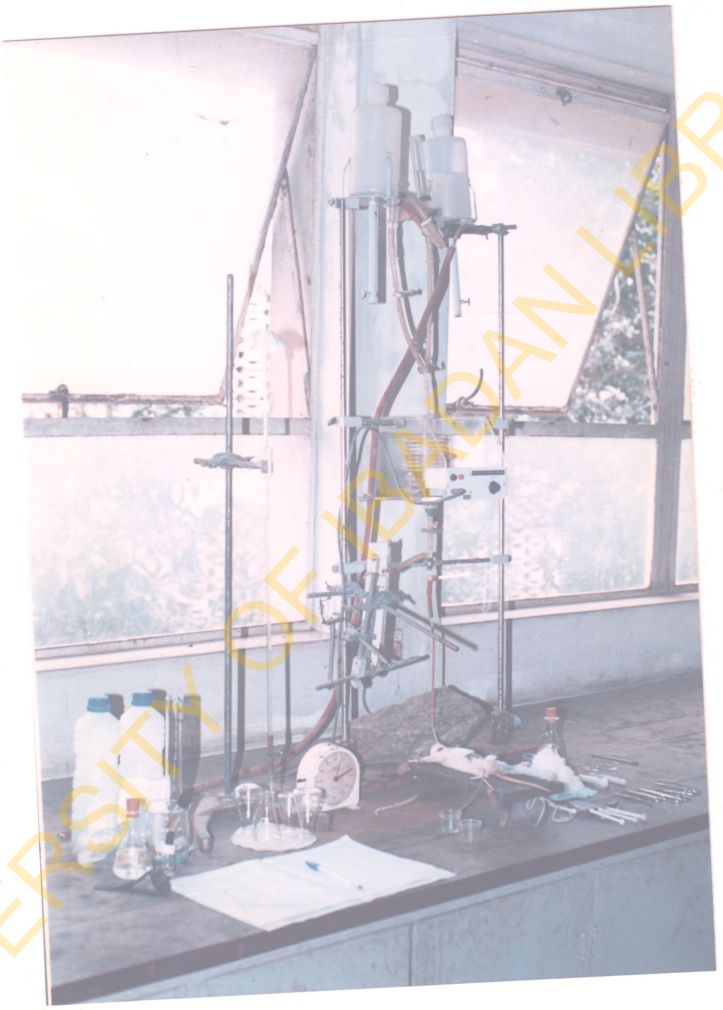


Plate 3.1: Langerdoff's apparatus

The gastric contents were then flushed out by warm saline through the esophageal tube. A constant flow of the perfusion fluid (0.15M NaCl at 31°C) was then maintained from a gravity feed monitored by a screw clip above the esophageal tube. The flow rate was adjusted to 1ml/min. 10ml of effluent collected every 10 minutes was tiltrated to end point against N/400 NaOH using phenophthalein as indicator. Basal gastric acid secretion was induced using 2% "Tween" 80.

Gpl: The effect of methyl angolensate alone (40mg kg^{-1}) and histamine alone (1.0mg kg^{-1} BW) was respectively carried out on each rat.

Gp II: The effect of methyl angolensate alone (40mg kg^{-1}BW), histamine alone (1.0mg kg^{-1}BW) and methyl angolensate (40mg kg^{-1}) plus histamine (1.0mg kg^{-1}) was respectively carried out on each rat.

Gp III: The effect of methyl angolensate alone (40mg kg^{-1}BW), carbachol alone (1.0mg kg^{-1}BW) and methyl angolensate (40mg kg^{-1}) plus carbachol (1.0mg kg^{-1}) was respectively carried out on each rat.

All injections were given intravenously slowly through a cannulated femoral vein.

3.15 Anti-Microbial Activity of Methyl Angolensate

The filter paper disc method was employed. Equal amount of the compound and antibiotics like streptomycin, tetracycline and chloramphenicol were impregnated into disc of diameter 6mm.

Each disc was placed aseptically on a nutrient agar plate which had been previously streaked with a five-hour culture of the test organism. Each plate comprising of methyl angolensate, and the antibiotic discs was tested against all the organisms by incubating for 24 hours at 37°C.

