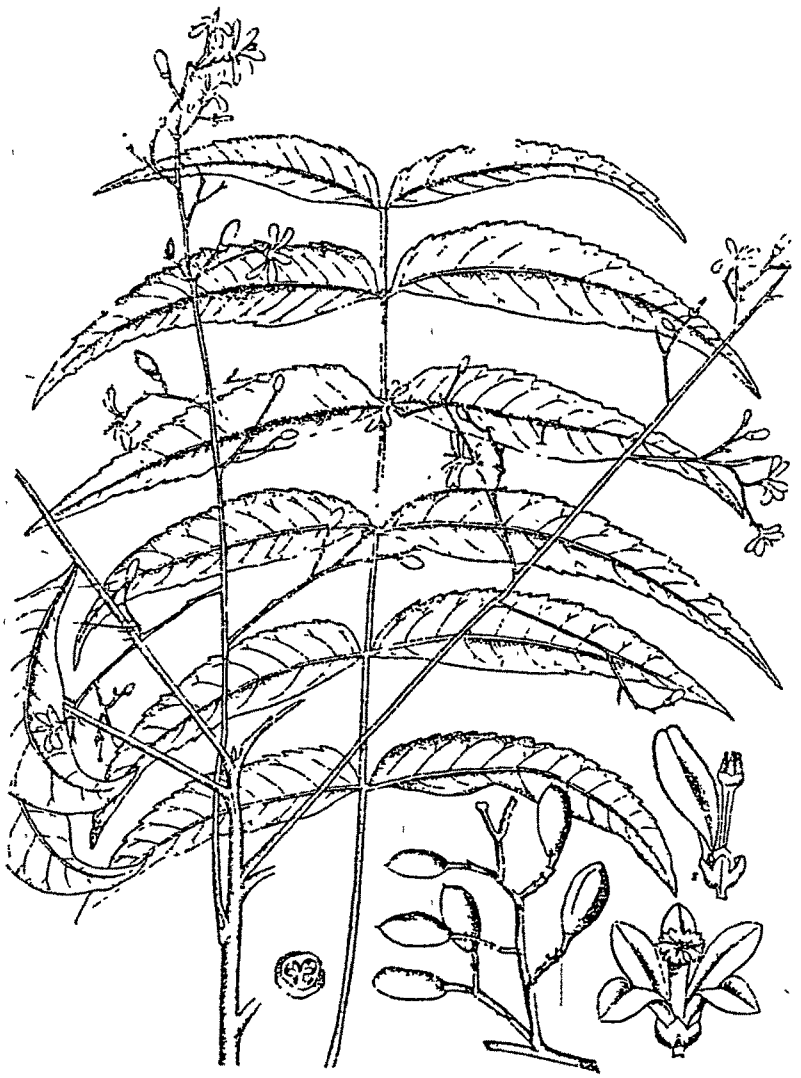


AZADIRACHTA INDICA

CHAPTER - 2

BIOLOGICALLY ACTIVE COMPOUNDS FROM AZADIRACHTA INDICA A. JUSS.

INTRODUCTION

Azadirachta indica A.Juss (Syn. Melia azadirachta Linn, Family Meliaceae), is commonly known as NEEM or MARGOSA tree. It is a large evergreen tree, a native of India and widely distributed in southeast Asia, tropical Australia and Africa[1,2]. Neem has a straight trunk and round-crown. It grows all over India, except at high altitudes and in thick forests. It is mostly cultivated as an avenue tree. It is a moderately salt resistant tree and, grows successfully in saline soil even in extreme draught. It can grow on alkaline 'usar' soil as well. The tree matures in 10 to 15 years.

Almost every part of the tree is bitter and has found applications in indigenous medicine[2]. Fifteen years old neem tree produces on an average about 400 kgs wood (per Cft=25 kgs). Leaves are about 22-32 cm long; leaflets 7-17 in number and about 6 to 7 cm long. A 7.5 to 8 metre tall neem tree on an average gives about 350 kgs leaves. Old leaves are shed in the month of February and March. Flowers appear in January to May along with new leaves. Fruits are smooth, ellipsoidal drups, 1.25 to 1.8 cm long and 1 cm wide, green when tender and greenish yellow when ripe; its pulp is bitter sweet and matures in May to August. The fruit contains one or two kernels enclosed in a tough shell. The kernel contains 45% oil on an average. Yield is about 50 kg per tree.

Medicinal properties and uses[3]

Neem is used in leprosy, intestinal worms, piles and urinary diseases. Root bark and young fruit are astringent, tonic and antiperiodic. Bark is bitter, tonic, astringent, antiperiodic and also vermifuge. Fruit is purgative

emollient and anthelmintic. Leaves are discutient, leaf juice is anthelmintic. Oil from nuts and leaves act as local stimulant, insecticide and antiseptic. Flowers are stimulant, tonic and stomachic. Gum from the bark is stimulant and demulcent tonic. The gum also possesses antispirochaetal and emmenagogue properties.

CHEMISTRY

Almost all parts of the tree have been investigated for the compounds having biological activity.

From seeds the compounds isolated are : azadirachtin[4], desacetylnimbin[5], nimbidic acid, nimbidinin[6], 17-hydroxyazadiradione[7], azadiradiones[8], 1- α -methoxy-1,2-dihydroepoxyazadiradione, 1β ,2 β -diepoxyazadiradione, 7-acetylneotrichilenone, 7-desacetyl-7-benzoylepoxyazadiradione, 7-desacetyl-7-benzoyl-gedunin,[9] 4-epinimbin[10], deacetylazadirachtinol[11].

Preliminary chemical examination of the oil from seeds indicated the presence of sulphur (0.42%)[12]. The principal constituents of the oil are : nimbin, nimbinin, nimbidin[13], vepinin[14], meldenin[15], azadirone, azadiradione, epoxyazadiradione, gedunin[16], (E)-2-methyl-2-butenoic acid (tiglic acid)[17], β -sitosterol, 24-methylenecycloartanol[18], nimbinene, 6-deacetylnimbinene, nimbandiol, 6-O-acetylnimbandiol[19], deacetylazadirachtinol[11], salannin, 3-desacetylsalannin[20], meliantriol[21], salannol, 1,3-diacetylvilasinin[22] and salannolide[23].

Azadirachtin[24,25], salanin[19,25], nimbochalcin, nimbocetin[26], cholesterol nimbocinol, 17-hydroxyazadiradione[27], nimolicinol[28], azadirachtol[29], nimocin, azadirone, epoxyazadiradione, gedunin, 7-deacetyl-7-benzoylazadiradione,

azadiradione[30] and few triterpenoids[31,32] are reported from fruit pulp of neem.

Compounds reported from wood oil are β-sitosterol, cycloeucalenol, 24-methylenecycloartanol[33].

Heartwood of Neem contains β-sitosterol, 24-methylenecycloartanol[34], nimatone[35] and triterpenes[36].

Important compounds isolated from trunk bark of the tree are : nimbin, nimbinin, nimbidin, nimbosterol[37], nimbiol, 7-oxoferruginol[38,39], tannin, a bitter principle margosine[37] and branch chain paraffin alcohols[40].

D-Glucosamine[40], aldobiouronic and aldotriouronic acids[41] are reported from gum-resin of Neem tree.

From green twigs of the Neem margosinolide and isomargosinolide[42] are reported.

Flavonoids such as kaempferol, quercetin, myricetin[43], quercetin-3-galactoside, kaempferol-3-glucoside[44] and other compounds like nonacosane and β-sitosterol[43] are reported from flowers of Neem.

Leaves mainly contain : nimbolide[45], β-sitosterol[46], nimbinen, 6-deacetylnimbinene, nimbandiol[47] chlorogenic acid[48], flavonoids such as quercetin[49], hyperoside, quercitrin, rutin[49], kaempferol[50], 4∝,6∝-dihydroxy-A-homoazadirone[51], nimocinol[52], nimbaflavone[53], azadirachtannin[54], 2',3'-dehydrosalannol[55], violaxanthin, butein, β-carotene, zeaxanthin, antheraxanthin, cryptoxanthin, neoxanthin[56], alkanes [$CH_3(CH_2)_nCH_3$, n=14,16,17,24,31,34][57,58] nimbocinone, stigmasterol[59], isonimbocinolide[60], nimocinolide, isonimocinolide[30], meldenindiol[61], isoazadirolide[62], n-hexacosanol, nonacosane,

xanthophyll[50], a viscous oil consisting of tri- and tetra cyclic sulfides of C_3, C_5, C_6, and C_9 units[63]. Leaves also contain glutamic acid, tyrosine, aspartic acid, alanine, proline, glutamine, cystine like amino acids.[64]. The average chemical composition (other than mentioned above), carbohydrates ($\sim$50%), crude proteins ($\sim$ 16%), fats ($\sim$2.3-7%), crude fibre (11.2-23.8%), calcium (0.8-2.4%) and phosphorus (0.13-0.24%).[100]

Neem as a source of pest control material

Use of neem as protection against damage by pests is known since antiquity. In India people often mix dried neem leaves with grain meant for storage and, with woollen clothes to protect them from ravages of insects. It has been reported to be (1) antifeedant (2) attractant (3) repellent (4) insecticide (5) nematicide (6) growth disruptor (7) antimicrobial (8) synergistic.

Antifeedant : The antifeedant activity of neem seems to be recorded by Mann & Burns (1927), who observed uniqueness of A. indica not being eaten by locusts (Schistocerca gregaria). Clear presence of deterrent in neem was first demonstrated[65] by Pradhan et al., (1962) to desert locust (S. gregaria) and migratory locust (Locusta migratoria), when they observed that as low as 0.1% neem seed powder (fresh) mixed in water was able to provide complete protection to the treated foliage. Since then, this has recommended by the Indian Agricultural Research Institute, New Delhi, for protection of agricultural crops from locust invasions. Satisfactory protection of stored wheat, maize, jowar, paddy and pulses over a period of one year has been achieved by mixing 1-2% neem kernel powder in grain from many species of stored grain insect.[66] Extracts of neem seed have also been reported to inhibit feeding in some field pests like P. brassicae and

C. trachypterous[67]. In laboratory and field trials at the International Rice Research Institute, Philippines, it has been demonstrated that 12% neem oil emulsion when sprayed on paddy suppresses feeding by brown plant hopper N. lugens Stal.[68]

It seems that more than one compounds are responsible for imparting antifeeding character. The most active compound has been identified as azadirachtin,[4,24,25,69,71] which is found to be 0.75 g/kg neem seed. The compound has been evaluated as a strong antifeedant to locust at 40 micrograms/litre concentration resulting in complete cessation of feeding.[69] The other antifeedant compounds identified are : meliantrol[21], a triterpenoid alcohol, salanin[72], 3-deacetylsalanin[22], salannol,[22] 1,3, diacetylvilasinin[22] and deacetylazadirachtinol[11].

At national chemical lab. of India, enriched neem extracts named Neemrich-I, II, and III were developed. Neemrich-I has high and persistent oviposition-deterring activity against adults and short-term but high antifeedant activity against 1st instar larvae of the potato tuber moth, P. operculella. Neemrich-II and III showed pronounced antifeedant and aphicidal activities respectively. Neemrich II at 5% concentration imparted 90% protection against S. litura feeding damage on caster, cabbage, cauliflower and tobacco leaves. A 1% emulsion of neemrich III gave a 100% kill of the aphids M. persicae and A. gossypii.[73]

Attractant : It is interesting that whereas neem acts as feeding deterrent to a variety of insect, it itself is attacked by atleast 42 insect species[2]. Still strange that neem leaves are strong food attractants to adults of white grub and have been very successfully utilized for control of this pest.[74]

Repellent : Neem contains several aromatic and odorescent principles which repel insect, white ants (Microtermes Sp.) and some species of ants do not like neem scent and move away from the treated vicinity. Neem cake has been practically used in clearing termite infested area in Madhya Pradesh. It has been reported that neem oil is a strong deterrent to egg laying by potato tuber moth[75].

Insecticide : Several neem products do have low to moderate toxicity particularly to softbodied insects. Toxicity of water suspension of neem seed kernel to A. gossypii, U. echinus and S. nigra was reported. Non-fatty alcohol extract of neem seed cake was found to possess appreciable aphicidal activity[75]. 'Neem oil extractive'-a resinous dark byproduct of neem oil refining has been found as an effective mosquito larvicide[76].

Nematicide : There has been a strong realization during the past 1-2 decades that nematodes constitute a limiting factor in the raising of agricultural crops in several pockets of the country. However, their control is extremely difficult due to the nature of the pest and prohibitive cost of nematicidal chemicals. Neem cake when applied 1800 kg/hectare resulted in significant reduction in the root galls of Okara (Hibiscus esculentus) and tomato caused by M. javonica[77]. Inhibitory effect on the larval emergence from the egg of M. incognita[78] and reduced egg-laying capacity of the females have been reported[79] as a result of neem cake application. It has been observed that application of N.P. and K alone as fertilizer to soil results in marked increase of plant parasitic nematodes. But if applied in combination with neem the population dwindles slowly and steadily[79]. Nimbidin and thionimone (the bitter principles) effectively killed the nematode and inhibited the larval growth.[80]

Growth disruptor : Certain compounds interfere in the normal hormonal balance of insects thereby causing disruption in normal growth. Such a chemical hold out promise in the pest control and have been emphatically advocated recently. That neem possesses growth disrupting activity, was first demonstrated by Gill[81], who reported that Pieris larvae feed for 48 hrs on foliage treated with neem products and subsequently reared on clean food, failed to develop to maturity and most of them died while moulting.

Mosquito larvae (C. fatigans) when reared on medium containing 0.005% neem oil extractive failed to emerge as adults.[76] Metamorphic disturbances due to azadirachtin were recorded when applied to larvae of diamond back moth (P. xylostella), cabbage caterpillar (P. brassicae) and army worm (H. virescens) at 0.025 mg per insect while at higher dosages 0.1 mg/larvae these died immediately[75].

Antimicrobial : Besides physico-chemical factors, pesticide degradation in soil is largely triggered by soil microorganisms. Inhibition of degradation of carbofuran, a popular soil insecticide particularly for paddy pests, by alcohol extract of fresh neem cake has been reported[82]. Carbofuran is known to convert most preferred form of soil and fertilizer nitrogen (ammoniacal) to least preferred form (nitrite and nitrate) with the result that even if carbofuran protected the crop for some period, the ultimate yield would still be poor due to non-availability of nitrogen. Neem cake is known to reverse this process[83] and is now an established nitrogen regulator. Therefore, while on one hand the neem cake prolongs the field life of carbofuran, on the other it increases the availability of nitrogen to the plant.

Growth regulating : Neem seed extract prolonged the development and caused loss in the body weight of Sitophilus oryzae[84]. It has been noted that neem seed suspension of various concentrations on a large number of insect and noted that it produced high mortality, significant reduction in weight, morphological deformation of pupae, inhibition of pupating, death of larvae and reluctance of feeding in larvae[75]. It is therefore, quite possible that leaf and kernel extracts of neem contain juvenile hormone (JH) like substances.

Synergistic : Neem extract had synergistic action in combination with custard apple against pulse beetle (C. chinensis), lesser grain borer (R. dominica L.) and housefly (M. domestica). Custard apple seed extract in combination with neem seed extract was half toxic against lesser grain borer and equitoxic as DDT to the housefly[85].

Medicinal evaluation of Neem products

Following activities have been reported from neem.

1. Anti-tubercular activity : Neem oil inhibited the growth of all the three strains[86] of Mycobacterium at a concentration of 12.5 mg/ml. Neem oil and nimbidiol in higher concentration exhibited partial inhibitory influence whereas nimbidin prolonged the survival period of mice affected with tuberculosis. Neem oil also depressed the hypersensitivity to tuberculinin sensitized guineapigs[87].

2. Anti-fungal activity : Nimbidin, nimbin, nimbidol and neem oil are very effective against various fungi at comparatively very low concentrations.[87-89]

3. Anti-protozoal : Nimbidin and sodium nimbidinate killed Paramaecium candatum in 1/500 dilution in 1 minute[90]. Nimbidol, however proved more

potent than nimbidin at a dose of 25 mg/100 g of body weight given orally in suppressing the infection by Plasmodium gellinaceum in chicks.[91]

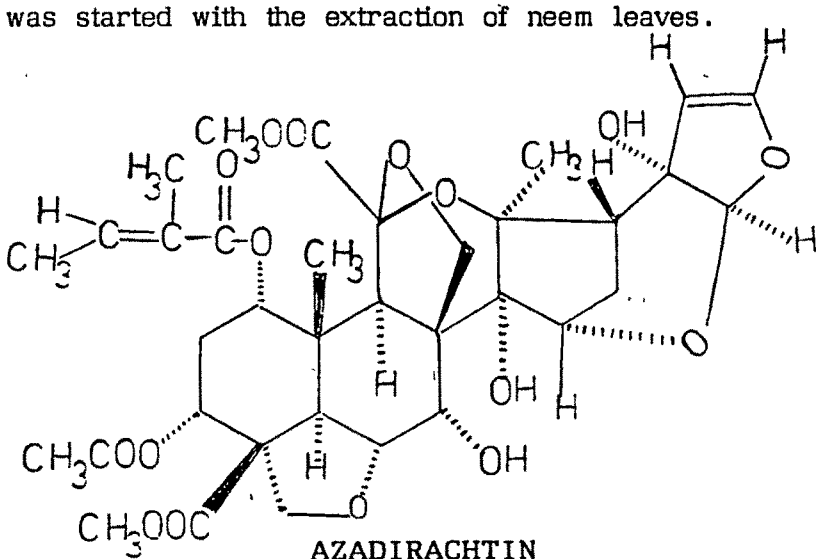
4. Dermatological diseases : Nimbidin is also effective against skin diseases such as eczema, scabies, arsenical dermitis, furunculosis and seborrhoeic dermatitis.[92]. The dried leaves are extracted with 70% ethanol, evaporated, the residue dissolved in propylene glycol in a ratio of 4:6 and the solution applied locally.[92]

5. Dental diseases : Nimbidin gargle and dentifrices have been found to be effective in bleeding gums and pyorrhoea. 'Silvose T' and 'Silvo TRS' which are patented extracts of the bark of Neem are intended as an active ingredient in toothpastes and other hygiene preparations. Neem extract when added to a toothpaste produced remarkable result, in preventing and healing the inflammation of the gum and paradontosisa.[75]

6. Other minor activities : Various neem products either alone or in combination with each other have shown some interesting activities like anti-bacterial[86], anti-viral[93], supermicidal[94], anti-ulcer[95], anti-inflammatory,[96] anti-pyretic[97], diuretic[98], cardiovascular[99] and anti-allergic[95].

RESULTS AND DISCUSSION

Azadirachtin a naturally occuring insect antifeedant[70] has become important for commercial usage in agriculture. Crude preparation of this compound have been commercially used in some countries, such as U.S.A. and China. This material has been isolated from neem seeds. The present work was undertaken with a view to isolate similar effective material from leaves. If successful, such a process would have advantage over the neem seeds process, because in contrast to seeds, which are available once in a year, neem leaves are available throughout the year. Our second objective was to isolate and identify maximum possible compounds from leaves and estimate their percentage composition, so that important compounds can be used commercially and also to develop or simplify procedure for extraction of commercially important compounds from leaves. With above objects the work was started with the extraction of neem leaves.



AZADIRACHTIN

Fresh leaves of Azadirachta indica (Neem) were collected from our campus in the month of April. Leaves free from twigs were dried under shade for 15 days. The dried leaves were powdered and repeatedly extracted (percolated) with 70% ethanol (aq.) at room temperature. Results are reported in Table-1.

TABLE-1 Extraction of A. indica leaves with 70% ethanol.

Expt. No.	Wt. of the fresh leaves* (dried leaves) (Kg)	Extraction	Time	Wt. of the extract (g) (%)+		Total extract (%)+ (g)
1	2.672 (0.888)	1	72 hr x 1 36 hr x 2	155.0	(17.45)	
		2	36 hr x 2	82.18	(9.25)	257.58
		3	24 hr x 2	16.40	(1.846)	(29)
		4	12 hr x 1	4.00	(0.45)	
2	4.5 (13.29)	1	72 hr x 1 36 hr x 4 24 hr x 2	1045.00	(23.22)	1066.00 (23.68)
		2	12 hr x 1	21.00	(0.466)	

* Free from twigs. + % on dry wt. basis of the leaves.

TABLE-2 Partitioning of 70% ethanolic extract with different solvents.

