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ISOLATION AND CHARACTERISATION OF THE ACTIVE PRINCIPLE OF SIDA CORDIFOLIA LINN

Submitted by

MD. WAHAB KHAN

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ABSTRACT

The work is divided into three chapters. The first chapter is entirely devoted to general reviews regarding the necessity of research into vast domain of the indigenous herbal medicinal plants. This part also deals with the description of the plant Sida cordifolia Linn and its medicinal uses. The objective of the project is also included in this chapter.

The interpretation of the results of the isolated products from Sida cordifolia Linn has been discussed in the second chapter. The ethanol extract was subjected to systematic study and from it the following compounds were isolated and characterized: a mixture of hydrocarbons, a mixture of higher fatty acid esters, β-sitosterol, palmitic acid, stearic acid, hexacosanoic acid and mixture of higher fatty acids. All the above compounds were characterized by i.r., p.m.r. and mass spectra. The identities of β-sitosterol, palmitic acid and stearic acid were confirmed by co-t.l.c., mixed melting point and superimposable i.r. spectra with corresponding authentic samples and also by converting them into known derivatives. Hexacosanoic acid was confirmed by its melting point and by the melting point of its methyl ester; a direct comparison with an authentic

sample of hexacosanoic acid was not possible because of its nonavailability with us. G. L. C. analysis as well as mass spectral fragmentation pattern established the presence of a number of alkanes in the hydrocarbon mixture; the mass spectra also revealed the presence of alkanes having even and odd number of carbon atoms. The fraction of fatty acid isolated during this study also behaved as a mixture as evidenced by g.l.c. analysis of its methyl ester; mass spectral analysis indicated similar results and also revealed the predominance of fatty acids having even number of carbon atoms. The fatty acid ester fraction was proved to be a mixture of ethyl esters of fatty acids by its p.m.r. spectra and also its mass spectral fragmentation pattern.

The third chapter enumerates all experiments performed for this work.

CONTENTS

CHAPTER 1
INTRODUCTION

1.1 General Introduction

The indigenous herbal drugs have great importance both from the professional and economic points of view. From time immemorial man has been using various plant materials in different forms for their curative and other effects. They were often used to get relief from pain, fatigue and various physical and mental ailments. How man has come to associate a particular hert with a particular disease is still as profound a mystery as is faith healing. Herbal cures alluded in the folklore have led to the advancement of systematic studies for obtaining drugs from plant materials. Systematic investigation of plant materials has yielded variety of substances. The chemistry of these substances, which has its root in the empirical knowledge of ancient medicine and finds its continuity in folk medicine even today, makes valuable contribution to the discovery of new drugs.

The original impetus to the chemistry of natural products came from medicine. They are found to be the only material available for treatment and prevention of diseases till as late as the middle of the nineteenth century. Throughout the middle ages the *materia medica* of plant origin was accepted as the only available ways and means of treatment of various diseases. Hundreds of years ago the medicinal plants in Indian subcontinent attracted the attention of the people of this region. Rigveda[1], one of the oldest scriptures, written in 4500-1600 B.C. contains informations on medicinal

plants of the subcontinent. For long time medicines in 'Ayurvedic' system are being extracted from various herbs. The Chinese were the first who recognised the usefulness of herbs. They extracted the drug 'Ma Huang[2]' from the crude preparation of Ephedra plant which is now used in the treatment of hay fever and bronchial asthma.

With an even increasing number of disorders in human body, more vigorous studies have been made in alleviating or eradicating diseases by administering chemicals obtained from plants or synthesized in the laboratory. Though plant kingdom is no more the source of insatiable demand for drugs except in some cases, it has come to serve another purpose. It provides newer models to use in the design of potential chemotherapeutic agents albeit the synthesis and modifications of known drugs continue to be an important aspect of drug design.

Chemists have been engaged in the isolation of the active constituents from the extracts of the curative parts of plant materials, and establish their structures by chemical and instrumental methods of analysis. Though rapid advances have been made in the chemistry of natural products recently, mainly due to the availability of various highly sophisticated instrumental techniques for structure determination, the unequivocal synthesis marks the proof of the structure of a compound. The skilful synthetic chemist, then creates

in the laboratory either totally or partially what is manufactured by plants. On the synthetic route to the natural product, many more variants are encountered and these too are screened for potential physiological activities. The isolation, structural elucidation and synthesis of quinine for example, have led to a host of antimalarials. Variants of penicillin, tetracycline, morphine, papaverine have found a way to medicine rack.

The use of drugs obtained from the medicinal plants have increased not only in our country but also over the most developed ones. Now a days about 25%[3] drugs, e.g. antibiotics, vitamins, hormones etc. come from the plant extracts through usual processing. In 1960 47% of drugs prescribed by the physicians in the United States were from natural sources, mostly antibiotics. In 1967 25% or 624 out of 3354 of the trade or generic name products, which appeared in the 1.05 billion prescriptions filled in the United States contained one or more ingredients derived from higher plants[3].

Research in indigenous drugs is particularly appropriate at the present moment, when the pharmaceutical companies of the world are emitting an unceasing flow of new synthetic drugs; attention should be turned to the possible remedies that may be found among indigenous herbs. The following examples of such research proving fruitful may be recalled. In Eastern Mediterranean countries and in Arabia the local

physicians often prescribe a decoction of the dried seeds of a local plant, Ammi Visnaga, as a diuretic and as an antispasmodic in renal colic. Investigations by G.V. Anrep and his colleagues in Cairo[4] showed the active constituent to be Khellin, which they found to be an effective vasodilator with a selective action on the coronary arteries. Subsequent clinical trials demonstrated the value of Khellin in the treatment of angina pectoris. From ancient times the root of an indigenous plant, Rauwolfia serpentina, has been widely used in India and Malaya as an antidote to insect and snake bites, as a febrifuge, as a stimulant to uterine contraction, and as a sedative. R.J. Vakil[5] investigated its use in hypertension and found it to have a marked hypotensive action. Even in the currently popular field of the chemotherapy of tuberculosis, indigenous plants are proving to be of interest. Thus Japanese workers have isolated from a vine named Stephania cepharantha, and from a wisteria like plant named S. Sasakii, the alkaloid cepharanthine which is being used for the treatment and the prophylaxis of tuberculosis in Japan[6]. Chinese workers have been investigating the anti-tuberculous activity of a series of local plants, and Virginia Wang[7] reports a 'prominent tuberculostatic activity' in the extracts of Copits root, Coptis chinensis this activity apparently residing in its alkaloid, berberine sulphate. It is clear that much remains to be learnt from close study of indigenous herbs.

A number of structural patterns have been isolated, identified and synthesized. Heterocycles occupy a predominant position due to their wide occurence in nature. Out of an estimated[8] 20,000 natural products, steroids and alkaloids are the most important groups of natural products that have profound therapeutic values. In plants, the isoprenoid units, present along with phenols give rise to various structural patterns such as coumarins, benzofurans, chromenes, chromones, flavonoids, isoflavonoids, xanthones and rotenoids[9,10]. Among nitrogeneous plant constituents, carbazole and indole alkaloids have attracted considerable interest because of their physiological activities. Nitrogenous constituent, acridone alkaloids have also created considerable interest among the scientists due to the reported[11] anti-tumor activity of acronycine (an acridone alkaloid), a component of the Rutanceous plants, Acronychia baueri and Vepris ampody. Hooper[12] isolated an alkaloid vasicine and a volatile oil from Adhatoda vosica (Beng-Basak). The plant is used as a remedy for cold, cough, bronchitis, dysentry etc.

Cryptopine[13] was isolated from the thebaine fraction of opium alkaloics[14]. It depresses the higher nervous centres, causes spinal paralysis in frogs, and convulsions in mammals[15]. Opium had been used for centuries in popular medicine, and both its analgesic and narcotic properties

were well known. In 1903, Derosine isolated a semi-pure alkaloid from opium and named it narcotine. Processes for the isolation of strychnine alkaloid[16] which are used as tonic, stimulant and febrifuge from seeds of Strychnos nux Vomica plants and berberine hydrochloride[16] which is used as a fibrifuge, carminative and antivomiting agent during pregnancy from the barks of Barberis aristata (Daru Haldi) have been developed at the BCSIR laboratories. Anwar and Ghani[17] have reported the isolation of a number of tropane and related alkaloids from the roots of Datura metel L var fastuosa. Hussain and Quaisuddin[18] reported the isolation of three compounds from dried powdered roots of Tacca aspera Roxb (varahikanda) which are reported to be excellent tonic and useful in haemorranagic diathesis, and believed to have curative effects in leprosy and in many other skin diseases. Hoque et al[19] reported the isolation of a number of pyrrolizidine alkaloid from Heliotropium Indicum Linn. Ali and his group[20] at the BCSIR laboratories, Dhaka isolated anti-leukaemic drugs vinblastine and vincristine from the plants Vinca rosea.

Dutch workers have recently reported the beneficial effects of extract of liquorice from Jashtimadhu for gastric ulcers. One of the components of liquorice is glycerhetic acid which is a polyterpene whose structural formula shows a striking resemblance to the cyclopentano-phenanthrene

steroid[21]. The interesting development in the recent study of digitalis is the emphasis on its cardiotonic rather than on its cardiotoxic properties and the reported discovery of a new glycoside digicorin. This glycoside, which has low toxicity is claimed to possess the curative action of digitalis as distinct from that of the better known glycosides which are largely cardiotoxic. It can be extracted from the leaves of D-purpurea and D-lanta[22].

Bangladesh is blessed with vast resources of medicinally important herbs and plants. Many of these are in wide use in folk medicines as well as in the Ayurvedic and Unani system of treatment. The financial condition of the people of our country often restricts them to use expensive chemotherapeutic drugs. The useful drugs obtained from the plant origin should be purified from inactive and harmful ones so as to bring them into use for mitigating the sufferings of the vast masses of humanity. Various medicinal plants in this country are still awaiting thorough and systematic examination. So an extensive and speedy exploitation of the medicinal plants is deemed necessary.

Some of the more useful medicinal herbs and plants of our country are: Terminalia arjuna Bedd (Arjun), used in heart diseases; Jatropha gossypifolia Linn (Lalbharenda), used against urinary complaints, ulcers; Cephalandra indica Naud (Telakucha), used for the treatment of glycosuria;

Abrus precatorus Linn (Kunch), used for the treatment of trachoma; Datura fastuosa Linn (Dhutura), used as poisons; Andrographis paniculatae Nees (Kalomegh), used for the treatment of irregular motions and of appetites; Tinospora cordifolia Miers (Gulancha), used for the treatment of chronic dysentry, chronic diarrhoea and also used against snake bite; Cassia angustifokia Vahl (Sonapata), used as laxative and purgative; Cinchona succirubra (Cinchona), used for the treatment of malaria; Rauwolfia serpentine Benth (Chandra), used for the treatment of high blood pressure; Ocium gratissimum Linn (Ramtulshi), used against rheumatism; Momordica chorantia Linn (Karala), used for the treatment of diabetes; Paederia foetida Linn (Gandha bhadulia), used in piles, inflammation of spleen, and pain in chest and liver; Tacca aspera Roxb (Varahikanda), reported to be excellent tonic and useful in haemorrahagic diathesis; and Sida cordifolia Linn (Brela), used in rheumatism and spermatorrhoea[1]. Some of these plants have been the subject of investigations of chemists in Bangladesh and number of articles have been published by them.

Sida cordifolia Linn is one of the wicely available medicinal herbs in Bangladesh. It grows abundantly along the road side of the rural areas in our country. In Bengali it is known as Brela[23]. The genus sida belongs to the

Malvaceae family and the plants belonging to this group are in Sanskrit by the general name 'bala'. There are some seven or eight species but Sanskrit writers make mention of five species of 'bala' under the name 'Pancha bala'. Sida cordifolia Linn also known as Sida herbacea Nicans and Rotundfolia Cav, Sida allthaifolia Swatzs known in English as country mallow.

Brela is a small, downy, erect shrub, about 1.5 m high, with long branches, sometimes rooting at the nodes. It is distributed in moist places throughout tropical and tropical India, Nepal, Pakistan and Srilanka. It is a perennial undershrub. The leaves are cordate, oblong, obtuse, crenate and very downy on both surfaces. The peduncles occur near the flower. The petioles are as large as the leaf; the stipules are linear measuring nearly half the length of the petiole. The flowers are small and tawny yellow or white. The bark is light yellowish-brown colour. The seeds are called 'bijband', grown in a pair on each carpel.

