STRUCTURAL ELUCIDATION AND ANTIPLASMODIAL ACTIVITIES OF SOME COMPOUNDS ISOLATED FROM THE RHIZOME OF *COCHLOSPERMUM TINCTORIUM*

A THESIS

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LEGON.

DEDICATION

To the omnipotent, the omniscient and to my parents for their
Love and care

DECLARATION

I declare that the experimental work described in this thesis was preformed by me in the Department of Chemistry, University of Ghana, Legon and the Immunology Unit of Noguchi Memorial Institute for Medical Research, University of Ghana, Legon under supervision and has not been presented for a degree in this University or any other University elsewhere for another degree.

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ABSTRACT

Cochlospernum tinctorium A. Rich was investigated for its chemical constituents and its antiplasmodial activities. The chemical constituents of petroleum ether, dichloromethane and ethyl acetate extracts of the rhizome of the plant were successively studied. The *in vitro* antiplasmodial activities for petroleum ether, dichloromethane, ethyl acetate, ethanol and water extracts as well as the isolated compounds were determined.

Three compounds isolated from the dichloromethane extract have been identified by spectroscopic methods to be triacontanol, triacontanyl p-coumarate and triacontanyl ferulate. This is the first report of the isolation of the two esters and the long chain saturated alcohol from the plant. Another compound was obtained from the dichloromethane extract and coded D9.

The petroleum ether extract of the rhizome yielded stigmasterol and two compounds which were coded PE6 and PE11. The ethyl acetate extract of the rhizome yielded two compounds which were coded E13 and E16$_6$

Using an *in vitro* technique, the inhibition of growth of *Plasmodium falciparum* (chloroquine sensitive, 3D7 and chloroquine resistance DD$_2$, strains,) were investigated based on the principle of uptake of ^{3}H-hypoxanthine by viable parasites on various extracts of *Cochlospermum tinctorium* and also on the isolated compounds with chloroquine as standard.

The percentage inhibition for all the extracts and isolated compounds were found to be dose-dependant, with the aqueous and dichloromethane extracts being more active with IC_{50} values 32.27 µg/ml and 24.34 µg/ml for the chloroqiune sensitive and 30.90 µg/ml and 23.45 µg/ml for the chloroquine resistant strains respectively.

CONTENTS

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER ONE

INTRODUCTION

Cochlospermum tinctorium is bushy, attains about 50 cm height with annual shoots arising from a perennial woody stock, and handsome golden yellow flowers which render it worthy of horticultural cultivation. Its time of flowering is during the rainy season. It is of widespread occurrence in savanna and scrubland throughout the drier parts of the West African Region, and, indeed seemingly in the devastated, rocky and annually burnt areas. It occurs also in Cameroun, Sudan and Uganda. In Ghana it is found in the savanna region.[1]

The Centre for Scientific Research into Plant Medicine at Akwapim-Mampong, Ghana has been vigorously investigating the therapeutic values of various plants used in traditional medicine against common diseases including malaria. In northern Nigeria, the rhizomes of *Cochlospermum tinctorium* A. Rich is used locally to treat febrile episodes including that due to malaria[2].

Malaria is an important public health problem, killing over one million people every year and infecting about half a billion in total. It remains endemic in 102 countries and more than half the world's population is at risk. There are probably more than 100 million cases of the disease throughout the world each year, of which perhaps a million are fatal. Inspite of control programmes in many countries, the malaria situation has shown little improvement within the past 15 years and this is partly due to world economic and/or political problems, notably in India and China.[3]

Malaria is the most common infectious disease reported at the outpatient departments of health institutions in Ghana and accounted for 40-42 % of all outpatients attendance from 1985-1987. It also accounted for 7-8% of all certified deaths in the 0-4 year age group[4]. This situation is aggravated by the development of resistance by various strains of *Plasmodium falciparum* to some commonly used antimalarials. Nonetheless in the absence of an effective vaccine against the disease prompt diagnosis and appropriate treatment using an effective antimalarial drug remains the bedrock of malaria control throughout the world.

In order to avoid the large financial effort to develop new drugs for prophylaxis and therapy, special attention has been paid in recent years to the use of traditional medicine.[5, 6, 7, 8] The great success in this field was the isolation of compounds from the plant *Artemisia annua*. This plant is used in traditional Chinese medicine.[9, 10]

The scientific basis for the use of *Cochlospermum tinctorium* against malaria has not been completely validated. The principal aim of the present study is to address this gap in knowledge.

1.1 ETHNOBOTANICAL USES OF *COCHLOSPERMUM TINCTORIUM* A. RICH

The pulped leaves of *Cochlospermum tinctorium* are used in Cote d'Ivoire to treat abscesses and furuncles. The oil from the seeds is used in treating leprosy. The unripe fruit capsules are however eaten by hunters to allay thirst. The fruit contains floss, which is used to stuff cushions. In Togo the plant is regarded as the father of cotton and it is spun into necklace cords. The Chambas of northern Nigeria use a drink from a mixture of the fruit and that of tamarindas as an antidote for snakebite.[1]

The powdered root is applied topically in Cote d'Ivoire and Burkina Faso to treat snakebite. The root along with other drug plant is used in Cote d'Ivoire and Burkina Faso for treating leprosy. The roots are also known to be used against ascites, beriberi and various oedemas in Burkina Faso. In Cote d'Ivoire and Burkina Faso the roots are used for oedematous conditions, for orchites, schistosomiasis, jaundice, fevers, epilepsy, pneumonia, intercostal pains and bronchial infections, in eye-instillations for conjunctivitis and for indigestion and stomach pains. [1]

In Gambia, the aqueous extract of the roots is given to women at childbirth. A root-infusion is used by the Fula cattlemen in Guinea to arrest diarrhoea in calves. The root is chewed in Nigeria as a tonic. In Senegal and Cote d'Ivoire the root has a reputation as an efficient decongestant, and as a venal vaso constrictor it is said to be effective in reducing haemorrhoids where surgery would normally be indicated. [1]

The root is used in Nigerian folk medicine to treat skin infections and water extracts of the roots have shown activity against Gram-positive organisms. The root may be crushed up with potassium salts obtained from vegetable ash and boiled, and with the addition of indigo a wider range of colour is achieved. A yellow or brownish –

yellow dye is obtained from the root which is used to colour shea butter and cooking oil and may perhaps also impart some flavour.[1]

The root is taken in baths for urino-gential disorders and kidney and intercostal pain. The young stem-bark yields a useful fibre. In Cameroun, cattle will not graze on related *Cochlospermum* species even in time of shortage of grass, thus suggesting that there may be some toxic substances in the other *Cochlospermum* species.[1]

1.2.ETHNOBOTANICAL USES OF SOME SPECIES OF COCHLOSPERMACEAE

1.2.1 *Cochlospermum vitifolium* (Willdenow) Sprengel

The plant is a small tree with attractive yellow flowers. It is found in Mexico, the Caribbean and savanna regions in West Africa. In Ghana it is under cultivation at the Cocoa Research Institute. In Brazil, the wood is used to make fishnet floats and also in Mexico the bark yields fibre, which is used to make rope. Its concoction is taken for jaundice.[1]

1.2.2. *Cochlospermum religiosum* (Linn.) Alston

The plant is a sparsely branched shrub, it is found in the drier parts of India, but now cultivated in several areas in West Africa. The dried leaves and flowers are said to be a stimulant. The floss surrounding the seeds is an inferior substitute for 'kapok'. The seeds contain non-drying oil reported in Indian material to amount to 14-15% and are used in soap-manufacture. The residual seed cake is used as manure. The bark contains a cordage fibre.[1]

The tree yields a gum, katira gum, which is insoluble in water. When mixed with gum arabic, it gives a water-borne adhesive paste. The gum is sweetish, cooling and sedative and it is used in cough medicines. The gum is used in cigar and ice cream manufacture and can be used as a substitute for gum tragacanth in various industrial processes.[1]

1.2.3 *Cochlospermum planchonii* Hook. f.

The plant is a shrub, which attains about 2 2.5m height. It is widespread in the region from Senegal to Western Camerouns and into Eastern Cameroun. It borne yellow flowers during its time of flowering in the rainy season.[1]

In Sierra Leone and Northern Nigeria the stem-bark is used in making string and rope. The seeds are also used as beads, while the root is a source of a yellow dye in Sudan and Northern Nigeria. In Lagos, Nigeria the root is used in cooking soup when oil is not available. A root decoction is drunk in northern Sierra Leone for the treatment of gonorrhoea. An extract of the root is said to control menstruation. The Fula of Northern Nigeria claim that a leaf-infusion bestows magical protection.[1]

1.3. Aim of the Project

The major aim of this project is two fold

(i) To evaluate the *in vitro* activity of the crude extracts from the rhizome of *Cochlospermum tinctorium* (Petroleum ether, Dichloromethane, Ethyl Acetate, Ethanol and Water) on strains of *Plasmodium falciparum*.

(ii). To isolate, purify and elucidate the structures of some natural products from the rhizome of *Cochlospermum tinctorium* and to evaluate their *in vitro* activity on the strains of *Plasmodium falciparum*.

CHAPTER TWO

LITERATURE REVIEW

Cochlospermum tinctorium A. Rich belongs to the family Cochlospermaceae. A review of the literature suggests that inspite of the fact that *C. tinctorium* has been used in folk medicine for the past century for treating malaria and other diseases, not much has been done to ascertain which compounds in the plant are active.

It is interesting to find in the literature that even though most parts of the plant such as fruit, leaves, stem and rhizomes are used extensively in folk medicine the bulk of scientific investigation has been done on the leaves and fruits with few reported studies on the rhizomes. A number of carotenoids and flavonoids have been isolated and characterized from the leaves and flowers. Some polyphenol compounds, tannins, triacylbenzenes triterpene and quercetin have also been isolated from the rhizome.

In this chapter, a brief description of the work done on the Cochlospermaceae species up to the year 2000 is discussed. The report includes chemical and biological studies.

2.1. *Cochlospermum tinctorium* A. Rich

Earliest work on this plant was by Benoit *et al* [11] in 1995 who analysed the antimalarial activity *in vitro* of *Cochlospermum tinctorium* tubercle extracts. The tubercle extracts were obtained by infusion and decoction and were tested *in vitro* on 2 strains of *Plasmodium falciparium*, (FcB1-Columbia) chloroquine resistant and (F32-Tanzania) chloroquine sensitive.

The 50% inhibitory concentration (IC_{50}) was determined by measuring [^{3}H]-hypoxanthine incorporation into parasite DNA and also by microscopical examination. The IC_{50} values obtained were of the order 1-2ug/ml, about one-tenth of those reported for the extract of neem leaves (*Azadirachta indica*) and about half the values reported for *Artemisia annua* extracts. Similar results were obtained with fresh extracts, frozen extracts and lyophilized extracts of *C. tinctorium*. The results also suggested that the traditional use of *C. tinctorium* extracts to treat malaria is based on a real anti-parasitic activity. The apparent stability of the anti malarial activity after either freezing or lyophilization is potentially valuable.

Françoise Benoit-Vical *et al* [12] analysed the *in vitro* antimalarial activity and cytotoxicity of *Cochlospermum tinctorium* and *C. planchonii* leaf extracts and essential oil. The leaf extracts were obtained by decoction. The oil components were extracted by hydrodistillation. The crude extracts and oils were tested for *in vitro* antimalarial activity on *Plasmodium falciparium* strain (FcBI-Columbia) chloroquine-resistant. The method used was the radioactive micromethod of Desjardins *et al.*[13]. The IC_{50} were evaluated after 24 and 72hr contacts between the oils and the parasite culture, and ranged from 22 to 500 μg/ml. *C. planchonii* leaf oil yielded the best antimalarial effect (IC_{50}: 22-35 μg/ml), while the most potent effect from crude leaf extracts was induced by *C.tinctorium* (IC_{50} 3.8 – 7.5 μg/ml)

The cytotoxicity of the leaf crude extracts and oils was assessed on the K562 cell line and showed IC_{50} values ranging between 33 and 2000μg/ml. For *C. tinctorium*, the essential oil characterized has a high proportion (40%) of oxygenated aliphatic components such as alcohols, aldehydes, ketones and esters and for *C. planchonii* the major component was sesquiterpenes (80%) with predominance of hydrocarbons: β-caryophyllene and farnesenes. The total yield obtained for the essential oil was 0.05% for C. *planchonii* and 0.2% for *C. tinctorium*. From the result it was suggested that since decoction is traditionally used *C. tinctorium* could be preferable to *C. planchonii* because *C. tinctorium* extract was more active than *C. planchonii*.

Diallo *et al* [14] also reported that *C. tinctorium* is used in African traditional medicine for the treatment of liver diseases. The hepatoprotective activity of the rhizome of *C. tinctorium* was investigated using carbon-tetrachloride toxicity on mouse and tert-butyl hydroperoxide *in vitro* induction of lipid peroxidation and hepatocyte lysis. Aqueous hydro-ethanolic and ethanolic extracts showed significant dose-dependent hepatoprotective actions. The ethanolic extract showed a hepatoprotective activity of lower doses. The ethanolic and hydro-ethanolic extracts established remarkable effects against the induction of lipid peroxidation and hepatocyte lysis; the aqueous extract showed comparatively weaker effects. These differences were related to the chemical composition of the extracts.

Carotenoids such as cochloxanthin (6-hydroxy-8`-apo-ϵ-caroten-3-one-8`-oic acid) and dihydrocochloxanthin (4,5-dihydro-6-hydroxy-8`-apo-ϵ-caroten-3-one-8`-oic acid), flavonoids such as 5,4^{1}-dimethlyquercetin and 7,3^{1}-dimethyldihydroquercetin) were found in both ethanolic and hydro-ethanolic extracts and only in minute amounts in the aqueous extract and this could be responsible for the protection against the t-BH intoxication. These compounds are antioxidant.

Quercetin derivatives (5,4'-dimethylquercetin and 7,3'-dimethylquercetin), arjunolic acid and major triterpene (aromadendrene, δ-cadinene, α-pinene α-selinene and α-humulene) were also isolated from the ethanolic extract of *C. tinctorium*, which showed antihepatotoxic and anti-inflammatory activities. Gallic acid, ellagic acid as well as ellagitannins were isolated from the three extracts studied. These take a prominent part in the antihepatotoxic activity of the traditional preparations from *C. tinctorium* rhizome.

Rabate[15] who investigated in 1938 reported the first critical examination of *C. tinctorium* that the rhizome contains a large quantity of starch, which resembles manioc (50-60 % of the dried drug). The friable powdered rhizomes readily yield starch on treatment with water.

Diallo and Vanhaelen [16] isolated cochloxanthin and dihydrocochloxanthin from the rhizome of *C. tinctorium* using high-speed counter current chromatography on a horizontal flow through coil-planet centrifuge. This method was used because carotenoids are well known for their instability. Analytical method such as HPLC and preparative TLC were also used to separate the constituents.

Diallo and Vanhaelen [17] did some chemical investigation on extract from the rhizome of *C. tinctorium*. Powdered dry rhizomes (500g) were extracted exhaustively with cold

MeOH by percolation. The extract was evaporated to yield a syrup, which was mixed with cellulose (100g). The mixture was percolated successively with

1. 1 Litre petroleum ether
2. 2.5 Litres ethyl acetate
3. 0.5 Litres $CHCl_3$
4. 350 ml $CHCl_3$–Me_2CO (1:1) mixture
5. 200 ml $CHCl_3$-MeOH (9:1) mixture
6. 200 ml $CHCl_3$-MeOH (8:2) mixture
7. 450 ml. CHCl3-MeOH (1:1) mixture

The $CHCl_3$ MeOH (1:1) fraction was evaporated and partitioned with $CHCl_3$-MeOH-H_2O (5:3.5:1.5, 600ml).

The residue of the chloroform extract was subjected to column chromatography on Silica gel (100g). The column elution was achieved with $CHCl_3$ containing increased amounts of MeOH. Arjunolic acid was detected in the $CHCl_3$-MeOH (9:1) fractions. It was further purified by preparative TLC on Silica gel in $CHCl_3$-EtOAc-HOAc (8:0.5:1.5) and finally on a C_{18} Silica gel column eluted with MeOH-H_2O (6:4, 8:2) to remove pigments. It was crystallized from EtOH / Me_2CO / H_2O. The R_f value of arjunolic acid on Silica gel in $CHCl_3$-MeOH (9.5:0.5) was 0.51.

The arjunolic acid, its triacetate derivative and its methyl ester were tested using the short-term *in vitro* assay on EBV-EA activation in Raji cells induced by 12-0-tetradeconylpho-bol-13-acetate (TPA). Their inhibitory effects on skin tumor promoters were found to be greater than previously studied natural products as 7-0-acetylcfromosin, retinoic and glycyrrhetinic acids.[18]

In 1987, Diallo *et al* [19] used a combination of column and preparative thin layer chromatography to isolate polyphenol compounds (gallic and ellagic acids) and carotenoids from the methanol extract of the rhizome of *C. tinctorium*. The methanol and ethanol extracts were investigated for antihepatotoxic activity using carbon tetrachloride and galactosamine – induced cytotoxicity in primary culture rat hepatocytes. It was detected that the extracts and the isolated compounds exhibited antihepatotoxic effect.

In 1991,Diallo *et al* [20] isolated some triacylbenzenes and long-chain volatile ketones from *C. tinctorium* rhizome. Eleven compounds were isolated from the volatile fraction through the use of steam distillation under N_2 from the air-dried and powdered rhizome. GC, NMR and GC-MS were used to investigate the compositions of the volatile fraction, among the 11 constituents detected, 8 were identified as straight chain ketonic compounds. The triacylbenzenes were isolated from a petroleum ether extract, separated by HPLC and identified by the NMR and MS.

2.2. *Cochlospermum angolense* (Welw)

An aqueous extracts of the roots of *C. angolense* is used for the treatment of icterus and in combination with other materials for the prophylasis of malaria.[21]

Presber *et al* [22] tested a red coloured crystalline product isolated from chloroform extract, on the growth of *P. falciparum* (M-25 / Zaire) and *P. bergbei* (Gdansk). The multiplication of *P. falciparum* was decreased to 50% of the control in the presence of 10μg/ml extracted material and there was a total inhibition at a concentration of 50μg/ml.

Mice erythrocytes infected by *P. bergbei* were incubated for 6 hours with 25ug/ml of the extract. The result showed that the DNA synthesis was depressed to nearly background level.

Nogueira Prista and Correia de Silva [23] found flavones and carotenoids in the bark of *C. angolense*. Also isolated from the plant are the following compounds, Oleanolic acid (mpt 308-309°), Quinone, $C_{12}H_2O$, (mpt 79-81°), a fatty substance (mpt 63-70°), Carotenoid and a Flavone.[24]

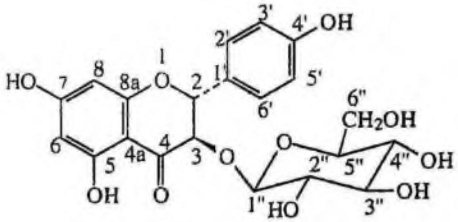
2.3. *Cochlospermum regium*

In 1995, Lima *et al* [25] isolated a dihydrokaempferol 3-0-glucopyranoside, $C_{21}H_{22}O_{11}$, melting point 177-179°C (0.4%) from the rhizome of *C. regium*. The following are the spectral peaks:

`H-NMR (200mHz, MeOH) 7.37 (2H, d, J = 8.6Hz), 6.81 (2H, d, J = 8.6Hz), 5.92 (1H, d, J = 2.1Hz) 5.89 (1H, d, J = 2.1Hz) 5.27 (1H, d, J =10.2Hz), 4.98 (1H, d, J= 10.2Hz), 3.79 (1H, d, J =7.52Hz), 3.76 (1H, dd, J =12.3, 2.2Hz), 3.60 (1H, dd, J =12.3, 5.7Hz), 3.07-3.35 (3H, m), 2.92-3.05 (1H, m);

^{13}C-NMR (50 MHz, MeOH-d_4): δ 83.55 (C-2), 71.52 (C-3), 196.11 (C-4), 103.06 (C-4a), 164.20 (C-5), 97.40 (C-6), 169.03 (C-7), 96.33 (C-8), 165.50(C-8a), 128.52 (C-1'), 130.40 (C-2'), 115.72 (C-3'), 159.30 (C-4'), 116.25 (C-5'), 130.47 (C-6'), 102.60 (C-1"), 74.55 (C-2"), 78.22 (C-3"), 71.24 (C-4"), 77.58 (C-5"), 62.60 (C-6");

DCI-MS (NH_3) m/z 468 [(M + 18), 64%], 451 (24), 342 (22), 306 (72), 298 (35), 271 (22), 256 (6), 198 (6), 180 (100), 162 (11), 127 (23).



Other compounds that have also been isolated from this plant include Quercetin derivatives, arjunolic acid, [14] apocarotenoids [17] and triacylbenzenes together with long chain volatile Ketones. [20]

2.4. *Cochlospermum gossypium* DL, syn *C. religiosum*

Ramachandraiah *et al* [26] in their investigation isolated β-sitosteryl-glucoside (0.3%) from the flowers of C. *gossypium*. The gum of *C. gossypium* is sweetish, cooling, sedative, useful in coughs, hoarse throat and scalding in the urine. It is also used for treating diarrhoea and dysentery,[27] the young leaves are used for cooling the hair, [27] while the dried leaves and flowers are used as stimulant.[28] Purification of the gum was done by precipitation by alcohol from acidified aqueous solutions. The ash-free gum is a white, amorphous, hygroscopic powder.[29] Tannins have been identified in the 50% alcoholic extract of the stem bark.[30] Naringenin has also been isolated from the flower of *C. gossypium*.[31]

2.5. *Cochlospermum planchonii* Hook. f

Aliyu *et al* [32] in their investigation isolated zinc formate from the rhizome of *C. planchonii* by using inhibition of two rat cytochrome P-450 enzymes, aminopyrine-N-demethylase and aniline hydroxylase, as bioassays to guide fractionation by solvent partitioning, polyamide column chromatography, preparative thin layer chromatography and fractional crystallization. The zinc formate was shown to be a hepatoprotective cytochrome P-450 enzyme inhibitor. The inhibitor did not melt at temperatures up to 300 ^{0}C and the ^{1}H-NMR spectrum in dimethylsulfoxide-d_6 contained broad signals (consistent with poor solubility) at 1.6-2.0 ppm (assigned to H-C=O) and at 3.0-4.0 ppm (water).

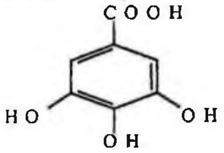
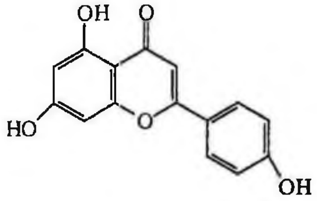
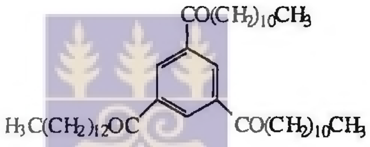
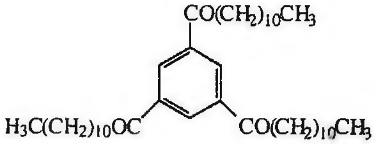
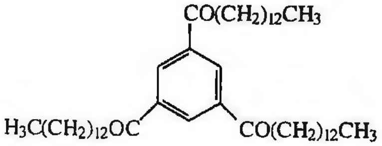
The ^{13}C-NMR spectrum contained a single signal at 165ppm (carbonyl). The infrared spectrum contained strong absorptions at 1370cm^{-1} and 1600cm^{-1} (carboxylate anion stretching). The Ultraviolet absorption spectrum lacked significant absorption at wavelength longer than 250mu. The FAB mass spectrum contained no significant peaks over 100m/e. Ashing in a gas flame left a copious white powder completely soluble in dilute hydrochloric acid. It was also analyzed by inductively coupled plasma atomic emission spectroscopy. All these results indicated that the crystalline inhibition was zinc formate.

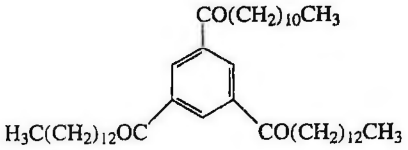
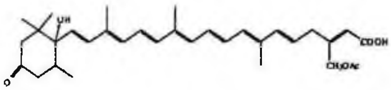
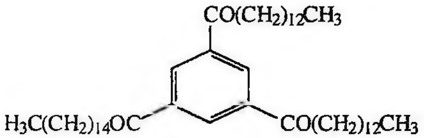
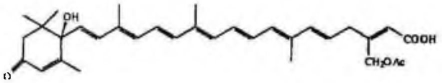
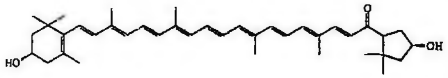
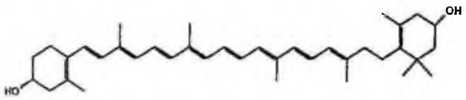
Addae-Mensah *et al* [33] in their investigation isolated four novel long-chain triacylbenzenes from the apolar fraction of *C. planchonii* rhizomes. The triacylbenzenes were separated by HPLC into 2 major compounds and 2 minor compounds, except for the MS all the compounds showed very similar spectroscopic behavior.

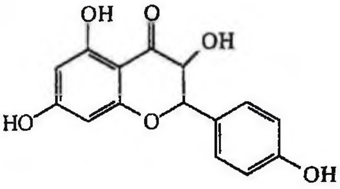
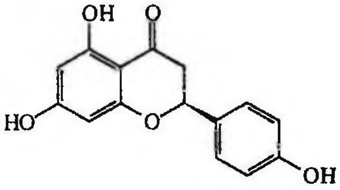
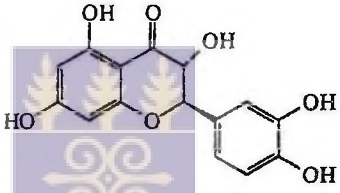
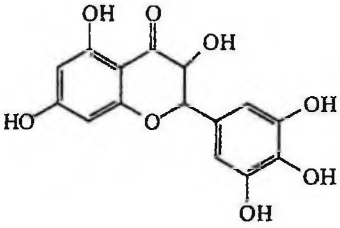
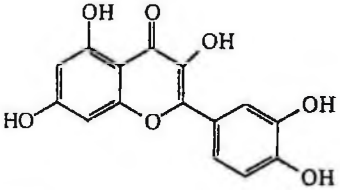
2.6. *Cochlospermum vitifolium*, Willdenow (Sprengel)

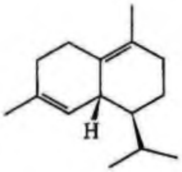
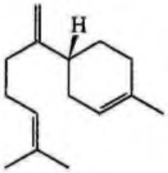
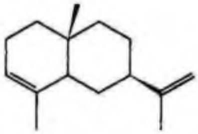
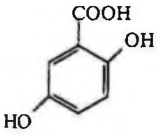
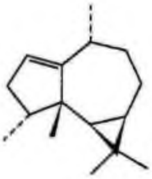
The root of *C. vitifolium* is used as a remedy against jaundice in middle American folk medicine and as a dye. Achenbach *et al* [34] isolated two new 7`-apocarotenoic acid (vitixanthin and dihydrovitixanthin) from the methanolic root extracts using repeated column chromatography and HPLC. The electronic spectra show maxima (MeOH) at 422, 398, 377 and 359 (sh) nm, which indicates a carotenoid heptaene chromophor. The Homo- and heteronuclear COSY experiments established structural details, which resemble cochloxanthin and dihydrocochloxanthin as far as part of the carotenoid polyene chain, are concerned.

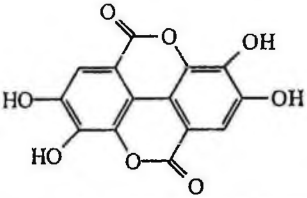
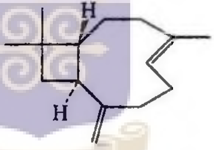
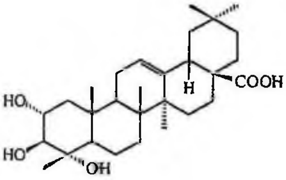
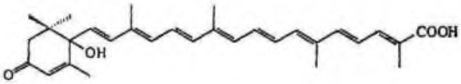
Table 1:COMPOUNDS ISOLATED FROM COCHLOSPERMUM SPECIES

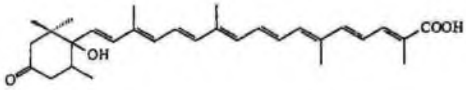
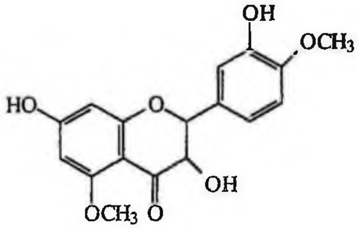
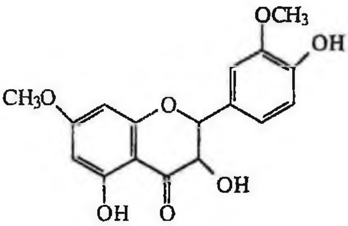
Name and Molecular formula	Structure	Source
Gallic acid (3,4,5-trihydroxybenzoic acid) $C_7H_6O_5$		Rhizome [19]
Apigenin 5,7-Dihydroxy-2- (4-hydroxyphenl)-4H-1-benzopyran-4-one $C_{15}H_{10}O_5$		Flower [36]
1,3-di(dodecanyl)-5-tetradecanoylbenzene $C_{44}H_{76}O_3$		Rhizome [20] and Rootbark [33]
1,3,5-tridodecanoyl-benzene $C_{42}H_{72}O_3$		Rhizome [20] and Rootbark [33]
1,3,5-tri(tetradecanoyl-benzene $C_{48}H_{84}O_3$		Rhizome [20] and Rootbark [33]

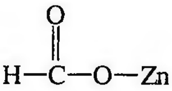
3,5-di(tetradecanoyl)-1-dodecanoylbenzene $C_{46}H_{80}O_3$		Rhizome [20] and Rootbark[33]
Dihydrovitixanthin $C_{33}H_{44}O_6$		Flower [34]
1,3-di(tetradecanoyl)-5-hexadecanoylbenzene $C_{50}H_{88}O_3$		Rhizome [20]
Vitixanthin $C_{33}H_{42}O_6$		Flower [34]
Capsanthin (3,3'-Dihydroxy-β,k-caroten-6'-one) $C_{40}H_{56}O_3$		Flower [36]
Zeaxanthin (β, β-carotene-3,3'-diol) $C_{40}H_{56}O_2$		Flower [36]

Kaempferol (3,4', 5,7-tetrahydroxy-flavone) $C_{15}H_{10}O_6$		Leaf [37]
Naringenin $C_{15}H_{12}O_5$		Entire plant[31]
Taxifolin (3',4',5',7-tetrahydroxy-dihydroflavonol) $C_{15}H_{12}O_7$		Entire Plant[38]
Myricetin (3,3',4',5',7-Hexahydroxyl-flavone) $C_{15}H_{10}O_8$		Leaf [37]
Quercetin (3',4',5,7-tetrahydroxy-flavonol) $C_{15}H_{10}O_7$		Leaf [37]

δ-Cadinene $C_{15}H_{24}$		Rhizome [35]
α-Pinene (2,6,6-Trimethylbicyclo-[3,1,1]hept-2-ene) $C_{10}H_{16}$		Rhizome [35]
β-Bisabolene (1-methyl-4-(5-methyl-1-methylene-4-hexenyl)cyclo-hexene) $C_{15}H_{24}$		Rhizome [20]
α-Selinene $C_{15}H_{24}$		Rhizome [35]
Gentisic acid (2,5-Dihydroxybenzenoic acid) $C_7H_6O_4$		Flower [36]
Aromadendrene $C_{15}H_{24}$		Rhizome [35]

Ellagic acid (2,3,7,8-Tetrahydroxy[1]benzo-pyrano[5,4,3-cde][1]benzo-pyran-5,10-dione) $C_{14}H_6O_8$		Leaf [19]
Lycopene ψ,ψ-carotene $C_{40}H_{56}$		Flower [36]
α-Humulene 2,6,6,9-Tetramethyl-1,4,8-cycloundecatriene $C_{15}H_{24}$		Rhizome [35]
Trans caryophyllene $C_{15}H_{24}$		Rhizome [35]
Arjunolic acid		Rhizome [14]
Cochloxanthin (6-hydroxy-8`-apo-ϵ-caroten-3-one-8`-oic acid) $C_{30}H_{38}O_4$		Rhizome [14]

Dihydrocochloxanthin (4,5-dihydro-6-hydroxy-8`-apo-ε-caroten-3-one-8`-oic acid) $C_{30}H_{40}O_4$		Rhizome [14]
5,4'-dimethylquercetin		Rhizome [14]
7,3'-dimethylquercetin		Rhizome [14]
2-Tridecanone ($C_{13}H_{26}O$)	$CH_3-(CH_2)_{10}-CO-CH_3$	Rhizome [20]
1-Dodecanol ($C_{12}H_{26}O$)	$CH_3-(CH_2)_{10}-CH_2OH$	Rhizome [20]
1-Tetradecanol ($C_{14}H_{30}O$)	$CH_3-(CH_2)_{12}-CH_2OH$	Rhizome [20]
3-Octadecanone ($C_{18}H_{36}O$)	$CH_3-(CH_2)_{14}-CO-CH_2-CH_3$	Rhizome [20]

1-Hydroxy-3-octadecanone ($C_{18}H_{36}O_2$)	$CH_3-(CH_2)_{14}-CO-CH_2-CH_2OH$	Rhizome [20]
3-Hexadecanone ($C_{16}H_{32}O$)	$CH_3-(CH_2)_{12}-CO-CH_2-CH_3$	Rhizome [20]
1-Hydroxy-3-hexadecanone ($C_{16}H_{32}O_2$)	$CH_3-(CH_2)_{12}-CO-CH_2-CH_2OH$	Rhizome [20]
1-O-Acetyl-3-hexadecanone ($C_{18}H_{36}O_2$)	$CH_3-(CH_2)_{12}-CO-CH_2-CH_2OAc$	Rhizome [20]
2-Pentadecanone ($C_{15}H_{30}O$)	$CH_3-(CH_2)_{12}-CO-CH_3$	Rhizome [20]
1-Nonadecanol ($C_{19}H_{40}O$)	$CH_3-(CH_2)_{17}-CH_2OH$	Rhizome [20]
Zinc formate HCOOZn	 $H-\overset{\overset{O}{\parallel}}{C}-O-Zn$	Rhizome [32]

2.7. THE DISEASE MALARIA

Malaria, a major public health problem worldwide, is caused by a protozoan parasite of the genus *Plasmodium*. The major species of this genus are *Plasmodium falciparum, Plasmodium vivax,* and *Plasmodium malariae*. The deadly *P. falciparum* is becoming increasingly resistant to available drugs and the number of drugs under development is dwindling.

Malaria is transmitted from one vertebrate host to another by the female mosquito vector the most efficient of this species being *Anopheles gambiae*. Parasites of the genus *Plasmodium* undergo the sexual portion of their life cycle in a mosquito. The sequence begins when a mosquito, after biting a vertebrate ingests a gametocyte-infected blood meal and ends with a second blood meal during which sporozoites are passed with the mosquito's saliva into a new vertebrate host (39,40).

The parasite must overcome a number of barriers to develop in a mosquito. Immediately after ingestion, gametocytes emerge from red blood cells and differentiate into male and female gametes. These gametes are fertilized and the resulting zygotes develop into ookinetes within the gut. The ookinetes enter the body cavity (hemocoel) by crossing the gut lining (peritrophic matrix) and the midgut epithelium, where they subsequently lodge on the hemocoel side of the gut epithelium, beneath the basal lamina. The ookinetes then develop into oocysts. Oocysts undergo many rounds of nuclear division and produce thousands of sporozoites that are released into the hemocoel. Sporozoites invade the salivary glands to be injected into a vertebrate host about 2 weeks after the mosquito ingested the first infected blood meal.[41]

2.7.1. Life cycle of the malaria parasite

The development of each of the four species of human plasmodia starts with the phase in which the direct progeny of sporozoites injected into the circulation by the bite of an infected female anopheles mosquito enter the liver where they grow and multiply in the parenchymatous cells. This pre-erythrocytic tissue schizogony is completed towards the end of the incubation period of the infection, which ranges from 7-37 days, when large numbers of tissue merozoites from ruptured tissue schizonts are released into the blood stream. In this form the parasite invades the erythrocytes, grows and multiplies asexually from trophozoites to mature blood schizonts, which release merozoites, and produces all the clinical symptoms of the disease. Some erythrocytic forms develop into two types of sexual parasites (gametocytes), which unite when taken up by a suitable female anopheles mosquito that has ingested the blood of the infected individual. Eventually, after the gradual stages of ookinete and oocyst, large numbers of sporozoites are produced and stored in the salivary glands of the female anopheles mosquito. These ensure the transmission of the disease when injected into a human host. The life cycle of the human malaria parasite is shown in Appendix VII.

In some species (*P. vivax*), the merozoites originating from pre-erythrocytic tissue schizogony re-enter liver cells and continue their development as secondary exoerythroctic forms which are responsible for producing relapses of malaria with clinical symptoms.[42.] There is evidence to suggest that the relapses may be due to the presence of two or more populations of parasites in the liver cells, one developing as the typical liver schizonts and the other (termed hypnozoites) persisting for some time as small, non-developing uninucleate parasites. The latter may initiate cycles of development weeks, months or years after the initial infection, giving rise to the so-called relapses.[43]

2.7.2. Clinical manifestations

After an incubation period ranging between 7 and 37 days for most cases of naturally transmitted malaria, the first signs of clinical malaria appear suddenly. These consist of headaches, malaise, anorexia, nausea, fatigue and dizziness.[44] A typical paroxysm starts with a feeling of cold accompanied by shivering, pallor and cyanosis. In children, it may present as a convulsive seizure. Other symptoms include dry cough, abdominal pain and vomiting. Irregular fever with the usual symptoms is not the only clinical picture of *falciparum* malaria. Because of the rapid multiplication of the *Plasmodia* of this species and their tendency to invade the internal organs, severe complications may appear suddenly. Drowsiness, coma, delirium, bloody diarrhoea, severe haemolytic anaemia, pulmonary oedema hyperpyrexia and renal failure indicate the involvement of various organs. Death may occur unless there is rapid diagnosis and adequate treatment.

2.7.3. Laboratory Diagnosis

Diagnosis of malaria is confirmed when a Giemsa-stained blood film obtained from the patient's thumb shows the presence of malaria parasites under a microscope. Most diagnosis can be made with a well-prepared and stained thick film but in the case of doubtful species diagnosis, or mixed infections, the thin film can often be the final arbiter.[45]

2.8. Antimalarials

2.8.1. Biological classification of antimalarials

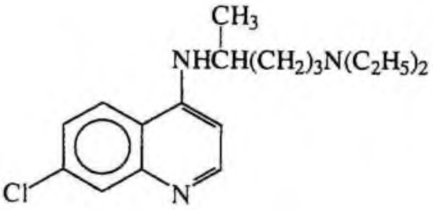
Since various stages in the life cycle of malaria parasites show different susceptibility to antimalarial drugs, these drugs have been classified into the following groups:

1. Tissue schizontocides (used for causal prophylaxis): These act on the pre-erythrocytic stages of the parasites (primary tissue forms or primary exo-erythrocytic forms) and thus completely preventing invasion of the blood cells.
2. Tissue schizontocides (used as antirelapse drugs): These act on the exoerythrocytic stages or tissue forms of *P. vivax* and *P ovale* and thus able to achieve radical cure of these infections. An example is Proguanil
3. Schizontocides (blood schizontocides or schizontocidal drugs): These act on the erythrocytic stages of parasites commonly associated with acute disease, though these stages may be present in some infections accompanied by few clinical symptoms. They also act on the sexual erythrocytic forms of *P.vivax*, *P.ovale* and *P malariae* but not directly on the mature gametocytes of *P.falciparum*. Examples are quinine, chloroquine and amodiaquine.
4. Gametocytocides (gametocytocidal drugs): These act on all sexual forms including those of *P.falciparum*, they also act on the developmental stages of malaria parasites in the anophelines. Examples quinine, primaquine.

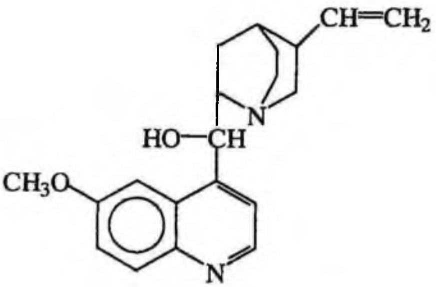
5. Sporontocides (sporontocidal drugs): These prevent or inhibit the formation of oocyts and sporozoites in anophelines that have fed on carriers of gametocytes. Proguanil and primaquine are examples.[46]

2.8.2. Pharmacological classification of Antimalarials

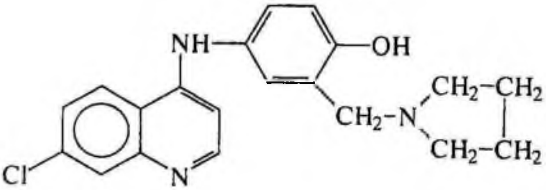
Antimalarial drugs may be broadly classified in two groups: lysosomotropic quinoline – containing drugs and antimetabolites. The lysosomotropic quinoline – containing drugs include quinine and quinidine, the 4-aminoquinolines, Chloroquine and amodiaquine, amopyroquine and the quinoline-methanol, mefloquine. The antimetabolities drugs include Lincomycin, actinomycin, cycloleucins, mitomycin and many others.



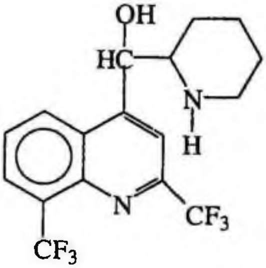
Chloroquine



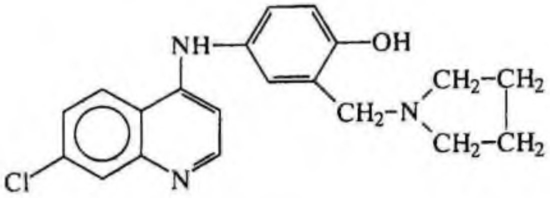
Quinine



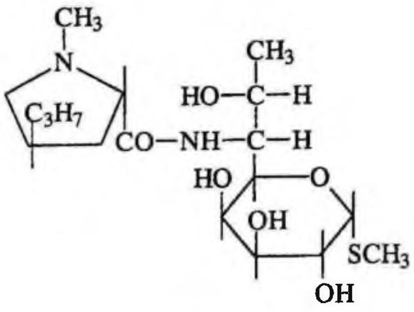
Amopyroquine



Mefloquine



Amopyroquine



Lincomycin

Quinoline-containing drugs have been the mainstay of antimalarial treatment for decades and are still widely used. Although the clinical use of quinolines is hampered by various degrees of parasite resistance [47, 48], there is still interest in drugs that share the same biochemical target.

The main target for quinoline antimalarial is haempolymerization, process whereby intraerythrocytic – stage malaria parasites detoxify haem in the digestive vacuole. Haem is a by-product of haemoglobin digestion and is used by the parasite as source of most of its essential amino acids [49]. It is potentially toxic to biological membrances and parasite enzymes [50, 51] and is thus sequestered in the form of an insoluble crystalline polymer haemozoin (or malaria pigment) [49].

Quinoline antimalarials have been shown to inhibit both synthetic and native polymerization [52,53,54]. Chloroquine appears to act by forming quinoline – haem complexes which terminate haemozin chain extension [55, 56]. The ability of drugs to inhibit haem polymerization is directly related to their antimalarial potency [53].

The classification of antimetabolites is complex and controversial. Their modes of action are very specific, three main mechanisms being in operation: inhibition of synthesis of the bacterial cell well, increased permeability of cytoplasmic membranes and interference with intracellular protein or nucleic acid synthesis. There are some correlations between the mode of action and the general spectrum of activity of antibiotics.[57]

2.8.3. Drug resistance in malaria

Drug resistance in malaria has been defined as the " ability of a parasite strain to survive and/ or to multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limit of the subject". Although the resistance of the drug embraces all species of malaria parasites and all acceptable dosages of blood or tissue schizontocides, gametocytocides and sporontocides, in practice it is most commonly applied to the effect of blood schizontocides on *falciparum* malaria.[57]

2.8.3.1 Resistance to the common antimalarial drugs

Human malaria parasite, *P. falciparum* has developed resistance to chloroquine and other drugs including amodiaquine and quinine. The other parasites have also developed resistance to proguanil, pyrimethamine and related compounds.

