CHAPTER - III

Observations

III. OBSERVATIONS

A. Observations on the Solvent Extraction and TLC Separation :

1) Fruit Extracts of Sapindus lurifolius :

The dried fruits after removal of seeds were powdered and the extraction was carried in five different solvents, petroleum ether, benzene, ethyl acetate, chloroform and ethanol, respectively and the five extracts obtained, their percentage composition and weight in grams is shown in the following table (No.2)

Table No.2

The percentage composition of compounds extracted in petroleum ehter, benzene ethyl acetate, chloroform and ethanol of Sapindus laurifolius.

No.	Material	Solvent	Weight in Gm.	Percentage
1.	Shade dried leaves	-	400.000	-
2.	Petroleum ether extract	Petroleum ether	5.616	1.40
3.	Benzene extract	Benzene	6.190	1.55
4.	Ethyl acetate extract	Ethyl acetate	4.135	1.03
5.	Chloroform Extract	Chloroform	2.112	0.53
6.	Ethanol extract	Ethanol	60.220	15.05
7.	Residue after extraction	-	321.747	80.44

2) TLC Observations :

TLC was carried out on the plates coated with silica gel G using chloroform : ethanol (85 : 15) solvent system. The five extracts (petroleum ether, benzene, ethyl acetate, chloroform and ethanol) were simultaneously spotted on the plates and chromatographs were developed by exposing the plates in Iodine chamber. The results of this experiment have been recorded in the Fig.No.3 and the components observed in each extract and their Rf values have been summarized in Table No.3.

Table No.3

The components and their Rf values of petroleum ether, benzene, ethyl acetate chloroform and ethanol extracts of the fruits of Sapindus laurifolius at 25.5°C.

No.	Extract	Components	Distance travelled		Rf Values
			Components cm	Solvent cm	
I	Petrolium ether extracts	PE_1	3	10.5	0.285
		PE_2	6	10.5	0.57
		PE_3	9	10.5	0.86
II	Benzene extract	BE_1	0.9	10.5	0.085
		BE_2	1.7	10.5	0.161
		BE_3	2.8	10.5	0.266
III	Ethyl acetate extract	EA_1	1.5	10.5	0.142
		EA_2	4.5	10.5	0.428
		EA_3	6	10.5	0.571
		EA_4	9.7	10.5	0.923
IV	Chloroform extract	CE_1	0.5	6.2	0.080
		CE_2	2	6.2	0.322
V	Ethanol extract	EE_1	0.6	6.2	0.096
		EE_2	2.1	6.2	0.338
		EE_3	3.9	6.2	0.629

TLC OF SOLVENT EXTRACTS OF THE FRUITS OF

Sapindus Laurifolius .

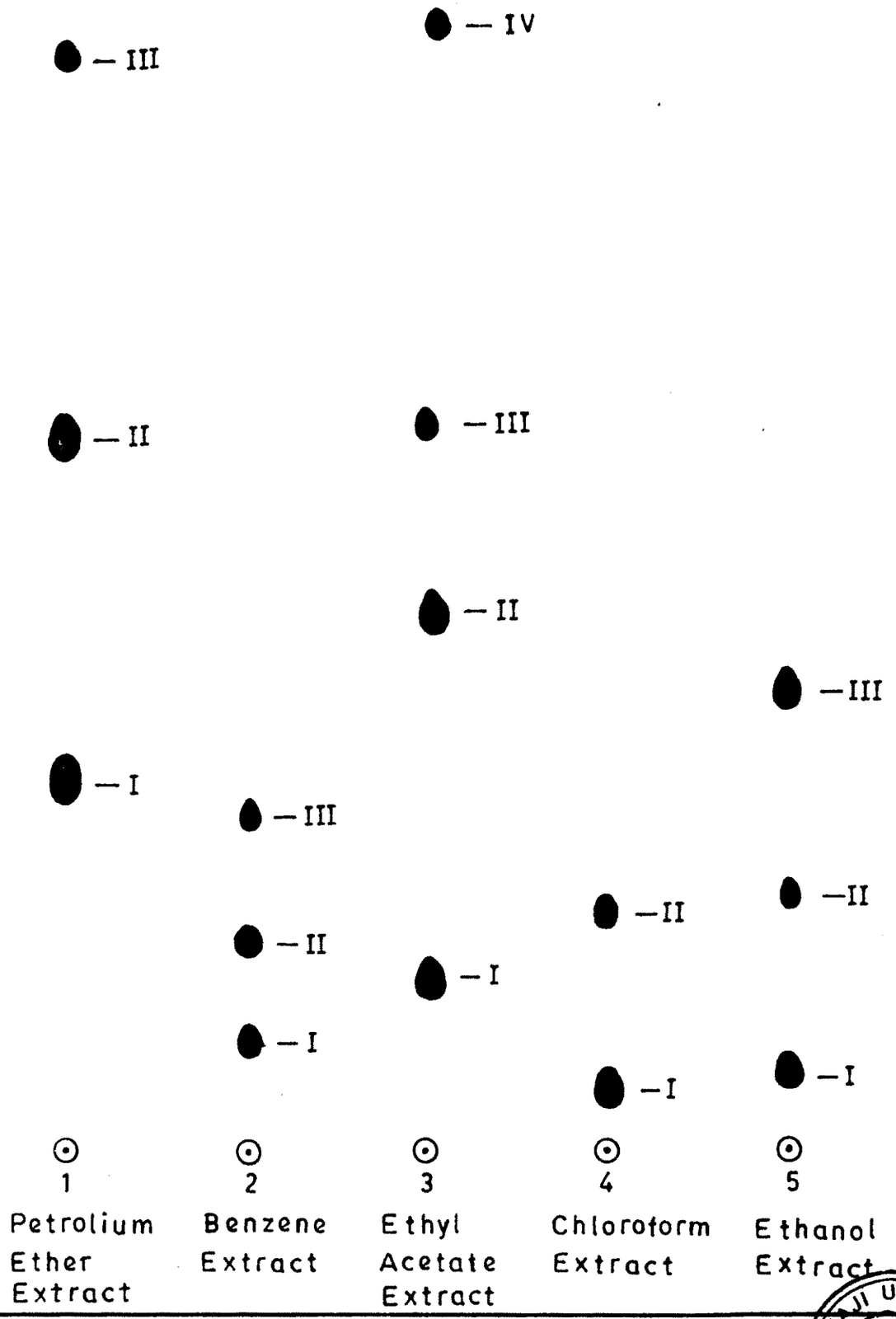


FIG. NO. 3

3. Melting Point :

i) The melting point of petroleum ether extract = $93^{o}C$

ii) The melting point of benzene extract = $96^{o}C$

iii) The melting point of ethyl acetate extract = $98^{o}C$

iv) The melting point of chloroform extract = $101^{o}C$

v) The melting point of ethanol extract = $99^{o}C$

B. Observations on the Phytochemical Analysis :

1) UV Spectral Observation :

UV spectra of the five extracts (petroleum ether, benzene, ethyl acetate, chloroform and ethanol) showed intense peaks at λ_{max} in between 220 nm to 290 nm. Fig.Nos. 4, 5, 6, 7 and 8 show the UV spectra for the petroleum ether, benzene, ethyl acetate, chloroform and ethanol soluble components respectively.

The UV spectrum of the petroleum ether extract showed two intense peaks at λ240 and λ260. The benzene extract contained only one peak at 290 nm. The ethyl acetate extract has λmaxima at 245 nm. The chloroform extract showed intense peak at λ280 nm. whereas the ethanol soluble components showed two peaks, the first maximum peak value at λ220 nm and second at λ285 nm.

2) NMR Spectral Observations :

The NMR spectra of the five extracts after dissolving in CCl_4 and with TMS as internal standard scanned on Perkin-Elmer 90 MH_z, R 32 spectrophotometer showed the following chemical shift expressed in δ ppm. Fig.No.9 shows the NMR spectrum of the petroleum ether extract of the fruits of S.laurifolius

U V SPECTRUM OF PETROLEUM ETHER EXTRACT OF FRUITS OF

Sapindus Laurifolius (Vahl).

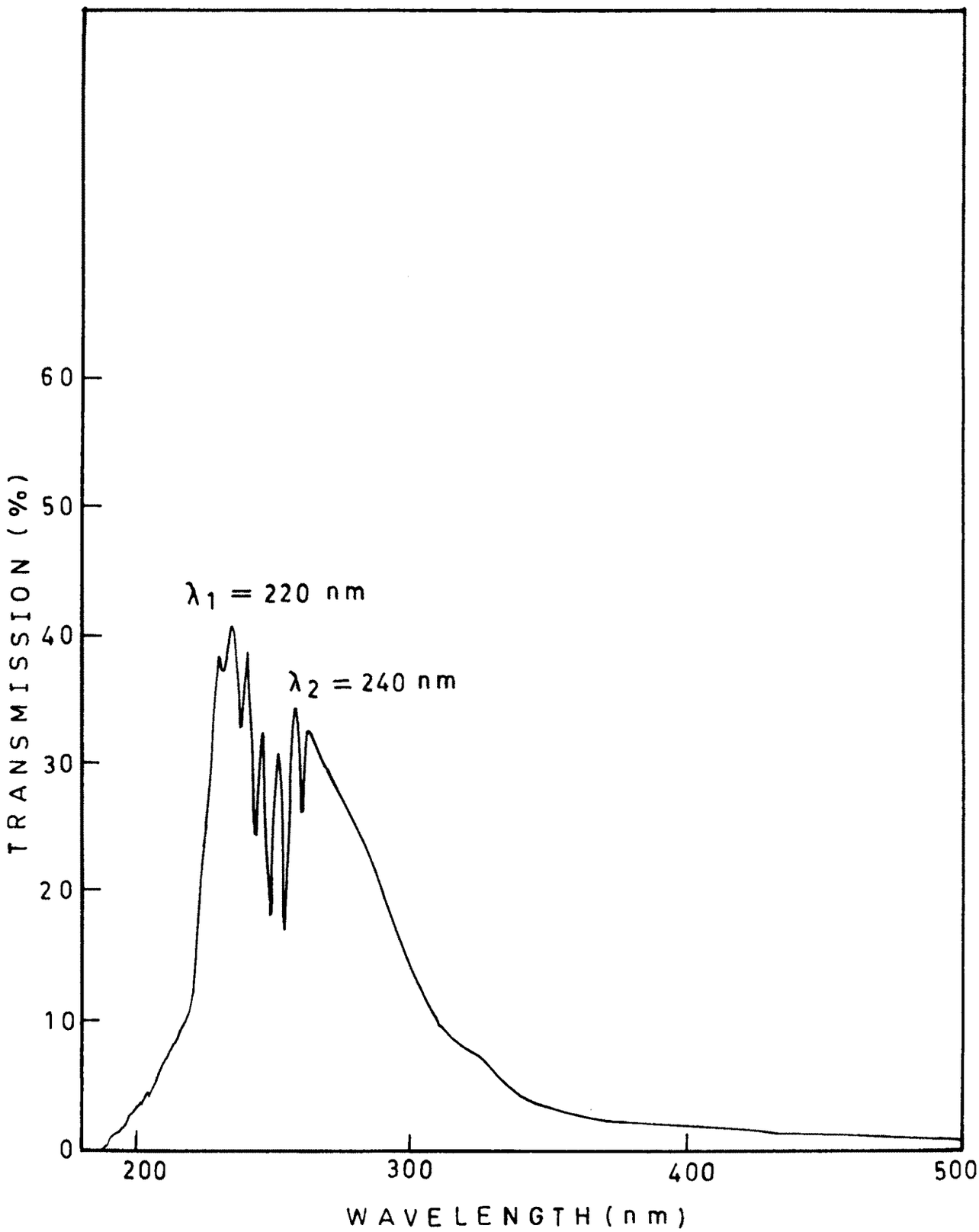


FIG.NO. 4

U V SPECTRUM OF BENZENE EXTRACT OF FRUITS OF

Sapindus Laurifolius (Vahl).

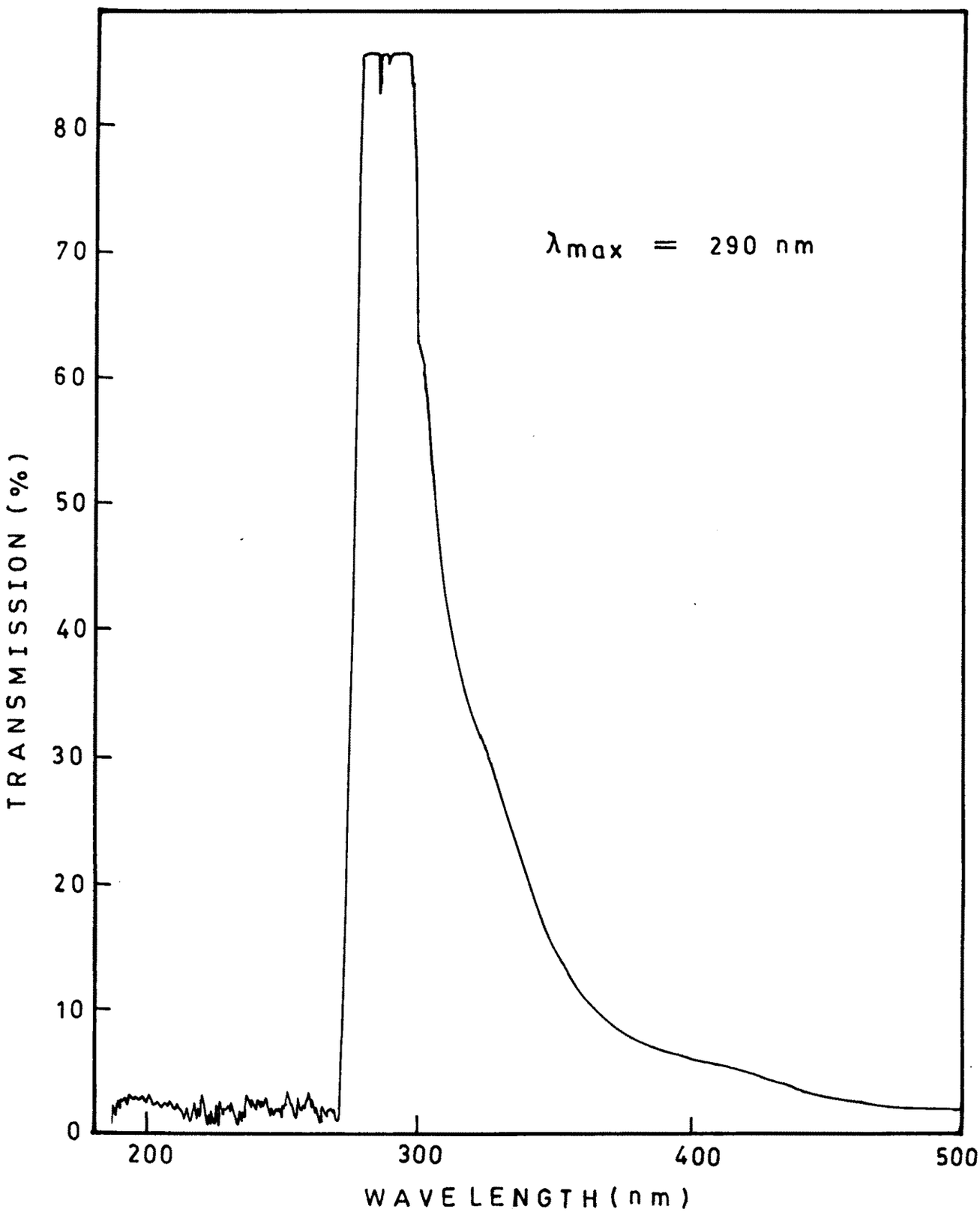


FIG. NO. 5

U V SPECTRUM OF ETHYL ACETATE EXTRACT OF FRUITS OF

Sapindus Laurifolius (Vahl).

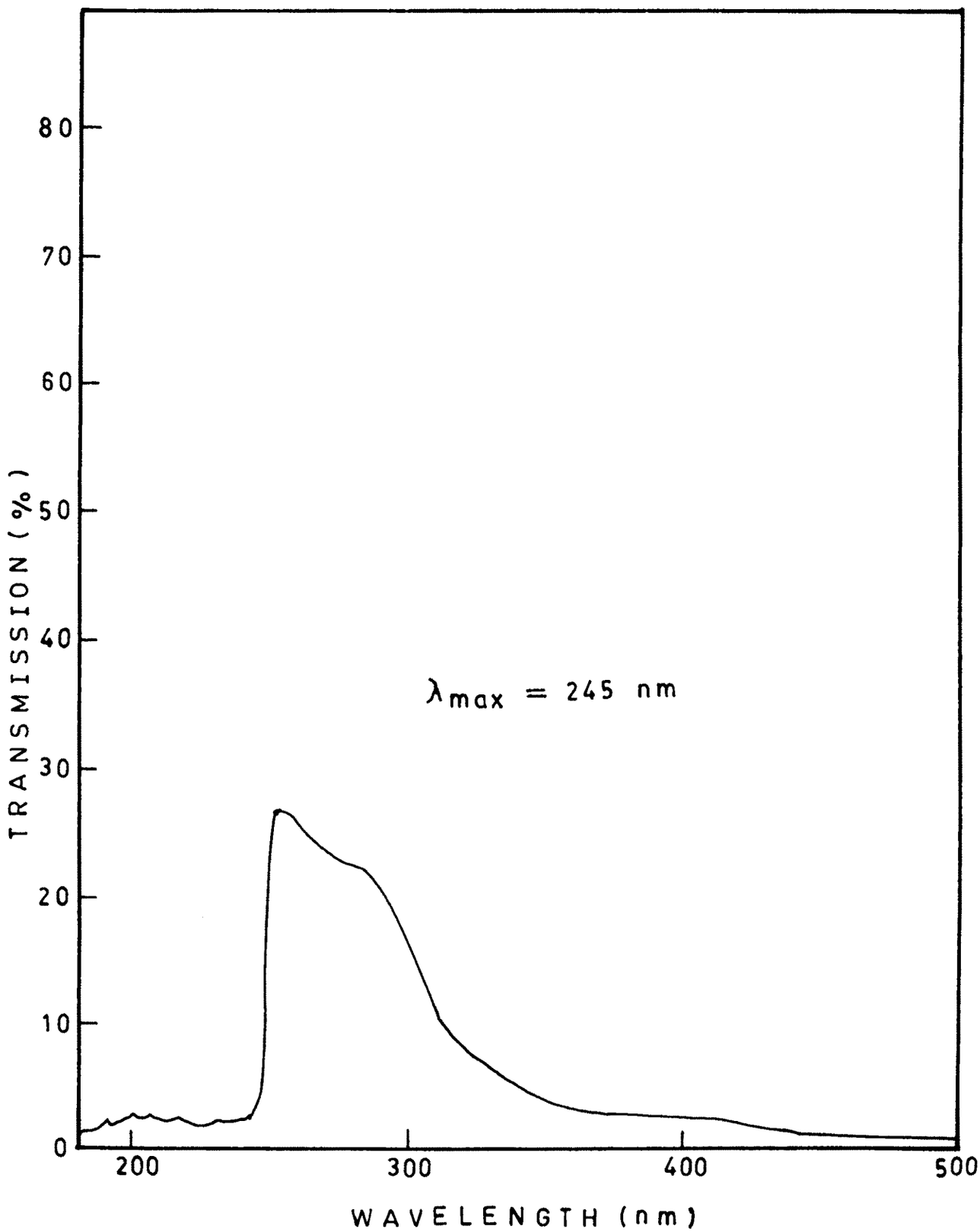


FIG. NO. 6

U V SPECTRUM OF CHLOROFORM EXTRACT OF FRUITS OF

Sapindus Laurifolius (Vahl).

