Aquaculture Practices – *Cyperus rotundus* L.

7.1 TAXONOMY

C. rotundus is a perennial plant native to Africa, central Europe and South Asia. There is a controversy regarding its origin in India though a broad sector of taxonomists believes its origin in all tropics and sub-tropics. It is considered as a deadly weed with a pernicious property of water retention from soil. Its invasion is recorded in more than 90 countries with harmful impacts on 52 different economically important crops. (Holm *et al.*, 1977). It is a curse for farmers due to its immense invasions and water fetching capacity by its underground tubers, presenting a threat of drought for the neighbouring agricultural crops thus reducing productivity. Reverse technology to reinstate their growth at adverse ambience was a disaster as it was subsequently impossible to eradicate once it is spread (USDA-NRCS, 2014). It is erect and rhizomatous terminated by spherical and elongated tubers. Leaves are linear with blunt tangent ends like triangle. It has terminal inflorescence, bisexual with leafy bracts and usually reddish to purplish in colour. Spikelet is compressed and linear and supported by 2-3 unequal rays with rosette of leaves arising out the sheets (Fig. 58). Underground tubers attach the shoots and spread eventually [Wikipedia].

Fig. 58 **Inflorescence of *Cyperus rotundus* L.**

7.2 MATERIALS AND METHODS

7.2.1 Extraction and isolation of bioactive compounds

The plants were seen to be grown at the edge of the wetland water bodies. The inflorescence of the plants was collected (approx 8Kg Fresh weight) without uprooting the whole plant. The fresh spikelet including both the florets and the glumes were isolated from the plant sample and sterilized in 5% Sodium hypochlorite for 15 minutes and rewashed with ddH_2O before further laboratory use. The blot dried samples were shade dried, powdered and processed for defatting. The defatted extract was dissolved in water and extracted with other organic solvents of low polarity to obtain a clear segregation of organic and inorganic fractions. The methanol fraction was made moisture free using anhydrous sodium sulphate as desiccant following which the fraction was concentrated and column run in basic alumina by the below tabulated solvent system. The F6 fraction was considered for further work which was repeatedly run through column with ascending methanol ratio.

Soxhlet extraction by Petroleum Ether (CrP) (non-polar), Chloroform(CrCh) and Methanol (CrM) (polar) for 18hrs with 3 refluxes/hr in average.

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Extract was filtered by vacuum filtration using vacuum pump with sintered disc funnel fitted in 250ml conical to retain unmixed particles and concentrated.

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Biochemical Analysis

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Bioactivity

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Methanol fraction subjected to Column Chromatography using Aluminium Oxide active, neutral I-II by the Solvent System as shown below

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Fractions	Solvent System	Ratio
Fraction 1 (F1)	H:EtOAc	8:2
Fraction 2 (F2)	H:EtOAc	7:3
Fraction 3 (F3)	CH_3Cl	10
Fraction 4 (F4)	CH_3Cl:EtOAc	7:3
Fraction 5 (F5)	CH_3Cl:MeOH	9:1
***Fraction 6 (F6)**	**CH_3Cl:MeOH**	**5:5**

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Thin Layer Chromatography using Silica Gel G

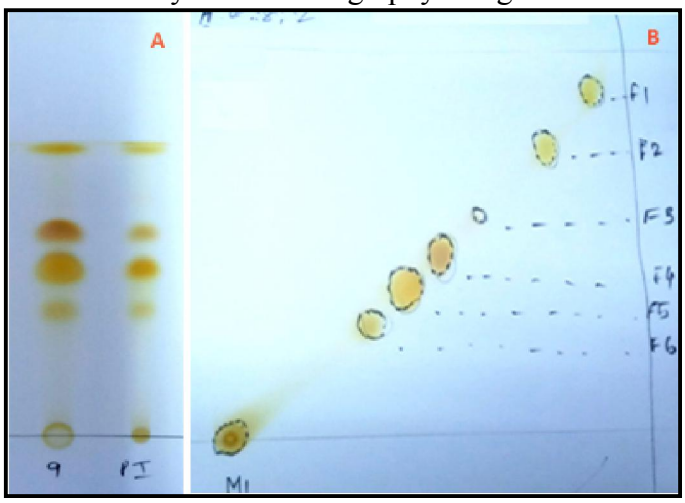

Fig. 59 **TLC showing 1D (A) and 2D (B) chromatogram of CrM fractions.**

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In-Vivo Bioassay

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Gas Chromatography Mass Spectroscopy (GC-MS)

7.2.2 BIOCHEMICAL TEST

Total Phenol Content and antioxidant assay by DPPH and ABTS was performed.

7.2.2 (A) Total Phenol Content

Folin-Ciocalteu method was used for the quantification of phenol in the three different polarity fractions of the inflorescence extract. 0.1ml of test sample was added to Folin-Ciocalteu reagent with sodium carbonate. The concentration range from 0.05mg/ml to 0.3mg/ml was test using Gallic acid as the standard phenol. After incubation for 30 mins, the absorbance at 765nm was noted and the results expressed as Gallic acid equivalents (GAE)/ gram of the test sample.

7.2.2 (B) Antioxidant activity:

(i) DPPH method:

The radical scavenging test was done using commercially available free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The DPPH solution was freshly prepared in ethanol at 0.1mM. 0.1 ml of plant sample dissolved in ethanol at concentration ranges 0.05mg/ml - 0.3 mg/ml was added to 3.9ml of the free radical solution and the absorbance read at 517 nm. Butylated Hydroxy Toluene (BHT) is a commercially available antioxidant and used as a standard. A trial with ascorbic acid as a standard was also applied but BHT showed a reliable reading and could be used in the same concentrations as those for the samples. The test sample scavenging potential and the IC50 are stated by the following equations:

$$DPPH\ radical\ scavenging\ (\%) = \{A_{blank} - A_{test\ samples}/A_{blank}\} \times 100$$

$$IC50 = \{\%\ Inhibition\ of\ the\ free\ radical/Concentration\ of\ the\ sample\} \times 50$$

(ii) ABTS method:

ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] is a commercially available free radical which can access the antioxidant activity of compounds of versatile polarity and chemical nature and at wide range of pH. The method described by Re *et al*. (1999) is used for the analysis.7mM ABTS solution was mixed with 2.4mM of potassium per sulphate in equal quantities. The mixture was allowed to incubate for 12hrs at room temperature. Following incubation the mixture was diluted with 60ml methanol/ml of ABTS. The plant extracts at concentrations 0.05mg/ml to 0.03mg/ml in different aliquots was tested with 0.1ml of the sample added with 3 ml of the ABTS solution. The absorbance reading was recorded after 7mins at 734nm. Butylated Hydroxy Toluene (BHT) was used as the standard and the scavenging capacity of the extract and the IC50 are stated by the following equation:

$$DPPH\ radical\ scavenging\ (\%) = \{A_{control} - A_{test\ samples}/A_{control}\} \times 100;$$

$$IC50 = \{\%\ Inhibition\ of\ the\ free\ radical/Concentration\ of\ the\ sample\} \times 50$$

7.2.3 IN-VITRO BIOLOGICAL ACTIVITY

7.2.3 (A) Antibacterial Screening

Well diffusion assay was applied to test the antimicrobial activity with the three fractions of the inflorescence extract (CrP, CrCh and CrM) of *C.rotundus* using tryptic soya agar plates. The plant samples at concentration range 1mg/ml to 0.01mg/ml were assayed for eight different fish pathogens viz; *Aeromonas hydrophila* (MK6) b. *A. veronii* (MK4) c. *A. popoffi* (MEE2) d. *Pseudomonas putida* (BGBG3) e. *Edwardsiella tarda* (CGH9) f. *Citrobacter freundii* (M7) g. *Bacillus safensis* (MOH1), and

Streptococcus agalactiae (TBT1). The culture media and glassware were sterilized at 121°C for 20 min. The annular radii of the zone of inhibition was noted and graphed.

7.2.3 (B) Cytotoxicity test – Brine shrimp Lethality Test

The plant extract was diluted for five ratios from 10000 ppm – 1 ppm with ethyl acetate as control. Brine shrimp (*Artemia salina*) eggs were obtained from Department of Mathematical biology, Jadavpur University, Kolkata, West Bengal. The eggs were reared in artificial seawater prepared by 35% - 38% of sea salt with a partition for dark (covered) and light effects for a period of two days. Once hatched the nauplii were attracted towards light.10 shrimps were added in each of the dilutions. After 24hrs the mortality of the shrimps were recorded in each dilutions. The fractions were assayed on the nauplii at exposure time of 1h, 6h, 12h, 18h and 24h using brine as the control and the organic solvent as the opposite.

7.2.3 (C) Anti-algal activity

The anti-algal activity was initiated after microscopic colony identification at two different experimental design each with four concentration ranges from 0.05mg/ml – 0.5mg/ml of the plant extract for treatment set and a control set under suitable algal growth parameters of 90 µmol m^{-2} s^{-1} of photons (light : dark = 14hrs:10 hrs) at 25°C for 96 hrs.Each treatment set in triplicate consisted of 3 ml of algal inoculums, 142 ml of BG11 culture medium and 2ml of methanol fraction [F6/CrM] of inflorescence of *C. rotundus*. The instance of adding the plant extract defined the experimental designs with the first being preventive (CmP) where it was added at 0hrs prior to algal inoculums and the latter, therapeutics (CmT), added after visible algal growth (approx. 48hrs). The exact measure of plant extract in the control set was substituted with distilled water. The anti-algal activity for each experimental design was estimated by their total and

segregated (Chl-a, Chl-b & Chl-c) chlorophyll content as per the protocol in APHA with negligible alterations. Post 96hrs, the algal cultures were filtered through solvent washed 47mm glass fibre filter (Whatman GF/C or Gelman AE) with Gelman polycarbonate filtration with vacuum <8 PSI and refrigerated in 10ml acetone. Premature phaeophytinization was prevented by addition of saturated $MgCO_3$ buffer solution prior to the filtration. The samples were tethered with a manual homogenizer and centrifuged at 2000rpm for 20mins. The supernatants containing the pigments were measured photometrically at 664 nm, 647 nm and at 630 nm. The turbidity correction was done at 750 nm. The standard equations for the calculation of Chlorophyll a, b and c are as follows:

- C_a = 11.85*(Abs 664) – 1.54*(Abs647)-0.08*(Abs 630)
- C_b = 21.03*(Abs 647) – 5.43*(Abs664)-2.66*(Abs 630)
- C_c = 24.52*(Abs 630) – 7.60*(Abs647)-1.67*(Abs 664)

C_a, C_b and C_c are concentrations of Chlorophyll a, Chlorophyll b and Chlorophyll c respectively, mg/L, and Abs at 664,647 and 630 is the corrected optical density at respective wavelength with light path of 1cm.

$$\text{Chl- (a/b/c), mg/m}^3 = \frac{C_a/C_b/C_c \ * \text{extract volume (L)}}{\text{Volume of the sample (m}^3\text{)}}$$

7.2.3 (D) Duckweed Assay

The duckweed assay was performed by root cuttings of *Spirodela polyrhiza*. The plant samples were collected from Cantonment pond, Barrackpore, Kolkata and cultured in laboratory using Steinberg medium. The roots of the laboratory grown *S. polyrhiza* were cut completely from the thallus and three fronds are left in each treatment applied in 20ml quantity in four different concentrations 1000ppm -10ppm including positive

control C+ as Double distilled water and negative control as methanol. The scissors used for the purpose and all the necessary glassware were sterilized at 121^0C for 15 minutes.

Pangasianodon hypophthalmus (Sauvage, 1878) is an extensively cultured fish species in aquaculture and are great economic resources to nation and diseases caused to this fish causes hefty loss to fish farmers. Hence this fish was taken for this research.

***Biological Classification**:*

Kingdom	Animalia
Phylum	Chordata
Class	Actinopterygii
Order	Siluriformes
Family	Pangasiidae
Genus	*Pangasianodon*
Species	*P.hypophthalmus*

Taxonomical features:

The fish is a native to Chao Phraya and Mekong of Thailand (Asia) and exotic to India. Posterior nostril located near anterior nostril, barbells very small or even absent & 9 pelvic-fin rays. It has a synonym of *Pangasius sutchi*. It is distinguished from similar species by the number of Pelvic-Fin rays which is 8-9 in number. It has 6 branched dorsal fin rays & two black stripes along and below lateral line respectively. Omnivorous and feeds on fish and crustaceans as well as on vegetable debris It is of non-aggressive behaviour and extensively cultured in cage cultures (FAO Species Identification field guide; Mekong River Commission).

Experimental set up:

The juvenile Pangasius fish used for experiment were maintained of almost uniform length of 4inch to 5inch with body weight of average 7±0.8gms. Glass aquariums 3ftx2ftx2ft were used holding around 25lts of water with 10 fish each. The feed was usually given at 3% of body weight of granular size of 0.8 mm with 41% protein content. The fish were challenged with 10µl/gm of body weight with 0.5 McFarland equivalent bacterial suspension of *Aeromonas veronii* corresponding to approx cell density of 1-1.5x10^8 CFU/ml. The 0.5 McFarland standard was prepared by mixing 0.05ml of 1% barium chloride with 9.95ml of 1% sulphuric acid, but as commercially available sulphuric acid are usually 98% stock hence 1% was enhanced to 1.2%. Four different experimental groups were maintained viz; a control group with normal feed {KP (+)} and without being challenged, a pre feeding group which was fed with a feed mixed with the compound at 80mg/kg of feed seven days prior to challenge (PF-I), a third group was included which was feed with the same mixture but after the challenge post infection symptoms (PI-F) and finally a negative control {KP (-)} which was challenged and maintained with normal feed.

Haematology:

After 96hrs of challenge, the fish were anesthetized with clove oil 50µl/l and blood sample was collected in 20µl heparin contained vials via caudal vein by BD insulin syringe. RBC and WBC diluting fluid was used to measure the Haemoglobin (Hb), White Blood Cells (WBC) and the Red blood Cells (RBC), and were measured by a haemocytometer Neubauer chamber under the light microscope blood. The Hb was measured using a plane haemometer by mixing the blood with 0.1N HCL solution and

diluting it until the colour matches exactly with the comparator tube provided as the sides of the haemometer.

Histopathology:

The histopathological observations were done on sections of fish liver and kidney tissues of the experimental fish population. Fish samples were excised after applying clove oil as anaesthetic agent at 2ml/5ml of water in the tank. As the fish had to be sacrificed, the blood was collected by tail ablation. The liver and kidney samples were collected in 10% neutral buffer formalin (NBF) for 24hr. Following this, tissues were excised and dehydrated in ascending alcohol series from starting from 50% for 5 times each for an hour followed by xylene for 15mins, xylene-paraffin 50% for 30mins, paraffin for 3hrs with three changes per hour and finally inserted in paraffin block. Microtome sectioning with size selection at 5 mm – 8mm was done. The staining protocol was done by downgrade and upgrades varying percentage of alcohol and finally stained with haematoxylin-eosin (HE). At least five continuous paraffin sections were obtained from each block and dispensed in hot water to relieve stretches and collected in albumin rubbed slides. The slides were observed under compound microscope after mounting with DPX. The presence of histopathological alterations for the two organs was evaluated for any changes caused due to infection.

7.2.5 STRUCTURAL IDENTIFICATION – GC-MS

The Gas Chromatography Mass spectrometry was outsourced from Krish Biotech (OECD GLP Certified Facility), Nadia, West Bengal. The GC-MS/MS with ID No KBR/CHM/GLP-92 of Agilent Technology was used for the analysis. Acetonitrile was used as the solvent. Samples were diluted in 2mlof the solvent followed by sonication for 20mins and vortex for 2mins. 1ml of the supernatant was filtered through 0.22µm

syringe filter. Each sample diluted 100times with the solvent and transferred to HPLC glass vial for the GCMS analysis. The references for the analysis was from NIST standard reference database 1A.

GC – Parameters:

Column	HP-5 Capillary Column
Carrier Gas Flow	He, 1.2ml/min
Injection Volume	2µl
Injection Mode	Spilt Mode; 2:1
Source Temperature	230°C
Electron Energy	70eV
Detector	Mass Spectrometry

7.3 STATISTICAL ANALYSIS

The interpretation of the biological activity of plant extracts and the purified compounds isolated from them needs to be summarised quantitatively in order to draw conclusive equations for any random variables.

The antioxidant assay model summary tabulates the Pearson's r correlation value for linearly correlated data and Spearman's Rho for non parametric data. The hypothesis examines the relationship graph of the percent scavenging/inhibiting activity of the free radicals in plant fraction with positive control through defined concentration from 0.05µg/ml to 1µg/ml. The R^2 value also known as shrunken R^2 is used as the unbiased estimator of the population. Its perceived utility is across varies research areas and time. Shapiro-Wilk significance test displayed by stem loop model predicts us the normal distribution of the data over a target variable. Kolmogorov-Smirnov Test (KS Test) assesses the circumstantial difference of data collection and their impact in outcome, mostly relevant in experiments with control set. Probit chi-square, Z-statistics and F-

value finds the goodness fit model and difference of means of the variables under consideration.

7.4 RESULTS

7.4.1 BIOCHEMICAL ASSAY

7.4.1 (A) Total Phenol Content (TPC):

The results are expressed in GAE/g of the sample (Fig. 60) using standard Gallic acid. The CrM fraction of the inflorescence was seen to have the highest phenol content followed by chloroform and Petroleum ether fraction. Phenol content represents as a concentration dependent parameter with highest readings at 0.3 mg/ml and lowest at 0.05 mg/ml. The coefficient of determination denoted as R^2= 0.982 showed the curve fit cubic model with equation $y = 0.197x^2+1.910x-2.941$.

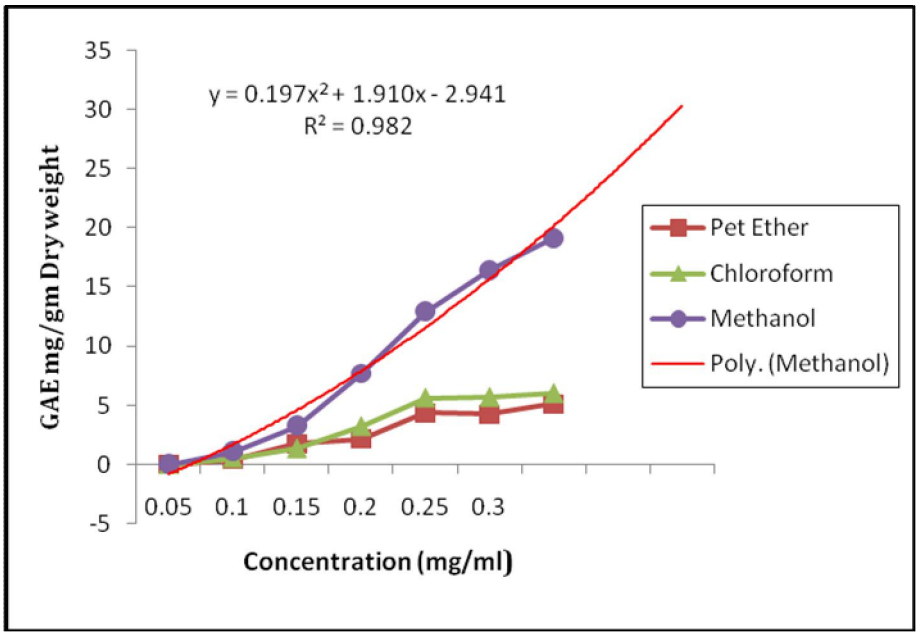


Fig. 60 **CrM, CrCh & CrP fraction of the inflorescence of *C.rotandus*.**

7.4.1 (B) Antioxidant Radical Scavenging Assay

(i) DPPH method

The antioxidant activity of the inflorescence extract followed similar graphical pattern as that of the phenols. The methanol fraction showed 98% scavenging activity followed by chloroform 94% and pet ether with less than 50% at highest concentrations. The collinear pattern of data representation of antioxidants and phenols in methanol fraction presumes that the phenols are the active scavengers of free radical. (Fig. 61). In all the cases, methanol was used as the blank taking in consideration the fact that DPPH was dissolved in methanol. The best fit curve estimation for methanol and chloroform followed a power equation $y = 5E\text{-}9x^{4.264}$ and $y = 3E\text{-}12x^{6.006}$ respectively and for pet ether fraction it followed an exponential equation $y = 0.027e^{0.030x}$. The IC50 was in the order of Methanol (CrM) < Chloroform (CrCh) <Pet ether (CrP) (Table 6). Hence CrM fraction was biochemically bioactive fraction of the inflorescence of *C.rotundus*.