Sida cordifolia Linn is considered to be one of the most valuable drugs in the Ayurvedic or Hindu medicine. In the Tibbi or the Mohammedan medicine it was used for its aphrodisiac effects. It has been largely used by the physicians from very ancient time.

The roots, leaves and seeds are all used in medicine and have a slightly bitterish taste. The juice of the plant

is mixed with the juice of Borassus flabellifer for local use in elephantiasis. The juice of the whole plant is used in rheumatism and spermatorrhoea.[24]

The mucilagenous leaves of Sida cordifolia Linn are used as a demulcent and their infusion is given in fever as a refrigerant. They are reported to be used against dysentry and for poulticing ulcers. A decoction of the leaves is said to possess emollient and diuretic properties[24]. The leaves are used in opthalmia.

In Cambodia and China, root is considered to possess astringent, diuretic and tonic properties. An infusion of it is given in uninary diseases, bilious disorders and gonorrhoea. It is also used in cystitis, strangury and haematuria. In nervous disorders such as hemiplegia, sciatica and facial paralysis, the root is administered internally in combination with asafoetida and rock salt. The root bark is powdered and administered with milk and sugar to relieve frequent micturition and leucorrhoea[25]. An infusion made from the roots are considered aromatic bitters having febrifuge, demulcent and diuretic properties. Mixed with makaradhwaja and musk it is used as a cardiac tonic.

Seeds are credited with demulcent and laxative properties and used in bowel complaints such as piles, colic, and tensemus[24]. Ethanol extract of the plant exhibits

antiprotozoal activity against Entamoeba histolytica strain STA and depresses blood pressure in cats and dogs[26].

Besides the above medicinal properties the plant is of great commercial value as it yields a fine white fiber, the cellulose content of which is 83 percent as against 75 percent in jute.

Sida cordifolia has been subject of chemical analysis from the end of the last century. It was reported to contain asparagin[27]. Ghosh and Dutta[28] carried out a systematic stuoy on Sida cordifolia and found the total alkaloid content in the plant body to be 0.085%. The seeds were found to contain about 4 time more alkaloid than either the stems, roots, or leaves. Extraction with petroleum ether, ether, absolute alcohol and water showed the presence of fatty oil, phytosterols, resins, resin acids, mucins, potassium nitrate and alkaloids, out no tannin or glycosides. The alcohol extract contained the whole of the alkaloid content. From the alcohol extract they were able to isolate two alkaloids which were identified as ephedrine and Ψ-ephedrine.

Dutta also reported[29] the presence of appreciable quantities of water soluble alkaloids but failed to isolate them. Reports on nitrogenous constituents of this family of over 700 species are very few. Gossypium is the only

other genus where the presence of two biogenic amines viz. 5-hydroxytryptamine[30], and histamine[31] has been reported. No true alkaloids have been previously described in the malvaceae family.

From the roots of this plant, Ghosal et al[32] isolated three β-phenethylamine, ephedrine and Ψ-ephedrine. Besides these they also isolated 2-caboxylated tryptamines, S(+)-N_b-methyltryptophan methyl ester and hypaphorine, along with three quinazoline alkaloids, viz. vascinone, vasicine and vasicinol. From the water-soluble alkaloid fraction they also isolated liberal amounts of choline and betaine. The works of this group of investigators further showed that the stems and leaves contain essentially the same alkaloids as are present in the roots, out in different amounts. Ephedrine and Ψ-ephedrine constitute the major bases in the aerial parts, but occur as minor components in the root.

Investigation on brela was carried out earlier by Dutta[33]. The roots of brela (various sida species, including S. rhombifolia, S. veronicaefolia, S. glutinosa, S. chinesis and S. corolifolia) contain steroids, alkaloids and fatty oils. The alkaloid content for various species ran about 0.05%. Various alkaloids were separated by paper chromatography; ephedrine is one of the minor alkaloids of the various species. The two chief alkaloids are highly water soluble and could not be extracted with organic solvents.

Chemical investigations of the leaves of Sida rhombifolia Linn was carried out by Bhatt and his group[34]. The leaves were found to contain lysine, histidine, phenylalanine, arginine, asparagine, glutamine, alanine, valine, leucine, aspartic acid, glutamic acid, glycine, serine, theonine and tyrosine. Total phytosterol content in the leaves of the plant was 0.052% (as cholesterol). Besides the aoove compounds myristic, palmitic, stearic, oleic and linoleic acid and an unidentified fatty acid were also isolated. The mixture of fatty acids showed high antibacterial activity. Seed oils of Sida rhombifolia contain sterculic acid and malvalic acid where determined by gas-liquid chromatography.

Investigation on Sida acute Burn has been reported by Khaleque and his colleagues[36]. Sequential extraction of dried roots of Sida acuta yielded oxalic acid and a compound having m.p. 222 -4°C which was not characterized; stem of this plant yielded β-sitosterol.

Alkaloidal constituents of Sida acuta, Sida humilis, Sida rhombifolia and Sida spinosa have been investigated by prakash and his colleagues[37]. These type of plants were found to contain three types of alkaloidal constituents, viz. β-phenethylamines, quinazolines and carboxylated tryptamines, same as that reported for Sida cordifolia. Ref As with Sida cordifolia choline and betaine were isolated

from these plants. The qualitative and quantitative variations in the alkaloidal constituents of roots and aerial portions at different stages of growth of these plants were also noted. Elaboration of the quinazoline alkaloids seems to be a characteristic feature of this genus. The favourable combination of sympathomimetic amines and vasicinone in these species probably accounts for their major therapeutic uses in the Indian systems of medicine

A polysaccharide which yielded an acidic xylan was isolated from dilute alkali treated roots of Sida acuta[38]. The xylan was methylated and the methylated xylan on hydrolysis gave mainly 2,3-di-o-methyl xylose and smaller quantities of 2,3,4-tri-o-methyl xylose, 2-o-methylxylose and an acidic substance.

Chemical examination of the seeds of Sida carpnifolia was carried out by C.S. pande and his colleague[39]. Incineration of the seeds gave 14.5% ash, containing Ca, Mg, K and Na as basic radicals and carbonate, sulfate and chloride as acidic radicals. Extraction of the seeds with petroleum ether gave 9% light yellowish green oil containing the fatty acids, oleic, linoleic, palmitic, stearic and arachidic acid. From the unsaponifiable matter only β-sitosterol could be identified. The mucilage on complete incineration gave 3.8% ash containing 0.77%. Fe and 2.02% Mg. Graded hydrolysis with 3% oxalic acid, 2N H_2SO_4 and concentrated

H_2SO_4 suggests the presence of maltose, galactose, arabinose, glucose, and maltobionic and galactouronic acids. The hydrolyzates of the first two hydrolysis gave 14 and 22.7% total reducing sugars; hydrolysis with concentrated sulphuric acid gave only glucose 20%.

I.2 Objective of the Project

From the above discussion it is revealed that extracts of different parts of the plant *Sida cordifolia* Linn have been used in the Ayurvedic system of medicine for a variety of purposes [40]. Chemical investigations on the whole species of the genus *Sida* [41,42] are quite appreciable and some study on *Sida cordifolia* Linn is also available in the literature. It has been reported [27,28,32] that the roots of the plant *Sida cordifolia* Linn contain asparagin, fatty oil, phytosterols, resins, resin acids, mucins, potassium nitrate and a number of known alkaloids. These compounds have also been detected in the stems and leaves of the plant. However, none of the compound so far isolated can explain properly the exact medicinal value of the plant body. Therefore a systematic study on the aerial parts of the plant *Sida cordifolia* Linn was undertaken.

CHAPTER 2
RESULTS AND DISCUSSIONS

2.1 Study on the Ethanol Extract

The aerial parts of the plant Sida cordifolia Linn were collected from Dhamrai, about 40 miles north of Dhaka city. The plant body was sun-dried, powdered (1.7 kg) and extracted with ethanol at room temperature. The ethanol extract was concentrated at reduced pressure when a dark greenish semi-solid mass was obtained (5.0 g). The crude mass was then triturated successively with petroleum ether (40-60°C), benzene, diethyl ether and methanol.

2.2 Study on the Petroleum ether Triturate

The petroleum ether triturate was evaporated to dryness at reduced pressure when a heavy light green mass (1.27 g) was obtained. The mass was chromatographed over a column of silica gel and eluted with hexane and mixtures of hexane-ethylacetate (19:1 and 9:1) in that order. Several fractions were collected and each of them was separately examined.

Fraction A, eluted with n-hexane gave a low melting waxy solid (0.32 g; m.p. 32-34°C) and was highly nonpolar. It gave only one spot on t.l.c. plates (R_f 0.92 in hexane) and appeared to be homogeneous . It showed i.r. absorptions at 2970, 2940, 2870, 1460, 1390, 730 and 720 cm^{-1}. The p.m.r. spectrum of the material showed only two absorptions, a triplet at δ0.88 and a sharp singlet at δ1.24. The nature of

the i.r. and the p.m.r spectra indicated it to be a saturated hydrocarbon. The melting point of the compound is similar to that for nonadecane ($C_{19}H_{40}$, m.p. 32°C)[43]. However the mass spectrum of the compound showed mass peak at as high as m/e 365. The mass spectrum exhibited fragmentation pattern expected of long chain alkanes i.e. m/e peaks at successive loss of 14 and 28 mass units. The spectrum also exhibited at least four sets of m/e peaks of decreasing intensity with increasing m/e which suggests the sample to be a mixture of at least four long chain alkanes. The m/e series 141, 127, 113, 99, 85, 71, 57; 211, 197, 183, 169, 155; 323, 309, 295, 281, 267, 253, 239, 225 and 365, 351 indicated the presence of $C_{10}H_{22}$(M^+-1, 141), $C_{15}H_{32}$ (M^+ -1, 211), $C_{23}H_{48}$(M^+ -1,323) and $C_{26}H_{54}$(M^+ -1,365) in the mixture. The assumption was readily borne out by the g.l.c. analysis of the material on a Shimadzu GC-3BF gas chromatograph which revealed the presence of five major and five to seven minor components. Unfortunately because of nonavailability of the authentic samples of higher alkanes the identity of the g.l.c. peaks could not be established.