Zones of inhibition were measured in mm.

3.16 Extraction of C. sanguinolenta

The air-dried roots of C. sanguinolenta were grounded into a powder. 200g of the pulverized root was subjected to cold extraction by soaking in cold methanol (500ml) for seven days. The mixture was filtered and a yellowish solution was obtained.

The solution was concentrated by evaporation under reduced pressure using rotary evaporator. The brownish residue was dried to a constant weight of 16.0g (8.0% yield).

3.17 Chromatographic Separation of C. sanguinolenta Extract

Analytical tlc of the Crude Extract

The analytical tlc of the crude extract was obtained by spotting methanolic solution of it on silica-gel-coated tlc plate and developed in a solvent system of chloroform:methanol 3:1 v/v. Two main spots were observed Rf values 0.30 and 0.27. On spraying with Dragendorff's reagent, the spots gave orange-red colouration suggesting that the compounds might be alkaloids.

Silica-Gel Packed Column Chromatography

The attempt to fractionate the crude extract by means of column chromatography packed with silica gel 60-120 mesh proved unsuccessful. The whole column was coloured yellow yet eluents on concentration gave solids which were too small to work with.

Preparative tlc of Crude Extract

Since the analytical tlc of the crude extract revealed only two major spots, it was thought that making a ptlc of the crude extract might give some tangible results.

The ptlc plates were prepared as previously described. The methanolic solution of the sample was neatly loaded on the plates using capillary tube. 0.50g of the crude extract was chromatographed on seven plates (1mm, 20x20cm) and developed in a solvent

system of chloroform:methanol 4:1 v/v. The two yellow bands were observed under the UV lamp and scrapped separately into micro-column previously packed with glass wool. The samples were eluted each with 100ml of 30% methanol in chloroform. The eluants were concentrated at about 40°C over water bath.

15mg (3% yield) of the compound designated CS-1 (Rf 0.30 in $CHCl_3$ MeOH 3:1) was obtained while the other compound designated CS-2 was 20mg (4% yield). Attempt to recrystallize these compounds failed and so spectra analysis was carried out on the amorphous compound.

Neutral Alumina-Pack Column Chromatography

The isolation of compounds CS-1 and CS-2 by ptlc as described above has two major problems which are: relatively low percentage yield of the compounds and secondly that the compounds obtained were shown to be impure by tlc. Hence it was thought that column chromatography packed with neutral alumina should be tried.

The column (2.5cm diameter by 18cm) was packed with a slurry of neutral alumina in hexane. 2.5g of methanolic crude extract of Cryptolepis sanguinolenta which was pre-adsorbed on 10g of neutral alumina and introduced on the column. Some granules of purified sand were sprinkled on the sample in the column so as to prevent any form of disturbance on the sample level during elution.

Elution was made with methylene chloride through gradual increase in polarity using methylene chloride methanol mixtures. The eluants were collected in 100ml portions and concentrated over water bath at about 40°C. Initial fractions eluted with 100% dichloromethane contain a reddish oil too small to work with. Fractions collected with 3% methanol in methylene chloride were deep purple in colour. Tlc examination showed a yellow spot. These fractions were pulled using ethylacetate solution. This exercise afforded the precipitation of a yellow coloured solid. This was filtered and treated with activated charcoal to give 0.20g (8% yield) of a yellow solid which was identified to be compound CS-1 by tlc examination. 0.15g of a second yellowish compound CS-2 was similarly obtained as a separate band from the alumina column. The two compounds CS.1 and CS.2 melted within similar range of 272-274°C. They are therefore identical.

ANTI MALARIAL TEST

3.18 Techniques of Blood Infection

One donor mouse infected with Yoeli nigeriensis was used for each experiment to avoid variation in parasitaemia of the mice used. In order to ensure that the donor mouse was reasonably infected with the parasite, blood sample was collected from the tail, and smeared on a slide and stained. The parasite count was made and

when the percentage parasitaemia was 46.99, it was used as donor mouse.

The donor mouse was slightly anaesthetized with chloroform and disected. Blood was then collected from it by cardiac puncture using a sterilized syringe containing small quantity of heparin. The blood was diluted with sterile physiological saline in such a way that 0.2ml of it contained the recommended number ($1x10^7$) of parasitized erythrocytes (75,76). 0.2ml of the diluted blood was then injected intraperitoneally into each mouse.