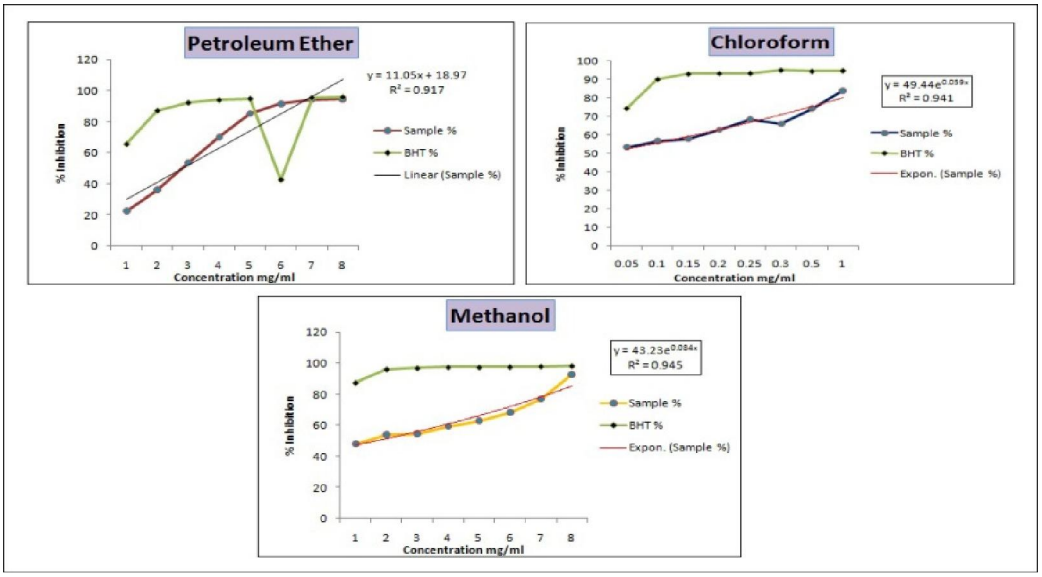


Fig. 61 **Antioxidant activity of CrP, CrCh and CrM fractions of the inflorescence of *C.rotandus* using DPPH.**

The relationship graph for % scavenging distributed through a range a concentration is statistically evaluated by IBM SPSS 20 which is detailed under Statistical analysis section.

Parameters	Pet Ether	Chloroform	Methanol
Curve Fit Model	Exponential	Power	Power
Equation	$y = 0.027e^{0.030x}$	$y = 3E\text{-}12x^{6.006}$	$y = 5E\text{-}9x^{4.264}$
IC50	0.121mg/ml	0.0479mg/ml	0.0878mg/ml

Table 6: **Antioxidant activity with IC50 value of CrP, CrCh & CrM of *C. rotundus***

(ii) ABTS Method:

The results in ABTS methods shows Cyperus inflorescence methanol fraction (Fig. 62) to possess the highest antioxidant with IC50 0.197µg/ml, followed by Chloroform with IC50 at 0.228µg/ml. The petroleum ether fraction had the least and weakest antioxidant activity at IC50 70.8µg/ml. For reference, the standard BHT had an IC50 at 0.0017µg/ml. Except for the non-polar fraction; the results have uniformity with that of the DPPH assay.

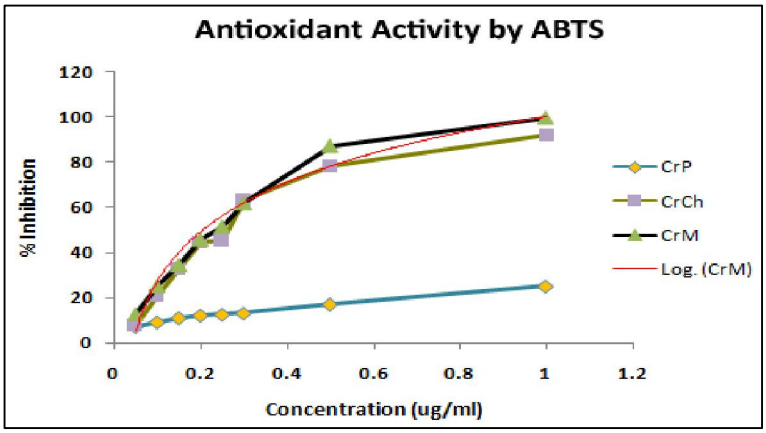


Fig. 62: **Antioxidant activity of CrP, CrCh and CrM Fraction of the inflorescence of *C.rotandus* using ABTS.**

7.4.2 IN-VITRO BIOLOGICAL ASSAY

7.4.2 (A) Antibacterial activity

The steady zone of inhibition was recorded for *C.freundii* for the entire three fractions with highest being 20mm of CrCh followed by CrM. CrCh (Fig. 63, 65) and CrP (Fig. 63, 66) showed a standard inhibition against *B.safensis* at 500ppm and 1000ppm but results were nullified for methanol. *A.hydrophila* and *A. Popoffi* were inhibited by 100ppm to 1000ppm of methanol extract. CrM exhibited an inhibition zone against *E.tarda* of 14mm at 500ppm which increased to 17mm at 1000ppm (Fig. 63, 64). The dose response curve is non linear cubic in nature following the polynomial equation. The bacterial species taken for microbial activity with their codes are *Aeromonas hydrophila* (MK6); *A. veronii* (MK4); *A. popoffi* (MEE2); *Pseudomonas putida* (BGBG3); *Edwardsiella tarda* (CGH9); *Citrobacter freundii* (M7) ; *Bacillus safensis* (MOH1) and *Staphylococcus aureus* (TBT1). The concentrations of the inflorescence extract are denoted by 100ppm (T1), 250ppm (T2), 500ppm (T3) and 1000ppm (T4).

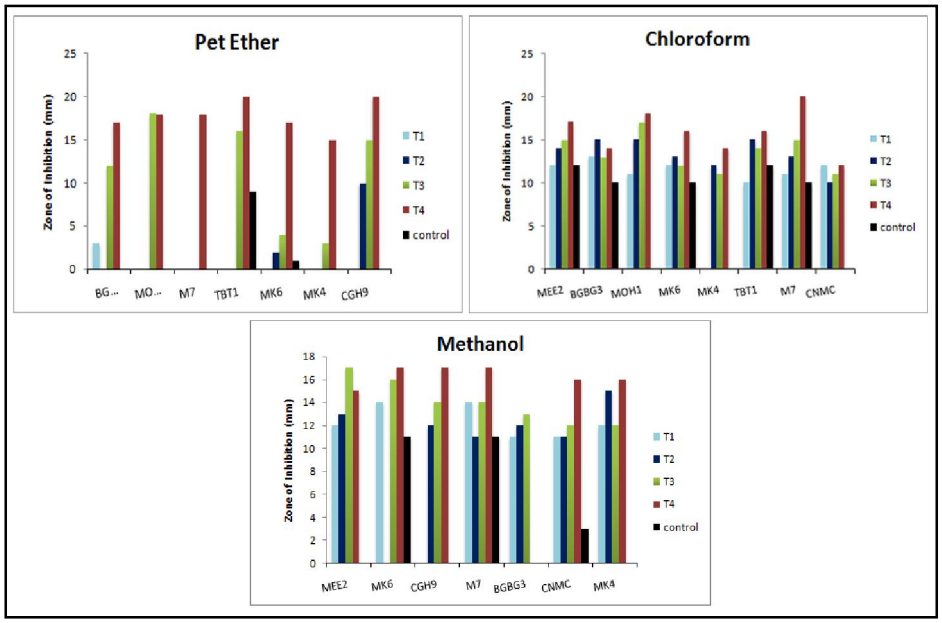


Fig 63: **Graphical representation of the zone of inhibitions against fish bacteria by CrP, CrCh and CrM of *C. rotandus*.**

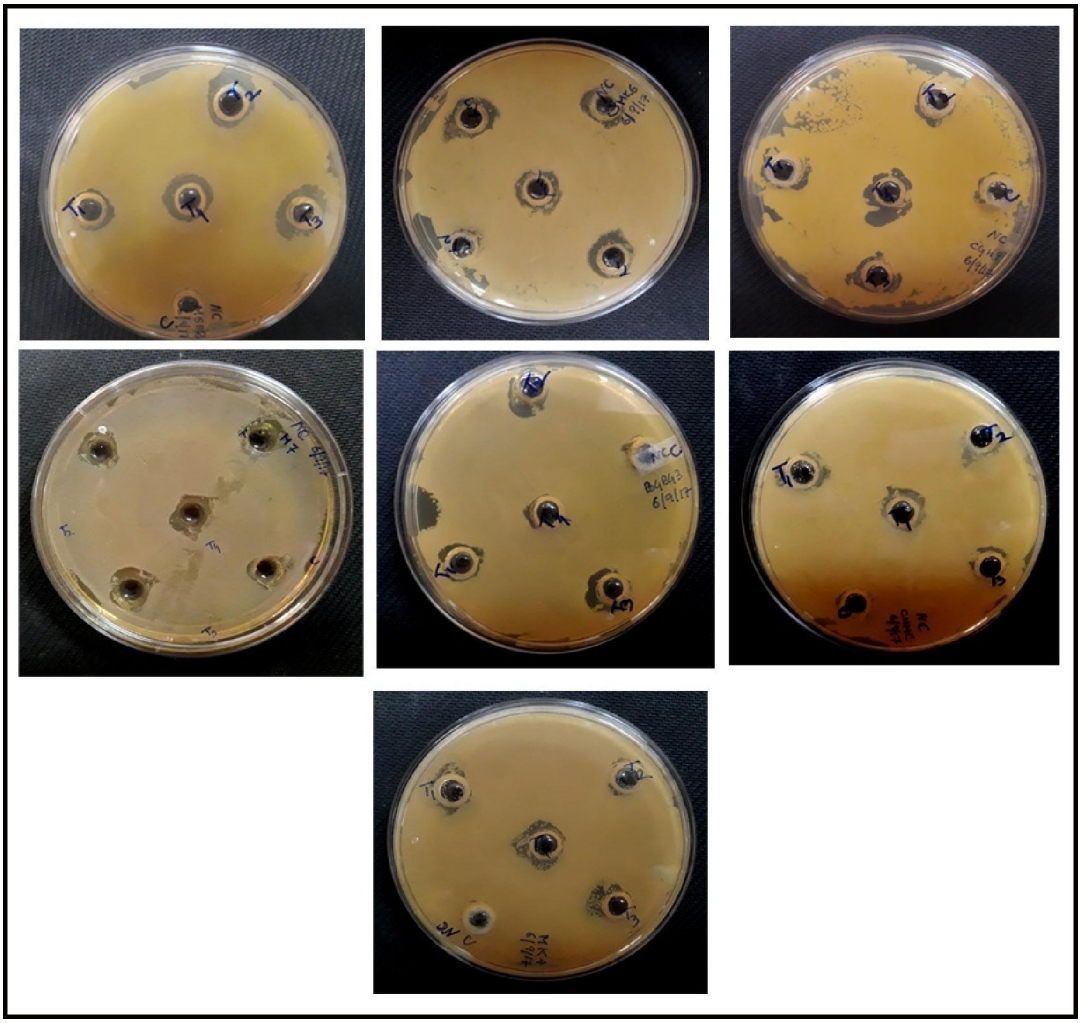


Fig 64: **The antimicrobial assay of methanol fraction (CrM) of *C. rotandus* against seven fish pathogenic bacteria**

Row1	*Aeromonas. popoffi* (MEE2), *A. hydrophila* (MK6), *Edwardsiella tarda* (CGH9)
Row2	*Citrobacter freundii* (M7), *Pseudomonas putida* (BGBG3), *Bacillus safensis* (MOH1)
Row3	*A. veronii* (MK4)

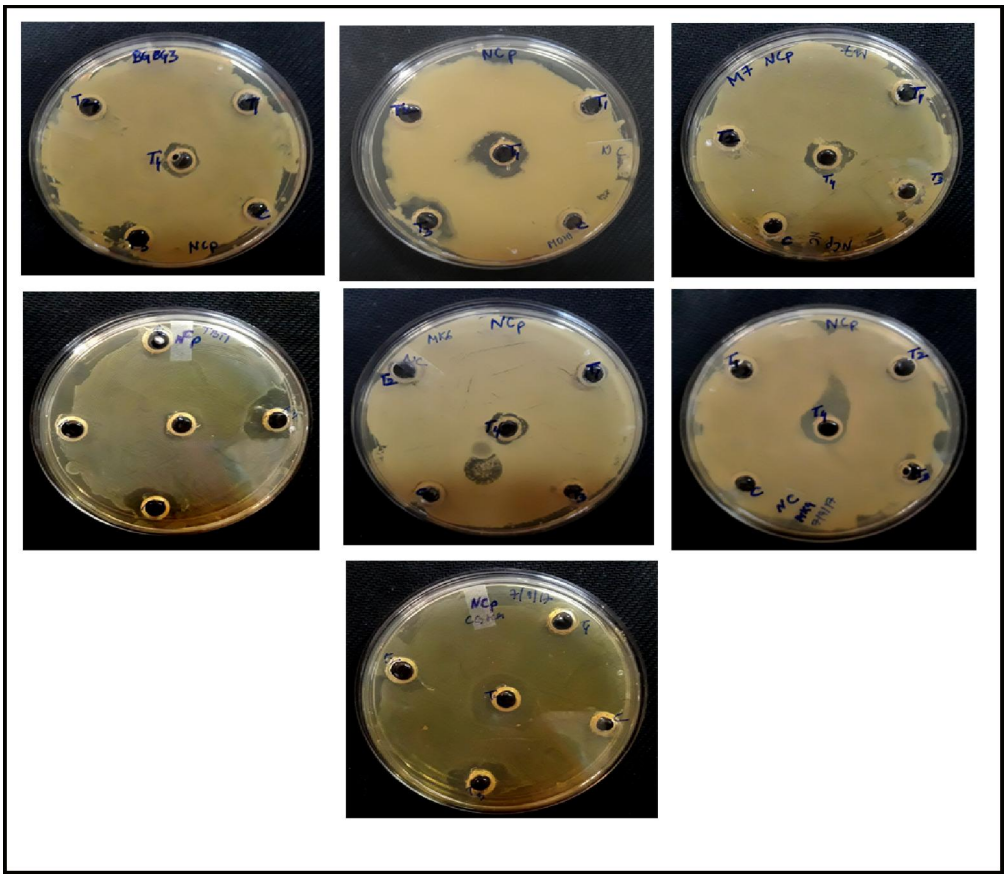


Fig 65: **The antimicrobial assay of Chloroform fraction (CrCh) of *C. rotandus* against seven fish pathogenic bacteria**

Row1	*Aeromonas. popoffi* (MEE2), *Pseudomonas putida* (BGBG3), *Bacillus safensis* (MOH1)
Row2	*A. hydrophila* (MK6), *A. veronii* (MK4), *Streptococcus aureus* (TBT1)
Row3	*Citrobacter freundii* (M7), *K.pneumonia* (CNMC)

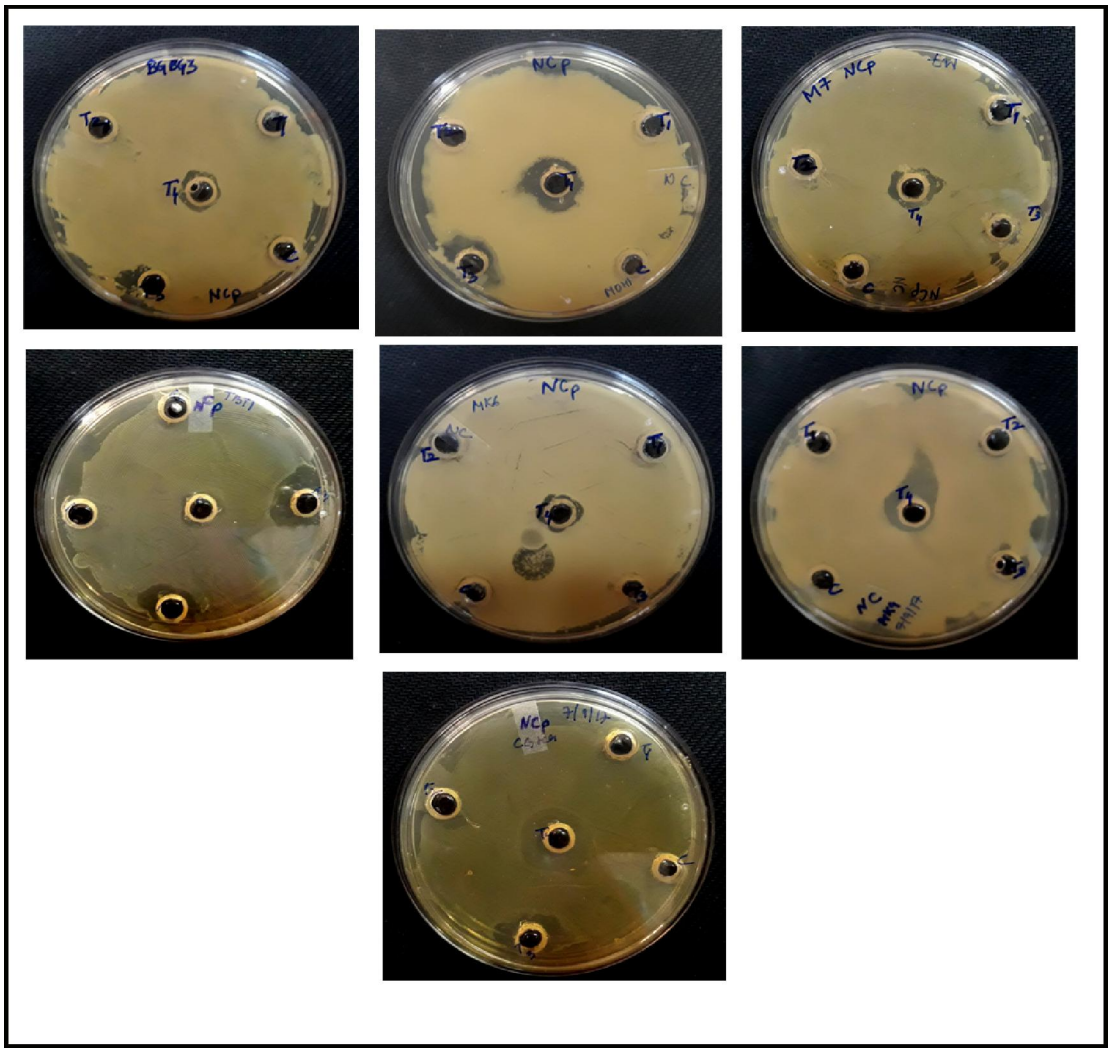


Fig 66: **The antimicrobial assay of Petroleum Ether fraction (CrP) of *C. rotandus* against seven fish pathogenic**

Row1	*Pseudomonas putida* (BGBG3), *Bacillus safensis* (MOH1), *Citrobacter freundii* (M7)
Row2	*Streptococcus aureus* (TBT1), *A. hydrophila* (MK6), *A. veronii* (MK4)
Row3	*Edwardsiella tarda* (CGH9)

7.4.2 (B) Cytotoxicity assay - Brine Shrimp Lethality Test

In case of *C.rotandus*, Pet Ether Fraction and methanol fraction with exposure time 1hr are found to be most toxic with LC25, LC50 and LC75 values at proximity (Fig. 67). Methanol fraction with 24hrs exposure time (M24) is found to be least toxic however chloroform and methanol fraction can be inferred to be gradually ineffective after 18hrs exposure time. 6h, 12h, 18h and 24h-LC50 test of Chloroform and Methanol is stated below. The petroleum ether fraction recorded the highest LC50 at 14195 µg/ml at 1h exposure which explains the non polar fraction could not pervade the nauplii initially and at 24 hrs exposure it records at 36.03 µg/ml. The most toxic was methanol 24hrs exposure with median lethality at 21.04 µg/ml.