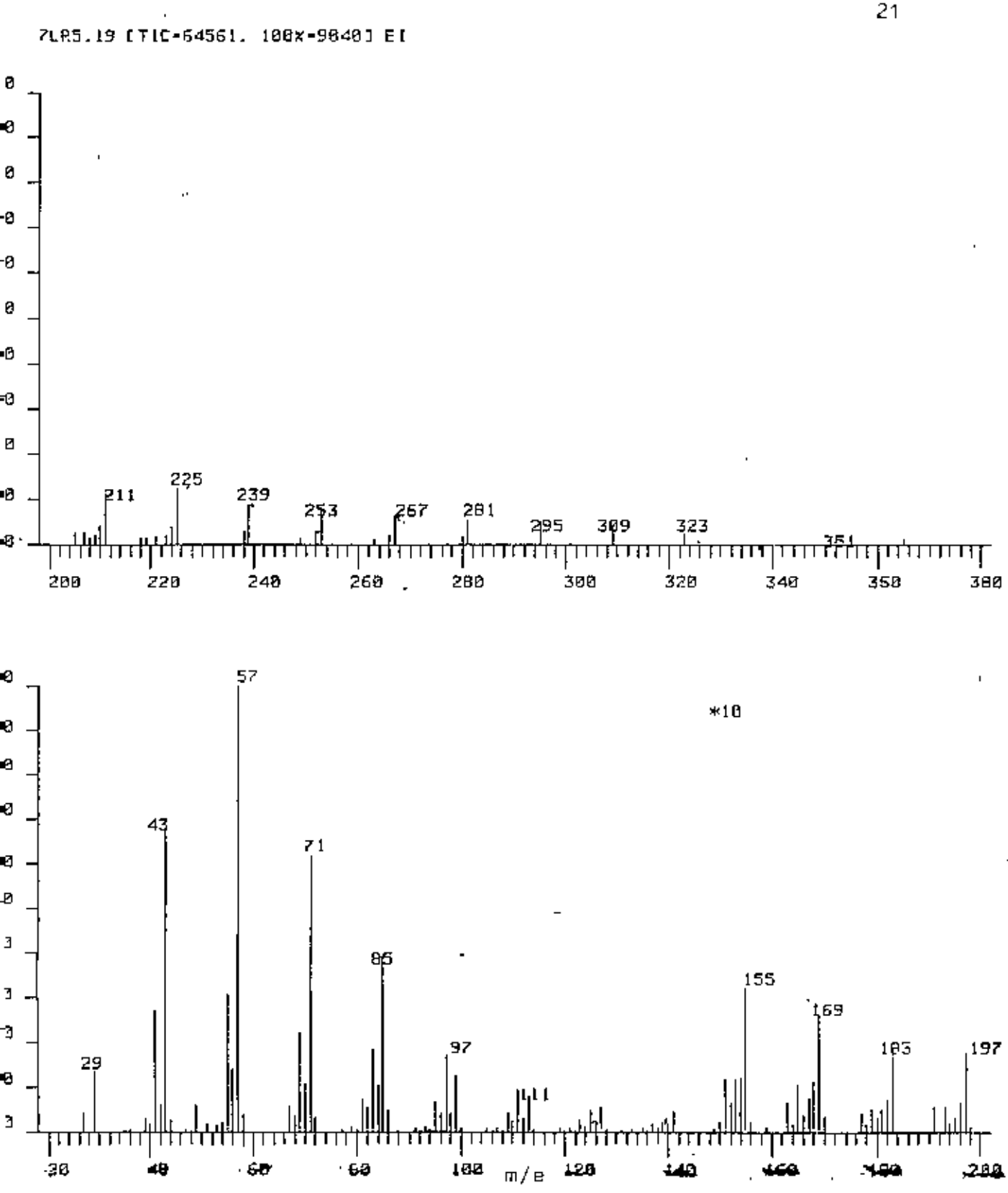


Fig. I Mass Spectrum of compound A.

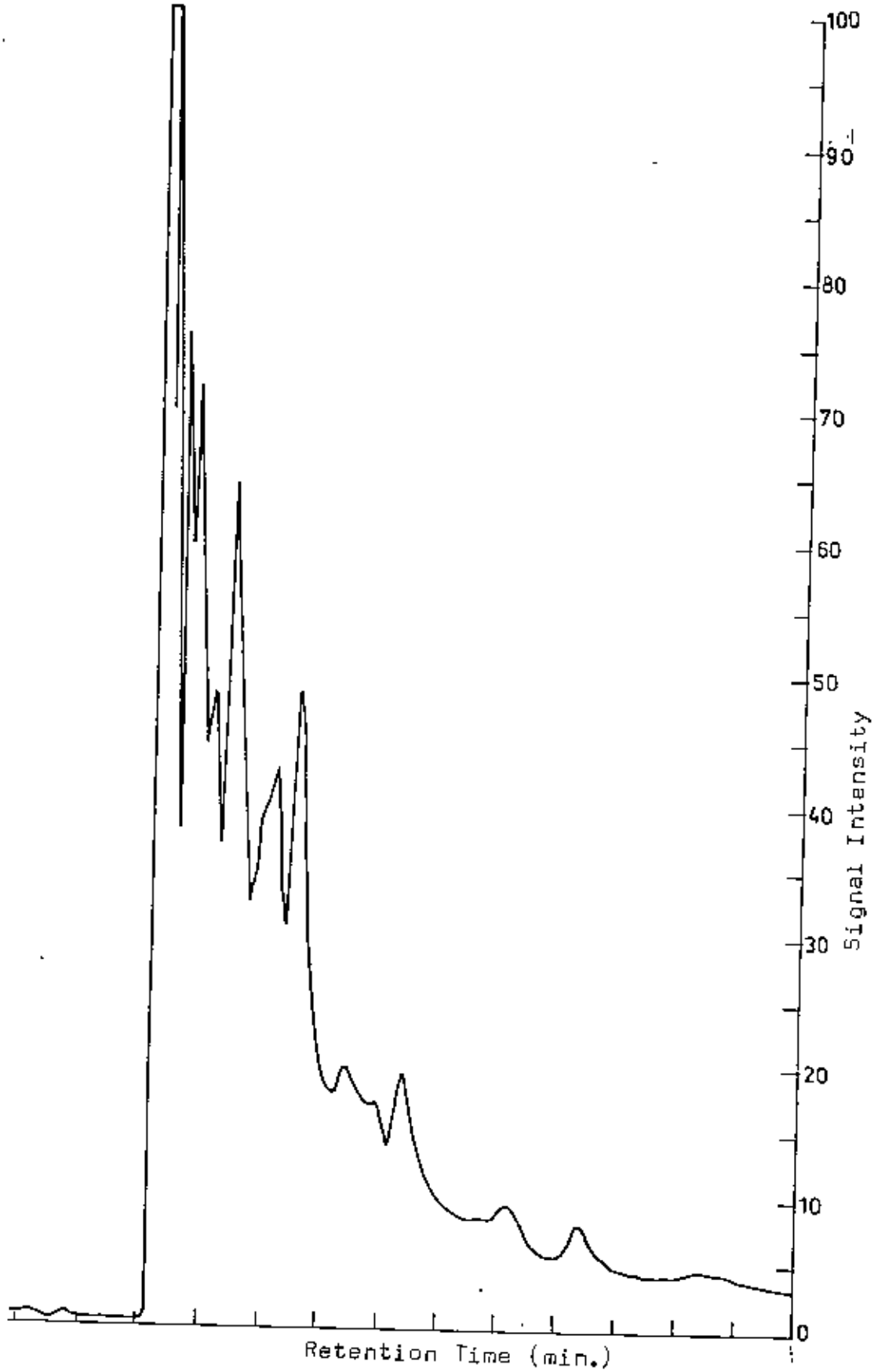
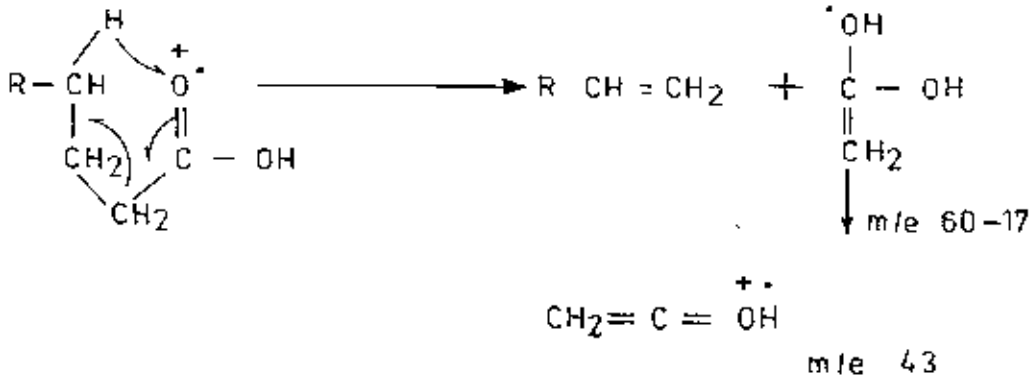


Fig. II Gas Liquid Chromotograph of Compound A.

Fraction 9 obtained by elution with hexane-ethylacetate (19:1) yielded a colourless gummy solid (0.36 g; m.p. 77-86°C) and showed two spots on the t.l.c. plates (R_f 0.48, 0.45 in hexane-ethylacetate, 4:1). The mixture was separated by preparative thin layer chromatography (chromatographed with silica gel) when two compounds B_1 and B_2 were obtained in the pure state. The one with R_f 0.48 (30 mg, B_1) was colourless solid and melted at 86-88°C. The i.r. absorptions at 3500-3300 (broad, OH) and 1700 cm^{-1} (strong, C = O for - COOH) indicated the compound to be a carboxylic acid. This was confirmed by converting it to its methyl ester with an ethereal solution of diazomethane. The ester melted at 63-65°C and showed the ester peak at 1730 cm^{-1} and did not show any absorption in the hydroxylic region. The p.m.r. spectrum of the compound showed a not too well defined triplet at $\delta(CDCl_3)$ 0.88, a sharp singlet of high intensity at 1.25 and a triplet at 2.32 which could be attributed to a terminal methyl group of a long alkyl chain, methylene groups of an alkyl chain and methylene group of the type $CH_2 - \overset{O}{\overset{\|}{C}} -$ respectively. The i.r. and p.m.r. spectra were thus characteristics of a long chain fatty acid. It was noted that the melting points of the compound and of its methyl ester are identical to those reported for hexacosanoic acid ($C_{25}H_{51}COOH$) and its methyl ester[44] respectively. The spectrum of the compound also revealed a number of mass

peaks showing successive loss of 14 mass units as expected from a compound possessing long alkyl chain. A closer examination of the mass spectrum showed the presence of all the fragment ions expected of hexacosanoic acid. The molecular ion ($RCOO^{+\cdot}H$) was observed at m/e 398 with moderate intensity and $RCOO^{+\cdot}H_2$ was also observed at m/e 397. Then one set of fragmentations could be observed from m/e 381($M-CH_3$) at 381, 353, 325, 297, 269, 241, 213, 185, 157, 129, 101, 73 arising from successive loss of 28 mass units for $CH_2=CH_2$ molecules. Another set of peaks at m/e 379, 351, 323, 295, 267, 239, 211, 183, 155, 127, were also noted with successive loss of 28 mass units starting from m/e 379 ($RCOO^{+\cdot}H_2-H_2O$). The mass peaks at m/e 127,113, 99, 85, 71, 57, 43 with successive loss of 14 mass units but with increasing intensity clearly revealed the presence of long chain alkyl system in the compound. The small mass peak at m/e 60 could arise from McLafferty rearrangement which on further fragmentation gave the base peak at m/e 43;



McLafferty Rearrangement of the Acid.

of course the high intensity of the latter may be due to the contribution of $C_3H_7^+$ formed by fragmentation of the alkyl chain. The compound thus appears to be hexacosanoic acid. Unfortunately the compound could not be compared with an authentic sample of hexacosanoic acid for non-availability of the latter with us.

The compound with R_f 0.45 (35.0 mg, B_2) was crystallised as white flakes from methanol, m.p. 63-64°C. Its i.r. and p.m.r. spectra were similar in nature as B_1 ν_{max} (nujol) 3500-2900 (OH), 1700 (acid C = O), 1360, 930 and 715 cm^{-1}; $\delta(CDCl_3)$:0.86(t), 1.25(S), 2.35(t) and is also a fatty acid. The methyl ester of the fatty acid was prepared by reacting it with diazomethane and was crystallised from methanol. It melted at 30 - 31°C. It showed no absorption in the hydroxylic region but a strong ester - C = O peak at 1735 cm^{-1} along with other peaks at 1180, 1105 and 710 cm^{-1}. The melting points of the acid and its ester suggested it to be palmitic acid, $C_{15}H_{31}COOH$[45]. The mass spectrum of the compound was also in agreement with that expected from palmitic acid. The molecular ion peak was observed at m/e 256 and the m/e peak at 257 could be attributed to $RCOOH_2^+$. As in the case of hexacosanoic acid as discussed above two sets of m/e peaks one at 213, 185, 157, 129, 101, 73 and the other at 211, 183, 155, 127

arising from m/e 241 (M-15) and m/e 239 (M+1-H_2O) respectively with successive loss of 28 mass units were observed. Mass peaks at m/e 113, 99, 85, 71, 57 and 43 showing loss of 14 mass units were also noted. Finally the compound was confirmed to be palmitic acid by co t.l.c., mixed m.p. and superimposable i.r. spectra with an authentic sample of palmitic acid.

Fraction C, eluted with hexane-ethylacetate (9:1) gave a waxy solid (0.40 g) on removal of the solvent. The solid melted at 70-73°C and showed three closely spaced spots on the t.l.c plate with R_f 0.48, 0.45, 0.43 when developed in hexane-ethylacetate (4:1) mixture. The material was subjected to separation by preparative thin layer chromatography and three relevant zones of silica gel were collected. The extraction of two of these gave very small quantity of material and were not sufficient for characterization. The other one yielded a colourless substance. It melted at 72-75°C and showed an elongated spot on t.l.c. plate (R_f 0.43 in hexane-ethylacetate, 4:1). The i.r. spectrum indicated it to be a carboxylic acid, ν_{max} (nujol) 3450-2800 (broad, O-H), 1700(acid, C=O). The p.m.r. spectrum was identical to those furnished by hexacosanoic acid and palmitic acid; $\delta(CDCl_3)$ 0.87(t), 1.22 (br.S) and 2.30(t).