Cannula, Needles and Syringes

The cannula used for oral administration of drugs to mice was the eosophageal type. It consisted of a steel cannula attached to a plastic syringe.

Sterile and non-pyrogenic disposable syringes and steel needles were used for injecting the animals.

Microscope and Oil Immersion

The Olympus research light standard microscope was used to read the slides. The eye piece magnification was x8 while the objective used was x100.

The oil immersion used was by RP Cargille with the following specification: nℓ = 1.518; nD = 1.515 at 20°C.

Giemsa Stain and Staining Technique

The powdered form of the Giemsa stain by Difeo Laboratories was used. A stock solution of the stain (15.2g/L) was made in glycerol-methanol (1:1). This was stored in a brown bottle in a refrigerator before use. 3% dilution of the stock solution was made with 0.01M phosphate buffer (pH 7.2) and was used for staining the blood smear on slides thus:

The tip of the mouse tail was cut with a pair of dissecting scissors to obtain a drop of blood on a clean microscope slide. The smooth edge of another slide (spreader) was placed on the blood at an angle of about 45° and moved initially slowly and then fastly such that the blood spread on the slide (77,78).

The dried blood film was then fixed in absolute methanol for a minute and stained with a 1in 10 dilution of the supplied Giemsa stain solution for 15 minutes. After which the slide was rinsed thoroughly with distilled water (79).

Preparation of Phosphate Buffer

0.01M phosphate buffer solution pH 7.2 was made as follows: 14ml of 0.2M NaH_2PO_4 was mixed with 36ml of 0.2M Na_2HPO_4 and 100ml of 1.5M NaCl. The mixture was diluted to 1 litre with distilled water. The buffer was kept in a well stoppered bottle and left in a refrigerator until time for use.

Preparation of Physiological Saline

Physiological saline was prepared by making 0.9% (w/v) solution of sodium chloride (Analar grade) using distilled water. The solution was poured into clean universal bottles and sterilized by autoclaving.

Preparation of Inoculum

In the preparation of the right inoculum size for the mice, the following calculation and steps were made:

80 small squares of haemocytometer had 450 diluted red blood cells (rbc). The volume of 1 small square of haemocytometer = $^1/_{4000}$ mm^3. Dilution factor is 200.

Thus 80/4000 mm^3 diluted blood contained 450 rbc.

$\therefore$ 1mm^3 undiluted blood contained 450x4000/80x200 rbc

$= 4.5 \times 10^9$ rbc.

Since percentage parasitaemia = 46.99%

$\therefore$ 1ml undiluted blood contained

$46.99/100 \times 4.5 \times 10^9$ parasitized rbc.

Since 1×10^7 parasitized red blood cells were required in 0.2ml of diluted blood for injecting each clean mouse, then 1ml of blood was required to contain 5×10^7 parasitized red blood cells.

Therefore the number of times the blood collected from the donor mouse had to be diluted with physiological saline so that 0.2ml of diluted blood would contain 1×10^7 parasitized rbc

$$= \frac{0.469 \times 4.5 \times 10^9}{5 \times 10^7} = 42.291$$

Total volume of blood needed for 30 mice = 0.2×30 = 6ml

∴ Volume of blood taken from donor mouse

$$= \frac{6}{42.291} = 0.142\text{ml}.$$

0.142ml of parasitized blood was taken from the donor mouse and diluted with normal saline to 6ml.

Preparation of Chloroquine Standard Drug

The chloroquine was administered at concentration of 5mg/kg/day.

Each mouse (average weight of 20g) required

$$\frac{5}{1000} \times \frac{20}{1} = 0.1 \text{ mg of chloroquine.}$$

0.2ml of chloroquine solution contained 0.1mg chloroquine.

∴ a 25ml stock solution required: 12.5mg of chloroquine.

Molecular wt. of chloroquine diphosphate = 515.9g

Molecular wt. of the phosphate groups = 195.94g

∴ Molar wt. of chloroquine base = (515.9 - 195.94)g

= 319.96g.