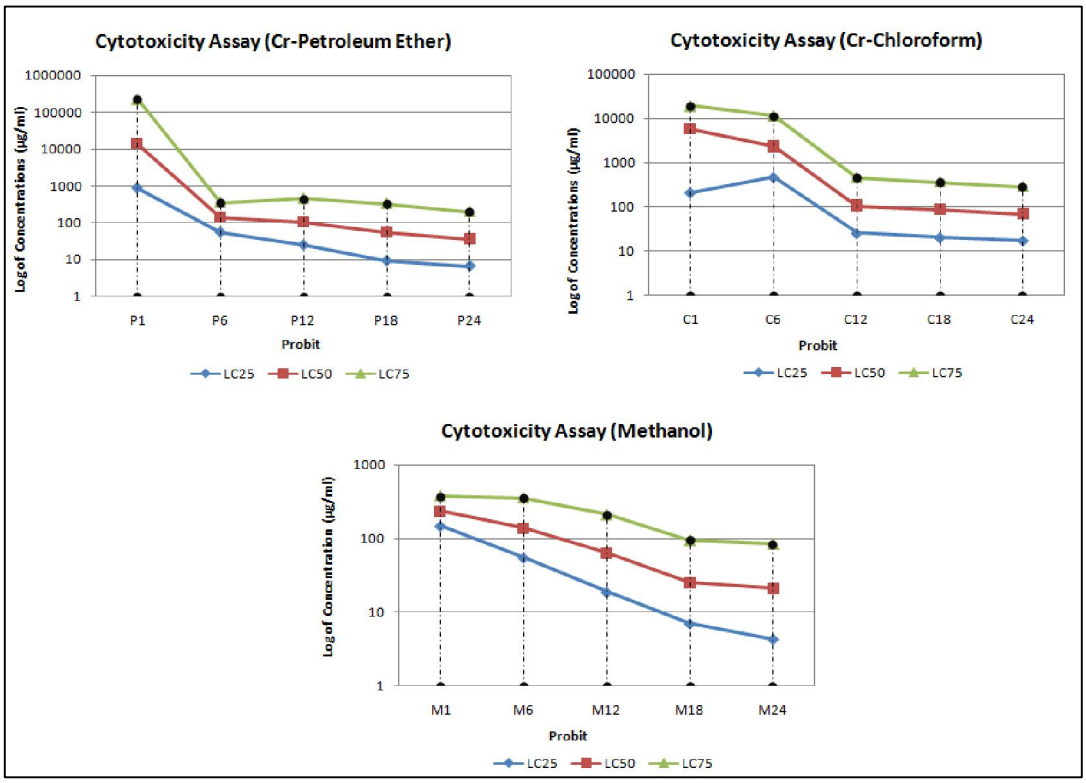


Fig 67: **Graphical representation of LC25, LC50 and LC75 against log of concentration for *C.rotandus* at 1hr, 6hrs, 12hrs, 18hrs and 24hrs exposure time.**

7.4.2 (C) Antialgal assay

The anti-algal activity by chlorophyll assessment displays a similar result like Vallisneria leaf extracts (Fig. 19, 20). The phenomenon of prophylaxis was not found to be as effective as lethality. The control sample with 0µg/ml of extract has the highest chlorophyll content of 0.727 mg/m^3. The chlorophyll content was inversely related to concentration gradient (Fig. 68).

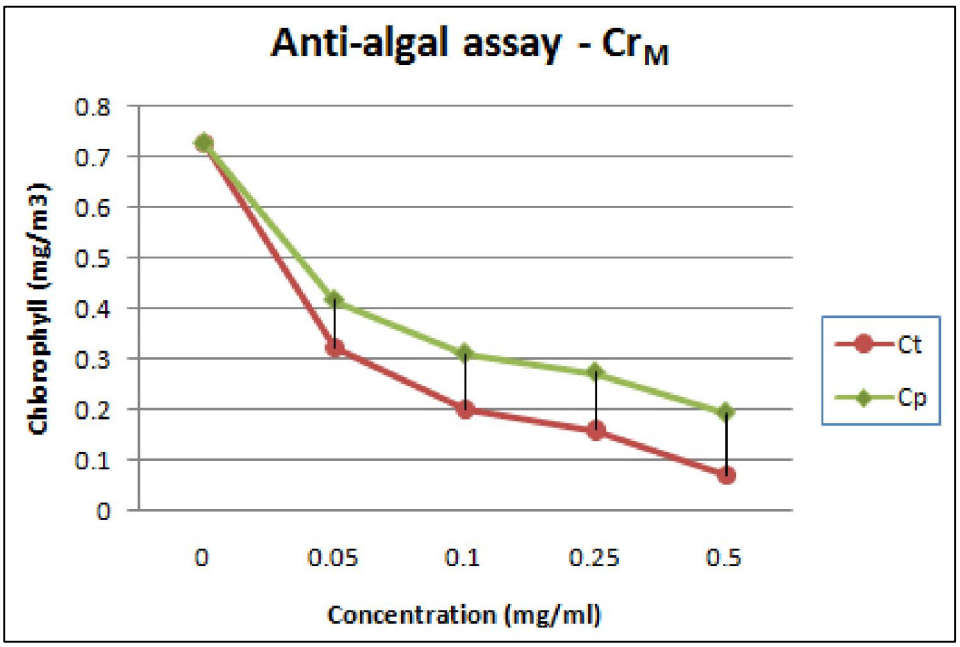


Fig 68: Anti-algal activity of CrM of Cyperus inflorescence extract.

The pigment quantification in the experimental sets infers that the chlorophyll content is inversely related to concentration gradient of the plant extract. The maximum chlorophyll content of 0.738 mg/m^3 was recorded for control with 0 mg/ml plant extract and the lowest at 0.081mg/m^3 and 0.2mg/m^3 in CmP and CmT respectively for 0.5mg/ml plant extract. Chl-a, Chl-b and Chl-c was found to be more in CmT with 60%, 7% and 5% respectively in comparison to 24%, 3% and 1% respectively in CmP (Fig. 69).

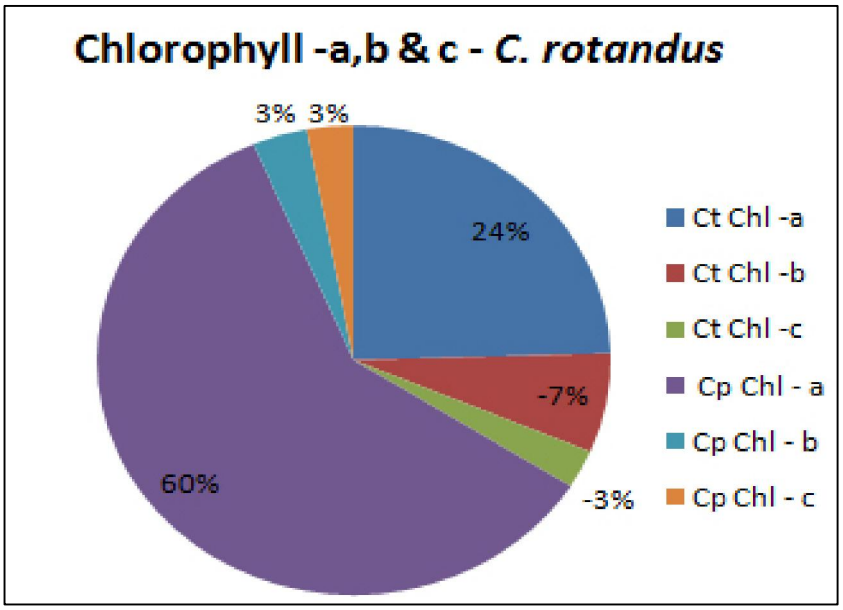
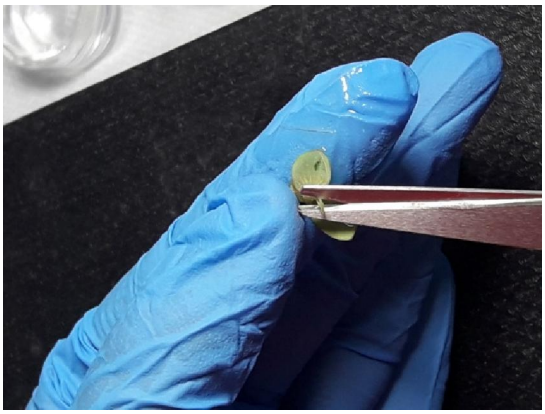
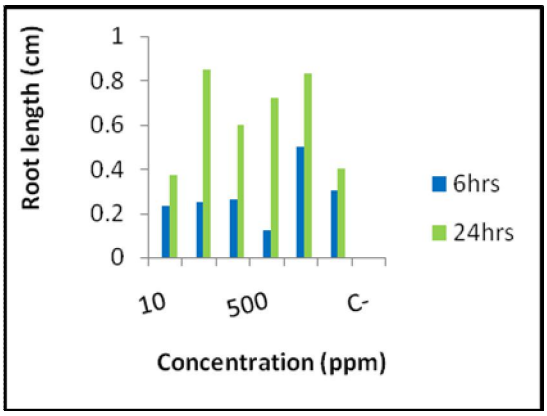


Fig 69: **A pie chart representation of the distribution of Chlorophyll a, b & c for Ct and Cp against *Microcystis aeruginosa*.**

7.4.2 (D) Duckweed Bioassay

The roots started growing after 6hrs of treatment with methanol fraction. The fraction seems to contain a root stimulatory factor with highest growth recorded at 1000ppm for 6hrs and 24hrs of 0.5 cm and 0.83cm respectively. The 50ppm concentration also proved to be noteworthy with 0.25cm and 0.85cm root growth which was higher than 100ppm & 500ppm with 0.60cm and 0.72cm respectively. The thallus toxicity of the treatments were noted which lost its chlorophyll within 2.35 hrs of application (Fig. 70)





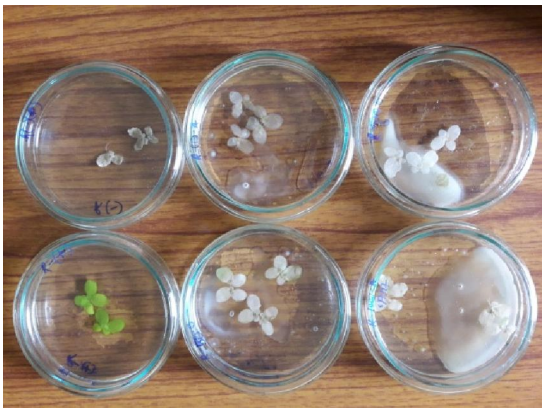


Fig 70: **Clockwise, Root cutting of *S.polyrhiza* under sterile condition; Graphical representation of the regenerated root length at five different concentrations of CrM of *C.rotandus*; *S. polyrhiza* bioassay and Laboratory grown *S. polyrhiza*.**

7.4.3 IN-VIVO FISH BIOASSAY

Fish sample analysis:

The fish samples were examined after 96hrs (Fig. 71).The tank KP(+) showed the uninfected fresh fish (A), Infected fish samples were collected from KP(-) with reddish lesions near the gills, fins and tail rot (D), the PI-F tank though was fed with prepared feed for 3days but it showed acute signs of infection with reddish patches all over the body (B) and finally the tank PF-I showed normal signs with no visible traits of infection (C).

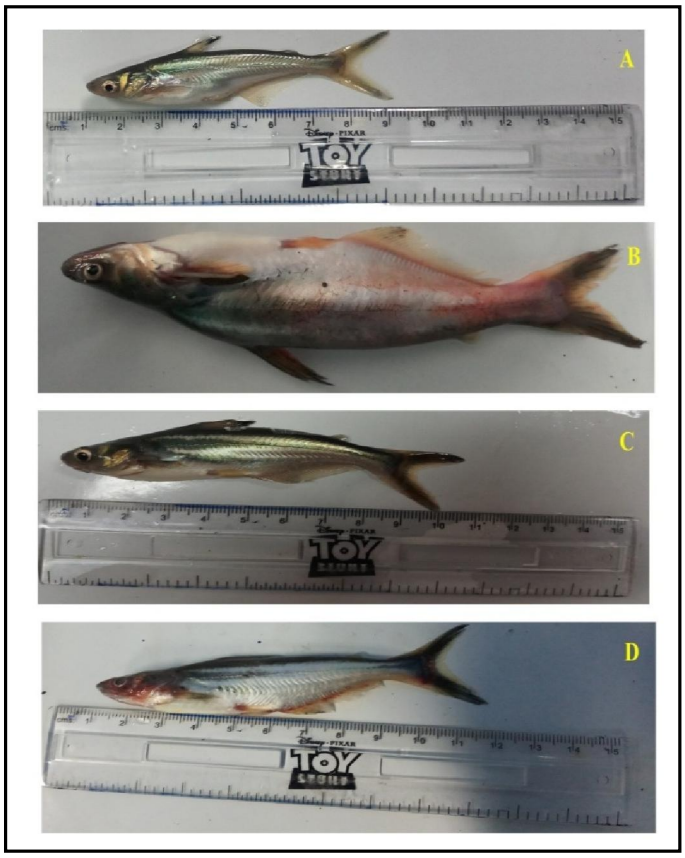


Fig 71: **A: Fish sample from KP (+); B: Fish sample from PI-F**

C: Fish sample from PF-I & D: Fish sample from KP (-)

Mortality Chart:

Treatment Sets	Total No	Mortality	Recovery Percentage
KP (+)	10	0	100
PF-I	10	2	80
PI-F	10	7	30
KP (-)	10	10	0

Haematological parameters:

Parameters	Fresh Fish	PF-I	PI-F	Infected fish
White Blood cells ($x10^3$ µl)	5.34±0.2	6.58±1.6	7.04±1.5	7.53±0.5
Red Blood Cells ($x10^6$ µl)	1.89±0.04	2.15±0.8	1.14±0.4	1.22±0.03
Haemoglobin (g/dL)	7.58±0.5	8.10±0.5	5.84±0.3	5.53±0.4

Histopathology:

Liver

The Normal Liver section (Fig. 72A) is seen with prominent and intact portal triad, bile duct & inter-lobular septum. The main alterations found in the liver are in PI-F (Fig. 72B) where loss of contact between the hepatocyte & pancerocyte, irregular shaped nucleus. In case of PF-I (Fig. 72C) there is not much irregularity seen other than excessive cytoplasmic vacuolation and in infected liver section (Fig. 72D) there are accumulation of melano-macrophage aggregation close to vessels, bile stagnation and nuclear degeneration.

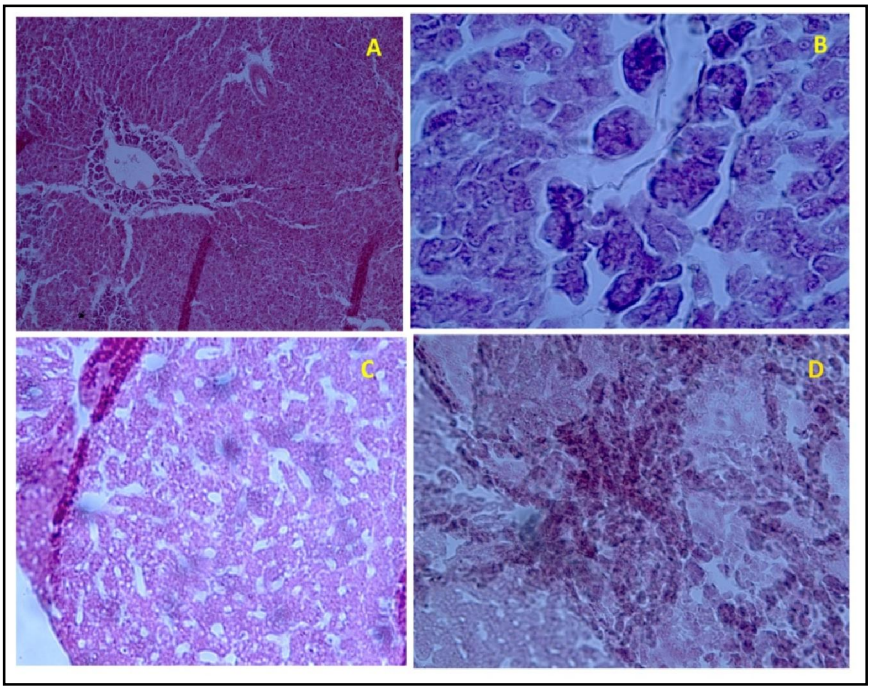


Fig 72: **Sections of *P.hypophthalmus* liver (40X) from four experimental sets. A: Normal Liver; B: PI-F liver; C: PF-I liver & D: Infected Liver**

Kidney

The Normal Kidney section (Fig. 73A) is seen with prominent Glomerulus in the Bowman's capsule space hematopoietic cells, proximal tubules. The main alterations found in the kidney are in PI-F (Fig. 73B) where there is absence of Bowman's space and deformed distal tubules. In case of PF-I (Fig. 73C) there is intact Glomerulus in the Bowman's capsule but there are accumulation of melano-macrophages (Fig. 73 D1 & D2) there are extensive melano-macrophage aggregation close to tubules and the tubules are highly dilated.