Expt. No.	Wt. of the extract (g) (10% ethanol used) ml	Solvent	Wt. of the fraction (g) (%)	
1	240 (1100)	pet.ether	18.50	(7.7)
		DCE	26.00	(10.23)
		ethylacetate	9.00	(3.75)
		n-butanol	22.68	(9.45)
2	1023 (4000)	pet.ether	68.00	(6.64)
		DCE	110.00	(10.75)
		ethylacetate	48.00	(4.69)
		n-butanol	96.00	(9.38)

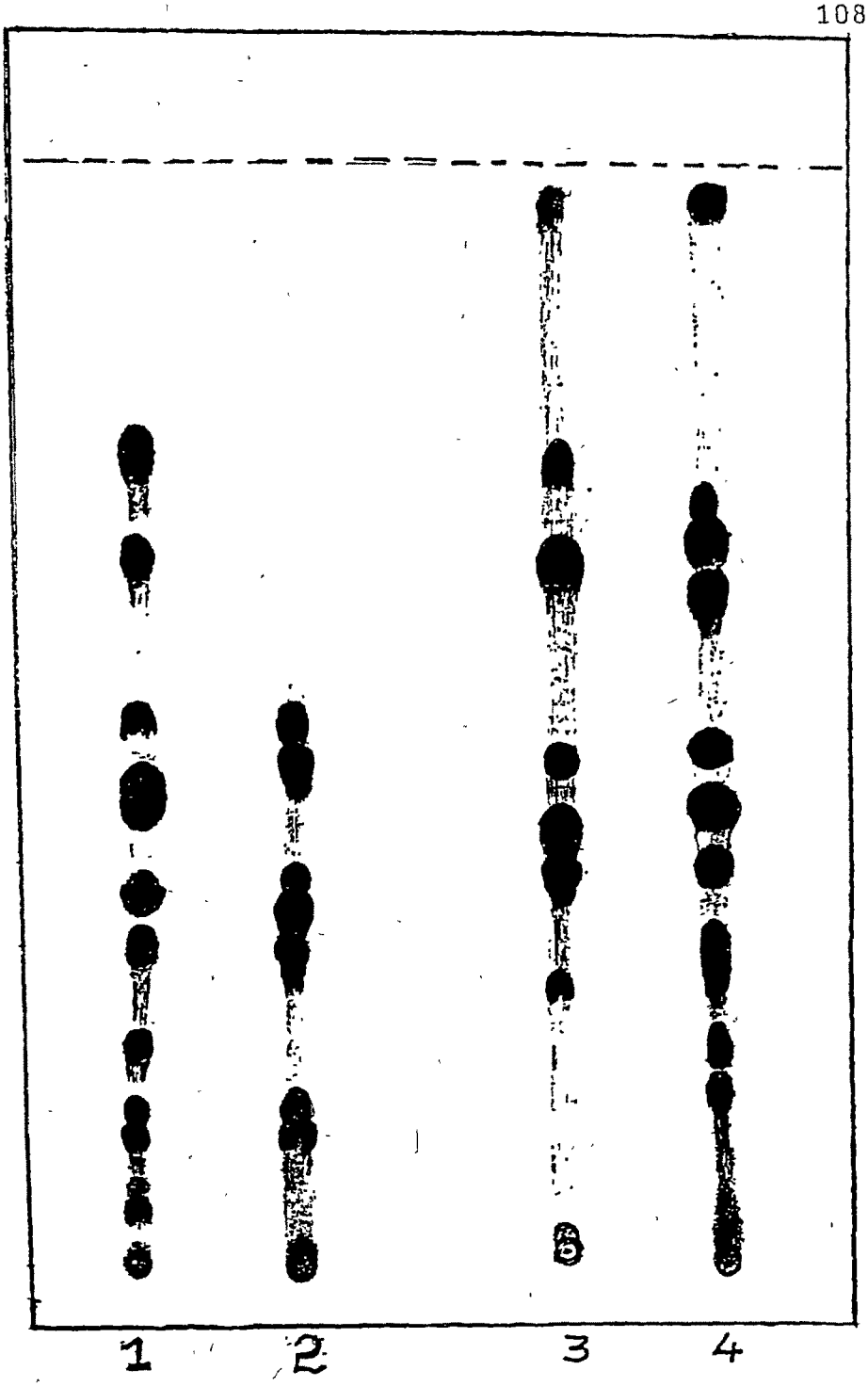
The alcohol extract of leaves after removal of solvent was dissolved in 10% ethanol (aq.) and partitioned with different solvents of increasing polarity. Solvent used were pet.ether (60-80°) dichloroethane (DCE), ethyl acetate and n-butanol (saturated with water). Results are reported in Table-2.

The extracts thus obtained were studied for antifeedant activity and for isolation of compounds. Fig.1 shows the TLC of various extracts of neem leaves.

Pet.ether extract (PE-1)

Pet.ether (PE-1) extract was a complex mixture. To reduce the complexity, it was separated into polar and comparatively non-polar compounds by counter current method.[102] The extract was dissolved in 90% aq. methanol and repeatedly extracted with pet.ether. Each of the pet.ether layer was separately extracted with 90% methanol. Solvent was removed from the combined pet.ether extract to afford the residue (PE-2) (44%). Similarly removal of solvent from combined MeOH layer gave methanol soluble fraction (56%) (Table-3), was further partitioned between benzene and 50% aq. MeOH as usual. The extract was dissolved in 50% aq. MeOH, which was repeatedly extracted with benzene. The benzene layers were separately extracted with 50% aq. MeOH. Usual work up yielded 90.41% benzene soluble portion and 9.69% MeOH soluble portion (Table-4).

Pet.ether extract (PE-2), benzene extract and 50% methanol extract (TLC, Fig. 2), thus obtained were submitted for the antifeedant activity testing and chromatographic separation of compounds.



Silica gel G plate.

Solvent system : (1) 10% EtOAc in benzene (for 1 & 2) (2) EtOAc:Butanone:H_2O:HCOOH (5:3:1:1)[101] (for 3 & 4).

Spray reagent:(1) 10% HNO_3 in H_2SO_4 (for 3 & 4).

(2) 1% vanillin in 50% H_3PO_4 (for 1 & 2).

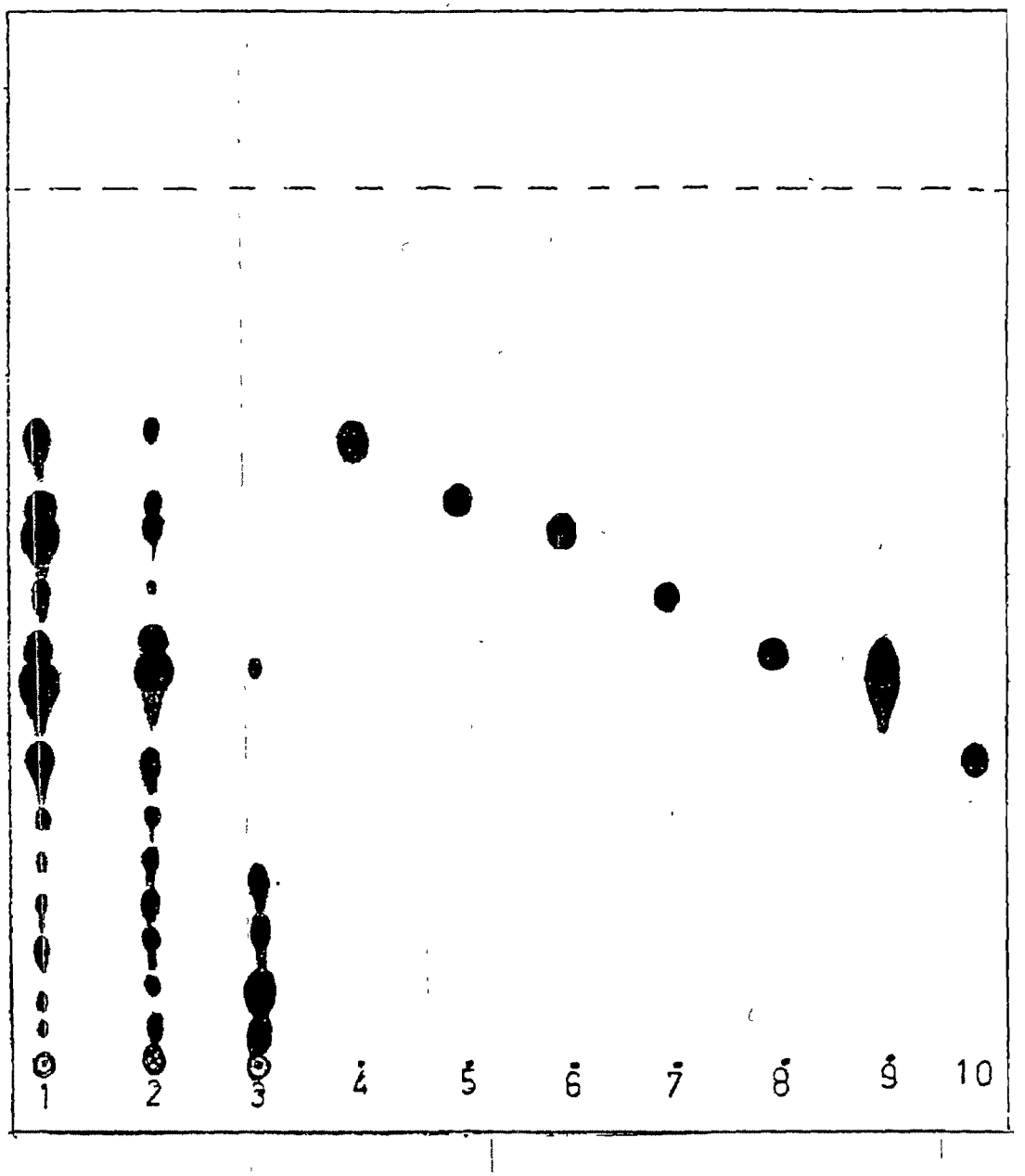
Fig. -1:-TLC of (1) pet.ether (2) dichloroethane (3) ethyl acetate and (4) n-butanol extract of Azadirachta indica leaves.

TABLE-3 Counter current distribution of pet.ether soluble portion between pet.ether and 90% methanol (aq.)

Expt. No.	Wt. of the pet.ether extract (g)(in 90% methanol, ml)	Pet.ether used for the extraction (ml)	90% methanol used for the extraction (ml)	Wt. of the pet.ether soluble portion (g) (%)	Wt. of the 90% methanol soluble portion(g) (%)
1	3.7576 (50)	50mlX5	50mlX4	1.6544 (44.02)	2.1032 (55.94)
2	28.5 (300)	300mlX5	300mlX4	13.4 (47.01)	14.8 (51.92)

TABLE-4 Counter current distribution of 90% methanolic extract between benzene and 50% methanol (aq.)

Expt. No.	Wt. of the 90% methanolic extract (g) (in 50% methanol, ml)	Benzene used for the extraction (ml)	50% methanol (aq.) used for the extraction (ml)	Wt. of the benzene soluble portion (g) (%)	Wt. of the 50% methanol soluble portion (g)(%)
1	2.1 (25)	50mlX5	25mlX4	1.8988 (90.41)	0.2035 (9.69)
2	14.8 (175)	350mlX5	175mlX4	13.9 (93.91)	0.793 (5.31)



(1) Pet.ether extract (2) Benzene extract (3) 50% Methanol (aq.) extract (4) Alkanes (5) Saturated esters (6) Unsaturated esters (7) α- Tocopherol (8) Phytol (9) Fatty acids (10) β-sitosterol plus stigmasterol.

Silica gel - G plate; Solvent system : 10% EtOAc in benzene.

Spray reagent : 1% vanillin in H_3PO_4.

Fig. 2 : TLC of compounds and extracts obtained from the pet.ether extract of A. indica leaves.

Pet.ether extract (PE-2)

The extract was subjected to broad cut column chromatography on SiO_2 gel/II-b and monitored by TLC. Elution with pet.ether followed by benzene in pet.ether afforded 17 fractions.

Fraction-1 Which was eluted with pet.ether was a brown viscous oil having a sulfur like odour. The viscous oil was distilled at 130-140°/3 mm The distillate exhibited two spots on TLC plate (solvent system : pet.ether, Rf 0.65 and 0.57). However, in GC (OV-1, 70-220°C) number of peaks were observed (Fig.3). Rechromatography of distillate failed to give any compound in pure form. IR and ^{1}H-NMR of the distillate showed absence of any functional groups. From its NMR spectrum, the fraction appeared to be consisting of hydrocarbons only, mainly alkanes. In GC-MS analysis (Fig. 3), five major peaks in the GC were scanned separately (Table-5). Peak-1 (5.82%) gave M^+ at m/e 156 corresponds to the hydrocarbon $C_{11}H_{24}$, peak-3 (8.12%) M^+ at m/e 170 corresponding to $C_{12}H_{26}$, peak-4 (5.5%) M^+ at m/e 184 corresponding to $C_{13}H_{28}$ and peak-5 (37.9%) M^+ at 212 corresponding to $C_{15}H_{32}$.

From the above data it was concluded that the fraction mainly consisted of saturated hydrocarbons.

Fraction-3 Which was also eluted with pet.ether was distilled at 240-60°/0.5 mm. The distillate (40%) was a light yellow oil. The residue as well as the distillate exhibited two spots on TLC (pet.ether, Rf 0.48 and 0.5). From the IR and NMR spectrum it was concluded that the fraction contained compounds without any functional groups, may be long chained hydrocarbon. When subjected to GC-MS (Fig. 4), there were atleast 10 distinct peaks

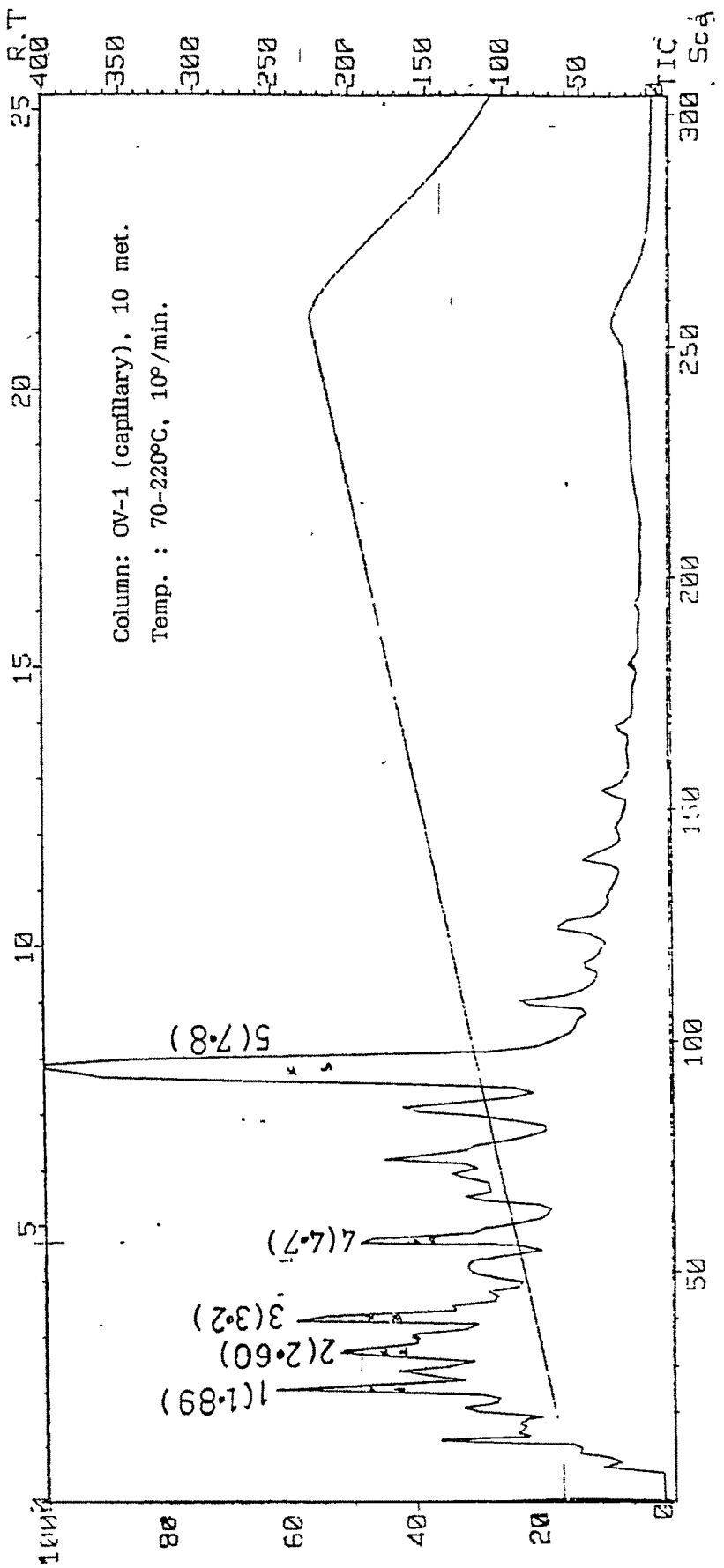


Fig.3 Gas chromatography of fraction-1 of the pet.ether extract.

TABLE-5 GC-MS data of the fraction-1 of pet.ether extract.

Peak (% in total mixture)	M^+ (Retention time, mins)	m/e (% intensity)	Molecular formula
1 (5.82)	156 (1.89)	85(100), 71(99.9), 41(95), 43(66), 56(55), 57(52), 70(42), 42(35), 55(30), 84(29).	$C_{11}H_{24}$
2 (7.265)	162 (2.6)	91(100), 119(58), 141(18.5), 162(18), 57(16.5), 128(16), 120(11), 79(8), 92(8), 98(6).	–
3 (8.125)	170 (3.2)	85(100), 71(76), 41(40), 170(36), 56(30), 70(25), 99(23), 98(28.5), 113(21), 84(21).	$C_{12}H_{26}$
4 (5.526)	184 (4.7)	71(100), 43(85), 57(71), 85(57), 41(42), 56(22), 184(19), 70(18), 55(17), 99(12).	$C_{13}H_{28}$
5 (37.9)	212 (7.8)	57(100), 71(62), 43(61), 85(43), 41(21), 133(120), 134(19), 91(13), 99(11.5), 56(11).	$C_{15}H_{32}$

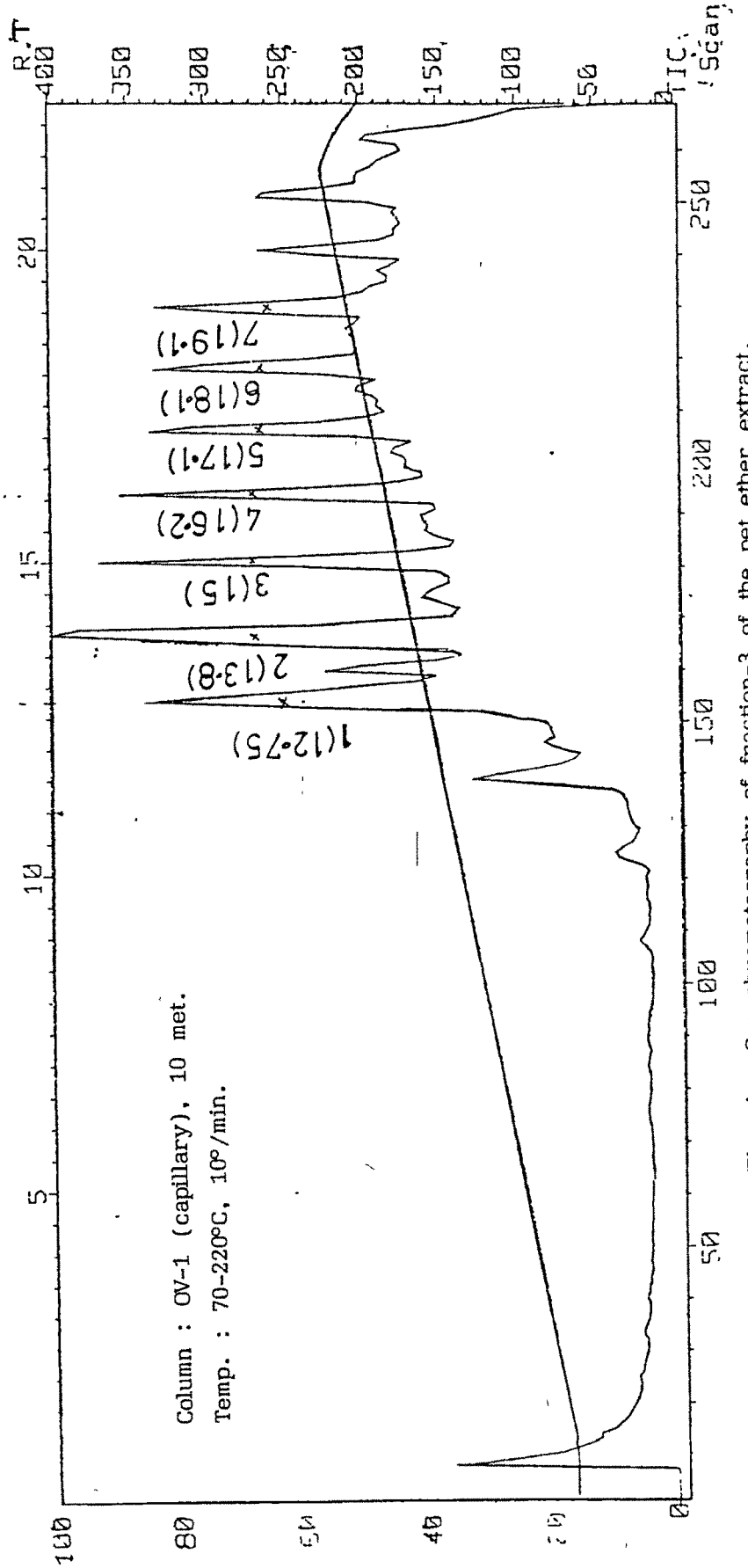


Fig. 4 : Gas chromatography of fraction-3 of the pet.ether extract.