In Ghana resistance of *P. falciparum* to chloroquine and other antimalarials has been reported.[58] Resistance by *P.falciparum* to chloroquine, as well as all species to proguanil and pyrimethamine, is attributable to selection under drug pressure of resistant mutants which survive by utilizing alternative metabolic pathways to those blocked by the particular drug. In respect of chloroquine, resistance is characterized by a decrease in high-affinity binding sites for the drug. Once selected, and provided that they escape the destructive action of the host immunity, the resistant parasites may be transmitted by local mosquitoes to other people in the immediate area, or may be carried by a migrant host to other places where mosquitoes may or may not be present to establish transmission. [57]

2.9. Plants as sources of antimalarials

The use of medicinal plants as a source of relief from malaria, which is one of the oldest infections mentioned in early writings in Egypt, India and China could be traced back to over 2000 years. Attempts were made to treat it by the use of roots, leaves and flowers of many plants, example was the use of powdered roots of Ch'ang shan (*Dichroa febrifuga*) in China for a least 2000 years. Today, plants are almost the exclusive source of drugs for the majority of the world's population. [57]

In industrialized countries, medicinal plants research has had its ups and downs during the last decades. However, substances derived from higher plants constitute about 25% of prescribed medicines. Extracts of medicinal plants have mainly been superseded by the pharmaceutical preparations of the developed countries. However, natural products from higher plants, fungi and bacteria continue to be used in pharmaceutical preparations either as pure compounds or as extracts. [59]

The structure-activity relationships of naturally derived antimalarials are studied with a view to improving the therapeutic effects of such drugs and reducing their toxic effects. The importance to medicine of natural product molecules lies not in their pharmaceutical or chemotherapeutic effects but also in their role as template molecules for the production of new drug substances. Quinine, which was isolated from cinchona bark was found to have undesirable toxic side effect such as impaired hearing on prolonged use. Attempts to modify the structure of the drug showed that the quinoline ring was essential for activity. Synthetic antimalarials such as chloroquine were developed in which the activity of quinine was retained but the toxicity reduced. [59]

Many tropical countries have a list of useful plants for treating malaria. Examples are Africa (Ghana, Nigeria), North America (Mexico) and South East Asian (Malaysia, Indian) countries. There have been numerous attempts to test plant extracts for antimalarial activity and in the most extensive programme reported in 1947, over 600 plants from 126 families were screened for *in vitro* activity against *P. gallinaceum* in chicks and against *P.lophurae* in ducklings. Species from some 33 genera gave positive results and the most significant levels of activity were found in extracts of species from the Amaryllidaceae and from the Simaroubaceae. The latter family contains a number of genera, which are used in indigenous medicine for a range of activities in many different countries. The active ingredients are degraded triterpenes known collectivity as quassinoids or simaroubolides. The biological activites of these compounds include anticancer, antiviral, insecticidal and anti-inflammatory activities. Several quassinoids are potent inhibitors of protein synthesis in *P falciparum in vitro*. [59]

The plants used medicinally for the treatment of malaria include *Brucca javanica* in Thailand, *Castela nichosoni*, which was used in Brownsville, Texas for treatment when quinine had failed and *Picrasma antidesma* in British Honduras. In another study, the antimalarial effect of the stem bark of three *Khaya* species was investigated. It showed that the order of activity of the three species was *K. lvorensis* > *K. grandifoila* > *K. senegalensis* while only *K. grandifolia* at 50 mg/kg/day was active against established infection.[59]

The antimalarial activities of several African plants used in treating malaria have been investigated. These plants include *Cassia siamea, Dialium guineense, Dichapetalum guineense, Gomphrena celosioides, Jatropha gossypiifolia, Nauclea latifolia, Paullinia pinnata* and *P. crassipes*. The aqueous extracts of *C. siamea, J. gossypiifolia* and *P. crassipes* were most effective against *P. falciparum* on a dose-dependent basis.[59]

An *in vitro* antimalarial test, utilising the inhibition of uptake of ^{3}H-hypoxanthine into *Plasmodium falciparum* cultured in human blood was used to assess the activity of four solvent extracts of *Nauclea latifolia*; aqueous, methanol, ethanol and chloroform. The percentage inhibition for all the extracts were found to be dose-dependent, with the methanol extract showing the strongest inhibition of 99% at a concentration of 500 μm/ml. All the extracts were found to possess moderate activity against *P. falciparum*.[59]

2.10. *In vitro* cultivation of *Plasmodium*.

For the estimation of the antimalarial effects of plant extracts, either *in vivo* or *in vitro* methods are used. In *in vivo* work, various dilutions of the extract based on LD $_{50}$ values are administered to rodents infected with the rodent malaria parasite, *P. berghei* and the following effects determined:

(1) Blood schizontocidal activity on early infection (4-day test).

(2) Repository effect, i.e. the ability of the extract to prevent infection or its prophylactic effect.

(3) Effect on established infection.

A number of studies have been published in which *in vivo* methods are reported. [60, 61] When *in vivo* methods are used, one is not certain whether an active ingredient in the extracts or a metabolite of such an active principle is responsible for any observed activity.

Briefly, *In vitro* work for the assessment of plant-derived antimalarials, includes incubation of the extracts or fractions with *P. falciparum* and using standard methods as discussed by Jensen and Trager [62] to measure the inhibition of growth and multiplication of the parasite. Microscopic examination of Giemsa-stained thin blood films can be used to determine the parasitaemia. Alternatively, the degree of inhibition of uptake of a radiolabelled nucleic acid precursor like ^{3}H-hypoxanthine by the parasites can be used to measure the extent of inhibition of growth of the parasites.[63]

Labeled hypoxanthine is used because a functional *de novo* purine biosynthetic pathway has not been demonstrated for the malaria parasite; consequently, they require an exogenous supply of preformed purines. The preferred purine for both *P. lophurae* and *P. falciparum* appears to be hypoxanthine, which is derived intraerythrocytically through the monophosphate → inosine monophosphate → inosine → hypoxanthine.

In contrast to the malaria parasite, the human host has the ability to synthesize purines *de novo*. The purine nucleotides are assembled from a variety of precursors. Glycine provides C-4, C-5 and N-7. The N-1 atom comes from aspartate. The other two nitrogen atoms, N-3 and N-9, come from the amide group of the side chain of glutamine. Activated derivatives of tetrahydrofolate furnish C-2 and C-8, whereas CO_2 is the source of C-6.

The use of the 96- well microtiter plate for such inhibition assays, together with the use of ^{3}H-hypoxanthine as an index of parasite growth provides a simple and convenient method which utilizes a minimum of scarce resources, as microlitre volumes are used. It also yields results rapidly (within 48 hours), large enough for statistical analysis and is easily reproducible. It is also more reliable, because it is based on the metabolism of purine in the parasite and hence more accurate. This method is usually preferred over microscopic methods because of the reasons mentioned. Scintillation counting is used in this method and it gives more reliable results and is less laborious. The microscopic method however is still of importance in studies in which the effect of drugs or extracts on the various growth stages is being studied. However, it is time consuming, strenuous and has a higher degree of subjectivity.

A new method that utilizes radioactive ethanolamine instead of hypoxanthine has been reported [64]. The major advantage of this method is that this precursor is incorporated into phospholipid, and the phospholipid incorporated can be used to evaluate *P. falciparum in vitro*. It is an ideal tool when compounds, which interfere with DNA and / or RNA metabolism, are to be investigated for their effect on *Plasmodium* growth.

The work of O'Neil *et al*, [63] shows that *in vitro* antimalarial testing can be carried out on crude plant extracts using *P. falciparum*. Initial information on activity may be obtained by using ten-fold dilutions of crude-extracts and solvent fractions. For accurate determination of IC_{50} values, further results are needed from two-fold dilutions. Unlike the *in vivo* methods, the *in vitro* approach indicates whether the plant extract as opposed to its metabolite has or does not have active antimalarial principles.

CHAPTER THREE

Results and Discussion

In the present investigation 1.0 kg of shade dried pulverised sample of the whole rhizome of *Cochlospermum tinctorium* was sequentially extracted with petroleum ether (40-60 ^{0}C), dichloromethane, ethyl acetate, ethanol and water for 48 hours each using cold percolation. Although some chemical investigations have been carried out on this plant, the aim of this project is to look at the antiplasmodial activities of the various crude extracts and the compounds isolated from the rhizome extracts.

The crude extracts from the petroleum ether, dichloromethane, ethyl acetate, ethanol and water were subjected to a series of phytochemical screening tests[71] in the hope of establishing the presence or absence of the various classes of organic compounds. The results are summarised in **Table 2** below. Crude extracts and compounds isolated were tested for antiplasmodial activity using a modification of the method of Jensen and Trager.[62]

Table 2: Summary of results of phytochemical screening tests on petroleum ether, dichloromethane, ethyl acetate, ethanol and water extracts of the rhizome of *Cochlospermum tinctorium*.

Class of compounds	Petroleum ether extract	Dichloro-methane extract	Ethyl Acetate extract	Ethanol extract	Water extract
Alkaloids	–	–	–	–	–
Terpenoids	+	–	–	–	–
Flavonoids	–	–	–	+	–

Leuco-anthocyanins	–	–	–	+	–
Tannins	–	–	+	+	–
Saponins	–	–	–	+	+
Cardiac glycosides	+	+	+	+	+
Anthraquinones and Anthracene derivatives	–	–	–	+	–

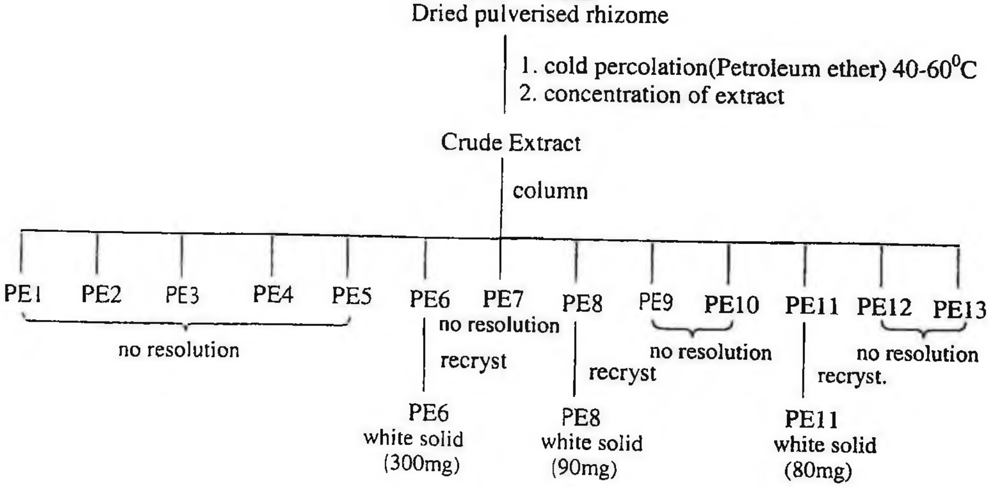
Symbols + = present/detected = absent/ not detected

The results showed the presence of cardiac glycosides in all the extracts, but no alkaloid was present in any of the extracts. Other compounds detected were anthraquinones and anthracene derivatives (ethanol extract), saponins (water and ethanol extracts), leuco-anthocyanins (ethanol extract), flavonoids (ethanol extract) and terpenoids (petroleum ether extract).

3.1. Fractionation of Petroleum ether extract

The petroleum ether extract was analysed as outlined in **Scheme 3.1**

Scheme 3.1



Three solid components: PE6, PE8 and PE11 were isolated. The percentage yields of the compounds based on the crude extract and also on the dry plant material are shown in **Table 3** below.

Table 3. Percentage yields of compounds isolated from the rhizome of *C. tinctorium*

Compound	**Mass/mg**	**% yield based on crude extract**	**% yield based on plant material**
PE6	300	17.241	0.030
PE8	90	5.844	0.009
PE11	80	7.843	0.008

3.1.1. Characterization of PE6

Fraction PE6 was evaporated to dryness and dissolved in a limited amount of petroleum ether. It was kept in a freezer at a temperature of –20^{0}C for 18 hours. A white solid precipitated from the solution. This was filtered and washed with cold petroleum ether. The sample coded as PE6 had a melting point of 70-72 ^{0}C.

The IR spectrum of PE6 (Appendix Ia) is shown in **Table 4** below

Table 4: Prominent peaks on IR spectrum of PE6

Frequency of signal (cm^{-1})	**Assignments**
3400 – 3200 (b)	O-H stretching vibrations
2960 – 2850 (s)	C-H stretching vibrations
1700 – 1680 (s)	C=O stretching vibrations
1670 – 1630 (s)	C=C bending vibrations
1470 – 1360 (m)	C-H bending vibrations
1350 –1000 (s)	C-O bending vibrations
970 –960 (v)	C-H bending vibration (trans alkene)
730 –675 (s)	C-H bending vibration (cis alkene)

The ^1H-NMR spectra (Appendix IIa, IIb and IIc) were assigned to PE6 as shown in **Table 5** below

Table 5: ^1H-NMR spectrum summary of PE6

Signal (δ, ppm)	**Assignment**
6.8 (1H, s)	-C=C$\underline{H}$–CO
5.0 (1H, s)	-R –O$\underline{H}$
2.9 (1H, d, J =2.1)	-CO–C$\underline{H}$-CH=
2.65 (2H, t, J = 7.3)	C=CH-C$\underline{H}$=CH- (cis)
2.58 (1H, d, J =2.1)	-CO–C$\underline{H}$–CH=
2.42 (1H, t, t, J =13.3, 13.3)	=CH-C$\underline{H}$=C$\underline{H}$-CH= (trans)
1.96 (1H, t, t, J =13.3, 13.3)	=CH-C$\underline{H}$=C$\underline{H}$-CH= (trans)
1.6 (3H, s)	$C\underline{H}_3$–CO-
1.3 (42H, bs)	CH_3-CH_2-$(C\underline{H}_2)_{21}$–C
0.9 (6H, t, J =6.6)	$C\underline{H}_3$–CH_2-C

The signals in the ^{13}C-NMR spectrum (Appendix IIIa) were assigned as shown in **Table 6** below

Table 6: Summary of ^{13}C-NMR spectrum of PE6

Chemical shifts (δ, ppm)	**Carbon Assignments**
211.53	C=O (carbonyl)
147.29	CH_2 (alkene)
139.10	CH_2 (alkene)
77.40-62.6	CH_2 (alkane)

51.34-22.56 13.98	CH_2 (alkane) CH_3

The ^{13}C-NMR spectrum gave 21 carbon atoms/peaks.

At this point it was not possible to deduce the exact structure of the sample PE6 because the mass spectrum and the expanded ^{1}H-NMR spectral were not obtained, but from the information available it suggests that the sample composes of a carbonyl group, an alcohol group, a saturated hydrocarbon group and a long chain unsaturated hydrocarbon group.

3.1.2. Characterization of PE8

Fraction PE8 was concentrated into a small volume and kept in a freezer at –20 ^{0}C for 8 hours. A white solid precipitated from the solution. The solid was filtered and washed with cold petroleum ether and coded as PE8. The melting point was found to be 123-125 ^{0}C.

The sample and an authentic sample of stigmasterol were dissolved in petroleum ether and spotted on the same TLC plate in various solvent systems and they gave the same R_f values. The IR spectrum (Appendix Ib), indicated the presence of hydrogen bonded O–H (3510cm^{-1}), an alkene C–H stretching vibration (2960-2850 cm^{-1}), an alkene C=C stretching vibration (1680-1620 cm^{-1}), a saturated hydrocarbon (C-H) stretching vibration (1600-1500 cm^{-1}), an alkane C–H bending vibration (1485-1350 cm^{-1}) and phenol C–O bending vibration (1350- 1000 cm^{-1}).

Table 7: Comparison of IR spectrum of PE8 and stigmasterol

PE8 IR peaks (cm^{-1})	Stigmasterol IR peaks (cm^{-1})	Interpretation
3500-3600	3500-3600	O-H stretch of alcohols
2960-2850	2950-2850	C-H stretch of alkanes
1680-1620	1670-1620	C=C stretch vibration of alkenes
1600-1500	1600-1500	C-H stretch of alkanes
1485-1350	1490-1360	C-H bending vibration of alkanes
1245	1245	O-H stretch of secondary alcohols
1015-1010	1020-1010	C-O stretch of alcohols

Comparison of the IR spectrum for PE8 and authentic sample of stigmasterol as shown in **Table 7** suggests that the sample PE8 was stigmasterol. The mixed melting point (123-124 ^{0}C) and the co-TLC of sample PE8 and the authentic sample of stigmasterol confirmed that the sample PE8 was stigmasterol.

3.1.3. Characterization of PE11

Fraction PE11 was kept in the freezer at –20^{0}C for 12 hours. A white solid was formed in the flask. This was filtered and washed several times with cold petroleum ether. The solid was labelled as PE11 and the melting point was 45-47 ^{0}C. The IR spectrum (Appendix Ic) is shown in **Table 8**.

Table 8: Summary of IR functional groups of PE11

Frequency of signal (cm^{-1})	**Assignments**
3100 – 3000 (m)	N-H stretching vibrations
2960 – 2850 (s)	C-H stretching vibrations
1700 – 1630 (s)	C=O stretching vibrations
1600 – 1500 (s)	C=C bending vibrations
1485 – 1445 (m)	C-H bending vibrations
1350 –1000 (s)	C-O bending vibrations
770 –730 (s)	Ar –H bending vibration

The ^{1}H-NMR spectra (Appendixes IId, IIe and IIf) were assigned as shown in **Table 9** below

Table 9: Summary of ^{1}H-NMR spectrum of PE11

Signal (δ, ppm)	**Assignment**
7.3 (2H, d, J =5.1)	Aromatic protons
7.05 (1H, m)	Aromatic protons
4.16 (1H, t)	$\mathrm{-CH{-}C\underline{H}{-}NH{-}CO{-}R}$
2.55 (4H, t, t, J = 15.0, 7.6)	$\mathrm{-CO{-}C\underline{H}_2{-}C\underline{H}_2{-}N{-}}$
2.00 (1H, m)	$\mathrm{-\ CH{-}C\underline{H}{-}CH_2{-}O{-}}$
1.60 (6H, t, J = 6.6)	$\mathrm{C\underline{H}_3{-}CH_2{-}O{-}}$
1.30 (64H, bs)	$\mathrm{-\ CH_2{-}(C\underline{H}_2)_{32}{-}CH_2{-}}$
0.91 (9H, t)	$\mathrm{C\underline{H}_3{-}CH_2\ -}$

The COSY spectra (Appendix VIa) indicated the coupling of 7.30ppm to 7.05ppm, 2.55ppm to 1.60ppm, 2.00ppm to 1.30ppm, 1.60ppm to 1.30ppm and 1.30ppm to 0.91ppm.

The signals in the ^{13}C-NMR spectrum (Appendix IIIb and IIIc) were assigned as shown in **Table 10** below

Table 10: Summary of ^{13}C-NMR spectrum of PE11.

Chemical shifts (δ, ppm)	**Carbon Assignments**
199.25	C=O (carbonyl)
138.51	CH_2 (alkene)
118.12	CH_2 (alkene)
77.59-76.74	CH_2 (alkane)
37.20-22.75	CH_2 (alkane)
14.16	CH_3

The ^{13}C-NMR spectrum gave 20 carbon atoms/peaks.

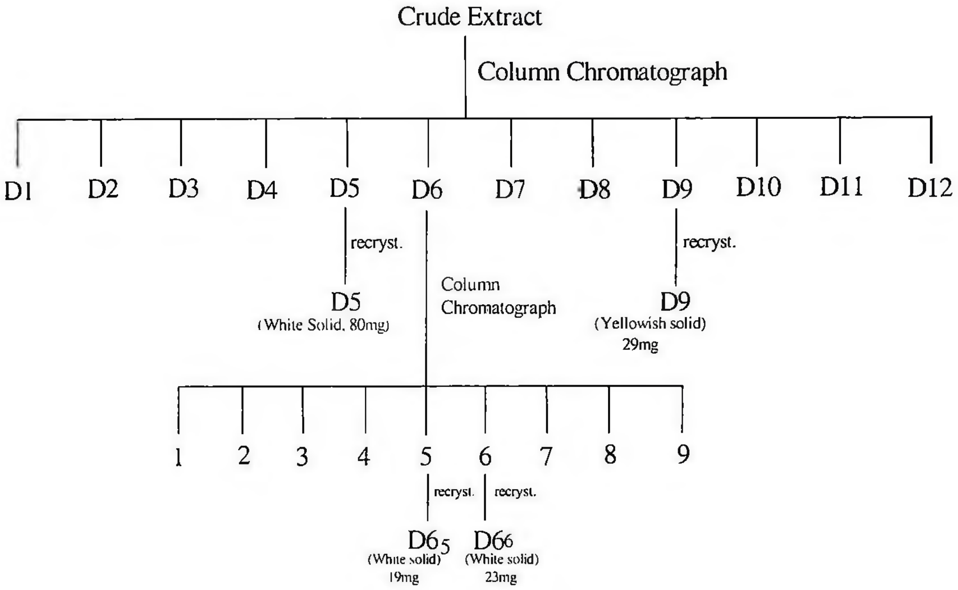
The UV spectra (Appendix Vc) showed two absorption maxima at 272nm, and 375nm. The data obtained was not sufficient to enable full characterisation of sample PE11, but the information available suggests that the sample has an aromatic part, an amide part, carbonyl part and a long chain unsaturated hydrocarbon part.

Fractions PE1 to PE5, PE7, PE9, PE10, PE12 and PE13 were not worked on because of the minute quantities and also the TLC was very complex.

3.2. Fractionation of Dichloromethane extract

Isolation and purification work done on the dichloromethane extract is outlined in **Scheme 3.2**.

Scheme 3.2



Four solid D5, $D6_5$, $D6_6$ and D9 were isolated. The percentage yields of these compounds based on the masses of the crude extract and also on the dry plant material are given in **Table 11** below.

Table 11: Percentage yields of compounds isolated from the rhizome of *C. tinctorium*

Compound	Mass/mg	% yield based on crude extract	% yield based on plant material
D5	80	19.51	0.0080
$D6_5$	19	3.33	0.0019
$D6_6$	23	4.04	0.0023
D9	29	11.60	0.0029

3.2.1. Characterization of D5

Fraction D5 on recrystallization from petroleum ether gave white crystals and was coded D5. This compound was found to be triacontanol with melting point 83-85^{0}C, literature melting point, 85-86^{0}C [65] The structure was identified by spectroscopic methods.

$HOCH_2(CH_2)_{28}CH_3$

triacontanol

The IR spectrum (Appendix Id) is shown in Table 12.

Table 12 Summary of IR interpretation of D5

Frequency of signal (cm^{-1})	Assignments
3400 – 3200 (b)	O-H stretching vibrations of hydrocarbon
2917	C-H stretching vibrations of hydrocarbon
2849	C-H stretching vibrations of hydrocarbon
1470- 1460	C-H bending vibrations of hydrocarbon
1070 – 1060	C-O bending vibrations of alcohol
730 – 720	CH_2 rocking vibrations of hydrocarbon

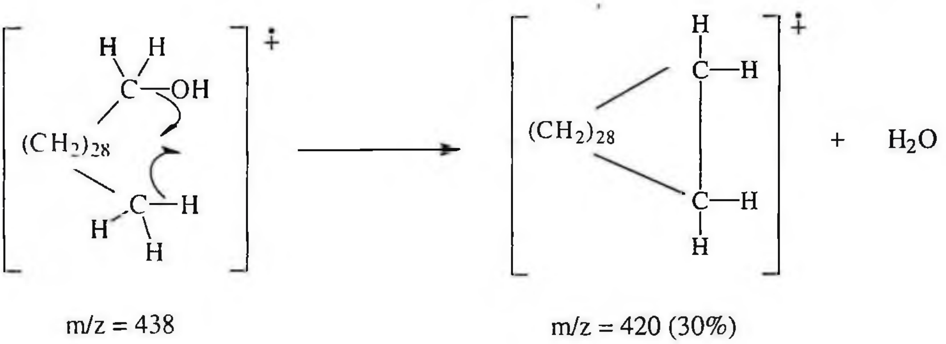
The ^{1}H-NMR spectra (Appendixes IIg, IIh and IIi) were assigned as shown in **Table 13**

Table 13: Summary of ^{1}H-NMR spectrum of D5.

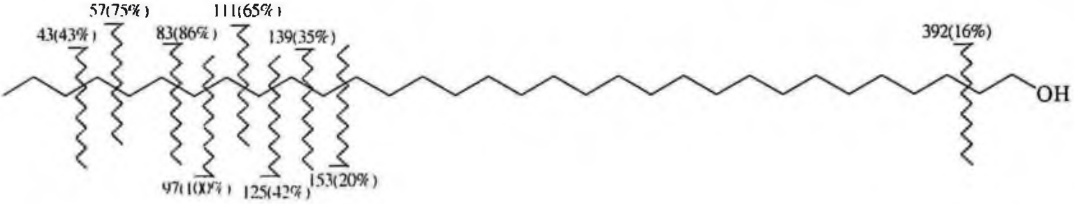
Signal (δ, ppm)	Assignment
3.64 (2H, t)	$R\text{-}CH_2\text{-}C\underline{H}_2\text{-}OH$
1.25 (2Hn, bs)	$R\text{-}(C\underline{H}_2)nCH_2\text{-}OH$
0.9 (3H, t)	$C\underline{H}_3\text{-}CH_2\text{-}(CH_2)_n\text{-}OH$

Integration of the ^{1}H-NMR spectrum and analysis of the mass spectrum (Appendix IVa) indicated that n is approximately 28. The molecular weight was 438. The mass spectrum (Appendix IVa) showed prominent peaks at m/z 420 (30%), 435 (2%) and a cluster of peaks occurring at 14 m/z units apart. A few spectral fragmentation patterns for D5 are accounted for in schemes 3.4, 3.5, 3.6 and 3.7.

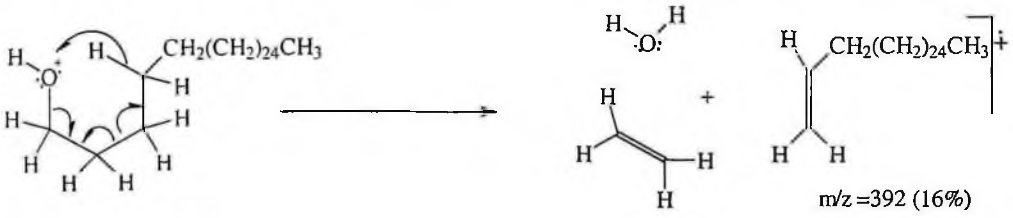
Scheme 3.4



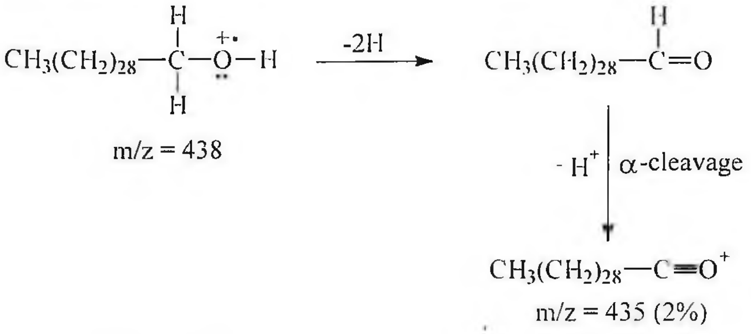
Scheme 3.5



Scheme 3.6



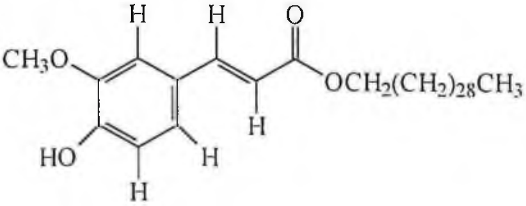
Scheme 3.7



3.2.2. Characterization of D6$_5$

The mother liquor from the dichloromethane extract of the rhizome of *C. tinctorium* on column chromatography over silica gel gave twelve fractions, D1, D2, D3, ... D12. Fraction D6 was rechromatographed and eluted with a mixture of petroleum ether / ethyl acetate. Nine fractions were obtained. These were labelled D6$_1$, D6$_2$, ... D6$_9$.

Fraction D6$_5$ on cooling in the freezer gave a white solid. The white solid was filtered and washed with cold petroleum ether. The white solid coded D6$_5$ gave a single spot on TLC plate, which was found to be triacontanyl ferulate (trans-triacontanyl-4-hydroxy-3-methoxycinnamate) mpt 81-83^{0}C. The structure of the compound was determined by spectroscopic methods and comparison of the data with those of an authentic sample. [66] This is the first time this compound has been isolated from this plant.



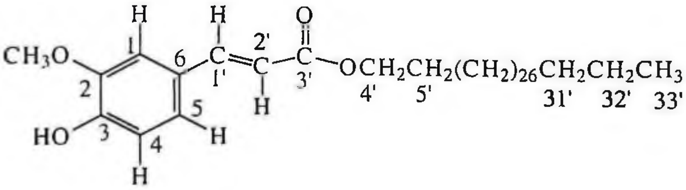
triacontanyl ferulate

The prominent peaks in the IR spectrum (Appendix Ie) are given in **Table 14** below.

Table 14:Summary of the IR functional groups of $D6_5$

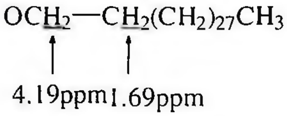
Frequency of signal (cm^{-1})	**Assignments**
3500 – 3200	O-H stretching vibration
2960 – 2850	C-H stretching vibration
1698	C=O stretching vibration (conjugated to carbonyl)
1674	C=C stretching vibration
1470 – 1350	C-H bending vibration
1070 – 1060	C-O bending vibration

^{1}H-NMR spectrum (Appendix IIj, IIk, III and IIm) gave a pair of doublets resonating at δ 7.61 and 6.29 (1H each, J = 16.0 Hz) were assigned to H-1` and H-2` respectively, representing vinyl protons. The signal at δ 7.11 (2H, d, d J= 8.1, 1.8 Hz) appeared as an AMX aromatic coupling system, which was assigned to H-4. The proton at H-3 appeared at δ 6.9 (H, d, J = 8.1 Hz). The appearance of a singlet at δ 5.80 is an indicative of the presence of a phenolic OH group. The oxygen bearing α-methylene protons appeared at δ 4.19 (2H, t. J = 6.8 Hz). The β-methylene proton appeared at δ 1.69 (2H, m). A broad singlet at δ 1.25, integrating for 54 protons was assigned to all other methylene protons in the straight chain while the terminal methyl appeared as a triplet at δ 0.88 (3H, t, J = 6.6H Hz).



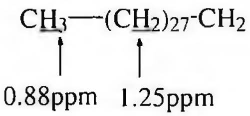
From the proton-proton correlation spectroscopy (homonuclear COSY) (Appendix VIb) it was identified that the following protons coupled to each other.

The β-methylene proton at 1.69 ppm is coupled to α-methylene protons at 4.19 ppm



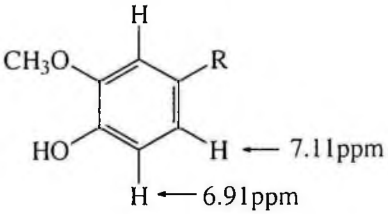
$$OC\underline{H}_2—C\underline{H}_2(CH_2)_{27}CH_3$$

The terminal methyl protons at 0.88 ppm are coupled to the methylene protons at 1.25 ppm

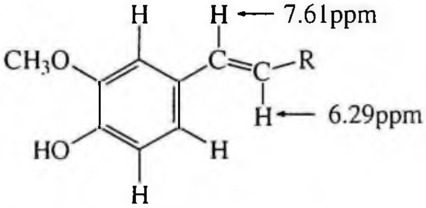


$$C\underline{H}_3—(C\underline{H}_2)_{27}-CH_2$$

The aromatic protons 6.91 ppm and 7.11 ppm are coupled to each other.



The vinyl protons 6.29 ppm and 7.61 ppm are coupled to each other.



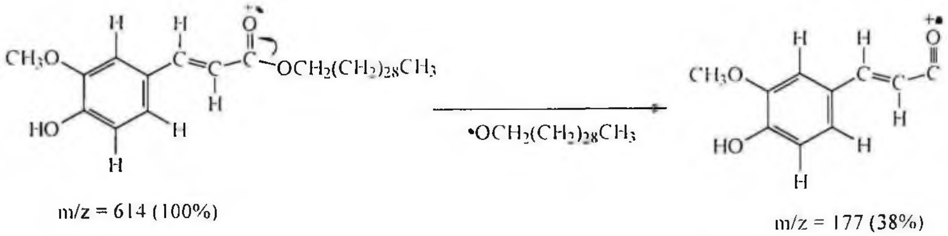
The signals in the ^{13}C-NMR spectra (Appendix IIId) were assigned as shown in **Table 15**.

Table 15: Summary of ^{13}C-NMR spectrum signals of D6$_5$.

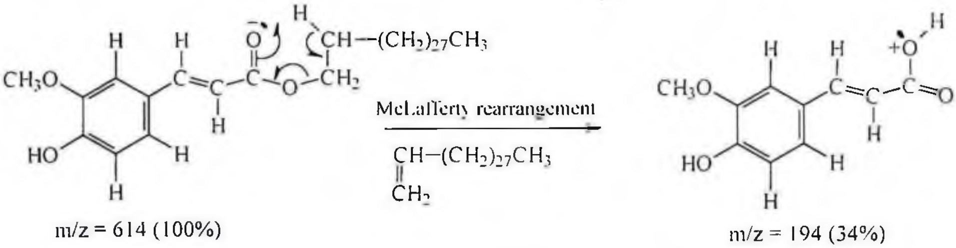
Chemical shifts (δ, ppm)	**Carbon Assignments**
14.1	C-33`
22.6	C-32`
28.6	C-31`
29.1	C-6` C-30`
109.3	C-5
115.7	C-2
70.1	C-4
123.1	C-3
76.9	C-6
115.7	C-1
114.7	C-1`
77.4	C-2`
144.7	C-3`
64.6	C-4`
55.8	C-5`

The mass spectrum (Appendix IVb) showed a molecular ion peak and a base peak at m/z 614 (100 %). Other significant peaks occurred at m/z 586 (27 %), 572 (2 %), 194 (34 %), 177 (38 %) 150 (12%) and 137 (18 %). Schemes 3.8, 3.9, 3.10, 3.11 and 3.12 represent the mass spectral fragmentation.

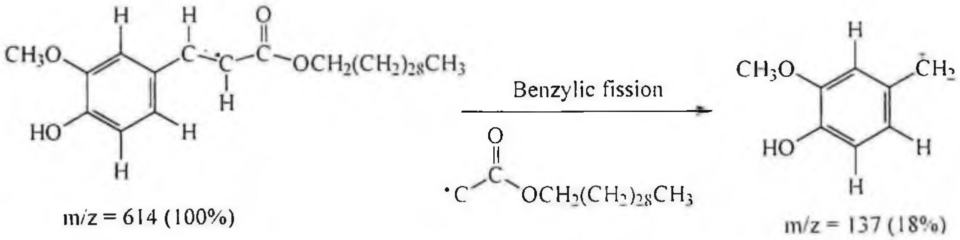
Scheme 3.8



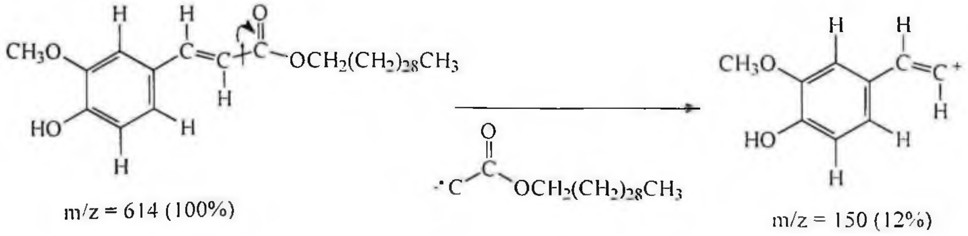
Scheme 3.9



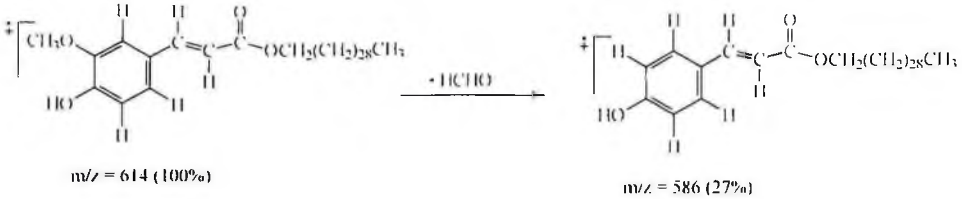
Scheme 3.10



Scheme 3.11

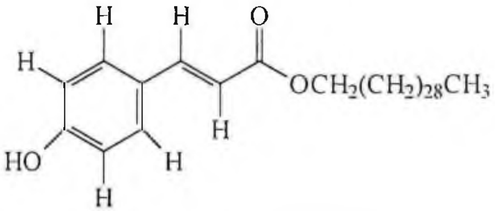


Scheme 3.12



3.2.3. Characterisation of $D6_6$

The residue from fraction $D6_6$ was dissolved in petroleum ether and kept in the freezer at -20^0C for 10 hours. A white solid recrystallised, filtered and washed with cold petroleum ether. The solid gave a single spot on TLC plate and was coded $D6_6$ It was identified on the basis of its spectral properties and comparison of the spectral data with literature data[67] to be triacontanyl p-coumarate (trans triacontanyl-4-hydroxyl-cinnamate). The melting point was found to be 79-81^{0}C.



triacontanyl p-coumarate

The IR spectra (Appendix If) indicated the presence of the following functional groups which are shown in **Table 16** below.

Table 16: Summary of IR functional groups of $D6_6$

Frequency of signal (cm^{-1})	Assignments
3350-3250 (b)	Ar-OH stretching vibration
1720-1710 (s)	C=O Stretching vibration of an ester

1640-1620 (w)	C=C Stretching vibration (conjugate to carbonyl)
1610-1590 (w)	C=C Aromatic ring
1520-1500 (w)	C=C Aromatic ring
1320-1260 (v)	C-H Bending vibration
1020-970 (v)	C-O Bending vibration
850-840 (s)	Ar-H Bending vibration
820-810 (s)	Ar-H Bending vibration

^{1}H-NMR spectrum (Appendixes IIn and IIo) gave signal exhibiting a typical AA`BB` system (2H, d, J = 6 Hz) at δ 7.4ppm and δ 6.8ppm for H-2, 6 and H-3, 5 respectively, as well as two coupled trans-olefinic protons (1H, d, J = 16 Hz) at δ 6.3ppm and δ 7.6ppm, which was assigned to H-2' and H-1' respectively. The methylene protons on the alcohol carbon atom forming the ester bond appeared at δ 4.2 ppm (2H, t, J = 7.0 Hz), while the adjacent methylene protons were at δ 1.8ppm (2H, m). A broad signal at δ 1.2ppm integrating for 54 protons was assigned to the other methylene protons in the straight chain while the terminal methyl group gave a triplet at δ 0.9ppm (3H, t, J = 6 Hz). A comparison of the ^{1}H-NMR of D6$_6$ and n-triacontanyl p-coumarate are shown in **Table 17**.

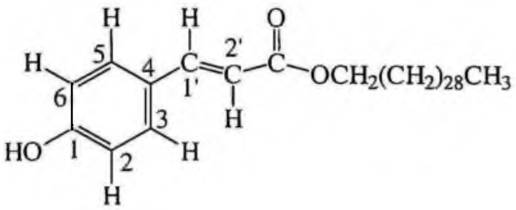
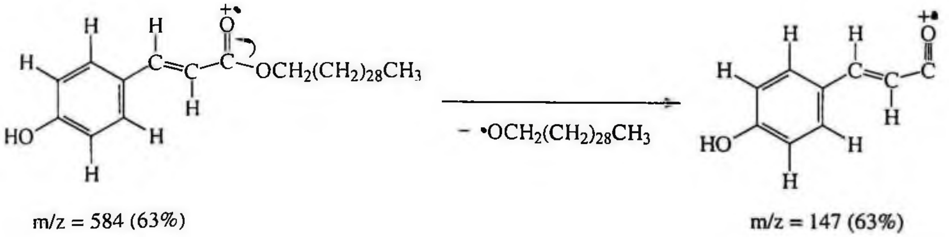


Table 17: Comparison of ^{1}H-NMR of D6$_6$ and n-triacontanyl p-coumarate

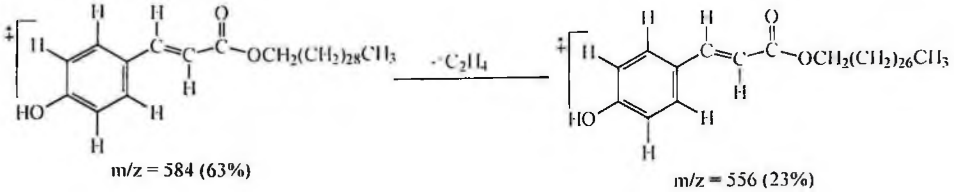
D6$_6$ signals (δ,ppm)	**Triacontanyl p-coumarate signals (δ,ppm)**	**Assignment**
7.60 (1H, d, J = 16.0Hz)	7.61 (1H, J = 16.0Hz)	Ar-C<u>H</u>=CH-CO (trans)
7.40 (2H, d, J = 6.0Hz)	7.39 (2H, J = 8.0Hz)	Aromatic protons
6.80 (2H, d, J = 6.0Hz)	6.83 (2H, J = 8.0Hz)	Aromatic protons
6.30 (1H, d, J = 16.0Hz)	6.27 (1H, J = 16.0Hz)	Ar-CH=C<u>H</u>-CO (trans)
4.20 (2H, t, J = 7.0Hz)	4.13 (2H, J = 7.0Hz)	$OC\underline{H_2}CH_2R$
1.80 (2H, m)	1.60 (2H, m)	$OCH_2C\underline{H_2}R$
1.20	1.23	54H, bs, $(CH2)_{27}$
0.90 (3H, t, J = 6.0Hz)	0.91 (3H, J = 6.0Hz)	$C\underline{H_3}$-CH_2-R

The mass spectrum (Appendix IVc) showed a molecular ion peak at m/z 584 (63 %) and a base peak at m/z 164 (100%). Other significant peaks occurred at m/z 556 (23%), 147 (63%), 120 (32%) and 107 (28%). Peaks occurring at m/z greater than 584 are probably due to an impurity. These are 614 (28%), 616 (10%) and 718 (2%). A few spectral fragmentation patterns for D6$_6$ have been accounted for in **Schemes 3.13, 3.14, 3.15, 3.16** and **3.17**.

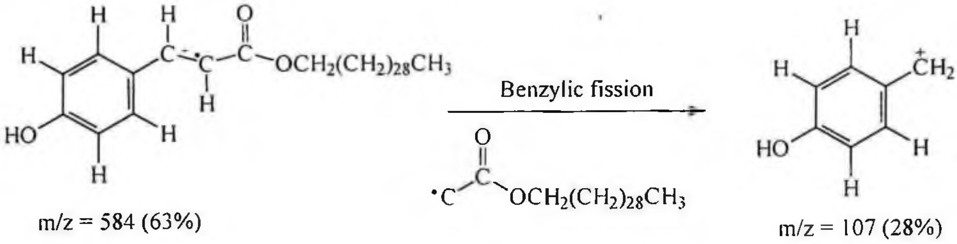
Scheme 3.13



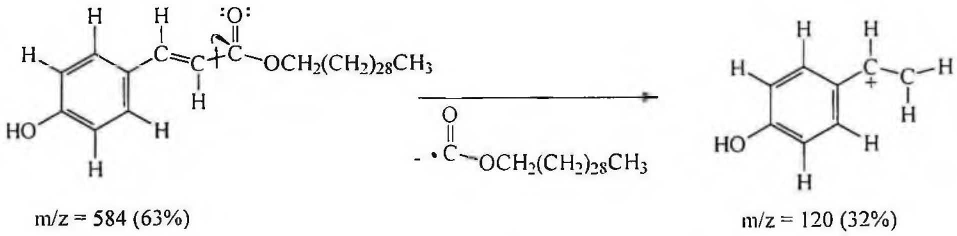
Scheme 3.14



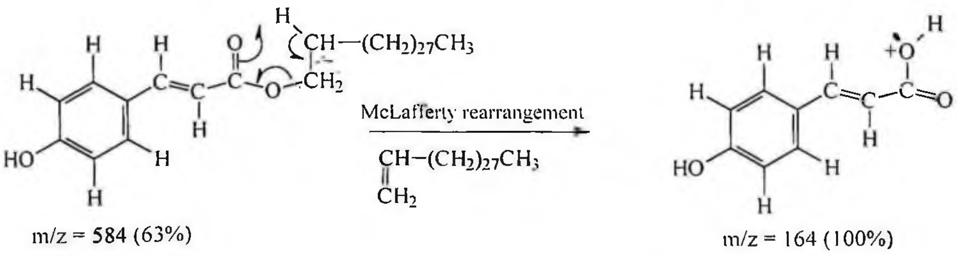
Scheme 3.15



Scheme 3.16



Scheme 3.17



3.2.4. Characterisation of D9

Fraction D9 was kept in the freezer at –20 ^{0}C for 24 hours. An orange solid was formed. The solid was filtered and washed with cold petroleum ether. The solid gave a single spot on TLC in different solvent system and was labelled as D9. The melting point was 200-202 ^{0}C.

The IR spectrum (Appendix Ig) indicated the presence of alkane C–H stretching vibrations (2960-2850 cm^{-1}), C=O stretching vibration for an ester (1725-1705 cm^{-1}), alkane C–H bending vibrations at (1485-1445 cm^{-1}), phenols or alcohol C–O bending vibrations at (1350-1000 cm^{-1}) and Ar–H bending vibrations at (770-690 cm^{-1}).

The ^{1}H-NMR spectrum (Appendixes IIp, IIq, IIr and IIs) were assigned as shown in **Table 18** below.

Table 18: Summary of ^{1}H-NMR spectrum of D9.

Signal (δ, ppm)	**Assignment**
7.64 (2H, d, J =1.3)	Aromatic proton (para)
6.68 (2H, d, J =8.4)	Aromatic proton (ortho)
6.54 (2H, d, J =6.4)	Aromatic proton (ortho)
4.92 (2H, q)	$-CH_2-C\underline{H}_2=CH-$
4.6 (2H, m)	$-CH_2-C\underline{H}_2-CH_2-$
4.55 (2H, d, d, J = 3.6, 3.2)	$-C-C\underline{H}=C\underline{H}-$
4.03 (2H, d, d, J = 4.5, 4.2)	$-C-C\underline{H}=C\underline{H}-$
3.25 (4H, t, t, J = 13.8, 6.6)	$-C\underline{H}_2-C\underline{H}_2-CH=C-$

2.95 (4H, t, t, J =13.8, 6.6)	$-C=CH-C\underline{H}_2-C\underline{H}_2-CH=C$
1.6 (2H, m)	$-CH_2-C\underline{H}_2-CH_2-$

The COSY spectra (Appendix VIc) indicated the presence of coupling of 6.68ppm to 4.92ppm, 6.68ppm to 4.6ppm, 4.92ppm to 3.25ppm, 4.55ppm to 4.03ppm, 4.55ppm to 2.95ppm and 3.25ppm to 2.95ppm. The UV spectra (Appendix Va) showed five absorption maxima at 290nm, 315nm, 420nm, 442nm and 471nm.