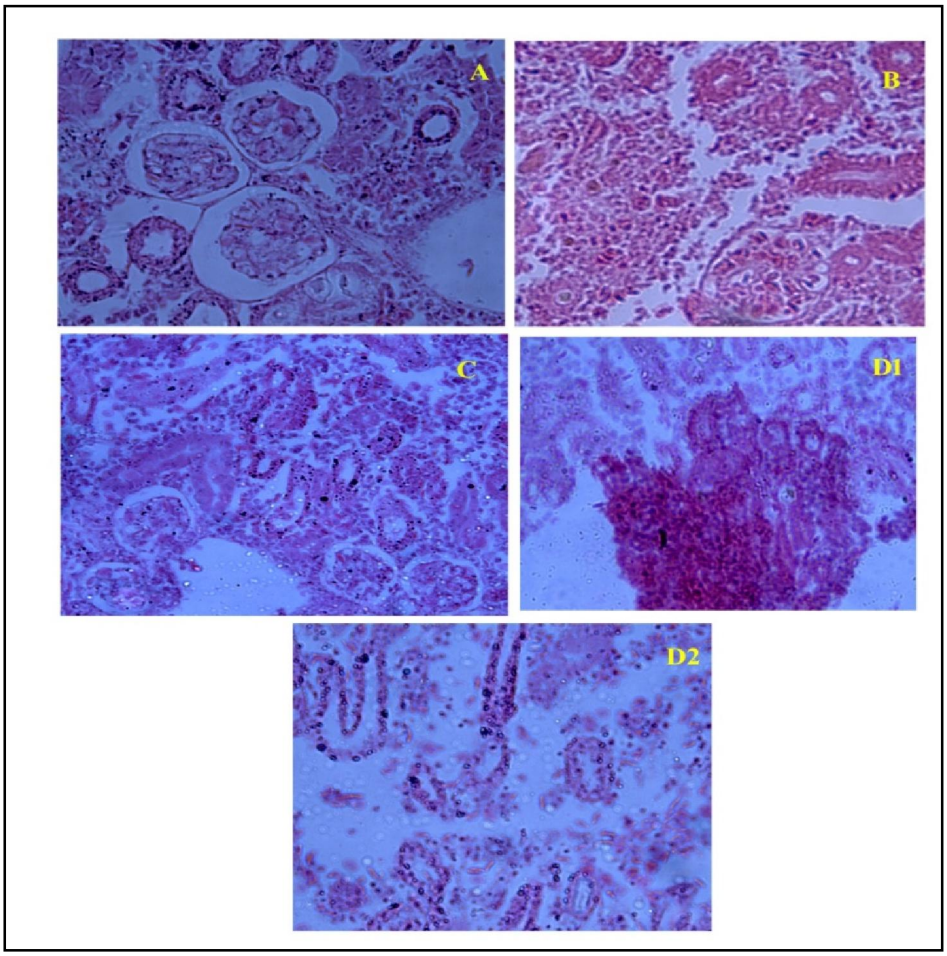


Fig 73: **Section of *P.hypophthalmus* kidney (40X) from four experimental sets. A: Normal Kidney; B: PI-F Kidney; C: PF-I Kidney & D1, D2: Infected Kidney**

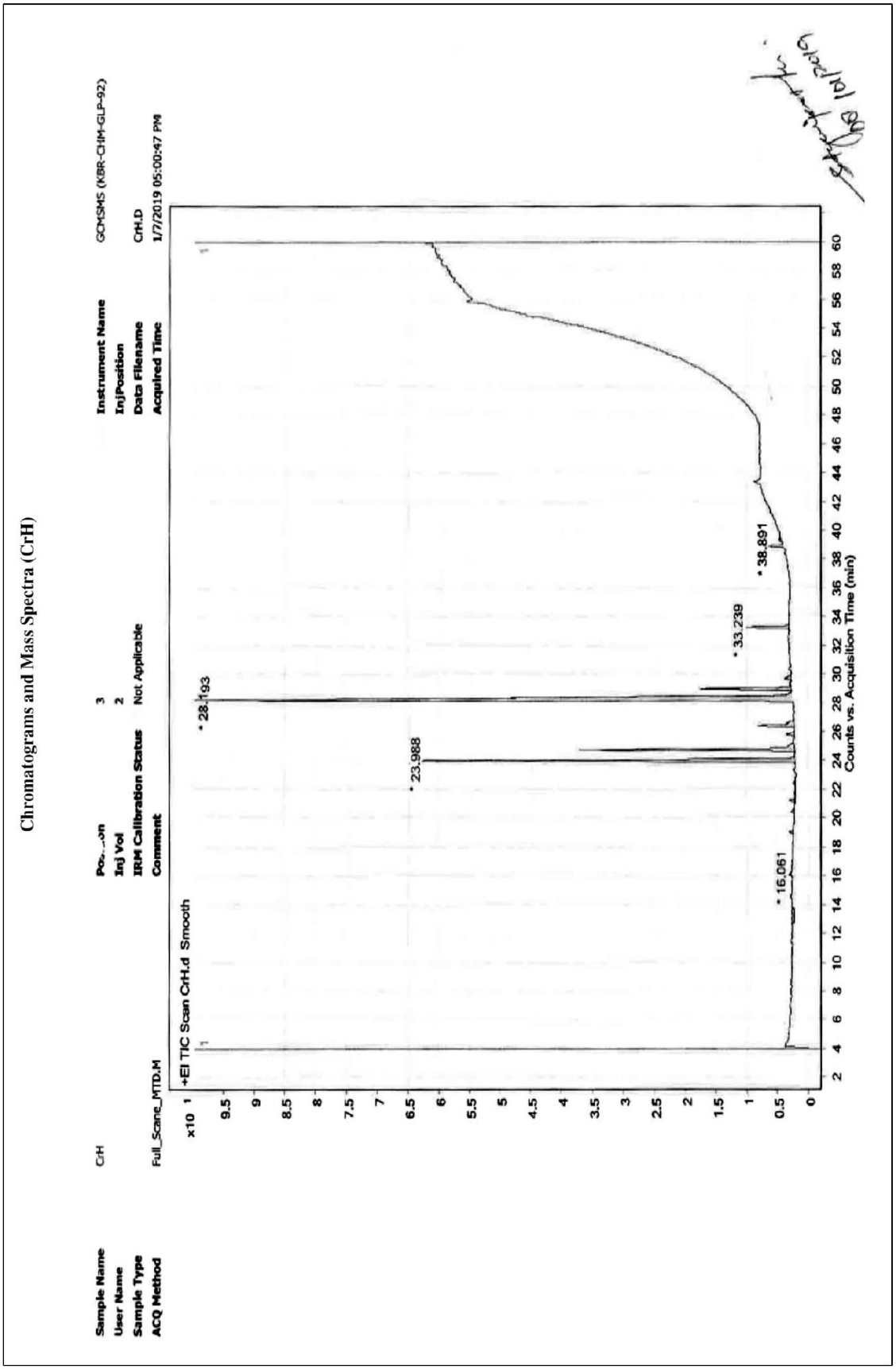
7.4.4 STRUCTURAL IDENTIFICATION – GCMS

The following peaks are identified as important constituent of the bioactive fractions CrP & CrM isolated from the inflorescence of the *Cyperus rotundus.*

Peaks: +Total Ion Chromatogram Scan – CrP							
Peaks	**RT (mins)**	**MF**	**RMF**	**Area %**	**MW**	**Chemical formula**	**Chemical Name**
9	23.988	871	880	43.24	270	$C_{17}H_{34}O_2$	Hexadecanoic Acid
12	26.401	732	734	5.53	248	$C_{14}H_{16}O_4$	Mycorradicin
13	28.193	858	870	100	294	$C_{19}H_{34}O_2$	Methyl linoleate
14	28.929	844	848	10.25	298	$C_{19}H_{38}O_2$	Methyl Stearate
16	33.239	687	747	5.12	326	$C_{21}H_{42}O_2$	Eicosanoic Acid, methyl ester
18	39.31	611	663	0.28	296	$C_{10}H_{28}O_4$	Slicic Acid

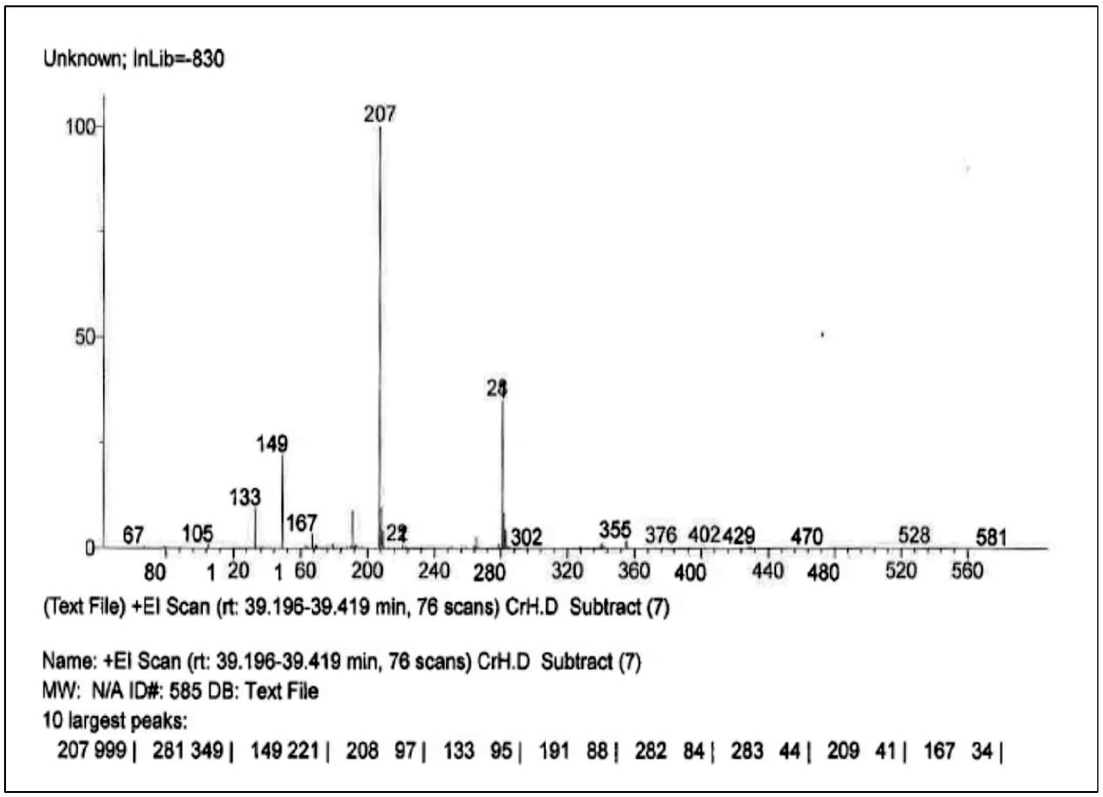
The Chromatogram and the Mass spectra of the important above mentioned peaks are given in the following pages:

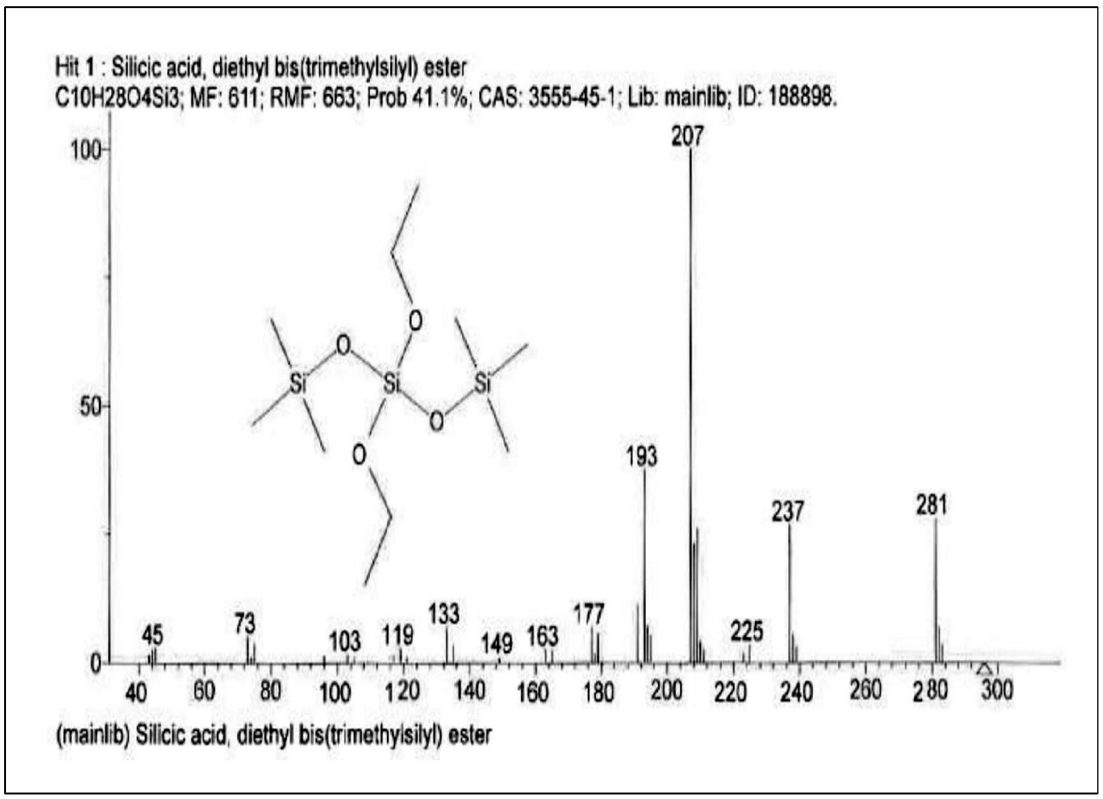
Chromatograms and Mass Spectra (CrH)

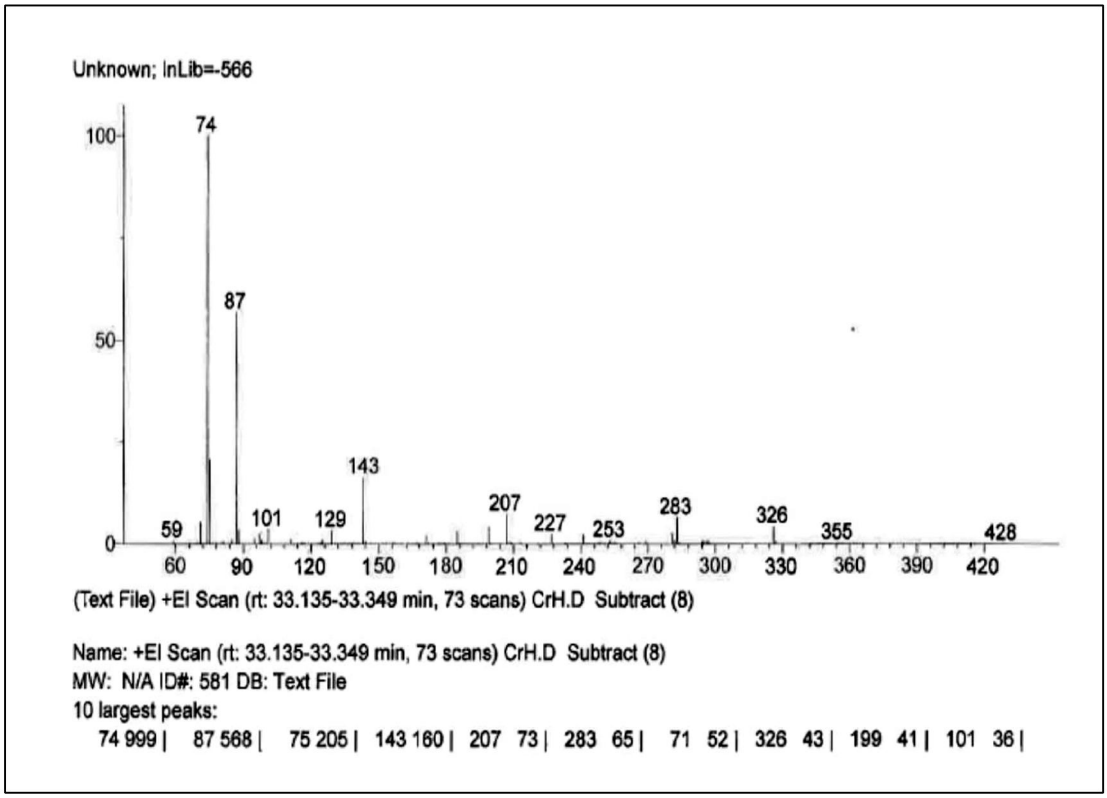


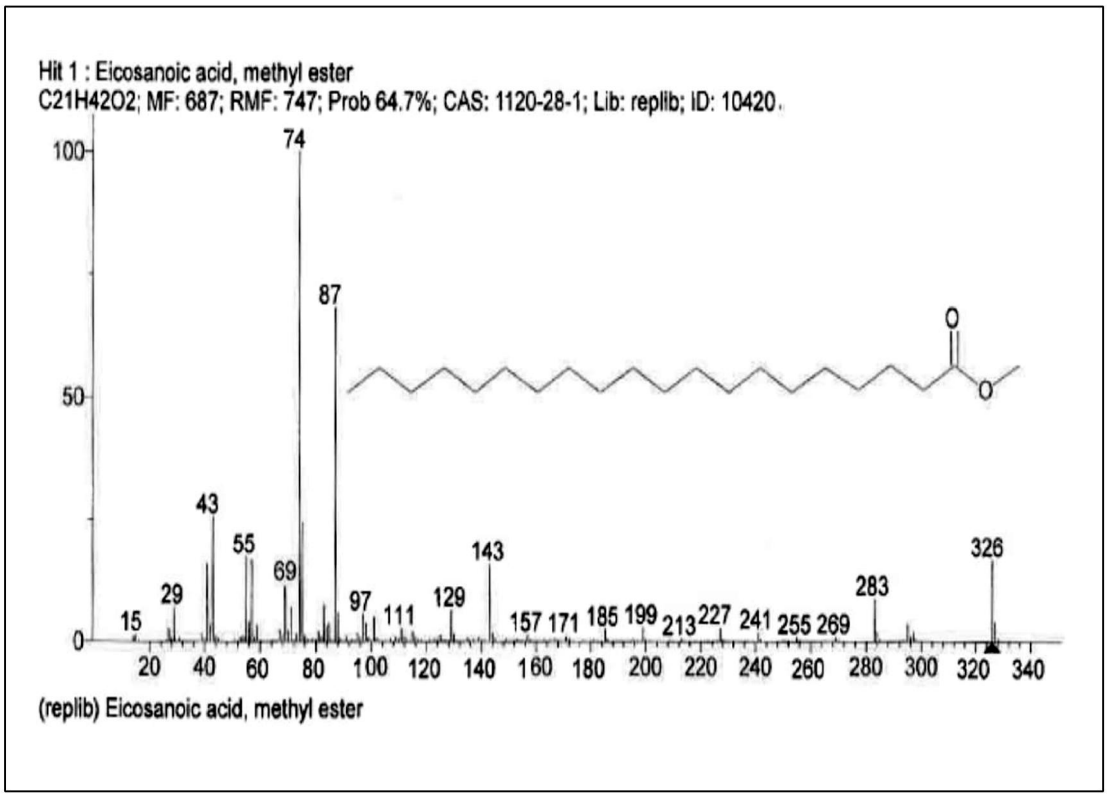
Peaks: + TIC Scan Smo - CrH.D (CrH.D)