in GC observed. In the mass spectrum, peak 1-7 with the molecular ion peaks at m/e 268, 282, 296, 310, 324, 338 and 352 respectively were assigned for hydrocarbons with formula C_nH_{2n+2}, where n=19,20,21,22,23,24, and 25 respectively (Table-6). The assignment was by comparision of the specfral data in the literature.[103,104]

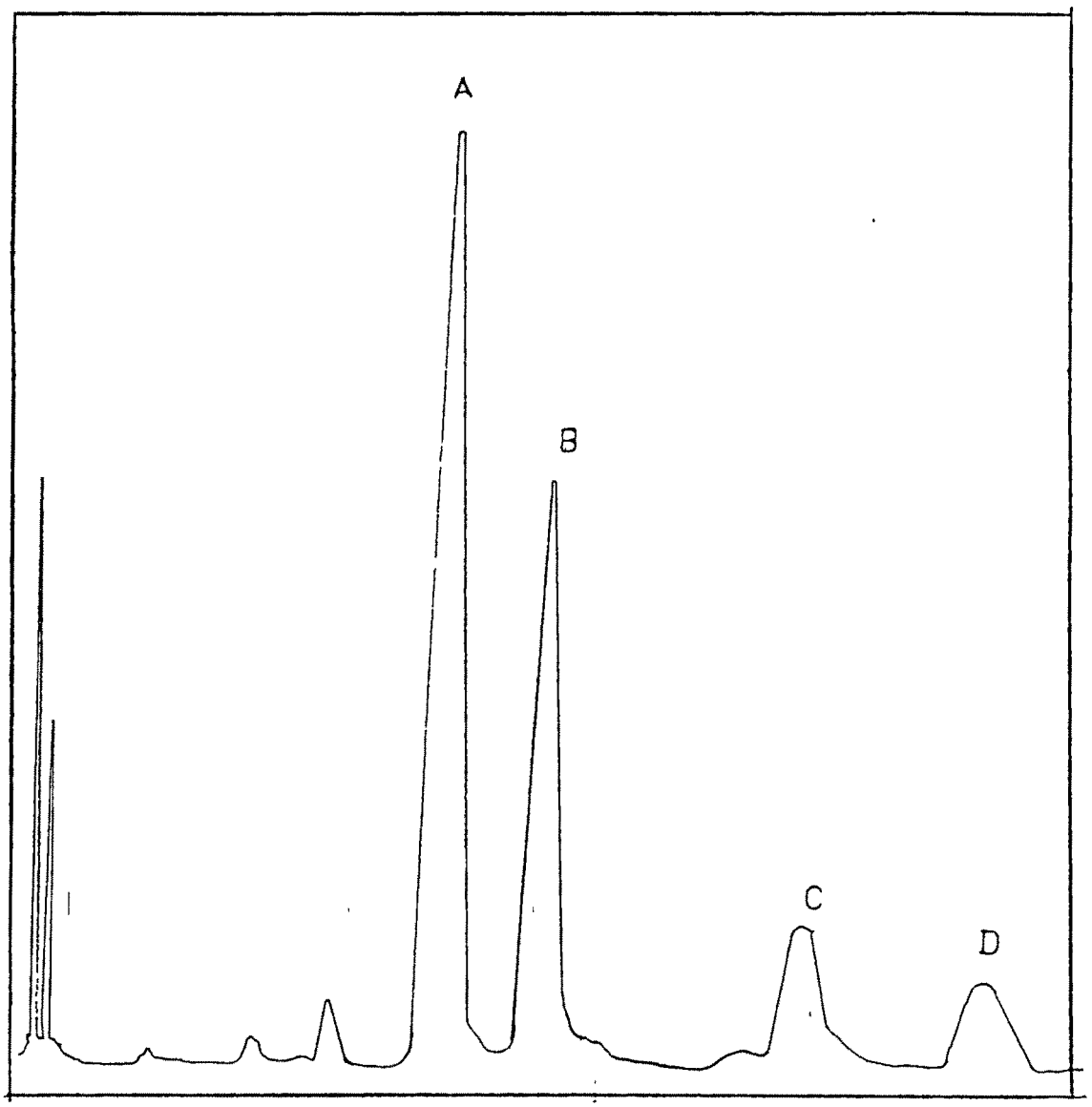
Fraction-4 eluted with 10% benzene in pet.ether exhibited two spots on TLC (benzene, Rf 0.55 and 0.48), which were separated by chromatography on silica gel. Fraction-4A (Rf 0.55) in GC (Fig. 5) showed four major peaks. From IR and ^{1}H-NMR the fraction was found to be a mixture of methyl and ethyl ester of long chain fatty acids. Methyl palmitate (1), ethyl palmitate (2), methyl stearate (3) and ethyl stearate (4) were identified by co-injection with the authentic samples in GC. Fraction-4B (Rf 0.48) consisting of two major components (GC) was found to be a mixture of methyl oleate (5) and ethyl oleate (6) by comparison (co-injection in GC, IR and NMR) with the authentic samples. In GC-MS of fraction-4, four compounds (Fig. 6) with molecular ion peaks at m/e 270, 284, 296 and 310 were identified as methyl palmitate, ethyl palmitate, methyl oleate and ethyl oleate respectively (Table-7). The identification was by comparison with the spectral data reported in literature.[103,104]

Fraction-6 eluted with 20% benzene in pet.ether was light brown oil. Distillation of oil at 155°(bath temp.) at 0.3 mm yielded TLC pure liquid (5% EtOAc in benzene, Rf 0.34). It was identified as ∝-tocopherol (7) by direct comparison with the authentic compound (NMR, IR).

Fraction-8 eluted with 30% ethyl acetate in pet.ether, was light brown

TABLE-6 GC-MS data of the fraction-3 of pet.ether extract.

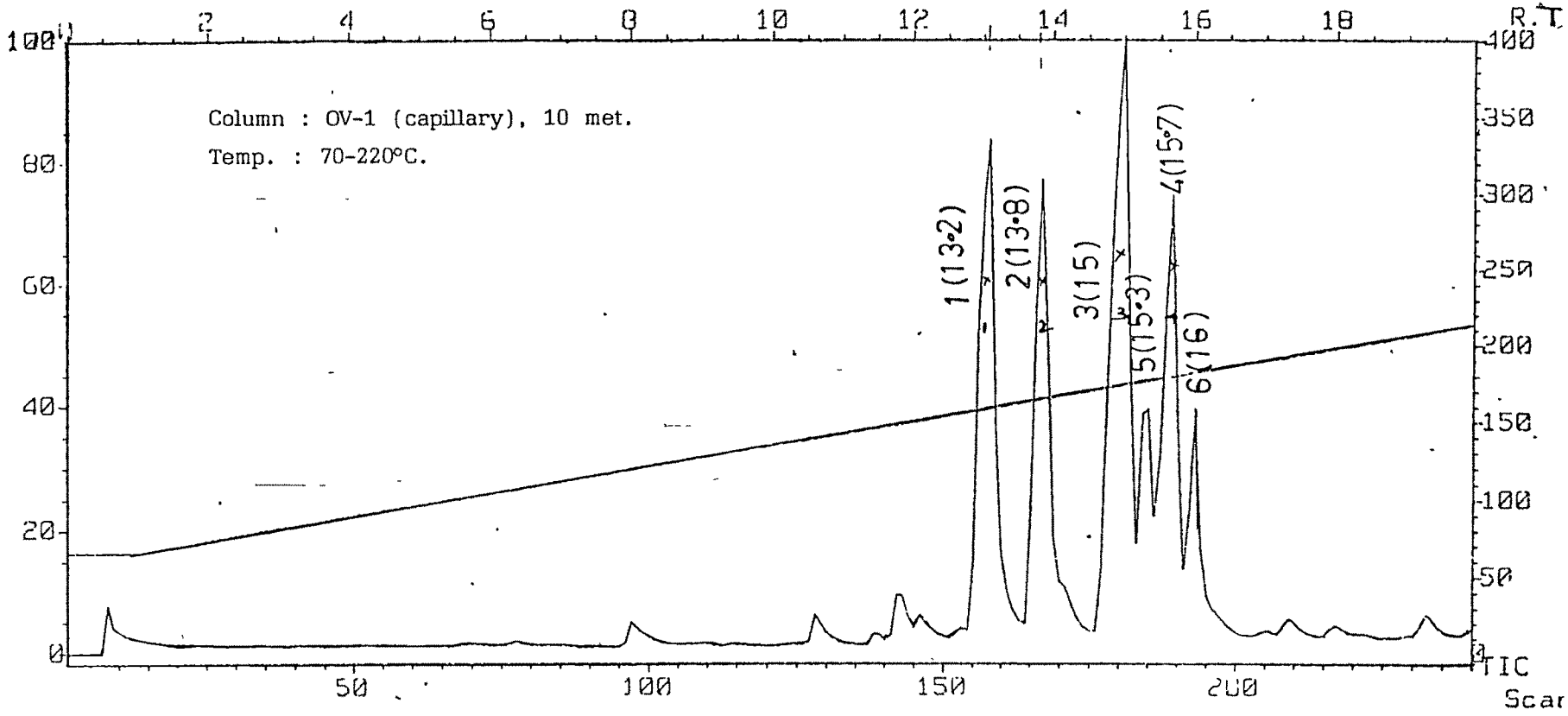
Peak (% in total mixture)	M^+ (Retention time, mins)	m/e (% intensity)	Molecular formula
1 (7.49)	268 (12.75)	57(100), 71(64), 43(64), 91(42), 85(40), 41(18), 175(14), 55(13.5), 99(12.5), 189(12).	$C_{19}H_{40}$
2 (8.38)	282 (13.8)	57(100), 71(70), 43(55), 85(44), 41(18), 55(16), 99(15.8), 113(11), 69(11), 127(9).	$C_{20}H_{42}$
3 (6.08)	296 (15)	57(100), 71(70), 43(62), 85(43), 41(17.5), 55(17), 99(16), 113(11), 56(11), 69(10).	$C_{21}H_{44}$
4 (6.33)	310 (16.2)	57(100), 71(68), 43(64), 85(43), 41(18), 55(18), 99(14.3), 56 (10.1), 113(10). 69(9).	$C_{22}H_{46}$
5 (7.83)	324 (17.1)	57(100), 71(71.5), 43(58), 85(48.5), 99(16.3), 41(16), 55(4), 113(11.4), 56(10), 69(9).	$C_{23}H_{48}$
6 (10.98)	338 (18.1)	57(100), 71(73), 43(58), 85(45), 55(16.5), 41(16.2), 99(16), 56(13), 113(11.5), 69(10.5).	$C_{24}H_{50}$
7 (10.84)	352 (19.1)	57(100), 7(69), 43(62), 85(41), 149(26), 55(20), 41(19), 113(14), 69(14), 99(12).	$C_{25}H_{52}$



Column : 10% SE-30, 6 feet, temp. 220°C.

A-Methyl palmitate, B-Ethyl palmitate, C-Methyl stearate, D-Ethyl stearate.

Fig. 5 : Gas chromatographic analysis of fraction 4A of the pet.ether extract.



(1) Methyl palmitate (2) Ethyl palmitate (3) Methyl oleate (4) Ethyl oleate (5) Methyl stearate (6) Ethyl stearate.

Fig. 6 : Gas chromatography of the fraction-4 of pet.ether extract.

TABLE-7 GC-MS data of the fraction-4 of pet.ether extract.

Peak (% in total mixture)	M^+ (Retention time, mins)	m/e (% intensity)	Molecular formula
1 (21.2)	270 (13.2)	43(100), 75(94), 270(84), 41(77), 55(73), 143(63), 57(58), 69(44), 227(32), 87(29).	$CH_3(CH_2)_{14}COOCH_3$
2 (15.4)	284 (13.8)	43(100), 284(88), 89(74), 57(65), 41(64.5), 55(62), 157(54), 101(53), 73(51.5), 61(44), 239(38).	$CH_3(CH_2)_{14}COOC_2H_5$
3 (30.02)	296 (15)	55(100), 41(76), 69(72), 74(68), 264(52), 83(50), 67(50), 41(48), 87(48), 88(44), 81(43), 296(18).	$CH_3-(CH_2)_7-CH=CH-(CH_2)_7COOCH_3$
4 (15.33)	310 (15.7)	55(100), 41(75), 69(75), 88(75), 264(53), 101(57), 83(54.4), 67(54.7), 81(50), 84(47), 43(46), 310(19).	$CH_3(CH_2)_7CH=CH-(CH_2)_7COOC_2H_5$

oil. Distillation of oil at 155° (bath temp.) at 0.3 mm yielded TLC pure (3% EtOAc in benzene, Rf 0.42) colourless liquid. GC showed 98% purity. It was identified as Phytol (8) by comparison with the authentic compound (mixed TLC and GC, NMR and IR).

Fraction-11 eluted with 40% benzene in pet.ether, was a gummy solid. Repeated crystallisation from pet.ether yielded white crystalline solid (m.p. 126-30°C). It showed a single spot on TLC but exhibited two peaks in GC (1% OV-17,260°). The two compounds were identified as β-Sitosterol (9) (63%) and stigmasterol (10)(37%) by direct comparison with the authentic compounds (mixed GC, NMR, IR).

Fraction-12 eluted with 50% benzene in pet.ether was a gummy solid. It was essentially a mixture of fatty acids (NMR, IR). It was converted to corresponding methyl esters with diazomethane.[105] Crude methyl esters were purified on chromatotron by eluting with 4% EtOAc in pet.ether and then distilled at 160-180°(bath temp.) at 3 mm. GC analysis of methyl esters on 10% OV-4 column (1.2 met) at 200° showed it to be a mixture of two compounds (Rt 2 and 4 mins). Both the compounds were separated by prep. GC on 20% SE-30 column (12 feet, H_2 flow 100 ml/min, temp. 230°). Methyl palmitate [m.p. 33-34°, b.p. 77-80°(bath temp.) at 1 mm] and Methyl oleate [b.p. 104-108°(bath temp.) at 0.44 mm] were identified by comparison with the authentic compounds (Mass, NMR, IR and mixed GC). Both the compounds were further confirmed by ^{13}C-NMR.

Methyl palmitate (Fig. 7)

^{13}C-NMR ($CDCl_3$) δ_c(ppm) : 29.12, 29.24, 29.31, 29.64, 29.88 (11x$\underline{C}H_2$), 24.88 ($\underline{C}H_2$-CH_2COOCH_3), 22.59 ($\underline{C}H_2$-CH_3), 13.94 ($\underline{C}H_3$), 33.94 ($\underline{C}H_2$-$COOCH_3$),

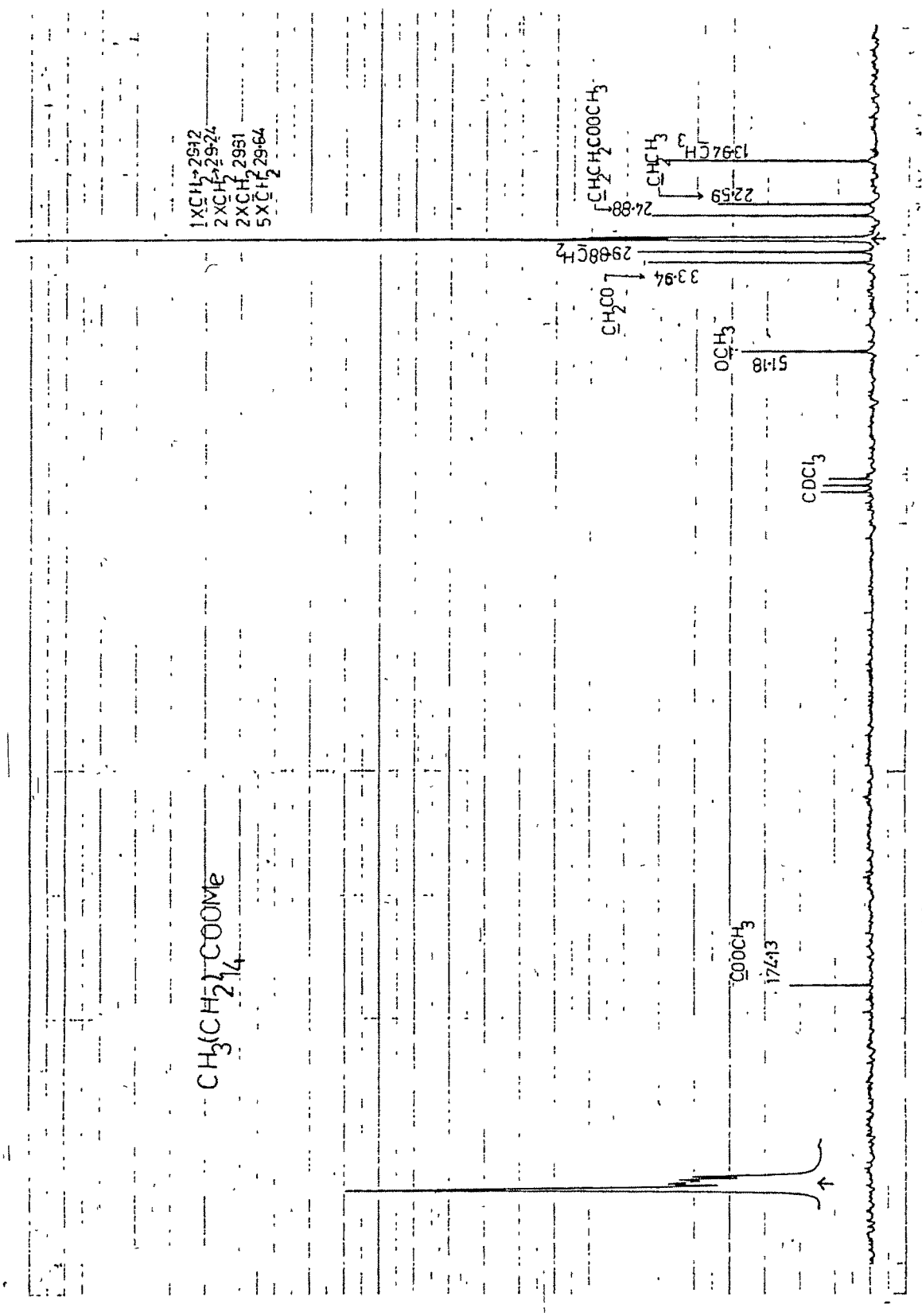


FIG. 7 : ^{13}C NMR SPECTRUM OF METHYL PALMITATE

51.18($COOC\underline{H}_3$), 174.13($\underline{C}OOCH_3$).

Methyl oleate (Fig. 8)

^{13}C-NMR ($CDCl_3$) δ_c(ppm): 29.64, 29.41, 29.23, 29.11 (8xCH_2), 27.06($\underline{C}H_2$-CH=CH-$\underline{C}H_2$), 24.88($\underline{C}H_2CH_2COOCH_3$), 22.53($\underline{C}H_2$-$CH_3$), 13.18($\underline{C}H_3$), 31.88($\underline{C}H_2CH_2CH_3$), 33.94($\underline{C}H_2COOCH_3$), 51.18($COO\underline{C}H_3$), 129.71, 129.94($CH_2$-$\underline{C}H$=$\underline{C}H$-$CH_2$)174.13($\underline{C}OOCH_3$).

Thus fatty acids present in leaves are palmitic acid and oleic acid.