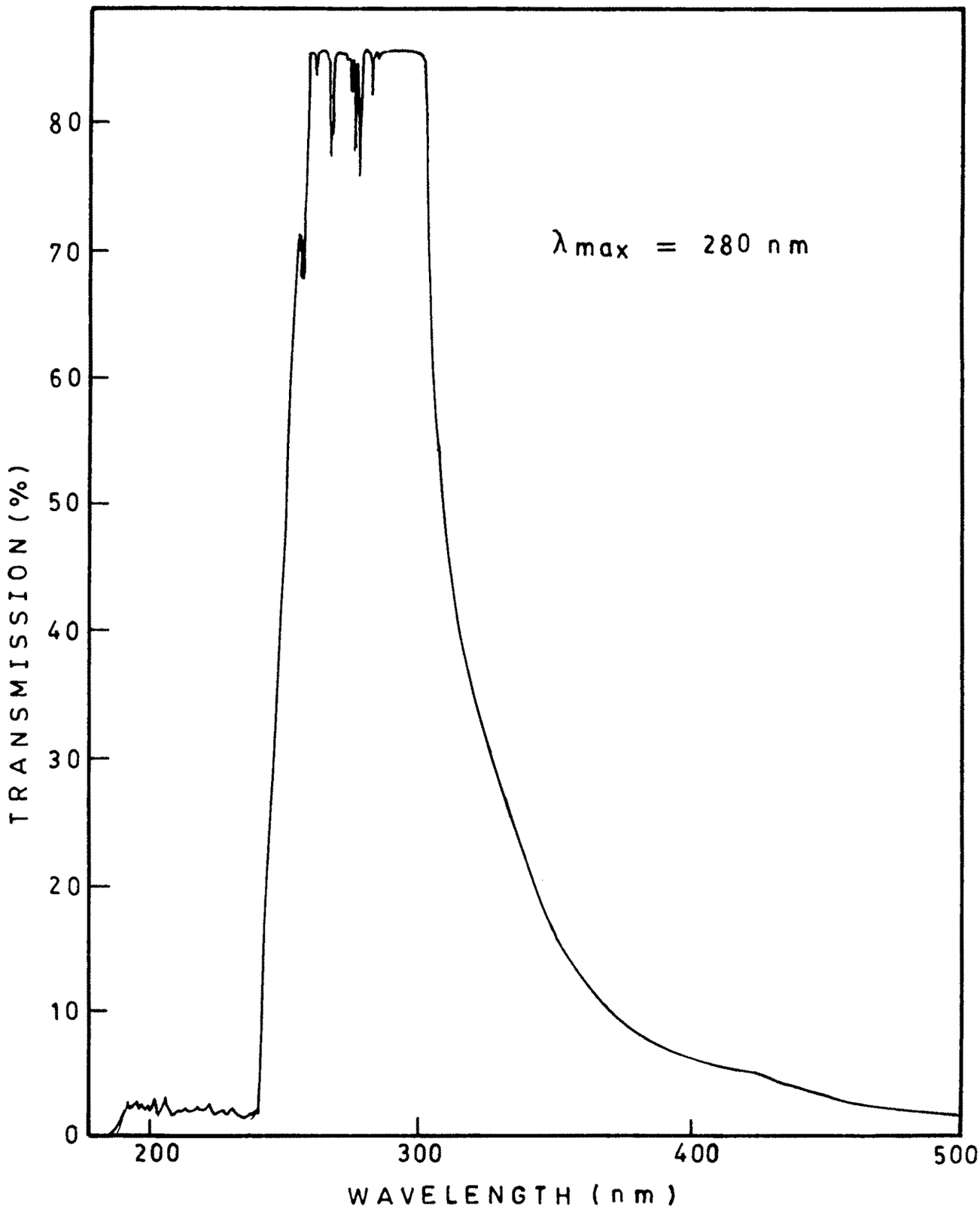


FIG NO. 7

UV SPECTRUM OF ETHANOL EXTRACT OF FRUITS OF

Sapindus Laurifolius (Vahl).

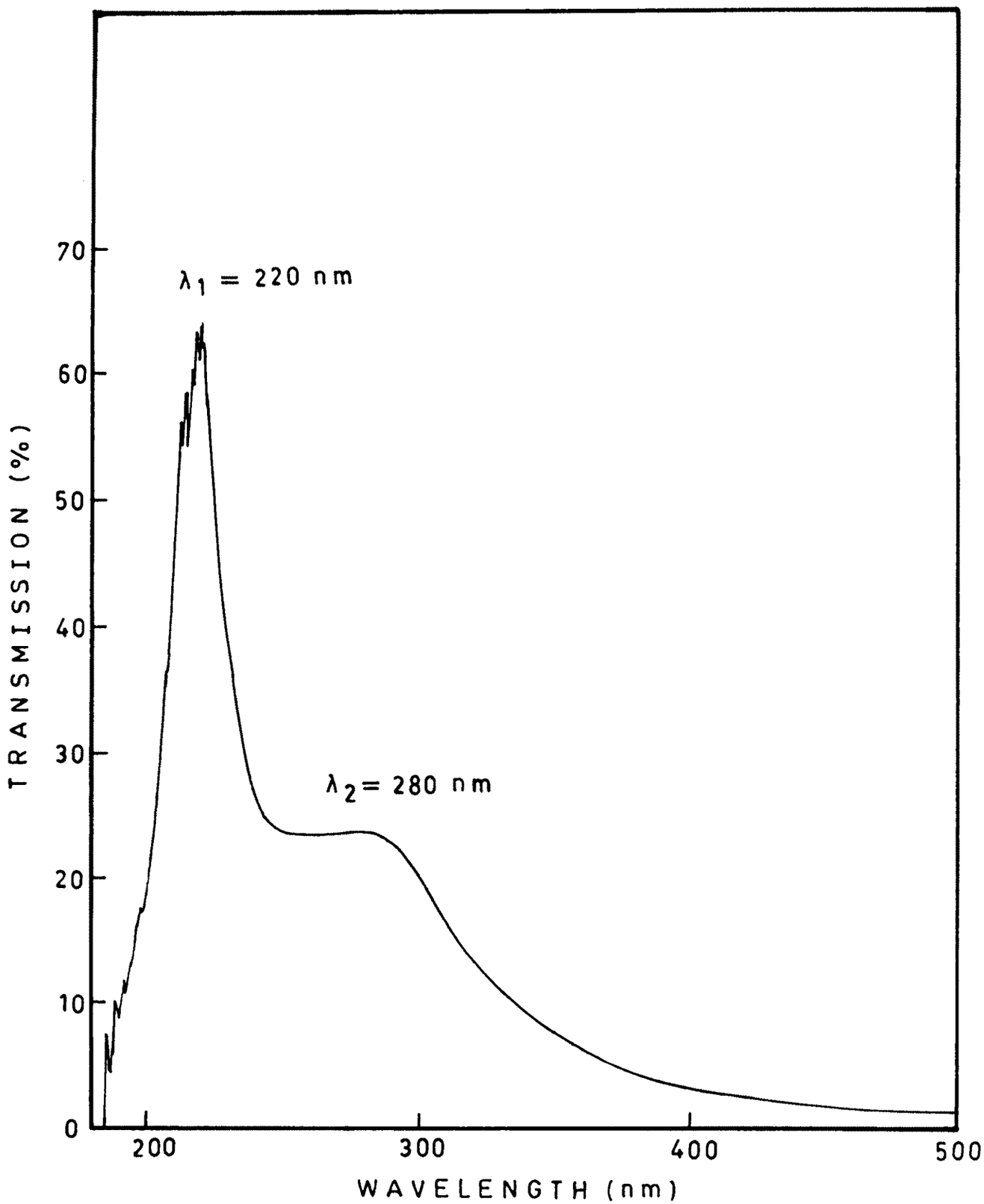


FIG. NO. 8

shows unresolved multiplet at 0.9, a singlet at 1.25, and unresolved multiplets at δ 1.6, δ 3.6, and δ 5.3. Fig. No.10 shows the NMR spectrum of the benzene extract showing singlets at δ 1.25 and δ 4.7 and unresolved multiplets at δ 0.9, δ 1.6, δ 3.6 and δ 5.3.

Fig.No. 11 shows the NMR spectrum of ethyl acetate soluble components in CCl_4. The values of the chemical shift expressed in δ ppm show a singlet at δ 1.25 and three multiplets at δ 0.9, 1.6 and 5.2. The component extracted in chloroform and dissolved in CCl_4 shows the five peaks on the NMR spectrum, a singlet at 1.25 and unresolved multiplets at δ 0.9, δ 1.6, δ 4.2 and δ 5.3.(Fig No.12). Fig.No.13 shows the NMR spectrum of ethanol extracted and TFA soluble components of the S. laurifolius. The values of the chemical shift expressed in δ ppm are given. The spectrum shows five peaks, all multiplets (unresolved) at 0.8, 1.2, 2.0, 3.9 and 5.0 δ ppm. Out of these peaks at 0.8, 1.2 and 3.9 are prominent, whereas a peak at 5.0 is small.

3) IR Spectral Observations :

IR spectra were scanned on PERKIN-ELMER-783 U.K. in nujol.

1. The petroleum ether extract (Fig.No.14) shows streching bands at 1745 cm^{-1} and 2910 cm^{-1}.
2. The benzene extract (Fig.No.15) shows three stretching bands at 1710 cm^{-1}, 1740 cm^{-1} and at 2850 cm^{-1}.
3. The ethyl acetate extract (Fig.No.16) shows four stretching bands at 1710 cm^{-1}, 1740 cm^{-1}, 2850 cm^{-1} and 3400 cm^{-1}.

NMR SPECTRUM OF PETROLEUM ETHER EXTRACT OF FRUITS OF Sapindus Laurifolius(Vahl) .

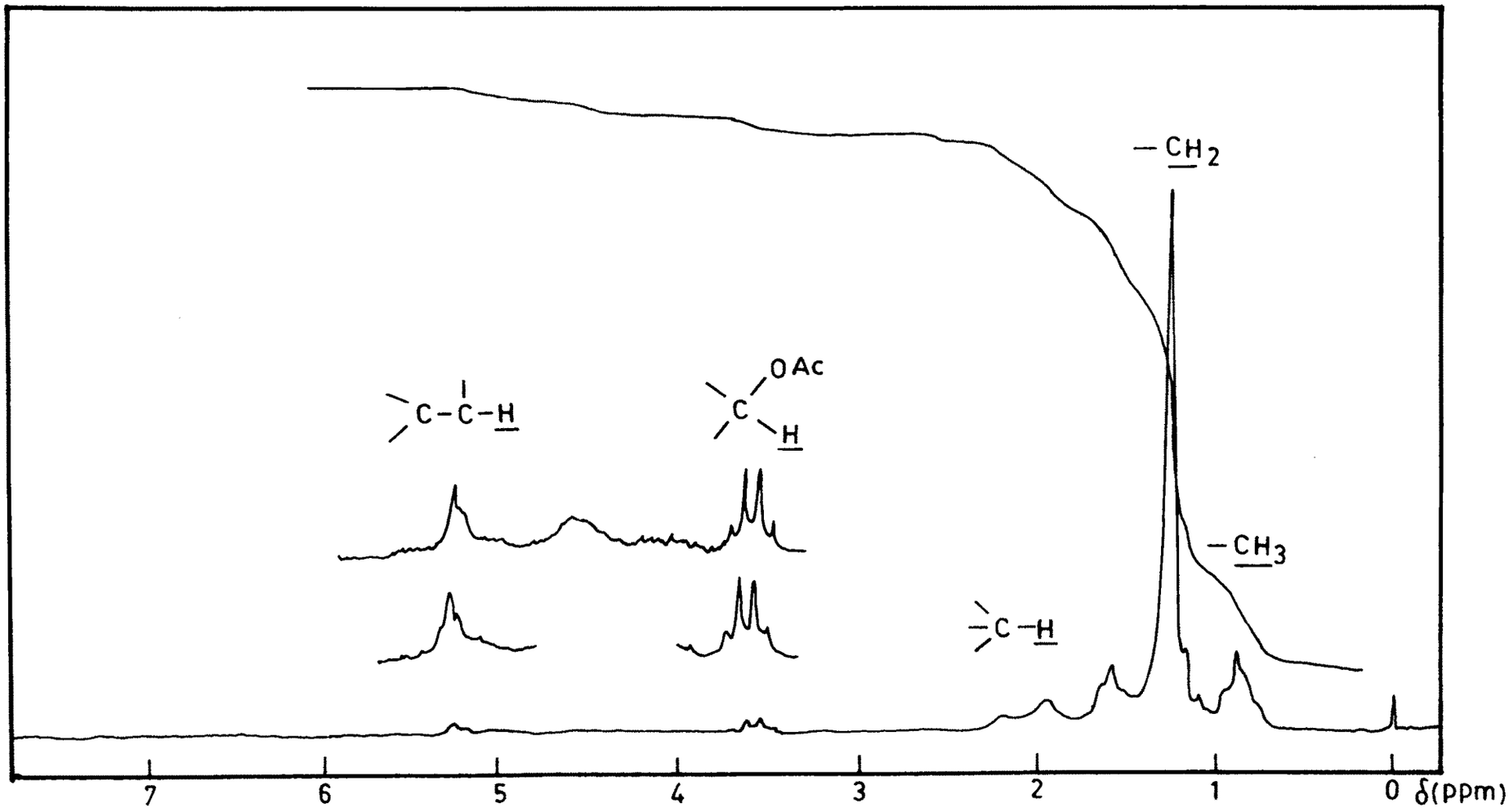


FIG. NO. 9

NMR SPECTRUM OF BENZENE EXTRACT OF FRUITS OF Sapindus Laurifolius (Vahl)

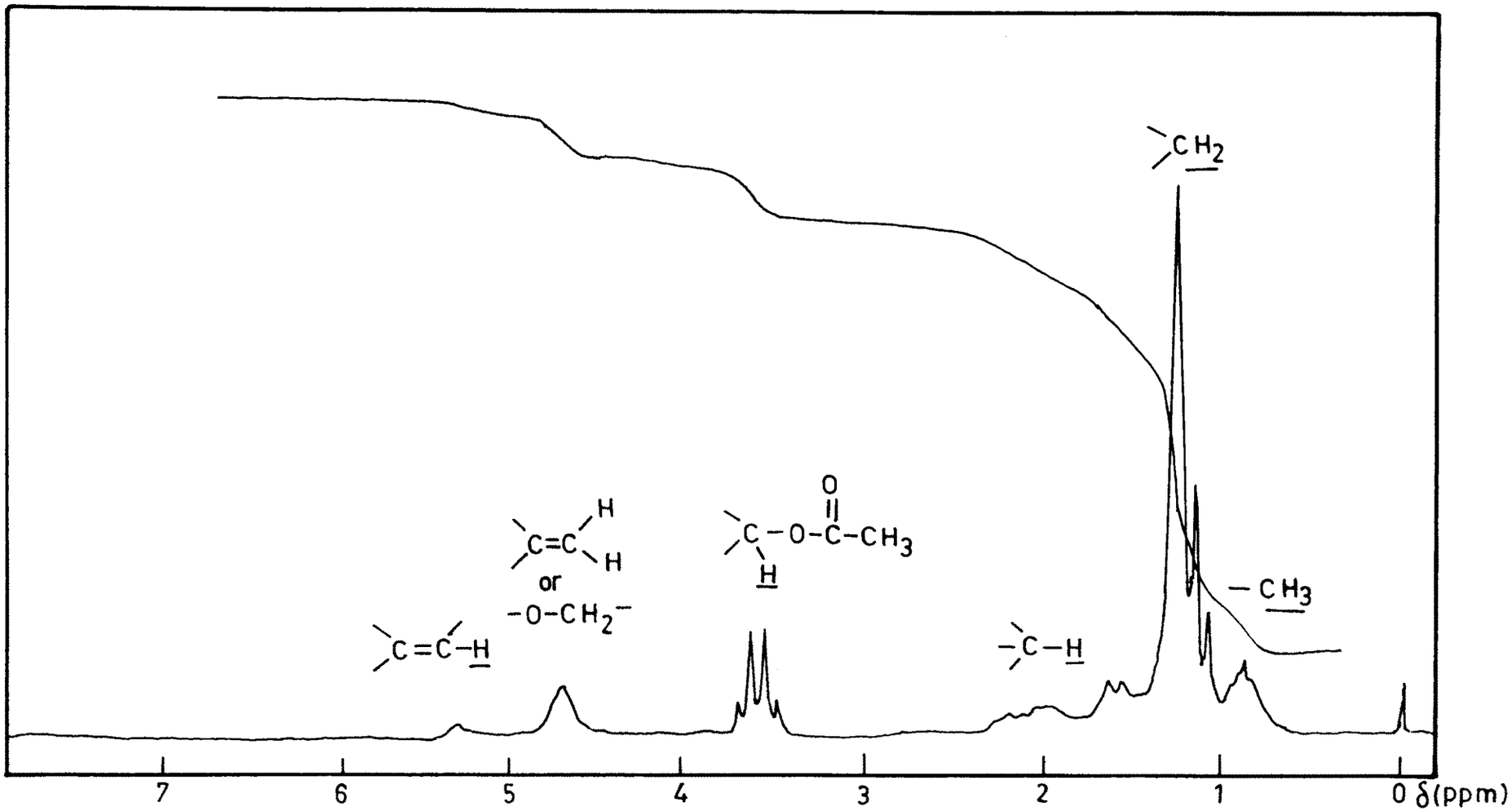


FIG. NO. 10

NMR SPECTRUM OF ETHYL ACETATE EXTRACT OF FRUITS OF Sapindus Laurifolius (Vahl).