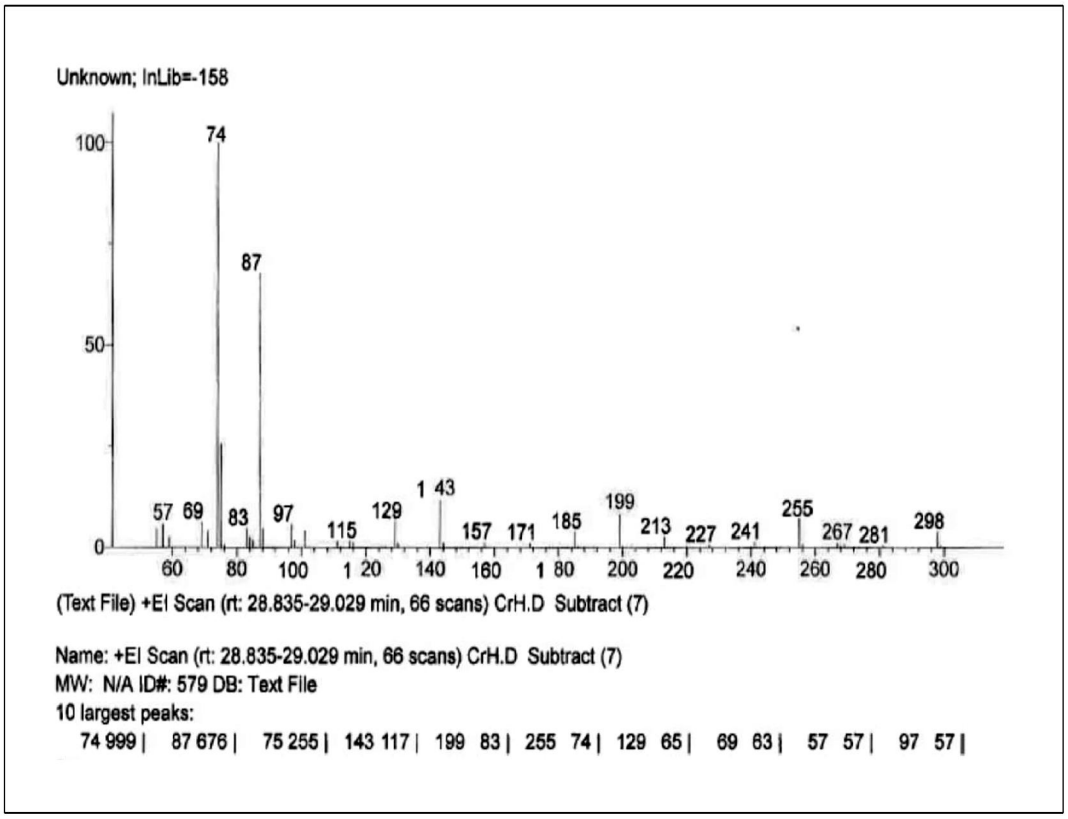
Peak	RT	Area	Area %	Height	Symmetry
1	13.025	8953352.42	1.67	183139.09	2.44
2	16.061	1398166.32	0.26	210142.65	1.09
3	19.02	3032662.46	0.56	347418.36	1.41
4	20.429	2413807.39	0.45	251137.17	1.78
5	21.223	3493795.23	0.65	439833.45	1.04
6	21.696	1021795.82	0.19	104201.56	1.93
7	22.409	1668417.65	0.31	169460.53	1.3
8	23.349	2022798.09	0.38	144692.08	0.77
9	23.988	232443789.4	43.24	28813081.9	1.05
10	24.735	152583817.7	28.38	16552026.8	1.17
11	25.805	5607634.11	1.04	637485.47	1.09
12	26.401	29732952.01	5.53	2820719.91	2.62
13	28.193	537576380.4	100	46252087.8	2.37
14	28.929	55104418.28	10.25	7023092.15	1.04
15	29.659	5836512.51	1.09	527029.84	2.36
16	33.239	27502598.71	5.12	3373332.42	1.06
17	38.891	10164988.64	1.89	1167684.67	1.29
18	39.31	1495051.87	0.28	178778.67	1.18

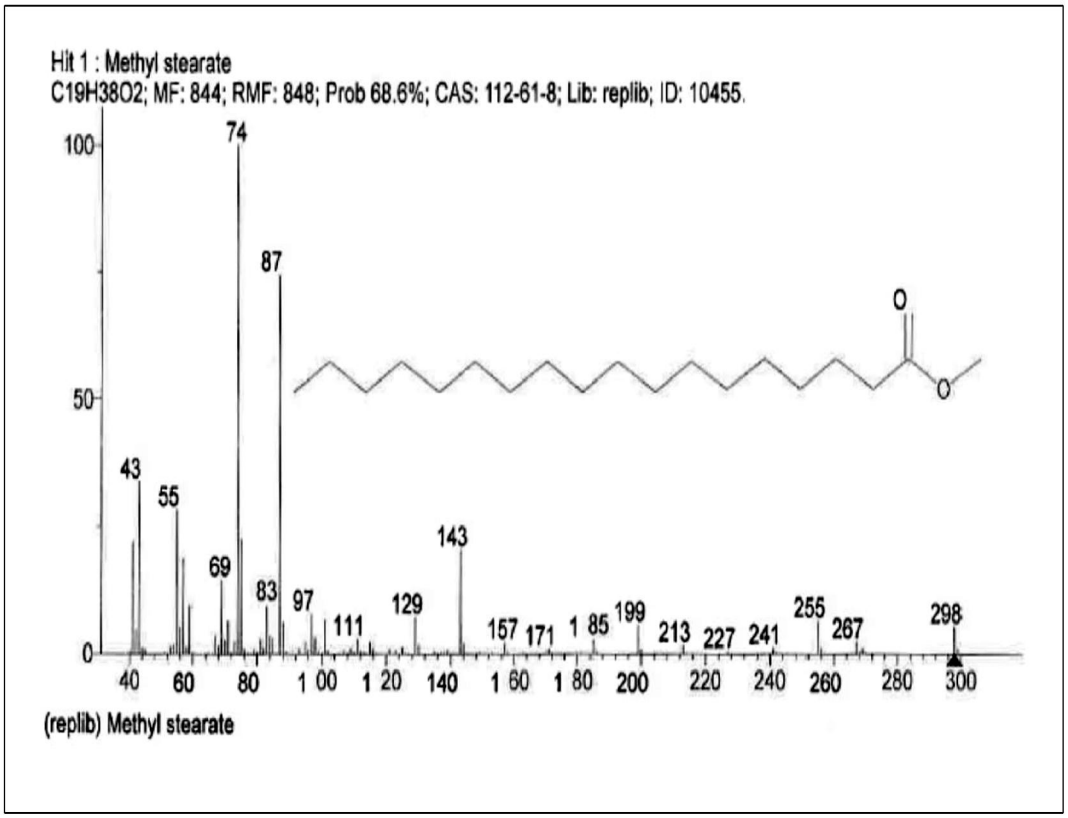

Unknown; InLib=-830
100
50
0
207
149
133
167
67
105
302
355
376
402
429
470
528
581
80
120
160
200
240
280
320
360
400
440
480
520
560
(Text File) +EI Scan (rt: 39.196-39.419 min, 76 scans) CrH.D Subtract (7)
Name: +EI Scan (rt: 39.196-39.419 min, 76 scans) CrH.D Subtract (7)
MW: N/A ID#: 585 DB: Text File
10 largest peaks:
207 999 | 281 349 | 149 221 | 208 97 | 133 95 | 191 88 | 282 84 | 283 44 | 209 41 | 167 34 |

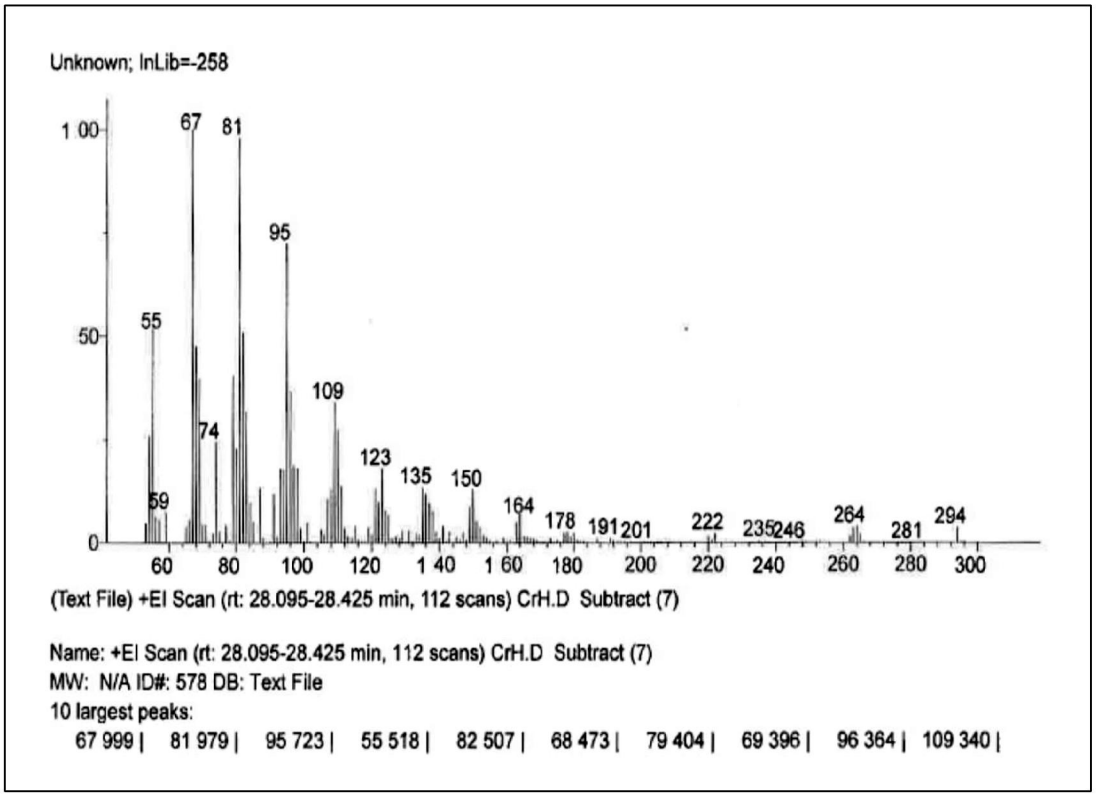

Hit 1 : Silicic acid, diethyl bis(trimethylsilyl) ester
C10H28O4Si3; MF: 611; RMF: 663; Prob 41.1%; CAS: 3555-45-1; Lib: mainlib; ID: 188898.
100
50
0
207
193
237
281
225
177
133
149
163
119
103
73
45
40
60
80
100
120
140
160
180
200
220
240
260
280
300
O
Si
(mainlib) Silicic acid, diethyl bis(trimethylsilyl) ester

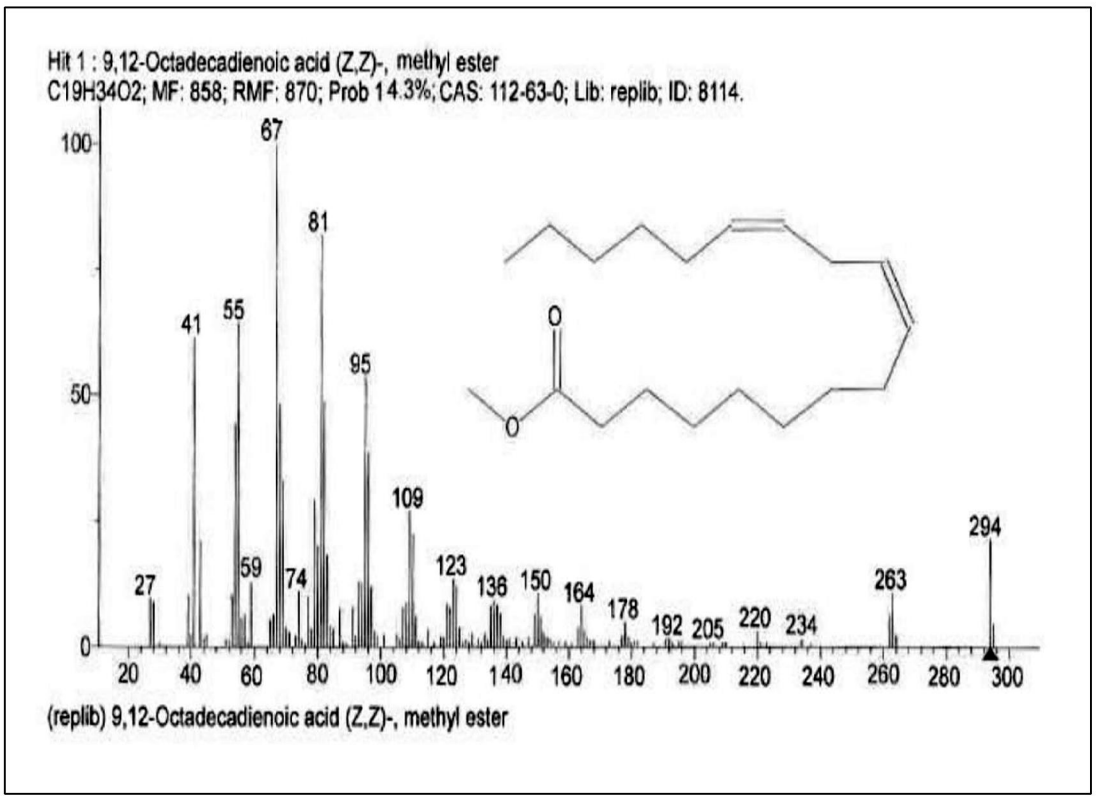

Unknown; InLib=-566
100
50
0
74
87
143
59
101
129
207
227
253
283
326
355
428
60 90 120 150 180 210 240 270 300 330 360 390 420
(Text File) +EI Scan (rt: 33.135-33.349 min, 73 scans) CrH.D Subtract (8)
Name: +EI Scan (rt: 33.135-33.349 min, 73 scans) CrH.D Subtract (8)
MW: N/A ID#: 581 DB: Text File
10 largest peaks:
74 999 | 87 568 | 75 205 | 143 160 | 207 73 | 283 65 | 71 52 | 326 43 | 199 41 | 101 36 |

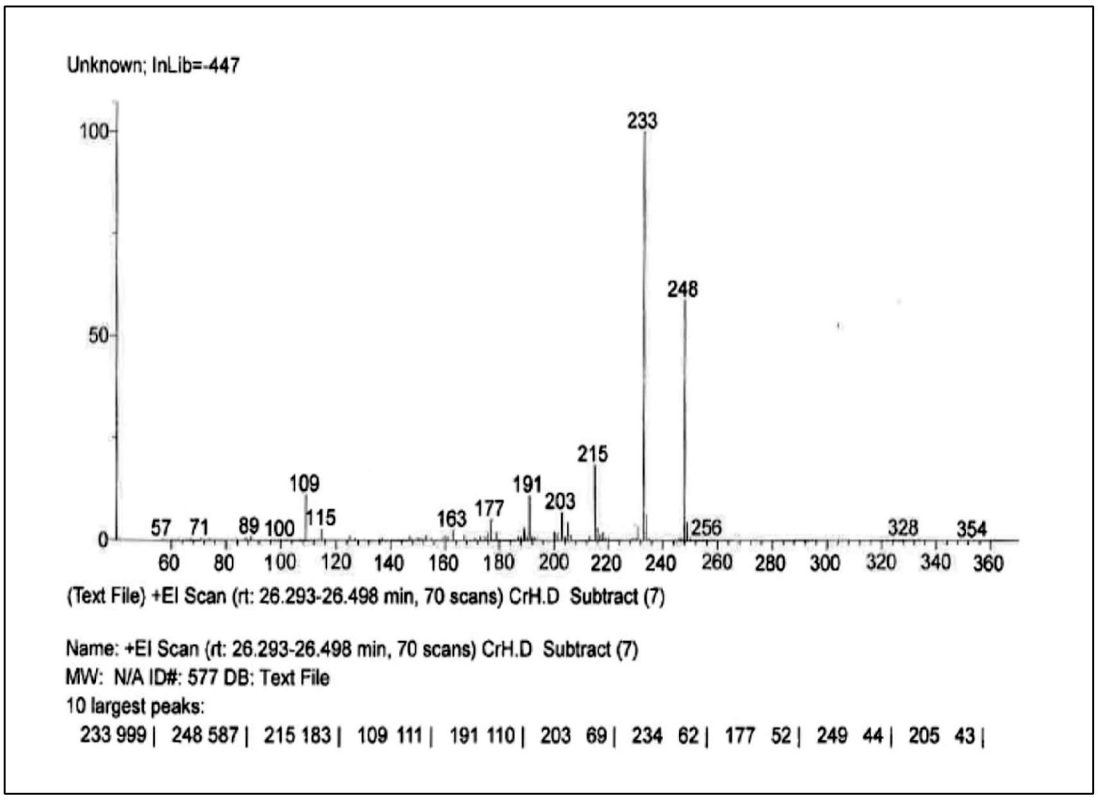

Hit 1 : Eicosanoic acid, methyl ester
C21H42O2; MF: 687; RMF: 747; Prob 64.7%; CAS: 1120-28-1; Lib: replib; ID: 10420
100
50
0
74
87
43
55
69
29
15
97
111
129
143
157 171 185 199 213 227 241 255 269
283
326
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
O
O
(replib) Eicosanoic acid, methyl ester

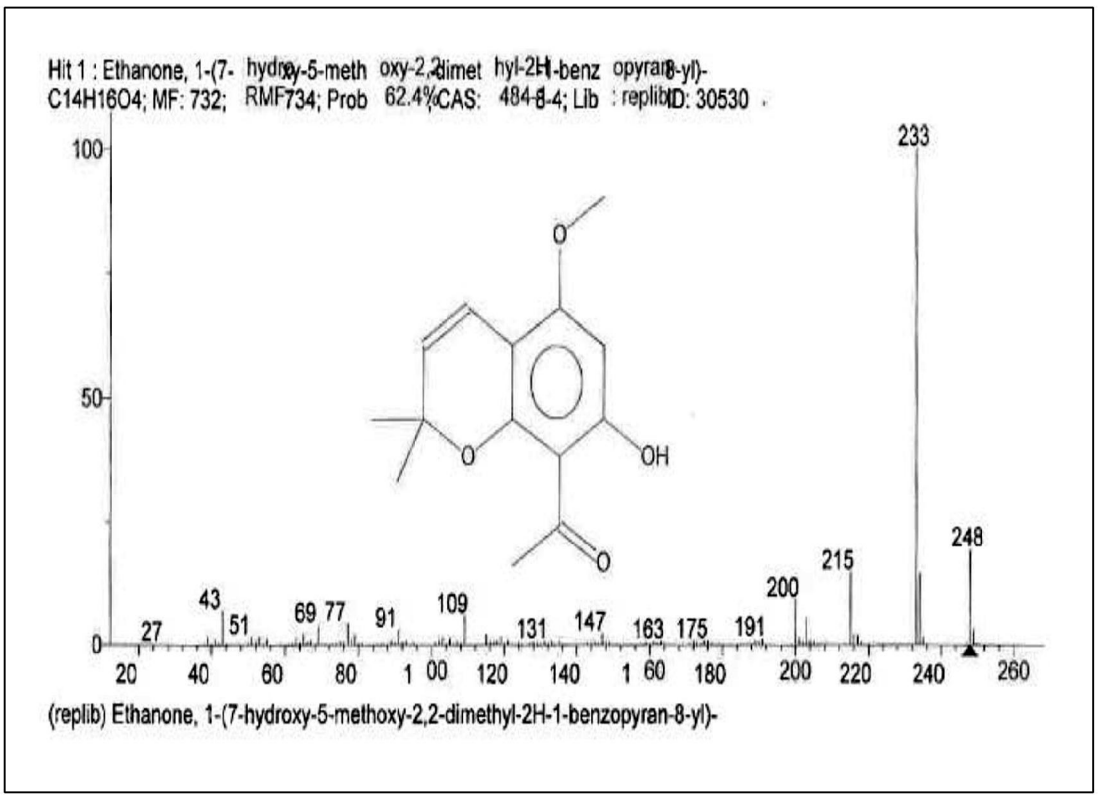

Unknown; InLib=-158
100
50
0
74
87
57 69 83 97 115 129 1 43 157 171 185 199 213 227 241 255 267 281 298
60 80 100 1 20 140 160 1 80 200 220 240 260 280 300
(Text File) +EI Scan (rt: 28.835-29.029 min, 66 scans) CrH.D Subtract (7)
Name: +EI Scan (rt: 28.835-29.029 min, 66 scans) CrH.D Subtract (7)
MW: N/A ID#: 579 DB: Text File
10 largest peaks:
74 999 | 87 676 | 75 255 | 143 117 | 199 83 | 255 74 | 129 65 | 69 63 | 57 57 | 97 57 |