Thus 319.96g chloroquine is contained in 515.9g of the salt

∴ Ratio of base to salt = 319.96:515.9 = 1:1.61.

∴ For 12.5mg of chloroquine, (12.5x1.61)mg of the diphosphate was dissolved in 25ml of distilled water.

Administration of Drugs

All drugs were administered to the mice orally using a metal cannular.

The control groups were given distilled water via the same route.

Evaluation of the Blood Schizontocidal Activity of the Aqueous Root Extract of C. sanguinolenta in-vivo Using the 4-Day Test

This method is based on that described by Peters (75,76). The blood schizontocidal activity of the aqueous root extract of C. sanguinolenta was tested against the drug sensitive Plasmodium yoeli nigeriensis in albino Swiss mice.

Thirty mice each received the standard dose of 10^7 parasitized red blood cells from one donor mouse. The day of inoculation was termed DO. Immediately after inoculation, the animals were given the aqueous root extract of C. sanguinolenta at different dose levels as shown below. Chloroquine and distilled water were also given to the respective controls.

Group	Drug	Dose (mg/kg)
1	C. sanguinolenta	200
2	C. sanguinolenta	100
3	C. sanguinolenta	50
4	C. sanguinolenta	25
5	Chloroquine	05
6	Distilled Water	-

Each group contained five mice. The administration of these drugs was repeated on D+1, D+2 and D+3.

A thin blood film of each mouse was made on D+4 and the percentage parasitaemia of each mouse was calculated.

The average percentage suppression of parasitaemia by each dose of drug was determined using the following formula:

$$\text{Average Percentage suppression} = \frac{\text{Average Percentage Parasitaemia in untreated control} - \text{Average Percentage Parasitaemia in treated groups}}{\text{Average percentage parasitaemia in untreated control}}$$

3.19 Anti Microbial Activity of C sanguinolenta

The method used is as described under section 3.15.

CHAPTER FOUR

CONCLUSION

The stem bark of Entandrophragma angolense was not toxic within the dose level tested. It therefore has a wide margin of safety, thus its oral administration as used traditionally may not have any immediate deleterious effect.

This stem bark extract has a pronounced anti-ulcer activity. This finding provides an experimental support for the use of the stem bark of E. angolense as an anti-ulcer drug. It is also established in this work that methyl angolensate is the main active principle in the stem bark extract of E. angolense.

The traditional belief that the stem bark of this plant is better than its root bark in ulcer treatment is also confirmed, since methyl angolensate (the active principle) is present at higher percentage in the stem bark.

Methyl angolensate has a poor activity towards Gram-negative bacteria and the fungus Candida albicans but it has some slight activity against the Gram-positive bacteria Staphylococcus aureus.

The aqueous root extract of Cryptolepis sanguinolenta showed an in vivo dose-related response against the malaria parasite

Plasmodium yoeli nigeriensis though at the dose levels used in this work, the extract only showed slight activity.

A new benzoquinoline alkaloid has been isolated from methanolic extract of C sanguinolenta.

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Antiulcer Activity of the Stem Bark Extract of *Entandrophragma angolense*

V. C. O. Njar* and J. K. Adesanwo
Department of Chemistry, University of Ibadan, Ibadan, Nigeria

J. M. Mahmud and O. B. Taiwo
Department of Pharmacology and Therapeutics, University of Ibadan, Ibadan, Nigeria

The effect of a methanol extract of the stem bark of *Entandrophragma angolense* was investigated on indomethacin-induced gastric ulcer in rats. The effect of the extract was dose-dependent, doses ranging from 0.4 to 1.6 g/kg produced significant effect ($p<0.05$). At the highest dose used (1.6 g/kg) complete inhibition of ulceration occurred. The probable mechanism of action of *E. angolense* is discussed.

Keywords: *Entandrophragma angolense* extract; indomethacin-induced gastric ulcers; cytoprotection.