The substance gave a methyl ester, which was obtained as a semi-solid (m.p. 55-67°C) mass and could not be crystallised. It showed no absorption in the hydroxylic region but a strong ester C=O peak at 1730 cm^{-1}, along with other peaks at 1180, 1110 and 710 cm^{-1}. The mass spectrum of the compound clearly indicated it to be a mixture of fatty acids, one of which was hexacosanoic acid (M.W. 396).

The mass peaks at m/e 480, 452, 424, 396, 382, 368, 354, 340 appear to be the molecular ions for dotricontanoic acid $CH_3(CH_2)_{30}COOH$, tricontanoic acid $CH_3(CH_2)_{28}COOH$, octacosanoic acid $CH_3(CH_2)_{26}COOH$, hexacosanoic acid $CH_3(CH_2)_{24}COOH$, pentacosanoic acid $CH_3(CH_2)_{23}COOH$, tetracosanoic acid $CH_3(CH_2)_{22}COOH$, tricosanoic acid $CH_3(CH_2)_{21}COOH$ and docosanoic acid $CH_3(CH_2)_{20}COOH$ respectively. The mass peaks expected from them could be identified in the mass spectrum of the compound and are tabulated below.

$CH_3(CH_2)_{30}COOH$ (M^+ 480) $\xrightarrow[-15]{-CH_3}$ 465 $\xrightarrow{-28}$ 437 $\xrightarrow{-28}$ 409 $\xrightarrow{-28}$ 381 $\xrightarrow{-28}$ 353 $\xrightarrow{-28}$ 325 $\xrightarrow{-28}$ 297 $\xrightarrow{-28}$ 269 $\xrightarrow{-28}$ 241 $\xrightarrow{-28}$ 213 $\xrightarrow{-28}$ 185 $\xrightarrow{-28}$ 157 $\xrightarrow{-28}$ 129 $\xrightarrow{-28}$ 101 $\xrightarrow{-28}$ 73.

$CH_3(CH_2)_{28}COOH$ (M^+ 452) $\xrightarrow[-15]{-CH_3}$ 437 $\xrightarrow{-28}$ 409 $\xrightarrow{-28}$ etc.

$CH_3(CH_2)_{26}COOH$ (M^+ 424) $\xrightarrow[-15]{-CH_3}$ 409 $\xrightarrow{-28}$ 381 $\xrightarrow{-28}$ etc.

$CH_3(CH_2)_{24}COOH$ (M^+ 396) $\xrightarrow[-15]{-CH_3}$ 381 $\xrightarrow{-28}$ 353 $\xrightarrow{-28}$ etc.

$CH_3(CH_2)_{23}COOH$ (M^+ 382) $\xrightarrow[-15]{-CH_3}$ 367 $\xrightarrow{-28}$ 339 $\xrightarrow{-28}$

311 $\xrightarrow{-28}$ 283 $\xrightarrow{-28}$ 255 $\xrightarrow{-28}$

227 $\xrightarrow{-28}$ 199 $\xrightarrow{-28}$ 171 $\xrightarrow{-28}$

143 $\xrightarrow{-28}$ 115 $\xrightarrow{-28}$ 87 $\xrightarrow{-28}$ 59.

$CH_3(CH_2)_{22}COOH$ (M^+ 368) $\xrightarrow[-15]{-CH_3}$ 353 $\xrightarrow{-28}$ 325 $\xrightarrow{-28}$ etc.

$CH_3(CH_2)_{21}COOH$ (M^+ 354) $\xrightarrow[-15]{-CH_3}$ 339 $\xrightarrow{-28}$ 311 $\xrightarrow{-28}$ etc.

$CH_3(CH_2)_{20}COOH$ (M^+ 340 $\xrightarrow[-15]{-CH_3}$ 325 $\xrightarrow{-28}$ 297 $\xrightarrow{-28}$ etc.

The mass spectral analyses thus indicate that the compound is probaoly a mixture of fatty acid, six possessing even number of carbon atoms and two possessing odd number

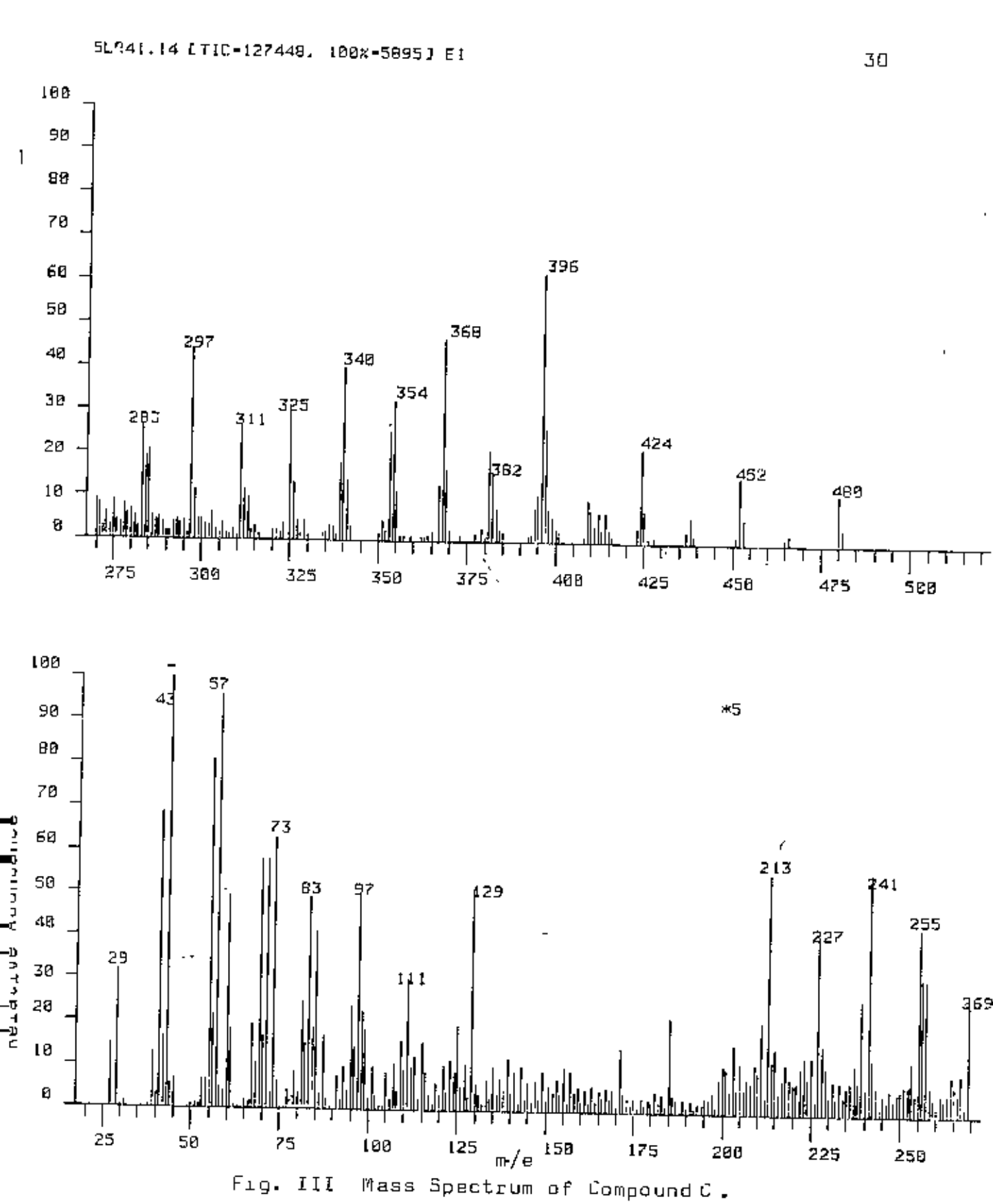


Fig. III Mass Spectrum of Compound C.

of carbon atoms. The methylester was subjected to g.l.c. analysis on silicone column at 210^{o}C, the sample showed a broad peak with a much higher retention time than those of the methylesters of palmitic acid and stearic acid. The broad peak tends to suggest that the column temperature was not sufficient to resolve the mixture of the esters in the sample. In the absence of suitable column which could bear a higher temperature further g.l.c. analysis of the ester could not be done.

2.3 Study on the Benzene Triturate

The benzene triturate of the crude mass obtained from ethanol extract gave a brown coloured residue (0.32g) on removal of the solvent. The material showed a distinct spot on t.l.c plate (R_f 0.56 in carbon tetrachloride-ethylacetate, 4:1) along with an elongated spot at the base. Column chromatography of the substance over silica gel and eluted with mixtures of different proportions of carbontetrachloride and ethyl acetate yielded a number of fractions. Fraction D eluted with carbon tetrachloride-ethylacetate (49:1) mixture showed one distinct spot on t.l.c. plate (R_f 0.55) with less distinct spots above and below this one. The other two fractions E and F did not give good t.l.c. picture. Fraction D was purified by preparative thin layer chromatography and yielded a colourless compound (60 mg, m.p. 60-67°C). The compound showed broad O-H absorption at 3500-2600 cm^{-1} and a sharp C=O absorption for carboxyl group at 1700 cm^{-1} in the i.r. spectrum. The p.m.r. spectrum showed a triplet at 0.88, a singlet at 1.26 and a triplet at 1.38. The compound gave a methyl ester with diazomethane and was a semi-solid mass. The ester showed the -C=O absorption for ester function at 1735 cm^{-1} and no absorption in the hydroxylic region. The compound thus also appeared to be

a fatty acid. The mass spectrum of the compound revealed it to be a mixture of higher fatty acids e.g. octacosanoic acid, $CH_3(CH_2)_{26}COOH$, hexacosanoic acid $CH_3(CH_2)_{24}COOH$, tetracosanoic acid $CH_3(CH_2)_{22}COOH$, docosanoic acid $CH_3(CH_2)_{20}COOH$, Octadecanoic acid $CH_3(CH_2)_{16}COOH$ and hexadecanoic acid $CH_3(CH_2)_{14}COOH$ for which molecular ion peaks were observed at m/e 424, 396, 368, 340, 284 and 256 respectively. The M-15 mass peaks for these were observed at m/e 409, 381, 353, 325, 269 and 241 respectively. The M-15 peaks then showed successive loss of 28 mass units and the mass peaks at m/e 213, 185, 157, 129, 101, 73 are common for all of them. The sequences are shown as below.

$$CH_3(CH_2)_{25}COOH \xrightarrow[-15]{-CH_3} 409 \xrightarrow{-28} 381 \xrightarrow{-28} 353 \xrightarrow{-28} \text{etc.}$$
$(M^+\cdot\ 424)$

$$CH_3(CH_2)_{24}COOH \xrightarrow[-15]{-CH_3} 381 \xrightarrow{-28} 353 \xrightarrow{-28} 325 \xrightarrow{-28} \text{etc.}$$
$(M^+\cdot\ 396)$

$$CH_3(CH_2)_{22}COOH \xrightarrow[-15]{-CH_3} 353 \xrightarrow{-28} 325 \xrightarrow{-28} 297 \xrightarrow{-28} \text{etc.}$$
$(M^+\cdot\ 368)$

$$CH_3(CH_2)_{20}COOH \xrightarrow[-15]{-CH_3} 325 \xrightarrow{-28} 297 \xrightarrow{-28} 269 \xrightarrow{-28} \text{etc.}$$
$(M^+\cdot\ 340)$