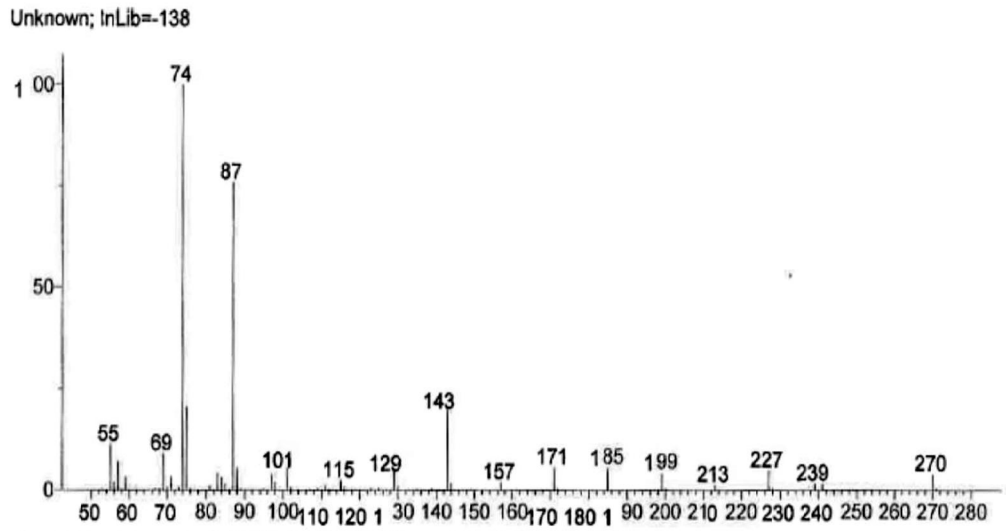

Hit 1 : Methyl stearate
C19H38O2; MF: 844; RMF: 848; Prob 68.6%; CAS: 112-61-8; Lib: replib; ID: 10455.
100
50
0
74
87
43
55
69
83
97
111
129
143
157 171 1 85 199 213 227 241 255 267 298
40 60 80 1 00 1 20 140 1 60 1 80 200 220 240 260 280 300
O
O
(replib) Methyl stearate


Unknown; InLib=-258
1 00
50
0
67
81
95
55
109
74
123
135
150
59
164
178
191
201
222
235
246
264
281
294
60
80
100
120
1 40
1 60
180
200
220
240
260
280
300
(Text File) +EI Scan (rt: 28.095-28.425 min, 112 scans) CrH.D Subtract (7)
Name: +EI Scan (rt: 28.095-28.425 min, 112 scans) CrH.D Subtract (7)
MW: N/A ID#: 578 DB: Text File
10 largest peaks:
67 999 | 81 979 | 95 723 | 55 518 | 82 507 | 68 473 | 79 404 | 69 396 | 96 364 | 109 340 |


Hit 1 : 9,12-Octadecadienoic acid (Z,Z)-, methyl ester
C19H34O2; MF: 858; RMF: 870; Prob 14.3%; CAS: 112-63-0; Lib: replib; ID: 8114.
100
50
0
67
81
41
55
95
109
27
59
74
123
136
150
164
178
192
205
220
234
263
294
O
O
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
(replib) 9,12-Octadecadienoic acid (Z,Z)-, methyl ester

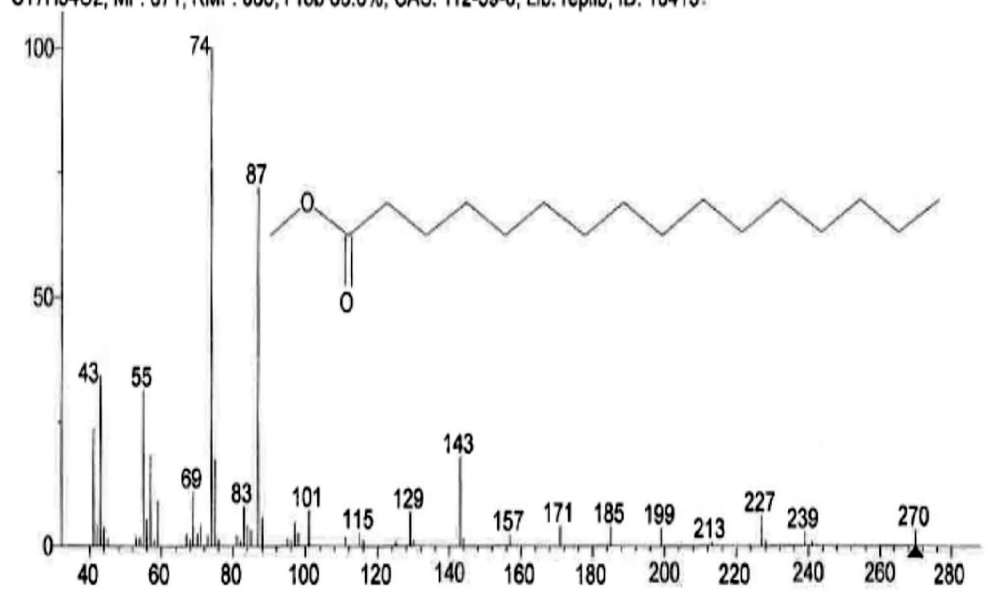

Unknown; InLib=-447
100
50
0
233
248
215
109
191
203
177
115
163
57 71 89 100
256
328 354
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360
(Text File) +EI Scan (rt: 26.293-26.498 min, 70 scans) CrH.D Subtract (7)
Name: +EI Scan (rt: 26.293-26.498 min, 70 scans) CrH.D Subtract (7)
MW: N/A ID#: 577 DB: Text File
10 largest peaks:
233 999 | 248 587 | 215 183 | 109 111 | 191 110 | 203 69 | 234 62 | 177 52 | 249 44 | 205 43 |


Hit 1 : Ethanone, 1-(7- hydroxy-5-meth oxy-2,2-dimet hyl-2H-1-benz opyran-8-yl)-
C14H16O4; MF: 732; RMF734; Prob 62.4%CAS: 484-8-4; Lib : replibID: 30530
100
50
0
233
248
215
200
109
43
51
69 77
91
131
147
163 175
191
27
20 40 60 80 1 00 120 140 1 60 180 200 220 240 260
O
O
OH
O
(replib) Ethanone, 1-(7-hydroxy-5-methoxy-2,2-dimethyl-2H-1-benzopyran-8-yl)-



(Text File) +EI Scan (rt: 23.887-24.095 min, 71 scans) CrH.D Subtract (7)

Name: +EI Scan (rt: 23.887-24.095 min, 71 scans) CrH.D Subtract (7)
MW: N/A ID#: 573 DB: Text File
10 largest peaks:
74 999 | 87 758 | 75 204 | 143 202 | 55 113 | 69 90 | 57 71 | 101 56 | 171 56 | 88 54 |

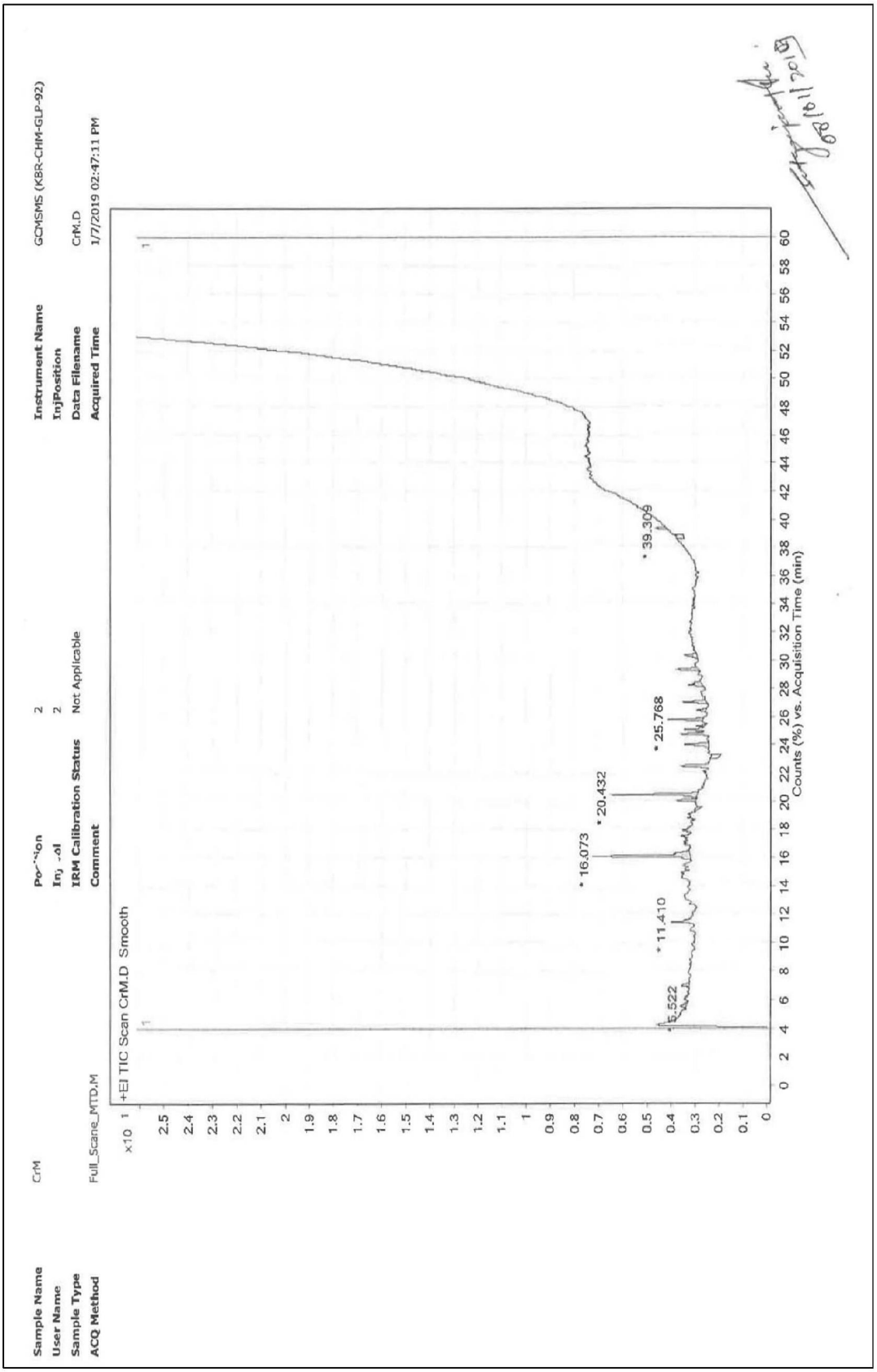


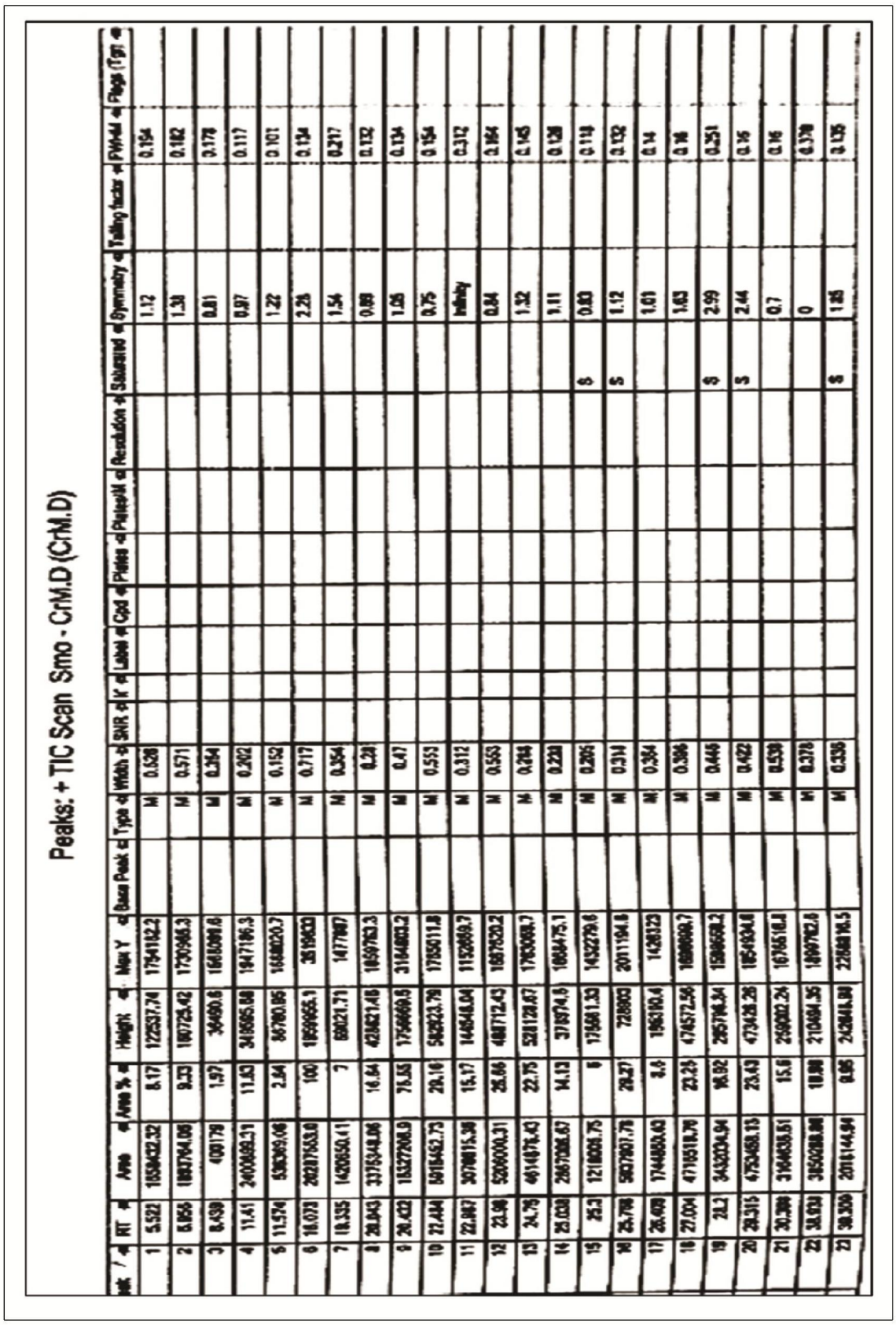
(replib) Hexadecanoic acid, methyl ester

Peaks: +Total Ion Chromatogram Scan – CrM							
Peaks	**RT (mins)**	**MF**	**RMF**	**Area %**	**MW**	**Chemical formula**	**Chemical Name**
13	24.75	747	772	22.75	248	$C_{14}H_{16}O_4$	Mycorradicin
14	25.038	729	878	14.43	362	$C_{22}H_{34}O_4$	Annosquamosin A
17	26.409	408	591	29.77	248	$C_{14}H_{16}O_4$	Mycorradicin
20	29.135	519	693	23.4	264	$C_{18}H_{16}O_2$	Cinnamylcinnamate

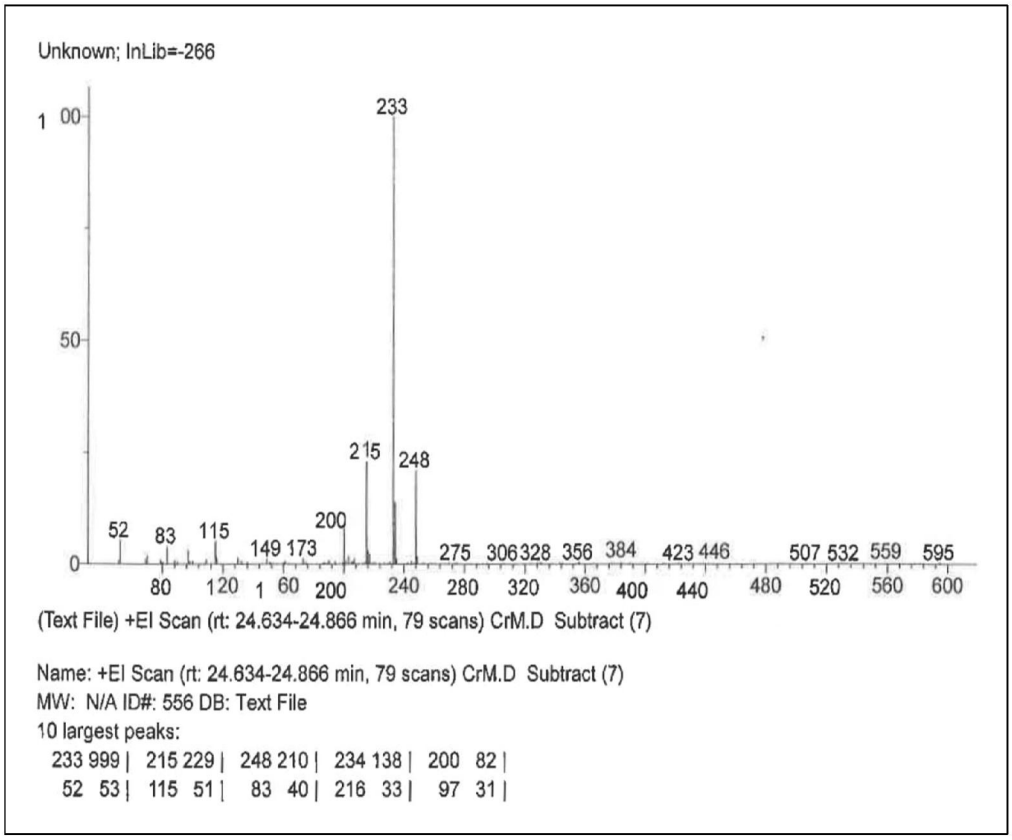
The Chromatogram and the Mass spectra of the important above mentioned peaks are given in the following pages:

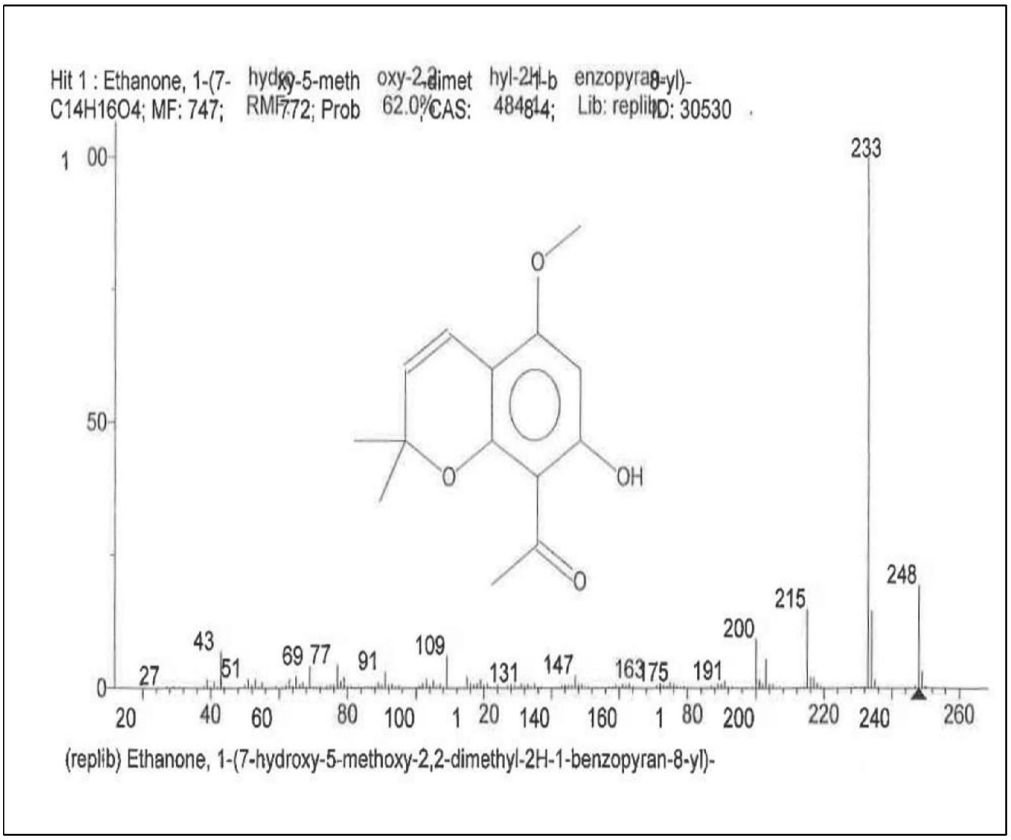
Chromatograms and Mass Spectra (CrM)