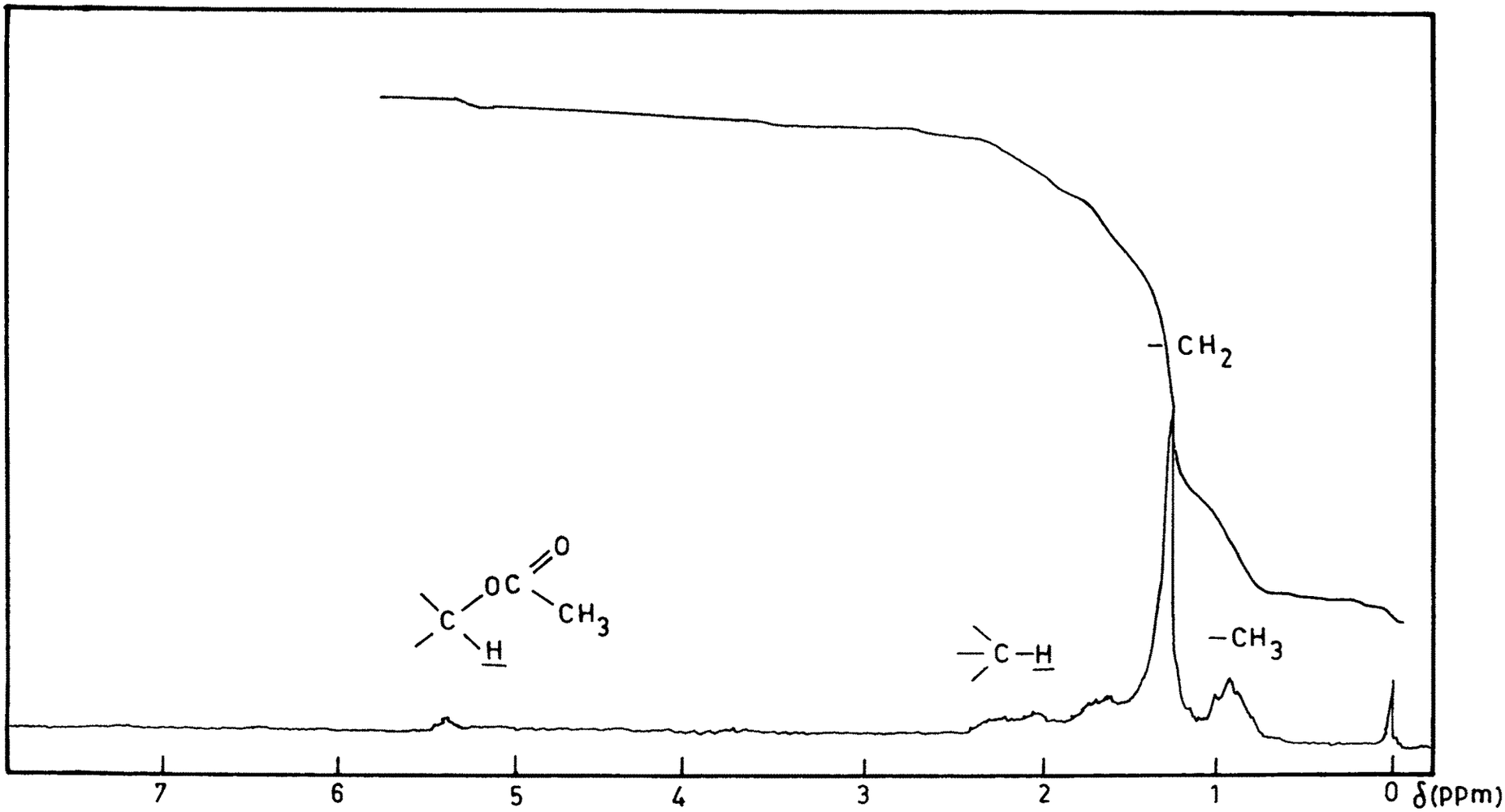


FIG. NO. 11

NMR SPECTRUM OF CHLOROFORM EXTRACT OF FRUITS OF Sapindus Laurifolius(Vahl).

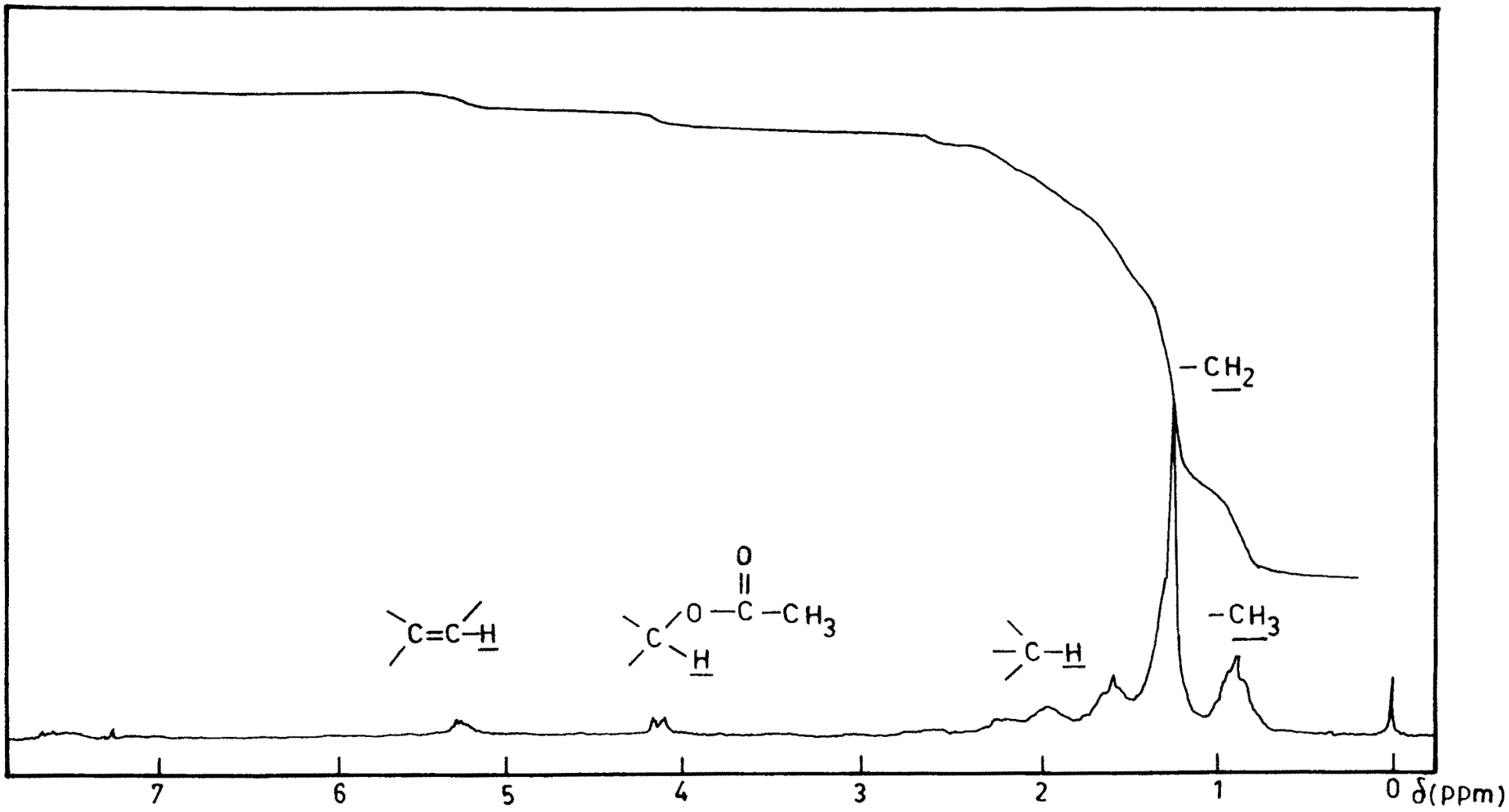


FIG. NO. 12

NMR SPECTRUM OF ETHANOL EXTRACT OF FRUITS OF Sapindus Laurifolius (Vahl)

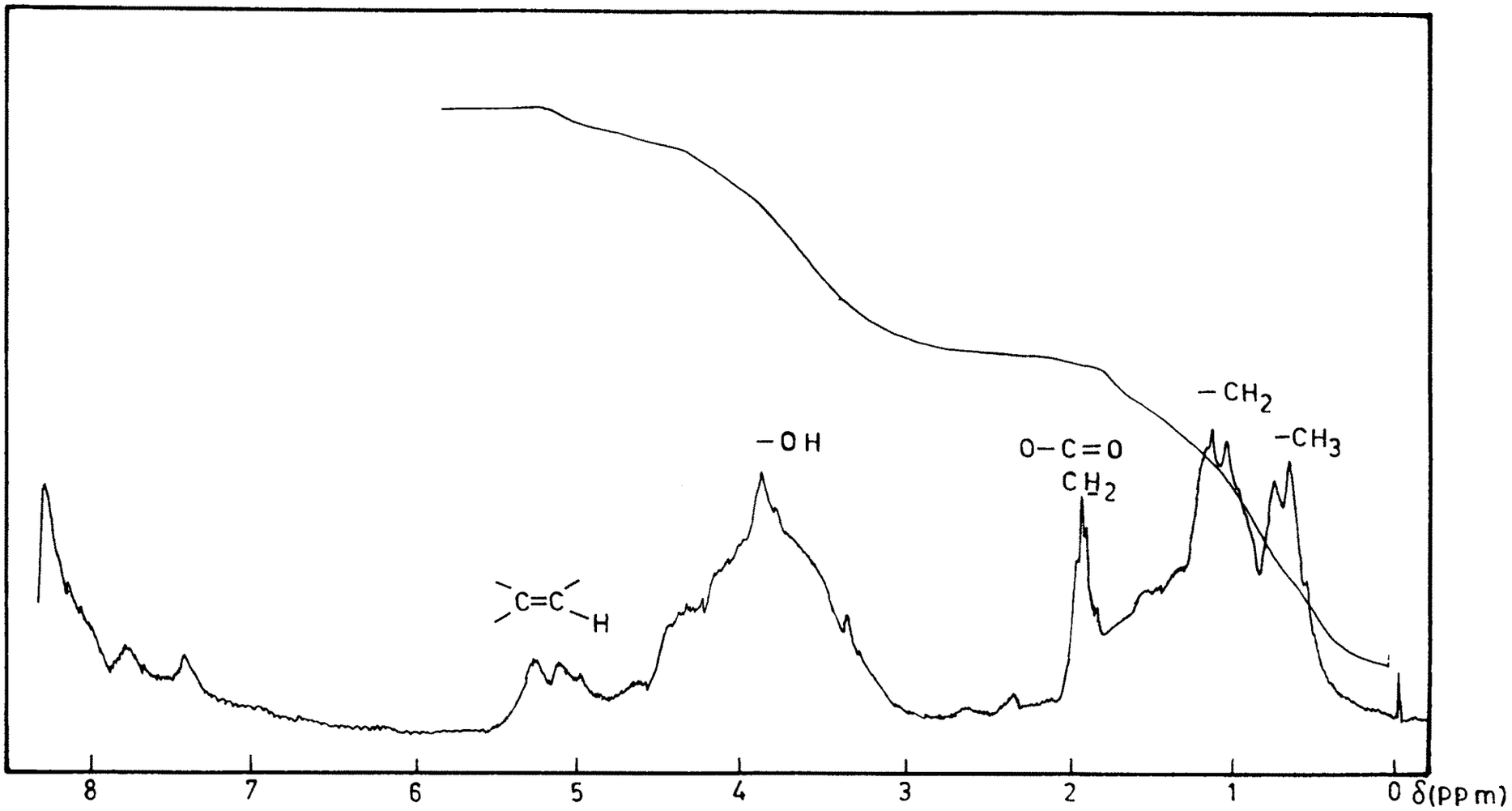
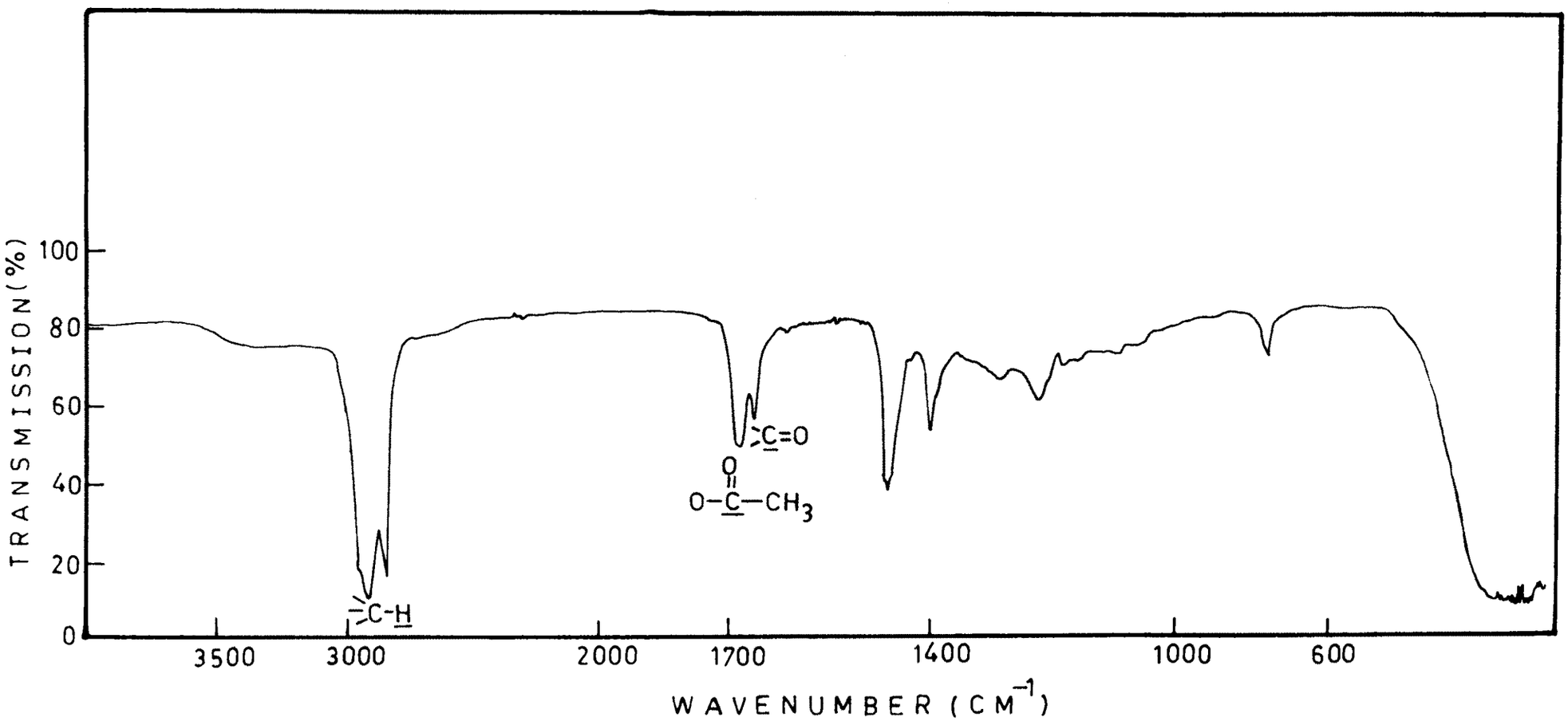


FIG. NO. 13

I R SPECTRUM OF PETROLEUM ETHER EXTRACT OF FRUITS OF Sapindus Laurifolius(Vahl).



FIG·NO·14

I R SPECTRUM OF BENZENE EXTRACT OF FRUITS OF Sapindus Laurifolius (Vahl).

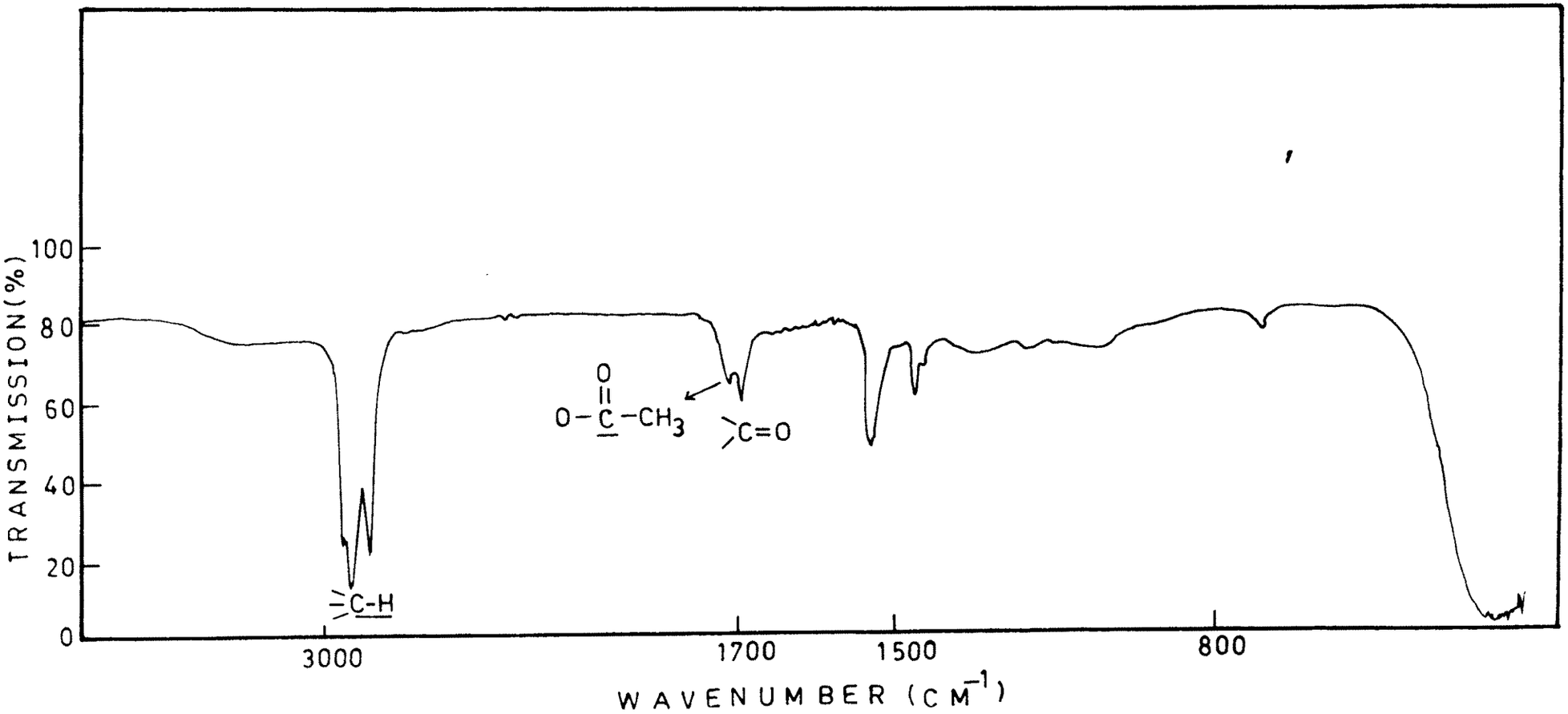


FIG. NO. 15

I R SPECTRUM OF ETHYL ACETATE EXTRACT OF FRUITS OF Sapindus Laurifolius (Vahl).