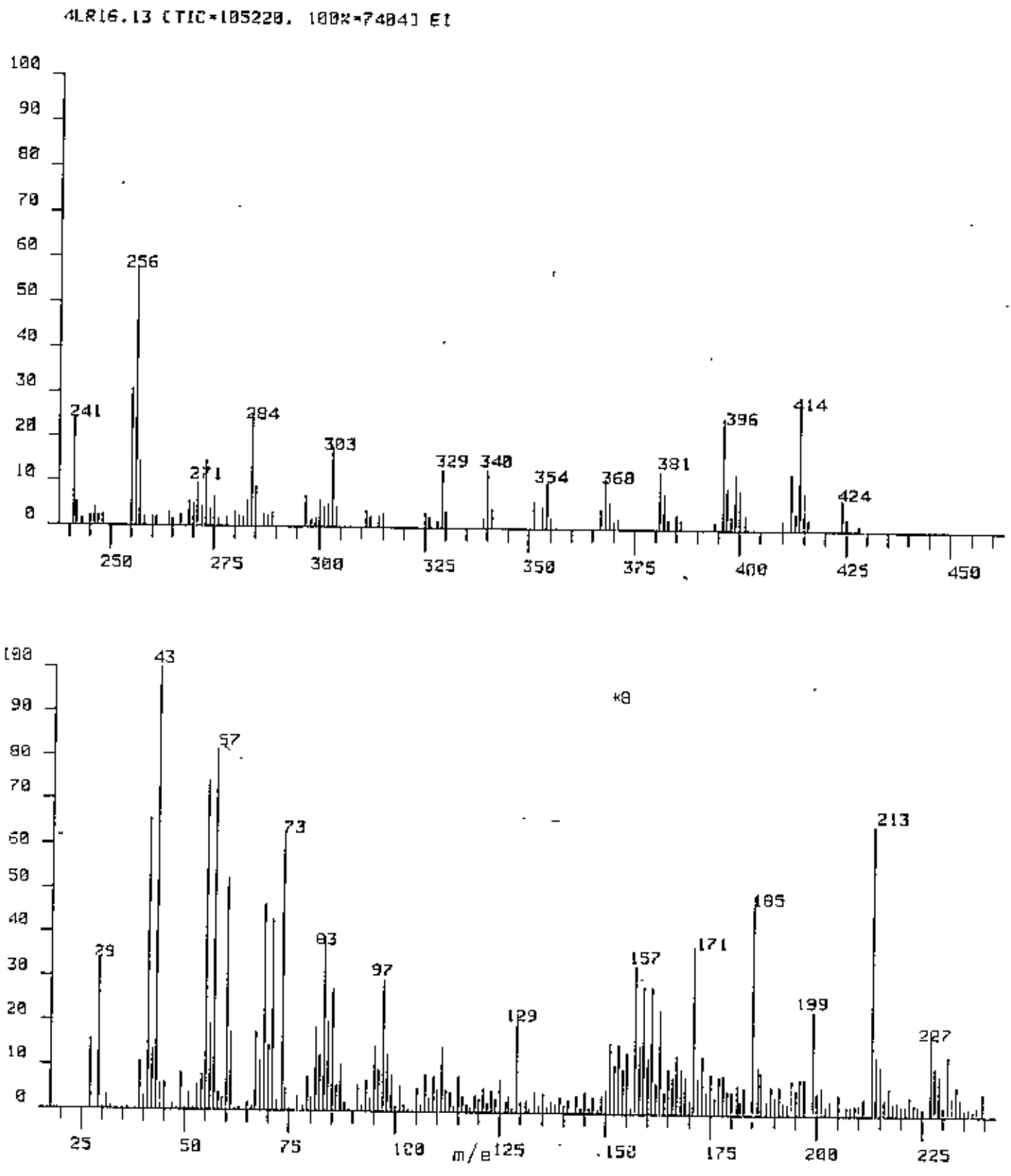


Fig. IV. Mass Spectrum of Compound D.

$$CH_3(CH_2)_{16}COOH \xrightarrow[-15]{-CH_3} 269 \xrightarrow{-28} 241 \xrightarrow{-28} 213 \xrightarrow{-28} \text{etc.}$$
(M^+ 284)

$$CH_3(CH_2)_{14}COOH \xrightarrow[-15]{-CH_3} 241 \xrightarrow{-28} 213 \xrightarrow{-28} 195 \xrightarrow{-28} \text{etc.}$$
(M^+ 256)

The compound thus in fact appears to be a mixture of fatty acids of even number of carbon atoms. The mass spectrum, however, shows the presence of at least one fatty acid with odd number of carbon atoms namely $CH_3(CH_2)_{21}COOH$. Its molecular ion is observed at m/e 354 and other expected mass peaks at M-15 and followed by successive loss of 28 mass units are also observed at m/e 339, 311, 283, 255, 227, 199, 171, 143, 115, 87 and 59 respectively. The long chain alkyl character of the compound was revealed by the nigher intensity of larger mass peaks with diminishing intensity for the lower mass peaks, specially on the lower side. The McLafferty rearrangement producing mass ion at m/e 60 is also observed which on further loss of 17 mass unit (OH) yielded the mass peak at m/e 43. The mass spectral data thus undoubtedly shows the compound to be a mixture of acids. The results could have been further confirmed by g.l.c. analysis of the methylester but the nonavailability of a suitable column did not allow us to proceed further

in this respect. It is thus observed that there is a good similarity in the acid composition of Fraction C and Fraction D.

2.4 Study on Diethyl Ether and Methanol Triturates

The diethylether triturate (0.86g) and methanol triturate (1.73g) gave complicated t.l.c pictures. Attempts to resolve them on the plates using different solvent systems proved abortive. No further work was therefore attempted on these triturates.

2.5 Study on the Mother Liquor of Ethanol Extract

The dark green mother liquor of ethanol extract was treated with an excess volume of distilled water when a black solid mass (6.0g) precipitated out. The semi-solid black mass (Q) was successively triturated with n-hexane, diethylether and methanol. The last two triturates revealed complex t.l.c behaviour and were not studied.

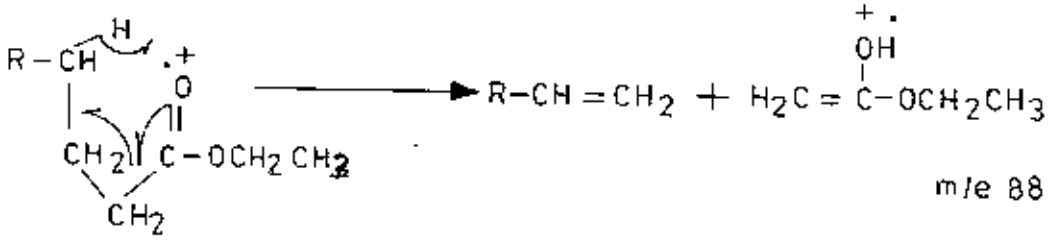
2.6 Study on n-hexane Triturate

The green coloured n-hexane triturate gave 3.5 g semi-solid mass on removal of the solvent. 1.75g crude product was chromatographed over a column of silica gel and eluted with carbontetrachloride-chloroform (3:1; 1:1) respectively. Several fractions were collected and each of them was separately examined.

Fraction G, eluted with carbon tetrachloride gave a waxy solid mass and was highly nonpolar (50.0mg, m.p. 32-34°C). It showed only one spot on t.l.c. plates (R_f 0.95 in carbon tetrachloride). Its i.r. spectra indicated it to be saturated hydrocarbon.

Fraction H, also eluted with carbontetrachloride gave a semi-solid mass (92 mg) and showed one spot on t.l.c plate (R_f 0.55 in carbon tetrachloride). The i.r. spectrum (liquid film) of the compound showed a strong absorption at 1735 cm^{-1} indicating it to be an ester. Two other bands were observed at 1180 cm^{-1}, 1105 cm^{-1} for -O-C=O of ester function. The triplet at δ 0.80 and the quarter at δ4.05 (J = 6.5 Hz) in the p.m.r. spectrum of the compound clearly showed it be an ethyl ester. The broad singlet at δ 1.22 and a not too well defined triplet at δ 2.25 showed the presence of large number of methylene groups and $-CH_2-C=O$ grouping respectively. The mass spectrum of the compound in general showed the presence of long chain alkyl system. However, the strong mass peaks at m/e 368, 312, and 284 which are in agreement with molecular ions of the ethyl ester of docosanoic acid $CH_3(CH_2)_{20}COOH$, stearic acid $CH_3(CH_2)_{16}COOH$ and palmitic acid $CH_3(CH_2)_{14}COOH$ strongly suggest that it is most probably a mixture of at least these three esters. The other important mass peaks can be explained from these structures. The presence of carbethoxy groups ($-COOCH_2CH_3$) in these compounds are clearly evidenced by the base peak at m/e 88 which can arise from all of them by McLafferty rearrangement. The mass peaks at m/e 295, 239 and 211 can also arise from M^+ 368, 312 and 284 respectively by the loss of $-COOCH_2CH_3$ group

from each of them. Other mass peaks expected from



McLafferty Rearrangement of the Esters.

fragmentation from both the sides i.e. the alkyl and ester sides were also observed. These are noted below.

Cleavage from the esterside:

$CH_3(CH_2)_{20}COOCH_2CH_3$ (M^+ 368) $\xrightarrow[-73]{-COOCH_2CH_3}$ 295 $\xrightarrow{-28}$ 267 $\xrightarrow{-28}$ 239 $\xrightarrow{-28}$ 211 $\xrightarrow{-14}$ 197 $\xrightarrow{-14}$ 183 $\xrightarrow{-14}$ 169 $\xrightarrow{-14}$ 155 $\xrightarrow{-14}$ 141 $\xrightarrow{-14}$ 127 $\xrightarrow{-14}$ 113 $\xrightarrow{-14}$ 99.

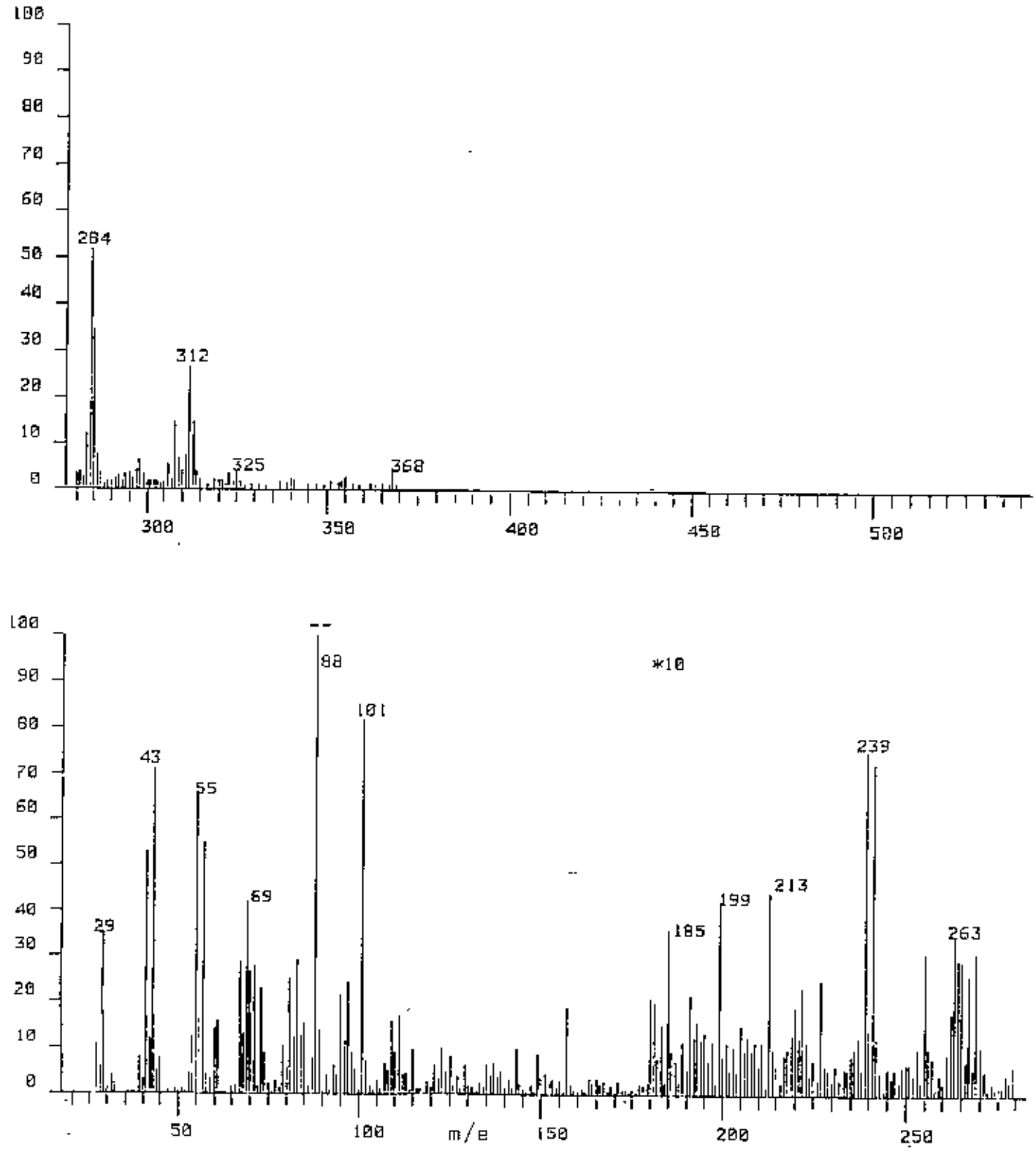


Fig. V Mass Spectrum of Compound H.