Fraction-14 eluted with benzene was a gummy solid. Its IR spectrum showed presence of OH [3400cm^{-1}(w)]>C=O [1720 cm^{-1}(s)] and COOH[2500-3000 cm^{-1} (w)] in the compound. Fraction was esterified with diazomethane and the esterified product was purified on chromatotron and eluted with 7% ethyl-acetate in pet.ether. Only 10% compound was eluted with this solvent system and was found to be methyl ester of palmitic and oleic acid. Rest of the compounds were polar and eluted with ethylacetate and methanol. Polar compounds have not yet been identified.

Benzene extract

Benzene soluble portion of the pet.ether extract was subjected to column chromatography on SiO_2 gel/II - b and eluted with pet.ether-benzene with increasing polarity. Fifteen fractions were collected. Earlier fractions contained the compounds already isolated from pet.ether extract (TLC, NMR). However, when eluent was changed to benzene-ethyl acetate after elution of fatty acids, different types of compounds were eluted. All fractions in their IR showed presence of hydroxy [3400 cm^{-1}(s)] as well as carbonyl [1720 cm^{-1}(s)] groups. All the fractions exhibited atleast 3-4 spots on TLC plate. Fraction-11 in its IR spectrum showed peaks due to OH [3400 cm^{-1} (s)]

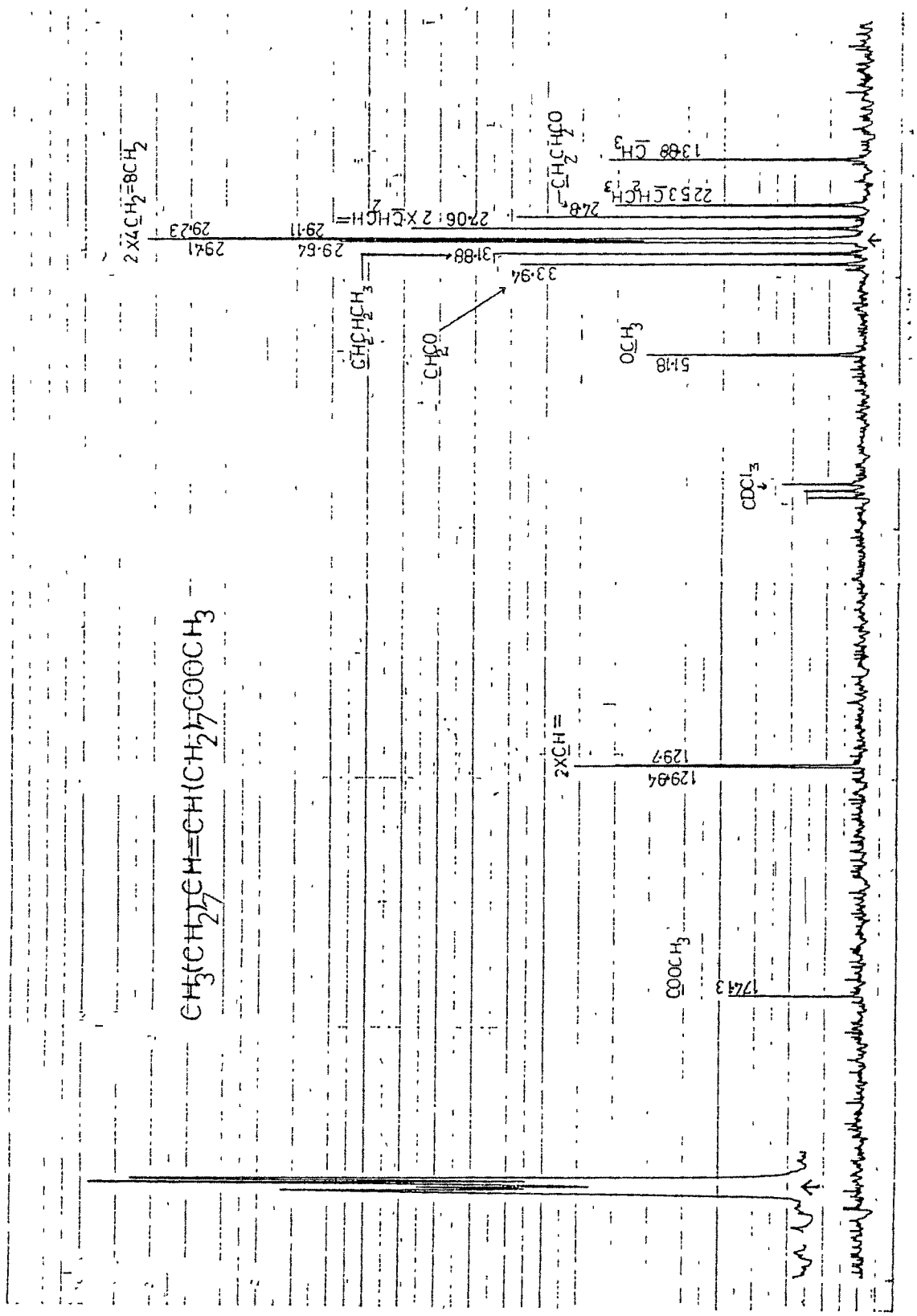


FIG. 8 ^{13}C NMR SPECTRUM OF METHYL OLEATE.

and >C=O [1720 cm^{-1}(s)]. It was assumed that the fraction contained some hydroxy ester. When NMR was recorded, it was found to be resembling the tetrol ester isolated from Commiphora mukul (Chapter-I). However, the said fraction was not identical, it was decided to do some more investigation on that fraction. The fraction was rechromatographed on chromatotron to afford a TLC pure (85-90%) compound in the form of a gummy material. IR spectrum showed both the hydroxyl and carbonyl functional groups and NMR indicated unsaturated long chain compound. The spectra resembled the spectra of the unsaturated tetrol esters isolated from Commiphora mukul.

It was then decided to saponify the compound into neutral and acid portion. As the quantity of pure compound was too less to carry out the experiment, the whole benzene extract was used for hydrolysis. Benzene extract was refluxed with 10% ethanolic KOH and worked up as usual. The acid portion (42%) and the neutral portion (18.5%) were collected. Acid fraction was esterified by diazomethane and the crude ester after purification on chromatotron was submitted for GC. Two peaks were obtained which were identified as methyl palmitate and methyl oleate (mixed GC with authentic samples).

The neutral portion consisting of a mixture of compounds (TLC) was subjected to chromatography on SiO_2 gel/II-b. Elution with EtOAc-benzene (1:1) afforded two pure compounds (12 and 10 mg). On TLC Rf values (60% EtOAc in pet. ether, 0.36 and 0.33) of both the compounds were close to the tetrol (Rf 0.28) but NMR of none of the two compounds matched with that of tetrol. Efforts to get more compounds in pure form, were abandonmented due to the complexity of the mixture.

Since the recovery of the neutral plus acids was only 60.5%, attempt was made to improve the recovery. For that the mixture obtained after hydrolysis was adsorbed on a mixture of Celite and Na_2SO_4 and continuously extracted with hot dichloroethane (DCE) in the Soxhlet apparatus for 16 hrs. DCE extract after usual work-up afforded dark brown viscous oil (65% yeild). TLC of the extract again showed absence of tetrol type compounds.

Further work on isolation of compounds from this extract was stopped as conclusive results were not forthcoming.

Estimation of compounds present in pet.ether extract

The compounds isolated from pet.ether extract are reported for the first time (except few alkanes and sterols). They are of commercial importance. Alkanes isolated from the leaves are known to possess mosquito larvicidal and insecticidal activity[106,107] and are used in making candles and waxed papers and boards.[109]. Methyl and ethyl esters of palmitic and oleic acids are widely used in cosmetics,[108] whereas palmitic and oleic acid find their application in the manufacture of surface active agents, detergents and soaps, also used in paints, lubricants, adhesives and rubber and textile industries.[108,110] α-Tocopherol is used as a constituent in multivitamin, single dose nutrient capsules and other liquid dietary supplements. It is also used as antioxidants to some extent by food technologists and pharmaceutical formulators.[111] Phytol is used in the synthesis of vitamin-E and vitamin-K.

Because of the commercial importance of the above mentioned compounds, it was decided to develop a method of estimation of the compounds in the total pet.ether estract of A. indica leaves. Since different solvents

are needed for the separation of the different types of compounds by partition, an alternative method was sought for the separation consisting of separation into neutrals and acids. The total pet.ether extract was dissolved in pet.ether and extracted with 5% NaOH [in methanol (50% aq.)]. The organic layer after usual work-up yielded the neutral (36.12%), whereas aq. methanolic extract after neutralisation with 50% phosphoric acid and after usual work-up gave acids (55.98%). The crude acid mixture was converted into methyl ester by treatment with diazomethane. The ester mixture was purified by two methods. In the 1st method the mixture was subjected to distillation to get colourless oil as distillate (44%). GC analysis of the distillate indicated it to be a mixture of methyl palmitate (31.3%) and methyl oleate (60.7%). In the 2nd method the ester mixture was chromatographed on SiO_2 gel/IIb The ester fraction was distilled and analysed by GC, which again showed mixture of methyl palmitate and methyl oleate in the ratio of 32.2:64.5, Total fatty acid content from the second method amounted to 20.09% of the pet.ether extract and ratio of palmitic acid and oleic acid, in acid portion was 33.12:64.88 (Table-8).

Neutral portion after removal of solvent was subjected to chromatography, which was monitored by TLC. Elution with pet.ether-benzene-ethylacetate afforded various fraction containing particular types of compounds like alkanes saturated esters, unsaturated esters, phytol. Percentage of each was calculated from the weight of the distillate after distillation of crude fraction. Results are reported in Table-8 and 9.

Isolation of phytol

Phytol which is a major constituent (~6%) is of commercial importance.

TABLE - 8 Percentage composition of compounds isolated from the pet.ether extract of A. indica leaves.

Wt. of the Pet.ether extract (g)	Wt. of the acids/neutral (%), gm	Wt. of each compound (% of each compound on the basis of total pet.ether extract)										
		From neutral									From acids	
		Alkanes	Methyl palmitate	Ethyl palmitate	Methyl stearate	Ethyl stearate	Methyl oleate	Ethyl oleate	Phytol	β-sitosterol + stigmasterol	Palmitic acid	Oleic acid
28.4	15.9 / 10.26	0.38	2.13	1.5	0.494	0.5112	3.02	1.47	1.718	0.4003	1.83	3.67
	(55.98) / (36.12)	(1.33)	(7.5)	(5.3)	(1.74)	(1.8)	(10.65)	(5.2)	(6.04)	(1.4)	(6.4)	(12.92)

TABLE - 9 GC analyses of the esters obtained from the neutral portion of pet.ether extract of A. indica leaves (column 10% SE-30, 6 feet, temp. 220°C).

Fraction	Methyl palmitate (%)	Ethyl palmitate (%)	Methyl stearate (%)	Ethyl stearate (%)	Methyl oleate (%)	Ethyl oleate (%)	Remarks
Saturated ester	43.52	29.57	10.75	7.39	-	-	
Unsaturated ester	11.11	8.96	-	-	50.17	24.01	Fraction contaminated with saturated ester (~20%).
Total ester (Fig.6)	20.99	14.75	4.82	5.1	29.5	14.46	

Attempts were done to isolate phytol from the pet.ether extract (PE-1) without involving chromatography. Our first choice was by partition between two immiscible solvents by counter current method. Pet.ether extract was dissolved in pet.ether and extracted with aq. methanol (90%). Fractions rich in phytol were combined and after usual work-up it was distilled. The distillate on distillation afforded colourless oil (7.62% of total). However, phytol thus obtained was contaminated with other impurities.

To increase yield and purity of phytol, second method was attempted. To hydrolyse chlorophyll which is a phytyl ester, the pet.ether extract was refluxed with 10% ethanolic KOH and the mixture after saponification was partitioned between 90% aq. methanol and pet.ether by counter current. The pet.ether fractions rich in phytol were combined, and the extract, a gummy liquid (14%) was distilled to give 90% pure phytol (TLC). Yield was 6.5%. The method was modified as follows. The mixture obtained after hydrolysis was diluted with water and extracted with solvent ether. The organic extract after usual work-up yielded dark brown oil (29.3%) which on distillation gave 90% pure phytol. Yield was 6.5%.

Dichloroethane extract

This extract was found to be most active (antifeedant) fraction. Our efforts were then directed to get a fraction, which was responsible for the antifeedant activity of the DCE extract or most active fraction of the DCE estract. In that connection the DCE extract was subjected to broad cut chromatography on SiO_2 gel/IIb and eluted with benzene-EtOAc with increasing polarity. On TLC, fraction-3 which was eluted with benzene-ethyl-acetate (7:3) was found to be comparable with the authentic sample of

azadirachtin rich fraction (Fig. 9). On removal of solvent, it was obtained as dark green fluffy solid and exhibited six spots on TLC. This fraction was most active amongst all the fractions collected so far. Fraction-4, which was eluted with EtOAc was dark green fluffy solid. It showed four compounds on TLC and was less active than fraction-3.

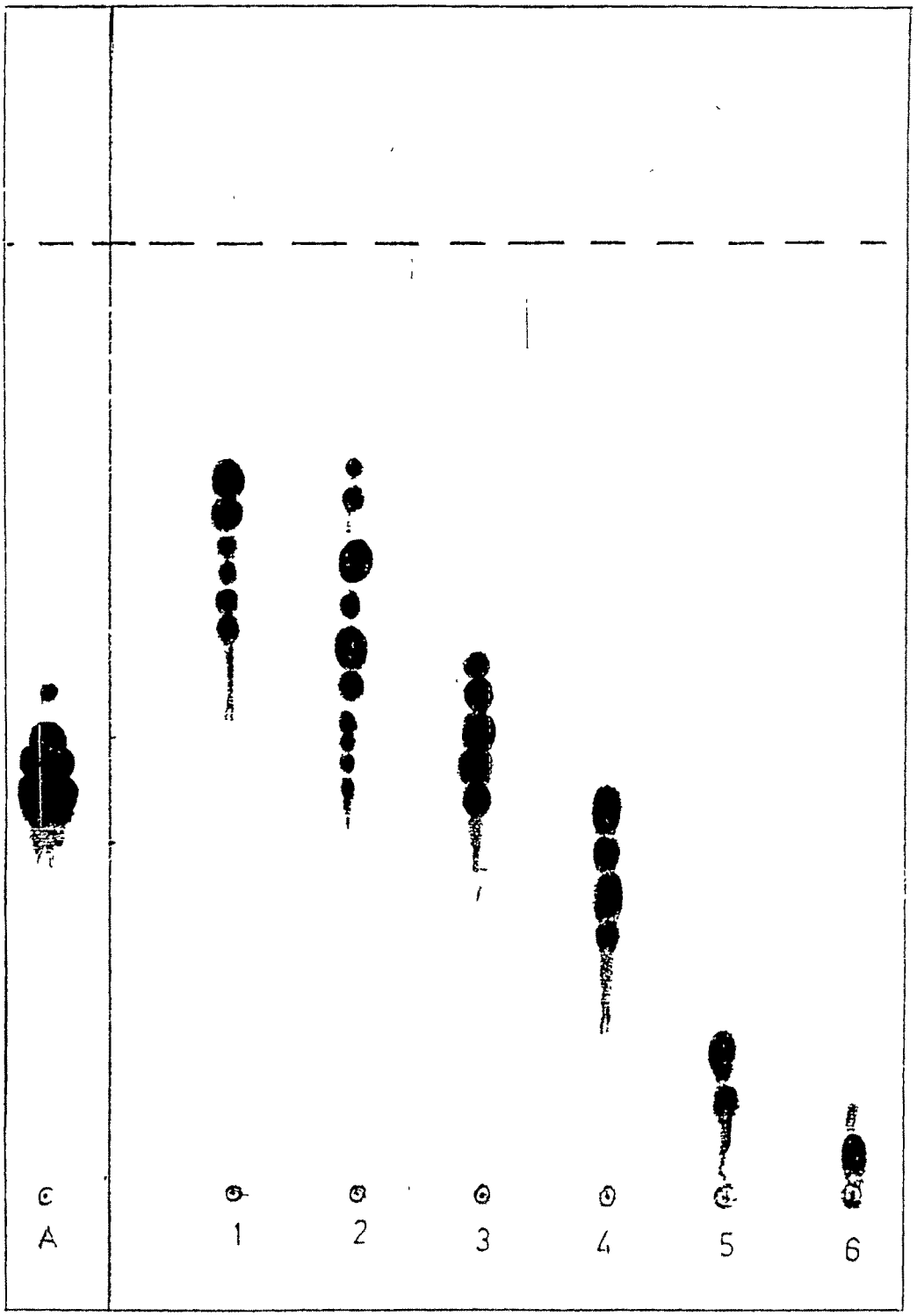
All the fractions obtained from column chromatography were contaminated with chlorophyll and other colouring matters. To get rid of it, the extract was separately passed through alumina grade I - II_A and grade III. The recovered extract (52-62%) was almost devoid of any colouring impurities and also retained its activity.

Ethylacetate extract

After removal of solvent, the extract was obtained as a yellow gummy solid, which on TLC (Fig. 10) showed major four spots. The extract was chromatographed on SiO_2 gel/II b and eluted with pet.ether-ethylacetate-methanol by gradient elution[113] (chromatogram-11).

Fraction-2 which was eluted with pet.ether-EtOAc (10:90→8:92) was a yellow solid and on TLC showed a nearly pure compound -1. Repeated crystallisation from methanol gave a yellow crystalline solid, TLC single spot, m.p. 296-300°. It was converted to trimethylsilyl ether[114] which on GC showed 98% purity. It was identified as Quercetin (11) by direct comparison with the authentic sample (m.p., mixed m.p., mixed TLC, CO-GC and superimposable IR, NMR and UV spectra).

Fraction-4 was eluted with ethylacetate-methanol (80:20 → 75:25)

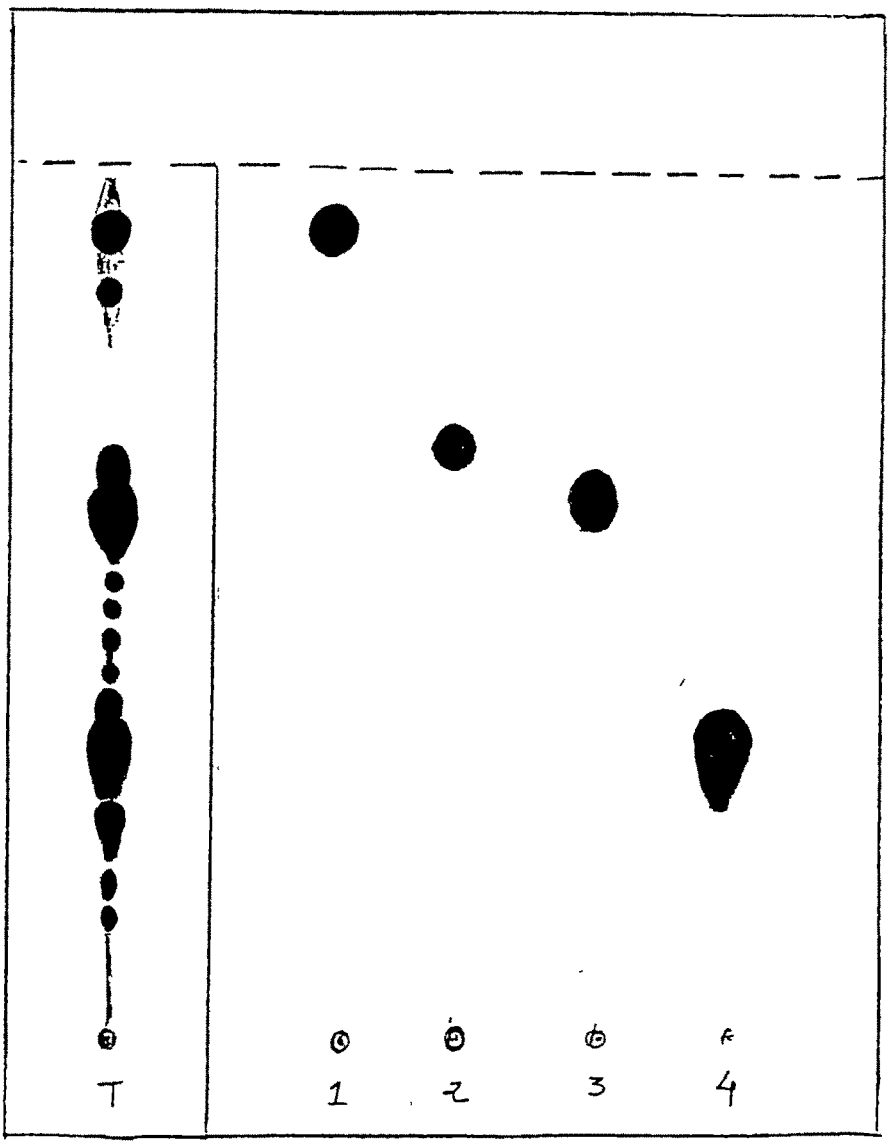


A : Azadirachtin rich fraction (authentic sample)

Silica gel - G plate; Solvent system :- 50% EtOAc in benzene

Spray reagent : 1% Vanillin-phosphoric acid.