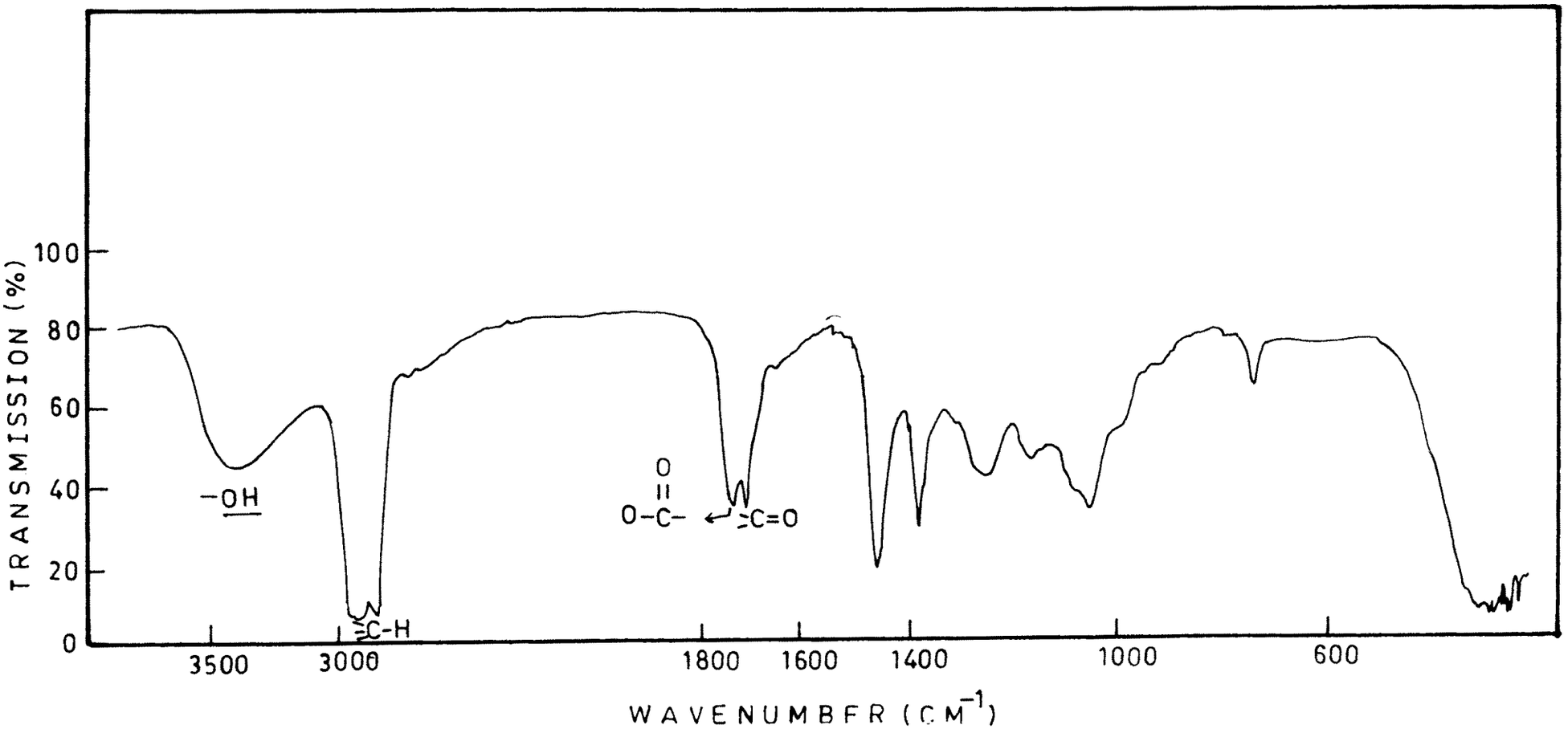


FIG. NO. 16

4. The chloroform extract (Fig.No.17) shows a broad band at 3400-3500 cm^{-1} and three more bands at 2900 cm^{-1}, 1750 cm^{-1} and 1710 cm^{-1}.

5. The ethanol extract (Fig.No.18) shows stretching broad band at 3300-3400 cm^{-1} and two bands between 2800-2900 cm^{-1} and 1700-1730 cm^{-1}.

4) Atomic Absorption Spectrophotometric Observations :

The petroleum ether, benzene, ethyl acetate, chloroform and ethanol extracts were analysed for inorgnic ions on Atomic Absorption Spectrophotometer (Perkins-Elmer, 3030 USA). The readings and the results are recorded in the Table No.4.

C. Observations on water quality analysis

The experimental procedures :

The fishes were kept in rectangular glass acquaria (25 liter capacity) and were acclimatized to the laboratory conditions for a week in the conditions similar to their natural habitat. The feeding was stopped before 24 hrs. of the commencement of the experiment. The fishes were not fed during the test period. For each experiment 10-20 fishes were transferred into a well cleaned test container. From the observations it seemed that there was no effect on the fish mortality on addition of the PE, BE, EAE and CE of the fruit extracts of S. laurifolius.

TABLE NO. 4

Inorganic ions in the petroleum ether, benzene, ethyl acetate chloroform and ethanol extracts of the fruits of Sapindus laurifolius

No.	Extracts	Inorganic ions*				
		Cu^{++}	Fe^{++}	Mg^{++}	Ca^{++}	Zn^{++}
1.	Petroleum ether extract (PE)					
	Readings	0.04	0.06	0.21	0.72	0.25
		0.04	0.06	0.22	0.72	0.25
		0.04	0.06	0.21	0.72	0.25
	Mean	0.04	0.06	0.21	0.72	0.25
2.	Benzene Extract (BE)					
	Readings	0.04	0.02	0.32	1.63	0.22
		0.04	0.02	0.32	1.63	0.23
		0.04	0.02	0.33	1.63	0.22
	Mean	0.04	0.02	0.32	1.63	0.22
3.	Ethyl acetate Extract (EAE)					
	Readings	0.04	0.04	0.23	2.34	0.31
		0.04	0.04	0.22	2.34	0.31
		0.03	0.04	0.23	2.34	0.31
	Mean	0.04	0.04	0.23	2.34	0.31
4.	Chloroform Extract (CE)					
	Readings	0.05	0.00	0.21	1.09	0.73
		0.05	0.00	0.21	1.08	0.73
		0.05	0.00	0.21	1.09	0.74
	Mean	0.05	0.00	0.21	1.09	0.73
5.	Ethanol Extract (EE)					
	Readings	0.03	0.01	0.23	0.69	0.16
		0.03	0.01	0.23	0.69	0.16
		0.03	0.02	0.23	0.68	0.16
	Mean	0.03	0.01	0.23	0.69	0.16

* N.B. All figures of inorganic ions are expressed in ppm.

I R SPECTRUM OF CHLOROFORM EXTRACT OF FRUITS OF Sapindus Laurifolius (Vahl).

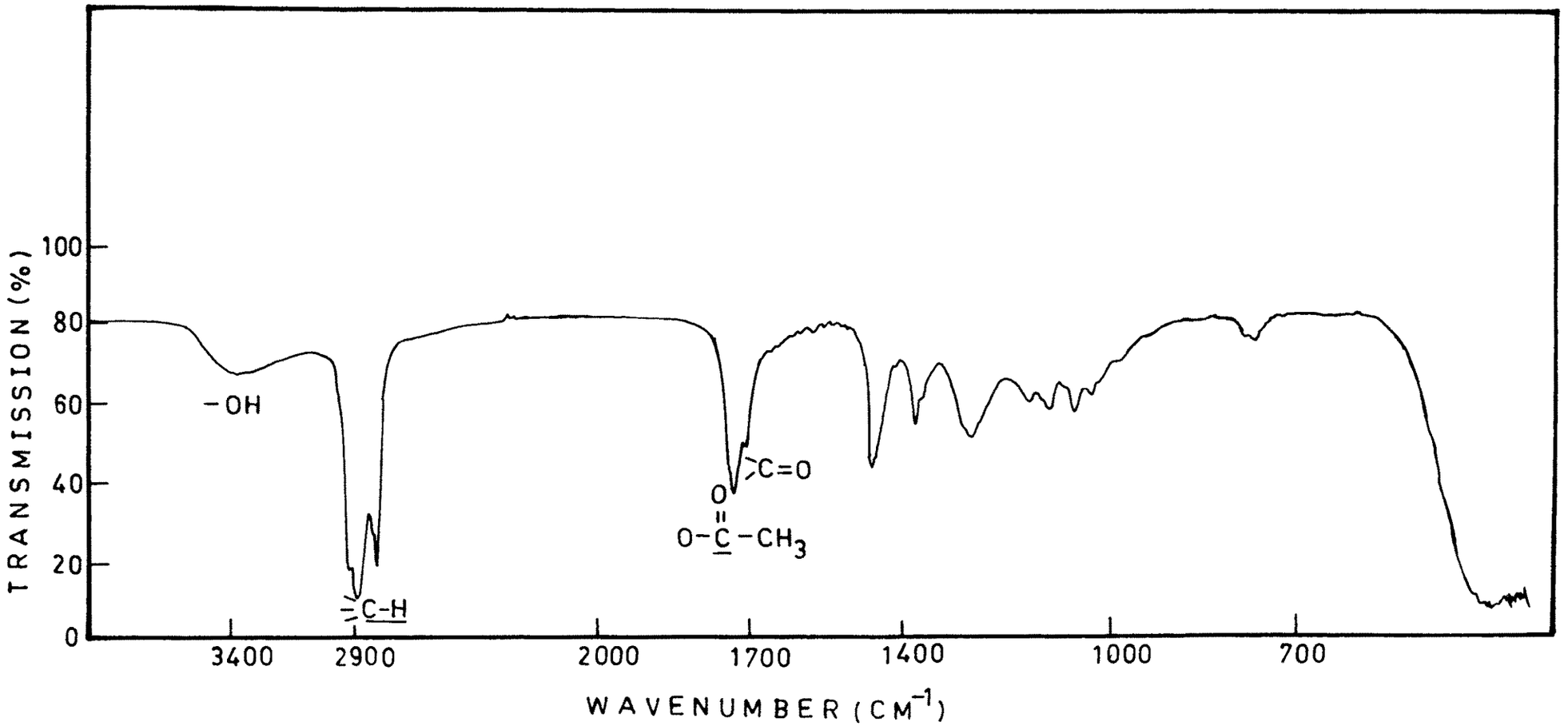


FIG. NO. 17

I R SPECTRUM OF ETHANOL EXTRACT OF FRUITS OF Sapindus Laurifolius (Vahl) .

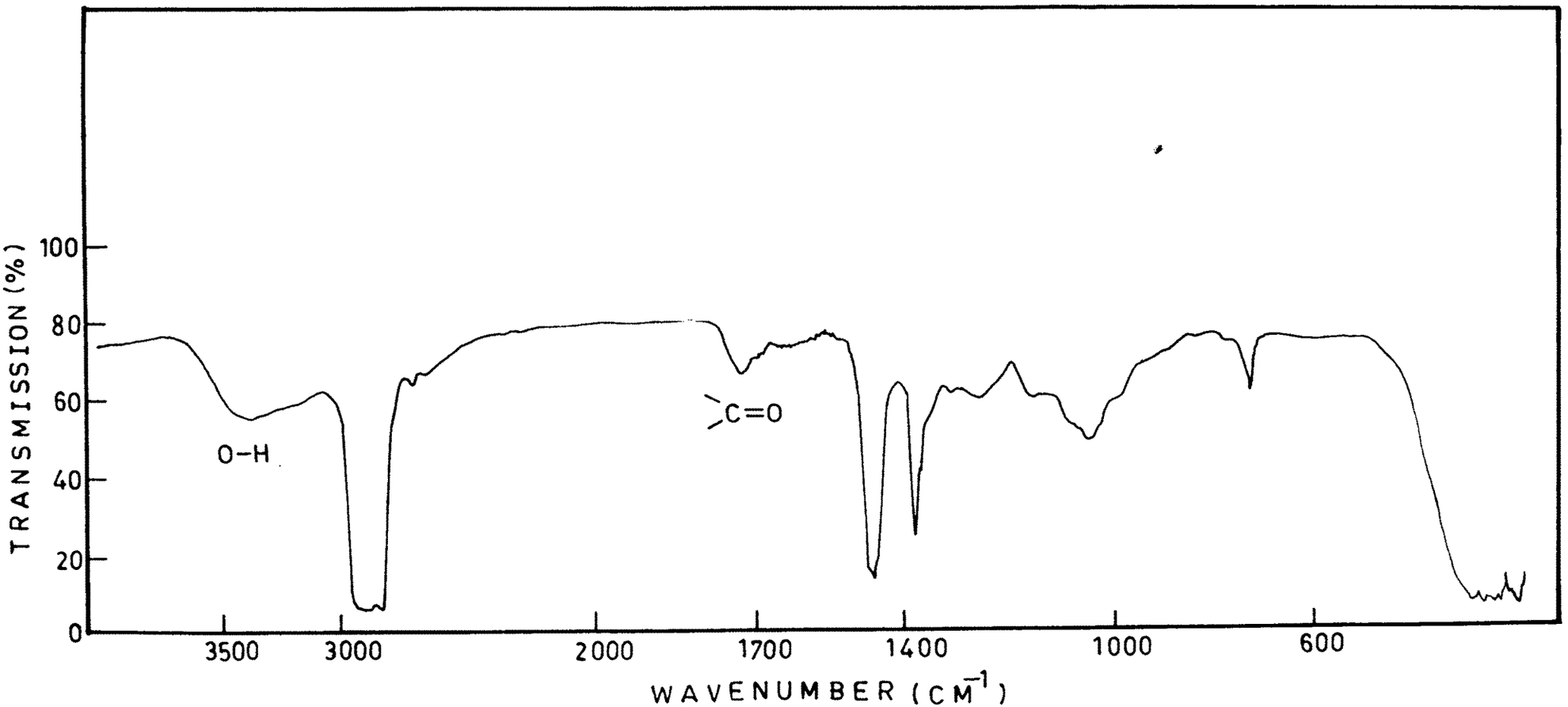


FIG. NO. 18

The ethanol extract (EE) was water soluble hence various concentrations were prepared by using chlorine-free tap water and desired concentrations (200, 225, 250, 275, 300 and 400 ppm) were made. The temperature, pH, DO, hardness of the experimental water were measured before the addition of fruit extracts and the same procedures were continued at regular interval for a period of 96 hrs.

Table No.5 provides the temperature range and Table No.6 gives the pH values of the experimental water. DO values have been tabulated in Table No.7 whereas Table No.8 gives the values of the total hardness.

D. Observations on the physiological responses to phytotoxin of S. laurifolius.

1. Mortality effects : LC_0, LC_{50} and LC_{100} .

The ethanol extract of the fruits of S. laurifolius showed lethal effects at various concentrations and at different time intervals. Table No.9 gives the LC_0, LC_{50} and LC_{100} values at different hrs. for the ethanol extract, which shows the percent mortality at different hours during the toxicological experiment.

2) Behavioural changes :

The behavioural responses of the fished during experimental procedures using ethanol extract (EE) were recorded as per visual observations. The responses to the other extracts PE, BE, EAE and CE did not change the behaviour of Tilapia mossambica. The reactions to EE were remarkable. The reaction time was different with different concentrations selected for the experimental procedures. At lower concentrations (200, 225 and 250 ppm) the behavioural changes

TABLE NO. 5

The temperatures of the experimental water during S. laurifolius fruit toxin intoxication to the fish, T. mossambica

No.	Concentration of fruit toxin* (ppm)	Duration of intoxication to fish in Hrs.								
		1	3	6	9	12	24	48	72	96
1.	Normal H_2O	25 ± 2	25±2	25±2	25±2	25+2	25±2	25±2	25+2	25±2
2.	200	26±2	26±2	26±2	26±2	26±2	26±2	25±2	26±2	26±2
3.	225	27±2	26±2	27±2	27±2	27±2	26±2	27±2	26±2	26±2
4.	250	25±2	25±2	25±2	25±2	26±2	25±2	25±2	25±2	25±2
5.	275	26±2	26±2	26±2	26±2	26±2	25±2	25±2	26±2	25±2
6.	300	26±2	26±2	26±2	26±2	26±2	27±2	26±2	27±2	26±2
7.	350	27±2	27±2	27±2	27±2	27±2	26±2	27±2	26±2	26±2
8.	400	25±2	25±2	25±2	25±2	25±2	24±2	25±2	24±2	25±2

* N.B. : 1) The ethanol extracted fruit powder was used as fruit toxin during the experiments.