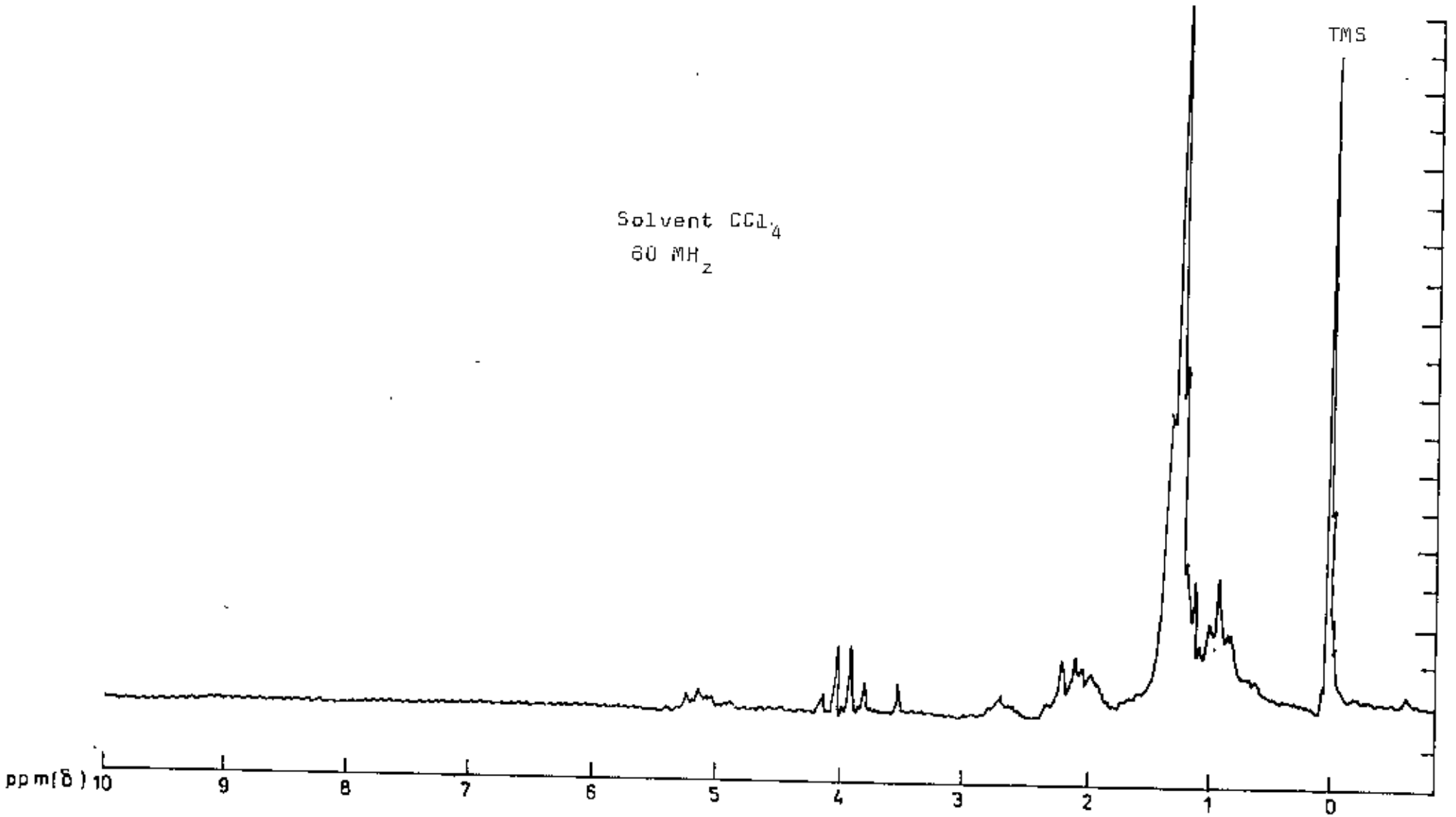


Fig. V I ^{1}HNMR Spectrum of Compound H.

$CH_3(CH_2)_{16}COOCH_2CH_3$ (M^+ 312) $\xrightarrow[-73]{-COOCH_2CH_3}$ 239 $\xrightarrow{-28}$ 211 $\xrightarrow{-14}$ etc.

$CH_3(CH_2)_{14}COOCH_2CH_3$ (M^+ 284) $\xrightarrow[-73]{-COOCH_2CH_3}$ 211 $\xrightarrow{-14}$ 197 $\xrightarrow{-14}$ etc.

Cleavage from alkyl side:

$CH_3(CH_2)_{20}COOCH_2CH_3$ (M^+ 368) $\xrightarrow[-15]{-CH_3}$ 353 $\xrightarrow{-28}$ 325 $\xrightarrow{-28}$ 297 $\xrightarrow{-28}$ 269 $\xrightarrow{-28}$ 241 $\xrightarrow{-14}$ 227 $\xrightarrow{-14}$ 213 $\xrightarrow{-14}$ 199 $\xrightarrow{-14}$ 185 $\xrightarrow{-14}$ 171 $\xrightarrow{-14}$ 157 $\xrightarrow{-14}$ 143 $\xrightarrow{-14}$ 129 $\xrightarrow{-14}$ 115 $\xrightarrow{-14}$ 101.

$CH_3(CH_2)_{16}COOCH_2CH_3$ (M^+ 312) $\xrightarrow[-15]{-CH_3}$ 297 $\xrightarrow{-28}$ 269 $\xrightarrow{-28}$ etc.

$CH_3(CH_2)_{14}COOCH_2CH_3$ (M^+ 284) $\xrightarrow[-15]{-CH_3}$ 269 $\xrightarrow{-28}$ 241 $\xrightarrow{-14}$ etc.

The mass spectrum of the compound showed some mass peaks at higher m/e values which appear to be due to the presence of some minor impurities of higher molecular weight. The p.m.r. spectrum of the compound showed an absorption in the olefinic region as a triplet at 5.25 which suggests the presence of olefinic compound. This may be due to the presence of some unsaturated fatty acid ester. Further confirmation about the nature of the mixture could not be established for want of suitable g.l.c. analytical facilities. The hydrolysis to convert the ester mixture to acids and analyse the acid mixture could not be done either because of the meagre quantity of material obtained. It may be noted that the petroleum ether triturate of the crude alcohol extract has been shown to contain free hexacosanoic acid (B_1) and free hexadecanoic acid (B_2) along with the mixture of other higher fatty acids (C).

Fraction K, eluted with carbon tetrachloride-chloroform (3:1) gave a yellowish solid mass (160 mg) on removal of solvent. It gave colourless fine needle shaped crystals (120 mg) on crystallisation from hexane. It showed one spot on t.l.c. plate (R_f 0.57 in carbon tetrachloride-ethyl acetate, 3:1). It melted at 136-137°C and gave positive Liebermann-Burchard and Salkowski test for steroids. The i.r. spectrum (nujol) showed O-H absorption at 3430, 1050, and 715 cm^{-1}. The compound was converted to its acetate by refluxing it with freshly distilled acetic anhydride and dry pyridine. The acetate melted at 124-26°C. The t.l.c. and i.r. showed different R_f and absorption than the parent compound; R_f 0.82 in carbon tetrachloride-ethyl acetate, 3:1, the absorption at 3430 cm^{-1} had disappeared and new bands at 1735 and 1120 cm^{-1} for C=O of acetate and acetate skeletal vibration were visible. The p.m.r spectrum of the compound showed the presence of six C-methyl signals appearing between δ 0.7-1.02. Out of these signals the one at δ 1.02 (3H,S) and δ0.7 (3H,S) are characteristics of the two angular methyl group (C_{19} and C_{18} respectively) in a steroid nucleus[46]. The other four C-methyl groups were observed at δ 0.9, 0.82, 0.8 and 0.78. A broad doublet (probably a doublet of a doublet) for one proton at δ 5.35 revealed the presence of an olefinic proton.

The compound showed the molecular ion peak at m/e 414 in agreement with the molecular formula of β-sitosterol, $C_{29}H_{50}O$. The mass spectrum also showed other important mass peaks at m/e 399 ($M-CH_3$), 396(M-18), 381($M-H_2O-CH_3$), 371(M-isopropyl group), 329($M-CH-CH_2CH_3$- isopropyl group), 273(M- side chain $C_{10}H_{21}$), 255(M- side chain $C_{10}H_{21}-H_2O$), 213(M- side chain $C_{10}H_{21}$ - ring D C_3H_5 - H_2O - H^+) which are characteristics for a hydroxy steroid[47]. The above spectral data and the observed melting point of the compound and of its acetate suggest its identity with β-sitosterol[48] (m.p. 136-37°C, acetate m.p. 124-26°C). The identity was finally confirmed by a comparison of its superimposable i.r. spectrum and undepressed mixed melting point with an authentic sample of β-sitosterol.

Fraction L, eluted with carbon tetrachloride - chloroform (1:1), yielded a pale yellow solid mass (550 mg) The fraction on repeated crystallisation from large volume of hexane gave fine crystals (250 mg). It melted at 70-71°C and was found to be homogeneous on t.l.c. plates in different solvent systems. The i.r. absorption at 3400 - 2550 and 1700 cm^{-1} indicated it to be a carboxylic acid. It was confirmed by converting it to methyl ester with diazomethane. The p.m.r spectrum showed a not too well resolved triplet at δ0.88 and a huge singlet at δ1.22 and a triplet at δ2.32, similar to that observed with palmitic acid and hexacosanoic acid. However, the m.p. of the compound and that of its methyl ester was similar to those reported for stearic acid (71 - 72°C) and its methyl ester (37 - 39°C)[49]. On comparison of the compound with the authentic sample of stearic acid it was found that their mixed melting point was undepressed, they produced superimposable i.r. spectrum and their R_f values were identical. The compound is therefore stearic acid.

2.7 Study on Aqueous Mother Liquor of Ethanol Extract

The brownish aqueous mother liquor of ethanol extract was extracted with diethyl ether and chloroform respectively. A brownish diethyl ether extract (1.50g) and a brown coloured chloroform extract (0.52g) were obtained. After basifying with 4% sodium hydroxide solution, the liquor was extracted with diethyl ether and chloroform and a negligible mass was obtained. The chloroform extract revealed complex t.l.c. behavior which was not further studied.

2.8 Study on Diethyl ether Extract of Aqueous Alcololic Mother Liquor

The orownish diethyl ether extract gave semi-solid mass (1.50g). This crude mass was chromatographed over a column of silica gel and eluted with carbon tetrachloride and the mixtures of carbon tetrachloride-ethyl acetate in the ratio of 19:1 and 4:1 respectively. Several fractions were collected and each of them was separately examined.

Fraction N, eluted with carbon tetrachloride gave a low melting semi-solid mass (225 mg) which was impure. It was separated by p.l.c. A yellowish low melting semi-solid mass (85 mg) was obtained. It showed one spot on t.l.c. plate (R_f . 0.54 in carbontetrachloride). It gave a similar i.r. and p.m.r. spectra to that of fraction H. The mass spectrum

showed also the same fragmentation to that of fraction H. The R_f values were identical to that of fraction H and were confirmed by co t.l.c. Therefore it is assumed that the fraction N is a mixture of esters like that of fraction H.

Fraction O, eluted with carbon tetrachloride-ethyl acetate (19:1) gave a white waxy solid. It showed one distinct spot with a long tail. It was separated by p.l.c. and yielded a white solid mass (60 mg). It showed one spot on t.l.c. plate (R_f 0.44 in carbon tetrachloride-ethyl acetate, 4:1) and melted at 73-75°C. The i.r., p.m.r and mass spectra indicated it to be a mixture of fatty acids like that of fraction D.