The signals in the ^{13}C-NMR spectra (Appendixes IIIe, IIIf and IIIg) were assigned as shown in **Table 19** below.

Table 19: Summary of ^{13}C-NMR spectrum of D9.

Chemical shifts (δ, ppm)	**Carbon Assignments**
173.33	C=O (carbonyl)
168.78	CH_2 (alkene)
168.56	CH_2 (alkene)
138.20-127.74	Carbons on aromatic ring
78.05-37.22	CH_2 (alkane)

The ^{13}C-NMR spectrum gave 18 carbon atoms/peaks.

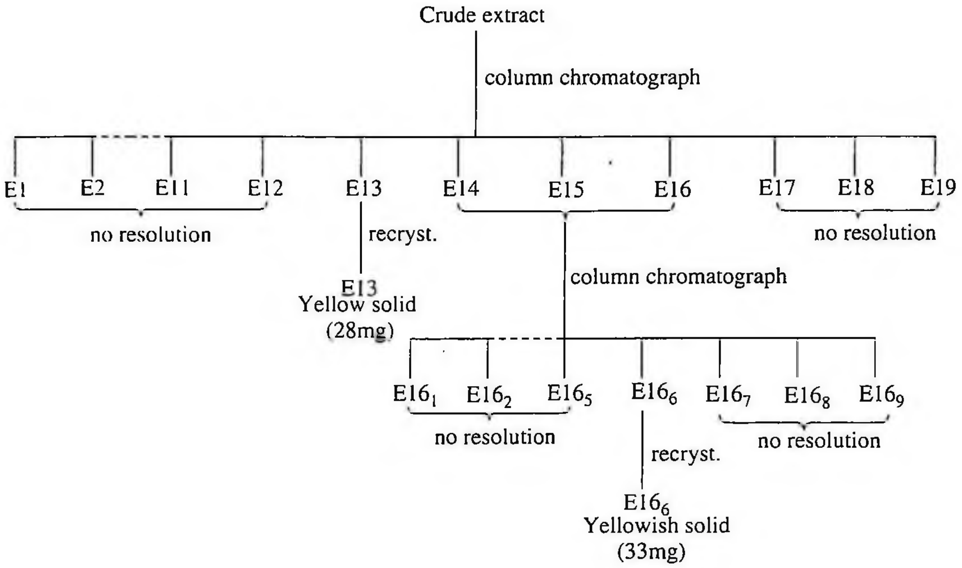
From the information available, the sample is suspected to have an aromatic ring, a long chain saturated hydrocarbon and a carbonyl groups. Full characterisation of the sample was not obtained because of lack of MS spectrum.

Fractions D1 to D4, D7, D8, D10 to D12, $D6_1$ to $D6_4$ and $D6_7$ to $D6_9$ were not worked on because of the minute quantities and also the TLC was very complex.

3.3. Fractionation of Ethyl acetate extract

Isolation and purification work was done on the ethyl acetate extract. The ethyl acetate extract was analysed as outlined in **scheme 3.18**

Scheme 3.18



Two solid components E13 and $E16_6$ were isolated. The percentage yields of the components based on the masses of the crude extract and the dry plant materials are shown in **Table 20** below.

Table 20: Percentage yields of compounds isolated from the ethyl acetate extract rhizome of *Cochlospermum tinctorium*

Compound	**Mass/mg**	**% yield based on crude extract**	**% yield based on plant material**
E13	28	12.73	0.0028
$E16_6$	33	4.93	0.0033

3.3.1. Characterization of E13

Fraction E13 on recrystallisation from petroleum ether yielded a yellow solid, which was coded E13. TLC of E13 gave one spot in different solvent systems. The melting point is 69-71 ^{0}C.

The IR spectrum signals (Appendix Ih) are shown in **Table 21** below.

Table 21: Summary of the IR functional groups of E13.

Frequency of signal (cm^{-1})	Assignments
2960 – 2850 (s)	C-H stretching vibrations
1720 – 1710 (s)	C=O stretching vibrations
1640 – 1620 (w)	C=C stretching vibrations
1470 – 1460 (s)	C-H bending vibrations
1320 –1260 (v)	C-H bending vibrations
730 –720 (s)	CH_2 rocking vibrations

The UV spectrum of E13 (Appendix Vb) showed two absorption maxima at 277nm and 380nm. The colour of the sample and UV spectrum suggests that it could have a carotenoid structure. With the information available, it suggests that the sample could have a carbonyl part and a saturated hydrocarbon part. Full characterisation of sample E13 was not obtained because of lack of ^{1}H-NMR, ^{13}C-NMR and MS spectrals.

3.3.2. Characterisation of E16$_6$

Fraction E16$_6$ was kept in the freezer at –20^{0}C for 24 hours. A yellowish solid was formed. The solid was filtered and washed with cold petroleum ether. The solid gave a single spot on TLC plates in different solvent systems and labelled $E16_6$. The melting point was found to be greater than 250 ^{0}C.

The IR spectrum signals (Appendix Ii) are shown in **Table 22** below.

Table 22: Summary of the IR functional groups of $E16_6$

Frequency of signal (cm^{-1})	**Assignments**
3400-3200 (b)	O –H stretching vibrations
2960 – 2850 (s)	C-H stretching vibrations
1680 – 1620 (v)	C=C stretching vibrations
1485 – 1370 (w)	C –H bending vibrations
1250 – 1150 (s)	C –O bending vibrations
1150 –1000 (v)	C –O bending vibrations

The information available suggests that the sample could have an alcohol and a saturated hydrocarbon parts, but full characterisation of the sample was not possible because the information obtained was not sufficient.

Fractions E1 to E12, E17 to E19, $E16_1$ to $E16_5$ and $E16_7$ to $E16_9$ were not worked on because of the minute quantities and also the TLC plates showed a lot of spots.

3.4. Antimalarial properties of crude extracts and compound isolated from the rhizome of *C.tinctorium* using *P.falciparium in vitro* culture.

Plasmodium falciparium was successfully cultured *in vitro* using a modification of the method of Jensen and Trager [62] and growth inhibition of *P falciparium* was used to study the antimalarial activity of the extracts and isolated compounds from the rhizome of *C.tinctorium*.

Growth Inhibition of *P.falciparium* was expressed as

$$\text{Percentage Inhibition} = \frac{C\,(\text{control}) - C\,(\text{test})}{C\,(\text{control})} \times 100$$

Where

C (control) = Count per minute of beta particles (CPM) for control wells (average)

C (test) = Counts per minute of beta particles (CPM) for wells with plant extracts/isolated compounds (average)

Graphs of standard chloroquine, various crude extracts and the isolated compounds are shown below

Fig.1: Growth inhibition of *P falciparum* (3D7) by chloroquine

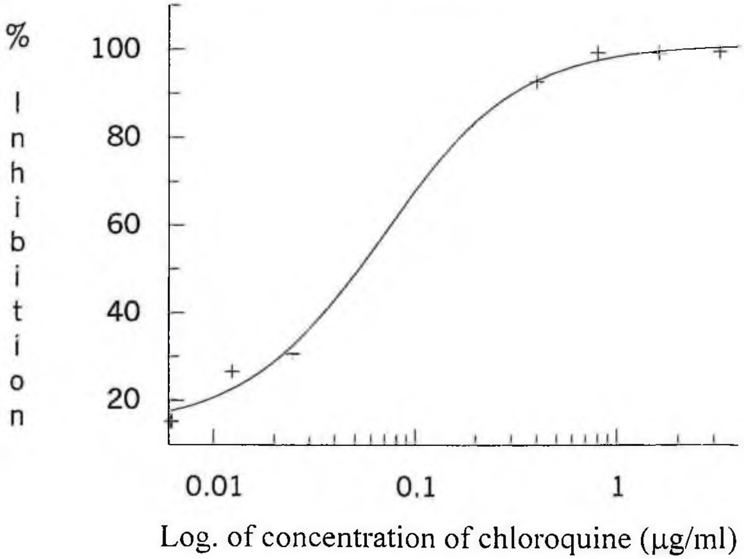


Fig.2: Growth inhibition of *P. falciparum* (DD_2) by chloroquine

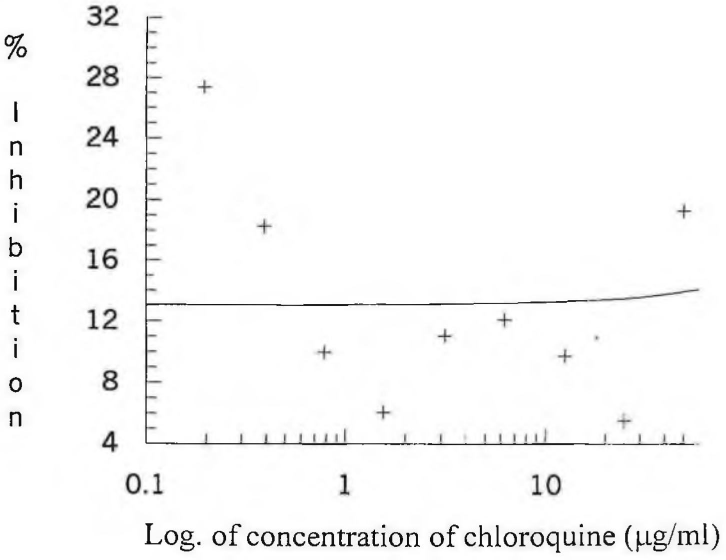


Fig.3: Growth inhibition of *P falciparum* (3D7) by Petroleum ether extract of *C.tinctorium*.

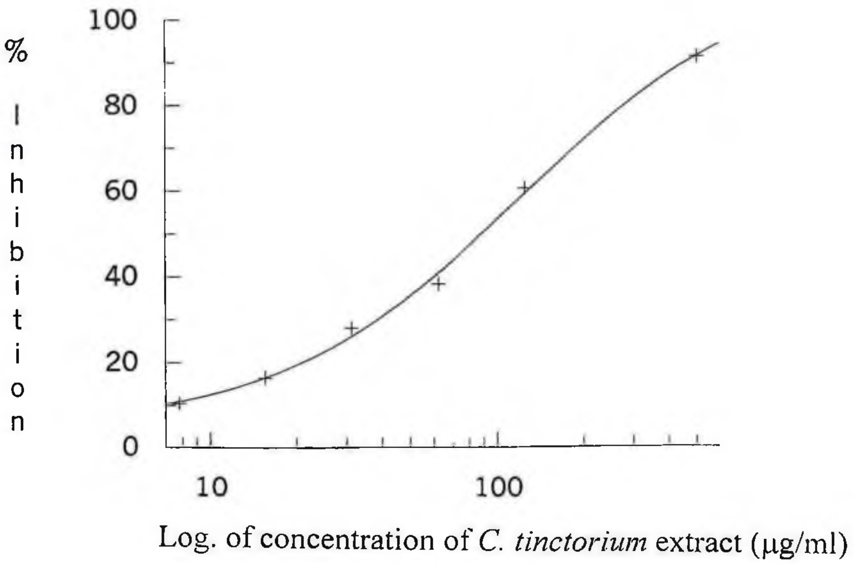


Fig.4: Growth inhibition of *P. falciparum* (DD$_2$) by Petroleum ether extract of *C. tinctorium*.

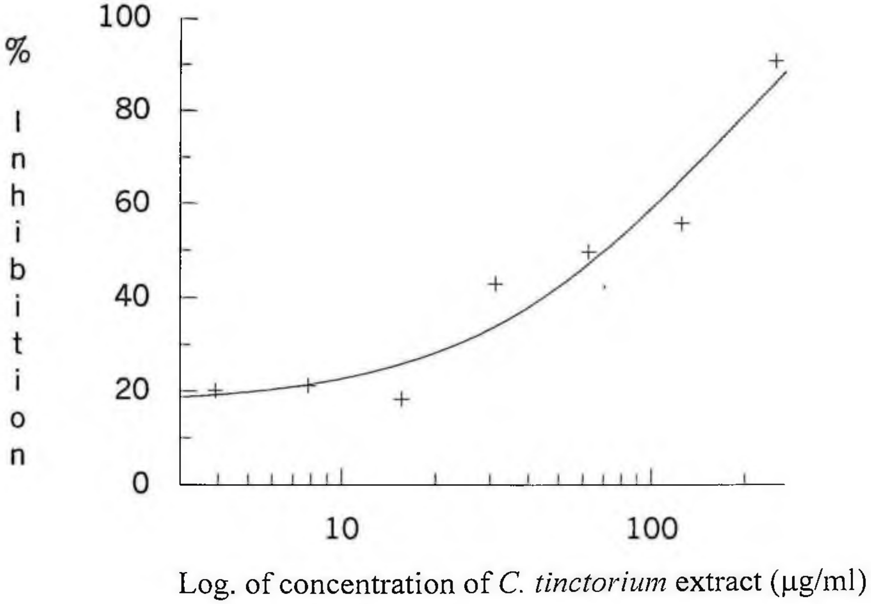


Fig.5: Growth inhibition of *P falciparum* (3D7) by Dichloromethane extract of *C. tinctorium*.

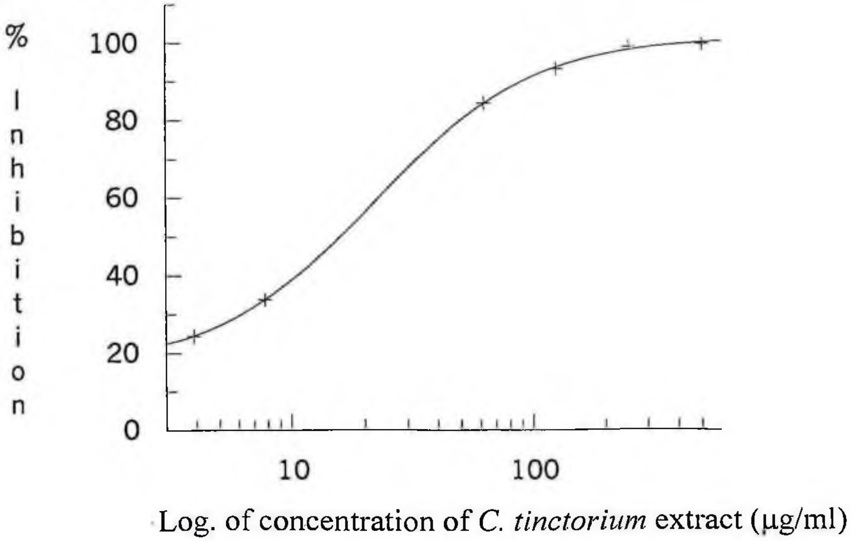


Fig.6: Growth inhibition of *P falciparum* (DD$_2$) by Dichloromethane extract of *C. tinctorium*.

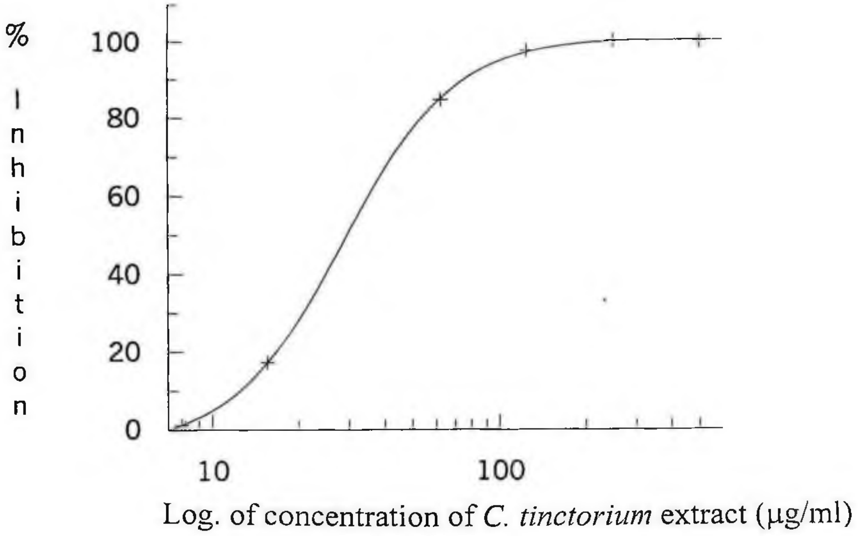


Fig.7: Growth inhibition of *P falciparum* (3D7) by Ethyl Acetate extract of *C. tinctorium*.

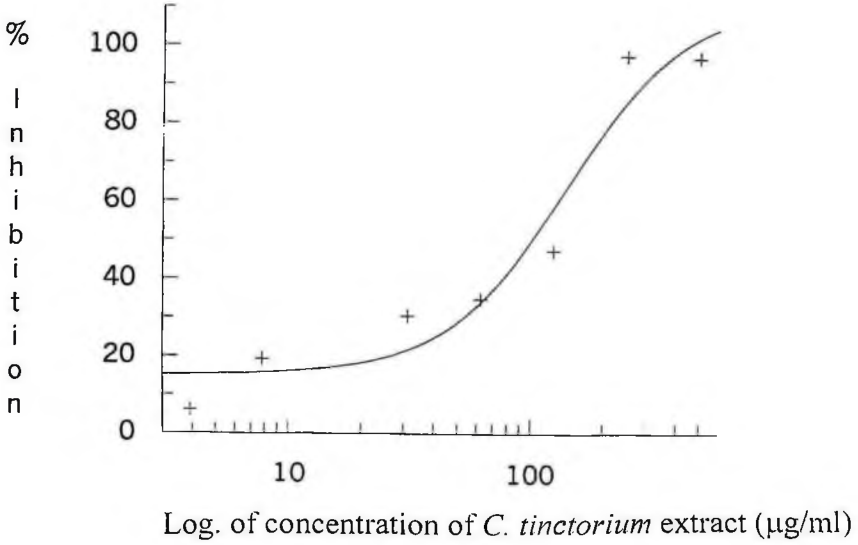


Fig.8: Growth inhibition of *P. falciparum* (DD_2) by Ethyl Acetate extract of *C. tinctorium*

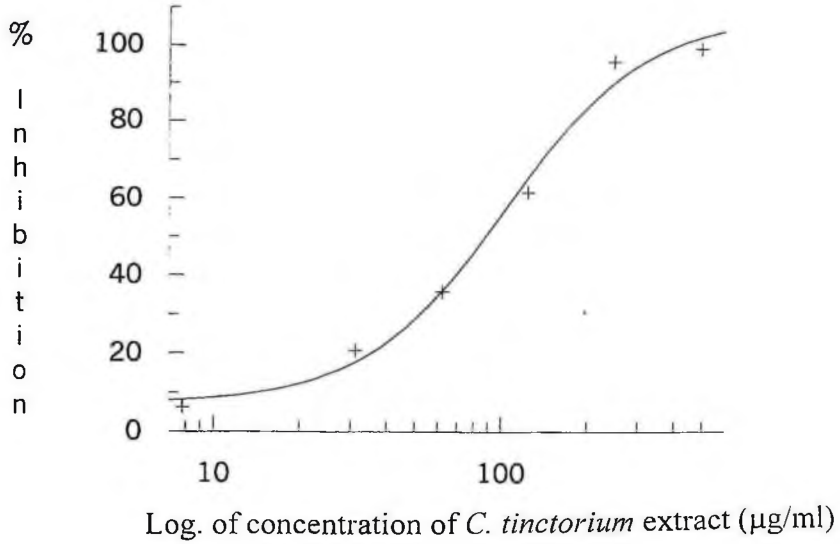


Fig.9: Growth inhibition of *P. falciparum* (3D7) by Ethanol extract of *C. tinctorium*.

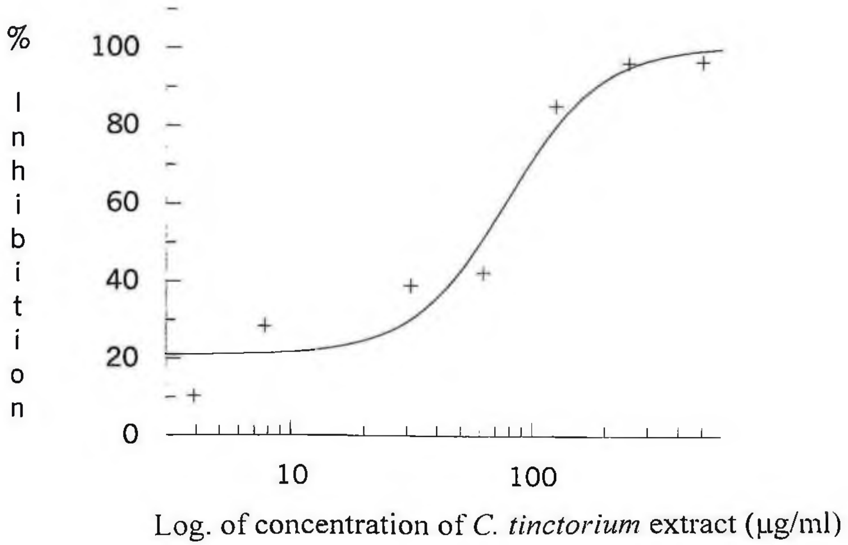


Fig.10: Growth inhibition of *P falciparum (*DD$_2$) by Ethanol extract of *C. tinctorium.*

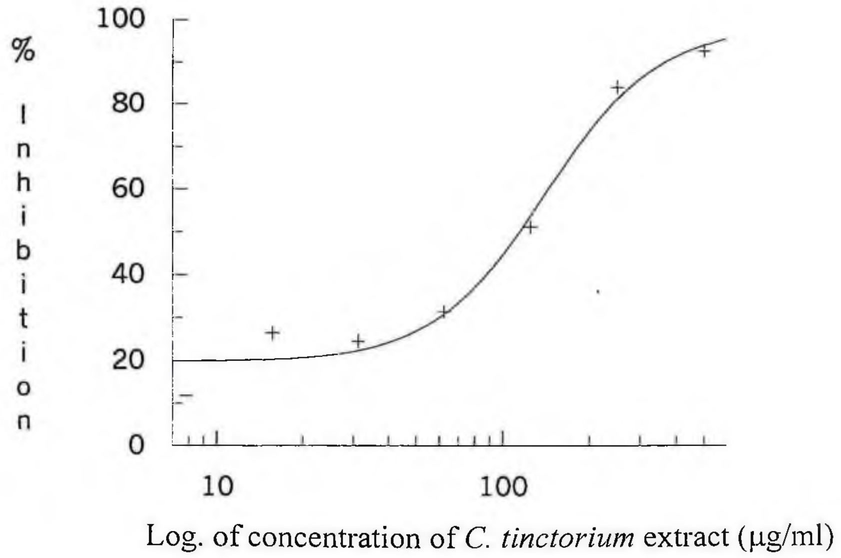


Fig.11: Growth inhibition of *P. falciparum* (3D7) by Water extract of *C. tinctorium.*

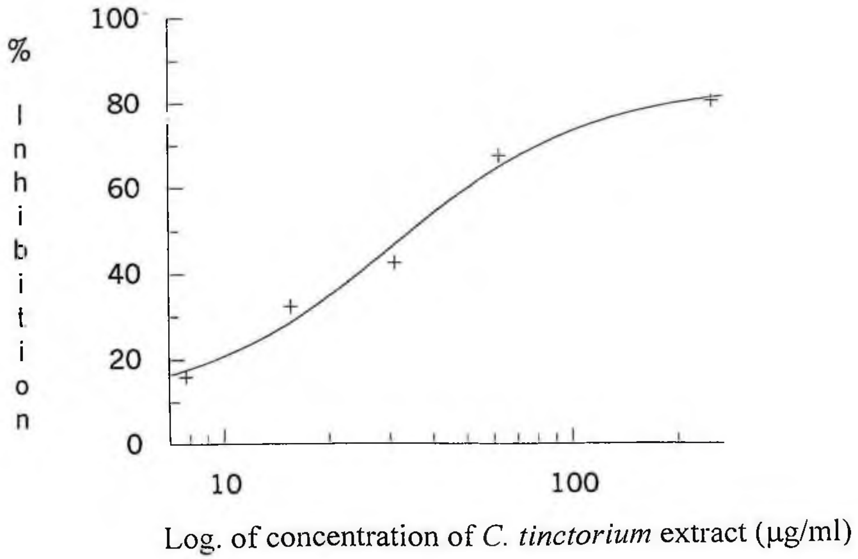


Fig.12: Growth inhibition of *P falciparum* (DD_2) by Water extract of *C. tinctorium*.

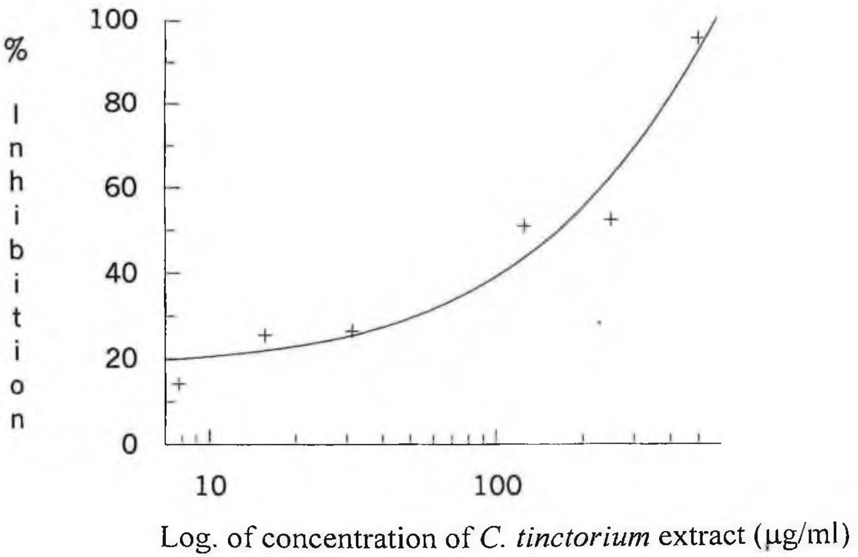


Fig.13: Growth inhibition of *P falciparum* (3D7) by triacontanol

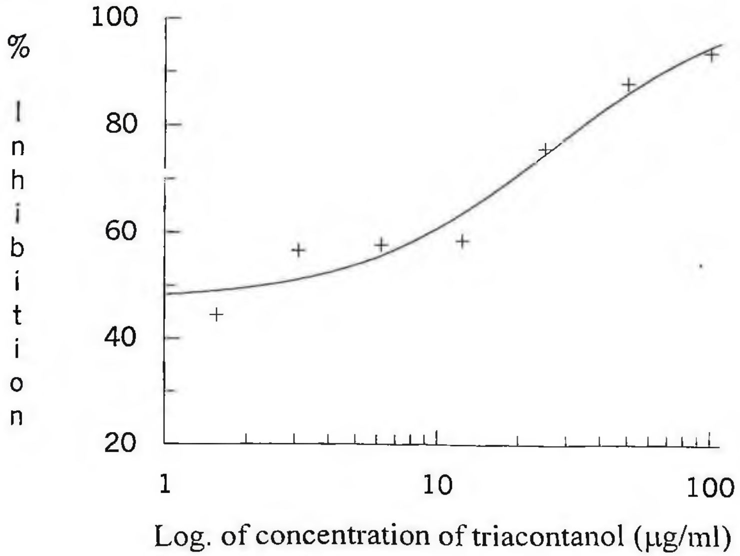


Fig. 14: Growth inhibition of *P falciparum* (3D7) by Fraction PE6

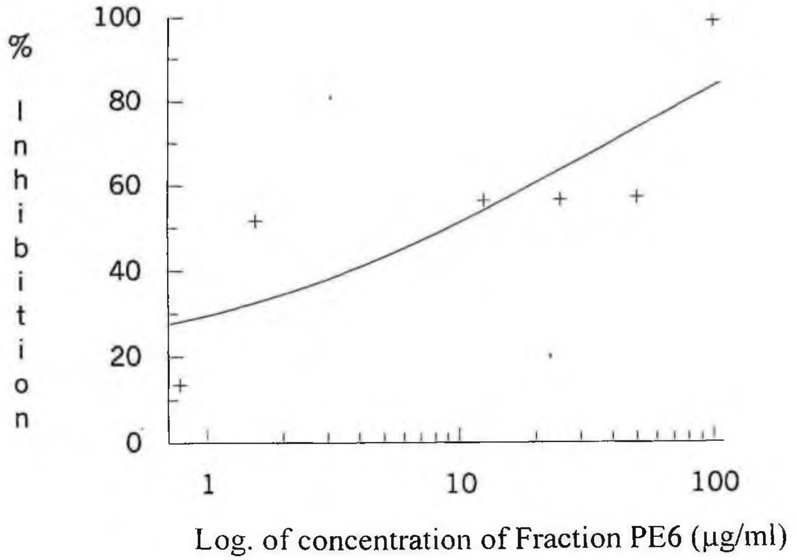


Fig. 15: Growth inhibition of *P. falciparum* (3D7) by Fraction PE8

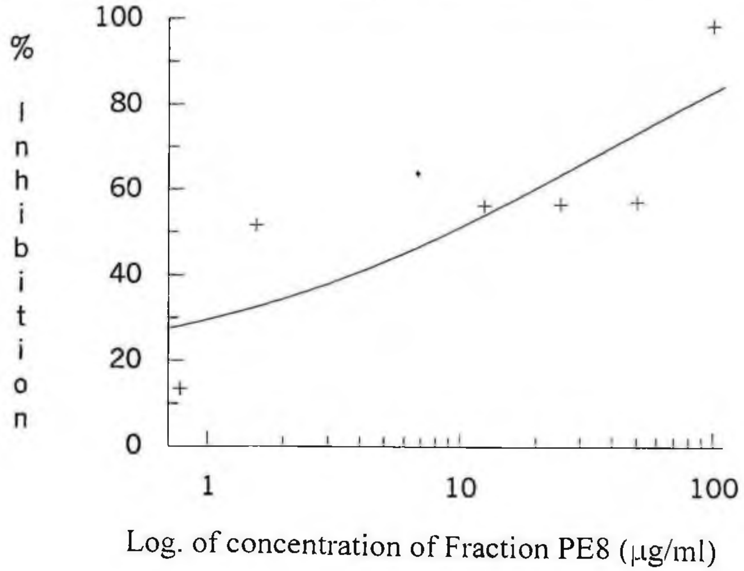


Fig. 16: Growth inhibition of *P falciparum* (3D7) by Fraction D9

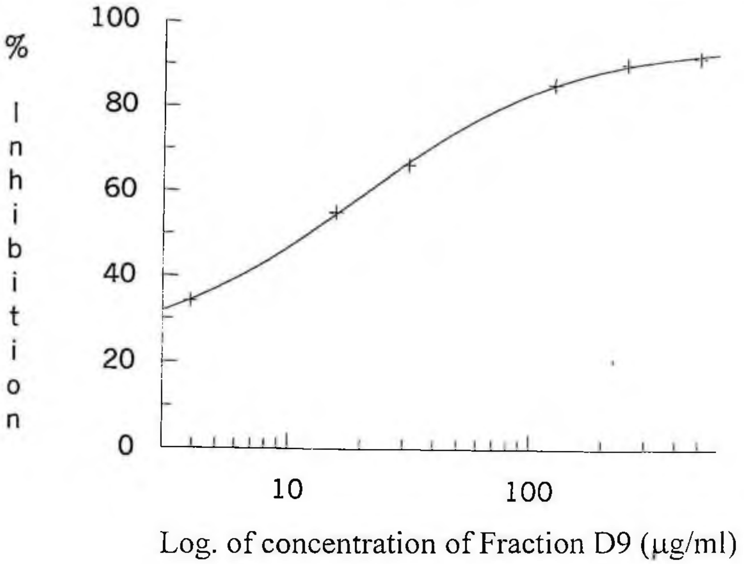


Fig. 17: Growth inhibition of *P falciparum* (3D7) by Fraction E13

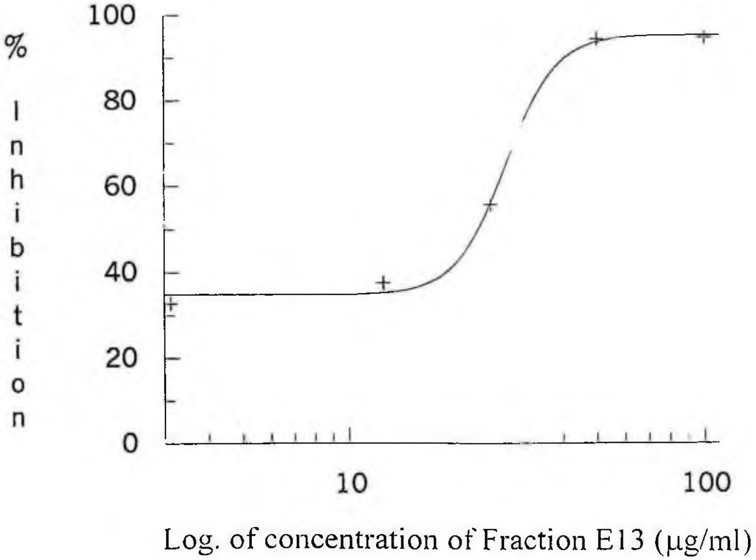
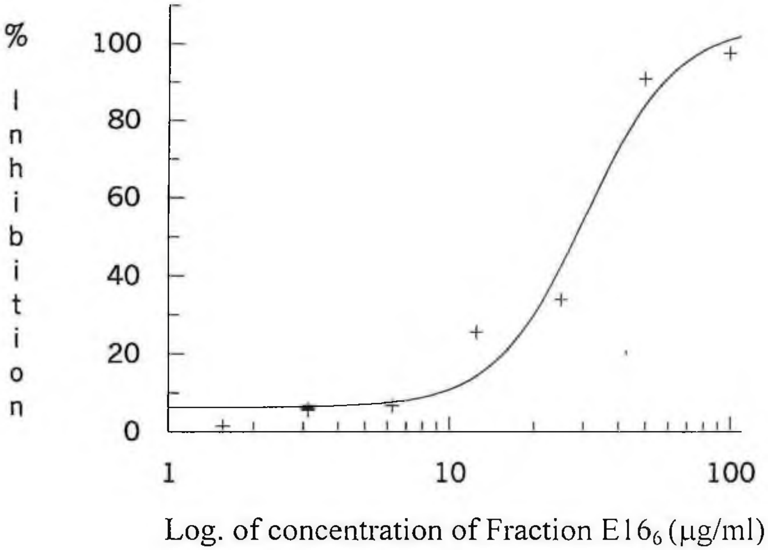


Fig. 18: Growth inhibition of *P falciparum* (3D7) by Fraction E16$_6$



Five different solvent extracts of the dried powdered rhizome of *Cochlospermum tinctorium* i.e. petroleum ether, dichloromethane, ethyl acetate, ethanol, water and six isolated compounds were tested for their *in vitro* antimalarial activity by assessing their ability to inhibit the uptake of ^{3}H-hypoxanthine by *P falciparum* (chloroquine sensitive strain, 3D7 and chloroquine resistant strain, DD_2) cultured in human red blood cells (O Rh^+) with chloroquine as a standard drug. The solvents used were selected so as to obtain extracts, which contain all possible active ingredients. The method of extraction, cold percolation, afforded maximum extraction while the potential activity of the active components remained unaffected by heat.

Seven different concentrations, each for the five extracts and the isolated compounds were used to determine the percentage inhibition and to calculate the 50% inhibitory concentration (IC_{50}). The assay for the crude extracts was replicated three times and that for the isolated compounds was replicated two times. The IC_{50} values were calculated from the average values. For the isolated compounds the activity was determined on only the chloroquine sensitive strain (3D7) of the *P. falciparum* because the percentage parasitaemia of the chloroquine resistant strain (DD_2) was not very high at the time of the determination.

The inhibition of growth *in vitro* of *P. falciparum* by all the extracts and isolated compounds were also found to be dose-dependent. These are shown in **Table 23.**

Table 23: Fifty- percent inhibitory concentrations (IC_{50}) of *Cochlospermum tinctorium* rhizome extracts and isolated compounds against *plasmodium falciparum in vitro.*

Extracts/isolated compounds	**Chloroquine-sensitive, 3D7 strain (µg/ml)**	**Chloroquine-resistant, DD_2 strain (µg/ml)**
Petroleum ether	148.18	239.27
Dichloromethane	24.34	23.45
Ethyl Acetate	108.94	127.23
Ethanol	57.02	167.86
Water	32.27	30.90
Triacontanol	26.84	--
Triacontanyl ferulate	>> 100	--
Triacontanyl p-coumarate	>> 100	--
PE6	22.15	--
PE8	62.4	--
PE11	>>100	--
D9	21.58	--
E13	27.7	--
$E16_6$	30.72	--

The dichloromethane extract exhibited the highest activity with IC_{50} values 24.34µg/ml and 23.45µg/ml for the 3D7 strain and DD_2 strain respectively. The water extract was also active against parasite growth of the 3D7 strain and DD_2 strain of *P. falciparum* with an IC_{50} values of 32.27µg/ml and 30.90µg/ml respectively. The IC_{50} values of the 3D7 strain and DD_2 strain for the ethyl acetate extract were 108.94µg/ml and 127.23µg/ml respectively. The ethanol extract at 57.02μg/ml and 167.86μg/ml was the 50% inhibitory concentration for the 3D7 and DD_2 strains of *P. falciparum* respectively. The petroleum ether extract exhibited the lowest activity with IC_{50} values 148.18μg/ml and 239.27μg/ml for *P. falciparum* strains, 3D7 and DD_2 respectively. For the isolated compounds, triacontanol, PE6, PE8, $E16_6$, E13 and D9 gave IC_{50} values of 26.84μg/ml, 22.15μg/ml, 62.4μg/ml, 30.72μg/ml, 27.7μg/ml and 21.58μg/ml respectively. The other compounds gave IC_{50} values greater than 100μg/ml.

A standard antimalarial, chloroquine, gave an IC_{50} value of 0.06μg/ml for the chloroquine sensitive strain (3D7) of the *P. falciparum*. Thus, all the extracts and the isolated compounds showed moderate antimalarial activity against *P. falciparum in vitro.*

The media used in the *in vitro* work usually contains no hypoxanthine. Hence when the radiolabelled hypoxanthine (^{3}H-hypoxanthine) is added, the parasites rapidly pick it up. Hypoxanthine is capable of crossing the malaria parasite membrane, unlike thymidine[68]. The hypoxanthine ultimately is incorporated into both the ribonucleic acid and deoxyribonucleic acid. This therefore provides a reasonably broad index of parasite metabolism. Only actively growing parasites are able to pick up the labelled marker. Parasites whose growth has been inhibited by the presence of the extract or isolated compounds do not pick up the labeled compound. Thus, the inhibition of uptake of ^{3}H-hypoxanthine is directly linked to the inhibition of growth the *P. falciparum* parasites.

Research has shown that some plant extracts exert their diverse effects in a synergistic fashion in which active constituents present in very small amounts act in concert. Some of these constituents may be novel compounds, each with a different mode of action on the *plasmodium* parasites. Hence, though they may be present in very small quantities the total effect of all these active constituents, each with its unique mode of action overwhelms the parasite, thus destroying it. This is confirmed in the dichloromethane extract that is very active as a whole but some of its isolated compounds (triacontanol and D9) being very active while triacontanyl p-coumerate and triacontanyl ferulate not very active. In the case of the petroleum ether extract the crude extract is least active, but PE8 which was isolated from it is slightly active with IC_{50} value of 62.4µg/ml.

This work has shown that the ethanol and water extracts of the rhizome of *C. tinctorium* has a greater inhibition of *P. falciparum* (3D7 strain) growth. This is an important point for us to do further work on it by isolating more compounds from them and testing their activity. It will be possible to get many effective antimalarials. The dichloromethane extract was able to inhibit equally, both the chloroquine-resisitant and sensitive strains of *P. falciparum* (3D7 and DD_2), indicating that the mechanism of action could be different from that of chloroquine, making it a potential for the treatment of chloroquine-resistant malaria, which is common in malaria endemic population such as ours.

The IC_{50} values which we obtained from the crude extracts and the isolated compounds were lower than that reported for extracts of a plant like *Azadirachta indica* (neem) IC_{50} = 0.05-0.4mg/ml[69], but higher than *Artemisia annua*, IC_{50} = 3.9µg/ml[70]

Toxicity of the isolated compounds could be a problem, and this needs to be addressed in *in vivo* studies, but the frequent use of *C. tinctorium* extracts in traditional practice is hopeful. There are a lot of constraints to the *in vitro* cultivation of *P. falciparum*. These include the high cost of ^{3}H-hypoxanthine, normal human serum (that is, serum without antibodies reactive to malaria parasites) and the gas for the culturing. In the case of the gas, a candle jar was used as a substitute, but this produced some problems such as production of too much smoke.

It is being suggested that in subsequent work on this plant, the parasites used for the assay need to be synchronized so that the testing is performed on each of the stages (that is, rings and schizonts).

CHAPTER FOUR

EXPERIMENTAL

4.1.GENERAL METHODS

Extractions of plant material with organic solvents and water were done using cold percolation. The organic extracts were concentrated and dried with a rotary evaporator while the water extracts were freeze-dried. Column chromatography and recrystallization were used in the isolation and purification of the various components of the crude extracts of petroleum ether, dichloromethane and ethyl acetate.

All analytical thin layer chromatography (TLC) was carried out with aluminum foil slides pre-coated with silica gel (thickness 0.2 mm) type Kiesegel $60F_{254}$ Merck. This was used to monitor the progress of the column and ascertain the purity of the components. Column chromatography was carried out using silica gel (fluka) $60F_{254}$ with particle size 0.063-0.200 mm as the main adsorbent, used in the ratio 20-50:1 g depending on complexion of the mixture being separated. As much as possible solvents used were of analar grade and where analar grade was not available, the solvent was distilled.

The solvents used included petroleum ether (40-60 ^{0}C), ethyl acetate dichloromethane and chloroform. Ratio of solvents mixtures are given by volumes. For example, petroleum ether/ethyl acetate (2:1) means 2 parts by volume of petroleum ether to 1 part by volume of ethyl acetate. Various solvent systems were prepared and transferred into developing tanks and left to stand for 20 minutes. This was to allow the solvent mixture to evaporate and saturate the developing chamber before running the chromatograms.

Visualization of spots on TLC was under ultra violet (UV) lamp or in iodine chamber or with anisaldehyde spray followed by heating. Melting points of pure compounds were determined using Gallenkamp melting point apparatus. Infrared (IR) spectra were recorded as either nujol mulls or dispersions in potassium bromide disc on either Shimadzu IR-408 or FI/IR-410-Jasco Spectrophotometer. Abbreviations used in describing IR signals includes s = strong; m = medium; w = weak; b = broad and sh = sharp. Ultraviolet (UV) spectra were recorded using Shimadzu UV-visible recording Spectrophotometer (UV-240).

NMR spectra were recorded in deuterated chloroform ($CDCl_3$) with tetramethyl silane (TMS) as internal reference on a Varian Gemini-2000 Spectrophotometer at a frequency of 300 MHz for ^1H and 75 MHz for ^{13}C. Chemical shifts (δ), are expressed in parts per million (ppm). Abbreviations used in interpreting the ^1H-NMR spectra include s = singlet d = doublet, t = triplet, q = quartet, m = multiplet and b = broad. Mass spectra (ms) were run on a Jeol AX505W Mass spectrometer.

Antimalarial properties of the various crude extracts and isolated samples of the rhizome of *C. tinctorium* using *Plasmodium falciparum in vitro* culture was preformed. A modification of the method of Jensen and Trager[62] was used for the bioassay. Growth inhibition of *P. falciparum* was determined and the IC_{50} obtained for the test materials.

4.2. Collection and Treatment of Plant Material

The rhizomes of *Cochlospermum tinctorium* were collected from the Brong Ahafo Region (9 km from Drobo towards Sampa) on the 7th December 1999. They were chopped into pieces, air dried in the shade for about two weeks and pulverised into a fine powder. A voucher specimen has been deposited at the Ghana Herbarium of the Department of Botany, University of Ghana, Legon under acquisition number GC 47680

4.3. REAGENTS

4.3.1. Wagner's reagent[71]: Potassium iodide (2.0 g) and iodine (1.27 g) were together dissolved in 20 ml distilled water in a volumetric flask and the solution made up to 100 ml with distilled water. The presence of alkaloids is indicated by the formation of large brown amorphous and flocculent precipitate after treating the hydrochloric acid solutions of the extracts with a few drops of the reagent.

4.3.2. Meyer's reagent: [71] Mercuric iodide (1.36 g) was dissolved in distilled water (60 ml) and the resulting solution added to the one obtained by dissolving potassium iodide (5 g) in distilled water (10 ml). The resulting solution was made up to 100 ml with distilled water. The formation of a pale yellow precipitate when a few drops of the reagent are added to the test solution is indicative of the presence of alkaloids.

4.3.3. Dragendorff's reagent: [71] Hydrated Bismuth nitrate (8 g) was dissolved in concentrated nitric acid (20 ml) and the resulting solution is slowly added to a solution of potassium iodide (27.2 g) in distilled water (50 ml) with stirring. Crystalline potassium nitrate which precipitates out was filtered off and the filtrate made to 100 ml with distilled water before being used as a reagent for testing for alkaloids. In the test, the solution of the extract is made distinctly acidic with sulphuric acid and completely freed of any ethanol in which the alkaloid precipitates are soluble. Two or three drops of the reagent are used since some of the alkaloid precipitates are soluble in excess reagent. Formation of brown precipitate is indication of presence of alkaloids.