INTRODUCTION

The stem bark of *Entandrophragma angolense* (Meliaceae) is widely used in some parts of Nigeria for the curative treatment of peptic ulceration in humans (Adelaja, 1990). A thorough literature search showed that no work has been done on the pharmacology of the plant. However, studies on wood constituents of the plant have been reported (Banerji and Nigam, 1984). Recently, a species of the genus *Entandrophragma utile* was reported (Johri and Onadanjo, 1990) to cause a 100% gastroprotection in experimental ethanol-induced gastric ulceration in rats. In this paper, the first report is presented on the gastroprotective effect of *E. angolense* in experimental indomethacin-induced ulcers.

MATERIALS AND METHODS

Animals. Male albino Sprague-Dawley rats (190–220 g) were obtained from the Animal House, College of Medicine, University of Ibadan, Ibadan, Nigeria. They were maintained under standard laboratory conditions and were fed normal rat chow and tap water *ad libitum*.

Plant material and extract preparation. The stem bark of *E. angolense* was collected from the Forest Reservation at Ijebu-Ode, Ogun State, Nigeria. The plant was identified by Dr Joyce Lowe of the Department of Botany, University of Ibadan, Ibadan, Nigeria.

The air-dried pulverized stem bark (200 g) was exhaustively extracted with methanol by means of a Soxhlet apparatus and the extract evaporated *in vacuo*. The residue was defatted and processed to give 15 g (7.5% yield) of powdered crude extract which was stored in a refrigerator for pharmacological studies.

Pharmacological tests.

Toxicity study. Twenty-five male rats (190–230 g) divided into five equal groups were used. Varying doses of the extract ranging from 20–200 g/kg were given orally each as a single dose. The control group received 0.5 mL of 2% Tween 80 (the vehicle for the extract). The mortality rate within 24 h period was recorded. All animals were observed for general behaviour over a period of 1 week.

Experimental gastric lesions. Male rats (190–220 g) of approximately the same age were randomly divided into six groups of seven animals each. One group served as control and received 2% Tween 80 (0.4 mL); a second group received propranolol (Sigma) while the remaining groups received different doses of the extract. All the test animals received approximately the same volume of extract in 2% Tween 80. Food and water were withdrawn 28 h and 2 h respectively before the start of the experiments. Apart from the group that received propranolol (40 mg/kg) intraperitoneally (i.p.), all other groups received their respective drugs orally. One hour after administering propranolol and 2 h after administration of 2% Tween 80 or extract, indomethacin (40 mg/kg, Merk, Sharp and Dohme) dissolved in 2% sodium carbonate in water was given intraperitoneally to all animals in all the groups. Four hours later, the animals were killed by a blow to the head. Their stomachs were opened along the greater curvature and washed with saline. Macroscopic and microscopic examinations of their stomachs were carried out, the presence of spots and scoring of gastric ulceration was done according to the methods of Elegbe (1978) and Zaidi and Mukerji (1958).

Statistical analysis. Statistical analysis was performed using Student's *t*-test and significance of difference was accepted at $p<0.05$. Data are presented as mean ± SEM.

* Author to whom correspondence should be addressed.

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RESULTS AND DISCUSSION

In this study, the efficacy of the methanol extract of *E. angolense* to protect against experimental indomethacin-induced gastric mucosal damage was investigated. An acute toxicity study was first conducted. A dose as high as 160 g/kg by mouth caused neither death nor any observable symptoms. In addition no symptoms of diarrhoea or stereotypic behaviour were observed over the 1 week period of study.

In the ulcer studies, indomethacin (40 mg/kg) administered intraperitoneally was effective in inducing acute gastric mucosal damage. This dose has been reported (Elegbe, 1978) to be effective in inducing gastric ulceration. The data presented in Fig. 1 and Table 1 show that the methanol extract of *E. angolense* produced a dose-dependent gastroprotective effect in indomethacin-induced ulceration in rats. Doses ranging from 400 to 800 mg/kg exerted a significant protective effect; total protection was exerted at a dose of 1600 mg/kg. The cytoprotection produced by propranolol (40 mg/kg) is lower than that caused by 800 mg/kg of the extract.