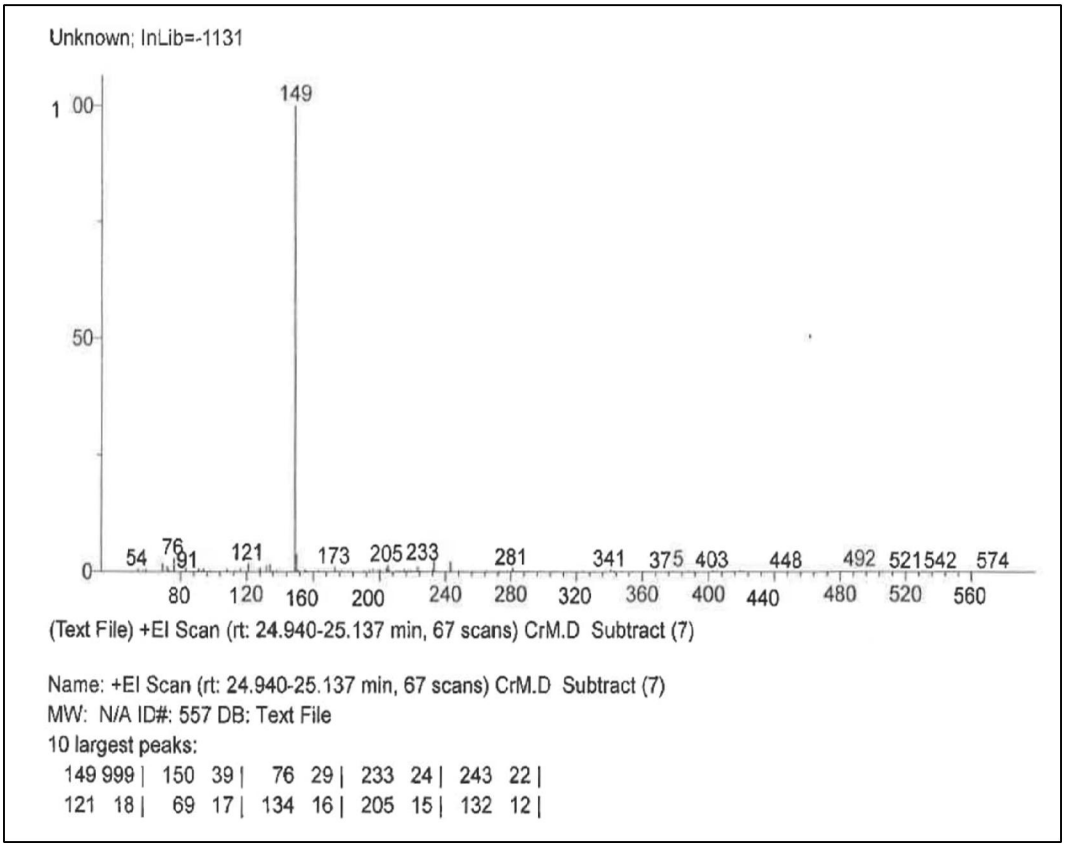


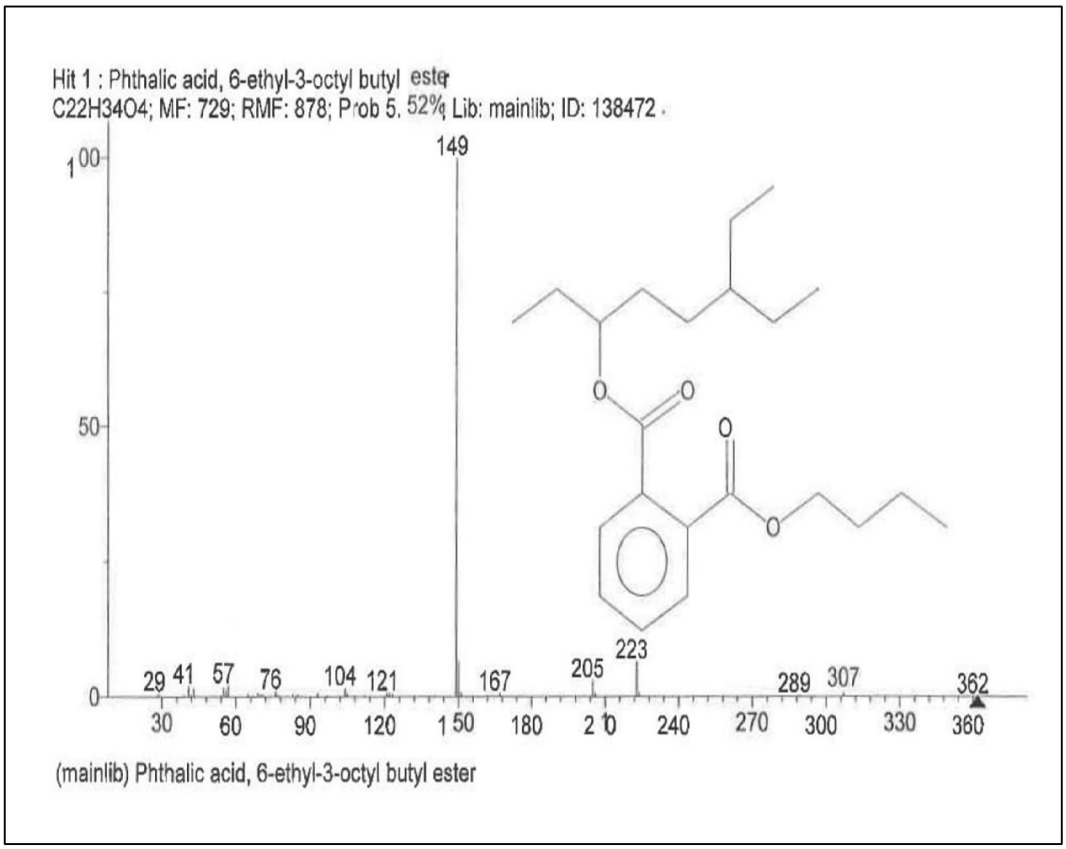


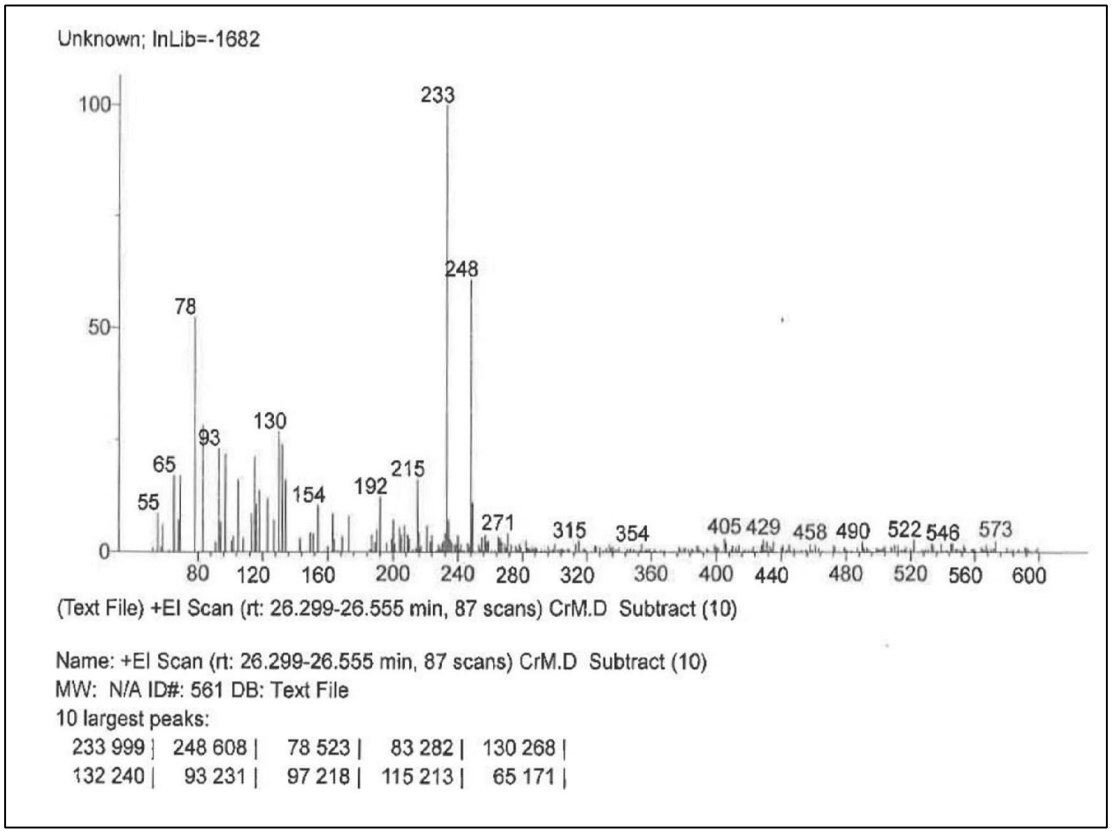
Peak #	RT	Area	Area %	Height	Max Y	Base Peak	Type	Width	SNR	Label	Cpd	Plates	Plates/M	Resolution	Saturated	Symmetry	Tailing factor	FWHM	Flags (Tgt)
1	5.522	1838422.32	8.17	122537.74	175491.2		MI	0.538								1.12		0.154	
2	8.856	1887764.08	8.39	180725.42	1790968.3		MI	0.571								1.38		0.182	
3	8.439	400179	1.97	36490.8	1661098.8		MI	0.384								0.81		0.178	
4	11.41	2400889.31	11.83	349695.88	1947186.3		MI	0.202								0.97		0.117	
5	11.574	536369.06	2.64	86780.85	1668020.7		MI	0.152								1.22		0.101	
6	16.073	2021755.8	100	1859655.1	2519603		MI	0.717								2.25		0.134	
7	19.335	1420650.41	7	69021.71	1477887		MI	0.364								1.54		0.217	
8	20.843	3375348.06	16.64	423621.48	1859763.3		MI	0.28								0.89		0.132	
9	20.422	1532720.9	75.56	1756669.5	3164803.2		MI	0.47								1.05		0.134	
10	22.444	8916452.73	28.16	582823.78	1785011.8		MI	0.553								0.75		0.154	
11	22.867	3078815.36	15.17	144648.84	1152859.7		MI	0.312								Infinity		0.312	
12	23.98	5200000.31	25.66	488712.43	1887529.2		MI	0.553								0.84		0.164	
13	24.76	4614078.43	22.75	528128.67	1765068.7		MI	0.284								1.32		0.145	
14	25.038	2867086.67	14.13	378974.6	1668475.1		MI	0.233								1.11		0.128	
15	25.3	1218009.75	6	175681.33	1432279.8		MI	0.205							S	0.83		0.118	
16	25.788	5807807.78	28.27	728803	2011194.8		MI	0.314							S	1.12		0.132	
17	26.409	1744800.62	8.6	196180.4	1438123		MI	0.384								1.01		0.14	
18	27.004	4719518.78	23.25	474572.56	1886699.7		MI	0.386								1.63		0.16	
19	28.2	3432039.94	16.92	295798.84	1586559.2		MI	0.446							S	2.99		0.251	
20	28.315	4753468.13	23.43	473428.28	1854934.8		MI	0.422							S	2.44		0.16	
21	30.388	3164838.61	15.6	259002.24	1676518.8		MI	0.538								0.7		0.16	
22	30.634	3850088.88	18.98	210494.36	1869762.8		MI	0.378								0		0.378	
23	30.956	2016144.94	9.95	242844.88	2286816.5		MI	0.339							S	1.85		0.135	

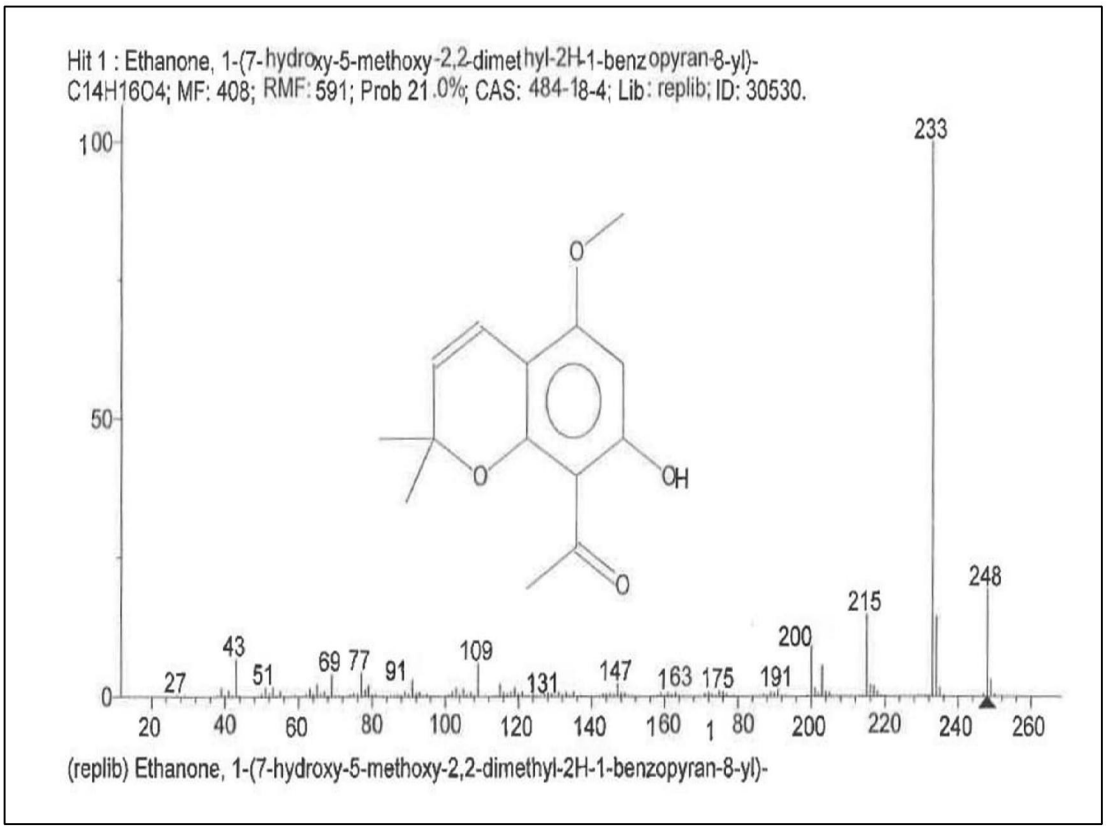

Unknown; InLib=-266
100
50
0
233
215
248
200
52
83
115
149 173
275 306 328 356 384 423 446
507 532 559 595
80 120 160 200 240 280 320 360 400 440 480 520 560 600
(Text File) +EI Scan (rt: 24.634-24.866 min, 79 scans) CrM.D Subtract (7)
Name: +EI Scan (rt: 24.634-24.866 min, 79 scans) CrM.D Subtract (7)
MW: N/A ID#: 556 DB: Text File
10 largest peaks:
233 999 | 215 229 | 248 210 | 234 138 | 200 82 |
52 53 | 115 51 | 83 40 | 216 33 | 97 31 |

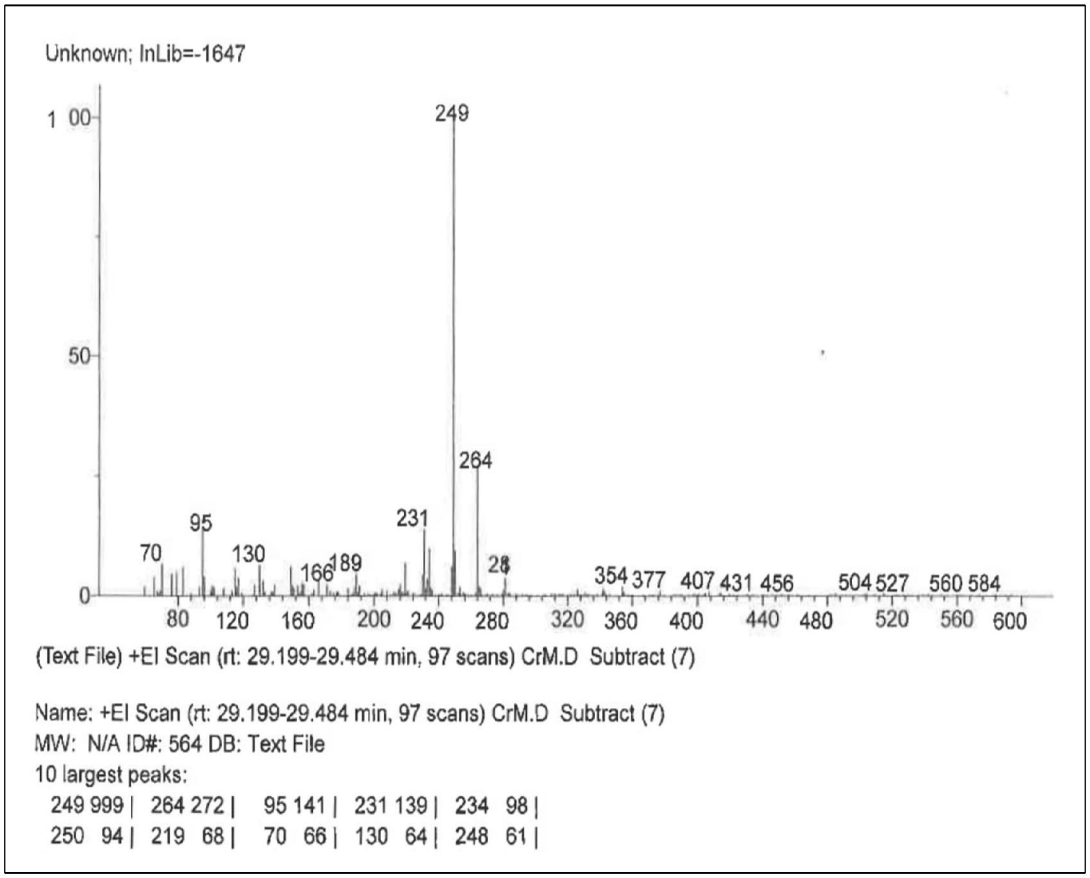

Hit 1 : Ethanone, 1-(7- hydroxy-5-meth oxy-2,2-dimet hyl-2H-b enzopyran-8-yl)-
C14H16O4; MF: 747; RMF: 772; Prob 62.0%; CAS: 484814; Lib: replib; ID: 30530
100
50
0
233
248
215
200
43
51
69 77
91
109
131
147
163 175
191
27
20 40 60 80 100 120 140 160 180 200 220 240 260
O
O
OH
O
(replib) Ethanone, 1-(7-hydroxy-5-methoxy-2,2-dimethyl-2H-1-benzopyran-8-yl)-