Fig. 9 : TLC of fractions obtained from DCE extract.



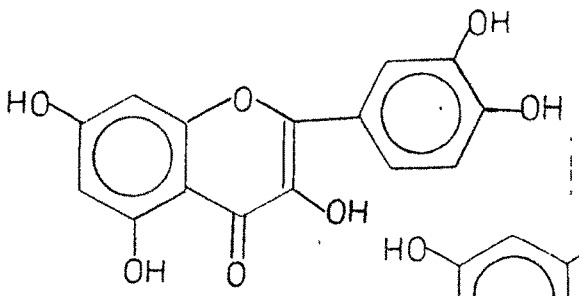
T-total EtOAc extract of A. indica leaves.

(1) Quercetin (2) Quercitrin (3) Isoquercitrin (4) Rutin, isolated from EtOAC extract.

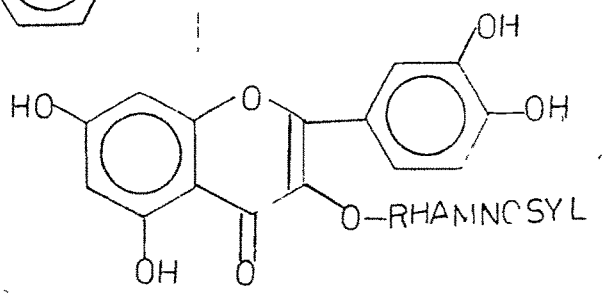
Silica gel - G plate ; Solvent system : EtOAc:MeOH:$CHCl_3$(10:4:6)

Spray reagent : H_2SO_4.

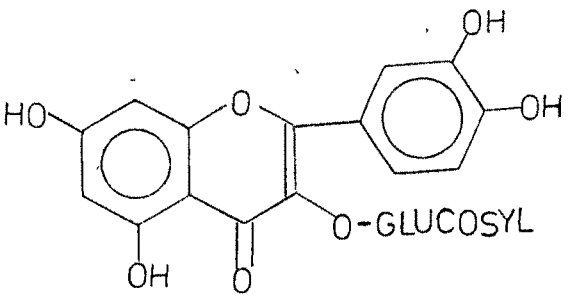
Fig. 10 : TLC of compounds isolated from EtOAc extract of A. indica leaves.



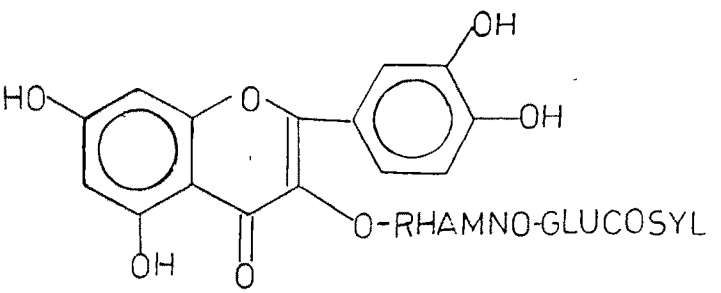
(11) Quercetin



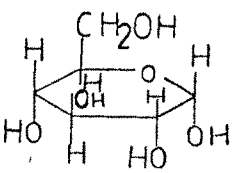
(12) Quercitrin



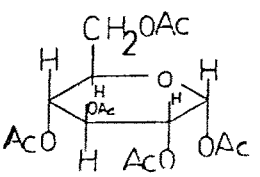
(13) Isoquercitrin



(14) Rutin



(15) D-Glucose



(16) D-Glucose pentaacetate

was a yellow gummy solid. On TLC it showed compound-2 contaminated with other impurity. Rechromatography of the fraction-4 on SiO_2 gel furnished a TLC pure (90%) compound-2, which was repeatedly recrystallised from aq. EtOH (80%) to get a yellow crystalline solid, TLC single spot, m.p. 173-75°. It was identified as Quercitrin (12) by comparison of m.p., UV and NMR (of TMS derivative) with the reported values in literature[114,115].

Fraction-6 eluted with EtOAc-MeOH (73:27 → 71:29) showed major compound-3 on TLC. It was rechromatographed on SiO_2 gel (chromatogram-13) to get a single spot compound, which on repeated recrystallisation gave a yellow crystalline solid, m.p. 196-98°. UV, IR and NMR (Fig. 11,12) spectra resembled with the spectra of flavanol glycosides. To find out the aglycone and the sugar, compound was hydrolysed with 2N HCl and the sugar portion and the aglycone were separated as usual. Aglycone was a yellow solid (single spot). It was crystallised from methanol as a yellow crystalline solid, m.p. 295-299°. It was identified as Quercetin (11) by direct comparison (mixed m.p., mixed TLC, superimposable IR and NMR). Sugar part after usual work-up gave a gummy liquid which showed single spot on TLC and paper chromatography corresponding to D-Glucose (15). It was converted to TMS derivative and analysed by GC, where the retention time matched with the TMS derivative of D-Glucose. When exposed to acetylation, the acetate derivative was a white solid (m.p. 109-111°). It was found to be glucose pentaacetate (16) by comparison with the authentic β-D-glucose pentaacetate (mixed TLC, mixed GC, identical IR and ^{1}H-NMR).

In the NMR a signal at δ 5.8 ppm confirmed the attachment of D-glucose at position-3 of quercetin[118], β -linkage of the sugar was also evident

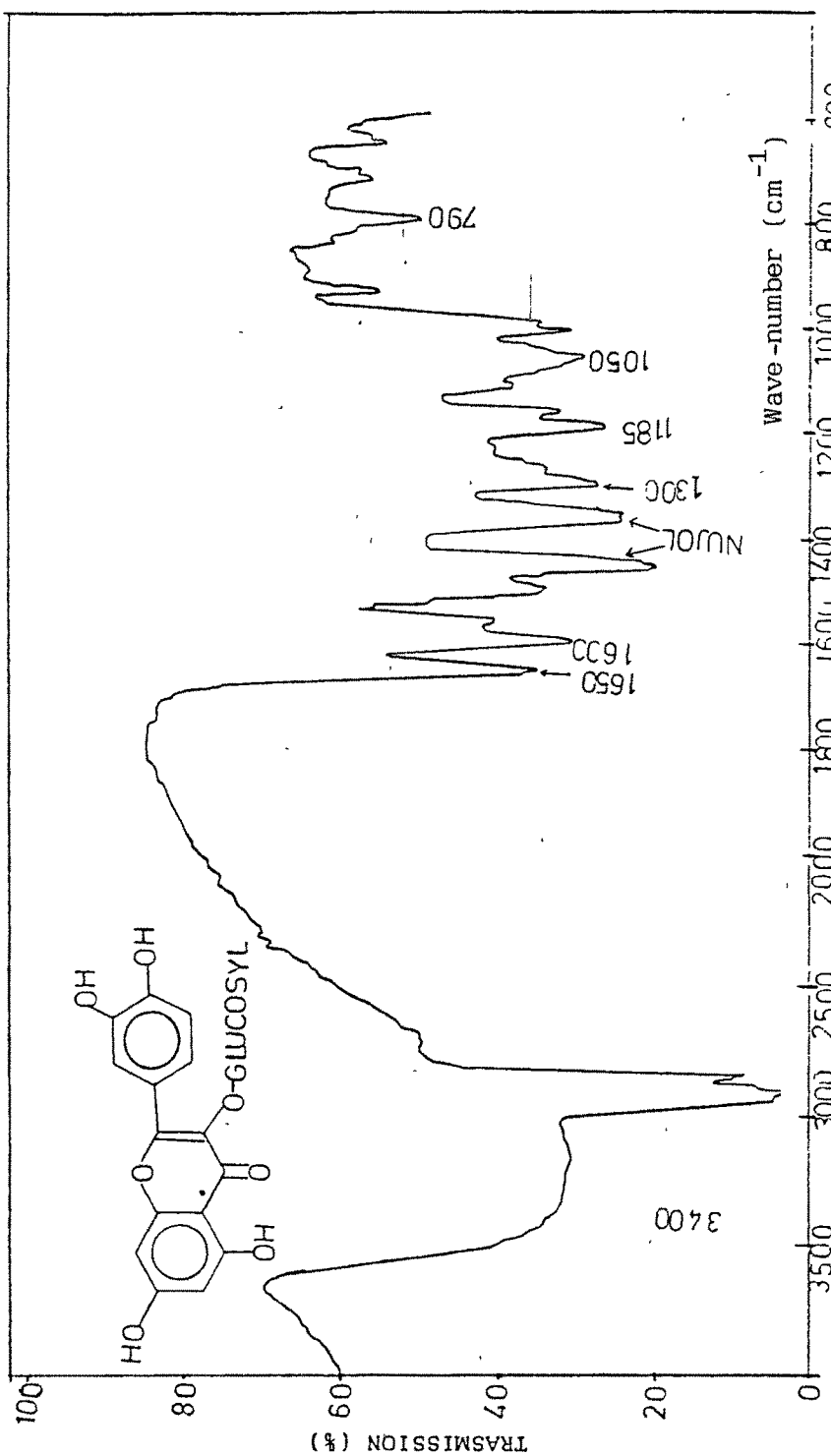


FIG. 11 : IR SPECTRUM OF ISOQUERCITRIN.

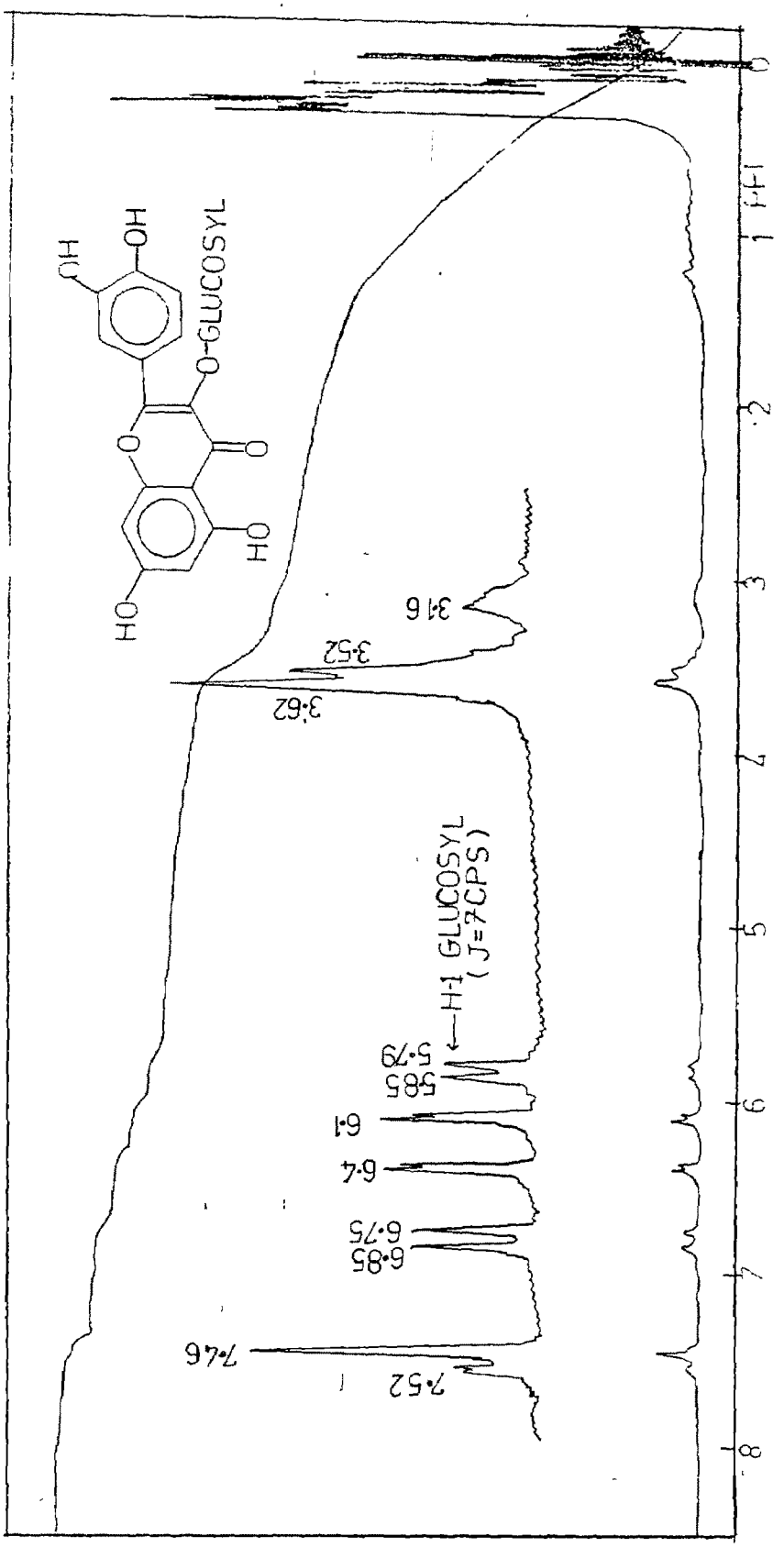


FIG. 12 : NMR SPECTRUM OF ISOQUERCITRIN (TMS ETHER)

from NMR (J=7 cps). Except arabinose, almost all sugars which are occuring naturally are in the pyranose form[119].

From the above studies the compound was characterised as Quercetin-3-O-β-D-glucopyranoside or Isoquercitrin (13). The compound was isolated for the first time from the leaves of A. indica.

Fraction-8 eluted with EtOAc-MeOH (69:31→ 67:33) was a yellow gummy mass showing nearly a single spot. It was purified further by rechromatography. A yellow coloured solid was obtained as single spot compound. Recrystallisation from aq. ethanol (80%) yielded compound-4 as crystalline solid, m.p. 184-186°C. Which was identified as Rutin (14) by direct comparison with the authentic sample [mixed m.p., mixed TLC, superimposable IR, UV and NMR (TMS-ether)].

Rutin and quercetin have been used in treatment of diseases states characterised by capillary bleeding associated with increased capillary fragility[117]. As these flavonoids are of commercial importance, it was decided to estimate percentage of each flavonoid in EtOAc extract. GC was found to be useless for the estimation.

High performance liquid chromatography (HPLC) of EtOAc extract.

The development of commercially available high pressure and medium pressure liquid chromatography system have opened up new horizones for the analysis of non-volatile complex molecules, which are difficult to analyze by gas chromatography, thin layer, paper and column chromatography. High pressure liquid chromatography overcomes many of these difficulties and

provides excellent resolution with high speed, high efficiency and sensitivity.

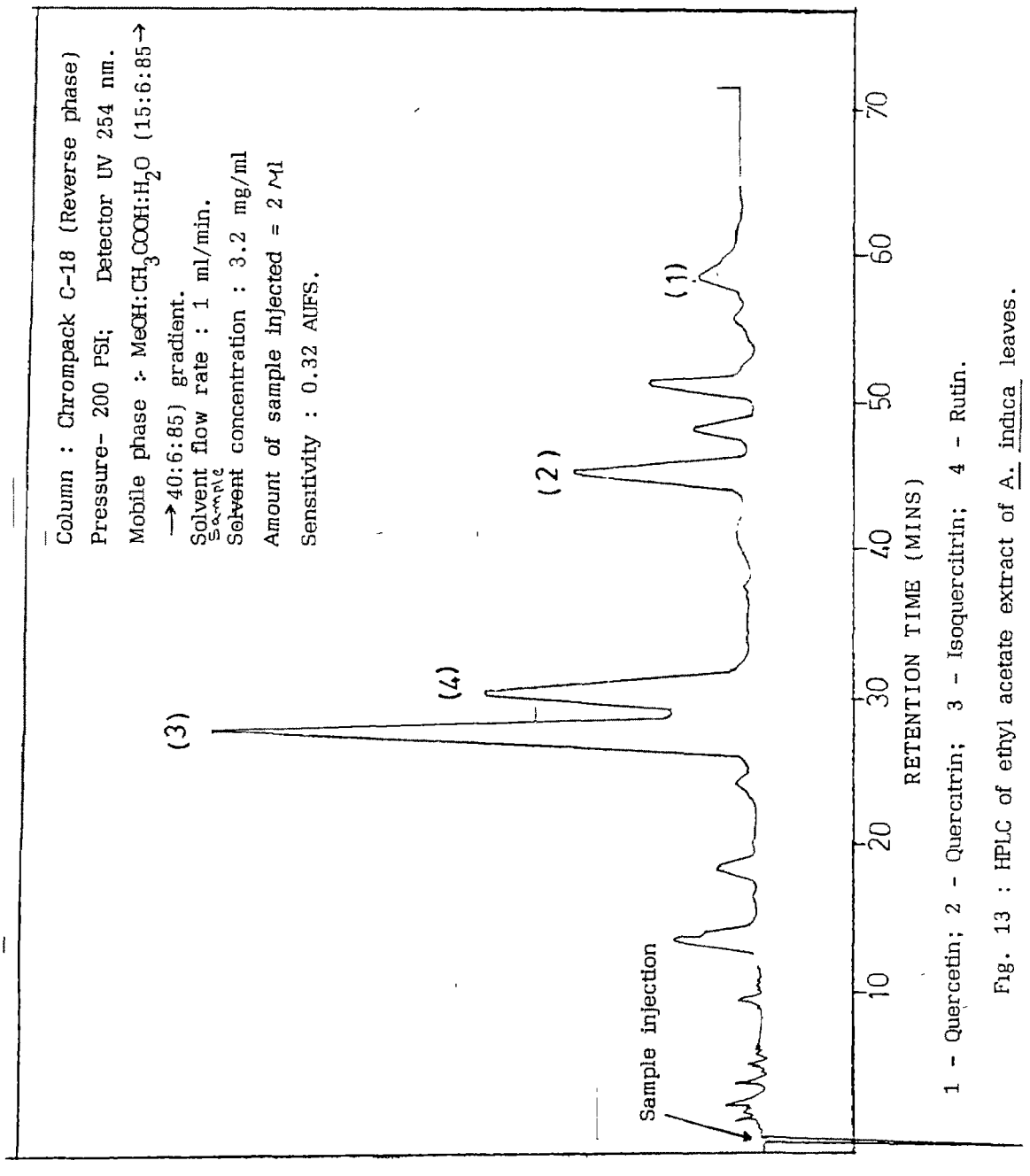
To find out percentage of each of the flavanoids in the extract it was decided to use HPLC for analysis. For the analysis a standard mixture was prepared by mixing accurately weighed quantities of quercetin, isoquercitrin, quercitrin and rutin, and subjected to HPLC (Fig. 13). The assignment of peaks were done by injecting the authentic samples and the area correction factor for each peak was calculated by the formula (Table-10).

$$\text{A.c.f.} = \frac{\text{Actual \% in the mixture}}{\text{Observed \% in HPLC}}$$

Exactly weighed sample of ethylacetate extract was passed through a column of SiO_2 gel/IIb and eluted with pet.ether-ethylacetate (1:1). The eluate was made free from solvent. Exactly weighed sample from the purified extract was dissolved in methanol and subjected to HPLC analysis. Percentage of each the component was then calculated using the area correction factor. Results are shown in Table-11.

Butanol extract

Butanol extract was obtained as a brownish gummy solid after removal of butanol under reduced pressure. The extract on TLC (solvent system EtOAc : MeOH : $CHCl_3$ - 5:2:3, spray reagent 10% HNO_3 in H_2SO_4) showed atleast five distinct spots (Fig. 14), termed as BE-1, BE-2, BE-3, BE-4 and BE-5 with decreasing polarity. Out of which BE-1, BE-4 and BE-5 imparted yellow colour, whereas BE-2 and BE-3 were brown. The extract was subjected to broad cut chromatography on SiO_2 gel/IIb (chromatogram-16) and eluted with ethylacetate-methanol with increasing polarity.



1 - Quercetin; 2 - Quercitrin; 3 - Isoquercitrin; 4 - Rutin.