2) The figures for temperature are in degree centigrade (^{o}C)

TABLE NO. 6

The pH values of the experimental water during S. laurifolius fruit toxin intoxication to the fish T. mossambica

No.	Concentration of fruit toxin* (ppm)	Duration of intoxication to fish in Hrs.								
		1	3	6	9	12	24	48	72	96
1.	Normal H_2O	7.0 ±0.03	7.0 ±0.03	7.0 ±0.03	7.0 ±0.03	7.0 ±0.03	7.0 ±0.04	7.0 ±0.03	7.0 ±0.03	7.0 ±0.03
2.	200	7.0 ±0.05	7.0 ±0.05	7.0 ±0.05	7.0 ±0.04	7.0 ±0.05	7.0 ±0.05	7.0 ±0.05	7.0 ±0.05	7.0 ±0.05
3.	225	7.1 ±0.01	7.1 ±0.01	7.1 ±0.01	7.1 ±0.01	7.1 ±0.01	7.0 ±0.06	7.0 ±0.06	7.0 ±0.06	7.1 ±0.06
4.	250	6.9 ±0.08	6.9 ±0.08	6.9 ±0.08	6.9 ±0.08	6.9 ±0.08	6.9 ±0.07	6.9 ±0.08	6.9 ±0.09	6.9 ±0.08
5.	275	7.2 ±0.03	7.2 ±0.03	7.2 ±0.03	7.2 ±0.03	7.2 ±0.03	7.1 ±0.05	7.1 ±0.05	7.1 ±0.05	7.1 ±0.05
6.	300	7.1 ±0.01	7.1 ±0.01	7.1 ±0.01	7.1 ±0.01	7.1 ±0.01	7.1 ±0.01	7.0 ±0.03	7.0 ±0.03	7.1 ±0.01
7.	350	7.3 ±0.02	7.3 ±0.02	7.3 ±0.02	7.3 ±0.02	7.3 ±0.02	7.2 ±0.03	7.2 ±0.03	7.2 ±0.08	7.2 ±0.08
8.	400	7.1 ±0.05	7.1 ±0.05	7.1 ±0.05	7.1 ±0.05	7.1 ±0.06	7.1 ±0.09	7.1 ±0.09	7.1 ±0.09	7.1 ±0.08

* N.B. : 1) The ethanol extracted fruit powder was used as fruit toxin during the experiments.

2) The figures under duration of intoxication are pH values.

TABLE NO. 7

The dissolved oxygen values of the experimental water during S. laurifolius fruit toxin intoxication to the fish T. mossambica

No.	Concentration of fruit toxin* (ppm)	Duration of intoxication to fish in Hrs.								
		1	3	6	9	12	24	48	72	96
1.	Normal H_2O	6.1 ±0.3	6.1 ±0.3	6.1 ±0.3	6.1 ±0.3	6.1 ±0.3	6.0 ±0.7	6.0 ±0.7	6.0 ±0.8	6.1 ±0.5
2.	200	5.8 ±0.8	5.8 ±0.8	5.8 ±0.8	5.8 ±0.8	5.8 ±0.8	5.7 ±0.6	5.8 ±0.7	5.8 ±0.2	5.8 ±0.4
3.	225	5.5 ±0.4	5.5 ±0.4	5.5 ±0.4	5.5 ±0.4	5.5 ±0.4	5.6 ± 0.2	5.6 ±0.2	5.6 ±0.3	5.5 ±0.2
4.	250	5.3 ±0.9	5.3 ±0.9	5.3 ±0.9	5.3 ±0.9	5.3 ±0.9	5.4 ±0.1	5.4 ±0.0	5.4 ±0.2	5.3 ±0.8
5.	275	6.5 ±0.2	6.5 ±0.2	6.5 ±0.2	6.5 ±0.2	6.5 ±0.2	6.6 ±0.1	6.6 ±0.2	6.4 ±0.2	6.5 ±0.5
6.	300	5.6 ±0.6	5.6 ±0.6	5.6 ±0.6	5.6 ±0.6	5.6 ±0.6	5.7 ±0.2	5.7 ±0.2	5.6 ±0.4	5.8 ±0.2
7.	350	6.0 ±0.9	6.0 ±0.8	6.0 ±0.9	6.0 ±0.0	6.0 ±0.2	6.1 ±0.3	6.1 ±0.3	6.1 ±0.2	6.1 ±0.5
8.	400	6.3 ±0.2	6.3 ±0.2	6.3 ±0.2	6.3 ±0.2	6.3 ±0.2	6.5 ±0.1	6.5 ±0.1	6.4 ±0.4	6.3 ±0.2

* N.B. : 1) The ethanol extracted fruit powder was used as fruit toxin during the experiments.

2) The D.O. values are expressed in mg/lit.

TABLE NO. 8

The values of hardness of the experimental water during S. laurifolius fruit toxin intoxication to the fish T. mossambica

No.	Concentration of fruit toxin* (ppm)	Duration of intoxication to fish in Hrs.								
		1	3	6	9	12	24	48	72	96
1.	Normal H_2O	31.24 ±1.1	31.24 ±1.2	31.24 ±1.2	31.24 ±1.2	31.24 ±1.2	30.50 ±2.0	30.50 ±2.0	30.50 ±2.0	31.25 ±1.5
2.	200	36.30 ±2.1	36.30 ±2.1	36.30 ±2.1	36.30 ±2.1	36.30 ±2.1	35.81 ±2.1	35.81 ±3.0	34.50 ±6.0	35.75 ±1.0
3.	225	41.61 ±2.2	41.81 ±3.0	41.20 ±2.2	42.10 ±1.3	41.12 ±1.4	40.30 ±2.1	41.43 ±2.4	41.20 ±2.1	41.20 ±2.1
4.	250	40.84 ±2.1	30.84 ±3.2	40.81 ±2.8	40.56 ±2.0	40.13 ±1.6	41.32 ±1.2	42.20 ±1.2	43.20 ±2.4	40.42 ±2.0
5.	275	48.52 ±3.1	48.30 ±2.0	48.30 ±2.0	47.30 ±1.6	47.00 ±2.6	46.50 ±1.0	47.1 ±3.0	46.9 ±2.2	46.2 ±1.2
6.	300	53.75 ±1.3	53.25 ±2.1	53.65 ±4.3	52.92 ±3.2	53.72 ±1.3	53.32 ±1.4	52.26 ±2.1	53.15 ±0.9	51.85 ±0.8
7.	350	48.27 ±2.7	48.90 ±1.2	48.65 ±2.1	48.20 ±0.8	47.90 ±1.7	47.55 ±2.7	47.13 ±3.1	48.32 ±2.1	47.22 ±1.6
8.	400	50.34 ±3.1	50.84 ±2.4	50.77 ±2.3	51.12 ±1.2	51.0 ±2.8	50.34 ±3.2	50.22 ±2.3	50.47 ±2.2	50.38 ±1.0

* N.B. : 1) The ethanol extracted fruit powder was used as fruit toxin during the experiments.

2) The hardness values are expressed in Mg $CaCO_3$/lit.

TABLE NO. 9

The values of per cent mortality of the fish T. mossambica during intoxication due to the fruit toxin of S. laurifolius

No.	Concentration of fruit toxin* (ppm)	Duration of intoxication to fish in Hrs.								
		1	3	6	9	12	24	48	72	96
1.	Normal H_2O	0	0	0	0	0	0	0	0	0
2.	200	0	5	10	25	40	50	80	65	80
3.	225	0	8	15	28	50	60	72	85	100
4.	250	0	10	20	35	50	70	80	95	100
5.	275	8	15	30	50	75	85	100	100	100
6.	300	15	35	50	85	100	100	100	100	100
7.	350	25	50	75	100	100	100	100	100	100
8.	400									

* N.B. : 1) The ethanol extracted fruit powder was used as fruit toxin during the experiments.

2) The figures under duration of intoxication to fish are per cent mortality.

were not noticeable but at higher concentrations (275, 300 and 400 ppm) behaviour of fish was changed. At higher concentrations, the first visible effects occurred after 10-60 minutes. The fish at first showed a brief period of high excitability, followed by a period of sluggishness. The opercular movements were increased along with the activity of the fish. The eye balls were bulged. The mouth remained completely open. The skin colour of the fish became completely dark. The fish bent on one side. It was also noticed that there was no response to different types of stimuli such as external movements, light, touch etc. Then after some time slowly the fish lost its equilibrium, turning on one side along their long axis and was lying horizontally. Finally the fish collapsed in upside down posture at the bottom of the acquarium.

Interestingly, fishes treated with EE (ethanol extract) secreted thick slimy mucus with blood clots, which were entangled in the gills.

3) Histopathological and Histochemical Observations :

I) Oral Cavity

a) Normal Histology of Oral Cavity :

The anterior chamber of the digestive tract of Tilapia mosambica is divided into an oral cavity and a pharynx. For the present study the earlier part is taken since the latter possesses the gill shifts. The oral (Buccal) cavity is lined with a thin stratified squamous epithelium which is supported by the connective tissue and muscular layer. The mucous membrane has several papillae, which are having lamina propria, a vascularized connective tissue core and

epithelial covering. These papillae are more or less with flat-top indicating fungiform type. Some times a few conical, shaped filiform papillae are also observed (Plate No.1, Figs.4,5,6,7,8). The histopathological, histochemical observations in normal and plant toxin treated tissues of oral cavity are photomicrographically illustrated in Plate No.1, Figs. 1 to 9.

The normal histology of oral cavity of T.mossambica has been described in detail by Harold (1987).

b) Histopathological alterations due to S.laurifolius fruit toxin :

The histological changes were clearly seen in the changes in the epithelium particularly in the thickness of the layer and in the diameter of the cells. The normal epithelium showed two types of cells in the epithelium small cuboidal cells (SC) in the outermost layer of the epithelium, scattered throughout the surface (Plate No.1, Figs.7,8) and the Large columnar cells (LC) continuously forming many layers of the stratified epithelium. At a comparative level the number of large cells were more than the small cells. The nuclei in the small cells were located at the centre whereas those of the large cells were at the base. During intoxication with low doses (200 ppm and 225 ppm) the number of small cells was found to be increased (Plate No.1, Figs.3,4). The number, the height and the size of the large cells were doubled. The staining capacity of these cells was also increased. In higher doses (400 ppm), the similar enhanced reactivity of the cells and the cell proliferation were distinctly observed (Plate No.1, Fig.6). In the normal connective tissue a few cells were randomly scattered. Their number was more towards the stratified

PLATE NO. 1, Fig. Nos. 1 to 9

(Impact of phytotoxin on mucins in the Buccal epithelium of T. mossambica)

Fig.No. 1 : A small portion of normal buccal epithelium stained with AB pH 2.5-PAS. Note large cells, stratified squamous epithelium (SEP) stained with PAS and alcianophilia towards luminal (L) side. Connective tissue (CT) show weak PAS reactivity. The lamina propria (LP) in papilla (P) was without any staining. x 240.

Fig.No. 2 : A small portion of buccal epithelium treated with 200 ppm ethanol extract of S. laurifolius fruit and stained with AB pH 2.5-PAS. Note increased staining in large cells (LC) and in connective tissue (CT). x 240.

Fig.No. 3 : The buccal epithelium treated with 350 ppm phytotoxin and stained with AB pH 2.5-PAS. Note decreased staining in large cells (LC) and in connective tissue (CT). But cell patches in the CT showed intense stain. x 240.

Fig.No. 4 : The normal buccal epithelium stained with H&E. Note large cells (LC) and secretory cells (SC) of the papilla (P). x 240.

Fig.No. 5 : The buccal epithelium treated with 250 ppm phytotoxin and stained with AB pH 1-PAS. Note increase in staining reactivity in stratified epithelium (SEP). x 240.

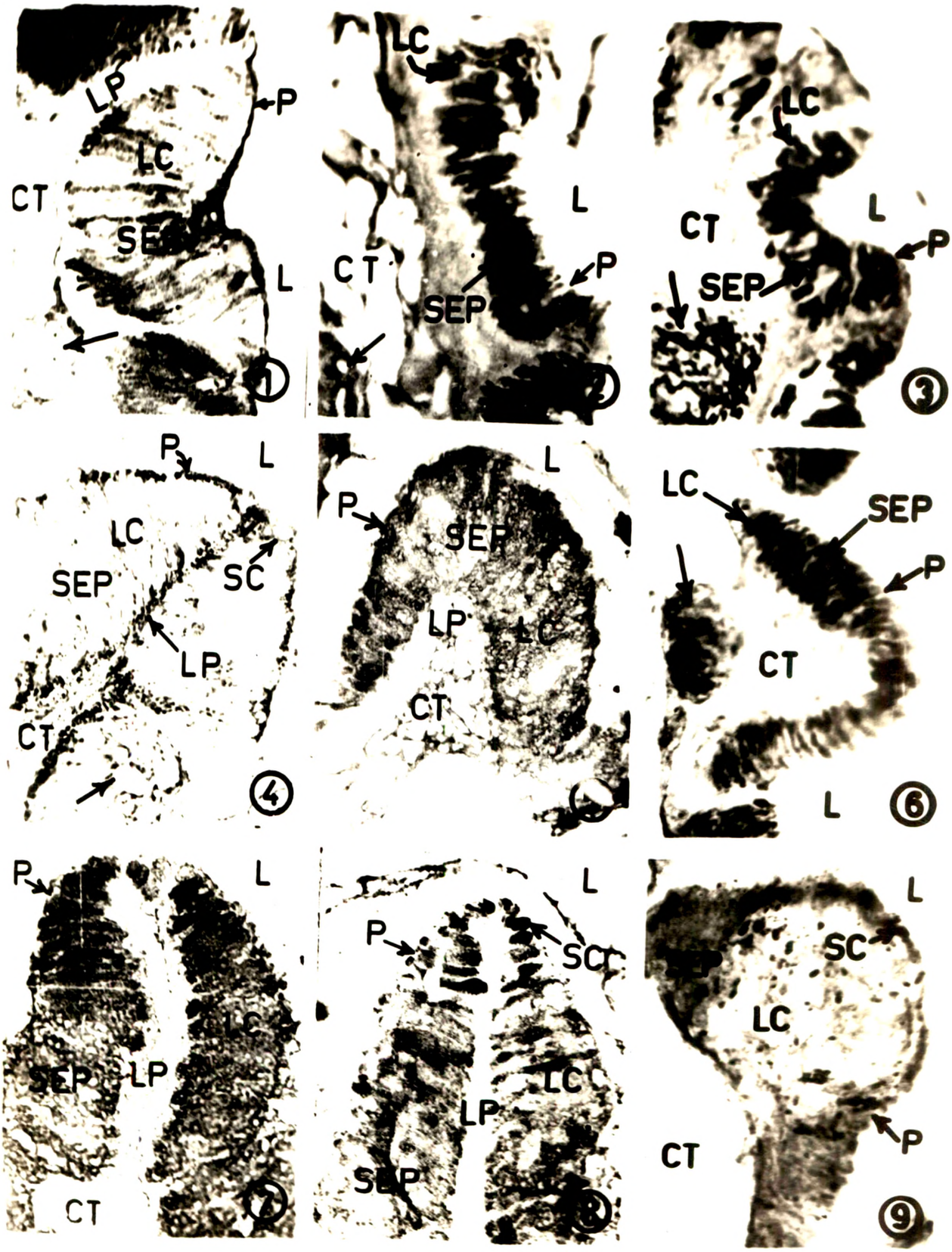
Fig.No. 6 : A single papilla in the buccal epithelium treated with high concentration (325 ppm) of phytotoxin. Note intense staining in cell patches in connective tissue (CT). x 240.

Fig.No. 7 : A single papilla (P) in buccal epithelium of normal fish stained with PAS. x 240.

Fig.No. 8 : A single papilla stained with PAS. Note reduced staining reactivities.

Fig.No. 9 : A destroyed papilla stained with PAS. Note loss of large cells (LC) and small cells (SC). x 240.

PLATE NO. 1


LP
P
LC
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SEP
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①
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SEP
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P
SC
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SEP
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L
SC
LC
CT
P
⑨

epithelium than the muscular side (Plate No.1, Fig.1,3). The cells and their sizes were increased in the toxin treatment (Plate No.1, Fig.3). There was no change in the muscle layer supporting the oral mucosa.

As pointed out earlier, there were two types of papillae (fungiform and filiform) in the normal oral cavity of Tilapia mosambica (Plate No.1, Figs.4,5, 6,7). During toxin treatment cells bordering these papillae showed remarkable changes in their size, number and staining reactivities. The number of small cells lying at the top of these papillae was moderate in 200 ppm and 300 ppm toxin treatment but it was increased enormously in 400 ppm toxin treatment. Sometimes they migrate in the lamina propria (Plate No.1, Fig.No.4). The large cells which were lying mainly on the sides of these papillae loose their columnar nature and became more or less round (Plate No.1, Figs.6,8). Their number was increased and staining reactivities were enhanced. Some time cells loose their boundaries and compact mass was observed (Plate No.1, Fig.No.9). The lamina propria of the papillae also showed increase in size and number of the cells. The connective tissue thickened and vacuolization were prominent in this tissue. As in case of stratified epithelium, there was no change observed in the histological structure of the muscles.

c) Histochemical Observations :

The histochemical data on some important staining reactions employed in the present investigation of the oral cavity of a fish, Tilapia mossambica, are recorded in Table No.10, according to the visually estimated staining intensity and shade with four plus (++++) representing the strongest activity. The

Table No. 10

Histochemical observations on mucosubstances in the various tissues of the oral cavity in normal and plant toxin (S. laurifolius) treated Tilapia mossambica (Peter's)

No.	Histochemical Methods	TISSUES											
		Epithelium						Connective tissues			Muscles		
		Large cells			Small cells								
		Normal	Low conc.	High conc.	Normal	Low conc.	High conc.	Normal	Low conc.	High conc.	Normal	Low conc.	High conc.
1	HE	N-blue E-ve	Staining moderate	Staining increased	N-blue E-+ve	Staining moderate	Staining increased	++	+++	++++	++	+++	++++
2	PAS	+++	++++	++++	+++	++++	++++	++	+++	++++	++	+++	++++
3	M Diastase-PAS	++	++	++	-	±	+	++	+++	++++	-	-	-
4	AB pH 1	++	+++	++++	-	++	++	-	-	-	-	-	-
5	AB pH 2.5	+++	+++	++++	-	++	++	-	-	-	-	-	-
6	AB pH 1 - PAS	++B ++P	+++B ++P	++++B +++P	++P -	+++P +B	+++P +B	++P	+++P	++++P	++P	+++P	++++P
7	AB pH 2.5-PAS	+++B ++P	++++B ++P	++++B +++P	++P	+++P +B	+++P +B	++P	+++P	++++P	++P	+++P	++++P
8	AF	++	+++	+++	-	-	-	-	-	-	-	-	-
9	AF-ABpH 2.5	++P ++B	+++P +++B	+++P +++B	-	-	-	-	-	-	-	-	-

N.B. : ++++ = Very intense reaction; +++ = Intense reaction; ++ = Moderate reaction; + = Poor reaction; - = No reaction.
Abbreviations : HE = Haematoxyline-Eosine; PAS = Periodic Acid Schiff; M.Diastase = Malt diastase;
AB = Alcian blue 8GX-300; AF = Aldehyde fuschin; P = pink; B = blue.

histochemical observations requiring further description and consideration, are presented hereafter along with the interpretations of the histochemical staining reactions. The distribution and alterations in the mucosubstances in various cellular elements in the oral cavity are photomicrographically illustrated in Plate No.1, Figs. 1 to 9.