4.3.4. Anisaldehyde Spray reagent; [72] Concentrated sulphuric acid (1 ml) was added to glacial acetic acid (50 ml). To the mixture was added anisaldehyde (0.5 ml). The reagent was always prepared fresh and used as a spray. The sprayed TLC plates are heated to 110 ^{0}C for 10 minutes before visualization in visible light. Most organic compound will stain as blue, violet, green, pink, yellow, red, cream and gray on a light pink background.

4.3.5 Liebermann-Buchard Reagent: [73] Redistilled acetic anhydride (5 ml) was cautiously mixed while cooling with concentrated sulphuric acid (5cm^3). The resulting mixture was again slowly added, also while cooling in ice, to absolute ethanol (50 ml). As a positive indication for the presence of terpenoids in an extract, this reagent when sprayed onto a TLC plate will produce pink-to-red spots after development followed by heating in an oven at a temperature of 110 ^{0}C for 15 minutes and then examination under U.V. lamp.

4.3.6. Iron (III) Chloride solution; [74] Iron (III) chloride solution (100 ml) was prepared by mixing iron (III) chloride (10 g) and distilled water (90 ml). Two to three drops of the solution were added to 3 ml a test solution. The presence of catechol-type tannins was indicated by the formation of a greenish-blue colouration.

4.4 EXTRACTION

Three different solvent extracts of the rhizome of *C. tinctorium* were made namely, petroleum ether extract, dichloromethane extract and ethyl acetate extract.

4.4.1. Petroleum ether extract of the rhizome

1.0 kg of the shade-dried pulverized plant material was extracted continuously for 24 hours with 7.5 litres of petroleum ether (40-60 ^{0}C) using cold percolation. After 24 hour, the solution was drained off and the solvent recovered. The process was repeated for 24 hours again using the previously recovered solvent. All the extracts were finally combined and then evaporated to dryness using the rotary vacuum evaporator. A yellowish-brown viscous residue (20.54 g) was obtained. TLC of this extract developed in petroleum ether/ethyl acetate (9:1) gave about 11 spots with anisaldehyde reagent. The yellowish-brown residue was stored in a deep-freezer until it was required.

4.4.2. Dichloromethane extract of the rhizome

After the petroleum ether extraction the plant material was dried and then soaked with 6 litres of dichloromethane in a percolator. After 24 hours, the solution was drained off and the solvent recovered on the rotary vacuum evaporator. This extraction was repeated once again for 24 hours. The two extracts were then combined and the solvent evaporated completely to obtain a yellowish residue (15.16 g). This was stored in a freezer. TLC of this extract developed in petroleum ether/ethyl acetate (9:1) gave about 12 spots with anisaldehyde spray.

4.4.3. Ethyl acetate extract of the rhizome

The plant material used for the dichloromethane extraction was dried and soaked with 6 litres of ethyl acetate for 24 hours. The same procedure used for the dichloromethane was followed. 10.29 g of extract was obtained. The TLC of this extract showed about 9 spots when developed in petroleum ether/ethyl acetate (9:1). The extract was stored in a deep-freezer until it was required.

4.5 PHYTOCHEMICAL SCREENING

Five extracts namely petroleum ether, dichloromethane, ethyl acetate, ethanol and water (crude) were each subjected to a series of phytochemical screening test as follows.

4.5.1 Test for alkaloids:

About 0.1g of each of the crude extracts in separate test tubes was added to 2M HCL solution (5 ml). This was stirred, warmed and filtered. The filtrate from each extract was then divided into three test tubes. To one portion of each test solution was added Dragendorff's reagent, to another portion, Meyer's reagent and to the remaining portion, Wagner's and observed. The formation of a yellowish or reddish brown precipitate in the test tubes indicated the presence of alkaloids.

4.5.2. Test for flavonoids and leucoanthocyanins.

To 0.1 g of each extracts was dissolved in 15 ml of 80% ethanol. The resulting solution was filtered and the filtrate divided into two portions. To one portion was added magnesium turnings followed by concentrated hydrochloric acid (0.5 ml) and observed for colour changes within 10 minutes. To the other portion was added concentrated hydrochloric acid (0.5 ml) and then warmed for about 5 minutes. The presence of any development of light pink colour in both extracts is an indication of the presence of flavonoids and leucoanthocyanins.

4.5.3. Salkowski test for cardiac glycosides:

To 0.5 g of each extract was dissolved in chloroform (2 ml) in a test tube. Concentrated sulphuric acid was carefully added down the side of the test tube to form a lower layer. Formation of a reddish brown colour at interface indicates the presence of the aglycone portion of cardiac glycoside.

4.5.4. Test for tannins:

To 0.5 g of each extract were dissolved in 80% of aqueous methanol (10 ml) and the solution divided into two portions. To one portion was added freshly prepared iron (III) chloride solution and to the other portion nothing was added to serve as a blank. The formation of a dark greenish-blue colouration on addition of iron (III) chloride signifies the presence of tannins.

4.5.5 Screening for saponins:

The presence of saponins in the extracts was evidenced by the fact that there was foaming when some amounts of the extracts were each shaken with water. The foams disappeared after the test tube had been set aside for about an hour.

4.5.6. Test for anthraquinones and anthracene derivatives

To 0.5 g of each extract was dissolved in 30 ml of distilled water and filtered. The filtrate was shaken-up with benzene (10 ml) in a separatory funnel and allowed standing. The benzene layer was transferred into a test tube and shaken with 2M ammonia solution (5 ml). The absence of a red colour in the ammonia layer was indicative of the absence of anthraquinones and anthracene derivatives in both extracts.

4.6. INVESTIGATION OF EXTRACT

4.6.1. Petroleum ether extract of the rhizome of *C. tinctorium*

The crude extract (20g) was introduced onto a column packed with silica gel (500 g). Elution was started with petroleum ether followed by petroleum ether/ ethyl acetate mixtures with increasing proportions of the ethyl acetate until 100% of ethyl acetate was used. This was followed by ethyl acetate/ ethanol mixture with increasing proportions of ethanol until 100% of ethanol was used. 20ml portions of the eluate were collected and monitored by TLC. Based on the TLC results the various fractions were combined into 13 main fractions labelled PE1, PE2, PE3, ...PE13. The fractional yields are shown in **Table 24**.

Table 24: Fractional yields of Petroleum ether extracts

Fraction	State	Colour	Yield/g
PE1	Oil	Colourless	0.44
PE2	Oil	Yellow	1.54
PE3	Oil	Yellow	0.87
PE4	Oil	Yellow	1.03
PE5	Oil and Solid	Yellow	0.97
PE6	Oil and Solid	Yellow	1.73
PE7	Oil and Solid	Yellow	0.98
PE8	Oil and Solid	Yellow	1.54
PE9	Syrup	Yellowish brown	1.06
PE10	Syrup	Yellowish brown	1.75
PE11	Oily semi-solid	Yellowish brown	1.02
PE12	Oily semi-solid	Dark brown	1.85
PE13	Oily semi-solid	Dark brown	3.05

4.6.1.1. Isolation of PE6

This fraction was kept in a freezer for 24 hours. It precipitated a white solid. The solid was filtered out and washed with cold petroleum ether. The TLC gave a single spot when an ansialdhyde spray was used. The white flaky solid (400mg) was labelled PE6 (Mpt 70-72 °C). The R_f values of sample PE6 in a mixture of petroleum ether/ethyl acetate (10:1) and (8:1) was 0.75 and 0.86 respectively.

The IR spectrum (Appendix Ia) of this compound showed peaks at 3425, 2956, 2915, 2851, 1699, 1666, 1467, 1377, 1266, 1133, 966 and 722 cm^{-1}. The ^{1}H-NMR spectrum (Appendices IIa, IIb and IIc) gave peaks at δ(ppm) 0.91(t), 1.30(bs), 1.60(s), 1.96(t, t), 2.25(m), 2.42(t, t), 2.58(d), 2.65(t), 2.91(d), 5.00(s) and 6.80(s).

In the ^{13}C-NMR spectrum (Appendix IIIa) signals appeared at δ(ppm) 13.98, 22.56, 23.49, 24.41, 26.63, 29.22, 29.38, 29.51, 29.54, 31.80, 34.92, 37.20, 40.54, 51.33, 62.61, 76.54, 76.97, 77.39, 139.09, 147.29 and 211.53.

4.6.1.2. Isolation of PE8

Fraction PE8 was kept in a deep-freezer for 24 hours. It precipitated a white solid. The solid (341 mg) was filtered out and washed with cold petroleum ether. The TLC gave two spots when anisaldehyde spray was used. The solid was recrystallized in petroleum ether and a white solid (90 mg) was obtained after it was filtered out and washed with cold petroleum ether. The TLC gave a single spot when an anisaldehyde reagent was used. The solid was labelled as PE8. The solid melted at 123-125 ^{0}C.

The R_f values of sample PE8 in a mixture of petroleum ether/ ethyl acetate are shown in **Table 25**.

Table 25: R_f values of PE8

Solvents System	R_f
Petroleum ether/ethyl acetate (8:1)	0.41
Petroleum ether/ethyl acetate (12:1)	0.25

The IR spectrum (Appendix Ib) gave peaks at 3510, 2955, 2920, 2850, 1725, 1640, 1604, 1519, 1463, 1376 and 1176 cm^{-1}

4.6.1.3. Isolation of PE11

Fraction PE11 was also kept in a freezer for 15 hours. It precipitated a yellowish solid. The solid (80.1 mg) was filtered out cold and washed cold petroleum ether. The TLC gave a single yellowish blue spot when viewed under an UV lamp and when an anisaldehyde reagent was used. The solid was labelled as PE11 and its melting point was 45-47 ^{0}C. The R_f values of the sample PE11 in a mixture of petroleum ether/ethyl acetate (8:1) and (3:1) were 0.19 and 0.85 respectively. The IR spectrum (Appendix Ic) of this compound showed peaks at 3255, 3184, 3061, 2957, 2920, 2852, 1609, 1509, 1468, 1176, 1084 and 721 cm^{-1}

The UV spectrum (Appendix Vc) showed maxima ($CHCl_3$) at 272 and 367 nm. The ^{1}H-NMR spectrum (Appendices IId, IIe and IIf) produced prominent signals at δ(ppm) = 7.30(d), 7.05(m), 4.16(t), 2.55(m), 2.00(m), 1.60(m), 1.33(bs) and 0.90(t). The two dimensional (2D)-NMR, COSY (Appendix VIa) gave a coupling of 1.60ppm to 1.33ppm, 1.33ppm to 2.00ppm, 1.33ppm to 0.90ppm, 1.33ppm to 4.16ppm, 1.60ppm to 2.55ppm and 7.05 to 7.30ppm.

In the ^{13}C-NMR spectrum (Appendices IIIb and IIIc) signals appeared at δ(ppm) 14.16, 22.75, 24.87, 25.64, 29.05, 29.43, 29.59, 29.67, 29.76, 29.81, 30.03, 31.99, 35.87, 37.20, 76.74, 77.17, 77.59, 118.12, 135.51 and 199.25.

4.6.2. Dichloromethane extract of the rhizome of *C.tinctorium*

The crude extract (10 g) was absorbed onto silica gel (20 g) and introduced onto a column packed with silica gel (500 g). Elution was started with petroleum ether followed by petroleum ether/ethyl acetate mixture with increasing proportions of the ethyl acetate until 100% of the ethyl acetate was used. Ethyl acetate/ethanol mixtures with increasing proportions of ethanol until 100% of ethanol was used followed this. 20ml portion of the elutes were collected and monitored by TLC. Based on the TLC results the various fractions were combined into 12 main fraction labelled D1, D2, D3...D12. The fractional yields are shown in **Table 26**

Table 26 Fractional yields of Dichloromethane extracts

Fraction	State	Colour	Yield/g
D1	Oil	Yellowish	0.25
D2	Oil	Yellowish	0.42
D3	Syrup	Yellowish	0.37
D4	Syrup	Yellowish	0.53
D5	Oil and Solid	Yellowish	0.41
D6	Oil and Solid	Yellowish	0.57
D7	Oil and Solid	Yellowish	0.35
D8	Oil and Solid	Yellowish	0.32
D9	Oil and Solid	Yellowish-brown	0.25
D10	Oil and Solid	Yellowish-brown	0.44
D11	Oil and Solid	Dark brown	1.33
D12	Oil and Solid	Dark brown	3.04

4.6.2. Dichloromethane extract of the rhizome of *C.tinctorium*

The crude extract (10 g) was absorbed onto silica gel (20 g) and introduced onto a column packed with silica gel (500 g). Elution was started with petroleum ether followed by petroleum ether/ethyl acetate mixture with increasing proportions of the ethyl acetate until 100% of the ethyl acetate was used. Ethyl acetate/ethanol mixtures with increasing proportions of ethanol until 100% of ethanol was used followed this. 20ml portion of the elutes were collected and monitored by TLC. Based on the TLC results the various fractions were combined into 12 main fraction labelled D1, D2, D3...D12. The fractional yields are shown in **Table 26**

Table 26 Fractional yields of Dichloromethane extracts

Fraction	State	Colour	Yield/g
D1	Oil	Yellowish	0.25
D2	Oil	Yellowish	0.42
D3	Syrup	Yellowish	0.37
D4	Syrup	Yellowish	0.53
D5	Oil and Solid	Yellowish	0.41
D6	Oil and Solid	Yellowish	0.57
D7	Oil and Solid	Yellowish	0.35
D8	Oil and Solid	Yellowish	0.32
D9	Oil and Solid	Yellowish-brown	0.25
D10	Oil and Solid	Yellowish-brown	0.44
D11	Oil and Solid	Dark brown	1.33
D12	Oil and Solid	Dark brown	3.04

4.6.2.1. Isolation of D5

Fraction D5 was kept in a freezer for 18 hours. It precipitated a white light solid. (72 mg) was filtered out and washed with cold petroleum ether. The TLC gave a single spot when an ansialdehyde reagent was used. The solid was labelled D5 (Mpt. 83-85 ^{0}C). The R_f values of sample D5 in a mixture of petroleum ether/ethyl acetate are shown in **Table 27.** It was however UV inactive, that is, the spot did not fluoresce under UV light.

Table 27 R_f values of D5

Solvents System	**R_f**
Petroleum ether/ethyl acetate (8:1)	0.60
Petroleum ether/ethyl acetate (9:1)	0.56
Petroleum ether/ethyl acetate (10:1)	0.44

The IR spectrum (Appendix Id) of D5 gave peaks at 3350, 2917, 2849, 1463, 1061 and 721 cm^{-1} The ^{1}H-NMR spectrum (Appendices IIg, IIh and IIi) produced prominent signals at δ(ppm)= 3.64(t), 1.56(m), 1.25(s) and 0.85(t).

The Mass spectrum (Appendix IVa) showed a molecular ion peak at m/z 435 (1.0%) with other prominent peaks occurring at m/z 420 (30%), 418 (4%), 392 (16%), 364 (5%), 350 (3%), 336 (4%), 321 (4%), 307 (4%), 293 (5%), 265 (7%), 237 (8%), 223 (8%), 209 (10%), 195 (12%), 167 (17%), 153 (20%), 139 (35%), 125 (42%), 111 (65%), 83 (86%), 57 (75%), 55 (55%) and 43 (43%). The base peak appeared at m/z 97 (100%).

4.6.2.2. Isolation of $D6_5$

Fraction D6 deposited white solid (250 mg), which showed 4 spots on a TLC plate when sprayed with anisaldehyde reagent and baked in an oven. This solid was put on a column and eluted with a mixture of petroleum ether/ethyl acetate. Fractions collected were combined into 9 main fractions and labelled $D6_1$, $D6_2$, $D6_3$...$D6_9$. All the fractions were put into a freezer.

A solid was formed in fraction $D6_5$. The white solid was filtered and washed with cold petroleum ether. The TLC gave a single spot when an ansialdehyde reagent was used. The white solid was coded $D6_5$ (mpt 81-83 ^{0}C). The R_f values of sample $D6_5$ in a mixture of petroleum ether/ethyl acetate are shown in **Table 28**. It was however UV inactive.

Table 28 R_f values of $D6_5$

Solvent System	$\mathbf{R_f}$
Petroleum ether/ethyl acetate (10:1)	0.35
Petroleum ether/ethyl acetate (9:1)	0.46
Petroleum ether/ethyl acetate (8:1)	0.50

The IR spectrum (Appendix Ie) of $D6_5$ gave peaks at 3488, 3445, 2958, 2924, 2853, 1696, 1674, 1466, 1378 and 1063 cm^{-1}. The ^{1}H-NMR spectrum (Appendices IIj, IIk, III and IIm) contained signals at δ(ppm) = 7.62(d), 7.06(d, d), 6.92(d), 6.30(d), 5.85(s), 4.20(t), 3.93(s), 1.68(q), 1.58(s), 1.38(m), 1.25(s) and 0.88(t). The two dimensional (2D)-NMR, COSY (Appendix VIb) gave a coupling of 1.68ppm to 4.20ppm, 0.88ppm to 1.25ppm, 6.92ppm to 7.06ppm and 6.30ppm to 7.62ppm.

In the ^{13}C-NMR spectrum (Appendix IIId) signals appeared at δ(ppm) 13.97, 22.55, 25.86, 28.63, 29.18, 29.23, 29.57, 31.80, 55.84, 64.55, 70.04, 76.54, 76.96, 77.39, 109.25, 114.69, 115.70, 123.08 and 144.69.

The Mass spectrum (Appendix IVb) showed a molecular ion peak and a base peak at m/z 614 (100%). Other prominent peaks occurred at m/z 586 (27%), 558 (4%), 194 (34%), 177 (38%), 150 (12%), 137 (18%), 97 (5%), 83 (6%), 57 (9%) and 43 (7%).

4.6.2.3. Isolation of D6$_6$

Fraction D6$_6$ was found to have deposited a white solid. This was filtered and washed several times with cold petroleum ether. The solid (23 mg) on TLC in different solvent systems gave a single spot and melted at 79-81 ^{0}C. This was coded D6$_6$. The R$_f$ values of the sample D6$_6$ in a mixture of petroleum ether/ethyl acetate are shown in **Table 29**. It was however UV inactive.

Table 29: R$_f$ values of D6$_6$

Solvent system	R$_f$
Petroleum ether/ethyl acetate (10:1)	0.28
Petroleum ether/ethyl acetate (9:1)	0.40
Petroleum ether/ethyl acetate (8:1)	0.45

IR spectrum of D6$_6$ (Appendix If) was determined and gave peaks at 3488, 3445, 2958, 2924, 2853, 1696, 1674, 1466, 1378 and 1063 cm^{-1}. The ^{1}H-NMR spectrum (Appendix IIn and IIm) produced signals at δ(ppm)= 7.62(d), 7.44(d), 6.84(d), 6.30(d), 4.2(t), 3.93(s), 1.68(q), 1.58(s), 1.38(m), 1.25(s) and 0.88(t).

The mass spectrum (Appendix IVc) showed prominent peaks at m/z 584 (63%), 556 (23%), 528 (3%), 194 (8%), 147 (63%), 120 (32%), 107 (28%), 83 (13%), 57 (19%) and 43 (15%). The base peak appeared at m/z 164 (100%). Other prominent peaks occurring at m/z greater than 584 are probably due to contamination by sample $D6_5$. These are 614 (28%) and 616 (10%).

4.6.2.4. Isolation of D9

The orange solid particles in fraction D9 was filtered and washed several times with cold petroleum ether. The solid (29 mg) showed a single spot on a TLC plate sprayed with anisaldehyde reagent and heated in an oven at a temperature of 110 ^{0}C. This was labeled D9 and gave a melting point of 200-202 ^{0}C. The solid was UV active.

The R_f values of the sample D9 in a mixture of petroleum ether/ethyl acetate are shown in **Table 30** below.

Table 30: R_f values of D9

Solvent system	**R_f**
Petroleum ether/ethyl acetate (3:1)	0.35
Petroleum ether/ethyl acetate (5:2)	0.40

IR spectrum of D9 (Appendix Ig) was determined and gave peak at 2917, 2847, 1712, 1463, 1266, 1171, 1037, 974 and 721 cm^{-1}. The UV spectrum of D9 (Appendix Va) showed maxima ($CHCl_3$) at 290, 315, 322, 422, 442 and 472 nm.

The ^{1}H-NMR spectrum (Appendices IIp, IIq, IIr and IIs) produced signals at δ(ppm) 1.3(s), 1.6(m), 2.9(t, t), 3.25(t, t), 4.01(d), 4.05(d), 4.55(d, d), 4.65(m), 4.9(q), 6.58(d) and 6.68(d). The two dimensional (2D)-NMR spectrum (Appendix VIc), COSY gave a coupling of 2.9 to 3.25, 4.05 to 4.55, 2.9 to 4.55, 4.9 to 3.25, 4.55 to 2.9, 4.65 to 6.68 and 4.9 to 6.58 ppm.

In the ^{13}C-NMR spectrum (Appendix IIIe, IIIf and IIIg) signals appeared at δ(ppm) 37.22, 37.49, 50.38, 54.64, 62.66, 76.76, 77.40, 78.05, 127.74, 127.99, 128.05, 128.33, 129.38, 129.64, 129.84, 130.13, 130.26, 132.37, 133.01, 134.35, 135.22, 136.79, 138.20, 168.56, 168.78 and 173.33.

4.6.3. Ethyl Acetate extract of the rhizome of *C. tinctorium*.

Ten grams of the crude extract was dissolved in a minimum volume of ethyl acetate and absorbed onto silica gel (25 g) and introduced onto a column packed with silica gel (500 g). The column was eluted using 100% petroleum ether / ethyl acetate with increasing proportions of the ethyl acetate until 100% of the ethyl acetate was used. This was followed by 100% ethanol. Twenty millilitre of the eluate was collected and monitored by TLC results the elutes were combined into 19 main fraction labeled E1, E2, E3, ... E19. The fractional yields are shown in **Table 31** below.

Table 31 Fractional Yields of Ethyl acetate extracts.

Fraction	State	Colour	Yield / g
E1	Oil	Colourless	0.15
E2	Oil	Yellowish	0.13

E3	Oil	Yellowish	0.13
E4	Oil	Yellowish	0.38
E5	Syrup	Yellowish-green	0.21
E6	Syrup	Yellowish-green	0.14
E7	Oil and Solid	Yellowish-green	0.12
E8	Oil and Solid	Yellowish-green	0.11
E9	Oil and Solid	Yellowish-green	0.15
E10	Oil and Solid	Yellowish-green	0.18
E11	Oil	Yellowish	0.19
E12	Oil and Solid	Yellowish-green	0.14
E13	Oil and Solid	Yellowish-green	0.22
E14	Oil and Solid	Yellowish-green	0.17
E15	Oil	Yellow	0.10
E16	Oil and Solid	Yellowish-brown	0.41
E17	Oil and Solid	Yellowish-brown	1.43
E18	Oil and Solid	Dark-Brown	1.78
E19	Oil and Solid	Dark-Brown	2.97

4.6.3.1. Isolation of E13

The fraction E13 was kept in a freezer for 24hours. It precipitated a yellowish solid. The solid (28 mg) was filtered and washed with cold petroleum ether. TLC of the solid showed as a single spot when sprayed with anisaldehyde spray reagent. This was labeled

E13 and gave a melting point of 69-71 ^{0}C. The solid was UV active. The R_f values of the sample E13 in a mixture of petroleum ether / ethyl acetate are shown in **Table 32** below.

Table 32. R_f values of E13

Solvent System	$\mathbf{R_f}$
Petroleum ether / ethyl acetate (3:1)	0.95
Petroleum ether / ethyl acetate (4:1)	0.59
Petroleum ether / ethyl acetate (8:1)	0.40
Petroleum ether / ethyl acetate (9:1)	0.31

The IR spectrum of E13 (Appendix Ih) was determined and gave peak at 2955, 2917, 2849, 1711, 1630, 1464, 1300, 1170 and 721 cm^{-1}. The UV spectrum of E13 (Appendix Vb) was determined.

4.6.3.2. Isolation of E16$_6$

Fraction E14, E15 and E16 were combined because they showed similar TLC profile. This was concentrated, relabeled E16. This solid (0.67 g) was put on a column (13.5 g silica gel) and eluted with 100% petroleum ether and mixture of petroleum ether/ethyl acetate with increasing proportion of ethyl acetate until 100% of the ethyl acetate was used. About 10 ml of the eluate were collected and monitored by TLC. Nine (9) main fractions were obtained and labeled E16$_1$, E16$_2$, ... E16$_9$. All the fractions were put into a freezer for 24 hours.

Fraction E16_6 precipitated a dark yellowish solid. The solid (33 mg) was filtered and washed with cold petroleum ether. The TLC gave a single spot when an anisaldehyde reagent was used. The solid was coded as E16_6 (mpt > 250 ^{0}C). The R_f value of the sample E16_6 in a mixture of petroleum ether/ethyl acetate are shown in Table 33 below.

Table 33:R_f value of E16_6

Solvent system	**R_f**
Petroleum ether/ethyl acetate (5:2)	0.35
Petroleum ether/ethyl acetate (2:1)	0.54

The IR spectrum of E 16_6 (Appendix Ii) was determined and gave peaks at 3408, 3073, 2942, 2870, 1689, 1642, 1451, 1377,1243, 1190 and 1049 cm^{-1}

4.7. ANTIMALARIAL ASSAY

4.7.1. Chemicals and Reagents

RPMI-1640 with Hepes modification and $NaHCO_3$ without glutamine (SIGMA, USA)

L-Glutamine (SIGMA, USA)

Gentamycin (stock 50mg/ml, GIBCO, Scotland)

Glycerol (BDH, England)

Sodium Chloride (SIGMA, USA)

Sorbitol (BDH, England)

Chloroquine diphosphate salt (SIGMA, USA)

^{3}H-hypoxanthine (NEN BOSTON, USA)

Ethanol (SIGMA, USA)

Methanol (SIGMA, USA)

Dimethylsulfoxide (DMSO) (SIGMA, USA)

Geimsa stain (BDH, England)

Immersion Oil (BDH, England)

4.7.2. Red Blood Cells and Serum

Blood group O Rh^+ (Volunteers)

Normal Human Serum (NHS) from blood group AB Rh^+ (SIGMA, USA)

4.7.3 Malaria Parasite Strains

Chloroquine sensitive *Plasmodium falciparum* 3D7 strain

Chloroquine resistant *Plasmodium falciparum* DD_2 strain (both strains were from the Centre for Medical Parasitology, Copenhagen, Denmark)

4.7.4. Materials

Candle jar

Pure candles

Flat bottom 96 well microtitre culturing plates

Glass filter fibers

Glass microscope slides

Millipore filters

25 cm^3 culture flasks

5 ml and 10 ml sterile disposable pipettes

5 ml and 50 ml sterile syringes

1.8 ml screw capped cryotubes

4.7.5. PLANT MATERIAL

The rhizomes of *Cochlospermum tinctorium* were collected from an area 9km from Drobo towards Sampa with the assistance of botanists from the Ghana Herbarium, Botany Department, University of Ghana, Legon. The plant material was air-dried for two weeks and ground by machine into coarse powder

4.8. METHODS

4.8.1. Preparation of Citrate-Phosphate Dextrose Buffer (CPD)

This was prepared by weighing 0.5 g citric acid, 2.647 g sodium citrate, 0.221 g sodium phosphate, 0.027 g Adenine (6-Aminopurine) and 3.154 g glucose in petri dishes transferring them into a 200 ml beaker and dissolving with 100 ml distilled water with stirring. The resulting solution was sterilized in an autoclave at 120 ^{0}C for 30 minutes after which it was stored at 4 ^{0}C. For every 20 ml of whole blood, 3 ml of CPD was added as an anticoagulant.

4.8.2. Preparation of Washing medium /Incomplete RPMI medium

This was prepared by adding 0.5 ml of gentamycin (50 mg/ml) and 4.0 ml of L-glutamine to 500 ml of RPMI solution. This was stored at 4 ^{0}C.

4.8.3. Preparation of Complete RPMI medium. (10% NHS)

This was prepared by adding 5 ml filtered (through 0.8 μm pore filter) NHS (O+) and 50 mg of D-glucose to 45 ml washing medium. This was stored at 4 ^{0}C and used for parasite cultivation.

4.8.4. Uninfected Red blood Cells

Human blood group O Rh+ was obtained from Korle-Bu Hospital and donors in CPD and stored at 4 ^{0}C. For cultivation, a volume of blood was centrifuged for 5 minutes at 2000 rpm. The plasma and buffy coat were removed using a sterile pipette. The packed cells remaining were washed several times (4x) with equal volumes of incomplete RPMI (that is, medium with no serum added) and the buffy coat on top of the cells removed. To the remaining packed cells was added an equal volume of incomplete RPMI medium to produce 50% PCV. This was stored at 4 ^{0}C and used for cultivation.

4.9. Extraction of the rhizome of *Cochlospermum tinctorium*

One hundred grams (100 g) of the dried and ground *Cochlospermum tinctorium* were sequentially extracted with 600 ml of Petroleum ether, dichloromethane, ethyl acetate, ethanol and water using cold maceration. Maceration was carried out in one-liter beaker for a period of 48 hours at room temperature with daily stirring of the plant material for each organic solvent. At the end of the 48 hours period, the extracts were obtained by filtration under pressure with a Bucher funnel and again using a funnel to filter it. Solvents were recovered from the extracts by using a rotary evaporator. These were stored at 4 ^{0}C in sealed round bottom flask. The aqueous extract was freeze-dried and stored in a dessicator in a sealed container. The TLC of the extracts, extracted for both the column chromatography and the antimalarial assay gave similar spots.

4.10 Preparation of various concentrations of crude extracts from the rhizome of *Cochlospermum tinctorium*

Each of the plant extracts (5 mg) was weighed into a cryotube. The aqueous extract was dissolved in 50 μl of water and the other extracts dissolved by shaking with 50 μl dimethylsulphoxide (DMSO). This gave a stock solution of $10^5 \mu$g/ml, which was filter-sterilized with a millipore filter (0.20 μl). Ten microlitre (10 μl) of this was added to 990 μl of complete RPMI in a lymphocyte tube to give a solution containing 1 μg/1μl. This was regarded as 1:1 dilution. Fifty microlitres (50 μl) of this solution was then added to 50 μl of parasitised red blood cells in the well to give a final concentration of 500 μg/ml.

This diluted solution (400 μl) was added to 400 μl of complete RPMI to give a 1:2 dilution. Fifty microlitres (50μl) of which was added to 50 μl of parasitised red cells in the well to give a concentration of 250 μg/ml. Similarly, Four hundred microlitres (400 μl) of the 1:2 dilution was added to 400 μl of complete RPMI to give a 1:4 dilution of which 50 μl was added to 50 μl of parasitised red blood cells in the well to give a final concentration of 125 μl/ml. Again 400 μl of the 1:4 dilution was added to 400 μl of complete RPMI to give a 1:8 dilution from which 50 μl was added to 50 μl of parasitised red blood cell in the well to give a concentration of 62.5 μg/ml.

Four hundred microlitres (400 μl) of the 1:8 dilution was added to 400 μl of complete RPMI to give a 1:16 from which 50 μl was added to 50 μl of parasitized red blood cells in the well to give a concentration of 31.25 μg/ml. Four hundred microlitres (400 μl) of the 1:16 dilution was added to 400 μl of complete RPMI to give a 1:32 dilution from which 50μl was added to 50μl of parasitised red blood cells in the well to give a concentration of 15.63 μl/ml. Four hundred microlitres (400 μl) of the 1:32 dilution was added to 400 μl of complete RPMI to give a 1:64 dilution from which 50 μl was added to 50 μl of parasitised red blood cells in the well to give a concentration of 7.81 μg/ml. Four hundred microlitres (400 μl) of the 1:64 dilution was added to 400 μl of complete RPMI to give a 1:128 dilution from which 50 μl was added to 50 μl of parasitised red blood cells in the well to give a concentration of 3.91 μg/ml.

The DMSO content of the 500 μg/ml, 250 μg/ml, 125 μg/ml, 62.5 μg/ml, 31.25 μg/ml, 15.63 μg/ml, 7.81 μg/ml and 3.91 μg/ml dilutions of test were respectively 0.5%, 0.25%, 0.125%, 0.0625%, 0.03125%, 0.0156%, 0.0078% and 0.0039%.

4.11 Preparation of various concentrations of isolated compounds from the rhizome of *C. tnctorium*

Each of the isolated compounds (1 mg) was weighed into cryotube. Fifty microlitres (50 μl) dimethylsulphoxide (DMSO) were used to dissolve the compounds by shaking. This gave a stock solution of 2×10^4 μg/ml, which was filter-sterilized with a millipore filter (0.20 μl). Ten microlitre (10 μl) of this was added to 990 μl of complete RPMI in a lymphocyte tube to give a solution containing 200 μg/ml. This was regarded as 1:1 dilution. Fifty microlitres (50μl) of this solution was then added to 50μl of parasitised red blood cells in the well to give a final concentration of 100 μg/ml.

This diluted solution (400μl) was added to 400μl of complete RPMI to give a 1:2 dilution, from which 50μl was added to 50μl of parasitised red cells in the well to give a concentration of 50μg/ml. Similarly, 400μl of the 1:2 dilution was added to 400μl of complete RPMI to give a 1:4 dilution of which 50μl was added to 50μl of parasitised red blood cells in the well to give a final concentration of 25μl/ml. Again 400μl of the 1:4 dilution was added to 400μl of complete RPMI to give a 1:8 dilution from which 50μl was added to 50μl of parasitised red blood cell in the well to give a concentration of 12.5μg/ml.

Four hundred microlitres (400μl) of the 1:8 dilution was added to 400μl of complete RPMI to give a 1:16 from which 50μl was added to 50μl of parasitised red blood cells in the well to give a concentration of 6.25μg/ml. Four hundred microlitres (400μl) of the 1:16 dilution was added to 400μl of complete RPMI to give a 1:32 dilution from which 50μl was added to 50μl of parasitised red blood cells in the well to give a concentration of 3.13μl/ml. Four hundred microlitres (400μl) of the 1:32 dilution was added to 400μl of complete RPMI to give a 1:64 dilution from which 50μl was added to 50μl of parasitised red blood cells in the well to give a concentration of 1.56μg/ml. Four hundred microlitres (400μl) of the 1:64 dilution was added to 400μl of complete RPMI to give a 1:128 dilution from which 50μl was added to 50μl of parasitised red blood cells in the well to give a concentration of 0.78μg/ml.

The DMSO content of the 100μg/ml, 50μg/ml, 25μg/ml, 1.25μg/ml, 6.25μg/ml, 3.13μg/ml, 1.56μg/ml and 0.78μg/ml dilutions of test were respectively 0.1%, 0.05%, 0.025%, 0.0125%, 0.00625%, 0.00313%, 0.00156% and 0.00078%.

4.12. *In Vitro* cultivation of malaria parasites

Parasitised red blood cells containing chloroquine sensitive (3D7) and resistant (DD_2) *P. falciparium* strains were obtained from the Immunology Unit, NMIMR, Legon and kept in continuous culture by the candle jar method of Trager and Jensen. [77] These parasites were chosen because they are sensitive to chloroquine (3D7) and resistant to chloroquine (DD_2), which is the standard drug, for treatment of malaria in Ghana. A fair comparison could therefore be made for their response to chloroquine and the plant extracts and isolated compounds.

To imitate cultivation cryopreserved parasites were removed from liquid nitrogen (-196 ^{0}C) and quickly disengaged and thawed by placing the tube in water bath set at 37 ^{0}C containing sterile water. It was immediately centrifuged at 1500rpm for 10 minutes and the supernant removed. An equal volume of the thawing medium (3.5% Sodium chloride in sterile water) was then added. It was again centrifuged at 1500rpm for 10 minutes and the supernatant removed. The cells were resuspended in 1.0 ml of RPMI 1640 medium supplemented with 10% normal human serum (AB^{+}), L-glutamine and gentamycin.[75]

This was washed once again as described and the cells added to a culturing flask containing 5ml of complete culture medium and 200μl of O+ packed red blood cells. This was then gassed with a special gas mixture (2.0% oxygen, 5.5% carbon dioxide and 92.5% nitrogen) for 30 seconds and incubated in an incubator set at 37^{0}C. Later, a candle jar was used. The flasks were flamed and placed in the candle jar containing a lighted candle. The jar was closed, the tap shut when the candle went off, and jar transferred into an incubator, kept at 37 ^{0}C.

The spent medium was changed everyday and slides were prepared and stained with Giemsa to monitor the growth of the parasites expressed as percentage parasitaemia. Five millilitres (5ml) of complete medium was added if the percentage parasitaemia is less than 3%. The cultures were maintained until the schizont stage with a parasitaemia of approximately 3.0% and it was used for the assay.

4.13. Fixation and Staining

Parasitaemia was determined to ascertain when the parasites in the culture flasks were ready for subculturing. Glass slides were soaked in 70% ethanol, cleaned well with cotton before the film was prepared. The dried blood films were fixed with methanol and air-dried. The films were stained using a 1:10 dilution of Giemsa stain for 10 minutes. The stained films were washed with water, dried and viewed under the microscope (Olympus BH2 Microscope) at 100X magnification. The parasitaemia was determined as a ratio of the number of parasitised erythrocytes, to the total number of erythrocytes in a microscope field.

4.14. Cryopreservation of Parasites

Cryopreservation was used to preserve some malaria parasites for future work. The ring stage of a culture of malaria parasites with parasitaemia between 3 and 5% was centrifuged at 1200rpm for 5 minutes. The supernatant fraction was then removed to obtain a pellet of parasites. An equal volume of freezing solution (28g glycerol, 5g sorbitol and 0.63g NaCl in 100ml distilled water and filter sterilised) was added to the pellet of parasites, gently mixed and transferred by sterile serological pipette into sterile cryotubes. The transfer was rapidly performed to avoid haemolysis during thawing. The cryotubes were rapidly closed and transferred into liquid nitrogen as described by Merryman and Hornblow.[76]. They were stored in liquid nitrogen until brought into *in vitro* culture.

4.15. Subculture

This was done when the parasitaemia in culture flasks reached between 3 and 5% from a starting parasitaemia of 0.25%. To 200μl of non-parasitised blood (O Rh+) was added 20μl of parasitsed blood in a 25ml culture flask to give a ratio of 10:1. To this was added 5ml of RPMI supplemented with 10% normal human serum. The subcultures were done for about three times to attain a stable growth before it was used for any assay.

4.16. *In Vitro* inhibition assay for extracts and isolated compounds from the rhizome of *C. tinctorium*

This was preformed using 96-well flat-bottomed microculture plates. The test procedure was based on the method of O'Neil *et al.*[63] Fifty microlitre aliquots of the various concentration of the extracts and isolated compounds of *C. tinctorium* (that is for extracts, 500μg/ml, 250μg/ml, 125μg/ml, 62.5μg/ml, 31.25μg/ml, 15.63μg/ml, 7.81μg/ml and 3.91μg/ml and isolated compound 100μg/ml, 50μg/ml, 25μg/ml, 12.5μg/ml, 6.25μg/ml, 3.13μg/ml, 1.56μg/ml and 0.78μg/ml) were introduced in triplicate into the wells. To each of these was added 50μl of parasitised blood of parasitaemia between 1 and 2% and cell suspension of 5×10^8/ml.

Positive control wells were free of any extract, while chloroquine (CQ) diluted with sterile complete RPMI in various concentrations were introduced into negative control wells. This was prepared from a stock chloroquine diphosphate salt (SIGMA, USA). The microculture plates were placed in a candle jar containing a lighted candle. The jar was closed and the tap shut when the candle went off. The jar was kept at 37^0C in an incubator. The plates were brought out after 24 hours and 20μl of ^{3}H-hypoxanthine (40μCi/ml) added to each well. They were incubated again at 37 ^{0}C for another 24 hours after which the cells were harvested on a glass-fibre filter using a Packard filter Mate 96 cell Harvester.

Radioactivity of ^{3}H-hypoxanthine incorporated in the DNA of the parasites were counted by Direct Beta Counter, Matrix 96 (Packard, USA) that uses gas mixture of helium gas (97.5%) and butane (2.5%) for scintillation. The glass-fibre filter was dried at 37 ^{0}C and later read using the counter.

4.17. Dimethylsulfoxide (DMSO) Trials

DMSO is normally used at a concentration range of 0.1%-0.5% for this type of inhibitor assay in the laboratory where this was performed. Two trial experiments were however performed to test for any possible effect it might have on the 3D7 parasites. This was performed using the method described under section 4.16.

Fifty microlitre aliquots of RPMI-1640 medium containing either 0.5% DMSO or 0.5% aqueous extract of *C. tinctorium* were introduced in triplicate into the wells. The 0.5% DMSO was the highest concentration of DMSO used in the inhibition assay and it was present in the 500μl/ml dilutions. Any inhibition at this level would add to the possible inhibitory effect of the plant extract.