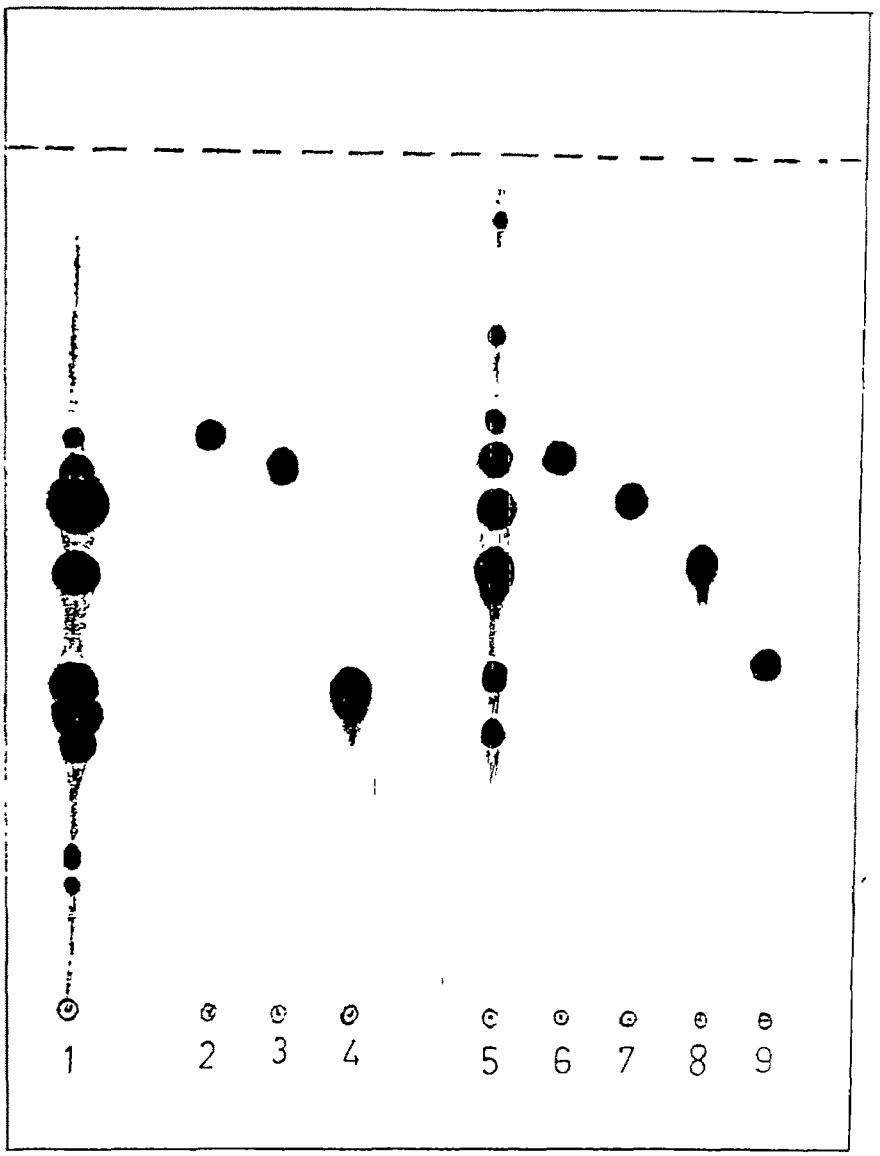
Fig. 13 : HPLC of ethyl acetate extract of A. indica leaves.

TABLE-10 : Analysis of known mixture of flavonoids by HPLC

Compound	Weight (g)	Actual % of compound in mixture	Observed % of compound in mixture	Area correction factor = Actual % / Observed %
1. Quercetin	0.002	16.12	9.09	1.773
2. Quercitrin	0.0031	25.00	22.26	1.123
3. Isoquercitrin	0.0017	13.70	25.26	0.542
4. Rutin	0.0056	45.14	43.124	1.042

TABLE-11 : Analysis of ethylacetat extract (purified) of A. indica leaves by HPLC.

Compound	Observed %	Actual % (i.e. Obs.% x correction factor)	Wt. of the each compound in purified extract (g)	% of each compound in total EtOAc extract
1. Quercetin	4.063	7.20	0.4392	4.90
2. Quercitrin	10.94	12.28	0.749	8.5
3. Isoquercitrin	37.24	20.19	1.23	13.99
4. Rutin	19.18	20.08	1.224	13.91
			T O T A L:	41.30



(1) Total butanol extract (2) Quercitrin (3) Isoquercitrin (4) Rutin (5) Total distilled acetate of butanol extract (6) Ethyl-α-D-glucoside tetraacetate (7) Ethyl-β-D-glucoside tetraacetate (8) D-(+)-Glucose (9) meso-Inositol.

Silica gel - G plate; Solvent system :- EtOAc:MeOH:$CHCl_3$ (5:2:3)

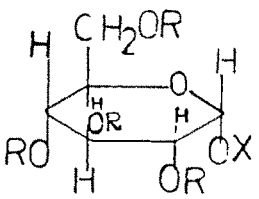
Spray reagent : 10% HNO_3 in H_2SO_4.

Fig. 14 : Compounds isolated from butanol extract of A. indica leaves.

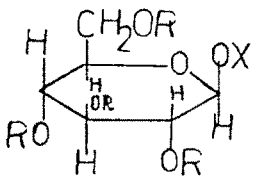
Fraction-3 was a yellow powder and showed two major yellow coloured spots corresponding to quercitrin (12) and isoquercetrin (13). Fraction-4 was a mixture of BE-1, BE-2, BE-3 and little BE-4, when rechromatographed on SiO_2 gel (chromatogram-17), it yielded BE-1, which turned out to be rutin (14). Fraction-4 (4) showed two spots. It was subjected to chromatography on chromatotron (chromatogram-18). Fraction-4 (4)/3 was a gummy syrup. From its NMR, it was apparent that it a mixture of ethylated sugar. In order to make the separation easier, the mixture was exposed to acetylation with acetic anhydride and pyridine at room temperature. The acetylated mixture exhibited major two spots on TLC (pet.ether-ethyl-acetate, 3:2) having Rf values 0.6 and 0.55. The two compounds were separated by chromatography on chromatotron and identified as ethyl-α-D-glucoside tetraacetate (Rf 0.6)(20) and ethyl-β-D-glucoside tetraacetate (Rf 0.55)(24) by direct comparison with the authentic samples [TLC (Fig. 14), IR, NMR]. Thus sugars isolated from n-BuOH extract were ethyl-β-D-gulcoside (23) and ethyl-α-D-glucoside (19).

Estimation of sugar in n-BuOH extract

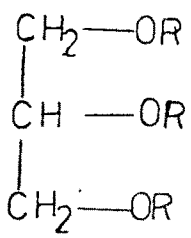
Since the n-BuOH extract contained flavonoids and sugars, to find out percentage of sugar in the total extract, the extract was acetylated using acetic anhydride in pyridine. The mixture obtained after usual work-up was distilled under vacuum. Fraction-1 distilled at 120-25°/100 mm was a colourless oil (17.4% of extract) with GC purity 95% and was identified as Triacetin (26) by direct comparison with authentic sample (superimposable IR, NMR and mixed GC). Second fraction (54%) which distilled at 210-250°/1 mm was a mixture (free from flavonoids) of four compounds (TLC, fig. 14), Rf values of three compounds matched with Rf values of ethyl-α-D-glucoside tetraacetate (20), ethyl-β-D-glucoside tetraacetate (24) and glucose penta-



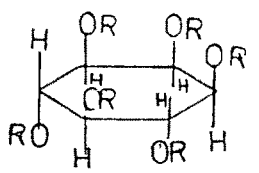
(17) R = H, X = H
(18) R = $COCH_3$, X = $COCH_3$
(19) R = H, X = CH_2CH_3
(20) R = $COCH_3$, X = CH_2CH_3



(21) R = H, X = H
(22) R = $COCH_3$, X = $COCH_3$
(23) R = H, X = CH_2CH_3
(24) R = $COCH_3$, X = CH_2CH_3



(25) R = H
(26) R = $COCH_3$



(27) R = H
(28) R = $COCH_3$

acetates* (18). The fourth compound was identified as meso-inositol hexa-acetate* (28). GC analysis of the total distillate was carried out on 10% DCQF-1 at 190°C. Percentage of all the compounds were calculated in the total extract (Table-12) by GC (Fig. 15).

Thus n-butanol extract contains glycerol (25) meso-inositol (27) glucose (17) and ethyl glucoside (19). These compounds are first time reported from leaves.

Aqueous extract

The aqueous extract was obtained as the remainder after extraction with the organic solvents. The extract was made free from water by removing water at reduced pressure. For easy handling, the extract was subjected to acetylation with acetic anhydride and pyridine at room temperature. The residue (53%) after usual work-up showed a complex mixture on TLC (Fig.17). It was chromatographed on SiO_2 gel and eluted with pet.ether-ethylacetate with increasing polarity (chromatogram-20). Fraction-3 eluted with pet.ether-ethylacetate (9:1), on TLC showed fairly pure compound, which was purified further on chromatotron to get ethyl-β-D-glucoside tetraacetate, identified by direct comparison with authentic sample. Fraction-4 was a mixture of four compounds, which was subjected to repeated chromatography to get a TLC pure compound, which in the GC analysis (10% DCQF-1, FID, 210°) showed it to be the mixture of α-D-glucose pentaacetate and β-D-glucose pentaacetate (by comparison with authentic samples and superimposable IR and NMR). Fraction-5 eluted with pet.ether-ethylacetate (85:15) was a white crystalline solid, which on repeated crystallization from chlorofom gave a white crystalline compound, m.p. 211-213°. The compound was identified as meso-inositol hexaacetate (28) by comparison with the authentic

* Isolated and identified from the aq. extract later on.

TABLE-12 : Percentage composition of compounds isolated from butanol and aq. extract of A. indica leaves.

Compound	Butanol extract		Aqueous extract	
	% in total distilled acetate*	% in butanol extract	% in total purified acetate*	% in aq. extract.
Glycerol	31.17	7.6	12.49	6.6
Ethyl- ∝ -D-glucoside and Ethyl- β -D-glucoside	22.53	5.519	8.15	4.3
∝ -D-glucose and β -D-glucose	11.5	2.817	28.93	15.47
Meso-Inositol	6.6	1.616	23.69	12.67

* % of corresponding acetate is given, analyzed by GC (column 10% DCQF-1 at 190°)

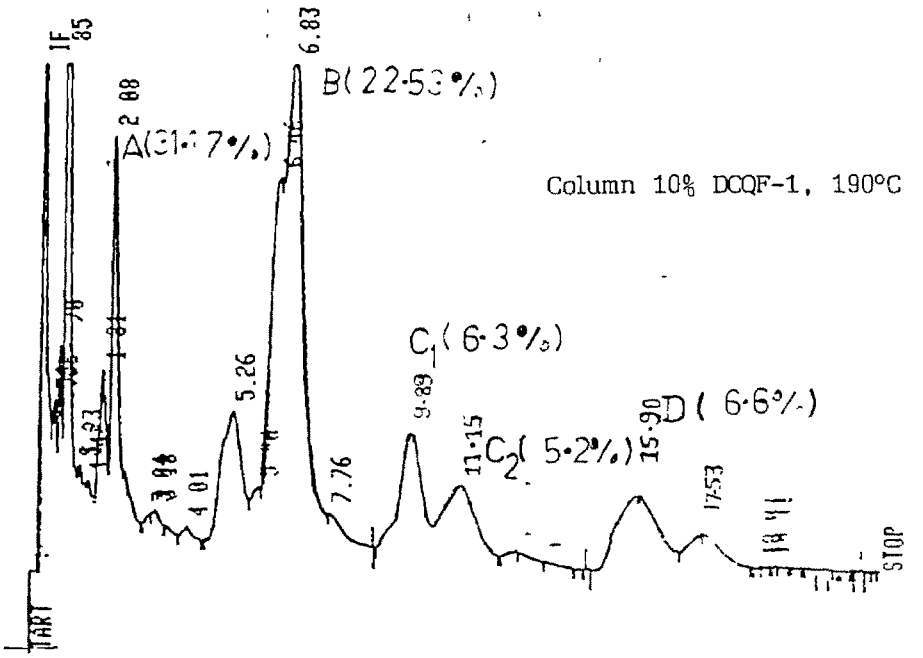
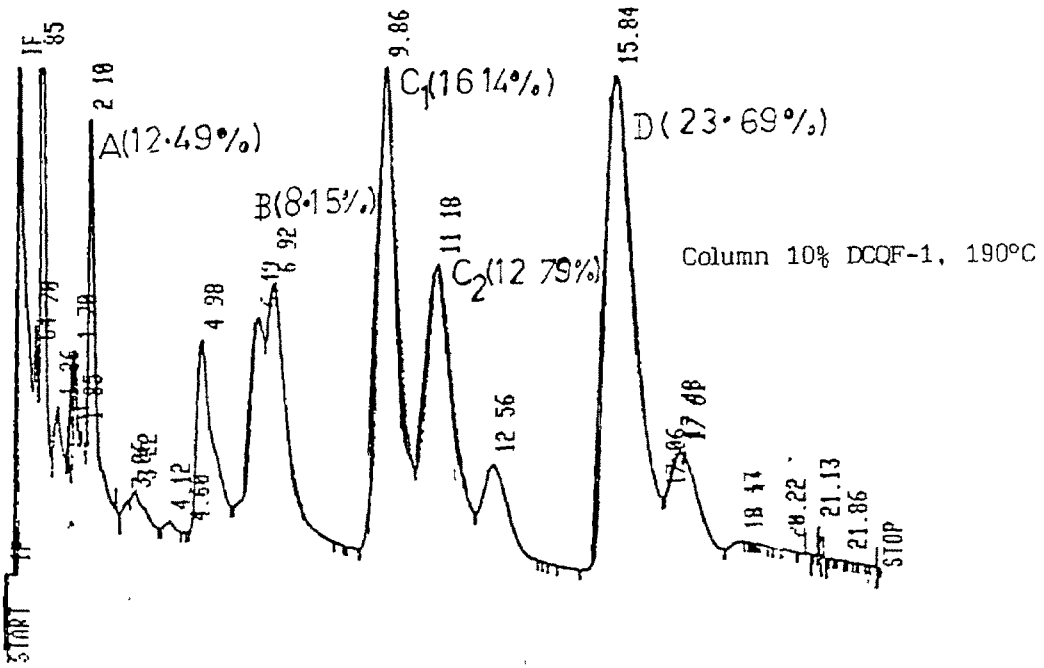


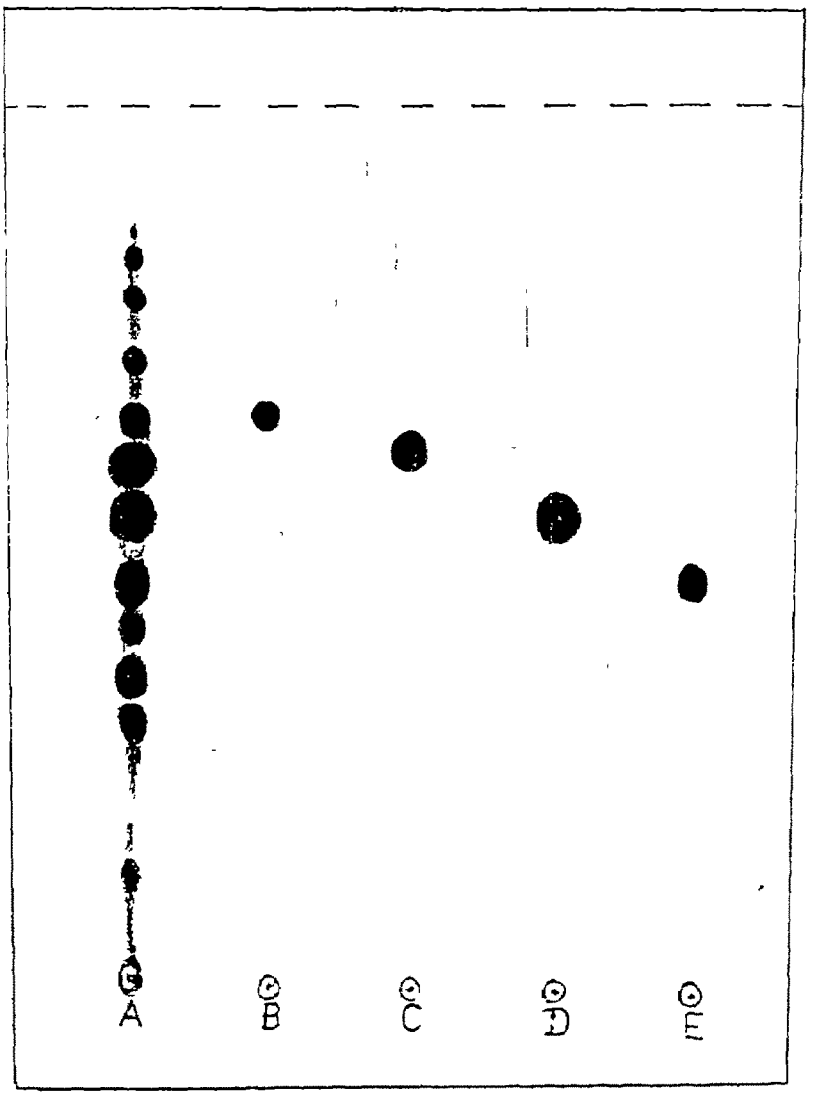
Fig. 15 : GC analysis of distilled acetylated product of butanol extract of A. indica leaves.



A-Triacetin, B-Mixture of α- and β -ethylglucoside tetraacetate C_1 and C_2-Glucose pentaacetate, D-meso-inositol hexaacetate.

(For Figs. 15 & 16)

Fig. 16 : GC analysis of purified acetylated product of aqueous extract of A. indica leaves.



(A) Total acetate of aq. extract of A. indica leaves. (B) Ethyl-α-D-glucoside tetracetate (C) Ethyl-β-D-glucoside tetracetate (D) D-glucose pentaacetate (E) meso-Inositol hexaacetate.

Silica gel - G plate; Solvent system : 40% EtOAc in pet.ether.

Spray reagent : H_2SO_4

Fig. 17 : Compounds isolated from acetylated aq. extract of A. indica leaves.

sample.

Ascorbic acid is present in many plants, was found to be absent in aq. extract, confirmed by TLC, spot test and paper chromatography with the authentic sample.

Thus compounds isolated from aq. extract were ethyl-β-D-glucoside (23), β-D-glucose (21), α-D-glucose (17) and meso-inositol (27).

For finding out percentage of the sugars in the total extract after acetylation, the acetate mixture (accurately weighed) was made free of polar impurities by passing it through a small column of SiO_2 gel and eluting with 40% ethylacetate in pet.ether. The eluate was weighed and analysed by GC (column 10% DCQF-1 at 190°, Fig. 16). Percentage of the each sugar and other compounds were calculated in the total extract (Table-12).

Chart 1 represents the compounds isolated from various extracts of leaves of A. indica and their percentages content.

Biological evaluation of A. indica leaves extracts for antifeedant activity.

Pet.ether (PE-1), dichloroethane (DCE), ethylacetate and n-butanol extracts obtained from 70% ethanolic extract of A. indica leaves were evaluated for antifeedant activity at various concentration against Spodoptera litura (tobacco armyworms) adults. Choice and no-choice test were performed and average of both the tests were taken for calculation of percentage deterrence (Table-13).

Aq. ethanolic extract of A. indica leaves (~ 26.34% of leaves)

Pet.ether extract (7.17%)	DCE extract (10.49%)	EtOAc extract (4.22%)	Butanol extract (9.41%)	Aq. extract (68.7%)
1. Alkanes (1.33%)	Most active	1. Quercetin (4.9%)	1. Ethyl-D-glucoside (5.5%)	1. Ethyl-β-D-glucoside (4.3%)
2. Methyl palmitate (7.5%)	fraction	2. Quercitrin (8.5%)	2. D-(+)glucose (2.8%)	2. D-(+)-Glucose (15.47%)
3. Ethyl palmitate (5.3%)	(22.04%)	3. Isoquercitrin (13.99%)	3. Meso-Inositol (1.6%)	3. Meso-Inositol (12.67%)
4. Methyl stearate (1.74%)		4. Rutin (13.91%)	4. Glycerol (7.6%)	4. Glycerol (6.6%)
5. Ethyl stearate (1.8%)			5. Flavonoids	
6. Methyl oleate (10.65%)				
7. Ethyl oleate (5.2%)				
8. Phytol (5.7%)				
9. Palmitic acid (3.2%)				
10. Oleic acid (12.69%)				
11. Mixture of β-sitosterol and stigmasterol (1.4%)				

CHART - 1

Percentage composition of compounds isolated from A. indica leaves

TABLE-13 Antifeeding effect of various extracts of A. indica leaves against Spodoptera litura in choice and no-choice test.

Extract	Concentration	% Deterrence
Pet.ether (PE-1)	5.0 %	100
	2.0 %	100
	1.0 %	30
DCE	5.0 %	100
	2.0 %	100
	1.0 %	100
	0.3 %	100
	0.1 %	65
EtOAc	5.0 %	100
	2.0 %	70
	1.0 %	0
BuOH	5.0 %	0

TABLE-14 Effect of sub-fractions of pet.ether extract of A. indica leaves on the feeding of Spodoptera litura.