The histochemical study on the oral cavity revealed the following significant facts about the elaboration of the mucosubstances - (1) on the basis of elaboration of the mucosubstances, the stratified epithelial cells showed two different histochemical reactivities in large cells and in the small cells. (2) These two types of cells showed distinct responses in elaboration of their muco - substances in plant toxin treated tissues. (3) The connective tissue showed very lower mucin secretion. (4) The muscles contained only glycogen in them.

i) Elaboration of mucosubstances by the large cells :

The histochemical reactions of these cells showed a moderate PAS reactivity, which was partially lost by diastase digestion indicating the presence of some glycogen in these cells. These cells showed alcianophilia with AB both at pH 1 and 2.5, the degree of intensity of staining at the latter pH level being somewhat higher than that at the former. The sequential AB (pH 1, 2.5)-PAS staining techniques indicated simultaneous presence of both the acidic and glycogen in these cells. AF and AF-AB pH 2.5 staining procedures also evidenced such simultaneous occurrence of these two types of mucosubstances in the cells.

Thus, the large cells of the stratified epithelium as well as in the oval papillae, are endowed with a capacity to elaborate the glycogen and acidic mucosubstance (especially sulfomucins as evidenced by positive reaction towards AB pH 1 and AF).

ii) Elaboration of mucosubstances by the small cells :

These cells exhibited intense PAS reactivity. The PAS reactivity was completely lost after prior malt diastase digestion. Towards other histochemical techniques, these cells showed negative reactivities indicating the presence of only glycogen in these cells.

iii) Elaboration of mucosubstances by the connective tissue :

The connective tissue showed negative reactivities towards the many of the histochemical staining techniques except trace PAS reaction in small cells scattered in between the connective tissue fibers. The PAS reactivity was resistant to prior diastase digestion indicating the presence of only neutral mucosubstances in it.

iv) Elaboration of mucosubstances by the muscular layer :

The PAS reactivity in the muscle cells was completely lost after diastase digestion. These cells showed negative reactivities towards other histochemical techniques indicating the presence of glycogen in this layer.

d) Mucosubstance alterations due to S.laurifolius fruit toxin :

The plant toxin studies revealed interesting alterations in the staining intensity and concentration of the mucosubstances in the large cells, small cells, connective tissue and muscle layer of the oral cavity of T.mossambica.

The large cells reflected variations in the intensity of staining and concentration of the mucosubstances synthesized by these cells. The cells synthesizing glycogen and acidic mucins showed minimum staining in the normal tissues. The staining intensity immediately after toxin treatment increased and reached its maximum but after higher doses the mucosubstances were decreased remarkably.

The small cells which contained only glycogen, showed interesting alterations in them after the toxin treatment. Their PAS reactivity was not completely abolished after prior diastase digestion indicating the transformation of glycogen into the neutral mucosubstances. Their intensity being moderate in lower doses of toxin treatment whereas reached a minimum in the higher doses.

The connective tissue showed the same staining reactivities even after the toxin treatment but in some PAS positive cells, which contained neutral mucosubstances in them the intensity of staining and concentration of the mucosubstances were increased in the tissues, treated with the plant toxin.

II) Gills

a) Normal Histology of Gills :

Each gill of Tilapia mossambica has two rows of primary gill lamellae. The primary gill lamellae internally supported by a bony structure and the spaces are filled with many blood cells (Plate No.2, Figs.4,5,6). The primary gill filaments give rise to several secondary gill lamellae placed at regular intervals. Externally, the primary gill lamellae are covered with epithelial layers from the both sides. The epithelium is followed by thin connective tissue

PLATE NO.2, Fig. Nos. 1 to 10

(Impact of phytotoxin on mucins in the gill of T. mossambica)

Fig.No. 1 : Small portion of normal gill stained with H&E, showing primary filament (PF) and secondary filament (SF). x 180.

Fig.No. 2 : Gills treated with phytotoxin (200 ppm) and stained with H&E. Note shortening and destoration in the secondary gill filament (SF). x 180.

Fig.No. 3 : Gills treated with higher concentration of phytotoxin (350 ppm) and stained with H&E. Note enhanced staining and curling of the secondary gill filaments. x 180.

Fig.No. 4 : Small portion of normal gills stained with PAS. Note intensely staining acidophils (AC) and tips of the secondary filaments (SF). x 180.

Fig.No. 5 : Gills treated with phytotoxin (250 ppm) and stained with AB pH 2.5-PAS. Note slogging off the secondary gill filament (SF), increase in the no. of pillar cells (PC). x 180.

Fig.No. 6 : Gills treated with phytotoxin (325 ppm) and stained with AB pH 2.5-PAS. Note decrease in pillar cells (PC). Shortening of the secondary filament (SF) and histolysis was evident in primary filament (PF). x 180.

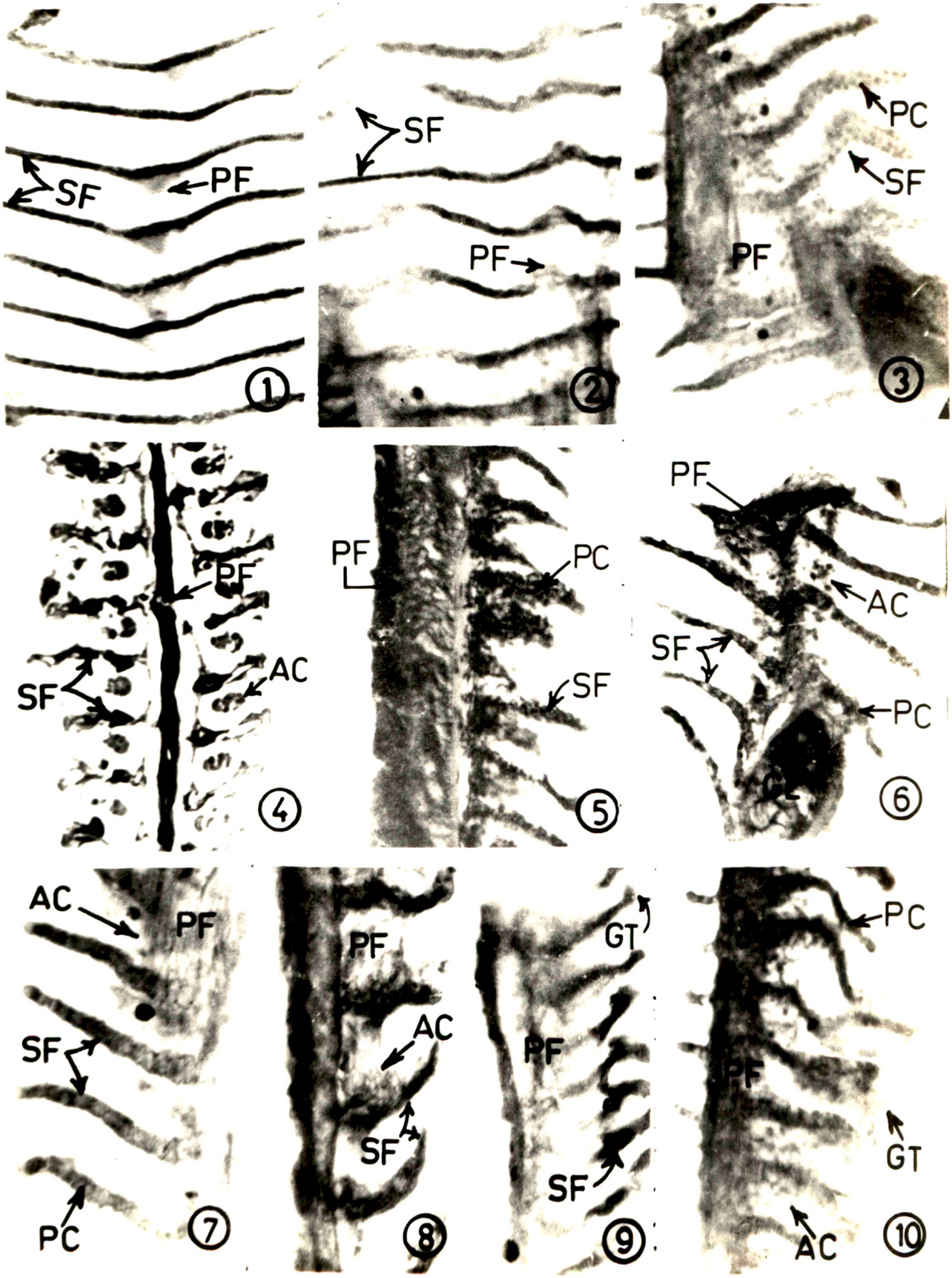
Fig.No. 7 : Normal gills stained with PAS showing normal histological picture in primary and secondary gill filament (PF and SF). x 180.

Fig.No. 8 : Gills treated with phytotoxin (200 ppm) and stained with PAS. Note intense staining and curling in secondary filaments and in acidophils (AC). x 210.

Fig.No. 9 : Gills treated with phytotoxin (250 ppm) stained with PAS. Note intense staining at the gill tip (GT) of secondary filaments (SF). x 180.

Fig.No. 10 : Gills treated with phytotoxin (300 ppm) stained with AB pH 1-PAS. Note baloon shaped gill tips (GT) of secondary gill filaments. x 180.

PLATE NO. 2


SF
PF
①
SF
PF
②
PC
SF
PF
③
PF
AC
SF
④
PF
PC
SF
⑤
PF
AC
SF
PC
⑥
AC
PF
SF
PC
⑦
PF
AC
SF
⑧
GT
PF
SF
⑨
PC
PF
GT
AC
⑩

and vascular layer. The mucous secreting cells and acidophilic cells are distributed in the epithelium of the primary gill lamellae.

The secondary gill lamellae are separated from each other by interbranchial septum and are free at their distal ends. These gill filaments are supported by cartilagenous tissue (Plate No.2, Figs. 3,4,6). The secondary lamellae are also supported internally by pillar cells (Plate No.2, Figs. 5, 6, 7, 8). These cells are stained in HE and are arranged in the lamellae in such a fashion that they lie just adjacent to the capillaries. The epithelial cells lining the secondary gill lamellae also consists of numerous mucous cells and the acidophils are seen at the bases of these lamellae.

The normal histology of gills of T.mossambica has been described in earlier work from this laboratory (Nikam, 1986; Harold, 1987).

b) Histopathological alterations due to S.laurifolius fruit toxin :

The effects of the ethanol extract of fruit of S.laurifolius showed very similar histopathological effects on the fish gills to those of Lasiosiphon eriocepholus (Harold, 1987). These changes observed only after the treatment of higher concentrations of the extract.

At lower doses (200-300 ppm), the effects were not prominent, however slight changes in the morphology and in the histology were observed. The staining reactivities were altered. These changes are summarized : the increase in interlamellar space, reduction in the primary gill lamellae, displacement of the epithelium from the basement membrane, initiation of the histolysis (Plate No.2 Fig.No.5) and increase in number of mucous secreting cells and acidophil cells in the primary gill lamellae.

The secondary gill lamellae were unevenly curved (Plate No.2, Figs.3,6, 8,10). Some had became thin and slightly shortened. The apical ends were bulged (Plate No.2, Figs.3,9,10), but basal regions of these lamellae were intact The blood spaces were reduced. The pillar cells were enlarged and their staining reactivities were enhanced. The number of mucous secreting cells in the epithelium and of the acidophil cells were very much increased.

The remarkable histological changes in the primary and secondary gill lamellae were observed in the treatment of higher concentrations (325-400 ppm) of toxin. The primary gill lamellae were affected and showed the alterations including reduction in the supporting bony element indicating degenerative changes, enlargement of distal gill lamellar blood spaces, increased subepithelial spaces, ruptured lamellar capillaries and detachment of epithelium.

The secondary gill lamellae also showed noticeable changes due to this plant toxin. The main alterations include the reduction and shortening of secondary gill lamellae (Plate No.2, Fig.3,5,8), accumulation of blood cells in the intercellular spaces, loss of pillar cells (Fig.7), formation of haematomas, overlapping of the filaments, uneven curling and discontinuation of the epithelium at the basal regions, increasing mucous secreting cells in the epithelium, increase in number and size of the acidophil cells. In secondary gill lamellae, there were many major degenerative changes occurred suggesting the histolysis in this tissue (Plate No.2, Figs.5, 8).

c) Histochemical observations :

The histochemical data on some important staining reactions employed in the present investigation of the gills of T.mossambica are recorded in

Table No.11, according to the visually estimated staining intensity and shade with four plus (++++) representing the strongest activity. The distribution and alterations in the various cellular elements and their mucosubstances in the gills are photomicrographically illustrated in Plate No.2, Figs.1 to 10.

The histochemical study on the gills revealed the following significant facts about the elaboration of the mucosubstances - (1) The mucosubstance elaborating cells showed a regular distribution along the free margins of primary gill filaments, secondary gill filaments, bases of the gill lamellae and interlamellar spaces. (2) The mucosubstance elaborating cells responded to the plant toxin treatment.

i) Mucosubstances elaboration by epithelial cells :

The epithelial cells exhibited PAS reactivities which was resistant to prolonged diastase digestion indicating absence of glycogen in them. The other histochemical techniques for demonstration of acid mucosubstances showed negative reactivities, indicating their absence from these sites. Thus, the gill epithelial cells of T.mossambica elaborated only neutral mucosubstances in them.

But some few cells in the epithelium showed PAS reactivity and slight alcianophilia at AB pH 2.5 and in the combined (AB 2.5-PAS) staining methods, indicating probable simultaneous occurrence of acidic mucosubstances along with the neutral mucins in them.

ii) Mucosubstances elaboration by Mucous cells :

There are different cells on the gill lamellae which stained deeply with various mucin demonstrating techniques employed in the present investigation.

Table No.11

Histochemical observations on mucosubstances in the various tissues of the Gills in normal and plant toxin (S. laurifolius) treated fish, Tilapia mossambica (Peter's)

No,	Histochemical Methods	TISSUES							
		Epithelial cells		Mucous cells				Basement Lamina	Gill rachis
		Neutral MPS cells	Mixed N+ Acidic cells	Neutral MPS cells	Neutral + Acidic cells	Sulfated	-CaOH containing cells		
1	HE	++++H ++E	++++H +++E	++++H ++E	++++H +++E	++++H +++E	++++H ++E	++++H +++E	++++H +E
2	PAS	++++P	++++P	++++P	++++P	++++P	++++P	++++P	++++P
3	M.Diastase-PAS	++++P	++++P	++++P	++++P	++++P	++++P	++++P	++++P
4	AB pH 1	-	+++B	-	++++B	++++B	-	++B	++++B
5	AB pH 2.5	-	+++B	-	++++B	++++B	++++B	+++B	++++B
6	AB pH 1-PAS	++++P	++++P +++B	++++P	++++P ++++B	++++B	++++P	++P ++B	++++B
7	A BpH 2.5-PAS	++++P	++++P +++B	++++P	++++P ++++B	++++B	++++B	++P ++B	++++B
8	AF	-	+++P	-	++P	++++P	-	±	++++P
9	AF-AB-pH 2.5	-	+++P	-	++P	++++P	-	±	++++P

N.B. :++++ = Very intense reaction; +++ = Intense reaction ++ = Moderate reaction; + = Poor reaction; - = No reaction.

Abbreviations : HE = Haematoxyline-Eosine; PAS = Periodic Acid Schiff; M.Diastase = Malt diastase; AB = Alcian blue 8GX-300; AF = Aldehyde fuschin; P = pink; B = blue.

All the mucous secretory cells were PAS positive. Their PAS positivity was resistant to prolonged diastase digestion, indicating absence of glycogen in them. In combined sequential staining techniques like AB pH 2.5-PAS and AB pH 1-PAS, some of these cells stained only purple (Plate No.2, Figs.4, 7) thus showing elaboration of neutral mucosubstances in them.

Some cells contained a mixture of neutral and sulfated mucosubstances. These cells showed PAS reactivity resistant to prior diastase digestion. In AB pH 1-PAS and AB pH 2.5-PAS sequential staining techniques, these cells showed mixed staining. The probable occurrence of sulfated mucosubstances were deduced from their alcianophilia both at pH 2.5 and pH 1.0 (Plate No.2, Figs.5 and 6). AF and AB pH 2.5-AF also showed mixed staining in these cells. Thus, these cells have capacity to synthesize and elaborate mixed, neutral and acidic (sulfated) mucosubstances.

There were some acidic mucosubstances secreting cells. These cells showed PAS reactivity resistant to diastase digestion. These cells showed intense alcianophilia both at pH 1.0 and pH 2.5. They stained blue with sequential staining techniques (AB pH 1-PAS and AB pH 2.5-PAS). Some of the acidic mucosubstances secreting cells were AF positive and in the combined sequential staining procedure, AF-AB pH 2.5 also showed pink staining in them. Thus some of the acidic mucosubstance secreting cells including the pillar cells have capacity to elaborate sulfomucins whereas other contained carboxyl containing acid mucosubstances in them (Plate No.2, Figs. 5, 6, 7, 8, 9, 10).

iii) Mucosubstances elaboration by the Basement lamina :

The epithelial cells rest on the basement lamina which showed moderate PAS reactivities. The PAS staining was not reduced by prior diastase digestion indicating absence of glycogen but the neutral mucins. The basement lamina, simultaneously, showed moderate alcianophilia both at pH 1 and pH 2.5. In the combined sequential staining procedures like AB (pH 1, 2.5)-PAS, this tissue stained bluish-pink indicating acid moieties along with the neutral mucins in it (Plate No.2, Figs.3,5,6,7 and 8).

iv) Mucosubstances elaboration by the Gill rachis :

The cartilagenous tissue present in the gill rachis showed strong PAS reactivity, deep alcianophilia both at pH 1 and pH 2.5, stained only blue in the combined sequential staining techniques like AB pH 1-PAS and AB pH 2.5-PAS, AF staining was prominent indicating sulfated acid mucosubstances in them (Plate No.2, Figs. 5,6,8) .

d) Mucosubstance alterations due to S.laurifolius fruit toxin :

The application of plant toxin S.laurifolius revealed several interesting alterations in the staining intensity and concentration of the mucosubstances in the epithelial cells, mucin elaborating cells, pillar cells, basement lamina and in the gill rachis of T.mossambica.