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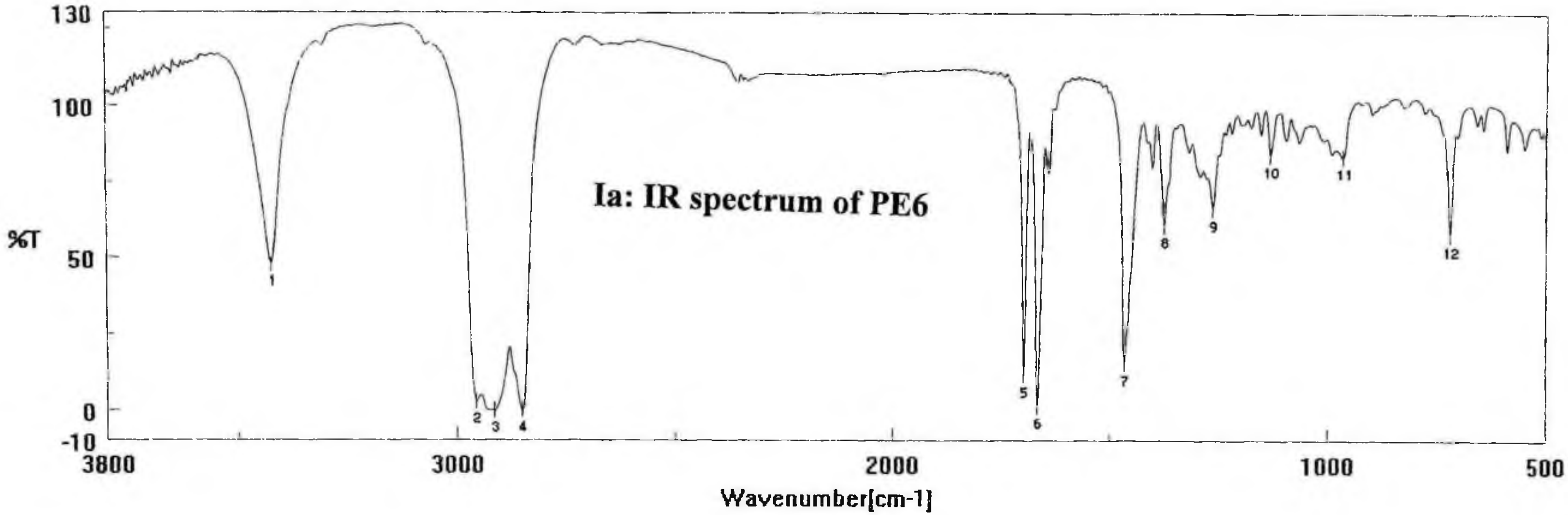
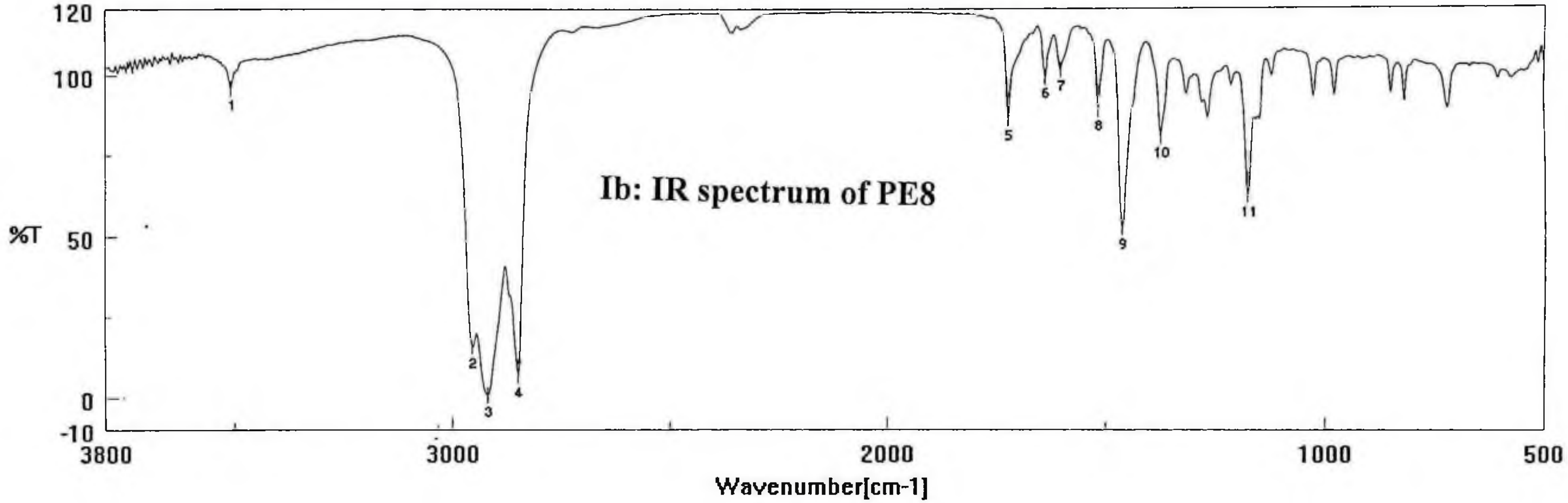
APPENDIX I

INFRARED SPECTRUM


Ia: IR spectrum of PE6

Accumulation	71	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	4	Scanning Speed	2 mm/sec
Date/Time	3/14/01 1:10PM		
Update	3/14/01 1:14PM		
Operator	WRP		
File Name	03_14_2001-006.jws		
Sample Name	PE 6		
Comment	Nujol Mull		

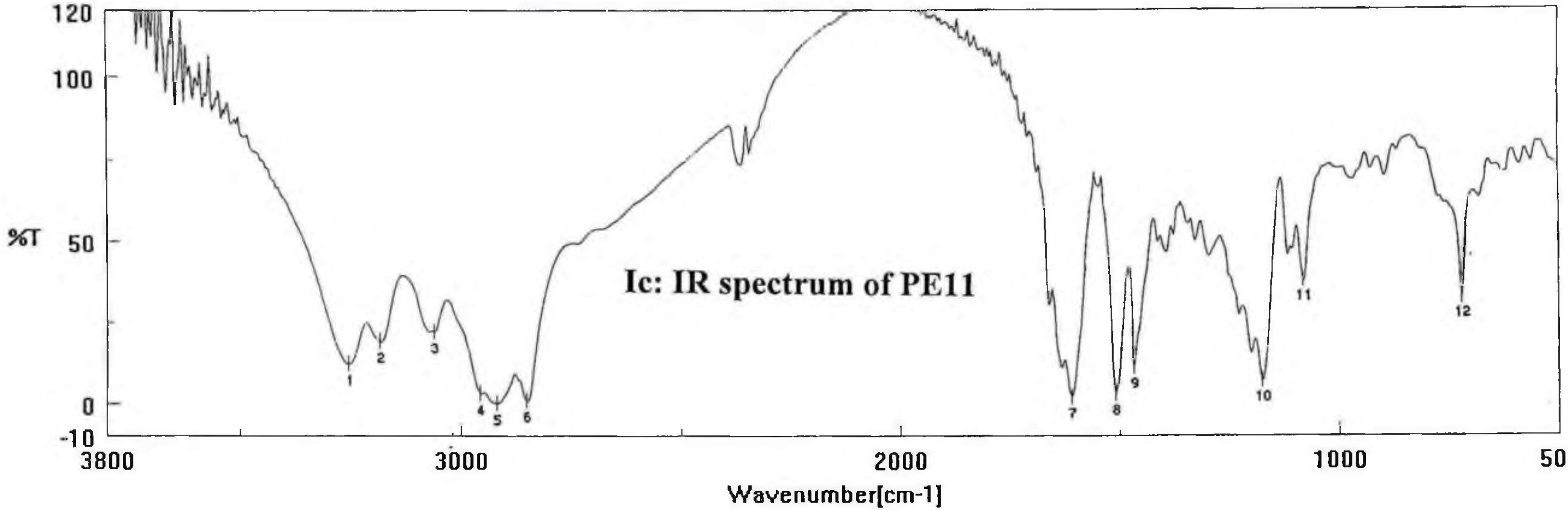
1: 3425, 47.86 2: 2956, 2.81 3: 2915, -0.15 4: 2851, 0.07
5: 1699, 11.47 6: 1666, 1.27 7: 1467, 15.27 8: 1377, 60.80
9: 1266, 66.14 10: 1133, 83.94 11: 966, 83.34 12: 722, 57.62


Ib: IR spectrum of PE8

Accumulation	64
Zero Filling	ON
Gain	4
Date/Time	3/14/01 1:23PM
Update	3/14/01 1:27PM
Operator	WRP
File Name	03_14_2001-007.jws
Sample Name	PE 8
Comment	Nujol Mull

Resolution	4 cm-1
Apodization	Cosine
Scanning Speed	2 mm/sec

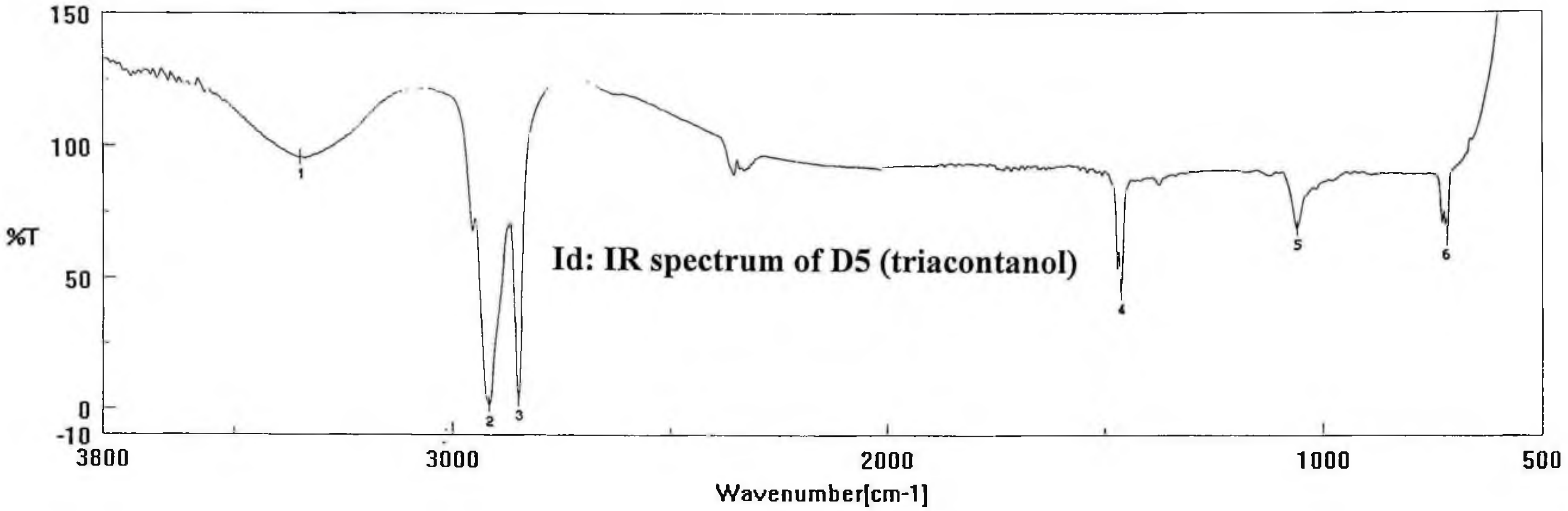
1: 3510, 95.82
2: 2955, 15.84
3: 2920, 0.75
4: 2850, 6.78
5: 1725, 86.07
6: 1640, 99.27
7: 1604, 101.68
8: 1519, 89.16
9: 1463, 52.55
10: 1376, 80.64
11: 1176, 62.42



Ic: IR spectrum of PE11

Accumulation	111	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	8	Scanning Speed	2 mm/sec
Date/Time	3/27/01 2:34PM		
Update	3/27/01 2:44PM		
Operator	WRP		
File Name	03_27_2001-006.jws		
Sample Name	PE 11		
Comment	KBr		

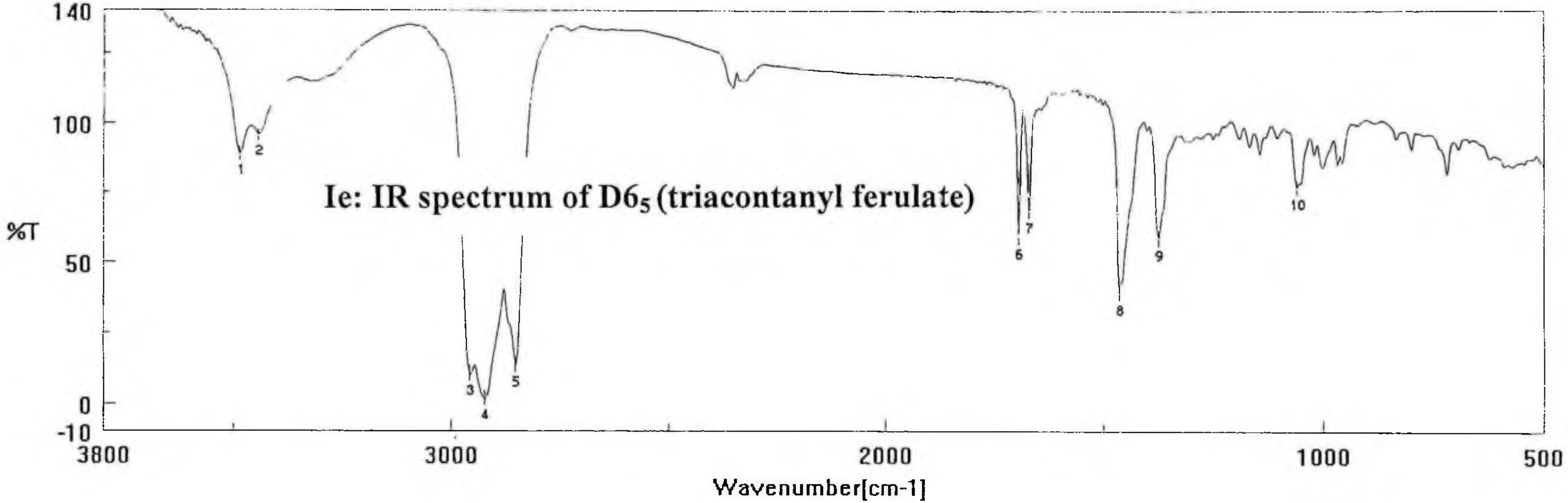
1: 3255, 12.20 2: 3184, 19.13 3: 3061, 22.10 4: 2957, 3.26
5: 2920, 0.07 6: 2852, 0.94 7: 1609, 2.06 8: 1509, 3.13
9: 1468, 11.30 10: 1176, 7.27 11: 1084, 38.10 12: 721, 32.80



Id: IR spectrum of D5 (triacontanol)

Accumulation	74	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	4	Scanning Speed	2 mm/sec
Date/Time	3/14/01 2:05PM		
Update	3/14/01 2:07PM		
Operator	WRP		
File Name	03_14_2001-010.jws		
Sample Name	D5		
Comment	KBr disk		

1: 3350, 95.74 2: 2917, 1.54 3: 2849, 3.83 4: 1463, 44.51
5: 1061, 68.55 6: 721, 64.83

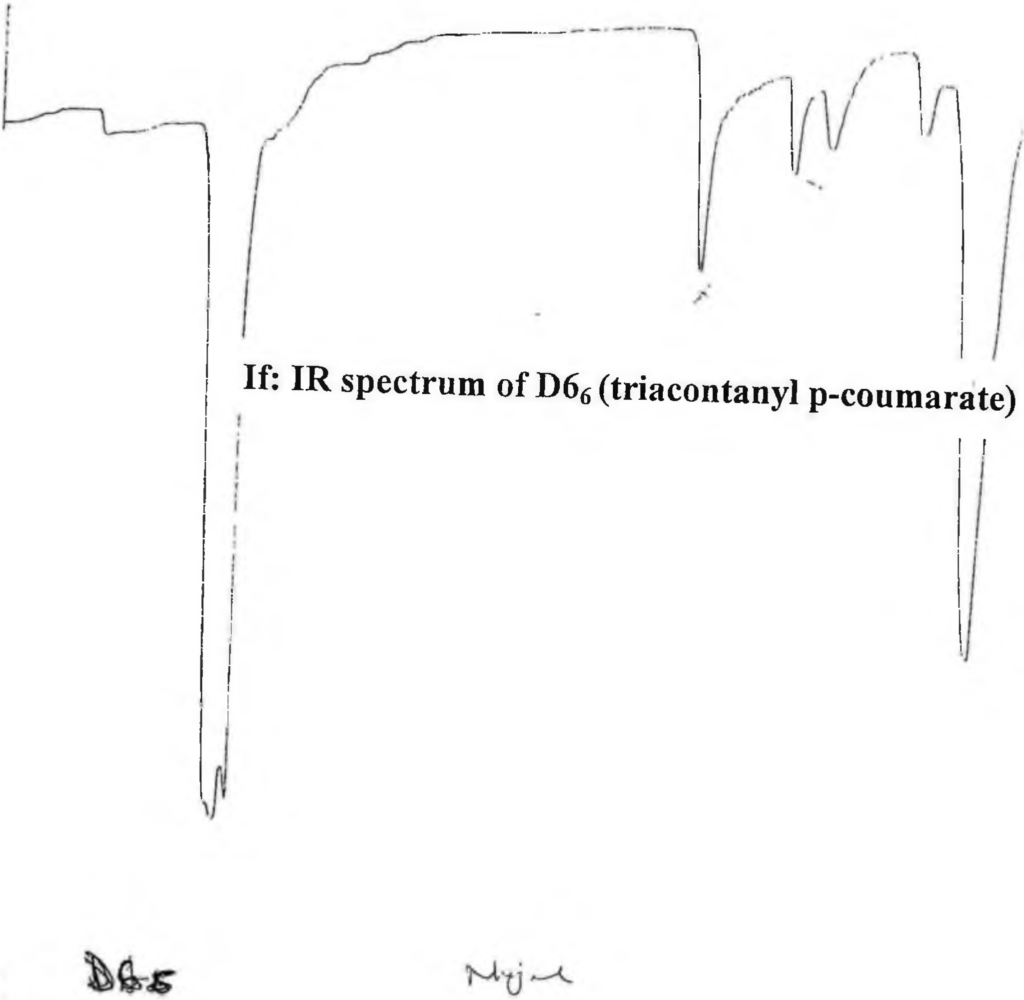


Ie: IR spectrum of D6$_5$ (triacontanyl ferulate)

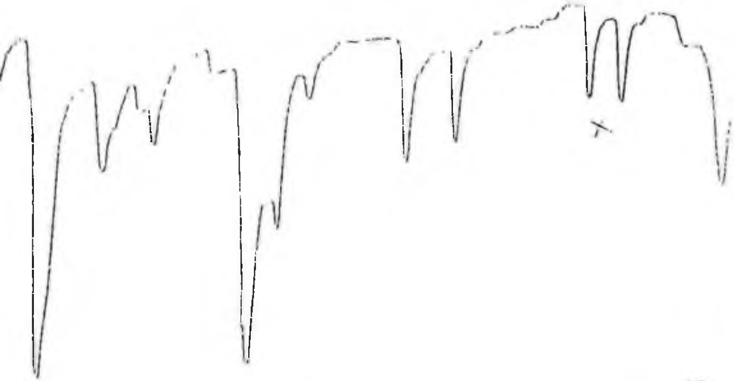
Accumulation	68
Zero Filling	ON
Gain	4
Date/Time	3/14/01 1:57PM
Update	3/14/01 2:01PM
Operator	WRP
File Name	03_14_2001-009.jws
Sample Name	D 6 (5)
Comment	Nujol Mull

Resolution	4 cm-1
Apodization	Cosine
Scanning Speed	2 mm/sec

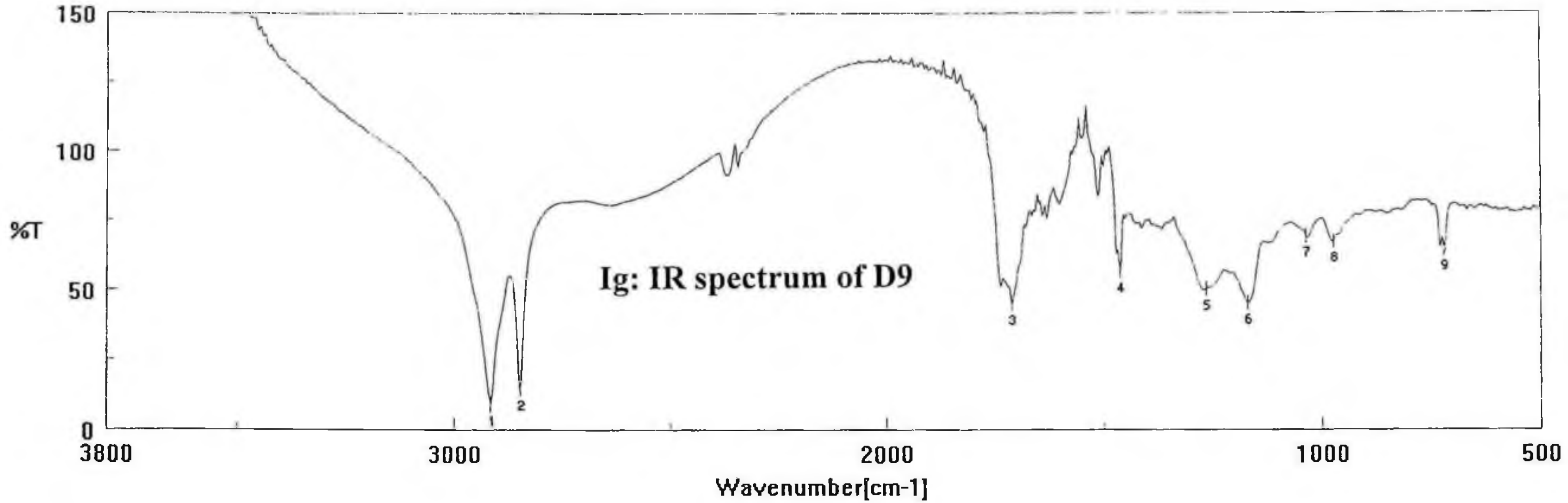
1: 3488, 88.96 2: 3445, 96.01 3: 2958, 10.60 4: 2924, 1.94
5: 2853, 13.62 6: 1698, 58.76 7: 1674, 68.07 8: 1466, 38.72
9: 1378, 58.28 10: 1063, 76.56



If: IR spectrum of D6$_6$ (triacontanyl p-coumarate)



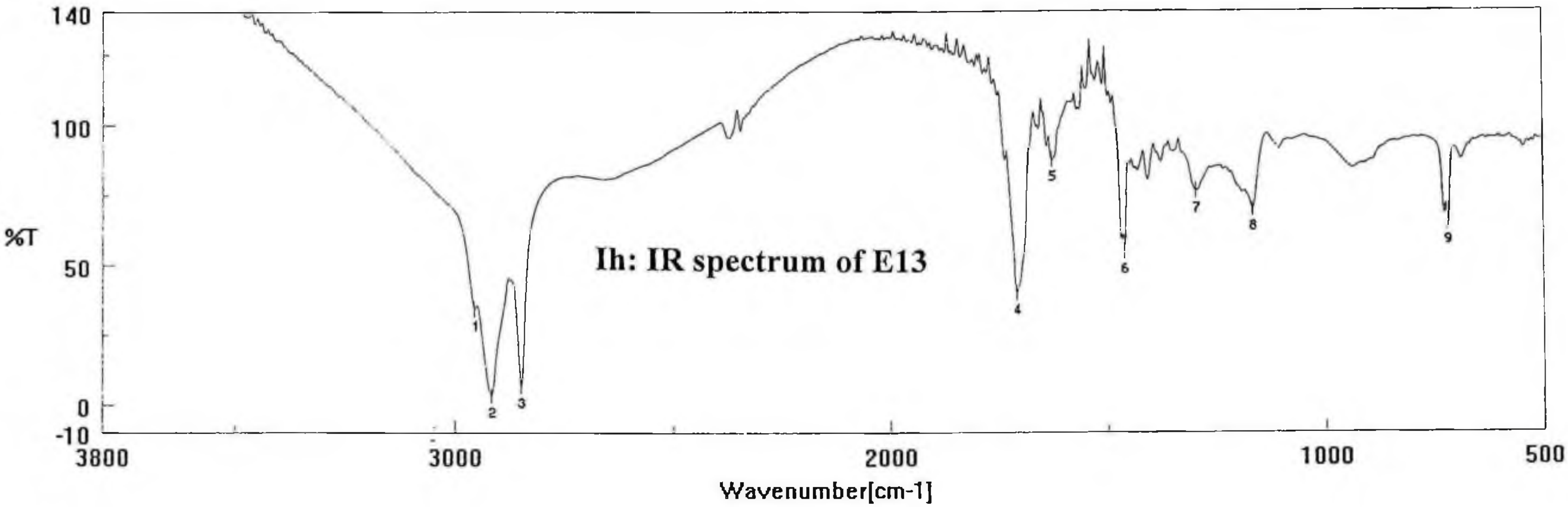
24/11/2000
Paul



Ig: IR spectrum of D9

Accumulation	83	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	8	Scanning Speed	2 mm/sec
Date/Time	3/27/01 1:56PM		
Update	3/27/01 2:01PM		
Operator	WRP		
File Name	03_27_2001-005.jws		
Sample Name	D 9		
Comment	KBr		

1: 2917, 9.25
2: 2847, 14.86
3: 1712, 44.95
4: 1463, 56.61
5: 1266, 50.14
6: 1171, 45.19
7: 1037, 69.12
8: 974, 67.40
9: 721, 64.29

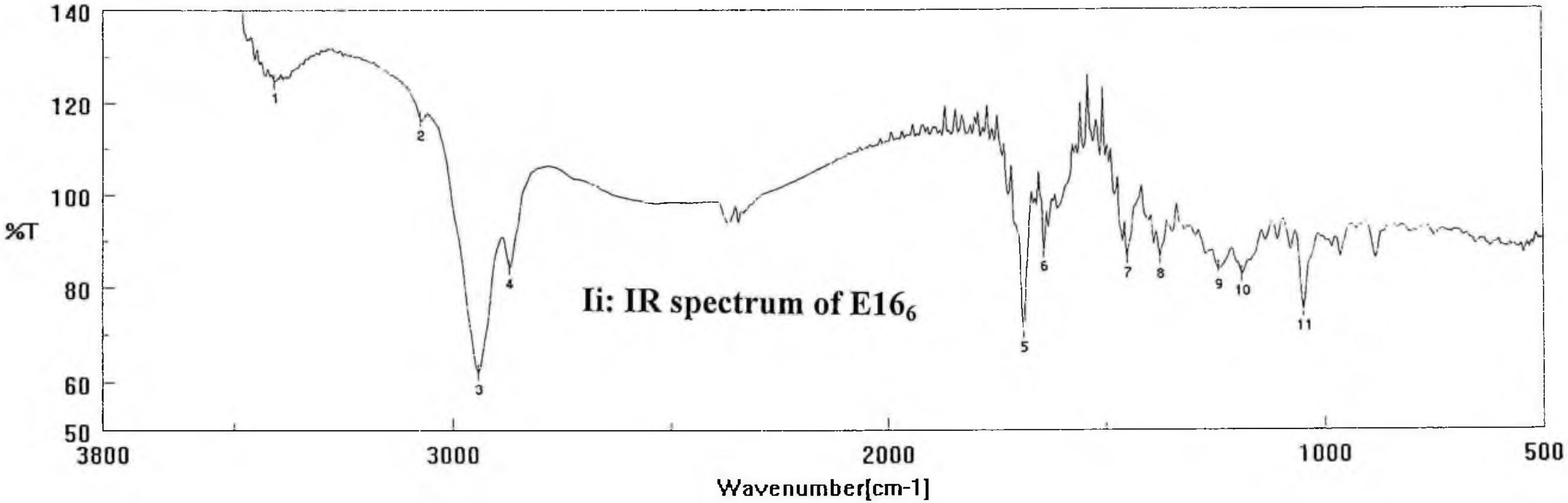


Ih: IR spectrum of E13

Accumulation	74
Zero Filling	ON
Gain	4
Date/Time	3/27/01 0:03PM
Update	3/27/01 0:07PM
Operator	WRP
File Name	03_27_2001-003.jws
Sample Name	E13
Comment	KBr

Resolution	4 cm-1
Apodization	Cosine
Scanning Speed	2 mm/sec

1: 2955, 34.22
2: 2917, 3.23
3: 2849, 6.66
4: 1711, 40.03
5: 1630, 87.51
6: 1464, 54.36
7: 1300, 76.29
8: 1170, 69.91
9: 721, 64.89



Ii: IR spectrum of $E16_6$

Accumulation	73
Zero Filling	ON
Gain	4
Date/Time	3/27/01 11:27AM
Update	3/27/01 11:32AM
Operator	WRP
File Name	03_27_2001-002.jws
Sample Name	E16-6
Comment	KBr

Resolution	4 cm-1
Apodization	Cosine
Scanning Speed	2 mm/sec

1: 3408, 124.71
2: 3073, 116.50
3: 2942, 62.18
4: 2870, 84.57
5: 1689, 70.92
6: 1642, 88.14
7: 1451, 86.68
8: 1377, 86.41
9: 1243, 84.19
10: 1190, 82.84
11: 1049, 75.38

APPENDIX II

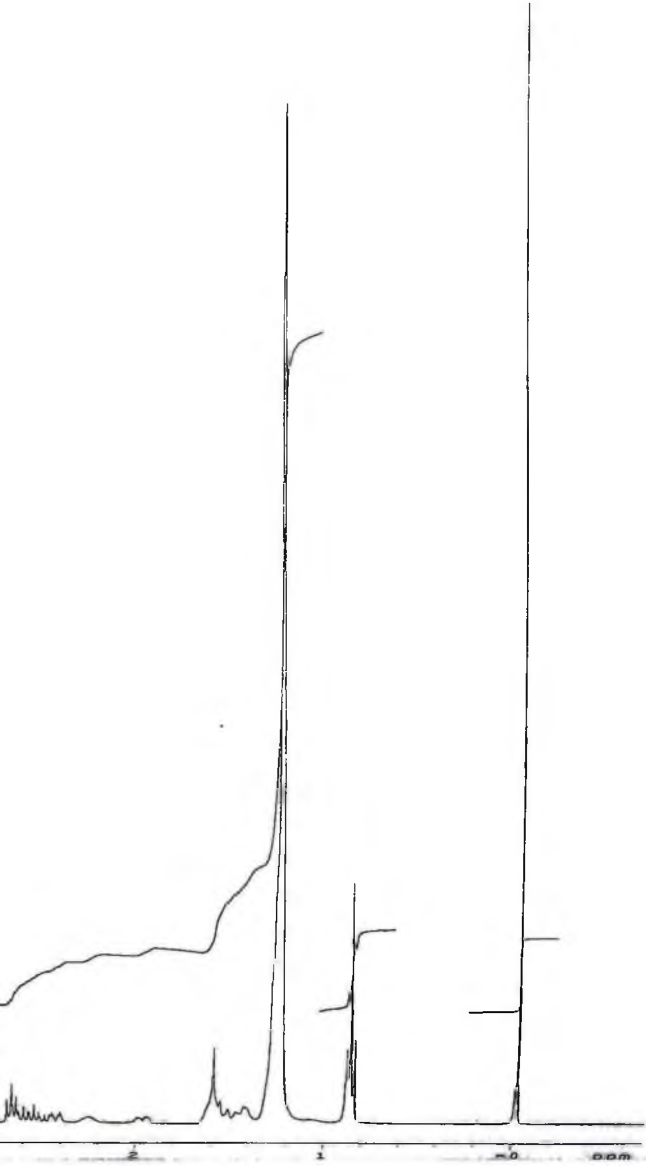
PROTON NMR SPECTRA

```
PE6

exp1  stdlh

      SAMPLE                DEC. & VT
date      Apr 23 01   dfrq        300.061
solvent       CDCl3   dn               H1
file            exp   dpwr             12
   ACQUISITION        dof               0
sfrq        300.061   dm              nnn
tn               H1   dmm               c
at            3.746   dmf             200
np            19008         PROCESSING
sw           2536.8   wtfile
fb             1400   proc             ft
bs               16   fn         not used
tpwr             50
pw              5.5   werr
d1                0   wexp
tof          -442.7   wbs
nt               16   wnt
ct               16
alock             n
gain       not used
      FLAGS
il                n
in                y
dp                y
     DISPLAY
sp           -210.8
wp           2536.8
vs              200
sc                0
wc              250
hzmm          10.15
is           500.00
rfl          2383.3
rfp          2172.4
th               20
ins           1.000
nm   cdc   ph
```

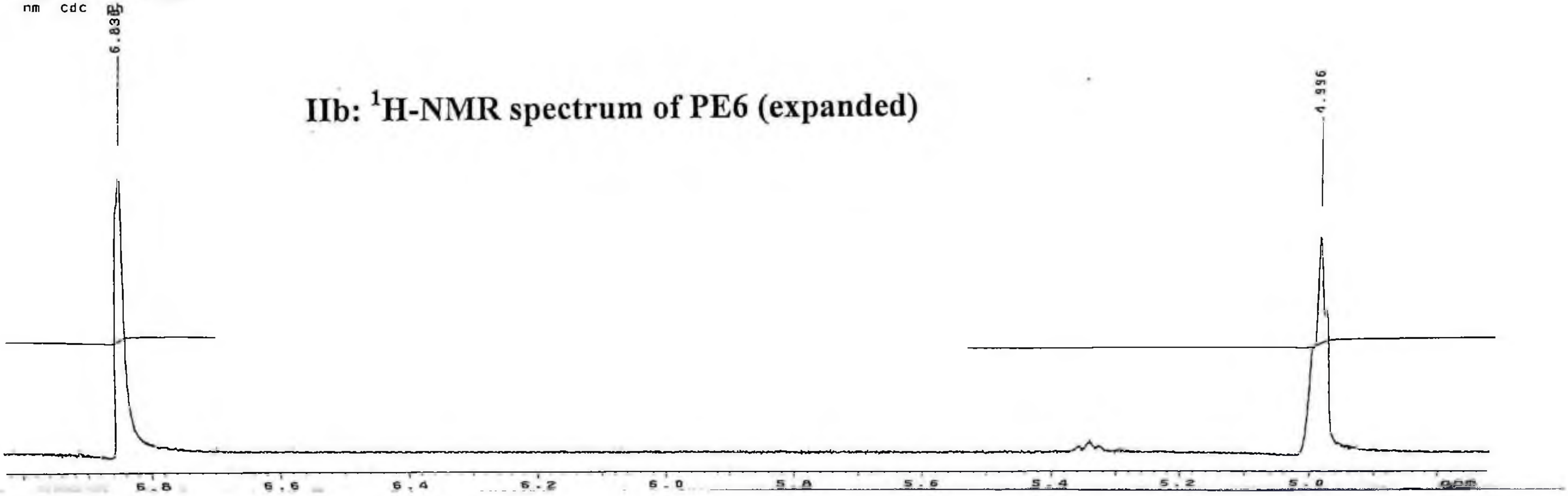
IIa: ^{1}H-NMR spectrum of PE6


2
1
0
ppm

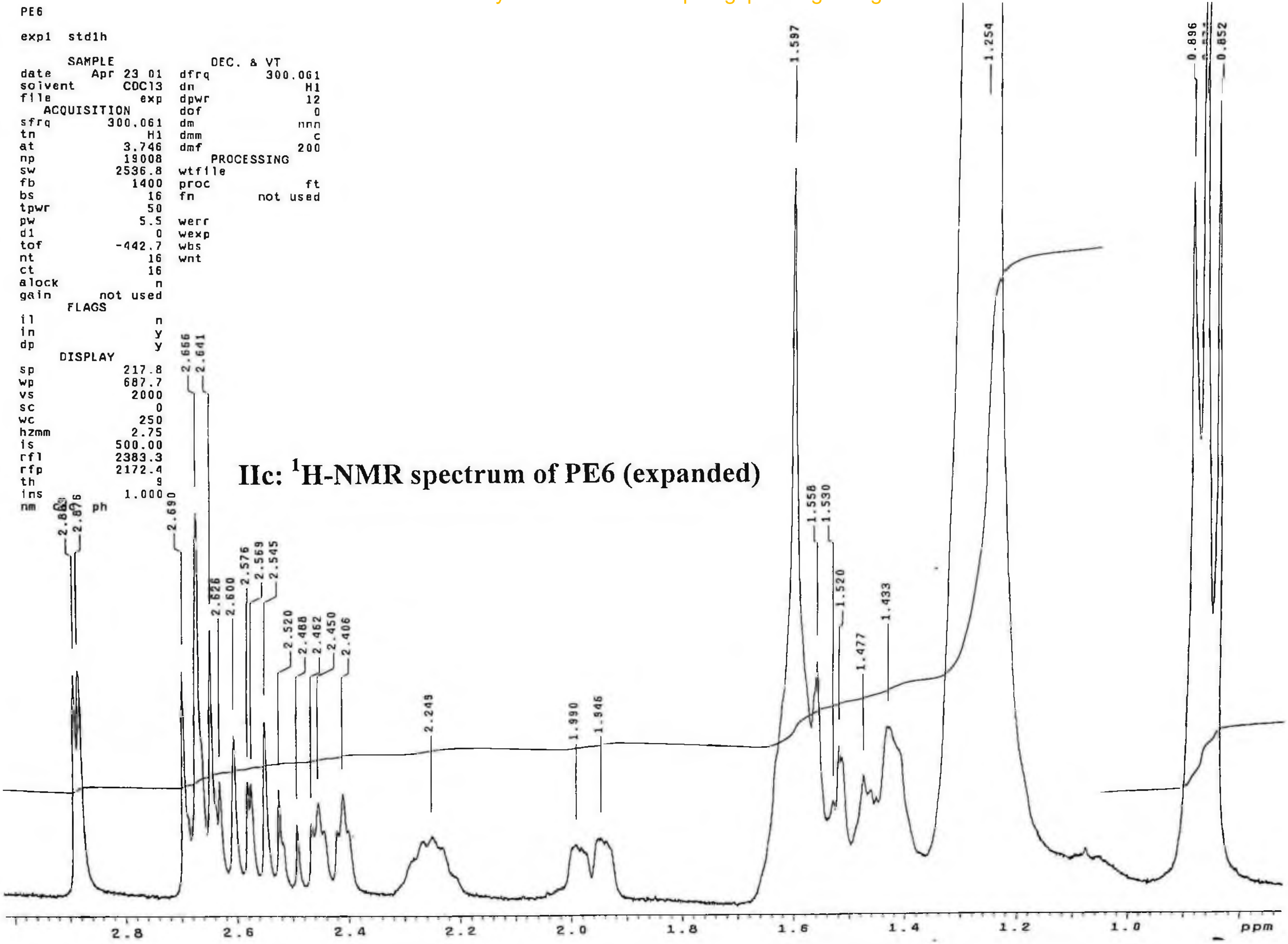
```
PE6

exp1  std1h

      SAMPLE                DEC. & VT
date       Apr 23 01    dfrq          300.061
solvent        CDC13    dn                 H1
file             exp    dpwr               12
   ACQUISITION          dof                 0
sfrq         300.061    dm                nnn
tn                H1    dmm                 c
at             3.746    dmf               200
np             19008        PROCESSING
sw            2536.8    wtfile
fb              1400    proc               ft
bs                16    fn           not used
tpwr              50
pw               5.5    werr
d1                 0    wexp
tof           -442.7    wbs
nt                16    wnt
ct                16
alock              n
gain        not used
     FLAGS
il                 n
in                 y
dp                 y
     DISPLAY
sp            1417.3
wp             690.9
vs              2000
sc                 0
wc               250
hzmm            2.76
is            500.00
rfl           2383.3
rfp           2172.4
th                20
ins            1.000
nm   cdc
```



IIb: ^{1}H-NMR spectrum of PE6 (expanded)

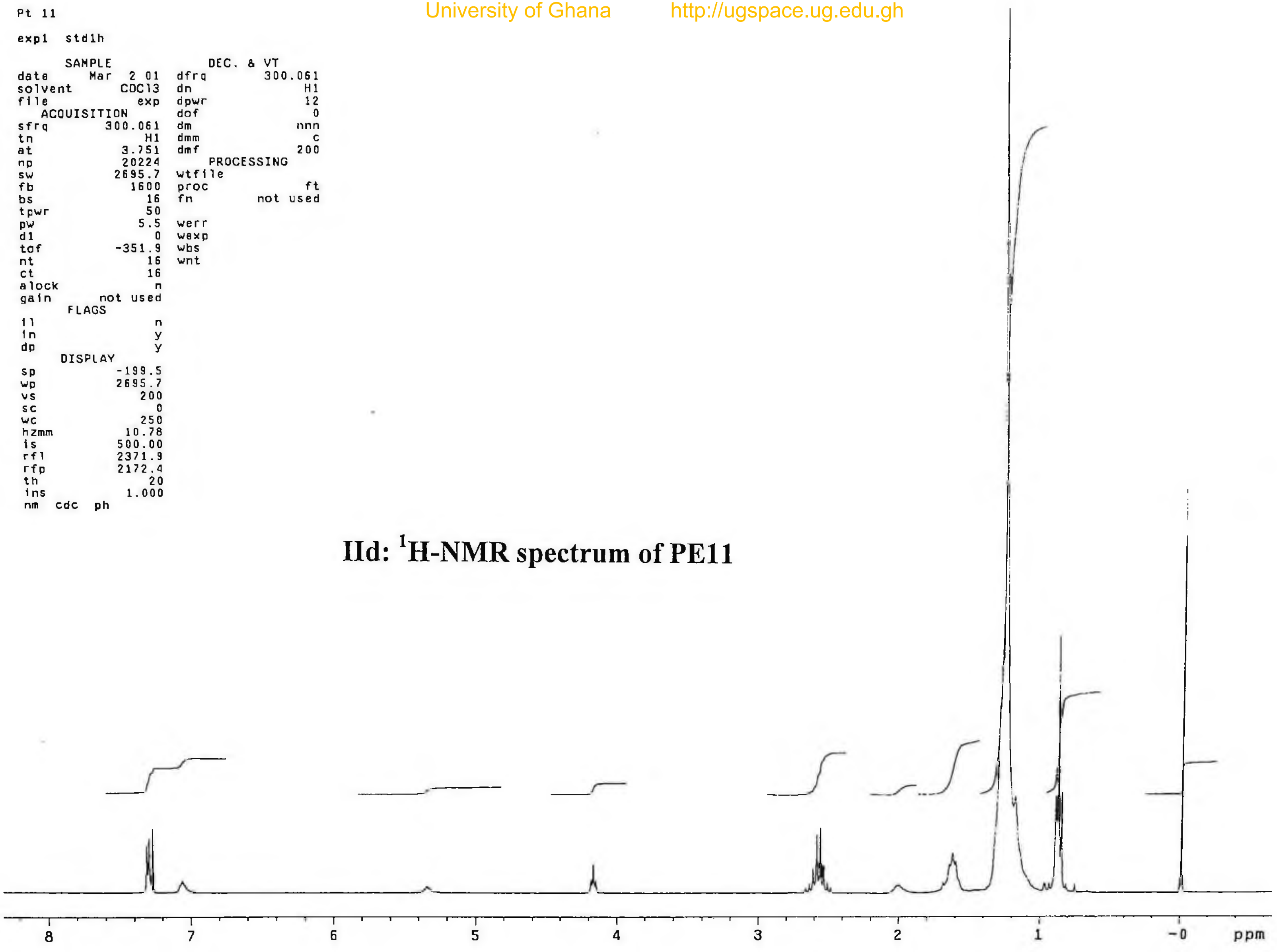


```
PE6

exp1  std1h

         SAMPLE                DEC. & VT
date      Apr 23 01    dfrq          300.061
solvent       CDCl3    dn                 H1
file            exp    dpwr               12
    ACQUISITION        dof                 0
sfrq        300.061    dm                nnn
tn               H1    dmm                 c
at            3.746    dmf               200
np            19008        PROCESSING
sw           2536.8    wtfile
fb             1400    proc               ft
bs               16    fn           not used
tpwr             50
pw              5.5    werr
d1                0    wexp
tof          -442.7    wbs
nt               16    wnt
ct               16
alock             n
gain       not used
         FLAGS
il                n
in                y
dp                y
        DISPLAY
sp            217.8
wp            687.7
vs             2000
sc                0
wc              250
hzmm           2.75
is           500.00
rfl          2383.3
rfp          2172.4
th                9
ins           1.000
nm      cdc  ph
```

IIc: ^{1}H-NMR spectrum of PE6 (expanded)



```
Pt 11

exp1  std1h

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solvent        CDCl3   dn                 H1
file             exp   dpwr               12
     ACQUISITION       dof                 0
sfrq         300.061   dm                nnn
tn                H1   dmm                 c
at             3.751   dmf               200
np             20224         PROCESSING
sw            2695.7   wtfile
fb              1600   proc               ft
bs                16   fn           not used
tpwr              50
pw               5.5   werr
d1                 0   wexp
tof           -351.9   wbs
nt                16   wnt
ct                16
alock              n
gain        not used
         FLAGS
il                 n
in                 y
dp                 y
       DISPLAY
sp            -199.5
wp            2695.7
vs               200
sc                 0
wc               250
hzmm           10.78
is            500.00
rfl           2371.9
rfp           2172.4
th                20
ins            1.000
nm   cdc   ph
```
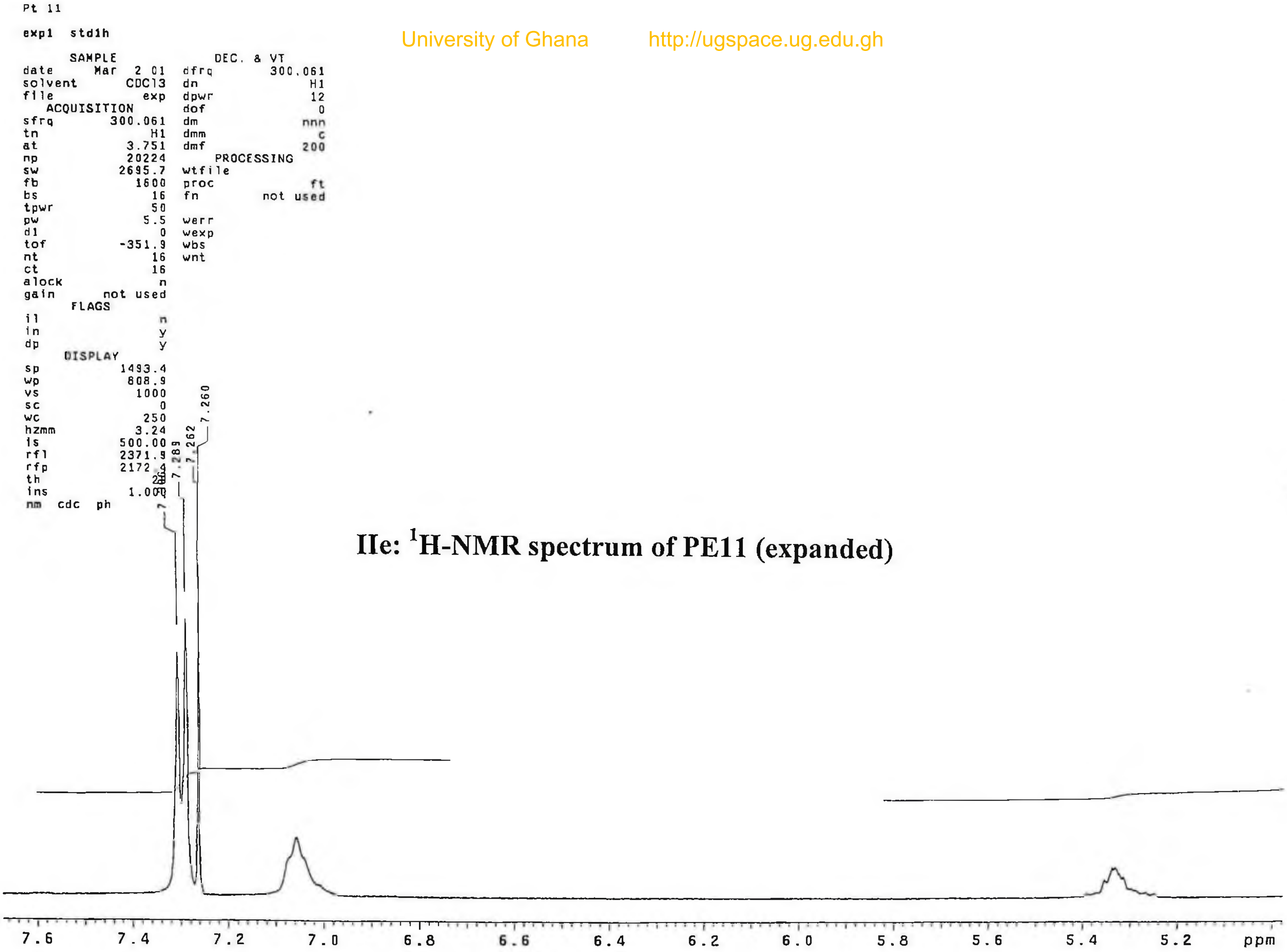

IId: ^{1}H-NMR spectrum of PE11



```
Pt 11

exp1  std1h

       SAMPLE                  DEC. & VT
date      Mar  2 01   dfrq          300.061
solvent       CDCl3   dn                 H1
file            exp   dpwr               12
    ACQUISITION       dof                 0
sfrq        300.061   dm                nnn
tn               H1   dmm                 c
at            3.751   dmf               200
np            20224       PROCESSING
sw           2695.7   wtfile
fb             1600   proc               ft
bs               16   fn           not used
tpwr             50
pw              5.5   werr
d1                0   wexp
tof          -351.9   wbs
nt               16   wnt
ct               16
alock             n
gain       not used
       FLAGS
il                n
in                y
dp                y
      DISPLAY
sp           1493.4
wp            808.9
vs             1000
sc                0
wc              250
hzmm           3.24
is           500.00
rfl          2371.9
rfp          2172.4
th               20
ins           1.000
nm  cdc   ph
```

IIe: ^{1}H-NMR spectrum of PE11 (expanded)

```
exp1  std1h

        SAMPLE                 DEC. & VT
date       Mar  2 01   dfrq          300.061
solvent        CDCl3   dn                 H1
file             exp   dpwr               12
   ACQUISITION         dof                 0
sfrq         300.061   dm                nnn
tn                H1   dmm                 c
at             3.751   dmf               200
np             20224       PROCESSING
sw            2695.7   wtfile
fb              1600   proc               ft
bs                16   fn           not used
tpwr              50
pw               5.5   werr
d1                 0   wexp
tof           -351.9   wbs
nt                16   wnt
ct                16
alock              n
gain        not used
        FLAGS
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in                 y
dp                 y
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wp             805.6
vs              1000
sc                 0
wc               250
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rfp           2172.4
th                20
ins            1.000
nm   cdc   ph
```