Extract (Sub-fractions)	Concentration	% Deterrence
Pet.ether (PE-2)	5.0 %	0
	2.0 %	0
	1.0 %	0
Benzene	5.0 %	50
	2.0 %	45
	1.0 %	0
50% Methanol	2.0 %	100
	1.0 %	100
	0.1 %	70

Pet.ether (PE-1) extract showed significant activity at 2% (100% activity) and 1% (30% activity) concentration of the extract. Pet.ether extract (PE-1) was further separated into pet.ether (PE-2), benzene and 50% methanol soluble portions and all the three sub-fractions were also evaluated for the antifeedant activity (Table-14). Only 50% methanolic extract showed good activity at 1% (100% activity) and 0.1% (70% activity) concentration (Table-14).

Only DCE extract showed very good activity at very low concentration. At 0.1% and 0.3% concentration it showed 65% and 100% activity respectively. DCE extract was further separated into six sub-fractions by column chromatography and all the fractions were tested for antifeedant activity against S. litura and Epilachna vijintioctopunctata adults in choice test (Table-15). Fraction-3, which is similar to Azadirachtin (a naturally occuring antifeedant) rich fraction on TLC (same Rf), showed the best activity (85-92.5%) at 0.1% concentration. Fraction-4 also showed 87% activity at 1% concentration. Remaining fractions did not show significant activity. Fraction 3, 4, and 5 of DCE extract were made free from chlorophyll and other colouring materials by passing through a column of neutral alumina of various grades also showed similar level of activity.

EtOAc extract showed 100% activity at very high concentration (5%) and n-butanol extract was found to be inactive.

Above result shows that major antifeedant activity of A. indica leaves is concentrated in DCE extract only. Fresh DCE extract itself can be used commercially as antifeedant.

TABLE-15 Effect of DCE extract fractions on the feeding of S. litura larve (3rd instars) and Epilachna vijintioctopunctata adults in a choice test.

Fractions	Concentration	Av. % Deterrence	
		S. litura	E. vijintioctopunctata
1	1.0	00	-
	0.1	00	-
2	1.0	15	-
	0.1	10	-
3	1.0	100	100
	0.1	92.5	85
4	1.0	85	88
	0.1	20	20
5	1.0	55	50
	0.1	20	18
6	1.0	00	-
	0.1	00	-

Comparison of DCE extract of A. indica leaves and Margosan-O.

0.3 gm DCE extract in 100 ml (EC) showed 100% feeding inhibition (24 hrs). A commercial formulation "Margosan-O" concentrate 0.3 ml in 100 ml H_2O showed 100% feeding inhibition (24 hrs). Margosan-O contains 300 ppm of Azadirachtin. It can be concluded that DCE extract contains similar level of active ingredients

EXPERIMENTAL

For general remark refer to experimental of Chapter-I. ^{13}C-NMR spectra were recorded on Jeol FX 90Q FT NMR (frequency ^{13}C- 22.5 MHz.) spectrometer in deuterochloroform using tetramethylsilane as a internal standard. GC-MS spectra were recorded on model Jeol JMS DX 303, GC column OV-1 (10 met, capillary). All the chromatographies were monitored by TLC.

Extraction of Azadirochta indica (Neem) leaves

Dry leaves of A. indica were powdered in a pulverizer and sieved through 18 mesh sieve. The powdered leaves (4.5 kg) were soaked in aqueous ethanol (70%, 8L) and kept at 30-35° for 72 hrs, after which the extract was withdrawn . The operation was repeated by adding appropriate quantity of fresh solvent for total 276 hrs. Extracts thus obtained were combined and freed of solvent to furnish 1066 gm of extract as a dark brown gummy mass.

Partition of 70% ethanolic extract

Ethanolic extract (1023 gm) was suspended in aq. ethanol (10%, 4L) and extracted successively with petroleum ether (200 ml X 6), dichloroethane (200 ml X 6), ethyl acetate (200 ml X 4) and n-butanol (saturated with water, 300 ml X 5). All the four extracts were freed from solvent under reduced pressure on rotary evaporator (~ 70°/100 mm). Aqueous layer was also freed from water on rotary evaporator at 90°/100 mm. Pet.ether extract (68 gm) was a dark brown viscous oil. Dichloroethane extract (100 g) was a dark green gummy solid. EtOAc extract (48 g) was a dark brown solid and n-butanol extract (96 g) was a brown fluffy solid, whereas aq. extract (68.7%) was a brown viscous syrup.

Counter current distribution of pet.ether (PE-1) extract

The pet.ether extract (3.75 g) was dissolved in aq. methanol (90%, v/v, 50 ml) and extracted with pet:ether (60-80°, 50 ml X 5). Each of the pet.ether layer was re-extracted with methanol (90% v/v, 50 ml X 5). All the pet. ether layers were combined, dried (Na_2SO_4) and solvent removed to get pet.ether extract as a viscous oil (PE-2, 1.65 gm). Removal of solvent from methanol layer (60°/90 mm) yielded methanolic extract as a gummy solid (2.1 g). Methanolic extract was dissolved in aq. methanol (50% v/v, 25 ml) and extracted with benzene (50 ml X 5). Each of the benzene layers was re-extracted with aq. methanol (50% v/v, 25 ml X 5). Removal of benzene from the combined benzene extract gave benzene extract as a gummy liquid (1.8 g). The alcoholic layers were combined and solvent was stripped off to get the methanolic extract (0.20 g) as a fluffy solid.

Pet.ether extract (PE-2) The extract was chromatographed on SiO_2/IIb using increasing amounts of benzene in pet.ether and then ethylacetate in benzene as the eluent.

TABLE-16 CHROMATOGRAM - 1

Material : 10.2 g (adsorbed on 20 g SiO_2 gel).

Adsorbent : 310 g SiO_2 gel/ IIb .

Column dimensions: 45 cm x 4.4 cm.

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
01.	Pet.ether (60-80°)	500ml X 7	0.1709	Brown oil having sulfur like odour.
02.	Pet.ether (60-80°)	500ml X 3	0.009	
03.	Pet.ether	500ml X 16	0.1960	Viscous liquid.

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
04.	10% benzene in pet.ether	500ml X 31	1.1943	Colourless liquid.
05.	10% benzene in pet.ether	500ml X 5	0.0283	
06.	10% benzene in pet.ether	500ml X 5	0.0623	Light brown oil.
07.	10% benzene in pet.ether	500ml X 25	0.1406	
08.	30% benzene in pet.ether	500ml X 29	1.0070	Light brown oil.
09.	30% benzene in pet.ether	500ml X 7	0.0838	
10.	30% benzene in pet.ether	300ml X 2	0.2021	
11.	40% benzene in pet.ether	500ml X 38	2.4716	Gummy solid.
12.	50% benzene in pet.ether	500ml X 54	1.4297	Gummy solid.
13.	50% benzene in pet.ether	500ml X 8	0.7062	
14.	Benzene	500ml X 61	0.6328	Gummy solid.
15.	20% ethylacetate in benzene	500ml X 15		
16.	Ethylacetate	500ml X 4	1.5442	
17.	Methanol	500ml X 3		
		T O T A L :	9.8788	

Lower alkanes Fraction-1 (20 mg) was distilled at 130-140° (bath temp.) at 3 mm to get 13 mg colourless oil. IR (neat) : 695, 885, 1170, 1380, 1470, 1650, 2860, 2930 and 2965 cm^{-1}. NMR δ: 0.9(t), 1.26(s), 1.67(s) ppm. Identified as a mixture of hydrocarbons, mainly alkanes (C_nH_{2n+2} n=11, 12, 13, 15) by IR, NMR and GC-MS.

Higher alkanes Fraction-2 (25 mg) was distilled at 240-260° (bath) at 0.5 mm furnishing 10 mg colourless liquid. Identified as mixture of saturated long chain hydrocarbon with formula C_nH_{2n+2}, where n=19 to 25 by IR, NMR and GC-MS.

Ester fraction Fraction-4 (1.19 mg) exhibited two spots on TLC. This mixture further chromatographed on SiO_2 gel/II b.

TABLE-17 CHROMATOGRAM-2

Material 1 gm

Adsorbent 60 g SiO_2 gel/II b.

Column dimension 48 cm x 1.8 cm

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
4(1)	Pet.ether	20ml x 10	0.0038	
4(2)	3% EtOAc in pet.ether	20ml x 5	0.3502	Colourless liquid.
4(3)	5% ethylacetate in pet.ether	20ml x 3	0.1203	Colourless liquid.
4(4)	7% ethylacetate in pet.ether	20ml x 9	0.3508	Colourless liquid.
4(5)	Methanol	100ml	0.1500	
		T O T A L :	0.9751	

Saturated esters Fraction-4(2)(350 mg) was distilled at ~110°(bath) at 0.4mm yielded 341 mg colourless liquid. It was identified as a mixture of saturated ester by GC, IR and NMR. Ethyl palmitate, methyl palmitate, ethyl stearate and methyl stearate were identified by GC-MS.

Unsaturated esters Fraction-4(4) (350 mg) was distilled at ~110°(bath) at 0.4 mm to get colourless liquid. The fraction was identified as mixture of unsaturated esters by GC, NMR and IR. Methyl oleate and ethyl oleate were identified by GC-MS.

α-Tocopherol Fraction-6 (62 mg) was slightly impure (TLC). It was purified on chromatotron (eluted with 3% EtOAc in pet.ether) to get 46 mg light brown oil. Distillation of oil at 155° (bath) at 0.3 mm yielded TLC pure liquid (28 mg). It was identified as α-tocopherol by direct comparison with the authentic sample (NMR, IR, mixed TLC).

Phytol Fraction-8 (1 g) was light brown oil. Distillation of oil (200 mg) at 155° (bath) at 0.3 mm yielded (180 mg) TLC pure liquid. Its GC analysis (10% DCQF-1, 180°) showed 98% purity. It was identified as phytol by comparison with the authentic compound (NMR, IR and CO-GC).

β-Sitosterol and Stigmasterol Fraction-11 (2.27 g) was gummy solid. Repeated crystallisation of gummy solid from pet.ether furnished white solid (203 mg, m.p. 126-130°). It showed a single spot on TLC (3% EtOAc in pet.ether, Rf 0.36) but exhibited two peaks in GC (1% OV-17, 260°). The two compounds were identified as β-sitosterol (63%) and stigmasterol (37%) by direct comparison with the authentic samples (CO-GC, NMR, IR).

Palmitic and Oleic acids Fraction-12 (1.42 g) was a gummy solid, identified as fatty acid (NMR, IR). It was converted to corresponding methyl esters with diazomethane (generated by reaction of nitrosomethyl urea and KOH in dry ether at 3-5°)[105]. Crude ester (1.43 g) obtained was purified on chromatotron (SiO_2 gel, 2 mm plate) by eluting with 4% EtOAc in pet.ether and distillation of purified ester at 160-180° (bath) at 3 mm furnished 1.2 gm colourless liquid. Its GC analysis (10% OV-4, 200°) showed presence of two esters, which (700 mg) were separated by preparative GC on 20% SE-30 (12 feet) column at 230°. Esters were identified as methyl palmitate [122 mg, m.p. 33-34°, b.p. 77-80° (bath) at 1 mm] and methyl oleate [310 mg, b.p. 104-107° (bath) at 0.4 mm] by comparison with the authentic samples (NMR, IR, CO-GC and ^{13}C-NMR).

Benzene extract Benzene extract was also a complex mixture of compounds (TLC). It was subjected to column chromatography on SiO_2 gel/II b.

TABLE-18 CHROMATOGRAM-3

Material 3.3 g (adsorbed on 6.6 g silica gel)

Adsorbent 120 g SiO_2 gel/II b.

Column dimensions 33 cm x 3.5 cm.

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
01.	20% benzene in pet.ether	500ml x 14	0.0516	Viscous liquid.
02.	40% benzene in pet.ether	500ml x 6	0.0360	Colourless liquid.
03.	40% benzene in pet.ether	500ml x 12	0.0653	Light brown oil.
04.	60% benzene in pet.ether	500ml x 7	0.0401	Light brown oil
05.	60% benzene in pet.ether	500ml x 4	0.0407	
6-8	80% benzene in pet.ether	500ml x 5		
	benzene	500ml x 12	0.7938	Gummy solid.
	5% ethylacetate in benzene	500ml x 12		
09.	10% ethylacetate in benzene	500ml x 13	0.1817	
10.	20% ethylacetate in benzene	500ml x 5	0.2484	Gummy liquid.
11.	20% ethylacetate in benzene	500ml x 10	0.1733	
12.	50% ethylacetate in benzene	500ml x 9	0.2446	
13.	ethylacetate	500ml x 11	0.899	
14.	50% methanol in ethylacetate	500ml x 1	0.2517	
15.	Methanol	500ml x 1	0.2066	

Alkanes (fraction-1), mixture of methyl oleate, methyl palmitate, methyl stearate, ethyl stearate, ethyl palmitate ethyl oleate (fraction-2), phytol (fraction-3), palmitic acid and oleic acid (fraction 6-8) were identified by comparison with the authentic samples (NMR, IR, CO-GC).

Fraction-10 This fraction was gummy liquid, on TLC (25% EtOAc in pet.ether) showed presence of atleast five compounds. It was further chromatographed on chromatotron.

TABLE-19 CHROMATOGRAM - 4

Material 0.232 g adsorbed on 1 g silica gel.

Adsorbent Silica gel

Thickness of rotor 1 mm.

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
10(1)	20% EtOAc in benzene	12ml X 5	0.026	
10(2)	20% EtOAc in benzene	12ml X 7	0.051	
10(3)	20% EtOAc in benzene	12ml X 15	0.071	Viscous gummy solid
10(4)	50% EtOAc in benzene	12ml X 7	0.035	
10(5)	Methanol	12ml X 7	0.028	
		T O T A L :	0.211	

Ester of poly-hydroxy compound : Fr 10(3) (71 mg) was a viscous gummy solid. It was identified as unsaturated ester of polyhydroxy (tetrol type) compound by NMR and IR spectra.

Hydrolysis of benzene extract

Benzene extract (1.3 g) and 6 ml 10% ethanolic KOH solution was refluxed on a boiling water bath ($\sim$ 95°) for 3 hrs. Ethanol was distilled off from the mixture and residue diluted with H_2O (15 ml) and then extracted with solvent ether (15 ml x 4). Ether extract after usual work-up furnished 260 mg (18.5%) neutral portion. Aqueous portion after acidification with H_3PO_4 (20% aq.), followed by extraction with solvent ether (15 ml x 6) yielded 590 mg (42%) acid portion as a green viscous liquid.

Acid portion (212 mg) was esterified with diazomethane, which after usual work-up and purification on chromatotron furnished 108 mg colourless liquid. It was identified as a mixture of methyl palmitate and methyl oleate by comparison with the authentic samples (NMR, IR, CO-GC).

Neutral portion on TLC showed presence of atleast 10 compounds (60% EtOAc in benzene). It was chromatographed on SiO_2 gel column.

TABLE-20 CHROMATOGRAM - 5

Material 0.259 g adsorbed on 1 g silica gel.

Adsorbent 10 g silica gel.

Column dimension 27 cm x 0.9 cm

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
1.	25% EtOAc in benzene	25ml x 3	0.1150	
2.	50% EtOAc in benzene	12ml x 2	0.054	
3.	50% EtOAc in benzene	12ml x 7	0.065	
4.	EtOAc	10ml x 2	0.012	Gummy solid
5.	EtOAc	10ml x 2	0.010	Gummy solid
6.	EtOAc	20ml x 2	0.002	
7.	Methanol	20ml x 2	0.006	
		T O T A L :	0.2647	

Fraction 4 (12 mg) and fraction 5 (10 mg) were gummy solid. On TLC both the fraction showed single spot (Rf 0.36 and 0.33, 60% EtOAc in pet.ether).

Separation of total pet.ether extract of A. Indica leaves into acids and neutral.

The pet.ether extract (28.4 g) was dissolved in 300 ml pet.ether and extracted with 5% NaOH (in 50% aq. methanol)(90 ml x 2, 60 ml x 2). The organic phase was washed with aq. methanol(50%, 90 ml x 1, 60 ml x 4) till neutral, brine (50mlX2) and dried (Na_2SO_4). Solvent removal furnished neutral material as a dark red liquid (10.26g, 36.12%).

The aq. phase was acidified with 50% (v/v) H_3PO_4 (45 ml). After saturation with NaCl, it was extracted with EtOAc (150 ml X 4, 90 ml X 3). The EtOAc layer (containing acids) was washed with brine (75 ml X 2) and dried (Na_2SO_4). Solvent removal furnished acidic material as a gummy viscous liquid (15.9 g, 55.98%). Acids (15.29 g) were treated with nitroso-methyl urea (2.9 g), which after usual work-up furnished crude ester as a dark brown liquid (16.23 g). Distillation of crude ester (20 mg) in a small bulb type distillation unit at 100-120°(bath) at 4 mm yielded colourless liquid (8.8 mg, 44%). GC showed presence of methyl palmitate (31.3%) and methyl oleate (60.07%) by comparison with the authentic samples (NMR, IR, CO-GC).

Crude ester was also purified by systematic chromatography on a SiO_2 gel/IIb column.

TABLE - 21 CHROMATOGRAM -6

Material	16.21 g
Adsorbent	37 g SiO_2 gel.
Column dimension	45 cm x 4.5 cm

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
1.	Pet.ether	500ml x 4	0.0249	
2.	2 % EtOAc in pet.ether	500ml x 1	0.0069	

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
3.	2 % EtOAc in pet.ether	500ml x 1	6.0111	Colourless liquid
4.	2 % EtOAc in pet.ether	500ml x 1	0.0102	
5.	Methanol	750ml x 1	8.5500	Viscous brown liquid
		T O T A L :	14.6031	

Methyl esters of palmitic and oleic acids: Fr. 3 (6 g) furnished TLC pure liquid. Distillation of the purified ester at 127-131°/1 mm yielded colourless liquid (5.7 g), GC analysis showed methyl palmitate (32.25%) and methyloleate (64.5%).

The neutral pet.ether extract on TLC (45% EtOAc in pet.ether) showed presence of atleast nine compounds. It was systematically chromatographed on SiO_2 gel/II b.

TABLE - 22 CHROMATOGRAM - 7

Material 10.26 g adsorbed on 12 g silica gel.

Adsorbent 240 g silica gel/II-b.

Column dimension 40 cm x 4 cm.

Fr. No:	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
1.	Pet.ether	500ml x 16	0.3801	viscous liquid.
2.	Pet.ether	500ml x 16	1.1235	colourless liquid.
3.	10% benzene in pet.ether	500ml x 18	1.6649	colourless liquid.
4.	10% benzene in pet.ether	500ml x 4	0.5243	
5.	30% EtOAc in benzene	500ml x 26	1.7183	light brown oil.
6.	40% EtOAc in benzene	500ml x 10	0.6209	gummy solid.
7.	Methanol	200ml x 1	4.023	
		T O T A L :	10.055	

Alkanes (Fr.1), ethyl stearate, ethyl palmitate, methyl palmitate, methyl stearate (Fr.2), ethyl oleate, methyl oleate (Fr.3), phytol (Fr.5), β-sitosterol, stigmasterol (Fr.6) were identified by comparison with the authentic samples.

Methods for isolation of phytol from pet.ether extract

METHOD-1 : Counter current extraction

Pet.ether extract (3.757 g) was dissolved in aq. methanol (90% v/v, 50 ml) and extracted with pet.ether (50 ml x 5). Each of the pet.ether layers was reextracted with aq. methanol (90% v/v, 50 ml x 5). Pet.ether extract 1-3 containing maximum phytol (TLC), were combined and solvent removal furnished crude phytol as brown liquid (1.48 g). Distillation of crude phytol at 190° (bath)/0.5 mm yielded light yellow liquid (0.286 g, 7.62% of the pet.ether extract).

METHOD-2 :

Pet.ether extract (2.4 g) and aq. ethanolic KOH (10%, 8.5 ml) was refluxed on a water bath (~85°) for 3 hrs. Solvent (80%) was distilled off on a rotary evaporator at 90°/100 mm and the residue was dissolved in aq. methanol (90%, 30 ml) and repeatedly extracted with pet.ether (30ml x 5) by counter current method as mentioned above. All the pet.ether extracts were combined, dried and solvent remove to get crude phytol as a brown liquid (0.3502 g). Distillation of crude phytol at 190°(bath)/0.5 mm furnished TLC pure (90%) phytol as a light yellow liquid (0.162 g, 6.5%).