The epithelial cells and mucous elaborating cells reflected variations in the intensity of staining and in the concentration of the mucosubstances elaborated by these cells. During lower doses of the plant toxins their mucin secretion was increased (Plate No.2, Fig.5) and during the treatment of higher doses

their elaboration reached maximum, covering the whole tissue (Plate No.2, Figs. 3 and 10).

The pillar cells of the secondary filaments showed reverse effects to that of the epithelial cells during the plant toxin treatment. In the low concentration the pillar cells showed maximum concentration of mucosubstances (Plate No. 2 Fig.3) while in the higher concentration due to the reduction in the number of pillar cells, their concentration and staining intensities were minimum (Plate No.2, Fig.7).

III - Liver

a) Normal Histology of Liver:

The Liver of Tilapia mossambica is composed of many lobules richly supplied with blood by many vessels. Each lobule consists of a central lumen surrounded by many cords of polygonal hepatic cells. The hepatic cells or hepatocytes contain granules of different sizes in the cytoplasm and a centrally placed nucleus. A number of blood spaces - sinusoids are scattered in the hepatis tissue. In between the hepatic cords of the hepatocytes, small bile canaliculi originate and many of them unite to form bile duct, which is seen in the hepatic lobules (Plate No.3, Figs. 1,4). In some sections the pancreatic tissue contains big and differently staining Islet-cells (Plate No.3, Fig. 4).

b) Histopathological alterations due to S.laurifolius fruit toxin :

The effects of the crude powder and of the ethanol extract showed very similar effects on the histology of the fish liver. The histological changes

PLATE NO. 3, Fig. Nos. 1 to 9

(Impact of phytotoxin on mucins in the Liver of T. mossambica)

Fig.No. 1 : Small portion of normal liver stained with PAS. Note staining in hepatocytes (HC). x 240.

Fig.No. 2 : Liver section treated with phytotoxin (250 ppm) and stained with H€. Note enlarged hepatocytes (HC) with ecentric nuclei (N). x 240.

Fig.No. 3 : Liver section treated with phytotoxin (300 ppm). Note rupturing of blood sinusoid (BS) with blood cells (BLC). x 240.

Fig.No. 4 : Liver treated with phytotoxin (225 ppm) stained with SB pH 1 - PAS. Note aggregation of cytoplasmic content of hepatocytes (arrows) : x 240.

Fig. No. 5 : Liver treated with phytotoxin (300 ppm) stained with H€. Note vacuolization. x 240.

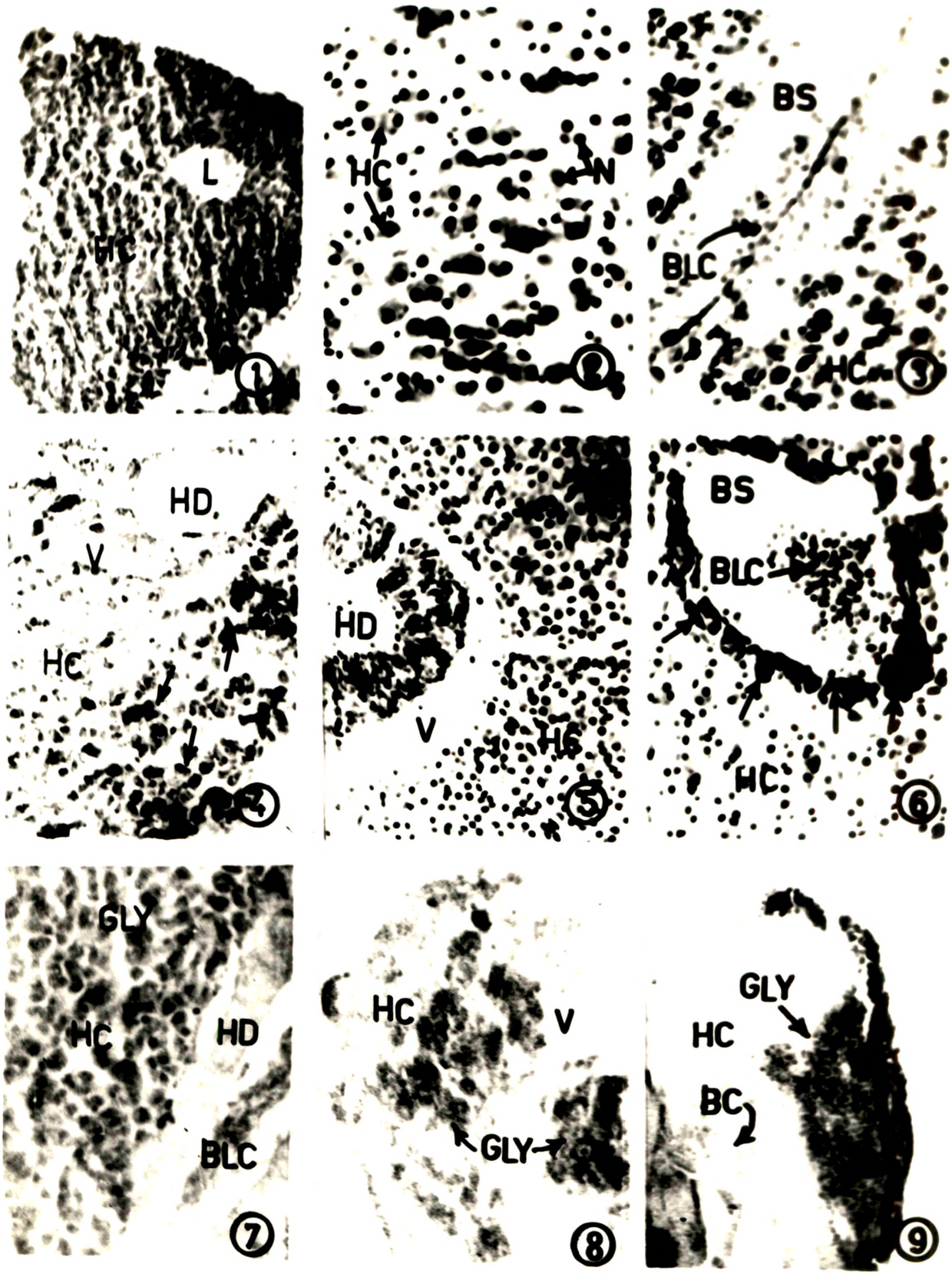
Fig.No. 6 : Liver treated with 350 ppm and stained weith AB pH 2.5 - PAS. Note enlarged sinusoids (BS) and intense staining at its periphary (arrows) and disarray of hepatocytes (HC). x 240.

Fig.No. 7 : Normal liver stained with PAS. Note intense staining of glycogen. x 240.

Fig. No. 8 : Liver treated with phytotoxin (200 ppm) stained with PAS. Note decrease in glycogen.

Fig.No. 9 : Liver treated with phytotoxin (350 ppm) Note only small patch of glycogen (Gly) at the periphary of the section. x 240.

PLATE NO. 3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observed in low and high doses of the toxin were distinct. The histopathologically distinct observable changes in the liver are summarized below :

The displacement of hepatocytes was the foremost effect of the plant toxin. The cells with the increasing doses started enlarging. There was aggregation of cytoplasmic contents. Due to this precipitation black patches could clearly be seen in this tissue (Plate No.3, Figs. 8). Some hepatocytes were swollen with large vacuoles. With higher concentrations (350 ppm) due to the swelling and vacuolization it was difficult to mark the cell boundaries. Disruption of sinusoids was also evident (Plate No.3, Fig. 3).

The other detectable histological change was observed in hepatocyte nuclei. Some cells were without nuclei, whereas in some the size of the nuclei was increased. The nuclei picnosis was observed. There were some binucleated hepatocytes. In some cells nuclei with mitotic divisions were observed (Plate No.3, Fig.2, 3).

The net result of the plant toxin showed the changes in the cordal arrangement, leading to deformation of liver histology (Plate No.3, Fig.3,6) showing large gaps in the hepatic tissues.

c) Histochemical observations :

The histochemical data on some important staining reactions employed in the present investigation of the liver of T.mossambica are recorded in Table No.12, according to the visually estimated staining intensity and shade with four plus (++++) representing the strongest activity. The histochemical observations requiring further description and consideration, are presented hereafter

Table No. 12

Histochemical observations on mucosubstances in the various tissues of the Liver in normal and plant toxin (S. laurifolius) treated fish, Tilapia mossambica (Peter's)

No.	Histochemical Methods	Hepatocytes		
		Normal	Low concentration of plant ploxin	High concentration of plant toxin
1	HE	++++H +++E	++++H +++E	++++H ++++E
2	PAS	++P	++ P	+P
3	M.Diastase-PAS	-	-	-
4	AB pH 1	+B	++B	+++B
5	AB pH 2.5	+B	++B	+++B
6	AB pH 1-PAS	+B ++P	++B ++ P	+++B ++P
7	AB pH 2.5-PAS	+B ++P	++B ++ P	+++B +P
8	AF	-	-	-
9	AF-AB pH 2.5	-	-	-

N.B. : +++ = Very intense reaction; +++ = Intense reaction; ++ = Moderate reaction; + = Poor reaction; - = No reaction.

Abbreviations : HE = Haematoxyline-Eosine; PAS = Periodic Acid Schiff; M.Diastase = Malt diastase;

AB = Alcian blue 8GX-300; AF = Aldehyde fuschin; P = pink; B = blue.

along with the interpretations of the histochemical staining reactions. The distribution and alterations in the mucosubstances in the liver are photomicrographically illustrated in Plate No.3, Figs. 1 to 9.

Elaboration of mucosubstances by Hepatocytes :

The liver cells exhibited intense PAS reactivities (Plate No.3, Figs.7,8 & 9). To test the nature of the PAS staining some of the sections were treated for enzymatic digestion by malt diastase. Such digestion indicated the glycogen nature of the PAS staining. Acid mucosubstances were very low in concentration as compared to Glycogen. The hepatic tissue contained glycogen and very few acidic mucosubstances.

The few patches of cells showed PAS positivity resistant to diastase digestion and moderate alcianophilia at pH 2.5 and all other reactions showed negative reactivities. Such reactivities indicated the presence of acidic mucosubstances in these cells.

Interestingly the cells bordering the blood sinusoids were highly PAS positive and contained high concentration of glycogen in them (Plate No.3, Fig. 6).

d) Mucosubstance alterations due to S.laurifolius fruit toxin :

The application of plant toxin, S.laurifolius to the T.mossambica revealed many striking changes in the intensity of staining and concentrations of the mucosubstances of the hepatic cells only.

The liver sections in the lower concentrations (200 ppm and 225 ppm) of plant toxin produced decrease in the staining intensity (Plate No.3, Fig. 7) whereas in concentration (300 ppm) it was found gradually decreased reaching lowest at 400 ppm (Plate No.3, Figs. 8,9). Thus, it indicated that with the high doses of toxin treatment glycogen content of the tissue was considerably depleated. The accumulation or aggregation of cells consequently formed, number of glycogen patches throughout the hepatic tissue.

IV - Kidney

a) Normal Histology of Kidney :

The kidney of Tilapia mossambica composed of many functional units the nephrons. The Malpighian bodies are clearly seen (Plate No.4, Figs.1,2,3) with Bowman's capsules in which glomeruli are enclosed. The Bowman's capsule has basement membrane. The Bowman's capsule leads to a narrow neck, with ciliated epithelium, continues into proximal and distal tubules. The proximal tubules are characterised by the tall columnar epithelium which shows brush border towards the luminal side, whereas the distal tubules are lined with epithelial cells only. The distal tubules end into collecting duct.

b) Histopathological alterations due to S.laurifolius fruit toxin :

The histology of the kidney was totally changed due to the toxin treatment even at lower doses also. The initial toxin dose affected the malpighian body. In the low concentration (225 ppm) the glomeruli of the kidney immediately increased in size. Subsequently at higher concentrations increase in capsular size, shrinkage in glomeruli, damage to the capilaries, degeneration of

PLATE No. 4, Fig. Nos. 1 to 9

(Impact of phytotoxin on mucins in the kidney of T. mossambica)

Fig. No. 1 : Small portion of normal kidney stained with PAS. Glomeruli (GL), Proximal tubule (PT), distal tubule (DT), are clearly seen. x 240.

Fig. No. 2 : Kidney section treated with phytotoxin (200 ppm) stained with AB pH 1 - PAS. x 240.

Fig. No. 3 : Kidney section treated with phytotoxin (200 ppm) and stained with AB pH 2.5 - PAS. x 240.

Fig. No. 4 : Kidney section treated with 250 ppm stained with PAS. Note enlarged blood capillaries (BLC). x 240.

Fig. No. 5 : Kidney section treated with (250 ppm) phytotoxin and stained with AB pH 1 - PAS. x 240.

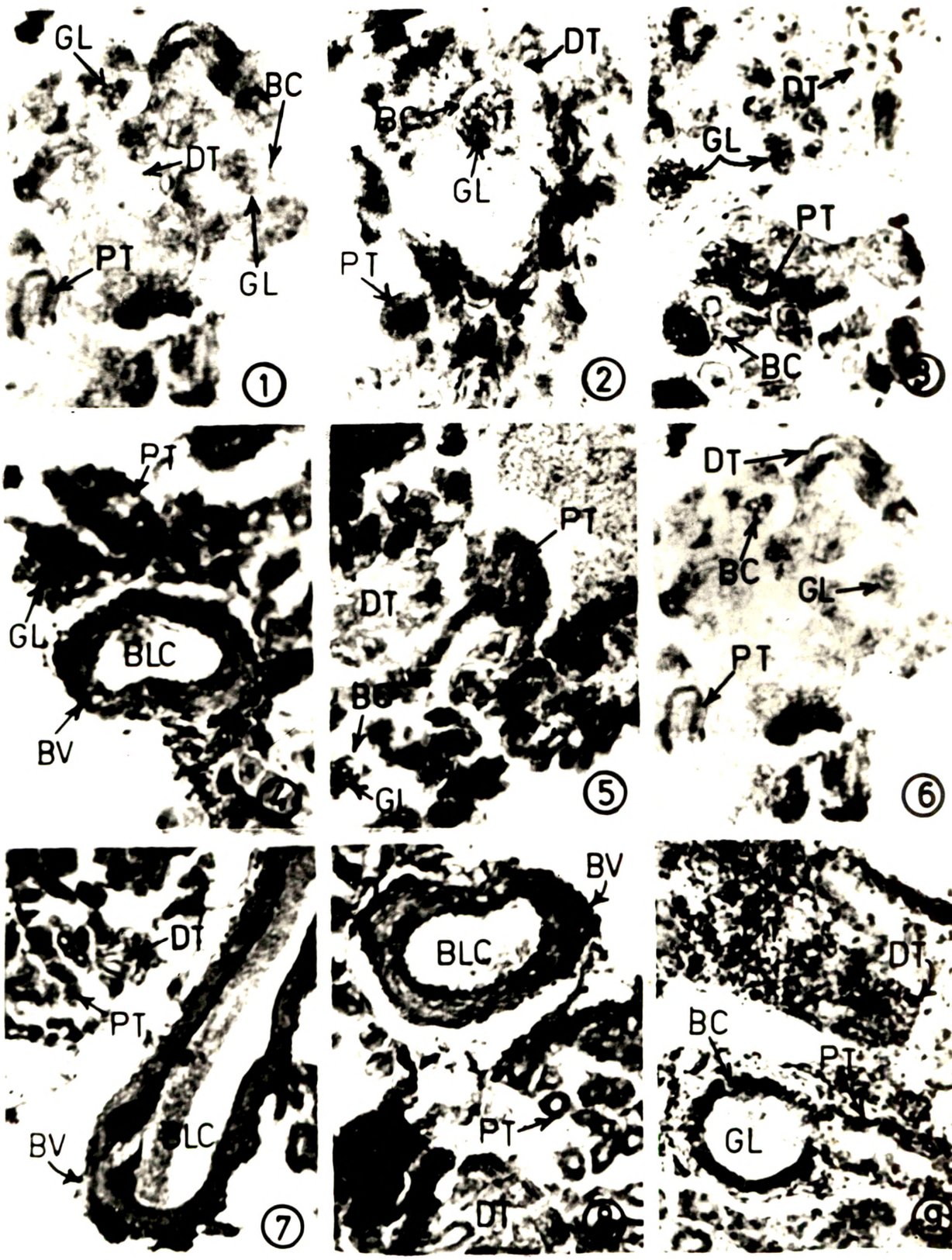
Fig. No. 6 : Kidney section treated with phytotoxin (250 ppm) and stained with AB pH 2.5. x 240.

Fig. No. 7 : Kidney section of phytotoxin (350 ppm) treated fish. Note coagulation of blood in the blood capillaries (BLC).

Fig. No. 8 : Small portion of kidney of toxin treated (350 ppm) Fish stained with AB pH 2.5 - PAS. x 240.

Fig. No. 9 : Kidney section of toxin treated (350 ppm) fish stained with AB pH 1 - PAS. Note enlarged glomeruli (GL) and destortion in kidney tubules. X 240.

PLATE NO. 4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basement membrane and endothelial cells, development of intertubular space, etc. took place particularly in the higher doses, the glomeruli became diffused (Plate No. 4, Figs.6, 9).

The lumen of the proximal tubules was reduced and the brush border showed disintegration. There was loss of cytoplasmic material in few proximal tubule cells. Some tubules showed distortion leading to change in the kidney structure. In some of the cells, nuclear material was lost. The edema in these tubules was prominent. The cytoplasm of the cells in these tubules showed increased eosinophilic activity.