The highest dose of the extract that was used in the acute toxicity study, and which did not cause any deaths was 120 g/kg. This dose is over a hundred times that of the extract (1.6 g/kg) which conferred total protection against indomethacin-induced gastric mucosal injury. This is an indication that the extract has a wide margin of safety and that oral administration of the plant extract as it is used traditionally may not have any immediate deleterious effect. In an earlier study (John and Onabanjo, 1990), 50 g/kg of the aqueous extract of *E. utile*, another species of the genus was reported to cause total protection in alcohol-induced gastric ulceration in mice and rats. In this present study, a dose of

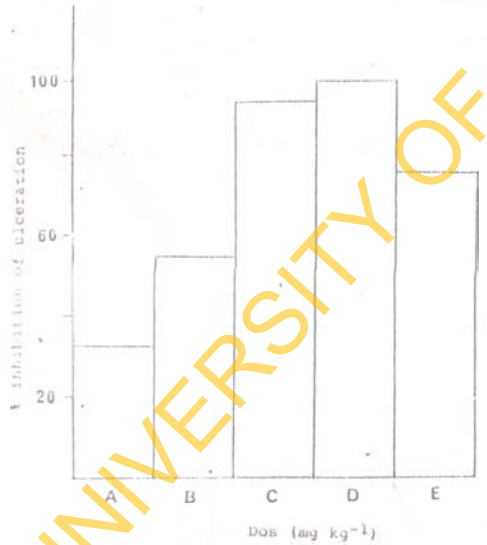

Figure 1. Percentage inhibition of ulceration at various doses of extract of propranolol. Dose of *E. angolense*; A, 200 mg/kg; B, 400 mg/kg; C, 800 mg/kg; D, 1600 mg/kg; dose of propranolol; E, 40 mg/kg.

Table 1. Effect of methanol extract of *E. angolense* on gastric mucosal lesion induced by indomethacin

Treatment[a]	Ulcer index[b]	Inhibition of ulceration[c] (%)
Control (2% Tween 80, 2 ml/kg, p.o.)	1.8 ± 0.2	–
E. angolense (mg/kg, p.o.)		
200	1.2 ± 0.1	33.3
400	0.6 ± 0.08[d]	66.6
800	0.1 ± 0.02[d]	94.4
1600	0.0 ± 0.00[d]	100.0
Propranolol (40 mg/kg, p.o.)	0.4 ± 0.07[d]	77.8

[a] Seven animals were used in each test.

[b] Ulcer index = $\frac{\text{Mean degree of ulceration} \times \text{\% of group ulcerated}}{100}$.

[c] % inhibition of ulceration = $\frac{\text{Ulcer index in control} - \text{Ulcer index in test}}{\text{Ulcer index in control}} \times 100$.

[d] Significant compared with control ($p < 0.05$).

1.6 g/kg of *E. angolense* conferred total protection against indomethacin-induced gastric mucosal damage. Thus it would appear that *E. angolense* is therapeutically superior to *E. utile*. This inference should, however, be taken with circumspection for the following reasons: 1. The method of inducing mucosal damage in the two studies was different. 2. Aqueous extract was used in their study while defatted methanolic extract was used in our study.

The mechanism by which this extract produces antiulcer preventive effect is not clear. However, since it has been reported (Robert, 1975; Whittle, 1977) that prostaglandins cytoprotect gastric mucosa against injury caused by indomethacin, it is probable that the cytoprotective effect of *E. angolense* observed in our study is related to the prostaglandin type of cytoprotection. The extract may act by stimulating the production of endogenous prostaglandins, which then protect. Another probable mechanism for the protective effect of *E. angolense* may be in connection with the sympathetic and the parasympathetic systems. This is based on the suggestions that the occurrence of indomethacin-induced ulceration may involve the sympathetic nervous system (Djatlang and Zamindast, 1973) or both sympathetic and parasympathetic nervous systems (Elegbe, 1978). Thus, it is probable that the extract of *E. angolense* acts by interfering with the sympathetic and/or the parasympathetic systems. A detailed study preferably with the active principle(s) of the extract will be necessary to establish the mechanism by which the extract produces the antiulcer preventive effect.

In view of the high efficacy of the crude methanol extract shown in this study, we are undertaking a phytochemical study of the stem bark of *E. angolense* aimed at isolating the antiulcer compound(s). The data presented in this paper provide experimental support for the use of the stem bark of *E. angolense* as an antiulcer drug.

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