METHOD-3 :

Pet.ether extract (3.1 g) and aq. ethanolic KOH (10%, 10 ml) was

refluxed on a water bath (85°) for 3 hrs. Solvent (80%) was removed and residue was diluted with water (15 ml). It was repeatedly extracted with solvent ether (200 mlx3), which after usual work-up furnished crude phytol as a brown liquid (0.9 g). Distillation of crude phytol at 190°(bath)/ 0.5 mm afforded TLC pure phytol (0.206 g, 6.6%).

Dichloroethane (DCE) extract

DCE extract was an extremely complex mixture (TLC) of compounds. The extract was chromatographed on a SiO_2 gel/IIb column.

TABLE-23 CHROMATOGRAM - 8

Material 41 g. adsorbed on 41 g SiO_2 gel.

Adsorbent 802 g SiO_2 gel/IIb.

Column dimension 44 cm x 6.2 cm.

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
1.	Benzene	1000ml x 15		
	10% EtOAc in benzene	1000ml x 22	6.3700	Dark green fluffy Solid.
	18% EtOAc in benzene	1000ml x 5		
2.	18% EtOAc in benzene	1000ml x 10		
	25% EtOAc in benzene	1000ml x 15	4.5200	Dark green fluffy solid.
	30% EtOAc in benzene	1000ml x 6		
3.	30% EtOAc in benzene	1000ml x 23	9.0400	Dark green fluffy Solid.
4.	EtOAc	1000ml x 5	6.9700	..do..
5.	30% MeOH in EtOAc	1000ml x 2	10.62	Dark green fluffy solid.
6.	MeOH	1000ml x 2	01.52	Dark green fluffy solid.
		T O T A L :	39.04	

All the fractions after solvent removal on rotary evaporator at 60°/100 mm furnished dark green fluffy solid.

To make the DCE extract free from chlorophyll and other colouring matters, it was broadly chromatographed over neutral alumina grade I-IIa.

TABLE-24 CHROMATOGRAM - 9

Material 0.725 g.
Adsorbent 11 g alumina grade I-IIa.
Column dimension 18 cm x 1.1 cm.

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
1.	Methanol	40ml x 1	0.258	Light green fluffy solid.
2.	Methanol	40ml x 1	0.042	Light green fluffy solid.
3.	Methanol	100ml x 1	0.029	Light green fluffy solid.
4.	Methanol	250ml x 1	0.048	Light green fluffy solid.
		T O T A L :	0.377 (g) (52%)	

DCE extract was also chromatographed on neutral alumina grade III.

TABLE - 25 CHROMATOGRAM - 10

Material 0.5619 g
Adsorbent 8.5 g alumina grade - III.
Column dimension 1.1 cm x 12.2 cm.

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
1.	Methanol	200ml x 1	0.3274	Light green fluffy solid.
2.	Methanol	300ml x 1	0.0233	Light green fluffy solid.
		T O T A L :	0.3507 (62.4%)	

Ethyl acetate extract

EtOAc extract was also a mixture of compounds (TLC) with a wide

range of polarity. It was chromatographed on SiO_2 gel/IIb and eluted with solvents of increasing polarity by gradient elution technique.

TABLE - 26 CHROMATOGRAM-11

Material 26.6 g

Adsorbent 780 g SiO_2 gel, grade IIb.

Column dimension 5.3 cm x 66 cm, Technique : Gradient elution.

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
1.	EtOAc in pet.ether			
	10 % ⟶ 50%	2,000ml		
	50% ⟶ 70%	500ml	0.3611	Yellow Powder.
	70% ⟶ 90%	500ml		
2.	EtOAc in pet ether	250ml	0.4245	
	90% ⟶ 92%			
3.	EtOAc in pet.ether	500ml	1.8854	Yellow gummy
	92% ⟶ 100%			Powder.
	Methanol in EtOAc	2,000ml	3.8728	
	1% ⟶ 20%			
4.	Methanol in EtOAc	500ml	3.8728	Yellow gummy solid
	20% ⟶ 25%			
5.	Methanol in EtOAc	500ml	2.4886	
	25% ⟶ 27%			
6.	Methanol in EtOAc	500ml	1.2926	Yellow solid
	27% ⟶ 29%			
7.	Methanol in EtOAc	500ml	0.6701	
	29% ⟶ 31%			
8.	Methanol in EtAOc	500ml	1.9203	Yellow fluffy solid
	31% ⟶ 33%			
9.	Methanol in EtOAc	3,000ml	3.9091	
	33% ⟶ 39%			
10.	Methanol	3,500ml	5.5568	Brown fluffy solid
		T O T A L :	22.3818	

TABLE - 29 CHROMATOGRAM - 14

Material 1.9283 g adsorbed on 2 g SiO_2 gel.

Adsorbent 60 g SiO_2 gel/IIb.

Column dimension 48 cm x 1.8 cm

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
8/1	80% EtOAc in pet.ether	250ml x 7	0.2625	
8/2	80% EtOAc in pet.ether	250ml x 1	0.0918	
8/3	90% EtOAc in pet.ether	250ml x 1	0.0141	
8/4	EtOAc	250ml x 1	0.1371	
8/5	0.5% MeOH in EtOAc	250ml x 1	0.5061	
8/6	0.5% MeOH in EtOAc	250ml x 1	0.1966	Yellow solid.
8/7	0.5% MeOH in EtOAc	250ml x 1	0.1420	
8/8	20% MeOH in EtOAc	250ml x 1	0.0955	
8/9	Methanol	250ml x 1	0.3166	
		T O T A L :	1.7622	

Rutin : Fr. 8 / 6 (196 mg) furnished yellow solid, which after repeated crystallisation from aq. ethanol (80%) furnished a yellow crystalline solid (66 mg, m.p. 184-186°). Identified as rutin by direct comparison with the authentic sample (NMR, IR).

High performance liquid chromatography of EtOAc extract of A. Indica leaves.

For HPLC analysis, the area correction factor for each pure compound was calculated. For this a standard mixture was prepared by mixing exactly weighed amount of quercetin (2 mg), quercitrin (3.1 mg), isoquercitrin (1.7 mg) and rutin (5.6 mg) and subjected to HPLC analysis and area correction factor was calculated by the given formula.

$$\text{Area correction factor} = \frac{\text{Actual percentage of the compound in the mixture}}{\text{Observed percentage of the compound in the mixture}}.$$

Observed percentage was calculated from the chart by taking weight of the tracing paper covered by the area of each peak.

For general HPLC analysis, exactly weighed ethyl acetate extract of the leaves was broadly chromatographed on SiO_2 gel as follow :

TABLE - 30 CHROMATOGRAM - 15

Material 8.8 g
Adsorbent 17.6 g SiO_2 gel/IIb.
Column dimension 37 cm x 3.6 cm

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
1.	50% EtOAc in pet.ether	500ml x 3	6.1	Yellow powder
2.	50% EtOAc in pet.ether	500ml x 3	2.2	Brown gummy solid.
	Methanol	500ml x 1		
		T O T A L :	8.3	

Fraction 1 (3.2 mg) containing flavonoid (yellow powder) was dissolved in spectroscopic grade methanol (1 ml) and subjected for HPLC analysis.

Butanol Extract

Butanol extract was a complex mixture of compounds (TLC). It was chromatographed on SiO_2 gel and eluted with solvent of increasing polarity by gradient elution technique.

TABLE - 31 CHROMATOGRAM - 16

Material 35 g adsorbed on 70 g SiO_2 gel.
Adsorbent 700 g SiO_2 gel/IIb.
Column dimension 60 cm x 7.1 cm

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
1.	MeOH in EtOAc (1%→12.5%)	2,000 ml	0.5163	
2.	MeOh in EtOAc (12.5%→15%)	500 ml	0.4814	
3.	MeOH in EtOAc (15%→20%)	1,000 ml	2.0024	Yellow solid
4.	MeOH in EtOAc (20%→23%)	1,000 ml	4.5676	Brown solid
5.	MeOH in EtOAc (23%→24%)	500 ml	2.7670	Brown solid
6.	MeOH in EtOAc (24%→26%)	1,000 ml	3.8098	Brown solid

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
7.	MeOH in EtOAc (26%→28%)	500 ml	1.8011	Brown solid.
8.	MeOH in EtOAc (28%→29%)	500 ml	0.5949	
9.	MeOH in EtOAc (29%→33%)	2000 ml	2.5126	Brown fluffy solid
10.	MeOH in EtOAc (33%→99%)	3500 ml	2.5603	Brown fluffy solid
11.	Methanol	2000 ml	3.5806	
		T O T A L :	25.1940	

Quercitrin and Isoquercitrin : Fraction 3 (0.738 gm) on TLC showed two yellow spots. Both the compounds were separated by chromatography as mentioned earlier and purified by crystallisation from aq. ethanol (80%), yielded yellow solid. Compounds were identified as quercitrin (40 mg) and isoquercitrin (60 mg) by comparison with the authentic samples [NMR (TMS ether), TLC, m.p. and IR].

Fraction 4 (4.5 g) was a brown solid, on TLC showed presence of at least four compounds. It was further separated by chromatography on a SiO_2 gel column.

TABLE - 32 CHROMATOGRAM - 17

Material 4.4 g adsorbed on 10 g SiO_2 gel.

Adsorbent 240 g SiO_2 gel/IIb.

Column dimension 45 cm x 3.8 cm

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
4(1)	70% EtOAc in pet.ether	100ml x 4		
4(2)	80% EtOAc in pet.ether	100ml x 10	0.6843	Dark red syrup.
4(3)	90% EtOAc in pet.ether	100ml x 4		
4(4)	Ethyl acetate	100ml x 9	1.1438	Yellow gummy solid
4(5)	Ethyl acetate	100ml x 9	1.3182	Yellow gummy solid
4(6)	5% MeOH in EtOAc	100ml x 3	0.6415	Brown fluffy solid

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
4(7)	10% MeOH in EtOAc	100ml x 7	0.2958	Yellow solid
4(8)	MeOH	250ml x 3	0.1247	Yellow solid
		T O T A L :	4.2083	

Rutin : Fr. 4 (7) (295 mg) was yellow solid, which was purified by crystallisation from aq. ethanol (80%), yielded yellow crystalline solid (50 mg). Identified as Rutin by comparison with the authentic sample (NMR, IR, m.p.)

Fraction 4 (4) (1.143 g) was yellow gummy solid. On TLC fraction showed presence of two compounds. It was further separated by chromatography on chromatotron.

TABLE - 33 CHROMATOGRAM - 18

Material 281 mg
Adsorbent Silica gel
Thickness of rotor 1 mm

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
4(4)/1	1% MeOH in EtOAc	4ml x 6	0.0050	
4(4)/2	1% MeOH in EtOAc	5ml x 2	0.0130	Yellow solid
4(4)/3	1% MeOH in EtOAc	5ml x 31	0.2310	Gummy syrup
		T O T A L :	0.249	

Isoquercitrin : Fr. 4(4)/2 furnished yellow solid (13 mg) identified as isoquercitrin by comparison with the authentic sample (NMR, TLC).

Ethylated sugars : Fr. 4(4)/3 (231 mg) was gummy syrup, identified as ethylated sugar by NMR and IR. It was acetylated with acetic anhydride in pyridine. Acetylated product (240 mg) on TLC showed major two spots (40% EtOAc in pet.ether). It was chromatographed on chromatotron.

TABLE - 34 CHROMATOGRAM - 19

Material 0.056 g

Adsorbent SiO_2 gel

Thickness of rotor 1 mm

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
1.	8% EtOAc in pet.ether	10ml x 6	0.006	
2.	16% EtOAc in pet.ether	10ml x 7	0.0195	Gummy solid.
3.	16% EtOAc in pet.ether	10ml x 1	0.0016	
4.	16% EtOAc in pet.ether	10ml x 4	0.0122	Gummy solid
5.	16% EtOAc in pet.ether	50ml x 1	0.0080	

T O T A L :

Ethyl- ∝ -D-glucoside tetraacetate : Fr. 2 (19.5 mg) was gummy solid, GC showed 99.43 % purity (3% SE-30, 180°). Identified as Ethyl- ∝ -D-glucoside tetraacetate by comparison with the authentic sample (NMR, IR, GC).

Ethyl- β -D-glucoside tetraacetate : Fraction 4 (12.2 mg) furnished gummy solid, GC showed 100% purity (3% SE-30, 180°). Identified as Ethyl- β -D-glucoside tetraacetate by comparison with the authentic compound (NMR, IR, GC, TLC).

Estimation of total sugar and other compounds in the butanol extract.

Butanol extract (12 g) was acetylated with acetic anhydride (12 ml) and pyridine (12 ml) at room temperature (14 hrs), furnished a dark brown gummy solid (13.5 g). Crude product was distilled using small fractionating column (5 cm) and pooled into two fractions. First fraction (B.P. 120-25°/100 mm) yielded colourless oil (2.36 g), GC showed 95% purity (3% SE-30, 100°). Identified as Triacetin by comparison with the authentic sample (NMR, IR, CO-GC etc.).Second fraction furnished (at 210-250°/1mm) light

yellow gummy liquid (7.3g). Acetate of glucose, ethyl glucoside ($\alpha + \beta$) and meso-inositol in 2nd fraction were identified by GC (co-injection with the authentic compounds, column 10% DCQF -1 at 190°) and TLC (EtOAc:MeOH: $CHCl_3$ :: 5:2:3). Percentage of each compounds in the butanol extract was claculated by GC analysis of total distillate.

Aqueous extract

Aq. extract (8.8 g) was acetylated with acetic anhydride (10 ml) and pyridine (10 ml) as usual, furnished 4.67 g (53.06%) dark yellow viscous liquid. TLC of the extract showed it to be a complex mixture of compound with a wide range of polarity (40% EtOAc in pet.ether). It was chromatographed on SiO_2 gel.

TABLE - 35 CHROMATOGRAM - 20

Material 3.264 g

Adsorbent 75 g SiO_2 gel/IIb

Column dimension 1.8 cm x 38 cm

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
1.	5% EtOAc in pet.ether	500ml x 9	0.1694	
2.	10% EtOAc in pet.ether	250ml x 2	0.1649	
3.	10% EtOAc in pet.ether	250ml x 2	0.0914	Gummy solid
4.	10% EtOAc in pet.ether	250ml x 2	1.0738	Gummy liquid
5.	15% EtOAc in pet.ether	250ml x 2	0.2221	White solid separated
6.	15% EtOAc in pet.ether	250ml x 2	0.1413	
7.	15% EtOAc in pet.ether	250ml x 2	0.0819	
8.	30% EtOAc in pet.ether	250ml x 1	0.2556	
9.	30% EtOAc in pet.ether	500ml x 1	0.4600	
10.	EtOAc	250ml x 2	0.4611	
11.	20% MeOH in EtOAc	500ml x 2	0.1008	
12.	MeOH	500ml	0.0341	
		T O T A L :	3.2564	

Ethyl- β -D-glucoside tetraacetate : Fraction 3 (91 mg) was gummy solid, identified as ethyl- β -D-glucoside tetraacetate by comparison with the authentic sample (NMR, IR and CO-GC).

Fraction 4 (1.07 g) furnished gummy liquid, on TLC it showed presence of at least four compounds. It was further chromatographed on SiO_2 gel.

TABLE - 36 CHROMATOGRAM - 21

Material	1.07 g
Adsorbent	70 g SiO_2 gel/IIb
Column dimension	1.8 cm x 38 cm

Fr. No.	Eluent	Vol. of Fraction	Wt. of Fraction (g)	Remarks
4(1)	10% EtOAc in pet.ether	250ml x 7	0.0194	
4(2)	12% EtOAc in pet.ether	100ml x 3	0.0591	
4(3)	12% EtOAc in pet.ether	100ml x 2	0.1356	
4(4)	12% EtOAc in pet.ether	100ml x 2	0.2200	
4(5)	15% EtOAc in pet.ether	100ml x 2	0.2553	Gummy liquid
4(6)	15% EtOAc in pet.ether	100ml x 2	0.1714	
4(7)	15% EtOAc in pet.ether	100ml x 2	0.0189	
4(8)	20% EtOAc in pet.ether	250ml x 2	0.0580	Cryst. comp. separa.
4(9)	EtOAc	500ml x 1	0.0256	
		T O T A L :	0.9633	

Fraction 4(5) (255 mg) yielded gummy liquid, showed two spots on TLC (40% EtOAc in pet.ether). It was further chromatographed on SiO_2 gel.

TABLE - 37 CHROMATOGRAM - 22

Material	240 mg
Adsorbent	12 g SiO_2 gel/IIb.
Column dimension	1.1 cm x 35 cm

Fr. No.	Eluent	Vol. of Fraction.	Wt. of Fraction (g)	Remarks
4(5)/1	8% EtOAc in pet.ether	100ml x 3	0.0103	
4(5)/2	8% EtOAc in pet.ether	50ml x 2	0.0072	
4(5)/3	8% EtOAc in pet.ether	50ml x 1	0.0532	Gummy liquid
4(5)/4	10% EtOAc in pet.ether	50ml x 1	0.0150	
4(5)/5	10% EtOAc in pet.ether	50ml x 1	0.0170	
4(5)/6	10% EtOAc in pet.ether	50ml x 1	0.0350	
4(5)/7	10% EtOAc in pet.ether	50ml x 1	0.0050	
4(5)/8	10% EtOAc in pet.ether	50ml x 1	0.0060	
4(5)/9	EtOAc	100ml	0.0300	
		T O T A L :	0.1787	

Glucose pentaacetate : Fr. 4(5)/3 (53 mg) was a gummy liquid, TLC single spot (40% EtOAc in pet.ether, Rf 0.52), GC analysis showed 91% purity (10% DCQF -1, 210°C). It was identified as a mixture of α- and β -D-glucose pentaacetate by direct comparison with the authentic compounds (NMR, IR, TLC, CO-GC).

meso-Inositol hexaacetate : Fr. 5 (222 mg) was a white solid. It was further purified by crystallisation from chloroform furnished white crystalline solid (100 mg, m.p. 211-213°), TLC single spot. Identified as meso-Inositol hexa-acetate by direct comparison with authentic compound (NMR, IR, GC and TLC).

Estimation of compounds (as acetate) present in butanol extract.

Crude acetylated product of butanol extract (1.4 g) was broadly chromatographed on SiO_2 gel/IIb (60g) column (dimension 2.1 cm x 29 cm) and eluted with 40% EtOAc in pet.ether (1 l) till all above isolated compounds came out of the column (TLC). Removal of solvent from eluent on rotavapor furnished light yellow liquid (749 mg). It was subjected to GC analysis (10%

DCQF-1 , 190°) and peaks were identified by co-injection with the authentic samples. Washing of column with methanol furnished brown fluffy solid (60 mg) which on TLC showed absence of above isolated compounds.

Insect Bioassay

Feeding deterrence was evaluated against tobacco armyworms (S. litura) and E. vijintioctopunctata. IIIrd instar larvae ware taken for the study from routine cultures of our laboratory. Two types of tests were performed against the armyworms.

(1) Choice test

(2) No choice test.

In choice test leaf disks of 2 cm^2 area were cut from castor leaves and dipped for 2 seconds in a solvent dissolved solution of test extracts. Five such disks were arranged along with another only solvent treated (controls) five disks concentrally in a petridish. Bottom of the petridish was provided with moist filter paper to avoid any leaf dessication. Ten larvae were placed in the centre of the petridish. After 24 hrs the disks were removed and measured on a graph paper (photodevice) to calculate percentage mean consumption of both types of disks.

Alternativel no-choice test was carried out in a similar fashion by deleting the untreated disks and using a separate untreated controls for comparison. In this experiments percentage feeding on treated material was recorded after 24 hrs and the consumed area expressed as percentage of the consumed area of controls.

In both the experiments % deterrence of more than 50% was recorded as positive effect, otherwise the test extracts were considered as totally inactive.

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ABSTRACT

The chemistry, medicinal properties and pest control activities of Azadirachta indica (neem) is discussed. Aqueous ethanolic (70%) extract of neem leaves was fractionated into pet.ether, dichloroethane (DCE), ethyl acetate and n-butanol solubles and aq. portion. All were tested for anti-feedant activity separately. Alkanes, esters, sterols, fatty acids, α-tocopherol and phytol were isolated and identified from the pet.ether soluble portion of leaves. Most active antifeedant fraction was obtained from DCE extract. EtOAc soluble portion furnished mainly flavonoids as (1) quercetin (2) quercitrin (3) isoquercitrin (4) rutin. Glycerol, glucose, ethylated glucose, meso-inositol were isolated and identified from n-butanol and aqueous fractions Estimation of all the isolated compounds from neem leaves extract either by GC, HPLC or column chromatography is also discussed.