The swelling and the damage of epithelial cells in the distal tubules were evidenced. The concentrated substances were observed towards the luminal side showing honey-comb like structure. The hyportrophy and edema led to disorientation in the distal tubules, thus causing distortion in the kidney structure. There were formation of spaces in between the intertubules, whereas lumen size was very much reduced. The severe necrotic changes were prominent in these tubules.

The cells of the collecting tubules were also enlarged. The vacuoles were observed in these tubule cells. The lumen size was very much reduced due to the enormous increase of the cells.

c) Histochemical Observations :

The histochemical data on some important staining reactions employed in the present investigation of the kidney of T.mossambica are recorded in Table No.13, according to the visually estimated staining intensity and shade

Table No. 13

Histochemical observations on mucosubstances in the various tissues of the kidney in normal and plant toxin S. laurifolius treated fish, Tilapia mossambica (Peter's)

No.	Histochemical Methods	TISSUES								
		Malphigian Body			Proximal Tubules			Distal tubules		
		Normal	Low dose of plant toxin	High doses of plant toxin	Normal	Low doses of plant toxin	High doses of plnat toxin	Normal	Low doses of plant toxin	High doses of plant toxin
1.	HE	++++H +++ E	++++H ++++E	++++H ++++E	++++H +++E	++++H +++E	++++H ++++E	+++H +++E	++++H ++++E	++++H ++++E
2.	PAS	+++P	++++P	++P	++P	+++P	++++P	++P	+++P	++++P
3.	M.Diastase-PAS	++P	+++P	+P	++P	+++P	++++P	-	-	-
4.	AB-pH 1	-	-	-	++B	+++B	++++B	++B	+++B	++++B
5.	AB pH 2.5	+++B	++++B	+B	++B	+++B	++++B	++B	+++B	++++B
6.	AB pH 1 - PAS	+++P	++++P	++P	++P ++B	+++P +++B	++++P ++++B	++P ++B	++P +++B	++++P ++++B
7.	AB pH 2.5-PAS	+++P +++B	++++P ++++B	++P +B	++P +++B	+++P +++B	++++ ++++B	++P ++B	+++P +++B	+++P ++++B
8.	AF	-	-	-	++P	+++P	++++P	++P	+++P	++++P
9.	AF-AB pH 2.5	++B	++++B	+B	++P ++B	++++P ++++B	++++P ++++B	++P	+++P	++++P

N.B. : +++ = Very intense reaction; +++ = Intense reaction; ++ = Moderate reaction; + = Poor reaction; - = No reaction.
Abbreviations : HE = Haematoxyline-Eosine; PAS = Periodic Acid Schiff; M.Diastase = Malt diastase; AB = Alcian blue 8GX-300; AF = Aldehyde fuschin; P = pink; B = blue.

with four plus (++++) representing the strongest activity. The histochemical observations requiring further description and consideration, are presented hereafter along with the interpretations of the histochemical staining reactions. The distribution and alterations in the mucosubstances in various parts of the kidney are photomicrographically illustrated in Plate No.4, Figs.1 to 9.

The histochemical study on the kidney revealed the following significant facts about the elaboration of the mucosubstances - (1) The mucosubstance elaboration exhibited by the malpighian bodies, proximal tubules and distal tubules. (2) There were very significant alterations in the mucosubstance elaborating tissues in response to the plant toxin.

i) Elaboration of mucosubstances by Malpighian bodies :

Both glomeruli and Bowman's capsule showed intense PAS reactivities (Plate No.4, Fig.1) which were partly susceptible to prior malt diastase digestion, indicating partial presence of some glycogen at both these sites. The non-susceptible chemical moiety was confirmed to be acidic mucosubstances by various alcian blue staining procedures. The basophilia (alcianophilia) with AB pH 2.5 gave a moderate staining reaction (Plate No.4, Fig. 2). In AB pH 1-PAS and AB 2.5 - PAS sequential staining procedures these parts showed mixed staining (Bluish-purple). Thus, Malpighian bodies indicated the presence of glycogen and acidic mucopolysaccharides in their cells.

ii) Elaboration of mucosubstances by Proximal tubules :

The PAS reaction was selectively intense in the luminal brush border of proximal tubules and cellular staining was slightly faint (Plate No.4, Fig. 2).

PAS positivity with prior diastase digestion had no effect indicating absence of glycogen in these tubules.

The luminal brush border, as in case of PAS showed intense alcianophilia at pH 2.5, indicating the presence of acidic mucins in these sites. AB 2.5-PAS combination produced a magenta colour only at the sites of luminal lining which showed intense reactivity for PAS whereas in the remaining cytoplasm AB staining was evident implying the presence of either neutral mucopolysaccharide or acidic mucins in these tubules (Plate No.4, Fig.3).

iii) Elaboration of mucosubstances by the distal tubules :

The distal tubules showed uniform PAS reactivity which had a slight suppressive effect with prior diastase digestion indicating the presence of glycogen in these tubules. Alcianophilia with AB pH 1 and AB pH 2.5 was also uniform but with very low intensity. AB (pH 1, 2.5)-PAS combined staining procedures showed mixed staining. AF staining was very faint and in the combined AF-AB pH 2.5 also AF reactivity was observed. Thus, the distal tubules were synthesing and elaborating glycogen and slightly sulfated acid mucosubstances.

d) Mucosubstance alterations due to S. laurifolius fruit toxin :

The application of plant toxin to fish, T. mossambica revealed interesting changes in the staining intensity and concentration of the mucosubstances elaborated by the kidney tissue.

The glomeruli and Bowman's capsule showed increasing and intensifying staining of the mucosubstances. These structures contained glycogen and acid mucopolysaccharide, immediately showed their concentration reaching maximum even in low doses of plant toxin (Plate No.4, Figs. 5, 8) and started decreasing as the concentrations of the plant toxin in the treatment increased (Plate No.4, Figs. 6 and 9). The mucosubstance form a ring surrounding the glomeruli near the Bowman's capsule (Plate No.4, Fig. 9). In the higher treatment accumulated mucosubstances slowly and steadily diminished (Plate No.4, Fig.9).

The proximal tubule mucosubstances were also increased immediately after the low doses treatment of the plant toxin (Plate No.4, Fig. 5) which were distinctly observed towards luminal brush borders. As against the Malpighian bodies, the increasing trend of mucosubstance accumulation or concentration and intensification in the sites of proximo tubules continued in the higher doses of the plant toxin treatment (Plate No.4, Figs. 5 and 8).

The distal tubule mucosubstances showed similar alteration pattern to those of the proximal tubule mucosubstances. Their concentration was moderate in the normal sections which slightly increased in the lower doses and continued in the higher concentration of plant toxin treatment. Thus, in distal tubules glycogen and sulfated acid mucosubstance varied with the concentration of plant toxin treatment, showed linear relationship as regards to their concengration and intensity of the mucosubstances.

(V) INTESTINE

a) Normal histology of intestine :

The intestine of Tilapia mossambica shows the usual four layered structure. The outermost serosa is followed by a muscular coat, comparatively better developed, consisting of an outer longitudenal and an inner circular layer. The submucosa is divisible into an outer stratum compactum, a dense connective tissue arranged in a wavy pattern and an inner stratum granulosum rich in capillary network. The latter merges with the tunica propria of the underlying mucosal coat, there being no muscularis mucosa. The epithelial lining of the mucosa consists of prismatic cells with basal nuclei. The nuclei of the intestinal cells are round with 2 to 3 nucleoli. The mucosal layer is thrown into many folds.

The intestinal epithelium of mucosal layer contains only two main types of cells. Goblet cells concerned with secretion and columnar cells which are generally lacking any definite secretory inclusions and which are assumed to be concerned with absorption. The goblet cells are of typical shape with a slender base, and permanent opening through the striated border. The columnar cells are tall and cylindrical having striations, free borders of it contain oval nuclei which are striated either centrally or towards the bases of the cells.

b) Histopathological alterations due to S. laurifolius :

In the phytotoxin treatment, the mucosa of intestine was seviourly damaged and different types of degenerative changes leading to complete

destruction of the cells (Plate No.5; Fig.2,4) were evident. In some areas the cells, were considerably swollen with granular cytoplasm (Plate No.5; Fig.2) and in others they had become extensively vacuolated (Plate No.5; Fig.4). In higher doses of toxin the mucosal lining cells were separated from one another and even different layers were separated from each other (Plate No.5; Fig.2,8). Some times some cells were completely destroyed and the dead cell debris was seen lying in the lumen (Plate No.5; Fig.4,7). The goblet cells were considerably enlarged. The blood capillaries were congested. Other layers were not much affected.

c) Histochemical observations :

The histochemical data on some important staining reactions employed in present investigation of the intestine of T. mossambica is recorded in table no.14, according to the visually estimated staining intensity and shade with 4 plus (++++) representing the strongest activity. The distributions and alteration in the various cellular elements and their mucosubstances in the intestine are photomicrographically illustrated in Plate No.5; Figs.1 to 8.

The histochemical studies on the intestine reveal the following significant facts about the elaboration of mucosubstances - (1) The mucosubstance elaborating cells were along the inner mucosal margin of the intestine. (2) Goblet cells show distinct mucosubstance content in them. (3) The plant toxin treatment showed induction to elaborate more mucosubstances by the different cellular elements of the intestine.

(i) Mucosubstances elaboration by epithelial cells :

The columnar epithelial cell showed the PAS reactivity which was resistant to prolonged diastase digestion and reacted weakly with alcian blue at

PLATE No. 5. Fig. Nos. 1 to 8 :

(Impact of phytotoxin on mucins in the Intestine of T. mossambica)

Fig. No. 1 : Small portion of normal intestine stained with HE with four layers - Serosa (S), Muscular coat (M), Submucosa (SM) and mucosal epithelium (ME). x 320.

Fig. No. 2 : Small portion of intestine of toxin treated (250 ppm) fish and stained weith HE. Note increase in number of epithelial cells and rupturing at some places. x 320.

Fig. No. 3 : Small portion of normal intestine stained with AB pH 2.5 - PAS. Note alcianophilia towards lumen in the epithelium (EP). x 320.

Fig. No. 4 : The section of intestine treated with phytotoxin (250 ppm) and stained with AB pH 1 - PAS. Note enhanced alcianophilia and PAS staining. x 320.

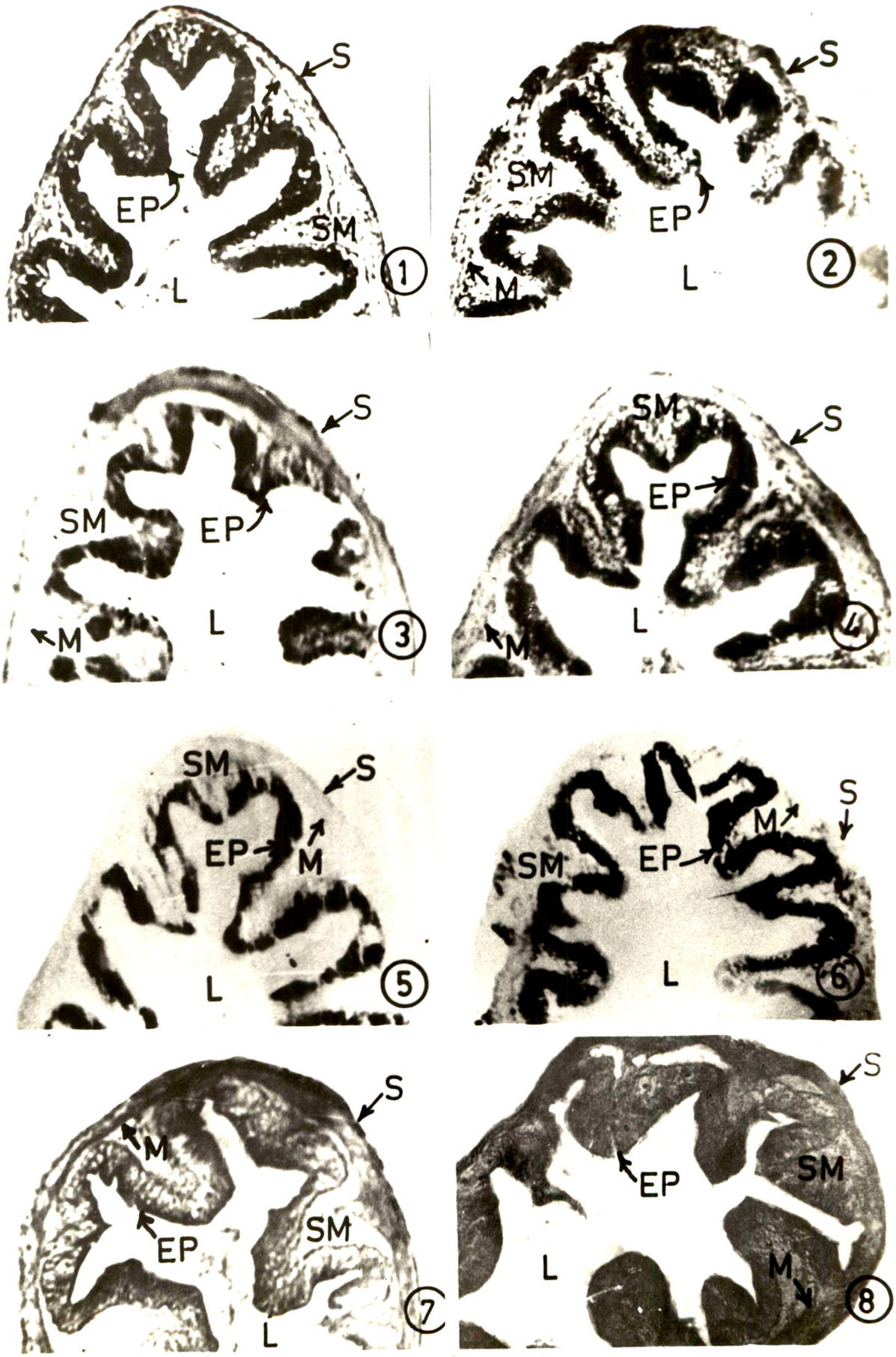
Fig. No. 5 : Small portion of normal intestine stained with AB pH 1. Note intensely stained goblet cells. x 320.

Fig. No. 6 : Small portion of intestine treated with phytotoxin (350 ppm) and stained with AB pH 2.5. Note intense alcianophilia. x 320.

Fig. No. 7 : Small portion of intestine of toxin treated (300 ppm) fish stained with AF. x 320.

Fig. No. 8 : Small portion of intestine of toxin treated (300 ppm) fish, stained with AF. Note increase in staining reactivity. x 320.

PLATE NO. 5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pH 2.5 and in the combined (AB pH 2.5 - PAS) staining method, indicating probable simultaneous occurrance of acidic mucosubstances along with the neutral mucins in them.

Goblet cells exhibited strong PAS reactivity resistant to prior diastase digestion. They reacted with AB both at pH 2.5 and pH 1 (Plate No.5; Figs.5,6) They stained blue with sequential staining techniques (AB pH 1 - PAS and AB pH 2.5 - PAS). Some of the acidic mucosubstances secreting cells were AF positive and in the combined sequential staining procedure AF - AB pH 2.5 showed pink staining in them. Thus, some of the acidic mucosubstance secreting goblet cells have capacity to elaborate sulfomucins whereas other contained carboxyle containing acid mucosubstances in them.

(ii) Mucosubstances elaboration by submucosa :

The submucosa layer of intestine showed PAS reactivity which was resistant to malt diastase digestion. In AB pH 1 - PAS and AB pH 2.5 - PAS techniques, this layer showed mixed staining. Other reactions showed the capacity of submucosa to synthasize and elaborate mixed, neutral and acidic (sulfated) mucosubstances.

(iii) Mucosubstances elaboration by muscle coat :

The muscle cells exhibited intense PAS reactivities (Plate No.5; Figs.7 and 8) which was labile to malt diastase digestion. Such enzymitic digestion tests indicated presence of glycogen in these cells.

TABLE NO. 14

Histochemical observations on mucosubstances in the various tissues of the Intestine in normal and phytotoxin (S. laurifolius) treated fish, Tilapia mossambica (Peters)

No.	Histochemical methods	INTESTINE TISSUES				
		MUCOSA		Sub-Mucosa	Muscular Coat	Serosa
		Columnar cells	Goblet cells			
1	HE	++++H +++E	++++H ++++E	+++H ++E	++H ++E	++H +E
2	PAS					
2	PAS	+++P	++++P	+++P	+++P	+++P
3	M.Diastase-PAS	+++P	++++P	+++P	-	+++P
4	AB pH 1	+++B	-	+++B	-	-
5	AB pH 2.5	+++B	++++B	+++B	-	-
6	AB pH 1-PAS	+++P +++B	++++P -B	+++P +++B	+++P -B	+++P -B
7	AB pH 2.5-PAS	+++P +++B	-P ++++B	+++P +++B	+++P -B	+++P -B
8	AF	+++P	-	+++P	-	-
9	AF-ABpH 2.5	+++P	++++B	+++P	-	-

N.B. : ++++ = Very intense reaction, +++ = Intense reaction,++ = Moderate reaction; + = Poor reaction, - = No reaction.

Abbreviations : HE = Herematoxyline-Eosine; PAS = Periodic Acid Schiff; M.Diastase = Malt diastase; AB = Alcian blue 8Gx - 300; AF = Adehyde fuschin; P = Pink; B = Blue.

(iv) Mucosubstances elaboration by serosa :

The serosa exhibited PAS reactivity which was resistant to prolonged malt diastase digestion indicating absence of glycogen in them. Other techniques for demonstration of acid mucosubstances showed negative reactivities, indicating their absence from this layer. Thus, the serosa of the intestine elaborated only neutral mucosubstances in them.

d) Mucosubstance alteration due to S. laurifolius fruit toxin :

The application of plant toxin of S. laurifolius reveal several interesting alterations in the staining intensity and concentration of mucosubstance in the four layers of the intestine.

During phyto-toxin treatment the number of the mucosubstance secreting cells in the intestine were enlarged and showed large amount of mucosubstance production, both in lower and higher doses of the phytotoxin. Especially, the columnar epithelial cells and the goblet cell showed maximum concentration and elaboration of mucosubstances (Plate No.5; Figs.No.4,5,6). Comparatively serosa, submucosa and muscular coat showed not much variations about their mucosubstance elaboration.