CHAPTER 3
EXPERIMENTAL

3.1 General Experimental

Melting Points (m.p.)

Melting points were determined on a Reichert hot-stage microscope and on a Fisher John's electrothermal melting point apparatus. Melting points were not corrected.

Infra-red Spectra (i.r.)

Infra-red spectra were recorded on a Perkin-Elmer 237 spectrometer and on a PYE UNICAM SP3 - 200 spectrometer using either chloroform or nujol mull or liquid film.

Nuclear Magnetic Resonance Spectra (n.m.r.)

Nuclear magnetic resonance spectra were recorded on a Perkin-Elmer R12A or Varian HA 100 instrument, using either deuterochloroform ($CDCl_3$) or carbon tetrachloride (CCl_4) as solvent with tetramethylsilane (TMS) as internal standard.

Mass Spectra (m.s.)

Mass spectra were recorded on a US - 55 Mass Spectrometer, RFI EASF 3.20.

Thin Layer Chromatography (t.l.c.)

The material used for thin layer chromatography was kiesel gel 60 GF_{254} (MERCK). The plates (7.5x2.5 cm) were prepared by drawing a suspension of kiesel gel 60 GF_{254} (8 g in 16 ml water) over the thoroughly cleaned plates. The plates were left in position at room temperature until the surface become completely dry. The plates were then allowed to stand for twenty four hours for activation and were ready for use.

Preparative Thin Layer Chromatography (p.l.c.)

Preparative thin layer chromatography was carried out on plates coated with kieselgel 60 GF_{254} (MERCK). The plates were prepared in the same manner as described above out using larger glass plates (23x20 cm) and a larger spreader allowing thicker coating (0.75 cm). The plates were air dried at room temperature over night and then further activated by warming them at 110°C for half an hour.

Column Chromatography

The column was prepared by slurry method, silica gel (60 - 120 mesh, BDH, England) being the stationary phase. The column was made half filled with the appropriate

solvent (the best running solvent was established by t.l.c.), and the slurry was poured into it so that the packing was compact and uniform. Air bubble was avoided by making the column as quickly as possible and allowing the solvent to fall drop by drop through the stopcock of the column. The solvent was allowed to pass through the column for sufficient time and then the column was allowed to settle for about one hour. The mixture of compounds was then taken as a solution and was allowed to fall on the surface of the column. The column was then eluted previously purified desired solvent system.

Gas Liquid Chromatography (g.l.c.)

The gas chromatographic analysis of the samples reported in the dissertation was performed on a Schimadzu GC - 3BF gas chromatograph with dual flame ionization detector using 2.1mx3mm glass column coated with 5% silicone GE SE-30 on AW-DMCS (60 - 80 mesh).

The following abbreviations were used in describing spectra:

I.R. : S, strong; m, medium; w, weak; b, broad.

P.M.R. : S, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; b, broad; sh, sharp.

3.2 Extraction of the Plant Sida Cordifolia Linn

Sida cordifolia Linn (brela) plants were collected from Dhamrai, 40 miles north of Dhaka. The aerial parts were dried in the sun, powdered (1.7 kg) and were extracted with ethanol for 24 hrs at room temperature. The extraction was continued until the extract became virtually colourless. The deep green alcoholic extract (ca 8 l) was concentrated to one fourth of its volume at reduced pressure. The extract was allowed to stand in a refrigerator when a light green solid mass deposited at the bottom. The solid mass was separated by decantation followed by centrifugation and dried. The filtrate was further reduced to one third of its volume and kept in the refrigerator and a further quantity of green solid mass was obtained. The two fractions of the residue were combined to give 5 g of a deep green, almost black mass, was denoted as P. The mother liquor, a heavy thick deep green solution (ca 500 ml) was poured into distilled water when a black solid precipitated out, was denoted as Q.

3.3 Isolation of the Components of Ethanol Extracts (Mass P)

The dried solid green mass (5g) obtained from ethanol extract was successively triturated with petroleum ether (40-60°C), benzene, diethyl ether and methanol. The diethyl ether and methanol triturates revealed complex t.l.c. behaviour and were not studied.

3.4 Examination of Petroleum ether Triturate

The petroleum ether triturate was concentrated to a small volume under reduced pressure and allowed to stand in the refrigerator. A light green coloured residue which deposited at the bottom of the container was separated, dried and weighed (8 mg). The deep green coloured mother liquor showed one spot on t.l.c. plate (R_f 0.92) along with an unresolved zone at the base line when developed in hexane. In hexane and ethyl acetate (9:1) mixture, the crude product exhibited three distinct spots at R_f 0.98, 0.47, 0.44 with an unresolved zone at the base line. The mother liquor was evaporated to dryness at reduced pressure and 1.27g of a crude green viscous material was obtained. This was chromatographed over a column of silica gel (160g, BDH, 60-120 mesh) and eluted with hexane and mixture of hexane and ethyl acetate in

the ratios of 19:1 and 9:1 in that order. Fractions of about 7 ml volume were collected and every fifth fraction was examined on t.l.c. plates. The fractions showing similar t.l.c. behaviour were combined. The results are shown in Table I.

Table I Column Chromatographic Separation of Petroleum ether Triturate

Fraction No.	Eluant	Yield	Remarks
Fraction A (collection nos. 3-30)	hexane	0.32g	One spot, m.p. 32-34°C R_f (hexane) 0.92
Fraction B (collection nos. 35-70)	hexane-ethyl acetate (19:1)	0.36g	Two spots, m.p. 77-86°C R_f (hexane-ethyl acetate, 4:1) 0.48, B_1; 0.45, B_2
Fraction C (collection nos. 72-160)	hexane-ethyl acetate (9:1)	0.40g	Three spots, m.p. 70-73°C R_f (hexane-ethyl acetate, 4:1) 0.48, 0.45, 0.43

3.4a Characterisation of Fraction A

Fraction A yielded a white low melting solid (0.32g, m.p. 32-34°C). It gave one spot on t.l.c. plate when developed in hexane (R_f 0.92).

IR ($CHCl_3$): ν_{max} 2970, 2940, 2870, 1460, 1390, 730, 720 cm^{-1}.

^{1}HNMR ($CDCl_3$): δ 0.88(q), 1.24 (s).

Mass Spectrum: m/e 365(M^+), 351, 323, 309, 295, 281, 267, 253, 239, 225, 211, 197, 183, 169, 155, 141, 127, 113, 99, 85, 71, 57.

G.L.C. analysis of Fraction A was carried out on silicone GE SE-30 at 150°C. It showed as many as twelve peaks, of which five were prominent.

3.4b Characterisation of Fraction B

Fraction B also gave a white solid mass (360 mg; m.p. 77-85°C) and showed two spots on t.l.c. plate (R_f 0.48, 0.45) when developed in hexane and ethyl acetate (4:1) mixture. The impure material was subjected to separation by preparative thin layer chromatography using carbon tetrachloride-ethyl acetate (12:1) mixture. Two compounds having R_f 0.48 and 0.45 in hexane-ethyl acetate (4:1) mixture were isolated; the total amount being 30 mg and 35 mg were denoted as B_1 and B_2 respectively.

Compound B_1 was a waxy solid and showed one spot on t.l.c. plates in different solvent systems. It melted at 86-88°C.

IR ($CHCl_3$): ν_{max} 3500-3300, 1700, 920, 720 cm^{-1}.

^{1}HNMR ($CDCl_3$): δ0.88(t), 1.25 (br.s), 2.32(t).

Mass Spectrum: m/e 397 (M+1), 396 (M^+), 381, 379, 353, 351, 325, 323, 297, 295, 269, 267, 241, 239, 213, 211, 185, 183, 157, 155, 129, 127, 101, 99, 85, 73, 71, 50, 57, 43.

Esterification of B_1

10 mg of the compound was dissolved in diethyl ether in presence of small amount of ethylalcohol. To this solution freshly prepared diazomethane in diethyl ether (prepared from the treatment of nitrosomethyl urea with caustic potash) was added dropwise until the yellow colour of diazomethane persisted. The reaction mixture was evaporated to dryness, redissolved in diethyl ether and dried over anhydrous sodium sulphate. On removal of the solvent a colourless compound was obtained. It melted at 63-65°C. The compound showed one spot on t.l.c. plates (R_f 0.90) when developed in hexane-ethylacetate (4:1) mixture.

IR (Film): ν_{max} 1730, 1190, 720 cm^{-1}.

Compound B_2 was crystallized from methanol. It melted at 63-64°C.

IR ($CHCl_3$): ν_{max} 3450-2900, 1700, 1370, 1350, 930, 715 cm^{-1}.

^{1}HNMR ($CDCl_3$): δ 0.85(t), 1.25,(br.s), 2.35(t).

Mass Spectrum: m/e 257(M+1), 256(M^+), 251, 241, 239, 213, 211, 185, 183, 157, 155, 129, 127, 113, 101, 99, 85, 73, 71, 60, 57, 43.

Esterification of B_2

10 mg of the compound was subjected to esterification with diazomethane following the same procedure as described earlier. The ester obtained as a semi-solid was crystallized from methanol. It melted at 30-31°C. It showed one spot on t.l.c. plate having R_f 0.77 in hexane-ethylacetate (4:1) mixture.

IR (Film): ν_{max} 1735, 1180, 1105, 710 cm^{-1}.

A mixed melting point of the compound B_2 with palmitic acid (m.p. 63-64°C).[45] remained undepressed and the i.r. spectrum of the compound was superimposable on that of an authentic sample of palmitic acid. The R_f value of the compound was also similar to that of palmitic acid.

3.4c Characterisation of the Fraction C

Fraction C, eluted with hexane-ethyl acetate (9:1) gave a white waxy solid (0.40g, m.p. 70-73°C) on removal of the solvent. It showed three spots on t.l.c. plate with R_f 0.48, 0.45, 0.43 in hexane-ethyl acetate (4:1). The material was subjected to separation by preparative thin layer chromatography using carbon tetrachloride-ethyl acetate (12:1) and three zones of silica gel corresponding to three different R_f values were collected. The extraction of two of these gave very small quantity of material. The other one yielded a colourless substance (30 mg). It melted at 72-75°C and showed an elongated spot on t.l.c. plate (R_f 0.43 in hexane-ethyl acetate 4:1).

IR ($CHCl_3$): ν_{max} 3450-2600, 1700 cm^{-1}.

^{1}HNMR ($CDCl_3$): δ 0.87(t), 1.22 (br.S) 2.30(t).

Mass Spectrum: m/e 480, 465, 453, 452, 437, 425, 424, 409, 397, 396, 383, 382, 381, 369, 368, 367, 355, 354, 353, 341, 340, 339, 325, 297, 269, 241, 213, 185, 183, 157, 129, 101, 53.

Esterification of C

10 mg of the compound was subjected to esterification with diazomethane following the same procedure as described earlier. Crystallization from different solvents proved abortion. The waxy mass melted at 55-57°C. It showed one spot on t.l.c. plate R_f (0.88) in hexane and ethyl acetate (4:1) mixture.

IR (Film) ν_{max} 1730, 1180, 1110, 710 cm^{-1}.

The ester on g.l.c. analysis at 210°C showed a broad peak having higher retention time than the esters of palmitic acid and stearic acid.

3.5 Examination of Benzene Triturate

The benzene triturate was evaporated to dryness when a deep green coloured viscous material was obtained (0.32g). It showed a spot on t.l.c. plate (R_f 0.56) along with a long tail at the base line when developed in carbon tetrachloride-ethyl acetate (4:1).

The crude benzene triturate was chromatographed over a column of silica gel (50g, BDH, 60-120 mesh) and was eluted with mixtures of carbon tetrachloride-ethyl acetate in the ratio 49:1, 23:2 and 4:1 respectively. Fractions of about 8 ml volume were collected and every fifth fraction was examined on t.l.c. plate. The fractions showing similar t.l.c. behaviour were combined. The results are shown in Table II.