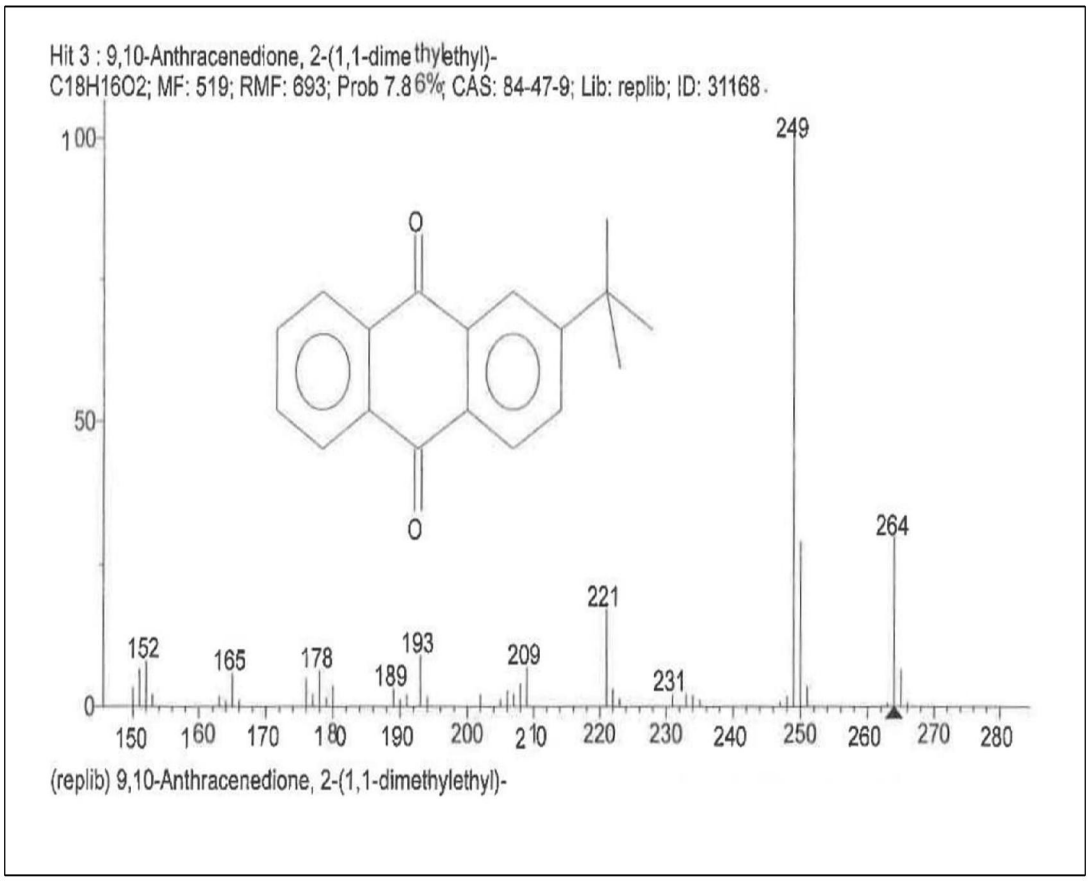

Unknown; InLib=-1131
100
50
0
149
54 76 91 121 173 205 233 281 341 375 403 448 492 521 542 574
80 120 160 200 240 280 320 360 400 440 480 520 560
(Text File) +EI Scan (rt: 24.940-25.137 min, 67 scans) CrM.D Subtract (7)
Name: +EI Scan (rt: 24.940-25.137 min, 67 scans) CrM.D Subtract (7)
MW: N/A ID#: 557 DB: Text File
10 largest peaks:
149 999 | 150 39 | 76 29 | 233 24 | 243 22 |
121 18 | 69 17 | 134 16 | 205 15 | 132 12 |


Hit 1 : Phthalic acid, 6-ethyl-3-octyl butyl ester
C22H34O4; MF: 729; RMF: 878; Prob 5.52%; Lib: mainlib; ID: 138472
100
50
0
149
29 41 57 76 104 121 167 205 223 289 307 362
30 60 90 120 150 180 210 240 270 300 330 360
O
O
O
O
(mainlib) Phthalic acid, 6-ethyl-3-octyl butyl ester


Unknown; InLib=-1682
100
50
0
233
248
78
130
93
65
215
192
154
55
271
315
354
405 429
458 490
522 546
573
80 120 160 200 240 280 320 360 400 440 480 520 560 600
(Text File) +EI Scan (rt: 26.299-26.555 min, 87 scans) CrM.D Subtract (10)
Name: +EI Scan (rt: 26.299-26.555 min, 87 scans) CrM.D Subtract (10)
MW: N/A ID#: 561 DB: Text File
10 largest peaks:
233 999 | 248 608 | 78 523 | 83 282 | 130 268 |
132 240 | 93 231 | 97 218 | 115 213 | 65 171 |


Hit 1 : Ethanone, 1-(7-hydroxy-5-methoxy-2,2-dimethyl-2H-1-benzopyran-8-yl)-
C14H16O4; MF: 408; RMF: 591; Prob 21.0%; CAS: 484-18-4; Lib: replib; ID: 30530.
100
50
0
233
O
O
OH
O
248
215
200
43
109
69 77
91
51
147
131
163 175
191
27
20 40 60 80 100 120 140 160 180 200 220 240 260
(replib) Ethanone, 1-(7-hydroxy-5-methoxy-2,2-dimethyl-2H-1-benzopyran-8-yl)-


Unknown; InLib=-1647
1 00
50
0
249
264
231
95
70
130
166
189
28
354 377 407 431 456
504 527 560 584
80 120 160 200 240 280 320 360 400 440 480 520 560 600
(Text File) +EI Scan (rt: 29.199-29.484 min, 97 scans) CrM.D Subtract (7)
Name: +EI Scan (rt: 29.199-29.484 min, 97 scans) CrM.D Subtract (7)
MW: N/A ID#: 564 DB: Text File
10 largest peaks:
249 999 | 264 272 | 95 141 | 231 139 | 234 98 |
250 94 | 219 68 | 70 66 | 130 64 | 248 61 |


Hit 3 : 9,10-Anthracenedione, 2-(1,1-dimethylethyl)-
C18H16O2; MF: 519; RMF: 693; Prob 7.86%; CAS: 84-47-9; Lib: replib; ID: 31168
100
50
0
249
264
221
152
165
178
193
189
209
231
150 160 170 180 190 200 210 220 230 240 250 260 270 280
(replib) 9,10-Anthracenedione, 2-(1,1-dimethylethyl)-

7.5 STATISTICAL ANALYSIS

The Spearman's Rho correlation for non parametric data of the antioxidant analysis shows a significant correlation $p<0.05$ in radical scavenging activity for the CrCh and CrM fraction for the test sample and the commercial antioxidant. The chloroform and methanol fraction of *C. rotandus* inflorescence extract followed power equation as the best fit curve estimation with $R^2 = 0.993$ for CrM followed by CrChl and CrP. The Standard Error of the Estimate is the deviation of the residuals, all at 95% confidence level. In principal increase in R^2 with decreasing SEE signifies less deviation from original data interpretation. As recorded the least SEE was for *C. rotandus* CrCh fraction of the inflorescence (3.4%).The SEE also decreased in CrM and was highest in CrP. Hence the data for CrM are the most relevant representative of the outcome followed by CrCh and least for CrP. The F value is found to be significant $p<0.05$ in all the three fractions, thus variance between the mean of two variables (% inhibition of sample and BHT) is significant concerning differential concentration (Fig. 74).

Pet Ether

Model Summary

R	R Square	Adjusted R Square	Std. Error of the Estimate
.994	.987	.978	4.203

The independent variable is Con.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Regression	5521.625	3	1840.542	104.169	.000
Residual	70.675	4	17.669		
Total	5592.301	7			

The independent variable is Con.

Correlations

			Con	Pinh	Control
Spearman's rho	Con	Correlation Coefficient	1.000	1.000**	.643
		Sig. (2-tailed)	.	.	.086
		N	8	8	8
	Pinh	Correlation Coefficient	1.000**	1.000	.643
		Sig. (2-tailed)	.	.	.086
		N	8	8	8
	Control	Correlation Coefficient	.643	.643	1.000
		Sig. (2-tailed)	.086	.086	.
		N	8	8	8

**. Correlation is significant at the 0.01 level (2-tailed).

Fraction: CrP

- Model: Exponential
- Eq.: $y = 0.027e^{0.030x}$
- F (3, 4) = 104.1, $p<0.05$
- ***IC50:0.121µg/***

Fraction: CrCh

- Model: Power
- Eq.: $y = 3E\text{-}12x^{6.006}$
- F (1, 6) = 129.4, p<0.05
- ***IC50:0.0479µg/ml***

Chloroform

Model Summary

R	R Square	Adjusted R Square	Std. Error of the Estimate
.978	.956	.948	.034

The independent variable is Con.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Regression	.153	1	.153	129.488	.000
Residual	.007	6	.001		
Total	.160	7			

The independent variable is Con.

Correlations

			percentage of inhibition	BHT as positive control
Spearman's rho	percentage of inhibition	Correlation Coefficient	1.000	.857**
		Sig. (2-tailed)	.	.007
		N	8	8
	BHT as positive control	Correlation Coefficient	.857**	1.000
		Sig. (2-tailed)	.007	.
		N	8	8

**. Correlation is significant at the 0.01 level (2-tailed).

Fraction: CrM

- Model: Power
- Eq.: $y = 5E\text{-}9x^{4.264}$
- F (3, 4) = 203, p<0.05
- ***IC50:0.0878µg/ml***

Methanol

Model Summary

R	R Square	Adjusted R Square	Std. Error of the Estimate
.997	.993	.989	1.558

The independent variable is Con.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Regression	1479.307	3	493.102	203.078	.000
Residual	9.713	4	2.428		
Total	1489.019	7			

The independent variable is Con.

Correlations

			Con	Pinh	Control
Spearman's rho	Con	Correlation Coefficient	1.000	1.000**	.994**
		Sig. (2-tailed)	.	.	.000
		N	8	8	8
	Pinh	Correlation Coefficient	1.000**	1.000	.994**
		Sig. (2-tailed)	.	.	.000
		N	8	8	8
	Control	Correlation Coefficient	.994**	.994**	1.000
		Sig. (2-tailed)	.000	.000	.
		N	8	8	8

**. Correlation is significant at the 0.01 level (2-tailed).

Fig 74: **Model summary and IC50 value of antioxidant activity of CrP, CrCh and CrM fraction of Inflorescence of *C.rotandus.***

The statistical analysis of the inflorescence extract of *C. rotundus* on brine shrimp lethality assay are given by the the stem and leaf model Shapiro-Wilk significance test with 6hrs time interval had p>0.05 by probit analysis. Hence CrM 12hr exposure is expressed as logit values (Fig. 77). In all the cases the p value for chi sqaure test is p≤ 0.15; hence a heterogeneity factor is calculated. The orders of median lethal concentration was highest for CrP (Fig. 75) fraction exposed for 1hour and lowest for methanol fraction exposed for 24hrs.Methanol fraction was found to be toxic compared to CrCh (Fig. 76) and pet ether

Petroleum Ether

Pet Ether

Tests of Normality

Time	1hrs-Pet Ether	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	1	.243	5	.200*	.894	5	.377

*. This is a lower bound of the true significance.

Tests of Normality

Time	6hrs-Pet Ether	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	6	.299	5	.164	.784	5	.060

a. Lilliefors Significance Correction

Tests of Normality

	12hrs-Pet Ether fraction of Cyperus	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	12	.322	5	.100	.732	5	.020

a. Lilliefors Significance Correction

Tests of Normality

Time	18hrs-Pet Ether	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	18	.256	5	.200*	.833	5	.145

*. This is a lower bound of the true significance.

Tests of Normality

Time	24hrs-Pet Ether	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	24	.273	5	.200*	.852	5	.201

*. This is a lower bound of the true significance.

Parameter Estimates

Time:1hr	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.555	.067	8.327	.000	.424	.685
	Intercept	-2.303	.202	-11.378	.000	-2.506	-2.101

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	14.628	3	.002[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter Estimates

Time:6hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	1.657	.140	11.820	.000	1.382	1.932
	Intercept	-3.556	.304	-11.702	.000	-3.860	-3.252

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	44.133	3	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter Estimates

Time:12hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	1.066	.075	14.220	.000	.919	1.212
	Intercept	-2.156	.162	-13.326	.000	-2.318	-1.995

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	100.828	3	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter Estimates

Time:18hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.865	.064	13.426	.000	.739	.991
	Intercept	-1.508	.131	-11.498	.000	-1.639	-1.376

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	92.885	3	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter Estimates

Time:24hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.916	.069	13.205	.000	.780	1.051
	Intercept	-1.425	.132	-10.821	.000	-1.557	-1.293

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	46.005	3	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Fig 75: **Test of normality (Shapiro Wilk) and Kolmogorov-Smirnov Test and Probit and logit analysis of CrP fraction of *C. rotandus* on brine shrimp lethality at 1hr, 6hrs, 12hrs, 18hrs and 24hrs interval.**

Chloroform:

Chloroform

Tests of Normality

Time	1hr-chloroform	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	1	.330	5	.079	.735	5	.021

a. Lilliefors Significance Correction

Tests of Normality

Time	6hrs-Chloroform	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	6	.277	5	.200*	.848	5	.190

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Tests of Normality

Time	12hrs-Chloroform	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	12	.322	5	.100	.732	5	.020

a. Lilliefors Significance Correction

Tests of Normality

Time	18hrs-Chloroform	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	18	.267	5	.200*	.773	5	.048

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Tests of Normality

Time	24hrs-chloroform	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	24	.254	5	.200*	.803	5	.086

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Parameter Estimates

Time:1hr	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	1.284	.150	8.582	.000	.991	1.577
	Intercept	-4.830	.527	-9.159	.000	-5.357	-4.303

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	2.335	3	.506[a]

a. Since the significance level is greater than .150, no heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter Estimates

Time:6hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	.968	.091	10.682	.000	.791	1.146
	Intercept	-3.263	.288	-11.345	.000	-3.550	-2.975

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	2.343	3	.504[a]

a. Since the significance level is greater than .150, no heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter Estimates

Time:12hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	1.066	.075	14.220	.000	.919	1.212
	Intercept	-2.156	.162	-13.326	.000	-2.318	-1.995

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	100.828	3	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter Estimates

Time:18hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	1.079	.077	14.070	.000	.928	1.229
	Intercept	-2.083	.160	-13.013	.000	-2.243	-1.923

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	75.431	3	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter Estimates

Time:24hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	1.099	.079	13.908	.000	.945	1.254
	Intercept	-2.022	.159	-12.702	.000	-2.181	-1.863

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	57.201	3	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Fig 76: **Test of normality (Shapiro Wilk) and Kolmogorov-Smirnov Test and Probit and logit analysis of CrCh fraction of *C. rotandus* on brine shrimp lethality at 1hr, 6hrs, 12hrs, 18hrs and 24hrs interval.**

Methanol:

Tests of Normality

Time	1hr-Methanol	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	1	.327	5	.087	.728	5	.018

a. Lilliefors Significance Correction

Tests of Normality

Time	6hrs-Methanol	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	6	.299	5	.164	.784	5	.060

a. Lilliefors Significance Correction

Tests of Normality

Time	12hrs-methanol	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	12	.249	5	.200*	.877	5	.298

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Tests of Normality

Time	18+hrs24hrs-Methanol	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Mortality	18+24	.220	5	.200*	.896	5	.390

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Parameter Estimates

Time:1hr	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	1.652	.146	11.347	.000	1.366	1.937
	Intercept	-5.276	.457	-11.532	.000	-5.733	-4.818

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	55.759	3	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter Estimates

Time:6hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	1.657	.140	11.820	.000	1.382	1.932
	Intercept	-3.556	.304	-11.702	.000	-3.860	-3.252

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	44.133	3	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter Estimates

Time:12hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LOGIT[a]	Concentration	2.087	.178	11.727	.000	1.738	2.436
	Intercept	-3.754	.354	-10.611	.000	-4.108	-3.400

a. LOGIT model: LOG(p/(1-p)) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
LOGIT	Pearson Goodness-of-Fit Test	43.730	3	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Parameter Estimates

Time:18hrs+ 24hrs	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration	1.168	.094	12.369	.000	.983	1.353
	Intercept	-1.641	.159	-10.302	.000	-1.801	-1.482

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Chi-Square Tests

Parameter		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	37.208	3	.000[a]

a. Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Fig 77: **Test of normality (Shapiro Wilk) and Kolmogorov-Smirnov Test and Probit and logit analysis of CrM fraction of *C. rotandus* on brine shrimp lethality at 1hr, 6 hrs, 12 hrs, 18 hrs and 24 hrs interval.**

7.6 DISCUSSION

Cyperus rotandus is a sedge which gas always been a hindrance to agriculture. With its immensely tolerant seeds, it pervades deep in the layers of soil after sprouting and germinates, diminishing or lowering the water table available for other neighbouring. It has strong erect roots which protects it from most adverse climatic conditions. In spite of all the reasons for its eradication, an attempt has been made to explain its existence in an aquatic habitat. The methanol fraction of the inflorescence of *C. rotandus* (CrM) was found to have a strong antioxidant activity with IC50 <150ug/ml. Added to the advantage that methanol is a polar fraction and is easily miscible with water; the toxicity of methanol can be easily avoided. The antibacterial activity of the inflorescence is remarkable in terms of inhibition zone displaying diameter of almost<

14mm in most cases at 0.1 – 0.5µg/ml. The anti-algal and antimicrobial trait could be utilized as a natural disinfectant may be utilized by sprouting of the seeds in the nearby water bodies or applied as chemoprophylaxis. The low toxicity and high antioxidant content of the methanol fraction of Cyperus favours its chances of being beneficial to fish health. The fact that it stimulates growth of regenerated roots at its higher doses could be preliminary used as sources to bio-fertilizers. The major constituents of CrM fraction is mycorradicin, a carotenoid cleavage product known for its phytoalexin antifungal activity (Carmen, 2007). Annosquamosin A and cinnamylcinnamate are diterpenoid and cinnamic acid derivative which are well known for antibacterial, anti-parasitic and insect anti-feedant (Jayaprakasha *et al*.; 2015). The anti-algal activity of the inflorescence of *C. rotundus* was found to possess a preventive property more effectively. The fraction potentially retarded the invasive growth of the algae after being inoculated in its favourable media, reducing its total chlorophyll content by 89% which was 13% more than most commercial algaecides added after visible algal mass. The algal mass was seen blemish and sediment at higher concentrations of the extract. The chl-a was found to be 36% more reduced in CrP which accounts for most pigmentation with EC50 at 0.014mg/ml. The main constituents of the fraction are Silicic acid, Eicosanoic acid methyl ester, Methyl Stearate, Methyl Linoleate and Mycorradicin (Abubakar and Majinda, 2016; Gomathi and Elango, 2015). The Silicic acid is known as a plant growth factor which enhances seedling growth (Neeru *et al*., 2016). The eicosanoic acid is known for its antifungal activity against *Candida albicans* which hosts on fish species; Tilapia, Zebra fish and Rainbow trout (Idowu, 2017). The methyl ester of linoleic acid shows strong antifungal activity against *Paracoccidioides brasiliensis* which known to cause skin infections to people who sustain their living on fishing and hunting (Martinez, 2015). As the fraction would be applied in an aquatic variable (Pan

et al., 2006) hence the evaluation of its antioxidant property would ensure safety against non-target toxicity.