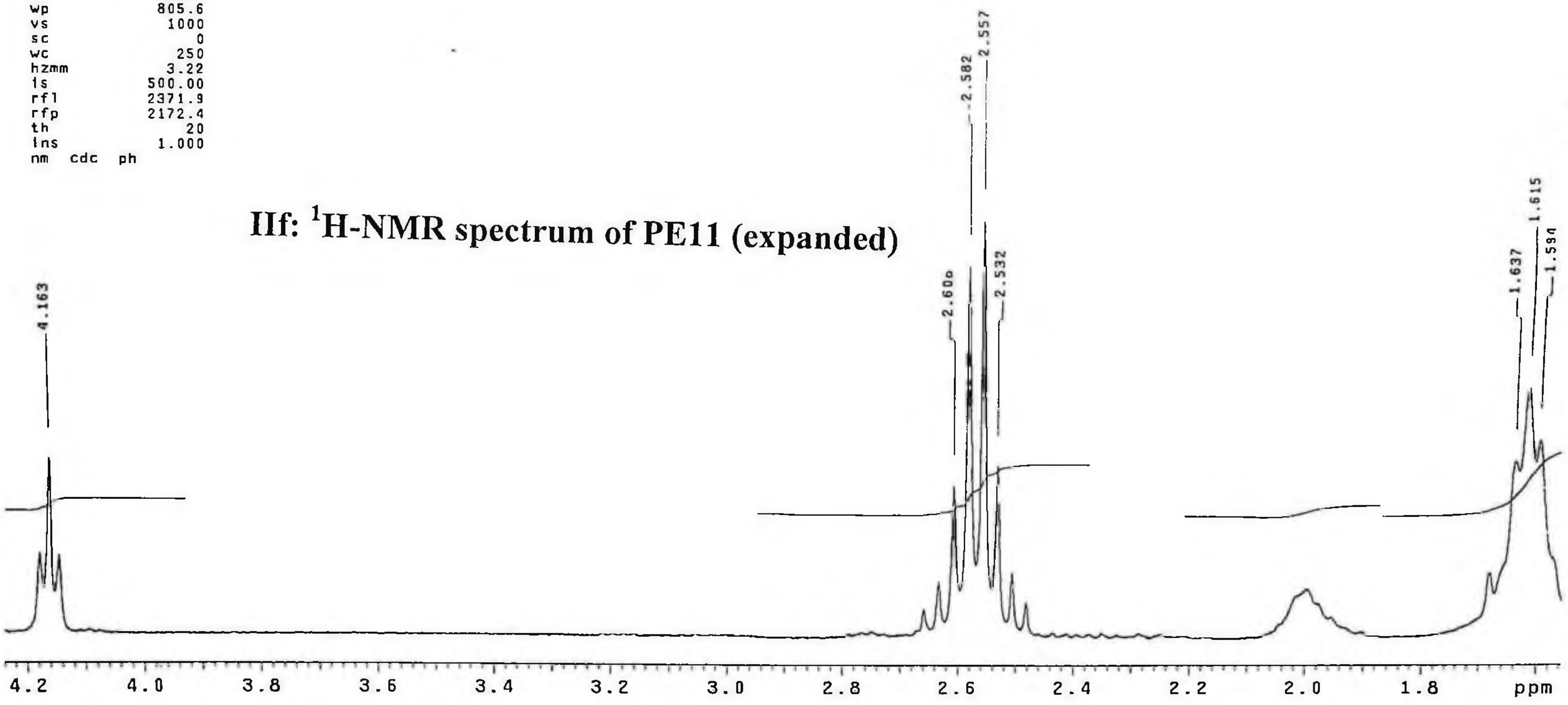

IIf: ^{1}H-NMR spectrum of PE11 (expanded)

```
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solvent       CDCl3  dn                H1
file            exp  dpwr              12
   ACQUISITION       dof                0
sfrq        300.061  dm               nnn
tn               H1  dmm                c
at            3.744  dmf              200
np            20736       PROCESSING
sw           2769.5  wtfile
fb             1600  proc              ft
bs  ‹            16  fn          not used
tpwr             50
pw              5.5  werr
d1                0  wexp
tof          -371.9  wbs
nt               16  wnt
ct               16
alock             n
gain       not used
      FLAGS
il                n
in                y
dp                y
     DISPLAY
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wp           2769.5
vs             1000
sc                0
wc              250
hzmm          11.08
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rfp          2172.4
th               20
ins           1.000
nm  cdc  ph
```

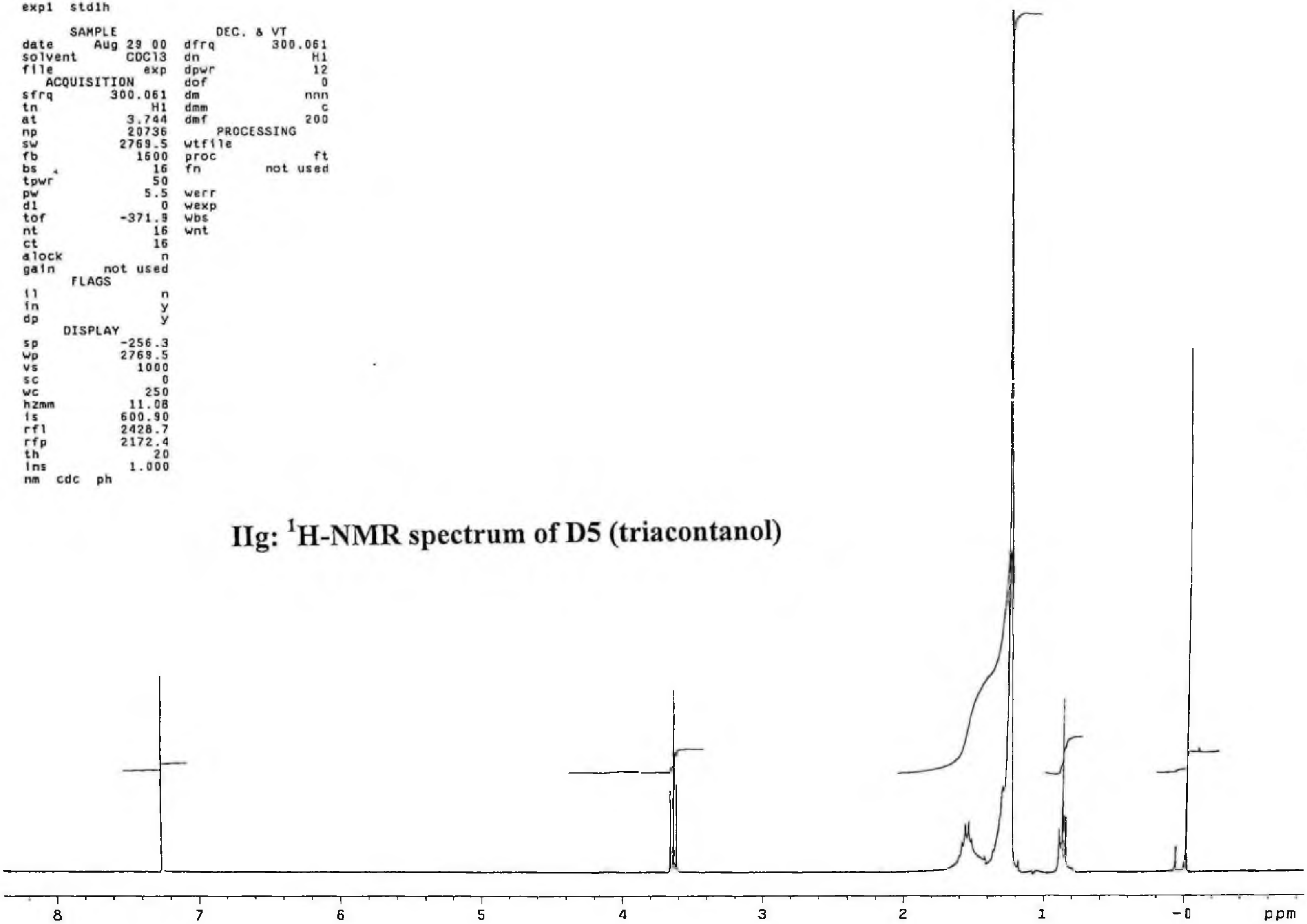

IIg: ^{1}H-NMR spectrum of D5 (triacontanol)

```
exp1  stdlh

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date      Aug 29 00  dfrq        300.061
solvent       CDCl3  dn               H1
file            exp  dpwr             12
   ACQUISITION       dof               0
sfrq        300.061  dm              nnn
tn               H1  dmm               c
at            3.744  dmf             200
np            20736       PROCESSING
sw           2769.5  wtfile
fb             1600  proc             ft
bs               16  fn         not used
tpwr             50
pw              5.5  werr
d1                0  wexp
tof          -371.9  wbs
nt               16  wnt
ct               16
alock             n
gain       not used
      FLAGS
il                n
in                y
dp                y
     DISPLAY
sp            669.1
wp            534.2
vs             1000
sc                0
wc              250
hzmm           2.14
is           600.90
rfl          2426.7
rfp          2172.4
th               12
ins           1.000
nm  cdc  ph
```
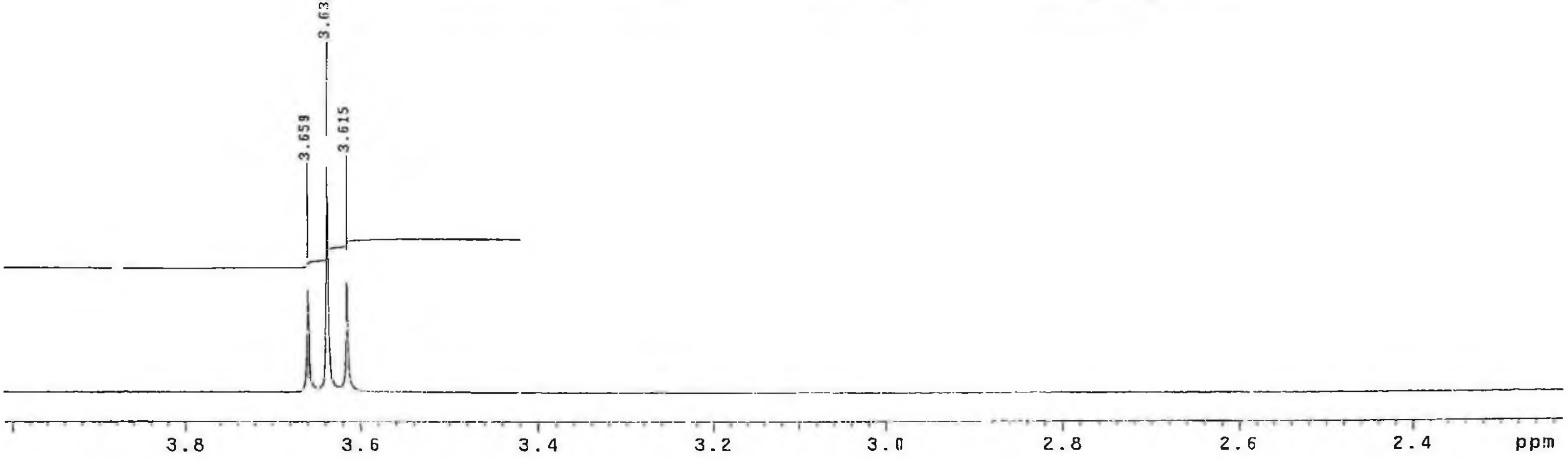
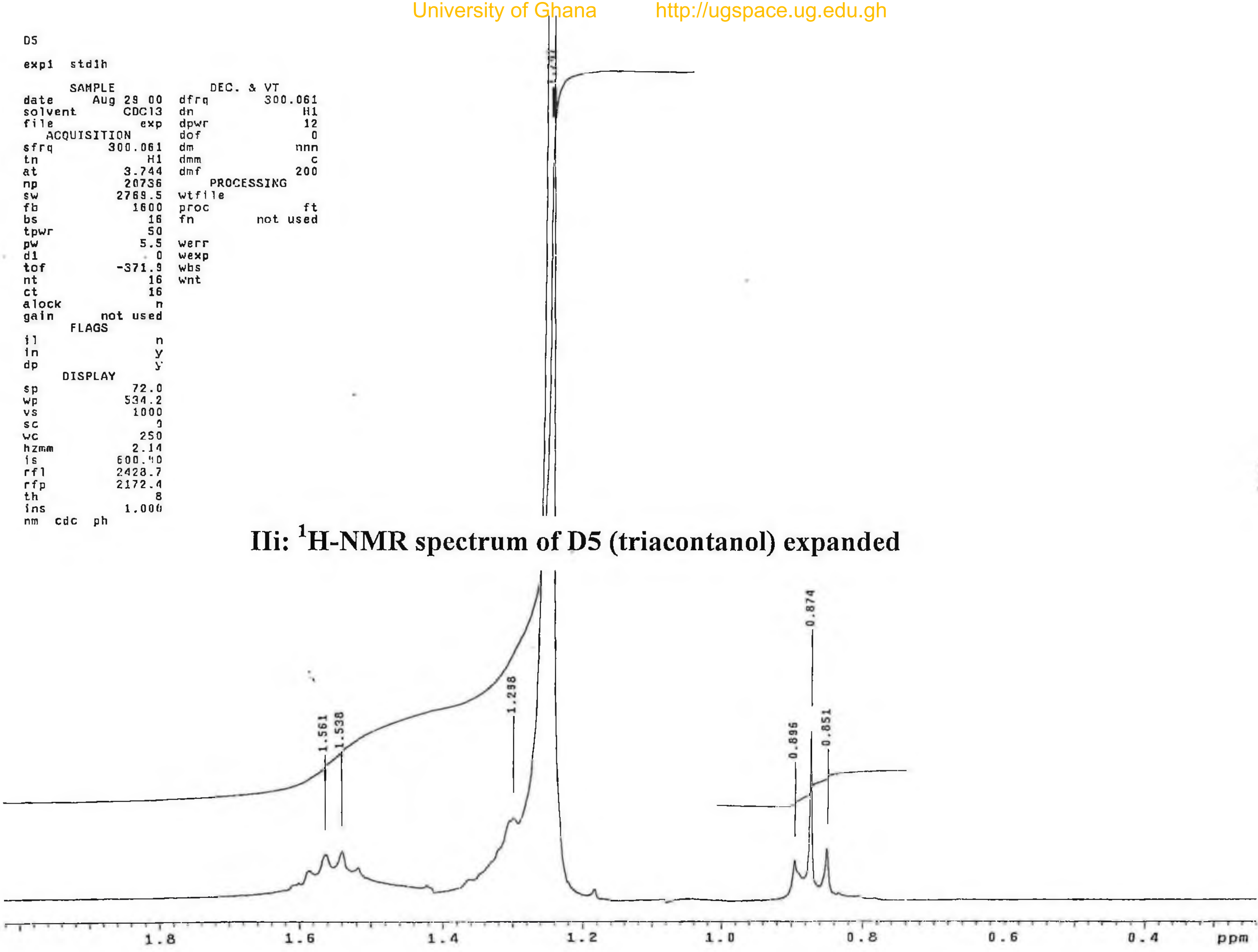
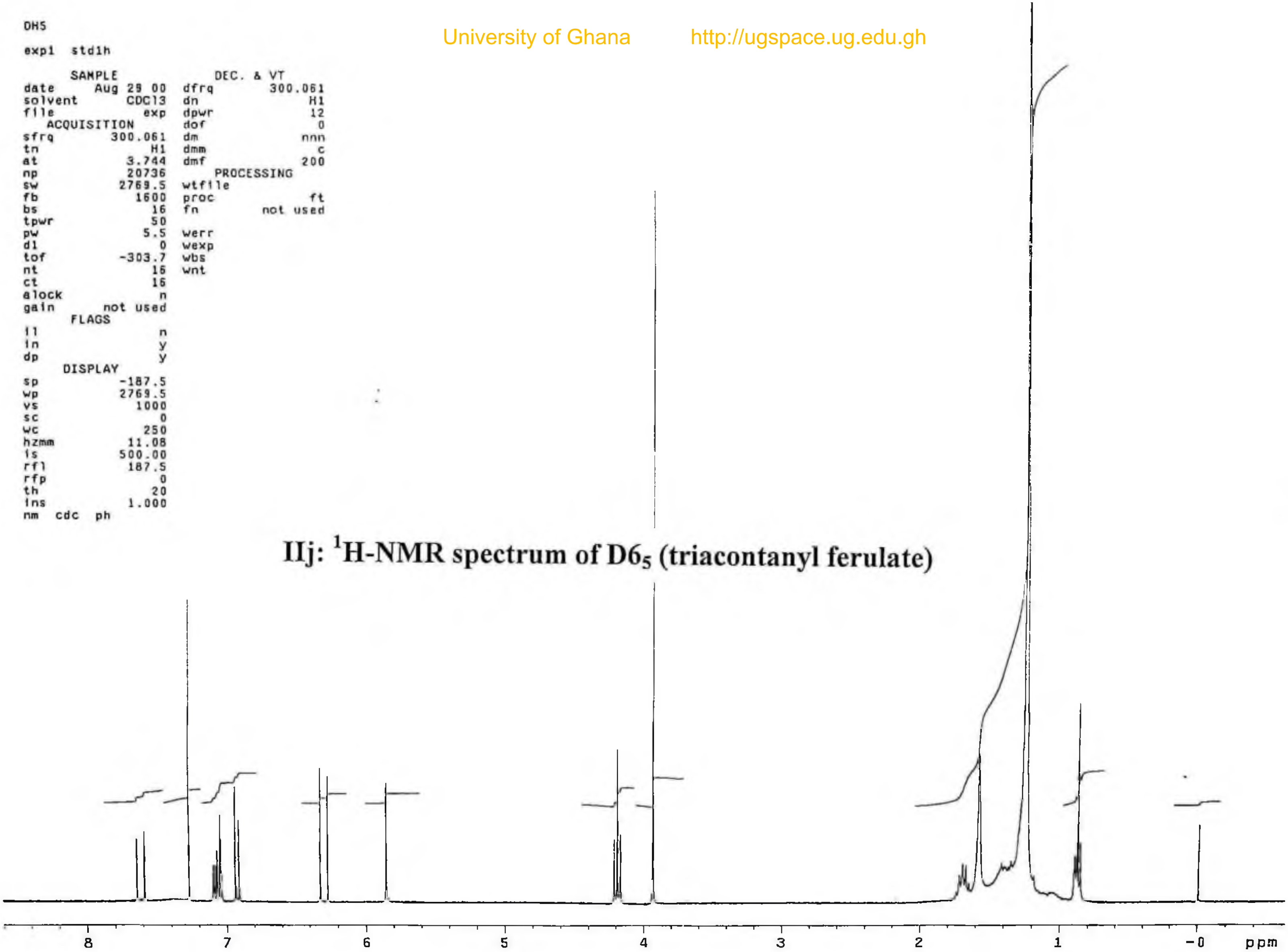

IIh: ^{1}H-NMR spectrum of D5 (triacontanol) expanded



```
D5

exp1  std1h

       SAMPLE               DEC. & VT
date       Aug 29 00  dfrq        300.061
solvent        CDCl3  dn               H1
file             exp  dpwr             12
    ACQUISITION       dof               0
sfrq         300.061  dm              nnn
tn                H1  dmm               c
at             3.744  dmf             200
np             20736      PROCESSING
sw            2769.5  wtfile
fb              1600  proc             ft
bs                16  fn         not used
tpwr              50
pw               5.5  werr
d1                 0  wexp
tof           -371.9  wbs
nt                16  wnt
ct                16
alock              n
gain        not used
       FLAGS
il                 n
in                 y
dp                 y
      DISPLAY
sp              72.0
wp             534.2
vs              1000
sc                 0
wc               250
hzmm            2.14
is            600.40
rfl           2428.7
rfp           2172.4
th                 8
ins            1.000
nm   cdc   ph
```

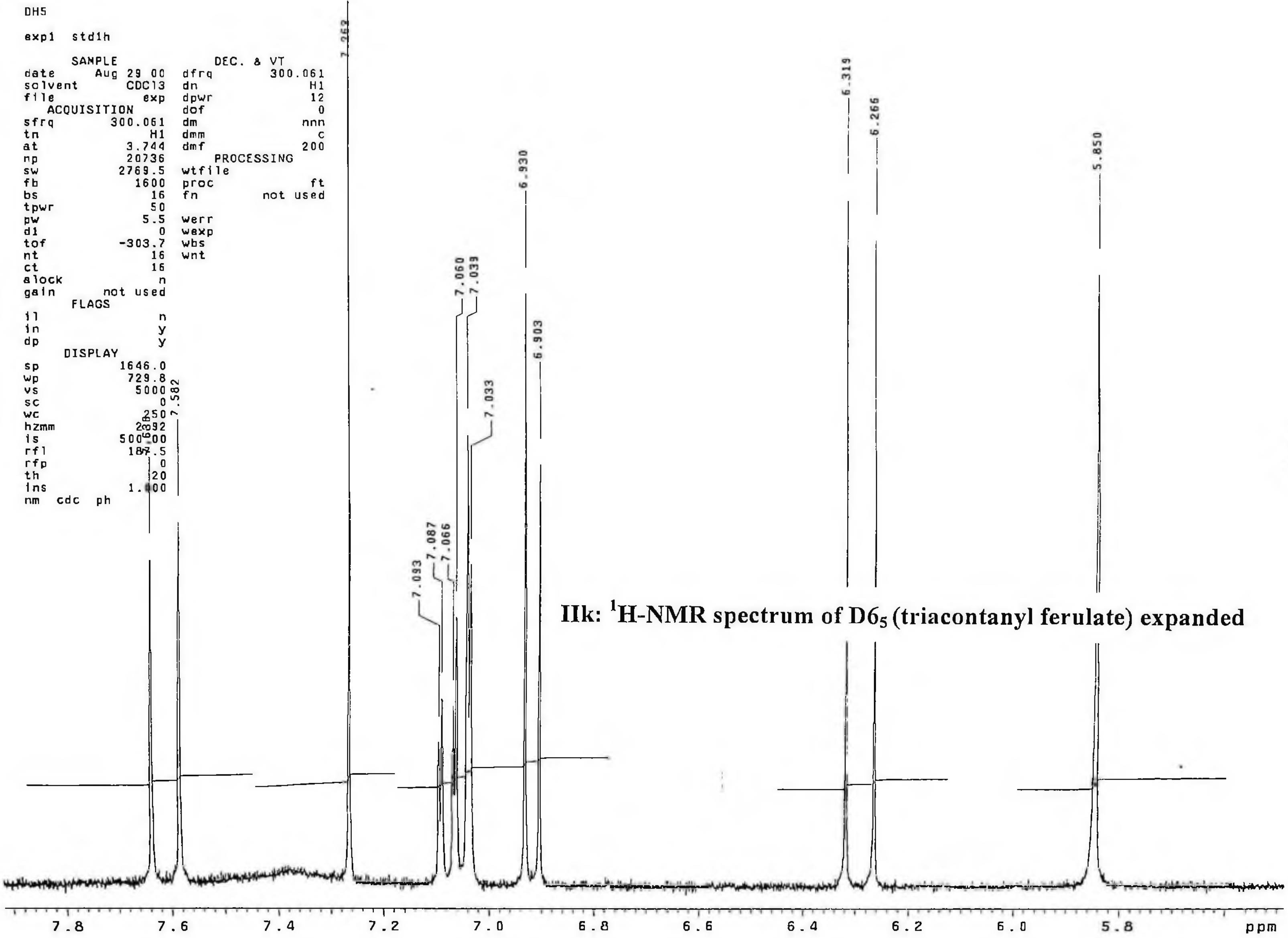


IIi: ^{1}H-NMR spectrum of D5 (triacontanol) expanded



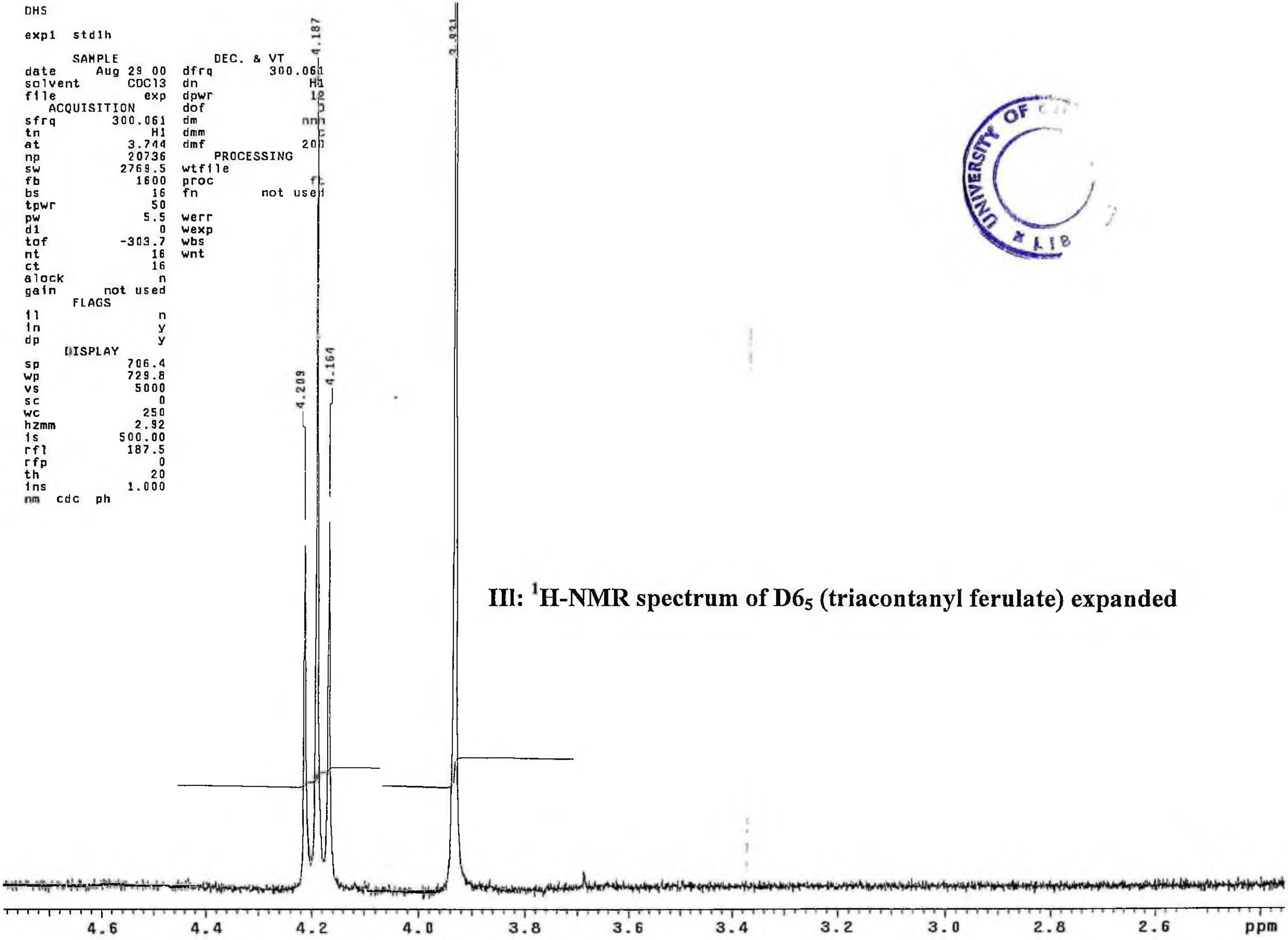
```
DH5

exp1  std1h

        SAMPLE               DEC. & VT
date     Aug 29 00   dfrq        300.061
solvent      CDCl3   dn               H1
file           exp   dpwr             12
   ACQUISITION       dof               0
sfrq       300.061   dm              nnn
tn              H1   dmm               c
at           3.744   dmf             200
np           20736       PROCESSING
sw          2769.5   wtfile
fb            1600   proc             ft
bs              16   fn         not used
tpwr            50
pw             5.5   werr
d1               0   wexp
tof         -303.7   wbs
nt              16   wnt
ct              16
alock            n
gain      not used
        FLAGS
il               n
in               y
dp               y
       DISPLAY
sp          -187.5
wp          2769.5
vs            1000
sc               0
wc             250
hzmm         11.08
is          500.00
rfl          187.5
rfp              0
th              20
ins          1.000
nm   cdc   ph
```

IIj: ^{1}H-NMR spectrum of $D6_5$ (triacontanyl ferulate)



```
DH5

exp1  stdih

      SAMPLE              DEC. & VT
date     Aug 29 00   dfrq       300.061
solvent      CDC13   dn              H1
file           exp   dpwr            12
   ACQUISITION       dof              0
sfrq       300.061   dm             nnn
tn              H1   dmm              c
at           3.744   dmf            200
np           20736        PROCESSING
sw          2769.5   wtfile
fb            1600   proc            ft
bs              16   fn        not used
tpwr            50
pw             5.5   werr
d1               0   wexp
tof         -303.7   wbs
nt              16   wnt
ct              16
alock            n
gain      not used
      FLAGS
il               n
in               y
dp               y
     DISPLAY
sp          1646.0
wp           729.8
vs            5000
sc               0
wc             250
hzmm          2.92
is          500.00
rfl          187.5
rfp              0
th              20
ins          1.000
nm  cdc  ph
```

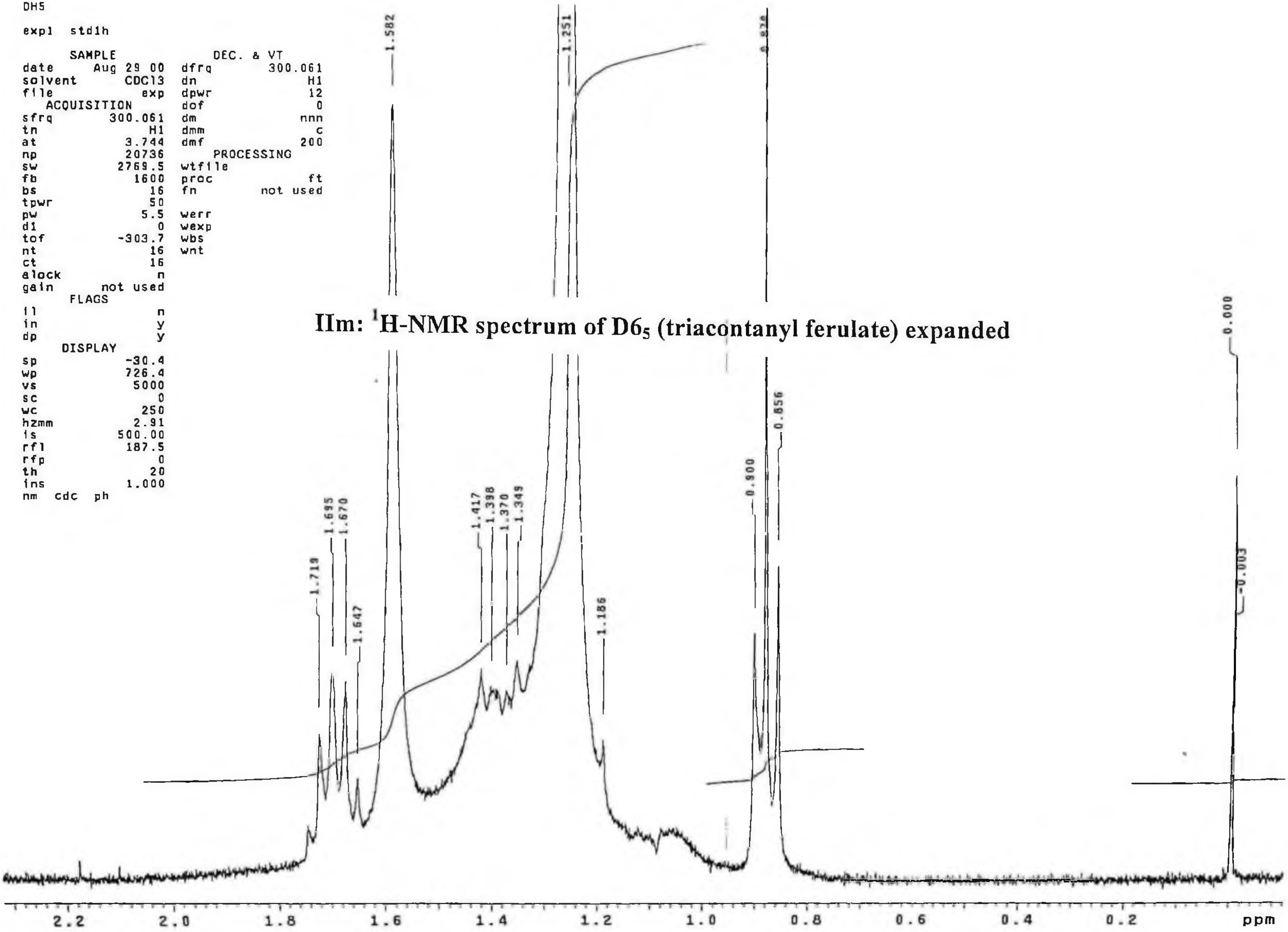
IIk: ^{1}H-NMR spectrum of D6$_5$ (triacontanyl ferulate) expanded



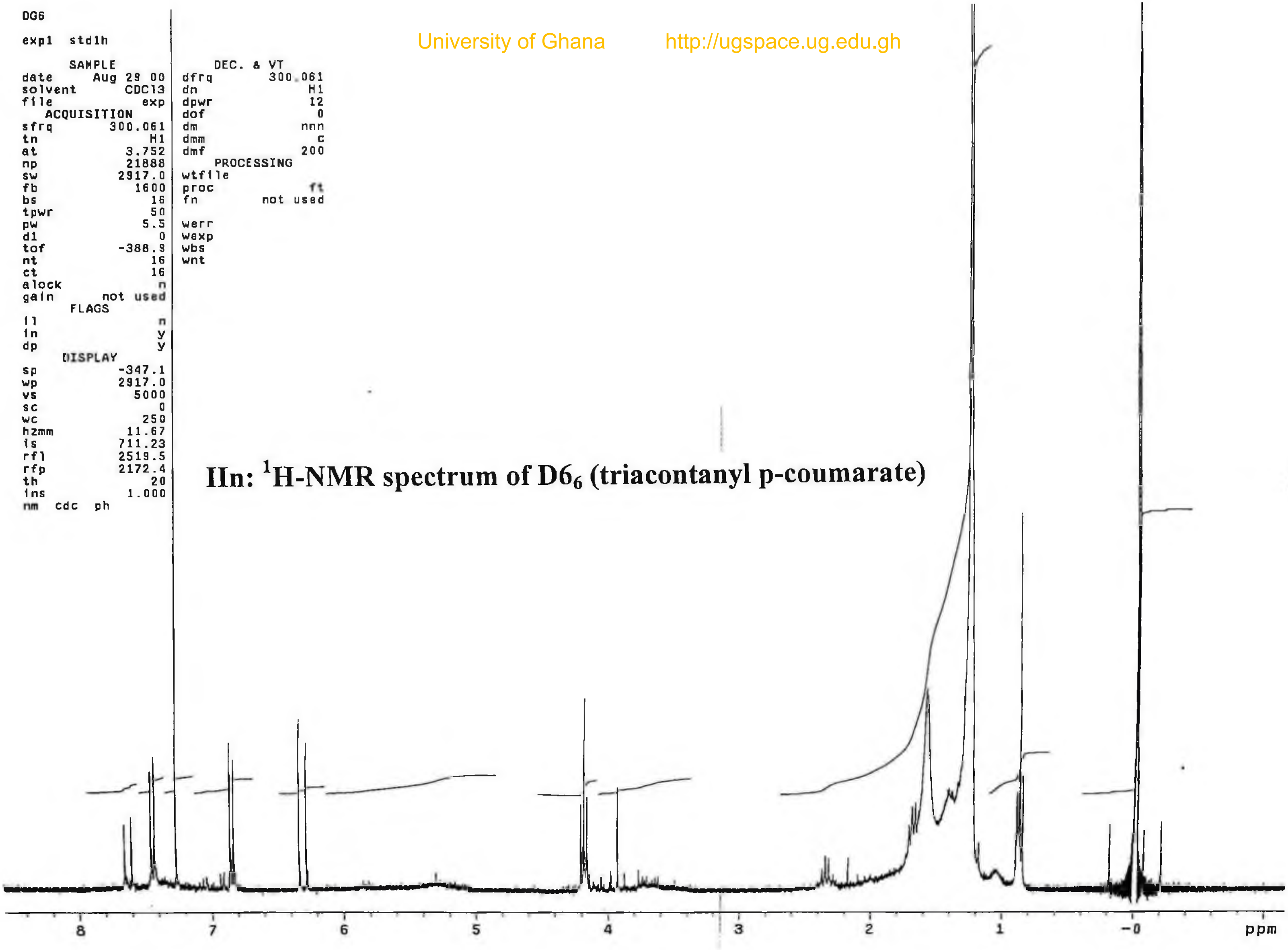
```
DHS

exp1   std1h

        SAMPLE               DEC. & VT
date      Aug 29 00   dfrq        300.061
solvent       CDCl3   dn               H1
file            exp   dpwr             10
     ACQUISITION      dof               0
sfrq        300.061   dm              nnn
tn               H1   dmm               c
at            3.744   dmf             200
np            20736        PROCESSING
sw           2769.5   wtfile
fb             1600   proc             ft
bs               16   fn         not used
tpwr             50
pw              5.5   werr
d1                0   wexp
tof          -303.7   wbs
nt               16   wnt
ct               16
alock             n
gain       not used
        FLAGS
il                n
in                y
dp                y
       DISPLAY
sp            706.4
wp            729.8
vs             5000
sc                0
wc              250
hzmm           2.92
is           500.00
rfl           187.5
rfp               0
th               20
ins           1.000
nm   cdc   ph
```

III: ^{1}H-NMR spectrum of D6$_5$ (triacontanyl ferulate) expanded

```
DH5

expl   std1h

        SAMPLE                DEC. & VT
date      Aug 29 00   dfrq         300.061
solvent       CDCl3   dn                H1
file            exp   dpwr              12
   ACQUISITION        dof                0
sfrq        300.061   dm               nnn
tn               H1   dmm                c
at            3.744   dmf              200
np            20736        PROCESSING
sw           2769.5   wtfile
fb             1600   proc              ft
bs               16   fn          not used
tpwr             50
pw              5.5   werr
d1                0   wexp
tof          -303.7   wbs
nt               16   wnt
ct               16
alock             n
gain       not used
        FLAGS
il                n
in                y
dp                y
      DISPLAY
sp            -30.4
wp            726.4
vs             5000
sc                0
wc              250
hzmm           2.91
is           500.00
rfl           187.5
rfp               0
th               20
ins           1.000
nm   cdc   ph
```

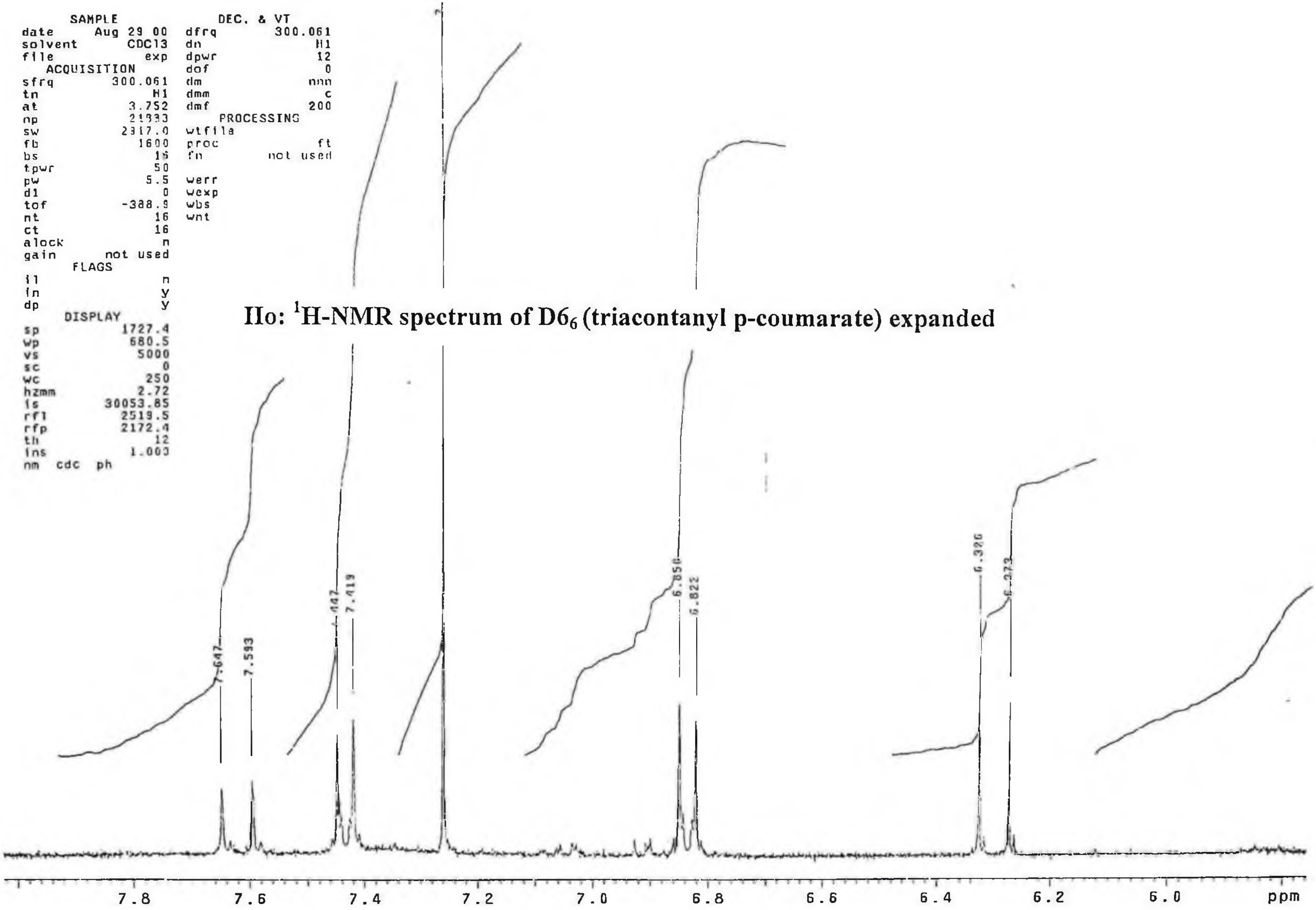


IIm: ^{1}H-NMR spectrum of D6$_5$ (triacontanyl ferulate) expanded



```
DG6

exp1  std1h

      SAMPLE               DEC. & VT
date       Aug 29 00  dfrq        300.061
solvent        CDCl3  dn               H1
file             exp  dpwr             12
   ACQUISITION        dof               0
sfrq         300.061  dm              nnn
tn                H1  dmm               c
at             3.752  dmf             200
np             21888       PROCESSING
sw            2917.0  wtfile
fb              1600  proc             ft
bs                16  fn         not used
tpwr              50
pw               5.5  werr
d1                 0  wexp
tof           -388.9  wbs
nt                16  wnt
ct                16
alock              n
gain        not used
      FLAGS
il                 n
in                 y
dp                 y
     DISPLAY
sp            -347.1
wp            2917.0
vs              5000
sc                 0
wc               250
hzmm           11.67
is            711.23
rfl           2519.5
rfp           2172.4
th                20
ins            1.000
nm    cdc   ph
```

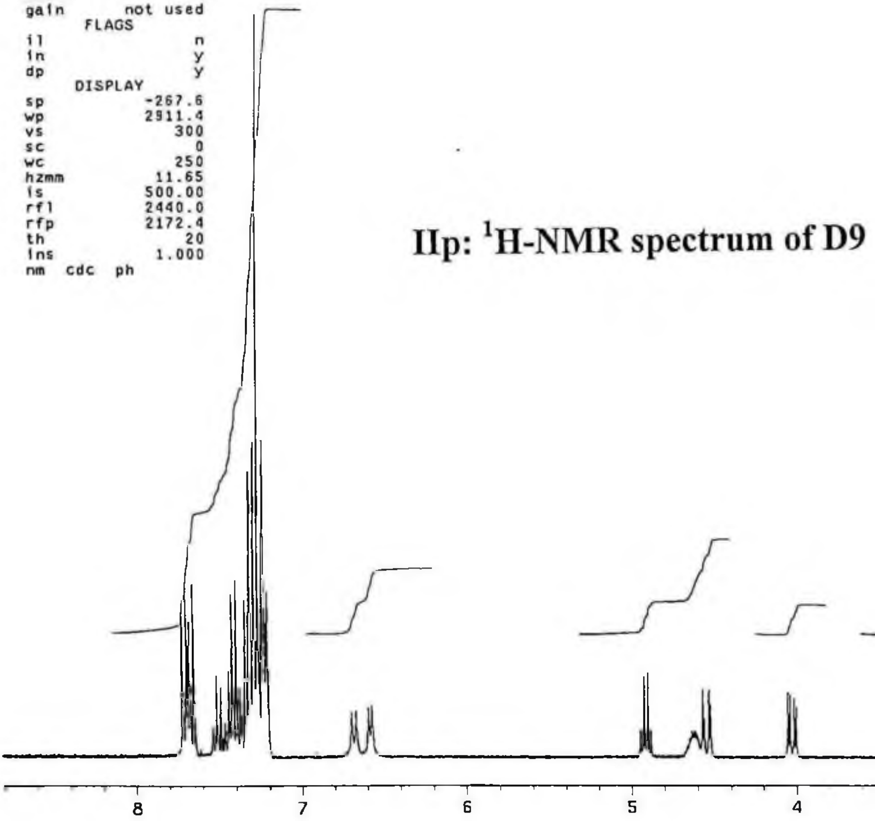
IIn: ^{1}H-NMR spectrum of D6$_6$ (triacontanyl p-coumarate)