Table II. Column chromategraphic Separation of Benzene Triturate

Fraction No.	Eluting solvent	Yield	Remarks
Fraction D (collection nos. 7-50)	Carbon tetrachloride: ethyl acetate(49:1)	0.125g	One distinct spot R_f(CCl_4:EtOAc,4:1) 0.56 and other minor spot. m.p. 55-63°C.
Fraction E (collection nos. 52-90)	Carbon tetrachloride: ethyl acetate(23:2)	0.10g	No distinct spot
Fraction F (collection nos.95-150)	Carbon tetrachloride: ethyl acetate(4:1)	0.085g	No distinct spot

3.5a Characterisation of Fraction D

Fraction D gave a greenish coloured solid mass (125 mg, m.p. 55-63°C) and it showed one distinct spot on t.l.c. plate (R_f 0.56) with less distinct spots above and below this one when developed in carbon tetrachloride-ethyl acetate (4:1). The sample was subjected to separation by preparative thin layer chromatography using the same solvent system. 60 mg of a compound having R_f 0.56 was obtained. It was a colourless solid and melted at 60-67°C.

IR ($CHCl_3$): ν_{max} 3500 - 2600, 1700 cm^{-1}.

^{1}HNMR ($CDCl_3$): δ 0.88(t), 1.26(s), 1.38(t).

Mass Spectrum: m/e 424, 409, 396, 381, 368, 354, 353, 340, 339, 325, 311, 284, 283, 269, 256, 255, 241, 231, 227, 199, 185, 171, 143, 137, 129, 115, 101, 87, 73, 59.

Esterification of D

10 mg of the compound was subjected to esterification with diazomethane following the same procedure as described earlier. Crystallisation from different solvents proved abortion. The waxy mass melted at 55-60°C. It showed one spot on t.l.c. plate (R_f 0.82 in hexane-ethyl acetate, 4:1).

IR (Film): ν_{max} 1735, 1180, 1110, 715 cm^{-1}

3.6 Examination of Mother Liquor of Ethanol Extract

The heavy dark green mother liquor of ethanol extract was treated with an excess volume of distilled water when a deep black coloured solid mass (6.0g) precipitated out. The water insoluble solid mass and the aqueous mother liquor were separately processed for further study.

3.7 Separation of the Components from the Water Insoluble Fraction

The semi-solid black mass (6.0g) was successively triturated with n-hexane, diethyl ether and methanol. The last two triturates appeared to be a complex mixture of a number of compounds as revealed by t.l.c. and as such no further study was attempted on them.

3.7a Examination of the Hexane Triturate

The green coloured n-hexane triturate gave 3.5g of a semi-solid mass on removal of the solvent. It showed two spots on t.l.c. plate (R_f 0.95, 0.55) along with a rather large size unresolved zone at the base line when developed in neat carbon tetrachloride. In a mixture of carbon tetrachloride-chloroform (3:1), the triturate showed one spot at the solvent front and two clear spots at R_f 0.56, 0.45 respectively along with an unresolved zone at the base line. 1.75g crude product was chromatographed over a column of silica gel (160 g, BDH, 60-120 mesh) and successively eluted with carbon tetrachloride and mixtures of carbon tetrachloride-chloroform in the ratio 3:1 and 1:1 respectively. Fraction of about 10 ml volume were collected. Every fifth fraction was examined on t.l.c. plate. The fractions showing similar t.l.c. behaviour were combined. The results are shown in Table III.

Table III Column Chromatographic Separation of Hexane Triturate

Fraction No.	Eluting solvent	Yield	Remarks
Fraction G (collection nos. 2-28)	Carbon tetrachloride	50 mg	One spot, $R_f(CCl_4)$ 0.95 White waxy solid m.p. 32-34°C.
Fraction H (collection nos. 30-80)	Carbon tetrachloride	92 mg	One spot, $R_f(CCl_4)$ 0.55 yellowish semi-solid.
Fraction K (collection nos. 90-130)	Carbon tetrachloride: chloroform (3:1)	160 mg	One spot, $R_f(CCl_4$-EtOAc, 3:1) 0.57 yellowish solid.
Fraction L (collection nos. 165-310)	Carbontetrachloride: chloroform(1:1)	550 mg	One spot with long tail $R_f(CCl_4$-EtOAc, 3:1) 0.30 yellowish solid.
Fraction M (collection nos. 312-425)	Carbon tetrachloride: chloroform (1:1)	425 mg	No distinct spot.

3.7b Characterisation of Fraction G

Fraction G, eluted with carbon tetrachloride gave a waxy solid mass on removal of the solvent. It showed only one spot on t.l.c plate (R_f 0.95 in carbon tetrachloride). It melted at 32 - 34°C.

IR ($CHCl_3$): ν_{max} 2970, 2940, 2870, 1460, 1390, 730, 720 cm^{-1}.

3.7c Characterisation of Fraction H

Fraction H yielded a yellowish low melting semi-solid mass (92 mg). It showed one splot on t.l.c. plate (R_f 0.55 in carbon tetrachloride).

IR (liquid film): ν_{max} 1735, 1180, 1105 cm^{-1}.

^{1}HNMR (CCl_4): δ 0.80(t), 1.22(br.s), 2.25(t), 4.05(q).

Mass Spectrum: m/e 368, 353, 325, 312, 297, 295, 284, 269, 267, 241, 239, 227, 213, 211, 199, 197, 185, 183, 171, 169, 157, 155, 143, 141, 129, 127, 115, 113, 101, 99, 88.

3.7d Characterisation of Fraction K

Fraction K, eluted with carbon tetrachloride-chloroform (3:1) mixture gave a yellowish solid mass (160 mg) on removal of the solvent. The solid mass was crystallised from hexane when fine needle shaped crystals (120 mg) were obtained. The compound showed one spot on t.l.c. plate (R_f 0.57) when developed in carbon tetrachloride-ethyl acetate (3:1) mixture. It melted at 136-37°C.

IR (nujol): ν_{max} 3430, 1050, 715 cm^{-1}.

^{1}HNMR ($CDCl_3$): δ 0.7, 0.78, 0.8, 0.82, 0.9, 1.02 and 5.35.

Mass Spectrum: m/e 399, 396, 381, 371, 329, 273, 255, 213.

Salkowski Reaction

A few mg of the compound was dissolved in chloroform and a few drops of concentrated sulphuric acid was added. A deep red colour was observed which indicated the positive test for sterol.

Liebermann-Burchard Reaction

A few mg of the compound was dissolved in chloroform and a few drops of concentrated sulphuric acid was added

to it. The 2-3 drops of acetic anhydride were added to the mixture. A green colour was observed which indicated it to be a sterol.

Acetylation of the Compound

A small quantity of the compound (14 mg) was taken in a round bottom flask and freshly distilled acetic anhydride (1.5 ml) was added to it followed by addition of 4-5 drops of distilled dry pyridine. The mixture was refluxed under anhydrous condition for three hours and then allowed to cool. The reaction mixture was poured into ice-water (20-25 ml). The residue which separated out was filtered and the product was crystallised from methanol. The acetate was dried in a vacuum desiccator.

It melted at 124 - 26°C and showed one spot on t.l.c. plate having R_f 0.82 in carbon tetrachloride-ethyl acetate (3:1) mixture.

IR (nujol): ν_{max} 1735, 1120 cm^{-1}.

A mixed melting point of the compound with β-sitosterol (136-37°C)[48] remained undepressed. The i.r. spectrum of the compound was superimposable on that of an authentic sample of β-sitostrol. The R_f value of the compound was also similar to that of β-sitosterol.

2.7e. Characterisation of Fraction L

Fraction L, eluted with carbon tetrachloride-chloroform (1:1) mixture gave a yellowish solid mass (550 mg) on removal of the solvent. The mass was crystallised several times from large volume of hexane, very fine white crystals (250 mg) were obtained. The compound showed one spot on t.l.c. plate (R_f 0.50) when developed in carbon tetrachloride-ethyl acetate (3:1) mixture. It melted at 70 - 71°C.

IR (nujol): ν_{max} 3400 - 2550 and 1700 cm^{-1}.

^{1}HNMR ($CDCl_3$): δ0.88 (b.t), 1.22 (sh.s), 2.32(t).

Esterification of the Compound

An ethanolic solution of the compound (15 mg) was treated with an excess of ethereal diazomethane. The ester obtained was crystallised from carbon tetrachloride. It melted at 37 - 39°C. It showed one spot on t.l.c. plate having R_f 0.75 in carbon tetrachloride-ethyl acetate (3:1) mixture.

IR (nujol): ν_{max} 1720, 1180, 1110, 715 cm^{-1}.

A mixed melting point of the compound with authentic sample of stearic acid (m.p. 71-72°C)[49] remained undepressed.

The i.r. spectrum of the compound was superimposable on that of an authentic sample of stearic acid. Its t.l.c. behaviour was also identical with that of stearic acid.

3.8 Examination of Aqueous Mother Liquor of Ethanol Extract

The brownish aqueous mother liquor of ethanol extract was extracted with diethyl ether and chloroform successively for three times. A brownish coloured diethyl ether extract (1.50 g) and a brown coloured chloroform extract (0.52 g) were obtained. After basifying with 4% sodium hydroxide solution, alkaline aqueous mother liquor was extracted successively with diethyl ether and chloroform and a negligible mass was obtained. The chloroform extract showed complicated t.l.c. picture. Therefore no further study was carried out.

3.9 Examination of Diethyl ether Extract of Aqueous Alcoholic Mother Liquor

A semi-solid brownish mass (1.50 g) was obtained from diethyl ether extract of aqueous alcoholic mother liquor. It showed two spots on t.l.c. plate (R_f 0.55, 0.32) along with an unresolved zone at the base line. The crude mass was chromatographed over a column of silica gel (150 g, BDH, 60 - 120 mesh) and eluted with carbon tetrachloride and mixtures of carbon tetra chloride and ethyl acetate in the ratio of 19:1 and 4:1 respectively. Fractions of about 10 ml volume were collected and every fifth fraction was examined on t.l.c. plates. The fractions showing similar t.l.c. behaviour were combined. The results are shown in Table IV.

Table IV. Column chromatographic separation of Diethyl ether Extract

Fraction No.	Eluant	Yield	Remarks
Fraction N (collection nos. 15-100)	Carbon tetrachloride	225 mg	Yellowish semi-solid one spot $R_f(CCl_4)$ 0.54
Fraction U (collection nos.110-220)	Carbon tetrachloride: ethyl acetate (19:1)	350 mg	Yellowish semi-solid m.p. 54-52°C. One distinct spot with long tail $R_f(CCl_4\text{-EtOAc},4:1)$ 0.44.
Fraction R (collection nos. 230-340)	Carbon tetrachloride: ethyl acetate (4:1)	425 mg	No distinct spot.

3.9a Characterisation of Fraction N

Fraction N yielded a yellowish low melting semi-solid mass (225 mg). The compound was separated by p.l.c. using carbon tetrachloride as solvent. A yellowish low melting mass (85 mg) was isolated. It showed one spot on t.l.c. plate (R_f 0.54 in carbon tetrachloride).

IR (Liquid film): ν_{max} 1733, 1180, 1110 cm^{-1}.

^{1}HNMR (CCl_4): δ 0.81(t), 1.23(br.s), 2.24(t), 4.06(q).

Mass spectrum: m/e 368, 353, 325, 312, 297, 295, 284, 269, 267, 241, 239, 227, 213, 211, 199, 197, 185, 183, 171, 169, 157, 155, 143, 141, 127, 127, 115, 113, 101, 99, 88.

3.9b Characterisation of Fraction O

Fraction O, eluted with carbon tetrachloride-ethyl acetate (19:1) gave a white waxy solid on removal of the solvent. It showed one distinct spot with a long tail when developed in carbon tetrachloride-ethyl acetate (4:1). The material was subjected to separation by p.l.c. using carbon tetrachloride-ethyl acetate (12:1). A white solid mass (50 mg) was isolated. It showed one spot on t.l.c. plate (R_f 0.44 in carbon tetrachloride-ethyl acetate, 4:1) and melted at 73-75°C.

IR ($CHCl_3$): ν_{max} 3450-2800, 1700 cm^{-1}

^{1}HNMR ($CDCl_3$): δ 0.86(t), 1.23(br.s), 2.31(t).

Mass spectrum: m/e 430, 415, 396, 381, 368, 354, 353, 340, 339, 325, 311, 284, 293, 269, 256, 255, 241, 231, 227, 199, 185, 171, 143, 137, 129, 115, 101, 87, 73, 59.

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