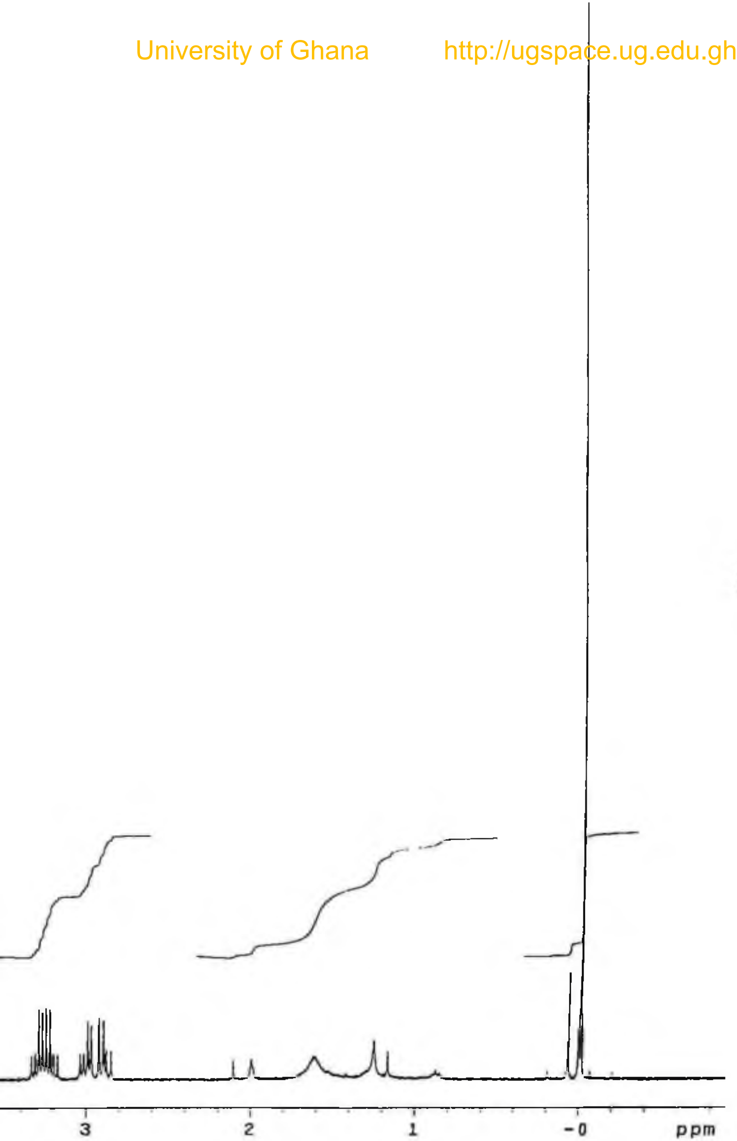
```
      SAMPLE                    DEC. & VT
date       Aug 29 00     dfrq          300.061
solvent        CDCl3     dn                 H1
file             exp     dpwr               12
   ACQUISITION           dof                 0
sfrq         300.061     dm                nnn
tn                H1     dmm                 c
at             3.752     dmf               200
np             21333          PROCESSING
sw            2317.0     wtfile
fb              1600     proc               ft
bs                16     fn           not used
tpwr              50
pw               5.5     werr
d1                 0     wexp
tof           -388.9     wbs
nt                16     wnt
ct                16
alock              n
gain        not used
       FLAGS
il                 n
in                 y
dp                 y
      DISPLAY
sp            1727.4
wp             680.5
vs              5000
sc                 0
wc               250
hzmm            2.72
is          30053.85
rfl           2519.5
rfp           2172.4
th                12
ins            1.000
nm   cdc   ph
```

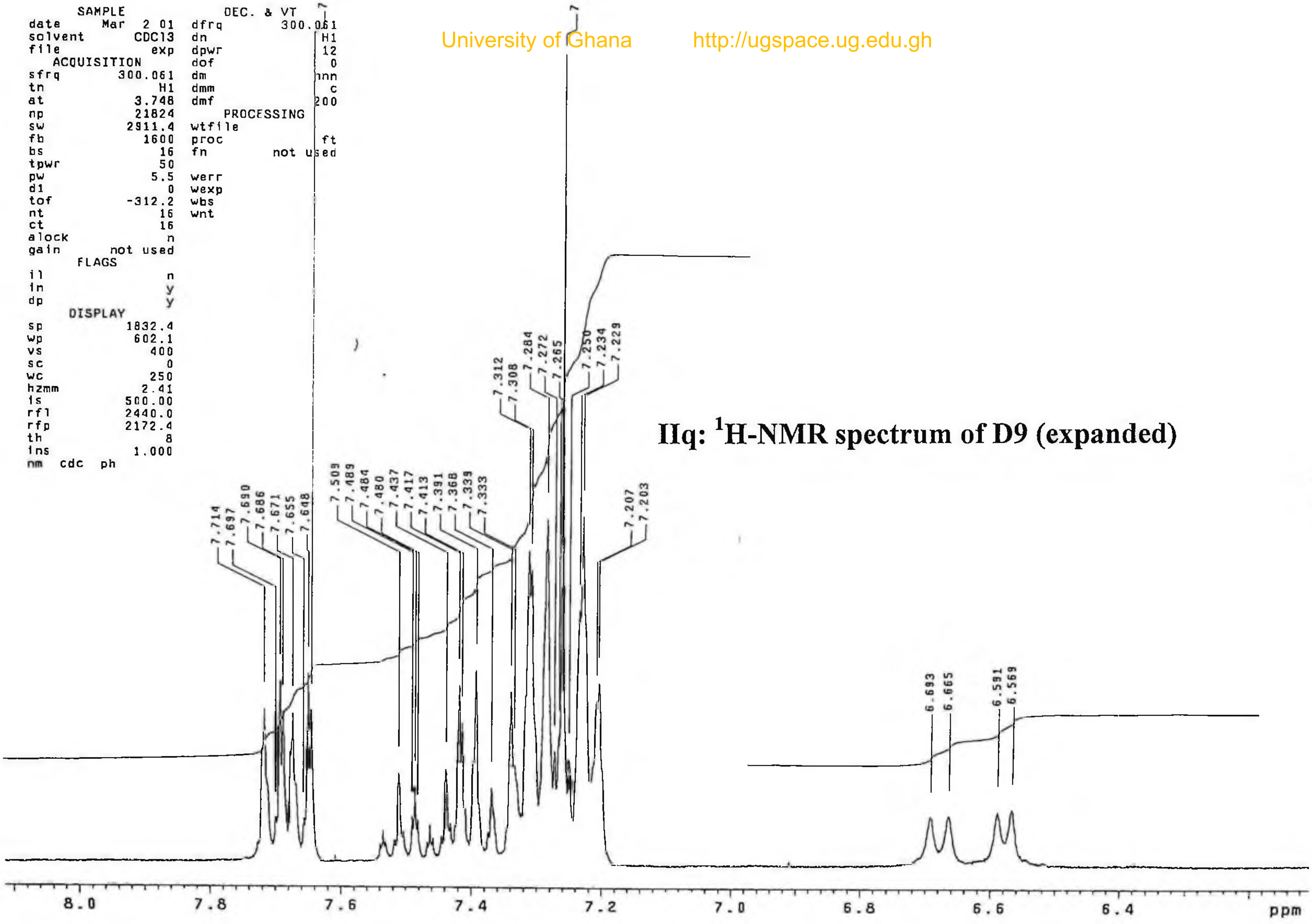
IIo: ^{1}H-NMR spectrum of D6$_{6}$ (triacontanyl p-coumarate) expanded

```
exp3   std1h

        SAMPLE                DEC. & VT
date        Mar  2 01    dfrq       300.061
solvent         CDC13    dn              H1
file              exp    dpwr            12
   ACQUISITION           dof              0
sfrq          300.061    dm             nnn
tn                 H1    dmm              c
at              3.748    dmf            200
np              21824        PROCESSING
sw             2911.4    wtfile
fb               1600    proc            ft
bs                 16    fn        not used
tpwr               50
pw                5.5    werr
d1                  0    wexp
tof            -312.2    wbs
nt                 16    wnt
ct                 16
alock               n
gain         not used
        FLAGS
il                  n
in                  y
dp                  y
       DISPLAY
sp             -267.6
wp             2911.4
vs                300
sc                  0
wc                250
hzmm            11.65
is             500.00
rfl            2440.0
rfp            2172.4
th                 20
ins             1.000
nm   cdc   ph
```



IIp: ^{1}H-NMR spectrum of D9


3
2
1
-0
ppm

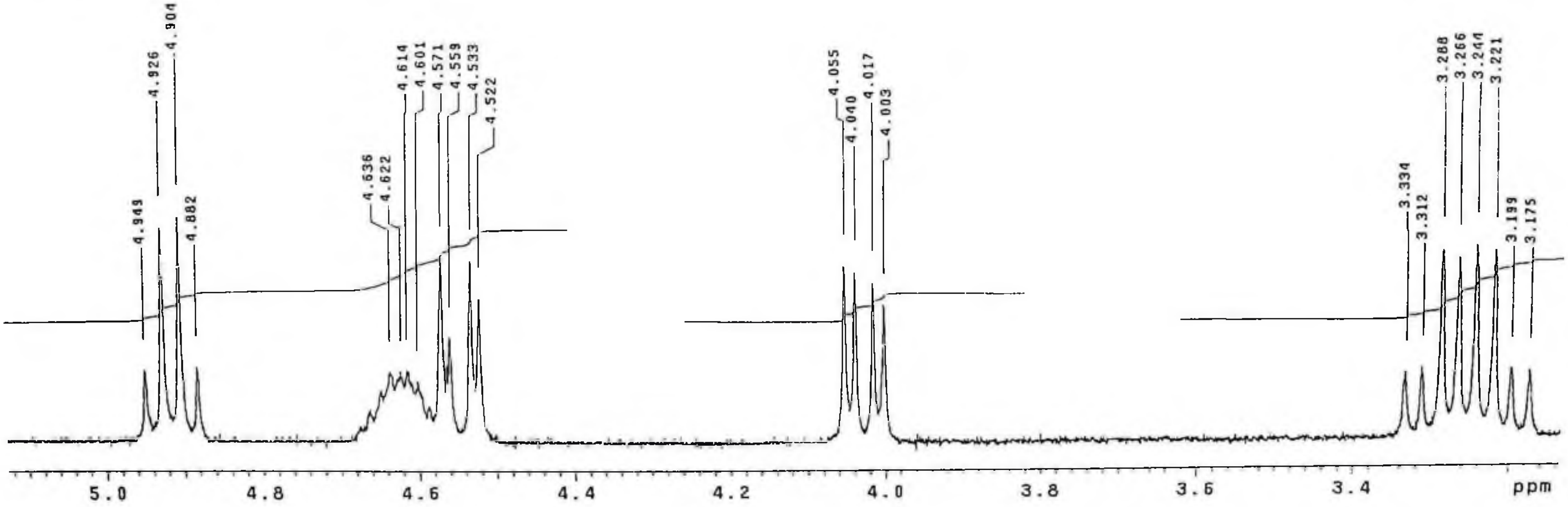


```
       SAMPLE                  DEC. & VT
date       Mar  2 01   dfrq          300.061
solvent        CDCl3   dn                 H1
file             exp   dpwr               12
   ACQUISITION         dof                 0
sfrq         300.061   dm                nnn
tn                H1   dmm                 c
at             3.748   dmf               200
np             21824       PROCESSING
sw            2911.4   wtfile
fb              1600   proc               ft
bs                16   fn           not used
tpwr              50
pw               5.5   werr
d1                 0   wexp
tof           -312.2   wbs
nt                16   wnt
ct                16
alock              n
gain        not used
      FLAGS
il                 n
in                 y
dp                 y
     DISPLAY
sp            1832.4
wp             602.1
vs               400
sc                 0
wc               250
hzmm            2.41
is            500.00
rfl           2440.0
rfp           2172.4
th                 8
ins            1.000
nm   cdc   ph
```

IIq: ^{1}H-NMR spectrum of D9 (expanded)

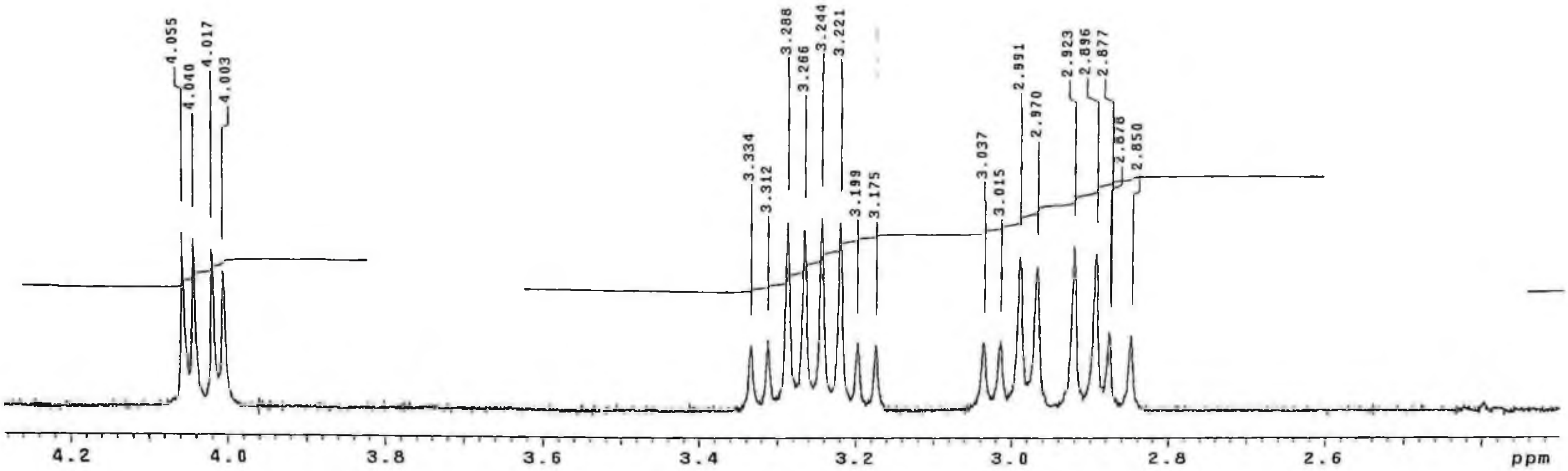
```
        SAMPLE                  DEC. & VT
date      Mar   2 01    dfrq          300.061
solvent        CDCl3    dn                 H1
file             exp    dpwr               12
   ACQUISITION          dof                 0
sfrq         300.061    dm                nnn
tn                H1    dmm                 c
at             3.748    dmf               200
np             21824        PROCESSING
sw            2911.4    wtfile
fb              1600    proc               ft
bs                16    fn           not used
tpwr              50
pw               5.5    werr
d1                 0    wexp
tof           -312.2    wbs
nt                16    wnt
ct                16
alock              n
gain        not used
       FLAGS
il                 n
in                 y
dp                 y
       DISPLAY
sp             940.1
wp             598.3
vs               800
sc                 0
wc               250
hzmm            2.39
is            500.00
rfl           2440.0
rfp           2172.4
th                 8
ins            1.000
nm   cdc   ph
```

IIr: ^{1}H-NMR spectrum of D9 (expanded)



```
       SAMPLE              DEC. & VT
date       Mar  2 01   dfrq          300.061
solvent       CDCl3    dn                 H1
file            exp    dpwr               12
    ACQUISITION        dof                 0
sfrq        300.061    dm                nnn
tn               H1    dmm                 c
at            3.748    dmf               200
np            21824        PROCESSING
sw           2911.4    wtfile
fb             1600    proc               ft
bs               16    fn           not used
tpwr             50
pw              5.5    werr
d1                0    wexp
tof          -312.2    wbs
nt               16    wnt
ct               16
alock             n
gain       not used
      FLAGS
il                n
in                y
dp                y
     DISPLAY
sp            690.6
wp            594.6
vs              800
sc                0
wc              250
hzmm           2.38
is           500.00
rfl          2440.0
rfp          2172.4
th                8
ins           1.000
nm   cdc   ph
```

IIs: ^{1}H-NMR spectrum of D9 (expanded)

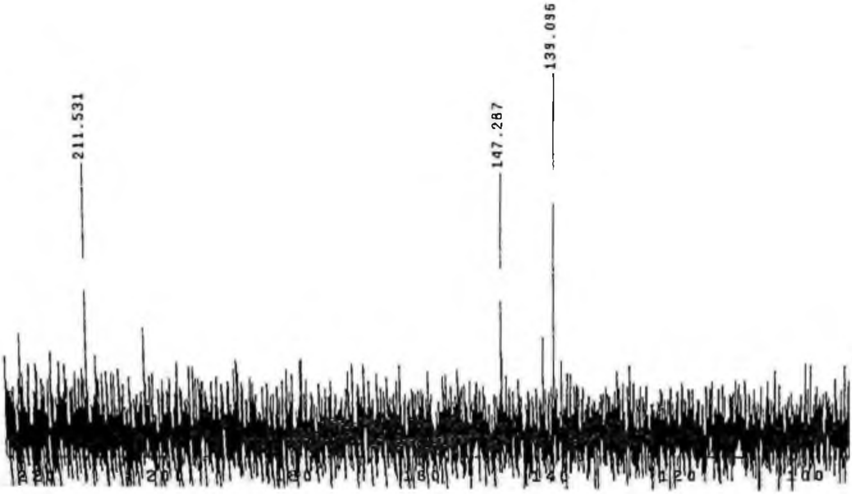


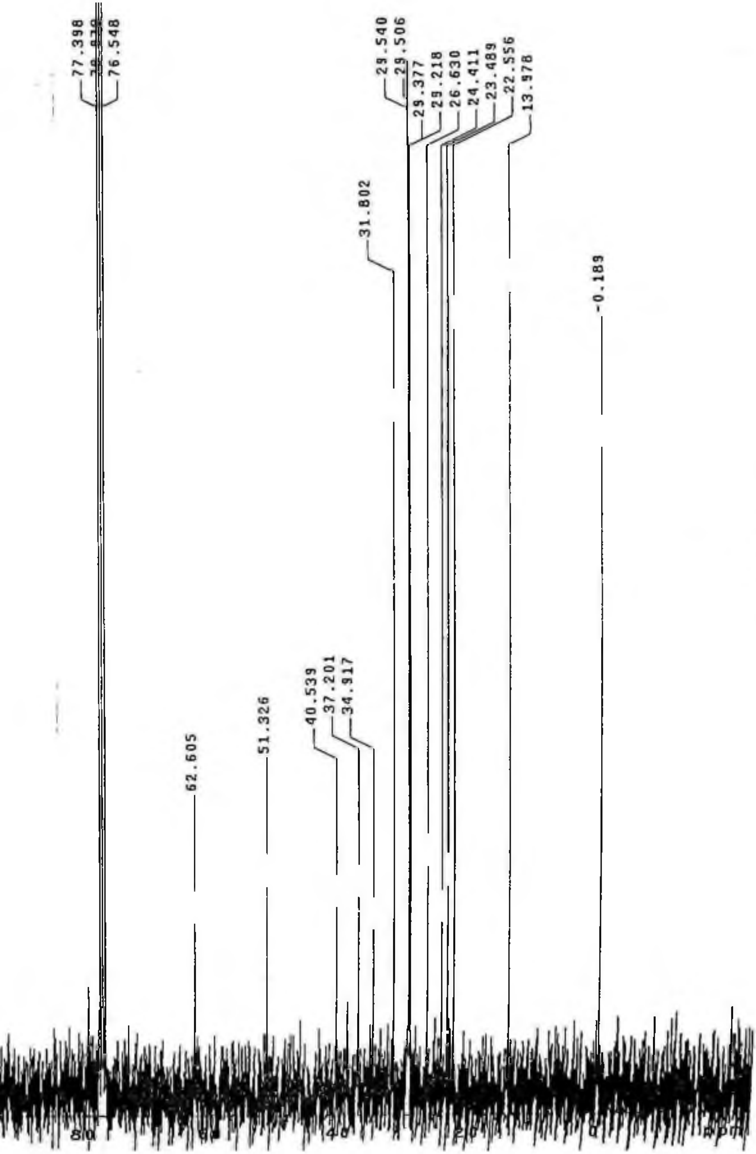
APPENDIX III

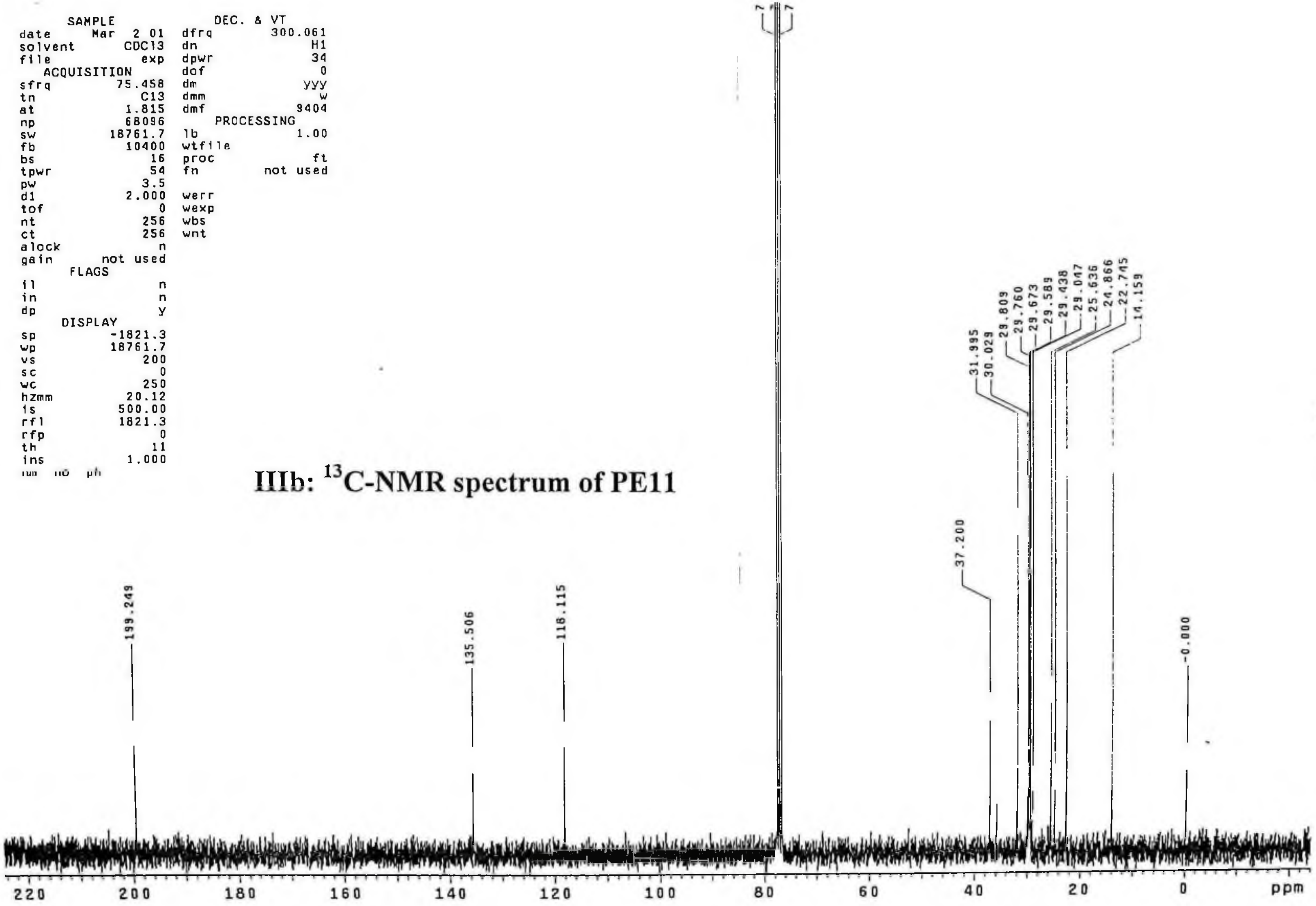
^{13}C-NMR SPECTRA

```
PE6

exp2   std13c

        SAMPLE                      DEC. & VT
date         Apr 23 01   dfrq           300.061
solvent          CDCl3   dn                  H1
file               exp   dpwr                34
    ACQUISITION          dof                  0
sfrq            75.458   dm                 yyy
tn                 C13   dmm                  w
at               1.815   dmf               9404
np               68096        PROCESSING
sw             18761.7   lb                1.00
fb               10400   wtfile
bs                  16   proc                ft
tpwr                54   fn            not used
pw                 3.5
d1               2.000   werr
tof                  0   wexp
nt                 256   wbs
ct                 160   wnt
alock                n
gain          not used
        FLAGS
il                   n
in                   n
dp                   y
       DISPLAY
sp             -1835.3
wp             18761.7
vs                 500
sc                   0
wc                 250
hzmm             20.12
is              500.00
rfl             7645.6
rfp             5810.2
th                  20
ins              1.000
nm   no   ph
```

IIIa: ^{13}C-NMR spectrum of PE6



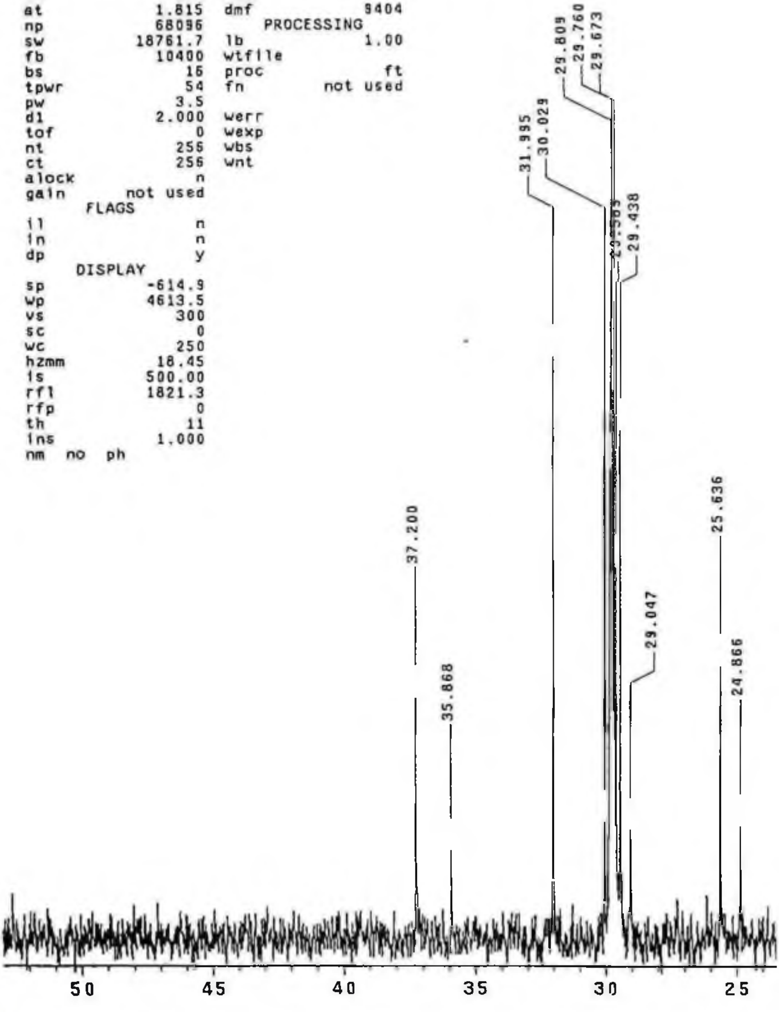

77.398
76.548
62.605
51.326
40.539
37.201
34.917
31.802
29.540
29.506
29.377
29.218
26.630
24.411
23.489
22.556
13.978
-0.189
80
60
40
20
0
ppm

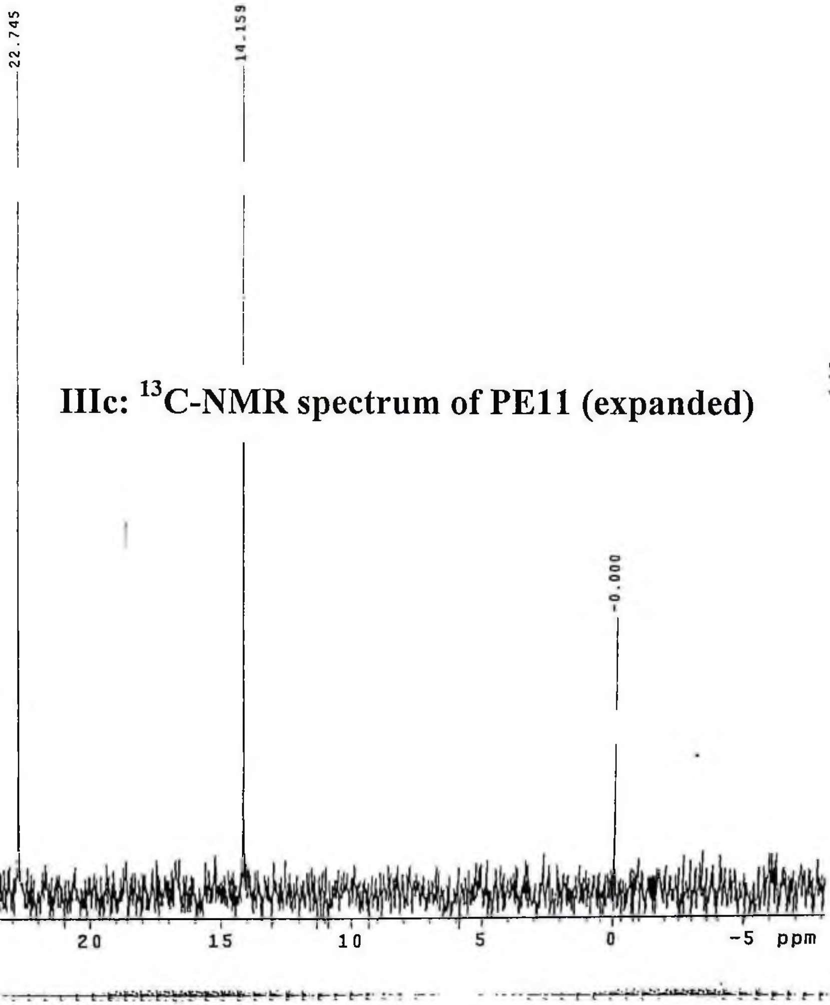


```
       SAMPLE                      DEC. & VT
date      Mar  2 01       dfrq           300.061
solvent       CDCl3       dn                  H1
file            exp       dpwr                34
     ACQUISITION          dof                  0
sfrq         75.458       dm                 yyy
tn              C13       dmm                  w
at            1.815       dmf               9404
np            68096             PROCESSING
sw          18761.7       lb                1.00
fb            10400       wtfile
bs               16       proc                ft
tpwr             54       fn            not used
pw              3.5
d1            2.000       werr
tof               0       wexp
nt              256       wbs
ct              256       wnt
alock             n
gain       not used
        FLAGS
il                n
in                n
dp                y
       DISPLAY
sp          -1821.3
wp          18761.7
vs              200
sc                0
wc              250
hzmm          20.12
is           500.00
rfl          1821.3
rfp               0
th               11
ins           1.000
nm    no    ph
```

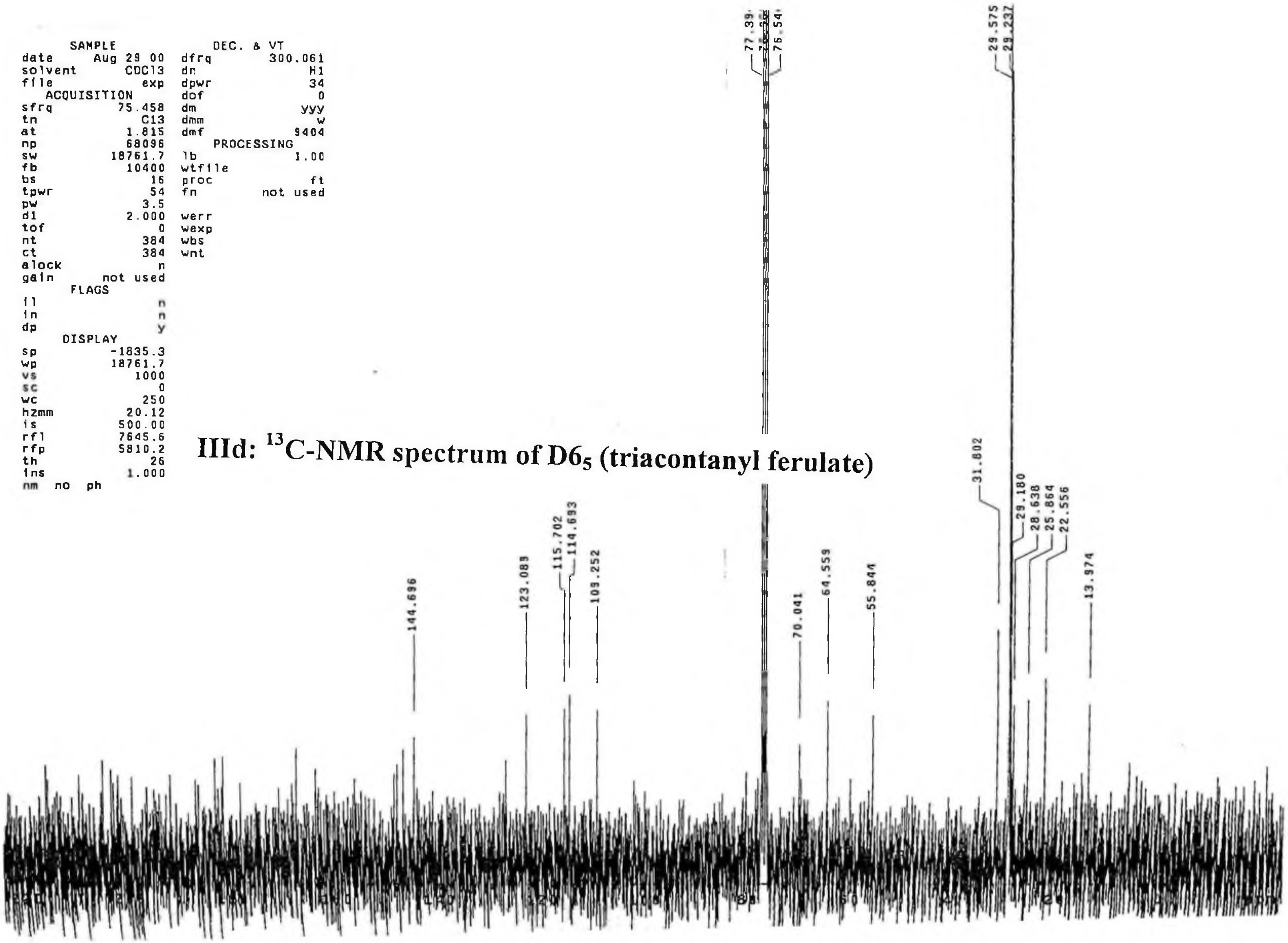
IIIb: ^{13}C-NMR spectrum of PE11

```
         SAMPLE                    DEC. & VT
date        Mar   2 01    dfrq           300.061
solvent          CDCl3    dn                  H1
file               exp    dpwr                34
      ACQUISITION         dof                  0
sfrq            75.458    dm                 yyy
tn                 C13    dmm                  w
at               1.815    dmf               9404
np               68096         PROCESSING
sw             18761.7    lb                1.00
fb               10400    wtfile
bs                  16    proc                ft
tpwr                54    fn            not used
pw                 3.5
d1               2.000    werr
tof                  0    wexp
nt                 256    wbs
ct                 256    wnt
alock                n
gain          not used
         FLAGS
il                   n
in                   n
dp                   y
        DISPLAY
sp              -614.9
wp              4613.5
vs                 300
sc                   0
wc                 250
hzmm             18.45
is              500.00
rfl             1821.3
rfp                  0
th                  11
ins              1.000
nm   no   ph
```



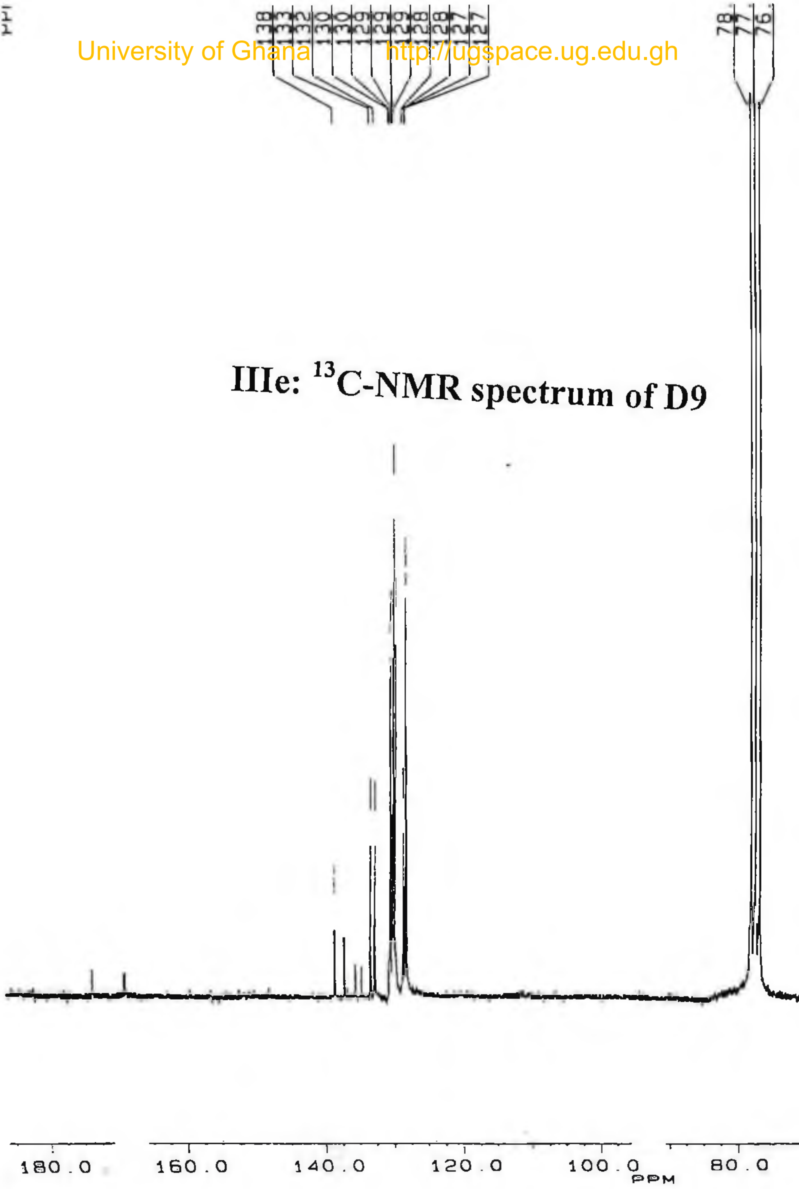


IIIc: ^{13}C-NMR spectrum of PE11 (expanded)



```
      SAMPLE                DEC. & VT
date      Aug 29 00   dfrq         300.061
solvent       CDCl3   dn                H1
file            exp   dpwr              34
   ACQUISITION        dof                0
sfrq         75.458   dm               yyy
tn              C13   dmm                w
at            1.815   dmf             9404
np            68096         PROCESSING
sw          18761.7   lb              1.00
fb            10400   wtfile
bs               16   proc              ft
tpwr             54   fn          not used
pw              3.5
d1            2.000   werr
tof               0   wexp
nt              384   wbs
ct              384   wnt
alock             n
gain       not used
      FLAGS
il                n
in                n
dp                y
     DISPLAY
sp          -1835.3
wp          18761.7
vs             1000
sc                0
wc              250
hzmm          20.12
is           500.00
rfl          7645.6
rfp          5810.2
th               26
ins           1.000
nm   no   ph
```

IIId: ^{13}C-NMR spectrum of D6$_5$ (triacontanyl ferulate)



IIIe: ^{13}C-NMR spectrum of D9

29

BRUKER

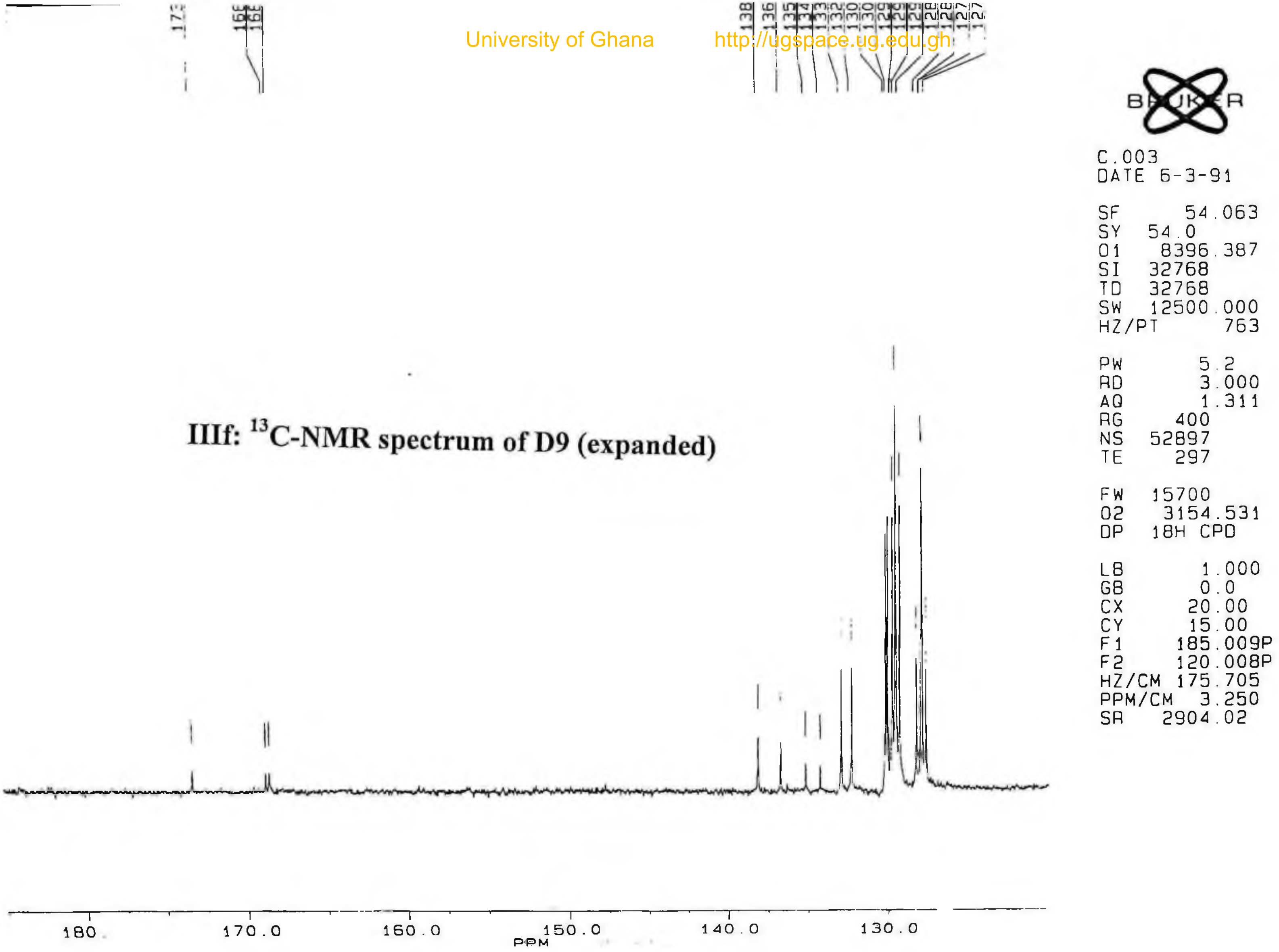
NITROB
DATE 6-3-91

SF 54.063
SY 54.0
O1 8396.387
SI 32768
TD 32768
SW 12500.000
HZ/PT 763

PW 5.2
RD 3.000
AQ 1.311
RG 400
NS 52897
TE 297

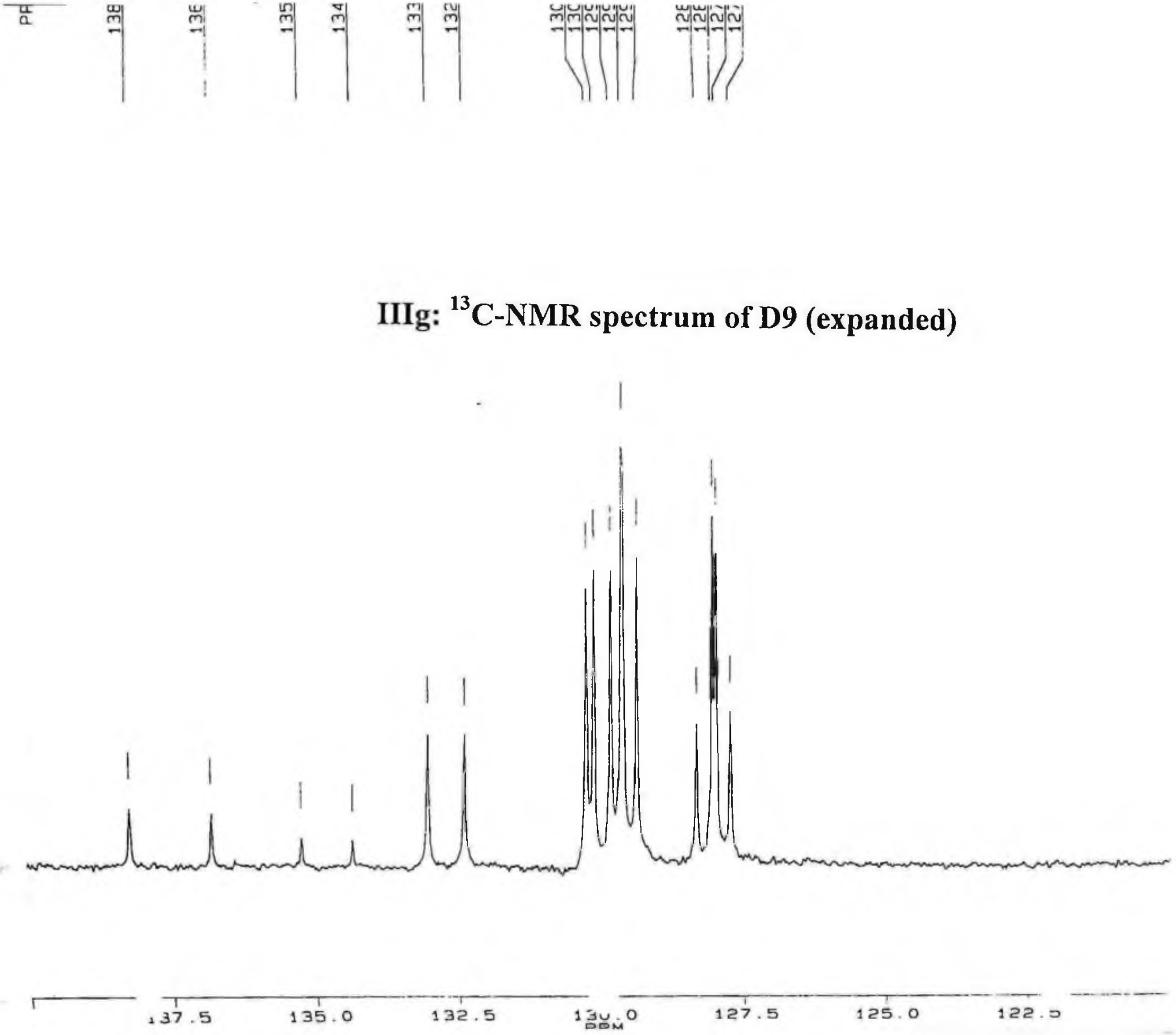
FW 15700
O2 3154.531
DP 18H CPD

LB 1.000
GB 0.0
CX 20.00
CY 15.00
F1 186.180P
F2 -.918P
HZ/CM 505.753
PPM/CM 9.355
SR 2904.02



```
C.003
DATE  6-3-91

SF        54.063
SY    54.0
O1      8396.387
SI     32768
TD     32768
SW     12500.000
HZ/PT        763

PW          5.2
RD          3.000
AQ          1.311
RG        400
NS      52897
TE        297

FW      15700
O2       3154.531
DP     18H  CPD

LB          1.000
GB          0.0
CX         20.00
CY         15.00
F1        185.009P
F2        120.008P
HZ/CM   175.705
PPM/CM    3.250
SR       2904.02
```

IIIf: ^{13}C-NMR spectrum of D9 (expanded)

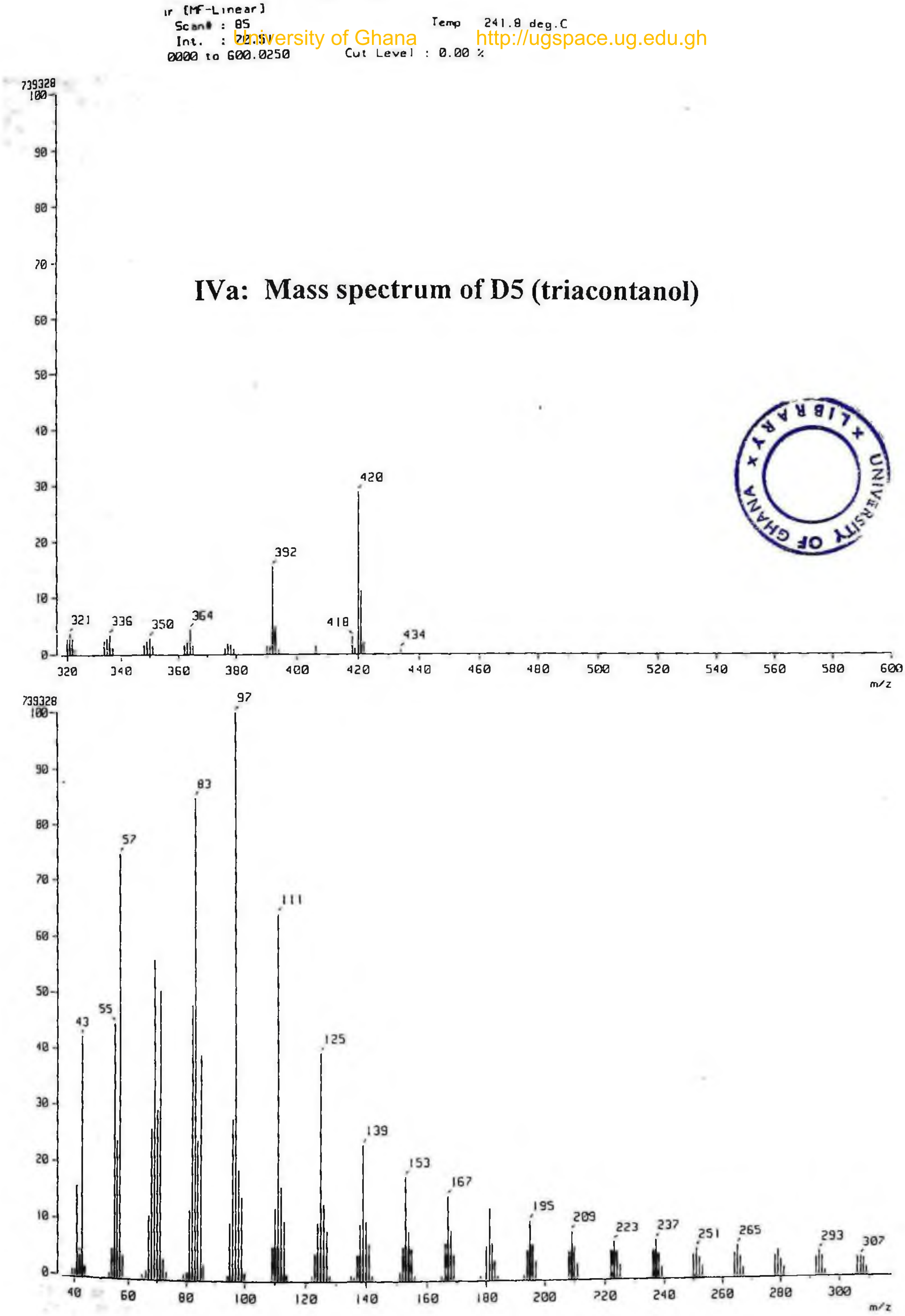


IIIg: ^{13}C-NMR spectrum of D9 (expanded)

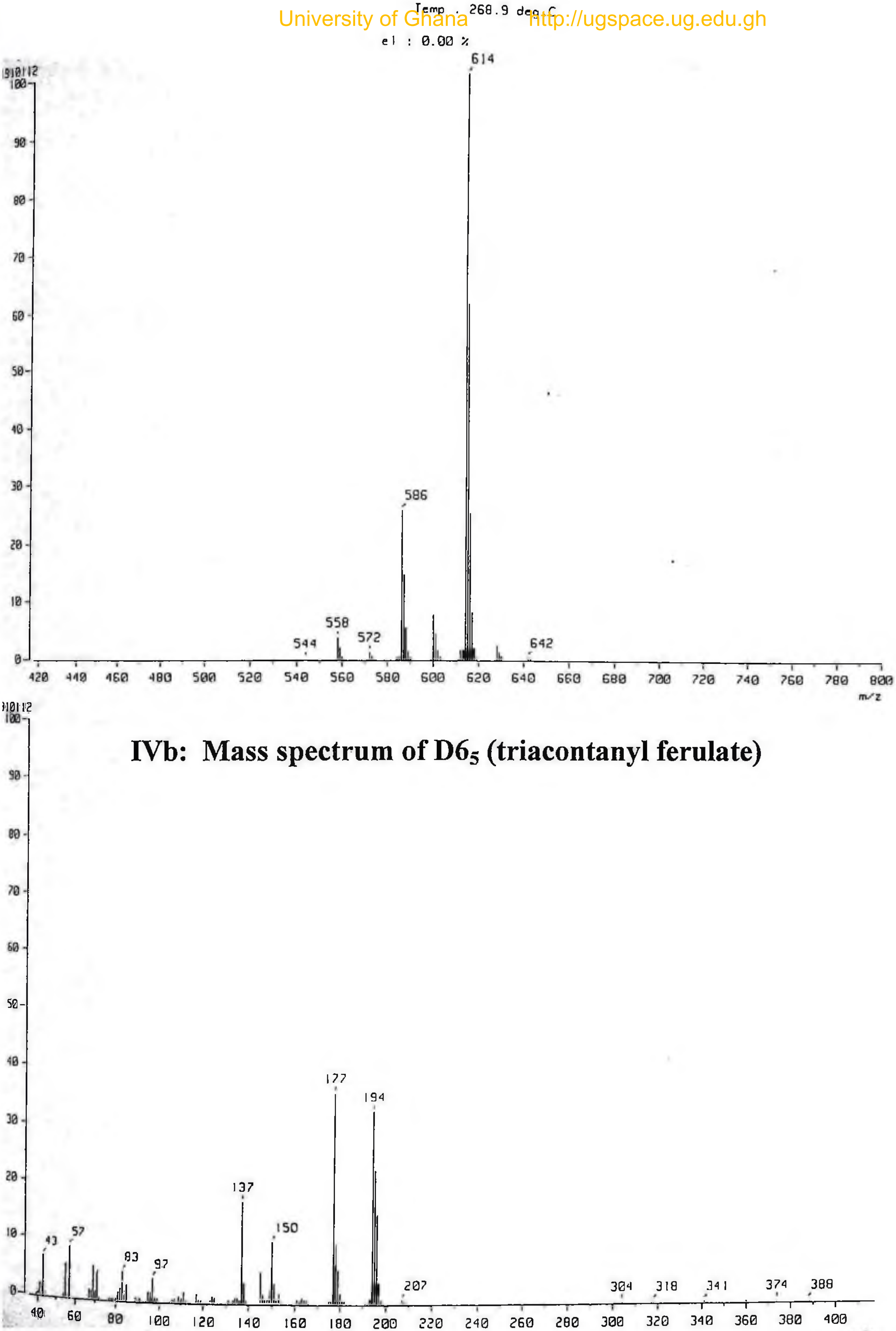
```
BRUKER

C.003
DATE  6-3-91

SF       54.063
SY    54.0
O1     8396.387
SI    32768
TD    32768
SW    12500.000
HZ/PT       763

PW          5.2
RD          3.000
AQ          1.311
RG        400
NS    52897
TE        297

FW    15700
O2     3154.531
DP    18H CPD

LB          1.000
GB          0.0
CX         20.00
CY         15.00
F1        140.005P
F2        120.023P
HZ/CM      54.016
PPM/CM       .999
SR     2904.02
```

APPENDIX IV

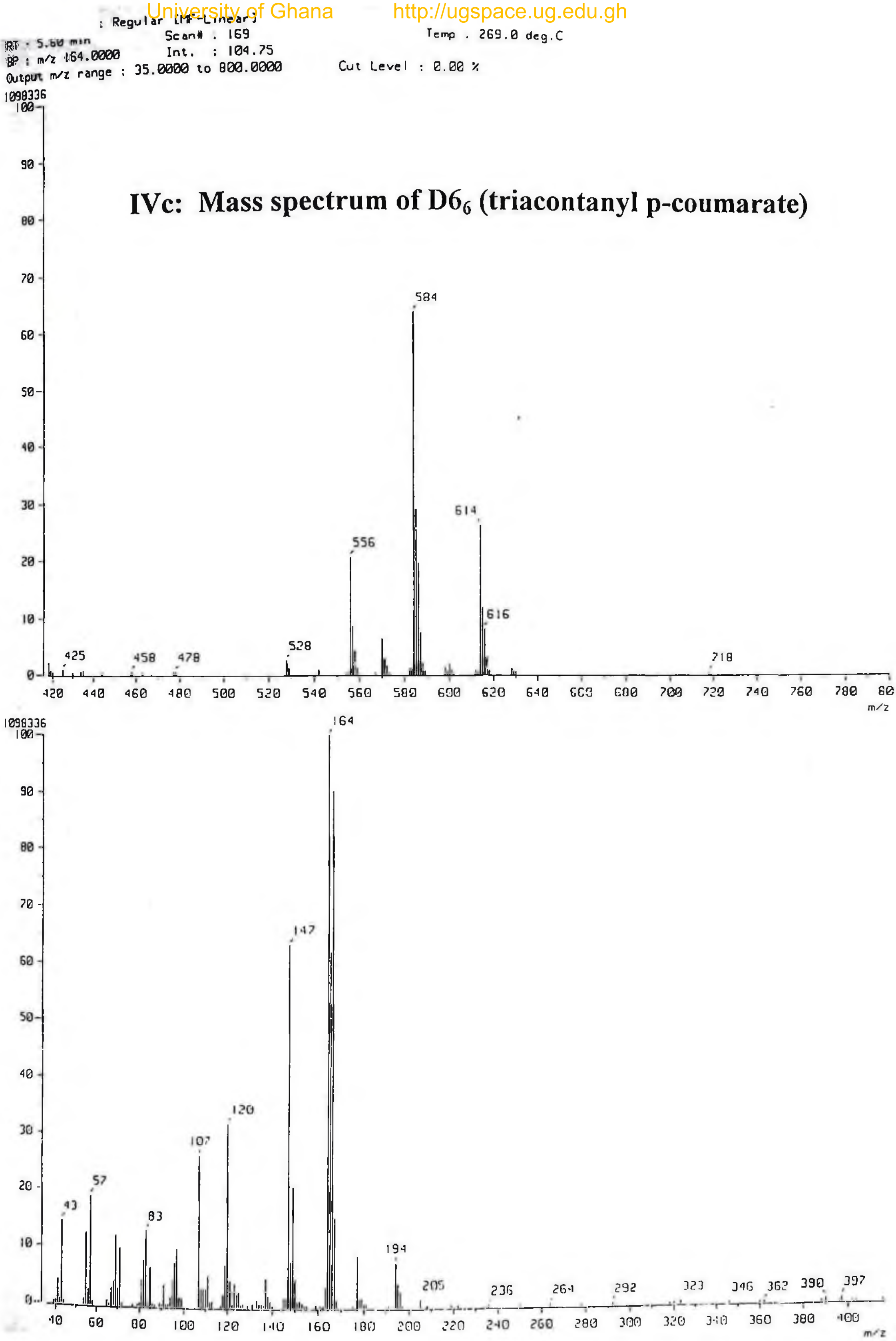
MASS SPECTRA



IVa: Mass spectrum of D5 (triacontanol)



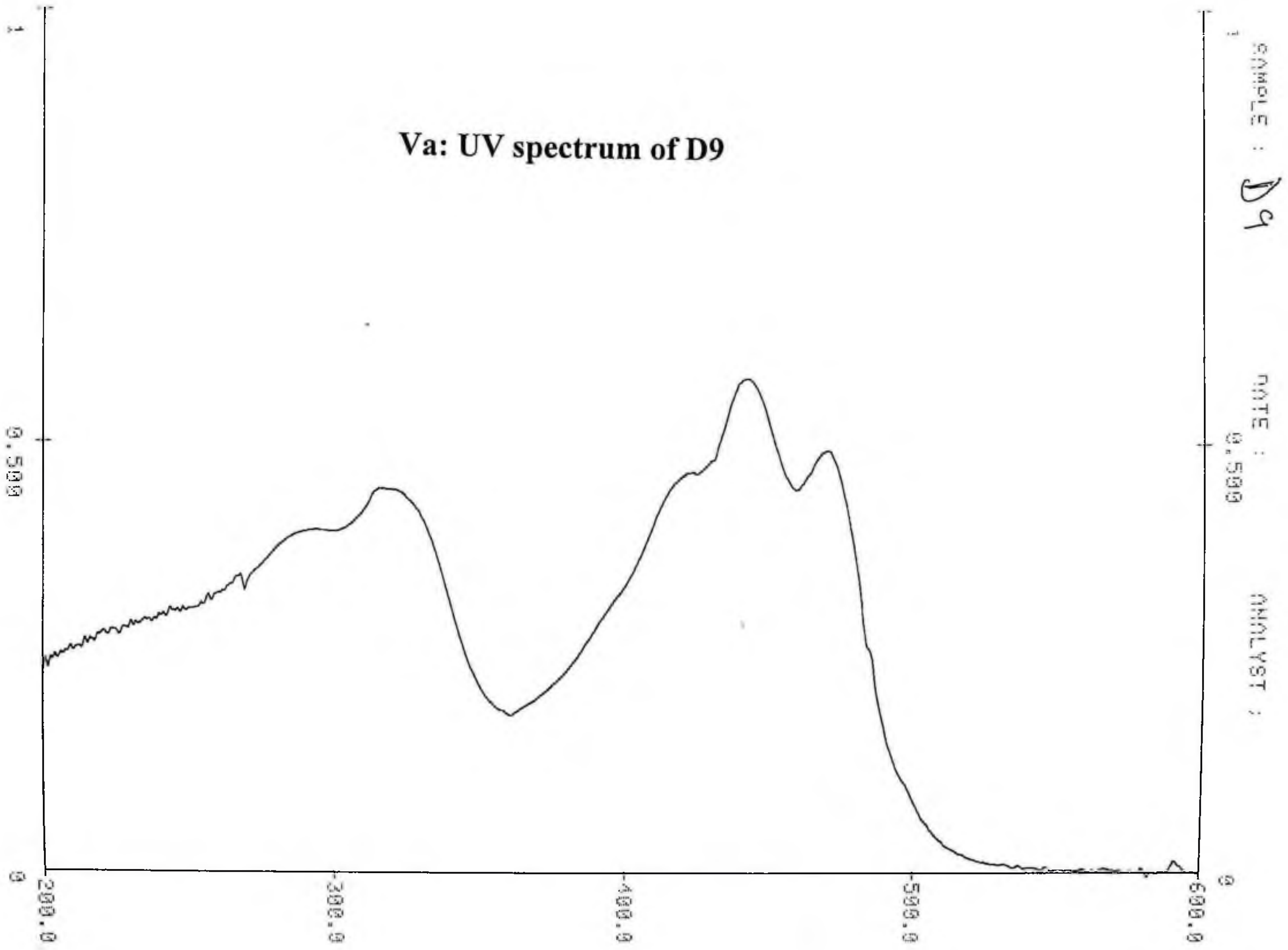
IVb: Mass spectrum of D6$_5$ (triacontanyl ferulate)



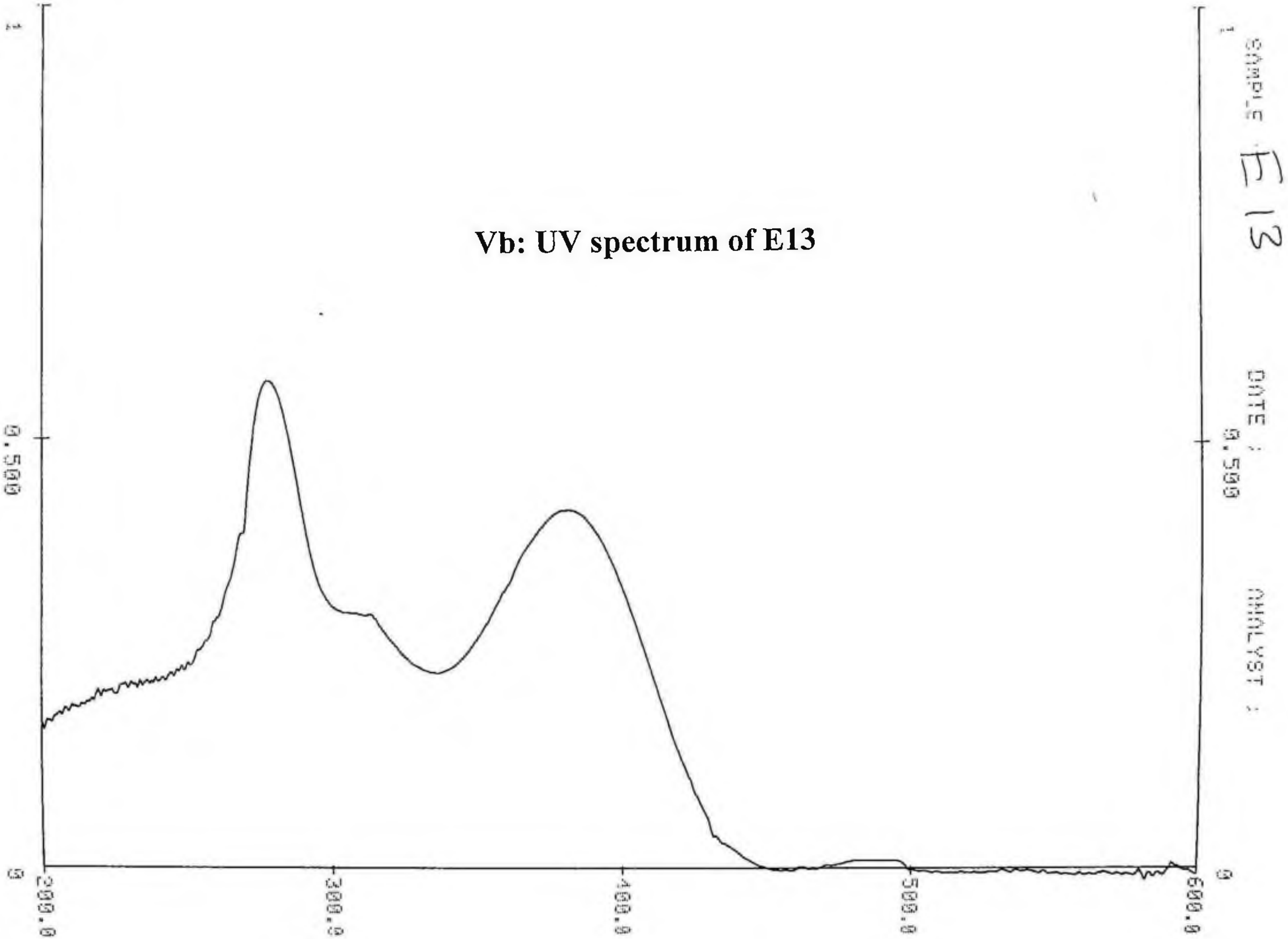
IVc: Mass spectrum of $D6_6$ (triacontanyl p-coumarate)

APPENDIX V

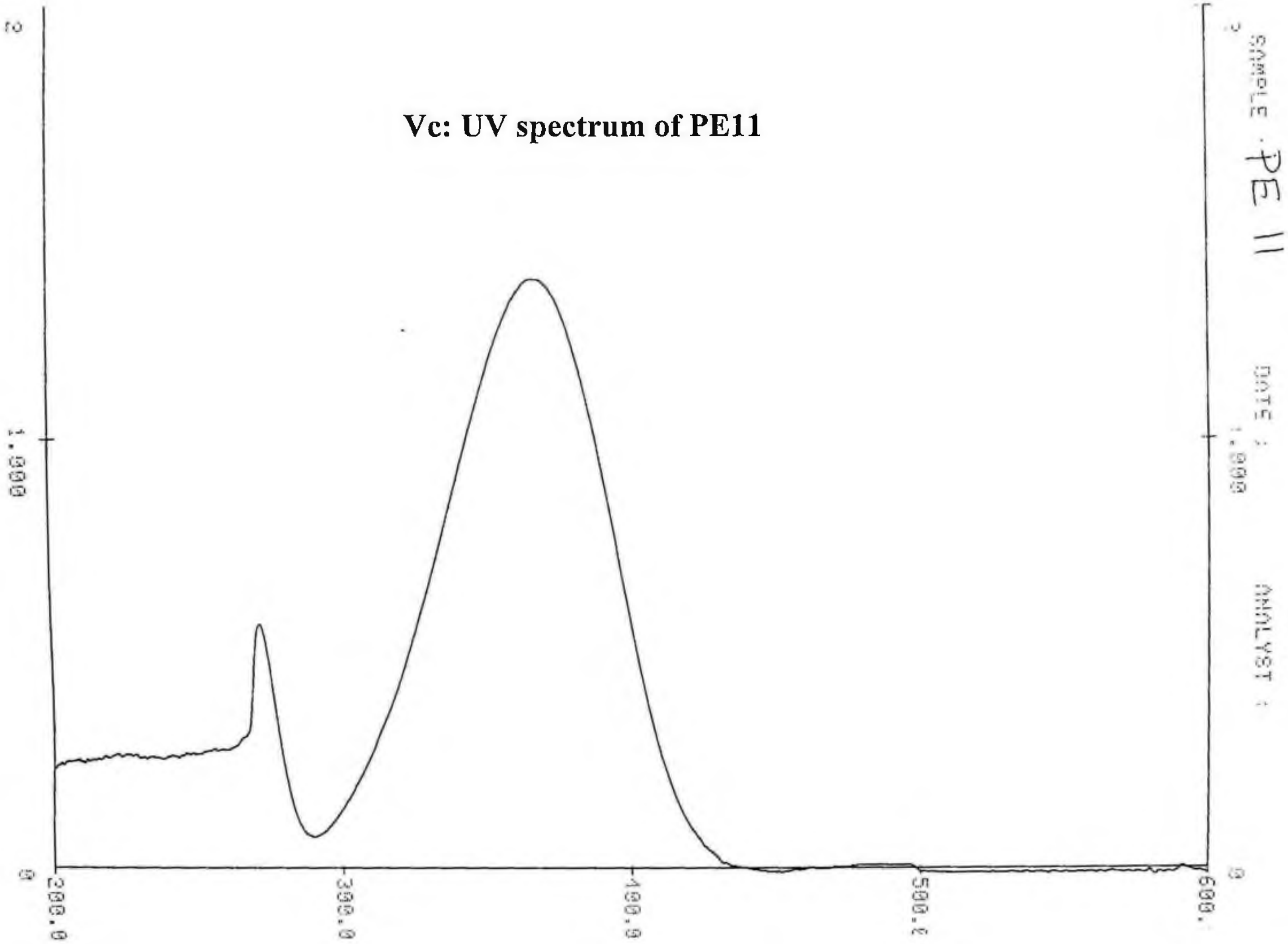
ULTRAVIOLET (UV) SPECTRA



Va: UV spectrum of D9



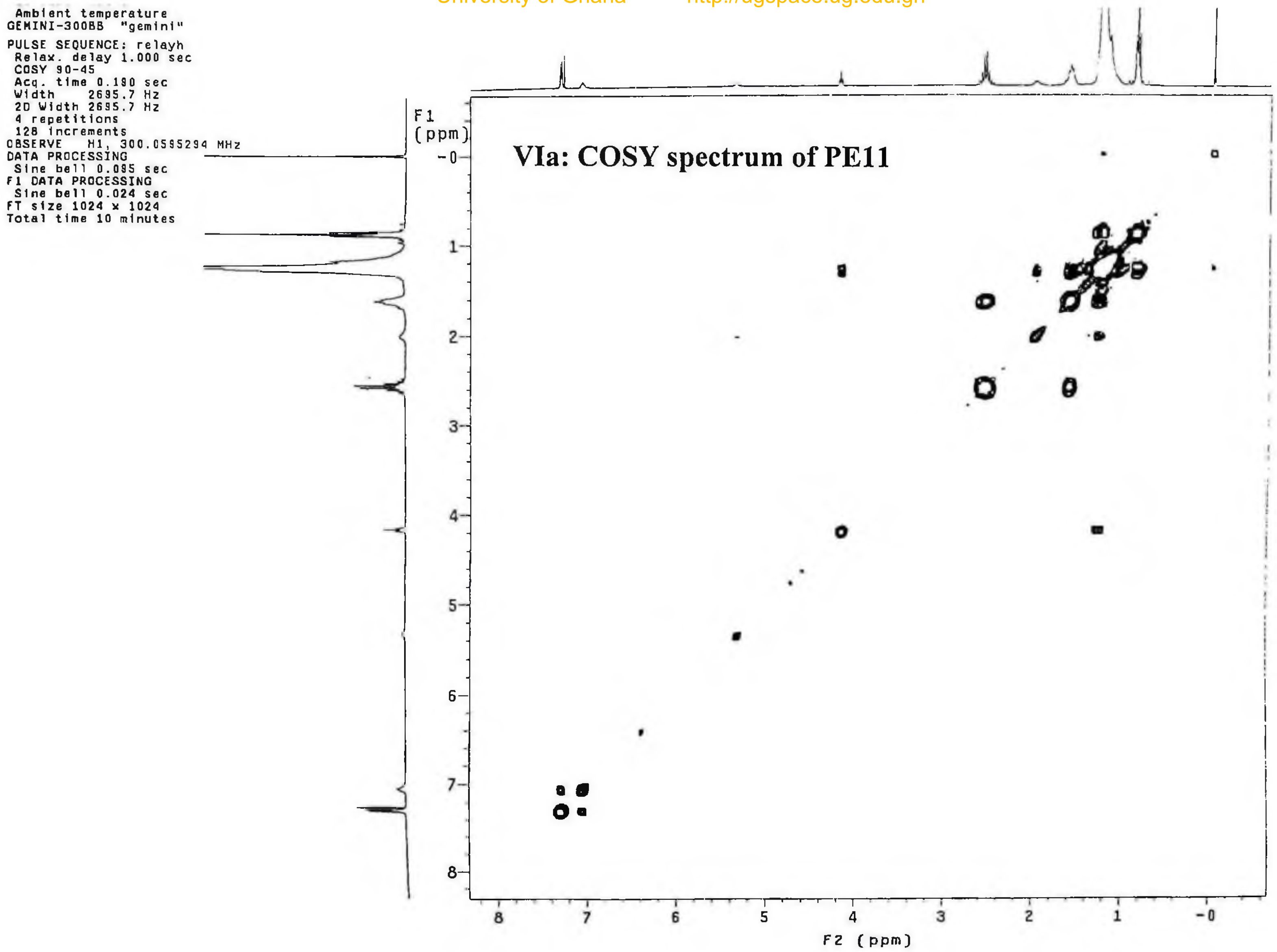
Vb: UV spectrum of E13



Vc: UV spectrum of PE11

APPENDIX VI

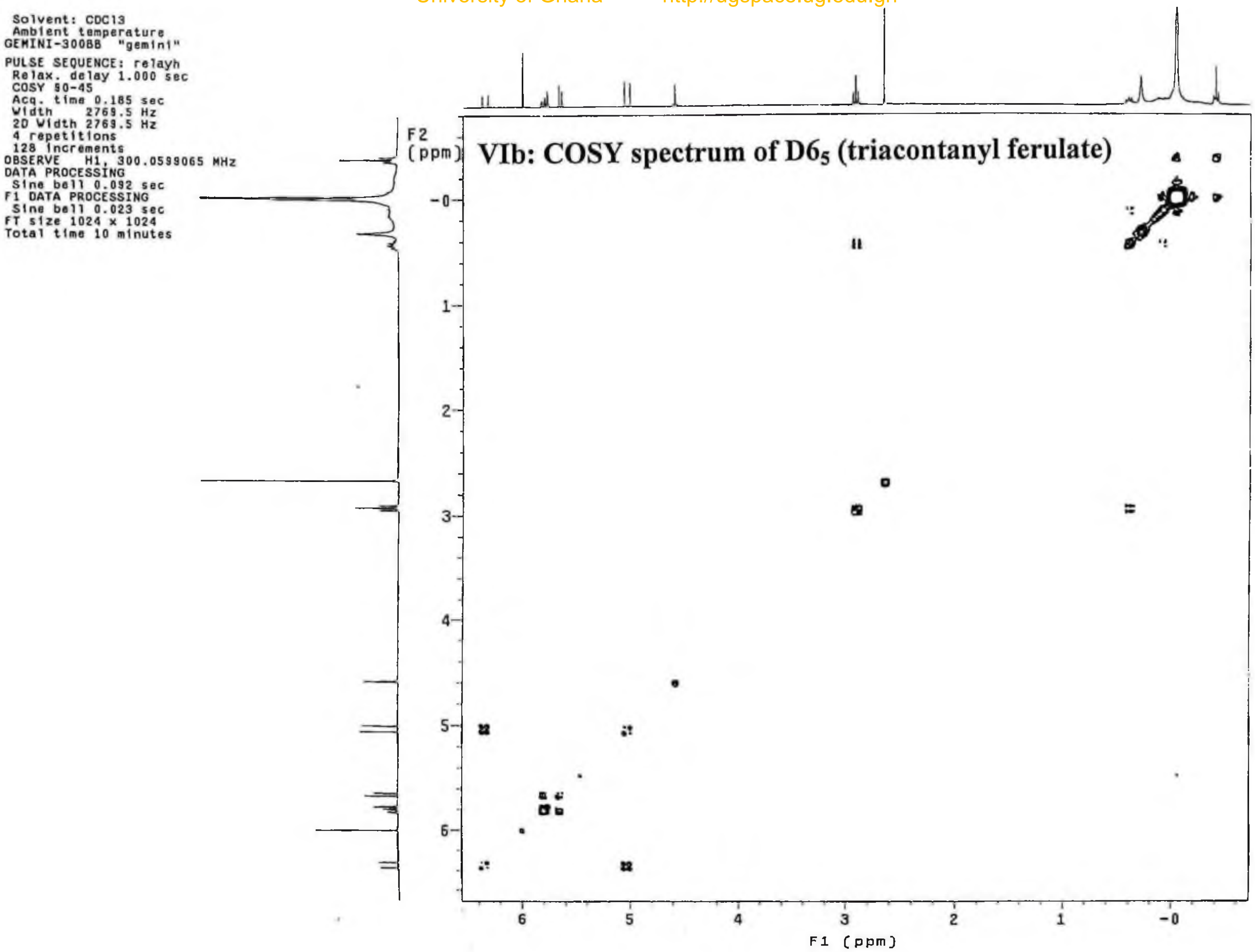
HOMONUCLEAR PROTON-PROTON SHIFT CORRELATION SPECTRA (COSY)



```
 Ambient temperature
GEMINI-300BB  "gemini"
PULSE SEQUENCE: relayh
 Relax. delay 1.000 sec
 COSY 90-45
 Acq. time 0.190 sec
 Width     2695.7 Hz
 2D Width 2695.7 Hz
 4 repetitions
 128 increments
OBSERVE   H1, 300.0595294 MHz
DATA PROCESSING
 Sine bell 0.095 sec
F1 DATA PROCESSING
 Sine bell 0.024 sec
FT size 1024 x 1024
Total time 10 minutes
```

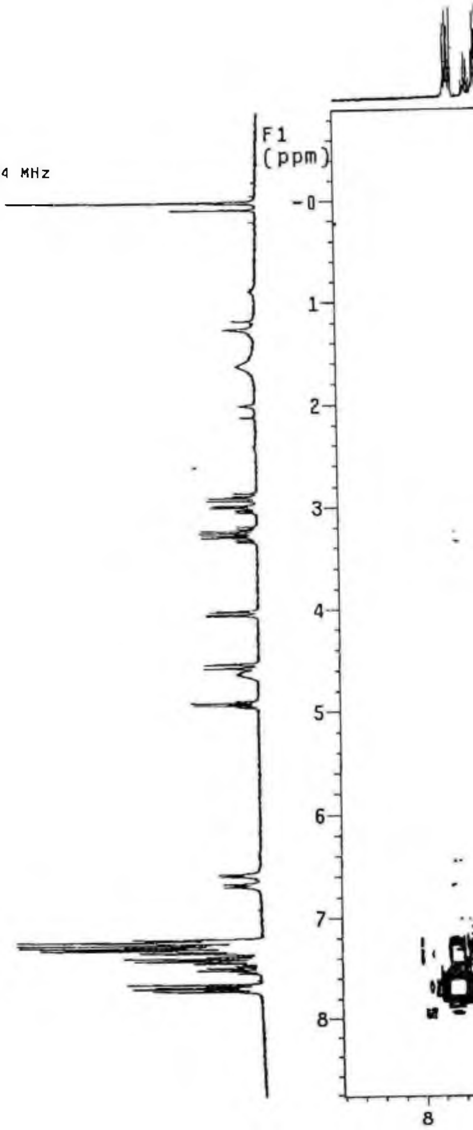
VIa: COSY spectrum of PE11

VIb: COSY spectrum of D6$_5$ (triacontanyl ferulate)

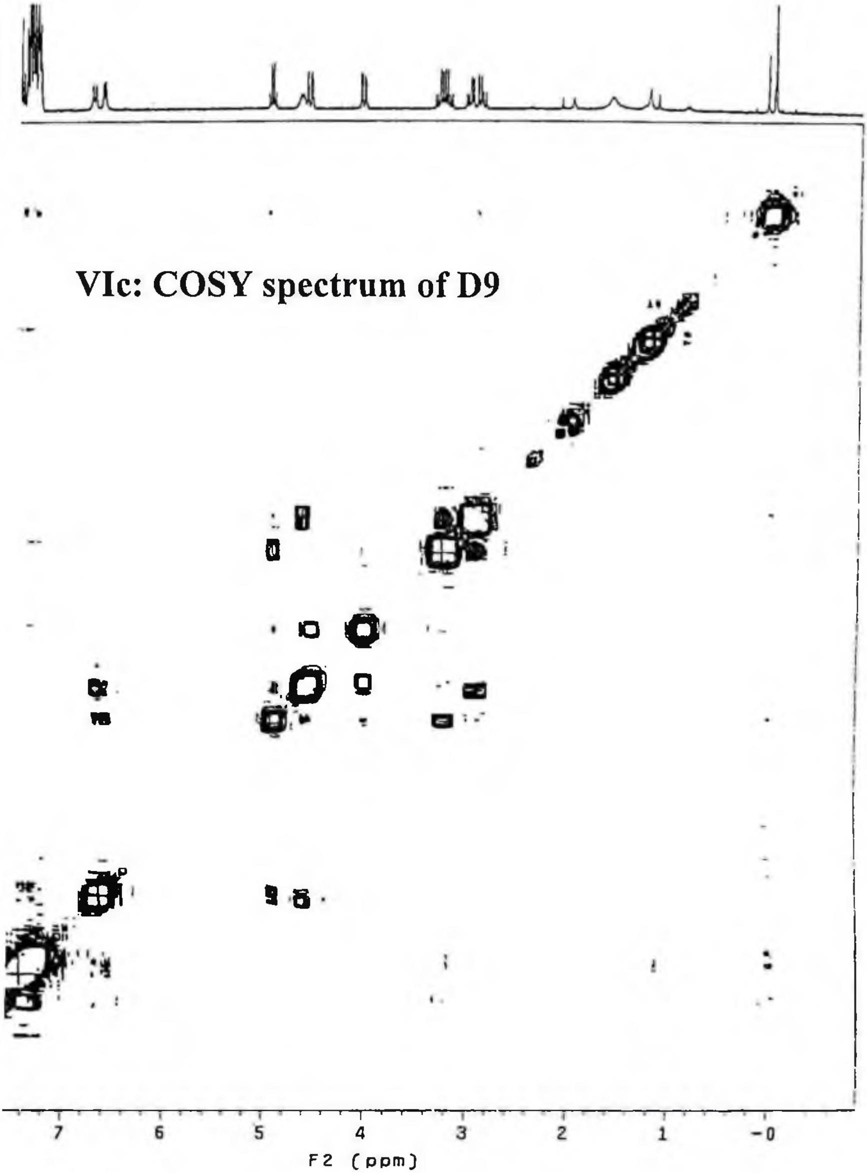


```
Solvent: CDCl3
Ambient temperature
GEMINI-300BB "gemini"
PULSE SEQUENCE: relayh
 Relax. delay 1.000 sec
 COSY 90-45
 Acq. time 0.185 sec
 Width     2769.5 Hz
 2D Width 2769.5 Hz
 4 repetitions
 128 increments
OBSERVE   H1, 300.0599065 MHz
DATA PROCESSING
 Sine bell 0.092 sec
F1 DATA PROCESSING
 Sine bell 0.023 sec
FT size 1024 x 1024
Total time 10 minutes
```

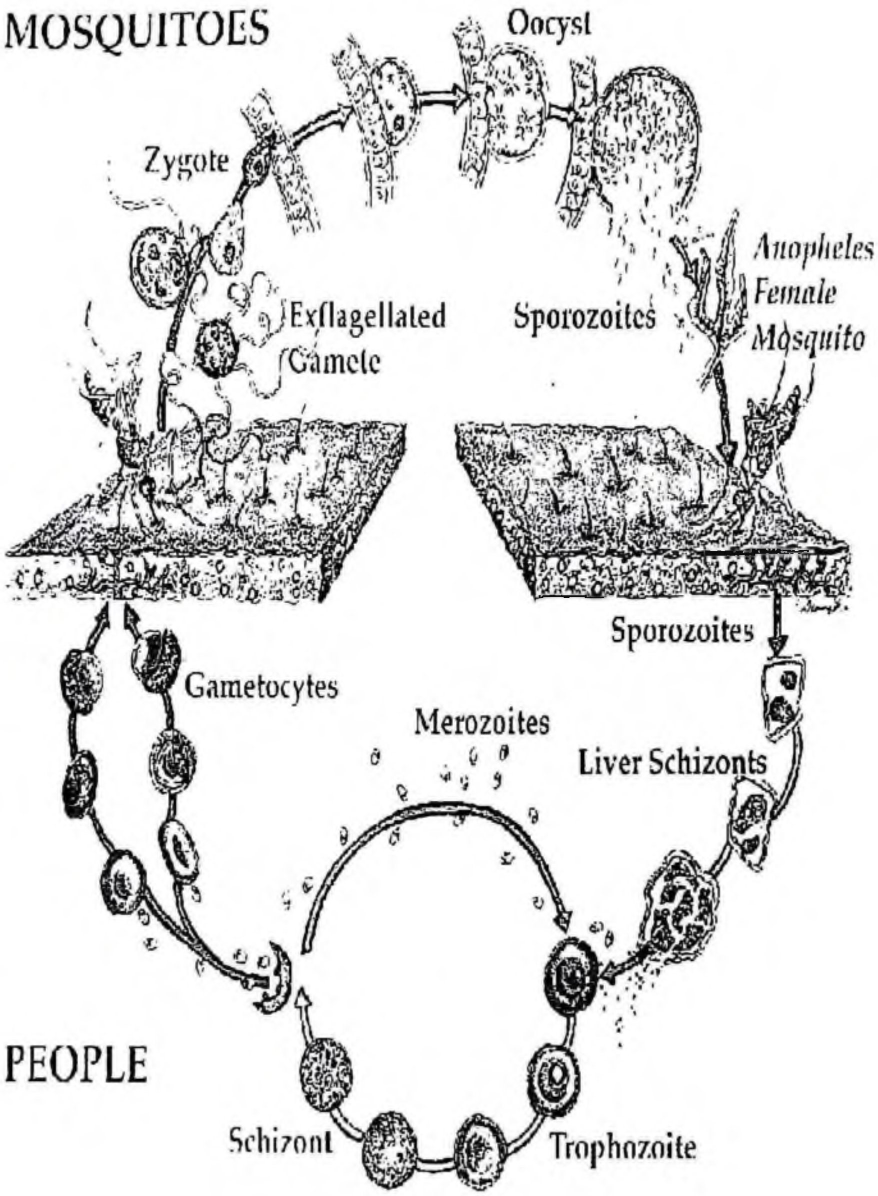
```
 Ambient temperature
GEMINI-300BB  "gemini"
PULSE SEQUENCE: relayh
 Relax. delay 1.000 sec
 COSY 90-90
 Acq. time 0.176 sec
 Width     2911.4 Hz
 2D Width 2911.4 Hz
 4 repetitions
 64 increments
OBSERVE   H1, 300.0595294 MHz
DATA PROCESSING
 Sine bell 0.088 sec
F1 DATA PROCESSING
 Sine bell 0.011 sec
FT size 1024 x 1024
Total time 5 minutes
```



VIc: COSY spectrum of D9



APPENDIX VII



Life Cycle of malaria Parasite (*Plasmodium falciparum*)
(http://www.malariatest.com/cycle.html)

APPENDIX VIII

Buffers and Solutions

(a) Washing Medium

RPMI – 1640	500.0ml
Gentamycin(10mg/ml)	2.5ml / 500 ml RPMI
L- glutamine	4.0ml / 500 ml RPMI

(b) Complete Parasite Medium

RPMI – 1640	500.0ml
Gentamycin(10mg/ml)	2.5ml / 500 ml RPMI
L- glutamine	4.0ml / 500 ml RPMI
NHS (A or O positive)	10% filtered through 0.8 m pore filter (to start with and later 5%)

Filter sterilize medium and store at 4°C

(c) CPD Buffer, pH 5-6

17mM	Citric acid	5.0 g
90mM	Sodium citrate	26.47 g
175mM	Glucose	31.54 g
16mM	NaH_3PO_4	2.21 g
2mM	Adenin (6-Aminopurine)	0.27 g
	Distilled water up to	1.0 litre

Filter sterilize medium and store at 4°C

(d) Giemsa Buffer, pH 7.2

$NaHPO_4$	1.0 g
KH_2PO_4	0.7 g
Distilled water up to	1.0 litre

Store at 4°C

(e) Parasites Thawing Mix

3.5% sodium chloride in distilled water
Filter sterilize and store at 4°C

(f) Freezing Mix

4.2% Sorbitol in 0.9% sodium chloride
Take 72 ml sorbitol solution and add 28 ml glycerol
Stir well and filter sterilize
Store at 4°C